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The long and short of antiviral defense: small RNA-based immunity in insects

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The host RNA interference (RNAi) pathway of insects senses virus infection and induces an antiviral response to restrict virus replication. Dicer-2 detects viral double-stranded RNA, produced by RNA and DNA viruses, and generates viral small interfering RNAs (vsiRNAs). Recent small RNA profiling studies provided new insights into the viral RNA substrates that trigger vsiRNA biogenesis. The importance of the antiviral RNAi pathway is underscored by the observation that viruses have evolved sophisticated mechanisms to counteract this small RNA-based immune response. More recently, it was proposed that another small RNA silencing mechanism, the piRNA pathway, also processes viral RNAs in *Drosophila* and mosquitoes. Here, we review recent insights into the mechanism of antiviral RNAi, viral small RNA profiles, and viral counter-defense mechanisms in insects.

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Introduction

All living organisms encounter a wide variety of microbial pathogens throughout their lifetime. A potent immune response is required to control or eliminate the pathogen and to ensure survival of the infected host. Mosquitoes and other blood-feeding insects transmit important human and animal viruses (arthropod-borne viruses, arboviruses), some of which are associated with debilitating disease and worldwide epidemics. With the increasing global threat of arboviruses, it is essential to understand the virus-vector interactions that determine virus transmission. Insights into the mechanisms of antiviral immunity will help us to understand arbovirus transmission cycles and to define novel strategies to restrict transmission and spread of pathogenic viruses.

RNA interference (RNAi) is a mechanism for small RNA-mediated gene silencing. Over the last years it has become apparent that RNAi is an important antiviral defense mechanism in insects, including the major vector mosquitoes and the model insect *Drosophila melanogaster* (fruit fly). Here, we review recent insights into this sophisticated and powerful antiviral defense system.

The RNAi pathway in insects

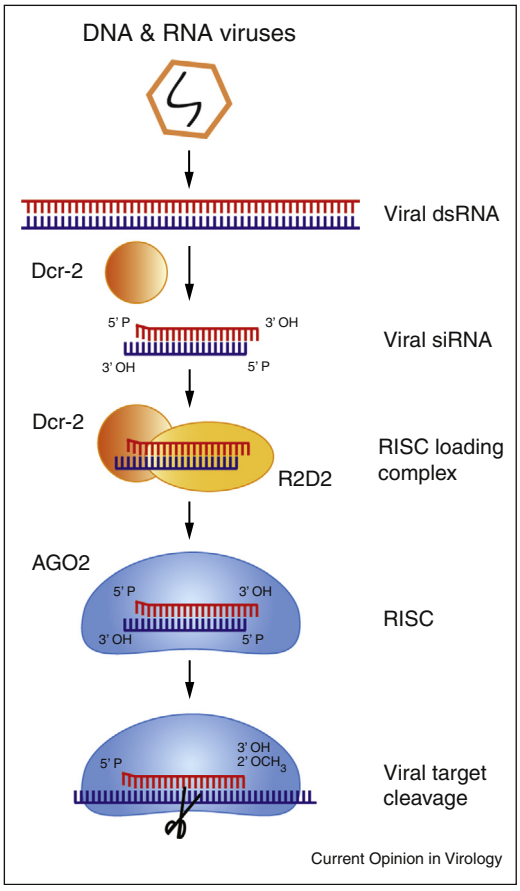
In RNAi, small interfering RNAs (siRNA) guide the Argonaute-2 (AGO2) protein onto target RNAs to induce their degradation. The central trigger of the RNAi pathway is double-stranded (ds) RNA. Endogenous dsRNA substrates, such as those derived from transposons, overlapping transcripts or long structured RNA loci, are processed by the RNase III enzyme Dicer-2 into 21-nt siRNA duplexes with 5′ monophosphates and 2-nt 3′ hydroxyl overhangs [1,2]. The dsRNA-binding proteins Loquacious PD isoform (Loqs-PD) and R2D2 are Dicer-2 co-factors that are required for efficient siRNA biogenesis and loading onto AGO2 within the RNA induced silencing complex (RISC) [3]. Arsenic resistance protein 2 (Ars2) is a Dicer-2 interacting protein that contributes to the efficiency of dsRNA processing [4]. Once loaded into RISC, one strand of the siRNA duplex (passenger strand) is eliminated from RISC [3]. This process requires the endonuclease activity of AGO2 to cleave the passenger strand as well as the endonuclease complex C3PO (Component 3 Promoter of RISC), a multimeric complex of Translin and Trax [5,6]. The guide strand is retained in RISC and is 2′-*O*-methylated at its 3′ terminal nucleotide by the RNA methyltransferase DmHen1, resulting in a mature, active RISC that is competent in inducing sequence-specific target cleavage by AGO2 (slicing) [7,8].

