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UNIVERSITY OF CALIFORNIA, SAN DIEGO

The embryonic *mir-35* microRNA cluster regulates development and RNAi efficiency in *C. elegans*

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in
Biological Sciences with Specialization in Bioinformatics
by
Katlin B. Massirer

Committee in charge:

Professor Amy E. Pasquinelli, Chair
Professor Vineet Bafna
Professor Richard Kolodner
Professor Maho Niwa
Professor Karen Oegema
Professor Michael G. Rosenfeld

2009

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The dissertation of Katlin B. Massirer is approved, and it is acceptable in quality and form for publication of microfilm and electronically:

Chair

University of California, San Diego

2009

Dedication

I dedicate my thesis to my very good friend and classmate Christina Chung (1980-2009). Christina was a true scientist inside and outside the lab and a wonderful friend. The hardest part of my whole PhD was losing Christina.

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ABSTRACT OF THE DISSERTATION

The embryonic *mir-35* microRNA cluster regulates development and RNAi efficiency in *C. elegans*

by

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Doctor of Philosophy in Biological Sciences with Specialization in Bioinformatics

UNIVERSITY OF CALIFORNIA, SAN DIEGO, 2009

Professor Amy E. Pasquinelli, Chair

MicroRNAs (miRNAs) are ~22 nucleotide small RNAs that regulate gene expression by pairing with partial complementarity to target mRNAs. While loss of specific miRNAs can result in distinct phenotypic abnormalities, very few miRNA genes have been shown to be essential for viability. In this dissertation, I demonstrate that the *mir-35* gene in *Caenorabditis elegans* has a vital role in

embryogenesis. This gene encodes a cluster of 7 paralogous miRNAs, mir-35-41, that are highly expressed in *C. elegans* embryos. I show that loss of the mir-35-41 cluster in the genetic mutant *mir-35(gk262)*, results in embryonic lethality, with defects in cytokinesis during the first embryonic cellular divisions and delays in cell cycle progression during subsequent embryonic cell divisions. Consistent with a role for *mir-35* miRNAs in early embryogenesis, the inviability of *mir-35(gk262)* worms was rescued maternally, suggesting that deposition of the miRNA gene products is sufficient for embryogenesis. Supporting this idea, the precursor and mature forms of mir-35 are detectable in female worms that produce only oocytes. Additionally, accumulation of mature mir-35 miRNA correlated with the production of embryos. Taken together, my results suggest that RNA products expressed by the *mir-35* gene are deposited in oocytes; following fertilization, increased maturation and synthesis of mir-35-41 miRNAs are then available to control early embryonic events, including cytokinesis and cell cycle progression.

In the second part of my dissertation, I investigated a surprising link between the *mir-35* gene and the RNAi pathway. While performing RNA interference (RNAi) experiments in *mir-35(gk262)* worms, I observed strong RNAi hypersensitivity of the strain. The hypersensitivity is dependent on the canonical RNAi pathway and is similar in levels to the described *lin-35* mutant. Additionally, microarrays indicated overlap in gene regulatory pathways for *mir-35* and *lin-35*. I found that LIN-35 protein levels are significantly reduced in *mir-*

35(gk262) embryos, indicating that the *mir-35-41* miRNAs positively regulate accumulation of LIN-35 protein. Although the regulation is probably indirect, the decreased level of LIN-35 likely explains the RNAi hypersensitive phenotype of *mir-35* mutant worms. Importantly, *lin-35* encodes the worm homolog of the human Rb retinoblastoma gene. Another connection between the *mir-35* gene and the RNAi pathway is the unexpected finding that nonspecific dsRNA affects the viability of *mir-35(gk262)* mutants. In contrast to almost complete lethality observed in the absence of *mir-35-41* miRNAs at the restrictive temperature, I found that the introduction of non-specific dsRNA could partially rescue this lethality. Since the rescue is dependent on RNAi pathway genes, my results indicate that *mir-35-41* is required for embryonic viability in a pathway that can be compensated by the initiation of RNAi.

In conclusion, my research demonstrates that the *mir-35-41* miRNAs are important for embryonic viability and regulate the efficiency of RNAi in *C. elegans*. Although the RNAi hypersensitivity of *mir-35* mutants may be largely through down-regulation of LIN-35 in these mutants, the *mir-35-41* miRNAs regulate *lin-35* and other genes in parallel pathways important for embryogenesis. In support of this model, embryos with loss of *lin-35* alone are viable, with loss of *mir-35* alone have reduced viability and with loss of both genes are inviable. My work establishes a new regulator of *lin-35* and demonstrates novel connections between RNAi pathway genes and embryonic viability.

Chapter 1: Introduction to *C. elegans*, microRNAs and RNAi

1.1 *Caenorhabditis elegans*: history of the model organism

The nematode *Caenorhabditis elegans* was established as a model organism by Sydney Brenner's publication in 1974 (Brenner 1974). At that time, after the central dogma of molecular biology had been established, Brenner envisioned that it was essential to understand the molecular mechanisms controlling gene expression and the complexity of cellular and genomic interactions. He believed that by using *C. elegans*, which has 302 neurons compared to *Drosophila* with 10^5 neurons, the scientific community would be able to answer many of the questions related to the complexity of the nervous system and to the spatial and temporal cell specification programs during development (Brenner 1974; Brenner 1974). Brenner started his *C. elegans* project with the use of electron microscopy to physically map every cell in the nervous system of the nematode. At the same time he used forward genetics to cause gene perturbations and selected behavioral mutants (Brenner 1974). Since then, *C. elegans* has been used extensively as a model organism for studies of basic cellular pathways, mapping of all neuronal connections, aging, cell death, development and various diseases (White, Horvitz et al. 1982; Hengartner and Horvitz 1994; Metzstein, Stanfield et al. 1998; Adam 2009; Panowski and Dillin 2009).

Several features have contributed to the widespread use of *C. elegans* as a model organism to investigate diverse biological problems. *C. elegans* worms are multicellular, self-fertilizing, hermaphrodite nematodes. The spontaneous occurrence of males permits the performance of genetic crosses. The worms and embryos are transparent and can be easily imaged, adults are only 1mm long, and worms can be grown in the laboratory on *Escherichia coli* with a short life cycle of 3 1/2 days at 20°C. The *C. elegans* cell lineage from the embryo first division to the adult animal was characterized as invariant, meaning that every individual has the same number of cells located in the same relative position and arising from the same genealogy (Sulston 1976; Deppe, Schierenberg et al. 1978; Horvitz, Sternberg et al. 1983). This characteristic is probably the most unique advantage for the study of development and allows researchers to follow and compare the development of a single cell lineage between wild type and mutant animals. All the synapses (about 5000 chemical synapses, 2000 neuromuscular junctions and about 500 gap junctions), neuronal connections and the entire neuronal circuit were also characterized (White, Horvitz et al. 1982; Sulston, Schierenberg et al. 1983).

The work using *C. elegans* as a model organism brought many scientific contributions, which have been recognized by the award of three Nobel Prizes during the last 10 years. In 2002, the *C. elegans* pioneers Sydney Brenner, Robert Horvitz and John Sulston received the Nobel Prize in Physiology or Medicine for their contributions to the understanding of genetic pathways

regulating organ development and apoptosis (Horvitz, Shaham et al. 1994; Metzstein and Horvitz 1999; Sulston 2003). In 2006, the Nobel Prize in Physiology and Medicine was awarded to Andrew Fire and Craig C. Mello, for the discovery of RNA interference in *C. elegans*, which allows reverse genetic studies by disrupting expression of specific genes (Fire, Xu et al. 1998; Montgomery and Fire 1998; Timmons and Fire 1998). In 2008, Martin Chalfie shared the Nobel Prize in Chemistry for his work using green fluorescence protein (GFP) for various *in vivo* applications in *C. elegans* (Chalfie, Tu et al. 1994). Currently there are more than 700 laboratories worldwide using the *C. elegans* system to answer an enormous variety of biological questions.

The future of the work in *C. elegans* continues to move towards Brenner's initial goal of understanding neurobiology networks and integrating cellular localization and specification with functions of gene products in a systems biology approach. There are still many questions to be addressed using *C. elegans*: for example, how do gene products interact and regulate the cell-to-cell signaling to form a 3D embryo structure, how is a given cell positioned relative to a specific neighbor, how are neuronal networks set up to transmit signals and regulate complex functions such as sensing chemicals or laying embryos, and how are the programs of developmental timing and larval molting coordinated and made irreversible.

1.2 *C. elegans* cell lineage and embryogenesis

Since the nineteenth century, biologists have been interested in studying cell division patterns in nematodes because of the simplicity and reproducibility in development. The concepts of cell lineage and germline stem cells were first defined during early developmental studies using the nematode *Ascaris*' eggs by Boveri, in 1892 (Ramalho-Santos and Willenbring 2007).

In 1976, the *C. elegans* pioneers presented the conclusion that *C. elegans* cell lineages (division patterns) and cell fates (cell differentiation) during embryogenesis are assigned in an invariant pattern (Sulston 1976). In 1983, mapping of the entire embryonic and post-embryonic *C. elegans* cell lineage was completed (Sulston, Schierenberg et al. 1983). Embryogenesis generates 671 cells and 113 of these undergo programmed cell death in the hermaphrodite and 111 in the male (Sulston, Schierenberg et al. 1983). The reason why one in every 6 cells die is probably because these cells have already accomplished their embryonic function. The whole cell lineage is derived from the 5 founder cells AB, MS, C, D, P4 (White, Southgate et al. 1976; Sulston, Schierenberg et al. 1983). P4 originates the germline while the remaining cells originate somatic tissues. The use of Nomarski microscopy coupled to photographs taken on 35mm film, rudimentary video taping systems and many hours of direct observation allowed this project to succeed. Direct observation was considered the only satisfactory method to follow cell divisions and cellular migration, mainly because cells move in three dimensions (Sulston, Schierenberg et al. 1983) and

sophisticated equipment, not available until recently, would be necessary for high resolution.

The determination of the cell lineage in wild-type worms opened the possibility to explore the very complex level of interactions between genes products and cell lineages by the use of genetic mutants and laser cell ablation, a process use to eliminate a single cell. These approaches were classically used to establish the apoptosis pathway in *C. elegans* (Horvitz and Sulston 1980). In addition to genetic mutations, the development of fluorescent *in vivo* techniques combined with confocal (time-lapse and z-series) and deconvolution microscopy led to higher resolution and reproducibility of cell positioning and cell structures (Stefansson, Eliceiri et al. 2005; Green, Audhya et al. 2008). Therefore, the use of these tools altogether can contribute to the understanding of a higher level of complexity relating cell lineages and gene products.

1.3 *C. elegans* fertilization

In adult worms, the gonad consists of two U-shaped arms, each terminating proximally at a spermatheca (Figure 1.1 B) (Hubbart et al, 2005). Germline stem cells in the gonad first differentiate to produce sperm during the L4 stage and then stop and switch to producing oocytes after the L4 molt. During oogenesis the undifferentiated nuclei are in a syncitium, sharing a common maternal cytoplasm. The maternal cytoplasm is packetd into oocytes, which

undergo maturation while they move through the proximal gonad arms. Shortly before ovulation the oocytes' nuclear envelope breaks down and the oocyte changes shape from a cube to a sphere. Fertilization occurs when mature oocytes pass through the spermatheca, generating embryos which continue to develop in the uterus until they reach 30 cells and are then laid (Hubbart et al.2005). The laid embryos develop *ex-utero* until hatching as L1 (larva stage 1) worms with about 550 cells. The worms progress through the larval stages L2, L3, L4 before reaching adulthood with about 800 somatic cells (Sulston 2003; Hubbard and Greenstein 2005).

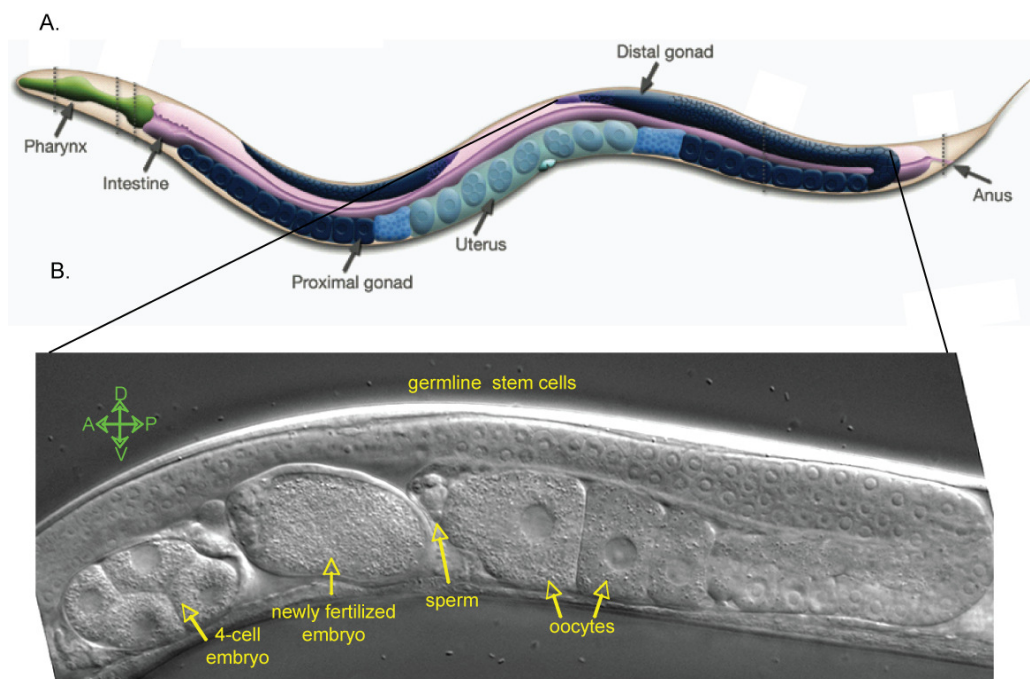


Figure 1.1 *C. elegans* anatomy and reproductive system. A. Adapted cartoon with arrowheads indicating the main worm anatomical structures. B. The reproductive system is amplified in a DIC micrograph of an adult hermaphrodite (400x). Cartoon courtesy of wormatlas.com.

As part of the efforts to characterize how gene products regulate cellular events, the detailed study of the first round of embryonic cell division is now possible with advanced imaging tools. In wild-type worms the first division includes a series of coordinated events. After the sperm enters the oocyte, meiosis ensues and an eggshell begins to form. Meiosis is completed with the extrusion of two polar bodies, after which the egg's cytoplasm is rearranged and the pro-nuclei become visible at opposite poles. During late G1 phase, the centrosome is duplicated. The maternal nucleus migrates towards the paternal nucleus, which has replicated its chromosomal DNA (S phase). The nuclei meet near the center of the embryo and rotate. Nuclear envelope breakdown occurs, mitotic spindles are assembled, and chromosomes align pairwise on the metaphase plate (M phase). Replicated genomes segregate to opposite poles (anaphase), a contractile ring assembles, and the mother cell divides asymmetrically (cytokinesis) into the large anterior AB cell and the small posterior P1 cell (Strome and Wood 1983; Miller, Nguyen et al. 2001).

Recently several collaborative high throughput projects generated important tools and gave functional insights into the analysis of early embryogenesis. Microarrays were used by the Hunter group to determine the temporal transcriptome of staged embryos starting at the first division through later time points (Baugh, Hill et al. 2003). Gene transcripts were categorized as maternal and zygotic according to their variation of expression during the different stages. In another project the use of a full genome embryonic RNAi

screen helped define all the genes essential for the first two mitotic cell divisions (Sonnichsen, Koski et al. 2005). In this study, after RNAi, the embryos were filmed in a spatial and temporal manner and uniformly categorized into functional groups, according to the observed phenotypes. Most recently, the cloning of early small RNAs from precisely staged embryos, combining fluorescence-activated cell sorting with next generation sequencing, defined regulatory RNAs important for embryogenesis (Stoeckius, Maaskola et al. 2009).

1.4 Discovery of miRNAs and the interest in the embryonic mir-35 cluster

MicroRNAs are a large family of ~22 nucleotide (nt) non-coding RNAs that were discovered in *C. elegans*, and are thought to exist in all multicellular organisms to regulate gene expression (Pasquinelli, Reinhart et al. 2000). In *C. elegans*, the *lin-4* miRNA regulates early larval stage transitions (Lee, Feinbaum et al. 1993; Wightman, Ha et al. 1993) and the *let-7* miRNA controls later larval to adult development (Reinhart, Slack et al. 2000; Slack, Basson et al. 2000; Pasquinelli 2002). These two miRNAs have genetically defined target genes that are negatively regulated by the miRNAs (Reinhart, Slack et al. 2000; Pasquinelli 2002). Other worm miRNAs have been implicated in controlling temporal patterning, egg-laying and left-right asymmetry (Reinhart, Slack et al. 2000; Lin, Johnson et al. 2003; Miska, Alvarez-Saavedra et al. 2007). However, most of the

174 miRNAs in *C.elegans* still have unknown functions. The majority of them are detectable at all times during development but a few appear specific to certain developmental stages (Lau, Lim et al. 2001; Lim, Lau et al. 2003). Surprisingly, deletions generated in 87 worm miRNA genes showed that individually most of these miRNAs are not essential for viability and development (Miska, Alvarez-Saavedra et al. 2007). Interestingly the mir-35-41 cluster of 7 paralogous miRNAs was initially identified by cloning as the only embryonic-specific group of miRNAs. The interest in understanding the mechanism and function of developmentally regulated miRNAs led us to study the mir-35-41 cluster.

1.5 Biogenesis of microRNAs

Generally, miRNAs are initially expressed as long primary RNA (pri-miRNA) transcripts by RNA polymerase II. The pri-miRNAs, which contain the mature miRNA sequence as part of a predicted hairpin, are recognized and processed by the Drosha/Pasha complex with combined RNaseIII and dsRNA binding functions (Figure 1.2) (Han, Lee et al. 2004; Kennedy, Wang et al. 2004). This results in the formation of ~60 nucleotide precursor miRNAs, also called pre-miRNAs (Lee, Ahn et al. 2003; Cullen 2004). The pre-miRNAs are exported to the cytoplasm, where they are processed to the mature ~22 nt miRNAs by the RNaseIII enzyme Dicer (Grishok, Pasquinelli et al. 2001; Hutvagner, McLachlan et al. 2001); (Ketting, Fischer et al. 2001). A putative helicase activity unwinds the duplex miRNA and a single strand is loaded into the miRNA-induced

silencing complex (miRISC). The miRISC complex, which has Argonaute proteins as core component, is responsible for guiding miRNAs to recognize their targets to inhibit their expression (Bartel and Chen 2004; Lin, Chang et al. 2005).

