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Stage-Specific Timing of the microRNA Regulation of *lin-28* by the Heterochronic Gene *lin-14* in *Caenorhabditis elegans*

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Abstract

In normal development, the order and synchrony of diverse developmental events must be explicitly controlled. In the nematode *Caenorhabditis elegans*, the timing of larval events is regulated by hierarchy of proteins and microRNAs (miRNAs) known as the heterochronic pathway. These regulators are organized in feedforward and

feedback interactions to form a robust mechanism for specifying the timing and execution of cell fates at successive stages. One member of this pathway is the RNA binding protein LIN-28, which promotes pluripotency and cell fate decisions in successive stages. Two genetic circuits control LIN-28 abundance: it is negatively regulated by the miRNA *lin-4,* and positively regulated by the transcription factor LIN-14through a mechanism that was previously unknown. In this report, we used animals that lack *lin-4* to elucidate LIN-14's activity in this circuit. We demonstrate that three *let-7*family miRNAs—miR-48, miR-84, and miR-241—inhibit *lin-28* expression. Furthermore, we show genetically that these miRNAs act between *lin-14* and *lin-28,* and that they comprise the pathway by which *lin-14* positively regulates *lin-28.* We also show that the *lin-4* family member *mir-237,* also regulates early cell fates. Finally, we show that the expression of these miRNAs is directly inhibited by *lin-14* activity, making them the first known targets of *lin-14* that act in the heterochronic pathway.

Keywords: *C. ELEGANS* MICRORNA HETEROCHRONIC GENES LIN-14 LIN-28 *LET-7*

TO grow from a single totipotent cell to a mature adult, every animal must carry out a succession of developmental programs. In the nematode *Caenorhabditis elegans*, a genetic pathway of heterochronic genes controls the proper timing and sequence of developmental programs during the animal's postembryonic stages. This pathway is composed of a number of microRNAs (miRNAs) and protein regulators, whose activation and repression over time is necessary for appropriate cell fate decisions during each of the animal's four larval stages (referred to as L1–L4) (Rougvie and Moss 2013). Some mutations in heterochronic genes cause stage-specific patterns to be skipped, resulting in precocious development, whereas other mutations cause such patterns to be reiterated, leading to retarded development (Ambros and Horvitz 1984). Several heterochronic genes have been conserved through evolution, which also have important roles in developmental timing (Tsialikas and Romer-Seibert 2015).

Early in the pathway, the miRNA *lin-4* down-regulates the expression of two proteins: LIN-14 and LIN-28 (Arasu *et al.* 1991; Lee *et al.* 1993; Moss *et al.* 1997; Feinbaum and Ambros 1999). Although LIN-14 is a putative transcription factor that promotes the succession of L1 and L2 cell fates, its targets in the heterochronic pathway have not been identified (Ambros and Horvitz 1987; Ruvkun *et al.* 1989; Hristova *et al.* 2005). LIN-28 is an RNA binding protein, best known for regulating the maturation of the miRNA *let-7* (Moss *et al.* 1997; Viswanathan *et al.* 2008; Lehrbach *et al.* 2009; Tsialikas and Romer-Seibert 2015). However, LIN-28's primary role in the heterochronic pathway, promoting L2 cell fates, does not require *let-7* activity (Vadla *et al.* 2012).

When *lin-4* is inactivated by a mutation, both *lin-14* and *lin-28* remain highly expressed late into development. However, if either *lin-14* or *lin-28* is removed in the absence of *lin-4*, the expression of the other gene drops (Arasu *et al.* 1991; Moss *et al.* 1997). This mutual dependence defines a *lin-14*–*lin-28* positive feedback loop (Seggerson *et al.*2002). Although we have known about this feedback loop for some time, how these two proteins each promote the expression of the other has remained elusive. The fact that each protein can be repressed in the other's absence, even when *lin-4* is already null, suggests that additional factors are negatively regulating their expression. Seggerson *et al.* (2002) found that this "lin-4-independent" repression of *lin-28* occurs after translation initiation, requires *lin-28*'s 3′ UTR, and contributes substantially to the down-regulation of *lin-28*, but they did not identify what factors are responsible.

Examination of *lin*-28's 3′ UTR reveals that in addition to one *lin-4* family binding site, there is at least one conserved *let-7* family binding site. This observation led several researchers to speculate that *lin-28* is regulated by *let-7* miRNAs, but this hypothesis has never been demonstrated experimentally (Reinhart *et al.* 2000; Resnick *et al.* 2010; Ambros 2011). The only evidence that *lin-28* acts downstream

of *let-7* family miRNAs comes from an investigation of three redundant *let-7* family members, miR-48, miR-84, and miR-241, referred to collectively as the "3let-7s" (Abbott *et al.* 2005). It was observed that the precocious phenotype of *lin-28* null mutants is completely epistatic to the retarded phenotype of a *3let-7s* null mutant, suggesting that the 3let-7s function upstream of *lin-28*. However, a *lin-28*::*GFP* reporter is still stage-specifically down-regulated even when the 3let-7s are absent. Not accounted for in these experiments was the contribution of *lin-4*, a known regulator of *lin-28*. Therefore, the significance of *let-7* family regulation of *lin-28* for developmental timing remains an open question.

In this report, we use a sensitized background in which *lin-4* is removed to resolve this issue. We show that the 3let-7s target *lin-28*, and that these are the missing factors in the *lin-14*–*lin-28* regulation. Moreover, we show that miR-237, another member of the *lin-4* family of miRNAs, is an additional regulator of early cell fates. Finally, we show that the expression of each of these miRNAs is negatively regulated at a transcriptional level by LIN-14. This result provides a mechanism for LIN-14's positive regulation of LIN-28and identifies LIN-14's first regulatory targets in the heterochronic pathway.

Materials and Methods

Worm strains and culture conditions

Nematodes were grown under standard conditions at 20° unless otherwise noted. Many strains carry the transgene *wIs78* that contains a seam cell nuclei marker (*scm*::*GFP*) and seam cell junction marker (*ajm*::*GFP*) to identify lateral hypodermal seam cells (Koh and Rothman 2001). The *maIs108 (lin-28*::*GFP)* transgene was used to track LIN-28 expression (Moss *et al.* 1997). MiRNA expression was followed using the transgenes *maIs134 (plin-4*::*GFP)*, *maIs135 (Pmir-237*::*GFP)*, *maIs138 (Pmir-84*::*GFP)*, *maIs140 (Pmir-241*::*GFP)*, and *maIs150 (Pmir-48*::*GFP)* (Martinez *et al.* 2008).

