Characterization of Virus-Encoded RNA Interference Suppressors in *Caenorhabditis elegans*

Xunyang Guo, Rui Lu

Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana, USA

In fungi, plants, and invertebrates, antiviral RNA interference (RNAi) directed by virus-derived small interfering RNAs (siRNAs) represents a major antiviral defense that the invading viruses have to overcome in order to establish infection. As a counterdefense mechanism, viruses of these hosts produce diverse classes of proteins capable of suppressing the biogenesis and/or function of viral siRNAs. This RNA-directed viral immunity (RDVI) in the nematode *Caenorhabditis elegans* is known to exhibit some unique features. Currently, little is known about viral suppression of RNAi in *C. elegans*. Here, we show that ectopic expression of the B2 protein encoded by Flock House virus (FHV) suppresses RNAi induced by either long double-stranded RNA (dsRNA) or an FHV-based replicon and facilitates the natural infection of *C. elegans* by Orsay virus but is not active against RNA silencing mediated by microRNAs. We report the development of an assay for the identification of viral suppressor of RNAi (VSR) in *C. elegans* based on the suppression of a viral replicon-triggered RDVI by ectopic expression of candidate proteins. No VSR activity was detected for either of the two Orsay viral proteins proposed previously as VSRs. We detected, among the known heterologous VSRs, VSR activity for B2 of Nodamura virus but not for 2b of tomato aspermy virus, p29 of fungus-infecting hy-povirus, or p19 of tomato bushy stunt virus. We further show that, unlike that in plants and insects, FHV B2 suppresses worm RDVI mainly by interfering with the function of virus-derived primary siRNAs.

Viral suppressors of RNA silencing (VSRs) are a group of virus-encoded proteins that facilitate virus infection by suppressing the antiviral immunity mediated by RNA interference (RNAi) (1). Small interfering RNAs (siRNAs) derived from replicating viruses guide sequence-specific antiviral RNAi in fungi, plants, and invertebrates (2). Accumulating evidence suggested that most of virus-derived siRNAs are processed from viral replication intermediates, in the form of double-stranded RNAs (dsRNAs), by Dicer proteins, a class of RNase III RNases (3). siRNA-mediated silencing of invading viruses culminates with the cleavage of viral transcripts by Argonaute (AGO) proteins which recruit siRNAs as a sequence guide for target RNA selection and slice the matching RNA molecules with their RNase H-like activity (2). In plants and the nematode worm *Caenorhabditis elegans*, RNA-dependent RNA polymerases (RdRPs) are essential for this RNA-directed viral immunity (RDVI) by amplifying siRNAs (4–6).

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. Therefore, one of the major strategies for the targeted viruses to establish successful infection is to produce VSRs. VSRs are diverse, in term of sequence and structure, classes of proteins encoded by fungus, plant, and animal viruses with DNA or RNA genome and target viral or host factors to suppress the biogenesis and/or function of siRNAs (7). 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, whereas 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 (8–14). 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 (15–17). In plants, VSRs are capable of interfering with the function of microRNAs (miRNAs), a class of endogenous small noncoding RNAs with important

functions in development and stress response (18–20). miRNAs are encoded by nuclear 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 (21, 22).

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 (1). The early assays surveyed suppression of transgene RNA silencing in plants by a candidate viral protein expressed from either an infectious recombinant virus or a transgene (23–25). RNAi targeting a cellular gene induced by synthetic long dsRNAs or siRNAs in animal cells was similarly used for the identification of VSRs (17). Subsequently, experimental systems were developed to directly assay for the suppression of RNA silencing that is induced by and specifically targets a replicating virus in a host cell (26). Development of these experimental systems was made possible by the detailed characterization of mutant viruses or virus-based replicons from which a cognate VSR was deleted. These VSR-deficient viruses and replicons exhibit no defect in replication but accumulate to only low levels in host cells because of robust and specific antiviral RNAi induced by virus replication. However, abundant accumulation of VSR-deficient viruses is restored in host cells defective in RDVI. Therefore, a candidate viral protein is identified as a VSR when its expression rescues the accumulation of a VSR-deficient mutant virus or replicon after replication in a host cell.

C. elegans has recently emerged as an important animal model

Received 22 January 2013 Accepted 25 February 2013

Published ahead of print 6 March 2013

Address correspondence to Rui Lu, rui.lu@lsu.edu.