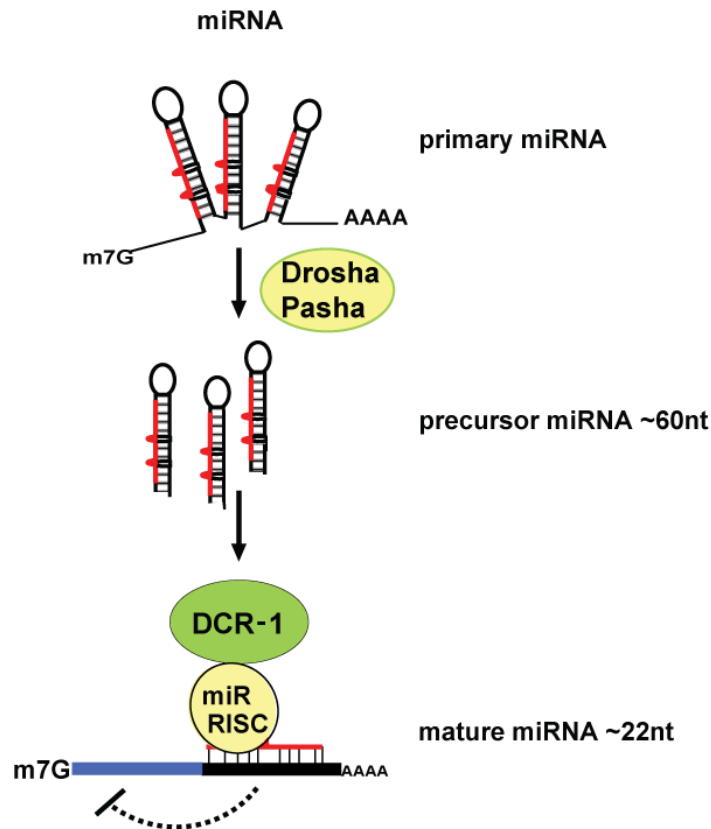


Figure 1.2 microRNA biogenesis. Long primary miRNA transcripts are processed into hairpin precursors, which are subsequently processed to mature miRNAs. Mature miRNAs inhibit target mRNA by imperfect complementarity binding.

MiRNAs are only partially complementary to specific sites in the 3'UTR regions of their target mRNAs, making the identification of targets by sequence complementarity complicated (Bartel and Chen 2004). Some rules for miRNA-target pairing were implemented in computational prediction programs, but it is

essential to biologically validate more targets in order to improve the accuracy of programs (Rajewsky and Socci 2004; Rajewsky 2006; Grimson, Farh et al. 2007; Bartel 2009).

Many mechanisms explaining how miRNAs inhibit expression of their target genes have been experimentally verified. miRNAs can mediate regulation through translation inhibition, mRNA degradation, mRNA de-adenylation and/or mRNA sequestration, possibly depending on each microRNA-target pair, condition, cell type or organism (Bagga, Bracht et al. 2005; Pillai, Bhattacharyya et al. 2005; Giraldez, Mishima et al. 2006; Maroney, Yu et al. 2006; Boutz, Chawla et al. 2007).

Since their discovery, miRNAs have been shown to regulate target mRNA genes in a variety of pathways, biological processes, and diseases, including development, the cell cycle, apoptosis, DNA damage, cell differentiation, cancer, diabetes, progressive kidney disease, immunological and mental disorders (Poy, Eliasson et al. 2004; Boehm and Slack 2005; Johnson, Esquela-Kerscher et al. 2007; Garzon, Volinia et al. 2008; Grishok, Hoersch et al. 2008; Stefani and Slack 2008; Kato, Paranjape et al. 2009; Pandey and Picard 2009),

1.6 Other small RNA pathways

In addition to the miRNA pathway, other small RNA pathways are present in worms. The exogenous RNA interference (exo-RNAi) and endogenous RNA interference (endo-RNAi) pathways process double stranded RNA into ~22nt small interfering RNAs (siRNAs) that silence homologous transcripts (Duchaine, Wohlschlegel et al. 2006; Lee, Hammell et al. 2006). miRNAs, exo-siRNAs and endo-siRNAs are produced by dicer nucleolytic cleavage of various forms of dsRNA. Dicer is the common component to these three pathways and loss of Dicer function generates unviable worms (Bernstein, Caudy et al. 2001; Ketting, Fischer et al. 2001; Duchaine, Wohlschlegel et al. 2006). The specificity of each pathway is given by the Argonaute proteins, but it is not known how the presence of 27 different Argonaute proteins in the worm can recognize different classes of small RNAs (Grishok, Pasquinelli et al. 2001; Vastenhouw, Fischer et al. 2003; Tijsterman and Plasterk 2004). In other species, the same Argonaute can function with both microRNAs and siRNAs (Williams and Rubin 2002; Meister, Landthaler et al. 2004; Okamura, Ishizuka et al. 2004). The exo-RNAi pathway, generally referred to as RNAi, is a mechanism of gene downregulation triggered by dsRNA from an exogenous source. The function of RNAi in the worms is not entirely clear, with some evidence for transposon silencing and viral defense (Tabara, Sarkissian et al. 1999; Schott, Cureton et al. 2005). RNAi is currently used as a powerful technique to achieve gene downregulation in many species. The gene *unc-22* encodes an abundant and non-essential myofilament protein

and its mutation causes worms to twitch or paralyze. In 1988, the Mello and Fire groups used injection of single and dsRNA against *unc-22* to study genetic interference. They observed that *unc-22* dsRNA could very efficiently produce the RNA interference effect and caused wild-type *C. elegans* worms to display the twitching phenotype (Fire, Xu et al. 1998). This effect resembled the genetic mutant phenotype for the *unc-22* gene. During the same year, Timmons and Fire showed that feeding worms *Escherichia coli* bacteria expressing dsRNA against *unc-22* also caused the twitching phenotype (Timmons and Fire 1998).

During the initial step of the RNAi pathway, long dsRNA from exogenous sources (exo-RNAi pathway) are recognized by the RNA binding protein RDE-4 together with DCR-1 to be processed into ~22 nt long siRNAs (Figure 1.3 A). The primary siRNAs bind to Argonaute protein RDE-1 and guide it to degrade mRNA targets of perfect complementary (Tabara, Sarkissian et al. 1999; Parrish and Fire 2001). The short siRNAs were first detected as products of exogenous RNAi in plants by the Baulcombe lab and in mammalian cells by the Tuschl lab (Elbashir, Harborth et al. 2001; Elbashir, Lendeckel et al. 2001). Worms, plants and fungi differ from other species in that they have an RNAi amplification mechanism triggered after the initial RNAi effect (Wassenegger and Krczal 2006). Amplification is dependent on the presence of RNA-directed RNA polymerases (RdRP), which have not been found in mammals (Zamore 2002). In worms, amplification occurs by binding of the primary siRNAs to target mRNAs and recruitment of the RdRP Family 1 (RRF-1) protein, which produces new

dsRNA. Secondary siRNA production involves recruitment of RdRP to an internal sequence around the primary siRNA target site for mRNA targeting, with unprimed synthesis of complementary RNA (Sijen, Steiner et al. 2007). Since secondary siRNAs are synthesized *de novo* using the target mRNA as a template, they can be complementary to sequence upstream in the mRNA to the primary siRNA trigger (Sijen, Fleenor et al. 2001; Sijen, Steiner et al. 2007). The newly synthesized RNA is processed by the Dicer complex to generate secondary siRNAs. Secondary siRNAs are loaded in part by RDE-1 but other Argonautes (SAGOs- synthetic secondary siRNA deficient Argonaute) seem to be involved in targeting the mRNAs (Yigit, Batista et al. 2006). This amplification is probably responsible for the potent RNAi gene silencing and may be related to RNAi inheritance in worms (Grishok, Tabara et al. 2000; Sijen, Fleenor et al. 2001; Sijen, Steiner et al. 2007).

In contrast to the exo-siRNAs, the endo-siRNAs originate from longer dsRNAs generated from transcription of exonic regions that also produce limited amounts of the complementary RNA strand (Lee et al, 2006) (Figure 1.3 B). The long dsRNAs are also guided by RDE-4, which acts with Dicer to generate endo-siRNAs. ERGO-1 is the functional Argonaute guided by endo-siRNAs to down-regulate mRNA targets with perfect complementarity. The endo-siRNA pathway also has an amplification step which is dependent on the RdRP RRF-3 and the exonuclease ERI-1 and specific SAGOs (Yigit, Batista et al. 2006).

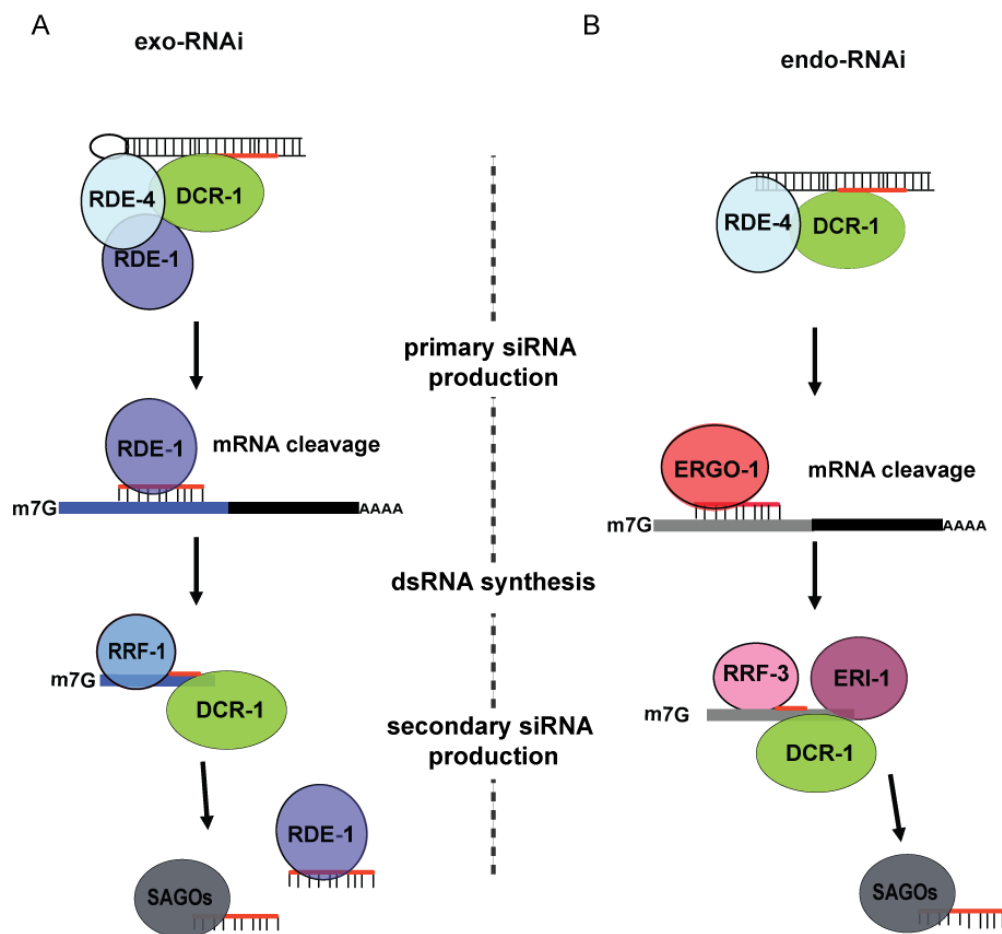


Figure 1.3 Small RNA pathways. A. Model for the exogenous RNAi pathway B. Model for the endogenous RNAi pathway.

Endo-siRNAs likely cause mild down-regulation of target genes since endo-siRNA targets are not significantly misregulated in *rde-4* adult mutant worms (Welker, Habig et al. 2007). Additionally, many of the predicted endo-siRNA targets cause embryonic lethality if knocked down by RNAi, indicating that these target mRNAs are normally present at a basal level (Asikainen, Heikkinen et al. 2008).

1.7 Genes that positively and negatively function in the RNAi pathways

The RNAi mechanism has been studied for about 10 years and key steps have been elucidated. Genes essential for eliciting an RNAi response were mainly discovered by genetic screens evaluating RNAi deficiency. RNAi deficient genes 1 to 4 (*rde-1*, *rde-2*, *rde-3* and *rde-4*) were mapped from a screen for resistance to the embryonic lethality caused by *pos-1* RNAi (Tabara, Sarkissian et al. 1999). These mutants are resistant to RNAi and do not display any phenotype when fed or injected with dsRNA against genes that otherwise would show loss of function phenotypes in wild-type worms.

On the other hand, genes that negatively regulate the RNAi pathway were identified as mutants that displayed stronger RNAi induced phenotypes than what was observed in wild-type worms introduced to the same dsRNA. The enhanced RNAi 1 (*eri-1*) and the RNAi dependent RNA polymerase 3 (*rrf-3*) genes were isolated from this kind of approach (Simmer, Tijsterman et al. 2002; Kennedy, Wang et al. 2004). Specifically, *rrf-3* was found in a screen for both enhanced RNAi and RNAi response in neurons (Simmer, Tijsterman et al. 2002). *rrf-3* displayed hypersensitivity to RNAi in many tissues, including neurons in the head, a tissue which normally is resistant to feeding RNAi (Simmer, Tijsterman et al. 2002); *eri-1* was also isolated in a screen specific for RNAi effectiveness in 23 of the GABAergic worm neurons (Kennedy, Wang et al. 2004). As mentioned in the previous section, the *rrf-3* and *eri-1* genes are directly involved in the endo-

siRNA pathway (Lee, Hammell et al. 2006). Another gene in the endo-siRNA pathway, the argonaute *ergo-1*, also shows increased sensitivity to RNAi when mutated. (Yigit, Batista et al. 2006).

The mechanism causing hypersensitivity in the endo-RNAi-related mutants *rff-3*, *eri-1* and *ergo-1* is not clear, but evidence for cross regulation between the small RNA pathways in these mutants has been suggested (Lee, Hammell et al. 2006). Competition for components between the exo and endo-RNAi pathways (such as *rff-3* and *rff-1*) was suggested by several studies (Lee, Hammell et al. 2006; Yigit, Batista et al. 2006; Sijen, Steiner et al. 2007), and higher levels of siRNAi products were detected in the hypersensitive mutants (Sijen, Steiner et al. 2007). In contrast, mutants of the RNAi dependent genes *rde-1*, *rde-4* and *rff-1*, do not result in enhancement of the endo-RNAi pathway, probably because the exo-RNAi pathway is under negative regulation (Lee, Hammell et al. 2006; Yigit, Batista et al. 2006)

Another class of mutations with enhanced RNAi consists of a group of genes related to the worm retinoblastoma (Rb) pathway (Ceron, Rual et al. 2007). The loss-of-function mutation of the Rb worm homolog *lin-35*, and some other the members in the pathway (*lin-15*, *dpl-1*, *lin-53*, *lin-9*, *lin-13* and *hpl-2*) result in RNAi hypersensitivity (Wang, Kennedy et al. 2005). The molecular mechanism for this effect is not known but indicates that the RNAi pathway can have other levels of regulation.

Chapter 2: Phenotypic characterization of mutant worms lacking the microRNA cluster mir-35-41

2.1 Introduction

MicroRNAs are ~22 nucleotide non-coding RNAs that regulate target mRNA genes in a variety of pathways (Grishok, Pasquinelli et al. 2001; Stefani and Slack 2008). In *C. elegans* most of the 174 miRNAs are expressed in all developmental stages (Miska, Alvarez-Saavedra et al. 2007). About a third of the worm miRNAs are differentially expressed during larval stages and are probably involved in controlling developmental timing and cellular specification (Lau et al 2001, Lim et al, 2003). *Lin-4* and *let-7* are examples of developmentally expressed miRNAs, and were the first to be discovered and extensively studied (Ambros 1989; Lee, Feinbaum et al. 1993; Olsen and Ambros 1999; Abbott, Alvarez-Saavedra et al. 2005; Bagga, Bracht et al. 2005). However many questions related to specific phenotypes, regulation of processing steps in biogenesis and the range of target genes remain to be answered for *let-7*, *lin-4* and other miRNAs. Another developmentally regulated group of miRNAs is the mir-35-41 cluster, which is highly expressed in worm embryos (Lau, Lim et al. 2001; Lim, Lau et al. 2003; Palakodeti, Smielewska et al. 2006). The mir-35-41 gene is required for embryonic viability but the biological pathways regulated by

this group of miRNAs have not been previously characterized. The seven miRNAs in this cluster share identical 5' sequences, indicating that they may target the same group of genes. Members of this cluster are conserved in the nematode *C. briggsae* and in planaria (Lau, Lim et al. 2001; Palakodeti, Smielewska et al. 2006). To understand the role of mir-35-41 in embryo development, I characterized the genetic mutant *mir-35(gk262)*, which lacks the entire mir-35-41 cluster. I found that deletion of the mir-35-41 cluster causes reduced brood size and results in an embryonic lethal temperature sensitive phenotype. In collaborations with the Oegema and Chisholm labs at UCSD, I used fluorescent embryonic markers in *mir-35(gk262)* worms combined with microscopy techniques to characterize phenotypic defects during the first embryonic division as well as later embryogenesis. I observed that loss of mir-35-41 resulted in early embryonic cytokinesis defects and delays in cell cycle progression during subsequent divisions. Because maternal rescue of the lethality of *mir-35(gk262)* embryos was observed, we propose that maternally loaded gene products are important for setting up initial divisions and cell specifications for normal embryo development. The phenotypic characteristics of *mir-35* mutants highlights the importance of the miR-35-41 cluster, and distinguishes it as one of the few miRNA gene loci associated with obvious phenotypes when mutated.

2.2 Results

2.2.1 Expression of mature miR-35 coincides with fertilization

The mature form of the *mir-35-41* cluster members were shown to be expressed in gravid adult worms and embryos (Lau et al., 2001). However, the precursor miRNAs (~60 nt) were also weakly detected by PAGE-Northern blot of RNA from L4 stage worms (Lau et al 2001, Lim et al, 2003). Additionally, expression of the *mir-35-41* cluster was reported to be dependent on the presence of germline (Lim et al, 2003). The developmentally regulated expression of the *mir-35-41* miRNAs and the possibility of regulated processing prompted me to further characterize the *mir-35* gene. I performed detailed analyses of the timing of expression of precursor and mature miR-35 RNAs and identified the primary transcripts expressed by the *mir-35* gene.

To investigate if expression of *mir-35* precursor and mature miRNAs correlated with specific events in the worm, such sperm or oocyte production and fertilization, worms were analyzed by microscopy at various time points and harvested for miRNA expression analyses. Wild-type (N2) worms were synchronized at 20°C and collected at different time points from mid third larval stage to adults. Before harvesting the worms, gonads were imaged to characterize the developmental stage and to evaluate the presence of sperm, oocytes or fertilized embryos. *C. elegans* embryos were also collected and all samples were subjected to RNA extraction. PAGE-Northern blot analyses

revealed that the miR-35 precursor is first detected at 40 hours of larval development, immediately after the L3 molt (Figure 2.1A pre-miR-35). This time point correlates with highly proliferative germ cells in the gonad (Hubbard et al, 2005). Interestingly mature miR-35 was not detectable until 10 hours later, at the 50h time point (Figure 2.1A miR-35), when the worms have the first fertilized embryos in the uterus (Figure 2.1B middle panel). These results suggest that precursors of the miR-35-41 miRNAs could be loaded in the oocytes and thus poised for maturation following fertilization. Experiments investigating the hypothesis that *mir-35-41* precursors accumulate in oocytes and fertilization is required for processing to the mature forms are presented in section 2.3.5. The panels in Figure 2.1B show representative worms just before and after fertilization, illustrating the correlation between the presence of embryos and detection of mature *mir-35* miRNA (Figure 2.1A). The temporal delay between the precursor expression and processing to mature miRNA led us to the question of when transcription of the gene corresponding to the primary miRNA is turned on in wild-type worms.

