Marquette University
e-Publications@Marquette

Master's Theses (2009 -) Dissertations, Theses, and Professional Projects

MIR-237 is Likely a Developmental Timing Gene that Regulates the L2-to-L3 Transition in C. Elegans

Xi Li
Marquette University

Recommended Citation
Li, Xi, "MIR-237 is Likely a Developmental Timing Gene that Regulates the L2-to-L3 Transition in C. Elegans" (2010). *Master's Theses (2009 -)*. Paper 65.
http://epublications.marquette.edu/theses_open/65

MIR-237 IS LIKELY A DEVELOPMENTAL TIMING GENE THAT REGULATES THE L2-TO-L3 TRANSITION IN *C. ELEGANS*

by

Xi Li, B.S.

A Thesis submitted to the Faculty of the Graduate School,
Marquette University,
In Partial Fulfillment of the Requirements for the Degree of Master of Science

Milwaukee, Wisconsin

August 2010

ABSTRACT

MIR-237 IS LIKELY A DEVELOPMENTAL TIMING GENE THAT REGULATES THE L2-TO-L3 TRANSITION IN *C. ELEGANS*

Xi Li, B.S.

Marquette University, 2010

Development is regulated in both the spatial and temporal dimensions. The developmental timing pathway in *C. elegans* is the most extensively studied timing mechanism. Many components of the pathway are conserved across phyla. Postembryonic development of *C. elegans* is comprised of four larval stages (L1 to L4). The *lin-4* microRNA regulates development from the L1 to L3 stages by repressing the expression of key developmental timing genes: *lin-14* and *lin-28.* Another microRNA, *mir-237,* shares sequence similarity with *lin-4* and they are grouped into one microRNA family. *mir-237* and *lin-4* display closely overlapped temporal expression in the hypodermis. I hypothesize that *mir-237* may function in the same developmental timing pathway as *lin-4.* I used a genetic approach to analyze the relationship between *mir-237* and other important developmental timing genes that regulate development at early stages. Data from genetic analysis suggests that *mir-237* likely functions downstream of *lin-14* and *lin-28,* and in parallel with *lin-46* to control L2-to-L3 transition.

mir-237 expression requires *lin-4* activity. In *lin-4(0)* mutants, *mir-237* expression is reduced. I used quantitative Real-Time PCR and a transcriptional GFP reporter transgene to analyze the mechanism for the regulation of *mir-237* in *lin-4(0)* mutants. My data suggest that this regulation of *mir-237* is largely transcriptional. *lin-14* likely mediates this regulation.

TABLE OF CONTENTS

CHAPTER

I. INTRODUCTION……………………………………………………………………………1

A brief introduction to developmental timing in *C. elegans* ……………………..……….1

Hypodermal cell division and differentiation are regulated by the developmental timing pathway……………………………………….………………………………...2

microRNAs are critical regulators of developmental timing……………….…………. 3

A brief overview of the early developmental timing pathway in *C. elegans* 8

lin-4 regulates the L1 to L3 development by repressing *lin-14* and *lin-28*. …………… 9

lin-4 independent mechanism to repress *lin-28*. ………………………………..……10

lin-14, lin-28, and *hbl-1* are key regulators of the L1 to L3 development …………...…11

The unknown: function of *mir-237* in developmental timing …………………….…..11

A brief summary of major findings …………………………………………………..13

II. MATERIALS AND METHODS…………………………………………………..……14

Nematode Strains …………………………………………………………………..…14

Phenotype Analysis …………………...……………………………………..………..9

RNA isolation, reverse transcription and quantitative Real-Time PCR ………………...10

III. RESULTS …………………………………………………………………………….…………..18

Part I. Genetic evidence reveals that *mir-237* is likely a developmental timing gene…...18

Loss of *mir-237* weakly suppresses *lin-14(0)* and *lin-28(0)* mutant phenotype...18

Loss of *mir-237* enhances the L2 repetition phenotype of *lin-46(0)* mutants…..23

Loss of *mir-237* enhances the bursting vulva phenotype of *mir-48 mir-241(0)* mutants, but not the retarded alae formation phenotype……………………..…24

Loss of *mir-237* does not suppress the precocious alae phenotype of *hbl-1(rf)* mutant…………………………………………………………………...……25

Part II. Repression of *mir-237* in *lin-4(0)* worms is largely transcriptional, and LIN-14 is likely involved in this repression.

Mature miR-237 level is greatly reduced in *lin-4* worms by qRT-PCR microRNA Taqman assay………………………………………..……………………..27

Repression of *mir-237* in *lin-4(0)* worms is largely transcriptional and is likely mediated by *lin-14* or *lin-28*………………………………………………..…..19

IV. DISCUSSION ………………………………………………..………………………...35

mir-237 is a developmental timing gene that regulates the L2-to-L3 transition 35

Repression of *mir-237* in *lin-4(0)* mutants is largely transcriptional……………………37

V. BIBLIOGRAPHY …………………………………………………………….................41

I. INTRODUCTION

A brief introduction to developmental timing in *C. elegans*

Animal development from a single cell requires precise coordination of developmental events such as cell division, differentiation and morphogenesis. In addition to genes that regulate spatial development, there are also genes that control development in the temporal dimensions: Developmental events must be executed in the correct sequence and at the appropriate times. These genes are called developmental timing, or heterchronic genes (Abbott, 2003; Moss, 2007). Developmental timing genes have been identified in a variety of animals as well as plants, and are most intensely studied in *C. elegans* due to its relatively simple and invariant cell lineages (Sulston and Horvitz, 1977). Many important developmental timing genes in the timing pathway of *C. elegans* are conserved across phyla, and their developmental expression patterns also implies conserved function in timing (Rougvie, 2001).

Postembryonic development of *C. elegans* is comprised of four larval stages (L1 to L4) distinguished morphologically by molts and the subsequent formation of the reproductive adult worm. At each stage, worms execute essentially invariant developmental programs that are regulated by developmental timing genes (Abbott, 2003; Ambros and Horvitz, 1984; Ambros and Horvitz, 1987). Misexpression of developmental timing genes can lead to either omission or reiteration of stage-specific developmental programs in multiple tissues, resulting in either precocious or retarded development of those tissues relative to other unaffected tissues. For example, *lin-14* activity is critical to proper execution of the L1 stage program. In *lin-14* loss of function mutants, the L1 stage program is skipped in many cell lineages relative to other normally developed tissues such as the gonad (Ambros and Horvitz, 1984; Ambros and Horvitz, 1987). As a result, certain developmental events that usually occur at the L2 stage, such as L2 stage specific cell division and differentiation pattern, dauer formation, and synaptic rewiring, will occur precociously at the L1 stage in mutant worms (Ambros, 2000). Whereas *lin-14* gain of function or

lin-4(0) worms keep reiterating the L1 stage developmental program in those tissues relative to the normally developing gonad (Ambros and Horvitz, 1984; Ambros and Horvitz, 1987).

<u>Hypodermal cell division and differentiation are regulated by the developmental timing pathway.</u>

Many of the developmental timing genes are studied by analyzing the effects of their mutations in the development of hypodermal tissue, especially a type of hypodermal cells called seam cells. The transparent cuticle of *C. elegans* allows us to keep track of seam cell lineages during development. Seam cells are located along the lateral side of the worm body (Sulston and Horvitz, 1977). The seam cell division and differentiation pattern is precisely regulated at each stage. Seam cells belong to three different cell lineages. Several V-lineage seam cells divide in a stem-cell-like manner with one daughter cell differentiating and the other daughter cell self-renewing (Figure 1). At the L2 stage, the self-renewing daughter cell divides twice. Due to the "L2 proliferative division", we can analyze the progression of developmental programs at the early stages by examining the number of seam cells. At the L4 stage, the self-renewing daughter cells will also terminally differentiate and generate an adult specific cuticle structure during L4 molt called "alae". Alae formation is used as an adult-specific marker to monitor the larva to adult transition.

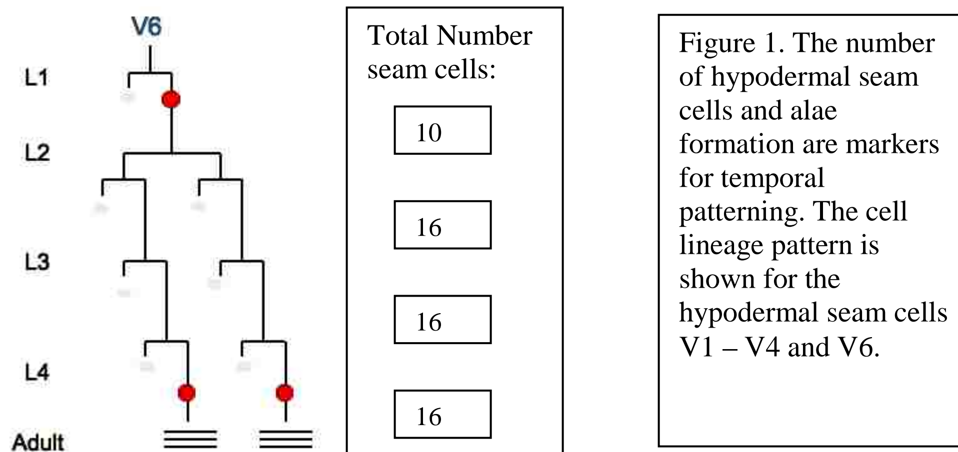


Figure 1. The number of hypodermal seam cells and alae formation are markers for temporal patterning. The cell lineage pattern is shown for the hypodermal seam cells V1 – V4 and V6.

<u>microRNAs are critical regulators of developmental timing.</u>

Some important heterochronic genes encode microRNAs. For example, the first two identified microRNAs, *lin-4* and *let-7,* are key players in the regulation of development at early and late stages, respectively (Lee et al., 1993; Reinhart et al., 2000). Studies on the developmental timing pathway also provide the opportunity for improving our understanding about microRNA functions during development.

microRNAs are short (20-23 nucleotide), endogenous, single-stranded RNA molecules. To briefly summarize microRNA biogenesis: 1) microRNA genes are transcribed by RNA polymerase II or RNA polymerase III into pri-microRNA, 2) The pri-microRNA is next endonucleolytically cleaved by Drosha to generate a shorter stem loop, the microRNA precursor (pre-microRNA), 3) The pre-microRNA is exported out of the nucleus, processed by the RNase Dicer. 4) The mature microRNA is then loaded into RNA-induced silencing complex (RISC) where it silences target mRNAs (Winter et al., 2009) (Figure 2).

miRNAs can silence target mRNAs through multiple mechanisms, including degradation of the target mRNA, inhibition of translation initiation, inhibition of translation elongation (Figure 7).

In most cases, the recognition of a microRNA and its target mRNAs is mediated by the complementary binding sites in the 3' UTR of the target mRNAs. One microRNA can regulate multiple target genes. For example, *lin-4* microRNA represses expression of both *lin-14* and *lin-28* through binding to the complementary sites in their 3' UTR (Wightman et al., 1993; Moss et al., 1997). An average microRNA is predicted to bind approximately 100 target sites (Brennecke et al., 2005).

