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# Post-embryonic expression of *C. elegans* microRNAs belonging to the *lin-4* and *let-7* families in the hypodermis and the reproductive system

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## Abstract

MicroRNAs (miRNAs) are regulatory molecules that negatively control gene expression by binding to complementary sequences on target mRNAs. The most thoroughly characterized miRNAs, *lin-4* and *let-7*, direct cell fate determination during the larval transitions in *C. elegans* and act as key regulators of temporal gene expression. *lin-4* and *let-7* are founding members of two distinct families of miRNA genes sharing strong sequence homology primarily in the 5′ end of the mature miRNAs. In this report, we characterize the temporal and spatial expression patterns of *lin-4* and *let-7* family members using Northern blot analysis and *mir::gfp* fusion studies. Our results show that *lin-4* and *let-7* homologues possess distinct temporal and spatial expression patterns during nematode development and that known heterochronic genes regulate their expression. We find that certain *lin-4* and *let-7* family members display overlapping expression patterns in the hypodermis and the reproductive system, suggesting that combinations of miRNAs from across families may control common developmental events.

### Keywords

microRNAs; *C. elegans*; development; *lin-4*; *let-7*; expression analysis

## Introduction

MicroRNAs (miRNAs), small noncoding RNAs of ~22 nucleotides, belong to a novel class of gene regulatory molecules found in plants and animals that negatively control gene expression by binding to complementary sequences on target messenger RNAs (mRNAs) (Bartel, 2004). Hundreds of miRNAs have recently been identified in worm, fly, and mammalian genomes through cloning and bioinformatic approaches (Ambros and Horvitz, 1984; Pasquinelli et al., 2000; Reinhart et al., 2000; Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001; Lagos-Quintana et al., 2002; Lee et al., 2002; Aravin et al., 2003; Brennecke et al., 2003; Johnston and Hobert, 2003; Lagos-Quintana et al., 2003; Lai

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et al., 2003; Lim et al., 2003a; Lim et al., 2003b; Sempere et al., 2003; Xu et al., 2003; Chang et al., 2004). Only a small subset of miRNAs identified have been characterized in animals and these control important developmental events involved in cellular differentiation, proliferation, apoptosis, and fat metabolism (Ambros and Horvitz, 1984; Reinhart et al., 2000; Brennecke et al., 2003; Johnston and Hobert, 2003; Xu et al., 2003; Chang et al., 2004; Chen et al., 2004; Johnson et al., 2005). The remaining uncharacterized miRNAs may also act as gene regulators that direct important biological processes during development and in the adult.

The first miRNAs to be discovered, *lin-4* and *let-7*, were identified through genetic analysis to act as developmental switches that control the timing of cell fate determination during the larval transitions in *C. elegans* (Chalfie et al., 1981; Ambros and Horvitz, 1984; Reinhart et al., 2000). *lin-4* and *let-7* loss-of-function mutations result in reiterations of the first larval stage (L1) and the fourth larval stage (L4) fates respectively, and these defects lead to disruptions in cell cycle exit and terminal differentiation. These early studies revealed that the *lin-4* and *let-7* miRNAs were key regulators in an emerging heterochronic pathway, which governs how tissues and organs are specified at the correct time and synchronized during development (Slack and Ruvkun, 1997; Banerjee and Slack, 2002). *lin-4* RNA accumulates during the late L1 stage and is responsible for the L1/L2 transition in nematodes by binding to imperfect complementary sites within the 3′UTRs of its gene targets, *lin-14* and *lin-28* (repressors of post-L1 fates), and down-regulating their expression at the level of translation (Lee et al., 1993; Wightman et al., 1993; Moss et al., 1997; Olsen and Ambros, 1999; Ambros, 2000). Likewise, *let-7* RNA accumulates during the L4 stage and is responsible for the L4/adult transition by inhibiting the expression of its target genes, *lin-41* and *hbl-1* (Reinhart et al., 2000; Slack et al., 2000; Pasquinelli and Ruvkun, 2002; Abrahante et al., 2003; Lin et al., 2003). The *lin-4* and *let-7* miRNAs are not exclusively used by nematodes to control developmental timing but are rather found to be evolutionarily conserved and temporally expressed in higher animals, implying a more universal role for these miRNAs during development (Feinbaum and Ambros, 1999; Pasquinelli et al., 2000; Reinhart et al., 2000; Lagos-Quintana et al., 2002; Pasquinelli and Ruvkun, 2002; Lim et al., 2003b; Sempere et al., 2003).

*lin-4* and *let-7* are members of two distinct miRNA families in *C. elegans* that include *lin-4* and *mir-237* in the *lin-4* family, and *let-7*, *mir-84*, *mir-241*, and *mir-48* in the *let-7* family based on sequence homology shared primarily at the 5′ end of the mature miRNAs (Lim et al., 2003b). In addition to *lin-4* and *let-7*, only one other miRNA from this group, *mir-84*, has been characterized. Recent work from our laboratory has shown that *mir-84*, the closest *let-7* homologue in nematodes, directs proper hypodermal seam cell and vulva morphogenesis, in part through the negative regulation of the *let-60*/RAS gene in these tissues (Grosshans et al., 2005; Johnson et al., 2005). Members of the *lin-4* and *let-7* families are found to be temporally regulated during nematode development (Lau et al., 2001; Lim et al., 2003b), however, little is known regarding how the temporal and spatial expression patterns of the *lin-4* and *let-7* family members overlap or if these genes direct similar biological functions as the known heterochronic miRNAs, *lin-4* and *let-7*.