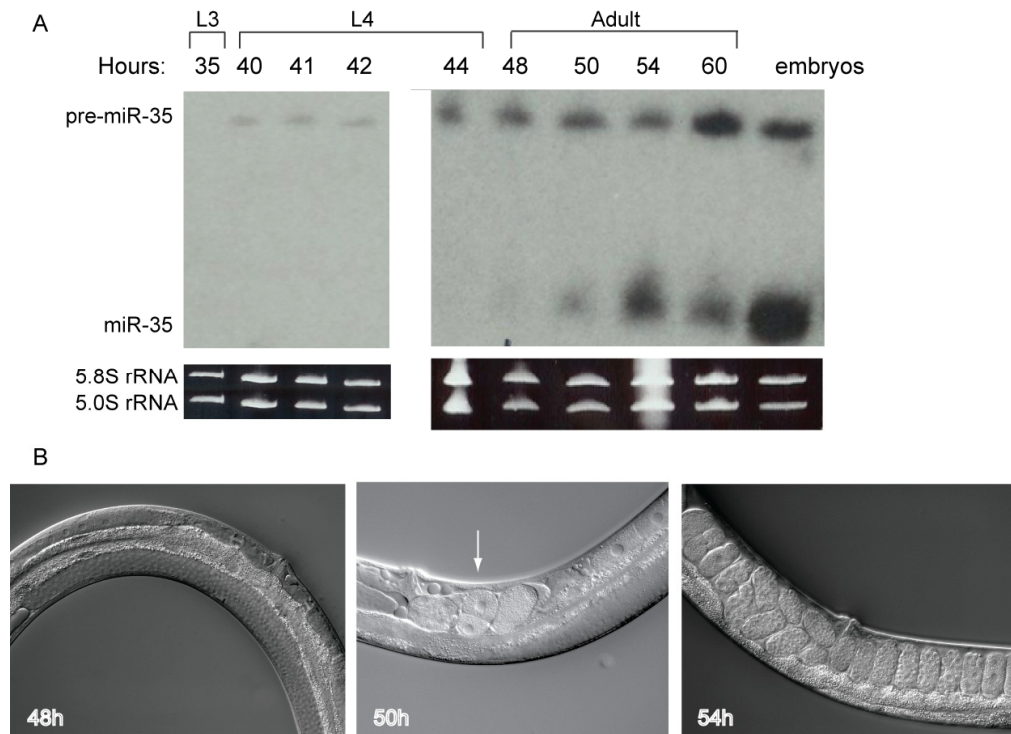


Figure 2.1 Northern blot for miR-35 during N2 development. A. Total RNA from synchronized N2 worms staged as indicated (hours on food) was analyzed by PAGE northern blotting for pre and mature miR-35 B. Micrographs of N2 worms corresponding to time points in panel A. At 48h germline is present but no fertilized embryos are observed, while at 50 hours the first embryos can be visualized (indicated by the arrowhead). At 54 hours many embryos can be visualized in the worm uterus. Pictures were taken at 400x.

2.2.2 The *mir-35* gene encodes a 1500-nt long polyadenylated primary transcript

Identification of the temporal and spatial expression patterns of primary miRNA transcripts is fundamental for understanding more the miRNA biogenesis and function. Moreover, expression of miRNAs in particular cell types or developmental stages can narrow down the classes of potential targets for a specific miRNA. To determine when and where *mir-35-41* primary transcripts are expressed endogenously, the full length primary transcript was first defined and its start site was used as reference for making a promoter-GFP gene reporter. Definition of the start site was also important for construction of the rescue transcript used in section 2.3.4.1. The miRNAs in the *miR-35* cluster are located on chromosome II (Figure 2.2A). We attempted to detect the primary transcript in wild type worms by RT-PCR using primers internal to the cluster, but due to low abundance or rapid processing of the transcript, we were not able to detect a PCR product. To accumulate enough primary *mir-35-41* transcript and facilitate detection, worms were grown on bacteria expressing dsRNA for Drosha or Vector control. Drosha is the RNaseIII enzyme involved in the first step of processing primary transcripts to precursor miRNAs, as defined in the section 1.4. Down-regulation of Drosha by RNAi was verified (Figure 2.2 B) resulting in the accumulation of primary transcripts (Figure 2.2 B). To enhance the Drosha RNAi effect, we used the RNAi enhanced worm strain *rrf-3(pk1426)*. *rrf-3* worms grown to adults on Drosha RNAi were harvested and total RNA was extracted.

After RT-PCR, accumulation of a band corresponding to the region flanking the mir-35-41 cluster was detected (Figure 2.2B), meaning that the sample was adequate for investigation of the primary transcript. The RNA was used for mapping of the 5' and 3' transcriptional ends by the RACE technique (Rapid amplification of cDNA ends). Using this method, identification of 5' end of transcripts is dependent on a cap structure in the intact mRNA and products obtained with the use of oligo deoxythymidine (oligo dT) as primer for the synthesis of the first cDNA strand reflect a polyadenylated transcript. In these conditions, we obtained one major product after the 5' Nested RACE (Figure 2.2C) and one product for 3' Nested RACE (Figure 2.2D). Cloning and sequencing of these products identified a ~1500 nucleotide long sequence corresponding to the mir-35-41 primary transcript (Figure 2.3D). The start site was located 142 nt upstream of the mature miR-35 and the 3' end is located 500nt downstream of miR-41. The mapping of the transcriptional start site allowed us to proceed with the gene reporter assays.

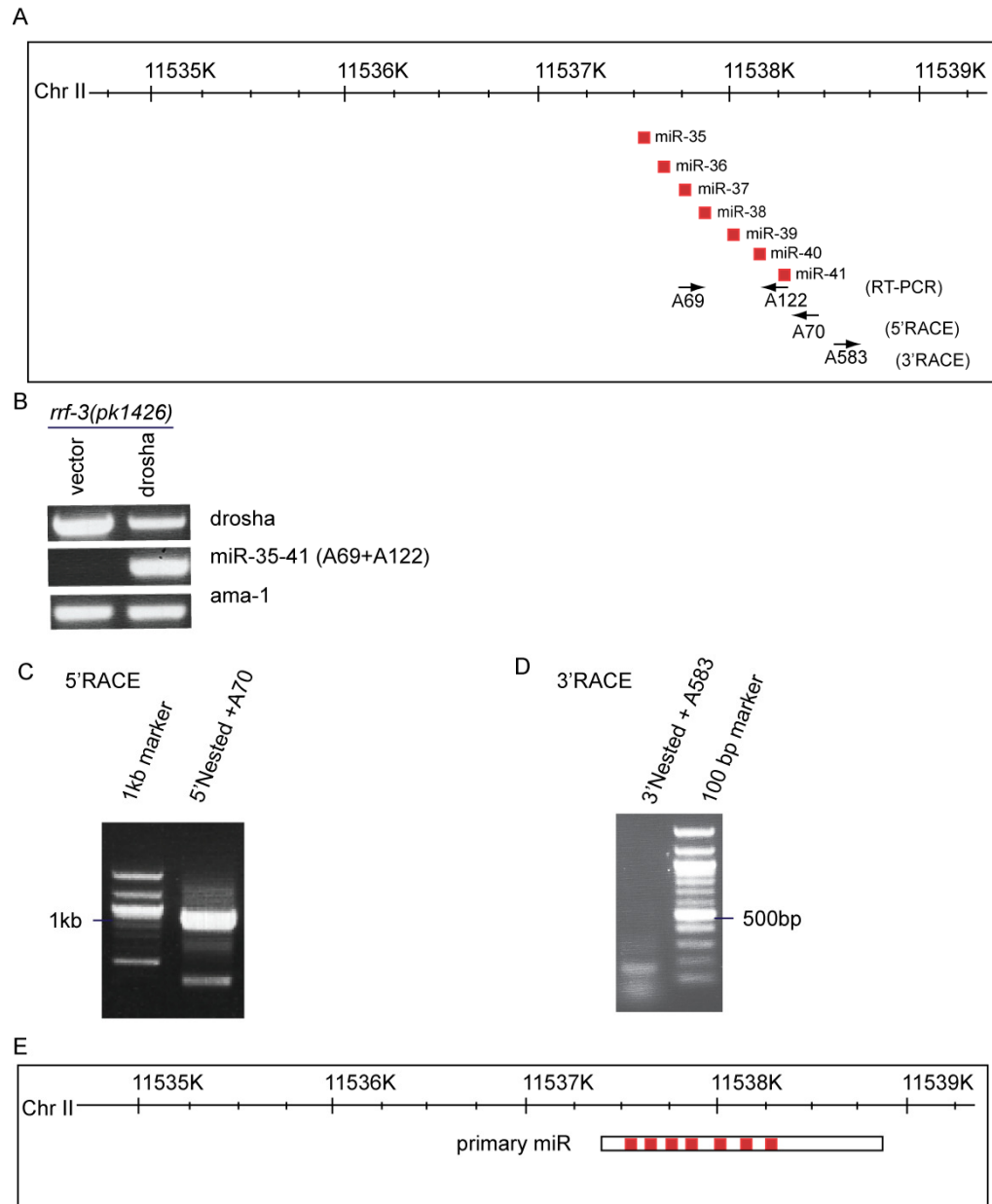


Figure 2.2 Cloning of the miR-35 primary transcript. A. Graphical representation of miR-35 to miR-41 located on chromosome II (drawn to scale). Primers used for RT-PCR and RACE are indicated. B. RT-PCR using RNA from adult *rrf-3(pk1426)* worms confirmed downregulation of Drosha mRNA after RNAi compared to vector. RT-PCR of the region flanking the miR-35 cluster (A69+A122). Ama-1 is the loading control. C. PCR product corresponding to the 5'RACE using the gene specific A70 oligo with the 5' Nested oligo is about 900-nt long. D. PCR product corresponding to the 3'RACE using the gene specific oligo A583 and the 3' Nested oligo. E. Graphical representation of the full length mir-35-41 primary transcript.

2.2.3 Sequences upstream of *mir-35* support transcription in embryos

To further characterize the spatial expression of *mir-35-41* in embryos, we used a promoter reporter. After the start site for the primary transcript was identified by RACE, we decided to use a region ~1.5 kb upstream of the start site as a putative promoter for the *mir-35-41* cluster, referred to as *pmir-35*. The promoter region of 1668bp (by convenience of primers) was PCR amplified and cloned upstream of the GFP–cDNA sequence (Figure 2.3A). This construct was transferred to the plasmid pPDMM016b which induces expression of the rescue fragment for the gene *unc-119* as a selection marker (Praitis et al 2001). The resulting transgene was used for ballistic bombardment. The strain used for bombardment is DP38 containing an *unc-119* mutation. DP38 worms are not able to move and also fail to enter the dauer stage. Integration of the transgene containing the *unc-119* rescue fragment allows transformed worms to move and survive starvation. The surviving worms are singled on separate plates to originate independent transgenic lines. Worms that are not transformed are unable to move and die.

Bombardment of the *pmir-35::GFP* reporter generated two independent transgenic lines. The GFP expression was examined across all the developmental stages and in embryos using fluorescent microscopy. In agreement with the expression of mature miR-35, the GFP was expressed in early embryos still inside the mother (up to 30 cells) and in later stage embryos

already laid (Figure 2.3 B, right and middle panel). This observation indicates that transcription of the *mir-35* gene in embryos may contribute to the robust accumulation of mature miRNAs at this stage. Almost no expression was detected during other developmental stages except for a few cells in early L1 (Figure 2.3 B, left panel) and one or two weak spots in the head during the other stages. From comparison of the GFP expression with previous Northern blot results we would also expect GFP signal in the L4 stage when precursor is already detectable. It is possible that the reporter was not detected earlier due to lack of important transcriptional regulatory elements in the promoter region chosen for the construct. Another possibility is that the transgene could be silenced in the germline and early embryogenesis. Transgene silencing is more commonly observed with extrachromosomal arrays but can also happen with integrated arrays (Praitis, Casey et al. 2001). Despite the lack of detectable GFP expression until embryogenesis, a construct using the same extent of promoter sequence was capable of partial rescue of the lethality in *mir-35(gk262)* embryos (see below). Thus, *pmir-35::GFP* transgenic worms can be used for RNAi screens of transcription factors that regulate primary transcript expression in embryos, which is at least partially sufficient for producing functional *mir-35* miRNAs. The possibility of transgene silencing could be approached with the recently established MosTIC (Mos1 excision-induced transgene-instructed gene conversion) technique of homologous recombination in the worms (Robert and Bessereau 2009; Robert, Katic et al. 2009).

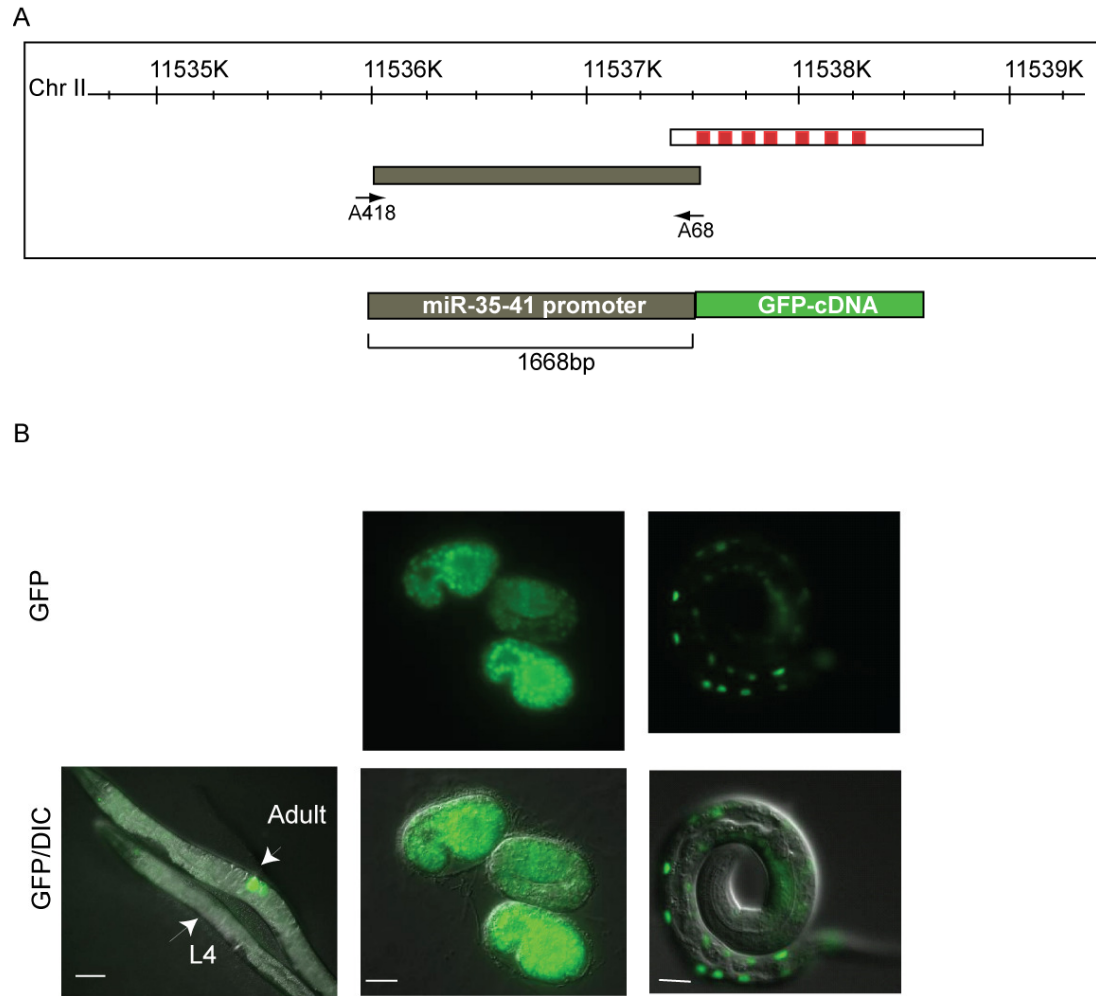


Figure 2.3 The miR-35 promoter drives expression in embryos. A. Graphical representation of the promoter fragment upstream of the mir-35-41 cluster (grey) used for the reporter construct (drawn to scale), with primer numbers indicated. The promoter was fused to *gfp*-cDNA. B. Micrograph showing GFP expression in one of two integrated promoter lines. Left panel showing one L4 and one adult worm (200x). The adult has a few green embryos in the uterus. Middle panel showing later stage embryos (400x). Right panel showing early L1 embryos (630x).

2.2.4 The mir-35-41 cluster is essential for worm viability and deletion results in embryonic phenotypes

Genetic mutants are an important tool for functional characterization of a pathway. In the case of miRNA, where the number of miRNA targets is variable, mutants showing a phenotype can provide insight about the functional classes of target genes. The *mir-35(gk262)* mutant is a deletion that removes 1270 nt including the entire miRNA cluster, (Figure 2.4A). The *mir-35(gk262)* worms were outcrossed three times in our lab before phenotypic characterization to eliminate other mutations in the genetic background of the strain. The *mir-35(gk262)* worms were evaluated for embryonic phenotypes of viability, temperature sensitivity and the embryonic lineage cellular divisions.

2.2.4.1 mir-35(gk262) worms have reduced brood size, low viability and are temperature sensitive

Since the mir-35-41 miRNAs are expressed in wild-type embryos, we hypothesized that this cluster may be involved in embryo development. Thus, we performed experiments to analyze embryonic phenotypes in the *mir-35(gk262)* strain.

On average, 250 embryos are laid by wild-type (N2) worms at the permissive temperature of 20°C and the brood size is reduced to about 200 at

25°C (Nayak 2004). We scored progeny produced by *mir-35(gk262)*, and found that significantly fewer embryos were laid at both 20°C and 25°C compared to the numbers produced by N2 worms (Figure 2.4B). *mir-35(gk262)* laid on average 1.6 and 2.6 times less embryos than N2 at 20°C and 25°C, respectively. The reduction of brood size could be either related to problems in sperm and oocytes or in embryo formation. If the latter would be the case, we would probably also observe lower viability of embryos. To test this possibility the embryos were followed for 3 days.

In my assays, viability was scored as the percent of embryos laid that hatch and grow up to L4 per single hermaphrodite parent. Wild type worms are 100% viable at both temperatures. Interestingly *mir-35(gk262)* were 30%-40% viable at 20°C and only 5% viable at 25°C (Figure 2.4C), suggesting that deletion of *mir-35-41* causes highly penetrant embryonic lethality. Taken together, the brood size and the viability results demonstrate that the *mir-35-41* cluster is important for embryo formation and viability. We also concluded that *mir-35(gk262)* mutants are temperature sensitive because these phenotypes are enhanced at 25°C.