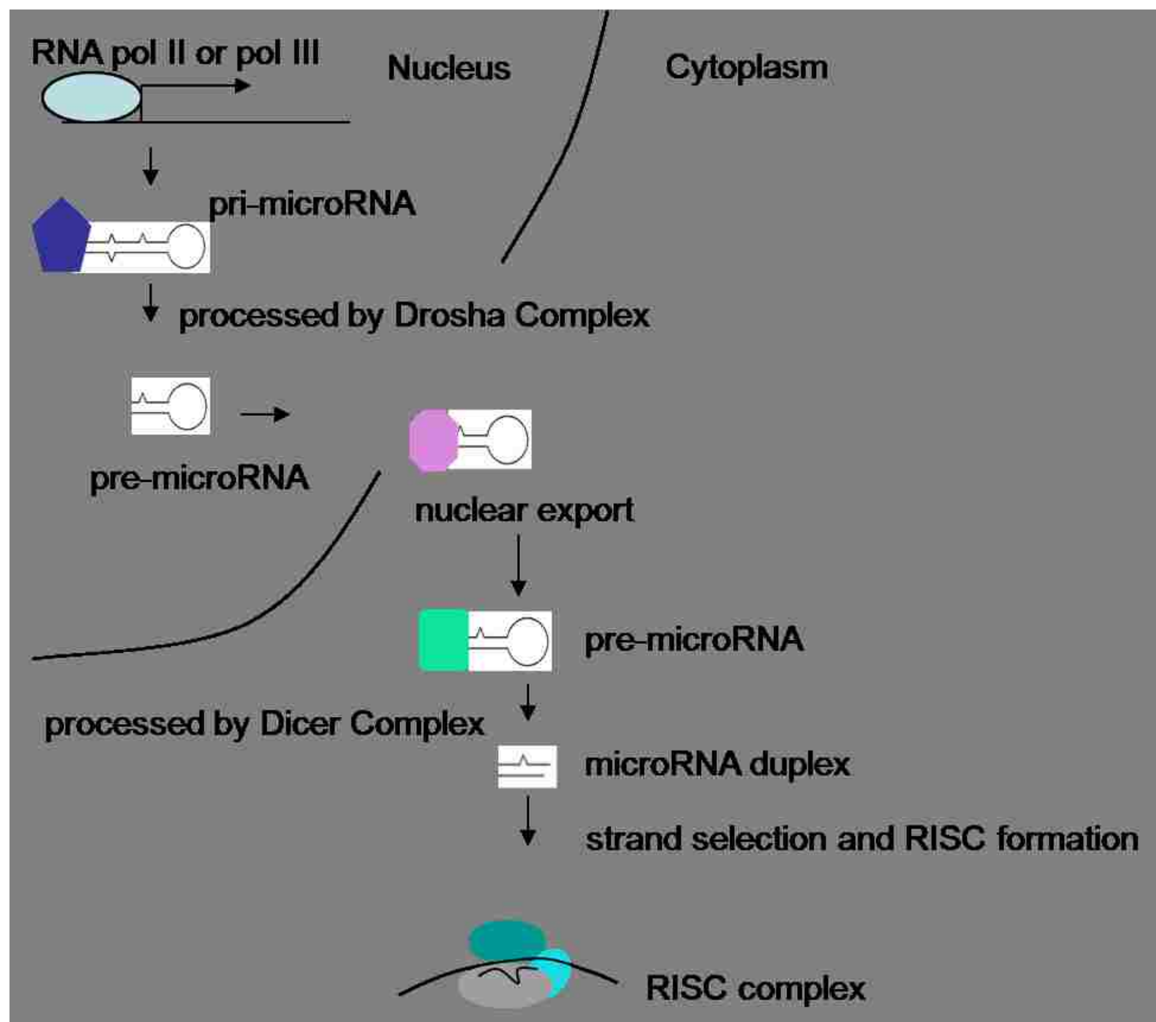
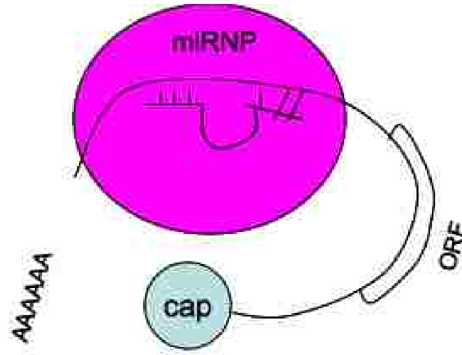
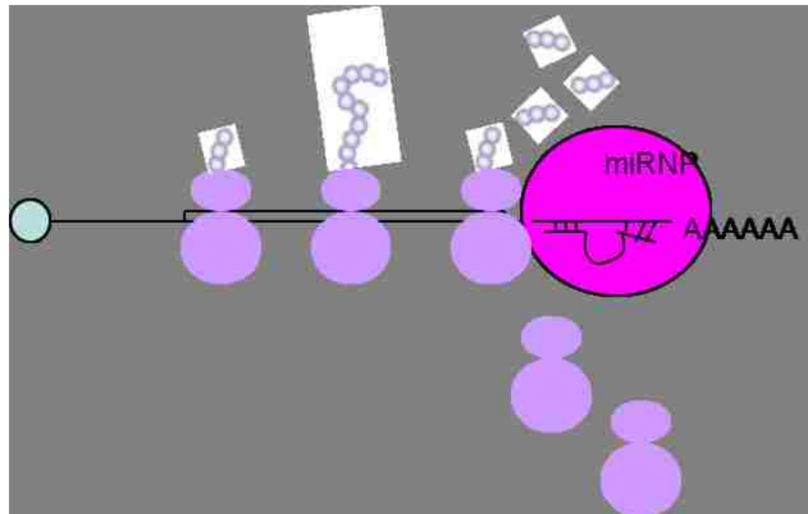


Figure 2. Biogenesis of miRNAs and formation of RISC complex.

(A)



(B)



(C)

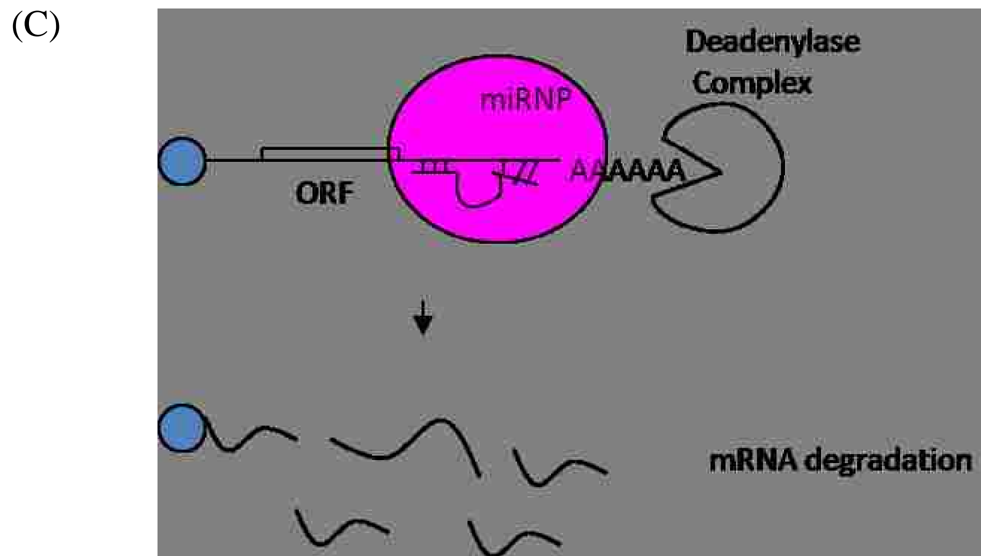


Figure 3. Possible mechanisms of microRNA mediated silencing. (miRNP : microRNAs function in the form of ribonucleoprotein complexes)

(A) Inhibition of translation initiation. (miRNP prevents the formation of translation initiation complex)
(B) Inhibition of translation elongation. (Such mechanisms include slowed or stalled elongation, ribosome drop-off/ premature termination, and cotranslational degradation of the nascent polypeptide.)
(C) Deadenylation of target mRNAs followed by mRNA degradation.

The 2-8 nucleotides sequence from the 5' region of the microRNAs are termed the "seed" region. microRNAs are grouped into families based on sharing a seed region (Bushati and Cohen, 2007). The microRNA-target recognition largely depends on the complementary binding between the microRNA 5' seed region and the target mRNA (Brennecke et al., 2005). In vitro studies suggest that the seed region interacts extensively with the PIWI domain of the argonaute protein, which is the central catalytic component of RISC. This interaction results in correct geometry to position the target strand within the catalytic site, which likely mediates the target strand degradation or translational repression (Bartel, 2009; van den Berg et al., 2008; Parker et al., 2006; Doench et al., 2003) (Figure 8). In vivo studies also confirmed that microRNA family members could function together in one common biological process and their function could be overlapping or redundant (Abbott et al., 2005).

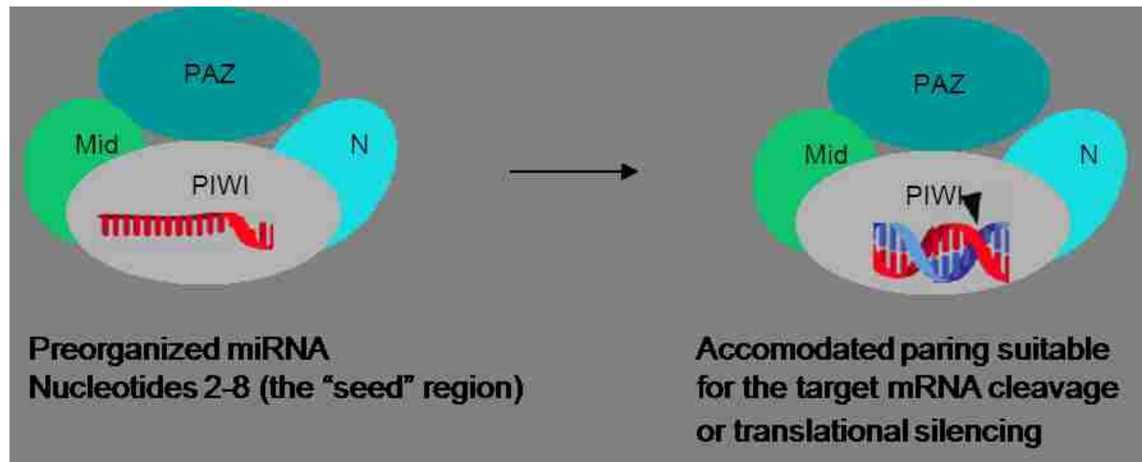


Figure 4. A schematic diagram showing the role of the "seed" region in microRNA-target recognition and microRNA mediated silencing.

However, the mechanism of microRNA mediated silencing is not fully understood. It is found that for some microRNAs, the 3' ends are determinants of target specificity within microRNA families (Brennecke et al., 2005). Possibilities remain that sequences other than the seed region are also responsible for the repression by a microRNA. Therefore, microRNA family members may have distinct targets and function. Whether microRNA family members are functionally equivalent needs further investigation.

A brief overview of the early developmental timing pathway in *C. elegans*

A simplified model of the heterochronic gene pathway in early developmental transitions from the L1 stage to the L3 stage is shown in Figure 5.

