RSD-2 mediates RDE-4-independent antiviral silencing in Caenorhabditis elegans

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RSD-2 MEDIATES RDE-4-INDEPENDENT ANTIVIRAL SILENCING IN
CAENORHABDITIS ELEGANS

A Thesis
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RNA interference (RNAi) is a phylogenetically conserved gene regulation mechanism that modulates a wide variety of biological functions through suppressing gene expression at transcriptional or posttranscriptional levels (Bass 2000; Sharp 2001). One of the major natural functions of RNAi is antiviral defense in cytosol. RNAi directed viral immunity (RDVI) targets viral transcripts for destruction using small interfering RNAs (siRNAs) processed from viral replication intermediates, in the form of double-stranded RNA (dsRNA), as sequence guide (Lu, Maduro et al. 2005). Accumulating evidence suggests RDVI in the nematode worm Caenorhabditis elegans begins with the biogenesis of virus-derived siRNAs (viRNAs) by DCR-1 (Bernstein, Caudy et al. 2001; Duchaine, Wohlschlegel et al. 2006), a type III ribonuclease, and RDE-4, a dsRNA binding protein (Grishok, Pasquinelli et al. 2001; Knight and Bass 2001). Efficient destruction of viral transcripts guided by viRNAs is then orchestrated by several host factors that form distinct classes. Some of the known host factors downstream of viRNA biogenesis include Argonaute proteins (e.g. RDE-1) (Tabara, Sarkissian et al. 1999; Hammond, Boettcher et al. 2001; Parrish and Fire 2001), RNA-dependent RNA polymerases (e.g. RRF-1) and putative RNA helicases (e.g. DRH-1) (Tabara, Sarkissian et al. 1999).

To better understand worm RDVI, we have recently performed a genetic screen aiming to isolate novel host factors in the RDVI pathway. RSD-2 is one of our top candidates whose function in RDVI has been confirmed using corresponding genetic mutants in this study. RSD-2 is a novel protein that is not conserved in fungi, plants, insects or vertebrates (Tijsterman and
Plasterk 2004). When the level of viral replication was accessed in double mutants that contain both the rsd-2 null allele and null allele corresponding to rde-1, rde-4 or drh-1, enhanced viral replication, as compared to respective single mutants, was observed. Since viRNAs can be readily detected in double mutants corresponding to rde-4;rsd-2, these observations together suggested that RSD-2 functions in a RDE-4-independent pathway for virus silencing. Since RSD-2 appears to be unique to the nematode kingdom, our study on the function and mechanism of RSD-2 may help reveal some unique features of the worm RDVI.
CHAPTER ONE: INTRODUCTION

1.1 C. elegans as a Model Organism

*C. elegans* is a type of free-living nematode, typically 1 mm in-length, which feeds on bacteria, usually *E. coli* species (Wood 1988). They can be maintained in the lab by growing on agar plates with the *E. coli* as the food source (Stiernagle 2006). *C. elegans* has been one of the model organisms for research on biological sciences since 1974 (Epstein, Waterston et al. 1974; Waterston, Epstein et al. 1974).

![Diagram of C. elegans anatomy]

**Figure 1.1** The anatomy of *C. elegans* (Girard, Fiedler et al. 2007). The hermaphrodites and males are different in their body size and structures such as the somatic gonad and tail. Numerous other tissues and organs are sexually dimorphous, especially the nervous system and musculature.

The basic anatomy of *C. elegans* includes mouth, pharynx, intestine, gonad, and collagenous cuticle (Riddle 1997). *C. elegans* has two sexes: hermaphrodites and males (Riddle 1997). Hermaphrodites have two ovaries, oviducts, spermatheca, and a single uterus (Riddle 1997). Males have a single-lobed gonad, vas deferens, and a tail specialized for mating. *C.
*elegans* has five pairs of autosomes and one pair of sex chromosomes (Riddle 1997). Hermaphrodite *C. elegans* has a pair of sex chromosomes (XX), but the males have only one sex chromosome (X0) (Riddle 1997). Hermaphrodites can be maintained by self-fertilizing, and males can be produced by heat shock at 42 °C for around 45 minutes and maintained by crossing with hermaphrodites. Self-fertilized hermaphrodites can lay approximately 300 eggs, while the number can be above 1,000 when fertilized by a male (Riddle 1997).

At 20 °C, *C. elegans* has an average life span of 2-3 weeks, and it needs around 14 hours, 12 hours, 8 hours, 8 hours, 10 hours, and 8 hours to grow from embryonic development-L1-L2-L3-L4-young adult-adult. For special, when stresses such as crowding or food limitation condition is encountered, L1 animals can enter into the dauer stage in which animals can survive without food for several months, and after the stresses disappear, the worms staying at the dauer stage can bypass both L2 and L3 stages and get into the L4 stage directly (Cassada and Russell 1975; Albert and Riddle 1988).

![Figure 1.2 The life span of *C. elegans* at 20 °C (Girard, Fiedler et al. 2007). *C. elegans* has a short life cycle. The life cycle is temperature-dependent. *C. elegans* goes through a reproductive life cycle (egg to adult) in 5.5 days at 15°C, 3.5 days at 20°C, and 2.5 days at 25°C.](image-url)
The genomic sequence of *C. elegans* was obtained and published in 1998 (1998), and the remaining gaps were finished by 2002. The *C. elegans* genome sequence is approximately 100 million base pairs long (Wood 1988).

Mutant *C. elegans* strains can be obtained through treatment of chemical mutagen such as Ethyl Methanesulfonate (EMS) or exposure to ionizing radiation (Epstein, Shakes et al. 1995; Jorgensen and Mango 2002), followed by genetically crossing with wild type *C. elegans* strain for several times to remove the possible undesired mutated genes based on the extent of used mutagenesis, and self-crossing to obtain the homozygous strain. Genetic cross among homozygous *C. elegans* with different genetic backgrounds can facilitate separation or combination of different genetic background to bring about new *C. elegans* strains with the genetic background we are interested in from their descendants. Transgenic *C. elegans* strains can be created through injection of the designed plasmid constructs to animals’ gonads followed by picking the desired strain from the next generation of treated animals (Mello, Kramer et al. 1991). There is a large collection of *C. elegans* genetic mutants, and they can be ordered from the Caenorhabditis Genetics Center (CGC) or be asked from the labs which generated those mutants (Rogers, Antoshechkin et al. 2008).

*C. elegans* genes can be down-regulated using many different procedures such as feeding worms with the bacteria producing corresponding dsRNAs, transforming the worm with the sequence which can be transcribed as dsRNAs, or artificially introducing synthesized dsRNAs into animals by either soaking or injection (Jose and Hunter 2007).
Figure 1.3 Illustration of strategies for down-regulating genes in *C. elegans* (Jose and Hunter 2007). (A) Feeding the worms with transgenic bacteria expressing dsRNAs complementary to the gene of interest. (B) Worms can be made transgenic to produce specific dsRNAs in vivo. (C) Synthesized dsRNAs can be introduced to worms through either injection or soaking.

1.2 RNA Interference

RNA interference (RNAi), or RNA silencing is a novel mechanism found in eukaryotic cells that regulates gene expressions (Hammond, Caudy et al. 2001; McManus and Sharp 2002; Tomari and Zamore 2005), and three main types of small RNAs, microRNA (miRNA), small interfering RNA (siRNA), and piwi-interacting RNA (piRNA), were found to play important roles in different RNAi pathways (Xie, Johansen et al. 2004; Ruby, Jan et al. 2006).

