RNAi pathways in the recognition of foreign RNA: antiviral responses and host–parasite interactions in nematodes

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Abstract

The nematode *Caenorhabditis elegans* was the first animal for which RNAi (RNA interference) in response to exogenous triggers was shown experimentally and subsequently the molecular components of the RNAi pathway have been characterized in some detail. However, the function of RNAi in the life cycle of nematodes in the wild is still unclear. In the present article, we argue that RNAi could be used in nematodes as a mechanism to sense and respond to foreign RNA that the animal might be exposed to either through viral infection or through ingestion of food sources. This could be of potential importance to the life cycle of parasitic nematodes as they ingest RNA from different hosts at different points during their life cycle. We postulate that RNA ingested from the host could be used by the parasite to regulate its own genes, through the amplification mechanism intrinsic to the nematode RNAi pathway.

Key features of the *Caenorhabditis elegans* exo-RNAi (RNA interference) pathway

C. elegans can mount RNAi responses to dsRNA (double-stranded RNA) originating either transcribed from its own genome (endo-RNAi) or from triggers derived independently of its own genome (exo-RNAi). Both pathways in *C. elegans* have been well characterized (for a recent review, see [1]). Notably, the *C. elegans* RNAi pathway displays several features that are unusual with regard to a number of other model organisms. These features as outlined below contribute to the effectiveness of RNAi in this organism and thus argue in favour of an important role for RNAi in its life cycle. In the present article, we briefly outline the RNAi pathway in *C. elegans*, focusing in particular on these exceptional features (Figure 1).

Unusually for animals, *C. elegans* displays both primary and secondary siRNA (short interfering RNA) pathways. The primary siRNA pathway involves the processing of dsRNA introduced by feeding with bacteria or injection to generate short (~23 bp) siRNAs. This pathway is dependent on the highly conserved ribonuclease III enzyme DICER [2]. There is only one DICER gene in *C. elegans*, which is responsible for the miRNA (microRNA) and endogenous siRNA pathways as well as exogenous RNAi [3]. The activity of DICER in these various different pathways is therefore thought to be controlled by the different complexes that it forms within the cell. The exo-RNAi complex consists of DICER, RDE-1, RDE-4 and DRH-1, whereas the endo-RNAi complex consists instead of DICER, DRH-3 and ERI proteins [4]. DRH-1 does not appear to be required for the response to exogenous dsRNA; its possible function will be considered further below. However, both *rde-4* and *rde-1* mutants are deficient in their response to exogenously supplied dsRNA. RDE-4 is a DICER partner protein that appears to enhance the activity of DICER on dsRNA substrates [5,6], whereas RDE-1 is the Argonaute responsible for binding primary siRNA [7]. Without RDE-1, primary siRNAs are still made, but they cannot be converted into secondary siRNAs [5].

Secondary siRNAs in *C. elegans*, downstream of RDE-1, are produced by RdRPs (RNA-dependent RNA polymerases) using the target of the primary siRNAs as a template [8]. This gives secondary siRNAs four crucial features that mark them out as distinct from primary siRNAs: first, they have a modal length of 22 nt; secondly, they possess a 5′ guanosine (G) (22Gs); thirdly, the 5′ nucleotide carries a triphosphate, as opposed to the monophosphate carried by DICER products; and finally, 22Gs show a strong antisense bias as opposed to DICER products that map equally to either strand [9]. Secondary siRNAs of this type are not known in *Drosophila* or mammals; additionally, in plants and fungi where secondary siRNAs are known, they are produced differently: from the generation of a long dsRNA by RdRP which is then a target for DICER cleavage.

