Detection of Orsay viral replication intermediates reveals spatial and regulatory links to

Caenorhabditis elegans **innate immune responses**

Supraja Ranganathan, P. Joseph Aruscavage, Brenda L. Bass

Department of Biochemistry, University of Utah, Salt Lake City, Utah, USA 84112-5650

Short title: Viral replication intermediates in *C. elegans*

Abstract

For a positive-strand RNA virus, the encoded viral RNA-dependent RNA polymerase (oRdRP)

synthesizes complementary antigenome strand and uses it as a template for amplifying the viral

genome, generating various replication intermediates. Structural proteins and viral genome are

packaged into virions, but the fate of replication intermediates is underexplored. Here, we investigate

Orsay Virus (OV) replication intermediates, including antigenome, oRdRP and double stranded RNA

(dsRNA), using PCR and fluorescence-based imaging in *C. elegans* intestines. As for other positive-

strand RNA viruses, we find that genome is in vast excess of antigenome. Antigenome is only

visualized in cells when using denaturation protocols, indicating basepaired intermediates. OV

antigenome is observed with distinct cytoplasmic and perinuclear localization patterns that depend on

factors required for generation of primary, but not secondary, siRNAs. In both wildtype and RNA

interference (RNAi) mutants, viral dsRNA is observed in the cytoplasm associated with oRdRP,

suggesting cytoplasmic virus replication hubs. Additionally, using antibodies to oRdRP, we observed

spherical structures of ~1μm in diameter defined by oRdRP at their surface; over 75% of infected

wildtype animals show these structures, which associate with mitochondria and autophagosomes in an

antiviral RNAi- and autophagy-dependent manner, respectively. Our study defines new features of OV

replication intermediates in wildtype animals, setting the stage for understanding their connection to the

viral life cycle and host antiviral pathways.

Author Summary

Viruses infect and cause disease in all organisms. Once they enter an organism, or host, viruses make

copies of themselves in a process called replication, and this process generates byproducts that are

recognized as foreign by the host. This signals the host to begin protecting itself using pathways such

as the innate immune response, underscoring the importance of understanding where and when

replication byproducts are generated. Here we utilize *Caenorhabditis elegans*, a microscopic

roundworm, to detect the viral replication intermediates of its naturally infecting pathogen, Orsay Virus

(OV). For the first time, we detect OV antigenome, a byproduct that is used as a template for viral

genome replication. We report a previously uncharacterized antigenome-containing perinuclear granule

that is partially dependent on the antiviral RNA interference (RNAi) pathway, an invertebrate immune

response. We also identify regions of cytoplasmic viral replication and unusually large (~1 micron)

spherical structures lined with the viral RNA-dependent RNA polymerase, an enzyme made by the virus

for its replication. By characterizing important viral replication byproducts, our studies set the stage for

understanding mechanistic connections between viral infection and the host response.

Introduction

Orsay Virus (OV) is a naturally-infecting *C. elegans* pathogen that closely resembles the *Nodaviridae* family of viruses. OV is a positive strand, single-stranded RNA (+ssRNA) virus, with a bipartite genome (oRNA1 and oRNA2) that preferentially infects *C. elegans* intestinal cells where it is predominantly restricted to anterior cells [1–3]. oRNA1 encodes an RNA-dependent RNA Polymerase (oRdRP), and oRNA2 encodes the capsid, δ (delta), and capsid-δ fusion proteins [4].

While details of OV replication and its intermediates are just emerging [3,5], it can be presumed that general features of positive-strand RNA viruses are operative [6,7]. Replication of a positive-strand RNA virus begins with translation of its RdRP from the genomic strand. The RdRP first synthesizes a complementary strand to the genome, the antigenome (-ssRNA), and this is the first RNA replication intermediate. The antigenome is then used as a template by the RdRP to amplify the viral genome [8]. The perfectly complementary strands of genome and antigenome can basepair to form a viral double-stranded RNA (dsRNA), another RNA intermediate of viral replication. Replication of closely related nodaviruses occurs inside membrane-enclosed compartments that protect viral RNA and other replication intermediates from the host antiviral machinery [7]. The structural proteins along with the viral genome they encapsulate are packaged and processed into a mature virion that is then egressed out of the cell.

Some viral replication intermediates are recognized by helicases to elicit an antiviral response. Among vertebrates, the RIG-I-like Receptor (RLR) family of helicases recognize dsRNA to elicit an interferon response. Invertebrates do not have interferon pathways, and invertebrates such as *C. elegans* instead utilize RNA interference (RNAi). In RNAi it is the enzyme Dicer that recognizes dsRNA, using a helicase domain related to that of RLRs. *C. elegans* Dicer (DCR-1) forms an antiviral RNAi complex with DRH-1, a Dicer-related helicase and RLR ortholog, and RDE-4 (RNAi-DEficient 4), a dsRNA binding protein. This antiviral complex cleaves viral dsRNA into 23H (H=A,U,C>G at 5' end) primary small-interfering RNAs (1° siRNAs). The 1° siRNA is then bound by RDE-1 (RNAi-deficient 1), an argonaute protein, and passed to other factors for generation and amplification of 22G (preferentially G at 5' end) secondary siRNAs (2° siRNAs) that are complementary to the viral genome [9,10].

The *C. elegans* antiviral response also involves OV-induced genes (OVIGs) [2,11], a subset of which are also the Intracellular Pathogen Response (IPR) genes [12], a transcriptional response somewhat analogous to the mammalian interferon response, and this requires DRH-1, but not DCR-1 or RDE-4 [13]. DRH-1-mediated OVIG induction occurs after viral replication, but the exact viral intermediate that DRH-1 recognizes to trigger OVIGs is unknown. However, recently OV dsRNA was visualized in association with DRH-1 in *C. elegans* intestines, raising the possibility that this recognition is important for triggering OVIGs [5]. *C. elegans* lacking RNAi factors have increased sensitivity to OV, and these mutant strains are widely used to understand OV replication dynamics and downstream RNAi responses [3]. Given that replication is required to elicit host antiviral responses, it is important to understand the temporal and spatial organization of replication intermediates. The predominantly studied viral replication byproduct is viral dsRNA; other replication intermediates are often ignored leading to an incomplete picture of replication dynamics and antiviral responses, especially in a wildtype background.

Here we detect and characterize three replication intermediates, oRdRP, oRNA1 antigenome, and OV dsRNA, in both wildtype and RNAi mutant backgrounds, capturing key steps of viral replication and their spatial organization. We show that OV is like other +ssRNA viruses in that the viral genomic strand is present in vast abundance relative to the antigenomic strand, in both wildtype and RNAi mutants. Using single molecule FISH (smFISH), we show that the OV antigenome is detectible in cells with genomic strand, and detection is facilitated with denaturation protocols, indicating most antigenome is basepaired. Unexpectedly, we observed antigenomic RNA within perinuclear regions that are partially depleted in strains defective for primary siRNA biogenesis, but not those aberrant for secondary siRNA amplification, setting the stage for connecting OV infection and replication with the downstream antiviral RNAi pathway. Surprisingly, we did not detect RNAi factors associating with the perinuclear-localized antigenome. We observed oRdRP-associated dsRNA puncta revealing possible replication hubs. Infected wildtype intestinal cells also showed oRdRP in lumen-containing spherical structures of ~1μm in diameter, in association with both mitochondria and autophagosomes, and dependent on antiviral RNAi and autophagy pathways. Together, the results reveal new hubs of viral

replication and processing and emphasize the importance of detecting and understanding viral

replication in a wildtype background.

Results

OV genomic strand accumulates significantly more than antigenomic strand in wildtype and

RNAi mutants

OV infection in *C. elegans* is typically measured by RT-qPCR in a strand-agnostic manner and

denoted as viral load, or by monitoring genomic strand accumulation using northern blot analysis. For a

plus-strand virus such as OV, these methods largely reflect levels of the more abundant genome but

offer little information about the less abundant antigenome [1]. To acquire this information, we first

monitored OV-infected animals with strand-specific RT-qPCR. We designed strand-specific probes for

oRNA1 genome or antigenome that each included a unique flanking sequence so that strand-specific

complementary DNA (cDNA) could be quantified with qPCR, using the unique flanking region as a

primer and a second downstream on the respective oRNA1 strand (**Fig 1A**). We also performed

northern blot analysis to complement our strand-specific RT-qPCR method and directly detect viral RNA

abundance.

We in vitro transcribed and purified fragments of genomic and antigenomic strands of oRNA1,

each ~500 nucleotides (nts) long, and used these transcripts to generate standard curves for

quantifying copy number in a strand-specific manner (**S1A and S1B Figs**); these transcripts were also

used to confirm that antigenomic probes were specific and able to detect 10^6 copies of purified ~500nt

antigenome ssRNA by northern blot assay (**S1C Fig**). We then monitored appearance of genome and

antigenome over time, using two infection protocols: one monitoring infection of L3 larvae at 12- and

24-hours post infection (hpi; **Fig 1B**), and a second aimed at quantifying strands after prolonged

infection, by infecting L1 larvae and assaying 48 and 72hpi (**Fig 1C**). For the latter, we chose infection

times that maintained animals in the same reproductive cycle (72hpi) so that populations would not be

contaminated with newly hatched larvae.

For each protocol, we compared infection of wildtype (N2) animals to that observed in antiviral

RNAi mutants, *drh-1(uu60), rde-4(uu71), rde-1(ne219)*, and a helicase point mutant (G492R*)* of DCR-1

(*eri-4(mg375)*) [14,15], referred to here as *dcr-1(mg375)* (**Figs 1D and 1E**). We observed significantly

higher levels of genome than antigenome for wildtype and RNAi mutant strains. Further, for each

infection protocol, with few exceptions, we observed a dramatic and significant increase in oRNA1

genome copy numbers at 24hpi and 72hpi relative to 12hpi or 48hpi, respectively, in both wildtype and

mutant animals. We observed a modest increase in oRNA1 antigenome relative to wildtype in *drh-1,*

rde-4 or *rde-1* mutants, but at early time points this was not observed in *dcr-1(mg375)* mutants (**Figs**

1D and 1E; points relative to pink dotted line). Quantification showed greater than 100-fold increase

in genome over antigenome at 12hpi, and over 1000-fold increase at all other infection times, for both

wildtype and RNAi mutants (**S1D and S1E Figs**). Northern blot analyses confirmed the low levels of

antigenome compared to genome (**S1F and S1G Figs**); controls established that if present antigenome

could be detected, but oRNA1 antigenome was below the limit of detection for wildtype and RNAi

mutants at all time points tested (12-72 hpi). oRNA1 genome was first detected at 24hpi for RNAi

mutants, and increased over time, but was only faintly detected at 72hpi for wildtype strains.