For siRNA-mediated RNAi pathway, RNAi is initiated by dsRNA (Fire, Xu et al. 1998). Subsequently, dsRNAs will be diced by Dicers into short fragments of 20-30 nts called small interference RNAs (siRNAs) (Ding and Voinnet 2007; Aliyari and Ding 2009). Thereafter, the passenger strand will be degraded and the guide strand will be incorporated into the RNA-induced silencing complex (RISC) for target selection, eventually resulting in either degradation
or translation arrest of the targets based on the extent of sequence complementarities between the guide strand and its targets (Hammond, Bernstein et al. 2000; Hammond 2005). Some organisms such as plants and *C. elegans* are able to produce RNA-dependent RNA polymerases (RdRPs), a class of enzymes responsible for generation of secondary siRNAs, which can also silence target transcript expression (Sijen, Fleenor et al. 2001; Pak and Fire 2007; Sijen, Steiner et al. 2007). As a result, RNAi in these organisms is very efficient despite the initially small amount of siRNAs, unlike organisms, such as mammals, which do not produce RdRPs. In addition, some organisms have structure channels between adjacent cells, e.g. plasmodesmata in plants, or protein channels in cell membranes, e.g. SID-1 protein in *C. elegans*, causing RNAi silencing signals being able to travel from cell to cell. Consequently, RNAi in these organisms is capable of spreading systemically.

Naturally, RNAi-related mechanisms contribute to development, transposon control, and antiviral silencing (Grishok, Pasquinelli et al. 2001; Boutet, Vazquez et al. 2003). Since the silencing effect is highly specific and robust, RNAi has been used as a genetic tool to suppress the expression of genes of our interest, mainly through introducing corresponding artificial dsRNAs into cell cultures or living organisms. Moreover, RNAi has also been used in reverse genetic to identify genes required for a particular cellular pathway or an event by systemically silencing each gene in the cell in a large-scale genetic screen.

In *C. elegans*, RNAi was known as efficient, transitive and systemic, and it can be induced by feeding, soaking, or injecting the organism with exogenous dsRNA, or through integrating the DNA sequence, which is able to generate dsRNA after transcription, into worm’s genome (Figure 1.3).
1.3 RNAi Directed Viral Immunity (RDVI)

Antiviral defense is one of the natural functions of RNAi (Boutet, Vazquez et al. 2003; Ding, Li et al. 2004; Deleris, Gallego-Bartolome et al. 2006; Wang, Aliyari et al. 2006). Infecting viruses in organisms can generate long dsRNAs during the viral replication. In plants and invertebrates, Dicers will cleave viral dsRNAs into short fragments of viral siRNAs (viRNAs), which are structurally similar to the siRNA duplex intermediate (Boutet, Vazquez et al. 2003). Afterward, one strand of the viRNA duplex will be loaded into RNA induced silencing complex (RISC) and used as sequence guide to find its complementary targets, the viral RNA transcripts. Consequently, the catalytic component of RISC, Argonaute protein will cleave the viral transcripts, resulting in the silencing of replicating viruses (Vastenhouw and Plasterk 2004).

In some organisms such as plants and nematodes, the production of secondary viRNAs by the organisms’ own RdRPs can bring about great increase of the amounts of viRNAs available to RISC, leading to the silencing effect of antiviral RNAi being further amplified (Aoki, Moriguchi et al. 2007; Diaz-Pendon, Li et al. 2007).

1.4 Flock House Virus (FHV)

We choose FHV as our model virus because FHV can replicate in yeast, plant, insect and mammalian cells and it is one of the well-studied viruses (Johnson and Ball 1999; Venter and Schneemann 2008). FHV contains two genomic segments, RNA1, which encodes the viral RNA dependent RNA polymerase (RdRP) (Figure 1.4A) and RNA2, which encodes precursor protein of the viral coating protein (Figure 1.4B). RNA1 can replicate autonomously in the absence of RNA2 whose replication is dependent on RNA1. At the 3’ end of RNA1, there is a subgenomic RNA3 which is transcribed during RNA1 replication from an internal site of the complementary,
replicative intermediate of RNA1 (Figure 1.4A). RNA3 is not required to initiate FHV infection and it encodes the RNAi suppressor protein B2.

![Figure 1.4 Genomic structure of Flock House Virus.](image)

**Figure 1.4 Genomic structure of Flock House Virus.** (A) Expression of wild type FHV RNA1 (FR1) produces FHV RdRP and expression of FHV RNA3 (FR3) produces RNAi suppressor B2 protein of FHV. (B) Expression of wild type FHV RNA2 (FR2) produces the precursor protein of FHV.

1.5 Orsay Virus

Orsay virus is a novel RNA virus distantly related to known nodaviruses, and it was isolated from wild *C. elegans* strain JU1580 with abnormal morphological phenotypes in intestinal cells (Felix, Ashe et al. 2011). RT-PCR assay and Illumina/Solexa high-throughput sequencing were utilized to confirm the presence of Orsay virus in the infected animals, and Orsay viral RNA was detected in intestine and somatic gonad, through fluorescent in situ hybridization (FISH) using a probe complementary to the sense RNA1 segment of Orsay virus (Felix, Ashe et al. 2011). The observation that Orsay virus infection invokes a small RNA response in JU1580 animals indicates RNAi mechanisms provide antiviral immunity to *C. elegans* and Orsay virus infection of mutant animals can be used to confirm genes essential for antiviral defense (Felix, Ashe et al. 2011). Also, the observation that Orsay virus accumulation increases in RNAi mutant strains, compared to that in wild type N2 worms, provides a completely natural setting to
demonstrate the roles of RDVI in *C. elegans*. Thus, all of our results were confirmed through Orsay virus infection.

The genomic structure of Orsay nodavirus is very similar to that of FHV. The genomic structure of Orsay nodavirus is very similar to that of FHV. Both viruses contain two genomic segments, RNA1 which encodes RNA dependent RNA polymerase, and RNA2 which encodes the precursor protein of viral coating protein at its 5' end. However, no predicted RNA3 sequence was found at the 3’ end of Orsay virus RNA1; instead, it seems that there is another open reading frame at 3’ end of Orsay virus RNA2. For detection of Orsay virus infection, we prepared cDNA probes that are complementary to the segment of Orsay virus RNA1.

![Figure 1.5 Schematic of genomic organization of Orsay virus (Felix, Ashe et al. 2011).](image)

The RNA1 segment (2,680 nucleotides) encoded a predicted open reading frame of Orsay virus RdRP, and the RNA2 segment was predicted to encode a capsid protein at its 5’ end as well as a second ORF at the other end.

### 1.6 The *rsd-2* Gene

Originally, *rsd-2* was screened out as a gene required for RNAi spreading from somatic cells to germline cells in *C. elegans* and the mutant animals showed no any development defect (Tijsterman and Plasterk 2004). The observation that *rsd-2* mutants showed phenotype when fed with dsRNA against somatic genes, but not germline genes, indicates that *rsd-2* mutants are sensitive to RNAi against somatic genes, but not to RNAi targeting germline genes. In other words, mutations in the *rsd-2* gene do not affect somatic RNAi (Tijsterman and Plasterk 2004).
They don’t appear defective in the initial uptake of dsRNA from the gut into somatic tissues but they fail to further distribute the dsRNA to the germline (Tijsterman and Plasterk 2004).

Later on, it was discovered that the function of \textit{rsd-2} in RNAi is very complex and environmentally regulated. First, \textit{rsd-2} gene has mosaic effect in both somatic RNAi and germline RNAi based on the observations after increasing the range of genes targeted by feeding RNAi. Second, the \textit{rsd-2} mutants show environmentally sensitive defects in cell autonomous RNAi elicited from transgene-delivered dsRNAs. Third, \textit{rsd-2} has essential roles in maintaining chromosome integrity, such as transposon silencing in some unfavorable environment (Wang, Aliyari et al. 2006).