In this report, we have begun to characterize the expression patterns of the miRNAs belonging to the *lin-4* and *let-7* families. We show that miRNAs, which are closely related on a sequence level, are not initially expressed at identical stages during *C. elegans* development based on Northern blot analysis, indicating that miRNA homologues may be functionally distinct. In support of this hypothesis, we observe unique temporal and tissue expression patterns in nematodes among the *lin-4* and *let-7* family members when miRNA promoter regulatory sequences are fused to the green fluorescence protein (*gfp)* reporter gene (*mir::gfp*). We note that certain members across the *lin-4* and *let-7* families are

expressed similarly in the developing hypodermal seam cells, the gonad, and the vulva, suggesting that unrelated miRNAs may control common developmental processes in these tissues.

## Results and Discussion

### The *lin-4* and *let-7* families are differentially expressed during nematode development

miRNAs identified within the *C. elegans* genome form distinct families based on sequence homology primarily at the 5′ portion of the mature miRNAs (Figs. 1A & 1C; Lim et al., 2003b). In order to explore the possibility that miRNAs within families are regulated in a similar manner, we compared the temporal expression pattern of the *lin-4* and *let-7* family members using developmental Northern blots. Our results demonstrate that despite the sequence homology shared among family members, their expression patterns were temporally dynamic and differed from one another (Figs. 1 & S1). For example, the *lin-4* miRNA is highly expressed during the late L1/eL2 transition through to adulthood with peak expression noted in mid-L3 (Feinbaum and Ambros, 1999; Figs. 1B & S1A). In contrast, we find by Northern blot that the *lin-4* homologue, miR-237, was detected at low levels during L2, was upregulated at early L3 and peaked during the L4 stage (Figs. 1B & S1A). We also note that our Northern blot expression data for the *lin-4* homologue, miR-237, and the *let-7* family members, miR-241, miR-48, and miR-84, differed from those reported by Lim et al., 2003, most likely due to the higher temporal resolution of our Northern blot data and a difference in the sensitivity of our probes. For instance, this previous study reported that the *let-7* family members, miR-84, miR-48, and miR-241, were expressed in a similar manner as the *let-7* RNA, which was first detected during the L3 stage (Reinhart et al., 2000; Lim et al., 2003b). However, our studies reveal that miR-241 and miR-48 both appeared one stage earlier than *let-7* at eL2 with robust expression by L3. miR-84 appeared at low levels in the early L1 stage, was upregulated to an intermediate level in mid-L2 and had a high level of expression in early L4 (Figs. 1D & S1B). It is interesting to note that all *let-7* family members showed maximal expression during the L4 stage. In addition, because the miRNA genes, *mir-241* and *mir-48*, are located within 1.7 kb of one another on chromosome V in the *C. elegans* genome and reside in the same orientation (Fig. S5C), it is assumed that *mir-241* and *mir-48* are co-transcribed and present within a single pri-miRNA transcript, which is later processed to form two independent 70 nucleotide pre-miRNA precursors. In that case, *mir-241* and *mir-48* could share common regulatory elements to direct their temporal and spatial expression, analogous to regulatory elements located in the introns of a variety of genes. In support of this hypothesis, we show in our Northern blot analysis that these genes are expressed similarly throughout development (Figs. 1D & S1B). Based on the unique expression patterns of closely related members belonging to the *lin-4* and the *let-7* families, we predict that these genes may direct distinct biological processes during development.

### Regulation of the *lin-4* and *let-7* families by the heterochronic genes, *lin-4* and *daf-12*

We tested whether miRNAs related to *lin-4* and *let-7* are regulated by the heterochronic genes during development. Our previous work showed that *let-7* miRNA expression is modulated by heterochronic genes that are genetically upstream of *let-7* in the heterochronic pathway, i.e. *lin-4*, *lin-14*, *lin-28* and *daf-12* (Johnson et al., 2003). In the present study, we similarly analyzed developmental Northern blots using RNA taken from animals with a loss-of-function mutation in the *lin-4* miRNA (*lin-4* (*e912*)), and a ligand-binding domain mutation in the *daf-12* nuclear hormone receptor (*daf-12* (*rh61*)) and found that both of these genes were essential for the proper regulation of all the *let-7* family members, as well as the regulation of the *lin-4* homologue, miR-237 (Figs. 2 & S2). The *lin-4* and *daf-12* genes are essential components of the heterochronic pathway and are normally required in

nematodes to direct the L1/L2 transition (Chalfie et al., 1981) and the L2/L3 transition (Antebi et al., 1998; Grosshans et al., 2005) respectively. As previously reported for *let-7* (Johnson et al., 2003), this work shows that mutations in *daf-12* also resulted in a decrease of miR-241, miR-48, and miR-84 RNA levels in the late larval and adult stages. However, while the loss of the *lin-4* miRNA caused a severe decrease in *let-7* RNA (Johnson et al., 2003), the loss of *lin-4* resulted in a much less pronounced effect on miR-241, miR-48 and miR-84 in the larval and adult stages. Our results also show that *mir-237* is regulated by its earlier expressed homologue, *lin-4*, and implies that these genes do not function redundantly. We further note that *daf-12* is also required for proper expression of *mir-237* specifically at the L3 stage, the time when miR-237 levels are upregulated during development in wild type animals (Figs. 1B & S1A). Taken together, these results reveal that the expression of the *lin-4* and *let-7* family members are regulated by known heterochronic genes and thus may indicate a role for these novel miRNAs in controlling developmental timing.

