

# Functional Analysis of MicroRNA Pathway Genes in the Somatic Gonad and Germ Cells During Ovulation in *C. Elegans*

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**FUNCTIONAL ANALYSIS OF MICRORNA PATHWAY GENES IN  
THE SOMATIC GONAD AND GERM CELLS DURING  
OVULATION IN *C. ELEGANS***

**by  
Carmela Rios**

**A Dissertation submitted to the Faculty of the Graduate School,  
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the Degree of Doctor of Philosophy**

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**ABSTRACT**  
**FUNCTIONAL ANALYSIS OF MICRORNA PATHWAY GENES IN**  
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**Carmela Rios**

**Marquette University, 2017**

MicroRNAs (miRNAs) are post-transcriptional regulators of gene expression that play critical roles in animal development and physiology, though functions for most miRNAs remain unknown. Worms with reduced miRNA biogenesis due to loss of Drosha or Pasha/DGCR8 activity are sterile and fail to ovulate, indicating that miRNAs are required for the process of oocyte maturation and ovulation. Starting with this penetrant sterile phenotype and using new strains created to perform tissue-specific RNAi, we characterize the roles of the *C. elegans* Pasha, *pash-1*, and two miRNA-specific Argonautes, *alg-1* and *alg-2*, in somatic gonad cells and in germ cells in the regulation of ovulation. Conditional loss of *pash-1* activity resulted in a reduced rate of ovulation and in basal and ovulatory sheath contractions. Similarly, knockdown of miRNA-specific Argonautes in the cells of the somatic gonad by tissue-specific RNAi results in a reduction of the ovulation rate and in basal and ovulatory sheath contractions. Reduced miRNA pathway gene activity resulted in a range of defects, including oocytes that were pinched upon entry of the oocyte into the distal end of the spermatheca in about 42% of the ovulation events observed following *alg-1* RNAi. This phenotype was not observed on worms exposed to control RNAi. In contrast, knockdown of *alg-1* and *alg-2* in germ cells results in few defects in oocyte maturation and ovulation. These data identify specific steps in the process of ovulation that require miRNA-specific Argonaute activity in the somatic gonad cells.

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Carmela Rios

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## CHAPTER ONE

### From microRNAs to Ovulation

#### I. Introduction

MicroRNAs (miRNAs) are one of several classes of small RNA molecules that have evolved to regulate the expression of genetic material. Other small RNAs include siRNAs and PIWI-interacting RNAs (piRNAs). The various small RNAs are distinguished from each other based on their length, the enzymes that process them, and the types of Argonaute (AGO) proteins with which they associate. Even still, there are areas of overlap among the different small RNAs, including enzymes that process them and the AGOs to which they associate (Table 1).

The first microRNA was discovered in *Caenorhabditis elegans* over 20 years ago, and since then this organism has been integral in the study of all small RNAs (Lee et al., 1993). Thanks to the work done in *C. elegans*, the role of miRNAs in humans is better understood. miRNAs are considered the dominant class of small RNAs in somatic tissue (Ha & Kim, 2014). They are estimated to target 60% of the protein coding genes (Friedman et al. 2009) in humans and their misregulation has been implicated in diverse diseases including heart disease (Xing et al., 2013), autism (Abu-Elneel et al., 2008), cancer (Calin et al., 2002) and antiviral defense (Lecellier et al., 2005). Continued miRNA research in *C. elegans* will lead to further insight of the function of these small RNA molecules.

#### II. Classes of small RNAs

The main classes of small RNAs that have evolved in animals to regulate genetic material include miRNAs, piRNAs, and endo-siRNAs. The majority of this chapter focuses on miRNAs, therefore in this section I will briefly describe the biogenesis and function of piRNAs and endo-siRNAs.

piRNAs are 21 nucleotides in length and are expressed in the animal gonads and function in genome surveillance (Claycomb 2014). piRNAs can act to regulate gene expression at both the transcriptional and post transcriptional level (Claycomb 2014). Targets for piRNAs are protein coding genes and transposable elements (Ketting 2011). In the germline, regulation somatically expressed protein coding genes is critical for the maintenance of germline integrity. Also, mobilization of transposable elements within the genome of an organism can be deleterious by interfering with protein coding genes and activating DNA damage response (Klattenhoff et al. 2007). piRNAs silence only a small group of known transposable elements in the *C. elegans* germline and have a larger role in the silencing of protein coding genes (Das et al. 2008).

Many of the details of piRNA biogenesis remain unknown. It is known that piRNAs are processed from a single stranded RNA precursors, that unlike miRNAs, contain no hairpin. The processing of the piRNAs does not require either Drosha or Dicer (Le Thomas et al. 2014). The only nuclease identified that processes piRNAs is Zucchini (the fly ortholog of the mouse MitoPLD). Activity of piRNAs utilizes a unique group of Argonautes (AGOs), Piwis (P-element induced wimpy testes), which are not used in either miRNA or siRNA activity (Table 1).

Endo-siRNAs (for exo-siRNAs see section III) are present in a variety of organisms and are diverse in both their biological functions and the factors required for their biogenesis (Table 1). In worms, endo-siRNA networks play a major role in regulating germline gene expression (Claycomb 2014). Two of the major classes of endo-siRNAs that exist in worms are the 26G-RNAs (26 nucleotide possessing a 5' guanine) and the 22G-RNAs. The 26G-RNAs function in regulating genes expressed during spermatogenesis and pseudogenes expressed during gametogenesis; the 22G-RNAs function in the majority of transposable element silencing (Das et al. 2008).

Table 1. Features and functions of small RNAs in *C. elegans*

small RNA	Length	AGO	Biogenesis factor	Function
miRNA	~22 nt.	ALG-1/2	Drosha/pasha Dicer	Post-transcriptional gene silencing in somatic tissue, mostly protein coding genes
piRNA	21 nt.	Piwi AGOs	Zucchini, Dicer independent	Germline genome surveillance, mostly by regulating protein coding gene and some transposable elements
Endo-siRNAs: 26G-RNAs	26 nt.	ERGO-1, ALG-3/4	Dicer, RdRP: RRF-3	Spermatogenesis mRNA regulation, pseudogenes
Endo-siRNAs: 22G-RNAs	22 nt.	Worm-specific Argonautes (WAGOs), CSR-1	Dicer independent, RdRPs: EGO-1, RRF-1	Silencing of transposable elements in germline, Chromosome segregation

*C. elegans* has an extensive and complex endo siRNA networks that are dependant on Dicer and RNA-dependent RNA-polymerases (RdRPs). The types of AGOs and RdRPs differ depending on if the siRNA is produced by an external trigger (exo-RNAi) or an endogenous one (Table 1).

### III. RNA Interference

RNA interference (RNAi) commonly refers to the process by which the introduction of an exogenous dsRNA with sequence complementarity to a gene leads to the silencing of that gene (exo-RNAi) (Fire et al., 1998; Rocheleau et al., 1997). Since the discovery of RNAi a variety of endogenous small RNA pathways have been described as well, which utilize overlapping machinery to promote gene silencing (Claycomb 2014; Ketting et al., 1998).

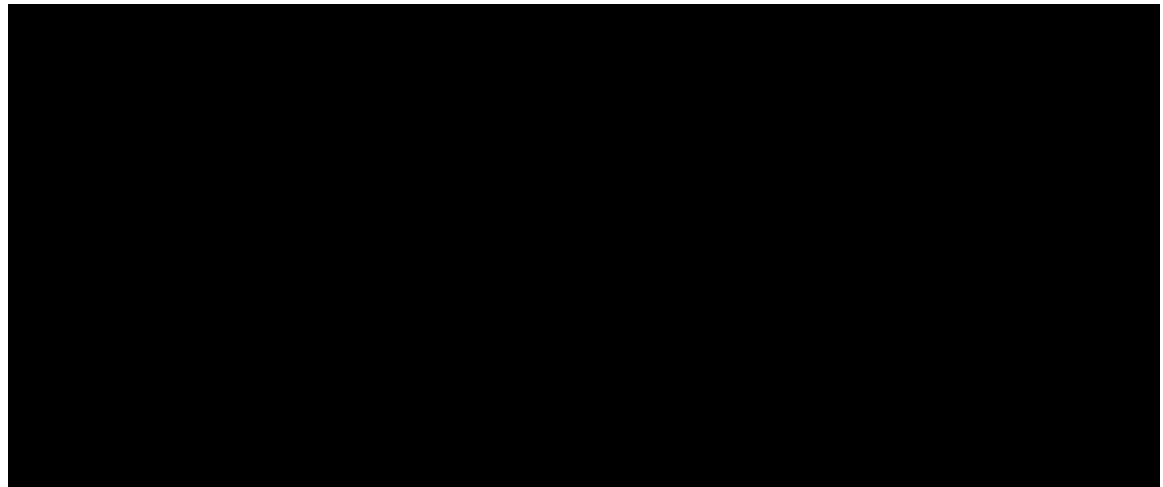
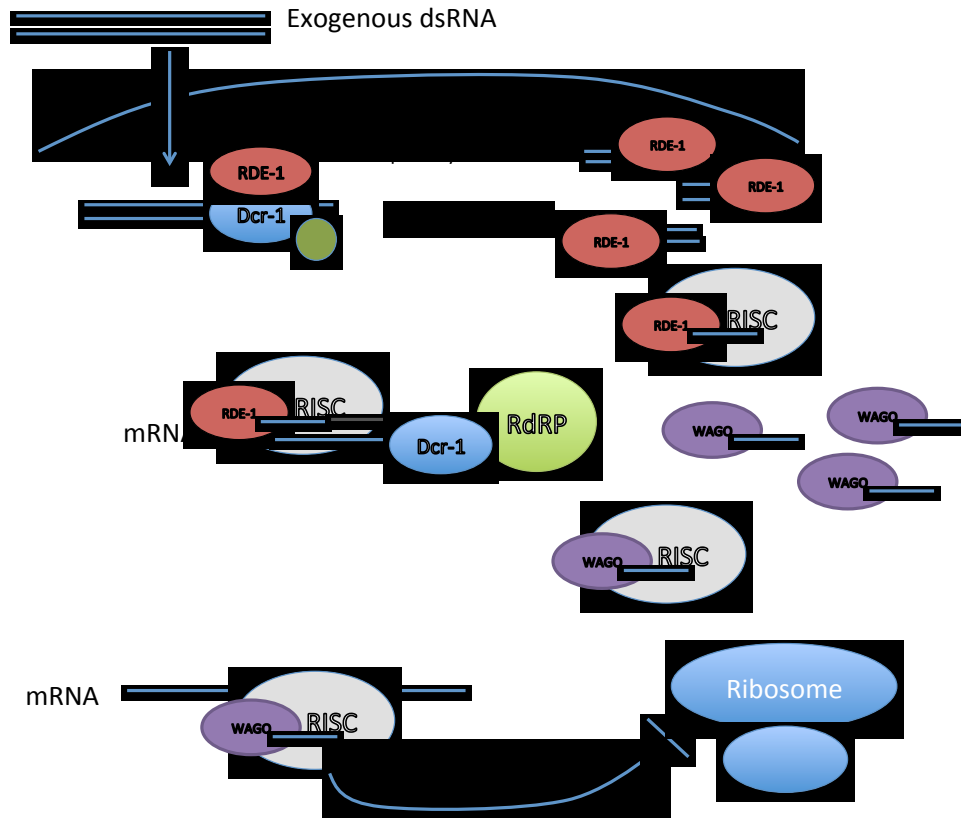
Andrew Fire, Craig Mellow and colleagues (Fire et al., 1998) observed that by injecting double stranded RNA (dsRNA) into *C. elegans*, the gene with complementarity was silenced. Interestingly it was observed that the double stranded RNA worked better than single stranded, it did not matter where the dsRNA was injected, or how little of the double stranded RNA was injected, silencing always occurred. It took years to tease the molecular mechanisms behind RNAi.

Exo-RNAi has a two-part mechanism, which is also found in the various endo-siRNA networks. Initially, the exogenous dsRNA is the substrate to produce primary siRNAs, which function as guides and primers in an amplification response, producing in the more abundant secondary siRNAs (Tsai et al., 2015). At this stage, Dicer in association with Argonaute RDE-1 interacts with the

exogenous dsRNA, producing ~21-nucleotide sections that constitute the primary siRNAs (Grishok et al., 2001; Ketting et al., 2001; Knight & Bass, 2001; Meister et al., 2004). The primary siRNAs are loaded onto the Argonaute RDE-1 to form the RISC complex (Tabara et al., 1999b), which associates with complementary mRNA to start the secondary or amplification response. In this stage, RNA polymerases (RdRPs) use the primary siRNA as primer and the mRNA as template to produce an antisense strand complementary to the mRNA (Pak & Fire, 2007). These secondary siRNAs are loaded to a set of other worm-specific Argonautes (WAGO), which mediate the silencing of the target gene (Yigit et al., 2006) (Fig. 1).

It is useful to point again that nematodes have 27 Argonautes (Youngman & Claycomb, 2014), which are key to the activity of the RISC complex in both RNAi and miRNA. The Argonaute activity are distinct. ALG-1 and ALG-2, which are required in miRNA activity are not required in response to RNAi and RDE-1 which is required for exo-RNAi is not a component of the miRNA pathway.

There are two known RdRp genes responsible for the amplification response of exo-RNAi. The first, *ego-1*, is expressed in germ cells and was identified by a germline RNAi defective loss-of-function mutant (Smardon et al., 2000). The second, *rrf-1*, is expressed in somatic cells and was identified by somatic RNAi-defective loss-of-function mutant (Sijen et al., 2001). *rrf-3* and *rrf-4* are other RdRP homologs that are not required for either somatic or germline exo-RNAi (Sijen et al., 2001).



Although RNAi has been widely used to describe the process by which the introduction of a dsRNA molecule complementary to gene leads to the silencing

of that gene (exo-RNAi), the term also refers to a variety of small RNA pathways (Claycomb 2014). These pathways require some of the same essential factors such as Argonautes and Dicer and are a means to regulate gene expression at both the transcriptional and post transcriptional level (Ketting 2011) (Table 1).

In my work, I take advantage of known genes required for miRNA biogenesis to knock down miRNA activity, by exo-RNAi, in a tissue specific manner. Upon knockdown of genes required for miRNA activity, I observe the miRNA-dependent process of ovulation. In order to perform RNAi in a tissue-specific manner, I had to generate novel mutants, which I discuss in the next two chapters.

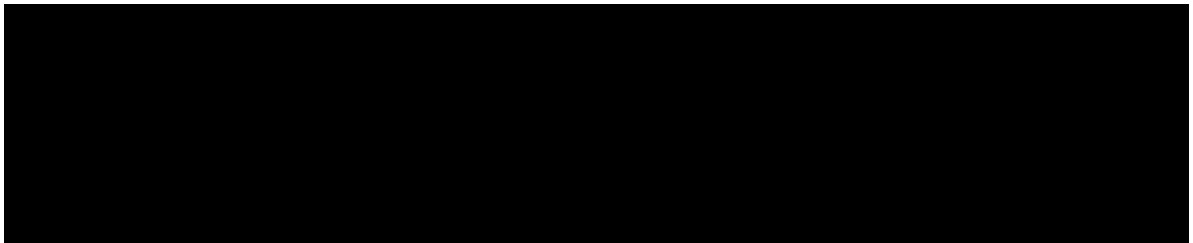
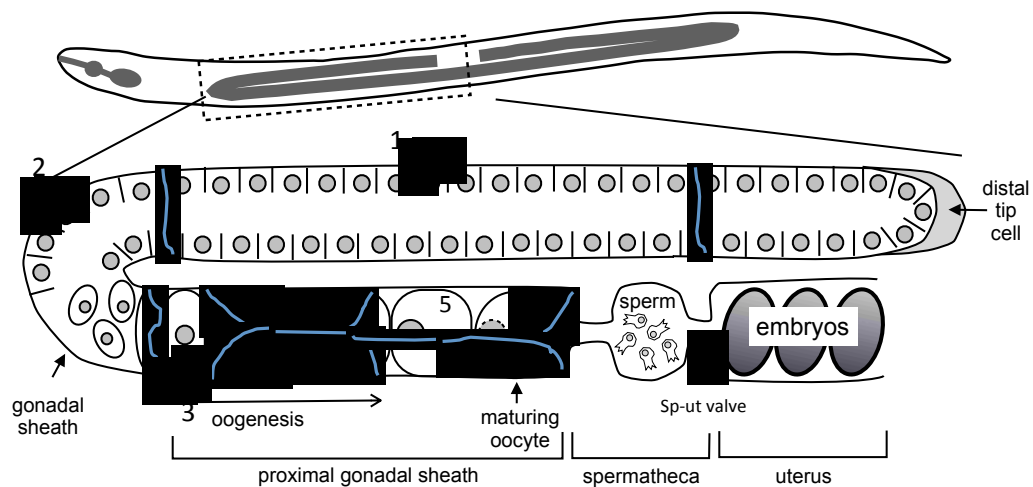
#### **IV. Anatomy of the Gonad**

A hermaphrodite worm contains two gonad arms, consisting of five somatic tissues, which form a tube-like structure around the germ cells (Fig. 2). The somatic tissues of the gonad include the sheath cells, distal tip cells, spermatheca, spermathecal-uterine valve and the uterus. All of these are derived from founder cells Z1 and Z4, which are present at the first larval stage. Founder cells Z2 and Z3 will give rise to the germ cells.

The germ line produces the gametes, egg and sperm. The spermatozoa are produced during the L4 stage, whereas oocytes are produced throughout the lifetime of the worm. In the adult hermaphrodite gonad the germ line shows progression in development starting at the distal end. Cells in the distal region near the DTC are in the mitotic zone; they are the stem cell population that form a syncytium, in which cells have only partial boundaries and surround a central

common cytoplasmic area, the rachis (Hirsh et al., 1976). As they move toward the bend of the gonad arm, cells begin meiosis I, progressing to diakinesis after the bend (Hubbard & Greenstein, 2000), where they arrest until oocyte maturation occurs in the most proximal oocyte (Fig. 2).

Each of the five somatic tissues has a specialized function. There are two distal tip cells, one on each distal end of the two gonad arms (Fig. 2). They have two main functions. The first is to lead the migration of the gonad arm during larval development. The second function is to regulate germ cell proliferation; this includes regulation of germ cell entry into mitosis and meiosis.





Five pairs of thin sheath cells surround the germ cells (Fig. 2). Gap junctions have been observed between each pair of sheath cells, adjacent sheath cells, and between sheath cells and oocytes (Hall et al., 1999). The proximal pairs of sheath cells (pair 3-5) are contractile, containing both thick (myosin) and thin (actin) filaments. The interaction of the actin and myosin filaments cause the sheath contractions that induce the movement of the oocytes through the gonad arm (Strome, 1986). The proximal sheath cells are similar to smooth muscle, but their contraction and relaxation is triggered by calcium sparks independent of nerve endings (Bui & Sternberg, 2002). The distal sheath cells (pair 1 and 2) also contain filaments, but in much lower quantity.

The spermatheca is an accordion-like structure composed of 26 cells (McCarter et al., 1997). The cells of the spermatheca contain microfilaments, but no myosin has been detected. When there is no oocyte in the spermatheca, the cell membranes are folded and the lumen is narrow. Once the oocyte enters the spermatheca, the membrane expands shaping around the oocyte. On the distal end of the spermatheca there is a syncytium made from the joining of four cells termed the sp-ut valve (Kimble & Hirsh, 1979), which dilates after fertilization and leads to the uterus.

## **V. Discovery of microRNAs**

A genetic screen searching for mutants with a lethality or sterility phenotype led to the identification of *let-7* (Meneely & Herman, 1979); shortly thereafter a screen searching for mutants defective in the normally invariant post-

embryonic cell lineages led to the identification of *lin-4* (Chalfie et al., 1981; Horvitz & Sulston, 1980). Both *let-7* and *lin-4* homozygous mutants have developmental timing defects, hence their reference early on as heterochronic genes. *let-7* mutants die as early as the 3<sup>rd</sup> larval stage (Meneely & Herman, 1979) and *lin-4* mutants exhibit the first larval stage cell lineage patterns in later larval stages, while also exhibiting an extra larval molt (Ambros & Horvitz, 1984).

Identification and cloning of the *lin-4* gene in *C. elegans* revealed that it does not code for a protein, but rather an RNA that exists in long and short forms (61 and 22 nucleotides) (Lee et al., 1993). In addition, the 22-nucleotide RNA product is complementary to repeated sequences of the 3'UTR on *lin-14* mRNA (Lee et al., 1993; Ruvkun et al., 1989). Interestingly, loss-of-function mutations in the *lin-14* gene were found to cause the opposite phenotype of the mutation in *lin-4*; among other observations, this led to the model that *lin-4* negatively regulates *lin-14* and *lin-28* (Wightman et al., 1993; Lee et al., 1993; Moss et al., 1997). In conclusion, the small RNA *lin-4* is key in the regulation of the L1-L3 larval stages.

*let-7* was brought to the forefront due to its sequence conservation across animal phyla and its importance in temporal development among several animal species; this conservation does not exist for *lin-4* (Pasquinelli et al., 2000). Due to the transient expression of both *let-7* and *lin-4* they were initially termed small temporal RNAs. It was soon realized however, that *lin-4* and *let-7* were only a small fraction of a large class of small RNAs, most of which were not expressed temporally, and were later named microRNAs (miRNAs). Soon thereafter, it was

discovered that some of the machinery used to process these temporal RNAs, was also used in the RNAi pathway (Grishok et al., 2001).

## **VI. MicroRNA Biogenesis**

My work investigates the role of miRNA-specific biogenesis genes in the process of ovulation. It is therefore critical to have a full understanding of the miRNA biogenesis process leading me to go into more depth in this section than in others.

### **A. Transcription**

miRNA genes are found throughout the genomes of plants, animals, and even viruses. They reside in intergenic (between protein-coding genes) regions, in antisense orientation to protein-coding genes, or in intronic (within protein coding genes) regions (Lee et al., 2004; Monteys et al., 2010). Intergenic miRNA genes are often found in clusters, in some cases with only a few nucleotides separating individual miRNA genes (Lau et al., 2001).