Consistently, levels of genomic RNA were higher in RNAi mutants than wildtype (**Figs 1D and**

1E), and trends matched the increase in viral load as determined in a strand-agnostic manner,

consistent with prior studies [10,13] (**S1H and S1I Figs).** In accordance with published studies, we

observed that the antiviral RNAi mutants, *drh-1*, *rde-4* and *rde-1*, were sensitive to OV and showed

increased viral load relative to wildtype at all timepoints tested, by all methods [10]. While previous

studies have reported the sensitivity of *dcr-1* using deletion mutants or knockdown (RNAi) [10,13], we

report the involvement of the helicase domain of DCR-1. The helicase mutant *dcr-1(mg375)* showed a

delayed response with a moderate increase in viral load, ~10-fold at 12hpi and 24hpi, and ~100-fold at

48hpi and 72hpi (**Figs 1D, 1E, S1H and S1I**).

Prior studies have looked at viral infection in the constitutively infected JU1580 strain, which has

a mutation in DRH-1 and is highly sensitive to OV infection [1,4]. To connect our studies to prior studies

we showed that a high viral load in the JU1580 strain relative to wildtype, as determined using strand-

agnostic protocols, correlated with a robust accumulation of oRNA1 genomic strand by both strand-

specific RT-qPCR and northern blot, while the oRNA1 antigenome levels remained significantly lower,

~1300-fold, (*p-value<0.0001*) (**Figs 1F, S1F and S1G**). This emphasizes that the antigenomic strand is

much lower than the genomic strand even with constitutive infection. To test if the ratio of genome to

antigenome was a property inherent to oRdRP mediated replication, we also monitored a heat shock-

inducible replicon system expressing wildtype (oRdRP^{WT}) or a replication-incompetent mutant

(oRdRP^{mut}) oRNA1 [16]. In the complete absence of infection, we observed a significant increase in

genome and antigenome upon inducing the expression of oRdRP^{WT} but not oRdRP^{mut}, and notably,

antigenome levels remained significantly lower (**Fig 1G**), which was confirmed by northern blot (**S1F**

Fig).

In summary, these studies indicate that the increase in viral load commonly measured using

strand-agnostic methods largely reflects an increase in genomic strand. At the time points tested, for

wildtype and RNAi mutant strains, we found that the genomic strand, the strand that is packaged into

virions, is in far excess of the antigenomic strand, which is only an intermediate, and these ratios are

intrinsic to the oRdRP and its tight regulation by host factors [17,18].

oRNA1 antigenome is visualized only with conditions that denature RNA basepairs

To gain insight into the localization pattern of OV genome and antigenome within infected cells, we

performed smFISH analyses by designing multiple strand-specific fluorescent deoxyoligonucleotide

probes for oRNA1 (27 probes, genomic strand; 48 probes, antigenomic strand). For initial optimization

of the protocol, we used OV infection-sensitive *jyIs8;rde-1* animals. *jyIs8* contains an integrated

transgene that expresses GFP under the control of the promoter of an OVIG, *pals-5*, and was used as

a marker for infection [19]. Uninfected controls established the absence of signal for *pals-5p::GFP* and

oRNA1 strands, while as in prior studies [19], a strong signal in the pharynx (asterisk) in the same

channel as antigenome was observed from fluorescence of the *myo-2p::mCherry* marker included in

the *jyIs8* transgene (**Fig 2A; uninfected**). At 24hpi we saw robust GFP expression in multiple intestinal

cells that typically extended beyond the cell(s) with abundant accumulation of oRNA1 genome. The

multi-cell GFP expression is consistent with prior studies showing distribution of DRH-1 and ZIP-1

beyond infected cells [5,20]. In these OV genome-containing intestinal cells, we observed ~95% of

infected animals had a diffuse cytoplasmic signal of antigenome without distinct puncta (**Figs 2A,**

infected (-) denature and 2B). We hypothesized that antigenome might be hybridized to the genome,

forming the viral dsRNA often observed during viral infection. To test this, we treated animals with

50mM NaOH for ~30 seconds to promote dsRNA denaturation prior to hybridization [21], hereafter

referred to as denature-smFISH (dsmFISH). Indeed, after denaturation we observed ~97% of infected

animals with oRNA1 antigenome punctate signal (**Figs 2A, infected (+) denature and 2B**). This

indicated that most of the oRNA1 antigenome was either basepaired with the genome or had extensive

intramolecular secondary structure. We used the dsmFISH denaturing conditions for subsequent

experiments.

Since the diffuse cytoplasmic signal observed using probes to the antigenome was similar to the

pattern of the oRNA1 genome signal, and because we anticipated that antigenome, as for other

positive-strand RNA viruses [7,22,23], would be localized to discrete replication compartments, we

considered the possibility that the diffuse antigenome signal was a consequence of bleed though from

the genome channel. Thus, we monitored antigenome in infected animals that had not been probed for

genome, and vice-versa (**Figs 2C and 2D)**. We observed the diffuse signal in the antigenome channel

even when we only probed for genome, indicating bleed-through. In subsequent experiments we

considered the diffuse cytoplasmic signal as nonspecific background. However, a lower level of

cytoplasmic signal, and importantly, distinct puncta, were specific to antigenomic probes (**Fig 2C, white**

arrowhead).

oRNA1 antigenome localizes to perinuclear foci upon infection

We focused on the understudied antigenomic strand and its distribution during OV infection. At

24hpi, in both *jyIs8* (wildtype) and *jyIs8;rde-1* mutant backgrounds, we observed infected intestinal cells

with robust genome signal as well as distinct antigenome puncta (**Fig 3A**). We observed that *rde-1*

mutants exhibited more infected cells than wildtype as quantified by cytoplasmic genome signal (**Fig**

3B). Strikingly, we observed a distinct perinuclear localization of antigenomic RNA, emphasized by line

scan analysis, in both wildtype and *rde-1* mutant strains (**Fig 3A**). Quantification showed that in

wildtype or *rde-1* mutant strains, ~83% or ~78% of infected animals exhibited perinuclear localization,

respectively (**Fig 3C**). The perinuclear antigenome puncta lacked an obvious colocalizing genome

signal, but the line scan showed a peak of genomic signal closely associated (**Figs 3D and 3E**). While

the perinuclear antigenome puncta was the most common pattern observed, multiple biological

replicates (≥10) revealed other patterns in wildtype infected animals (**Figs 3F-J**). For example, close

association of antigenomic puncta with genome was observed in the cytoplasm (**Figs 3F and 3G**). In

other cases, antigenomic RNA was observed as concentrated cytoplasmic foci surrounded by genome

signal (**Figs 3H, white arrowhead and 3I**). Given that antigenomic strand is used as a template for

viral replication to generate viral genome, possibly the closely associated genome and concentrated

antigenome puncta could indicate active replication hubs in the infected cytoplasm. However, in

infected animals that only exhibited genomic signal in the lumen **(Fig 3J)**, as expected in animals that

have released the packaged virions [2], we did not observe any cells with antigenomic signal, indicating

antigenomic strands may be degraded, processed through efficient antiviral defense mechanisms, or

below limits of detection. These studies allowed the first visualization of the antigenomic strand in OV-

infected *C. elegans* and also revealed its distinct localization patterns.

Primary siRNA biogenesis, but not secondary siRNA amplification, is partially required for

perinuclear localization of antigenome

A key aspect of antiviral defense in *C. elegans* is cleavage of viral dsRNA by the antiviral

complex, comprised of DCR-1, DRH-1, and RDE-4 [24]. The antiviral complex processes viral dsRNA

to produce 1° (23H) siRNAs [25], which are bound by RDE-1 for presentation to 2° (22G) siRNA

amplification factors. Mutations or deletions in DCR-1, DRH-1 or RDE-4 prevent accumulation of both

1° and 2° siRNAs, while RDE-1 mutants accumulate primary siRNAs but lack secondary siRNAs [10].

Given the key role of these RNAi factors in antiviral defense, we wondered about their relationship to

OV replication intermediates. We infected synchronized L1 larvae of RNAi mutants, *dcr-1(mg375), drh-*

1(uu60), rde-4(uu71), rde-1(ne219), as well as wildtype (*N2*), for 24 hours and performed dsmFISH. As

observed previously (**Fig 2A, uninfected**), a minimal background of diffuse fluorescence in the

antigenome channel in uninfected animals was observed for all strains (**S2 Fig**). At 24hpi, both wildtype

and RNAi mutants showed abundant genome signal in one or more intestinal cells, with significantly

more infected cells in RNAi mutants (**Figs 4A, G+AG column 1, and 4B**). Consistent with our previous

experiments, we observed distinct perinuclear localization of antigenome in wildtype (~81% animals) or

rde-1 mutants (~83% animals) (**Figs 4A and 4C, *N2, rde-1***). In antiviral RNAi mutant animals, we

observed a partial reduction in animals exhibiting perinuclear localization of antigenome (**Figs 4A and**

4C, *dcr-1, drh-1, rde-4*). While antiviral RNAi mutants cannot process viral dsRNA as efficiently as

wildtype, some non-processive activity remains, as evidenced by siRNA reads that map to ends of the

viral genome/antigenome [10,26]. Possibly the partial reduction in perinuclear puncta relates to this

incomplete loss of activity. MUT-16 is a scaffolding component required for mutator foci formation in the

germline, sites where secondary siRNA amplification occurs [27,28]. MUT-16 is also required for

secondary siRNA amplification during OV infection [29]. We used a *mut-16(pk710)* strain to test if this

strain phenocopies *rde-1* mutants with respect to perinuclear localization. We observed that over 85%

of infected *mut-16* mutants at 24hpi exhibited perinuclear localization of antigenome, not significantly

different from wildtype or *rde-1* mutant backgrounds (**Figs 4A and 4C, *mut-16***). Since *rde-1* or *mut-16*

mutant animals, which accumulate 1° siRNAs but lack 2° siRNAs, show robust perinuclear localization

of antigenome, our experiments suggest perinuclear localization of the antigenome requires wildtype

accumulation of 1° siRNAs but is unaffected by aberrant accumulation of 2° siRNAs. Together these

results indicate that the signal from the perinuclear-localized antigenome in some way depends on

proper function of the three factors of the antiviral RNAi complex.

