Genetic Identification of Development Pathways Regulated by Conserved microRNAs in Caenorhabditis elegans

John L. Brenner
Marquette University

GENETIC IDENTIFICATION OF DEVELOPMENTAL PATHWAYS REGULATED BY CONSERVED MICRORNAS IN *Caenorhabditis elegans*

by

John L. Brenner, B.Sc.

A Dissertation submitted to the Faculty of the Graduate School,
Marquette University,
in Partial Fulfillment of the Requirements for
the Degree of Doctoral Philosophy

Milwaukee, WI

May 2012

ABSTRACT
GENETIC IDENTIFICATION OF DEVELOPMENTAL PATHWAYS REGULATED BY CONSERVED MICRORNAS IN *Caenorhabditis elegans*

John L. Brenner, B.Sc.

Marquette University, 2012

microRNAs (miRNAs) are approximately 22 nucleotide non-coding RNAs that function to repress genes by binding to complementary sites in target mRNAs and play critical roles in development and disease. It is predicted that more than 60% of human genes are regulated by miRNAs, however, little is known about the individual functions of miRNAs. I used the nematode worm, *Caenorhabditis elegans*, as a model to identify developmental processes and pathways regulated by conserved miRNAs. Genetic examination of miRNA function is hindered by lack of obvious phenotypes attributed to loss of individual miRNA genes. Phenotypes attributable to loss of individual miRNA genes were identified by examining worms mutant for individual miRNA genes and *alg-1,* which encodes an Argonaute protein that functions in the miRNA pathway in *C. elegans*. This analysis identified functions for 80% of miRNA genes examined. miRNAs were found to regulate diverse processes, including embryonic development, directional migration of the gonad, and developmental timing. The goal of the second half of this study was to determine the mechanism whereby loss of members of the *mir-51* miRNA family suppresses the developmental timing defects of *alg-1* mutant worms. Genetic evidence indicates the *mir-51* family regulates the L2 to L3 transition through regulation of *hbl-1* expression. Interestingly, the *mir-51* family genetically interacts in pathways regulated by the let-7 and miR-35 families, as well as lsy-6, miR-240/786, and miR-1. Evidence herein indicates that the *mir-51* family does not regulate these pathways through miRNA biogenesis or activity. Instead it is possible that the miR-51 family regulates multiple targets in diverse developmental pathways.

ACKNOWLEDGEMENTS

John L. Brenner, B.Sc.

First and foremost I would like to thank my advisor and mentor, Dr. Allison Abbott. Her guidance has been instrumental in my growth as a scientist and as a person. Not only did she teach me how to think scientifically, but also helped me to learn how to communicate better as a scientist. Her focus and drive has won my admiration, and I hope that I can emulate her ability in the future. She was simply a great mentor.

I am grateful for my lab mate, Dr. Benedict Kemp, who has been a great source of knowledge. His energy and humor made work enjoyable. Furthermore, I very much valued his guidance through a very difficult time.

I also thank the current and former members of the Abbott Lab. I am grateful to the current graduate student in the lab, Mitchell Romens, for providing good conversation of both work and life while in the lab. It made work life a little easier. I am grateful for all the undergraduates in the lab, past and present, for providing me mentorship opportunities in a lab setting. These interactions were always positive and only served to reinforce my desire to teach.

I was fortunate to have four excellent committee members: Dr. James Anderson, Dr. Stephen Munroe, Dr. Rosemary Stuart, and Dr. Gail Waring. I thank them for thought stimulating meetings and guidance through my project. I also thank Dr. Waring specifically for the opportunity to be a part of the Summer

Research Program. The experience reinforced my career aspirations. I also want to thank Dr. Stuart for guiding me through my first teaching assistant assignment, which was an incredibly positive experience that served to reinforce my desire to teach.

I am extremely grateful to my family and friends, both close and distant. Their support has helped me through this process. I want to specifically thank my brother Chris for being a true big brother and my brother Garrett for introducing me to Biology in a little lab at Humboldt State. I would not be here if it weren't for that trip. I am extremely grateful to my Dad who has supported me throughout my life. I strive to continually make him proud on a daily basis.

I express my deepest thanks to Christine Mengel for her care, understanding, and love. Her help through the last phase of this dissertation is extremely appreciated. I also thank her for her expertise and knowledge on document formatting which allowed the final phases of this document manageable.

Lastly, I would like to thank members of the scientific community: Robert Shaw and Eric Miska, Luisa Cochella and Oliver Hobert, Tomoaki Mizuno and Kunihiro Matsumoto, and the *C. elegans* genetics center (CGC) for reagents and worm strains. I thank Eric Moss and Victor Ambros for their input and advice on aspects of my project. I acknowledge the financial support by the Department of Biological Sciences, Marquette University, and the GAANN Fellowship. My work was funded in part by the NIH.

LIST OF PUBLICATIONS

Brenner, J L, Jasiewicz, K L, Fahley, A F, Kemp, B J, and Abbott, A L. Loss of Individual microRNAs causes mutant phenotypes in sensitized genetic backgrounds in C. elegans. (2010) *Current Biology* 20, 1321-1325.

Brenner, J L, Kemp, B J, and Abbott, A L. The mir-51 Family of microRNAs Functions in Diverse Regulatory Pathways in Caenorhabditis elegans. *PLoS One* (In Press).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1: Background and Significance

Overview

microRNAs (miRNAs) are approximately 22 nucleotide non-coding RNAs that post-transcriptionally regulate target genes and are critical regulators in development and disease. miRNAs interact with target mRNAs in a sequence specific manner, and post-transcriptionally regulate target expression. The importance of miRNA regulation of gene expression is highlighted by the observation that fish, flies, worm, and mice fail to develop in the absence of critical miRNA biogenesis components (Grishok et al., 2001; Ketting et al., 2001; Bernstein et al., 2003; Wienholds et al., 2003; Lee et al., 2004b; Giraldez et al., 2005). However, the individual functions of most miRNAs are not known. This introduction will review the current understanding of miRNA biogenesis, the mechanisms of miRNA post-transcriptional gene regulation, the strategies that have been used to identify miRNA targets and functions, and finally, the known functions for individual miRNAs. The central goal of this study was to identify functions for individual miRNA genes in development of the nematode worm *Caenorhabditis elegans*.

1.1 miRNA Biogenesis

An outline of microRNA biogenesis is shown in Figure 1.1. The typical animal miRNA is produced by sequential processing of longer RNA precursors (Reviewed in Kim, 2005). First, RNA Polymerase II transcribes most miRNA genes as long, primary transcripts (pri-miRNA). Similar to protein coding transcripts, these pri-miRNAs are both capped and polyadenylated (Lee et al., 2004a). While in the nucleus, the pri-miRNA is trimmed by the Drosha Microprocessor Complex to a 60-80 nucleotide hairpin, called the precursor miRNA (pre-miRNA) (Lee et al., 2003; Denli et al., 2004). A subset of pre-miRNAs are not processed by Drosha but are instead the products of splicing (Ruby et al., 2007). The pre-miRNA is specifically exported to the cytoplasm (Lund et al., 2004), where it is recognized and cleaved by the enzyme Dicer (Bernstein et al., 2001; Grishok et al., 2001; 2001; Hutvágner et al., 2001; Ketting et al., 2001; Knight and Bass, 2001). This miRNA duplex is associated with a complex of proteins termed the miRNA-Induced Silencing Complex (miRISC) (Reviewed in Kim et al., 2009). The miRNA duplex is unwound, leaving the mature, approximately 22 nucleotide, single-stranded miRNA to serve as a guide for the miRISC to bind and regulate target transcripts.

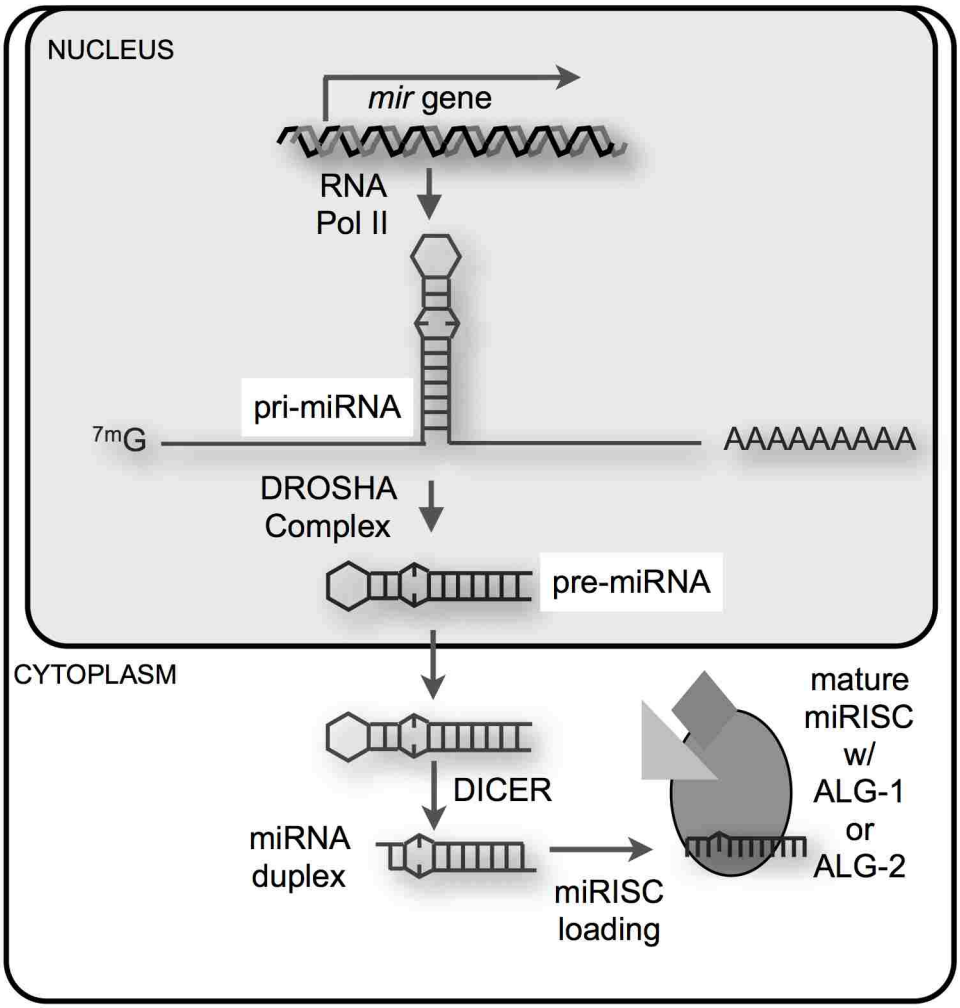


Figure 1.1 Cartoon of miRNA biogenesis. RNA Pol II, RNA Polymerase II; Pri-miRNA, primary miRNA transcript; pre-miRNA, precursor miRNA; ^{7m}G, methylated cap.

1.2 An Argonaute family protein forms the core component of the miRISC

A core component of the miRISC is an Argonaute protein. *alg-1* and *alg-2* encode Argonaute proteins that are specific to the miRISC in *C. elegans* (Grishok et al., 2001). Knockdown of *alg-1* and *alg-2* by RNAi causes embryonic lethality and developmental defects similar to mutant worms lacking *let-7* miRNA family members. Mature miRNA levels are reduced when *alg-1* and *alg-2* are knocked down (Grishok et al., 2001). In contrast, mutations in the other 25 Argonautes in *C. elegans* (Yigit et al., 2006) do not affect miRNA function. Conversely, loss of *alg-1* and *alg-2* do not obviously affect the function of the small interfering RNA (siRNA) pathway (Grishok et al., 2001). ALG-1 and ALG-2 complexes bind almost exclusively to miRNAs (Zhang et al., 2007; Corrêa et al., 2010), but miRNAs can also associate with RDE-1 (Steiner et al., 2007; Corrêa et al., 2010), the Argonaute required for exogenous siRISC function. RDE-1 complexes loaded with miRNAs are likely non-functional since loss of *rde-1* does not result in phenotypes expected for loss or reduction of miRNA function in *C. elegans* and RDE-1 cannot compensate for loss of *alg-1* and *alg-2* (Grishok et al., 2001). miRNA loading specifically into a miRISC containing either ALG-1 or ALG-2 is therefore necessary for miRNA regulation of its targets in *C. elegans*.

1.3 Mechanism of post-transcriptional regulation by miRNAs.

The consequence of a miRNA binding to its target mRNA is, most often, reduction in target protein levels. There are multiple mechanisms for miRNA-mediated gene regulation, including inhibition of translation or destabilization of the target transcript (Reviewed in Filipowicz et al., 2008; Huntzinger and Izaurralde, 2011; Figure 1.2). Inhibition of translation is thought to occur through prevention of translation initiation or through a block of translation elongation. miRISC binding to a target prevents the initiation complex from assembling onto the transcript, likely through direct interactions with the 5' cap (Humphreys et al., 2005; Wang et al., 2006; Kiriakidou et al., 2007; Mathonnet et al., 2007; Thermann and Hentze, 2007; Wakiyama et al., 2007). miRNAs may block translation elongation as evidenced by miRNA targets residing in polysomes (Olsen and Ambros, 1999; Seggerson et al., 2002; Maroney et al., 2006; Nottrott et al., 2006; Petersen et al., 2006), but a precise mechanism for how the miRISC prevents the ribosome from elongating remains unknown. Large scale proteome and transcriptome analysis indicate that reduction of miRNA target protein levels is often coupled with a reduction in miRNA target transcript levels, indicating that miRNAs promote the degradation of their target transcript (Baek et al., 2008; Selbach et al., 2008; Hendrickson et al., 2009; Guo et al., 2010). Degradation begins with removal of the poly-A tail, which is mediated by enzymes recruited by the miRISC (Behm-Ansmant et al., 2006; Giraldez et al., 2006; Wu et al., 2006). The resulting transcript without the poly-A tail is then susceptible to 3' to 5' exonucleases. However, poly-A tail removal does not condemn a transcript to be

destroyed, as some deadenylated miRNA targets are relatively stable (Wu et al., 2010). Each mechanism of post-transcriptional gene regulation by a miRNA may not be mutually exclusive. Instead, a miRNA binding to its target might begin with a block on translation initiation triggered by deadenylation and miRISC interaction with the 5’ cap, followed by degradation (Djuranovic et al., 2011). Although it is still not certain that miRNA mediated target regulation is a coupled process of translation block and mRNA decay, the typical effect seems to be a reduction in protein levels, and often reduction in transcript levels.

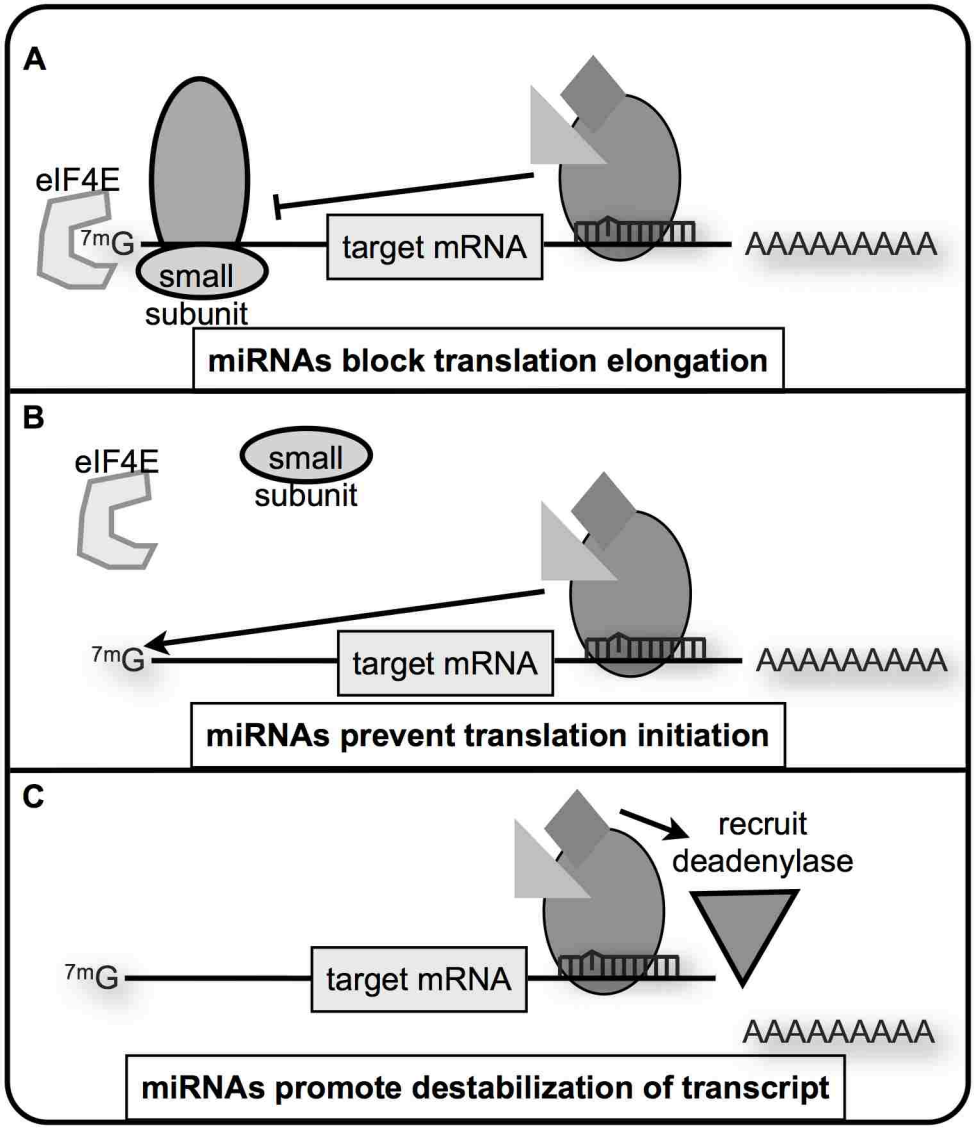


Figure 1.2 Cartoon Diagram for Mechanisms of miRNA mediated post transcriptional repression. miRISC interacts with target UTR, which: A) blocks translation elongation, B) blocks the initiation of translation, or C) promotes the destabilization of the transcript through recruitment of additional enzyme complexes. Small subunit refers to the 40S ribosomal subunit. ^{7m}G refers to the modified 5' cap of a mature transcript. The oval bound to the triangle and diamond is a cartoon representation of the miRISC, along with a miRNA attaching to the 3' end of the target mRNA. Not drawn to scale.

1.4 Determinants of miRNA target recognition

With few exceptions (Yekta et al., 2004; Davis et al., 2005), most animal miRNAs do not bind perfectly to their target mRNAs. Imperfect pairing precludes identification of miRNA targets through simple genomic searches for complementary sites. In order to identify miRNA targets, a number of algorithms have been created to identify putative binding sites in the 3' UTRs of mRNAs (Reviewed in Bartel, 2009).

1.4.1 The importance of the "seed" to miRNA target recognition

A specific region of the miRNA, called the seed sequence, is a primary determinant of miRNA target recognition (Doench and Sharp, 2004; Brennecke et al., 2005; Nielsen et al., 2007). The seed sequence represents nucleotides 2-7 of the mature miRNA sequence. Mispairing between a single nucleotide of the seed sequence and the target often eliminates miRNA mediated repression, whereas mispairing outside the seed sequence is often tolerated (Doench and Sharp, 2004; Brennecke et al., 2005; Nielsen et al., 2007). Mis-pairing in the seed sequence can be compensated by extensive pairing between the 3' end of the miRNA and its target (Doench and Sharp, 2004; Brennecke et al., 2005; Krek et al., 2005; Grimson et al., 2007; Nielsen et al., 2007). Other factors also contribute to target recognition (Doench and Sharp, 2004; Brennecke et al., 2005; Krek et al., 2005; Grimson et al., 2007; Nielsen et al., 2007). These factors include multiple binding sites within the 3' UTR, structure and accessibility of the 3' UTR, and binding at the 3' end of the miRNA (Bartel, 2009).

1.4.2 miRNAs are grouped into families

MicroRNAs have been grouped together primarily based on sequence similarity at their 5' end (Ambros et al., 2003; Grad et al., 2003; Lim et al., 2003). Since these miRNAs share a common seed sequence, they are predicted to regulate shared targets. In mouse, *mir-133a-1* and *mir-133a-2* are individually not essential, but mice lacking both of these miRNAs have severe heart defects resulting in early lethality or heart failure in older mice (Liu et al., 2008). These two miRNAs regulate the same targets, SRF and cyclin D, whose mis-regulation partially accounts for the cardiac defects in mice lacking *mir-133a-1* and *mir-133a-2*. However, *mir-133a-1* and *mir-133a-2* are identical in sequence allowing them to regulate identical targets. miRNA families often contain multiple members with unique sequences at their 3' ends. Thus, although it is recognized that miRNA families can regulate shared targets and have similar functions, it is unknown to what extent they have non-overlapping functions. One known non-overlapping function for members of a miRNA family is known in *C. elegans*, where let-7 but not its family members miR-48, miR-84, and miR-241 regulate *lin-41* (Reinhart et al., 2000; Abbott et al., 2005). Differences at the 3' end of the miRNAs may account for the inability for miR-48, miR-84, and miR-241 to regulate *lin-41* (Abbott et al., 2005). Identification of functions for individual members of miRNA families will allow for further analysis of possible non-overlapping roles for miRNAs that share common 5' seed sequences.

1.5 Identification of miRNA Targets

The first miRNAs were discovered through genetic dissection of *C. elegans* mutants. Although forward genetic analyses revealed the function of additional miRNAs, the identification of let-7, which is perfectly conserved in humans (Pasquinelli et al., 2000), paved the way for biochemical attempts to identify the small RNAs present in animals. The first biochemical identification of miRNAs (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001) revealed the existence of many miRNAs without revealing their corresponding targets.

1.5.1 Genetic Identification of miRNA targets

The first miRNA targets were identified genetically in *C. elegans*. lin-4 binds to sequences in the *lin-14* mRNA to block production of LIN-14 (Lee et al., 1993; Wightman et al., 1993). let-7 blocks production of LIN-41 through binding partially complementary sites in the *lin-41* 3' untranslated region (UTR) (Reinhart et al., 2000; Slack et al., 2000). Identification of both targets was made possible through examination of mutant worms with obvious, penetrant phenotypes that were opposite to those of mutant worms lacking *lin-4* or *let-7* (Lee et al., 1993; Wightman et al., 1993; Slack et al., 2000). An additional developmental timing gene, *lin-28*, was also found to be a target of the lin-4 miRNA through identification of complementary sites within its 3' UTR (Moss et al., 1997).

Reverse genetic approaches have also been applied to identify individual miRNA function. However, as will be discussed in more detail later in this

chapter, loss of individual miRNA genes often has no negative consequence on development (Miska et al., 2007). Further work is then needed to identify developmental consequences attributable to the loss of individual miRNAs and identify biologically relevant targets.

1.5.2 Computational identification of miRNA targets

Computational approaches predict targets for many of the known miRNAs. As was observed with lin-4 and let-7, animal miRNAs typically do not bind with perfect complementarity to their targets (Lee et al., 1993; Wightman et al., 1993; Reinhart et al., 2000; Slack et al., 2000). Although there is significant overlap between lin-4 and let-7 to their targets, extensive pairing may not be necessary. Complementary binding of the seed sequence to its target may be sufficient to confer regulation (Bartel, 2009). Searching for perfect complementary binding sites between a miRNA seed sequence and a putative target is one way to determine if a specific gene is a target of a specific miRNA. However, this approach may generate many false-positives (Bartel, 2009). Instead, target prediction algorithms generate refined target lists by focusing on parameters of miRNA target binding beyond seed pairing, such as multiple binding sites within a 3' UTR, structural accessibility to regions within the UTR, or compensatory binding between the 3' end of the miRNA and its target (Bartel, 2009). Some of these predictions generate a broad landscape of putative miRNA targets. For example, greater than 60% of human protein coding genes are now predicted miRNA targets (Friedman et al., 2009). These predictions suggest many targets

can be regulated by a miRNA, but experimental evidence is still needed to validate these miRNA targets.

1.5.3 Biochemical Identification of miRNA targets

Multiple biochemical approaches have been used to identify miRNA targets. These approaches include identification of RNAs and/or proteins whose levels are decreased when a miRNA is overexpressed, identification of RNAs and/or proteins whose levels are increased when a miRNA is lost or antagonized, and identification of RNAs that immunoprecipitate with proteins associated with the miRISC (Thomas et al., 2010).

First, transfection of miRNAs into cells that normally lack the miRNA has been used to identify targets. Microarray profiling of cells transfected with brain-specific miR-124 revealed targets for the miRNA, and also revealed that a function of this miRNA is to reinforce a brain cell-specific expression profile (Lim et al., 2005). Quantitative proteomics further supports this role for miR-124 (Baek et al., 2008). However, expression of a miRNA into cells that don't normally express it can generate indirect effects on gene expression. Also, overexpression of the transfected miRNA might allow it to interact with genes that are not targets in normal physiological contexts (Thomas et al., 2010).

An alternative to determining the effect on the transcriptome or the proteome by miRNA overexpression is to examine the effect of loss or reduction of miRNA expression. This approach can also identify many targets, however, the effect on gene expression in the absence of miRNA function can be small

compared to the effect of overexpression (Linsley et al., 2007; Selbach et al., 2008). Since the level of repression of a miRNA on its targets can be modest (Baek et al., 2008), this approach may not be sensitive enough to identify targets.

miRNA targets have also been identified through high throughput sequencing of RNAs that immunoprecipitate with individual protein components of the miRISC machinery (Beitzinger et al., 2007; Easow et al., 2007; Karginov et al., 2007; Zhang et al., 2007; Hendrickson et al., 2009; Zhang and Emmons, 2009; Zisoulis et al., 2010). mRNAs identified through this approach are likely to be targeted by miRNAs in vivo. Recent work using RIP-SEQ (RNA immunoprecipitation followed by sequencing), has identified approximately 100bp fragments of mRNAs that associate with the miRISC component ALG-1 (Zisoulis et al., 2010). Biochemical identification of targets has been coupled with computational approaches to enhance the accuracy of computational predictions (Hammell et al., 2008).

Collectively, biochemical approaches reveal that many genes are miRNA targets, and also reveal that most miRNAs modestly reduce target gene expression (Baek et al., 2008; Selbach et al., 2008). However these approaches generally do not reveal the biological significance of the miRNA target relationship. Further work beyond these biochemical methods are needed to identify the physiological pathways in which a miRNA might regulate its target and the significance of the regulation.

1.6 Identification of miRNA Functions

1.6.1 *lin-4* and the *let-7* family miRNAs control developmental timing in *C. elegans*.

A set of cells in the worm called the lateral hypodermal seam cells go through several rounds of division in larval development (Figure 1.3). The timing and pattern of these divisions is precisely controlled in wild type worms. In each of the four larval stages (L1 - L4) the seam cells undergo a single round of asymmetric cell division with one daughter fusing with the hypodermal syncytial cell hyp7, wherease the other daughter cell maintains the stem cell like fate of the seam. In the L2 stage, the asymmetric division is preceded by a symmetric division that increases the total number of seam cells from 10 to 16. At the end of larval development, the seam cells terminally differentiate, fuse, and produce the adult specific cuticle structure called alae. Repetition or omission of any of these specific programs gives a heterochronic phenotype and worms that display these phenotypes are classified as developmental timing mutants (Ambros and Horvitz, 1984).

miRNAs regulate developmental timing in *C. elegans*. *lin-4*, as previously discussed, was identified due to its highly penetrant phenotype in the worm. In the absence of *lin-4*, LIN-14 remains high resulting in worms continually repeating the L1 stage program (Lee et al., 1993; Wightman et al., 1993, Figure 1.3). *lin-14* is completely epistatic to *lin-4* (Ambros and Horvitz, 1987; Ambros, 1989). Worms lacking *lin-14* have an opposite phenotype to that of loss of *lin-4*, and skip the first larval program (Ambros and Horvitz, 1984, Figure 1.3). Another

miRNA, let-7, regulates the larval-to-adult switch in *C. elegans* (Reinhart et al., 2000). Worms lacking *let-7* repeat a later larval program, and the lateral seam fail to terminally differentiate at the L4-to-adult transition (Reinhart et al., 2000; Figure 1.3). This phenotype is opposite to that of worms lacking the let-7 target, *lin-41*, which skip a larval program, resulting in the seam terminally differentiating a single stage earlier (Reinhart et al., 2000; Slack et al., 2000; Figure 1.3). *let-7* family members, *mir-48*, *mir-84*, and *mir-241*, regulate the L2-to-L3 transition in worms (Abbott et al., 2005). Interestingly, worms lacking these miRNAs individually do not obviously display developmental timing defects, but worms lacking all three repeat the L2 specific symmetric division of the lateral seam cells (Abbott et al., 2005; Figure 1.3). This repetition of the L2 specific division results in an increase of the total number of seam cells in later development. Worms lacking *mir-48*, *mir-84*, and *mir-241* fail to down-regulate their target, *hbl-1*, whose down-regulation is necessary to exit the L2 program (Abrahante et al., 2003; Abbott et al., 2005). It is unknown if other miRNAs play a role in developmental timing.

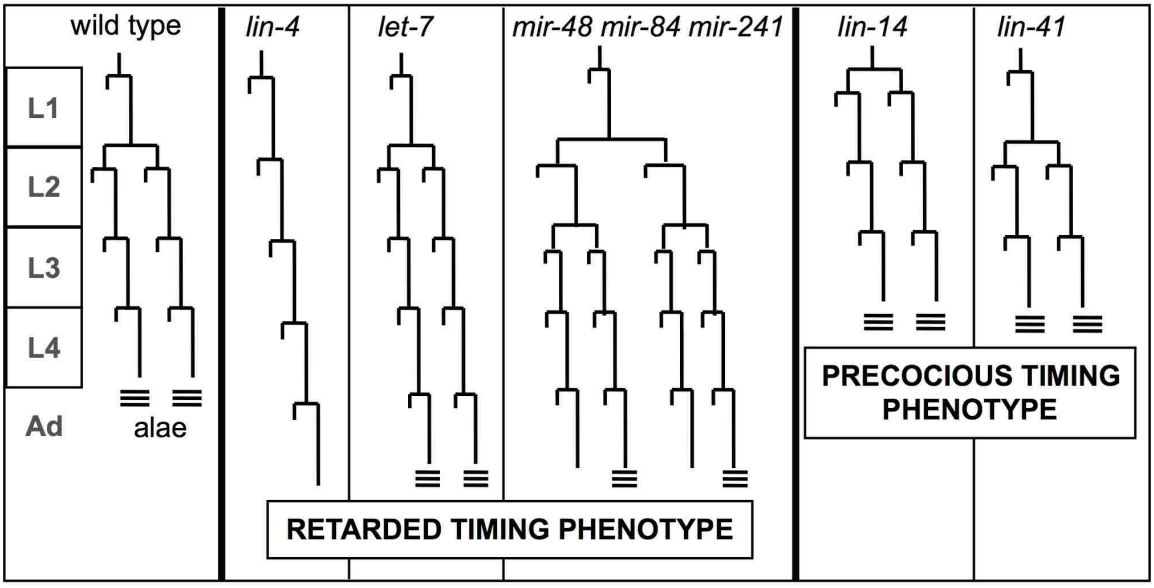


Figure 1.3 Summary of developmental timing defects in the seam cell division pattern of lin-4 and let-7 family mutants and their targets. Each branch represents a division of the lateral hypodermal seam cells. Discontinuation of a line indicates terminal differentiation of the cell. The three horizontal lines represent the formation of the adult specific cuticle structure, alae. The pattern of seam cell division is shown for wild type, *lin-4*, *let-7*, *mir-48 mir-84 mir-241*, *lin-14*, and *lin-41* worms.

Table 1.1. Summary of functions identified for miRNAs in *C. elegans*

miRNA gene/family	Target(s)	Observed function	References
lin-4	*lin-14, lin-28*	Developmental timing, lifespan, HSN axon outgrowth	Ambros 1989 Moss et al. 1997 Olsson-Carter and Slack, 2010 Wightman et al. 1993 Boehm and Slack, 2003
let-7, mir-48, -84, -241	*daf-12, hbl-1, let-60, lin-41*	Developmental timing, vulval cell fate specification	Reinhart et al. 2000 Abbott et al. 2005 Abrahante et al. 2003 Grosshans et al. 2005 Johnson et al. 2005 Lin et al. 2003
mir-61	*vav-1*	vulval cell fate specification	Yoo and Greenwald, 2005.
lsy-6	*cog-1*	ASEL/R neuron fate specification	Johnston and Hobert, 2003
mir-273	*die-1*	ASEL/R neuron fate specification	Chang et al. 2004.
mir-1	*unc-29, unc-63, mef-2*	Synaptic transmission	Simon et al. 2008
mir-51, -52, -53, -54, -55, -56	cdh-3	embryogenesis, pharyngeal attachment	Alvarez and Horvitz, 2010 Shaw et al., 2010
mir-35, -36, -37, -38, -39, -40, -41, -42		embryogenesis	Alvarez and Horvitz, 2010
mir-240		defecation	Miska et al. 2007
mir-786		defecation	Miska et al. 2007
mir-58		Locomotion, body size, egg laying, dauer entry	Alvarez and Horvitz, 2010
mir-80, -81, -82		Locomotion, body size, egg laying, dauer entry	Alvarez and Horvitz, 2010

1.6.2 Multiple miRNAs, including let-7 family members, regulate vulval cell fate specification in *C. elegans*.

Vulva formation is preceded by specification of hypodermal cells called the Vulval precursor cells (VPCs). The specification of the VPCs is controlled, in part, through an inductive signal involving the LET-60/RAS signaling pathway and a lateral signal involving the LIN-12/Notch signaling pathway (Sternberg, 2005). The cell that will become the vulva, the 1˚ VPC, is high in LET-60/RAS activity but low in LIN-12/Notch activity. In contrast, the neighboring 2˚ VPCs both have low LET-60/RAS activity, but high LIN-12/Notch activity. The proper activity of these signaling pathways is important to properly specify the VPCs and lead to the formation of a single vulva (Sternberg, 2005).

Two miRNAs function in vulval cell fate specification, miR-61 (Yoo and Greenwald, 2005) and the let-7 family member, miR-84 (Johnson et al., 2005). miR-61 is activated in 2˚ VPCs by LIN-12 and regulates *vav-1* (Yoo and Greenwald, 2005). Downregulation of VAV-1 contributes to promoting the 2˚ VPCs by reinforcing LIN-12 activity (Yoo and Greenwald, 2005). miR-84 also functions in the 2˚ VPC. *mir-84* is expressed in the 2˚ VPCs and regulates the let-7 family target, *let-60/RAS* (Johnson et al., 2005). In this role, miR-84 reinforces the 2˚ VPC fate by maintaining low LET-60/RAS activity in these cells. Interestingly, loss of either of these miRNAs individually has no negative consequence on vulval cell fate specification (Miska et al., 2007), indicating they are auxiliary to other regulators of vulval cell fate specification.

1.6.3 miRNAs specify the left/right asymmetry in a pair of neurons in *C. elegans*.

