Misexpression of the *Caenorhabditis elegans* miRNA *let-7* Is Sufficient to Drive Developmental Programs

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The *Caenorhabditis elegans* microRNAs (miRNAs) *lin-4* and *let-7* promote transitions between stage-specific events in development by down-regulating the translation of their target genes. Expression of *let-7* is required at the fourth larval stage for the proper transition from larval to differentiated, adult fates in the hypodermis; however, it was not known whether expression of *let-7* is sufficient to specify these adult fates. To test this, we created fusion genes between *lin-4* and *let-7* that direct the expression of *let-7* two stages early, at the L2 stage. We find that animals bearing the fusion genes show precocious adult development at the L4 stage, indicating that temporal misexpression of *let-7* is sufficient to direct the larval-to-adult transition. Additionally, an RNA interference (RNAi)-based screen for enhancers of the precocious phenotype identified the *period* ortholog *lin-42*, among other genes, which are candidate modulators of the effects of *let-7* expression. *let-7* is conserved throughout bilaterian phylogeny, and orthologs of its targets have roles in vertebrate development, suggesting the importance of understanding how *let-7* promotes terminal differentiation in *C. elegans* and other organisms.

The first two miRNAs discovered, *lin-4* and *let-7*, regulate the timing of certain developmental events that occur in particular stages of *C. elegans* development. *lin-4* is required for the transition from L1 to L2 and from L2 to L3 developmental programs in the hypodermal seam cells, among other tissues. Animals lacking *lin-4* reiterate in each larval stage and, at the nominal adult stage, seam cell division patterns that are normally confined to the L1 stage (Chalfie et al. 1981; Ambros and Horvitz 1984). *lin-4* promotes the normal progression through developmental programs by down-regulating translation of its target genes, *lin-14* and *lin-28* (Lee et al. 1993; Wightman et al. 1993; Moss et al. 1997). Inactivation of the *lin-4* targets causes the opposite heterochronic phenotype as loss of *lin-4*: Developmental programs appropriate to later stages occur precociously.

Similarly, *let-7* acts to trigger a transition from larval to adult patterns of development. In *let-7* mutants, the hypodermal seam cells continue to divide in adults, rather than fusing and secreting the adult-specific cuticular structure known as alae (Reinhart et al. 2000). *let-7* acts by inhibiting the translation of its targets *lin-41* and *hbl-1* (Slack et al. 2000; Abrahante et al. 2003; Lin et al. 2003), and down-regulation of these targets in turn allows synthesis of the transcription factor encoded by *lin-29* (Rougvie and Ambros 1995), the most downstream gene discovered in the heterochronic pathway, the set of miRNAs and their protein-coding targets that regulate developmental timing (Ambros and Horvitz 1984). At the larval-to-adult transition, LIN-29 directs expression of genes (such as collagens) involved in synthesis of the adult cuticle and inhibits transcription of those required for synthesis of larval cuticle (Liu et al. 1995; Rougvie and Ambros 1995). Recently, *let-7* was shown to target additional genes at the larval-to-adult transition, including the gene encoding the nuclear hormone receptor DAF-12 as well as several other transcription factors (Grosshans et al. 2005).

Although a number of other genes that regulate developmental timing in *C. elegans* have been identified, it remains unclear how transitions between developmental programs following the L1 stage are promoted. Work on the *let-7* paralogs *mir-48*, *mir-84*, and *mir-241* has suggested that they have a role in the transition to L3 developmental programs (Abbott et al. 2005; Hayes 2005; Li et al. 2005). It was not clear whether any outputs of the heterochronic pathway that are independent of *let-7* expression are required to promote the larval-to-adult transition.

Understanding how *let-7* promotes the transition to terminal differentiation in *C. elegans* is important because of the roles that *let-7* has in many animals. *let-7* is perfectly conserved throughout bilaterian phylogeny, and roles for its target, *lin-41*, has also been identified in vertebrate development (Pasquinelli et al. 2000; Kloosterman et al. 2004; Lancman et al. 2005; Schulman et al. 2005). Analogously to its role in *C. elegans*, up-regulation of *let-7* is associated with transition from the larval to pupal stage in *Drosophila* (Sempere et al. 2002, 2003; Bashirullah et al. 2003). Reduced expression of *let-7* has also been observed in a variety of tumors, suggesting it may have a broader role in promoting terminal differentiation (Takamizawa et al. 2004; Johnson et al. 2005; Karube et al. 2005).