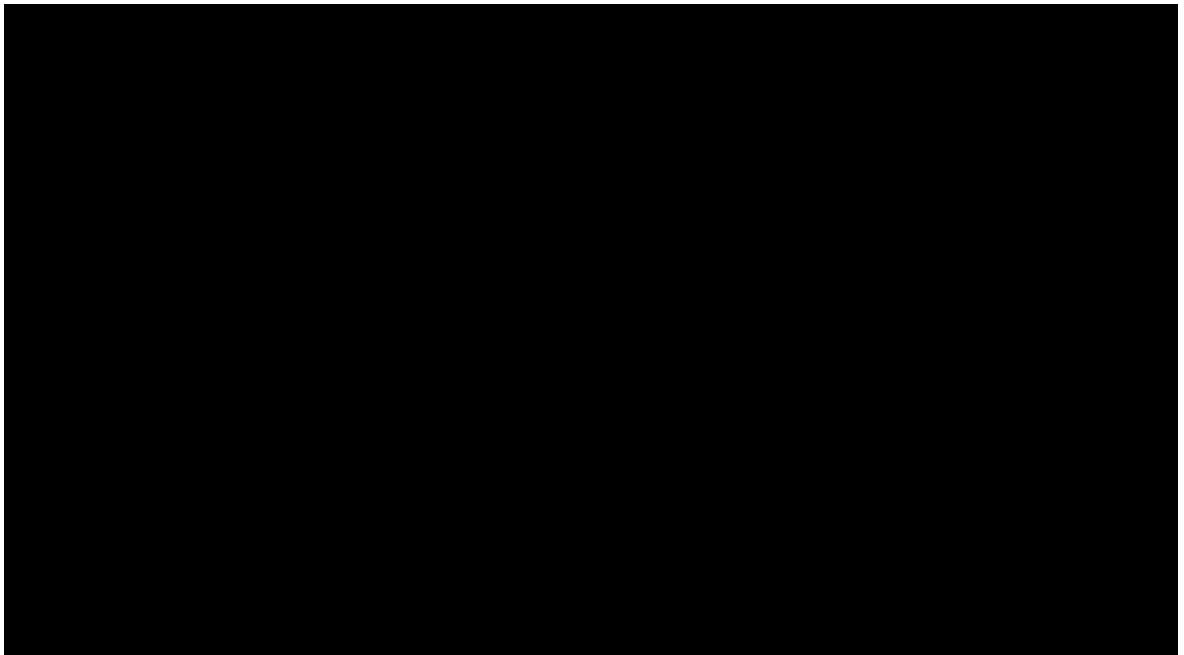
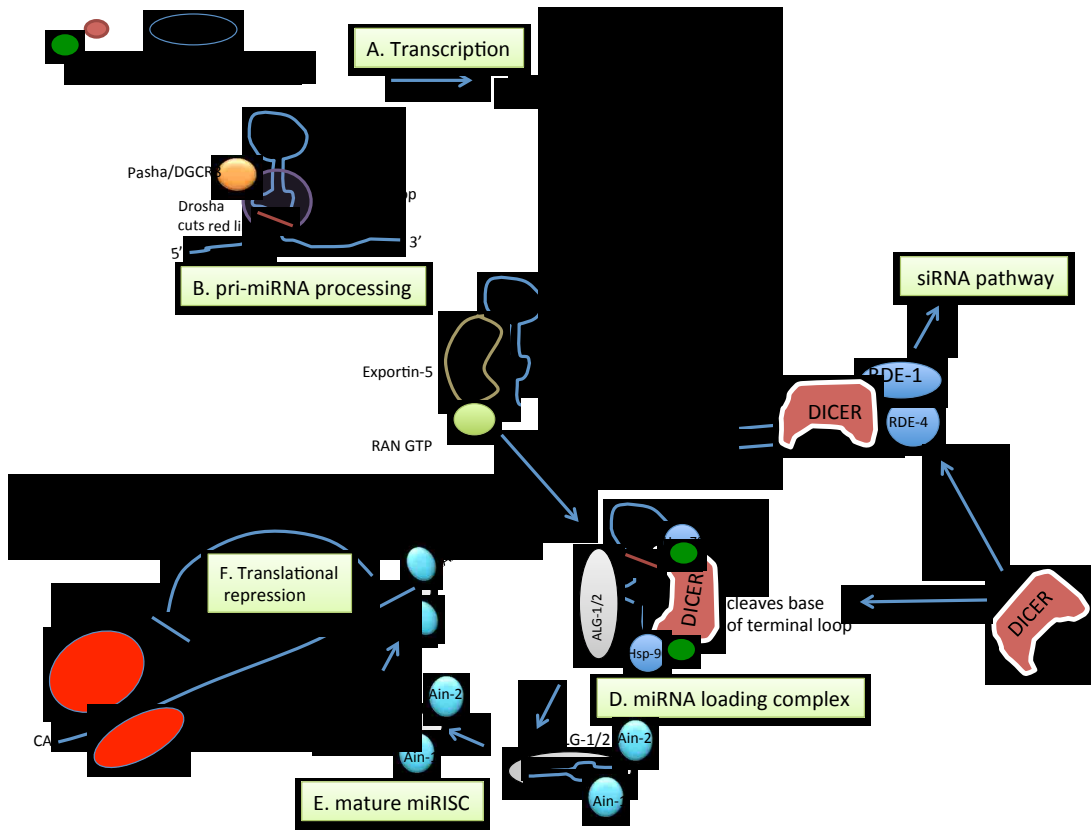
Although there is some knowledge of how miRNA expression is controlled, much uncertainty remains. Clusters of miRNA genes may be transcribed together under the control of a single promoter (Lau et al., 2001). Intronic miRNA genes were originally thought to be under the regulation of the host promoter; while this is true for most, about one third have upstream regulatory elements consistent with a promoter region (Monteys et al., 2010).

The transcription of most miRNA genes is performed by RNA Polymerase II (Lee et al., 2004) (Fig. 3). Exceptions include viral miRNAs (Pfeffer et al., 2005) and microRNAs produced via a non-canonical biogenesis pathway (Babiarz et

al., 2008), which are transcribed by RNA Polymerase III. Similar to protein coding genes, these initial transcripts contain a cap and poly A tail, but lack the long open reading frame that codes for the protein product (Cai et al., 2004; Lee et al., 2004b). The initial transcript is referred to as the primary miRNA transcript (pri-miRNA), which has to undergo nuclear processing before it can be recognized and exported out of the nucleus (Lee et al., 2002). The pri-miRNA can be longer than 1 kilobase. It contains a central stem of approximately 35 base pairs in length that houses the miRNA sequence, a terminal loop, and single-stranded RNA segments at both the 5' and 3' ends (Ha & Kim, 2014) (Fig. 3).

### **B. Nuclear Processing**

In the nucleus, the pri-miRNA is processed by the Microprocessor complex, consisting of Drosha, its cofactor Pasha (mammalian DGCR8) and other components into a ~60 nucleotide pre-miRNA (Denli et al., 2004; Han et al., 2004; Lee et al., 2002). Drosha is an RNase III-type enzyme of 160 kDa, which binds to the open ended region of the pri-miRNA hairpin (Lee et al. 2003; Ha and Kim 2014). RNase III enzymes are double stranded RNA (dsRNA)-specific and produce cuts on both sides of an RNA helix (Zamore 2001). DGCR8/Pasha, a 90KD dsRNA-binding protein, is recruited by Drosha and provides additional RNA-binding activity (Ha and Kim 2014). Key components of the pri-miRNA required for Drosha activity include the 10bp terminal loop and the ~30 bp stem (Zeng et al. 2005). Drosha cleaves approximately 22 bp down from the



terminal loop, leaving a 2-nucleotide, 3' overhang (Zeng et al., 2005) (Fig. 3). The product of Drosha cleavage, the pre-miRNA, is exported out of the nucleus by a specialized export factor Exportin 5, which binds in the presence of Ran-GTPase (Lund et al., 2004; Yi et al., 2003) (Fig. 3). The hydrolysis of Ran-GTP to Ran-GDP induces release of the pre-miRNA in the cytoplasm (Yi et al., 2003).

### **C. Cytoplasmic Processing and Activity**

Once in the cytoplasm, the pre-miRNA is recognized and processed by a second RNAase III endonuclease, Dicer (200kDa). Whereas Drosha and cofactor DGCR8/Pasha exclusively process pri-miRNAs, Dicer functions in the processing of pre-miRNAs and other siRNAs (Ha & Kim, 2014; Ketting et al., 2001) (Fig. 3). Nematodes and mammals have only one Dicer gene; other organisms such as *Drosophila* have two: Dicer-1, which functions in miRNA biogenesis, and Dicer-2 for siRNA biogenesis (Lee et al., 2004). Because worms have only one Dicer, it has been observed that competition for Dicer activity exists among the different small RNA pathways (Zhuang et al., 2012). Dicer's PAZ domain recognizes the 2 nucleotide 3' overhang, and cleavage occurs approximately 21-25 nucleotides from the overhang (MacRae et al., 2007). The cleavage results in a 3' 2 base pair overhang at the base of the terminal loop, which is discarded, leaving a ~22nt dsRNA consisting of the guide and "passenger" (Fig. 3).

After processing by Dicer, the dsRNAs containing miRNAs or other siRNAs will associate with AGO proteins, which are an essential component of all currently known small RNA pathways (Hutvagner & Simard, 2008). AGOs

form the catalytic center of the RNA-Induced Silencing Complex (RISC). They contain two characteristic PAZ domains (also found in Dicer), which bind to the 2-nt, 3' overhang end generated by RNase III enzymes (Cerutti et al., 2000).

Individual AGOs associate specifically with a subset of small RNAs. In *Drosophila*, AGO-1 associates with miRNAs and AGO-2 with siRNAs (Hammond et al., 2001; Okamura et al., 2004). Nematodes have 27 AGO genes (Youngman & Claycomb, 2014); out of these, genetic and biochemical studies have demonstrated that only ALG-1 and ALG-2, are required for the miRNA pathway, (Grishok et al., 2001; Hutvagner et al., 2004). Also in worms, the Argonaute RDE-1, functions exclusively in the RNAi pathway (Tabara et al., 1999).

The specificity by which individual AGOs associate with small RNAs suggests there are characteristics in the dsRNAs that make this specificity possible. One of the determinants of AGO selection is the physical structure of the small RNA (Jannot et al., 2008; Steiner et al., 2007). The “bulge” found on the dsRNA, consisting of unpaired nucleotides, prevents the ~22-nt dsRNA duplex from entering the RNAi pathway and increases its ability to enter the miRNA pathway (Steiner et al., 2007; Jannot et al., 2008). A second factor that directs a small RNA to a specific pathway are the binding proteins that associate with Dicer. For example, the loading protein R2D2 in *Drosophila* associates with Dicer-2 to load the dsRNA onto AGO-2, forming a siRNA-induced silencing complex (siRISC). Another example is the association of *Drosophila's* Dicer-1 with one of two isoforms of Loquacious to load the dsRNA duplex onto AGO-, forming a miRNA-induced silencing complex (miRISC) (Forstemann et al., 2005; Fukunaga et al., 2012; Saito et al., 2005). In worms, the RNA binding proteins

that facilitate loading onto ALG-1 and ALG-2 are unknown, but in the siRNA pathway, RDE-4 associates with RDE-1 (Tabara et al., 1999). Human small RNA pathways do not fit into this paradigm because the four existing AGO proteins (AGO1-4) can associate with both siRNAs and miRNAs (Liu et al., 2004; Meister et al., 2004; Yoda et al., 2010).

In *C. elegans*, the dsRNA duplex is initially loaded onto ALG-1 or ALG-2 in a complex with Dicer and other protein factors, including chaperone proteins Hsp70/Hsp90 (Iwasaki et al., 2010); this is often referred to as the miRNA loading complex (Jannot et al., 2008; Liu et al., 2012). It is in this complex where the “guide” strand is retained and the “passenger” (also known as the “star” or \* ) strand is released and degraded (Yoda et al., 2010). In *C. elegans* the sequence and position of the duplex facilitates guide strand selection; there does not seem to be a strong bias for either the 3 prime or 5 prime strand of the duplex to become the passenger or the guide strand and even for a specific miRNAs the roles can change (Zinovyeva et al., 2015). In addition, it has been observed that the passenger strand also has some silencing ability (Ha & Kim, 2014). Recently, ALG-1 was identified as having a key role in both selection of the guide strand and disposal of passenger strand (Ha & Kim, 2014; Zinovyeva et al., 2015). Once the guide strand is retained and the passenger strand is released and degraded, the mature miRISC complex is formed (Fig. 3).

The miRISC functions to repress translation, with the guide strand identifying the target mRNA through imperfect base complementarity. The mechanism by which miRISC repress translation is not completely understood.



While different models have been proposed, evidence supports that miRISC reduces mRNA abundance by repressing translation first and subsequently initiating mRNA degradation (Catalanotto et al., 2016). mRNA degradation is thought to largely be accomplished by deadenylation of the target mRNA (Filipowicz et al., 2008). On the other hand, AGOs that function in the siRNA pathway contain a PIWI domain with active slicer activity, which gives the complex the ability to cleave the target mRNA (Song, Smith, Hannon, & Joshua-Tor, 2004). These “slicer” acting AGOs include RDE-1 in worms, ALG-2 in *Drosophila*, and AGO2 in humans. In humans however, AGO2 can also associate with miRNAs as part of the miRNA-RISC complex (Ha & Kim, 2014). The mechanism of translational repression is less clear. There is evidence that translation is repressed at initiation and post-initiation steps (Chekulaeva & Filipowicz, 2009).

AGOs recruit proteins AIN-1 and AIN-2 (orthologs to human and *Drosophila* GW182) into the *C. elegans*-miRISC via their tryptophan binding pockets (Jannot et al., 2008). However, the role of AIN-1 and AIN-2 in the miRISC is not well understood. Embryonic lethality resulting from loss-of-function mutation in *alg-1* and *alg-2* can be rescued by an *alg-1* allele with a mutation in both tryptophan residues, this suggests that AIN-1 and AIN-2 are not required in the miRISC during embryonic development (Jannot et al., 2016).

#### **D. Non-canonical miRNA biogenesis pathways**

Production of some animal miRNAs occurs via a pathway that bypasses Drosha. In these cases, the pre-miRNA is spliced directly from introns during

mRNA processing; the products are referred to as mirtrons (Berezikov et al., 2007). There are 4 known mirtrons in worms and 11 in flies. After processing by debranching enzymes, the initial spliced intron forms a fold similar to that of a pre-miRNA. This fold enables association with Exportin-5, allowing its export from the nucleus, where it is then cleaved by Dicer and loaded onto an AGO (Westholm & Lai, 2011).

Deep sequencing of small RNAs in Drosha, DGCR8 and Dicer mutants has identified miRNAs that bypass Drosha separate from mirtrons. These miRNAs include endogenous short-hairpin RNAs produced through transcription that can also be processed by Dicer and form a functional RISC (Chong et al., 2010). Deep sequencing analysis of small RNAs from *dcr-1(0)* homozygous population derived from heterozygous parents revealed the existence of “pseudo-miRNAs” carrying 2-5nt. extensions on either the 5' or the 3' end of miRNAs (Drake et al., 2014).

## **VII. Functions of miRNAs**

### **A. *lin-4* and *let-7* miRNAs function in developmental timing.**

The development of the adult body plan requires tight control of cell division and differentiation. In *C. elegans*, the genes that control developmental timing, referred to as heterochronic genes, have been well characterized (Rougvie, 2001). Ambros and Horvitz (Ambros & Horvitz, 1984) were the first to describe heterochronic mutants with abnormal cell fate phenotypes. Two of these

phenotypes were the result of mutations of miRNAs *lin-4* and *let-7* (see section I).

Early in post-embryonic development, *lin-4* regulates the transition through the first three larval stages by downregulating *lin-14* and *lin-28* (Lee et al., 1993; Moss et al., 1997; Wightman et al., 1993). Later, during the fourth larval stage, the worm must transition into the adult form. This transition is facilitated by the expression of miRNA *let-7*, which downregulates *hbl-1*, *lin-41*, and *daf-12*, allowing for the expression of the adult-specific transcription factor *lin-29* (Abrahante et al., 2003; Grosshans et al. 2005; Lin et al., 2003; Slack et al., 2000).

Initial *let-7* targets were identified upon finding complementary sequences in the 3' UTR and genetic analysis of loss-of-function phenotypes. For example, *hbl-1* and *lin-41* loss-of-function mutations cause early expression of seam-cell fate, and both partially suppress *let-7* loss-of-function mutation (Grosshans et al., 2005; Lin et al., 2003; Slack et al., 2000; Vella et al., 2004). The first direct demonstration of a miRNA bound to its target in vitro was with *let-7* binding to *lin-41* (Vella et al., 2004).

### **B. miRNAs belonging to the same family may act redundantly.**

Loss-of-function phenotypes also helped demonstrate redundancy in miRNA activity. For example, mutations in the adult transcription factor *lin-29* results in a heterochronic defect whereby the fourth larval stage is repeated indefinitely. A mutation in miRNA *let-7* results in a less severe defect whereby the fourth larval stage is only repeated once. This observation suggests that *let-7*

may not be the only negative regulator in this genetic pathway (Abbott et al., 2005).

This functional redundancy often occurs between miRNAs of the same family; miRNA family members contain the same sequence in ~ 8 nucleotides on the 5' end (the 'seed') (Bartel, 2009; Lim et al., 2003). The seed is the most important factor in determining target regulation (Bartel, 2009), which is likely why miRNAs within the same family often exhibit functional redundancy. *mir-48*, *mir-84*, and *mir-241* belong to the same family as *let-7* (Lau et al., 2001; Lim et al., 2003) and are examples of miRNAs that act redundantly. The regulators of a *let-7* target, *hbl-1*, were identified genetically as the *let-7* family members: *mir-48*, *mir-84*, and *mir-241* (Abbott et al., 2005; Li et al., 2005).

miRNAs of the same family do not always exhibit functional redundancy. Although the seed sequence is an important factor in determining target regulation, there are several others. miRNA targets can also be impacted by pairing at the miRNA 3' end. Differences in spatiotemporal expression and abundance of miRNAs are all factors that impact regulation (Brennecke et al., 2005; Didiano & Hobert, 2008; Grimson et al., 2007; Martinez et al., 2008; Mukherji et al., 2011)

Analysis of strains with mutations in 15 of the 23 known *C. elegans* miRNA families was conducted to investigate redundancy in activity among miRNAs (Alvarez-Saavedra & Horvitz, 2010). Of the strains analyzed, most exhibited no observable phenotypes (Alvarez-Saavedra & Horvitz, 2010). Exceptions were the *mir-35*, *mir-51* and *mir-58* families. The *mir-35* family, consisting of *mir-35-mir-42* (Lau et al., 2001), is required for embryonic

development and individual family members exhibit functional redundancy (Alvarez-Saavedra & Horvitz, 2010). Single mutants of the *mir-51* family members, which consists of *mir-51-mir-56* (Lau et al., 2001), produces no observable defects (Alvarez-Saavedra & Horvitz, 2010), but strains with mutations in all *mir-51* family members die during late embryogenesis. The embryonic lethality phenotype can be rescued by transgene expression of any one of the *mir-51* family members, demonstrating their functional redundancy (Alvarez-Saavedra & Horvitz, 2010). The *mir-58* family consisting of *mir-58*, *mir-80-mir-82* and *mir-1834* (Lau et al., 2001) are expressed during all stages of development and are orthologues to *Drosophila bantam* miRNA, which controls cell proliferation and apoptosis (Brennecke et al., 2003). Again, functional redundancy is inferred due to single mutants having no readily observable phenotype, but mutants lacking all *mir-58* family members except *mir-1834* are sluggish, small, exhibit egg retention, and have slower rates of egg laying (Alvarez-Saavedra & Horvitz, 2010);

#### **D. Few single miRNA mutants exhibit penetrant mutant phenotypes**

After the initial identification of the role of *lin-4* and *let-7* in development, *lisy-6* was identified to function in neuronal patterning by controlling left/right neuronal asymmetry by downregulating *cog-1* mRNA (Johnston & Hobert, 2003). Researchers continued the search for functions for miRNAs with the generation of 87 mutants with deletion mutations in individual miRNA genes (Miska et al., 2007). This brought the total number of individual miRNA mutants to 95 out of the roughly 115 identified miRNAs in *C. elegans* (Bartel, 2009; Lau et al., 2001; Lee

& Ambros, 2001; Lim et al., 2003; Ruby et al., 2006). Surprisingly, phenotypic analysis resulted in the majority of the single miRNA mutants not being essential for viability or development (Miska et al., 2007). The lack of mutant phenotypes in single miRNA mutants supports a hypothesis of functional redundancy, including miRNAs within the same family, whereby the elimination of one does not result in an observable mutant phenotype.

Alternatively, when deletions occurred in miRNA clusters (these are not clusters of miRNAs of a family) some penetrant mutant phenotypes were observed (Miska et al., 2007). Mutations in the *mir-240-mir-786* cluster resulted in a long defecation cycle (Miska et al., 2007). Further analysis of the *mir-240-mir786* cluster resulted in identifying *mir-786* as necessary for pacemaker activity required in the posterior intestinal cell for the rhythmic defecation cycle. miR-786 functions in controlling calcium wave initiation in part through the regulation of *elo-2* (Kemp et al., 2012).

#### **E. Identification of miRNA functions through the use of stress conditions and sensitized backgrounds**

Identifying functions for individual miRNAs is challenging because loss of individual miRNAs mostly do not display observable mutant phenotypes (Miska et al., 2007). This may result from miRNAs having redundant roles, their modest regulation of targets or miRNAs having specialized roles that are difficult to observe in broad phenotypic analysis. The redundancy may not be isolated to miRNAs of the same family, as worms with an entire miRNA family deleted do not always exhibit strong phenotypes (Alvarez-Saavedra & Horvitz, 2010). There may also be redundancy among miRNAs in different families. One way to

overcome these obstacles is to analyze individual miRNA mutants under stress conditions or altered genetic backgrounds. Some of these sensitized genetic backgrounds include mutants with reduced processing and activity of all miRNAs (Brenner 2010) or reduced activity of a variety of regulatory pathways (Lehner et al., 2006). These genetic backgrounds make a viable, albeit stressed, organism more susceptible to the removal of a single miRNA and thus more likely to exhibit a mutant phenotype.

One stress condition that has been successfully used to investigate miRNAs is age. Deep sequencing of small RNA in aged worms that differ in life span led to the observation that miR-71, miR-238, miR-243 are overexpressed in aged animals and function to increase life span (de Lencastre et al., 2010). In the same study miR-239 was found to reduce longevity (de Lencastre et al., 2010).

Worms exposed to radiation have been used to identify miRNA functions. The role for *mir-34* in DNA damage response was identified by analyzing *mir-34* mutant worms exposed to radiation (Kato et al., 2009). This work revealed that *mir-34* is required for normal cellular response to DNA damage caused by radiation, a role thought to be conserved in humans (Kato et al., 2009).