The antiviral RNAi pathway in insects

DsRNA is not detectable in uninfected, healthy cells, but accumulates upon virus infection [9]. Viral dsRNA may thus feed into the RNAi machinery to restrict virus replication (Figure 1). Genetic studies underscore the essential role of the RNAi response in controlling virus infection. *Drosophila* mutants for core components of the RNAi pathway (*Dicer-2*, *R2D2*, or *AGO2*) are highly sensitive to RNA virus infection, which correlates with an increase in viral RNA accumulation [10–13]. Similarly, in arbovirus-infected mosquitoes, higher viral loads are observed upon knockdown or inactivation of RNAi pathway components [14–18]. Recent studies demonstrated that DNA viruses are also targets of the antiviral RNAi response in insects [19•,20•,21•,22•,23].

Dicer-2 processes viral dsRNA into 21-nt viral small interfering RNAs (vsiRNAs). Interestingly however,

Figure 1



The antiviral RNAi pathway in insects. Dicer-2 (Dcr-2) detects viral double-stranded RNA, produced by RNA and DNA viruses, and generates viral small interfering RNAs (vsiRNAs). Dicer-2 and R2D2 are required for vsiRNA incorporation in Argonaute-2 (AGO2), within the RNA-induced silencing complex (RISC). The strand that remains incorporated in RISC guides the recognition and cleavage of target viral RNA to restrict virus replication.

Dicer-2 does not require its cofactors Loqs-PD and R2D2 for production of vsiRNAs during RNA virus infection [24•,25•], but R2D2 is required for their loading into RISC [10,11,13,24•,25•]. The Dicer-2 associated protein Ars2 seems to contribute to antiviral defense, but its direct role in vsiRNA biogenesis remains elusive [4].

Viral dsRNA is an essential intermediate in replication of single-stranded (ss) RNA viruses and it has been suggested that Dicer-2-mediated cleavage of such intermediates is sufficient to explain the antiviral activity of RNAi. Several lines of evidence suggest that viruses are also restricted by RISC-mediated cleavage of viral ssRNAs. *R2D2* and *AGO2* mutant flies are more sensitive to virus infection [10,11,13,24•,25•], which implies a role for vsiRNA-loaded RISC in antiviral defense. Indeed, viral siRNAs are resistant to beta elimination treatment (which shortens RNA containing non-modified 2′ and 3′ hydroxyl groups at the 3′ terminus), suggesting that at least some vsiRNAs are AGO2-associated and 2′-*O*-methylated at their 3′ end [26,27]. More recently, it was demonstrated that slicer-incompetent *AGO2* mutants are highly susceptible to infection with the negative strand (−) RNA virus Vesicular stomatitis virus [24•]. Another line of support comes from the observation that two unrelated insect RNA viruses (Cricket paralysis virus and Nora virus) have evolved antagonists that inhibit slicer activity of pre-assembled RISC [28••,29•]. Together these studies indicate that antiviral defense depends on dicing and slicing of viral RNAs.

