Viral Suppression of Antiviral RNAi in Insects

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Viral Suppression of Antiviral RNAi in Insects

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Chapter 1

General introduction and outline of the thesis

Adapted from:

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Defense and counterdefense in the RNAi-based antiviral immune system in insects

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In our daily lives we are exposed to numerous micro-organisms, ranging from benign commensal species to pathogenic microbes. Crucial for the fitness of a host organism is an immune system that can discriminate self from non-self, and distinguishes harmful pathogens from harmless microbes. In vertebrates, the immune response is divided into an innate and an adaptive immune response. The innate immune response forms the first line of defense that recognizes conserved pathogen associated molecular patterns (PAMPs). The innate immune response can in turn activate a highly specific adaptive immune response that generates immunological memory. Invertebrates, however, lack the adaptive immune responses that mediate immune defense in vertebrate animals, and they are thought to rely solely on their innate immune response to fight pathogens.

Viruses form a major challenge to the host immune system, as they are one of the most abundant biological entities on the planet and are believed to infect all living organisms [1]. Arthropods are no exception, and some viruses are of specific human interest as they can be pathogenic to arthropods of economical value, like honeybees and shrimp [2]. In addition, mosquitoes and other blood-feeding arthropods can transmit important viruses to humans and other animals (arthropod-borne (arbo) viruses) [3]. To limit the impact of these infections on public health and health care, it is important to understand the interplay between insect immune defense and viral counter-defense.

Drosophila as a model to study innate immunity

Drosophila melanogaster, generally referred to as fruit flies, are used for more than one hundred years as a model organism by experimental biologists [4]. Fruit flies have a short generation time along with a high production of progeny and can be easily and inexpensively maintained in the laboratory [5]. Together with the completion of the *Drosophila* genome sequence and an unprecedented variety of genetic methods and tools, these characteristics makes *Drosophila* a powerful model organism to analyze gene function and regulation *in vivo* [6,7].

Since *Drosophila* shares conserved developmental and cellular processes with vertebrates, studies in flies made seminal contributions to our understanding of fundamental biological processes in invertebrates as well as vertebrates [8-10]. Studies in *Drosophila* also provided important insights into the innate immune response against bacteria and fungi. One significant contribution was the discovery of Toll as an immune receptor, which also proved to play key roles in the immune system of mammals [11-14]. Although initial studies focused on the immune response against bacteria and fungi, increasing efforts are made to decipher the antiviral immune response in *Drosophila* using a selection of insect viruses.

Model viruses in *Drosophila*

Only a limited set of viruses have been described as natural pathogens of *Drosophila*. Nevertheless, this model organism is permissive to experimental infection with various insect viruses, including arboviruses [15-17]. In combination with the wide array of *Drosophila* mutants available, this creates a powerful system to identify host factors required for viral infection as well as factors that are important for antiviral defense.

A well-characterized natural pathogen of fruit flies is Drosophila C virus (DCV), a member of the *Dicistroviridae* family (order Picornavirales) [18-20]. In nature, DCV causes a non-pathogenic persistent infection with no obvious symptoms, and it is transmitted horizontally through feeding or close contact [20,21]. In contrast, experimental infection by injection of DCV in the body cavity of *Drosophila* leads to high mortality [22-25]. After injection, DCV rapidly disseminates to several organs, including the fat body, cells of the digestive tract, thoracic muscle fibers, and the epithelial sheath surrounding the egg chamber [22,24,26]. Cricket paralysis virus (CrPV) is a *Dicistroviridae* family member that is closely related to DCV. CrPV was originally isolated from field crickets, in which it causes a fatal paralysis [27]. Although obtained from a different host, CrPV replicates efficiently in *Drosophila* and causes high mortality after experimental infection [28-30]. Therefore, both DCV and CrPV can be used to model acute viral infections.

Flock House virus (FHV) (*Nodaviridae*), originally isolated from New Zealand grass grubs, is regularly used to study virus-host interactions in *Drosophila* [30-34]. Similar to DCV and CrPV, intrathoracic injection of FHV in adult flies causes high lethality [23,30,32,35]. The fat body, muscles, and trachea are the main target tissues during FHV infection [32]. Interestingly, FHV also efficiently infects cardiomyocytes, the heart muscle cells, of *Drosophila* [23]. Since viral infections are a major cause of acute myocarditis (inflammation of the heart muscle) in humans, FHV may become an important model to study cardiotropic virus infections [36].

Nora virus is another natural pathogen of *Drosophila*. It was recently identified as a non-pathogenic, persistent infection in *Drosophila* laboratory stocks and wild-caught flies [37]. Despite its recent discovery, Nora virus was found to be associated with Drosophila stocks for at least twenty years [37]. Nora virus is excreted in the feces of infected flies, and horizontally transmitted through the fecal-oral route [38]. Although Nora virus particles are mainly found in the intestine of infected flies, the exact target cells remain elusive. In contrast to DCV, injection of Nora virus into *Drosophila* mirrors the natural infection in its persistent, non-pathogenic character [38]. This makes Nora virus a suitable model to study the interplay between the innate immune system and persistent infections.

Insect innate immunity

The innate immune response relies on germline-encoded pathogen recognition receptors that recognize conserved molecular patterns of microbial pathogens. This response is composed of a cellular and humoral component. Three types of circulating blood cells, called plasmatocytes, lamellocytes and crystal cells, mediate the cellular response in *Drosophila* [39]. The plasmatocytes phagocytose invading pathogens, while lamellocytes are responsible for the encapsulation of objects too large to be phagocytosed, such as parasitic wasp eggs [40]. Both phagocytosis and encapsulation lead to the destruction of the invading pathogen, although the mechanisms are poorly understood [39]. Lamellocytes are not found in the embryo or adult and are restricted to the larval stage [40]. Crystal cells are non-phagocytic cells that mediate melanization needed for wound healing and encapsulation [39,41,42].

The humoral response against bacteria and fungi is characterized by the synthesis and secretion of antimicrobial peptides (AMPs) by the fat body. AMPs can insert into the membrane of pathogens to form transmembrane pores that induce lysis of microbial cells. Alternatively, some AMPs can translocate to the pathogen's cytoplasm to inhibit cell-wall synthesis, nucleic-acid synthesis, protein synthesis, or enzymatic activity [43]. The strong induction of AMPs upon infection with bacteria or fungi is regularly used as a marker of an activated immune system, and mainly relies on the Toll and immune deficiency (IMD) pathway [11,44-46].

The Toll receptor, initially identified for its role in embryonic development, is important for the innate immune response against Gram-positive bacteria and fungi [11,46]. Unlike the vertebrate Toll-like receptors, the *Drosophila* Toll receptor is not directly activated by microbial motifs [47,48]. Instead, soluble receptors like Gram-negative binding protein (GNBP)-1 and -3, and peptidoglycan recognition protein (PGRP)-SA and -SD recognize molecular motifs of fungi and Gram-positive bacteria, respectively [49-51]. Activation of the GNBP and PGRP receptors initiates a proteolytic signaling cascade that results in the maturation of pro-Spätzle into the active cytokine Spätzle [47,48]. Binding of Spätzle to the Toll recepotor activates an intracellular signaling pathway eventually leading to the translocation of the NF-κB like transcription factors Dorsal and Dorsal-related immunity factor (Dif) to the nucleus. Translocated Dorsal and Dif then induce the expression of AMPs, such as Drosomycin and Defensin [52-54].

The IMD pathway is activated by Diaminopimelic acid (DAP)-type peptidoglycans (PGN) of Gram-negative bacteria. Recognition of the DAP-type PGN by the peptidoglycan-recognition protein-LC (PGRP-LC) or PGRP-LE leads to intracellular recruitment of IMD [55-58]. Subsequently, IMD initiates two distinct signaling cascades that both lead to the activation of the NF-κB transcription factor Relish. Translocation of Relish

to the nucleus induces the expression and secretion of another set of AMPs, which includes Diptericin and Cecropin [59,60].
The Janus kinase/signal transducers and activators of transcription (Jak/Stat) pathway also plays a role in innate immune defense [39,61]. Signaling of this pathway is triggered by damage and stress signals, and is initiated by the binding of a cytokine from the Unpaired (Upd) family to the domeless receptor (Dome) [62,63]. Activation of the Dome receptor induces phosphorylation of Stat92E via the JAK kinase hopscotch, leading to Stat92E dimerization and translocation to the nucleus [62]. Activated Stat92E subsequently induces expression of genes with Stat binding sites in their promoter [62,63].
Although the Toll, IMD, and Jak-Stat pathways are mainly implicated in the defense against bacteria and fungi, they were also reported to play a role during infection with some viruses [64-67]. In contrast to bacterial and fungal infection, however, virus infections do not consistently induce a robust AMP response [66]. In addition, transcriptional induction of pathway components differed between studies and seemed to be specific for a virus or virus family [66]. Due to this lack of consistency, the roles of the Toll, IMD, and Jak-Stat pathway in antiviral defense remain poorly understood.
Over the last years, it has become clear that RNA interference (RNAi) mediates a robust antiviral response in insects, nematodes, fungi and plants [68]. RNA interference is a small RNA-based mechanism for gene silencing that is triggered by the presence of double-stranded RNA (dsRNA). Double stranded RNA, which cannot be detected in uninfected cells [69], is produced during virus infections and sensed as a danger signal that triggers the antiviral RNAi response. Viral dsRNA is processed by an RNase of the Dicer family into 21-nt small interfering RNAs (siRNAs). These viral siRNAs (vsiRNAs) are incorporated in an Argonaute (AGO)-containing RNA-induced silencing complex (RISC), where they guide the recognition and cleavage of viral target RNAs and thereby restrict viral replication.

RNA silencing in insects

RNA silencing refers to a family of gene silencing mechanisms that are guided by small RNAs in association with an Argonaute family member. There are three major RNA silencing pathways in animals: the siRNA/RNAi pathway, the microRNA (miRNA) pathway and the piwi-associated RNA (piRNA) pathway. These pathways differ in the biogenesis of the small RNA, the Argonaute to which the small RNA associates, their targets, and their effector functions.

The siRNA pathway

The RNAi pathway is initiated by the ribonuclease Dicer-2 (Dcr-2) which processes long dsRNA into siRNAs [70] (Figure 1). Dicer proteins are type III members of the RNaseIII family that contain a DExD/H ATPase domain, a DUF283 domain, a Piwi/Argonaute/Zwille (PAZ) domain, two tandem RNaseIII domains, and a dsRNA-binding domain. The PAZ domain interacts with the terminus of long dsRNA, which is then positioned along the surface of the protein towards the processing center of Dicer [71]. An intramolecular dimer of the two RNaseIII domains forms the processing center of the enzyme. Each RNaseIII domain cleaves one strand of the long dsRNA molecule, thereby generating 21-nt siRNAs with 2-nt 3′ overhangs that bear characteristic 5′ monophosphate and 3′ hydroxyl moieties [72-74]. The distance between the PAZ domain and the RNaseIII active sites determines the characteristic 21-nt size of siRNAs [74,75]. The DExD/H ATPase domain converts ATP to provide energy required for processive cleavage of long dsRNA [76,77]. In contrast to *Drosophila* Dcr-2, the activity of human Dicer is not enhanced by the addition of ATP [78,79]. No functions have thus far been assigned to the other Dcr domains. Efficient processing of dsRNA by *Drosophila* Dcr-2 requires Loquacious isoform PD (Loqs-PD). Loqs-PD probably acts as an adaptor molecule that enhances the affinity of Dcr-2 for long dsRNA [80-83]. A similar activity has been proposed for Arsenic resistance protein 2 (Ars2), which promotes the efficiency and fidelity of Dcr-2-mediated cleavage [84].

Following dsRNA cleavage, the resulting siRNA is bound by Dcr-2 and its dsRNA-binding protein partner R2D2, generating a RISC loading complex (RLC). Binding of R2D2 to the siRNA is enhanced by the phosphate group at the 5′ terminus of the siRNA, thereby ensuring that only authentic siRNAs are efficiently bound by the complex [78,80,85]. The RLC is located in specific cytoplasmic foci termed D2 bodies. Presumably, the D2 bodies form the site where the RLC loads the siRNA duplex into an Argonaute-2 (AGO2) containing RISC [86].

RISC loading is an ATP dependent process that involves, in addition to the RLC, heat shock protein 90 (Hsp90), which presumably induces a conformational change in AGO2 needed to accommodate the siRNA duplex [87,88]. Within RISC, the PAZ domain of AGO2 binds the 3′ terminus of the strand of the siRNA duplex that will be retained in RISC (the guide strand) at the 2-nt overhang [89-91]. The 5′ phosphate of the guide strand is bound in a pocket in the AGO2 middle (Mid) domain [92,93]. Upon loading of the siRNA into RISC, the endonucleolytic activity of the Piwi domain of AGO2 cleaves the phosphodiester bond between nucleotides 9 and 10 of the strand that will be excluded from RISC (the passenger strand) [94-96]. The adaptor molecule C3PO degrades the 9- and 12-nt RNA fragments that result from passenger strand cleavage [97]. The fate of the two strands of an siRNA duplex is determined by its binding orientation to R2D2 and Dcr-2 in the RLC. R2D2 binds the thermodynamically most stable end of the siRNA, whereas Dcr-2 binds the other end. The strand that

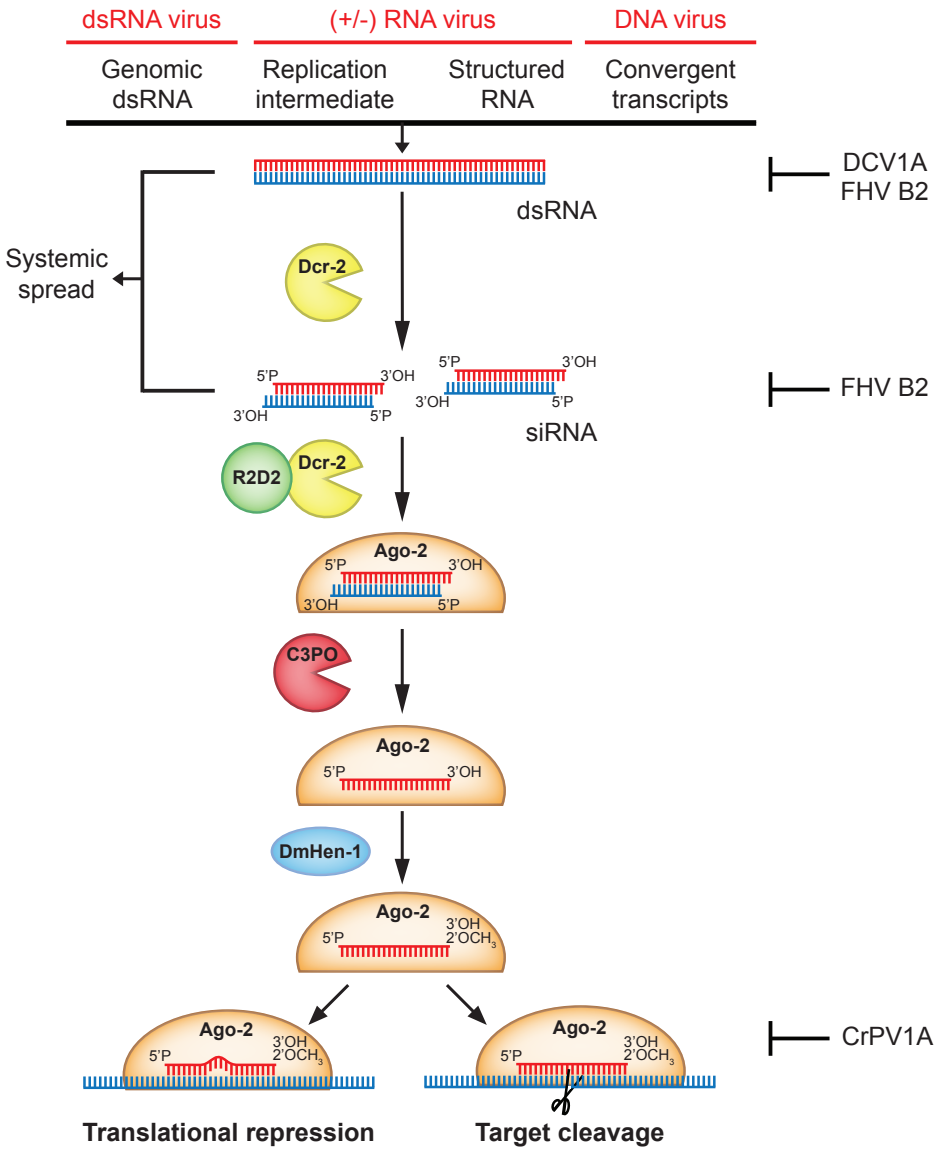

Figure 1: Schematic overview of the RNAi pathway in insects. Viral dsRNA is processed by Dicer-2 (Dcr-2) into 21-nt small interfering RNAs (siRNAs), which are incorporated in an Argonaute-2 (AGO2) containing RNA-induced silencing complex (RISC). Within RISC, these siRNAs guide the recognition and cleavage of viral target RNAs and thereby restrict viral replication. Suppressors of RNAi from Cricket paralysis virus (CrPV 1A), Drosophila C virus (DCV 1A), and Flock House virus (FHV B2) interfere at different stages of the antiviral RNAi pathway (indicated on the right). Systemic spread of RNAi is thought to be essential for effective antiviral defense. The nature of the sequence specific silencing signal (dsRNA or siRNA) is still unknown.

interacts with R2D2 at its 3′ terminus will become the guide strand [85].
After passenger strand cleavage and elimination, the guide strand is 2′-O-methylated at the 3′ terminal nucleotide by the S-adenosylmethionine-dependent methyltransferase Hen-1 (DmHen-1), resulting in the formation of a mature RISC [98]. The mature RISC uses the guide strand to bind complementary RNA sequences. When a fully complementary RNA is bound by RISC, the RNase activity of the AGO2 Piwi domain cleaves the target RNA (Slicer activity), thereby inducing its degradation [99,100]. Alternatively, AGO2 may induce translational repression if central mismatches between guide strand and target RNA prevent Slicer activity [101].

The miRNA pathway

miRNAs are central regulators of gene expression that inhibit translation and/or induce degradation of their target mRNAs [102]. miRNAs derive from genomically encoded stemloop RNA transcripts produced by all eukaryotic cells. Canonical miRNA biogenesis starts with RNA polymerase II dependent production of primary miRNAs (pri-miRNAs) in the nucleus [103]. These pri-miRNAs form RNA structures with one or more stemloop structures. Typically, a pri-miRNA consists of an imperfect base-paired dsRNA stem with a terminal loop and flanking sequence [104]. In the nucleus, the flanking sequences of the pri-miRNA are removed through processing by the RNaseIII enzyme Drosha and its binding partner Pasha, producing a precursor miRNA (pre-miRNA) [105,106]. Pre-miRNAs are subsequently transported to the cytoplasm by Exportin-5 [107]. In the cytoplasm, the RNaseIII enzyme Dicer-1 (Dcr-1), together with the PB isoform of loquacious (loqs-PB), cleaves pre-miRNAs to produce the mature miRNAs [108,109]. Mature miRNAs are imperfect RNA duplexes of approximately 22-nt in length that contain a 5′ phosphate and a 2-nt 3′ overhang characteristic of an RNaseIII product [110]. Although the majority of miRNAs is produced by this canonical miRNA pathway, alternative biogenesis mechanisms that are independent of Drosha and/or Dicer exist [111,112].
To exert their regulatory function, mature miRNA duplexes generally associate with an Argonaute-1 (AGO1) containing RISC complex. Central mismatches in the miRNA duplex facilitate sorting of the miRNA into AGO1, whereas mismatches in the seed region (guide positions 2-8) or the middle of the 3′ region (guide positions 12-15) promote unwinding of the duplex. Duplex unwinding leads to a mature miRNA RISC (miRISC), in which one miRNA strand is retained, whereas the other strand is discarded [113-115]. Although both strands can potentially be loaded into AGO1, the strand most frequently associated with AGO1 is termed the mature miRNA strand, while the other is called the miRNA* strand. The mature miRNA serves as a sequence specific guide to target miRISC to messenger RNAs (mRNAs). In animals, the miRNA target sequences reside in general in the 3′ untranslated region (3′UTR), although some miRNA target sites have been identified in the 5′ UTR and open reading frame

of RNA transcripts [102]. miRNAs bind their target mRNAs through imperfect base-pairing. Base-pairing between nucleotides 2-8 (the seed sequence) of the miRNA and the target mRNA is generally required for recognition [116]. Upon target binding, miRISC can either inhibit translation, facilitate deadenylation, and/or initiate the degradation of the mRNA [102].

In *Drosophila*, the miRNA and siRNA pathways were considered to be separate pathways, with Dcr-2 and AGO2 dedicated to the RNAi pathway, and Dcr-1 and AGO1 dedicated to the miRNA pathway. Although the biogenesis of siRNAs and miRNAs are indeed separate processes, some cross-talk occurs at the level of RISC loading. More specifically, some miRNAs, especially those with more extensive base pairing, can be loaded into AGO2 [117,118]. Recently, it was shown that both the mature miRNA and miRNA* strands are frequently loaded into AGO2 by a *Dcr-2* and *R2D2*-dependent mechanism [117,119]. These findings render the mature miRNA/miRNA* strand nomenclature somewhat ambiguous. Therefore, the strand nomenclature changed to 5-p and 3-p, referring to the strands originating from the 5′ arm or the 3′ arm of the pre-miRNA, respectively.

The piRNA pathway

Piwi-interacting RNAs (piRNAs) are small silencing RNAs of ~24-30 nucleotides in size that interact with members of the Piwi subfamily of the AGO family [120]. The Piwi subfamily comprises Piwi, Aubergine (Aub) and Argonaute-3 (AGO3), which are predominantly expressed in germline tissues. In these tissues, the piRNA pathway prevents the activation of transposons. The biogenesis of piRNAs remains poorly understood, but in contrast to siRNAs and miRNAs, piRNAs are generated in a Dicer-independent manner. They most likely arise from long single-stranded precursor RNAs that originate from intergenic regions, termed piRNA loci, which contain sequences of transposons and their defective remnants. How these precursors are processed into mature piRNAs is not completely clear. Recently, the endoribonuclease Zucchini was implicated in cleaving the 5′ end of the single-stranded precursor RNA, thereby determining the 5′ end of the piRNA. Piwi and Aub proteins preferentially bind cleavage products that contain a 5′ uridine after which the Piwi and Aub-associated RNA molecules are trimmed from their 3′ ends by an unknown nuclease. In the presence of a target transposon RNA, these primary piRNAs engage in an amplification loop, known as the ping-pong mechanism. According to the ping-pong model, Piwi and Aub associated antisense piRNAs target transposon mRNAs, which are subsequently cleaved. The cleavage products are then transferred onto AGO3 and further processed into sense piRNAs. The AGO3 associated piRNAs direct the cleavage of antisense transposon RNAs, leading to a feedback loop that results in amplification of the initial pool of piRNAs.

The ping-pong model was deduced from the characteristic features of the piRNAs

that are associated with the different Piwi proteins. First, piRNAs bound to Piwi and Aub are in general antisense to the transposon RNAs, whereas piRNAs bound to AGO3 are most often of sense orientation. Second, Piwi- and Aub-associated piRNAs have a strong bias for a uridine at position one, whereas AGO3-associated piRNAs have a strong preference for an adenine at position ten. Third, the first ten nucleotides of Piwi- and Aub-associated piRNAs are frequently complementary to the 5′ ten nucleotides of AGO3-associated piRNAs.

RNAi as an antiviral defense mechanism in insects

Viral dsRNA is a strong non-self signature. An appealing hypothesis is that viral dsRNA is sensed and processed by Dcr-2 into vsiRNAs. Once incorporated into AGO2/RISC, these vsiRNAs may guide the recognition and cleavage of single-stranded viral target RNAs. This model predicts that the RNAi pathway exerts antiviral activity at two levels, by Dicer-mediated cleavage of viral dsRNA and by AGO2-mediated targeting of viral single-stranded RNA.

Three major lines of evidence support a crucial role for the RNAi pathway in antiviral defense in insects. First, RNAi-deficient flies (*Dcr-2*, *R2D2*, or *AGO2* null mutants) are more sensitive than wild-type flies to infection with several viruses, such as DCV, CrPV, FHV, vesicular stomatitis virus (VSV) (*Rhabdoviridae*), and Invertebrate Iridescent virus 6 (IIV-6) (*Iridoviridae*). Virus infections in these RNAi mutants result in higher viral RNA copy numbers, higher viral titers, and increased mortality [17,25,30,32,65,121,122]. *AGO2* and *R2D2* mutants are also hypersensitive to the dsRNA virus Drosophila X virus (*Birnaviridae*), but, surprisingly, *Dcr-2* mutants are not [121]. The observation that *AGO2* and *R2D2* mutants (in which Dcr-2 is fully functional) are also hypersensitive to virus infection indicates that cleavage of viral dsRNAs by itself is not sufficient to control acute virus infection. RNAi also proved to be essential for controlling replication of several arthropod-borne (arbo-) viruses in mosquitoes. Knockdown of *Dcr-2* or *AGO2* expression in *Aedes aegypti* results in higher viral titers after infection with either Dengue virus (*Flaviviridae*) or Sindbis virus (*Togaviridae*) [123,124]. Comparable results were obtained upon infections of *Anopheles gambiae* mosquitoes with O'nyong-nyong virus (*Togaviridae*) after *AGO2* depletion [125]. Second, the accumulation of vsiRNAs during virus infections in both *Drosophila* and mosquitoes provides direct evidence for processing of viral RNAs by Dcr-2 [31,33,126-130] (discussed below). Third, insect viruses encode viral suppressors of RNAi (VSRs) as a counter-defense to the RNAi-based immune response [25,33,131] (discussed below).

In addition to cell-autonomous silencing, activation of the RNAi pathway can lead to systemic silencing in plants. In the setting of a viral infection, non-cell autonomous

RNAi may thus generate systemic protective immunity in non-infected tissues. Systemic RNAi in plants is a composite of short-range and long-range movement of a silencing signal. Movement of a silencing signal to distal sites in the plant requires amplification of the signal by a cellular RNA-dependent RNA polymerase (RdRP) [132]. In contrast, local spread of RNAi to 10-15 neighboring cells is independent of RdRP activity. The mobile silencing molecules in plants were recently identified as siRNAs; it remains to be established whether they are protein-bound or not [133]. Virus infections in *Drosophila* also trigger a systemic RNAi response, which depends on a mechanism for active uptake of dsRNA [134]. Accordingly, fly mutants with defects in dsRNA uptake are hypersensitive to virus infection. These observations suggest that, in addition to the cell-autonomous response, a systemic RNAi response is essential for effective antiviral immunity in flies. The nature of the mobile silencing signal remains unknown. In addition, it remains elusive whether flies express a protein with RdRP activity to amplify the silencing signal for systemic spread.

Small RNA profiles during virus infection

The antiviral RNAi pathway in plants and insects is triggered by viral dsRNA molecules, which are processed by Dcr-2 into vsiRNAs. In theory, there are several potential sources of viral dsRNA that can serve as a substrate for Dcr-2. These sources include genomic dsRNA, structural RNA elements in the viral genome or in viral transcripts, dsRNA replication intermediates and convergent transcripts of viral genes. The recent introduction of massive parallel sequencing created the opportunity to gain in-depth insight into the origin of the viral small RNAs for different classes of insect viruses (Table 1).

Viral siRNA profiles

The first insect vsiRNA profiles came from a study designed to profile endogenous small RNAs from a *Drosophila* S2 cell line [135]. In addition to endogenous small RNAs, a large subset of AGO2-associated siRNAs was found to match the FHV genome. These vsiRNAs arose due to a persistent infection of the S2 cell line. Alignment of the available FHV-derived vsiRNAs to the viral genome indicated that they mapped in roughly equal proportions to both the positive (+) and negative (-) strand of the genome [136]. During (+) strand RNA virus infection, the (+) and (-) RNA strands accumulate asymmetrically, with the genomic (+) strands being approximately 50- to 100-fold more prominent than the (-) strands [137]. The equal distribution of the vsiRNAs over the (+) and (-) RNA strands of the FHV genome, therefore, suggests that dsRNA replication intermediates are the major substrates for vsiRNA biogenesis. Interestingly, although the vsiRNAs mapped across the entire viral genome, there

Table 1: Overview of insect viruses of which vsiRNA profiles have been determined by massive parallel sequencing.

Virus	Family	Genome	Host	References
Drosophila A virus[1]	*Tetraviridae*	(+) RNA	*Drosophila*	[130]
Drosophila C virus	*Dicistroviridae*	(+) RNA	*Drosophila*	[130,138,145]
Nora virus	Unassigned	(+) RNA	*Drosophila*	[130,192]
Sindbis virus	*Togaviridae*	(+) RNA	*Aedes aegypti/Drosophila*	[128,140]
Semliki Forest virus	*Togaviridae*	(+) RNA	*Aedes aegypti/Aedes albopictus*	[143]
Chikungunya virus	*Togaviridae*	(+) RNA	*Aedes aegypti/Aedes albopictus*	[141]
Mosquito nodavirus	*Nodaviridae*	(+) RNA	*Aedes aegypti*	[130]
West Nile virus	*Flaviviridae*	(+) RNA	*Culex pipiens quinquefasciatus/Drosophila*	[126,140]
Dengue virus	*Flaviviridae*	(+) RNA	*Aedes aegypti*	[142,155]
Flock House virus[2]	*Nodaviridae*	(+) RNA (segmented)	*Drosophila*	[31,127,136,,139,144]
American nodavirus[2]	*Nodaviridae*	(+) RNA (segmented)	*Drosophila*	[130]
Vesicular stomatitis virus	*Rhabdoviridae*	(-) RNA	*Drosophila*	[17,145]
Rift Valley Fever virus	*Bunyaviridae*	(-) RNA (segmented)	*Aedes aegypti/Aedes albopictus/Drosophila*	[145,148]
Schmallenberg virus	*Bunyaviridae*	(-) RNA (segmented)	*Culicoides sonorensis/ Aedes aegypti*	[149]
La Crosse virus	*Bunyaviridae*	(-) RNA (segmented)	*Drosophila*	[140]
Blue Tongue virus	*Reoviridae*	dsRNA (segmented)	*Culicoides sonorensis/ Aedes aegypti*	[149]
Drosophila totivirus	*Totiviridae*	dsRNA	*Drosophila*	[130]
Drosophila X virus	*Birnaviridae*	dsRNA (segmented)	*Drosophila*	[130,138]
Drosophila birnavirus	*Birnaviridae*	dsRNA (segmented)	*Drosophila*	[130]
Invertebrate iridescent virus 6	*Iridoviridae*	dsDNA	*Drosophila*	[65,122]
Culex tritaeniorhynchus densovirus	Unassigned	dsDNA	*Culex pipiens molestus*	[150]

[1]Drosophila A virus was referred to as Drosophila tetravirus by Wu *et al.* [130].
[2]Given the close similarity between Flock House virus and American nodavirus at the nucleotide level, these viruses likely represent two variants of the same virus species within the *Nodaviridae* family.

were a few specific hotspots from which the majority of the vsiRNAs were derived. These observations suggest that certain regions within the viral genome are more accessible to Dcr-2 than others. Results from this first study were confirmed by other reports on FHV small RNA profiles [31,127,138,139]. The equal distribution of vsiRNAs over the (+) and (-) strands of the viral genome seem to be a general theme in (+) strand RNA virus infection, as similar vsiRNA profiles were obtained during other (+) strand RNA virus infections in either *Drosophila* or mosquito [126,128,130,140-143]. Therefore, it seems that the dsRNA replication intermediate is a common target of the insect antiviral RNAi pathway during infection with (+) strand RNA viruses. For some RNA viruses, dicing of structural elements within the viral genomes have been suggested to contribute to vsiRNA production. For example, some studies reported a bias of vsiRNAs mapping to the genomic strand during FHV or DCV infection [138,144,145]. The reason for this positive-strand bias is unclear, but might be due to processing of the asymmetrically produced viral (+) RNA strand, presumably at structured RNA elements.

Interestingly, in contrast to most insect (+) strand RNA viruses, structural RNA elements within the viral genome have been identified as the main source for vsiRNA biogenesis for some plant (+) strand RNA viruses, such as Cymbidium ringspot virus (CymRSV) (*Tombusviridae*) [146]. This discrepancy might reflect differences in substrate-specificity between insect Dcr-2 and the plant Dcr-like enzymes.

In analogy to (+) strand RNA viruses, (+) and (-) strands accumulate asymmetrically during VSV infection, a (-) strand RNA virus, with the genomic (-) strand being five to ten fold more abundant than the antigenomic (+) strand [147]. In contrast to (+) strand RNA viruses though, (-) strand RNA viruses do not produce detectable amounts of dsRNA during infection [17,69]. Nevertheless, vsiRNAs are evenly distributed between the genomic and antigenomic strands in infections with VSV, Rift Valley fever virus (RVFV), La Crosse virus, and Schmallenberg virus [17,140,145,148,149]. Together with the uniform distribution of vsiRNAs along the entire length of the genome, these observations indicate that also in (-) strand RNA viruses the dsRNA replication intermediate is a substrate for Dcr-2.

Recently, Wu *et al.* identified seven distinct RNA viruses in two *Drosophila* cell lines [130]. Among these viruses, of which some had not been identified before, are three viruses with a dsRNA genome (Drosophila totivirus, Drosophila birnavirus and Drosophila X virus). Interestingly, for all of the analyzed dsRNA viruses in both cell lines, the vsiRNAs mapped in roughly equal ratios to both strands of the viral genome. Furthermore, vsiRNAs mapped all along the genome without clustering at specific hotspots. Similar results were obtained for the segmented dsRNA virus Blue tongue virus in a *Culicoides* midge derived cell line [149]. Although dsRNA viruses are believed to shield their genome in the viral core, these results suggest that the genomic dsRNA is the predominant substrate for Dcr-2.

DNA viruses also produce viral dsRNA during infection, presumably through structured RNA transcripts or overlapping convergent transcripts [69]. These dsRNA species are potential substrates for Dcr-2. Indeed, vsiRNAs corresponding to the dsDNA virus IIV-6 were identified in IIV-6 infected flies [65,122]. The IIV-6 derived vsiRNAs clustered in hot spots and mapped equally to both strands of the viral genome. Interestingly, sense and antisense transcripts were detected at loci covered by the vsiRNA hotspots. Therefore, vsiRNAs were suggested to originate from overlapping convergent transcripts. Also in mosquitoes, vsiRNAs corresponding to a DNA virus, *Culex tritaeniorhynchus* virus, were reported [150]. The vsiRNAs were mainly derived from the viral RNA transcripts, although the mechanism of vsiRNA production in this infection remains unclear.
Taken together, vsiRNA profiling shows that all major classes of viruses are targeted by the insect antiviral RNAi response. To prevent activation of the innate immune defenses of their hosts, viruses protect their dsRNAs from immune sensors. For example, (+) strand RNA viruses shield their dsRNA replication intermediates in virus-induced membrane vesicles, whereas the dsRNA genomes of dsRNA viruses are protected in viral cores [151]. Similar to (+) strand RNA viruses, non-segmented (-) strand RNA viruses like VSV seem to replicate their genome in membranous vesicles [152,153]. Nevertheless, vsiRNA profiles indicate that the viral dsRNAs are available for Dcr-2-mediated cleavage. This suggests that Dcr-2 is capable of protruding the compartments in which viral dsRNAs are shielded or that, at certain stages of the viral replication cycle, viral dsRNA is released into the cytoplasm where it is exposed to Dcr-2.