OV antigenome perinuclear puncta do not colocalize with antiviral RNAi factors

Given the partial loss of perinuclear antigenome signal in animals with deficiencies in the antiviral

complex, we considered the possibility that factors of the antiviral RNAi pathway (**Fig 5A**) might colocalize

with the perinuclear antigenome signal. We took advantage of existing strains for DCR-1, RDE-1 and

DRH-3, a helicase related to DRH-1 that is involved in 2° siRNA amplification, that were tagged at the

endogenous locus with 3XFLAG::GFP. We observed a diffuse distribution of DCR-1, RDE-1 and DRH-3

without infection (**Figs 5B, 5C and 5D**). As expected from prior studies [28], in uninfected and infected

animals we observed DRH-3 localized at perinuclear foci in the germline which served as an internal

control (**Fig 5D**).

For all GFP-tagged proteins, DCR-1, RDE-1 and DRH-3, at 24hpi we observed distinct

cytoplasmic puncta in cells with genomic signal (**Figs 5E, 5G and 5H**), and these puncta were clearly

separated from perinuclear antigenome puncta, as confirmed by line scan for DCR-1 (**Figs 5E and 5F**).

Notably, DRH-1, a known interactor of DCR-1, also exhibits cytoplasmic punctate distribution in OV

infected cells [5]. The pattern for DCR-1 was observed in 100% of infected animals, and for RDE-1 and

DRH-3, in around 50% of infected animals. Thus, although we observed that mutations in factors

involved in the production of 1° siRNAs, but not those required for 2° siRNA amplification, showed

reduced number of infected animals with perinuclear antigenome (**Fig 4C**), at least for the factors

tested, we do not observe the factors themselves localizing to the perinuclear region.

dsRNA accumulates during OV infection and associates with oRdRP

Positive-strand viruses amplify their genome using the antigenome as template [8], and viral

dsRNA arises during this process. dsRNA is often visualized using dsRNA-specific antibodies [30], and

to understand if dsRNA accumulation varies in wildtype and RNAi mutants, we used the J2 dsRNA-

specific antibody with a dot blot assay, first showing that we could detect as low as 50pg of purified 800

basepair dsRNA (**S3 Fig**). We then collected total RNA from OV-infected (72hpi) and uninfected

samples and again used the dot blot assay to monitor dsRNA accumulation. Control experiments

showed that dsRNA was not detected without OV infection, and consistent with the fact that wildtype

animals have efficient antiviral RNAi, we also did not observe dsRNA accumulation in wildtype animals

(**Fig 6A**); however, given the limited sensitivity of this assay, we cannot rule out the existence of low

levels of dsRNA in the wildtype background. Importantly, we detected dsRNA accumulation in all RNAi

mutants tested (**Fig 6A**).

To evaluate whether the dsRNA signal was from viral or endogenous dsRNA, we looked for oRdRP

colocalized with dsRNA in infected cells. We infected *jyIs8* (wildtype) or *jyIs8;rde-1* animals, picked

GFP-positive infected animals and performed intestinal immunofluorescence assays using antibodies

against oRdRP and dsRNA (J2). We observed that *pals-5p::GFP* expressing infected intestinal cells

showed both oRdRP and dsRNA puncta, colocalized, in both *jyIs8* and *jyIs8;rde-1* animals (**Fig 6B; red**

arrowhead). We also observed a strong nuclear dsRNA signal in oRdRP containing *pals-5::GFP*

expressing *jyIs8* intestinal cells that was not observed in uninfected animals or *jyIs8;rde-1* mutants (**Fig**

6B, merge zoom). The dsRNA that is colocalized with oRdRP is consistent with viral dsRNA being

produced during replication, while the nuclear dsRNA more likely reflects endogenous intronic dsRNA

induced in infected animals [25,31].

Interestingly, apart from oRdRP puncta, we noticed distinct oRdRP-containing spherical structures

in the *pals-5p::GFP* positive intestinal cells (**Fig 6B, merge zoom, white arrowhead**). Over 75% of

pals-5p::GFP positive wildtype animals exhibited at least one spherical oRdRP signal, with the rest only

showing RdRP puncta (*p=0.0046*); less than 50% of *rde-1* mutant animals exhibited oRdRP spheres.

We quantified the total number of oRdRP spheres per animal and observed that wildtype animals

showed between 5 to 58 spheres with an average diameter of 1.023μm (**Figs 6C and 6D**). The number

of spheres per animal was significantly lower in *jyIs8;rde-1* animals (*p=0.0424)*, with 0 to 25 spheres

per animal and an average diameter of 1.122μm (*p=0.0154*), slightly higher than the observed diameter

in wildtype animals. While we did not observe any dsRNA puncta inside the oRdRP spherical

structures, we did find a few spheres closely associated with dsRNA puncta (**Fig 6B, merge zoom,**

yellow arrowhead).

oRdRP spherical structures associate with mitochondria and autophagosomes

Given that the oRdRP signal was present at the periphery of the spheres, we predicted that oRdRP,

similar to other *Nodaviridae* RdRPs [32], has transmembrane domains. We utilized four different

transmembrane domain prediction programs [33–37] and observed two distinct N-terminal

transmembrane domains, suggesting oRdRP is associated with membranes in infected cells (**S4 Fig)**.

To understand the nature of the spheres, we first tested whether the spheres associated with lipid

droplets. In *C. elegans* intestines, lipid droplets are ~1μm in diameter with a phospholipid monolayer

[38,39]. Using a neutral lipid stain, we observed a reduction in the number of lipid droplets in infected

intestines at 24hpi, relative to uninfected controls, consistent with other studies in the literature (**S5 Fig**)

[1,40]. However, while we observed lumen-containing oRdRP spheres in infected intestines, we did not

observe lipid stain within lumens, indicating that oRdRP does not associate with the membrane of lipid

droplets.

The *Nodaviridae* family of viruses utilize mitochondrial membranes to form compartments that aid in

replication [7,23]. To test whether oRdRP spheres were mitochondrial associated, we infected strains

containing the inner membrane protein *timm-50* tagged with *wrmScarlet* [41] and performed

immunofluorescence using oRdRP antibody at 24hpi. In infected intestinal cells, we observed distinct

oRdRP along with closely associated *wrmScarlet::timm-50* signal, also confirmed by line scan analysis,

consistent with an association of oRdRP spheres with mitochondrial membrane (**Figs 7A, 7B**).

Since oRdRP does not get packaged into virions, it seemed possible that oRdRP spheres

associated with autophagosomes as part of their degradation. Autophagosomes are multi-membrane

structures that are induced during autophagy, a degradation pathway for organelle turnover, as well as

for clearing viruses and viral proteins [42,43]. An important step of this degradation pathway is the

formation of spherical autophagosomes mediated by ATG-3 [44,45]. ATG-3 also plays a role in OV

infection at a step downstream of replication [46]. To test if the formation of oRdRP spheres is

dependent on ATG-3 we used the *atg-3(bp412)* mutant. At 24hpi, we observed significantly fewer

oRdRP spheres per infected animal, relative to wildtype (**Fig 7C**), phenocopying results from *rde-1*

mutants (**Fig 6B**). This is consistent with a connection between oRdRP spheres and the autophagy

pathway.

We then wanted to test whether the oRdRP spheres were themselves autophagosomes. We

utilized an intestinal autophagy flux reporter that expresses *GFP::LGG-1::mKate2* to mark

autophagosomes by the presence of *GFP::LGG-1* and absence of mKate2 [44]. Without infection, we

did not observe oRdRP staining but saw multiple *GFP+/mKate2-* puncta in *C. elegans* intestines,

indicating autophagosome formation (**Fig 7D, uninfected**). We chose a GFP+ punctum at random and

showed with a line scan a clear GFP signal, but no oRdRP signal in uninfected intestines (**Fig 7E, top**

panel). At 24hpi we observed abundant GFP+/mKate2- puncta, consistent with previous studies [19],

and lumen-containing oRdRP spheres in intestines (**Fig 7D, OV infected**). Using line scans and

quantification, we observed that ~50% of oRdRP spheres contained GFP signal in their lumen (**Fig 7E,**

bottom panels, a), while remaining spheres lacked this signal (**Fig 7E, bottom panels, b**). There were

also GFP::LGG-1 puncta that were not associated with oRdRP signal (**Fig 7E, OV infected, white**

arrowhead). Thus, a portion of oRdRP spheres are associated with autophagosomes, possibly

containing oRdRP destined for degradation, but as yet, the reason for the unique spherical structures is

unclear.

Discussion

The abnormal morphology of the intestine in RNAi mutant *C. elegans* after infection with OV has

been visualized with transmission electron microscopy [1], and the OV genome and oRdRP have been

visualized using smFISH and immunofluorescence [2,3]. Here we add to this understanding by

characterizing three OV replication intermediates: oRdRP, the antigenome, and dsRNA (**Fig 8**). Using

quantitative, strand-specific methods, we demonstrate that genome is in vast excess of antigenome

(**Fig 1**), as found in other positive-strand RNA viruses [1,21,47–49]. By utilizing a helicase mutant of

DCR-1 (*dcr-1(mg375)*), we confirm the importance of helicase function to antiviral RNAi and efficient

antiviral defense (**Figs 1 and S1**), as suggested previously with in vitro experiments [24]. By applying

denaturation protocols, we visualize the lowly abundant antigenome in intestinal cells (**Fig 2**), observing

both cytoplasmic and perinuclear localization (**Figs 3D-J**), with the latter affected by deficiencies in

factors that affect generation of 1°, but not 2°, siRNAs (**Fig 4**). While genomic RNA was not observed to

localize in the perinuclear region, it exhibited close association with the antigenome (**Fig 3**). The closely

associated genome-antigenome puncta in the cytoplasm possibly indicate replication hubs, as does the

observation of oRdRP associating with dsRNA puncta in the cytoplasm of infected cells (**Fig 6B**).

Notably, in wildtype animals, we also observed oRdRP at the surface of lumen-containing spheres, and

a decrease in the number of spheres in autophagy mutants suggests spheres may be related to host

clearance of viral byproducts (**Figs 6B and 7C-7E**). Our study offers an example of an invertebrate viral

infection model and sets the stage for a more complete spatial and temporal understanding of the OV

life cycle.

Intercellular communication during OV infection

In vertebrates, viral infection triggers the production and extracellular release of interferons,

which bind to cell surface receptors of neighboring cells to activate intracellular kinases that

phosphorylate STAT transcription factors, leading to their dimerization and translocation to the nucleus

where they induce transcription of Interferon Stimulated Genes (ISGs) to elicit antiviral defense [50,51]. In our studies of the invertebrate *C. elegans*, we also observed an intercellular response, whereby cells that did not contain viral RNA but were proximal to those that did, showed expression of *pals-5*, a member of the OVIGs, whose transcription is induced by OV infection (**Figs 2A and 3A**). *sta-1*, an ortholog of mammalian STATs, also plays a role in OV infection by regulating the expression of antiviral genes [52]. While DRH-1 and ZIP-1 have been observed to be activated in neighboring cells [5,20], we speculate that, as for STATs in mammalian cells, *sta-1* may also be activated in neighboring cells to regulate OVIGs, thereby conferring resistance to OV infection in these cells.