Strains used are as follows: *N2 WT* (Bristol), *RG733 wIs78*, *ME348 lin-4(e912) II*; *wIs78*, *ME349 lin-4(e912) II*; *lin-14(n179ts) X*; *wIs78*, *ME357 lin-4(e912) II*; *mir-48 mir-241(nDf51) V*; *lin-14(n179ts) mir-84(n4037) X*; *wIs78*, *ME358 lin-4(e912) II*; *mir-48 mir-241(nDf51) V*; *lin-14(n179ts) X*; *wIs78*, *ME359 lin-4(e912) II*; *lin-14(n179ts) mir-84(n4037) X*; *wIs78, ME390 lin-4(e912) II*; *mir-48 mir-241(nDf51) V*; *lin-14(n179ts) mir-84(n4037) X*; *maIs108, ME394 lin-4(e912) II*; *maIs150, ME396 lin-14(n179ts) X*; *maIs150, ME398 lin-14(n179ts) X*; *maIs140, ME399 lin-14(n179ts) X*, *maIs138, ME400 lin-4(e912) II*; *maIs140, ME402 maIs140, ME404 maIs138, ME405 maIs150, ME407 lin-4(e912) II*; *maIs138, RF167 lin-14(ma135) X*; *wIs78, RF173 mir-237(n4296) lin-14(ma135) X*; *wIs78, RF204 lin-28(n719) I*, *wIs78, RF205 lin-28(n719) I*, *mir-237(n4296) X*; *wIs78, RF303 lin-46(ma164) V*; *wIs78, RF304 lin-46(ma164) V*; *mir-237(n4296) X*; *wIs78, RF404 hbl-1(ve18) X*; *wIs78, RF405 hbl-1(ve18) mir-237(n4296) X*; *wIs78, RF455 mir-237(n4296) X*; *wIs78, RF472 lin-4(e912) II*; *wIs78, RF477 lin-14(n179ts) X*; *wIs78, RF478 mir-237(n4296) lin-14(n179ts) X*; *wIs78, RF479 lin-4(e912) II*; *mir-237(n4296) X*; *wIs78, RF486*: *ctIs39, RF488 lin-14(n355n679ts) X*; *wIs78, RF492 lin-28(n719) I*; *lin-46(ma164) V*; *wIs78, RF493 lin-28(n719) I*; *lin-46(ma164) V*; *mir-237(n4296) X*; *wIs78, RF503 mir-237(n4296) lin-14(n355n679ts) X*; *wIs78, RF576 lin-4(e912) II*; *maIs135, RF585 lin-14(n179ts) X*; *maIs135, RF742*: *lin-4(e912) II*; *mir-237(n4296) lin-14(n179ts) X*; *ctIs39, RF743*: *lin-4(e912) II*; *lin-14(n179ts) X*; *ctIs39, RF744*: *mir-237(n4296) lin-14(n179ts) X*; *ctIs39, RF745*: *lin-14(n179ts) X*; *ctIs39, DR441 lin-14(n179ts) X*, *DR721 lin-4(e912) II*, *VT573 lin-4(e912) II*, *lin-14(n179ts) X*, *VT808 maIs108*, *VT892 lin-4(e912) II*; *lin-14(n179ts) X*; *maIs108, VT1066 mir-48mir-241(nDf51) V*, *mir-84(n4037)*

X, *VT1113* *maIs135*, *VT1207* *lin-4(e912) II*; *mir-48* *mir-241(nDf51) V*; *lin-14(n179ts)* *mir-84(n4037) X*, *MT355* *lin-14(n355) X*.

Microscopy and phenotype analysis

Nomarski DIC and fluorescence microscopy were used to count seam cell nuclei and score adult lateral alae. Developmental stage was assessed by the extent of gonad and germ line development. When scoring percent animals with GFP fluorescence, the animal was positive if it had fluorescence in any seam cell, and no distinction was made in the fluorescence intensity. All images were taken with a ×100 objective on a Zeiss Axioplan2 microscope.

RNA interference

Bacterially mediated RNA interference (RNAi) was performed as previously described (**Timmons *et al.* 2001**). The RNAi vectors contained a 3.5-kb region of ***hbl-1*** genomic sequence or 740 bp of the ***lin-28*** ORF in the L440 vector backbone. ***hbl-1*** RNAi was administered by feeding postembryonically: gravid adults were dissected and embryos allowed to hatch on double strand RNA (dsRNA)-expressing bacteria. Bacteria were induced in culture and seeded on NGM plates containing 1 mM IPTG, 50 μg/ml ampicillin and 12.5 μg/ml tetracycline. Empty vector was used as a negative control. ***lin-28*** RNAi was administered by injection of dsRNA. A 950-bp fragment including the 740 bp of the ***lin-28*** ORF and T7 promoters was amplified using PCR from the ***lin-28*** RNAi vector. A total of 150 ng of this fragment was used for *in vitro* transcription in the MEGAscript T7 Transcription Kit (Ambion). The transcription reaction was purified using the MEGAclear Transcription Clean-Up Kit (Ambion). Purified dsRNA was injected into the gonad of L4 animals, and F_1's were scored for phenotype.

Protein extraction and Western blot

Synchronous cultures were prepared by bleaching 5–10 10-cm plates of gravid adults for each strain according to standard protocols to isolate unhatched eggs (**Hope 1999**). Eggs were hatched overnight (12–16 hr) in M9, and starved L1's were placed onto NGM plates seeded with AMA1004 *Escherichia coli* at 25°. Worms were collected at the L1 and L3 molts, when the majority of worms entered lethargus (the exact timing varied between lines and was confirmed visually in a dissection microscope). Worms were collected using chilled M9, and the resulting worm pellet was flash frozen in liquid nitrogen. Samples were stored at −80° until further processing. We found that the 2-ml RNase-Free Elution Tubes (Ambion, AM12480) produced the best worm pellets with the least loss due to worms sticking to the walls. Frozen pellets were ground in a mortar and pestle on dry ice, resuspended in an equal volume of RIPA buffer [150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% Tris pH 8.0, 1× complete protease inhibitor (Roche Diagnostics, Indianapolis, IN) and 1 mM PMSF], incubated for 30–60 min at 4°, and then centrifuged at top speed for 20 min at 4°. The resulting supernatant was transferred to a fresh tube, and protein estimation was performed using the Bradford assay (Bio-Rad, Hercules, CA).

A total of 20 μg of protein for each sample was used for Western blot. **LIN-28** antisera (**Seggerson *et al.* 2002**) was used at a 1:2000 dilution. Secondary antibody was HRP-conjugated donkey anti-rabbit Ig (Amersham, Piscataway, NJ), used at a 1:2000 dilution. As a gel loading control, mouse anti-actin

monoclonal antibody (MP Biomedicals) was used at 1:1000, and HRP-conjugated goat anti-mouse Ig (Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1:25,000.

RNA extraction and qRT-PCR assays

Synchronous cultures were prepared as describe above. For each strain, 200 lethargic animals were handpicked at the L1 and L3 molts. Animals were kept in 250 µl RNA*later*(Applied Biosystems, Foster City, CA) overnight at 4°, and then stored at −20° until further processing. RNA was extracted using the mirVana miRNA Isolation Kit (Ambion) following the total RNA isolation procedure. Isolated RNA was then treated with TURBO DNase (Ambion). All samples were analyzed on an Applied Biosystems 7500 Real-Time PCR System.

