Antiviral RNAi against Orsay virus is neither systemic nor transgenerational in *Caenorhabditis elegans*

Alyson Ashe\(^1,2,4,\#\), Peter Sarkies\(^1,2,5\), Jérémie Le Pen\(^1,2,3\), Mélanie Tanguy\(^1,3\), Eric A. Miska\(^1,2,3,\#\)

\(^1\) Wellcome Trust Cancer Research UK Gurdon Institute and
\(^2\) Department of Biochemistry, University of Cambridge, Tennis Court Rd, Cambridge CB2 1QN, UK
\(^3\) Department of Genetics, University of Cambridge, Downing St, Cambridge CB2 3EH, UK
\(^4\) School of Molecular Bioscience, Molecular Bioscience Building, University of Sydney, NSW 2006, Australia
\(^5\) MRC Clinical Science Section, Imperial College London, Du Cane Rd, London W12 0NN, UK

\# co-corresponding authors

Abstract word count: 242

Manuscript word count: 4667
Abstract

Antiviral RNA-mediated silencing (RNAi) acts as a powerful innate immunity defence in plants, invertebrates and mammals. In C. elegans RNAi is systemic, i.e. RNAi silencing signals can move between cells and tissues. Furthermore RNAi effects can be inherited transgenerationally and may last for many generations. Neither the biological relevance of systemic RNAi nor transgenerational RNAi are currently understood. Here we examined the role of both pathways to protect C. elegans from viral infection. We studied the Orsay virus, a positive strand RNA virus related to Nodaviridae, and the first and only virus known to infect C. elegans. Immunity to Orsay virus infection requires the RNAi pathway. Surprisingly, we found that genes required for systemic or transgenerational RNAi did not have a role in antiviral defence. Furthermore, we found that Orsay virus infection did not elicit a systemic RNAi response even when a target for RNAi was provided using transgenes. Finally, we show that viral siRNAs, the effectors of RNAi, are not inherited to a level that provides any significant resistance to viral infection in the next generation. We conclude that systemic or transgenerational RNAi does not play a role in the defence to natural Orsay virus infection. Furthermore, our data suggest that there is a qualitative difference between experimental RNAi and antiviral RNAi. Our data are consistent with a model of systemic and transgenerational RNAi that requires a nuclear or germline component which is lacking in almost all RNA virus infections.

Importance
47 Since its discovery in *Caenorhabditis elegans*, RNAi has proven a valuable scientific tool in many organisms. In *C. elegans* exogenous RNAi spreads throughout the entire organism and can be passed between generations, however there has been controversy as to the endogenous role/s that the RNAi pathway plays. One endogenous role for which spreading both within the infected organism and between generations would be advantageous is a role in viral defence. In plants antiviral RNAi is systemic, and the spread of RNAi between cells provides protection against subsequent viral infection. Here we investigate this using the only naturally occurring virus known to infect *C. elegans*, Orsay virus, and surprisingly find that in contrast to the exogenous RNAi pathway, the antiviral RNAi response targeted against this virus does not spread systemically throughout the organism and cannot be passed between generations. These results suggest that there are differences between the two pathways which remain to be discovered.

**Introduction**

RNA interference (RNAi) is a mechanism of gene silencing which is broadly conserved across eukaryotes. RNAi is initiated by cleavage of long double-stranded RNA (dsRNA) by the RNase III enzyme Dicer into short 20-24 nucleotide (nt) siRNAs (1). These siRNAs are bound by Argonaute proteins and act as a guide to the complementary mRNA, which is subsequently destroyed by the slicer action of the Argonaute protein (2).

In *C. elegans* there is an additional amplification step to the pathway. siRNAs generated by Dicer (DCR-1) form complexes with Argonaute proteins which recruit RNA dependent RNA polymerases (RdRP) to the target mRNA. The RdRPs produce an abundant class of siRNAs (3, 4), which are almost exclusively 22 nt and possess a
guanine (G) as the 5' nucleotide, hence are referred to as 22G-RNAs. 22G-RNAs are more abundant than siRNAs produced by Dicer and are required for effective gene silencing. However, they are not able to recruit RdRPs to the target, thus are unable to initiate generation of further 22G-RNAs (5).

The exogenous RNAi pathway in *C. elegans* is systemic (6, 7). Uptake of double-stranded RNA into the intestine by the transporter protein SID-2 and the transfer between cells by SID-land SID-5 is capable of silencing gene expression in most tissues (8-11). The exact mobile RNA species remains elusive, but there is some evidence to suggest that dsRNA molecules (probably DCR-1 products) are mobile and can be exported and imported by proteins required for systemic RNAi (12).

