THE ARGONAUTE FAMILY OF GENES IN *CAENORHABDITIS ELEGANS*

A Dissertation Presented

By

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

CSR	chromosome segregation and RNAi defective
DCR-1	*C. elegans* Dicer protein
DNA	deoxyribonucleic acid
DRH	Dicer related helicase
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
endo-RNAi	endogenous RNAi
ERGO	endogenous RNAi deficient Argonaute
ERI	enhancer of RNAi
exo-RNAi	exogenous RNAi
MAGO	multiple-Argonaute mutant
miRNA	micro RNA
mRNA	messenger RNA
piRNA	Piwi-interacting RNA
pre-miRNA	miRNA precursor
pri-miRNA	primary miRNA
rasiRNA	repeat-associated small interfering RNA
RDE	RNAi defective
RdRP	RNA-dependent RNA polymerase
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA-mediated interference or RNA interference
RSD	RNA spreading defective
SAGO	synthetic secondary-siRNA defective Argonaute
siRNA	short (small) interfering RNA
ssDNA	single-stranded DNA
tncRNA	tiny-noncoding RNA

ABSTRACT

Members of the Argonaute family of proteins, which interact with small RNAs, are the key players of RNAi and other related pathways. The *C. elegans* genome encodes 27 members of the Argonaute family. During this thesis research, we sought to understand the functions of the members of this gene family in *C. elegans.* Among the Argonaute family members, *rde-1* and *alg-1/2* have previously been shown to be essential for RNAi and development, respectively. In this work, we wanted to assign functions to the remaining members of this large family of proteins.

Here, we describe the phenotype of 31 deletion alleles representing all of the previously uncharacterized Argonaute members. In addition to *rde-1,* our analysis revealed that two other Argonaute members *csr-1* and *prg-1* are also essential for development. *csr-1* is partially required for RNAi, and essential for proper chromosome segregation. *prg-1,* a member of PIWI subfamily of Argonaute genes, exhibits reduced brood size and temperature-sensitive sterile phenotype, implicating that it is required for germline maintenance.

Additionally, we showed that RDE-1 interacts with trigger-derived sense and antisense siRNAs (primary siRNAs) to initiate RNAi, while several other Argonaute proteins, SAGO-1, SAGO-2, and perhaps others, functioning redundantly, interact with amplified siRNAs (secondary siRNAs) to mediate downstream silencing. Moreover, our analysis uncovered that another member of Argonaute gene family, *ergo-1*, is essential for the endogenous RNAi pathway.

Furthermore, we built an eight-fold Argonaute mutant, MAGO8, and analyzed its developmental phenotype and sensitivity to RNAi. Our analysis revealed that the genes deleted in the MAGO8 mutant function redundantly with each other, and are required for RNAi and the maintenance of the stem cell totipotency.

CHAPTER I

GENERAL INTRODUCTION

Contributors to the work presented in Chapter I:

Niraj H. Tolia from Leemor Joshua-Tor's lab at the Cold Spring Harbor Laboratory made the phylogenetic tree in Figure I-2.

RNA-mediated interference (RNAi) is a sequence specific posttranscriptional gene silencing mechanism that is triggered by double stranded RNA (dsRNA). The phenomenon of RNAi was discovered in the nematode *C. elegans* by Andrew Fire and Craig Mello in 1998, who were honored with the Nobel Prize in Physiology or Medicine in 2006. In the years following the initial discovery, RNAi has become an indispensable research tool for scientists to address gene function by reducing the expression of a gene of interest. More recently, scientists have learned that RNAi has a biological role in regulating gene expression, and counteracting transposable elements and viruses in plants and animals. Since these findings, interest in RNAi has flourished with the hope of understanding how the molecular mechanism of RNAi works so that RNAi may be used in medicine and agriculture.

The Discovery of RNAi

RNA-mediated interference is a homology-dependent posttranscriptional gene silencing phenomenon. The history of homology-dependent gene silencing mechanisms goes back to the early 1990s. The first excample of homology-dependent gene silencing was observed in plants. While scientists were trying to increase the amount of purple pigment in petunia flowers by over-expressing *chalcone synthaseA (chsA),* they unexpectedly found that the purple flowers actually lost their pigment and became white flowers. This silencing phenomenon was named "cosuppression" (Napoli et al., 1990; van der Krol et al., 1990). The underlying

mechanism of cosuppression remained a mystery until the discovery of RNAi (Fire et al., 1998).

The history of RNAi in *C. elegans* started in 1995 with a study from the laboratory of Ken Kemphues. In this study, he and his student, Su Guo, used antisense RNA, complementary to mRNA, to inhibit the expression of the maternally expressed *par-1* gene. Surprisingly, both antisense and the control sense RNA preparations (identical in sequence to the mRNA) were equally effective in inhibiting *par-1* expression (Guo and Kemphues, 1995). At the time there was no explanation for the effectiveness of sense RNA in gene silencing. After this report, the laboratory of Craig Mello began to use RNA transcribed from cDNA as a reverse genetics tool due to its efficiency. The injection of RNA into *C. elegans* phenocopied the loss of function phenotypes of the targeted genes. The RNA silencing had different properties than canonical antisense RNA inhibition, and it was clear that it had to be a new phenomenon. Therefore, the RNA silencing phenomenon was named "RNA-mediated interference" or "RNAi" (Rocheleau et al., 1997).

Andrew Fire and Craig Mello collaborated to discover the mechanism of RNAi. They published their historical paper in 1998, demonstrating that dsRNA was the trigger molecule of RNAi. The silencing of genes by the injection of single-stranded RNA (ssRNA) was explained by the contamination of in vitro transcripts by dsRNA molecules, due to the production of ectopic transcripts by RNA polymerases during the in vitro transcription reactions (Fire et al., 1998). Furthermore, the original study described three key properties of RNAi: 1) RNAi was induced by dsRNA

homologous to only exons 2) mRNA levels of the targeted gene was reduced, similar to the observation made during cosuppression in plants (Napoli et al., 1990; van der Krol et al., 1990) 3) when dsRNA was injected in an animal, RNAi effect was amplified and observed in its progeny.

The potency and the ease of use of RNAi in humans, animals, and plants made it a popular tool among scientists. Over the past several years, enormous amounts of data have suggested that RNAi is a naturally occurring gene silencing mechanism involved in various cellular processes. In their original paper, Fire and Mello speculated about the biological role, mechanism and use of RNA-mediated gene silencing as follows:

> "*...Genetic tools are available for only a few organisms. Double stranded RNA could conceivably mediate interference more generally in other nematodes, in other invertebrates, and, potentially, in vertebrates. RNA interference might also operate in plants: several studies have suggested that inverted-repeat structures or characteristics of dsRNA viruses are involved in transgene dependent cosuppression in plants. There are several possible mechanisms for RNA interference in C. elegans. A simple antisense model is not likely: annealing between a few injected RNA molecules and excess endogenous transcripts would not be expected to yield observable phenotypes. RNA targeted processes cannot, however, be ruled out, as they could include a catalytic component. Alternatively, direct RNA-mediated interference at the level of chromatin structure or transcription could be involved. Interactions*

between RNA and the genome, combined with propagation of changes along chromatin, have been proposed in mammalian X-chromosome inactivation and plant gene cosuppression. If RNA interference in C. elegans works by such a mechanism, it would be new in targeting regions of the template that are present in the final mRNA (as we observed no phenotypic interference using intron or promoter sequences). Whatever their target, the mechanisms underlying RNA interference probably exist for a biological purpose. Genetic interference by dsRNA could be used by the organism for physiological gene silencing. Likewise, the ability of dsRNA to work at a distance from the site of injection, and particularly to move into both germline and muscle cells, suggests that there is an effective RNA-transport mechanism in C. elegans."

An Overview of the RNAi Pathway

RNAi and related pathways have been found to occur in organisms from *S. pombe* to mammals. As the rate of RNAi research grows, various functions of RNAi-related pathways are being discovered, including antiviral defense, silencing of transposons, miRNA regulation of development, transcriptional silencing of heterochromatin, and even the elimination of genomic DNA. Our current knowledge of mechanism of RNAi has predominantly come from studies in *C. elegans*, *D. melanogaster*, plants, and cultured human cells. Genetic studies combined with biochemical analysis elucidated the major protein and RNA components of the RNAi pathway. Although

RNAi and related mechanisms differ from one organism to another, the major components of the RNAi pathway are evolutionary conserved.

When exogenous dsRNA is introduced into a cell, it is recognized by a dsRNA-specific ribonuclease called Dicer, which cleaves the trigger dsRNA into ~21-23 nt long short-interfering RNA molecules (siRNAs). Inside the cell, Dicer proteins function as subunits of larger protein complexes. After siRNAs are produced, they are loaded onto a multisubunit protein complex called the RNA-Induced Silencing Complex (RISC), which contains an Argonaute protein as the catalytic subunit. RISC removes the sense strand (passenger strand) from the siRNA duplex and retains the antisense strand (guide strand). In the effector step of the pathway, Argonaute cleaves the target mRNA in a guide-strand dependent manner (Figure I-1). The catalytic Argonaute proteins are also known as "Slicer". In the following sections, I describe the key components and steps of the RNAi pathway in greater detail.

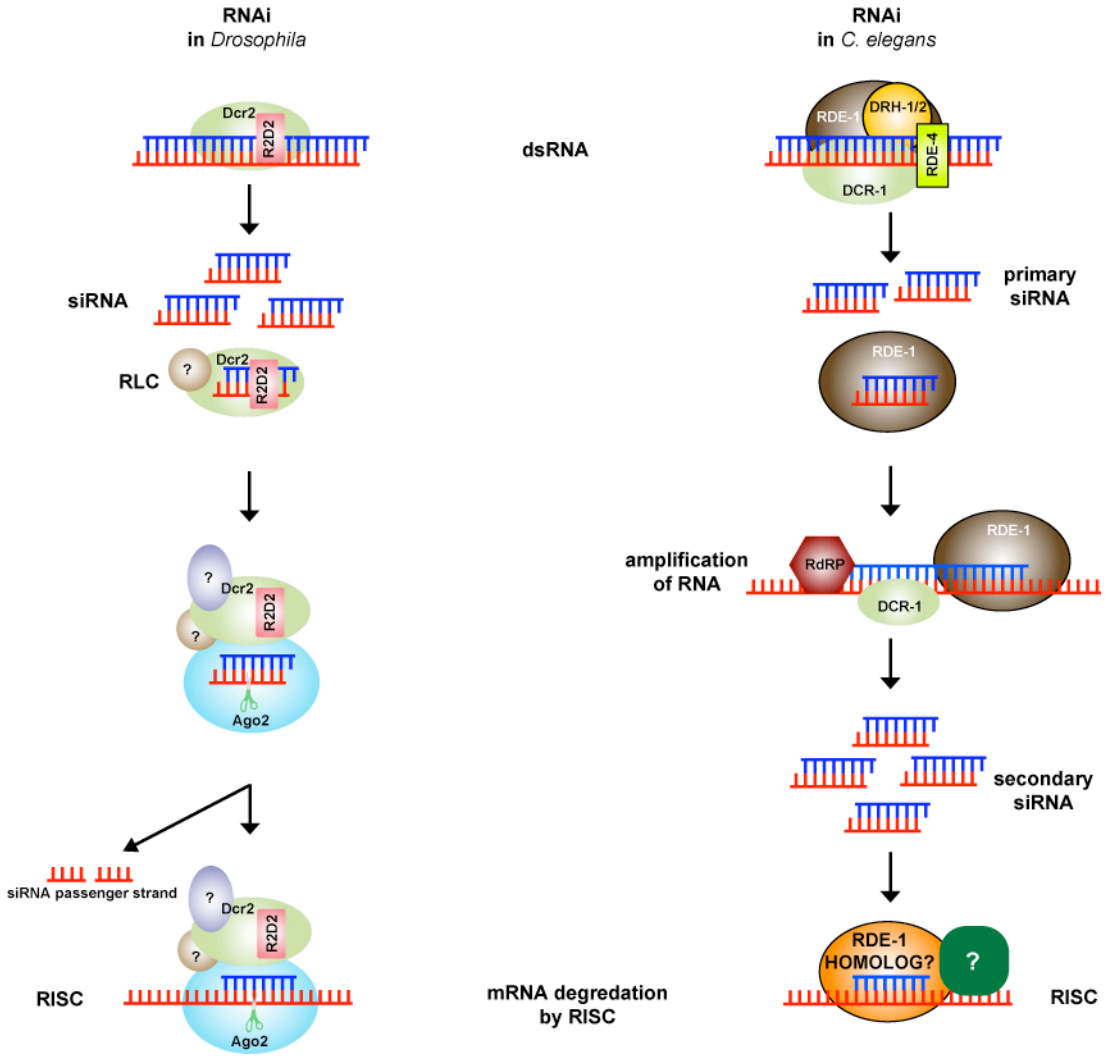


Figure I-1. Comparison of RNAi pathways in *D. melanogaster* and *C. elegans*. In *Drosophila,* siRNAs are bound to RISC-loading complex (RLC), which loads siRNAs onto pre-RISC complex. Ago2 removes passenger strand from pre-RISC to form RISC, which cleaves the target mRNA. In *C. elegans*, primary siRNAs are generated by RNAi initiation complex, which contains RDE-1, DCR-1, RDE-4 and DRH-1/2. RdRP is thought to replicate target mRNA to amplify RNAi signal. More siRNAs are generated from amplified target. Amplified siRNAs are called secondary siRNAs. An Argonaute/secondary siRNA complex is proposed to mediate target mRNA degredation. Unlike *C. elegans*, human and *Droshophila* genome do not encode any RdRP; therefore, RNAi signal is not amplified in these organisms. RdRP, RNA-dependent RNA polymerase. RISC, RNA-induced silencing complex. RLC, RISC-loading complex. The *Drosophila* RNAi pathway is adapted from from Kim et al. **RNA,** 2006.

Key Components and Steps in the RNAi Pathway

Dicer Proteins and Production of Small RNAs

After dsRNA was discovered as a trigger of RNAi (Fire et al., 1998; Kennerdell and Carthew, 1998; Ngo et al., 1998; Waterhouse et al., 1998), several research groups collectively showed that trigger dsRNA was processed into small RNA molecules during RNAi. Hamilton and Baulcombe (1999) reported for the first time that a small RNA species of ~25-nt long accumulated during posttranscriptional gene silencing in plants. Subsequently, trigger dsRNA was shown to be diced into small RNA species (~21-23 nt) during RNAi in *D. melanogaster* extracts (Hammond et al., 2000; Zamore et al., 2000). Furthermore, the latter work showed that both strands of dsRNA were cleaved, suggesting that trigger dsRNA was cleaved by a dsRNA-specific enzyme. Interestingly, the cleavage of target mRNA followed a similar pattern (every 21-23 nt) in the regions that were homologous to the trigger dsRNA, suggesting that each siRNA mediated the sequence specific cleavage of the target mRNA.

Once trigger dsRNA was found to be the precursor of siRNA molecules (Hammond et al., 2000; Zamore et al., 2000), researchers turned their focus toward finding the ribonuclease responsible for making siRNAs from the trigger dsRNA. The identification of this ribonuclease was expedited by the observation that the RNAse III class of nucleases were the only enzymes known to use dsRNA as a substrate and cleave it into a particular size of dsRNA fragments (Rotondo et al., 1997; Abou Elela and Ares, 1998). The potential role of an RNAse III class of

enzyme in RNAi was proposed by Brenda Bass (2000) based on the ATP requirement of mRNA cleavage during RNAi (Zamore et al., 2000). Soon after, several research groups showed that siRNAs were produced from dsRNA trigger by an RNAse III class of enzyme called Dicer (Bernstein et al., 2001; Elbashir et al., 2001a; Elbashir et al., 2001b; Grishok et al., 2001; Ketting et al., 2001; Knight and Bass, 2001; Xie et al., 2004). In addition to two RNAse III domains, Dicer contains a dsRNA-binding domain (dsRBD), a DEXH-box RNA helicase domain, a PAZ domain (Carmell and Hannon, 2004), and a domain of unknown function (DUF283), which has been recently proposed to be homologous to the dsRNA-binding domains (Dlakic, 2006; Macrae et al., 2006).

Dicer makes staggered siRNA duplexes with 3′ 2-nt protruding ends, bearing 5′ phosphate and 3′ hydroxy termini (Elbashir et al., 2001a). Two RNAse III domains of Dicer are called RNAse IIIa and RNAse IIIb, which form an internal heterodimer (Zhang et al., 2004b; Macrae et al., 2006). The former domain is larger and closer to the N-terminus. Dicer appears to contain a single processing center with two catalytic sites, each located on different RNase III domains (Macrae et al., 2006). The catalytic sites are made of highly conserved acidic residues which coordinate a pair of divalent cations (Macrae et al., 2006). The RNAse IIIa domain produces siRNA 5′ end, whereas the RNAse IIIb domain produces siRNA 3′ end (Zhang et al., 2004a). Dicer prefers to produce siRNAs from free ends of the dsRNA substrate (Zhang et al., 2002; Lee et al., 2003). The crystal structure of the *Giardia intestinalis* Dicer showed that the PAZ domain of Dicer has similar structure to the PAZ domain of human

Argonaute1 (hAGO1) (Macrae et al., 2006), which binds to 3′ 2 nt overhang of siRNA molecules (Lingel et al., 2003; Song et al., 2003; Yan et al., 2003; Ma et al., 2004). Modeling of dsRNA with *Giardia* Dicer indicated that the 3′ overhang of the dsRNA sits in an RNA binding pocket in the PAZ domain (Macrae et al., 2006). It was proposed that Dicer measures dsRNA like a ruler starting from the PAZ domain (Macrae et al., 2006). The length of dsRNA cut by Dicer is determined by the positions of the PAZ and RNAse III domains on dsRNA (Zhang et al., 2004b; Macrae et al., 2006).

The number of Dicer paralogs varies by organism. While the *C. elegans* and human genomes encode only one Dicer, the *D. melanogaster* genome encodes two Dicers, and the *A. thaliana* genome encodes four Dicers (called DCL1-4). Each Dicer seems to allow for a diversification of small RNA-mediated pathways. For example, In *D. melanogaster,* Dicer-1 makes miRNAs, and Dicer-2 makes siRNAs. In plants, DCL function is more diversified, and each DCL appears to have a major function in a distinct small RNA-mediated pathway, and they can produce small RNAs of different sizes. For instance, DCL1 makes miRNAs (Park et al., 2002; Kurihara and Watanabe, 2004), DCL3 makes 24-nt repeat associated siRNAs (rasiRNA)(Xie et al., 2004; Xie et al., 2005), and DCL4 makes 21-nt long trans-acting siRNAs (ta-siRNAs) (Gasciolli et al., 2005; Xie et al., 2005). Although distinct DCL proteins appear to be dedicated to distinct small RNA pathways in *A. thaliana*, some DCL proteins exhibit partial redundancy; for example, in the absence of DCL4, ta-siRNAs are produced by

DCL2 and DCL3 (Gasciolli et al., 2005; Henderson et al., 2006). Moreover, these small RNA-mediated pathways seem to intersect (reviewed in Vazquez, 2006).

DsRNA Binding Proteins and Loading of the RISC with siRNAs

Although recombinant Dicer protein can cleave dsRNA into siRNA molecules in vitro (Provost et al., 2002; Zhang et al., 2002; Zhang et al., 2004a), Dicer proteins are found as components of larger protein complexes in vivo. Dicer proteins appear to partner with at least one dsRNA binding protein in the RNAi pathway. For example, *C. elegans* Dicer homolog DCR-1 was found to be associated with the dsRNA binding protein RDE-4, one of the first RNAi genes identified from genetic screens (Tabara et al., 2002). RDE-4 contains two dsRNA binding domains, and forms a stable homodimer in solution with the C-terminal domain of the protein being required for dimerization. The homo-dimerization of RDE-4 does not appear to be required for RNA binding and is proposed to be involved in facilitating the interaction with Dicer (Parker et al., 2006). RDE-4 preferentially binds to long dsRNA rather than siRNAs in a sequence independent manner (Tabara et al., 2002; Parker et al., 2006).

Although RDE-4 interacts with DCR-1 and binds to long dsRNAs, the *D. melanogaster* homolog, R2D2, forms a complex with DCR-2 that binds to siRNA duplexes (Liu et al., 2003; Tomari et al., 2004). In *D. melanogaster* siRNA strand with lower thermodynamic stability at its 5′ region is often loaded on the RISC, whereas the other strand is degraded. Then, the question is how this asymmetry is

achieved? Studies found that R2D2 protein binds to the more stable end of siRNA duplex, allowing separation of strands from the less stable end, and passing the strand with less stable 5′ terminus onto Argonaute protein (Tomari et al., 2004; Liu et al., 2006). Recent studies showed that once siRNA duplex associates with the RISC, the passenger strand (sense strand) is cleaved by Ago2 protein, and dissociated from the complex (Matranga et al., 2005; Rand et al., 2005; Leuschner et al., 2006). However, it is known that the cleavage of a passenger strand requires a perfect duplex, especially at the center. Then, how can miRNA/RISC form when there are mismatches and bulges in miRNA/miRNA* duplexes? How can passenger strands be removed from RISC complex that contains catalytically inactive Argonaute proteins? Is there an alternative mechanism to remove the passenger strand? To test this idea Matranga et al. used a chemically modified passenger strand that cannot be cleaved by Ago2. They found that chemically modified passenger strand was still dissociated from RISC, but took longer. This finding suggests that the dissociation of the passenger strand from RISC does not necessarily require the cleavage of the passenger strand (Matranga et al., 2005). Then, the question is what removes the modified passenger strand from the complex? One explanation for this can be the involvement of an Argonaute protein other than Ago2; and this Argonaute protein can be catalytically inactive.

On the other hand, *D. melanogaster* R2D2 paralog, Loquacious (Loqs) makes a complex with Dcr-1. While Dcr-1 can cleave dsRNA alone, like Dcr-2, it cannot cleave miRNA precursor without help of Loqs (Saito et al., 2005). Thus the activity

of Loqs is necessary for miRNA maturation (Forstemann et al., 2005; Jiang et al., 2005; Saito et al., 2005), R2D2 activity is not required for the Dcr-2 processing of dsRNA into siRNAs (Liu et al., 2003).

In addition, dsRNA binding proteins have been identified as Dicer partners in humans (Han et al., 2004; Chendrimada et al., 2005; Haase et al., 2005; Maniataki and Mourelatos, 2005; Lee et al., 2006b), plants (Hiraguri et al., 2005; Nakazawa et al., 2007), and worms (Duchaine et al., 2006). A recent work showed that DCR-1 interacts with nearly 80 different proteins in *C. elegans* (Duchaine et al., 2006). In *D. melanogaster* and *A. thaliana,* Dicer proteins seem to be diversified and contribute to the specialization of small RNA-mediated pathways. However, humans and *C. elegans* genome encodes only single Dicer. Therefore, it is conceivable that in these organisms different Dicer partners contribute to the specialization of small RNA-mediated pathways. Identification and characterization of more Dicer partners will shed light on the molecular mechanisms of small RNA-medicated pathways.

Effector Step of RNAi and Argonaute Family of Proteins

The *D. melanogaster piwi* gene is the founding member of the Argonaute family of genes. Cox et al. (1998) found that the *piwi* gene encodes a highly basic protein, with orthologs in *C. elegans*, *A. thaliana* and humans. The *piwi* orthologs in *C. elegans prg-1* and *prg-2,* were shown to be essential for stem cell self-renewal (Cox et al., 1998), and the *A. thaliana* orthologs, *ZWILLE (ZLL), argonaute (ago)* and *argonaute-like* were shown to be required for cell division in the meristem (Moussian

et al., 1998). In addition, *D. melanogaster* ortholog *aubergine (aub)* was shown to be required for posterior body patterning and pole cell formation (Harris and Macdonald, 2001). The gene family was named "Argonaute" because the leaves of *A. thaliana argonaute1* mutant alleles looked like a small squid (Bohmert et al., 1998).

The initial sequence alignment of the *piwi* homologs indicated a conserved domain of 43 residues called the "piwi box" (Cox et al., 1998). Detailed analysis of these proteins indicated that the piwi box was actually part of a larger domain (~300 amino acids) which is now called the "Piwi domain". This domain is also found in Prokaryotes and *Schizosaccharomyces pombe* (Cerutti et al., 2000). Further analysis of the central region of *piwi* proteins identified a second conserved domain called "PAZ" for Piwi, Argonaute, and Zwille/Pinhead (~110 amino acids). In addition to the Argonaute family, the PAZ domain is also contained in Dicer homologs.

The PAZ Domain

After the Argonaute proteins were shown to catalyze mRNA cleavage in the RISC complex (Liu et al., 2004a; Meister et al., 2004), biochemical and structural studies on these proteins gained momentum. Nucleic acid binding experiments and structural analysis of the PAZ domain of *D. melanogaster* AGO1 and AGO2 by nuclear magnetic resonance spectroscopy (NMR) revealed that PAZ was a nucleic acid binding domain (Lingel et al., 2003; Yan et al., 2003; Lingel et al., 2004). PAZ is able to bind to both single and double-stranded RNA of different sizes, albeit with a higher affinity for dsRNA. The affinity of the PAZ domain for the two-nucleotide

3′overhang in an siRNA duplex was shown to be higher than that for blunt ended siRNA duplexes (Lingel et al., 2003; Yan et al., 2003). The PAZ domain not only binds to RNA, but also binds to DNA (Lingel et al., 2003; Yan et al., 2003). The binding of the PAZ domain to DNA may have biological significance in vivo. This is discussed under the "Piwi domain" section below.

To gain a deeper understanding of the interaction between the PAZ and RNA, Ma and collaborators co-crystallized the PAZ domain of human eIF2C with a 9-nucleotide RNA molecule. This single-stranded small RNA molecule unexpectedly formed an siRNA-like A-form duplex with two-nucleotide 3′ overhangs between two PAZ domains that make contact with each RNA strand along the length of the duplex. Based on this structure, it was suggested that the PAZ domain favors and stabilizes the duplex structure. The two-nucleotide 3′ overhangs were buried inside the RNA binding pockets in the PAZ domain (Ma et al., 2004). Interestingly, small chemical modifications at the 3′ overhang ends, such as a 2′-*O*-methyl, did not affect the binding affinity of the PAZ, suggesting that the 3′ end can support small modifications (Ma et al., 2004). Similarly, the physiological occurrence of siRNA modifications at the 3′ ends has been observed in plants and animals (Chan et al., 2004; Ruby et al., 2006; Vagin et al., 2006).

The Piwi Domain

The Piwi domain confers the catalytic activity of Argonaute proteins. Determination of the crystal structure of the Piwi domain demonstrated that this domain forms an RNAse H fold, which cleaves the RNA strand in a DNA/RNA hybrid (Liu et al., 2004a; Parker et al., 2004; Song et al., 2004; Parker et al., 2005). During RNAi, the Piwi domain cleaves the target mRNA in an siRNA/mRNA hybrid, between bases that are complementary to the 10th and 11th position of the siRNAs (Song et al., 2003; Ma et al., 2005; Parker et al., 2005). The cleavage activity requires Mg^{+2} ions and yields a product with 5′-phosphate and 3′-OH ends (Schwarz et al., 2004). Moreover, while the PAZ domain recognizes 3′ ends of siRNAs (Lingel et al., 2004; Ma et al., 2004), the Piwi domain recognizes 5′ ends (Ma et al., 2005; Parker et al., 2005). According to the studies done in the *D. melanogaster* (Nykanen et al., 2001) and mammalians cell lysates (Schwarz et al., 2002), 5′ phosphorylation of siRNAs was necessary for mRNA cleavage. However, in vitro studies in *Arabidopsis thaliana* and mammalians showed that purified RISC complexes do not necessarily require 5′ phosphate for target mRNA cleavage, but it is important for complex stability and cleavage fidelity (Qi et al., 2005; Rivas et al., 2005).

Structural analysis of archaeabacterium *Pyrococcus furiosus* Argonaute (PfAgo) (Song et al., 2004; Rivas et al., 2005) and biochemical analysis of mammalian Argonaute proteins (Liu et al., 2004a; Rivas et al., 2005) showed that this protein was the catalytic subunit of RISC. Three metal-coordinating residues (DDH) in the Piwi domain have been shown to be essential for the catalytic activity (Liu et al., 2004a;

Song et al., 2004; Rivas et al., 2005). However, not all the Argonaute proteins contain DDH motifs. The Argonaute proteins containing naturally or experimentally mutated catalytic sites were found to be inactive. For instance, among the four human Argonaute proteins, only human Ago2 is catalytically active (Liu et al., 2004a; Meister et al., 2004). Human Ago1 and Ago4, which are missing some of the putative metal-coordinating residues, are catalytically inactive proteins. Although human Ago3 has all of the metal-coordinating residues, it is found to be catalytically inactive for unknown reasons. The physiological functions of the Argonaute proteins that naturally lack of metal-coordinating residues are not yet understood.

Although the function of Argonaute proteins in eukaryotes is understood, their function in prokaryotes is not yet known. Studies in archaeabacterium *Archaeoglobus fulgidus* and eubacterium *Aquifex aeolicus* showed that these Argonaute proteins bind to DNA more tightly than to RNA molecules (Ma et al., 2005; Yuan et al., 2005). Moreover, prokaryotic Argonaute can cleave the target mRNA in a DNA-dependent manner (Yuan et al., 2005). Biological significance of these observations is not known yet. One possibility is that prokaryotes may be using either small RNA or DNA molecules to regulate gene expression. If this true, it will be very interesting to know if they can cut the nucleic acids. Argonaute proteins can also bind to DNA molecules in eukaryotes (Lingel et al., 2003; Yan et al., 2003). Biological importance of this is not clear either. However, RNAi is involved in modification of chromatin and transcriptional silencing in eukaryotes. It is possible that these proteins may interact with DNA in vivo to perform their function.

The RNAi Pathway in *C. elegans*

Initial Identification and Characterization of RNAi Deficient *(rde)* Mutants

After the discovery of RNAi, scientists turned their focus towards understanding the mechanism of RNAi. Large scale genetic screens became feasible in *C. elegans* after the invention of "soaking RNAi" in concentrated dsRNA solutions (Tabara et al., 1998) and "feeding RNAi" (see below) by the ingestion of bacteria expressing dsRNAs (Timmons and Fire, 1998).

In order to uncover the genetic mechanism of RNAi and to study its biological role, Tabara et al. (1999b) designed the first dedicated genetic screen in *C. elegans*. In this study, animals mutagenized with ethyl methyl sulfonate (EMS) were exposed to bacteria expressing *pos-1* dsRNA, and the F2 progeny resistant to *pos-1* RNAi were identified. The *pos-1* gene encodes a cytoplasmic zinc-finger protein, and plays an essential role in the development of germline blastomers (Tabara et al., 1999a). Therefore, worms exposed to *pos-1* RNAi laid dead eggs. However, mutants that were deficient in RNAi gave viable embryos. Among the RNAi deficient mutants *(rde)* recovered, *rde-1, rde-2*, *rde-3* and *rde-4* were further characterized. While *rde-1, rde-3,* and *rde-4* were found to be resistant to both germline and somatic RNAi, *rde-2* was found to be resistant to only germline RNAi. In parallel, existing mutants deficient in the silencing of transposons *(mut)* were also tested for RNAi resistance because some *rde* alleles mapped close to known *mut* alleles. Among these mutants, *mut-2* was found to be resistant to both germline and somatic RNAi, while *mut-7* was found to be resistant to only germline RNAi

Besides the *rde* phenotype, the developmental phenotype of these mutants was analyzed. Among those mutants, *rde-1* and *rde-4* were quite healthy and had no other phenotype beyond the RNAi deficiency. On the other hand, *rde-2* and *rde-3* were essential for transposon silencing and showed mutator phenotypes, which were also found in *mut-2* (Collins et al., 1987) and *mut-7* (Ketting et al., 1999) strains. The observation of a strong mutator phenotype in some of the *rde* mutants suggested that RNAi plays a role in the silencing of transposons. Furthermore, the mutator strains *rde-2*, *rde-3*, *mut-2* and *mut-7* also showed a high incidence of male *(him)* phenotype caused by the increased X chromosome non-disjuction during meiosis, suggesting that RNAi somehow affects proper chromosome segregation. Later, *rde-3* and *mut-2* were found to be alleles of the same gene (Chen et al., 2005). The *rde* mutants were also tested for their potential role in transgene silencing. Among those, *mut-7* and *rde-2* reactivated otherwise silenced transgenes in the germline, whereas *rde-1* did not. In conclusion, some of the RNAi deficient mutants exhibit tissue specificity and additional phenotypes. The data suggest that RNAi, transposon silencing, and transgene silencing pathways are distinct, yet somehow mechanistically related.

Inheritance of RNAi

A remarkable feature of RNAi in *C. elegans* is that the interference effect can be transmitted to subsequent generations (Fire et al., 1998; Montgomery et al., 1998; Tabara et al., 1999b). The genetic requirement for RNAi genes in inheritance was examined by the Mello lab (Grishok et al., 2000). In this study, the functions of the

rde genes *rde-1*, *rde-2*, *rde-4,* and *mut-7* were tested. Genetic analysis of these mutants showed that *rde-1* and *rde-4* were required for the initiation of RNAi, but not for inheritance of RNAi to subsequent generations. On the other hand, *rde-2* and *mut-7*, functioning downstream in the RNAi pathway, were required for the transmission of the interfering agent, but not for the initiation of the RNAi signal. Additionally, this study showed that *rde-1* function was depended on the activity of *rde-4,* implicating that these two genes function collaboratively at the initiation step in the RNAi pathway. However, the activity of *rde-1* required neither the activity of *rde-2* nor the activity of *mut-7*. Because of the additional phenotypes of *rde-2* and *mut-7* mutant animals, Grishok et al. hypothesized that *rde-2* and *mut-7* might be representing a downstream step where several RNAi-related pathways converge. In addition to *rde-1,* there are 26 more Argonaute family of genes in *C. elegans*. Therefore, additional Argonaute genes might be involved in proper chromosome segregation, transposon and transgene silencing pathways.

RNAi is Target-Dependent in *C. elegans*

Homology-dependent gene silencing mechanisms in plants and *D. melanogaster* associate with accumulation of short interfering RNA molecules (siRNA), detectable by Northern blot analysis (Hamilton and Baulcombe, 1999; Zamore et al., 2000). When silencing is triggered by transgene expression or dsRNA exposure, 21-23 nt long small RNAs that are homologous to the trigger region accumulate in both sense and antisense polarities. The accumulation of siRNAs in both orientations indicates

that they cannot simply be mRNA degradation products. If these small RNA molecules do not originate from mRNA, then what is the source of small RNA molecules?