Sensitized genetic backgrounds have been successful in identifying functions of miRNAs. 25 strains containing single miRNA deletions and the loss-of-function *alg-1(gk214)* allele were generated and analyzed for developmental, morphological and motility defects (Brenner et al., 2010). There were novel, enhanced or synthetic phenotypes observed in 19 of the 25 strains, including: embryonic lethality, gonad morphology defects, adult lethality, as well as suppression of the *alg-1* phenotype (Brenner et al., 2010). Loss of the individual

*mir-51* family members partially suppresses the developmental phenotypes observed in *alg-1* mutant worms (Brenner et al., 2010). *mir-51* family members regulate the developmental timing pathway in the L2 stage upstream of *hbl-1* (Brenner et al., 2012). A second sensitized background was used that knocked-down 'hub' genes by RNAi. 'Hub' genes encode chromatin regulatory proteins and interact with a range of signaling pathways required in development (Lehner et al., 2006). 11 strains with a miRNA deletion alleles were used and exposed to RNAi targeting one or more of the 'hub' genes. Four of the 11 strains showed synthetic sterility phenotypes with the most severe belonging to the *mir-240* and *mir-786* mutants. miR-786 was later implicated in calcium wave initiation in the posterior intestinal cell (Brenner et al., 2010; Kemp et al., 2012).

#### **F. microRNA Biogenesis Mutants**

Among the various approaches to investigate functions of miRNAs, analyzing mutants that have a mutation in one or more of the genes in the biogenesis pathway, which likely reduces levels of essentially all miRNAs, would logically be the most probable to produce observable phenotypes. In my work, this is the approach I used to identify roles for miRNAs as a class of regulators in ovulation and to tease apart in what cells or tissues they act.

Dicer is required for miRNA biogenesis and siRNA biogenesis (Table 1, Fig. 3) (Denli et al., 2004; Grishok et al., 2001; Ketting et al., 2001). Because miRNAs are required for embryonic development, to analyze Dicer and other miRNA biogenesis mutants, homozygous mutant offspring are isolated from heterozygote parents. Some of these zygotic mutants contain maternal activity of



the essential gene, which can allow survival to adulthood, at which point phenotypic analysis can occur. Zygotic loss of *C. elegans* Dicer (*dcr-1*) results in sterility with endomitotic oocytes (Grishok et al., 2001; Knight & Bass, 2001). Analysis of *dcr-1* zygotic mutant worms identified a requirement for Dicer activity in the somatic gonad not the germ line for normal fertility (Drake et al., 2014). It is not clear how this finding translates into the requirement for miRNAs in the germ line for normal fertility; differences in miRNA abundance between worms that have zygotic deletion of *dcr-1* and wild-type animals was found to be modest (Drake et al., 2014; Grishok et al., 2001; Knight & Bass, 2001). It is clear that both Dicer and miRNAs are required after fertilization for zygotic development (Tang et al., 2007).

In mice, loss of Dicer in oocytes inhibits meiosis, at meiosis II (Flemr et al., 2013; Murchison et al., 2007). Transcriptome analysis comparing mRNAs of Dicer mutants did not find higher levels of mRNAs containing miRNA binding sites compared to wild-type; this suggests that miRNAs are not acting to reduce mRNA expression in the maternal transcriptome (Ma et al., 2010). Analysis of miRNA levels in mouse oocytes of Dicer mutants do not show reduction in miRNA levels; this suggests that it is siRNAs not miRNAs that function in meiotic progression (Flemr et al., 2013). Like worms, mice require Dicer in somatic cells of the gonad for fertility (Nagaraja et al., 2008). Teasing out miRNA functions by the use of Dicer mutants is difficult due to its role in both siRNA and miRNA pathways.

On the other hand, mutant phenotypes that result from zygotic elimination of *drsh-1* (Drosha) should be due to the depletion in miRNAs not siRNAs.

Analysis of the Drosh mutant, *drsh-1(tm0654)*, *C. elegans* reveals a sterility phenotype with no other visible defects (likely because of maternal rescue) (Denli et al., 2004). This suggests that miRNAs are required for the process of ovulation.

In worms, zygotic loss of miRNA-specific Argonautes *alg-1* and *alg-2* leads to embryonic lethality due to developmental arrest (Vasquez-Rifo et al., 2012). The embryonic lethality only occurs if both Argonautes are eliminated (Vasquez-Rifo et al., 2012). Likewise, RNAi targeting *alg-1* and *alg-2* genes causes worms to arrest at embryonic stage (Grishok et al., 2001; Vasquez-Rifo et al., 2012). Additional phenotypes observed upon *alg-1* and *alg-2* knockdown include: burst vulva phenotype, gonad arm development, and defects in germ cell differentiation (Grishok et al., 2001). Loss of function mutations in *alg-1/2* genes results in reduced number of progeny and a reduced mitotic region in the gonad arm that indicates a reduced number of proliferative cells (Bukhari et al., 2012). Rescue experiments determine that expression of *alg-1* and *alg-2* is required in the somatic distal tip cell for germ cell development (Bukhari et al., 2012).

### **G. miRNAs in germ cells**

The role for miRNAs in the regulation of germ cells is unclear. While translational regulation is essential for meiotic maturation in animals (Mendez and Richter, 2001), the activity of miRNAs may not be required for germ cell development in some organisms.

Mouse oocytes undergo a massive degradation of transcripts during meiotic maturation (Su et al., 2007). Dicer is essential for the process of oocyte

maturation in mice, where its loss results in sterility due to defects in meiosis (Murchison et al., 2007; Tang et al., 2007). The sterility observed in *Dicer* mutant mice may be due to the inhibition of siRNAs and not miRNAs. Transcriptome analysis comparing *Dicer* mutant oocytes to ES cells indicates that there is limited miRNA-associated mRNA degradation in oocytes and it is likely the inhibition of the endo-RNAi pathway that is the major cause of the transcriptome changes (Ma et al., 2010). This conclusion is supported by analysis of *Dgcr8* mutant oocytes. *Dgcr8* is only involved in the miRNA biogenesis pathway, whereas *Dicer* is required for both the siRNA and miRNA pathways, which showed a similar depletion of miRNAs compared to the *Dicer* mutant oocytes (Suh et al., 2010). In addition, the mRNA levels in the *Dgcr8* mutant oocytes were almost identical to those of wild-type animals, which suggests limited miRNA activity in mouse oocytes (Suh et al., 2010).

It is not known if, like mice, worms lack miRNA activity in oocytes. Like mice, loss of *Dicer* in worms inhibits meiotic maturation (Knight & Bass, 2001). *Dicer* is phosphorylated and localized to the nucleus during most of oogenesis, precluding its normal miRNA and siRNA processing function in the cytoplasm. Additionally, mosaic analysis indicates that *Dicer* is dispensable in the germ line for the process of oocyte maturation and ovulation (Drake et al., 2014). Mosaic analysis indicates that *Dicer* is dispensable in the germ line but not in the somatic gonad for the processes of oocyte maturation and ovulation (Drake et al., 2014; Tops et al., 2006). Whether and how these observations relate to miRNA activity are questions that require further investigation that I address in my research. The differences in miRNA abundance between worms that have zygotic deletion

of Dicer and wild-type animals were found to be modest (Drake et al., 2014; Grishok et al., 2001; Knight & Bass, 2001); this is surprising because Dicer is required for the cytoplasmic processing of miRNAs. A possible explanation for the continued presence of miRNAs in the *dcr-1(0)* zygotic mutants is the presence of maternal Dicer.

In contrast, there are data from other organisms indicating that miRNAs are required in germ cells for their development and activity. In flies, miRNA biogenesis genes, including *ago1*, *dicer-1*, *drosha* and *pasha*, are necessary for oocyte development and germ cell proliferation (Azzam et al., 2012). Also in flies, several studies have ascribed functions for individual miRNAs in germline development or proliferation (reviewed in Carthew et al., 2016).

In the *C. elegans* germline, many genes are translationally regulated through sequences in the 3' UTR of the mRNA (Merritt et al., 2008), which could reflect a role for miRNAs. In addition, many miRNAs are present in mature oocytes of worms (Gu et al., 2009) and recently, 13 miRNAs were found to be enriched in the germline (McEwen et al., 2016). The *mir-35* family members are highly abundant in *C. elegans* oocytes (Gu et al., 2009, McEwen et al., 2016). There are many functions attributed to the *mir-35* family including early development (Alvarez-Saavedra and Horvitz, 2010), germ cell proliferation (Liu et al., 2011) and sperm production (McJunkin and Ambros, 2014). While oocyte miRNAs such as the *mir-35* family may largely act after fertilization in early embryonic development, it remains possible that a subset of these miRNAs function earlier to control oocyte maturation and ovulation.

#### **H. miRNAs in the Somatic Gonad**

There is evidence that miRNA biogenesis genes are required in cells of the somatic gonad for gonad development, germ cell proliferation and oocyte maturation. miRNA-specific Argonautes ALG-1 and ALG-2 together with a set of miRNAs expressed in the distal tip cell of the somatic gonad are required for germ cell proliferation, oocyte abundance and brood size (Bukhari et al., 2012; Drake et al., 2014). Genetic mosaic analysis indicates that Dicer is required in the somatic gonad for the processes of oocyte maturation and ovulation (Drake et al., 2014; Tops et al., 2006). Whether the requirement for Dicer in the somatic gonad is due to its role in miRNA or siRNA biogenesis is not clear. For what events during ovulation and in what cells of the somatic gonad miRNA biogenesis mutants are required is still unknown. My work addresses these unknowns.

Some individual miRNAs are known to function in gonad development and function. A screen searching for individual miRNAs that function in development, analyzed worms with single miRNA deletions in a sensitized genetic background consisting of an *alg-1* mutation. The search identified 6 miRNAs involved in gonad morphogenesis (Brenner et al., 2010). Of these, *mir-83* and *mir-34* were found to function in distal tip cell migration during the development of the gonad (Burke et al., 2015). Out of 70 genetic fusions using miRNA promoters that drive the expression of GFP (Martinez et al., 2008), 8 were found to be expressed in the somatic distal tip cell (*plet-7*, *plin-4*, *pmir-80*, *pmir-237*, *pmir-247-797*, *pmir-359*, *pmir-53* and *pmir-71*). Analysis of mutant worms containing single mutations in each of these eight miRNA genes identified 5 miRNAs (*let-7(n2853)*, *lin-*

*4(e912)*, *mir-237(n4296)*, *mir-359(n4540)* and *mir- 247(n4505)* play a role in maintaining brood size and correct mitotic region in the germ cells (Bukhari et al., 2012).

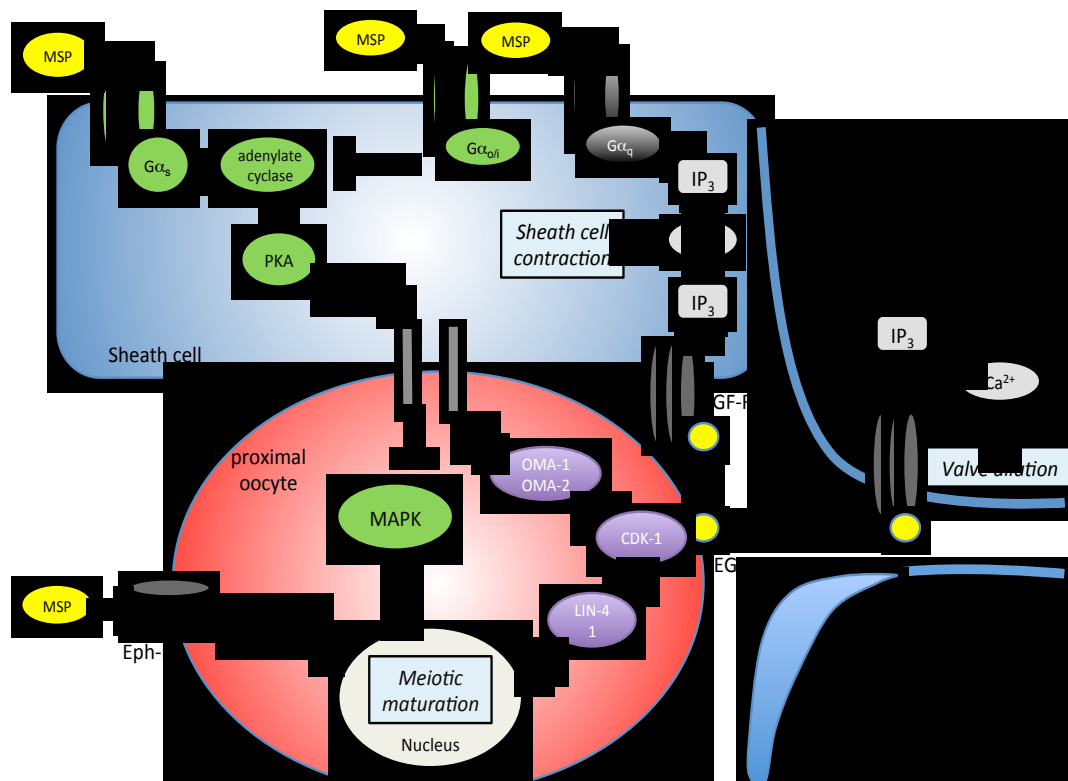
### **VIII. The Ovulation Process**

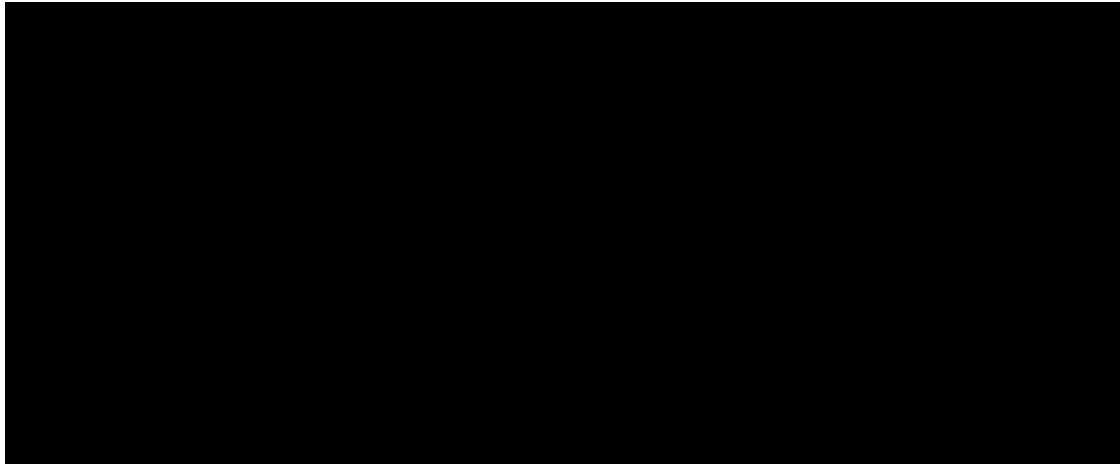
Ovulation is a rhythmic behavior that occurs every 20-25 min in each gonad arm and is preceded by an increase in contraction frequency of sheath cells, from a basal rate of 10 contractions per minute to an ovulatory rate of ~23 per minute (McCarter et al. 1999). Ovulation occurs in the gonad, two U-shaped arms with germ cells that divide mitotically at the distal (relative to the spermatheca) end of the tube and then mature as they reach the most proximal position of the gonad arm (Fig. 2).

The process of ovulation requires the coordinated activity of multiple cells to occur normally. Ovulation requires meiotic resumption, which is regulated by both positive and negatively acting pathways between the somatic cells and germ cells. Oocytes undergo meiotic resumption in a process referred to as meiotic maturation. Meiotic maturation is often regulated by hormonal signaling and soma-germline interactions and it involves the transition from prophase I to metaphase I. Sheath cells surrounding the proximal oocyte function to inhibit oocyte maturation (McCarter et al., 1999). Major Sperm Protein (MSP), released from sperm cells, interacts with somatic sheath cells to release this inhibition, initiating both meiotic maturation and, through an independent pathway, sheath cell contractions. In the presence of MSP, MAPK is activated in the oocyte, inducing meiotic maturation. Upon meiotic resumption, the oocyte produces LIN-

3/EGF that is released and interacts with LET-23/EGF receptors in the sheath cells, thereby increasing the rate and intensity of contractions, while causing dilation in the distal spermatheca valve, both via IP<sub>3</sub>-mediated calcium release (Fig. 4).

Meiotic resumption and ovulation require the activation of proteins OMA-1 and OMA-2, which function redundantly (Detwiler et al., 2001). Although the exact mechanism by which the OMA proteins promote meiotic maturation has yet





to be determined, they are known to function upstream of CDK-1 activation that suppresses expression of *lin-41*. LIN-41 is a highly conserved TRIM-NHL RNA binding protein that is best known for its role in developmental timing pathway in somatic cells as the target of let-7 miRNA (Reinhart et al., 2000; Slack et al., 2000; Vella et al., 2004). *lin-41* inhibits meiotic maturation and enables oocytes to grow by functioning in 3'UTR mediated translational repression (Spike et al., 2014). *lin-41* promotes oocyte growth and inhibits M-phase entry to maintain oocyte quality. LIN-41 and LIN-41::GFP begin to be eliminated from oocytes as they undergo meiotic maturation, which in the *C.elegans* gonad occurs in the most proximal oocyte (Spike et al., 2014). For my work it is important to note that *let-7* miRNA is not known to be expressed in germ cells (Lau et al., 2001). In the absence of sperm, inhibitory pathways of meiotic maturation are activated;  $Ga_{o/i}$  in the sheath cells, and the VAB-1 Eph receptor in the oocyte (Greenstein unpublished data).



## CHAPTER TWO

### Materials and Methods

#### Strains and maintenance

*C. elegans* strains were grown on NGM plates seeded with *E. coli* strain AMA1004 at 20°C unless otherwise indicated. Strains used are listed in Table 2.

#### RNAi by feeding

All bacteria for RNAi experiments were isolated from the Ahringer RNAi library (Kamath et al., 2003). To knock down *alg-1* activity, the X-6D15 clone (Source Bioscience) was used. *alg-1* and *alg-2* are 80% identical at the nucleotide level, this level of similarity is within a range where partial cross-interference in RNAi assays is expected, therefore it is likely that *alg-1* knockdown results in knockdown of *alg-2* (Grishok et al., 2001; Schubert et al., 2000). RNAi bacteria were cultured in Luria-Bertani (LB) broth supplemented with 100 µg/ml ampicillin, and 50 µg/ml tetracycline. Overnight cultures of RNAi bacteria were used to seed NGM plates supplemented with 1mM IPTG and 100 µg/ml ampicillin. Plates were kept at room temperature for 24 hours to allow for induction of dsRNA expression. Worms were transferred to RNAi plates at the L4 stage and F1 progeny were analyzed as young adults. Alternatively, for post-embryonic RNAi, worms were transferred at approximately the L2 stage and subsequently analyzed as young adults, approximately 42 hours later. Bacteria

that have the pPD129.36/L4440 plasmid, which is an empty RNAi plasmid, was used as a negative control for RNAi.

Table 2

Strain	Genotype	Description
*N2	wild-type	Wild-type
PD8753	<i>dcr-1(ok247) III/hT2 [bli-4(e937) let-?(q782) qls48] (I;III).</i>	Dicer mutant
*RF103	<i>rrf-1(ok589) I</i>	RNAi in the germline
RF104	<i>rrf-1(ok589) I; alg-2(ok304) II</i>	RNAi in the germline with alg-2 mutation
*RF849	<i>alg-2(ok304) II; rde-1(ne219) V; xwEx189 [mir786<sup>prom</sup>::rde-1(+):unc-54<sup>3' UTR</sup>]</i>	RNAi in the somatic gonad
*RF851	<i>alg-2(ok304) II; rde-1(ne219) V</i>	Control for RF849
*RF855	<i>pash-1(mj100) I</i>	Pasha mutants; <i>pash-1(mj100)</i> mutants isolated from SX1359 strain without the rescuing array.
RF862	<i>unc-119(ed3) III; rde-1(ne219) V; xwTi2[inx-8<sup>prom</sup>::rde-1(+):unc-54<sup>3' UTR</sup>] X</i>	RNAi in the sheath cells; <i>xwTi2</i> location X 8805171bp (intron)
*RF864	<i>xwTi1 [inx-8<sup>prom</sup>::rde-1(+):unc-54<sup>3' UTR</sup>]; unc-119(ed3) III, rde-1(ne219) V</i>	RNAi in the sheath cells; <i>xwTi1</i> location III 3994771bp
*RF878	<i>oxTi76 IV; rde-1(ne219) V; unc-119(ed3) xwTi1 III</i>	Control strain to validate tissue specific RNAi in the sheath cells
RF884	<i>oxTi76 IV; rde-1(ne219) V; xwTi2 X; unc-119 III</i>	Control strain to validate tissue specific RNAi in the sheath cells
RF885	<i>xwTi5 [pie-1<sup>prom</sup>::rde-1(+):tbb-2<sup>3' UTR</sup>]IV; rde-1(ne219) V</i>	RNAi in the germline; <i>xwTi5 IV</i> 172,923,205bp
*RF922	<i>alg-2(ok304) II; rde-1(ne219) V; xwTi5 [pie-1<sup>prom</sup>::rde-1(+):tbb-2<sup>3' UTR</sup>]</i>	RNAi in germline
*SX1359 <sup>a</sup>	<i>pash-1(mj100) I; mjEx331</i>	Pasha temperature sensitive mutant with rescue array
VC1138	<i>drsh-1(ok369) I/hT2 [bli-4(e937) let-?(q782) qls48] (I;III).</i>	Drosha mutant
*RF102	<i>alg-2(ok304) II</i>	alg-2 mutant
*DG3913	<i>lin-41 (tn1541[GFP::tev::s::lin-41]) I</i>	GFP::lin-41 fusion
*WM27	<i>rde-1(ne219) V</i>	RNAi resistant

<sup>a</sup> Strain provided by Eric Miska, Lehrbach et al, 2012 \* Strains used for publication, Rios et al. 2017

## Microscopy

Nomarski DIC and epifluorescence microscopy was performed using a Nikon 80i compound microscope equipped with a CoolSNAP HQ2 monochrome camera (Roper Scientific, CA). Images were captured with a 60x Plan Apo objective lens using Elements software (Nikon). For time-lapse observations of sheath contraction and ovulation, day 1 young adult worms were anesthetized for 20-25 min in M9 solution with 0.1% tricaine and 0.01% tetramisole (Sigma-Aldrich, St. Louis, MO) before viewing (McCarter et al., 1997; Reese, Dunn, Waddle, & Seydoux, 2000b). Anesthetized worms were mounted on a 2% agarose pad.