Here, we show that precocious expression of *let-7* under the control of the *lin-4* promoter is sufficient to drive premature execution of adult developmental programs. At the fourth larval stage, animals carrying a *lin-4::let-7* fusion gene show precocious adult differentiation of the hypodermis that is dependent on *lin-29* activity. We also report results of an RNAi-based screen to identify enhancers of the precocious phenotype that results from early *let-7* expression. Inactivation of certain genes involved in developmental timing and RNA metabolism causes an enhanced precocious phenotype in animals bearing the *lin-4::let-7* fusion gene.

SECTION THEMES
miRNA Fusion Genes

To address whether expression of the miRNA *let-7* is sufficient to direct the larval-to-adult transition in the lateral hypodermal seam cells of *C. elegans*, we expressed *let-7* in the first larval stage, two stages earlier than it is normally expressed. We designed two constructs, both placing the *let-7* precursor and downstream sequence immediately 3' to the putative *lin-4* promoter and precursor (Fig. 1A). We chose to use the *lin-4* promoter because mutations in *lin-4* and *let-7* affect the same tissues, suggesting that they are expressed in the same places. In addition, although it has now been shown that miRNA-coding genes are transcribed by RNA polymerase II (Cai et al. 2004; Lee et al. 2004), it was not originally clear how

miRNA expression is controlled. We included the *lin-4* precursor because of the possibility that elements regulating *lin-4* transcription could be embedded in the precursor sequence. Construct A (*ExA*) included 573 nucleotides upstream of the *lin-4* precursor, which was shown to be sufficient to rescue a *lin-4* mutant (Lee et al. 1993). Construct B (*ExB*) was nearly identical, except that it included 1668 nucleotides upstream of the *lin-4* precursor, and the sequence 3' of the *lin-4* precursor was extended by 8 nucleotides before the fusion point to *let-7*.

The *lin-4* Promoter Can Drive
Precocious Expression of *let-7*

Northern blot analysis demonstrated that both constructs *Ex[lin-4A::let-7]* (data not shown) and *Ex[lin-*

Figure 1. Fusions to *lin-4* sequence drive precocious expression of *let-7*. (A) Schematics of the *lin-4A::let-7* and *lin-4B::let-7* fusion constructs. (B) Blot of RNA isolated from *lin-4B::let-7* and control lines at the L2 and L4 stages probed for *let-7*. Information on the transgenic arrays is presented in Table 1. (C) The same blot reprobed for *lin-4*.

4B::let-7] (Fig. 1B) express the mature *let-7* miRNA by the beginning of the L2 stage. This was also the case with *Ex[lin-4::let-7(n2853)]*, in which the *n2853* point mutation was included. Surprisingly, when *let-7* was overexpressed under its own promoter from a 2459-bp genomic fragment beginning 1576 nucleotides 5' of the *let-7* precursor, it was also detected by early L2. The method used here normally reveals *let-7* expression at a low level in the L3 stage in wild-type *C. elegans* (Reinhart et al. 2000), although a more sensitive method has detected low-level expression at the L2 stage (Abbott et al. 2005). It is possible that a negative regulatory element was not included in the *let-7* overexpression construct or that increased expression from the multicopy array reveals *let-7* earlier than is the case when it is expressed from the endogenous *let-7* locus. The temporal response element described by Johnson et al. (2003) as necessary for proper regulation of *let-7* was included. Quantitation of the signal from the 22-nucleotide band in each lane and normalization of loading relative to U6 small nuclear RNA (snRNA) signal revealed that *let-7* was expressed at similar levels in the L2 stage in all lines, although the highest level of expression was achieved under the *lin-4* promoter (Fig. 1B). The apparently slightly lower expression level of the *n2853* control construct could be due to imperfect hybridization between the probe and the mutated target sequence. In all