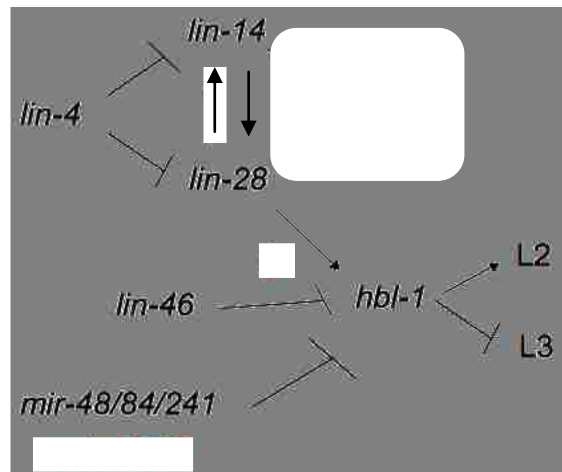


Figure 5. A simplified model of the heterochronic gene pathway in early developmental stages (L1 to L3)

lin-14 is the major regulator of the L1 stage development. *lin-4* microRNA accumulates at early stages, which causes down-regulation of *lin-14* (Feinbaum and Ambros, 1999). Reduction of LIN-14 levels trigger the L1 to L2 transition (Ambros and Horvitz, 1987; Holtz and Pasquinelli, 2009). *lin-14, lin-28, lin-46,* and the three *let-7* family members *mir-48/84/241* all function to regulate the L2 to L3 development (Ambros and Horvitz, 1987; Ambros and Horvitz,

1984; Pepper et al., 2004; Abbott et al., 2005). *lin-14* and *lin-28* positively regulate each other through a feedback loop (Seggerson et al., 2002). *lin-28* positively regulates the downstream gene *hbl-1*. *lin-46* and *lin-28* have opposing activities and they may converge on *hbl-1.* (Pepper et al., 2004). The three *let-7* family members *mir-48/84/241* function in parallel to *lin-28* and *lin-46* to regulate *hbl-1* (Abbott et al., 2005). At the L2 stage, *lin-4* expression is further up-regulated and LIN-14 level is further lowered. Due to the regulation by *lin-4* and *lin-14,* LIN-28 level is reduced, and HBL-1 level is decreased, which triggers the L2 to L3 development (Abrahante et al., 2003; Moss et al., 1997)

lin-4 regulates the L1 to L3 development by repressing *lin-14* and *lin-28.*

lin-4 microRNA down-regulates the expression of its targets *lin*-14 and *lin-28* through their 3'UTR (Wightman et al., 1993; Moss et al., 1997). *lin-14* mRNA 3'UTR contains seven complementary sites for *lin-4* and its repression is closely associated with the increased expression of *lin-4* (Wightman et al., 1993; Holtz and Pasquinelli, 2009). The expression of *lin-4* is not detected at hatching, increases during the L1 to L2 transition, and peaks at the mid L3 stage (Feinbaum and Ambros, 1999). LIN-14 protein expression is highest after hatching, decreases during the L1 to L2 transition, and becomes almost undetectable by the early L2 stage (Feinbaum and Ambros, 1999; Ruvkun et al., 1989).

lin-4 represses *lin-28* through a single binding site in its 3'UTR (Moss et al., 1997), which may account for the difference in the temporal pattern of *lin-14* and *lin-28* down-regulation. LIN-28 protein expression is abundant at L1, decreases during L2 to L3 transition, and becomes undetectable by L3 stage (Moss et al., 1997).

The temporal expression patterns of *lin-4, lin-14* and *lin-28* are shown in Figure 6.

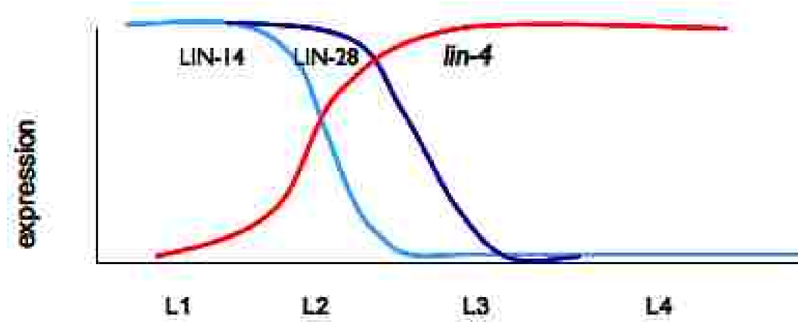


Figure 6. Temporal expression patterns of *lin-4, lin-14* and *lin-28.*

<u>*lin-4* independent mechanism to repress *lin-28*</u>

lin-28 is also indirectly regulated by *lin-14,* independently of *lin-4.* In *lin-4(0); lin-14(ts)* mutants, at the restrictive temperature of 20˚C, LIN-14 level is similar to that in the wild type worms, and LIN-28 level is down-regulated despite the absence of *lin-4.* The mechanism for this repression is not clear. The LIN-28 protein level decreases to a much bigger extent compared to the reduction of the *lin-28* mRNA level (Bagga et al., 2005, Seggerson et al., 2002). The sucrose gradient profile of *lin-28* mRNAs associated with polysome remains unchanged in *lin-4(0); lin-14(ts)* mutants, suggesting that this repression of *lin-28* by *lin-14* may occur after translation initiation (Seggerson et al., 2002). A GFP reporter transgene assay showed that the 3'UTR of *lin-28* is required for this *lin-4* independent regulation by *lin-14* (Seggerson et al., 2002). The above evidence leads to the hypothesis that a microRNA may be involved in the *lin-4* independent repression of *lin-28.*

lin-14, lin-28, and *hbl-1* are key regulators of the L1 to L3 development

lin-14 plays a crucial role in regulating the L1 stage development. It encodes a DNA binding protein that likely functions as a transcription factor (Hristova et al., 2005). High level of LIN-14 ensures the execution of the L1 stage program. There are three differentially spliced LIN-14A and LIN-14B1/B2 protein isoforms. They show the same temporal expression pattern and function, but they are different in the amount of expression and activity (Reinhart and Ruvkun, 2001). The overall level of LIN-14 protein is the critical determinant of temporal cell fate: at the L1 stage, the total amount of LIN-14A and LIN-14B activity is robust and this allows the execution of the L1 stage program. At the L2 stage, the level of LIN-14 goes down to a moderate level. And this level helps to maintain high level of *lin-28* expression, which is required for execution of the L2 stage program. *lin-28* encodes an RNA binding protein that could function to regulate translation or RNA processing (Winter et al., 2009). In *lin-28(0)* mutants*,* the L2 stage program is skipped and worms show adult characteristics precociously at the L4 stage (Ambros and Horvitz, 1984). In *lin-28(gf)* mutants*,* the L2 stage program is reiterated and worms display retarded development (Moss et al., 1997).

hbl-1 is the most downstream key regulator of the L2 to L3 stage development. Knockdown of *hbl-1* by RNAi causes the worms to display reduced number of seam cells, indicating a partial omission of the L2 stage program (Abrahante et al., 2003).

The unknown: function of *mir-237* in developmental timing

mir-237 is a *lin-4* microRNA family member in *C. elegans.* A study using transcriptional *mir::gfp* reporter showed that *lin-4* and *mir-237* display distinct but overlapping temporal and spatial expression patterns (Esquela-Kerscher et al., 2005).

In hypodermal cells, *lin-4* expression is first detected at the late L1 stage, and is up-regulated at the early L2 stage with peak expression at the L3 stage. In contrast, *mir-237* is first

detected at the early L2 stage, and is upregulated at the early L3 stage with peak expression at the L4 stage (Figure 7).

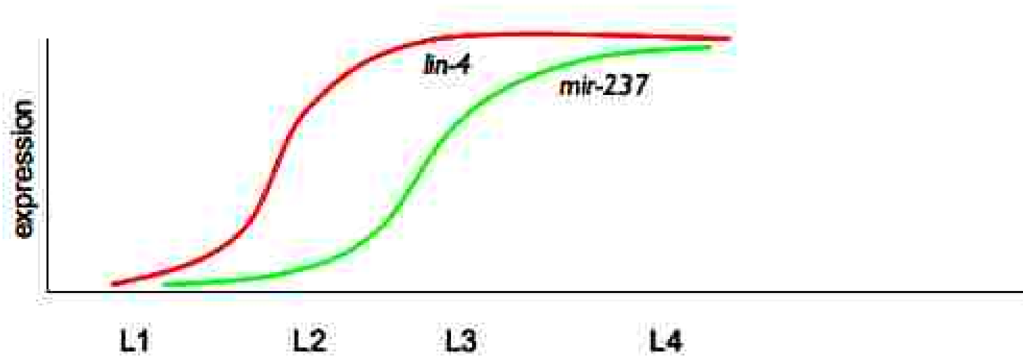


Figure 7. Temporal expression of *lin-4* and *mir-237* in the hypodermis.

The overlapping temporal and spatial expression patterns of the two *lin-4* microRNA family members imply that they may have common targets and may function in the same pathway. The *lin-4* microRNA is a key regulator in the early developmental timing pathway. It is unknown whether *mir-237* also controls early developmental timing. Loss of *mir-237* alone does not cause an observable mutant phenotype (Miska et al., 2007). Evidence is lacking so far that supports its role in regulating developmental timing at the early stages. Based on the temporal expression profile of *mir-237,* I hypothesized that *mir-237* may regulate the L2 to L3 transition. I used a genetic approach to analyze the genetic relationship between *mir-237* and other developmental timing genes at the early stages.

In addition, northern blot showed that in *lin-4(0)* mutants, mature miR-237 level is significantly reduced. Other microRNAs such as the three *let-7* family microRNAs *mir-48/84/241* are not affected as much, suggesting that the repression of *mir-237* in *lin-4(0)* mutants is specific. But the underlying mechanism is not known yet. One microRNA affecting the activity of its own

family member hasn't been investigated so far. It is unknown whether this *mir-237* repression in *lin-4(0)* mutants is at the transcriptional level or post-transcriptional level. *lin-14* and *lin-28* are the targets identified for *lin-4.* Both LIN-14 and LIN-28 level are high in *lin-4(0)* mutants (Wightman et al., 1993; Moss et al., 1997). It is unknown whether either LIN-14 or LIN-28 can regulate *mir-237* levels. I used quantitative Real-Time PCR and a transcriptional GFP reporter transgene to address these questions.

A brief summary of major findings

My data indicate that *mir-237* is likely a developmental timing gene in the early timing pathway to regulate the L2-to-L3 transition. In addition, the repression of *mir-237* in *lin-4(0)* mutants is largely at transcriptional level.

II. MATERIALS AND METHODS:

Nematode Strains

C. elegans strains were grown under standard conditions as described (Wood,1988). Strains used are listed in Table 1. The wild type strain used was var. Bristol N2 (Brenner, 1974). Strains were grown at 20˚C, except where otherwise indicated. Double and triple mutant strains were built by standard genetic crossing. For genotyping mutant strains, mutant alleles were identified by performing PCR reactions that amplified the genomic region flanking the mutations. Then the amplified region was either sequenced (Functional Biosciences) or characterized by its size to confirm that it was the mutant allele. For the sequences of primers used, see Table 2.

Phenotype Analysis

1. L4 molt lethality: the number of L4m-stage worms that burst at the vulva were scored.
2. Seam cell assays: larval stages were assessed by gonad morphology. Worms were anesthetized with 1mM levamisole and then mounted on agarose pads for viewing. The number of lateral hypodermal seam cells was counted at specific larval stages using fluorescence microscopy. Seam cells were identified by GFP expression from *wIs78 IV,* which contains the seam cell specific marker *scm-1:gfp* that marks seam cell nuclei (Terns et al., 1997). *t*-test is preformed to analyze the results of the seam cell assays.
3. Alae formation assays: Adult lateral alae formation was scored using Nomarski DIC microscopy. Alae and seam cells were scored on one side of individual animals. Chi-square is performed to analyze the results of the alae formation assays.

4. Transcriptional GFP reporter assays: To analyze transcriptional regulation of *mir-237,* strains were built with a transgene that contained the 2kb promoter region for *mir-237* fused to gfp *(mals135, Pmir237::gfp)* (Martinez et al., 2008a). Images of the *mir-237* transcriptional GFP reporter in the hypodermal tissue for all strains were taken at the same exposure for each stage. 0.982, 0.39, 0.055 are the used exposure for L1, L3 and L4 stage, respectively. For hypodermal tissue of each worm, DIC and fluorescence images were taken at the same plane of focus.

RNA isolation, reverse transcription and quantitative Real-Time PCR

Total RNA was isolated from staged populations of worms as previously described (Lee and Ambros, 2001). Two sets of RNA samples were staged and collected independently. The integrity of the isolated RNA was examined by agarose gel electrophoresis. The concentration of the RNA was measured by NanoDrop spectrophotometer. Mature *mir-237* level was measured by TaqMan MicroRNA Assays (Applied Biosystems) according to the manufacturer's protocol. 1 to 10 ng of total RNA was used in each 15 - μL RT reaction. To prepare the Real-Time PCR reaction, the cDNA product was diluted 1:15. All reactions were run in triplicate. snoRNA (sn2429) was the endogenous control used to normalize the expression levels of *mir-237.* For both sets of RNA, Real-Time PCR assay was performed using both BioRad MyiQ Single-Color Real-Time PCR detection system and the Applied Biosystems StepOnePlus Real-Time PCR Systems. Real-Time PCR results were analyzed using the comparative C_T method (Thomas D Schmittgen & Kenneth J Livak, 2008).

