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Virus-derived siRNAs and piRNAs in immunity and pathogenesis

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Abstract

Cellular organisms have evolved related pathways for the biogenesis and function of small interfering RNAs (siRNAs), microRNAs and PIWI-interacting RNAs (piRNAs). These distinct classes of small RNAs guide specific gene silencing at both transcriptional and posttranscriptional levels by serving as specificity determinants. Small RNAs of virus and host origins have been found to modulate virus-host interactions by RNA interference (RNAi), leading to antiviral immunity or viral pathogenesis. Deep sequencing-based profiling of virus-derived small RNAs as products of host immune recognition not only allowed us to gain insight into the expansion and functional specialization of host factors involved in the antiviral immunity, but also made it possible to identify new viruses in a culture-independent manner. Here we review recent developments on the characterization and function of virus-derived siRNAs and piRNAs in eukaryotic hosts.

Introduction

Small RNAs determine specificity in RNA silencing or RNA interference (RNAi) by recognizing complementary RNA and DNA targets [1–3]. These small RNAs are products of distinct biogenesis pathways and include small interfering RNAs (siRNAs), microRNAs (miRNAs) and PIWI-interacting RNAs (piRNAs). Both siRNAs and miRNAs are 20 to 24 nucleotides in length and are produced by the dsRNA-specific Dicer nucleases whereas piRNAs are longer (23 to 30 nt) and Dicer-independent. However, siRNAs, miRNAs and piRNAs all load into Argonaute proteins (AGOs) to mediate RNA cleavage, translational repression, RNA destabilization or epigenetic modifications to the histones or DNA. Accumulating evidence suggested specificity of distinct AGO proteins in binding small RNAs according to their duplex structure and the identity of their first nucleotide [1–3].

In addition to Dicer and AGO proteins, several families of proteins contribute to the biogenesis and function of small RNAs [1–3]. These include the family of dsRNA-binding proteins with tandem dsRNA-binding domains (e.g., RDE-4, R2D2 and TRBP) required for the biogenesis and/or AGO loading of small RNAs by direct interactions with the Dicer family of type III ribonucleases. RNA silencing in fungi, plants and nematodes depends on the amplification of siRNAs by cellular RNA-dependent RNA polymerases (RdRP) found also in ticks, but not in fruit flies, mosquitoes or mammals. In addition, RNA helicases (e.g.,

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SDE-3, Armitage and DRH-1) play important roles in diverse small RNA pathways, although the molecular mechanism underlying the biological function of these RNA helicases remains largely unknown. Moreover, the steady state accumulation, therefore the function, of small RNAs is also influenced by other host factors that regulate the small RNA metabolism.

Virus infection induces the production of virus-derived small RNAs (vsRNAs) with properties of siRNAs [4–6], miRNAs [7] and/or piRNAs [8] in diverse eukaryotic organisms. These viral small RNAs modulate a wide spectrum of virus-host interactions, leading to the establishment of antiviral immunity or development of viral disease. As recently reviewed comprehensively [9–12], viral siRNAs processed from dsRNA replicative intermediates guide specific antiviral immunity by RNAi in fungi, plants and invertebrates (Fig. 1) and herpesviral miRNAs modulate pathogenicity and immune responses in mammals. It is important to point out that fruit fly mutants (e.g., *dicer-2* and *r2d2* RNAi-defective mutants) severely defective in antiviral immunity are not compromised in bacterial immunity by Toll and Imd signaling pathways and that direct proof of either production or antiviral activity of viral siRNAs in mammalian cells has yet been demonstrated. In this article, we will review the new developments on the siRNA-directed antiviral immunity and highlight the recent discoveries of vsRNAs in mammalian hosts and viral piRNAs in insect cells as well as the application of vsRNA deep sequencing in culture-independent virus discovery.