Substrates for viral siRNA biogenesis

A hallmark of an antiviral RNAi response is the Dicer-2-dependent production of vsiRNAs. Putative Dicer-2 substrates include viral replication intermediates of ssRNA viruses, viral genomes of dsRNA viruses, structured RNA elements in viral ssRNA genomes or viral transcripts, and dsRNA that is formed by hybridization of overlapping transcripts of DNA viruses. Several recent studies used deep sequencing to analyze small RNAs in virus-infected samples. The detection of vsiRNAs provides direct support for Dicer-mediated processing of viral dsRNA. Moreover, vsiRNA sequences can be mapped to the viral genome and this information can be used to deduce the viral substrates that are processed by Dicer-2.

Small RNA profiles from (+) RNA viruses

During replication of positive-strand (+) RNA viruses, the viral (+) RNA strand serves as a template for negative strand (−) RNA synthesis. The (−) RNA strand in turn serves as a template for the production of (+) viral RNA progeny [30]. Viral RNA replication is in general asymmetric: the viral (+) RNA strand greatly outnumbers the viral (−) RNA strand. Nevertheless, upon infection of insect hosts with different (+) RNA viruses, vsiRNAs of positive and negative polarity were recovered in roughly equal numbers. This observation, together with the finding that vsiRNAs map along the entire length of the viral genome (Table 1), indicates that viral dsRNA replication intermediates are a major substrate for Dicer-2 during (+) RNA virus infection.

Nevertheless, in some (+) RNA virus infections, Dicer-2 may process additional substrates. In Flock House virus (FHV) infection of *Drosophila*, for example, vsiRNAs mainly derive from the (+) RNA strand across the two segments of the viral RNA genome [25•]. This suggests that intramolecular base pairing within viral ssRNA genomes form dsRNA substrates for Dicer. FHV encodes the RNAi antagonist B2 that binds dsRNA (Table 2, discussed below) and markedly affects vsiRNA profiles [31–33]. Infection with a B2 deficient mutant FHV (FHV ΔB2) resulted in the production of vsiRNAs that mapped in similar numbers to the (+) and (−) strands at the 5′

Table 1

Small RNA profiles from naturally and experimentally infected insects

Virus	Virus family	Genome segments (number)	Virus (abbr.)	Infected host	Experimental system	Proposed Dicer substrates	Reference
(+) RNA virus	*Togaviridae*	1	Sindbis virus	*D. melanogaster*; *Ae. aegypti*; *Ae. albopictus*	Cell; *in vivo*	dsRNA replication intermediates; structured ssRNA	[17,24•, 38,51••]
			O'nyong-nyong virus	*An. gambiae*	*In vivo*	dsRNA replication intermediates; structured ssRNA	[68]
			Semliki Forest virus	*Ae. albopictus*; *Ae. aegypti*	Cell	dsRNA replication intermediates	[52,69]
			Chikungunya virus	*Ae. aegypti*; *Ae. albopictus*	Cell; *in vivo*	dsRNA replication intermediates	[50••]
	Dicistroviridae	1	Drosophila C virus (DCV)	*D. melanogaster*	Cell	dsRNA replication intermediates; structured ssRNA	[22•,34, 44•]
	Flaviviridae	1	Dengue virus	*Ae. aegypti*; *Ae. albopictus*	Cell; *in vivo*	dsRNA replication intermediates	[48,49]
			West Nile virus	*D. melanogaster*; *Ae. albopictus*; *C. quinquefasciatus*	Cell; *in vivo*	dsRNA replication intermediates; structured ssRNA	[38,70]
			Cell fusing agent virus	*Ae. aegypti*; *Ae. albopictus*	Cell	dsRNA replication intermediates	[48]
	Nodaviridae	2	Flock House virus (FHV)	*D. melanogaster*	Cell; *in vivo*	dsRNA replication intermediates; defective RNAs; structured ssRNA	[2,25•,27, 34,44•,71]
			American Nodavirus	*D. melanogaster*	Cell	dsRNA replication intermediates; defective RNAs	[34,44•]
	Tetraviridae	1	Drosophila A virus[a]	*D. melanogaster*	Cell	dsRNA replication intermediates	[44•]
	Unassigned	1	Nora virus	*D. melanogaster*	Cell; *in vivo*	dsRNA replication intermediates	[29•,44•]
(-) RNA	*Bunyaviridae*	3	Rift Valley fever virus	*D. melanogaster*; *Ae. aegypti*; *Ae. albopictus*	Cell	dsRNA replication intermediates; intergenic RNA hairpin	[22•,39]
			La Crosse virus	*D. melanogaster*; *Ae. albopictus*	Cell	dsRNA replication intermediates; structured ssRNA	[38]
			Schmallenberg virus	*Ae. aegypti*; *Culicoides sonorensis*	Cell	dsRNA replication intermediates	[40]
	Rhabdoviridae	1	Vesicular stomatitis virus	*D. melanogaster*	Cell; *in vivo*	dsRNA replication intermediates; viral genome-transcript hybrids	[10,22•, 24•]
dsRNA	*Reoviridae*	10	Bluetongue virus	*Ae. aegypti*; *Culicoides sonorensis*	Cell	genomic dsRNA	[40]
	Birnaviridae	2	Culex Y Virus[b]	*C. tarsalis*; *An. sinensis*	Cell; *in vivo*	genomic dsRNA	[72] Unp. obs.
		2	Drosophila X virus	*D. melanogaster*	Cell	genomic dsRNA	[34,44•]
		2	Drosophila birnavirus	*D. melanogaster*	Cell	genomic dsRNA	[44•]
	Totiviridae	1	Drosophila totivirus	*D. melanogaster*	Cell	genomic dsRNA	[44•]
ssDNA	*Parvoviridae*	1	Culex tritaeniorhynchus virus	*C. pipiens molestus*	*In vivo*	structured ssRNA; inverted repeats at non-coding genomic termini	[73]
dsDNA	*Iridoviridae*	1	Invertebrate Iridescent virus type 6	*D. melanogaster*	Cell; *in vivo*	convergent overlapping transcripts	[19•,20•, 23]
	Poxviridae	1	Vaccinia virus	*D. melanogaster*	Cell	convergent overlapping transcripts; tandem repeats at genomic termini; structured ssRNA	[22•]