Copyright © 2013, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JVI.00148-13

for the study of virus-host interaction, especially the antiviral immunity, in single-Dicer invertebrates (11, 27–31). RDVI in *C. elegans* exhibits several distinct features. Current studies on *C. elegans* RDVI suggest that the worm RDVI is initiated upon the processing of viral dsRNAs into primary siRNAs by the single worm Dicer, DCR-1, with the aid of a dsRNA binding protein termed RDE-4 (30–32). Subsequently, RDE-1, an AGO protein, recruits primary siRNAs as sequence reference for the target viral transcript selection (33–35). As found in plants, the worm RDVI also requires an RdRP termed RRF-1 (27, 30, 32). However, unlike the plant RdRPs that produce secondary siRNAs with the help from Dicer, RRF-1 functions downstream of RDE-1 and directs unprimed synthesis of 22-nt single-stranded siRNAs with triphosphate group at the 5' end in a Dicer-independent manner (36–38). In addition to AGO and RdRP proteins, the worm RDVI also requires some components, such as RSD-2 and DRH-1, that are not conserved in plants or insects. RSD-2 is a novel protein known to contribute to chromosomal functions probably through facilitating the accumulation of secondary siRNAs (39, 40). DRH-1 is a putative DEAD box RNA helicase that shares significant sequence homology with RIG-I, a mammalian cytosolic virus sensor in interferon-mediated antiviral immunity (32, 41). Interestingly, DRH-1 appears to be a dedicated factor of RDVI in *C. elegans* since RNA silencing targeting cellular transcripts occurs in a DRH-1-independent manner (32). Besides, worm RDVI seems to be negatively regulated by a mechanism that involves the degradation of siRNAs (42).

Little is known about viral suppression of RDVI in *C. elegans*. Recent studies have discovered naturally occurring virus that infects and induces RDVI in *C. elegans* (27). However, it is unknown whether the virus encodes VSR or VSR expression enhances virus infection in *C. elegans*. Previously, B2 of FHV has been shown to be required for efficient viral replication in wild-type worms but not in worm mutants defective in RDVI (11), suggesting a function for FHV B2 in worm RDVI suppression. However, it remains unknown whether ectopic expression of FHV B2 suppresses RNAi induced by synthetic dsRNA or worm RDVI induced by FHV or heterologous viruses. Here, using FHV B2 as a reference VSR, we developed a robust RDVI suppression assay that allows for the identification of VSRS with RDVI suppression activity in *C. elegans*. Using this assay, we 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 our 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, we 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, our observations suggested 21-nt primary siRNAs may not make major contribution to worm RDVI.

MATERIALS AND METHODS

Worm genetics. The Bristol isolate of *C. elegans*, N2, was used as the reference strain in the present study. Other N2-derived mutants used in the present study include *rde-1* (*ne300*) and *rrf-1* (*pk1417*). The genotype of *rde-1* worms was confirmed using *skn-1* feeding RNAi combined with genomic DNA sequencing. The genotype for *rrf-1* allele *pk1417* was identified using PCR as described previously (29). All worm strains were main-

tained using NGM plates seeded with *Escherichia coli* strain OP50 except otherwise indicated. Standard genetic cross was used to deliver various transgenes into different genetic backgrounds.

Plasmid constructs and transgenic worms. All constructs utilizing the heat-inducible promoter were developed by inserting the target gene into pPD49.83 utilizing the XmaI and SacI site. All constructs utilizing the *sur-5* promoter were developed by inserting the target gene into LR50 described previously (29). The coding sequences for TBSV p19 and TAV 2b were PCR amplified from corresponding T-DNA expression binary constructs described previously (43, 44). 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. The construct used to drive *gfp* dsRNA expression in *E. coli* was described previously (29).

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* at final concentration 40 ng/μl and injected into the gonads of target worms. Generation of corresponding chromosomal integrants and assay for viral replication were as described previously (29).

Infectious filtrate preparation and Orsay virus inoculation. Orsay virus was maintained using the JU1580 isolate at room temperature following a protocol described previously (27). To prepare Orsay virus inoculum, JU1580 worms infected with Orsay virus were washed off, using M9 buffer, from slightly starved 10-cm plates, 5 ml per plate. The virus-containing liquid was then filtered through a 0.22-μm-pore-size filter unit (Millipore), and the filtrate was used to resuspend pelleted OP50 *E. coli* for NGM plate seeding.

RNAi experiments. The *skin-1* and *gfp* feeding RNAi assay was performed using a bacterial feeding protocol described previously (45). Briefly, NGM agar plates containing 5 mM IPTG and 100 mg of carbenicillin/ml were seeded with *E. coli* HT115 expressing *skn-1* or *gfp* dsRNAs.