For germline RNAi experiments using worms expressing the *pie-1<sup>prom</sup>::rde-1(+)* and *GFP::tev::s::lin-41* transgene, day 1 young adult worms were immobilized by placing worms on 2  $\mu$ l of Polybead Microspheres 0.10 $\mu$ m (Polysciences) on a 5% agarose pad. A small amount of petroleum jelly was placed around the coverslip to prevent desiccation. Only worms that displayed movement indicating viability on the pad were analyzed for ovulation events.

## Ovulation rate assay

The method used to calculate ovulation rate was modified from McCarter et al. (1999). The ovulation rate is calculated as the number of ovulation events per gonad arm per hour, which reflects the rate of meiotic maturation. Day 1 young adults were placed onto individual plates and viewed using a Nikon SMZ-1500 stereomicroscope to determine the initial number of embryos present in the uterus. Worms were then transferred to a 20°C incubator for an average of 3

hours; at which point the final number of embryos inside the uterus was counted. The number of ovulations for each individual worm was determined by counting the number of embryos or live progeny produced. These values were placed into the formula [(Final number of embryos in uterus – Initial number of embryos in uterus) + number of progeny or embryos produced] / [(2 gonad arms) (3 hours)] = number of ovulation events per hour per gonad arm.

### **In vivo analysis of sheath cell contractions.**

Time-lapse DIC images of the proximal gonad and oocytes were captured (10 frames/sec) during individual ovulation events prior to rounding of the proximal oocyte through entry of the fertilized embryo into the uterus. Worms were observed until ovulation occurred or for 60 minutes. If the proximal oocyte did not resume meiosis and no ovulation occurred within 60 minutes, they were categorized as failing to resume meiosis. Worms in which the oocyte resumed meiosis but failed to fully enter or exit the spermatheca were observed for a minimum of 10 minutes after nuclear envelope breakdown or entry into the spermatheca, respectively. To measure the rate of gonadal sheath contractions, the number of lateral displacements were counted, focusing on one side of the -1 oocyte that included the junction to -2 oocyte. The rate of basal contractions was calculated during a 3-5 minute interval prior to the initiation of ovulatory contractions. The rate of ovulatory contractions was determined by the maximum number of contractions that occurred in the -3 to 0 minute period, with time 0 indicating when the oocyte was fully in the spermathecal (D'Agostino et al., 1999b).

## **In vivo analysis of worms expressing the *GFP::tev::s::lin-41* transgene**

DIC and fluorescent images of the proximal gonad and oocytes of worms expressing *GFP::tev::s::lin-41* were captured during individual ovulation events. Images were captured prior to rounding of the proximal oocyte through entry of the fertilized embryo into the uterus. Analysis was accomplished by measuring the ratio of the average fluorescence (F) in a region of interest (ROI) in the most proximal oocyte ( $F_1$ ) and the corresponding fluorescence of the next oocyte ( $F_2$ ). The ratio  $F_1/F_2$  quantifies the relative expression of LIN-41 in the proximal oocyte. The second oocyte,  $F_2$ , is used as a stable reference to normalize quantification as LIN-41 is reduced in the proximal oocyte. Measurements were taken starting around 8 min prior to ovulation and continuing until approximately 4 min after ovulation.

## **Construction of transgenic strains**

Gibson assembly (NEB) was used to generate the pCR4 plasmid containing the *inx-8<sup>prom</sup>::rde-1(+):unc-54<sup>3'utr</sup>* transgene in the pCFJ909 plasmid backbone for use in MiniMOS insertion (Frøkjær-Jensen et al., 2014; Reece-Hoyes et al., 2007). PCR amplification of a 1067 bp genomic fragment upstream of the *inx-8* start site was performed using primers AA1225 and AA1229 and N2 genomic DNA as a template. Primer sequences are provided in Table 3. PCR amplification of *rde-1(+):unc-54<sup>3'utr</sup>* was performed using AA1052 and AA1222 primers and the pXXY2004.1 plasmid (Denli et al., 2004; Espelt et al., 2005; Grishok et al., 2001) as a template. pXXY2004.1 was kindly provided by Dr. Keith Nehrke (University of Rochester, NY). PCR products (0.05 pmoles) were

mixed with a 5.1 kb PstI-SpeI pCFJ909 fragment (0.05 pmoles) in a Gibson assembly reaction (NEB). pCR4 construction was confirmed by sequencing. To generate strains that contain the *inx-8<sup>prom</sup>::rde-1(+)::unc-54<sup>3'utr</sup>* transgene

Table 3 List of oligonucleotides

name	sequence
AA1052	ATGTCCTCGAATTTTCCCGAATTG
AA1222	CGTGGATCCAGATATCCTGCAAACAGTTATGTTTGGTATATTGGG
AA1225	CGCCAAGCTACGTAATACGACTCACGAAGGAAGGACACATGGAACCTCTC
AA1229	TCGGGAAAATTTCGAGGACATTCTGCCCTTCGAACCCGAGTG
AA1282	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAAAAATGTCCTCGAATTTTCCCGAATTGG
AA1283	GGGGACCACTTTGTACAAGAAAGCTGGGTTTATGCGAACGACATTCCAGGG
AA1309	CTGCCTACCGTAGAAGCAGT
AA1310	AAAGTTTTGAAGCCAATGTTTGCTC
Ocf1588	GGTGGTTTCGACAGTCAAGGT

inserted in the genome, an injection mix containing pCR4 (10ng/ul), along with coinjection plasmids pGH8 (10ng/ul), pCFJ90 (2.5ng/ul), pCFJ104 (10ng/ul), pMA122 (5 ng/ul) and pCFJ601 (50 ng/ul) was injected into *unc-119(ed3)* worms. Worms that contained a transgene insertion were obtained as described in Frokjaer-Jensen et al. (Frøkjær-Jensen et al., 2014). Progeny were screened for rescue of the Unc phenotype and for the absence of coinjection marker expression (detected by RFP expression), which indicated the presence of an extrachromosomal array. For the *xwTi1 [inx-8<sup>prom</sup>::rde-1(+)::unc-54<sup>3'utr</sup>]* insertion, inverse PCR was performed to determine the genomic location (Table 1). Worms with *inx-8<sup>prom</sup>::rde-1(+)::unc-54<sup>3'utr</sup>* transgenes were crossed with *rde-1(ne219)V* mutant worms using standard genetic approaches, using PCR to follow the *xwTi1*

insertion (AA1309 with Ocf1588, and AA1310 with AA1309 primers). The presence of the *ne219* allele was confirmed by sequencing.

MultiSite Gateway cloning (Life Technologies) was used to generate the pCR6 plasmid containing the *pie-1<sup>prom</sup>::rde-1(+)::tbb-2<sup>3'utr</sup>* transgene. First, PCR was used to amplify the *rde-1(+)* coding region using primers AA1282 and AA1283 with pXXY2004.1 as a template. The necessary *attB1* and *attB2* sequences were added using PCR primers. The PCR product was recombined with the Gateway donor vector pDONR221 to make the pCR5 *rde-1(+)* entry clone. pCR5 construction was confirmed by sequencing. To generate the *pie-1<sup>prom</sup>::rde-1(+)::tbb-2<sup>3'utr</sup>* transgene, pCR5 along with entry clones pCM1.127 and pCM1.36, which supplied the *pie-1* promoter and the *tbb-2* 3' UTR sequences (Addgene plasmid #21384, #17249), respectively, were recombined with destination vector pCFJ907 to generate pCR6. pCR6 junctions were confirmed by sequencing. *rde-1(ne219)* mutant worms were injected with pCR6 (10ng/μl) along with coinjection plasmids as described above. Worms that contained a transgene insertion were obtained as described in Frøkjær-Jensen et al. (Frøkjær-Jensen et al., 2014; Gilchrist et al., 2016; Gu et al., 2009; M. Lee et al., 2014; Ma et al., 2010; McEwen et al., 2016). Progeny were screened for resistance to G418 (Invitrogen), which was added to NGM plates to a final concentration of 0.4 mg/ml, along with the absence of coinjection marker expression, which indicated the presence of an extrachromosomal array. Inverse PCR was performed to determine the genomic location of the *xwTi5* insertion (Table 1).

### **Brood size assay**

For most strains, 3 L4-stage P0 worms were placed onto RNAi plates. From these plates 10, L4-stage F1 hermaphrodites were selected and placed onto individual RNAi plates. Worms were transferred to new RNAi plates each day for four days and plates were scored three days later for number of F2 progeny present. For post-embryonic RNAi experiments, 20 L2-stage P0 worms were placed onto an RNAi plate. Eighteen hours later 10 L4-stage worms were picked and placed onto individual RNAi plates and the number of embryos produced were counted. For these worms, embryos were counted because of the penetrant embryonic lethality observed. For all strains, worms that died during the 4 day period or burrowed into the plate were excluded from analysis. For *pash-1(mj100ts)*, *dcr-1(ok247)*, *drsh-1(ok369)*, and wild-type worms no RNAi plates were used. Instead, 10 L4-stage P0 hermaphrodites were selected and placed onto individual NGM seeded plates. Worms were transferred to new plates each day for four days and plates were scored three days later for number of F1 progeny present.

### **Clones for tissue-specific RNAi**

The clones used for *pos-1*, *itr-1*, *lin-14*, *unc-15*, and *dpy-13* tissue-specific RNAi were taken from the Ahringer Library. The clone used for GFP RNAi was the L4417 plasmid, which was gift from Andrew Fire (Addgene plasmid #1649).



## CHAPTER THREE

### Generation and Validation of Genetic Tools for Tissue-Specific RNAi

#### Introduction and Background

I investigated the requirement for miRNA-specific biogenesis genes in the cells of the somatic gonad and germ cells during the process of ovulation. One of the approaches was to knock down the miRNA-specific Argonaute *alg-1* using RNAi. RNAi is widely used in *C. elegans* for the rapid knockdown of most genes of interest (Fire et al., 1998). Tissue-specific RNAi can be performed using a mosaic system of expressing wild-type *rde-1*, in a *rde-1* mutant genetic background (Qadota et al., 2007). The Argonaute RDE-1 was the first Argonaute identified in worms and functions in the RNAi pathway (Tabara et al., 1999b). *rde-1* mutants are resistant to RNAi and *rde-1* functions in a cell-autonomous manner (Tabara et al., 1999b). Therefore, RNAi sensitivity is conferred only in the tissues expressing *rde-1* (Qadota et al., 2007), which is accomplished by using the many tissue-specific promoters that are available in *C. elegans*. To identify the requirement of miRNA biogenesis genes in the somatic gonad and germ cells I have generated novel genetic tools using cell-specific promoters to drive the expression of *rde-1* in an otherwise *rde-1* mutant background.

In addition to tissue-specific rescue of *rde-1* activity, there exists a *C. elegans* deletion mutant, *rrf-1*, that exhibits RNAi sensitivity in the germline but not in most somatic cells. *rrf-1* encodes an RNA-dependent RNA polymerase thought to be required for the amplification of dsRNA during exogenous-RNAi in

somatic tissues (Sijen et al., 2001). *rrf-1* deletion mutants show no obvious defects in development and physiology. These *rrf-1* mutants are sensitive to RNAi targeting genes expressed in germline but resistant to muscle specific RNAi (Sijen et al., 2001). *rrf-1* mutant strains have become a widely used tool in *C. elegans* for the investigation of genes acting in the germline. However, they have been found to display RNAi in some somatic tissues, including the intestine and the hypodermis. Importantly, no RNAi was observed in cells of the somatic gonad (Kovacevic et al., 2012).

In my work, I initially used *rrf-1(ok589)* mutants to identify the role of miRNA-specific Argonaute *alg-1* in the germline. Knockdown of *alg-1* by RNAi in *rrf-1(ok589)* worms resulted in a reduced ovulation rate (Fig. 10C). However, due to the possibility that the observed phenotype was the result of somatic knockdown of *alg-1*, I generated a new tool for tissue-specific knockdown of genes in the germ cells.

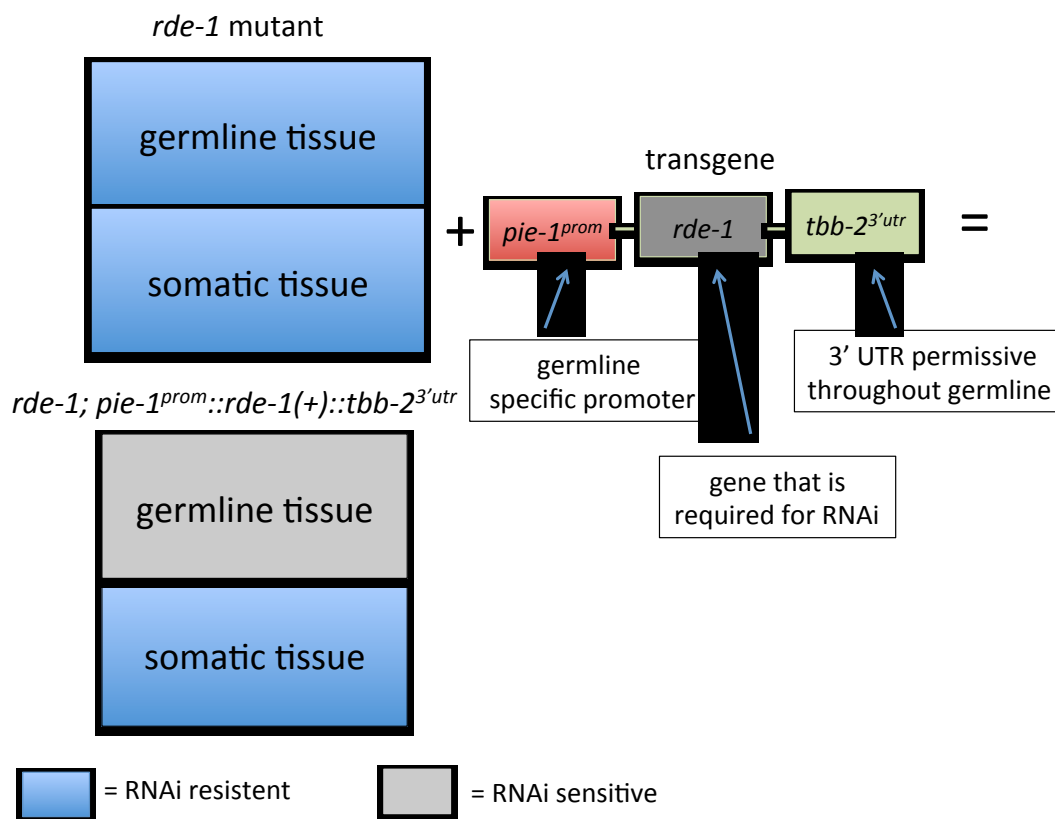
To generate a mutant that is sensitive to RNAi in germ cells but resistant in somatic cells, I used the promoter of the *pie-1* gene to drive a wild-type copy of *rde-1(+)*, followed by a *tbb-2* 3'UTR, which is permissive in the germline. The *pie-1* gene encodes a zinc finger protein that is essential for germline cell fate (Reese et al., 2000a; Yin et al., 2004). The *pie-1* promoter allows expression in all germ cells (D'Agostino et al., 2006). This transgene was integrated into the *C. elegans* genome (Table 2) of an *rde-1(ne219); alg-2(ok304)* mutant. This mutant should rescue RNAi sensitivity in the germ cells while maintaining RNAi resistance in somatic cells (Fig.5).

In worms, ovulation is a complex behavior, regulated by multiple signaling pathways between somatic and germ cells. To unravel some of this complexity, in addition to generating mutants with RNAi sensitivity in germ cells, we developed mutants to investigate the role of miRNA biogenesis genes in cells of the somatic gonad. Two mutants were generated for this purpose. The first utilized the *mir-786* promoter. The miRNA *mir-786* is expressed in the somatic gonadal sheath cells, spermatheca, uterus, as well as in the posterior cells of the intestine (Kemp et al., 2012; Ma et al., 2010). The *mir-786::rde-1(+):unc-54<sup>3'utr</sup>* transgene was expressed as an extrachromosomal array in a *rde-1(ne219); alg-2(ok304)* genetic background. The *alg-2(ok304)* allele was introduced to further reduce Argonaute activity.

The second mutant utilized the promoter of the *inx-8* gene, so that RNAi sensitivity would be restricted to the sheath cells of the somatic gonad. The somatic innexin *inx-8* gene is expressed in the proximal sheath cells as a component of the gap junctions that connect sheath cells to developing oocytes (Drake et al., 2014; Starich et al., 2014). The *inx-8<sup>prom</sup>::rde-1(+):unc54<sup>3'utr</sup>* transgene was integrated (Table 1) into a *rde-1(ne219)* genetic background. After the mutants were constructed, RNAi sensitivity upon expression of wild-type *rde-1* in specific tissues was validated by a procedure modified from (Qadota et al., 2007).

### **Validation of RNAi tissue-specificity using embryonic lethal and somatic required genes**

To confirm RNAi sensitivity in the germ cells, we chose a gene, *pos-1*, that is required for embryonic development and causes a sterile phenotype if knocked down by RNAi in the germline (Hodgkin et al., 1979). *pos-1* encodes a CCCH-



**Figure 5. Tissue-specific RNAi achieved using *rde-1* mutants.**

Blue represents tissue in the worm that becomes resistant to RNAi upon mutation in *rde-1* gene. Upon the introduction of *pie-1<sup>prom</sup>::rde-1(+):tbb-2<sup>3'utr</sup>* transgene into an *rde-1* mutant worm, expression of a wild copy of the *rde-1* gene is driven by *pie-1* (a germline specific promoter). This intervention restores a functional copy of *rde-1* in germline tissue, which then becomes sensitive to RNAi (depicted in red). This approach was also used to restore RNAi capability in the somatic gonad, using the *inx-8* and the *mir-786* promoters.

type zinc-finger protein; during embryogenesis, maternally provided POS-1 is essential for proper cell fate specification (Tabara et al., 1999a). When exposed to *pos-1* RNAi both strains containing the *pie-1::rde-1(+):tbb-2* transgene in an *rde-1(ne219)* mutant, displayed 100% sterility, compared to 0% sterility for *rde-1(ne219)* controls, and 100% sterility for wild-type worms (Table 2). This clearly demonstrates that RNAi sensitivity is present in germ cells of the mutant strains.

To confirm RNAi resistance in somatic tissue we chose two somatically expressed genes that cause strong penetrant phenotypes upon RNAi knockdown. The first, *unc-15*, encodes a paramyosin ortholog expressed in body wall muscle (Hoppe & Waterston, 2000). Animals exposed to RNAi targeting *unc-15* exhibit problems with self-propelled movement compared to wild-type worms (Cronin et al., 2005). When exposed to RNAi targeting the *unc-15* gene, neither strain expressing the *[pie-1::rde-1(+):tbb-2];rde-1(ne219)* transgene in *rde-1(ne219)* worms had any individuals that displayed an *unc*-phenotype and were virtually indistinguishable from those exposed to control RNAi (Table 4). 100% of wild-type worms exposed to *unc-15* RNAi displayed an Unc phenotype (Table 2).

The second gene used to test RNAi resistance in somatic tissues was *dpy-13*. *dpy-13* encodes a member of the collagen superfamily (Johnstone & Barry, 1996). Animals exposed to RNAi targeting *dpy-13* display a “dumpy” phenotype (Dpy) characterized by a shorter and more stout body than that of their wild-type counterparts (Brenner, 1974). When strains expressing the *pie-1::rde-1(+):tbb-2* transgene in an *rde-1(ne219)* mutant were exposed to RNAi targeting the *dpy-13* gene, 0% of worms displayed a Dpy phenotype. The

exposed worms were virtually indistinguishable from the same strains exposed to control RNAi (Table 4). On the other hand, 100% of wild-type worms exposed to *dpy-13* RNAi displayed a Dpy phenotype (Table 4). From these results, we conclude that the expression of *rde-1* under the control of the *pie-1* promoter can rescue the *rde-1* mutation exclusively in the germ cells.

To confirm the ability of the *mir-786* promoter to rescue the expression of *rde-1* in cells of the somatic gonad but not in germ cells, we again chose the gene *pos-1* to test for germ cell resistance to RNAi and *itr-1* to test for somatic gonad sensitivity. *itr-1* encodes the IP<sub>3</sub> receptor in worms and it is expressed in the cells of the somatic gonad (Dal Santo et al., 1999). Upon exposure of *itr-1* RNAi worms exhibit severe phenotypes including sterility (Yin et al., 2004). Upon RNAi testing for germline resistance both the *rde-1(ne219);alg-2(ok304)* worms expressing the *mir-786<sup>prom</sup>::rde-1(+):(+)* *unc-54<sup>3utr</sup>* transgene and their respective *rde-1(ne219);alg-2(ok304)* strain controls exhibited 0% sterility when exposed to *pos-1* RNAi and 5% and 0% sterility upon exposure to control RNAi, respectively (Table 4). Upon RNAi testing for somatic gonad sensitivity, *rde-1(ne219);alg-2(ok304)* worms expressing the *mir-786<sup>prom</sup>::rde-1(+):(+)* *unc-54<sup>3utr</sup>* transgene and their respective *rde-1(ne219);alg-2(ok304)* strain controls exhibited 80% and 0% sterility when exposed to *itr-1* RNAi, respectively (Table 4). From these results, we conclude that the expression of *rde-1* under the control of the *mir-786* promoter can rescue the *rde-1* mutation in cells of the somatic gonad while maintaining the germline resistant to RNAi. 0% sterility when exposed to *itr-1*

**Table 4: Strain validation using tissue-specific RNAi**

Strain	Genotype	Phenotype					
		% Sterility <sup>a</sup>			% Observable defect <sup>b</sup>		
		Control RNAi	<i>pos-1</i> RNAi	<i>itr-1</i> RNAi	<i>lin-14</i> RNAi	<i>unc-15</i> RNAi	<i>dpy-13</i> RNAi
RF851	<i>alg-2(ok304) II; rde-1(ne219) V</i>	0% (n=20)	0% (n=20)	0% (n=20)	N/D	N/D	N/D
RF849	<i>alg-2(ok304) II; rde-1(ne219) V; xwEx189 [mir786prom::rde-1(+):unc-543' UTR]</i>	5% (n=20)	0% (n=20)	80% (n=20)	N/D	N/D	N/D
N2	wild-type	0%* (n=20)	100%* (n=20)	100% (n=20)	100% (n=136)	100%** (n=15)	100%** (n=15)
RF104	<i>rrf-1(ok589)I; alg-2(ok304)II</i>	5%* (n=20)	100%* (n=20)	N/D	15% (n=166)	N/D	N/D
WM27	<i>rde-1(ne219)V</i>	0% (n=20)	0% (n=20)	0% (n=20)	5% (n=224)	0%	0%
	xwTi5 [ <i>pie-1::rde-1(+):tbb-2</i> ]; <i>rde-1(ne219)V</i>	N/D	100%* (n=15)	N/D	N/D	0%** (n=15)	0%** (n=15)
RF886	xwTi5 [ <i>pie-1::rde-1(+):tbb-2</i> ]; <i>rde-1(ne219)V</i>	N/D	100%* (n=20)	N/D	N/D	0%** (n=15)	0%** (n=15)

\*L4's were placed on RNAi plates, and young adults were analyzed

\*\* L2's placed on RNAi plates, and young adults were analyzed

No asterisk indicates parental L4's were placed on plates and F1's were analyzed

<sup>a</sup>Sterility was assessed by cloning single worms onto RNAi plates and counting live progeny

<sup>b</sup>Observable defects for *lin-14* RNAi: developmental timing defects (including size of worm and precocious vulva formation). Observable defects for *unc-15* RNAi: worms were unable to move normally (Unc). Observable defects for *dpy-13* RNAi: worms were short and wide (Dpy).

