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The Function of Secondary Argonautes in the ALG-3/4 Sperm Small RNA pathway in C. elegans

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Abstract

The mechanism of RNA interference is one that contains a great amount of complexity and is dependent upon many proteins and enzymes within the cytoplasm of living cells. The nematode, *C. elegans*, proves to be a great experimental model because it contains a complex repertoire that is close in relation to mammals, but resides in different forms that are not 22- and 26G- RNAs. Scientists have identified a total of 24 argonautes in *C. elegans*. However, there is still uncertainty as to the specific function of some of these worm-specific argonautes. Since we already know that primary siRNAs and argonautes lead to the production of secondary argonautes, the question remains as to which secondary argonautes are involved in the ALG-3/4 pathway and can the method for how they regulate their targets be uncovered. From male and sperm gene expression datasets, we have identified candidate secondary Argonautes that could be involved in the downstream function of the ALG-3/4 pathway (Unpublished data, Mello Lab). Here we report that the addition of the genetic mutants *wago-1*, *wago-9,* and *wago-10* produce significantly lower broods and their compounded effect can be seen in the overall phenotype of the worm. In addition, further investigation is needed to determine whether any of the WAGO mutant strains in this study have a similar temperature sensitive male sterility due to a sperm defect in spermiogenesis, which emulates that of the *alg-3/4* double mutant phenotype.

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1 Introduction

1.1 Introduction to RNAi

The world of molecular medicine is constantly growing and can be largely attributed to the discovery of new scientific and biological mechanisms. These discoveries allow scientists to think of new engaging questions and even question certain beliefs that were once thought to be absolutely true. RNA interference is one particular scientific finding that has shaped the way molecular biologists view and postulate about gene regulation. The hope is that this extremely complex mechanism can be fully understood and used for therapeutic purposes through its ability to decrease the expression of pathogenic genes, most notably oncogenes (Mello, 2004). For this purpose, it is imperative that the numerous pathways that exist within the realm of RNA interference are extensively studied and understood.

1.2 Discovery of RNAi

The discovery of RNA interference came about from experiments in the early 1990's that incorporated scientists testing color manipulation of petunias. Scientists were attempting to change the color of the petunia from a normal shade of purple to a very intense, dark shade of purple by injecting mRNA encoding a pigmentation gene that would exaggerate the color if overexpressed. In a strange turn of events, the color of the petunia turned either partially white or completely white. This suggested that the gene suppressed the production of the pigment rather than amplified it. In the late 1990s, Dr. Craig Mello and Dr. Andrew Fire first discovered RNA interference in animals while using the nematode, *C. elegans*, to study other cellular processes. Through several experiments of theirs, Dr. Mello and Dr. Fire determined that this newly founded gene-silencing mechanism was triggered by double-stranded ribonucleic acid (dsRNA) and existed in many living organisms (Mello, 2004). Soon after this discovery, Dr. Mello and Dr.

Fire labeled this mechanism as RNA interference (RNAi) and in 2006 were awarded The Nobel Prize in Physiology or Medicine for their discovery.

1.3 *Caenorhabditis elegans*

Caernorhabditis elegans, the free-living nematode for which experiments on RNA interference are easily conducted, possesses many notable features that make it such a widely used experiment model system (**Figure 6A & 6B**). *C. elegans* has proved to be a very important experimental model system in studying cell differentiation due to its simple and easily manageable genome. The genome of the nematode possesses approximately one hundred million base pairs, which is roughly 1/30 the size of the human genome. The determining of the complete sequence for *C. elegans* genome has proved to be very beneficial in RNAi research, especially since its 20,000 genes are equal in number to the human genome. The nematode can be easily grown and genetically manipulated making research more convenient for researchers. Another important trait that makes the *C. elegans* such a desirable experimental model system is that it's transparent, which makes it a powerful tool for cell biology research (Cooper, 2009). Interestingly, *C. elegans* are comprised of both hermaphrodites and males and recognition of the males is only possible by observing their distinctive arrow-shaped tail.

1.4 The RNAi mechanism

The RNAi mechanism occurs in the nematode and exists within many living organisms and plays an important role in defending living cells against parasitic genomic elements such as transposons and viroids, as well as regulating gene expression. The mechanism of RNA interference is one that contains a great amount of complexity and is dependent upon many proteins and enzymes within the cytoplasm of living cells. The "Central Dogma" in the world of molecular biology is the genetic flow of information from the DNA to the RNA, which then is used as a template to synthesize proteins. It was found that RNA can be regulated post-

transcriptionally by RNAi and small RNAs to control of gene expression (Joshua-Tor, 2010). The RNAi mechanism is initiated by introduction and recognition of dsRNA by an enzyme called Dicer. Dicer can then cleave this dsRNA into 21-22 nucleotide (nt) small interfering RNAs (siRNAs). siRNAs are loaded onto Argonaute (AGO) proteins and then target specific messenger RNAs (mRNAs) that have sequences complementary to the siRNA by base pairing (Fischer, 2010). These Argonaute proteins can be classified into three paralogous groups: Argonaute-like proteins, which are similar in comparison to *Arabidopsis thaliana* AGO1 and human AGOs; Piwi-like proteins, which are more closely related to *D. melanogaster* PIWI (P-element induced wimpy testis), but only in animals and expressed in the germline; and the *Caenorhabditis elegans*-specific argonautes WAGO (worm-specific Argonaute) (Simard, 2008) (**Figure 3**). Argonaute proteins have been identified in gene silencing in most organisms, which include plants, fungi, protozoans and metazoans including humans (Conine, 2010).

Soon after the discovery of RNAi, by supplying exogenous dsRNA it was determined that many organisms, including plants and mammals, could produce antisense small RNAs (~20 – 30nts) targeting mRNAs and other genomic sequences naturally (Mello, 2004). Rather than living cells receiving dsRNA by either viral dsRNA or injection of dsRNA, dsRNA can come from within by naturally occurring processes. It has been recently discovered that there are triggers other than dsRNA, most notably single-stranded RNA (ssRNA). These endogenous small RNAs include miRNAs, piRNAs and endo-siRNAs (Vasale, 2010). microRNA (miRNA) act as the post-transcriptional regulators and RNA sequences that bind to target mRNAs via complementary sequences to induce gene silencing. miRNAs are genomically encoded genes that encode for RNAs that form double-stranded hairpins (Fischer, 2010). These hairpins are recognized by a series of enzymes to create the mature 21-22 nucleotide miRNA that can be

loaded into miRNA-specific argonautes to form miRISC complexes. The miRISC complex can then target mRNAs through imperfect complementary base pairing to regulate the mRNAs either post-transcriptionally or translationally (Fischer, 2010).

1.5 Pathways involved in Endogenous RNAi

The Piwi-interacting RNAs (piRNAs) are a class of 24-31 nucleotide RNAs that are processed in a Dicer-independent manner to regulate targets in animals. This class of small endogenous RNAs associates with Piwi proteins and play a significant role in transposon silencing in flies, while they are restricted mostly to male germ cells. The generation of piRNAs relies instead on the endonuclease activity of Piwi-like proteins. It has been observed through experiments involving the experimental model system, *D. melanogaster*, that PIWI can promote the production of piRNAs by increasing the transcription of piRNA loci that are localized in the subtelomeric regions (Simard, 2008). piRNAs, in *C. elegans* known as 21U-RNAs, are a class of germline specific RNAs that target and repress selfish genomic elements (Batista, 2006). Other endogenous small pathways have been described in a variety of organisms. However, the organism to date with the largest complement of these pathways is *C. elegans*. There are many Endo-RNAi pathways in *C. elegans* including the miRNA pathway, the 21U-RNA pathway or piRNA pathway, the CSR-1 22G-RNA pathway required for chromosome segregation and the WAGO 22G-RNA pathway required for genome surveillance (**Figure 5**).

microRNAs, piRNAs, and endogenous siRNAs are conserved across the phyla. Of all the small RNAs, endo-siRNAs are the least well studied. The nematode, *C. elegans*, proves to be a great experimental model because it contains a complex repertoire. Through scientific research experiments using worms, it is possible to better understand the role and interactions of the endo-siRNAs in the worm that may have analogous functions in mammals, including humans.