Table 1. List of strains

Strain	Genotype
RG733	*wIs78 IV* (It contains the seam cell specific marker *scm-1::gfp*)
RF168	*mir-237(n4296)X; wIs78 IV*
RF173	*lin-14(ma135)X; wIs78 IV*
RF167	*lin-14(ma135) mir-237(n4296)X; wIs78 IV*
RF204	*lin-28(n719)I; wIs78 IV*
RF205	*lin-28(n719)I; mir-237(n4296)X; wIs78 IV*
RF190	*lin-28(n719)I*
RF191	*lin-28(n719)I; mir-237(n4296)X*
RF194	*mir-48 mir-241(nDf51)V*
RF195	*mir-48 mir-241(nDf51)V; mir-237(n4296)X*
RF200	*lin-46(ma164)V*
RF201	*lin-46(ma164)V; mir-237(n4296)X*
RF303	*lin-46(ma164)V; wIs78 IV*
RF304	*lin-46(ma164)V; mir-237(n4296)X; wIs78 IV*
RF404	*hbl-1(ve18)X*
RF405	*hbl-1(ve18) mir-237(n4296)X*
VT1113	*Pmir-237::gfp*
RF305	*lin-4(e912)Π;Pmir-237::gfp*
RF306	*lin-4(e912)Π; lin-14(n179)X;Pmir-237::gfp*
RF366	*lin-14(n179)X;Pmir-237::gfp*
RF446	*lin-14(n536)X;Pmir-237::gfp*

Table 2. List of sequences of primers used in genotyping the mutant alleles.

Primer Name	mutant allele	Primer Position[a]	Sequence
AA415	*mir-237(n4296)*	F1	5' – gaatgtacaaaaagttaatgccgactc – 3'
AA416		R1	5' – aagatttaaaaatgagagatcacatgg – 3'
AA417		R2	5' – ccgtcgacgattatctaacacttacta – 3'
AA153	*lin-46(ma164)*	F1	5' – tcggaacaaagagagagatcg – 3'
AA154		R1	5' – cccttagaaacttcgcttcg – 3'
AA155		S1	5' – gtcgaaacggtgaagtttcc – 3'
AA167	*hbl-1(ve18)*	F1	5'- accacgagggagagtttgtg-3'
AA170		R1	5; - tgaatcttttctccgggttg – 3'
AA169		S1	5'- caacctctcctatttgcatgg-3'
AA34	*lin-14(n536)*	F1	5'- taactatatggatgccacgc-3'
AA35		R1	5'- tgcttctgaatgaggtgaag-3'
AA37		R2	5'- caatcctaagcaatagaggt-3'

[a]. For all deletion alleles, F1, R1, and R2 were used: F1 was the 5' forward primer. R1 was the 3' reverse primer that localized outside of the deletion region. R2 was the 3' reverse primer that localized inside the deletion region. For other alleles, F1 and R1 were used to amplify the region covering the mutation site, and S1 was used as the sequencing primer.

III. RESULTS

Part I. Genetic evidence reveals that *mir-237* is likely a developmental timing gene.

Loss of *mir-237* weakly suppresses *lin-14(0)* and *lin-28(0)* mutant phenotype

I first tested whether *mir-237* interacted genetically with *lin-14.* I proposed that if *mir-237* functions downstream of *lin-14,* then loss of *mir-237* may partially suppress the precocious phenotype of *lin-14(0)* mutant worms.

lin-14(0) worms showed complete alae formation at the L3 molt. In contrast, in *lin-14(0)mir-237(0)* mutants, about 10% of worms showed incomplete alae formation at the L3 molt. Representative pictures of the incomplete alae in those double mutants are shown in Figure 8. The incomplete alae formation could result from defect in timing, or defect in seam cell differentiation or collagen generation. If complete alae formation is observed later at the L4 molt, then that indicates that the incomplete alae formation in the double mutants is a developmental timing defect. Future work is needed to show whether complete alae is formed in the double mutants at the L4 molt. The suppression of the precocious alae formation phenotype in *lin-14(0)mir-237(0)* double mutants suggests that *mir-237* may function downstream of *lin-14.*

lin-14(0) mutants have increased seam cell number at the L1 stage due to precocious execution of the L2 proliferation seam cell division program. I found that there was no difference in the number of seam cells between the single mutant and the double mutants (Table 3). It is possible that seam cell division is regulated by a more robust network of genes that can tolerate small fluctuations. Therefore, loss of *mir-237* isn't sufficient to cause changes in the seam cell division program in the *lin-14(0)* mutants, but is sufficient to cause small changes in alae formation.

I next tested whether *mir-237* interacted genetically with *lin-28.* I proposed that *mir-237* functions downstream of *lin-28.* Thus, loss of *mir-237* may partially suppress the precocious phenotype of *lin-28(0)* mutant worms.

lin-28(0) worms showed complete alae formation at the L3 molt. In contrast, I found that in *lin-28(0)mir-237(0)* mutants, about 19% of worms displayed incomplete alae at the L3 molt (Table 3). Representative pictures of the incomplete alae in those double mutants are shown in Figure 9. Future work is needed to show whether the double mutants form complete alae at the L4 molt.

The seam cell number in the *lin-28(0)* mutants is reduced due to omission of the L2 proliferation program (Abbott et al., 2005). I found that there was no difference in the number of seam cells between the single mutant and the double mutants. Loss of *mir-237* alone wasn't sufficient to affect the seam cell division program in the *lin-28(0)* mutants (Table 3).

Occasionally, branched alae was found in *lin-14; mir-237* mutants. Branched alae formation is mostly due to defective collagen genes or defects in collagen secretion (Zugasti et al., 2005; Eschenlauer and Page, 2003; Kostrouchova et al., 1998). This suggests that *lin-14* and *mir-237* may regulate certain collagen genes or genes that regulate collagen secretion.

My data indicate that *lin-14(0)* or *lin-28(0)* precocious phenotype is partially dependent on *mir-237* activity, and that *lin-14* and *lin-28* may function upstream of *mir-237*.

Table 3. Phenotype Analysis

Strain	Genotype		Alae Synthesis (% of worms) @L3 molt			Alae Synthesis (# of worms) @L4 molt			Average Seam Cell Number (n): number of worms scored			
			none	gapped	complete	none	gapped	complete	L1	L2	L3	L4
RG733	*wIs78 IV*	*wIs78 IV*	100	0	0	0	0	100	10(7)	16(12)	16(10)	16(11)
RF168	*mir-237(n4296)X; wIs78 IV*	*mir-237(0)X; wIs78 IV*	100	0	0	0	0	100	10(19)	16(20)	16(19)	16(19)
RF173	*lin-14(ma135)X; wIs78 IV*	*lin-14(0)X; wIs78 IV*	0	0	100				16(20)	15(18)	16(20)	15(21)
RF167	*lin-14(ma135) mir-237(n4296)X; wIs78 IV*	*lin-14(0) mir-237(0)X; wIs78 IV*	0	10	90				15(23)	16(15)	15(19)	16(19)
RF190	*lin-28(n719)I*	*lin-28(0)I*	0	0	100							
RF191	*lin-28(n719)I; mir-237(n4296)X*	*lin-28(0)I; mir-237(0)X*	0	19	81							
RF204	*lin-28(n719)I; wIs78 IV*	*lin-28(0)I; wIs78 IV*							10(19)	11(19)	12(21)	11(21)
RF205	*lin-28(n719)I; mir-237(n4296)X; wIs78 IV*	*lin-28(0)I; mir-237(0)X; wIs78 IV*							10(15)	11(21)	11(20)	11(19)
RF194	*mir-48 mir-241(nDf51)V*	*mir-48 mir-241(0)V*				0	86	14				
RF195	*mir-48 mir-241(nDf51)V; mir-237(n4296)X*	*mir-48 mir-241(0)V; mir-237(0)X*				0	89	11				
RF200	*lin-46(ma164)V* @15˚C	*lin-46(0)V* @15˚C				0	89	11				
RF201	*lin-46(ma164)V; mir-237(n4296)X* @ 15˚C	*lin-46(0)V; mir-237(0)X* @ 15˚C				0	100	0				
RF303	*lin-46(ma164)V; wIs78 IV*@ 15˚C	*lin-46(0)V; wIs78 IV*@ 15˚C									18(21)	18(21)
RF304	*lin-46(ma164)V; mir-237(n4296)X; wIs78 IV* @ 15˚C	*lin-46(0)V; mir-237(0)X; wIs78 IV* @ 15˚C									20(21)	21(21)
RF404	*hbl-1(ve18)X; wIs78 IV*	*hbl-1(rf)X; wIs78 IV*									16(36)	23(19)
RF405	*hbl-1(ve18) mir-237(n4296)X; wIs78 IV*	*hbl-1(rf) mir-237(0)X; wIs78 IV*									16(37)	22(20)

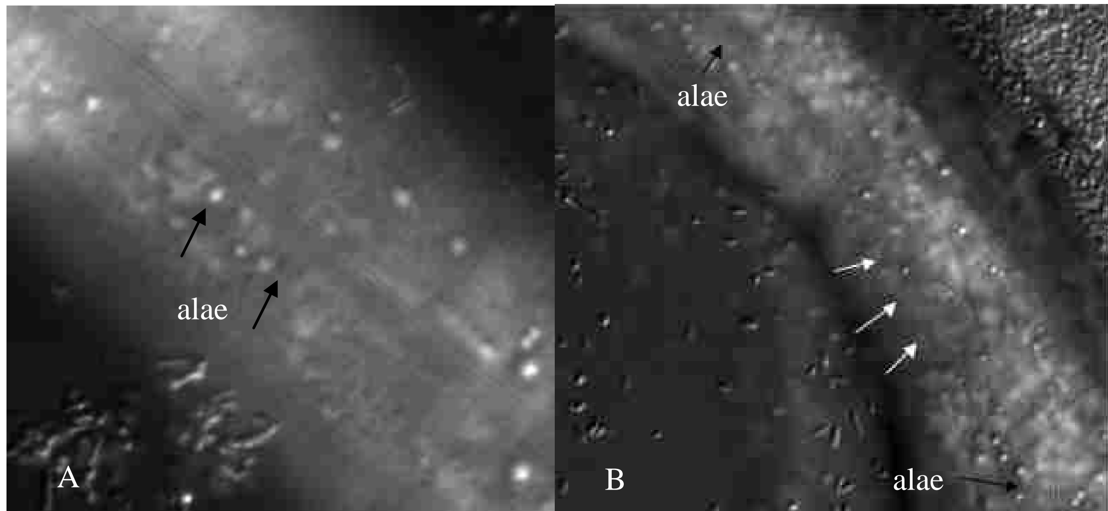


Figure 8. (A) An example of complete alae. (B) Incomplete alae in *lin-14(0)mir-237(0);wls78* mutants at the L3 molt. (Black arrows point to the region where alae formed; white arrows point to the region where alae didn't form)

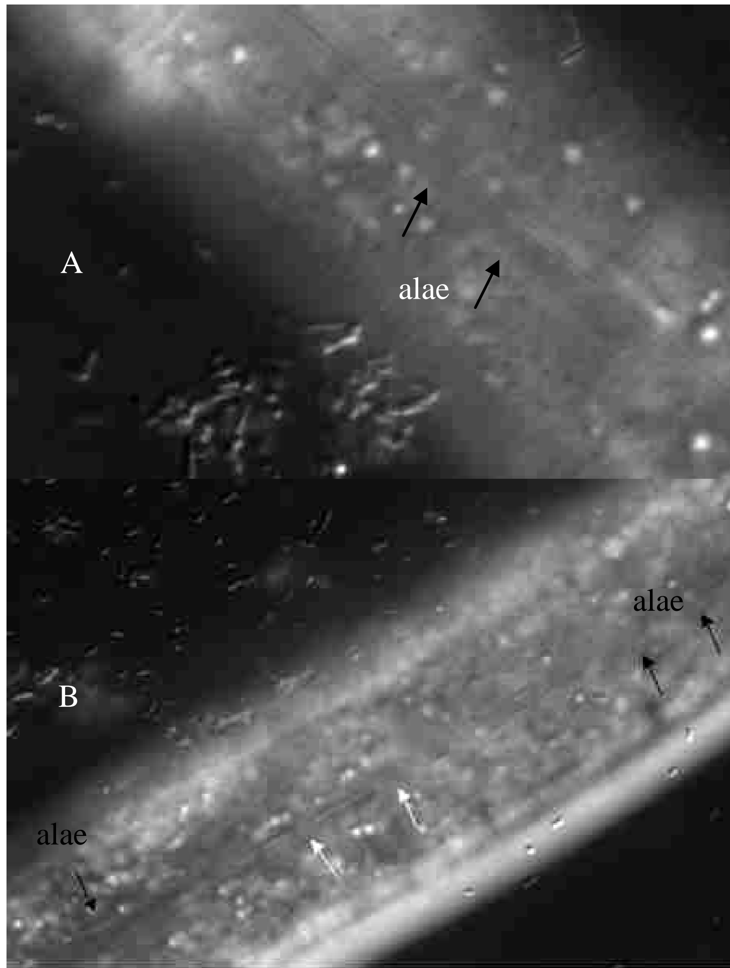


Figure 9. (A) An example of complete alae. (B) Incomplete alae in *lin-28(0)mir-237(0)* mutants at the L3 molt. (Black arrows point to the region where alae formed; white arrows point to the region where alae didn't form)