Assay for miRNA biogenesis/function suppression activity. Worms used in this assay contain a let-7 function reporter transgene, which contains a green fluorescent protein (GFP) coding sequence fused with the 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, and thus enhanced green fluorescence can be observed in the pharynx tissue in worms defective in miRNA biogenesis and/or function (46). 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). The PRF4 construct was coinjected to produce the roller phenotype as a visual mark for the VSR transgene. After 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.

Protein and RNA gel blot analysis. Total proteins were extracted from worms of mixed stages treated with or without heat induction and were resolved on an 8% SDS-PAGE gel, electroblotted, and subjected to protein gel blot analysis using either anti-HA (Cell Signaling Technology) or anti-actin primary antibody (Sigma-Aldrich) and goat anti-rabbit secondary antibody (Cell Signaling Technology). Total RNA extraction, small RNA enrichment, viral genomic and subgenomic RNA detection, and siRNA and miRNA detection were performed using protocols described previously (29). The detection of viral high molecular transcripts used probe derived from the full-length of *gfp* cDNA. For FR1gfp siRNA detection, the probes were prepared using 32 DNA oligonucleotides covering the entire GFP region of FR1gfp. The detection of miR-58Dna used digoxigenin (DIG)-labeled oligonucleotide ATTGCGTACTGAACGATCTCA as a probe. Four DNA oligonucleotides of 19, 21, 23, or 25 nt were detected

FIG 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, B1 coding sequence of FHV; fB2, B2 coding sequence of FHV; GFP, the coding sequence of green fluorescence 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 rRNA serves as an 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 h after virus inoculation. A 1.2-kb cDNA fragment derived from the 3' end of Orsay virus RNA1 was used to prepare probes for hybridization. Methylene blue-stained rRNA serves as an equal loading control.

using DIG-labeled cDNA oligonucleotides and, together with miR-58, served as a size reference.

Terminator treatment of small RNA samples. The terminator treatment was carried out by mixing 20 μ g of small RNA sample with 4 U of terminator exonuclease (Epicentre, Inc.) in 50- μ l reaction mix containing 1 $\times$ buffer and 1 U of RNase inhibitor. The reaction mix was then incubated at 30°C for 60 min. For both treatments, the treated small RNA samples were cleaned through extracting with phenol-chloroform and then precipitated with ethanol.

Imaging microscopy. The green and red fluorescence images were recorded using a Nikon digital camera p7000 mounted on a Nikon SMZ1500 microscope.

RESULTS

FHV B2 suppresses dsRNA-triggered RNAi and RDVI targeting a natural viral pathogen of *C. elegans*. Thus 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 (8, 26, 47). 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 (11), 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 (Fig. 1A). The B1 protein of FHV, which is translated from the same subgenomic RNA as B2, is known to be inactive in RNAi suppression (47) and thus served as control to FHV B2 in our test. As shown in Fig. 1B, 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 (27). 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 the Orsay virus infection in transgenic worms constitutively expressing FHV B2. As shown in Fig. 1C, compared to that in nontransgenic 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*.

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 GFP coding sequence in the place of B2 coding sequence (Fig. 2A), was described previously (32). 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 whether the FR1gfp-induced RDVI was suppressed and whether the 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* (26, 47). In our experimental system, two plasmid constructs were comicroinjected into the gonads of young adult worms containing the FR1gfp replicon (32), 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 (Fig. 2A). The second construct directed mcherry expression in the pharynx tissue and was used to generate a visible marker for transgene transformation. Most of the extra-chromosomal 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.

As shown in Fig. 1B, we found that all transgenic progenies carrying the HIP::fB2 extrachromosomal arrays, marked by red fluorescence 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 (47). 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

FIG 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, replicase of FHV; Rz, self-cleaving ribozyme sequence from hepatitis delta virus; UTR, 3' end untranslated sequence of the worm gene *unc-54*; Pmyo-2, 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, at 48 h after 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 at 48 h after heat induction. The total RNA was hybridized with probes prepared from full-length *gfp* cDNA. Methylene blue-stained rRNA serves as an 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).

worms using Northern blot hybridization. As shown in Fig. 2C, 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 the 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 whether 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 ex-

FIG 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 Fig. 2 for experiment details.

pressing the B1. However, in comparison, the expression of FHV B2 utilizing the heat-inducible promoter achieved a stronger rescue on FR1gfp replication (Fig. 2D).

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. 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 Fig. 2. Our gonad microinjection of plasmid constructs containing either the capsid protein or the delta protein coding sequence driven by the heat-inducible promoter (Fig. 3A) generated 17 and 13 lines of transgenic worms, respectively. However, none of these transgenic lines showed enhanced GFP fluorescence after heat induction (Fig. 3B). This result suggests that none of the two putative proteins encoded by Orsay virus RNA2 possesses worm RDVI suppression activity.