Why is the antigenome localized to the perinuclear region?

During infection, the antigenome is typically restricted to regions of viral replication [53]. Surprisingly, using dsmFISH, we observed ~80% of OV-infected wildtype animals with perinuclear localized antigenome, without the presence of other signatures of replication, such as oRdRP, genome, or dsRNA (**Figs 3A and 6B**). In the *C. elegans* germline, perinuclear regions are hubs for RNAi machinery [28,54], but so far, such perinuclear granules have not been observed in somatic tissues. However, given the overlap of factors observed in germline perinuclear puncta and those involved in antiviral RNAi [27,28,55], it is perhaps not surprising that we observed a connection of the intestinal perinuclear-localized antigenome and the antiviral RNAi pathway. Specifically, we observed a decrease in the percentage of infected worms with perinuclear-localized antigenome in animals with deficiencies in factors of the antiviral complex, known to affect levels of 1° siRNAs (DCR-1, DRH-1, RDE-4) (**Fig 4**). Although the antiviral complex is necessary for efficient antiviral defense [24], mutations of *drh-1* or *rde-4* typically do not completely eliminate production of 1° siRNAs [10]. For example, mutations in *drh-1* often show siRNAs at the termini of the genome and antigenome, with loss of internal siRNAs, consistent with a loss of processive cleavage [10,26]. So, the partial reduction in antigenome perinuclear localization could be due to the diminished siRNA production of these mutant animals. By contrast, animals with deficiencies in antiviral RNAi factors that affect levels of 2° siRNAs (RDE-1, MUT-16) showed wildtype levels of perinuclear localized antigenome (**Fig 4**); this is noteworthy given

that MUT-16, a scaffolding protein, is required for mutator foci formation, a perinuclear granule in the

germline [28].

We also did not observe the factors themselves, DCR-1 or RDE-1 (**Figs 5E-H**) localized in the

perinuclear region, suggesting that the perinuclear puncta are not hubs for generation of siRNAs.

Consistent with previous reports [28], we also observed that DRH-3, required for 2° siRNA

amplification, did not localize to perinuclear foci in infected intestinal cells even with OV infection (**Fig

5H**). There are other factors involved in steps in between RDE-1 loading and 2° siRNA amplification,

such as RDE-8 [55], and also in the subsequent targeting of the genome for degradation, which

requires SAGO-2 [56]; it remains possible that these factors or others will be associated with the

perinuclear antigenome.

As evident from our strand-specific RT-qPCR assays, we observed a moderate increase in the

antigenomic strand abundance in RNAi mutants relative to WT (**Figs 1D and 1E**). Thus, it is also

possible that the loss of antigenomic perinuclear signal in primary RNAi mutants might correlate with an

increased antigenomic signal in the cytoplasm, suggesting an impaired localization (**Fig 4A**). While

images appear to suggest this, further quantification is necessary to conclude whether the increased

cytoplasmic signal compensates for the signal from the perinuclear region in these mutants.

Finally, we note that the smFISH probes we used would, in theory, also be complementary to

siRNAs. During antiviral RNAi, 1° siRNAs are generated from both strands of dsRNA during cleavage

by the DCR-1-DRH-1-RDE-4 complex, followed by association of RDE-1 with one strand of the siRNA.

Since we observed a perinuclear signal only with the antigenome probe (**Figs 3D and 3E**), if the

perinuclear signal resulted from hybridization to siRNAs, it seems most likely to arise from the single-

stranded siRNA that is delivered by RDE-1 to the viral genome for subsequent 2° siRNA amplification

by the host RdRP, RRF-1 [29,57]. However, we did not observe an effect on perinuclear antigenome

signal in animals lacking RDE-1 (**Fig 4),** nor a colocalization of RDE-1 with the perinuclear antigenome

puncta (**Fig 5G).** Further, while we cannot rule out the possibility that the perinuclear signal is due to

hybridization with an antisense siRNA, the 48 probes used to detect antigenome were uniformly

distributed along the length of the antigenome, and if it occurred, spurious complementarity to 2°

siRNAs would likely be to only part of the siRNA, and thus, unlikely to generate a robust signal. That

said, we cannot completely rule out this possibility.

What is the nature of dsRNA induced during OV infection?

During infection by both DNA and RNA viruses, dsRNA accumulates in infected cells, typically in

the cytoplasm [58]. The large majority of existing studies detect dsRNA using commercially available

antibodies [30,59], and the dsRNA is inferred to be of viral origin because it correlates with infection,

and in some cases is observed associated with the viral RdRP [60]. Indeed, using an antibody to

dsRNA, we and others have observed dsRNA accumulation in OV-infected intestinal cells (**Fig 6B;** [5]).

In our experiments, we were only able to detect antigenome upon denaturation (**Fig 2A**), suggesting

the antigenome is basepaired, either intramolecularly or in dsRNA formed between the genome and

antigenome. Previous observations of oRdRP show a cytoplasmic distribution in OV-infected cells [3],

and in our studies, in wildtype and *rde-1* mutant animals, we find cytoplasmic dsRNA puncta associated

with oRdRP, consistent with a viral origin (**Fig 6B**). These dsRNA-associated oRdRP puncta are

possible viral replication hubs, similar to previous observations in other +ssRNA virus infections [47,60].

We also observed closely associating genome and antigenome cytoplasmic puncta suggestive of

replication hubs (**Figs 3F and 3H**). Future studies will also focus on identifying colocalized antigenome

and oRdRP to confirm the presence of cytoplasmic replication hubs. dsRNA puncta that lack

association with oRdRP could be partially processed dsRNAs or siRNAs.

As in prior studies that used antibodies to detect dsRNA after viral infection of mammalian cells

[58], we also observed dsRNA in the nucleus. A robust nuclear J2 signal was observed only after viral

infection (**Fig 6B, jyIs8, dsRNA**), in *pals-5p::GFP* positive cells, but not in neighboring *pals-5p::GFP*

negative cells. While it is possible that this dsRNA is of viral origin, we favor the idea that it derives from

infection-induced transcription of endogenous genes that contain intronic dsRNA, which are prevalent

in many animals [31]. Among the differentially-expressed genes produced during OV infection [2,11],

some do contain introns that encode dsRNA, such as *zip-1* and *uggt-2*, as defined in Reich et.al. [25].

Interestingly the nuclear dsRNA signal is observed in wildtype-infected nuclei but not in *rde-1* animals

(**Fig 6B**), possibly due to their inefficient antiviral response. This also highlights the importance of

investigating antiviral responses in a wildtype background, since unique host responses to OV may not

always be evident in mutant strains.

What are oRdRP spheres?

Prior studies have only visualized oRdRP in RNAi mutant backgrounds at late larval stages,

possibly explaining why these spherical structures were missed previously [3]. While typical viral

replication organelles are ~300nm in diameter, we observed oRdRP-rimmed mitochondrially-associated

spheres (**Figs 7A, 7B**), that were larger, ~1μm in diameter (**Fig 6D**), and in contrast to replication

organelles, lacked association with dsRNA (**Fig 6B**). Further, even though *rde-1* mutant animals are

more permissive to virus, they exhibited reduced oRdRP spheres (**Figs 6B and 6C**), consistent with the

idea that spheres are not replication organelles. While higher resolution analyses are required to

determine the nature and function of oRdRP sphere interaction with cellular organelles, one intriguing

possibility is that these are structures associated with viral clearance.

Endoplasmic reticulum (ER) networks, specifically rough ER, are predominantly found in the

region surrounding the nucleus, often connected to the outer nuclear membrane. ER and ER quality

control (ERQC) mechanisms are hijacked by viruses to aid in their replication and assembly [61,62].

Notably, oRNA1 and capsid proteins also associate with ER during infection [63], suggesting ER as a

site for translation of viral proteins and its packaging. Consistent with this, at 24hpi, we observed a

distinct peak in genomic strand surrounding the nucleus (**Fig 3**). Given that oRdRP does not get

packaged, it must be cleared by the host. One possibility is that the oRdRP spheres we observe at

24hpi (**Fig 6B**) are formed from the remnants of replication sites targeted by the host for clearance. In

other viral infection models, viral RdRP is ubiquitinated and degraded through autophagy or

proteosome degradation pathways [43], and between 12hpi and 24hpi with OV, genes related to

ubiquitin-proteasome pathways are induced [2,11]. We observed that mutation of ATG-3, a protein

involved in the formation of autophagosomes, or RDE-1, a protein involved in ER-associated silencing

(ERAS), caused a reduction in the number of oRdRP spheres (**Figs 6C and 7C**), suggesting a link to

autophagy and RNAi/ERAS, respectively. In *C. elegans*, infection with OV also induced GFP::LGG-1

puncta, which showed partial association with ubiquitin puncta [19]. Given that some oRdRP spheres

themselves associate with autophagosomes (**Figs 7D and 7E**), it will be important in future studies to

determine whether or not the oRdRP in spheres is ubiquitinated by the host machinery and targeted for

degradation through autophagy post viral replication.

Materials and Methods

***C. elegans* growth and maintenance**

All *C. elegans* strains were cultured at 20°C under standard conditions unless specified [64]. The list of strains used in this study can be found in S1 Table. Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440).

Orsay virus filtrate preparation

OV filtrate was prepared, and viral titer calculated, as described [16]. Briefly, JU1580 strain was cultured in S medium [55] and infected with OV for 3 days. Following infection, supernatant was collected and passed through a 0.22μm filter. The resulting filtrate was aliquoted and flash frozen at -80°C for long term storage. To calculate viral titer, *rde-1(ne219)* animals (1000 L1 per plate) were infected with serial dilutions (10-fold dilutions prepared in M9 buffer) of the viral filtrate.

Orsay virus infection

For viral load, strand-specific PCR (ss-qPCR), northern blot and dot blot experiments: Strains were grown to gravid adults in liquid medium. Gravid adults were bleached (10% NaOH, 20% bleach), and embryos were synchronized in M9 buffer overnight at 20°C. For L3 infection experiments, 2000 synchronized L1s were plated in OP50-containing NGM plates and grown for 39 hours at 20° and infected with 0.1X OV for either 12 hours or 24 hours. For L1 infection experiments, 2000 synchronized L1s were plated in OP50-containing NGM plates and infected with 0.1X OV for 48 or 72 hours. The resulting infected and uninfected animals were washed in M9 buffer 4 times and collected in Trizol for RNA extraction.