Virus-derived piRNA profiles

Massive parallel sequencing of small RNAs from infected insect material showed that vsiRNAs are produced during infection with insect viruses from all classes. In association with RISC, these vsiRNAs are thought to cleave complementary viral RNAs, allowing the RNAi pathway to act as a major antiviral response in insects. Interestingly, while analyzing vsiRNAs in a cell line derived from the ovarian somatic sheet (OSS), Wu *et al.* [130] implicated another class of small RNAs in antiviral defense. In addition to vsiRNAs, high levels of viral piRNAs were identified for two viruses: DCV and American Nodavirus (ANV) [130]. Although less abundant, viral piRNAs were also identified for other viruses that persist in this cell line. These observations suggest that viruses may be targeted by both the siRNA and piRNA pathway in insects.
The viral piRNAs detected in OSS cells were 24-30 nucleotides in length with peaks at 27 and 28 nucleotides [130]. The OSS cell line expresses *Piwi*, but not *Aub* and *AGO3*. Primary piRNAs are therefore produced, but the lack of Aub and AGO3 precludes ping-pong amplification [154]. In agreement, viral piRNAs resemble primary piRNAs with a strong bias for a uridine at position one, but no enrichment for an adenine at

position ten [154]. Moreover, the viral piRNAs were almost exclusively of (+) polarity for both (+) strand RNA and dsRNA viruses. These data suggest that the genomic RNAs of the (+) strand RNA viruses and the sense transcripts of dsRNA viruses are processed by the primary piRNA pathway in *Drosophila*. These observations are not restricted to *Drosophila*, as viral piRNAs were also detected in different mosquitoes and mosquito cell lines using viruses from diverse families [138,140-142,148,155-157]. In contrast to the *Drosophila* OSS cell line, mosquito cells produce piRNAs that map to both strands of the viral genome. Furthermore, viral piRNAs show a bias for uridine at position one, and enrichment for adenosine at position ten, indicative of the ping-pong-dependent amplification mechanism [138,141,148,156,157]. Strikingly, small RNA profiles from head and thorax of infected *Aedes albopictus* mosquitoes suggest that the ping-pong-dependent viral piRNA production also occurs in somatic tissues, which is in stark contrast to *Drosophila* where the piRNA machinery is believed to be restricted to the germline [141,158,159].
Maintenance of germline integrity is of crucial importance to ensure the proper development of offspring. The germline-specificity of the piRNA pathway and the detection of viral piRNAs in *Drosophila* led to the hypothesis that the piRNA pathway may protect the germline from invasion by viruses [160]. The detection of viral piRNA-like RNAs with a ping-pong signature in the mosquito germ line and soma suggests that the mosquito piRNA pathway provides another layer of small RNA-mediated antiviral defense, additional to the antiviral siRNA pathway. A recent finding that Semliki Forest virus (SFV) infection is enhanced upon knockdown of piRNA components supports this hypothesis [156].

Viral suppression of RNAi

Despite the potent antiviral activity of the RNAi pathway in plants and insects, many viruses manage to persist in these organisms. Thus, viruses seem to be able to avoid recognition by the RNAi pathway or to counteract its antiviral activity. Indeed, plant and insect viruses encode VSRs that allow them to replicate in the presence of a potent RNAi-based antiviral immune response [30,68,144,161-164]. Since dsRNA is recognized as a “non-self” immune activator, it bears little surprise that many viruses prevent detection of dsRNA by the immune system. Many VSRs bind dsRNA in a sequence-independent manner, thereby shielding it from Dicer cleavage [25,163,165]. Other VSRs, such as P19 from Cymbidium Ringspot Tombusvirus (CymRSV), are able to specifically bind siRNAs [163,165-168]. In biochemical assays, these VSRs sequester siRNAs and prevent their incorporation into RISC. The mechanism of RNAi suppression in viral infection, however, may be more complex. P19, for example, is dispensable for virus accumulation in primary infected cells, but

prevents cell-to-cell movement of the virus-induced silencing signal. Accordingly, in the absence of P19, the virus accumulates normally within vascular bundles, but is unable to establish systemic infection of the leaves [168,169].
Binding of dsRNA or siRNA is a feature that is shared by many VSRs. Nevertheless, in analogy to the multitude of mechanisms by which mammalian viruses suppress innate and adaptive immunity, some plant and insect viruses suppress RNAi by a mechanism that is independent of dsRNA or siRNA binding. These mechanisms are discussed in the next sections.

RNAi suppressors encoded by plant viruses

Although dsRNA binding appears to be a common mechanism to suppress RNAi, some VSRs employ other mechanisms to counteract the RNAi pathway (Table 2). These VSRs rely on protein-protein interaction with key components of the RNAi pathway. One example of such a VSR is the P0 protein of *Poleroviruses* (*Luteoviridae*) [170-172]. Beet western yellows virus (BWYV) P0, for example, interacts with and induces degradation of AGO1, the main antiviral RISC component in plants. P0 contains an F-box motif, which is commonly found in proteins within the E3 ubiquitin ligase complex. This suggests that P0 induces ubiquitination and subsequent proteosomal degradation of AGO1 [173]. Indeed, the VSR activity of P0 depends on an interaction with a protein from the E3 ubiquitin ligase complex. However, blocking proteosomal degradation did not prevent AGO1 degradation. These data suggest an ubiquitin-dependent, proteosomal-independent mechanism for P0 VSR activity.
Through an interaction with part of the PAZ and Piwi domains of AGO1, the Cucumber mosaic virus (CMV) (*Bromoviridae*) 2b protein is able to suppress Slicer activity of a pre-assembled RISC *in vitro* [174]. In addition, CMV 2b binds small RNAs *in vitro*, which is suggested to contribute to VSR activity [175,176]. Which activity predominates during an authentic virus infection remains to be established.
The P38 capsid protein of Turnip crinkle virus (TCV) (*Tombusviridae*) employs yet another mechanism of RNAi suppression. P38 contains two glycine-tryptophane (GW) motifs. Different cellular proteins use linear GW or WG motifs as an "AGO hook" for functional interactions with AGO proteins [177]. P38 mimics this cellular GW motif-based interaction; its GW motifs allow P38 to bind AGO1 *in vitro* and to suppress RNAi *in vivo* [178,179]. Similarly, the sweet potato mild mottle virus (SPMMV) (*Potyviridae*) P1 protein uses three GW/WG motifs to bind AGO1 and suppress its function [180].

RNAi suppressors encoded by insect viruses

Plant viruses are extensively studied and most, if not all, viruses seem to encode one or multiple VSRs [163,181]. In contrast, few VSRs have thus far been identified and characterized in insect viruses. The B2 protein of FHV was the first VSR identified in an invertebrate virus [33]. Homodimers of B2 bind to dsRNA independent of sequence

Table 2: Viral suppressors of RNAi (VSRs) of selected plant and insect viruses.

Host	Virus	VSR	Mechanism of suppression	Ref.
Plant	Cymbidium ringspot virus	P19	siRNA binding	[166]
	Pothos latent virus	P14	siRNA and dsRNA binding	[193]
	Beet western yellows virus	P0	Interaction with and degradation of AGO1	[170-172]
	Turnip crinckle virus	P38	GW motif-based interaction with AGO1	[178]
	Cucumber mosaic virus	2b	siRNA binding and AGO1 interaction, inhibition of Slicer activity	[174-176]
Insect	Flock House virus	B2	siRNA and dsRNA binding	[33,182]
	Drosophila C virus	1A	dsRNA binding	[25]
	Cricket paralysis virus	1A	Interaction with AGO2, inhibition of Slicer activity	[131]

and length. Indeed, viral dsRNA replication intermediates co-immunoprecipitate with B2 from FHV-infected S2 cells, confirming an interaction of B2 with dsRNA *in vivo* [31]. B2 seems to exert a dual mode of RNAi suppression. Binding of long dsRNA inhibits Dcr-2 cleavage; binding of siRNAs prevents their incorporation into AGO2 [33,182]. The importance of a VSR for virus replication is genetically well-supported in the case of FHV. Its RNAi suppressor B2 allows the virus to replicate in the presence of a functional antiviral RNAi response, but it is dispensable for replication in RNAi mutants [30,144]. The VSR activity of the DCV 1A protein also depends on dsRNA binding. As a member of the *Dicistroviridae*, DCV encodes two polyproteins from two distinct open reading frames. The first open reading frame, ORF1, encodes the nonstructural proteins, whereas ORF2 encodes the viral capsid proteins [2]. DCV 1A maps to the N-terminal part of ORF1. In contrast to FHV B2, DCV 1A binds long dsRNA, but not siRNAs, with high affinity *in vitro*, thereby inhibiting Dcr-2 cleavage of dsRNA [25]. Therefore, DCV 1A likely binds the viral replication intermediate to protect its degradation by Dcr-2. Protection of the viral dsRNA from Dcr-2 cleavage by VSRs is, however, not complete, as indicated by the detection of vsiRNAs in FHV and DCV infection.

CrPV is the closest relative of DCV within the *Dicistroviridae* family. The VSR of CrPV, 1A, maps to the same genomic location as DCV 1A. Interestingly, whereas CrPV and DCV share a high degree of protein sequence identity within ORF1 (~55%), the ORF1 N-terminal region that contains the VSRs is not well conserved. It is, therefore, no surprise that CrPV 1A suppresses RNAi through a different mechanism as DCV 1A. CrPV 1A directly interacts with AGO2, without affecting RISC assembly or stability. Since CrPV 1A is able to inhibit the activity of a pre-assembled RISC, it most likely

interferes with the Slicer activity of AGO2 [131].
The difference in mechanism and potency of RNAi suppression was suggested to explain the difference in pathogenicity between DCV and CrPV. DCV establishes a non-lethal, persistent infection, whereas CrPV causes high mortality [2,183]. This hypothesis was supported by expression of DCV 1A (Sindbis-DCV 1A) or CrPV 1A (Sindbis-CrPV 1A) from a recombinant Sindbis virus, which is thought not to suppress RNAi by itself [184,185]. In adult flies, Sindbis-CrPV 1A replicates to higher titers and causes higher mortality than Sindbis-DCV 1A, which correlates with the more potent RNAi suppression of CrPV 1A. Thus, VSRs may be important determinants of viral pathogenicity (Figure 2).

RNAi suppression by arboviruses

Arboviruses are maintained in a cycle that requires transmission by haematophagous arthropod vectors (mainly mosquitoes and ticks) to vertebrate hosts. After ingestion of a virus-infected blood meal, arboviruses replicate in the midgut epithelium of their vector. The virus then spreads through the hemolymph into the salivary glands for further amplification and transmission to a naive vertebrate host. Several arboviruses, including Dengue virus, Sindbis virus and O'nyong-nyong virus, are suppressed by RNAi in their mosquito vectors [123-125].
Arbovirus infection in the insect vector is typically persistent and non-pathogenic. The mosquito's RNAi pathway thus restricts arbovirus replication, but is unable to fully clear these viruses. Since arboviruses rely on survival of their mosquito vector for transmission from host to host, the non-pathogenic phenotype is thought to be

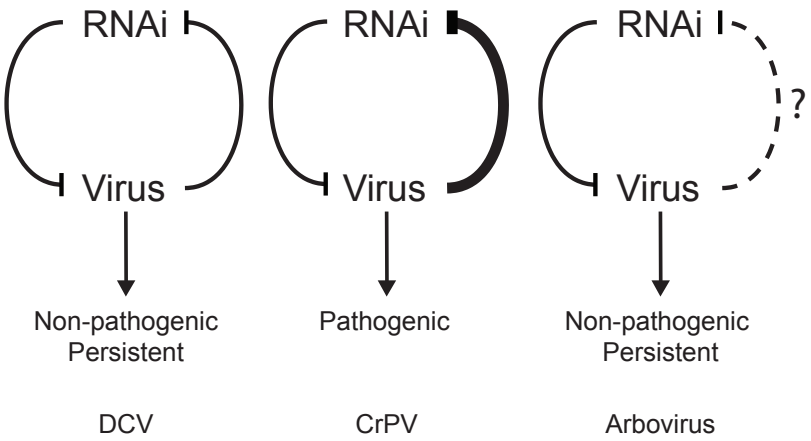

Figure 2: Defense and counter-defense in virus infection of insects. An antiviral RNAi response restricts virus replication, whereas viruses suppress the antiviral RNAi response via dedicated RNAi suppressor proteins (VSRs). VSR activity may be an important determinant of viral pathogenicity. A potent VSR may render a virus pathogenic to its host (for example, Cricket paralysis virus, CrPV). Absent or mild VSR activity may result in non-pathogenic persistent infections, such as observed in natural Drosophila C virus (DCV) infections or in arbovirus infection of their invertebrate vector.

of key importance for efficient virus spread. Hence, an arbovirus that effectively kills its vector would therefore be selected against in nature, since it would decrease the chance of spread to vertebrate hosts. Consequently, it was suggested that it might be deleterious for the survival of arboviruses to encode a potent VSR [184,186] (Figure 2).

Nevertheless, in recent years, suppressors of RNAi have been identified in arboviruses. The first suppressor identified in an arbovirus is the B2 protein of Nodamura virus (NoV, *Nodaviridae*) [187,188]. Like the B2 protein from FHV, the NoV B2 protein can bind long dsRNA and siRNAs, preventing the use of these RNA molecules in the RNAi pathway. In addition, the non-coding subgenomic flavivirus RNA (sfRNA) of West Nile virus and Dengue virus was recently shown to interfere with the RNAi response in mosquitoes [189]. Results from this study suggest that the sfRNA saturates Dicer in a concentration-dependent manner, thereby blocking the RNAi pathway. These data indicate that arboviruses might employ different strategies for RNAi suppression to tune the delicate balance of antiviral defense and virus counter-defense.

Concluding remarks and open questions

Viruses and RNAi share an intricate relationship, in which the cellular RNAi machinery restricts virus replication and viruses suppress the antiviral RNAi response. In the current model, viruses produce dsRNAs that, after processing by Dicer into vsiRNAs, guide RISC to cleave viral target RNAs in a sequence specific manner. The advent of deep-sequencing technology has led to unanticipated insights into the biogenesis of vsiRNAs. Viral replication intermediates and genomic dsRNA seem to be the main targets for Dcr-2 in infections with single stranded RNA viruses (either of (+) or (-) polarity) and dsRNA viruses, respectively. Since viral replication intermediates and genomic dsRNA are crucial entities in the viral life cycle, it was proposed that Dicing of these dsRNA molecules is sufficient to control persistent infections [127]. The potency and mechanism of viral RNAi suppressors has also been suggested to play a role in viral pathogenesis. This notion was based on the observation that the RNAi suppressor of the pathogenic CrPV was more potent than that of the relatively benign DCV [131]. Yet, with only few insect RNAi suppressors identified, it remains unclear whether this hypothesis is valid for all insect VSRs. More generally, the identification of additional RNAi suppressors may illuminate the full extent by which viruses suppress RNAi. Moreover, characterization of immune antagonists sheds light on the critical steps of antiviral mechanisms and may result in the identification of new components and regulators of antiviral immune responses [190,191].

During co-evolution of viruses and their hosts, dynamic fine-tuned interactions have emerged that are best studied in natural virus-hosts combinations. Of special interest

in this regard are *Drosophila* pathogens, as virus-host interactions can be studied using the expansive experimental toolbox available in *Drosophila*. In addition, the use of recombinant *Drosophila* viruses would establish a system where both virus and natural host are amenable to genetic manipulation.
The interaction between (arbo-)viruses and their mosquito hosts remain poorly understood. Arboviruses are targets of the RNAi pathway in their mosquito vector. Nevertheless, arboviruses in general do not seem to generally express RNAi suppressor proteins. This raises important questions as to whether some arboviruses and mosquito viruses lack, or only mildly suppress RNAi to prevent a pathogenic course of infection in their vector.

Aim and outline of this thesis

The RNAi pathway is the major antiviral defense mechanism in insects. The aim of this thesis is to gain more insight into the interaction between the antiviral RNAi pathway and viral counter-defense mechanisms. To identify viral proteins that suppress the RNAi pathway in insects, we describe in **chapter 2** an RNAi sensor assay in *Drosophila* S2 cells. In **chapter 3** we investigate whether Nora virus, which persistently infects *Drosophila*, is a target of the antiviral RNAi pathway. Using the RNAi sensor assay of chapter 2 and other functional and biochemical assays, we identify viral protein 1 (VP1) of Nora virus as a viral suppressor of RNAi. We extended the characterization of VP1 in **chapter 4**, in which we explore the possibility to use transgenic expression of VP1 as a tool to study RNAi *in vivo*. In **chapter 5** we describe the interaction of VP1 with AGO2, the core component of RISC. Using Nora virus VP1 sequences isolated from different *Drosophila* species, we report a species-specific suppression of RNAi by VP1. Therefore, we postulate that viral RNAi suppressors may act as a host-specificity factor. In **chapter 6** we provide evidence that the entomobirnaviruses Culex Y virus, recently isolated from wild-caught mosquitoes, and Drosophila X virus, encode an RNAi suppressor. Finally, in **chapter 7** the results of this thesis are discussed and future prospects are presented.

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Chapter 2

Identification of viral suppressors of RNAi by a reporter assay in *Drosophila* S2 cell culture

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Abstract

The RNA interference (RNAi) pathway plays an important role in antiviral immunity in insects. To counteract the RNAi-mediated immune response of their hosts, several insect viruses, such as Flock house virus, Drosophila C virus and Cricket paralysis virus, encode potent viral suppressors of RNAi (VSRs). Because of the importance of RNAi in antiviral defense in insects, other insect viruses are likely to encode VSRs. In this chapter, we describe a detailed protocol for an RNAi reporter assay in Drosophila S2 cells for the identification of VSR activity.

1. Introduction

During prolonged co-evolution of virus and host, viruses have developed various sophisticated strategies to evade the immune defenses of their hosts. In insects, RNA interference (RNAi) is an important antiviral defense mechanism (reviewed in [1-3]). The RNAi machinery is triggered by viral double-stranded RNA (dsRNA), which is cleaved by Dicer-2 into viral small interfering RNAs (v-siRNAs). The v-siRNAs are incorporated into an RNA-induced silencing complex (RISC) where they guide the recognition and cleavage of complementary viral RNAs by Argonaute-2 (Ago-2) and thereby restrict viral replication. To interfere with the antiviral RNAi defense system, several insect viruses encode potent viral suppressors of RNAi (VSRs). These VSRs include Flock house virus (FHV) B2 [4], Drosophila C virus (DCV) 1A [5], and Cricket paralysis virus (CrPV) 1A [6]. The VSRs target different steps in the RNAi pathway. For example, both FHV B2 and DCV 1A block Dicer-mediated cleavage of long dsRNA into siRNAs by binding long dsRNA molecules, whereas FHV B2 also sequesters siRNAs to prevent their incorporation into RISC [5,7-10]. In addition to these dsRNA-binding activities, several VSRs interact with components of the RNAi machinery directly. For instance, FHV B2 interacts with Dicer in order to suppress siRNA biogenesis [11], whereas CrPV 1A inhibits RISC activity via an interaction with Ago-2 [12].

Given the importance of RNAi as an antiviral defense mechanism in insects, many more insect viruses are likely to encode VSRs. This chapter provides a detailed protocol that can be used to routinely screen potential VSRs for their ability to suppress RNAi in the *Drosophila* Schneider 2 (S2) cell line. In brief, S2 cells are first co-transfected with a plasmid that expresses the potential VSR and copper-inducible expression plasmids for the firefly and *Renilla* luciferases. Two days after transfection, the cells are treated with dsRNA to silence expression of the firefly luciferase reporter (dsRNA feeding). The *Renilla* luciferase reporter is not silenced and functions as an internal control which can be used to normalize the data. Several hours after dsRNA treatment, expression of the luciferase reporters is induced with $CuSO_4$. The cells are lysed the next day and luciferase activity is quantified by dual-luciferase reporter (DLR) assays. The data are presented as firefly/*Renilla* ratios and, therefore, increased ratios indicate RNAi suppression by a potential VSR. Once a VSR has been identified, additional reporter assays and biochemical experiments can be performed to determine which step in the RNAi pathway is targeted by the VSR.

A flow chart of the RNAi reporter assay and two variants thereof is shown in **Figure 1**. In experiments to identify VSR activity, we routinely induce RNAi by adding dsRNA to the culture supernatant (dsRNA feeding, *see* **Note 1**). In **Figure 2**, a representative example is presented of an experiment that demonstrates the VSR activities of both DCV 1A and CrPV 1A, using the standard RNAi reporter assay and the two variants. In one of the variants, siRNAs are co-transfected with the plasmids

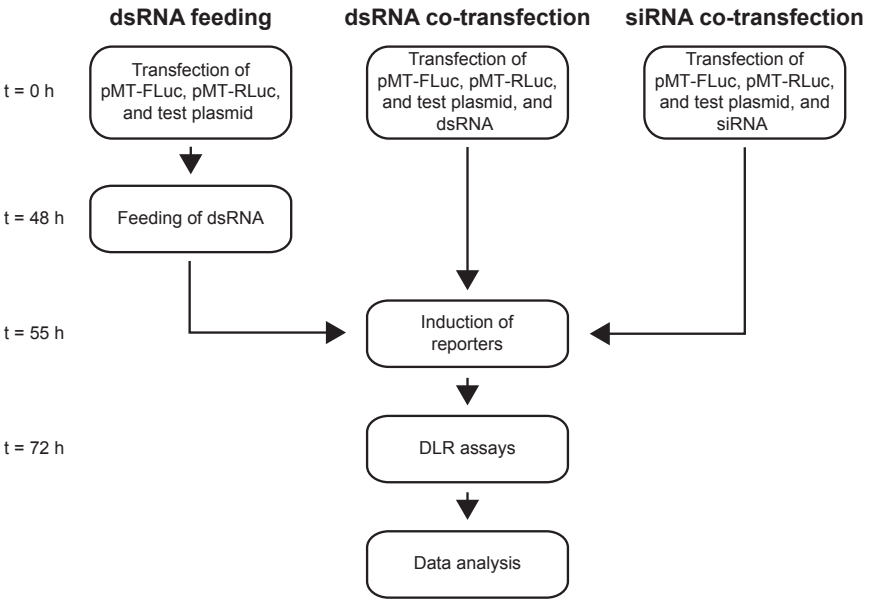

Figure 1: Flow chart of the RNAi reporter assay. See text for details.

(siRNA co-transfection). In contrast to dsRNA, siRNAs do not require processing by Dicer-2. Successful suppression of RNAi in this variant therefore implies that the VSR interferes with steps downstream of Dicer-2 cleavage. In the other variant, dsRNA is co-transfected with the plasmids, rather than added to the culture supernatant (dsRNA co-transfection). Notably, whereas DCV 1A inhibits Dicer-2 cleavage of dsRNA [5], the protein is unable to suppress RNAi in this experimental set-up. Presumably, the co-transfected dsRNA is processed into siRNAs before the VSR is expressed at sufficient levels to suppress Dicer-2 cleavage. The main protocol (**Sections 2** and **3**) describes the RNAi reporter assay using dsRNA feeding to induce RNAi; details on the variants are described in the Notes section.

2. Materials

2.1. Cell culture

1. *Drosophila* S2R+ cells (Drosophila Genomics Resource Center, https://dgrc.cgb.indiana.edu) (*see* **Note 1**).
2. Culture medium: Schneider's *Drosophila* Medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated Fetal Calf Serum (Biochrom, Berlin, Germany) and 1% Penicillin/Streptomycin (Invitrogen). Store at 4°C.
3. Cell scrapers (Corning, Corning, NY).
4. 25 cm^2 cell culture flasks (Corning).

5. 96 well cell culture plates (Corning).
6. Hemocytometer.

2.2. Generation of templates for in vitro transcription

1. Phusion High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland), including 5x Phusion HF Buffer (*see* **Note 2**).
2. 10 mM dNTP mix (Roche, Mannheim, Germany).
3. Plasmid templates for polymerase chain reaction (PCR) containing the GL3 firefly luciferase and green fluorescent protein (GFP) target sequences.
4. The following primers (10 µM):
   T7-FLuc-F: 5'-TAATACGACTCACTATAGGGAGATATGAAGAGATACGCCCTGGTT-3'
   T7-FLuc-R: 5'-TAATACGACTCACTATAGGGAGATAAAACCGGGAGGTAGATGAGA-3'
   T7-GFP-F: 5'-TAATACGACTCACTATAGGGAGAAGCTGACCCTGAAGTTCATCTG-3'
   T7-GFP-R: 5'-TAATACGACTCACTATAGGGAGAGGTGTTCTGCTGGTAGTGGTC-3'
5. Thermal cycler.

2.3. In vitro transcription and dsRNA formation

1. T7-promoter-flanked firefly luciferase and GFP PCR products (*see* **Section 3.2.**).
2. RiboMAX Large Scale RNA Production System-T7 (Promega, Madison, WI), containing T7 Transcription 5x Buffer, rNTPs (25 mM each), and T7 Enzyme Mix (*see* **Note 3**).
3. GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO) (*see* **Note 4**).
4. Heating block.
5. Spectrophotometer.

2.4. Transfection

1. S2R+ cells cultured in a 96 well plate, seeded one day prior to transfection at a density of $5x10^4$ cells per well (*see* **Section 3.1.**). The cells should be 40-80% confluent on the day of transfection.
2. Effectene Transfection Reagent (Qiagen, Hilden, Germany), including Buffer EC and Enhancer.
3. Plasmids pMT-FLuc and pMT-RLuc, which express the firefly (pMT-FLuc) and *Renilla* (pMT-RLuc) luciferases from the copper-inducible metallothionein promoter [5] (*see* **Note 5**).
4. Plasmids that express the potential VSRs which are to be tested for VSR activity as well as the corresponding empty plasmid (*see* **Note 6**).
5. A plasmid that expresses a known VSR (*see* **Note 7**).

2.5. dsRNA feeding

1. Transfected S2R+ cells in a 96 well plate (*see* **Section 3.4.**).
2. Firefly luciferase and GFP dsRNA (*see* **Section 3.3.**).
3. Culture medium (*see* **Section 2.1**, item 2).

2.6. Induction of the reporters

1. Transfected S2R+ cells in a 96 well plate that are fed with dsRNA (*see* **Section 3.5.**).
2. 50 mM $CuSO_4$: Dissolve 2.5 g of $CuSO_4.5H_2O$ in 200 ml of H_2O. Filter sterilize, aliquot, and store at -20°C.
3. Culture medium (*see* **Section 2.1**, item 2).

2.7. DLR assays

1. Transfected S2R+ cells in a 96 well plate that are fed with dsRNA and of which the reporters have been induced (*see* **Section 3.6.**).
2. Phosphate-buffered saline (PBS): Dissolve 80 g of NaCl, 2 g of KCl, 11.5 g of $Na_2HPO_4.7H_2O$, and 2 g of KH_2PO_4 in 1 l of H_2O to prepare a 10x stock solution. Adjust the pH to 7.3 with HCl, sterilize by autoclaving, and store at room temperature. To prepare a working solution, add nine volumes of H_2O to one volume of 10x PBS stock solution.
3. Dual-Luciferase Reporter Assay System (Promega), containing Passive Lysis Buffer (PLB), Luciferase Assay Reagent II (LAR II), and Stop & Glo Reagent (*see* **Note 8**).
4. Rocking platform or orbital shaker.
5. Luminometer tubes.
6. Luminometer.

3. Methods

3.1. Cell culture

1. Culture S2R+ cells in 25 cm^2 flasks at 25°C without CO_2. The cells should be passaged when approaching confluency.
2. To split the cells, scrape the cells with a cell scraper in their culture supernatant and resuspend them by gently pipetting up and down several times.
3. Transfer the desired amount of cells to new cell culture flasks and/or plates in an appropriate volume of fresh culture medium. Split the cells 1:5 into 25 cm^2 flasks for routine maintenance. For RNAi reporter assays, count the cells using a hemocytometer and seed $5x10^4$ cells in 100 µl of culture medium per well in a 96 well plate. Since the RNAi reporter assays are performed in triplicate,

seed three wells for each potential VSR that is to be tested as well as for all the controls (*see* **Section 3.4.**).

3.2. Generation of templates for in vitro transcription

1. Perform PCR reactions to generate T7-promoter-flanked templates for in vitro transcription (*see* **Section 3.3.**). Primers T7-FLuc-F and T7-FLuc-R are used for the amplification of firefly luciferase; primers T7-GFP-F and T7-GFP-R are used for the amplification of GFP. Both primer sets introduce a T7 promoter sequence at both the 5' and 3' end of the amplified fragment. Prepare 50-µl PCR reactions containing:
   a. 1 pg to10 ng of plasmid template DNA
   b. 10 µl of 5x Phusion HF Buffer
   c. 1 µl of 10 mM dNTP mix
   d. 2.5 µl of 10 µM forward primer
   e. 2.5 µl of 10 µM reverse primer
   f. 0.5 µl of 2 U/µl Phusion DNA Polymerase
   g. H_2O to 50 µl

   Mix by gently pipetting up and down.
2. Place the PCR reactions in a thermal cycler. To amplify the target sequences, start with an initial denaturation at 98°C for 30 sec, followed by 30 cycles of denaturation at 98°C for 10 sec, annealing at 61°C for 20 sec, and extension at 72°C for 20 sec. Finish with a final extension of 10 min at 72°C.
3. Analyze 5 µl of the PCR product by standard agarose gel electrophoresis and ethidium bromide staining. The T7-promoter-flanked firefly luciferase and GFP PCR fragments should have lengths of 489 and 483 bp, respectively (*see* **Note 9**). The PCR products can be used directly in subsequent in vitro transcription reactions without purification.

3.3. In vitro transcription and dsRNA formation

1. Firefly luciferase and GFP dsRNA is generated by in vitro transcription reactions on their corresponding T7-promoter-flanked PCR fragments (*see* **Section 3.2.**). Since the PCR fragments contain a T7 promoter sequence at both the 5' and 3' end, both strands of the dsRNA duplex are generated in a single reaction. Set up 20-µl in vitro transcription reactions containing:
   a. 5 µl of T7-promoter-flanked PCR product
   b. 4 µl of T7 Transcription 5x Buffer
   c. 6 µl of rNTPs (25 mM each)
   d. 2 µl of T7 Enzyme Mix
   e. 3 µl of H_2O

   Mix by gently pipetting up and down.

2. Incubate at 37°C for 2-4 h.
3. Place the reaction mixture in a heating block pre-heated to 80°C and incubate for 10 min.
4. Switch off the heating block, but do not take out the reaction mixture.
5. Let the reaction mixture slowly cool to room temperature in the heating block to allow dsRNA formation.
6. Clean up the in vitro transcribed dsRNA using the GenElute Mammalian Total RNA Miniprep Kit according to the manufacturer's instructions (*see* **Note 10**).
7. Verify the integrity of the dsRNA by standard agarose gel electrophoresis and ethidium bromide staining, and determine its concentration and purity using a spectrophotometer.
8. Aliquot the purified dsRNA preparations and store at -80°C (*see* **Note 11**).

3.4. Transfection

1. Transfections are done in triplicate for the following experimental conditions:
    1) Non-specific dsRNA control (no VSR; GFP dsRNA)
    2) Specific dsRNA control (no VSR; firefly luciferase dsRNA)
    3) Positive VSR control (known VSR; firefly luciferase dsRNA)
    4) Test samples (each of the potential VSRs; firefly luciferase dsRNA)

    The non-specific and specific dsRNA controls are included to monitor the efficiency and specificity of the dsRNA-induced silencing of the firefly luciferase reporter, whereas the positive VSR control is incorporated to determine whether the sensitivity of the assay allows detection of VSR activity.
2. For transfection of S2R+ cells in a well of a 96 well plate, combine the following amounts of plasmid DNA (*see* **Notes 12** and **13**):
    a. 50 ng of one of the following plasmids:
        1) The empty plasmid (non-specific and specific dsRNA control)
        2) The plasmid that expresses a known VSR (positive VSR control)
        3) A plasmid that expresses a protein which is to be tested as a VSR (test sample)
    b. 12.5 ng of pMT-FLuc
    c. 3 ng of pMT-RLuc
3. Adjust the volume to a total of 30 µl with Buffer EC.
4. Add 0.8 µl of Enhancer to condensate the DNA. Mix by vortexing for 1 sec.
5. Incubate at room temperature for 2-5 min.
6. Briefly spin down.
7. Dilute the Effectene Transfection Reagent 8x in Buffer EC and add 2.5 µl of the dilution to the DNA-Enhancer mixture to create condensed Effectene-DNA complexes. Mix by vortexing for 10 sec.
8. Incubate at room temperature for 5-10 min.

9. Add the transfection mixture to the culture supernatant of the S2R+ cells. There is no need to refresh the medium prior to transfection.
10. Incubate the cells at 25°C for 48 h.

3.5. dsRNA feeding

1. Dilute the purified dsRNA preparations in culture medium to a final concentration of 20 ng/µl.
2. At 48 h after plasmid transfection, add 10 µl of the diluted dsRNA targeting either firefly luciferase (specific dsRNA control, positive VSR control and test samples) or GFP (non-specific dsRNA control) to the culture supernatant of the cells. There is no need to refresh the medium prior to dsRNA feeding (*see* **Note 14**).
3. Incubate the cells at 25°C for 7 h.

3.6. Induction of the reporters

1. Dilute the 50 mM $CuSO_4$ stock solution 10x in culture medium and induce expression of the firefly and *Renilla* luciferase reporters by adding 16 µl of the $CuSO_4$ dilution to the culture supernatant of the cells at 7 h after dsRNA feeding.
2. Incubate the cells at 25°C for 17 h.

3.7. DLR assays

1. At 17 h after induction of the reporters, completely remove the culture supernatant from the cells.
2. Add 100 µl of PBS to the cells.
3. Gently swirl the culture plate.
4. Completely remove the PBS from the cells.
5. Apply 100 µl of 1x PLB to the cells.
6. Place the culture plate on a rocking platform or orbital shaker and shake gently at room temperature for 15 min to ensure complete lysis of the cells (*see* **Note 15**).
7. Pre-dispense 25 µl of LAR II into the number of luminometer tubes required to complete the desired number of DLR assays.
8. Program a luminometer to perform a pre-measurement delay of 2 sec followed by a measurement period of 10 sec (*see* **Note 16**).
9. Transfer 10 µl of the cell lysate into a luminometer tube pre-dispensed with LAR II and mix by pipetting up and down several times (*see* **Note 17**). It is not necessary to clear the lysate of residual cell debris first.
10. Place the tube in the luminometer and measure the firefly luciferase reporter activity.
11. Remove the tube from the luminometer.

12. Add 25 µl of Stop & Glo Reagent and mix by vortexing briefly.
13. Place the tube in the luminometer and measure the *Renilla* luciferase reporter activity.
14. Discard the tube and proceed with the next DLR assay (*see* **Note 18**).