Genetic screens in *N. crassa*, *A. thaliana,* and *C. elegans* identified several genes encoding cellular RNA-dependent RNA Polymerases or RdRP, distinct from viral RNA-dependent RNA polymerases, in the RNAi silencing pathway. RdRP enzymes seem to play important roles in RNAi-related silencing pathways by generating dsRNA. It has been reported that RdRPs can replicate dsRNA by both primer dependent and independent manner (Schiebel et al., 1993b, 1993a; Schiebel et al., 1998). However, RdRP activity is not essential for RNAi in every organism, for instance *D. melanogaster* and humans do not have known cellular RdRPs (Stein et al., 2003).

The siRNAs that accumulate during RNAi in *C. elegans* have different characteristics from siRNAs in some other organisms. While *A. thaliana*, *D. melanogaster,* and humans accumulate both sense and antisense strands of siRNAs, *C. elegans* accumulate only antisense strands (Grishok, 2001; Pak and Fire, 2007; Sijen et al., 2007). Interestingly, accumulation of the antisense strand is target dependent (Grishok, 2001). For instance, when transgenic worms carrying a transgene expressing green fluorescent protein (GFP) from jellyfish as a target were fed bacteria expressing *gfp*-dsRNA, only antisense siRNAs corresponding to the GFP gene accumulated. However, when wild type animals (with no GFP target) were fed bacteria expressing *gfp*-dsRNA, no siRNA accumulation was detected. This

observation suggests either a selective replication of the sense strand or a selective retention of the antisense strand by worms. Subsequent studies indicated that the RNAi signal was amplified in *C. elegans*. Antisense siRNAs that accumulate during somatic RNAi was found be dependent on the activity of *rrf-1* (RdRP). As expected no siRNA accumulation was detected in an *rrf-1* mutant (D. Conte and C. Mello unpublished data).

Once RNAi was triggered by dsRNA, silencing was not only limited to the target region, but also spread to the 5′ adjacent region of the target (secondary region) by the activity of RdRPs. However, no silencing was detectable in the 3′ adjacent region of the target during RNAi (Sijen et al., 2001; Alder et al., 2003). Only was trace amount of siRNAs corresponding to the 3′ adjacent region of the target detected in large-scale siRNA sequencing experiments (Pak and Fire, 2007). While siRNAs originated from secondary region are called "secondary siRNAs", siRNAs directly derived from trigger dsRNAs are called "primary siRNAs." Until this thesis study, it was unknown what kind of protein complexes interacted with primary and secondary siRNAs (see Chapter II for results and Yigit et al., 2006). Interestingly, the RNAi signal spreads bidirectionally in plants in a SDE1/SGS2 (RdRP) dependent manner (Klahre et al., 2002; Vaistij et al., 2002; Petersen and Albrechtsen, 2005). Spreading of RNAi signal into the 3′ region of the target suggests that RdRP might be replicating mRNA starting from the 3′ end of mRNA or it might be replicating an existing complementary RNA (cRNA) strand (Yamada et al., 2003). Taken together these observations suggest that an amplification step is essential for RNAi in *C. elegans.*

Spreading of RNAi silencing signals outside of the target region has been termed "transitive RNAi" (Sijen et al., 2001).

Systemic RNAi

RNA-mediated interference spreads from tissue to tissue in *C. elegans*. For example, when dsRNA is injected into the gut or head, it can silence the target genes in the germline. Conversely, when a dsRNA is injected into the germline it can silence a somatic gene (Fire et al., 1998). Moreover, in addition to RNAi induction by feeding the worms bacteria expressing dsRNA (Timmons and Fire, 1998), the same result can be achieved simply by soaking the worms in a concentrated dsRNA solution (Tabara et al., 1998).

First, these observations beg several questions. What is the mechanism of dsRNA uptake and how does dsRNA enter into the body of the animals, cross the cell boundaries, and travel from one tissue to another? Second, what is the nature of the spreading molecule: is it a long dsRNA or an siRNA? Third, what are the genes required for spreading of the RNAi signal?

Unfortunately, we still do not have answers to most of these questions. However, genetic screens have provided some clues. To find the genes essential for systemic RNAi, but not for the cell autonomous RNAi, Winston et al. (2002) designed a genetic screen using a transgenic strain expressing GFP in the pharyngeal and body-wall muscle cells. In this screen, a myo-2::GFP-dsRNA triggered a cell autonomous silencing of myo-2::GFP in pharyngeal muscle cells and a systemic silencing of myo-

3::GFP in body-wall muscle cells. After the animals were mutagenized, mutants that failed to silence the GFP in body-wall muscle cells, but silenced the GFP in pharyngeal muscle cells were isolated. These mutants were called systemic RNA interference deficient *(sid)* mutants. Among the three major complementation groups identified, *sid-1* was found to encode a conserved multispan transmembrane protein, which is now known to be essential for systemic RNAi and not for cell autonomous RNAi (Winston et al., 2002). To test whether *sid-1* transports dsRNA into cells, SID-1 was ectopically expressed in *D. melanogaster* S2 cells which have no *sid-1* orthologs. It is important to note that *D. melanogaster* S2 cells can also take up the exogenous dsRNA by receptor-mediated endocytosis (Saleh et al., 2006; Ulvila et al., 2006). The expression of SID-1 enhanced the efficiency of RNAi in these cells by increasing the import of the dsRNA molecules. Interestingly, SID-1 imported longer dsRNA more efficiently than shorter siRNAs across the membrane (500 bp long-dsRNA versus 100 bp or 50 bp long-dsRNA), and this transport did not require hydrolysis of ATP molecules, suggesting that dsRNA diffuses through the SID-1 channel by a passive diffusion mechanism. The silencing agent therefore seems to be long dsRNA molecules rather than short Dicer products. To date, the functions of the remaining *sid* genes isolated in the Winston screen remain unclear.

Subsequently, Tijsterman et al. (2004) identified two major classes of systemic RNAi deficient mutants that were resistant to RNAi by feeding, but not by injection. Although they called these mutants RNA spreading defective (*rsd)*, phenotypes were not analyzed in detail. From studies in the Mello lab, we know that the RNAi

resistance of some of the *rde* mutants is dependent on the injected dsRNA concentrations. These kinds of mutants appear sensitive to RNAi when a high dose of dsRNA is injected, but are resistant when low dose of dsRNA is injected. Therefore, the so-called *rsd* mutants may not be defective in spreading but may simply be sensitive to the injected doses of dsRNA. For the reason, these mutants require more careful mechanistic analysis. Nonetheless, two different classes of *rsd* mutants were isolated. Class I mutants (*rsd-4, rsd-8/sid-1*) were resistant to both germline and somatic RNAi, whereas class II mutants *(rsd-2, rsd-3, rsd-6)* were resistant to only germline RNAi. The *rsd* genes, with the exception of *rsd-3*, do not have close homologs in mammals, and their molecular functions are not yet understood.

Beyond *C. elegans*, systemic RNAi has also been shown in plants, however *D. melanogaster* does not appear to have systemic RNAi (Roignant et al., 2003). Plants seem to have two types of spreading: a long range, tissue to tissue, and short range, cell to cell spreading. Long range spreading occurs through the phloem transport system while cell to cell spreading is mediated by plasmodesmata, channels that connect the cytoplasm of cells together (Voinnet and Baulcombe, 1997; Himber et al., 2003). In animals, spreading seems to be species specific. For example, *C. briggsae*, *C. remanei*, and *C. sp. PB2801* are resistant to RNAi by feeding and soaking, but sensitive to RNAi by injection (Descotte and Montgomery, 2006). However, it is possible that in these species systemic RNAi occurs, but may be active only under certain conditions such as viral infections. This idea is supported by the fact that

some plant viruses can induce systemic RNAi silencing (Voinnet et al., 2000; Yoo et al., 2004). Moreover, some of viruses have evolved suppressors that are specific for systemic RNAi (Voinnet et al., 1999; Voinnet et al., 2000; Guo and Ding, 2002).

miRNA Pathway

miRNAs are found in animals, plants, and viruses (The miRBase Registry 9.1) The founding members of the miRNA family, *lin-4* and *let-7*, discovered using forward genetic approaches in *C. elegans* (Lee et al., 1993; Reinhart et al., 2000), were shown to be regulators of developmental timing. The cloning of *lin-4* and *let-7* revealed that they did not encode protein, but rather non-coding small antisense RNAs, about 22 nt long, which are complementary to the 3′ untranslated regions (UTR) of their target genes (Lee et al., 1993; Wightman et al., 1993; Reinhart et al., 2000). Subsequently, discovery of similar siRNA species in *A. thaliana* (Hamilton and Baulcombe, 1999), and cloning of a second miRNA, let-7, suggested that the function of miRNA and RNAi pathways could be related and evolutionary conserved. The loss of function phenotype of the *C. elegans dcr-1* was similar to the phenotype of heterochronic genes *let-7* and *lin-4* (Grishok et al., 2001; Ketting et al., 2001). Surprisingly, *Dicer* protein were found to be essential for the miRNA pathway in both *C. elegans* (Grishok et al., 2001; Ketting et al., 2001) and *D. melanogaster* Hutvagner et al. (2001), suggesting that miRNA and RNAi pathways intersect in these organisms. Moreover, RNAi depletion of *C. elegans rde-1* homologs, *alg-1/2* exhibited a heterochronic phenotype similar to the *dcr-1*, *let-7*, and *lin-4* mutants, indicating that

a subgroup of Argonaute family of genes was involved in the miRNA pathway. Northern blot analysis of *let-7* and *lin-4* miRNAs demonstrated that *dcr-1* and *alg-1/2* proteins were required for the biogenesis of these miRNAs. Interestingly, *alg-1/2* genes were not essential for the RNAi pathway in *C. elegans* (Grishok et al., 2001). Data collectively suggest that miRNA and RNAi pathways are related and converge on Dicer protein. Furthermore, the data suggest that distinct Argonaute family of proteins are specialized for different small RNA mediated pathways.

Biogenesis of miRNAs

Recent studies showed that RNA Polymerase II (RNAP II) generates eukaryotic miRNAs (Cai et al., 2004; Lee et al., 2004) although RNA Polymerase III (RNAP III) is shown to generated several miRNAs in mouse γ-herpesvirus 68 (MHV68) (Pfeffer et al., 2005; Borchert et al., 2006). Each primary transcript (pri-miRNAs) forms a hairpin stem-loop structure. They are usually clustered in the genome, and generated from a single polycistronic transcript (reviewed in Bartel, 2004). Pri-miRNAs are then processed by an RNAse III enzyme, Drosha, into miRNA precursors (pre-miRNAs), which are approximately 70-nt long (Lee et al., 2003). In *D. melanogaster* and *C. elegans,* Drosha partners with a dsRNA binding protein called Pasha/DGCR8 (Denli et al., 2004; Han et al., 2004). Once pre-miRNAs are made, they are transported to the cytoplasm via exportin-5 (Exp5), which is a RanGTP-dependent dsRNA binding receptor (Yi et al., 2003; Bohnsack et al., 2004; Lund et al., 2004; Ma et al., 2004) (Zeng and Cullen, 2004). In the cytoplasm, pre-miRNA is processed by

RNAse III enzyme, Dicer, into ~ 22 nt long miRNA duplexes (Bernstein et al., 2001; Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001). miRNA duplex is incorporated into RISC complex to form a miRNA-charged RISC complex (miRISC), containing an Argonaute member as a subunit. The non-miRNA strand of miRNA duplex is called microRNA* (miRNA*) and is degraded quickly during RISC assembly.

Mechanism of miRNA Silencing

Animal miRNAs usually function through imperfect base pairing to the 3′ untranslated region (UTR) of mRNAs (reviewed in Carrington and Ambros, 2003), which usually contain multiple binding sites for a miRNA. Initially, miRNAs were thought to function by repressing translation because there was no significant change in *lin-14* mRNA level by *lin-4* miRNA, whereas the *lin-14* protein level was decreased (Wightman et al., 1993). Consistent with this idea, recent biochemical studies have shown that miRNAs can regulate gene expression either at initiation or during translation of the target mRNA (Humphreys et al., 2005; Nottrott et al., 2006; Petersen et al., 2006). In contrast, several reports suggested that miRNAs could also cause destablization and degradation of target mRNAs (Bagga et al., 2005; Lim et al., 2005; Behm-Ansmant et al., 2006; Wu et al., 2006).

In contrast to the animal miRNAs, plant miRNAs make perfect or near-perfect base pairing with their targets, which usually reside a single miRNA-binding site (Reinhart et al., 2002). Moreover, plant miRNAs can have binding sites outside of

the 3′ UTR. In plants, the high degree of sequence complementarity between a miRNA and its target allows miRISC complex to cleave its target in a similar manner to siRNA-RISC (siRISC) during RNAi (Tang et al., 2003; Qi et al., 2005). Nonetheless, perfect base pairing between miRNA and its target does not necessarily mean that a miRNA will execute its action only through cleavage. Some miRNAs, such as plant miR172, which makes perfect base pairing with its target, was shown to cleave the target mRNA (Aukerman and Sakai, 2003; Chen, 2004), and to repress its translation at the same time (Schwab et al., 2005).

Additional Classes of Small RNAs in *C. elegans* and Other Organisms

TncRNA, endo-siRNA and X cluster siRNA in *C. elegans*

The discovery of miRNAs (Lee et al., 1993) and siRNAs (Hamilton and Baulcombe, 1999), and the overlapping functions of both pathways in *C. elegans* (Grishok et al., 2001) suggested that miRNAs and their function might be evolutionarily conserved. cDNA cloning and bioinformatic studies in model organisms such as *C. elegans* (Lau et al., 2001; Lee and Ambros, 2001), *A. thaliana* (Park et al., 2002; Reinhart et al., 2002; Rhoades et al., 2002), *D. melanogaster* (Lagos-Quintana et al., 2001), and humans (Lagos-Quintana et al., 2001) not only provided enough evidence to support this hypothesis, but also identified several novel classes of small RNAs.

Although miRNAs *lin-4* (Lee et al., 1993), *let-7* (Reinhart et al., 2000), and *lsy-6* (Johnston and Hobert, 2003) in *C. elegans* were cloned using forward genetic approaches, the identification of miRNAs using forward genetics is a rare event

because of the small size of miRNAs and the redundancy between the conserved miRNA families. Each miRNA family represents a group of highly homologous miRNA sequences. Two other strategies that are used to identify miRNAs are direct cloning of miRNAs isolated from biological samples, and computational predictions. In the direct cloning approach, the cloning of miRNAs is biased towards more abundant miRNA species. Also, computational analysis of miRNAs is not straightforward because of the nature of imperfect base pairing. For instance, miRNA precursors contain imperfect base pairing within the secondary stem loop structure, and most miRNAs also make imperfect base pairing with their target mRNA sequences. Therefore, computational predictions become hard to interpret, unless they are validated experimentally. Nevertheless, plant miRNAs are highly complementary to the their target mRNAs and easier to predict by computational analysis (Rhoades et al., 2002; Jones-Rhoades and Bartel, 2004). Recently, the development of a large scale sequencing technology or high-throughput pyrosequencing (Margulies et al., 2005) has allowed the discovery of additional miRNAs and novel endogenous small RNA species.

Computational and short cDNA cloning (independent of 5′ phosphate) approaches used by the Ambros lab identified 21 new miRNAs and three novel classes of small RNAs with similar properties to miRNAs and siRNAs in *C. elegans* (Ambros et al., 2003). These small RNA classes are: tiny noncoding RNAs (tncRNAs), endogenous siRNAs (endo-siRNAs), and X-cluster derived small RNAs (X-cluster). A total of 33 distinct small RNAs, which are approximately 20-21 nt long, were classified as

tncRNAs in *C. elegans*. These small RNAs were called tncRNAs because they originated from noncoding regions of the *C. elegans* genome. The computational analysis indicated that tncRNAs do not make a perfect base pairing with predicted or existing mRNA sequences, suggesting that tncRNAs might be functioning like miRNAs. In contrast to miRNA precursors, tncRNAs were not predicted to form a fold-back stem loop secondary structure. Interestingly, most of the tncRNAs were developmentally regulated like miRNAs; however, they did not seem to be conserved in sequence beyond *C. elegans* (Ambros et al., 2003).

Cloning of short cDNA sequences identified 746 distinct antisense sequences (~ 22-nt long) corresponding to the coding region of 541 different *C. elegans* genes. Interestingly, antisense sequences often originated from transposon sequences; consistent with previous reports that RNAi plays a role in transposon silencing (Ketting et al., 1999; Tabara et al., 1999b). Furthermore, these small RNAs make perfect base pairing with their target, similar to siRNAs derived from trigger dsRNA during RNAi.

X cluster siRNAs derive from a 2 kb upstream region of the F47E1.1 gene on the X chromosome, and contain 41 individual cDNA sequences which are oriented in the same direction. Surprisingly, the X cluster locus does not contain or overlap any predicted gene in *C. elegans* genome. Also, it is not yet known if X cluster siRNAs are developmentally regulated (Ambros et al., 2003).

21U-RNA and 26-mer endo-siRNAs in *C. elegans*

A recent work using high throughput pyrosequencing in *C. elegans* identified additional novel classes of *C. elegans* small RNAs (Ruby et al., 2006). This study, unlike Ambros et al. (2003), used a cDNA cloning strategy that was dependent on the presence of a 5′ monophosphate on small RNA molecules.

Two prominent subclasses of these novel small RNAs were called "21U-RNAs and "26-mer siRNAs" (Ruby et al., 2006). The 21U-RNA, as inferred from its name, is 21-nt long and starts with a 5′ Uridine. The 21U-RNA derives from 5,302 loci on three distinct regions of the Chromosome IV, mapping to both strands of DNA. However, they do not appear to be originated from a pre-miRNA-like precursor. The majority of the 21U-RNAs originate from intergenic regions and from introns. One of the most interesting features of the 21U-RNA is their conserved upstream sequences. Each 21U-RNA gene contains two conserved upstream motifs. The small motif is located adjacent to the 21U-RNA and contains a consensus YRNT sequence, in which T corresponded to 5′ U of the 21U-RNA. The large motif is located upstream from the small motif, and separated by an approximately 20-nt long spacer sequence. Interestingly, upstream motifs are conserved in *C. briggsae,* and they are proposed to be the promoter sequences for each 21U-RNA. The function of the 21U-RNAs are not yet known; however, their involvement in regulation of chromatin structure has been proposed (Ruby et al., 2006).

The 26-mer siRNAs are considered as a subclass of endo-siRNAs that ranges from 18-mer to 26-mer. The Bartel library (Ruby et al., 2006) identified 2378 distinct

endo-siRNA sequences using high throughput pyrosequencing. The 26-mer long RNAs represented the most prominent species in the cDNA library. Like the Ambros library (Ambros et al., 2003), the Bartel library (Ruby et al., 2006) frequently contained RNAs corresponding to the transposons sequences. Interestingly, 55% of the 26-mer RNAs in the Bartel library corresponded to sperm specific genes.

The chemical identity of small RNA ends can give information about how they are produced. Some siRNAs in plants (Li et al., 2005) and in animals including *D. melanogaster* rasiRNAs (Li et al., 2005; Vagin et al., 2006) and exogenous-siRNAs (produced by exogenous dsRNA during RNAi) (Pelisson et al., 2007) can be modified at the 3′ ends. To have more insight into the production of endogenous small RNAs, Ruby et al. (2006) did chemical analysis of 5′ and 3′ ends of "siRNA26-1" and "21U-1" RNAs, which are representatives from each 26-mer and 21-U classes, respectively. The analysis of 5′ ends by kinase and phosphatase treatment found that the 5′ ends carried monophosphates. Moreover, the analysis of the 3′ ends found that these small RNAs were resistant to periodate treatment, indicating that either 2′ or 3′ ends are modified (Ruby et al., 2006).

An important difference between the Ambros and the Bartel library is the proportion of miRNAs to endo-siRNAs. The Ambros library contained almost equal amounts of miRNAs and siRNAs, whereas the Bartel library contained 100 times more miRNA than siRNAs. It is worth noting that the Ambros library was independent of 5′ phosphates, whereas the Bartel cDNA library was dependent on 5′ phosphate. miRNAs bear 5′ phosphates and 3′ hydroxy termini. Therefore, the

difference between the two libraries could be due to the lack of 5′ monophosphates in the 21U-RNA and other endo-siRNAs. Although chemical analysis showed that "siRNA26-1" and "21U-1" RNAs carry 5′ monophosphates, 5′ monophosphate carrying-small RNAs could simply be representing a small portion of the total 26-mer-siRNAs and 21U-RNAs. In agreement with this idea, the capping reaction using total small RNAs from worms indicated the existence of a small RNA population that carried 5′ di- or triphosphates (Ruby et al., 2006).

The *C. elegans* genome contains four putative RdRP proteins, which generate products that bear 5′ triphosphate. The three *C. elegans* RdRPs, *ego-1, rrf-1, and rrf-3,* were already implicated in small RNA silencing pathways and appear to be required for the production of small RNAs. Therefore it is conceivable that endogenous siRNAs are products of RdRPs in *C. elegans*. Currently, we neither know the biogenesis nor the function of these novel siRNA species. It would be interesting to know if the production of these small RNAs is dependent on the Dicer or RdRP genes. Also, it is great interest to us to know which of 27 *C. elegans* Argonaute family of proteins interacts with these small RNA species, and how these RNAs function.

Repeat-Associated Small Interfering RNAs (rasiRNAs)

In *D. melanogaster,* Dcr-1 produces miRNAs, and Dcr-2 produces siRNAs. A third group of small RNAs, named repeat-associated siRNAs (rasiRNAs), enriched in germline, were identified in the *D. melanogaster* genome (Aravin et al., 2001; Aravin

et al., 2003; Vagin et al., 2006). rasiRNAs derive from repetitive sequence elements in the genome such as telomeric regions, transposons and *Su(Ste)* locus. Small RNAs produced by Dcr-1 and Dcr-2 are ~22-nt in size; however, the rasiRNAs range from 24 to 29 nt. In addition, rasiRNAs primarily originate from the antisense strand (Aravin et al., 2003; Vagin et al., 2006). A work from the Zamore lab showed that accumulation of rasiRNAs was not dependent on Dcr-1 and Dcr-2 proteins, suggesting that rasiRNAs are made by a different mechanism than miRNAs and siRNAs in *D. melanogaster*. However, it is possible that Dcr-1 and Dcr-2 function redundantly in the production of rasiRNAs. In *C. elegans*, RdRP proteins seem to be responsible for producing endo-siRNAs; however, *D. melanogaster* genome does not encode a known RdRP protein. At the present time, the enzymes making rasiRNAs and their precursors are not yet known. Based on the molecular analysis, rasiRNAs appear to be a distinct class of small RNA. *D. melanogaster* genome has five members of the Argonaute family of genes, *ago1, ago2*, *ago3, aub,* and *piwi.* In *D. melanogaster*, miRNAs function through Ago1, and siRNAs function through Ago2. Vagin et al. (2006) hypothesized that rasiRNAs might be functioning through a different Argonaute member. Indeed, Vagin et al. found that the production of the *Sut(Ste)* and *roo* rasiRNA (most abundant retrotransposon in *D. melanogaster*) were dependent on the wild type activities of the Piwi subfamily of genes, Piwi and Aub, but were not dependent on the genes essential for canonical RNAi and miRNA pathways. Moreover, they showed that *piwi* and *aub* directly associated with *roo* and

Su(Ste) rasiRNAs. Taken together, this data suggest that distinct small RNAs act through different Argonautes in the RNAi-related pathways in *D. melanogaster*.

Piwi-interacting RNAs (piRNAs)

Recently, a novel class of small RNAs called Piwi-interacting RNAs (piRNAs) were characterized from mouse, rat, and human testes (Girard et al., 2006; Lau et al., 2006). piRNAs are approximately 30-nt long and expressed at very high levels, more than any other endogenous small RNAs in these organisms. In mouse, there are four members of the AGO subgroup, AGO1-AGO4, and three members of the Piwi subgroup, MIWI, MILLI, and MIWI2. The Piwi subgroup of Argonaute proteins is known to be predominantly expressed in the germline. Biochemical experiments found that piRNAs co-purifies with MIWI, but not with Ago2 in mouse (Girard et al., 2006). Moreover, biochemical fractionation assays in rat found that piRNAs co-purifies with the rat Piwi homolog, Riwi, and the human RecQ1 homolog, rRecQ1 (Lau et al., 2006). The Riwi/piRNA fraction was found to form a cleavage competent RISC, called piRC. The *Neurospora crassa* homolog of rRecQ1, QDE-3, is required for quelling as is the Argonaute family member, QDE-2 (Fagard et al., 2000). Human RecQ1 functions as an ATP dependent DNA helicase (Sharma et al., 2005).

Interestingly, *C. elegans* genome encodes two Piwi related genes, PRG-1 and PRG-2. The depletion of the activity of these two proteins by RNAi results in smaller brood size (Cox et al., 1998). The reduction in the brood size could be due to defective sperms in *C. elegans*. In addition, more than half of the endogenous

siRNAs in the Bartel library (Ruby et al., 2006) was sperm specific. Taken together, PRG-1 and PRG-2 may have evolved to function through sperm specific small RNAs in the *C. elegans*.

Enhancers of RNAi in *C. elegans*

Recently, several research groups have identified a specific class of *C. elegans* mutants which exhibit increased sensitivity to dsRNA. This class of mutants was named *eri* for enhancer of RNAi (Kennedy et al., 2004). The first *eri* mutant, *rrf-3,* identified by reverse genetics, encodes one of the four putative RdRP proteins in *C. elegans*; the other three putative RdRP proteins being *ego-1, rrf-1,* and *rrf-2* (Sijen et al., 2001). In *C. elegans*, some genes, particularly in the nervous system, are refractory to dsRNA, giving either weak or nonexistent RNAi phenotype. However, mutations in *rrf-3* gene significantly enhances RNAi in both neuronal and non-neuronal tissues (Simmer et al., 2002). While *ego-1* and *rrf-1* mutants are respectively resistant to germline and somatic RNAi, the *rrf-2* mutant did not show any abnormal phenotype in RNAi assays (Sijen et al., 2001). This implies that *rrf-2* either functions redundantly with other RdRPs or is involved in other small RNA mediated cellular pathways.

To identify additional *eri* mutants, Kennedy et al. (2004) took advantage of the inefficient RNAi in the nervous system and designed a genetic screen using a strain carrying the *unc-47::gfp* fusion gene, expressed in the 26 γ-aminobuttyric acid (GABA)-containing neurons in *C. elegans.* In a wild type background, *unc-47::gfp*

expression exhibited weak or no silencing when worms were fed bacteria expressing *gfp* dsRNA. After mutagenesis with EMS, the mutant strains were fed bacteria expressing *gfp* dsRNA. Then, strains which expressed little or no *gfp* was isolated as *eri* mutants. Among the identified *eri* mutants, *eri-1, eri-3, and eri-5,* were cloned and further characterized (Kennedy et al., 2004; Duchaine et al., 2006). In addition to increased sensitivity to RNAi, *eri* mutant strains exhibit other common phenotypes such as temperature sensitive sperm-defective sterility at 25 ºC, high incidence of male (Him) phenotype due to increased X-chromosome disjunction, and spontaneous silencing of the somatic transgenes (Duchaine et al., 2006). These additional phenotypes suggested that *eri* genes might be involved in different endogenous processes. Interestingly, Duchaine et al. (2006) found that *C. elegans* Dicer, DCR-1, co-purified with ERI-1, ERI-3, ERI-5, and RRF-3. Further biochemical analysis of these proteins found that the interaction between ERI-1 and DCR-1 was abolished in *eri-3* mutant extracts, and reduced in *eri-5* mutant extracts, suggesting that DCR-1 forms a complex with ERI proteins. In agreement with this data, an ERI-1 immunocomplex contained ERI-3, ERI-5, DCR-1, and DRH-3 (a DEAH/D RNA Helicase) (Duchaine et al., 2006).

As mentioned above, the single *C. elegans* Dicer homolog, DCR-1, is essential for both RNAi and miRNA pathways. In the RNAi pathway, DCR-1 forms an initiation complex together with RDE-1, RDE-4 and DRH-1 (Tabara et al., 2002). In miRNA pathway, DCR-1 interacts with ALG-1 and ALG-2 (Duchaine et al., 2006). DCR-1/ALG complex seems to be necessary for the maturation of miRNAs (Grishok et al.,

2001). Interaction of DCR-1 with proteins that are involved in different small RNAi-mediated pathways suggested that DCR-1/ERI protein complex might be functioning in a small RNA-mediated pathway. To test this hypothesis, Duchaine et al. (2006) checked the accumulation of three novel classes of endogenous small RNA species in *dcr-1* and *eri* mutant strains. These novel classes of small RNAs are tiny noncoding RNAs (tncRNAs), derived from noncoding region of *C. elegans* genome; X-cluster, derived from a locus in X-chromosome; and endogenous siRNAs (endo-siRNAs) that are complementary to the proteins coding regions (Lee et al., 2006a). Northern blot analysis showed that DCR-1, DRH-3, RRF-3, ERI-1, and ERI-3 were required for the accumulation of tncRNA, X-cluster RNA, and endo-siRNA species that are complementary to the protein coding regions. In the *eri-5* mutant strain, the amount of the small RNA species were less than in wild type animals, consistent with the weak phenotype of *eri-5* mutant (Duchaine et al., 2006).

Data from Duchaine et al. demonstrated the involvement of *dcr-1* and *eri* genes in endogenous siRNA (endo-siRNA) biogenesis. However, the question remains as to why *eri* mutant strains exhibit enhanced RNAi? The clues came from more careful analysis of *rrf-3* and *eri-1* mutant strains. As mentioned above *rrf-3* and *eri-1* mutant strains exhibit hypersensitive RNAi phenotypes. The enhanced RNAi phenotypes of these two mutant strains were shown to be associated with over-accumulation of exogenous siRNAs (exo-siRNAs) compare to the wild type strain (Sijen et al., 2001; Simmer et al., 2003). (Henceforth, siRNAs originated from experimentally introduced trigger dsRNA are called exogenous siRNAs or exo-siRNAs, whereas siRNAs

originated from the genome itself are called endogenous or endo-siRNAs; unless otherwise stated, siRNAs always refer to exo-siRNAs. Similarly, RNAi triggered by an exogenous dsRNA trigger is called exogenous or exo-RNAi, whereas RNAi triggered by an endogenous dsRNA are called endogenous RNAi or endo-RNAi; unless otherwise stated RNAi always refers to exo-RNAi). Moreover, Lee et al. (2006a) found that the targets of endo-siRNAs were subject to ongoing silencing in wild type worms. In other words, in *rrf-3* and *eri-1* mutant strains, expression of endo-siRNA targets was elevated. Based on these observations two models were proposed for the increased RNAi phenotype of *eri* mutants. The first model predicted that some of the RNAi genes would be subject to ongoing silencing by endo-siRNAs, and derepression of this silencing in *eri* mutants would upregulate the exo-RNAi pathway. However, the analysis of expression profiles of *C. elegans* genes from *eri* strains showed no evidence for the upregulation of genes involved in the exo-RNAi pathway (Lee et al., 2006a). In a second model, a competition between the common factors of exo- and endo-siRNA pathways was proposed. This model predicts that there is a limiting common factor or factors that are essential for both exo- and endo-RNAi pathways. Duchaine et al. tested this model by checking the level of K02E2.3 endo-siRNAs when worms are exposed to *sel-1* dsRNA. Interestingly, wild type worms subjected to exogenous dsRNA accumulated less K02E2.3 endo-siRNAs than wild type worms not exposed to sel-1 RNAi. However, when the same experiments was done with the *rde-1(ne300)* mutant strain, deficient in exogenous RNAi, no

change in K02E2.3 endo-siRNA level was observed (Duchaine et al., 2006), suggesting that exo- and endo-RNAi pathways compete for a common limiting factor at the downstream of *rde-1*.

RNAi and Antiviral Defense

Over the past years, RNAi has been established as an innate antiviral defense system in plants and animals (Hamilton and Baulcombe, 1999; Li et al., 2002; Lu et al., 2005; Voinnet, 2005; Wilkins et al., 2005). The early indications of RNAi as an antiviral defense system came from the studies in plants in which siRNAs derived from viral genomes were detected after infection (Hamilton and Baulcombe, 1999). These siRNAs carried the hallmarks of Dicer processing and the generation of the dsRNA substrate was hypothesized to form by one of two ways: intrinsic self-complementary folding of viral mRNA that create a dsRNA structures, or viral or cellular RdRPs that generate dsRNA during viral replication.

Some of the RNAi deficient mutants in plants were found to be more susceptible to viral infections. For example, the RdRP mutant *rdr6/sgs2,* also involved in transgene silencing, cannot restrict viral replication (Mourrain et al., 2000) and the *dcl2*/*dcl4* double mutant exhibits dramatically increased viral replication (Deleris et al., 2006). Interestingly, there are no known natural viral pathogens for *C. elegans*. However, three groups have recently demonstrated that at least two different viral species can replicate in *C. elegans*. Studies with vesicular somatitis virus (VSV) in *C. elegans* primary cell culture showed that the replication of this virus was increased

in cells isolated from RNAi deficient mutants *rde-1*, *rde-4* and *rrf-1,* while it was reduced in cells isolated from *rrf-3* and *eri-1* mutants, in which the RNAi response was enhanced (Schott et al., 2005; Wilkins et al., 2005). Moreover, siRNAs corresponding to VSV were detectable in wild type *C. elegans* cells, but not in *rde-4* mutant cells (Wilkins et al., 2005). Furthermore, Flock house virus (FHV) can replicate in *C. elegans* in an *rde-1* dependent manner. Surprisingly, in *rde-1* mutant animals, the FHV RNAi suppressor, B2, can still suppress RNAi. In other words, antiviral RNAi is still active in *rde-1* animals, albeit weaker than in wild type, suggesting that antiviral RNAi might be mediated by one or more of the remaining 26 Argonaute members in *C. elegans* (Lu et al., 2005).

In addition to FHV, many other viruses contain RNAi suppressors. Biochemical and three-dimensional structural studies revealed viral RNAi suppressors act by different mechanisms. For example, while Tomato Bushy Stunt Virus (TBSV) p19 protein binds to 21-nt long siRNA duplexes and prevents them from entering the RISC complex (Vargason et al., 2003; Ye et al., 2003; reviewed in Zamore, 2004), B2 protein of FHV binds to dsRNA, and prevents Dicer from making siRNAs from its substrate (Chao et al., 2005; Lu et al., 2005). Furthermore, adenovirus VA1, a highly structured 160-nt-long noncoding RNA, seems to function by competing for Dicer binding (Lu and Cullen, 2004; Andersson et al., 2005). The presence of RNAi suppressors in viral genomes is one of the strongest pieces of evidence that RNAi has evolved as a natural antiviral defense system in living organisms.