Small RNAs derived from hepatitis C and influenza A viruses

Infection of mammalian hosts with several families of nucleus-replicating DNA viruses triggers production of viral miRNAs [7,12]. However, there has been no direct evidence of production of viral siRNAs in mammalian cells infected with RNA viruses, unlike in fungi, plants and invertebrates [9–11]. Use of deep sequencing platforms in a recent study revealed low abundant vsRNAs in mammalian cells infected with several positive-strand RNA viruses exhibiting key features of viral siRNAs [6]. For example, vsRNAs from hepatitis C virus (HCV) and poliovirus have approximately equal strand ratios and contain paired strands with 2-nt 3' overhangs, suggesting that these vsRNAs are Dicer products processed from a dsRNA precursor. Moreover, co-immunoprecipitation demonstrated loading of HCV vsRNAs into human Argonaute proteins [6]. However, it is unknown why these vsRNAs do not exhibit a size preference as expected for siRNAs or piRNAs, which have been found in *Drosophila* (Fig. 2), and if they mediate specific silencing of viral RNAs in the infected mammalian cells. It also remains to be determined if similar siRNA-like vsRNAs are produced in mammalian cells in response to infection of other related and unrelated RNA viruses.

Profiling small RNAs in mammalian cells infected with influenza A virus identified an unusual class of vsRNAs [13,14]. These vsRNAs correspond to the precise 5' ends of all eight segments of the viral negative-strand genomic RNA (vRNA), exhibit random size distribution within the range of 18–27 nt because of their heterogeneous 3' ends, and accumulate to levels readily detectable by Northern blots 2 to 8 hours post infection [13,14]. In contrast to AGO-loaded mammalian miRNAs and siRNAs that contain 5' monophosphate, the influenza vsRNAs bear a 5'-terminal triphosphate and physically associate with the viral RdRP complex [13]. Therefore, the influenza vsRNAs may result from either premature termination during vRNA synthesis by the viral RdRP or non-specific endonucleolytic cleavages of vRNA.

These influenza vsRNAs were referred as leader RNAs (leRNAs) similar to the short leader RNAs derived from the 5' ends of the genomic RNA of several negative-strand RNA viruses

such as vesicular stomatitis virus (VSV) [14]. The VSV leRNAs range from 44 to 54 nt in length and have been proposed to regulate the switch from mRNA transcription to genomic RNA replication. Consistent with a regulatory role, the influenza vsRNAs do not induce the autonomous antiviral defense responses in mammalian cells [13]. Notably, introduction of a locked nucleic acid (LNA) complementary to the segment 4 leRNA specifically inhibited the accumulation of the segment vRNA in single-cycle infection assays, but had little effect on the accumulation of either the complementary vRNA (cRNA) or the mRNA from segment 4 [13]. Since LNA treatment is expected to deplete the complementary leRNA as shown for miRNA-complementary LNAs, this finding suggests a role for leRNA in the virus genome replication [13]. However, additional studies are necessary to establish the specificity of the LNA oligo because the segment 4 vRNA, unlike its positive-strand cRNA or mRNA, also has the potential to bind to the LNA oligo.

How potent is the insect RNA-based immunity?

Infection of *Drosophila* with positive-, negative- and double-strand RNA viruses induces Dicer2-dependent production of viral siRNAs, which are predominantly 21-nt in length with approximately equal strand ratios (Fig. 2A) and load into AGO2 (reviewed in [9,10]). Specific AGO2-dependent silencing guided by viral siRNAs has been recently detected in *Drosophila* cells infected with VSV [15]. This finding supports the earlier genetic studies showing that active dicing to produce viral siRNAs without AGO2-dependent slicing in mutant *Drosophila* cells, embryos or flies is insufficient to suppress virus replication [9,10].

Viral suppressors of RNA silencing (VSRs) B2 and A1 encoded by members in the *Nodaviridae* and *Dicistroviridae* respectively act by distinct mechanisms [9, 16–18]. Viruses from both families contain a positive-strand RNA genome. A1 is translated directly from the genomic RNA as a polyprotein and proteolytically cleaved from the N-terminal region. In contrast, B2 expression depends on the production of the subgenomic RNA from RNA1 of the bipartite nodaviral genome, which self-replicates independently of RNA2 since it also encodes the viral RdRP (Fig. 3, top right) [19]. B2-deficient mutant RNA1 (R1ΔB2) of Flock house virus (FHV) and Nodamura virus (NoV) fails to replicate to detectable levels in cultured *Drosophila* and mosquito cells so that AGO2 depletion is required for rescue of R1ΔB2 accumulation [5,20]. Similarly, either B2 expression or presence of a loss-of-function allele in *DCR2* or *AGO2* is essential to ensure robust replication of FHV RNA1 in *Drosophila* embryos or transgenic flies [5,21,22]. Recent studies have further shown that heterologous expression of B2 and A1 from the genome of Sindbis virus dramatically enhances virus accumulation and virulence in adult mosquitoes and fruit flies, respectively [18,23]. Notably, injection of dsRNA targeting *DCR2* or *AGO2* increased replication of Dengue virus-2 (DENV2) in the mosquito vector and decreased the extrinsic incubation period required for virus transmission [24]. These findings together demonstrate that RNA-based immunity plays a major role in insect defense against virus infection, unlike bacterial and fungal immunity that depends on the transcriptional induction of the antimicrobial peptide genes by the closely related Toll and Imd signaling pathways [9].