The cell fates between a pair of neurons in the head, the ASEL and ASER, are controlled by the miRNAs lsy-6 (Johnston and Hobert, 2003) and miR-273 (Chang et al., 2004). The lsy-6 miRNA is expressed solely in the ASEL and is required for its specification (Johnston and Hobert, 2003). Worms lacking *lsy-6* specify two ASER neurons due to the lsy-6 target, *cog-1*, remaining high in the cell normally fated to become the ASEL. *mir-273*, in contrast, is expressed primarily in the ASER. mir-273 negatively regulates *die-1*, which is necessary for lsy-6 expression in the ASEL (Chang et al., 2004). Bilateral expression of miR-273 is sufficient to promote the specification of two ASER, consistent with a lack of lsy-6 in the ASEL. Interestingly, loss of *mir-273* has no effect on the bilateral specification of the ASEL and ASER (Chang et al., 2004), suggesting it is auxiliary to other regulators of ASER specification.

1.6.4 The muscle-specific *mir-1* regulates synaptic transmission in *C. elegans*.

The muscle specific *mir-1* is highly conserved in both sequence and expression pattern from *C. elegans* to humans (Nguyen and Frasch, 2006). *mir-1* is necessary for normal development in both flies and mice (Sokol and Ambros, 2005; Zhao et al., 2007). In the worm, *mir-1*, although not essential for viability (Miska et al., 2007), regulates activity at the neuromuscular junction by regulating multiple muscle specific targets including subunits of the acetylcholine receptor, *unc-29* and *unc-63*, and the MEF-2 transcription factor (Simon et al., 2008).

Misregulation of these targets allows *mir-1* mutant worms to be partially resistant to levamisole, which causes paralysis in *C. elegans*.

1.6.5 *mir-35* family and *mir-51* family regulate embryonic development in *C. elegans.*

Two miRNA families are required for embryonic development in C. elegans, the *mir-35* family and the *mir-51* family (Alvarez-Saavedra and Horvitz, 2010). Worms lacking individual members of the *mir-35* or *mir-51* families develop normally (Miska et al., 2007). However, worms lacking either the entire *mir-35* or *mir-51* family arrest during embryogenesis (Alvarez-Saavedra and Horvitz, 2010), indicating the individual members of the family function redundantly in embryonic development. In further support that these miRNA families function redundantly, transgenic expression of any one family member is sufficient to rescue the embryonic lethality associated with loss of the entire family (Alvarez-Saavedra and Horvitz, 2010). A direct target of the *mir-35* family is unknown. Misregulation of the *mir-51* family target, *cdh-3*, is in part responsible for the failure of the pharynx to attach to the mouth in *mir-51* family mutant worms (Shaw et al., 2010). However reduction of *cdh-3* expression fails to suppress embryonic lethality observed in worms lacking the *mir-51* family members, suggesting involvement of additional targets (Shaw et al., 2010).

1.7 In most cases, loss of individual miRNAs has no obvious consequence on development in *C. elegans*

The identification of the vast majority of miRNAs was achieved through cloning and sequencing small RNAs from animals. Thus, the function of most miRNAs is unknown. In order to identify the functions of individual miRNAs, deletion alleles were generated for the majority of the known miRNA genes in *C. elegans* (Miska et al., 2007). Interestingly, in most cases, loss of individual miRNA genes has no obvious effect on development (Miska et al., 2007).

1.7.1 miRNAs function redundantly

miRNA families can function together to regulate common targets. For example, the *let-7* family members, *mir-48*, *mir-84*, and *mir-241* function together to down-regulate their target, *hbl-1*, for normal progression from the L2 to the L3 larval program (Abbott et al., 2005). While worms missing individual *let-7* family member miRNAs do not repeat the L2 larval stage, double and triple mutant worms display penetrant developmental timing defects, indicating the family members redundantly regulate *hbl-1*. Similarly, the *mir-35* and *mir-51* families are collectively required for embryonic development in *C. elegans*. Loss of individual members of these families has no obvious adverse effect on embryonic development (Alvarez-Saavedra and Horvitz, 2010). Except for these families and the *mir-58/80* family (Table 1.1), most miRNA families are not necessary for normal development in *C. elegans* (Alvarez-Saavedra and Horvitz, 2010). This

indicates redundancy between miRNA families alone cannot account for lack of developmental defects for mutants of individual miRNA genes.

miRNAs unrelated by sequence may also regulate common targets. For example, the *lin-28* 3' UTR contains binding sites for both the *lin-4* and *let-7* family of miRNAs (Moss et al., 1997; Moss and Tang, 2003). Many other genes are predicted to contain multiple binding sites for miRNAs that do not belong to the same family. Therefore different miRNAs or different miRNA families could coordinately regulate a common target, such that loss of regulation at any one site could be tolerated.

1.7.2 miRNAs have cell-specific functions

Certain cell-specific functions may have been missed in the phenotypic analysis carried out by Miska et al. (2007). For example, the analysis would not have identified a function for lsy-6, which specifies the ASEL neuronal fate, since worms lacking *lsy-6* develop otherwise normally (Johnston and Hobert, 2003). Worms lacking *lsy-6* also display a subtle chemosensation defect, which is also likely to have been missed in broad-based phenotypic assays. A function was not identified for *mir-1* through phenotypic analysis (Miska et al., 2007), although it was later found that *mir-1* regulates synaptic activity (Simon et al., 2008). For this reason, more cell specific assays could be employed to assess if individual miRNAs play a role in cell-specific functions.

1.7.3 miRNAs fine-tune target expression

miRNAs can behave like switches or like fine-tuners of target protein levels. lin-4 behaves as a “genetic switch” and virtually eliminates protein levels of its target, *lin-14* (Lee et al., 1993; Wightman et al., 1993). In contrast, miR-8 in *D. melanogaster* “fine tunes” the protein levels of its target, *atrophin*. Both increase and loss of *atrophin* is detrimental to development, indicating that miR-8 functions to maintain *atrophin* protein levels within an optimal range (Karres et al., 2007). Although loss of *mir-8* has an obvious effect on Drosophila development, fine-tuning relationships may be less likely to result in obvious developmental defects.

Recent evidence indicates that most miRNAs function to fine-tune the expression of their targets (Baek et al., 2008; Selbach et al., 2008; Hendrickson et al., 2009; Guo et al., 2010). Therefore, loss of an individual miRNA may result in modest changes in target expression. This modest misregulation may be insufficient to cause an obvious developmental defect.

The ability of a miRNA to switch off or fine-tune target gene expression is likely not an intrinsic property of a miRNA (Mukherji et al., 2011). Instead the stoichiometry and pairing between a miRNA and its respective targets dictates the extent to which it can down-regulate its target (Mukherji et al., 2011). Therefore, if the expression of a miRNA target gene is low, a miRNA may act to keep target expression low or off. However, when target gene expression is elevated, the miRNA may elicit a smaller effect and act to only mildly diminish the

expression of its targets. Therefore, the loss of an individual miRNA could result in loss of both fine-tuning and switch regulation of target expression.

1.8 Hypothesis and Goal

The hypothesis that miRNAs unrelated by sequence can regulate common targets or pathways can explain the lack of developmental defects in worms lacking individual miRNA genes. To test this and identify functions for individual miRNA genes, *alg-1(gk214)* was used as a genetically sensitized mutant background. *alg-1* encodes an Argonaute protein that functions specifically in the miRNA pathway in *C. elegans* (Grishok et al., 2001). Mature miRNAs are reduced in *alg-1* mutant worms (Grishok et al., 2001), indicating that overall miRNA levels and likely activity is reduced. In order to reveal the function of individual miRNAs, 25 mutant worm strains were generated that are each homozygous for a different miRNA deletion allele and the *gk214* allele of *alg-1.* A primary aim of this study was accomplished through phenotypic characterization of these mutant worms: to identify functions for individual miRNAs in development.

The second aim of this study was to further characterize the mechanism whereby loss of an individual miRNA resulted in phenotypes identified in Aim 1. Chapter 2 contains the results from phenotypic analysis of these *mir; alg-1* mutant worms. Since 80% of the *mir; alg-1* mutans displayed phenotypic differences relative to a control *alg-1* strain, attention was focused on characterizing phenotypes attributed to loss of members of the *mir-51* family.

Loss of members of the *mir-51* family suppressed developmental timing defects of *alg-1* mutant worms. This family was selected because loss of the *mir-51* family member, *mir-52*, resulted in the most robust suppression of *alg-1* developmental timing defects. The *mir-51* family functioned in diverse developmental pathways in *C. elegans*, including developmental timing, neuronal and vulval cell fate specification, the defecation motor program, and synaptic transmission.

The final aim of this study was to identify target genes of the *mir-51* family that are required for suppression of developmental timing defects. Four genes, *cul-1*, *lin-66*, *tlp-1*, and *vhp-1*, were identified as candidate downstream targets of the *mir-51* family. However, molecular experiments are inconclusive in the identification of these genes as direct targets of the *mir-51* family in developmental timing. Instead, it is possible that the *mir-51* family may regulate distinct target sets in diverse developmental pathways in *C. elegans*, possibly to fine-tune or buffer protein levels to an optimal range.

The examination of these aims allowed for the identification of functions for individual miRNA genes by identifying phenotypes attributable to loss of individual miRNA genes. Further analysis of these phenotypes allows for dissection of specific developmental pathways regulated by miRNAs in *C. elegans* and the identification of biologically relevant targets of individual miRNAs.

Chapter 2: Identifying phenotypes attributable to the loss of individual miRNA genes in the nematode worm, *C. elegans*.

2.1 Introduction

In most cases, no phenotypic defects have been identified associated with the loss of an individual miRNA gene in *C. elegans* (Miska et al., 2007). Functional redundancy among related miRNA genes, which share a 6 nucleotide 5' 'seed' sequence, can partially account for the lack of phenotypes (Alvarez-Saavedra and Horvitz, 2010). It is also possible that unrelated miRNAs, which have distinct seed sequences, may regulate shared targets and pathways. For example, *lin-28* has recognition sites for both the lin-4 and let-7 families of miRNAs (Lee et al., 1993; Reinhart et al., 2000). Therefore, we hypothesized that miRNAs unrelated by sequence could regulate common targets or pathways. To address this hypothesis and to identify phenotypes attributed to loss of individual miRNA genes, the *alg-1(gk214)* mutant background was utilized. *alg-1* encodes an Argonaute protein that functions in the miRNA pathway (Grishok et al., 2001). *gk214* is a loss-of-function allele that deletes 220 basepairs at the 5' end of *alg-1*, which eliminates the first exon and part of an intron. Worms homozygous for *gk214* display pleiotropic defects, including developmental timing defects, gonad migration defects, and embryonic lethality (Table 2.1). These phenotypes are likely due to reduced overall miRNA activity (Grishok et al., 2001). 25 strains with miRNA deletion alleles along with the *gk214* allele were generated. These miRNA deletion alleles represent 31 miRNAs, which are either conserved

through mammals or display developmentally-regulated expression (Lim et al., 2003). Strains carrying individual miRNA deletion alleles were first backcrossed with the wild type N2 to eliminate background mutations. Most of these mutants lack an individual miRNA gene, although a few lack the sequences for multiple mature miRNA sequences that are found in a cluster in the genome. For example, the *nDf58* allele lacks a 1805 bp region containing *mir-54, mir-55,* and *mir-56,* referred to as *mir-54/55/56*, which are located in close proximity to one another in the genome, and may be derived from a common transcript (Shaw et al., 2010). These 25 *mir; alg-1* mutants were examined for defects in developmental timing, gonad migration, embryonic lethality, adult lethality, and gross morphology defects. 24 of the 31 miRNAs examined in this way resulted in phenotypes (Table 1).

2.2 Seven *mir; alg-1* mutant strains display enhanced embryonic lethality

Each *mir; alg-1* strain was examined for embryonic lethality. While 3% of *alg-1* mutant worm embryos fail to hatch, seven *mir; alg-1* mutant strains had significantly increased embryonic lethality (between 7-13%, Table 2.1). These seven *mir; alg-1* strains represent eight miRNA genes: *mir-51*, *mir- 57*, *mir-59*, *mir-77*, *mir-228*, *mir-240 mir-786*, and *mir-246*. This analysis indicates these eight miRNAs may function in embryonic development.

Table 2.1. Phenotypic Characterization of miRNA mutants in *alg-1* sensitized genetic background

		Developmental Timing	Gonad Migration	Embryonic Lethality	Adult Lethality
Strain	Genotype	% Incomplete alae formation[a]	% Abnormal[b]	% Unhatched[c]	% Dead at 72hr[d]
N2	wild type	0%	0%	0%	0%
RF54	*alg-1(gk214)*	61%	8%	3%	63%
RF70	*mir-1(n4102); alg-1(gk214)*	57%	**25%****	3%	53%
RF129	*mir-34(n4276) alg-1(gk214)*	59%	13%	5%	72%
RF420	*mir-51(n4473); alg-1(gk214)*	**31%****	7%	**8%***	51%
RF411	*mir-52(n4114); alg-1(gk214)*	**3%****	0%	6%	**16%****
RF398	*mir-53(n4113); alg-1(gk214)*	60%	17%	1%	57%
RF410	*mir-54/55(nDf45) alg-1(gk214)*	**4%****	11%	2%	**12%****
RF89	*mir-54/55/56(nDf58) alg-1(gk214)*	**23%****	4%	3%	**5%****
RF133	*mir-57(gk175); alg-1(gk214)*	51%	5%	**8%****	73%
RF137	*mir-59(n4604); alg-1(gk214)*	69%	**23%****	**10%****	**83%****
RF153	*mir-72(n4130); alg-1(gk214)*	56%	2%	5%	48%
RF81	*mir-73/74(nDf47) alg-1(gk214)*	75%	7%	3%	**40%****
RF178	*mir-77(n4285); alg-1(gk214)*	54%	8%	**12%****	59%
RF65	*mir-83(n4638); alg-1(gk214)*	51%	**25%****	3%	77%
RF141	*mir-85(n4117); alg-1(gk214)*	48%	4%	3%	54%
RF77	*mir-124(n4255); alg-1(gk214)*	69%	**18%***	2%	69%
RF145	*mir-228(n4382); alg-1(gk214)*	**39%****	5%	**13%****	53%
RF93	*mir-234(n4520); alg-1(gk214)*	53%	5%	3%	57%
RF182	*mir-235(n4504); alg-1(gk214)*	56%	7%	0%	**80%****
RF85	*mir-237(n4296) alg-1(gk214)*	47%	12%	3%	75%
RF163	*mir-238(n4112); mir-239a/b(nDf62) alg-1(gk214)*	**22%****	3%	1%	**11%****
RF60	*mir-240 mir-786(n4541) alg-1(gk214)*	50%	13%	**7%****	**45%***
RF186	*mir-244(n4367); alg-1(gk214)*	**13%****	9%	5%	53%
RF149	*mir-246(n4636); alg-1(gk214)*	44%	7%	**8%****	71%
RF368	*mir-247 mir-797(n4505) alg-1(gk214)*	41%	**25%****	4%	57%
RF343	*mir-259(n4106); alg-1(gk214)*	**34%****	**28%****	4%	59%

* $p < 0.05$, ** $p < 0.01$ by the chi-square test, as compared to *alg-1* single mutants.

[a] Alae were scored at the L4m using DIC microscopy. n > 39 (range: 39-204) worms scored for each strain

[b] Gonad morphology was scored in young adult worms using DIC microscopy. n > 41 (range: 41-262) worms scored for each strain

[c] Embryos were transferred to a new plate and scored after 16-24 hours for the presence of unhatched embryos. n > 78 (range: 78-548) embryos scored for each strain.

[d] Synchronized L1-stage worms were transferred to plates to initiate development. Lethality was scored 72 hours after plating at 20^{o}C, n > 76 (range: 76-172) worms scored for each strain.

2.3 Six *mir; alg-1* mutant strains display enhanced gonad migration defects

Each *mir; alg-1* strain was examined for defects in gonad morphology. In wild type worms, the distal tip cell guides gonad migration to form a U-shaped tube by late larval development (Figure 2.1). In 8% of *alg-1* mutant worms, either the anterior or posterior gonad arm undergoes an additional turn, away from the midline (Figure 2.1, Table 2.1). These defects were found predominantly, but not exclusively in the posterior gonad arm. The penetrance of this phenotype is significantly increased up to 18-28% in six *mir; alg-1* strains (Table 2.1). These six *mir; alg-1* strains represent 7 miRNA genes: *mir-1*, *mir-59*, *mir-83*, *mir-124*, *mir-247 mir-797*, and *mir-259*.

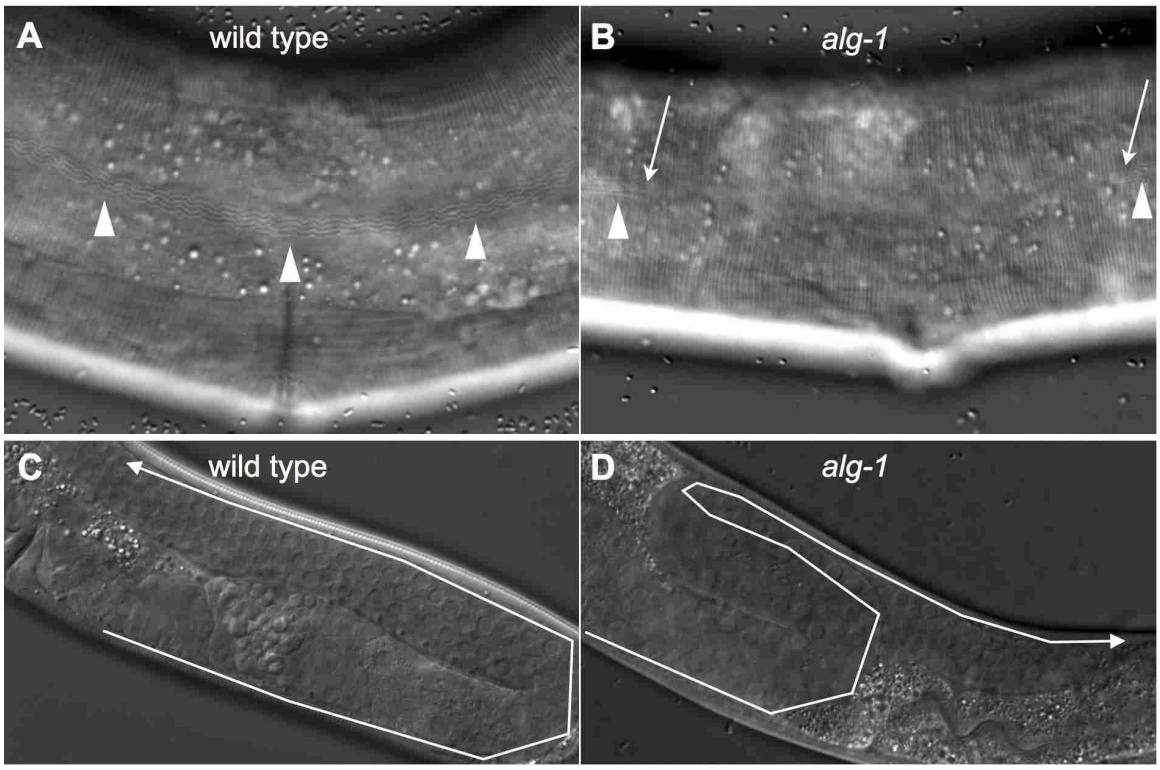


Figure 2.1. Developmental Timing and Gonad Migration Phenotypes of *alg-1*. DIC images of alae in wild type (A) and *alg-1(gk214)* mutant (B) at the young adult stage. White triangles point to alae. Area between white arrows in B contain no alae. DIC images of posterior gonad arm in wild type (C) and *alg-1(gk214)* mutant (D) at the young adult stage. White line in C and D trace the migration path of the gonad arm, with the distal tip cell near the location of the arrowhead.

2.4 Twelve *mir; alg-1* strains display enhanced or suppressed developmental timing or adult lethality defects

Each *mir; alg-1* strain was examined for adult lethality and developmental timing defects, as determined by alae formation. 63% of *alg-1* mutants die early during adulthood (Table 2.1). This lethality is primarily due to a failure to exit the molting cycle as an adult and subsequent failure of embryos to exit the vulva. This results in a bag of worms phenotype (Table 2.2). Two strains, *mir-59; alg-1* and *mir-235; alg-1* had enhanced lethality compared to *alg-1* (Table 2.1), which

was confirmed to be due to failure to exit the molting cycle and display the bag-of-worms phenotype (Table 2.2). In contrast, six other *mir; alg-1* strains had a lower percentage of adult lethality compared to *alg-1* (Table 2.1). Five of the six *mir; alg-1* strains with reduced adult lethality also had reduced alae formation defects compared to *alg-1* (Table 2.1). Alae, which form on the adult cuticle, can be used as a marker for the terminal differentiation of the seam cells (see Section 1.5). In retarded developmental timing mutants, alae formation is delayed and do not form properly at the L4-to-adult transition (Ambros and Horvitz, 1984). 61% of *alg-1* worms show retarded alae formation (Figure 2.1, Table 2.1). Three additional *mir; alg-1* strains (for a total of 9 *mir; alg-1* strains) showed reduced alae formation defects compared to *alg-1* (Table 2.1), indicating that loss of these miRNAs partially suppressed the retarded developmental timing defect of *alg-1* mutants. These twelve *mir; alg-1* strains represent 17 miRNA miRNAs: *mir-51*, *mir-52*, *mir-54*, *mir-55*, *mir-56*, *mir-59*, *mir-73*, *mir-74*, *mir-228*, *mir-235*, *mir-238*, *mir-239a*, *mir-239b*, *mir-240*, *mir-786*, *mir-244*, and *mir-259*.

Table 2.2. Analysis of enhancement or suppression of *alg-1* lethality phenotype

Strain	Genotype	% Total adult lethality	% Adults that enter lethargus[a]	% Lethality of worms that enter lethargus[b]	% Adult bursting at vulva	% Non-lethargic Bag of worms[c]
N2	wild type	3%	0%	--	3%	0%
RF54	*alg-1(gk214)*	67%	68%	90%	3%	3%
RF411	*mir-52(n4114); alg-1(gk214)*	**17%****	**0%****	--	5%	12%
RF410	*mir-54-55(nDf45) alg-1(gk214)*	**33%****	**21%****	100%	3%	8%
RF89	*mir-54-56(nDf58) alg-1(gk214)*	**18%****	**4%****	100%	3%	12%
RF137	*mir-59(n4604); alg-1(gk214)*	**87%****	**88%****	92%	3%	4%
RF81	*mir-73-74(nDf47) alg-1(gk214)*	55%	**53%***	95%	2%	4%
RF182	*mir-235(n4504); alg-1(gk214)*	70%	72%	90%	2%	3%
RF163	*mir-238(n4112); mir-239a-b(nDf62) alg-1(gk214)*	**27%****	**17%****	100%	1%	9%
RF60	*mir-240 mir-786(n4541);alg-1(gk214)*	**27%****	**14%****	100%	7%	5%

* $p < 0.05$, ** $p < 0.01$ by the chi-square test, as compared to *alg-1* single mutants.
[a] plates were examined every hour from 12 to 20 hours after the L4m for worms that entered lethargus as defined by cessation of pharyngeal pumping and reduced locomotion.
[b] Worms that entered lethargus were transferred to a new plate and scored after 16 hours for lethality. Worms died 28-36 hours after the L4m with embryos that hatched within the adult worm. Represented as # of worms that entered lethargus and died / total # of worms that entered lethargus.
[c] Worms that did not enter a supernumerary lethargus but died (36 hours after the L4m) with embryos that hatched within the adult worm ("bag of worms")

2.5 Rescue of observed *mir; alg-1* phenotypes by transgenic expression of miRNA genes

In order to validate that the phenotypes observed in *mir; alg-1* strains are due specifically to loss of the miRNA gene, transgenic rescue experiments was performed. To accomplish this, extrachromosomal arrays that contained the genomic fragment for individual miRNA genes were generated. *mir; alg-1* worms that were identified to have the extrachromosomal array, by expression of a fluorescent marker in the pharynx of the worm, were examined for defects in

gonad migration, and defects in alae formation at the L4 to Adult transition. In most cases, multiple extrachromosomal arrays for each miRNA gene were examined. Expression of the miRNA(s) from an extrachromosomal array which contained the miRNA stem and flanking sequences was sufficient to rescue the increased penetrance of gonad migration defects and alae phenotypes for most *mir; alg-1* strains, summarized in Table 2.3. Criteria for rescue is detailed below.

To examine the ability for a given miRNA expressed from an extrachromosomal array to rescue the observed gonad migration defects observed in Table 2.1, the percent of worms with gonad migration defects in *mir; alg-1* strains carrying extrachromosomal arrays for different miRNA genes was compared to the percent of gonad migration defects observed in the *mir; alg-1* strain lacking the array (Table 2.4). For example, 25% of *mir-1; alg-1* worms display gonad migration defects. This is reduced to 8% in *mir-1; alg-1* worms expressing *mir-1* from the *xwEx65* extrachromosomal array (Table 2.4). This is similar to the percentage of *alg-1* single mutant worms that display the gonad migration defect (Table 2.1, Table 2.4). This supports that loss of *mir-1* is responsible for the gonad migration defects observed in *mir-1; alg-1* worms. Similar results were found for rescue using extrachromosomal arrays for *mir-59*, *mir-83*, *mir-124*, *mir-247 mir-797*, and *mir-259* (Table 2.4).

Table 2.3. Transgenic rescue of mutant phenotypes in *mir; alg-1* strains.

miRNA in transgene	Genomic region used for transgene rescue	Phenotype	# lines rescued/total lines
mir-1	T09B4, 22804-25946	Gonad migration	2/6
mir-59	B0035, 14594-17759	Gonad migration	1/4
mir-83	C06A6, 13348-16443	Gonad migration	1/3
mir-124	C29E6, 5404-8667	Gonad migration	2/5
mir-247 mir-786	C39E6, 16210-18288	Gonad migration	3/4
mir-259	F25D1, 9049-10772	Gonad migration	1/1
mir-54, -55, -56	F09A5, 17121-20817	Alae formation	1/1[a]
mir-228	T12E12, 22144-24235	Alae formation	0/4
mir-238	K01F9, 2771-5902	Alae formation	3/3
mir-244	T04D1, 14821-17172	Alae formation	2/4
mir-259	F25D1, 9049-10772	Alae formation	1/1

[a] *mjEx160* transgene provided by R. Shaw and E. Miska

Table 2.4. Transgenic rescue of *mir; alg-1* gonad migration defects

Strain	gonad migration defects	n	p-value[a]
RF54 *alg-1*	8%	262	
RF70 *mir-1; alg-1*	25%	56	
RF388 *mir-1; alg-1; xwEx60[mir-1]*	16%	91	0.295
RF389 *mir-1; alg-1; xwEx61[mir-1]*	**9%**	**88**	**0.019**
RF390 *mir-1; alg-1; xwEx62[mir-1]*	23%	22	0.934
RF391 *mir-1; alg-1; xwEx64[mir-1]*	23%	30	0.927
RF392 *mir-1; alg-1; xwEx65[mir-1]*	**8%**	**90**	**0.008**
RF393 *mir; alg-1; xwEx66[mir-1]*	14%	90	0.168
RF137 *mir-59; alg-1*	23%	62	
RF381 *mir-59; alg-1; xwEx51[mir-59]*	17%	65	0.563
RF382 *mir-59; alg-1; xwEx52[mir-59]*	20%	30	0.991
RF383 *mir-59; alg-1; xwEx53[mir-59]*	16%	55	0.541
RF421 *mir-59; alg-1; xwEx76[mir-59]*	**9%**	**97**	**0.036**
RF65 *mir-83; alg-1*	25%	56	
mir-83; alg-1; xwEx72[mir-83][b]	17%	81	0.376
mir-83; alg-1; xwEx70[mir-83][b]	17%	76	0.372
RF414 *mir-83; alg-1; xwEx73[mir-83]*	**2%**	**45**	**0.004**
RF77 *mir-124; alg-1*	18%	56	
RF384 *mir-124; alg-1; xwEx54[mir-124]*	**7%**	**144**	**0.041**
RF385 *mir-124; alg-1; xwEx55[mir-124]*	21%	34	0.966
RF386 *mir-124; alg-1; xwEx57[mir-124]*	15%	67	0.847
RF387 *mir-124; alg-1; xwEx58[mir-124]*	18%	28	0.763
RF394 *mir-124; alg-1; xwEx59[mir-124]*	**6%**	**86**	**0.045**
RF368 *mir-247 alg-1*	25%	59	
RF426 *mir-247/797 alg-1; xwEx78[mir-247/797]*	**3%**	**29**	**0.027**
RF427 *mir-247/797 alg-1; xwEx79[mir-247/797]*	**5%**	**44**	**0.011**
mir-247/797 alg-1; Ex[mir-247/797][b]	6%	17	0.160
RF428 *mir-247/797 alg-1; xwEx80[mir-247/797]*	**0%**	**29**	**0.007**
RF343 *mir-259; alg-1*	28%	60	
RF425 *mir-259; alg-1; xwEx77[mir-259]*	**6%**	**32**	**0.026**

[a] χ^2 test statistical analysis was performed comparing mir;alg-1 worms ± extrachromosomal array for given miRNA gene. $P < 0.05$ were considered significant.
[b] worms of given genotype not given strain name

Similar analysis was performed to determine if miRNA expression from an extrachromosomal array was able to rescue the alae formation phenotypes observed in *mir; alg-1* mutant worms. The percent of worms with alae defects in *mir; alg-1* strains carrying an extrachromosomal array for a given miRNA was quantified (Table 2.5). For example, 14% of *mir-238; alg-1* mutant worms display incomplete alae at the L4-to-adult transition, which is increased to 52% in *mir-*

238; alg-1 worms expressing *mir-238* from the *xwEx16* extrachromosomal array (Table 2.5). The 52% of *mir-238; alg-1; xwEx16* worms displaying incomplete alae is similar to the 61% of *alg-1* mutant worms observed to display incomplete alae (Table 2.5). This supports that loss of *mir-238* specifically causes the difference in alae formation defects between *mir-238; alg-1* and *alg-1* mutant worms. Similar results were observed for rescue using extrachromosomal arrays for *mir-54/55/56*, *mir-244*, and *mir-259* (Table 2.5). A transgene previously demonstrated to rescue loss of the whole *mir-51* family of miRNAs (Shaw et al., 2010) was used to rescue *mir-54/55/56 alg-1* mutants. Since the *mir-51* family has been shown to function redundantly (Alvarez-Saavedra and Horvitz, 2010; Shaw et al., 2010), rescue for *mir-51*, *mir-52*, or *mir-54/55* was not performed. Extrachromosomal arrays generated for *mir-228* failed to rescue the alae formation phenotype of *mir-228; alg-1* worms (Table 2.5).

Since the enhancement of embryonic lethality was modest in *mir; alg-1* strains described above (≤ 10% increase), rescue of this phenotype via expression of the miRNA from an extrachromosomal array was not attempted.

Table 2.5. Transgenic rescue of *mir; alg-1* alae formation phenotype

Strain	% Incomplete Alae formation at L4-to-Adult	n	p-value[a]
RF54 *alg-1*	61%	204	
RF89 *mir-54/55/56 alg-1*	23%	57	
RF403 *mir-54/55/56 alg-1; mjEx160[mir-54/55/56]*	**60%**	**35**	**0.001**
RF145 *mir-228; alg-1*	37%	68	
mir-228; alg-1; xwEx[mir-228][b]	16%	19	0.146
mir-228; alg-1; xwEx[mir-228][b]	28%	18	0.665
mir-228; alg-1; xwEx[mir-228][b]	32%	37	0.818
mir-228; alg-1; xwEx[mir-228][b]	32%	22	0.869
RF161 *mir-238; alg-1*	14%	43	
RF251 *mir-238; alg-1; xwEx14[mir-238]*	**39%**	**41**	**0.018**
RF252 *mir-238; alg-1; xwEx15[mir-238]*	**38%**	**42**	**0.022**
RF253 *mir-238; alg-1; xwEx16[mir-238]*	**52%**	**44**	**0.000**
RF186 *mir-244; alg-1*	13%	48	
RF396 *mir-244; alg-1; xwEx74[mir-244]*	8%	39	0.705
RF395 *mir-244; alg-1; xwEx74[mir-244]*	18%	34	0.739
RF416 *mir-244; alg-1; xwEx74[mir-244]*	**89%**	**9**	**0.000**
RF417 *mir-244; alg-1; xwEx75[mir-244]*	**95%**	**19**	**0.000**
RF343 *mir-259; alg-1*	34%	59	
RF425 *mir-259; alg-1; xwEx77[mir-259]*	**61%**	**31**	**0.023**

[a] χ^2 test statistical analysis was performed comparing mir;alg-1 worms ± extrachromosomal array for given miRNA gene. $P < 0.05$ were considered significant.

[b] worms of given genotype not given strain name

Chapter 3: The *mir-51* family likely functions upstream of *hbl-1* to specify the L2 to L3 transition in *C. elegans*

3.1 Introduction

80% of the miRNAs examined in the previous chapter caused quantifiable differences in phenotypes of *alg-1* mutant worms (Table 1). One interesting finding was that loss of different miRNAs suppressed the alae formation defects of *alg-1* mutants. These miRNAs included members of the *mir-51* family (*mir-51*, *mir-52*, and *mir-54/55/56*), *mir-228*, the *mir-238* family (*mir-238*; *mir-239a/b*), *mir-244*, and *mir-259*. Further examination of the *mir-51* family in developmental timing was chosen since loss of one family member, *mir-52*, showed the strongest suppression of *alg-1* developmental timing defects (Table 2.1).

This observed suppression of developmental timing defects by loss of *mir-51* family members was unexpected. The *mir-51* family is part of the larger miR-99/100 family that shows deep conservation from cnidarians through humans (Grimson et al., 2008). The *mir-51* family is comprised of six members in *C. elegans*, miR-51 through miR-56. Loss of the entire *mir-51* family in *C. elegans* results in embryonic lethality (Alvarez-Saavedra and Horvitz, 2010; Shaw et al., 2010). Loss of multiple members of the *mir-51* family results in pleiotropic effects including larval lethality and slow growth (Alvarez-Saavedra and Horvitz, 2010; Shaw et al., 2010). The *mir-51* family are expressed broadly and abundantly throughout the life of the worm (Lim et al., 2003; Ruby et al., 2006; Kato et al., 2009a; Shaw et al., 2010). These features are unlike those of many developmental timing genes. In this chapter, genetic interactions between *mir-51*

family members and known developmental timing genes were examined in order to better identify a mechanism whereby loss of *mir-51* family members can suppress *alg-1* developmental timing phenotypes.

3.2 Loss of *mir-51* family members partially suppress retarded developmental timing phenotypes

3.2.1 Loss of *mir-51* family members, individually or multiply, has no effect on developmental timing

In order to determine the mechanism whereby loss of *mir-51* family members results in suppression of *alg-1* developmental timing defects, worms individually mutant for *mir-51* family members were examined for developmental timing phenotypes. Mutants lacking individual members of the *mir-51* family mutants did not display developmental timing abnormalities such as alae formation defects or defects in seam cell divisions (Table 3.1 and Table 3.2). Furthermore, worms lacking 5 of 6 members of the *mir-51* family, *mir-52* through *mir-56*, did not display alae defects (Table 3.1 and Table 3.2), despite displaying other mutant phenotypes including larval lethality and slow growth (Alvarez-Saavedra and Horvitz, 2010). This indicates the *mir-51* family is not required for normal progression of developmental time.

