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RNAi-mediated Antiviral Immunity in Caenorhabditis elegans

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RNAI-MEDIATED ANTIVIRAL IMMUNITY IN *CAENORHABDITIS ELEGANS*

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Biological Sciences

by

Xunyang Guo
B.S., Sun Yat-sen University, 2009
December 2013
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I would like to show my gratitude to my committee, for their continuous kind support and warm help during the Ph.D. program.

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<table>
<thead>
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>aa</td>
<td>amino acids</td>
</tr>
<tr>
<td>AGO</td>
<td>Argonaute</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>C. elegans</td>
<td>Caenorhabditis elegans</td>
</tr>
<tr>
<td>C. briggsae</td>
<td>Caenorhabditis briggsae</td>
</tr>
<tr>
<td>CP</td>
<td>coat protein</td>
</tr>
<tr>
<td>DRBP</td>
<td>double-stranded RNA binding proteins</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double-stranded RNA</td>
</tr>
<tr>
<td>endo-siRNA</td>
<td>endogenous small interfering RNA</td>
</tr>
<tr>
<td>FHV</td>
<td>Flock house virus</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>LGP2</td>
<td>Laboratory of genetics and physiology 2</td>
</tr>
<tr>
<td>MDA5</td>
<td>Melanoma differentiation associated gene 5</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>N. benthamiana</td>
<td>Nicotiana benthamiana</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>OV</td>
<td>Orsay virus</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>piRNA</td>
<td>PIWI-interacting RNA</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>PTGS</td>
<td>posttranscriptional gene silencing</td>
</tr>
<tr>
<td>RdRP</td>
<td>RNA-dependent RNA polymerase</td>
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</table>
RDVI RNAi-directed viral immunity
RIG-I Retinoic acid inducible gene I
RISC RNA-induced silencing complex
RLH RIG-I like RNA helicase
RMVI RNAi-mediated viral immunity
RNAi RNA interference
SAGO secondary Argonautes
shRNA short hairpin RNA
siRNA small interfering RNA
sRNA small RNA
ssRNA single-stranded RNA
TAV Tobacco aspermy virus
TBSV Tomato bushy stunt virus
T-DNA transfer DNA
viRNA virus derived RNAs
viRNA virus-derived siRNA
VSR viral suppressors of RNAi
WAGO worm Argonautes
### LIST OF WORM STRAINS

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
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<tr>
<td>N2</td>
<td>wild type</td>
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<tr>
<td>rde-1(ne300)</td>
<td>RNAi defective-1</td>
</tr>
<tr>
<td>rde-4 (ne299)</td>
<td>RNAi defective-4</td>
</tr>
<tr>
<td>rrf-1(pk1417)</td>
<td>RNA-dependent RNA Polymerase Family-1</td>
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<tr>
<td>ergo-1(tm1860)</td>
<td>Endogenous RNAi deficient Argonaute-1</td>
</tr>
<tr>
<td>drh-1(tm1329;ok3495)</td>
<td>Dicer Related RNA Helicase-1</td>
</tr>
<tr>
<td>drh-2 (ok951)</td>
<td>Dicer Related RNA Helicase-2</td>
</tr>
<tr>
<td>drh-3 (ne4253)</td>
<td>Dicer Related RNA Helicase-3</td>
</tr>
<tr>
<td>rsd-2 (tm1429; pk3307)</td>
<td>RNAi Spreading Defective-2</td>
</tr>
<tr>
<td>sid-1(qt2)</td>
<td>systemic RNAi Defective-1</td>
</tr>
<tr>
<td>JU1580</td>
<td>C.elegans isolates</td>
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ABSTRACT

Viruses are tiny intracellular parasites that often cause devastating diseases on cellular organisms. To suppress viral infection, cellular organisms have evolved a wide spectrum of antiviral defense mechanisms. Antiviral RNA interference (RNAi) is one of the antiviral mechanisms conserved in eukaryotes. During antiviral RNAi, the destruction of invading viral RNAs is mediated by small interfering RNAs derived from the viral replication complex, in the form of double-stranded RNA (dsRNA). Because of its sequence-specific nature, antiviral RNAi can also target host homologous transcripts, for instance leading to disease syndromes in plants. As a counter-defense mechanism, many viruses produce RNAi suppressors that suppress RNAi through distinct mechanisms. So far, RNAi-mediated virus-host interactions have remained largely unexplored in the nematode worms which are known to have a unique RNAi gene constitution. As a result, whether virus-derived siRNAs (viRNAs) are able to direct worm gene silencing, whether heterologous viral suppressors are still functional in nematode worms and how the worm-specific RNAi genes contribute to antiviral RNAi remain open questions.

In this thesis I describe my exploration of several aspects of RNAi-mediated virus-host interaction in the nematode Caenorhabditis elegans. Through the study of virus-induced gene silencing in C. elegans I found that viRNAs can target and silence host genes. This is the first demonstration that viRNAs have the potential to silence host gene expression in the animal kingdom. Since certain viral suppressors that inhibit viRNA function without a size reference become resistant to RNAi but some suppressors that specifically suppress the function of 21-nucleotide (nt) viRNAs still sensitive to RNAi, I conclude that 21-nt viRNAs do not play a major role in worm antiviral RNAi. My study on the function of a worm-specific RNAi gene, called rsd-2 (RNAi spreading defective 2), suggested that antiviral RNAi in C. elegans can be initiated
in the absence of a dsRNA binding protein, which is in sharp contrast to that in plant and insect systems. Through functional domain swap and viRNA profiling, I found that RIG-I-like RNA helicases contribute to worm antiviral RNAi through distinct mechanisms with one of them contributing to virus detection.
CHAPTER 1 GENERAL INTRODUCTION

1.1 Antiviral RNA interference

RNA interference (RNAi), or RNA silencing, is a phylogenetically conserved gene regulation mechanism mediated by several classes of small non-coding RNAs (1, 2). One of the major biological functions of RNAi is antiviral defense which serves as major innate antiviral mechanism in fungi, plants and invertebrates (3-14). Accumulating evidence suggests that RNA directed viral immunity (RDVI) is initiated upon the processing of viral double-stranded RNAs (dsRNAs), formed through intramolecular base-pairing or during viral replication, into small interfering RNAs (siRNAs) by type III ribonuclease called Dicer. Apparently, Dicer processing of viral dsRNA on its own is not sufficient to curb viral infection, because efficient RDVI also requires other RNAi factors, such as Argonaute (AGO) proteins, RNA-dependent RNA polymerases (RdRPs), dsRNA binding proteins (DRBPs) and putative RNA helicases (Figure 1.1) (7, 15-20).

siRNA mediated silencing of invading viruses culminates with the cleavage of viral transcripts by AGO proteins which recruit siRNAs as sequence guide for target RNA selection and slice the matching RNA molecules with their RNase H-like activity (20). In plants and the nematodes Caenorhabditis elegans, RNA-dependent RNA polymerases (RdRPs) contribute to RDVI by amplifying siRNAs (17, 21, 22) through generating secondary viral siRNAs. The plant RdRPs convert cleaved viral transcripts into dsRNAs which are further processed into secondary siRNAs, whereas the worm RdRPs initiate de novo synthesis of secondary siRNAs using the cleaved transcripts as template in a dicer-independent manner (16, 17, 21, 22). Double stranded binding proteins (DRBPs) are essential components of RDVI in plants, insects and nematode worms. Whereas some DRBPs play important role in Dicer processing of dsRNA into siRNAs some other DRBPs are found to facilitate the loading siRNA into AGO proteins (23-27). Putative
RNA helicases are implicated in various siRNA-mediated gene silencing pathways. However, how they contribute to antiviral RNAi remains largely unknown.

Plant RDVI also involves an intercellular signal that is believed to restrict systemic spreading of the invading virus by priming an antiviral status prior to virus arrival (28, 29). Recently, siRNAs were found to serve as the physical carrier of this mobile silencing signal in plants (30, 31). In fruit fly RDVI also spreads systemically although the mechanism involved is different (32).

Figure 1.1 Schematic pathway of RNAi-mediated antiviral silencing. Exogenous long double stranded RNA (dsRNA) is processed by Dicer into small interfering RNAs (siRNA) with 21-24 nt and 3’end overhang, Argonaute proteins (AGO) recruit the passenger strand of siRNA and cleave the targets which are complimentary to siRNAs. RNA dependent RNA polymerase (RdRP) amplifies the RNAi by converting the cleavage products into secondary siRNA duplex. The image was adapted from review (33).

1.2 Viral Suppression of RNAi

Since RDVI is mediated by siRNAs processed from replicating viral genomes, the chance for the targeted viruses to evade RDVI through generating genome variants is low. As a counterdefense mechanism, many viruses encode diverse, in term of sequence and structure, classes of proteins capable of suppressing RNAi. Viral suppressors of RNAi (VSRs) can be
encoded by fungus, plant and animal viruses with DNA or RNA genomes. Accumulating evidence suggests that VSRs often target viral or host factors to suppress the biogenesis and/or function of siRNAs (34). For example, the B2 proteins produced by flock house virus (FHV) or nodamura virus (NoV) have been shown to bind long dsRNAs, thereby suppressing the processing of dsRNAs into siRNAs by Dicer. Nonetheless, the p19 protein of tomato bushy stunt virus (TBSV) and the 2b protein of tomato aspermy virus (TAV) specifically bind and inhibit the function of 21-nucleotide (nt) siRNAs (9, 35-40). The fact that TBSV p19 is an active suppressor of RNAi in both insect and mammalian cells suggests that its function does not require host factors (41-43). In plants, VSRs are capable of interfering with the function of microRNAs (miRNAs), a class of endogenous small noncoding RNAs with important function in development and stress response (44-46). miRNAs are encoded by endogenous genes and their biogenesis requires Dicer proteins and cofactors. Accumulating evidence suggests that suppression of miRNA function by VSRs is responsible for the developmental defects induced by plant virus infection (47, 48).

Development of RDVI suppression assays has played an important role in the discovery and characterization of VSRs and the study of VSR-mediated virus-host interaction. In plants, the fastest and most popular way is through agrobacterium-mediated transient expression assay (Figure 1.2) (33). Usually, the candidate VSR was co-infiltrated with a reporter gene, such as green fluorescent protein (GFP) encoding gene, using agrobacterium infiltration to the plants, then the VSR can be identified if plants were resistant to the local silencing triggered and targeted by the reporter gene (49). Another strategy is to deliver the candidate VSR into the reporter plants through genetic cross, thus, the on-going silencing will be reversed (6). However, using those methods, it was difficult to test systemic silencing transported between cells through
this assay. Thus, a grafting assay was adopted to identify the suppression of systemic silencing by visualizing the silencing signal generated from the rootstock which can spread to scion and silence the transgene in the scion (29, 49). In the cell culture system, the strategy to identify VSRs was based on rescue of the mutant virus defective of replication. Briefly, a known virus genome was modified to obtain an un-translated suppressor, thus, viral replication would be suppressed in wild type animals, but would be restored if a suppressor is provided in trans or from heterologous virus (12). Therefore, a candidate viral protein is identified as VSR when its expression rescues the accumulation of a VSR-deficient mutant virus or replicon after replication in a host cell.

![Image of leaf samples](image_url)

Figure 1.2 Identification of VSR through co-agroinfiltration. The transgenic GFP *Nicotiana benthamiana* leaves were co-infiltrated with a mixture of two agrobacterium *tumefaciens* strains, one directs the expression of GFP and thereby induces GFP mRNA silencing, and the other simultaneously expresses the FHV-encoded B2 (left leaf), B1 (middle leaf), or the plant cucumoviral 2b (right leaf). The leaves were detached and photographed under UV illumination 6 days after infiltration. GFP silencing is visualized in the middle leaf as a bright red color zone surrounding the infiltrated patch caused by chlorophyll fluorescence. The image was adapted from (7).

So far, little is known about viral suppression of RDVI in *C. elegans*. Recent studies have discovered a naturally occurring virus that infects and induces RDVI in *C. elegans* (50). However, it is unknown if the virus encodes VSR or VSR expression enhances virus infection in *C. elegans*. Previously, the B2 protein of FHV has been shown to be required for efficient viral
replication in wild type worms but not in worm mutants defective in RDVI (9), suggesting a function for FHV B2 in worm RDVI suppression. However, it remains unknown if ectopic expression of FHV B2 suppresses RNAi induced by synthetic dsRNA or worm RDVI induced by FHV or heterologous viruses.

1.3 Virus induced gene silencing

Owing to its sequence-specific nature, RDVI in plants can be redirected to target host transcripts with matching sequences (51). For example, potato virus X (PVX) and tobacco rattle virus (TRV) with modified genomes have been found to be able to induce potent silencing of homologous genes upon establishing successful infection (52-55). Based on this observation, virus induced gene silencing (VIGS) has been adopted as a genetic tool for the dissecting of diverse biological pathways in plants (55, 56). Very recently, it has been shown that the same mechanism involved in VIGS is also responsible for the induction of viral diseases (57, 58).

Currently, although animal virus encoded microRNAs (miRNAs), processed from viral transcripts with hairpin like secondary structures, have been shown to modulate host gene expression thereby to facilitate virus infection (59-61), it remains to be an open question whether viRNAs also have the potential to modulate host gene expression in the animal kingdom. The nematode worm C. elegans would be an animal model of particular interest to address this question, considering the fact that, like plants, C. elegans genome also encodes RdRP as important component of RDVI (19, 62).

1.4 Worm-specific antiviral RNAi genes

In addition to Dicer, AGO and RdRP proteins, the worm RDVI also requires some unique components, such as DRH-1 (Dicer related RNA helicase 1) and RSD-2 (RNAi spreading defective 2) (62). DRH-1 encodes a putative DExD/H box RNA helicase that shares significant sequence homology with RIG-I (retinoic acid-inducible gene I), a cytosolic virus sensor that
initiates interferon-mediated viral immunity upon virus detection in mammals (63). Intriguingly, although essential to RDVI, DRH-1 appears to be dispensable in RNAi triggered by artificial dsRNAs, suggesting a dedicated role of DRH-1 in antiviral defense (62). Previously, it was reported that viRNAs detected in drh-1 mutants became undetectable in drh-1;rde-4 double mutants (62), suggesting that DRH-1, together with RDE-1 and RRF-1, functions in the same genetic pathway as RDE-4.

RSD-2 together with SID-1 (systemic RNA interference-deficient 1) was originally identified as one the key factors that enable systemic spreading of RNAi (1, 64, 65). A recent study suggested that RSD-2 contributes to the accumulation of secondary siRNAs in both exogenous and endogenous RNAi pathways (66). Since rsd-2 mutants displayed defects in transposon silencing under unfavorable condition it is believed that RSD-2 plays an important role in maintaining chromosome integrity (67). SID-1 contains multiple putative transmembrane domains and has been shown to be required for dsRNA intake (65, 68, 69). Interestingly, a recent study suggested that at least two classes of silencing dsRNA RNAs that move between C. elegans tissues can act as or generate the systemic silencing signal (70). Currently, whether SID-1 contributes to systemic RNAi by transporting these two classes of silencing RNAs remains largely unknown. Previously, high concentration of dsRNAs were found to trigger efficient RNAi in the absence of RDE-4 (71), but the biological significance of this observation remains poorly understood.

RSD-2 was implicated in RDVI in a genetic screen that selected for genes whose downregulation by dsRNA feeding led to loss of RDVI (62). Since rsd-2 acting as an RDVI genes is unique to nematode worms it would be interesting to ask whether worm-specific genes direct antiviral silencing in the absence of RDE-4.
1.5 RIG-I-like RNA helicases and RDVI

Intriguingly, antiviral RNAi in *C. elegans* is also regulated by Dicer-related helicase 1 (DRH-1), which is highly homologous to mammalian RIG-I (retinoic acid-inducible gene I) and is largely dispensable for exogenous RNAi (62). RIG-I is a key component of mammalian antiviral innate immunity (72-74). This finding thus suggests that RIG-I-like RNA helicases (RLHs) contribute to distinct antiviral mechanisms across kingdoms. Whereas antiviral RNAi induced by a Flock house virus (FHV)-based RNA replicon is dependent on *drh-1*, a reduced replication of the FHV replicon is detected in mutant nematodes defective in *drh-2*, suggesting that *drh-2* is a negative regulator of RDVI in worms. The DRH family of genes in *C. elegans* is absent in insects and plants. Each of the DRH proteins contains homologous helicase domain and C-terminal regulatory domain (CTD) originally identified in RIG-I. A unique N-terminal domain is found in DRH-1 and DRH-3 but not in DRH-2 (62, 75). Currently, how DRH-1 contributes to antiviral silencing, through which mechanism DRH-2 negatively regulates antiviral silencing, and whether DRH-3, which shares similar domain structure with DRH-1, plays a role in antiviral silencing remains largely unknown.

Previous studies showed that RIG-I functions as virus sensor in interferon mediated antiviral immunity in mammals (63, 72, 76-78). Pathogen sensing by RIG-I leads to the transcriptional activation of effector genes in the nucleus (72). In contrast, DRH-1 functions in RNAi-mediated antiviral immunity. Specifically, detection of viral double-stranded RNA (dsRNA) by Dicer endonucleases is associated with the production of small interfering RNAs (siRNAs), which subsequently direct sequence-specific antiviral immunity through RNA interference (RNAi) in cytoplasm (79-82). Currently, how DRH-1 contributes to RDVI remains largely unknown. As a result, whether RLHs confer similar biological function in distinct antiviral mechanisms remains an open question.
Figure 1.3 Schematic structure of RIG-I-like RNA Receptors family. CARD: caspase activation and recruitment domain; RD: regulatory domain. The image was adapted from review (83).

The homologous DExD/H box helicase domain of mammalian RIG-I is flanked by the N-terminal caspase activation and recruitment domains (CARDs) and a C-terminal regulatory domain (CTD) (Figure 1.3). RIG-I binding of the viral 5’-triphosphate dsRNA through the helicase domain and CTD activates the downstream signaling events, leading to the induction of interferon-dependent antiviral immunity (84-86). The mammalian genomes also encode two additional RLHs, melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). Both MDA5 and LGP2 contain CTD, but the N-terminal CARDs are found only in MDA5 (Figure 1.3). MDA5 senses viruses that are not detected by RIG-I via a distinct strategy whereas LGP2 seems to function as a regulator of RIG-I and MDA5 activities through currently unknown mechanism (76, 87-90). The worm RLHs and mammalian RLHs share not only similar functional domain organization but also similar functionality (Figure 1.4) (62). Mechanistic study of worm RLHs is expected to have direct input to our understanding of mammalian virus sensing mediated by RLHs.

1.6 *C. elegans* and RDVI

*C. elegans* has recently emerged as an attractive model organism to study RDVI. Since RNAi triggered by artificial dsRNA mechanistically recapitulates RDVI, previous studies on artificial dsRNA-triggered RNAi in *C. elegans* have significantly improved our understanding of
RDVI in *C. elegans* nematodes. Current models envision that, as shown in Figure 1.5, the worm RDVI is initiated upon the processing of viral dsRNA into primary viRNAs by the worm Dicer called DCR-1 (19, 62, 92). DCR-1 also processes precursor microRNAs (miRNAs) into mature miRNAs, a class of endogenous small RNAs with important role in development (93). Whereas efficient processing of viral dsRNA into primary viRNA requires a dsRNA binding protein termed RDE-4 (RNAi defective 4), the processing of precursor miRNAs into mature miRNAs appears to be RDE-4 independent (8, 62, 75, 93, 94). Interestingly, RDE-4 as a key factor of RDVI is also conserved in the fruit fly whose genome is known to encode two Dicer proteins with dedicated function in the biogenesis of siRNA and miRNA respectively (75, 94-97). So far, at least two closely related AGO proteins, RDE-1 (RNAi defective 1) and C04F12.1, have been found to play important role in RDVI in *C. elegans* (8, 9, 19, 62). RDE-1 is one of the 27 worm Argonauts that has the “slicer” activity, an RNase H like activity that cleaves the RNA molecule with perfect sequence match to the guide strand of siRNAs (98, 99). Interestingly, the “slicer” activity of RDE-1 is only required for the cleavage of the passage-strands of primary viRNAs but
not the cleavage target (100). However, the binding of target by the guide strand of primary siRNAs, together with RDE-1, is believed to trigger the RdRP activity of RRF-1 which initiates the de novo synthesis of secondary siRNAs using the target viral RNAs as templates. Unlike primary siRNAs, all secondary siRNAs are single-stranded and are known to carry a triphosphate group at the 5’ end (5’ ppp) (101, 102). These secondary siRNAs are believed to bind secondary AGO proteins that destroy target viral RNAs through currently unknown mechanisms (103). The fact that RDE-1 is required for production of secondary siRNAs suggests that RRF-1 functions downstream of RDE-1 in the same genetic pathway (104) (103). Currently, how C04F12.1 and secondary AGO proteins contribute to RDVI remains largely unknown.

1.7 Objectives of my study

The aim of my study is to better understand how RNAi regulates virus-nematode interactions using *C. elegans* as a model system. The *C. elegans* genome encodes RdRPs like the plant genomes. It also encodes single Dicer as mammals do. Thus, findings from my study are expected to have direct input to the study of RDVI in diverse organisms. My study on the function and mechanism of worm-specific genes is also expected to unravel some unique features of worm RDVI.