Scientists have identified a total of 24 argonautes in *C. elegans* including 12 worm-specific. However, there is still uncertainty as to the specific function of some of these worm-specific argonautes. The one dilemma faced by scientists is the redundancy and specific function of these argonautes across the scope of Endo-RNAi pathways. We do know that exogenous RNAi trickles into the WAGO pathway. Scientists know this by eliminating all 12 WAGOs in the exogenous RNAi pathway, thus inducing no RNAi. This mechanism involves dsRNA triggering the Dicer enzyme to cleave the dsRNA, followed by the loading of the anti-sense strand onto the RDE-1 Argonaute. RDE-1 is thought to recruit an RNA-dependent RNA-polymerase (RdRP) to then exploit the target mRNA as a template for the production of secondary small RNAs, also termed 22G-RNAs. The 22G-RNAs are then loaded onto WAGOs to be brought to either the cytoplasm for mRNA turnover or to the nucleus for transcriptional silencing (Yigit, 2006). One of the most interesting molecular actions is the involvement of the worm argonautes that leads scientists to question how involved is the WAGO pathway in the plentiful downstream steps of RNAi.

1.5.1 CSR (22G-RNAs)

As mentioned previously, the CSR pathway is responsible for the production of 22G-RNAs in the germline of *C. elegans.* The CSR-1-interacting small RNAs (22G-RNAs) are members of a class of endogenous small RNAs that are neither microRNAs nor piRNAs. Through experimental research it has been deduced that the CSR-1-interacting small RNAs are antisense to thousands of germline-expressed protein-coding genes. CSR-1 and other cofactors such as the RNA-dependent RNA polymerase EGO-1, DRH-3, and a Tudor-domain protein EKL-1 localize to chromosomes and are required for proper chromosome segregation (Claycomb, 2009). These profuse small RNAs are typically 22 nucleotides in length and contain a 5’ triphosphate and a strong affinity for a 5’ Guanosine (Ambros et al., 2003). The genes that

are usually targeted by the CSR-1 22G-RNAs are genes that have mRNAs expressed in the germline, embryos, and oocytes. CSR-1 and other protein components of the 22G-RNA pathway localize to P granules, which are found in close proximity on the cytoplasmic face of nuclear pores. The localization to P granules is a very similar action that takes place with the worm-specific Argonaute 1 (WAGO-1) of the WAGO pathway. It is also now known that WAGO-1 interacts with a nonoverlapping set of 22G-RNAs that function to target transposons and other pseudogenes. This suggests that the 2 separate germline 22G-RNA pathways share core components while both functioning to physically maintain the genome by chromosome segregation and suppression of transposons and other harmful elements (Claycomb, 2009). The worm-specific Argonaute (WAGO) pathway is primarily responsible for silencing transposons, pseudogenes, cryptic loci, and certain protein-encoding genes.

1.5.2 Piwi-interacting RNAs (21U-RNAs)

piRNAs in *C. elegans* primarily function to mediate the genome-wide surveillance of germline transcripts and seek out foreign sequences while, simultaneously, endogenous germline-expressed genes are actively protected from piRNA-induced silencing (Shirayama, 2012). It is known that the PRG-1 protein is vital for initiation in silencing transgenes that are contrived to contain complementarity to endogenous 21U-RNAs (Gu, 2012). The *prg-1* gene itself is the regulator for germline development and fertility, but is not fully involved in transposon silencing in the germline. The *prg-1* gene has been important in discovering the correlation between the 21U-RNA and WAGO pathways. Through examining any changes in 22G-RNA levels it was determined that the worm-specific Argonaute (WAGO) pathway was the pathway primarily affected by the *prg-1* mutant gene (Gu, 2012). The increased mRNA expression of WAGO mRNA targets *prg-1* mutant worms also indicates that the 21U-RNA and WAGO endogenous pathways are interconnected. This leads scientists to further believe that the

21U-RNA pathway, though separated, progresses into the WAGO pathway and affects the functions of these worm-specific argonautes. Though PRG-1 produces 21U-RNAs that target transposons there is a secondary step, which leads to the production of 22G-RNAs that are loaded into the worm-specific argonautes (WAGO) to target mRNAs (Gu, 2012).

1.5.3 Worm Argonautes (WAGOs) (22G-RNAs)

The WAGO pathway is involved in the production of 22G-RNAs. These worm-specific argonautes mainly function in the cytoplasm, though NRDE-3, WAGO-9, and WAGO-10 can serve to function in the nucleus. Therefore, these are some of the few known argonautes that can bind siRNAs produced by RNA-dependent RNA Polymerases that act upon mRNA templates within the cytoplasm and reallocates to the nucleus of the cell. It was only recently discovered that WAGO-9 and WAGO-10 are found in the nucleus as well (Buckley, 2011). This is extremely important for the execution of RNAi in the nuclei of *C. elegans*. With a total of 24 distinct Argonaute proteins in *C. elegans*, many carry out different functions in the germline. These 22G-RNAs that target mRNA, including those in the CSR endogenous pathway, are referred to as Secondary argonautes.

1.5.4 ERI (26G-RNAs)

A class of 26 nucleotide small RNAs called the 26G-RNAs were first identified in deep-sequencing datasets in *C.elegans* as part of the previously described Dicer-ribonuclease-dependent endogenous small RNA pathway, called the ERI endo-RNAi pathway, and a subset of these were noted to be enriched for spermatogenesis-expressed mRNA targets (Conine, 2010). The ERI pathway can be broken down into an embryonic pathway and a sperm pathway. Both the embryonic and sperm pathways involve RNA-dependent RNA polymerase-mediated small RNA biogenesis; these small RNAs are loaded into different argonautes (ERGO-1 embryo)

ALG-3/4 sperm (Pavelec, 2009). ALG-3/4 is required for the biogenesis of 26G-RNAs and components of the ERI pathway (Han, 2009).

1.6 Project mission

It is well known that these endogenous RNAi pathways (the 21U-RNA, CSR, WAGO, and ERI pathways), including the ALG-3/4 pathway, contain primary small RNAs and argonautes that result in the production of 22G-RNAs. One interesting similarity between all of these endogenous RNAi pathways is that they ultimately channel into the worm-specific argonautes in downstream steps. One of the questions to be answered is since it is already known that primary siRNAs and argonautes lead to the production of secondary argonautes, which secondary argonautes are involved in the ALG-3/4 pathway and uncovering how these argonautes regulate their targets. From male and sperm gene expression datasets, candidate secondary argonautes that could be involved in the downstream function of the ALG-3/4 pathway have been identified (Unpublished data, Mello Lab). Using a combination of genetics and molecular biology can provide further insight into which of these secondary argonautes are required for the function of the ALG-3/4 sperm small RNA pathway and identify those that are temperature-sensitive sterile.

2 Materials and Methods

2.1 Worm Strains

Caernorhabditis elegans culture and genetics were essentially as described (Brenner, 1974). The Bristol strain (N2) was used as the standard wild-type strain. *fog-2* (q71) was used during propagation in order to increase the population of males in each strain. *fog*-2 is a germline specific sex determination gene that is required for hermaphrodite spermatogenesis. Other alleles used in this study were as follows: *wago-1* (*tm1414*), *wago-9* (*tm1200*), *wago-10* (*tm1132*), *c14b1.7* (*tm1119*), and *csr-1*.