Table 3.1. Genetic interactions of *mir-51* family with retarded developmental timing mutants

		Alae at L4 to adult transition				Lethality		
Strain[a]	seam cells[b]	complete	gapped	none	n	% burst	% bag of worms	n
RG733 *wild type*	16.0	100	0	0	20	0	0	208
RF481 *wild type*	16.1	100	0	0	20	0	0	109
RF491 *mir-51*	16.2	100	0	0	20	0	0	151
RF499 *mir-52*	15.9	100	0	0	20	0	0	181
RF483 *mir-53*	16.1	100	0	0	20	0	0	176
RF399 *mir-54/55/56*	16.1	99	1	0	98	0	0	228
RF692 *mir-52/53/54/55/56*	--[c]	100	0	0	16	--	--	--
RF554 *mir-48/84/241*	22.6	0	100	0	40	56	37	111
RF556 *mir-52; mir-48/84/241*	17.7[e]	49[k]	51	0	39	3[k]	77	90
RF553 *mir-48/84/241*	22.7	0	100	0	37	66	26	125
RF555 *mir-51; mir-48/84/241*	21.8	0	100	0	37	42[l]	41	112
RF557 *mir-53; mir-48/84/241*	22.2	0	100	0	38	49[l]	39	134
RF558 *mir-54/55/56; mir-48/84/241*	20.6[f]	21[l]	79	0	38	25[l]	57	141
VT1064 *mir-48/84*	--	--	--	--	--	0	69	236
RF451 *mir-51; mir-48/84*	--	--	--	--	--	0	30[n]	101
RF469 *mir-52; mir-48/84*	--	--	--	--	--	0	5[n]	148
RF454 *mir-53; mir-48/84*	--	--	--	--	--	0	62	106
RF451 *mir-54/55/56; mir-48/84*	--	--	--	--	--	0	2[n]	93
MT7626 *let-7ts* @25˚C	--	0	50	50	16	100	--	103
RF447 *mir-51; let-7* @25˚	--	0	80	20	20	100	--	119
RF448 *mir-52; let-7ts* @25˚	--	7	73	20	15	96	--	114
RF449 *mir-53; let-7* @25˚	--	0	53	47	17	99	1	92
RF442 *mir-54/55/56; let-7* @25˚	--	7	21	71	14	99	1	91
RF568 *lin-46 @15˚*	19.4	5	95	0	40	--	--	--
RF569 *mir-52; lin-46 @15˚*	17.8[h]	23[m]	77	0	39	--	--	--
RF504 *lin-46 @15˚*	18.1	19	81	0	59	--	--	--
RF594 *mir-51; lin-46 @15˚*	17.6	38	62	0	37	--	--	--
RF599 *mir-53; lin-46 @15˚*	18.1	24	76	0	21	--	--	--
RF505 *mir-54/55/56; lin-46 @15˚*	17.4	8	92	0	39	--	--	--
RF619 *mir-48/241*	19.1	5	95	0	21	31	49	144
RF730 *mir-48/241; mjEx160[mir-54/55/56]*	22.1[g]	9	91	0	32	66[d]	24[d]	136[d]
VC894 *puf-9*	--	29	71	0	34	--	--	--
RF578 *mir-52; puf-9*	--	34	66	0	50	--	--	--
RF620 *mir-52; mir-48/241*	16.6[i]	85	15	0	20	--	--	--
RF625 *mir-48/241; puf-9*	19.2	0	100	0	19	--	--	--
RF626 *mir-52; mir-48/241; puf-9*	16.2[j]	0	100	0	17	--	--	--

[a] Full genotype information, include alleles used, can be found in Table 7.1.
[b] seam cells counted in L4-stage worms using *wIs78* or *wIs79[scm::gfp]*, n ≥ 18 (range 19 - 30).
[c] indicates results not determined.
[d] population scored for lethality is a mix of worms ± for *mjEx160*.
[e] indicates significant difference compared to RF554 *mir-48/84/241* (student's t-test, $p < 0.05$), which contain *wIs79*.
[f] indicates significant difference compared to RF553 mir-48/84/241 (student's t-test, $p < 0.05$), which contain *wIs78*.
[g] indicates significant difference comparing worms from the same strain ± for *mjEx160* (student's t-test, $p < 0.05$).
[h] indicates significant difference compared to RF568 *lin-46* (student's t-test, $p < 0.05$).
[i] indicates significant difference compared to RF619 *mir-48/241* (student's t-test, $p < 0.05$).
[j] indicates significant difference compared to RF625 *mir-48/241; puf-9* (student's t-test, $p < 0.05$).
[k] indicates significant difference compared to RF554 *mir-48/84/241* (χ^2, $p < 0.05$) which contain *wIs79*.
[l] indicates significant difference compared to RF553 *mir-48/84/241* (χ^2, $p < 0.05$) which contain *wIs78*.
[m] indicates significant difference compared to RF568 *lin-46* (χ^2, $p < 0.05$).
[n] indicates significant difference compared to VT1064 *mir-48/84* (χ^2, $p < 0.05$).

3.2.2 Loss of *mir-51* family members suppresses retarded developmental timing phenotypes of *let-7* family mutants

Since *alg-1* developmental timing defects are similar to those associated with the loss of the *let-7* family miRNAs (Grishok et al., 2001), the effect of loss of individual *mir-51* family members on *let-7* family timing defects was examined to determine if a similar suppression occurs outside the *alg-1* mutant background (Table 3.1). The let-7 family members, *mir-48*, *mir-84*, and *mir-241*, function together to control the timing of the L3 stage program through down-regulation of their target, *hbl-1* (Abbott et al., 2005). In the L2 stage, a subset of hypodermal seam cells undergo two rounds of cell division resulting in an increase in the number of seam cells from 10 to 16. In mutants lacking *mir-48*, *mir-84* and *mir-241* (hereafter referred to as *mir-48/84/241)*, the L2 stage program is repeated thereby producing extra seam cells (Abbott et al., 2005). At the L4-to-adult transition, *mir-48/84/241* mutant worms fail to produce cuticles with complete adult alae formation. In addition, many of these mutant worms burst at the L4 to adult transition or fail to exit the molting cycle, which leads to the “bag-of-worms” phenotype (Abbott et al., 2005). *mir-52; mir-48/84/241* had fewer seam cells than *mir-48/84/241* worms, indicating a suppression of the L2 reiteration phenotype. Additionally, loss of *mir-52* suppressed the alae formation defects and bursting phenotypes of *mir-48/84/241:* 100% of *mir-48/84/241* mutants displayed incomplete alae and 56% of *mir-48/84/241* mutant worms burst at the L4-to-Adult transition reduced to 51% and 3% in *mir-52; mir-48/84/241*, respectively (Table 3.1). However, 77% of *mir-52; mir-48/84/241* worms showed the bag of worms

phenotype, indicating an extra adult-stage molt. This likely reflects a partial suppression of the *mir-48/84/241* phenotype, rather than an inability to suppress molting since loss of *mir-52* strongly suppressed the ectopic molting phenotype of *alg-1* worms (Table 2.2) as well as *mir-48/84* double mutant worms (Table 3.1).

mir-48/84/241 developmental timing defects were suppressed by loss of other *mir-51* family members, though to a lesser extent than *mir-52* (Table 3.1). It is likely that the differences in the ability to suppress the *mir-48/84/241* phenotype observed between family members reflects differences in their overall expression levels since *mir-52* and *mir-54/55/56* are expressed at higher levels compared to *mir-51* and *mir-53* (Lim et al., 2003; Ruby et al., 2006; Kato et al., 2009a).

let-7 regulates later stages in developmental timing relative to its family members *mir-48*, *mir-84*, and *mir-241*. At 25˚C, *let-7(n2853)* mutants display a repetition of a late larval program with failure to form complete alae and lethality due to bursting at the vulva at the L4 to adult transition (Reinhart et al., 2000; Table 3.1). Loss of *mir-51* family members had no significant effect on the phenotype of *let-7ts* worms (Table 3.1). This indicates that later larval stages are insensitive to loss of *mir-51* family members. Interestingly, a few *mir-52; let-7ts* and *mir-54/55/56 let-7ts* worms survived into adulthood at 25˚C, whereas no *let-7ts* worms survived in this characterization (Table 3.1). These small differences were not significant, but it may reflect modest suppression.

These data indicate that early timing events are most sensitive to loss of *mir-51* family members.

3.2.3 Expression of *mir-51* family members from an extrachromosomal array enhances retarded developmental timing phenotypes of *let-7* family mutants

While loss of *mir-51* family members resulted in suppression of retarded developmental timing defects, we wondered if the opposite was true: can elevated expression of *mir-51* family members enhance retarded developmental timing defects? To test this, the effect of elevated expression of *mir-51* family members on *mir-48 mir-241* (*mir-48/241*) mutant worms, which display slightly less penetrant developmental timing defects relative to *mir-48/84/241* mutants (Abbott et al., 2005), was examined. To elevate the expression of *mir-51* family members, *mjEx160*, which is an extrachromosomal array with the genomic fragment for *mir-54/55/56* that was previously shown to rescue the embryonic lethality of *mir-51* family mutant worms (Shaw et al., 2010) and the developmental timing phenotypes in *mir-54/55/56 alg-1* mutant worms (Table 2.5), was used. *mjEx160* enhanced developmental timing defects of *mir-48/241* mutant worms: *mir-48/241* worms had 19.1 seam cells on average which was increased to 22.1 in *mir-48/241; mjEx160* worms (Table 3.1). This indicates elevated expression of *mir-51* family members enhances the L2 repetition phenotype. This also indicates that this early timing event is sensitive to both loss and increase of *mir-51* family expression.

3.2.4 Loss of *mir-52* partially suppresses *lin-46*, but not *puf-9*, retarded developmental timing phenotypes

Since loss of *mir-51* family members suppressed the retarded developmental timing defects of *let-7* family mutants, it is possible that loss of *mir-51* family members is also able to suppress the phenotypes of two additional mutants, *lin-46* and *puf-9,* that display retarded developmental timing defects (Pepper et al., 2004; Nolde et al., 2007). *lin-46* functions in parallel to the *let-7* family to control the timing of the L3 program (Pepper et al., 2004; Abbott et al., 2005). *lin-46* mutants fail to properly execute the L3 stage program and show reiteration of the L2 program at 15˚C (Pepper et al., 2004). Loss of *mir-52* partially suppressed *lin-46* developmental timing defects: *mir-52; lin-46* mutant worms had fewer seam cells and displayed weaker alae defects compared to *lin-46* mutant worms (Table 3.1). Loss of the other *mir-51* family members had no significant effect on *lin-46* (Table 3.1). *puf-9* is a pumilio family homolog that negatively regulates *hbl-1* in a 3‘UTR dependent fashion (Nolde et al., 2007). *puf-9* mutant worms failed to form complete alae at the L4 to adult transition. Loss of *mir-52* did not suppress the *puf-9* alae defects (Table 3.1). This suggests that *puf-9* may function downstream of the *mir-51* family to regulate developmental timing.

To test whether *puf-9* was necessary for *mir-52*-mediated suppression of the *let-7* family developmental timing defects, worms multiply mutant for *mir-52*, *puf-9*, and *let-7* family miRNAs, *mir-48* and *mir-241* (*mir-48/241*) were examined. *mir-52; mir-48/241* mutant worms had reduced seam cell numbers in L4

compared to *mir-48/241* mutant worms (Table 3.1). *puf-9* was not required for *mir-52* mediated suppression of the extra seam cell phenotype of *mir-48/241* mutant worms (Table 3.1). However, *puf-9* activity was required for the *mir-52*-mediated suppression of alae formation defects: *mir-52; mir-48/241; puf-9* mutant worms failed to form complete alae at the L4 to adult transition (Table 3.1). Together, these data indicate that the *mir-51* family functions to regulate the execution of the L3 stage program, acting either downstream or in parallel to the *let-7* family miRNAs and *lin-46*.

3.3 Loss of *mir-51* family members can enhance precocious developmental timing phenotypes

Suppression of retarded developmental timing defects by loss of *mir-51* family members suggests that this family of miRNAs acts to oppose normal progression through larval development. To further examine this possibility, the genetic interactions between *mir-51* family members and a set of precocious developmental timing genes were examined (Table 3.2). It is predicted that, if the *mir-51* family acts to oppose larval transitions, then loss of *mir-51* family members should result in enhancement of precocious developmental timing defects. Consistent with this, loss of *mir-52* enhanced the precocious development of *mir-48(ve33), hbl-1*, and *lin-14* mutant worms (Table 3.2). First, *mir-48(ve33)* mutant worms display early accumulation of miR-48 and precocious formation of adult-specific alae in L4 stage worms (Li et al., 2005). Loss of *mir-52* enhanced the precocious alae phenotype of *mir-48(ve33)* worms (Table 3.2). Second, *hbl-1* is a central regulator of the L2 to L3 cell fate decision (Abrahante

et al., 2003; Lin et al., 2003). Loss of *mir-52* enhanced the precocious alae phenotype of *hbl-1(ve18)* mutants: 76% of *hbl-1(ve18)* mutants displayed either complete or gapped alae in the L4 stage compared to 97% of *mir-52; hbl-1* worms (Table 3.2). Enhancement of *hbl-1(ve18)* may reflect reduced activity of *hbl-1* itself, since *ve18* is a reduced function, not a null, allele (Abrahante et al., 2003). *lin-14* functions to regulate both L1 to L2 and L2 to L3 cell fate decisions (Ambros and Horvitz, 1987). At 25˚C, 34% of *lin-14(n179)* worms form complete alae during larval development compared to 76% of *mir-52; lin-14(n179)* worms (Table 3.2). Loss of no other *mir-51* family member significantly enhanced the precocious development of *mir-48(ve33), hbl-1(ve18)*, and *lin-14(n179ts)*, except for *mir-51* which significantly enhanced *mir-48(ve33)* (Table 3.2). In contrast to the enhancement of the precocious phenotypes described above, enhancement of the precocious phenotypes of *lin-41, lin-42, or lin-28* was not observed (Table 3.2). This enhancement of the precocious development observed in *mir-48(ve33), hbl-1(ve18)*, and *lin-14(n179ts)* mutant worms is consistent with the *mir-51* family functioning to oppose the execution of L3 stage program.

Table 3.2. Genetic interactions of *mir-51* family with precocious developmental timing mutants

Strain[a]	Precocious Alae[b]			
	complete	gapped	none	n
RF481 *wild type*	0	0	100	12
RF499 *mir-52*	0	0	100	13
RG733 *wild type*	0	0	100	9
RF491 *mir-51*	0	0	100	14
RF483 *mir-53*	0	0	100	15
RF399 *mir-54/55/56*	0	0	100	13
RF692 *mir-52/53/54/55/56*	0	0	100	15
RG490 *mir-48(ve33)*	0	55	45	47
RF582 *mir-51; mir-48(ve33)*	0	85[d]	15	34
RF583 *mir-52; mir-48(ve33)*	0	88[d]	12	34
RF584 *mir-53; mir-48(ve33)*	0	76	24	34
RF587 *mir-54/55/56; mir-48(ve33)*	0	43	57	28
RF534 *hbl-1*	0	76	24	41
RF535 *mir-52; hbl-1*	2[e]	95	2	44
RF510 *hbl-1*	7	77	17	30
RF530 *mir-51; hbl-1*	20	71	9	35
RF512 *mir-53; hbl-1*	11	77	11	35
RF511 *mir-54/55/56; hbl-1*	3[f]	80	17	30
RF563 *lin-14 @25˚C*	34	66	0	29
RF588 *mir-52; lin-14 @25˚C*	76[g]	20	4	25
RF500 *lin-41*	0	37	63	35
RF536 *lin-41*	0	11	89	38
RF529 *mir-51; lin-41*	0	41	59	32
RF537 *mir-52; lin-41*	0	14	86	36
RF539 *mir-53; lin-41*	0	27	73	37
RF501 *mir-54/55/56; lin-41*	0	11	89	37
RF538 *lin-42*	0	89	11	37
RF541 *mir-52; lin-42*	3	93	3	29
RF508 *lin-42*	13	80	7	46
RF527 *mir-51; lin-42*	8	83	8	36
RF526 *mir-53; lin-42*	3	86	11	36
RF509 *mir-54/55/56; lin-42*	6	91	3	33
VT517 *lin-28*[c]	5	90	5	20
RF572 *mir-51; lin-28*[c]	0	95	5	20
RF573 *mir-52; lin-28*[c]	0	100	0	20
RF574 *mir-53; lin-28*[c]	5	95	0	20
RF575 *mir-54/55/56; lin-28*[c]	0	100	0	20

[a] full genotype information, including alleles used, can be found in Table 7.1.
[b] alae were scored in L3 molt or early L4-stage worms, except where otherwise noted
[c] alae were scored in the L2 molt
[d] significantly different compared to RG490 mir-48(ve33) (χ^2, $p < 0.05$).
[e] significantly different compared to RF534 *hbl-1* (χ^2, $p < 0.05$).
[f] significantly different compared to RF510 *hbl-1* (χ^2, $p < 0.05$).
[g] significantly different compared to RF563 *lin-14* (χ^2, $p < 0.05$).

3.4 Loss of *mir-51* family members suppresses *hbl-1* mis-expression in *mir-48/84/241* mutant worms

Genetic interactions between *mir-51* family members and *let-7* family members as well as *hbl-1(ve18)* suggest that the *mir-51* family may act upstream of *hbl-1* expression. *hbl-1* is robustly expressed in the hypodermis during embryonic and early larval development, then is subsequently down-regulated through its 3' UTR by the late L3 stage (Abrahante et al., 2003). The down-regulation of *hbl-1* in the hypodermis requires the *let-7* family members, *mir-48*, *mir-84*, and *mir-241* (Abbott et al., 2005). Therefore it is possible that the observed suppression of developmental timing defects in *mir-52; mir-48/84/241* reflects a suppression of *hbl-1* mis-regulation. Indeed, loss of *mir-52* partially suppressed the *hbl-1* misexpression phenotype of *mir-48/84/241* mutant worms: in 91% of *mir-48/84/241* worms, *hbl-1::gfp::hbl-1* expression remained high in L3, whereas only 62% of *mir-52; mir-48/84/241* displayed high *hbl-1::gfp::hbl-1* expression (Figure 3.1A-E). Consistent with the *mir-51* family having redundant function, loss of *mir-54/55/56* also suppressed the *hbl-1* misexpression phenotype of *mir-48/84/241* mutant worms (Figure 3.1E). This indicates that the *mir-51* family acts in opposition to the *let-7* family activity, and acts upstream of *hbl-1* expression.

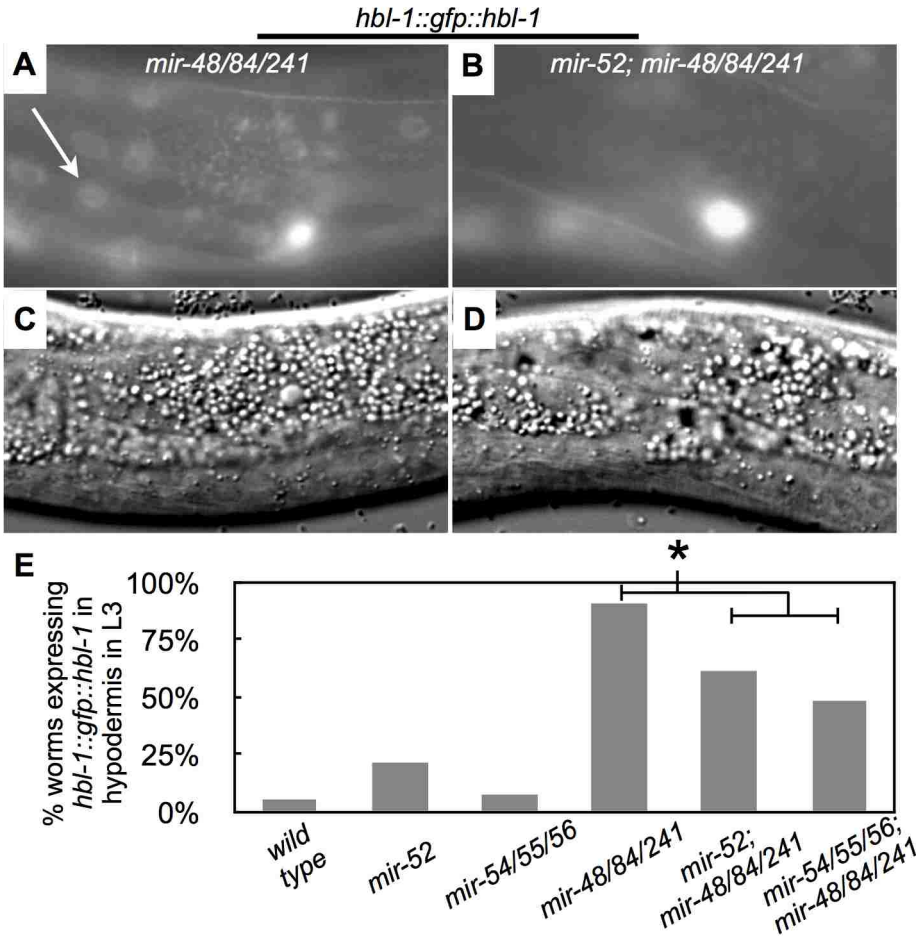


Figure 3.1. Loss of *mir-52* or *mir-54/55/56* restores *hbl-1* regulation in *mir-48/84/241* mutants. (A-E) Effect of *mir-52* and *mir-54/55/56* on *hbl-1::gfp::hbl-1* expression. Representative fluorescent image of *hbl-1::gfp::hbl-1* transgene expression in (A) *mir-48/84/24*1, with expression on in hyp7 nuclei, and (B) *mir-52; mir-48/84/24*1 mutant worms, with expression off in hyp7 nuclei, at the L3 stage with corresponding DIC images (C and D, respectively). White arrow in A pointing to a hyp7 nuclei. (E) Percentage of worms with *hbl-1::gfp::hbl-1* expression in hypodermis of L3 stage worms, n ≥ 33 (range 33 – 37). * indicates significant difference (χ^2, $p < 0.01$).

3.5 The *mir-51* family does not regulate *lin-28* expression

Like *hbl-1*, *lin-28* is also a critical regulator of L3 cell fate decisions. Interestingly, loss of *mir-51* family members had no effect on the precocious development of *lin-28* (Table 3.2), which is consistent with the *mir-51* family functioning upstream of *lin-28*. To test if the *mir-51* family functions upstream of *lin-28* to regulate the execution of the L3 stage program, worms multiply mutant for *mir-52*, *lin-46*, *lin-28*, and *mir-48/84/241* were examined. *lin-28; lin-46; mir-48/84/241* mutant worms continually repeat the L2 stage-specific symmetric seam cell division (Abbott et al., 2005). Loss of *mir-52* had no effect on the extra seam cell phenotype of *lin-28; lin-46; mir-48/84/241* mutant worms (Figure 3.2A). This is consistent with the *mir-51* family acting upstream or in parallel to *lin-28* to regulate the execution of the L3 stage program.

If the *mir-51* family functions upstream of *lin-28*, then it might be that *mir-51* family members indirectly regulate *lin-28* expression. We used a *lin-28::gfp::lin-28* transgene to determine whether the *mir-51* family functions to promote *lin-28* expression. However, no difference was observed in *lin-28::gfp::lin-28* expression between *mir-48/84/241* and *mir-52; mir-48/84/241* worms (Figure 3.2B,C). Thus, mis-regulation of *lin-28* does not account for the observed suppression of developmental timing defects in *mir-52; mir-48/84/241* worms. These data together are consistent with the *mir-51* family functioning in parallel to *lin-28*, *lin-46* and the *let-7* family, but upstream of *hbl-1* to regulate the execution of the L3 stage program.

A

Strain	seam cell number	
	mid L3	mid L4
RF690 *lin-28; lin-46; mir-48/84/241*	29.3	50.1
RF689 *mir-52; lin-28; lin-46; mir-48/84/241*	29.2	49.8

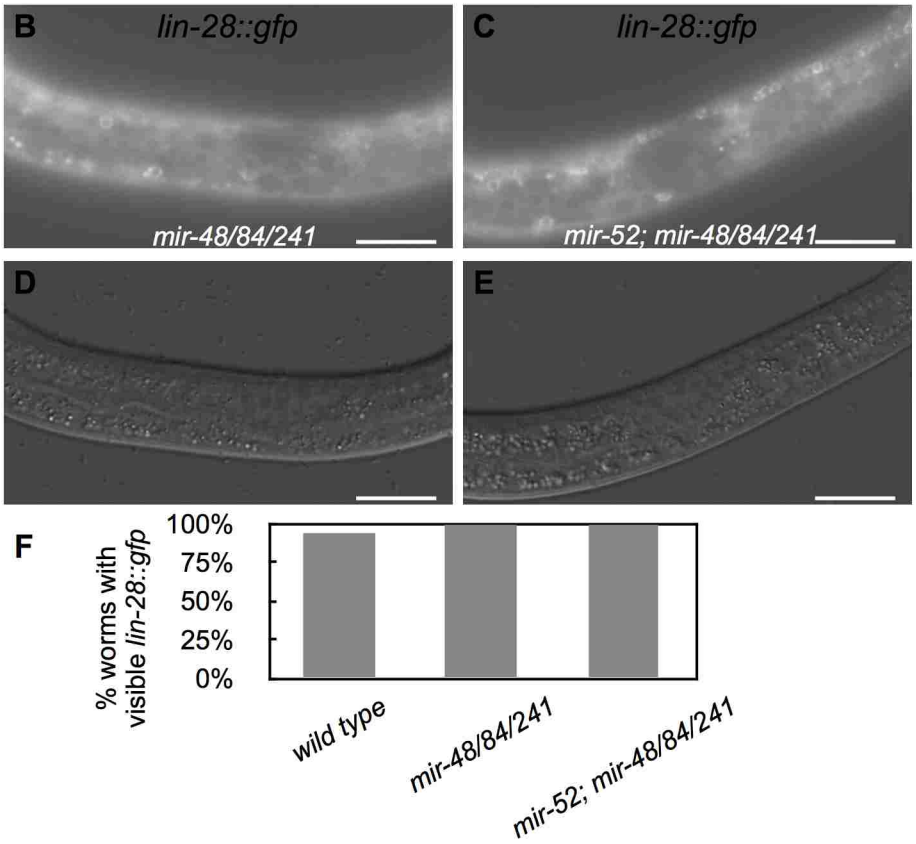


Figure 3.2. *mir-52* does not alter *lin-28* expression. (A) *mir-52* has no effect on seam cell number of *lin-28; lin-46; mir-48/84/241* mutant worms when scored in L3 or L4 stages. (B, C) Representative fluorescent image of *lin-28::gfp* transgene expression at the L2 molt stage in (B) *mir-48/84/241* and (C.) *mir-52; mir-48/84/241* with corresponding DIC images, (D and E, respectively). (F) Percentage of worms of given genotype scored with visible *lin-28::gfp* expression. No significant difference was observed between strains (χ^2, $p > 0.05$).

Chapter 4: The *mir-51* family interacts genetically in many miRNA-dependent developmental pathways

4.1 Introduction

Loss of *mir-51* family members results in suppression of developmental timing defects of *alg-1* mutant worms (Table 2.1). Suppression of *alg-1* mutant developmental timing defects by loss of *mir-51* family members may reflect a specific function for this family to regulate targets in the developmental timing pathway. Experiments in the previous chapter indicate that the *mir-51* family acts in the developmental timing pathway, primarily in the L2 to L3 transition. However, this role in developmental timing may be indirect. For example, the developmental timing defects observed in mutants of individual miRISC components, *alg-1* or *ain-1*, are due to lower overall miRNA activity, including the lin-4 and let-7 family miRNAs (Grishok et al., 2001; Ding et al., 2005). Also unlike other developmental timing genes, the *mir-51* family are expressed broadly and abundantly throughout the life of the worm (Lim et al., 2003; Ruby et al., 2006; Kato et al., 2009a; Shaw et al., 2010). Loss of the entire *mir-51* family in *C. elegans* results in embryonic lethality (Alvarez-Saavedra and Horvitz, 2010; Shaw et al., 2010). Loss of multiple members of the *mir-51* family results in pleiotropic effects including larval lethality and slow growth (Alvarez-Saavedra and Horvitz, 2010; Shaw et al., 2010). These phenotypes indicate that the *mir-51* family regulates multiple downstream targets and pathways. Together these observations may indicate a broader function for the *mir-51* family in miRNA activity or biogenesis. If the *mir-51* family has a broader role in miRNA activity,

then it is predicted that loss of *mir-51* family members could suppress other miRNA mutant phenotypes that are distinct from developmental timing, including *lsy-6* regulation of neuronal asymmetry, *let-7* family regulation of vulva cell fate specification, *mir-240/786* regulation of defecation, *mir-35* family regulation of embryonic development and *mir-1* regulation of neuromuscular function, which represent the known pathways affected by loss of miRNAs in *C. elegans*. The effect of loss of *mir-51* family members on these known miRNA pathways was examined to test this prediction.

4.2 *mir-51* family members genetically interact with *lsy-6* to specify the ASEL neuron

The *lsy-6* miRNA specifies the ASEL cell fate through down-regulation of its target, *cog-1* (Johnston and Hobert, 2003). *lsy-6* repression of *cog-1* is necessary for *lim-6::gfp* reporter expression in the ASEL (Johnston and Hobert, 2003; Figure 4.1A). 100% of worms homozygous for *ot149*, a loss-of-function allele of *lsy-6*, display mutant *lim-6::gfp* expression (Figure 4.1B; Johnston and Hobert, 2003). In contrast, only 14% of worms homozygous for the *lsy-6(ot150)* allele display this mutant *lim-6::gfp* expression (Figure 4.1B). Although the molecular nature of the *lsy-6(ot150)* allele is unknown, the phenotype suggests it represents a *lsy-6* reduced function. Therefore the *lsy-6(ot150)* allele is referred to as *lsy-6rf* and *lsy-6(ot149)* as *lsy-6lf* for loss-of-function. *lsy-6rf* is significantly enhanced by *alg-1.* 14% of *lsy-6rf* worms have mutant *lim-6::gfp* expression compared to 27% of *lsy-6rf; alg-1* worms (Figure 4.1B). This is consistent with further reduced *lsy-6* activity. Neither *mir-238* nor *mir-244,* two miRNA genes

found to suppress developmental timing defects of *alg-1* mutant worms (Table 2.1), had an effect on the mutant *lim-6::gfp* expression in *lsy-6rf; alg-1*. In contrast, *mir-54/55/56* significantly suppressed mutant *lim-6::gfp* expression in *lsy-6rf; alg-1*: 27% of *lsy-6rf; alg-1* mutant worms displayed mutant *lim-6::gfp* compared to 16% of *lsy-6rf; alg-1; mir-54/55/56* (Figure 4.1B).

Since *mir-54/55/56* are part of the larger *mir-51* family, the role of another *mir-51* family member, *mir-52,* on ASEL specification was examined. *mir-52* had no significant effect on *lsy-6rf* (Figure 4.1C). Since such a low proportion of *lsy-6rf* mutants display the mutant *lim-6::gfp* expression, worms heterozygous for two *lsy-6* alleles, *lsy-6lf* and *lsy-6rf*, hereafter referred to *lsy-6rf/lsy-6lf*, were used to achieve a genetic background with optimally compromised lsy-6 activity. 85% of these *lsy-6rf/lsy-6lf* worms fail to express *lim-6::gfp* in the ASEL compared to 100% of worms homozygous for *lsy-6lf* (Figure 4.1C). Loss of *mir-52* partially suppressed mutant *lim-6::gfp* expression in *lsy-6rf/lsy-6lf*: 85% of *lsy-6rf/lsy-6l*f worms displayed mutant *lim-6::gfp* compared to 61% of *mir-52; lsy-6rf/lsy-6lf* worms (Figure 4.1C). This observed suppression is consistent with lsy-6 activity being elevated in the absence of *mir-51* family members and may indicate a broader role for this family in regulation of miRNA biogenesis or activity.

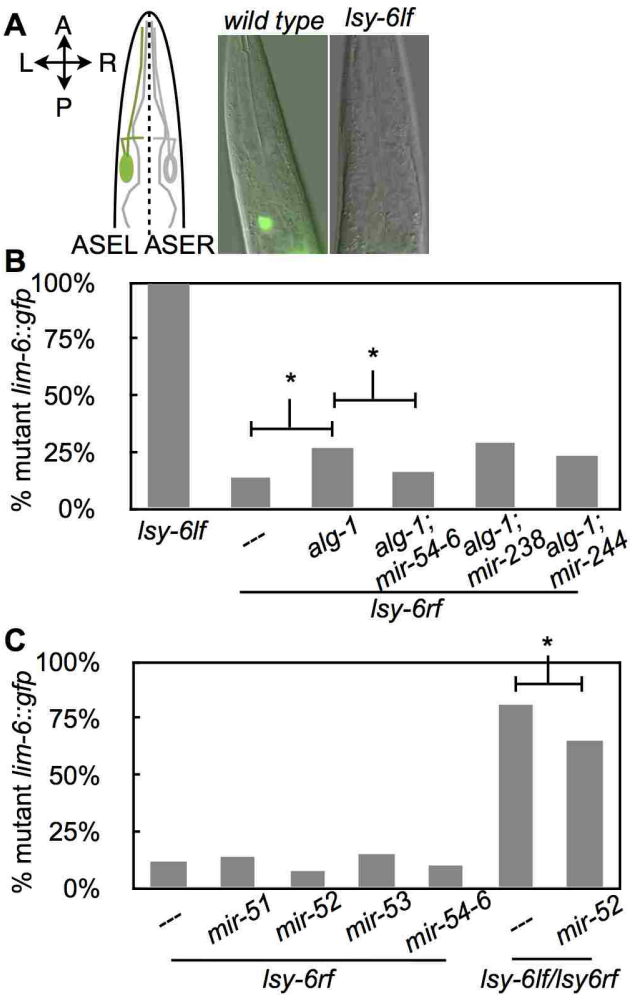


Figure 4.1. Loss of *mir-51* family members suppress ASEL mis-specification in *lsy-6* mutants. (A, B) *mir-52* suppresses ASEL specification defects of *lsy-6(rf)/lsy-6(lf)* worms. (A) Cartoon of *lim-6::gfp* expression. *lim-6::gfp* is normally expressed in the ASEL, but remains off in the ASER. In *lsy-6lf* worms, *lim-6::gfp* is not expressed in the ASEL. A, anterior; P, posterior; L, left; R, right. (B, C) Worms of indicated genotypes were scored for *lim-6::gfp* expression in late larval and young adult stages, n ≥ 169. * indicates significant difference (χ^2, $p < 0.01$). All strains above the horizontal line in B and the horizontal line on the left in C are homozygous for *lsy-6rf* allele, with the strain represented by the dashed line being otherwise wild type. Strains above the right horizontal line in C are heterozygous for two alleles of *lsy-6*: *lsy-6rf* and *lsy-6lf*, with the strain represented by the dashed line being otherwise wild type.

4.3 *mir-51* family members genetically interact with *let-60/RAS* in vulva specification

Like neuronal cell fate specification, vulva cell fate specification is also regulated by miRNAs. The *let-7* family regulates *let-60/RAS* which plays an essential role in vulva development (Johnson et al., 2005). Each vulva precursor cell is sensitive in the levels of *let-60*. The primary (1˚) VPC has high levels of *let-60*, which promotes the formation of a mature vulva. Worms with a gain-of-function mutation in *let-60* often produce multiple vulvas (Muv) due to ectopically high LET-60 activity in cells normally fated to produce secondary (2˚) VPCs (Sternberg, 2005). Overexpression of *let-7* family members has been shown to partially suppress *let-60gf* Muv phenotype (Johnson et al., 2005). If the *mir-51* family negatively regulates miRNA biogenesis or activity, then loss of *mir-51* family members should suppress the *let-60gf* Muv phenotype, consistent with increased *let-7* family levels or activity.