3.8. Presentation of the data

1. To normalize the data from the DLR assays, calculate the firefly/*Renilla* ratio for each sample.
2. Determine the mean firefly/*Renilla* ratio as well as the standard deviation of the triplicates for each experimental condition.
3. Present the data in diagrams similar to those shown in **Figure 2** (*see* **Note 19**). The controls provide important information regarding the quality of the

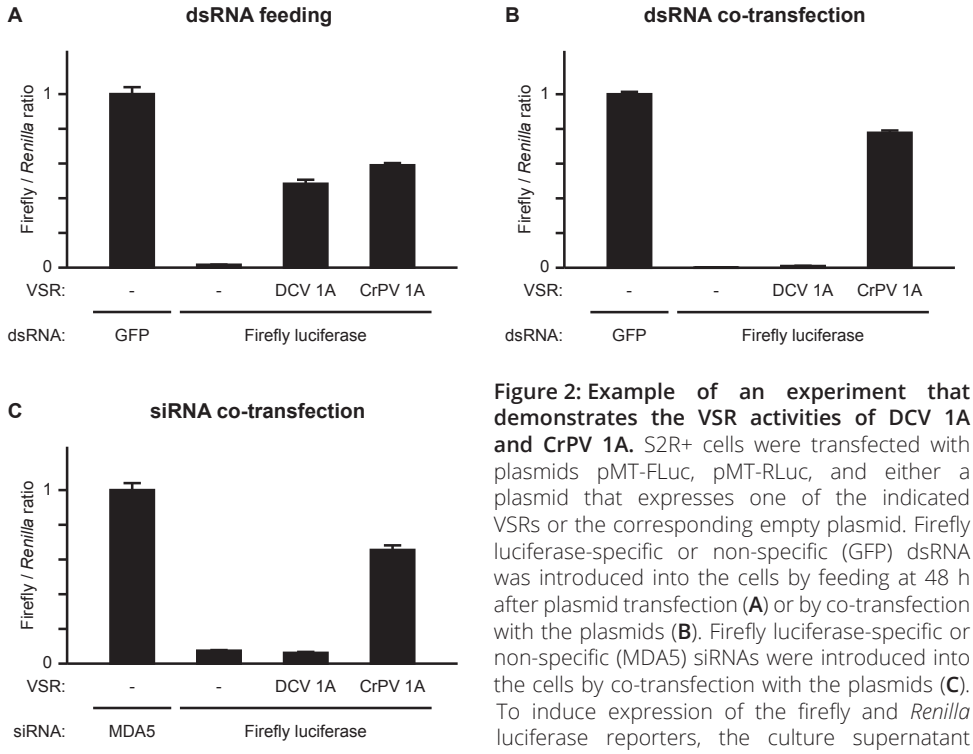

Figure 2: Example of an experiment that demonstrates the VSR activities of DCV 1A and CrPV 1A. S2R+ cells were transfected with plasmids pMT-FLuc, pMT-RLuc, and either a plasmid that expresses one of the indicated VSRs or the corresponding empty plasmid. Firefly luciferase-specific or non-specific (GFP) dsRNA was introduced into the cells by feeding at 48 h after plasmid transfection (**A**) or by co-transfection with the plasmids (**B**). Firefly luciferase-specific or non-specific (MDA5) siRNAs were introduced into the cells by co-transfection with the plasmids (**C**). To induce expression of the firefly and *Renilla* luciferase reporters, the culture supernatant was supplemented with $CuSO_4$ at 55 h after transfection. At 72 h after transfection, DLR assays were performed to determine the activity of the reporters. The firefly/*Renilla* ratios were calculated and the data were normalized to the non-specific dsRNA or siRNA controls. Error bars represent the standard deviations of three independent samples. DCV 1A interferes with RNAi at the level of Dicer and only suppresses RNAi if the luciferase reporter is silenced by dsRNA feeding after plasmid transfection. Notably, whereas DCV 1A inhibits Dicer-2 cleavage of dsRNA [5], the protein is unable to suppress RNAi after dsRNA transfection. Presumably, the co-transfected dsRNA is processed into siRNAs before the VSR is expressed at sufficient levels to suppress Dicer-2 cleavage. CrPV 1A, which interferes with RNAi at the level of RISC, is active in all variants of the RNAi reporter assay.

experiment. First, successful silencing of the firefly luciferase reporter should be evident from a lower firefly/*Renilla* ratio of the specific dsRNA control than of the non-specific dsRNA control (*see* **Notes 20** and **21**). Second, when compared to the specific dsRNA control, the positive VSR control must present a higher firefly/*Renilla* ratio (*see* **Notes 22** and **23**).

4. Notes

1. To silence the firefly luciferase reporter, we add dsRNA to the culture supernatant of S2R+ cells (dsRNA feeding, or soaking). The dsRNA is taken up by the cells and processed by the RNAi machinery [13]. The S2 cell line is highly heterogeneous in morphology, growth rate, and other characteristics. Be aware that, due to variable passage history and culture conditions, not all sub-lines of the S2 cell line possess the ability to efficiently take up dsRNA from the culture supernatant. When using an S2 cell line other than S2R+, make sure that the cells are capable to do so. Different S2 cell lines might require optimization of the protocol. When using S2 cells that do not take up dsRNA from the culture supernatant, you can consider transfection of the dsRNA into the cells.
2. Phusion DNA Polymerase works very well in our hands, but any thermostable DNA polymerase (such as Taq) can be used to amplify the templates for in vitro transcription. Keep in mind that the indicated cycling conditions are optimized for amplification with Phusion DNA Polymerase. Use of other polymerases may require optimization of the PCR reaction.
3. The RiboMAX Large Scale RNA Production System-T7 is specifically designed to produce large amounts of in vitro transcribed RNA. However, other T7 RNA polymerase-based in vitro transcription methods can also be used.
4. The GenElute Mammalian Total RNA Miniprep Kit is designed to isolate total RNA from mammalian cells and tissues, but the kit can also be used to clean up RNA. Although the kit is not optimized for dsRNA, we obtain good results using the RNA clean up procedure of the kit. When using other commercial kits or methods to clean up dsRNA, verify its functionality.
5. Plasmids pMT-FLuc and pMT-RLuc are derived from vector pMT/V5-His B (Invitrogen).
6. We generally express our proteins of interest from vector pAc5.1/V5-His (Invitrogen). The *Drosophila* actin 5C (Ac5) promoter in this vector allows high-level, constitutive expression in S2 cells. In addition, the vector contains a C-terminal V5 epitope and a polyhistidine (6xHis) tag which can be used to confirm expression of the protein.
7. As a positive control, you can include a known VSR in your experiment. Essentially

all established VSRs can be used as a positive control when the firefly luciferase reporter is silenced by dsRNA feeding two days after plasmid transfection. However, when the reporter is silenced by co-transfection of dsRNA or siRNAs with the plasmids, it is important to use a VSR that interferes with RNAi at steps downstream of Dicer (for example, CrPV 1A, *see* **Figure 2**).

8. Instructions for preparation and storage of 1x PLB, LAR II, and the Stop & Glo Reagent are described in the manufacturer's technical manual. It is important that all reagents and samples are at ambient temperature when performing the DLR assays, since the activity of the luciferase reporters is temperature sensitive.
9. The firefly luciferase and GFP PCRs should generate single T7-promoter-flanked fragments with the indicated sizes. If no products or non-specific products are observed on the agarose gel, optimization of the PCR reaction may be required. Alternatively, it might be necessary to purify the correct fragment from gel before continuing with in vitro transcription.
10. It is not necessary to remove the DNA template by digestion with DNase.
11. Avoid multiple freeze-thaw cycles and keep the RNA on ice whenever it is thawed for use.
12. Since the experiments are performed in triplicate, it is convenient to prepare a master mix for each experimental condition.
13. Instead of inducing RNAi by dsRNA feeding at 48 h after plasmid transfection, the firefly luciferase reporter can be silenced by co-transfection of dsRNA or siRNAs with the plasmids. For dsRNA co-transfection, add 10 ng of either firefly luciferase (specific dsRNA control, positive VSR control and test samples) or GFP (non-specific dsRNA control) dsRNA to the mixture of plasmids during the transfection procedure. For siRNA co-transfection, add 2 µl of a 1 µM stock solution to the mixture of plasmids during the transfection procedure. We purchase our firefly luciferase-specific and non-specific control (MDA5) siRNAs from Dharmacon (Lafayette, CO). When performing the dsRNA or siRNA co-transfection variants of the assay, omit the dsRNA feeding step (*see* **Section 3.5.**).
14. Some researchers use FCS-free culture medium during dsRNA feeding. In our experiments, we do not observe any difference in the efficiency of dsRNA-mediated silencing when feeding is performed in either the presence or absence of FCS.
15. The cell lysates can be stored at -20°C for up to one month if you wish to continue with the DLR assays later. For long-term storage, the lysates should be stored at -80°C. Prevent multiple freeze-thaw cycles, since this can cause gradual loss of reporter activity.
16. Single-sample, multiple-sample, and plate-reading luminometers can be used

to perform the DLR assays. It is recommended that multiple-sample and plate-reading luminometers are equipped with reagent injectors. This is not required for single-sample luminometers.

17. It is important not to mix by vortexing, but by pipetting up and down. Vortexing can create a microfilm of the luminescent solution along the sides of the tube which can escape mixing with the Stop & Glo Reagent in subsequent steps.
18. It is more convenient to first measure the firefly luciferase activities in all the samples, before measuring the *Renilla* luciferase activities.
19. As an alternative, you can present the data as fold silencing relative to the non-specific dsRNA control. To calculate the fold silencing for a specific experimental condition, divide the mean firefly/*Renilla* ratio of the non-specific dsRNA control by that of the experimental condition.
20. If you do not observe silencing in the specific dsRNA control, make sure that the dsRNA preparations are of sufficient quality (*see* **Section 3.3.**). It is important to work under RNase-free conditions to prevent degradation of the RNA.
21. Low absolute firefly and *Renilla* luciferase counts may indicate a low transfection efficiency and may require optimization of the assay.
22. If you do not observe suppression of RNAi by either the positive VSR control or the test samples, confirm their expression (for example, by Western blot analysis).
23. When interpreting data from the RNAi reporter assay, it is important to realize that virtually any dsRNA-binding protein can suppress RNAi when over-expressed [14]. Where possible, confirm the activity of an identified VSR in cells infected with the virus carrying the VSR.

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Chapter 3

Convergent evolution of Argonaute-2 Slicer antagonism in two distinct insect RNA viruses

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Abstract

RNA interference (RNAi) is a major antiviral pathway that shapes evolution of RNA viruses. We show here that Nora virus, a natural *Drosophila* pathogen, is both a target and suppressor of RNAi. We detected viral small RNAs with a signature of *Dicer-2* dependent small interfering RNAs in Nora virus infected *Drosophila*. Furthermore, we demonstrate that the Nora virus VP1 protein contains RNAi suppressive activity *in vitro* and *in vivo* that enhances pathogenicity of recombinant Sindbis virus in an RNAi dependent manner. Nora virus VP1 and the viral suppressor of RNAi of Cricket paralysis virus (1A) antagonized Argonaute-2 (AGO2) Slicer activity of RNA-induced silencing complexes pre-loaded with a methylated single-stranded guide strand. The convergent evolution of AGO2 suppression in two unrelated insect RNA viruses highlights the importance of AGO2 in antiviral defense.

Author summary

Multi-cellular organisms require a potent immune response to ensure survival under the ongoing assault by microbial pathogens. Co-evolution of virus and host shapes the genome of both pathogen and host. Using *Drosophila melanogaster* as a model, we study virus-host interactions in infections by Nora virus, a non-lethal natural pathogen of fruit flies. Insects depend on the RNA interference (RNAi) pathway for antiviral defense. A hallmark of the antiviral RNAi response is the production of viral small RNAs during infection. We detected Nora virus small RNAs during infection of *Drosophila*, demonstrating that Nora virus is a target of the antiviral RNAi pathway. Furthermore, we show that Nora virus viral protein 1 (VP1) inhibits the catalytic activity of Argonaute-2, a key protein of the RNAi pathway. The 1A protein of Cricket paralysis virus suppresses RNAi via a similar mechanism. Importantly, whereas Nora virus persistently infects *Drosophila*, Cricket paralysis virus induces a lethal infection. Our findings thus indicate that two distantly related viruses independently evolved an RNAi suppressor protein that targets the Argonaute-2 protein. Altogether, our results emphasize the critical role of Argonaute-2 in insect antiviral defense, both in lethal and persistent infections.

Introduction

An efficient antiviral immune response is essential for the control or elimination of virus infection and for survival of the infected host. The immune system exerts a strong evolutionary pressure that shapes the genetic makeup of viral pathogens. Indeed, viruses evolved counter-defense mechanisms to evade, suppress or inactivate host immunity. Studying these mechanisms provides important insight in the critical steps of antiviral responses and may uncover novel components and regulators of immune pathways.

Plants, fungi, and invertebrate animals rely on the RNA interference (RNAi) pathway for antiviral defense [1,2]. The initial trigger of an antiviral RNAi response is the recognition and cleavage of viral double-stranded RNA (dsRNA) into viral small interfering RNAs (vsiRNAs), in insects by the ribonuclease Dicer-2 (Dcr-2). These vsiRNAs act as specificity determinants of the Argonaute-2 (AGO2) containing effector nuclease complex RISC (RNA-induced silencing complex). RISC maturation involves a number of sequential steps: loading of the vsiRNA into AGO2, cleavage and elimination of the passenger RNA strand, and 2'-O-methylation of the 3'-terminal nucleotide of the retained guide strand. It is thought that vsiRNA-loaded RISC subsequently cleaves viral target RNA (Slicer activity). The hypersensitivity to viral infections of *AGO2* mutant flies and of *AGO2* knockdown mosquitoes provides genetic support for this hypothesis [3-7]. Nevertheless, direct evidence supporting this model, for example by the detection of viral Slicer products, is lacking.

The evolution of viral suppressors of RNAi (VSRs) is a testament to the antiviral potential of the RNAi pathway in plants and insects. Given the central role of dsRNA and siRNAs as initiators and specificity determinants of the RNAi pathway, it is not surprising that many VSRs sequester dsRNA. For instance, the Drosophila C virus (DCV) 1A protein binds long dsRNA and shields it from processing by Dcr-2 [6]. Flock House virus (FHV) B2 displays a dual RNA binding activity: it binds long dsRNA as well as siRNAs, thereby preventing their incorporation into RISC [8-10]. Similarly, many plant VSRs display dsRNA binding activities, leading to the hypothesis that dsRNA or siRNA binding is a general mechanism for RNAi suppression [11,12]. Nevertheless, other mechanisms have been reported [1]. The RNAi suppressive activity of the Cricket paralysis virus (CrPV) 1A protein, for example, relies on a direct interaction with AGO2 [13].

VSRs have been identified in dozens of plant viruses from all major virus families [1]. In contrast, VSRs have thus far been identified in only three insect RNA viruses (FHV, CrPV, and DCV). These VSRs were characterized using genetic and biochemical approaches in the model organism *Drosophila melanogaster*. While these viruses indeed efficiently infect *Drosophila* laboratory stocks and cell lines, DCV is the only natural *Drosophila* pathogen among these three viruses [14,15]. Although FHV and

CrPV have a remarkable broad host range in the laboratory, they were originally isolated from non-Drosophilid host species: the New Zealand grass grub (*Costelytra zealandica*) and field crickets (*Teleogryllus oceanicus* and *T. commodus*), respectively [16-19].

Since viral counter-defense mechanisms co-evolve with the antiviral immune responses of the host species, it is essential to characterize a VSR within the correct evolutionary context. We therefore set out to identify an RNAi suppressor in Nora virus, a positive sense (+) RNA virus that persistently infects *Drosophila* laboratory stocks as well as *Drosophila* in the wild [20] (D.J. Obbard, personal communication). The genome organization and phylogeny suggest that Nora virus is the type member of a novel virus family within the order of *Picornavirales* [20]. Here we show that Nora virus VP1, the protein product of open reading frame 1 (ORF1), suppresses RNAi in cell culture as well as in flies. In accordance, VP1 is an RNAi-dependent viral pathogenicity factor. In a series of biochemical assays, we show that both Nora virus VP1 as well as CrPV 1A inhibit Slicer activity of a pre-assembled RISC loaded with a methylated guide strand. The lack of amino acid sequence similarity between CrPV 1A and Nora virus VP1 suggests that their Slicer antagonistic activities resulted from convergent evolution, providing direct support for the critical role of AGO2 Slicer activity in antiviral defense.

Results

Nora virus is a target of RNAi *in vivo*

Nora virus is an enteric (+) RNA virus that successfully establishes a persistent infection in flies [20]. The mechanism by which this virus establishes persistent infections is unknown. To determine whether Nora virus is a target for Dcr-2, we analyzed the presence of Nora virus small RNAs in the w^{1118} *Drosophila* strain that is widely used as a recipient strain for transgenesis. We isolated and sequenced 19-29 nt small RNAs from body (abdomen and thorax), thorax and head of adult w^{1118} flies. Sequence reads that perfectly matched the *Drosophila* genome were annotated and discarded. Of the remaining reads, 396.646 (7,8%, body), 237.265 (10,6%, thorax), and 1.099.496 (7,7%, head) matched the published Nora virus sequence (NC_007919.3), indicating that the w^{1118} strain was infected by Nora virus (Table 1). As RNA viruses rapidly evolve, viral small RNA sequences may have been missed in this initial matching step. We therefore reconstituted the Nora virus genome through an iterative alignment/consensus treatment of the viral small RNA sequences in our libraries [21]. The reconstituted Nora virus genome (rNora virus) differed at only 3.2% of the nucleotides from the published genome sequence. Aligning small RNAs to the rNora virus genome instead of the published Nora virus sequence resulted

Table 1: Annotation of small RNA sequences in libraries from body (abdomen and thorax), thorax, and head of Nora virus infected w^{1118} adult flies.

	Body	Thorax	Head
Total library	18.296.275	17.280.520	49.633.458
Match to D. melanogaster*	13.184.119	15.033.831	35.435.546
Unmatched*	5.112.156	2.246.689	14.197.912
Nora virus (NC_007919.3)*	396.646	237.265	1.099.496
Nora virus (reconstituted)*	479.572	291.045	1.329.336

*The number of reads matching the *Drosophila* genome, reads that fail to map to the *Drosophila* genome (unmatched), and reads mapping to the Nora virus genome (isolate Umea 2007) and the reconstituted Nora virus genome are indicated for each library.

in an increased number of viral reads in the three libraries (~121%, Table 1). We therefore used the reconstituted genome as a reference genome in further analyses. In all three libraries, Nora virus-derived small RNAs were predominantly 21-nt long, the typical size of Dicer-2 products. The size distribution of small RNAs derived from the (+) RNA strand, however, were noticeably wider than those derived from the (-) RNA strand (Figure 1A). For 21-nt viral RNA reads, there was only a slight bias towards (+) small RNAs (ratio (+) RNA / total RNA ~0.58), whereas small RNAs of other sizes were predominantly derived from the (+) strand (Figure 1B). In all three libraries, the 21-nt Nora virus-derived RNAs are distributed across the genome, covering both the (+) and (-) viral RNA strands with approximately equal numbers (Figure 1C). These data suggest that dsRNA replication intermediates of Nora virus are processed into 21-nt long siRNAs. The origin of the other size classes of viral small RNAs remains unclear. However, as the predominance of (+) over (–) small RNA reads is reminiscent of the excess of (+) over (-) viral (full-length) RNA that is typically observed in (+) RNA virus infection, they may be due to non-specific RNA degradation.

Drosophila Dcr-2 generates 21-nt duplex siRNAs in which 19 nucleotides are base-paired leaving a 2-nt 3' overhang at each end. For each library, we collected the 21-nt RNA reads whose 5' ends overlapped with another 21-nt RNA read on the opposite strand of the Nora virus genome. Then, for each possible overlap of 1 to 21-nt, the numbers of read pairs were counted and converted into Z-scores (Figure 1D). This analysis revealed that 21-nt Nora virus-derived RNAs in body and thorax libraries tend to overlap by 19-nt, which is a typical feature of siRNA duplex precursors. This siRNA duplex signature was observed to a lesser extent in head libraries. Very little Nora virus RNA can be detected in the head [22], yet vsiRNA levels were similar in head, thorax, and body (Table 1). The origin of the vsiRNAs in the head and the reason for the less pronounced vsiRNA signature of those small RNAs remain unclear. Altogether, our results strongly suggest that Nora virus double-stranded replication intermediates are processed by Dcr-2 into vsiRNAs that trigger an RNAi response in infected flies.

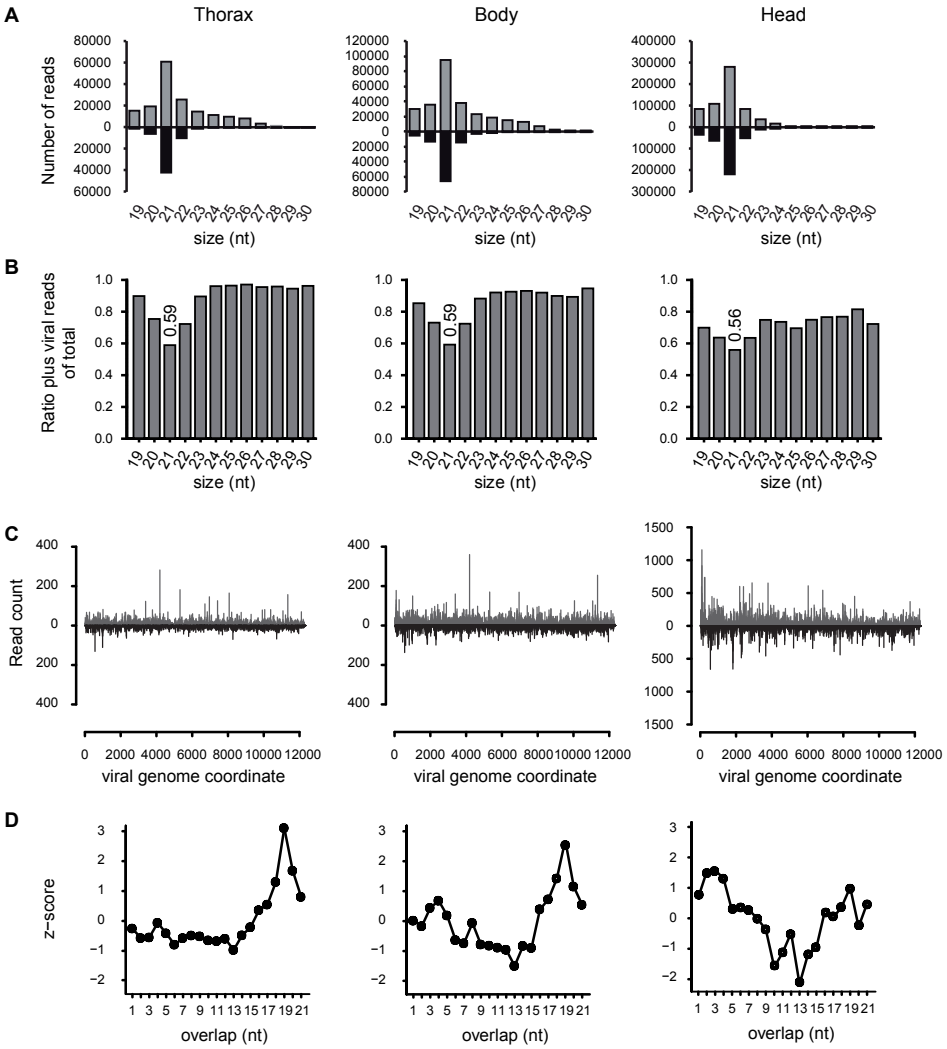


Figure 1: Nora virus is targeted by RNAi in adult flies. (**A**) Size distribution of Nora virus-derived small RNAs in libraries from thoraxes, bodies and heads of w^{1118} flies. Read counts of small RNAs matching the (+) and (-) viral RNA strands are in gray and black, respectively. (**B**) Proportion of (+) Nora virus small RNA reads of total viral reads. Frequencies were computed from the distributions in panel A for each size class. (**C**) Viral siRNA distribution across the viral genome. The abundance of 21-nt small RNAs matching the (+) and (-) viral RNA strands of the reconstituted Nora virus (rNora) reference genome is shown in gray and black, respectively. (**D**) Z-scores for the number of overlapping pairs of sense and antisense 21-nt Nora virus small RNAs matching the rNora virus reference genome. For each possible overlap of 1 to 21-nt, the number of read pairs was counted and converted into a Z-score.

Nora virus VP1 suppresses RNAi *in vitro*

Our small RNA profiles indicate that Nora virus is targeted by Dcr-2. Nevertheless, the virus efficiently establishes a persistent infection, suggesting that it is able to

evade or suppress the antiviral RNAi response. The Nora virus genome contains four open reading frames (ORFs) (Figure 2A). Nora virus ORF2 is predicted to encode the helicase, protease, and polymerase domains that together form a picornavirus-like replication cassette. ORF4 encodes three proteins that make up the Nora virus capsid (VP4A, VP4B, and VP4C) [23]. To determine whether the Nora virus genome encodes an RNAi suppressor, we analyzed the four ORFs in an RNAi sensor assay in *Drosophila* cell culture (Figure 2B-2D). In this assay, S2 cells are transfected with firefly (FLuc) and *Renilla* luciferase (RLuc) reporter plasmids and a plasmid that expresses one of the four viral ORFs. Subsequently, FLuc expression is silenced using specific dsRNA, and FLuc and RLuc activity is monitored. As expected, DCV 1A, a well characterized VSR that binds long dsRNA, efficiently suppressed RNAi, whereas the inactive DCV 1A K73A mutant was unable to do so (Figure 2C and [6]). Cotransfection of the ORF1 expression plasmid also resulted in de-repression of FLuc, suggesting that VP1, the protein product of ORF1, is a suppressor of RNAi. Expression of ORF3 and ORF4 did not affect FLuc activity (Figure 2C). However, since expression of ORF2 and the production of mature capsid proteins from ORF4 were not detectable on

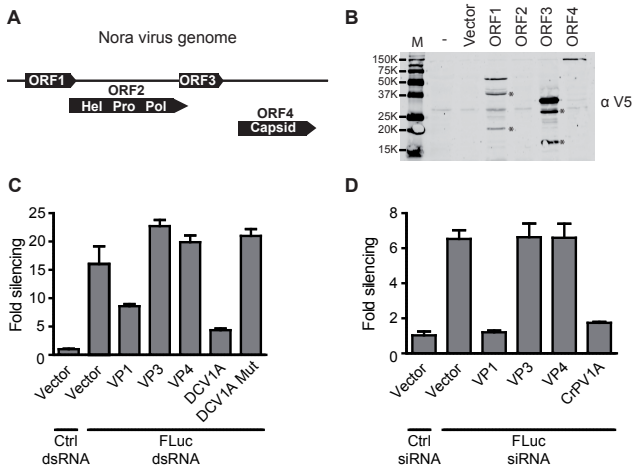


Figure 2: Nora virus VP1 suppresses RNAi *in vitro* (**A**) Schematic representation of the Nora virus genome with its four predicted ORFs in three different reading frames. There is a 7-nt overlap between ORF1 and ORF2 and a 26-nt overlap between ORF2 with ORF3. An intergenic region of 85 nt separates ORF3 and ORF4. (**B**) Western blot analysis of V5-epitope tagged Nora virus expression constructs. Two days after transfection of the indicated plasmids into S2 cells, expression of the constructs was analyzed by Western blot using the V5 antibody (αV5). Asterisks (*) indicate additional bands that do not correspond to the expected size of the full-length protein product. (**C**) RNAi reporter assay in *Drosophila* S2 cells. Copper-inducible plasmids encoding FLuc and RLuc were transfected into S2 cells together with a construct expressing Nora virus ORF1, 3, and 4, encoding viral protein 1 (VP1), VP3, and VP4, respectively. Two days after transfection, dsRNA targeting FLuc or GFP (Ctrl) was added to the medium. Seven hours later, expression of FLuc and RLuc was induced and luciferase activity was measured the next day. FLuc counts were normalized to RLuc counts and presented as fold silencing relative to the control GFP dsRNA. Plasmids encoding DCV 1A and the K73A mutant (DCV 1A mut) were used as controls. (**D**) siRNA-based RNAi reporter assay. The experiment was performed as described in panel C, but 21-nt FLuc siRNAs were cotransfected with the reporter plasmids to silence gene expression. An siRNA targeting the human MDA5 gene was used as a non-silencing control (Ctrl). Bars in panel C represent averages and standard deviations of five independent samples; bars in panel D represent averages and standard deviations of three independent samples. Panel C and D are representative for two and three independent experiments, respectively.

western blot, we cannot exclude the possibility that these protein products are able to suppress RNAi as well (Figure 2B).

Next, we tested whether VP1 inhibits the production of siRNAs by Dcr-2 or a subsequent step in the RNAi pathway. To this end, we repeated the RNAi sensor assay using a synthetic siRNA that does not require Dcr-2 cleavage for its silencing activity. Also under these conditions, Nora virus VP1 suppressed silencing of the FLuc reporter. Furthermore, VP1 suppressed RNAi to a similar extent as CrPV 1A, which was previously shown to suppress the effector stage of the RNAi machinery [13] (Figure 2D).

In *Drosophila*, the microRNA (miRNA) and siRNA pathways are separate processes, with Dcr-1 and AGO1 dedicated to the miRNA pathway and Dcr-2 and AGO2 to the siRNA pathway. Nevertheless, crosstalk between the miRNA and RNAi pathways occurs. Using miRNA sensor assays in S2 cells, in which FLuc expression is silenced by endogenous miRNAs or co-expressed primary miRNAs, we observed that VP1 does not suppress miRNA activity (Text S1 and Figure S1). Together, these data indicate that VP1 is able to suppress the RNAi, but not the miRNA pathway, at a step after dsRNA processing by Dcr-2.

The C-terminus of VP1 is essential for its suppressor activity

VP1 is highly conserved among different Nora virus isolates (Figure S2). We were unable to predict a protein domain in VP1 suggestive of a mechanism of action. Furthermore, we did not obtain a significant alignment to any other protein from

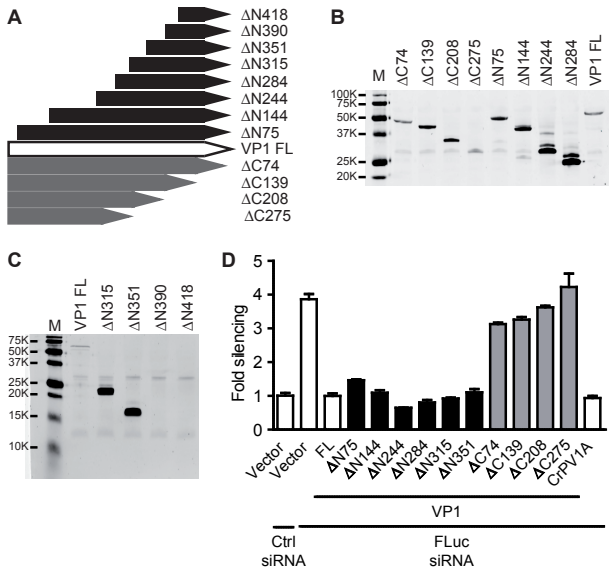


Figure 3: The C-terminus of Nora virus VP1 is essential for RNAi suppressor activity (**A**) Schematic presentation of full-length (FL) and N- and C-terminal deletion mutants (ΔN and ΔC) of VP1. (**B,C**) Western blot analysis of VP1 expression constructs. V5 epitope tagged expression constructs were transfected into *Drosophila* S2 cells and expression of $VP1^{FL}$ and the deletion mutants was analyzed by Western blot using a V5 antibody (αV5). (**D**) RNAi reporter assay in S2 cells. The experiment was performed as described in the legend to Figure 2D, using plasmids encoding either CrPV 1A, $VP1^{FL}$ or the VP1 deletion mutants. Bars represent averages and standard deviations of three independent samples. The graph is representative for two independent experiments.

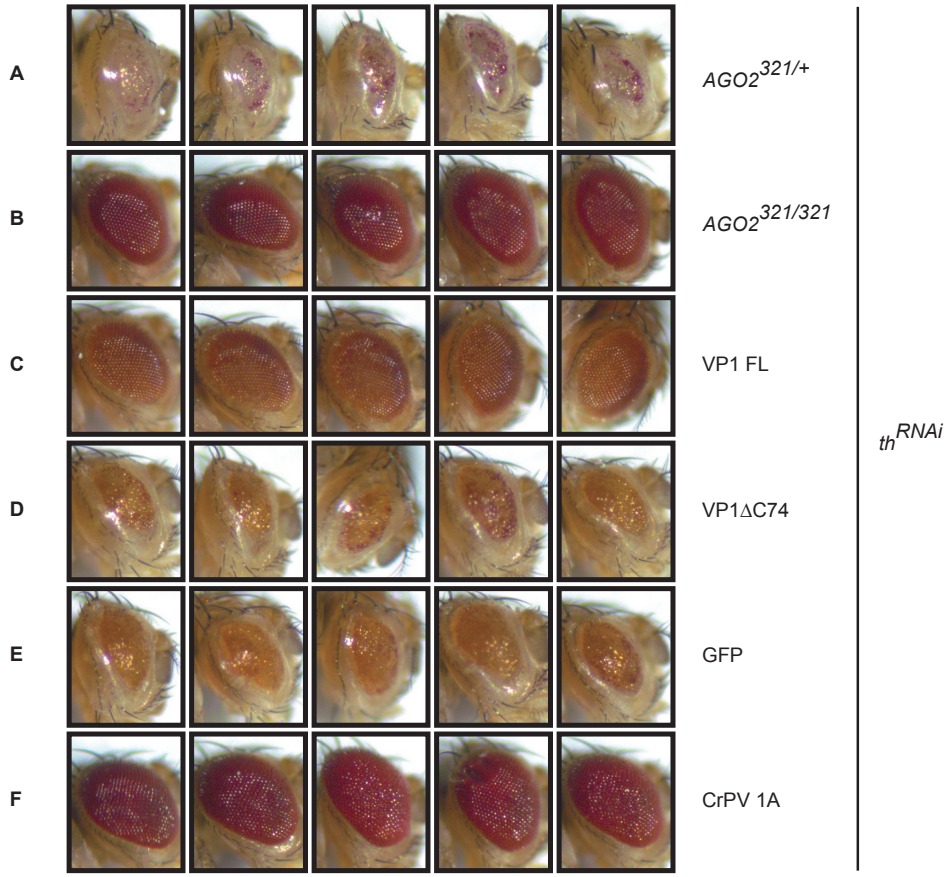


Figure 4: VP1 suppresses RNAi *in vivo*. (**A-F**) RNAi of *Drosophila Inhibitor of Apoptosis1 / thread* (*th*) in the eye of adult flies in the indicated genetic background or in the presence of several transgene constructs. RNAi-mediated knockdown of *th* results in a reduced size and pigmentation of the eye and roughening of the eye surface in $AGO2^{321}$ heterozygotes (**A**), but not in $AGO2^{321}$ homozygotes (**B**). Eye phenotype of transgenic flies co-expressing the th^{RNAi} construct and Nora virus full-length VP1 (VP1 FL, **C**), a C-terminal deletion mutant of VP1 ($VP1^{\Delta C74}$, **D**), GFP (**E**) or CrPV 1A (**F**). Maximum silencing of *th* was examined in the presence of the GFP control transgene (**E**). For each line, five representative pictures of eyes of two- to four-day-old male flies are presented. Pictures are representative for three independent experiments.

the non-redundant protein sequence database. To map the VSR region of VP1, we generated a series of N- and C-terminal (ΔN and ΔC) truncations and tested them in the RNAi reporter assay in S2 cells (Figures 3A and S3). With the exception of the $VP1^{\Delta N390}$ and $VP1^{\Delta N418}$ mutants, in which no protein could be detected on Western blot, all $VP1^{\Delta N}$ and $VP1^{\Delta C}$ constructs produced proteins of the expected size (Figure 3B, C). Deletion of 74 amino acids (aa) or more from the C-terminus of VP1 resulted in loss of suppressor activity (Figure 3D). This suggests that the active domain of VP1 resides in its C-terminal region. Indeed, deleting up to 351 aa from the N-terminus ($VP1^{\Delta N351}$), out of a total of 475 aa, did not affect VSR activity. These results show that

the RNAi suppressor activity of VP1 maps to the C-terminal 124 aa.

VP1 is an RNAi suppressor *in vivo*

We next evaluated the VSR activity of Nora virus VP1 *in vivo* using transgenic flies in which *thread* (*th*), also known as *Drosophila inhibitor of apoptosis 1*, can be silenced by expression of dsRNA targeting this gene (th^{RNAi} [24,25]) (Figure 4). Eye-specific expression of th^{RNAi} using the GMR-GAL4 driver leads to severe apoptosis in the developing eye. As a consequence, th^{RNAi} flies display a reduced eye size, loss of eye pigmentation, and roughening of the eye surface (Figure 4A, results are shown for $AGO2^{321}$ heterozygotes; th^{RNAi} in a wildtype background shows the same phenotype, data not shown and [25]). Silencing of *th* in the eye of th^{RNAi} flies is fully dependent on the RNAi pathway, since the phenotype is lost in an *AGO2* null mutant background (Figure 4B). These results indicate that the th^{RNAi} sensor fly is a robust system to monitor RNAi activity *in vivo*.