The *gk262* deletion described above also eliminates part of the Y625A.9 transcript, encoded in the opposite direction to *mir-35-41* gene (Figure 2.4A). Since the phenotype observed for *mir-35(gk262)* could be caused by disruption of this transcript, it was crucial to test if the *mir-35-41* cluster sequence was sufficient to rescue *mir-35(gk262)* embryonic lethality. To evaluate this possibility

I generated a PCR fragment of ~2.6 kb (2640 bp) containing the same upstream region used for the miR-35 promoter GFP (section 2.3.3), and the mir-35-41 sequence including a putative polyA signal. The transgene sequence was only long enough to rescue the mir-35-41 gene but not encode the Y625A.9 transcript, which is opposite to the mir-35-41 cluster (figure 2.4A). This rescue fragment was co-injected with the pharynx marker *myo-2::GFP* into *mir-35(gk262)* worms. The injection technique generates multi-copy extrachromosomal arrays and transgenic worms are identified by GFP expression in the pharynx. When *mir-35(gk262)* worms were injected they generated very few progeny and no transgenic lines were obtained. We then used a different approach of injecting N2 worms to generate lines from which males could be produced. Transgenic N2 males were crossed into *mir-35(gk262)*. Once transgenic *mir-35(gk262);Exmir-35-41* worms were obtained, the viability was assessed. We observed rescue of 50% of viability in the *mir-35(gk262)* transgenic worms (Figure 2.4D). This confirms that absence of the mir-35-41 cluster is at least partially responsible for the reduced viability in *mir-35(gk262)* mutants. In agreement with these conclusions, RNAi experiments against the Y625A.9 transcript did not result in phenotypes (Phenobank). We also analyzed miR-35 expression in the rescued worms by Northern blot and confirmed that the rescue fragment recovered expression of miR-35. The miR-35 levels in the rescued worms were lower than in N2 and this may explain why the rescue of viability was not higher than 50%. Lower expression is potentially caused by lack of some regulatory elements in the region used as promoter for the rescue fragment. Another possibility is that

the array is not expressed in all cells or at the right time in the worms or that it can be silenced in the germline. In conclusion, I have observed reduced brood size and low viability when *mir-35-41* is deleted. The *gk262* worms are temperature sensitive with almost complete embryonic lethality at 25°C. A rescue fragment containing the same promoter region that was sufficient for driving expression in embryos, successfully rescued *mir-35(gk262)* embryonic lethality.

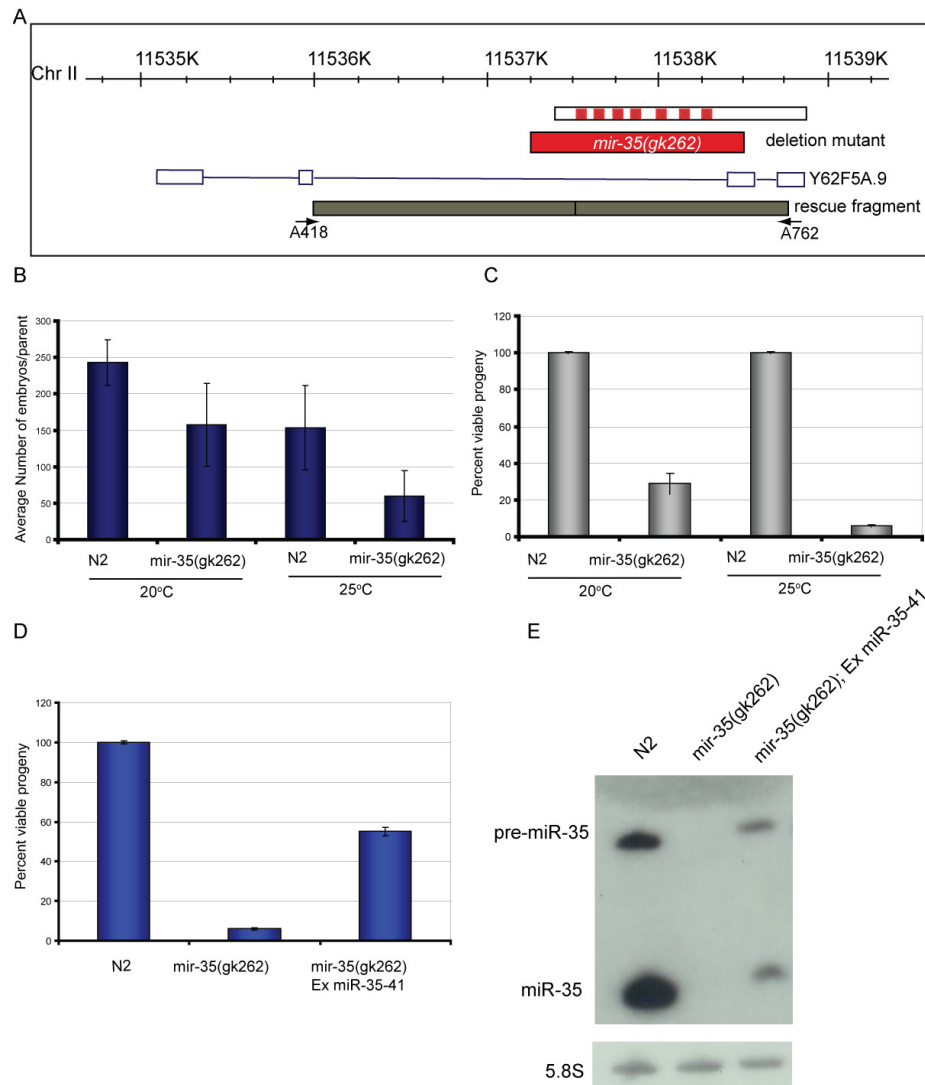


Figure 2.4 *mir-35(gk262)* have reduced brood size and reduced viability that can be rescued by transgenic expression of the miRNAs. **A.** Graphical representation of the *mir-35-41* cluster primary transcript, the *mir-35(gk262)* deletion mutants, the Y62F5A.9 coding sequences (boxes represent exons and lines represent introns) and the *mir-35* rescue fragment (drawn to scale). **B.** Average number of embryos laid by *mir-35(gk262)* and N2 worms at 20°C and 25°C. **C.** Percent viable progeny of N2 and *mir-35(gk262)* at 20°C and 25°C, representing the average number of embryos laid that reached the L4 stage, per parent **D.** Percent viable progeny of N2, *mir-35(gk262)* and *mir-35(gk262);Ex mir-35-41* at 25°C. **E.** Northern blot for miR35 expression in N2, *mir-35(gk262)* and *mir-35(gk262);Ex mir-35-41* embryos. **A.** Total RNA from embryos was analyzed by PAGE northern blotting for pre and mature miR-35. 5.8S is used as a loading control. Error bars for graphs represent the standard error of the mean for three independent experiments.

2.2.4.2 Deletion of the mir-35-41 cluster results in embryonic cytokinesis defects

During *C. elegans* self-fertilization, the newly formed embryo goes through many important events that end in the first embryonic cleavage and specification of a larger somatic cell (AB) and a smaller germline cell (P1), which will undergo several rounds of mitotic cell divisions (Brenner 1974). Specific phenotypes observed during this period have been functionally categorized by a full genome RNAi screen, and can be assigned to not previously characterized mutants (Sonnichsen, Koski et al. 2005).

Because we observed that the mir-35-41 cluster is essential for embryonic viability, we hypothesized that misregulation of a group of targets with related functions could be the cause of embryonic lethality in worms lacking the miR-35 cluster. Furthermore, we expected that phenotypic defects during the first division could narrow down candidates for regulation by the mir-35-41 cluster. One possibility is that members of the cluster target many genes in the embryo in order to set up a specific condition such as to promote embryo development or downregulate maternal mRNAs.

In collaboration with Rebecca Green in Karen Oegema's laboratory at UCSD, we performed imaging of the first embryonic cellular division with worms containing the fluorescent markers mCherry-histone to mark chromosomes and a GFP fusion with a PH domain (GFP-PHPLCd1) to mark the plasma membrane.

This strain was used as wild-type control and was crossed into *mir-35(gk262)* for phenotypic evaluation. We imaged embryos using spinning disc confocal microscopy combined with time-lapse and z-sections. Wild-type and *mir-35(gk262)* embryos were imaged for 20 minutes at the restrictive temperature of 25°C, with controlled stage temperature. We observed that all five N2 embryos showed wild type first division. Nine out of twenty-one *mir-35(gk262)* embryos showed abnormalities: two embryos failed to extrude the polar body, and 7 had variable defects in cytokinesis. Figure 2.5 is a snapshot of one wild-type (N2) embryo and one *mir-35(gk262)* embryo during cytokineses. The upper panel shows a DIC picture and the lower panel shows overlapping of GFP and RFP (PH-GFP domain and m-cherryHistone). In the N2 embryo cytokinesis is finishing with the cellular furrow almost completely ingressed and DNA migrating away from the center of the embryo. In the *mir-35(gk262)* mutant the DNA is still positioned in the center of the cell while the furrow is ingressing. Chromosome bridging during furrow ingression was another defect observed during cytokinesis. We concluded that deletion of *mir-35-41* causes problems very early in embryo development. The phenotypes were pleiotropic and somewhat related to the categories of defective meiosis (polar body extrusion), protein synthesis (pleiotropy) and actin/myosin (cytokinesis). The variety of defects observed during the first cell division of *mir-35* mutants is unusual because genetic mutants or RNAi-treated worms typically display consistent phenotypes that can be further characterized. The pleiotropy did not allow us to pinpoint a specific group

of genes whose mis-regulation could explain the mir-35(gk262) embryonic lethality.

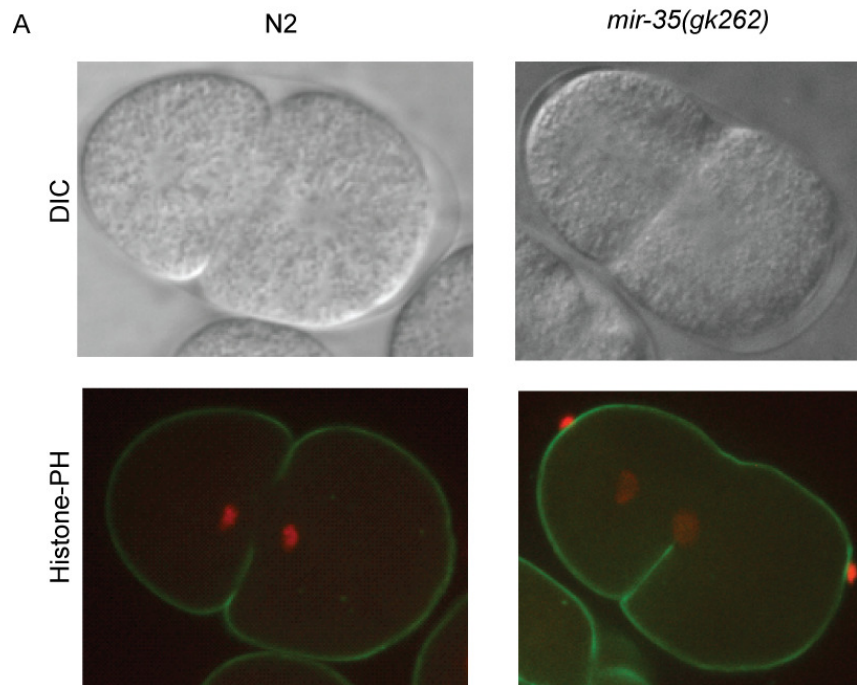


Figure 2.5 *mir-35(gk262)* mutants have defects in cytokinesis. A. Selected frame from time lapse confocal imaging. DIC and fluorescent composite of *C. elegans* early N2 (left) and *mir-35(gk262)* (right) embryos expressing mCherry-histone (chromosome) and a GFP-PH domain (plasma membrane) 630x.

2.2.4.3 Deletion of the mir-35-41 cluster results in delayed cell cycles during embryogenesis

The *C. elegans* embryos develop from fertilization to 550 cells at the time of hatching in about 800 minutes at 20°C (Deppe et al 1978). Most of the cell divisions and specifications happen during the first half of embryogenesis. The different fates in early blastomers are assigned by varying amounts of maternal factors and by cell-cell interaction modulation (Sulston 1976; Hird and White 1993). During the second half of development, the epidermis is formed and the worm body encloses and elongates. Formation of the ventral cleft is an important event during this period and consists of internalizing precursor cells of the gut, pharynx and muscles (Sulston, Schierenberg et al. 1983; Sulston 2003). After enclosure, the worm's circumference is reduced by a factor of three and elongation increases by a factor of four (Sulston, 1983).

To define the developmental stage at which *mir-35(gk262)* embryos were arrested, we performed live imaging of embryos using DIC time lapse and z-section (4D-Nomarski analysis). This approach was possible through a collaboration with Claudiu Giurumescu in the Chisholm lab at UCSD. Because *mir-35(gk262)* embryos show high lethality at 25°C, embryos were imaged at this temperature starting at the 2-4 cell stage for 10 hours. After imaging we observed that all the N2 embryos were able to hatch, showing that the imaging conditions were not damaging the embryos. Compared to N2, the *mir-35(gk262)* embryos showed severe defects in gastrulation with mass of cells, eventual cellular

detachment, and incorrect cell positioning. Twelve out of twenty embryos died later between the coma and the 2 fold stage.

In order to characterize the cell lineage and cell migration in the embryos, nuclear fluorescent GFP and confocal were used. The *mir-35(gk262)* strain was crossed to the *zuls178* strain, which contains the GFP-histone marker (H3 histone). The nuclear fluorescent GFP signal was detected by fluorescent confocal time lapse imaging (at one minute resolution) combined with z-sectioning (35 slices that are 0.85 μm apart). All the embryos were positioned in the same orientation for imaging (ventral side close to microscope objective). Recordings were done at 22.5°C as measured by a thermosensor placed in the oil between the coverslip and the objective. We had evaluated *mir-35(gk262)* lethality at 25°C, but wild type embryos did not survive during confocal imaging at this temperature, probably due to exposure to the laser allied to the temperature. The imaging at 22.5°C allowed the *zuls178* (wild-type reference) embryos to survive (4/4) while all of the *mir-35(gk262)* embryos arrested during imaging at different stages (7/7). The imaging system recorded the nuclei positions, since nuclei are resolved more clearly than cell boundaries. The movies were subjected to computational reconstruction of cell dynamics by software developed in Andrew Chisholm's laboratory. Briefly, nuclei are treated as spheres with a radius magnitude that depends on the number of cell cycles each lineage underwent. Position of each nucleus is recorded and new nuclei are detected based on the closest neighbor to pre-existing nuclei with manual

curation of nuclei localization. The computational analysis results in a 3D image of the embryo where we can determine distance between cells, cell migration, timing of cell division and specific lineage arrangements. Apoptotic cells were identified from the specific aspect of histone-GFP fluorescence associated with condensed chromatin.

The cleavage pattern was normal in mir-35(gk262) with the six stem cells generated in the same order as in wild type embryos. Interestingly, mir-35(gk262) can complete proliferation but are unable to complete morphogenesis (Figure 2.6A) Worms that can reach 3 fold stage are usually still able to contract muscles, often have very irregular shape, in two embryos the pharynx was not attached to the mouth opening. Compared to the wild-type rate, mir-35(gk262) mutants had abnormal timing of cell division or delayed cell cycle. Previous cell lineage studies in embryonic mutants have pointed out the importance of timing for embryonic cell divisions (Deppe et al 1978, Miwa et al 1980, Schierenberg et al 1980). In these studies all temperature sensitive mutants with timing defects during embryo development were at least maternally sufficient, indicating that division rates of cell lines are preprogrammed in the embryo by maternal genes. This was called the mode of intracellular preprogramming (Deppe et al, 1978). The preprogramming by maternal genes involves the overall rate as well as the relative rates between cell lines. Due to maternal control, timing defects could be causative of later defects. This could explain why mir-35(gk262) have defects very early in the first cleavage and later on. Probably by the time the embryos die

they have already deviated from normal development but continued cell divisions until a crisis point (Schierenberg et al 1980, Miwa et al 1980).

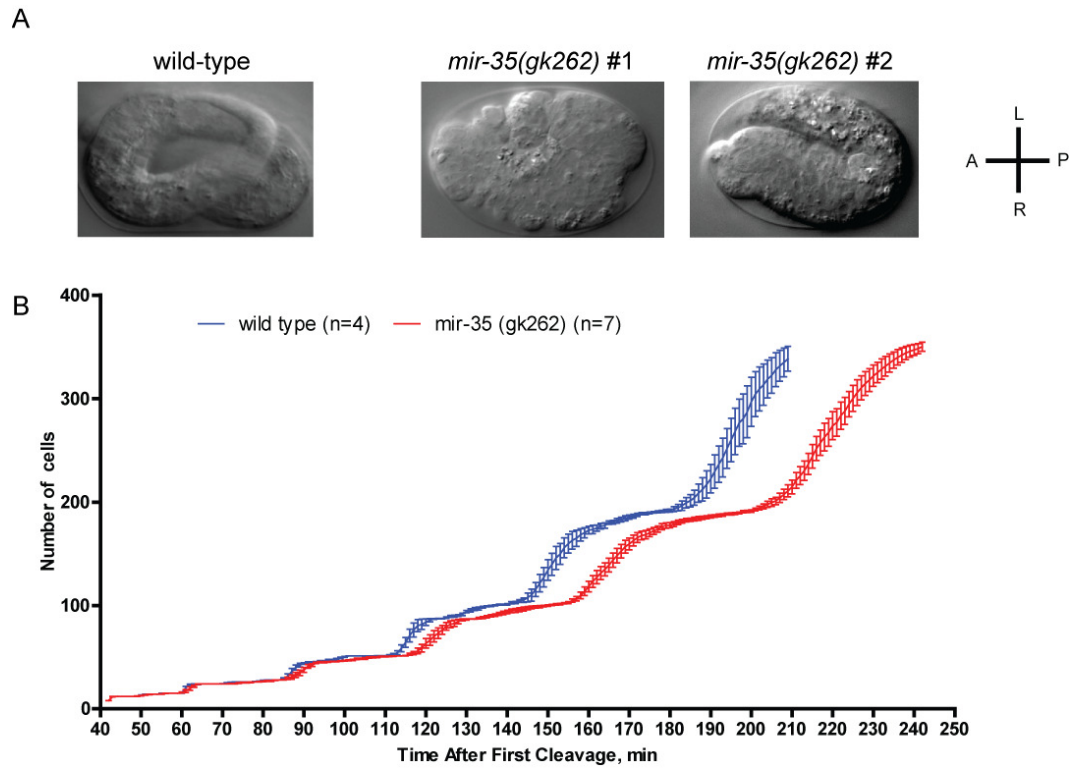


Figure 2.6 *mir-35(gk262)* deletion causes delay in cell cycle. A. Micrograph of one wild-type (zuls178) and two *mir-35(gk262)* embryos after fluorescent confocal imaging (anterior is to the left) 630x. B. Total number of cells along time after first division.

2.2.5 Maternal or zygotic expression of the mir-35-41 cluster can rescue mir-35(gk262) embryonic lethality

Maternal rescue occurs if one maternal wild-type allele is sufficient to allow survival of homozygous mutant progeny. To determine if maternal contribution of *mir-35-41* is required for embryonic viability, wild-type males containing a body muscle gfp marker (PD4251) were crossed to mutant *mir-35-/-mir-35-* hermaphrodites at 25°C (restrictive temperature). After 24 hours parents were removed from the mating plate. The presence of green F1 marks the cross progeny and, thus, heterozygous *mir-35-/-mir-35+*. Successful cross and presence of heterozygous also means that the zygotic contribution, transmitted by the male DNA, is sufficient to rescue. Heterozygous F1 were singled and F2 progeny was genotyped. Genotyping of the F2 progeny showed a Mendelian distribution with 1/4 of the F2 worms being homozygous *mir-35-/-mir-35-* (total 62 worms), confirming maternal rescue by the microRNA gene product.

Because heterozygous *mir-35-/-mir-35+* were rescued from both wild-type male parent (cross 1) and from a heterozygous hermaphrodite parent (self cross) we also conclude that presence of one *mir-35-41* allele is sufficient to rescue *mir-35(gk262)* embryonic lethality.