<u>Loss of *mir-237* enhances the L2 repetition phenotype of *lin-46(0)* mutants</u>

lin-46 regulates developmental timing at the L2 to L3 transition. It encodes a scaffolding protein that may function to potentiate the activity of a developmental timing complex (Pepper et al., 2004). The molecular function of the LIN-46 protein is not clear. The developmental timing phenotypes of *lin-46(0)* worms are cold sensitive. This cold sensitivity is not a property of the allele but rather the process regulated by the LIN-46 protein. At 20˚C, some *lin-46(0)* worms form incomplete alae at the L4 molt. At 15˚C, a greater percentage of the *lin-46(0)* worms display incomplete alae. In addition, although at 20˚C *lin-46(0)* worms have normal number of seam cells, at 15˚C they have increased number of seam cells.

To determine whether *mir-237* functions with *lin-46* to regulate the L2 to L3 development, I compared the alae formation at the L4 molt as well as the number of seam cells at L3 and L4 stages in *lin-46(0)* strain and in *lin-46(0); mir-237(0)* strain.

At 15˚C, I found that 10% of *lin-46(0)* worms had complete alae at the L4 molt, whereas none of the double mutants formed complete alae (Table 3). And the gapped regions in the double mutant worms were mostly much bigger than that in the single mutants. This suggests that the retarded alae formation phenotype in *lin-46(0)* worms was enhanced in *lin-46(0); mir-237(0)* mutants.

At 15˚C, *lin-46(0)* worms had an average of 18 seam cells after L2 stage, whereas the double mutants displayed about 20 seam cells (Table 3). Statistical analysis of both the alae formation and the seam cell number indicated that the two strains were significantly different ($p<0.001$).

These data suggest that *mir-237* likely functions in parallel with *lin-46* to regulate the L2-to-L3 transition.

<u>Loss of *mir-237* enhances the bursting vulva phenotype of *mir-48 mir-241(0)* mutants, but not the retarded alae formation phenotype.</u>

The *let-7* family microRNAs *mir-84, mir-48* and *mir-241* regulate development from the L2 to the L3 stage (Abbott et al., 2005). Loss of *mir-48* and *mir-241* leads to partial reiteration of the L2 stage program. Thus, the mutant worms display incomplete alae formation at the L4 molt and an increased number of seam cells. *mir-48 mir-241(0)* mutants also have bursting vulva phenotype at adult stage. It is not exactly clear what can cause the vulva to burst. It can result from improper connection among vulva tissue and nearby tissue such as hypodermis and gonad, leading to vulva morphogenesis defects at L4 molt. Timing defects in the hypodermis can cause vulva to burst (Reinhart et al., 2000; Li et al., 2005).

To determine whether *mir-237* functions together with *mir-48 mir-241* to control early development, I compared developmental timing defects between *mir-48 mir-241(0)* mutants and *mir-48 mir-241(0);mir-237(0)* triple mutants.

About 89% of the triple mutants formed gapped alae at the L4 molt, compared to 86% in the *mir-48 mir-241(0)* worms (Table 3). No significant difference was found between the defects of those two strains, suggesting that loss of *mir-237* activity does not enhance the retarded alae formation phenotype of *mir-48 mir-241(0)* mutants. Seam cells were not scored in these strains.

On the other hand, about 24% of *mir-48 mir-241(0)* mutants displayed the bursting vulva phenotype as young adults, whereas 43% of the triple mutant worms had bursting vulva. The enhancement of bursting vulva phenotype in the triple mutants suggests that *mir-237* functions together with *mir-48* and *mir-241* to regulate vulva formation. It is possible that *mir-237* functions together with *mir-48 mir-241* to regulate the L2 to L3 development in the hypodermal tissue which ensures normal vulva morphogenesis later at the L4 molt.

<u>Loss of *mir-237* does not suppress the precocious alae phenotype of *hbl-1(rf)* mutant</u>

hbl-1 is also an important regulator of the L2 to L3 development. *hbl-1(rf)* reduction of function mutants form complete alae precociously at the L3 molt. The seam cells in these mutants, however, do not permanently exit from the division cycle at the L3 molt, and they divide again at the L4 stage, resulting in an increase in the number of seam cells.

To determine whether there is a genetic interaction between *hbl-1* and *mir-237,* I compared the number of seam cells in *hbl-1(rf)* strain and *hbl-1(rf) mir-237(0)* strain at the L3 and L4 stages. At the L3 stage, both strains had about 16 seam cells. At the L4 stage, *hbl-1(rf)* mutants had an average of 22.5 seam cells and *hbl-1(rf) mir-237(0)* mutants had an average of 22.4 seam cells (Table 3). Statistical analysis indicated that there was no significant difference between the two. These data are consistent with the model that *mir-237* functions upstream of *hbl-1*.

The above genetic data suggest that *mir-237* functions downstream of *lin-14* and *lin-28,* in parallel to *lin-46,* and upstream of *hbl-1* (Figure 10). Next, I investigated the mechanism of the repression of *mir-237* expression in *lin-4(0)* mutants. Consistent with my genetic data, I proposed that *lin-4* may regulate *mir-237* through *lin-14* or *lin-28*: *lin-4* negatively regulates *lin-14* or *lin-28* activity, and *lin-14* or *lin-28* negatively regulates *mir-237* expression. First I analyzed whether this repression of *mir-237* in *lin-4(0)* mutants is at transcriptional level or post-transcriptional level. Then I tested whether *lin-14* or *lin-28* is likely involved in this repression.

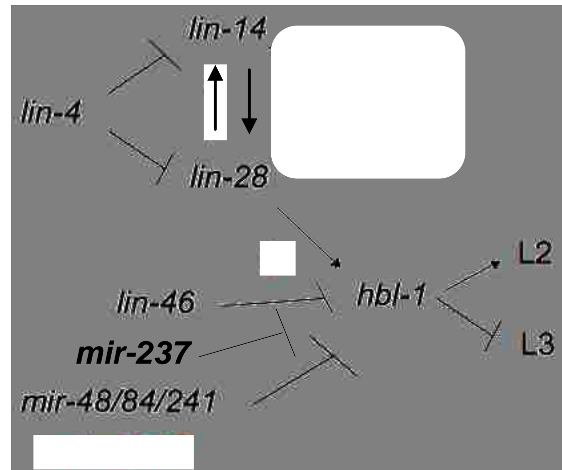


Figure 10. Model for the role of *mir-237* in the early developmental timing pathway based on the above genetic analysis. Depicted are the regulatory relationships among those early developmental timing genes.

Part II. Repression of *mir-237* in *lin-4(0)* worms is largely transcriptional, and LIN-14 is likely involved in this repression.

Mature miR-237 level is greatly reduced in *lin-4(0)* worms by qRT-PCR microRNA Taqman assay.

A previous study using northern blot analysis showed that *mir-237* accumulation was significantly reduced in *lin-4(0)* worms in comparison to wild type worms (Esquela-Kerscher et al., 2005). To quantify *mir-237* expression, I used quantitative Real-Time PCR to measure the mature miR-237 level at L3 and L4 stages in both strains. I found about 5 fold less miR-237 in *lin-4(0)* worms than wild-type worms at both L3 and L4 stage (Figure 11).

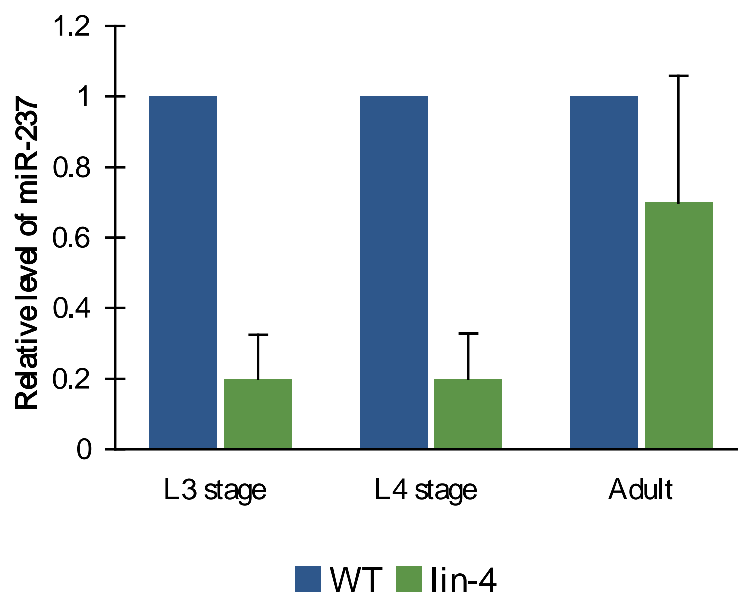


Figure 11. *lin-4(0)* worms display a reduced level of miR-237 relative to wild type worms. The analysis was performed twice using two independently isolated RNA preparations.

<u>Repression of *mir-237* in *lin-4(0)* worms is largely transcriptional and is likely mediated by *lin-14* or *lin-28.*</u>

qRT-PCR measures the ~22 nucleotide mature miR-237. Reduced mature miR-237 could result from reduced transcription of the *mir-237* primary transcript or from reduced Drosha or Dicer processing of the pri-microRNA or pre-microRNA. To differentiate between these two possibilities, I used an integrated transcriptional GFP reporter of *mir-237* (*Pmir-237::gfp*) that was shown to closely mimic the transcription level of the endogenous *mir-237* gene (Martinez et al., 2008a) to detect the level of *mir-237* transcription in the *lin-4(0)* worms compared to that in the wild type worms. The expression of *Pmir-237::gfp* closely matches the temporal expression profile of endogenous *mir-237* established by northern blot (Esquela-Kerscher et al., 2005). At the L3 stage, only 10% of *lin-4(0)* worms showed *Pmir-237::gfp* expression in the hypodermis, whereas 87% of the wild type worms displayed *Pmir-237::gfp* expression in the hypodermis. At the L4 stage, 75% of *lin-4(0)* worms showed *Pmir-237::gfp* expression in the hypodermis, compared to 100% of wild type worms (Table 4). Representative micrographs of the L3 and L4 stage worms are shown in Figure 12. For each stage, the same exposure was used for all strains (see Methods for details). The intensity of *Pmir-237::gfp* expression in *lin-4(0)* worms was significantly lower than in the wild type worms at L3 and L4 stages, although this has not been quantified. These data together with the quantitative Real-Time PCR data indicate that the repression of *mir-237* in *lin-4(0)* worms is largely due to transcriptional control.