Primary miRNA transcripts:

A total of 300 ng RNA was used in the iScript cDNA Synthesis Kit (Bio-Rad). A total of 12.5 ng cDNA was used in each SYBR green (Applied Biosystems) reaction. Primers were designed with the forward primer 5′ of the Drosha cleavage site and reverse primer in the pre-miRNA stem (Supplemental Material, Table S1). *ama-1* was used for normalization. Dissociation/melting curves were determined after each run. Relative changes in pri-miRNA levels were determined by the ΔΔCt method using *ama-1* levels for normalization.

Mature miRNA transcripts:

The miRNA–qRT-PCR (Taqman Assay, Applied Biosystems) was performed using Taqman probes for *lin-4*, miR-48, miR-237, miR-241, and small nucleolar RNA (snRNA) sn2841 according to the manufacturer's instructions. Relative changes in mature miRNA levels were determined by the ΔΔCt method using sn2841 levels for normalization.

Data and reagent availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article. All raw data and reagents are available upon request.

Results

The 3let-7s act in the lin-14–lin-28 feedback loop

The 3let-7s act downstream of lin-14 to regulate L2 specific cell fates:

To assess the contribution of the *3let-7s* to *lin-28* regulation, we employed a genetic background that lacked functional *lin-4*, a known regulator of *lin-28*. *lin-4(e912)* single mutants reiterate L1 cell fates indefinitely and exhibit high constitutive expression of both LIN-14 and LIN-28 (Arasu *et al.* 1991; Moss *et al.* 1997). Loss-of-function alleles of *lin-14* suppress this severely retarded mutant, so that *lin-4(e912)*; *lin-14(n179ts)* animals progress through the larval stages to produce adult cuticle and show stage-specific down-regulation of LIN-28 (Ambros 1989; Moss *et al.* 1997).

The temporal progression of larval development in *C. elegans* may be followed through the divisions and differentiation of lateral hypodermal cells, called seam cells. These cells divide asymmetrically during each larval stage, producing a cell that joins the hypodermal syncytium and one that remains a seam cell. In the L2, six seam cells undergo a symmetric division, doubling their number. Therefore, WT animals are born with 10 seam cells per side, which increases to 16 in the L2 and stays at this number thereafter (see Figure S1A and Table 1, line 1, WT). By counting seam cells, we can assess whether the L2 stage was normal, skipped, or reiterated in heterochronic mutant combinations. In addition, all seam cells normally differentiate at the end of the L4, when they fuse and secrete a cuticle structure called alae (Table 1, line 1). Precocious development may be scored by determining whether adult alae formed early in the L3, or conversely, retarded development may be scored by determining whether alae failed to form in the L4.

Table 1 The *3let-7s* act downstream of *lin-14* in the heterochronic pathway

Average number of seam cells[a,b]			**Percentage of animals with alae formation at the L4 molt[a,c]**						
Line	**Strain**	**Genotype[d,e]**	**L1**	**L2**	**L3**	**L4**	**No alae (%)**	**Gapped (%)**	**Complete (%)**
1	RG733	WT	10.05	16	16	16	0	0	100
2	ME348	*lin-4(e912)*	—	—	—	11.5	100	0	0
3	ME349	*lin-4(e912)*; *lin-14(n179ts)*	—	—	—	41	37	63	0
4	ME349	*lin-4(e912)*; *lin-14(n179ts)*, 25°	—	—	—	21.8	0	60	40
5	ME357	*lin-4(e912)*; *mir-48 mir-241(nDf51)*; *lin-14(n179ts) mir-84(n4037)*	—	—	—	110.5[f]	100[f]	0[f]	0[f]
6	ME357	*lin-4(e912)*; *mir-48 mir-241(nDf51)*; *lin-14(n179ts) mir-84(n4037)*, 25°	—	—	—	132.4[f]	100[f]	0[f]	0[f]
7	ME358	*lin-4(e912)*; *mir-48 mir-241(nDf51)*; *lin-14(n179ts)*	—	—	—	100.4[f]	100[f]	0[f]	0[f]
8	ME359	*lin-4(e912)*; *lin-14(n179ts) mir-84(n4037)*	—	—	—	45	60[a]	40[a]	0[a]
9	ME359	*lin-4(e912)*; *lin-14(n179ts) mir-84(n4037)*, 25°	—	—	—	23.1	0	53	47

↵a Number of animals (*n*) scored for each genotype is at least 30.
↵b *P*-values determined using Student's *t*-test.
↵c *P*-values determined using Fisher's exact test.
↵d All strains contain *wIs78* (*ajm-1*::*GFP*, *SCMp*::*GFP*) to mark seam cells.

[e] Strains were maintained at 20° unless otherwise indicated.
[f] $P < 0.001$ compared to ME349 at corresponding temperature.
[g] $P < 0.05$ compared to ME349 at 20°.

lin-4(e912) animals display a retarded heterochronic phenotype: they make no adult alae and have fewer seam cells than WT (because they reiterate the L1 patterns and never undergo the symmetric division of the L2) (Table 1, lines 1 and 2) (Chalfie *et al.*1981). The *lin-14(n179ts)* allele is a recessive mutation in the protein coding region of the *lin-14* gene that causes a reduction in both activity and protein abundance (Ambros and Horvitz 1987; Ruvkun *et al.* 1989). This temperature-sensitive allele has more severe phenotypes at the restrictive temperature of 25° than at the permissive temperature of 20° (Ambros and Horvitz 1987). Accordingly, its suppressive effect in the *lin-4(e912)*; *lin-14(n179ts)* double mutant is stronger at 25°. *lin-4(e912)*; *lin-14(n179ts)* animals at 20° often produce some alae and a greater than WT number of seam cells (because they reiterate the symmetric division of the L2) (Table 1, line 3). At 25°, this strain makes nearly WT number of seam cells and all animals made at least some alae (Table 1, line 4).

In this *lin-4(e912)*; *lin-14(n179ts)* background, we removed the *3let-7s* and found strong enhancement of the retarded phenotype: a large increase in seam cell number and complete absence of adult alae (Table 1, lines 5 and 6) (Figure S1). This effect was primarily due to loss of *mir-48* and *mir-241* (Table 1, line 7). In contrast, the loss of *mir-84* alone did not cause any significant change to seam cell number, although it did enhance the alae defect at 20° (Table 1, compare lines 3 and 4 to lines 8 and 9, $P > 0.05$). Thus, removal of the *3let-7s* appeared to enhance the reiteration of L2 cell fates in a strain lacking *lin-4*.