cases, the abundance of the mature form of the *let-7* miRNA increased at the L4 stage. Interestingly, in each line, an accumulation of the precursor form of *let-7* relative to the abundance of the mature product was observed in the L2, but this accumulation was resolved by the L4 stage (Fig. 1B and data not shown). This suggests the possibility that processing of *let-7* by the Dicer-ALG-1/2 complex (Grishok et al. 2001; Hutvagner et al. 2001), as well as the transcription of *let-7*, could be temporally regulated. No similar L2-stage accumulation of *lin-4* precursor was observed, demonstrating that miRNA processing in general was not disrupted (Fig. 1C).

Both of the *lin-4::let-7* fusion constructs drove production of functional *let-7*, as demonstrated by their ability to rescue a *let-7* null mutant (Table 1). However, all of the lines that were tested showed at most weak rescue of a *lin-4* null (data not shown). In addition, when RNA derived from animals carrying the *lin-4B::let-7* transgenes *mgEx719* and *mgEx720* in a *lin-4* null background was probed, no clear *lin-4* expression was detected (data not shown). These observations suggest that the *lin-4* precursor produced from these fusion genes may be degraded rather than processed to mature *lin-4*. Many miRNAs are found in genomic clusters (such as *mir-35-mir-41* in *C. elegans* and *let-7* and the *lin-4* homolog, *miR-125*, in *Drosophila*) and are processed from a single primary transcript (Lau et al.

Table 1. Phenotypes Generated by *let-7* Precocious- and Overexpression Transgenes

Strain/Array	Construct	Injection concentration (ng/μl)	Precocious alae at L4 stage (% [n])	Rescue of <i>let-7</i> (% [n])
<i>mgEx704</i>	<i>lin-4A::let-7</i>	40	0 (5)	n.d.
<i>mgEx705</i>	<i>lin-4A::let-7</i>	40	n.d.	20 (10)
<i>mgEx706</i>	<i>lin-4A::let-7</i>	20	n.d.	77 (22)
<i>mgEx707</i>	<i>lin-4A::let-7</i>	20	n.d.	80 (10)
<i>mgEx708</i>	<i>lin-4A::let-7</i>	20	n.d.	100 (26)
<i>mgEx709</i>	<i>lin-4A::let-7</i>	20	6 (17)	71 (14)
<i>mgEx710</i>	<i>lin-4A::let-7</i>	40	0 (7)	<5 (>20)
<i>mgEx711</i>	<i>lin-4B::let-7</i>	15	80 (5)	n.d.
<i>mgEx712</i>	<i>lin-4B::let-7</i>	15	33 (12)	n.d.
<i>mgEx713</i>	<i>lin-4B::let-7</i>	15	40 (10)	40 (10)
<i>mgEx714</i>	<i>lin-4B::let-7</i>	10	0 (3)	59 (12)
<i>mgEx715</i>	<i>lin-4B::let-7</i>	10	29 (7) ^b	80 (5)
<i>mgEx716</i>	<i>lin-4B::let-7</i>	10	69 (26)	n.d.
<i>mgEx717^a</i>	<i>lin-4B::let-7</i>	5	0 (7)	100 (9)
<i>mgEx718^a</i>	<i>lin-4B::let-7</i>	5	35 (17)	n.d.
<i>mgEx719^a</i>	<i>lin-4B::let-7</i>	5	43 (7)	n.d.
<i>mgEx720^a</i>	<i>lin-4B::let-7</i>	1	38 (8)	100 (6)
<i>mgEx721^a</i>	<i>lin-4B::let-7</i>	1	45 (31)	n.d.=
<i>mgEx722^a</i>	<i>lin-4B::n2853</i>	5	0 (12)	n.d.=
<i>mgEx723^a</i>	<i>lin-4B::n2853</i>	1	0 (20)	n.d.=
<i>mgEx724^a</i>	<i>lin-4B::n2853</i>	1	4 (24)	0 (9)
<i>mgEx692^a</i>	<i>let-7++</i>	1	0 (14)	100 (8)
<i>mgEx693^a</i>	<i>let-7++</i>	1	0 (16)	n.d.=
<i>lin-41(ma104)</i>	n.a.	n.a.	50 (12)	n.d.
<i>mgEx719^a</i>	<i>lin-4B::let-7</i>	5	78 (9)	n.d.
<i>mgEx719;</i> <i>lin-29 (RNAi)^a</i>	<i>lin-4B::let-7</i>	5	0 (9)	n.d.
<i>mgEx720; vector (RNAi)</i>	<i>lin-4B::let-7</i>	1	26 (19) ^b	n.d.
<i>mgEx720;</i> <i>lin-29(RNAi)</i>	<i>lin-4B::let-7</i>	1	0 (14) ^b	n.d.