Unp. obs., our unpublished observations.

[a] Referred to as Drosophila tetravirus in this study, but high sequence identity to Drosophila A virus (DAV) suggest that it is a strain of DAV [74].

[b] Referred to as Mosquito X virus by Huang *et al*. [72]. High sequence identity between Culex Y virus [75] and Mosquito X virus suggests that they are variants of a single virus species within the Entomobirnavirus genus.

terminal region of RNA segment 1 [25•,27]. Similar to wildtype FHV infection, vsiRNAs also mainly derive from the viral (+) RNA strand in infections with the (+) RNA virus Drosophila C virus (DCV) [22•,34]. Like FHV, DCV encodes a dsRNA binding RNAi suppressor protein (1A) that is thought to shield the replication intermediates from Dicer [11]. These results might suggest that the 1A and B2 proteins shield replication

Table 2

Viral RNAi suppressors in insect viruses and arboviruses

Family	Virus (abbr.)	Host/Vector[a,b]	Experimental insect host[c]	Suppressor	Mechanism	Replication defect of VSR mutant virus[d]	Rescue of FHV ΔB2 replicon[e]	Increased replication of recombinant Alphavirus[f]	References
Ascoviridae	Heliothis virescens ascovirus-3e	*Heliothis virescens*	*Spodoptera frugiperda*; *Heliothis virescens*	Orf 27 (RNase III)	Degradation of dsRNA	Yes[g]	n.t.	n.t.	[60]
Birnaviridae	Drosophila X virus	*D. melanogaster*	*D. melanogaster*	VP3	Long dsRNA binding prevents Dicer-2 cleavage; siRNA binding	n.a.	Yes	n.t.	[76] Unp. obs.
	Culex Y virus	*Culex pipiens*	*Culex tarsalis*	VP3	Long dsRNA binding prevents Dicer-2 cleavage; siRNA binding	n.a.	Yes	n.t.	Unp. obs.
Dicistroviridae	Drosophila C virus (DCV)	*D. melanogaster*	*D. melanogaster*	1A	Long dsRNA binding prevents Dicer-2 cleavage; interferes with RISC assembly	n.a.	Yes	Yes	[11,28••,77]
	Cricket paralysis virus	*Teleogryllus* sp.	*D. melanogaster*	1A	Inhibition of AGO2 endonuclease activity	n.a.	Yes	Yes	[13,28••,29•]
Flaviviridae	Dengue virus	*Ae. aegypti*; *Ae. albopictus*	*Spodoptera frugiperda*	NS4B	Inhibition of (human) Dicer activity	n.t.	n.t.	n.t.	[62]
	West Nile virus	*Culex* spp.	*Ae. Albopictus*; *D. melanogaster*	sfRNA	Inhibition of (human) Dicer activity	n.t.	n.t.	Yes	[63]
	Dengue virus	*Ae. aegypti*; *Ae. albopictus*	*Ae. albopictus*	sfRNA	–	n.t.	n.t.	n.t.	[63]
Nodaviridae	Flock House virus (FHV)	*Costelytra zealandica*	*D. melanogaster*; *Spodoptera frugiperda*; *Ae. aegypti*; *Ae. albopictus*	B2	Long dsRNA binding prevents Dicer-2 cleavage; siRNA binding prevents RISC incorporation; Dicer-2 binding	Yes	Yes	Yes	[13,17,18,27, 31–33,50••, 54,57,64,77]