2.2 Screening for Candidates

The genes selected for use in this study were selected as candidates from genome wide mRNA and small RNA sequencing datasets from males and sperm that focused on locating the genes involved in the spermatic pathway. These secondary argonaute candidates are thought to play a role in the downstream steps of ALG-3/4 included: *wago-1* (*tm1414*), *wago-9* (*tm1200*), *wago-10* (*tm1132*), and *c14b1.7* (*tm1119*).

2.3 PCR amplification for mutant verification

Amplifications were conducted in 20 µL reactions containing 13.4 µL of deionized water, 2.5 µL of 10x Ex Taq Buffer, 2.0 µL of dNTPs, 1.0 µL of Forward and Reverse primer each, and 0.1 µL of Ex Taq polymerase. Samples were amplified by the process; *wago-1* primers: 59.0°C Annealing temperature, 2:00 min. Elongation time; *wago-9* primers: 55.0°C Annealing temperature, 1:20 min. Elongation time; *wago-10* primers: 55.0°C Annealing temperature, 1:30 min. Elongation time; *c14* primers: 55.0°C Annealing temperature, 1:30 min.

Elongation time and then repeated for 30 cycles. Following amplification, 5 µL of 6x sample loading dye containing bromophenol blue tracking dye was added to each sample, which contained 25 µL before addition. Samples were then ran on a 1% agarose gel with a 100bp and 1kb ladder at 100V until the tracking dye had migrated three fourths of the length of the gel. Upon completion, the 1% agarose gel was observed under the UV light and a digital photo was taken showing the ethidium bromide stained DNA in each lane.

2.4 Creating the *wago-9* (*tm1200*); *wago-10* (*tm1132*) double mutant

Crosses were performed on NGM (Nematode Growth Medium) mating plates using 7 males of the *wago-9* strain and 3 hermaphrodites of the *wago-10* strain, as well as vice versa. Crosses were placed at 20°C for approximately 24 hours. The hermaphrodites for each cross were picked from each mating plate and single-picked to separate OP50 plates. The hermaphrodites were then placed back at 20°C for approximately 24 hours. At the end of the 24 hours, plates were observed and scanned for males to determine whether crosses were successful or not. For final verification that the desired strain was acquired, 4 – 5 L1 progeny from the single-picked hermaphrodite OP50 plates were placed in 5 µL of solution for worm lysis. The worm lysis solution consisted of 2 µL protease K and 100 µL worm lysis buffer. Worm lysis polymerase chain reaction (WL-PCR) for the *wago-9; wago-10* strain was prepared in 5 µL reactions of a worm lysis buffer/Protease K mixture in order to lyse the worms. Each tube contained 4 -5 young progeny (L1 or L2) worms that were picked into PCR tubes filled with the worm lysis buffer/Protease K mixture and was number labeled (i.e. #1-10).

2.5 Creating the *wago-9;c14;wago-10* triple mutant

Crosses were performed on NTGD mating plates using 7 males of the *wago-9;c14* strain and 3 hermaphrodites of the *wago-10* strain, as well as vice versa. Crosses were placed at 20°C for approximately 24 hours. The hermaphrodites for each cross were picked from each mating plate and single-picked to separate OP50 plates. The hermaphrodites were then placed back at 20°C for approximately 24 hours. At the end of the 24 hours, plates were observed and scanned for males to determine whether crosses were successful or not. For final verification that the desired strain was acquired, 4 – 5 L1 progeny from the single-picked hermaphrodite OP50 plates were placed in 5 µL of solution for worm lysis. The worm lysis solution consisted of 2 µL protease K and 100 µL worm lysis buffer. Worm lysis polymerase chain reaction (WL-PCR) for the *wago-9; c14;wago-10* strain was prepared in 5 µL reactions of a worm lysis buffer/Protease K mixture in order to lyse the worms. Each tube contained 4 -5 young progeny (L1 or L2) worms that were picked into PCR tubes filled with the worm lysis buffer/Protease K mixture and was number labeled (i.e. #1-10).

2.7 Creating the *wago-9;c14;wago-10;wago-1* quad mutant

Crosses were performed on NTGD mating plates using 7 males of the *wago-9;c14;wago-10* strain and 3 hermaphrodites of the *wago-1* strain, as well as vice versa. Crosses were placed at 20°C for approximately 24 hours. The hermaphrodites for each cross were picked from each mating plate and single-picked to separate OP50 plates. The hermaphrodites were then placed back at 20°C for approximately 24 hours. At the end of the 24 hours, plates were observed and scanned for males to determine whether crosses were successful or not. For final verification that the desired strain was acquired, 4 – 5 L1 progeny from the single-picked hermaphrodite OP50 plates were placed in 5 µL of solution for worm lysis. The worm lysis solution consisted of 2 µL

protease K and 100 µL worm lysis buffer. Worm lysis polymerase chain reaction (WL-PCR) for the *wago-9; c14;wago-10;wago-1* strain was prepared in 5 µL reactions of a worm lysis buffer/Protease K mixture in order to lyse the worms. Each tube contained 4 -5 young progeny (L1 or L2) worms that were picked into PCR tubes filled with the worm lysis buffer/Protease K mixture and was number labeled (i.e. #1-10).

2.8 Brood size counting for all strains

Once all desired genotypes and mutant strains were acquired (*wago-1*, *wago-9*, *wago-10*, *c14*, *wago-9;c14*, *wago-9;wago-10*, *wago-9;wago-10;c14*, and *wago-1;wago-9;c14; wago-10*, each strain was grown synchronously to maintain the population. Simultaneously, 15-20 hermaphrodites were single picked onto OP50 plates for each strain. The hermaphrodites were placed at both 20°C and 25°C for a period of 24 hours. The hermaphrodite for each strain on every plate was picked to a new OP50 plate after the initial 24-hour time had elapsed; the previous plate that the hermaphrodite resided on to lay eggs was then counted and recorded. This process was repeated until each hermaphrodite (original mother) had stopped laying eggs for a period of two consecutive days. For all strains, the brood size counting for each hermaphrodite was recorded daily and tallied at the end of the brood size counting process for comparative analysis.

3 Results

From genome wide mRNA and small RNA sequencing datasets from males and sperm, candidate secondary argonautes were identified that could be involved in the downstream function of the ALG-3/4 pathway (Unpublished data, Mello Lab). Each WAGO strain used in this study, as well as CSR-1, was selected from an initial experiment (Conine) that observed levels of gene expression in the Endogenous RNAi Pathway. These worm argonautes were also selected due to their proximity to one another according to the Argonaute Tree (**Figure 3**). In order to test the brood size for each of the proposed mutant strains, each mutant strain needed to be created. Using standard genetic cross techniques, the mutant strains were successfully created and propagated to use in multiple rounds of brood size counting.

3.1 Schema and brood analysis: *wago-9; wago-10* (Double) mutant

The genetic cross map (**Figure 4A**) was used to generate the desired phenotype of *wago-9*;*wago-10*. In the first type of cross, *wago-9* males were mated with *wago-10* hermaphrodites. The reciprocal involved *wago-10* males that mated with *wago-9* hermaphrodites. *wago-9* and *wago-10* mutant strains were both propagated to acquire males used for both types of crosses. This particular cross was especially important because it established a base in setting up the rest of the mutant crosses in this experiment.

The brood size analysis (**Figure 7**) clearly shows that the *wago-9* and *wago-10* single mutant strains showed a much higher brood at 25^{o}C than at 20^{o}C. *wago-9* single mutant strain produced an average brood count of approximately 212 at 20^{o}C while the same strain at 25^{o}C produced an average count of 130, a reduction of over 40%. Similarly, *wago-10* single mutant strain exhibited a dramatic decrease from 20^{o}C to 25^{o}C: 211 to 117, respectively. The *wago-*

9;wago-10 double mutant strain produced an almost identical brood size to that of both *wago-9* and *wago-10* single strains at 20^{o}C, 214. However, over a 4-fold decrease in brood size was observed in the double mutant at 25^{o}C (average brood size of 51) when compared to the *wago-9* and *wago-10* single mutant strains. The more severe decrease in brood size means that they may act in synergy and can be considered related in the same pathway. As these 2 genes, *wago-9* and *wago-10*, are believed to be linked and participate in the same pathway, it is worth investigating whether the addition of a third mutant gene will lower the brood size counts at 25^{o}C and generate a similar yet more severe phenotype.