In summary, maternal or zygotic expression was sufficient to rescue *mir-35(gk262)* lethality. The rescue by either one copy of the DNA allele or by the miR-35 gene product from the mother indicates that mature miRNAs can be

deposited in the embryos by the mother or can be expressed in the embryo. To complement this finding, we investigated miR-35 expression in strains containing only oocytes or sperm compared to hermaphrodites. To obtain RNA from males, we hand picked 1000 males from a *him-8* (*tm611*) population; for females we used the temperature sensitive strain *fem-1*(*hc17*). *Fem-1* mutants grown at 25°C only produce oocytes and are thus sterile females, while they are fertile hermaphrodites at 20°C. By northern blot analysis we were able to detect precursor and mature miR-35 in hermaphrodites (N2 and *fem-1* at 20°C) but miR-35 was undetectable in *him-8* males. The presence of the precursor and mature miR-35 in oocytes may represent maternal transmission of the miRNA. The lack of detection of mature in the males is contradictory with rescue by one allele from the male, but one possibility is that males could still transmit the primary miR-35 transcript (that we have not tested).

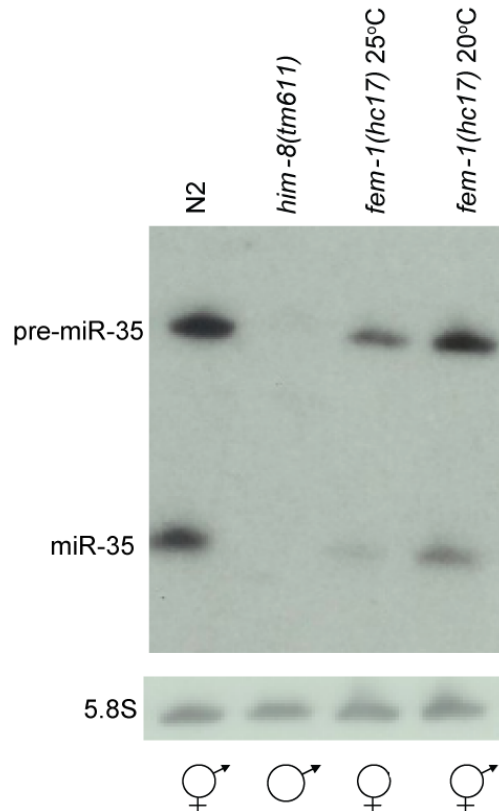


Figure 2.7 Northern blot for precursor and mature miR-35 in sex-related mutants. Synchronized adult worms were used. 5.8S is used as loading control.

2.2.6 Potential miR-35 targets

The various developmental problems observed in *mir-35(gk262)* during embryogenesis suggest that the *mir-35-41* cluster may target many genes for downregulation. We investigated target candidates in a highthroughput approach. Based on our previous observation that target mRNA levels can be regulated by miRNAs (Bagga 2005), I performed microarray experiments using *C. elegans* Affymetrix chips. I compared total RNA extracted from N2 embryos to *mir-35(gk262)* embryos for the microarray. I expected to see potential targets

upregulated in *mir-35(gk262)*, when the miR-35 cluster is not expressed, in comparison to the wild type. After microarray normalization, 125 genes were upregulated in *mir-35(gk262)* embryos compared to N2 ($p < 0.005$ and ≥ 2 fold change). These candidates were computationally evaluated for complementary sites for miR-35-miR-41 in their 3'UTR sequence, but there was no enrichment of potential miRNA sites. We then evaluated the upregulated gene candidates based on functional categories and interactions. The open source program Osprey, which allows visualization of complex network interactions was used for these analyses. Given a list of genes, the program searches the genes in *C. elegans* databases for direct genetic and yeast-two-hybrid interactions. In a second step, interactors for the first group of genes are found. For example, in Figure 2.6 the Rb/cell cycle node contains the *C. elegans* Rb homolog lin-35; lin-35 did not significantly change in our microarray, but three interactors of lin-35 (*crn-6*, T21D12.2, W09G12.3) were upregulated, bringing up a lin-35 network as candidates for regulation by the miR-35-41 cluster. We observed that genes upregulated in *mir-35(gk262)* were grouped in networks mainly related to embryonic development (Figure 2.8). Specifically, the miR-35-miR-41 target candidates were enriched in interactions with the functional categories: cell migration/muscle development/DNA binding, germline/body polarity, proteasome complex, and Rb/cell cycle.

We hypothesize that over expression of genes negatively regulated by *mir-35* may contribute to *mir-35(gk262)* embryo lethality. Thus RNAi depletion of

these genes could suppress the *mir-35(gk262)* embryonic lethality and could be used to evaluate target candidates. For target validation I focused of the lin-35/Rb network because of its functional relationship to cell cycle. This will be discussed in chapter 3.

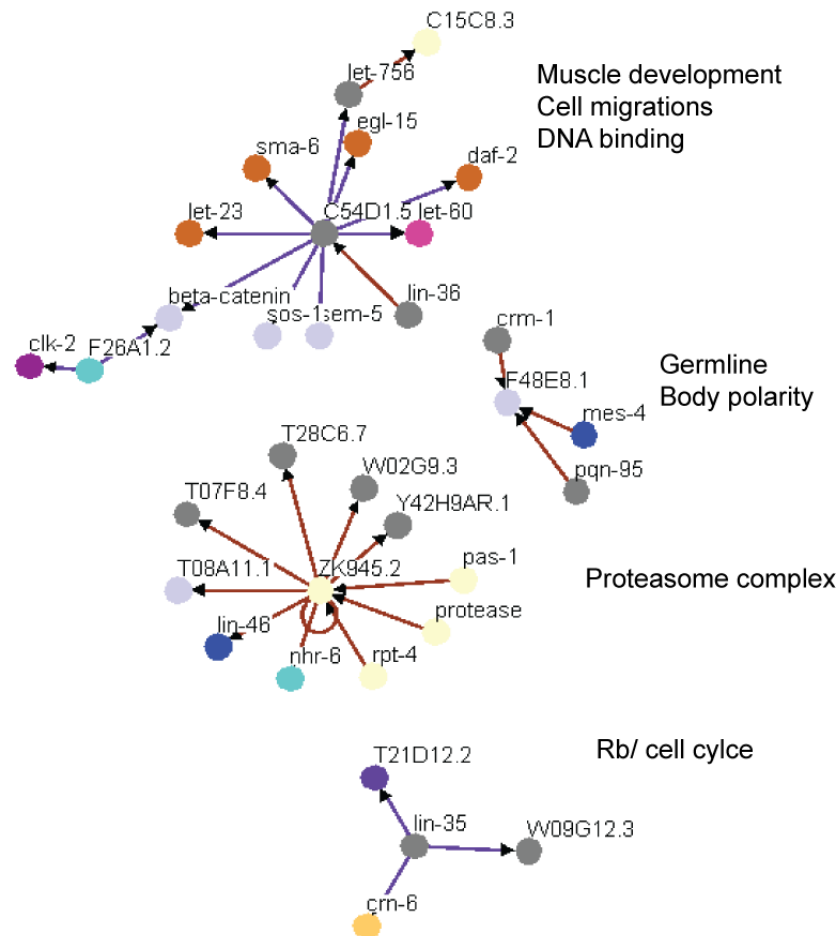


Figure 2.8 *mir-35-41* interaction candidates. Graphical representation of interaction network among genes up regulated in *mir-35(gk262)* and other interactors. On the right side, functional categories for each network (Osprey Network Visualization System).

2.3 Discussion

The results described here demonstrate that the *mir-35-41* cluster of miRNAs is essential during embryogenesis in worms. The *mir-35(gk262)* deletion allele removes all 7 of the miRNAs, and growth of *mir-35(gk262)* mutants at 20°C or 25°C results in 60-70% or 90-95% embryonic lethality, respectively. Reduced viability of the mutants indicates that the *mir-35-41* cluster regulates important target genes during embryogenesis. Temporal and spatial expression of the primary, precursor and mature miRNA forms provide key information for the elucidation of miRNA regulation and processing. To date, only primary miRNA transcripts for only a few miRNA genes have been defined (Bracht, Hunter et al. 2004). I identified the *mir-35-41* full length primary transcript as a sequence of ~1500 nucleotides long. Sequence upstream of the miR-35 start site was used for a reporter construct and showed expression in embryos. This result agrees with detection of mature functional miR-35 by Northern blot at this stage in development. Recently, miRNA promoters driving GFP expression for 73 miRNA genes were studied (Martinez, Ow et al. 2008). In this article the *mir-35-41* promoter used was 400bp longer (upstream) than the one used for my work. Besides predominant expression in embryos, *mir-35-41* promoter activity was also detected in the other larval stages and in adults including the vulva, seam cells, head neurons, and the rectum (EDGEdb for visualization). The broader expression found by Martinez indicates that the promoter used in my work lacked regulatory regions located in an upstream region. Nonetheless, the promoter used in my studies contained sufficient sequence to drive expression in embryos

and rescue *mir-35(gk262)* embryonic lethality. The differences between expression of mature miRNA and miRNA promoter activity observed by Martinez indicates there may be regulation of transcript stability or processing of either pri-miRNA, the precursor miRNA, or the mature miRNA post-transcriptionally. Studies combining these different promoters will contribute to the understanding mir-35-41 regulation.

I have also shown that maternal and zygotic contribution of one copy of the mir-35-41 allele as well as maternal contribution of the miR-35 gene product are sufficient to rescue *mir-35(gk262)* temperature sensitive inviability. We observed defects in cytokinesis during the first cell divisions in *mir-35(gk262)* embryogenesis and delayed cell cycles during embryo progression with most of the embryos dying after gastrulation around the 1.5-2 fold embryonic stage. Early studies of embryonic lethal temperature sensitive mutants proposed a model for timing in development. Given that divisions and migrations in embryonic and post-embryonic lineages are strictly ordered, the precise timing of events is very important (Schierenberg, Miwa et al. 1980). In many genetic studies it was observed that all mutants with cell cycle timing defects were at least maternally sufficient (Deppe, Schierenberg et al. 1978; Schierenberg, Miwa et al. 1980). These observations resulted in the proposed model in which autonomous division rates of cell lines are pre-programmed in the *C. elegans* embryo by maternal genes. Maternal components seem sufficient to direct the embryo through the initial cleavage rounds up to approximately the onset of gastrulation.

Recently embryonic small RNAs were characterized in precisely staged embryos by fluorescence-activated cell sorting followed by second generation sequencing. Members of mir-35-41 cluster showed a peak in expression during the early embryos (Stoeckius, Maaskola et al. 2009). The pre-programmed cell model seems to apply well to how mir-35-41 can regulate its targets. In summary we know from Stoeckius et al that mir-35-41 is expressed very early, in combination with our data showing embryonic defects already during the first cell division, but later delays in embryonic cell cycle rounds. These data support the idea that mir-35-41 cluster may be transcribed before fertilization but is also important later during larval development.

2.4 Methods

***C. elegans* worm strains**

Worms were maintained on NGM worm plates seeded with OP50 bacteria. The following strains were used: wild type Bristol strain N2, *mir-35(gk262)* VC514, *zuls718*, *him-8(tm611)*, *fem-1(hc17)*, *mir-35(gk262); zuls718*,

Brood size assay

Synchronized worms were singled as L4 and allowed to lay embryos for 24h. Parents were then transferred to a new plate for 3 days or until they start to lay oocytes.

Viability assay

Worms were hatched at 20°C and starved L1s were plated at the indicated temperature for the assay. Worms were singled as L4 and allowed to lay embryos for 24h. Parents were then removed from the plates and embryos were counted. 40 hours later worms that grew up to L4 were counted as viable.

PAGE Northern blot

Total RNA was prepared by homogenization of frozen embryos or worm pellets in Trizol Reagent according to manufacturer protocol (Invitrogen). 8µg of total RNA were subjected to Northern analysis. The total RNA was separated by electrophoresis in 11% denaturing polyacrylamide gels and transferred to nylon

membranes (Zeta-Probe GT, Biorad, Hercules, CA). The 5' kinase labeled oligo probe for miR-35 was hybridized to the blot in 5XSSC, 7% SDS, 0.02 m sodium phosphate, 1XDenhardt's solution for approximately 12 h at 50°C and then washed in 3XSSC, 5% SDS, 0.025 m sodium phosphate, 10XDenhardt's solution at 50°C. A 5.8S probe was used as control.

First cell division

Adult embryos were cut and embryos were mounted on a 2% agarose pad covered with a coverslip for confocal imaging (Oegema et al., 2001). Imaging were obtained at 25°C using a 60× 1.4 NA PlanApochromat lens on a spinning disk confocal mounted on a Nikon TE2000-E inverted microscope equipped with a krypton-argon 2.5 W water-cooled laser (Spectra-Physics, Mountain View, CA) and a Hamamatsu Orca ER CCD camera was used. Acquisition parameters, shutters, and focus were controlled by MetaMorph software (Molecular Devices, Downington, PA). Exposure times were 100 and 200 ms for GFP and RFP images, respectively (laser power = 0.8 W).

2.5 Acknowledgment

Chapter 2, in part, is in preparation for publication of the material, Massirer K.B., Giurumescu C., Green R., Oegema K., Pasquinelli A..

Chapter 3: The embryonic mir-35-41 cluster regulates RNAi sensitivity

3.1 Introduction

During the discovery of RNA interference (RNAi) it was first proposed that RNAi would work through an anti-sense mechanism (Fire, Albertson et al. 1991). More careful studies using injection of single and double stranded RNAs in *C. elegans* showed that dsRNA was much more efficient in causing gene silencing than single stranded RNA (Fire, Xu et al. 1998). Later observations showed that feeding or soaking worms in dsRNA would also result in an RNAi effect and this effect could be transmitted to the next generation (Tabara, Grishok et al. 1998; Timmons and Fire 1998; Vastenhouw, Brunschwig et al. 2006). The RNAi mechanism is now known to be conserved in most eukaryotes (Cerutti and Casas-Mollano 2006; Shabalina and Koonin 2008). The RNAi technique of gene silencing is widely used and many highthroughput tools such as lentiviral libraries, shRNA libraries, were developed for different model organisms, including plants, drosophila and mammalian systems (Fraser, Kamath et al. 2000; Kamath, Fraser et al. 2003; Berns, Hijmans et al. 2004). One important tool developed for *C. elegans* was a feeding RNAi library, containing the coding sequence for each gene in the genome (Fraser, Kamath et al. 2000; Kamath, Fraser et al. 2003). In the library, bacterial strains contain plasmids with the genomic sequence of the gene to be targeted cloned between two T7 promoter

sites in opposite directions. The bacterial strain expresses T7 polymerase to produce dsRNA from the plasmid (Timmons, Court et al. 2001). The dsRNA is ingested by the worms, resulting in activation the gene silencing pathway and down-regulation of the targeted gene.

Many of the steps involved in the RNAi mechanism were elucidated using *C. elegans* genetics and the RNAi library itself to screen for resistance or sensitivity to RNAi. During RNAi, dsRNA is recognized by the RNA binding enzyme RDE-4 and guided for cleavage by the RNaseIII Dicer (Timmons, Court et al. 2001; Tabara, Yigit et al. 2002; Vazquez, Gascioli et al. 2004). The resulting small interfering RNAs (siRNAs) are bound by the argonaute protein RDE-1 and used as guides to find the homologous sequence and degrade it. (Tabara, Yigit et al. 2002). Because of their function in key steps in the pathway, *rde-1*, *rde-4* and *dcr-1* were defined as genes essential for RNAi.

During genetic screens, mutations in genes causing enhancement in the efficiency of RNAi were also observed. These genetic mutants are currently used as potent tools to study RNAi against genes that cause weak phenotypes in wild-type worms and for achieving an RNAi effect in neurons. The best characterized enhanced RNAi mutants are the exonuclease 'enhanced RNAi -1' (*eri-1*), the RNA directed RNA polymerase 3 (*rrf-3*) (Simmer, Tijsterman et al. 2002; Kennedy, Wang et al. 2004), the argonaute *ergo-1* and *lin-35*, the worm homolog of Rb (Lehner et al, 2006). *Eri-1*, *rrf-3* and *ergo-1* are involved in production of endogenous small RNAs (endo-siRNA pathway) and most probably affect the

efficiency of RNAi by competing with components in the RNAi pathway. The *C. elegans lin-35* gene is responsible for vulva formation and intestinal cell differentiation in worms (Lu and Horvitz 1998) . The protein is also highly expressed in embryos with unknown function. The RNAi sensitivity of *lin-35* was mainly observed in studies characterizing enhancement of the synthetic multivulva phenotype. A *lin-35* mutant strain was shown to have a gene expression profile that is enriched in endogenous siRNA targets, suggesting decreased levels or function of endo-siRNAs in this mutant.

While using RNAi in our experiments with *C. elegans*, we also came across a mutation that causes hypersensitivity to RNAi. Surprisingly, deletion of the mir-35-41 cluster of 7 miRNAs resulted in enhanced RNAi phenotypes. The region containing the miR-35- miR-41 cluster is about 700 nucleotides long and the 7 members of the cluster show conservation in their 5' region, where the miRNA is predicted to bind its targets. These 7 miRNAs are thus predicted to bind the same group of targets and regulation may depend on the combined expression of the members in the cluster. Expression of the miR-35 members is specific to embryos in *C. elegans* and its deletion causes embryonic lethality (described in Chapter 2). To understand the regulation between the mir-35-41 cluster and the RNAi pathway I compared the mutant allele *gk262*, which deletes the mir-35-41 cluster, with the previously mentioned RNAi hypersensitive mutants. I also used these and the mutants in essential genes for RNAi to generate double mutants with *gk262*. I found that *mir-35(gk262)* shows high

sensitivity to RNAi, comparable to the highest reported single mutant RNAi enhanced strain *lin-35(n745)*. Based on characterization of the double mutants, I concluded that the RNAi hypersensitivity was dependent on canonical exo-RNAi pathway genes. Unexpectedly, I also observed that introduction of nonspecific dsRNA resulted in significant rescue of the *mir-35(gk262)* lethality. Because I also observed overlapping regulated genes between the two RNAi hypersensitive mutants, *lin-35(n745)* and *mir-35(gk262)*, I further characterized LIN-35 protein levels and genetic interaction with *mir-35(gk262)*. The observation of reduced levels of LIN-35 protein in *mir-35(gk262)* mutants indicates that mir-35-41 positively regulate *lin-35*. Taken together, my results show that the mir-35 miRNA gene regulates RNAi sensitivity, at least in part by affecting LIN-35 protein levels. Additionally, I found that loss of *lin-35* enhances the embryonic lethality of mir-35 mutants, revealing a new role for the Rb homolog in this stage of worm development.

Finally, my observation that nonspecific dsRNA can partially compensate for the loss of mir-35-41 during embryogenesis is an important consideration for RNAi based screens and raises many questions about the role of this miRNA family. While future experiments are needed to solve the mystery of how nonspecific dsRNA rescues *mir-35* mutant worms, my working model is that in the absence of the mir-35 miRNAs, the level or activity of an RNA binding protein is inappropriately increased, which results in mis-regulation of genes important for embryogenesis. The nonspecific dsRNA remedies this problem by titrating

the binding or activity of the factor up-regulated in *mir-35(gk262)*. A good candidate for this factor is the Dicer enzyme but future work will be needed to test this hypothesis.

