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Abstract:

In eukaryotes, antiviral RNA interference (RNAi) is a widespread antiviral mechanism that is known to share several key genes with classical RNAi triggered by artificial double-stranded RNAs (dsRNAs). Current studies suggest that antiviral RNAi requires extra genes to keep virus under control compared to classical RNAi.

To better understand antiviral RNAi we have recently carried out a genetic screen that aimed to identify novel worm genes with dedicated function in antiviral RNAi. 1026A and 1026D are two of the loss of antiviral RNAi alleles isolated from this screen. To find out whether classical RNAi is also compromised by mutations in these two alleles we tested worms containing these two alleles in RNAi response triggered *skn-1* dsRNA feeding. We found that although capable of supporting high-level viral replication, worms containing the 1026D allele remain sensitive to classical RNAi triggered by *skn-1* dsRNA feeding. In contrast, worms containing the 1026A allele support high-level viral replication and are resistant to the *skn-1* dsRNA feeding. This finding once again confirms that antiviral RNAi is mechanistically different from classical RNAi and requires extra genes to suppress the replication of viral pathogens. Mechanistic study of the worm gene corresponding to 1026D is expected to improve our understanding on worm antiviral RNAi.

Introduction

RNA interference (RNAi) is a unique mechanism found in some eukaryotic organisms. Andrew Fire and Craig Mello demonstrated that dsRNAs are the triggers of RNAi in their paper entitled *Potent and Specific Genetic Interference by Double-Stranded RNA in Caenorhabditis elegans*. Fire and Mello found that certain endogenous genes of *Caenorhabditis elegans*(C.elegans) could be shut down through the introduction of dsRNA. Although they observed that ssRNA could do the job in a less profound fashion, Fire and Mello realized the importance of dsRNA as trigger. Observing that it only took a few molecules to do the job, they proposed that an amplification process might occur (**1**).This started a new trend in research and effort into discovering the mechanisms behind RNA interference. From this original study, Fire and Mello proposed the first ideas behind several underlying parts of RNA interference phenomena: a dsRNA model drives the process, there is an underlying complex mechanism behind the process, and that this process can shut down target genes. In addition, several protocols for RNAi induction were created that are still used today for RNA interference research, such as gonad microinjection and bacterial feeding(**1**). However, Fire and Mello did not realize that RNA interference would be more complex and have more to discover than they could have imagined.

In modern times, RNA interference has two different classifications: Endogenous RNAi and Exogenous RNAi. In endogenous RNAi, small-interfering RNA (siRNA) and microRNA (miRNA) are generated from internal sources. This type of RNAi is generated inside of an organism's body by native RNA to regulate multiple functions inside of the body, some of which still are not known (**2**). Exogenous RNAi refers to the introduction of an outside dsRNA trigger into an organism, such as artificial dsRNA or viral dsRNA. dsRNA can be made through transcription of a DNA sequence,RNA replication, or viral genome replication (**3**). While these processes are separate from one another, it is known that these systems do interact with one

another at certain points (**2**). However, to what extent these RNAi pathways interact with each other remains largely unknown.

RNAi can be found in many different types of organisms. Currently, RNAi activity has been seen in fungi, plants, *C. elegans*, and *Drosophila melanogaster* (*Melanogaster*). All have the ability to use class III endoribonucleases, known as Dicers, and RNA-Dependent RNA Polymerases(RDRP) to create primary and secondary small interfering RNAs (siRNAs) varying in 21-25 nt(nucleotide) length (**4**). My research focuses on the *C. elegans* model organism and RNA interference. Specifically, I study the suppression of viral replication in a process known as RNA directed viral immunity (RDVI). Although sharing some basic components with classical RNAi (RNAi triggered by artificial dsRNA), RDVI has been shown to be a separate pathway from classical RNAi. In a unique fashion, RDVI has its own unique genes and pathways triggered by the introduction of viral dsRNA into an organism such as *C.elegans* (**5**). The process of delivery of dsRNA depends not only on the organism, but the particular virus as well.