To study virus-nematode host interaction mediated by VSRs, I developed a robust RDVI suppression assay using FHV B2 as a reference VSR. This assay has allowed for the identification of VSRs with RDVI suppression activity in *C. elegans*. Using this assay I not only demonstrated that the Orsay virus RNA2 encodes no RDVI suppression activity but also identified NoV B2 as a VSR that retained the RDVI suppression activity in worm. It was also clear from my study that FHV B2 mainly inhibits the function, rather than the biogenesis, of virus-derived primary siRNAs in *C. elegans* but is unable to suppress the function of worm miRNAs which use the same Dicer for biogenesis. Intriguingly, I found that TBSV p19 is not an
active RDVI suppressor in *C. elegans*. Since TBSV p19 is known to specifically bind and inhibit the function of 21 nt siRNAs, my observations suggested 21-nt primary siRNAs do not make major contribution to worm RDVI.

![Diagram](image)

Figure 1.5 Current working model for RNAi-mediated antiviral immunity in the nematode worm *C. elegans*. DCR-1: Dicer-1. RDE-1: RNAi deficient 1, an Agonaute protein. RDE-4: RNAi deficient 4, a double-stranded RNA binding protein. The passenger strands of primary siRNAs are in green whereas the guide strands are in red.

By analyzing viral replication and viRNA accumulation in single and double worm mutants defective in *rsd-2*, I found that RSD-2, as an important component of worm RDVI, functions downstream of primary viRNA biogenesis. Most importantly, with an increase in viral replication, primary viRNAs accumulated to a higher level in *rsd-2;rde-4* double mutants compared to that in *rsd-2* single mutants, suggesting an *rde-4*-independent mechanism for the biogenesis of primary viRNAs in *C. elegans*. I further showed that RRF-1, together with RDE-1, also contribute to the *rde-4*-independent RDVI. Notably, although playing an essential role in
facilitating the spreading of RNAi triggered by artificial dsRNAs, SID-1 appears dispensable in RDVI targeting a natural viral pathogen of *C. elegans*.

To find out whether replicating virus can induce silencing of host genes in nematodes, I tested the silencing of cellular transcripts triggered by replicating virus in *C. elegans*. The results suggested that viRNAs derived from a modified flock house virus (FHV) can mediate potent silencing of cellular transcripts in a sequence-specific manner. Importantly, VIGS in *C. elegans* is inheritable, suggesting that profound epigenetic consequences in the progeny populations can be induced by invading viruses. Therefore, my study not only confirmed that viRNAs can modulate host gene expression in animal kingdom but also paved the way for in-depth study of novel virus-animal host interactions mediated by viRNAs.

RLHs are unique antiviral RNAi gene as they are only found in nematode worms but not in fungi, plants or insects. The *C. elegans* genome encodes altogether 3 RLHs. Currently, how they contribute to worm RDVI remains largely unclear. In this study I showed that *drh-1* and *drh-3* of *C. elegans* are essential for antiviral RNAi, but exhibit distinct antiviral activities. Using a transgenic approach developed to assay for the antiviral function of *drh-1* in whole animals, I examined the antiviral activity of the predicted domains in DRH-1 and determined if *C. elegans* antiviral RNAi could be mediated by any of the conserved domains of human RIG-I protein. My results indicate that *C. elegans* antiviral RNAi requires an essential activity of DRH-1 to detect viral dsRNA in a manner analogous to virus dsRNA sensing by RIG-I in mammals. Using the same strategy I found that the putative DRH-2 domains can functionally replace the corresponding domains of DRH-1 in antiviral silencing, suggesting that DRH-2 may act as a competitor of DRH-1 in worm RDVI.
CHAPTER 2 MATERIALS AND METHODS

2.1 Maintenance of C. elegans strains

The Bristol isolate of C. elegans N2 was used as the reference wild-type strain throughout this study. All C. elegans strains except some temperature sensitive mutants are maintained at room temperature on Nematode Growth Medium (NGM) in petri dishes with a diameter of 35mm, 60mm or 100mm. A Wheaton dispenser, unispense, was used to prepare all of those dishes. The E. coli food lawn was produced by applying E. coli OP50 liquid culture to those dishes under sterile condition. Approximately 100 L4 larvae were transferred to new plates for maintenance of fresh worm stock every other day.

2.2 Worm genetics

All alleles used in this study are derived from N2 and include drh-1 (tm1329 and ok3495), drh-2 (ok951), drh-3 (ne4253), rsd-2 (tm1429 and pk3307), rde-1 (ne300), rde-4 (ne337), rrf-1 (pk1417) and sid-1 (qt2). The genotypes of pk3307, sid-1, rde-1 and rde-4 worms were confirmed using skn-1 feeding RNAi and genomic DNA sequencing. The drh-3 mutant allele was confirmed through genomic DNA sequencing, combined with 25°C sterile phenotype. The genotypes of rrf-1, drh-1, drh-2, rsd-2 were confirmed by PCR using primer sets shown in Table 1 (62). All worm strains are maintained in the room temperature, except drh-3, which is stored in 19°C incubator.

2.3 Genetic crosses

Males are generated through heat shock (37°C for 45min). All genetic crosses were carried out on empty NGM petri dish with 100mM Ampicillin using one virgin hermaphrodite with 4-6 males for cross. Heterozygous F1s were singled out when the cross is successful. Subsequently, 16 F2s were picked up and examined using feeding RNAi or PCR.
Table 2.1 PCR primer sets for mutant alleles amplification

<table>
<thead>
<tr>
<th></th>
<th>Upstream primer</th>
<th>Downstream primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>drh-1 (tm1329)</strong></td>
<td>tm1329_internal_b</td>
<td>Tm1329minus</td>
</tr>
<tr>
<td></td>
<td>ATACTCTG CCTCGAGCCGAT</td>
<td>TCAGTCG TATCTCCAATTTTCGA</td>
</tr>
<tr>
<td><strong>drh-2(ok951)</strong></td>
<td>drh-21780plus</td>
<td>Ok951minus</td>
</tr>
<tr>
<td></td>
<td>AGTAGCAT TTCGAGAGGT</td>
<td>TTGCTTTT CCTGGACATGAAGTG</td>
</tr>
<tr>
<td><strong>rrf-1(pk1417)</strong></td>
<td>rrf-1EcoRV</td>
<td>rrf-1NsiI</td>
</tr>
<tr>
<td></td>
<td>AGGAGAGCATAGAAGGATATCA</td>
<td>TCGACAATGCATCCTGACATGA</td>
</tr>
<tr>
<td><strong>rsd-2 (tm1429)</strong></td>
<td>tm1429intf</td>
<td>tm1429intb</td>
</tr>
<tr>
<td></td>
<td>C TACTAAACCGGTACGTGT</td>
<td>CCACAGGGATTTTGTAGGGA</td>
</tr>
</tbody>
</table>

2.4 *C. elegans* chromosomal integration

40 young adult worms carrying extrachromosomal transgene arrays are treated with 3,500 rad of gamma-ray from a 137Cs source. Eggs are collected upon 4 hours after induction, then approximately 500 F1s are picked up for screening integrated animals in the F3 generation. Once an integrant is confirmed, the worms will be back-crossed 4 times to reduce the non-specific genetic mutations.

2.5 Plasmid constructs

FR1gfp replicon was a derivative of pFR1-3 by replacing the NcoI-SacI fragment of FHV RNA1 with the full length GFP coding sequence as described in (62). This created a translational fusion of GFP with the N-terminal 23 amino acids of B2 and deletion of approximately 200 nucleotides from the B2 open reading frame (ORF). HDV ribozyme sequences were added in the 3’end of the viral genome so that all non-viral sequences at the 3’ end of FR1gfp transcripts will be removed after transcription.

The FR1fp replicon construct was modified from FR1gfp, by replacing the full-length *gfp* coding sequence with its half 3’ end. The start codon for the B2 protein was disrupted so that no
translation will be initiated from the B2 ORF. The FR1fp was created by digestion of the FR1fp construct with BamHI, followed by T4 polymerase end-filling and re-ligation.

The DI634 replicon construct was created using the same strategy as that used for the development of FR1gfp. To facilitate the insertion of foreign sequences, a multiple cloning site (MCS) corresponding to AscI-XhoI-NotI was introduced into the 3’ end of DI634 at the position where the original viral sequence has been removed. The DI634unc22 construct was created by inserting a 552 nt sequence derived from the exon 7 of unc-22 into DI634 utilizing the AscI and NotI sites. Similarly, the DI634skn-1 construct was created by inserting 380 bp skn-1 coding sequence into the MCS of DI634.

All constructs used for RNAi suppression activity assay were developed utilizing either a heat inducible promoter or a sur-5 promoter. To express RNAi suppressors using the heat inducible promoter, the candidate RNAi suppressor coding sequences were inserted into pPD49.83 utilizing the XmaI and Sac I sites. To express RNAi suppressors using the sur-5 promoter the candidate RNAi suppressor coding sequences were inserted into LR50 described in (105). The coding sequences for TBSV p19 and TAV 2b were PCR amplified from corresponding T-DNA expression binary constructs described previously (49, 106). The point mutations in p19m and 2bm were introduced through PCR amplification of wild type genes using primers containing desired mutations. All resulting constructs were confirmed through DNA sequencing.

In RSD-2 function rescue assay the Psur5::RSD-2 construct was developed by inserting wild type RSD-2 coding sequence, amplified through PCR, into the LR50 vector described in (105). The RSD-2 coding sequence in the resulting construct was confirmed by DNA sequencing.
The plasmid construct used to drive gfp dsRNA expression in E. coli was described in (105). Full length of eGFP coding sequence was amplified through PCR, and inserted into the L4440 vector through XbaI and SacI sites. The plasmid was first confirmed with genomic sequencing and then transformed into HT115 competent cells. Feeding RNAi targeting skn-1 construct was built through the same strategy.

2.6 Transgenic worms

Transgenic animals were generated through gonadal microinjection of the target constructs. Briefly, the target plasmid constructs, each at a final concentration of 10 ng/µl, were mixed with the 2-log DNA ladder (New England Biolabs Inc.) at final concentration of 100 ng/µl and the reporter plasmid Pmyo-2::mCherry or PRF4 at final concentration 40 ng/µl and injected into the gonads of young adult worms. Normally 30-40 F0s were injected, extrachromosomal array were randomly passed down from F1 to progenies. Worms carrying reporter genes were maintained for chromosomal integration or function assay.

2.7 RNAi experiments

The skn-1 or gfp feeding RNAi assay was performed using a bacterial feeding protocol (107). Briefly, NGM agar plates containing 5 mM IPTG and 100 mg/ml carbenicillin were seeded with E. coli. HT115 strain expressing skn-1 or gfp dsRNAs. The skn-1 feeding RNAi was scored from the next generation of worms, while the gfp feeding RNAi result was determined after 48 hours induction. All RNAi experiments are carried at room temperature.

2.8 Infectious filtrate preparation and Orsay virus inoculation

Orsay virus was maintained using the JU1580 isolate at room temperature following protocol described previously (50). To prepare Orsay virus inoculums, infected JU1580 worms were washed off from slightly starved 10 cm plates using M9 buffer, 5ml per plate. The virus-
containing liquid was then filtered through 0.22 μm filter unit (Millipore) and used to resuspend pelleted OP50 *E. coli* for NGM plate seeding.

### 2.9 RNA preparation and Northern blot analysis

To extract total RNA, 4-6 medium plates of adult worms were washed with ddH$_2$O to 14ml Falcon tubes, after removing supernatant, 5 volume of TRI reagent (Sigma aldrich, Inc.) was added and followed by homogenization. 1/5 volume of chloroform was added and the sample was spun with maximum speed for 10min at 4°C. Then, add equal volume of isopropanol was added to the supernatant to precipitate total RNA. To collect total RNA, the content was spun at 5000rpm for 5min. The pellet was then washed with 70% Ethanol and resuspended in DEPC H$_2$O.

To detect high molecular weight RNA, including the viral genomic and subgenomic RNAs and the cellular transcripts, 4 to 6 μg total RNA of each sample was denatured, loaded in the 1.2% agarose gel, which was then transfer to the Hybond N+ membrane (GE Healthcare Inc.) followed by UV crosslinking using $1.8 \times 10^5 \mu$J/CM$^2$ as the output power (SpectroLinker). Then the membranes were hybridized with alkaline phosphatase-labeled cDNA probes derived from the target RNAs at 65°C overnight (AlkPhos Direct Labeling module, GE Healthcare). After washing twice at 65°C, then another twice at room temperature, the target RNAs were then be detected using CDP-Star Detection Reagent following the manufacture’s instruction (GE Healthcare). The probes for FR1gfp transcript detection were prepared using cDNA fragments amplified from the gfp coding sequence. The probes used to detect Orsay virus RNA1 were prepared using cDNA fragments amplified, through RT-PCR, from the 3’ end of Orsay virus RNA1 genome.
To prepare small RNA samples for Northern blot analysis, total RNAs were extracted from 10 large plates of worms following the procedure described above. Small RNAs were then enriched using the mirVana kit (Ambion). For small RNA detection, 10 to 20 µg of samples containing enriched small RNAs was resolved in 15% acrylamide denaturing gel (PAGE). After electrophoreses, all RNA samples were transferred into Hybond Nylon membranes followed by crosslinking.

The detection of small RNAs, viRNAs or miRNAs, adopted a miRNA detection protocol with minor modifications (108). Briefly, following the UV crosslinking, the membranes were hybridized with DIG-labeled DNA oligo probes in PerfectHyb buffer (Sigma Aldrich) overnight at 37°C. The DNA oligos that would detect all minus-stranded viRNAs derived from the gfp region of FR1gfp were labeled using the DIG Oligonucleotide Tailing Kit (Roche Applied Science). The blots were then washed 3 times at 42°C using the DIG Wash and Block Buffer Set (Roche Applied Science). The small RNAs were then detected using Alkaline Phosphatase linked Anti-Digoxigenin antibody together with the CSPD substrate (Roche Applied Science). For FR1fp siRNA detection, the probes were prepared using 32 DNA oligos covering the entire FP region of FR1fp. miR-58 detection was reprobed with the same membrane with DNA oligos ATTGCCGTACTGAACGATCTCA. The 4 DNA oligos that served as size references for viRNAs and miRNAs were detected using 4 DIG-labeled 4 DNA oligos with complementary sequences.

2.10 Protein extraction and western blot analysis

Total proteins were extracted from 5 medium plates of worms using lysis buffer (Boston Bioproduct, Inc.) with protease inhibitor cocktail (Thermo Scientific™). The mixture was homogenized for 1min, followed by centrifuge at maximum speed at 4°C for 15min. The
supernatant was the collected and heat denatured. 20-30µg protein sample was resolved into 8% SDS-PAGE gel. After being transferred to Hybond-P PVDF membrane (GE Healthcare Life Science), the target proteins were detected with anti-HA as primary antibody (Cell Signaling Technology) and goat anti-rabbit secondary antibody (Cell Signaling Technology). As equal loading control, the same set of membranes was re-blotted using HRP linked anti-β-actin antibody (Cell Signaling Technology).

2.11 Small RNA sequencing

RNA extracts containing enriched small RNAs were used for the construction of small RNA libraries. To capture virus-derived secondary siRNAs, all input RNA samples were treated with tobacco acid pyrophosphatase (Epicentre) before being used for small RNA library construction. All small RNA libraries were generated using TruSeq RNA Sample Preparation Kit (Illumina) following the manufacture’s instruction and sequenced using the Illumina HiSeq2000. After the adaptor being removed, the small RNA sequences were analyzed with in-house pipelines described previously (109).

2.12 DRH-1 and RSD-2 function rescue assay

The DRH-1/RSD-2 function rescue assay utilized ectopic expression of DRH-1/RSD-2 coding sequence in drh-1 (tm1329) / rsd-2 (tm1429) null mutants carrying a nuclear transgene corresponding to the FR1gfp replicon. The sur-5 promoter, known to be active in most of the worm somatic tissues (110), was used to drive the expression of DRH-1/RSD-2. A construct that directs mCherry expression in the pharynx tissue was used to produce a visible mark for the Psur5::RSD-2 transgene. The function rescue assay began with microinjection of target construct into the gonad of mutant worms together with the mCherry reporter construct. Because of the nature of worm transformation through gonad injection, most of the transgenic extrachromosomal arrays are randomly passed onto the next generations. As a result, some
progenies are free of the extrachromosomal arrays and thus can serve as internal negative control. Upon heat induction, which will initiate the replication of FR1gfp replicon, transgenic worms marked with red fluorescence in the pharynx will show no or decreased green fluorescence if the target transgene is able to restore the function of its derived mutants.

2.13 Assay for miRNA biogenesis/function suppression activity

Worms used in this assay contain a let-7 function reporter transgene, which contains GFP coding sequence fused with let-7 target sequence, namely the 3’ end untranslated region of lin-41, and a transgene expressing the let-7 miRNAs. Both transgenes are driven by the myo-2 promoter thus enhanced green fluorescence can be observed in the pharynx tissue in worms defective in miRNA biogenesis and/or function (111). The assay began with microinjection of plasmid constructs containing candidate VSR coding sequence driven by the myo-2 promoter into the reporter worm strain SX333 (obtained from the Caenorhabditis Genetics Center (CGC)). The PRF4 construct was co-injected to produce the roller phenotype as visual mark for the VSR transgene. Following microinjection, transgenic lines carrying transmittable extrachromosomal arrays were picked up and maintained at room temperature. For each transgenic line, the green fluorescence in the pharynx tissue was compared, at different developmental stages, in between worms that carry the extrachromosomal array and worms that do not.

2.14 Terminator treatment of small RNA samples

The terminator treatment was carried out by mixing 20 µg small RNA sample with 4 units of Terminator exonuclease (Epicentre Inc.) in 50 µl reaction mix containing 1x buffer and 1 unit of RNase inhibitor. The reaction mix was then incubated at 30°C for 60 minutes. For both treatments, the treated small RNA samples were cleaned through extracting with phenol-chloroform and then precipitated with ethanol.
2.15 Imaging microscopy

The green and red fluorescence images were recorded using the same exposure for each set of images. A Nikon digital camera p7000 mounted on a Nikon SMZ1500 microscope was used to record all images.
CHAPTER 3 CHARACTERIZATION OF VIRUS-ENCODED RNAI SUPPRESSORS IN CAENORHABDITIS ELEGANS

3.1 Introduction

In fungi, plants and insects, antiviral RNAi mediated by virus-derived small interfering RNAs (siRNAs) represents a major antiviral defense mechanism that invading viruses have to overcome in order to establish infection. As a counterdefense mechanism, viruses of these hosts produce diverse classes of proteins, termed viral suppressors of RNAi (VSR) that suppress the biogenesis and/or function of viral siRNAs. So far, more than 30 VSRs have been identified (112). Accumulating evidence suggests that these VSRs suppress RNAi through distinct mechanisms. For instance, the B2 proteins of Flock house virus (FHV) binds dsRNAs without size references and suppress RNAi in both plants and insect kingdoms. It can be further inferred from these findings that B2 suppresses RNAi without the aid of host factors. Interestingly, the p19 protein of tomato bushy stunt virus (TBSV) was found to specifically bind 21-nucleotides siRNAs thereby to suppress their function. Further, certain viral suppressors can suppress the function of miRNAs, leading to plant diseases. RNAi-directed viral immunity (RDVI) is also conserved in the nematode Caenorhabditis elegans (C. elegans) which is known to possess some unique features, in term of RNAi gene constitution (9). Previous study suggested that in C. elegans the B2 protein of FHV is required for efficient viral replication but becomes dispensable in the RNAi defective mutant worms, such as rde-1, suggesting a function of B2 in RNAi suppression (9). Currently, whether HV B2 is able to suppress RNAi when produced in trans and how FHV B2 suppresses worm RNAi through a similar mechanism remain largely unknown. Moreover, Orsay virus, the very first natural viral pathogen of C. elegans, is known to replicate

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1 “Part of this chapter previously appeared as [Guo X and Lu R, Characterization of Virus-Encoded RNA Interference Suppressors in Caenorhabditis elegans, March 2013]. It is reprinted by permission of [Copyright © American Society for Microbiology—see appendix]”
at an enhanced level in RNAi defective mutants, suggesting a deficiency of Orsay virus in RNAi suppression. It is thus interesting to find out whether wild type Orsay virus contains functional suppressors. In this chapter, I developed an assay, based on rescue of a suppressor free FHV replicon, to identify viral suppressors of RNAi using FHV B2 as a reference VSR and characterized the function and mechanism of several VSRs identified in heterologous systems. Findings from this research not only identified the mode of action of FHV B2 in RNAi suppression but also revealed some unique features of worm antiviral RNAi.

