LETTERS

Animal virus replication and RNAi-mediated antiviral silencing in *Caenorhabditis elegans*

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The worm *Caenorhabditis elegans* is a model system for studying many aspects of biology, including host responses to bacterial pathogens[1,2], but it is not known to support replication of any virus. Plants and insects encode multiple Dicer enzymes that recognize distinct precursors of small RNAs and may act cooperatively[3–7]. However, it is not known whether the single Dicer of worms and mammals is able to initiate the small RNA-guided RNA interference (RNAi) antiviral immunity as occurs in plants[8] and insects[9]. Here we show complete replication of the Flock house virus (FHV) bipartite, plus-strand RNA genome in *C. elegans*. We show that FHV replication in *C. elegans* triggers potent antiviral silencing that requires RDE-1, an Argonaute protein[10,11] essential for RNAi mediated by small interfering RNAs (siRNAs) but not by microRNAs. This immunity system is capable of rapid virus clearance in the absence of FHV B2 protein, which acts as a broad-spectrum RNAi inhibitor[9,12] upstream of *rde-1* by targeting the siRNA precursor. This work establishes a *C. elegans* model for genetic studies of animal virus–host interactions and indicates that mammals might use a siRNA pathway as an antiviral response.

We chose animal nodavirus FHV to determine whether *C. elegans* supports virus replication, because FHV replicates in yeast, plant, insect and mammalian cells[13]. The first genome segment of FHV, RNA1, encodes the entire viral contribution to the viral RNA-dependent RNA polymerase (RdRP) and replicates autonomously in the absence of RNA2, which depends on RNA1 for replication[13] (Fig. 1a). Worm strains were generated to carry either a chromosomally integrated FR1 (for FHV RNA1) or FR2 (for FHV RNA2) transgene, designed to yield transcripts in the soma after heat induction that are identical in sequence to FHV genomic RNAs 1 and 2, respectively (Fig. 1b). Northern blot hybridizations detected a high-level accumulation of FHV RNA1 in worms carrying the FR1-3 transgene after transcription induction (Fig. 1c, lane 3). FHV RNA2 accumulated to high levels in strain FR1-3/FR2 carrying both FR1-3 and FR2 combined by genetic crosses (Fig. 1c, lane 5) but was undetectable in worms carrying FR2 alone (Fig. 1c, lane 6). Thus, the abundant viral RNAs detected in transgenic worm strains resulted from active RNA replication, because in the absence of RNA replication the initial heat-inducible transcripts were below the limit of detection in FR2 worms two days after induction.

Both FR1-3 and FR1-3/FR2 worms also contained abundant RNA3 (Fig. 1c, lanes 3 and 5). RNA3 is a subgenomic RNA transcribed during RNA1 replication from an internal site of the complementary, replicative intermediate of RNA1, (−)-RNA1 (Fig. 1a), and is not required in the initiation of FHV infection unlike the genomic RNAs. RNA1 replication initiated from FR1-2 transcripts was inefficient and became detectable only in the presence of RNA2 replication (Fig. 1c, lanes 2 and 4), which directs the expression of the pre-capsid protein (pre-CP) essential for virion packaging (Fig. 1a). Taken together, the detection of self-replication of RNA1 and *trans* replication of RNA2 as well as the production of a subgenomic RNA in these worm strains provide evidence that *C. elegans* supports complete replication of the FHV RNA genome.

To investigate a possible induction of antiviral silencing by FHV RNA replication in worms, we first examined whether FHV accumulation in *C. elegans* requires expression of the FHV-encoded B2 protein, an RNAi suppressor that is active across the animal and plant kingdoms[9,12] and is not known to have a direct role in FHV RNA replication[13]. We created a derivative of the FR1-3 transgene, FR1-3ΔB2 (Fig. 1b), containing a point mutation that abolished the B2 open reading frame but had no effect on the out-of-frame overlapping viral RdRP open reading frame (Fig. 1b). In contrast to high levels of RNA1 and RNA3 in FR1-3 worms (Fig. 1c, lane 3), we