Virions of the B2-deficient FHV mutant, FHVΔB2, are unable to initiate a productive infection in either *Drosophila* S2 cells or adult *Drosophila* [21,25]. However, FHVΔB2 replicated to high levels and induced high mortality rates in *dcr2* and *ago2* mutant flies and FHVΔB2 also efficiently infected *r2d2* mutant flies [25]. Therefore, RNA-based immunity is sufficiently potent to terminate viral infection in the adult insect so that the virus-encoded VSR activity is essential for establishing infection. These findings suggest that as is known for plant viruses [26,27], VSRs may constitute an indispensable functional module for viruses that can establish a productive infection in insects. Deep sequencing revealed presence of a low abundant, yet typical population of viral siRNAs in wildtype flies

accompanying the arrest of FHV Δ B2 infection [25]. When RNAi was suppressed, however, viral small RNAs became strongly biased for positive strands at later stages of infection [25]. This suggests that degradation of the asymmetrically produced viral positive-strand RNAs associated with abundant virus accumulation may contribute to the positive-strand bias of viral small RNAs.

A new small animal model for viral immunity and pathogenesis

Caenorhabditis elegans has served as a genetically tractable model organism for studying many aspects of biology, including host responses to bacterial pathogens [28]. It grows on agar plates with *Escherichia coli* lawns as a food source, has a 3-day life cycle with a transparent body, produces ~300 progeny from each animal, and is often the most popular animal species for whole genome forward genetic screens. In addition to the classical mutagenesis protocols, whole genome screens are readily achieved by feeding RNAi (worms are fed with a library of *E. coli* strains expressing dsRNA targeting *C. elegans* genes), which directly identifies the genes without genetic mapping.

The recent discovery of Orsay and Santeuil viruses that naturally infect and cause diseases in *C. elegans* and *C. briggsae* respectively [29] has reignited the idea of using worms as a model to study viral immunity and pathogenesis [30–33]. Interestingly, both viruses belong to the *Nodaviridae* that includes the broad host-range FHV used in one of the first *C. elegans* viral immunity studies [30]. These pioneering worm studies utilized infection of primary cultured cells with VSV or replication of FHV in whole animals launched from an inducible and integrated FHV transgene. The replication cycle of FHV in the transgenic model begins with translation of the viral RdRP in the cytosol from nuclear transcripts and thus bypasses the initial steps in virus entry that occur during infection of worm cells by VSV. However, both viruses are restricted by the same set of RNAi pathway genes such as *rde-1* and *rde-4* [30–32], indicating that the immunity is triggered by viral RNA replication in the cytoplasm.

Orsay virus readily infected *rde-1* and *rde-4* mutants and yielded higher levels of viral RNA and visible disease symptoms as compared to the wildtype N2 strain of *C. elegans* [29]. Moreover, the infected worms produced viral siRNAs as shown by deep sequencing although only the negative-strand vsRNAs have a preferred size of 22-nt [29], in contrast to siRNAs derived from B2-deficient FHV mutant that are predominantly 23-nt in length for both strands [8] (Fig. 3, bottom left). These results demonstrate induction of RNA-based immunity in the infected worms that protects worms from virus infection and are therefore consistent with the early studies [30–32].