Consistent with reduced *let-7* family activity, *alg-1* enhanced *let-60gf* Muv phenotype (Figure 4.2B). Consistent with enhanced *let-7* family activity, loss of *mir-54/55/56*, but not miRNA genes that were also found to suppress *alg-1* developmental timing defects (Table 2.1), *mir-238* or *mir-244*, suppressed the *alg-1*-dependent enhancement of *let-60gf.* 54% of *alg-1; let-60gf* worms display the Muv phenotype compared to 40% of *mir-54/55/56; alg-1; let-60gf* worms (Figure 4.2B). Interestingly, loss of *mir-54/55/56* enhanced the Muv phenotype of *let-60gf* (Figure 4.2C). This is not consistent with increased *let-7* family activity. In contrast, *mir-52* partially suppressed the multivulva phenotype of *let-60gf* worms

(Figure 4.2C), which is consistent with a role in microRNA activity. This suggests ectopic LET-60 levels are reduced in *mir-52; let-60gf* animals, whereas it suggests that LET-60 levels are increased in *mir-54/55/56; let-60gf* worms. This may reflect distinct activities of individual *mir-51* family members in the control of vulva development. The loss of *mir-52* resulting in suppression of the Muv phenotype of *let-60gf* worms is consistent with let-7 family activity being elevated in vulva precursor cells. This may indicate a broad role for *mir-52* in negatively regulating miRNA biogenesis or activity. Loss of *mir-54/55/56* resulting in enhancement of the Muv phenotype of *let-60gf* worms is not consistent with elevated let-7 family activity in vulva precursor cells. This may indicate that the *mir-51* family does not have a broad role in negatively regulating miRNA biogenesis or activity.

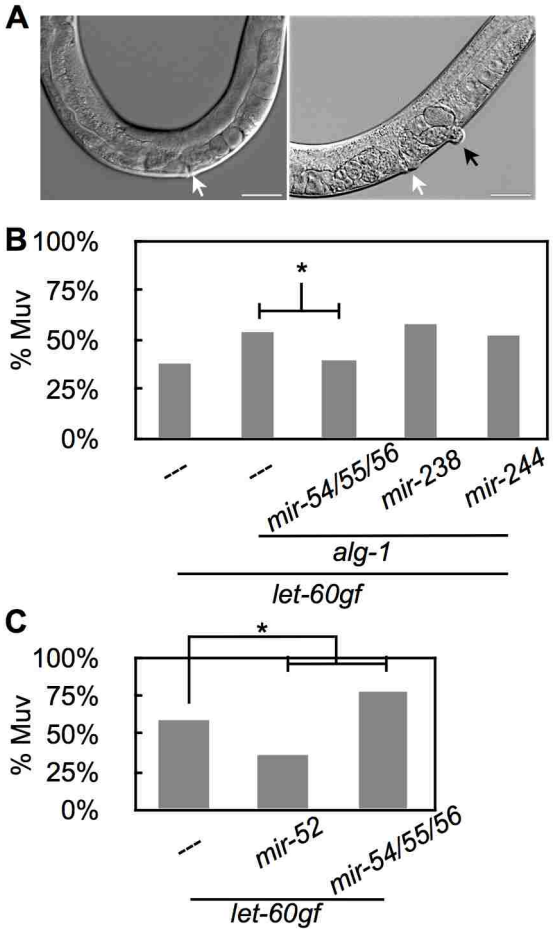


Figure 4.2. The *mir-51* family functions in vulva cell specification. (A) Left panel - A wild type worm with one normal vulva, white arrow. Right panel - A *let-60gf* worm with one normal vulva, white arrow, and one ectopic vulva, black arrow. Bars represent 100 µm. (B) *mir-54/55/56*, but not *mir-238* or *mir-244* suppress the multiple vulva (Muv) phenotype of *alg-1; let-60gf* worms. (C.) *mir-52* partially suppresses, while *mir-54/55/56* enhances the multivulva phenotype of *let-60gf* worms. For both B ad C, strains were maintained at 20˚ prior to synchronized L1 worms of the indicated genotype were allowed to develop at 25˚C for 2-3 days and then scored as young adults for presence of multiple vulva (Muv), n ≥ 100. * indicates significant difference (χ^2, $p < 0.01$). Strains above the top horizontal line in B are homozygous for *alg-1*, above the bottom horizontal line are homozygous for *let-60gf*, with strains represented by dashed lines being otherwise wild type. All strains in C are homozygous for *let-60gf*, with the strain represented by the dashed line being otherwise wild type.

4.4 *mir-52* genetically interacts with *mir-240/786* to regulate the defecation motor program

mir-240/786 is necessary for the normal rhythmicity of the defecation motor program (Miska et al., 2007). Worms initiate a defecation motor program, which comprises three events: a posterior body contraction, an anterior body contraction and an enteric muscle contraction resulting in an expulsion, approximately every 50 seconds (Thomas, 1990). In worms carrying the *n4541* mutation, which deletes both *mir-240* and *mir-786* sequences, defecation cycle time is dramatically increased and the time between cycles is irregular (Miska et al., 2007; Figure 4.3). Loss of *mir-52*, but not other members of the *mir-51* family, significantly reduced mean defecation cycle time of *mir-240/786* worms (Figure 4.3). The mechanism whereby loss of *mir-240/786* results in defecation cycle defects is unknown, but partial suppression of this phenotype by loss of *mir-52* may reflect a broader role for miR-52 in negatively regulating miRNA activity.

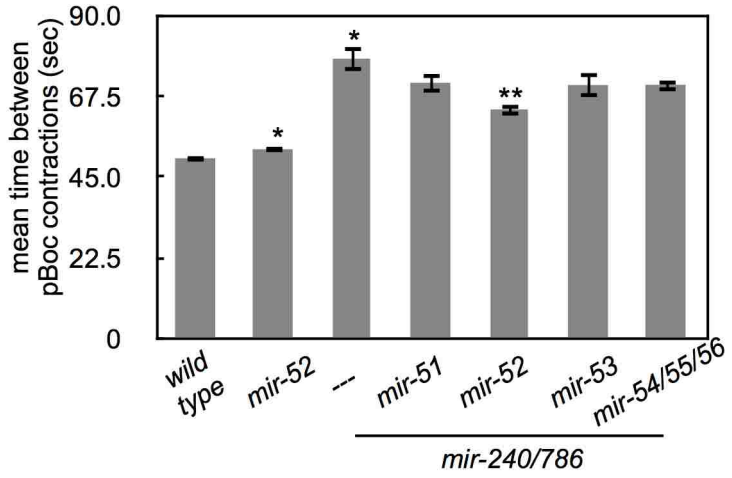


Figure 4.3. Loss of *mir-52* mildly suppresses defecation cycle defect of *mir-240/786* mutant worms. Graph represents mean time between consecutive pBoc contractions for n ≥ 5 worms. * indicates significant difference compared to wild type (student's t-test, $p < 0.01$). ** Indicates significant difference compared to *mir-240/786* mutants (student's t-test, $p < 0.01$). All strains above horizontal line are *mir-240/786*, with the strain represented by the dashed line being otherwise wild type. Error bars represent ± Standard Error of the Mean.

4.5 Loss of *mir-54/55/56* enhances embryonic lethality of *mir-35* family mutants

The *mir-35* family consists of *mir-35* through *mir-42*. The family members are redundantly required for embryonic development (Alvarez-Saavedra and Horvitz, 2010). Mutants lacking *mir-35* thru *mir-41* exhibit temperature sensitive embryonic lethality. Loss of *mir-54/55/56* did not significantly suppress the embryonic lethality phenotype of *mir-35/41* mutants, but rather enhanced this phenotype (Figure 4.4). This is not consistent with the activity of the remaining *mir-35* family member, *mir-42*, being elevated in the absence of *mir-51* family members.

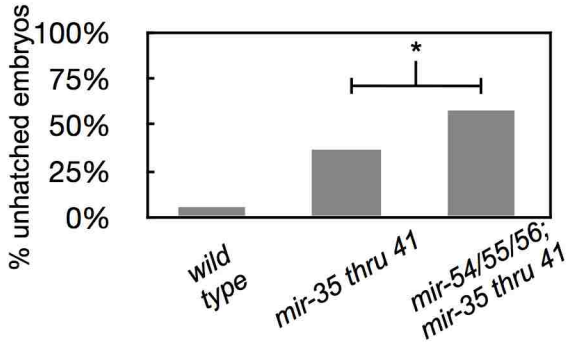


FIgure 4.4. Loss of *mir-54/55/56* enhances embryonic lethality of *mir-35* family mutant. *mir-54/55/56* enhances the embryonic lethality of *mir-35* thru *41* mutant worms. L4 worms of the indicated genotypes were shifted to 25˚ and the next day embryos from these worms were collected. After 24 hours, unhatched embryos were counted. Percent of unhatched out of total embryos plated are listed for each genotype scored (n > 148). * indicates significant difference (χ^2, p < 0.01).

4.6 *mir-52* modestly suppresses *mir-1* resistance to levamisole

mir-1 is necessary for normal neuromuscular function (Simon et al., 2008). *mir-1* mutants display a resistance to levamisole-induced paralysis due to an increase in levels of its targets, UNC-29 and UNC-63 (Simon et al., 2008). Loss of *mir-52* modestly increased the sensitivity of *mir-1* worms to levamisole. After 140 minutes on 200 µM levamisole *mir-52*; *mir-1* worms were slightly less resistant to levamisole-induced paralysis compared to *mir-1* mutant worms (Figure 4.5). In addition, *mir-52* worms are more sensitive to levamisole compared to wild type (Figure 4.5).

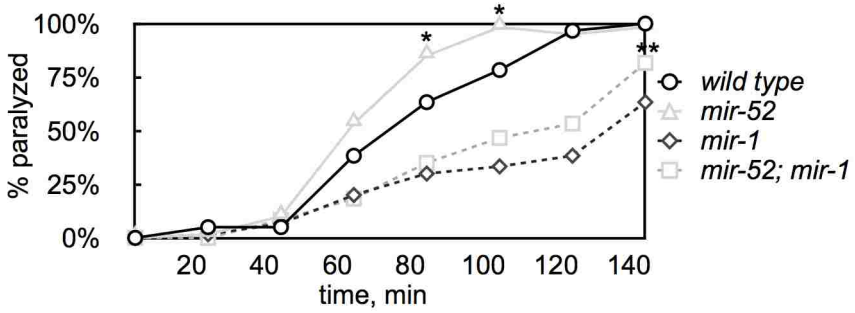


Figure 4.5. Loss of *mir-52* modestly suppresses *mir-1* mutant worm resistance to levamisole. *mir-52* modestly increases sensitivity to levamisole. Graph shows percent of total worms paralyzed after transfer to NGM plates supplemented 200 µM with levamisole. * indicates significant difference compared to wild type at the indicated time point (χ^2, $p < 0.05$). ** indicates significant difference compared to *mir-1* at the indicated time point (χ^2, $p < 0.05$).

4.7 Loss of *mir-51* family members does not effect mature miRNA levels

The observation that loss of *mir-52* suppressed multiple miRNA-dependent phenotypes in diverse pathways is consistent with the *mir-51* family acting broadly to regulate the miRNA pathway. Therefore, *mir-52* might act to broadly regulate miRNA biogenesis. To examine if the *mir-51* family regulates the miRNA pathway, levels of a set of miRNAs that display different expression and biogenesis characteristics were quantitated. Specifically, the levels of mature miRNAs for let-7, a developmentally-regulated miRNA that functions in the developmental timing pathway in the hypodermis (Reinhart et al., 2000), miR-58, a highly abundant miRNA (Kato et al., 2009a), miR-244, a miRNA that is expressed at lower levels primarily in hypodermal seam cells (Martinez et al., 2008b), and miR-62, a miRtron that displays Drosha independent biogenesis (Ruby et al., 2007), were examined. The mature levels of these miRNAs are unchanged in *mir-52* mutants as well as in mutant worms lacking 5 of 6 members of the *mir-51* family, *mir-52* through *mir-56*, *mir-52/53/54/55/56* (Figure 4.6). *mir-52/53/54/55/56* mutant worms display inpenetrant embryonic lethality, slow growth, and mating defects (Alvarez-Saavedra and Horvitz, 2010; Shaw et al., 2010) indicating that *mir-51* family targets are sufficiently misregulated to result in observed mutant phenotypes. However, no change in mature miRNA levels was observed. These results indicate that the *mir-51* family does not function to broadly regulate miRNA biogenesis.

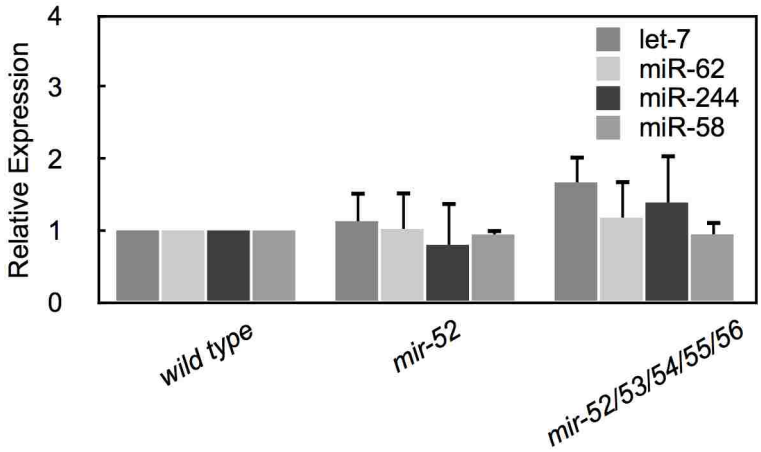


Figure 4.6. Loss of *mir-51* family members does not alter mature miRNA expression. Expression of let-7, miR-62, miR-244, and miR-58 in wild type, *mir-52*, and *mir-52/53/54/55/56* mutant worms relative to the average of two control RNAs, U18 and sn2343. The graph represents the level of mature miRNAs relative to wild type. No differences in mature miRNA expression was observed (student's t-test, $p > 0.24$).

4.8 Loss of *mir-52* has no effect on the ability of *lsy-6* to regulate its target, *cog-1*

Loss of *mir-51* family members had no obvious effect on the mature levels of a panel of miRNAs (Figure 4.6), suggesting the *mir-51* family does not act broadly to regulate miRNA biogenesis. Instead the *mir-51* family may act broadly to regulate miRNA activity. Previous analysis with the *hbl-1::gfp* reporter (Figure 3.1) is consistent with this model. However, this result cannot distinguish between the *mir-51* family acting broadly to regulate the miRNA pathway versus acting specifically to regulate the developmental timing pathway. To distinguish between these models and determine if the regulation of miRNA targets was affected by loss of *mir-52*, the activity of ectopically expressed lsy-6 in the repression of a *cog-1::gfp::cog-1* reporter (Johnston and Hobert, 2003) was examined. Ectopic expression of *lsy-6* under control of the *cog-1* promoter allows for examination of the activity of lsy-6 miRNA in cells where it is normally not found, including uterine and vulva cells (Johnston and Hobert, 2003). 40% of worms with ectopic expression of the *lsy-6* miRNA still show visible *cog-1::gfp* expression in uterine cells (Figure 4.7A-E). If *mir-52* negatively regulates miRNA activity, then loss of *mir-52* would result in fewer worms showing *cog-1::gfp* expression in uterine cells. However, loss of *mir-52* had no effect on the ability of ectopic *lsy-6* to downregulate expression of *cog-1* (Figure 4.7E). These data indicate that *lsy-6* activity is not enhanced in the absence of *mir-52*, thereby suggesting that the *mir-51* family does not function broadly to regulate the activity of miRNAs.

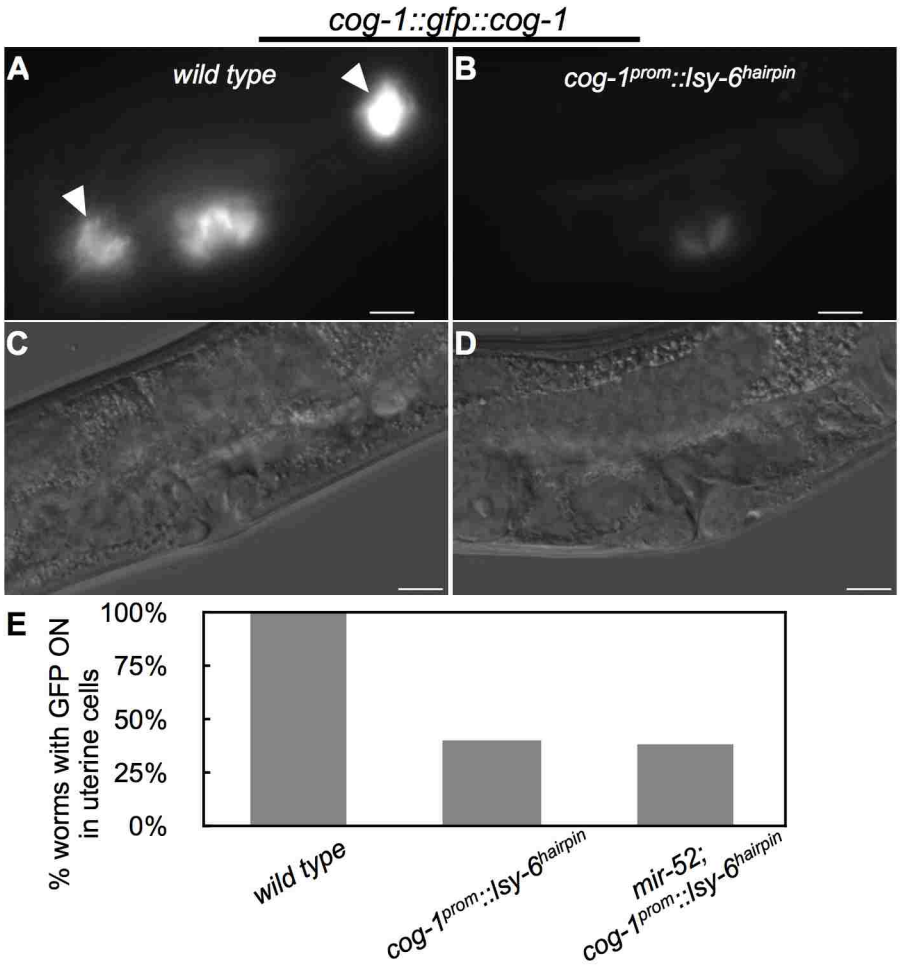


Figure 4.7. Loss of *mir-52* has no effect on the ability of lsy-6 to regulate its target, *cog-1*. (A-E) Effect of *mir-52* on *lsy-6* mediated regulation of *cog-1::gfp::cog-1* expression. Representative fluorescent image of *cog-1::gfp::cog-1* transgene expression in (A) wild type worms and (C.) worms with *cog-1::lsy-6* transgene with corresponding DIC images (B and D, respectively). White triangles point to uterine cells. Bars represent 10μm. (E) Percentage of worms of given genotype without *cog-1::gfp* expression in either uterine cell, n ≥ 20 (range 20 – 68). Worms were scored in mid-to-late L4 stage.

Chapter 5: Identification of miR-51 Family Targets

5.1 Identification of miR-51 family targets in developmental timing

The analysis from the previous two chapters indicates the *mir-51* family acts in broad developmental pathways in *C. elegans*. Evidence from the previous chapter also indicates that the *mir-51* family likely does not act to broadly regulate miRNA biogenesis or activity. Therefore the mechanism whereby the *mir-51* family acts in these broad developmental pathways remains unclear.

miRNAs typically act to down-regulate their direct targets. If the *mir-51* family mediates suppression of developmental timing defects through misregulation of a key target, then knocking down that target should result in loss of suppression.

In an attempt to identify a direct target or targets of the *mir-51* family, three different target prediction algorithms were used: Targetscan (version 4.2), PicTar, and mirWIP (Lall et al., 2006; Hammell et al., 2008; Friedman et al., 2009). This analysis collectively identified 319 genes that are predicted to be regulated by the *mir-51* family. Only those mRNAs that immunoprecipitated with the ALG-1 protein (Zisoulis et al., 2010) were selected. 127 of 319 predicted targets of the *mir-51* family contained ALG-1 binding sites (Zisoulis et al., 2010). Of those 127 candidates, 51 predicted targets were found to contain a perfect recognition site for the first six nucleotides of the *mir-51* family seed sequence within the ALG-1 binding site. To test whether these genes are downstream targets of the *mir-51* family, RNAi was used to knockdown the activity of these 51 candidates genes in

mir-52; mir-48/84/241 worms. In these worms, activity of key target genes is expected to be elevated due to the loss of miR-52. Their knockdown should therefore result in developmental timing defects similar to *mir-48/84/241* worms, indicating a loss of *mir-52* mediated suppression.

Four candidate target genes for the *mir-51* family were identified. Knockdown of these genes*, lin-66*, *vhp-1, cul-1*, and *tlp-1,* resulted in an increase in the number of *mir-52; mir-48/84/241* worms that display a bursting phenotype (Figure 5.1). The effect of RNAi knockdown of *lin-66*, *vhp-1, cul-1*, and *tlp-1* on seam cell divisions and alae formation in *mir-52; mir-48/84/241* worms was determined (Table 5.1).

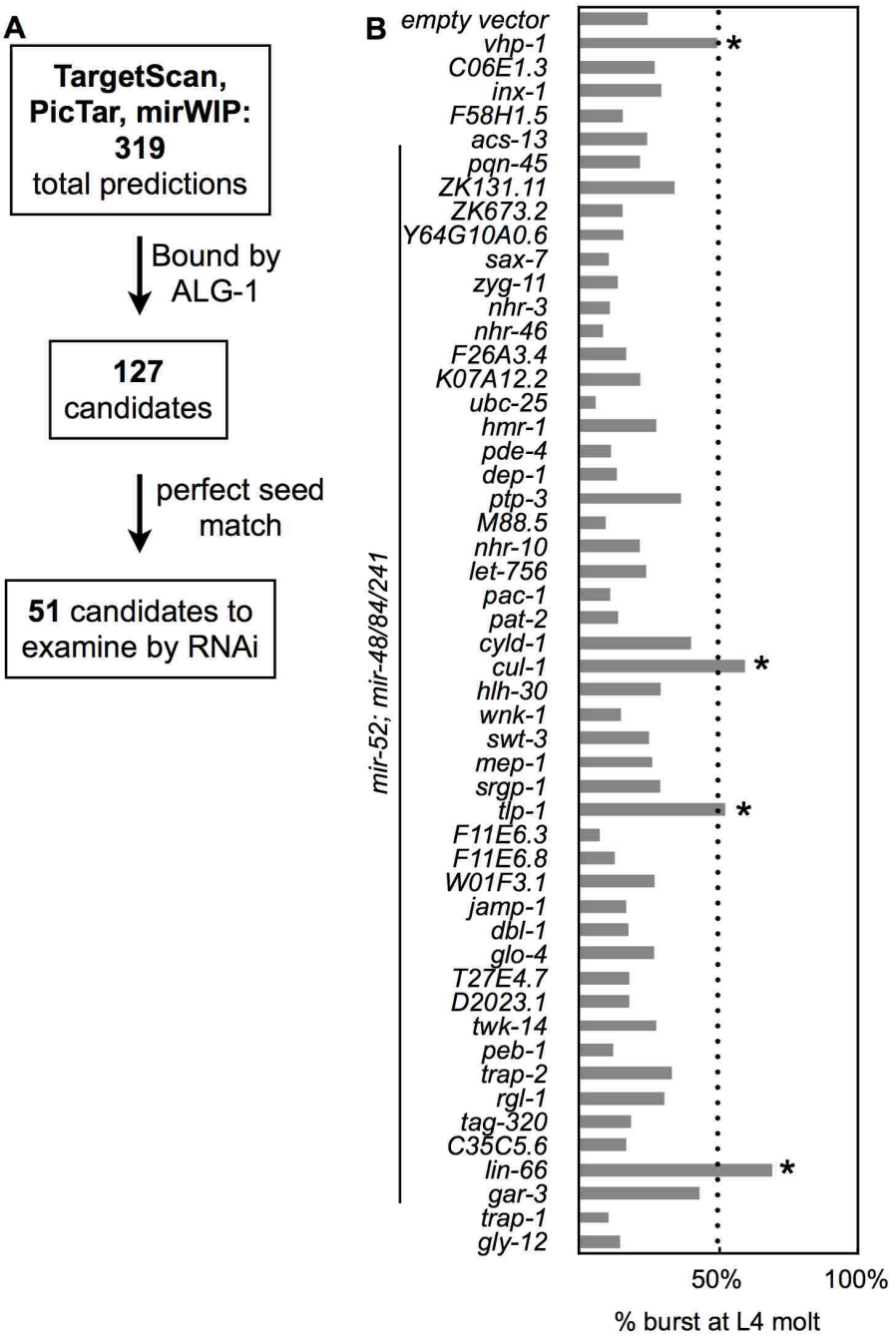


Figure 5.1. Identifying predicted miR-51 family targets using RNAi knockdown. (A) Schematic for narrowing target predictions to screen by RNAi. (B) Percent of *mir-52; mir-48/84/241* worms that burst at the L4 to adult transition following RNAi knockdown of 51 predicted miR-51 family targets , n ≥ 64. * marks the 4 RNAi clones that caused over 50% bursting as marked by the dashed line.

Table 5.1. Effect of RNAi knockdown of candidate miR-51 family targets on developmental timing.

				Alae[c]		
line	Strain[a]	RNAi	Seam[b]	Complete	Gapped	None
1	RF481 wild-type	*empty vector*	16.0	100%	0%	0%
2		*lin-66*	19.5*	53%	47%	0%
3		*vhp-1*	16.0	0%	22%	78%
4		*cul-1*	--[d]	36%	64%	0%
5		*tlp-1*	15.9	95%	5%	0%
6	RF551 *mir-52*	*empty vector*	16.0	100%	0%	0%
7		*lin-66*	17.4*	80%	20%	0%
8		*vhp-1*	16.1	6%	0%	94%
9		*cul-1*	--	57%	43%	0%
10		*tlp-1*	15.9	90%	10%	0%
11	RF554 *mir-48/84/241*	*empty vector*	21.4	0%	95%	5%
12		*lin-66*	25.7*	0%	40%	60%
13		*vhp-1*	22.0	0%	0%	100%
14		*cul-1*	--	0%	100%	0%
15		*tlp-1*	22.7	0%	70%	30%
16	RF556 *mir-52; mir-48/84/241*	*empty vector*	17.9	20%	80%	0%
17		*lin-66*	21.9*	21%	79%	0%
18		*vhp-1*	19.2*	0%	0%	100%
19		*cul-1*	--	15%	85%	0%
20		*tlp-1*	18.4	0%	100%	0%

[a] full genotype information can be found in Table S1.
[b] average number of GFP+ seam cells in L4 stage, n ≥ 12 (range 12 - 40).
[c] percentage of scored worms with adult alae at the L4 to adult transition, n ≥ 7 (range 7 - 40).
[d] unable to score worms accurately for seam cell number
* significantly different compared to empty vector control (student's t-test $p < 0.01$).

Knockdown of *lin-66*, *vhp-1* and *cul-1* in wild-type worms caused defects in alae formation (Table 5.1). Knockdown of *tlp-1* only rarely caused alae formation defects (Table 5.1). Knockdown of *lin-66* resulted in increased number of seam cells in wild-type worms, consistent with earlier work (Morita and Han, 2006). This indicates that *lin-66*, *vhp-1,* and *cul-1* activities are all required for developmental timing in hypodermal cells. Loss of *mir-52* suppressed the alae formation defects caused by knockdown of *lin-66* and *cul-1* (Table 5.1, lines 7 and 9) but not defects caused by knockdown of *vhp-1* nor *tlp-1* (Table 5.1, lines 8 and 10).

To determine if the activities of candidate targets were required for the observed suppression of *mir-48/84/241* developmental timing defects, the effect of RNAi of the four targets in *mir-48/84/241* and *mir-52; mir-48/84/241* was examined *(*Table 5.1, lines 11-20). If the suppression phenotype is due to elevated levels of targets, then knockdown of target activity would result in no suppression and therefore would result in similar defects in these two strains. Knockdown of *lin-66, vhp-1,* and *tlp-1* all enhanced the alae defects of *mir-48/84/241* worms while only knockdown of *lin-66* enhanced the extra seam cell defect (Table 5.1). Loss of *mir-52* suppressed the defects observed in *mir-48/84/241* following target knockdown for *cul-1, lin-66,* and *tlp-1.* Although loss of *mir-52* suppressed the extra seam cell defects, the alae formation defects following *vhp-1* were the same between *mir-48/84/241* and *mir-52; mir-48/84/241* worms (Table 5.1, compare lines 13 and 18), indicating that the suppression of alae defects by loss of *mir-52* requires *vhp-1* activity. The partial suppression of developmental timing defects in *mir-52; mir-48/84/241* following knockdown of *cul-1, lin-66,* and *tlp-1* may reflect the regulation of multiple targets by the *mir-51* family miRNAs or that these targets function in parallel to the *mir-51* family and are not direct targets.

The suppression of developmental timing phenotypes in *mir-52; mir-48/84/241* is reduced following knockdown of *lin-66*, *vhp-1, cul-1*, and *tlp-1* by RNAi, providing genetic evidence that these genes may be direct targets of the *mir-51* family. If so, then their regulation would be expected to require the putative *mir-51* family miRNA binding site in the 3’ UTRs of these genes. To test

this, worms that express *gfp-PEST* transgenes (Frand et al., 2005) under the control of the *col-10* promoter, followed by the sequence for the 3‘UTR of *lin-66*, *vhp-1, cul-1*, and *tlp-1*, were generated. The *col-10* promoter drives expression specifically in the hypodermis during embyronic and larval development. The *mir-51* family of miRNAs are expressed in multiple tissues throughout development, including the hypodermis (Shaw et al., 2010). Therefore, if *lin-66*, *vhp-1, cul-1*, and *tlp-1* are direct miRNA targets, then it would be expected to see a reduction of *gfp* expression compared to a control *col-10::gfp-PEST* trangene under the regulation of the *unc-54* 3’ UTR. In addition, this 3’ UTR dependent down-regulation would also require the predicted miR-51 family binding site. The reporters for *cul-1*, *lin-66* and *tlp-1* have reduced *gfp* expression relative to the *unc-54* control transgene (Figure 5.2A, C, G, K). The transgene regulated by the *vhp-1* 3’ UTR showed no detectable reduction compared to the *unc-54* control (Figure 5.2A, M), suggesting that *vhp-1* is not negatively regulated by its 3‘UTR in the hypodermis. If the reduction in GFP expression for the *lin-66*, *cul-1*, and *tlp-1* reporters is dependent on the presence of miR-52, then it is predicted that loss of *mir-52* would result in elevated GFP expression of these reporters. Consistent with this, the expression of the *cul-1* 3’ UTR transgene was modestly increased compared to wild type worms (Figure 5.2D). Although there appeared to be a modest difference in the expression of the *lin-66* 3’UTR transgene expression in the *mir-52* mutant background (Figure 5.2H), quantitative analysis of these worms indicated no significant difference between the mean expression of the *lin-66* 3’UTR transgene in wild type compared to *mir-52* mutant worms.

This indicates that *lin-66* is not significantly regulated by miR-52. GFP expression for the *tlp-1* reporter did not appear significantly different in *mir-52* mutant worms relative to wild-type worms (Figure 5.2L), indicating that *tlp-1* is not regulated by miR-52.

cul-1 contains a single predicted binding site for the *mir-51* family of miRNAs. To test if this binding site is necessary for the observed differences in *gfp* expression between wild type and *mir-52* mutant worms, a *gfp* reporter transgene that lacked the 6 nucleotides corresponding to the recognition sequence for the *mir-51* family seed sequence in the *cul-1 (cul-1*Δ) UTR was created. Expression of this reporter in both wild type and *mir-52* mutant worms was examined. The reporter under control of the *cul-1*Δ *3'*UTR was down-regulated compared to the *unc-54* control (Figure 5.2A, E), indicating additional regulatory sites present in the *cul-1 3'*UTR. However, there were no differences between the expression of the reporter under the control of the *cul-1*Δ *3'*UTR between wild-type and *mir-52* mutant worms (Figure 5.2E, F). This indicates the deleted nucleotides are necessary for miR-52 dependent regulation of the *cul-1* reporter.

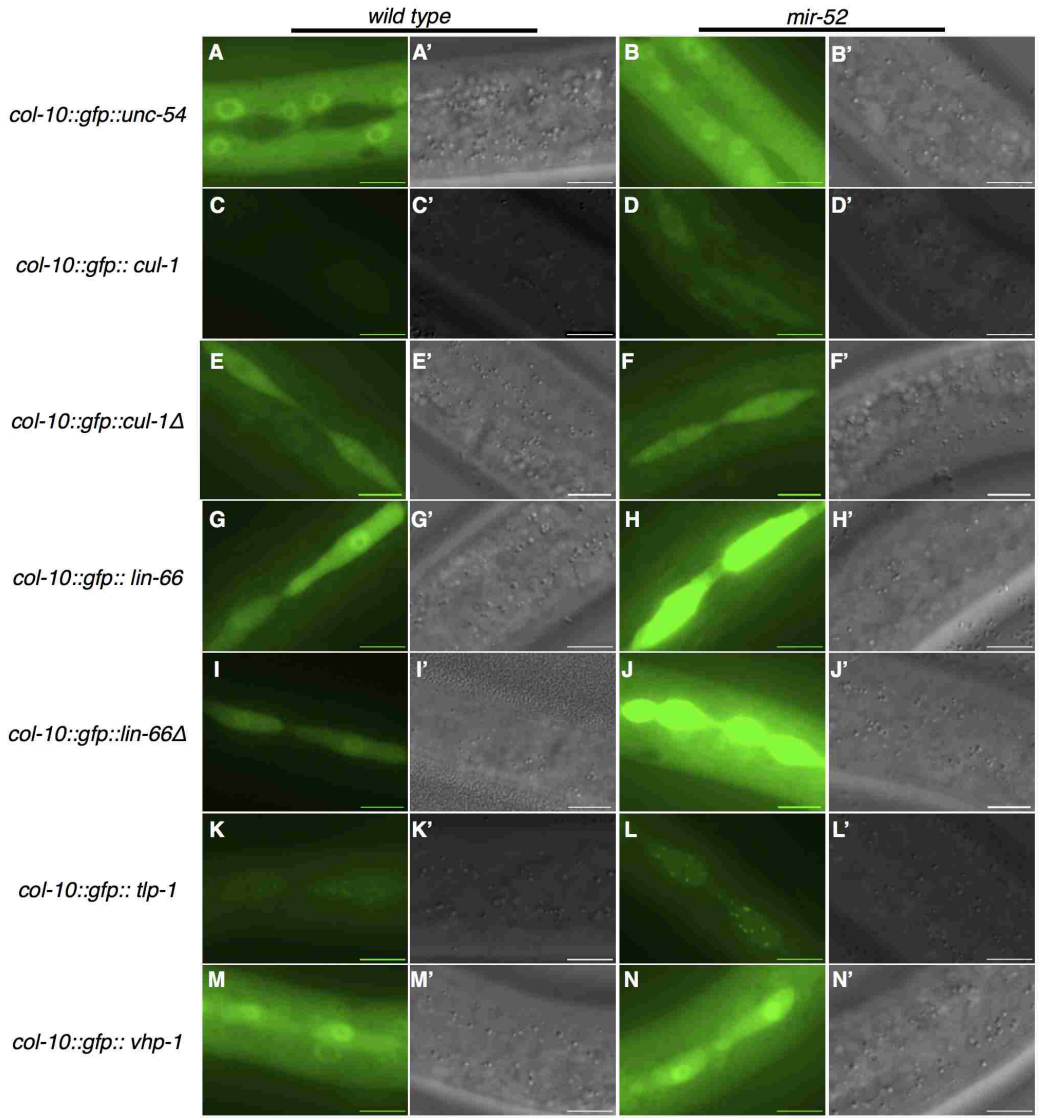


Figure 5.2. *3'utr* reporter transgene analysis of *cul-1*, *lin-66*, *tlp-1*, and *vhp-1*. Representative fluorescent images of L3 staged worms for wild type (A, C, E, G, I, K, M) with corresponding DIC images (A', C', E', G', I', K', M') and *mir-52* mutant worms (B, D, F, H, J, L, N) with corresponding DIC images (B', D', F', H', J', L', N'). All worms examined showed similar expression of *col-10::gfp::unc-54* in either wild type (A) or in *mir-52* (C). Images in A and C taken with a 10 ms exposure time. 86% of wild type worms had *col-10::gfp::cul-1* expression similar as in (C), n = 7. 86% of *mir-52* worms had *col-10::gfp::cul-1* expression as in (D), n = 7. Images of C and D taken with a 50 ms exposure time. 56% of wild type worms showed *col-10::gfp::cul-1Δ* expression as in (E), n = 9. 75% of *mir-52* mutant worms showed *col-10::gfp::cul-1Δ* as in (F), n = 8. Images in E and F were taken with a 100 ms exposure time. 38% of wild type worms showed *col-10::gfp::lin-66* expression as in (G), n = 8. 43% of *mir-52* worms showed *col-10::gfp::lin-66* expression as in (H), n = 7. Images of G and H taken with a 50 ms exposure time. 80% of wild type worms showed *col-10::gfp::lin-66Δ* expression as in (I), n = 6. 83% of *mir-52* worms showed *col-10::gfp::lin-66Δ* expression as in (J), n = 6. Images in I and J were taken with a 10 ms exposure time.80% of wild types worms showed *col-10::gfp::tlp-1* expression as in (K), n = 5. 86% of *mir-52* worms showed *col-10::gfp::tlp-1* expression as in (L), n = 7. Images of M and O taken with a 40 ms exposure time. All worms examined showed similar expression of *col-10::gfp::vhp-1* in either wild type (M) or *mir-52* worms (N). Images in M and N taken with a 50 ms exposure time. All DIC images (A'-N') taken with a 3 ms exposure time. Bars represent 10 µm.