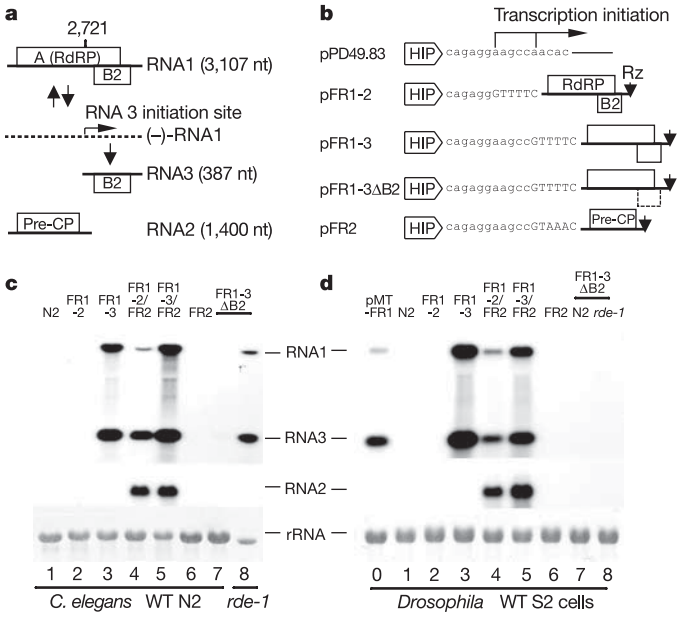

Figure 1 | Replication and silencing of FHV in *C. elegans*. **a**, Structure, replication and expression of FHV genome. **b**, Structure of FHV transgenes. HIP, heat inducible promoter. The junction region between HIP (lower-case letters) and FHV cDNA (capital letters) is shown. Transcriptional initiation sites from HIP were indicated. Rz is a self-cleaving ribozyme used to reduce non-viral extensions at the 3′ termini of heat-induced transcripts. **c**, **d**, Northern blot detection of FHV RNA accumulation in *C. elegans* (**c**) and fruitfly S2 cells (**d**). Each lane in **c** and **d** was loaded with 4 μg of total RNA, except for lane 8 of **c**. WT, wild-type.

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detected no accumulation of FHV RNA1 in worms carrying an integrated FR1-3ΔB2 transgene (Fig. 1c, lane 7). Thus, B2 is also essential for FHV accumulation in *C. elegans* as has been shown in cultured insect cells[9,12].

If the lack of FHV RNA accumulation in FR1-3ΔB2 worms was indeed due to a loss of B2 suppression of RNAi, we would expect to find FR1-3ΔB2 accumulation rescued in worm mutants defective in support of RNAi. To test this hypothesis, worm strains carrying FR1-3 and FR1-3ΔB2 were each crossed into an *rde-1 (ne219)* mutant background[10]. Unlike many RNAi mutants such as *dcr-1/Dicer*, which exhibit multiple developmental defects due to the disruption of microRNA (miRNA) function[6], *rde-1* mutants are otherwise very healthy[10]. Northern blot analysis revealed that, whereas FHV RNA1 was undetectable in wild-type N2 worms carrying FR1-3ΔB2 (Fig. 1c, lane 7), the same FR1-3ΔB2 transgene directed abundant accumulation of FHV RNAs 1 and 3 in the *rde-1* mutant background (Fig. 1c, lane 8). In fact, the accumulation level of FHV RNAs in FR1-3ΔB2/*rde-1* worms was similar to that in FR1-3 worms (Fig. 1c, compare lanes 3 and 8; note that the amount of total RNAs loaded in lane 8 was about one-third of that loaded in lane 3; see also Fig. 2). Thus, the mutant RNA1 from the FR1-3ΔB2 transgene is not defective in self-directed replication, indicating that the reduced accumulation of viral RNA in FR1-3ΔB2/N2 worms (Fig. 1c, lane 7) was due to the induction and clearance of viral RNAs by antiviral silencing in an *rde-1*-dependent siRNA pathway.

