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The Function and Mechanism of RRF-1 in Antiviral RNAi in C. elegans

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by

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Introduction

This experiment focused on the rrf-1 gene of *Caenorhabditis elegans*. The rrf-1 gene is found on chromosome I in the worm. The goal of the experiment was to discover whether or not rrf-1 is required in antiviral RNAi. Also, how rrf-1 interacts with genes that play key roles in antiviral RNAi, such as rde-4, was a probing question.

Caenorhabditis elegans as a Model Organism

Caenorhabditis elegans is a non-parasitic roundworm that has become an important model organism in biological research. It was first introduced as a model organism in 1963 by Sydney Brenner (9). When it was first introduced, it was used for the study of development and neurobiology (9). It has since been used to study many different biological processes.

There are several advantages to using *C. elegans* in research. Firstly, the worm has a very short life cycle of about two weeks. This poses an advantage when doing research because results can be obtained relatively quickly. Also, the maintenance of the nematodes is extremely cost-effective and simple. The nematodes are maintained on agar plates that contain *Escherichia coli*, which serves as the worm's food source (8). Many worms can be maintained on a single plate due to their small size. Other features that make *C. elegans* advantageous to use as a model organism are its simplistic body plan and unique behavior.

There are also some genomic features that make *C. elegans* a great model system. Firstly, the cell lineage was completed by John Sulston (1). Also, it was the first multicellular organism to have its genome sequenced (1). The genome of *C. elegans* is 40% homologous to the human genome (1), and it was discovered that the nematode contains many of the disease genes and pathways that are found in humans (9). Therefore, the worm has become an important model for the study of human disease. There are several ways in which genes can be down-regulated in the worm, which is quite convenient. The worm genes can be down-regulated by feeding of bacteria that contains dsRNA, transcription of hairpin dsRNA in vivo, soaking the worms in a solution that contains dsRNA, or injection of the dsRNA into the gut of the worm. Finally, there are many mutants available for study. Some of these mutants can cause an easily observable behavioral change.

The last characteristic of C. elegans that is unique and important in biological research is the reproduction process. *C. elegans* contains two sexes, males and hermaphrodites. Reproduction can occur in two ways. The most common and natural reproduction method is self-fertilization by the hermaphrodites. The progeny will be genetically identical to the mother when this happens. The second way that reproduction can occur is of particular importance to researchers. Sexual reproduction can occur via the mating of male and female worms. This type of reproduction is the key to performing genetic crosses and introducing mutant genes into the worms. The only caveat about this type of reproduction is that male worms are relatively difficult to come by naturally. Therefore, heat induction is used to increase the chance that male worms will be produced. Once males are obtained, they are easy to maintain since mating between males and females will produce progeny that is half male and half female.

RNA Interference

RNA interference was discovered by Andrew Fire and Craig Mello in 1998 (11). It was actually first characterized in *C. elegans* as a post-transcriptional gene silencing mechanism (10, 11). Its main function lies in defense from foreign dsRNAs, viruses, and transposable elements.

RNAi produces sequence-specific disintegration of homologous mRNA sequences (11). Since the nematodes do not have any natural pathogens, RNAi has been studied using those that infect mammals such as Vesicular Stomatitis Virus, or VSV (10). The process of RNAi in *C. elegans* has been extensively studied and worked out over the last few years.

RNAi is triggered by long double-stranded RNA. DsRNA will be processed into primary small-interfering RNAs (10). Next, the RNA-induced silencing complex (RISC) will target homologous RNA. Specific sequences will be cleaved, which will stimulate post-transcriptional gene silencing (10). Finally, a host RNA-dependent RNA polymerase (RdRP) will amplify the primary siRNAs and produce secondary siRNAs. Several genes have been discovered to be involved in the classical RNAi process in *C. elegans*.