**3.2 FHV B2 suppresses dsRNA triggered RNAi and RDVI targeting a natural viral pathogen of *C. elegans***.

So far, no VSR has been shown to suppress classical RNAi triggered by long dsRNA in *C. elegans*. FHV B2 retains its RNAi suppression activity when produced in trans in both plant and insect system (7, 12, 38). Despite its insect origin, FHV B2 was shown to be required for efficient replication of the cognate virus in the wild type *C. elegans* but became dispensable in worm mutants defective in RDVI (9), suggesting that FHV B2 retains its RDVI suppression activity in worms. To find out whether FHV B2 suppresses classical RNAi in *C. elegans* we assayed the silencing of a *gfp* transgene triggered by ingestion of *gfp* dsRNA in worms carrying constitutively active nuclear transgene corresponding to FHV B1 and FHV B2 (Figure 3.1 panel A). The B1 protein of FHV, which is translated from the same subgenomic RNA as B2, is known to be inactive in RNAi suppression (7) and thus served as a control to FHV B2 in our test. As shown in Figure 3.1 panel B, silencing of the *gfp* transgene was suppressed in transgenic worms expressing FHV B2 but not in wild type N2 worms or worms expressing FHV B1, confirming that FHV B2 indeed suppresses long dsRNA triggered RNAi in worms.

Orsay virus is a naturally occurring viral pathogen of *C. elegans* that was originally isolated from a worm mutant defective in RDVI (50). Interestingly, the replication of Orsay virus
in wild type N2 worms is significantly weaker compared to that in RDVI-defective mutants. The fact that Orsay virus is still sensitive to RDVI suggested that its replication would be further enhanced in worms expressing a functional VSR. To test this hypothesis, we checked Orsay virus infection in transgenic worms constitutively expressing FHV B2. As shown in Figure 3.1 panel C, compared to that in non-transgenic N2 worms and N2 worms expressing FHV B1, the replication of Orsay virus was significantly enhanced in transgenic N2 worms expressing FHV B2. These results together suggested that FHV B2 is able to suppress both long dsRNA triggered RNAi and RNAi triggered by natural viral infection in C. elegans.

Figure 3.1 FHV B2 suppresses long dsRNA triggered RNAi and RDVI targeting Orsay virus. A. Schematic structure of the sur-5 promoter based transgenes. Psur-5, the promoter for worm gene sur-5. UTR, the 3’ end untranslated sequence of the worm gene unc-54. fB1, the B1 coding sequence of FHV. fB2, the B2 coding sequence of FHV. GFP, the coding sequence of green fluorescent protein. B. FHV B2 suppresses dsRNA triggered RNAi. All worms used in this test contain a nuclear transgene corresponding to Psur-5::GFP. Shown here is the accumulation of gfp transcripts in wild type N2 worms or worms containing Psur-5::fB1 or Psur-5::fB2 transgene as indicated. Asterisks denote total RNA samples extracted from worms fed on E. coli food expressing GFP dsRNA. Methylene blue-stained ribosomal RNA serves as equal loading control. C. FHV B2 enhances the replication of Orsay virus. Shown here is the accumulation of Orsay virus RNA1 in C. elegans isolate JU1580, wild type N2 worms, rde-1 mutants and worms expressing FHV B1 and B2 respectively. Total RNA was prepared 72 hours after virus inoculation. A 1.2kb cDNA fragment derived from the 3’ end of Orsay virus RNA1 was used to prepare probes for hybridization. Methylene blue-stained ribosomal RNA serves as equal loading control.
3.3 Development of an assay for the identification of VSRs in *C. elegans*

To facilitate the discovery and characterization of VSRs in *C. elegans*, we developed an RDVI suppression assay based on the induction of RDVI by the self-replication of the genomic RNA1 of FHV. The construction of FR1gfp, an FHV RNA1 based replicon that contains a green fluorescent protein (GFP) coding sequence in the place of B2 coding sequence (Figure 3.2 panel A), was described previously (62). As shown previously, the replication of FR1gfp launched from a chromosomally integrated transgene is suppressed by RDVI in wild type worms but is restored to yield green fluorescence expressed from the subgenomic RNA produced after RNA1 replication in RNAi-defective mutant worms such as rde-1 and drh-1 mutants. We determined if the FR1gfp-induced RDVI was suppressed and expression of GFP was activated following ectopic expression of a functional VSR. We chose the FHV B2 as the VSR since it exhibits RNAi suppression activity following ectopic expression in both plant and insect cells and suppresses FHV RNA1-induced RDVI when encoded in cis (7, 12). In our experimental system, two plasmid constructs were co-microinjected into the gonad of young adult worms containing the FR1gfp replicon (62) and GFP expression was monitored in the next generation of worms. The first constructs directed expression of the FHV B2 driven by the same heat inducible promoter used to initiate FR1gfp replication (Figure 3.2 panel A). The second construct directed mCherry expression in the pharynx tissue and was used to generate a visible marker for transgene transformation. Most of the extrachromosomal transgenic arrays generated through gonad injection are randomly passed on to the next generations so that there are always some worms within each generation of the transgenic lines that are free of the transgene, marked by the absence of mCherry expression. Therefore, progenies from a transformed parent often include individuals that do not inherit the transgene, and thus can serve as an internal negative control.
Figure 3.2 Ectopic expression of FHV B2 suppresses worm RDVI targeting FR1gfp replicon. A. Schematic structure of the FR1gfp replicon, the Pmyo-2::mCherry reporter construct, and the FHV B1 and B2 transgenes utilizing the heat inducible promoter. HIP, heat inducible promoter. Protein A, the replicase of FHV. Rz, the self-cleaving ribozyme sequence from hepatitis delta virus. UTR, the 3' end untranslated sequence of the worm gene unc-54. Pmyo-2, the promoter for the worm gene myo-2, which directs target gene expression in the pharynx tissue. B. Visualization of green fluorescence in worms carrying the FR1gfp nuclear transgene and extrachromosomal array corresponding to FHV B1 or FHV B2, as indicated, 48 hours post heat induction. Shown here are merged images recorded using the same exposure under white light, red fluorescence and green fluorescence. Worms showing red or orange (merged from red and green) color are transgenic for FHV B1 or FHV B2. C. Accumulation of FR1gfp genomic and subgenomic RNAs in wild type N2 worms or worms carrying the rde-1 null allele (ne300) or integrated transgene corresponding to FHV B1 or B2 as indicated. Total RNA was prepared 48 hours post heat induction. The total RNA was hybridized with probes prepared from full length gfp cDNA. Methylene blue stained ribosomal RNA serves as equal loading control. D. Accumulation of FR1gfp transcripts in N2 worms expressing FHV B1 or B2 utilizing the sur-5 promoter or the heat inducible promoter (indicated with asterisks).

As shown in Figure 3.1 panel B, we found that all transgenic progenies carrying the HIP::fB2 extrachromosomal arrays, marked by red fluorescent in head, produced bright full body green fluorescence in response to induction of FR1gfp replication through heat treatment. However, no full body green fluorescence was observed in the progenies carrying the transgenic arrays that directed expression of FHV B1, which was encoded by the same subgenomic RNA as B2 but showed no VSR activity (7). To further verify these findings, we generated chromosomal
integrants for the FHV B1 and B2 transgenes and checked the accumulation of FR1gfp transcripts in response to heat induction in respective transgenic worms using Northern blot hybridization. As shown in Figure 3.2 panel C, enhanced FR1gfp replication was detected in FHV B2 chromosomal integrants as found in the rde-1 mutant worms defective in RDVI, but not in the integrants containing FHV B1 transgene. These results together showed that ectopic expression of FHV B2 suppressed the replicon-induced RDVI in adult worms, indicating that rescue of the VSR-deficient FR1gfp replicon could serve as an RDVI suppression assay to identify VSRs in *C. elegans* following heat inducible expression.

We next determined if RDVI was suppressed by FHV B2 driven by a constitutive promoter. We found that the replication of FR1gfp was also significantly enhanced in worms constitutively expressing FHV B2 compared to that in worms constitutively expressing the B1. However, in comparison, the expression of FHV B2 utilizing the heat inducible promoter achieved a stronger rescue on FR1gfp replication (Figure 3.2 panel D).

### 3.4 The genomic RNA2 of Orsay virus encodes no detectable RDVI suppression activity

The fact that the replication of Orsay virus in wild type N2 worms is suppressed by RDVI and can be rescued by FHV B2 suggested that Orsay virus encodes weak or no RDVI suppression activity. As an effort to test this hypothesis we subjected the putative capsid protein and the delta protein encoded by Orsay virus genomic RNA2 to the RDVI suppression assay described in Figure 3.2. Our gonad microinjection of plasmid constructs containing either the capsid protein or the delta protein coding sequence driven by the heat inducible promoter (Figure 3.3 panel A) generated 17 and 13 lines of transgenic worms respectively. However, none of these transgenic lines showed enhanced GFP fluorescence after heat induction (Figure 3.3 panel B). This result suggests that none of the two putative proteins encoded by Orsay virus RNA2 possesses worm RDVI suppression activity.
Figure 3.3 The genomic RNA2 of Orsay virus encodes no RDVI suppression activity. A. Schematic structure of heat inducible transgenes corresponding to the putative coat protein (CP) and delta protein of Orsay virus respectively. B. Visualization of green fluorescence in worms carrying the FR1gfp nuclear transgene and extrachromosomal arrays generated through gonad injection of HIP::CP or HIP::delta. Worms showing red or orange (merged from red and green) color carry the HIP::CP or HIP::delta transgene. See Figure 3.2 for experiment details.

3.5 NoV B2, but not TBSV p19, TAV 2b or hypovirus p29, suppresses RDVI in C. elegans

Both TBSV p19 and TAV are well-characterized VSRs of plant origin that specifically bind and suppress the function of 21 nt siRNA duplexes (35-37, 41). In particular, TBSV p19 is known to be functional in heterologous systems such as insect and mammalian (41-43) and, owing to its target specificity, has been used as a genetic tool to explore the molecular mechanism of 21 nt siRNAs or miRNAs (31, 41). The p29 protein encoded by fungus-infecting hypovirus is another VSR known to be able to suppress RNAi in heterologous system such as plants (113). Currently, how p29 suppresses RDVI remains largely unknown. To find out whether these three VSRs retain their RDVI suppression activity in worm, we subjected all of them to the RDVI suppression assay described in Figure 3.2. Our assay also included NoV B2 which, despite sharing limited sequence identity with FHV B2, adopts a similar mechanism as FHV B2 to suppress RNAi (40) thus was expected to retain the RDVI suppression activity in
worms. Indeed, as shown in Figure 3.4 panel A&B, ectopic expression of NoV B2 utilizing the heat inducible promoter led to successful rescue of FR1gfp replication, manifested as significantly enhanced GFP fluorescence, in wild type N2 worms. Surprisingly, such a rescue was not observed for ectopic expression of TBSV p19, TAV 2b, a p19 loss of function mutant termed p19m, that contains the R72G point mutation (114), or hypovirus p29 (data not shown). Consistent with this observation, enhanced FR1gfp replication was detected by Northern blotting only in chromosomal integrants containing the heat inducible NoV B2 transgene (Figure 3.4 panel C).

Previous study suggested that TBSV p19 can be tagged with an HA epitope tag to the carboxyl-terminus (C-terminus) without losing its RNAi suppression activity (41). To rule out the possibility that the failure of TBSV p19 in suppressing worm RDVI is a result of instability of this suppressor when produced in worm we checked the accumulation of HA-tagged p19 expressed from a heat inducible transgene, termed HIP::p19HA (Figure 3.4 panel D, upper left panel), using Western blotting. As shown in the lower left panel of Figure 3.4 panel D, the production of HA-tagged p19 can be readily detected 48 hours post heat induction in chromosomal integrant containing the HIP::p19HA transgene. Like that found for wild type TBSV p19, no RDVI suppression activity was detected for this HA-tagged variant (Figure 3.4 panel D, right panel). These results thus confirmed that the failure of TBSV p19 in RDVI suppression is not a result of instability in worm.

To find out whether TBSV p19, TAV 2b and NoV B2 are able to suppress dsRNA triggered RNAi and RDVI targeting Orsay virus we checked the gfp transgene silencing triggered by gfp dsRNA ingestion (see Figure 3.1 for details) and Orsay virus replication in wild
Figure 3.4 NoV B2, but not TBSV p19 or TAV 2b, suppresses long dsRNA triggered RNAi and RDVI targeting Orsay virus.

A. Schematic structure of heat inducible transgenes corresponding to NoV B2 (nB2), TBSV p19 (p19), TBSV p19m (p19m), TAV 2b (2b) and p29.

B. Visualization of green fluorescence in worms carrying the FR1gfp replicon transgene and extrachromosomal arrays generated through gonad injection of constructs shown in A. Shown here are merged images recorded under white light, red fluorescence and green fluorescence with the same exposure 48 hours after heat induction.

C. Accumulation of FR1gfp transcripts in transgenic N2 worms carrying the integrated transgenes corresponding to NoV B2, TBSVp19, TBSV p19m and TAV 2b. p19m, a p19 variant that contains the R72G point mutation and is known to be deficient in RNAi suppression.

D. HA-tagged TBSV p19 is deficient in RDVI suppression. Upper left panel, the structure of a heat inducible transgene expressing TBSV p19 tagged with HA at the C-terminus. Lower left panel, Western blot detection of HA-tagged p19 produced in wild type N2 worms and worms containing the heat inducible transgene shown in upper left panel. Right panel, the HA-tagged TBSV p19 is deficient in RDVI suppression. Shown here is the accumulation of FR1gfp transcripts detected in wild type N2 worms and worms containing the heat inducible transgene corresponding to HA-tagged p19. Asterisks denote samples prepared using heat induced worms.

E. NoV B2, but not TBSV p19 or TAV 2b, suppresses dsRNA triggered RNAi targeting a gfp transgene.

F. NoV B2, but not TBSV p19 or TAV 2b, enhances the replication of Orsay virus. Shown here is the accumulation of Orsay virus RNA1 in worms containing NoV B2, TBSV p19 and TAV 2b transgenes, as indicated, utilizing the constitutively active promoter of the sur-5 gene.
type N2 worms constitutively expressing each of these suppressors. As shown in Figure 3.4 panel E, the suppression on dsRNA triggered gfp transgene silencing was only observed in NoV B2-expressing worms. Consistent with this observation, enhanced Orsay virus replication, compared to that in wild type N2 worms, only occurred in worms transgenic for NoV B2 (Figure 3.4 panel F). These observations together suggested that NoV B2, but not TBSV p19 or TAV 2b, suppresses dsRNA triggered RNAi and RDVI triggered during natural viral infection in C. elegans.

3.6 FHV B2 targets a step downstream of primary siRNA biogenesis to suppress RDVI

To better understand the molecular mechanism underlying the success/failure of the nodavirus B2s, TBSV p19 and TAV 2b in worm RDVI suppression we examined the accumulation of FR1gfp-derived siRNAs in worms containing heat inducible transgene corresponding to each of these VSRs. Consistent with previous deep sequencing analysis (115), our Northern blotting analyses detected discrete primary siRNA bands with the major one showing up at the position corresponding to 23 nt in the rde-1 mutants which are known to accumulate only primary siRNAs (Figure 3.5 panel A) (62, 103, 116). As indicated by arrows in Figure 5 panel A, our Northern blot analyses also detected an siRNA band with a size falling between 21 and 22 nt in wild type N2 worms and worms containing heat inducible transgene corresponding to FHV B1. However, such a siRNA band was not detected in single or double mutants containing the rrf-1 null allele. In the light of the fact that rrf-1 functions downstream of rde-1 to produce 22 nt secondary siRNAs in a Dicer-independent manner (101, 102, 116, 117) we believed that this band represents the rrf-1-dependent secondary siRNAs. The faster migration rate of this band compared to the 22 nt primary siRNAs may be a manifestation of the fact that the rrf-1-dependent siRNAs carry a triphosphate group at the 5’ end. FR1gfp-derived siRNAs were also detected in FHV B2 and NoV B2 transgenic worms with a pattern similar to
that detected in *rde-1* mutants (Figure 3.5 panel A&B), suggesting that FHV B2 and NoV B2 inhibit the function, rather than the biogenesis of primary siRNAs to suppress RDVI. In agreement with this notion, the *rrf-1*-dependent secondary siRNAs were not detected in the presence of FHV B2 (Figure 3.5 panel A & B). As shown in Figure 3.5 panel A, the accumulation of miR-58 was not reduced in response to FHV B2 expression compared to that in wild type N2 worms or worms expressing FHV B1, suggesting that FHV B2 does not suppress the biogenesis of worm miRNAs.

As shown in Figure 3.5 panel C, the *rrf-1*-dependent siRNAs were also detected in worms expressing TBSV p19, TAV 2b or their loss of function mutants with a pattern similar to that detected in wild type N2 worms. However, it is interesting to note that despite of the fact that none of TBSV p19, TAV 2b and their loss of function mutants exhibited RDVI suppression activity in our FR1gfp replication rescue assays (Figure 3.4), a slightly enhanced accumulation of 22 nt primary siRNAs was detected in worms expressing wild type TBSV p19 or TAV 2b compared to worms expressing respective loss of function mutants (Figure 3.5 panel C).

To find out whether FR1gfp-derived siRNAs detected in the presence of FHV B2 are bona fide primary siRNA duplexes produced by worm Dicer we treated our small RNA samples with Terminator exonuclease which destroys single-stranded RNAs carrying mono-phosphate group at the 5’ end but is much less efficient in digesting siRNA duplexes carrying monophosphate at the 5’ end (118). As expected, our Terminator treatment destroyed the miR-58 miRNAs in all small RNA samples (compare the treated samples with the untreated fB1 and fB2 small RNA samples) (Figure 3.5 panel D). However, such a treatment generated no detectable impact to the abundance of siRNAs detected in *rde-1* mutants or worms expressing the FHV B2.
Figure 3.5 FHV B2 targets a step downstream of the primary siRNA biogenesis to suppress RDVI. A. The accumulation of FR1gfp-derived siRNAs, detected by Northern blotting, in worms containing heat inducible transgene corresponding to FHV B1 or FHV B2 and wild type N2 worms and single and double mutants defective in rde-1 and/or rrf-1 function as indicated. After the detection of the virus-derived siRNAs, the same filter membrane was reused, after stripping, for the detection of miR-58 miRNAs. M, four DNA oligos with different sizes as indicated. They were detected using DIG-labeled complementary DNA oligos and, together with miR-58, served as size reference. The accumulation of miR-58 in each sample also served equal loading control. Arrows indicate the rrf-1-dependent siRNAs. B. Shown here is the accumulation of FR1gfp-derived siRNAs in rde-1 mutants and worms containing heat inducible FHV B2 or NoV B2 transgenes. C. Shown here is the accumulation of FR1gfp-derived siRNAs in rrf-1 mutants and worms containing heat inducible transgenes corresponding to, as indicated, TBSV p19, TAV 2b or their loss of function mutants. 2bm, a loss of function mutant of TAV 2b, which contains the P41A point mutation (36). To get size reference and equal loading control, the same filter membrane was re-probed for the detection of miR-58 miRNAs. D. Northern blot detection of FR1gfp-derived siRNAs in transgenic N2 worms containing heat inducible transgenes corresponding to various VSRs, as indicated, after treatment with the Terminator exonuclease. After the detection of the virus-derived siRNAs, the same filter membrane was reused for the detection of miR-58 miRNAs to generate size reference and equal loading control and Terminator treatment effectiveness control. Asterisk denotes samples not treated with the Terminator exonuclease.
This result thus suggested that the siRNAs detected in the presence of FHV B2 are bona fide primary siRNA duplexes.

3.7 FHV B2 does not inhibit the function of worm miRNAs

In plants, VSRs are capable of interfering with the function of miRNAs, leading to development defects (44, 47, 48). The *C. elegans* genome encodes a single Dicer that is required for the biogenesis of both siRNAs and miRNAs (93, 119). The fact that the accumulation of miR-58 is not affected in worms expressing FHV B2 (Figure 3.5 panel A&B) suggested that FHV B2 does not suppress the biogenesis of worm miRNAs. However, heat inducible expression of FHV B2 exhibited stronger suppression on RDVI, compared to constitutive expression (Figure 3.2 panel D), suggested a hypothesis that constitutive expression of FHV B2 leads to the suppression on miRNA function such that worms constitutively expressing FHV B2 at high level failed to develop and thus were selected out. To test this hypothesis we checked the suppression activity of FHV B2 on miRNA function using an assay system developed previously (111). This assay system features a *myo-2* promoter driven *gfp* transgene containing the let-7 target sequences within the 3’ end untranslated region. Thus, suppression of miRNA function will result in enhanced green fluorescence in the pharynx tissue which can be easily identified.

We used the *myo-2* promoter to drive the expression of FHV B2 and the control protein FHV B1 in our assay to ensure that both transgenes will have the same tissue-specific expression pattern as the *gfp* reporter gene (Figure 3.6 panel A). To ensure that functional FHV B2 proteins are produced in the pharynx tissue, we checked the suppression activity of FHV B2 on RNAi triggered by two transgenes that produce complementary transcripts within the pharynx tissue. As shown in Figure 3.6 panel A, the Pmyo-2::GFP construct contains a GFP coding sequence under the control of *myo-2* promoter whereas the Pmyo-2::PFG construct contains a sequence complementary to the GFP coding sequence under the control of the same *myo-2* promoter. Thus,
co-delivery of Pmyo-2::GFP and Pmyo-2::PFG constructs is expected to trigger gfp silencing in wild type N2 worms. Indeed, as shown in Figure 3.6 panel B, a transgenic locus containing both Pmyo-2::GFP and Pmyo-2::PFG transgenes produced weak green fluorescence in wild type N2 worms but bright green fluorescence in rde-4 mutants. Enhanced green fluorescence produced by the same transgenic locus was also observed in wild type N2 worms containing the Pmyo-2::fB2 transgene. However, such an enhancement in green fluorescence was not observed in the presence of the Pmyo-2::fB1 transgene, confirming that the Pmyo-2::fB2 transgene produces functional FHV B2 in the pharynx tissue.