Both FHV RNAs 1 and 2 produced in worms were biologically active after transfection into cultured *Drosophila* S2 cells (Fig. 1d, lanes 3–5), which are known to initiate potent RNAi-mediated antiviral silencing after challenge with FHV[9]. In contrast, no viral RNA was detected in S2 cells transfected with total RNA extracted from either FR1-3ΔB2/N2 or FR1-3ΔB2/*rde-1* worms (Fig. 1d, lanes 7 and 8), in spite of the fact that the total RNA sample extracted from FR1-3ΔB2/*rde-1* worms contained abundant FHV RNA1 (Fig. 1c, lane 8). This finding shows that progeny RNA1 of FR1-3ΔB2 produced in FR1-3ΔB2/*rde-1* worms remained defective in B2 expression and incapable of preventing virus clearance by RNAi-mediated antiviral silencing in S2 cells[9]. We therefore conclude that abundant accumulation of FHV RNAs detected in FR1-3ΔB2/*rde-1* worms does not result from a genetic reversion in the progeny of FR1-3ΔB2 but is due to the genetic suppression of viral B2 deletion by the loss-of-function mutation of *rde-1* in the worm genome. This genetic complementation of loss-of-function mutations in a viral RNAi suppressor gene and a host RNAi pathway gene provides the first direct evidence that viral RNAi suppressors enhance virus accumulation and facilitate viral infection by suppressing the host antiviral silencing mechanism.

Comparative analysis of the viral RNA accumulation among FR1-3/*rde-1*, FR1-3/N2 and FR1-3ΔB2/*rde-1* worms reveals two interesting results. First, the accumulation of FHV RNA1 derived from the same FR1-3 transgene array was much lower in N2 worms than in *rde-1* worms (Fig. 2, compare lanes 3 and 4 with lanes 5 and 6). This difference could be due to an incomplete B2 suppression of the worm *rde-1*-dependent antiviral silencing in FR1-3 worms, or alternatively it might result from the recently discovered, *rde-1*-dependent transcriptional transgene silencing in the soma[14]. Second, FHV RNA1 accumulated to much higher levels in FR1-3/*rde-1* worms than in FR1-3ΔB2/*rde-1* worms (Fig. 2, compare lanes 5 and 6 with lanes 9 and 10), indicating that B2 expression also enhances viral accumulation in absence of *rde-1*. Because B2 is a broad-spectrum RNAi suppressor active in both the animal and plant kingdoms[9,12] but does not influence the rate of FHV RNA replication[13], our data indicate the possible presence of active antiviral RNAi in *rde-1* worms, which might be mediated by one or more of the remaining 26 worm Argonaute (AGO) genes, among which differential requirements for *alg-1*, *alg-2*, *ppw-1* and *ppw-2* in various RNAi processes have been documented[15].

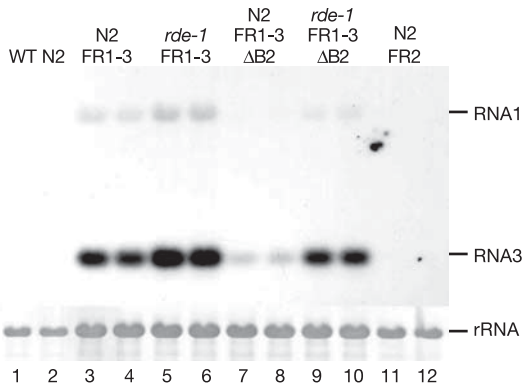

Figure 2 | FHV RNAi suppressor is active in *rde-1* worms. Total RNA was extracted from worms of either wild-type (WT) or *rde-1* genotype carrying an integrated FR1-3 or FR1-3ΔB2 transgene, two days after transcriptional induction. Northern blot hybridizations were performed as in Fig. 1c, d.