Recent construction of a transgenic *C. elegans* strain carrying a modified FHV replicon has facilitated the genetic analysis of the animal antiviral immunity pathways [34]. The B2 gene in FHV RNA1 is removed and replaced with GFP in the replicon, FR1gfp (Fig. 3, top right). B2 removal dramatically sensitizes the viral replicon to host immunity so that intense GFP expression associated with robust viral replication is observed only in mutant worms such as *rde-1* defective in the antiviral immunity pathway [34] (Fig. 3, top left). Use of the FR1gfp worms in a small-scale feeding RNAi screen has identified 35 genes that may play a role in antiviral immunity. The identified genes include many well-characterized RNAi factors as well as *drh-1*, which is dispensable for RNAi targeting cellular genes and highly homologous to the mammalian RIG-I family of immune receptors for RNA viruses [35–37] (Fig. 3, bottom right). Thus, it is likely that genome-wide genetic screens in the animal model by either feeding RNAi or classical mutagenesis approaches will provide a genome-view of viral immunity genes of *C. elegans* and identify homologous mammalian genes and pathways with an antiviral function.

Functional expansion and specialization in plants

RNA-based immunity against RNA viruses in *Arabidopsis thaliana* is controlled by multiple cooperative siRNA pathways involving two Dicer-like proteins (DCL) and three AGOs in contrast to a single DCR2-AGO2 siRNA pathway in *D. melanogaster* (reviewed in [9,10]). In addition, potent antiviral silencing requires amplification of viral siRNAs by either *A. thaliana* RdRP 1 (RDR1) or RDR6 (Fig. 4A), homologs of which are found in fungi, *C. elegans* and ticks, but not in humans or *D. melanogaster*; an earlier report of the D-elpl subunit of the Drosophila RNA polymerase II elongation complex as a non-canonical RdRP was recently retracted [38]. Notably, recent studies have revealed functional specialization in different members of DCL, RDR and AGO families by using virus mutants deficient in the expression of the cognate VSR [39–44]. For example, RDR1 and RDR6 appear to target different genomic regions of cucumber mosaic virus (CMV) for siRNA amplification even though either RDR alone is sufficient to confer virus resistance [40,42,43]. Similarly, loss of DCL4 alone partially rescues systemic infection of VSR-defective mutants of CMV, turnip crinkle virus (TCV) and turnip mosaic virus (TuMV), but virulent infection is fully restored only in mutant plants defective for both *DCL2* and *DCL4* [39,40,42,43].

Both DCL2 and DCL4 appear to have access to the viral dsRNA products of the viral and host RdRPs, leading to production of viral primary and secondary siRNAs, respectively [40,42,43,45]. In contrast to DCL4-dependent 21-nt viral siRNAs, however, 22-nt siRNAs produced by DCL2 in response to the CMV mutant, which accumulate to high levels in *dcl4* mutant plants, are unable to direct effective antiviral silencing [44]. One hypothesis to explain the qualitative difference is that the longer siRNA does not guide efficient cleavages of the target RNA by RNA-induced silencing complex (RISC). Interestingly, RDR6-dependent dsRNA synthesis and subsequent siRNA biogenesis is triggered by the cleavage of the target mRNA guided by 22-nt miRNAs, but not by 21-nt miRNAs [46,47], suggesting a similar qualitative difference between 21- and 22-nt miRNAs.

A recent study also indicates that AGO1 and AGO2 of *A. thaliana* exhibit unique and cooperative antiviral properties [44]. *A. thaliana* mutants defective in either AGO1 or AGO2 displayed enhanced virus susceptibility and additive effects between *ago1* and *ago2* mutant alleles were revealed by examining 25 single, double and triple mutants impaired in nine AGO genes [44,48,49]. Viral siRNAs including viral secondary siRNAs accumulated to high levels in the infected *ago1*, *ago2* and *ago1 ago2* mutant plants and co-immunoprecipitated with AGO1 and AGO2 [44,48] (Fig. 4B). Deep sequencing showed similar enrichment of both 21- and 22-nt viral siRNAs in AGO1 and AGO2. However, approximately 90% of AGO1- and AGO2-loaded viral siRNAs contain a 5'-terminal U and A, respectively, as found for plant endogenous small RNAs [3]. These findings indicate that AGO1 and AGO2 are both dispensable for the biogenesis of viral siRNAs and may cooperatively cleave viral RNAs guided by two overlapping sets of viral siRNAs (Fig. 4B).