Figure 3.6 FHV B2 does not inhibit miRNA function. A. Schematic structure of the myo-2 promoter based constructs. PFG, the antisense of GFP coding sequence. B. FHV B2 suppresses RNAi in the pharynx tissue. Shown here is the green fluorescence in pharynx tissue in wild type N2 worms, rde-4 mutants and worms containing Pmyo-2::fB1 or Pmyo-2::fB2 transgene as indicated. All worms used in this test contain a nuclear transgene generated by co-injection of Pmyo-2::GFP and Pmyo-2::PFG. The images were produced by merging images recorded using the same exposure under white light, red fluorescence or green fluorescence. C. FHV B2 does not suppress miRNA function in C. elegans. Shown here is the green fluorescence in the pharynx tissue in wild type N2 worms and worms containing extrachromosomal array corresponding to Pmyo-2::fB1 or Pmyo-2::fB2 as indicated. The images were produced by merging images recorded under white light or green fluorescence with the same exposure. The insets are images recorded under white light.

To find out whether FHV B2 suppresses the function of miRNAs, we injected the wild type N2 worms carrying the gfp reporter transgene with Pmyo-2::fB1 or Pmyo-2::fB2 constructs.
Subsequently, we checked the production of green fluorescence in worms containing extrachromosomal arrays corresponding to Pmyo-2::fB1 or Pmyo-2::fB2. As shown in Figure 3.6 panel C, no enhanced green fluorescence was observed in response to ectopic expression of either FHV B1 or FHV B2, suggesting that FHV B2 does not interfere with the function of miRNAs in *C. elegans*.

### 3.8 Conclusions

By developing an assay, based on rescue of mutant Flock house virus replication described above, I have successfully identified Nodovirus B2, which shares limited sequence homology with Flock house virus B2 but uses similar mechanism in RDVI suppression, as another viral suppressor that retains RDVI suppression activity in the worm system, confirming the robustness of this assay system. Notably, no VSR activity was detected for either of the coat protein or delta protein of Orsay virus proposed previously as VSRs. Among other known heterologous VSRs, no functional suppressor activity detected for 2b of tomato aspermy virus (TAV), p29 of fungus-infecting hypovirus, or p19 of tomato bushy stunt virus (TBSV). Since p19 is known to specifically bind 21 nt siRNA to suppress RDVI, I concluded that siRNAs of 21 nt do not make major contribution in RDVI. Further, unlike that in plants and insects, FHV B2 suppresses worm RDVI mainly by interfering with the function of virus-derived primary siRNAs, but not affecting the primary siRNA biogenesis. Furthermore, ectopic expression of Flock house B2 in the pharynx of worms with miRNA sensor construct indicated that B2 does not affect miRNA function. Thus, this assay system for the first time makes it possible to identify VSRs with worm RDVI suppression activity. It can be expected that functional and mechanistic characterization of VSRs identified using this assay will help unravel some unique features of VSR-mediated virus-nematode worm interaction.
CHAPTER 4 SILENCING OF HOST GENES DIRECTED BY VIRUS-DERIVED SIRNAS IN CAENORHABDITIS ELEGANS

4.1 Introduction

Small interfering RNAs (siRNAs) processed from viral replication intermediates, by ribonuclease III-like enzyme Dicer, guide sequence-specific antiviral silencing in fungi, plants and invertebrates. In plants, virus-derived siRNAs (viRNAs) can target and silence cellular transcripts and in some cases are responsible for the induction of plant diseases. Currently, it remains an open question whether viral derived siRNAs have the potential to modulate host gene expression in the animal kingdom as well. The nematode worm C. elegans would be an animal model of particular interest to address this question, considering the fact that, like plants, C. elegans produces four RNA-dependent RNA Polymerases (rrf-1~3, and ego-1) as important amplifying components of RDVI. In this chapter, I developed a modified Flock house virus replicon (FR1FP), in which only half of 3’ end GFP sequence is used to replace B2, and tested whether it replicates to trigger antiviral silencing and whether the antiviral silencing triggered by this particular replicon can be redirected to target homologous transgene and host transcripts (unc-22, skn-1) in the nematode C. elegans.

4.2 Development of an FHV RNA1 based replicon for the test of VIGS in C. elegans

FHV is a representative member of the nodavirus family that features bipartite genomes of plus-polarity. The genomic RNA1 of FHV can replicate autonomously, producing a subgenomic RNA, the RNA3 (Figure 4.1 panel A). The RNA3 encodes an RNA silencing suppressor called B2, which suppresses RDVI in diverse organism species, including C. elegans (7, 9). The genomic RNA2 of FHV encodes the viral coat protein and its replication requires the

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1 “Part of this chapter previously appeared as [Guo X, Li WX, and Lu R, Silencing of Host Genes Directed by Virus-Derived Short Interfering RNAs in Caenorhabditis elegans, August 2012]. It is reprinted by permission of [Copyright © American Society for Microbiology—see appendix]”
protein A, the viral replicase produced by the genomic RNA1. Recently, two nodaviruses have been found to naturally infect the nematode worms *C. elegans* and *C. briggsae* respectively, making FHV an ideal model virus for the study of virus-host interaction in nematodes (50). Importantly, when delivered through a transgene strategy, the FHV RNA1, or its derivative, can replicate autonomously to trigger potent RDVI in *C. elegans*, making it a perfect, owing to its small size and simple structure, viral agent for the study of cytosolic antiviral mechanism, such as RDVI, in the nematodes (9, 62).

Previously, an FHV RNA1 based replicon, named FR1gfp, has been shown to replicate and trigger potent RDVI in *C. elegans* (62). The FR1gfp replicon was created by replacing the B2 coding sequence with that coding for enhanced green fluorescence protein (GFP). In order to test VIGS in *C. elegans* using a *gfp* transgene as a reporter, we generated a new FHV RNA1 based replicon called FR1fp. The FR1fp features the 3’ end half of *gfp* coding sequence, 341 bp in length, in the place of B2 coding sequence (Figure 4.1 panel B). To find out whether FR1fp can perform self-replication to trigger RDVI in *C. elegans*, we generated a chromosomal integrant carrying the FR1fp replicon and checked its replication in different genetic backgrounds. As shown in Figure 4.1 panel C, FR1fp replication, manifested as the production of the subgenomic RNA3, was detectable in wild type N2 worms but became significantly enhanced in worm mutants corresponding to *rde-1*, *rde-4* and *rrf-1*. Since *rde-1*, *rde-4* and *rrf-1* are known genes with important functions in worm RDVI (8, 19, 62), this result confirmed that FR1fp indeed replicates and triggers potent RDVI in *C. elegans*.

In *C. elegans*, *rde-4*, but not *rde-1*, is required for the biogenesis of discrete classes of primary viRNAs that can be detected by northern blot (62). To find out whether the FR1fp replication leads to the accumulation of viRNAs derived from the FP region, we performed
Figure 4.1 Development of the FR1fp replicon. A. Schematic structure and replication of FHV RNA1 and RNA2. Protein A, the replicase of FHV. B2, the RNA silencing suppressor. RNA3, the subgenomic RNA of RNA1. B. Structure of the FR1fp replicon. HIP, the heat inducible promoter from worm gene hsp-16.41. FP, the 3’ end half of GFP coding sequence. Rz, the self-cleaving ribozyme sequence of hepatitis delta virus. UTR, the 3’ end untranslated region of unc-54. C. Accumulation of FR1fp genomic (RNA1) and subgenomic (RNA3) RNAs in different genetic backgrounds as indicated in response to heat induction. The FR1fp transcripts were detected using probes corresponding to the FP region of FR1fp. Methylene blue stained ribosomal RNA (rRNA) serves as equal loading control. D. Accumulation of FR1fp-derived viRNAs in different genetic backgrounds as indicated. The viRNAs were detected using DIG-labeled DNA oligos corresponding to the FP region of FR1fp. The accumulation of miR-58 detected by northern blot serves as both size reference and equal loading control.

viRNA detection using DNA oligo probes that cover the entire FP region. As shown in Figure 4.1 panel D, in agreement with previous findings, high-level accumulation of viRNAs derived from the FP region was detected in rde-1 mutants but not in rde-4 mutants. The viRNAs also accumulated in rrf-1 mutants, at a lower level, with a similar size distribution as that in rde-1 worms. It is worth noting that the viRNAs were also detected in the wild type N2 worms with a major band appearing at around 22nt. Considering the fact that the abundance of primary
viRNAs in *C. elegans* peaks at 23 nt (62, 115), the 22 nt viRNAs detected in N2 background may represent the secondary siRNAs produced by RRF-1.

4.3 FR1fp replication triggers potent silencing of a *gfp* transgene

To have a quick test on VIGS targeting a constitutively expressed *gfp* transgene in *C. elegans*, we injected the FR1fp construct, together with an mCherry reporter construct, into the gonads of worms carrying a nuclear *gfp* transgene driven by the *sur-5* promoter (Figure 4.2 panel A). The mCherry reporter construct directs mCherry expression in worm pharynx tissue thus will serve as a visual mark for the FR1gfp transgene. Since both the *sur-5* promoter and the promoter driving FR1fp primary transcription are active in most *C. elegans* cells (110, 120), the silencing of the *gfp* transgene is expected to occur in response to heat induction if *C. elegans* indeed supports VIGS. Indeed, of 13 transmittable lines obtained, most adult worms carrying the extra-chromosomal arrays corresponding to the FR1fp, manifested as red fluorescence in the pharynx area, exhibited reduced *gfp* expression, compared to worms that do not carry the transgene, 48 hours post heat induction. To confirm that the reduction in *gfp* expression is resulted from RNA silencing, we generated a chromosomal integrant for the FR1fp replicon and checked *gfp* expression in worm mutants defective in RNA silencing after heat induction. As shown in Figure 4.2 panel B, the expression of the *gfp* transgene, manifested as green fluorescence, was markedly reduced in wild type N2 worms in response to heat induction. However, such a reduction did not occur in RNA silencing defective mutants corresponding to *rde-1* or *rde-4* despite of high-level replication of the FR1fp in both mutants (Figure 4.2 panel C). *rde-4* and *rde-1* are known to play essential roles in primary siRNA biogenesis and function respectively. These results thus confirmed that the down-regulation of *gfp* expression observed in this test is indeed mediated by RNA silencing. The *gfp* silencing also occurred in the *rrf-1* knockouts but to a lesser extent compared to that in wild type N2 worms (Figure 4.2 panel B&C). Since the *rrf-1* products are
known to be responsible for the production of secondary siRNAs, this test further suggested that both primary and secondary viRNAs mediate target destruction in VIGS in C. elegans.

Figure 4.2 The FR1fp replication triggers potent silencing of a gfp transgene. A. The structure of the transgene expressing enhanced GFP and the Pmyo-2::mCherry reporter construct. Psur-5, the promoter of worm gene sur-5. Pmyo-2, the promoter of the myo-2 gene. B. The comparison of green fluorescence intensity in between worms that carry the FR1fp replicon, manifested as red fluorescence in head, and those that do not in different genetic backgrounds as indicated after heat induction. All worm strains carry the same nuclear transgene corresponding to the Psur-5::GFP. Shown here are merged images recorded under white light, red fluorescence and green fluorescence. C. The accumulation of the gfp transcripts and FR1fp RNAs in the worm strains shown in B before and after heat induction. Phosphotase labeled DNA probes corresponding to the FP region of FR1fp were used for viral RNA detection. The gfp transcripts were detected using probes derived from the 5’ end of GFP coding sequence that does not overlap with the FP region of the FR1fp. D. The accumulation of the gfp transcripts and FR1fp RNAs in sid-1 knockouts as compared to that in N2 background in response to heat induction.

In C. elegans, RNA silencing involves an intercellular silencing signal that guides sequence-specific silencing in distant tissues/cells, a phenomenon termed systemic silencing (121, 122). A recent study further suggested that it is the long dsRNAs and/or primary siRNAs that act as or generate the mobile silencing signal (70). To find out whether the gfp silencing
triggered by FR1fp replication involves systemic silencing, we checked the FR1fp induced gfp silencing in the sid-1 knockout mutants. SID-1 is a C. elegans transmembrane protein that functions as a dsRNA-selective dsRNA-gated channel in systemic silencing (68, 69). We reasoned that if the FR1fp triggered gfp silencing in some cells is initiated by an intercellular silencing signal rather than by FR1fp derived viRNAs, the gfp silencing will be compromised in sid-1 knockouts as compared to that in wild type N2 worms. As shown in Figure 4.2 panel D, the gfp silencing triggered by FR1fp in sid-1 mutants containing the qt2 allele is comparable to that in wild type N2 worms. It was also clear that the FR1fp replicated to comparable levels in the sid-1 knockouts and the wild type N2 worms. Based on this test, we concluded that the gfp silencing triggered by FR1fp in this report is mainly intracellular silencing.

4.4 The replicating FR1fp is the only trigger of the VIGS targeting the gfp transgene

In plants, RNA silencing can be triggered by transient expression of homologous sequences. It is believed that dsRNAs formed through overlapped bidirectional transcription of the same sequences serve as the trigger (123). To confirm that the gfp silencing described in Figure 4.2 is indeed triggered by viRNAs derived from replicating FR1fp rather than transient over-production of the FR1fp primary transcripts, we changed the trigger replicon from FR1fp to a replication deficient mutant named FR1fpfs (Figure 4.3 panel A). The FR1fpfs was created by introducing a frame-shift mutation into the protein A coding sequence in FR1fp. Apparently, the FR1fpfs retains all of the biological properties needed for being replicated by a functional replicase provided in trans, since co-injection of FR1fpfs and the HIP::protein A construct (Figure 4.3 panel A), which carries wild type FHV protein A coding sequence under the control of the same heat inducible promoter, can rescue the replication of FR1fpfs (data not shown). To find out whether transient production of the FR1fpfs primary transcripts can trigger the silencing of the gfp transgene, we injected the FR1fpfs construct, together with the mCherry reporter
construct (Figure 4.2 panel A), into the N2 worms carrying the Psur-5::GFP transgene. Of 7 transgenic lines carrying the FR1fp<sup>fs</sup> extra-chromosomal arrays, no gfp silencing was observed after heat induction (Figure 4.3 panel B). To rule out the possibility that the lack of gfp silencing is due to the failure in the FR1fp<sup>fs</sup> primary transcript production, we checked the accumulation of FR1fp<sup>fs</sup> transcripts in response to heat induction using northern blot. As shown in Figure 4.3 panel C, FR1fp<sup>fs</sup> transcripts were detected 12 hours post heat induction. These results together suggested that the gfp silencing in our setup is indeed induced by replicating FR1fp that produces viRNAs in an RDE-4-dependent manner.

Figure 4.3 The gfp silencing shown in Figure 4.2 is triggered by replicating FR1fp. A. The schematic structure of FR1fp<sup>fs</sup> and FR1RdRP. Protein A, the replicase of FHV, an RdRP. B. Green fluorescence visualized in gfp transgenic worms carrying extrachromosomal arrays corresponding to FR1fp and FR1fp<sup>fs</sup> respectively before and after heat induction. All worms in this test contained the same gfp transgene as described in Figure 4.2 panel B. The extrachromosomal arrays were generated through gonad injection of target constructs together with reporter construct Pmyo-2::mCherry (Figure 4.2 panel A). Shown here are merged images recorded under white light, red fluorescence and green fluorescence 48 hours post heat induction. C. The comparison of gfp silencing in response to heat induction in between worm strains carrying the extrachromosomal arrays corresponding to FR1fp or FR1fp<sup>fs</sup>. D. Upper panel, structure of the FR1ΔB2 replicon. Lower panel, the comparison of gfp silencing in response to heat induction in between worm strains carrying the extrachromosomal arrays corresponding to FR1fp or FR1ΔB2 replicon.
To confirm that the gfp silencing is indeed induced by viRNAs derived from replicating FR1fp rather than an artifact resulted from the replication of non-specific viruses, we switched the triggering replicon from FR1fp to FR1ΔB2. FR1ΔB2 contains a point mutation that disrupted the start codon of the B2 ORF thereby to sensitize the virus to RDVI (Figure 4.3 panel D, upper panel) (9). As a result, replicon of FR1ΔB2 in wild type N2 worms will induce the production of viRNAs that are not complementary to the gfp mRNAs. Thus, no gfp silencing should occur in response to FR1ΔB2 replication. As shown in Figure 4.3 panel D, lower panel, the gfp silencing did not occur in response to FR1ΔB2 replication. These results together suggested that the gfp transgene silencing described in Figure 4.2 is indeed triggered by viRNAs derived from replicating FR1fp.

4.5 VIGS is inheritable in C. elegans

In C. elegans, RNA silencing induced by exogenous dsRNAs is inheritable (124). A recent report demonstrated that antiviral silencing in C. elegans also features a non-Mendelian, multigenerational inheritance and that it is the viRNAs that serve as physical carrier of the silencing signal across generations (125). Considering the fact that viRNAs are capable of mediating potent silencing of cellular transcripts as described above, it would be of interest to ask whether VIGS can be transmitted to the next generation. To address this question, we initiated gfp silencing by heat inducing the transgenic worms carrying both the gfp transgene and the FR1fp replicon, as described in Figure 4.2, and checked the gfp silencing in the next 2 generations (see Figure 4.4 panel A for details). We observed gfp silencing, albeit to a lesser extent compared to that in the mothers, in the F1 generation. In agreement with this observation, the gfp transcripts of the F1 generation accumulated to a lower level compared to the non-induced worms as confirmed by northern blot (Figure 4.4 panel B). In the F2 generation, the gfp silencing was much less evident compared to that in the F1 generation and accordingly the
northern blot failed to detect a decrease in gfp transcript level (Figure 4.4 panel B). As expected, no gfp silencing was detected in heat induced mothers or the next two generations when the same test was carried out using the rde-1 knockouts. Based on these results, we concluded that VIGS in the nematode worms is inheritable.

Figure 4.4 VIGS is inheritable in C. elegans. A. The strategy used to test whether VIGS in C. elegans is inheritable. B. The accumulation of the gfp transcripts in heat induced mothers (P0) and next two generations (F1 and F2) in wild type N2 or rde-1 knockouts. All worms used in this test contained the same gfp nuclear transgene and the same FR1fp replicon as described in Figure 4.2 panel B. The gfp transcripts were detected 48 hours post heat induction for the mothers.

4.6 VIGS can target and silence endogenous gene in C. elegans

unc-22 encodes an abundant myofilament protein required for muscle structure and function and reduction in unc-22 expression, e.g. as a result of RNA silencing, produces severe twitching phenotype that can be easily identified (1). The fact that VIGS in C. elegans is inheritable prompted us to ask whether VIGS is able to target endogenous genes, such as the unc-22, thereby to produce RNA silencing phenotypes in the next generation. To address this question, we developed a new replicon by replacing the FP region of the FR1fp with an unc-22 sequence, derived from the exon 7, of the same length. Unfortunately, upon heat induction, the
transgenic worms containing the extra-chromosomal arrays corresponding to the new replicon did not produce progenies with discernible twitching phenotype (data not shown). We believed that the lack of viral replication, thus the accumulation of viRNAs, is responsible for the failure of unc-22 silencing since the replication of the new replicon was extremely weak (data not shown). Very likely, modification in the RNA3 region of the new replicon has rendered it much less efficient in self-replication.

FHV replication is often associated with the production of an RNA2-derived defective-interfering RNA called DI-634 (126). Importantly, the DI-634 can be modified for the expression of heterologous genes (127). Thus, as a strategy to circumvent the requirement of self-replicating RNA1 to trigger unc-22 silencing, we created a DI-634 based replicon, termed DI634 (Figure 4.5 panel A). Like that in the FR1fp replicon, the transcription of the DI634 replicon is initiated by the same heat inducible promoter and the same self-cleaving ribozyme sequence was attached to the 3’ end of the DI634 genome to remove the non-viral sequence after transcription. When the DI634 replicon and the FR1∆B2 replicon were co-injected into the drh-1 knockouts, high-level accumulation of the DI634 derived RNAs were detected using probes specific to the DI634 genome (Figure 4.5 panel B).