3.2 Schema and brood analysis: *wago-9; c14; wago-10* (Triple) mutant

Similarly to the double mutant strain, the triple mutant was created by crossing *wago-9;c14* and *wago-10* mutant strains. The genetic cross map (**Figure 4B**) was used to generate the desired phenotype of *wago-9;wago-10;c14*. *wago-9;c14* and *wago-10* worms both propagated to acquire males used for both types of crosses. In the first type of cross, *wago-9;c14* males were mated with *wago-10* hermaphrodites. The reciprocal involved *wago-10* males that mated with *wago-9;c14* hermaphrodites. Though this is a similar schematic to how the double mutant strain was created, it is a necessary construct due to the known proximity of the genes. The *c14* gene is known to be located almost directly next to the *wago*-9 gene on chromosome III within the *C.* elegans genome. Verification was done using wago-9 and wago-10 primers for the same set of samples/worm candidates (**Figure 1A & 1B**). A discrete band seen near 400 base pairs indicated a homozygous mutant, thus indicating that worm candidate is the Double mutant.

The brood sizes for each of the *wago-9;c14* and *wago-10* mutant strains were both well above 200 (224 and 211, respectively) at 20^{o}C (**Figure 7**). The same strains at 25^{o}C both saw decreases of approximately 50% as *wago-9;c14* brood dropped from 224 to 124 at the elevated

temperature while *wago-10* decreased from 211 to 117 (same data from the comparative analysis for the double mutant). The newly engineered *wago-9;c14;wago-10* triple mutant strain shows remarkably different counts in brood size. Triple mutant at 20^{o}C produced at baseline brood count of 221. At 25^{o}C a dramatic decrease in number of progeny was seen in the Triple mutant at 25^{o}C. This decrease in brood size was nearly four-fold as the counts at 20^{o}C (221) dropped to 61 at 25^{o}C. As previously demonstrated in the double mutant, the compounded decrease in brood size counts by adding mutant genes translates that they act in sync with one another within the same pathway. One item to take note of is the slight increase in brood size from the double to the triple mutant at the elevated temperature. However, the resulting dramatic decrease in progeny count in the Triple mutant strain still proves that these genes are related and function in the same pathway. To examine whether the addition of another mutant strain has a compounded effect on the phenotype of these *C. elegans*, the addition of another mutant gene was conducted.

3.3 Schema and brood analysis: *wago-9; c14; wago-10; wago-1* (Quad) mutant

The last of the *C. elegans* worms to be genetically engineered and constructed was the Quad mutant. The genetic cross map (**Figure 4C**) was used to generate the desired phenotype of *wago-9;wago-10;c14;wago-1* (Quad). *wago-9;c14;wago-10* (Triple) and *wago-1* worms both propagated to acquire males used for both types of crosses. In the first type of cross, *wago-9;c14;wago-10* males were mated with *wago-1* hermaphrodites. The reciprocal involved *wago-1* males that mated with *wago-9;c14;wago-10* hermaphrodites. However, this genetic cross was executed by mating Triple mutant males with *wago-1* hermaphrodites. Contrary to the double and triple, only crossing Triple mutant males with wago-1 mutant hermaphrodites created the Quad mutant. Verification for the Quad mutant was conducted by PCR analysis and used wago-1, wago-9, and wago-10 primers for the same set of samples/worm candidates (**Figure 2**). The

clear, lone band in lane 8 of the samples using the wago-1 primers indicated the desired homozygous mutant or Quad mutant strain.

Having known the brood size from the newly genetically engineered Triple mutant, the thought was that the brood size for the Quad mutant would be significantly lower than that of the Triple and Double mutant strains. The Triple mutant brood of 221 was significantly higher than that of the *wago-1* at 20^{o}C, which was 93. At an elevated temperature of 25^{o}C, the *wago-1* single mutant strain produced a lower average brood size compared to the Triple mutant. Triple had produced an average brood size of 61 while *wago-1* generated an average progeny count of 44. The brood size analysis for the Quad mutant showed that the addition of the *wago-1* mutant gene has decreased the average brood. At 20^{o}C the Quad mutant produced an average brood of 83 while decreasing in brood size by over 50% at 25^{o}C to result in an average of 40.

4 Discussion

4.1 Key findings

The purpose of this study was to analyse and determine the role of a group of particular secondary argonautes and how they are regulated in the ALG-3/4 sperm small RNA pathway. Through genetically engineering the *C. elegans*, each mutant strain phenotype was able to be monitored and provide insight into the possible interactions and consequences of mutating these particular secondary worm argonautes. Key findings from this study include: 1) In creating the quad mutant, a successful genetic cross could only be obtained by crossing triple males with *wago*-1 hermaphrodites, 2) the brood sizes did not progressively decrease from the double to triple to quad mutant strain as was originally hypothesized, however, the combination of the mutations generally resulted in a lower brood size than the single mutant strains on their own, 3) the phenotype for each of the genetically engineered mutant strains appeared to become more "sluggish" and "sick" with each mutant cross made (double→ triple→ quad), and 4) that *wago-1* showed a very similar phenotype to that of the quad mutant in brood size, mortality rate, and physical appearance.

4.2 The Quad mutant genetic cross was successful only one particular way

The genetic crosses for creating the double and triple mutant strains proved to be relatively easy compared to designing the quad mutant. Designing the double mutant strain was made possible by crossing *wago-9* males with *wago-10* hermaphrodites and vice versa. The same concept could be used for creating the triple mutant strain: crossing *wago-9*;*c14* males with *wago-10* hermaphrodites and vice versa. It was noticed when trying to create the quad mutant that the *wago-1* strain appeared very unhealthy and would become increasingly unhealthy with each day that passed while propagating the population to the maintain the population. Initial attempts at crossing *wago-1* into the triple mutant were unsuccessful and took significantly

longer to engineer the quad because crossing wago-1 hermaphrodites into triple mutant males could only successfully make the cross. The lack in ability for the *wago-1* males to fertilize the triple mutant hermaphrodites could signify that there is a large defect in the male sperm. This could be an important finding for scientists that are interested in a better understanding for the ALG-3/4 pathway and how it promotes thermotolerant sperm development. Consequently, this could also preface uncovering the ways in which *wago-1, wago-9, wago-10* and *c14* are regulated for fertility, germline maintenance, and development in a multitude of organisms (Conine, 2010).

4.3 Brood sizes did not decrease with each genetic cross

The most glaring finding in this study concerned the brood sizes for each mutant strain. Each of the mutant strains used were temperature-sensitive sterile and showed lower brood counts at elevated temperatures. This parallels a prior finding that core body temperatures for most mammals are lethal to sperm and external male gametogenesis (Conine, 2010). Since each of the mutant strains produced a lower amount of progeny the finding was not unexpected. However, the lack of pattern or correlation between the double, triple, and quad brood counts was extremely fascinating. Though the triple mutant contained an additional mutation compared to the double mutant, it generated a higher brood at 25^{o}C. This means that the addition of the *c14*-mutated gene did not increase the defectiveness in the male sperm and rather encouraged a slight increase in sperm production and effectiveness. The slight increase in brood size count from the Double to Triple mutant indicates that *c14* mutant gene, though close in proximity to *wago-9* on chromosome III, does not increase defectiveness in male sperm (C.C. Conine, personal communication).

