Small RNA-based antimicrobial immunity

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Abstract | Protection against microbial infection in eukaryotes is provided by diverse cellular and molecular mechanisms. Here, we present a comparative view of the antiviral activity of virus-derived small interfering RNAs in fungi, plants, invertebrates and mammals, detailing the mechanisms for their production, amplification and activity. We also highlight the recent discovery of viral PIWI-interacting RNAs in animals and a new role for mobile host and pathogen small RNAs in plant defence against eukaryotic pathogens. In turn, viruses that infect plants, insects and mammals, as well as eukaryotic pathogens of plants, have evolved specific virulence proteins that suppress RNA interference (RNAi). Together, these advances suggest that an antimicrobial function of the RNAi pathway is conserved across eukaryotic kingdoms.

Small interfering RNAs (siRNAs). Short double-stranded RNAs of 20–23 nucleotides in length with 2-nucleotide 3′ overhangs that have 5′-monophosphate and 3′-hydroxyl termini and are cleaved from long perfectly complementary double-stranded RNA precursors by Dicer. The guide strand of siRNA in the RNA-induced silencing complex directs RNA interference in a sequence-specific manner.

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Host defence against microorganisms is mediated by early innate immune responses and the later activation of adaptive immunity, which confer broad-spectrum and highly specific antimicrobial activity, respectively. The discovery of small interfering RNAs (siRNAs), microRNAs (miRNAs) and PIWI-interacting RNAs (piRNAs) in eukaryotes revealed novel means to regulate gene expression and host antimicrobial immunity. Known collectively as small silencing RNAs, siRNAs, miRNAs and piRNAs are 20–30 nucleotides in length and all guide sequence-specific gene silencing in complex with an Argonaute protein (AGO protein) and cofactors[1]. This process of gene silencing is commonly known as RNA interference (RNAi) and generally involves the degradation of mRNA molecules, thereby preventing gene expression. When the siRNAs are derived from viruses, they function as guides to specifically target the invading viruses for RNAi and thereby inhibit viral replication and infection.

The RNAi pathway is initiated by the Dicer family of class 3 RNase III enzymes, which generate both siRNAs and miRNAs. However, piRNAs are produced through a Dicer-independent pathway. To mediate RNAi, siRNAs and miRNAs are integrated into the RNA-induced silencing complex (RISC) by uniting with members of the AGO subfamily. Pairing of the siRNA or miRNA guide strand with a complementary sequence in an RNA molecule then directs degradation or translational repression of that RNA by the AGO protein. The production and gene silencing activity of piRNAs require AGO proteins that belong to the PIWI subfamily, which are found in animals but not in fungi or plants[1].

Over the past 20 years, numerous studies have documented Dicer-dependent production of virus-derived siRNAs (vsiRNAs) in fungal, plant, insect, nematode, rodent and human cells after infection with a wide range of RNA viruses[2–4]. Plant and insect cells also accumulate vsiRNAs in response to DNA virus infection, and the genomes of some mammalian DNA viruses encode their own miRNAs. In addition, virus-derived piRNAs (vpiRNAs) are readily detectable in insect cells after RNA virus infection and from the integrated endogenous viral elements present in the genomes of mosquitoes, chickens, rodents and primates. Moreover, recent studies have provided evidence for a new role of small silencing RNAs in host immune responses to eukaryotic pathogens[5–7]. An antimicrobial function for the RNAi pathway is supported by the finding that diverse plant and animal viruses, as well as plant eukaryotic pathogens, have evolved specific virulence proteins that suppress RNAi.

In this Review, we summarize and compare the current understanding of the antimicrobial activities of small silencing RNAs in fungi, plants, invertebrates and mammals. We focus on the mechanisms used by plants and animals to produce and amplify vsiRNAs and discuss the effector mechanisms and viral suppression of antiviral RNAi. We highlight recent evidence for an antiviral function of the RNAi pathway in mammals[8–11] and for an antimicrobial function of mobile host and pathogen small RNAs against eukaryotic pathogens in plants[5–7]. Finally, we discuss the biogenesis and function of vpiRNAs discovered recently in animals.

Functions of Dicer enzymes

Pathogen recognition either at the cell membrane or in the cytosol by innate immune receptors frequently triggers downstream protein signalling cascades, leading to the transcriptional induction of effector genes that have broad-spectrum antimicrobial activities[12,13]. In antiviral

microRNAs
(miRNAs). Small (~21–23 nucleotides in length), single-stranded RNA molecules that have 5′-monophosphate and 3′-hydroxyl termini, are cleaved from long hairpin RNA precursors by Dicer, and specifically inhibit gene expression in the RNA-induced silencing complex in a sequence-specific manner.

PIWI-interacting RNAs
(piRNAs). Single-stranded RNAs of 23–33 nucleotides in length that have 5′-monophosphate and 3′-hydroxyl termini and are produced from single-stranded RNA precursors in a Dicer-independent manner. They are found in animals and not in plants, possibly because plant genomes do not encode any Argonaute (AGO) protein of the PIWI subfamily that is necessary for piRNA biogenesis. piRNAs guide specific cleavages of target RNAs by PIWI proteins.

Argonaute protein
(AGO protein). A member of a family of proteins that associate with small interfering RNAs, microRNAs or PIWI-interacting RNAs to mediate RNA interference. AGO proteins contain an amino-terminal PAZ domain and a central domain that bind the 3′ end and 5′ phosphate of the guide strand small RNA, respectively, as well as a carboxy-terminal PIWI domain that has structural similarity to RNase H. A subset of AGO proteins have endonuclease activity, whereas most mammalian AGO subfamily members only silence translation.

RNAi responses, Dicer enzymes mediate the detection of virus-specific double-stranded RNA (dsRNA) and the immediate processing of the long dsRNA into siRNAs[2–4]. Once integrated into the RISC, vsiRNAs function as the specificity determinants of the induced antiviral defence mechanism and, by base pairing with complementary viral RNAs, direct specific virus gene silencing. Therefore, Dicer enzymes have a dual function in antiviral RNAi by functioning as both the immune receptor and the specificity producer. As further distinctions from the known innate and adaptive immune mechanisms, both the non-self inducer and the specificity determinants of antiviral RNAi are RNA molecules and can be readily identified by deep sequencing of the total small RNAs produced by infected cells.

Dicer proteins in insects and filamentous fungi. Flock house virus (FHV) infection of *Drosophila melanogaster* S2 cells and adult flies has proved to be a valuable model for understanding antiviral RNAi mechanisms[14–21]. FHV is a member of the Nodaviridae family and has a positive-sense single-stranded RNA (ssRNA) genome that encodes a viral suppressor of RNAi (VSR), the B2 protein (BOX 1). The genome of *D. melanogaster* encodes two Dicer proteins, Dicer-1 and Dicer-2, that initiate two genetically distinct pathways for the biogenesis and function of miRNAs and siRNAs, respectively (BOX 2). Early findings indicated an antiviral role for Dicer-2 because FHV-induced viral RNA clearance in *D. melanogaster* S2 cells required AGO2 from the siRNA pathway initiated by Dicer-2 (REF.[14]) (FIG. 1). Use of genetic loss-of-function mutant flies in subsequent studies of FHV infection led to the identification of Dicer-2 as the first Dicer protein that is essential for the production of both vsiRNAs and antiviral RNAi[15,16] (FIG. 1). *dicer-2* mutant flies have no obvious developmental defects[22] and support active innate immune Toll signalling and IMD signalling induced by Gram-positive and Gram-negative bacteria, leading to the transcriptional induction of antimicrobial peptide genes[15]. However, *dicer-2* mutant flies are defective in the production of vsiRNAs and are highly susceptible to FHV infection compared with wild-type flies[15,16,19]. Consistent with a key role for RNAi in antiviral responses, a B2-deficient FHV mutant (FHVΔB2) is cleared rapidly in wild-type flies, whereas the same mutant virus accumulates to high virus titres and is highly virulent in mutant flies lacking Dicer-2 or the dsRNA-binding protein (dsRBP) R2d2 (REF.[19]) (FIG. 1). Deep sequencing of vsiRNAs from the infected S2 cells and adult flies has further identified long dsRNA replicative intermediates of FHV to be the precursors of vsiRNAs. Accordingly, in viral infection without interference by the B2 VSR protein, fly vsiRNAs are predominantly 21 nucleotides in length and are divided approximately equally into the sense and antisense strands[17–19]. These findings demonstrate that the long dsRNA–siRNA pathway initiated by Dicer-2, and not the Dicer-1-dependent miRNA pathway, mediates antiviral RNAi defence against FHV in fruitflies. Similarly, only one of the two Dicer genes encoded by the filamentous fungi *Colletotrichum higginsianum* and *Cryphonectria parasitica* is necessary for antiviral RNAi against RNA viruses[23,24].