Silencing initiated by the exogenous RNAi pathway can spread not just within the treated animals, but also to their offspring in what is known as transgenerational inheritance (13-16). Transgenerational inheritance initiated by RNAi does not occur at every locus, and is not fully penetrant: *i.e.* not all offspring inherit the silenced phenotype. The mechanisms responsible for both the transmission and establishment of transgenerational silencing remain cryptic, although it seems that both small RNA pathways and chromatin modifiers are required (13-18).

RNAi acts as a potent defense mechanism against viruses in plants and animals (19-23). In plants, long viral dsRNA precursors are processed into 21 nt long siRNAs by Dicer-like 4 (24, 25). These 21 nt siRNAs are capable of moving from cell to cell and directing silencing in the recipient cell (26, 27). The spread of siRNAs ahead of viral infection confers resistance on recipient cells. 24 nt Dicer-like 3 products are also capable of systemic spreading (26, 28). Similarly, *Drosophila* also utilize a siRNA pathway in viral defence. In this case, both Dicer 2 and Argonaute 2 are required for
an effective antiviral response (29). Again, systemic spreading throughout the organism is important in antiviral immunity, although in this case it seems to be dsRNA molecules that are mobile (29). RNAi pathways have also recently been suggested to play a role in viral defence in mammals (20, 21), although to date it is not known if the antiviral silencing can spread between cells.

The RNAi pathway also acts in viral defence in *C. elegans*. The initial trigger is a dsRNA viral replication intermediate that is recognized by DRH-1 (30). This recognition allows DCR-1 and accessory proteins to produce siRNAs and subsequently 22G-RNAs in a manner that appears to utilize the same pathway as classical RNAi gene silencing (19, 30-32): indeed the level of our current knowledge suggests that it is only the viral recognition factor DRH-1 that differs between the two pathways.

Despite the similarities to the canonical RNAi pathway it is still unclear whether the *C. elegans* antiviral siRNA pathway gives rise to systemic effects. In the case of a virus that infects somatic cells such as the Orsay virus, indirect evidence to support systemic antiviral RNAi could be taken from transgenerational inheritance of silencing, since this implies that the RNAi response must have spread into the germline. Transgenerational silencing of a Flock House Virus transgene under the control of a heat shock promoter has been observed (33) however, this may occur as a result of the presence of the transgene in all cells of the animal. More recently, it was reported that parental exposure to Orsay virus can protect offspring from infection (34). However Guo and colleagues reported that *sid-1* mutants are not any more
susceptible to Orsay virus infection than N2, suggesting that systemic RNAi is not important in viral defence (35).

In this study, we test for the existence of a systemic RNAi response against the Orsay virus using a sensor for antiviral siRNA to report on the spread of antiviral silencing between cells. Surprisingly, in contrast to the exogenous RNAi pathway in *C. elegans* and viral defence pathways in both plants and *Drosophila*, we show that RNAi following infection with Orsay virus is not systemic. Consistently, we show that there is no transgenerational inheritance of Orsay virus-induced silencing. Together these results suggest partitioning between intermediates in RNAi induced by the Orsay virus and exogenous dsRNA and challenge the assumption that systemic RNAi evolved as an antiviral defense mechanism.

**Materials and Methods**

**Genetics**

*C. elegans* were grown under standard conditions at 20 °C on HB101 bacteria unless otherwise indicated. The wild-type strain was var. Bristol N2 (36). Strains used in this study were: HC75 *ccIs4251; sid-1(qt2)*, HC271 *ccls4251; qtIs3; sid-2(qt42); mIs11*, RB2519 *drh-1(ok3495)*, SX2836 *mjIs242[psur-5::GFP::OrsayRNA2::tbb-2 + psur-5::mCherry::unc-54]*, SX2839 *mjIs242[psur-5::GFP::OrsayRNA2::tbb-2 + psur-5::mCherry::unc-54]; sid-1(qt2)*, SX2838 *mjIs242[psur-5::GFP::OrsayRNA2::tbb-2 + psur-5::mCherry::unc-54]; drh-1(ok3495)*, SX2813 *mjEx565[psur-5::GFP::OrsayRNA1::tbb-2]; mjEx566[psur-5::mCherry::unc-54]*

**Molecular Biology**

Sensor generation
psur-5::GFP::OrsayRNA2::tbb-2 and psur-5::GFP::OrsayRNA1::tbb-2

These constructs were generated using the MultiSite Gateway Three-fragment Vector Construction Kit (Life Technologies). psur-5 in the first position was a gift from the Ahringer laboratory, and GFP (cloned from pPD95.75) was placed in the second position. PCR fusion was used to generate the OrsayRNA::tbb-2 fragments which were placed in the third position. All three fragments were combined in an LR reaction into the pCFJ150 vector. Primer sequences for amplifying viral segments from cDNA are listed in Table S1 (available on request).