Though the brood size counts did not progressively decrease with each mutant cross, the difference in counts between the single mutant worms and the combined mutant worms were especially significant. At an elevated temperature of 25^{o}C, both *wago-9* and *wago-10* single mutant strains had average progeny counts of over 117 while *wago-9*;*c14* mutant had an average count of 124, which is significantly higher than *wago-1* single mutant's brood of 44. Meanwhile each of the combined mutant strains obtained brood counts of 61 or lower. This dramatic decrease shows that the combined interactions of these genetic mutations lead to a more temperature-sensitive sterile worm. Interestingly, *csr-1* mutants produced the lowest brood size of all strains in this study with an average count of 1.5 at 25^{o}C. Prior studies showing that CSR-1 interacts with chromatin at target loci and protein-coding domains to promote their proper organization within the holocentric chromosomes of *C. elegans* are consistent with these findings since the *csr-1* mutants have also been proven to have severe chromosome segregation defects. The ultimate result of this chromosome segregation defect is embryonic lethality, which can lead to a very low brood count.

4.4 Quad mutant phenotype is due to the loss of *wago-1*

From the brood size analysis in the combined WAGO mutants and *wago-1* single mutant a comparison to the *alg-3*;*alg-4* and MAGO (multiple Argonaute) strains can be drawn. The *alg-3*;*alg-4* double mutant was reported to cause complete temperature-sensitive sterility, which is the same for the MAGO-12 mutant. Since both MAGO-12 and *alg-3*;*alg-4* mutant strains cause complete sterility at elevated temperatures and *csr-1* mutants produce an almost completely sterile worm with known chromosome segregation defects, it can be inferred that the combination of the mutations that exist within both the quad mutant and *wago-1* single mutant interfere with proper chromosome segregation as well, but to a lesser degree. Though this comparison can be made, it does not shown that *wago-1* is involved at the ALG-3/4 pathway.

During the propagation process, before the brood size analysis was conducted, initial observations of the phenotypes for each of the strains was noted. *wago-9* and *wago-10* single mutants did not display any signs of significant morphological issues. These would include overall size of the worm and changes in organelle structure. In addition, *wago-9*; *c14* did not display any eye-catching deformities or abnormalities even with the additional *c14* mutated gene. The first of the notable morphological issues and abnormalities were initially observed in the *wago-9*;*wago-10* double mutant. The double mutant's organelles were intact and were of proper size in relation to the other single mutants. However, the double mutant strain appeared to be a bit less mobile than the previously mentioned single mutant strains. The most notable of the mutant strains were both the *wago-1* single mutant and the quad mutant. These strains were both very "sluggish" and "sick" in appearance, which could translate to something else happening within the worm's genome that is causing these strains to appear very dysfunctional. The quad mutant and *wago-1* single mutant also had the highest mortality rates, which was not exhibited by any of the other strains in the study. The high death rate for both can be attributed to the sickness and abnormalities that were initially seen in the two strains during propagation.

4.5 Future Experiments

The collective findings and results of this study can direct scientists towards further research and experiments in order to better understand the roles that these worm argonautes play in the endo-RNAi pathway. Though initially planned as part of this study, immunocytochemistry could be used to find where these WAGOs are localized in the male sperm and germline of each mutant strain. This would be important since the phenotype for these strains have been acquired and have a better understanding for what is occurring on the "outside"; the next step is acquiring information as to what is happening "inside". Another experiment worth considering is to cross the double, triple, and quad mutant strains with the N2 wild-type strain to observe whether there

is an increase in the broods or not. This would provide insight as to whether there is a partial rescue observed from mating these mutant strains with the wild-type strain, as previously conducted with MAGO-12 mutant by Conine et al., 2010. Lastly, it has been shown that *wago-1* mutants have a sterility phenotype, but not yet known if this is male-specific. Further experimentation into the gender specificity of the *wago-1* mutant phenotype can be conducted to close this gap.

The creation of the double, triple, and quad mutant strains have shown that there is not a compounded effect on the thermotolerance of male sperm. Though there is a dramatic decrease in the number of progeny for all of the genetically engineered mutant strains, the brood size counts tell us that the compounded mutations in these genes lead to what appears to be a defect in the male sperm, but cannot be confirmed until further experiments are conducted. In conclusion it has been shown that the phenotype of the quad is very similar to *wago-1* mutant, which means that the phenotype observed in the quad mutant is due to the loss of *wago-1*. Further experimentation is required in order to answer whether any of the WAGO mutants have a similar phenotype to that of the ALG-3/4 mutants, which is temperature-sensitive male sterility due to a sperm defect in spermiogenesis and if the ALG-3/4 pathway is feeding into the WAGO pathway in downstream steps that regulated the function of these small RNAs.

5 References

Brenner, S. (1974, May). The Genetics of Caenorhabditis Elegans. *Genetics*, 77, 71-94.

Buckley, B. A., Burkhart, K. B., Gu, S. G., Spracklin, G., Kershner, A., Fritz, H., ... (2012). A nuclear argonaute promotes multigenerational epigenetic inheritance and germline immortality. *Nature, 489*(7416), 447. doi: 10.1038/nature11352

Claycomb, J. M., Ketting, R. F., Conte, D., Mello, C. C., Batista, P. J., Pang, K. M., ... (2009). The argonaute CSR-1 and its 22G-RNA cofactors are required for holocentric chromosome segregation. *Cell, 139*(1), 123-134. doi: 10.1016/j.cell.2009.09.014

Conine, C. C., Batista, P. J., Gu, W., Claycomb, J. M., Chaves, D. A., Shirayama, M., & Mello, C. C. (2010). Argonautes ALG-3 and ALG-4 are required for spermatogenesis-specific 26G-RNAs and thermotolerant sperm in caenorhabditis elegans. *Proceedings of the National Academy of Sciences of the United States of America, 107*(8), 3588-3593. doi: 10.1073/pnas.0911685107

Fischer, S. E. J. (2010). Small RNA-mediated gene silencing pathways in C. elegans. *The International Journal of Biochemistry & Cell Biology, 42*(8), 1306-1315. doi: 10.1016/j.biocel.2010.03.006

Guang, S., Bochner, A. F., Pavelec, D. M., Burkhart, K. B., Harding, S., Lachowiec, J., & Kennedy, S. (2008). An argonaute transports siRNAs from the cytoplasm to the nucleus. *Science (New York, N.Y.), 321*(5888), 537-541. doi: 10.1126/science.1157647

Han, T., Manoharan, A. P., Harkins, T. T., Bouffard, P., Fitzpatrick, C., Chu, D. S., ... (2009). 26G endo-siRNAs regulate spermatogenic and zygotic gene expression in caenorhabditis elegans. *Proceedings of the National Academy of Sciences of the United States of America, 106*(44), 18674-18679. doi: 10.1073/pnas.0906378106

Hutvagner, G., & Simard, M. J. (2008). Argonaute proteins: Key players in RNA silencing. *Nature Reviews Molecular Cell Biology, 9*(1), 22-32. doi: 10.1038/nrm2321

Joshua-Tor, L., & Hannon, G. J. (2011). Ancestral roles of small RNAs: An ago-centric perspective. *Cold Spring Harbor Perspectives in Biology, 3*(10), a003772-a003772. doi: 10.1101/cshperspect.a003772

Lee, H., Gu, W., Shirayama, M., Youngman, E., Conte, J., Darryl, & Mello, C. C. (2012). C. elegans piRNAs mediate the genome-wide surveillance of germline transcripts. *Cell, 150*(1), 78.