NoV B2, but not TBSV p19, TAV 2b, or hypovirus p29, suppresses RDVI in *C. elegans*. Both TBSV p19 and TAV 2b are well-characterized VSRs of plant origin that specifically bind and suppress the function of 21-nt siRNA duplexes (9, 13, 14, 17). In particular, TBSV p19 is known to be functional in heterologous systems such as insect and mammalian (15–17) and, owing to its target specificity, has been used as a genetic tool to explore the molecular mechanism of 21 siRNAs or miRNAs (17, 48). The p29 protein encoded by fungus-infecting hypovirus is another VSR known to be able to suppress RNAi in heterologous system such as plants (49). 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 Fig. 2. Our assay also included NoV B2 which, despite sharing limited sequence identity with FHV B2, adopts a mechanism similar to that of FHV B2 to suppress RNAi (12), was thus expected to retain the RDVI suppression activity in worms. Indeed, as shown in Fig. 4A and 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

FIG 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 panel A. Shown here are merged images recorded under white light, red fluorescence and green fluorescence with the same exposure 48 h 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. The upper left panel shows the structure of a heat-inducible transgene expressing TBSV p19 tagged with HA at the C terminus. The lower left panel shows Western blot detection of HA-tagged p19 produced in wild-type N2 worms and worms containing the heat-inducible transgene shown in the upper left panel. In the 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. Asterisks denote total RNA samples extracted from worms fed on *E. coli* food expressing *gfp* dsRNA. (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.

TBSV p19, TAV 2b, a p19 loss-of-function mutant termed p19m, that contains the R72G point mutation (50), or hypovirus p29. Consistent with this observation, enhanced FR1gfp replication was detected by Northern blotting only in chromosomal integrants containing the heat-inducible NoV B2 transgene (Fig. 4C).

A previous study suggested that TBSV p19 can be tagged with an hemagglutinin (HA) epitope tag to the carboxyl terminus (C terminus) without losing its RNAi suppression activity (17). 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 (Fig. 4D, upper left panel), using Western blotting. As shown in the lower left panel of Fig. 4D, the production of HA-tagged p19 can be readily detected 48 h after heat induction in a 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 (Fig. 4D, right panel). These

results thus confirmed that the failure of TBSV p19 in RDVI suppression is not a result of instability in worms.

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 Fig. 1 for details) and Orsay virus replication in wild-type N2 worms constitutively expressing each of these suppressors. As shown in Fig. 4E, 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 (Fig. 4F). 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*.

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

FIG 5 FHV B2 targets a step downstream of the primary siRNA biogenesis to suppress RDVI. (A) 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 oligonucleotides with different sizes as indicated. They were detected using DIG-labeled cDNA oligonucleotides and, together with miR-58, served as size references. The accumulation of miR-58 in each sample also served as an equal loading control. Arrows indicate the *rrf-1*-dependent siRNAs. (B) Accumulation of FR1gfp-derived siRNAs in *rde-1* mutants and worms containing heat-inducible FHV B2 or NoV B2 transgenes. (C) Accumulation of FR1gfp-derived

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 (51), 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 (Fig. 5A) (32, 35, 52). As indicated by arrows in Fig. 5A, 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 an 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 (36–38, 52), 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 (Fig. 5A and 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 (Fig. 5A and B). As shown in Fig. 5A, 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 Fig. 5C, 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 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 (Fig. 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 (Fig. 5C).

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 a monophosphate group at the 5' end but is much less efficient in digesting siRNA duplexes carrying monophosphate at the 5' end (53). As expected, our Terminator treatment destroyed

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 that contains the P41A point mutation (9). To obtain a size reference and equal loading control, the same filter membrane was reprobed 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.

the miR-58 miRNAs in all small RNA samples (compare the treated samples to the untreated fB1 and fB2 small RNA samples) (Fig. 5D). However, such a treatment generated no detectable impact to the abundance of siRNAs detected in *rde-1* mutants or worms expressing the FHV B2. This result thus suggested that the siRNAs detected in the presence of FHV B2 are bona fide primary siRNA duplexes.