## Using *mir::gfp* fusions to analyze temporal and spatial expression patterns of the *lin-4* and *let-7* families

In order to determine whether the *lin-4* and *let-7* family members exhibit distinct temporal and spatial expression patterns during nematode development, we examined the expression of these miRNAs *in vivo* by fusing *mir* promoter regulatory sequences to the *gfp* reporter gene followed by the heterologous 3′UTR of the *unc-54* gene (*mir::gfp*). (Promoter regions chosen for the *lin-4* and *let-7* families are graphically depicted in Figs. S3, S4, & S5.) In general, we defined the promoter region of a miRNA as follows: (1) If a rescuing construct was known, we used the minimal DNA sequences in the rescuing construct residing upstream of the mature miRNA sequence, e.g. *lin-4* and *let-7* (Lee et al., 1993;Reinhart et al., 2000). (2) We used approximately 2.0 kb of genomic DNA residing upstream of the mature miRNA sequence or most genomic sequence up to the next gene, whichever was less, e.g. *mir-84* (Johnson et al., 2005), or (3) We chose upstream sequences that possessed regions of conservation between *C. elegans* and *C. briggsae*. Our previous work has shown that similar *mir::gfp* constructs made for *let-7* in *C. elegans*, which relied solely on the *let-7* promoter for regulation, were sufficient to drive temporal expression and suggested that miRNA regulation is controlled at the level of transcription and not by RNA processing and/or miRNA stability (Johnson et al., 2003). This technology has also been used to successfully detect the expression of certain *C. elegans* neural-specific miRNAs (Johnston and Hobert, 2003;Chang et al., 2004). Furthermore, we found that injection of the empty vector, pPD95.75, which contains only the *gfp* gene and the *unc-54* 3′UTR, did not result in background GFP expression (data not shown). Taken together, the *mir::gfp* constructs made for the novel *lin-4* and *let-7* families likely indicate at what time and in which areas these miRNAs are normally expressed. Using this approach, our expression analysis showed that the *lin-4* and *let-7* families exhibited interesting overlapping expression patterns in the developing hypodermal seam cells, the gonad and the vulva.

## *lin-4* and *let-7* family expression in the hypodermal seam cells during development

Our *mir::gfp* expression studies revealed that the *lin-4* and *let-7* family members were differentially expressed in the hypodermal seam cells during nematode development. Seam cells, lateral hypodermal cells responsible for secreting the worm cuticle, undergo a characteristic pattern of cell divisions at each larval molt and terminally differentiate by the adult stage (Fig. 3; Rougvie, 2001). The heterochronic genes, including *lin-4* and *let-7*, control the timing of seam cell development and mutations in these genes result in either precocious or retarded seam cell terminal differentiation (Rougvie, 2001). In the present study, we found that *lin-4* was temporally expressed in the hypodermal seam cells at the early L2 stage and expression persisted in adulthood (Figs. 3 & S6). These temporal

expression patterns closely mirrored those observed for *lin-4* by Northern blot analysis (Figs. 1B & S1A;Feinbaum and Ambros, 1999). Furthermore, the spatial patterning of *lin-4* in the hypodermal seam cells correlated with the requirement of this gene at the L1/L2 transition to direct normal seam cell development (Ambros and Horvitz, 1984;Feinbaum and Ambros, 1999). However, the *lin-4* homologue, *mir-237*, was not expressed in the seam cells until eL3, which mirrored the up-regulation of this gene at this stage by Northern blot (Figs. 1B & S1A) and implicates a distinct role for *mir-237* during seam cell development. We previously reported that the 1.8 kb *let-7* promoter directed temporal *gfp* expression in the seam cells of transgenic animals at the early L4 stage and in the adult (Figs. 3 & S6;Johnson et al., 2003), consistent with the detection of *let-7* RNA by Northern blot analysis (Figs. 1D & S1B) and the requirement for *let-7* during later stages of seam cell development (Reinhart et al., 2000). We also found that *mir-84* temporal expression was also observed in the hypodermal seam cells at the early L4 stage, similar to *let-7* seam cell expression (Figs. 3 & S6;Johnson et al., 2005), the time when miR-84 RNA expression begins to peak as shown in our Northern studies (Figs. 1D & S1B). Thus, we find that *mir-84* and *let-7* display partially overlapping expression patterns and suggest a common role for *let-7* and *mir-84* in seam cell development. However, animals carrying the *mir-48::gfp* construct showed temporal expression in the hypodermal seam cells two stages earlier than its *let-7* homologues at the L2 stage (Figs. 3 &S6) correlating with the onset of miR-48 RNA detection by Northern blot analysis (Figs. 1D & S1B). These findings support the notion that closely related miRNA family members may be functionally distinct in select tissues. Our results also suggest that miRNAs may be required at each stage of larval development to control the timing of seam cell division and terminal differentiation.

## miRNA expression in the reproductive system

The *lin-4* and *let-7* miRNA family members also exhibited unique temporal and spatial expression patterns in the reproductive system using *mir::gfp* fusion techniques. Our studies revealed that highly related miRNAs were not expressed identically in the gonad during nematode development. For example, *lin-4* expression was first observed within the developing gonad at the beginning of L3, specifically in the distal tip cells and the anchor cell of the somatic gonad (Figs. 4A & S7A) and expression in the distal tip cells continued in the adult (data not shown). However, the *lin-4* homologue, *mir-237*, was detected two stages earlier at L1 in the somatic gonad progenitor cells, Z1 and Z4 (Figs. 4B & S7B). This result differs from our Northern blot data, which showed that miR-237 RNA was first detected at L2 (Figs. 1B & S1A). We believe that the *mir-237::gfp* expression at the L1 stage is accurate and since *mir-237* is only expressed in the Z1 and Z4 cells at this stage, miR-237 RNA is below the levels of detection by Northern blot analysis. Once the Z1 and Z4 somatic gonad derivatives expand at L2, miR-237 RNA can be seen by Northern blot. By the L3 stage, and persisting into the adult (data not shown), *mir-237* was expressed in the developing gonad, including the anchor cell, a subset of unidentified uterine cells, and the distal tip cells (Figs. 4B & S7B). The up-regulation of *mir-237* expression in animals carrying the *mir-237::gfp* construct at the L3 stage in a variety of tissues, such as the gonad and the hypodermis, mirrors the increased miR-237 RNA levels observed at L3 by Northern blot analysis (Fig. 1B & S1A). Our studies reveal that *mir-237* is expressed in a distinct temporal and spatial pattern compared to *lin-4* and supports our hypothesis that although family members share strong sequence homology, they may direct unique developmental events.