The effect of deleting the 6 nucleotide complementary site for the miR-51 family in the UTR of *lin-66* was also examined in both wild type and *mir-52* mutant worms. GFP expression from the reporter under the control of the *lin-66*Δ 3'UTR was down-regulated relative to the *unc-54* control (Figure 5.2A, I). Surprisingly, *gfp* reporter expression under the control of the *lin-66*Δ *3'*UTR was found to be elevated in *mir-52* mutant worms compared to wild type worms (Figure 5.2I, J). This indicates that the deleted nucleotides may allow miR-52 to regulate the *lin-66* UTR. The sequence does not contain other recognition sites for the miR-52 seed sequence, which may indicate that this effect is indirect.

Since miRNAs can function to promote the degradation of target transcripts (Bartel, 2009), mRNA levels for *cul-1*, *lin-66*, *tlp-1*, and *vhp-1* in wild type, *mir-52*, and *mir-52/53/54/55/56* mutant worms were measured using quantitative RT-PCR. *vhp-1* levels were significantly increased in *mir-52/53/54/55/56* mutants, while *cul-1*, and *lin-66*, transcript levels remained unchanged (Figure 5.3). *tlp-1* transcript levels were undetected in wild type, or in either *mir-52*, or *mir-52/53/54/55/56* mutant worms (data not shown). The increase in *vhp-1* transcript levels is consistent with *vhp-1* being negatively regulated by the *mir-51* family, though this regulation was not observed using *gfp* reporter transgenes (Figure 5.2M, N).

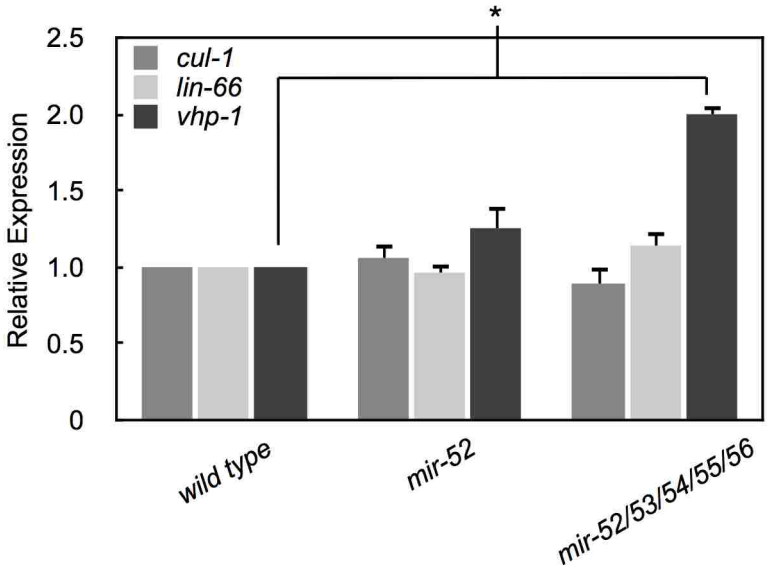
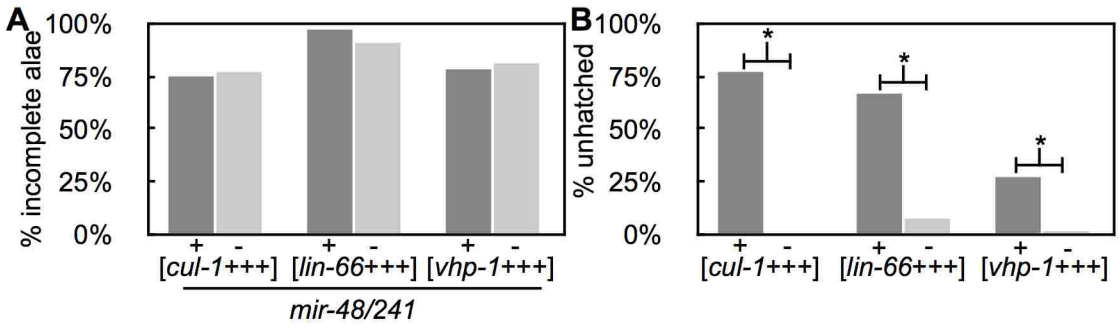


Figure 5.3. *vhp-1* transcript level is elevated in the absence of multiple *mir-51* family members. Expression of *cul-1*, *lin-66*, and *vhp-1* in wild type, *mir-52*, and *mir-52/53/54/55/56* mutant worms relative to the average of two control RNAs, *ama-1* and *cdc-42*. The graph represents fold change in expression relative to wild type. Error bars represent the standard deviation for fold change calculated between two biological replicates. * indicates significant difference (student's t-test, $p < 0.05$).

5.2 Overexpression of *lin-66*, *cul-1*, *vhp-1* is not sufficient to suppress retarded developmental timing defects

Combined genetic, reporter *gfp*, and quantitative RT-PCR data does not clearly indicate that *cul-1*, *lin-66*, or *vhp-1* are the relevant target of miR-52 in developmental timing. If indeed one of these genes is a relevant target of miR-52 in developmental timing, then it is predicted that up-regulation of one of these targets could suppress retarded developmental timing phenotypes similar to the loss of *mir-52*. To test this, transgenes containing the coding sequences for *cul-1*, *lin-66*, and *vhp-1* were generated. Expression of *cul-1*, *lin-66*, and *vhp-1* from these arrays should result in elevated expression relative to wild type. The effect of these transgene arrays on the retarded development of worms lacking *mir-48*

and *mir-241* (*mir-48/241*) was examined. Expression of *cul-1*, *lin-66*, or *vhp-1* from a transgene array had no effect on the formation of alae of *mir-48/241* mutant worms (Figure 5.4A). This indicates that expression of these genes individually from high-copy transgenes is not sufficient to suppress retarded developmental timing phenotypes. Intriguingly, both *mir-48/241* mutant and wild-type worms carrying these arrays often displayed pleiotropic defects, including embryonic lethality and body morphology defects (Figure 5.4B-F). This suggests that expression levels of *cul-1*, *lin-66*, and *vhp-1* are significantly increased in these transgene-containing worms to generate abnormal phenotypes. Therefore it is unlikely that overexpression of any one of these genes individually is responsible for suppressed developmental timing phenotypes of *mir-52; mir-48/241*.



C

	post-embryonic phenotypes			
	larval lethal	slow growth	gross body morphology	wild type
[*cul-1*+++]	13%	20%	3%	63%
[*lin-66*+++]	19%	2%	2%	77%
[*vhp-1*+++]	2%	0%	14%	84%

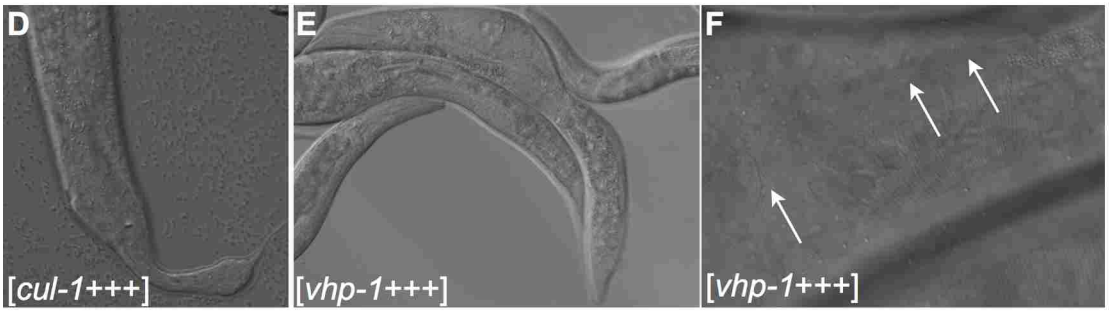


Figure 5.4. Overexpression of *cul-1*, *lin-66*, or *vhp-1* does not suppress developmental timing defects, but can cause pleiotropic defects. (A) Percent of worms with incomplete alae at the L4 to adult transition in *mir-48/241* mutant worms that carry the corresponding array, +, or do not carry the array, -, as determined by expression of *sur-5::gfp*, n ≥ 16 (range 16 – 37). There is no difference in alae formation between worms that carry the array compared to worms that do not (χ^2, $p > 0.05$) (B) Percent of unhatched embryos in wild type worms that carry the corresponding array, +, or do not carry the array, -, as determined by the expression of *sur-5::gfp*, n ≥ 60 (range 60 – 166). * Indicates a significant difference between worms that carry the array compared to worms that do not (χ^2, $p < 0.05$). (C) Quantification of post-embryonic defects observed in worms carrying the corresponding array as determined by expression of *sur-5::gfp*, n ≥ 30 (range 30 – 105). Worms were assessed as slow growth if stage was younger than the 4th larval stage three days after hatching. Any worm with abnormal body shape were assessed as having gross body morphology defects. (D-F) Representative DIC images of worms with gross body morphology defects overexpressing *cul-1* (D) or *vhp-1* (E, F). White arrows in F point to branchpoints in the cuticle structure called alae.

5.3 *mir-52* regulates distinct target sets in multiple miRNA-regulated developmental pathways

RNAi knockdown was used to determine if a candidate set of predicted *mir-51* family targets were required for the suppression of developmental timing defects by the loss of *mir-51* family members. Four genes, *lin-66*, *vhp-1, cul-1*, and *tlp-1* appeared to be required, in part, for the *mir-52*-mediated suppression of *mir-48/84/241* developmental timing defects. It is also possible that one or all of these predicted targets could also be required for the *mir-52*-mediated suppression of other miRNA dependent phenotypes. To test, RNAi was used to knockdown the levels of *lin-66*, *vhp-1, cul-1*, and *tlp-1* in *mir-52; let-60gf* worms (Figure 5.5A). *cul-1* activity is required for the observed suppression of the Muv phenotype in *mir-52; let-60(ga89ts)* worms. *mir-52; let-60gf; cul-1(RNAi)* worms displayed a stronger Muv phenotype than *mir-52; let-60gf* worms on empty vector control RNAi. This supports that *cul-1* acts downstream or in parallel of *mir-52* to regulate vulva cell fate specification. Interestingly, RNAi knockdown of *vhp-1* further suppressed the Muv phenotype of *mir-52; let-60gf* worms (Figure 5.5A). This suggests a more complex role for *vhp-1* in the vulva specification pathway. RNAi knockdown of *lin-66,* or *tlp-1* had no effect on the vulva specification pathway (Figure 5.5A).

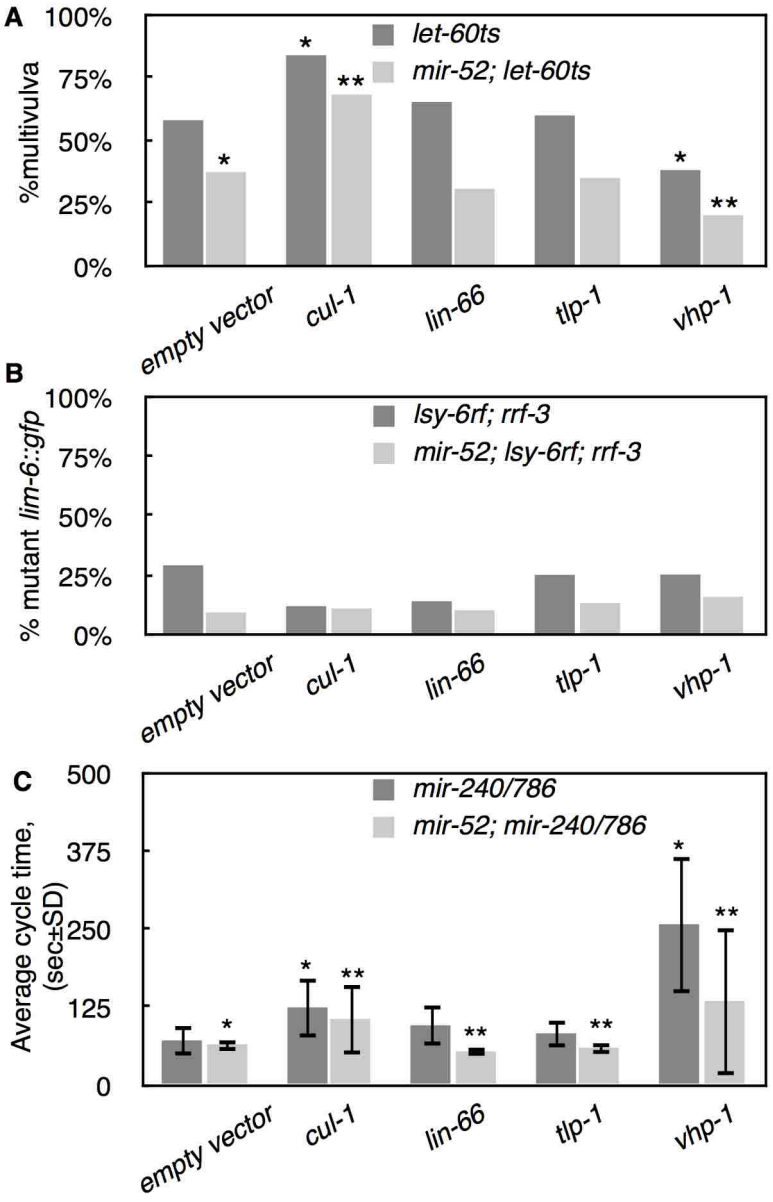


Figure 5.5. RNAi of *cul-1*, *lin-66*, *tlp-1*, and *vhp-1* in developmental pathways regulated by *mir-52*. (A) Percent of *let-60ts* and *mir-52; let-60ts* worms with multiple vulva (multivulva), after RNAi knockdown of *cul-1*, *lin-66*, *tlp-1*, and *vhp*-1, along with empty vector RNAi control, n ≥ 25 (range 25 – 163). * Indicates significant difference compared to *let-60ts* on empty vector control (χ^2, $p < 0.01$). **Indicates significant difference compared to *mir-52; let-60ts* on empty vector control (χ^2, $p < 0.01$). (B) Percent of *lsy-6rf* (reduced function)*; rrf-3* and *mir-52; lsy-6rf; rrf-3* worms with mutant *lim-6::gfp* expression following knockdown of *cul-1*, *lin-66*, *tlp-1*, and *vhp*-1 with empty vector RNAi control, n ≥ 82 (range 82 – 382). (C) Average cycle time between pBoc contractions of *mir-240/786* and *mir-52; mir-240/786* worms following RNAi knockdown of *cul-1*, *lin-66*, *tlp-1*, and *vhp*-1 with empty vector RNAi control, n ≥ 6, minimum 3 cycles per worm. *Indicates significant increase compared to *mir-240/786* on empty vector control (student's t-test, $p < 0.001$). **Indicates significant increase compared to *mir-52; mir-240/786* on empty vector control (student's t-test, $p < 0.00001$). SD, standard deviation.

Next RNAi was used to knockdown *lin-66*, *vhp-1, cul-1*, and *tlp-1* expression in *mir-52; lsy-6rf; rrf-3* worms. An *rrf-3* loss of function mutation was used to increase the effectiveness of RNAi in neurons (Simmer et al., 2002). While 29% of *lsy-6rf; rrf-3* worms displayed a mutant *lim-6::gfp* expression pattern with a failure of ASEL specification, only 9% of *mir-52; lsy-6rf; rrf-3* worms showed this mutant expression pattern, which is unchanged following knockdown of *lin-66*, *vhp-1, cul-1*, and *tlp-1* (Figure 5.5B).

Lastly RNAi was used to knockdown *lin-66*, *vhp-1, cul-1*, and *tlp-1* in *mir-52; mir-240/786* worms to determine if any of these genes are required for the *mir-52* mediated suppression of *mir-240 mir-786* defecation defects. The suppression of the *mir-240/786* defecation phenotype required *vhp-1* and *cul-1* activity. Knockdown of *vhp-1* and *cul-1* significantly increased the average defecation cycle time of *mir-52; mir-240/786* worms (Figure 5.5C). Knockdown of *tlp-1* or *lin-66* reduced the average defecation cycle time of *mir-52; mir-240/786* worms (Figure 5.5C).

Chapter 6: Discussion

6.1 Overview

The goal of this research was to identify developmental pathways regulated by conserved or developmentally regulated miRNAs in *Caenorhabditis elegans*. To accomplish this, strains were generated that were homozygous for individual miRNA deletion alleles and *alg-1(gk214)*. Although worms mutant for individual miRNA genes have no obvious developmental abnormalities (Miska et al., 2007), 80% of the *mir; alg-1* worms display observable phenotypic differences compared to *alg-1* single mutants. This analysis identified phenotypes attributable to loss of individual miRNA genes for 25 of the 31 miRNAs examined. Furthermore, this suggests roles for these miRNAs in developmental timing, embryonic development and gonad migration.

Among the observations of the *mir; alg-1* worms was that loss of *mir-51* family members suppressed developmental timing defects of *alg-1* worms. Further genetic analysis of the *mir-51* family revealed that this family functions upstream of *hbl-1* to regulate execution of the L3 stage program in developmental timing. Since the *mir-51* family displays broad and abundant expression (Lim et al., 2003; Ruby et al., 2006; Kato et al., 2009a; Shaw et al., 2010), it is possible that this family may regulate pathways other than developmental timing *mir-51* family members genetically interacted in diverse, miRNA-dependent developmental pathways in *C. elegans*, including specification of neuronal asymmetry, specification of vulval cell fate, the defecation motor

program, and synaptic activity. Loss of *mir-51* family members had no effect on the mature levels of let-7, miR-58, miR-62, or miR-244, and loss of *mir-52* had no effect on lsy-6 regulation of its target in uterine cells, indicating that the *mir-51* family likely does not regulate these broad pathways through a common mechanism of regulating miRNA biogenesis or activity. Instead, it is likely that *mir-51* family regulates distinct targets in diverse developmental pathways.

6.2 Use of *alg-1* as a sensitized genetic background to reveal miRNA function.

The *alg-1(gk214)* allele was used as a genetically sensitized background to reveal the functions of individual miRNA genes. 25 of the 31 miRNAs analyzed in this background resulted in quantifiable developmental phenotypes. This supports that these 25 miRNAs act in pathways that regulate developmental programs including embryogenesis, developmental timing, and gonad morphogenesis. This study identified a limited number of phenotypes attributed to loss of miRNA genes, which may indicate that this background is only sensitive to miRNA loss in a subset of developmental pathways. Further reduction of miRNA activity by knocking down *alg-2*, which encodes an Argonaute that also functions in the miRNA pathway, may increase the sensitivity of this background. However, loss of *alg-1* and *alg-2* results in embryonic lethality (Grishok et al., 2001). Further analysis is needed to specify how these miRNAs regulate different developmental programs.

Interestingly, this analysis indicates that miRNAs may act together to regulate these different developmental pathways. One model is that these

miRNAs regulate overlapping targets in a developmental pathway. For example, *mir-1*, *mir-124*, and *mir-259* were each found to enhance the gonad migration defects of *alg-1*. By Targetscan (Lewis et al., 2005; Jan et al., 2011), each share a predicted target, *lin-26*. *lin-26* encodes a zinc-finger transcription factor that is necessary to specify epidermal cell fates in *C. elegans* (Labouesse et al., 1994), but a role in gonad morphogenesis is unknown. Therefore, Targetscan and additional prediction algorithms provide a platform to identify shared targets of these miRNAs to assess if misregulation of these shared targets leads to a similar phenotypic result. Alternatively, these miRNAs could regulate distinct targets that converge in a developmental pathway.

The developmental timing pathway is strongly compromised in the *alg-1* mutant background. 17 of the miRNAs analyzed in this study were found to significantly enhance or suppress the developmental timing phenotypes of *alg-1* mutant worms. Surprisingly, since loss of a subset of individual miRNA genes resulted in suppression of *alg-1* developmental timing defects, then these miRNAs may act in opposition to the *let-7* family in developmental timing.

The mechanism whereby loss of miRNAs results in suppression of *alg-1* is unknown. One model is that these miRNAs regulate specific components of the timing pathway. For example, *mir-238* is predicted, by Targetscan (Lewis et al., 2005; Jan et al., 2011), to bind known developmental timing genes *daf-12* (Antebi et al., 1998) and *lin-29* (Rougvie and Ambros, 1995). Alternatively, these miRNAs could regulate specific targets in the miRNA pathway. In this model, loss of miRNA regulation of miRNA pathway genes results in elevated miRISC activity.

In support of this, *alg-2* is a predicted target of *mir-228* and *mir-259*. It is also possible that the observed suppression is due to a more general effect on miRISC availability. In the absence of abundantly expressed miRNAs, such as *mir-52* (Lim et al., 2003; Ruby et al., 2006; Kato et al., 2009a), limiting miRISC can be utilized by the remaining miRNAs, such as the let-7 family. However, it does not account for the observed suppression of all *mir; alg-1* worms, as loss of some abundantly expressed miRNAs did not result in suppression in all cases. Also, strong suppression for miRNAs that are relatively weakly expressed, such as *mir-238* and *mir-259* (Ruby et al., 2006), was observed.

The use of the *alg-1* genetic background revealed functions for individual miRNAs, and also suggested that unrelated miRNAs may regulate common pathways. The ability for unrelated miRNAs to regulate common targets or pathways may explain the lack of developmental phenotypes in worms lacking individual miRNA genes (Miska et al., 2007) or whole miRNA families (Alvarez-Saavedra and Horvitz, 2010). The use of the *alg-1* background can be expanded to additional miRNAs not included in this study. In addition to using genetically sensitized backgrounds, environmental stresses can reveal individual miRNA function (Kato et al., 2009b; de Lencastre et al., 2010). Alternatively, genetic backgrounds with more cell-specific phenotypes can be used. For example, as is discussed in more detail later, worms homozygous for the *ga89* gain-of-function allele of *let-60/RAS* display a weakly penetrant multiple vulva phenotype, which is sensitive to loss of *mir-51* family members. Mutations in genes involved in the Ras pathway, such as *gap-1*, which encodes a G-protein activating protein that

activates the GTPase domain of LET-60/RAS, affect the penetrance of the multivulva phenotype of *let-60(ga89)*, while not displaying a multivulva phenotype in an otherwise wild type background (Eisenmann and Kim, 1997). Since a primary function for miRNAs may be to confer robustness to developmental programs (Hornstein and Shomron, 2006), then miRNA function might be revealed under conditions of environmental or genetic fluctuation, such as the *let-60(ga89)* mutant background.

A broad analysis of all individual miRNAs may prove exhaustive for analysis in a specific genetic background such as *let-60*. Previous analysis of the spatial expression pattern of individual miRNAs (Martinez et al., 2008b) can assist in chosing individual miRNAs to examine in specific genetic backgrounds. For example, a *gfp* reporter for *mir-235* shows expression in vulva cells (Martinez et al., 2008b). *mir-235* and other miRNAs that are expressed in the vulva could be examined in the *let-60(ga89)* mutant background for an effect on vulva cell fate specification. Genetic examination of individual miRNAs that display spatially restricted expression patterns will allow for further placement of individual miRNAs in developmental pathways and assist in identification of their individual functions.

6.3 The *mir-51* family regulates diverse developmental pathways in *C. elegans*

The goal of the analysis presented in Chapter 3 and Chapter 4 was to identify a mechanism whereby loss of *mir-51* family members suppressed developmental timing phenotypes of *alg-1* mutant worms. Genetic data indicated that the *mir-51* family acts to oppose the execution of the L2 to L3 transition in developmental timing (Figure 5.1). Loss of *mir-51* family members suppressed the retarded developmental timing defects of worms lacking genes that regulate early timing events: *mir-48, mir-84*, and *mir-241* and *lin-46*. However loss of *mir-51* family members had no effect on retarded developmental timing defects of later acting timing genes, *let-7* and *puf-9*. Furthermore, loss of *mir-51* family members enhanced the precocious developmental timing defects for genes necessary for early fates: *hbl-1*, *lin-14*, and *mir-48(ve33)*. However loss of *mir-51* family members had no effect on the later acting *lin-41*. Lastly, misexpression of *hbl-1* in *mir-48/84/241* is suppressed by loss of *mir-51* family members. This indicates the L2 to L3 developmental timing program is the most sensitive to loss of *mir-51* family members.

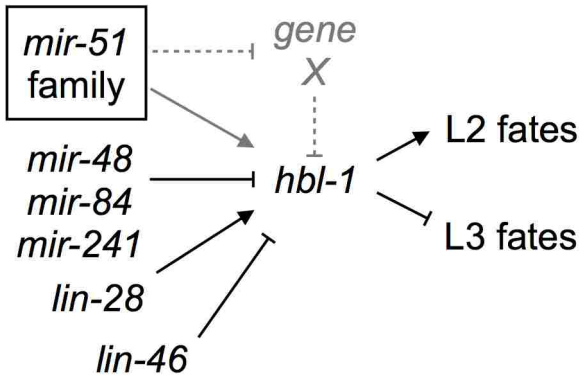


Figure 6.1 Genetic Model for *mir-51* family in developmental timing. Lines in gray represent the proposed genetic role for the *mir-51* family in developmental timing.

The *mir-51* family members are atypical developmental timing genes. First, the *mir-51* family is broadly and abundantly expressed throughout development (Lim et al., 2003; Kim, 2005; Ruby et al., 2006; Kato et al., 2009a; Shaw et al., 2010). In contrast, *lin-4* and *let-7* show developmentally regulated expression (Wightman et al., 1993; Reinhart et al., 2000; Lee et al., 2004a). Second, mutant worms lacking individual or multiple members of the *mir-51* family do not display developmental timing defects (Table 3.1 & 3.2; Alvarez-Saavedra and Horvitz, 2010). Therefore, the *mir-51* family miRNAs are not themselves developmental timing genes, but instead they may act in the execution phase of the larval transition.

Surprisingly, *mir-51* family members genetically interacted with additional miRNA genes that have broad individual functions. These miRNAs regulate diverse pathways including neuronal asymmetry, *let-60/RAS*-dependent vulval cell specification, the defecation motor program, and synaptic activity. Loss of the *mir-51* family member, *mir-52*, resulted in partial suppression of neuronal fate

defects of *lsy-6rf/lsy-6lf* worms, vulval specification defects of *let-60gf* worms, defecation cycle defects of *mir-240/786* worms, and levamisole resistance of *mir-1* worms. In contrast, other miRNA genes that were also found to suppress developmental timing defects of *alg-1*, *mir-238* and *mir-244*, had no effect on neuronal fate defects or vulval specification defects. The ability of *mir-52* to genetically interact in diverse pathways could reflect regulation of an individual target that functions broadly, or regulation of multiple, pathway specific targets. A direct miR-51 family target in developmental timing was not conclusively identified. Identification of direct miR-51 family targets will distinguish between these models.

An alternative model to explain the observed suppression of miRNA-dependent phenotypes by loss of *mir-52* is that loss of this abundant miRNA (Lim et al., 2003; Ruby et al., 2006; Kato et al., 2009a) frees up a limited pool of available miRISC. In this model, miRNAs compete for a limited pool of available miRISC. When an abundant miRNA is lost, then other miRNAs are able to utilize the available miRISC to regulate their targets. Suppression of mutant phenotypes in *alg-1* by loss of the abundantly expressed *mir-52* fits with this model. In genetic backgrounds where miRISC components are lost, such as in *alg-1*, miRISC is limiting. miRNA precursors accumulate in worms with reduced *alg-1* (Grishok et al., 2001; Lund et al., 2004) or in human cells with reduced Argonautes (Diederichs and Haber, 2007). Increased expression of human Argonaute is capable of increasing the levels of exogenously expressed miRNAs (Diederichs and Haber, 2007). Also consistent with limited RISC availability in human cells,

siRNA transfection results in elevation of miRNA targets (Khan et al., 2009). It is unknown if miRISC is limiting in a wild type worm. miRNA precursors are generally low in abundance in wild type worms for most miRNAs (Grishok et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). The presence of these miRNA precursors could indicate competition for miRISC loading of these precursors, or could indicate the steady-state level of miRNA precursor during miRNA biogenesis. If this model were true, then it might be expected that mature miRNA levels would be elevated in the absence of an abundant miRNA. However, the mature miRNA levels of let-7, miR-58, miR-62, and miR-244 are unchanged in *mir-52* or in *mir-52/53/54/55/56* worms. Additionally, if this model were true, then it is predicted that miRNA activity would be elevated in the absence of *mir-52*. However, loss of *mir-52* had no effect on the ability of ectopic *lsy-6* to regulate its target, *cog-1* in uterine cells. Neither of these results is consistent with loss of *mir-52* mediating suppression via freeing up miRISC. This also suggests that miRISC is not limited in wild type backgrounds. Further examination of miRISC competition and availability in wild type backgrounds is needed to determine if miRISC availability may contribute to *mir-51* family-dependent phenotypes.

An alternative model to explain a role for the *mir-51* family in diverse developmental pathways is that this family negatively regulates a gene that acts to promote miRNA biogenesis or activity. No change in mature miRNA levels between wild type and *mir-52* or *mir-52/53/54/55/56* worms suggests that miRNA biogenesis is unchanged in the absence of *mir-51* family members (as discussed above). Furthermore, loss of *mir-52* had no effect on ectopic *lsy-6* activity in

uterine cells, suggesting the *mir-51* family does not broadly regulate miRNA biogenesis or activity. It remains possible that the *mir-51* family regulates miRNA biogenesis and/or activity in a cell-specific fashion. Identification of direct targets of the *mir-51* family may reveal if the *mir-51* acts in specific pathways to regulate miRNA biogenesis or activity.

Since evidence provided in this study is inconsistent with the *mir-51* family functioning broadly through regulation of miRNA biogenesis or activity, it is possible that the *mir-51* family regulates many, distinct targets in diverse developmental pathways in *C. elegans*. Identification of direct targets of the miR-51 family will be key to testing this model.

6.4 The *mir-51* family in vulval cell specification

One unexpected finding in this study is that loss of *mir-52* suppressed the multivulva phenotype of *let-60(ga89gf)*, while loss of *mir-54/55/56* enhanced the multivulva phenotype of *let-60(ga89gf)*. These opposing interactions could indicate distinct activities for members of the *mir-51* family members in vulva cell fate specification. The interaction between *mir-51* family members and *let-60* was examined since *let-60* is a *let-7* family target and that *let-60(ga89gf)* multivulva phenotype is sensitive to *let-7* family expression (Johnson et al., 2005) and miRNA activity (Hammell et al., 2009). The loss of *mir-52* suppressing *let-60(ga89gf)* is consistent with elevated *let-7* family activity, while loss of *mir-54/55/56* suppressing *let-60(ga89gf)* is not. The multivulva phenotype in *let-60(ga89gf)* worms is due to ectopically high LET-60 activity in cells that were not

induced to adopt the primary (1˚) Vulva precursor cell fate (VPC). Activation of EGF/Ras/MAP kinase pathway is critical for specification of the 1˚ VPC and likely coordinates the lateral signal received by cells neighboring the 1˚ VPC, called the secondary (2˚) VPCs (Sternberg, 2005). LIN-12 activity is elevated in the 2˚ VPCs and *lin-12* is both necessary and sufficient for 2˚ VPC specification (Sternberg, 2005). Interestingly, *lin-12* contains two predicted binding sites for the *mir-51* family according to Targetscan (Lewis et al., 2005; Jan et al., 2011). The observation that loss of *mir-52* partially suppressed *let-60(ga89gf)* multivulva phenotype is consistent with elevated LIN-12 preventing formation of ectopic vulva in 2˚ VPCs. If *lin-12* is a target of the *mir-51* family, then why does loss of *mir-54/55/56* enhance the multivulva phenotype of *let-60(ga89gf)*? Worms homozygous for some *lin-12* gain-of-function alleles display a multivulva phenotype and is due to elevated *lin-12* activity (Greenwald et al., 1983; Greenwald and Seydoux, 1990). Therefore the differences between *mir-52* and *mir-54/55/56* might be related to differences in *lin-12* misregulation in each mutant background. Differences in individual miRNA expression and differences between the sequences at the 3' end of each miRNA within the family could result in each miRNA contributing unequally to regulation of a common target. Consistent with being a possible miRNA target, the *lin-12* RNA immunoprecipitates with the miRISC protein, AIN-1 (Zhang et al., 2007). However, *lin-12* RNA did not immunoprecipitate with ALG-1 (Zisoulis et al., 2010). By Targetscan, *lin-12* is one of six total predictions for the *mir-51* family which contains more than one binding site for this family within its 3' UTR and

both sites show relatively high seed pairing (Lewis et al., 2005; Jan et al., 2011). However, *lin-12* is not a predicted target of the *mir-51* family by mirWIP (Hammell et al., 2008). This may indicate that, although the *mir-51* family sites are conserved, the UTR may be inaccessible based on predicted structure. Therefore, it remains unclear if *lin-12* is a direct target of the miR-51 family. Future experiments can be directed at determining if *lin-12* is a *mir-51* family target and if differential misregulation of *lin-12* is responsible for the difference in penetrance of the multivulva phenotype between *mir-52; let-60(ga89gf)* and *mir-54/55/56; let-60(ga89gf)*.