Previous studies investigating temporal control of seam cell development have noted high seam cell numbers in retarded mutants that reiterate L2 cell fates. However, the number of seam cells observed in *lin-4(e912)*; *lin-14(n179ts)*; *3let-7s(0)* animals was remarkably high compared to such mutants (Moss *et al.* 1997; Abbott *et al.* 2005). If the L2 double cell division were simply reiterated during the L3 and L4, one would expect ~46 seam cells at the end of the L4 (Figure S1). However, this strain produced on average 110.5 seam cells by the end of the L4, with a range between 52 and 166 seam cells. To understand this better, we followed worms in this strain from hatching until adulthood. We found that they executed L2 cell fates during the L1, when they had on average 15.7 seam cells (compared to 10 in the WT) (Figure S1B). This precocious execution of L2 is due to the loss of *lin-14* function at 25° in *lin-14(n179ts)* animals (Ambros and Horvitz 1987). We observed that they continued to execute L2 cell fates during each larval stage through the L4 (Figure S1, A and B). However, several animals underwent a complete second round of double cell divisions during the L4 and sometimes earlier in the L3. It is also notable that the cells appeared in clusters rather than along the lateral midline as is typical of seam cells (Figure S1C). We conclude that the L2 fates are indeed reiterated in this strain, but we have no explanation for the additional seam cells from late stage extra divisions.

lin-28 and hbl-1 act downstream of the 3let-7s:

The above genetic data show that the *3let-7s* act downstream of *lin-14* to regulate L2 cell fates. Since *lin-28* plays a critical role in the specification of L2 fates, the simplest explanation is that the *3let-7s* act in the *lin-14*–*lin-28* feedback loop, and might even comprise the pathway by which *lin-14* positively regulates *lin-28*. If so, then the severely retarded phenotypes seen in *lin-4(e912)*; *lin-14(n179ts)*; *3let-7s(0)* animals might be due to an up-regulation of *lin-28* expression, causing the reiteration of L2 fates.

To determine whether *lin-28* is required for the retarded phenotype of animals lacking *lin-4* and the *3let-7s*, we knocked down *lin-28* by RNAi. When WT animals are injected with *lin-28* RNAi, they exhibit a precocious phenotype, in which the L2 stage is skipped, seam cell numbers are reduced, and precocious alae form at the end of the L3 stage (Table 2, line 3). When *lin-4(e912)*; *lin-14(n179ts)*; *3let-7s(0)* animals were injected with *lin-28* RNAi, they displayed a similar precocious phenotype with reduced seam cell numbers and alae formation at the end of the L3 stage (Table 2, line 4). Therefore, the *lin-28*(RNAi) phenotype is completely epistatic to the retarded phenotype of *lin-4(e912)*; *lin-14(n179ts)*; *3let-7s(0)* animals. This result is consistent with the *3let-7s* acting upstream of *lin-28* to inhibit its activity.

Table 2 *lin-28* and *hbl-1* are genetically downstream of the *3let-7s*

			Average seam cell number[a,b]		***Precocious alae[a,d,g]***	***Adult alae[c,d]***			***Percentage of animals with alae formation***
Line	***Strain***	***Genotype/ treatment[e,f]***	***L3***	***L4***		***No alae***	***Gapped***	***Complete***	***N***
1	*RG733*	*WT*	*16(6)*	*16(16)*	*0(6)*	*0*	*0*	*100*	*12*
2	*ME357*	*lin-4(e912); mir-48 mir-241(nDf51); lin-14(n179ts) mir-84(n4037)*	*—*	*110.5(30)*	*—*	*100*	*0*	*0*	*30*
3	*RG733*	*lin-28(RNAi)*	*11.5(12)*	*11.5(30)*	*100(19)*	*—*	*—*	*—*	*—*
4	*ME357*	*lin-4(e912); mir-48 mir-241(nDf51); lin-14(n179ts) mir-84(n4037); lin-28(RNAi)*	*11(12)[h]*	*11.3(20)[h]*	*100(7)[h]*	*—*	*—*	*—*	*—*
5	*RG733*	*hbl-1(RNAi)*	*13.6(61)*	*15.8(59)*	*98(51)*	*—*	*—*	*—*	*—*
6	*ME357*	*lin-4(e912); mir-48 mir-241(nDf51); lin-14(n179ts) mir-84(n4037); hbl-1(RNAi)*	*18.4(45)[i]*	*28.4(78)[i,i]*	*2 (61)[k]*	*41.2[i]*	*41.2[i]*	*17.6[i]*	*68*

[a] Number of animals (*n*) scored for each genotype is indicated in parenthesis.

↲b *P*-values determined using Student's *t*-test.
↲c Animals were scored at the end of the L4 stage for both seam cells and alae formation.
↲d *P*-values determined using Fisher's exact test.
↲e All strains contain *wIs78* (*ajm-1*::*GFP*, *SCMp*::*GFP*) to mark seam cells.
↲f Strains were maintained at 20°.
↲g Animals were scored at the beginning of the L4 stage for precocious alae formation.
↲h $P > 0.05$ compared to RG733 treated with *lin-28* RNAi at the corresponding stage.
↲i $P < 0.001$ compared to RG733 treated with *hbl-1* RNAi at the corresponding stage.
↲j $P < 0.001$ compared to ME357-untreated L4 animals.
↲k $P > 0.05$ compared to RG733 treated with *hbl-1* RNAi at the corresponding stage.

Since *lin-28* is suspected to act upstream of *hbl-1* in the heterochronic pathway (Vadla *et al.* 2012), and *hbl-1* is a known target of the *3let-7s* (Abbott *et al.* 2005), we wanted to know whether removing *hbl-1* could also suppress the retarded phenotype in *lin-4(e912)*; *lin-14(n179ts)*; *3let-7s(0)* animals. Again, we assayed this by knocking down *hbl-1*using RNAi. Like *lin-28* RNAi, WT animals fed *hbl-1* RNAi display a precocious phenotype, in which the L2 stage is skipped, causing reduced seam cell numbers and precocious alae formation at the end of the L3 (Table 2, line 5) (Fay *et al.* 1999; Abrahante *et al.*2003; Lin *et al.* 2003). When *lin-4(e912)*; *lin-14(n179ts)*; *3let-7s(0)* animals were fed *hbl-1*RNAi, they displayed a similar precocious phenotype of reduced seam cell numbers at the end of the L3, although the strength of this suppression was less than with the *lin-28* RNAi (Table 2, compare lines 4 and 6). However, they did not form alae at the end of the L3 stage (Table 2, line 6). Instead, *hbl-1* RNAi treated animals formed alae at the end of the L4 stage, which is never seen in untreated *lin-4(e912)*; *lin-14(n179ts)*; *3let-7s(0)*animals (Table 2, compare lines 2 and 6). These data suggested that the retarded phenotype seen in *lin-4(e912)*; *lin-14(n179ts)*; *3let-7s(0)* animals is caused, at least in part, by the misregulation of *hbl-1*.

LIN-28 is constitutively expressed in the absence of the 3let-7s:

Since the retarded phenotype seen in *lin-4(e912)*; *lin-14(n179ts)*; *3let-7s(0)* animals required *lin-28*, we assayed for changes in LIN-28 expression directly by immunoblot (Figure 1). In WT animals, LIN-28 is highly expressed upon hatching and during the L1 stage, and expression decreases throughout development, so that by the L3 it reduced 10- to 20-fold (Figure 1, lanes 1 and 2) (Seggerson *et al.* 2002). *lin-4(e912)* single mutants express high levels of LIN-28 late into development, but this effect is reversed by a temperature-sensitive mutation in *lin-14* grown at the restrictive temperature (Figure 1, lanes 3–6). Removal of the *3let-7s* alone does not increase LIN-28 expression late in development (Figure 1, lanes 7 and 8). However, the loss of both *lin-4* and the *3let-7s*caused LIN-28 to be expressed late in development, despite the reduction in *lin-14*activity (Figure 1, lanes 9 and 10).