Mechanism of viral disease symptoms

Three recent papers published by *PLoS Pathogens* provide some fascinating insights on how virus-host interactions are responsible for the induction of disease symptoms [50–52]. Co-infection of CMV with a 339-nt single-stranded satellite RNA (satRNA) induces a bright yellow leaf symptom in *Nicotiana* species including *N. benthamiana* plants [53]. satRNA replication by the helper virus triggers massive production of satRNA-derived siRNAs, which unlike the biogenesis of the helper viral siRNAs, does not depend on either RDR1 or RDR6 [42,54,55]. Two groups of investigators now show that the yellow symptom is caused by specific silencing of a host gene encoding the subunit I of magnesium protoporphyrin chelatase (ChII) involved in chlorophyll biosynthesis, which is mediated by a siRNA derived

from the previously mapped pathogenicity region of the yellow isolate of satRNA [51,52]. Minimal substitutions introduced into satRNA that disrupted the complementarity with the ChII mRNA of *N. benthamiana* prevented induction of the yellow symptom (Fig. 5), whereas those substitutions that restored the complementarity with tomato and *A. thaliana* ChII mRNAs gained the activity to induce the yellow symptom [51].

Stable expression of VSRs under the control of broad tissue specificity promoters in transgenic plants can interfere with the functionality of endogenous miRNAs and siRNAs and induce severe developmental abnormalities similar to disease symptoms (reviewed in [56]). The study by Voinnet and colleagues showed that introduction of a mutant allele for *Auxin Response Factor 8* (ARF8), a transcriptional factor targeted by miRNA 167, largely eliminated the developmental abnormalities associated with VSR expression in transgenic plants [50]. Notably, leaf serration, which is part of the disease complex induced by TuMV infection in wildtype *A. thaliana* plants, was also hardly discernable in the infected *arf8* mutant plants [50]. These findings suggest that VSR-mediated misregulation of host genes targeted by endogenous small RNAs contributes to the development of disease symptoms. It should be pointed out that additional viral factors must play key roles in the induction of viral diseases since VSR-defective TCV, CMV and TuMV mutants are highly virulent in silencing-defective plants [39,40,42,43] (Fig. 4A).

Viral piRNAs from insects

First viral piRNAs were detected in *Drosophila* ovarian somatic sheet (OSS) cells persistently infected with six RNA viruses [8] (Fig. 2B). The OSS cells produce typical populations of viral siRNAs targeting four of these viruses, including two positive-strand RNA viruses and two dsRNA viruses. However, the population of 21-nt viral siRNAs is not as predominant for the remaining two positive-strand RNA viruses, Drosophila C virus (DCV) and American nodavirus (ANV, ~88% nucleotide sequence identity with FHV). Instead, a distinct population of vsRNAs of 23 to 30 nucleotides in length was more abundant for both DCV and ANV (Fig. 2B). These longer vsRNAs are defined as viral piRNAs because they are almost exclusively in one polarity (Fig. 2B) and have a strong preference for uridine at the 5' terminus (Fig. 2C), which are the two notable features of the PIWI-loaded endogenous primary piRNAs detected in OSS cells [57,58]. OSS cells are related to ovarian follicle cells that ensheath oocytes and express PIWI, but not AGO3 or Aubergine (AUB), all of which are members from the PIWI subfamily of AGOs conserved in animals but absent in plants [1–3].

Recent deep sequencing studies revealed similar populations of viral piRNAs in the cultured C6/36 mosquito cell line originally derived from embryos of *Aedes albopictus* mosquitos [59,60]. vsRNAs detected in C6/36 mosquito cells mix infected with Dengue virus-2 (DENV2) and cell fusing agent virus (CFAV, an insect flavivirus) are almost exclusively positive strands and predominantly 27 nucleotides in length without an obvious peak at 21-nt [59]. This is in contrast to a typical DENV2 siRNA population detected in *A. aegypti* mosquitoes and Aag2 cell culture infected with DENV2. Interestingly, the longer vsRNAs of DENV2 and CFAV from C6/36 cells exhibit a modest preference for A at nucleotide position 10, a feature of AGO3-loaded piRNAs in *Drosophila* [1, 2]. vsRNAs in the size range of 23 to 28 nucleotides were also found in C6/36 cells infected with either Sindbis virus or La Crosse virus although further characterization of these vsRNA populations is necessary to determine if these longer vsRNAs have preferences for a 5'-terminal U or for A at nucleotide position 10 [60].