To find out whether VIGS can target and silence the unc-22 gene, we inserted 552 bp unc-22 coding sequence, derived from the exon 7, into the multiple cloning site (MCS) of the DI634 replicon and delivered the new replicon, termed DI634unc22 (Figure 4.5 panel A), together with the FR1∆B2 replicon, into wild type N2 worms through gonad injection. Upon heat induction, the accumulation of DI634unc22-derived transcripts became detectable 8 hours post heat induction (phi) and was further enhanced at 24 hours hpi (Figure 4.5 panel C). Accordingly, a decrease at the unc-22 mRNA level was detected at 24 hours phi using northern blot. As a result
of the decrease in the *unc-22* expression, we observed twitching phenotype on approximately 52% progenies produced 24 hpi by the N2 mothers whereas no twitching phenotype was observed for progenies produced by non-treated mothers or the *rde-1* mothers that carry the same transgene (Figure 4.5 panel D). Using the same strategy, we also tested the silencing of another endogenous gene *skn-1*, whose function is required for intestine development of early embryos.

Figure 4.5 Down-regulation of endogenous gene expression by VIGS. A. Schematic structure of the DI634 and the DI634-based replicons DI634unc22 and DI634skn-1. MCS, multiple cloning site corresponding to AscI-XhoI-NotI. unc-22, a cDNA fragment derived from the exon 7 of the *unc-22* gene. Skn-1, a cDNA fragment derived from the skn-1 coding sequence. B. Replication of DI634 in the presence of a functional protein A produced by FR1ΔB2 in *drh-1* knockouts. The replication of FR1ΔB2 was detected using cDNA probes derived from the RNA3 of FR1ΔB2. The replication of DI634 was detected using cDNA probes derived from the DI634 genome. C. Down-regulation of the *unc-22* expression triggered by replicating DI634unc22. The accumulation of the DI634unc22 replication products and the *unc-22* transcripts were detected using *unc-22*-specific cDNA probes at different time point as indicated. hpi, hours post heat induction. D. The VIGS phenotype resulted from DI634unc22 replication in the F1 progenies. Shown here are the percentages of twitching F1 progenies produced by heat induced mother containing the DI634unc22 replicon 24 hpi. The error bars indicate standard deviation for the twitching phenotype. E. viRNAs mediated potent silencing of the endogenous gene *skn-1*. Shown here are the embryos and worm larvae produced by heat induced N2 and *rde-1* mothers carrying the same FR1ΔB2 and DI634skn-1-replicons.
Our result, as shown in figure 4.5 panel E, clearly showed that VIGS can target and silencing
skn-1, leading to the production of dead eggs from heat induced worms carrying the DI634s
replicon (Figure 4.5 panel A). Based on these results, we concluded that VIGS can target and
silence endogenous genes in C. elegans.

4.7 Conclusions

In this chapter, I show that viral siRNAs derived from FR1FP replicon indeed triggered
potent silencing of homologous GFP transgene in C. elegans, whereas a replication deficient
FR1FP replicon failed to induce silencing, confirming that only replicating virus triggers host
gene silencing in this study. Like that found in plants, virus-induced gene silencing (VIGS) in C.
elegans also involves RRF-1, a worm RNA-dependent RNA polymerase (RdRP) that is known
to produce single-stranded secondary siRNAs in a Dicer-independent manner. I further
demonstrated that VIGS in C. elegans is inheritable, suggesting that VIGS has the potential to
generate profound epigenetic consequences in future generations. In addition, by using modified
Flock house virus RNA2 genome that contains part of C. elegans unc-22 or skn-1 coding
sequence, I demonstrated that viral derived siRNAs are able to modulate endogenous gene
expression. Altogether, these findings, for the first time, confirmed that viRNAs have the
potential to regulate host gene expression in the animal kingdom. Most importantly, the success
in uncoupling the trigger and the target of the antiviral silencing would allow for the exploration
of novel features of virus-host interactions mediated by viRNAs in the animal kingdom.
CHAPTER 5 ANTIVIRAL RNA SILENCING INITIATED IN THE ABSENCE OF RDE-4, A DOUBLE-STRANDED RNA BINDING PROTEIN, IN CAENORHABDITIS ELEGANS

5.1 Introduction

Small interfering RNAs (siRNAs) processed from double-stranded RNA (dsRNA) of virus origin mediate potent antiviral defense through a process referred to as RNA interference (RNAi) or RNA silencing in diverse organisms. In the simple invertebrate *Caenorhabditis elegans*, RNAi machinery is initiated by single Dicer, which partners with the dsRNA binding protein RDE-4 to process dsRNA into primary viral derived siRNAs (viRNAs), then one of the strands of primary viRNA duplex is loaded into the Argonaute proteins (AGO) by thermodynamic mechanism, and binds to its complementary mRNA as target. The Argonaute proteins perform “slicer” activity for mRNA cleavage and recruit host RNA-dependent RNA polymerase (RdRP) to generate secondary viral siRNAs with tri-phosphate groups in the 5’end. Notably, In *C. elegans* this RNA-directed viral immunity (RDVI) also requires a number of worm-specific genes for its full antiviral potential. Previous genetic screen through feeding RNAi suggested that RSD-2 (RNAi spreading defective 2) is a worm-specific component of RDVI that contributes to RDVI through unknown mechanism. Interestingly, RSD-2 was originally identified as a requirement for uptaking dsRNA signal into the germline. Later on it was found to be required for chromosome segregation. Since RSD-2 is specific to nematodes, it would be interesting to determine through what a unique mechanism this worm-specific gene contributes to RDVI. In this chapter, I tested whether ectopic expression of wild type RSD-2 is able to rescue RSD-2 function in *rsd-2* mutant (tm1429). By comparing Flock house virus and Orsay virus replication levels in double mutants

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1 “Part of this chapter previously appeared as [Guo X, Zhang R, Wang J and Lu R, Antiviral RNA silencing initiated in the absence of RDE-4, a double-stranded RNA binding protein, in Caenorhabditis elegans. July 2013]. It is reprinted by permission of [Copyright © American Society for Microbiology—see appendix]”
corresponding to rsd-2 and other known RDVI genes, such as rde-1, rde-4, and rrf-1, together with small RNA profiling, I found that RSD-2 contributes to rde-4-dependent and rde-4-independent RDVI in *C. elegans*.

### 5.2 rsd-2 is not essential for the biogenesis of primary viRNAs

*rsd-2* of *C. elegans* was first implicated in RDVI through an RNAi screen that selected for genes whose down-regulation by dsRNA feeding led to loss of RDVI (62). To confirm that the loss of RDVI in worms fed with *rsd*-2-targeting dsRNA was indeed caused by partial loss of *rsd*-2 function, we examined the replication of FR1gfp replicon in the mutant worms containing *tm1429* or *pk3307* alleles of *rsd*-2 respectively. The *tm1429* allele contains a 251 nt deletion, spanning from nt #1376 to #1626 of *rsd*-2 cDNA, and is hence predicted to produce a truncated RSD-2 that contains only the first 458 amino acid (aa) residues of the 1266 aa wild type RSD-2. Conversely, the *pk3307* allele contains a premature stop codon in the RSD-2 coding sequence that caused the truncation of the last 542 aa of RSD-2. Thus, both *rsd*-2 alleles are considered as null allele. The FR1gfp replicon was developed from flock house virus (FHV) RNA1 by replacing the coding sequence of B2, the FHV-encoded RNAi suppressor, with that of green fluorescent protein (GFP). As a result, the replication of FR1gfp is subdued by RDVI in wild type N2 worms but rescued in RDVI defective worms such as the *rde*-1 mutants. As shown in Figure 5.1 panel A, similar to the *rde*-1 mutants, both *rsd*-2 mutants supported elevated accumulation of FHV RNAs (Figure 5.1 panel A, compare lanes 3 and 4 with 1 and 2), thus confirming a critical role of wild type RSD-2 in FHV-targeting RDVI. Enhanced accumulation was also observed in the *rsd*-2 mutants for Orsay virus, a natural viral pathogen of *C. elegans* that is closely related to FHV (Figure 5.1 panel B, compare lanes 2 and 3 with lane 1) (50). These results together strongly suggested that RSD-2 plays an important role in RDVI.
To reconfirm that the loss of RDVI in *rsd-2* mutants indeed resulted from the *rsd-2* null alleles but not any other closely linked genetic alleles we checked if ectopic expression of wild type RSD-2 restores RDVI in *rsd-2* mutants (*tm1429*) containing the FR1gfp replicon. The ectopic expression of wild type RSD-2 coding sequence was achieved through gonad microinjection of plasmid construct containing RSD-2 coding sequence driven by the *sur-5* promoter (figure 5.1 panel C) (110). A plasmid construct that directs mCherry expression in pharynx tissue was co-injected to produce a visual reporter for the transgene. As shown in Figure 5.1 panel C, FR1gfp replication, manifested as green fluorescence, was suppressed in *rsd-2* mutants containing the Psur-5::RSD-2 transgene (compare the worms that showed red fluorescence in the heads with worms that did not). Consistent with this observation, FR1gfp transcripts were detected at reduced level in worms containing the Psur-5::RSD-2 transgene (Figure 5.1 panel D, compare lane 2 with lane3). These results thus confirmed that RSD-2 indeed plays an important role in worm RDVI.

In *C. elegans*, the same gene can play distinct roles in different RNAi pathways. For example, *ergo-1* plays essential role in endogenous RNAi but contributing to the biogenesis of endogenous siRNAs but appears to negatively regulate RDVI (62). *rsd-2* is known to contribute to the accumulation of secondary, but not primary, endogenous siRNAs (66). Currently, whether RSD-2 contributes to RDVI through a similar mechanism remains largely unknown. To address this question, we checked the accumulation of viRNAs in *rsd-2* mutants (*tm1429*) using Northern blotting. Both *rde-1* and *rde-4* play essential roles in worm RDVI. However, virus-derived siRNAs accumulate to readily detectable level in *rde-1* but not *rde-4* mutants (8, 62). Thus, as a control, the accumulation of viRNAs in *rde-1* and *rde-4* mutants was also detected in this test. As shown in Figure 5.1 panel E, although not detectable in wild type N2 worms or *rde-4* mutants,
FR1gfp-derived viRNAs was detected at high levels in \textit{rde-1} mutants with a major band detected at the position corresponding to 23-nucleotide (nt). FR1gfp-derived siRNAs were also detected.

Figure 5.1 \textit{rsd-2} is not essential for the biogenesis of primary viRNA. A. Northern blot to show the accumulation of FR1gfp genomic and subgenomic transcripts in different genetic backgrounds as indicated. Total RNA was extracted from heat induced worms 48 hours post heat induction. The viral transcripts were detected using probes prepared from full length \textit{gfp} cDNA. RNA1, the genomic RNA of FR1gfp. RNA3, the subgenomic RNA of FR1gfp. rRNA, methylene blue stained ribosomal RNA serving as equal loading control. B. Accumulation of Orsay virus RNA1 (ovRNA1) in wild type N2 worms and \textit{rsd-2} mutants carrying \textit{tm1429} or \textit{NL3307} allele as indicated. Total RNA was prepared 72 hours post viral inoculation. Orsay virus RNA1 was detected using cDNA probes prepared using a cDNA fragment amplified from the 3’ end of Orsay virus RNA1. C. Upper panel, the structure of Psur-5::\textit{rsd-2}. Psur-5, the promoter for endogenous gene \textit{sur-5}. RSD-2, the coding sequence of wild type \textit{rsd-2}. UTR, the 3’ end untranslated region of \textit{unc-54}. Lower panel, visualization of green fluorescence in \textit{rsd-2} mutants (\textit{tm1429}) carrying the FR1gfp replicon transgene and extrachromosomal array corresponding to Psur-5::RSD-2 48 hours post heat induction. Shown here are merged images recorded, using the same exposure, under red fluorescence and green fluorescence. Worms showing red color are transgenic for Psur-5::RSD-2. D. Accumulation of FR1gfp transcripts in wild type N2 worms, \textit{drh-1} mutants and \textit{rsd-2} mutants (\textit{tm1429}) with/without the extrachromosomal assay derived from Psur-5::RSD-2 construct. RSD-2\textsuperscript{wt} denotes the Psur-5::RSD-2 transgenic array. E. Accumulation of FR1gfp-derived siRNAs in wild type N2 worms and RDVI defective mutants as indicated. M, four DNA oligos with sizes of 19, 21,23 and 25 nt detected and used, together with miR-58, as size references.
in rsd-2 mutants with a pattern similar to that in rde-1 mutants but at a visibly lower level. Previously, it has been shown that primary siRNAs produced by worm Dicer are predominantly 23-nt in size and accumulate at high level in rde-1 mutants (103, 115, 128) (92). This result thus suggested that rsd-2 is not essential for the biogenesis of primary viRNAs.

5.3 rsd-2 contributes to rde-4-independent antiviral silencing

We previously reported that FR1gfp-derived siRNAs detected in drh-1 mutants become undetectable in drh-1;rde-4 double mutants, suggesting that drh-1 and rde-4 function in a linear genetic pathway (62). To find out whether rsd-2 also functions in the same genetic pathway, we compared the accumulation of primary viRNAs in double mutants corresponding to drh-1;rde-4, rsd-2;rde-4 and rsd-2;drh-1. As shown in Figure 5.2 panel A, although not detectable in drh-1;rde-4 double mutants, FR1gfp-derived primary siRNAs were detected in both rsd-2;drh-1 and rsd-2;rde-4 double mutants (compare lane 3 with lanes 1 and 4). Most importantly, an enhanced replication for both Orsay virus and FR1gfp was observed in rsd-2;rde-4 and rsd-2;drh-1 double mutants compared to rde-4 and drh-1 single mutants respectively (Figure 5.2 panel B&C, left panels). These observations together suggested an rde-4-independent mechanism that directs antiviral silencing in an rsd-2-dependent manner in C. elegans. RDE-1 specifically recruits primary siRNAs, but not secondary siRNAs, for target RNA selection and is believed to function downstream of RDE-4 (103). Thus, we expected to see enhanced viral replication in rsd-2;rde-1 double mutants compared to rde-1 single mutants if rsd-2 indeed contributes to RDVI in an rde-4-independent manner. As shown in Figure 5.2 panel B&C, right panel, enhanced viral replication, although to a lesser extent, was observed in rsd-2;rde-1 double mutants compared to rde-1 single mutants. As expected, FR1gfp-derived siRNAs accumulated in rsd-2;rde-1 double mutants (Figure 5.2 panel A, lane 1). Taken together, these results strongly suggest that there is an rde-4-independent but rsd-2-dependent pathway contributing to RDVI in C. elegans.
Figure 5.2 rsd-2 contributes to rde-4-independent antiviral silencing. A. Accumulation of FR1gfp-derived siRNAs in double mutants, as indicated, defective in RDVI. See Figure 5.1 panel E for experimental details. B. Accumulation of Orsay virus RNA1 (ovRNA1) in wild type N2 worms and RDVI defective mutants carrying single or double mutations as indicated. See Figure 5.1 panel B for experimental details. C. Accumulation of FR1gfp transcripts in various genetic backgrounds as indicated. See Figure 5.1 panel A for experimental details.

5.4 rsd-2 and rrf-1 function in the same RDVI pathways

rrf-1 encodes an RdRP that initiates de novo synthesis of secondary siRNAs using the targeted transcript as template in RNAi (101, 102, 116, 129). To find out whether rsd-2 functions in the same genetic pathway as rrf-1, which is known to function downstream of rde-4 and rde-1 (103, 104), we checked viral replication in rsd-2;rrf-1 double mutant as compared to the single
mutants. We expected to see enhanced viral replication in the double mutants if \textit{rsd}-2 and \textit{rrf}-1 function in separate genetic pathways. However, as shown in Figure 5.3 panel A, an enhancement in the replication of FR1gfp or Orsay virus was not observed in the double mutants compared to the single mutants (compare lane 4 with lanes 2 and 3 in the left panel and lane 5 with lanes 3 and 4 in the right panel). Consistently, FR1gfp-derived 23-nt primary siRNAs accumulated to comparable levels in the \textit{rsd}-2;\textit{rrf}-1 double mutants and in the single mutants (Figure 5.3 panel B, compare lanes 1 and 2 with lane 7). This result, together with the results shown in Figure 5.2, suggests that both \textit{rrf}-1 and \textit{rsd}-2 function in two independent genetic pathways that mediate \textit{rde}-4-dependent and \textit{rde}-4-independent antiviral silencing respectively.

![Figure 5.3 rsd-2 and rrf-1 function in the same antiviral silencing pathways. A. Left panel: accumulation of FR1gfp transcripts in various genetic backgrounds as indicated. See Figure 5.1 panel A for experimental details. Right panel: accumulation of Orsay virus in single or double mutants containing \textit{rsd}-2 and/or \textit{rrf}-1 null alleles. C. The accumulation of FR1gfp-derived siRNAs in single or double mutants containing \textit{rsd}-2 and/or \textit{rrf}-1 null alleles. See Figure 5.1 panel E for experimental details.](image)

\textbf{5.5 rrf-1 contributes to \textit{rde}-4-independent antiviral silencing}

To confirm that \textit{rrf}-1 indeed contributes to \textit{rde}-4-independent RDVI, we first compared the replication of both FR1gfp and Orsay virus in \textit{rrf}-1;\textit{rde}-4 and \textit{rrf}-1;\textit{drh}-1 double mutants to
Figure 5.4 *rrf-1* contributes to *rde-4*-independent antiviral silencing. A. Accumulation of Orsay virus RNA1 (ovRNA1) in wild type N2 worms and RDVI defective mutants carrying single or double mutations as indicated. B. Accumulation of FR1gfp transcripts in wild type N2 worms and RDVI defective mutants carrying single or double mutations as indicated. C. Accumulation of FR1gfp-derived siRNAs in double mutants, as indicated, defective in RDVI in the single mutants corresponding to *rrf-1*, *drh-1* and *rde-4*.

that in the single mutants corresponding to *rrf-1*, *drh-1* and *rde-4*. We expected to see enhanced viral replication in the double mutants if *rrf-1* indeed contributes to *rde-4*-independent RDVI. As shown in Figure 5.4 panel A&B, both FR1gfp and Orsay virus replicated to higher levels in the double mutants compared to the single mutants. Most importantly, we detected FR1gfp-derived primary siRNAs in the *rrf-1;rde-4* and *rrf-1;drh-1* double mutants in contrast to that in *drh-1;rde-4* double mutants (Figure 5.4 panel C, compare lanes 2 and 4 with lane 2). As shown in Figure 5.4 panel A&B, an increase in FR1gfp replication was observed in the *rrf-1;rde-1* double mutants as compared to the single mutants although such an increase was not clear for Orsay virus. As expected, FR1gfp-derived primary siRNAs also accumulated in *rde-1;rrf-1* double
mutants (Figure 5.4 panel C, lane 3). These observations thus confirmed that **rrf-1** plays a role in RDE-4-independent RDVI.

**5.6 rde-1 plays a role in rde-4-independent RDVI**

Previously, an enhanced viral replication was observed in **drh-1;rde-1**, but not in **drh-1;rde-4**, double mutants compared to the single mutants (62), suggesting a role for **rde-1** in **rde-4**-independent RDVI. To find out whether **rde-1** indeed contributes to **rde-4**-independent RDVI, we compared both viral replication and primary viRNA accumulation in **rde-1;rde-4** double mutants and the single mutants. We expected to see enhanced viral replication and accumulation of primary viRNAs if **rde-1** indeed plays a role in **rde-4**-independent RDVI. As shown in Figure 5.5 panel A, both Orsay virus and FR1gfp replicated to higher levels in the double mutants compared to the single mutants. Most importantly, FR1gfp-derived primary siRNAs, although not detectable in the **drh-1;rde-4** double mutants, became readily detected in the **rde-1;rde-4** double mutants (Figure 5.5 panel B, compare lane 3 with lane 2). These results together suggested that **rde-1** also plays a role in **rde-4**-independent RDVI.

Figure 5.5 **rde-1** contributes to **rde-4**-independent antiviral silencing. A. Viral replication is further enhanced in **rde-1;rde-4** double mutants compared to the single mutants. Shown here is the accumulation of Orsay virus (left panel) and FR1gfp transcripts in single or double mutants containing **rde-1** and/or **rde-4** null alleles. B. Accumulation of FR1gfp-derived primary siRNAs in single or double mutants containing **rde-1** and/or **rde-4** null alleles.
5.7 *sid-1* is not required for RDVI targeting Orsay virus

In *C. elegans*, RNAi triggered by artificial long dsRNA spreads systemically and causes sequence-specific silencing in distant tissues that are not exposed to the dsRNA trigger (1). This observation raised a question whether, like that in plants (28, 29), worm RDVI involves an intercellular signal that prevents viruses from spreading systemically. Previously, we have shown that RDVI triggered by an FHV replicon was not compromised in *sid-1* mutants (105) defective in RNAi spreading (65, 68, 130). However, since potent RDVI will have been triggered in every single cells that contain the replicon transgene a systemic silencing signal may not be needed to silence such a viral replicon that is not known to move systemically. To find out whether worm RDVI indeed spreads systemically to restrict virus spread under natural condition, we compared Orsay virus infection in between wild type N2 worms and worms defective in *sid-1*. As shown in Figure 5.6, an increase in Orsay virus replication was not detected in *sid-1* mutants as compared to that in wild type N2 worms (compare lane 4 with lane 2). Based on this observation, we concluded that *sid-1* mediated systemic RNAi is not required for restricting Orsay virus infection in *C. elegans*.