Rde -4 is a dsRNA-binding protein (7). Rde-4 acts with dcr-1, an RNase III enzyme present in the worm, to cleave the dsRNA into primary small interfering RNAs (7, 10, 11). These siRNAs will be 22-26 nucleotides long (12). The primary siRNAs will then be loaded into the RNA-induced silencing complex, also known as RISC (11). RISC contains an Argonaute protein, Ago. The function of Ago is to activate RISC by cleaving and discarding the siRNA passenger strand (11). The guide strand of the primary siRNA then works to lead RISC to homologous RNA so that it may be cleaved (11). Rde-1 is a member of the argonaute gene family (10). It acts downstream of primary siRNA production to assist in its interaction with target mRNA (7). It also recruits rrf-1, an RNA-dependent RNA polymerase. Rrf-1 uses the mRNA as a template to produce secondary siRNAs. This will amplify the RNAi response as well as ultimately direct the targeted cleavage of the mRNA (7).

In my experiment, I was particularly interested in RNAi Directed Viral Immunity (RDVI). The mechanism is very similar to classical RNAi, but there are some differences. The first difference is the starting product. The process begins with viral genomic RNA. A dsRNA replication intermediate is then produced. Dicer will cleave the dsRNA into primary viral siRNAs. RISC, which consists of the primary viral siRNA and Ago, will target and cleave the viral RNA. Lastly, the host RdRP will use the viral RNA as a template to produce secondary viral siRNAs. Another key difference is that a gene, drh-1, is present and specific to RDVI. Dr. Lu and his colleagues discovered that drh-1 directs antiviral RNAi downstream of viral RNA biogenesis (3). They also revealed that if drh-1 is depleted, antiviral RNAi will be suppressed. Therefore, this process is especially dependent on rde-4, which plays a role in the sensing of viral RNA and virus-derived siRNA (viRNA) biogenesis, drh-1, and dcr-1 (3).

FHVRNA1eGFP Transgene

Flock House Virus (FHV) is a positive-strand RNA virus (2). The natural host for the virus is the grass grub, *Costelytra zealandica*. However, Dr. Lu and his colleagues have shown that FHV can replicate in *C. elegans*, as well as induce gene silencing. This virus has a very small genome, which makes it very easy to modify. There are two RNAs in the FHV genome. RNA1 codes for a viral RdRP. RNA3 is a product of the replication of RNA1. RNA3 contains the B2 protein, which is a known RNAi suppressor. RNA2 codes for a precursor of the capsid protein (pre-CP) (3). Using FHV, Dr. Lu and his colleagues developed a strategy for initiating FHV replication in *C. elegans*. First, a heat-inducible promoter is added to the FHV RNA1. Also, a self-cleaving ribozyme sequence is added. The self-cleaving ribozyme will cleave the poly-A tails of the RNA1. After this cleavage event, viral replication will take place. The RdRP and RNA3 with the B2 protein will be produced.

The FHVRNA1eGFP transgene was developed by Dr. Lu and his associates as a reporter system for the study of RDVI. This transgene was an important component of the experiment presented in this paper. The FHV RNA1 with the heat-inducible promoter and self-cleaving ribozyme sequence is utilized. Most of the B2 coding sequence is replaced with the eGFP coding sequence (3). Since the B2 protein is no longer produced, RNAi will not be suppressed under normal conditions. However, when mutant genes that are essential to antiviral RNAi are inserted into the worm, the RNAi will be suppressed due to the loss of those genes and disruption of RNAi (3). Heat induction of worms that contain this transgene will allow GFP to be visible. Dr. Lu has been a pioneer for this RNAi work and has introduced this transgene into several genetic backgrounds.

Materials and Methods

Maintenance of Worms

The worms in this experiment were maintained on plates that contained NGM media. These plates are made by combining 3 g of NaCl, 10 g of agar, 8 g of agarose, 2.5 g of bactopeptone, and 975 mL of ddH_2O. This mixture is then autoclaved. After, 1 μL of $CaCl_2$, 1 μL of $MgCl_2$, 0.5 μL of cholesterol, and 25 mL of PPK is added to the mixture. The mixture is then put into clean, empty plates. Once the plates have cooled and hardened, *E. coli* is added to serve as food for the nematodes.