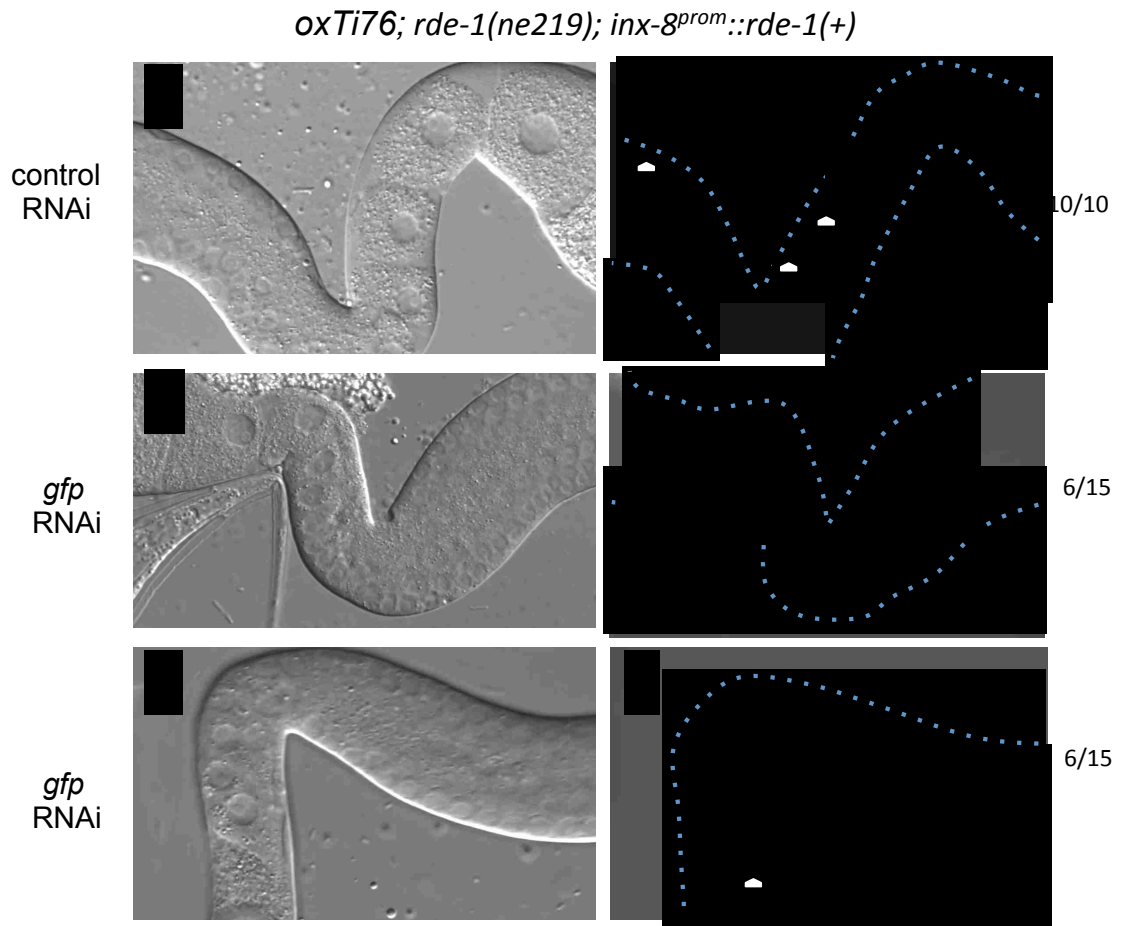
RNAi (Table 4). From these results, we conclude that the expression of *rde-1* under the control of the *mir-786* restores the RNAi pathway in the somatic gonad and not the germ cells.

### Examination of tissue specificity using GFP markers

To confirm *rde-1* rescue by the *inx-8* promoter specifically in the sheath cells of the somatic gonad, we utilized an approach that would allow for visualization of nuclear GFP knockdown by RNAi in these cells. To do this we crossed the *rde-1(ne219); inx-8<sup>prom</sup>::rde-1(+):unc-54<sup>3'utr</sup>* mutants with worms that had ubiquitous expression of nuclear *gfp* (*oxTi76*). These worms were exposed to *gfp* RNAi and viewed under DIC and fluorescence imaging after different times of exposure (Fig. 6). 100% of *oxTi76; rde-1(ne219); inx-8<sup>prom</sup>::rde-1(+)* worms exposed to control RNAi had visible GFP in the sheath cell nuclei after (.5 second exposure). Upon exposure to *gfp* RNAi, 60% of the of the *oxTi76; rde-1(ne219); inx-8<sup>prom</sup>::rde-1(+)* mutants had no or very faint GFP visible after a 2 second exposure (Fig. 6). In spite of some uncertainty due to the presence of minor GFP fluorescence after the 2 second exposure, we conclude from these results that the expression of *rde-1* under the control of the *inx-8* promoter can rescue the *rde-1* mutation in cells of the sheath.

The *mir-786::rde-1(+):unc-54<sup>3'utr</sup>* transgene was expressed as an extrachromosomal array in a *rde-1(ne219); alg-2(ok304)* genetic background. The *alg-2(ok304)* allele was introduced to further knockdown Argonaute activity. The second mutant utilized the promoter of the *inx-8* gene, so that RNAi sensitivity would be restricted to the sheath cells of the somatic gonad. The





**Figure 6. Tissue-specific RNAi in sheath cells shown in dissected gonad**  
 (A) A representative DIC image of an *oxTi76; rde-1(ne219); inx-8<sup>prom</sup>::rde-1(+)* gonad arm exposed to negative control RNAi (empty vector). (B) A representative fluorescent image of an *oxTi76; rde-1(ne219); inx-8<sup>prom</sup>::rde-1(+)* gonad arm exposed to control RNAi. 10/10 gonad arms exposed to control RNAi contained visible GFP in the sheath cell nuclei (.5 second exposure). No gonad arm exposed to *gfp* RNAi had GFP visible after a .5 second exposure (data not shown). (C) A representative DIC image of an *oxTi76 IV; rde-1(ne219); inx-8<sup>prom</sup>::rde-1(+)* exposed to *gfp* RNAi. (D) A representative fluorescent image of an *oxTi76; rde-1(ne219); inx-8<sup>prom</sup>::rde-1(+)* gonad arm exposed to *gfp* RNAi. 6/15 gonad arms exposed to *gfp* RNAi had no GFP visible (2 sec exposure). (E) A representative DIC image of an *oxTi76; rde-1(ne219); inx-8<sup>prom</sup>::rde-1(+)* gonad arm exposed to *gfp* RNAi. (F) A representative fluorescent image of an *oxTi76; rde-1(ne219); inx-8<sup>prom</sup>::rde-1(+)* gonad arm exposed to *gfp* RNAi (2 sec. exposure). With this longer exposure, 6/15 gonad arms had highly visible GFP and 3/15 had faint GFP visible.

somatic innexin *inx-8* gene is expressed in the proximal sheath cells as a component of the gap junctions that connect sheath cells to developing oocytes (Drake et al., 2014; Starich et al., 2014). The *inx-8<sup>prom</sup>::rde-1(+):unc54<sup>3'utr</sup>* transgene was integrated (location identified, Table 1) into a *rde-1(ne219)* genetic background. After the mutants were constructed, RNAi sensitivity upon expression of wild-type *rde-1* in specific tissues was validated by a procedure modified from (Qadota et al., 2007).

## CHAPTER FOUR

### Functional Analysis of microRNA pathway genes in the Somatic Gonad and Germ Cells During Ovulation in *C. elegans*

#### Introduction

MicroRNAs (miRNAs) are ~22 nt small non-coding RNAs that function to repress the translation of target mRNAs, typically through binding to sites in their 3' untranslated region (UTR) (Bartel, 2009; McCarter et al., 1999a). Through their association with Argonaute proteins, they serve as guide molecules for activity of the miRNA-induced silencing complex (miRISC) (Ha & Kim, 2014). Worms have two Argonautes that are required in the miRISC, but not other small interfering RNA (siRNA) pathways, and are encoded by *alg-1* and *alg-2* (Grishok et al., 2001; Ha & Kim, 2014; Hutvagner, 2001; Hutvagner et al., 2004; Ketting et al., 2001). Most mature miRNAs are generated through the canonical miRNA biogenesis pathway, consisting of a nuclear processing step, to generate a stem-loop pre-miRNA structure, and a cytoplasmic processing step, to generate the mature, active miRNA. Nuclear processing requires the RNase III enzyme Drosha, along with its cofactor, DGCR8/Pasha, whereas cytoplasmic processing requires the Dicer RNase III enzyme (Ha & Kim, 2014). Dicer processes both miRNAs and siRNAs while Drosha/DGCR8/Pasha is only known to process miRNAs (Grishok et al., 2001; Hutvagner, 2001; Ketting et al., 2001) .

Although miRNAs are essential for worm, fly, fish and mouse development (Grishok et al., 2001; Ketting et al., 2001), the identification of specific biological functions and direct downstream targets for miRNAs remains a critical gap in our

knowledge. Individual miRNAs for which functions have been described in *C. elegans* include the pioneering *lin-4* and *let-7*, which act to regulate larval developmental timing. The identification of *lin-4* and *let-7* was achieved through the strong, penetrant phenotypes induced by their mutation (Lee et al., 1993; Reinhart et al., 2000). However, most loss of function mutations in individual miRNA genes do not result in readily observable mutant phenotypes (Miska et al., 2007). One approach to identify and characterize miRNA-regulated processes is to examine the effects of inhibiting the activity of the miRNA biogenesis pathway. Loss of the miRNA-specific Argonaute genes *alg-1* and *alg-2* during early development causes embryonic lethality, demonstrating an essential role for miRNAs during embryogenesis (Grishok et al., 2001).

Interestingly, worms that have maternal, but not zygotic, activity of miRNA biogenesis genes, including *drsh-1* (Drosha), *pash-1* (Pasha/DGCR8) and *dcr-1* (Dicer), are sterile with endomitotic oocytes, indicating strong, penetrant ovulation defects (Denli et al., 2004; Grishok et al., 2001; Knight & Bass, 2001). In addition, mice with conditional loss of Dicer activity in the somatic cells of the gonads (Nagaraja et al., 2008), and of Pasha ortholog DGCR8 activity in the female reproductive tract are sterile (Kim et al., 2016). These results indicate that miRNAs are required in the mouse somatic gonad for normal fertility. In worms, Dicer/*dcr-1* activity is required in the somatic gonad for fertility (Drake et al., 2014) and Argonaute/*alg-1* acts in the somatic distal tip cell to control germline proliferation (Bukhari et al., 2012; Denli et al., 2004; Drake et al., 2014; Grishok et al., 2001; Knight & Bass, 2001). However, the specific events of oocyte

maturation and ovulation that require miRNA activity in the somatic gonad cells in worms remain unknown.

A role for miRNAs in the control of ovulation in germ cells is less clear. While translational regulation is essential for meiotic maturation in animals (Lehrbach et al., 2012; Mendez & Richter, 2001), the activity of miRNAs may not be required for germ cell development in all organisms (Lehrbach et al., 2012; Ma et al., 2010; Suh et al., 2010). In worms, Dicer is phosphorylated and localized to the nucleus during most of oogenesis, thereby preventing its normal cytoplasmic processing role (Drake et al., 2014). However, miRNA biogenesis likely functions at an early stage of germ cell development because mature, processed miRNAs are present in oocytes (Gu et al., 2009; McEwen et al., 2016). It is clear that some maternal miRNAs that are present in oocytes, including the miR-35 family, are essential for early development (Abbott et al., 2005; Alvarez-Saavedra & Horvitz, 2010; Brenner et al., 2010). Mosaic analysis indicates that Dicer activity is not essential in the germ line for the processes of oocyte maturation and ovulation to occur (Drake et al., 2014; Tops et al., 2006). However, it remains possible that maternal miRNAs act in the oocyte to more finely regulate the processes of oocyte maturation and ovulation.

Notably, the differences in miRNA abundance between worms that have zygotic deletion of Dicer and wild-type animals were found to be modest (Drake et al., 2014; Grishok et al., 2001; Knight & Bass, 2001). This is surprising because Dicer is required for the cytoplasmic processing of miRNAs. A possible explanation for the continued presence of miRNAs in the *dcr-1* zygotic mutants is presence of maternal Dicer activity. Because of the modest reduction of miRNA

levels in a zygotic *dcr-1* mutant, we analyzed the function of the miRNA-specific Argonautes, *alg-1* and *alg-2*, to determine if miRNAs can act to regulate oocyte maturation and ovulation, since these proteins act downstream of Dicer and are necessary for miRISC activity.

In worms, ovulation is a complex, rhythmic behavior that is regulated by multiple signaling pathways between the soma and the germ cells. The gonad arms contain germ cells that divide mitotically at the distal end and mature into oocytes as they reach the most proximal position of the gonad arm (Greenstein, 2005; Vasquez-Rifo et al., 2012). The somatic distal tip cell (DTC) controls the mitotic zone and ten somatic sheath cells surround the rest of the germ cells with the six most proximal sheath cells capable of contraction. The spermatheca is also contractile with a constricted distal end preventing the oocyte from entering the spermatheca until ovulation (Kemp et al., 2012; McCarter et al., 1999a). Major sperm protein (MSP) is released from sperm and interacts with receptors on the somatic sheath cells to initiate contractions of the proximal sheath cells and activate meiotic maturation in the oocyte. Upon meiotic resumption (transition from prophase I to metaphase I), the oocyte signals to the sheath cells and spermatheca resulting in an increase in sheath contraction rate and intensity, termed ovulatory contractions, and dilation of the distal end of the spermatheca. The mature oocyte is thus propelled into the spermatheca where it is fertilized (Iwasaki et al. 1996; McCarter et al., 1999a; Starich et al., 2014; Yin et al., 2004). Both sheath cell contraction and spermatheca dilation are dependent upon IP<sub>3</sub>-mediated calcium release (Clandinin et al., 1998; Iwasaki et al., 1996; McCarter et al., 1999a). Meiotic maturation is the rate-limiting step in the production of

embryos (McCarter et al., 2001). Thus, the rate of ovulation typically reflects the rate of meiotic maturation.

In order to investigate the role of miRNAs in specific events of oocyte maturation and ovulation more directly in both germ cells and the somatic gonad, we assessed the effects of conditional knockdown of miRNA pathway genes, including *pash-1* and the two miRNA-specific Argonautes, *alg-1* and *alg-2*, on ovulation events, including ovulation rate, sheath cell contractility, movement of oocyte through the spermathecal. We found that *pash-1* as well as *alg-1* and *alg-2* activities are not essential in germ cells for ovulation. However, *alg-1* and *alg-2* are important in the cells of the somatic gonad for control of sheath cell contraction and dilation of the distal end of the spermatheca. Also, our data indicates that *alg-1* and *alg-2* activity in the somatic gonad acts to maintain the rate of meiotic maturation in the oocyte. There was no evidence that meiotic maturation marker *lin-41* was mis-regulated in worms with knockdown of *alg-1*.

## RESULTS

Sterility in miRNA biogenesis mutants has been previously observed. We start by showing that the basis of sterility in *drsh-1(ok369)* mutant worms is the failure of ovulation, accompanied by and likely due to the absence of sheath contractions. Then we demonstrate the consequences that the knockdown of the *pash-1* gene has for various steps of ovulation. Finally, we use tissue-specific knockdown of miRISC genes *alg-1* and *alg-2* in somatic gonad and germ cells, as a way to determine the function of miRNA pathway genes that act further down the miRNA pathway in the regulation of oocyte maturation and ovulation.

### miRNA-specific biogenesis genes are required for ovulation

While sterility has been described for miRNA biogenesis mutants, including *dcr-1* and *drsh-1* mutants (Denli et al., 2004; Grishok et al., 2001; Knight & Bass, 2001), specific defects in the process of ovulation have not been described. *drsh-1(ok369)* homozygous mutants were derived from balanced, heterozygous worms and therefore had maternal activity of *drsh-1(+)*, allowing for worms to complete embryonic and larval development. Zygotic *drsh-1* mutant worms have a sterile phenotype with endomitotic oocytes (Denli et al., 2004; Drake et al., 2014; Grishok et al., 2001; Gu et al., 2009; Knight & Bass, 2001; McEwen et al., 2016). To examine this defect further, ovulation rate analysis and video microscopy was performed. Zygotic *drsh-1* mutants produced no progeny (Fig. 7A) and had few, if any, ovulation events (Fig. 8A). In agreement, video microscopy revealed essentially no gonadal sheath contractions in ~1 hour of observation (data not shown). These results provide quantitative analysis of the observed sterile phenotype and further support a requirement for miRNA biogenesis genes in the process of ovulation.

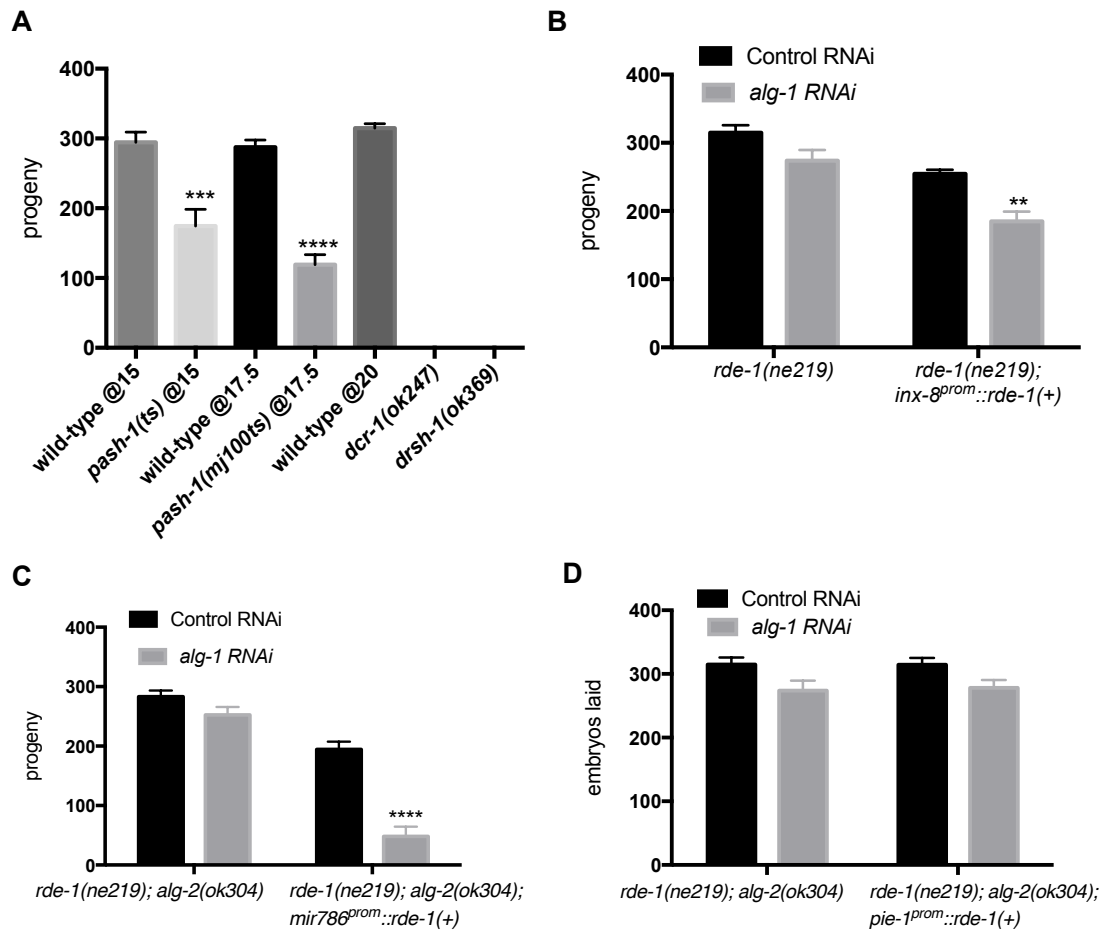
To identify the specific steps in ovulation for which miRNA biogenesis is required, we used the *mj100* conditional allele of the *pash-1* gene, which results in reduced, but not eliminated, miRNA biogenesis activity. We used *pash-1(mj100ts)* worms, with and without the *mjEx331 (eft-3<sup>prom</sup>::pash-1(+))* extrachromosomal rescue array (Lehrbach et al., 2012; McJunkin & Ambros, 2014). *pash-1(mj100ts)* mutant worms are viable and develop essentially normally at 15°C but are not viable at 25°C, with severe defects observed in