Consistent with its RNAi suppressive activity in cell culture, expression of full-length VP1 ($VP1^{FL}$) in th^{RNAi} flies resulted in eyes with a normal size and a rescue of the rough eye phenotype (Figure 4C). The phenotype of th^{RNAi} flies expressing the $VP1^{\Delta C74}$ mutant was similar to that of flies expressing GFP as a negative control, confirming that this mutant is functionally inactive (Figure 4D, E). Notably, while VP1 only partially rescued the RNAi-dependent phenotype, CrPV 1A fully reverted the th^{RNAi}-induced phenotype (Figure 4F). Whether this difference is due to a more robust RNAi suppressive activity of CrPV 1A or to a difference in expression level remains to be established.

VP1 enhances viral pathogenicity *in vivo*

Having established that VP1 displays RNAi suppressive activity *in vitro* and *in vivo*, we next analyzed the effect of VP1 on viral pathogenicity in adult flies. To this end, we generated recombinant Sindbis virus (SINV) expressing the functional $VP1^{\Delta N351}$ (SINV-VP1) or GFP (SINV-GFP) from a second subgenomic promoter (Figure 5A). Although arboviruses are a target of the RNAi pathway during infection in insects [3,5,26], we and others have not detected VSR activity in infections with SINV and the related alphavirus Semliki Forest virus [27,28] (data not shown). Indeed, SINV recombinants expressing the viral RNAi suppressors FHV B2 and CrPV 1A were significantly more pathogenic than their controls in mosquitoes and *Drosophila*, respectively [13,28].

We injected wildtype w^{1118} flies with the SINV recombinants and monitored survival over time. SINV-GFP (and the parental SINV virus, data not shown) induced only modest mortality in these flies with a fully functional RNAi response. After 36 days of infection, 73% of the SINV-GFP infected flies and all mock infected flies were still alive. In contrast, SINV-VP1 infection resulted in more severe mortality. SINV-VP1 infected flies died faster and only 9% of the flies survived the 36-days follow up period (Figure 5B). Although these results indicate that VP1 enhances viral pathogenicity,

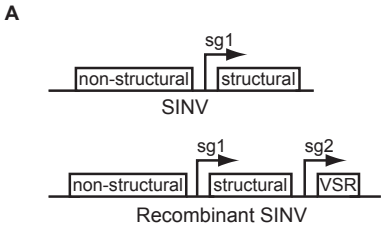
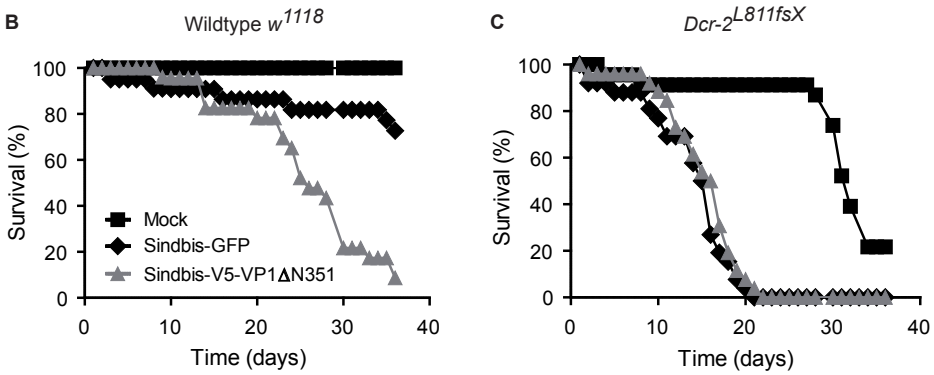


Figure 5: VP1 enhances viral pathogenicity via its RNAi suppressive activity. (**A**) Schematic representation of Sindbis virus (SINV) and SINV recombinant containing a duplicated subgenomic promoter (sg1 and sg2) driving expression of a viral suppressor of RNAi (VSR). (B and C) Survival curves of *w*^{1118} wildtype flies (**B**) and *Dcr-2*^{L811fsX} mutants (**C**) infected with SINV recombinants expressing either GFP (black diamond) or VP1$^{\Delta N351}$ (gray triangle), or mock infected (black square). Survival curves are representative of two independent experiments.



they fail to show that this effect depends on its VSR activity. Viral proteins are often multifunctional and the effect of VP1 on the course of infection might be attributed to another, as yet unknown, activity of VP1. We therefore performed recombinant SINV infections in RNAi deficient *Dcr-2* mutant flies. In this genetic background, an RNAi suppressor is not expected to enhance pathogenicity of the virus. Upon infection with SINV-GFP, the *Dcr-2* mutants died much faster than wild-type flies, confirming that SINV is indeed a target of the RNAi pathway. In contrast to infections in RNAi competent flies, the course of infection of SINV-VP1 and SINV-GFP was remarkably similar in *Dcr-2* mutants, with 100% mortality at 22 days after infection in both cases (Figure 5C). We therefore conclude that VP1 enhances virulence of an RNA virus *in vivo* through its RNAi suppressive activity.

Nora virus VP1 interferes with the effector phase of RNAi

To further characterize the VSR activity of Nora virus VP1, we next analyzed the activity of VP1 in a series of biochemical assays that monitor individual steps of the RNAi pathway. To this end, we fused the active VP1$^{\Delta N284}$ mutant to the maltose binding protein (MBP-VP1) and purified it from *Escherichia coli*. We verified that MBP-VP1 fusion proteins are fully functional in VSR assays in S2 cells to exclude the possibility that MBP interferes with VP1 VSR activity (data not shown).

The ability of VP1 to suppress siRNA-initiated RNAi in S2 cells (Figure 2D) suggests

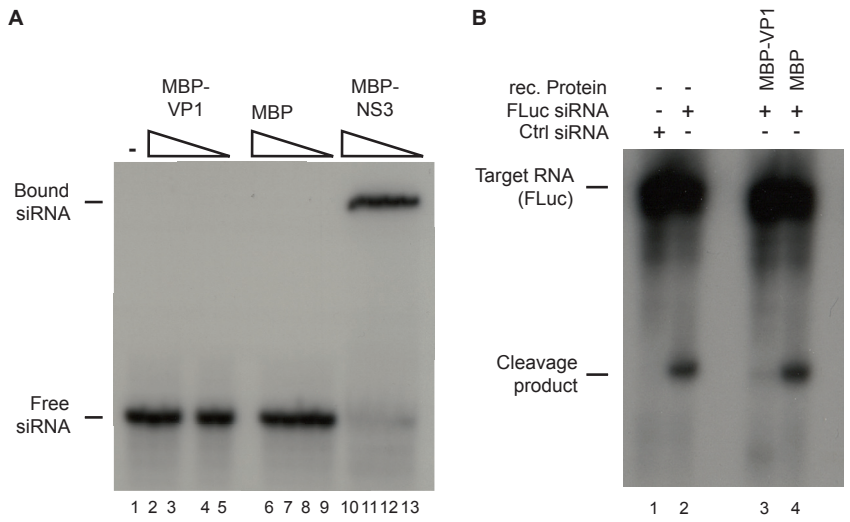


Figure 6: (**A**) Mobility shift assays for binding of viral RNAi suppressor proteins to siRNAs. Radiolabeled siRNAs were incubated in buffer (lane 1) or with decreasing amounts of recombinant MBP-VP1$^{\Delta N284}$ (lanes 2-5), MBP (lanes 6-9), and MBP-NS3 (lane 10-13). Ten-fold dilutions were used, starting at 2 µM for MBP-VP1$^{\Delta N284}$ (lane 2) and 2.6 µM for MBP (lane 6). MBP-NS3 was tested in two-fold dilutions (highest concentration of 8 µM, lane 10). RNA mobility shifts were analyzed on an 8% native polyacrylamide gel. (**B**) RISC Slicer assay in *Drosophila* embryo lysate. Lysates were incubated with non-targeting control siRNA (Ctrl, lane 1) or with FLuc siRNA (lanes 2-4) in the absence (lane 2) or presence of recombinant MBP-VP1$^{\Delta N284}$ (lane 3) or MBP (lane 4). RISC cleavage products were analyzed on an 8 % denaturing polyacrylamide gel. Slicer assay is representative for two independent experiments.

that VP1 inhibits a step downstream of siRNA production by Dcr-2. In accordance, recombinant VP1 was unable to bind long dsRNA in gel mobility shift assays and could not interfere with Dcr-2 mediated processing of long dsRNA into siRNAs in S2 cell extract (Figure S4A, B). We next analyzed whether VP1 is able to bind siRNAs in a gel mobility shift assay. As a positive control, we used a fusion protein of MBP and the Rice hoja blanca virus non-structural protein 3 (NS3), which binds duplex siRNAs with high affinity [29]. Whereas NS3 efficiently bound siRNAs in our assays, we were unable to observe a shift in mobility of siRNAs after incubation with VP1, even at the highest concentrations used (Figure 6A).

Since VP1 is incapable of interfering with the initiator phase of the RNAi pathway, we next examined the effect of VP1 on the effector phase of RNAi. For this purpose, we used an *in vitro* RNA cleavage assay (Slicer assay) in *Drosophila* embryo extract [30], in which a sequence-specific siRNA triggers cleavage of a target RNA. Since the 5′ cap of the target RNA is radioactively labeled, the 5′ cleavage product can be visualized by autoradiography after separation on a denaturing polyacrylamide gel. Indeed, a cleavage product of the expected size was detected if embryo extract was incubated with a target RNA and a specific siRNA. Specific cleavage products were not generated in the presence of a non-specific control siRNA (Figure 6B, lanes 1

and 2). Recombinant VP1 protein, but not control MBP protein, efficiently inhibited the production of cleavage product (Figure 6B, lanes 3 and 4). We note, however, that a minor fraction of the target RNA is still cleaved in the presence of VP1 (Figure 6B, lane 3). Together, these experiments show that VP1 does not affect the initiator phase of the RNAi pathway, but interferes with RISC activity.

Nora virus VP1 inhibits RISC activity of pre-assembled mature RISC

To discriminate between RISC assembly and target RNA cleavage by a pre-assembled RISC complex, we performed Slicer assays under two experimental conditions (Figure 7A). In the first approach, a purified suppressor protein is added 30 minutes before the siRNA, which allows us to analyze the effect of the VSR on both RISC loading and target cleavage. In the second approach, the embryo extract is incubated with siRNAs for 30 minutes before addition of recombinant protein. This second protocol allows a mature RISC to form prior to the addition of a VSR, thereby allowing us to assess the effect of the VSR on slicing only. As CrPV 1A was previously shown to affect the effector phase of the RNAi pathway [13], we generated recombinant GST-CrPV 1A as well as control GST. These proteins were included in our assays.

Using the first protocol, cleavage of the target RNA was suppressed by VP1 (Figure 7B, lane 3). Strikingly, VP1 was also able to inhibit target cleavage when added to an embryo lysate containing pre-loaded RISC (Figure 7B, lane 7). The observed suppression of slicing was VP1 specific, since MBP alone did not inhibit RNA cleavage (lane 4 and 8). Recombinant CrPV 1A also suppressed slicing in both experimental procedures (Figure 7B, lanes 5 and 9).

To determine if VP1 affects the protein stability of AGO2, we incubated the recombinant proteins in *Drosophila* embryo extract and analyzed endogenous AGO2 protein levels by Western blot. Neither VP1 nor CrPV 1A affected AGO2 protein levels in embryo lysate, indicating that these two proteins do not mediate RNAi suppression through degradation of AGO2 (Figure 7C).

To further confirm the inhibitory effect of VP1 on Slicer activity rather than RISC assembly, we performed Slicer assays using different siRNA guides. During RISC maturation, guide strands in AGO2 are 2'-O-methylated at their 3' terminal nucleotide by the *Drosophila* methyltransferase Hen1 [31]. This modification protects AGO2 associated siRNAs from degradation by trimming and tailing events that occur when there is extensive base-pairing of the guide RNA with a target RNA [32]. To overcome a requirement for Hen1, an siRNA bearing a 2'-O-methylated 3'-terminal nucleotide on the guide strand was used in Slicer assays. Similar to the non-methylated siRNA, the methylated siRNA produced a specific cleavage product of the expected size (Figure 7D, lane 2). Both Nora virus VP1 and CrPV 1A inhibited the cleavage activity of RISC that was pre-loaded with the methylated siRNA (Figure 7D, lane 3 and 5). Again, the GST and MBP control proteins were unable to affect Slicer activity (Figure

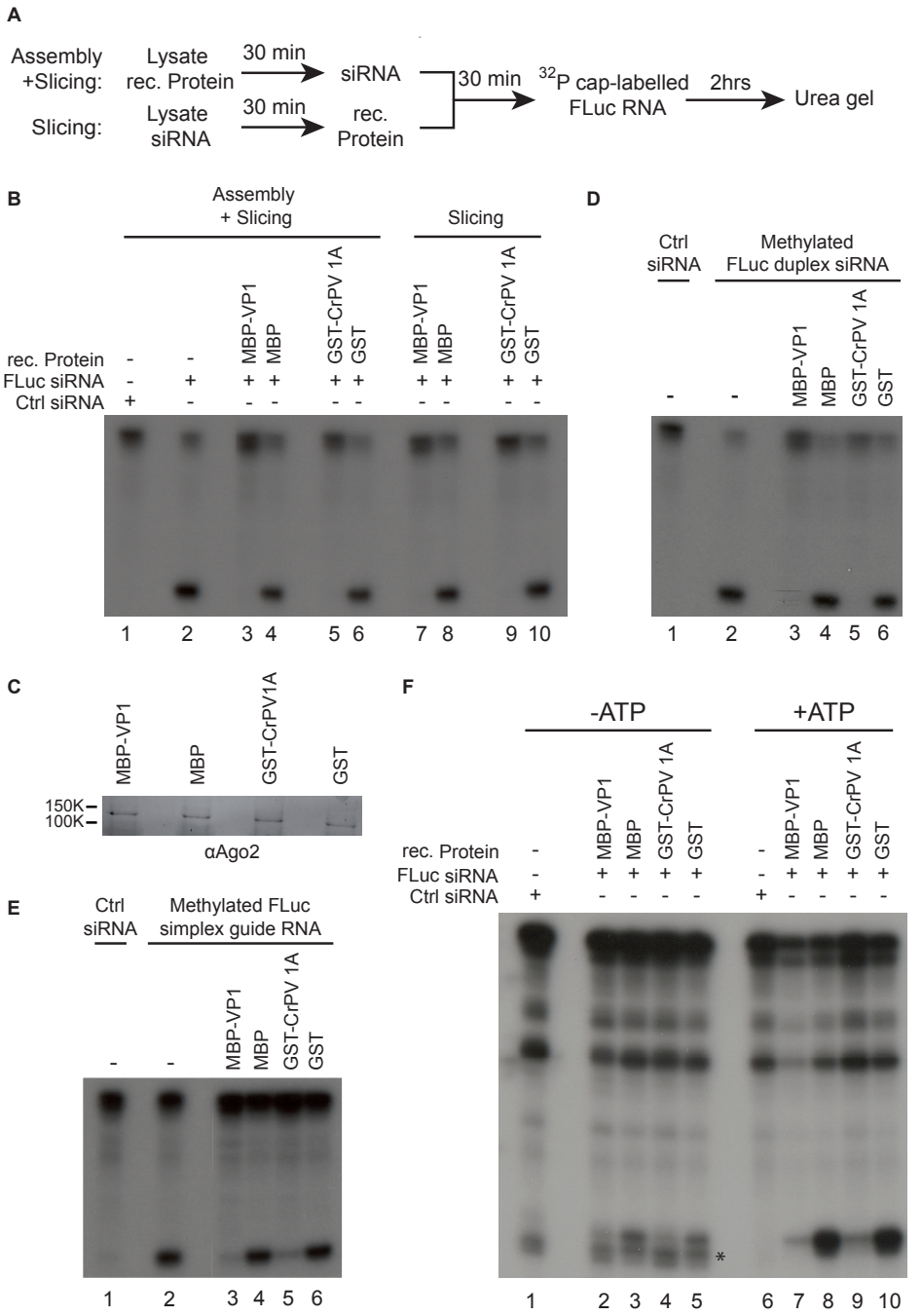


Figure 7: VP1 inhibits Slicer activity of pre-assembled mature RISC. (**A**) Schematic overview of the two experimental conditions of the Slicer assay designed to monitor the effect of recombinant (rec.) proteins on RISC assembly and Slicer activity (top) or on Slicer activity of pre-assembled RISC (bottom) (**B**) Slicer assays in *Drosophila* embryo lysates. RISC activity was analyzed in the presence of a non-targeting control siRNA (lane 1) or a specific FLuc siRNA (lane 2-10). Recombinant proteins were added before

(lanes 3-6) or after (lanes 7-10) assembly of RISC as indicated. As a control for possible buffer effects, recombinant protein was substituted by protein storage buffer (lanes 1 and 2). (**C**) Western blot showing the endogenous AGO2 protein levels in embryo lysate after incubation for 2 hours with the indicated recombinant proteins. The blot was developed with AGO2 antibody 4D2. (**D**) Slicer assay using an siRNA with a 2′-O-methylated guide strand. A non-modified control siRNA (lane 1) or a FLuc siRNA duplex containing a 2′-O-methyl group at the 3′ terminal nucleotide of the guide strand (lanes 2-6) was added to embryo lysate 30 minutes prior to the addition of the indicated recombinant proteins. (**E**) Slicer assay using a 2′-O-methylated simplex guide RNA. A control siRNA duplex (lane 1) or a single-stranded FLuc specific guide strand with a 2′-O-methyl group at the 3′ terminal nucleotide (lane 2-6) was added prior to the addition of the indicated recombinant proteins. (**F**) Slicer assays in the presence or absence of ATP. Embryo lysate was incubated with a control siRNA (lanes 1 and 6) or a specific FLuc siRNA (lanes 2-5 and 7-10). ATP was then depleted (lanes 1-5) or depleted and subsequently regenerated (lanes 6-10) and Slicer activity was monitored. An asterisk (*) indicates a non-specific band appearing in RISC assays under ATP depleted conditions.

7D, lane 4 and 6).

After loading of the siRNA as a duplex, AGO2 cleaves the passenger strand which is then degraded by the C3PO nuclease complex [33]. To circumvent canonical loading of RISC, we induced RISC formation with a single-stranded methylated guide RNA. Although less efficient, loading of single-stranded guide strands into AGO2 is possible via a bypass mechanism [34,35]. Indeed, at high concentrations, methylated single-stranded guide RNA-induced specific cleavage of cap-labeled target RNA (Figure 7E, lane 2). Interestingly, single-stranded guide RNA-induced target cleavage was specifically inhibited both by Nora virus VP1 and by CrPV 1A (Figure 7E, lanes 3 and 5). These results indicate that both CrPV 1A and Nora virus VP1 inhibit Slicer activity of mature RISC rather than RISC assembly.

Following maturation, RISC binds, cleaves, and releases complementary target RNA, and returns to a Slicer-competent state. *Drosophila* RISC is a multiple turnover complex, in which release of the cleaved target RNA is a rate-limiting step that is greatly enhanced by ATP [36]. We therefore analyzed suppression of Slicer activity under ATP-limiting conditions with a 20-fold molar excess of siRNA over target RNA. RISC was loaded in the presence of ATP, after which creatine kinase was inactivated by NEM, and ATP was depleted (-ATP) by addition of hexokinase and glucose (Figure S5). In parallel, ATP levels were restored (+ATP) after NEM treatment by adding back creatine kinase, and omitting hexokinase treatment. As expected, RISC shows a lower cleavage rate in –ATP conditions than in +ATP conditions (Figure 7F, compare lanes 3 and 5 with lanes 8 and 10). Even under –ATP conditions, Nora virus VP1 and CrPV 1A were able to inhibit Slicer activity (Figure 7F, lanes 2 and 4), suggesting that these two VSRs inhibit the catalytic target cleavage by AGO2.

Discussion

The mechanisms by which RNA viruses evade sterilizing immunity and establish chronic persistent infections remain poorly understood [37]. Nora virus successfully establishes a persistent infection in *Drosophila*, providing an excellent model to study mechanisms of persistence. We show here that Nora virus is a target of the antiviral RNAi machinery and that it encodes a potent suppressor of RNAi. Of note, Nora virus RNA levels are unaffected by mutations in the RNAi pathway [38]. These observations therefore suggest that dynamic interactions between the antiviral RNAi response and viral counter-defense mechanisms determine viral persistence.

The production of viral siRNAs is a hallmark of an antiviral RNAi response. By detection of Nora virus-derived vsiRNAs in infected fly stocks, we provide direct evidence that Nora virus is a target of Dcr-2. Nora virus vsiRNAs are distributed across the viral genome, with similar amounts derived from the (+) and (-) RNA strands. During (+) RNA virus infection, (+) viral RNA accumulates in large excess over (-) viral RNA (~50-100 fold). Cleavage of structured RNA elements by Dcr-2 is therefore expected to produce viral small RNAs that mirror this asymmetric distribution. Thus, similar to other RNA viruses, our results imply that Dcr-2 targets the dsRNA intermediates in Nora virus replication [2,4,39-41].

The current model proposes that the antiviral RNAi response relies on dicing of viral dsRNA and on slicing of viral target RNAs using vsiRNAs as a guide. Genetic analyses support the role of AGO2 in antiviral defense: *AGO2* mutants are hypersensitive to a number of RNA virus infections [3-7,42]. Yet, interpretation of this *AGO2* phenotype is complicated by other cellular functions of AGO2, such as regulation of cellular gene transcription and control of transposon activity [43-45]. An alternative model proposes that dicing of double-stranded replication intermediates plays an important role in latent virus infection [46]. Dicing of an essential replication intermediate by Dicer-2 should theoretically be sufficient to abort a productive virus replication cycle. The convergent evolution of VSRs that suppress the catalytic activity of AGO2 in two distantly related RNA viruses, Nora virus and CrPV, underlines the essential role of AGO2 Slicer activity in antiviral defense, also in persistent infections *in vivo*. Importantly, these two viruses display a strikingly different course of infection – CrPV causes a lethal infection, whereas Nora virus establishes a non-lethal, persistent infection – suggesting that the interaction between a VSR and the host RNAi machinery is not the main determinant for viral pathogenicity.

Materials and Methods

Small RNA sequencing and analysis

Total RNA was extracted from dissected heads, bodies (abdomens and thoraxes) and thoraxes from w^{1118} male flies using Trizol reagent (Invitrogen), and RNA quality was verified on a Bioanalyzer (Agilent). Small RNAs were then cloned using the DGE-Small RNA Sample Prep Kit and the Small RNA v1.5 Sample Preparation Kit (Illumina) following the manufacturer's instructions. Libraries were sequenced on the Illumina HiSeq platform.

Sequence reads were clipped from 3' adapters using fastx_clipper (http://hannonlab.cshl.edu/fastx_toolkit/). Reads in which the adapter sequence (CTGTAGGCACCATCAATCGT) could not be detected were discarded. Only the clipped 19-30 nt reads were retained. Sequence reads were first matched against the *Drosophila* genome (v5.37) using Bowtie (http://bowtie-bio.sourceforge.net/index.shtml). Reads not matching the *Drosophila* genome were then matched against the published Nora virus sequence (NC_007919.3, isolate Umeå 2007), allowing one mismatch during alignment. Viral small RNAs were then used to reconstitute a small RNA-based consensus genome sequence (rNora virus, JX220408) using Paparazzi [21] with NC_007919.3 as a starting viral reference genome. Distributions of Nora virus small RNA sizes were computed by parsing the Bowtie outputs with a python script (available upon request). Small RNA profiles were generated by collecting the 21-nt reads that matched the rNora virus sequence allowing one mismatch, and their frequency relative to their 5' position in the rNora virus (+) or (-) genomic strand was plotted in R. siRNA duplex signatures were calculated according to an algorithm developed to calculate overlap in piRNA sequence reads [47,48]. The distribution of siRNA overlaps was computed by collecting the 21-nt rNora virus RNA reads whose 5' ends overlapped with another 21-nt read on the opposite strand. For each possible overlap of 1 to 21 nt (i), the number of read pairs (O) was counted and converted to a Z-score with the formula Z(i) = (O(i)-mean(O))/standard deviation (O). Small RNA sequences were deposited to the Sequence Read Archive (SRA) at the National Center for Biotechnology Information (NCBI) under accession number SRA054241.

Cell culture and viruses

Drosophila S2 cells were cultured at 25°C in Schneider's medium (Invitrogen) supplemented with 10% heat inactivated fetal calf serum, 50 U/mL penicillin, and 50 µg/mL streptomycin (Invitrogen). DCV was cultured and titered on S2 cells as described previously [6]. For the production of recombinant SINV, the coding sequence of either GFP or the N-terminal V5 epitope tagged $VP1^{\Delta N351}$ was cloned into the XbaI site of the double subgenomic pTE3'2J vector [49]. The resulting plasmids were linearized by XhoI restriction, purified and used as template for *in*

vitro transcription using the mMESSAGE mMACHINE SP6 High Yield Capped RNA Transcription kit (Ambion). *In vitro* transcribed RNA was purified using the RNeasy kit (Qiagen) and transfected into BHK cells. Viral titers in the supernatant were determined by plaque assay on BHK cells.

RNAi reporter assay in S2 cells

RNAi reporter assays were performed as described previously using 25 ng pMT-GL3, 6 ng pMT-Ren, and 25 ng suppressor plasmid per well of a 96-well plate [50]. Plasmids encoding Nora virus cDNA constructs were generated as described in Protocol S1.

Flies and fly injections

Flies were maintained on standard medium at 25°C with a light/dark cycle of 12 hours/12 hours. Fly stocks that were used for Sindbis virus infection and for preparation of embryo lysate were cleared of *Wolbachia* and endogenous virus infection (see Protocol S1).

We used the following fly stocks and alleles: *UAS-CrPV 1A* [13,51], $AGO2^{321}$ [52], $Dcr\text{-}2^{L811fsX}$ [53], th^{RNAi} [24,25]. The coding sequences of the full-length VP1 and the inactive $VP1^{\Delta C74}$ mutant with an N-terminal V5 epitope tag were cloned into the pUAST vector using the SacII and XbaI restriction sites [54]. The resulting plasmids were microinjected into *Drosophila* w^{1118} embryos to generate transgenic fly lines (Bestgene Inc). Virus infections of adult female flies were performed as described previously using 5,000 PFU of recombinant SINV [6]. Survival was monitored daily. *In vivo* RNAi experiments were performed by crossing *GMR-GAL4*, *UAS-thRNAi/CyO* virgins [25] with *UAS-VSR/TM3 Sb* flies. The eye phenotype was monitored in two- to four-day-old male F1 offspring lacking the *CyO* and *TM3 Sb* balancers.

Production of recombinant proteins in *E. coli*

The GST and MBP fusion proteins were purified from *E. coli* as described in Protocol S1. Purified recombinant proteins were dialyzed against dialysis buffer (20 mM Tris-HCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 140 mM NaCl, 2.7 mM KCl) Recombinant proteins were stored as aliquots at -80°C in dialysis buffer containing 30% glycerol.

Gel mobility shift, Dicer and Slicer assays

Gel mobility shift assays were performed as described [6]. Briefly, uniformly radio-labeled 113 nt long dsRNA (50 cps/reaction) or end-labeled siRNAs (200 cps/reaction) were incubated with purified recombinant protein for 30 minutes at room temperature. Samples were then separated on an 8% native polyacrylamide gel and exposed to a Kodak Biomax XAR film.

Dicer and Slicer assays were performed according to the protocol of Haley and colleagues with minor modifications, described in Protocol S1 [30]. For Slicer assays

with the methylated duplex, FLuc guide strand 5'- UCG AAG UAC UCA GCG UAA GU[mU] and passenger strand 5'- CUU ACG CUG AGU ACU UCG AUU were annealed by incubating 20 μM of each siRNA strand in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate) for 1 min at 90°C, followed by incubation for 1 hour at 37°C. For guide strand loading of RISC, embryo lysates were incubated with FLuc single-stranded guide strand RNA at a final concentration of 10 μM. Radiolabeled probes and target RNA for gel shift and Slicer assays are described in Protocol S1.

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Supporting information

Text S1.

Nora virus VP1 is unable to suppress the miRNA pathway

Several plant virus RNAi suppressors influence the miRNA pathway, thereby inducing strong developmental defects in transgenic plants that express RNAi suppressors during development [55,56]. This effect may be due to convergence of the antiviral RNAi and miRNA pathways on Argonaute-1 (AGO1) in plants. In *Drosophila*, the miRNA and siRNA pathways are parallel pathways. Nevertheless, there is crosstalk between these pathways with miRNA and miRNA-star sequences being loaded into AGO2 and, conversely, with siRNAs being loaded into AGO1 [57,58]. To determine whether VP1 suppresses the miRNA pathway, we used a miRNA sensor assay in S2 cells (Protocol S1). In this assay, an FLuc reporter containing the 3'UTR of the *Drosophila par6* gene (FLuc-par6), a target for miRNA1, is co-transfected with a plasmid expressing the primary miRNA1 (pri-miR1), or a control plasmid expressing pri-miR12 [59,60]. Co-transfection of pri-miR1 led to specific silencing of the FLuc-par6 gene (Figure S1). We verified whether the reporter was suppressed in an *AGO1* dependent manner, by cotransfection of dsRNA targeting *AGO1* or, as a control, *AGO2*. As expected, the miRNA reporter assay monitors the canonical miRNA pathway, since knockdown of the *AGO1* gene by dsRNA led to de-repression of FLuc-par6 expression (although this did not reach statistical significance, p=0.09). In contrast, co-transfection of AGO2 dsRNA did not lead to de-repression, but even enhanced silencing of the miRNA reporter, perhaps reflecting more efficient AGO1 loading under conditions in which AGO2 is depleted. Expression of Nora virus VP1 did not de-repress the FLuc-par6 construct, indicating that VP1 does not suppress the miRNA pathway. Similarly, VP1 did not affect silencing of a miRNA sensor consisting of a luciferase construct containing two perfect complementary target sites for the endogenous miR2 in its 3'UTR (data not shown) [6]. In addition, transgenic flies expressing VP1 driven by a strong ubiquitous promoter (Tubulin-GAL4) are viable and fertile, lending further support to the conclusion that VP1 does not inhibit miRNA biogenesis and function (data not shown).

Protocol S1.

Extended Experimental Procedures and Supplemental Methods

Molecular cloning

To construct plasmids encoding C-terminal V5 epitope tagged proteins of Nora virus, cDNA prepared from Nora virus infected flies was amplified using primers 5'-AGT GGT ACC AAC ATG ATT AAC AAT CAA ACA AAC and 5'-GGT GGG CCC TTG ACA TTG TTG TTT CTG CG for ORF1, primers 5'-AGT GGT ACC AAC ATG TTA ATT GAA GCT TTC ATC and 5'-GGT GGG CCC TCC AAG ATC TCC TCT TTT AAT G for ORF2, primers 5'-AGT

GGT ACC AAC ATG GCA TTA AAA GAG GAG ATC and 5'-GGT GGG CCC TTG CAT AGA GTC ATA AAT TAC for ORF3, and primers 5'-AGT GGT ACC AAC ATG CAG AAT CCA ACA CAA ACC and 5'-GGT GGG CCC CTG CTG CCT CAC GGA AGG GAA for ORF4. Amplified products were cloned as KpnI and ApaI fragments into pAc5.1-V5-His-A (Invitrogen). For the expression of VP1 mutants tagged at the N-terminus with the V5-His epitopes, the pAc5.1-V5-His-Ntag plasmid was constructed. This plasmid was created by annealing and cloning the oligonucleotides 5'-CAA CAT GGG TAA GCC TAT CCC TAA CCC TCT CCT AGG TCT CGA TTC TAC GCG TAC CGG TCA TCA TCA CCA TCA CCA TG and 5'-AAT TCA TGG TGA TGG TGA TGA TGA CCG GTA CGC GTA GAA TCG AGA CCT AGG AGA GGG TTA GGG ATA GGC TTA CCC ATG TTG GTA C into the EcoRI and KpnI restriction sites of pAc5.1-V5-His-A. The sequences of all VP1 deletion mutants were cloned into pAc5.1-V5-His-Ntag using the EcoRI and SacI restriction sites. For mutant sequences see supplemental Figure S3.

miRNA sensor assay

The miRNA sensor assay and its plasmids were described previously [59,60] . Briefly, 5x104 S2 cells were seeded per well in a 96-well plate one day before transfection. Subsequently, the cells were transfected with 54.5 ng suppressor plasmid, 6.8 ng pMT-FLuc-par6, 1.6 ng pMT-Ren, and 2.7 ng pMT-miR1 or pMT-miR12 using the Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. To knockdown *AGO1* or *AGO2* expression, 5.4 ng of dsRNA was cotransfected with the plasmids. Expression of the reporter constructs was induced with $CuSO_4$ at 48 hrs post transfection and luciferase activities were measured at 72 hrs post transfection.

Clearance of *Wolbachia* and endogenous viruses from fly stocks

Fly stocks used for Sindbis virus infection and preparation of embryo lysates were cleared from endogenous viruses by collecting eggs on apple-juice agar plates, followed by a treatment with 50% household bleach for 5 minutes. Subsequently, the bleached eggs were washed three times in a large volume of water, after which they were transferred to clean vials containing standard fly food. After culturing the fly stocks for two generations we confirmed the absence of Nora virus and Drosophila C virus (DCV) by RT-PCR. Fly stocks were then cleared from *Wolbachia* infection by raising the flies for two generations on standard fly food supplemented with 0.05 mg/mL tetracycline hydrochloride (Sigma). To verify the clearance of *Wolbachia* infection, PCR amplification was performed with *Wolbachia* specific primers on DNA extracts of adult flies, as described earlier [61].

Production of recombinant proteins in *E. coli*

To fuse the $VP1^{\Delta N284}$ protein to the C-terminus of maltose binding protein (MBP), the coding sequence of the $VP1^{\Delta N284}$ mutant was cloned as an EcoRI-SalI fragment

into the pMal-C2X vector (New England Biolabs). The resulting pMal-C2X-VP1$^{\Delta N284}$ and parental pMal-C2X plasmids were transformed into the *E.coli* BL21 (DE3) strain. Expression of the recombinant fusion proteins was induced at 1.0 OD_{600} by adding 0.2 mM IPTG followed by incubation at 37°C for 3 hours. MBP and MBP-VP1$^{\Delta N284}$ fusion proteins were purified using amylose resin (New England Biolabs) according to the manufacturer's protocol.
The coding sequence of CrPV 1A (amino acids 1-148) was amplified using primers 5'- CGG GAA TTC ATG TCT TTT CAA CAA ACA AAC AAC and 5'- AGA GTC GAC TTA GAA GGC TCT GCA TT and cloned into the pGEX-4T-1 plasmid (GE healthcare) as an EcoRI-SalI fragment. After transformation of the *E. coli* BL21 (DE3) strain with the resulting pGEX-CrPV 1A plasmid, expression was induced at 1.0 OD_{600} using 0.2 mM IPTG. Protein production was allowed to continue overnight at 20°C. The GST-CrPV 1A fusion protein was purified using glutathione sepharose 4 fast flow (GE healthcare) according to the manufacturer's protocol. GST (pGEX-4T-1) and GST-DCV 1A (pGEX-DCV 1A) fusion proteins were purified using the same method, after induction of protein expression at 37°C for 3 hours [6].

Radioactively labeled probes and target RNA
Uniformly radio-labeled 113 bp long dsRNA was generated by *in vitro* transcription in the presence of α-[^{32}P]-UTP using a T7 promoter flanked firefly luciferase (GL3) PCR product as a template. T7 promoter flanked PCR products were generated with primers 5'-TAA TAC GAC TCA CTA TAG GGA GAT ATG AAG AGA TAC GCC CTG GTT and 5'-TAA TAC GAC TCA CTA TAG GGA GAA TAG CTT CTG CCA ACC GAA C. Unincorporated nucleotides were removed using a G-25 sephadex column (Roche) followed by purification of the dsRNA from an 8% polyacrylamide gel. Gl3 siRNAs (Dharmacon) were ^{32}P end-labeled using T4 polynucleotide kinase (Roche) after which unincorporated nucleotides were removed using a G-25 sephadex column (Roche).
To generate target RNA for the Slicer assay, a 492 bp region of the GL3 luciferase gene was PCR amplified using the primers 5'-TAA TAC GAC TCA CTA TAG GGA GAA TGG AAG ACG CCA AAA ACA T and 5'-CAT CGA CTG AAA TCC CTG GT. The GL3 PCR product was used as a template for *in vitro* transcription using the Ampliscribe T7 flash transcription kit (Epicentre). After purification from an 8% urea-polyacrylamide gel, the RNA was cap-radiolabeled with the Scriptcap m^{7}G capping system (Epicentre) according to the manufacturer's protocol. The capped RNA was purified from an 8% Urea-polyacrylamide gel before use in the Slicer assay.