Crossing Experiments

The purpose of this experiment was to determine the function of rrf-1 in RNAi directed viral immunity. To accomplish this goal, crossing experiments were performed. FHVRNA1eGFP;*rde-4* hermaphrodites were crossed with *rrf-1* males. The cross was performed on a C plate. This plate is essentially a plate that contains media but not food. A small amount of food is transferred to this plate from another plate. The small amount of food ensures that the worms will be in the same area to feed, which makes it more likely for mating to occur. The ratio of worms for the cross was one hermaphrodite to four males. This also increases the probability that mating will occur and be successful.

The FHVRNA1eGFP;*rde-4* worms were the result of a successful cross performed by a member of the lab, Xunyang Guo. FHVRNA1eGFP worms are essentially wild-type N2 worms that contain the transgene. These worms have a distinct roller phenotype which is quite different from wild-type N2 worms. Therefore, it is very easy to test for the presence of the FHVRNA1eGFP transgene in offspring. If the offspring have a roller phenotype, they contain the transgene. The *rde-4* worms are rde-4 deficient. *Rde-4* mutant animals will have a deficiency in antiviral RNAi since rde-4 is important in sensing the viral RNA as well as viRNA biogenesis (3, 7).

The *rrf-1* males that were used were obtained via heat induction and maintained over time. These worms are rrf-1 deficient. Rrf-1 is an RNA-dependent RNA polymerase in *C. elegans*. This is the gene that I was most interested in. The goal of this experiment was to discover the role of rrf-1 in antiviral immunity.

When the FHVRNA1eGFP;*rde-4* hermaphrodites were crossed with *rrf-1* males, the cross was deemed successful when the presence of male rollers was confirmed. F1 roller hermaphrodites that were smaller than the F1 roller males were chosen. These F1 hermaphrodites were placed on individual agar plates to self-fertilize and produce progeny. Once the

hermaphrodite laid at least 50 eggs, PCR was used to confirm the presence of the *rrf-1* gene in the F1 hermaphrodite.

PCR and Electrophoresis

The Polymerase Chain Reaction is used to amplify specific sequences of DNA or RNA. This will allow for an exponential increase in the target RNA. Forward and reverse primers are used to select the specific sequence of RNA that needs to be amplified.

Electrophoresis is separation of RNA based on size. Large RNA fragments will move more slowly than smaller ones. By using electrophoresis, we can visualize the results of PCR. A kB ladder is loaded into either the first or last well to serve as the control.

In my experiment, PCR and Electrophoresis were used to test for the presence of the *rrf-1* mutant gene. The test is to make sure that the nematode is heterozygous for the rrf-1 gene, rather than homozygous. By using a kB ladder as a guide, we can compare the relative sizes of the RNA. If the worm is homozygous for the rrf-1 gene, it will be larger (3 kB) and move slower. If the worm is heterozygous and contains the mutant *rrf-1*, it will be smaller (1 kB) and move faster. Therefore, size comparisons can be used to determine whether or not the worm contains the mutant or not. Indeed, when PCR and electrophoresis were performed, the sample was determined to be 1 kB.

Skn-1 Feeding

Skn-1 is an endogenous worm gene that is responsible for small intestine development. Therefore, if skn-1 is knocked out, no progeny will develop. Skn-1 feeding RNAi was used to test for the presence of the *rde-4* transgene. Once it has been confirmed by PCR that the F1 hermaphrodite did indeed contain the *rrf-1* transgene, progeny from that worm are placed on individual skn-1 plates. The progeny should be FHVRNA1eGFP;*rde-4*;*rrf-1* double mutants. There should be 50 to 100 skn-1 plates each with one FHVRNA1eGFP;*rde-4*;*rrf-1* worm. There should also be two control plates. The first skn-1 control plate should contain around 5 FHVRNA1eGFP;*rde-4* hermaphrodites. The second skn-1 control plate should contain around 5 *rrf-1* hermaphrodites. These controls will ensure that the skn-1 plates are effective and working properly.