Subsequent studies have shown that diverse positive-sense ssRNA, negative-sense ssRNA and dsRNA viruses, as well as DNA viruses, are all targeted by Dicer-2 for the production of vsiRNAs and antiviral RNAi in fruitflies[15,16,18,20,25–28]. The production of similar populations of vsiRNAs has also been extensively documented in mosquitoes[29,30], as well as in honeybees[31], silkworms[32,33], leafhoppers[34] and cabbage looper moths[35], after infection with diverse RNA viruses, which indicates that the induction of antiviral RNAi is a conserved feature of insect virus infection[4,30,36]. Bioinformatic analysis indicates that these vsiRNAs are processed by Dicer from viral dsRNA molecules that are either synthesized by replication of RNA viruses or formed between complementary transcripts from convergent transcription in DNA viruses (FIG. 1). Interestingly, although fly, mosquito and leafhopper vsiRNAs are predominantly 21 nucleotides in length[17–19,29,30,34], dominant 20-nucleotide and 22-nucleotide vsiRNAs are produced in silkworms[32] and honeybees[31], respectively. The antiviral function of mosquito Dicer-2 and its homologous protein in silkworms and leafhoppers has been verified by either genetic knockout or RNAi knockdown[32,34,37].

Dicer proteins are multidomain enzymes[1] that cleave both strands of their dsRNA substrates. They form a pseudodimer comprising tandem RNase III domains plus a carboxy-terminal dsRNA-binding domain. Dicer enzymes also contain a central PAZ domain, which is also present in AGO proteins, binds specifically to dsRNA termini with the characteristic 2-nucleotide 3′-terminal overhang and is essential for cleaving the Drosha-processed precursor miRNA (pre-miRNA) into mature miRNA. However, *D. melanogaster* Dicer-2 has an additional activity that is lacking in Dicer-1. Dicer-2 can recognize dsRNA substrates that lack the 2-nucleotide 3′-terminal overhang, which enables ATP-dependent processive generation of many siRNA duplexes from a single long dsRNA substrate[1]. A recent study has shown that the amino-terminal helicase domain of Dicer-2 has the unexpected activity

Box 1 | **The Nodaviridae**

The genus *Alphanodavirus* includes flock house virus (FHV) and other viruses that infect insects, as well as nodamura virus, which is transmissible to suckling mice by mosquitoes and lethal to suckling mice, suckling hamsters and insects. Nodamura virus infection of infant mice causes flaccid paralysis of the limbs, neuronal necrosis and degeneration of paravertebral and limb skeletal muscles; these symptoms are considered to be similar to those of mice infected with coxsackie viruses.

Nodaviruses contain a bipartite (composed of two segments — RNA1 and RNA2) positive-sense single-stranded RNA genome. Viral genomic RNA1 and RNA2 encode RNA-dependent RNA polymerase and capsid protein, respectively. RNA3, a subgenomic RNA that is transcribed after RNA1 replication, encodes B2 protein, which is a viral suppressor of RNA interference. Nodavirus RNA1 can self-replicate in the absence of RNA2. As a double-stranded RNA (dsRNA)-binding protein, B2 suppresses Dicer-mediated processing of long dsRNA into small interfering RNAs. Both dsRNA binding and Dicer suppression are defective if the conserved arginines at position 54 in the FHV B2 protein and position 59 in the nodamura virus B2 protein are substituted with glutamines. Orsay, Santeuil and Le Blanc viruses isolated from *Caenorhabditis* spp. are tentative members of the Nodaviridae family because of the close sequence similarity among their RNA-dependent RNA polymerases, but the genomes of these viruses do not encode the B2 protein.

Box 2 | **Small silencing RNA pathways in fruitflies and mammals**

Fruitflies

The genome of *Drosophila melanogaster* encodes two Dicer proteins and five Argonaute (AGO) proteins, which are divided into the AGO subfamily (AGO1 and AGO2) and the PIWI subfamily (AGO3, Aub and Piwi). Exogenous and endogenous double-stranded RNAs (dsRNAs) are processed into small interfering RNAs (siRNAs) of ~21 nucleotides by Dicer-2 in association with the PD isoform of Loquacious (Loqs-PD), which contains tandem dsRNA-binding domains. Duplex siRNAs are loaded into AGO2 by a protein complex composed of Dicer-2 and the dsRNA-binding protein (dsRBP) R2d2. Subsequently, one strand of the siRNA duplex is cleaved by AGO2 and ejected to generate a mature siRNA-induced silencing complex (siRISC) for slicing target RNAs that are complementary to the remaining guide strand siRNA.

Primary microRNAs (miRNAs) transcribed in the nucleus are processed into ~60-nucleotide precursor miRNAs (pre-miRNAs) by the ribonuclease Drosha in complex with the dsRBP Pasha. After nuclear export, pre-miRNAs are further processed by Dicer-1 in complex with the dsRBPs Loqs-PA and Loqs-PB into ~22-nucleotide mature miRNAs, which are assembled with AGO1, GW182 and other cofactors into the miRNA-induced silencing complex (miRISC).

PIWI-interacting RNAs (piRNAs), which are found almost exclusively in gonadal tissues of fruitflies, are 23–33 nucleotides in length. The RNA transcripts of *D. melanogaster* piRNA clusters are processed into primary piRNAs that are assembled into the piRNA-induced silencing complex (piRISC) with Piwi or Aub and then translocated to the nucleus to direct transcriptional silencing of transposons. Primary piRNAs are amplified by a feedforward RNA cleavage cycle (known as the ping-pong amplification loop) mediated by AGO3 and Aub. Aub-bound and AGO3-bound piRNAs are complementary over ten nucleotides from their 5′ end and contain uracil at position 1 and adenine at position 10, respectively. Secondary piRNA processing is cytoplasmic and uses RNA transcripts of transposons as substrates for RNA slicing, thereby silencing transposons by a post-transcriptional mechanism.

Mammals

The genomes of mammals encode one Dicer protein and four AGO proteins that belong to the AGO subfamily. Similar to fruitfly miRNAs, mammalian miRNAs are produced by two sequential RNA cleavage steps by Drosha and Dicer enzymes, which function in complex with the dsRBPs DGCR8 and RISC-loading complex subunit TARBP2, respectively. Mammalian miRNAs are enriched for uracil at position 1 and are non-selectively assembled into the miRISC with AGO1–AGO4. The mRNAs that are complementary to the miRNA seed region and other regulatory sites are targeted for decay and translational repression by TNRC6, which is an orthologue of fly GW182 and is recruited by AGO proteins in the miRISC. If pairing with the miRNA is sufficiently extensive, the target mRNA can be sliced by AGO2, which retains the active site tetrad and other properties essential for the slicer activity.

Less is known about the endogenous siRNAs that are found in certain mouse cells, including oocytes, embryonic stem cells and male germ cells. Although Drosha-produced precursor miRNAs are ideal substrates, mammalian Dicer also carries out noncanonical cuts of endogenous and artificial short-hairpin RNAs to produce functional small silencing RNAs.

The piRNA pathway is highly conserved between flies and mammals. The mouse genome encodes three PIWI proteins, as found in flies, and primate genomes encode four PIWI proteins. However, expression of mouse PIWI proteins is largely restricted to the testes.

of recognizing dsRNA substrates with blunt ends or long 5′-terminal overhangs[21]. The ATP-dependent processive dicing of these dsRNA substrates may be crucial for the induction of antiviral RNAi, as the non-self viral dsRNA molecules that are either synthesized by replication of RNA viruses or formed between complementary transcripts from convergent transcription in DNA viruses do not contain the characteristic 2-nucleotide 3′-terminal overhang found in pre-miRNAs (BOX 2).

Dicer-like proteins in plants. The mechanism of antiviral RNAi in plants is best understood for the model plant *Arabidopsis thaliana*[4], which has a genome that encodes four Dicer-like proteins (DCLs) (BOX 3). Unlike insects and fungi, in which a selected Dicer protein has antiviral activity, multiple plant DCLs function redundantly or cooperatively in the immune detection of plant RNA and DNA viruses (FIG. 2). Thus, antiviral RNAi against diverse positive-sense ssRNA viruses becomes consistently defective in *A. thaliana* plants only if more than one DCL gene is inactivated[38–42]. For example, although turnip crinkle virus accumulates to higher levels and induces more severe disease symptoms in *dcl2*-mutant plants than in wild-type plants, loss of DCL2 has only a transient effect on the accumulation of turnip crinkle virus vsiRNAs. Similarly, virus titres, disease symptoms and vsiRNA levels are similar among *dcl2*-mutant plants and wild-type plants after infection with cucumber mosaic virus or turnip mosaic virus[38]. By contrast, loss of both 21-nucleotide and 22-nucleotide vsiRNAs in *dcl2* and *dcl4* double-mutant plants markedly increases virus titres and the severity of disease symptoms for all of the RNA viruses examined so far[39–42] (including VSR-deficient mutants of turnip crinkle virus, cucumber mosaic virus and turnip mosaic virus, in addition to potato virus X)[41–45]. Consistent with the genetic studies[39–42], small RNA deep sequencing has demonstrated that the 21-nucleotide class of vsiRNAs produced by DCL4 is the most dominant species of vsiRNAs produced that target RNA viruses in plants[45,46]. DCL2-dependent 22-nucleotide vsiRNAs accumulate at much lower levels when DCL4 is functional and seem to be less effective at mediating antiviral RNAi than 21-nucleotide vsiRNAs[47].

