Published in final edited form as:

FEBS Lett. 2005 October 31; 579(26): 5965–5973.

Antiviral silencing in animals

Hong-Wei Li and Shou-Wei Ding*

Center for Plant Cell Biology, Institute for Integrative Genome Biology, and Department of Plant Pathology, University of California, Riverside, CA 92521, USA

Abstract

RNA silencing or RNA interference (RNAi) refers to the small RNA-guided gene silencing mechanism conserved in a wide range of eukaryotic organisms from plants to mammals. As part of this special issue on the biology, mechanisms and applications of RNAi, here we review the recent advances on defining a role of RNAi in the responses of invertebrate and vertebrate animals to virus infection. Approximately 40 miRNAs and 10 RNAi suppressors encoded by diverse mammalian viruses have been identified. Assays used for the identification of viral suppressors and possible biological functions of both viral miRNAs and suppressors are discussed. We propose that herpesviral miRNAs may act as specificity factors to initiate heterochromatin assembly of the latent viral DNA genome in the nucleus.

1. Introduction

Homology-dependent gene silencing was discovered in transgenic plants in the form of co-suppression between introduced transgenes or between a transgene and its homologous endogenous gene [1–4]. Similar gene silencing phenomena have subsequently been described in a wide range of eukaryotic organisms such as fungi [5], worms [6], flies [7], and mammals [8]. Both RNA silencing and RNA interference (RNAi) have been used as generic terms to describe these related gene silencing mechanisms guided by small RNAs such as small interfering RNAs (siRNA) and microRNAs (miRNA).

A core feature of RNA silencing detected in all organisms is the production of 21- to 26-nt small RNAs by the endoribonuclease Dicer [9]. In *Drosophila melanogaster*, dicing of the imperfectly base-paired precursor miRNAs (pre-miRNAs) and the perfectly base-paired double-stranded RNA (dsRNA) are carried out by two distinct Dicers, Dicer-1 and Dicer-2, respectively. In contrast, both pre-miRNAs and dsRNA are processed by the single Dicer in mammals and worms. The resulting miRNAs and siRNAs are asymmetric [10] when they are assembled into effector complexes such as the RNA-induced silencing complex (RISC). All effector complexes described to date contain a member of the Argonaute (Ago) protein family, containing the PAZ and PIWI domains [11]. siRNA and miRNA control the specificity of RNA silencing by recruiting the effector complex to a cognate single-stranded RNA target, leading to either slicing, translational arrest, or nascent RNA synthesis [9]. RNA synthesis by RNA-dependent RNA polymerases (RdRP) amplifies RNAi as it provides a new source of dsRNA for the production of secondary siRNA and may also be essential for the spread of RNAi to distal tissues in some organisms [12]. Unlike Dicer and Ago, RdRP is found only in fungi, plants and worms and the multiple-turnover RISC may mediate RNAi in the absence of a cellular RdRP in *D. melanogaster* and mammalian cells [9].

Pioneering work by plant virologists has established that plants respond to virus infection by inducing RNAi, leading to specific recognition and destruction of the invading virus RNAs

*Corresponding author., E-mail address: dingsw@ucr.edu.

and homologous host RNAs [13,14]. Plants infected with either RNA or DNA viruses accumulate virus-derived siRNAs in both plus and minus polarities, suggesting a role for dsRNA in the initiation of antiviral RNAi. Furthermore, diverse viral proteins essential for plant infection have been identified as suppressors of RNAi [15].

In contrast to the widespread recognition of a natural antiviral role for RNA silencing in higher plants, no experimental evidence was available until recently on whether or not RNA silencing plays a similar antiviral role in the animal kingdom. Here we review recent progress in establishing RNA silencing as a novel nucleic acid-based antiviral immunity in invertebrates and discuss features and possible functions of miRNAs and RNAi suppressors encoded diverse mammalian viruses.

2. Insect viruses induce and suppress antiviral silencing

Following the 1998 demonstration of dsRNA-mediated RNAi in *Caenorhabditis elegans* [16], many arthropod species have been found to support RNAi. These include fruit fly [17], medfly [18], milkweed bug [19], mosquito [20,21], giant silkworm [22], red flour beetle [23], Noctuid moth [24], tick [25], honeybee [26], and spider [27]. However, the first direct evidence establishing a natural antiviral role for RNAi in an animal species was not reported until 2002 [28]. The experimental system was based on infection of cultured *D. melanogaster* S2 cells with flock house virus (FHV), a member of the *Nodaviridae* family.

Nodaviruses contain a bipartite plus-strand RNA genome, and are pathogens of insects and fishes although one member, Nodamura virus (NoV), is known to be pathogenic also in mammals [29]. RNA1, the 1st genome segment of nodaviruses, encodes the entire viral contribution to the viral RNA-dependent RNA polymerase (RdRP) and replicates autonomously in the absence of RNA2, which depends on RNA1 for replication. RNA2 directs the expression of the pre-capsid protein (pre-CP) essential for virion packaging. Induction of antiviral silencing in *Drosophila* cells upon FHV infection is supported by several lines of evidence. First, FHV infection initiated by FHV virions results in the rapid accumulation of the virus-specific 22-nt RNAs in infected cells, which were detected by both RNA blot hybridization [28] and direct small RNA cloning (HW Li and SW Ding, unpublished data). These small RNAs correspond to both plus and negative strand FHV RNAs, suggesting recognition and dicing of double-stranded replicative intermediates into viral siRNAs by the *Drosophila* RNAi machinery.

Second, increased accumulation of FHV RNAs was observed in the fly cells after the RNAi pathway was made partially defective by depleting Ago2, a core component of the siRNA-loaded RISC [9]. Thus, a functional RNA silencing pathway naturally restricts FHV accumulation in infected fruit fly cells. Third, the B2 protein of FHV was identified as a suppressor of antiviral silencing. B2 is encoded at the 3'-end of FHV RNA1, but unlike the viral RdRP that is translated directly from RNA1, B2 is translated from RNA3, a subgenomic RNA transcribed during RNA1 replication. The silencing suppressor activity of B2 was first revealed using an RNA silencing assay established in transgenic plants [28]. Further work showed that an FHV RNA1 mutant that does not express B2 failed to accumulate to detectable levels in wild type *Drosophila* cells, but the same mutant accumulated to wild type levels in S2 cells defective for RNAi due to Ago2 depletion. Thus, the essential role of B2 in FHV infection is suppression of the Ago2-dependent antiviral silencing induced by FHV replication in *Drosophila* cells [28].

Recent work showed that NoV RNA replication also triggers the RNAi-mediated antiviral response in *D. melanogaster* cells [30]. Abundant accumulation of NoV RNAs in cultured *Drosophila* cells is dependent on either Ago2 depletion or expression of B2 of either NoV or FHV. Although B2 of NoV shares limited sequence similarity with FHV B2 (identity <19%),

it also suppresses RNA silencing in plants and rescues the infection of the B2-deletion mutant of FHV in fruit fly cells [30]. Therefore, induction and suppression of RNAi-mediated antiviral silencing in *Drosophila* cells is a conserved feature of two distantly related nodaviruses, implicating RNAi as an antiviral immunity in fruit flies [13].