Feeding dsRNA targeting skn-1 allows one to detect whether worms are resistant or sensitive. If the worm is sensitive to the food, RNAi has not been compromised and the skn-1 gene is targeted. If the worm is resistant to the food, RNAi has been compromised and the skn-1 gene cannot be targeted. When wild-type N2 worms are placed on skn-1 plates, they will produce eggs that will die and not hatch. Likewise, *rrf-1* worms are also sensitive to skn-1 feeding RNAi. However, *rde-4* worms are resistant to skn-1 feeding. The eggs of these worms will hatch. Therefore, when the *rde-4* transgene is present in the double mutants, the worms will be resistant as well since loss of the rde-4 gene results in the loss of RNAi. This allows us to confirm the presence of *rde-4*.

GFP and Northern Blotting

GFP stands for green fluorescence protein. It was first discovered in the jellyfish, *Aequorea victoria*. GFP is a very useful research tool because it can be expressed in organisms at high levels without causing damaging effects to the organism (13). In this experiment, GFP was used to visualize the replication of the virus in the worms. The FHVRNA1eGFP transgene is

essential in visualizing GFP. The worms underwent heat induction for 3-4 hours so that the GFP could be visualized.

Pictures to show the GFP were obtained by paralyzing the worm. 1 M NaN_3 was diluted to 50 mM in a 1.5 mL tube. A small tube with 20µL of the sodium azide dilution was used to paralyze around 20 worms. Once the worms were submerged in solution, the solution was transferred to a plate that contained no food using a pipette.

Northern blotting was performed after GFP. Northern blotting is the process of blotting RNA to nitrocellulose paper. This procedure allows us to view the amount of virus replication in the nematodes.

Results

GFP was used to compare the relative amounts of viral replication in different strains in order to infer the function of rrf-1. The FHVRNA1eGFP transgene was used in all of the strains to visualize GFP. A scale of 0-5 was used to rate the amount of GFP observed in adult worms. A score of 0 indicated that no fluorescence was seen. A score of 1 indicated that fluorescence was only witnessed in the head region of the worm. A score of 2 indicated that fluorescence was observed in the head region and midsection. A score of 3 designated that fluorescence was detected all over the body, but not at a bright level. A score of 4 indicated that rather bright fluorescence was detected all over the body. Finally, a score of 5 signified that the worm contained very bright fluorescence everywhere in the body.

The strains that were observed were FHVRNA1eGFP;N2, FHVRNA1eGFP;*rrf-1*, FHVRNA1eGFP;*rde-4*, and FHVRNA1eGFP;*rde-4*;*rrf-1*. The results are visualized in Figure 1. The FHVRNA1eGFP;N2 strain was given a score of 1. GFP was seen mainly in the head region of most adult worms. The FHVRNA1eGFP;*rrf-1* worms were given a score of 2. Bright fluorescence was seen in the head of the worm and dim fluorescence was seen in the rest of the body of some worms. The FHVRNA1eGFP;*rde-4* worms were given a score of 3. These worms contained GFP all over the body. Finally, the FHVRNA1eGFP;*rde-4*;*rrf-1* worms were given a score of 4. Most adult worms contained bright fluorescence all over their body.

Northern blotting was used to gauge the replication of the virus. The results from the northern blot can be seen in Figure 2. These results support the GFP results. In N2 worms, no virus replication is seen. This is due to the fact that RNAi is working properly. In the *rde-4* mutant worms, some virus replication is seen. There was a significant amount of virus replication observed in the *rde-4*;*rrf-1* double mutant. Finally, there was also a substantial amount of virus replication detected in the *drh-1*;*rrf-1* double mutant.