In addition to 21-nucleotide and 22-nucleotide vsiRNAs, *A. thaliana* plants accumulate high levels of 24-nucleotide vsiRNAs that are made by DCL3 in response to infection with DNA viruses from the geminivirus and pararetrovirus groups[48–50] (FIG. 2). Both groups of viruses contain small circular DNA genomes that are replicated in the nucleus by host enzymes. Notably, viral siRNAs of the three size classes densely cover the entire viral genome in both polarities[48–50], which suggests that

RNA interference
(RNAi). A process of RNA sequence homology-dependent gene silencing guided by small silencing RNAs such as small interfering RNAs, microRNAs or PIWI-interacting RNAs bound to an Argonaute (AGO) protein-containing multicomponent ribonucleoprotein complex.

RNA-induced silencing complex
(RISC). A multicomponent ribonucleoprotein complex, comprising the guide strand of microRNAs or small interfering RNAs, Argonaute (AGO) proteins and cofactors, that silences the expression of proteins from target mRNAs by either RNA cleavage or RNA decay and/or translational repression depending on the complementarity of mRNA sequences to the packaged small RNAs.

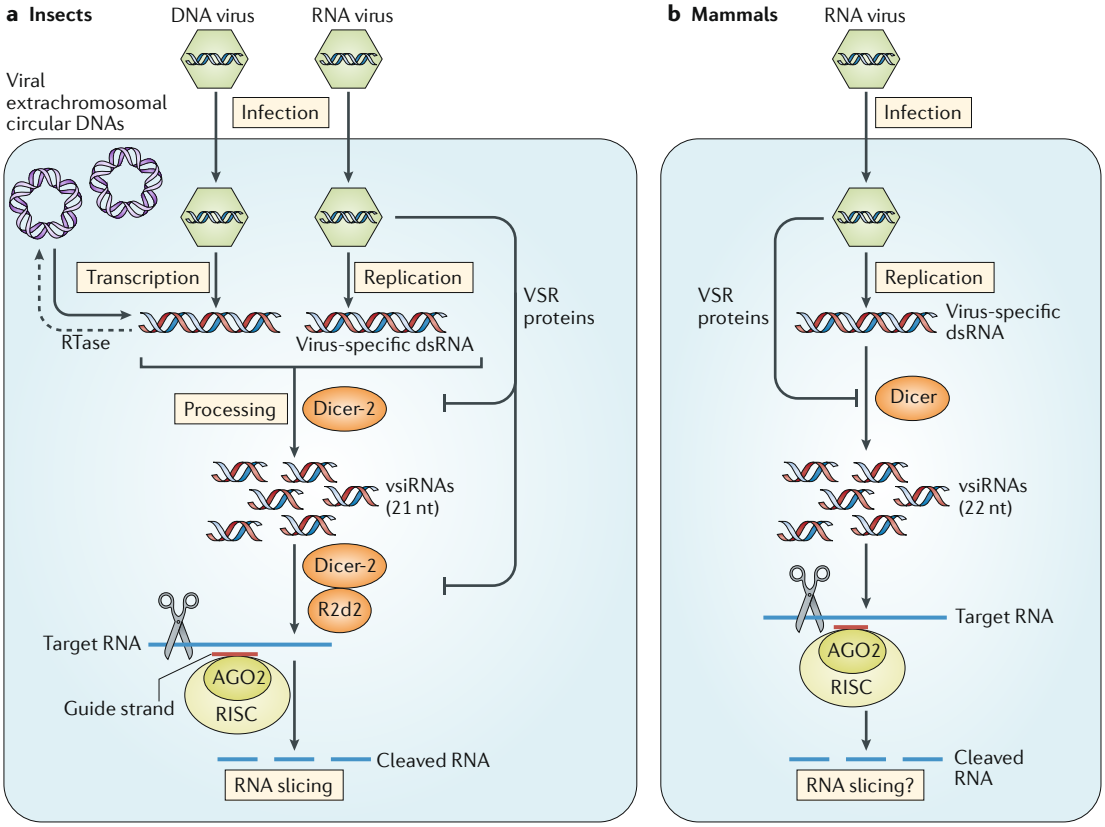

Fig. 1 | **Antiviral RNAi in insects and mammals. a** | In response to RNA virus infection of fruitflies and mosquitoes, Dicer-2 produces two distinct populations of 21-nucleotide virus-derived small interfering RNAs (vsiRNAs). They are processed from double-stranded RNA (dsRNA) precursors synthesized by viral RNA-dependent RNA polymerases or bidirectional transcription from viral extrachromosomal circular DNAs that are reverse transcribed from undefined viral RNA templates. Insect DNA viruses also trigger Dicer-2-dependent processing of virus-specific dsRNAs from sense and antisense transcripts of convergent transcription. Fly antiviral RNA interference (RNAi) requires R2d2, but not Loqs-PD, which is essential for endogenous biogenesis of small interfering RNAs (siRNAs). **b** | Mammalian vsiRNAs produced by Dicer are predominantly 22 nucleotides in length. Antiviral RNAi in both fruitflies and mammalian cells depends on the slicing activity of Argonaute protein 2 (AGO2). Three verified mammalian viral suppressor of RNAi (VSR) proteins are all unrelated dsRNA-binding proteins and suppress the biogenesis of vsiRNAs[136], whereas insect VSR proteins target distinct steps in antiviral RNAi[36]. RISC, RNA-induced silencing complex; RTase, reverse transcriptase (encoded by host retrotransposons).

vsiRNAs are processed from dsRNA precursors generated by bidirectional transcription of the entire viral circular DNA genomes[50]. Genetic characterization of *A. thaliana* infection with wild-type and VSR-deficient mutant geminiviruses has revealed a novel antiviral mechanism whereby DCL3-dependent vsiRNAs induce transcriptional gene silencing to target the DNA virus[51–53].

DCL2 also functions redundantly with DCL3 to suppress infection with potato spindle tuber viroid (PSTVd) in *Nicotiana benthamiana* plants[54]. PSTVd belongs to the unique dicot plant-specific class of small non-coding circular RNA pathogens that are replicated through a rolling-circle mechanism by host enzymes in the nucleus or in chloroplasts[55]. Viroid infection induces the production of mostly 21-nucleotide and 22-nucleotide viroid-derived siRNAs (vd-siRNAs), although 24-nucleotide vd-siRNAs are also abundant in plants infected with PSTVd and other viroids that replicate in the nucleus[56–58]. Computer-assisted assembly of viroid genomes from the sequencing of overlapping vd-siRNAs yields head-to-tail repeats of the viroid RNA genome[58], which not only suggests that the concatemeric sense and antisense viroid RNAs produced during replication form direct repeat dsRNAs to serve as substrates for DCL2, DCL3 and DCL4, but also provides the first culture-independent and homology-independent approach for viroid discovery.

Dicer proteins in nematodes and mammals. The nematode *Caenorhabditis elegans* and mammals have genomes that encode a single Dicer protein for the biogenesis of both miRNAs and siRNAs (BOX 2). In *C. elegans*, Dicer-1 processes long dsRNA into siRNAs in a complex with three other proteins, RNAi-defective 1 (RDE-1), RDE-4 and Dicer-related helicase 1 (DRH-1), none of which is necessary for miRNA function. Viral RNA replication induces antiviral RNAi in *C. elegans*; accordingly, *rde-1*-, *rde-4*- and *drh-1*-mutant worms showed significantly increased replication of both FHV and Orsay virus compared with wild-type worms[59–64] (FIG. 2). Orsay virus naturally infects *C. elegans* and is most similar to nodaviruses[61] (BOX 1).

Deep sequencing of small RNAs has shown that nematodes produce predominantly 23-nucleotide vsiRNAs in a Dicer-1-dependent manner from the viral dsRNA

IMD signalling
One of two innate immune nuclear factor-κB signalling pathways in *Drosophila melanogaster*. The IMD pathway responds to DAP-type peptidoglycan from Gram-negative, and some Gram-positive, bacteria. This leads to the rapid and robust production of antimicrobial peptides.

Box 3 | **Small silencing RNA pathways in plants**

The genome of *Arabidopsis thaliana* encodes four Dicer-like proteins (DCL1–DCL4), six RNA-dependent RNA polymerases (RDRs) and eight Argonaute (AGO) proteins. Plant AGO proteins belong to the AGO subfamily and are all active in RNA slicing. Plant microRNAs (miRNAs) are typically produced by DCL1, via sequential cleavage of nuclear transcripts, and guide post-transcriptional silencing of the target mRNAs by both RNA slicing and translational repression. Many plant species produce two major endogenous small interfering RNA (siRNA) populations, known as phased siRNAs and heterochromatic siRNAs. Phased siRNAs are mostly 21 nucleotides in length and are processed by DCL4 in regular increments from a well-defined terminus of long double-stranded RNAs (dsRNAs), which are synthesized de novo by RDR6 following RNA slicing by an miRNA-induced silencing complex (miRISC) or a siRNA-induced silencing complex (siRISC). Some phased siRNAs are *trans*-acting siRNAs.