3.2 Results

3.2.1 Control RNAi feeding conditions can rescue *mir-35(gk262)* embryonic lethality

Many studies in plant and animal systems have established that specific miRNAs can regulate a wide range of mRNA targets in diverse biological pathways. Thus, the finding that individual deletion of the majority of miRNA genes in *C. elegans* results in undetectable phenotypes came as a surprise (Miska, Alvarez-Saavedra et al. 2007). Within the 10% of worm miRNA genes that exhibit phenotypes when deleted is the *mir-35-41* locus. I have shown that *mir-35(gk262)* mutation results in 70% embryonic lethality at 20°C and the lethality is increased to 95% at the more restrictive temperature of 25°C. In the best studied cases, miRNAs negatively regulate expression of target protein-coding genes (Olsen and Ambros 1999; Bagga, Bracht et al. 2005). Thus, I predicted that the lethality associated with loss of the *mir-35-41* miRNAs was due to up-regulation of specific target genes and RNAi depletion of the targets could rescue this phenotype. Based on work described in Chapter 2, candidates

functionally related to embryonic development were chosen to test for suppression of the embryonic lethality of *mir-35* mutants.

To test if down-regulation of potential miR-35 targets could suppress the embryonic lethality of *mir-35(gk262)* worms, I attempted to use the *C. elegans* feeding RNAi library. This RNAi technique consists of feeding *C. elegans* with engineered bacteria that produce dsRNA against a gene of interest, which results in knockdown of the corresponding endogenous worm gene. In general, the knockdown is sufficient to cause loss of function phenotypes comparable to genetic mutants for the tested gene. The RNAi library consists of a bacterial strain deficient in RNase III, containing the vector L4440 with two opposing T7 promoters flanking a multiple cloning site (MCS) (Figure 3.2A). Any sequence cloned between the T7 promoters will be transcribed to generate dsRNA upon induction of T7 polymerase production in the bacteria by addition of IPTG to the media (Timmons and Fire 1998). One important negative control used for comparison with specific gene knockdowns is the empty RNAi vector, which lacks worm sequences in the L4440 plasmid. This standard control, called Vector RNAi, is routinely included in RNAi experiments and has not previously been reported to cause any phenotypes.

Since I had established that *mir-35(gk262)* worms grown on regular *E. coli* OP50 food were 5% viable at 25°C (Figure 3.1 column 1), I expected that worms fed the vector RNAi clone would likewise exhibit this level of lethality but RNAi against target genes would rescue the lethality. However, during preliminary tests

with the Vector RNAi, I observed that this control RNAi condition resulted in 35-40% rescue of lethality of *mir-35(gk262)* worms at 25°C (Figure 3.1). This result indicated that the viability of *mir-35* mutant worms is affected by double stranded RNA (dsRNA) produced from the Vector clone, despite the absence of worm sequences in the plasmid. To test the hypothesis that dsRNA produced from the MCS by the Vector RNAi clone was able to rescue *mir-35(gk262)* lethality, I first evaluated *mir-35(gk262)* growth on a panel of RNAi feeding conditions. Standard RNAi plates are normal worm plates (NGM) with the addition of the antibiotic carbenicillin for bacterial selection and of IPTG, which is necessary for induction of T7 expression for transcription of dsRNA. The presence or absence of carbenicillin in the worm plates did not have an effect on the *mir-35(gk262)* rescue. To test if IPTG itself could be responsible for rescuing the worms, IPTG containing NGM plates were seeded with OP50 food. No rescue of embryos laid by the *mir-35(gk262)* worms was observed (Figure 3.1 column 2), indicating that rescue was dependent on the RNAi bacterial strain HT115. The HT115 strain lacking the dsRNA producing plasmid L4440, also did not rescue (Figure 3.1 column 3), meaning that the presence of the plasmid L4440 was essential for *mir-35(gk262)* rescue. By plating *mir-35(gk262)* worms on bacteria containing the Vector RNAi in the presence or absence of IPTG (Figure 3.1 column 3, 4, 5 and 6), I observed rescue of 30-50% of the *mir-35(gk262)* lethality only in the presence of IPTG. Finally, a Vector RNAi strain from a different laboratory in the presence of IPTG (Figure 3.1 column 7), caused similar rescue, thereby, excluding a particular abnormality in our laboratory's Vector containing bacterial

clone. I next tested if the short dsRNA produced from the MCS sequence in the Vector clone was specifically required for the rescue of *mir-35(gk262)* lethality. The *mir-35(gk262)* worms were fed bacteria containing a plasmid with sequence corresponding to GFP (green fluorescence protein) cloned into the MCS (Figure 3.1 column 8). The GFP RNAi results agreed with the Vector RNAi results, showing rescue of *mir-35(gk262)* lethality. The dsRNA rescue appears specific to the *mir-35(gk262)* worm strain, since growth on Vector RNAi does not rescue phenotypes of other miRNA mutants such as *let-7 (n2853)* and *lin-4(e912)*. I concluded that nonspecific RNAi feeding conditions can partially rescue the lethality associated with deletion of the miR-35-41 cluster of miRNAs.

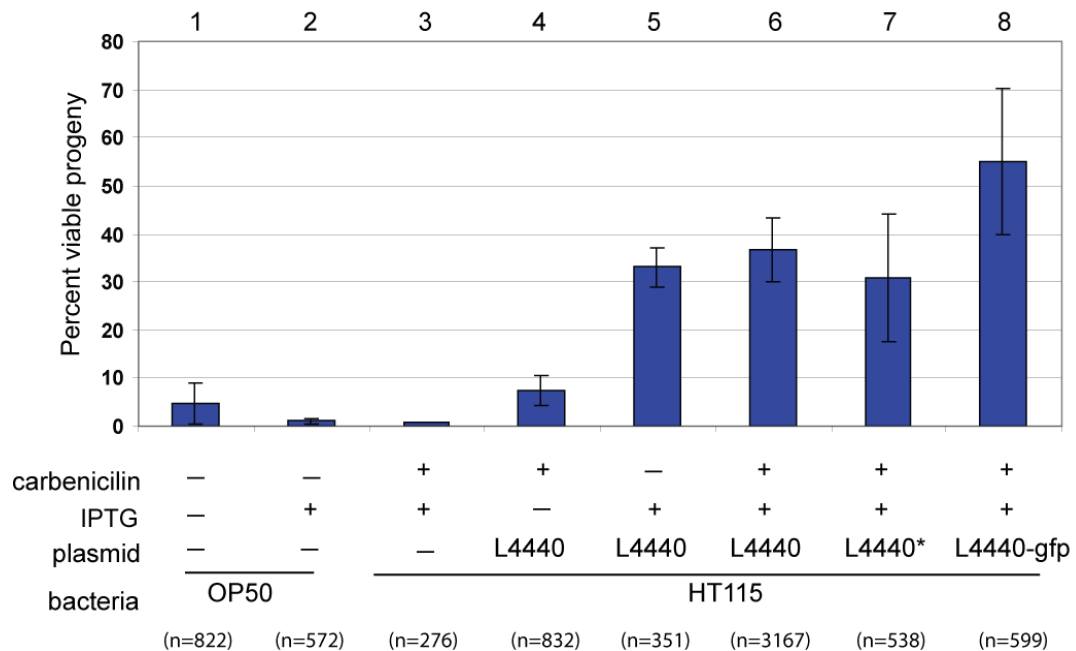


Figure 3.1 Control RNAi feeding conditions can rescue *mir-35(gk262)* embryonic lethality. Worms were grown on regular worm plates supplemented with carbenicilin and IPTG and seeded with different bacterial strains. OP50 is the regular *E. coli* food. HT115 is the bacteria used for RNAi that hosts the RNAi plasmid L4440 for production of dsRNA. Percent viable progeny represents the average number of embryos laid that reached the L4 stage, per *mir-35(gk262)* parent at 25°C. Error bars represent the standard error of the mean for at least two independent experiments. n: total number of embryos counted for each experimental condition.

3.2.2 dsRNA can be generated from the L4440 Vector RNAi clone

Since the rescue of *mir-35(gk262)* embryonic lethality by RNAi feeding conditions requires the addition of IPTG, I predicted that dsRNA produced by T7-mediated transcription of the vector plasmid was responsible for this effect. The L4440 Vector RNAi has two T7 promoter sites oriented in opposite directions for dsRNA production (Figure 3.2 A). Since the vector does not have T7 termination

sequences, some dsRNA is made from the MCS and from the vector backbone. A previous publication showed that worms containing a GFP-lacZ DNA transgene integrated into the genome became silenced after feeding of Vector RNAi (Grishok and Sharp 2005). In that specific case a region of 2kb with high identity between the transgene and the L4440 vector caused the observed GFP silencing. However, no previous study had specifically shown that transcription of the MCS sequence alone produced detectable dsRNA. Figure 3.2 shows a graphical representation of the vector L4440 (panel A) and the plasmid sequence corresponding to the MCS flanked by opposing T7 promoters (panel B). The MCS region located between the two T7 promoters in L4440 is 190 nt long. This size was confirmed by double digestion (Bgl II + Kpn I) of the L4440 vector, which excised a band of the expected size (171 nt) (Figure 3.2 C, lane 4). For comparison, digestion of the L4440-GFP plasmid excised the expected 700 nt band corresponding to the GFP coding sequence (Figure 3.2 C, lane 6). To determine if the Vector RNAi bacteria produced dsRNA from the MCS, Vector RNAi and the comparative GFP RNAi strains were grown in the presence or absence of IPTG. Total RNA was extracted from the bacteria and treated with RNaseA to remove single-stranded RNA. The digestion was performed in a high salt buffer to maintain dsRNA structure, protecting it from RNaseA cleavage. I confirmed that dsRNA was made by visualization of a ~195nt band for the MCS (Figure 3.2 D lane 8) and a ~720 nt band for GFP from bacteria in the presence of IPTG (Figure 3.2 D lane 10). These results show that dsRNA accumulates in bacteria that contain the "empty" L4440 vector control plasmid.

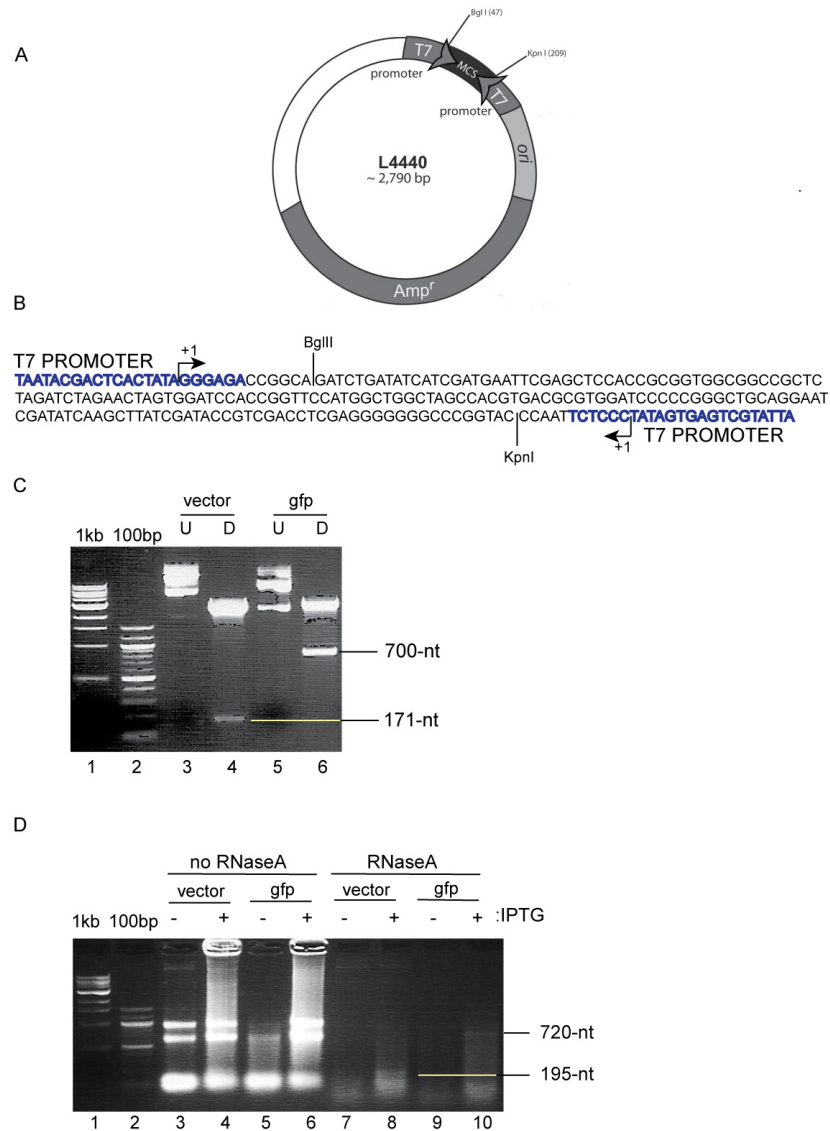


Figure 3.2 dsRNA can be generated from the L4440 Vector RNAi. A. L4440 Plasmid Map depicts the two T7 promoter sequences oriented in opposite directions and flanking the multiple cloning site (MCS). B. Sequence corresponding to the 180 nt MCS flanked by T7 promoter sequences. T7 transcription initiation sites are labeled +1. C. L4440-vector and L4440-GFP DNAs were run on a gel undigested (U) (lanes 3 and 5) or digested (D) with BglIII and KpnI (lanes 4 and 6). D. 5 ug of RNA from HT115 bacteria containing L4440-vector (lanes 3 and 4, 7 and 8) or L4440-GFP (lanes 5 and 6, 9 and 10), grown in the absence (-) or presence (+) of IPTG were run on a gel before and after RNaseA treatment. Lanes 8 and 10 show the band corresponding to the dsRNA produced. Double bands in lanes 3-6 represent the abundant 16S and 23S ribosomal RNAs.

3.2.3 Non-specific dsRNA can rescue *mir-35(gk262)* embryonic lethality

To verify that the dsRNA produced by the RNAi feeding plasmids was solely responsible for the rescue of *mir-35(gk262)* lethality, I directly injected dsRNA into the mutants and scored embryonic viability. dsRNA corresponding to GFP was synthesized in vitro from a PCR template that consisted of GFP sequence flanked by T7 promoter sequences. First, I injected the GFP dsRNA into the GFP-expressing strain PD4251 to confirm the effectiveness of my technique and the dsRNA functionality. Injection of dsRNA corresponding to GFP, but not vector, resulted in strong down-regulation of GFP expression in most of the muscle body cells (Figure 3.3 A). I then crossed this GFP marker into *mir-35(gk262)* worms, generating the strain called PQ299. The use of PQ299 allowed me to verify that my injections produced an RNAi response (GFP silencing) and test if the injected dsRNA rescued *mir-35(gk262)* lethality at 25 °C. In agreement with the feeding RNAi experiments, injection of GFP-dsRNA into PQ299 L4 worms, rescued 30% of their progeny (Figure 3.3 B). The buffer injection control resulted in the same low viability as non-injected PQ299 worms. Consequently, non-specific dsRNA introduced into *mir-35(gk262)* worms by feeding or injection can partially rescue the lethality associated with deletion of the miR-35 cluster of miRNAs.

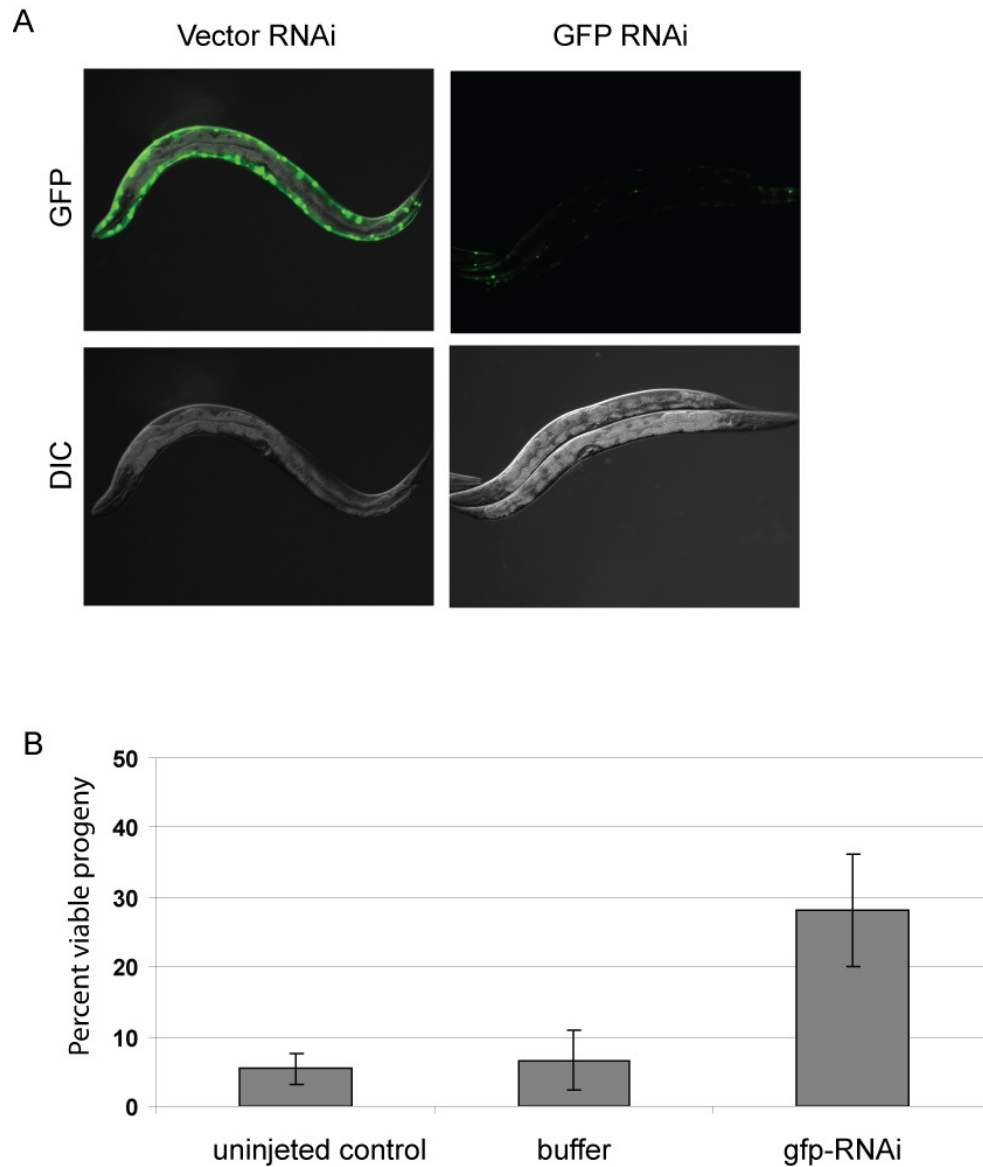


Figure 3.3 Non-specific dsRNA can rescue mir-35(gk262) embryonic lethality. A. Micrograph of the PD4251 worm strain containing a transgenic GFP after injecting MCS dsRNA or GFP dsRNA. Upper panel shows GFP and lower panel DIC images taken at 200x magnification. B. Percent viable progeny represents the average number of embryos laid that reached the L4 stage per mir-35(gk262);PD4251 parent for worms not injected, injected with buffer control, injected with GFP dsRNA. Injected parents were placed at 25°C.

3.2.4 dsRNA does not cause significant changes in overall endogenous mRNA levels

Once dsRNA is introduced into worms, it is processed into small interfering RNAs (siRNAs) by the RNAi machinery. The siRNAs target the corresponding endogenous mRNAs for degradation, resulting in down-regulated expression of the target gene. Although vector or GFP dsRNA does not correspond to any worm genes, general introduction of dsRNA could induce gene expression changes that affect the viability of *mir-35(gk262)* worms. One possibility was that the presence of dsRNA could result in upregulation of specific genes in the RNAi pathway. This hypothesis was tested by microarray hybridization experiments, using total RNA from three independent sets of wild type (N2) and *mir-35(gk262)* embryos produced by worms cultured in the presence (+IPTG) or absence (-IPTG) of vector dsRNA induction. The rationale for this experiment is that changes in mRNA expression profiles in the presence versus absence of IPTG would indicate genes that specifically respond to the uptake of nonspecific dsRNA.