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 (19, 21, 22). The *C. elegans* genome encodes a single Dicer that is required for the biogenesis of both siRNAs and miRNAs (54, 55). The fact that the accumulation of miR-58 is not affected in worms expressing FHV B2 (Fig. 5A and B) suggested that FHV B2 does not suppress the biogenesis of worm miRNAs. However, the fact that heat-inducible expression of FHV B2 exhibited stronger suppression on RDVI, compared to constitutive expression (Fig. 2D), 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 (46). 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 (Fig. 6A). 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 Fig. 6A, 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, codelivery of Pmyo-2::GFP and Pmyo-2::PFG constructs is expected to trigger *gfp* silencing in wild-type N2 worms. Indeed, as shown in Fig. 6B, 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.

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 Fig. 6C, 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*.

FIG 6 FHV B2 does not inhibit miRNA function. (A) Schematic structure of the *myo-2* promoter-based constructs. PFG, 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 coinjection 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.

DISCUSSION

RDVI represents a major antiviral mechanism in fungi, plants, and insects (2). To survive, many viruses produce diverse classes, in terms of sequence and structure, of VSRs that suppress RDVI through distinct mechanisms (1, 7). 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 (7, 15, 22). The nematode worm *C. elegans* has recently emerged as an important animal model for the study of virus-host interaction in single-Dicer invertebrates. Thus far, the study on VSR-mediated virus-nematode host interaction, especially the interaction that leads to disease development, has remained to be 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 sup-

pression, 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 interaction.

Unlike plants and insects, the nematode worm *C. elegans* uses a single Dicer to initiate both RDVI and other RNAi-related pathways. Moreover, the worm RDVI pathway features some unique components, such as RSD-2 and DRH-1 (32), and is known to require RRF-1, an RdRP that produces 22-nt single-stranded secondary siRNAs in a Dicer-independent manner (32, 37, 38, 52). 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, in addition to FHV B2, 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 (Fig. 1, 2, 3, and 4). Interestingly, however, 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 (Fig. 5A and 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 an siRNA degradation mechanism (42), the abundance of virus-derived siRNAs is extremely low in *C. elegans*. Using a newly developed Northern blotting protocol (29), we managed to detect virus-derived siRNAs at an unprecedented resolution. Consistent with previous deep-sequencing analysis (51), our Northern blotting analyses detected several viral siRNA bands, with the major one detected with a size of 23 nt, in *rde-1* mutants (Fig. 5A). 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 (56), *rde-1* mutants are known to accumulate only primary siRNAs (35), 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 (Fig. 5D). Our Northern blotting analyses also detected an *rrf-1*-dependent siRNA band with a size falling between 21 and 22 nt (Fig. 5A and C). The unique migration pattern of these *rrf-1*-dependent siRNAs may simply reflect the fact that, although 22 nt in size (36–38), *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 22-nt primary siRNAs which carry monophosphate group at the 5' end.

FHV B2 is a versatile VSR that suppresses RNAi in diverse organisms (16, 26, 47). Previous biochemical and structural studies suggested that FHV B2 forms homodimers and binds to dsRNA without a length preference (8, 10, 11). 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 (8). Previously, it has been shown that the major mode of action of B2 in plants and insects is to suppress the biogenesis of siRNAs (47, 57). 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 *rde-1* mutants (Fig. 5A). Since *rde-1* mutant is known to be defective in the biogenesis, but not the function, of primary siRNAs (35), 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 *rrf-1*-dependent secondary siRNAs became undetectable in worms expressing FHV B2 (Fig. 5A and 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 here.

TBSV p19 is a well-characterized VSR of plant origin that can suppress RNAi in heterologous systems such as insects and mammals (17, 26). 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 (9, 13, 14). 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 (17, 48). We show here 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, which, unlike the 22- and 23-nt primary siRNAs, can only be detected in some RDVI mutants (Fig. 5A and C), do not make major contribution to RDVI in *C. elegans*.

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 (19, 22). Animal miRNAs and their passenger strands often do not form near-perfect sequence matches and thus have been shown to be resistant to the inhibitory effect of VSRs produced in *Drosophila* (15, 16). 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* (Fig. 5 and 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 (15). 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 (58). 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 Fig. 5, will induce developmental defects in worms. Thus 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 FR1gfp replication rescue compared to those carrying heat-inducible FHV B2 transgene (Fig. 2D). 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.

ACKNOWLEDGMENTS

We thank the *Caenorhabditis* Genetics Center for some of the worm strains used in this study. We also thank E. Jorgensen for the Pmyo-2::mcherry construct, M. Félix, E. Miska, and D. Wang for the JU1580 worm and the Orsay virus, and D. Nuss for the p29 clone.

This study was supported by the Board of Regents, Louisiana [LEQSF(2011-14)-RD-A-09 and LEQSF-EPS(2012)-PFUND-277], and by the National Institutes of Health (1R03AI092159-01A1).