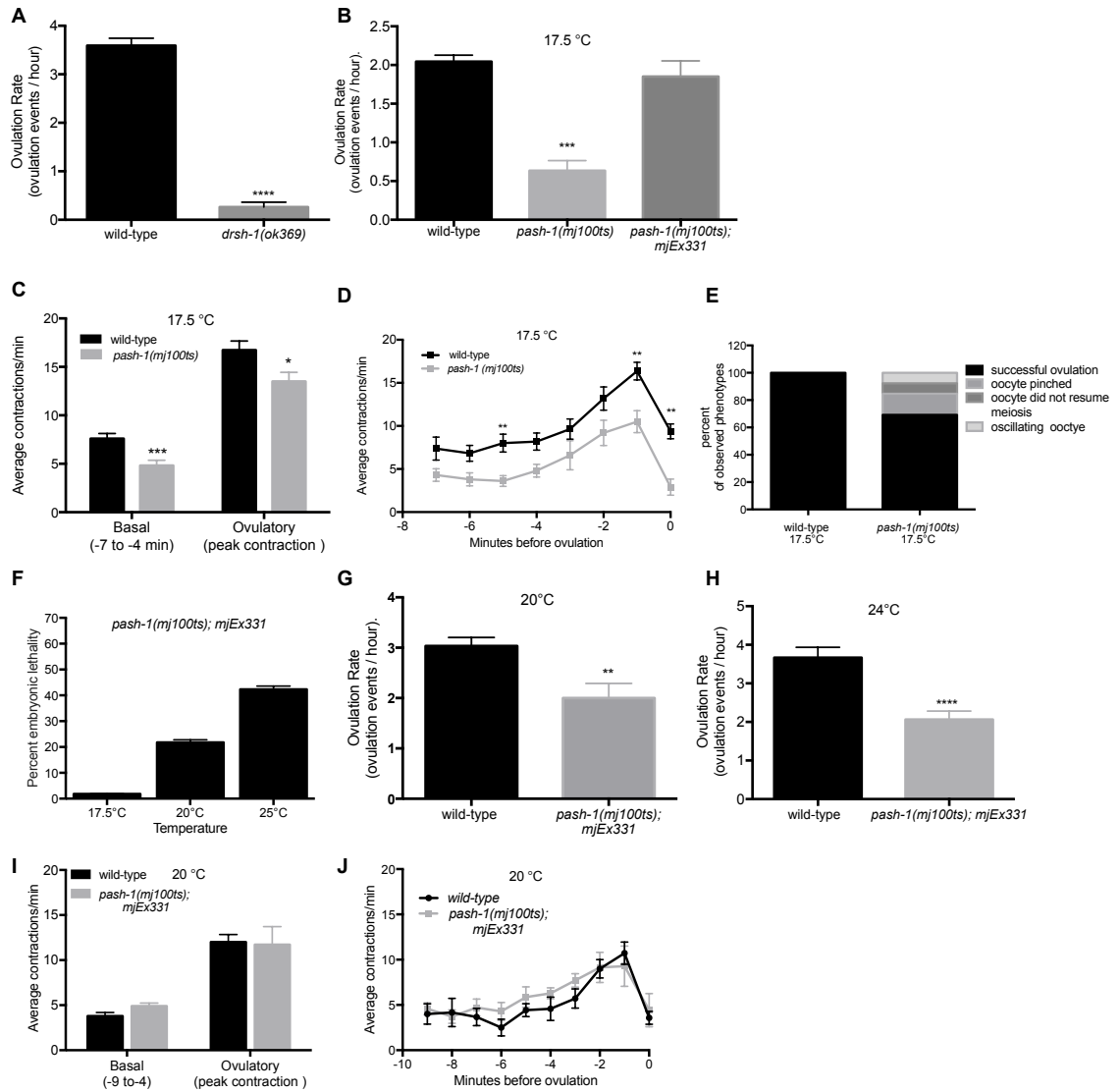


miRNA biogenesis (Lehrbach et al., 2012; Merritt et al. 2008). It is important to note that *pash-1(mj100ts)* worms display an early aging phenotype with shortened lifespan and altered metabolism at the restrictive temperature of 25°C ( Lee & Schedl, 2006; Lehrbach et al., 2012). It is possible that worms may display a weaker early aging phenotype at the intermediate temperatures. To minimize any potential indirect effects from early aging, all analysis was performed in young adult worms, within 24 hours of the L4 molt.

Compared to wild-type worms, *pash-1(mj100ts)* mutants had significantly reduced brood size (Fig. 7A) and ovulation rate at 15° C (data not shown), and these defects were even more pronounced at the intermediate temperature of 17.5° C (Fig. 8B). The temperature of 17.5°C was chosen for further analysis because worms showed a reduced ovulation rate but remained viable. When worms are grown at 17.5° C, *pash-1* activity is expected to be moderately reduced (Lehrbach et al., 2012) so that phenotypes observed demonstrate the effects of a reduction, but not an absence, of miRNAs. When *pash-1(mj100ts)* mutant worms are grown at 20°C or higher, the worms are not viable, therefore analysis of ovulation events was not possible at elevated temperatures.

Next, we analyzed whether decreased *pash-1* activity resulted in other observable ovulation phenotypes. Video microscopy of *pash-1(mj100ts)* worms grown at 17.5°C was performed to observe individual ovulation events (n = 13). Gonadal sheath cells were monitored and found to have a reduced rate for basal and ovulatory contractions (Fig. 8C-D) compared to wild-type. There were no ovulation defects observed in wild-type worms grown at 17.5°C (Fig. 8E). In





**Figure 8. miRNA pathway genes are required for the regulation of the ovulation rate, somatic sheath contractions and distal spermathecal dilation during ovulation.** (A-B) Ovulation rates were measured by total progeny and embryo production in populations of wild-type, miRNA biogenesis mutant worms and miRNA biogenesis mutant worms expressing a rescue array at different temperatures. (A) Ovulation rates (ovulation events / hour) for wild-type and *drsh-1(ok369)* worms at normal growing conditions of 20° C. *drsh-1(ok369)* were zygotic mutants, collected from heterozygous hermaphrodites and therefore had maternal *drsh-1* activity (n = 14-30). (B) Wild-type, *pash-1(mj100ts)* and *pash-1(mj100ts)* carrying an extrachromosomal rescue array, *mjEx331*, grown at the permissive temperature of 17.5°C ovulation rates (ovulation events / hour) (n = 10-25). (C-E) Individual ovulation events were analyzed using time-lapse microscopy. (C) Average basal and ovulatory sheath contractions were determined for wild-type and *pash-1(mj100ts)* mutants grown at 17.5°C. Average basal contractions were determined using the -7 to -4 minute interval with time 0 corresponding to when the oocyte is inside the spermatheca. Average peak ovulatory contractions were determined using the single highest contraction rate at 17.5°C (n = 10). (D) Wild-type and *pash-1(mj100ts)* sheath contraction rates were analyzed as single minute intervals from -7 minutes until 0 minutes at 17.5°C (n = 10). (E) A summary of ovulation phenotypes observed for individual ovulation events in wild-type (n=10) and *pash-1(mj100ts)* (n =13 events) worms. (F-J) *pash-1(mj100ts)* mutant worms carrying an extrachromosomal rescue array, *mjEx331*, were grown at different temperatures (17.5-25°C). Worms grown at restrictive temperatures lost activity from the *pash-1(mj100ts)* allele. The rescue array is only expressed in the soma due to transgene silencing. (F) Embryonic lethality was measured at 17.5°C, 20°C and 25°C (n > 200). (G-H) The ovulation rate (ovulation events / hour) was measured by counting total progeny and embryos produced for wild-type and *pash-1(mj100ts)*, *mjEx331* worms grown at the restrictive temperatures of 20°C and 24°C respectively. (I-J) Sheath contractility was analyzed for individual ovulatory events using time-lapse microscopy in wild-type and *pash-1(mj100ts)*, *mjEx331* worms. The average rate of basal contractions was determined from the -9 to -4 minute interval. Time 0 corresponds to when the oocyte is inside the spermatheca. The average rate of ovulatory sheath contraction was determined by using the single highest contraction rate observed in individual worms for wild-type and *pash-1(mj100ts)*, *mjEx331* worms at 20°C (n=7). *pash-1(mj100ts)*; *mjEx331* worms grown at 20°C. There were no ovulation defects observed for wild-type worms grown at 20°C (n = 10, data not shown), or for *pash-1(mj100ts)*; *mjEx331* worms grown at 20°C (n = 7, data not shown). Error bars indicate SEM. Statistical analysis was performed using unpaired, non-parametric, t-test \* p < 0.05, \*\*\* p < 0.001, \*\*\*\* p < 0.0001.

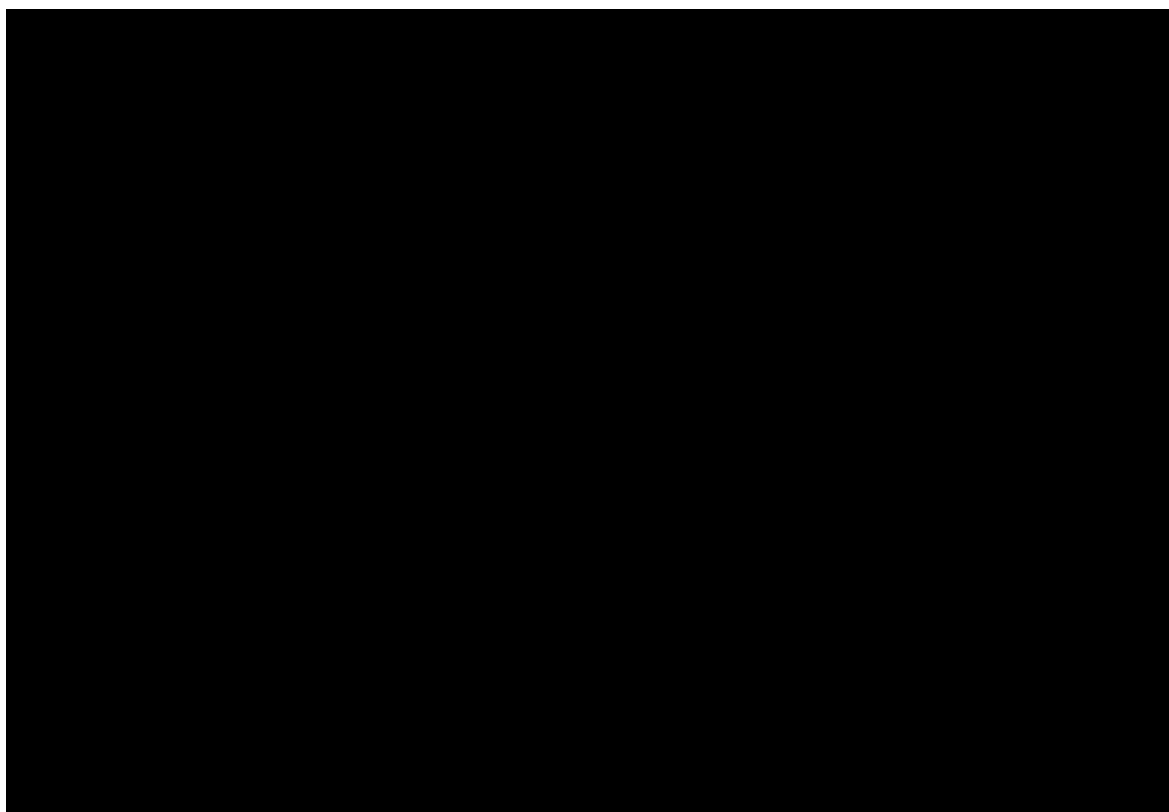
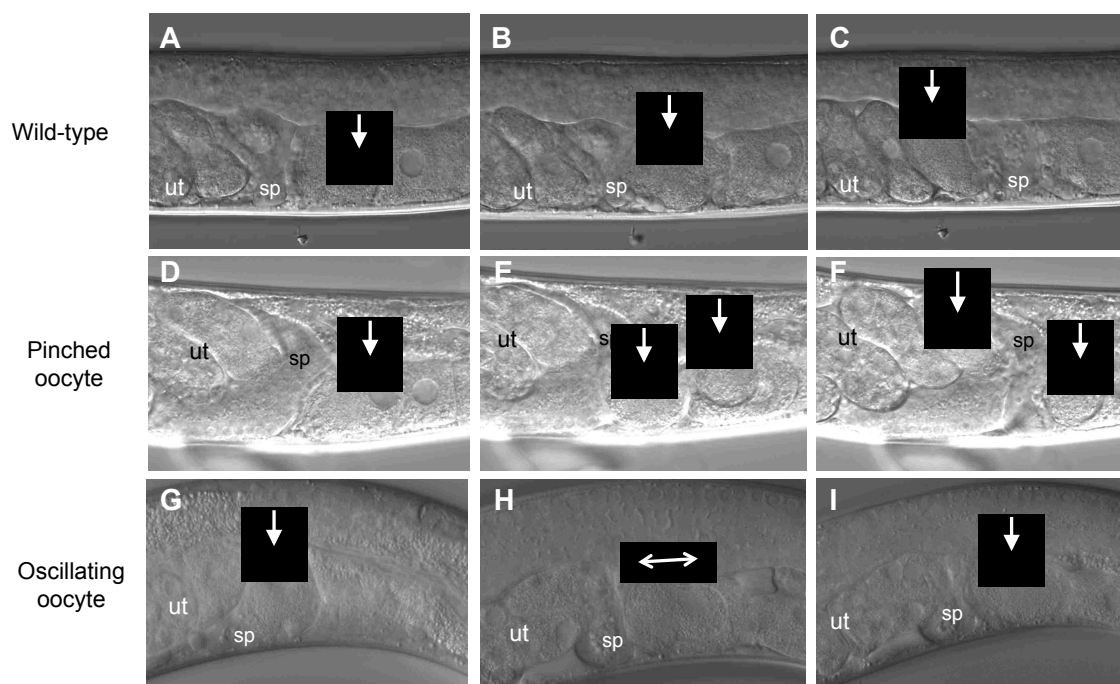
contrast, in over 30% of the recorded ovulation events in *pash-1(mj100ts)* worms ovulation defects were observed (Fig. 8E). In 15% of the recorded ovulation events, the proximal oocyte was pinched by the distal end of the spermatheca while entering the spermatheca (Fig. 8E and Fig. 9D-F). In addition, in 8% of the ovulation events the oocyte failed to resume meiosis, and in another 8% of the events the oocyte oscillated in and out of the spermatheca (Fig. 8E and Fig. 9G-I). These data indicate that miRNAs are required either in the somatic cells or in the germ cells for regulation of sheath cell contractions and spermatheca dilation in the process of ovulation.

In order to determine if germ cells require *pash-1* activity for ovulation, *pash-1(mj100ts)* worms with the *mjEx331* rescuing extra-chromosomal array were analyzed at elevated temperatures. Because extra-chromosomal arrays are typically silenced in the germ line, the *mjEx331* array provides somatic, but not germ cell, *pash-1(+)* rescuing activity. First, compared to wild-type worms, *pash-1(mj100ts)* with *mjEx331* at 17.5° C had no significant difference in the rate of ovulation (Fig. 8B). This result indicates that expression of wild-type *pash-1(+)* activity in somatic cells is sufficient to restore ovulation rate at the intermediate temperature of 17.5° C (Fig. 8B). To validate knockdown of maternal miRNAs in the germline, we analyzed the rate of embryonic lethality in *pash-1(mj100ts);mjEx331* grown at elevated temperatures. It is known that loss of maternal miRNAs, including the *mir-35* family, results in a fully penetrant embryonic lethality phenotype (Alvarez-Saavedra and Horvitz, 2010). Embryonic lethality increased with temperature in *pash-1(mj100ts);mjEx331* worms (Fig. 8F), suggesting reduced levels of maternal miRNAs in the oocyte. *pash-*

*1(mj100ts)* worms without the rescuing array that are shifted to the restrictive 25°C show 100% embryonic lethality and mature miR-35 is not detectable (Lehrbach et al., 2012). Because the observed embryonic lethality phenotype was not fully penetrant in *pash-1(mj100ts);mjEx331* at 24°C (Fig 8F), it is likely that there some activity of the *pash-1(ts)* allele is still present in worms grown at 24°C for many generations.

Next we tested ovulation rates for the *pash-1(mj100ts);pash-1::gfp* with the *mjEx331* rescue array at elevated temperatures (Fig. 8G-H). It was not possible to analyze *pash-1(mj100ts)* worms without the rescue array at elevated temperatures because viability is reduced when worms are grown at 20°C or higher. The ovulation rate was reduced in *pash-1(mj100ts);mjEx331* grown at 20°C and even further at 24°C compared to wild-type worms (Fig. 8G-H). The ovulation rate of worms grown at 24°C was reduced from 3.7 ovulation events/hour in wild-type to 2.1 ( $p < 0.0001$ ) in the *pash-1(mj100ts);mjEx331* (Fig. 8H). The decrease in the ovulation rate of the *pash-1(mj100ts)* with the *mjEx331* rescue array at elevated temperatures suggests a possible role for miRNAs in germ cells in the process of ovulation, or alternatively is a result of incomplete *pash-1 (+)* somatic rescue of the array.

Video microscopy was performed to analyze ovulation events ( $n = 13$ ) in *pash-1(mj100ts);mjEx331* worms with somatic *pash-1(+)* rescue. Worms were analyzed at 20° C. In spite of the decreased ovulation rate (Fig. 8G), there were no observable differences in the rate of basal or ovulatory contractions compared



to wild-type (Fig. 8I-J). In addition, there were no defects in transit through the spermatheca observed during ovulation events in *pash-1(mj100ts); mjEx331* (n=13 successful ovulation events). Together, our results suggest a role for *pash-1* activity in somatic cells and germ cells for the ovulation rate, and in somatic cells for sheath contractions and transit of the oocyte through the spermatheca.

### **miRNA-specific Argonaute activity is required in somatic gonad cells, but not germ cells, for ovulation**

Having identified ovulation rate, sheath contractions, and distal spermatheca defects in the *pash-1(mj100ts)* worms, we next investigated the role for miRNA-specific Argonautes in the cells of the somatic gonad during ovulation by knocking down *alg-1* and *alg-2*. The miRISC functions downstream of Pasha/*pash-1* in the miRNA pathway and is required for miRNA activity. *C. elegans* have 27 Argonaute genes (Youngman & Claycomb, 2014). Out of these, only *alg-1* and *alg-2*, have been shown to be required for the miRNA pathway, (Abbott et al., 2005; Alvarez-Saavedra & Horvitz, 2010; Bartel, 2009; Brenner et al., 2010; Grishok et al., 2001; Hutvagner et al., 2004; Miska et al., 2007), and not RNAi (Grishok et al., 2001). To validate the RNAi knockdown of *alg-1*, we exposed L4-stage *alg-2(ok304)* mutant worms to *alg-1* RNAi and verified 100% embryonic lethality (data not shown), in parallel with every RNAi experiment. First, tissue-specific knockdown of *alg-1* was performed in the cells of the somatic gonad. Expression of *alg-1* and *alg-2* has been analyzed extensively (Tops et al., 2006; Vasquez-Rifo et al., 2012). *alg-1* is expressed from early embryogenesis to adulthood in most, if not all, cells (Tops et al., 2006)



and both *alg-1* and *alg-2* are expressed in the cells of the somatic gonad (Vasquez-Rifo et al., 2012).

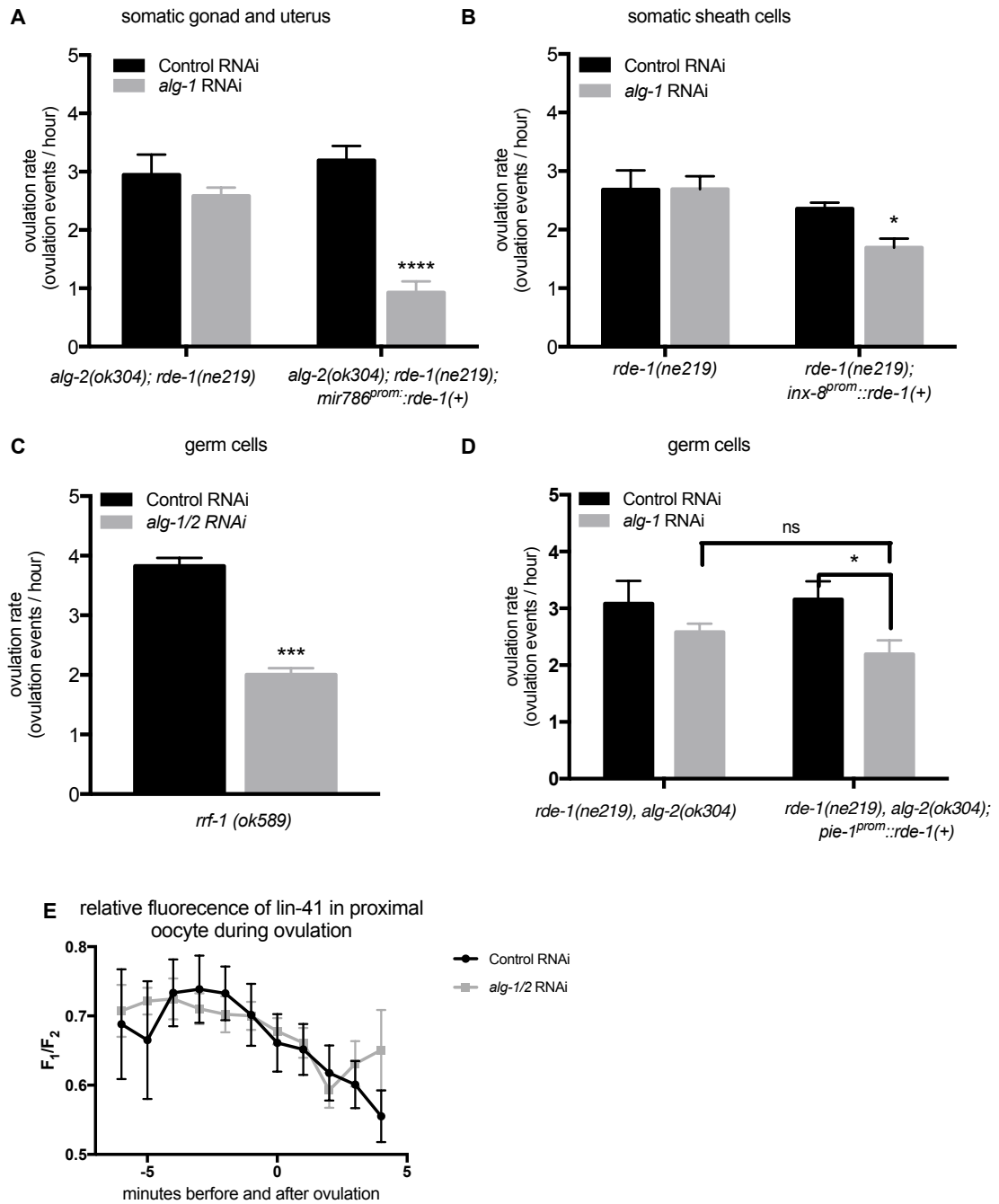
To test the role of miRNA-specific Argonautes in the somatic gonad we generated two strains for tissue-specific RNAi. The first, *alg-2(ok304);rde-1(ne219); mir-786<sup>prom</sup>::rde-1(+)*, targeted the entire somatic gonad, and the second, *rde-1(ne219);inx-8<sup>prom</sup>::rde-1(+)*, the sheath cells of the somatic gonad (see Ch. 3 for promoter information and validation procedure). The *rde-1(ne219);inx-8<sup>prom</sup>::rde-1(+)* transgenic worms and the *rde-1(ne219)* control strain were exposed to *alg-1* RNAi and a control RNAi, while the *alg-2(ok304);rde-1(ne219); mir-786<sup>prom</sup>::rde-1(+)* and the *alg-2(ok304);rde-1(ne219)* control strain were exposed to post-embryonic *alg-1* RNAi and a control RNAi (see Materials and Methods, Ch. 2). Compared to *alg-2(ok304);rde-1(ne219)* controls, *alg-2(ok304);rde-1(ne219); mir-786<sup>prom</sup>::rde-1(+)* worms showed a significantly reduced rate of ovulation from a rate of 3.2 ovulation events/hour following control RNAi to 0.9 ovulation events/hour following *alg-1* RNAi (Fig. 10A). There was also a significant decrease in overall brood size ( $p < 0.0001$ , Fig. 7).