Plant genomes encode unique nuclear RNA polymerases IV and V that are essential for RNA-directed DNA methylation. RDR2 physically associates with RNA polymerase IV and uses RNA polymerase IV transcripts as templates to synthesize dsRNA for dicing into 24-nucleotide heterochromatic siRNAs by DCL3. After loading into AGO4, AGO4–siRNA complexes are recruited to the chromatin target sites by using nascent transcripts of RNA polymerase V as RNA scaffolds. Subsequent interaction of AGO4 with RNA polymerase V transcripts recruits the DNA methyltransferase DRM2, leading to de novo cytosine methylation of the adjacent DNA and histone modifications to shut down gene transcription. Both miRNAs and siRNAs are selectively loaded into distinct AGO proteins according to their 5′-terminal nucleotide and protected from degradation by 2′-*O*-methylation catalysed by the methyltransferase HEN1.

replicative intermediates[18,62,63,65]. However, *rde-1*-, *rde-4*- and *drh-1*-mutant nematodes produce abundant 23-nucleotide vsiRNAs following FHV RNA replication or Orsay virus infection[62–64,66,67]. Therefore, similar to Dicer-2 of fruitflies[15–17,21], nematode Dicer-1 alone is sufficient to detect virus infection and process the viral long dsRNA precursors into vsiRNAs in the absence of RDE-1, RDE-4 or DRH-1. A role of DRH-1 in antiviral RNAi downstream of virus sensing is interesting, as it is highly homologous to mammalian retinoic acid inducible gene-I (RIG-I)-like receptors[62,68], which detect intracellular viral RNA to trigger interferon-regulated innate immunity against RNA virus infection[12,13]. Notably, diverse cellular dsRNAs of *C. elegans* are edited by adenosine deaminases that act on RNA to avoid Dicer-1 processing and subsequent silencing by the antiviral RNAi pathway[69], which suggests adaptation to antiviral RNAi in nematodes.

Two studies in 2013 reported the first detection of abundant 22-nucleotide vsiRNAs that are highly enriched for canonical siRNA duplexes with 2-nucleotide 3′ overhangs in mammalian cells after infection with two different positive-sense ssRNA viruses[8,9] (FIG. 1). The 22-nucleotide vsiRNAs in both polarities were readily detectable in baby hamster kidney 21 (BHK-21) cells, mouse embryonic stem cells (ESCs) and suckling mice infected with nodamura virus that lacked the functional VSR B2 protein[8,9]. By contrast, infection with wild-type encephalomyocarditis virus (a *Cardiovirus* in the Picornaviridae family) induces Dicer-mediated biogenesis of vsiRNAs in undifferentiated mouse ESCs, but this is markedly reduced after ESC differentiation[9]. These findings suggest that mammalian vsiRNA biogenesis is suppressed by either a cognate VSR protein or a cellular pathway in differentiated cells, either of which would explain why previous attempts to identify vsiRNAs by simple bulk sequencing of small RNAs from mature mammalian cells infected with a range of wild-type RNA viruses were unsuccessful[70–77].

Recent studies have revealed a prominent role for viral suppression of vsiRNA biogenesis during virus infection of human cells[10,11] (FIG. 1). Similar to the B2 protein of nodamura virus, both the non-structural 1 (NS1) protein of influenza A virus (IAV) (a negative-strand RNA virus in the Orthomyxoviridae family) and the 3A protein of human enterovirus 71 (HEV71) (an *Enterovirus* in the Picornaviridae family) potently suppress the production of vsiRNAs to target their respective cognate viruses in mature mammalian cells[10,11]. Although unrelated in sequence, these three mammalian VSR proteins are all dsRBPs, with B2 and NS1 proteins being known previously to suppress antiviral RNAi in insect cells[78] and synthetic dsRNA-induced RNAi in mammalian cells[79–81]. In the absence of VSR activity, abundant 22-nucleotide vsiRNAs processed from the viral dsRNA replicative intermediates accumulate in IAV-infected human 293T and lung epithelial (A549) cell lines, as well as in monkey epithelial cell lines, and in HEV71-infected human 293T cells and rhabdomyosarcoma cells[10,11]. Thus, the induction of mammalian antiviral RNAi does not have to depend on the infection of stem cells, which may not occur in the infection cycle of many viruses. Use of *Dicer*-knockout human 293T cells further showed that wild-type human Dicer mediates the biogenesis of both IAV vsiRNAs and HEV71 vsiRNAs[10,11]. Therefore, in addition to the type I interferon response (BOX 4), RNA virus infection induces antiviral RNAi in wild-type differentiated human cells. These findings suggest that the observed decrease in vsiRNA abundance on differentiation of mouse ESCs might be specific to stem cell differentiation induced in cell culture.

Amplification of vsiRNAs

Host RNA-dependent RNA polymerases. Antiviral RNAi in both plants[45,46] and nematodes[62,63,66] requires amplification of vsiRNAs by a related family of host RNA-dependent RNA polymerases known as RDRs in plants and RRFs in nematodes. In the current model[4,63] (FIG. 2), the production of primary vsiRNAs processed from viral dsRNA replicative intermediates is necessary to trigger the RDR-dependent or RRF-dependent biogenesis of secondary vsiRNAs. *C. elegans* RRF-1 catalyses the de novo synthesis of secondary vsiRNAs with a 5′-triphosphorylated guanosine that are predominantly antisense to the viral genomic RNAs and 22 nucleotides in length[62,63] and thus differ from the 23-nucleotide primary vsiRNAs made by Dicer-1. By contrast, primary and secondary vsiRNAs in plants are biochemically indistinguishable because the secondary vsiRNAs are processed by Dicer from long dsRNAs that are synthesized by RDRs. Genetic studies have indicated a role for RDR2 and RDR6 in the gene silencing induced by geminivirus infection in *A. thaliana*[51,82,83] (FIG. 2). However, geminivirus vsiRNAs accumulate to similar levels in wild-type plants and in *rdr1, rdr2* and *rdr6* triple-knockout mutant plants[50], which indicates that vsiRNA amplification might occur by RDR3, RDR4 or RDR5, which are homologous to the RDR that confers disease tolerance to geminivirus infection in tomato plants[84].