Our genetic analysis indicates that the viral RNAi suppressor is active in both wild-type and *rde-1* worms, indicating that B2 might act upstream of AGO. AGO has a key function in the RNA-induced silencing complex by binding to siRNA for recognition and cleavage of the mRNA target[6,11]. No known protein domain was recognizable in the 106-residue FHV B2. We found that B2 synthesized as a glutathione *S*-transferase (GST) fusion was able to bind *in vitro* to a 21-nucleotide (nt) siRNA duplex (Fig. 3a, lanes 1–6) independently of its overhang nucleotides (data not shown); siRNA binding has been reported for p19, a silencing suppressor encoded by the plant tombusvirus[16]. However, unlike p19, which binds much more weakly to double-stranded RNA (dsRNA) longer than 23 nt, B2 also bound 25-nt siRNA and dsRNA 44 nt (Fig. 3d) and 100 nt (data not shown) in length. Furthermore, competition experiments indicated a much higher affinity of B2 for long dsRNA than for siRNA duplexes because the 100-nt dsRNA was approximately 30-fold more effective than 21-nt siRNA in inhibiting the formation of the siRNA–B2 complex (Fig. 3a, c).

A mutational analysis identified the replacement of Arg by Gln at position 54 (R54Q) of B2, which, when introduced into FHV RNA1, led to at least a 20-fold reduction in the accumulation of FHV RNAs in comparison with wild-type FHV RNA1 (data not shown). The same R54Q mutation completely abolished the B2 activity to bind long dsRNA (Fig. 3d, lanes 8–19). The effect of the R54Q mutation on siRNA binding was less marked: whereas the upper migrating siRNA–B2 complexes disappeared, the faster-migrating siRNA–B2 complexes remained visible (Fig. 3b, compare lanes 1, 2, 5 and 6 with lanes 3, 4, 7 and 8). Furthermore, we found that Dicer processing of a labelled 500-nt dsRNA into siRNAs was inhibited by FHV B2 fused with GST beginning at 800 nM (Fig. 3e, lane 9) but not by GST up to 10,000 nM (Fig. 3e, lane 6), and that the inhibitory effect was essentially eliminated by the R54Q mutation (data not shown). These findings indicate a new mechanism of viral suppression of antiviral silencing by targeting the dsRNA precursor of siRNAs, although they do not rule out a possible role of siRNA binding[16–18]. This model is consistent with the genetic analysis placing B2 upstream of AGO and explains why B2 is active in both the animal and plant kingdoms.

Endogenous mammalian gene silencing by miRNAs starts in the nucleus with the cleavage by Drosha of primary miRNA transcripts into precursor miRNAs, which are then exported to the cytoplasm, where they are further processed into mature miRNAs by Dicer[6]. Much less is known about the initiation of antiviral RNA silencing in any organism. For RNA viruses, present models consider both dsRNA produced during RNA replication and highly structured elements in single-stranded viral RNAs as potential initiators by means of the siRNA and miRNA pathways, respectively[16,18,19]. Our genetic analyses (ref. 9 and this study) indicate a possibly more

prominent role for the siRNA pathway in the recognition of the viral silencing initiators because neither *Drosophila* AGO2 nor *C. elegans* RDE-1 is essential for miRNA function[20], in spite of their requirement in antiviral silencing. Furthermore, with a few exceptions such as influenza viruses[12], and unlike DNA viruses[21], most RNA viruses replicate exclusively in the cytoplasm and may escape detection by the miRNA pathway initiating in the nucleus. Nevertheless, potent antiviral silencing detected in a single Dicer organism rules out an essential role for multiple Dicers found in plants and insects[3–7], indicating an antiviral potential for the mammalian RNAi machinery through the siRNA pathway in addition to targeting viral mRNAs by cellular miRNAs[22]. The *C. elegans* model established in this work will facilitate genetic studies of animal virus–host interactions, many aspects of which cannot be addressed in the alternative model in *Saccharomyces cerevisiae*[23], a unicellular organism that does not seem to encode an RNAi pathway[6]. Although most nodaviruses are pathogens of insect and fish hosts, Nodamura virus[12] infects and kills sucking mice and sucking hamsters[13], indicating a potential of the worm model for studying the pathogenesis of mammalian viral diseases.