Preparation of RNAi constructs

Viral segments were PCR amplified from cDNA using primers with appropriate tails for BP cloning into pDONR221 (Gateway cloning). LR reactions were performed to place viral segments in a gateway modified RNAi vector L4440. Primer sequences are listed in Table S1 (available on request).

Detection of viral infection

Virus filtrate was prepared as described previously (19).

Infection of strains of interest. For all strains, two or three young adults were inoculated with 20 µl of viral filtrate for 4 days at 20 °C in 55 mm plates.

Detection of viral RNA. Four days after infection, all animals were collected in M9; RNA extraction and qRT-PCR was performed as described previously (19, 30).

Aliquots of RNA were kept apart for small RNA libraries (see below).

Comparison of infection methods

Infection of strains of interest.
Liquid culture based infection: 200 L2s were rotated for 1 hour at 20 °C in 300 µl M9, 100 µl HB101 in LB broth and 100 µl of Orsay Virus filtrate (non-diluted, or diluted in M9 10 or 100 times). After 1 hr, the animals were collected and washed three times in M9 before transfer to 50 mm plates.

Agar based infection: 200 L2s were transferred to a 50 mm plate per individual infection. 100 µl of Orsay Virus filtrate was added (non-diluted, or diluted in M9 10 or 100 times).

Infections were performed in five biological replicates.

Detection of viral RNA. For all strains 200 L2 animals were infected and collected 48 hours post-infection for detection of viral RNA as described above.

RNAi

RNAi bacteria were grown for 6 hr with shaking at 37 °C. Bacteria were then seeded onto 55 mm NGM plates containing IPTG (1 mM) and carbenicillin (25 µg/ml). After drying overnight, 2-3 animals were added and then grown at 20 °C for 4 days, at which point they were either collected for RNA extraction (for measuring Orsay viral load, or for small RNA sequencing), or their phenotype was scored (for the viral sensor experiments). For the analysis of phenotype of animals treated with RNAi against unc-22 (ZK617.1) or dpy-11 (F46E10.9) genes (37), 5 plates of 4 L4 animals were plated on RNAi plates against the relevant gene and the progeny grown up on the same plates. Adult animals were then transferred onto non-RNAi plates and the progeny were scored for the phenotype to measure the percentage of animals displaying the phenotype. The experiment was repeated in triplicate.

Small RNA sequencing

RNA extraction, library preparation and sequencing
Extraction of RNA for libraries, library preparation and sequencing was performed as described previously (30). P0 animals were grown at 20 °C on 3 10 cm plates and collected as a mixed stage population of predominantly adults 4 days after viral infection or RNAi treatment. F1s were obtained by bleaching of P0s, and assayed at three different ages – as embryos (immediate collection), after 24 hr or 72 hr. P0 infection was performed in biological duplicates, with one replicate used for the embryos, and the other used for both 24 hr and 72 hr timepoints. P0 replicates were compared to ensure equality.

**Sequencing analysis.** Small RNA libraries were sequenced using the Illumina MiSeq and/or HiSeq. Processing and alignment of high-throughput sequencing data to the Orsay virus was carried out as described previously, using Bowtie for all sequence alignments allowing up to one mismatch to compensate for divergence in the viral sequence (30). To generate plots of small RNAs aligning to *unc-22* and *dpy-11* coding sequences in fasta format for *dpy-11* and *unc-22* genes were downloaded from wormbase (WS236) and were used to build genomes using Bowtie-build to which small RNAs were aligned allowing 0 mismatches.

**Results**

*Weak transgenerational transmission of antiviral siRNAs.*

Exposing *C. elegans* to dsRNA through feeding, injection or viral infection, results in the generation of two classes of siRNAs that bring about RNAi (Fig 1A). The first class of siRNAs are generated by DCR-1 activity on dsRNA, possess 5' monophosphates, have a modal length of 23 nucleotides (nt) and no overall first nucleotide bias. The second class of siRNAs (22G-RNAs) are generated by the...
activity of RNA dependent RNA polymerase and have 5' triphosphates, with a strong preference for G at the first nucleotide. Standard small RNA library preparation cannot detect 22G-RNAs unless the 5' triphosphate is removed by enzymatic treatment. We performed polyphosphatase treatment of the RNA before library preparation, which enables the detection of both direct Dicer products and 22G-RNAs.

To better understand the antiviral RNAi response we used high-throughput sequencing to assess the small RNAs present in biological duplicates of N2 animals infected with Orsay virus (N2 P0) and compared with their uninfected offspring (N2 F1) (raw data available from GEO accession number GSE60020). As shown previously (30) the N2 P0 sample (Fig 1B) shows Dicer products mapping both sense and antisense to the viral genome and abundant 22G-RNAs mapping antisense to the viral RNA. There are considerably less viRNAs in F1 animals than their parents and they decrease over time (Fig 1 C-E, Fig 2A).