Once synthesized, 22Gs are incorporated into a number of secondary Argonaute proteins that appear to function

Key words: evolution, nematode, parasite, RNA interference (RNAi), virus.
Abbreviations used: dsRNA, double-stranded RNA; FHV, Flock house virus; GFP, green fluorescent protein; miRNA, microRNA; RdRP, RNA-dependent RNA polymerase; RIG-I, retinoic acid-inducible gene I; RNAi, RNA interference; siRNA, short interfering RNA.
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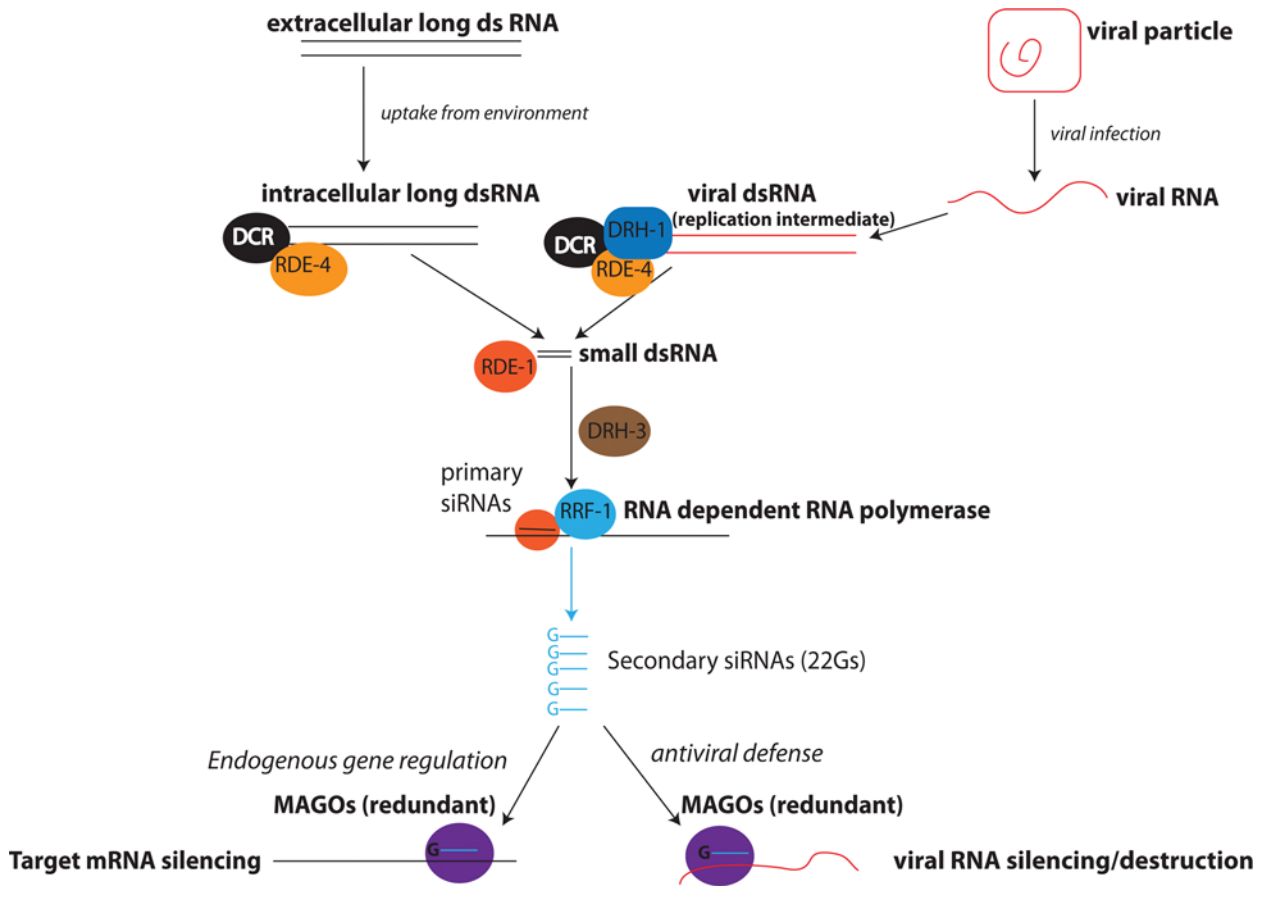
Figure 1 | The *C. elegans* RNAi response to exogenous RNA



redundantly to bring about target silencing [10]. Importantly, the whole process will give rise to amplification of the initial primary siRNAs generated by DICER, making silencing in response to exogenous dsRNA more potent.