\textit{RSD-2} protein is a novel protein with no close homolog found in other organisms, thus no indication of its molecular function so far. RSD has an N-terminal domain that exists in three copies (Tijsterman and Plasterk 2004). RSD-2 protein was found to exist in multiple cellular compartments, including the nucleolus and cytoplasmic compartments, and to most frequently interact with RSD-6 (Wang, Aliyari et al. 2006).

The \textit{rsd-2} gene is located on chromosome IV. Two alleles of \textit{rsd-2} were used in my study: \textit{tm1429}, which contains 451 base pair (bp) deletion, and \textit{pk3307}, which contains a point mutation (c to t) (Figure 1.6). The \textit{tm1429} allele was isolated using TMP/UV as mutagen by Shohei Mitani’s lab at the Tokyo Women’s Medical College, and the \textit{pk3307} allele was isolated using EMS as mutagen by Hubrecht Laboratory (Rogers, Antoshechkin et al. 2008).

The wild type RSD-2 coding sequence is shown below. Pink colored sequences are flanking sequences, and capitalized sequences are deleted sequences in the \textit{tm1429} allele.
The sequences of the plus strand of mutant *rsd*-2 in strain tm1429 are as followings.

tcaacccaaaatctcacaatactctttgcattgtaagaaaccattcccgtgttcattttgggaagccgagagataacttttcgcgccaagac
cattttctgcattgataaatgttatctcaatattccccactactggaagtttatttttttgcatttttgcagcattatacaacatcaaaaacaacagttaagcagatcagataatgatccaatcagtcatcgaattgtcttttaagtggagctact
gctttgaaacatgcttttcagtggatcaccgtattgtaccctgaaaaatccaaaaattaacttttttttcattttgcagcattatacaacataaaaaa
cagttttcaagattagttgaaacgaacgatcagaaatagctcagaaagatgtgatctatccaaacaagaatctacaatttttaagtggagctact
gctttgaaacatgcttttcagtggatcaccgtattgtaccctgaaaaatccaaaaattaactttttttgcattttgcagcattatacaacataaaaaa

cacgttttcaagattagttgaaacgaacgatcagaaatagctcagaaagatgtgatctatccaaacaagaatctacaatttttaagtggagctact
gctttgaaacatgcttttcagtggatcaccgtattgtaccctgaaaaatccaaaaattaactttttttgcattttgcagcattatacaacataaaaaa

(Received July 2008)

The sequences of the plus strand of mutant *rsd*-2 in strain tm1429 are as followings.
Figure 1.6 Genomic positions of the wild type \textit{rsd-2} gene and the \textit{rsd-2} mutant alleles (Rogers, Antoshechkin et al. 2008). The tm1429 mutant allele contains a 451 bp deletion and the pk 3307 mutant allele contains a nucleotide substitution of c to g.
CHAPTER TWO: MATERIALS AND METHODS

2.1 C. elegans Genetics and Culture

All worm strains were propagated and maintained using standard protocols. The food for the worms is prepared using *E. coli* strain OP50 or HT115. The Bristol strain N2 was used as the standard wild-type strain. Alleles used in this study are all derived from N2 and include *rde-1* (ne300), *rde-4* (ne337), *drh-1* (tm1329), *drh-2* (ok951), *rrf-1* (pk1417), *ergo-1* (tm1860), and *rsd-2* (tm1429 and pk3307). The genotypes of the worm strains containing single or double mutations were confirmed by PCR and/or feeding RNAi targeting *skn-1*. The strain used to maintain Orsay virus is JU1580 from Dr. David Wang’s lab.

2.2 Transgenic Construct and Transgenic Worms

The FR1gfp replicon was derived from pFR1-3 through replacing most of the B2 coding sequence (the nucleotides 2,802–3,001 of RNA1) by the full length enhanced GFP coding region as described previously (Li, Li et al. 2004; Lu, Maduro et al. 2005). This method created a translational fusion of GFP with N-terminal 23 amino acids of B2 and around 200 nucleotides (nts) deletion from the B2 open reading frame. Transgenic animals carrying the FR1fp replicon was generated by microinjecting into wild type strain with FR1fp, in which the 3’ end of enhanced GFP coding sequence was used to replace B2 coding sequence in pFR1-3 instead of the full length enhanced GFP sequence. Psur-5::GFP contains a constitutive sur-5 promoter followed by full length enhanced GFP coding sequence. Similarly, Psur-5::RSD-2 contains wild type RSD-2 coding sequence driven by the sur-5 promoter.

Animals were made transgenic by gonadal microinjection following standard protocol (Mello, Kramer et al. 1991). Briefly, FR1gfp plasmid (final concentration 5 mg/ml) was mixed
with the rol-6D plasmid pRF4 (final concentration 100 mg/ml) for injection into wild-type N2 animals. Integrated lines were then generated by treating about 50 transgenic hermaphrodites with 3,500 rad of γ-rays from a $^{137}$Cs source, followed by screening for integrated animals in the F2 generation.

### 2.3 Orsay Virus Infections

The Orsay virus stock was maintained using *C. elegans* isolate JU1580. To prepare Orsay virus inoculum, Orsay virus particles was extracted by first washing off infected JU1580 animals from 6 cm culture plates using distilled water. Then, the animals were precipitated by centrifugation. Next, the virus containing supernatant solution was collected and filtered through 0.2 µm filters to remove other possible contaminants. At last, 5-10 times concentrated *E. coli* solution was mixed with the filtered liquid, and aliquots were dropped on the middle of the agar plates for culturing *C. elegans* strains. As soon as the Orsay virus containing *E. coli* food in the culture plates becomes dry, worms can be grown in these plates for 2 to 3 days to be infected by Orsay virus.

### 2.4 RNAi Experiments

Feeding RNAi targeting *gfp* or *skn-1* was performed by feeding worms with *E. coli* food which can express dsRNAs corresponding to *gfp* or *skn-1* coding sequence respectively. IPTG at final concentration of 2 mM was used for the induction of dsRNA expression.

### 2.5 RNA Preparation and Northern Blot Analysis

Total RNA was prepared using the TRI Reagent method (MRC, Inc.). RNA concentrations were normalized and used for northern blot analysis according to standard protocols described previously (Li, Li et al. 2002) using labeled cDNA probe corresponding to the 3’ end 387 nts of
GFP, which will hybridize to both RNA1 and RNA3 of FHV. For high molecular weight viral RNA detection, 3 to 6 µg total RNA per lane was loaded in 1.2% agarose gel. For small RNA analysis, 10 to 20 µg of enriched small RNA samples per lane was resolved using 15% acrylamide denaturing gel along with chemically synthesized DNA oligos as size references. After electrophoreses, the RNA samples were transferred onto Hybond N+ membrane (Amersham Bioscience) and UV cross-linked using 1800 µJ/cm² as output power (SpectroLinker). For northern blot detection of FHV and full length GFP transcripts, cDNA probes were prepared by labeling GFP DNA fragments using AlkPhos Direct Labeling Reagents (GE Healthcare Company). For (-)viRNA analysis, the membranes were hybridized with synthesized oligo DNA probes, which have the same polarity of FHV RNA1 and were labeled using DIG kit (Roche Company), in the hybridization buffer. Sequence for oligo probes used for the detection of miR-58 is ATTCGGTACTGAACGATCTCA.

2.6 RSD-2 Functional Rescue Experiment

The RSD-2 functional rescue was carried out by injecting the rsd-2 mutants (tm1429) carrying the FR1gfp replicon with the Psur-5::RSD-2 construct. The F1 progenies were then examined for GFP expression after being maintained at 25 °C for 36 hours post induction of the FR1gfp replicon replication. In the F1 progenies that carry the wild type rsd-2 extrachromosomal array, complete loss or significant reduction of green fluorescence expression was considered as successful rsd-2 functional rescue.