	Nodamuravirus (NoV)	*Culex tritaeniorhynchus*	*D. melanogaster*; *An. gambiae*; *Ae. albopictus*	B2	Binding of long dsRNA and siRNA; inhibition of (human) Dicer activity	Yes	Yes	Yes	[17,25•,27, 50••,54,56]
	Wuhan Nodavirus (WhNV)	*Pieris rapae*	*Pieris rapae*; *D. melanogaster*	B2	Long dsRNA binding prevents Dicer-2 cleavage; siRNA binding prevents RISC incorporation; Dicer-2 binding	n.t.	n.t.	n.t.	[55,58]

Unassigned	Nora virus	*D. melanogaster*	*D. melanogaster*	VP1	Inhibition of AGO2 endonuclease activity	n.t.	Yes	Yes	[29•] Unp. obs.
	Dimm Nora-like virus	*D. immigrans*	*D. immigrans*	VP1	Inhibition of AGO2 endonuclease activity	n.a.	n.t.	Yes	Unp. obs.

Unp. obs, unpublished observations

[a] The natural host range of most insect viruses is poorly defined. We report the species from which the virus was first isolated. For the arthropod-borne viruses from the *Flaviviridae*, we report the mosquito vector.

[b] Common names: *Aedes aegypti*, yellow fever mosquito; *Aedes albopictus*, tiger mosquito; *Anopheles gambiae*, African malaria mosquito; *Costelytra zealandica*, grass grub; *Culex pipiens*, common house mosquito; *Culex tarsalis*, – (no common name); *Culex tritaeniorhynchus*, –; *Drosophila melanogaster*, common fruit fly; *Drosophila immigrans*, –; *Heliothis virescens*, tobacco budworm; *Pieris rapae*, the small white (butterfly); *Spodoptera frugiperda*, Fall Army worm; *Teleogryllus oceanicus* and *Teleogryllus commodus*, Australian field crickets.

[c] The experimental insect host that was used to show RNAi suppressive activity and/or study the mechanism of action.

[d] Replication defect in VSR mutant virus/replicon and rescue of the defect by genetic inactivation of RNAi pathway components. n.t., not tested; n.a., infectious cDNA not available.

[e] VSR-mediated rescue of replication defect of RNAi suppressor-defective FHV replicon (FHV ΔB2). n.t., not tested.

[f] Increased replication and/or pathogenicity of VSR-expressing recombinant Alphavirus.

[g] Less viral DNA upon vRNase III knockdown in HvAV-3e infection.

intermediates for Dicer-mediated cleavage and that under these conditions other viral RNA substrates are processed into vsiRNAs. Remarkably however, infection of *Aedes aegypti* mosquitoes with recombinant Sindbis virus (which is thought not to encode an RNAi suppressor) engineered to encode the FHV B2 protein resulted in strongly reduced vsiRNA levels, without a concomitant shift towards (+) viral small RNAs [17]. These data suggest that viral RNAi suppressor proteins might affect vsiRNA profiles, but that this might be virus and host species dependent.