Comparative genomic analysis has revealed a conserved RNAi pathway between *D. melanogaster* and the malaria mosquito *Anopheles gambiae* [31,32]. As observed in *D. melanogaster*, NoV RNA replication in cultured *An. gambiae* cells also triggers RNAi-mediated antiviral silencing [30]. B2 expression is essential for the accumulation of NoV RNAs in transfected cells but it became dispensable in *An. gambiae* cells depleted of the mosquito Ago2 by RNAi. Thus, the RNAi machinery of malaria mosquitoes has the capacity to detect the replicating virus RNA and to mount an effective antiviral response.

gypsy is an endogenous retrovirus of *D. melanogaster*. Pelisson and colleagues show that sensor reporter constructs carrying part of the *gypsy* sequence are silenced in fly ovaries in a manner dependent on the *piwi* argonaute gene [33]. Notably, fly ovaries accumulate *gypsy*-specific small RNAs of 25–27 nt, indicating that the endogenous retrovirus is also targeted for silencing in *D. melanogaster* by an RNAi mechanism [33]. We found recently that infection of *D. melanogaster* cells with Cricket paralysis virus (CrPV) leads to accumulation of CrPV-specific siRNAs and that CrPV encodes the activity to suppress the antiviral silencing response in *D. melanogaster* cells (R Aliyari and SW Ding, unpublished data). CrPV is an insect RNA virus that is distinct to nodaviruses, but is closely related to animal picornaviruses [34]. Taken together, available data show that RNAi represents a common antiviral response in invertebrates and that effective suppression of RNAi immunity may be a key feature of invertebrate viruses.

3. Do arboviruses induce and suppress antiviral silencing in arthropod vectors?

Arthropod-borne viruses (arboviruses) include many dangerous human pathogens and most have an RNA genome such as dengue viruses (DENV) and alphaviruses. However, arboviruses are not considered true insect viruses because they are typically not pathogenic to their insect hosts [35]. Early studies to engineer pathogen-derived resistance against DENV have suggested active antiviral silencing in *Aedes aegypti* [36,37]. Cultured *Ae. aegypti* cells or female adults became resistant to DENV after transfection with recombinant Sindbis alphavirus (SINV) expressing a structural protein of DENV. Resistance to DENV is serotype-specific and independent of the expression of the DENV protein, indicating that it is RNA-mediated [36]. Insertion of part of an endogenous gene into the SINV vector also leads to silencing of the corresponding gene in infected mosquitoes. All of these features are reminiscent of the specific RNA silencing of host endogenous genes and transgenes induced by plant virus expression vectors [38,39].

O'nyong-nyong virus (ONNV) is a human pathogenic alphavirus vectored by *An. gambiae*. Olson and colleagues showed that co-injection of ONNV with dsRNA targeting *An. gambiae* Ago2 resulted in higher virus titer and wider virus distribution in adult female mosquitoes in comparison with co-injection of a non-specific dsRNA [40]. Since the role of *An. gambiae* Ago2 to mediate antiviral RNAi is known [30], this finding suggests an antiviral role for RNAi against an arbovirus in its arthropod vector [40]. Demonstration of the accumulation of ONNV-specific siRNAs in infected *An. gambiae* will further support this conclusion.

As in mosquitoes, insertion of host genes into SINV resulted in specific host gene silencing in the commercially used silkworm *Bombyx mori* infected with the recombinant virus [41]. Similar

virus-induced gene silencing was also recently documented in cultured tick (*Ixodes scapularis*) cells infected with a gene expression vector based on another alphavirus, Semliki forest virus (SFV) [42]. Notably, the accumulation of alphavirus-specific small RNAs was detected in insect cells infected with either alphavirus, indicating that alphaviral RNA replication induces antiviral RNAi in these insects. However, it has not been addressed in any of these systems whether or not the accumulation of the alphavirus is down-regulated by induction of the insect RNAi.

It remains unknown what is the molecular basis for the difference between arboviruses and insect viruses since both are recognized by the RNAi machinery of insects. Our recent work indicates that the dengue viral genome may not encode a suppressor of antiviral silencing since none of the mature dengue viral proteins is able to rescue the infection of the B2-deletion mutant of FHV in fruit fly cells (WX Li and SW Ding, unpublished data). Thus, one attractive hypothesis is that unlike true insect viruses, arboviruses do not encode a suppressor of antiviral silencing. Alternatively, arbovirus-encoded RNAi suppressors are not active or less active in insect hosts than in their vertebrate hosts. As a result, replication and infection of arboviruses may be restricted to insect tissues where antiviral silencing is weak.

4. RNAi-mediated antiviral silencing in *C. elegans*

The RNAi machinery of the nematode *C. elegans* contains features of RNA silencing found unique in both vertebrates and plants [6]. As found in mammals, *C. elegans* contains a single Dicer that processes precursors of both miRNA and siRNA. However, *C. elegans* also supports transitive RNAi and systemic RNAi, which are active in plants but apparently not in mammals or *D. melanogaster* [12]. Until recently [43,44], no experimental evidence was available on whether or not the worm RNAi pathway is able to recognize and mount an effective defense against virus infection, although dsRNA-mediated RNAi was first demonstrated in *C. elegans* [16]. This is at least partly because *C. elegans* is not known to support replication of any virus.

Recently, Wilkins and colleagues showed that Vesicular stomatitis virus (VSV), which has a nonsegmented, negative-strand RNA genome, is able to infect primary cell cultures of *C. elegans*. VSV accumulation is enhanced in cell cultures derived from RNAi-defective worm mutants, but is inhibited in cell cultures from mutants with an enhanced RNAi response [44]. Lu and colleagues have shown complete replication of FHV in worms after transcriptional induction of chromosomally integrated transgenes coding for full-length cDNA copies of FHV genomic RNAs [43]. Further analysis showed that FHV replication in *C. elegans* triggers potent antiviral silencing that must be suppressed by FHV B2. A B2-deficient mutant of FHV, which does not replicate in wild-type animals, is efficiently rescued in a mutant carrying a loss-of-function mutation in *rde-1*, which encodes a worm AGO essential for RNAi mediated by siRNAs but not by miRNA [6,45]. This genetic complementation of loss-of-function mutations in a viral RNAi suppressor gene and a host RNAi pathway gene provides the first direct evidence in any host systems for a counter-defense role of a virus-encoded RNAi suppressor. This complementation has not been demonstrated in plants despite the facts that plant viruses carrying loss-of-function mutations in their suppressor gene exhibit defects in plant infections and that plant viral suppressors target various steps in the RNA silencing pathway [15].

B2 expression was also found to enhance virus accumulation in the *rde-1* mutant, indicating the presence of active antiviral silencing in the *C. elegans* mutant that is possibly mediated by an RDE-1 homolog. Notably, the *C. elegans* genome encodes 27 AGOs in total, among which differential requirements for the Agos Alg-1, Alg-2, PPW-1 and PPW-2 in various RNAi processes have been documented [46]. The number of AGO genes in *C. elegans* is significantly higher than the 5 found in *Drosophila* and 10 in *Arabidopsis* [47], suggesting an evolutionary

strategy of host adaptation to viruses through expansion of the AGO family. Although tobamoviruses and nepoviruses use plant-parasitic nematodes as their transmission vector [48], there are no known natural viral pathogens of *C. elegans*.