C. elegans has been shown to support the replication of two types of viruses, the artificial Flock House Virus (FHV) and the Orsay virus that naturally infects *C. elegans* (**5, 6**). These viruses replicate in very specific ways. Both are classified as Class IV viruses according to the Baltimore classification. Class IV viruses are single-stranded RNA (ssRNA) viruses whose genome is made up of plus-stranded RNA((**7**). Although they are members of the *Nodavirdae* family, both FHV and Orsay virus share the same structure and replication mode as the majority of (+) sense ssRNA viruses. Orsay and FHV are non-enveloped viruses with icosahedral capsid symmetry (**8**). Their genomes have a capped 5’ end and a non-polyadenylated 3’ end. The genome encodes an RDRP (RNA1) and a second RNA that makes up the capsid proteins (RNA2).Upon penetration of a cell after external delivery into *C. elegans*, the genome is injected and both the RNA molecules are transcribed. The RNA2 produces an alpha protein that is broken down into beta and gamma subunits to produce the capsid. These viral proteins surround new genomic version of the RNA 1 and RNA2 genes. The genomic RNAs are used both for production of new particles and immediate infection (**9**). In *C. elegans*, these viruses are seen in the area where they penetrate, the intestinal epithelial cells. Now that the replication process is clear, the explanation of the pathways and how the siRNAs are produced will be a little clearer.

Several genes are shared between classical RNAi and RDVI in *C. elegans*: DCR-1, RDE-4, RDE-1, RSD-2, RRF-1, and DRH-3. However, RDVI includes genes that are unique only to antiviral situation. At this time, only one gene on importance is known: DRH-1(**10**). Each gene serves a unique function.

Primary viral siRNA production involves the interaction of three genes in both pathways: DCR-1, RDE-4, and DRH-1. DCR-1 is a class III RNase. The main function of DCR-1 is to cleave dsRNA to form primary siRNAs (**11**). In *C. elegans*, it proves to be the most essential gene for survival (lacking DCR-1 means automatic death for *C.elegans* mutants). These primary siRNAs are not made by DCR-1 alone, but through complexing with RDE-4 (11,**12**). RDE-4 is a double-stranded RNA binding protein. Upon introduction of dsRNA into *C. elegans*, RDE-4 functions by binding dsRNA through the use of its two dsRNA binding motifs. Upon binding to the dsRNA, RDE-4 will complex with DCR-1 to cleave the dsRNA into primary siRNAs (**12**). While classical RNAi requires RDE-4 to create primary siRNAs, RDVI does not fully require this gene (**6**). RDE-1 is an Argonaut protein essential for the RISC Complex (more on this later) in RNAi. RDE-1 acts as an acceptor for the primary siRNAs produced by DCR-1 and RDE-

4(**11**). It will use primary siRNAs to trigger either degradation of a homologous RNA target or to produce secondary siRNAs. While the primary siRNA complex remains the same in both pathways, it has to be regulated somehow. That is the function of DRH-2. DRH-2 is a DEADBOX-like helicase (**11**). Like most helicases, it functions in the unwinding of dsRNA. However, it acts more to regulate the production of primary siRNAs. Experimental data shows that the production of primary siRNAs is decreased through the activation of DRH-2 (**10**). This protein is only activated through a frame shift mechanism. DRH-2 is located upstream of DRH-1, which functions only in RDVI. Although DRH-1 only acts in RDVI, DRH-2 can be activated in either pathway. This shows that activation functions to regulate production. DRH-1 is found to only be essential in RDVI. DRH-1 is DEAD box-like helicase (**11**). DRH-1 is unique to the RDVI pathway and functions to unravel dsRNA and complexes with both RDE-4 and DCR-1 to produce primary siRNAs. However, it only functions to some extent in siRNA processing in regards to RDVI (**6**).

Secondary siRNA production is more or less the same in both pathways. When RDE-1 accepts primary siRNA from DCR-1: RDE-4: RDE-1 complex (DRH-1 added in RDVI), it moves towards the RISC complex to produce secondary siRNA. This interaction involves several genes: DRH-3, RSD-2, and RRF-1. DRH-3 is another type of dead box-like helicase. Unlike DRH-1 and DRH-2, it is found on a separate chromosome (DRH-3 is found on *C.elegans* chromosome I, DRH-1 and DRH-2 on chromosome IV) and functions only in the production of secondary siRNA (**10,11**). DRH-3 acts to unravel primary siRNA for the creation of secondary siRNAs (22nt in length). RSD-2, also known as RNAi spreading defective 2, involves itself in secondary siRNA by complexing with DRH-3 and RRF-1. RSD-2 allows the spreading of siRNA particles in both exogenous and RDVI pathways (**6**). Then, RRF-1 comes into play. RRF-1 is an RDRP (RNA-dependent RNA polymerase). RRF-1 functions to copy the produced primary siRNA and produce a new secondary copy from it. The only difference from these two transcripts lies in the unique production sequence: secondary siRNA always begins with a Guanine nucleotide. This means that all secondary production is transcribed from an associated Cytosine on the adjacent primary siRNA. Now that we understand these genes better, we need to see how they act in the unique RDVI pathway.