Mello, C. C., & Conte, D. (2004). Revealing the world of RNA interference. *Nature, 431*(7006), 338-342. doi: 10.1038/nature02872

Pavelec, D. M., Lachowiec, J., Duchaine, T. F., Smith, H. E., & Kennedy, S. (2009). Requirement for the ERI/DICER complex in endogenous RNA interference and sperm development in caenorhabditis elegans. *Genetics, 183*(4), 1283-1295. doi: 10.1534/genetics.109.108134

Shirayama, M., Seth, M., Lee, H., Gu, W., Ishidate, T., Conte, J., Darryl, & Mello, C. C. (2012). piRNAs initiate an epigenetic memory of nonself RNA in the C. elegans germline. *Cell, 150*(1), 65.

Vasale, J. J., Gu, W., Thivierge, C., Batista, P. J., Claycomb, J. M., Youngman, E. M., ... (2010). Sequential rounds of RNA-dependent RNA transcription drive endogenous small-RNA biogenesis in the ERGO-1/Argonaute pathway. *Proceedings of the National Academy of Sciences of the United States of America, 107*(8), 3582-3587. doi: 10.1073/pnas.0911908107

WormAtlas. (n.d). *Introduction to C. elegans anatomy.* Retrieved Apr 4, 2013, from WormAtlas: http://wormatlas.org/hermaphrodite/introduction/Introframeset.html

Yigit, E., Mello, C. C., Batista, P. J., Bei, Y., Pang, K. M., Chen, C. G., ... (2006). Analysis of the C. elegans argonaute family reveals that distinct argonautes act sequentially during RNAi. *Cell, 127*(4), 747-757. doi: 10.1016/j.cell.2006.09.033

Figures

A)

B)

Figure 1. Verification of the Triple mutant strain by Polymerase Chain Reaction
PCR pictures showing verification for confirming the Triple mutant strain. The red arrows are directed at candidate worm #34, which is the appropriate candidate. Figure 1A displays a PCR gel using wago-10 primers. The red box encompasses the distinctive band that represents the desired homozygous mutant. Figure 1B displays a PCR gel using the same samples as in Figure 1A with the exception of using wago-9 primers and, therefore, no such distinctive bands should be seen.

Figure 2. Verification of the Quad mutant strain by Polymerase Chain Reaction

Picture of PCR showing the verification for the Quad mutant strain. Eight candidates were analyzed using 3 different primers sets (wago-1, wago-9, and wago-10). The red arrow indicates the correct worm candidate due to the distinctive band using the wago-1 primer set.

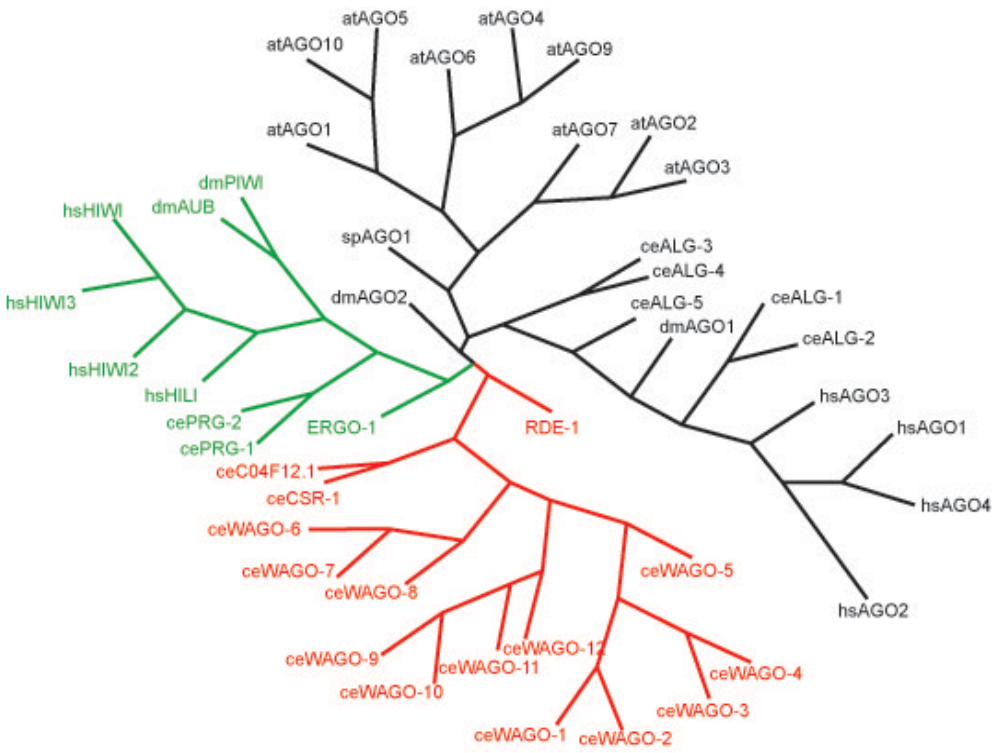
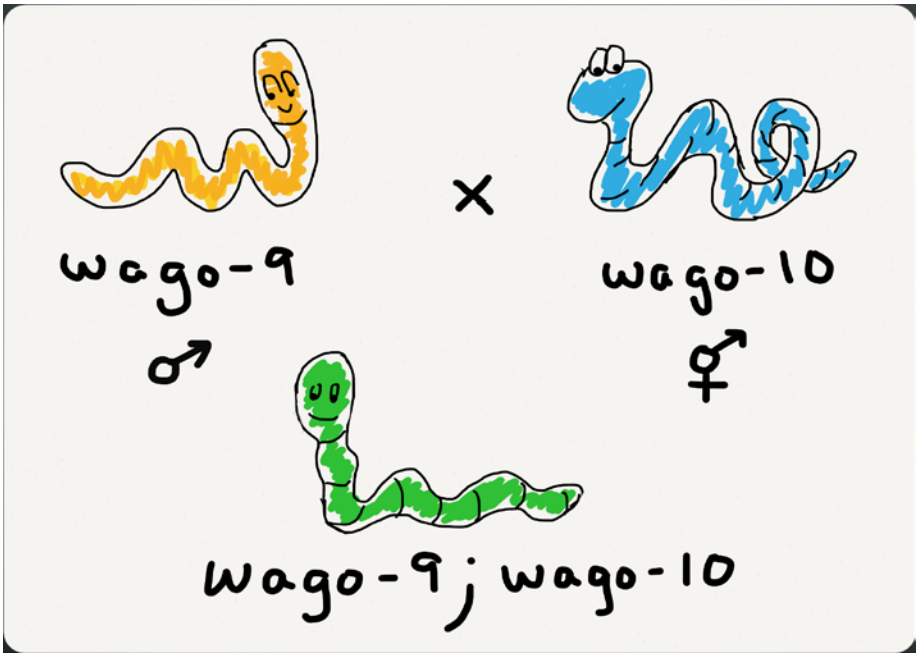