5. Mammalian virus-encoded miRNAs

The RNAi machinery of invertebrates and vertebrates shares many features and neither may contain the RdRP component found in fungi, plants and *C. elegans*. It has been controversial whether or not RNAi acts as an antiviral immunity in vertebrates as has been established in plants and invertebrates. In 2004, Tuschl and colleagues reported the cloning and detection of five virus-specific small RNAs of 21–23 nucleotides from Burkitt's lymphoma cell line latently infected with Epstein-Barr virus (EBV), a human gammaherpesvirus [49]. These RNAs are considered miRNAs, not siRNAs, because of the detection of both the fold-back structures flanking the cloned RNAs in the genomic sequence and the approximately 60-nt precursor miRNAs (pre-miRNAs) in the infected cells. Furthermore, EBV-infected cells accumulate an EBV-specific miRNA*, which corresponds to the opposite strand of an miRNA in the predicted fold-back structure [49]. Thus far, a total of 34 miRNAs (plus 7 corresponding miRNA*s), all of which are encoded by human herpesviruses, have been cloned and verified by northern blot analysis [50–52]. These include 11 miRNAs from Kaposi's sarcoma-associated gammaherpesvirus (KSHV) and 9 miRNAs each from mouse gammaherpesvirus 68 (MGHV) and human cytomegalovirus (HCMV), a betaherpesvirus. In addition, viral miRNAs have also been identified from cells infected with Simian virus 40 (SV40) and adenovirus by RNase protection assays and/or northern blot hybridizations [53,54].

These viral miRNAs are at least as abundant as cellular miRNAs as indicated by the frequency of cloning and northern blot analysis. Like cellular miRNAs, most of the viral miRNAs are likely to be processed from DNA-dependent RNA polymerase II (Pol II) transcripts and editing of the viral pre-miRNAs can occur [52]. However, all of the 9 MGHV miRNAs are predicted to be transcribed by Pol III [52]. All of the viral miRNAs identified so far are encoded by DNA viruses of which both DNA replication and mRNA transcription occur in the nucleus. It will be of interest to determine if miRNAs are produced by poxviruses that contain large DNA genomes but replicate entirely in the cytoplasm. RNA transcripts from these cytoplasmic viruses would not have access to the nuclear protein Drosha, which is required for the production of pre-miRNA.

Production and detection of virus-specific small RNAs demonstrate that the single mammalian Dicer is capable of sensing the dsRNA feature of viral RNAs in infected cells as found in *C. elegans*, which also encodes a single Dicer [43]. Two classes of viral miRNAs have the antiviral potential in targeting important virus genes. First, four of the cloned herpesviral miRNAs are perfectly complementary to viral mRNAs transcribed in the opposite orientation [49,53]. Since it is known that miRNAs can act like siRNAs to cleave their target mRNAs [55,56], these viral miRNAs may have an antiviral role to direct cleavages of their target viral mRNAs. Indeed, Ganem and colleagues showed that both the miRNA and its miRNA* of SV40 mediate *in vivo* cleavages of the perfectly complementary SV40 early transcripts, which encode the viral T antigens [53]. Intriguingly, down-regulation of T antigens by SV40 miRNAs had no effect on the production of infectious virus particles in cell cultures. Instead, wild-type SV40-infected cells became less sensitive to lysis by cytotoxic T cells than a mutant lacking miRNAs. Thus, miRNA production is considered beneficial for SV40 under the conditions examined as the viruses maintain replicative efficiency by reducing excess antigen production [53]. It will be important to determine if this is reproduced in an animal model. Second, five known viral miRNAs are located in the mature mRNA of viral protein-coding genes [49,52]. These include three EBV miRNAs found within the 5' and 3' untranslated region of the EBV protein-coding gene BHRF1, HCMV-miR-U112-1 in the viral gene UL114, and KSHV-miR-10 in the kaposin

mRNA transcripts. Thus, excision of pre-miRNA from these transcripts would also disrupt expression of the viral protein, which may occur at the latent stage [49].

For most of the known viral miRNAs, however, bioinformatics searches detected no highly complementary sites within the coding regions of the corresponding viral genomes [49–52]. Thus, a popular hypothesis is that viral miRNAs act like true miRNAs to down regulate expression of cellular and/or viral genes by inhibiting their translation, which may play a critical role in specific aspects of virus biology such as the establishment and/or maintenance of herpesvirus latency [49,50]. However, the 35 miRNAs identified from the three closely related gammaherpesviruses and one betaherpesvirus share no substantial sequence homology with one another [52]. Thus, it is difficult to reconcile how conserved host genes/functions are targeted by such diverse viral miRNAs.

In addition to translational inhibition and cleavage of target mRNAs, Arabidopsis miRNAs also guide chromatin modification by inducing DNA methylation downstream of the miRNA complementary sites [57,58]. In this regard, miRNAs can be functionally equivalent to the centromeric siRNAs found in fission yeast that act as specificity factors to initiate heterochromatin assembly [59]. The role of siRNAs in directing repressive chromatin and/or DNA modifications appears to be conserved in plants, *Drosophila* and mammals [60,61]. Furthermore, it is known that the genomic DNA of nucleus-replicating viruses is assembled into mini-chromatin with host histones and other chromatin proteins and that DNA methylation targets and represses genome expression of human DNA viruses [62]. Thus, an entirely untested but intriguing possibility is that viral miRNAs may induce *in cis* viral DNA methylation and/or methylation of cellular histones associated with the viral DNA in the nucleus, in a manner similar to heterochromatin siRNAs.

It is interesting to note that viral miRNAs cloned from cells latently infected with three herpesviruses are clustered in latency-associated regions, whereas HCMV miRNAs cloned from lytically infected cells are spread across the viral genome [50–52]. It is possible that the highly clustered, latency-associated viral miRNAs play a role in the initiation and/or maintenance of the latent state of herpesviral infection in a position-dependent manner. This model can be easily tested by determining if the mammalian RNAi machinery and/or viral suppressors of RNAi play any role in virus latency.

6. Mammalian antiviral immunity directed by cellular miRNAs

A recent study from the Voinnet lab discovered a novel type of mammalian antiviral immunity mediated by a cellular miRNA [63]. A miR-32 complementary site was identified and mapped to the 3' UTR shared by five mRNAs of the retrovirus primate foamy virus type 1 (PFV-1). A PFV-1 mutant carrying mutations that disrupt the partial complementation with miR-32 accumulated to much higher levels than the unmodified virus in infected cells. A defensive role for the RNAi pathway in mammalian cells against PFV-1 is further supported by the observation that the Tas protein encoded by PFV-1 suppresses RNA silencing mediated by either miRNAs or siRNAs [63].