As stated previously, classical exogenous RNAi and RDVI share similar genes, but function differently. Classical exogenous RNAi is only triggered by artificial RNA that is injected or introduced from outside the body. RDVI is only triggered by viruses and viral particles. In addition, RDVI requires genes not found in classical exogenous RNAi (many of which have yet to be discovered). But the RDVI pathway also differs in how it produces siRNA.

From a previous study, it is hypothesized that RDVI can occur in one of two ways: with RDE-4 or without RDE-4 (**Fig1**). This is very different from classical exogenous RNAi. From the experiments done by Xunyang Guo, Rui Zhang, Jeffery Wang, and Rui Lu, two routes have been hypothesized to exist in *C. elegans* (**6**). This makes the pathway very different in how primary siRNAs are produced. Using the proposed RDE-4 dependent pathway, dsRNA from both Orsay and FHV gets introduced into *C.elegans* by feeding or microinjection. The dsRNA is bound by RDE-4 and brought into the DCR-1: RDE-4: DRH-1 complex. After being unwound by DRH-1, DCR-1 cleaves the RNA into 23nt segments. These segments are transferred to RDE-1 through another complex between DCR-1: RDE-4: RDE-1. RDE-1 transfers the primary siRNA to the DRH-3: RSD-2: RRF-1 complex. DRH-3 unwinds the primary siRNA and then

RRF-1 makes a complimentary copy to the sequence. Either particle are able to be used for degradation. Looking at the other proposed pathway, RDE-4 independent, we see the similar production with only DCR-1 acting by itself without a complex. However, this results in a lower production of siRNA (**6**). This gives us another possibility in the production of primary and secondary siRNAs for RDVI. While there is evidence, more studies will be needed to confirm this. At this time, RDE-4 dependent is the main pathway model for RDVI.

With the basic genetics and pathway mechanisms out of the way, the focus needs to shift to how we test for genes essential for RDVI. To test for these essential RDVI genes, we use a few basic methods: reporter strains, EMS mutagenesis, and RNAi feeding tests. These techniques are essential to the discovery of unique genes for RDVI.

Reporter strains are *C.elegans* strains that have been injected with transgenes for the purpose of visualizing an item of interest for study. My research uses reporter strains that have two features to allow for the study of RDVI in *C.elegans*: an mCherry protein that allows us to ensure delivery into target organism and a Green Fluorescent Protein (GFP) with a heat induced promoter that allows for the visualization of viRNA particles in the body of the target organism. Delivery of these transgenes into an organism is done by one of two mechanisms: micro gonad injection or crossing in of the transgene through a particular cross scheme. The method usually depends on what purpose the gene is needed. The lab uses two transgenes: 48 and 11. The 48 transgene is designed with the FR1gfp replicon based off the RNA 1 of the FHV (**13**). In addition, it contains an mCherry protein gene to allow for observation of delivery. However, this transgene was created by Tianyun Long to also deliver several functional genes into the target organism: RDE-1, RDE-4, DRH-1, and RSD-2(**Fig2 A**). These genes serve to rescue the essential functional genes of RDVI and classical Exogenous RNAi to ensure that the main functional genes are operational. This is essential to ensure the worm has all primary functional genes in working order. For the purpose of researching RDVI, the 48 transgene was injected into the Bristol isolate, N2, to create a reporter strain through mircogonad injection. This strain, known as 48N2, was then used to create lab mutant strains through EMS mutagenesis (described later). The 11 transgene only contains the FR1gfp replicon and the mCherry protein(**Fig 2 B**). Unlike the 48 transgene, this transgene is crossed into *C.elegans* using a particular crossing scheme (**14**). The 11 transgene serves to enhance the visualization of GFP to a greater extent than what could be seen with the 48 transgene. The two genes are can be seen in different spots on the *C.elegans* body: 48 shows in the head only, 11 in the body only. These genes both help to improve our ability to visually monitor RDVI activity.