Figure 5.6 *sid-1* is not required for RDVI in *C. elegans*. Shown here is the accumulation of Orsay virus RNA1 in wild type N2 worms and *sid-1* mutants. See Figure 5.1 panel B for experimental details.
5.7 A working model for rde-4-dependent and rde-4-independent antiviral silencing in C. elgans. WAGO, worm-specific AGO (103). Here we propose that the processing of viral dsRNAs into primary viRNAs occurs in rde-4-dependent or rde-4-independent manner. drh-1 contributes to the rde-4-dependent, but not rde-4-independent, antiviral silencing. We believe that rsd-2 and rrf-1 contribute to both rde-4-dependent and rde-4-independent antiviral silencing while rde-1 plays a major function in the rde-4-dependent antiviral silencing and a minor function in rde-4-independent antiviral silencing. We also believe that the major function of rrf-1 is to direct the synthesis of secondary viRNAs, rsd-2 contributes to the function of primary viRNAs, thereby initiating the synthesis of secondary viRNAs. As proposed previously, some WAGOs may direct the cleavage of cognate viral transcripts using the secondary viRNAs as sequence guide (103).

5.8 Conclusions

In this chapter, I first established an antiviral role of RSD-2 by showing that rsd-2 null mutants (tm1429 and pk3307) permitted higher levels of viral RNA accumulation, and that this enhanced viral susceptibility was reversed by ectopic expression of wild type RSD-2 open reading frame. I then examined the relationship of rsd-2 with other known components of RNAi pathways and established that rsd-2 functions in a novel pathway that is independent of rde-4 but likely requires the RNA-dependent RNA polymerase RRF-1, suggesting a critical role for RSD-2
in secondary viRNA biogenesis, likely through coordinated action with RRF-1. Together these results suggest that RDVI in the single-Dicer organism *C. elegans* depends on the collective actions of both RDE-4-dependent and RDE-4-independent mechanisms to produce RNAi-inducing viRNAs. My study reveals, for the first time, a novel siRNA-producing mechanism in *C. elegans* that bypasses the need for a dsRNA-binding protein.
CHAPTER 6 HOMOLOGOUS RIG-I-LIKE HELICASE PROTEINS DIRECT RNAI-MEDIATED ANTIVIRAL IMMUNITY IN C. ELEGANS BY DISTINCT MECHANISMS

6.1 Introduction

In the previous chapter, I described that the worm-specific gene *rsd*-2 plays an essential role in RNAi-directed viral immunity (RDVI) in *Caenorhabditis elegans*. In this chapter I will focus on another worm-specific RDVI component, termed Dicer-related helicase 1 (DRH-1), which encodes the helicase and C-terminal regulatory domains homologous to the mammalian RIG-I-like helicase (RLH) family of cytosolic immune receptors. Previous studies through small RNA analysis indicated that DRH-1 contributes to RDVI in the RDE-4 (a double stranded RNA binding protein) dependent pathway. However, how DRH-1 regulates the ancient antiviral innate immunity through RNAi remains largely unknown. Interestingly, *C. elegans* DRH family include two additional members, named DRH-2 and DRH-3. DRH-2, a close homologue of DRH-1, seems to be a negative regulator of RDVI, but the molecular and biochemical mechanism of DRH-2 in RDVI remains to be defined. DRH-3, which shares a more divergent sequence homologue with DRH-1 and DRH-2, is known to be required for worm development and contribute to classic RNAi, including germline RNAi. Currently, whether DRH-3 also plays a role in RDVI is unknown. To address these questions, I first developed a DRH-1 function rescue assay that would allow me to determine the requirement of each of DRH-1 domain in RDVI. Using this assay, I found that the helicase domain and C-terminal regulatory domain (CTD) of DRH-1 can be independently replaced by the corresponding RIG-I domains known to contribute to virus sensing, suggesting a role of DRH-1 in virus detection. I further showed that DRH-2

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1 “Part of this chapter previously appeared as [Guo X, Zhang R, Wang J, Ding SW and Lu R, Homologous RIG-I-like helicase proteins direct RNAi-mediated antiviral immunity in C. elegans by distinct mechanisms, September 2013]. It is reprinted by permission of [Copyright © PNAS—see appendix]”
may negatively regulate RDVI by acting as a competitor of DRH-1 and DRH-3 functions downstream of primary, but upstream of secondary siRNA, biogenesis in worm RDVI.

6.2 DRH-1 is required for nematode defense against infection by a natural virus pathogen

An antiviral role of DRH-1 was first identified through the characterization of antiviral RNAi induced by replication of FHV, which does not naturally infect *C. elegans* (62). A recent report described identification of a natural *C. elegans* virus, Orsay virus, which is closely related to FHV and shown to accumulate to significantly enhanced levels in RNAi-defective worm mutants such as *rde-1* and *rde-4* (50). We found that Orsay virus infection was also markedly enhanced in worm mutants carrying either of the two *drh-1* loss-of-function alleles (Fig. 6.1 panel A). This finding indicates that DRH-1 is also required for natural antiviral defense in *C. elegans*.

To verify the antiviral RNAi function of DRH-1, we carried out a transgene complementation assay in *drh-1* mutant animals as illustrated in Figure 6.2 panel A. In this assay, plasmid Psur-5::DRH-1 that directed DRH-1 expression under the constitutively active *sur-5* promoter was injected into *drh-1* mutant animals that carried the chromosomally integrated transgene FR1gfp controlled by a heat-inducible promoter (62). Heat treatment of *drh-1*:FR1gfp worms is expected to induce replication of the FHV-based RNA replicon and expression of the replicon-encoded green fluorescence protein (GFP) from a subgenomic mRNA synthesized by the FHV replicase. Since FR1gfp does not encode the RNAi suppressor protein B2 (62) green fluorescence would be undetectable if antiviral RNAi is restored in *drh-1* mutant animals by ectopic expression of DRH-1. We found that green fluorescence indeed became undetectable in *drh-1*:FR1gfp worms following gonad microinjection with Psur-5::DRH-1 (compare animals carrying Psur-5::DRH-1 marked by the red fluorescence expressed from the co-injected mCherry
reporter plasmid with animals showing no red fluorescence in heads, Fig. 6.2 panel B). This finding illustrated that ectopic expression of DRH-1 restored antiviral RNAi in the drh-1 mutants.

Figure 6.1 Both the N- and C-terminal domains of DRH-1 are indispensable for antiviral RNAi. A. Accumulation of Orsay virus RNA1 detected by Northern blot in wild type worms and genetic mutants as indicated. N2, the Bristol isolate of wild type C. elegans. JU1580, an isolate of C. elegans that is defective in antiviral RNAi. Methylene blue-stained ribosomal RNA (rRNA) serves as equal loading control. B. Schematic structure of DRH-1 and its derivatives used in the DRH-1 function rescue assay. NTD, N-terminal domain. DExD/H, the DEAD-box superfamily domain. Helicase C, the RNA helicase superfamily C terminal domain. CTD, C-terminal regulatory domain originally identified in RIG-I. C. Northern blot detection of FR1gfp transcripts in drh-1 mutants carrying the integrated transgenes corresponding wild type DRH-1 or its derivatives as indicated 48 hours post heat induction. Methylene blue stained ribosomal RNA serves as equal loading control. D. Orsay virus RNA1 detected by Northern blot in drh-1 mutants carrying the integrated transgenes corresponding wild type DRH-1 or its derivatives as indicated 72 hours post virus inoculation.

To further confirm this result, *drh-1*::FR1gfp worms carrying a stably integrated Psur-5::DRH-1 transgene were generated and used for the heat induction of FR1gfp or for Orsay virus infection. Northern blotting analysis showed that FR1gfp replication and Orsay virus infection were both suppressed in *drh-1* mutants containing the Psur-5::DRH-1 transgene (Fig. 6.1 panel
These results together show that DRH-1 plays an essential role in the worm antiviral RNAi induced by either FHV replication or Orsay virus infection.

6.3 Both the N- and C-terminal domains of DRH-1 are indispensable for antiviral RNAi

The DRH-1 function rescue assay established above made it possible for us to map the domain requirement in the antiviral function of DRH-1. In addition to the DEAD-box RNA helicase domain, DRH-1 contains a worm-specific N-terminal domain (NTD) and a conserved C-terminal regulatory domain (CTD) originally identified in RIG-I (131). We generated four domain deletion mutants of DRH-1 (Fig. 6.1 panel B), named DRH-1NTD, DRH-1DHC, DRH-1NDH and DRH-1NDHp respectively, and examined their antiviral activity using the DRH-1 function rescue assay. We found that ectopic expression of none of these four DRH-1 mutants restored antiviral RNAi in drh-1;FR1gfp worms (Fig. 6.2 panel B), indicating that both of the terminal domains of DRH-1 are essential for its antiviral function. As a further confirmation, we generated chromosomal integrants carrying nuclear transgenes corresponding to each of these DRH-1 deletion mutants and assayed for both FR1gfp replication and Orsay virus infection in the selected animal lines. Northern blot analysis (Fig. 6.1 panel C&D) showed that neither FR1gfp replication nor Orsay virus infection was suppressed in integrants carrying any of the DRH-1 domain deletion mutants, in contrast to the control integrants carrying the wild type DRH-1 transgene. Western blotting suggested stable expression of both DRH-1NTD and DRH-1NDH examined in transgenic worms by C-terminal tagging with an HA epitope (Fig. 6.2 panel C&D). These results together indicate that, in addition to the central helicase domains, both NTD and CTD of DRH-1 are indispensable for antiviral RNAi.
Figure 6.2 Both the N- and C-terminal domains of DRH-1 are indispensable for antiviral RNAi. 
A. Upper panel, schematic structure of the plasmid constructs Psur-5::DRH-1 and Pmyo-2::mCherry. Psur-5, the promoter of the endogenous gene sur-5. Pmyo-2, the promoter for the endogenous gene myo-2. UTR, the 3’ end untranslated region of the endogenous gene unc-54. 
Lower panel, schematic strategy of DRH-1 function rescue assay. Red dots indicate red fluorescence in the pharynx tissue. Green color represents green fluorescence produced by replicative FR1gfp. 
B. Visualization of green fluorescence in drh-1 mutants (tm1329) carrying the FR1gfp replicon transgene and the extrachromosomal arrays corresponding to wild type DRH-1 or its derivatives, as indicated. Expression of mCherry in pharynx tissue directed by the myo-2 promoter serves as visual mark of the transgenes. 
C. and D. Western blot detection of HA-tagged DRH-1 and its derivatives in corresponding transgenic worms. M, molecular weight references. 
1, non-transgenic N2 worms. 2, N2 worms transgenic for HA-tagged DRH-1 or its derivatives as indicated. NTD-HA, HA-tagged DRH-1NTD. NDH-HA, HA-tagged DRH-1NDH. The expression of β-actin was detected and used as equal loading controls.
6.4 The predicted domains of DRH-2 were functional in a fusion protein with the N-terminal domain of DRH-1

The predicted DRH-2 protein is highly homologous to DRH-1 (Fig. 6.3) but lacks the worm-specific NTD plus one of the three motifs in the DEAD-box helicase domain. We determined if use of the predicted protein domains of DRH-2 to replace the corresponding domains of DRH-1 produced a chimeric protein capable of mediating antiviral RNAi in *drh-1* mutant background. We found that ectopic expression of such a chimeric protein, D1D2 (Fig. 6.4 panel A), was associated with loss of GFP expression in *drh-1*:FR1gfp worms (Fig. 6.5 panel A), indicating rescue of DRH-1 function in antiviral RNAi by D1D2 driven by the constitutive *sur-5* promoter. We next generated stable animal lines expressing D1D2 driven by either *sur-5* or the heat-inducible promoter in *drh-1*:FR1gfp background. Northern blot analysis showed that expression of D1D2 driven by the *sur-5* promoter suppressed the replication of both FR1gfp and Orsay virus in the *drh-1* mutant background (Fig. 6.4 panel B&C). Notably, expression of D1D2 utilizing the heat inducible promoter conferred stronger antiviral RNAi targeting FR1gfp replicon (Fig. 6.4 panel B). However, transgenic expression of DRH-2, the region of DRH-1 equivalent to DRH-2 (DRH-1DHC*), or the N-terminal region of DRH-1 absent in DRH-2 (DRH-1NTD*), was insufficient to rescue DRH-1 function (Fig. 6.4 panel A&B&C/Fig 6.5 panel A). Epitope-tagging and Western blotting assay suggested stable expression of DRH-1NTD*, DRH-1DHC* and DRH-1 in transgenic worms (Fig. 6.5 panel B). Thus, these findings indicate that when expressed as a fusion protein with the NTD of DRH-1, the predicted helicase and CTD domains of DRH-2 mediate antiviral RNAi as effectively as the corresponding domains of DRH-1.
Figure 6.3 DRH-1 and DRH-2 share high-level sequence homology. Shown here is the sequence alignment between DRH-2 and DRH-1as generated by Clustal W. Sequence marked with red box represents the conserved DEAD-box domain. Sequence marked with green box represents the conserved helicase C terminal. Sequence marked with blue box represents the conserved C-terminal regulatory domain of RIG-I.
Figure 6.4 The putative domains encoded by DRH-2 mediate antiviral RNAi when fused with the NTD of DRH-1. A. Schematic structure of DRH-1, DRH-2 and their domain variants. See Figure 6.1 panel B and main text for more details. B. Accumulation of FR1gfp transcripts detected by Northern blot in drh-1 mutant carrying the integrated transgenes corresponding to wild type DRH-1, putative DRH-2 or their domain variants as indicated. HI denotes DRH-1 function rescue utilizing heat inducible promoter for candidate gene expression. C. Accumulation of Orsay virus RNA1 detected by Northern blot in drh-1 mutants carrying the integrated transgenes corresponding to wild type DRH-1, putative DRH-2 or their domain variants as indicated. OK3495, a DRH-1 variant encoded by the ok3495 allele.

6.5 The helicase and CTD domains of RIG-I were functional in a fusion protein with the N-terminal domain of DRH-1

An essential role for both the N- and C-terminal domains of DRH-1 in the antiviral defense is analogous to the domain requirement of mammalian RIG-I (132). DRH-1 shares significant sequence homology in the RNA helicase and CTD domains with RIG-I (Fig. 6.6) although by comparison DRH-1 and RIG-I are more distantly related than between DRH-1 and DRH-2. We found that transgenic expression of human RIG-I or a truncation mutant removing the N-terminal CARD domains (RIG-IDHC) in drh-1;FR1gfp worms did not suppress replication of FR1gfp (Fig. 6.7 panel A/6.8 panel A). Since the worm-specific NTD of DRH-1 was indispensable in
antiviral RNAi, we next determined the antiviral activity of a DRH-1/RIG-I chimeric protein termed D1RIG-I, in which the N-terminal CARD domains of human RIG-I were replaced with the NTD of DRH-1 (Fig. 6.7 panel A). As shown in Figure 6.7 panel A, ectopic expression of D1RIG-I, but not D1RIG-IND, effectively restored antiviral RNAi in drh-1;FR1gfp worms, indicating that the helicase and CTD domains of RIG-I confer similar biological function as the corresponding DRH-1 domains do. Similarly, antiviral RNAi was also restored by another DRH-1/RIG-I chimeric protein, D1RIG-ID1C, in which only the central helicase domain of DRH-1 was replaced by the corresponding domain of RIG-I (Fig. 6.7 panel A/6.8 panel A). This finding showed that antiviral RNAi was mediated by the helicase domain of RIG-I in the context of the N- and C-terminal domains of DRH-1.

Figure 6.5 The predicted domains of DRH-2 were functional in a fusion protein with the N-terminal domain of DRH-1. A. Visualization of green fluorescence in drh-1 mutants (tm1329) carrying both FR1gfp nuclear transgene and extrachromosomal arrays corresponding to wild type DRH-1, putative DRH-2 or their domain variants as indicated. See Figure S1B for experimental details. B. Western blot detection of HA-tagged DRH-1, DRH-1NTD* and DRH-1DHC* in transgenic worms generated through co-injection of three plasmid constructs corresponding to HA-tagged DRH-1, DRH-1DHC* and DRH-1NTD* respectively. M, molecular weight references. 1, non-transgenic N2 worms. 2, N2 worms transgenic for HA-tagged DRH-1, DRH-1NTD* and DRH-1DHC*. DRH-1-HA, HA-tagged DRH-1. DHC*-HA, HA-tagged DRH-1DHC*. NTD*-HA, HA-tagged DRH-1NTD*. The expression of β-actin was detected and used as equal loading controls.
Figure 6.6 DRH-1 and RIG-I share significant sequence homology within the RNA helicase domain, including the DEAD-box subdomain and the helicase C-terminal subdomain, and the CTD domain. Shown here is the sequence alignment between DRH-1 and RIG-I as generated by Clustal W. Sequence marked with red box represents the conserved DEAD-box domain. Sequence marked with green box represents the conserved helicase C terminal. Sequence marked with blue box represents the conserved C-terminal regulatory domain of RIG-I.
As described above, we further generated stable animal lines carrying each of these DRH-1/RIG-I chimeric constructs and assayed for both FR1gfp replication and Orsay virus infection in the selected animal lines. Consistent with the above observations, replication of both FR1gfp and Orsay virus was significantly inhibited in stable transgenic animals constitutively expressing D1RIG-I and heat inducible expression of D1RIG-ID1C restored antiviral RNAi to an extent comparable to that by D1RIG-I (Fig. 6.7 panel B/C/D). As observed above for D1D2, the heat inducible promoter directed more efficient rescue on DRH-1 function in antiviral FR1gfp silencing than the constitutive promoter (Fig. 6.7 panel B). These results together indicated that either of the RNA helicase and CTD domains of RIG-I is competent to functionally replace the homologous domain of DRH-1 in antiviral RNAi in *C. elegans*.

Previous structural studies showed that a KWK motif in the CTD of RIG-I mediates physical interaction between RIG-I and dsRNA although it is unknown if the interaction is functionally important (84, 85, 133). Since the KWK motif is conserved in DRH-1 CTD (Fig. 6.8 panel B upper panel), we next determined if this motif is critical for antiviral RNAi in *C. elegans*. To this end, we introduced the alanine substitutions into the KWK motif in the CTD domains of both DRH-1 and D1RIG-I, and subjected the resulting mutant constructs, DRH-1AAA and D1RIG-IAAA, to DRH-1 function rescue assay. As shown in Figure 6.8 panel B lower panel and Figure 6.8 panel C, expression of either mutant was unable to restore DRH-1 function in antiviral RNAi against FR1gfp. These findings strongly suggest that antiviral RNAi mediated by DRH-1 involves an essential activity of DRH-1 to detect viral dsRNA in a manner analogous to virus dsRNA sensing by RIG-I in mammals.
Figure 6.7 The RNA helicase and CTD domains of RIG-I functionally replace the corresponding domains of DRH-1 in antiviral RNAi. A. Schematic structure of RIG-I and its domain variants. B. Accumulation of FR1gfp transcripts detected by Northern blot in *drh-1* mutants carrying the integrated transgenes corresponding to wild type DRH-1 or RIG-I domain variants as indicated. HI denotes DRH-1 function rescue utilizing heat inducible promoter for candidate gene expression. C. Accumulation of Orsay virus RNA1 detected by Northern blot in *drh-1* mutants carrying the integrated transgenes corresponding wild type DRH-1 and D1RIG-I. D. Accumulation of FR1gfp transcripts detected by Northern blot in *drh-1* mutants carrying the integrated transgenes corresponding wild type DRH-1, D1RIG-I and D1RIG-ID1C.

6.6 DRH-3 regulates antiviral RNAi by a mechanism distinct from DRH-1

DRH-3 shares a similar domain structure with DRH-1 but contains more divergent sequences compared to DRH-1 and DRH-2 (Fig. 6.9). Recent studies have shown that DRH-3 is required for germline development and RNAi by participating in the biogenesis of 22G RNAs (134-136), suggesting a possible role for DRH-3 in antiviral RNAi. We investigated FHV
Figure 6.8 The helicase and CTD domains of RIG-I were functional in a fusion protein with the N-terminal domain of DRH-1. A. Visualization of green fluorescence in \textit{drh-1} mutants carrying both FR1gfp replicon transgene and extrachromosomal arrays corresponding to wild type RIG-I or its domain variants, as indicated. See Figure 6.1 C for experimental details. B. Upper panel, sequence alignment between the C-terminal sequences of DRH-1 and RIG-I. The KWK motifs are highlighted with bold fonts. Lower panel, visualization of green fluorescence in \textit{drh-1} null mutants (\textit{tm1329}) carrying both FR1gfp replicon transgene and extrachromosomal arrays corresponding to DRH-1AAA or D1RIG-IAAA. DRH-1AAA, a DRH-1 variant that contains K988A, W989A and K990A point mutations. D1RIG-IAAA, a D1RIG-I variant that contains the K982A, W983A and K984A point mutations. C. Accumulation of FR1gfp transcripts detected by Northern blot in \textit{drh-1} mutants carrying heat inducible transgenes corresponding to DRH-1AAA or D1RIG-IAAA.

replication and Orsay virus infection in worms containing the \textit{ne4253} allele of \textit{drh-3}. A single amino acid change occurred in the helicase C domain of DRH-3 encoded by the \textit{ne4253} allele and, as a result, worms containing this allele are defective in dsRNA-induced RNAi in the somatic tissues (136). We found that \textit{drh-3} mutant worms were highly susceptible to Orsay virus infection (Fig. 6.10 panel A, top). Northern blot analysis detected similarly high level replication of Orsay virus in \textit{drh-3}, \textit{drh-1} and \textit{JU1580} mutant worms. Moreover, we found that FR1gfp also replicated to higher levels in \textit{drh-3} mutant worms compared to wild type N2 worms (Fig. 6.10
panel A, bottom). These findings indicate that DRH-3 indeed plays a role in worm antiviral RNAi.