RNAi, Cosuppression, Transgene, and Transposon Silencing

The phenomenon known as "cosuppression" is often mistaken for "transgene silencing." While both silencing pathways rely on the presence of an exogenously-introduced copy of a given gene, cosuppression (van der Krol et al., 1990; Jorgensen et al., 1996) refers to the simultaneous silencing of both exogenous and endogenous gene copies, whereas transgene silencing refers to the silencing of only the transgene. Cosuppression and transgene silencing are not unique to plants, and also are seen in fungi and animals.

Early in our understanding, it was not clear what the underlying mechanism of homology-dependent silencing in plants was. However, subsequent studies, particularly the discovery of RNAi in *C. elegans*, showed that dsRNA was the common trigger of homology-dependent silencing pathways. Transformation of cells by different methods causes the formation of multicopy extrachromosomal arrays in many organisms. For example, in *C. elegans,* injected plasmids form long tandem arrays (several hundred copies) and undergo complex rearrangement, which are inheritable (Mello et al., 1991). Thus, the expression of dsRNA or sense transcripts from transgenes or viral vectors triggers homology-dependent gene silencing in organisms. Although dsRNA can be formed directly from inverted transgenic repeats and viral vectors, the activity of the RDR6 (RNA dependent RNA polymerase) appears to be required for the formation of dsRNA from single copy transgenes in *A. thaliana* (Dalmay et al., 2000). In plants, after siRNAs are made from the dsRNAs, they are incorporated into a cleavage competent AGO1/RISC (Baumberger and

Baulcombe, 2005; Qi et al., 2005). Furthermore in plants, the AGO1/RISC associates with small RNAs originated from different RNAi-related pathways such as miRNAs and transacting siRNAs (ta-siRNAs) (Vaucheret et al., 2004; Baumberger and Baulcombe, 2005).

In *C. elegans* various RNAi-related pathways seems to protect the genome against invading nucleic acids. For example, transposons are normally silenced in the germline of the animals; however, they are mobilized when there are mutations in the mutator class of genes *(mut)* as described above. Analysis of the *mut* class of genes revealed that some of these genes, *mut-2/rde-3, mut-7, mut-8/rde-2 and mut-9,* were also required for cosuppression, and RNAi (Ketting and Plasterk, 2000; Tops et al., 2005). This observation indicates that transposon silencing, cosuppression, and RNAi pathway share common components in *C. elegans.*

Among the 27 Argonaute genes, *rde-1* was shown not to have any phenotype other than RNAi deficiency, while *alg-1* and *alg-2* were shown to be essential for the miRNA pathway, but not the RNAi pathway. Recently, RNAi-based screens implicated additional members of this gene family in both transposon and transgene silencing pathways (Vastenhouw et al., 2003; Robert et al., 2005). However, the molecular functions of these Argonaute genes in these pathways are not clear. Furthermore, it is unclear the extent to which these Argonautes participate in RNA itself. Further molecular and genetic analysis of the Argonaute family of genes will help to understand the mechanism and complicated relationships between these siRNA-mediated silencing pathways.

RNAi and Transcriptional Silencing

The proteins composing the Argonaute family are key components of discrete RISC complexes. As mentioned above, Argonaute/siRNA and Argonaute/miRNA complexes regulate gene expression at the posttranscriptional level, by cleaving mRNA and repressing translation, respectively. Recent studies showed that small RNAs could also regulate gene expression at the transcriptional level in eukaryotes such as *S. pombe,* plants, and mammals through the cytosine methylation within chromosomal DNA and histone methylation. It has been well established that DNA and histone methylation (H3K9) is associated with transcriptional silencing. The RNAi mediated transcriptional silencing is best investigated in plants and *S. pombe.* Although both DNA and histone methylation occurs in plants, only histone methylation is detected in *S. pombe*.

RNA-directed methylation of chromosomal DNA was identified as early as 1994 in plants (Wassenegger et al., 1994; Pelissier and Wassenegger, 2000) and several subsequent studies in plants firmly established that RNA-directed methylation of the homologous DNA was indeed mediated by RNAi components. In plants, the expression of transgenes from viral vectors corresponding to promoter regions was found to induce the transcriptional silencing of the targeted genes. This silencing was associated with the generation of ~23 nt long siRNA molecules corresponding to the transgene sequence and with the de novo methylation of cytosines within the corresponding chromosomal DNA sequences (Mette et al., 2000; Jones et al., 2001). Subsequently, Sijen et al. (2001) showed that targeting the promoter region of

chalcone synthaseA (*chsA*) by a corresponding dsRNA caused transcriptional silencing, while targeting the coding region of the same gene caused posttranscriptional silencing (PTGS). Interestingly, siRNAs corresponding to both targeted regions accumulated during silencing and caused the methylation of cytosine within the genomic locus. Then, Volpe et al. (2002) showed for the first time that H3K9 methylation and heterochromatin formation in the centromeres of *S. pombe* was mediated by RNAi.

In *A. thaliana,* there are there are ten members of the Argonaute gene family. To date, only AGO1 and AGO4 have been characterized. While AGO1 is involved in transgene silencing and the miRNA pathway, AGO4 seems to affect RNA-mediated chromatin silencing. The *ago4-1* allele was originally isolated (Zilberman et al., 2003) as a suppressor of *clark kent* (*clk*) mutants, which were overmethylated, silenced epigenetic alleles of the *SUPERMAN (SUP)* gene (Jacobsen and Meyerowitz, 1997). Two other suppressors of *clark kent* (*clk*) were *chromomethylase3 (cmt3),* which encodes a DNA methyl transferase, and *kryptonite (kyp),* which encodes an H3K9-specific protein methyltransferase (Lindroth et al., 2001; Jackson et al., 2002). In the *ago4-1* mutant background, both cytosine and histone H3K9 methylation were reduced in *SUP* and several other loci including a 180-nt centromeric repeat *(CEN)* sequence, and the Ta3 retrotransposon; however, they were still present in the some of the tested loci, indicating that *AGO4* plays a role in chromatin silencing (Zilberman et al., 2003). Although CpNpG and asymmetric methylation was reduced in the *ago4-1* mutant, CpG methylation was not affected.

The analysis of some of the additional loci indicated the presence of *AGO4*-independent methylation, suggesting that additional members of the Argonaute family of genes may function in the methylation process or an alternative pathway. In addition, H3K9 methylation was reduced in *SUP, Ta3,* and *AtSN1* loci. In wild type *A. thaliana* plants, the *AtSN1* locus produces siRNAs. Interestingly, in an *ago4-1* mutant strain, siRNAs associated with the *AtSN1* locus was not detectable (Zilberman et al., 2003). Taken together, these data suggest a strong interaction between DNA methylation, H3K9 methylation, and RNAi.

The methylation of DNA and histone at the same locus raise the question whether the two types of methylation are sequential? Studies of H3K9 methylation in an *A. thaliana met1* mutants implied that DNA methylation was necessary prior to H3K9 methylation (Soppe et al., 2002; Tariq et al., 2003). These observations were also supported by reports suggesting that histone modifications were necessary to maintain de novo DNA methylation (Aufsatz et al., 2002; Aufsatz et al., 2004). Moreover, in a *kryptonite (kyp)* mutant strains, RNA directed de novo methylation of DNA still occurred (Malagnac et al., 2002). However, in the fungus *N. crassa,* H3K9 methylation occurs before DNA methylation (Tamaru and Selker, 2001). Additionally, in *A. thaliana,* at least CMT3 dependent CnG methylation appears to be downstream of H3K9 methylation (Jackson et al., 2002).

RNAi-mediated transcriptional silencing is not unique to plants. In human cell lines, siRNAs were shown to induce transcriptional silencing in a methylation-dependent (Morris et al., 2004; Weinberg et al., 2006) and independent manner (Ting

et al., 2005). In a recent study, Kim et al.(2006) reported that AGO1, but not AGO2 binding, was enriched on the promoters of both exogenous and endogenous genes when they were targeted by dsRNA. The silencing of these genes was associated with H3K9 methylation in a methyltransferase-dependent manner. Moreover, this silencing required the activity of the TRBP2 protein, a dsRNA binding protein known to be associated with the Dicer/AGO2 complex (Chendrimada et al., 2005).

Transcriptional regulation by RNAi in *C. elegans* is not yet well established. However, a recent report suggested that dsRNA could induce transcriptional silencing of a somatically expressed transgene (Grishok et al., 2005). Interestingly, transcriptional silencing was found to be dependent on the canonical RNAi pathway genes *dcr-1, rde-1, rde-4* and *rrf-1*. In addition, the transcriptional silencing was stronger in an RNAi enhancer mutant *rrf-3*. In addition, RNAi-based genome-wide screens have identified genes that are involved in chromatin structure and transcriptional silencing, and at the same time, involved in either RNAi or cosuppression (Grishok et al., 2005; Kim et al., 2005; Robert et al., 2005). Taken together these findings suggest that RNAi has a transcriptional component in *C. elegans* or at least a fraction of RNAi related pathways lead to TGS of some sort in *C. elegans*.

RNAi and Heterochromatin

Most of our knowledge about RNAi-mediated transcriptional silencing comes from studies from *S. pombe* centromeres, which are known to have low levels of

transcription. *S. pombe* has a well-defined, yet complex centromeric region similar to mammalian centromeres (Martienssen et al., 2005). *S. pombe* centromeric DNA is composed of two structural domains; the central core region *(cnt)* and the centromeric flanking repeats. The central core region is flanked by immediate symmetrical *imr* sequences. *imr-cnt-imr* region is also flanked by outside *otr* repeat sequences, which forms a centromeric structure like *otr-imr-cnt-imr-otr.* Furthermore, symmetrical *otr* sequences are made of inverted *dg* and *dh* repeats. Biochemical and genetic studies showed that central core *(cnt)* region and flanking repeat region *imr/otr* associate with different sets of proteins. For example, while *cnt* domain associates with histone H3 variant Cnp1, *imr/otr* domain associates with, Swi6, Chp1, and Abp1. Swi6 and Chp1 are chromodomain proteins, which bind to methylated histone tails. Abp1 is a CENP-B homolog, which binds to centromeric DNA. Moreover, both domains are associated with different functions. While the central core *(cnt)* serves as a kinetochore attachment domain and mediates spindle attachment, flanking heterochromatin domains are necessary for sister chromatid cohesion (reviewed in Ekwall, 2004).

An intact centromeric heterochromatin structure is essential for cohesion between sister chromatids. In *S. pombe,* analysis of a *swi6* mutant strain showed that lack of *swi6* specifically disrupts cohesion of sister chromatids at centromeric regions, but not at the arms (Bernard et al., 2001). Furthermore, Swi6 is required for Rad21 and Psc3 association with centromeres, which are subunits of a large cohesin complex (Bernard et al., 2001; Nonaka et al., 2002). *Swi6, rad21,* and *psc3* mutant strains

show chromosome segregation defects which are similar to loss of function phenotypes of RNAi pathway components in *S. pombe* (Bernard et al., 2001; Nonaka et al., 2002; Volpe et al., 2002) and *Trypanosoma brucei* (Durand-Dubief and Bastin, 2003)

S. pombe is unique in having a single member of each of the Dicer, Argonaute, and RdRP genes. Volpe et al. (2002) showed that deletions in *dcr1*$^+$, *ago1*$^+$, or *rdp1*$^+$ lead to chromosome segregation defects, and de-silencing of the genes located in the *imr/otr* domain, accompanied by loss of both H3K9 methylation and Swi6 recruitment to the centromeres, suggesting for the first time that there is a link between RNAi and heterochromatin formation. Further, biochemical and genetic studies found that Chp1 forms a complex with Ago1 and Tas3, a novel protein with unknown function. This complex has been called RNA-Induced Transcriptional Silencing complex (RITS) and directly associates with siRNAs originated from centromeric transcripts. The RITS complex appears to be recruited to centromeric region by siRNAs in a sequence specific manner, and interacts with chromosomal DNA through Chp1 in a Clr4 dependent manner, which specifically methylates H3K9 (Verdel et al., 2004).

In the *Rdp1* background, no siRNAs associate with RITS, suggesting that enzymatic activity of this protein is important to produce siRNAs from centromeric regions (Volpe et al., 2003). Moreover, immunoprecipitation experiments with Rdp1 showed that Rdp1 interacts with Hrr1 (an RNA helicase) and Cid12 (a poly-A polymerase), an ortholog of the *C. elegans* RNAi protein, *rde-3* (Motamedi et al.,

2004; Chen et al., 2005; Sugiyama et al., 2005). This second complex has been called RNA-Directed RNA polymerase complex (RDRC) and thought to be required for the production of dsRNA from nascent transcripts in centromeric region (Motamedi et al., 2004). In the *clr4* deletion strain, RITS complexes possess very little siRNA. Furthermore, RITS and RDRC complexes no longer interact with each other in *clr4* deletion strain, suggesting that the wild type activity of *clr4* is necessary for siRNA generation by the RITS/RDRC complex (Motamedi et al., 2004).

S. pombe RNAi requires the components of the canonical RNAi pathway genes *ago1, dcr1* and *rdp1*, but does not require the components of the RITS complex Chp1 and Tas3. The wild type activity of *clr4* is also found to be required for efficient production of siRNAs during RNAi (Schramke and Allshire, 2003; Sigova et al., 2004); however, the function of *clr4* during RNAi is not yet understood.

RNAi and Genome Elimination

Genome elimination is an extraordinary phenomenon occuring in many species including ciliates, nematodes and insects. One of the most studied ciliate is the *Tetrahymena*, which carries a somatic macronucleus and a germline micronucleus. During vegetative phase of growth, the macronucleus is transcriptionally active, whereas the germline micronucleus is transcriptionally inert. During conjugation or mating between individual Tetrahymena, which is induced by starvation, the micronucleus undergoes mitosis, meiosis, and cross-fertilization to give rise to new micro- and macronuclei. After this, the old macronucleus is degraded and the new

macronucleus undergoes genome rearrangements during which approximately 15% of the genome is deleted.

Studies found that the *Tetrahymena* Argonaute protein Twi1p was required for successful genome elimination following conjugation. Additionally, small RNAs homologous to the eliminated DNA sequences, called scan RNAs (scRNAs), were detected in wild type, but not in the *Twi1* mutant background (Mochizuki et al., 2002). The involvement of Twi1p and scRNAs strongly suggest that genome elimination is mediated by an RNAi-like mechanism. To test whether or not Tethrahymena also responded to an exogenous dsRNA, Yao et al. (2003) injected dsRNA corresponding to macronuclear-destined-segments (MDS) in conjugating cells. The dsRNA successfully led to elimination of MDS sequences in the new macronucleus. In addition, heterokaryons carrying the bacterial transposon Tn5 in germline micronuclei eliminated the transgene in new macronuclei after mating (Yao et al., 2003).

RNAi leads to heterochromatin formation by methylation of H3K9 in plants, humans and *S. pombe*. In *Tetrahymena*, H3K9 methylation has also been implicated in the DNA elimination process. *PDD1* encodes an abundant chromodomain protein that can bind to methylated H3K9. Disruption of the wild type activity of *PDD1* eliminates scRNA accumulation and H3K9 methylation (Mochizuki et al., 2002; Taverna et al., 2002; Liu et al., 2004b). Thus, an RNAi-mediated establishment of heterochromatin in the targeted region is necessary before the elimination of chromosomal DNA from the macronucleus can occur. Normally, dsRNA derived

from chromosomal DNA controls gene expression by promoting heterochromatin formation, and this is sufficient for silencing. However, Tetrahymena takes the regulation of gene expression one step further and eliminates the silenced heterochromatin region within macronucleus. This phenomenon indicates that the formation of heterochromatin at specific loci across species seems to be highly conserved in its dependence on the RNAi machinery.

Introduction to My Thesis Research

Argonaute proteins are key players in RNAi and other small RNA-mediated pathways. Genome sequencing predicts that *C. elegans* contains 27 members of the Argonaute proteins family, the largest known Argonaute family among model organisms. Phylogenetic analysis indicated that the Argonaute family of proteins could be divided into two major subgroups. The first is the AGO subgroup related to *A. thaliana* AGO1 protein, and the second is the PIWI subgroup related to *D. melanogaster* PIWI protein (Carmell et al., 2002). Our own phylogenetic analysis indicates the existence of a third subclass of the Argonaute family of proteins that is specific to *C. elegans* and equally distant from the PIWI and AGO subgroups (Figure I-2, shown in red). Interesting questions arise from this discovery: 1) Why does *C. elegans* encode so many Argonaute genes? 2) Why does *C. elegans* have a third subclass of the Argonaute protein family?

C. elegans naturally inhabits the soil, where it is exposed to unstable environmental changes (e.g. temperature, moisture, salt), and encounters many

viruses and bacteria which can be potentially pathogenic. These environmental conditions may explain why the *C. elegans* Argonaute family have evolved more than that of other organisms. Diversification of the Argonaute gene family appears to be common among the higher eukaryotes (Figure I-2). Multicellular organisms are more complex and may have evolved Argonaute gene family for different cellular processes. For example in D. melanogaster, Ago1 functions in the miRNA pathway, whereas Ago2 functions in the RNAi pathway, and Piwi, Aub and Ago3 function in the rasiRNA pathway. Similarly, in *C. elegans, rde-1* functions in the exo-RNAi pathway, whereas *alg-1* and *alg-2* function redundantly in the miRNA pathway. Thus, it seems that each Argonaute member may have evolved specifically for a distinct small RNA-mediated pathway.

The observation that Argonautes are small RNA binding proteins, and that different members of this gene family are involved in distinct pathways inspired us to hypothesize that remaining Argonaute members in *C. elegans* may be involved in separate small RNA-mediated pathways. These small RNA-mediated pathways include exo-RNAi, endo-RNAi, miRNA pathways, and novel small RNA-mediated pathways.

Moreover, multiple Argonaute members may function in the same small RNA-mediated pathway. Genetic and biochemical studies found that, unlike *D. melanogaster* and humans, *C. elegans* requires activity of an RdRP (RNA-dependent-RNA polymerase) during RNAi (see Figure I-1). The need for RdRP suggests that there is an RNA replication step during RNAi. In *C. elegans* once exogenous dsRNA

enters into cell, it is recognized and processed into siRNAs by an RNAi initiation complex containing RDE-1, RDE-4, DCR-1, and DRH-1/2 (Tabara et al., 1999b). siRNAs derived directly from exogenous dsRNA trigger are called primary siRNAs, which lead to the amplification of target mRNA by an RdRP. siRNAs that are generated by an RdRP are called secondary siRNAs. Generation of two different siRNA species during RNAi pathway lead us to hypothesize that Argonaute members function twice during RNAi: first binding to primary siRNAs, then to secondary siRNAs. Previous studies from our own lab indicate that *rde-1* protein functions at the initiation step of RNAi. Therefore, we propose that RDE-1 binds to primary siRNAs, and any of remaining Argonaute member(s) bind to secondary siRNAs. However, we cannot rule out the possibility that RDE-1 functions twice during RNAi, binding to both primary and secondary siRNAs itself. We favor former possibility because RDE-1 is a part of the RNAi initiation complex, which produces primary siRNAs (Grishok, 2001; Tabara et al., 2002). A two steps RNAi mechanism appears to be unique to *C. elegans* because humans and *D. melanogaster* appear to have lost their RdRp genes during evolution. Based on the genetic and biochemical data it is tempting to speculate that Argonaute proteins in *C. elegans* (see Figure I-2) may have evolved specifically for a two steps RNAi mechanism. Interestingly, most Argonaute proteins represented in the red clade are lack of the catalytic residues in the PIWI domain, which is required for the cleavage activity of the RISC (Figure II-7). It is worth noting that *A. thaliana* genome also encodes RdRP genes. Therefore, a similar mechanism may be exist in this organism as well.

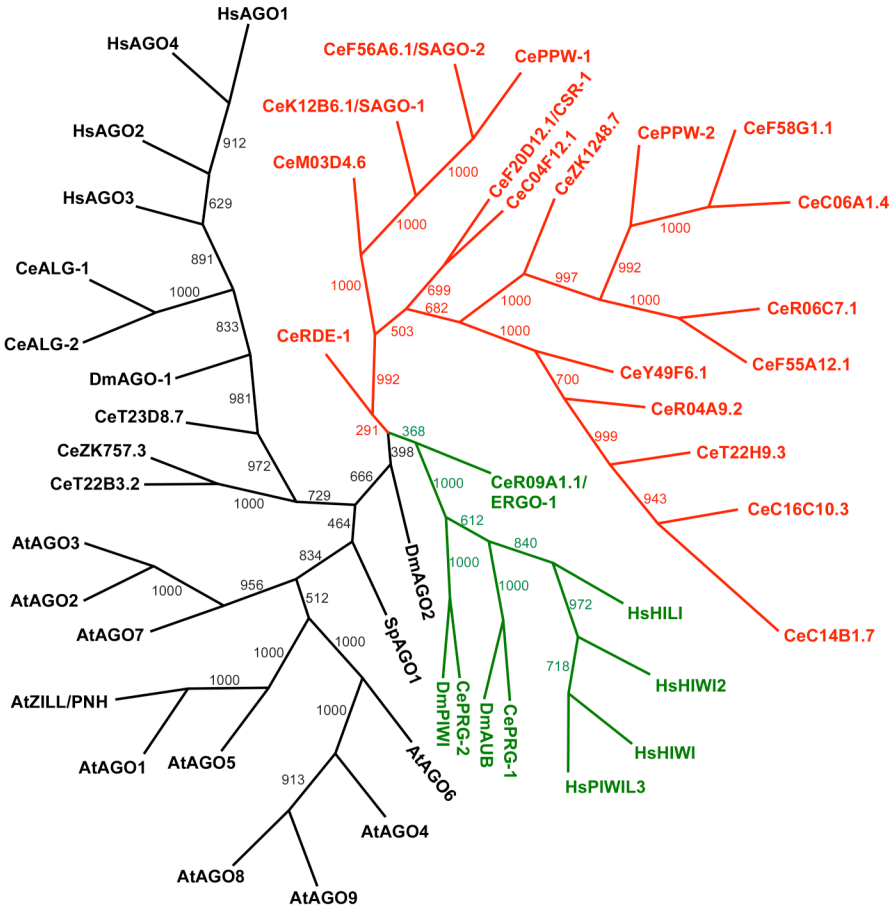


Figure I-2. Phylogenetic tree of representative Argonaute proteins from plants, animals and fungi. The AGO group with representatives in all three kingdoms is indicated in black, the PIWI group common to all metazoans is indicated in Green. An expanded group of *C. elegans* proteins about equally distant from the PIWI and AGO subgroups is shown in red. ClustalW was used for the alignment and the tree was created by bootstrapping and neighbor-joining methods using Phylip® software. Ce *(Caenorhabditis elegans)*, At *(Arabidopsis thaliana),* Hs *(Homo sapiens)*, Sp *(Schizosaccharomyces pombe)*.

If additional Argonaute proteins function in RNAi in *C. elegans*, why have they not been isolated from genetic screens designed to find *rde* mutants? I believe this question can be explained by the following reasons: 1) Genetic screens may not have been saturated to find every single RNAi gene. This does not seem likely because genetic screens from our and other laboratories suggest that RNAi screens are close to saturation. 2) Additional Argonaute members that function in RNAi may be essential for viability; therefore, it would not be possible to isolate these genes in the RNAi screens that were designed to find non-essential *rde* genes. 3) Additional Argonaute members involved in RNAi may be redundant with each other. In this case, inactivation of an Argonaute gene by a mutation would be unlikely to give a noticeable *rde* phenotype.

Another important point to consider is that Argonaute members may be expressed in the different tissues and stages during development. For example, in the case of RdRP proteins, *rrf-1* is required for RNAi in somatic tissues, whereas *ego-1* is required for RNAi in the germline tissue. Therefore it is conceivable that additional Argonautes may be functioning in the different tissues and at the different developmental stages. Unfortunately, expression pattern of Argonaute proteins are not yet known.

In addition to their potential role in RNAi and in the miRNA pathway, remaining Argonaute members may be involved in endogenous small RNA pathway. This class of small RNAs was first discovered in *C. elegans* and excludes miRNAs. Although a number of genes, including Dicer, were shown to be needed for the accumulation of

some small RNA species, biogenesis and function of which are not yet known. Also, we do not know if all the endogenous small RNA species are discovered in *C. elegans*. Exploration of the small RNA world in *C. elegans* continues. Functionally distinct small RNA subclasses can potentially be interacting with distinct Argonaute protein(s).

One exciting discovery in the RNAi field was the involvement of RNAi in chromatin modifications that leads to transcriptional gene silencing (Mochizuki et al., 2002; Volpe et al., 2002; Fukagawa et al., 2004; Pal-Bhadra et al., 2004; Haussecker and Proudfoot, 2005; Kanellopoulou et al., 2005). Transcriptional silencing by RNAi has been extensively studied in *S. pombe,* in which mutations in RNAi pathway genes causes defects in centromeric heterochromatin and chromosome segregation. Several *C. elegans* Argonaute members have been implicated in transcriptional silencing (Grishok et al., 2005), however mutations in these genes do not cause any chromosome segregation defect. It is not known how and how many of Argonaute members are involved in transcriptional silencing. Recently, Duchaine et al. (2006) reported that a putative RNA helicase, DRH-3, is essential for RNAi and proper chromatin segregation. DRH-3 interacts with DCR-1. DRH-1 and DRH-2, the closest paralogs of DRH-3, were found in the RNAi initiation complex together with RDE-1, which is an Argonaute member, DCR-1, and RDE-4 (Tabara et al., 2002). Therefore, it is conceivable that DRH-3 and DCR-1 may function together with an Argonaute member to produce small RNAs that mediate proper chromatin structure and other necessary functions required for proper chromosome segregation.

Finally, Argonaute members may function in transposon silencing and antiviral defense. In fact, mutations in some RNAi genes mobilize transposons in *C. elegans* germline (Ketting et al., 1999; Tabara et al., 1999b; Sijen and Plasterk, 2003), suggesting that RNAi protects the genome against invading nucleic acids. Moreover, recent studies showed that, although there is no known virus naturally infecting *C. elegans*, viruses can replicate in *C. elegans* in RNAi dependent manner. One of these studies indicated that antiviral RNAi is still active in the *rde-1* background, albeit weaker, suggesting that additional Argonaute members are involved in antiviral defense.

Here, I have discussed the likely function of Argonaute proteins in distinct small RNA-mediated pathways in *C. elegans*. As mentioned above, out of total 27 Argonaute members in *C. elegans*, only three of them characterized. My goal in this thesis project was to uncover the function of Argonaute family of genes in *C. elegans,* with particular focus on the Argonautes functioning in the RNAi pathway.

To study the function of Argonaute gene family, we first inactivated the Argonaute members by RNAi, and tested their involvement in RNAi and development. Based on our RNAi experiments, we systematically started generating deletion alleles of Argonaute genes, and eventually obtained at least one deletion allele of each Argonaute gene with the exception of *rde-1,* of which we have more than 20 alleles. Analysis of individual members of Argonaute members showed that different members of this family functioned at the different steps in the exo-RNAi pathway. Furthermore, we found that Argonaute proteins were involved in the endo-RNAi

pathway. In a similar manner to the exo-RNAi pathway, different members of this family functioned at the different steps in the endo-RNAi pathway. Interestingly, although the exo- and endo-RNAi pathways employed distinct Argonaute members at the initiation step, these pathways shared the same Argonaute members at the downstream of RdRP proteins, and functioned redundantly with each other. In addition, we found that at least one Argonaute member was required for both RNAi and proper chromosome segregation, suggesting a link between RNAi and chromatin structure. We also found that a PIWI related Argonaute member, *prg-1,* was required for proper germline development. Finally, analysis of an eight fold Argonaute mutant indicated that Argonaute proteins are also involved in stem cell totipotency in *C. elegans*. In the following chapters, I present and discuss the importance of these findings.

CHAPTER II

ANALYSIS OF THE *C. ELEGANS* ARGONAUTE FAMILY REVEALS THAT DISTINCT ARGONAUTES ACT SEQUENTIALLY DURING RNAi

Contributors to the work presented in Chapter II:

Pedro Batista and Martin Simard from the Mello lab did the experiments in Figure II-1 and II-2, except that Erbay Yigit built the MAGO strain. Pedro Batista did the experiments in Figure II-5A.

Erbay Yigit discovered the RNAi phenotype of *csr-1* in Figure II-3B, and did the experiments in Figure II-4A, II-4D, II-9D, and did the experiments in Figure II-4B and II-4C, except that RDE-1::HA IP was done by Pedro Batista from the Mello lab.

Ka Ming Pang from the Mello lab built the Histone- and tubulin-GFP; *csr-1* strain, and made the microscopic photograph. Pedro Batista from the Mello lab did the experiments in Figure II-3C. Erbay Yigit did the experiments in Figure II-3D and II-3E, except that *tm119* and *tm1200* were out-crossed by Pedro Batista.

Erbay Yigit did the experiments in Figure II-5B and II-5C, except that Northern blot hybridization was done by Pedro Batista from the Mello lab.

Niraj H. Tolia from Leemor Joshua-Tor's lab made Figure II-7. Shohei Mitani generated the deletion alleles of Argonaute genes. Erbay Yigit made Figure II-8.

Erbay Yigit did the experiments in Figure II-9A, II-9B and II-9C, except that *csr-1; pie-1::gfp::h2b* strain was built and microscopic photography was done by Ka Ming Pang.

Erbay Yigit did the experiments in Figure II-10, except that Pedro Batista and Yanxia Bei built the C14 C16 double, and the *ppw-1;* C14 C16 triples.

In table II-1, the following strains were out-crossed by Erbay Yigit: C04F12.1*(tm1637, 4x),* C04F12.1*(tm1637, 4x),* F56A6.1/*sago-2(tm0894, 7x),* D2030.6/*prg-1(tm0872, 7x),* T23D8.7*(tm1163, 4x),* Y110A7A.18*(tm 1120, 5x, tm1065, 0x),* C06A1.4*(tm0887, 5x),* F58G1.1*(tm1019, 7x),* F20D12.1/*csr-1(tm0892, 6x),* M03D4.6*(tm1144, 5x),* C01G5.2/*prg-2(tm1094, 5x),* K12B6.1/*sago-1(tm1195, 5x)*. The following strain was out-crossed by Yanxia Bei: T22H9.3*(tm1332, 0x, tm1186, 5x).* The following strains were out-crossed by Pedro Batista: R04A9.2*(tm1116, 5x),* C14B1.7*(tm1119, 5x),* C16C10.3*(tm1200, 5x).* Chun-Chieh G. Chen alleles from the Mello lab isolated the following *rde-1* alleles: ne4085, ne4086.

In Table II-2, Erbay Yigit made all the strains, except that Pedro Batista from the Mello lab made WM127, WM128, and WM125 .

SUMMARY

Argonaute proteins interact with small RNAs to mediate gene silencing. *C. elegans* contains 27 Argonaute genes, raising the question of what roles these genes play in RNAi and related gene-silencing pathways. Here we describe 31 deletion alleles representing all of the previously uncharacterized Argonaute genes. Analysis of single- and multiple- Argonaute mutant strains reveals functions in several pathways including: (i) chromosome segregation, (ii) fertility, and (iii) at least two separate steps in the RNAi pathway. We show that RDE-1 interacts with trigger-derived sense and antisense RNAs to initiate RNAi, while several other Argonaute proteins interact with amplified siRNAs to mediate downstream silencing. Overexpression of downstream Argonautes enhances silencing, suggesting that these proteins are limiting for RNAi. Interestingly, these Argonaute proteins lack key residues required for mRNA cleavage. Our findings support a two-step model for RNAi, in which functionally and structurally distinct Argonautes act sequentially to direct gene silencing.

INTRODUCTION

The term RNA interference (RNAi) was initially coined to describe a gene-silencing mechanism induced by the experimental introduction of RNA into the nematode *C. elegans* (Rocheleau et al., 1997; Fire et al., 1998). Subsequent work in numerous organisms revealed that key steps in the RNAi pathway are shared by a diverse and truly remarkable set of endogenous gene regulatory mechanisms (for review see Zamore and Haley, 2005). Among others, these include; mechanisms that down-regulate endogenous genes and restrain the expression of selfish or exogenous genetic material; mechanisms that direct transcriptional gene silencing and alter chromatin to promote kinetochore function and chromosome segregation; and, perhaps most remarkable of all, a mechanism in Tetrahymena in which the genomic content of nuclei are compared within a shared cytoplasm prior to chromatin modification and targeted DNA elimination. The term RNAi is often used now to refer to the shared portion of all of these diverse pathways.

During RNAi, members of the Dicer family of proteins process dsRNA to initiate gene silencing (reviewed in Carmell and Hannon, 2004). Dicer can process dsRNAs derived from either exogenous or endogenous sources, generating small interfering (si) RNAs of approximately 21 nucleotides that guide sequence-specific silencing. In addition to processing dsRNA substrates, Dicer copurifies with a large complex that loads the siRNAs into the RNA-induced silencing complex (RISC) (Liu et al., 2003; Chendrimada et al., 2005).

Several studies, including recent elegant structural and functional studies, suggest that members of the Argonaute protein family are key components of RISC (Liu et al., 2004a; Meister et al., 2004; Song et al., 2004). In *C. elegans,* the Argonaute protein RDE-1 is required for silencing in response to experimentally-introduced dsRNA (Tabara et al., 1999b). Argonaute proteins have also been implicated in gene silencing in fungi, plants, protozoans and metazoans including humans (reviewed in Carmell et al., 2002). Most organisms have multiple members of the Argonaute protein family, and several studies suggest that these proteins are specialized to perform distinct functions. For example, two closely related *C. elegans* Argonaute proteins, ALG-1 and ALG-2, are not required for silencing in response to exogenous or transgene-derived dsRNA but are essential for the processing and function of the Dicer-derived, developmentally-important small RNA species termed microRNAs or miRNAs (Grishok et al., 2001).

Biochemical studies indicate that Argonaute proteins interact with Dicer (Hammond et al., 2001; (Tabara et al., 2002; Chendrimada et al., 2005), and that small RNAs generated by Dicer are loaded directly onto Argonaute proteins to form active RISC (Reviewed in Filipowicz, 2005). Once charged with a small RNA, Argonaute proteins are thought to mediate the target-sensing and effector steps in all RNAi-related mechanisms. Two distinct RNA-binding domains in Argonaute proteins, the PAZ and PIWI domains, appear to facilitate interactions with the 3′ and 5′ termini (respectively) of the small single-stranded RNA guides, leaving internal nucleotides available for base-pairing (reviewed in Song and Joshua-Tor, 2006).

Upon target recognition, base-pairing interactions and helix formation are predicted to place the phosphodiester backbone of the target RNA in proximity to the catalytic center of the RNase H-related PIWI domain. In the case of siRNA RISC (siRISC), this interaction is thought to lead directly to target mRNA cleavage. In other RISC complexes, such as the majority of miRISC complexes in animals, helix formation is interrupted by imperfect base pairing, preventing direct cleavage of the target RNA and allowing other forms of regulation, such as inhibition of mRNA translation.