^a All observations reported on this line of the table were done in a *wIs54[scm::gfp] V; let-7(mn112) unc-3(e151)* X background.

^b Scored in a *let-7(mn112) unc-3(e151)* background.

= This transgene rescued *let-7(mn112)* (only at 15°C for *lin-4::n2853* lines), but the penetrance of rescue was not determined. n.d. indicates not determined; n.a. indicates not applicable.

2001; Bashirullah et al. 2003). However, the mature *lin-4* and *let-7* sequences are positioned more closely (7–15-bp separation) in our fusion genes than the miRNAs in these naturally occurring clusters (>30-bp separation).

Early Expression of *let-7* Generates Precocious Development

It was expected that early expression of *let-7* would cause precocious down-regulation of its target, LIN-41, causing a phenotype similar to that seen in animals bearing loss-of-function mutations in *lin-41*. Animals lacking LIN-41 are dumpy (Dpy) and partly sterile. In wild-type *C. elegans*, the lateral hypodermal seam cells fuse and secrete a cuticular structure known as alae at the L4-to-adult molt (Sulston and Horvitz 1977). In *lin-41* loss-of-function mutants, both seam cell fusion and production of alae occur one stage early, at the L3-to-L4 molt (Slack et al. 2000). Animals expressing both *Ex[lin-4A::let-7]* and *Ex[lin-4B::let-7]* were Dpy and had a partially penetrant egg-laying defect (data not shown). However, animals expressing *Ex[lin-4A::let-7]* only very rarely produced alae precociously, whereas those expressing *Ex[lin-4B::let-7]* produced alae with a penetrance similar to that of *lin-41(ma104)*; 29–80% of animals bearing *Ex[lin-4B::let-7]* and 50% of *lin-41* mutants produced alae covering at least one of the lateral hypodermal seam cells at the L3-to-L4 molt (Table 1). As shown in Figure 2, both *lin-41(ma104)* animals and those expressing *Ex[lin-4B::let-7]* often produced “patchy” alae during the L4 stage, with some seam cells precociously expressing the adult fate, while others continued to express the larval fate. The “patchy alae” phenotype has also been observed for other heterochronic mutants (Ambros 1989).

Consistent with the results for alae formation, animals bearing *lin-4B::let-7* transgenes showed precocious expression of a reporter for *col-19*, which encodes a collagen that is a component of the adult cuticle. *col-19*, which is positively regulated by LIN-29, is expressed only in the adult stage in wild-type *C. elegans* (Abrahante et al. 1998); 75% ($n = 12$) of animals carrying *mgEx719[lin-4B::let-7]* expressed the *vels13[col-19::gfp]* reporter in the L4 stage. Animals bearing *lin-4B::let-7* transgenes only rarely expressed *col-19::gfp* as early as the L3 stage (Fig. 3).

Precocious Alae Result from Early Expression of *let-7*

To test whether the precocious phenotype generated by the *Ex[lin-4B::let-7]* transgenes was due to expression of *let-7*, we introduced the *n2853* point mutation into the *lin-4B::let-7* construct. Introduction of the *n2853* mutation was expected to block *let-7* function, because *let-7(n2853)* mutant worms have a temperature-sensitive lethal phenotype similar to that of *let-7(mn112)* null mutants. In fact, animals bearing the *Ex[lin-4B::let-7(n2853)]* constructs only very rarely (0–4%) produced alae precociously (Table 1). Those animals bearing *mgEx[lin-4B::let-7(n2853)]* that did produce alae precociously produced only faint alae, not distinct patches as observed with animals bearing the *Ex[lin-4B::let-7]*