To define a role for DRH-3 in antiviral RNAi, we compared the accumulation of FR1gfp-derived viral siRNAs in \textit{drh-1} and \textit{drh-3} mutants using control mutants known to be defective in the biogenesis of primary siRNAs (\textit{rde-4}) or secondary siRNAs (\textit{rde-1} and \textit{rrf-1}) (92, 103, 115). Consistent with previous deep sequencing results (115, 128), several species of viral siRNAs were detected in the \textit{rde-1} mutants with a major band detected at the position corresponding to 23 nucleotides (nt) (Fig. 6.10 panel B). Viral siRNAs also accumulated to readily detectable levels in both \textit{drh-3} and \textit{rrf-1} mutants with a dominant 23-nt band similar to that in \textit{rde-1} mutants (Fig. 6.10 panel B/6.11), indicating robust production of viral primary siRNAs in \textit{drh-3}, \textit{rde-1}, and \textit{rrf-1} mutants. By comparison, viral siRNAs including the 23-nt species reproducibly accumulated to much lower levels in both \textit{drh-1} and \textit{rde-4} mutants than in \textit{rde-1} and \textit{drh-3} mutants even though the FR1gfp replicated to comparable levels in \textit{rde-1}, \textit{drh-1} and \textit{drh-3} mutants (Fig. 6.10 panel A&B/6.11).

Our findings suggested a major defect of \textit{drh-1} mutant animals in the biogenesis of primary viral siRNAs as in \textit{rde-4} mutant animals. In contrast, loss of antiviral RNAi in \textit{drh-3} mutant animals was not associated with defective biogenesis of viral primary siRNAs, suggesting that DRH-3 may regulate the biogenesis of viral secondary siRNAs as demonstrated for its role in exogenous RNAi (136). To test this idea, we compared the viral siRNA profiles in \textit{drh-3} and \textit{rrf-1} mutant animals through deep sequencing using a cloning protocol capturing both primary and secondary siRNAs. As found in studies on exogenous RNAi (92, 103, 115), \textit{rrf-1} mutant animals produced a typical population of primary siRNAs since the sequenced viral siRNAs were predominantly 23 nucleotides in length, contained similar reads number for positive and negative
strands, and were not enriched for 22-nt siRNAs with 5’-terminal guanine nucleotide (Fig. 6.10 panel C, left). We found that the profile of viral siRNAs sequenced from *drh-3* mutant animals

Figure 6.9 DRH-3 contains more divergent sequences compared to DRH-1 and DRH-2. Shown here is the sequence comparison in between DRH-1, DRH-2 and DRH-3. Clustal W was used for the sequence alignment.
(Fig. 6.10 panel C, right) were highly similar to that from *rrf-1* mutant animals and lacked a population of 22G RNAs. These observations together suggested a role for DRH-3 in the biogenesis of viral secondary siRNAs.

Figure 6.10 DRH-3 regulates antiviral RNAi by a mechanism distinct from DRH-1. A. Upper panel, accumulation of Orsay virus RNA1 detected by Northern blot in JU1580, wild type N2 worms and genetic mutants defective in *drh-3* or *drh-1* as indicated. Lower panel, accumulation of FR1gfp transcripts detected by Northern blot in wild type N2 worms and genetic mutants defective in *drh-3*, *rde-1* or *drh-1* as indicated. B. Accumulation of FR1gfp-derived siRNAs detected by Northern blot in different genetic backgrounds as indicated. Shorter exposure image of the same blot is shown in the lower panel. C. Primary and secondary FR1gfp-derived siRNA populations in *rrf-1* and *drh-3* mutants. The reads are grouped based on polarity and according to length and the identity of the first nucleotide.
Figure 6.11 DRH-3 regulates antiviral RNAi by a mechanism distinct from DRH-1. Left panel, accumulation of FR1gfp transcripts detected by Northern blot in wild type N2 worms and genetic mutants defective in \(drh-1\), \(rrf-1\) or \(drh-3\). Right panel, accumulation of FR1gfp-derived siRNAs detected by Northern blot in \(rrf-1\) and \(drh-3\) mutants. The accumulation of miR-58 was detected by Northern blot and used as both size reference and equal loading control.

### 6.7 DRH-1 is dispensable for RNAi targeting cellular transcripts

In contrast to antiviral RNAi, DRH-1 is largely dispensable in RNAi induced artificially to target cellular transcripts of endogenous genes such as \(skn-1\), \(dpy-13\) and \(unc-22\) (62) or transgene (Fig. 6.12). Our recent study has detected RNAi of cellular mRNAs targeted by virus-derived siRNAs in \(C.\ elegans\) (105). Since low levels of FR1gfp-derived viral siRNAs remained detectable in \(drh-1\) mutants (Fig. 6.10 panel B), we next determined if these viral siRNAs were active in RNAi targeting cellular transcripts in the absence of DRH-1. To test this hypothesis, we utilized a transgenic worm strain carrying a Psur-5::GFP transgene and a previously described replicon transgene, FR1fp, which does not express GFP due to deletion of the 5’-half of the GFP coding sequence but triggers potent silencing of the homologous cellular GFP transcripts (105) (Fig. 6.13 panel A). As expected, FR1fp replication triggered potent \(gfp\) silencing, manifested as reduction in the intensity of green fluorescence in the wild type N2 worms, but not in RNAi defective mutants corresponding to \(rde-1\) or \(rde-4\) (Fig. 6.13 panel B). Consistently, FR1fp-derived siRNAs accumulated at lower levels in \(drh-1\) mutants than in \(rde-1\) mutants (Fig. 6.14)
We detected \textit{gfp} silencing also in \textit{drh-1} mutants albeit to a lesser extent (Fig. 6.13 panel B). Northern blot analysis showed that the \textit{gfp} transcripts were markedly reduced in wild type N2 worms and \textit{drh-1} mutants but not in \textit{drh-1} double mutants with \textit{rde-1} or \textit{rde-4} (Fig. 6.14 panel B). In contrast, both RNAs 1 and 3 from replicon FR1fp accumulated to high levels in all of the examined \textit{drh-1}, \textit{rde-1}, and \textit{rde-4} single and double mutants (Fig. 6.14 panel B). These findings showed that \textit{drh-1} was essential for silencing a replicating viral replicon, but not a homologous cellular mRNA targeted by the same pool of siRNAs in the same animals. In an independent set of experiments, we compared the susceptibility of the replicating FR1fp and cellular GFP mRNA to feeding RNAi and found that cellular transcripts remained highly susceptible unlike the viral replicon (Fig. 6.15). Therefore, we propose that unlike \textit{drh-3} that is required for both exogenous RNAi and antiviral RNAi, participation of an additional \textit{drh-1} pathway is necessary to inhibit virus infection.

Figure 6.12 DRH-1 is not required for long dsRNA triggered RNAi. A. Structure of the Psur-5::GFP transgene. Psur-5, the promoter of the worm gene \textit{sur-5}. GFP, the coding sequence of enhanced green fluorescent protein. UTR, the 3’ end untranslated region of the worm gene \textit{unc-54}. B. Northern blot detection of \textit{gfp} transcripts in response to \textit{gfp} dsRNA ingestion in different worm strains as indicated. The accumulation of \textit{gfp} transcripts was detected at 0 hour (left panel) and 48 hours (right panel) after \textit{gfp} dsRNA ingestion as indicated. The full length \textit{gfp} cDNA was used to prepare probes for hybridization. Methylene blue-stained ribosomal RNA serves as equal loading control.
Figure 6.13 DRH-1 is dispensable for RNAi targeting cellular transcripts. A. Schematic of experiment strategy used to test whether DRH-1 is required for the silencing of cellular transcripts mediated by viral siRNAs. HIP, heat inducible promoter. Protein A, the replicase of FHV. FP, the 3’ end half of GFP coding sequence. Rz, self-cleaving ribozyme sequence derived from hepatitis D virus, which functions to remove all non-viral sequence at the 3’ end of the FR1fp primary transcripts. B. Visualization of gfp silencing triggered by replicating FR1fp in different genetic backgrounds as indicated. All worm strains carry the same Psur-5::GFP transgene and FR1fp replicon transgene which uses mCherry expressed in pharynx as visual mark.

Figure 6.14 DRH-1 is not required for the silencing of cellular transcripts mediated by viral siRNAs. A. Accumulation of FR1fp-derived siRNAs detected by Northern blot in wild type N2 worms and genetic mutants defective in rde-1, rde-4 and drh-1 as indicated. B. Northern blot detection of GFP silencing triggered by FR1fp replication in different genetic backgrounds as indicated 48 hours post heat induction. The GFP transcripts were detected using oligo probes that would not hybridize to the FP region of FR1fp.
Figure 6.15 Replicating viruses are less sensitive to long dsRNA triggered RNAi compared to cellular transcripts. A. Schematic of the strategy used to test whether replicating viruses are less sensitive to long dsRNA triggered RNAi compared to cellular transcripts. B. Heterologous siRNAs are capable of mediating virus silencing in *C. elegans*. Transgenic N2 strain carrying the Psur-5::GFP and the FR1fp nuclear transgenes was used in this test. Shown here is the accumulation of FR1fp transcripts in response to heat induction after the worms have been fed on OP50 food or HT115 food expressing *gfp* dsRNA. Methylene blue stained ribosomal RNA serves as equal loading control. C. Accumulation of FR1fp transcripts in response to heat induction in worm strains as indicated. All worm strains used in this test contain the same Psur-5::GFP and FR1fp transgenes as described in B and have been fed on HT115 food expressing the *gfp* dsRNA. The FR1fp transcripts were detected using probes derived from the FP region of FR1fp. The GFP transcripts were detected using probes corresponding to the 5’ half of GFP coding sequence that does not overlap with the FP region of FR1fp.

6.8 Conclusions

Based on the DRH-1 function rescue assay, here I show that ectopic expression of wild type DRH-1 suppresses both Flock house virus and Orsay virus replication in *drh-1* mutant (tm1329) background. The antiviral function of DRH-1 requires the homologous RIG-I domains
as well as its worm-specific N-terminal domain (NTD). I also showed that the helicase and C-terminal domains encoded by either worm DRH-2 or human RIG-I can functionally replace the corresponding domains of DRH-1 to mediate antiviral RNAi in *C. elegans*. Notably, substitutions in a three-residue motif (KWK) of the C-terminal regulatory domain (CTD) of RIG-I that physically interacts with viral dsRNA abolish the antiviral activity of CTDs of both RIG-I and DRH-1 in *C. elegans*. Genetic analysis revealed an essential role for both DRH-1 and DRH-3 in *C. elegans* antiviral RNAi targeting a natural viral pathogen. However, Northern blot and small RNA deep sequencing analyses indicate that DRH-1 acts to enhance production of viral primary siRNAs whereas DRH-3 regulates antiviral RNAi by participating in the biogenesis of secondary siRNAs after Dicer-dependent production of primary siRNAs. Based on these observations, I propose that DRH-1 facilitates the acquisition of viral dsRNA by the worm dicing complex for the subsequent processing into primary siRNAs. The strong parallel for the antiviral function of RLHs in worms and mammals suggests that detection of viral dsRNA may activate completely unrelated effector mechanisms, or alternatively, that the mammalian RLHs have a conserved activity to stimulate production of viral siRNAs for antiviral immunity by an RNAi effector mechanism.
CHAPTER 7 DISCUSSION

7.1 Viral suppression of RDVI in C. elegans

RDVI represents a major antiviral mechanism in fungi, plants and insects (20). To survive, many viruses produce diverse classes and small proteins, termed VSRs, to suppress RDVI through distinct mechanisms (34, 112). Since VSRs can target and suppress RNAi directed by endogenous siRNAs and miRNAs, studies on the VSR-mediated virus-host interactions have not only significantly improved our understanding of the evolutionary arm race between viruses and their natural hosts but also allowed us to gain insight into the mechanistic basis underlying disease induction by virus infection in the aforementioned systems (34, 42, 47). The nematode worm C. elegans has recently emerged as an important animal model for the study of virus-host interaction in single-Dicer invertebrates. So far, the study on VSR-mediated virus-nematode host interaction, especially the interaction that leads to disease symptoms, is largely an unexplored field mainly owing to the fact that a robust RDVI suppression assay has yet to be developed for the identification of VSRs with worm RDVI suppression activity. Here we reported a worm RDVI suppression assay system developed using FHV B2 as a reference VSR. Because a viral replicon is used as both trigger and target of RDVI, our assay system is expected to identify VSRs with true function in RDVI suppression. Using this assay we have successfully identified NoV B2, which shares limited sequence homology with FHV B2 but uses similar mechanism in RDVI suppression, as another VSR that retains RDVI suppression activity in the worm system, confirming the robustness of our assay system. Thus, our assay system for the first time makes it possible to identify VSRs with worm RDVI suppression activity. It can be expected that functional and mechanistic characterization of VSRs identified using our assay will help unravel some unique features of VSR-mediated virus-nematode worm interactions.
Unlike plants and insects, the nematode worm *C. elegans* uses a single Dicer to initiate both RDVI and other RNAi-related pathways. Besides, the worm RDVI pathway features some unique components, such as RSD-2 and DRH-1 (62), and is known to require RRF-1, an RdRP that produces 22 nt single-stranded secondary siRNAs in a Dicer-independent manner (62, 101, 102, 116). These observations make it interesting to ask whether the worm RDVI responds differently to VSRs identified in heterologous systems. To address this question we assayed the RDVI suppression activity for VSRs encoded by fungus, plant and insect viruses in *C. elegans*. Our results clearly showed that, FHV B2 and NoV B2, but not the p19 or 2b proteins encoded by plant viruses or the p29 protein encoded fungus virus, suppresses RDVI triggered by replicating viruses (Figure 3.1, 3.2, 3.3, 3.4 and data not shown). Interestingly, unlike that in plants and insects, FHV B2 appears to target a step downstream of primary siRNA, but upstream of secondary siRNA, biogenesis to suppress RNAi (Figure 3.5 panel A & D). These results together not only confirmed that worm RDVI indeed responds differently to heterologous VSRs but also shed light on some unique mechanistic features of worm RDVI as discussed below.

Probably due to a siRNA degradation mechanism (137) the abundance of virus-derived siRNAs is extremely low in *C. elegans*. Using a newly developed Northern blotting protocol (105) we managed to detect virus-derived siRNAs at an unprecedented resolution. Consistent with previous deep sequencing analysis (115), our Northern blotting analyses detected several viral siRNA bands, with the major one detected with a size of 23-nt, in *rde-1* mutants (Figure 3.5 panel A). We believed that these viral siRNAs are bona fide primary siRNA duplexes produced by the worm Dicer, considering the facts that the primary siRNAs produced by worm Dicer are predominantly 23-nt in size (92), *rde-1* mutants are known to accumulate only primary siRNAs (103), and the detected viral siRNAs are resistant to Terminator exonuclease, which destroys
single-stranded RNA molecules with 5’ end monophosphate group, such as miRNAs, but is much less efficient in digesting siRNA duplexes (Figure 3.5 panel D). Our Northern blotting analyses also detected an \textit{rrf-1}-dependent siRNA band with a size falling between 21 and 22nt (Figure 3.5 panel A & C). The unique migration pattern of these \textit{rrf-1}-dependent siRNAs may simply reflect the fact that, although 22 nt in size (101, 102, 117), \textit{rrf-1}-dependent siRNAs carry a triphosphate, instead of a monophosphate, group at the 5’ end and thus are expected to migrate faster than the 22nt primary siRNAs which carry monophosphate group at the 5’ end.

\textbf{FHV B2 is a versatile VSR that suppresses RNAi in diverse organisms (7, 12, 43).} Previous biochemical and structural studies suggested that FHV B2 forms homodimers and binds to dsRNA without length preference (9, 38, 39). These observations suggested that FHV B2 could have dual modes of action in RDVI suppression: inhibiting Dicer-processing of dsRNAs or interfering with the function of siRNAs (38). Previously, it has been shown that the major mode of action of B2 in plants and insects is to suppress the biogenesis of siRNAs (7, 138). Currently, it remains unclear whether FHV B2 actively suppresses RDVI by inhibiting the function of siRNAs. Here we show that FR1gfp-derived siRNAs in worms expressing FHV B2 can be detected at a comparable amount and with a similar pattern as that in \textit{rde-1} mutants (Figure 5 panel A). Since \textit{rde-1} mutant is known to be defective in the biogenesis, but not the function, of primary siRNAs (103), this observation suggests that the major mechanism of FHV B2 in worm RDVI suppression is to inhibit the function, thus the biogenesis of secondary siRNAs, of primary siRNAs. In supporting this notion, the \textit{rrf-1}-dependent secondary siRNAs became undetectable in worms expressing FHV B2 (Figure 3.5 panel A & B). Worm RDVI pathway contains some unique components such as DRH-1. Considering the fact that the mammalian counterparts of DRH-1 function as cytosolic virus sensors, it is possible that DRH-1 functions as a virus sensor.
to facilitate the viral dsRNA acquisition by worm Dicer. As a result, the biogenesis of viral primary siRNAs can be significantly enhanced even in the presence of FHV B2. However, since FHV B2 can inhibit the function of primary siRNAs and the biogenesis of secondary siRNAs, the targeted viruses will still be able to replicate efficiently in the presence of FHV B2 as revealed in this study.

TBSV p19 is a well characterized VSR of plant origin that can suppress RNAi in heterologous systems such as insects and mammals (12, 41). Previous studies suggested that both TBSV p19 and TAV 2b specifically bind and inhibit the function of 21 nt siRNAs to suppress RNAi. Although siRNA duplexes of other sizes can also be bound by these two VSRs, the binding affinity diminishes rapidly with increasing size differences (35-37). In fact, because of its target specificity TBSV p19 has been used as a universal RNAi suppressor to explore the molecular mechanism of 21 nt siRNAs and miRNAs (31, 41). Here we show that, although successfully expressed in C. elegans, TBSV p19 failed to suppress long dsRNA triggered RNAi, RDVI triggered by two unrelated viruses and the biogenesis of RRF-1-dependent secondary siRNAs. This finding suggests that virus-derived 21-nt primary siRNAs, do not make major contribution to RDVI in C. elegans (Figure 3.5 panel A&C).

In plants, VSRs can interfere with the function of miRNAs, which usually form near-perfect sequence matches with their passenger strands, resulting in developmental defects or diseases (44, 47). Animal miRNAs and their passenger strands often do not form near-perfect sequence matches thus have been shown to be resistant to the inhibitory effect of VSRs produced in Drosophila (42, 43). Currently, it remains unclear whether VSRs are able to interfere with the biogenesis and/or function of miRNAs in the nematode kingdom which uses a single Dicer to initiate both siRNA and miRNA pathways. In this report we show that FHV B2 is unable to
suppress the biogenesis and function of miRNAs in *C. elegans* (Figure 3.5&3.6). This is the first demonstration that VSRs selectively suppress siRNA, but not miRNA, function in organisms that use single Dicer to produce both siRNAs and miRNAs.

Previously, endogenous siRNAs that function in transposon control in *Drosophila* have been shown to be susceptible to the inhibitory effect of VSRs (42). Recently, worm endogenous siRNAs have been shown to contribute to normal cellular function by maintaining wide-spread gene silencing together with piRNAs, a class of endogenous small RNAs whose biogenesis does not require Dicer (139). Thus, it was expected that functional inhibition of these endogenous siRNAs by nodavirus B2 proteins, which appears to inhibit the function of virus-derived siRNAs as shown in Figure 3.5, will induce developmental defects in worms. So far, we have not observed any developmental defects associated with constitutive expression of the B2 VSRs. However, it remains possible that the inhibitory effect of the B2 proteins on worm endogenous siRNAs takes much longer time to develop or needs a specific bioassay to identify. Alternatively, strong inhibition of endogenous siRNA function may have resulted in lethal embryos and, as a result, only transgenic animals expressing the B2 proteins at low level can survive. In supporting this hypothesis, transgenic lines carrying constitutively expressed FHV B2 transgene showed weaker Fr1 gfp replication rescue compared to those carrying heat inducible FHV B2 transgene (Figure 3.2 panel D). Nevertheless, our study suggested that some heterologous VSRs, such as the FHV B2, can retain their functional specificity in the worm system, making it possible to use these VSRs as genetic tools to study the biogenesis and function of worm endogenous siRNAs.