Small RNA profiles from (−) RNA viruses

Analogous to (+) RNA viruses, the viral genomic RNA strand is template for the production of complementary RNA strands during (−) RNA virus replication. Because of asymmetric replication, the genomic strand of (−) RNA viruses is more abundant than the antigenome (+) RNA during infections [35,36]. Genome replication of (−) RNA viruses occurs in ribonucleoprotein complexes, which requires the initial assembly of the viral RNA with the viral nucleocapsid protein (N) [35,37]. The formation of the RNA-N protein complex might explain why dsRNA is not readily detectable in (−) RNA virus infection [9]. An additional RNA species in (−) RNA virus infections is the viral messenger RNA, which may be expressed at different relative levels during infection. For example, a gradient of mRNA transcripts (N > P > M > G > L) is produced by the viral RNA polymerase of the non-segmented Vesicular stomatitis virus, which is the net result from the sequential mode of transcription based on gene order [35].

Small RNA profiling in (−) RNA virus infections, demonstrated that vsiRNAs are generally equally distributed over genomic and antigenomic RNA strands and that they are produced from the full-length genome [10,22•,24•,38–40]. These observations suggest that replication intermediates are the predominant Dicer-2 substrate in (−) RNA virus infection of adult flies and of *Drosophila* or mosquito cells (Table 1). For Vesicular stomatitis virus, it was suggested that viral genome-transcript hybrids provide a putative dsRNA template that can be recognized by Dicer-2 [24•]. An additional Dicer-2 substrate was suggested for the tri-segmented Rift Valley fever virus. Here, Dicer-2-dependent vsiRNAs were unevenly distributed across the S segment and were most likely derived from an intergenic hairpin [22•].

Small RNA profiles from dsRNA viruses

Genome replication of most dsRNA viruses occurs within the viral capsid that encapsulates the viral dsRNA [41]. Some dsRNA viruses, such as members of the *Birnaviridae* family, protect their genome by forming ribonucleoprotein complexes [42,43]. Nevertheless, several studies indicate that viral dsRNA genomes are accessible for Dicer-2. For example, small RNA deep-sequencing of

persistently infected *Drosophila* cell lines revealed roughly equal numbers of (+) and (−) vsiRNAs for three different dsRNA viruses (Drosophila totivirus, Drosophila X virus, Drosophila birnavirus), indicating that the viral dsRNA genome is cleaved by Dicer-2 [34,44•]. Similarly, vsiRNAs of both polarities were recovered in equal proportions from *Aedes* mosquito-derived cell lines infected with Bluetongue virus, with vsiRNAs evenly distributed across the viral genome [40].

Small RNA profiles from DNA virus infections

In contrast to RNA viruses, DNA viruses do not replicate via a dsRNA replication intermediate. Nevertheless, we and others showed that the RNAi machinery contributes to control of DNA virus infection in insects [19•,20•,21•] and therefore other dsRNA sources must be processed by Dicer-2 (Table 1). Indeed, overlapping bidirectional transcripts base pair to form dsRNA substrates for Dicer-2 dependent vsiRNA biogenesis in *Drosophila* [19•,20•,23]. A number of recent publications have now analyzed vsiRNA profiles in DNA virus infections in different invertebrate model systems and these studies further underscore that convergent transcripts are a major source of vsiRNA production, but that other substrates, such as structured RNA elements [22•], can also contribute to the vsiRNA profile [23].