2.7 Imaging Microscopy

GFP fluorescence images were collected using an AmScope MT1000 camera mounted on an Olympus IMT-2 microscope.
CHAPTER THREE: STRATEGIES TO STUDY RDVI IN C. ELEGANS AND RESULTS

3.1 Initiate FHV Replication in C. elegans

The FHV replication in C. elegans was initiated using a strategy illustrated in Figure 3.1. It has been shown that Northern blot hybridizations could detect high level accumulation of FHV RNA1 and RNA3 in worms carrying the FR1-3 transgene described previously (Lu, Maduro et al. 2005).

![Figure 3.1 Strategy to trigger FHV replication in C. elegans. Heat induction can activate the promoter of the FR1 transgene so that the transcription of the following sequence will be driven. Then the ribozyme will be produced. After the ribozyme cleaves itself, the FHV plus strand will be produced and translated to FHV RdRP. Next, with the help of FHV RdRPs, the transgenic FHV will be able to replicate.]

The high level accumulation of viral RNAs detected in transgenic worm strains arise from active RNA replication because in the absence of RNA replication the initial heat-induced transcripts were below the limit of detection in worms two days post heat induction. FHV RNA2 accumulation can also be detected in FR1-3;FR2 transgenic worms after heat induction. Therefore, detection of FHV genomic RNA1, RNA2 and subgenomic RNA3 in these worm
strains provide strong evidences that *C. elegans* supports complete replication of the FHV RNA genome (Lu, Maduro et al. 2005).

To further confirm that antiviral RNAi contributes to FHV silencing in *C. elegans*, FR1-3 ΔB2 transgene was introduced to well-known RNAi deficient mutant worm strains such as *rde-1* mutant strain, which is very healthy, unlike many other RNAi mutant strains (Lu, Maduro et al. 2005). In contrast to undetectable accumulation of FHV RNA1 and 3 in wild type N2 worms carrying FR1-3 ΔB2 transgene, northern blot analysis showed abundant accumulation of FHV RNA1 and 3 in the FR1-3 ΔB2 transgenic *rde-1* mutant strain (Lu, Maduro et al. 2005). Thus, the mutant FHV RNA1 produced from FR1-3 ΔB2 transgene is not defective in self-replication, suggesting that the decreased accumulation of FHV RNA1 and 3 in N2 worms resulted from induction and clearance of viral RNAs by antiviral silencing in an *rde-1*-dependent siRNA pathway. Taken together, all these evidences confirmed that antiviral RNAi contributes to FHV silencing in *C. elegans*, making FHV transgenic *C. elegans* strains ideal model system to study the antiviral RNAi.

### 3.2 Identify Genes Required for Antiviral RNAi in *C. elegans* by Feeding RNAi

Previously, a derivative of the infectious full-length cDNA clone of FHV RNA1, FR1gfp (Li, Li et al. 2004), in which the B2 coding sequence is replaced by GFP coding sequence has been shown to replicate autonomously and produce green fluorescence (Figure 3.2). The inserted GFP coding sequence fused with the N-terminal 23 codons of B2 is expressed only from the recombinant RNA3 produced during FR1gfp replication, but not directly from FR1gfp because its initiation codon is more than 2.7 kb away from the 5’-terminus of FR1gfp RNA (Figure 3.2).
FR1gfp is defective in RNAi suppression because of the loss of B2 production but not in replication.

**Figure 3.2 Strategy to identify genes required for antiviral RNAi in C. elegans.** In the FR1gfp transgene, GFP sequence was used to replace part of RNA3 sequence so that FHV replication levels can be visualized. Next, feeding RNAi was used to down-regulate specific C. elegans genes. In the normal condition, after heat induction FHV would not replicate well in the absence of RNAi suppressor B2, while in case the C. elegans antiviral RNAi system does not function appropriately either, the FHV transgenic viruses should restore their ability to replicate; thus, through comparing the GFP expression levels of the worms, the antiviral RNAi gene components can be selected out.

As a result, the FR1gfp replication and gfp expression from the FHV replicon occur only after antiviral RNAi is suppressed by either B2 co-expression or genetic disruption of the antiviral RNAi pathway in both cultured fruit fly and mosquito cells (Li, Li et al. 2004). To develop a model for genetic screens to identify new genetic components in antiviral RNAi, we generated C. elegans strains bearing a chromosomally integrated FR1gfp transgene under the control of a heat-inducible promoter (Figure 3.2).

We found that very little green fluorescence was observed in FR1gfp worms after the heat induction of the FHV replicon transgene (Figure 3.3 top left). In contrast, bright green fluorescence was observed throughout the worm body after FR1gfp transgenic worms were fed
on *E. coli* food that expresses *rde-1* dsRNA (Figure 3.3 top middle), which depletes the mRNA of *rde-1* in a process called feeding RNAi (Timmons, Court et al. 2001). Bright green fluorescence was also observed in FR1gfp worms after a loss-of-function *rde-1* allele was introduced into FR1gfp worms by genetic crosses (Lu, Yigit et al. 2009). Northern blot hybridizations confirmed the abundant accumulation of the chimeric FHV RNA1 and RNA3 in FR1gfp worms after *rde-1* depletion, but FR1gfp replication was inhibited in wild-type N2 worms (Lu, Yigit et al. 2009). Thus, productive FR1gfp replication and abundant GFP expression in FR1gfp transgenic worms depend on the genetic disruption of the antiviral RNAi pathway, suggesting that FR1gfp transgenic worms could be used to screen for new genetic components in the antiviral RNAi pathway by feeding RNAi (Lu, Yigit et al. 2009). *Rsd-2* was identified as one of the candidates using this strategy (Figure 3.3).

![Image](image_url)

**Figure 3.3** *Rsd-2* was screened out as a gene required for antiviral RNAi in *C. elegans* (Lu, Yigit et al. 2009). Green fluorescence was detected in FR1gfp transgenic worms after feeding RNAi targeting specific genes or the commonly used L4440 vector. Photographs were taken 48 hours after induction of the replicon replication.
3.3 *Rsd-2* Is Required for Antiviral RNAi in *C. elegans*

3.3.1 *Rsd-2* Is Required for RNAi Targeting FHV in *C. elegans*

To confirm that *rsd-2* is indeed required for RDVI, the FR1gfp replicon transgene was introduced to *rsd-2* mutants containing different alleles through genetic crosses. After heat induction, the *rsd-2* mutant strains showed the similar brightness of GFP as the *drh-1* mutant strain which is defective in RDVI (Lu, Yigit et al. 2009) (Figure 3.4A, compare the top two with the right bottom), suggesting that *rsd-2* is indeed required for RDVI targeting FR1gfp. Northern blot hybridization further confirmed that FHV accumulates to similar levels in *rsd-2* mutants as those in *drh-1* mutants (Figure 3.4B). All these observations confirmed that *rsd-2* is indeed a gene required for RDVI in *C. elegans*.