Another important and unusual feature of RNAi in *C. elegans* is its ability to act systemically throughout the animal [11]. This requires that at least one silencing signal must be mobile, so that dsRNA taken up in the intestine or introduced through injection can give rise to silencing in recipient cells. One factor of major importance in systemic RNAi is *sid-1*, which is required for the import of the silencing signal into recipient cells [12]. The SID-1 protein is a channel with specificity for dsRNA [13], suggesting that the mobile signal is double-stranded. Moreover, evidence from mosaic animals mutant for RNAi pathway proteins in specific tissues suggests that the signal is downstream of DICER activity, but upstream of secondary siRNA generation [14]. Thus the signal is likely to be some form of primary siRNA; however, its exact nature is still undefined and is a question of major importance for the field.

An even more remarkable feature of the *C. elegans* RNAi pathway is the ability for *C. elegans* to take up and process dsRNA from its food source [15]. Experimentally, this is exploited by using *Escherichia coli* that express dsRNA against a particular gene; this is particularly effective if the *E. coli* used is a strain that lacks dsRNA digestion activity [16]. Another dsRNA transporter, *sid-2*, which is expressed in intestinal cells, is required specifically for this uptake process and is thought to take up long dsRNA via an ATP-dependent process [17,18]. The importance of *sid-2* in the uptake of dsRNA from the environment is emphasized by the fact that introducing a *sid-2* transgene into the related nematode *Caenorhabditis briggsae*, which lacks a functional *sid-2* gene, makes *C. briggsae* sensitive to RNAi by feeding [17].

The function of RNAi in nematodes

The elaborate pathway of RNAi including its special features suggests that it may play an important role in the life cycle of *C. elegans* in the wild. However, exactly what this role might be has been difficult to establish. Part of this difficulty comes from the fact that despite the prominence of *C. elegans* as a model organism, very little is known about its ecology. In the following sections, we first consider the evidence that RNAi in *C. elegans* is important in antiviral defence before

considering how RNAi might play a more general role in the interaction between nematodes and their environment.

RNAi and antiviral defence in *C. elegans*

RNAi was initially discovered in plants as an antiviral defence mechanism and has subsequently been shown to be important in antiviral defence in *Drosophila* and other insects (reviewed in [19]). Indeed, comparative genomics of *Drosophila* species suggests that proteins involved in the RNAi pathway has been evolving at a similar rate to genes involved in innate immunity, in contrast with their paralogues in the miRNA pathway which are evolving at a much lower rate, indicating that antiviral defence may be a primary function of RNAi [20]. It is therefore an attractive possibility that RNAi in *C. elegans* also plays a role in antiviral defence.

In order to establish whether *C. elegans* RNAi could defend against viral infection, a transgenic model of viral infection was established using a replicon of the insect-specific FHV (Flock house virus) and comprising the coding regions of the FHV RdRP and GFP (green fluorescent protein). When expressed, the RdRP recognizes its RNA and allows an intracellular replication cycle to take place. This artificial model mimics some of the features of the replication of a positive-strand RNA virus. Components of the RNAi pathway, such as *dcr-1* and *rde-1* are required to limit the accumulation of GFP [21]. It could be argued though that this is just a different way to generate intracellular dsRNA, already known to be a substrate for the RNAi pathway. An alternative system including viral entry into cells was achieved by using VSV (vesicular stomatitis virus) to infect primary cells from *C. elegans*; this again suggested a requirement for the RNAi pathway in limiting virus infection [22]. However, this is not a natural pathogen for *C. elegans* and primary cells are not an ideal system for utilizing the power of *C. elegans* genetics, thus limiting the mechanistic insight that can be obtained.