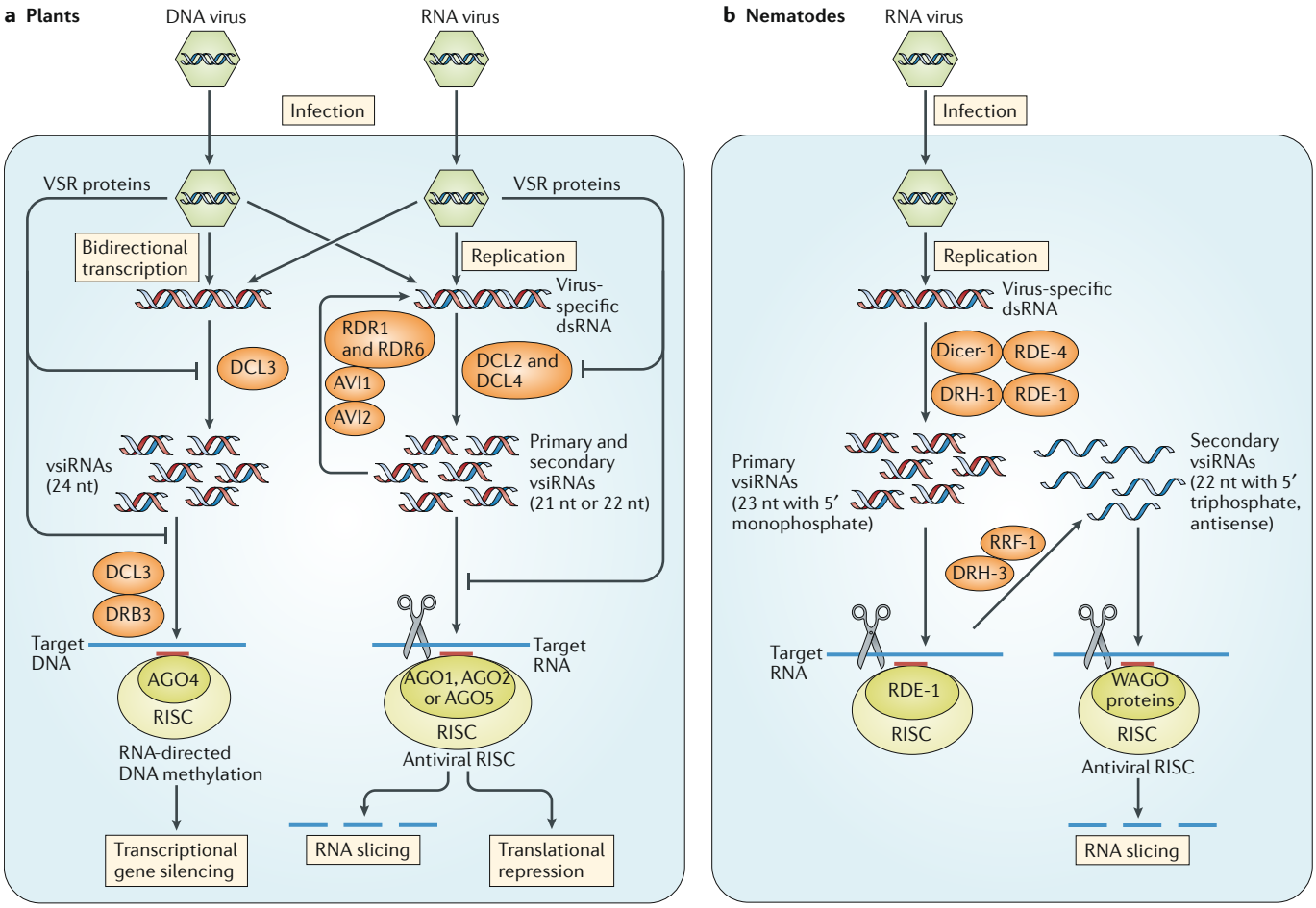

Fig. 2 | **Antiviral RNAi in plants and nematodes. a** | In plants, multiple Dicer-like enzymes (DCLs) produce distinct size classes of virus-derived small interfering RNAs (vsiRNAs) from double-stranded RNA (dsRNA) precursors produced as RNA virus replicative intermediates or from bidirectional transcription of circular DNA virus. With the help of antiviral RNA interference (RNAi)-defective 1 (AVI1) (a phospholipid flippase) and AVI2, plant RNA-dependent RNA polymerases RDR1 and RDR6 synthesize new virus-specific long dsRNAs that are also diced into secondary vsiRNAs. Genomic DNA of plant geminiviruses is further targeted by DCL3-dependent 24-nucleotide vsiRNAs for RNA-directed DNA methylation and transcriptional gene silencing. The genomes of all plant RNA and DNA viruses encode at least one viral suppressor of RNAi (VSR) protein to suppress various steps in antiviral RNAi[137,177]. **b** | *Caenorhabditis elegans* Dicer-1 processes the precursors of vsiRNAs in complex with three additional proteins. Both RNAi-defective 4 (RDE-4) and Dicer-related helicase 1 (DRH-1) increase the production of 23-nucleotide primary vsiRNAs, whereas RDE-1 is essential for the subsequent biogenesis of 22-nucleotide secondary vsiRNAs by the RNA-dependent RNA polymerase RRF-1 together with DRH-3. Multiple plant Argonaute (AGO) proteins and worm AGO (WAGO) proteins with RNA slicing activity participate in antiviral RNAi. Although worm antiviral RNAi can be suppressed by the VSR B2 protein of flock house virus, the natural worm pathogen Orsay virus does not seem to have a genome that encodes VSR activity[178]. DRB3, double-stranded RNA-binding protein 3; RISC, RNA-induced silencing complex.

RNA replication by viral RNA-dependent RNA polymerases occurs inside vesicle-like membrane invaginations induced in subcellular membrane domains following enrichment of specific phospholipids. Interestingly, forward genetic screens carried out in independent studies[85–87] identified two new host genes in plants, antiviral RNAi-defective 1 (*AVI1*) and *AVI2* (FIG. 2), that are required for the biogenesis of both secondary vsiRNAs by RDR1 and RDR6 to target RNA viruses and endogenous virus-activated siRNAs by RDR1 to target thousands of plant genes. *AVI1* encodes the related phospholipid transporters ALA1 and ALA2, which are dispensable for RDR6-dependent biogenesis of the endogenous *trans*-acting siRNAs (ta-siRNAs); these ta-siRNAs are much less abundant than vsiRNAs and thus may not depend on the formation of a specialized membrane structure for the synthesis of the precursor dsRNA.

Transcriptional induction of *RDR1* is a common response to virus infection of diverse plant species[88,89]. A recent study identified a unique signalling cascade that regulates the antiviral function of *RDR1* in rice plants[90] (FIG. 3). *RDR1* expression is normally repressed in healthy plants because the *RDR1* promoter contains specific elements for binding by MIKC^{C}-type MADS box proteins. Virus infection induces *RDR1* expression and antiviral RNAi by increasing the production of miR-444, which targets these *RDR1*-specific repressor genes for silencing[90].

MIKC^{C}-type MADS box proteins
A group of transcription factors that contain the MADS box, which is involved in DNA binding and dimerization with other MADS box proteins, and three additional conserved domains — the intervening domain, the keratin domain and the carboxy-terminal domain.

Box 4 | **Interferon-mediated and RNAi antiviral responses in mammals**

Virus infection in mammals triggers the production of type I interferons upon sensing of viral nucleic acids by pattern recognition receptors[12,13]. Subsequent type I interferon signalling induces the expression of numerous interferon-stimulated genes (ISGs) to establish an antiviral state. Because virus-derived double-stranded RNA (dsRNA) is a shared pathogen-associated molecular pattern that triggers both type I interferon-mediated and RNA interference (RNAi) antiviral responses, various models for their potential interaction have been proposed but had not been rigorously examined until recently[10,11]. For example, antiviral RNAi was initially considered to be inactive in mammals because simple bulk small RNA sequencing did not reveal a dominant peak of virus-derived small interfering RNAs (vsiRNAs) after infection with a range of wild-type RNA viruses, and these viruses did not replicate to higher levels following *Dicer* knockout in human 293T cells[70–77]. An antagonistic interaction between type I interferon-mediated and RNAi antiviral responses was also proposed on the basis of studies of infection in embryonic stem cells, cellular microRNA (miRNA) silencing or heterologous expression of *Drosophila melanogaster* proteins[9,73,171,172]. However, recent studies have revealed robust production of abundant vsiRNAs in mature mammalian cells and newborn mice after infection with RNA viruses when the cognate viral suppressor of RNAi (VSR) protein is rendered non-functional. Importantly, activation of neither type I interferon response nor cell differentiation inhibits the biogenesis of vsiRNAs processed from viral dsRNA replicative intermediates. Moreover, inactivation of type I interferon responses does not increase the biogenesis of vsiRNAs from viral dsRNA precursors, whereas Dicer-dependent processing of artificial long dsRNA molecules is increased[80,119,173,174]. This suggests that there is differential recognition of the viral and artificial dsRNAs in type I interferon-mediated and RNAi responses.

Silencing activity of vsiRNAs has been demonstrated during virus infection of human 293T cells in the presence and absence of type I interferon signalling[11]. Thus, although a previous study suggested that cellular miRNA silencing is defective in 293T cells upon induction of type I interferon responses[73], activation of type I interferon responses does not inhibit the activity of vsiRNAs from the natural immune response. This finding is consistent with the observation of efficient inhibition of virus replication by synthetic small interfering RNAs (siRNAs) in a range of mammalian cells[175]. In addition, the slicing activity of Argonaute protein 2 (AGO2) potently suppresses virus infection in mouse embryonic fibroblasts (MEFs) regardless of the presence or absence of type I interferon signalling[10]. The levels of type I interferons and induction of ISGs are similar in wild-type and RNAi-defective (*Ago2*^{D597A}) MEFs after infection with multiple viruses[10]. Interestingly, blocking both type I interferon-mediated and RNAi antiviral responses further increases virus titres compared with the inhibition of either response alone[10,11]. Together, these findings indicate that antiviral RNAi confers an interferon-independent antiviral function in mammals. Accordingly, a recent study found that suppression of antiviral RNAi in human cancer cells by nodamura virus B2 protein increases virus titres, which can be further increased by blocking the type I interferon response[176]. However, antiviral RNAi has so far been characterized only in cell culture and newborn mice, which are known to induce weaker interferon responses than adult mammals[170]. Thus, it remains to be determined whether antiviral RNAi is also active and necessary in adult mammals.

Retrotransposons
A subclass of transposons that amplify themselves in a genome through a process that involves the reverse transcription of RNA to DNA by a reverse transcriptase that is encoded by a retrotransposon.

Transposons
Also known as 'jumping genes' and 'selfish DNA'; DNA sequences that encode transposases, the enzymes that are required to excise the transposon from its original chromosomal location and to integrate it in a different position within the genome. The ends of transposons consist of DNA repeats that function as recognition sites for the transposase itself.

Haemocytes
Cells found within the haemolymph of an insect that are equivalent to the blood cells in vertebrates. Different types of haemocyte are plasmatocytes, crystal cells and lamellocytes. These cells have important roles in immunity through the secretion of cytokines and the phagocytic clearance of invaders.

Extrachromosomal circular DNA. Neither fruitflies nor mammals have genomes that encode RNA-dependent RNA polymerase homologues[1]. However, the accumulation of vsiRNAs triggered by RNA virus infection is detectable by northern blot hybridization more readily in insect and mammalian cells than in *C. elegans* cells[8,10,11,19,62,66,91], which indicates that insect and mammalian vsiRNAs are at least as abundant as nematode vsiRNAs. Characterization of non-retroviral RNA virus infection in fruitflies and mosquitoes has uncovered DNA fragments that are reverse transcribed from viral RNAs by enzymes encoded by endogenous retrotransposons and that are embedded in transposons[92,93]. Preventing viral DNA synthesis with reverse transcriptase inhibitors decreases the biogenesis of vsiRNAs and increases the susceptibility of these insects to RNA virus infection, which suggests that viral DNA production is part of the insect antiviral RNAi response.