For intestinal dissection: ~200 synchronized L1 animals were transferred to a plate containing OP50. Animals were incubated for 44-48 hours until they reached late larval stage (L3/L4). Animals were infected with OV (0.1X or 1X) for 24 hours and collected for dissections.

For smFISH experiments: ~300-500 synchronized L1s of respective strains were plated on OP50-containing plates and infected with OV (1X) at 20°C. Infected animals were collected after 24 hours of infection.

RNA extraction, cDNA synthesis and qPCR

Total RNA from worms was collected using Trizol-chloroform-isopropanol extraction. Briefly, animals

frozen in Trizol were thawed, and chloroform was added. Aqueous layer was separated, and RNA was

precipitated using 100% isopropyl alcohol by incubating at room temperature for 30 minutes. The

resulting pellet was washed and treated with DNase. DNase-treated RNA was then purified using Zymo

RNA pure column as per manufacturer's instructions. The resulting total RNA was used for all

downstream analyses. cDNA was synthesized using Applied Biosystems cDNA synthesis kit as per

manufacturer's protocol for all experiments involving random hexamers. qPCR was performed using a

QuantStudio3 instrument. Primers for qPCR are listed in S2 Table. Ct values were normalized to levels

of *cdc-42*, and fold change was calculated using the $\Delta\Delta$Ct method [66].

Strand-specific RT-qPCR

We used strand-specific probes designed to bind the middle of one of the two Orsay RNA1 (oRNA1)

strands; each probe also included a unique adapter sequence that did not bind within the viral genome

or antigenome. To synthesize first strand cDNA, we incubated the probe with RNA and 10mM dNTP at

60°C for 10 mins, followed by reverse transcription at 37°C for 1 hour using the Applied Biosystems

cDNA synthesis kit; enzyme was inactivated at 85°C for 20 minutes. We then used this strand-specific

cDNA for qPCR. The unique adapter sequence was used as a forward primer combined with a reverse

primer complementary to a region on the same strand of oRNA1, for qPCR. Primers are listed in S3

Table.

Dot blot assay

Dot blot assays were performed as described [30] with slight modifications. Briefly, 500ng of total RNA

was diluted to 4μl and carefully spotted onto a supercharged nylon membrane. Once dried, the

membrane was crosslinked under UV light (UV Stratalinker 1800, auto-crosslink setting). The cross-

linked membrane was blocked in blocking buffer (1X PBS, 0.05% Tween-20, 50mg/ml sheared salmon

sperm DNA, 5% skim milk) for 1 hour at room temperature (RT). The membrane was incubated with

primary antibody (Anti-dsRNA:J2, 1:2000) overnight at 4°C. The membrane was then washed thrice

with 1X PBST (1X PBS, 0.05% Tween-20) for 10 minutes at RT. The membrane was then incubated

with secondary antibody (Anti-mouse IR dye 800CW, 1:20,000) for 1 hour at RT. The membrane was

washed twice with 1XPBST for 10 minutes at RT, followed by two washes with 1X PBS for 10 minutes

at RT. The membrane was then imaged using a LI-COR Odessey Infrared Imager. Antibodies were

diluted in antibody dilution buffer (1X PBS, 0.05% tween-20, 2% skim milk). In-vitro synthesized and

gel-purified ~800bp dsRNA was used as control for the dot blot assay.

Intestine dissection, immunostaining

Dissection and immunostaining protocols were based on [56] with modifications as below. Briefly, GFP+

infected animals were picked onto 20μl of 5μM levamisole. Worms were dissected using sterilized

surgical blades and transferred to 1X M9 buffer. Dissected intestines were washed twice with 1X M9,

twice with 1X PBST and fixed in 4% paraformaldehyde at RT for 15 minutes. Fixed intestines were

washed thrice with 1X PBST and blocked using a blocking solution (1X PBS, 0.5% Tween-20, 5%

Bovine Serum Albumin, 0.5% Sodium azide) at 4°C overnight. Following blocking, intestines were

incubated with Anti-oRdRP antibody (gift from Dr. David Wang [3]) and Anti-dsRNA (J2) antibody for 1-2

hours at RT. Unbound primary antibodies were washed off with 1X PBST, followed by incubation with

secondary antibodies for 1-2 hours at RT. This was followed by two washes with 1X PBST and one

wash with 1X PBST containing Hoechst (0.1ng/mL). For lipid droplet staining, LipidSpot™ 610 Lipid

droplet stain was diluted in 1X PBST and incubated for 30mins before Hoechst staining.

Immunostained intestines were stored in 1X PBST at 4°C until mounting and imaging. Antibodies were

diluted in blocking solution as per manufacturer's recommendations. Antibody information is listed in S3

Table.

smFISH

~200 synchronized L1s of *jyIs8* or *jyIs8;rde-1* were plated on OP50-containing plates and infected with

OV at 20°C. Infected animals were collected after 24 hours of infection. Animals were washed with 1X

M9 buffer and 1X PBST (1X PBS, 0.05% Tween-20) and then fixed in 4% paraformaldehyde for 15

minutes at RT. Fixed animals were washed thrice with 1X PBST, followed by permeabilization in 70%

ethanol in 1X PBST for at least 2 hours at 4°C. Permeabilized worms were washed thrice with 1X PBST

to remove residual ethanol. If applicable, denaturation of dsRNA was performed by treating worms with

50mM sodium hydroxide for ~30 seconds at RT [21] and immediately washing twice with 1X PBST. This

was followed by a wash with 2X SSC buffer (Saline-Sodium Citrate), and then animals were pre-

hybridized in 1 mL of Wash Buffer A (Stellaris® RNA FISH Wash Buffer A with nuclease-free water and

10% formamide). Samples were then hybridized to probes for the genome (27 probes) and antigenome

(48 probes) (1μl of 12.5μM of each probe mix) in 100μl of hybridization buffer (Stellaris® RNA FISH

Hybridization buffer with freshly added 10% formamide) per sample. Samples were incubated at 37°C

overnight at 1200rpm (vortex). Post-hybridization, Wash Buffer A was directly added to samples

followed by incubation at 37°C for 30 minutes (vortex). Pellet was washed with Wash Buffer A with

Hoechst (0.1ng/mL) at 37'C or 30 minutes (vortex), followed by Stellaris® RNA FISH Wash Buffer B for

5 mins at RT on the nutator and then washed once with 1X PBST. Hybridized samples were stored in

1X PBST at 4°C until imaging. The sequences of the smFISH probes are listed in S4 Table.

Imaging

Animals were mounted onto 2% agarose pads using Vectashield mounting solution. Images were

captured using a DeltaVision Widefield Microscope with a 60X (oil) objective, Numerical Aperture

(NA=1.42), equipped with a sCMOS digital camera using the Blue-Green-Orange-Far Red channels or

Blue-Green-Red-Far Red channels. Multiple Z-slices spanning the entire thickness of the intestine with

Z-stacks 0.2μM apart were captured. Images were captured and deconvolved using the softWoRx

image processing software using standard settings. Individual channels were minimally adjusted in FIJI

for better visualization. Unless otherwise specified, a single Z-slice was used to represent data.

Acquisition setting for smFISH: Genome (Far-red), 5% transmission, 0.015-0.075 second exposure.

Antigenome (Orange), 30-50%transmission, 0.200-0.500 second exposure. Note that antigenome

channel was exposed for at least 10X longer than the genome channel.

Quantification of perinuclear puncta distribution of oRNA1 antigenome

Deconvolved and processed images were opened on ImageJ, and colors were assigned for genome

(cyan) and antigenome (magenta) channels. A composite image was created with a merge of these

channels. Animals with at least one perinuclear puncta enriched for antigenome and negative for

genome were considered a positive hit (visualized through line scan analysis). Randomization and blind

quantification were performed with the help of Bass lab members to account for user bias.

Quantification of oRdRP spheres

Deconvolved and processed images were used to perform oRdRP sphere diameter quantification on minimally adjusted Z-slices for the oRdRP channel. Progressive z-slices showing a point progressing to a circle and ending back in a point, was considered a sphere. Center z-slice of the sphere was determined, and the diameter (from a single z-slice) was calculated using the line tool on ImageJ. Randomization and blind quantification were performed with the help of Bass lab members to account for user bias.

Quantification of association of oRdRP with mitochondria and autophagosomes

Deconvolved and processed images were used to perform line scan analyses on minimally adjusted Z-slices of widefield images for oRdRP and wrmScarlet (mitochondria) or GFP (autophagosome) channels.

Heat shock inducible transgenic replicon system: heat shock induction protocol

Transgenic array, GFP positive (in pharynx) animals (4-5 L4s) were picked onto a fresh OP50-containing plate. Animals were incubated at 20°C for 3 days. Once they reached L4 larval stage, animals were heat shocked at 34°C for 2 hours. Animals were collected after 24 hours of recovery at 20°C for strand-specific RT-qPCR and northern blot assays.

Standard curve for strand-specific RT-qPCR

In the region where the strand-specific primers were designed to bind oRNA1, a 500bp region was amplified using primers flanking a T7 promoter (genome) or SP6 promoter (antigenome). The purified PCR product was then used for in-vitro transcription to generate the genomic or antigenomic strand using T7 or SP6 RNA polymerase, respectively. Known numbers of purified ssRNA strands, ranging from 10^9 to 10^2 copies, were used for reverse transcription using strand-specific primers to generate cDNA. The resulting cDNA was used for qPCR to generate a standard curve with copy number on the x-axis and cycle threshold (Ct) values on the y-axis. Standard curve was generated, and the equation was obtained by fitting points to a semi-log line using GraphPad Prism software. The resulting equation was then used to determine copy number for strand-specific RT-qPCR analysis of infection experiments.

Northern blot

Total RNA purified from early and late infected animals was heated at 65°C for 10 minutes in northern

loading buffer containing ethidium bromide. Buffers were prepared and northern blot was performed as

described in [57]. Membrane was then imaged under short UV light to capture ethidium bromide

staining for the loading control. Membrane was pre-hybridized using hybridization buffer at 42°C for 20-

30 minutes. 50μL purified, and end-labeled probes were mixed with hybridization buffer and added to

the membrane for hybridization overnight at 42°C; membranes were then washed with northern wash

buffer thrice. Antigenome hybridized membranes were exposed to a PhosphorImager screen for 3

days; genome hybridized membranes were exposed for 2-3 hours; 72hpi sample containing membrane

was only exposed for 15 minutes. PhosphorImage screens were imaged with a Typhoon Biomolecular

Imager.