Here we show that Argonaute proteins not only function in several different pathways in *C. elegans* but that, surprisingly, distinct Argonautes function sequentially during RNAi. Our findings support a model in which the RDE-1 protein engages siRNAs derived from Dicing of the trigger dsRNA (primary siRNAs), while a set of several other Argonaute proteins interact with siRNAs that are amplified during the silencing process (secondary siRNAs). Overexpression of the downstream (or secondary) Argonaute proteins causes the accumulation of high levels of siRNAs, and results in animals that are hypersensitive to RNAi. These findings suggest that secondary Argonaute protein levels are limiting for RNAi in *C. elegans*. The secondary Argonaute proteins lack key metal-coordinating residues in their RNase H-related PIWI domains, perhaps explaining why siRISC-mediated cleavage activity has not been detected to date in *C. elegans*. Finally, we provide evidence that endogenous (endo) RNAi pathways also utilize Argonaute proteins at two steps and appear to converge on the same secondary Argonautes that function in the exogenous dsRNA-induced, or exo-RNAi, pathway. In summary, our findings point to diverse

roles for Argonaute proteins in *C. elegans*, and support an Argonaute-relay mechanism involving structurally and functionally distinct Argonautes that act sequentially during the initiation and effector steps of RNAi.

RESULTS

RDE-1 Interacts with Trigger-Derived Single-Stranded RNA

Genetic and biochemical studies place the *C. elegans* Argonaute protein RDE-1 at an upstream step in the RNAi pathway (Grishok et al., 2000). To ask if RDE-1 interacts with siRNAs derived directly from the processing of the exogenous trigger dsRNA, which are present at very low levels (Parrish et al., 2000), we utilized a sensitive assay that employs a 2′-*O*-methylated RNA affinity matrix to trap sequence-specific Argonaute/siRNA-mediated RNA binding events (Hutvagner et al., 2004). When whole animal lysates are exposed to this matrix, siRNA protein complexes are able to interact with the 2′-*O*-methylated RNA through sequence-specific base pairing but are unable to cleave the modified RNA backbone and are therefore retained on the affinity matrix (See Figure II-1A).

We found that, after exposure of animals to dsRNA, the RDE-1 protein exhibits sequence-specific interactions with both the sense and antisense 2′-*O*-methylated RNA matrices. These interactions were specific for the trigger dsRNA sequence to which the animals were exposed (Figure II-1B). This interaction was not detected when animals were exposed to the bacterially expressed dsRNA trigger for 1

hour or less (Figure II-1C), suggesting that internalization and processing of the trigger dsRNA in the animal is required to form an RDE-1 complex capable of sequence-specific binding to the affinity matrix.

Consistent with processing of the original dsRNA trigger into single-stranded guide RNAs, we found that pretreatment of the extracts with the single-stranded ribonucleases RNase A/T1, but not with the dsRNA-specific nuclease RNase V1, dramatically reduced the interaction of RDE-1 with the 2′-*O*-methyl target RNA matrices (Figure II-1D). The sequence-specific retention of RDE-1 on the 2′-*O*-methylated matrices occurred with similar efficiency regardless of whether a target mRNA was expressed in the strain (Figure II-1E, compare lanes 1 and 2).

To further analyze the step at which RDE-1 functions in RNAi, we tested the binding of the RDE-1 protein to the 2′-*O*-Methyl matrices in various RNAi-deficient mutant backgrounds. In the strong loss-of-function *sid-1(ne328)* mutant, which has defects in dsRNA uptake and systemic transport to tissues in the body (Winston et al., 2002), RDE-1 exhibited a markedly reduced interaction with the 2′-*O*-Methyl target sequences (Figure II-1E, lane 4). In contrast, in an RNAi-deficient, multiple-Argonaute mutant (MAGO) strain (described below), and in a strain deficient in *rrf-1* which encodes an RNA-dependent RNA polymerase (RdRP) related protein that is thought to amplify the silencing signal (Smarden et al., 2000; Sijen et al., 2001; Conte and Mello unpublished), the RDE-1 protein was still recruited to the 2′-*O*-Methyl matrices (Figure II-1E, lanes 5 and 6). These findings support the placement of RDE-1 downstream of the systemic transport of dsRNA into tissues, and upstream of the

amplification of the silencing signal.

RDE-1 Does Not Interact with Secondary siRNAs

During RNAi in *C. elegans* the target mRNA appears to serve as a template for the RdRP-dependent amplification of the silencing signal (Sijen et al., 2001). The secondary siRNAs produced through this amplification process are abundant enough to detect by Northern blot analysis and consist of the antisense polarity only (Grishok and Mello Unpublished; Sijen et al., 2001).

To ask whether RDE-1 interacts with these amplified secondary siRNAs we exposed animals to dsRNA and examined RDE-1 immune complexes for associated small RNAs by Northern blot analysis. For this analysis we targeted a GFP-transgenic strain that produces abundant and easily detected secondary siRNAs after exposure to GFP dsRNA. In these studies, neither sense nor antisense siRNAs were detected in RDE-1 immunoprecipitates (Craig Mello). To ask if low levels of the siRNAs corresponding to the amplified region interact with RDE-1, we used sense and antisense 2′-*O*-Methyl matrices complementary to GFP sequences located 5′ of the region targeted by the dsRNA trigger (Regions p2 and p1 in Figure II-2A). After triggering RNAi with dsRNA targeting region p3, we confirmed by Northern blot analysis that secondary siRNAs could be detected with a probe derived from region p2 (Figure II-2B). Although RDE-1 was readily recovered on the 2′-*O*-Methyl matrix corresponding to the trigger, RDE-1 was not recovered on the 2′-*O*-Methyl matrix corresponding to the upstream region, region p2 (Figure II-2C, top panel). When

RNAi was initiated using a trigger dsRNA targeting region p2, we found that RDE-1 was readily recovered on the region-p2-specific affinity matrix (Figure II-2C, bottom panel), demonstrating that the p2 matrix is functional. These data suggest that the RDE-1 protein only interacts with the very low abundance primary siRNAs, and not with the much more abundant secondary siRNAs derived from the amplification process.

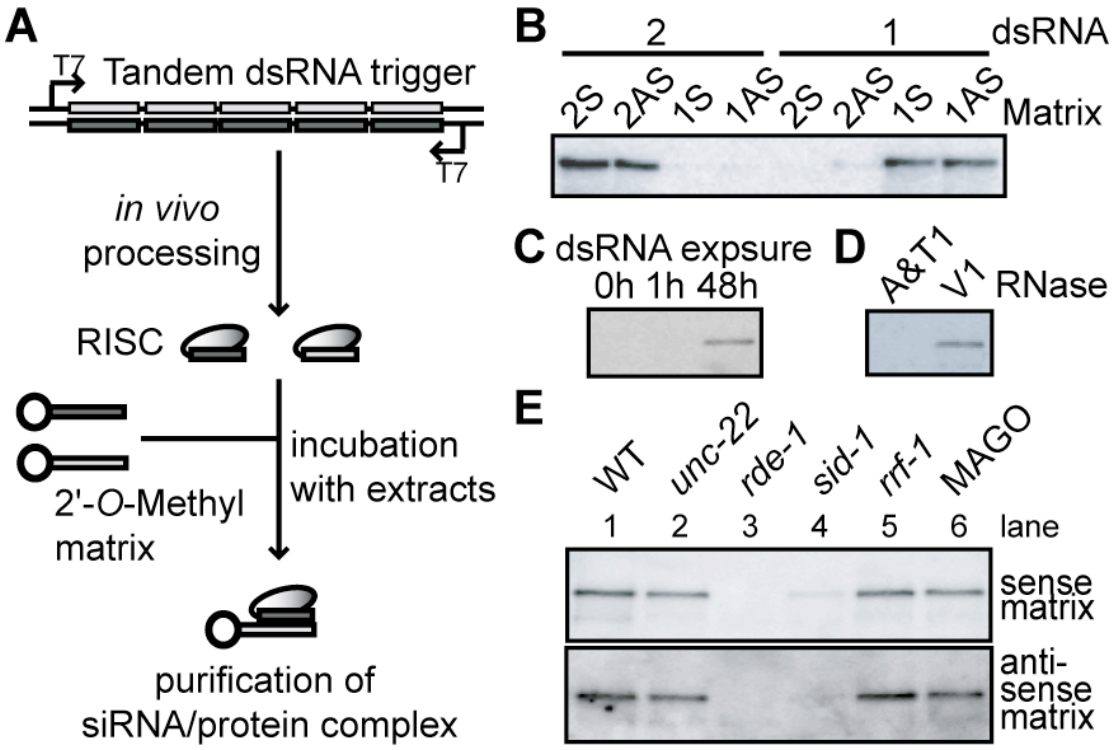


Figure II-1(A,B,C,D,E) Sequence specificity and genetics of RDE-1/RNA affinity matrix binding. (A) Schematic representation of the strategy used to recover proteins interacting with low-abundance (primary) siRNAs. (B-E) Western blot analysis to detect HA::RDE-1 (B-D) or endogenous RDE-1 protein (E) in lysates prepared from worms treated as diagramed in (A), using non-overlapping 40 nt segments of GFP as dsRNA triggers. (B) RDE-1 exhibits sequence specific interactions with the 2′-*O*-Methyl matrices. (C) The association of RDE-1 with trigger-derived RNA requires prolonged exposure of worms to the dsRNA-expressing E. coli. Animals were either not exposed to *E. coli*-expressing dsRNA (0h), or were allowed to feed on the *E. coli* for 1 hour or 48 hours as indicated. (D) The RDE-1 interaction with the 2′-*O*-methyl matrix depends on single-stranded RNA. Prior to exposure to the affinity matrix, worm lysates were pretreated with either the dsRNA-specific nuclease RNase V1 (V1), or with the single-stranded RNA-specific nucleases RNase A and RNase T1 (A&T1). Under these conditions, unmodified control RNAs were totally degraded while the 2′-*O*-Methyl modified oligonucleotides were unaffected (Sproat et al., 1989; Tabara et al., 2002). (E) Genetic analysis of RDE-1 affinity-matrix binding. dsRNA triggers and 2′-*O*-Methyl affinity matrices were prepared using a 40 nt region of the *unc-22* gene that is deleted in *unc-22(st528),* a functionally-wild type allele that harbors an in-frame deletion. The RNAi-deficient mutant strains analyzed are *unc-22(st528), rde-1(ne300), sid-1(ne328),* and *rrf-1(pk1417)*.

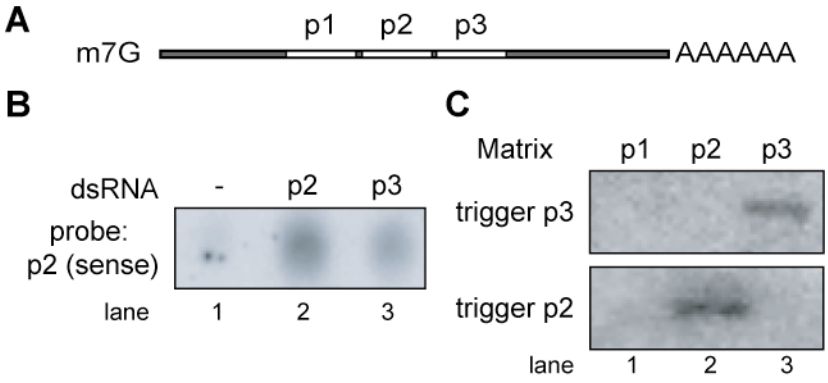


Figure II-2(A,B,C). RDE-1 does not interact with secondary siRNAs. (A) Schematic representation of the GFP transcript, showing the relative positions of targeted regions. The dsRNA triggers and 2′-*O*-Methyl affinity matrices were prepared as described in Figure II-1A, using sequences corresponding to the three 40 nt regions of GFP indicated in the diagram. Lysates prepared from GFP-transgenic animals exposed to the dsRNA triggers (p2 and p3) were used for (B) Northern blot analysis of small RNA species, and (C) Western blot analysis for RDE-1 protein after exposure to affinity matrices (as indicated). In (B) the RNA probe used was derived from region p2. Note that small RNAs corresponding to region p2 are detected even when region p3 is used as the trigger.

Genetic Analysis of Argonaute Mutants in *C. elegans*

Since RDE-1 does not appear to interact with secondary siRNAs, we reasoned that one or more of the numerous RDE-1 homologs in the *C. elegans* genome might play this downstream role in the RNAi pathway. The *C. elegans* genome contains a set of 27 annotated Argonaute-related genes (Figure I-2). To begin to assign functions to these genes we first used RNAi to target each gene for silencing. In addition, we generated deletion alleles for all of these genes, except for *rde-1* and *alg-2*, for which alleles were already available (Figure II-8).

The two most highly conserved members of the *C. elegans* Argonaute family, *alg-1* and *alg-2*, have overlapping functions in the miRNA pathway and are essential for development (Grishok et al., 2001). Our analysis revealed that two additional Argonautes, F20D12.1 which we have renamed *csr-1,* and *prg-1*, are also essential for development. Depletion of *csr-1* by RNAi resulted in penetrant embryonic lethality with defects in the organization of chromosomes at metaphase of each early embryonic cell cycle, and the formation of anaphase DNA bridges (Figure II-3B). Most *csr-1* deletion homozygotes are sterile but some hermaphrodites produce a few embryos with chromosome segregation defects identical to those observed in *csr-1(RNAi)* embryos. The *csr-1* mutant is also partially deficient in germline RNAi (Figures II-9A and II-9B). Thus *csr-1* defines a new gene class, *csr,* (pronounced 'caesar') whose members exhibit loss-of-function phenotypes with defects in both chromosome segregation and RNAi. A mutation in *prg-1(tm872)*, a member of the metazoan-specific Piwi subfamily of Argonaute genes, exhibited a reduced brood size

and a temperature-sensitive sterile phenotype (Figure II-9C), consistent with previous findings linking *prg-1* to germ line maintenance (Robert et al., 2005).

A single mutant, R09A1.1, which we have renamed *ergo-1* for endogenous RNAi deficient Argonaute mutant, exhibited an enhanced sensitivity to RNAi (Figure II-3C). This enhanced RNAi phenotype was partially rescued by the introduction of an *ergo-1* wild type transgene, supporting the idea that the enhanced RNAi phenotype is due to a loss of *ergo-1* activity (Figure II-3C). As implied by its name, *ergo-1* activity is required for an endogenous RNAi pathway (See Below).

Multiple Argonautes Contribute Incrementally to RNAi

We assayed each viable Argonaute mutant allele for sensitivity to RNAi. We also used a sequential RNAi assay to search for potential involvement of each Argonaute in RNAi (Figure II-9D). These assays defined *ppw-1* (also observed in Tijsterman et al., 2002) and F58G1.1 as partially deficient in RNAi. These two genes represent divergent members of an expanded clade of Argonautes present in *C. elegans* (Figure I-2, red branches). To more carefully examine the activities of the other members of this clade we analyzed mutant alleles of these genes using a more sensitive microinjection assay optimized for detecting deficiencies in RNAi.

In this more sensitive assay we targeted the muscle-specific *unc-22* gene and set the dose of dsRNA for microinjection at 20 µg/ml which is sufficient to induce approximately 50% paralyzed and 50% motile twitching animals after injection into wild type animals. These assays revealed that while two mutants, *ppw-1* and

F58G1.1, were partially deficient in germline RNAi (Figure II-3D, blue bars), four mutants, K12B6.1, F56A6.1, C04F12.1 and F58G1.1, were partially deficient in RNAi targeting the somatic gene *unc-22* (Figure II-3D, green and black bars). For reasons described below, we have renamed K12B6.1 and F56A6.1 *sago-1* and *sago-2,* respectively.

We next examined the consequences of creating a multiple mutant including alleles of four genes implicated in RNAi by their single-mutant phenotypes (*ppw-1*, *sago-1*, *sago-2* and F58G1.1). In this multiple-Argonaute mutant (MAGO) strain we also included alleles of two additional genes, C06A1.4, a close homolog of F58G1.1, and M03D4.5 a close homolog of *sago-2* and *ppw-1*. Both C06A1.4 and M03D4.5 are now predicted to be pseudogenes and, perhaps consistent with this designation, their inclusion in multiple mutant strains did not appear to result in any enhancement of the RNAi defect in our assays (Figure II-10). The MAGO strain, comprised of the *ppw-1(tm914)*, *sago-1(tm1195), sago-2(tm894),* F58G1.1*(tm1019)*, C06A1.4*(tm887)*, and M03D4.6*(tm1144)* alleles, was resistant to both germline and somatic RNAi (Figure II-3D). This strain was still weakly sensitive to RNAi in response to injected dsRNA at concentrations of 1mg/ml (Figure II-3E). Nevertheless, this strain was strongly deficient in RNAi by feeding, and was suitable for the functional studies described below. The MAGO strain also exhibits a temperature-dependent reduction in fertility when cultured at 25°C, but has no other easily discernable phenotypes.

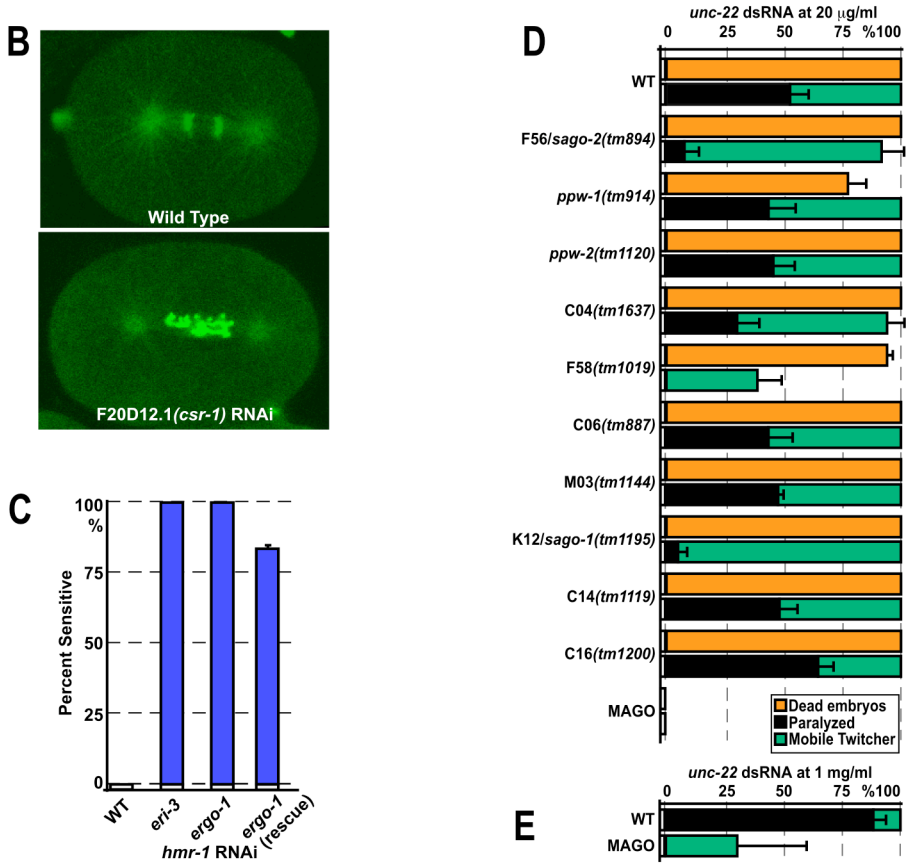


Figure II-3(B,C,D,E). Argonaute genes are required for RNAi and development. (B) *csr-1*/F20D12.1 is required for chromosome segregation. Histone- and tubulin-GFP fluorescence images of wild-type and *csr-1*/F20D12.1(RNAi) embryos at anaphase of the first cell division. (C) ergo-1(tm1860) exhibits enhanced RNAi. The broods of between 7 and 10 animals (~80 embryos per animal) were scored per genotype and the percent of embryos sensitive to RNAi targeting the hmr-1 E-cadherin gene is shown. Expression of wild-type ERGO-1 from a transgene (ergo-1 rescue) partially restores resistance to RNAi. Failure to see a more robust rescue may reflect the poor expression of the ergo-1(+) high-copy number transgene in the germ line. (D-E) Multiple red-clade AGOs contribute to RNAi. For ger_line RNAi, 9 to 10 animals were exposed to pos-1(RNAi) by feeding and the percent pos-1 embryonic lethal embryos produced is shown (Orange bars). For somatic RNAi, between 4 and 10 animals were injected with 20µg/ml unc-22 dsRNA (D), or with 1mg/ml unc-22 dsRNA (E), and the percent paralyzed progeny (Black bars) or twitching but motile progeny (Green bars) are shown. The error bars (C-E) represent the 95% confidence interval.

Argonautes required for RNAi exhibit qualitatively distinct activities

To compare the activities of Argonaute genes we performed rescue assays in which we used the potent muscle-specific *myo-3* promoter to over-express individual Argonautes in the muscles of the *rde-1* and MAGO strains. Consistent with the idea that RDE-1 and the MAGO components are not interchangeable, we found that overexpression of RDE-1 rescued the *rde-1* mutant, but failed to rescue RNAi in the MAGO strain (Figure II-4A). Conversely, overexpression of wild type or GFP-tagged alleles of the MAGO components, *sago-1, sago-2* and *ppw-1*, strongly rescued the MAGO strain but failed to rescue the RNAi defect of the *rde-1* mutant strain (Figure II-4A). These findings suggest that *sago-1, sago-2* and *ppw-1* encode functionally interchangeable proteins whose overexpression can compensate for the collective RNAi defect of the MAGO strain. RDE-1, on the other hand, appears to have a qualitatively distinct activity. We also attempted to rescue the *rde-1* and MAGO strains using other AGO family members. The microRNA-Argonaute *alg-1*, as well as *prg-1* and *csr-1*, failed to rescue either *rde-1* or the MAGO strain (Figure II-4A).

SAGO-1 and SAGO-2 Interact with Secondary siRNAs

The findings that at least three Argonautes, SAGO-1, SAGO-2 and PPW-1, appear to differ functionally from RDE-1 in our muscle-specific rescue assays prompted us to ask whether these Argonautes might interact with secondary siRNAs. To address this question, Northern blot analysis was performed to detect small RNAs associated with

GFP-tagged SAGO-1 and SAGO-2. Indeed, secondary siRNAs derived both from within the trigger region (Figure II-4B, probe p2) and from the region upstream of the trigger dsRNA (Figure II-4B, probe p1) were detected in GFP-immune complexes recovered from the corresponding MAGO-rescued strains (Figure II-4C, lanes 9 and 10). We did not detect siRNAs using a probe located just downstream (3′) of the trigger dsRNA (probe p3 in Figure II-4B), and we did not detect sense siRNAs associated with these immune complexes using probes from any of the three regions (p1, p2 or p3).

Interestingly, we noticed that strains over-expressing GFP::SAGO-1 exhibited an enhanced level of RNAi overall. For example, 100% (n=76), of the myo-3p::GFP::SAGO-1 transgenic animals exhibited a paralyzed *unc-22* RNAi phenotype, whereas wild type animals failed to exhibit paralyzed twitchers and were instead strong, but still motile, twitchers after 36 hours of exposure to *unc-22* RNAi (n=54).

Consistent with the increased level of silencing in these strains, we found that the levels of secondary siRNAs were substantially increased relative to wild type levels in strains over-expressing SAGO-1 (Figure II-4D, compare lane 3 to lanes 5). The over-accumulation of siRNAs was less evident in the GFP::SAGO-2 transgenic strain (Figure II-4D, compare lanes 3 and 6). This appears to reflect relatively weaker expression from the GFP::SAGO-2 transgene (see Western Blot, lower panel in Figure II-4C). As expected from previous studies (Grishok and Mello unpublished, Sijen et al., 2001), only siRNAs of the antisense polarity were detected in these assays.

Taken together the findings; (i) that mutations in *sago-1* and *sago-2* lead to reduced RNAi activity, (ii) that these mutations appear to disrupt RNAi downstream of the interaction of RDE-1 with primary siRNAs, (iii) that overexpression leads to increased RNAi activity and to the rescue of secondary siRNA levels, and (iv) that the rescuing proteins co-immunoprecipitate with secondary siRNAs, strongly support the notion that at least these two Argonautes (and likely others) interact with and stabilize secondary siRNAs to direct silencing during RNAi.

Consistent with the idea that RDE-1 is functionally distinct from these Argonautes we found that, although HA::RDE-1 fully rescues the RNAi defect of *rde-1(ne300),* its overexpression does not lead to any observable increase in secondary siRNA levels (Figure II-4D, lane 7), and does not result in any detectable interaction between HA::RDE-1 and secondary siRNAs (Figure II-4C, lane 3). Finally, consistent with the placement of SAGO-1 and SAGO-2 either at the same step, or downstream of, RdRP-dependent secondary-siRNA production, we found that overexpression of SAGO-2 failed to rescue the RNAi-deficient phenotype of an *rrf-1/*RdRP-mutant strain, and as expected also failed to rescue secondary siRNA accumulation in the *rrf-1* mutant background (Figure II-4D, top panel, lane 8).

Based on the strong genetic and physical criteria linking *sago-1* and *sago-2* to secondary siRNAs, we propose to define this gene class as *sago* (pronounced *say-go*), for synthetic secondary-siRNA defective Argonaute mutants. This class of Argonautes is likely to include *ppw-1,* a close homolog of *sago-1* and *sago-2,* as well as other members of the expanded clade of Argonaute genes in *C. elegans* (See

Figure I-2 and Discussion).

Rescue assays distinguish *rde-1* and the MAGO activities:

	Mutant Strain			
Transgene	***rde-1(ne300)***	**(n)**	**MAGO**	**(n)**
none	**-**	**(72)**	**-**	**(82)**
myo-3::GFP	**-**	**(66)**	**-**	**(80)**
myo-3::RDE-1::HA	**+**	**(58)**	**-**	**(78)**
myo-3::GFP::SAGO-1	**-**	**(61)**	**+**	**(49)**
myo-3::GFP::SAGO-2	**-**	**(76)**	**+**	**(75)**
myo-3::PPW-1	**-**	**(46)**	**+**	**(73)**
myo-3::GFP::ALG-1	**-**	**(63)**	**-**	**(69)**
myo-3::PRG-1	**-**	**(50)**	**-**	**(63)**
myo-3::CSR-1	**-**	**(77)**	**-**	**(47)**

Figure II-4A. GFP::SAGO-1 and GFP::SAGO-2 rescue the MAGO strain. (A) Rescue of the RNAi deficient phenotypes of the *rde-1* and MAGO strains via *myo-3*-promoter-driven expression of Argonaute genes (as indicated). Transgenic animals were cultured on *unc-22* dsRNA-expressing bacteria. Animals were scored for the *unc-22(RNAi)* phenotype. The (+) indicates Unc (RNAi-responsive) animals while (-) indicates NonUnc (RNAi-deficient) animals. One hundred percent of the animals scored (n) showed the indicated phenotype.

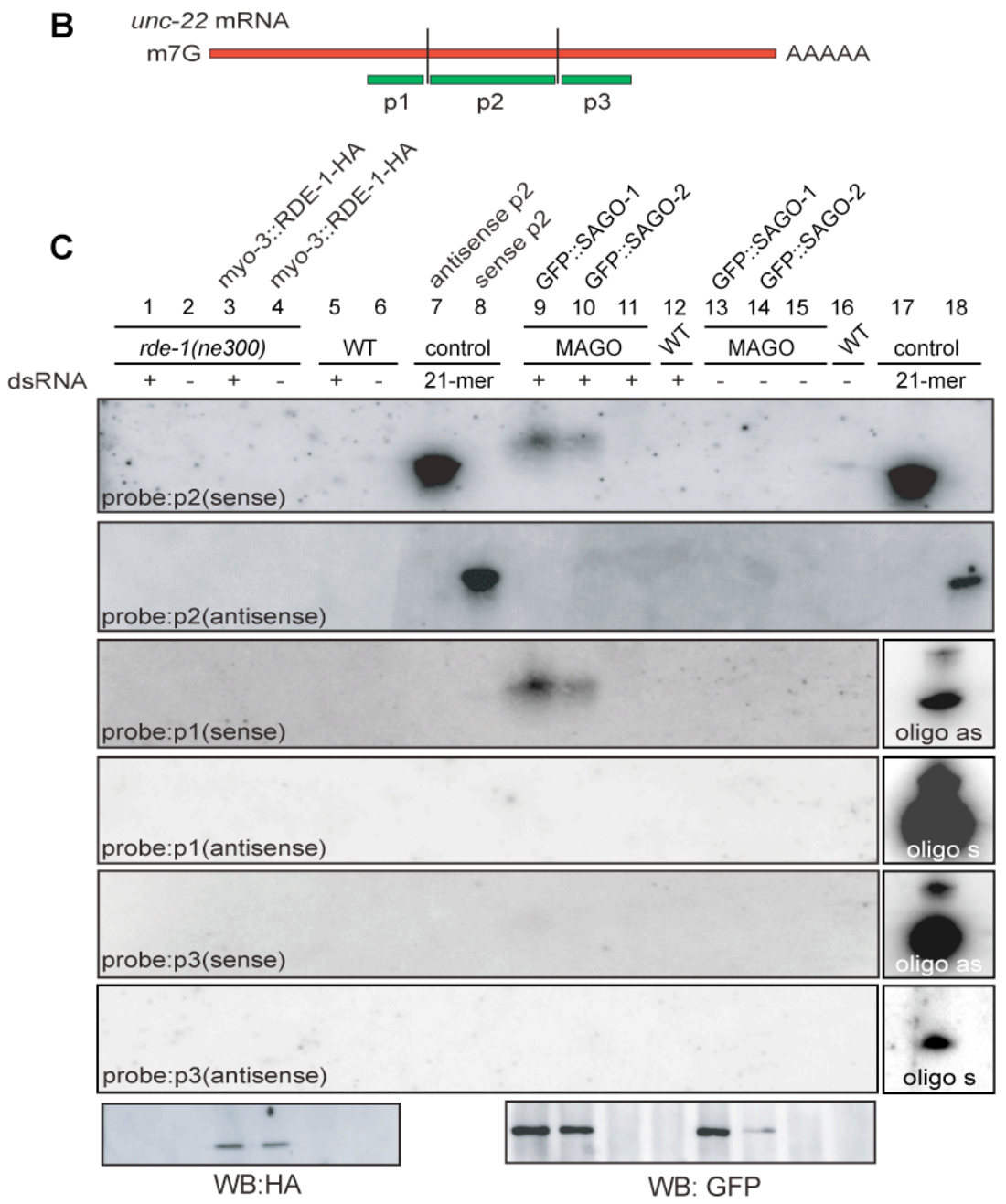


Figure II-4 (B,C). GFP::SAGO-1 and GFP::SAGO-2 interact with secondary siRNAs. (B) Schematic diagram indicating the regions within the *unc-22* gene used to prepare RNA probes. (C-D) Northern blot analysis of small RNAs in (C) GFP::AGO immune complexes and (D) total lysates. The strains and probes are as indicated; the dsRNA trigger was derived from region p2. The lower panel in (C) is a Western blot probed with a GFP-specific monoclonal antibody.

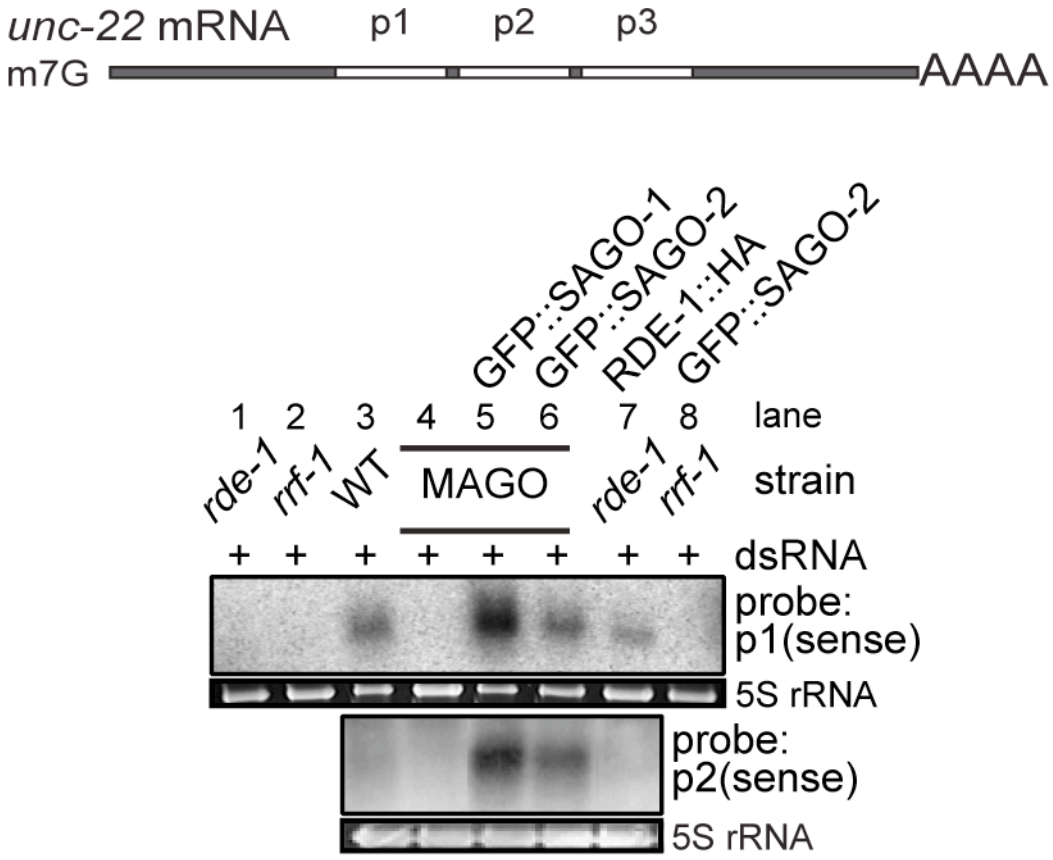


Figure II-4D. siRNAs overaccumulate in overexpressing GFP::SAGO-1 and GFP::SAGO-2 strains. In (D) the RNAi-deficient alleles analyzed are *rde-1(ne300),* and *rrf-1(pk1417)*, the 5S ribosomal RNA is shown as a loading control. In the upper panel of (D) the p1-specific probe is a Starfire™ probe comprised of a 40nt segment of region p1.

An Endogenous Small RNA Pathway Requires ERGO-1 and the SAGO Proteins

The finding that increasing the levels of the SAGO proteins increases RNAi activity suggests that these Argonautes are present in limited supply. In *C. elegans,* silencing in response to exogenous, experimentally-delivered, dsRNA (exo-RNAi) is increased when certain endogenous-RNAi (endo-RNAi) pathways are compromised by mutation (Duchaine et al., 2006). These findings suggest that the exo-RNAi and endo-RNAi pathways may converge on, and compete for, an unknown limiting factor shared by both pathways. Because the SAGO proteins are limiting for exo-RNAi we wondered if they might encode components of this shared limiting activity. Consistent with this idea, we found that siRNAs derived from an endogenous *C. elegans* gene, K02E2.6, and from an apparently non-coding X-chromosome cluster are both reduced in the MAGO strain (Figure II-5A and B).