Dicer assay
Dicer assays were performed in a final volume of 12 µL containing 4µL S2 cell extract, 3 µL dicer buffer, 1 µL uniformly labeled dsRNA (200 cps), and 4 µL purified

recombinant protein. Dicer buffer contained 0.175 µg/µL creatine kinase (Roche), 16.7 mM DTT, 0.02 mg/µL creatine monophosphate (Roche), 3.3 mM MgAc, 50 mM Hepes-KOH, 33.3% glycerol, 0.67 U/µL RNasin (Roche), and 3.3 mM ATP. Reactions were incubated for 3 hrs at 27°C after which they were deproteinized by proteinase K and phenol extracted [30]. After precipitation, the RNA was dissolved in Ambion loading buffer II and loaded on a 12% denaturing polyacrylamide gel. Dicer products were visualized by exposing the polyacrylamide gel to a Kodak Biomax XAR film.

Slicer assay

Drosophila embryo lysates were produced from w^{1118} flies as described [30]. Slicer reactions contained 5 µL embryo lysate, 3 µL cleavage buffer, 100 nM siRNA, 0.3 µM recombinant protein, and 1 µL capped target RNA (~1000 cps) in a final volume of 11 µL. The GL3 siRNA (Dharmacon) was used to induce cleavage of the firefly luciferase target RNA, whereas the negative control siRNA (Qiagen) was used as a negative control. After assembly of the reaction, samples were incubated for 2 hours at 25°C. Samples were then treated with proteinase K, extracted with phenol, and precipitated as described [30]. Precipitated RNA was dissolved in Loading buffer II (Ambion) and analyzed on an 8% urea-polyacrylamide gel. Slicer products in ATP depleting conditions were analyzed on a 6% urea-polyacrylamide gel. Kodak Biomax XAR films were used to visualize the radioactive Slicer products.

Supplemental figures

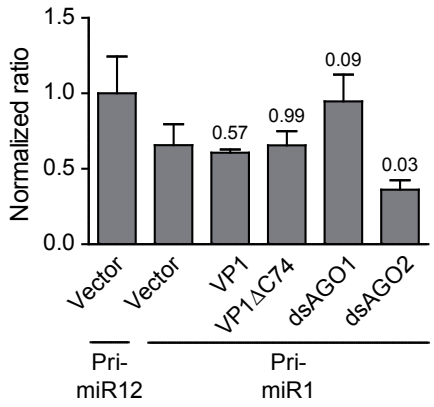

Figure S1: VP1 is unable to suppress the miRNA pathway. A firefly luciferase (FLuc) construct containing the par6 3'UTR, a target for miRNA1 (FLuc-par6), was co-transfected with plasmids encoding *Renilla* luciferase (RLuc) and either Nora virus VP1 or the inactive $VP1^{\Delta C74}$ mutant. FLuc-par6 expression was silenced by co-transfecting a plasmid encoding pri-miRNA1, whereas a pri-miRNA12 expressing construct was used as a negative control. *AGO1* or *AGO2* gene expression was knocked-down by co-transfection of dsRNA targeting these genes (dsAGO1 and dsAGO2, respectively). Expression of FLuc and RLuc was induced two days after transfection, and reporter activities were measured three days after transfection. RLuc activity was used to normalize FLuc activity within each sample, and data were normalized to the pri-miR12 treated sample. Bars represent averages and standard deviations of biological triplicates. A representative graph of two independent experiments is shown. The numbers represent p-values relative to pri-miR1 treated vector control samples in a two-tailed Student's t-test assuming equal variances.

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NoraV Umea 2007          MINNQTNKKGPQLERVHFGSTQVVGKSTKRRQRGTKLDIEYTVRRNDAPKEQKFLISEIF 60
NoraV NL1                MINNQTNKKGPQLERVHFGSTQVVGKSTKRRQRGTKLDIEYTVRRNDAPKEQKFLISEIF 60
NoraV NL2                MINNQTNKKGPQLERVHFGSAQVVGKSTKRRQRGTKLDIEYTVKRNDAPKEQKFLVSEIF 60
NoraV reconstituted FR1  MINNQTNKKGPQLERVHFGSAQVVGKSTKRRQRGTKFDIEYTVKRNDAPKEQKFLVSEIF 60
                         ********************:***************:******:***********:****

NoraV Umea 2007          DEKLDKQIKYEKKQNHTFIKPKLNLVIKEEQHITKKVLRGKERAATHAFMKEMVESNKIQ 120
NoraV NL1                DEKLDKQIKYEKKQNHTFIKPKLNLVIKEEQHIIKKVLRGKERAATHAFMKEMVESNKIQ 120
NoraV NL2                DEKLDKQIKYEKKQNHTFIKPKLNLVTREEQHMTKKVLRGKERAATHAFMKEMVESNKIQ 120
NoraV reconstituted FR1  DEKLDKQIKYEKKQNHTFIKPKLNLVTREEQHVTKKVLRGKERAATHAFMKEMVESNKIQ 120
                         ************************** :****: **************************

NoraV Umea 2007          PSWNVEYEKEIDEVDLFFMKKKTKPFSGFSIKELRDSLIVQSDDKNMAQPTVMSSIDEIV 180
NoraV NL1                PSWNVEYEKEIDEVDLFFMKKKTKPFSGFSIKELRDSLIVQSDDKNMAQPTVMSSIDEIV 180
NoraV NL2                PSWNVEYEKEIDEVDLFFMKKKTKPFSGFSIKELRDSLIVQSDDKNMAQPTVMSSINEIV 180
NoraV reconstituted FR1  PSWNVEYEKEIDEVDLFFMKKKTKPFSGFSIKELRDSLIVQSDDKNMAQPTVMSSTNEIV 180
                         ************************************************** :***

NoraV Umea 2007          TPREEISVSAISEQLASLMERVDKLEKMNAALEEENKQLKKEREATIKSVKKEAKKIKQE 240
NoraV NL1                TPREEISVSAISEQLASLMERVDKLEKMNAALEEENKQLKKEREATIKSVKKEVKKIKQE 240
NoraV NL2                TPREEISVSAISEQLASLMERVDKLEKMNAALEEENKQLKKEREATIESVKKEAKRTKQE 240
NoraV reconstituted FR1  TPREEISVSAISEQLASLMERVDKLEKMNAALEEENKQLKKEREATIKSVKKEAKRTKQE 240
                         ***********************************************:*****.*: ***

NoraV Umea 2007          KPQIVKKTQHKSLGVNLKITKTKVVGQEQCLEIENTQHKKFVEKPSMPLKVSKKMTEHQL 300
NoraV NL1                KPQIVKKTQHKSLGVNLKITKTKVVGQEQCLEIENTQHKKFVEKPSMPLKVSKKMTEHQL 300
NoraV NL2                KPQIAKKTQHKSLGVNLKITKIKVVGQEQCLEIENTQHKKFVEKPSMPSKVSKKMKGQQL 300
NoraV reconstituted FR1  KPQIAKKTQHKSLGVNLKITKTKVVGQEQCLEIENTQHKKFVEKPSMPSKVSKKMKGQQL 300
                         ****.**************** ************************** ******. :**

NoraV Umea 2007          KKTIRTWYEFDPSKLVQHQKEVLNSVVTNTTFADKVRETGIPKQKIRYVAKPPAEEKRSI 360
NoraV NL1                KKTIRTWYEFDPSKLVQHQKEVLNSVVTNTTFADKVRETGIPKQKIRYIAKPPAEEKRSI 360
NoraV NL2                KKTIRTWYEFDPSKLVQHQKEVLNSVVTNTTFADKVRETGIPKQKIRYTAKPPAEEKRSI 360
NoraV reconstituted FR1  KKTIRTWYEFDPSKLVQHQKEVLNSVVTNTTFADKVRETGIPKQKIRYTAKPPAEEKRSI 360
                         ********************************************** ************

NoraV Umea 2007          HFYGYKPKGIPNKVWWNWVTTGTAMDAYEKADRYLYHQFKREMMIYRNKWVKFSKEFNPY 420
NoraV NL1                HFYGYKPKGIPNKVWWNWVTTGTAMDAYEKADRYLYHQFKREMMIYRNKWVKFSKEFNPY 420
NoraV NL2                HFYGYKPKGIPNKVWWNWVTTGTAMDAYEKADRYLYHQFKREMMVYRNKWVKFSKEFNPY 420
NoraV reconstituted FR1  HFYGYKPKGIPNKVWWNWVTTGTAMDAYEKADHYLYHQFKREMMVYRNKWVKFSKEFNPY 420
                         *********************************:************:***************

NoraV Umea 2007          LSKPKMVWEENTWEYEYKTDVPYNFILKWRQLVQTYKPNTPIQADWYKISQKQQC 475
NoraV NL1                LSKPKMVWEENTWEYEYKTDVPYNFILKWRQLVQTYKPNTPIQADWYKISQKQQC 475
NoraV NL2                LSEPKMVWEENTWEYEYKTDVPYNFILKWRQLVQTYKPNTPIQADWYKISQKQQC 475
NoraV reconstituted FR1  LSEPKMVWEENTWEYEYKTDVPYNFILKWRQLVQTYKPNTPIQADWYKISQKQQC 475
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Figure S2: Alignment of VP1 sequences from different Nora virus isolates. Alignment of VP1 sequences of Nora virus isolate Umeå 2007 (accession number GQ257737) and Nora virus sequences from infected fly stocks from our own laboratory (isolates NL1 and NL2, GenBank accession number JQ288019 and JQ288020). We analyzed VP1 sequences in a total of eight Nora virus infected fly stocks. Five VP1 sequences were identical to NL1, one was the NL2 sequence, and two stocks contained a mixed population of Nora virus sequences. These eight stocks were obtained from five different laboratories or stock centers. However, they have been maintained in our laboratory before we tested them for Nora virus infection, and we cannot exclude the possibility that they became infected in our laboratory. Although we therefore cannot infer overall virus diversity from these data, they do indicate that VP1 is a conserved protein. The FR1 isolate is the Nora virus genome that was reconstituted from small RNA sequences from wildtype w^{1118} flies from a laboratory based in France (GenBank accession number JX220408).

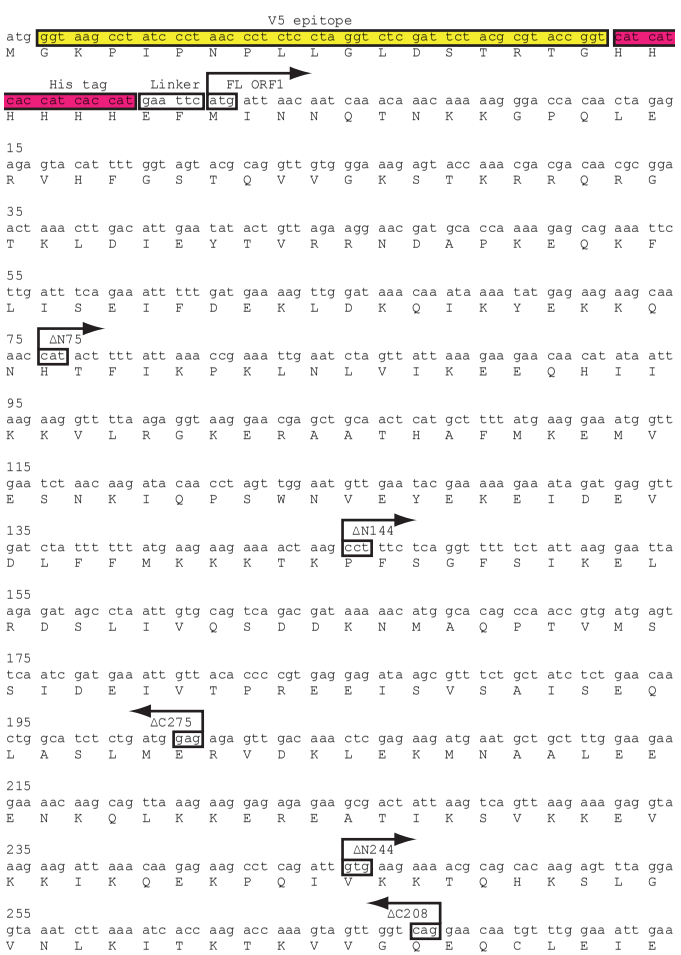

Figure S3: Nucleotide and protein sequence of full-length and VP1 mutants fused to V5-His tag at the N-terminus. Nucleotide and amino acid sequence of V5 epitope and Histidine (His) tagged full-length VP1 sequence (VP1^{FL}). A linker sequence between the His tag and VP1 was created to facilitate cloning. Start and stop sites of the respective N- and C-terminal deletion mutants of VP1 are indicated. The VP1 deletion mutants were fused to the V5-His tag in an identical way as the VP1^{FL} construct.

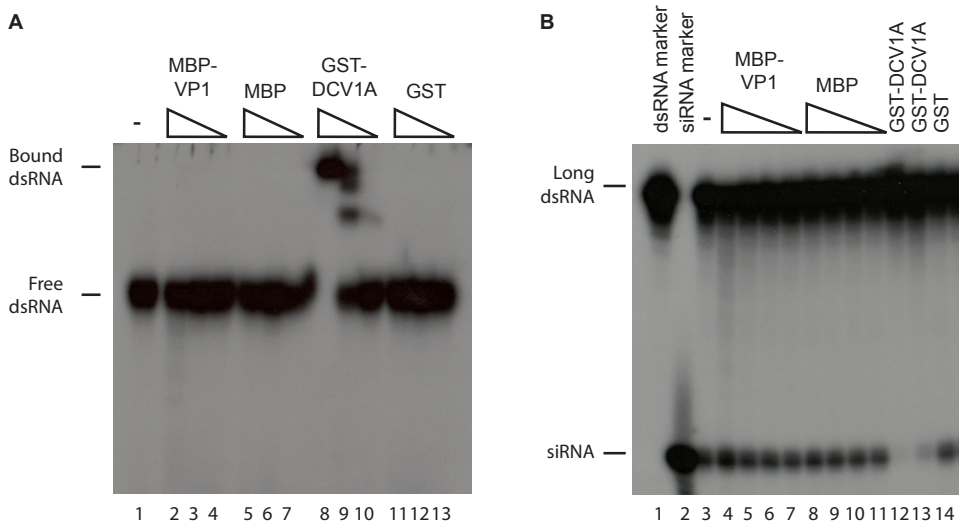

Figure S4: Nora virus VP1 is unable to bind long dsRNA or to interfere with Dcr-2 activity. (**A**) Mobility shift assay of suppressor proteins with long dsRNA. Uniformly radiolabeled long dsRNA was incubated for 30 minutes with buffer (lane 1) or recombinant MBP-VP1$^{\Delta N284}$ (lanes 2-4) , MBP (lanes 5-7), GST-DCV 1A (lanes 8-10) or GST (lanes 11-13). Ten-fold dilutions of recombinant protein were used starting from the following concentrations: MBP-VP1$^{\Delta N284}$ (2 µM, lane 2), MBP (2.6 µM, lane 5), GST-DCV 1A (1 µM, lane 8), and GST (2.24 µM, lane 11). RNA mobility shifts were analyzed on an 8 % native polyacrylamide gel. (**B**) Dicer activity in S2 cell extract in the presence of viral suppressor proteins. Uniformly radiolabeled long dsRNA was incubated in S2 cell extract for 3 hours with buffer (lane 3) or the indicated recombinant proteins. Two-fold dilutions were used for MBP-VP1$^{\Delta N284}$ (lanes 4-7, highest concentration 1.1 µM) and MBP (lanes 8-11, highest concentration 4.2 µM). Two independent preparations of GST-DCV 1A were used (lane 12, concentration of 0.54 µM and lane 13, concentration of 0.03 µM). GST was used at a concentration of 1.2 µM (lane 14). As size markers, dsRNA input (lane 1) and end-labelled siRNAs (lane 2) were used. Dicer products were analyzed on a 12% denaturing polyacrylamide gel.

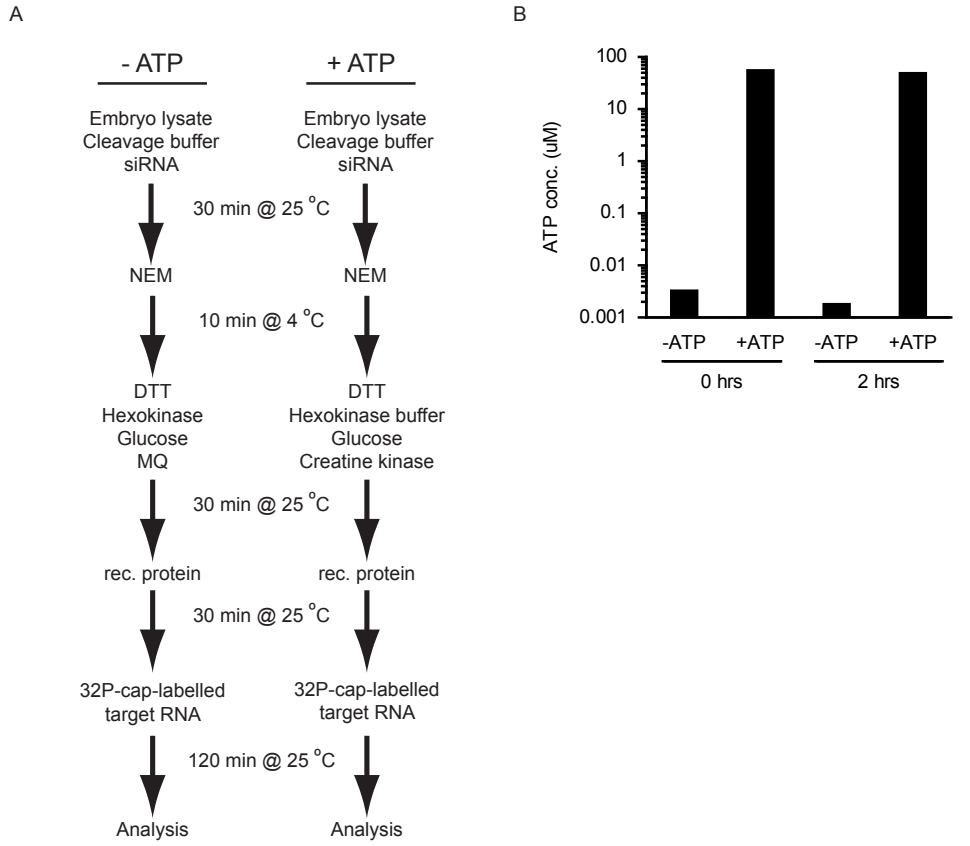

Figure S5: ATP depletion during Slicer assay. (**A**) Schematic representation of the protocol used to deplete (-ATP) or to regenerate ATP after initial depletion (+ATP) for Slicer assays of Figure 7F. For RISC loading, *Drosophila* embryo lysate was incubated with an siRNA for 30 minutes under standard conditions. Subsequently, N-ethylmaleimide (NEM) was added in both conditions to inhibit the ATP regenerating activity of creatine kinase. After incubating the reactions for 10 minutes on ice, DTT was added to quench the NEM in both conditions. Hexokinase, glucose, and milliQ water (MQ) were added in the –ATP protocol to deplete the pool of ATP. For the +ATP condition, Hexokinase was substituted by hexokinase buffer, and MQ was substituted for Creatine kinase to restore the ATP regenerating activity. Subsequently, the reactions were incubated for 30 minutes after which recombinant protein (rec. protein) was added. Following another 30 minutes incubation period, the ^{32}P-cap-labelled RNA was added to the reaction, after which the incubation was continued for another 2 hours. Subsequently, reactions were analyzed on a polyacrylamide gel. (**B**) ATP concentrations before and after the Slicer assay under –ATP and +ATP conditions. ATP levels were measured at the moment of target RNA addition (0 hrs) or after 2 hours of incubation with target RNA. For ATP concentration measurements, recombinant protein was substituted for protein storage buffer, and target RNA was substituted for MQ. ATP levels were measured using the Celltiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer's protocol.

Chapter 4

Inhibition of RNAi by transgenic expression of Nora virus VP1 protein in adult *Drosophila*

Manuscript in preparation:

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Abstract

Plants and insects rely on the RNA interference (RNAi) pathway – gene silencing mediated by small interfering RNAs (siRNAs) – for antiviral defense. Consequently, plant and insect viruses express viral suppressors of RNAi (VSRs) to counteract the antiviral RNAi pathway. The identification and characterization of viral RNAi suppressors provides important insights into virus-host interactions. In addition, these proteins may be used as powerful experimental tools to study RNAi function. We recently identified the viral protein 1 (VP1) of Nora virus as an RNAi suppressor. In this report, we further characterize the activities of VP1 in transgenic *Drosophila melanogaster*. We show that the VP1$^{\Delta N351}$ protein, a mutant lacking the 351 N-terminal amino acids, efficiently suppresses the siRNA pathway *in vivo* without affecting the related microRNA pathway. Ubiquitous or fat body specific expression of VP1$^{\Delta N351}$ sensitized flies to Drosophila C Virus (DCV) infection. In addition, ubiquitous expression of VP1$^{\Delta N351}$ led to an increase in viral RNA specifically in the fat body tissue. These results establish VP1$^{\Delta N351}$ as a useful tool to study the RNAi pathway in *D. melanogaster* and to inhibit RNAi in a tissue specific manner.

Introduction

Small RNAs are important regulators of gene expression that play critical roles in many cellular processes in eukaryotes. In association with Argonaute proteins, small RNAs can silence gene expression in a sequence specific manner. Currently, small RNAs are divided in three main classes, small interfering RNAs (siRNAs), microRNAs (miRNAs), and piwi-interacting RNAs (piRNAs).

MicroRNAs are encoded in the genome of eukaryotes, and function as important regulators of endogenous gene expression. In the fruit fly *Drosophila melanogaster*, miRNA biogenesis depends on the nuclear RNaseIII enzyme Drosha and its double stranded RNA (dsRNA) binding partner Pasha. In the cytoplasm, the RNaseIII enzyme Dicer-1 (Dcr-1) and its dsRNA binding partner protein Loquacious (Loqs) are responsible for subsequent miRNA maturation. Mature miRNAs then associate with an Argonaute 1 (AGO1) containing protein complex to exert their regulatory function [1]. In contrast, piRNAs are generated in a Dicer-independent manner, and associate with the PIWI subclass of Argonaute proteins to suppress the activity of transposons in the germline [2]

RNA interference (RNAi) is initiated by processing of long double stranded (ds) RNA into siRNAs in the cytoplasm [3]. dsRNA is readily detectable in infections with viruses with dsRNA, positive-stranded RNA, or DNA genomes [4]. As a consequence, these viruses have the potential to trigger the siRNA pathway. Indeed, viral dsRNA is recognized and cleaved by the RNaseIII enzyme Dicer-2 (Dcr-2) into viral siRNAs (vsiRNAs) which are loaded into an Argonaute-2 (AGO2) containing RNA-induced silencing complex (RISC) [5-9]. Subsequently, one strand of the vsiRNA duplex is retained in RISC, and guides recognition and cleavage of complementary viral sequences thereby restricting virus replication [10-14].

Plant and insect viruses express viral suppressors of RNAi (VSRs) that allow them to replicate in the face of the antiviral RNAi response. Many of these proteins bind either long dsRNA molecules and/or small RNA duplexes, precluding their use as trigger and guides of the RNAi pathway [15-17]. However, suppression of RNAi through interaction with key proteins of RNA silencing pathways has also been reported in both insect and plant viruses [8,18-25].

The critical role of RNAi in antiviral defense is supported by the observation that RNAi deficient flies (*Dcr-2* or *AGO2* null mutants) are hypersensitive to virus infection [17,26-28]. Although these null mutants are viable and fertile, multiple defects have been reported. For instance, genetic loss of *AGO2* leads to the activation of mobile genetic elements and the deregulation of Drosophila fragile X protein (dFMR1) expression, the latter resulting in neurogenesis and oogenesis defects [29-31]. In addition, Dcr-2 and AGO2 were shown to operate in the nucleus, contributing to transcriptional control probably by influencing the processivity of RNA polymerase II

[32]. Recently, AGO2 was also implicated in the regulation of pre-mRNA splicing [33] . These defects complicate the interpretation of the phenotype of RNAi mutants upon virus infection. Spatio-temporal controlled inactivation of RNAi would be a useful approach to minimize these effects.

Recently, we identified an RNAi suppressor, viral protein 1 (VP1), in the positive-stranded RNA virus Nora virus [8,34]. We mapped the RNAi suppressor activity to the C-terminal 124 amino acids of VP1. Furthermore, we showed that Nora virus VP1 inhibits slicer activity of a mature RISC. Although a full-length VP1 ($VP1^{FL}$) transgene suppressed RNAi in transgenic flies, its suppressive activity was only mild. Therefore, we characterized in this report the RNAi suppressor activity of the N-terminal deletion mutant $VP1^{\Delta N351}$, which retains 124 C-terminal amino acids, *in vivo*. We show that the $VP1^{\Delta N351}$ transgene is a potent suppressor of the RNAi pathway *in vivo*, and that it does not affect the miRNA pathway. Furthermore, we show that transgenic flies have an increased sensitivity to Drosophila C virus (DCV) when $VP1^{\Delta N351}$ is expressed ubiquitously in all tissues or specifically in the fat body. Altogether, our results imply that the VP1 transgene is a useful reagent to probe RNAi regulated processes *in vivo*.

Results

Nora virus $VP1^{\Delta N351}$ suppresses RNAi *in vivo*

Recently, we identified Nora virus VP1, the protein product from open reading frame 1, as a viral suppressor of RNAi [8]. The RNAi antagonistic activity mapped to the C-terminus of VP1, since a 74 amino acid C-terminal deletion mutant ($VP1^{\Delta C74}$) is inactive. In support, we showed that the N-terminal deletion mutant $VP1^{\Delta N351}$, which codes for the 124 C-terminal amino acids, is fully functional in RNAi suppressor activity *in vitro* [8].

To further characterize the RNAi suppressor activity of VP1 and its mutants in adult flies, we made use of the GAL4/UAS system [35]. In this system, a responder fly line, containing a transgene of interest under the control of an Upstream Activating Sequence (UAS), is crossed with a driver line that expresses the yeast transcriptional activator GAL4. In the F1 offspring, GAL4 induces expression of the UAS-regulated transgene. Therefore, regulated expression of GAL4 in the driver line can be used to control transgene expression in a spatiotemporal manner. Recently, we described transgenic fly lines expressing the $VP1^{FL}$ or the inactive $VP1^{\Delta C74}$ sequence [8]. In this report, we generated responder flies containing the V5-tagged $VP1^{\Delta N351}$ sequence downstream of the UAS sequence. Crossing the $VP1^{FL}$ and $VP1^{\Delta C74}$ responder lines with *actin-GAL4* (*act-GAL4*) or *tubulin-GAL4* (*tub-GAL4*) driver lines resulted in expression of the VP1 transgenes in the offspring (Figure 1A). Although the *tub-GAL4* driver induced higher expression levels of both the $VP1^{FL}$ and $VP1^{\Delta C74}$ construct, it produced only

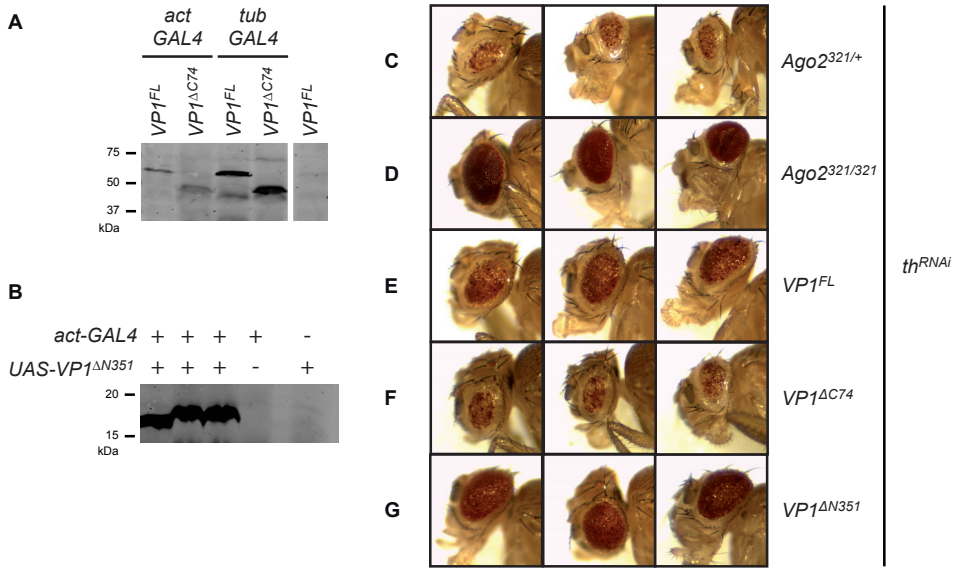

Figure 1: VP1$^{\Delta N351}$ suppresses RNAi *in vivo*. (**A,B**) Western blot analysis of VP1 expression. Transgenic flies containing full-length VP1 (*UAS-VP1^{FL}*), a C-terminal deletion mutant of VP1 (*UAS-VP1$^{\Delta C74}$*), and a N-terminal deletion mutant of VP1 (*UAS-VP1$^{\Delta N351}$*, three independent transgenic lines) behind the GAL4 responsive UAS element were crossed with the *actin-GAL4* (*act-GAL4*) or the *tubulin-GAL4* (*tub-GAL4*) driver lines. Expression of VP1 in the F1 progeny was analyzed by western blot using the anti-V5 antibody. (**C-G**) Knockdown of *thread* by RNAi (*th^{RNAi}*) in the eyes of adult flies in the background of an *AGO2* heterozygote (*AGO2$^{321/+}$*) (**C**), an *AGO2* homozygous mutant (*AGO2$^{321/321}$*) (**D**) or in flies co-expressing VP1^{FL} (**E**), the inactive VP1$^{\Delta C74}$ mutant (**F**), and the active VP1$^{\Delta N351}$ mutant (**G**). For every cross, three representative pictures of eyes of two- to four-day-old male flies are shown.

limited offspring for the VP1^{FL} cross. Therefore, we chose to use the *act-GAL4* driver in further experiments. Crossing three independent VP1$^{\Delta N351}$ responder fly lines with the *act-GAL4* driver resulted in expression of the VP1$^{\Delta N351}$ protein (Figure 1B). Since all VP1$^{\Delta N351}$ fly lines were viable and express the VP1$^{\Delta N351}$ protein in equal amounts, we choose one line for further analysis. We will refer to this transgenic line as 'VP1$^{\Delta N351}$' in the rest of the manuscript.

Having established that the *VP1^{FL}*, *VP1$^{\Delta C74}$*, and the *VP1$^{\Delta N351}$* transgenes are expressed, we determined the RNAi suppressor activity of these proteins *in vivo*. To this end, we used a transgenic RNAi sensor line, in which the inhibitor of apoptosis *thread* (*th*) is silenced by expression of dsRNA targeting this gene (*th^{RNAi}*) [36,37]. Expression of *th^{RNAi}* in the developing eye results in severe apoptosis leading to a reduced eye size and loss of pigmentation together with roughening of the eye surface (Figure 1C). In line with earlier reports [8,37], the *th^{RNAi}* phenotype depends on functional RNAi, since no developmental defects were observed in an *AGO2* null background (Figure 1D). Likewise, suppressing AGO2 activity by expression of VP1^{FL} resulted in larger eyes size and an increase in pigmentation, whereas expression of VP1$^{\Delta C74}$ did not restore the RNAi phenotype (Figure 1E,F). Expression of VP1$^{\Delta N351}$ fully restored

the eye size and increased pigmentation in the eye (Figure 1G). In addition, VP1$^{\Delta N351}$ reduced roughening of the eye surface to a greater extent than VP1^{FL}. These results indicate that the *VP1$^{\Delta N351}$* transgene is a more potent RNAi suppressor than *VP1^{FL}*, which is likely a result of the higher expression level of VP1$^{\Delta N351}$.

Nora virus VP1 does not affect miRNA function *in vivo*

The miRNA pathway regulates expression of many endogenous transcripts. Transgenic plants expressing RNAi suppressor proteins from plant viruses often show developmental phenotypes, suggesting that these VSRs affect the miRNA pathway [38,39]. Transgenic flies expressing Nora virus VP1$^{\Delta N351}$ are viable and fertile, and appear normal (data not shown). Since defects in miRNA pathway genes are associated with developmental defects, these observations suggest that VP1 does not affect miRNA biogenesis and function. To directly assess the effect of VP1 on the miRNA pathway *in vivo*, we used a previously described miRNA sensor fly [40,41]. In

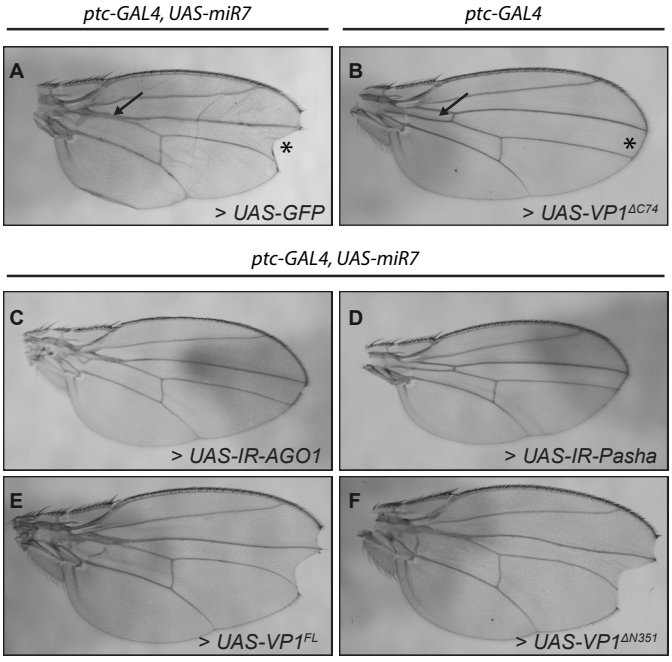

Figure 2: VP1 does not affect miRNA function *in vivo*. (**A**) Ectopic expression of miR7 in the wing leads to distal notching of the wing (*) and loss of the proximal L3/L4 domain (arrow). (**B**) Ectopic expression of VP1$^{\Delta C74}$ in the absence of the miR7 construct results in normal wing development. (**C-F**) Wing phenotype of flies expressing miR7 along with an RNAi inducing inverted repeat (IR) targeting *Argonaute-1* (*AGO1*) (C), and *Pasha* (**D**), or transgenes encoding VP1^{FL} (**E**), or VP1$^{\Delta N351}$ (**F**). For all constructs, expression in the wing was driven by the *patched-GAL4* (*ptc-GAL4*) driver. Pictures are representative of 10-15 wings examined.

this sensor, ectopic expression of miR7 inhibits *Notch* target gene activity, leading to notched wings and a proximal growth defect in the wings of these miR7 sensor flies. Indeed, ectopic expression of miR7 along with a GFP control transgene in the developing wing resulted in a clear distal wing notch and loss of the proximal L3/L4 domain (Figure 2A). We verified that the Notch phenotype is miR7 dependent, since in the absence of miR7 expression, a normal wing phenotype was observed

(Figure 2B). In contrast, expression of miR7 under conditions in which expression of *AGO1* or *Pasha* is knocked-down did not induce wing notching and growth defects (Figure 2C,D). These results indicate that the wing phenotype of the miR7 sensor indeed depends on a functional miRNA pathway. We then tested if VP1 expression could affect the miR7-induced phenotype. Neither the full-length, nor the *VP1$^{\Delta N351}$* construct altered the wing phenotype induced by ectopic expression of miR7 (Figure 2E,F). These results demonstrate that VP1 is unable to affect the miRNA pathway *in vivo*, which is in line with our previous observations in cell culture [8].