We have also shown that miRNAs across families display similar spatial expression patterns in the developing gonad, suggesting that unrelated miRNAs could direct common biological processes in this tissue. The *lin-4* family member, *mir-237*, and the *let-7* family member, *mir-84*, were both expressed at the L1 stage in the Z1 and Z4 cells of the gonad, and during

the L3 stage in the distal tip cells and uterine cells of the somatic gonad (Fig. 4B, 4C, S7B & S7C). These results suggest that *mir-237* and *mir-84* may have overlapping functions or work in a similar pathway to control proper gonad formation at early larval stages. However, by the adult stage, *mir-84* and *mir-237* exhibited distinct expression patterns in the somatic gonad, and *mir-84* expression was additionally observed in the sheath cells, the spermatheca, the uterine cells surrounding the eggs, as well as the distal tip cells, where-as *mir-237* expression was only detected in the distal tip cells at this stage (data not shown), implying that these two genes may have distinct roles in the gonad in the adult. Strikingly, we found that the other *let-7* family members were expressed quite differently to *mir-84* in the gonad, and *let-7* was observed in the anchor cell at L3 (Figs. 5 & S8) and in the distal tip cells at the adult stage (data not shown), and *mir-48* was not detected in the gonad at any stage (data not shown). Again, these results imply that the *let-7* homologues may not be functionally redundant.

## Overlapping miRNA expression patterns in vulval cells

During our analysis of the *lin-4* and *let-7* miRNA families using *mir* promoter::*gfp* fusion constructs, we were struck by the unique expression patterns these miRNAs exhibited in the vulval precursor cells (Figs. 4 & S8; Johnson et al., 2005). Our results suggest that a complex circuitry of miRNAs specifies vulval cell fates. It is well established that at the L3 stage, signaling from the anchor cell of the somatic gonad (via LIN-3) is responsible for the specification of six hypodermal precursor cells (P3.p-P8.p) to adopt either 1°, 2° or 3°cell fates. The 1° and 2° cells will later differentiate into the vulva, a specialized hypodermal structure that allows the passage of eggs from the gonad into the external environment. The 3° non-vulval cells will fuse with the multinucleated hypodermal cell, hyp7, and become part of the epidermis (Fig. 4;Sulston and Horvitz, 1977;Sulston and White, 1980;Kornfeld, 1997). Past work has revealed that the miRNAs, *lin-4*, *let-7* and *mir-84*, are important for normal vulval morphogenesis (Chalfie et al., 1981;Ambros and Horvitz, 1984;Feinbaum and Ambros, 1999;Reinhart et al., 2000;Slack et al., 2000;Johnson et al., 2005). We observed partially overlapping expression patterns of the *lin-4* and *let-7* family members at L3 in the anchor cell and the vulval precursor cells (VPCs) (Figs. 4 & S8;Johnson et al., 2005). Intriguingly, both *lin-4* and *let-7* were weakly expressed in the anchor cell, which is required for proper vulval patterning, and in the P5.p, P6.p, and P7.p cells that will later differentiate into the mature vulva. Moreover, specific expression of *mir-48* was observed in the P5.p and P7.p cells that will assume 2° vulval cell fates. In addition, the *lin-4* family member, *mir-237*, and the *let-7* family member, *mir-84*, were both expressed in the anchor cell and in the VPCs except P6.p at the mL3 stage. However, expression of *mir-84* in the vulval precursor cells appeared dynamic in nature and was detected in the P5.p, P6.p, and P7.p descendants but down regulated in the P3.p, P4.p, and P8.p descendents by the end of the L3 stage (Johnson et al., 2005). It is interesting to note that expression of *mir-237*, *mir-84*, and *mir-48* was absent in the P6.p cell during the mid- to late L3 stage, and expression of *mir-237* and *mir-84* but not *mir-48* was present in the anchor cell. The partially overlapping expression patterns of the *lin-4* and *let-7* family members seen in the vulva precursor cells and the anchor cell may reveal a combinatorial regulating code, a “miRNA code”, reminiscent of the LIM homeobox code that specifies neuronal differentiation in the vertebral spinal cord (Tsuchida et al., 1994;Thor et al., 1999;Hobert, 2004). We propose that these miRNAs and their homologues act to fine-tune vulval patterning by controlling gene targets in the vulval precursor cells.

## Conclusion

This report reveals that miRNAs belonging to the *lin-4* and *let-7* families are regulated in specific temporal and spatial patterns during *C. elegans* development. The dynamic expression of the *lin-4* and *let-7* family members, detected by Northern blot analysis and

*mir::gfp* technology, suggests that these miRNA homologues may not act redundantly but rather perform distinct biological functions. Moreover, our studies imply that miRNAs, which are unrelated and belong to different families, are similarly expressed and may work together to direct common developmental processes such as seam cell, gonad and vulva formation. We also present evidence that the *lin-4* paralogue, *mir-237*, and the *let-7* family members are regulated by a subset of heterochronic genes that control developmental timing in nematodes. Interestingly, we found that *lin-4* is important for the proper expression of *mir-237*, further supporting the notion that these miRNAs possess distinct developmental functions. These studies are an important step in understanding which developmental processes miRNAs control and identifying candidate gene targets.