A more compelling argument implicating RNAi in antiviral defence would come from the use of a naturally occurring *C. elegans* virus in various mutants without the RNAi pathway. However, *C. elegans* has been maintained as a laboratory strain for 50 years and little is known about its ecological niche. It was therefore exciting when the first virus known to infect nematodes in the wild was discovered inside a wild strain of *C. elegans* isolated in Orsay, France. In a collaboration between the Felix, Miska and Wang laboratories, the novel virus, named Orsay, was found to be a positive-stranded ssDNA (single-stranded DNA) virus with a bipartite genome, distantly related to the FHV. Purified virus isolated from the infected wild strain can also infect the laboratory strain of *C. elegans*, although, interestingly, not to the same extent as the wild host strain [23].

Three lines of evidence suggest that the RNAi pathway is involved in defending against infection with the Orsay virus. First, mutants defective in the Argonaute protein RDE-1 required for secondary siRNA accumulation are infected approximately 100-fold more than the N2 laboratory strain. Secondly, in infected N2 animals, both primary and secondary siRNA are found against the virus, suggesting a canonical small RNA response to viral infection [23]. Thirdly, the naturally more sensitive wild host strain, when infected with the Orsay virus, demonstrates an unusual small RNA response, indicating failure of the primary processing step of RNAi (P. Sarkies, A. Ashe, J. Le Pen and E.A. Miska, unpublished work).

How might a canonical RNAi response be generated downstream of viral infection of N2 animals? During its replication, the ssRNA (single-stranded RNA) Orsay virus will generate a dsRNA replication intermediate, which could potentially be attacked by DICER. It seems likely that DICER acts on such a dsRNA substrate, from the fact that the primary antiviral siRNAs in N2 can be seen to map to both strands of the viral genome to roughly equal quantities ([23] and P. Sarkies, A. Ashe, J. Le Pen and E.A. Miska, unpublished work). Although the activity of DICER on the viral genome would be expected to help tackle viral infection directly, secondary siRNAs generated in N2 are clearly also important because *rde-1* mutants, with no secondary siRNAs but still able to generate primary siRNAs, show increased sensitivity to viral infection. Thus the effectors of RNAi are likely to be both primary and secondary siRNAs in conjunction with their Argonaute proteins.

One important further point that needs to be considered is the extent to which the *C. elegans* RNAi pathway is segregated from other small RNA pathways. In plants and *Drosophila*, dedicated DICER enzymes are responsible for the antiviral siRNA pathway, distinct from the miRNA pathway and, in plants, the endogenous siRNA pathway (reviewed in [19]). However, as mentioned above, there is only one *C. elegans* DICER existing in at least three different complexes. Which of these complexes recognizes the Orsay virus?

Given that the RNAi response to the Orsay virus requires *rde-1*, it seems likely that the DICER–RDE-1–RDE-4–DRH-1 exo-RNAi complex is required for the recognition of viral dsRNA rather than the ERI–DICER complex involved in endo-RNAi. This raises an intriguing possibility to explain the function of DRH-1 within this complex. DRH-1 is homologous with the mammalian protein RIG-I (retinoic acid-inducible gene I), a cytosolic sensor of viral RNA, which acts to stimulate the interferon response. RIG-I carries a C-terminal domain, conserved in DRH-1, which can bind to dsRNA with a 5′ triphosphate, causing a conformational change in the protein and the activation of downstream signalling pathways [24,25]. Thus the role of DRH-1 in the complex might be specifically to sense viral dsRNA.

Previous studies have shown that *drh-1* mutant animals are deficient in repressing the FHV-based transgene, while retaining sensitivity to RNAi induced by injection of dsRNA [26]. As this system does not recapitulate the dynamics of viral infection, however, it is of major importance that *drh-1* animals are also sensitive to infection with the Orsay virus. Moreover, analysis of deep sequencing data suggests that DRH-1 is required for generation of a strong primary siRNA response to the virus, whereas secondary siRNA

production is unaffected. Thus it seems likely that, analogous to mammals, DRH-1 is able to recognize dsRNA with a 5′ triphosphate, thus activating it specifically in response to viral infection (P. Sarkies, A. Ashe, J. Le Pen and E.A. Miska, unpublished work) (Figure 1). Whether the antiviral RNAi aspect of DRH-1's function is conserved in mammals, or indeed whether any part of the signalling function of DRH-1 is present in nematodes is a fascinating question for future study.