Detection of viral piRNAs in insect cells suggests an antiviral role for the piRNA pathway, which may represent a distinct functional specialization of RNA silencing specific to

animals. This hypothesis is consistent with the early observations that *piwi* and *aub* *Drosophila* mutants exhibit enhanced virus susceptibility [61,62]. However, piRNA pathway mainly acts to repress transposons and repeat elements in the germline which RNA viruses often do not invade with a few exceptions [63]. Does this suggest a role for the piRNA pathway to prevent viral invasion into the germline and to inhibit viral vertical transmission, or in antiviral defense in the soma? In addition, the viral piRNAs detected in *Drosophila* and mosquito cells are mostly positive strands. These viral piRNAs are not expected to target the viral positive-strand viral genomic RNA and mRNA although processing the viral RNAs into piRNAs itself may be antiviral. Future studies in adult flies and mosquitoes will be necessary to determine if viral antisense piRNAs are produced and if the piRNA pathway have an antiviral role in the soma and germline.

Virus discovery by deep sequencing and assembly of vsRNAs

The first reported deep sequencing study of vsRNAs showed surprisingly that viral siRNAs produced by the host immune system overlap in sequence [21], in contrast to plant endogenous trans-acting siRNAs and *Drosophila* hairpin RNA transgene-derived siRNAs that are in 21-nt phases [1–3, 64–65]. Subsequent studies found that Dicer cleavage may occur at every nucleotide position of the replicating viral genome and that viral piRNAs also overlap [8, 66–68]. Based on this overlapping feature of vsRNAs, a novel culture-independent approach for virus discovery has been developed and termed vdSAR for virus discovery by deep sequencing and assembly of total small RNAs [8,69].

In vdSAR (Fig. 6A), total small RNAs were isolated from a host, sequenced in a single Illumina lane, and assembled into contigs by the Velvet program developed for genome assembly from short reads. Virus-specific contigs are identified by searching the non-redundant nucleotide sequence entries of NCBI both before and after in silico translation using BlastN and BlastX, respectively. The complete genomes of the viruses identified could subsequently be recovered by PCR and cloned. Use of vdSAR led to the discovery of two new geminiviruses from sweet potato and five new positive-strand RNA and dsRNA viruses from *Drosophila* and mosquitoes [8,69]. Notably, four of the identified invertebrate viruses exhibit only low sequence similarities to known viruses so that none could be assigned into an existing virus genus (Fig. 6B). Thus, vdSAR is capable of discovering new viruses that are only distantly related to known viruses similar to metagenomic approaches used for discovery of viruses in environmental and clinical samples [70].

Metagenomic approaches require partial purification of viral particles and random amplification of viral nucleic acid sequences prior to sequencing. Importantly, vdSAR involves sequencing of the fraction of host small RNAs and data mining of only those small RNAs that can assemble into contigs so that both the amount of sequencing and data complexity are greatly reduced. Importantly, vdSAR identifies only replicating viruses since it assembles viral genomes from the products of an active host immune response to infection. However, neither approach is capable of discovering complete novel viruses that exhibit no detectable homology to known viruses, which represents a major challenge in virus discovery [70].

Concluding remarks

Viral siRNAs processed from virus-derived dsRNA direct specific clearance of the invading viral RNAs by RNA silencing in fungi, plants and invertebrates. Consistent with previous studies in insect cell culture and in whole plants, a recent study shows that in the absence of a virus-encoded activity to suppress RNAi, RNA-based immunity induced by viral RNA replication is sufficiently potent to terminate infection in adult fruit flies. Importantly, this major antiviral immunity in *Drosophila* is independent of the Toll and Imd signaling

pathways essential for bacterial and fungal immunity [37, 71–72]. RNA-based immunity appears less potent in adult female mosquitoes where arthropod-borne RNA viruses replicate to high levels even though they may not encode a VSR. However, RNA silencing inhibits the accumulation of arboviruses in mosquitoes and increases the extrinsic incubation time required for their transmission to the vertebrate hosts.

SiRNA-mediated resistance to RNA viruses in plants is controlled by at least two members of the Dicer, AGO and RdRP protein families. Recent genetic studies further indicate functional diversification in antiviral defense for different Dicer, AGO and RdRP proteins of *A. thaliana*. For example, 21-nt viral siRNAs produced by DCL4 is much more potent in defense than 22-nt viral siRNAs processed by DCL2 whereas AGO1 and AGO2 act cooperatively by loading distinct sets of viral siRNAs with 5'-terminal U and A, respectively. By contrast, a single siRNA pathway plays an essential role in defense against RNA viruses in *D. melanogaster*. However, recent detection of viral piRNAs in *Drosophila* and mosquito cells suggests an alternative approach to expand the antiviral role of small RNAs in animals.