Our studies show that miRNA homologues exhibit distinct temporal and tissue specific expression patterns during development and that unrelated miRNAs belonging to different families exhibit overlapping expression patterns. If miRNAs are expressed with such varying spatial and temporal expression patterns, then even a small number of miRNAs could achieve a diverse level of regulation. We predict that combinations of miRNAs may be required to regulate a single gene target and initiate a given biological response, such as vulva formation (see above). Many examples exist in which the 3′ UTRs of *bona fide lin-4* and *let-7* targets contain multiple miRNA complimentary sites belonging to different families. For instance, the 3′ UTRs of *lin-14* and *lin-28*, both known targets of *lin-4*, have conserved *let-7* complementary sites that are thought to be functionally important. The converse is true with the 3′ UTRs of *lin-41* and *hbl-1*, targets of *let-7*, which also contain *lin-4* complementary elements. Moreover, additional members of the *lin-4* and *let-7* families may bind to the *lin-4* and *let-7* complementary sites in these 3′ UTRs, or possibly complementary sites for unrelated miRNAs may also be present. We propose that distinct family members may bind with different affinities to the same or multiple complementary sites in a given 3′ UTR, which could achieve varying degrees of translational regulation in the tissues where the miRNAs are expressed. Both *lin-4* and *let-7* are evolutionarily conserved in mammals, with humans possessing three *lin-4* homologues, *mir-125a*, *mir-125b1*, and *mir-125b2*, and over ten *let-7* homologues (Lagos-Quintana et al., 2002; Lim et al., 2003b). Due to the apparent complex regulation observed for the *lin-4* and *let-7* family members in *C. elegans*, there is a potential for an even higher level of miRNA-mediated gene regulation in mammals. Taken together, our results suggest an intricate system of negative regulation directed by miRNAs to specify the proper patterning, differentiation, and morphogenesis of a variety of structures such as the seam cells, gonad and vulva in the nematode. The next challenge will be to determine exactly which genes the *lin-4* and *let-7* miRNA families are controlling during these developmental processes.

## Experimental Procedures

### Northern blot analysis

Approximately 20.0 μg of total RNA was obtained from N2 wild type worms, *lin-4* loss-of-function mutants (*lin-4(e912)*), and *daf-12* ligand binding domain mutants (*daf-12(rh61)*) animals for Northern blot analysis using methods described previously by Reinhart et al., 2000. Probes used to detect RNA levels of *lin-4* (5′-TCACACTTGAGGTCTCAGGGA-3′), *mir-237* (5′-AAGCTGTTCGAGAATTCTCAGGGA-3′), *mir-84* (5′-TACAATATTACATACTACCTCA-3′), *mir-241* (5′-TCATTTCTCGCACCTACCTCA-3′), and *mir-48* (5′- TCGCATCTACTGAGCCTACCTCA-3′) were made using the StarFire Oligonucleotide Labeling System (IDT). Probes p249N (5′-AACTATACAACCTACTACCTCACCGGATCC-3′) and pU6 (5′-GCAGGGGCCATGCTAATCTTCTCTGTATTG-3′), used to detect *let-7* and U6 RNAs respectively (Reinhart et al., 2000), were 5′-end labeled with γ-$^{32}$P ATP using the KinaseMax Kit (Ambion). Northern blots analyzing miRNA expression compared *lin-4* and

*daf-12* mutant samples to wild type (N2) samples at the identical developmental stage in order to derive the relative intensity of the labeled probe bound to the miRNA band. All Northern blots probed with a given miRNA were subsequently stripped and reprobed with U6 to normalize lanes for loading.

**Expression analysis in animals carrying *mir::gfp* constructs—***mir::gfp* constructs for the miRNA genes *lin-4*, *mir-237*, *let-7*, *mir-84*, *mir-241*, and *mir-48* were constructed to include the miRNA promoter upstream of the *gfp* gene followed by the heterologous 3′ UTR from the *unc-54* gene. Lin4GFPAS (*lin-4::gfp*) was made by amplifying 507 bp of genomic sequence (base pairs −513 to −7) upstream of the mature *lin-4* sequence from N2 genomic DNA and adding a *Sma*I site and an *Age*I site to the 5′ and 3′ ends, respectively, using the polymerase chain reaction (PCR) with primers LIN4LB3 and LIN4LB1 (Table 1). This product was digested with *Sma*I and *Age*I and then cloned into the pPD95.70 vector (gift from A. Fire), which was also digested with *Sma*I and *Age*I. Digestion of pPD95.70 with *Sma*I and *Age*I removed the nuclear localization signal (NLS) from this vector. PSJ840 (*mir-84::gfp*) was made using a similar cloning strategy as described and consisted of 2.2 kb of genomic sequence (base pairs -2201 to −9) upstream of the *mir-84* mature sequence and was amplified using the PCR primers MIR84UP and MIR84DN (Table 1). PSJ11 (*let-7::gfp*) was made by amplifying 1.8 kb of genomic sequence (base pairs -1762 to −1) upstream of the mature *let-7* sequence as previously described (Johnson et al., 2003) using the PCR primers LET7SMJ2 and LET7SMJ3 (Table 1). MIR241-2kb (*mir-241::gfp*) consisted of 2.0 kb of genomic sequence (-2036 to −1) upstream of the mature *mir-241* sequence and was amplified from N2 genomic DNA using the PCR primers JRP7 and JRP2 (Table 1), which added a *Bam*H1 site to the 5′ and 3′ fragment ends. The resulting PCR product was then digested with *Bam*H1 and cloned into the pPD95.70 vector. A similar cloning strategy as described for *mir-241::gfp* was used to create MIR48-1KB (*mir-48::gfp*), which consisted of 1.1 kb of genomic sequence (-1147 to −1) upstream of the mature *mir-48* sequence amplified using the PCR primers JRP6 and JRP3 (Table 1). Animals carrying the *mir-241::gfp* showed non-temporal expression patterns, which varied between lines and were not further characterized in this study. Due to the close proximity of *mir-241* and *mir-48* in the genome, *mir-241* and *mir-48* may share regulatory elements located between these two genes in order to direct their proper expression. Our *mir-241::gfp* construct would have lacked these shared regulatory sequences. The *mir-237::gfp* construct was generated using the PCR-Fusion-Based Protocol as previously described (Hobert, 2002), which consisted of 1.7 kb of genomic sequence (base pairs -1749 to −1) upstream of the mature *mir-237* sequence. In short, a PCR fragment of 1.9 kb consisting of the *gfp* gene and the 3′ UTR from the *unc-54* gene was amplified from the pPD95.75 vector (gift from A. Fire) using the PCR primers GFPAK9 (5′-AGCTTGCATGCCTGCAGGTCGACT-3′) and GFP2C (5′-GGAAACAGTTATGTTTGGTATATTGGG-3′). In parallel, a PCR fragment of 1.7 kb was amplified using the PCR primers MIR237AK5 and MIR237AK6 (Table 1) from N2 genomic DNA (base pairs −1749 to −1) that consisted of the *mir-237* promoter region. The 3′ PCR primer (MIR237AK6) for the *mir-237* promoter contained a 24-nucleotide overhang for the *gfp* gene (underlined in Table 1). In the second PCR reaction, equal amounts of the above PCR products were added for primer extension using the PCR primers MIR237AK5 and GFP2C, resulting in a 3.6 kb *mir237::gfp* fusion construct. Recent annotation for the *C. elegans* genome in WormBase revealed two tRNA genes approximately 1kb upstream of the mature *mir-237* sequence that were included in the original *mir-237* promoter construct (Fig. S4B). Since the tRNA genes reside in the opposite orientation to the *mir-237* gene, their presence is not believed to drive GFP expression.