Several important predictions can be made based on this discovery. First, the large numbers of miRNAs identified for each animal species (e.g. >500 in human) and the broad spectrum of genes targeted by each miRNA (up to 200 human genes/miRNA) [64,65] predict that many cellular miRNAs may have antiviral potential. Second, it is likely that the expression pattern of cellular miRNAs may play a role in the pathogenesis, tissue tropism, and/or host range of individual viruses. Third, the combined specificity repertoire of host miRNAs may influence the rate and direction of virus evolution. This could be particularly significant for viruses with an RNA genome because of high error rates in RNA replication. It has been documented that both human immunodeficiency virus (HIV) and poliovirus readily escape highly effective,

artificially introduced siRNAs through unique point mutations within the targeted regions [66–69]

7. Mammalian viral suppressors of RNAi

The first indication for an antiviral potential of the mammalian RNAi pathway came from the identification of RNAi suppressors encoded by mammalian viruses [28,30]. RNAi suppression by B2 of FHV and NoV, vaccinia E3L, and NS1 of human influenza A, B, and C viruses were first demonstrated in insect cells [30] and their activities in suppressing RNAi mediated by siRNAs and miRNAs were later confirmed in mammalian cells ([70]; Li HW and Ding SW, unpublished data). In addition, VA1 of adenovirus [54,71], NSs of La Crosse virus [72], $\sigma 3$ of reovirus [73], Tat of HIV [74], and Tas of PFV-1 [63] also exhibit RNAi suppressor activity in mammalian cells. VA1 is a highly structured RNA of about 160 nt transcribed from a viral Pol III promoter during viral infection. Notably, these RNAi suppressors are encoded by genetically diverse viruses whose genome types include dsDNA (vaccinia and adenovirus), dsRNA (reovirus), segmented plus-strand (nodaviruses) and negative-strand (influenza and La Crosse viruses) RNA, and plus-strand RNA replicated via DNA intermediates (HIV and PFV-1 retroviruses). Many mammalian viruses are sensitive to inhibition of RNAi mediated by introduced siRNAs [75], which, however, does not necessarily argue against the possibility that they may encode a RNAi suppressor. Engineered virus resistance based on RNAi is also effective against a wide range of plant viruses carrying diverse RNAi suppressors [76]. This is probably because viral suppression of RNAi is always partial or is effective against only one of the antiviral silencing pathways [77]. Thus, the evolutionarily fine tuned viral counter-defense may become ineffective upon up-regulation of antiviral silencing by artificial means.

RNAi suppression assays

Two types of assays have been used to identify mammalian viral suppressors of RNA silencing according to whether or not the silencing inducer is a replicating virus. Replication of the RNA1 mutant of either FHV or NoV expressing no B2 does not lead to a readily detectable accumulation in cultured *Drosophila* S2 cells because of antiviral silencing in a RISC-dependent manner [28,30]. Rescue of either RNA1 mutant as determined by Northern blot detection of virus RNAs occurs when S2 cells are co-transfected with a plasmid directing expression of an RNAi suppressor. The readout of this silencing suppression assay can also be expression of green fluorescent protein (GFP) after the GFP coding sequence is fused in frame with the initiation codon of B2 in RNA1 of either FHV or NoV [30]. A similar assay that is based on suppression of the RISC-dependent silencing of the replicating NoV RNA1 has been developed in cultured mosquito cells using a constitutive promoter to drive the *in vivo* transcription of the viral RNA1 [30].

B2 of NoV also enhances the accumulation of both RNA1 and the RNA1-derived RNA3 of NoV in HeLa cells [78] and B2 of both NoV and FHV can inhibit RNAi triggered artificially in human cells ([70]; Li HW and Ding SW, unpublished data). However, it is not known if the enhanced NoV accumulation in HeLa cells is due to a RISC-dependent RNAi triggered by NoV RNA replication. Therefore, suppression of antiviral silencing by many of the known mammalian viral suppressors such as B2, NS1, and E3L were first shown in heterologous systems established either in insect cells or in plants [28,30]. For that reason, demonstration of the RNAi suppression activity in mammalian cells relies on induction of RNAi mediated by synthetic siRNAs, short hairpin RNAs (shRNAs) transcribed from transgene constructs, or cellular miRNA.

The most commonly used inducer of RNAi in mammalian cells is the chemically synthesized siRNAs of 21-nt that are 5'-phosphorylated and contain 2-nt overhangs at the 3'-ends [79]. However, identification of many viral RNAi suppressors in mammalian cells involves the use

of shRNAs [54,70,71,74]. shRNAs is analogous to pre-miRNAs because it requires *de novo* processing into siRNAs followed by RISC assembly. Thus, use of shRNAs as RNAi triggers will identify suppressors that target those early steps. Indeed, several known suppressors are inactive when synthetic siRNAs were used to induce RNAi (see below). In addition, perfectly and imperfectly complementary sites for cellular miRNAs cloned into the 3'-UTR of reporter constructs can sense RNA cleavage and translational inhibition respectively mediated by the cognate miRNAs. This type of sensor constructs has been successfully used to identify the RNAi suppressor activity of Tas in mammalian cells [63].

Both endogenous genes and transgenes encoding GFP or luciferase have been used as the reporter target gene in RNAi suppression assays. An eGFP-based reporter constructed recently was shown particularly useful for this purpose [70]. The plasmid encodes an engineered PEST sequence that destabilizes eGFP to allow real time correlation between fluorescence and mRNA levels of GFP [80]. In addition, six extra copies of the shRNA target site were inserted into the 3'-UTR to enhance sensitivity to RNAi [70].

Inducible silencing of a reporter GFP transgene integrated in the genome of *Nicotiana benthamiana* plants was used in the identification of the first animal viral suppressor of RNAi [28,81]. Among the known mammalian viral suppressors, E3L, Tat, NSs and VA have not been assayed in plant systems; however, B2 [30], NS1 [82,83], and Tas [63] are all active suppressors of plant RNA silencing. $\sigma 3$ of reovirus also suppresses RNA silencing in plants although this activity has yet to be confirmed in animal cells [73]. This is important as it appears that over expression of any dsRNA-binding protein including an *Escherichia coli* protein results in suppression of transgene silencing in this plant assay [73]. In contrast, antiviral silencing induced by the replicating FHV RNA in *Drosophila* cells is insensitive to over expression of many cellular dsRNA-binding proteins [30]

RNAi suppression mechanisms

Most of the mammalian viral suppressors reported to date are dsRNA-binding proteins. E3L and $\sigma 3$ contain a canonical dsRNA-binding domain (dsRBD). However, the structural motif of NS1 involved in dsRNA binding forms a symmetric homodimer with a six-helix chain fold [84,85] that is distinct from either the canonical dsRBD or p19 [86,87]. Similarly, both B2 and Tat share no homology at the primary sequence level with any of the known dsRNA-binding proteins. Tat binds to perfect base-paired dsRNA as well as a 59-nt stem-loop structure called trans-activation (TAR) response element of HIV RNA [88]. Both NS1 [30,83] and B2 [43, 70] bind siRNAs in addition to long dsRNA, but siRNA binding has not been tested for any of the other suppressors and it is also unknown if Tas or NSs binds long dsRNAs. Interestingly, B2 also binds to the imperfectly base-paired human pre-miRNAs [70] and exhibits a 30-fold higher affinity to long dsRNA than siRNAs [43]. A single amino acid replacement at position 54 of FHV B2 (R54Q) largely eliminated its activity to suppress antiviral silencing and to bind long dsRNA but it did not completely abolished siRNA-binding [43]. Thus, B2 may inhibit antiviral silencing by targeting the precursor of siRNAs [43], in contrast to p19 of the plant tombusvirus, which has much weaker affinity to siRNA duplexes longer than 23 nt and inhibits the assembly of siRNAs into RISC [86,87,89]. dsRNA as a cellular target of B2 perhaps explains why B2 can suppress RNAi in a wide range of host species including plants, fruit flies, mosquitoes, worms and mammals [28,30,43,70].