Figure 2. A typical L4 larva bearing the *lin-4B::let-7* transgene showing precocious alae formation. This animal expressed a mix of larval and adult fates in the hypodermis, indicated by the break in the alae (arrow).

transgenes (data not shown). The fact that the introduction of the *n2853* point mutation abrogates the precocious phenotype induced by expression of *Ex[lin-4B::let-7]* demonstrates that the precocious phenotype results from early expression of *let-7*, not overexpression of *lin-4*.

The precocious phenotype generated by the *lin-4::let-7* constructs was not a product of simple overexpression of *let-7*. Animals bearing the *Ex[let-7⁺⁺]* transgenes never formed alae precociously (Table 1), even though they produced *let-7* at the L2 stage at a level comparable to that of animals bearing the *lin-4::let-7* constructs (Fig. 1B).

The Heterochronic Pathway Mediates the Effects of Precocious *let-7* Expression through *lin-29*

To confirm that the precocious phenotype produced by expression of *Ex[lin-4B::let-7]* is mediated by the established heterochronic pathway, we inactivated the terminal gene in this pathway, *lin-29*, by feeding RNAi. Animals bearing either of two *lin-4B::let-7* transgenes produced alae at the L3-to-L4 molt when fed vector RNAi, but they never produced alae when fed *lin-29(RNAi)* (Table 1), indicating that the precocious phenotype produced by expression of the *lin-4::let-7* fusion gene is mediated by *lin-29*.

RNAi Screen for Enhancers of the Precocious *lin-4::let-7* Phenotype

Although the *lin-4B::let-7* construct drives precocious production of *let-7* two stages early, by the beginning of the L2 stage, its expression results in production of alae and expression of *col-19::gfp* only one stage early, at the L3-to-L4 molt. It seemed possible that some factor might inhibit the early action of *let-7* or that some other factor necessary for *let-7* function might not normally be present as early as the L2 stage.

To identify such factors, we conducted a feeding RNAi screen using a library of 438 clones selected because they were expected to have a role in RNA metabolism, RNAi, genome stability and transposon silencing, miRNA function, or control of developmental timing. Animals bearing *mgEx719[lin-4B::let-7]* in a *col-19::gfp*; *let-7(mn112)* *unc-3(e151)* background were fed each clone, and they and their progeny were scored for expression of *col-19::gfp* earlier than the L4 stage; 128 clones emerged that either appeared to cause expression of *col-19::gfp* earlier than the L4 stage or that caused other phenotypes (such as sickness or sterility) specifically in the animals bearing the *lin-4::let-7* transgene (data not shown). None of the seven vector clones included and scored blindly was noted to cause precocious *col-19::gfp* expression nor any other phenotypes in larvae. 25 of the 128 clones appeared to enhance precocious expression of *col-19::gfp* in animals bearing the *lin-4::let-7* transgene upon a single retest (Table 2; data not shown). Four of the 25 positive clones that caused the most potent or penetrant precocious expression of *col-19::gfp* were examined by Nomarski microscopy to determine the age of the green animals. The clones corresponding to *lin-42* (*period*), Y57G11A.5 (which encodes a predicted RNA-binding protein that may be involved in splicing), and Y77E11A.7 and T26A5.5, which are required for genome stability, were all confirmed to cause *col-19::gfp* expression by the L3 stage in animals bearing *mgEx719[lin-4B::let-7]* (Fig. 3A; Table 2).

Figure 3. Enhancement of the *mgEx725[lin-4B::let-7]* precocious *col-19::gfp* expression phenotype by RNAi. (A) *lin-42(RNAi)* enhanced the precocious *col-19::gfp* expression phenotype of animals bearing the *lin-4B::let-7* transgene. On the bottom is an L3 animal bearing *mgEx725[lin-4B::let-7]* and expressing *col-19::gfp* precociously. Above it is an L4 animal without the *mgEx725* transgene and showing only background fluorescence. (B) Effect of feeding RNAi against the indicated clone on the age distribution of animals expressing *col-19::gfp* in the F₁ generation.