The transcriptional repression of *mir-237* in *lin-4(0)* worms could be mediated by LIN-14 or LIN-28, as these proteins are both elevated in *lin-4(0)* worms (Wightman et al., 1993; Moss et al., 1997). To determine whether *lin-14* or *lin-28* is involved in this repression of *mir-237* observed in *lin-4(0) worm*s, I used the above transcriptional reporter to monitor the transcription of *mir-237* in the following strains: *lin-4(0);lin-14(ts);Pmir-237::gfp, lin-14(ts);Pmir-237::gfp,* and *lin-14(gf);Pmir-237::gfp.*

Pmir-237::gfp expression data and representative micrographs are shown in Table 4, Figure 12 and Figure 13. If *Pmir-237::gfp* mis-expression in *lin-4(0)* worms is due to elevated levels of LIN-14, then a similar pattern of expression is expected in *lin-14(gf)* gain of function mutants. Indeed, in *lin-14(gf)* worms, I observed repression of *Pmir-237::gfp* expression similar to what was observed in the *lin-4(0)* worms. At L1 and L2 stages, none of *lin-14(gf)* mutants showed *Pmir-237::gfp* expression in the hypodermis whereas 14% of wild type worms showed *Pmir-237::gfp* expression. At the L3 stage, 33% of *lin-14(gf)* worms had *Pmir-237::gfp* expression compared to 87% of the wild type worms. At the L4 stage, 89% of *lin-14(gf)* worms had *Pmir-237::gfp* expression compared to 100% of the wild type worms (Table 4).

In addition, I observed no repression of *Pmir-237::gfp* expression in *lin-14(ts)* worms but rather up-regulation of *Pmir-237::gfp* expression at the L1 to L3 stages. At the L1 and L2 stages, 74% of *lin-14(ts)* mutants showed *Pmir-237::gfp* expression in the hypodermis compared to 14% of the wild type worms. At the L3 and L4 stages, 100% of *lin-14(ts)* worms displayed *Pmir-237::gfp* expression compared to 87% at L3 and 100% at L4 in the wild type worms (Table 4).

In *lin-4(0); lin-14(ts)* mutants, I did not observe the repression of *Pmir-237::gfp* expression that was observed in the *lin-4(0)* worms. At the L1 and L2 stages, none of *lin-4(0)* worms had any *Pmir-237::gfp* expression in the hypodermis, whereas about 68% of *lin-4(0); lin-14(ts)* mutants displayed *Pmir-237::gfp* expression. At the L3 and L4 stage, 100% of *lin-4(0); lin-14(ts)* worms showed *Pmir-237::gfp* expression compared to 10% at L3 stage and 75% at L4 stage in the wild type worms (Table 4).

These data indicate that LIN-14 or LIN-28, which are coordinately regulated in these strains, can directly or indirectly repress the transcription of *mir-237.*

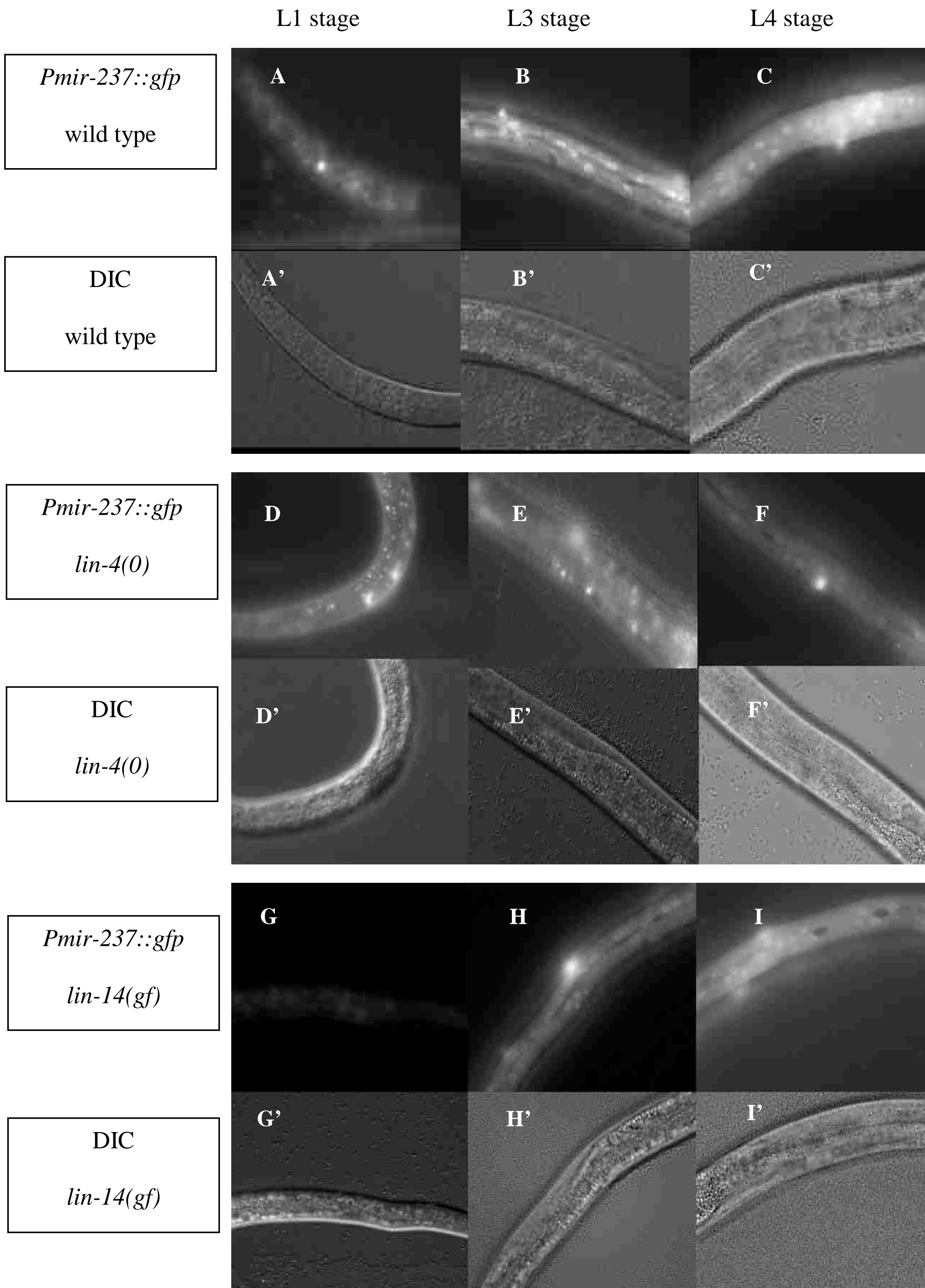

L1 stage
L3 stage
L4 stage
Pmir-237::gfp
wild type
DIC
wild type
Pmir-237::gfp
lin-4(0)
DIC
lin-4(0)
Pmir-237::gfp
lin-14(gf)
DIC
lin-14(gf)
A
B
C
A'
B'
C'
D
E
F
D'
E'
F'
G
H
I
G'
H'
I'

Figure 12. *Pmir-237::gfp* expression is reduced in *lin-4(0)* worms and *lin-14(gf)* worms compared to wild type worms.
(A)~(C): *Pmir-237::gfp* expression in wild type worms at L1, L3 and L4 stages.
(D)~(F): *Pmir-237::gfp* expression in *lin-4(0)* worms at L1, L3 and L4 stages.
(G)~(I): *Pmir-237::gfp* expression in *lin-14(gf)* worms at L1, L3 and L4 stages.
Corresponding DIC images are also shown.
In A, D, and E, no hypodermal GFP was observed in the worms, the fluorescence is from autofluorescent gut granules. In addition there is *Pmir-237::gfp* expression in the somatic gonad in every worm.

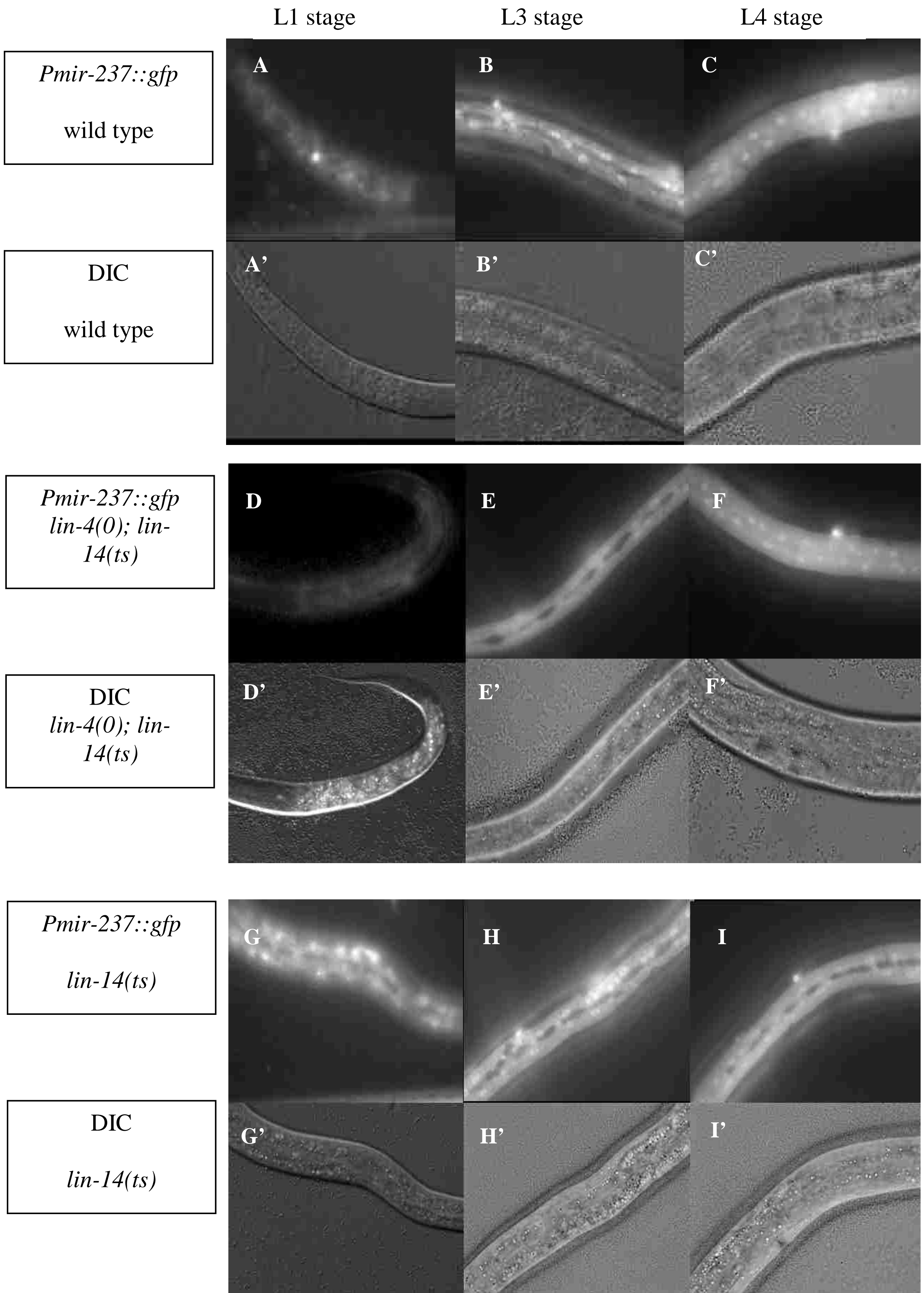

L1 stage
L3 stage
L4 stage
Pmir-237::gfp
wild type
DIC
wild type
Pmir-237::gfp
lin-4(0); lin-14(ts)
DIC
lin-4(0); lin-14(ts)
Pmir-237::gfp
lin-14(ts)
DIC
lin-14(ts)
A
B
C
A'
B'
C'
D
E
F
D'
E'
F'
G
H
I
G'
H'
I'

Figure 13. *Pmir-237::gfp* is precociously expressed in *lin-4(0); lin-14(ts)* worms and *lin-14(ts)* worms compared to wild type worms.
(A)~(C): *Pmir-237::gfp* expression in wild type worms at L1, L3 and L4 stages.
(D)~(F): *Pmir-237::gfp* expression in *lin-4(0); lin-14(ts)* worms at L1, L3 and L4 stages.
(G)~(I): *Pmir-237::gfp* expression in *lin-14(ts)* worms at L1, L3 and L4 stages.
Corresponding DIC images are also shown.
In A and D, no hypodermal GFP was observed in the worms, the fluorescence is from autofluorescent gut granules. In addition there is *Pmir-237::gfp* expression in the somatic gonad in every worm.