Nora virus VP1 enhances DCV pathogenicity *in vivo*

A functional RNAi pathway is important for control of virus infection, as inactivation of crucial components of this pathway renders flies more sensitive to virus infection [17,26-28]. Since Nora virus VP1^{FL} and VP1$^{\Delta N351}$ specifically suppress the RNAi pathway *in vivo*, we investigated whether these transgenes alter the sensitivity to DCV infection, a natural pathogen of *Drosophila* [42,43]. Therefore, we injected transgenic VP1 flies with DCV and monitored survival thereafter.

Two genetic control lines, one expressing no VP1 transgene (*act-GAL4* x wt), the other expressing the inactive VP1$^{\Delta C74}$ protein (*act-GAL4* x *UAS-VP1$^{\Delta C74}$*), showed a median survival of 8 days post infection (dpi) (Figure 3A). Flies expressing VP1^{FL} or VP1$^{\Delta N351}$ died faster than these controls, with a median survival of 6 and 4 dpi, respectively (Log-rank, $p<0.0001$). Because flies expressing an active VP1 protein show a higher mortality after DCV infection, we determined whether DCV replicated to higher titers in these flies. To this end, we injected flies with DCV, and analyzed viral load over time. In all transgenic lines, titers of DCV initially increased at 24 and 48 hours post infection (hpi) before reaching a plateau at 72 hpi (Figure 3B). Surprisingly, we did not detect a significant difference in viral load at any time point in flies expressing VP1$^{\Delta N351}$ relative to flies expressing the inactive VP1$^{\Delta C74}$ protein, or to two fly lines that do not express a VP1 transgene (wt x *act-GAL4* and wt x *VP1$^{\Delta N351}$*).

Since the fat body is one of the main target tissues of DCV, we reasoned that expression of VP1$^{\Delta N351}$ might increase viral replication in this tissue [44,45]. To examine this possibility, we injected VP1$^{\Delta N351}$ expressing flies and two genetic controls with DCV. Subsequently, we determined viral RNA levels in the whole fly and, in parallel, in isolated fat body tissue. Consistent with our observations on infectious virus titers, similar levels of viral RNA were detected in the three different crosses when using total RNA from whole flies (Figure 3C). We did, however, observe an increase in viral RNA levels in the fat body of VP1$^{\Delta N351}$ expressing flies, although this difference did not reach statistical significance ($p = 0.096$, one-way ANOVA). This result suggests that ubiquitous expression of VP1$^{\Delta N351}$ during DCV infection specifically increases viral loads in the fat body.

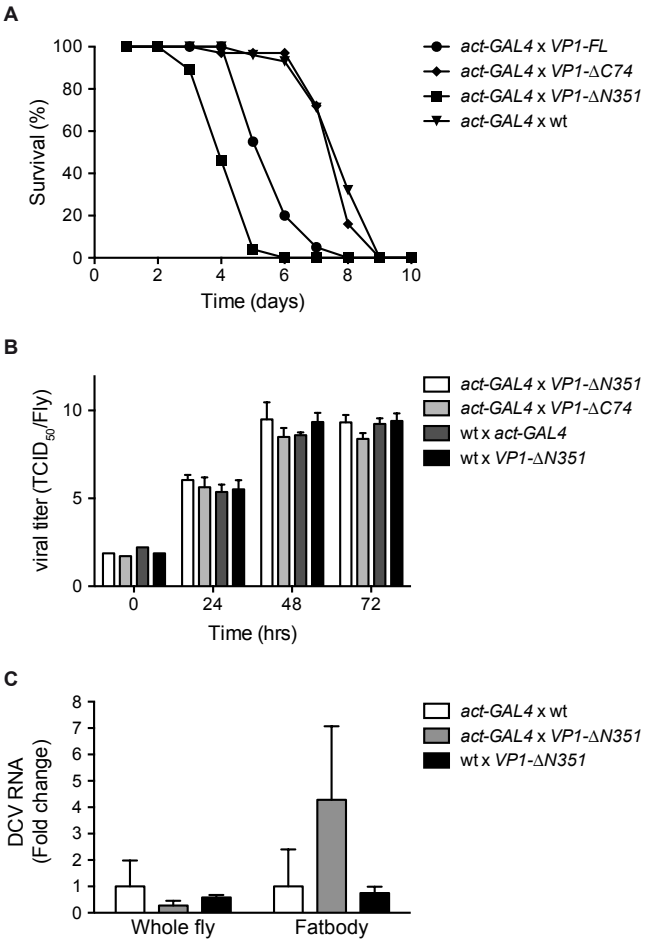

Figure 3: VP1$^{\Delta N351}$ expression sensitizes flies to DCV infection. (**A**) Survival curves of DCV infected flies expressing the VP1^{FL} (circle), VP1$^{\Delta C74}$ (diamond) and VP1$^{\Delta N351}$ (squares), or in control flies that only express the GAL4 driver (triangle). At least 20 flies were injected with DCV and survival was monitored daily. Survival curves are representative of two independent experiments. (**B**) DCV titers in the progeny of the indicated crosses at 0, 24, 48, and 72 hours post infection (hpi). Input levels were determined at 0 hpi in one pool of five flies. Three pools of 4-5 flies were collected at the other time points, except for the wt x *act-GAL4* cross at 48 hpi, and the wt x *VP1$^{\Delta N351}$* cross at 24 and 48 hpi (n=2 pools). Bars represent means and standard deviation. (**C**) DCV RNA levels in three pools of 5 whole flies or in isolated fat body tissue from 5 flies collected at 48 hpi. DCV RNA levels were determined by quantitative RT-PCR. Data are normalized to RP49 and presented as fold-change relative to the *act-GAL4* x wt control.

VP1 expression in fat body tissue enhances DCV pathogenicity

Fat body and hemocytes are considered the main immune organs of insects. The fat body plays an important role in the humoral immune response by producing various immune effector molecules. Hemocytes are involved in the cellular immune response through phagocytosis, melanisation, and encapsulation of invading pathogens [46]. In addition, hemocyte-mediated phagocytosis has been implicated in the defense against viral infections [47]. Since VP1$^{\Delta N351}$ expressing flies showed an increased mortality and higher viral RNA levels in fat body tissue compared to control flies, we wondered if specific expression of VP1 in immune tissues enhances DCV pathogenicity. To achieve expression of VP1$^{\Delta N351}$ and VP1$^{\Delta C74}$ in the fat body and hemocytes, we crossed the responder flies with the *c564-GAL4*, and the *hemolectin-GAL4* (*hml-GAL4*) driver lines, respectively [48-50]. As a negative control we drove expression of VP1$^{\Delta N351}$ and VP1$^{\Delta C74}$ in the nervous system, which is not implicated in immune defense, using the *Nervana2-GAL4* (*nrv2-GAL4*) driver [51]. As genetic

controls, we crossed wild-type flies with the respective driver lines.

To assess the effect of tissue specific VP1 expression on survival, we injected the offspring of all crosses with DCV. Similar to the genetic control, flies expressing the inactive VP1$^{\Delta C74}$ in the fat body showed a median survival of 7 days after DCV injection (Figure 4A). In contrast, VP1$^{\Delta N351}$ expressing flies had a median survival of 4 days and showed a higher mortality rate compared to VP1$^{\Delta C74}$ flies and the genetic control that does not express VP1, which showed a median survival of 6 and 7 days, respectively (Log-rank, $p<0.0001$). In contrast, VP1$^{\Delta N351}$ expression in hemocytes or the nervous system did not affect survival, compared to flies expressing the inactive VP1$^{\Delta C74}$ in these tissues (Figure 4B,C). These results indicate that specific expression of VP1$^{\Delta N351}$ in the fat body can sensitize flies to DCV infection to a similar extent as ubiquitous expression of VP1$^{\Delta N351}$.

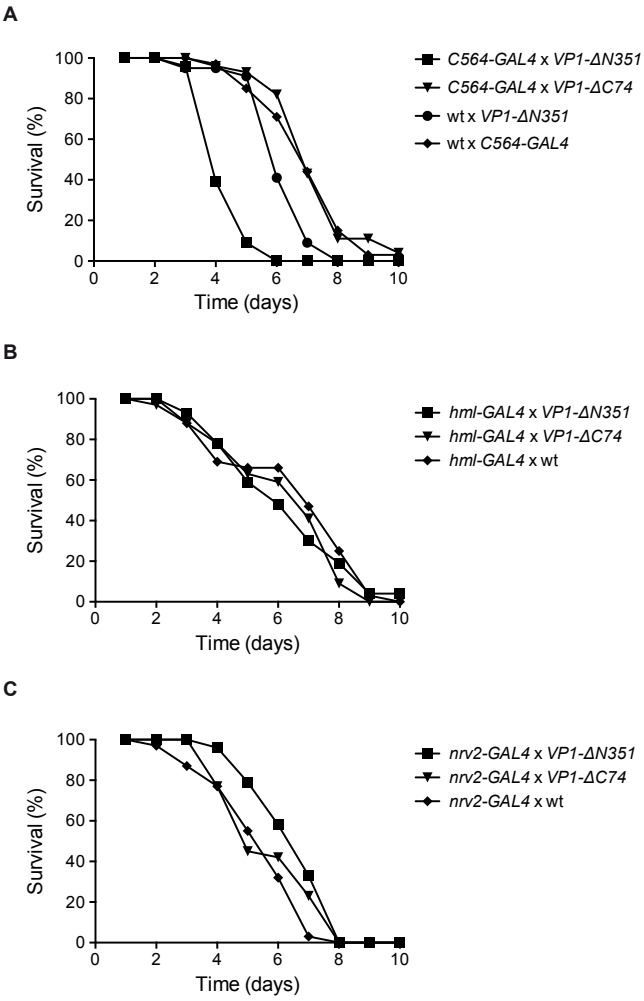

Figure 4: Fat body specific expression of VP1$^{\Delta N351}$ enhances sensitivity to DCV. (**A-C**) Survival curves of DCV infected flies with the indicated genotypes. Tissue-specific expression of VP1 transgenes in fat body (**A**), hemocytes (**B**), and nervous systems (**C**) was induced by the *C564-GAL4*, *hml-GAL4*, and *nrv2-GAL4* drivers, respectively. Survival of flies expressing the VP1$^{\Delta N351}$ (squares) or VP1$^{\Delta C74}$ (triangles) transgenes and control flies expressing the GAL4 driver, but no VP1 transgene (diamonds) and control flies in which the VP1 transgene is not induced due to the lack of a GAL4 driver (circles) were analyzed. For survival analysis, at least 22 flies were injected with DCV and survival was monitored daily.

Discussion

The RNAi pathway is the major antiviral defense system in *Drosophila*. Although genetic inactivation of key components of this pathway have yielded crucial insights into virus-host interactions, multiple defects have been identified in genetic null mutants [29,32]. To minimize these defects, inactivation of RNAi by viral suppressors of RNAi in a spatiotemporal manner would be a useful complementary approach.
Here, we show that expression of the Nora virus VP1$^{\Delta N351}$ transgene leads to potent suppression of the RNAi pathway *in vivo*. VP1$^{\Delta N351}$ suppressed RNAi more potently than VP1^{FL}, which is likely due to the higher expression level of the VP1$^{\Delta N351}$ construct. Furthermore, VP1$^{\Delta N351}$ did not affect miRNA function, which is important to prevent global changes in gene expression. Similar to earlier reports on *AGO2* knockout flies, we show that antagonizing AGO2 function by expression of VP1$^{\Delta N351}$ induced hypersensitivity to DCV infection [17]. In contrast to *AGO2* knockout lines, however, we were unable to detect a higher viral load in whole flies expressing VP1$^{\Delta N351}$ compared to genetic controls. However, we could identify an increase in viral RNA levels in VP1$^{\Delta N351}$ flies in the fat body, the main target tissue of DCV. In line with this observation, we showed that specific expression of VP1$^{\Delta N351}$ in fat body tissue decreased survival to a similar extent as ubiquitous expression of VP1$^{\Delta N351}$. These results underline the importance of the fat body in DCV infection, and suggest that a tissue specific increase in viral titer can reduce the fitness of the host.
RNAi is important for posttranscriptional gene silencing in the cytoplasm, as well as for control of nuclear processes, such as transcriptional regulation and splicing of host genes [32,33]. Consequently, *AGO2* mutants display defects at the transcriptional as well as the post-transcriptional level [29,32]. Our unpublished observations indicate that VP1 localizes exclusively in the cytoplasm in S2 cells. Thus, the VP1$^{\Delta N351}$ transgene efficiently inhibits cytoplasmic RNAi, but is unlikely to affect nuclear RNAi. Altogether, we propose that the VP1$^{\Delta N351}$ transgenic fly is a useful experimental tool to study RNAi regulated processes in *Drosophila*.

Materials and Methods

Fly stocks and cell lines

Drosophila S2 cells were cultured at 25ºC in Schneider's medium (Invitrogen), supplemented with 10% heat inactivated fetal calf serum, 50 µg/mL streptomycin, and 50 U/mL penicillin (Invitrogen). Fly stocks were maintained on standard medium at 25ºC using a light/dark cycle of 12 hours/12 hours. All fly stocks used in survival experiments were cleared of Wolbachia and endogenous viruses as described previously [8].

The following fly stocks and alleles were described previously: *tub-GAL4* [52], *C564-GAL4* [49], *hml-GAL4* [48,50], *nrv2-GAL4* [51], *AGO2*^{321} [53], *UAS-th*^{RNAi} [36,37], *UAS-GFP* (C. antoniewski and C. Carré, unpublished), *UAS-mir7* [41], *UAS-VP1*^{FL}, and *UAS-VP1*$^{\Delta C74}$ [8]. The *Act-GAL4*, *ptc-GAL4*, and *UAS-IR-AGO1* transgenic flies were obtained from the Bloomington Stock Center (http://flystocks.bio.indiana.edu), and *UAS-IR-Pasha* was obtained from the Vienna Drosophila RNAi Center (http://stockcenter.vdrc.at). *w*^{1118} flies were used as wild-type control. To generate *UAS-VP1*$^{\Delta N351}$ transgenic flies, the VP1$^{\Delta N351}$ sequence, fused to a V5-tag at its the N-terminus, was cloned into the XbaI and SacII restriction sites of the pUAST vector [35]. The resulting plasmid was micro-injected into *Drosophila w*^{1118} embryos (Bestgene).

Virus infections

For survival and titration experiments, two- to four-day-old female flies were injected with 1000 $TCID_{50}$ units of DCV. Subsequently, survival was monitored daily, and flies were transferred to fresh food every two days. Differences in survival were evaluated with a Log-rank (Mantel-Cox) test as implemented in Graph pad prism (version 5).

For DCV titration, three groups of 4-5 flies were collected at 24, 48, and 74 hours post infection (hpi). Flies were homogenized in 300 µL PBS and fly debris was removed twice by centrifugation for 5 minutes at 13.000 rpm. Subsequently, 20 µL supernatant was used to determine the viral titer by end-point dilution on *Drosophila* S2 cells (Invitrogen) [54].

Viral RNA levels in whole flies or fat bodies were determined at 48 hpi using quantitative RT-PCR (qRT-PCR). To this end, RNA from five whole flies or from dissected fat body tissue of five flies was isolated using isol-RNA lysis reagent according to the manufacturer's protocol (5 PRIME). After DNase I treatment, the RNA was used as a template for cDNA synthesis using the Taqman reverse transcription kit (Roche). Subsequently, qPCR was performed using the primer pair DCV-F, 5'-AAAATTTCGTTTTAGCCCAGAA-3' and DCV-R, 5'-TTGGTTGTACGTCAAAATCTGAG-3'. DCV levels were normalized to RNA levels of ribosomal protein 49 (RP49). Primers for amplification of RP49 were RP49-F, 5'-ATGACCATCCGCCCAGCATAC-3' and RP49-R, 5'-CTGCATGAGCAGGACCTCCA-3'.

Western blot detection

For expression of the VP1 constructs in transgenic fly lines, *act-GAL4* or *tub-GAL4* driver lines were crossed with the VP1 responder lines. From the F1 offspring, ten flies lacking the *CyO* and *TM3 Sb* balancers were homogenized in Laemli buffer. Homogenates were centrifuged for 5 minutes at 13.000 rpm to remove insoluble fly material. The supernatant was then analyzed by SDS-PAGE followed by western blot using a V5 antibody (Life technologies) followed by a goat-α-mouse IRdye680 antibody (LI-COR). Western blots were imaged using the LI-COR Odyssey imager.

***In vivo* RNAi assay**

The *th*^{RNAi} experiment was performed as described [8]. Briefly, to assess the effects of VP1 transgenes on RNAi-mediated silencing of *thread*, a *th*^{RNAi} construct was co-expressed with VP1 in the developing eye by crossing *GMR-GAL4*, *UAS-th*^{RNAi}*/CyO* virgins with *UAS-VP1/TM3 Sb* flies. To establish the RNAi dependence of the assay, the *GMR-GAL4*, *UAS-th*^{RNAi}*/CyO* construct was crossed into an *AGO2*^{321} background using standard genetic methods. From the F1 offspring, the eye-phenotype of two- to four-day-old male flies, lacking the *CyO* and *TM3 Sb* balancers, was determined using a Leica EC3 camera mounted on a Leica S6D stereo microscope.

***In vivo* miRNA sensor assay**

For the miRNA sensor assay in flies, the *ptc-GAL4* and *UAS-miR7/CyO* alleles were recombined using standard genetic methods. Subsequently, *ptc-GAL4*, *UAS-miR7/CyO* virgins were crossed with either the *UAS-VSR/TM3 Sb* male flies, or with *UAS-GFP*, *UAS-IR-AGO1*, or *UAS-IR-Pasha* male flies. Two-day-old adult flies lacking the *CyO* balancer were incubated at -20ºC for one day, after which the wings were mounted with Euparal for examination of the phenotype.

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Chapter 5

Species-specific interaction between Argonaute-2 and viral suppressors of RNAi from novel *Drosophila* Nora viruses

Manuscript in preparation:

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Abstract

The ongoing conflict between viruses and their hosts can drive the co-evolution of host immune genes and viral immune suppressors. It has previously been suggested that an evolutionary 'arms race' could be occurring between rapidly evolving components of the *Drosophila* antiviral RNAi pathway and viral genes that antagonize it. Recently, we have shown that *Drosophila melanogaster* Nora virus (DmelNV) is a target of the antiviral RNA interference (RNAi) pathway. As a counter-defense, DmelNV viral protein 1 (VP1) suppresses the RNAi pathway by antagonizing Argonaute-2 (AGO2)-mediated RNA cleavage (slicer activity). Here we show that Nora virus VP1 can develop a species-specific RNAi suppressor activity that depends on the ability to interact with AGO2. We have identified novel Nora-like viruses in wild-caught populations of *D. immigrans* (DimmNV) and *D. subobscura* (DsubNV) that are 36% and 26% divergent from DmelNV at the amino acid level, and have analyzed their interactions with the RNAi machinery from host and non-host species. We show that DimmNV and DsubNV VP1 are unable to suppress RNAi in *D. melanogaster* S2 cells, which is in contrast to the potent RNAi suppressive activity of DmelNV VP1 in these cells. Moreover, we show that the RNAi suppressor activity of DimmNV VP1 is restricted to its natural host species, *D. immigrans*. Specifically, we find that DimmNV VP1 interacts with *D. immigrans* AGO2, but not with *D. melanogaster* AGO2, and suppresses slicer activity in embryo lysates from *D. immigrans*, but not from *D. melanogaster*. Surprisingly, the activity of DmelNV VP1 does not seem to be restricted to its host species, as it interacts with both *D. melanogaster* and *D. immigrans* AGO2 and suppresses slicer activity in both *D. melanogaster* and *D. immigrans* embryo lysates. Our results emphasize the importance of analyzing viral RNAi suppressor activity in the relevant host species. Moreover, these results suggest that rapid co-evolution between Nora viruses and their hosts may result in host species-specific activities of RNAi suppressor proteins, and therefore that viral RNAi suppressors could be host-specificity factors.

Introduction

As obligate intracellular parasites, viruses modulate and exploit the host cellular environment for their replication. The host antiviral defense system restricts virus infections, and in turn, viruses dedicate a significant fraction of their coding capacity to produce virulence factors that antagonize the antiviral immune response. Co-evolution of virus and host may lead to a host-specific adaptation of viral counter-defense mechanisms to the host antiviral defense system, which can contribute to host specificity of the virus [1].

The RNA interference (RNAi) pathway is a major antiviral defense system in plants, arthropods, nematodes and fungi [2-5]. Double stranded RNA (dsRNA), which is typically produced during virus infection but absent from non-infected cells [6], triggers the RNAi pathway. In insects, cleavage of viral dsRNA by the ribonuclease Dicer-2 (Dcr-2) generates viral small interfering RNAs (vsiRNAs) [7-19]. Dcr-2 and its binding partner R2D2 bind these vsiRNAs and subsequently load the small RNA duplexes into an Argonaute-2 (AGO2) containing RNA-induced silencing complex (RISC) [20]. One strand of the vsiRNA is retained and guides the recognition and cleavage of complementary viral RNAs by AGO2 [7,21-24]. In response, insect and plant viruses encode suppressors of RNAi (VSRs) to counteract the antiviral RNAi pathway. Different mechanisms for RNAi suppression have been identified, for example some VSRs bind long dsRNA and/or siRNAs to shield them from Dicer cleavage or prevent their loading into Argonaute, respectively [7,25-33]. Other suppressors interact with Argonaute proteins to inhibit their activity or induce their degradation [10,34-40].

The ongoing arms race with viruses can impose a strong selective pressure on immune genes of the host [41]. Strikingly, *Dcr-2*, *R2D2*, and *AGO2* belong to the 3% fastest evolving genes in *D. melanogaster* and *D. simulans*, showing very high rates of adaptive amino acid substitution and recent selective sweeps in multiple *Drosophila* species [42-44]. It has been hypothesized that this rapid adaptive evolution may be driven by antagonistic co-evolution with viral suppressors of RNAi [45], as the RNAi pathway continues to evolve new ways to escape viral antagonists, leading to counter-adaptations by viruses that require further adaptations in the RNAi pathway of the host. A potential outcome of this ever-lasting antagonistic co-evolution is that viral RNAi suppressors become specialized to suppress RNAi in their host species, while losing this activity in non-host species. This may be unlikely for viral antagonists that bind dsRNA, which often efficiently suppress RNAi in both host and non-host species, and in some cases even across kingdoms [46-50]. However, when viruses antagonize protein components of the RNAi pathway, there is ample opportunity for co-evolution and the evolution of host-specificity.

Nora virus of *Drosophila melanogaster* (DmelNV) is a natural fruit fly pathogen

that causes a persistent infection in laboratory stocks as well as in wild caught flies [51]. Persistent infections are thought to reflect a dynamic equilibrium between host defense responses and viral counter-defense mechanisms [52]. The widespread abundance and persistent nature of DmelNV infections may suggest a metastable equilibrium between antiviral RNAi responses and viral counter-defense mechanisms. Indeed, we recently showed that DmelNV is a target and a suppressor of the antiviral RNAi pathway [10]. We identified viral protein 1 (VP1), the product of open reading frame 1, as an RNAi suppressor that counteracts AGO2-mediated target RNA cleavage (Slicer activity).

Here we present two novel Nora-like viruses identified by metagenomic sequencing of wild populations of *D. immigrans* (DimmNV) and *D. subobscura* (DsubNV), and use these viral genomes to study RNAi antagonism from an evolutionary perspective. We find that DmelNV VP1 does not display host specificity: DmelNV VP1 co-localizes and interacts with the AGO2 protein from its natural host *D. melanogaster*, as well as from non-host *D. immigrans*. In accordance, DmelNV VP1 suppressed slicer activity in embryo lysates from *D. melanogaster* as well as from *D. immigrans*. In contrast, we find that the RNAi suppressor activity of DimmNV VP1 is restricted to its natural host species, *D. Immigrans*: DimmNV VP1 was unable to interact with *D. melanogaster* AGO2 (Dmel AGO2), but specifically interacted with AGO2 from its natural host, *D. immigrans* (Dimm AGO2). In line with this observation, we show that recombinant DimmNV VP1 protein inhibits Slicer activity in embryo lysates of its host *D. immigrans*, but not in lysates of its non-host *D. melanogaster*. We conclude that co-evolution between Nora viruses and their *Drosophila* hosts can result in a host species-specific interaction with, and antagonism of, AGO2, and therefore that viral suppressors of RNAi are candidate host specificity determinants.

Results

Dmel Nora virus VP1 co-localizes with Dmel AGO2

We recently showed that DmelNV VP1 inhibits RNA cleavage (slicer) activity of a pre-assembled RISC in *D. melanogaster* [10], which suggests that VP1 and mature RISC reside in the same cellular compartment. To determine the subcellular localization of VP1 and AGO2, the catalytic core component of RISC, we generated recombinant full-length VP1 protein fused to the enhanced green fluorescent protein at its C-terminus (VP1-EGFP). We first verified that VP1-EGFP was competent in suppressing RNAi using a reporter assay in S2 cells. In contrast to a control vector, VP1-EGFP protein potently suppressed siRNA-induced silencing of a firefly luciferase reporter, indicating that this fusion protein is a functional RNAi suppressor (Figure S1). We next determined the subcellular localization of DmelNV VP1 and Dmel AGO2 in S2 cells after co-

transfection of the VP1-EGFP expression plasmid along with AGO2 expression plasmids. We used two AGO2 constructs, one containing the genomic sequence [53], the other containing the cDNA sequence of AGO2 [54], both fused to a FLAG epitope at their N-termini. VP1-EGFP localized in perinuclear foci in the cytoplasm (Figure 1). Both FLAG-AGO2 constructs showed a diffuse cytoplasmic pattern with enrichment at cytoplasmic foci. Merging the two detector channels demonstrated that some of the VP1-EGFP foci overlap with the FLAG-AGO2 foci. This co-localization suggests that VP1 may directly interact with AGO2 to exert its suppressor activity.

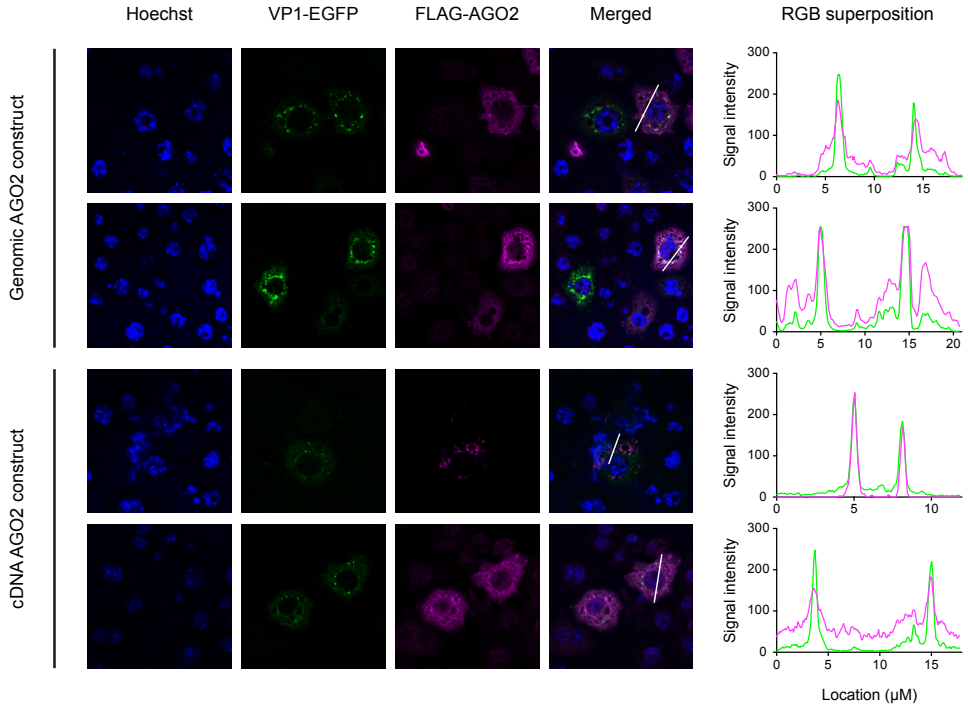

Figure 1: DmelNV VP1 co-localizes with Dmel AGO2 in S2 cells. Confocal images of S2 cells expressing full-length VP1-GFP fusion protein together with FLAG-tagged Dmel AGO2 protein. AGO2 was expressed either from a cDNA sequence or from a genomic sequence. After transfection, AGO2 was detected by immunofluorescence using the anti-FLAG antibody. Nuclei were visualized using Hoechst staining. Right panels show superposition of red and green signal along the marked line in the merged pictures.

Dmel Nora virus VP1 interacts with Dmel AGO2

To investigate a physical interaction between VP1 and AGO2, we analyzed DmelNV VP1 immunoprecipitations (IPs) for the presence of AGO2. To this end, we transfected S2 cells with an active V5 epitope-tagged VP1 construct (V5-VP1) that encodes the C-terminal 124 amino acids of VP1 along with a FLAG-tagged AGO2 cDNA construct. IP of V5-VP1 resulted in specific co-precipitation of the FLAG-AGO2 protein (Figure 2A). In contrast, a V5-control vector failed to co-purify FLAG-AGO2. To confirm the interaction between VP1 and AGO2, we performed the reverse experiment. IP of

FLAG-AGO2 protein co-precipitated V5-VP1, while a FLAG-control vector was unable to do so (Figure 2B). Although the interaction between VP1 and AGO2 is evident, only a minor fraction of VP1 was immunoprecipitated along with AGO2. This observation is in agreement with our microscopic analyses, in which only a small fraction of FLAG-AGO2 protein co-localizes with VP1-EGFP (Figure 1). To confirm these results, we immunoprecipitated V5-VP1 protein and probed for endogenous AGO2 in the IP fraction. As expected, we observed a strong enrichment of endogenous AGO2 protein after VP1 IP, whereas IP of cells transfected with V5-control plasmid did not co-precipitate AGO2 protein (Figure 2C). These results indicate that DmelNV VP1 interacts with Dmel AGO2 in *Drosophila* S2 cells.

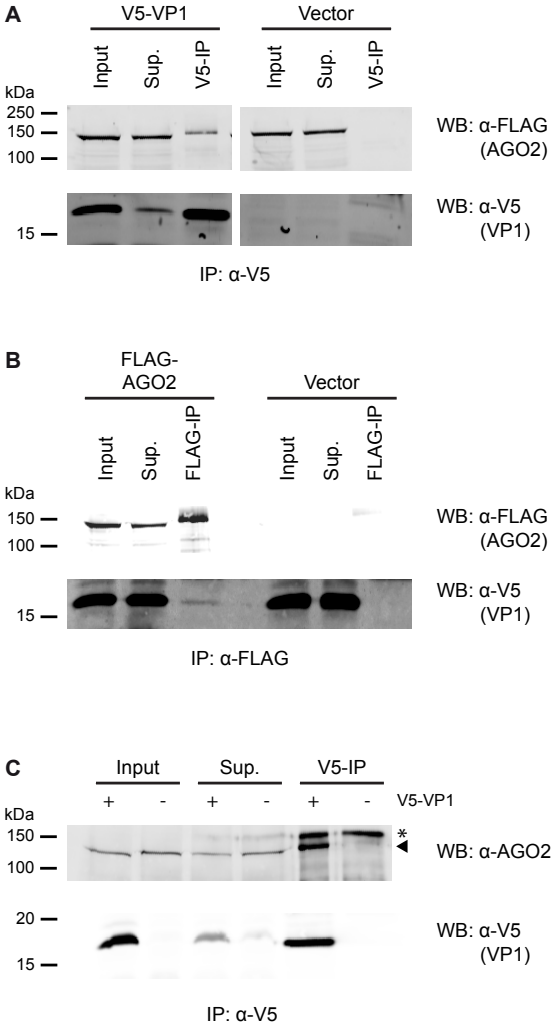

Figure 2: DmelNV VP1 interacts with Dmel AGO2 in S2 cells. (**A**) Western blot (WB) analysis of V5 immunoprecipitation on lysates from S2 cells transfected with a FLAG-AGO2 expression plasmid and either V5-tagged DmelNV VP1$^{\Delta N351}$ (V5-VP1) or V5-control plasmid (Vector). The epitope-tagged proteins were detected in the input, supernatant after IP (Sup), and the immunoprecipitate (V5-IP) with the indicated antibodies. (**B**) FLAG immunoprecipitation of lysates from S2 cells transfected with V5-tagged DmelNV VP1$^{\Delta N351}$ (V5-VP1) and either FLAG-AGO2 or FLAG-control plasmids (Vector), followed by SDS-PAGE, and western blotting with the indicated antibodies. (**C**) V5 immunoprecipitation of lysates from S2 cells transfected with V5-tagged DmelNV VP1$^{\Delta N351}$ (+) or V5-control (-) plasmids. After SDS-PAGE, endogenous AGO2 or DmelNV VP1$^{\Delta N351}$ proteins were detected by western blot using anti-AGO2 (α-AGO2) and anti-V5 (α-V5) antibody, respectively. Asterisk (*) indicates a non-specific background band, and triangle indicates AGO2.

Identification of novel Nora-like viruses from *D. immigrans* and *D. subobscura*

AGO2 evolves rapidly and adaptively in multiple species of *Drosophila* [42,43]. We therefore hypothesized that the AGO2-VP1 interaction may also evolve rapidly as Nora-like viruses adapt to different hosts. In particular, optimization of host AGO2-VP1 interaction may come at the cost of losing efficient AGO2-VP1 interactions in non-host species. As a consequence, viral suppressors of RNAi are candidate host specificity determinants. To test whether the AGO2-VP1 interaction is host-specific, we set out to identify novel Nora-like viruses from divergent *Drosophila* species.