To get an idea of how these very low levels of inherited viRNAs compared to inheritance following exogenous RNAi, we also prepared libraries from animals feeding on RNAi food against the endogenous loci dpy-11 (Fig 1F-I) and unc-22 (Fig 1J-M), and their offspring who were fed on standard HB101 bacteria. Both the dpy-11 and unc-22 genes are expressed in somatic cells; however, the dpy-11 phenotype conferred by RNAi can be inherited into the F1 generation whilst the unc-22 phenotype cannot (Fig 2B) (38). siRNAs complementary to both dpy-11 and unc-22 were approximately as abundant as viRNAs in infected P0 animals (Fig1 F,J). Both dpy-11 and unc-22 siRNAs were retained to considerably higher levels than the viRNAs in the F1 offspring (Fig 2A). Notably 22G-RNAs are still clearly visible in
the offspring of both *unc-22* and *dpy-11* treated animals at all time points sampled; *dpy-11* 22G-RNAs were higher than *unc-22* 22G-RNAs consistent with the inheritance of the *dpy-11* phenotype. Thus the viral siRNAs are inherited into the F1 generation at a reduced level compared to endogenous, somatically expressed loci, even those for which a phenotype is not detected in the F1.

No evidence for a protective “vaccination” effect in F1s

The fact that viRNAs appear to be inherited at such low levels strongly suggested that they would be unable to protect against future viral infection in the F1 generation. To test this directly we grew wild-type animals in the presence or absence of virus, “bleached” them to remove infected P0 animals and the Orsay virus from the culture, and then reinjected the F1 generation. If the small number of inherited viRNAs could protect against viral infection in the subsequent generation we would expect to see lower levels of viral replication in the offspring of infected parents compared to uninfected parents. We detected viral load in F1 animals four days after infection by qPCR for Orsay RNA. There was no difference in Orsay RNA levels between the animals whose parents had been exposed to Orsay virus and those with no prior exposure (Fig 2C, D). These results suggest that the few viRNAs detected in the F1 generation are insufficient to induce viral silencing. This lack of F1 “vaccination” by the Orsay virus contrasts with recently published work by Sterken *et al.* (34). One main difference between the two studies is the infection procedure – infection in liquid culture for 1 hour, followed by growth on agar (Sterken) vs infection and growth on agar (this study). It is plausible that liquid based infection may result in higher levels of viral infection and thus make F1 “vaccination” possible. To address this issue we tested the levels of infection produced by the two methods in both N2
and *drh-1* animals and found that agar-based infection was more reproducible and resulted in higher levels of infection than liquid culture based infection over a range of viral concentrations (Fig 2E).

**Generation of an antiviral 22G sensor to detect viral infection**

To try and understand the reasons for the failure of viRNAs to be inherited into the F1 generation we developed a GFP sensor capable of detecting antiviral 22G-RNAs. The sensor consists of an integrated, multi-copy transgenic array of the ubiquitous *sur-5* promoter driving GFP expression, followed by approximately 600bp of Orsay RNA2 before the *tbb-2* 3’ UTR (*psur-5::GFP::OrsayRNA2::tbb-2*). The animals also carry the same promoter driving mCherry expression with an alternate 3’ UTR (*psur-5::mCherry::unc-54*) (Fig 3A). In this system uninfected animals should express both GFP and mCherry, resulting in “orange” animals. Upon Orsay virus infection, viRNAs produced in the infected cells should silence the GFP transgene resulting in red cells. As RNAi is systemic in *C. elegans*, the mobile silencing signal generated after or during the dicing of viral dsRNA should spread systemically through the organism. When, in an uninfected cell, the mobile species encounters the mRNA produced from the sensor transgene the mobile signal should trigger the production of 22G-RNAs and silence the sensor (Fig 3B).

GFP and mCherry expression in uninfected animals is ubiquitous, but predominately intestinal (Fig 3C). To confirm that the sensor is responsive to silencing in all cells we performed RNAi against GFP or against the Orsay RNA2 fragment. This treatment silences the sensor robustly in all animals, with residual GFP expression in the pharynx only (the pharynx is known to be somewhat RNAi resistant) (Fig 4), confirming that exogenous RNAi silences this sensor systemically. Surprisingly,
however, when we exposed these sensor animals to the Orsay virus, GFP silencing was not observed systemically: although 40% of animals displayed at least one silenced intestinal cell, less than 3% failed to silence all intestinal cells (Fig 3D).