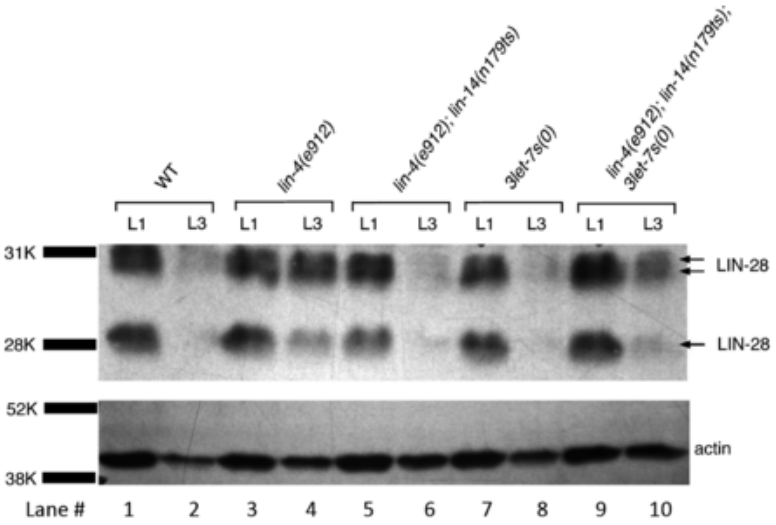

Figure 1. The 3let-7s repress LIN-28 expression. Representative Western blot shows anti-LIN-28 antisera at the L1 and L3 molts in different strain backgrounds grown at 25°. The antisera recognize three LIN-28 isoforms: a doublet at ~36 kDa and a single band at 28 kDa, which are the products of alternative splicing and post-translational modification (Seggerson *et al.* 2002). Actin was used as a loading control. *3let-7s(0)* represents the *mir-48 mir-241(nDf51)*; *mir-84(n4037)* triple mutant.

Previously, a *lin-28*::*GFP* reporter was used to monitor *lin-28* expression and regulation (Moss *et al.* 1997; Abbott *et al.* 2005). This reporter reflects *lin-28*'s temporal down-regulation from L1 to L3 (Table 3, line 1). When *lin-4* is removed, the reporter shows high *lin-28* expression late in development (Moss *et al.* 1997), but when *lin-14* activity is also reduced, the normal temporal regulation is restored (Table 3, line 2). When only the *3let-7s* are removed, no reduction in reporter expression occurs (Abbott *et al.* 2005). However, when we removed the *3let-7s* in a *lin-4(e912)*; *lin-14(n179ts)* mutant background, we observed late expression of the *lin-28*::*GFP* reporter: 100% of *lin-4(e912)*; *lin-14(n179ts)*; *3let-7s(0)* animals expressed *lin-28*::*GFP* during the L3 compared to 0% of WT or *lin-4(e912)*; *lin-14(n179ts)* at the same stage (Table 3, compare L3 values). Taken together, these data show that the *3let-7s* are required for the repression of *lin-28* that occurs when *lin-14* activity is reduced.

Table 3 The *3let-7s* repress expression of *lin-28*::*GFP*

		% of animals expressing *lin-28*::*GFP* in hypodermis[a,b]	
Line	**Genotype[c]**	**L1**	**L3**
1	WT	100	0
2	*lin-4(e912)*; *lin-14(n179ts)*	100	0
3	*lin-4(e912)*; *lin-14(n179ts)*; *3let-7s(0)*	100	100[d]

↵a Individual animals were scored for the presence of GFP expression in the hypodermis.
↵b Number of animals scored for each time point (*n*) is at least 30.
↵c All strains were grown at 25°.
↵d $P < 0.001$ compared to WT at the corresponding stage. *P*-value determined using Fisher's exact test.

The lin-4 family member mir-237 functions in the heterochronic pathway

mir-237 is not individually required for developmental timing, but loss of mir-237 enhances and suppresses timing defects of other heterochronic mutants:

Because *lin-28*'s 3′ UTR contains a *lin-4* binding site, it is possible that it could be bound by other miRNAs that are related to *lin-4*. *C. elegans* encodes one other *lin-4* family member, *mir-237* (Lim *et al.* 2003). To determine whether *mir-237* has a role in the heterochronic pathway, we first analyzed *mir-237(n4296)* mutant worms. *n4296* is a null allele which deletes the entire *mir-237* gene, but does not affect viability or cause gross abnormalities in the worm (Miska *et al.* 2007). Animals mutant for *mir-237* alone do not differ from WT animals in seam cell number or alae formation (compare Table 4, line 1 and Table 1, line 1).

Table 4 *mir-237* acts downstream from *lin-14* in the heterochronic pathway

			Average number of seam cells[a,c]				Percentage of animals with alae formation[a,b]					
							L3 molt			L4 molt		
Line	**Strain**	**Genotype[d,e]**	**L1**	**L2**	**L3**	**L4**	**No alae (%)**	**Gapped (%)**	**Complete (%)**	**No alae (%)**	**Gapped (%)**	**Complete (%)**
1	RF455	*mir-237(n4296)*	10.15	16	16	16.05	100	0	0	0	0	100
2	RF477	*lin-14(n179ts)*	—	—	15.9	15.6	0	5	95	—	—	—
3	RF478	*mir-237(n4296) lin-14(n179ts)*	—	—	15.55	15.9	0[f]	40[f]	60[f]	—	—	—
4	ME368	*lin-4(e912)*; *lin-14(n179ts), 25°*	—	—	–	19.8	—	—	—	0	10	90
5	ME389	*lin-4(e912)*; *mir-237(n4296)lin-14(n179ts), 25°*	—	—	–	23.3[g]	—	—	—	40[g]	26.7[g]	33.3[g]
6	RF303	*lin-46(ma164), 15°*	—	—	18.3	18.2	—	—	—	0	100	0
7	RF304	*lin-46(ma164); mir-237(n4296), 15°*	—	—	23.1[g]	22.4[g]	—	—	—	5	90	5

8	RF492	*lin-28(n719)*; *lin-46(ma164)*	—	—	15.9	16.1	—	—	—	0	0	100
9	RF493	*lin-28(n719)*; *lin-46(ma164)*; *mir-237(n4296)*	—	—	16.1[f]	16.2	—	—	—	0	0	100
10	RF204	*lin-28(n719)*	10	10	11.4	11.1	0	18	82	—	—	—
11	RF205	*lin-28(n719)*; *mir-237(n4296)*	10	10	11	10.9	6	6	88	—	—	—

↲a Number of animals (*n*) scored for each genotype is >20.
↲b *P*-values determined using Fisher's exact test.
↲c *P*-values determined using Student's *t*-test.
↲d All strains contain *wIs78* (*ajm-1*::*GFP*, *SCMp*::*GFP*) to mark seam cells.
↲e Strains were maintained at 20° unless otherwise indicated.
↲f $P < 0.05$ compared to line above.
↲g $P < 0.001$ compared to line above.