Table 4. *Pmir-237::gfp* expression in the hypodermis

Strain	Genotype	% of total worms that displayed GFP expression (n)		
		L1 and early L2	L3	L4
VT1113	*Pmir-237::gfp*	14 (36)	87 (15)	100 (20)
RF305	*lin-4(0); Pmir-237::gfp*	0 (18)	10 (20)	75 (16)
RF306	*lin-4(0); lin-14(ts);Pmir-237::gfp*	68 (37)	100 (18)	100 (20)
RF366	*lin-14(ts);Pmir-237::gfp*	74 (23)	100 (18)	100 (20)
RF446	*lin-14(gf);Pmir-237::gfp.*	0 (18)	33 (18)	89 (18)

IV. DISCUSSION

Expression of developmental timing genes is strictly regulated spatially and temporally. Previous study using transcriptional *mir::gfp* reporters showed that *lin-4* family microRNAs in *C. elegans, lin-4* and *mir-237*, displayed overlapping but distinct temporal and spatial expression patterns. In the hypodermis, *lin-4* expression is first detected at the L1 stage whereas *mir-237* expression is first detected at the L2 stage; at the L2 stage and beyond, both of them are expressed (Esquela-Kerscher et al., 2005). microRNA family members can function in the same pathway. *mir-237* shares sequence similarity with *lin-4,* and also displays closely overlapping temporal and spatial expression pattern with *lin-4. lin-4* is a key regulator of the developmental timing at early stages, from the L1 stage to the L3 stage. I investigated the possibility that *mir-237* also functions in the early developmental timing pathway. Previous work showed that loss of *mir-237* alone does not lead to an observable developmental timing phenotype, suggesting that the role of *mir-237* in the timing pathway, if any, is not essential, or that other genes function redundantly to *mir-237*. Here I used a genetic approach to analyze the relationship between *mir-237* and other early developmental timing genes. My data indicate that *mir-237* is likely a developmental timing gene that can regulate stage-specific cell fate choices between the L2 stage and the L3 stage.

In addition, northern blot showed that *mir-237* is repressed in *lin-4(0)* mutants. My data suggest that this repression is largely due to transcriptional control.

mir-237 is likely a developmental timing gene that regulates the L2-to-L3 transition

Data from genetic analysis showed that loss of *mir-237* weakly suppressed the precocious alae phenotype of both *lin-14(0)* and *lin-28(0)* mutants, suggesting that *mir-237* may function downstream of *lin-14* and *lin-28* (Figure 8, Figure 9 and Table 3). Loss of *mir-237* enhanced the

retarded alae phenotype as well as the abnormal seam cell division phenotype of *lin-46(0)* mutants, suggesting that it functions in parallel with *lin-46* to control L2-to-L3 transition (Table 3). Loss of *mir-237* enhanced the bursting vulva phenotype of *mir-48 mir-241(0)* mutants, suggesting that it may function in parallel with *mir-48* and *mir-241* to control developmental timing in the hypodermis. Together these data provide the evidence that *mir-237* functions in the developmental timing pathway, regulating the L2-to-L3 transition.

In the precocious mutants *lin-14(0)* and *lin-28(0),* genes that regulate the larva-to-adult switch, such as the most downstream developmental timing gene *lin-29* (Rougvie and Ambros, 1995), are precociously expressed. *mir-237* weakly suppressed the precocious alae phenotype of both *lin-14(0)* and *lin-28(0)* mutants. This suggests that *mir-237* affects the expression of those downstream genes. Future work is needed to show when, where, and how *mir-237* activity influences the expression of the downstream genes, resulting in the observed effects on alae formation.

The key evidence that shows that *mir-237* regulates the L2-to-L3 transition is that loss of *mir-237* enhances the L2 repetition phenotype of *lin-46(0)* mutants, including both retarded alae formation and the extra number of seam cells. *lin-46* was shown to likely function only once in the timing pathway during the L2-to-L3 transition. *mir-237* may function in parallel with *lin-46* to tip the balance of cell fate choices between the L2 stage and the L3 stage. *lin-46(0) mir-237(0)* worms displayed about two more seam cells than *lin-46(0)* worms. Thus, not every seam cell is affected by the loss of *mir-237*. This is because the regulation of seam cell division is cell autonomous, and *mir-237* may play a subtle role in regulating seam cell division. In most seam cells, the fluctuation caused by loss of *mir-237* activity is not sufficient to overcome the robust regulation by other developmental timing genes that control the seam cell division, such as *lin-4* and the *let-7* family microRNAs.

It is not exactly clear what can cause the bursting vulva phenotype. Previously it has been shown that timing defects in the *let-7(0)* mutants leads to the bursting vulva phenotype (Reinhart

et al., 2000). It has also been shown that the connections between hypodermal cells and vulva cells are important for the structural integrity during vulva morphogenesis (Newman et al., 2000). *mir-237, mir-48,* and *mir-241* show overlapped temporal expression pattern in the hypodermis. It is possible that *mir-237, mir-48,* and *mir-241* function in the early timing pathway in hypodermal tissue and indirectly influence vulva morphogenesis later. However, *mir-237, mir-48,* and *mir-241* are also expressed in the vulva precursor cells. Future work is needed to show whether division or differentiation of vulva precursor cells is defective in those mutants, which may also account for the bursting vulva phenotype.

The above genetic analysis suggests that *mir-237* is likely a developmental timing gene that can regulate L2-to-L3 transition. Based on my data, I position *mir-237* in the early developmental timing pathway downstream of *lin-14* and *lin-28,* and in parallel with *lin-46* (Figure 10). *lin-4* and *mir-237* share the same seed sequence and have the potential ability to regulate common targets. 3'UTR of *lin-14, lin-28* and *hbl-1* all have complimentary sites to the *lin-4* family seed sequence (Esquela-Kerscher et al., 2005). Future work is needed to identify the targets of *mir-237,* and to test whether *lin-4* and *mir-237* activity is functionally equivalent, or whether they have distinct roles in the pathway. Answering those questions will advance our understanding about the functions of microRNA family members *in vivo*.

Repression of *mir-237* in *lin-4(0)* mutants is largely transcriptional

My data using quantitative Real-Time PCR is consistent with published northern blot data (Esquela-Kerscher et al., 2005). In *lin-4(0)* mutants, mature miR-237 level is highly reduced. The expression of *Pmir-237::gfp* in *lin-4(0)* mutants compared to wild type closely matches the temporal expression profile of endogenous *mir-237* established by northern blot and my quantitative Real-Time PCR data (Table 4, Figure 11 and Figure 12), suggesting that the

repression of *mir-237* in *lin-4(0)* mutants is largely transcriptional. In addition, my data showed that in *lin-4(0); lin-14(ts)* mutants, the repression of *mir-237* transcription is released and *mir-237* transcription is at wild type levels. In *lin-14(ts)* mutants where *lin-14* activity is reduced at 20˚C compared to wild type, I found mis-regulation of *mir-237* transcription, with *mir-237* expression observed one stage earlier than in wild type worms. Whereas in *lin-14(gf)* mutants where *lin-14* activity is increased, *mir-237* transcription is reduced compared to wild type. These data suggest that *lin-14* activity may regulate *mir-237* transcription. However, since in *lin-14(ts)* or *lin-14(gf)* mutants the worms execute precocious or retarded developmental programs, many stage-specifically expressed genes may also be mis-regulated. Future work is needed to show whether mis-regulation of *mir-237* in *lin-14(ts)* or *lin-14(gf)* mutants is direct.

LIN-14 has been shown to contain a putative consensus DNA binding site, GAACRY (Hristova et al., 2005). Sequence analysis of the 2 kb *mir-237* promoter region reveals two candidate LIN-14 binding sites. They are located at about 200 nucleotides upstream of the region encoding the ~70 nucleotide pre-miR-237 stem loop. One of these sites is conserved in the promoter of *mir-237* in *C. briggsae.* Future work is needed to show whether LIN-14 indeed binds *mir-237* promoter directly *in vivo*. If so, then this would provide an *in vivo* example of a positive feedback loop between microRNAs and transcription factors as discussed in Martinez et al (2008b).

If LIN-14 directly represses *mir-237* expression *in vivo*, then it is likely that *mir-237* functions in the *lin-4* independent feedback loop between *lin-14* and *lin-28* (Figure 14). The *lin-28* 3' UTR contains a binding site for the *lin-4* family microRNAs and thus is a candidate target for miR-237. In addition, the temporal expression of *mir-237* is consistent with the model that *mir-237* functions in the *lin-4* independent feedback loop between *lin-14* and *lin-28*.

I propose whereby at the L1 stage, *mir-237* is repressed by LIN-14, and the high levels of LIN-14 and LIN-28 promote the L1 stage program. However, at the L2 stage, accumulation of *lin-4* activity leads to down-regulation of LIN-14 and subsequent weak expression of *mir-237.*

mir-237 may then contribute to the down-regulation of *lin-28* along with *lin-4* and the *let-7* family microRNAs. Thus LIN-28 level is maintained relatively high due, in part, to the absence of *mir-237* activity, which allows for execution of the L2 stage program.

mir-237 is up-regulated at the L2-to-L3 transition, and it may promote the development through the L2-to-L3 transition by down-regulating the key developmental timing regulators (*lin-14, lin-28* and/or *hbl-1*). Loss of *mir-237* does not cause observable defects in developmental timing, suggesting that *mir-237* plays a cooperative role rather than an essential one in the pathway. This data indicates that other genes function redundantly to *mir-237* in regulating the L2-to-L3 transition. I speculate that at the L2-to-L3 transition, a further up-regulation of *lin-4* causes LIN-14 level to fall below the threshold necessary for *mir-237* repression, and then the system will switch to another state in which *mir-237* and *lin-4* together stably repress LIN-14 and LIN-28 to allow for proper execution of L3 stage program. In this way, *mir-237* expression subtly affects the developmental decision between execution of the L2 stage program and the L3 stage program (Figure 14 and Figure 15).

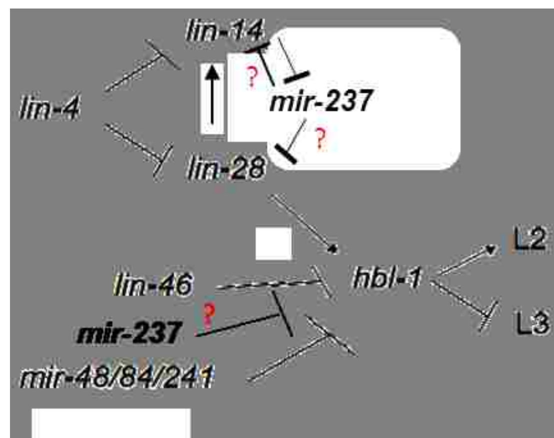


Figure 14. Model for *mir-237* activity in the regulation of the L2-to-L3 transition.

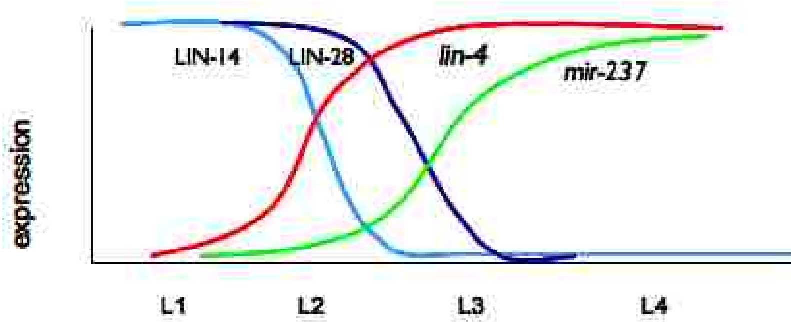


Figure 15. Temporal expression profile of *lin-4, lin-14, lin-28* and *mir-237* in the hypodermis.