*Orsay virus infection does not produce a mobile siRNA signal*

Given the limited ability of the antiviral RNAi response generated against the Orsay virus to spread between cells, we hypothesized that systemic RNAi may not be important in defending against viral infection. Supporting this view, Guo et al. recently reported results suggesting that indeed Orsay virus levels accumulated to similar levels in N2 and a *sid-1* mutant (35). We confirmed this result, and additionally showed that *sid-2* is dispensable for viral defence (Fig 5A). In order to further study the role of *sid-1* in viral defence we assayed the siRNAs present in Orsay infected animals and their offspring by small RNA sequencing as described above for N2 animals (Fig 5B,C). We could detect no difference in small RNAs between the *sid-1* strain and N2, further confirming that *sid-1* transport of mobile siRNAs is not required for their generation in the context of Orsay virus infection.

If RNAi is not moving from cell-to-cell during infection with the Orsay virus, cases where we observe sensor silencing in more than one cell would only occur due to the virus directly infecting each cell. To test this hypothesis we crossed the sensor into the *drh-1* background, which displays increased susceptibility to viral infection (Fig 6A). *drh-1* sensor animals displayed an increased number of animals with many silenced cells (Fig 6C), suggesting that silencing of the sensor is driven by infection rather than mobile RNAi. While *drh-1* animals are defective in the production of antiviral siRNAs, they do produce them at low levels (30), enough to silence the sensor in infected cells.
To test the lack of cell-to-cell spreading further we crossed the sensor into the *sid-1* background. As *sid-1* are as susceptible to viral infection as N2 (Fig 5A), there should be no difference between the number of silenced cells within individual animals between N2 and *sid-1* sensor animals. However, when we crossed the sensor into *sid-1* mutants (Fig 6B) there was a significant change in the number of animals with multiple silenced cells in the *sid-1* background compared to N2 (Fig 6C). This might indicate that a low level of spreading of silencing from one cell to its direct neighbour does occur in N2. To address this issue in all three strains we followed eight partially silenced animals individually over three days and the GFP silencing never became systemic (Fig 7). Over the three days that we followed the N2 and *sid-1* sensor animals the number of GFP silenced cells rarely changed, indicating that if there is a silencing signal passing from one cell to another it happens very slowly or infrequently. In the *drh-1* background the number of silenced cells increased in more than half of the animals over the three day period. Although not significantly different to N2, this trend is consistent with an increased number of infected cells due to the the higher levels of infection known to be sustained in this background (30).

Taken together these results indicate that, unlike in exogenous RNAi, viral infection does not result in large numbers of dsRNA intermediates able to be trafficked by *sid-1*.

Orsay-derived transgenes are not sufficient to enable inheritance of antiviral siRNAs against the Orsay virus

The lack of inheritance of the antiviral RNAi response against the Orsay virus was surprising given that Flock house virus transgene silencing was reported to be transgenerationally inherited (33). One explanation for this discrepancy could be that
the Flock house virus transgene is carried in all cells, whilst the Orsay virus only
infects intestinal cells. Transgenerational silencing might therefore not occur in the
Orsay virus because there is a requirement for a template, either RNA or DNA, in the
germline in order to transmit a silencing signal. To test this, we asked whether Orsay
virus infection could transmit silencing of the viral sensor transgene, carried in all
cells, into the next generation.

First, we tested whether RNAi induced silencing against either GFP or the Orsay
RNA2 fragment in the P0 generation could result in transgenerational sensor
silencing. We subjected P0 Orsay RNA2 sensor animals to RNAi treatment with
either GFP or Orsay RNA2. Either treatment results in complete sensor silencing as
described previously (Fig 4). Adults were then removed from the RNAi treatment and
the phenotype of the resultant F1 progeny scored after 4 days. Both treatments
resulted in silencing of the GFP transgene in the F1 generation (Fig 8A), showing that
the sensor is capable of being silenced in a transgenerational manner.

To test for transgenerational silencing of the sensor following Orsay virus exposure,
we scored the GFP status in the offspring of eight animals that themselves displayed
partial sensor silencing (and were thus infected with Orsay virus). Orsay virus is not
transmitted vertically (19), so the only virus present on these plates is that carried
within the parent. We performed this experiment in the wildtype sensor, sid-1 and
drh-1 genetic backgrounds. The offspring of wildtype and sid-1 infected parents
showed almost no GFP silencing (and were not significantly different from each
other), whereas drh-1 F1 animals showed significantly more GFP silencing compared
to the wildtype sensor (p<0.001) (Fig 8B). These data are consistent with no
inheritance of transgene silencing following viral infection in any genotype and
horizontal transmission of viral infection and thus de novo sensor silencing in the case of drh-1.

No protection against infection with the Orsay virus in the F1 generation following RNAi

We have shown that exposure of parents to the Orsay virus does not confer resistance in F1 progeny and that Orsay virus-induced sensor silencing does not seem to be inherited. RNAi-induced silencing against the viral sensor can however be passed on to the F1 generation, so therefore we asked whether RNAi-induced silencing in the presence of the Orsay sensor could protect against Orsay virus in the P0 generation, or provide protection against Orsay virus infection in F1 offspring.