![Figure 3.4](image)

**Figure 3.4** *Rsd-2* is required for RDVI targeting FHV in *C. elegans*. (A) Detection of green fluorescence in FR1gfp transgenic worms 48 hours after induction of the replicon replication. (B) Accumulation of FR1gfp genomic (RNA1) and subgenomic RNA (RNA3) by northern blotting in FR1gfp transgenic worms 48 hours post heat induction. Methylene blue staining of total RNA was provided to show equal loading.
3.3.2 *Rsd-2* Is Required for RDVI Targeting Orsay Virus in *C. elegans*

Orsay virus is a natural viral pathogen of *C. elegans*. To find out whether *rsd-2* is a gene required for RDVI under natural condition, we inoculated wild type N2 worms, *rsd-2* mutants containing distinct alleles (*tm1429* and *pk3307*), and *drh-1* mutants with Orsay virus. As shown in Figure 3.5, Orsay virus accumulated to high levels in *rsd-2* mutants and *drh-1* mutants but low levels in the wild type N2 worms (Figure 3.5A, compare the lane 2, 3 and 4 with the lane 1). RT-PCR detection also confirmed the high-level accumulation of Orsay virus in *rsd-2* mutants (Figure 3.5B). Taken together, these results confirmed that *rsd-2* is required for RDVI under natural condition.

![Figure 3.5](image)

**Figure 3.5** *Rsd-2* is required for RDVI targeting Orsay virus in *C. elegans*. (A) Detection of the Orsay virus genomic RNA1 accumulation levels by northern blotting in the worms grown on Orsay virus particle containing food for 2-3 days. Methylene blue staining of total RNA was provided to show equal loading. (B) Confirmation of the Orsay virus replication in worms by using RT-PCR to get two different DNA segments which were transcribed reversely from Orsay viral RNAs, using two different pairs of primers. Following RT-PCR, DNA gel electrophoresis was performed and photographed. The sizes of these two DNA fragments are 1.2 and 1.3 kb respectively.
3.3.3 Wild Type *rsd-2* Can Rescue RDVI in *rsd-2* Knockout Mutants

To further confirm that it is the *rsd-2* null alleles, but not any other null alleles, that are responsible for the loss of RDVI in the *rsd-2* mutants used in the test described in Figure 3.5, we checked FR1gfp replication in *rsd-2* mutants (*tm1429*) transgenic for Psur-5::RSD-2 (Materials and Methods 2.2). We reasoned that if it were the *rsd-2* null alleles, but not any other null alleles, that are responsible for the loss of RDVI in the *rsd-2* mutants, ectopic expression of wild type RSD-2 coding sequence in the *rsd-2* mutants will restore RDVI.

To test this hypothesis, the wild type *rsd-2* expressing construct (Psur-5::RSD-2) was co-infected with an mCherry reporter construct, which directs mCherry expression in pharynx, into the *rsd-2* mutants carrying the FR1gfp replicon transgene. Since, in most cases, the extrachromosomal arrays produced through gonad injection can only be randomly passed on to the next generation, as a result, within each generation of the transgenic lines, there are worms that do not carry the extrachromosomal arrays thus can serve as internal negative control. As shown in Figure 3.6A, within each transgenic line, clear correlation between reduced GFP expression and red fluorescence in pharynx produced by the reporter transgene was observed, suggesting that ectopic expression of wild type RSD-2 coding sequence restored RDVI in the *rsd-2* knockout mutants.

As a reconfirmation, we checked FR1gfp replication in the chromosomal integrants corresponding to the wild type *rsd-2* transgene using northern blot. We observed that, in contrast to the high-level replication of FR1gfp in the *rsd-2* mutants, the FR1gfp replication was significantly suppressed in *rsd-2* mutants containing the integrated transgenes corresponding to the wild type RSD-2 coding sequence (Figure 3.6B). These observations together confirmed that it is the *rsd-2* null alleles that results in the loss of antiviral silencing in the *rsd-2* mutants.
Figure 3.6 Wild type *rsd-2* gene rescued RDVI in *rsd-2* mutants carrying the FR1gfp transgene. (A) Detection of green fluorescence in FR1gfp transgenic worms with and without the extrachromosomal array that has copies of wild type *rsd-2* gene which rescue a loss-of-function mutation in the endogenous copies of *rsd-2* 48 hours after induction of the replicon replication. The array also expresses mCherry as a marker gene, from the mal2::mCherry construct in which mal2 is a promoter that only functions in the pharyngeal muscle cells of *C. elegans* and mCherry is a protein that can emit red fluorescence. Based on which animal’s pharynx shows red under the fluorescence microscope, the marker allows us to differentiate which animal contains the extrachromosomal array and which animal does not. (B) Accumulation of FR1gfp genomic (RNA1) and subgenomic RNA (RNA3) by northern blotting in the FR1gfp transgenic N2, *rsd-2* mutant, *rsd-2* mutant carrying integrated wild type *rsd-2*, *drh-1* mutant, and *rsd-2* mutant carrying non-integrated wild type *rsd-2* worms 48 hours post heat induction. Methylene blue staining of total RNA was provided to show equal loading.

### 3.3.4 *Rsd-2* Is Not Required for viRNA Biogenesis

*Rsd-2* was originally identified as a host factor responsible for systemic spreading of RNAi in *C. elegans* (Tijsterman and Plasterk 2004). Currently, how *rsd-2* contributes to RDVI remains largely unknown. As an effort to address this question, we decided to determine whether *rsd-2* is required for viRNA biogenesis. To this end, the small RNAs from N2, *rrf-1*, *rde-1*, *rde-4*, *drh-1* and *rsd-2* strains were extracted and northern blot hybridizations were performed to check the
viRNA levels in these strains. We probed for viRNAs of the antigenomic polarity, instead of viRNAs of genomic polarity, because probing for viRNAs of the genomic polarity resulted in a smear and no discrete bands were detected in any of the worm strains tested either before or after transcriptional induction of FR1gfp (Lu, Yigit et al. 2009).

![Image](image.png)

**Figure 3.7 ViRNAs accumulated in the rsd-2 mutants.** Analysis of the (-)viRNAs accumulation levels in the FR1gfp transgenic wild type and single knockout worm mutants 48 hours post heat induction. 15 µg of total small RNAs was loaded in each lane. DIG-labeled DNA oligos complementary to viRNA and miRNA were used as the probes. The same membrane was probed for miR-58 after stripping as the loading control.

The viRNAs were undetectable in wild-type worms, which may be due to the viral replication inhibition and thus lower levels of viral dsRNA for dicing in these worms. However, we detected viRNAs in the rsd-2 strains (Figure 3.7), indicating that the rsd-2 gene is dispensable for viRNA biogenesis.

### 3.4 Delineate an rsd-2-dependent Genetic Pathway That Contributes to RDVI in *C. elegans*

The predominant model for antiviral silencing is the canonical dsRNA-siRNA pathway of RNAi, which is initiated by viral double-stranded replicating intermediates. The Sole RNase III
enzyme Dicer of *C. elegans* will cleave the viral dsRNAs into primary viRNAs, which will be loaded into the RISC complex and direct the silencing of viral transcripts. The RdRP in *C. elegans* can use the cleaved viral RNAs as templates to produce secondary viRNAs, which can be further used to silence viral transcripts, so that antiviral RNAi effects can be amplified. The followings are some discoveries on the antiviral RNAi pathway in *C. elegans*. First, the observation that Dicer was required for anti-VSV in *C. elegans* cells (Schott, Cureton et al. 2005) suggests that Dicer is required for antiviral silencing in *C. elegans*. Second, dsRNA-binding proteins (dsRBPs) are also required by Dicer to play its role in RNAi. For example, in the exogenous dsRNA initiated RNAi (exo-RNAi) pathway in *C. elegans*, the dsRBP, RDE-4, cooperates with Dicer during cleaving long dsRNAs to siRNAs, although it is not essential in the following steps (Parker, Eckert et al. 2006). Furthermore, RDE-4 was found to be required for viRNAs biogenesis in the RDVI pathway of *C. elegans* (Lu, Yigit et al. 2009). Third, a DEXH-box helicase protein DRH-1 and an Argonaute protein RDE-1 were pulled down together with RDE-4 and DCR-1 in the exo-RNAi pathway in *C. elegans*, through immunoprecipitation (Tabara, Yigit et al. 2002), suggests that DCR-1, RDE-4, DRH-1, and RDE-1 may interact with each other to function in the exo-RNAi pathway in *C. elegans*. What’s more, DRH-1 and RDE-1 were found as requirements for activity of viRNAs, and both were found working downstream of RDE-4 in RDVI in *C. elegans* (Lu, Yigit et al. 2009). Fourth, Dicer-related helicase protein DRH-2 and Argonaute protein ERGO-1 were found as negative regulators of antiviral RNAi in *C. elegans* (Lee and Sinko 2006; Lu, Yigit et al. 2009).