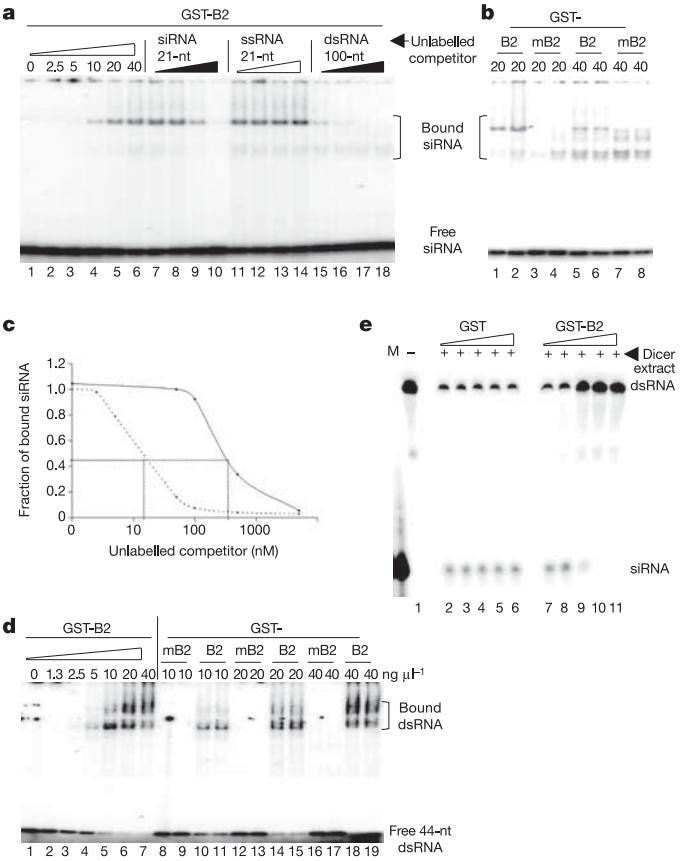

Figure 3 | FHV B2 is a dsRNA-binding protein and inhibits siRNA production *in vitro*. **a**–**d**, GST-tagged B2 protein (GST-B2) binds both 21-nt siRNA duplex (**a** and **b**) and 44-nt dsRNA (**d**) *in vitro*. Volumes of the top siRNA–B2 band in lanes 7–10 and 15–18 of **a** were quantified by phosphorimaging and plotted against the concentrations of the unlabelled competitor RNAs (**c**); dotted line, 100-nt dsRNA as unlabelled competitor; solid line, 21-nt siRNA as unlabelled competitor. Note the presence of non-specific signals in lanes 1, 8, 13 and 16 of **d**. The final concentration for all labelled RNAs was 50 nM whereas 4 different concentrations (50, 100, 500 and 5,000 nM) were used for each unlabelled competitor RNA (lanes 7–18 of **a**). The concentrations of GST-B2 or GST fused with the mutant B2 (GST-mB2) ranged from 0 to 40 ng μl^{-1} in all RNA binding assays as indicated at the top of each lane, except for lanes 7–18 of **a** where GST-B2 was constant at 40 ng μl^{-1}. **e**, B2 inhibits *in vitro* processing of a labelled long dsRNA by the Dicer extracts from fruitfly S2 cells. Concentration gradients of GST or GST-B2 used were 50, 200, 800, 3,200 and 10,000 nM. The labelled dsRNA was incubated in buffer without Dicer extract (lane −). A labelled 21-nt siRNA was used as a marker (M) at the left.

METHODS

Transgene constructs and transgenic worms. Constructs were generated by standard methods by using the vector pPD49.83 (a gift from A. Fire) which contains the promoter of the *hsp16-41* gene[24]. Full-length cDNAs to FHV RNA1 and RNA2 together with a self-cleaving ribozyme from the tobacco ringspot virus satellite RNA were obtained from the infectious FHV cDNA clones previously constructed for infection of *Drosophila* S2 cells[9].