We tested a series of regions of the Orsay RNA genome for their ability to protect against Orsay virus replication in RNAi feeding experiments (Fig 8C). Intriguingly, Orsay levels were most affected after RNAi against Orsay RNA1 compared with Orsay RNA2 (Fig 8D). This difference in RNAi efficacy between RNA1 and RNA2 could be due to the fact that RNA1 encodes the RNA dependent RNA polymerase and RNA2 the capsid protein. RNAi against the RdRP is more likely to have a direct effect on viral RNA accumulation within infected cells than RNAi against the capsid, which is more likely to affect later steps of the viral life cycle such as assembly.

We then asked whether there was also a reduction in Orsay RNA levels following concurrent RNAi and viral infection in N2 or an Orsay sensor background (Fig 8E). Because viral levels were most significantly altered in animals feeding on RNAi clones targeting RNA1 rather than RNA2, for this experiment we used an Orsay RNA1 sensor instead of the previously used Orsay RNA2 sensor. The sensor was extrachromosomal instead of integrated but otherwise identically constructed.
Interestingly, while Orsay RNAi reduced the levels of Orsay RNA detected in N2, the presence of the RNA1 sensor resulted in even more RNAi-induced protection against Orsay replication (Fig 8F). It is tempting to speculate that this sensor-associated RNAi “boost” is due to the presence of siRNA molecules (generated from the sensor) already in the cell before the viral entry, thus enabling immediate viral RNA destruction.

To test whether the combination of RNAi against the Orsay virus and the sensor could confer protection in the F1 offspring we bleached the adults from the previous experiment to generate uninfected embryos and then infected them with the Orsay virus (Fig 8E). Despite the large difference in Orsay infection levels between RNAi treated and non-treated sensor animals in the P0 generation, there was no difference in the infection levels of their offspring (Fig 8G, grey). There was also no difference in viral infection levels in the N2 F1 offspring of parents fed on either empty or RNA1 RNAi (Fig 8G, black).

These data show that even in the most extreme case of RNAi and a transgenic viral portion, there is still no evidence for deposition into the F1 offspring of functional small RNA molecules that can protect against Orsay virus infection.

**Discussion**

Two of the most notable aspects of the siRNA pathway in *C. elegans* initiated in response to exposure to dsRNA matching endogenous genes are its ability to spread throughout the animal and its ability to act transgenerationally. There has been much speculation on what the function of these properties is for animals in the wild, with the proposed role of RNAi in antiviral silencing a key candidate. Here we have shown that infection of *C. elegans* with the Orsay virus instigates neither transgenerational
nor systemic silencing. In the absence of any other known naturally occurring
*Caenorhabditis elegans* virus, it is possible that systemic RNAi may function in defense against an
as yet undiscovered infection. Nevertheless our results have important implications
both for the mechanism of systemic RNAi and the biology of small RNA pathways in
*Caenorhabditis elegans*.

The fact that antiviral siRNA induced by Orsay virus infection, in contrast to
siRNA induced by exposure to dsRNA, does not spread between cells may be
explained by differences in the intermediates produced by the two pathways.
Importantly, 22G-RNAs, produced by both pathways, are unlikely to transfer RNAi in
*Caenorhabditis elegans* (12), perhaps because they cannot themselves trigger production of further
22G-RNAs in somatic cells (5, 39). However, there is evidence that a small RNA
species generated by the activity of Dicer/RDE-4 in response to dsRNA generated
from endogenous genes or taken up from the environment is able to spread between
cells (12). Both RDE-4 and Dicer are active on viral dsRNA, thus their activity must
somehow be different when acting on viral dsRNA as compared to other sources of
dsRNA. It is possible that this difference might be due to the requirement of DRH-1
specifically for activity on viral dsRNA, perhaps reflecting a different subcellular
localization of the DRH-1/Dicer complex. An alternative possibility is that the Orsay
virus itself prevents systemic RNAi. Such a situation is well known to occur in plant
viruses, many of which encode suppressors of silencing that prevent cell-to-cell
spreading of silencing (40). Further work will be required to distinguish between
these two possibilities.

Our observations on the non-systemic nature of the antiviral RNAi response to
infection with the Orsay virus are fully consistent with the fact that we detected no
transgenerational protection against infection, and did not detect a strong small RNA signal mapping to the virus in the embryos derived from infected individuals. This is in contrast to robust inheritance of both small RNAs and the silencing phenotype in the case of RNAi-induced silencing of the \textit{dpy-11} gene and inheritance of small RNAs, albeit without an observable phenotype, in silencing of the \textit{unc-22} gene. The lack of systemic RNAi after infection with the Orsay virus thus prevents small RNAs from entering the embryos to sufficient extent to transmit the silencing effect.