During an exploratory RT-PCR survey of Nora virus prevalence in wild *Drosophila*, we identified the presence of two novel Nora-like viruses in wild populations of *D. immigrans* (DimmNV) and *D. subobscura* (DsubNV). Following this, we took a

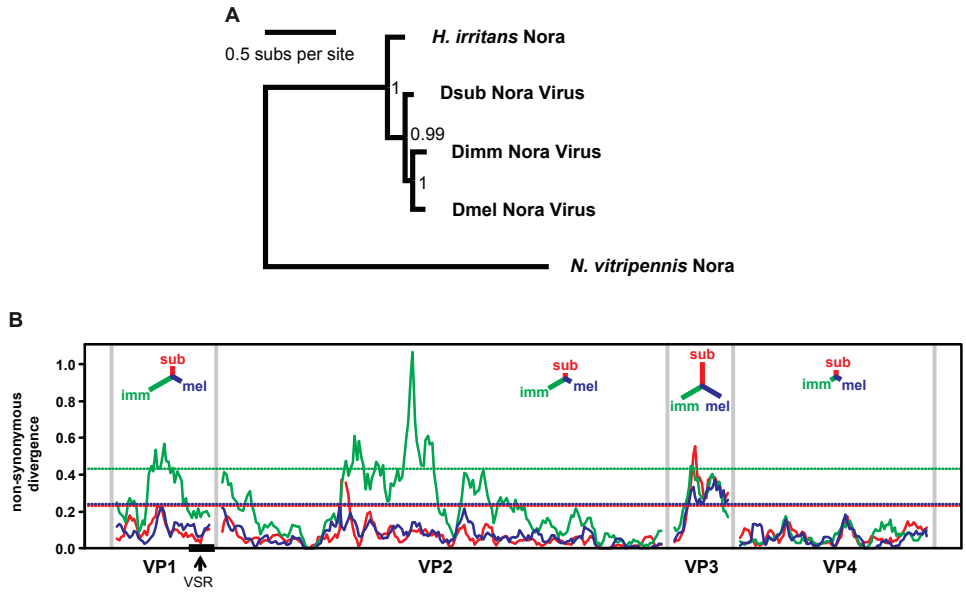

Figure 3: Phylogenetic analysis and non-synonymous divergence between Nora viruses. (**A**) Phylogenetic analysis of the most conserved Nora virus gene (VP4) suggests that the three *Drosophila* Nora viruses are each other's closest relatives, and that they are all closely related to the Nora-like sequence derived from *Haematobia irritans*. Note that DimmNV is much more divergent from DsubNV and DmelNV in VP1 and VP2 (which are not available for the other two viruses), and that although DimmNV appears to be most closely related to DmelNV based on VP4, the extreme divergence from the *Nasonia* Nora sequence may make the rooting unreliable. The tree presented is the mid-point rooted Bayesian maximum *a posteriori* tree (99% of the posterior set) inferred under a protein substitution model. (**B**) A sliding-window analysis of nonsynonymous divergence between the three *Drosophila* Nora viruses, calculated as the number of nonsynonymous substitutions per nonsynonymous site in windows of 50 codons wide, is plotted against the position along the protein-coding regions of the viral genome. Dashed lines show a nominal 95% significance threshold for genome-wide peaks in divergence derived from randomisation tests, such that peaks crossing the line are unlikely to occur by chance, given the overall divergence for that virus. In addition, inset for each viral protein is an unrooted tree with branch lengths proportional to overall divergence for that gene. Note that DimmNV is more divergent for VP1 and VP2 than for VP3 or VP4, while VP3 shows the highest overall divergence between the three viruses.

metagenomic RNA-sequencing approach to recover near-complete viral genomes for both viruses (lengths 12,265 nt and 12,276 nt) from population samples of *D. immigrans* and *D. subobscura* collected in the United Kingdom. These novel viruses are more closely related to the Nora virus originally identified in *D. melanogaster* (DmelNV) [51] than to the Nora-like virus recently described from the fly *Haematobia irritans* [55], or the much more distantly related Nora-like virus described from the wasp *Nasonia vitripennis* [56] (Figure 3A). Overall, DmelNV is more divergent from DimmNV than it is from DsubNV (65% vs. 71% overall base identity, respectively), but phylogenetic analysis based on the coat protein VP4 suggests that DmelNV and DimmNV may be each other's closest relatives. Divergence between the three viruses is too high to reliably estimate the true number of synonymous substitutions per codon [57,58], and the low nonsynonymous to synonymous substitution ratio (dN/dS=0.076, SE=0.003) may therefore represent an upper limit. Amino-acid divergence between the viruses varies substantially between genes (Figure 3B). For example, amino-acid identity between DimmNV and DmelNV varies from 82% for VP4 to only 43% for VP3 (unknown function), with VP1 showing an intermediate level of conservation (51% amino acid identity). A sliding-window analysis of nonsynonymous divergence shows that DmelNV and DsubNV are more similar to each other than to DimmNV in VP1 and VP2, but that the three viruses are equidistant from each other in VP3 and VP4. This may be a result of host-mediated selection, reflecting the closer relationship between *D. melanogaster* and *D. subobscura*, or it may be a result of recombination in the history of these three viruses.

VP1 of Dimm and Dsub Nora-like virus do not suppress RNAi in *D. melanogaster* S2 cells.

To test whether there is host-specificity in the interaction between antiviral RNAi components and viral RNAi antagonists, we analyzed whether the DimmNV and DsubNV VP1 proteins are able to suppress RNAi in the S2 cell line from *D. melanogaster*. To this end, we cloned the full-length (FL) VP1 sequences and N- and C-terminal deletion mutants thereof (ΔN and ΔC) as N-terminal fusions to the V5 epitope in an insect expression plasmid (Figure S2). We verified expression of the DimmNV VP1 constructs by western blot after transfection in *Drosophila* S2 cells (Figure 4A). With the exception of the DimmNV VP1$^{\Delta N362}$, all DimmNV VP1 constructs were expressed at least at the level of DmelNV VP1^{FL} that efficiently suppresses RNAi in reporter assays in S2 cells [10]. We then analyzed the ability of the DimmNV VP1 constructs to suppress RNAi in reporter assays. We transfected S2 cells with FLuc reporter plasmids, along with VP1 expression plasmids and induced silencing of the FLuc reporter by soaking the cells in FLuc specific dsRNA. As reported earlier [10], all DmelNV VP1 constructs, except DmelNV VP1$^{\Delta C74}$, suppressed RNAi-mediated silencing of the FLuc reporter. In contrast, none of the DimmNV VP1 constructs

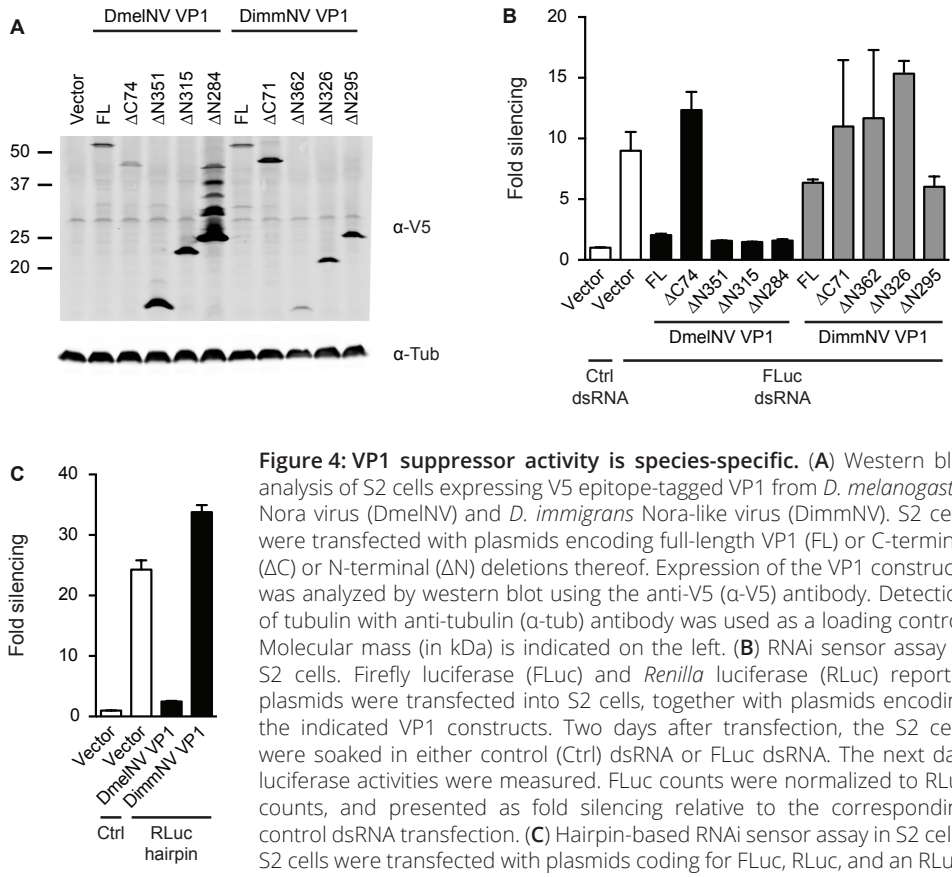

Figure 4: VP1 suppressor activity is species-specific. (**A**) Western blot analysis of S2 cells expressing V5 epitope-tagged VP1 from *D. melanogaster* Nora virus (DmelNV) and *D. immigrans* Nora-like virus (DimmNV). S2 cells were transfected with plasmids encoding full-length VP1 (FL) or C-terminal (ΔC) or N-terminal (ΔN) deletions thereof. Expression of the VP1 constructs was analyzed by western blot using the anti-V5 (α-V5) antibody. Detection of tubulin with anti-tubulin (α-tub) antibody was used as a loading control. Molecular mass (in kDa) is indicated on the left. (**B**) RNAi sensor assay in S2 cells. Firefly luciferase (FLuc) and *Renilla* luciferase (RLuc) reporter plasmids were transfected into S2 cells, together with plasmids encoding the indicated VP1 constructs. Two days after transfection, the S2 cells were soaked in either control (Ctrl) dsRNA or FLuc dsRNA. The next day, luciferase activities were measured. FLuc counts were normalized to RLuc counts, and presented as fold silencing relative to the corresponding control dsRNA transfection. (**C**) Hairpin-based RNAi sensor assay in S2 cells. S2 cells were transfected with plasmids coding for FLuc, RLuc, and an RLuc-hairpin together with a control vector (Vector) or constructs expressing N-terminal deletion mutants of DmelNV VP1 (DmelNV VP1$^{\Delta N285}$) or DimmNV VP1 (DimmNV VP1$^{\Delta N281}$). RLuc counts were normalized to FLuc counts, and presented as fold silencing over non-hairpin control transfections. Bars in Panels B and C represent means and standard deviations of three independent biological replicates.

efficiently suppressed silencing of the reporter (Figure 4B). To confirm these results, we used an RNAi sensor assay that is independent of dsRNA uptake by S2 cells. In this sensor assay, the RLuc reporter is silenced by expression of an inverted repeat that folds into an RLuc-specific RNA hairpin. In line with the previous RNAi sensor assay, DimmNV VP1 did not suppress hairpin-induced silencing of the RLuc reporter in *D. melanogaster* S2 cells, whereas DmelNV VP1 efficiently suppressed RNAi (Figure 4C). In addition, we tested if the VP1 constructs can suppress RNAi in a sensor assay in which silencing is induced by co-transfection of siRNAs. Also in this assay, DimmNV VP1 was unable to suppress silencing of the FLuc reporter, whereas DmelNV VP1 efficiently suppressed RNAi-based silencing (Figure S3C). Similarly, the DsubNV VP1 constructs were unable to suppress long dsRNA- or siRNA-induced RNAi in *D. melanogaster* derived S2 cells (Figure S3A-C).

Dimm Nora virus VP1 inhibits Slicer activity in its natural host species, *D. immigrans*

The inability of DimmNV VP1 and DsubNV VP1 to suppress RNAi in *Drosophila* S2 cells may be explained in two ways. First, viral RNAi suppressors may have a species-specific activity, following the prediction that RNAi antagonists efficiently suppress RNAi in host species, but not in non-host species. Second, some Nora-like viruses may either be unable to suppress RNAi, or they may encode RNAi suppressor activity in different regions of the viral genome, as was previously observed for members within a single plant virus family [59-61]. To address the first possibility, we tested the ability of DimmNV VP1 and DmelNV VP1 to suppress RNAi in both host species using *in vitro* RNA cleavage (Slicer) assays [62] in lysates of embryos from *D. melanogaster* and *D. immigrans*. We excluded *D. subobscura* and DsubNV from this and subsequent analyses, as members of the obscura group encode multiple AGO2-like proteins, for which the biochemical and antiviral activity remain to be established [63].

In slicer assays, RNAi dependent cleavage of a 5′-^{32}P cap-labelled target RNA is induced by the addition of a target specific siRNA. Since the target RNA is radio-labeled at its 5′ cap, the 5′ cleavage product can be visualized by autoradiography after polyacrylamide gel electrophoresis. As expected, in both *D. melanogaster*

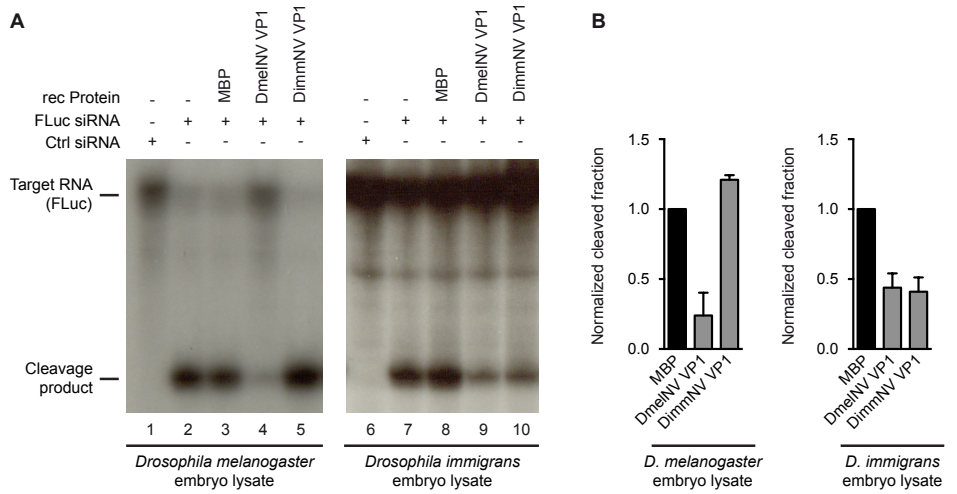

Figure 5: Species-specific inhibition of AGO2 slicer activity. (**A**) In vitro RNA cleavage (slicer) assays in lysates from *D. melanogaster* embryos (left panel) or *D. immigrans* embryos (right panel). Radioactively cap-labelled target RNA was incubated in embryo lysate together with a non-specific control siRNA (lanes 1,6) or a target specific siRNA (lanes 2-5,7-10). Target cleavage was determined either in the absence of recombinant protein (lanes 2,7) or in the presence of 0.3 µM of MBP (lanes 3,8), MBP-DmelNV VP1$^{\Delta N284}$ (lanes 4,9), or DimmNV VP1$^{\Delta N295}$ protein (lanes 5,10). (B) Quantification of target cleavage in *D. melanogaster* and *D. immigrans* embryo lysate in the presence of MBP, DmelNV VP1$^{\Delta N284}$, or DimmNV VP1$^{\Delta N295}$ protein. The fraction of cleaved RNA was normalized to MBP and determined by dividing the intensity of the cleavage product by the total intensity of cleavage product and non-cleaved target. Bars represent means and standard deviations of two independent experiments.

and *D. immigrans* embryo lysates a specific cleavage product was observed after incubation with a target specific siRNA (Figure 5A, lanes 2 and 7). In line with our earlier report [10], recombinant DmelNV VP1 protein potently inhibited cleavage of the target RNA in *D. melanogaster* embryo lysate, whereas the control, Maltose Binding Protein (MBP), was unable to do so (Figure 5A, compare lanes 3 and 4). In contrast, recombinant DimmNV VP1 protein did not inhibit Slicer activity in *D. melanogaster* embryo lysate (Figure 5A, lane 5), which is in line with our observation that DimmNV VP1 did not suppress RNAi in cell-based reporter assays in *D. melanogaster* cells (Figure 4). Surprisingly, in the *D. immigrans* embryo lysate both the DmelNV VP1 and the DimmNV VP1 protein inhibited target RNA cleavage (Figure 5A, lanes 9 and 10). As expected, the MBP control protein did not inhibit Slicer activity (Figure 5A, lane 8). Quantification of independent experiments indicates that both DmelNV and DimmNV VP1 proteins suppressed Slicer activity to a similar extent in the *D. immigrans* embryo lysate (Figure 5B). These results, together with those from the cell-based reporter assays indicate that DimmNV VP1 inhibits Slicer activity in its natural host *D. immigrans*, but is unable to suppress RNAi in a heterologous *D. melanogaster* background. In contrast, DmelNV VP1 inhibits Slicer activity in both a *D. melanogaster* and a *D. immigrans* background.

Species-specific interaction between DimmNV VP1 and Dimm AGO2

The results shown in figures 1 and 2 indicate that DmelNV VP1 interacts with Dmel AGO2 to suppress its Slicer activity. Similarly, given the observation that DimmNV VP1 suppresses slicer activity in *D. immigrans* lysates, it is likely that DimmNV VP1 interacts with Dimm AGO2. We hypothesized that the inability of DimmNV VP1 to suppress RNAi in *D. melanogaster* may then be due to an inefficient interaction with Dmel AGO2. To test these hypotheses, we analyzed VP1 interactions with host and non-host AGO2 proteins by co-immunoprecipitations. First, we co-expressed DmelNV VP1 or DimmNV VP1 with a Dmel FLAG-AGO2 construct in S2 cells and immunopurified the VP1 proteins using V5 affinity beads. As controls, we analyzed IPs of cells transfected with empty vector. As observed above (Figure 2), IP of DmelNV VP1 co-precipitated Dmel FLAG-AGO2 protein. In contrast, IP of DimmNV VP1 did not enrich for Dmel FLAG-AGO2 in the IP fraction, compared to IP of the V5-control vector (Figure 6A). To confirm these results, we analyzed the interaction between VP1 proteins and endogenous *D. melanogaster* AGO2. Similar to our observations in Figure 2, DmelNV VP1 co-immunoprecipitated endogenous Dmel AGO2, whereas the control vector failed to do so (Figure 6B). In contrast, DimmNV VP1 failed to co-IP Dmel AGO2, which mirrors our observation with epitope-tagged Dmel AGO2. These observations imply that the inability of DimmNV VP1 to suppress RNAi in *D. melanogaster* is due to its inability to efficiently interact with Dmel AGO2.

We then analyzed the interaction of DimmNV VP1 with Dimm AGO2. To this end,

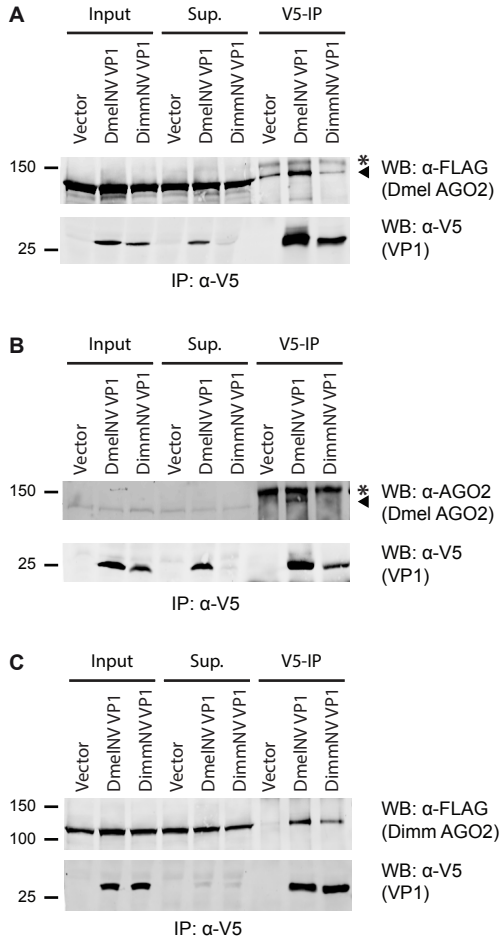

Figure 6: Species-specific interaction between VP1 and AGO2. (**A**) V5 Immunoprecipitation (V5-IP) of lysates from S2 cells transfected with FLAG-tagged Dmel AGO2 expression plasmid and either V5-tagged DmelNV VP1$^{\Delta N284}$, DimmNV VP1$^{\Delta N295}$, or V5-control plasmids. Input, supernatant after IP (Sup.), and the immunoprecipitate (V5-IP) were analyzed by western blot (WB) using anti-V5 (α-V5) or anti-FLAG (α-FLAG) antibodies. (**B**) V5 immunoprecipitation of S2 cells transfected with plasmids encoding V5-tagged DmelNV VP1$^{\Delta N284}$, DimmNV VP1$^{\Delta N295}$, or V5-control vector. Fractions were analyzed by western blot (WB) using antibodies for endogenous AGO2 (α-AGO2) and V5 (α-V5). (**C**) V5 immunoprecipitation on lysates from S2 cells co-transfected with plasmids encoding FLAG-tagged Dimm-AGO2 and either V5-tagged DmelNV VP1$^{\Delta N284}$, DimmNV VP1$^{\Delta N295}$, or V5-control vector. VP1 and Dimm-AGO2 proteins were detected on western blot (WB) using anti-V5 (α-V5) and anti-FLAG (α-FLAG) antibodies, respectively. Asterisk (*) indicates a non-specific background band, and triangle indicates AGO2.

we cloned the *D. immigrans* AGO2 cDNA sequence downstream of FLAG (Dimm FLAG-AGO2). As expected, the predicted protein domains of Dimm FLAG-AGO2 are similar to those of Dmel AGO2, suggesting that the overall protein structure of Dimm and Dmel AGO2 are alike. We thus analyzed the interaction of DmelNV VP1 or the DimmNV VP1 with Dimm FLAG-AGO2 in co-IP. Both DmelNV VP1 and DimmNV VP1 efficiently co-purified the Dimm-AGO2 protein (Figure 6C). These results show that AGO2-VP1 interactions correlate with RNAi suppressor activity: DmelNV VP1 interacts with both Dmel and Dimm AGO2 and suppresses slicer activity of these hosts; DimmNV VP1 interacts with Dimm AGO2, but not Dmel AGO2, and suppresses slicer activity in *D. immigrans*, but not in *D. melanogaster*.

DimmNV VP1 specifically suppresses Dimm AGO2 activity

The species-specific interaction of DimmNV VP1 with Dimm AGO2 suggests that this interaction is the major determinant for the observed species specificity

in Slicer activity. To test this hypothesis, we set out to reconstitute Dimm AGO2-based silencing in *D. melanogaster* S2 cells and analyze whether DimmNV VP1 could suppress this reconstituted pathway. To this end, we knocked-down endogenous AGO2 expression in *D. melanogaster* S2 cells, and rescued its activity with either the Dmel AGO2 or Dimm AGO2 cDNA construct.

First, we assessed the efficacy of knockdown of AGO2 expression in S2 cells using dsRNA against the coding sequence (CDS) or the 3' untranslated region (3' UTR) of the endogenous Dmel AGO2 transcript. To monitor AGO2 activity in these S2 cells we induced RNAi with the RLuc-specific RNA hairpin. Compared to a non-specific dsRNA control, dsRNAs against the CDS or the 3'UTR of AGO2 severely reduced hairpin-induced silencing of the RLuc reporter (Figure 7A). This experiment thus creates the opportunity to knockdown endogenous AGO2 expression with UTR-targeting dsRNA and rescue silencing defects with Dmel AGO2 or Dimm AGO2 cDNA constructs, which lack the AGO2 3'UTR sequence and are therefore not targeted by the RNAi approach. Strikingly, both Dmel AGO2 and Dimm AGO2 rescued silencing activity in *D. melanogaster* cells, whereas Dmel AGO1 or the control vector were unable to do so (Figure 7B). These results indicate that Dimm AGO2 is fully functional in a *D. melanogaster* background and that the limited sequence identity to Dmel AGO2 (only 63% amino-acid identity, excluding the glutamine-rich region) does not impede its ability to interact with Dmel Dcr-2 and R2D2 or other components of the *D. melanogaster* RISC complex.

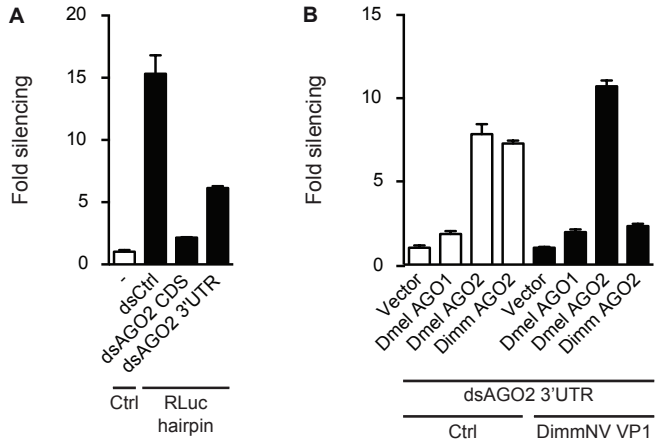

Figure 7: DimmNV VP1 inhibits Dimm AGO2 function. (**A**) RNAi reporter assay using hairpin-induced silencing of an RLuc reporter that shows the AGO2 dependence of the RLuc hairpin RNAi reporter assay. The experiment was performed as described in Figure 3D, only a non-specific control dsRNA (dsCtrl) or dsRNA targeting the coding sequence or the 3'UTR of Dmel AGO2 (dsAGO2 CDS and dsAGO2 3'UTR, respectively) was co-transfected along with the reporter plasmids. (**B**) Rescue of endogenous AGO2 knockdown in RLuc hairpin RNAi reporter assay. Endogenous AGO2 expression was knocked-down by transfection of dsRNA targeting the AGO2 3'UTR along with the reporter plasmids (described in Figure 3D) and with control plasmid (Vector), or expression plasmids encoding *D. melanogaster* AGO1 (Dmel AGO1) or AGO2 (Dmel AGO2), or *D. immigrans* AGO2 (Dimm-AGO2). Control vector (-) or a plasmid encoding *D. immigrans* Nora virus VP1 (DimmNV VP1$^{\Delta N281}$) was co-transfected. Bars in all panels represent means and standard deviations of three biological replicates.

Using this AGO2 rescue assay, we investigated whether DimmNV VP1 suppressed Dmel AGO2 and Dimm AGO2-mediated silencing. DimmNV VP1 expression did not impede Dmel Ago2-mediated RNAi (Figure 7B), which is in line with our observations that DimmNV VP1 did not inhibit RNAi in *D. melanogaster* S2 cells (Figure 4A). In contrast, we observed that Dimm AGO2-mediated silencing was efficiently suppressed by DimmNV VP1 (Figure 7B).
We were unable to analyze DmelNV VP1 in this assay, as its potent RNAi suppressive activity would impede silencing of endogenous Dmel AGO2, which is required for this assay. Together, these results indicate that the interaction of VP1 with AGO2 is the major determinant for its RNAi suppressive activity. Moreover, these data imply that the VP1-AGO2 interaction is a major determinant for the species-specific effects of VP1.

Discussion

Viruses and hosts engage in an ongoing arms race in which viral counter-defense mechanisms drive the adaptive evolution of host immune genes, which in turn requires ongoing counter-adaptations in viral immune antagonists [1,41]. This ever-lasting cycle of adaptations and counter-adaptations may result in species-specific interactions between virus and host [41,64].
The antiviral RNAi genes *R2D2*, *Dcr-2* and *AGO2* belong to the 3% fastest evolving genes of *Drosophila melanogaster* and show evidence of positive selection in multiple species [42]. Strikingly, rapid evolution is observed in the antiviral RNAi pathway, whereas the microRNA pathway does not show evidence for rapid evolution. It is therefore possible that antagonistic host-parasite interactions – either through prolonged coevolution or through invasion by novel pathogens – are responsible for the observed rapid adaptive evolution in RNAi genes.
Nora virus is a positive stranded RNA virus that was recently identified in laboratory stocks of *Drosophila melanogaster* [51]. Its unique genome organization and capsid structure suggests that Nora virus is the founding member of a novel virus family [65]. We report here that divergent Nora-like virus sequences are found in wild-caught *D. immigrans* and *D. subobscura* flies. Together with the recent isolation of Nora-like virus sequences from the horn fly *Haematobia irritans* and the parasitoid wasps *Nasonia vitripennis* and *N. giraulti*, these observations suggest that Nora virus is a member of a large family of widespread pathogens that infects multiple insect species [55,56].
Although little is known regarding the natural host range of Nora viruses, it is worth noting that neither of our population samples of *D. immigrans* and *D. subobscura* showed sequences derived from the alternative viral lineages (i.e. DmelNV was

not identified in *D. immigrans* or *D. subobscura*, and similarly for the other Nora-like viruses), despite being collected in mixed samples containing multiple species of *Drosophila*. It is therefore possible that, as is the case for the purely vertically transmitted Sigma viruses, Nora viruses move between hosts very rarely [66].

Plant and insect viruses can suppress the antiviral RNAi pathway of their hosts via a variety of mechanisms [7,10,25,30,33,38,67,68]. Recently, we showed that Nora virus VP1 suppresses RNAi by inhibiting AGO2 slicer activity of a pre-assembled RISC [10]. In this report we show that the RNAi suppressor activity of VP1 from Nora-like viruses can be species-specific and that its RNAi suppressive activity correlates with its ability to interact with AGO2. DimmNV VP1 efficiently interacts with Dimm AGO2 and suppresses AGO2-mediated target cleavage (slicer) activity in *D. immigrans* embryo lysates. In contrast, DimmNV VP1 was unable to suppress RNAi in *D. melanogaster* cells, did not interact with Dmel AGO2, and did not inhibit Slicer activity in *D. melanogaster* embryo lysates. These results are consistent with a model in which adaption and co-evolution of DimmNV with its host resulted in a species-specific AGO2-VP1 interaction.

Our findings have important practical implications. Experimentally amenable model systems, such as *Drosophila melanogaster* or *Arabidopsis thaliana* are often used to identify and characterize viral suppressors of RNAi, including in viruses that naturally do not infect these hosts. Our observation that RNAi suppressor proteins may have species-specific activity, indicates that it is important to take into account the correct evolutionary context in experiments aimed at the identification of viral suppressors of RNAi. For example, we note that we would not have been able to detect RNAi suppressive activity in DimmNV, if we had solely relied on experiments in *D. melanogaster*.

In striking contrast to DimmNV, DmelNV VP1 did not show a species-specific interaction. It can engage in an interaction with both Dimm and Dmel AGO2 and, accordingly, it inhibited slicer activity in both *D. immigrans* and *D. melanogaster* embryo lysates. We suggest that there are two potential explanations for this. First, it may be that the viruses differ in natural host range, and if DmelNV has a wider host range than DimmNV then the broader-spectrum functionality of its VSR across divergent hosts could be maintained by selection. Although none of these three viruses was identified from the other host species, DmelNV has been isolated from wild *Drosophila simulans* (DJO, unpublished data). Second, if there is not a substantial trade-off associated with host-specialisation and if DmelNV has colonised *D. melanogaster* quite recently, it could just be a matter of time until DmelNV loses its broad-spectrum VSR.

Strikingly, the species-specific interaction between AGO2 and VP1 is the sole determinant for the species-specific activity of DimmNV VP1. As we were able to reconstitute Dimm AGO2-based silencing in *D. melanogaster* cells, it indicates that

Dimm AGO2 is fully functional in a D. melanogaster background and that the limited amino acid identity with Dmel AGO2 (~64%) does not impede its ability to interact with Dmel Dicer-2 and R2D2 or other components of the RISC complex. Thus, even though RNAi genes are rapidly evolving and show high rates of adaptive substitution, these results imply that this diversification has not impeded cross-species interactions of RNAi genes, even over the tens of millions of years that separate *D. melanogaster* and *D. immigrans*. This conservation of function may imply that the need for interaction between Dicer-2, R2D2, AGO2, and other RNAi-pathway genes imposes a constraint on the evolution of these genes, and thus their opportunity to evolve in response to virus-mediated selection.

Materials and Methods

Identification and sequencing of novel Nora-like viruses

While surveying wild *Drosophila* populations for the prevalence of Nora virus using RT-PCR (unpublished data; PCR primers: forward 5'-GACCATTGGCACAAATCACCATTTG-3', reverse 5'-TCTTAGGCCGGTTGTCTTCACCC-3') we unexpectedly identified Nora Virus-like PCR products from *D. immigrans* and from members of the obscura group (sampled in Edinburgh, UK; longitude 55.928N, latitude 3.170W). Following this, we took a metagenomic approach to obtain near-complete viral genomes. Flies were collected from elsewhere in the UK, and samples pooled by species for RNA extraction and Illumina double-stranded nuclease normalized RNA-sequencing. For *D. subobscura*, only male flies were used as females are difficult to distinguish morphologically from close relatives. RNA was extracted from each collection using a standard Trizol (Invitrogen) procedure, according to the manufacturer's instructions, and pooled in proportion to the number of contributing flies. In total, the two pools comprised 338 male *D. subobscura* (60 flies July 2011 Edinburgh 55.928N, 3.170W; 60 flies October 2011 Edinburgh 55.928N, 3.170W; 38 flies July 2011 Sussex 51.100N, 0.164E; 180 flies August 2011 Perthshire 56.316N, 3.790W) and 498 *D. immigrans* (63 flies, July 2011 Edinburgh 55.928N, 3.170W; 285 flies July 2011 Edinburgh N55.921, W3.193; 150 flies July 2011 Sussex 51.100N, 0.164E). Total RNA was provided to the Beijing Genomics Institute (Hong Kong) for normalization and 100nt paired-end Illumina sequencing. Paired-end reads were quality trimmed using ConDeTri version 2 [69] and assembled *de novo* using the Trinity transcriptome assembler with default settings (r2011-08-20; [70]). We used tblastn with a DmelNV protein query to identify two partially overlapping Nora-like contigs from *D. immigrans*, and a single contig from *D. subobsura*. Quality-trimmed paired-end reads were mapped back to these contigs using stampy (version 1.0.21: [71]) to obtain a consensus sequence, based

on majority-calls at each position. In total, 286,242 reads mapped to DimmNV (0.45% of all reads, median read depth 1200-fold) and 68,914 reads mapped to DsubNV (0.13% of all reads, median read depth 133-fold). Consensus sequences have been submitted to Genbank under accession numbers KF242510 (DsubNV) and KF242511 (DimmNV).

Tree inference and sequence analysis

The relationship between *Nasonia vitripennis* Nora-like virus (Genbank FJ790488; [56]), *Haematobia irritans* Nora-like virus (Genbank HO004689, HO000459, and HO000794; [55]), DmelNV (Genbank NC_007919.3; [65]), DsubNV, and DimmNV was inferred from VP4 (capsid protein), which is the most conserved gene and the one with the most coverage in the non-Drosophila sequences. For the *N. vitripennis* Nora-like virus we selected the longest sequence (FJ790488) for inclusion here. Phylogenetic inference used MrBayes (v3.2.1: [72]) with discrete gamma-distributed rate variation and model-jumping between fixed-rate amino acid models. Two parallel runs of four heated chains were used, and convergence was assessed by examination of the potential scale reduction factor and the variance in split-frequencies between runs (PSRF ~1 for all parameters; variance in split-frequencies <0.0001). The nonsynonymous divergence along each of the branches leading to DmelNV, DsubNV, and DimmNV (Figure 3B) was inferred using the method of Li [73], relative to an ancestral sequence inferred by maximum likelihood. Sliding windows were 50 codons wide, and placed every 30 codons. Nominal genome-wide 'significance' thresholds were derived by repeating the sliding-window analysis on 1000 randomizations of codon order.

Cloning

The following constructs were described previously: all DmelNV VP1 constructs [10], pCasper_FLAG-HA-AGO2 [53], pAFW-AGO2 [54], pAFW (Drosophila Genomics Resource Center, http:// https://dgrc.cgb.indiana.edu), pMT-FLuc [33], pMT-RLuc [33], pRmHa-*Renilla*-hairpin [74], pAc5-V5-His-A (Invitrogen), and pAc5-V5-His-Ntag [10].

For the localization of VP1 in S2 cells, the EGFP sequence was amplified using primer pair 5'-AGTAAGCTTATGGTGAGCAAGGGCGAG-3' and 5'-GGTTCTAGATTACTTGTACAGCTCGTCCATGC-3'. Subsequently, the HindIII and XbaI restriction sites were introduced into pAc5-V5-His-VP1^{FL} [10], and used to insert the EGFP fragment, generating pAc5-VP1^{FL}-EGFP.

cDNA of *D. immigrans* and *D. subobscura* was made using Promega MMLV-RT together with Promega RNasin Plus according to manufacturer's instructions. Subsequently, DimmNV VP1 and DsubNV VP1 sequences were PCR amplified from *D. immigrans* and *D. subobscura* cDNA and cloned as full-length and deletion constructs downstream

of the V5-His tag in pAc5-V5-His-Ntag.
The *D. immigrans* AGO2 cDNA sequence (Genbank KF362118), including parts of the 5′ and 3′ UTRs, was PCR amplified using the primer pair 5′-TGCAGCAAAAATTAGAAGCAAA-3′ and 5′-AGCCGTACCTAGAACCAGCA-3′. The resulting PCR product was used as a template in a nested PCR using primer pair 5′-AGTTCTAGACCGCGGGAATGGGTAAAAAGAACAAGTTCAAACCA-3′ and 5′-AGTTCTAGACCGCGGGAAGCGCTGTGGCACAGCTTCCGC-3′. The nested PCR product was subsequently cloned into the pAFW vector using the SacII and SalI restriction sites. To fuse the DimmNV VP1$^{\Delta N295}$ protein to the C-terminus of the maltose binding protein (MBP), we PCR amplified the VP1 coding sequence from pAc5.1-Ntag-DimmNV VP1^{FL} with primer pair 5′-AGTGGATCCCCAAAACTTCCAAGTGTACCTTCAAAG -3′ and 5′-GGTGTCGACTTAGTTTTGTTTATTTTTGTACCAATCGTTGG -3′. The resulting PCR product was cloned into the pMal-C2X vector (New England biolabs) using BamHI and SalI restriction sites, producing the pMal-C2X-DimmNV VP1$^{\Delta N295}$ vector.