To determine the genetic pathway that involves *rsd-2*, six double mutants carrying FR1gfp transgene by genetic crosses, *rsd-2;rede-4*, *rsd-2;drh-1*, *rsd-2;rede-1*, *rsd-2;rrf-1*, *rsd-2;drh-2*, *rsd-2;ergo-1* were created. We observed that both FHV and Orsay virus replication levels in the *rsd-
2; rde-4 and rsd-2; drh-1 double mutants were enhanced compared to corresponding single mutants, suggesting that rsd-2 works in an RDVI pathway different from that involves rde-4 and drh-1. In contrast to the discovery of no viRNAs detected in the rde-4 single mutants and rde-4; drh-1 double mutants (Lu, Yigit et al. 2009), we found viRNAs accumulated in the rsd-2; rde-4 double mutants, further corroborating our conclusion that rsd-2 works in an rde-4-independent RDVI pathway. Furthermore, our observations that both FHV and Orsay virus replication levels were not enhanced in rsd-2; rrf-1 double mutants, suggested rsd-2 and rrf-1 may function in the same RDVI pathway. In addition, our detection that both FHV and Orsay virus replication level were decreased in rsd-2; drh-2 and rsd-2; ergo-1 double mutants, further confirmed both drh-2 and ergo-1 negatively regulate rde-4-dependent RDVI pathway in C. elegans.

3.4.1 Both FHV and Orsay Virus Replication Were Enhanced in rsd-2; rde-4 Double Mutants Compared to Either rsd-2 or rde-4 Single Mutants

To investigate the rsd-2-dependent genetic pathway in C. elegans, FR1gfp transgene was introduced to rsd-2; rde-4 double mutants through genetic crosses. After heat induction of the FR1gfp transgenic N2, rde-4, rsd-2, and rsd-2; rde-4 strains, the GFP expression level in the rsd-2; rde-4 double mutants was found to be enriched compared to respective single mutants (Figure 3.8, compare picture 4 with 2 and 3), indicating that FHV replication level in the rsd-2; rde-4 double mutants was enhanced compared to either rsd-2 or rde-4 single mutants.

Total RNAs were extracted from all of the above four heat inducted strains, and northern blot hybridizations were performed to check FHV replication levels. We observed FHV replication level was enhanced in rsd-2; rde-4 double mutants (Figure 3.9A, compare the lane 4 with 2 and 3), demonstrating a further loss of RDVI in rde-4; rsd-2 double mutants compared to
either *rde-4* or *rsd-2* single mutants. This result indicated that FHV was targeted by two parallel antiviral RNAi pathways in *C. elegans* and that *rsd-2* works in an *rde-4*-independent antiviral RNAi pathway.

**Figure 3.8** GFP expression level was enhanced in *rsd-2;rde-4* double mutants. Detection of green fluorescence in the FR1gfp transgenic wild type, single and double knockout worm mutants 48 hours post replicon replication.

**Figure 3.9** FHV and Orsay virus replication levels were enhanced in *rsd-2;rde-4* double mutants. (A) Accumulation of the FR1gfp replicon RNAs in wild type, single and double knockout worm mutants 48 hours after replicon replication. (B) Accumulation of the Orsay virus RNA1 in wild type, single and double knockout worm mutants after growing them on Orsay virus particle containing food for 3 days.
To further confirm our conclusion that \textit{rsd-2} works in a separate antiviral RNAi pathway compared to \textit{rde-4}, we used Orsay virus to infect the N2, \textit{rde-4}, \textit{rsd-2}, and \textit{rsd-2; rde-4} mutant strains for 2 days, and then total RNAs were extracted for northern blot analyses. We observed Orsay virus replication level in \textit{rsd-2; rde-4} double mutants was enhanced (Figure 3.9B, compare the lane 4 with 2 and 3), further reinforcing our conclusion that \textit{rsd-2} functions in an antiviral RNAi pathway that does not involve \textit{rde-4}.

\textbf{3.4.2 Both FHV and Orsay Virus Replication Were Enhanced in \textit{rsd-2; drh-1} Double Mutants Compared to Corresponding Single Mutants}

If \textit{rsd-2} indeed works in a separate antiviral RNAi pathway, both FHV and Orsay virus replication should be enhanced in \textit{rsd-2; drh-1} double mutants because \textit{drh-1} is known to function downstream of \textit{rde-4} down the same genetic pathway. To test this hypothesis, FR1gfp transgene was introduced to \textit{rsd-2; drh-1} double mutants through genetic crosses. After heat inducting the FR1gfp transgenic N2, \textit{drh-1}, \textit{rsd-2}, and \textit{rsd-2; drh-1} strains, the GFP expression level was shown enhanced in \textit{rsd-2; drh-1} double mutants compared to either \textit{rsd-2} or \textit{drh-1} single mutants (Figure 3.10, compare image 4 with 2 and 3), implying that FHV replication was enhanced in the \textit{rsd-2; drh-1} double mutants.

Total RNAs were extracted from the heat inducted strains for northern blot hybridizations to check FHV replication levels. The observation that FHV replication level was enhanced in \textit{rsd-2; drh-1} double mutants (Figure 3.11A, compare the lane 4 with 2 and 3) further upheld our conclusion that \textit{rsd-2} functions in an antiviral RNAi pathway that does not involve \textit{rde-4} or \textit{drh-1}.
As a reconfirmation, we used Orsay virus to infect the N2, rde-4, drh-1, and rsd-2;drh-1 mutants for 2 days, and then total RNAs were extracted for northern blot analyses. Our observation that Orsay virus replication level in rsd-2;drh-1 double mutants was enhanced (Figure 3.11B, compare the lane 4 with 2 and 3) compared to each single mutants once again confirmed rsd-2 functions in a genetic pathway that does not involve rde-4 and drh-1.

Figure 3.10 Rsd-2 and drh-1 function in separate RDVI pathways. Detection of green fluorescence in the FR1gfp transgenic wild type, single and double knockout worm mutants 48 hours post replicon replication.

Figure 3.11 FHV and Orsay virus replication levels were enhanced in drh-1;rsd-2 double mutants. (A) Accumulation of the FR1gfp replicon RNAs in wild type, single and double knockout worm mutants 48 hours after replicon replication. (B) Accumulation of the Orsay virus RNA1 in wild type, single and double knockout worm mutants after growing them on Orsay virus particle containing food for 3 days.
3.4.3 Both FHV and Orsay Virus Replication Levels Were Enhanced in rsd-2;rde-1 Double Mutants Compared to Either rsd-2 or rde-1 Single Mutants

*Rde-1* encodes an Argonaute protein that functions downstream of siRNA biogenesis in RNAi. To find out whether *rsd-2* and *rde-1* function in the same genetic pathway, FR1gfp transgene was introduced to *rsd-2;rde-1* double mutants by genetic crosses. After heat induction of the FR1gfp transgenic N2, *rde-1*, *rsd-2*, and *rsd-2;rde-1* strains, the GFP expression level was shown enhanced in *rsd-2;rde-1* double mutants compared to either single mutants (Figure 3.12, compare picture 4 with picture 2 and 3), suggesting that FHV replication level was enhanced in the *rsd-2;rde-1* double mutants.