Table 2. Possible Enhancers of the Precocious *col-19::gfp* Expression in Animals Bearing *mgEx719[lin-4B::let-7]*

Developmental timing	Nucleases
F47F6.2 (<i>lin-42</i>)	R17.2
F11A1.3 (<i>daf-12</i>)	Y39G8C.1 (<i>xrn-1</i>)
Argonaute orthologs	F31E3.4
F55A12.1	C50C3.7
C35D6.3	ZK1098.3
Nucleic acid binding	Helicases
C08H9.2	Y54E2A.6
C56G2.1	Genome stability / transgene silencing
C44E4.4	R06C7.7 (<i>lin-61</i>)
R05H10.2	T26A5.5
M4.2 (<i>puf-4</i>)	Y77E11A.7
Y57G11A.5	AC8.1 (<i>pme-6</i>)
Y116A8C.32	Unknown function
C30G7.1 (<i>hil-1</i>)	C28A5.1
mRNA nuclear export	Y54E2A.7
F10G8.3 (<i>npp-17</i>)	

The 25 apparent enhancers were tested a final time with a strain bearing a red fluorescent protein (*rfp*)-marked *lin-4B::let-7* transgene that eliminated background green fluorescence from the original *tub-1::gfp*-marked *lin-4B::let-7* transgene. Animals bearing the *lin-4B::let-7* transgene showed *col-19::gfp* expression by the L3 stage when grown in synchrony and fed RNAi against the following genes beginning at the L1 stage: *lin-42*, 7 of 22; C08H9.2, 3 of 23; C56G2.1, 3 of 27; *hil-1*, 4 of 29; R17.2, 2 of 22; Y54E2A.6, 2 of 18; Y54E2A.7, 3 of 18; *lin-61*, 6 of 21. No animals lacking the *lin-4B::let-7* transgene showed expression of *col-19::gfp* before the L4 stage. One of 20 *lin-4B::let-7* transgene-bearing animals fed vector RNAi dimly expressed *col-19::gfp* at the L3 stage. For clones that induced relatively penetrant precocious expression of *col-19::gfp* in the P₀ generation, the F₁ progeny were washed from the well, and the age of *col-19::gfp*-expressing animals that bore the *lin-4::let-7* transgene was determined (Fig. 3B). Inactivation of *lin-42* (*period*) and R17.2 (which encodes a nuclease) in particular increased the proportion of L3 animals that expressed *col-19::gfp* as compared to vector. Targeting of Y54E2A.6 (which encodes a helicase) and C08H9.2 (which encodes a KH-domain protein) had less effect at the L3 stage, but increased the proportion of animals expressing *col-19::gfp* before the adult stage.

A final category of clone that emerged from the screen was those that allowed *let-7* null animals to live in the absence of the *lin-4::let-7* transgene. Among the six suppressors was *lin-41*, a known suppressor of *let-7* and one of its established targets (Slack et al. 2000). Three of the six clones (those targeting F55F8.3, K07C5.4, and F17C11.9) corresponded to genes required for assembly of the ribosome or translation. The significance of this finding is unclear, but it may be that the lethality of *let-7* derives from derepression of the translation of *lin-41* or other *let-7* targets and that this lethality is prevented by defects in translation. Two additional suppressors were *crn-1*, which encodes an endonuclease (Parrish et al. 2003), and *puf-3*, which encodes an ortholog of Pumilio, an RNA-binding protein implicated in posttranscriptional regulation (Wickens et al. 2002).

CONCLUSIONS

The data presented here demonstrate that early expression of the *let-7* miRNA is sufficient to drive adult differentiation of the hypodermis and that this precocious phenotype is mediated by the heterochronic gene *lin-29*. It is perhaps surprising that animals bearing the *Ex[let-7⁺⁺]* transgenes failed to produce alae precociously, given that animals expressing both *Ex[lin-4B::let-7]* and *Ex[let-7⁺⁺]* produced similar levels of mature *let-7* miRNA by the L2 stage. One explanation is that the *lin-4* and *let-7* promoters may drive *let-7* expression in different tissues and that expression of *let-7* in the L2 stage in tissues where the *lin-4* promoter is active is required to yield the precocious phenotype. It could be that some factor prevents the early activity of *let-7* in the tissues where it is expressed under its own promoter but that this factor is not present in additional tissues where *Ex[lin-4B::let-7]* drives *let-7* expression. Candidates for such a factor include molecules such as LIN-42 and others identified in the enhancer screen.