CRISPR-Cas9 for *rde-4(uu71)* generation

rde-4(uu71) allele was generated with the CRISPR/Cas9 system as described [25].

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References

1. Félix M-A, Ashe A, Piffaretti J, Wu G, Nuez I, Bélicard T, et al. Natural and Experimental Infection of Caenorhabditis Nematodes by Novel Viruses Related to Nodaviruses. PLOS Biol. 2011;9: e1000586. doi:10.1371/journal.pbio.1000586

2. Castiglioni VG, Olmo-Uceda MJ, Villena-Giménez A, Muñoz-Sánchez JC, Legarda EG, Elena SF. Story of an infection: Viral dynamics and host responses in the Caenorhabditis elegans-Orsay virus pathosystem. Sci Adv. 2024;10: eadn5945. doi:10.1126/sciadv.adn5945

3. Franz CJ, Renshaw H, Frezal L, Jiang Y, Félix M-A, Wang D. Orsay, Santeuil and Le Blanc viruses primarily infect intestinal cells in *Caenorhabditis* nematodes. Virology. 2014;448: 255–264. doi:10.1016/j.virol.2013.09.024

4. Félix M-A, Wang D. Natural Viruses of Caenorhabditis Nematodes. Annu Rev Genet. 2019;53: 313–326. doi:10.1146/annurev-genet-112618-043756

5. Batachari LE, Dai AY, Troemel ER. Caenorhabditis elegans RIG-I-like receptor DRH-1 signals via CARDs to activate antiviral immunity in intestinal cells. Proc Natl Acad Sci. 2024;121: e2402126121. doi:10.1073/pnas.2402126121

6. de Beijer NL, Snijder EJ, Bárcena M. A Cool Look at Positive-Strand RNA Virus Replication Organelles: New Insights from Cryo-Electron Microscopy. Annu Rev Biochem. 2024;93: 163–187. doi:10.1146/annurev-biochem-052521-115736

7. Stancheva VG, Sanyal S. Positive-strand RNA virus replication organelles at a glance. J Cell Sci. 2024;137: jcs262164. doi:10.1242/jcs.262164

8. Ball LA. Replication of the genomic RNA of a positive-strand RNA animal virus from negative-sense transcripts. Proc Natl Acad Sci U S A. 1994;91: 12443–12447.

9. Guo X, Zhang R, Wang J, Ding S-W, Lu R. Homologous RIG-I–like helicase proteins direct RNAi-mediated antiviral immunity in C. elegans by distinct mechanisms. Proc Natl Acad Sci U S A. 2013;110: 16085–16090. doi:10.1073/pnas.1307453110

10. Ashe A, Bélicard T, Le Pen J, Sarkies P, Frézal L, Lehrbach NJ, et al. A deletion polymorphism in the Caenorhabditis elegans RIG-I homolog disables viral RNA dicing and antiviral immunity. eLife. 2013;2: e00994. doi:10.7554/eLife.00994

11. Chen K, Franz CJ, Jiang H, Jiang Y, Wang D. An evolutionarily conserved transcriptional response to viral infection in Caenorhabditis nematodes. BMC Genomics. 2017;18. doi:10.1186/s12864-017-3689-3

12. Reddy KC, Dror T, Sowa JN, Panek J, Chen K, Lim ES, et al. An Intracellular Pathogen Response Pathway Promotes Proteostasis in C. elegans. Curr Biol CB. 2017;27: 3544-3553.e5. doi:10.1016/j.cub.2017.10.009

13. Sowa JN, Jiang H, Somasundaram L, Tecle E, Xu G, Wang D, et al. The C. elegans RIG-I homolog DRH-1 mediates the Intracellular Pathogen Response upon viral infection. J Virol. 2019 [cited 7 Nov 2019]. doi:10.1128/JVI.01173-19

14. Welker NC, Pavelec DM, Nix DA, Duchaine TF, Kennedy S, Bass BL. Dicer's helicase domain is required for accumulation of some, but not all, C. elegans endogenous siRNAs. RNA. 2010;16: 893–903. doi:10.1261/rna.2122010

15. Pavelec DM, Lachowiec J, Duchaine TF, Smith HE, Kennedy S. Requirement for the ERI/DICER
complex in endogenous RNA interference and sperm development in Caenorhabditis elegans.
Genetics. 2009;183: 1283–1295. doi:10.1534/genetics.109.108134

16. Jiang H, Chen K, Sandoval LE, Leung C, Wang D. An Evolutionarily Conserved Pathway Essential
for Orsay Virus Infection of Caenorhabditis elegans. mBio. 2017;8. doi:10.1128/mBio.00940-17

17. Ahlquist P, Noueiry AO, Lee W-M, Kushner DB, Dye BT. Host Factors in Positive-Strand RNA Virus
Genome Replication. J Virol. 2003;77: 8181–8186. doi:10.1128/JVI.77.15.8181-8186.2003

18. Jakubiec A, Jupin I. Regulation of positive-strand RNA virus replication: The emerging role of
phosphorylation. Virus Res. 2007;129: 73–79. doi:10.1016/j.virusres.2007.07.012

19. Bakowski MA, Desjardins CA, Smelkinson MG, Dunbar TA, Lopez-Moyado IF, Rifkin SA, et al.
Ubiquitin-Mediated Response to Microsporidia and Virus Infection in C. elegans. PLOS Pathog.
2014;10: e1004200. doi:10.1371/journal.ppat.1004200

20. Lažetić V, Wu F, Cohen LB, Reddy KC, Chang Y-T, Gang SS, et al. The transcription factor ZIP-1
promotes resistance to intracellular infection in Caenorhabditis elegans. Nat Commun. 2022;13:
17. doi:10.1038/s41467-021-27621-w

21. Genoyer E, Wilson J, Ames JM, Stokes C, Moreno D, Etzyon N, et al. Exposure of negative-sense
viral RNA in the cytoplasm initiates innate immunity to West Nile virus. Mol Cell. 2025;85: 1147-
1161.e9. doi:10.1016/j.molcel.2025.01.015

22. Fernández de Castro I, Tenorio R, Risco C. Virus Factories. Encycl Virol. 2021; 495–500.
doi:10.1016/B978-0-12-814515-9.00001-1

23. Miller DJ, Schwartz MD, Ahlquist P. Flock House Virus RNA Replicates on Outer Mitochondrial
Membranes in Drosophila Cells. J Virol. 2001;75: 11664–11676. doi:10.1128/JVI.75.23.11664-
11676.2001

24. Consalvo CD, Aderounmu AM, Donelick HM, Aruscavage PJ, Eckert DM, Shen PS, et al. C.
elegans Dicer acts with the RIG-I-like helicase DRH-1 and RDE-4 to cleave dsRNA. eLife.
2024;13. doi:10.7554/eLife.93979.2

25. Reich DP, Tyc KM, Bass BL. C. elegans ADARs antagonize silencing of cellular dsRNAs by the
antiviral RNAi pathway. Genes Dev. 2018;32: 271–282. doi:10.1101/gad.310672.117

26. Coffman SR, Lu J, Guo X, Zhong J, Jiang H, Broitman-Maduro G, et al. Caenorhabditis elegans
RIG-I Homolog Mediates Antiviral RNA Interference Downstream of Dicer-Dependent Biogenesis
of Viral Small Interfering RNAs. mBio. 2017;8. doi:10.1128/mBio.00264-17

27. Uebel CJ, Anderson DC, Mandarino LM, Manage KI, Aynaszyan S, Phillips CM. Distinct regions of
the intrinsically disordered protein MUT-16 mediate assembly of a small RNA amplification
complex and promote phase separation of Mutator foci. PLOS Genet. 2018;14: e1007542.
doi:10.1371/journal.pgen.1007542

28. Phillips CM, Montgomery TA, Breen PC, Ruvkun G. MUT-16 promotes formation of perinuclear
Mutator foci required for RNA silencing in the C. elegans germline. Genes Dev. 2012;26: 1433–
1444. doi:10.1101/gad.193904.112

29. Lowe D, Shukla A, Kennedy S. Viral RNA pUGylation Promotes Antiviral Immunity in C. elegans.
bioRxiv; 2025. p. 2025.07.09.663919. doi:10.1101/2025.07.09.663919

30. de Faria IJS, Imler J-L, Marques JT. Protocol for the analysis of double-stranded RNAs in virus-
infected insect cells using anti-dsRNA antibodies. STAR Protoc. 2023;4: 102033.
doi:10.1016/j.xpro.2022.102033

31. Reich DP, Bass BL. Mapping the dsRNA World. Cold Spring Harb Perspect Biol. 2019;11: a035352.
doi:10.1101/cshperspect.a035352

32. Miller DJ, Ahlquist P. Flock House Virus RNA Polymerase Is a Transmembrane Protein with Amino-
Terminal Sequences Sufficient for Mitochondrial Localization and Membrane Insertion. J Virol.
2002;76: 9856–9867. doi:10.1128/JVI.76.19.9856-9867.2002

33. Hallgren J, Tsirigos K, Pedersen MD, Almagro Armenteros JJ, Marcatili P, Nielsen H, et al.
DeepTMHMM predicts alpha and beta transmembrane proteins using deep neural network. 2022.
doi:10.1101/2022.04.08.487609

34. Tusnády GE, Simon I. The HMMTOP transmembrane topology prediction server. Bioinforma Oxf
Engl. 2001;17: 849–850. doi:10.1093/bioinformatics/17.9.849

35. Cserzö M, Wallin E, Simon I, von Heijne G, Elofsson A. Prediction of transmembrane alpha-helices
in prokaryotic membrane proteins: the dense alignment surface method. Protein Eng. 1997;10:
673–676. doi:10.1093/protein/10.6.673

36. Sonnhammer EL, von Heijne G, Krogh A. A hidden Markov model for predicting transmembrane
helices in protein sequences. Proc Int Conf Intell Syst Mol Biol. 1998;6: 175–182.