Isolation of RNA viruses that naturally infect *C. elegans* establishes a novel small animal model to explore innate antiviral mechanisms. Use of the high throughput genetic and genomic tools available in this model host may lead to the identification of host factors required for the recognition of virus-derived dsRNA by Dicer, which may occur either in membrane-enclosed compartments where viral replication takes place or after its release from the damaged membrane structures.

Expression of host genes may be altered by VSRs that interfere with host miRNA activities or silenced directly by viral siRNAs (reviewed in [73]). Surprisingly, however, expression of several distinct VSRs contributes to the development of viral disease symptoms by acting on a common miRNA-regulated transcriptional factor of the host. Recent studies also establish a specific role for a pathogen-derived siRNA in the induction of a well-known yellow leaf symptom. Similarly, hepatitis C virus infection requires a host miRNA that binds to the 5' terminus of the viral genomic RNA with 3' overhanging nucleotides, which may protect the 5' terminal viral sequences from nucleolytic degradation or from inducing innate immune responses to the RNA terminus [74]. These findings indicate a dual function for RNA silencing in both viral immunity and viral pathogenesis. In this regard, it is interesting to note that RNA-based immunity is not always beneficial to the host. A recent study shows that loss of the RNA silencing machinery is essential to maintain the beneficial killer virus in *Saccharomyces cerevisiae* [75].

Detection of siRNA-like vsRNAs in mammalian cells suggests a possible antiviral role for the siRNA pathway in mammals, which has been under debate. It will be critical in future studies to establish a mammalian system for virus infection in which there is production of a vsRNA population with a clear length bias expected for the products (siRNA) of Dicer or for piRNAs. Such a system will make it possible to determine if the viral siRNAs or piRNAs are active in directing specific silencing of the complementary RNAs and if the activity of mammalian VSRs identified in exogenous RNAi assays is required for infection [16,26].

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* of special interest

** of outstanding interest

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yielded higher levels of viral RNA and obvious disease symptoms as compared to infection of the corresponding wild-type N2 strain, demonstrating an antiviral role for RNAi in worms. [PubMed: 21283608]

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Highlights

- RNA-based antiviral immunity increases the extrinsic incubation period required for virus transmission by mosquitoes, and is sufficiently potent to terminal infection but independent of the Toll and Imd signaling pathways essential for bacterial and fungal immunity in adult *Drosophila*
- Discovery of natural viral pathogens of *C. elegans* establishes a new small animal model for innate immunity studies
- Functional expansion and specialization of gene families in RNA-based antiviral immunity occur in plants
- Viral disease symptoms can be caused by specific silencing of an important host gene guided by a pathogen-derived siRNA
- Virus-derived small RNAs are produced in mammalian cells infected with hepatitis C and influenza A viruses, but it remains unclear if the RNAi pathway has a natural antiviral role in mammals
- Virus-derived piRNAs are produced in infected fruit fly and mosquito cells, but it is unclear if there is an antiviral function for viral piRNAs and/or the piRNA pathway, which is highly conserved between insects and mammals
- A novel culture-independent approach for virus discovery has been established by deep sequencing and assembly of virus-derived siRNAs and piRNAs.

Fig. 1.

Innate & adaptive properties of RNA-based antiviral immunity. Infection of RNA viruses in fungi, plants, insects and mosquitoes triggers biogenesis of virus-derived siRNAs, which are then assembled with an Argonaute protein and others into an effector complex to guide specific clearance of viral RNAs in the infected cell. Effective antiviral immunity in plants also depends on the amplification of viral siRNAs by host RNA-dependent RNA polymerase (not shown). Recognition of pathogen-associated molecular patterns (PAMP) by pattern recognition receptors (PRR) in *Drosophila* and mammalian innate immunity resembles the detection of the viral dsRNA by the Dicer nuclease except that the latter generates pathogen-derived siRNAs, which are used as specificity determinants in a manner analogous to the mammalian adaptive immunity.

Figure 2, A&B

Figure 2C

Fig. 2.