The introduction of vector dsRNA to N2 worms resulted in minor overall changes in gene expression profiles. When we compared the profiles of embryos from +/-IPTG treated N2 parents, the great majority of mRNA transcripts did not change significantly ($p > 0.005$). Most of the significant changes were less than 1.5 fold in either direction. In the presence of dsRNA, 140 genes were significantly up-regulated, although 90% showed less than a 1.1 fold difference.

Of the 147 genes that showed down-regulation, 85% changed less than 1.5 fold. Nullifying our prediction, none of the transcripts corresponding to known genes in the RNAi pathways were significantly changed in the presence of dsRNA. Due to the small changes and because the differentially expressed genes were not enriched in specific gene ontology categories, we conclude that the presence of vector dsRNA does not cause a significant effect on the general transcriptome profile of N2 embryos.

The number of genes affected by the presence of dsRNA in *mir-35(gk262)* was greater than that detected in N2, but there was also no enrichment for specific gene categories. From the comparison of *mir-35(gk262)* embryos produced in the presence or absence of dsRNA (+/-IPTG), 258 genes were significantly up-regulated and 257 were down-regulated by the dsRNA inducing conditions. There was significant overlap in the gene expression profiles of N2 and *mir-35(gk262)* embryos in the presence of dsRNA. In conclusion, the presence of dsRNA did not cause dramatic changes in the embryonic transcriptome profiles and did not reveal obvious candidates that might contribute to the suppression of embryonic lethality by vector RNAi in *mir-35(gk262)* worms.

3.2.5 Expression of mir-42, a member of the miR-35 family is not affected in *mir-35(gk262)*

Since the miR-35 family of miRNAs has one more member, miR-42, it is possible that these miRNAs share common target mRNAs. We proposed that miR-42 expression could be upregulated during the rescue of *mir-35(gk262)* and compensate for the absence of miR-35-41. I showed that wild type *C. elegans* embryos contain high levels of the mature forms of miR-35-41 (Figure 2.1). Thus, misregulation of many specific targets by miR-35-41 could cause the embryonic lethality observed by deletion of this cluster. The miR-42 sequence is 350kb away from the miR-35 cluster of miRNAs on chromosome II and it is not deleted in *mir-35(gk262)* worms. The eight nucleotides of the 5' end of miR-42 are conserved with the miR-35-41 cluster miRNAs (Figure 3.4A) and miR-42 is also normally expressed in embryos. Since only protein-coding transcripts were profiled by the array analyses, I used Northern blotting to directly test if miR-42 levels were affected in *mir-35(gk262)* mutants and or in the presence of dsRNA. These experiments demonstrated that the levels of mature miR-42 and another unrelated miRNA, miR-2, were unchanged in N2 versus *mir-35(gk262)* mutants in the presence or absence of Vector dsRNA (Figure 3.4 B). These results suggest that rescue of the embryonic lethality of *mir-35(gk262)* worms by dsRNA is through a mechanism other than up-regulation of a miRNA family member that could re-establish regulation of common targets.

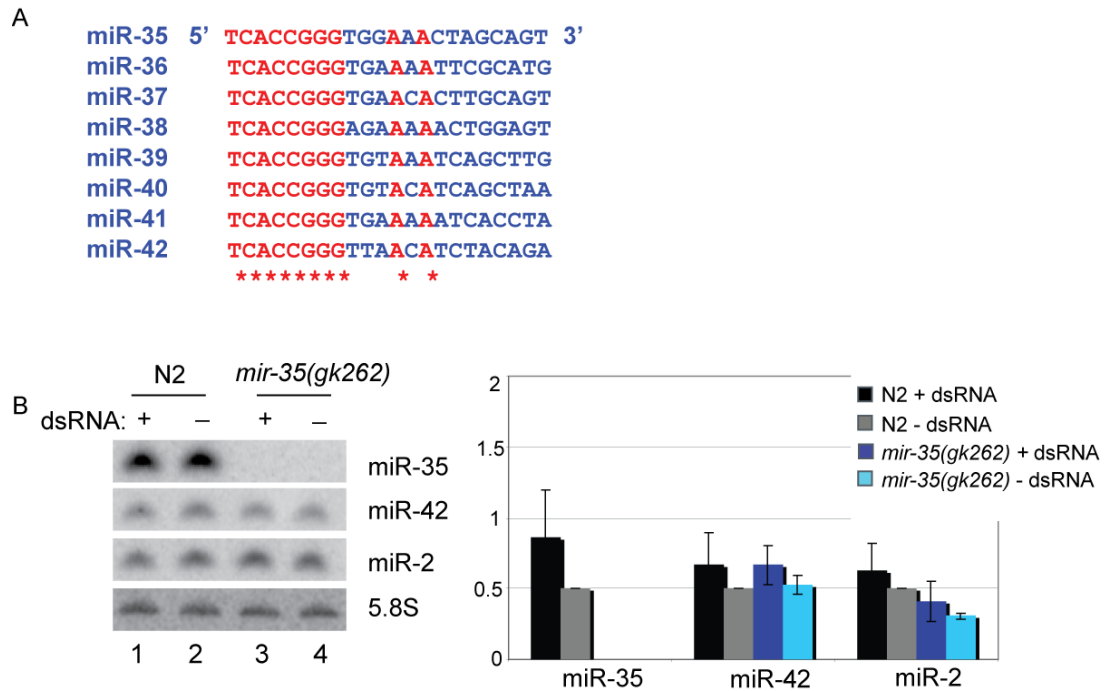


Figure 3.4 Expression of endogenous miRNAs is not affected in the absence of miR-35-41 cluster. A. Alignment of mature sequences of the miR-35 family showing miR-42 5'end conservation. Asterisks represent nucleotides conserved in all members. B. Total RNA was isolated from wild type (N2) or *mir-35(gk262)* worms grown in the presence (+) or absence (-) of IPTG to induce dsRNA. RNA was analyzed by PAGE-northern blotting with probes for miR-35, miR-42 or miR-2. C. Levels of miR-35, miR-42 and miR-2 relative to 5.8S were plotted for 3 independent experiments. miR-35 is completely absent in *mir-35(gk262)*.

3.2.6 Specific RNAi pathway genes affect the embryonic viability of *mir-35(gk262)* worms

Another way of testing if the rescue by dsRNA is related to the RNAi pathway is by crossing *mir-35(gk262)* to genetic mutants for components in the pathway. The simplest hypothesis is that mutation of other genes in the RNAi pathway would enhance *mir-35* lethal phenotypes and mutation of genes required for RNAi would suppress *mir-35(gk262)* phenotypes. We generated genetic doubles of *mir-35(gk262)* with key components in the small RNA pathways. Using single and double mutants, I assessed the viability of each strain at 20°C and 25°C. The single mutants defective in RNAi (*rde-1*, *rde-4* and *rrf-1*) showed no embryonic inviability as seen on Table 3.1. The combination of these mutants with *mir-35(gk262)* did not rescue the embryonic lethality of *mir-35(gk262)* single mutants. Like *mir-35(gk262)*, the RNAi enhanced mutants, *eri-1* and *rrf-3* exhibit temperature sensitive phenotypes. The *eri-1* and *rrf-3* mutants show wild type phenotypes at 20°C with reduced brood size and sterility at 25°C. The combination of *mir-35(gk262)* with *eri-1(mg366)* or *rrf-3(pk1426)* results in the *mir-35* phenotype of reduced embryonic viability at 20°C (33% for *mir-35*, 100% for *eri-1* and 37% for *mir-35;eri-1* doubles, 100% for *rrf-3* and 35% for *mir-35(gk262);rrf-3(pk1426)* Table 3.1. On the other hand, the *eri-1* and *rrf-3* phenotypes of sterility linked to sperm defects at 25°C were also seen in the double mutants with *mir-35(gk262)* (no embryos produced for *eri-1* and *rrf-3* or *mir-35;eri-1* and *mir-35;rrf-3* (Table 3.2). In contrast to *rrf-3* and *eri-1* mutants,

loss of *ergo-1*, an argonaute protein, results in RNAi hypersensitivity without obvious fertility or viability defects. I found that embryos from *ergo-1* mutants are completely viable, but when combined with the *mir-35(gk262)* allele the double mutants display the embryonic lethality typical of *mir-35* single mutants. Taken together, these results indicate that loss of *eri-1*, *rrf-3* or *ergo-1* does not affect the *mir-35(gk262)* phenotype at 20°C; at 25°C, the sterility associated with *eri-1* (*mg366*) or *rrf-3(pk1426)* obscures any downstream effects on the *mir-35(gk262)* embryonic lethal phenotype.

Additionally, we have crossed *mir-35(gk262)* to the RNAi enhanced strain *lin-35(n754)*. *Lin-35* encodes the worm homolog to the Rb (retinoblastoma) gene but it is not clear how deletion of *lin-35* is related to the RNAi hypersensitivity. Given that we were not able to obtain double mutants of *lin-35(n745); mir-35(gk262)* for viability analysis, we suspected that these genes could interact and we explore this later.

Table 3.1 Effect of essential RNAi pathway genes on *mir-35(gk262)* embryonic viability

Worm strains	% Viability	
	20°C	25°C
Wild type (N2)	100	100
<i>rde-1(ne300)</i>	100	100
<i>rde-4(ne301)</i>	100	100
<i>rrf-1(pk1417)</i>	100	100
<i>mir-35(gk262)</i>	33 ± 11	6 ± 2
<i>mir-35(gk262);rde-1(ne300)</i>	35 ± 9	3 ± 2
<i>mir-35(gk262);rde-4(ne301)</i>	34 ± 21	3 ± 1
<i>mir-35(gk262);rrf-1(pk1417)</i>	47 ± 18	5 ± 5

Percent viability represents embryos that grew up to L4
Average of at least 2 independent experiments

Table 3.2 Effect of RNAi hypersensitive genes on *mir-35(gk262)* embryonic viability

Worm strains	% Viability	
	20°C	25°C
Wild type (N2)	100	100
<i>rrf-3(pk1426)</i>	100	Ste
<i>eri-1(mg366)</i>	100	Ste
<i>ergo-1(tm1860)</i>	100	100
<i>lin-35(n745)</i>	88 ± 29	Ste
<i>mir-35(gk262)</i>	33 ± 11	6 ± 2
<i>mir-35(gk262);eri-1(mg366)</i>	37 ± 10	Ste
<i>mir-35(gk262);rrf-3(pk1426)</i>	45 ± 2	Ste
<i>mir-35(gk262);ergo-1(tm1860)</i>	21 ± 10	5 ± 2
<i>mir-35(gk262);lin-35(n745)</i>	Inviabile	Inviabile

Percent viability represents embryos that grew up to L4
Average of at least 2 independent experiments

3.2.7 The susceptibility of *mir-35(gk262)* to RNAi is dependent on the canonical RNAi pathway

To identify if specific steps in the RNAi pathway are required for rescue of *mir-35* mutants, the single and double mutants related to the RNAi pathway were evaluated for response to dsRNA. In contrast to the almost complete embryonic lethality observed for *mir-35(gk262)* worms cultured at 25°C, 30% of *mir-35(gk262)* embryos from parents raised on Vector RNAi control bacteria are viable. As described previously, this result is dependent on induction of dsRNA

synthesis. The viability of the single mutants defective in RNAi (*rde-1*, *rde-4* and *rrf-1*) was not affected by the presence of dsRNA (Table 3.3). As expected, the rescue by Vector RNAi was lost in *mir-35(gk262); rde-1(ne300)* and in *mir-35(gk262); rde-4(ne301)* double mutants that are incapable of activating an RNAi response. This result demonstrates that the rescue by dsRNA is dependent on the RNAi pathway. Interestingly, when the *mir-35* deletion was combined with deletion of *rrf-1*, an RNA dependent RNA polymerase, functioning downstream in the RNAi pathway, the embryonic rescue was maintained. From these results, I conclude that the miR-35-41 miRNAs are required for embryonic viability in a pathway that can be compensated by initiation of an RNAi response via nonspecific dsRNA. Moreover the rescue by dsRNA is not dependent on downstream steps in the RNAi pathway.

The *rrf-3*, *eri-1* and *ergo-1* genes result in increased sensitivity to RNAi. These genes function in the endogenous RNAi pathway, whereby encoded small interfering RNAs (endo-siRNAs) regulate the expression of worm genes. The hypersensitivity associated with mutations in these genes is believed to result from lessened competition between the exogenous and endogenous RNAi pathways. Culture of the single and double mutants on Vector dsRNA at 25°C did not substantially affect their phenotypes. Strains with mutations in *eri-1* and *rrf-3* alone or in combination with *mir-35* were largely sterile, while embryos from *ergo-1* mutants were 100% viable and the *ergo1;mir-35* double mutants showed viability similar to *mir-35(gk262)* embryos alone on Vector

Table 3.3 Rescue of *mir-35(gk262)* by dsRNA is dependent on the initial steps of the RNAi pathway

Worm strains	%Viability	
	Vector - IPTG	Vector + IPTG
wild-type (N2)	100	100
<i>rde-1(ne300)</i>	100	100
<i>rde-4(ne301)</i>	100	100
<i>rrf-1(pk1417)</i>	100	100
<i>mir-35(gk262)</i>	11 ± 8	36 ± 11
<i>mir-35(gk262);rde-1(ne300)</i>	2 ± 1	9 ± 3
<i>mir-35(gk262);rde-4(ne301)</i>	8 ± 1	11 ± 2
<i>mir-35(gk262);rrf-1(pk1417)</i>	4 ± 2	46 ± 12

Percent viability represents embryos that grew up to L4. Ste: sterile
Average of at least 2 independent experiments

Table 3.4 RNAi hypersensitive mutants do not enhance the rescue of *mir-35(gk262)* by dsRNA

Worm strains	%Viability	
	Vector - IPTG	Vector + IPTG
wild-type (N2)	100	100
<i>rrf-3(pk1426)</i>	Ste	Ste
<i>eri-1(mg366)</i>	Ste	Ste
<i>ergo-1(tm1860)</i>	100	100
<i>lin-35(n745)</i>	Ste	Ste
<i>mir-35(gk262)</i>	11 ± 8	36 ± 11
<i>mir-35(gk262);rrf-3(pk1426)</i>	Ste	Ste
<i>mir-35(gk262);eri-1(mg366)</i>	Ste	Ste
<i>mir-35(gk262);ergo-1(tm1860)</i>	7 ± 5	38 ± 11
<i>mir-35(gk262);lin-35(n745)</i>	inviable	inviable

Percent viability represents embryos that grew up to L4
Average of at least 2 independent experiments

3.2.8 Loss of the miR-35 cluster results in hypersensitivity to RNAi

While using forward genetic approaches to identify genes that contributed to the embryonic lethal phenotype of *mir-35(gk262)* worms, I made the surprising discovery that this strain exhibits enhanced sensitivity to RNAi. While diverse protein coding genes have been implicated as negative regulators of the RNAi pathway in *C. elegans*, my results were the first to link specific miRNAs to a role in controlling the efficiency of RNAi. Thus, to evaluate the sensitivity of *mir-*

35(gk262) mutants to RNAi, I first used feeding RNAi against the gene *unc-22* (UNCoordinated-22), which is known to cause a partially penetrant twitching phenotype in wild type (N2) worms in comparison to *unc-22* genetic mutants, which are completely paralyzed. With this assay we expected that RNAi enhanced strains would become paralyzed. To determine the degree of sensitivity in *mir-35(gk262)* worms, we compared the RNAi effect to the mutant strain *rrf-3(pk1426)*, which is one of the established RNAi hypersensitive worm strains (Simmer et al, 2002).

Remarkably, 83% of *mir-35(gk262)* worms became paralyzed (Table 3.5) in contrast to only 2% of N2 and 17% of *rrf-3(pk1426)*. Since *unc-22* genetic mutants exhibit paralysis, this result suggests that *mir-35(gk262)* worms are RNAi hypersensitive. To further characterize the RNAi hypersensitivity of *mir-35(gk262)* mutants, feeding RNAi against genes with somatic and embryonic functions was similarly performed.

Knockdown of the somatic gene *lin-1* (abnormal cell LINEage) causes almost no detectable phenotype in N2, while genetic mutants show a Muv (multivulva) phenotype. After *lin-1* RNAi, *mir-35(gk262)* and *rrf-3(pk1426)* mutants showed significantly more Muv worms (54% of *rrf-3(pk1426)* and 69% of *mir-35(gk262)*) than N2 (1%) (Table 3.1). RNAi treatment of another somatic gene *sqt-1*(QUaT) caused 80% of *rrf-3(pk1426)* and 84% of *mir-35(gk262)* to roll around the axis of their own bodies (Rol) compared to 7% of N2 (Table 3.5). Inhibition of the germline specific gene *pos-1* and the embryonic specific gene

sex-1 (Signal Element on X) by RNAi caused complete embryonic lethality (Emb) in *rrf-3(pk1426)* and *mir-35(gk262)* strains, while N2 embryos hatched to some degree (Table 3.5). With these phenotypic analyses I confirmed the *mir-35(gk262)* enhanced sensitivity to RNAi and I showed that worms lacking the miR-35-41 cluster are at least as sensitive to RNAi as the *rrf-3* genetic background.

To determine if the enhanced RNAi phenotype of *mir-35(gk262)* mutants was dependent on the canonical RNAi pathway, *unc-22* RNAi treatment was performed in the worms with mutations in essential genes for the RNAi pathway. As expected, the single mutants defective in RNAi (*rde-1*, *rde-4* and *rrf-1*) showed no twitching phenotype in response to *unc-22* RNAi (Table 3.6). Comparing the single and double mutant strains, I found that mutations in *rde-1*, *rde-4* or *rrf-1* render *mir-35(gk262)* mutants completely RNAi defective, resulting in loss of the *unc-22* phenotypes observed in the *mir-35(gk262)* single mutant. The data show that *mir-35(gk262)* mutants require *rde-1*, *rde-4* and *rrf-1* to exhibit an RNAi response. While this might have been expected, there is precedence for an RNAi hypersensitive strain *lin-15(n745)* being independent of *rrf-1* activity (Wang et al, 2005).

Although the RNAi hypersensitivity of *mir-35* mutants is already relatively strong, I asked if it could be further enhanced when combined with other RNAi enhanced strains. To test this *unc-22* RNAi was performed in the strains *eri-1* and *ergo-1* single mutants and combinations with *mir-35(gk262)*. The paralysis

rates in *eri-1* and *ergo-1* worms after *unc-22* RNAi were similar to *rff-3* (ranging around 25%) in relation to N2 (2%) and thus less sensitive than the 83% seen for *mir-35(gk262)* (Table 3.7). When the enhanced RNAi strains were combined with *mir-35(gk262)*, the already high penetrance of paralysis of *mir-35(gk262)* single mutants increased to about 90%. Since *mir-35(gk262)* alone have high penetrance of paralyzed worms the experiment only allows us to conclude that *mir-35(gk262)* doubles are as sensitive as *mir-35(gk262)* single mutants. The other RNAi hypersensitive mutant, *lin-35(n745)*, was reported as more sensitive to RNAi than *eri-1* and *rff-3* (Lehner, Calixto et al. 2006). Our experiments evaluating *lin-35* response to *unc-22* RNAi are in agreement with this observation and the high penetrance of the paralysis phenotype is comparable to the *mir-35(gk262)* mutant (Table 3.7).