EMS mutagenesis is done according to a standard protocol developed by Brenner (**15**). EMS mutagenesis functions through the addition of an ethyl group to a Guanine. This causes inappropriate pairings between a guanine and an thymine, causing formations of premature stop codons and inducing a mutation. Typically a loss-of–function mutation or null allele occurs from this process, which is random and affects only a single base pairing(16). As such, it is a great technique to use to cause random mutations to discover the possibility of new genes for RDVI. The process in the lab involves using the 48N2 reporter strain. Since essential RDVI genes are carried by the transgene, we mutate this reporter through EMS. The actual mutations will not appear until the F2 generation is developing. In this generation, the abnormal phenotype will present itself with some sort of obvious physical mutation (abnormal growth, heat sensitivity,

developmental problems, etc.)(**16**). This process allows us to mutate one random gene and discover its location and function.

The final method is RNAi feeding. RNAi feeding involves delivering a dsRNA through bacterial feeding. Bacterial feeding involves the transformation of bacterial strains by introducing a gene into them that will be transferred into another organism. Similar to a way that we introduce viruses into the target organism, bacterial cells are transformed to have a vector containing the gene of interest(**17**).This is done by cloning the gene of interest into the bacterial cell genome(**18**). In my research, *Escherichia coli* (*E.coli*.) strain OP50 transformed to express *C.elegans* gene *skn-1*. *Skn-1* is a gene involved in the development of intestinal and pharageyal cells of *C.elegans* (**19**). When this type of dsRNA is introduced, it knocks down the *skn-1* gene. This is very important to RDVI research.*Skn-1* not only is involved in the development of intestinal cells, but it is also a component of the classical exogenous RNAi pathway. Knocking down this gene will give us one of two results in offspring: sterility or no reaction to the food. This is very important. To see if a gene is unique to RDVI, we must see if the classical exogenous pathway is functioning. An organism that is sensitive to the food and lays sterile eggs means that the mutated gene is only important to RDVI, not classical exogenous RNAi. However, no sensitivity to the food means that the classical exogenous pathway is not fully functioning, thus the gene is not unique to RDVI. While the gene may still serve a purpose to RDVI, it is still shared with the other pathway. This method allows to gauge what direction research on a gene needs to go or if we need to study the gene at all for a particular scientific aspect.

Though the previous techniques are used to created and test mutants, we have to find a way to isolate mutants. Although some mutants can be distinguished by physical mutations, many mutants with the 48 or 11 transgenes look exactly alike before RNAi testing is done. To isolate these mutants, we need to start after the F2 offspring is collected from the EMS mutagenesis(**15**)(**Fig 3**). Twenty-five percent of the F2 offspring should contain a mutant phenotype from the EMS mutagenesis. This is observed under fluorescent microscopy either by physical, observable mutations or heightened GFP fluorescence under specialized light. However, the GFP illumination is lowered under the presence of 48 transgene. In order to better see the GFP accumulation, the 11 transgene is crossed in using a particular cross scheme(**14**)(**Fig 4**).To accomplish this, male *C.elegans* must be made through heat induction of virgin hermaphrodites in the L3-L4 stage. Fifteen hermaphrodites are placed in 37*C for 45 minutes. *C.elegans* heated at this temperature will suffer nondisjunction events in the offspring. This results in males as *C.elegans* gender is determined by two sex types, hermaphrodite(XX) and male(XO)(**20**).*Upon* the crossing of 11N2 wild type males with virgin mutant hermaphrodites, offspring will be seen with both 48(head) and 11(body) transgenes. Fifteen of these are isolated into fifteen separate agar plates. These plates are allowed to self-replicate. Three phenotypes will be seen under the microscope: head only(48 transgene), body only(11 transgene), and head and body(both transgenes). Of these, 100 of the body only will be isolated into 100 different plates and be allowed to self-replicate. Of these, 25%-30% will be mutant only plates. This is checked by comparison to the wild type 11N2, where a higher GFP illumination will be the mutant strain. For these, the 20-25 with the highest GFP florescence will be used to create a DNA sequence of the worms with a large number of mutant worms with minor genetic differences.

For my study, two lab created mutant strains, 1026A and 1026D, underwent the cross scheme and were isolated to be sequenced to create a genomic map. The two strains were also subjected to RNAi feeding to test for the presence of an RDVI unique genes. The study was followed to answer one question: do either 1026A or 1026D have mutated genes unique to RDVI and if so, where are they located on the *C.elegans* genome?