Expression of GFP::SAGO-1 and GFP::SAGO-2 in the muscles of MAGO animals rescued the accumulation of the X-cluster and K02E2.6 endo-siRNA species (Figure II-5B). As with the secondary exo-siRNAs (see Figure II-4C), these endo-siRNA species accumulate to levels that are higher than wild type levels in strains over-expressing these Argonautes (Figure II-5B). Note that the level of endo-siRNA accumulation correlates with the level of SAGO-protein expression as measured in the Western blot (Figure II-5B, lower panel). Like the secondary exo-siRNAs, we found that the endo-siRNAs also co-immunoprecipitate with GFP-SAGO-1 and GFP-SAGO-2 (Figure II-5C).

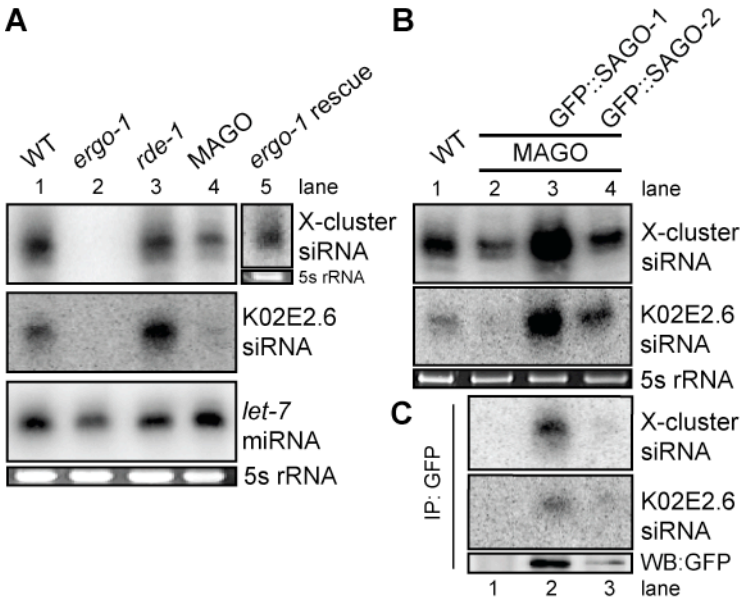


Figure II-5(A,B,C). *ergo-1(tm1860)* and the MAGO strain are deficient in endo-siRNA expression. (A, B) Northern Blot analysis of endogenous small RNAs in wild type and various mutant and transgenic rescued strains, as indicated. The 5S ribosomal RNA blots are provided as loading controls. In (A) the RNAi-deficient alleles analyzed are *rde-1(ne300),* and *ergo-1(tm1860)*. (C) IP-Northern blot analysis (top two panels), and IP-Western blot analysis (Bottom panel) of GFP-immune complexes recovered from rescuing GFP::SAGO-1 and GFP::SAGO-2 transgenic strains. Probes for the K02E2.6 and the X-cluster endo-siRNAs, and for the *let-7* miRNA are described in (Duchaine et al., 2006).

Interestingly, endo-siRNA levels were even more dramatically reduced in *ergo-1(tm1860)* Argonaute mutant animals (Figure II-5A, lane 2), in which exo-RNAi is enhanced (see Figure II-3C, and Discussion). Furthermore, consistent with competition between the ERGO-1 and RDE-1 pathways, the levels of K02E2.5 endo-siRNAs were increased in animals deficient for *rde-1* (Figure II-5A, See Discussion). There were no significant changes in the level of *let-7* miRNA expression in these strains (Figure II-5A). Expression of a partially rescuing *ergo-1(+)* transgene in the *ergo-1(tm1860)* mutant strain partially restored the expression of the X-cluster-derived endogenous siRNA species (Figure 5A, right panel).

DISCUSSION

Through a combination of forward genetics, reverse genetics and proteomics we have arrived at a model for RNAi (Figure II-6) that explains how multiple small RNA-mediated silencing pathways interact with each other and converge on shared components of the RNAi-machinery. This model explains how RNA-silencing pathways can achieve both specificity and amplification. According to this model, upon exposure to *E. coli* expressing dsRNA, intestinal cells take up and disseminate small quantities of dsRNA to other tissues via a systemic mechanism that depends in part on the SID-1 channel protein (Winston et al., 2002; Feinberg and Hunter, 2003). The dsRNA is then processed by a Dicer complex that includes the dsRNA binding protein RDE-4 and the Argonaute protein RDE-1 (Tabara et al., 2002). A scanning

phase of RNAi follows, in which RDE-1::primary siRNA complexes search for target mRNA sequences. RDE-1 then recruits RdRP, perhaps indirectly through an initial round of target mRNA cleavage. This initial targeting by RDE-1 is sufficient to initiate amplification but insufficient, by itself, to cause silencing (due to the low levels of the primary siRNAs).

The target mRNA is proposed to act as a template for the primer-independent synthesis of new dsRNA (see also Duchaine et al., 2006). RdRPs related to those involved in RNAi have been shown to catalyze primer-independent RNA synthesis (Schiebel et al., 1993b; Makeyev and Bamford, 2002; Tang et al., 2003). Recruitment of RdRP directly to the target mRNA, without the need for priming, would permit new dsRNA synthesis without consuming the original trigger-derived siRNAs. This process would allow each of the rare RDE-1/siRNA complexes to be recycled to target multiple transcripts and would thus permit multiple rounds of RdRP-dependent amplification. According to this model, a second Dicer complex would then act to process the RdRP products and to load the amplified secondary siRNAs onto members of a group of partially redundant "secondary" Argonautes that include SAGO-1, SAGO-2 and likely other related proteins.

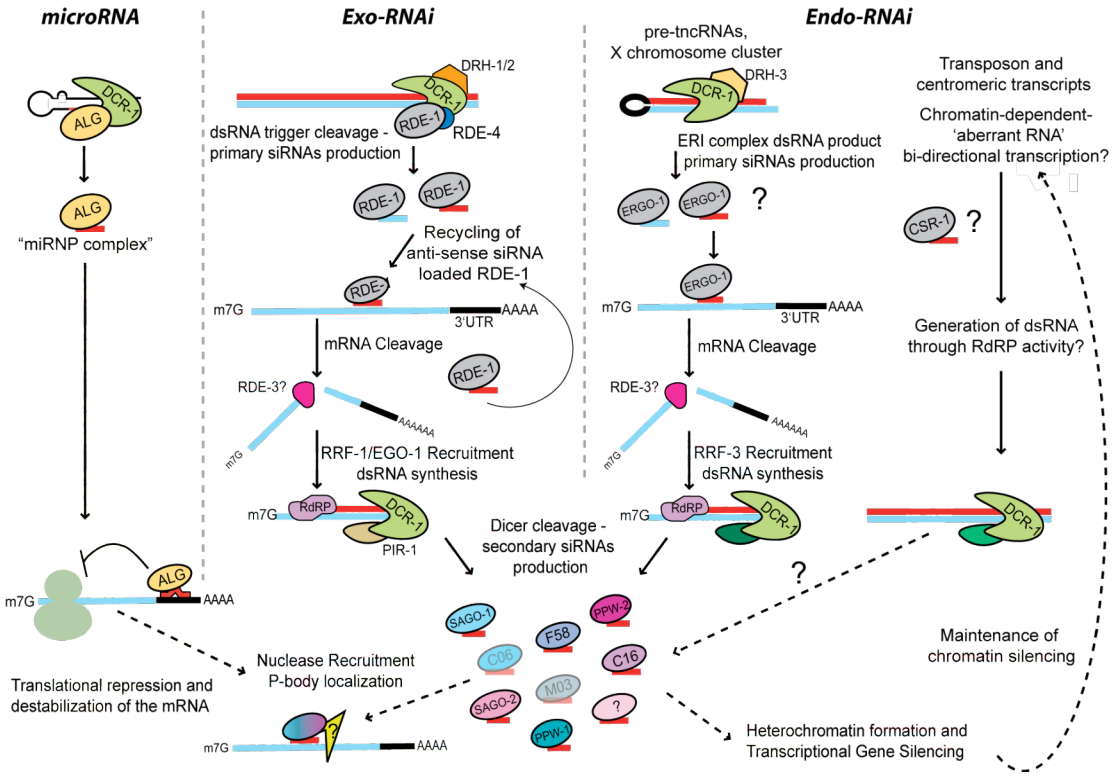


Figure II-6. Model. Schematic representations of RNAi-related pathways in C. elegans. Exo- and Endo- RNAi pathways are proposed to involve sequential rounds of Argonaute action involving primary-siRNA containing Argonaute complexes (Grey ovals), and secondary-siRNA containing Argonaute complexes (Colored ovals). The miRNA pathway is proposed to involve a single Argonaute-mediated step. Distinct DCR-1 complexes are proposed to recognize the dsRNA substrates illustrated in the diagram. Evidence exists for several of these complexes, including the ALG, RDE-1, ERI and PIR-1 containing DCR complexes (Tabara et al., 2002; Duchaine et al., 2006). After primary-siRNA-directed cleavage, a protein complex potentially containing RDE-3 (Chen et al., 2005, pink object) is proposed to mark the 3′ end of the 5′ cleavage product and to recruit RdRP. The question marks and dashed lines indicate speculative elements in the model.

The RDE-1 and the SAGO proteins exhibit structural differences that may help explain their distinct biological activities. An alignment of members of the Argonaute protein family reveals that most members of this family, including RDE-1 and ERGO-1, exhibit conservation of key metal-coordinating residues in the RNase H-related PIWI domain (D,D, and H residues in Figure II-7). SAGO-1, SAGO-2 and several other members of the expanded *C. elegans* Argonaute clade (Red branches in Figure I-2),

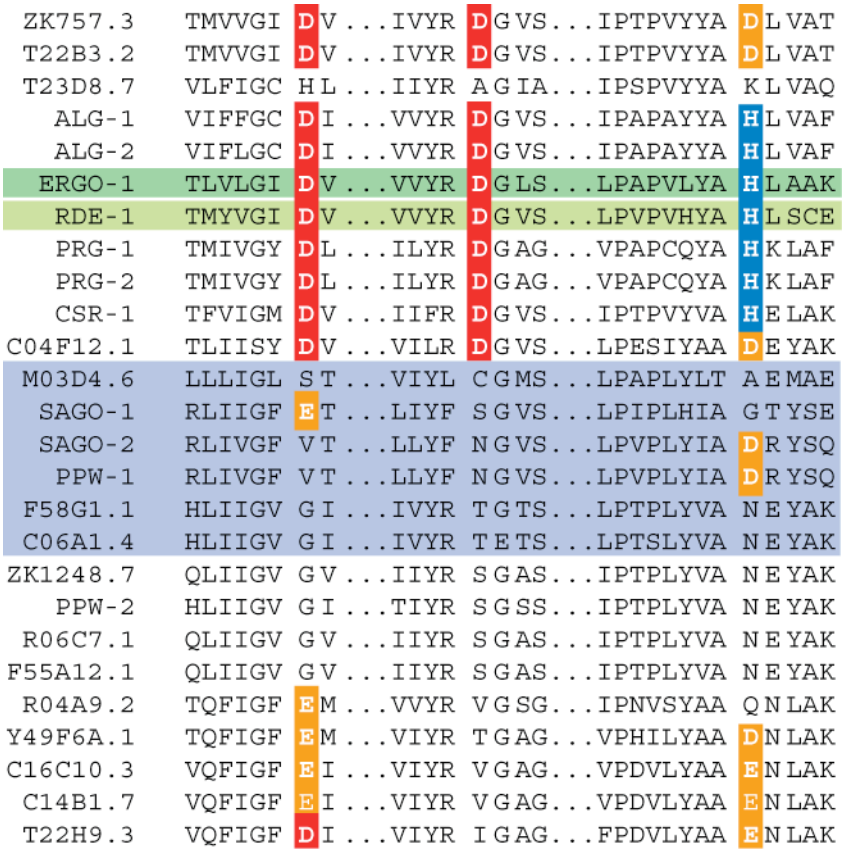


Figure II-7. Secondary Argonautes lack key catalytic residues. Alignment of *C. elegans* Argonaute proteins in three regions with similarity to the catalytic center of RNase H. Within these regions two key aspartic acid residues (highlighted in red) and a histidine residue (highlighted in dark blue) coordinate a magnesium ion at the catalytic center of the RNase H enzyme. Substitutions compatible with metal binding are indicated in brown. The RDE-1 and ERGO-1 amino-acid sequences are highlighted in shades of green, while those of the MAGO strain components are highlighted in blue.

including the other components of the RNAi-deficient MAGO strain (Figure II-7, blue shaded sequences), conspicuously lack these residues. Thus, while RDE-1 might be expected to retain catalytic activity, the SAGO proteins would very likely require accessory factors to mediate target mRNA turnover (Model, Figure II-6).

The model for RNAi proposed above provides two opportunities for amplification. First the RDE-1/siRNA complex, although low in abundance, is proposed to work repeatedly to generate multiple templates for RdRP. Second, Dicer is proposed to process each RdRP-derived dsRNA product into several secondary siRNAs. Acting together, these two steps [(i) repeated mRNA targeting by the RDE-1/primary-siRNA complex, followed by (ii) RdRP-dependent dsRNA synthesis, and Dicer processing] could generate potentially thousands of secondary siRNA for each original primary siRNA.

While amplification of the silencing signal would have obvious benefits for suppressing viral gene expression, this is balanced against a danger of amplifying off-target silencing. Conceivably, any off-target cleavage events mediated by the primary-siRNA/RDE-1 complex could lead to a chain reaction of silencing with obvious deleterious consequences. The model for silencing proposed here could safeguard against off-target amplification in three ways. First, since RDE-1 does not need to silence the target mRNA by itself, the target-scanning step mediated by RDE-1 can afford to incorporate a very high degree of selectivity. Second, since the downstream Argonautes lack catalytic residues required for mRNA cleavage, they may be unable to generate cleaved substrates for further amplification. And finally,

the downstream Argonaute proteins are present in limited supply, and thus provide limited capacity to support multiple simultaneous silencing reactions.

Perhaps consistent with the idea that safeguards exist to prevent the initiation of off-target silencing, the injection of concentrated dsRNA, or even the promoter-driven expression of dsRNA, cannot bypass the requirement for *rrf-1,* the RdRP required for amplification. Furthermore, although, we have shown that RDE-1 still appears to interact with primary siRNAs in *rrf-1* mutants, neither the primary nor the secondary siRNAs are detectable in *rrf-1* mutants, even in the presence of abundant promoter-driven dsRNA (Sijen et al., 2001; Conte and Mello, unpublished). These results suggest that the processing of trigger dsRNA and loading into the RDE-1 complex may be inherently inefficient. Alternatively, mechanisms may exist that function to limit the formation of the RDE-1/primary-siRNA complex, even in the presence of large quantities of trigger dsRNA. Such mechanisms could be important to limit the pioneering round of target recognition by RDE-1 and thus to minimize the risk of amplifying off-target silencing reactions.

Intersecting RNAi Pathways in *C. elegans*

Several of our findings suggest that ERGO-1 may function in the endo-RNAi pathway in a manner analogous to the role of RDE-1 in the exo-RNAi pathway. Furthermore, our findings support the hypothesis that the ERGO-1 and RDE-1 pathways converge on the SAGO proteins (Figure II-6). Consistent with this model, the MAGO strain, which includes lesions in *sago-1* and *sago-2,* exhibits defects in

both secondary siRNA accumulation and in the accumulation of endogenous siRNA species. The convergence of several pathways on members of the secondary group of Argonautes may provide selective pressure for the maintenance of this amplified gene family.

ERGO-1 is required for endo-siRNA accumulation, and lesions in *ergo-1* enhance exo-RNAi. These findings support the placement of ERGO-1 upstream of the convergence between the endo- and exo-RNAi pathways in the model (Figure II-6). Accordingly, while mutations in *ergo-1* prevent the accumulation of endo-siRNAs, they do not interfere with exo-siRNA production. Instead, by eliminating an abundant endo-siRNA species that would otherwise compete with exo-siRNAs for loading onto the limiting SAGO proteins, lesions in *ergo-1* enhance the exo-RNAi pathway (Figure II-6).

The ERI proteins, and the RdRP RRF-3, may function along with ERGO-1 in the production of endo-siRNAs (Duchaine et al., 2006). ERGO-1 has a potentially intact catalytic domain, and in this respect is structurally similar to RDE-1 (Figure II-7). Conceivably, low levels of dsRNA synthesis from endogenous loci could provide precursors for the production of primary endo-siRNAs that are loaded onto ERGO-1. ERGO-1, through RNA-scanning, target-cleavage, and RRF-3-recruitment, may then direct the accumulation of abundant secondary endo-siRNA species that interact with, and compete for, the SAGO proteins.

Argonautes and Transcriptional Gene Silencing

Transcriptional silencing appears to be an important mode of RNAi-directed silencing in *C. elegans*. While this has been best studied in Fungi (reviewed in Grewal and Rice, 2004), elements of a transcriptional silencing pathway exist in a variety of organisms (Reviewed in Wassenegger, 2005). In *C. elegans*, transgene silencing and cosuppression, which are maintained in part by chromatin-related silencing pathways (Grishok et al., 2005; Robert et al., 2005), require a subset of the genes implicated in exogenous-dsRNA-induced RNAi.

Here we have shown that CSR-1, an essential Argonaute protein, is required, directly or indirectly, for chromosome segregation in *C. elegans*. In addition CSR-1 appears to contribute to germline RNAi. Expression of CSR-1 in the muscle failed to rescue the secondary-Argonaute defect in our assays, raising the possibility that CSR-1 functions at yet another step in the RNAi pathway, or requires specific co-factors that are not present in muscle cells. One interesting possibility is that germline RNAi has a strong transcriptional silencing component and that CSR-1 plays a role in mediating chromatin effects important for both germline RNAi and chromosome segregation (Model, Figure II-6).

An emerging theme from this and several other recent studies is the remarkable importance of Argonaute proteins for germline maintenance and function. In *C. elegans* at least 4 distinct groups of Argonaute genes are required for fertility. These include *csr-1*, *prg-1*/*prg-2, alg-1*/*alg-2* and the multiple Argonaute mutant strain (MAGO) that includes *sago-1* and *sago-2*. In the mouse, all three members of

the Piwi/*prg* Argonaute family, Miwi (Deng and Lin, 2002), Mili (Kuramochi-Miyagawa et al., 2004) and Miwi2 (G.J. Hannon, personal communication) are required for male fertility. Two recent reports have shown that an abundant species of ~30 nucleotide siRNAs (named piRNAs) interacts with Mili in meiotic spermatocytes (Aravin et al., 2006; Girard et al., 2006). Interestingly, piRNAs accumulate asymmetrically in a manner analogous to the secondary and X-cluster-derived siRNAs found in *C. elegans*. Clearly, there is still much to learn about the production and function of small RNAs. The paradigms of sequential Argonaute action and of intersection between Argonaute-mediated silencing pathways are likely to be important for understanding the diversity and complexity of RNAi-related mechanisms in numerous organisms.

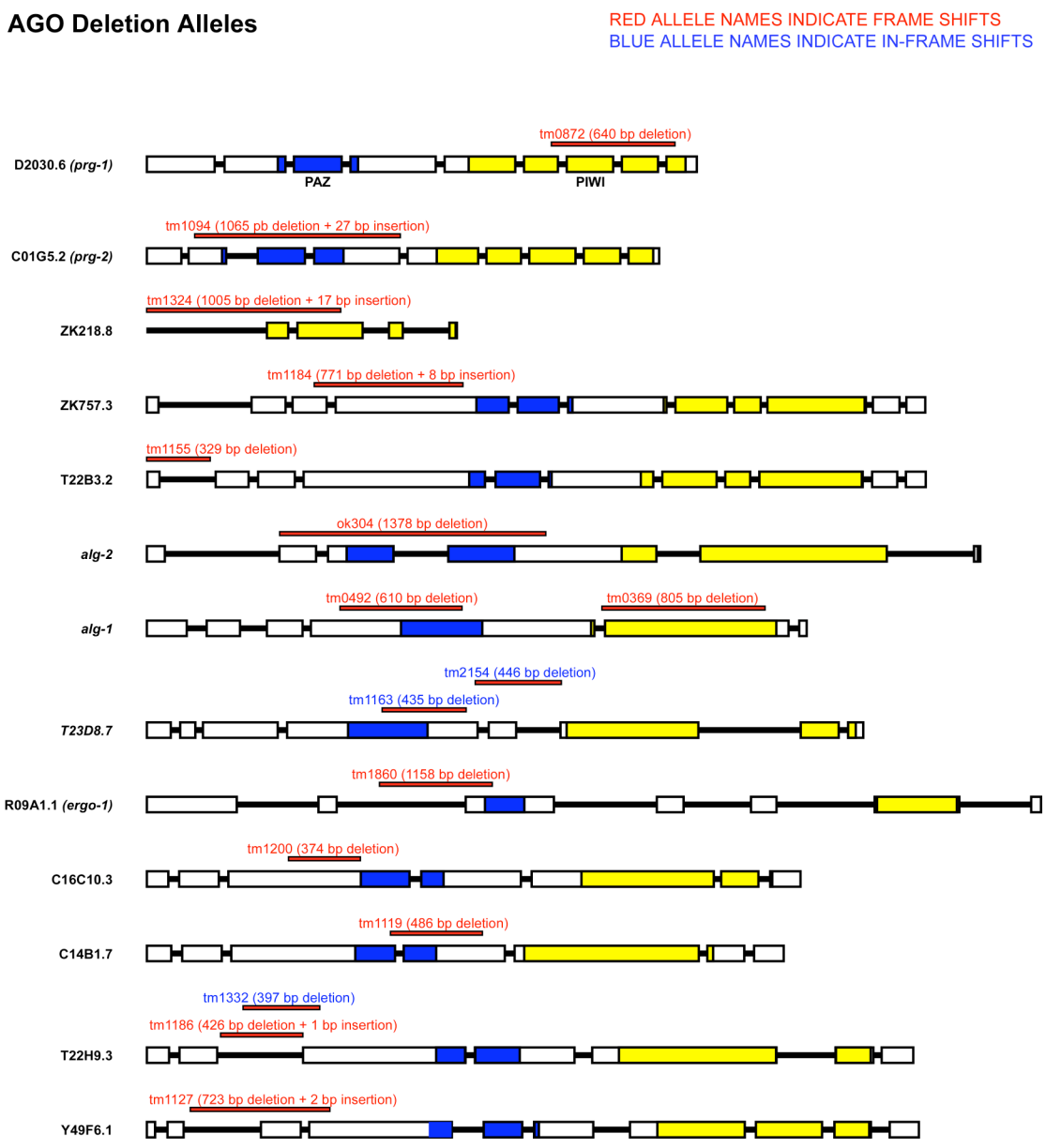


Figure II-8. Argonaute deletion alleles. Figure is drawn in scale. The scale of R09A1.1 is twice smaller than others. The figure continues on the next page.

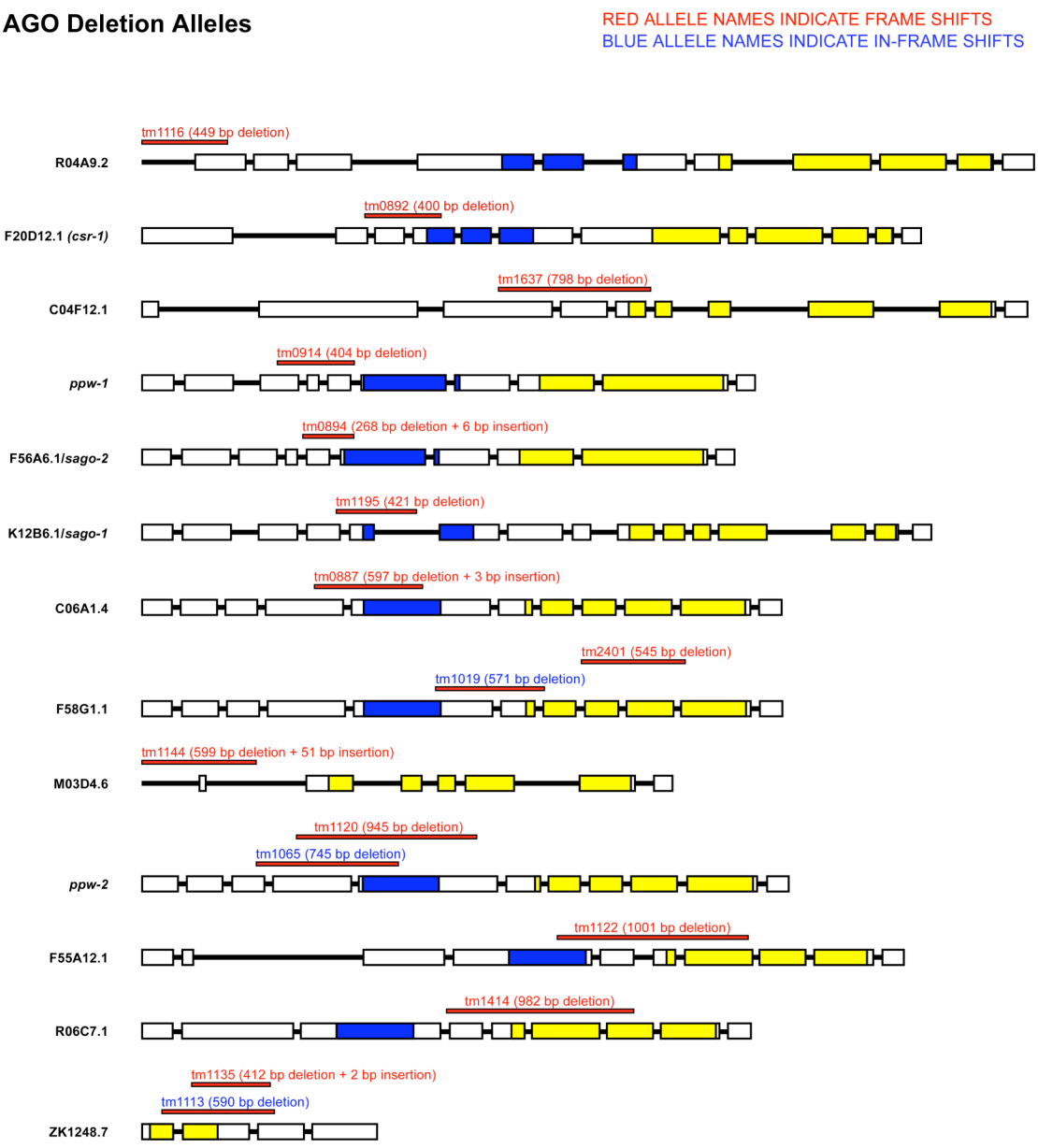


Figure II-8. Argonaute deletion alleles (continued from previous page). Figure is drawn in scale.

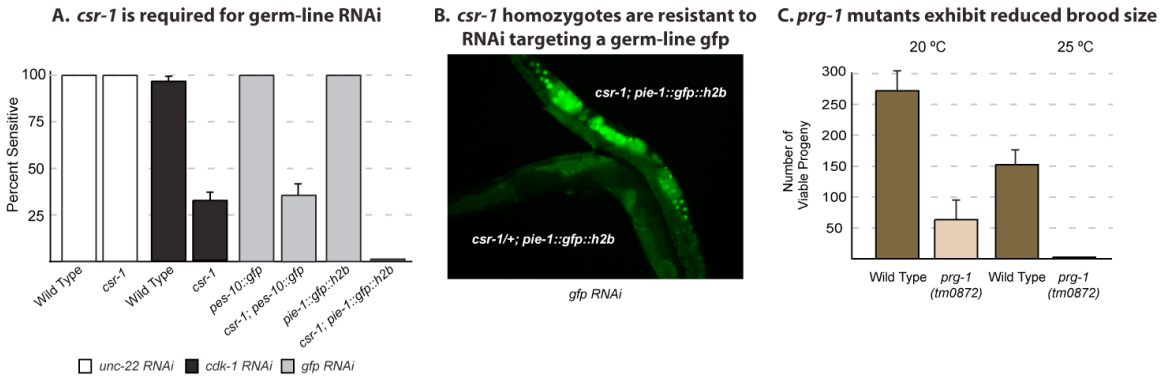


Figure II-9(A,B,C). Phenotypic analysis of argonaute genes. (A–B) *csr-1(tm892)* exhibits a partial defect in germline and early zygotic RNAi. (A) Graph showing the percent of animals sensitive to RNAi targeting two different gfp transgenes, and the endogenous genes *cdk-1* and *unc-22,* (as indicated). For the assays targeting *unc-22* and *pie-1::gfp::h2b;* homozygous-*csr-1* and homozygous-*pie-1::gfp::h2b*-transgenic-*csr-1* adults were compared to corresponding wild-type and wild type-*pie-1::gfp::h2b*-transgenic adults. Expression of GFP in the germ lines of the adult animals was scored as an indication of resistance to RNAi. For the assays targeting *cdk-1* and *pes-10::gfp;* homozygous-*csr-1* and homozygous-*pes-10::gfp*-transgenic *csr-1* adults were allowed to produce progeny, and these progeny were compared to those produced by the corresponding wild-type and wild-type-*pes-10::gfp*-transgenic animals. Expression of GFP in the embryos was scored as an indication of resistance to RNAi. For the *cdk-1* assays, it was possible to score the *csr-1* embryos because the arrest point for cdk-1 at the one-cell stage is prior to that of csr-1. For unc-22 RNAi, 20 P0-animals were tested per strain. For *cdk-1* and gfp RNAi in *pes-10::gfp; csr-1* strain, 80 F1-embryos from five homozygous *csr-1* animals were scored. The experiments were repeated three times. For gfp RNAi in *csr-1; pie-1::gfp:h2b* strain, twenty P0-animals were tested. (B) Fluourescence microgrph showing expression of *pie-1::gfp::h2b* expression in a *csr-1* homozygous adult (upper worm), and a silenced heterozygote (lower worm). Expression of histone::GFP is visible in the nuclei of many proximal oocytes, and is also visible in fertilized embryos present in the uterus of the csr-1 homozygote. (C) *prg-1(tm872)* exhibits a temperature-dependent sterile phenotype.Wild-type and *prg-1(tm872)* animals were cultured for one generation at the indicated temperatures, and the progeny produced by eight animals were scored for viability.

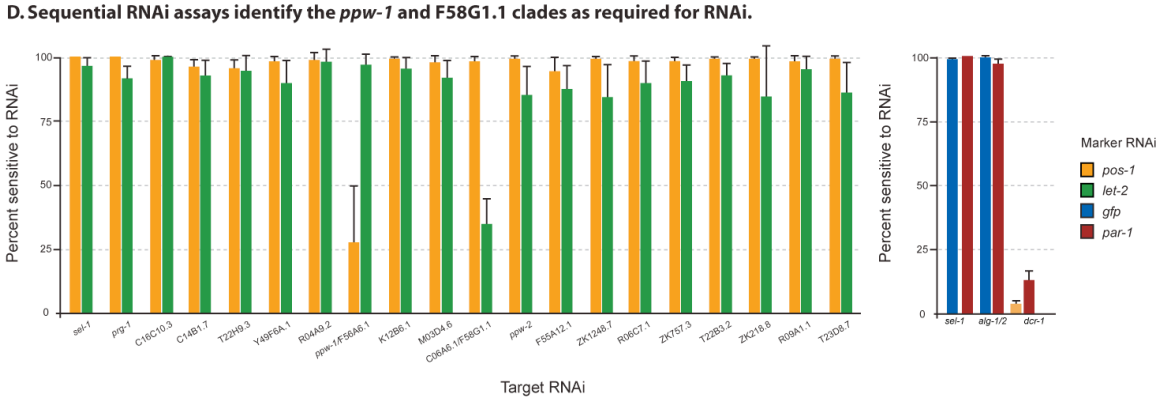


Figure II-9D. Analysis of RNAi defects by sequential dsRNA injection. Either a control dsRNA *(sel-1)* or a dsRNA targeting each Argonaute gene (400 mg/ml) was injected, followed after 6-10 hours by a dsRNA targeting one of four marker genes (as indicated in the key at right). The progeny of eight to ten injected animals were scored, and the error bars indicate the 95% confidence interval.

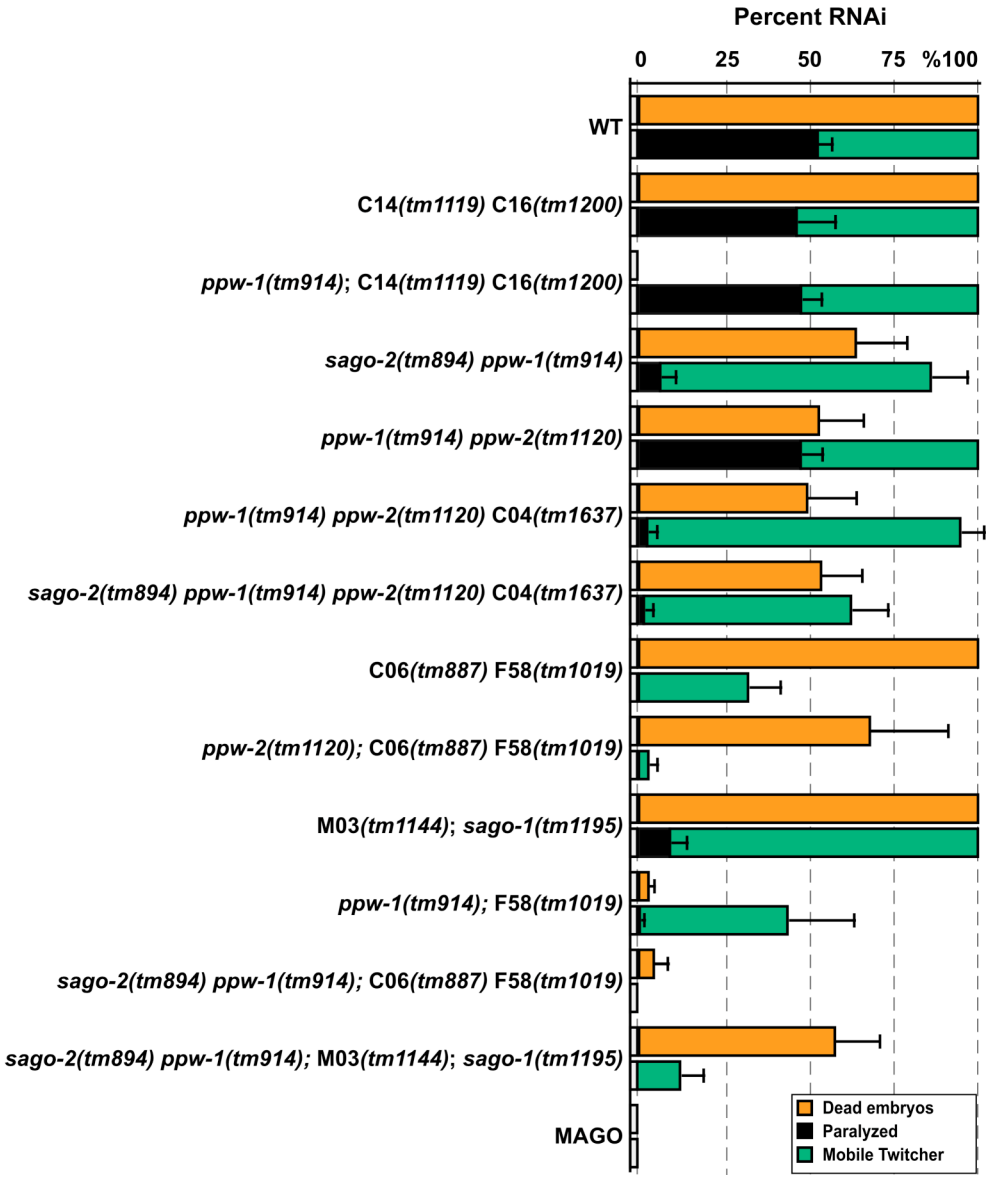


Figure II-10. RNAi sensitivity in Argonaute multiple mutants. Red bars represent the percent of embryos exhibiting the *pos-1(RNAi)* embryonic lethal phenotype after exposure to dsRNA by feeding. The Black and Green bars indicate the percent of animals that are paralyzed (black shaded area) or twitching but motile (green area) after injection of 20 µg/ml *unc-22* dsRNA. For *pos-1 RNAi,* the entire broods of between nine and ten animals were scored per strain. For *unc-22* RNAi, ~40 to 60 progeny of four to ten injected animals were scored per strain. Error bars indicate the 95% confidence interval.