In insects, this viral DNA synthesis occurs in macrophage-like haemocytes, and there is evidence for the secretion of insect vsiRNAs in exosome-like vesicles, which circulate in the haemolymph and generate systemic antiviral immunity[94]. Interestingly, the viral DNA fragments in infected fruitfly and mosquito cells have recently been shown to exist in a circular form[95], similar to the extrachromosomal circular DNA (eccDNA) that has been discovered in a wide range of species, including normal and cancer cells in humans[96]. Notably, injection of the total eccDNA isolated from FHV-infected S2 cells into naive fruitflies triggers virus-specific protective immunity and the production of a population of vsiRNAs that are typical of those made by Dicer-2 in fruitflies[95]. The fly vsiRNAs that are templated by the viral eccDNAs in the absence of FHV infection are mapped across the full-length genomic RNA1 and RNA2 of FHV in both polarities and at high densities. These findings reveal a novel mechanism in fruitflies for Dicer-2-dependent biogenesis of a distinct population of vsiRNAs from dsRNA substrates that are not synthesized by viral RNA replication[95] (FIG. 1). Bidirectional transcription of the viral eccDNAs may be responsible for the generation of dsRNA substrates in a manner similar to the biogenesis of vsiRNAs that target plant circular DNA viruses[48–50] (FIG. 2). It is not known whether the insect vsiRNAs templated by eccDNAs include vsiRNAs with 5′ triphosphates, such as those cloned recently from fruitflies infected with Sindbis virus[94]. As human cells synthesize viral DNA from distinct positive-sense and negative-sense ssRNA viruses[97], it will be interesting to determine whether mammals also boost the abundance of vsiRNAs through the production of viral eccDNAs.

Antiviral RNAi effector mechanisms

Antiviral activity of vsiRNAs. Infection with RNA or DNA viruses triggers homology-dependent RNAi that specifically targets viral RNAs in infected plants[98–101], invertebrates[20,26,28,102] and mammalian cells[11], demonstrating the antiviral activity of the vsiRNAs. The RISC, which is the

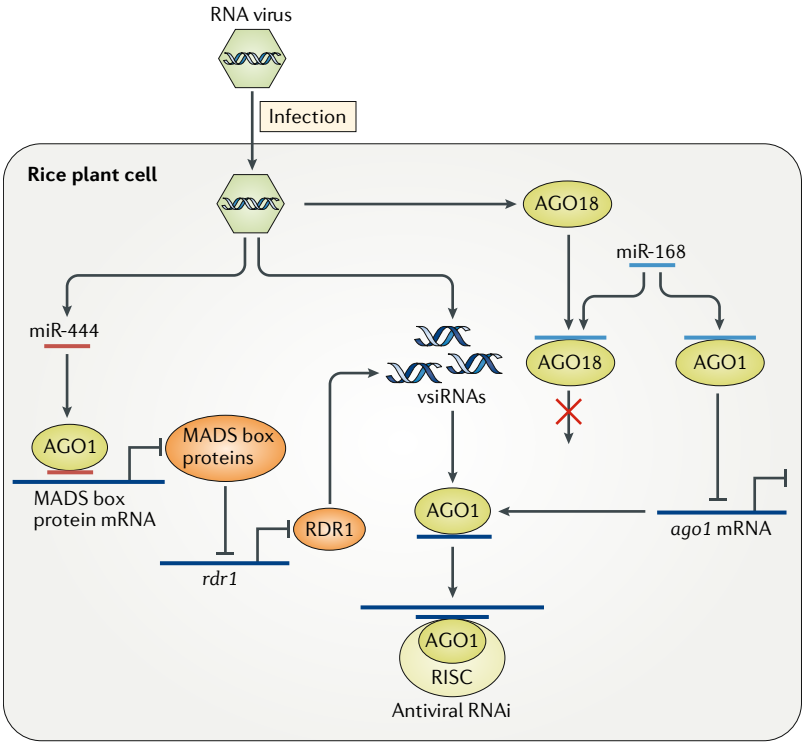

Fig. 3 | **Regulation of antiviral RNAi in rice plants.** The expression of *ago1* (encoding Argonaute protein 1) and *rdr1* (encoding RNA-dependent RNA polymerase 1) in rice plants is normally negatively regulated by microRNA-168 (miR-168) and MADS box proteins, respectively. RNA virus infection in rice plants induces the expression of *ago18* and miR-444, which subsequently activate strong antiviral RNA interference (RNAi). miR-444 represses the expression of MADS box proteins, resulting in the derepression of *rdr1* and amplification of the virus-derived small interfering RNAs (siRNAs). Similarly, AGO18 specifically sequesters miR-168, resulting in the derepression of *ago1* and increased antiviral RNA-induced silencing complex (RISC) activity. The pathway leading from AGO18 is marked by an X in the figure, as it is inactive in mediating suppression of the miRNA targets.

effector complex of RNAi, is composed minimally of a single-stranded siRNA and a member of the AGO protein subfamily[1,103–105]. Genetic studies have shown that a single AGO protein mediates antiviral RNAi in insects, fungi and mammals[9,10,14,24,78,106–108] (FIG. 1). By contrast, multiple AGO proteins confer overlapping and cooperative antiviral activities in plants[44,47,51,109–113] or both sequential and redundant functions in nematodes[59–61,63] (FIG. 2). The loading of vsiRNAs in the specific antiviral AGO proteins has been verified by co-immunoprecipitation experiments in insect, plant and mammalian cells[9–11,17,47]. The dsRBP R2d2 is essential for the loading of siRNA into the RISC[114] in antiviral RNAi, but both R2d2 and the dsRBP Loqs-PD (BOX 2) are dispensable for the biogenesis of vsiRNAs in fruitflies[15,19,107] (FIG. 1). Moreover, the defective antiviral RNAi response of *ago*-mutant plants and *AGO*-mutant animals to RNA virus infection is frequently accompanied by the production of abundant vsiRNAs[17,19,47,63,66]. Together, these findings illustrate that effective antiviral RNAi requires the function of the AGO–vsiRNA effector complex.

It is interesting to note that the production of abundant vsiRNAs in *ago*-mutant plants and *AGO*-mutant animals does not have an obvious effect on viral infection[17,47,62,63,66,107,110]. In plants and nematodes, the most abundant population of vsiRNAs is the secondary vsiRNAs that are not directly processed from viral replicative intermediates[42,45,46,63], which may explain why dicing alone to generate vsiRNAs is not inhibitory for viral replication. Accordingly, this model predicts that vsiRNAs templated by the viral eccDNAs in insects may be more abundant than the primary vsiRNAs, which remains to be investigated.

RNA slicing. In addition to the AGO domains that anchor the siRNA, the PIWI domain of some AGO proteins catalyses RNA slicing of the target RNA in the region bound by the siRNA[103,104,115]. Alanine substitutions of the metal-coordinating triad (DDD) in the PIWI domain of AGO2 disrupt antiviral RNAi in *A. thaliana*[110], indicating an antiviral role for the RNA slicing activity in RNAi. Similarly, antiviral RNAi is defective in *Ago2*-mutant *D. melanogaster*, in which AGO2 contains an amino acid substitution at position 966 (V→M) that impairs slicing activity of the protein[107]. Indeed, endonucleolytic cleavage of target RNAs and inhibition of viral RNA replication directed by the vsiRNA-loaded RISC have been demonstrated in a plant in vitro system[116].

The available evidence suggests that mammalian antiviral RNAi also involves RNA slicing guided by vsiRNAs. The specificity of homology-dependent viral RNA degradation, which is detectable in both the presence or the absence of type I interferon signalling in human 293T cells after infection with the mutant HEV71 (BOX 4), is determined by vsiRNAs produced by wild-type human Dicer[11]. A recent study[10] has also characterized IAV infection of primary mouse embryonic fibroblasts (MEFs) that express a knock-in allele of *Ago2* containing an alanine substitution in the DDH triad ($Ago2^{D597A}$) and that are inactive in RNA slicing[117]. In mammals, of the four AGO proteins, only AGO2 has endonucleolytic activity (BOX 2), which is essential for RNA slicing triggered by siRNAs but is dispensable for miRNAs to cause translational repression and mRNA decay through association with any of the four AGO proteins[1].