The *period* homolog *lin-42* has been previously shown to cause precocious development of the hypodermis when it is inactivated (Jeon et al. 1999). Loss of other genes that cause precocious heterochronic phenotypes, such as *lin-28*, has been shown to up-regulate levels of *let-7* (Johnson et al. 2003). It seemed possible that the precocious phenotype of *lin-42* mutants could also depend on such up-regulation of *let-7*. However, the fact that inactivation of *lin-42* strongly enhances the precocious phenotype of animals bearing *lin-4B::let-7* transgenes suggests that up-regulation of *let-7* is not primarily responsible for the precocious phenotype of *lin-42* mutants.

It was recently shown that the abundance of mature *let-7* and many other miRNAs in vertebrates is controlled at the level of processing of the primary transcript by Drosha (Thomson et al. 2006). No similar regulation has been shown in *C. elegans*; indeed, transcription of a *let-7* reporter (Johnson et al. 2003) and processing of the primary transcript (Bracht et al. 2004) are closely correlated with the appearance of mature *let-7*. However, regulation of processing, at the level of either the primary transcript or the hairpin precursor, is one reason the precocious phenotypes generated by *let-7* expression under the *lin-4* promoter could be weaker than anticipated. Indeed, the accumulation of the hairpin precursor form of *let-7* at the L2 stage (Fig. 1B) suggested that such regulation might occur.

Additional candidates for factors that could act together with *let-7* to direct the larval-to-adult transition were the *let-7* paralogs *mir-48*, *mir-84*, and *mir-241* (Lau et al. 2001; Lim et al. 2003). Indeed, *let-7* and *mir-84* act together to promote cessation of molting in adults, and overexpression of *mir-84* causes precocious execution of the L3-stage pattern of seam cell divisions in the L2 stage (Hayes 2005). However, when *lin-4::mir-48* and *lin-4::mir-84* constructs analogous to the *lin-4B::let-7* construct were injected, they caused no obvious precocious phenotypes (data not shown). Animals injected with combinations of these constructs and the *lin-4B::let-7* construct showed precocious development, but not to a greater extent than animals bearing *Ex[lin-4B::let-7]* alone (data

not shown). Similarly, no effect on the L2-stage seam cell divisions was noted for the *lin-4B::let-7* transgenes, even in combination with the enhancer clones identified here (data not shown). These results suggest that despite their sequence similarity, *let-7* and its paralogs have at least partly distinct targets in promoting developmental progression. This finding is in agreement with the observation that the effects of *mir-84* overexpression are similar to but distinct from those of *mir-48* overexpression (Hayes 2005; Li et al. 2005) and also with the observation by Leaman et al. (2005) that inactivation of individual *miR-310* family members caused similar, but not identical, phenotypes in *Drosophila* larval development.

The results of the RNAi screen for enhancers of the precocious phenotype caused by expression of *lin-4B::let-7* point to possible roles in the regulation of *let-7* (at least as expressed from the fusion transgene) for molecules implicated in various aspects of RNA metabolism. One caveat is that the verification of these observations remains incomplete. Retesting with the *rfp*-marked *lin-4::let-7* transgene failed to detect precocious *col-19::gfp* expression in the P₀ generation of animals for many of the clones. Greater penetrance of precocious phenotypes is expected upon screening of the F₁ progeny of animals fed each clone. Determining whether these clones enhance precocious *col-19::gfp* expression in heterochronic mutant backgrounds (such as *lin-14*, *lin-28*, *lin-42*, and *hbl-1*) will point to roles the corresponding genes might have in the heterochronic pathway. It would also help clarify the relationship of the enhancers to the regulation of endogenous *let-7*.

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