Although B2 inhibits RNAi induced by either shRNA or siRNA in mammalian cells, shRNA-induced RNAi is several-fold more sensitive to B2 [70]. Moreover, both Tat and VA1 suppress RNAi induced by shRNAs but not siRNA-induced RNAi [71,74]. Accumulation of VA1 appears to inhibit both the processing and export of shRNA transcripts and pre-miRNA in mammalian cells [54,71]. Over expression of B2 in human cells also increase the accumulation of shRNA transcripts and at least one endogenous pre-miRNA examined [70]. These data

suggest that B2, Tat, and VA1 may act upstream of Dicer cleavage. Indeed, all three suppressors inhibit the *in vitro* cleavage of dsRNA by human Dicer [70,71,74] and FHV B2 also blocks siRNA production from dsRNA by *Drosophila* Dicer extracts [43].

Available data further indicate that these suppressors may target the dicing step by distinct mechanisms. VA1 RNA binds Dicer *in vitro* [54,71] and serves as a substrate of Dicer *in vitro* and *in vivo*, leading to production of VA-specific miRNAs [54], suggesting a mechanism of RNAi suppression by sequestering Dicer. In contrast, B2 inhibition of siRNA production by *Drosophila* Dicer extracts is correlated with its ability to bind long dsRNA because the inhibitory effect of B2 was essentially eliminated by the R54Q mutation [43]. Thus, B2 may inhibit RNAi by mainly preventing the processing of the siRNA precursor, which is distinct to that proposed for p19 via sequestering siRNAs [89].

Tat inhibition of the Dicer activity may be distinct from its activity in either RNA binding or transcriptional activation, as suggested by the contrasting activities of two Tat mutants [74]. Replacement of Lys by Ala at position 51 (K51A) abolished the RNAi suppression activity of Tat without any effect on its transcriptional activity whereas the opposite was true when the same replacement was made at position 41 of Tat (K41A). Notably, the transcriptionally active, RNAi suppression-deficient TatK51A is fully active for RNA binding [74]. However, it remains to be determined if Tat or TatK51A binds siRNAs.

Functional roles of RNAi suppression in virus infection

Introduction of the TatK51A mutation into HIV led to a modest decrease in the accumulation of the recombinant HIV in infected human cells in comparison with the wild type [74]. Similarly, an NoV mutant that does not express B2 exhibits a defect in virus accumulation as severe in some human cell lines as in fruit fly and mosquito cell lines [30,78]. In fact, all of the virus-encoded RNAi suppressors identified to date are required for virus infection in mammalian cells. However, most of these suppressors, including NS1, E3L, Tat, and VA1, are also known to function in their mammalian hosts as inhibitors of the innate antiviral immunity regulated by the interferon system [90]. In particular, inhibition of the IFN/PKR-mediated immunity by NS1 and E3L [90] requires the dsRNA binding motif implicated in RNAi suppression [30,43]. Thus, it will be critical to establish if the requirement of these RNAi suppressors in the infection of mammalian hosts is due to a specific RNAi suppression and independent of or in addition to, their active suppression of IFN/PKR-mediated immunity. In this regard, it may be particularly informative to know if TatK51A, deficient in RNAi suppression but active in RNA binding [74], remains active in the suppression of the IFN response.

This issue has been resolved in invertebrates, which do not contain the IFN/PKR system, because virus mutants carrying loss-of-function mutations in the RNAi suppressor gene are specifically rescued in cell cultures [28,30,44] and whole organisms [43] either by RNAi depletion of Ago2 or by a genetic loss-of-function mutation in an RNAi pathway gene. Use of similar genetic knock-out mouse cell lines [91] or knocking-down expression of RNAi pathway genes by siRNAs should help clarify this controversial issue in mammalian systems. A complementary approach is to determine if expression of mammalian RNAi suppressors enhances virus accumulation in mammalian cell lines (e.g., Vero cells) or animal models deficient in IFN/PKR-mediated immunity.

Identification of viral suppressors effective against RNAi induced by shRNAs but not siRNAs may have provided a clue for their biological function as viral regulators of the miRNA pathway. Indeed, Tas, VA1, B2 and NS1 also suppress RNAi mediated by miRNAs ([63,71] Li HW and Ding SW, unpublished data). Thus, viral RNAi suppressors may play a counter-defensive role in modulating the targeting of viral genes or host genes by either cellular and/

or viral miRNAs. Interference of miRNA-regulated expression of host genes may also provide a new mechanism of mammalian viral pathogenesis as has been proposed for plant viral RNAi suppressors [92,93]. Notably, NS1 is localized in the nucleus of infected cells and may thus have a potential to disrupt the biogenesis of mammalian miRNAs and the RNAi-directed assembly of heterochromatin in the nucleus.

8. Conclusions and perspectives

Available data clearly support a naturally antiviral role for RNAi in invertebrate animals. As has been demonstrated for plant viruses, invertebrate viruses induce RNAi that specifically targets the infecting virus RNAs and they encode RNAi suppressors that are essential for virus infection. Moreover, the rescue of nodaviral mutants deficient in RNAi suppression in insect cells and worms deficient in RNAi provides direct evidence for a specific requirement of viral suppression of the RNAi antiviral immunity during virus infection. This type of genetic complementation has not been demonstrated for any of the several dozens of plant viral suppressors. It is evident that RNAi also protects arthropod vectors against arboviruses because of the detection of viral siRNAs and homology-dependent silencing of RNA molecules in infected insects. However, most of these observations have been made in cell cultures and it is largely unknown if adult invertebrates will respond to virus infection in a similar manner. It is possible that antiviral silencing is mediated by the siRNA pathway because antiviral silencing in *D. melanogaster* and *C. elegans* requires an Ago member not essential for miRNA function. This predicts an involvement of *D. melanogaster* Dicer-2 in the production of viral siRNAs, which, however, remains to be confirmed. Given the availability of the fruit fly and worm mutants carrying genetic mutations in genes involved in well defined steps of RNAi pathways, it is likely that these host models will facilitate further genetic and molecular dissection of the RNAi-mediated antiviral silencing pathway in invertebrates.

Several lines of data suggest that miRNAs play a role in mammalian responses to virus infection. These include the demonstration of antiviral silencing directed by a cellular miRNA and the identification of RNAi suppressors encoded by diverse mammalian viruses. In addition, production of viral miRNAs demonstrates recognition of the nucleus-replicating DNA viruses by the mammalian RNAi machinery. Moreover, specific virus RNA silencing has been demonstrated for two SV40 miRNAs and proposed for nine herpesviral miRNAs. Much less is clear about the role of siRNAs in mammalian responses to viruses. dsRNA is produced during replication of viruses with an RNA genome and of some DNA viruses due to symmetrical transcription of both viral DNA strands [94]. Induction of antiviral silencing by two cytoplasmic replicating RNA viruses recently demonstrated in a single Dicer organism, *C. elegans*, suggests an antiviral potential of the human Dicer. However, large scale RNA cloning projects had not identified viral miRNAs or siRNAs from human cells infected with Hepatitis C virus (HCV), yellow fever virus (YFV), or HIV [52]. Two and four small RNAs were each cloned once from HIV and YFV infected cells, respectively, but all are in plus-sense orientation and from genomic regions not predicted to form structures similar to pre-miRNAs. In addition, infection of HCV did not interfere with RNAi of a host mRNA mediated by either siRNAs or shRNAs, suggesting that infection of these plus-strand RNA viruses in cell cultures may escape detection of the mammalian RNAi machinery so that active viral suppression of RNAi is not required [52]. Nevertheless, the siRNA hypothesis has not been examined rigorously for many mammalian viruses and use of RNAi-deficient cell lines coupled with ectopic expression of well-defined RNAi suppressors may prove informative.