Material and Methods

Worm Strains

The Bristol strain N2 was used as the standard wild type strain. The Argonaute alleles and strains used in this study are described in the text and are listed in (Table III.1). Additional alleles used in this study are; *rrf-1(pk1417)* I, *alg-2(ok304)* II, *sid-1(ne328)* V, *unc-22(st528)* IV. Deletions mutations were obtained as previously reported (Gengyo-Ando and Mitani, 2000). *C. elegans* culture and genetics were as described in (Brenner, 1974).

Table II-1. Outcrossing Status of Argonaute Deletion Alleles

The (x) indicates how many times each mutant was outcrossed.

Chromosome	ORF and Allele Names
LGI	C04F12.1*(tm1637, 4x)*
	C18E3.7*(tm914, 6x)*
	F55A12.1*(tm1122, 1x, ok1078 0x)*
	F56A6.1/*sago-2(tm0894, 7x)*
	D2030.6/*prg-1(tm0872, 7x)*
	R06C7.1*(tm1414, 0x, ok1074 0x)*
	T23D8.7*(tm1163, 4x)*
	Y110A7A.18*(tm 1120, 5x, tm1065, 0x)*
LGII	T07D3.7/*alg-2(ok304)*
	C06A1.4*(tm0887, 5x)*
	F58G1.1*(tm1019, 7x)*
	Y49F6A.1*(tm1127, 5x)*
	ZK1248.7*(tm1113, 0x, tm1135, 0x)*
LGIII	C14B1.7*(tm1119, 5x)*
	C16C10.3*(tm1200, 5x)*
	ZK757.3A*(gk188 0x, ok1041 0x, tm1184, 1x)*
LGIV	F20D12.1/*csr-1(tm0892, 6x)*
	M03D4.6*(tm1144, 5x)*
	C01G5.2/*prg-2(tm1094, 5x)*
	T22B3.2*(tm1155, 0x)*
LGV	K12B6.1/*sago-1(tm1195, 5x)*
	rde-1(ne300,ne4085,ne4086)
	T22H9.3*(tm1332, 0x, tm1186, 5x)*
	R09A1.1/*ergo-1(tm1860, 5x)*
	ZK218.8*(tm1324/+, 0x)*
LGX	*alg-1(gk214, tm0369, 0x, tm492, 0x)*
	R04A9.2*(tm1116, 5x)*

Table II-2. Strains Used in This Study

Strain	**Genotype** (All strains were generated using out-crossed alleles)
WM127	C14B1.7*(tm1119)* C16C10.3*(tm1200)* III
WM128	*ppw-1(tm0914)* I; C14B1.7*(tm1119)* C16C10.3*(tm1200)* III
WM129	*sago-2(tm0894) ppw-1(tm0914)* I
WM130	*ppw-1(tm0914) ppw2(tm1120)* I
WM131	*ppw-1(tm0914) ppw-2(tm1120)* C04F12.1*(tm1637)* I
WM132	*sago-2(tm0894) ppw-1(tm0914) ppw-2(tm1120)* C04F12.1*(tm1637)* I
WM133	C06A1.4(tm0887) F58G1.1(tm1019) II
WM134	*ppw-2(tm1120)* I; C06A1.4*(tm0887)*, F58G1.1*(tm1019)* II
WM135	M03D4.6*(tm1144)* IV; *sago-1(tm1195)* V
WM136	*ppw-1(tm914)* I; F58G1.1*(tm1019)* II
WM137	*sago-2(tm0894) ppw-1(tm0914)* I, C06A1.4*(tm0887)* F58G1.1*(tm1019)* II
WM138	*sago-2(tm0894) ppw-1(tm0914)* I; M03D4.6*(tm1144)* IV; sago-1*(tm1195)* V
WM126 (MAGO)	*sago-2(tm0894) ppw-1(tm0914)* I; C06A1.4*(tm0887)*, F58G1.1*(tm1019)* II; M03D4.6*(tm1144)* IV; *sago-1(tm1195)* V
WM118	neIS9[myo-3::HA::RDE-1] in *rde-1(ne300)*
WM119	neIS10[myo-3::GFP::sago-2] in WM126
WM120	neIS11[myo-3::GFP::sago-1] in WM126
WM121	neEx7[myo-3::C18E3.7] in WM126
WM122	neEx8[myo-3::GFP::ALG-1] in WM126
WM123	neEx9[myo-3::PRG-1] in WM126
WM124	neEx10[myo-3::GFP::unc-54 3′utr]
WM125	neEX11[ERGO-1] in ergo-1(tm1860)

Worm Lysis and Single Worm PCR

In this study, the deletion alleles were identified by PCR amplifications. PCR amplifies a shorter band in a deletion strain than wild type strain. The size difference between the deletion and wild type bands were analyzed by agarose gel electrophoresis. PCR amplifications were done with single worm DNA. For rapid and efficient outcrossing and multiple fold mutant building, the crude worm lysates were used as PCR templates. In my hand, Taq DNA Polymerase (Roche) gave the most robust PCR results. DNA of single worms was prepared in 2.5 µl of lysis buffer (10 mM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 0.45% NP40, 0.45% Tween20, 0.01% gelatin, 100 µg/ml Proteinase K) and incubated at -70 ºC for 15 minutes, 90 minutes at 65 ºC, and then 15 minutes at 95 ºC. The following program was used for single worm PCR reactions: 94 ºC for 2 minutes, 94 ºC for 15 seconds, 56 ºC for 30 seconds, 72 ºC for 1 minute/1 kb, 34 cycles with 10 minutes final elongation step. PCR amplifications were performed in total volume of 25 µl. One third of single worm lysate was used as template in PCR reaction. If nested PCR was necessary, one tenth of single worm lysate was used as template in the first PCR amplification, and 1:100 dilution of the first PCR product was used as a template in the second PCR reaction. The PCR products were analyzed by agarose gel electrophoresis.

Building Multiple Fold Argonaute Mutants

Following GFP-positive balancer strains were used in this study to build multiple fold mutants:

***tag-319(ok1420)/mIn1[mIs14 dpy-10(e128)]* II.**

mIs14[myo-2::gfp; pes-10::gfp].

***dis-3(ok357)/nT1[qIs51]*; *+/ nT1[qIs51]* (IV; V).**

qIs51[myo-2::gfp; pes-10::gfp].

***rrf-1(ne4075) ego-1(om71)/hT2[bli-4(e937) let-?(q782) qIs48]* (I; III).**

qIs48[myo-2::gfp; pes-10::gfp]

***avr-1* (I)*; avr-15(ad1051) glc-1(pk54)/DnT1* (IV; V).**

Single Argonaute deletion alleles that are used for making multiple fold mutant strains were outcrossed at least four times to remove background mutations from mutagenized worms (Table II-1). But, Argonaute deletion alleles in *pha-1(ts)* background were not outcrossed before mating with *pha-1(ts)*. In some cases *him-8(e1489)*, which exhibits high incidence of males phenotype, was used to easily find males for mating.

For the schematic representation of the crosses designed to build multiple fold mutants please see Figure II-11.

Singles:

***sago-2(tm894)* I (7-times outcrossed).**

Wild type males were mated with *sago-2(tm894) ppw-1(tm914) unc-29* hermaphrodites. Non-Unc F1 hermaphrodites were picked onto individual plates. Non-Unc F2 hermaphrodites were picked onto individual plates. Plates segregating Non-Unc progeny were selected, and mothers were harvested for single worm PCR to determine the *sago-2(tm894)* genotype. After the recombination, this strain lost a background mutation that was linked to *sago-2(tm894)* on the same chromosome.

F58G1.1*(tm1019),* unlinked from *dpy-10* C06A1.7*(tm887)* F58G1.1*(tm1019)* II.

Wild type males were mated with *dpy-10* C06A1.7*(tm887)* F58G1.1*(tm1019)* hermaphrodites. Non-Dpy F1 hermaphrodites were picked onto individual plates. Non-Dpy F2 hermaphrodites were picked onto individual plates. Plates segregating Non-Dpy progeny were selected, and mothers were harvested for single worm PCR to determine the F58G1.1*(tm1019)* genotype. F58G1.1*(tm1019)* was partially resistant to germline RNAi at 20 ºC. C06A1.7*(tm887)* F58G1.1*(tm1019)* double was sensitive to germline RNAi, suggesting that C06A1.7*(tm887)* mutation suppresses F58G1.1*(tm1019)* germline RNAi phenotype. To rule out the possibility that F58G1.1*(tm1019)* had a background mutation in the same chromosome before the double was made, it was unlinked from *dpy-10* C06A1.7*(tm887)* F58G1.1*(tm1019)*. After unlinking, F58G1.1*(tm1019)* gained its original germline RNAi deficient

phenotype, confirming C06A1.7*(tm887)* suppressed F58G1.1*(tm1019)* germline RNAi phenotype.

Doubles:

***sago-2(tm894)* I; *pha-1(ts)* III**

sago-2(tm894) males were mated with *pha-1(ts)* hermaphrodites at 15 ºC. F1 hermaphrodites were picked onto individual plates. After F1 hermaphrodites reproduced progeny at 15 ºC, F1 hermaphrodites were shifted to 25 ºC to distinguish cross progeny from self progeny. After F1 cross progeny was distinguished, their progeny was picked onto individual plates. About 60 F2 progeny was picked onto individual plates at 15 ºC carrying bacteria expressing *pos-1* dsRNA to select RNAi resistant animals. Once animals resistant to *pos-1* RNAi reproduced, the mothers were shifted to 25 ºC distinguish *pha-1(ts)* genotype.

The *tm894* allele had background mutation on LGI that made the strain resistant to both germline and somatic RNAi. This background mutation was lost in the 7-times outcrossed *sago-2(tm894)* strain that is now only partially defective in somatic RNAi, observed only by injection of *unc-22* dsRNA at 20 ng/µl concentration.

F58G1.1*(tm1019)* II; *pha-1(ts)* III

F58G1.1*(tm1019)* males were mated with *pha-1(ts)* hermaphrodites at 15 ºC. F1 hermaphrodites were picked onto individual plates. After F1 hermaphrodites reproduced about 60 progeny at 15 ºC, they were shifted to 25 ºC to distinguish cross

progeny from self progeny. After F1 cross progeny was distinguished, their progeny was picked onto individual plates. After F2 hermaphrodites reproduced, they were shifted to 25 ºC to distinguish *pha-1(ts)* genotype, and they were also harvested for single worm PCR to determine the F58G1.1*(tm1019)* deletion.

***ppw-1(tm914)* I; *pha-1(ts)* III**

ppw-1(tm914) males were mated with *pha-1(ts)* hermaphrodites at 15 ºC. F1 hermaphrodites were picked onto individual plates. After F1 hermaphrodites reproduced about 60 progeny at 15 ºC, they were shifted to 25 ºC to distinguish cross progeny from self progeny. After F1 cross progeny was distinguished, their progeny was picked onto individual plates. After F2 hermaphrodites reproduced, they were shifted to 25 ºC to distinguish *pha-1(ts)* genotype, and they were also harvested for single worm PCR to determine the *ppw-1(tm914)* deletion.

***pha-1(ts)* III; *rde-1(ne300)* V**

rde-1(ne300) males were mated with *pha-1(ts)* hermaphrodites at 15 ºC. F1 hermaphrodites were picked onto individual plates. After F1 hermaphrodites reproduced progeny at 15 ºC, F1 hermaphrodites were shifted to 25 ºC to distinguish cross progeny from self progeny. After F1 cross progeny was distinguished, their progeny was picked onto individual plates. About 60 F2 progeny was picked onto individual plates at 15 ºC carrying bacteria expressing *pos-1* dsRNA to select RNAi

resistant animals. Once animals resistant to *pos-1* RNAi reproduced, the mothers were shifted to 25 ºC distinguish *pha-1(ts)* genotype.

***ppw-1(tm914) unc-29* I**

ppw-1(tm914) males were mated with *unc-29* hermaphrodites. Non-Unc F1 hermaphrodites were picked onto individual plates. Unc F2 hermaphrodites were picked onto individual plates. After Unc F2 worms reproduced, mothers were harvested for single worm PCR to determine the *ppw-1(tm914*) deletion.

***sago-2(tm894) ppw-1(tm914)* I**

sago-2(tm894) ppw-1(tm914) unc-29; him-8(e1489) males were mated with *ppw-1(tm914)* hermaphrodites. F1 hermaphrodites were picked onto individual plates. After F1 hermaphrodite reproduced, mothers were harvested for single worm PCR to determine the cross progeny (F1) carrying *sago-2(tm894)* deletion. Non-Unc F2 progeny was picked onto individual plates. Plates segregating Non-Unc F3 progeny were selected, and mothers were harvested for single worm PCR to determine *sago-2(tm894) ppw-1(tm914).*

***dpy-10* C06A1.7*(tm887)* II**

C06A1.7*(tm887)* males were mated with *dpy-10 unc-4* hermaphrodites. Non-Unc F1 hermaphrodites were picked onto individual plates. Dpy Non-Unc F2 hermaphrodites

were picked onto individual plates, and after they reproduced, they were harvested for single worm PCR to determine C06A1.7*(tm887)* deletion.

C06A1.7*(tm887)* F58G1.1*(tm1019)* II

C06A1.7*(tm887)* males were mated with *sago-2(tm894) ppw-1(tm914)*; *dpy-10* C06A1.7*(tm887)* F58G1.1*(tm1019)*; *him-8(e1489)* hermaphrodites. Non-Dpy F1 hermaphrodites were picked onto individual plates. Non-Dpy F2 hermaphrodites were picked onto individual plates. Plates segregating Non-Unc F3 progeny were selected, and mothers were harvested for single worm PCR to determine C06A1.7*(tm887)* F58G1.1*(tm1019)*. Among C06A1.7*(tm887)* F58G1.1*(tm1019)* strains, Non-Him worms were selected.

M03D4.6*(tm1144)* IV *sago-1(tm1195)* V

M03D4.6*(tm1144)* males were mated with *sago-1(tm1195)* hermaphrodites. F1 progeny was picked onto individual plates. After F1 mothers reproduced, they were harvested for PCR to determine F1 cross progeny, which carried M03D4.6*(tm1144)* deletion. After F1 cross progeny was determined, 64 F2 hermaphrodites were picked onto individual plates. After F2 hermaphrodites reproduced, they were harvested for PCR to determine M03D4.6*(tm1144) sago-1(tm1195)* genotype.

ppw-1(tm914)* F58G1.1*(tm1019)

ppw-1(tm914) males were mated with F58G1.1*(tm1019)* hermaphrodites. F1 progeny was picked onto individual plates. After F1 mothers reproduced, they were harvested for PCR to determine F1 cross progeny, which carried *ppw-1(tm914)* deletion. After F1 cross progeny was determined, 64 F2 hermaphrodites were picked onto individual plates. After F2 hermaphrodites reproduced, they were harvested for PCR to determine *ppw-1(tm914)* F58G1.1*(tm1019)* genotype.

***ppw-1(tm914) ppw-2(tm1120)* I**

ppw-2(tm1120) males were mated with *ppw-1(tm914) unc-29* hermaphrodites. Non-Unc F1 hermaphrodites were mated with *rrf-1(ne4075) ego-1(om71)/hT2[bli-4(e937) let-?(q782) qIs48]; him-8(e1489)* males. GFP positive hermaphrodites were picked onto individual plates. After mothers reproduced, they were harvested for PCR to determine *ppw-1(tm914) ppw-2(tm1120)* genotype. Then, Non-GFP worms were picked to establish *ppw-1(tm914) ppw-2(tm1120)* homozygous strain.

Triples:

***sago-2(tm894) ppw-1(tm914)* I; *pha-1(ts)* III**

sago-2(tm894) ppw-1(tm914); *him-8(e1489)* males were mated with *pha-1(ts)* hermaphrodites at 15 ºC. F1 hermaphrodites were picked onto individual plates. After F1 hermaphrodites reproduced about 60 progeny at 15 ºC, F1 mothers were shifted to 25 ºC to distinguish cross progeny mother from self progeny mother. After F1 cross progeny was distinguished, their progeny (F2) was picked onto individual

plates. After F2 hermaphrodites reproduced, F2 mothers were shifted to 25 ºC to distinguish *pha-1(ts)* woms. From plates carrying *pha-1(ts)* homozygous animals, worms were harvested for single worm PCR to determine the *sago-2(tm894) ppw-1(tm914)* deletions. Note: This strain was not tested for germline and somtic RNAi resistance. It is possible that the background mutation linked to *sago-2(tm894)* was still in the strain (please see "*sago-2(tm894)* I; *pha-1(ts)* III" strain for details).

***sago-2(tm894) ppw-1(tm914) unc-29* I**

sago-2(tm894) males were mated with *ppw-1(tm914) unc-29* hermaphrodites. Non-Unc hermaphrodite progeny (F1) was mated with *rrf-1(ne4075) ego-1(om71)/hT2[bli-4(e937) let-?(q782) qIs48]*; *+/hT2[bli-4(e937) let-?(q782) qIs48]*, *him-8(e1489)* males. GFP hermaphrodite cross progeny (F_1') from this second cross was picked onto individual plates. Plates segregating Unc progeny was selected, and mothers were harvested for single worm PCR to determine the *sago-2(tm894) ppw-1(tm914)* deletions. *sago-2(tm894) ppw-1(tm914) unc-29* triple was segregated from *sago-2(tm894) ppw-1(tm914) unc-29/hT2[bli-4(e937) let-?(q782) qIs48]* strains. Finally, Non-Him strains were identified on the basis of the incidence of males segregated from *sago-2(tm894) ppw-1(tm914) unc-29/hT2[bli-4(e937) let-?(q782) qIs48]* strains.

***sago-2(tm894) ppw-1(tm914)* I, *him-8(e1489)* IV**

sago-2(tm894) males were mated with *ppw-1(tm914) unc-29* hermaphrodites. Non-Unc hermaphrodite progeny (F1) was mated with *rrf-1(ne4075) ego-1(om71)/hT2[bli-4(e937) let-?(q782) qIs48]*; *+/hT2[bli-4(e937) let-?(q782) qIs48]*, *him-8(e1489)* males. GFP hermaphrodite cross progeny (F′) from this second cross was picked onto individual plates. Plates segregating Unc progeny was selected, and mothers were harvested for single worm PCR to determine the *sago-2(tm894) ppw-1(tm914)* deletions. *sago-2(tm894) ppw-1(tm914) unc-29* triple was segregated from *sago-2(tm894) ppw-1(tm914) unc-29/hT2[bli-4(e937) let-?(q782) qIs48]* strains. Finally, strains that carried *him-8(e1489)* were identified on the basis of the Him (high incidence of males) phenotype characteristic of *him-8(e1489)* strains.

***dpy-10* C06A1.7*(tm887)* F58G1.1*(tm1019)* II**

F58G1.1*(tm1019)* males were mated with *dpy-10* C06A1.7*(tm887)* hermaphrodites. Non-Dpy F1 progeny was picked onto individual plates. Dpy F2 hermaphrodites was picked onto individual plates. After they reproduced, mothers were harvested for single worm PCR to determine F58G1.1*(tm1019)* and C06A1.7*(tm887)* deletions.

***ppw-2* I; C06A1.7*(tm887)* F58G1.1*(tm1019)* II**

ppw-2(tm1120) males were mated with *tag-319(ok1420)/mIn1[mIs14 dpy-10(e128)]* hermaphrodites. Individual GFP positive F1 males were mated with C06A1.7*(tm887)* F58G1.1*(tm1019)* hermaphrodites. After hermaphrodites reproduced, F1 males were harvested for single worm PCR to determine *ppw-2(tm1120)* deletion. From plates

carrying *ppw-2(tm112)* F1 males, GFP positive F2 hermaphrodites were picked onto individual plates. After GFP positive F_2' hermaphrodites reproduced, they were harvested for PCR do determine *ppw-2(tm112)* deletion. After homozygous *ppw-2(tm112)* worms were distinguished, Non-GFP progeny of GFP positive were harvested for PCR to determine C06A1.7*(tm887)* F58G1.1*(tm1019)* deletions.

***ppw-1(tm914) ppw-2(tm1120)* I; *unc-32(e189)* III**

ppw-1(tm914) ppw-2(tm1120) males were mated with *dpy-5*; *rol-6*; *unc-32(e189)* hermaphrodites. Non-Dpy, Non-Rol, and Non-Unc F1 hermaphrodites were picked onto individual plates. Unc, Non-Dpy, and Non-Rol F2 hermaphrodies were picked onto individual plates. After Unc F2 animals reproduced, they were harvested for PCR to determine *ppw-1(tm914)* and *ppw-2(tm1120)* deletions.

***ppw-1(tm914) ppw-2(tm1120)* C04F12.1*(tm1637)* I**

C04F12.1*(tm1637)* males were mated with *ppw-1(tm914) ppw-2(tm1120)* ; *unc-32(e189)* hermaphrodites. Non-Unc F1 hermaphrodites were mated with *hT2[bli-4(e937) let-?(q782) qIs48]/rrf-1(ne4075) ego-1(om71); him-8(e1489)* males. GFP positive F′ hermaphrodites were picked onto individual plates. After F′ hermaphrodites reproduced, they were harvested for PCR to determined C04F12.1*(tm1637), ppw-1(tm914), and ppw-2(tm1120)* deletions. After hT2[bli-4(e937) let-?(q782) qIs48]/C04F12.1*(tm1637) ppw-1(tm914) and ppw-2(tm1120)* worms were distinguished, Non-GFP progeny worms were segregated from balance

worms, and finally the Non-Him strains were identified on the basis of the incidence of males.

Quadruples:

***avr-14(ad1302)* I; *csr-1(tm892)/DnT1* IV; *avr-15(ad1051) glc-1(pk54)/DnT1* V**

Wild type (N2) males were mated with *csr-1/unc-24* heterozygous hermaphrodites. Individual Non-Unc F1 male progeny was mated with *avr-1; +/DnT1; avr-15(ad1051) glc-1(pk54)/DnT1* hermaphrodites. After hermaphrodites reproduced, males were harvested for PCR to determine *csr-1(tm892)* deletion. Then, individual Non-Unc F_1' male progeny was backcrossed with *avr-1; +/DnT1; avr-15(ad1051) glc-1(pk54)/DnT1* hermaphrodites. Unc hermaphrodite progeny (F_1'') was picked onto individual plates. The, plates segregating Non-Unc sterile worms were selected because *csr-1(tm892)* homozygotes are sterile worms, which were later harvested for PCR to determine *csr-1(tm892)* deletion. From plates which carried *csr-1(tm892)* deletions, Non-Unc worms at L1-L3 stage were picked onto individual plates containing ivermectin. Ivermectin plates that carried viable worms were distinguished. Unc mothers of these viable worms were selected and kept to maintain the strain *avr-14(ad1302)*; *csr-1(tm892)/DnT1*; *avr-15(ad1051) glc-1(pk54)/DnT1*.

***sago-2(tm894) ppw-1(tm914) unc-29* I, *him-8(e1489)* IV**

sago-2(tm894) males were mated with *ppw-1(tm914) unc-29* hermaphrodites. Non-Unc hermaphrodite progeny (F1) was mated with *rrf-1(ne4075) ego-*

1(om71)/hT2[bli-4(e937) let-?(q782) qIs48]; *+/hT2[bli-4(e937) let-?(q782) qIs48]*, *him-8(e1489)* males. GFP hermaphrodite cross progeny (F_1') from this second cross was picked onto individual plates. Plates segregating Unc progeny was selected, and mothers were harvested for single worm PCR to determine the *sago-2(tm894) ppw-1(tm914)* deletions. *sago-2(tm894) ppw-1(tm914) unc-29* triple was segregated from *sago-2(tm894) ppw-1(tm914) unc-29/hT2[bli-4(e937) let-?(q782) qIs48]* strains. Finally, strains that carried *him-8(e1489)* were identified on the basis of the Him (high incidence of males) phenotype characteristic of *him-8(e1489)* strains.

***sago-2(tm894) ppw-1(tm914)* I; C06A1.7*(tm887)* F58G1.1*(tm1019)* II**

C06A1.7*(tm887)* males were mated with *sago-2(tm894) ppw-1(tm914)*; *dpy-10* C06A1.7*(tm887)* F58G1.1*(tm1019)*; *him-8(e1489)* hermaphrodites. Non-Dpy F1 hermaphrodite progeny was picked onto individual plates. Then, Non-Dpy F2 hermaphrodites were picked onto individual plates. After F2 hermaphrodites reproduced, they were harvested for PCR to determine *sago-2(tm894), ppw-1(tm914),* C06A1.7*(tm887),* and F58G1.1*(tm1019)* deletions. After strains was homozygouzed for *sago-2(tm894), ppw-1(tm914),* C06A1.7*(tm887),* and F58G1.1*(tm1019)* deletions, the Non-Him strains were identified on the basis of the incidence of males.

***sago-2(tm894) ppw-1(tm914)* I; M03D4.6*(tm1144)* IV *sago-1(tm1195)* V**

dis-3(ok357)/nT1[qIs51]; *+/ nT1[qIs51]* males were mated with *sago-2(tm894) ppw-1(tm914)* hermaphrodites. GFP positive F1 males were mated with

M03D4.6*(tm1144) sago-1(tm1195)* hermaphrodites. GFP positive F_1' hermaphrodites were picked onto individual plates. After GFP positive F_1' hermaphrodites reproduced, they were harvested for PCR do determine *sago-2(tm894)* and *ppw-1(tm914)* deletions. Non-GFP worms were segregated from GFP worms, and hermaphrodites were picked onto individual plates. After Non-GFP hermaphrodites reproduced, they were harvested for PCR to determine homozygous *sago-2(tm894), ppw-1(tm914),* M03D4.6*(tm1144),* and *sago-1(tm1195)* deletions.

***sago-2(tm894) ppw-1(tm914)* I; C06A1.7*(tm887)* F58G1.1*(tm1019)* II**

rrf-1(ne4075) ego-1(om71)/hT2[bli-4(e937) let-?(q782) qIs48]; *him-8(e1489)* males were mated with C06A1.7*(tm887)* F58G1.1*(tm1019)*. Individual GFP positive F1 male progeny was mated with *sago-2(tm894) ppw-1(tm914)* hermaphrodites. GFP positive F_1' hermaphrodites were picked onto individual plates. After GFP positive F_1' hermaphrodites reproduced, they were harvested for PCR to determine C06A1.7*(tm887)* and F58G1.1*(tm1019)* deletions. Then, GFP F_2' progeny was picked onto individual plates. GFP positive F_2' worms were harvested for PCR to determine homozygous C06A1.7*(tm887)* and F58G1.1*(tm1019)* deletions. After that Non-GFP F3′ worms were picked onto individual plates. After Non-GFP F3′ hermaphrodites reproduced, they were harvested for PCR to confirm the existence of *sago-2(tm894)* and *ppw-1(tm914)* deletions.

***ppw-1(tm914) ppw-2(tm1120)* C04F12.1*(tm1637)* I; *unc-32(e189)* III**

C04F12.1*(tm1637)* males were mated with *ppw-1(tm914) ppw-2(tm1120)* ; *unc-32(e189)* hermaphrodites. Non-Unc F1 hermaphrodites were mated with *rrf-1(ne4075) ego-1(om71)/hT2[bli-4(e937) let-?(q782) qIs48]/; him-8(e1489)* males. GFP positive F_1' hermaphrodites were picked onto individual plates. Plates segregating Unc worms were selected, and mothers were harvested for PCR to determined C04F12.1*(tm1637), ppw-1(tm914), and ppw-2(tm1120)* deletions. After *hT2[bli-4(e937) let-?(q782) qIs48]/ppw-1(tm914) ppw-2(tm1120)* C04F12.1*(tm1637)* carrying worms were distinguished, Non-GFP, Unc hermaphrodites were segregated from balanced strain, and finally the Non-Him strains were identified on the basis of the incidence of males.

***sago-2(tm894) ppw-1(tm914) ppw-2(tm1120)* C04F12.1*(tm1637)* I**

sago-2(tm894) males were mated with *ppw-1(tm914) ppw-2(tm1120) C04F12.1(tm1637)*; *unc-32(e189)* hermaphrodites. Individual F1 males were mated with *rrf-1(ne4075) ego-1(om71)/hT2[bli-4(e937) let-?(q782) qIs48]*; *him-8(e1489)* hermaphrodites. GFP positive F_1' progeny was picked onto individual plates. After GFP positive F_1' worms reproduced, they were harvested for PCR to determine *sago-2(tm894), ppw-1(tm914), ppw-2(tm1120), and* C04F12.1*(tm1637)* deletions. Then, Non-GFP F_2' hermaphrodites were picked onto individual plates. After F_2' hermaphrodites reproduced, they were harvested for PCR do confirm all the deletions again.

***sago-2(tm894) ppw-1(tm914)* I; *him-8(e1489)* IV; *sago-1(tm1195)* V**

sago-2(tm894) ppw-1(tm914); him-8(e1489) males were mated with individual *sago-2(tm894) ppw-1(tm914)*; M03D4.6*(tm1144)*; *sago-1(tm1195)* hermaphrodites. F1 progeny was picked onto individual plates. After F1 hermaphrodites reproduced, they were harvested for PCR do determine heterozygous worms for *sago-1(tm1195)* deletion. The heterozygous F1 worms for *sago-1(tm1195)* deletion had to be cross progeny. After hermaphrodites F1 cross progeny was distinguished, F2 hermaphrodites were picked onto individual plates. After F2 hermaphrodites reproduced, they were harvested for PCR to determine *sago-2(tm894), ppw-1(tm914), and sago-1(tm1195)* deletions, and wild type allele of M03D4.6. After these alleles were homozygouzed, strains that carried *him-8(e1489)* were identified on the basis of the Him (high incidence of males) phenotype characteristic of *him-8(e1489)* strains.

***sago-2(tm894) ppw-1(tm914)* I; F58G1.1*(tm1019)* II; *sago-1(tm1195)* V.**

sago-2(tm894) ppw-1(tm914); him-8(e1489); sago-1(tm1195) males were mated with *ppw-1(tm914)* I; F58G1.1*(tm1019)* hermaphrodites. F1 hermaphrodites were picked onto individual plates. After F1 hermaphrodites reproduced, they were harvested for PCR to determine heterozygous F58G1.1*(tm1019)* deletion, which indicates F1 cross progeny. After F1 cross progeny was distinguished, F2 hermaphrodites were picked onto individual plates. After F2 hermaphrodites reproduced, they were harvested for PCR to determine *sago-2(tm894), ppw-1(tm914), F58G1.1(tm1019),* and *sago-*

1(tm1195) deletion alleles. After worms were homozygouzed for these alleles, Non-Him worms were selected based on the frequency of males on the plates.

Quintuples:

***avr-14(ad1302)* I; *csr-1(tm892)/DnT1* IV; *avr-15(ad1051) glc-1(pk54)/DnT1* V; *pes-10::gfp* X.**

pes-10::gfp males were mated with *avr-14(ad1302)*; *csr-1(tm892)/DnT1*; *avr-15(ad1051) glc-1(pk54)/DnT1* hermaphrodites. Single GFP positive, Unc, hermaphrodite F1 progeny was mated with single Non-Unc F1 male. GFP positive, Unc F_1' hermaphrodites were picked onto individual plates. Plates that segregated sterile worms were selected because *csr-1/csr-1* homozygous worms are maternal effect sterile. Then, GFP positive F_2' hermaphrodites progeny was picked onto individual plates. 16 F3′ hermaphrodite progeny were checked for GFP signal from each plate to find F_2' mother who was homozygous for *pes-10::gfp* transgene. Note: Some of the ivermectin resistance mutations still might be in this strain.

***sago-2(tm894) ppw-1(tm914)* I; *dpy-10* C06A1.7*(tm887)* F58G1.1*(tm1019)* II.**

rrf-1(ne4075) ego-1(om71)/hT2[bli-4(e937) let-?(q782) qIs48]; him-8(e1489) males were mated with *dpy-10* C06A1.7*(tm887)* F58G1.1*(tm1019)* hermaphrodites. GFP positive, Non-Dpy F1 males were mated with *sago-2(tm894) ppw-1(tm914)* hermaphrodites. GFP positive F_1' hermaphrodites were picked onto individual plates. Dpy segregating plates were selected. GFP positive Dpy worms were picked onto

individual plates. After GFP positive Dpy worms reproduced they were harvested for PCR to determine C06A1.7*(tm887)* and F58G1.1*(tm1019)* deletions. After these genes were homozygouzed, Non-GFP Dpy worms were picked onto individual plates. After these worms reproduced, adults were harvested for PCR to confirm *sago-2(tm894)* and *ppw-1(tm914)* deletions. Finally, Non-Him worms were selected based on the frequency of males on the plates.