Methods and Materials:

Worm Genetics. The wild type strain 11N2, a lab created reporter strain acted as the control reference strain for this study. Other strains used were 1026A and 1026D, both derived from the 48N2 reporter strain. Worm strains were maintained on NGM plates containing OP50 bacteria. 11 transgene was delivered into the various backgrounds through standard genetic crossing.

Worm Mutagenesis. Mutant worms created from 48N2 wild type worms through EMS mutagenesis (**15**). L4 or young adult hermaphrodites are washed with M9 buffer inside 15mLtube. Centrifuge at 700rpm for 1 min and remove supernant. Wash and spin three more times. Resuspend with 2mL buffer and add 2X stock EMS(100mM).Incubate at 20*C for 4 hours with constant rotation. Wash 5x with M9 buffer to remove EMS. Allow to reproduce on standard NGM plate. Take F1 offspring and allow to gravid. Bleach to recover F2 eggs and look for mutation(**Fig 3**).

Plasmid Constructs and Transgenic Worms. FR1gfp replicon creation was described in an earlier experiment (**13**).This was developed from Flock House Virus (FHV) RNA1 by replacing B2 sequencing with GFP protein. Worms were made transgenic through gonad microinjection protocol (**13**). Transgenes 48 and 11 were created previously by Tianyun Long. Transgene 48 contains Psur-5::rde1, Psur-5::rde4, Psur-5::drh-1, Psur-5::rsd-2, HIP::RdRP::GFP, Myo2::Mcherry::UTR gene sequences(**Fig 2A**). Psur acts as promoter for four genes, HIP is heat induced promoter for GFP activation, and Myo2 acts for mCherry. UTR are untranslated regions of the genes. Transgene 11 contains only HIP::RdRP::GFP, Myo2::Mcherry::UTR(**Fig 2B**).

Genetic Crossing and Screening. Worm genes were crossed into strains using a cross scheme outlined by Zuryn and Jarriault (**14**)(**Fig 4**). Primary screenings consisted of observation for 11 transgene presence. 100 plates created using mutant phenotype offspring previously screened. 20 plates selected from GFP accumulation screening. GFP screening consisted of incubation at 33*C for 3 hours and incubation overnight for 18-24 hours at 25*C. This allows for GFP to be activated through HI promoter(**Fig 5**). High GFP brightness is equivalent to high viral RNA accumulation. Plates with the brightest GFP were selected as samples for sequencing.

RNAi Experiments. Targeting of *skn*-1 gene was done through *E.coli feeding* according to a previous protocol (**18**). NGM agar plates containing 5 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and 50 mg/ml carbenicillin were used to prepare RNAi food. All feeding RNAi experiments were carried out at room temperature using *skn-1* seeded NGM agar plates. Plates analyzed for RDVI response.

RNA Northern Blot Analysis. Northern blot analysis performed by Fei Meng according to previous protocol (**13**). Viral RNA particles extracted form 1026A and 1026D as whole and run to compare to N2 wild type.

DNA Extraction. DNA was extracted from worm strains 1026A and 1026D according to protocol developed by Lamitina Labs(**21**).20mL large agarose plates created using standard NGM agar with concentrated OP50 food created with Terrific Broth. Worms placed and allowed to grow and reproduce til starved. Rinsed with M9 buffer then centrifuged for 30 seconds. Supernant removed and worm pellet washed in 4ml of TEN. Supernant removed and resuspend pellet in .5 ml TEN. Frozen at 20*C. Transfer to 1.5ml tube and add 25ul 10%SDS and 2.5ul 20mg/ml Proteinase K. Incubate at 50*C-60*C for 1 hour. Resuspend at 10,20, and 30 min. Add 2.5ul Proteinase K and incubate another hour. Extract with .5ml phenol. Mix by inversion. Phenol/CIA extract solution. Add 45ul 3M NaOAc and 0.8 ml ethanol at room temperature. Precipitate should appear(DNA).Spin lightly for few seconds. Drain ethanol and wash with ice cold 70% ethanol. Drain liquid. Resuspend pellet in 0.5ml TEN. Add 2ul of 10mg/ml RNase A. Incubate at 37*C for one hour. Phenol/CIA extract solution. Add 45ul of 3MNaOAc/0.8ml ethanol mixture. Invert and mix well. Spin for 30 seconds. Drain ethanol and wash with 70% ethanol that is ice cold. Drain and let pellet dry completely. Resuspend in 100ul of TE.