Acknowledgements

We thank Drs. Olivier Voinnet and Morris Maduro for critical review of this manuscript. Research on RNA silencing in this lab was supported by grants from NIH, USDA, California Citrus Research Board, and the University of California BioSTAR program.

References

1. Matzke MA, Primig M, Trnovsky J, Matzke AJM. Reversible Methylation and Inactivation of Marker Genes in Sequentially Transformed Tobacco Plants. *EMBO J* 1989;8:643–649. [PubMed: 16453872]
2. Napoli C, Lemieux C, Jorgensen R. Introduction of a chimeric chalcone synthase gene into *Petunia* results in reversible co-suppression of homologous genes in trans. *Plant Cell* 1990;2:279–289. [PubMed: 12354959]
3. Van der Krol AR, Mur LA, Beld M, Mol JN, Stuitje AR. Flavonoid genes in *petunia*: addition of a limited number of gene copies may lead to a suppression of gene expression. *Plant Cell* 1990;2:291–299. [PubMed: 2152117]
4. Smith CJ, Watson CF, Bird CR, Ray J, Schuch W, Grierson D. Expression of a truncated tomato polygalacturonase gene inhibits expression of the endogenous gene in transgenic plants. *Mol Gen Genet* 1990;224:477–481. [PubMed: 2266949]
5. Nakayashiki, H. (2005). RNA silencing in fungi: mechanisms and applications. *FEBS Lett*.
6. Grishok, A. (2005). RNAi mechanisms in *C. elegans*. *FEBS Lett*.
7. Kavi, H.H., Fernandez, H., Xie, W., and Birchler, J.A. (2005). RNA silencing in *Drosophila*. *FEBS Lett*.
8. Aravin, A., and Tuschl, T. (2005). Identification and characterization of small RNAs involved in RNA silencing. *FEBS Lett*.
9. Hammond, S.M. (2005). Dicing and Slicing: The core machinery of the RNA interference pathway. *FEBS Lett*.
10. Hutvagner, G. (2005). Small RNA asymmetry in RNAi: function in RISC assembly and gene regulation. *FEBS Lett*.
11. Collins, R.E., and Cheng, X. (2005). Structural Domains in RNAi. *FEBS Lett*.
12. Voinnet, O. (2005). Non-cell autonomous RNA silencing. *FEBS Lett*.
13. Ding SW, Li H, Lu R, Li F, Li WX. RNA silencing: a conserved antiviral immunity of plants and animals. *Virus Res* 2004;102:109–115. [PubMed: 15068886]
14. Voinnet, O. (2005). Induction and suppression of RNA silencing: insights from viral infections. *Nat Rev Genet*.
15. Qu, F., and Morris, TJ. (2005). Suppressors of RNA silencing encoded by plant viruses and their role in viral infections. *FEBS Lett*.
16. Fire A, Xu SQ, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 1998;391:806–811. [PubMed: 9486653]
17. Kennerdell JR, Carthew RW. Use of dsRNA-mediated genetic interference to demonstrate that *frizzled* and *frizzled 2* act in the wingless pathway. *Cell* 1998;95:1017–1026. [PubMed: 9875855]
18. Pane A, Salvemini M, Delli Bovi P, Polito C, Saccone G. The transformer gene in *Ceratitidis capitata* provides a genetic basis for selecting and remembering the sexual fate. *Development* 2002;129:3715–3725. [PubMed: 12117820]
19. Hughes CL, Kaufman TC. RNAi analysis of Deformed, proboscipedia and Sex combs reduced in the milkweed bug *Oncopeltus fasciatus*: novel roles for Hox genes in the hemipteran head. *Development* 2000;127:3683–3694. [PubMed: 10934013]
20. Levashina EA, Moita LF, Blandin S, Vriend G, Lagueux M, Kafatos FC. Conserved role of a complement-like protein in phagocytosis revealed by dsRNA knockout in cultured cells of the mosquito, *Anopheles gambiae*. *Cell* 2001;104:709–718. [PubMed: 11257225]
21. Adelman ZN, Sanchez-Vargas I, Travanty EA, Carlson JO, Beaty BJ, Blair CD, Olson KE. RNA silencing of dengue virus type 2 replication in transformed C6/36 mosquito cells transcribing an inverted-repeat RNA derived from the virus genome. *J Virol* 2002;76:12925–12933. [PubMed: 12438618]
22. Bettencourt R, Terenius O, Faye I. Hemolin gene silencing by ds-RNA injected into *Cecropia* pupae is lethal to next generation embryos. *Insect Mol Biol* 2002;11:267–271. [PubMed: 12000646]
23. Bucher G, Scholten J, Klingler M. Parental RNAi in *Tribolium* (Coleoptera). *Curr Biol* 2002;12:R85–R86. [PubMed: 11839285]