REFERENCES

- Li F, Ding SW. 2006. Virus counterdefense: diverse strategies for evading the RNA-silencing immunity. *Annu. Rev. Microbiol.* 60:503–531.
- Ding SW, Voinnet O. 2007. Antiviral immunity directed by small RNAs. *Cell* 130:413–426.
- Roghih Aliyari S-WD. 2009. RNA-based viral immunity initiated by the Dicer family of host immune receptors. *Immunol. Rev.* 227:176–188.
- Dalmay T, Hamilton A, Rudd S, Angell S, Baulcombe DC. 2000. An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. *Cell* 101:543–553.
- Garcia-Ruiz H, Takeda A, Chapman EJ, Sullivan CM, Fahlgren N, Bremel KJ, Carrington JC. 2010. *Arabidopsis* RNA-dependent RNA polymerases and dicer-like proteins in antiviral defense and small interfering RNA biogenesis during turnip mosaic virus infection. *Plant Cell* 22:481–496.
- Wang X-B, Wu Q, Ito T, Cillo F, Li W-X, Chen X, Yu J-L, Ding S-W. 2010. RNAi-mediated viral immunity requires amplification of virus-derived siRNAs in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U. S. A.* 107:484–489.
- Wu Q, Wang X, Ding S-W. 2010. Viral suppressors of RNA-based viral immunity: host targets. *Cell Host Microbe* 8:12–15.
- Chao JA, Lee JH, Chapados BR, Debler EW, Schneemann A, Williamson JR. 2005. Dual modes of RNA-silencing suppression by Flock House virus protein B2. *Nat. Struct. Mol. Biol.* 12:952–957.
- Chen H-Y, Yang J, Lin C, Yuan YA. 2008. Structural basis for RNA-silencing suppression by Tomato aspermy virus protein 2b. *EMBO Rep.* 9:754–760.
- Lingel A, Simon B, Izaurralde E, Sattler M. 2005. The structure of the flock house virus B2 protein, a viral suppressor of RNA interference, shows a novel mode of double-stranded RNA recognition. *EMBO Rep.* 6:1149–1155.
- Lu R, Maduro M, Li F, Li HW, Broitman-Maduro G, Li WX, Ding SW. 2005. Animal virus replication and RNAi-mediated antiviral silencing in *Caenorhabditis elegans*. *Nature* 436:1040–1043.
- Shaik Syed Ali P, Chen JCH. 2009. Structure of the RNA-binding domain of Nodamura virus protein B2, a suppressor of RNA interference. *Biochemistry* 48:2307–2309.
- Vargason JM, Szittyta G, Burgan J, Tanaka Hall TM. 2003. Size selective recognition of siRNA by an RNA silencing suppressor. *Cell* 115:799–811.
- Ye K, Malinina L, Patel DJ. 2003. Recognition of small interfering RNA by a viral suppressor of RNA silencing. *Nature* 426:874–878.
- Berry B, Deddouche S, Kirschner D, Imler J-L, Antoniewski C. 2009. Viral suppressors of RNA silencing hinder exogenous and endogenous small RNA pathways in *Drosophila*. *PLoS One* 4:e5866. doi:10.1371/journal.pone.0005866.
- Chou YT, Tam B, Linay F, Lai EC. 2007. Transgenic inhibitors of RNA interference in *Drosophila*. *Fly* 1:311–316.
- Dunoyer P, Lecellier CH, Parizotto EA, Himber C, Voinnet O. 2004. Probing the microRNA and small interfering RNA pathways with virus-encoded suppressors of RNA silencing. *Plant Cell* 16:1235–1250.
- Carrington JC, Ambros V. 2003. Role of microRNAs in plant and animal development. *Science* 301:336–338.
- Chapman EJ, Prokhnevsky AI, Gopinath K, Dolja VV, Carrington JC. 2004. Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step. *Genes Dev.* 18:1179–1186.
- Sunkar R, Chinnusamy V, Zhu J, Zhu JK. 2007. Small RNAs as big players in plant abiotic stress responses and nutrient deprivation. *Trends Plant Sci.* 12:301–309.