Nematodes were propagated and maintained by standard protocols[1]. The following strains were used: N2 (wild type), *unc-119(ed4) III* and *rde-1(ne219) V*. Animals were made transgenic by gonadal microinjection[25]. FHV plasmids were mixed with either the *rol-6^D* plasmid pRF4 for injection into wild-type animals[25], or the *unc-119(+)* plasmid pDP#MM016B for injection into *unc-119* mutants[26]. Integrated lines were generated by treating about 30 transgenic hermaphrodites with 3,500 rad of γ-rays from a ^{137}Cs source, followed by screening for integrated animals in the F_2 generation.

rol-6^D and *unc-119(+)* transgenes were combined as described[26]. To generate *rde-1;rol-6^D* strains, *rde-1(ne219)* males were crossed with *rol-6^D* transgene hermaphrodites. F_2 animals were grown individually on *Escherichia coli* HT115 expressing *unc-22* dsRNA (ref. 27), and plates generating no Unc-22 animals were assumed to be homozygous for *rde-1(ne219)*.

Assay for FHV replication in *C. elegans*. Expression of FHV transgenes was achieved for each strain as follows. First, ten young adult hermaphrodites were placed on a seeded 10-cm plate and allowed to grow to the next generation for 5 days at 20 °C. Plates were incubated for 2 h at 33 °C and returned to 23 °C for two days, after which they were washed off plates into water and stored at −80 °C. Total RNA was obtained by homogenizing thawed worm pellets with a Tissue Tearor (BioSpec Products) followed by extraction with TRIzol (Invitrogen), then a column-based RNA purification with the RNeasy kit (Qiagen) in accordance with the manufacturer's instructions. RNA concentrations were normalized and used for northern blot analysis in accordance with standard protocols[9] with a ^{32}P-labelled cDNA probe that corresponded either to FHV RNA2 or to the last 387 nt of FHV RNA1, thus hybridizing to both RNAs 1 and 3.

To verify the biological activities of FHV RNAs produced in *C. elegans*, cultured *Drosophila* S2 cells were transfected with 2 µg of total RNA extracted from individual worm strains two days after heat induction. Three days after transfection, total RNA was extracted from S2 cells for northern blot detection of FHV RNA accumulation. pMT-FR1 was used as a control and contained the full-length cDNA of FHV RNA1 under the transcriptional control of the $CuSO_4$-inducible metallothionein promoter as described[9].

Fusion protein expression, dsRNA binding and Dicer activity assays. Expression of GST-tagged B2 proteins (GST-B2) in *E. coli* and RNA binding assays were performed as described previously[12,28]. Both single-stranded and duplex siRNAs were synthesized chemically and end-labelled by exchange reaction. Long dsRNA (44 nt and 100 nt) was transcribed and annealed *in vitro*, either with or without kinase end-labelling after dephosphorylation. The final concentration for all labelled RNAs was 50 nM, whereas four different concentrations (50, 100, 500 and 5,000 nM) were used for each unlabelled competitor RNA. mB2 contained an Arg → Gln substitution at position 54 of FHV B2. The concentrations of GST-B2 or GST-mB2 used in RNA binding ranged from 0 to 40 ng μl^{-1} as indicated. Binding reactions were resolved by native 6% polyacrylamide gel electrophoresis (PAGE) for binding to the 44-nt dsRNA or 8% for siRNA binding. Gels were dried before autoradiography.

Preparation of Dicer extracts from *Drosophila* S2 cells, labelling of long dsRNA, and the Dicer activity assay were as described[7]. A ^{32}P-labelled 500-nt dsRNA at 7.5 nM was incubated with the Dicer extracts in the presence of either GST or GST-B2 in five different concentrations, namely 50, 200, 800, 3,200 and 10,000 nM. After incubation, RNAs were fractionated by 15% PAGE along with a chemically synthesized 21-nt siRNA labelled with [γ-^{32}P]ATP as a marker.

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