In summary, the results presented here suggest a role for the *lin-4* family microRNA *mir-237* in the developmental timing pathway to control the L2-to-L3 cell fate transitions in the hypodermis. *lin-4* indirectly affects *mir-237* expression largely at transcriptional level and possibly through regulation of *lin-14.* Future work is needed to show whether LIN-14 directly represses *mir-237* transcription, and whether *mir-237* is a component that acts in the *lin-4* independent genetic circuit between *lin-14* and *lin-28.*

BIBLIOGRAPHY

Abbott, A. L. Heterochronic genes. *Curr Biol.* 13, 824-825 (2003).

Abbott, A. L., Alvarez-Saavedra, E., Miska, E. A., Lau, N. C., Bartel, D. P., Horvitz, H. R., and Ambros, V. The let-7 MicroRNA Family Members mir-48, mir-84, and mir-241 Function Together to Regulate Developmental Timing in Caenorhabditis elegans. *Dev Cell* 9, 403-414 (2005).

Abrahante, J. E., Miller, E. A. and Rougvie, A. E. Identification of heterochronic mutants in Caenorhabditis elegans. Temporal misexpression of a collagen::green fluorescent protein fusion gene. *Genetics* 149, 1335-1351 (1998).

Abrahante, J.E., Daul, A.L., Li, M., Volk, M. L., Tennessen, J. M., Miller, E. A., and Rougvie, A. E. The Caenorhabditis elegans hunchback-like gene lin-57/hbl-1 controls developmental time and is regulated by microRNAs. *Dev Cell* 4, 625-637 (2003).

Ambros, V. and Horvitz, H. R. Heterochronic mutants of the nematode Caenorhabditis elegans. *Science* 226, 409-416 (1984).

Ambros, V. and Horvitz, H. R. The lin-14 locus of Caenorhabditis elegans controls the time of expression of specific postembryonic developmental events. *Genes & Development* 1, 398-414 (1987).

Newman, A. P., Inoue, T., Wang, M., and Sternberg, P. W. The *Caenorhabditis elegans* heterochronic gene *lin-29* coordinates the vulval-uterine-epidermal connections. *Current Biology* 10, 1479-1488 (2000)

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

van den Berg, A., Mols, J. and Han, J. RISC-Target interaction: cleavage and translational suppression. *Biochim Biophys Acta.* 11, 668-677 (2008).

Banerjee, D. and Slack, F. Control of developmental timing by small temporal RNAs: a paradigm for RNA-mediated regulation of gene expression. *Bioessays* 24, 119-129 (2002).

Bartel, D. P. MicroRNAs: target recognition and regulatory functions. *Cell* 136, 215-233 (2009).

Reinhart, B. J., Slack, F. J., Basson, M., Pasquinelli, A. E., Bettinger, J. C., Rougvie, A.E., Horvitz, H.R., and Ruvkun, G. The 21-nucleotide *let-7* RNA regulates developmnetal timing in *Caenorhabditis elegans. Nature.* 403, 901-906 (2000)

Brennecke, J., Stark, A., Russell, R. B. and Cohen, S. M. Principles of microRNA-target recognition. *PLoS Biol* 3, e85 (2005).

Bushati, N. and Cohen, S. M. microRNA Functions. *Annu Rev Cell Dev Biol* (2007).

Esquela-Kerscher, A., Johnson, S.M., Bai, L., Saito, K., Partridge, J., Reinert, K.L., and Slack, F.J. Post-embryonic expression of C. elegans microRNAs belonging to the lin-4 and let-7 families in the hypodermis and the reproductive system. *Dev Dyn* 234, 868-877 (2005).

Feinbaum, R. and Ambros, V. The timing of lin-4 RNA accumulation controls the timing of postembryonic developmental events in Caenorhabditis elegans. *Dev Biol* 210, 87-95 (1999).

Holtz, J. and Pasquinelli, A. E. Uncoupling of lin-14 mRNA and protein repression by nutrient deprivation in Caenorhabditis elegans. *RNA* 15, 400-405 (2009).

Hristova, M., Birse, D., Hong, Y. and Ambros, V. The Caenorhabditis elegans heterochronic regulator LIN-14 is a novel transcription factor that controls the developmental timing of transcription from the insulin/insulin-like growth factor gene ins-33 by direct DNA binding. *Mol Cell Biol* 25, 11059-11072 (2005).

Doench, J.G., Petersen, C.P., and Sharp, P.A. siRNAs can function as miRNAs. *Genes & Development* 17, 438-442 (2003).

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

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

Lee, R. C. and Ambros, V. An extensive class of small RNAs in Caenorhabditis elegans. *Science* 294, 862-864 (2001).

Lehrbach, N. J., Armisen, J., Lightfoot, H. L., Murfitt, K. J., Bugaut, A., Balasubramanian S, and Miska EA. LIN-28 and the poly(U) polymerase PUP-2 regulate let-7 microRNA processing in Caenorhabditis elegans. *Nat Struct Mol Biol* 16, 1016-1020 (2009).

Lin, S.Y., Johnson, S.M., Abraham, M., Vella, M.C., Pasquinelli, A., Gamberi, C., Gottlieb, E., and Slack, F.J. The C elegans hunchback homolog, hbl-1, controls temporal patterning and is a probable microRNA target. *Dev Cell* 4, 639-650 (2003).

Kostrouchova, M., Krause, M, Kostrouch, Z., and Rall, J.E. CHR3: a Caenorhabditis elegans orphan nuclear hormone receptor required for proper epidermal development and molting. *Development.* 125, 1617-1626 (1998)

Martinez, N. J., Ow, M. C., Reece-Hoyes, J. S., Barrasa, M. I., Ambros, V. R., and Walhout, A.J. Genome-scale spatiotemporal analysis of Caenorhabditis elegans microRNA promoter activity. *Genome Res.* 18, 2005-2015 (2008a).

Martinez, N. J., Ow, M.C., Barrasa, M.I., Hammell, M., Sequerra, R., Doucette-Stamm, L., Roth, F. P., Ambros, V. R., and Walhout, A.J. A C. elegans genome-scale microRNA

network contains composite feedback motifs with high flux capacity. Genes & Development 22: 2535–2549 (2008b).

Li, M., Jones-Rhoades, M. W., Lau, N. C., Bartel, D. P., and Rougvie, A.E. Regulatory mutations of *mir-48,* a *C. elegans let-7* Family MicroRNA, cause developmental timing defects. *Developmental Cell*. 9, 415-422 (2005).

Miska, E. A., Alvarez-Saavedra, E., Abbott, A. L., Lau, N.C., Hellman, A.B., McGonagle, S. M., Bartel, D.P., Ambros, V. R., and Horvitz, H.R. Most Caenorhabditis elegans microRNAs are individually not essential for development or viability. *PLoS Genet* 3, e215 (2007).

Morita, K. and Han, M. Multiple mechanisms are involved in regulating the expression of the developmental timing regulator lin-28 in Caenorhabditis elegans. *EMBO J* 25, 5794-5804 (2006).

Moss, E. G., Lee, R. C. and Ambros, V. The cold shock domain protein LIN-28 controls developmental timing in C. elegans and is regulated by the lin-4 RNA. 8, 1539-49 *Cell* 88, 637-646 (1997).

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

Moss, E. G. Heterochronic genes and the nature of developmental time. *Curr Biol* 17, R425-34 (2007).

Newman, M. A., Thomson, J. M. and Hammond, S. M. Lin-28 interaction with the Let-7 precursor loop mediates regulated microRNA processing. *RNA* (2008).

Zugasti,O., Rajan, J., and Kuwabara P.E. The function and expansion of Patched- and Hedgehog-related homologs in *C. elegans. Genome Res.* 15, 1402-1410 (2005.)

Olsen, P. H. and Ambros, V. The *lin-4* regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. *Dev Biol* 216, 671-680 (1999).

Parker, J.S., Roe, S.M.and and Barford, D. Molecular mechanism of target RNA transcript recognition by Argonaute-Guide Complexes. *Cold Spring Harb Symp Quant Biol.* 71, 45-50 (2006).

Pasquinelli, A. E., Reinhart, B. J., Slack, F., Martindale, M.Q., Kuroda, M.I., Maller, B., Hayward, D.C., Ball, E. E., Degnan, B., Müller, P., Spring, J., Srinivasan, A., Fishman, M., Finnerty, J., Corbo, J., Levine, M., Leahy, P., Davidson, E., and Ruvkun, G. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature* 408, 86-89 (2000).

Pepper, A. S., McCane, J. E., Kemper, K., Yeung, D. A., Lee, R. C., Ambros, V., and Moss, E. G.The C. elegans heterochronic gene lin-46 affects developmental timing at two larval stages and encodes a relative of the scaffolding protein gephyrin. *Development* 131, 2049-2059 (2004).

Reinhart, B. J., Slack, F. J., Basson, M., Pasquinelli, A. E., Bettinger, J. C., Rougvie, A. E., Horvitz, H. R., and Ruvkun, G. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature* 403, 901-906 (2000).

Reinhart, B. J. and Ruvkun, G. Isoform-specific mutations in the Caenorhabditis elegans heterochronic gene lin-14 affect stage-specific patterning. *Genetics* 157, 199-209 (2001).

Rougvie, A. E. Control of developmental timing in animals. *Nat Rev Genet* 2, 690-701 (2001).

Rougvie, A. E. Intrinsic and extrinsic regulators of developmental timing: from microRNAs to nutritional cues. 132, 3787-3798 (2005).

Rougvie, A.E., and Ambros, V. The heterochronic gene *lin-29* encodes a zinc finger protein that controls a terminal differentiation event in Caenorhabditis elegans.121, 2491-500. (1995)

Roush, S. and Slack, F. The let-7 family of microRNAs. *Trends Cell Biol* (2008).

Roush, S. F. and Slack, F. J. Transcription of the C. elegans let-7 microRNA is temporally regulated by one of its targets, hbl-1. *Dev Biol* 334, 523-534 (2009).

Ruvkun, G., Ambros, V., Coulson, A., Waterston, R., Sulston, J., and Horvitz, H. R. Molecular genetics of the Caenorhabditis elegans heterochronic gene lin-14. *Genetics* 121, 501-516 (1989).

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

Roush, S., and Slack, F. J. The *let-7* family of microRNAs. *Trends in cell biology*. 18, 505-16 (2008).

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

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

Sulston, J. E. and Horvitz, H. R. Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. *Dev Biol.* 56, 110-56 (1977).

Eschenlauer, S. C. P., and Page, A. P. The *Caenorhabditis elegans* ERp60 homolog protein disulfide isomerase-3 has disulfide isomerase and transglutaminase-like cross-linking activity and is involved in the maintenance of body morphology. *J Biol Chem.* 278, 4427-4237 (2003)

Terns, R. M., Kroll-Conner, P., Zhu, J., Chung, S. and Rothman, J. H. A deficiency screen for zygotic loci required for establishment and patterning of the epidermis in Caenorhabditis elegans. *Genetics* 146, 185-206 (1997).

Thummel, C. S. Molecular mechanisms of developmental timing in C. elegans and Drosophila. *Dev Cell* 1, 453-465 (2001).

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

Wood, W. B. and the Community of *C. elegans* Researchers. The Nematode *Caenorhabditis elegans.* 1988 by Cold Spring Harbor Laboratory Press.

Winter, J., Jung, S., Keller, S., Gregory, R. I. and Diederichs, S. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat Cell Biol* 11, 228-234 (2009).

Wu, L. and Belasco, J. G. Micro-RNA regulation of the mammalian lin-28 gene during neuronal differentiation of embryonal carcinoma cells. *Mol Cell Biol* 25, 9198-9208 (2005).

Yu, J., Vodyanik, M. A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J. L., Tian, S., Nie, J., Jonsdottir, G. A., Ruotti, V., Stewart, R., Slukvin, II., and Thomson, J. A. Induced pluripotent stem cell lines derived from human somatic cells. *Science* 318, 1917-1920 (2007).