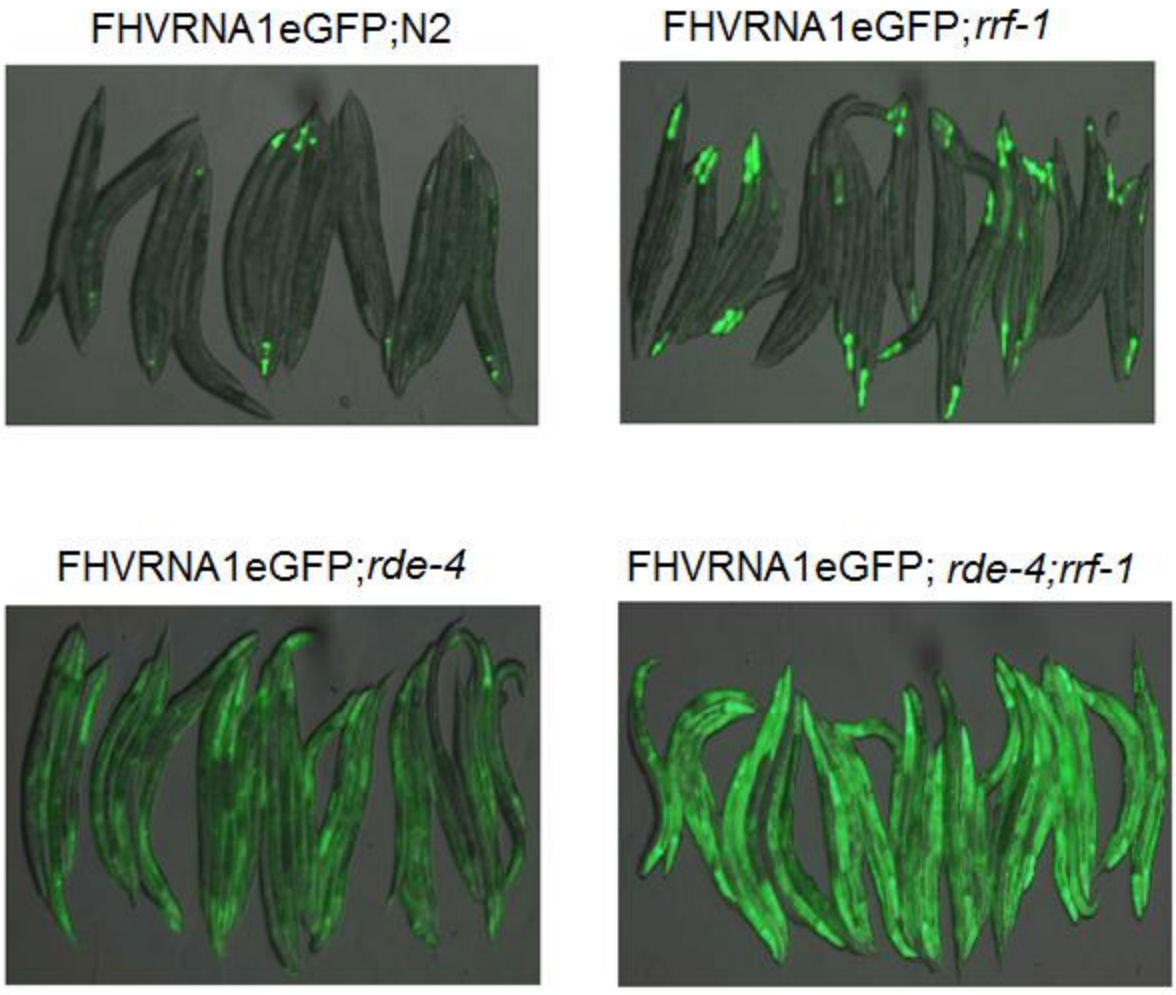

Figure 1: Visualization of GFP fluorescence in worm strains

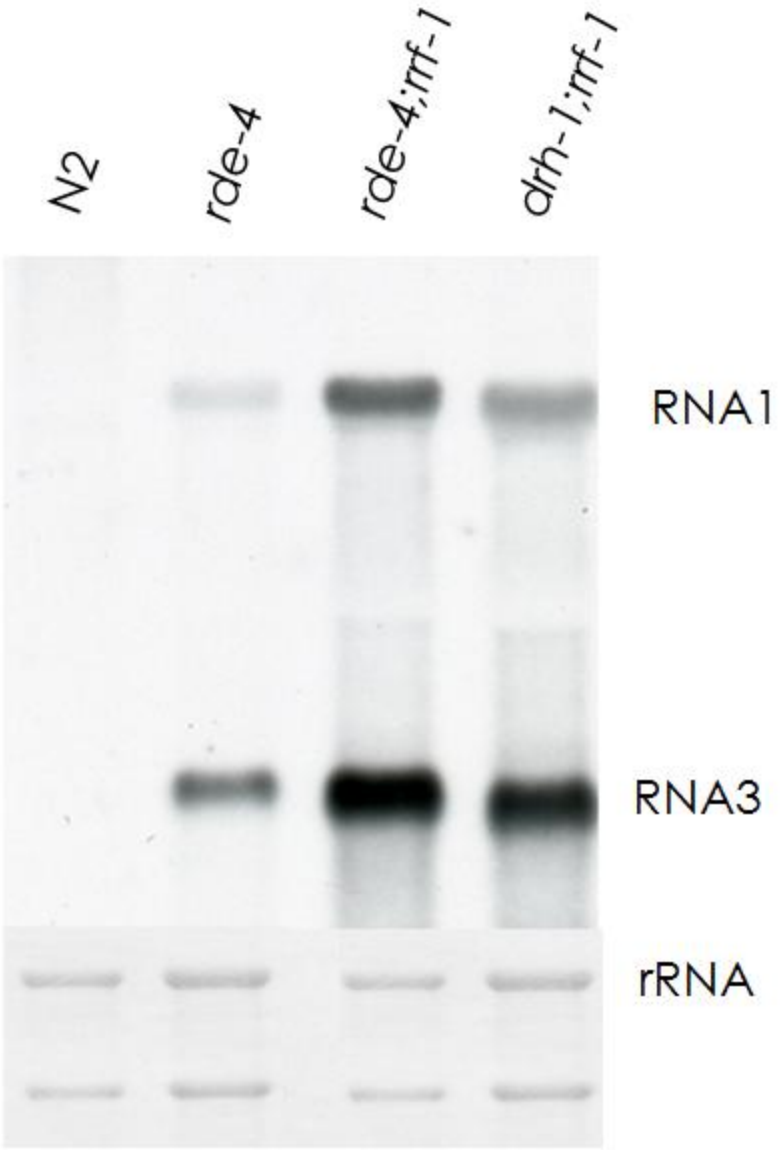

Figure 2: Northern blot analysis of virus replication with rRNA control

Conclusion

Firstly, *rrf-1* worms are sensitive to RNAi targeting skn-1 while *rde-4* worms are not. Rde-4 is required for primary siRNA production. Therefore, *rde-4* worms that lack the gene have completely compromised RNAi. Since classical RNAi is compromised, the worms are resistant to the skn-1 food. However, rrf-1 is not involved in the production of primary siRNAs. The primary siRNAs are still present, and classical RNAi is only partially compromised. This is why *rrf-1* worms are sensitive to skn-1 feeding. This indicates that rde-4 plays a stronger role in classical RNAi than rrf-1.

Also, the amount of GFP indicates the amount of replication of the virus in the worm. Therefore, as seen in the results section, rrf-1 does play a role in antiviral RNAi. This can be seen in the FHVRNA1eGFP;*rrf-1* worms as well as the FHVRNA1eGFP;*rde-4*;*rrf-1* worms. Compared to FHVRNA1eGFP;N2 worms, the FHVRNA1eGFP;*rrf-1* worms do show brighter

fluorescence, which indicates that it does function in antiviral RNAi. Also, the double mutant worms, FHVRNA1eGFP;*rde-4*;*rrf-1*, contain slightly brighter fluorescence than the FHVRNA1eGFP;*rde-4* worms. Since the fluorescence is brighter in the double mutant worms, it can be inferred that rrf-1 actually interacts with rde-4. The loss of both of these genes greatly affects RNAi. However, by examining the fluorescence of just the FHVRNA1eGFP;*rrf-1* worms, it can be assumed that rrf-1 is a weak requirement for antiviral RNAi compared to other genes, such as rde-4.