All experimental constructs were sequenced and injected at 50 ng/μl together with the co-transformation marker, pRP4 [*rol-6(su1006)*] (100 ng/μl) into the gonads of early Adult (eAd) stage N2 worms and transgenic lines were obtained. At least three independent lines

for each *mir::gfp* were examined at every stage of nematode development (L1-Adult) and the temporal and spatial expression patterns were compared.

**Visualization of Worms—**Worms were mounted on agarose pads for viewing as described (Schnabel, 1999). Worms were viewed on an Axioplan2 imaging microscope (Zeiss) using either Normarski optics and Kohler illumination or florescent imaging for GFP. All pictures were taken with an AxioCam using AxioVision version 2.0.5 (Zeiss).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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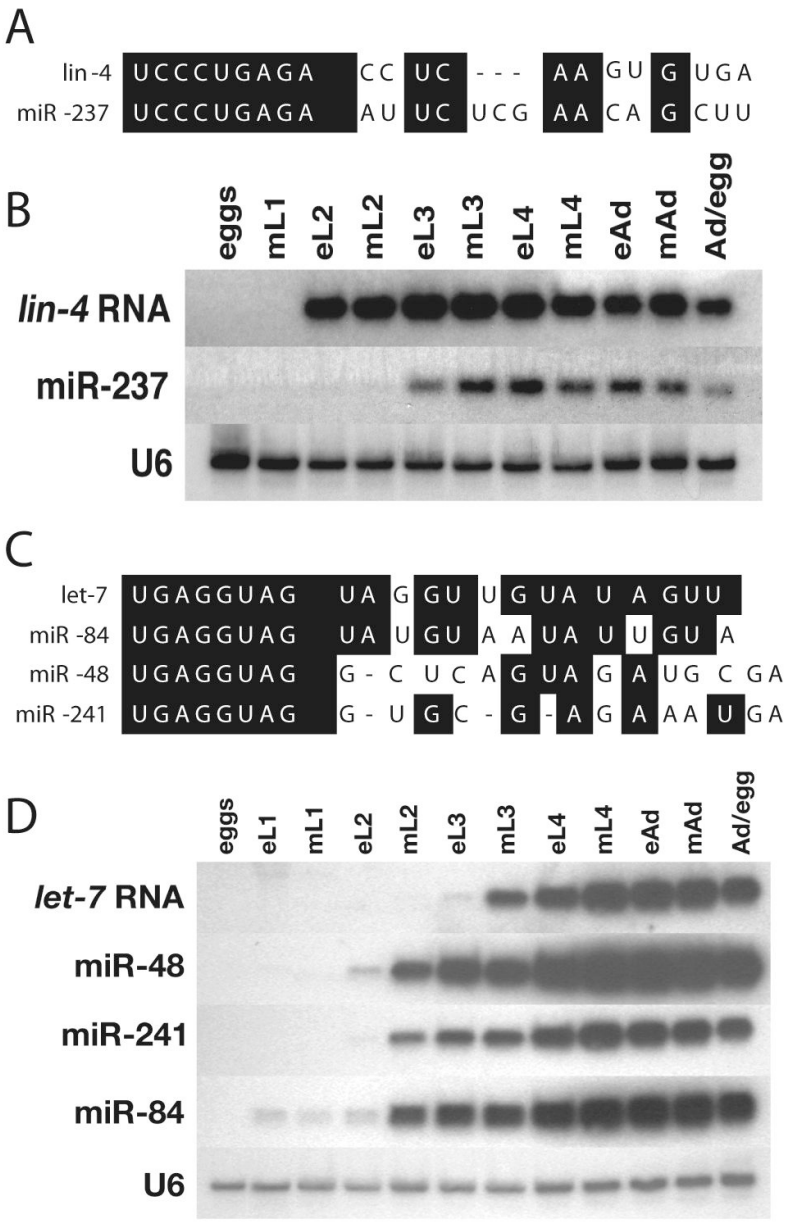