Importantly, almost all cellular miRNAs are present at similar levels in differentiated $Ago2^{D597A}$ MEFs and wild-type MEFs, which contrasts with the marked loss of endogenous miRNAs that occurs in *Ago2*-knockout MEFs[118]. This difference suggests that $Ago2^{D597A}$ cells are better suited than *Ago2*-knockout cells for antiviral RNAi studies. IAV replicates to significantly higher levels and induces more cytopathy in $Ago2^{D597A}$ cells than in wild-type cells[10]. By comparison, abolishing the slicing activity of AGO2 is significantly more effective in increasing the accumulation of an NS1-deficient mutant of IAV than of wild-type IAV, regardless of the presence or absence of type I interferon signalling. Moreover, both encephalomyocarditis virus and vesicular stomatitis virus accumulate to higher levels in $Ago2^{D597A}$ cells than in wild-type cells[10], which shows that the RNA slicing activity of mouse AGO2 has a broad-spectrum antiviral function. Nevertheless, it is important to note that unlike in the primary MEF cell lines, deletion of *Ago2* in immortalized MEFs lacking MAVS (also known as IPS1 or VISA), which do not mount a type I

RNA slicing
The specific endonucleolytic cleavage of mRNA molecules that contain a sequence complementary to the guide strand small RNA (small interfering RNA, microRNA or PIWI-interacting RNA) in the RNA-induced silencing complex by the PIWI domain of a subset of Argonaute (AGO) proteins. The cleavage occurs in the middle of the region that is base paired with the guide strand.

interferon response, does not increase susceptibility to virus infection[119]. Similarly, the production of abundant vsiRNAs in human 293T cells is not associated with the inhibition of IAV replication[10,120], which indicates that the use of specific infection models is crucial for determining the activity of vsiRNAs.

Translational repression. The effector mechanisms of antiviral RNAi may also include vsiRNA-guided translational repression of viral mRNAs. This is supported by the finding that *ago1-27*-mutant *A. thaliana* has increased susceptibility to various RNA viruses[47,121,122]. Hypomorphic *ago1-27*-mutant plants have near normal RNA slicing activity but are defective in translational repression guided by miRNAs and siRNAs[123–125]. There is also evidence for translational repression of viral mRNAs by AGO1 in *N. benthamiana*[126]. Moreover, *N. benthamiana* AGO2 can direct both RNA slicing and translational repression, and its catalytic tetrad DEDD is indispensable for both activities[127]. Thus, further studies are necessary to define the antiviral role for the slicing activity of the identified antiviral AGO proteins, which may be necessary for the slicing and removal of the passenger strand of duplex vsiRNA to activate vsiRNA–RISC for translational repression instead of viral RNA slicing[127].

Interestingly, preventing expression of the VSR protein B2 during FHV replication in mammalian BHK-21 cells induces translational inhibition of viral RNAs[128]. The specific translational repression of the viral RNAs may be directed by the vsiRNAs, which have been shown to accumulate following nodaviral RNA replication in BHK-21 cells[63,64]. Consistent with the proposed model, suppression of the specific translational repression by B2 protein requires its dsRNA-binding activity[128], which is also essential for its VSR activity[17,59,79,129]. Moreover, there was no evidence of other nonspecific mechanisms of translational inhibition, such as the induction of global translational shutdown mediated by protein kinase R activation, viral RNA degradation by RNase L activation or the formation of stress granules[128].

Derepression of AGO1. Rice AGO18 has a novel antiviral activity that is distinct from those described above[112] (FIG. 3). AGO1, which is essential for antiviral RNAi, is targeted for specific silencing by miR-168 in both *A. thaliana* and rice plants, thereby repressing antiviral RNAi[112,130]. However, AGO18 sequesters miR-168 to alleviate the repression of AGO1. Thus, the small RNA-binding activity, but not the slicing activity, of AGO18 is required for its antiviral function. Accordingly, expression of a modified *ago1* transgene that is resistant to miR-168-mediated regulation rescues the deficiency of *ago18*-mutant plants in terms of virus resistance[112].

RNA-directed DNA methylation. In addition to post-transcriptional gene silencing (or RNAi), DNA virus infection of plants triggers homology-dependent RNA-directed DNA methylation and transcriptional gene silencing[131–133] (FIG. 2). Recent studies have shown that nuclear geminivirus minichromosomes accumulate DNA methylation and histone repressive marks and that DCL3, double-stranded RNA-binding protein 3 (DRB3) and AGO4 proteins, which are necessary for the biogenesis and function of 24-nucleotide siRNAs, are required for DNA methylation-dependent antiviral defence in *A. thaliana*[51–53,134]. Because *A. thaliana* mutants of the RNA-directed DNA methylation pathway have no obvious developmental defects[135] (BOX 3), the markedly defective defence against geminiviruses of these mutants therefore provide a convenient phenotypic assay[52].

Viral suppressors of RNAi

The discovery of VSR proteins encoded by diverse plant, insect and mammalian viruses provided strong evidence for a natural and conserved antiviral function of the RNAi pathway[2–4,136]. As reviewed recently[36,137], VSR proteins are widespread among RNA viruses that infect plants and animals. VSR proteins have also been identified in the two plant DNA virus families and insect DNA viruses of the Ascoviridae, Iridoviridae and Baculoviridae families.

VSR proteins were initially identified in reporter systems that assay for the suppression of transgene RNA silencing in plants[138–140]. However, many bacterial and plant dsRBPs were also found to suppress transgene silencing in the same assays[141,142], revealing a requirement for direct evidence to establish a physiological function for the identified RNAi suppressor activity in virus infection. The B2 protein of FHV was the first VSR protein shown to suppress not only transgene silencing in plants but also AGO2-dependent antiviral RNAi in insect host cells triggered by viral RNA replication[14]. In contrast to wild-type FHV, the mutant FHV that lacks functional B2 protein is cleared rapidly in *D. melanogaster* cells and adult fruitflies, but it replicates to high levels and becomes highly virulent when the function of Dicer-2, R2d2 or AGO2 in the dsRNA–siRNA pathway is compromised[14,16,17,19]. Efficient genetic rescue of the defects in host infection has also been demonstrated in *A. thaliana* mutants that have single or multiple loss-of-function alleles in antiviral RNAi pathways for VSR-deficient beet curly top virus (a DNA virus)[51,52] and for VSR-deficient mutant RNA viruses[41,42,45–47,110], including turnip crinkle virus, cucumber mosaic virus and turnip mosaic virus. It is important to note that genetic inactivation of multiple antiviral RNAi pathways in *A. thaliana* plants does not further increase the accumulation levels of wild-type turnip mosaic virus and cucumber mosaic virus (Fny strain) compared with wild-type plants[45,85], indicating near-complete suppression of antiviral RNAi by the encoded VSR proteins. Therefore, infection with less pathogenic strains or VSR-deficient virus mutants may be necessary to define the antiviral function of a particular RNAi pathway. Interestingly, cucumber mosaic virus may have evolved a self-attenuation mechanism, in which the activity of the VSR protein B2 is antagonized by the viral coat protein[143].

Unlike wild-type nodamura virus, nodamura virus mutants that are defective in RNAi suppression owing to either *B2* gene deletion or a single-residue substitution in B2 protein are rapidly cleared in BHK-21 cells, mouse ESCs and suckling mice[8,9]. By comparison, *Ago2* deletion in mouse ESCs is significantly more effective in increasing the accumulation of B2-mutant nodamura virus than wild-type nodamura virus, suggesting

Protein kinase R
One of the cytosolic sensors of viral and artificial double-stranded RNA in mammals, which, upon activation, can phosphorylate the eukaryotic translation initiation factor eIF2α, leading to global translation shutdown and apoptosis.

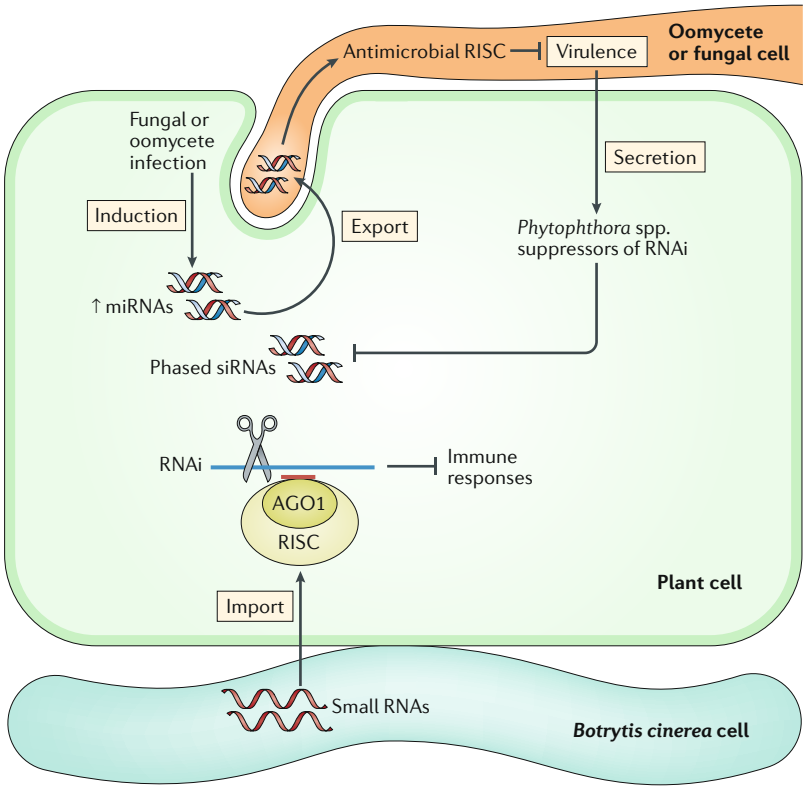

Fig. 4 | **Antimicrobial RNAi by mobile small silencing RNAs.** Plant infection with fungi or oomycetes increases the production of endogenous microRNAs (miRNAs) and phased small interfering RNAs (siRNAs). Some of these miRNAs are exported into fungal hyphae to inhibit infection by silencing the virulence genes using the fungal RNA interference (RNAi) machinery. Oomycete pathogens secrete *Phytophthora* spp. suppressors of RNAi into plant cells that block the biogenesis of host siRNAs and miRNAs, thereby facilitating infection. By contrast, plants import fungal small RNAs to facilitate infection by silencing host immunity genes using the host RNAi machinery. AGO1, Argonaute protein 1; RISC, RNA-induced silencing complex.

specific suppression of an AGO2-mediated antiviral RNAi response by B2 in mouse cells and young mice[63,64]. Similarly, genetic rescue of *NS1*-deletion mutant IAV in *Ago2*^{D597A} MEFs and of VSR-deficient mutant HEV71 in *Dicer*-knockout human 293T cells also indicates suppression of an antiviral RNAi response by human VSR proteins[10,11].