6.5 The *mir-51* family in specification of neuronal asymmetry

The mutant ASEL to ASER phenotype in worms homozygous for *lsy-6(ot150rf)* is sensitive to reduced miRNA activity (Hammell et al., 2009). Loss of *mir-51* family members resulted in suppression of ASEL mis-specification defects of *lsy-6rf/lsy-6lf* and *lsy-6rf; alg-1* mutant worms. This is consistent with elevated *lsy-6* activity in these worms (Johnston and Hobert, 2003). This elevation of *lsy-6* activity in the absence of *mir-51* family members, in addition to the models already presented, could be mediated through regulation of *die-1* (Didiano et al., 2010). *die-1* is expressed in the ASEL and promotes *lsy-6* expression (Chang et al., 2004). The *die-1* 3'UTR is regulated by miR-273, which is specifically expressed in the ASER. Although *mir-273* is not necessary to keep *die-1* repressed in the ASER, ectopically high expression of *mir-273* in the ASEL can cause it to adopt the ASER fate via *die-1* repression (Chang et al., 2004).

Interestingly, the *mir-51* family is partially related in sequence to *mir-273* (Didiano et al., 2010). Although *mir-273* and the *mir-51* family are not necessary for the bilateral specification of the ASEL and ASER (Didiano et al., 2010), loss of the *mir-51* family could result in elevated *die-1* expression in the ASEL. Elevated expression of *die-1* in the ASEL may have no effect in a wild type background, but could assist in promoting the ASEL fate in worms heterozygous for *lsy-6(ot150rf)/lsy-6(ot150lf)*. Future experiments will be directed at determining if *die-1* misregulation mediates the observed suppression of *lsy-6(ot150rf)/lsy-6(ot150lf)* by loss of *mir-52*.

6.6 The direct targets of the *mir-51* family

The *mir-51* family genetically interacted in multiple, diverse pathways. However, the mechanism whereby it acts in each of these pathways is unknown. The phenotypes observed in this analysis are expected to be due to elevation of protein levels of direct miR-51 family targets. In order to identify direct, downstream targets of the miR-51 family involved in developmental timing RNAi was used to knockdown the activity of a set of predicted miR-51 family targets whose RNAs had previously been identified to immunoprecipitate with ALG-1 (Zisoulis et al., 2010). This analysis indicated that three genes, *lin-66*, *tlp-1*, and *vhp-1,* may act downstream of the *mir-51* family in developmental timing. However data from reporter transgene experiments and RT-PCR experiments do not conclusively support that any of these genes are direct miR-51 family targets

whose misregulation mediates suppression of retarded developmental timing defects.

This approach to identifying direct targets of the *mir-51* family in developmental timing required that the gene be a predicted target of this family by either Targetscan (Lewis et al., 2005; Jan et al., 2011) or mirWIP (Hammell et al., 2008), and had to have been immunoprecipitated with ALG-1 (Zisoulis et al., 2010). This generated a list of 51 candidates that were likely miR-51 family targets. It is possible that restricting analysis to these 51 candidates resulted in a failure to identify a relevant target of the *mir-51* family in developmental timing. This analysis can be expanded to include genes not examined in this study.

Analysis of these 51 candidate genes identified four candidates, *cul-1*, *lin-66*, *tlp-1*, and *vhp-1*. Knockdown of *cul-1*, *lin-66*, and *vhp-1* in wild-type worms resulted in retarded alae formation or extra seam cell divisions indicating that the activities of these genes are necessary for the proper execution of developmental fate decisions. Although knockdown of *tlp-1* had no effect on wild type alae formation, it significantly enhanced the alae formation defects of *mir-52; mir-48/84/241* worms. These results indicate that they may function as a downstream target of the *mir-51* family or alternatively may function in parallel to *mir-51* family members to regulate developmental timing. Analysis of reporter transgenes and qPCR, however, do not provide sufficient evidence to distinguish between these models. Furthermore, overexpression of *cul-1*, *lin-66*, or *vhp-1* is not sufficient to account for the observed suppression. This suggests that these genes are not the critical targets of the *mir-51* family in developmental timing. Alternatively, it

may indicate that the observed suppression of developmental timing defects requires the misexpression of multiple *mir-51* family targets. Misregulation of *cul-1*, *lin-66*, *tlp-1*, and *vhp-1*, along with other yet identified targets, may contribute to the observed suppression of developmental timing phenotypes.

cul-1 encodes a cullin protein that interacts with Skp1 related proteins and is required for cell cycle progression (Kipreos et al., 1996; Nayak et al., 2002). Loss of *cul-1* results in hyperplasia of all tissues with abnormally small cells due to fast G1 to S phase progression (Kipreos et al., 1996). CUL-1 acts in a complex with DRE-1 and SKR-1 and functions in parallel with *daf-12* activity (Fielenbach et al., 2007). Interestingly, knockdown of *cul-1* by RNAi causes both precocious seam cell fusion but also retarded alae formation (Fielenbach et al., 2007), suggesting complex regulation of targets in the developmental timing pathway by CUL-1. These results showed that the *cul-1* 3' UTR was sufficient to mediate repression of the *col-10::gfp* transgene. This repression required both the presence of *mir-52* and the predicted *mir-51* family binding site, indicating it is a direct target of the *mir-51* family. In the absence of miR-51 family members, elevated *cul-1* activity could act to slow or delay cell cycle progression thereby promoting cell cycle exit, terminal differentiation, and alae formation in seam cells at the L4 to adult transition. No effect was observed on the L2 reiteration phenotype, which indicates that misexpression of *cul-1* alone cannot account for the observed suppression of developmental timing defects by loss of *mir-51* family members.

lin-66 is required for the temporal down-regulation of *lin-28* in the regulation of the L2 to L3 transition though its molecular function remains unknown (Morita and Han, 2006). Suppression of the *mir-48/84/241* timing defects by loss of *mir-52* is reduced following *lin-66* RNAi. The *lin-66* 3' UTR is sufficient to direct the repression of a *col-10::gfp* reporter. However, this reporter is not significantly misexpressed in the absence of *mir-52*, which indicates it may not be a direct target of the *mir-51* family. The presence of the remaining family *mir-51* members may be sufficient to maintain the downregulation of this reporter in the absence of *mir-52*. If LIN-66 levels were elevated in the absence of *mir-51* family members, then the levels of LIN-28 should be reduced in a 3' UTR-dependent manner. No reduction was observed in *lin-28::gfp::lin-28* expression in worms scored at the L2 to L3 transition, which suggests that LIN-66 may not be significantly elevated in the absence of *mir-52* at this stage. It is possible that LIN-66 levels may be elevated at later stages in the absence of *mir-51* family members. If so, elevated LIN-66 levels may contribute to the observed suppression of developmental timing defects in the absence of *mir-51* family members.

tlp-1 encodes a transcription factor that functions in the T cell lineage to specify asymmetric cell fates and control cell fusion (Zhao et al., 2002). A role for *tlp-1* in developmental timing is unknown, and *tlp-1* expression, although largely absent from the seam cells, is expressed in posterior cell lineages of the hypodermis (Zhao et al., 2002). Knockdown of *tlp-1* had no effect on developmental timing of wild type worms, however knockdown of *tlp-1* did

significantly enchance the alae formation defects of *mir-52; mir-48/84/241* worms. This suggests that *tlp-1* may function in the terminal differentiation of the seam cells. Based on its role in cell fusion in the T cell lineage (Zhao et al., 2002), elevated TLP-1 may promote the terminal differentiation and fusion of the seam cells in the absence of *mir-51* family members. The *tlp-1* 3'UTR was sufficient to down-regulate a *col-10::gfp* reporter, but loss of *mir-52* had no significant effect on its expression. It is possible that the remaining *mir-51* family members are sufficient to down-regulate the *tlp-1* UTR. *tlp-1* transcripts were not detected in RNA isolated from adult hermaphrodites by qPCR. *tlp-1* may be a target of the miR-51 family that functions to mediate the suppression of developmental timing phenotypes. However, since knockdown of *tlp-1* had no effect on the L2 reiteration phenotype, then misexpression of *tlp-1* alone cannot account for the suppression of developmental timing defects by loss of *mir-51* family members.

Lastly, *vhp-1* encodes a MAPK phosphatase that regulates stress resistance and axon regeneration (Kim et al., 2004; Mizuno et al., 2004; Nix et al., 2011) through the regulation of its downstream targets, *pmk-3* and *kgb-1* (Nix et al., 2011). In the germline, VHP-1's target, KGB-1, promotes the degradation of DCR-1 and GLH-1 (Orsborn et al., 2007; Beshore et al., 2011). DCR-1 is an RNaseIII riboendonuclease that is required for miRNA biogenesis (Grishok et al., 2001). However, no changes in miRNA levels were observed in *mir-51* family mutants, which may be expected if DCR-1 levels were elevated. GLH-1 is a DEAD box RNA helicase that is a P granule component (Orsborn et al., 2007),

which are complexes of protein and RNA aggregates in the germline and may function like somatic P bodies (Buchan and Parker, 2009). Reduced activity of GLH-1 results in defects in P granule localization (Beshore et al., 2011). VHP-1 may function to modulate the activity of P bodies in somatic cells through the regulation of KGB-1. Elevated levels of VHP-1 would result in lower activity of KGB-1 and subsequently enhance P body-dependent mRNA degradation or storage. Because genetic interactions consistent with elevated miRNA activity were observed in some, but not all, miRNA dependent pathways, this regulation of miRNA activity by VHP-1 may only occur in specific cells or at specific times in development. This would be similar to the activity of the TRIM-NHL protein, NHL-2, and the DEAD-box RNA helicase, CGH-1, that function to modulate miRISC activity acting as a cofactor for a subset of miRNAs, including the *let-7* family (Hammell et al., 2009). Although the *vhp-1* 3' UTR does not mediate repression of the *col-10::gfp* transgene, elevated levels of *vhp-1* mRNA were detected in the *mir-51* family multiply mutant worms (*mir-52/53/54/55/56*). It is possible that binding sites for miR-51 family regulation lie outside of the 3' UTR used in this analysis. Interestingly, an ALG-1 binding site (Zisoulis et al., 2010) was identified in the first exon of the coding region of *vhp-1*.

It is unlikely that misregulation of any of these genes alone is sufficient to result in the observed suppression of developmental timing defects by loss of *mir-51* family members. First, knockdown of any of the four did not fully eliminate the suppression of developmental timing defects by loss of *mir-52*. If any of these were solely responsible, then it is expected that knockdown would completely

eliminate this effect. Second, expression of *cul-1*, *lin-66*, or *vhp-1* from a transgene array, which is expected to increase the levels of these genes, did not result in suppression of phenotypes. Therefore, misregulation of these, and likely additional, targets may mediate suppression of developmental timing defects.

In addition to developmental timing, the *mir-51* family functioned in vulva and neuronal cell fate specification, regulation of the defecation cycle, and regulation of neuromuscular function. The targets of the *mir-51* family in these pathways remain unknown. Interestingly, knockdown of *cul-1* and *vhp-1* affected the penetrance of multivulva phenotype and mean defecation cycle time, consistent with these genes having a function in these pathways. The mechanism whereby either gene might function in these pathways is unclear. *vhp-1* may interact in these pathways through regulation of *kgb-1* as discussed above. However, reduction of *vhp-1* resulted in reduced penetrance of the multivulva phenotype of both *let-60(ga89gf)* and *mir-52; let-60(ga89gf)* worms, which is inconsistent with our prediction if KGB-1 levels were elevated, and thus DCR-1 levels reduced, in vulva precursor cells. This observation also indicates that misregulation of *vhp-1* likely cannot account for the role of the *mir-51* family in vulval cell fate specification. Further analysis is needed to determine the role of *cul-1* in vulval cell fate specification and the defecation cycle. Future work can also be directed at determining if other targets of the *mir-51* family are necessary for the observed phenotypes in these broad developmental pathways.

cdh-3 is a confirmed target of the *mir-51* family (Shaw et al., 2010) that was not examined in this analysis by RNAi knockdown since it was not identified

as a target pulled down by ALG-1. *cdh-3* is a target of the *mir-51* family during embryogenesis, whereas ALG-1 bound transcripts were identified from late larval stage worms (Zisoulis et al., 2010), possibly explaining the lack of *cdh-3* from their analysis. *cdh-3* encodes a cadherin protein, which is part of a large family of proteins that are important for epithelial morphogenesis (Pettitt et al., 1996). Worms carrying a presumptive loss-of-function of *cdh-3* display weakly penetrant tail tip defects (Pettitt et al., 1996). In worms lacking the *mir-51* family, *cdh-3* levels are elevated, which leads to failure of the pharynx to properly attach to surrounding tissue, resulting in early embryonic lethality (Shaw et al., 2010). However, misexpression of *cdh-3* alone does not account for the pleiotropic phenotypes observed in the absence of multiple members of the *mir-51* family (Shaw et al., 2010). This indicates that additional *mir-51* family targets are sufficiently misexpressed in absence of multiple family members to result in observable phenotypes, such as slow growth and larval lethality. It is unlikely that misexpression of *cdh-3* alone can account for the phenotypes observed in this study. Cadherins typically function to form cell-to-cell contacts in adherens junctions (Sopko and McNeill, 2009), and it is unclear how *cdh-3* might function in the diverse pathways described in this study. Although a role for *cdh-3* in these diverse developmental pathways can not be ruled out, it is unlikely that misexpression of *cdh-3* alone can account for all the observed phenotypes in the absence of *mir-51* family members.

Based on the current understanding of both the known *mir-51* family target, *cdh-3*, and the predicted targets analyzed in this study, it is possible that

the *mir-51* family regulates distinct targets sets in diverse developmental pathways in *C. elegans*, and the phenotypes observed in this study are due to misregulation of multiple targets. Future work can be directed at identifying all genes misregulated in the absence of *mir-51* family members. Since the *mir-51* family functions in diverse developmental pathways throughout development, then identification of genes misexpressed in the absence of *mir-51* family members at various developmental stages will be necessary to identify the relevant targets of the *mir-51* family.

6.7 The *mir-51* family likely fine-tunes target gene expression in *C. elegans*

Based on the analysis of predicted *mir-51* family targets, it is possible that the *mir-51* family acts to regulate multiple and distinct target sets in *C. elegans*. But what does this regulation entail, and what types of relationships do the *mir-51* family miRNAs have with their targets?

The type of regulation mediated by the *mir-51* family is not like that of lsy-6, which acts like a genetic switch to shut down the expression of a target in a specific cell. *lsy-6* is expressed specifically in the ASEL and acts to regulate a specific target, *cog-1*, to mediate the proper specification of this cell (Johnston and Hobert, 2003). Similarly, miR-124 is expressed specifically in neuronal cells of the mammalian brain and targets non-neuronal genes, which is thought to reinforce the proper expression profile of these cells (Lim et al., 2005). In contrast, the *mir-51* family is expressed in most, if not all, tissue types throughout *C. elegans* (Martinez et al., 2008b; Zhang and Emmons, 2009; Shaw et al.,

2010). This broad expression pattern makes it unlikely that the *mir-51* family functions to reinforce the cell fate of an individual cell type. Instead, the *mir-51* family may regulate a broad set of targets in distinct cell types. Additionally, the target sets may be distinct between cell types.

It is also unlikely that the *mir-51* family acts to switch off the expression of their targets genes. lin-4, for example, is expressed beginning in larval development and functions to down-regulate and eliminate LIN-14 levels (Lee et al., 1993; Wightman et al., 1993). This temporal expression of lin-4 allows it to function as a switch. In contrast, the *mir-51* family is abundantly expressed throughout *C. elegans* development starting in embryogenesis (Lim et al., 2003; Ruby et al., 2006; Kato et al., 2009a). This temporal expression makes this family less well suited to act as a temporal switch during developmental transitions, such as the L2 to L3 transition. However it is possible that the *mir-51* family could act to switch off gene expression very early in development, possibly right after the maternal-to-zygotic transition which immediately precedes the onset of abundant *mir-51* family expression. A role for the *mir-51* family in early embryogenesis is unknown, and most defects in worms lacking the whole *mir-51* family are observable in the later stages of embryogenesis (Alvarez-Saavedra and Horvitz, 2010; Shaw et al., 2010). It is possible that *mir-51* family targets that are misexpressed during early embryogenesis do not mediate an effect until later stages. Aside from this small window in development, it is unlikely that the *mir-51* family serves as a switch in later stages of development. Instead the *mir-51*

family may act in later developmental decisions to primarily fine-tune or buffer the expression levels of its target genes.

Recent evidence from Mukherji et al. (2011) indicates that miRNA regulation is not an inherent property of the miRNA. Instead the level of repression effected by a miRNA represents the cumulative effect of the extent of miRNA pairing to its target, including both number of sites and strength of binding, and the stoichiometry between the miRNA and its target (Mukherji et al., 2011). The abundance and broad spatial expression pattern of the *mir-51* family suggests that it is likely to be co-expressed with its targets, and may therefore act as a switch to strongly repress its targets. However, close examination of genes predicted to be targets of the *mir-51* family show that very few of these genes contain multiple sites complementary to the *mir-51* family seed. Of the 293 conserved targets predicted by Targetscan (Lewis et al., 2005; Jan et al., 2011), only six have more than one conserved binding site for the miR-51 family. This may preclude strong repression by the abundant *mir-51* family.

Many genes that are switched off by miRNAs contain multiple binding sites in its 3'UTR for a given miRNA. Furthermore, robust repression by miRNAs depends on cooperation of miRNA regulation at multiple sites (Wu et al., 2010). *lin-14* has seven sites within its 3'UTR for lin-4 (Lee et al., 1993; Wightman et al., 1993). In addition, many of these sites can be considered strong binding sites, with full complementarity between the miRNA seed sequence and the UTR. In contrast, the *mir-51* family predicted targets do not contain any genes with more than two conserved target sites within their 3'UTR. Therefore, based on

Targetscan predictions, only a handful of genes are predicted to be strongly repressed by the *mir-51* family.

Although the results from the Targetscan predictions for the *mir-51* family do not obviously point to a target that may be switched off by this family, it cannot exclude the possibility of the *mir-51* family strongly repressing six of its targets. These six genes contain two target sites for the miR-51 family, which might be sufficient to mediate strong repression. The known miRNA target *cog-1*, for example, has two predicted sites for lsy-6, of which one is necessary for strong repression by lsy-6 (Johnston and Hobert, 2003). lsy-6 can switch off *cog-1* despite the presence of only two complementary sites. However, a primary feature of the two sites in *cog-1* is that the seed binds with full complementarity and the 3' end of the miRNA also may pair considerably (Johnston and Hobert, 2003). This is generally lacking from the *mir-51* family target predictions. Six genes have two sites, and only one gene, *lin-12*, has both sites classified as '8mers' with full complementarity to the *mir-51* family seed and conserved neighboring nucleotides adjacent to the seed (Bartel, 2009). Interestingly, as was mentioned previously, *lin-12* was not identified as a gene pulled down by ALG-1 (Zisoulis et al., 2010), and therefore it is unclear if it is a genuine miRNA target. Additionally, high LIN-12 activity, and presumably expression, is necessary in vulva precursor cells (Sternberg, 2005) where the *mir-51* family members are expressed (Martinez et al., 2008a; Zhang and Emmons, 2009; Shaw et al., 2010). It is expected that the miR-51 family would interact with the *lin-12* transcript in these cells. This may reflect insufficient pairing at the 3' end between

miR-51 family members and *lin-12*, or inaccessibility to the target sites. The other five predicted targets that contain two conserved sites are a mix of '8mer' and '7mer' or '6mer' sites, and may also have the potential to be strongly repressed by the miR-51 family.

While only six of the predicted targets of the miR-51 family contain multiple sites, seventy-seven predicted targets contain a single '8mer' site. Such a high number of genes with an individual conserved site may suggest that the miR-51 family can interact with a large number of genes. Furthermore, since these sites show conservation among various species of *Caenorhabditis*, it may indicate these sites have been preferentially retained through evolution. Unfortunately little is known about the consequence of having a single miR-51 family site present in a UTR. The *mir-51* family target, *cdh-3* is among these seventy-seven target predictions, and the individual site is sufficient to mediate down-regulation by the miR-51 family (Shaw et al., 2010). The quantitative effect of the miR-51 family on CDH-3 is unknown. Although it seems unlikely that it generally switches off *cdh-3*, since *cdh-3* expression is observed in many cell types in later development (Pettitt et al., 1996), where members of the *mir-51* family are also expressed (Shaw et al., 2010).

Based on the expression profile of *mir-51* family members and their broad function in diverse developmental pathways, it may be that this family regulates distinct sets of target genes, likely acting to buffer or fine-tune the levels of these genes to an optimal range. The *mir-51* family seems well suited to perform this role in diverse cell types. It is abundantly expressed, making it highly likely that a

miR-51 family member will bind any genuine direct target. For this reason, it is unlikely for UTRs to acquire multiple sites for the miR-51 family though evolution, since multiple sites for an abundantly expressed miRNA would likely eliminate its expression. However, retention of a single site might be favorable to fine-tune gene expression, or possibly to prevent stochastic expression. This subtle role for the *mir-51* family would allow it to function in diverse developmental pathways, possibly serving as a general mechanism in the canalization of these developmental programs (Hornstein and Shomron, 2006).

Chapter 7: Methods

7.1 General Methods and Strains.

All strains were maintained under standard conditions as previously described (Wood, 1988). Worms were kept on NGM plates seeded with *E. coli* strain AMA1004 (Casadaban et al., 1983). The wild type strain used was var. Bristol N2 (Brenner, 1974). A full list of strains used in this study is in Table 7.1. All strains were kept at 20˚C unless otherwise indicated. miRNA mutant strains were first outcrossed to wild type N2. For the generation of multiply mutant strains, the miRNA deletion allele were identified by performing PCR with primers that amplified the genomic region flanking the deletion mutation. Sequences for primers used in genotyping reactions can be found in Table 7.2.

Table 7.1. Strains used in this study

Strain	**Genotype**
N2 *wild type*	wild type
RF54 *alg-1*	*alg-1(gk214)X*
RF70 *mir-1; alg-1*	*mir-1(n4102)I; alg-1(gk214)X*
RF129 *mir-34; alg-1*	*mir-34(n4276) alg-1(gk214)X*
RF420 *mir-51; alg-1*	*mir-51(n4473)IV; alg-1(gk214)X*
RF411 *mir-52; alg-1*	*mir-52(n4114)IV; alg-1(gk214)X*
RF398 *mir-53; alg-1*	*mir-53(n4113)IV; alg-1(gk214)X*
RF410 *mir-54/55 alg-1*	*mir-54/55(nDf45) alg-1(gk214)X*
RF89 *mir-54/55/56 alg-1*	*mir-54/55/56(nDf58) alg-1(gk214)X*
RF133 *mir-57; alg-1*	*mir-57(gk175)II; alg-1(gk214)X*
RF137 *mir-59; alg-1*	*mir-59(n4604)IV; alg-1(gk214)X*
RF153 *mir-72; alg-1*	*mir-72(n4130)II; alg-1(gk214)X*
RF81 *mir-73/74 alg-1*	*mir-73/74(nDf47) alg-1(gk214)X*
RF178 *mir-77; alg-1*	*mir-77(n4285)II; alg-1(gk214)X*
RF65 *mir-83; alg-1*	*mir-83(n4638)IV; alg-1(gk214)X*
RF141 *mir-85; alg-1*	*mir-85(n4117)II; alg-1(gk214)X*
RF77 *mir-124; alg-1*	*mir-124(n4255)IV; alg-1(gk214)X*
RF145 *mir-228; alg-1*	*mir-228(n4382)IV; alg-1(gk214)X*
RF93 *mir-234; alg-1*	*mir-234(n4520)II; alg-1(gk214)X*
RF182 *mir-235; alg-1*	*mir-235(n4504)I; alg-1(gk214)X*
RF85 *mir-237 alg-1*	*mir-237(n4296) alg-1(gk214)X*
RF163 *mir-238; mir-239a/b alg-1*	*mir-238(n4112)III;*

Table 7.1. Strains used in this study

Strain	Genotype
	mir-239a/b(nDf62) alg-1(gk214)X
RF60 *mir-240/786 alg-1*	*mir-240 mir-786(n4541) alg-1(gk214)X*
RF186 *mir-244; alg-1*	*mir-244(n4367)I; alg-1(gk214)X*
RF149 *mir-246; alg-1*	*mir-246(n4636)IV; alg-1(gk214)X*
RF368 *mir-247/797 alg-1*	*mir-247 mir-797(n4505) alg-1(gk214)X*
RF343 *mir-259; alg-1*	*mir-259(n4106)V; alg-1(gk214)*
RF392 *mir-1; alg-1; Ex[mir-1 rescue]*	*mir-1(n4102)I; alg-1(gk214)X; xwEx65*
RF421 *mir-59; alg-1; Ex[mir-59 rescue]*	*mir-59(n4604)IV; alg-1(gk214)X; xwEx76*
RF414 *mir-83; alg-1; Ex[mir-83 rescue]*	*mir-83(n4638)IV; alg-1(gk214)X; xwEx73*
RF384 *mir-124; alg-1; Ex[mir-124 rescue]*	*mir-124(n4255)IV; alg-1(gk214)X; xwEx54*
RF394 *mir-124; alg-1; Ex[mir-124 rescue]*	*mir-124(n4255)IV; alg-1(gk214)X; xwEx59*
RF426 *mir-247/786 alg-1; Ex[mir-247 rescue]*	*mir-247 mir-786(n4505) alg-1(gk214)X; xwEx78*
RF427 *mir-247/786 alg-1; Ex[mir-247 rescue]*	*mir-247 mir-786(n4505) alg-1(gk214)X; xwEx79*
RF428 *mir-247/786 alg-1; Ex[mir-247 rescue]*	*mir-247 mir-786(n4505) alg-1(gk214)X; xwEx80*
RF425 *mir-259; alg-1; Ex[mir-259 rescue]*	*mir-259(n4106)V; alg-1(gk214)X; xwEx77*
RF403 *mir-54/55/56 alg-1; mjEx160[mir-54/55/56 rescue]*	*mir-54/55/56(nDf58) alg-1(gk214)X; mjEx160*
RF251 *mir-238; alg-1; Ex[mir-238 rescue]*	*mir-238(n4114)III; alg-1(gk214)X; xwEx14*
RF252 *mir-238; alg-1; Ex[mir-238 rescue]*	*mir-238(n4114)III; alg-1(gk214)X; xwEx15*
RF253 *mir-238; alg-1; Ex[mir-238 rescue]*	*mir-238(n4114)III; alg-1(gk214)X; xwEx16*
RF416 *mir-244; alg-1; Ex[mir-244 rescue]*	*mir-244(n4367)I; alg-1(gk214)X; xwEx74*
RF417 *mir-244; alg-1; Ex[mir-244 rescue]*	*mir-244(n4367)I; alg-1(gk214)X; xwEx75*
MT7626 *let-7ts*	*let-7(n2853)X*
RF448 *mir-52; let-7ts*	*mir-52(n4114)IV; let-7(n2853)X*
RF554 *mir-48/84/241*	*mir-48 mir-241(nDf51)V; mir-84(n4037) wIs79X*
RF556 *mir-52; mir-48/84/241*	*mir-52(n4114)IV; mir-48 mir-241(nDf51)V; mir-84(n4037) wIs79 X*
RF730 *mir-48/241; mjEx160[mir-54/55/56]*	*mir-48 mir-241(nDf51)V; mjEx160*
RF504 *lin-46*	*wIs78IV; lin-46(ma164)V*
RF568 *lin-46*	*lin-46(ma164)V;wIs79X*
RF594 *mir-51; lin-46*	*mir-51(n4473) wIs78IV; lin-46(ma164)V*
RF569 *mir-52; lin-46*	*mir-52(n4114)IV; lin-46(ma164)V; wIs79X*
RF599 *mir-53; lin-46*	*mir-53(n4113) wIs78IV; lin-46(ma164)V*
RF505 *mir-54/55/56; lin-46*	*wIs78IV; lin-46(ma164)V; mir-54/55/56(nDf58)X*
VT1064 *mir-48/84*	*mir-48(n4097) maIs105V; mir-84(n4037)X*
RF451 *mir-51; mir-48/84*	*mir-51(n4473)IV; mir-48(n4097) maIs105V; mir-84(n4037)X*
RF469 *mir-52; mir-48/84*	*mir-52(n4114)IV; mir-48(n4097) maIs105V; mir-84(n4037)X*
RF454 *mir-53; mir-48/84*	*mir-53(n4113)IV; mir-48(n4097) maIs105V; mir-84(n4037)X*
RF451 *mir-54/55/56; mir-48/84*	*mir-48(n4097) maIs105V; mir-84(n4037) mir-54/55/56(nDf58)X*
VC894 *puf-9*	*puf-9(ok1136)X*
RF578 *mir-52; puf-9*	*mir-52(n4114); puf-9(ok1136)X*
RF619 *mir-48/241*	*mir-48/241(nDf51)V; wIs79X*
RF620 *mir-52; mir-48/241*	*mir-52(n4114)IV; mir-48/241(nDf51) wIs79X*
RF625 *mir-48/241; puf-9*	*mir-48 mir-241(nDf51)V; puf-9(ok1136) wIs79X*

Table 7.1. Strains used in this study

Strain	Genotype
RF626 *mir-52; mir-48/241; puf-9*	*mir-52(n4114)IV; mir-48/241(nDf51)V; puf-9(ok1136) wIs79X*
RG490 *mir-48(ve33)*	*mir-48(ve33)V*
RF582 *mir-51; mir-48(ve33)*	*mir-51(n4473)IV; mir-48(ve33)V*
RF583 *mir-52; mir-48(ve33)*	*mir-52(n4114)IV; mir-48(ve33)V*
RF584 *mir-53; mir-48(ve33)*	*mir-53(n4113)IV; mir-48(ve33)V*
RF587 *mir-54/55/56; mir-48(ve33)*	*mir-48(ve33)V; mir-54/55/56(nDf58)X*
RF510 *hbl-1*	*wIs78IV; hbl-1(ve18)X*
RF534 *hbl-1*	*hbl-1(ve18) wIs79X*
RF530 *mir-51; hbl-1*	*mir-51(n4473) wIs78IV; hbl-1(ve18)X*
RF535 *mir-52; hbl-1*	*mir-52(n4114)IV; hbl-1(ve18) wIs79X*
RF512 *mir-53; hbl-1*	*mir-53(n4113) wIs78IV; hbl-1(ve18)X*
RF511 *mir-54/55/56; hbl-1*	*wIs78IV; hbl-1(ve18) mir-54/55/56(nDf58)X*
RF563 *lin-14*	*lin-14(n179) wIs79x*
RF588 *mir-52; lin-14*	*mir-52(n4114)IV; lin-14(n179) wIs79X*
RF500 *lin-41*	*lin-41(ma104)I; wIs78IV*
RF536 *lin-41*	*lin-41(ma104)I; wIs79X*
RF529 *mir-51; lin-41*	*lin-41(ma104)I; mir-51(n4473) wIs78IV*
RF537 *mir-52; lin-41*	*lin-41(ma104)I; mir-52(n4114)IV; wIs79X*
RF539 *mir-53; lin-41*	*lin-41(ma104)I; mir-53(n4113) wIs78IV*
RF501 *mir-54/55/56; lin-41*	*lin-41(ma104)I; wIs78IV; mir-54/55/56(nDf58)X*
RF508 *lin-42*	*lin-42(n1089)II; wIs78IV*
RF538 *lin-42*	*lin-42(n1089)II; wIs79X*
RF527 *mir-51; lin-42*	*lin-42(n1089)II; mir-51(n4473) wIs78IV*
RF541 *mir-52; lin-42*	*lin-42(n1089)II; mir-52(n4114)IV; wIs79X*
RF526 *mir-53; lin-42*	*lin-42(n1089)II; mir-53(n4113) wIs78IV*
RF509 *mir-54/55/56; lin-42*	*lin-42(n1089)II; wIs78IV; mir-54/55/56(nDf58)X*
VT517 *lin-28*	*lin-28(n719)I*
RF572 *mir-51; lin-28*	*lin-28(n719)I; mir-51(n4473)IV*
RF573 *mir-52; lin-28*	*lin-28(n719)I; mir-52(n4114)IV*
RF574 *mir-53; lin-28*	*lin-28(n719)I; mir-53(n4113)IV*
RF575 *mir-54/55/56; lin-28*	*lin-28(n719)I; mir-54/55/56(nDf58)X*
RG733 *wild type*	*wIs78[scm::gfp]IV*
RF481 *wild type*	*wIs79[scm::gfp]X*
RF491 *mir-51*	*mir-51(n4473) wIs78*
RF499 *mir-52*	*mir-52(n4114)IV; wIs79X*
RF483 *mir-53*	*mir-53(n4113) wIs78IV*
RF399 *mir-54/55/56*	*wIs78IV; mir-54/55/56(nDf58)X*
RF692 *mir-52/53/54/55/56*	*mir-52(n4100) mir-53(n4114)IV; mir-54/55/56(nDf58)X*
RF447 *mir-51; let-7*	*mir-51(n4473)IV; let-7(n2853)X*
RF449 *mir-53; let-7*	*mir-53(n4113)IV; let-7(n2853)X*
RF442 *mir-54/55/56; let-7*	*let-7(n2853) mir-54/55/56(nDf58)X*
RF553 *mir-48/84/241*	*wIs78IV; mir-48 mir-241(nDf51)V; mir-84(n4037)X*
RF555 *mir-51; mir-48/84/241*	*mir-51(n4473) wIs78IV; mir-48 mir-241(nDf51)V; mir-84(n4037)X*
RF557 *mir-53; mir-48/84/241*	*mir-53(n4113) wIs78IV; mir-48 mir-241(nDf51)V; mir-84(n4037)X*
RF558 *mir-54/55/56; mir-48/84/241*	*wIs78IV; mir-48 mir-241(nDf51)V; mir-84(n4037) mir-54/55/56(nDf58)X*
RF486 *hbl-1::gfp::hbl-1*	*ctIs39[hbl-1::gfp::hbl-1]IV*