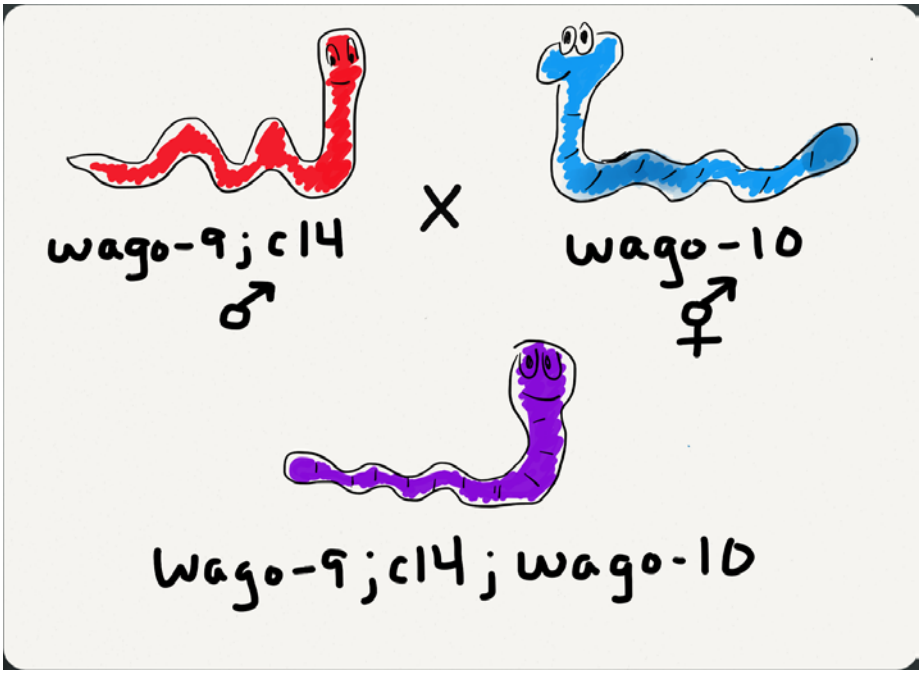
Figure 3: Argonaute Tree

Phylogenetic tree of representative Argonaute proteins in fungi, plants, and animals (proposed by Colin Conine). The Argonaute group with representatives in all three kingdoms is labeled in black font. There is an expanded group of Argonaute proteins that found in *C. elegans* are labeled in red while the PIWI group common to all metazoans is labeled in green. (ce: *Caenorhabditis elegans*, at: *Arabidopsis thaliana*, hs: *Homo sapiens*, sp: *Schizosaccharomyces pombe*).

A)


wago-9
♂
×
wago-10
♀
wago-9; wago-10

B)


wago-9;c14
♂
×
wago-10
♀
wago-9;c14; wago-10

C)

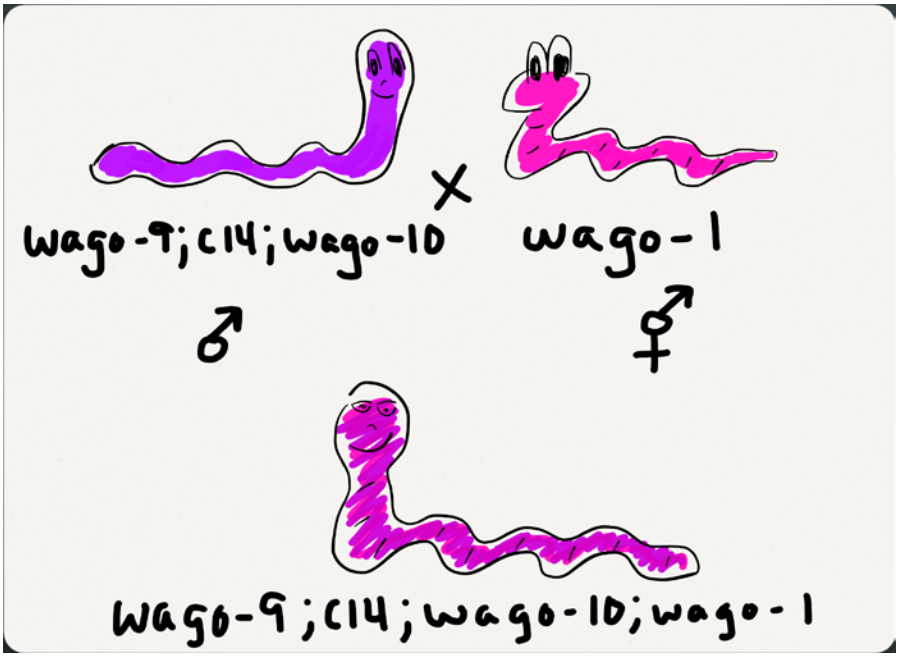


Figure 4. Schematic sketches for creating the Double (A), Triple (B), and Quad (C) mutant strains

A) Picture drawing of the genetic cross between *wago-9* males and *wago-10* hermaphrodites in order to create the *wago-9;wago-10* (Double) mutant strain. B) Picture Drawing of the genetic cross between *wago-9;c14* mutant males and *wago-10* mutant hermaphrodites, which resulted in the *wago-9;c14;wago-10* (Triple) mutant strain. C) Picture drawing of the genetic cross between *wago-9;c14;wago-10* (Triple) mutant males and *wago-1* mutant hermaphrodites, thus creating the *wago-9;c14;wago-10;wago-1* (Quad) mutant strain.

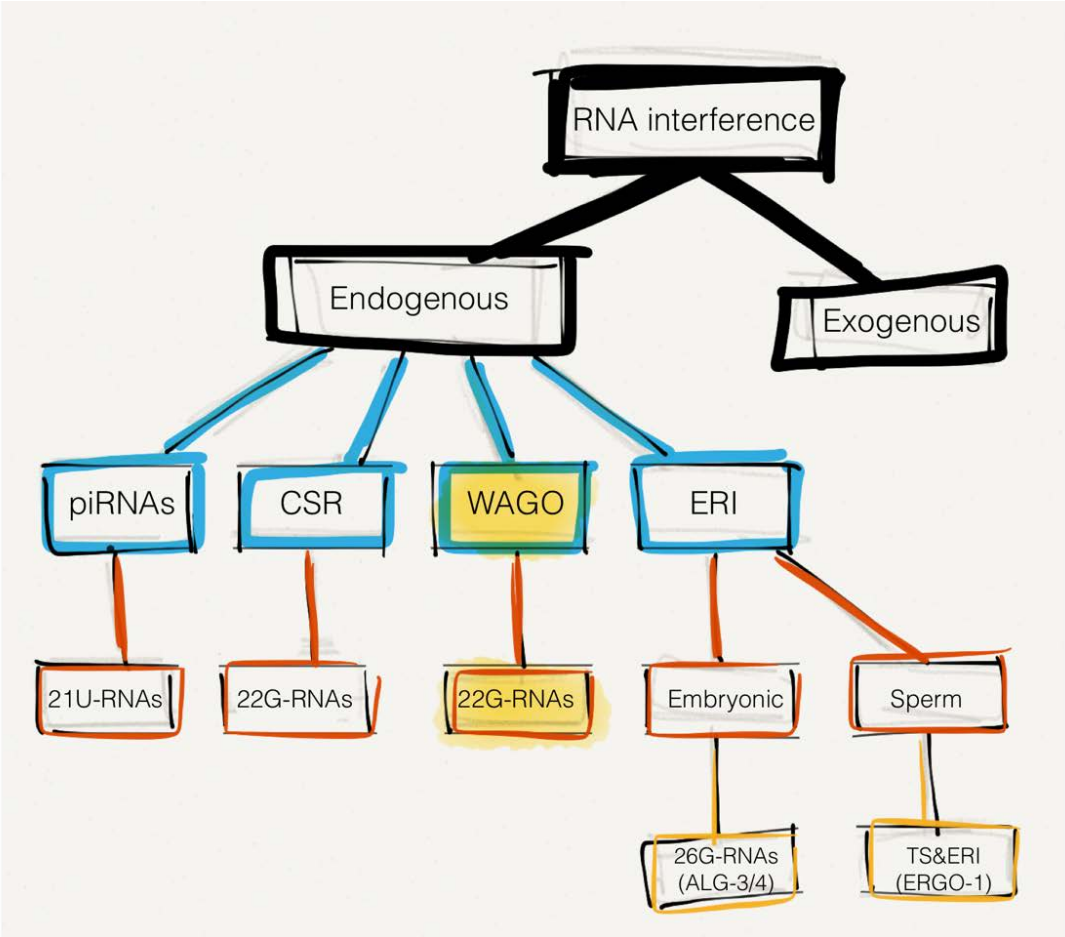
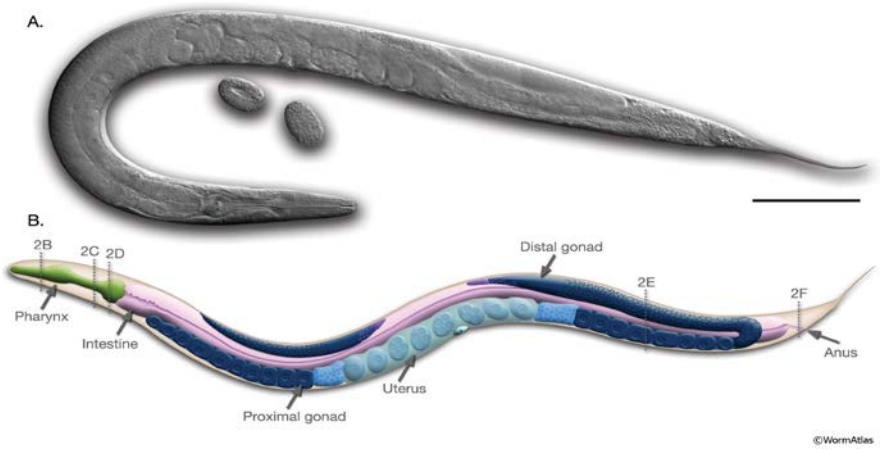