![Figure 3.12 GFP expression level was enhanced in rsd-2;rde-1 double mutants. Detection of green fluorescence in the FR1gfp transgenic wild type, single and double knockout worm mutants 48 hours post replicon replication.](image)

Northern blot hybridization also confirmed that FHV accumulated to higher levels in *rsd-2;rde-1* double mutants than those in either *rde-1* or *rsd-2* single mutants (Figure 3.13A, compare lane 4 with lane 2 and 3). Thus, we concluded *rsd-2* and *rde-1* function in separate antiviral RNAi pathways.
To affirm this conclusion, Orsay virus was used to infect the N2, rsd-2, rde-1, and rsd-2;rde-1 mutant strains for 2 days, and then total RNAs were extracted and northern blot hybridizations were performed to check the Orsay virus replication levels. The observations that the Orsay virus replication level in rsd-2;rde-1 double mutants was enhanced compared to either single mutants (Figure 3.13B, compare lane 4 with 2 and 3) further bolstered our conclusion that rsd-2 and rde-1 work in separate antiviral RNAi pathways.

Figure 3.13 FHV and Orsay virus replication levels were enhanced in rsd-2;rde-1 double mutants. (A) Accumulation of the FR1gfp replicon RNAs in wild type, single and double knockout worm mutants 48 hours after replicon replication. (B) Accumulation of the Orsay virus RNA1 in wild type, single and double knockout worm mutants after growing them on Orsay virus particle containing food for 3 days.

3.4.4 ViRNAs Accumulated in rsd-2;rde-4 Double Mutants

It is known that rde-4 is responsible for viRNA biogenesis in its antiviral RNAi pathway based on the observation of no viRNAs detected in rde-4 single mutants (Lu, Yigit et al. 2009) and that rsd-2 is not required for viRNA biogenesis based on the observation of viRNAs accumulation in rsd-2 single mutants compared to wild type strain. Thus, if it is true that rsd-2 works in a different antiviral RNAi pathway from rde-4 and drh-1, then viRNAs would accumulate in rsd-2;rde-4 double mutants because mutant rde-4 should not affect the viRNA
biogenesis of the antiviral RNAi pathway in which $rsd$-2 involves and mutant $rsd$-2 does not affect the viRNA biogenesis of its own antiviral RNAi pathway since $rsd$-2 gene is dispensable for viRNA biogenesis.

That northern blot hybridization analysis detected viRNAs accumulated to high levels in $rsd$-2;$rde$-4 double mutants (Figure 3.14), further substantiated $rsd$-2 works in an $rde$-4 independent antiviral RNAi pathway.

![Figure 3.14 ViRNAs accumulated in $rsd$-2;$rde$-4 double mutants](image)

**Figure 3.14 ViRNAs accumulated in $rsd$-2;$rde$-4 double mutants.** Analysis of the (-)viRNAs accumulation levels in the FR1gfp transgenic double knockout worm mutants 48 hours post heat induction. 15 µg of total small RNAs was loaded in each lane. DIG-labeled DNA oligos complementary to viRNA and miRNA were used as the probes. The same membrane was probed for miR-58 after stripping as the loading control.

**3.4.5 Neither FHV nor Orsay Virus Replication Levels in $rsd$-2;$rrf$-1 Double Mutants Were Enhanced Compared to Those in $rsd$-2 Single Mutants**

To investigate the antiviral RNAi pathway that involves $rsd$-2, FR1gfp transgenic $rsd$-2;$rrf$-1 double mutants were constructed through genetic crosses. Northern blot analyses showed that both FHV and Orsay virus replicated to similar levels in $rsd$-2;$rrf$-1 double mutants as those
in *rsd-2* single mutants (Figure 3.15, compare lane 3 with 4), suggesting *rsd-2* and *rrf-1* may work in the same genetic pathway for antiviral RNAi.

![Image](image.png)

**Figure 3.15** FHV and Orsay virus replication levels in *rsd-2;rrf-1* double mutants were similar to those in *rsd-2* single mutants. (A) Accumulation of the FR1gfp replicon RNAs in wild type, single and double knockout worm mutants 48 hours after replicon replication. (B) Accumulation of the Orsay virus RNA1 in wild type, single and double knockout worm mutants after growing them on Orsay virus particle containing food for 3 days.

### 3.4.6 Both FHV and Orsay Virus Replication Levels Were Decreased in the *rsd-2;drh-2* Double Mutants Compared to Those in *rsd-2* Single Mutants

To investigate the *rsd-2*-dependent genetic pathway for antiviral RNAi, we constructed FR1gfp transgenic *rsd-2;drh-2* double mutants by genetic crosses. The observations that both FHV and Orsay virus replicated to lower levels in *rsd-2;drh-2* double mutants compared to those in *rsd-2* single mutants (Figure 3.16, compare lane 3 with 4), indicating the *drh-2* mutant allele enhanced antiviral RNAi in the mutant *rsd-2* background. This finding confirmed that the *rde-4*-dependent RDVI pathway was targeted by *drh-2* for negative regulation.
Figure 3.16 FHV and Orsay virus replication levels were decreased in rsd-2;drh-2 double mutants compared to those in rsd-2 single mutants. (A) Accumulation of the FR1gfp replicon RNAs in wild type, single and double knockout worm mutants 48 hours after replicon replication. (B) Accumulation of the Orsay virus RNA1 in wild type, single and double knockout worm mutants after growing them on Orsay virus particle containing food for 3 days.

3.4.7 Both FHV and Orsay Virus Replication Levels Were Decreased in rsd-2;ergo-1 Double Mutants Compared to Those in rsd-2 Single Mutants

To determine the rsd-2-dependent genetic pathway for antiviral RNAi, we also constructed FR1gfp transgenic rsd-2;ergo-1 double mutants by genetic crosses. We found that the accumulations of both FHV and Orsay virus were decreased in rsd-2;ergo-1 double mutants compared to those in rsd-2 single mutants (Figure 3.17, compare lane 3 and 4), indicating that the ergo-1 mutant allele enhanced antiviral RNAi function in the rsd-2 mutants. This result confirmed that the rde-4-dependent RDVI pathway was also targeted by ergo-1 for negative regulation.
Figure 3.17 FHV and Orsay virus replication levels were decreased in rsd-2; ergo-1 double mutants compared to those in rsd-2 single mutants. (A) Accumulation of the FR1gfp replicon RNAs in wild type, single and double knockout worm mutants 48 hours after replicon replication. (B) Accumulation of the Orsay virus RNA1 in wild type, single and double knockout worm mutants after growing them on Orsay virus particle containing food for 3 days.

3.5 Other Biological Functions of rsd-2 in C. elegans

3.5.1 Transgene Can Be Silenced by viRNAs in the rsd-2 Mutant Strain

To test whether rsd-2 is required for transgene silencing mediated by viRNAs, we developed several FR1fp:gfp transgenic strains that contain both an FR1fp replicon transgene and a gfp transgene. The FR1fp replicon transgene is the same as the FR1gfp except that the 3’ end of the gfp sequence was used to substitute for the whole gfp sequence. GFP expression levels were examined 48 hours post heat induction of the transgenic N2, rsd-2, and drh-1 mutant strains, and we found that FR1fp replicon replicated in the rsd-2 mutant strain and GFP expression of this strain was suppressed accordingly (Figure 3.18). This result demonstrated that viRNAs produced from the FR1fp replicon were able to silence gfp transgene in the absence of the rsd-2 gene. We thus concluded that rsd-2 is not essential for transgene silencing mediated by viRNAs.
Figure 3.18 Rsd-2 is not important for viRNAs mediated transgene silencing. (A) FHV replication levels in the FR1fp:gfp transgenic worms 48 hours post heat induction. (B) GFP expression levels in the FR1fp:gfp transgenic worms 48 hours post heat induction.