RNAi in the response to the environment: host–parasite interactions in nematodes

Although it seems likely that a key function of the RNAi response to exogenous dsRNA is in antiviral immunity, a consideration of the features of RNAi mentioned in the previous sections suggests that it is unlikely to be its sole function. First, the role of DRH-1 appears to be specific for viral infection and is not important in generating a robust response to dsRNA introduced by either feeding or injection. Thus the exogenous RNAi pathway in the absence of DRH-1 is likely to have an important function distinct from antiviral RNAi. Secondly, dsRNA taken up by feeding requires the specific transporter SID-2, which is not required for the introduction of dsRNA into cells after Orsay virus infection, as the viral dsRNA is generated intracellularly. Therefore what is the role of the response to dsRNA taken up from the environment?

One obvious possibility is that dsRNA could be used by the nematode as a sensitive environmental trigger. RNA taken up from the environment would trigger the generation of primary siRNAs and, if any of these primary siRNAs showed sequence identity with sections of nematode transcripts, they could be amplified to generate secondary siRNAs and the transcript could be silenced. This could be used to regulate genes in response to the different food sources with which the nematodes would be in contact. One tantalizing suggestion was made recently on the basis that a 50 bp non-coding RNA expressed in *E. coli* can alter the behaviour of *C. elegans* feeding on the bacteria. This effect appeared to be dependent on the small RNA machinery, including *rde-4*, although *sid-2* was not apparently required, suggesting that there may be an alternative uptake mechanism for this particular RNA [27].

Addressing these questions properly in *C. elegans*, however, is difficult because we have no idea what *C. elegans* actually eats in the wild. *E. coli*, for example, is unlikely to be found in the same habitats as *Caenorhabditis* nematodes. An alternative approach is therefore to switch attention to parasitic nematodes, as they have a much better characterized natural ecology. Parasitism is widespread among nematodes, having arisen several times independently, with parasitic and free-living nematodes often existing in the same clade [28]. This means that many parasitic nematodes are quite closely related to *C. elegans*, thus are likely to share many features of the RNAi pathway in common with them. Moreover, they often switch between different hosts, offering the opportunity to study whether different hosts could instigate different small RNA responses in the parasite.

In the hope of investigating this possibility, we have begun to sequence the small RNAs from parasitic nematodes at different phases of their life cycle in order to look for small RNAs that could originate directly from the host. Preliminary evidence suggests that indeed certain parasitic nematodes, in particular the potato cyst worm *Globodera pallida*, can be shown to make secondary siRNAs that map to their host genome. A subset of these map antisense to the animal's own transcriptome as well, making them possible candidates for endogenous gene regulation in response to exogenous RNA uptake (P. Sarkies, J. Jones and E.A. Miska, unpublished work). Further research will be required to establish whether this is generally the case for parasitic nematodes and indeed whether *C. elegans* uses this same strategy in the wild.

An intriguing further possibility raised by this line of thought is the idea that hosts may use the RNAi machinery of their parasite as a defence mechanism. So, for example, parasitized animals may produce dsRNA themselves that could silence essential nematode genes. Although there is no evidence for this occurring in the wild at present, the idea that this could work in principle was supported by transgenic *Arabidopsis* plants that could be used to cause embryonic lethality in *Meloidogyne incognita* nematodes feeding on the plant, by inserting a dsRNA transgene into *Arabidopsis* targeting an essential gene in the nematode [29]. The potency of the RNAi machinery appears to vary significantly between different nematode species [30]; perhaps an 'RNAi arms race' between host and parasite might explain why.

Conclusions

Although study of the function of the RNAi pathway in *C. elegans* has lagged behind mechanistic analyses, the emerging field of *C. elegans* virology offers a promising route to advance our knowledge. Additionally, the RNAi pathway may allow nematodes to sense their environment directly through the uptake of dsRNA from their food source. Together, these areas of study may help us to understand the selective advantage offered by the remarkable molecular gymnastics of primary and secondary siRNA generation in the nematode.

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