24. Beck M, Strand MR. RNA interference silences *Microplitis demolitor* bracovirus genes and implicates glcI. 8 in disruption of adhesion in infected host cells. *Virology* 2003;314:521–535. [PubMed: 14554081]
25. Aljamali MN, Sauer JR, Essenberg RC. RNA interference: applicability in tick research. *Exp Appl Acarol* 2002;28:89–96. [PubMed: 14570119]
26. Beye M, Hartel S, Hagen A, Hasselmann M, Omholt SW. Specific developmental gene silencing in the honey bee using a homeobox motif. *Insect Mol Biol* 2002;11:527–532. [PubMed: 12421410]
27. Stollewerk A, Schoppmeier M, Damen WG. Involvement of Notch and Delta genes in spider segmentation. *Nature* 2003;423:863–865. [PubMed: 12815430]
28. Li HW, Li WX, Ding SW. Induction and suppression of RNA silencing by an animal virus. *Science* 2002;296:1319–1321. [PubMed: 12016316]
29. Ball LA, Johnson KL. Reverse genetics of nodaviruses. *Adv Virus Res* 1999;53:229–244. [PubMed: 10582101]
30. Li WX, Li H, Lu R, Li F, Dus M, Atkinson P, Brydon EW, Johnson KL, Garcia-Sastre A, Ball LA, Palese P, Ding SW. Interferon antagonist proteins of influenza and vaccinia viruses are suppressors of RNA silencing. *Proc Natl Acad Sci U S A* 2004;101:1350–1355. [PubMed: 14745017]
31. Hoa NT, Keene KM, Olson KE, Zheng L. Characterization of RNA interference in an *Anopheles gambiae* cell line. *Insect Biochem Mol Biol* 2003;33:949–957. [PubMed: 12915186]
32. Zdobnov EM, von Mering C, Letunic I, Torrents D, Suyama M, Copley RR, Christophides GK, Thomasova D, Holt RA, Subramanian GM, Mueller HM, Dimopoulos G, Law JH, Wells MA, Birney E, Charlab R, Halpern AL, Kokoza E, Kraft CL, Lai Z, Lewis S, Louis C, Barillas-Mury C, Nusskern D, Rubin GM, Salzberg SL, Sutton GG, Topalis P, Wides R, Wincker P, Yandell M, Collins FH, Ribeiro J, Gelbart WM, Kafatos FC, Bork P. Comparative genome and proteome analysis of *Anopheles gambiae* and *Drosophila melanogaster*. *Science* 2002;298:149–159. [PubMed: 12364792]
33. Sarot E, Payen-Groschene G, Bucheton A, Pelisson A. Evidence for a piwi-dependent RNA silencing of the gypsy endogenous retrovirus by the *Drosophila melanogaster* flamenco gene. *Genetics* 2004;166:1313–1321. [PubMed: 15082550]
34. Johnson KN, Christian PD. A molecular taxonomy for cricket paralysis virus including two new isolates from Australian populations of *Drosophila* (Diptera: Drosophilidae). *Arch Virol* 1996;141:1509–1522. [PubMed: 8856030]
35. Higgs S. (2004) How do mosquito vectors live with their viruses. In *Microbe-vector interactions in vector-borne diseases*, S.H Gillespie, G.L Smith and A Osbourn, eds (Cambridge University Press), pp 103–137.
36. Sanchez-Vargas I, Travanty EA, Keene KM, Franz AW, Beaty BJ, Blair CD, Olson KE. RNA interference, arthropod-borne viruses, and mosquitoes. *Virus Res* 2004;102:65–74. [PubMed: 15068882]
37. Olson KE, Higgs S, Gaines PJ, Powers AM, Davis BS, Kamrud KI, Carlson JO, Blair CD, Beaty BJ. Genetically engineered resistance to dengue-2 virus transmission in mosquitoes. *Science* 1996;272:884–886. [PubMed: 8629025]
38. Kumagai MH, Donson J, della-Cioppa G, Harvey D, Hanley K, Grill LK. Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. *Proc Natl Acad Sci U S A* 1995;92:1679–1683. [PubMed: 7878039]
39. Lindbo JA, Silva-Rosales L, Proebsting WM, Dougherty WG. Induction of a Highly Specific Antiviral State in Transgenic Plants: Implications for Regulation of Gene Expression and Virus Resistance. *Plant Cell* 1993;5:1749–1759. [PubMed: 12271055]
40. Keene KM, Foy BD, Sanchez-Vargas I, Beaty BJ, Blair CD, Olson KE. RNA interference acts as a natural antiviral response to O'nyong-nyong virus (Alphavirus; Togaviridae) infection of *Anopheles gambiae*. *Proc Natl Acad Sci U S A* 2004;101:17240–17245. [PubMed: 15583140]
41. Uhlirova M, Foy BD, Beaty BJ, Olson KE, Riddiford LM, Jindra M. Use of Sindbis virus-mediated RNA interference to demonstrate a conserved role of Broad-Complex in insect metamorphosis. *Proc Natl Acad Sci U S A* 2003;100:15607–15612. [PubMed: 14668449]
42. Garcia S, Billecocq A, Crance JM, Munderloh U, Garin D, Bouloy M. Nairovirus RNA sequences expressed by a Semliki Forest virus replicon induce RNA interference in tick cells. *J Virol* 2005;79:8942–8947. [PubMed: 15994788]

43. Lu, R., Maduro, M., Li, F., Li, H., Broitman-Maduro, G., Li, W.X., and Ding, S.W. (2005). Animal virus replication and RNAi-mediated antiviral silencing in *Caenorhabditis elegans*. *Nature In press*.
44. Wilkins, C., Dishongh, R., Moore, S.C., Whitt, M.A., Chow, M., and Machaca, K. (2005). RNAi is an antiviral defence mechanism in *Caenorhabditis elegans*. *Nature In press*.
45. Tabara H, Sarkissian M, Kelly WG, Fleenor J, Grishok A, Timmons L, Fire A, Mello CC. The rde-1 gene, RNA interference, and transposon silencing in *C.-elegans*. *Cell* 1999;99:123–132. [PubMed: 10535731]
46. Vastenhouw NL, Plasterk RH. RNAi protects the *Caenorhabditis elegans* germline against transposition. *Trends Genet* 2004;20:314–319. [PubMed: 15219396]
47. Carmell MA, Xuan ZY, Zhang MQ, Hannon GJ. The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. *Genes & Development* 2002;16:2733–2742. [PubMed: 12414724]
48. MacFarlane, S.A., and Robinson, D.J. (2004). Transmission of plant viruses by nematodes. In *Microbe-vector interactions in vector-borne diseases*, Volume 63, S.H Gillespie, G.L Smith and A Osbourn, eds (Cambridge University Press), pp 263–285.
49. Pfeffer S, Zavolan M, Crasser FA, Chien M, Russo JJ, Ju J, John B, Enright AJ, Marks D, Sander C, Tuschl T. Identification of virus-encoded microRNAs. *Science* 2004;304:734–736. [PubMed: 15118162]
50. Cai X, Lu S, Zhang Z, Gonzalez CM, Damania B, Cullen BR. Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells. *Proc Natl Acad Sci U S A* 2005;102:5570–5575. [PubMed: 15800047]
51. Samols MA, Hu J, Skalsky RL, Renne R. Cloning and identification of a microRNA cluster within the latency-associated region of Kaposi's sarcoma-associated herpesvirus. *J Virol* 2005;79:9301–9305. [PubMed: 15994824]
52. Pfeffer S, Sewer A, Lagos-Quintana M, Sheridan R, Sander C, Grasser FA, van Dyk LF, Ho CK, Shuman S, Chien M, Russo JJ, Ju J, Randall G, Lindenbach BD, Rice CM, Simon V, Ho DD, Zavolan M, Tuschl T. Identification of microRNAs of the herpesvirus family. *Nat Methods* 2005;2:269–276. [PubMed: 15782219]
53. Sullivan CS, Grundhoff AT, Tevethia S, Pipas JM, Ganem D. SV40-encoded microRNAs regulate viral gene expression and reduce susceptibility to cytotoxic T cells. *Nature* 2005;435:682–686. [PubMed: 15931223]
54. Andersson MG, Haasnoot PC, Xu N, Berenjian S, Berkhout B, Akusjarvi G. Suppression of RNA interference by adenovirus virus-associated RNA. *J Virol* 2005;79:9556–9565. [PubMed: 16014917]
55. Llave C, Kasschau KD, Rector MA, Carrington JC. Endogenous and silencing-associated small RNAs in plants. *Plant Cell* 2002;14:1605–1619. [PubMed: 12119378]
56. Yekta S, Shih IH, Bartel DP. MicroRNA-directed cleavage of HOXB8 mRNA. *Science* 2004;304:594–596. [PubMed: 15105502]
57. Bao N, Lye KW, Barton MK. MicroRNA binding sites in *Arabidopsis* class III HD-ZIP mRNAs are required for methylation of the template chromosome. *Dev Cell* 2004;7:653–662. [PubMed: 15525527]
58. Herr, A. J. (2005). Pathways through the small RNA world of plants. *FEBS Lett. In press*.
59. Verdel, A., and Moazed, D. (2005). RNAi-directed assembly of heterochromatin in fission yeast. *FEBS Lett*.
60. Wassenegger M. The role of the RNAi machinery in heterochromatin formation. *Cell* 2005;122:13–16. [PubMed: 16009128]
61. Kapoor, A., Agius, F., and Zhu, J.K. (2005). Preventing Transcriptional Gene Silencing by Active DNA Demethylation. *FEBS Lett*.
62. Kalantari M, Calleja-Macias IE, Tewari D, Hagmar B, Lie K, Barrera-Saldana HA, Wiley DJ, Bernard HU. Conserved methylation patterns of human papillomavirus type 16 DNA in asymptomatic infection and cervical neoplasia. *J Virol* 2004;78:12762–12772. [PubMed: 15542628]
63. Lecellier CH, Dunoyer P, Arar K, Lehmann-Che J, Eyquem S, Himber C, Saib A, Voinnet O. A cellular microRNA mediates antiviral defense in human cells. *Science* 2005;308:557–560. [PubMed: 15845854]
64. Bentwich, I. (2005). Prediction and validation of microRNAs and their targets. *FEBS Lett*.