37. Krogh A, Larsson B, von Heijne G, Sonnhammer ELL. Predicting transmembrane protein topology
with a hidden markov model: application to complete genomes1. J Mol Biol. 2001;305: 567–580.
doi:10.1006/jmbi.2000.4315

38. Mak HY. Lipid droplets as fat storage organelles in Caenorhabditis elegans. J Lipid Res. 2012;53:
28–33. doi:10.1194/jlr.R021006

39. Shi X, Li J, Zou X, Greggain J, Rødkær SV, Færgeman NJ, et al. Regulation of lipid droplet size
and phospholipid composition by stearoyl-CoA desaturase. J Lipid Res. 2013;54: 2504–2514.
doi:10.1194/jlr.M039669

40. Casorla-Perez LA, Guennoun R, Cubillas C, Peng B, Kornfeld K, Wang D. Orsay Virus Infection of
Caenorhabditis elegans Is Modulated by Zinc and Dependent on Lipids. J Virol. 2022;96: e01211-
22. doi:10.1128/jvi.01211-22

41. Valera-Alberni M, Yao P, Romero-Sanz S, Lanjuin A, Mair WB. Novel Imaging Tools to Study
Mitochondrial Dynamics in Caenorhabditis elegans. bioRxiv; 2024. p. 2024.07.16.603730.
doi:10.1101/2024.07.16.603730

42. Choi Y, Bowman JW, Jung JU. Autophagy during viral infection — a double-edged sword. Nat Rev
Microbiol. 2018;16: 341–354. doi:10.1038/s41579-018-0003-6

43. Liang S, Wu Y-S, Li D-Y, Tang J-X, Liu H-F. Autophagy in Viral Infection and Pathogenesis. Front
Cell Dev Biol. 2021;9. doi:10.3389/fcell.2021.766142

44. Dawson ZD, Sundaramoorthi ,Hemalatha, Regmi ,Suk, Zhang ,Bo, Morrison ,Stephanie, Fielder
,Sara M., et al. A fluorescent reporter for rapid assessment of autophagic flux reveals unique
autophagy signatures during C. elegans post-embryonic development and identifies compounds

that modulate autophagy. Autophagy Rep. 2024;3: 2371736.
doi:10.1080/27694127.2024.2371736

45. Fujita N, Itoh T, Omori H, Fukuda M, Noda T, Yoshimori T. The Atg16L complex specifies the site of
LC3 lipidation for membrane biogenesis in autophagy. Mol Biol Cell. 2008;19: 2092–2100.
doi:10.1091/mbc.e07-12-1257

46. Kalugotla G, Marmerstein V, Schriefer LA, Wang L, Morrison S, Perez LC, et al. ATG-3 limits Orsay
virus infection in C. elegans through regulation of collagen pathways. bioRxiv. 2025;
2025.01.13.632696. doi:10.1101/2025.01.13.632696

47. Shulla A, Randall G. Spatiotemporal analysis of hepatitis C virus infection. PLoS Pathog. 2015;11:
e1004758. doi:10.1371/journal.ppat.1004758

48. Novak JE, Kirkegaard K. Improved method for detecting poliovirus negative strands used to
demonstrate specificity of positive-strand encapsidation and the ratio of positive to negative
strands in infected cells. J Virol. 1991;65: 3384–3387. doi:10.1128/JVI.65.6.3384-3387.1991

49. Warncke SR, Knudsen CR. Detection methods targeting the positive- and negative-sense RNA
transcripts from plus-stranded RNA viruses. APMIS Acta Pathol Microbiol Immunol Scand.
2022;130: 284–292. doi:10.1111/apm.13202

50. Ivashkiv LB, Donlin LT. Regulation of type I interferon responses. Nat Rev Immunol. 2014;14: 36–
49. doi:10.1038/nri3581

51. Schreiber G. The molecular basis for differential type I interferon signaling. J Biol Chem. 2017;292:
7285–7294. doi:10.1074/jbc.R116.774562

52. Tanguy M, Véron L, Stempor P, Ahringer J, Sarkies P, Miska EA. An Alternative STAT Signaling
Pathway Acts in Viral Immunity in Caenorhabditis elegans. mBio. 2017;8.
doi:10.1128/mBio.00924-17

53. den Boon JA, Nishikiori M, Zhan H, Ahlquist P. Positive-strand RNA virus genome replication
organelles: structure, assembly, control. Trends Genet TIG. 2024;40: 681–693.
doi:10.1016/j.tig.2024.04.003

54. Huang X, Feng X, Yan Y, Xu D, Wang K, Zhu C, et al. Compartmentalized localization of perinuclear
proteins within germ granules in C. elegans. bioRxiv; 2024. p. 2024.03.25.586584.
doi:10.1101/2024.03.25.586584

55. Tsai H-Y, Chen C-CG, Conte D, Moresco JJ, Chaves DA, Mitani S, et al. A ribonuclease coordinates
siRNA amplification and mRNA cleavage during RNAi. Cell. 2015;160: 407–419.
doi:10.1016/j.cell.2015.01.010

56. Seroussi U, Lugowski A, Wadi L, Lao RX, Willis AR, Zhao W, et al. A comprehensive survey of C.
elegans argonaute proteins reveals organism-wide gene regulatory networks and functions.
James DE, Pasquenelli A, Kennedy S, editors. eLife. 2023;12: e83853. doi:10.7554/eLife.83853

57. Tabara H, Sarkissian M, Kelly WG, Fleenor J, Grishok A, Timmons L, et al. The rde-1 Gene, RNA
Interference, and Transposon Silencing in C. elegans. Cell. 1999;99: 123–132.
doi:10.1016/S0092-8674(00)81644-X

58. Son K-N, Liang Z, Lipton HL. Double-Stranded RNA Is Detected by Immunofluorescence Analysis
in RNA and DNA Virus Infections, Including Those by Negative-Stranded RNA Viruses. J Virol.
2015;89: 9383–9392. doi:10.1128/jvi.01299-15

59. Corbet GA, Burke JM, Bublitz GR, Tay JW, Parker R. dsRNA-induced condensation of antiviral
proteins modulates PKR activity. Proc Natl Acad Sci. 2022;119: e2204235119.
doi:10.1073/pnas.2204235119

60. Andronov L, Han M, Zhu Y, Balaji A, Roy AR, Barentine AES, et al. Nanoscale cellular organization
of viral RNA and proteins in SARS-CoV-2 replication organelles. Nat Commun. 2024;15: 4644.
doi:10.1038/s41467-024-48991-x

61. Romero-Brey I, Bartenschlager R. Endoplasmic Reticulum: The Favorite Intracellular Niche for Viral
Replication and Assembly. Viruses. 2016;8: 160. doi:10.3390/v8060160

62. Byun H, Gou Y, Zook A, Lozano MM, Dudley JP. ERAD and how viruses exploit it. Front Microbiol.
2014;5: 330. doi:10.3389/fmicb.2014.00330

63. Efstathiou S, Ottens F, Schütter L-S, Ravanelli S, Charmpilas N, Gutschmidt A, et al. ER-associated
RNA silencing promotes ER quality control. Nat Cell Biol. 2022;24: 1714–1725.
doi:10.1038/s41556-022-01025-4

64. Brenner S. The Genetics of CAENORHABDITIS ELEGANS. Genetics. 1974;77: 71–94.
doi:10.1093/genetics/77.1.71

65. Maintenance of C. elegans. doi:10.1895/wormbook.1.101.1

66. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative
PCR and the 2(-Delta Delta C(T)) Method. Methods San Diego Calif. 2001;25: 402–408.
doi:10.1006/meth.2001.1262

67. Batachari LE, Sarmiento MB, Wernet N, Troemel ER. Orsay Virus Infection in Caenorhabditis
elegans. Curr Protoc. 2024;4: e1098. doi:10.1002/cpz1.1098

68. He SL. Northern blot. Methods Enzymol. 2013;530: 75–87. doi:10.1016/B978-0-12-420037-
1.00003-8

69. Guo YR, Hryc CF, Jakana J, Jiang H, Wang D, Chiu W, et al. Crystal structure of a nematode-
infecting virus. Proc Natl Acad Sci. 2014;111: 12781–12786. doi:10.1073/pnas.1407122111

70. Pavelec DM, Lachowiec J, Duchaine TF, Smith HE, Kennedy S. Requirement for the ERI/DICER
Complex in Endogenous RNA Interference and Sperm Development in Caenorhabditis elegans.
Genetics. 2009;183: 1283–1295. doi:10.1534/genetics.109.108134

Fig 1: Dynamics of oRNA1 strand-specific accumulation upon OV infection.

(A) Schematic of strand-specific RT-qPCR of OV RNA1. Primers with unique adapters (green colors) were used for reverse transcription to generate cDNA specifically from oRNA1 strands. Resulting cDNA was used for qPCR with primers that recognize the unique adapter and a downstream region (in black, half arrows). Schematics of (B) late larval (L3) and (C) early larval (L1) OV infection protocols. Number of copies (per ng of total RNA) of oRNA1 genome and antigenome at (D) L3 or (E) L1 infection of indicated strains and times. Data, mean with standard deviation (N=3, biological replicates). Black asterisks, significance between indicated infection time point and previous time point for oRNA1 genomic strand; Two-way ANOVA, Uncorrected Fischer's LSD test. Pink asterisks, significance between genome and antigenome at indicated timepoint as shown for N2; Ratio paired t-test. No asterisks, no significant difference (See also S1 Fig). Number of copies of oRNA1 genome or antigenome as measured by strand-specific RT-qPCR in (F) constitutively OV-infected JU1580 animals (Dotted lines, copy number of genome or antigenome in N2 animals at 12hpi. Ratio paired t-test), and (G) in strains expressing oRNA1^{WT} or oRNA1^{mut} with or without heat shock. Data are mean with standard deviation (N=4, biological replicates). Ratio paired t-test. *, p-value<0.05; **, p-value<0.01; ***, p-value<0.001; ****, p-value<0.0001*.

Fig 2: oRNA1 antigenome is only detectible upon RNA denaturation

(A) Representative smFISH images of uninfected (denatured) and infected animals (*jyIs8;rde-1*) with or without dsRNA denaturation. *pals-5p::GFP* (green), oRNA1 genome (cyan), oRNA1 antigenome (magenta), scale bar, 25μm. Column 4 shows an enlarged image of region boxed in column 3. White arrowhead points to antigenome puncta. Pharynx signal (*) is from *mCherry*, a co-expression marker of the integrated transgene (*jyIs8*). (N>3, biological replicates). Solid white lines outline animals, dashed white lines outline infected intestinal cells. (B) Distribution of percent animals exhibiting antigenomic signal (puncta) with or without denaturation (N=3, biological replicates). Total number of infected animals is indicated. Representative images of OV-infected animals with or without denaturation using either (C) antigenome or (D) genome smFISH probes only. oRNA1 genome (cyan), oRNA1 antigenome

(magenta), scale bar, 25μm. Solid white lines outline an animal, dashed white lines outline infected

intestinal cells, and white arrowhead points to antigenome puncta in column 3 (antigenome zoom of C).