The northern blot results further support these conclusions about the rrf-1 gene. Virus replication can be seen in the *rde-4* worms. This indicates that it plays a role in antiviral RNAi. There is even more virus replication seen in the *rde-4*;*rrf-1* double mutant. This again supports the conclusion that rrf-1 and rde-4 interact and further suppress RNAi. Finally, the *drh-1*;*rrf-1* double mutant does not show as much virus replication as the *rde-4*;*rrf-1* double mutant. This suggests that while drh-1 is a key player in RDVI, the interaction between *rde-4*;*rrf-1* is much stronger.

Future endeavors into this matter include performing this experiment with other genes, such as ergo-1, rde-1, and drh-1. This would allow me to discover how rrf-1 interacts with these other genes and how the absence of rrf-1 and these genes affects antiviral RNAi. Another future endeavor would involve using small RNA detection to test whether or not the rrf-1 mutants interact with other RNAi genes.

References

1. "A Short History of *C. elegans* Research." WormClassroom. Laboratory for Optical and Computational Instrumentation at the University of Wisconsin-Madison. 1 Apr. 2011.
2. Dasgupta, Ranjit, Li-Lin Cheng, Lyric C. Bartholomay, and Bruce M. Christensen. "Flock House Virus Replicates and Expresses Green Fluorescent Protein in Mosquitoes." *Journal of General Virology* (2003): 1789-1797. 7 Apr. 2011.
3. Ding Shou-Wei, et al. "An RIG-I-Like RNA Helicase Mediates Antiviral RNAi Downstream of Viral siRNA Biogenesis in Caenorhabditis elegans." *PLoS Pathogens* 5.2 (2009): 1-11. *Academic Search Complete*. EBSCO. Web. 30 Mar. 2011.
4. Fischer, Sylvia E.J. "Small RNA-Mediated Gene Silencing Pathways in C. elegans." *The International Journal of Biochemistry and Cell Biology* (2010): 1306-1315.
5. Grishok, Alla, Jina L. Sinskey, and Phillip A. Sharp. "Transcriptional silencing of a transgene by RNAi in the soma of C. elegans." *Genes & Development* 19.6 (2005): 683-696. *Academic Search Complete*. EBSCO. Web. 30 Mar. 2011.
6. Grishok, Alla, Sebastian Hoersch, and Phillip A. Sharp. "RNA interference and retinoblastoma-related genes are required for repression of endogenous siRNA targets in Caenorhabditis elegans." *PNAS* 105.51 (2008): 20386-20391.
7. Habig, Jeffrey W., P. Joseph Aruscavage, and Brenda L. Bass. "In C. elegans, High Levels of dsRNA Allow RNAi in the Absence of RDE-4." *PLoS ONE* 3.12 (2008): 1-10. *Academic Search Complete*. EBSCO. Web. 30 Mar. 2011.
8. "Introduction to *C. elegans* Anatomy." Worm Atlas. 2011. Cold Spring Harbor Laboratory Press. 31 Mar. 2011 <http://www.wormatlas.org/hermaphrodite/introduction/mainframe.htm>.
9. Kaletta, Titus, and Michael O. Hengartner. "Finding function in novel targets: C. elegans as a model organism." *Nature Reviews Drug Discovery* 5.5 (2006): 387-399. *Academic Search Complete*. EBSCO. Web. 30 Mar. 2011.

10. Khaled Machaca, et al. "RNA interference is an antiviral defence mechanism in Caenorhabditis elegans." *Nature* 436.7053 (2005): 1044-1047. *Academic Search Complete*. EBSCO. Web. 30 Mar. 2011.
11. RNAi Web. 2004-2008. 5 Apr. 2011. <http://www.rnaiweb.com>.
12. Tabara, Hiroaki, Erbay Yigit, Haruhiko Siomi, and Craig C. Mello. "The dsRNA Binding Protein RDE-4 Interacts with RDE-1, DCR-1, and a DExH-Box Helicase to Direct RNAi in *C. elegans*." *Cell* 109.7 (2002): 861-871.
13. "The Green Fluorescent Protein: Discovery, Expression, and Development." The Royal Swedish Academy of Sciences. 8 Oct. 2008. <http://nobelprize.org/nobel_prizes/chemistry/laureates/2008/chemadv08.pdf>.