### 7.2 Virus induced gene silencing in *C. elegans*

Viruses are obligate intracellular parasites that absolutely rely on the macromolecule synthesis and metabolism pathways of their hosts for replication. Thus, virus-host interaction represents one of the most intimate pathogen-host interactions that are tightly regulated by
various cellular pathways. RDVI is mediated by one such cellular pathway that is conserved in fungi, plants and invertebrates. Previous studies have demonstrated that it is the viRNAs processed from the invading viral genomes, in the form of dsRNA, that confer the target specificity of RDVI (20). Owing to its sequence-specific nature, RDVI can be redirected to target cellular transcripts in plants and in some cases accounts for the induction of plant diseases (57, 58). Currently, it remains unclear whether viRNAs can guide the silencing of cellular transcripts thereby to mediate another layer of virus-host interaction in the animal kingdom. In this report, we probed the possibility of VIGS in the nematode worm C. elegans. Our results clearly showed that viRNAs can mediate potent silencing of homologous cellular genes, endogenous gene and transgene (Figure 4.2 and Figure 4.5). Thus, for the first time, we demonstrated that viRNAs can modulate host gene expression in the animal kingdom. Most importantly, the success in uncoupling the RDVI trigger and the RDVI target will not only allow us to revisit the virus-animal host interaction from a new perspective but also facilitate our exploration of the unique features of worm RDVI.

In plants, VIGS targeting the promoter sequence of a gfp transgene resulted in both methylation of the targeted sequence and inheritable transcriptional gene silencing that is independent of the virus trigger (140). Interestingly, VIGS targeting the coding region of the same gfp transgene resulted in sequence-specific gene silencing and DNA methylation that was not inherited. This is in sharp contrast to the VIGS in C. elegans, in which inheritable silencing of host genes, endogenous gene and transgene, can be readily triggered when the coding sequences were targeted by VIGS. Currently, it remains unclear whether the inheritance of VIGS in worm is associated with any epigenetic modifications to the targeted sequences.
Silencing of host genes mediated by virus-derived siRNAs has been shown to be responsible for disease induction in plants (57, 58). miRNAs encoded by both DNA and RNA viruses can also modulate host gene expression, thereby facilitating virus infection in the animal kingdom (59-61). Since miRNAs and siRNAs use similar factors for their biogenesis and function, it will be of great interest to see whether viRNAs can modulate host gene expression thereby to facilitate virus infection in animal kingdom. Since viRNAs can target and induce transgenerational silencing in *C. elegans* (Figure 4.4&4.5), our study, for the first time, made it possible to test this hypothesis in *C. elegans*. The fact that RNA silencing in *C. elegans* can readily target and down-regulate genes required for RNA silencing makes the genes involved in RNA silencing ideal candidates for this test. The *drh-1* gene will be of particular interest in this regard since *drh-1* is known to play an essential role in RDVI but appears dispensable in RNA silencing targeting cellular transcripts.

DRH-1 as key component of RDVI appears to be unique to the nematode worm. Thus, function and mechanism study of DRH-1 may help unravel some unique features of worm RDVI. The fact that DRH-1 selectively mediates the silencing of invading viral RNAs suggests a hypothesis that DRH-1 is a RIG-I function analog that senses and mediates the silencing of invading viral RNAs. However, currently it remains possible that DRH-1 specifically mediates viRNA-guided silencing irrespective of the origin of silencing targets. To rule out this possibility, one will need to present both viral transcripts and homologous cellular transcripts to the same set of viRNAs to see whether DRH-1 selectively mediates the silencing of viral RNAs but not the cellular transcripts. Now, with the success in uncoupling the RDVI trigger and the RDVI target, we can have a straightforward test on this hypothesis by performing VIGS in *drh-1* null mutants.
We reasoned that if DRH-1 selectively mediates virus silencing, VIGS should occur in a DRH-1-independent manner.

In plants, RDVI features a systemic signal that is believed to prime an antiviral status prior to virus arrival (28). Two recent reports further suggested that the 21 nt siRNAs serve as the physical carrier of the systemic signal in plants (30, 31). Systemic antiviral silencing also occurs in insects although the mechanism involved differs (32). Previous studies have demonstrated that the systemic gene silencing can be triggered by artificial dsRNAs in *C. elegans*. Currently, it remains an open question whether RDVI also features a systemic antiviral signal in *C. elegans* which are known to share RdRP with plants. Although it is not appropriate to use our current VIGS setup to address this question, mainly due to the fact that the heat inducible promoter used to initiate FHV replication can be activated in most of somatic tissues, a modified version of our setup may help address this question. For example, if we produce the virus trigger and the *gfp* target in distinct tissues, we would be able to tell whether RDVI in *C. elegans* involves a systemic signal based on the status of the *gfp* silencing in response to viral replication.

In *C. elegans*, exactly how viral RNA targets are sliced by AGO-containing complexes remains largely unknown. The large number of *C. elegans* AGO proteins makes the dissection of the mechanisms involved even more challenging. The dilemma in studying the slicing mechanism of RDVI resides in the fact that the slicing of the viral targets can not be uncoupled from the dicing of the RDVI trigger and, as such, it is impossible to evaluate respective contributions to the destruction of invading viral RNAs. Apparently, our success in testing VIGS will facilitate the study on mechanisms involved in viral RNA slicing in that the dicing of the trigger and the slicing of the target are completely uncoupled in VIGS and, as such, the accumulation of the VIGS target is solely affected by the slicing mechanism. Moreover, since the
slicing of the VIGS target is mediated by both primary and secondary viRNAs (Figure 4.2 panel B&C), our study on the slicing mechanisms will be further facilitated by the fact that, in \textit{rrf-1} knockouts, the slicing mediated by primary viRNAs can be further uncoupled from the slicing by secondary viRNAs. C04F12.1 is another \textit{C. elegans} AGO protein that contributes to RDVI and, like RDE-1, features the key catalytic residues of RNase H (62, 103). Currently, it remains unclear whether C04F12.1 recruits primary viRNAs for viral target cleavage. Now, with the success in testing VIGS in \textit{C. elegans}, this question can be easily addressed by assaying the efficiency of VIGS in double mutant corresponding to \textit{C04F12.1;rrf-1}.

For currently unknown reason, irrespective of the worm genetic backgrounds, FHV RNA1 and its derivatives replicate less efficiently in worm larvae compared to that in adult worms. As a result, the VIGS phenotypes observed in this report were less prominent in worms developed from heat induced larvae. This may have also prevented us from observing more pronounced VIGS phenotypes that take longer time to develop.

Orsay virus naturally infects \textit{C. elegans} and exhibits enhanced replication only in mutant worms defective in RDVI or in the presence of a functional RNA silencing suppressor (50), indicating that the current Orsay virus isolate may be a natural mutant deficient in RNA silencing suppression. If proved true, this Orsay virus isolate together with its RNA2 based replicon may serve us well as an ideal VIGS trigger in \textit{C. elegans}. As shown in this report, the RNA2 of FHV can be modified to function as a trigger of VIGS without compromising the viral replication. Very likely, the same strategy can be used for developing an Orsay virus RNA2 based replicon as the trigger of VIGS. The genomic RNA2 of Orsay virus contains two ORFs. One of the ORFs encodes the viral coat protein whereas the other encodes a putative protein, named delta protein, with unknown function (50). It is likely that one of the ORFs can be replaced with foreign
sequence for VIGS. It can be expected that an Orsay virus based trigger will allow for prolonged observations of VIGS phenotypes since Orsay virus infects worm larvae and, unlike FHV whose optimal replication temperature is above 25°C, replicates efficiently at room temperature.

7.3 RSD-2 and RDE-4-independent RDVI in C. elegans

rsd-2 was first identified as a gene that enables RNAi spreading from soma to germline (64). A role of rsd-2 in RDVI was then revealed in a genetic screening that utilized RNAi mediated gene knockdown to phenocopy genetic mutants (62). Recently, a genetic study further suggested that rsd-2 helps maintain normal chromosomal function, such as transposon control, under unfavorable conditions (67). Currently, how rsd-2 contributes to those biological processes remains poorly understood. rsd-2 is unique to nematode worms, such as C. elegans which are known to encode single Dicer required for the biogenesis of both siRNAs and miRNAs. Thus, functional and mechanistic study of rsd-2 is expected to not only shed light on mechanism whereby rsd-2 helps maintain chromosomal integrity in response to environmental stresses but also reveal the unique aspects of RDVI in single-Dicer invertebrates. Here we show that rsd-2 functions in two parallel genetic pathways that mediate antiviral silencing in rde-4-dependent and rde-4-independent manner respectively. In Arabidopsis, dsRNA binding protein regulates RDVI by facilitating the processing of viral dsRNA into siRNAs by one of the four Dicers (23, 24). In Drosophila, the RDE-4 homologue R2D2 contributes to the function of viRNAs produced by one of the two Drosophila Dicers (95, 96, 141). Therefore, by demonstrating the rde-4-independent mechanism for primary viRNA biogenesis, our study, suggested that RDVI in single-dicer invertebrates can be initiated in the absence of a dsRNA binding protein.

Previously, it has been shown that dsRNA of high concentration triggers RNAi in C. elegans in the absence of RDE-4 (71). This observation suggests that viruses, as powerful replicators that
rapidly produce large amount of dsRNA replication intermediates, may trigger RDVI in the absence of RDE-4. Here we show that, with an increase in viral replication level, primary viRNAs were detected at high level in rde-4 mutants containing either rsd-2 or rrf-1 null allele, suggesting an rde-4-independent mechanism for the generation of primary viRNAs (Figure 5.2&5.4). Apparently such an increase in viral replication and viRNA accumulation can not be attributed to residual rde-4 function in the rde-4 allele used in this study since FR1gfp-derived siRNAs were not detected in rde-4 single or drh-1;rde-4 double mutants under the same condition (62) (Figure 5.1 panel E, Figure 5.2 panel A, Figure 5.4 panel C and Figure 5.5 panel B). Thus, by demonstrating the existence of an rde-4-independent pathway for antiviral silencing, we explained why dsRNAs of high levels triggers RNAi in the absence of RDE-4 in C. elegans (71). Notably, although readily detectable in worms, such as rde-1, rrf-1 and rsd-2 mutants, that are defective in secondary viRNA biogenesis, virus-derived primary siRNAs were hardly detectable in wild type N2 worms or worms defective in rde-4 (Figure 5.1 panel E, 5.2 panel A, 5.3 panel C&5.4 panel C ). This may simply reflect the fact that antiviral silencing in both rde-4-dependent and rde-4-independent mechanisms is amplified by the secondary viRNAs and, as a result, much less viral dsRNAs will be processed into primary viRNAs by Dicer in the presence of secondary viRNAs.

Notably, our observations suggested that rde-1 also plays a role in rde-4-independent RDVI like rsd-2 does (Figure 5.5). Interestingly, an increase in FHV replication level was observed in rde-1;rsd-2 double mutants compared to rde-1 single mutants. Probably, rde-1 plays a major role in rde-4-dependent but a minor role in rde-4-independent RDVI and as a result, antiviral silencing in rde-1 mutants is further compromised in the presence of an rsd-2 null allele. Nevertheless, by analyzing the accumulation of viral transcripts and primary viRNAs in various
single and double worm mutants defective in RDVI in our study, for the first time, demonstrated that, in addition to the conventional rde-4-dependent pathway, there is an rde-4-independent pathway for antiviral silencing in C. elegans (Figure 5.7). Previously, we have shown that another RDE-1-related worm AGO, termed C04F12.1, plays a role in RDVI. It would be interesting to test whether C04F12.1 plays a role in rde-4-independent RDVI. C. elegans is known to encode a large number of AGO proteins. Thus, testing the rde-4 dependence of C04F12.1 may allow us to address the question whether different worm AGO proteins specifically function in mechanistically distinct RDVI pathways in C. elegans.

rsd-2 is known to be required for the accumulation of secondary, but not primary, endogenous siRNAs (66). Currently, it remains unclear whether rsd-2 contributes to RDVI through similar mechanism. Here we show that FR1gfp-derived primary siRNAs accumulated in rsd-2 with a pattern similar to that in rrf-1 or rde-1 single mutants which are known to accumulate primary viRNAs (Figure 5.1 panel E, 5.2 panel A&5.3 panel C). This finding suggests that, similar to that in endogenous RNAi, rsd-2 enhances cell autonomous antiviral silencing by contributing to the accumulation of secondary, but not primary, viRNAs. This finding explained why rsd-2 mutants are resistant to low dosage but sensitive to high dosage of dsRNAs (66). Presumably, when the trigger dsRNAs are present at high concentration sufficient primary siRNAs will be produced to trigger efficient RNAi in the absence of secondary siRNAs. However, when the trigger dsRNAs are introduced at low level, efficient RNAi will require those dosage-sensitive RNAi factors, such as RSD-2, RDE-10 and RDE-11, which amplify RNAi by promoting the production/accumulation of secondary siRNAs (66). Notably, the replication of both FHV and Orsay virus was further enhanced in rsd-2 mutants compared to that in rrf-1 mutants, suggesting that rsd-2 may also contribute to the function of primary viRNAs. RSD-2
contains 3 tandem domains of unknown function at the N-terminus. Functional characterization of these domains may yield further insight into the mechanistic basis of RSD-2 in RDVI.

Like that in plants, RNAi in *C. elegans* involves an intercellular signal that is responsible for systemic spread of RNAi (1, 142, 143). A recent study further suggested that long dsRNAs or their processed intermediates may be the physical carrier of this intercellular signal (70). Consistent with this finding, systemic spreading of the intercellular signal requires the transmembrane protein SID-1 that transports dsRNAs without a size preference and thus may facilitate the intercellular transportation of the silencing signal (65, 68, 130). Previously, it has been shown that SID-1 is dispensable for RDVI triggered by artificial viruses, such as vesicular stomatitis virus and FHV (19, 105). Currently, whether SID-1 contributes to RDVI under natural conditions remains largely unknown. To address this question, we compared Orsay virus infection between wild type N2 worms and *sid-1* mutants and found that Orsay virus infection was not further enhanced in the *sid-1* mutants (Figure 5.6). Probably, worm RDVI involves an intercellular silencing signal that is transported through a SID-1-independent mechanism. Consistent with this hypothesis, SID-1 was shown to be dispensable for the export of the mobile silencing signal in *C. elegans* (144). Alternatively, SID-1 as a key component of systemic RNAi may be active in non-intestine tissues and as a result, its role in systemic RDVI can not be identified using Orsay virus which mainly targets the intestine cells for replication (50). Moreover, probably due to the lack of an RDVI suppression activity, Orsay virus is known to be highly sensitive to RDVI (104). It is thus possible that the replication of Orsay virus in *sid-1* mutants is suppressed by intracellular antiviral silencing, making the intercellular antiviral silencing redundant in keeping this particular virus under control.
Unlike RDE-4 which plays an important role in the biogenesis of endogenous siRNAs, DRH-1 appears to be a dedicated factor of worm RDVI (62, 94). DRH-2 shares significant sequence homology with DRH-1 but appears to negatively regulate worm RDVI (62). These observations together raised an interesting question whether DRH-2 specifically targets and regulates the function of DRH-1 thereby to negatively regulate the *rde-4*-dependent RDVI. Apparently, by addressing this question we will be able to find out whether worms have evolved such a mechanism that would allow for specific regulation of RDVI without affecting the normal cellular function under unfavorable environments.

### 7.4 RIG-I-like RNA helicases and antiviral innate immunity

It is intriguing that, as an essential component of the nematode antiviral RNAi, DRH-1 encodes the helicase and C-terminal domains homologous to mammalian RLHs, since RLHs initiate the IFN-dependent antiviral immunity that is absent in *C. elegans* (62). In this study, we developed an assay to dissect the domain requirement of DRH-1 in antiviral RNAi against both the FHV replicon and Orsay virus by transgenic expression of the wild type and mutant forms of DRH-1 in animals carrying a loss-of-function allele of *drh-1*. Our results show that the antiviral activity of DRH-1 requires its helicase domain and CTD as well as the worm-specific NTD. We also demonstrate that the homologous helicase and CTD domains encoded by either the worm DRH-2 or human RIG-I can functionally replace the corresponding domains of DRH-1 to mediate antiviral RNAi in *C. elegans*. Strikingly, three amino acid substitutions in the KWK motif predicted to prevent the physical interaction of RIG-I with viral dsRNA abolished the antiviral activity of the CTDs of both RIG-I and DRH-1 in *C. elegans*. These findings strongly suggest that antiviral RNAi in *C. elegans* requires an activity of DRH-1 to detect viral dsRNA known to be essential for the virus sensing by RIG-I.
Available data illustrates an identical genetic requirement for antiviral RNAi triggered by either the replication of FHV or the infection of Orsay virus (8, 9, 19, 50, 62). This is probably because induction of nematode antiviral RNAi requires the recognition and processing (into siRNAs) of dsRNA produced during RNA virus replication and Orsay virus is closely related to FHV (50). Consistently, we found in this study that DRH-1 identified in a feeding RNAi screen based on the FHV replicon-induced antiviral RNAi is also necessary for the nematode defense against Orsay virus infection. Moreover, we show that antiviral RNAi induced by both the FHV replicon and Orsay virus requires DRH-3. We note that DRH-3 is known to participate in exogenous and endogenous RNAi of \textit{C. elegans} (134, 136), in contrast to DRH-1 that is largely dispensable for RNAi targeting cellular transcripts whether or not the silencing siRNAs are processed from a replicating viral RNA or an exogenous long dsRNA. Using an improved protocol for Northern detection of small RNAs (105), we found that the 23-nt primary siRNAs targeting the FHV replicon reproducibly accumulated to higher levels in \textit{drh-3} mutants than in \textit{drh-1} mutants although both mutants supported similar replication levels of the replicon. Deep sequencing further indicated that the abundant viral siRNAs produced in \textit{drh-3} mutants did not include a population of secondary siRNAs. These observations together indicate that DRH-3 regulates RNAi targeting both cellular transcripts and viruses by participating in the biogenesis of secondary siRNAs after Dicer-dependent production of primary siRNA biogenesis, as proposed previously for its role in endogenous RNAi (136). By contrast, DRH-1 may act to specifically enhance production of viral primary siRNAs by facilitating the recruitment of the heterodimer DCR-1/RDE-4 to the viral dsRNA bound by DRH-1 for the subsequent processing into primary siRNAs by DCR-1. Our model is consistent with a previous study that detected
physical interaction of RDE-4 with DRH-1, but not with DRH-3 (75), and would also explain why the function of DRH-1 is essential only for RNAi against virus infection.

Viral RNA replication occurs in discrete subcellular compartments such as the spherules on the outer membrane of mitochondria shown for FHV (145). Virus dsRNA replicative intermediates are also found in complexes with viral replicase and host co-factor proteins. These physical barriers may prevent DCR-1/RDE-4 complex from gaining access to viral dsRNA. DRH-1-mediated detection of the unique viral dsRNA for the production of primary siRNAs may be particularly important to ensure effective RNAi against the replicating viral RNAs in *C. elegans*, which encodes only one Dicer, unlike multi-Dicer plant and insect hosts that process distinct siRNA and miRNA precursors with dedicated Dicer proteins (81). However, production of primary siRNAs in the absence of DRH-1 may still be sufficient for RNAi to target the non-replicating cellular transcripts.

Rapid progress has been made to understand the interferon-regulated effector mechanism of the mammalian antiviral immunity initiated by RIG-I, MDA5 and LGP2 in the cytosol (146). Our study provides an interesting parallel for a three-member family of RLHs to regulate antiviral defense in *C. elegans* using RNAi as the effector mechanism. Analogous to the role of the N-terminal CARDs of RIG-I in the downstream immune signaling (146), the interaction of DRH-1 with RDE-4 may involve the worm-specific NTD of DRH-1, which is critical for the use of RNAi as the effector mechanism. Therefore, detection of viral dsRNA by related RLHs in nematodes and mammals activates unrelated effector mechanisms, illustrating functional diversification of RLHs during evolution via acquisition of specific N-terminal signaling domains. An alternative model is that the mammalian RLHs have a conserved activity to recruit
DCR-1 for the production of viral siRNAs to activate antiviral immunity by an RNAi effector mechanism.

7.5 Finale

Virus-host interaction represents one of the most intimate pathogen-host interactions in that viruses are intracellular parasites that obligate rely on host for reproduction. To suppress viral infection, cellular organisms have evolved a wide spectrum of cytosolic antiviral mechanisms. RNAi is such an antiviral mechanism that has shaped many aspects of virus-host interaction, including virus clearance and viral pathogenesis. To date, exactly how antiviral RNAi protect single-Dicer organisms from virus attack remains largely unknown. My study described in this thesis represents the very first attempt that aims to address this question. Findings from my study suggest that 23-nt siRNAs play a major role in antiviral silencing and host gene silencing in the single-dicer organism *C. elegans*. It was also clear from my study that some worm-specific genes allow for antiviral RNAi to be initiated in the absence of dsRNA binding proteins, which is in sharp contrast to that in plants and insects which are known to produce more than one Dicer protein. My study further suggests that RIG-I-like RNA helicases as virus sensors are conserved in mechanistically unrelated antiviral immune responses. Thus, my research efforts not only revealed some unique features of antiviral RNAi in single-dicer organisms but also developed *C. elegans* as a powerful animal model for the study of many aspects of virus-animal host interaction, including cytosolic virus detection by RIG-I-like RNA helicases.
REFERENCES


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VITA

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