***sago-2(tm894) ppw-1(tm914)* I; C06A1.7*(tm887)* F58G1.1*(tm1019)* II; *him-8(e1489)* IV.**

rrf-1(ne4075) ego-1(om71)/hT2[bli-4(e937) let-?(q782) qIs48]; *him-8(e1489)* males were mated with C06A1.7*(tm887)* F58G1.1*(tm1019)*. Individual GFP positive F1 male progeny was mated with *sago-2(tm894) ppw-1(tm914)* hermaphrodites. GFP positive F_1' hermaphrodites were picked onto individual plates. After GFP positive F_1' hermaphrodites reproduced, they were harvested for PCR to determine C06A1.7*(tm887)* and F58G1.1*(tm1019)* deletions. Then, GFP F_2' progeny was picked onto individual plates. GFP positive F_2' worms were harvested for PCR to determine homozygous C06A1.7*(tm887)* and F58G1.1*(tm1019)* deletions. After that Non-GFP F3′ worms were picked onto individual plates. After Non-GFP F3′ hermaphrodites reproduced, they were harvested for PCR to confirm the existence of *sago-2(tm894)* and *ppw-1(tm914)* deletions. At the end, Him strains were selected based on the frequency of males on the plates.

Sextuples:

***sago-2(tm894) ppw-1(tm914)* I; C06A1.7*(tm887)* F58G1.1*(tm1019)* II M03D4.6*(tm1144)* IV *sago-1(tm1195)* V.**

csr-1(tm892)/nT1[qIs51]; *+/ nT1[qIs51]* males were mated with *sago-2(tm894) ppw-1(tm914)* I; C06A1.7*(tm887)* F58G1.1*(tm1019)* hermaphrodites. GFP positive F1 male progeny was mated with *sago-2(tm894) ppw-1(tm914);* M03D4.6*(tm1144); sago-1(tm1195)* hermaphrodites. GFP positive F_1' hermaphrodites were picked onto individual plates. After GFP positive F_1' hermaphrodites reproduced, they were harvested for PCR to distinguish worms carrying C06A1.7*(tm887)* and F58G1.1*(tm1019)* deletion. Then, GFP positive F_2' hermaphrodites were picked onto individual plates. After GFP positive F_2' hermaphrodites reproduced, they were harvested for PCR to determine *sago-2(tm894), ppw-1(tm914), ppw-2(tm1120),* C06A1.7*(tm887);* and F58G1.1*(tm1019)* deletions. After these deletion alleles were homozygouzed, Non-GFP worms were segregated. These worms were harvested for PCR to confirm M03D4.6*(tm1144)* and *sago-1(tm1195)* deletion after they reproduced.

Septuples:

***sago-2(tm894) ppw-1(tm914)* I; C06A1.7*(tm887)* F58G1.1*(tm1019)* II M03D4.6*(tm1144)* IV *sago-1(tm1195)* V; *pes-10::gfp* X.**

pes-10::gfp males were mated with *sago-2(tm894) ppw-1(tm914)* I; C06A1.7*(tm887)* F58G1.1*(tm1019)*; M03D4.6*(tm1144); sago-1(tm1195)* hermaphrodites. GFP

positive F1 hermaphrodite progeny was picked onto individual plates. GFP positive F2 hermaphrodite progeny was picked onto individual plates. After GFP positive F2 mothers reproduced, they were harvested for PCR to determine *sago-2(tm894), ppw-1(tm914)*, C06A1.7*(tm887),* F58G1.1*(tm1019)*, M03D4.6*(tm1144),* and *sago-1(tm1195)* deletions. After these deletion alleles were homozygouzed, *pes-10::gfp* locus was homozygouzed by finding a GFP positive worm that segregates only GFP positive progeny.

***sago-2(tm894) ppw-1(tm914) ppw-2(tm1120)* C04F12.1*(tm1637)* I; F58G1.1*(tm1019)*; M03D4.6*(tm1144)* IV; *sago-1(tm1195)* V.**

sago-2(tm894) ppw-1(tm914); C06A1.7*(tm887)* F58G1.1*(tm1019)*, M03D4.6*(tm1144)*; *sago-1(tm1195)* males were mated with *sago-2(tm894) ppw-1(914) ppw-2(tm1120)*; C04F12.1*(tm1637);* F58G1.1*(tm1019)* hermaphrodites. F1 hermaphrodite progeny was picked onto individual plates. After F1 worms reproduced, they were harvested for PCR to determine C06A1.7*(tm887)* deletion, which indicates that F1 worm was a cross progeny. Enough number of F2 hermaphrodites were picked onto individual plates. After F2 worms reproduced, they were harvested for PCR to determine *ppw-2(tm1120)*, C04F12.1*(tm1637),* C06A1.7*(tm887)* , M03D4.6*(tm1144)*, and *sago-1(tm1195)* deletions. After that strains looked homozygote for all the deletions were selected, 20 F3 progeny was harvested for PCR to confirm that worms were homozygotes.

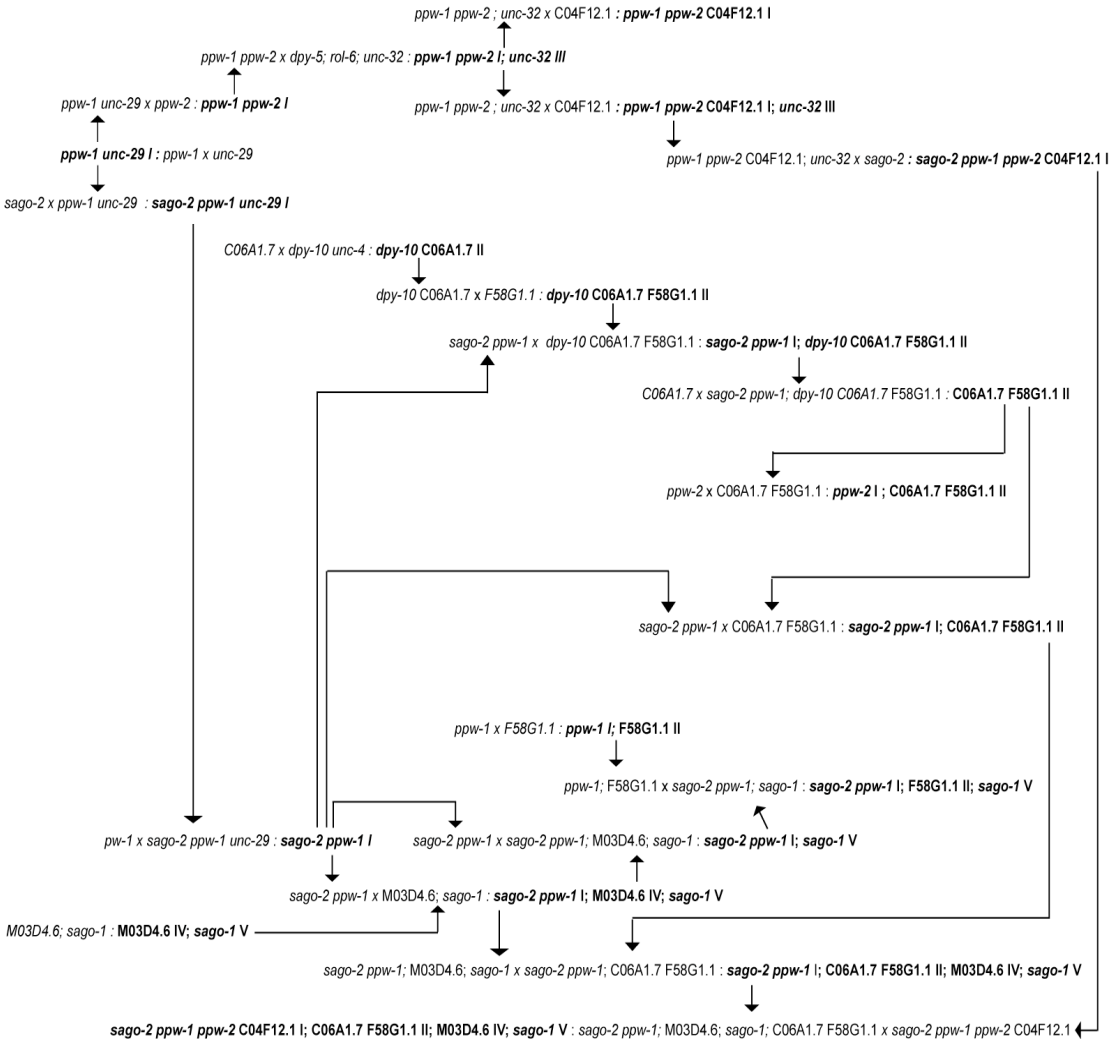


Figure II-11. Schematic representation of genetic crosses designed to build multiple fold mutants. For allele names and the details of the genetic crosses, please see the material and methods section of Chapter II.

Rescue Experiments

For *myo-3* promoter-driven expression in muscle, AGO ORFs were cloned into pPD96.52. Transgenic animals were generated by co-injection of the plasmid constructs at 10 µg/ml with the marker plasmid pRF4 (Mello et al., 1991) at 100 µg/ml. Extra chromosomal arrays were integrated by UV treatment (Evans, 2006). *ergo-1* rescued lines were generated by co-injecting a genomic PCR fragment produced using forward primer: ATG TTT CAA AAA AAG TTA TGG CC, and reverse primer: GAA AAA GAA TGA ATG AAC TGC, at a 5 µg/ml concentration, along with the marker plasmid pTG96 (Yochem et al., 1998), at 100µg/ml.

RNAi experiments

RNAi was carried out as previously reported (Fire et al., 1998; Timmons et al., 2001). Worms were grown on NGM plates containing 1 mM IPTG unless otherwise stated. The sequences used to generate short tandem RNAi triggers, as well as the complementary 2′-*O*-Methyl affinity matrices were: 5′-AAG GTA TTG ATT TTA AAG AAG ATG GAA ACA TTC TTG GAC A-3′ and 5′-TGT CCA AGA ATG TTT CCA TCT TCT TTA AAA TCA ATA CCT T-3′ (GFP food region 1); 5′-AAG TTT GAA GGT GAT ACC CTT GTT AAT AGA ATC GAG TTA A-3′ and 5′-TTA ACT CGA TTC TAT TAA CAA GGG TAT CAC CTT CAA ACT T-3′ (GFP food region 2); 5′-TTT CAA AGA TGA CGG GAA CTA CAA GAC ACG TGC TGA AGT C-3′ and 5′-GAC TTC AGC ACG TGT CTT GTA GTT CCC GTC ATC TTT GAA A;3′ (GFP food region 3); 5′-GGA TAT GTC GTT GAA CGT TTT GAG AAG AGA

GGT GGC GGT G-3′; 5′-CAC CGC CAC CTC TCT TCT CAA AAC GTT CAA CGA CAT ATC C-3′ (for unc-22 RNAi trigger). The non-specific 2′-*O*-Methyl oligonucleotide had the following sequence: 5′-CAU CAC GUA CGC GGA AUA CUU CGA AAU GUC-3′. The 2′-*O*-Methyl-modified RNA oligonucleotides were obtained from IDT. Biotin was attached to the 5′ end of the modified oligonucleotides via a six-carbon spacer arm.

Biochemistry and Molecular Biology

Protein and RNA purifications were performed as previously described (Duchaine et al., 2006). Western Blot analysis, imunoprecipitation of GFP tagged protein complexes, as well as 2′-*O*-Methyl oligonucleotide affinity matrix studies were performed as reported in (Hutvagner et al., 2004). To remove non-specific 2′-*O*-methyl oligonucleotide interactors the clarified worm lysate was pre-incubated for 45 min with an unrelated 2′-*O*-Methyl oligonucleotide.

Antibodies used in this study are as follows: (i) monoclonal HRP conjugated anti-HA (Roche), (ii) an affinity-purified polyclonal anti-RDE-1 antibody (Tabara et al., 2002) or (iii) Full-Length A.v. Polyclonal Antibody (BD Bioscience). Images were collected on a LAS-3000 Intelligent Dark-Box (Fujifilm). Northern Blot analysis was performed as described in (Duchaine et al., 2006).

Imaging and Video Microscopy

DIC and fluorescence images were collected as reported in (Duchaine et al., 2006).

Determining Start Codon (ATG) Sites by Splice Leaders

To confirm the start site of each predicted gene, we did 5′ RACE with SL1 and SL2 oligos. The PCR products were sequenced by additional gene specific oligos to find ATG codon.

***csr-1* 5′ RACE:**

csr-1 transcript receives SL2 sequence:

ggttttaacccagttactcaaggttaga**atg**cagtcggataatgtcggtcgtggccgtggaagccgtggtggttctcgtgga ggtagtggacgtggtagaggacgtggaggatacggagactcgtatcgtccggatttagaaacgagagaaacccatgtaa cgtatc

***ppw-1* 5′ RACE:**

ppw-1 transcript receives SL1 sequence:

ggtttaattacccaagtttgagagtctctcgaacc**atg**gaaaaacaactagaagctatgttcgtctcggacagacctgctgcc ccagctgcccaaaagcttggtaccgctccgctcgctgcaaaaaagacgagaaatgtggagaggggaaccaaggtcaata tcgataccaacatt

***ppw-2* 5′RACE**

ppw-1 transcript receives SL1 sequence:

ggtttaattacccaagtttgagagn...n**atg**cctgctacaccggttccaccggtaacaatgccaccagtgccaccagttggt
tttccaccggtcaccgctccgccaggacttcatccaccaccaccagtcccaccggttccggttccgactcttcctgtcacctc
ggaacacaaaactgctcacgatg

***sago-2* 5′ RACE:**

sago-2 transcript receives SL1 sequence.

ggtttaattacccaagtttgagagagtctctcgaact**atg**gaaaaacaactaaaagctatgtccgtctcggacaaacctgctg
ccccagctgcccaaaagcttggtaccgctccgctcgctgcaaaaaagacgagaaatgaggagtggggaaccaaggtca
atatcgataccaacattcgcaaattgacgatcaaaccgaatcagccaatttacaagtacgctgtgcaagtgaactacgtcttc
cggaaacctgatggaactgaggcgacaatcgaaatgtccaaatcagccaaaaagggaacggagcacgacaacgacaa
a

***sago-1* 5′ RACE:**

sago-2 transcript receives SL1 sequence.

nnnnnnnnnncccaagtttgagagagctactcctcacacacaacactcgcc**atg**tccaatatcacccaagtcaccagca
gcatggcttcagcctcactctccaacaaggctcctcttcccgtcggacatcaaccacttgcagagaaaaaaccaaaagagg
tcaatcaagaaggcacaccagtccaaatcgtcacaaatatgcggaaaatcaatctcgagaagaaccactcaatcttcaaat
actcggtgcaagtccttttgtttaccaaaagtcggacggcaccgagctcgtcctggaaaagtcaaagtccgtcggttctgg
atgtgaccatgagcgaagcaagagtcactgcctccgcgtc

C06A1.4 5′ RACE

C06A1.4 transcript receives SL1 sequence. The sequencing of a fragment of C06A1.4 cDNA with gene specific primers showed that there is an out of frame-shift mutation in exon 3 which causes an premature stop codon.

ggtttaattacccaagtttgnnngaggacaaaatngaaaag**atg**ccagcccttccatcagtctacacgccttctggagctcc atcgagcgtccacgcaccacctgccgttccaccagttccagttccaactcaaccgctaagatcggagtatcagacgtctaa cgacgcttgcatcaagagactggaagagctcaatatcgctc

M03D4.6 5′ RACE

M03D4.6 is predicted by the Wormbase as a gene encoding only PIWI domain containing protein. The predicted gene is only 116 bp long (371 amino acid long peptide). Our attempts to do 5′ race with the primers which is supposed amplify ~ 500 bp 5′ region failed. Therefore, we decided to sequence cDNA clone (yk224) with gene specific primers. The sequencing results showed that yk224 includes region upstream of predicted start site suggesting that the Wormbase prediction is wrong shown in bold cases below. The translation of sequenced fragmen of yk224 is always out of frame suggesting that M03D4.6 encoding an out of frame cDNA. However, to find real M03D4.6 ORF it is necessary to sequence remaining parts of this yk and to do 5′ RACE under new conditions.

Sequenced portion of yk224:

aactgactcctggaaagaaaatatacgtgcaccatgtaacggtgaactatgtgttcagaaaaaaagaccgctcacatgcttc tttcaaattgtccagttcaaggagaaggtcagcagaatacaacaggacagtttttcgtcacaacggatatgaaatccagccta

ttccatcctgatttccaaccactttccgagctgataatgtcgtataacctctattatcatggaataaaatgcaattccagagatgg

aaagaagctgtcacaagcattcaaaggactgaacatctctgtcaactatggcaaaaataaaaatctacaggaagatgcagt

gatgtttaagataaaggggttttcacattcttcaagagaacaaagatttgtgaacgaaggagtcgaaattactgtggagtctta

ttttagaagaaaattcggaattcatctaagatatccggaacttatgacagttgtggctgaaggaagaacatctttgctctactttc

caccggaattgatgttgtgtagtccatcgcagaaggtgaacagataga**atgattaacaacgaaggaaaggatcttgtca**

agatggcatctgcatctccattcattcgccatgatgtaacagaaagacttgttgaagaagtcggattgaaatcgaat

tacttcaacgattttatcactgtcggagaatccgttgaagtcgatggaattgtgcttccaactccgcgtattttcttccg

agatggacaagaaacttccttgaataaccaatccttcagaaacccaaccgattttgctcaaacgggatttttcgttga

cgccaagcagcagtacaatttccttcgattgattttattcacaataacgacgttcatgccgttttccggagagccaaa

aactcgggaaagcaaatcatattcctggtcactagaaagcattacgactatcataacttttttcaaaagtttggagca

ggaatacgatgttcttacccaagagattcatttcgagacagctagaaatttgtaccagagagctgaaacttgttcca

acattattaataaaactaatgtcaggcttggtggattgaattatgtggtcaacagtgagacttggaatgattctggat

tgttgttgattggactttcgacagcaccttatctcaattcgtattctagtgaaaatgtcacgacaattggctttgtctca

aacaccatggatcatccacaaaagtttgctggaggctacaaatacgtaaaatcaggaagtgacgttttcggtcagg

taatgcctgaaattcttctcaattccctcagaagtgcccgaaaagctcgaaaaatcaagcctatgaacattgttattt

acttgtgtgggatgagtgaaagtcggttcagtatcgtcaaggaagagtacgtcagaaattgtcattcggttttcaag

acgctcggagaaaaatatagtccccagttgacgattattgtgggatccaaaggccacagtacgagactttacgcga

gaggggaacgagaccaaatatcaaacctgcagccaggaaccattgttgactctgtaattgtgtctccagattataat

aaatttttccattgtggggcagttgcccgtcaagggacatgcaaagcaacgaaatatacagttttgtatccggagtc

accaaaaatggaatggattcagcggatgactaacgatttttgttatatgcatgagatcgtttttcatccagtcagcctt

ccagggccactataccttacagccgagatggccgagcgtggaactaaaaatctcgcggagaaaaatgaacctatc

attttccaaggaatcgttgattttgatgcgacaaatgcgaaatacgggtacagaaataaaggccttgccgacacta

gatttaacgcataagcccagtatttcaacacgtttttttttcagaaagtatttgtattttgcaaatttttttatttcgattttccttttttg aaattgattttcactgaaaagtaaattttcgatgggtttcccctgggtttttaattatttcaattgacttttttaaccaacatgttgtttg ttaagcttatttctttacacctgattttaacatttgaaccctttacaatcataaatctcaatattcaatattcaattccccacataaata tatttt

cDNA Clones Used in This Study:

yk233f7 (C06A1.4), yk54g10 (F20D12.1/csr-1), yk447b12 (F15B10.2), yk226c6 (C01B10.1)

Plasmid Used in This Study

myo-3::RDE-1::HA (pCCM867): Plasmid pCCM 865 (pHIT1) was amplified by oligos CMo7972 and CMo7974, PCR product was digested by Nhe I/Apa I and cloned into pPD96.52.

myo-3::GFP::K12B6.1/SAGO-1 (pCCM868): YAC containing GFP::K12B6.1/SA♂1 gene was amplified by oligos CMo7789 and CMo9534, digested by Nhe I/Apa I and cloned into pPD96.52.

myo-3:: GFP::SAGO-2/SAGO-2 (pCCM869): Y65B4A YAC DNA was amplified by CMo7793 and CMo7794, digested by Nhe I/Apa I and cloned into pPD96.52. Then, GFP cassette from pCCM115 was cloned into the resulting plasmid at Not I site.

myo-3::PPW-1 (pCCM870): Not I/Apa I fragment was removed from pEY137, and replaced with shorter Not I/Apa I fragment of pEY159.

myo-3::C04F12.1 (pCCM871): Genomic DNA was amplified by CMo11306 and CMo11307, digested by Nhe I/Age I, and cloned into pPD96.52.

myo-3::ALG-1 (pCCM872): pCCM863 was amplified by CMo7789 and CMo9535, digested by Nhe I/Apa I and cloned into pPD96.52.

myo-3::CSR-1 (pCCM873): Genomic DNA was amplified by CMo10389 and CMo9533, digested by Nhe I/Apa I and cloned into pPD96.52.

myo-3::PRG-1 (pCCM874): Genomic DNA was amplified by CMo10407 and CMo10408, digested by Nhe I/Apa I and cloned into pPD96.52.

myo-3::GFP::3′-UTR-unc-54 (pCCM875): pCCM115 was amplified by CMo7789 and CMo10222, digested by Nhe I/ Sac I and cloned into pPD96.52.

myo-3::F58G1.1 (pCCM876): YAC DNA containing F58G1.1 gene was amplified by CMo10687 and CMo10688, digested by Nhe I/Age I, cloned into pPD96.52.

myo-3::F58G1.1 (pCCM877): Pedro made this one.

CHAPTER III

ANALYSIS OF AN EIGHT-FOLD ARGONAUTE MUTANT (MAGO8) INDICATES THAT RNAI CONTRIBUTES TO THE MAINTENANCE OF STEM CELL TOTIPOTENCY IN *C. ELEGANS*

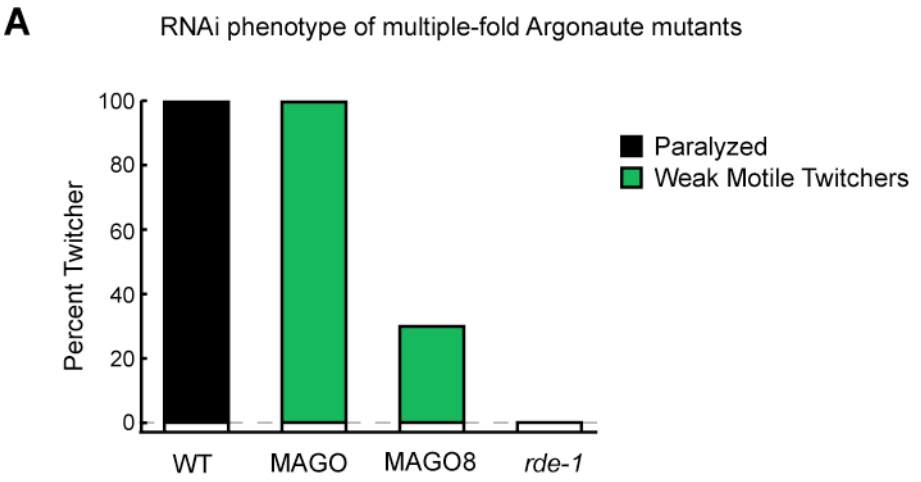
SUMMARY

Argonaute proteins function sequentially in the RNAi pathways. Argonaute member RDE-1 functions in the exo-RNAi pathway and binds to primary siRNAs. The components deleted in the six-fold Argonaute mutant (MAGO) function redundantly downstream of both exo- and endo-siRNA pathways, and bind to secondary siRNAs. While *rde-1* is strongly resistant to RNAi, the six-fold MAGO mutant exhibits resistance to dsRNA in a concentration dependent manner. Here we describe the phenotype of an eight-fold Argonaute mutant (MAGO8) that is more resistant to RNAi than the MAGO mutant, but is still weaker than *rde-1*, suggesting that more members of Argonaute gene family function at the downstream step of the exo- and perhaps the endo-RNAi pathways. Surprisingly, we found that there is somatic cell differentiation in the germline of the MAGO8 mutant, suggesting that RNAi may play an important role in maintaining the totipotency of germ cells.

RESULTS AND DISCUSSION

We previously showed that Argonaute proteins function sequentially in the RNAi pathways. RDE-1 is essential for the initiation of RNAi in *C. elegans,* and binds to primary siRNAs, derived from trigger dsRNA. A second subgroup of Argonaute proteins (MAGO) are functionally redundant downstream of *rde-1,* and are required for both exo- and endo-RNAi pathways. The components of the MAGO mutant bind to secondary siRNAs, downstream of RdRP amplification.

While *rde-1* mutant is strongly resistant to dsRNA, the MAGO mutant shows a weaker, dose dependent sensitivity to dsRNA, suggesting that additional Argonaute protein(s) function at the downstream step of RNAi pathways. To test this idea, we built an eight-fold Argonaute mutant called "multiple fold Argonaute mutant eight" or MAGO8, which is comprised of the original MAGO components (*sago-1, sago-2, ppw-1,* C06A1.4, F58G1.1, M03D4.6) along with *ppw-2,* and C04F12.1. We included these two Argonaute components in MAGO8 because PPW-2 resides in the same branch as F58G1.1 and C06A1.4, two components of MAGO, in the phylogenetic tree of Argonaute proteins, while C04F12.1 single mutant is weakly resistant to somatic RNAi (Figure I-2, Figure II-3D, and Figure II-10).



B RNAi rescue assays

	Mutant Strain					
Transgene	*rde-1(ne300)*	(n)	MAGO	(n)	MAGO8	(n)
none	-	(25)	-	(25)	-	(50)
myo-3::RDE-1::HA	+	(36)	-	(35)	n.t.	
myo-3::GFP::SAGO-2	-	(35)	+	(25)	+	(50)

Figure III-1(A,B). RNAi sensitivity of the MAGO8 mutant. (A) Sensitivity of wild type, MAGO, MAGO8, and *rde-1* strains to RNAi. Gravit adults were scored for the *unc-22(RNAi)* phenotype. (B) GFP::SAGO-2 rescues both MAGO and MAGO8 strains. L4 larvae were scored for the *unc-22(RNAi)* phenotype. Animals were cultured on *unc-22* dsRNA-expressing bacteria on the plates containing 0.1 mM IPTG. The (+) indicates Unc (RNAi-responsive) animals while (-) indicates NonUnc (RNAi-deficient) animals. One hundred percent of the animals scored (n) showed the indicated phenotype.

To test if the MAGO8 mutant is resistant to RNAi, we used *unc-22* feeding RNAi, which allowed us to score intermediate RNAi phenotypes. When wild type animals were exposed to *unc-22* RNAi at L2-L3 stage, all the animals became paralyzed once they reached adulthood (Figure III-1A). On the other hand, the MAGO animals did not show any sign of *unc-22* twitching phenotype until they became gravid adults, hours after they reached adulthood. Once the MAGO animals reached adulthood, 100% of the animals became weak mobile twitchers (Figure III-1A). In contrast, only 30% of the MAGO8 animals exhibited a weak mobile twitching phenotype after they became gravid adults, and the remaining 70% were not affected by *unc-22* RNAi (Figure III-1A). Overexpression of a single component of the MAGO mutant was sufficient to rescue the RNAi deficiency of the MAGO mutant. For example, *myo-3*::GFP::SAGO-2, whose expression in *rde-1* did not rescue RNAi deficient phenotype of *rde-1*, rescued the RNAi deficiency of the MAGO mutant. To test if *myo-3*::GFP::SAGO-2 transgene rescues the MAGO8 RNAi deficiency, we crossed *myo-3*::GFP::SAGO-2; MAGO transgenic strain into the MAGO8 mutant, and generated a *myo-3*::GFP::SAGO-2; MAGO8 transgenic strain. The *myo-3*::GFP::SAGO-2; MAGO8 transgenic strain was totally paralyzed on *unc-22* RNAi food in a similar manner to the *myo-3*::GFP::SAGO-2; MAGO transgenic strain (Figure III-1B). Thus, overexpression of a single component of the MAGO8 mutant could rescue the RNAi deficiency of the MAGO8 mutant.

In addition to the RNAi defects, we also observed developmental defects in the MAGO8 mutant. At low 15 ºC, the MAGO8 mutant did not exhibit any visible

developmental abnormality, and can be maintained indefinitely. However, when raised at 25 ºC, the MAGO8 animals had smaller brood size and became totally sterile after two generations. The *C. elegans* hermaphrodite has two mirror-image U-shaped gonad arms. Each arm contains proliferating germ cells in the distal arm, followed in the middle by meiotic germ cells, which enter gametogenesis in the proximal arm. The size of the MAGO8 gonad arms and the number of germ cells were comparable to that of the wild type, suggesting that germ cell proliferation (self-renewal) in the MAGO8 mutant was not disturbed. However, we found that some "germ cells" in the middle of the gonad, where normally the meiotic germ cells are, had abnormal cellular and nuclear morphology. For example, these "germ cells" were unlike normal germ cells whose nuclei were larger and flatter and were surrounded by a granular cytoplasm. Furthermore, about 5% (n=42) of the MAGO8 animals had gut granules, an intestine specific, birefringent, autofluorescent, lysosome-related organelles, in middle of the gonad (Hermann et al., 2005) (Figure III-2). Taken together, these "germ cells" in the MAGO8 mutant most likely lost their germ cell identity and instead "transdifferentiated" into somatic cells; for example, the intestinal cells described above. Our preliminary observations suggest that Argonaute proteins may be important for the maintenance of germ cell totipotency. Apparently, we need to perform further experiments to address that, first of all, if these soma cells are really derived from germ cells. To answer this question, we can put *glp-4 (bn2),* a germ-cell-deficient mutant, into MAGO8 background and examine the presence of these "transdifferentiated" cells in the gonads. Second, we need to examine if these "germ

cells" really lost their germ cell identity although they lost their germ cell morphology. We can stain these "germ cells" with antibodies that recognize P-granules, cytoplamic RNA-rich organelles that are specifically present only in germ cells. Third, although almost all the MAGO8 animals had "germ cells" with abnormal morphology in the middle of the gonad, only 5% of them had gut granule expressing cells. Two explanations for this discrepancy are that the "germ cells" are not germ cells any more but are not committed to other cell type either, or these "germ cells" "transdifferentiated" into other types of somatic cells, such as neurons, muscle or hypodermal cells other than intestinal cells. We can answer this question by staining these "germ cells" with antibodies that are specific for different cell types. Additionally we asked the question: are the germ cell "differentiation" phenotype caused by the accumulative effect of missing all the Argonaute components or by missing only *ppw-2* and C06A1.4? Construction of *ppw-2;* C04F12.1 double mutant, and examination of its phenotype would help answer this question.

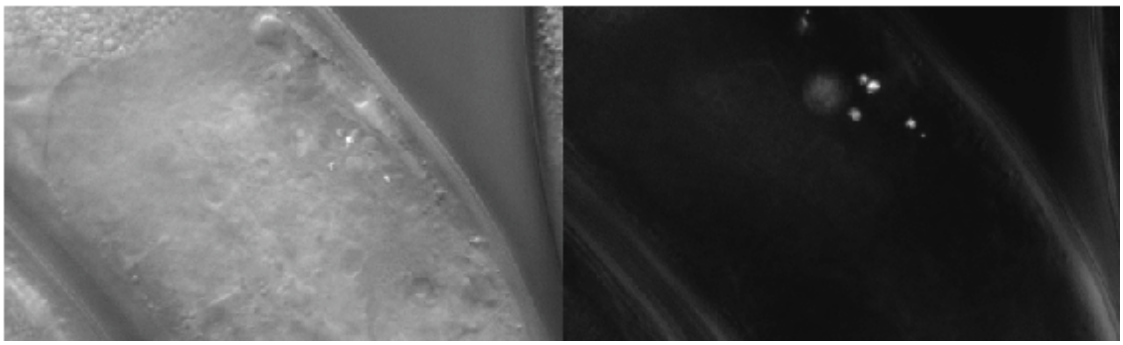


Figure III-2. Ectopic somatic cells in the germline of the MAGO8 strain. The panels show light (left) and fluorescence (right) micrographs of adult gonad with autofluorescent gut granules.

Future Experiments

We built an eight-fold Argonaute multiple fold mutant (MAGO8), and showed that this mutant was more resistant to RNAi than the original six-fold MAGO mutant. However, the MAGO8 mutant was still less resistant to RNAi than the *rde-1* mutant. Residual RNAi activity in the MAGO8 mutant suggests that additional Argonaute members are involved in the secondary steps of the RNAi pathway proposed in our model in Chapter II. Among the Argonautes deleted in the MAGO8 background, only C04F12.1 has the non-canonical catalytic residues required for the cleavage activity of the Argonaute proteins while others totally lack these residues (Figure II-7). There are at least 5 more Argonaute proteins in the *C. elegans* Argonaute family lacking these residues (Figure II-7). It is tempting to speculate that at least some of them are required in the secondary step of RNAi.

Argonaute family of proteins has been shown to be involved in germline maintenance in multiple organisms including *C. elegans* (Cox et al., 1998; Deng and Lin, 2002; Houwing et al., 2007). They have been shown to be required for germline stem cell self-renewal and sperm development. One interesting phenotype exhibited in the MAGO8 mutant background is the "differentiation" of the "germ cells" in the middle of the gonad. This result raises the question of how Argonaute proteins can regulate the totipotency of germ cells in *C. elegans*? Recent studies identified many endogenous small RNAs and miRNAs with unknown functions in *C. elegans* (Ambros et al., 2003; Ruby et al., 2006). We have shown in Chapter II that the MAGO components are required for the endo-siRNA production as MAGO either

failed to accumulate or dramatically reduced endo-siRNAs. Unpublished results from our laboratory (Pedro Batista and Craig Mello) have shown that the MAGO8 mutant completely failed to accumulate endo-siRNAs. The difference in the amount of endo-siRNA in MAGO and MAGO8 might explain why the MAGO8 background produced "transdifferentiated" "germ cells" while MAGO mutant did not, or there are yet to be identified classes of small RNAs that are missing in the MAGO8 but not in the MAGO mutant. It is worth noting that when the only Dicer homolog in *C. elegans*, *dcr-1*, is mutated, worms are sterile; however, they do not have "transdifferentiated" "germ cells" either. It has been proposed that endo-siRNAs are derived from dsRNA precursors that are generated by RdRP amplification, bi-directional transcription, or internal folding, and Dicer is required for processing these dsRNAs into ~22-nt siRNAs. The reason is that *dcr-1* mutant does not exhibit the "transdifferentiated" "germ cells" phenotype could possibly be because that in the *dcr-1* homozygous mutant, the maternal load is sufficient to compensate for its activity just enough to prevent the germ cells to "transdifferentiate." Alternatively, the production of the endo-siRNAs responsible to maintain the germ cell totipotency is independent of Dicer activity. The maternal DCR-1 activity theory is supported by the fact that homozygous *dcr-1* null allele develop further (sterile adult) than the F1 progeny of *dcr-1 (RNAi)* progeny (dead larvae), in which both the maternal and zygotic *dcr-1* activities are compromised (Grishok et al., 2001). It has been recently shown by two different groups that in *D. melanogaster,* the repeat associated small RNAs (rasiRNAs) are generated independent of Dicer activity, instead via a step wise

production of sense- and antisense- siRNAs from two Argonaute proteins, Ago3 and Aub (Brennecke et al., 2007; Gunawardane et al., 2007). So it is possible that the small RNAs responsible for germ cell totipotency are produced independent of DCR-1 activity. Nonetheless, it is important that these small RNAs be identified. Pyrosequencing, a high-throughput sequencing technology (Margulies et al., 2005) could be utilized to sequence the total small RNAs isolated from the MAGO8 and the MAGO animals. The small RNAs that are missing or dramatically reduced in the MAGO8 background compared to MAGO animals would be candidates involved in germ cells totipotency maintenance.