Properties of viral siRNAs and piRNAs from *Drosophila*. **A & B.** Distribution of sense and antisense viral siRNAs and piRNAs cloned respectively from *Drosophila* S2 cells and ovary somatic sheet cells and sequenced by the Illumina platform. The persistently infected viruses include four with +RNA genomes (ANV, DAV, DCV and Nora virus) and three with dsRNA genomes (DBV, DXV and DTV). **C.** Aggregate nucleotide composition of total sense and antisense 21nt viral siRNAs and 27–28nt viral piRNAs cloned from *Drosophila* ovary somatic sheet cells persistently infected with the six RNA viruses. Note that the strong 5'-terminal uridine bias is observed for the +-strand piRNAs only.

Fig. 3.

RNA-based antiviral immunity in *Caenorhabditis elegans*. **A.** A loss of function allele of *rde-1* restored GFP expression from an FHV-based replicon, FR1gfp, whose replication is suppressed in wild-type worms. **B.** Genome structure of FHV RNA1 and FR1gfp. FHV RNA1 encodes protein A, the catalytic subunit of the viral RdRP which also directs transcription of RNA3, the mRNA for VSR protein B2. FR1gfp was created by replacing the majority of B2 coding sequence with that of GFP such that the GFP was produced as a fusion protein with the N terminus of B2. **C.** Size distribution of sense and antisense viral siRNAs cloned from *rde-1* null mutants. RDE-1 is an AGO required for the biogenesis of secondary siRNAs in RNAi induced by exogenous dsRNA. **D.** The helicase domains of three DRH proteins are highly homologous to those of the RIG-I family of human immune receptors. CARD, caspase activation and recruitment domain; DEAD-box, the DExD/H-box helicase domain; CTD, C terminal regulatory domain. Note: DRH-1 and DRH-3 also feature an N terminal domain (in blue) with currently unknown function.

Fig. 4. Antiviral immunity directed by viral primary and secondary siRNAs in *Arabidopsis thaliana*. **A.** Distinct virulence phenotypes of cucumber mosaic virus (CMV) and its VSR-deficient mutant (CMV-Δ2b) six weeks post infection in wild-type (Col-0) and mutant plants as indicated. In the absence of viral suppression of RNA silencing, CMV-Δ2b is avirulent in wild-type plants, but becomes as virulent as CMV in *DCL2* and *DCL4* double knockout plants (*dcl2/4*), which produce neither primary nor secondary viral siRNAs. The viral primary siRNAs processed from viral RdRP products in *RDR1* and *RDR6* double knockout plants (*rdr1/6*) provide a basal defense so that virulence of CMV-Δ2b is only partially rescued. **B.** Selective enrichment of viral siRNAs in AGO1 and AGO2. Left panel, relative abundance of different categories of small RNAs obtained and deep sequenced from plants infected with CMV-Δ2b before and after co-immunoprecipitation (IP) with AGO1 or AGO2. Right panel, properties of viral siRNAs loaded in AGO1 and AGO2 sequenced from plants infected with CMV-Δ2b.

Fig. 5. Disease symptom induction by an siRNA derived from Y satellite RNA (Y-sat) of Cucumber mosaic virus (CMV). Shown are *N. benthamiana* plants 14 days post infection by CMV together with Y-sat or Y-sat mut-Tom at as indicated. Y-sat mut-Tom is a Y-sat mutant that contains two point mutations to disrupt a highly complementary region of 22 nucleotides between Y-sat and a host mRNA, which encodes a key protein involved in chlorophyll synthesis. Reprinted with the authors' permission from ref 52.

Fig. 6.

Virus discovery through deep sequencing and assembly of vsRNAs **A.** Work flow of virus discovery by deep sequencing and assembly of virus-derived small RNAs. **B.** Phylogenetic analysis of the two new nodaviruses using the Clustal W method. The viral CP sequences were used as input for this analysis. Abbreviations of virus names are as follows: ANV, American nodavirus; TCLV, Tn5 cell line virus; FHV, Flock house virus; BBV, Black beetle virus; BoV, Boolarra virus; NoV, Nodamura virus; PaV, Pariacato virus; WNV, Wuhan nodavirus; MNV, Mosquito nodavirus; RgNNV, Redspotted grouper nervous necrosis virus; SjNNV, Striped Jack nervous necrosis virus.