Ovulation also requires the normal development and physiology of germ cells to proceed normally. Signals from sperm, including MSP, act to trigger meiotic maturation in the proximal oocyte, while signals from the oocyte act to trigger increased sheath contractility and dilation of the distal end of the spermatheca (Drake et al., 2014; K. Iwasaki et al., 1996; McCarter et al. 1999a). The decrease in the ovulation rate observed in *pash-1(mj100ts)* worms with the rescue array, lead us to further assess the role of miRNAs in the germ cells. Our approach was to again knockdown miRNA-specific Argonautes. First,

knockdown of *alg-1* was performed in *rrf-1* mutant worms. *rrf-1* encodes an RNA dependent RNA polymerase that is required for RNAi in somatic tissue (McCarter et al. 1999a; Sijen et al., 2001; Yin et al., 2004).

*rrf-1* mutants are sensitive to RNAi in the germline, but are resistant in somatic cells (Bui & Sternberg, 2002; Clandinin et al., 1998; Sijen et al., 2001; Yin et al., 2004). However, *rrf-1* mutants have been found to display RNAi in some somatic tissues including the intestine and the hypodermis, but, importantly, no RNAi was observed in cells of the somatic gonad (Kovacevic et al., 2013; Kumsta & Hansen, 2012). RNAi was performed in *rrf-1* worms at the L4 stage and their F1 progeny were analyzed as young adults. In *rrf-1(ok589)* mutants, knockdown of *alg-1* by RNAi resulted in a reduced ovulation rate (Fig. 10C), supporting the results observed in the *pash-1(ts)* worms with the rescue array.

Because the phenotypes observed upon *alg-1* knockdown in the *rrf-1(ok589)* mutants may reflect indirect involvement of somatic tissues, a tissue-specific RNAi strain was constructed, using the *pie-1* promoter to drive *rde-1(+)* activity, followed by the *tbb-2* 3'UTR (see Ch. 3 for promoter information and validation procedure). For these experiments, post-embryonic RNAi was performed starting in L2 stage worms in an effort to avoid potential knockdown in any somatic lineages (Reece-Hoyes et al., 2007). Exposure to *alg-1* RNAi beginning at the L2



**Figure 10. miRNA-specific Argonautes *alg-1* and *alg-2* are required in the somatic gonad, but likely not in the germ cells, for the regulation of ovulation rate.**

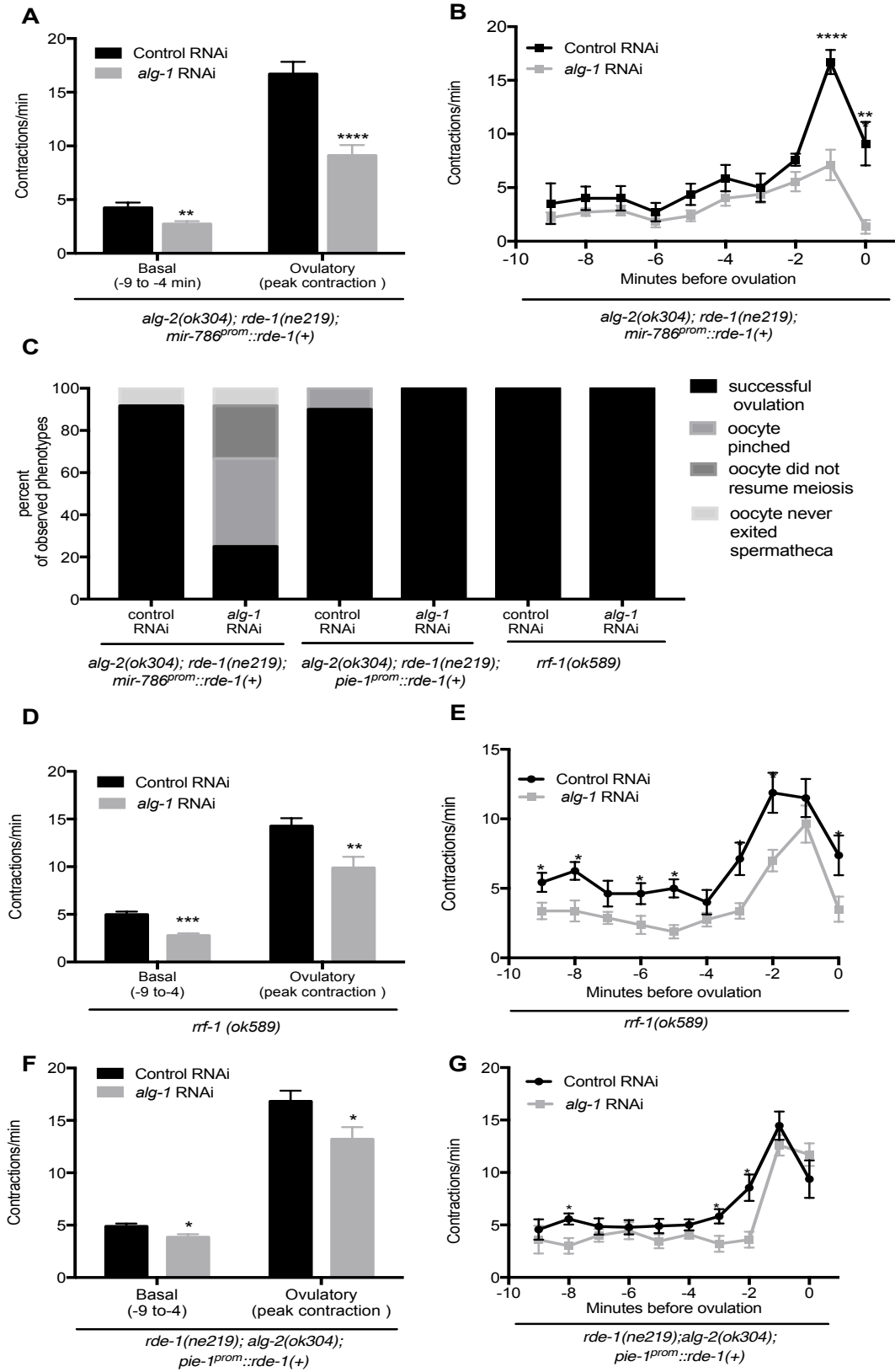
Ovulation rate was analyzed following RNAi to knockdown *alg-1* or a control RNAi (empty vector). For each group, 10-20 young adult worms were analyzed. To verify *alg-1* knockdown, we exposed *alg-2(ok304)* L4 stage worms to *alg-1* RNAi and confirmed that they exhibited 100% embryonic lethality. (A) Ovulation rates (ovulation events / hour) measured by total progeny and embryo production in *alg-2(ok304); rde-1(ne219)* and *alg-2(ok304); rde-1(ne219); mir-786<sup>prom</sup>::rde-1(+)* following post-embryonic RNAi. The *mir-786* promoter drives the expression of rescuing *rde-1(+)* activity throughout the somatic gonad. (B) Ovulation rates (ovulation events / hour) measured by total progeny and embryo production in *rde-1(ne219)* control strain and *rde-1(ne219); inx-8<sup>prom</sup>::rde-1(+)* following RNAi. The *inx-8* promoter drives expression of rescuing *rde-1(+)* activity in the somatic sheath cells. (C-D) Tissue-specific gene knockdown in germ cells was performed using *rrf-1(ok589)* mutants and *rde-1(ne219); alg-2(ok304); pie-1<sup>prom</sup>::rde-1(+)* transgenic worms following RNAi to knockdown *alg-1* or with control (empty vector) RNAi. (C) Ovulation rates (ovulation events / hour) for *rrf-1(ok589)* worms following *alg-1* or control (empty vector) RNAi (n = 10-20). (D) Ovulation rates (ovulation events / hour) for *rde-1(ne219); alg-2(ok304)* control strain and *rde-1(ne219); alg-2(ok304); pie-1<sup>prom</sup>::rde-1(+)* transgenic worms following post-embryonic *alg-1* or control RNAi. The *pie-1* promoter drives rescuing *rde-1(+)* expression in germ cells. (E) Average fluorescent measurements of the proximal oocytes of worms expressing *GFP::tev::s::lin-4* on *alg-1* and control RNAi prior to rounding of the proximal oocyte through entry of the fertilized embryo into the uterus. Analysis was accomplished by measuring the ratio of the average fluorescence (F) in a region of interest (ROI) in the most proximal oocyte (F<sub>1</sub>) and the corresponding fluorescence of the next oocyte (F<sub>2</sub>). The ratio F<sub>1</sub>/F<sub>2</sub> quantifies the relative expression of *lin-41* in the proximal oocyte. Error bars indicate SEM. Statistical analysis was performed using unpaired, non-parametric, t-test for C,E and two-way ANOVA, Tukey's multiple comparison for A-B and D. \* p < 0.05, \*\*\* p < 0.001, \*\*\*\* p < 0.0001

stage in *alg-2(ok304); rde-1(ne219); pie-1<sup>prom</sup>::rde-1(+)* hermaphrodite worms, resulted in 100% embryonic lethality indicating a strong, penetrant reduction in miRNA biogenesis in the germline. When worms of this genotype were exposed to post-embryonic *alg-1* RNAi, a decreased rate of ovulation was observed compared to control RNAi (p = 0.0474) (Fig. 10D). However, the rate of ovulation was not significantly different from *rde-1(ne219); alg-2(ok304)* without the *pie-1<sup>prom</sup>::rde-1(+)* transgene (Fig. 10D) indicating that the modest effect could be attributed to a variation in the *rde-1(ne219); alg-2(ok304)* background and was not due to the specific knockdown of miRNA biogenesis in the germline. In

addition, there was no significant decrease in the total number of embryos produced (Fig. 7D).

Having identified a decrease in ovulation rate in the *rrf-1(ok589)* mutants exposed to RNAi, but not in *alg-2(ok304); rde-1(ne219); pie-1<sup>prom</sup>::rde-1(+)* mutants, we sought to investigate if *alg-1* knockdown was associated with a misregulation of meiotic maturation marker *lin-41*. LIN-41 is a highly conserved TRIM-NHL RNA binding protein and is best known for its role in developmental timing pathway in somatic cells as the target of *let-7* miRNA oocyte (Spike et al., 2014). LIN-41 inhibits meiotic maturation and enables oocytes to grow by functioning in 3'UTR mediated translational repression (Spike et al., 2014). LIN-41 and LIN-41::GFP begin to be eliminated from oocytes as they undergo meiotic maturation, which in the *C.elegans* gonad occurs in the most proximal oocyte (Spike et al., 2014). By measuring the change in fluorescence of the proximal oocyte in *lin-41 [GFP::tev::s::lin-41]* mutant worms exposed to *alg-1* RNAi and comparing them to worms exposed to control RNAi, we can determine if knockdown of *alg-1* results in the misregulation of marker *lin-41*.

*lin-41 [gfp::tev::s::lin-41]* worms were placed on post-embryonic *alg-1* and control RNAi. Images of the proximal gonad and oocytes of worms expressing *GFP::tev::s::lin-4* were captured during individual ovulation events prior to rounding of the proximal oocyte through entry of the fertilized embryo into the uterus. There was no significant difference observed in fluorescence of oocytes in worms exposed to *alg-1* RNAi to those to those exposed to control RNAi (Fig.10E, n=8). This suggests that *lin-41* is not misregulated during meiotic



**Figure 11.** miRNA-specific Argonautes *alg-1* and *alg-2* are required in the somatic gonad for sheath contractility and distal spermathecal dilation. (A-B) Sheath contractility was analyzed during individual ovulatory events using time-lapse microscopy in *alg-2(ok304); rde-1(ne219); mir-786<sup>prom</sup>::rde-1(+)* following post-embryonic *alg-1* and control RNAi (n = 12). (B) The average rate of basal contractions was determined from the -9 to -4 minute interval with time 0 corresponding to when the oocyte is inside the spermatheca. Average peak ovulatory sheath contraction rate was determined by using the single highest contraction rate observed in individual worms. The average peak ovulatory contractions were determined by using the single highest contraction rate observed in individual worms. (B) Contraction are shown as single minute intervals prior to ovulation. (C) A summary of ovulation phenotypes observed for individual ovulation events in *rde-1(ne219); alg-2(ok304); mir786<sup>prom</sup>::rde-1(+)* (n=12), *rde-1(ne219); alg-2(ok304); pie-1<sup>prom</sup>::rde-1(+)* (n=10), and *rff-1(ok589)* (n=10) mutant worms following control and *alg-1* RNAi. For descriptions of ovulation defects, see Figure 2. (D-G) *alg-1* and control (empty vector) RNAi was performed on *rff-1* and post-embryonically on *rde-1(ne219); alg-2(ok304); pie-1<sup>prom</sup>::rde-1(+)* mutant worms. (D) The average rate of basal contractions was determined using the -9 to -4 minute interval with time 0 corresponding to when the oocyte is inside the spermatheca. The average ovulatory sheath contraction rate was determined by using the single highest contraction rate observed in individual worms (n = 10). (E) Contraction rates are shown in single minute intervals prior to ovulation. (F) The average rate of basal contractions was determined using the -9 to -4 minute interval. The average ovulatory sheath contraction rate was determined by using the single highest contraction rate observed in individual worms (n = 10). (G) Contraction rates are shown in single minute intervals prior to ovulation for *rde-1(ne219); alg-2(ok304); pie-1<sup>prom</sup>::rde-1(+)* worms following RNAi. Error bars indicate SEM. Statistical analysis was performed using unpaired, non-parametric, t-test for (A-B, D-G). \* p < 0.05, \*\*\* p < 0.001, \*\*\*\* p < 0.0001

maturation. Taken together, the results of our various approaches indicate that germ cells do not require *alg-1* and *alg-2* to maintain the normal ovulation rate.

### ***alg-1* and *alg-2* are required in the somatic gonad for sheath contraction and spermatheca dilation**

We used our tissue-specific RNAi strains to investigate whether reduction in *alg-1* and *alg-2* activity in cells of the somatic gonad and germ cells would result in reduced sheath contractions and distal spermathecal dilation, as observed in *pash-1(mj100ts)* mutants. Video microscopy of *alg-2(ok304); rde-1(ne219); mir-786<sup>prom</sup>::rde-1(+)* worms demonstrated that the average basal

contractions were reduced from 4.2 to 2.7 contractions/minute ( $p < 0.05$ ) while the average ovulatory contractions had a larger reduction from 16.7 to 9.1 contractions/min. ( $p < 0.0001$ ) following *alg-1* RNAi compared to control RNAi (Fig. 11A-B). Similar, but more severe defects to *pash-1(ts)* mutants were observed during video microscopy of individual ovulation events ( $n = 12$ ): 25% showed successful ovulations (Fig. 9A-C, Fig. 11C), 42% showed pinching by the distal spermatheca (Fig. 9D-F, Fig. 11C), 25% failed to resume meiosis and did not ovulate within 1 hour of observation (Fig. 11C), and 8% entered but failed to exit the spermatheca (Fig. 11C). *alg-2(ok304);rde-1(ne219); mir-786<sup>prom</sup>::rde-1(+)* worms on control RNAi showed few ovulation defects: 92% showed successful ovulations (Fig. 9A-C, Fig. 11C), and 8% entered but failed to exit the spermatheca (Fig. 11C,  $n = 12$ ). These data support a role for miRNAs acting in the somatic gonad to regulate the rate of meiotic maturation, rate of sheath cell contractions, and the dilation of the distal spermatheca.

To assess the role of *alg-1* and *alg-2* in germ cells in individual ovulation events, we used our tissue-specific RNAi strains that allow for knockdown in germ cells but not in somatic gonad cells. In *rrf-1(ok589)* mutants, knockdown of *alg-1* by RNAi resulted in reduced basal and ovulatory sheath contractions (Fig. 11D-E), however the pattern of sheath contractions closely mirrored that of worms placed on control RNAi. Importantly, *rrf-1(ok589)* worms exposed to *alg-1* RNAi and control RNAi showed wild-type ovulation events (Fig. 2A-C, Fig. 11C) with no defects in oocyte transit through the spermatheca observed ( $n > 10$ , Fig. 11C).

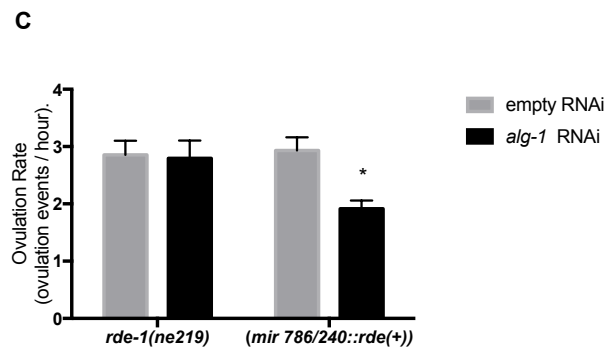
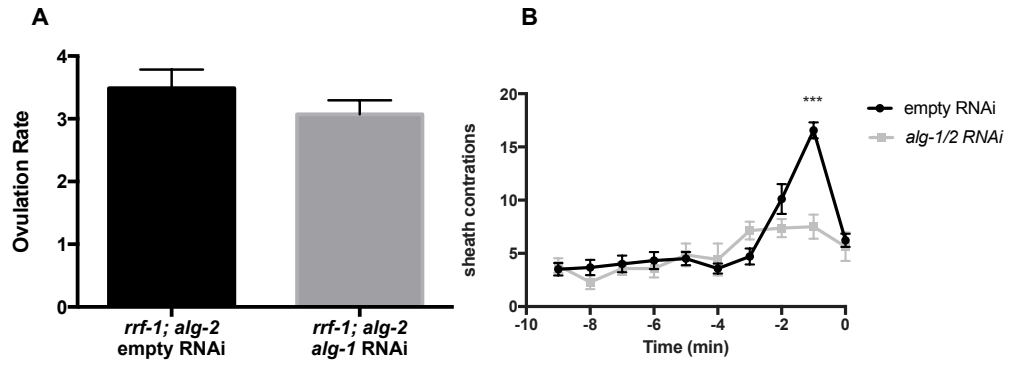


We next performed video microscopy on the *alg-2(ok304); rde-1(ne219); pie-1<sup>prom</sup>::rde-1(+)* worms to on both *alg-1* and control RNAi. A modest decrease in the rates for basal and ovulatory contractions was observed following *alg-1* RNAi, but the pattern of sheath contractions mirrored even more closely those of worms exposed to control RNAi (Fig. 11F-G). Importantly, knocking down *alg-1* in the *alg-2(ok304); rde-1(ne219); pie-1<sup>prom</sup>::rde-1(+)* mutants did not result in any defects in oocyte transit through the spermatheca (n > 10, Fig. 11C). We conclude that miRNA pathway genes are not essential in germ cells for the process of ovulation in *C. elegans*.

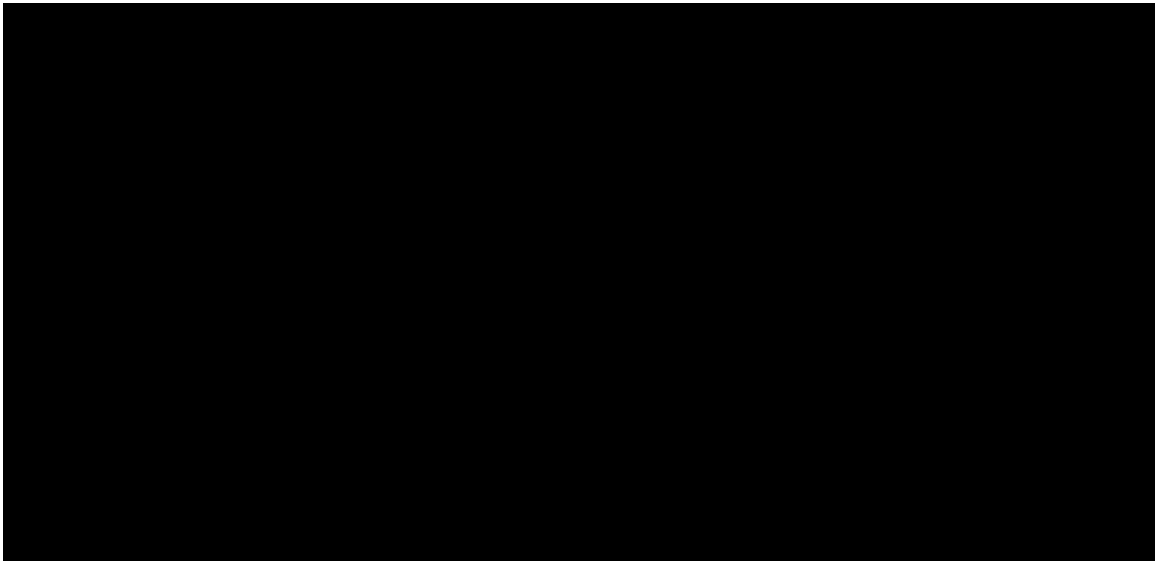
#### **Additional mutants with knockdown of *alg-1* in the germline and somatic gonad**

An *alg-2* mutation was added to *rrf-1* mutants to further knockdown miRISC activity in the germ cells. Upon exposure to empty and *alg-1* RNAi there was no significant reduction in ovulation rate observed (Fig. 12A). Analysis of sheath contraction revealed a significant decrease in the peak ovulatory contraction, but no difference in the basal rate of contraction compared to control (Fig. 12B). Due to the findings that *rrf-1* mutants have RNAi capabilities in the somatic tissues and that *alg-2* is not expressed in the germ cells these results were not published.