The function of RNAi in antiviral defense in plants, fungi and animals, including mammals, has led to the proposal that viral infection was the major driving force behind its evolution. Systemic silencing, which potentially allows the antiviral siRNAs to move ahead of the spread of virus, and transgenerational vaccination of the next generation might seem to be ideal components of an effective antiviral pathway. It is therefore interesting that neither of these two aspects of the \textit{C. elegans} RNAi pathway are employed in targeting the Orsay virus, despite an absolute requirement for cell-autonomous RNAi in antiviral defense. It remains possible that other naturally occurring viruses will be discovered which can instigate systemic or transgenerational responses, in particular viruses that could infect the germline or DNA viruses, which, in plants, appear to be targeted by transgenerational silencing through RNA-directed DNA methylation. It is also possible that, as noted above, the Orsay virus itself could have evolved to prevent systemic silencing, although it is worth noting that the RNAi-competent strain N2 is unlikely to be the natural host of the Orsay virus. Orsay virus was discovered in the strain JU1580, which is deficient for antiviral RNAi (19, 30): thus there may not have been strong selective pressure to evolve resistance to systemic silencing in this virus. However, it remains an interesting possibility that systemic RNAi in \textit{C. elegans} evolved for completely different reasons, linked
potentially to its unusual ability to take up dsRNA from its environment. The answers
to these questions will require deeper sampling of *C. elegans* in its natural
environment to understand better the selective forces acting on the RNAi pathway in
the wild.

**Acknowledgements**

We thank Sylviane Moss for managing high-throughput sequencing. Some strains
were provided by the CGC. This work was supported by Cancer Research UK, the
Wellcome Trust and an ERC Starting Grant to E.A.M. A.A. was supported by a
Herchel-Smith postdoctoral fellowship. P.S. was supported by a Gonville and Caius
College fellowship. M.T. was supported by Cancer Research UK. JLP was supported
by a Wellcome Trust/University of Cambridge 4-year PhD programme in
Developmental Biology. Some strains were provided by the CGC, which is funded by
NIH Office of Research Infrastructure Programs (P40 OD010440).

**References**

1. Tijsterman M, Plasterk RHA. 2004. Dicers at RISC: The Mechanism of
   Annual review of biophysics 42:217-239.
   Result from Unprimed RNA Synthesis and Form a Distinct Class. Science
   315:244-247.
6. Feinberg EH, Hunter CP. 2003. Transport of dsRNA into cells by the
   elegans requires the putative transmembrane protein SID-1. Science
   295:2456-2459.


Figure Legends

Figure 1. Deep sequencing of viRNAs after Orsay virus infection and RNAi. (A) Cartoon showing the pathway of 23 nt Dicer product and 22G production in C. elegans. (B-M) 5' independent small RNA sequencing of P0 and F1 animals after either Orsay virus exposure (B-E) or the indicated RNAi treatment (F-M). P0 animals were assayed as a mixed stage population of predominantly adults, and F1s were synchronized and assayed at three different ages as indicated. Data are shown as sense or antisense and ordered according to the size of the RNA molecule. Y axis is reads per million.

Figure 2. No evidence for inheritance of viRNAs after Orsay virus infection. (A) Shown are the 23 nt sense (left) Dicer products and 22G antisense (right) secondary RNAs from Figure 1 normalized to library size and the level in the P0 generation. (B) Shown is the percentage of P0 and F1 animals displaying dpy or unc phenotype following exposure to RNAi. Error bars show the standard deviation from three (P0) or four (F1) biological replicates. ** p<0.005, t-test. (C) The experimental design for the data shown in (D). (D) qRT-PCR data for the relative levels of Orsay virus four days after exposure in animals whose parents were either infected (+V+V) or uninfected (-V+V) with Orsay virus (Orsay infection of parents was confirmed by qRT-PCR). Data were normalized to gapdh levels and the level of infection in the +V parents. Error bars show the SEM. (E) This graph shows a comparison between Orsay virus infection levels between liquid and agar-based infection protocols in both drh-1 and N2 strains with three different concentrations of virus. Each condition was performed with 5 biological replicates and error bars represent the SEM.
Figure 3. Development of a viRNA sensor. (A) Schematic of viRNA sensor. (B) Cartoon showing the expected phenotype of sensor animals in the absence of Orsay virus (top), and the presence of Orsay virus with (middle) or without (bottom) systemic silencing. Yellow indicates both GFP and mCherry expression. Red indicates only mCherry expression. (C) Orsay sensor showing representative uninfected (left panel) and infected (right panel) animals. Infected and uninfected animal images were taken at the same intensity. (D) The percentage of sensor animals showing the indicated amount of silenced cells in the absence (grey) or presence (black) of Orsay virus. Error bars indicate the SEM for six biological replicates.

Figure 4 Orsay sensor with RNAi. Orsay sensor showing representative animals treated with the indicated RNAi clone. All images were taken at the same intensity.