However, many miRNAs act redundantly within genetic pathways (Abbott et al. 2005; Miska et al. 2007), prompting us to explore whether mir-237 might act on lin-4 targets in the heterochronic pathway in concert with other miRNAs. We examined many genetic relationships (Table S2) and found two where mir-237(n4296) either enhanced or suppressed a heterochronic phenotype (Table 4). We found that loss of mir-237 suppressed the lin-14(n179ts) precocious alae phenotype (Table 4, lines 2 and 3). Consistent with this result, it also enhanced the retarded seam cell number and alae defect of lin-4(e912); lin-14(n179ts) (Table 4, lines 4 and 5). Together, these data suggest that mir-237 acts downstream of lin-14 to promote later cell fates.

We further explored mir-237's relationship with the heterochronic pathway by testing its relationship to lin-28 and lin-46. lin-46 encodes a putative protein–protein interaction factor whose role in the heterochronic pathway is still poorly understood; previous work in our lab showed that lin-46 acts at the same step as lin-28, but in a nonlinear fashion, to promote L3 cell fates (Pepper et al. 2004). Accordingly, lin-46(ma164) mutants reiterate the L2, causing an increased seam cell number, and form gapped alae at the end of the L4 (Table 4, line 6) (Pepper et al. 2004). We found that loss of mir-237 enhances the lin-46 extra seam cell phenotype (Table 4, compare lines 6 and 7). This result suggests that mir-237 is acting at the same step as lin-46 in the heterochronic pathway. To further investigate mir-237's relationship to lin-46, we utilized lin-28(n719); lin-46(ma164) double mutant animals. Mutations in both lin-28 and lin-46 cause mutual suppression of their respective heterochronic defects, making lin-28(n719); lin-46(ma164) double mutants nearly WT (compare Table 1, line 1, to Table 4, line 8) (Pepper et al. 2004). In this background, loss of mir-237 caused no change in seam cell number at the L4 or in alae formation (Table 4, line 9). Furthermore, loss of mir-237 had no effect on the precocious phenotype seen in lin-28(n719) single mutants (Table 4, compare lines 10 and 11), making lin-28(n719) completely epistatic. Taken together, these results suggest that mir-237 regulates cell fates in the L2 and that it may act upstream of lin-28.

mir-237 regulates the expression of hbl-1:

To confirm that *mir-237* regulates L2 cell fates, we investigated the effect of loss of *mir-237* on the expression of *hbl-1* using a GFP reporter. *hbl-1* contains *lin-4* family binding sites in its 3' UTR, making it a potential direct target of *mir-237* (Abrahante *et al.* 2003; Lin *et al.* 2003). *hbl-1* also appears to be the most proximal regulator of L2 cell fates, making its expression a valuable tool for studying this larval stage (Abbott *et al.* 2005; Karp and Ambros 2012; Vadla *et al.* 2012). During WT development, *hbl-1*::*GFP* is highly expressed in the hypodermis upon hatching and throughout the L1 (Figure 2, bar 1), but is down-regulated as development proceeds, becoming undetectable by the L3 (Fay *et al.* 1999; Abrahante *et al.* 2003). In *lin-14(n179ts)* animals, *hbl-1*::*GFP* is precociously down-regulated, such that only 42% of animals express the reporter during the L1 (Figure 2, bar 2). We found that 80% of *lin-4(e912)*; *lin-14(n179ts)* and 74% of *mir-237(n4296)* *lin-14(n179ts)* animals expressed *hbl-1*::*GFP* during the L1 (Figure 2, bars 3 and 4). When both *lin-4* and *mir-237* are removed, 90% of animals expressed the reporter during the L1 in the *lin-14(n179ts)* background (Figure 2, bar 5). Taken together, these data suggest that *mir-237* acts upstream of *hbl-1* to block its expression. Additionally, this effect may be additive with its other family member, *lin-4*.

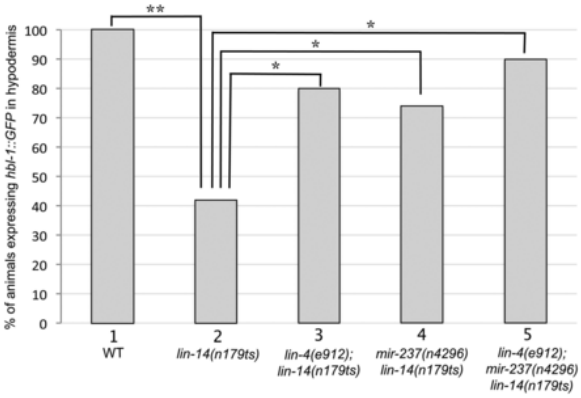

Figure 2. miR-237 represses expression of downstream targets. Bar graph depicts the percentage of animals expressing the *hbl-1*::*GFP* reporter during the L1. Individual worms were scored for the presence of GFP expression in the hypodermis. All experiments were performed at 20°. * $P < 0.05$, ** $P < 0.001$. *P*-values were determined using Fisher's exact test, $n \geq 20$ for each time point.

LIN-14 negatively regulates the 3let-7s and mir-237

The promoter regions of the 3let-7s and mir-237 contain putative LIN-14 binding sites:

lin-14 encodes a sequence-specific DNA-binding transcription factor (Hristova *et al.*2005). However, previous reports had not identified any targets for LIN-14 that regulate the timing of cell fates. Since our data indicated that the *3let-7s* and *mir-237* act genetically downstream of *lin-14*, we asked whether they could be direct transcriptional targets of LIN-14.

A previous study identified a short consensus sequence for LIN-14 binding, GAAC(RY) (Hristova *et al.* 2005). We first searched 2 kb upstream of each miRNA for this site and found numerous potential binding sites. We then determined which of these sites were conserved between *C. elegans* and *C. remanei*, and identified several promising LIN-14binding sites in each promoter (Figure S2). The presence of these sites within conserved promoter regions, combined with our genetic results, made these miRNAs candidates for direct regulation by LIN-14.

LIN-14 negatively regulates the abundance of miR-48, miR-241 and miR-237:

To determine whether LIN-14 controls the abundance of miR-48, miR-241, and miR-237, we measured levels of the mature form of each miRNA using qRT-PCR in wild-type (WT) and mutant animals. In WT animals, the three miRNAs were expressed at very low levels during the L1, when LIN-14 is normally expressed, and increased in abundance 20- to 30-fold by the L3 when LIN-14 is down-regulated (Figure 3, A–C, WT). In two different strains in which LIN-14 was constitutively expressed, *lin-14(n355)* and *lin-4(e912)*, this sharp increase in abundance in the L3 was not seen (Figure 3, A–C, compare WT and mutant L3 bars). Rather, the relative amount of each miRNA was significantly lower in the L3 in these strains compared to WT ($P < 0.01$). In a strain where LIN-14 activity was reduced, *lin-14(n179ts)* at 25°, each miRNA was expressed precociously, between 8- and 15-fold higher in the L1 compared to WT [Figure 3, A–C, compare WT and *lin-14(n179ts)*L1 bars]. Taken together, these data reveal an inverse correlation between the presence of a functional LIN-14 and the abundance of miR-48, miR-241, and miR-237. This correlation is specific to these miRNAs, as mutations in *lin-14* do not affect the abundance of another miRNA in the heterochronic pathway, *lin-4* (Figure 3D).