Virus-derived piRNAs

Recent small RNA profiling studies have implicated the piRNA pathway in antiviral defense in mosquitoes. Piwi-associated RNAs (piRNAs) are 25–30 nt small RNAs that associate with the PIWI class of Argonaute proteins [45,46]. Piwi proteins and their associated piRNAs are highly enriched in germline tissues of model organisms, such as *Drosophila*, zebrafish and mice, in which they suppress the mobilization of transposable elements [45,46]. Two mechanisms for piRNA biogenesis have been characterized: primary piRNA biogenesis and a complex feed-forward amplification loop, termed the ping-pong amplification cycle [46]. A defining feature of ping-pong amplified piRNAs, which stems from their unique biogenesis, is an enrichment for Uridine at position 1 (U1 bias) of antisense piRNAs and an enrichment for Adenine at position 10 (A10 bias) for sense piRNAs [46]. The potential of the piRNA pathway to process viral RNAs was first suggested by the detection of piRNA-sized viral RNAs in persistently infected *Drosophila* ovarian sheet cells [44•,47]. Several studies that detected arboviral small RNAs of positive polarity within the piRNA size-range suggested that the piRNA pathway also processes viral RNAs in mosquitoes [38,48,49].

More recently, small 25–30 nt RNAs with clear features of ping-pong dependent piRNAs were recovered from *Aedes* mosquitoes or mosquito-derived cells infected with (+) RNA arboviruses from the *Togaviridae* family (Sindbis virus, Semliki Forest virus and Chikungunya virus) and (−) RNA arboviruses from the *Bunyaviridae* family (La Crosse virus, Schmallenberg virus and Rift Valley fever virus). Virus-derived piRNAs (vpiRNAs) of positive polarity showed a non-uniform distribution across the viral genome and presented an A10 bias, whereas vpiRNAs of negative polarity were enriched for U1 [39,40,50••,51••,52]. Together, these results indicate that *de novo* vpiRNAs are produced in a ping-pong dependent manner upon RNA virus infection of major vector mosquitoes (Table 1). In *Aedes* mosquitoes, the PIWI gene family greatly expanded and expression of at least some of the eight PIWI family members does not seem to be restricted to germ line tissues [50••,51••,53], raising the possibility that the piRNA pathway has functionally diversified in mosquitoes.

The intriguing identification of viral piRNAs in mosquitoes raises important questions about their biogenesis and functions. Interestingly, knockdown of Piwi4 in *A. aegypti* cells resulted in increased Semliki Forest virus viral loads, suggesting a role of the piRNA pathway in restricting virus replication [52]. Similarly, knockdown of AGO3 in *Anopheles* mosquitoes leads to increased O'nyong-nyong virus titres [14], although production of vpiRNAs has yet to be demonstrated in this mosquito species.

Viral suppression of RNAi

Viruses have evolved suppressors of RNAi (VSRs) to antagonize the RNAi-based antiviral immune response. These VSRs may target different critical steps of the antiviral RNAi pathway (Table 2). It is not surprising that many VSRs sequester dsRNA or siRNAs, as these molecules are the initiators and sequence specificity determinants of RNAi. For example, the natural fly pathogen DCV encodes the 1A protein that binds long dsRNA and protects it from Dicer-2 processing. It was thus proposed that 1A protects replication intermediates from Dicer-2 mediated processing [11], although more recently it was reported that DCV 1A may also interfere with RISC loading [28••]. Members of the *Alphanodavirus* genus (*Nodaviridae family*), including FHV, Nodamura virus (NoV) and Wuhan nodavirus (WhNV), encode the B2 protein that suppresses RNAi via a multimode mechanism. B2 binds long dsRNA and siRNAs, thereby inhibiting Dicer-2 processing and RISC loading, respectively [33,54–56]. Additionally, the FHV and WhNV B2 proteins directly associate with Dicer-2, which likely contributes to their RNAi suppressive activity [57,58]. Despite the similar mechanism of action, only limited amino acid identity exists between the B2 proteins of these viruses (23.5% identity between WhNV and FHV; 26.4% identity between NoV and FHV). Nevertheless, the structures of NoV and FHV are strikingly similar [59], suggesting that they derive from a common ancestral sequence.