**Figure 1. Temporal expression of the *lin-4* and *let-7* miRNA families throughout *C. elegans* development**

A. Sequence alignment of the *lin-4* family designated in the 5′ to 3′ orientation. B. Northern blot analysis of *lin-4* and miR-237 shows distinct expression patterns at early stages of nematode development. U6 RNA is shown as a loading control. C. Sequence alignment of the *let-7* family. *mir-84* shares the closest sequence homology with *let-7* compared to the other family members. D. Northern blot analysis of *let-7*, miR-84, miR-48 and miR-241 reveals distinct temporal expression patterns for these closely related miRNAs. U6 is shown as a loading control. Abbreviations: eL1, early larval stage 1; mL1, mid-L1; eL2, early L2; mL2, mid-L2; eL3, early L3; mL3, mid-L3; eL4, early L4, mL4, mid-L4; eAd, early adult stage; mAd, mid-Ad; Ad/egg, late stage adults carrying eggs.

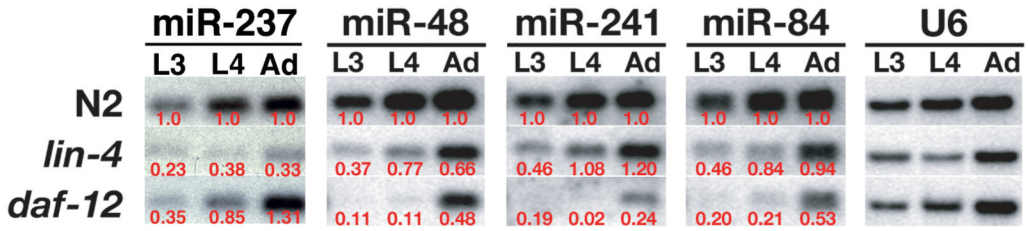


**Figure 2. Regulation of *mir-237* and the *let-7* family members by the heterochronic genes, *lin-4* and *daf-12***

Northern blot analysis of miR-237, miR-84, miR-48, and miR-241 expression during the third larval (L3) stage 3, the forth larval (L4) stage, and in the adult (Ad) with total RNA taken from wild-type worms (N2), *lin-4 (e912)* loss-of-function mutants, and *daf-12 (rh61)* ligand binding domain mutants. All the miRNAs analyzed showed some degree of regulation by both heterochronic genes. U6 is shown as a loading control. The numbers shown in red are the normalized ratios of miRNA expression in heterochronic mutants vs. wild-type N2 animals.

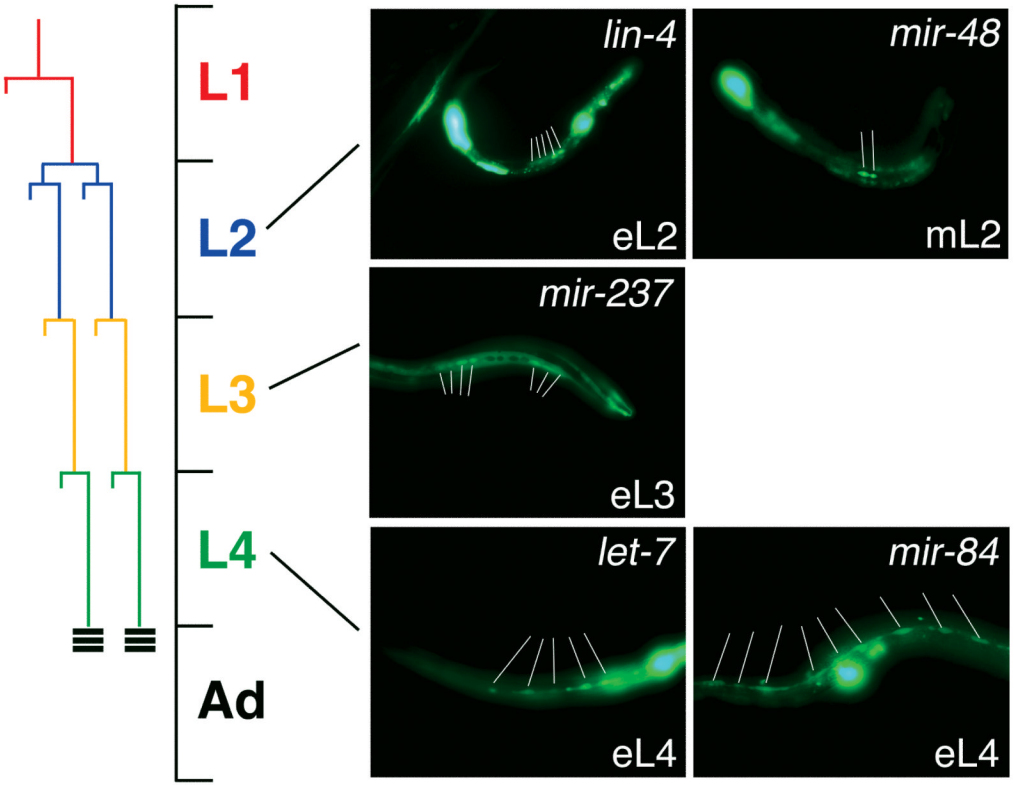


**Figure 3. Temporal expression of *lin-4* and *let-7* family members in the hypodermal seam cells during nematode development**

Transgenic animals carrying *mir::gfp* fusion constructs for *lin-4*, *mir-237*, *let-7*, *mir-84*, and *mir-48* were analyzed throughout *C. elegans* development for seam cell expression. The larval stages shown in each panel denotes the earliest stage at which seam cell expression was first detected for the specified *mir::gfp* construct. White lines indicate positive expressing seam cells. The schematic diagram to the left depicts the characteristic division pattern of the hypodermal seam cells during larval development, in which after each seam cell division, one daughter cell fuses with the hypodermis (hyp7) and the other daughter cell divides again, terminally differentiating at the adult stage (indicated by the horizontal black bars) (Rougvie, 2001).