3.5.2 GFP Was Silenced in the FR1fp; gfp;rsd-2 Mutant Strain by Exogenous GFP dsRNA

To test whether rsd-2 plays a role in RNAi triggered by artificial dsRNAs, a process often been referred to classical RNAi, feeding RNAi strategy was used to check whether feeding the FR1fp:gfp:rsd-2 mutant strain with the E. coli food producing the GFP dsRNA can down-regulate GFP expression of this strain.

Figure 3.19 GFP was silenced in the rsd-2 mutant strain. (A) GFP expression levels of the worms fed with the regular E. coli strain. (B) GFP expression levels of the worms which were fed with the GFP dsRNA producing E. coli strain for 24 hours and 48 hours.
After 24 and 48 hours feeding RNAi treatment, we found both green fluorescence and northern blot hybridization showed silencing of GFP expression in the FR1fp;gfp;rsd-2 mutant strain (Figure 3.19B), suggesting that rsd-2 is dispensable for artificial dsRNA triggered RNAi in C. elegans. This test further suggested that antiviral RNAi and classical RNAi pathways in C. elegans are genetically distinct although they share some genetic components such as DCR-1, RDE-1 and RDE-4.

3.5.3 Rsd-2 Is Not Required for miRNA Biogenesis

To test whether rsd-2 is involved in miRNA mediated RNAi pathway, total small RNAs were extracted from the rsd-2 mutant strain and northern blot hybridization analyses were performed. The observation that miRNA-58 expression levels were similar between the wild type N2 and mutant rsd-2 strains (Figure 3.20) suggested that miRNA expression levels were not affected in rsd-2 mutants because miR-58 is one of the representative miRNAs of C. elegans. Together with our observation that the rsd-2 mutant strain did not show any development defect, we concluded that rsd-2 is dispensable for miRNA biogenesis or function.

Figure 3.20 MiRNA-58 expression levels were not affected in rsd-2 mutants. Analysis of the miRNAs expression levels in wild type, single and double knockout worm mutants. 15 µg of total small RNAs was loaded in each lane. DIG-labeled DNA oligos complementary to miRNA were used as the probes. Ethidium bromide staining of tRNAs was provided to show equal loading.
CHAPTER FOUR: CONCLUSIONS

The observations that both Orsay and FHV viruses accumulated to high level in rsd-2 knockout mutants and that the wild type rsd-2 gene can readily rescue the antiviral silencing in rsd-2 mutants confirmed rsd-2 is the gene required for antiviral silencing in C. elegans.

The fact that FHV replicon replicated to a much higher level in the rsd-2;rde-4 double mutants compared to that in corresponding single mutants indicates that rsd-2 and rde-4 work in separate genetic pathways for antiviral silencing in C. elegans. Consistent with this observation, viRNAs were detected in rde-4;rsd-2 double mutants and rsd-2 single mutants, but not in rde-4 mutants.

We detected the FHV RNA1 and RNA3 accumulation and the FHV-specific viRNAs of the antigenomic polarity, (-)viRNAs, in rsd-2 mutants. This is in sharp contrast to the fact that no viRNAs were detected in rde-4 mutants. These observations ruled out a direct role of rsd-2 gene in virus sensing or viRNA biogenesis. Since the high-level accumulation of (-)viRNAs in rsd-2 mutants failed to inhibit the replication level of the FHV replicon suggests that rsd-2 is essential for activity of viRNAs.

We found that the FHV replicon replicated to lower level in rsd-2;drh-2 double mutants compared to that in rsd-2 single mutants. This result showed that the drh-2 null allele enhanced antiviral silencing when rde-4-mediated antiviral silencing was functional, substantiating that the rde-4-initiated antiviral silencing pathway was targeted by drh-2 for negative regulation. By analog, we believe ergo-1 also negatively regulates the rde-4-mediated antiviral silencing.

Based on these findings, we conclude that rsd-2 is required for antiviral silencing in C. elegans, by contributing to rde-4-independent antiviral silencing. The fact that viRNAs can still
be detected in *rsd-2* mutants suggests that *rsd-2* is required for function, but not the biogenesis, of viRNAs.
CHAPTER FIVE: DISCUSSIONS

Prior research has demonstrated that viruses can infect and replicate in *C. elegans* and their replication can trigger RDVI (Lu, Maduro et al. 2005; Schott, Cureton et al. 2005; Wilkins, Dishongh et al. 2005; Liu, Lin et al. 2006). These findings suggest that *C. elegans* could become an important model for understanding basic aspects of virus–host interactions. In this work, we discovered that (i) the antiviral silencing induced by either FHV or Orsay virus requires *rsd-2*, (ii) the production of FHV siRNAs in the *rsd-2* mutant strain is *rde-4*-independent, and (iii) *rsd-2* is not essential for the sensing or biogenesis of viRNAs. These findings suggested a role of *rsd-2* in an *rde-4* independent antiviral silencing pathway. In addition, my study also demonstrated that *rsd-2* does not play important roles in miRNA, exo-RNAi, or viRNA mediated silencing of cellular transcripts, suggesting a virus-specific function.

*Rsd-2* Is One of the Genetic Requirements of Antiviral RNAi in *C. elegans*

Our study confirmed that *rsd-2* is one of the known RNAi factors that contribute to worm RDVI. The fact that the antiviral silencing against FHV and Orsay virus was further enhanced in both *drh-2* and *ergo-1* mutant worms but decreased in both *rsd-2;drh-2* and *rsd-2;ergo-1* double mutants confirmed that *drh-2* and *ergo-1* are negative regulators of antiviral silencing in *C. elegans*.

*C. elegans* RSD-2 May Control the Target Specificity of Distinct siRNA Pathways

Several lines of evidence suggested that *rsd-2* has a specific role in the *rde-4*-independent antiviral RNAi pathway in *C. elegans*. First, a further loss of the worm antiviral RNAi against both FHV and Orsay virus was observed in *rsd-2;rde-4*, *rsd-2;drh-1* double mutants compared to either single mutants, indicating that *rsd-2* and *rde-4* work in separate antiviral RNAi pathways in *C. elegans*. Second, viRNAs, which are undetectable in *rde-4* single mutants, became
detectable in rsd-2; rde-4 double mutants, suggesting that the production of viRNAs in the rsd-2 mutant strain occurs in an rde-4-independent manner. This observation further confirmed rsd-2 regulates a distinct siRNA pathway.

RSD-2 Contributes to the Function of viRNAs in Worm Antiviral RNA Silencing

The observation that viRNAs could be detected in the rsd-2 mutants carrying the FR1gfp transgene suggested that rsd-2 is not essential for viRNA biogenesis in the antiviral RNAi pathway and that rsd-2 does not play a critical role in the sensing of the viral dsRNA triggers. Thus, rsd-2 could act downstream of both steps of sensing viral dsRNA triggers and processing viral dsRNA triggers into viRNAs. Because an RNAi factor can contribute to virus sensing, viRNA biogenesis or function of viRNAs, we believe that rsd-2 is required for the function of viRNAs.

Uniqueness of RSD-2 in C. elegans

So far, no RSD-2 homologue has been identified in other organisms, suggesting the uniqueness of RSD-2 mediated RDVI in C. elegans.
REFERENCES


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