- Chen J, Li WX, Xie D, Peng JR, Ding SW. 2004. Viral virulence protein suppresses RNA silencing-mediated defense but upregulates the role of microRNA in host gene expression. *Plant Cell* 16:1302–1313.
- Jay F, Wang Y, Yu A, Taconnat L, Pelletier S, Colot V, Renou J-P, Voinnet O. 2011. Misregulation of auxin response factor 8 underlies the developmental abnormalities caused by three distinct viral silencing suppressors in *Arabidopsis*. *PLoS Pathog.* 7:e1002035. doi:10.1371/journal.ppat.1002035.
- Anandalakshmi R, Pruss GJ, Ge X, Marathe R, Smith TH, Vance VB. 1998. A viral suppressor of gene silencing in plants. *Proc. Natl. Acad. Sci. U. S. A.* 95:13079–13084.
- Brigneti G, Voinnet O, Li WX, Ji LH, Ding SW, Baulcombe DC. 1998. Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO J.* 17:6739–6746.
- Kasschau KD, Carrington JC. 1998. A counterdefensive strategy of plant viruses: suppression of posttranscriptional gene silencing. *Cell* 95:461–470.
- Li WX, Li H, Lu R, Li F, Dus M, Atkinson P, Brydon EW, Johnson KL, Garcia-Sastre A, Ball LA, Palese P, Ding SW. 2004. Interferon antagonist proteins of influenza and vaccinia viruses are suppressors of RNA silencing. *Proc. Natl. Acad. Sci. U. S. A.* 101:1350–1355.
- Félix M-A, Ashe A, Piffaretti J, Wu G, Nuez I, Bécicard T, Jiang Y, Zhao G, Franz CJ, Goldstein LD, Sanroman M, Miska EA, Wang D. 2011. Natural and experimental infection of *Caenorhabditis* nematodes by novel viruses related to nodaviruses. *PLoS Biol.* 9:e1000586. doi:10.1371/journal.pbio.1000586.
- Franz CJ, Zhao G, Félix M-A, Wang D. 2012. Complete genome sequence of Le Blanc virus, a third *Caenorhabditis* nematode-infecting virus. *J. Virol.* 86:11940.
- Guo X, Li W-X, Lu R. 2012. Silencing of host genes directed by virus-derived short interfering RNAs in *Caenorhabditis elegans*. *J. Virol.* 86:11645–11653.
- Schott DH, Cureton DK, Whelan SP, Hunter CP. 2005. An antiviral role for the RNA interference machinery in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. U. S. A.* 102:18420–18424.
- Wilkins C, Dishongh R, Moore SC, Whitt MA, Chow M, Machaca K. 2005. RNA interference is an antiviral defence mechanism in *Caenorhabditis elegans*. *Nature* 436:1044–1047.
- Lu R, Yigit E, Li WX, Ding SW. 2009. An RIG-I-Like RNA helicase mediates antiviral RNAi downstream of viral siRNA biogenesis in *Caenorhabditis elegans*. *PLoS Pathog.* 5:e1000286. doi:10.1371/journal.ppat.1000286.
- Steiner FA, Okihara KL, Hoogstrate SW, Sijen T, Ketting RF. 2009. RDE-1 slicer activity is required only for passenger-strand cleavage during RNAi in *Caenorhabditis elegans*. *Nat. Struct. Mol. Biol.* 16:207–211.
- Tabara H, Sarkissian M, Kelly WG, Fleenor J, Grishok A, Timmons L, Fire A, Mello CC. 1999. The *rde-1* gene, RNA interference, and transposon silencing in *Caenorhabditis elegans*. *Cell* 99:123–132.
- Yigit E, Batista PJ, Bei Y, Pang KM, Chen CC, Tolia NH, Joshua-Tor L, Mitani S, Simard MJ, Mello CC. 2006. Analysis of the *Caenorhabditis elegans* Argonaute family reveals that distinct Argonautes act sequentially during RNAi. *Cell* 127:747–757.
- Gent JI, Lamm AT, Pavelec DM, Maniar JM, Parameswaran P, Tao L, Kennedy S, Fire AZ. 2010. Distinct phases of siRNA synthesis in an