Imagining Microscopy. GFP and mcherry fluorescence images were taken with a Nikon p7000 digital camera mounted on a Nikon SMZ1500 microscope.GFP and mcherry observed under special fluorescent lighting. Images merged to show abundance of transgene and GFP at same time in *C.elegans* strains 1026A and 1026D. RNAi experiments recorded under standard lighting.

Results:

***C.elegans* strains 1026A and 1026D both have heat sensitive mutations.** Mutant strains 1026A and 1026D have both shown sensitivity to heat. Normal protocols dictate heat induction at 33*C for 3.5 hours in order to allow the activation of GFP. However, mutants have shown sensitivity to prolonged heat (**Fig 6**).After exposure to 33*C for 3.5 hours, mutants have trouble surviving in order to be observed or reproduce further. This type of mutation has been seen before by David Hirsh (**22**). It may be possible that the affected RNAi genes may be linked to genes coding for proteins that regulate *C.elegans* body temperature. However, we cannot place the mutation in 1026D into any of the major Hirsh categories, unlike 1026A, which will be explained later. In order to allow for visualization of the viRNA particles, heat induction was lowered to a maximum of 3 hours. This allowed for efficient GFP visualization and the survival of the specimens. This type of heat sensitive mutation has cause the creation of a new lab protocol to deal with future physical mutations of this type (See Materials and Methods).

***C.elegans* strain 1026A has delayed growth from mutation.** The mutated gene of strain 1026A causes delayed growth of *C.elegans* specimens. Unlike the average lifespan of the 11N2 wild type worms, 1026A has altered lifecycles. Full development into adult hood can take 5-7 days, longer than the average 4 days demonstrated by 11N2 and most mutants (**Fig 7**). This affects its life cycle by allowing shorter reproduction time. If we look at a previous study done by Hirsh, we see a classification of heat sensitive mutations involving reproduction and growth (**22**).This may give some insight into the nature of the mutated gene in 1026A. Hirsh categories it as a

class six mutation interfering with the reproductive lifecycle of an organism.1026A requires addition time to lay eggs and will produce less eggs than the average wild type or other comparable mutant strains. Since this sort of classification is linked to heat sensitivity, it gives some evidence of a separate mutation in the 1026A and the 1026D strains separate from RNAi feeding experiments.

***C.elegans* strain 1026A has mutant gene essential for RDVI and Classical Exogenous RNAi.** 1026A was subject to *skn-1* feeding tests in order to test for sensitivity to *skn-1* food. The tests showed that 1026A is not sensitive to the food (**Fig 8B**). As such, the mutated gene of 1026A is not unique to RDVI. When this type of RNAi testing is done, we are testing to see if the mutated non-functioning gene is unique for RDVI by sensitivity to the RNAi food. In a strain like 1026A, the introduction of dsRNA through feeding producing fertile eggs means the organism has an incomplete classical RNAi pathway. As such the strain cannot be unique for RDVI, or the classical pathway would be complete. However, viral accumulation is evident in 1026A through heat induction and PCR using the 11 transgene, accumulation of GFP was able to be visualized using the HIP:: RdRP::GFP gene crossed into the 1026A strand(**Fig 10A**). This was monitored through the Myo2::Mcherry::UTR reporter crossed along with the other gene. 1026A accumulated more viral particles than 1026D.Up regulation of viral particles is seen in infection by both Orsay and FHV(**Fig 9 A,B**).This shows that the mutated gene in 1026A is essential for both RDVI and Classical Exogenous RNAi. However, the exact nature and location of the gene cannot be determined without DNA sequencing and further testing on RNAi response.