An alternative mechanism to interfere with the initiation of the antiviral RNAi response is used by Heliothis

virescens ascovirus-3e. This virus encodes an RNase III enzyme (orf 27) that degrades dsRNA [60]. Thus, the orf27 protein may compete with Dicer-2 for dsRNA substrates or degrade siRNA duplexes to prevent their incorporation into AGO2 in a manner analogous to the RNase III protein of the plant virus sweet potato chlorotic stunt virus [61]. Viruses may also target the effector phase of the antiviral RNAi response. For example, Nora virus VP1 and Cricket paralysis virus 1A interact with AGO2 and thereby inhibit RISC-mediated slicing of target RNAs [28••,29•].

The transmission of arboviruses relies on mosquito vectors that support a productive virus infection. Despite a functional RNAi-response, arboviruses establish a persistent, non-pathogenic infection in mosquitoes. Although Nodamura virus, a putative arbovirus, was known to encode an RNAi suppressor (Table 2), it was for a long time believed that most arboviruses do not encode a VSR, as this would lead to pathology and killing of the vector thus preventing efficient virus transmission. Indeed, the introduction of a VSR (FHV B2) in recombinant Sindbis virus induced pathogenicity and led to mortality of infected mosquitoes [17,18].

Recently, it was suggested that the Dengue virus NS4B protein suppresses RNAi by inhibiting Dicer processing independent of dsRNA-binding [62]. Alternatively, flaviviruses may use non-coding ssRNA molecules to counteract RNAi in mammals and insects. West Nile virus and Dengue virus, for example, produce large amounts of subgenomic flavivirus (sf) RNA that contains extensive secondary structures. It was proposed that sfRNAs compete with other dsRNA substrates, such as essential viral replication intermediates, for Dicer-mediated cleavage to inhibit an antiviral RNAi response [63].

Strong genetic evidence supports the importance of B2 protein in FHV infection. Indeed, in the absence of a suppressor protein (ΔB2), abundant vsiRNAs are produced and, as a consequence, no potent FHV infection was established in wildtype embryos and adult flies. Importantly, the replication defect of FHV ΔB2 was rescued by genetic inactivation of RNAi genes [13,25•,27,64]. However, such genetic evidence is lacking for VSRs of most other viruses, in some cases due to the lack of a reverse genetics system to engineer these viruses. For some VSRs, proof-of-concept for a role in suppressing RNAi was provided by rescuing replication of the FHV ΔB2 replicon or by analyzing virus replication of a recombinant Alphavirus engineered to express the VSR of interest (Table 2). Nevertheless, future studies are needed to provide insights into the role of the identified VSRs in viral pathogenesis and transmission.

Many insect viruses encode suppressors of RNAi; yet in other viruses, including some well-characterized arboviruses, attempts to identify RNAi suppressive activities have not been successful (unpublished observations). Although this might mean that those viruses do not encode RNAi suppressors, an alternative explanation might be that RNAi reporter assays that are frequently used to detect RNAi suppressive activity fail to identify *bona fide* VSRs. This could be due to technical limitations of the assay, the use of non-host systems, or the inability of reporter assays to identify suppressors that work *in cis* [65••]. Also, RNAi reporter assays may not address all aspects of the antiviral RNAi response that are putative targets for viral interference. For example, systemic spread was proposed to be essential for efficient antiviral RNAi [66], but no reporter assays have been established that would identify suppressors of systemic antiviral RNAi responses.

Concluding remarks

It is evident that RNAi is an important antiviral defense system against parasitic nucleic acids, including transposable elements and the major classes of viruses. Yet, important questions regarding the biogenesis and function of vsiRNAs and vpiRNAs remain. For example, an open question pertains to the recognition of viral dsRNA: when and where is viral dsRNA accessible to Dicer-mediated processing. An exciting new twist to the field of antiviral RNAi is the recent observation that viral persistence is mediated by the combined activities of a cellular reverse transcriptase and the RNAi pathway [67••]. Specific viral fragments are reverse transcribed and integrate into the host genome to generate transcripts that are processed into vsiRNAs. How these observations should be incorporated in the conceptual framework of antiviral RNAi remains a challenge for future investigation. Clearly, we are only beginning to understand the complexity of this sophisticated antiviral machine.

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- • of special interest
- •• of outstanding interest

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