- endogenous RNAi pathway in *Caenorhabditis elegans* soma. *Mol. Cell* 37: 679–689.
37. Pak J, Fire A. 2007. Distinct populations of primary and secondary effectors during RNAi in *Caenorhabditis elegans*. *Science* 315:241–244.
 38. Sijen T, Steiner FA, Thijssen KL, Plasterk RH. 2007. Secondary siRNAs result from unprimed RNA synthesis and form a distinct class. *Science* 315:244–247.
 39. Han W, Sundaram P, Kenjale H, Grantham J, Timmons L. 2008. The *Caenorhabditis elegans* *rsd-2* and *rsd-6* genes are required for chromosome functions during exposure to unfavorable environments. *Genetics* 178: 1875–1893.
 40. Zhang C, Montgomery TA, Fischer SE, Garcia SM, Riedel CG, Fahlgren N, Sullivan CM, Carrington JC, Ruvkun G. 2012. The *Caenorhabditis elegans* RDE-10/RDE-11 complex regulates RNAi by promoting secondary siRNA amplification. *Curr. Biol.* 22:881–890.
 41. Jiang Q-X, Chen ZJ. 2012. Structural insights into the activation of RIG-I, a nanosensor for viral RNAs. *EMBO Rep.* 13:7–8.
 42. Kennedy S, Wang D, Ruvkun G. 2004. A conserved siRNA-degrading RNase negatively regulates RNA interference in *Caenorhabditis elegans*. *Nature* 427:645–649.
 43. Lu R, Folimonov A, Shintaku M, Li WX, Falk BW, Dawson WO, Ding SW. 2004. Three distinct suppressors of RNA silencing encoded by a 20-kb viral RNA genome. *Proc. Natl. Acad. Sci. U. S. A.* 101:15742–15747.
 44. Voinnet O, Pinto YM, Baulcombe DC. 1999. Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. *Proc. Natl. Acad. Sci. U. S. A.* 96:14147–14152.
 45. Kamath RS, Ahringer J. 2003. Genome-wide RNAi screening in *Caenorhabditis elegans*. *Methods* 30:313–321.
 46. Lehrbach NJ, Armisen J, Lightfoot HL, Murfitt KJ, Bugaut A, Balasubramanian S, Miska EA. 2009. LIN-28 and the poly(U) polymerase PUP-2 regulate let-7 microRNA processing in *Caenorhabditis elegans*. *Nat. Struct. Mol. Biol.* 16:1016–1020.
 47. Li H, Li WX, Ding SW. 2002. Induction and suppression of RNA silencing by an animal virus. *Science* 296:1319–1321.
 48. Dunoyer P, Schott G, Himber C, Meyer D, Takeda A, Carrington JC, Voinnet O. 2010. Small RNA duplexes function as mobile silencing signals between plant cells. *Science* 328:912–916.
 49. Segers GC, van Wezel R, Zhang X, Hong Y, Nuss DL. 2006. Hypovirus papain-like protease p29 suppresses RNA silencing in the natural fungal host and in a heterologous plant system. *Eukaryot. Cell* 5:896–904.
 50. Qiu W, Park JW, Scholthof HB. 2002. Tombusvirus P19-mediated suppression of virus-induced gene silencing is controlled by genetic and dosage features that influence pathogenicity. *Mol. Plant-Microbe Interact.* 15:269–280.
 51. Wu Q, Luo Y, Lu R, Lau N, Lai EC, Li W-X, Ding S-W. 2010. Virus discovery by deep sequencing and assembly of virus-derived small silencing RNAs. *Proc. Natl. Acad. Sci. U. S. A.* 107:1606–1611.
 52. Aoki K, Moriguchi H, Yoshioka T, Okawa K, Tabara H. 2007. *In vitro* analyses of the production and activity of secondary small interfering RNAs in *Caenorhabditis elegans*. *EMBO J.* 26:5007–5019.
 53. Szittyá G, Moxon S, Pantaleo V, Toth G, Rusholme Pilcher RL, Moulton V, Burgyn J, Dalmay T. 2010. Structural and functional analysis of viral siRNAs. *PLoS Pathog.* 6:e1000838. doi:10.1371/journal.ppat.1000838.
 54. Grishok A, Pasquinelli AE, Conte D, Li N, Parrish S, Ha I, Baillie DL, Fire A, Ruvkun G, Mello CC. 2001. Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *Caenorhabditis elegans* developmental timing. *Cell* 106:23–34.
 55. Knight SW, Bass BL. 2001. A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in *Caenorhabditis elegans*. *Science* 293:2269–2271.
 56. Ketting RF, Fischer SE, Bernstein E, Sijen T, Hannon GJ, Plasterk RH. 2001. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *Caenorhabditis elegans*. *Genes Dev.* 15:2654–2659.
 57. Aliyari R, Wu Q, Li HW, Wang XH, Li F, Green LD, Han CS, Li WX, Ding SW. 2008. Mechanism of induction and suppression of antiviral immunity directed by virus-derived small RNAs in *Drosophila*. *Cell Host Microbe* 4:387–397.
 58. Bagijn MP, Goldstein LD, Sapetschnig A, Weick E-M, Bouasker S, Lehrbach NJ, Simard MJ, Miska EA. 2012. Function, targets, and evolution of *Caenorhabditis elegans* piRNAs. *Science* 337:574–578.