Table 7.1. Strains used in this study

Strain	Genotype
RF473 *mir-52; hbl-1::gfp::hbl-1*	*mir-52(n4114) ctIs39[hbl-1::gfp::hbl-1]IV*
RF487 *mir-54/55/56; hbl-1::gfp::hbl-1*	*ctIs39IV; mir-54/55/56(nDf58)X*
RF464 *mir-48/84/241; hbl-1::gfp::hbl-1*	*ctIs39IV; mir-48 mir-241(nDf51)V; mir-84(n4037)X*
RF494 *mir-52; mir-48/84/241; hbl-1::gfp::hbl-1*	*mir-52(n4114) ctIs39IV; mir-48 mir-241(nDf51)V; mir-84(n4037)X*
RF467 *mir-54/55/56;mir-48/84/241; hbl-1::gfp::hbl-1*	*ctIs39IV; mir-48 mir-241(nDf51)V: mir-84(n4037) mir-54/55/56(nDf58)X*
VT1102 *lin-28; lin-46; mir-48/84/241*	*lin-28(n719)I; lin-46(ma164) mir-48 mir-241(nDf51)V; mir-84(n4037) wIs79X*
RF689 *mir-52; lin-28; lin-46; mir-48/84/241*	*lin-28(n719)I; mir-52(n4114)IV; lin-46(ma164) mir-48 mir-241(nDf51)V; mir-84(n4037) wIs79X*
VT808 *lin-28::gfp*	*maIs808*
VT1138 *mir-48/84/241; lin-28::gfp*	*mir-48 mir-241(nDf51)V; mir-84(n4037)X; maIs108[lin-28::gfp]*
RF691 *mir-52; mir-48/84/241; lin-28::gfp*	*mir-52(n4114)IV; mir-48 mir-241(nDf51)V; mir-84(n4037)X; maIs108*
SD551 *let-60gf*	*let-60(ga89)IV*
RF319 *let-60gf; alg-1*	*let-60(ga89)IV: alg-1(gk214)X*
RF321 *let-60gf; mir-54/55/56 alg-1*	*let-60(gk214)IV; alg-1(gk214) mir-54/55/56(nDf58)X*
RF320 *let-60gf; mir-238; alg-1*	*mir-238(n4114)III; let-60(ga89)IV; alg-1(gk214)X*
RF370 *let-60gf; mir-244; alg-1*	*mir-244(n4367)I; let-60(ga89)IV; alg-1(gk214)X*
RF462 *mir-52; let-60gf*	*mir-52(n4114) let-60(ga89)IV*
RF440 *let-60; mir-54/55/56*	*let-60(ga89)IV; mir-54/55/56(nDf58)X*
OH3646 *lsy-6rf; lim-6::gfp*	*otIs114I; lsy-6(ot150)V*
RF323 *lsy-6rf; alg-1; lim-6::gfp*	*otIs114I; lsy-6(ot150)V; alg-1(gk214)X*
RF325 *lsy-6rf; mir-54/55/56 alg-1; lim-6::gfp*	*otIs114I; lsy-6(ot150)V; alg-1(gk214) mir-54/55/56(nDf58)X*
RF324 *lsy-6rf; mir-238; alg-1; lim-6::gfp*	*otIs114I; mir-238(n4112)III; lsy-6(ot150)V; alg-1(gk214)X*
RF397 *lsy-6rf; mir-244; alg-1; lim-6::gfp*	*mir-244(n4367) otIs114I; lsy-6(ot150)V; alg-1(gk214)X*
RF531 *mir-51; lsy-6rf; lim-6::gfp*	*otIs114I; mir-51(n4473)IV; lsy-6(ot150)V*
RF532 *mir-52; lsy-6rf; lim-6::gfp*	*otIs114I; mir-52(n4114)IV; lsy-6(ot150)V*
RF546 *mir-53; lsy-6rf; lim-6::gfp*	*otIs114I; mir-53(n4113)IV; lsy-6(ot150)V*
RF367 *mir-54/55/56; lsy-6rf; lim-6::gfp*	*otIs114I; lsy-6(ot150)V; mir-54/55/56(nDf58)X*
OH3645 *lsy-6lf; lim-6::gfp*	*otIs114I; lsy-6(ot149)V*
RF565 *lsy-6lf; lim-6::gfp*	*otIs114I; lsy-6(ot149) wwIs5V*
RF590 *mir-52; lsy-6lf; lim-6::gfp*	*otIs114I; mir-52(n4114)IV; lsy-6(ot149) wwIs5V*
RF61 *mir-240/786*	*mir-240 mir-786(n4541)X*
RF542 mir-51; mir-240/786	*mir-51(n4473)IV; mir-240 mir-786(n4541)X*
RF543 *mir-52; mir-240/786*	*mir-52(n4114)IV; mir-240 mir-786(n4541)X*
RF544 mir-53; mir-240/786	*mir-53(n4113)IV; mir-240 mir-786(n4541)X*
RF552 mir-54/55/56; mir-240/786	*mir-240 mir-786(n4541) mir-54/55/56(nDf58)X*
MT14119 *mir-35 thru 41*	*mir-35/36/37/38/39/40/41(nDf50)II*
RF210 *mir-54/55/56; mir-35 thru 41*	*mir-35/36/37/38/39/40/41(nDf50)II; mir-54/55/56(nDf58)X*

Table 7.1. Strains used in this study

Strain	Genotype
RF753 *mir-1*	*mir-1(n4102)I*
RF754 *mir-52; mir-1*	*mir-1(n4102)I; mir-52(n4114)IV*
PS3662 *cog-1::gfp*	*syIs63IV*
OH7310 *cog-1::gfp; cog-1^{prom}::lsy-6^{hairpin}*	*syIs63 otIs193IV*
RF622 *mir-52; cog-1::gfp; cog-1^{prom}::lsy-6^{hairpin}*	*mir-52(n4114) syIs63 otIs193IV*
RF663 *lsy-6; rrf-3; lim-6::gfp*	*otIs114I; rrf-3(pk1426)II; lsy-6(ot150)V*
RF662 *lsy-6; mir-52; rrf-3; lim-6::gfp*	*otIs114I; rrf-3(pk1426)II; mir-52(n4114)IV; lsy-6(ot150)V; rrf-3(pk1426)II*
RF45 *mir-1*	*mir-1(n4102)I 4x outcrossed*
RF124 *mir-34*	*mir-34(n4276)X 4x outcrossed*
VT1553 *mir-57*	*mir-57(gk175)II 4x outcrossed*
VT1555 *mir-59*	*mir-59(n4604)IV 4x outcrossed*
RF154 *mir-72*	*mir-72(n4130)II*
RF48 *mir-73/74*	*mir-73/74(nDf47)X 4x outcrossed*
RF164 *mir-77*	*mir-77(n4285)II 4x outcrossed*
RF1 *mir-83*	*mir-83(n4638)IV 4x outcrossed*
RF125 *mir-85*	*mir-85(n4117)II 4x outcrossed*
RF46 *mir-124*	*mir-124(n4255)IV 4x outcrossed*
RF127 *mir-228*	*mir-228(n4382)IV 4x outcrossed*
RF47 *mir-234*	*mir-234(n4520)II 4x outcrossed*
RF165 *mir-235*	*mir-235(n4504)I 4x outcrossed*
RF57 *mir-237*	*mir-237(n4296)X 4x outcrossed*
RF157 *mir-238*	*mir-238(n4112)III 4x outcrossed*
RF158 *mir-239a/b*	*mir-239a/b(nDf62)X 4x outcrossed*
RF166 *mir-244*	*mir-244(n4367)I 4x outcrossed*
RF126 *mir-246*	*mir-246(n4636)IV 4x outcrossed*
RF24 *mir-247/797*	*mir-247 mir-797(n4505)X 4x outcrossed*
RF326 *mir-259*	*mir-259(n4106)V 4x outcrossed*
RF716 *cul-1+++*	*xwEx135*
RF717 *cul-1+++*	*xwEx136*
RF718 *cul-1+++*	*xwEx137*
RF732 *mir-48/241; cul-1+++*	*mir-48 mir-241(nDf51)V; xwEx135*
RF707 *lin-66+++*	*xwEx128*
RF708 *lin-66+++*	*xwEx129*
RF709 *lin-66+++*	*xwEx130*
RF710 *lin-66+++*	*xwEx131*
RF733 *mir-48/241; lin-66+++*	*mir-48 mir-241(nDf51)V; xwEx131*
RF719 *vhp-1+++*	*xwEx138*
RF720 *vhp-1+++*	*xwEx139*
RF721 *vhp-1+++*	*xwEx140*
RF722 *vhp-1+++*	*xwEx141*
RF723 *vhp-1+++*	*xwEx142*
RF734 *mir-48/241; vhp-1+++*	*mir-48 mir-241(nDf51)V; xwEx138*
RF642 *col-10::gfp::unc-54 utr*	*xwEx94*
RF643 *col-10::gfp::unc-54 utr*	*xwEx95*
RF644 *col-10::gfp::unc-54 utr*	*xwEx96*
RF667 *mir-52; col-10::gfp::unc-54 utr*	*mir-52(n4114)IV; xwEx96*
RF645 *col-10::gfp::vhp-1 utr*	*xwEx97*
RF646 *col-10::gfp::vhp-1 utr*	*xwEx98*
RF647 *col-10::gfp::vhp-1 utr*	*xwEx99*
RF668 *mir-52; col-10::gfp::vhp-1 utr*	*mir-52(n4114)IV; xwEx97*
RF648 *col-10::gfp::cul-1 utr*	*xwEx100*
RF649 *col-10::gfp::cul-1 utr*	*xwEx101*
RF650 *col-10::gfp::cul-1 utr*	*xwEx102*

Table 7.1. Strains used in this study

Strain	**Genotype**
RF662 *mir-52; col-10::gfp::cul-1 utr*	*mir-52(n4114)IV; xwEx102*
RF653 *col-10::gfp::tlp-1 utr*	*xwEx103*
RF654 *col-10::gfp::tlp-1 utr*	*xwEx104*
RF655 *col-10::gfp::tlp-1 utr*	*xwEx105*
RF663 *mir-52; col-10::gfp::tlp-1 utr*	*mir-52(n4114)IV; xwEx105*
RF660 *col-10::gfp::lin-66 utr*	*xwEx109*
RF661 *col-10::gfp::lin-66 utr*	*xwEx110*
RF680 *mir-52; col-10::gfp::lin-66 utr*	*mir-52(n4114)IV; xwEx109*
RF677 *col-10::gfp::lin-66Δ utr*	*xwEx118*
RF678 *col-10::gfp::lin-66Δ utr*	*xwEx119*
RF679 *col-10::gfp::lin-66Δ utr*	*xwEx120*
RF686 *mir-52; col-10::gfp::lin-66Δ utr*	*mir-52(n4114)IV; xwEx118*
RF670 *col-10::gfp::cul-1Δ utr*	*xwEx111*
RF671 *col-10::gfp::cul-1Δ utr*	*xwEx112*
RF672 *col-10::gfp::cul-1Δ utr*	*xwEx113*
RF684 *mir-52; col-10::gfp::cul-1Δ utr*	*mir-52(n4114)IV; xwEx111*

Table 7.2. primers used for miRNA allele detection

gene	allele	description	primer - WT/deletion (5')
mir-1	*n4102*	primer - WT/deletion (5')	GCAAAGAAATATTAAAGTTGTGCTG
		primer - WT/deletion (3')	ATCATTCTCGCTCTCTCTAGTTCTCTT
		primer - WT only (3')	GACGGAGGAATGAGTAGAAAAAGAC
mir-34	*n4276*	primer - WT/deletion (5')	CTAGACGAGTTTAACAACAACAACAAA
		primer - WT/deletion (3')	AGTAAGAGGACAGGAACAGG
		primer - WT only (5')	ACAAGTTGATCTTTTCCTTCTCTTTTT
		primer - WT only (3')	ACTTTTTCCAGTTACAGTGTCAAACTT
mir-51	*n4473*	primer - WT/deletion (5')	TTAATACTTATCAGAAAACTGATGTGG
		primer - WT/deletion (3')	GATTGTTGTTTTTGTAATATTTTCTCG
		primer - WT only (5')	TATTACTAAACGCATGTCAGAAGTTTG
mir-52	*n4114*	primer - WT/deletion (5')	AAGGTTCGACGTTTCTCCTG
		primer - WT/deletion (3')	GTTTATGAACGCCGCTTGG
		primer - WT only (5')	TTCCGTGCTTGACAGCGAAGC
mir-53	*n4113*	primer - WT/deletion (5')	AACCTGGGAGTATAAGAATGAAAGG
		primer - WT/deletion (3')	TCTCTGCGTATCTTTGTCTACATTG
		primer - WT only (3')	ATGCATCAAAATTGGTCAGTTATTC
mir-54-55	*nDf45*	primer - WT/deletion (3')	TATATGAGCAGGGCTGCCCAGCGTTC
		primer - WT/deletion (3')	CGCGCTCTGACTAGGATATGAGACGACGA
		primer - WT only (5')	GATGTGGTATGTGTCTCTCCACAC
		primer - WT only (3')	AAATCTAGACATGCTTCGCAATAAG
mir-54-56	*nDf58*	primer - WT/deletion (5')	TATATGAGCAGGGCTGCCCAGCGTTC
		primer - WT/deletion (3')	GAACTAAAGTATGTTGCTAGTGGCAACCA
		primer - WT only (3')	CAGTAGGTGAGTTGGAACGGAGCCAG

Table 7.2. primers used for miRNA allele detection

gene	allele	description	primer - WT/deletion (5')
mir-57	*gk175*	primer - WT/deletion (5')	CCGACAAATCCTCAAAGCAT
		primer - WT/deletion (3')	AGACGTGATTTCTTGGCTCC
		primer - WT only (5')	TCCCAGAATAAAAGAAGAAGTTCAG
mir-59	*n4604*	primer - WT/deletion (5')	CTAGGTAATCTAGGCGTTCACACGATTAAC
		primer - WT/deletion (3')	GTTACCATGGTTTGCAAATAGCTTGTC
		primer - WT only (3')	GTCACCTTGCTTCTTCTTCTTCTTTTCTTC
mir-72	*n4130*	primer - WT/deletion (5')	TTTAGGATTTAAAGGATTAAATACATTTCC
		primer - WT/deletion (3')	CTACGAAGTTTGTATGCCTACAGTAACC
		primer - WT only (3')	GCAGAATAGTAGTAGTAGAATAGGGGAATC
mir-73-74	*nDf47*	primer - WT/deletion (5')	GTCATTTCTACTTTTAATATTTGGGAAAGG
		primer - WT/deletion (3')	ACTAATCTTGAGCCTAATATGGATAAAAAG
		primer - WT only (5')	GATTAATCCTCTATATTTTCCTCCCTTC
mir-77	*n4286*	primer - WT/deletion (5')	AAGAATTGAGAAAACTGATATATAGTGCAA
		primer - WT/deletion (3')	GTATAATTTACCTTTGACAATTTGGAATAA
		primer - WT only (3')	AAACTCTACCTTTGTCTAACTCCAATAACT
mir-83	*n4638*	primer - WT/deletion (5')	TCTAAGGTCCTTTCCATTTCTTTCT
		primer - WT/deletion (3')	AAAACAGAGCTTTTCTCGTTGTCTA
		primer - WT only (5')	CAAACAGCACATACCTCTTTCTTTT
mir-85	*n4117*	primer - WT/deletion (5')	TATTTTAAAAGAGATTATCAAGATGCTTTG
		primer - WT/deletion (3')	AGAATAAAAGTGTAGAACGTGGAATATACA
		primer - WT only (3')	AGAAGAAGATCATGTAAGAATTCATTTTC
mir-124	*n4255*	primer - WT/deletion (5')	CTCTCTTCTACCAAACAACCAGTATTC
		primer - WT/deletion (3')	AAGTGTTTGTTCTAAAATTCAAGTCGT
		primer - WT only (5')	CTTTAGTGGACATCTAAGTCTTCCAAC
mir-228	*n4382*	primer - WT/deletion (5')	TTTGCTGTTCTCACGTATATAGAAAATAAT

Table 7.2. primers used for miRNA allele detection

gene	allele	description	primer - WT/deletion (5')
		primer - WT/deletion (3')	GAAATTATTAAACTTGTTCAATTTTTACGG
		primer - WT only (3')	AATAAAAGAATTTTTGAAGGCAGTAGTAGT
mir-234	*n4520*	primer - WT/deletion (5')	TACCAACAAAAAGAACGGGC
		primer - WT/deletion (3')	TTTGATTTTGCTGACGAAGC
		primer - WT only (3')	GCACATTTGAAACGACAGG
mir-235	*n4504*	primer - WT/deletion (5')	AATGAGCATGCTTTTACACTATAAATCTAC
		primer - WT/deletion (3')	ATACTAGTTCAGAACAATTTTAGTCCTGTG
		primer - WT only (3')	CTATCTATTCCTTATTCTTCCAAGTGCTAT
mir-237	*n4296*	primer - WT/deletion (5')	GAATGTACAAAAAGTTAATGCCGACTC
		primer - WT/deletion (3')	AAGATTTAAAAATGAGAGATCACATGG
		primer - WT only (3')	CCGTCGACGATTATCTAACACTTACTA
mir-238	*n4112*	primer - WT/deletion (5')	CATTAACAATATATAGGTTTTGCTCGTAAG
		primer - WT/deletion (3')	TTATAAAGTAAACTTGGAGAACTACAACCA
		primer - WT only (3')	TTAGATTCAGATATGTAACCAATCAAAAGT
mir-239a/b	*nDf62*	primer - WT/deletion (5')	AAATTTGAAGTAAATTGGACAATAACAATA
		primer - WT/deletion (3')	GTTATACTTGCTCTGATAATGAAATGGTAG
		primer - WT only (3')	TAAAATATACTAGACTTGGTGCACTTTTTC
mir-240 mir-786	*n4541*	primer - WT/deletion (5')	TCTGCAAGTACAATAAGAGAGAAAACA
		primer - WT/deletion (3')	TCTACATCAACATTTTGCATAAAGAAC
		primer - WT only (3')	AAAAAGTTTCTAACAACGAAATAGCAA
mir-244	*n4367*	primer - WT/deletion (5')	ATGTATCCTTAAAAACAAATGTAGCAGTTA
		primer - WT/deletion (3')	TAGTACTGTAATTGTTGTTCAAACCTTTTT
		primer - WT only (3')	CCTAAAAACGCAAAAATAGAATACACTTTA
mir-246	*n4636*	primer - WT/deletion (5')	GTTGATTGGTTAAATGATGAAAGTACACTA
		primer - WT/deletion (3')	TACAGTAATGTTTTGAAATGTTTTGTTATG

Table 7.2. primers used for miRNA allele detection

gene	allele	description	primer - WT/deletion (5')
		primer - WT only (3')	ATTTAGTTGTGAACAAAGAAAAATGATAAA
mir-247 mir-797	*n4505*	primer - WT/deletion (5')	AAGATGTTTTCTTTTCCACTACCAGT
		primer - WT/deletion (3')	ATTTGAATTTCTCTCCTGACTCTGTT
		primer - WT only (5')	AATTCAAAACAACACCTGTGAATATG
mir-259	*n4106*	primer - WT/deletion (5')	ATCACTTGATATCTACGTCAAAAAGAGTAT
		primer - WT/deletion (3')	ATTAATACGAATGATTTATTATCAGCACAT
		primer - WT only (3')	TCAAATTATACTTCTTTGATCTTCTCAAAA

7.2 Molecular Biology

All basic molecular biology techniques including PCR, restriction enzyme digestion of DNA, DNA purification, gel purification of DNA, DNA ligation, preparation of *E. coli* competent cells, transformation of *E. coli*, and plasmid DNA extractions were done according to Abbott Lab protocols or to manufacturers specifications where applicable.

7.3 Synchronization of worm cultures

Worm cultures were synchronized using the hypochlorite treatment as described in the Abbott Lab protocol manual. Typically, four plates containing high populations of embryos and/or gravid worms were collected by adding 2 mL of sterile water to each plate and collection of liquid from each plate into a 15 mL conical centrifuge tube. After centrifugation at slow speed, worm/embryo mix was treated with approximately 6 mL of bleach solution (0.1% hypochlorite, 1.25 M sodium hydroxide) for no more than 6 minutes with vigorous shaking using a

vortex. After bleaching, remaining “pellet” was washed at least twice with 15 mL of sterile water, and excess liquid was discarded from the pellet. After wash, 5-7 mL of S Medium was added and the resulting mix was transferred to a sterile petri plate and placed on a shaker at low speed. Worms were collected the next day. Concentration of L1s was estimated by counting L1s from a 2 µl drop.

7.4 Microscopy

Fluorescence and DIC microscopy was performed using a Nikon Eclipse 80i equipped with a Photometrics CoolSNAP HQ2 monochrome digital camera and RS Image software (Roper Scientific) or NIS Elements software (Nikon). Worms were anesthetized in 1mM levamisole and mounted onto a glass slide with a small pad made with 2% agarose in M9 media.

7.5 Extraction of total RNA from *C. elegans*

1000 Late L4 and L4 molt worms were collected for wild type, *mir-52*, and *mir-52/53/54/56* mutant worms in M9 Media. After collection worms were washed twice with fresh M9, allowed to incubate at room temperature (RT) for 15 min, and washed again with M9. Excess media was removed and volume was estimated. 1 volume of Trizol (Invitrogen) was added to the worm slurry and mixed using a pellet mixer (VWR). Trizol was added to a final 1 mL volume. Samples were frozen at −80˚C at this step. After samples thawed, 1/6th total volume of chloroform was added, followed by vortexing for 30 sec to 1 min. Samples were allowed to incubate for 3 min at RT prior to centrifugation at 10k x

g for 15 min at 4˚C. Aqueous layer was transferred to a new tube and volume was estimated. Equal volume of chloroform was added, samples were vortexed then centrifuged at 12k x g for 5 min at RT. Aqueous layer was transferred to a new tube, volume was estimated, and an equal volume of isopropanol and 20 ng glycogen was added. Sample was allowed to incubate at −20˚C for 1 hour prior to centrifugation at 10k x g for 10min at 4˚C. The supernatant was discarded and the RNA pellet was washed using 1 mL of 75% Ethanol in DEPC water solution. The pellet was allowed to dry at RT for 10min prior to resuspension in 200 µl preheated 68˚C DEPC Water. RNA samples were DNase treated (DNA-free Kit, Ambion) per manufacturers specifications. 1/10th volume of 3M sodium acetate, pH 5.2 and 1 volume phenol:chloroform was added. The sample was vortexed for 30 sec to 1 min, allowed to incubate at RT for 5 min, then centrifuged at 12k x g for 5 min at RT. Aqueous layer was transferrred to a new tube, equal volume of chloroform was added, the sample was vortexed, allowed to incubate at RT for 5 min, then centrifuged at 12k x g for 5 min. Aqueous layer was transferred to a new tube, 1.5 volumes of isopropanol and 20ng of glycogen was added. before incubation at −20˚C for 1 hour. Sample was allowed to incubate at −20˚C for 1 hour prior to centrifugation at 10k x g for 10min at 4˚C. The supernatant was discarded and the RNA pellet was washed using 1 mL of 75% Ethanol in DEPC water solution. The pellet was allowed to dry at RT for 10min prior to resuspension in 30-50 µl preheated 68˚C DEPC Water. RNA quality and quantity was determined using NanoDrop ND-1000 Spectrophotometer. RNA quality was

also assessed by gel electrophoresis with 100-1000 ng of total RNA loaded onto a 2% Agarose Gel in TBE Buffer plus ethidium bromide.

7.6 qRT-PCR

For analysis of *cul-1*, *lin-66*, and *vhp-1* transcript levels, reverse transcription of 500 ng of total RNA was prepared using the iScript cDNA synthesis kit (BioRad). cDNA was diluted with TE buffer before use or storage at −20˚C. 10 ng total RNA was used in 20 µl qPCR using iQ Sybr green Super Mix (BioRad). Primers, listed in Table 7.3, were used at a final concentration of 400nM. Primer efficiency was determined to be above 90% for each primer pair at various concentrations of cDNA obtained from wild type worms. Primer specificity was confirmed in each reaction by melting temperature analysis. Data was analyzed using $2^{-\Delta\Delta C}{}_t$ method with the mean of *ama-1* and *cdc-42* as reference (Livak and Schmittgen, 2001; Hoogewijs et al., 2008). 10 ng of total RNA was used to analyze the levels of mature miRNAs with Applied Biosystems Taqman miRNA assays following manufacturers protocol. Data was analyzed using $2^{-\Delta\Delta C}{}_t$ method with the mean of U18 and sn2343 as reference. Statistical analysis was performed comparing the mean fold change in expression from two independent biological replicates relative to wild type using student's t-test.

Table 7.3 Primers for molecular biology experiments

Use	Location/ Description[d]	Amplicon size	Sequence
mir-1 rescue	T09B4, 22804-25946	3143 bp	ATCTGCAGATTTCACATCCTTCGAACTTCTTC
			ATCCCGGGTTTTTAATGTGTTTGTCAGGTGTG
mir-59 rescue	B0035, 14594-17759	3166 bp	ATCCCGGGAGCTCTAGGTAATCTAGGCGTTCA
			ATCTGCAGTCCTCCTTTAGTTCAGCTTTCACT
mir-83 rescue	C06A6, 13348-16443	3096 bp	ACTGGTCGACCGTTGCTCTCAATTCTAAAAACCT
			ACTGCTGCAGATATTTGAAAGGAAAAAGGGTTCC
mir-124 rescue	C29E6, 5404-8667	3264 bp	ATCCCGGGCCAGTTTCTCATTATCTTCGGATT
			ATCTGCAGGGTTTGTCTGATCTTCATCACATC
mir-247/797 rescue	X: 4757213 - 4755181	2079 bp	CACCATATTCACAGGTGTTGTTTT
			TTGGAAGAAGAAAAATCAATCAAA
mir-259 rescue	F25D1, 9049-10772	1724 bp	ATCACTTGATATCTACGTCAAAAAGAGTAT
			CTTTAAAAGTCTTCTGGAAAAAGTGG
mir-228 rescue	T12E12, 22144-24235	2092 bp	ATCAAGCTTTTCCAAAACAGTTCCAAATTTCT
			ATCAAGCTTTAGGTGGCCGAGTTTTTGTATT
mir-238 rescue	K01F9, 2771-5902	3132 bp	ATCCCGGGTTCAATTTTCCAATCAACAATCAG
			ATCTGCAGTACTCAATCGTGCAATTTCTTCAT
mir-244 rescue	T04D1, 14821-17172	2352 bp	ACTGATGCGGCCGCTATTTTTGCGTTTTTAGGCTTAGG
			ACTGATGCGGCCGCGAAGAAGTCCAAACATCCTTGATT
*cul-1 3'*UTR	III: 10474775 - 10475541	767 bp	GTACCTCGCCTAATTCATTTCATT
			TCGAAGAAAATTACACAAAAACGA
*lin-66 3'*UTR	IV: 13892325 - 13893051	727 bp	GGTAACTGAGTGATTGTACATACCAAA
			TAAGGGAGAGAGCGAGAGACATA
*tlp-1 3'*UTR	IV: 13701131 - 13701749	619 bp	TGTTTTTCTAGCATTTTTCTGTCTG
			TTGAATTTTGTTTGATACTTTTAGTGG
*vhp-1 3'*UTR	II: 5340231 - 5341828	1598 bp	TTTTGTGAACATCATTCTCTAGTCCA
			CGTCACGCCCTTCTTCAATA
cul-1+++	III: 10469397 - 10475541	6145 bp	TTTCTTCCATACTGATTCTGACCA
			TCGAAGAAAATTACACAAAAACGA

Table 7.3 Primers for molecular biology experiments

Use	Location/ Description[d]	Amplicon size	Sequence
lin-66+++	IV: 13884127 - 13893051	8925 bp	TCAATGAACACTTTTCTCGAACAT
			TAAGGGAGAGAGCGAGAGACATA
tlp-1 qPCR	Bridges exon 3 and 4 of T23G4.1	83 bp	TGGCAAGCCATGTTAAGAAA
	Within exon 4 of T23G4.1		AGCTTTTCCCGTTGATGTTG
cul-1 qPCR	Bridges exon 4 and 5 of D2045.6	108 bp	CGGAAGAGCGTGATTAGTGC
	Within exon 6 of D2045.6		GAATCGACCGGAAAGTTGTG
lin-66 qPCR	Bridges exon 6 and 7 of B0513.1	145 bp	GTTTCTCGCCATCCAATCAT
	Within exon 7 of B0513.1		AAAGGGTCATAGTAGCGCTGAG
vhp-1 qPCR	Within exon 5 of F08B1.1a	105 bp	ATTATGCGCTACATGAAAATGG
	Within exon 6 of F08B1.1a		TTCAAGCAATTGTCCCATAAAA
B0513.1/*lin-66* RNAi	IV: 13888187 - 13892331	5857 bp	TGTCTTACGAAATGAATAGTCTCTT
			AGTTACCAATACGGAGTGAGTT
C06E1.3 RNAi[b]	III: 8581168 - 8583238	2071 bp	TGAAACTCAAAGAGGGATCATGT
			TTCCTTAGGTTAATGTGAGCCAA
C08B11.1/*zyg-11* RNAi[a]	II: 8018734 - 8019916	1183 bp	CAACTCCACTCGACTCGTCA
			CTCCAAGAGCTTTTCGCAAG
C16E9.4/*inx-1* RNAi[b]	X: 6947911 - 6948565	655 bp	CACATGGAAGACGCTCTTGA
			CTTTTTCACCGACTGCCTGT
C18F3.2/*sax-7* RNAi[a]	IV: 8077840 - 8078931	1092 bp	ACTCCACCTCATATCGTGCC
			CAGCCGGATAAAAATCCAGA
C45E5.6/*nhr-46* RNAi[a]	IV: 5748852 - 5749910	1059 bp	TCTGAGCCCGAAGAGTTTGT
			ATGTCATTGTTGCACGGTGT
F56F3.1/*pqn-45* RNAi[a]	III: 4472662 - 4473833	1172 bp	ATGGAACCACAGGTTGGTGT
			AAACGTGGCTAATCCAATGC
F58H1.5 RNAi[a]	V: 11960146 - 11962444	2299 bp	CGTTGCTGCTCTTTCAGTTTTAT
			TTCCATTGTAAACTTGGAGCTGT

Table 7.3 Primers for molecular biology experiments

Use	Location/ Description[d]	Amplicon size	Sequence
H01A20.1/*nhr-3* RNAi[a]	X: 14558660 - 14559803	1144 bp	GAGGCATCCGGAAGACATTA
			GGGTTTCGATCGACAAAAGA
Y64G10A.6 RNAi[a]	IV: 14277864 - 14280226	2363 bp	TCATTATGAAATTTGGGATTTCG
			TTCCCTGTATCTCCTACTCACCA
Y65B4BL.5/*acs-13* RNAi[c]	I: 511215 - 512142	928 bp	AAGGTGAGGGAAAATGGAAATAA
			ACCGGTTTCAATACATTTGTGAC
ZK131.11 RNAi[a]	II: 13814452 - 13817223	2772 bp	TGTTTCAAGTTTTCCTTCTTCCA
			GATTTAAGAAGATGGGGATGAGG
ZK673.2 RNAi[b]	II: 10448222 - 10449118	897 bp	TAATGGAATAATCGCCGAGG
			TCGAGTGCTTTTGAGTGGTG

[a] Primers from Kamath et al., 2003
[b] primers from Sönnichsen et al., 2005
[c] primers from Fraser et al., 2000)
[d] location is given as position within cosmid sequence or genomic position by linkage group.

7.7 Transgene Rescue experiments

To create transgenic animals, germline transformation was performed as described (Mello et al., 1991; Mello and Fire, 1995). Injection mixes contained 5-25 ng/µl of the rescue plasmid, 25-100ng/µl of a co-injection marker (*myo-2^{prom}::gfp* or *myo-2^{prom}::dsRed*) containing plasmid, and pRS413 plasmid for a final DNA concentration of 150 ng/µl. Transgenic animals expressing GFP or dsRed in the pharynx were assayed for alae formation or for gonad migration defects.

7.8 Transgene 3' UTR reporter experiments

The *syIs63* transgene was used to monitor *cog-1* repression in the presence of ectopic *lsy-6* expression driven from a transgene array expressing *cog-1*^{prom}*::lsy-6*^{hairpin} as described in Johnston and Hobert (2003). This array was chromosomally integrated to generate *otIs193* (kindly provided by L. Cochella and O. Hobert).

For *col-10*^{prom}*::gfp-PEST::3'UTR* constructs, the *cul-1*, *lin-66*, *tlp-1*, and *vhp-1* 3'UTRs were amplified by PCR using primers listed in Table 7.3. PCR products were inserted into the pAM1 vector containing gfp-PEST (Pro-Glu-Ser-Thr) (Frand et al., 2005). pAM1 was created by subcloning the gfp-PEST sequence from pAF207 (Frand et al., 2005) into the bluescript vector, prior to adding the *col-10*^{prom} sequence upstream of the gfp-PEST sequence. *col-10*^{prom}*::gfp-PEST::3'UTR* constructs for *cul-1Δ* and *lin-66Δ* were generated using Quikchange site directed mutagenesis (Stratagene) to remove the 6 nucleotide miR-51 family seed recognition sequence. Injection mixes contained 20 ng/µl of the *col-10*^{prom}*::gfp-PEST::3'UTR* construct along with 5-10 ng/µl of *myo-2*^{prom}*::dsRed* and 75-80 ng/µl 1kb DNA Ladder (New England Biolabs) for a final DNA concentration of 105 ng/µl. Multiple lines were identified for each reporter. Strains representing the different isolated lines are listed in Table 7.1. One representative transgenic line was used to compare GFP expression between wild type and *mir-52(n4114)* mutant worms.

7.9 RNAi experiments

Bacteria for RNAi experiments were isolated from the Ahringer RNAi library (Kamath et al., 2003). Bacterial clones were confirmed by sequencing, except for analyses performed in Appendix Figure 1. For clones not isolated from the RNAi library, genomic DNA was amplified using gene-specific primers previously used (Fraser et al., 2000; Kamath et al., 2003; Sönnichsen et al., 2005), cloned into the EcoRV site of pPD129.36 (Timmons and Fire, 1998), and transformed into *E. coli* strain HT115. RNAi bacteria was used to seed NGM plates supplemented with IPTG (1 mM) and ampicillin (100 µg/ml). Plates were allowed to incubate at room temperature for 24-48 hours before use. For analysis of *mir-51* family targets, synchronized L1s were transferred to RNAi bacteria and scored at the L4 molt. For additional experiments with RNAi of *cul-1*, *lin-66*, *tlp-1*, and *vhp-1*, L4 worms were plated onto RNAi bacteria and the progeny of these worms were scored.

7.10 DNA Sequencing

All DNA sequencing was performed by Functional Biosystems Company (Madison, WI).