Fig 3: oRNA1 antigenome localizes to perinuclear foci

(A) Representative images of *jyIs8(wt)* or *jyIs8;rde-1(ne219)* animals infected with OV for 24 hours

along with corresponding line scan analysis (yellow arrow shows path of scan). Columns 1-3 depict

pals-5p::GFP (green), oRNA1 genome (cyan), antigenome (magenta), respectively. Merge zoom

(column 4) shows an enlarged image of the region boxed in columns 2 and 3 with signal from genome

and antigenome. Scale bar, 25μm. Infected cells are outlined with a white solid line and nuclei

(Hoechst) with a white dotted line. Pharynx signal (*) is from *mCherry,* a co-expression marker of the

integrated transgene (*jyIs8*). (B) Violin plot showing number of nuclei surrounded by genome smFISH

signal in indicated strains. Unpaired t-test with Welch's correction, ****; p=0.0003* (C) Bar graph showing

percentage of infected animals exhibiting perinuclear localization of oRNA1 antigenome in infected cells

of *jyIs8(wt)* or *jyIs8;rde-1(ne219).* N=4, biological replicates. Total number of infected animals is

indicated in gray above each bar. (D-J) Representative images and line scans (yellow arrow shows

path of scan) of *jyIs8 (wt)*-infected cells (white solid line) showing distribution pattern of oRNA1

antigenome (magenta) in (D, E) perinuclear, (F, G) cytoplasmic, or (H, I) cytoplasmic foci, in genome-

containing infected cells, or (J) lumen (cyan, white solid line outlines infected animal). Dotted lines

indicate nuclei in G+AG (column 4) to enable visualization of nuclei in other columns. In (H), showing

cytoplasmic antigenome puncta, nucleus is unmarked since it is on a different z-plane.

Fig 4: Perinuclear localization of oRNA1 antigenome is partially dependent on primary siRNA

biogenesis but not secondary siRNA amplification

(A) Representative images of OV-infected cells with distribution patterns of genome (G, cyan),

antigenome (AG, magenta) and nuclei (Hoechst, blue) at 24hpi. In column 1, infected worm is outlined

(solid white line) with total number (n) of infected animals visualized in all replicates. Scale bar, 25μm.

Columns 2, 3 & 4 show enlarged images of region boxed in column 1. Dotted lines indicate nuclei in

G+AG (column 4) to enable visualization of nuclei in other columns. (B) Violin plot showing number of

nuclei surrounded by genome smFISH signal in indicated strains. Kruskal-Wallis Multiple comparison

test, **;p<0.01, ***; p<0.001, ****;p<0.0001. (C) Quantification of percent infected animals exhibiting

perinuclear localization of oRNA1 antigenome at 24hpi in indicated strains. Data are mean with

standard deviation, N≥3, biological replicates. One-way ANOVA with Tukey's multiple comparison test.

*;p<0.05, ***;p<0.001.

Fig 5: Perinuclear localized oRNA1 antigenome does not colocalize with known antiviral RNAi

factors

(A) Schematic of antiviral RNAi pathway. Maximum Intensity Projection images as labeled for DCR-

1::3XFLAG::GFP, GFP::3XFLAG::RDE-1, DRH-3::GFP::3XFLAG, genome (cyan), antigenome

(magenta) and merge in intestinal cells. Shown are localization patterns for uninfected animals (B, C,

D) or at 24hpi (E, G, H). Boxed region in (D) shows an enlarged image of perinuclear localized DRH-3

in germline (inset). Columns 2,3 & 4 in E, G and H show infected cell (white solid line) in region boxed

in column 1. White arrowheads point to GFP puncta. Number in column 3 (n) indicate the fraction of

infected animals with GFP puncta in infected cells. (F) Line scan analysis of DCR-1::3XFLAG::GFP

infected cell showing distribution pattern of genome, antigenome and DCR-1::3XFLAG::GFP. Yellow

arrow shows path of scan. Over 40 infected animals were scored among 2 independent replicates for

each strain. Scale bar, 25μm.

Fig 6: oRdRP is present in spherical structures or dsRNA-associated puncta in OV infected cells

(A) Representative dot blot spotted with total RNA from uninfected and OV infected animals at 72hpi;

probed with J2 anti-dsRNA (J2) antibody. (N=3, biological replicates). (B) Representative images of

uninfected or infected (24hpi) jyIs8 (wt) or jyIs8;rde-1 dissected intestines (white solid outline). Infection

was signaled by expression of pals-5p::GFP (green) and also visualized were oRdRP (gray), dsRNA

(red), merge (oRdRP and dsRNA). Nuclei (as marked by Hoechst) outlined with white dotted line. Red

arrowheads, possible co-localization of oRdRP and dsRNA; white arrowheads, oRdRP spherical

structures; yellow arrowhead, oRdRP sphere near dsRNA puncta. Scale bar, 25μm; merge zoom,10μm.

N≥4 biological replicates with at least 5 animals per replicate for jyIs8; N=3 biological replicates with at

least 5 animals per replicate for jyIs8;rde-1. (C) Quantification of number of oRdRP spheres per

infected animal in indicated strains. Unpaired t-test with Welch's correction; *, p=0.0424. (D) Distribution

of diameter of each sphere measured from the infected animals. Unpaired t-test with Welch's

correction; *, p=0.0154.

Fig 7: oRdRP spheres associate with mitochondria and autophagosomes during OV infection.

(A) Representative image of infected dissected intestines (solid white outline) showing oRdRP

distribution (gray) along with wrmScarlet::timm-50 signal (red) and merge panels. Scale bar, 25μm. The

second row (inset) is zoomed-in image of the outlined region in OV infected panel. White arrowheads

point to oRdRP sphere associating with wrmScarlet::timm-50. N=3. (B) Line scan analysis showing

distribution patterns of oRdRP and wrmScarlet::timm-50 as indicated. Yellow arrow shows path of scan.

(C) Quantification of number of oRdRP spheres per infected animal in indicated strains. Unpaired t-test

with Welch's correction. *, p=0.0379. N=3 biological replicates. (D) Representative images of infected

intestine (solid white outline) of wildtype animal showing oRdRP distribution (gray) along with

autophagosome marker (green) and merge panels. Scale bar, 25μm. (E) Line scans showing

association of oRdRP (gray) and autophagosomes (green) as indicated in the boxed region from (D).

Yellow arrow shows path of scan.

Fig 8: Viral replication intermediates observed in infected intestinal cells.

Cartoon shown observed replication intermediates in context of a viral infection. Among the various

intermediates generated during viral replication, we highlight three intermediates that do not get

packaged into virions. (1) oRdRP, which we observed associated with dsRNA or in spherical structures

associated with mitochondria and/or autophagosomes, in an RDE-1- and ATG-3-dependent manner. (2)

Antigenome, generated as a template for replication, which we observed associated with genome in the

cytoplasm or present as distinct perinuclear puncta, devoid of genome, and dependent on the

generation of primary siRNAs but independent of secondary siRNA amplification. (3) dsRNA, which

gets processed by the antiviral RNAi complex to elicit antiviral RNAi response, which we observed as

puncta associating with oRdRP. OV capsid structure was adapted from PDB: 4NWV [69]. The antiviral

RNAi complex is to scale and was adapted from Consalvo et. al. [24].

Supporting information

S1 Fig: Increase in viral load corresponds to increase in genomic strand in wildtype and RNAi mutants.

Strand-specific RT-qPCR of purified Genomic (A) or Antigenomic (B) strands of oRNA1 were used to generate standard curves. Equations written in plot were used to determine copy number from strand-specific RT-qPCR shown in Figs 1 and S1. (C) Representative PhosphorImage of dot blot spotted with serial dilutions (10^8 to 10^2 copies) of an ~500nt fragment of purified genome or antigenome RNA and probed using DNA oligonucleotides (end-labeled with ^{32}P γ-ATP) complementary to oRNA1 strands. Fold change (Genome/Antigenome) of wildtype and RNAi mutants of L3 infected (D) or L1 infected (E) animals at 12 or 24 hpi, as measured by strand-specific RT-qPCR. Data, mean with standard deviation. Representative northern blots of L3 (F) or L1 (G) animals for indicated times of infection points along with controls (JU1580, WUM32, WUM33) probed with DNA oligonucleotides (end-labeled with ^{32}P γ-ATP) specific to oRNA1 genome or antigenome. Ribosomal RNA (rRNA) as loading control was visualized with ethidium bromide after transfer to nylon membrane. Strand-agnostic Orsay viral load in L3 (H) or L1 (I) animals after 12 or 24 hpi. Data, mean with standard deviation. One-way ANOVA with Tukey's multiple comparison test; *, p-value<0.05; **, p-value<0.01, N=3 . Related to Fig 1.

S2 Fig: Establishing background signal in uninfected animals using genome or antigenome smFISH probes.

Representative images of uninfected animals indicating minimal to no background signal of smFISH probes for oRNA1 genome (cyan), antigenome (magenta) and merge (genome and antigenome). Scale bar, 25μm. Related to Fig 4.

S3 Fig: J2 anti-dsRNA antibody can detect as low as 50pg of purified dsRNA

Representative image of dot blot spotted with serial dilutions (50 ng to 5 pg) of in vitro synthesized and purified 800-bp dsRNA, probed with J2 anti-dsRNA antibody (N>3). Related to Fig 6.

S4 Fig: oRdRP open-reading frame showing predicted transmembrane domains and RdRP motifs

Schematic at bottom shows amino acid numbers from N to C terminus with regions of predicted

transmembrane domains (7-30 and 51-74) and conserved viral RdRP motifs (422-612) in black.

Hydrophobicity plot above used a window size of 17 amino acids and was calculated using the Kyte

and Doolittle method. Regions with hydrophobicity of >1.5 are shown in brackets.

S5 Fig: oRdRP spheres do not contain lipids

Representative images of uninfected and OV-infected (24hpi) intestines showing oRdRP (gray, anti-

oRdRP), lipid droplets (red, LipidSpot™ 610 Lipid Droplet Stain), merge and inset (region boxed in

merge column). White arrowheads point to lipid droplets (inset). N=3, biological replicates; Scale bar,

10μm.

S1 Table: List of *C. elegans* strains used in this study.

S2 Table: List of primers used in this study.

S3 Table: List of antibodies used in this study.

S4 Table: List of smFISH probes used in this study.

 Fig 2

Fig 4

Fig 5

972   **Fig 6**

A

B

Average diameter ± S.E.M
jyIs8 = 1.02µm ± 0.01µm
jyIs8;rde-1 = 1.12µm ± 0.03µm

 Fig 7

976   **Fig 8**