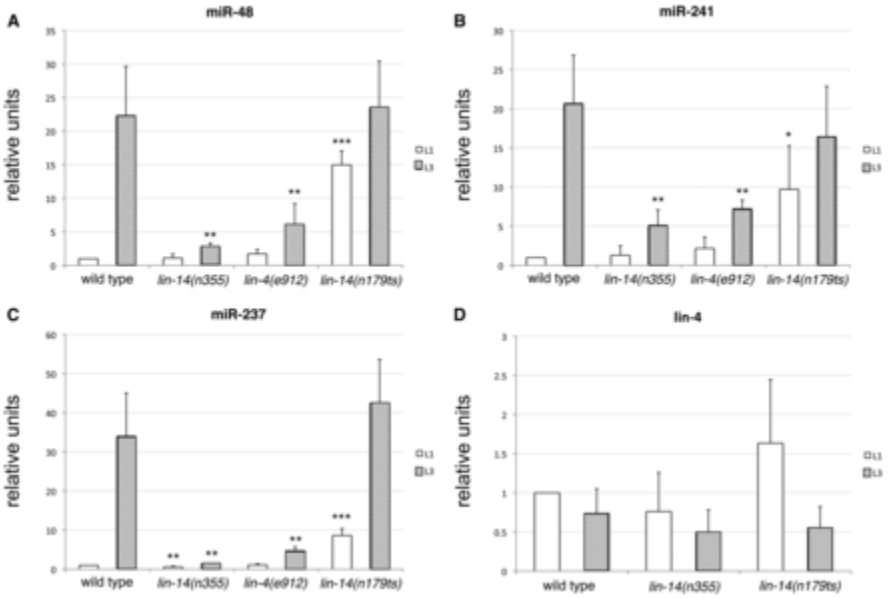

Figure 3. LIN-14 inhibits the expression of miR-48, miR-241, and miR-237. Bar graphs depict relative abundance of mature miRNAs at the L1 and L3 molts, determined using Taqman qRT-PCR. Fold changes are relative to WT L1M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to WT at the corresponding stage. *P*-values were determined using Student's *t*-test, on at least three biological replicates. Mature *lin-4* was measured as a negative control. Animals were grown at 25°.

LIN-14 mutations affect the timing of expression of the 3let-7s and mir-237 transcriptional reporters:

As a proxy for measuring the abundance of the primary transcripts of the *3let-7s* and *mir-237*, we utilized a set of *Pmir*::*GFP* reporters (Martinez *et al.* 2008). These reporter fusions contain the promoter region of each miRNA and the coding region of GFP. As previously reported for WT animals, each

reporter was undetectable in the hypodermis and seam cells during the L1, but abundantly expressed during the L3 (Figure 4, A–D, WT).

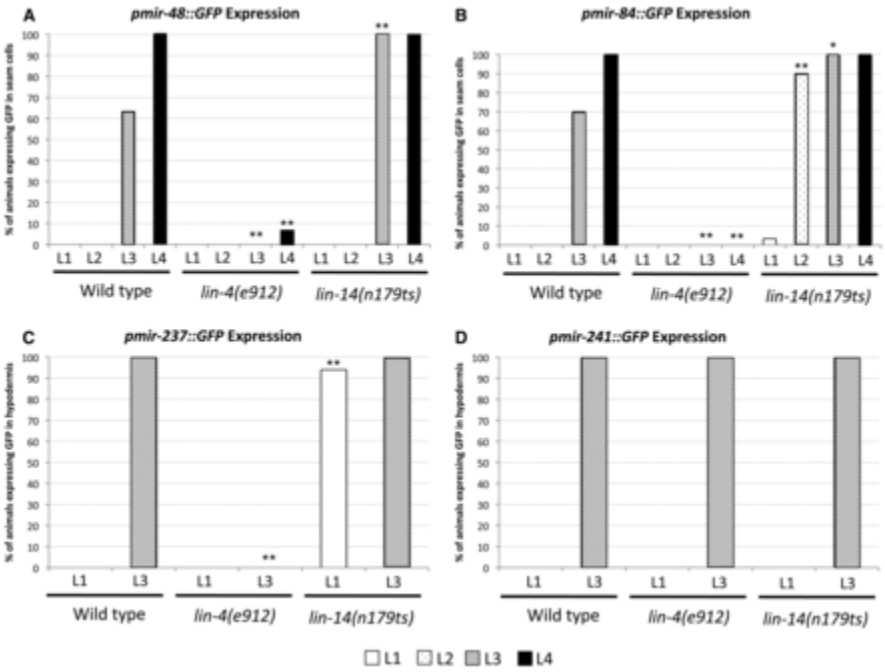

Figure 4. LIN-14 inhibits the expression of the 3let-7s and mir-237 GFP reporters. Bar graphs show the percentage of animals expressing each *Pmir*::*GFP* construct at the indicated larval stages. Animals in A, B, and D were grown at 25°, and animals in C were grown at 20°. Individual worms were scored for the presence of GFP expression in the hypodermis and/or seam cells. * $P < 0.01$, ** $P < 0.001$ compared to WT at the corresponding stage. *P*-values were determined using Fisher's exact test, $n \geq 20$ for each time point.

To test the effect of constitutive expression of *lin-14* on the expression of each miRNA, we utilized the *lin-4(e912)* mutant. In contrast to WT animals, where expression begins in the L3, 0% of the *lin-4(e912)* L3 animals expressed the *mir-48*, *mir-84*, or *mir-237* GFP reporters (Figure 4, A–C). Even during the L4, where 100% of WT animals expressed the *mir-48* and *mir-84* GFP reporters, only 6% of *lin-4(e912)* mutants expressed *Pmir-48*::*GFP*, and 0% expressed *Pmir-84*::*GFP* (Figure 4, A and B). These data strongly suggest that LIN-14 negatively regulates the expression of *mir-48*, *mir-84*, and *mir-237* at the level of transcription.

We also tested whether loss of *lin-14* would affect the expression of these miRNAs. Indeed, the *mir-48*, *mir-84*, or *mir-237* GFP reporters were all expressed precociously in *lin-14(n179ts)* mutants compared to WT animals (Figure 4, A–C). These results further demonstrate LIN-14's strong inhibitory effect on the transcription of *mir-48*, *mir-84*, or *mir-237*.

Surprisingly, the expression of *Pmir-241*::*GFP* was unaffected by the constitutive expression or absence of *lin-14*. This was unexpected, given that the abundance of mature miRNA was dramatically altered in these backgrounds compared to WT (Figure 4D). There is currently no defined promoter sequence for *mir-241*, so it is possible that there are regulatory regions located outside of the sequence contained in the *Pmir-241*::*GFP* reporter.