***C.elegans* strain 1026D mutated gene is need for RDVI.** When subjected to *skn-1*, 1026D was shown to be sensitive to the food(**Fig 8C**). Sensitivity to the food indicates a shutdown of *skn-1*, leading to sterility in the subjected organism. S*kn-1* is necessary for the development of worm intestines (**19**). When *skn-1* food in ingested, dsRNA is introduced into host organism. In a strain like 1026D, sterile eggs are laid. This means strains have completed classical RNAi pathways, meaning the mutated gene is unique to the RDVI pathway. Sterility of offspring means completed delivery into the parental organism and introduction of *Skn-1* gene knockdown, where *Skn*-1 plays a role in the classical RNAi pathway. Using the same reporter gene and GFP transferred into 1026A, visualization of the viRNA particles showed accumulation in 1026D, although to a lesser extent than1206A(**Fig10B**). Northern blot analysis showed up regulation of reproduction of viral particles upon introduction of both Orsay and FHV into the 1026D(**Fig 9 A,B**).This was to a greater extent than 1026A.

Transgene 48 affects Exogenous RNAi and RDVI measurement. Upon attempting to visualize original 48N2 derived strains of 1026A and 1026D, viRNA particles do not give adequate GFP fluorescence(**Fig 10C**). In addition, when attempting RNAi feeding to 1026D without the 11 transgene, 1026D is not sensitive(**Fig 8D**). It is possible that since 48 is a transgene used to rescue function, the delivery of these genes may affect the ability for delivery of the *skn-1* dsRNA into the classical RNAi pathway, thereby giving a false result. It is only after delivery of the 11 transgene that the GFP fluorescence is able to be seen accurately(**Fig 10C**). In addition, testing for the classical pathway in 1026D is able to be performed to show the completion of the pathway(**Fig 8C**). The exact effect of the 48 transgene is not known at this time.

Discussion:

RDVI is a unique and complicated mechanism of eukaryotic organisms. Previous lab studies have shown essential genes to RDVI and production of siRNA from viral RNA (**6, 10, 13**). From those studies one unique gene was found(DRH-1) that is not also used in classical exogenous RNAi (**10,13**).To further search for genes essential to RDVI and those unique only to RDVI, I subjected *C.elegans* strains 1026A and 1026D to both GFP visualization and RNAi feeding experiments. From these experiments, I was able to conclude that the mutated gene in 1026D is unique to RDVI, while the mutated gene in 1026A is not unique to RDVI, yet serves an essential function in both RDVI and classical exogenous RNAi.

The discovery that the mutated gene in 1026D is unique only to RDVI is very important. It offers the chance to discover another gene that serves not only a major function to RDVI, but that is only found in this pathway. However, we do not currently know where this gene is located on the *C.elegans* genome. To isolate this location, 20-25 sub strains from crossing of the 11 transgene into the 1026D strain and isolation of different sub strains will have their DNA extracted and sent off for sequencing. Once the location of this gene is discovered, further experimentation will be able to be done and a new gene will be introduced to the scientific community.

However, the discovery of the 1026A mutated gene cannot be ignored. Although not unique to RDVI, exposure to both Orsay and FHV viruses show that the gene has some function is the suppression of viRNA upon infection.We know that this gene is essential to both pathways. Similar to 1026D, the 20-25 isolated sub strains will have their DNA extracted to be sequenced. Finding the location of this gene will not only give the location on the *C.elegans* genome, but also gives some insight into any linked genes. From the results, we can see that the heat sensitivity and developmental delay of this strain may be due to linkage to a heat-sensitive gene (**22**). Isolating the location may give a direction for multiple scientific fields to explore in *C.elegans*.

This study has given some insights into the world of RNAi and RDVI in *C.elegans*. These studies give us insight into the mysteries of eukaryotic organisms. RNAi has been shown in plants, fungi, *C.elegans*, and *D.melanogaster* (**4**). In addition, RNAi activity has been seen in humans. Based on other lab studies (**10, 13**), we know that genes homologous to those found in humans exist in *C.elegans*. The DRH-1 gene is homologous to the RIG-helicase found in humans. Although humans have a much more complicated system of RNAi, we may be able to find genes similar to the other *C.elegans* genes, which will allow the scientific community to develop a hypothesis on the RNAi and RDVI pathways in humans. In particular, developing a pathway hypothesis for RDVI in humans would allow the scientific community to begin to develop antivirals methods to directly treat viral infection through regulation of RDVI genes.

Future directions for lab research will be working on both discovering new genes for RDVI and discovering their counterparts in humans. Hopefully with time, advances can be made that will allow the treatment of humans with precise antivirals that will utilize the RDVI pathway in conjunction with other human immune responses. For now, the lab will continue to discover new genes and mutations that will further the development of the RDVI pathway and our understanding of how RDVI functions in *C.elegans*.

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