In mammals a few transcription factors including Oct4 and Sox2 have been implicated in the maintenance of pluripotency of embryonic stem cells (reviewed in Surani et al., 2007). No such factors have been identified so far in *C. elegans.* Interestingly, Ciosk et al. (2006) reported that when two RNA binding proteins, GLD-1 and MEX-3, are missing, germ cells transdifferentiated into somatic cells in *C. elegans,* a phenotype similar to MAGO8 described earlier in this chapter. It will be interesting to examine if MAGO8 is required for GLD-1 and MEX-3 protein localization in the germ cells and vice versa provided that MAGO8 antibodies are available. Both GLD-1 and MEX-3 are KH-domain containing RNA binding proteins implicated in the repression of protein translation. A few targets of these two proteins have been identified, one of which is histone H3 methytransferase (Fong et al., 2002). It is possible that in both *gld-1 mex-3* double mutant and MAGO8 mutant, the change of epigenetic status in germ cells cause them to transdifferentiate into

somatic cells. Interestingly, when a group of histone deacetylase complex genes are mutated, somatic cells exhibit germ cell like properties including expressing P-granules. These mutants are more sensitive to RNAi (Unhavaithaya et al., 2002; Wang et al., 2005) a phenotype contrary to that of MAGO8.

Material and Methods

Building the eight-fold Argonaute Mutant (MAGO8): *sago-2(tm894) ppw-1(tm914) ppw-2(tm1120)* C04F12.1*(tm1637)* I; C06A1.4*(tm887)* F58G1.1*(tm1019)*; M03D4.6*(tm1144)* IV; *sago-1(tm1195).*

sago-2(tm894) ppw-1(tm914); C06A1.7*(tm887)* F58G1.1*(tm1019)*, M03D4.6*(tm1144)*; *sago-1(tm1195)* males were mated with *sago-2(tm894) ppw-1(914) ppw-2(tm1120)*; C04F12.1*(tm1637);* F58G1.1*(tm1019)* hermaphrodites. F1 hermaphrodite progeny was picked onto individual plates. After F1 worms reproduced, they were harvested for PCR to identify the ones that had C06A1.7*(tm887)* deletion, which indicates that F1 worm was a cross progeny. Enough number of F2 hermaphrodites from F1 with C06A1.7 (tm887) deletion was picked onto individual plates. After F2 worms reproduced, they were harvested for PCR to identify worms that were homozygous for *ppw-2(tm1120)*, C04F12.1*(tm1637),* C06A1.7*(tm887)* , M03D4.6*(tm1144)*, and *sago-1(tm1195)* deletions. After that strains looked homozygote for all the deletions selected, 20 F3 progeny was harvested for PCR to confirm that worms were homozygotes.

CHAPTER IV

GENERAL DISCUSSION

Sequential Function of the Argonaute Family of Proteins in the Intersecting RNAi Pathways

In this thesis we examined the function of the Argonaute family of genes in *C. elegans*. Our data revealed that different Argonaute members are involved in distinct small RNA mediated pathways. Among those, we especially focused on the role of the Argonaute proteins involved in the exo- and endo-RNAi pathways. Our analysis of the Argonaute members involved in RNAi revealed that these proteins function in a sequential manner in the RNAi pathways, and interact with different species of small RNAs at each step. Interestingly, both exo- and endo-RNAi pathways employ distinct Argonaute proteins at the upstream step of each pathway, however they converge on a pool of the Argonaute proteins, which function redundantly at the downstream step.

The experiments designed in the Chapter II have clearly shown that RDE-1 binds to primary siRNAs, while SAGO-1 and SAGO-2, and perhaps the additional Argonaute proteins, bind to secondary siRNAs. Data from (Sijen et al., 2007) is in agreement with our own observation. It has been accepted that primary siRNAs derives directly from dsRNA trigger in the exo-RNAi pathway (Sijen et al., 2001; Tabara et al., 2002). However, the origin of secondary siRNAs has been elusive. Two models have been proposed for the generation of secondary siRNAs. In the first model, RdRP proteins, using either primary siRNA or mRNA as template, produces individual ~21-23 nt long siRNAs. In the second model, RdRP proteins replicate long stretches of target mRNA, which is then processed by Dicer into 21-23 nt long

siRNAs. It is unlikely that primary siRNAs are templates for RdRP proteins because it has been shown that the production of secondary siRNAs are dependent on the presence of target mRNA (Grishok, 2001; Sijen et al., 2001). Then the question remains is how secondary siRNAs are produced from target mRNA? Soon after this thesis project was finished, Plasterk and Fire labs reported that secondary siRNAs carry 5′ triphosphates, suggesting that secondary siRNAs are directly produced by the enzymatic activity of RdRPs (Pak and Fire, 2007; Sijen et al., 2007). However, data from Plasterk and Fire labs does not necessarily rule out the possibility that Dicer is involved in the production of secondary siRNAs. Moreover, data from Plasterk lab has shown that RDE-1::primary siRNA complex cuts mRNA, and RdRP replicates mRNA in a primer independent manner during RNAi, in agreement with our own data (see Model, Figure II-6).

The chemical structure of these secondary siRNAs suggests that they can be substrate for *pir-1*, an essential gene for RNAi, which encodes a putative phosphatase that specifically removes gamma and beta phosphates from RNA molecules (Deshpande et al., 1999). The wild type activity of *pir-1* may be required for the function of secondary siRNAs. We can test this idea, first, by comparing the phosphorylation status of secondary siRNAs in wild type and *pir-1* mutant animals. Second, we can check the phosphorylation status of secondary siRNAs in wild type and *pir-1* animals that makes complexes with SAGO proteins. An additional function for *pir-1* has been proposed by (Sijen et al., 2007). In their model, PIR-1 removes the phosphates from secondary siRNAs and feeds back these siRNAs (tertiary siRNAs)

to RDE-1 complex where they function as primary siRNAs to contribute to the production of secondary siRNAs.

In our model in the Chapter II, we depicted four parallel RNAi pathways. Each pathway is triggered by a different double stranded RNA molecule, which is processed into small RNAs by Dicer. However, it is worth noting that *C. elegans* may produce Dicer independent small RNAs as well. Our model represents that the Argonaute proteins have specifically evolved for each pathway. Recently, cloning of endogenous small RNAs indicated the existence of many different endogenous small RNA species. However, we do not know the function of these novel classes of small RNA species, and what Argonaute proteins interact with them. It is likely that the some of the small RNAs enter into the endogenous RNAi pathway and interact with the Argonaute members we described in our model. Alternatively, it is likely that these small RNA species interact with distinct Argonaute members and represent a new RNAi-related pathway that is not defined yet. For example, a member of Piwi subfamily, *prg-1,* exhibits reduced brood size and temperature sensitive sterility phenotype, but it is not involved in RNAi. Mammalian homologs of *prg-1* have been shown to interact with sperm specific small RNAs (Girard et al., 2006; Lau et al., 2006). Therefore, it is likely that *prg-1* protein may interact with sperm specific RNAs in *C. elegans*, and represent a new small RNA pathway.

A remarkable feature of the Argonaute proteins that function at the downstream step in the RNAi pathways is that they lack of the catalytic residues. However, Argonaute proteins that function at the upstream steps in the RNAi pathways contain

catalytic residues (DDH). We have built a multiple fold mutant (MAGO8) that contains mutations in 8 different Argonaute genes that encodes catalytically incompetent proteins. This mutant still sensitive to RNAi, albeit weakly, suggesting that more Argonaute members are involved in RNAi. All the RNAi pathways appear to converge on catalytically incompetent Argonautes. We do not know how many Argonaute protein function at the downstream step of RNAi pathways. In *C. elegans*, there are total 16 Argonaute members that lack DDH motif (Figure II-7). It is conceivable that several RNAi-related pathways may converge on these 16 Argonaute members. We can perhaps test this notion by building a 16-fold Argonaute mutant and analyzing its phenotype.

Does RNAi have a transcriptional component?

RNAi is associated with transcriptional silencing in various organisms; however, this association in *C. elegans* is not clear. If RNAi has a transcriptional component, what the function of the Argonaute proteins in this process is, and which Argonaute members are involved in this process remains unclear. Although this is discussed briefly in the Chapter II in the context of *csr-1* mutant phenotype, I believe this subject deserves more discussion in the context of the entire Argonaute gene family.

According to the two recent reports, RNAi in *C. elegans* can lead to the transcriptional silencing of a gene that is targeted by dsRNA (Grishok et al., 2005; Vastenhouw et al., 2006). This silencing effect can be inherited and requires the activity of canonical RNAi genes, *rde-1* and *rde-4,* and chromatin modifying

enzymes (Grishok et al., 2005; Vastenhouw et al., 2006). We have shown that CSR-1 is essential for chromosome segregation and is required for RNAi in germline. It is possible that, similar to the *S. pombe* Ago, CSR-1 interacts with small RNAs originated from chromatin and leads to the histone modifications that are required for proper chromatin structure. *csr-1* is also implicated in cosuppresion along with two other Argonaute members, *ppw-2* and C14B1.7/C16C10.3 (Robert et al., 2005). This is particularly interesting since we found that these three genes are also needed for RNAi (Figure II-10). It has been known that transgene silencing and co-suppression are closely associated with transcriptional repression (Kelly et al., 2002; Robert et al., 2005). The eight-fold Argonaute mutant, MAGO8, described in the Chapter III, is comprised of the original MAGO components (*sago-1, sago-2, ppw-1,* C06A1.4, F58G1.1, M03D4.6) along with *ppw-2,* and C04F12.1. According to our RNAi rescue experiments *ppw-1* and C04F12.1 appear to function at the downstream of the exo- and endo-RNAi pathways. This data suggests to me that both *ppw-2* and C04F12.1 may be executing their function at the transcriptional level. I envision a model where the secondary Argonautes, such as SAGO::secondary siRNA complex mediate mRNA degradation, while PPW-2::secondary siRNA and C04F12.1:: secondary siRNA complexes repress the transcriptional activity of the same gene. We can test this model by following experiments performed in the animals exposed to dsRNA. First, we can do nuclear run-on assays and compare the transcriptional initiation rates between wild type and mutant animals such as *ppw-2*, C04F12, MAGO, and MAGO8 animals. Second, we can do RT-PCR to compare pre-mRNA

and mRNA levels between wild type and mutant animals. The transcriptional repression should affect the levels of pre-mRNA. Conversely, post-transcriptional silencing should affect only mRNA levels, but not pre-mRNA levels. Third, we can perform chromatin immunoprecipitation (ChIP) experiments using antibodies against acetylated histone H3 or H4, and antibodies against the C-terminal domain (CTD) of polymerase II. While acetylated H3 and H4 normally associate with actively transcribed genes, histone deacetylation associates with transcriptional silencing. Additionally, we can check modifications at 5′ and 3′ termini of siRNAs that interact with PPW-2 and C04F12 to test if these siRNAs have characteristics of secondary siRNAs.

A genetic approach can initially be used to test the potential function of *ppw-2* and C04F12.1 in transcriptional silencing. In this experiment, we can simply inject *ceh-13* dsRNA or any other dsRNA described by Vastenhouw et al (2006) into wild type and mutant animals, and score the long-term Ceh RNAi phenotype after generations. I favor this approach because it allows not only the testing of *ppw-2* and C04F12.1, but also the remaining members of Argonaute gene family in a shorter time (Figure II-10 and Figure II-3D). Moreover, relevant multiple-fold mutants that we have generated can be tested by the same strategy. The experiments proposed above should help understand the function of Argonaute members in transcriptional silencing.

Conclusion

Over the past several years, there has been remarkable progress in the understanding of the function of RNAi and other small RNA-mediated pathways. These pathways regulate crucial biological processes in many organisms. However, we still do not know much about these small RNA-mediated pathways, and how their components interact with each other. Argonautes family of proteins are the key players of small RNA mediated pathways, and study of this family of proteins will greatly increase our knowledge on the RNAi-related pathways. *C. elegans* is a great model organism to study RNAi-related pathways because numerous Argonaute genes appear to evolve specifically for distinct small RNA-mediated pathways. Large scale sequencing of small RNAs has become possible with the advances in the sequencing technology (Margulies et al., 2005). I think we should take advantage of new high-throughout sequencing technologies, combined with genetics and biochemistry, and dissect the small RNA-mediated pathways. Studies of the small RNA mediated pathways in *C. elegans* should help us uncover the similar pathways in humans and other organisms, and hopefully lead us to the development of the new therapeutics that will improve human life.

CHAPTER V

APPENDIX

Table V-1. Primers used for 5′ RACE experiments. Primers are used with either splice ladder sequences (SL1 and SL2) for PCR or for sequencing of the PCR products.

Gene	Oligo Used
sago-1	CMo4790, CMo4791, CMo4792
M03D4.6	CMo4793, CMo4794, CMo4795
C06A1.4	CMo4796, CMo4797, CMo4798
F58G1.1	CMo4799, CMo4800, CMo4801
F55A12.1	CMo4802, CMo4803, CMo4804
ZK1248.7	CMo4805, CMo4806, CMo4807
R06C7.1	CMo4808, CMo4809, CMo4810
ZK757.3a	CMo4811, CMo4812, CMo4813
T22B3.2a	CMo4814, CMo4815, CMo4816
R09A1.1	CMo4817, CMo4818, CMo4819
T23D8.7	CMo4820, CMo4821, CMo4822
prg-2	CMo4823, CMo4824, CMo4825
C04F12.1	CMo4826, CMo4827, CMo4828
C14B1.7	CMo4829, CMo4830, CMo4831
C16C10.3	CMo4832, CMo4833, CMo4834
prg-1	CMo4835, CMo4836, CMo4837
csr-1	CMo4838, CMo4839, CMo4840
R04A9.2	CMo4841, CMo4842

Table V-2. Primers used to make dsRNA.
T7 promoter sequence was "ATTGCTAATACGACTCACTATAGGG."

Gene	Template	External Primers	T7-tagged primers
prg-1 (D2030.6)	yk15d12	n/a	CMo2599, CMo2601
prg-2 (C01G5.2)	yk561a4	n/a	CMo2599, CMo2601
ZK218.8	cDNA	CMo5156, CMo5157	CMo5158, CMo5159
rde-1	cDNA	n/a	CMo5514, CMo5515
ZK757.3	yk36h10	n/a	CMo2599, CMo2601
T22B3.2	cDNA	CMo8261, CMo8262	CMo5512, CMo5513
alg-1	cDNA	n/a	CMo5055, CMo5056
alg-2	cDNA	n/a	n/a
T23D8.7	cDNA	CMo4660, CMo4661	CMo4662, CMo4663
ergo-1	cDNA	CMo8263, CMo8264	CMo5510, CMo5511
C16C10.3	cDNA	CMo8283, CMo8284	CMo5502, CMo5503
C14B1.7	cDNA	CMo8265, CMo8266	CMo8267, CMo8233
T22H9.3	cDNA	CMo8234, CMo8235	CMo5504, CMo5505
Y49F6A.1	cDNA	CMo4644, CMo4645	CMo4646, CMo4647
R04A9.2	cDNA	CMo4648, CMo4649	CMo4650, CMo4651
csr-1	yk54g10	n/a	CMo2599, CMo2601
C04F12.1	cDNA	n/a	CMo4654, CMo4655
ppw-1	yk225a9	n/a	CMo2599, CMo2601
sago-2	cDNA	n/a	CMo5406, CMo5407
sago-1	yk227f8	n/a	CMo2599, CMo2601
M03D4.6	cDNA	CMo8236, CMo8237	CMo5506, CMo5507
C06A1.4	cDNA	CMo8238, CMo8239	CMo8240, CMo8241
F58G1.1	yk233f7	n/a	CMo5410, CMo5411
ppw-2	cDNA	CMo5152, CMo5153	CMo5154, CMo5155
F55A12.1	cDNA	CMo8242, CMo8243	CMo8244, CMo8245
R06C7.1	yk125b7	n/a	CMo2599, CMo2601
ZK1248.7	cDNA	CMo8246, CMo8247	CMo8248, CMo5418
par-1	cDNA	CMo4530, CMo4531	CMo4532, CMo4533

Table V-3. Argonaute deletion lesions.

Gene	Allele	Deletion Site	External Primer	Internal Primer
prg-1	*tm0872*	21432/21433-22072/22073 (640 bp deletion)	CMo7393 CMo7395	CMo7394 CMo7396
prg-2	*tm1094*	14468/14469-AAACAAGTGTTTAACAATTAAACAAGT-15533/15534 (1065 bp deletion + 27 bp insertion)	CMo8168 CMo8169	CMo8170 CMo8171
ZK218.8	*tm1324*	24010/24011-TTCGATTTCTACCTGAA-25015/25016 (1005 bp deletion + 17 bp insertion)	n/a	n/a
ZK757.3	*tm1184*	24969/24970-ttttcttc-25740/25741 (771 bp deletion + 8 bp insertion)	n/a	CMo11132 CMo11133
T22B3.2	*tm1155*	7953/7954-8282/8283 (329 bp deletion)	n/a	CMo12535 CMo12536
alg-1	*tm0369*	(805 bp deletion)	CMo7385 CMo7387	CMo7386 CMo7388
alg-1	*tm0492*	(610 bp deletion)	CMo7385 CMo7387	CMo7386 CMo7388
T23D8.7	*tm1163*	28137/28138-28572/28573 (435 bp deletion)	CMo8180 CMo8183	CMo8181 CMo8182
T23D8.7	*tm2154*	28607/28608-29053/29054 (446 bp deletion)	n/a	n/a
R09A1.1	*tm1860*	7750/7751-8908/8909 (1158 bp deletion)	n/a	CMo10536 CMo10537
C16C10.3	*tm1200*	32390/32391-32764/32765 (374 bp deletion)	n/a	CMo10372 CMo10373
C14B1.7	*tm1119*	22488/22489-22974/22975 (486 bp deletion)	CMo10523 CMo10524	n/a
T22H9.3	*tm1186*	14409/14410-G-14835/14836 (426 bp deletion + 1 bp insertion)	n/a	11196 11197
T22H9.3	*tm1332*	14521/14522-14918/14919 (397 bp deletion)	n/a	n/a
Y49F6A.1	*tm1127*	16762/16763-GN-17485/17486 (723 bp deletion + 2 bp insertion)	n/a	CMo11400 CMo11401
R04A9.2	*tm1116*	300/301-749/750 (449 bp deletion)	n/a	CMo11398 CMo11399
csr-1 /F20D12.1	*tm0892*	26731/26732-27131/27132 (400 bp deletion)	CMo7381 CMo7383	CMo7382 CMo7384
C04F12.1	*tm1637*	3597/3598-4395/4396 (798 bp deletion)	n/a	CMo8174 CMo8175
ppw-1	*tm0914*	2238/2239-2642/2643 (404 bp deletion)	CMo7389 CMo7391	CMo7390 CMo7392
sago-2	*tm0894*	40599/40600-GGGGGG-40867/40868	n/a	CMo8407

/F56A6.1		(268 bp deletion + 6 bp insertion)		CMo8408
ppw-1	*tm0914*	2238/2239-2642/2643 (404 bp deletion)	CMo7389 CMo7391	CMo7390 CMo7392
sago-2/ F56A6.1	*tm0894*	40599/40600-GGGGGG-40867/40868 (268 bp deletion + 6 bp insertion)	n/a	CMo8407 CMo8408
sago-1/ K12B6.1	*tm1195*	27251/27252-27672/27673 (421 bp deletion)	CMo8588 CMo8589	CMo8590 CMo8591
M03D4.6	*tm1144*	37469/37470- TCCAAANCNNNCCCTGGANCTTG GCCCGATGAG GAATNGAA TNCCAACCTT-38068/38069 (599 bp deletion + 51 bp insertion)	CMo8584 CMo8586	CMo8585 CMo8587
C06A1.4	*tm0887*	17503/17504-18100/18101 (597 bp deletion)	n/a	CMo8403 CMo8404
F58G1.1	*tm1019*	5 9529/9530-CTG-10100/10101 (571 bp deletion + 3 bp insertion)	CMo7832 CMo7833	CMo7834 CMo7835
F58G1.1	*tm2401*	10282/10283-10827/10828 (545 bp deletion)	n/a	n/a
ppw-2	*tm1065*	60230/60231-60975/60976 (745 bp deletion)	n/a	CMo11132 CMo11133
ppw-2	*tm1120*	59817/59818-60762/60763 (945 bp deletion)	n/a	CMo8176 CMo8178
F55A12.1	*tm1122*	31785/31786-32786/32787 (1001 bp deletion)	CMo7828 CMo7829	CMo7830 CMo7831
R06C7.1	*tm1414*	4662/4663-5644/5645 (982 bp deletion)	CMo11513 CMo11514	CMo11515 CMo11516
ZK1248.7	*tm1113*	3509/3510-4099/4100 (590 bp deletion)	n/a	n/a
ZK1248.7	*tm1135*	3536/3537-TT-3948/3949 (412 bp deletion + 2 bp insertion)	n/a	n/a

Table V-4. Primer sequences used in this study

CMo2599	TTG TAA AAC GAC GGC CAG
CMo2601	GGG AAC AAA AGC TGG AGC
CMo4530	CTC CAT CGT CAG GAT CTT CG
CMo4531	CAG CTC TTG GGC TAT TTT CG
CMo4532	CGC CGA GTT ATA AGC CTT CC
CMo4533	CAG CTC TTG GGC TAT TTT CG
CMo4644	CAT CAC CAA GAT GCC CAA AC
CMo4645	TTA TTT CTT CCC CGC TTT GC
CMo4646	GGG AAA ATG GGT CCA CAG AG
CMo4647	AGC AGA CCT GAT GAG CGA AC
CMo4648	TGG GTG AGA TGG GCT CTA AG
CMo4649	GAG CTC GAT TGC TCG TAA GC
CMo4650	AAG CTC AAT GCC TGC TCT TC
CMo4651	TTG TGC AAA CGA CTT TCC TG
CMo4654	AGC TGA CTT GAC GGA AAT CG
CMo4655	TTG GCA TGA CGC TTA GTG AC
CMo4660	TTC CGT CGA AAA AGC TGA AC
CMo4661	ATA CTG GCG AAG GAA TGG
CMo4662	TCG AGT GCC CGT TAG TTT TC
CMo4663	CGA TCA TAG CAC ATG CAT CC
CMo4790	GTT GTG GTT TGG AAC GAT
CMo4791	ACT CGT CAT TTT TGA GCT
CMo4792	AGA GAC AAC CTT GGC TGT
CMo4793	TGG CGA ATG AAT GGA GAT
CMo4794	CTG TCA TAA GTT CCG GAT
CMo4795	TTC CGA ATT TTC TTC TAA
CMo4796	AAC ACC TCC AGC TTG ATA
CMo4797	ACC CTT GG AAA AGA TCCA
CMo4798	GAA TGC TTT GAC CAT CGT
CMo4799	GTC TTT ACT GCG CCT TCT
CMo4800	ACT CCT CAT AGT CAC CCA
CMo4801	GAA TCG CAC AGC ACT TGT
CMo4802	CGT GGG GCA TAA ATC TCG
CMo4803	TGG CAT CCA GTT CTG AGA
CMo4804	TTG TGT AGA GAG TTG ATT
CMo4805	GCC ATT GTA TCC TGT CCT
CMo4806	GTC CTT TGT TAT CCA TCA
CMo4807	CAG CCG GTG GTG CGG AAA
CMo4808	TTG GCA TCC AGT TCT GAG
CMo4809	TCT TCT CAA CCG CAA GGA
CMo4810	AAC ACT TAT CGC GAC GAT
CMo4811	TCG AGA CGT GCA ACT GTA
CMo4812	TTA TAC GCA TTT CGT CTT
CMo4813	TAC ATC CCG GAT GA TGAA

CMo4814	TGA TCA TCC GGG AAT TCG
CMo4815	CAG CTTT CCA AAA AA TGA
CMo4816	CTT TGT CCA ATT TTC TAC
CMo4817	GCA AAC TCC TGG GCG TTG
CMo4818	CGG TTC GGC CAC CAC CGA
CMo4819	CTC CTC CGC GGT TTC CAC
CMo4820	TTG AAT AGA TTA GGT GAT
CMo4821	TGT GAA TTC CA TCGA AAA
CMo4822	CGA TAA GTC CGG GAT TCT
CMo4823	CTG ACA AGA GCG AAG TTC C
CMo4824	GCA TCG AAG CTT CGA CG
CMo4825	ATC GTA TTG AAG ATG TTG ATG G
CMo4826	GGA TGA TCA GAC GGA ATT GG
CMo4827	CAG ATT TCC AAG GCC TTC C
CMo4828	GAG CAA CTT TCA CGA TTT CC
CMo4829	GCG TCA CCA GTG TAG ACT CC
CMo4830	AAG AAA GTG GTG GCT TTT TCC
CMo4831	GGA ACA GAT GAT GAC GTC AAC
CMo4832	TTC GAC ACG AAT TTC CAT TC
CMo4833	GTC GTA AGC AAG GAA GTG GTG
CMo4834	GGA GTA GAG AAC ATT GGT GCA C
CMo4835	GCA CAG CAC GAG AAT CTC C
CMo4836	TTG TTT GCT TGA ATC GGA TG
CMo4837	CAA TCG GGA TGA AGT TGG
CMo4838	CGG AAA ATC GTT CGC TC
CMo4839	ATT TAG GAT CCA CGG TAG TGG
CMo4840	AAA TCA TGA CGG TCT CCT CC
CMo4841	AAC GAT TTT CCA TTC TTG TGG
CMo4842	AAA GTCA GCT TCT GTC AGA TGG
CMo4843	CAG TGG CTT TCG TTT TGG
CMo4844	CGA GGA ACC TTT AAT AGG ATC C
CMo4845	GCT GGA CAG CAA AAT GTG A
CMo4846	GAG AAT CTC GCC AAG AGA GG
CMo4847	AAA GAT TCA TGC TGG TGA AGA G
CMo4848	GGC GGT CAT GAA CAA TGA AG
CMo4849	CAT CGT GAG CAG TTT TGT G
CMo4850	GGC ATC ACT CAG AGA ATT TGG
CMo4851	ACG GGT TTC CAT GGA GAG
CMo4852	CCT GAT TTG GTC GCA CAC
CMo5055	GGT CGC TCA ATT CTT CTT CG
CMo5056	CTG CTG GTG GAT GAG TGA
CMo5152	AGT GCC ACC AGT TGG TTT TC
CMo5153	ATT GAG CTC CCA TGG TTT TG
CMo5154	CCG ACT CTT CCT GTC ACC TC
CMo5155	CGG ATT GAG AAT TCC TCT GC
CMo5156	ACT AGG CCT CTA CCC CTT CG

CMo5157	GCC AGC ACT GGA ATA GCT TG
CMo5158	TCA GTG CTA CTG GCT GTG TG
CMo5159	AGG AAG GGA GAT GGG TTT TC
CMo5502	TCG AAG TTG TCT CGC AGA AAG G
CMo5503	CTG CCG CAT AAA GAA CAT CTG G
CMo5514	TTC GGC ATG AGA AGA AGC AGA C
CMo5515	CGA CAT TCC AGG GTA CTTC ACG
CMo5931	GAA AAA CAA CTA AAA GCT ATG TCC GTC
CMo7381	GGC CTT TCT CTG CGT TTA GT
CMo7382	GCC CTG AAC ATC TTC GGG CT
CMo7383	CGC AGC GTT TCG GTC AGG AT
CMo7384	TCA GGA TGA GCA ACG TCC AT
CMo7389	CGT ACT TTT TTG GGC CAG AG
CMo7390	CGA ATG GCG CCA TTC TGT TA
CMo7391	TTG GTTA CCG GCG AAC GCA A
CMo7392	CTT CGA GGT TGA CTG GGT CT
CMo7393	CTG TAG AGG ATC AAG CGG CT
CMo7394	AGT CGT GGT ACA GAT CGT AG
CMo7395	GGG ATA TCT CGC AAC GAC TA
CMo7396	GAG AGG CCG TGG TTC AGG AT
CMo7789	AAA GCT AGC ATG GCG GCC GCG AGT AAA G
CMo7793	AAA GCT AGC ATG GCG GCC GCG
CMo7794	AAA GGG CCC AAT TAT TTA ATT GCC TTT TTT GTT CG
CMo7828	CCC TAG TAA GGA AGT GAC GT
CMo7829	CCG ATG CTC CAC TCC TGT AG
CMo7830	CGT TCT TGA CCG TCG CGA TA
CMo7831	CTG TGT TGA AGG AGC CAA GA
CMo7832	CAT TGG GGT TTA GAA CTC CT
CMo7833	GTT CCG ACT CAA CCG TTA AG ATA CTT
CMo7834	ATA CTT GGT TCC AGC AGG TG
CMo7835	ACG TCT AAC GAC GCT TGC AT
CMo7972	AAA GCT AGC ATG TCC TCG AAT TTT CCC G
CMo7974	AAA GGG CCC CAC GGG TTC TGA AAT TAC AGA TTT C
CMo8168	CAG CAC CAT CTC TGT AGA GA
CMo8169	CTG GGA ACT ATC CAA CCC GA
CMo8170	ATC TTA CTT CGG ACG AAG TG
CMo8171	CGG GCC AAC CAT CAA AAT GA
CMo8174	TCG TTAC GAC GTC AGT GTT C
CMo8175	AAC GCA GCT TGG CTC GTC AC
CMo8176	CGC AGC CTC ACG CAA ACG AT
CMo8178	ACA ATG CCT GCT ACA CCG GT
CMo8233	GAA CAC TTC ACC AAG CTT TTG C
CMo8234	CAT TTA CAC CAG TGG TGA GG AAG
CMo8235	CGC ATC TCC TCG CTA ACA AGA
CMo8236	CGT CAC AAC GGA TAT GAA ATC C
CMo8237	TGT CGG CGA GGC CTT TAT TTC

CMo8238	CGT TGT TCT ATC AGT ACA GCG TTC
CMo8239	GCA GAT GAT GCT GGT CAA CTC TC
CMo8240	TGA AGT TAC TTT CAC CAA GAA GGG A
CMo8241	AAC ACT TGG AAT GTT ATC CA TGGT
CMo8242	GTG ACC CAC CAT GTG AAA CG
CMo8243	GCT TCT CGT TGA AAT CAG TTT GC
CMo8244	TTT TCC ATA CGG ATA AAA TCA TGG
CMo8245	TGT AAC CGA TTT CAT TCG TCA AC
CMo8246	GCT TGC AAG AAG AGA GGA ATG ATA
CMo8247	GGA AAC ATC CAT CTT CTC GGA
CMo8248	GTT TTG CAT ACT CGT TGG CA
CMo8261	GCT GAC TAG TTC AGG ATC TGG TTC AC
CMo8262	CAT TCA TCC AGA GAA CGT GAT ATC TC
CMo8263	GAT AAG GGT CTC GGC CAG AAG
CMo8264	GCC GAT CTG TGT GGA
CMo8265	GTT CAT CGC TCA ACA GGA CC
CMo8266	TCT CGA GTG CTC TTT GTA
CMo8267	CGG ACA TGC ATG AAC CGT CA
CMo8283	CCG AAG GAA GAA TGG AAA TTC
CMo8284	CTT TGA TGA TGC GGT TCT CA
CMo8584	GAT CTG CCT AGT TTT CCC CA
CMo8586	ACT CGT TCC CCT CTC GCG TA
CMo8585	CCA GCC TAT TCC ATC CTG AT
CMo8587	TCC GAG CTG ATA ATGT CGT A
CMo8588	CAC AAC ACT CGC CAT GTC CA
CMo8589	CCG GTT TTG GAA GAA CGA GT
CMo8590	AAG TCA CCA GCA GCA TGG CT
CMo8591	CGA GTC CAG TCA CAC TCT CT
CMo9533	AAA GGG CCC AAG AAA TCT TCC AGT GAA CAT ATG TG
CMo9534	AAA GGG CCC TCA GAA AAG TTG CCT AAA ACC CC
CMo9535	AAA GGG CCC TTG GAC AAT GTA ATC AAC TGC GAG
CMo10222	AGA GCT CTT AAG CGG CCG CTT TGT ATA GTTC
CMo10372	CAT GGC CGA CTT GCT CGA CA
CMo10373	TTG GAC AGC TTG TCG CAG AA
CMo10389	GCTAGCGTTAGAATGGCGGCCGCACAGTCGGATAATGTCGGTCG
CMo10407	AGC TAG CAT GGC GGC CGC AGC ATC TGG AAGTGGTCGCGG
CMo10408	AGG GCC CCT CAA GCA TGG TAA ATT ACC CTC
CMo10523	TTC ACC TAC TCA GCC TGT CT
CMo10524	CAG ACC AAA ATG GAC GAC GT
CMo10536	ACC CGA GCT TCC GGA TGT GT
CMo10537	TCT CGG TGG ATC ATG AGT AA
CMo10687	CTCTCGCTAGCATGGCGGCCGCGCCAGCTCTTCCTCCAGTCTACAC
CMo10688	CTC TCA CCG GTT CAT GCG TTG ACA CGA CGA CC
CMo11132	CTT CCT TCG ATC TTC CGT AC
CMo11133	GTT AGG CTA GCT GTA AGC AT
CMo11306	GAAGGGCTAGCATGGCGGCCGCACCATCAAAGAAGAACAAAAAG

	GCC
CMo11307	GAAGGA CCG GTT TAA GCA ATT CTC TTA TTT TTG AAA GAGC
CMo11400	ATC AAG AGC CAT ACC GCA TCC
CMo11401	TTC CCC ATC TAC AAG TCT GAC
CMo12535	CGT ACT TCT CGG AGA AGT AG
CMo12536	CGC AAT GGT TAG AAG TCG CA

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