As mentioned before, we also attempted to combine *lin-35* with the *mir-35(gk262)* strain. We isolated heterozygous but not homozygous worms for the *mir-35(gk262)* mutation in the *lin-35(n745)* background. Since *lin-35* mutants exhibit reduced brood size but no embryonic lethality at 20°C, loss of *lin-35* may enhance the *mir-35* phenotype from ~40% to complete embryonic lethality at this temperature. Supporting this hypothesis, RNAi against *lin-35* in *mir-35(gk262)* mutants showed high embryonic inviability resulting from the loss of these two negative regulators of the RNAi pathway. The *lin-35* gene encodes the worm homolog of the mammalian retinoblastoma protein pRB, a tumor suppressor that represses G1 to S transition in the cell cycle by interaction with the E2F

transcription factor. In worms, the *lin-35* gene is highly expressed in embryos, but its function during this stage of development is not well characterized other than having an important role in pharynx formation in late embryogenesis (Fay et al, 2003). My results demonstrate that *lin-35* activity partially compensates for the loss of the mir-35 cluster in embryos.

Table 3.5 *mir-35(gk262)* are hypersensitive to RNAi

Gene	RNAi phenotype	Worm strains			Tissue
		N2	<i>rrf-3(pk1426)</i>	<i>mir-35(gk262)</i>	
<i>unc-22</i>	% Pri	2 ± 2	17 ± 5	83 ± 14	somatic
<i>lin-1</i>	% Mvu	1 ± 0	54 ± 7	69 ± 8	somatic
<i>sqt-1</i>	% Rol	7 ± 1	80 ± 4	84 ± 6	somatic
<i>pos-1</i>	% Emb	10 + 4	100	100	germline
<i>sex-1</i>	% Emb	36 ± 11	98 ± 1	100	embryonic

Pri: paralyzed, Mvu: multivulva, Rol: roller, Emb: embryonic lethal. Average of at least 2 independent experiment

Table 3.6 The susceptibility of *mir-35(gk262)* is dependent on the RNAi pathway

Worm strains	Phenotype %		
	Wt	Twitching	Paralyzed
wild-type (N2)	10 ± 1	88 ± 3	2 ± 2
<i>rde-1(ne300)</i>	100	0	0
<i>rde-4(ne301)</i>	100	0	0
<i>rrf-1(pk1417)</i>	100	0	0
<i>mir-35(gk262)</i>	0	17 ± 5	83 ± 4
<i>mir-35(gk262);rde-1(ne300)</i>	100	0	0
<i>mir-35(gk262);rde-4(ne301)</i>	100	0	0
<i>mir-35(gk262);rrf-1(pk1417)</i>	100	0	0

Percent of adult worms with the Wt, twitching or paralyzed phenotype

Average of at least 2 independent experiment

Table 3.7 The response of *mir-35(gk262)* to RNAi is similar to the effect in *lin-35(n745)*

Worm strains	Phenotype %		
	Wt	Twitching	Paralyzed
wild-type (N2)	10 ± 1	88 ± 3	2
<i>rrf-3 (pk1426)</i>	0	83 ± 14	17
<i>eri-1 (mg366)</i>	0	73 ± 3	27
<i>ergo-1 (tm1860)</i>	0	66 ± 4	34
<i>lin-35 (n745)</i>	0	8 ± 2	95
<i>mir-35 (gk262)</i>	0	17 ± 5	83
<i>mir-35 (gk262); rrf-3 (pk1426)</i>	0	11 ± 4	89
<i>mir-35 (gk262); eri-1 (mg366)</i>	0	10 ± 1	90
<i>mir-35 (gk262); ergo-1 (tm1860)</i>	0	5 ± 7	95
<i>mir-35 (gk262); lin-35 (n745)</i>	inviabile		

Percent of adult worms with the Wt, twitching or paralyzed phenotype

Average of at least 2 independent experiment

3.2.9 The expression LIN-35 protein is down-regulated in *mir-35(gk262)* embryos

To identify changes in gene expression that could help explain why *mir-35(gk262)* mutants are RNAi hypersensitive and embryonic lethal, our embryonic

mir-35(gk262) microarray data was compared with results from published arrays using *lin-35(n745)* embryos (Kirienko & Fay, 2007). There was statistically significant overlap in genes up-regulated in the two mutant strains. Since the loss of *miR-35-41* or *lin-35* results in worms with RNAi hypersensitivity and some similarities in gene expression profiles, these two genes may positively regulate each other in a redundant pathway important for embryogenesis and regulation of the RNAi pathway. The mRNA corresponding to *lin-35* was not differentially expressed between *mir-35(gk262)* and N2 embryos. We confirmed this result by RT-PCR (Figure 3.5 B). To evaluate the LIN-35 protein level, Western blotting for LIN-35 was performed on protein extracts from N2 versus *mir-35(gk262)* embryos. LIN-35 protein levels were 2.8-fold reduced in the mutants relative to N2 (Figure 3.5), suggesting that miR-35-41 positively regulates the accumulation of LIN-35 protein in embryos. In contrast, miR-35 miRNA levels were unaffected in *lin-35(n745)* mutant embryos as seen by Northern analysis (Figure 3.5C). The decreased levels of LIN-35 in *mir-35(gk262)* worms could explain the RNAi hypersensitivity but do not fully account for the embryonic viability phenotype. Instead, miR-35-41 likely regulates *lin-35* and other genes in parallel pathways important for embryogenesis. In support of this model, embryos with the loss of *lin-35* alone are viable, while the loss of *mir-35* alone reduces viability and the loss of both genes causes unviability. This work identifies miR-35-41 as a new regulator of *lin-35* and demonstrates novel connections between RNAi pathway genes and embryonic viability.

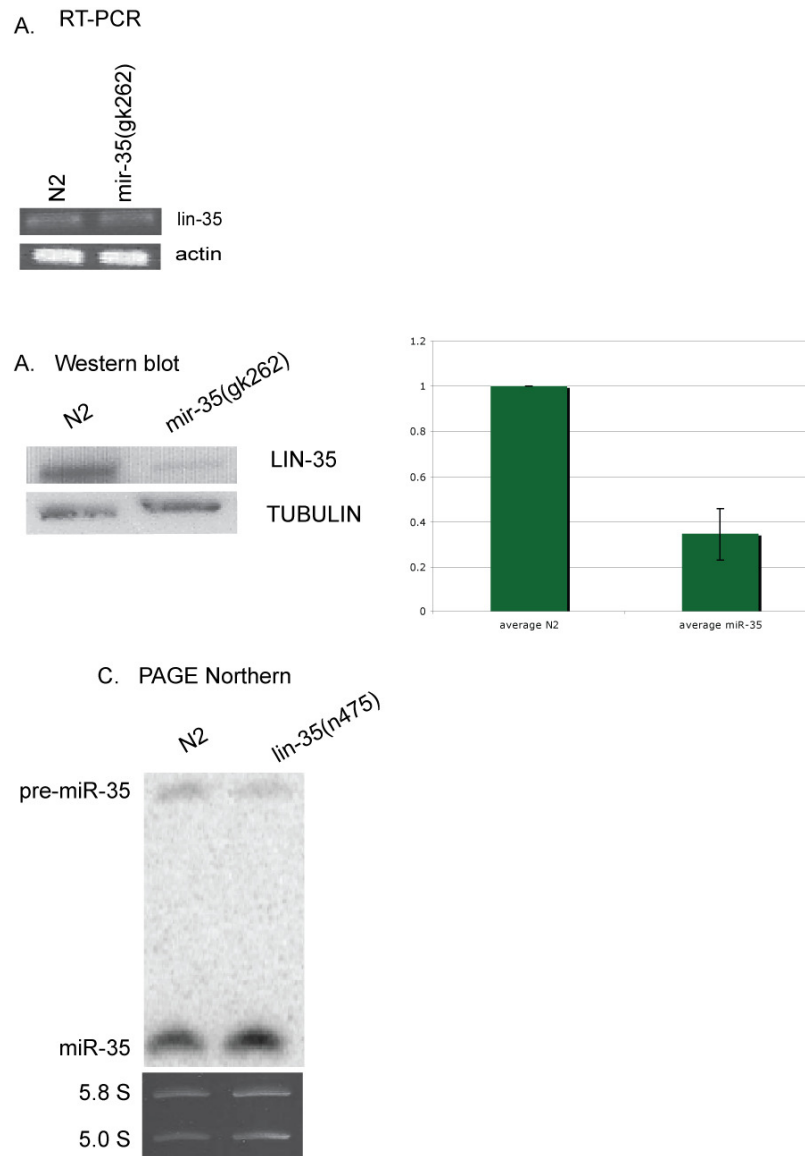


Figure 3.5 *mir-35(gk262)* have decreased LIN-35 protein. A. RT-PCR for *lin-35* mRNA in N2 and *mir-35(gk262)* worms and actin loading control. Graph of 2 replicates. B. 20 ug total protein lysates from N2 and *mir-35(gk262)* embryos were subjected to western blot analysis of LIN-35 and the control alpha-TUBULIN. Graph represents the average and standard error from 3 independent experiments, comparing levels of LIN-35 relative to TUBULIN in N2 versus *mir-35(gk262)* ($p < 0.005$). B. PAGE Northern analyses of total RNA from N2 and *lin-35(n475)* embryos to detect miR-35 precursor and mature miRNA levels. D. Graph for 3 independent experiments showing microRNA levels relative to 5.8S.

3.3 Discussion

Products of the small RNA pathways control a wide range of endogenous target genes in *C. elegans*. The choice for targets depends on the initial dsRNA source that originates the small RNA and of their interacting proteins. Recent work has shown evidence for cross regulation among the miRNA, endo-RNAi and exo-RNAi pathways (Lee, Hammell et al. 2006; Yigit, Batista et al. 2006). For example, mutants in the components of the endo-siRNA pathway *rrf-3*, *ergo-1* and *eri-1* show hypersensitivity to RNAi (Simmer, Tijsterman et al. 2002; Kennedy, Wang et al. 2004; Yigit, Batista et al. 2006). The *rrf-3* and *eri-1* mutants, when exposed to exogenous RNAi, show increased amounts of processed small RNAs (Lee, Hammell et al. 2006; Yigit, Batista et al. 2006). This demonstrates the existence cross regulation between the two pathways. The worm homolog of Rb, *lin-35*, and some other members in the *lin-35* pathway also show RNAi enhancement (Lee, Hammell et al. 2006; Ceron, Rual et al. 2007). Additionally, microarray analyses of *lin-35* mutants revealed enrichment of endosiRNA targets in the up-regulated gene set, suggesting that *lin-35* is required for repression of endo-siRNA (Grishok, Hoersch et al. 2008). Our surprising observation that *mir-35(gk262)* exhibit enhanced sensitivity to RNAi indicates that the miR-35 cluster can regulate the RNAi pathway. In contrast to the *mir-35(gk262)* lethality at 25°C, we observed that a significant percentage of *mir-35(gk262)* embryos from parents raised on Vector RNAi control bacteria are viable at this temperature. This result is also dependent on induction of dsRNA synthesis indicating that nonspecific dsRNA can partially rescue the lethality

associated with deletion of the miR-35 cluster of miRNAs and this effect was also dependent on genes that initiate the RNA response.

Testing of RNAi sensitivity to *unc-22* in *mir-35(gk262)* in comparison to the *rrf-3*, *lin-35* and *eri-1* mutants showed that *mir-35(gk262)* sensitivity is comparable to *lin-35*. Crosses between *mir-35(gk262)* and the genes *rde-1* and *rde-4*, required for the RNAi pathway, caused loss of the RNAi sensitivity, showing its dependence on an active pathway. We tested if the absence of *mir-35-41* in embryos could result in higher amounts of endo-siRNAs based on the report that siRNAs target mostly genes related to embryonic lethality. An excess of endo-siRNAs could be responsible for the lethality on *mir-35(gk262)* mutants. If that were the case, cross of *mir-35(gk262)* with the endo-siRNA argonaute specific gene *ergo-1* would rescue *mir-35* lethality, but we did not observe improvement in the *mir-35* phenotype after this genetic test. The lack of viable progeny from both the *mir-35(gk262)* cross to *lin-35* and by *lin-35* RNAi in *mir-35* worms strongly indicated that these two genes interact. The additional observation that *mir-35(gk262)* has very low *lin-35* levels agrees with a model where miR-35-41 positively regulate *lin-35* in a redundant pathway important for embryogenesis and regulation of the RNAi pathway. Because microRNAs usually regulate genes negatively, we propose the model illustrated in Figure 3.6, where mature miRNAs of the miR-35 cluster would inhibit a factor which negatively regulates *lin-35*. Thus, the repression of LIN-35 in *mir-35* mutants would result in the observed RNAi hypersensitivity. However, the decreased embryonic viability

of *mir-35* mutants cannot be fully explained by down-regulation of LIN-35 because genetic null mutants of *lin-35* are 100% viable at 20°C, in contrast to the 30% viability of *mir-35(gk262)* at this temperature. Loss of both *lin-35* and *mir-35* results in apparently complete inviability. Thus, we propose a pathway where *mir-35* and *lin-35* act in parallel to promote embryonic viability. Since miRNAs and the LIN-35 transcriptional repressor typically negatively regulate their targets, we predict intermediate genes that are mis-regulated in these mutants and, thus, contribute to the embryonic inviability when both arms of the pathway are deficient.

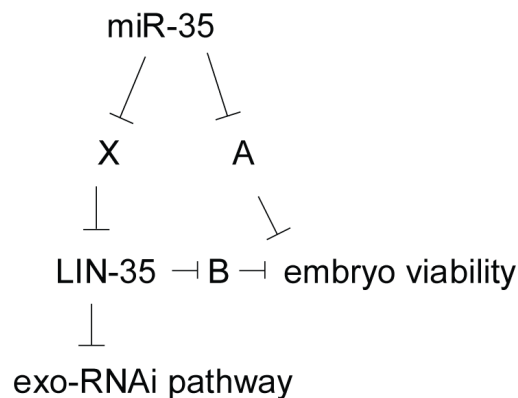


Figure 3.6. Model: *mir-35-41* positive regulation of *lin-35*.

3.4 Methods

Worm Strains

Worms were maintained on NGM worm plates seeded with OP50 bacteria. The following strains were used: wild type Bristol strain N2, mir-35(gk262) VC514, rrf-3 (pk1426) II NL2099, rrf-1(pk1417) I NL2098, lin-35(n745) I MT10430, ergo-1(tm1860) V WM158, rde-1(ne300) V WM27, rde-4(ne299) III WM49, eri-1(mg366) IV GR1373. Integrated array: PD4251 ccls4251I [myo-3::Ngfp-lacZ, pSAK4(myo-3::Mtgfp)]; dpy-20 (e1282)IV.

Oligos

A68- GCCACTGCTAGTTTCCACCCGGTGA, A69-GGATCAGATCGAGCCATTGCTGG, A122-TAGGTGATTTTTCCACCCGGTGATAGCGAG, A583-GATCCTAATTGTGCGCTCTTTTGCTTCC, A70- GAGCAGGAGGCTCCAGACCTAGG

Feeding RNAi

RNAi plates were prepared using carbenicillin 25 ug/ml and 6mM IPTG (Isopropyl β -D-1-thiogalactopyranoside, Apex™). RNAi strains were streaked out from the Ahringer feeding RNAi library on LB plates added of ampicillin and tetracycline. A single colony of bacteria containing vector (L4440) or the RNAi clones was inoculated in LB-Ampicilin overnight at 37°C. On the next day the cultures were concentrated 9x and seeded on RNAi plates. Worms were synchronized as L1 after hypochlorite treatment of gravid hermaphrodites

followed by embryos hatched ON in M9 buffer at 20°C. Worms were then plated on OP50 until reaching L4 (40 hours) when washed and transferred to RNAi. Adults were scored the next day for *unc-22*, *lin-1* and *sqt-1* phenotypes. For *pos-1* and *sex-1*, L1 parents were directly plated on RNAi food and allowed to grow to adults. Parents were then singled and their embryos were counted after about 50 embryos had been laid. The plates were monitored over the next few days to determine the percent of embryos that were embryonic lethal.

dsRNA synthesis for microinjection

cDNA corresponding to *gfp* was PCR amplified using a pair of primers containing T7(A2007-T7) and SP6(A2008-SP6) promoter sequences. The PCR product was gel purified. Two separate reactions were performed for in vitro transcription, one containing T7 RNA polymerase and one containing SP6 RNA polymerase. 200ng of PCR purified template + 1X transcription buffer, rNTP mix at final 0.05 mM, dTT 2mM and 20 U T7 or SP6 in 50 uL reaction (Epicentre). 5ug/uL each strand were mixed in 1x injection buffer and anneal at 68°C for 10 minutes and 37°C for 40 minutes.

dsRNA microinjection

For dsRNA injection *mir-35(gk262)* worms were crossed into PD4251 containing an integrated array made by three plasmids: pSAK2 (*myo-3* promoter driving nuclear targeted GFP-LacZ fusion); pSAK4 (*myo-3* promoter driving mitochondrially targeted GFP) and *dpy-20* subclone as a selectable marker. The

PD4251 strain produces GFP in the nucleus and mitochondria of all body muscle cells. Microinjections were done in both gonad arms of L4 *mir-35(gk262); PD4251* worms grown at 25°C from starved L1s (hatched on at 20°C). After a two hour recovery period, the injected worms were singled on NGM plates and allowed to lay embryos for 24 hours. The injected parents were picked off the plates and their progeny was count. Two days later the non-green progeny who reached the L4 stage was scored as viable and rescued by the *gfp* RNAi. As control we injected *mir-35(gk262); PD4251* with injection buffer.

Detection of dsRNA produced by RNaseA Digestion

RNAi plates were prepared as mentioned before with the addition or not of IPTG. Plates were seeded with 600uL of concentrated vector of *gfp* bacteria and incubated overnight at RT for T7 polymerase induction by IPTG and production of dsRNA. On the next day, one plate for each RNAi was washed with LB to a 1.5 mL microcentrifuge tube and span at 4°C, 8kxg for 4 minutes. Longer times have been shown to cause bacterial RNA degradation. Total RNA was extracted by addition of 1mL Trizol® to bacteria pellet. Samples were homogeneized by short vortex and incubated at 68°C for 5 minutes and RNA was extracted according to manufacturer protocol.

For RNaseA digestion the RNaseA enzyme (Fermentas #EN0531) was diluted in its original buffer composition (Tris-Cl pH7.4 + glycerol to final concentration 50%v/v) to 1ng/uL. A 10 uL reaction mix containing 5ug of total bacterial RNA, a final concentration of 0.32M NaCl and final concentration of

0.1ng/uL of RNase was incubated for exactly 5 minutes at 37°C in heat block. Samples were purified by phenol: chlorophorm extraction according to lab protocol (volume to 200 uL with H₂O + 20 uL NaOAc 3M + 1ul glycogen 20mg/mL).

Western Blot

To prepare protein for Western blotting, frozen worms were boiled in 2× SDS buffer and concentrations were determined by Qubit (Invitrogen). Samples were run on denaturing 4%–20% gradient gels and electroblotted to Immun-Blot PVDF membranes (Bio-Rad). Western analysis for LIN-35 protein (was done using 1:500). Anti-Tubulin (1:1,000, Sigma) was used as controls. Protein levels were quantified using Quantity One (Bio-Rad) software.

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