Figure 5. sid-1 and sid-2 are not required for viral resistance. (A) qRT-PCR showing the relative levels of Orsay virus four days after infection in N2, drh-1, sid-1 and sid-2. drh-1 animals show significantly higher levels of Orsay virus RNA than N2 (p<0.05, t-test), but there is no significant difference between either sid-1 or sid-2 and N2. Data were normalized to gapdh and then N2. (B) Shown are the 23 nt sense (left) Dicer products and 22G antisense (right) secondary RNAs from (C) (sid-1) and Figure 1 (N2) normalized to library size and the level in the P0 generation. (C) 5’ independent small RNA sequencing of P0 and F1 sid-1 animals after either Orsay virus exposure. P0 animals were assayed as a mixed stage population of predominantly adults, and F1s were synchronized and assayed at three different ages as indicated. Data are shown as sense or antisense and ordered according to the size of the RNA molecule. Y axis is reads per million. The 5’ nucleotide is indicated by colour. Red = A, green = C, blue = G and pink = U. (D)
Figure 6. Orsay sensor in sid-1 and drh-1 backgrounds. (A, B) Orsay sensor showing representative uninfected (left panel) and infected (right panel) animals. Infected and uninfected animal images were taken at the same intensity. (C) The percentage of sensor N2 (light grey), sid-1 (grey) or drh-1 (black) animals showing the indicated amount of silenced intestinal cells in the presence of Orsay virus. The amount of intestinal cell silencing differs significantly between both the sid-1 and drh-1 backgrounds and the wildtype background (p< 0.001 in both cases. Fishers exact test). Error bars represent the SEM over six biological replicates.

Figure 7. No systemic sensor silencing following Orsay virus. Horizontal blocks indicate individual infected animals followed over three days – 5 (black), 6 (light grey) and 7 (dark grey) dpi. The x axis indicates the percentage of the animal silenced. Eight animals each were followed for N2 (A), sid-1 (B) and drh-1 (C).

Figure 8. RNAi-induced Orsay virus silencing. (A) This graph shows the percentage of F1 animals with inherited sensor silencing after parental exposure to feeding RNAi against either Empty vector (black), GFP (light grey) or Orsay RNA2 (dark grey). Error bars represent the SEM for two biological replicates. (B) Sensor silencing in the F1 offspring of infected animals (as judged by sensor silencing). The x axis shows the percentage of N2 (light grey), sid-1 (dark grey) and drh-1 (black) animals with the indicated amount of sensor silencing in the intestine. *** p< 0.0005, Fishers exact test. (C) Cartoon showing the position on the Orsay genome of the RNAi clones used in D. and the position of the qPCR amplicons (D) This graph shows the relative levels of Orsay virus (4 dpi) as measured by qRT-PCR in N2 animals fed with the indicated RNAi clone. Error bars represent the SEM for n=3 (R1-1, R1-2, R2-1), n=6 (R1-3, R1-4, R2-2, R2-3) or n=12 (empty) biological replicates (E) Schematic illustrating the experimental design to test for the presence or
absence of viral resistance in the F1 generation caused by previous viral exposure, viral RNAi, or both. (F) This graph shows the relative Orsay virus levels measured by qRT-PCR in P0 animals 4 days after Orsay virus exposure. Animals were either wildtype or carried an Orsay RNA1 sensor transgene and were exposed to either empty vector or Orsay RNA1 RNAi. Exposure to Orsay RNAi in the P0 generation causes resistance to Orsay viral infection in both genetic backgrounds, although the effect is more significant in the sensor background. Data were normalized to gapdh and then N2 empty vector. * indicates P<0.05 *** indicates P<0.0005, t-test. Error bars represent the SEM for three biological replicates. (G) Relative Orsay RNA1 levels in F1 animals. The x-axis shows the RNAi treatment and/or Orsay exposure of their parents. N2 animal are shown in black and RNA1 sensor animals in light grey. There is no significant difference between any treatment or strain. Data were normalised to gapdh and then N2 F1 empty vector. Error bars represent the SEM for three biological replicates.
A double stranded viral RNA

DICER → primary siRNAs: 23mers (sense and antisense)

RdRP → secondary siRNAs: 22Gs (antisense)

B

N2 + Orsay virus

C

F1 0 hrs (embryo)

D

F1 24 hrs (L1 larvae)

E

F1 72 hrs (L4/YA)

F

N2 + dpy-11 RNAi

G

H

I

J

N2 + unc-22 RNAi

K

L

M
A) Percentage of GFP silenced F1 animals

B) Percentage of F1 animals

C) Orsay RNA1

D) Relative Orsay RNA1 level

E) Orsay RNA1 RNAi

F) Relative Orsay RNA1 level

G) Relative Orsay RNA1 level