65. Bentwich I, Avniel A, Karov Y, Aharonov R, Gilad S, Barad O, Barzilai A, Einat P, Einav U, Meiri E, Sharon E, Spector Y, Bentwich Z. Identification of hundreds of conserved and nonconserved human microRNAs. *Nat Genet* 2005;37:766–770. [PubMed: 15965474]
66. Gitlin L, Stone JK, Andino R. Poliovirus escape from RNA interference: short interfering RNA-target recognition and implications for therapeutic approaches. *J Virol* 2005;79:1027–1035. [PubMed: 15613331]
67. Westerhout EM, Ooms M, Vink M, Das AT, Berkhout B. HIV-1 can escape from RNA interference by evolving an alternative structure in its RNA genome. *Nucleic Acids Res* 2005;33:796–804. [PubMed: 15687388]
68. Das AT, Brummelkamp TR, Westerhout EM, Vink M, Madiredjo M, Bernards R, Berkhout B. Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. *J Virol* 2004;78:2601–2605. [PubMed: 14963165]
69. Dunoyer P, Voinnet O. The complex interplay between plant viruses and host RNA-silencing pathways. *Curr Opin Plant Biol* 2005;8:415–423. [PubMed: 15939663]
70. Sullivan CS, Ganem D. A virus-encoded inhibitor that blocks RNA interference in mammalian cells. *J Virol* 2005;79:7371–7379. [PubMed: 15919892]
71. Lu S, Cullen BR. Adenovirus VA1 noncoding RNA can inhibit small interfering RNA and MicroRNA biogenesis. *J Virol* 2004;78:12868–12876. [PubMed: 15542639]
72. Soldan SS, Plassmeyer ML, Matukonis MK, Gonzalez-Scarano F. La Crosse virus nonstructural protein NSs counteracts the effects of short interfering RNA. *J Virol* 2005;79:234–244. [PubMed: 15596819]
73. Lichner Z, Silhavy D, Burgyan J. Double-stranded RNA-binding proteins could suppress RNA interference-mediated antiviral defences. *J Gen Virol* 2003;84:975–980. [PubMed: 12655099]
74. Bennasser Y, Le SY, Benkirane M, Jeang KT. Evidence that HIV-1 encodes an siRNA and a suppressor of RNA silencing. *Immunity* 2005;22:607–619. [PubMed: 15894278]
75. Uprichard, S.L. (2005). The therapeutic potential of RNA interference. *FEBS Lett*.
76. Roth BM, Pruss GJ, Vance VB. Plant viral suppressors of RNA silencing. *Virus Res* 2004;102:97–108. [PubMed: 15068885]
77. Mlotshwa S, Voinnet O, Mette MF, Matzke M, Vaucheret H, Ding SW, Pruss G, Vance VB. RNA silencing and the mobile silencing signal. *Plant Cell* 2002;14:S289–S301. [PubMed: 12045284]
78. Johnson KL, Price BD, Eckerle LD, Ball LA. Nodamura virus nonstructural protein B2 can enhance viral RNA accumulation in both mammalian and insect cells. *J Virol* 2004;78:6698–6704. [PubMed: 15163762]
79. Amarzguioui, M., Rossi, J.J., and Kim, D. (2005). Approaches for chemically synthesized siRNA and vector-mediated RNAi. *FEBS Lett*. *In press*.
80. Li X, Zhao X, Fang Y, Jiang X, Duong T, Fan C, Huang CC, Kain SR. Generation of destabilized green fluorescent protein as a transcription reporter. *J Biol Chem* 1998;273:34970–34975. [PubMed: 9857028]
81. Li WX, Ding SW. Viral suppressors of RNA silencing. *Current Opinion in Biotechnology* 2001;12:150–154. [PubMed: 11287229]
82. Delgadillo MO, Saenz P, Salvador B, Garcia JA, Simon-Mateo C. Human influenza virus NS1 protein enhances viral pathogenicity and acts as an RNA silencing suppressor in plants. *J Gen Virol* 2004;85:993–999. [PubMed: 15039541]
83. Bucher E, Hemmes H, de Haan P, Goldbach R, Prins M. The influenza A virus NS1 protein binds small interfering RNAs and suppresses RNA silencing in plants. *J Gen Virol* 2004;85:983–991. [PubMed: 15039540]
84. Chien CY, Tejero R, Huang Y, Zimmerman DE, Rios CB, Krug RM, Montelione GT. A novel RNA-binding motif in influenza A virus non-structural protein 1. *Nat Struct Biol* 1997;4:891–895. [PubMed: 9360601]
85. Liu J, Lynch PA, Chien CY, Montelione GT, Krug RM, Berman HM. Crystal structure of the unique RNA-binding domain of the influenza virus NS1 protein. *Nat Struct Biol* 1997;4:896–899. [PubMed: 9360602]
86. Vargason JM, Szitty G, Burgyan J, Tanaka Hall TM. Size selective recognition of siRNA by an RNA silencing suppressor. *Cell* 2003;115:799–811. [PubMed: 14697199]

87. Ye K, Malinina L, Patel DJ. Recognition of small interfering RNA by a viral suppressor of RNA silencing. *Nature* 2003;426:874–878. [PubMed: 14661029]
88. Partidos CD, Hoebeke J, Moreau E, Chaloin O, Tunis M, Belliard G, Briand JP, Desgranges C, Muller S. The binding affinity of double-stranded RNA motifs to HIV-1 Tat protein affects transactivation and the neutralizing capacity of anti-Tat antibodies elicited after intranasal immunization. *Eur J Immunol* 2005;35:1521–1529. [PubMed: 15789358]
89. Silhavy D, Burgyan J. Effects and side-effects of viral RNA silencing suppressors on short RNAs. *Trends Plant Sci* 2004;9:76–83. [PubMed: 15102373]
90. Garcia-Sastre A. Mechanisms of inhibition of the host interferon alpha/beta-mediated antiviral responses by viruses. *Microbes Infect* 2002;4:647–655. [PubMed: 12048034]
91. Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, Mills AA, Elledge SJ, Anderson KV, Hannon GJ. Dicer is essential for mouse development. *Nat Genet* 2003;35:215–217. [PubMed: 14528307]
92. Kasschau KD, Xie Z, Allen E, Llave C, Chapman EJ, Krizan KA, Carrington JC. PI/HC-Pro, a Viral Suppressor of RNA Silencing, Interferes with Arabidopsis Development and miRNA Function. *Dev Cell* 2003;4:205–217. [PubMed: 12586064]
93. Chen J, Li WX, Xie D, Peng JR, Ding SW. Viral Virulence Protein Suppresses RNA Silencing-Mediated Defense but Upregulates the Role of MicroRNA in Host Gene Expression. *Plant Cell* 2004;16:1302–1313. [PubMed: 15100397]
94. Maran A, Mathews MB. Characterization of the double-stranded RNA implicated in the inhibition of protein synthesis in cells infected with a mutant adenovirus defective for VA RNA. *Virology* 1988;164:106–113. [PubMed: 3363861]