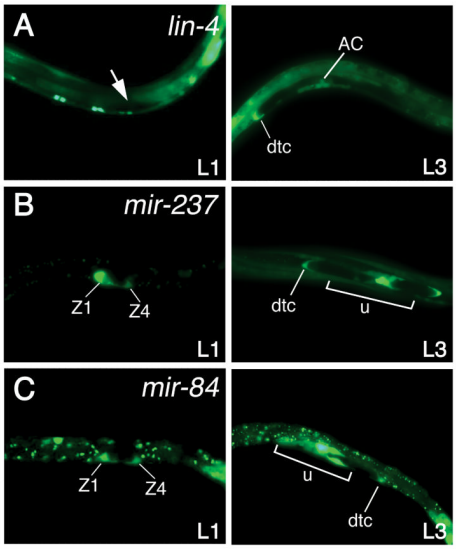


**Figure 4. Distinct temporal and spatial patterning of the *lin-4* and *let-7* family members in the gonad throughout nematode development**

Animals carrying *mir::gfp* fusion constructs for *lin-4* (A), the *lin-4* homologue, *mir-237* (B), and the *let-7* homologue, *mir-84* (C), were analyzed for expression in the developing gonad at the first larval (L1) stage and the third larval (L3) stage 3. Note that *lin-4* was not detected in the gonad at the L1 stage (white arrow). Abbreviations: AC, anchor cell; dtc, distal tip cell; u, uterine cells including the anchor cell; Z1 and Z4, somatic gonad progenitor cells.

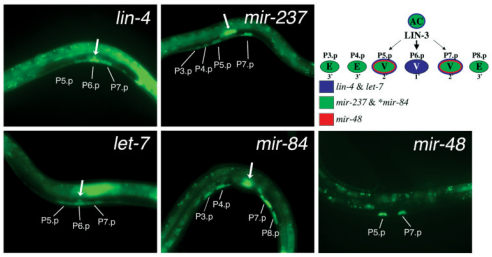


**Figure 5. Differential expression patterns of the *lin-4* and *let-7* family members in the developing vulva and anchor cell**

Animals carrying *mir::gfp* fusion constructs for *lin-4*, the *lin-4* homologue, *mir-237*, *let-7*, and the *let-7* family members, *mir-84* and *mir-48* were analyzed for expression in the vulval precursor cells (VPCs), P3.p-P8.p, and in the anchor cell of the somatic gonad (white arrows) at third larval (L3) stage. A schematic diagram of the spatial expression patterns for the *lin-4* and *let-7* family members during vulva development at the L3 stages is shown to the right. The morphogenic signal, LIN-3, secreted by the anchor cell (AC) induces the closest vulva precursor cell, P6.p, to adopt the 1° cell fate and the two adjacent VPCs, P5.p and P7.p, to adopt the 2° cell fates. 1° and 2° cells will later differentiate into the mature vulva (V). VPCs that are not induced by the anchor cell (P3.p, P4.p, and P8.p) will take on non-vulval 3° cell fates, fuse with the hyp 7 cell, and become part of the outlaying epidermis (E). Our *mir::gfp* fusion studies for *lin-4* (blue), *let-7* (blue), *mir-237* (green), *mir-84* (green), and *mir-48* (red) showed unique and partially overlapping expression patterns in both the anchor cell of the somatic gonad and the VPCs, indicating that these miRNAs may play an important role during cell fate specification and vulva formation. The (*) indicates that *mir-84* shows dynamic expression in the VPCs and by the very late L3 stage, *mir-84* expression was noted exclusively in the P5.p, P6.p, and P7.p cells (Johnson et al., 2005). Note that Pn.p cells depicted in panels for *lin-4*, *let-7*, and *mir-48* have undergone one cell division.

## Table 1
**Oligonucleotide sequences of PCR primers used for the *mir::gfp* constructs**

| PCR primer | Name | Sequence (5′ - 3′) |
|---|---|---|
| *lin-4* forward | LIN4LB3 | CAACAACCCGGGGTCGACGAGACGCCGAGTCTCCC |
| *lin-4* reverse | LIN4LB1 | CAACAAACCGGTAGGCCGGAAGCATAAACTCATAAACC |
| *mir-237* forward | MIR237AK5 | CTGAATCGACTTCTCTAGGAATCC |
| *mir-237* reverse + *gfp* overhang | MIR237AK6 | AGTCGACCTGCAGGCATGCAAGCTCCACGCAATGTAGAAGTTTTGAAC |
| *let-7* forward | LET7SMJ2 | GGTACCCTCCCTCTTTTAAGCCTG |
| *let-7* reverse | LET7SMJ3 | CAACAAACCGGTCCGGATCCACAGTGTAGACCGTCC |
| *mir-84* forward | MIR84UP | CAACAACCCGGGGTGCAACGAGCTCTGGAGCATAAG |
| *mir-84* reverse | MIR84DN | CAACAAACCGGTAGGCAGACGTATGATGAATAGTAG |
| *mir-241* forward | JRP7 | CGGGATCCCGGTGTCGTCGTGTTCTAAATGTTCC |
| *mir-241* reverse | JRP2 | CGGGATCCACTTTGACACCCCCGCGGTTTG |
| *mir-48* forward | JRP6 | CGGGATCCCGCCATATTTTCGATAGCACAGGAAGG |
| *mir-48* reverse | JRP3 | CGGGATCCAGTTCCCGGGAGTTTCAATTGG |