Much is known about the molecular targets of VSR proteins[36,137]. These include the RNA substrates and products of Dicer and the protein components of the RNAi pathway that are required for the biogenesis and activity of vsiRNAs. However, diverse viral structural and non-structural proteins that function at specific stages of the viral life cycle or in counter defence have been identified as VSR proteins. Further studies are needed to examine the specific VSR activity that facilitates virus infection. For example, although several VSR proteins have been shown to inhibit the cell-to-cell spread of RNAi in plants[144–146], it is unknown whether vsiRNAs spread to confer resistance in plants as described for plant endogenous siRNAs and fly vsiRNAs[94,147–149]. Because both Dicer and AGO2 are essential for animal development[1,117,150], examining the in vivo function of mammalian VSR proteins will require the development of conditional knockout mice, in particular adult mice with fully functional interferon-regulated innate immunity[12].

Ping-pong amplification
A model proposed for the biogenesis of animal primary and secondary PIWI-interacting RNAs.

Oomycete pathogens
A distinct phylogenetic lineage of filamentous fungus-like eukaryotic microorganisms, which include important plant pathogens such as those that cause devastating diseases of potato plants.

Virus-derived PIWI-interacting RNAs

Since the first report in *D. melanogaster* ovarian somatic sheet cells[18], vpiRNAs have been identified in mosquito cell lines and in adult *Aedes* mosquitoes (specifically in somatic tissues) infected with various RNA viruses[151–154]. Interestingly, a resistant locus in the genome of domestic chickens targeting an infectious retrovirus and the endogenous virus elements from non-retroviral RNA viruses integrated in mosquito, rodent and primate genomes are also prominent sources of vpiRNAs[155–158]. Similar to the endogenous piRNAs produced by animals to target transposable elements (BOX 1), the biogenesis of vpiRNAs requires members of the PIWI subfamily of AGO proteins and is Dicer-independent[151,159,160]. Mosquito sense and antisense vpiRNAs are loaded in distinct PIWI proteins and enriched for adenosine at the tenth nucleotide position and uridine at the first nucleotide position, respectively, which are the signatures of ping-pong amplification of piRNAs[18,151,160]. However, studies carried out so far, mostly using mosquito cell culture and RNAi knockdown, have not yet provided direct evidence that vpiRNAs have antiviral activity[152,159–161]. Thus, future studies should develop new hypotheses and experimental systems to characterize the function of vpiRNAs.

RNAi against eukaryotic plant pathogens

Pioneering studies of oomycete pathogens have suggested that the plant RNAi pathway has an antimicrobial function against eukaryotic pathogens[5], which encode their own RNAi machinery[1]. The oomycete genus *Phytophthora* includes important crop pathogens such as *Phytophthora infestans*, which famously caused the 1845 Irish potato famine. Among the effector proteins that are secreted by *Phytophthora* spp. to enter host cells, two unrelated proteins exhibit RNAi suppressor activity in plants and can increase host susceptibility to infection with *Phytophthora* spp. and viruses but not with bacterial pathogens[5,162,163]. One *Phytophthora* spp. suppressor of RNAi functions to potently inhibit the biogenesis of endogenous miRNAs and siRNAs in *A. thaliana*[5,162,163]. These findings suggest the existence of a novel counter-defence strategy evolved by eukaryotic pathogens (FIG. 4). Accordingly, *Phytophthora sojae* infection of soybeans was found to increase the accumulation of endogenous miRNAs and phased siRNAs that target host defence genes for silencing[164].

Recent studies found that infection with the fungal plant pathogen *Verticillium dahlia* significantly increases the production of endogenous miR-166 and miR-159 in cotton plants and *A. thaliana*, and both miRNAs are subsequently exported into the fungal hyphae to inhibit the expression of specific target genes essential for pathogenicity[7] (FIG. 4). Infection of *A. thaliana* with the fungus *Botrytis cinerea* also induces the export of endogenous host siRNAs to silence fungal virulence genes, which occurs via exosome-like extracellular vesicles[165], as found for fly vsiRNAs[94]. The presence of a natural mechanism for specific host small RNAs to

translocate into fungal cells for gene silencing explains why the genomes of *Phytophthora* spp. encode RNAi suppressors to inhibit the biogenesis of host small RNAs[5] and why in planta production of long dsRNAs can trigger RNAi of essential fungal genes and confer fungal resistance[166]. Interestingly, after infection with *B. cinerea*, *A. thaliana* genes and tomato plant genes that are involved in immunity are targeted for AGO1-mediated silencing by the pathogen-encoded 21-nucleotide small RNAs produced by fungal Dicer proteins[6]. Parasitic dodder plants also produce a distinct set of miRNAs to induce RNAi against specific host genes in *A. thaliana* and tobacco plants[167], which suggests a virulence function of parasite small RNAs. Therefore, expression of pathogen-encoded suppressors of RNAi and bidirectional trafficking of functional miRNAs and/or siRNAs between plants and eukaryotic pathogens during infection may have a key role in host resistance against eukaryotic pathogens (FIG. 4).

Concluding remarks

Antiviral RNAi is an important defence mechanism against viral infection in fungi, plants, insects and nematodes. Recent studies have shown that infection with four different sense and antisense ssRNA viruses from three families also triggers the production of abundant 22-nucleotide vsiRNAs in cultured mammalian cells and suckling mice. Surprisingly, the genomes of three of these viruses encode distinct VSR proteins that potently suppress the biogenesis of vsiRNAs. Inactivation of VSR protein activity derepresses Dicer-mediated production of vsiRNAs in both undifferentiated and differentiated mammalian cells. Importantly, VSR-deficient mutant viruses become highly susceptible to viral clearance by antiviral RNAi, which requires Dicer-dependent vsiRNAs, AGO2 or the RNA slicing activity of AGO2 and can occur independently of the type I interferon antiviral response. Among the known mechanisms of innate and adaptive immunity[12,13,168,169], therefore, antiviral RNAi is the only mechanism that is broadly conserved in all of the eukaryotic kingdoms.

However, many important questions remain to be addressed regarding the functions and mechanisms of action of antiviral RNAi in mammals. For example, the biogenesis and antiviral activity of mammalian vsiRNAs have so far been characterized only in cell culture and newborn mice, which are known to induce weaker type I interferon responses than adult mammals[170]. Thus, it will be crucial to determine whether antiviral RNAi is also active and necessary in the presence of rapid immune responses mediated by type I, type II and type III interferons. It is currently unknown whether antiviral RNAi directs virus clearance in vivo by specific slicing and/or translational repression and mRNA decay. To address this question, it is necessary to develop infection models for the functional characterization of vsiRNAs and RNAi pathway genes under conditions that do not compromise the essential functions of cellular miRNAs. In this regard, the identification of host genes that are essential for antiviral RNAi but dispensable for miRNA function will be beneficial. Moreover, it remains unclear whether viral suppression of antiviral RNAi is a widespread counter-defence strategy of mammalian viruses, as it is known to be for plant and insect viruses. It may also be worth assessing whether viral suppression of antiviral RNAi inhibits or improves any of the other known innate and adaptive immune responses. Interestingly, constitutive upregulation of the antiviral RNAi pathway in cucumber and rice plants is associated with broad-spectrum virus resistance[89,112], suggesting novel approaches for crop breeding. Exploring the potential impact of this newly recognized mammalian antiviral mechanism and its viral suppression may also lead to new hypotheses for the treatment and vaccination of human viral diseases. Another idea worth exploring is that vpiRNAs may confer transgenerational immune memory[156]. Finally, future studies will investigate the functions and mechanisms of mobile host and fungal small RNAs in disease and resistance. Together, the available data indicate that the conserved RNAi pathway directs small RNA-based antimicrobial immune responses across eukaryotic kingdoms.

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All authors contributed to researching the content for the manuscript and editing before submission. S.-W.D. was responsible for writing the manuscript.

Competing interests
S.-W.D and Y.L. declare competing interests. They are named on one patent application, which is pending, regarding the use of small interfering RNAs as a new mechanism of mammalian antiviral immunity.