Figure 5. Endogenous RNAi Pathway and substituent pathways

Diagram depicting the branches and extensions that represent RNA interference and Post-Transcriptional Gene-Silencing (PTGS). The diagram displays RNAi broken into two distinct pathways: Endogenous and Exogenous. The Endogenous RNAi pathway involves four separate, yet cooperative pathways: the piRNA pathway, CSR pathway, WAGO pathway, and the ERI pathway. The yellow highlight represents the pathway that is the primary focus and where the worm Argonaute proteins used in this study are derived.

A)



B)

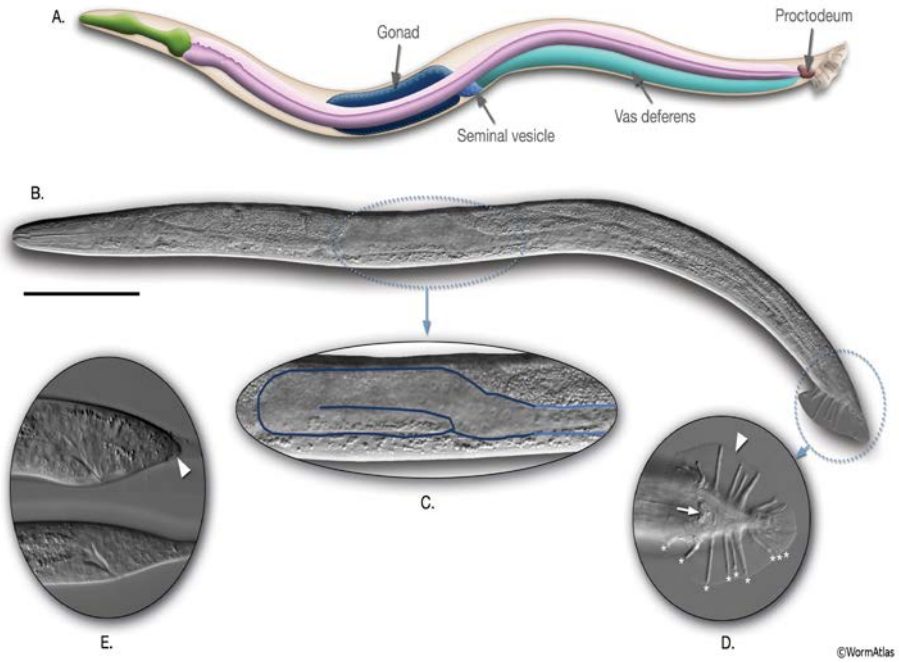


Figure 6: Adopted from WormAtlas (Altun & Hall, 2009)

Figure 6. Anatomy of *Caenorhabditis elegans*

A) The anatomy of a *C. elegans* hermaphrodite in both detailed drawing and image from microscopic view. B) The anatomy of a *C. elegans* male in both detailed drawing and image from microscopic view, which indicates the distinctive tail that allows the male to be easily recognized.

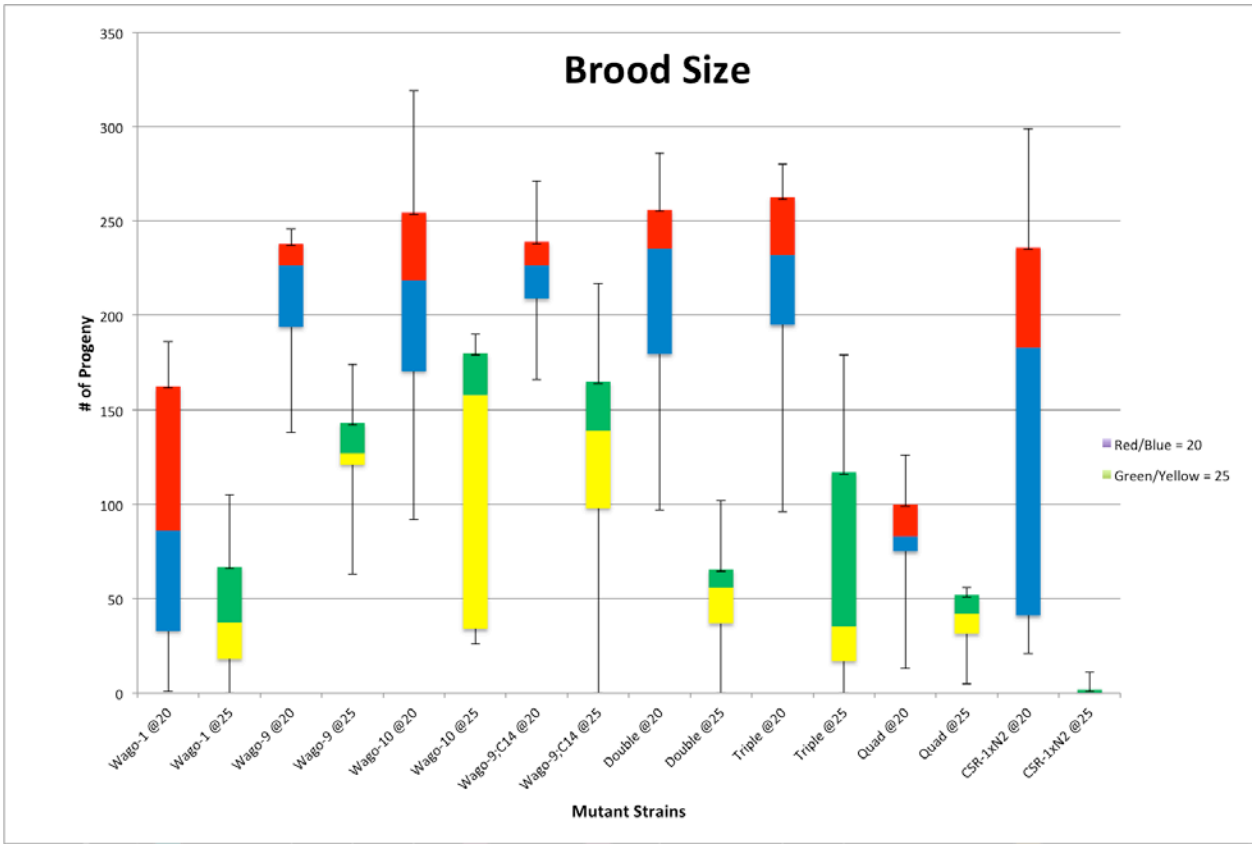


Figure 7. Brood size analysis

The box and whisker plot shows the brood size numbers for all strains used in this study. The y-axis represents the number of progeny produced by each particular mutant strain. Brood size analysis conducted at 20C used red to represent the 75% percentile and blue to represent the 25% percentile. For 25C, green was used to indicate the 75% percentile and yellow was used for the 25% percentile. The line that separates red/blue and green/yellow is the average for each brood. Error bars were used to show the range of the brood counts for each strain.