APPENDIX

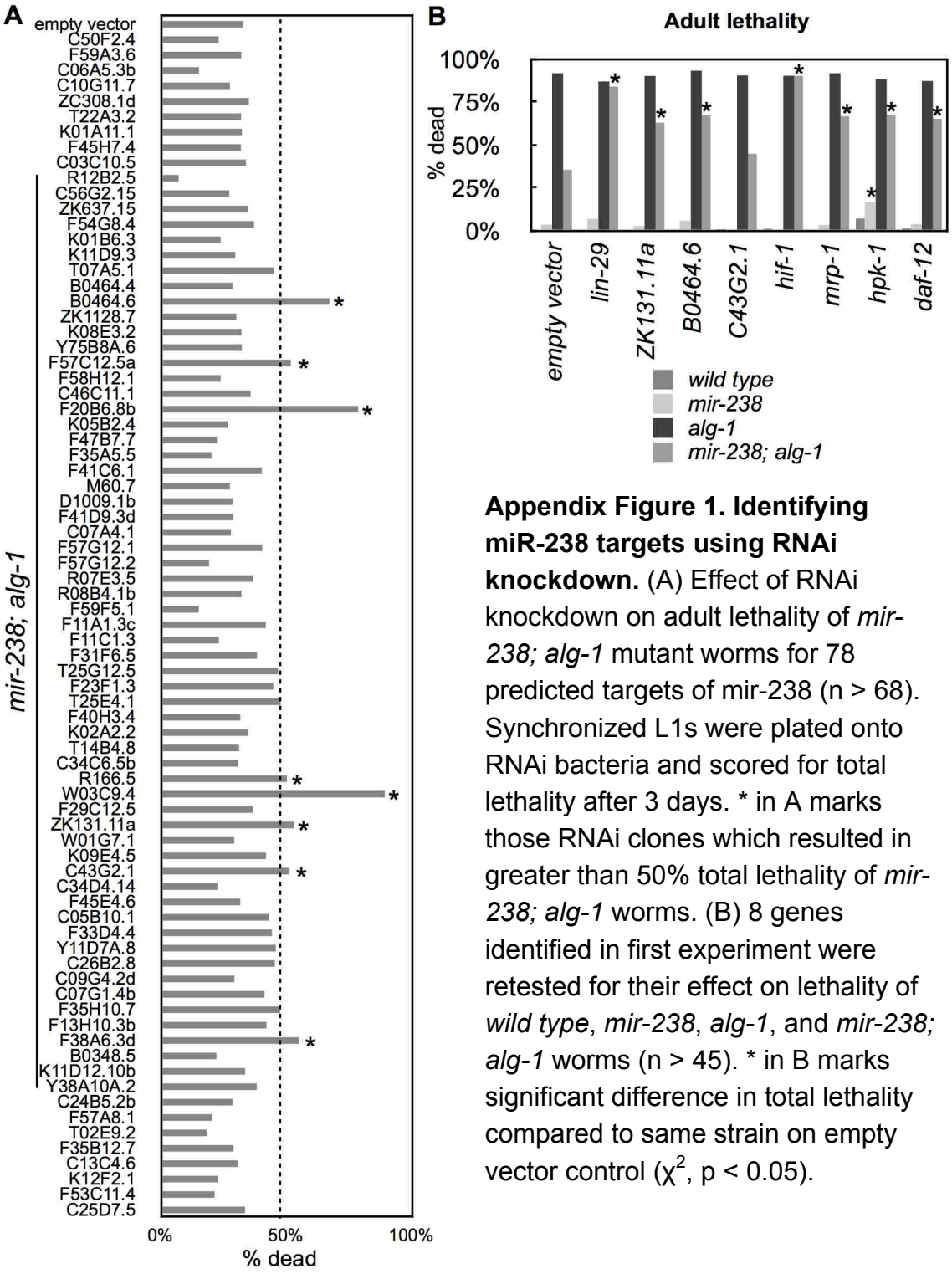
Identification of targets of miR-238

Results/Discussion

mir-238; alg-1 mutant worms displayed significantly less alae formation defects and adult lethality compared to *alg-1* mutants (Table 1). In order to identify and validate targets of *mir-238*, PicTar and Targetscan (version 3.0) algorithms were used to compile a list of 78 targets. To determine if these predicted targets function downstream of *mir-238* to mediate suppression of *alg-1*, RNAi was used to knockdown the activity of these 78 genes in *mir-238; alg-1* worms. In these worms, activity of key target genes is expected to be elevated due to the loss of miR-238. Their knockdown should therefore result in defects similar to *alg-1* worms, indicating a loss of *mir-238* mediated suppression. Knockdown of 8 candidates by RNAi enhanced the lethality of *mir-238; alg-1* mutants to greater than 50% (Appendix Figure 1A): *lin-29*, *ZK131.11a*, *B0464.6*, *C43G2.1*, *hif-1*, *mrp-1*, *hpk-1*, and *daf-12*. The effect of RNAi knockdown on these 8 genes on viability of *wild type*, *mir-238*, *alg-1,* and *mir-238; alg-1* worms was examined. Only knockdown of one of 8 had significant impact on viability of *mir-238* worms, *hpk-1* (Appendix Figure 1B). Knockdown of these 8 genes had no obvious impact on lethality of *alg-1* mutant worms. All, except *C43G2.1*, significantly increased the lethality observed in *mir-238; alg-1* (Appendix Figure 1B). These results are consistent with *lin-29*, *ZK131.11a*, *B0464.6*, *hif-1*, *mrp-1*,

hpk-1, and *daf-12* functioning downstream of *mir-238* to mediate suppression of *alg-1*. The identity of these RNAi clones has not been confirmed.

Further work is needed to support these results, including characterization of the effect of knockdown of these genes on formation of adult specific alae. Evidence is also needed to support that any of these genes are direct targets of miR-238. Two of these genes have previously characterized roles in developmental timing, *lin-29* and *daf-12*. *lin-29* is necessary to specify adult cell fates in the hypodermis (Rougvie and Ambros, 1995). LIN-29 levels may be increased in *mir-238; alg-1* compared to *alg-1* worms. However, this effect might be indirect through regulation of genes that function upstream of *lin-29*. For example, let-7 promotes *lin-29* expression through repression of *lin-41* (Reinhart et al., 2000). Further analysis is needed to determine if miR-238 regulates the *lin-29* UTR.



Appendix Figure 1. Identifying miR-238 targets using RNAi knockdown. (A) Effect of RNAi knockdown on adult lethality of *mir-238; alg-1* mutant worms for 78 predicted targets of mir-238 (n > 68). Synchronized L1s were plated onto RNAi bacteria and scored for total lethality after 3 days. * in A marks those RNAi clones which resulted in greater than 50% total lethality of *mir-238; alg-1* worms. (B) 8 genes identified in first experiment were retested for their effect on lethality of *wild type*, *mir-238*, *alg-1*, and *mir-238; alg-1* worms (n > 45). * in B marks significant difference in total lethality compared to same strain on empty vector control (χ^2, $p < 0.05$).

daf-12 regulates both entry into the dauer diapause and developmental timing (Antebi et al., 1998). If *mir-238* regulates *daf-12*, then it is expected that DAF-12 would be elevated in *mir-238; alg-1* worms. Elevated DAF-12 in the presence of dafachronic acid promotes the expression of the *let-7* family of miRNAs (Bethke et al., 2009). Increased expression of the *let-7* family members in *alg-1* mutant worms may alleviate the developmental timing defects in these worms, including the lethality due to ectopic entry into the molting cycle as adults. Future work can be directed at determining if levels of *let-7* family members are upregulated in *mir-238* mutant worms and whether miR-238 regulates the *daf-12* 3' UTR.

None of the other six genes have characterized roles in developmental timing. *hif-1* encodes a transcription factor whose expression is induced in stressful conditions, including low oxygen, and promotes a long lifespan in *C. elegans* (Mehta et al., 2009). *mrp-1* encodes the *C. elegans* homolog to the multidrug resistance protein, found to be highly expressed in many human cancers, which plays a role in early development for entry into the dauer diapause (Yabe et al., 2005). *hpk-1* encodes a kinase with homology to the dual-specificity protein kinase DYRK1A/*minibrain* but its function is unknown in *C. elegans* (Raich et al., 2003; Manning, 2005). The functions of *ZK131.11a*, and *B0464.6* are unknown. However, since knockdown of these genes enhance the adult lethality of *mir-238; alg-1* worms, its possible these genes have yet undescribed roles in developmental timing. Future work can be directed at

determining if these targets are direct targets of miR-238, and identifying their role, if any, in developmental timing.

REFERENCES

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

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

Alvarez-Saavedra, E., and Horvitz, H.R. (2010). Many families of C. elegans microRNAs are not essential for development or viability. Curr Biol *20*, 367–373.

Ambros, V. (1989). A hierarchy of regulatory genes controls a larva-to-adult developmental switch in C. elegans. Cell *57*, 49–57.

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

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

Ambros, V., Lee, R.C., Lavanway, A., Williams, P.T., and Jewell, D. (2003). MicroRNAs and other tiny endogenous RNAs in C. elegans. Curr Biol *13*, 807–818.

Antebi, A., Culotti, J.G., and Hedgecock, E.M. (1998). daf-12 regulates developmental age and the dauer alternative in Caenorhabditis elegans. Development *125*, 1191–1205.

Baek, D., Villén, J., Shin, C., Camargo, F.D., Gygi, S.P., and Bartel, D.P. (2008). The impact of microRNAs on protein output. Nature *455*, 64–71.

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

Behm-Ansmant, I., Rehwinkel, J., Doerks, T., Stark, A., Bork, P., and Izaurralde, E. (2006). mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. Genes Dev *20*, 1885–1898.

Beitzinger, M., Peters, L., Zhu, J.Y., Kremmer, E., and Meister, G. (2007). Identification of human microRNA targets from isolated argonaute protein complexes. RNA Biol *4*, 76–84.

Bernstein, E., Caudy, A.A., Hammond, S.M., and Hannon, G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature *409*, 363–366.

Bernstein, E., Kim, S.Y., Carmell, M.A., Murchison, E.P., Alcorn, H., Li, M.Z., Mills, A.A., Elledge, S.J., Anderson, K.V., and Hannon, G.J. (2003). Dicer is essential for mouse development. Nat Genet *35*, 215–217.

Beshore, E.L., McEwen, T.J., Jud, M.C., Marshall, J.K., Schisa, J.A., and Bennett, K.L. (2011). C. elegans Dicer interacts with the P-granule component GLH-1 and both regulate germline RNPs. Developmental Biology *350*, 370–381.

Bethke, A., Fielenbach, N., Wang, Z., Mangelsdorf, D.J., and Antebi, A. (2009). Nuclear hormone receptor regulation of microRNAs controls developmental progression. Science *324*, 95–98.

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

Brenner, S. (1974). The genetics of Caenorhabditis elegans. Genetics *77*, 71–94.

Buchan, J.R., and Parker, R. (2009). Eukaryotic Stress Granules: The Ins and Outs of Translation. Mol Cell *36*, 932–941.

Casadaban, M.J., Martinez-Arias, A., Shapira, S.K., and Chou, J. (1983). Beta-galactosidase gene fusions for analyzing gene expression in escherichia coli and yeast. Meth. Enzymol. *100*, 293–308.

Chang, S., Johnston, R.J., Frøkjaer-Jensen, C., Lockery, S., and Hobert, O. (2004). MicroRNAs act sequentially and asymmetrically to control chemosensory laterality in the nematode. Nat Cell Biol *430*, 785–789.

Corrêa, R.L., Steiner, F.A., Berezikov, E., and Ketting, R.F. (2010). MicroRNA-directed siRNA biogenesis in Caenorhabditis elegans. PLoS Genet *6*, e1000903.

Davis, E., Caiment, F., Tordoir, X., Cavaillé, J., Ferguson-Smith, A., Cockett, N., Georges, M., and Charlier, C. (2005). RNAi-mediated allelic trans-interaction at the imprinted Rtl1/Peg11 locus. Curr Biol *15*, 743–749.

de Lencastre, A., Pincus, Z., Zhou, K., Kato, M., Lee, S.S., and Slack, F.J. (2010). MicroRNAs both promote and antagonize longevity in C. elegans. Curr Biol *20*, 2159–2168.

Denli, A.M., Tops, B.B.J., Plasterk, R.H.A., Ketting, R.F., and Hannon, G.J. (2004). Processing of primary microRNAs by the Microprocessor complex. Nature *432*, 231–235.

Didiano, D., Cochella, L., Tursun, B., and Hobert, O. (2010). Neuron-type specific

regulation of a 3'UTR through redundant and combinatorially acting cis-regulatory elements. Rna *16*, 349–363.

Diederichs, S., and Haber, D.A. (2007). Dual role for argonautes in microRNA processing and posttranscriptional regulation of microRNA expression. Cell *131*, 1097–1108.

Ding, L., Spencer, A., Morita, K., and Han, M. (2005). The developmental timing regulator AIN-1 interacts with miRISCs and may target the argonaute protein ALG-1 to cytoplasmic P bodies in C. elegans. Mol Cell *19*, 437–447.

Djuranovic, S., Nahvi, A., and Green, R. (2011). A parsimonious model for gene regulation by miRNAs. Science *331*, 550–553.

Doench, J.G., and Sharp, P.A. (2004). Specificity of microRNA target selection in translational repression. Genes Dev *18*, 504–511.

Easow, G., Teleman, A.A., and Cohen, S.M. (2007). Isolation of microRNA targets by miRNP immunopurification. Rna *13*, 1198–1204.

Eisenmann, D.M., and Kim, S.K. (1997). Mechanism of activation of the Caenorhabditis elegans ras homologue let-60 by a novel, temperature-sensitive, gain-of-function mutation. Genetics *146*, 553–565.

Fielenbach, N., Guardavaccaro, D., Neubert, K., Chan, T., Li, D., Feng, Q., Hutter, H., Pagano, M., and Antebi, A. (2007). DRE-1: an evolutionarily conserved F box protein that regulates C. elegans developmental age. Dev Cell *12*, 443–455.

Filipowicz, W., Bhattacharyya, S.N., and Sonenberg, N. (2008). Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet *9*, 102–114.

Frand, A.R., Russel, S., and Ruvkun, G. (2005). Functional genomic analysis of C. elegans molting. PLoS Biol *3*, e312.

Fraser, A.G., Kamath, R.S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M., and Ahringer, J. (2000). Functional genomic analysis of C. elegans chromosome I by systematic RNA interference. Nature *408*, 325–330.

Friedman, R.C., Farh, K.K.-H., Burge, C.B., and Bartel, D.P. (2009). Most mammalian mRNAs are conserved targets of microRNAs. Genome Research *19*, 92–105.

Giraldez, A.J., Cinalli, R.M., Glasner, M.E., Enright, A.J., Thomson, J.M., Baskerville, S., Hammond, S.M., Bartel, D.P., and Schier, A.F. (2005). MicroRNAs regulate brain morphogenesis in zebrafish. Science *308*, 833–838.

Giraldez, A.J., Mishima, Y., Rihel, J., Grocock, R.J., van Dongen, S., Inoue, K., Enright, A.J., and Schier, A.F. (2006). Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. Science *312*, 75–79.

Grad, Y., Aach, J., Hayes, G.D., Reinhart, B.J., Church, G.M., Ruvkun, G., and Kim, J. (2003). Computational and experimental identification of C. elegans microRNAs. Mol Cell *11*, 1253–1263.

Greenwald, I., and Seydoux, G. (1990). Analysis of gain-of-function mutations of the lin-12 gene of Caenorhabditis elegans. Nature *346*, 197–199.

Greenwald, I.S., Sternberg, P.W., and Horvitz, H.R. (1983). The lin-12 locus specifies cell fates in Caenorhabditis elegans. Cell *34*, 435–444.

Grimson, A., Farh, K.K.-H., Johnston, W.K., Garrett-Engele, P., Lim, L.P., and Bartel, D.P. (2007). MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol Cell *27*, 91–105.

Grimson, A., Srivastava, M., Fahey, B., Woodcroft, B.J., Chiang, H.R., King, N., Degnan, B.M., Rokhsar, D.S., and Bartel, D.P. (2008). Early origins and evolution of microRNAs and Piwi-interacting RNAs in animals. Nature *455*, 1193–1197.

Grishok, A., Pasquinelli, A.E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D.L., Fire, A., Ruvkun, G., and Mello, C.C. (2001). Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. Cell *106*, 23–34.

Guo, H., Ingolia, N.T., Weissman, J.S., and Bartel, D.P. (2010). Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature *466*, 835–840.

Hammell, C.M., Lubin, I., Boag, P.R., Blackwell, T.K., and Ambros, V. (2009). nhl-2 Modulates microRNA activity in Caenorhabditis elegans. Cell *136*, 926–938.

Hammell, M., Long, D., Zhang, L., Lee, A., Carmack, C.S., Han, M., Ding, Y., and Ambros, V. (2008). mirWIP: microRNA target prediction based on microRNA-containing ribonucleoprotein-enriched transcripts. Nat Methods *5*, 813–819.

Hendrickson, D.G., Hogan, D.J., McCullough, H.L., Myers, J.W., Herschlag, D., Ferrell, J.E., and Brown, P.O. (2009). Concordant regulation of translation and mRNA abundance for hundreds of targets of a human microRNA. PLoS Biol *7*, e1000238.

Hoogewijs, D., Houthoofd, K., Matthijssens, F., Vandesompele, J., and Vanfleteren, J.R. (2008). Selection and validation of a set of reliable reference genes for quantitative sod gene expression analysis in C. elegans. BMC Mol. Biol. *9*, 9.

Hornstein, E., and Shomron, N. (2006). Canalization of development by microRNAs. Nat Genet *38 Suppl*, S20–S24.

Humphreys, D.T., Westman, B.J., Martin, D.I.K., and Preiss, T. (2005). MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. Proc Natl Acad Sci USA *102*, 16961–16966.

Huntzinger, E., and Izaurralde, E. (2011). Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nat Rev Genet *12*, 99–110.

Hutvágner, G., McLachlan, J., Pasquinelli, A.E., Bálint, E., Tuschl, T., and Zamore, P.D. (2001). A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science *293*, 834–838.

Jan, C.H., Friedman, R.C., Ruby, J.G., and Bartel, D.P. (2011). Formation, regulation and evolution of Caenorhabditis elegans 3'UTRs. Nature *469*, 97–101.

Johnson, S.M., Grosshans, H., Shingara, J., Byrom, M., Jarvis, R., Cheng, A., Labourier, E., Reinert, K.L., Brown, D., and Slack, F.J. (2005). RAS is regulated by the let-7 microRNA family. Cell *120*, 635–647.

Johnston, R.J., and Hobert, O. (2003). A microRNA controlling left/right neuronal asymmetry in Caenorhabditis elegans. Nature *426*, 845–849.

Kamath, R.S., Fraser, A.G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M., et al. (2003). Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. Nature *421*, 231–237.

Karginov, F.V., Conaco, C., Xuan, Z., Schmidt, B.H., Parker, J.S., Mandel, G., and Hannon, G.J. (2007). A biochemical approach to identifying microRNA targets. Proc Natl Acad Sci USA *104*, 19291–19296.

Karres, J.S., Hilgers, V., Carrera, I., Treisman, J., and Cohen, S.M. (2007). The conserved microRNA miR-8 tunes atrophin levels to prevent neurodegeneration in Drosophila. Cell *131*, 136–145.

Kato, M., de Lencastre, A., Pincus, Z., and Slack, F.J. (2009a). Dynamic expression of small non-coding RNAs, including novel microRNAs and piRNAs/21U-RNAs, during Caenorhabditis elegans development. Genome Biol *10*, R54.

Kato, M., Paranjape, T., Müller, R.U., Ullrich, R., Nallur, S., Gillespie, E., Keane, K., Esquela-Kerscher, A., Weidhaas, J.B., and Slack, F.J. (2009b). The mir-34 microRNA is required for the DNA damage response in vivo in C. elegans and in vitro in human breast cancer cells. Oncogene *28*, 2419–2424.

Ketting, R.F., Fischer, S.E., Bernstein, E., Sijen, T., Hannon, G.J., and Plasterk, R.H. (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes Dev *15*, 2654–2659.

Khan, A.A., Betel, D., Miller, M.L., Sander, C., Leslie, C.S., and Marks, D.S. (2009). Transfection of small RNAs globally perturbs gene regulation by endogenous microRNAs. Nat Biotechnol *27*, 549–555.

Kim, D.H., Liberati, N.T., Mizuno, T., Inoue, H., Hisamoto, N., Matsumoto, K., and Ausubel, F.M. (2004). Integration of Caenorhabditis elegans MAPK pathways mediating immunity and stress resistance by MEK-1 MAPK kinase and VHP-1 MAPK phosphatase. Proc Natl Acad Sci USA *101*, 10990–10994.

Kim, V.N. (2005). MicroRNA biogenesis: coordinated cropping and dicing. Nat Rev Mol Cell Biol *6*, 376–385.

Kim, V.N., Han, J., and Siomi, M.C. (2009). Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol *10*, 126–139.

Kipreos, E.T., Lander, L.E., Wing, J.P., He, W.W., and Hedgecock, E.M. (1996). cul-1 is required for cell cycle exit in C. elegans and identifies a novel gene family. Cell *85*, 829–839.

Kiriakidou, M., Tan, G.S., Lamprinaki, S., De Planell-Saguer, M., Nelson, P.T., and Mourelatos, Z. (2007). An mRNA m7G cap binding-like motif within human Ago2 represses translation. Cell *129*, 1141–1151.

Knight, S.W., and Bass, B.L. (2001). A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in Caenorhabditis elegans. Science *293*, 2269–2271.

Krek, A., Grün, D., Poy, M.N., Wolf, R., Rosenberg, L., Epstein, E.J., MacMenamin, P., da Piedade, I., Gunsalus, K.C., Stoffel, M., et al. (2005). Combinatorial microRNA target predictions. Nat Genet *37*, 495–500.

Labouesse, M., Sookhareea, S., and Horvitz, H.R. (1994). The Caenorhabditis elegans gene lin-26 is required to specify the fates of hypodermal cells and encodes a presumptive zinc-finger transcription factor. Development *120*, 2359–2368.

Lagos-Quintana, M., Rauhut, R., Lendeckel, W., and Tuschl, T. (2001). Identification of novel genes coding for small expressed RNAs. Science *294*, 853–858.

Lall, S., Grün, D., Krek, A., Chen, K., Wang, Y.-L., Dewey, C.N., Sood, P., Colombo, T., Bray, N., MacMenamin, P., et al. (2006). A genome-wide map of conserved microRNA targets in C. elegans. Curr Biol *16*, 460–471.

Lau, N.C., Lim, L.P., Weinstein, E.G., and Bartel, D.P. (2001). An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science *294*, 858–862.

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

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

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Rådmark, O., Kim, S., et al. (2003). The nuclear RNase III Drosha initiates microRNA processing. Nature *425*, 415–419.

Lee, Y., Kim, M., Han, J., Yeom, K.-H., Lee, S., Baek, S.H., and Kim, V.N. (2004a). MicroRNA genes are transcribed by RNA polymerase II. Embo J *23*, 4051–4060.

Lee, Y.S., Nakahara, K., Pham, J.W., Kim, K., He, Z., Sontheimer, E.J., and Carthew, R.W. (2004b). Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. Cell *117*, 69–81.

Lewis, B.P., Burge, C.B., and Bartel, D.P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell *120*, 15–20.

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

Lim, L.P., Lau, N.C., Garrett-Engele, P., Grimson, A., Schelter, J.M., Castle, J., Bartel, D.P., Linsley, P.S., and Johnson, J.M. (2005). Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature *433*, 769–773.

Lim, L.P., Lau, N.C., Weinstein, E.G., Abdelhakim, A., Yekta, S., Rhoades, M.W., Burge, C.B., and Bartel, D.P. (2003). The microRNAs of Caenorhabditis elegans. Genes Dev *17*, 991–1008.

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

Linsley, P.S., Schelter, J., Burchard, J., Kibukawa, M., Martin, M.M., Bartz, S.R., Johnson, J.M., Cummins, J.M., Raymond, C.K., Dai, H., et al. (2007). Transcripts targeted by the microRNA-16 family cooperatively regulate cell cycle

progression. Mol Cell Biol *27*, 2240–2252.

Liu, N., Bezprozvannaya, S., Williams, A.H., Qi, X., Richardson, J.A., Bassel-Duby, R., and Olson, E.N. (2008). microRNA-133a regulates cardiomyocyte proliferation and suppresses smooth muscle gene expression in the heart. Genes Dev *22*, 3242–3254.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25*, 402–408.

Lund, E., Güttinger, S., Calado, A., Dahlberg, J.E., and Kutay, U. (2004). Nuclear export of microRNA precursors. Science *303*, 95–98.

Manning, G. (2005). Genomic overview of protein kinases. WormBook : the Online Review of C Elegans Biology 1–19.

Maroney, P.A., Yu, Y., Fisher, J., and Nilsen, T.W. (2006). Evidence that microRNAs are associated with translating messenger RNAs in human cells. Nat Struct Mol Biol *13*, 1102–1107.

Martinez, N.J., Ow, M.C., Barrasa, M.I., Hammell, M., Sequerra, R., Doucette-Stamm, L., Roth, F.P., Ambros, V.R., and Walhout, A.J.M. (2008a). A C. elegans genome-scale microRNA network contains composite feedback motifs with high flux capacity. Genes Dev *22*, 2535–2549.

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

Mathonnet, G., Fabian, M.R., Svitkin, Y.V., Parsyan, A., Huck, L., Murata, T., Biffo, S., Merrick, W.C., Darzynkiewicz, E., Pillai, R.S., et al. (2007). MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F. Science *317*, 1764–1767.

Mehta, R., Steinkraus, K.A., Sutphin, G.L., Ramos, F.J., Shamieh, L.S., Huh, A., Davis, C., Chandler-Brown, D., and Kaeberlein, M. (2009). Proteasomal regulation of the hypoxic response modulates aging in C. elegans. Science *324*, 1196–1198.

Mello, C., and Fire, A. (1995). DNA transformation. Methods Cell Biol *48*, 451–482.

Mello, C.C., Kramer, J.M., Stinchcomb, D., and Ambros, V. (1991). Efficient gene transfer in C.elegans: extrachromosomal maintenance and integration of transforming sequences. Embo J *10*, 3959–3970.

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

Mizuno, T., Hisamoto, N., Terada, T., Kondo, T., Adachi, M., Nishida, E., Kim, D.H., Ausubel, F.M., and Matsumoto, K. (2004). The Caenorhabditis elegans MAPK phosphatase VHP-1 mediates a novel JNK-like signaling pathway in stress response. Embo J *23*, 2226–2234.

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

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

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

Mukherji, S., Ebert, M.S., Zheng, G.X.Y., Tsang, J.S., Sharp, P.A., and van Oudenaarden, A. (2011). MicroRNAs can generate thresholds in target gene expression. Nat Genet *43*, 854–859.

Nayak, S., Santiago, F.E., Jin, H., Lin, D., Schedl, T., and Kipreos, E.T. (2002). The Caenorhabditis elegans Skp1-related gene family: diverse functions in cell proliferation, morphogenesis, and meiosis. Curr Biol *12*, 277–287.

Nguyen, H.T., and Frasch, M. (2006). MicroRNAs in muscle differentiation: lessons from Drosophila and beyond. Curr Opin Genet Dev *16*, 533–539.

Nielsen, C.B., Shomron, N., Sandberg, R., Hornstein, E., Kitzman, J., and Burge, C.B. (2007). Determinants of targeting by endogenous and exogenous microRNAs and siRNAs. Rna *13*, 1894–1910.

Nix, P., Hisamoto, N., Matsumoto, K., and Bastiani, M. (2011). Axon regeneration requires coordinate activation of p38 and JNK MAPK pathways. Proc Natl Acad Sci USA *108*, 10738–10743.

Nolde, M.J., Saka, N., Reinert, K.L., and Slack, F.J. (2007). The Caenorhabditis elegans pumilio homolog, puf-9, is required for the 3'UTR-mediated repression of the let-7 microRNA target gene, hbl-1. Developmental Biology *305*, 551–563.

Nottrott, S., Simard, M.J., and Richter, J.D. (2006). Human let-7a miRNA blocks protein production on actively translating polyribosomes. Nat Struct Mol Biol *13*, 1108–1114.

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

Orsborn, A.M., Li, W., McEwen, T.J., Mizuno, T., Kuzmin, E., Matsumoto, K., and Bennett, K.L. (2007). GLH-1, the C. elegans P granule protein, is controlled by the JNK KGB-1 and by the COP9 subunit CSN-5. Development *134*, 3383–3392.

Pasquinelli, A.E., Reinhart, B.J., Slack, F., Martindale, M.Q., Kuroda, M.I., Maller, B., Hayward, D.C., Ball, E.E., Degnan, B., Müller, P., et al. (2000). Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature *408*, 86–89.

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

Petersen, C.P., Bordeleau, M.-E., Pelletier, J., and Sharp, P.A. (2006). Short RNAs repress translation after initiation in mammalian cells. Mol Cell *21*, 533–542.

Pettitt, J., Wood, W.B., and Plasterk, R.H. (1996). cdh-3, a gene encoding a member of the cadherin superfamily, functions in epithelial cell morphogenesis in Caenorhabditis elegans. Development *122*, 4149–4157.

Raich, W.B., Moorman, C., Lacefield, C.O., Lehrer, J., Bartsch, D., Plasterk, R.H.A., Kandel, E.R., and Hobert, O. (2003). Characterization of Caenorhabditis elegans homologs of the Down syndrome candidate gene DYRK1A. Genetics *163*, 571–580.

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

Rougvie, A.E., and Ambros, V. (1995). The heterochronic gene lin-29 encodes a zinc finger protein that controls a terminal differentiation event in Caenorhabditis elegans. Development *121*, 2491–2500.

Ruby, J.G., Jan, C., Player, C., Axtell, M.J., Lee, W., Nusbaum, C., Ge, H., and Bartel, D.P. (2006). Large-scale sequencing reveals 21U-RNAs and additional microRNAs and endogenous siRNAs in C. elegans. Cell *127*, 1193–1207.

Ruby, J.G., Jan, C.H., and Bartel, D.P. (2007). Intronic microRNA precursors that bypass Drosha processing. Nature *448*, 83–86.

Seggerson, K., Tang, L., and Moss, E.G. (2002). Two genetic circuits repress the

Caenorhabditis elegans heterochronic gene lin-28 after translation initiation. Developmental Biology *243*, 215–225.

Selbach, M., Schwanhäusser, B., Thierfelder, N., Fang, Z., Khanin, R., and Rajewsky, N. (2008). Widespread changes in protein synthesis induced by microRNAs. Nature *455*, 58–63.

Shaw, W.R., Armisen, J., Lehrbach, N.J., and Miska, E.A. (2010). The conserved miR-51 microRNA family is redundantly required for embryonic development and pharynx attachment in Caenorhabditis elegans. Genetics *185*, 897–905.

Simmer, F., Tijsterman, M., Parrish, S., Koushika, S.P., Nonet, M.L., Fire, A., Ahringer, J., and Plasterk, R.H.A. (2002). Loss of the putative RNA-directed RNA polymerase RRF-3 makes C. elegans hypersensitive to RNAi. Curr Biol *12*, 1317–1319.

Simon, D.J., Madison, J.M., Conery, A.L., Thompson-Peer, K.L., Soskis, M., Ruvkun, G.B., Kaplan, J.M., and Kim, J.K. (2008). The MicroRNA miR-1 Regulates a MEF-2-Dependent Retrograde Signal at Neuromuscular Junctions. Cell *133*, 903–915.

Slack, F.J., Basson, M., Liu, Z., Ambros, V., Horvitz, H.R., and Ruvkun, G. (2000). The lin-41 RBCC gene acts in the C. elegans heterochronic pathway between the let-7 regulatory RNA and the LIN-29 transcription factor. Mol Cell *5*, 659–669.

Sokol, N.S., and Ambros, V. (2005). Mesodermally expressed Drosophila microRNA-1 is regulated by Twist and is required in muscles during larval growth. Genes Dev *19*, 2343–2354.

Solomon, A., Mian, Y., Ortega-Cava, C., Liu, V.W.T., Gurumurthy, C.B., Naramura, M., Band, V., and Band, H. (2008). Upregulation of the let-7 microRNA with precocious development in lin-12/Notch hypermorphic Caenorhabditis elegans mutants. Developmental Biology *316*, 191–199.

Sopko, R., and McNeill, H. (2009). The skinny on Fat: an enormous cadherin that regulates cell adhesion, tissue growth, and planar cell polarity. Curr Opin Cell Biol *21*, 717–723.

Sönnichsen, B., Koski, L.B., Walsh, A., Marschall, P., Neumann, B., Brehm, M., Alleaume, A.-M., Artelt, J., Bettencourt, P., Cassin, E., et al. (2005). Full-genome RNAi profiling of early embryogenesis in Caenorhabditis elegans. Nature *434*, 462–469.

Steiner, F.A., Hoogstrate, S.W., Okihara, K.L., Thijssen, K.L., Ketting, R.F., Plasterk, R.H.A., and Sijen, T. (2007). Structural features of small RNA precursors determine Argonaute loading in Caenorhabditis elegans. Nat Struct Mol Biol *14*, 927–933.

Sternberg, P.W. (2005). Vulval development. WormBook : the Online Review of C Elegans Biology 1–28.

Thermann, R., and Hentze, M.W. (2007). Drosophila miR2 induces pseudo-polysomes and inhibits translation initiation. Nature *447*, 875–878.

Thomas, J.H. (1990). Genetic analysis of defecation in Caenorhabditis elegans. Genetics *124*, 855–872.

Thomas, M., Lieberman, J., and Lal, A. (2010). Desperately seeking microRNA targets. Nat Struct Mol Biol *17*, 1169–1174.

Timmons, L., and Fire, A. (1998). Specific interference by ingested dsRNA. Nature *395*, 854.

Wakiyama, M., Takimoto, K., Ohara, O., and Yokoyama, S. (2007). Let-7 microRNA-mediated mRNA deadenylation and translational repression in a mammalian cell-free system. Genes Dev *21*, 1857–1862.

Wang, B., Love, T.M., Call, M.E., Doench, J.G., and Novina, C.D. (2006). Recapitulation of short RNA-directed translational gene silencing in vitro. Mol Cell *22*, 553–560.

Wienholds, E., Koudijs, M.J., van Eeden, F.J.M., Cuppen, E., and Plasterk, R.H.A. (2003). The microRNA-producing enzyme Dicer1 is essential for zebrafish development. Nat Genet *35*, 217–218.

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

Wood, W.B. (1988). The Nematode Caenorhabditis Elegans (Cold Spring Harbor Laboratory Pr).

Wu, E., Thivierge, C., Flamand, M., Mathonnet, G., Vashisht, A.A., Wohlschlegel, J., Fabian, M.R., Sonenberg, N., and Duchaine, T.F. (2010). Pervasive and cooperative deadenylation of 3'UTRs by embryonic microRNA families. Mol Cell *40*, 558–570.

Wu, L., Fan, J., and Belasco, J.G. (2006). MicroRNAs direct rapid deadenylation of mRNA. Proc Natl Acad Sci USA *103*, 4034–4039.

Yabe, T., Suzuki, N., Furukawa, T., Ishihara, T., and Katsura, I. (2005). Multidrug resistance-associated protein MRP-1 regulates dauer diapause by its export activity in Caenorhabditis elegans. Development *132*, 3197–3207.

Yekta, S., Shih, I.-H., and Bartel, D.P. (2004). MicroRNA-directed cleavage of HOXB8 mRNA. Science *304*, 594–596.

Yigit, E., Batista, P.J., Bei, Y., Pang, K.M., Chen, C.-C.G., Tolia, N.H., Joshua-Tor, L., Mitani, S., Simard, M.J., and Mello, C.C. (2006). Analysis of the C. elegans Argonaute family reveals that distinct Argonautes act sequentially during RNAi. Cell *127*, 747–757.

Yoo, A.S., and Greenwald, I. (2005). LIN-12/Notch activation leads to microRNA-mediated down-regulation of Vav in C. elegans. Science *310*, 1330–1333.

Zhang, H., and Emmons, S.W. (2009). Regulation of the Caenorhabditis elegans posterior Hox gene egl-5 by microRNA and the polycomb-like gene sop-2. Dev Dyn *238*, 595–603.

Zhang, L., Ding, L., Cheung, T.H., Dong, M.-Q., Chen, J., Sewell, A.K., Liu, X., Yates, J.R., and Han, M. (2007). Systematic identification of C. elegans miRISC proteins, miRNAs, and mRNA targets by their interactions with GW182 proteins AIN-1 and AIN-2. Mol Cell *28*, 598–613.

Zhao, X., Yang, Y., Fitch, D.H.A., and Herman, M.A. (2002). TLP-1 is an asymmetric cell fate determinant that responds to Wnt signals and controls male tail tip morphogenesis in C. elegans. Development *129*, 1497–1508.

Zhao, Y., Ransom, J.F., Li, A., Vedantham, V., Drehle, von, M., Muth, A.N., Tsuchihashi, T., McManus, M.T., Schwartz, R.J., and Srivastava, D. (2007). Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. Cell *129*, 303–317.

Zisoulis, D.G., Lovci, M.T., Wilbert, M.L., Hutt, K.R., Liang, T.Y., Pasquinelli, A.E., and Yeo, G.W. (2010). Comprehensive discovery of endogenous Argonaute binding sites in Caenorhabditis elegans. Nat Struct Mol Biol *17*, 173–179.