Discussion

For many years we have known that *lin-14* positively regulates *lin-28*, but the mechanism underlying this regulation was unknown. Here, we show that *lin-14* inhibits the expression of two families of miRNAs, a subset of which in turn negatively regulates *lin-28* (Figure 5). We believe that the structure of this regulation ensures a robust transition from L1- to L2-specific cell fates in all cells at the appropriate time.

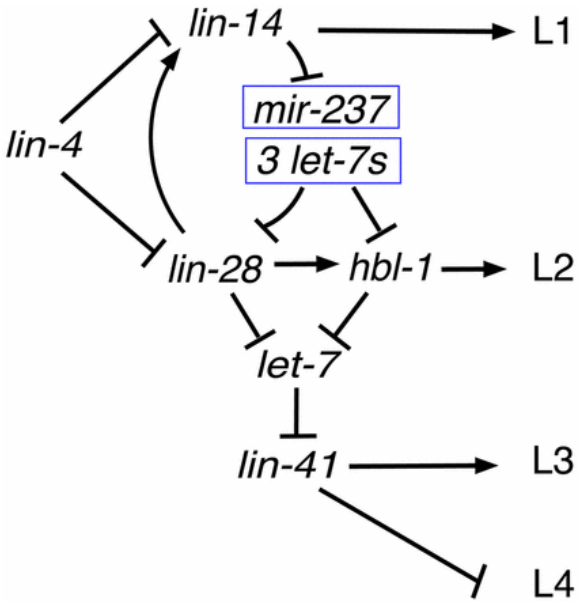

Figure 5. A model for the heterochronic pathway. Depicted are the genetic relationships among key regulators controlling the early- and late-stage phenotypes discussed. The miRNAs whose positions in the pathway are defined here are boxed.

The discovery that the *3let-7s* acted redundantly to regulate L2 cell fates presented the first promising candidates for *lin-28* regulation (Abbott *et al.* 2005). The 3' UTR of *lin-28*contains at least one putative *let-7* family binding site, and these three miRNAs were the first *let-7* family members known to act early enough in development to regulate *lin-28*. Indeed, by genetic epistasis analysis, Abbott *et al.* (2005) showed that the *3let-7s* act upstream of *lin-28*. However, they did not observe any effect on *lin-28* expression in a *3let-7s* mutant. We believe they did not see an effect because *lin-4* (and *mir-237*) were still present, allowing *lin-28* to be down-regulated, even in the absence of the *3let-7s*.

By utilizing a genetic background that lacks *lin-4*, we have revealed that the *3let-7s* do regulate *lin-28* expression (Table 1). By removing *lin-4* and *lin-14*, the only genes regulating *lin-28* were its additional regulators. We hypothesized that these regulators were the *3let-7s*, and their removal produced extremely retarded animals. We further confirmed that this retarded phenotype resulted from increased *lin-28*. LIN-28 was highly expressed late into development as measured by Western blot and *lin-28*::*GFP*reporter.

Additionally, *lin-28* expression was required, as removing it by RNAi completely suppressed the retarded phenotype. Interestingly, while *lin-28* RNAi was completely epistatic (the worms were entirely precocious), *hbl-1* RNAi only partially so (the worms were still slightly retarded). While this could be due to differing efficacies of the RNAi, it could also give insight into the relationship between *lin-28* and *hbl-*

1. A number of small- and large-scale studies have been performed in *C. elegans* and other systems to identify *lin-28* targets when it acts independently of *let-7* (Polesskaya *et al.* 2007; Xu and Huang 2009; Xu *et al.* 2009; Balzer *et al.* 2010; Qiu *et al.* 2010; Peng *et al.* 2011; Cho *et al.* 2012; Wilbert *et al.* 2012; Hafner *et al.* 2013; Stefani *et al.* 2015; Yang *et al.* 2015). Vadla *et al.*(2012) identified *hbl-1* as a downstream target by monitoring *hbl-1* reporter expression in *lin-28* mutants. While removing *hbl-1* could suppress most of the retarded phenotype of *lin-28* overexpression in the experiments reported here, it could not do it completely. This suggests that *lin-28* may have other targets that also promote L2 cell fates. Which of the thousands of predicted targets are biologically relevant to *lin-28*'s let-7-independent activity remains to be determined.

We also used this background lacking *lin-4* and *lin-14* to identify *mir-237* as an additional regulator of early cell fates. We found that the *mir-237* mutant suppressed the precocious alae phenotype of *lin-14* loss-of-function animals and enhanced the retarded phenotypes of *lin-4(e912)*; *lin-14(n179ts)*. Also, we were able to reveal that *mir-237* regulated *hbl-1* by following *hbl-1*::*GFP* expression in different *mir-237* mutants. Whether this regulation is direct or indirect, possibly through regulation of *lin-28*, remains unknown.

This work is also the first to identify biologically relevant targets for *lin-14* in the heterochronic pathway. Previous reports found LIN-14 was a nuclear localized protein, with little to no sequence similarity to other known proteins (Ruvkun and Giusto 1989; Wightman *et al.* 1991). Hristova *et al.* (2005) reported that *lin-14* encoded a sequence-specific, DNA-binding transcription factor. However, the target they focused on was the *ins-33* insulin-like growth factor, which is not implicated in developmental timing (Hristova *et al.* 2005). Here, we provide genetic evidence that the *3let-7s* and *mir-237* act downstream of *lin-14*. Furthermore, LIN-14 activity directly affects the abundance of their mature transcripts and the expression of their corresponding transcriptional reporters. In the case of *mir-241*, the relationship was less clear. Although the mature miRNA was strongly affected by *lin-14* activity, the transcriptional reporter was not. It is possible that the transcriptional reporter lacked the relevant sequences/structural signals for *lin-14* binding and regulation. It is also possible that *lin-14* utilizes a mechanism we do not yet understand, possibly acting on the RNA to decrease stability, rather than as a traditional transcription factor. In any case, the molecular details of LIN-14 protein's activity remained to be resolved.

This work also provides a possible explanation for the unusual nature of *lin-4* mutants. miRNAs are relatively weak effectors of protein abundance, and as such, very few have discernible effects when mutated on their own (Miska *et al.* 2007). *lin-4* has always been an exception to this paradigm, having profound effects on the course of the worm's developmental timeline, as well as causing 10- to 20-fold effects on the protein levels of its targets (Olsen and Ambros 1999; Seggerson *et al.* 2002). This work has uncovered the reason for this unusually catastrophic phenotype: removing *lin-4* effectively removes five miRNAs from two different miRNA families from the heterochronic pathway. When *lin-4* is mutated, *lin-14* is not down-regulated, meaning it continues repressing the expression of the *3let-7s* and *mir-237* long after the L1 is over. The combined loss of *lin-4*, the *3let-7s*, and *mir-237* is profound, committing the worm to executing L1-specific fates indefinitely.

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Footnotes

- Supplemental material is available online at www.genetics.org/lookup/suppl/doi:10.1534/genetics.116.195040/-/DC1.
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- Received August 19, 2016.
- Accepted October 20, 2016.
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