The ovulation rate of *mir786<sup>prom</sup>::rde-1* mutant worms on *alg-1* RNAi was significantly decreased compared to worms on empty RNAi (Fig. 12C). To further knockdown miRISC activity in the somatic gonad an *alg-2* mutation was added and further analysis was performed (Fig. 11A-C).



n=20



## CHAPTER FIVE

### DISCUSSION

Worms with mutation in genes involved in the miRNA-specific biogenesis pathway including *drsh-1* and *pash-1*, are sterile (Denli et al., 2004; Grishok et al., 2001). In them, we observed essentially no successful ovulations. In spite of this evidence for their necessary involvement, the functional roles of miRNAs in the complex process of ovulation remain unknown. Using a conditional *pash-1* temperature sensitive mutant and new strains created to perform tissue-specific RNAi, I characterized the roles of miRNA biogenesis gene *pasha* in the whole worm and Argonautes *alg-1* and *alg-2* in somatic gonad and germ cells. The knockdown of the miRNA-specific Argonautes interferes with the miRNA biogenesis pathway downstream of previously characterized miRNA biogenesis mutants (Denli et al., 2004; Drake et al., 2014; Grishok et al., 2001; Knight & Bass, 2001) and impacts the activity of the miRNAs. I conclude that *alg-1*, *alg-2* and likely miRNA activity are not essential in germ cells for ovulation but are required in the cells of the somatic gonad for proper sheath cell contraction and distal spermathecal dilation. In addition, my results suggest that miRNAs function in cells of the somatic gonad for the control of meiotic maturation in oocytes, as evidenced by the decrease in ovulation rate, which requires oocyte maturation to occur.

In contrast to *drsh-1* zygotic mutants, we did not observe a penetrant sterile phenotype in any of our experimental conditions. This may reflect the

conditional or partial knockdown of miRNA biogenesis. The limited penetrance could be due in part to incomplete knockdown of Argonaute activity. Also, worms have 27 Argonaute genes, including *T23D8.7/hpo-24*, which is closely related to *alg-1* and *alg-2*; therefore it remains possible that additional Argonaute proteins could function to mediate miRNA regulation in the germline (Youngman & Claycomb, 2014), though such activity has never been described. In addition, worms that allowed for knockdown of *alg-1* and *alg-2* in the entire somatic gonad (*mir-786<sup>prom</sup>::rde-1(+)* transgene in an *rde-1*-null genetic background) expressed *rde-1(+)* activity from an extrachromosomal array, which can have some mosaicism in transgene expression. Lastly, worms with the *pash-1* temperature sensitive allele likely have some residual protein activity at the semi-permissive temperature of 17.5°C.

### **miRNAs in germ cells appear not to have an essential role in ovulation**

miRNAs are found in germ cells across the animal kingdom, including worms, flies, mice, and cows (Gilchrist et al., 2016; Gu et al., 2009; M. Lee et al., 2014; Ma et al., 2010; McEwen et al., 2016). In worms, several miRNA families are abundantly expressed in the oocyte, including some that are known to be required for embryonic development and others with unknown functions (Gu et al. 2009). The *mir-35* family is one of several found to be highly enriched in germ cells, specifically in oocytes (Gu et al., 2009; McEwen et al., 2016). Like other miRNAs expressed in oocytes, the *mir-35* family is required for embryonic development but has no known function in maturation or ovulation (McJunkin & Ambros, 2014). The *mir-35* family functions to control the number of progeny

produced by adult hermaphrodites (McJunkin & Ambros, 2014), but the mechanism remains unknown. Our results give weight to the possibility that the miRNAs present in the oocyte do not function in maturation or regulating the signaling pathways required for ovulation, but rather are poised to act after fertilization during embryonic development.

In previous work with zygotic-null Dicer (*dcr-1(0)*) worms, levels of miRNAs in the germline were surprisingly high, making their activity possible, even in the *dcr-1(0)* genetic background. To eliminate this possibility, I took an alternative approach, knocking down miRNA-specific genes *alg-1* and *alg-2* in the germ cells. ALG-1 and ALG-2 act downstream of Dicer in the biogenesis pathway and function in miRNA silencing as a core component of the miRISC. This approach allowed me to examine the function of miRNAs in germ cells in the process of oocyte maturation and ovulation.

There was reason to suspect that the miRNAs are active in the germ cells. Post-transcriptional control of gene expression is essential in the *C. elegans* germline for the development of mature oocytes. Analysis of reporter transgene expression demonstrated that the 3'UTRs of mRNAs in the germline and developing oocytes play a larger role than the promoter regions (Merritt et al., 2008). RNA binding proteins, miRNAs, and siRNAs can all function through sites in the 3' UTR to control translation. Many RNA binding proteins are essential for normal germline development, including PUF proteins, GLD-1, and NOS-3 (Lee & Schedl, 2006). In addition various siRNAs function in the germ cells to maintain genomic integrity (Gu et al. 2009, Lee et al. 2012; Lee et al., 2012).

To test whether miRNAs found in germ cells act as 3' UTR regulators in the process of ovulation, I knocked down miRNA biogenesis in these cells. There was strong embryonic lethality upon exposure to *alg-1* RNAi the mutants that have the RNAi pathway rescued exclusively in the germline (*pie-1<sup>prom</sup>::rde-1(+)*) and in the *pash-1* temperature sensitive mutants with *pash-1* rescue in somatic cells, indicating strong, penetrant knockdown of miRNA activity. Surprisingly, despite this strong knockdown, I did not observe similarly strong or penetrant ovulation defects. My study therefore indicates that germ cell miRNAs do not play an essential role for the control of oocyte maturation or ovulation, but are required soon after ovulation for early embryonic development.

The role of miRNA activity in the germ cells of animals is unclear. While translational regulation is essential for meiotic maturation in animals (Mendez & Richter, 2001), the activity of miRNAs may not be required for germ cell development in some organisms. Indeed, it has been demonstrated that miRNA activity is suppressed in mouse oocytes (Ma et al., 2010) though it is not clear if such suppression occurs in worms. Also in mice, conditional loss of Dicer results in sterility due to defects in meiosis (Murchison et al., 2007; Tang et al., 2007). However, this phenotype is due to loss of siRNAs and not the loss of miRNAs (Ma et al., 2010; Suh et al., 2010). In worms, Dicer is phosphorylated and localizes to the nucleus during most of oogenesis, precluding its normal miRNA processing function in the cytoplasm. Moreover, mosaic analysis indicates that Dicer is dispensable in the germ line for the process of oocyte maturation and ovulation (Drake et al., 2014). These observations indicate lack of involvement of Dicer in germ cells up to and including ovulation.

The observation that miRNA activity is largely dispensable in the germline prior to fertilization is consistent with research in mice but inconsistent with research in flies. In mice, gene regulation by miRNAs is inactive in both oocytes and early embryos before the maternal-to-zygote transition (Svoboda, 2010). Also in mice, oocytes with a deletion in the miRNA biogenesis-specific *Dgcr8* exhibit normal maturation. In addition, limited miRNA-directed mRNA degradation occurs in mouse oocytes (Ma et al., 2010). On the other hand, in flies, miRNA biogenesis genes, including *ago1*, *dicer-1*, *drosha* and *pasha*, are necessary for oocytes to form and for germ cell proliferation (Azzam et al., 2012). Also in flies, several studies have ascribed functions for individual miRNAs in germline development or proliferation (reviewed in Carthew et al., 2016).

Why has miRNA function in germ cells diversified within the animal kingdom? The small RNA biogenesis pathways are interconnected as a result of shared enzymes that process them and the AGOs to which they associate. This overlap may provide an explanation for the suppression of miRNA activity in the germline of mice and possibly worms. The importance of maintaining germline integrity for current and future generations by funneling resources to siRNA biogenesis and activity may give a teleological reason for suppression of miRNAs. Interestingly, nematodes and mammals have only one Dicer gene; other organisms such as *Drosophila* have two: Dicer-1, which functions in miRNA biogenesis, and Dicer-2 for siRNA biogenesis (Lee et al., 2004a). In worms, it has been observed that competition for Dicer activity exists among the different small RNA pathways (Zhuang & Hunter, 2012). Competition is also attributed to the phenotype that occurs in worms with mutations in endo-RNAi

specific genes (*rrf-3* and *eri-1*), which results in enhancement of some RNAi pathways and elimination of others (Lee et al., 2006). Suppression of miRNA activity may be required to ensure a robust activity of siRNA's in the germ cells. As organisms evolve towards parsimony, proteins gain broader roles, which leads to increased regulation of their substrates.

The experiments that demonstrated suppression of miRNA activity in mouse oocytes can be performed in worms to determine whether oocyte miRNAs are suppressed. The activity of miRNAs in mouse oocytes was assessed, in part, by expressing in oocytes cognate mRNAs with luciferase reporter genes that contained 3' binding site for miRNAs or oocyte siRNAs (Ma et al. 2010). These tests demonstrated that endogenous oocyte miRNAs had poor ability to repress the reporter mRNAs compared to siRNAs, leading to the conclusion that siRNAs rather than miRNAs are active in the oocytes. It would be very important and decisive to perform these same experiments in worms.

### **Germline roles of miRNAs that have not been demonstrated but remain possible**

In our work, the knockdown of *alg-1*, *alg-2* and *pasha* in the germ line did not result in strongly penetrant ovulation defects. With these observations, it is important to note that I never assayed miRNAs in the germ cells. To validate knockdown of maternal miRNAs in the germline, I assayed embryonic lethality, because it is known that loss of maternal miRNAs, including the *mir-35* family, results in a fully penetrant embryonic lethality phenotype (Alvarez-Saavedra and Horvitz, 2010). Because the observed embryonic lethality phenotype was not fully penetrant in *pash-1* temperature sensitive mutants with the *pash-1* rescue



array at 24°C (Fig 8F), some activity of the *pash-1(ts)* allele likely persisted in worms grown at 24°C. Therefore, the lack of a sheath contraction phenotype in the *pash-1(ts)* mutants with the rescue array (Fig. 8J) may be the result of residual activity of the temperature sensitive allele in the germline. On the other hand, post-embryonic RNAi was applied to the mutants with *alg-1* and *alg-2* knockdown in the germline because they exhibit 100% embryonic lethality. Even this strong phenotype does not assure lack of maternal miRNAs. Therefore, one cannot interpret the lack of phenotypes observed in these mutants as evidence of absence of involvement of miRNA in any part of the ovulation process.

A strong phenotype was observed in the *pash-1* temperature sensitive mutants with the *pash-1* rescue array at 24°C (Fig 8H). The ovulation rate in these mutants was reduced to 2 ovulation events per hour compared to almost 3.5 per hour in wild-type worms (Fig. 8H). One explanation for this phenotype is the mosaicism of the rescue array in the somatic cells. This may leave some somatic cells not expressing the rescue, resulting in a reduced ovulation rate. Another interpretation is that the decrease in ovulation rate at 24°C is due to the reduced biogenesis of miRNAs due to the *pash-1(ts)* allele. miRNAs may indeed function as “fine tuners” in pathways involved in meiotic maturation in the germ cells. It is important to point out, however, that there was no reduction in ovulation rate observed in the *rde-1(ne219); alg-2(ok304); pie-1<sup>prom</sup>::rde-1(+)* mutants on *alg-1* RNAi (Fig. 10D).

It is important to point out that a significant reduction in ovulation rate was observed in the *rrf-1* mutants (RNAi ability in the germ cells) on *alg-1* RNAi (Fig. 10C) and in the *pash-1* temperature sensitive mutants with the rescue array at

24°C (Fig 8F). However, this same phenotype was not observed in the mutant we generated to knockdown *alg-1* and *alg-2* in the germ cells (*rde-1(ne219); alg-2(ok304); pie-1<sup>prom</sup>::rde-1(+)*) (Fig. 10D). We interpreted these conflicting results to reflect some somatic RNAi capability in the *rrf-1* mutant and lack of *pash-1* rescue in the cells of the somatic gonad, due to the mosaicism of the array. Another possibility still remains, that of miRNAs in the germ cells function in the process of meiotic maturation.

Lastly, there is the possibility that additional *C. elegans* AGOs function in the miRISC. Worms have 27 Argonaute genes, including *T23D8.7/hpo-24*, which is closely related to *alg-1* and *alg-2*; making it possible that these proteins could function to mediate miRNA regulation in the germline (Youngman & Claycomb, 2014). Therefore, by targeting the *alg-1* gene, other Argonautes were left to act in the miRISC, although this has never been documented. To test this unknown, analysis of ovulation in mutants exposed to RNAi targeting *alg-1* and *T23D8.7/hpo-24* may be helpful.

### **Ovulation events that require ALG-1 and ALG-2 in the somatic gonad involve calcium signaling**

The basal and the more intense ovulatory sheath contractions that propel the mature oocyte into the spermatheca require calcium release via IP<sub>3</sub> receptor channels (McCarter et al., 1999a; Yin et al., 2004). Upon meiotic resumption, the oocyte produces LIN-3/EGF that interacts with the LET-23/EGFR on the distal spermatheca cells, causing dilation, probably by initiating IP<sub>3</sub>-dependent calcium release (Bui & Sternberg, 2002; Clandinin et al., 1998; Yin et al., 2004). After fertilization, directional constriction of the spermatheca propels the embryo into

the uterus; this constriction also requires calcium release through IP<sub>3</sub> receptors (Kovacevic et al., 2013).

Decreased activity of the IP<sub>3</sub> signaling pathway results in ovulation defects similar to those observed when I reduced miRNA biogenesis in the somatic gonad. Worms with reduced IP<sub>3</sub>-dependent calcium release exhibit a decrease in both basal and ovulatory sheath contractions in a pattern similar to what I observed (Yin et al., 2004). Worms exposed to *plc-3* RNAi and *itr-1(sa73)* loss-of-function mutants both exhibit pinching of the proximal oocyte by the distal spermatheca during ovulation. I observed a similar pinching of the proximal oocyte in worms with reduced *alg-1* and *alg-2* in the somatic gonad. These observations support a role for miRNAs in the regulation of the IP<sub>3</sub> signaling pathway in somatic gonad cells.

Previously, our group identified a role of *mir-786* in the control of the IP<sub>3</sub> mediated rhythmic behavior of defecation (Kemp et al., 2012). Interestingly, *mir-786* shows high expression in the somatic gonad (Kemp et al., 2012) and its deletion causes synthetic sterility in a sensitized genetic background (Brenner et al., 2010; Nagaraja et al., 2008), suggesting that miR-786 is one of the miRNAs that control ovulation from the somatic gonad. However, loss of *mir-786* alone does not result in severe ovulation defects (unpublished data) indicating that additional miRNAs likely act in sheath cells and the spermatheca to control ovulation.

Future work to address the function of miRNAs in the control of calcium signaling in the somatic gonad would benefit from the development of novel imaging tools based on some of my constructs. Kovacevic et al imaged calcium

in the spermatheca during ovulation with GCaMP, a genetically encoded calcium indicator or biosensor (Kovacevic et al., 2013). Their strain, crossed with our mutant with the RNAi pathway rescued in the somatic gonad would allow for the visualization of calcium in a worm that has a knockdown of *alg-1* and *alg-2* in the somatic gonad. In addition, mutant worms can be generated that result in tissue-specific expression of calcium biosensors as a single copy transgene by utilizing the somatic gonad-specific promoters *mir-786* and *inx-8*.

If calcium signaling is identified as a miRNA-dependent pathway in the somatic gonad, the identification of specific miRNAs (in addition to the proposed *mir-786*) involved is essential. I hypothesize that multiple miRNAs function to regulate calcium-dependent contractions in the sheath cells and spermatheca during ovulation. I believe this because of the discrepancy between the lack of sheath cell contractions in miRNA biogenesis mutants and the weak phenotype observed in the *mir-786* loss-of-function mutants. Small RNA sequence can be used to identify miRNAs expressed in the sheath cells and spermatheca. This can be accomplished by comparing small RNA profiles of a *pash-1(mj100)* temperature sensitive mutant at a restrictive temperature with one that expresses a *pash-1* rescue transgene exclusively in the somatic gonad. A rescue transgene consisting of *mir-786<sup>prom</sup>::pash-1(+):gfp*, would rescue miRNA biogenesis in the entire somatic gonad. Once the miRNAs expressed in the sheath and spermathecal are identified, analysis of ovulation in loss-of-function mutants would identify miRNAs involved.

**ALG-1 and ALG-2 function in the somatic gonad to regulate the rate of meiotic maturation**

We use the rate of ovulation as readout for the rate of meiotic maturation. The control of meiotic maturation involves signaling between MSP and Gas receptors on the somatic sheath cells, which activate the adenylate cyclase (*acy-4*) –PKA pathway. The activation is required for resumption of meiosis in the proximal oocyte (Govindan et al. 2009; Kim et al., 2016) ALG-1 and ALG-2 together with a set of miRNAs expressed in the distal tip cell of the somatic gonad maintain germ cell proliferation, oocyte abundance and brood size (Bukhari et al., 2012; Drake et al., 2014). Therefore, the decrease in ovulation rate and brood size that is observed following knock down of ALG-1 and ALG-2 in the somatic gonad may result from reduced miRNA activity in the distal tip cell. It is possible that miRNAs function in the sheath cells to directly or indirectly control the activation of *acy-4* and the induction of meiotic maturation of the proximal oocyte. We did investigate the expression meiotic maturation marker LIN-41 upon *alg-1* knockdown and did not observe any misregulation (Fig. 10E), therefore I suspect that the decrease in ovulation rate is a result of decrease in germ cell proliferation.

**Genetic mosaic analysis may provide valuable insight to cell types that require miRNA activity for ovulation**

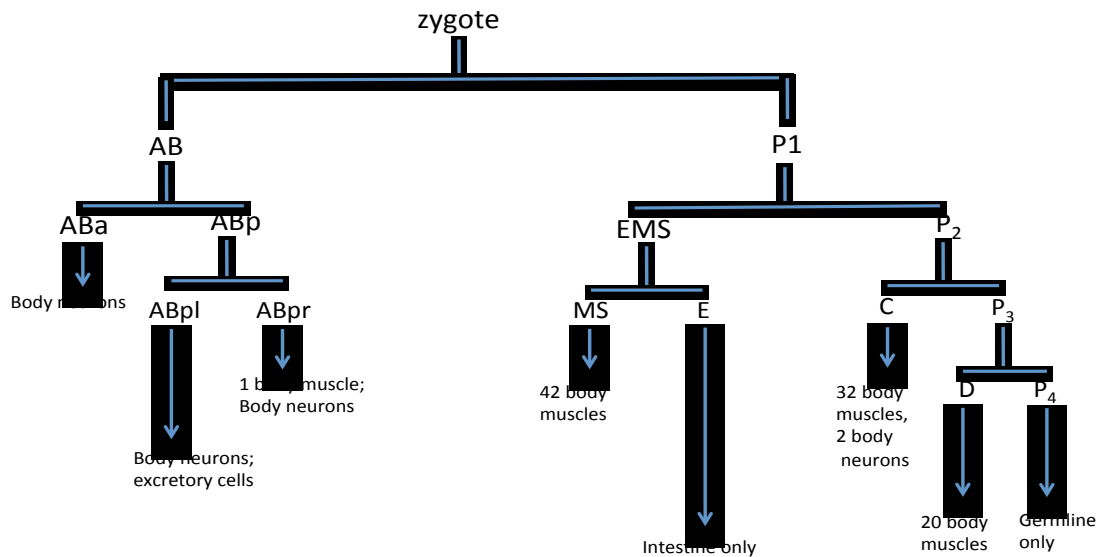
I conclude this chapter with a discussion of an experiment that I attempted, but did not succeed in executing. Worms with mutations in genes involved in the miRNA-specific biogenesis pathway, including *drsh-1* and *pash-1*, are sterile (Denli et al., 2004; Grishok et al., 2001). Accordingly, we observed essentially no successful ovulations in these mutants. In our work, we never

observed sterility due to defects in meiotic maturation or ovulation upon knockdown of miRNA biogenesis genes in either the germ cells or the somatic gonad. We did observe significant reductions in ovulation, in the rate of sheath cell contraction, and defects in oocyte entry into spermatheca upon knockdown of *alg-1* and *alg-2* in cells of the somatic gonad. Although I have discussed plausible explanations for why sterility did not occur, I believe that the analysis of *drsh-1* genetically mosaic worms would definitively answer the question.

The analysis of genetically mosaic worms, where some cells carry the *drsh-1* wild-type genes in an otherwise mutant *drsh-1* organism, can reveal in what cells this essential gene acts to prevent the sterility phenotype. I spent a significant amount of time attempting genetic mosaic analysis. I attempted to make a genetically mosaic worm by injecting a wild-type *drsh-1* gene into *drsh-1* null (*drsh-1(ok369)*). The presence of *drsh-1* can be followed by co-injecting *RFP* (red fluorescent protein) with a nuclear localization signal driven by a strong constitutively expressed promoter (*eft-2::rfp*).

If the *drsh-1* construct that I applied is expressed in all cells of the first generation homozygote *drsh-1*-null, the worms will exhibit a completely wild-type phenotype. This however should not occur in all progeny because the extrachromosomal arrays with which I applied the gene construct are partially unstable in mitosis. When the array fails to be transmitted to one of the daughter cells during the process of cell division, all the descendants of that cell will be homozygous mutant. Because worm lineage is essentially invariant (Fig. 12), the founder cell in which the array was lost can be determined. For example, if it is found that germ cells are RFP minus, as are a subset of body muscle cells, it will

follow that upon P2 cell division the array was not inherited in the P3 cell (Fig. 12).



**Figure 13. The embryonic progenitors of several cell types in *C. elegans* hermaphrodite.**

Ovulation is triggered by signaling between somatic gonad and germ cells, which makes these two cell types the most likely candidates for the requirement of miRNA expression in the process. Had this experiment been successful, I would have screened RFP positive worms to identify any that inherited the rescuing array and were sterile. These worms would have failed to inherit the rescuing array in the subset of cells that require miRNAs for the process of ovulation. Because this was not achieved, I can only speculate that any sterile

worms will have lost the array in the MS lineage that gives rise to the somatic gonad (Fig. 13).

A major obstacle in genetic mosaic analysis lies in expressing the rescue transgene, in this case *drsh-1*, in the germline (Kelly et al., 1997). To address this challenge, I used sonicated *E. coli* genomic DNA as the carrier DNA in the injection mix. In spite of my efforts, after several months of injections and modifications to the injection mix I was not able to obtain a strain of worms that had a *drsh-1* rescuing array expressed in the germline. This is still the ideal experiment to identify cells that require miRNA biogenesis to facilitate ovulation.



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