Cell culture and immunostaining

Drosophila S2 cells were cultured as described previously [10]. For the localization of VP1 and AGO2 proteins, S2 cells were seeded in a 24-well plate at a density of 3x10^5 cells per well. The next day, cells were transfected with 800 ng of pAc5-VP1^{FL}-EGFP plasmid together with 800 ng of either pAFW-AGO2 or pCasper_FLAG-HA-AGO2 plasmid using Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. Three days after transfection, the cells were resuspended and seeded on concanavalin A (ConA) coated coverslips [75]. After a 2-hour incubation, the cells were fixed for 5 minutes with 4% paraformaldehyde, and permeabilized in PBS/0.1% Triton for 10 minutes, followed by incubation in blocking buffer (PBS/0.1% Triton/5% FCS) for 1 hour. For AGO2 detection, the cells were incubated with anti-FLAG antibody (Sigma), diluted 1:200 in blocking buffer, for 1 hour. Subsequently, the samples were stained with Alexa Fluor 594-conjugated secondary goat anti-rabbit antibody (1:400 dilution) for 1 hour. Nuclei were visualized using Hoechst staining (1:15000 dilution) in PBS/0.1% Triton, for 10 minutes, after which the coverslips were mounted with Mowiol. Samples were analyzed using an Olympus FV1000 confocal laser scanning microscope.

RNA silencing reporter assays

Double-stranded RNA was generated by *in vitro* transcription using T7 promoter-flanked PCR fragments as a template, as described previously [76]. For production of AGO2 dsRNA, a part of the coding sequence or 3′ untranslated region of Dmel AGO2 was PCR amplified using primer combination 5′-TAATACGACTCACTATAGGGAGATACTATGGTGAAGAACGGGTCG-3′ and 5′-TAATACGACTCACTATAGGGAGAGAACATGTCCTCAATCTCCTCC-3′, or primer

combination 5'-TAATACGACTCACTATAGGGAGAGCAACGTATTGAATCTTATT-3' and 5'-TAATACGACTCACTATAGGGAGAAGAACAATATTTGGCGGACC-3', respectively.
miRNA and RNAi sensor assays were performed as described [10,76]. For hairpin-induced silencing of the RLuc reporter, $5x10^4$ S2 cells were seeded per well in a 96-well plate. The seeded cells were co-transfected with 10 ng pMT-FLuc, 10 ng pMT-RLuc, 50 ng pRmHa-*Renilla*-hairpin, and 50 ng of expression plasmids encoding VP1 and/or AGO per well using Effectene transfection reagent (Qiagen). The pAc5-Ntag-DmelNV VP1$^{\Delta 284}$ and pAc5-Ntag-DimmNV VP1$^{\Delta N295}$ plasmids were used for VP1 expression. For knockdown of endogenous AGO2, 5 ng of AGO2 dsRNA or control dsRNA was co-transfected along with reporter plasmids. Two days after transfection, the expression of the luciferase reporter and the pRmHA-*Renilla*-hairpin was induced by the addition of 0.5 mM $CuSO_4$ per well. The next day, cells were lysed and FLuc and RLuc activity was measured with the Dual luciferase reporter assay system (Promega) according to manufacturer's protocol.

Immunoprecipitation and western blotting

For immunoprecipitations, S2 cells were seeded in 6-well plates at a density of $2x10^6$ cells per well. The next day, cells were transfected with AGO2 and/or VP1 expression plasmids using Effectene transfection reagent (Qiagen). Expression plasmids encoding DmelNV VP1$^{\Delta N351}$, DmelNV VP1$^{\Delta N284}$, or DimmNV VP1$^{\Delta N295}$ were used for co-immunoprecipitation experiments. Three days post transfection, cells were washed twice with PBS and subsequently resuspended in lysis buffer (30 mM HEPES-KOH, 150 mM NaCl, 2 mM Mg(OAc), 0.1% NP-40, 5 mM DTT) supplemented with protease inhibitor cocktail (Roche). After incubation on ice for 10 minutes, the samples were passed forty times through a 25-gauge needle, followed by incubation on ice for 10 minutes. Subsequently, cell lysates were centrifuged at 13,000 rpm for 30 minutes and a sample of the supernatant was taken to analyze the input for IP. To remove proteins that non-specifically bind to the IP beads, the remaining supernatant was incubated with Pierce protein G agarose at 4 ºC for 5 hours while mixing end-over-end. Next, the protein G agarose was separated from the supernatant by centrifugation, after which the supernatant was incubated overnight with anti-V5 agarose affinity gel (Invitrogen) at 4 ºC while mixing end-over-end. The next day, the anti-V5 agarose was separated from the supernatant by centrifugation, and a sample was taken from the supernatant. After the remaining supernatant was removed, the V5-agarose was washed three times with lysis buffer, and three times with either wash buffer 150 (25 mM Tris-Cl, 150 mM NaCl) or wash buffer 200 (25 mM Tris-Cl, 200 mM NaCl). All wash steps were done with 40 to 60 times beads volume of wash buffer. Subsequently, the beads were boiled in SDS sample buffer at 95 ºC for 10 minutes, followed by a brief centrifugation step to collect the beads at the bottom of the tube. The proteins in the supernatant were then separated on a SDS-PAGE gel, after which they were

transferred onto a nitrocellulose membrane by western blot. Primary antibodies used for western blot detection were anti-FLAG-M2 (1:1000 dilution; Sigma), anti-V5 (1:5000 dilution; Invitrogen), anti-AGO2 (1:500 dilution; generously provided by the Siomi lab), and anti-tubulin-alpha (1:1000 dilution, Sanbio), and secondary antibodies were goat anti-mouse-IRdye680 (1:15000 dilution; LI-COR), and goat anti-rabbit-IRdye800 (1:15000 dilution; LI-COR). All western blots were scanned using an Odyssey infrared imager (LI-COR biosciences)

Purification of recombinant protein

To purify recombinant MBP-DimmNV VP1$^{\Delta N295}$ protein, the pMal-C2X-DimmNV VP1$^{\Delta N295}$ plasmid was transformed into the *Escherichia coli* BL21 (DE3) strain. Subsequently, expression of the recombinant protein was induced by addition of 0.2 mM IPTG. Recombinant protein expression was allowed to proceed overnight at 18 ºC. The next day, recombinant MBP-DimmNV VP1$^{\Delta N295}$ was purified using amylose resin (New England biolabs) according to the manufacturer's protocol. The purified protein was subsequently transferred to a dialysis membrane (MWCO 12-14 kDa) and incubated overnight in dialysis buffer (20 mM Tris-Cl, 0.5 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 140 mM NaCl, 2.7 mM KCl) at 4 ºC, followed by a second dialysis step for 5 hours at 4 ºC. The dialyzed protein solution was stored at -80 ºC in dialysis buffer containing 30% glycerol.

Slicer assays

A new *D. immigrans* line was established from flies collected in June 2012 in Edinburgh (Coordinates 55.921N, 3.193W). *D. immigrans* was cultured similarly as *D. melanogaster* on standard media. Embryo lysates were generated from *D. immigrans* and from an RNAi-competent *D. melanogaster* laboratory control strain (w^{1118}). *In vitro* target RNA cleavage assays in *D. melanogaster* embryo lysates were performed as described [10]. Minor changes were incorporated for the slicer assay in *D. immigrans* embryo lysate: the reaction contained 0.9 mM $MgCl_2$ and was allowed to proceed for 5 hours at 25 ºC before RNA extraction. Suppressor activities of MBP-DmelNV VP1$^{\Delta N284}$ and MBP-DimmNV VP1$^{\Delta N295}$ proteins were tested in these Slicer assays.

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Supporting information

Supplemental figures

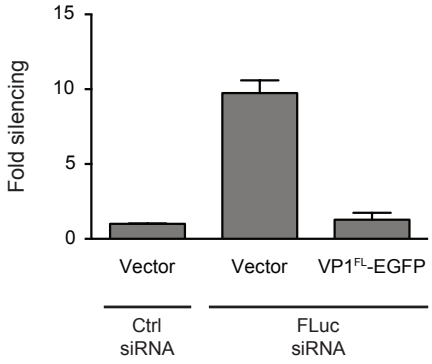

Figure S1: Nora virus VP1-EGFP fusion protein suppresses RNAi. An RNAi sensor assay in *Drosophila* S2 cells was performed to analyze the ability of a VP1-EGFP fusion protein to suppress RNAi. S2 cells were transfected with plasmids encoding Firefly luciferase (FLuc) and *Renilla* luciferase (RLuc) reporter along with a control vector (Vector) or a plasmid encoding the full-length VP1 sequence fused at its C-terminus to enhanced green fluorescent protein ($VP1^{FL}$-EGFP). Control siRNA (Ctrl siRNA) or a siRNA targeting FLuc (FLuc siRNA) were cotransfected to induce silencing of FLuc. FLuc counts were normalized to RLuc counts and presented as fold silencing relative to Ctrl siRNA. Bars represent means and standard deviations of three independent biological replicates.

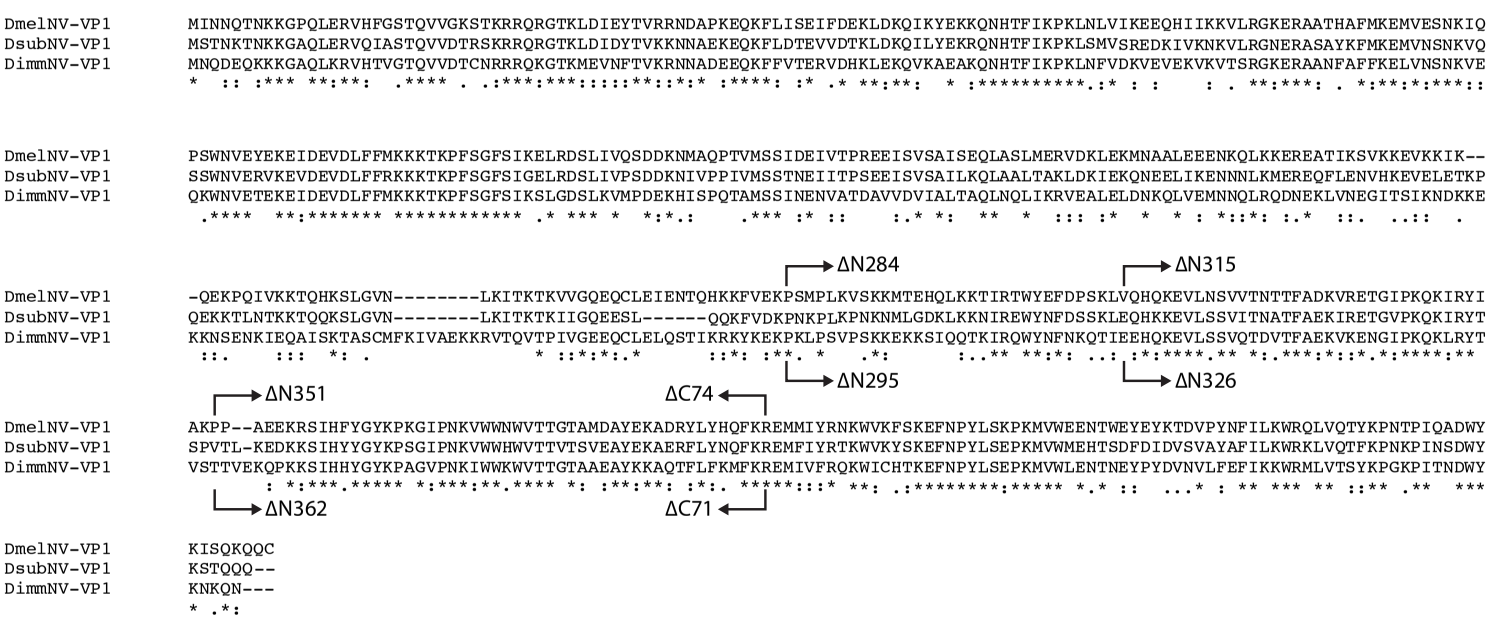

Figure S2: Alignment of the VP1 sequence of Nora viruses from different Drosophila species. VP1 sequences of *D. melanogaster* Nora virus (DmelNV), *D. subobscura* Nora-like virus (DsubNV), or *D. immigrans* Nora-like virus (DimmNV) were aligned with Clustal Omega using default settings. Arrows indicate the first amino acid of the N-terminal deletion mutants (ΔN) and the last amino acid of the C-terminal deletion mutants (ΔC) of DmelNV VP1 (above alignment) and DimmNV VP1 (below alignment) that were used in this study.

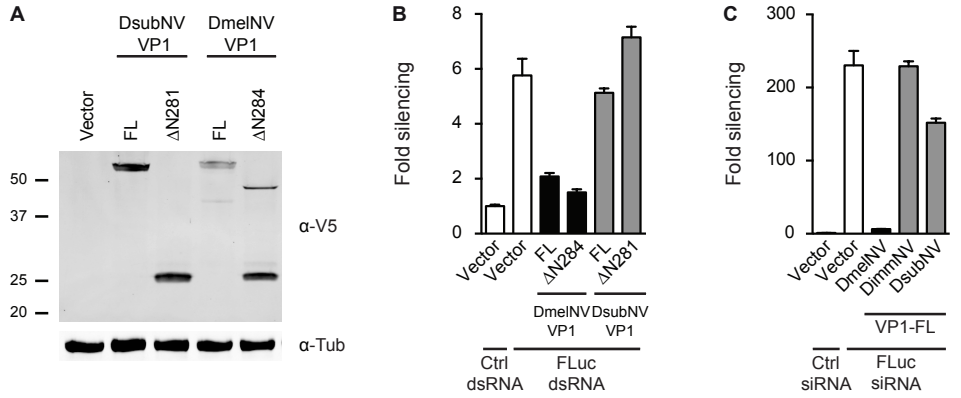

Figure S3: DsubNV VP1 and DimmNV VP1 do not suppress RNAi in *D. melanogaster* cells. (**A**) Western blot analysis of V5-tagged full-length (FL) or N-terminal deletion (ΔN) constructs of DsubNV VP1 or DmelNV VP1. VP1 proteins were detected with anti-V5 (α-V5) antibody. Tubulin (α-tub) was used as a loading control. (**B**) RNAi sensor assay in S2 cells. Firefly luciferase (FLuc) and *Renilla* luciferase (RLuc) reporter plasmids were cotransfected with plasmids encoding either DmelNV VP1, DsubNV VP1, or a control vector (Vector). Two days after transfection, cells were soaked in medium containing FLuc dsRNA or control dsRNA. One day later, luciferase activities were measured and FLuc counts were normalized to RLuc counts and expressed as fold silencing relative to the non-hairpin control transfection (**C**) RNAi sensor assay in S2 cells using full-length (FL) constructs of DmelNV VP1, DimmNV VP1, and DsubNV VP1. The assay was done as described in panel B, only siRNAs against FLuc (FLuc siRNA) or a control siRNA (Ctrl siRNA) were cotransfected with the plasmids instead of soaking the cells in dsRNA. Bars represent means and standard deviations of three independent biological replicates.

Chapter 6

Mosquito and entomobirnaviruses suppress dsRNA and siRNA-induced RNAi

Manuscript under revision at Nucleic Acid Research (NAR):

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Abstract

RNA interference (RNAi) is a crucial antiviral defense mechanism in insects, including the major mosquito species that transmit important human viruses. To counteract the potent antiviral RNAi pathway, insect viruses encode RNAi suppressors. However, whether mosquito viruses generally suppress the RNAi pathway remains unclear. We therefore set out to study RNAi suppression by Culex Y virus (CYV), a member of the *Birnaviridae* family that was recently isolated from *Culex pipiens* mosquitoes. Using well-established RNAi reporter assays, we show that RNAi is suppressed in cells infected with CYV and that the viral VP3 protein is responsible for RNAi antagonism. In contrast, VP3 did not affect the microRNA (miRNA) pathway, nor were expression levels of endogenous miRNAs affected by CYV infection. We used recombinant VP3, purified from *Escherichia coli*, to investigate the RNAi-suppressive activity of VP3 in more detail. We demonstrate that VP3 binds long double-stranded RNA (dsRNA) as well as small-interfering RNAs (siRNAs) in electrophoretic mobility shift assays. In agreement with its dsRNA-binding activity, VP3 interfered with Dicer-mediated cleavage of long dsRNA into siRNAs in cell extracts. Slicing of target RNAs by a pre-assembled RNA-induced silencing complex was not affected by VP3. Finally, we show that the RNAi-suppressive activity of VP3 is conserved in Drosophila X virus, a birnavirus that persistently infects *Drosophila* cell cultures. Together, our results suggest that the antiviral RNAi machinery drives the evolution of RNAi antagonists in mosquito viruses.

Introduction

RNA interference (RNAi) is a cellular mechanism that regulates gene expression in a broad range of eukaryotes. In plants, insects, nematodes, and fungi, the RNAi pathway also acts as an antiviral defense mechanism [1]. For insects, the antiviral RNAi pathway is most extensively studied in *Drosophila melanogaster*. The current model for antiviral RNAi in *Drosophila* is that virus-derived double-stranded RNA (dsRNA) is processed by Dicer-2 (Dcr-2) into viral small-interfering RNA (vsiRNA) duplexes that associate with an Argonaute-2 (AGO2) containing RNA-induced silencing complex (RISC). One strand of the vsiRNA duplex is retained in RISC to guide the identification and cleavage (slicing) of complementary viral RNAs [2]. The detection of virus-derived small RNAs during infections in *Drosophila* supports this model [3-11]. Moreover, the hypersensitivity of *Drosophila* RNAi pathway mutants to virus infections confirms the important role of RNAi in antiviral defense [3,5,6,12-15]. In turn, insect viruses encode viral suppressors of RNAi (VSRs) to counteract the antiviral RNAi pathway. For example, the Flock House virus (FHV) B2 and Drosophila C virus (DCV) 1A proteins bind and shield long dsRNA from Dcr-2 cleavage [13,16-18]. FHV B2 additionally binds siRNA duplexes, which inhibits their loading into RISC [17]. Recently, the 1A protein of Cricket paralysis virus (CrPV) and viral protein 1 (VP1) of Nora virus were shown to inhibit AGO2 Slicer activity [8,19]. Most VSRs that target the same step in the RNAi pathway do not share sequence identity or structural conservation. Thus, insect viruses independently evolved a diverse set of RNAi antagonists that suppress the antiviral RNAi pathway by distinct mechanisms.

Mosquitoes are important vectors for the transmission of arthropod-borne viruses (arboviruses) that can cause serious diseases in humans, such as Dengue virus (DENV), West Nile virus (WNV), Japanese encephalitis virus, and Yellow fever virus [20]. In addition, mosquitoes are hosts to a diverse array of viruses that are not transmitted to vertebrates [21-24]. As was observed in virus infections of *Drosophila*, virus-derived small RNAs are detected in mosquitoes and mosquito cell lines infected with arboviruses and mosquito-restricted viruses [22,25-30]. In addition, knockdown of RNAi pathway components in mosquitoes results in higher virus titers after infection with different arboviruses [31-35]. These results show that also in mosquitoes, the RNAi pathway serves as an important antiviral defense mechanism. Despite the antiviral activity of the RNAi pathway against a broad range of viruses, reports on VSR activity in viruses that infect mosquitoes are limited to the genera *Alphanodavirus* (*Nodaviridae* family) and *Flavivirus* (*Flaviviridae* family).

Nodamura virus, like FHV a member of the *Alphanodavirus* genus, was first isolated from *Culex tritaeniorhynchus* mosquitoes near the Japanese village Nodamura [36]. Successful experimental infections in a range of vertebrates in combination with the detection of neutralizing antibodies in Japanese pigs, suggest that Nodamura

virus is a mosquito-transmitted arbovirus [37]. The B2 protein of Nodamura virus, like FHV B2, inhibits RNAi by binding long dsRNA as well as siRNA duplexes [38,39]. More recently, the non-coding subgenomic flavivirus RNA (sfRNA) of WNV and DENV was shown to suppress RNAi [40]. sfRNA is abundantly produced during flavivirus infection as a result of incomplete degradation of the genomic RNA [41]. Probably because of their stem-loop structure, sfRNA molecules compete with Dicer substrates, thereby decreasing Dicer activity. DENV non-structural protein 4B (NS4B) was also shown to interfere with Dicer function, by a mechanism independent of dsRNA binding [32].

The identification and characterization of viral immune antagonists may provide important insight into the mechanisms, components, and regulators of immune pathways [42,43]. For example, the Slicer antagonism of two unrelated RNA viruses indicates that slicing of viral target RNAs is an important aspect of the antiviral RNAi response [8,19]. To begin to understand the diversity of RNAi suppressive activities in mosquito viruses, we set out to identify an RNAi suppressor in the mosquito-restricted Culex Y virus (CYV). CYV is a bisegmented dsRNA virus of the *Entomobirnavirus* genus of the *Birnaviridae* family that was recently isolated from hibernating *Culex pipiens* complex mosquitoes in Germany [23]. The non-enveloped CYV particles contain two dsRNA genome segments that are predicted to encode five viral proteins (VPs). Isolation of the highly related Espirito Santo virus and Mosquito X virus in Brazil and China, respectively, indicates that these birnaviruses are widely distributed in nature [22,24]. The high degree of similarity between Espirito Santo virus, Mosquito X virus, and CYV suggests that they are strains of a single species within the *Entomobirnavirus* genus.

Here, we show that CYV and Drosophila X virus (DXV), like CYV a member of the *Entomobirnavirus* genus, suppress the RNAi pathway during infection. We mapped the RNAi suppressor activity to VP3, and demonstrate that VP3 is able to bind long dsRNA as well as siRNAs and that it inhibits Dcr-2-mediated cleavage of long dsRNA. To our knowledge, we describe the first VSR from a mosquito-restricted virus. Moreover, together with the VSR activities identified in Nodamura virus, WNV and DENV, these data show that the antiviral activity of the RNAi pathway in mosquitoes drives the evolution of VSRs in both arboviruses and mosquito-restricted viruses.

Results

The CYV VP3 protein suppresses RNAi

The identification of vsiRNAs in infections with Mosquito X virus, DXV, and Drosophila birnavirus as well as the enhanced sensitivity of *R2D2* and *AGO2* mutant flies to DXV infection indicates that entomobirnaviruses are a target of the antiviral RNAi

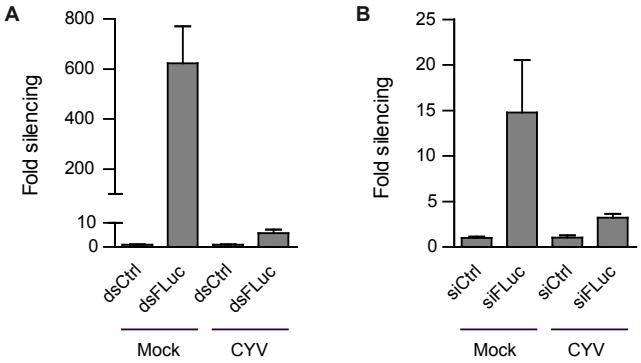

Figure 1: RNAi is suppressed during CYV infection. (**A**) dsRNA-induced RNAi reporter assay in infected S2 cells. Mock- and CYV-infected S2 cells were transfected with firefly (FLuc) and *Renilla* luciferase (RLuc) expression plasmids together with a non-silencing control dsRNA (dsCtrl) or FLuc dsRNA (dsFLuc). The next day, luciferase activities were measured. FLuc counts were normalized to RLuc counts and presented as fold silencing relative to dsCtrl. (**B**) siRNA-induced RNAi reporter assay in infected S2 cells. The experiment was performed as in (A), but RNAi was induced by co-transfection of non-silencing control siRNA (siCtrl) or FLuc siRNA (siFLuc). Bars in (A) and (B) represent averages and standard deviation of three independent samples.

machinery [11,15,22]. Hence, we deemed it likely that members of this genus would encode VSRs. We therefore used well-established reporter assays in S2 cells to determine whether CYV counteracts RNAi [44]. In these assays, the effect of virus infection or expression of individual viral proteins on RNAi-mediated silencing of a firefly luciferase (FLuc) reporter is monitored. We first determined whether RNAi is suppressed in cells that are infected with CYV. To this end, we measured luciferase activities in mock- and CYV-infected cells that were co-transfected with the FLuc reporter plasmid and 113-nt *in vitro* transcribed FLuc dsRNA. A *Renilla* luciferase (RLuc) reporter plasmid was included as a normalization control. As expected, in mock-infected cells, the FLuc reporter was efficiently silenced (~600-fold) by dsRNA treatment (Fig. 1A). However, dsRNA-mediated silencing of the FLuc reporter was strongly suppressed (to ~6-fold) in CYV-infected cells (Fig. 1A). CYV infection also suppressed silencing of the FLuc reporter (from ~15-fold to ~3-fold) when we induced RNAi with 21-nt synthetic siRNA duplexes (Fig. 1B). These data show that CYV infection inhibits RNAi induced by dsRNA as well as siRNAs.

To identify the viral proteins responsible for CYV-mediated RNAi suppression, we generated expression constructs for the five proteins (VP1 to VP5) that are predicted to be encoded by the CYV genome (Fig. 2A). Expression of all five proteins in transfected cells was confirmed by Western blot analysis (Fig. 2B). We then tested the individual viral proteins for VSR activity in our RNAi reporter assays. Cells were co-transfected with the FLuc and RLuc reporter plasmids and an expression plasmid for one of the viral proteins. The FLuc reporter was silenced by dsRNA feeding two days after transfection to allow expression of the viral proteins before the induction of RNAi. Of the five viral proteins, only VP3 suppressed silencing of the FLuc reporter

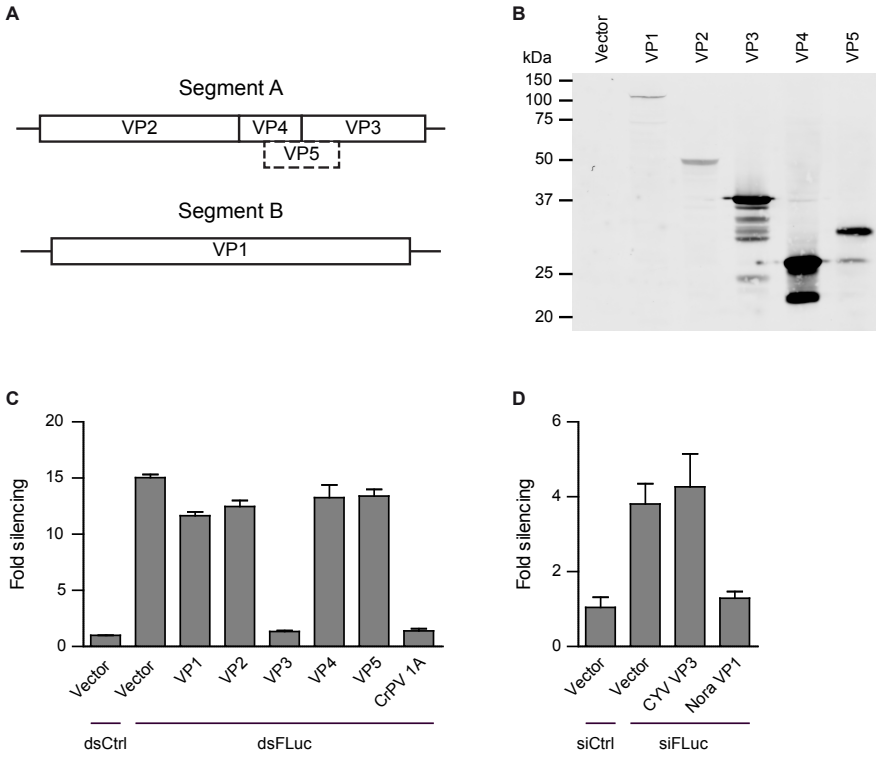

Figure 2: CYV VP3 suppresses RNAi. (**A**) Schematic representation of the bisegmented dsRNA genome of CYV. Genome segment A encodes a polyprotein precursor of VP2 (capsid), VP4 (protease) and VP3 (ribonucleoprotein). A VP5 protein, homologous to DXV VP5, may be expressed in the -1 reading frame. Unlike DXV VP5, which initiates with a canonical AUG codon, expression of CYV VP5 would require initiation by a non-AUG codon. To ensure efficient expression, we introduced an AUG start codon in the CYV VP5 expression plasmid. Genome segment B encodes VP1 (RNA-dependent RNA polymerase). (**B**) Western blot analysis of CYV protein expression. *Drosophila* S2 cells were transfected with plasmids encoding the CYV VP1 to VP5 proteins fused to the V5-epitope tag at their N-termini. Cells were harvested and lysed four days after transfection and expression was analyzed by Western blot using anti-V5 antibodies. (**C**) dsRNA-induced RNAi reporter assay in S2 cells. Firefly (FLuc) and *Renilla* luciferase (RLuc) expression plasmids were co-transfected into *Drosophila* S2 cells together with an empty control vector (Vector) or a plasmid encoding one of the CYV proteins (VP1 to VP5). A plasmid expressing the CrPV 1A protein was used as positive control. Two days after transfection, the cells were soaked in medium containing non-specific control dsRNA (dsCtrl) or FLuc dsRNA (dsFLuc). The next day, luciferase activities were measured. FLuc counts were normalized to RLuc counts and presented as fold silencing relative to dsCtrl. (**D**) siRNA-induced RNAi reporter assay in S2 cells. The experiment was done as in (C), except that non-specific control siRNA (siCtrl) or FLuc siRNA (siFLuc) were co-transfected with the plasmids to induce silencing. Nora virus VP1 (Nora VP1) was used as a positive control. Bars in (C) and (D) represent averages and standard deviation of three independent samples.

to a similar extent as the positive control CrPV 1A [19] (from ~15-fold to background levels, Fig. 2C). However, VP3 did not inhibit silencing of the FLuc reporter when RNAi was induced by co-transfection of siRNAs along with the luciferase and VP3 expression plasmids (Fig. 2D). This is in contrast to the AGO2 antagonists Nora virus VP1 and CrPV 1A that effectively suppress siRNA-induced RNAi in reporter

assays (Fig 2D and [8,19]. The inability of CYV VP3 to suppress siRNA-induced RNAi under these conditions seems at odds with the reduced efficiency of siRNA-induced RNAi in infected cells (Fig 1B). However, in Figure 1, the cells were infected prior to transfection, whereas in Figure 2D, siRNAs were cotransfected along with the expression plasmids. Most likely, the siRNAs are incorporated into RISC before VP3 is expressed at sufficiently high levels. These data therefore suggest that VP3 suppresses the RNAi pathway at a step that precedes target cleavage by AGO2.

CYV VP3 does not inhibit the miRNA pathway

The RNAi pathway shares basic features with the microRNA (miRNA) pathway. Both pathways depend on Dicer proteins for the generation of small RNAs that guide the recognition and silencing of complementary RNAs by Ago proteins. The Dicer and Ago proteins in the RNAi and miRNA pathways are, however, different. The RNAi pathway depends on Dcr-2 and AGO2 for small RNA biogenesis and function, whereas the miRNA pathway predominantly depends on Dcr-1 and AGO1. To investigate whether the miRNA pathway is affected by CYV infection, we compared the expression levels of the mature miRNAs miR-2b and miR-252 in mock- and CYV-infected cells. The miR-2b and miR-252 levels were not altered after CYV infection (Fig. 3A), which indicates that CYV does not affect miRNA biogenesis. To confirm and extend these results,

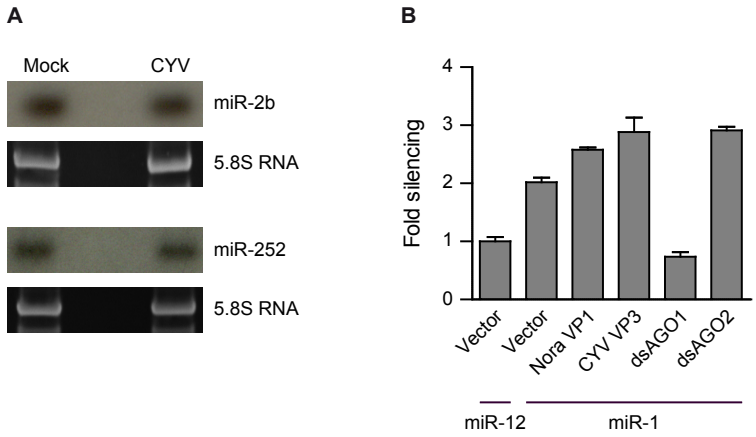

Figure 3: CYV does not affect miRNA biogenesis and function. (**A**) Northern blot analysis of mature miRNAs in mock- and CYV-infected S2 cells. Mature miR-2b and miR-252 were detected with radiolabelled probes. Ethidium bromide staining of the 5.8S ribosomal RNA band was used as a loading control. (**B**) miRNA sensor assay in S2 cells. A plasmid encoding the firefly luciferase (FLuc) reporter fused to the Par6 3'UTR, a target for miR-1, was co-transfected along with plasmids encoding the primary (pri) miR-1 transcript or the non-silencing control pri-miR-12 and a *Renilla* luciferase (RLuc) reporter plasmid. In addition, an empty control plasmid (Vector) or plasmids encoding either Nora virus VP1 (Nora VP1) or CYV VP3 were co-transfected. Three days after transfection, luciferase activities were measured. FLuc counts were normalized to RLuc counts and presented as fold silencing relative to the miR-12 control. To verify that the reporter was miRNA pathway-dependent, dsRNAs targeting *AGO1* (dsAGO1) or, as a negative control, *AGO2* (dsAGO2) were co-transfected to silence the respective genes. Bars in (B) represent the averages and standard deviation of three independent samples.

we used a miRNA sensor assay to determine whether the CYV VP3 protein inhibits miRNA function. Cells were transfected with an expression plasmid encoding a FLuc reporter with miR-1 target sites in its 3' UTR along with plasmids encoding a primary miR-1 transcript and the CYV VP3 protein. Again, an RLuc reporter plasmid was co-transfected for normalization purposes. As anticipated, the FLuc reporter was silenced (~2-fold) by miR-1 in a miRNA pathway-dependent fashion, since silencing was fully suppressed by knockdown of *AGO1*, but not by knockdown of *AGO2* (Fig. 3B). Silencing of the FLuc reporter was not affected by co-expression of either CYV VP3 or Nora virus VP1 (Fig. 3B). These data indicate that the VP3 VSR activity does not affect the biogenesis nor the function of miRNAs.

The VSR activity of VP3 is conserved in DXV

Having identified CYV VP3 as a suppressor of RNAi, we analyzed whether the VSR activity of this protein is conserved in DXV, an entomobirnavirus that infects *Drosophila*. To this end, we first studied RNAi suppression in cells infected with DXV. As observed for CYV, dsRNA-mediated silencing of the FLuc reporter was strongly suppressed (from ~1400-fold to ~150-fold) in cells infected with DXV (Fig. 4A). We then tested the DXV VP3 protein for VSR activity. DXV VP3 suppressed silencing of the FLuc reporter (from ~15-fold to background levels) when RNAi was induced by dsRNA feeding two days after transfection of the luciferase and VP3 expression plasmids (Fig. 4B), similar to our observations with CYV VP3. In addition, DXV VP3 mildly suppressed silencing of the FLuc reporter (from ~23-fold to ~9-fold) when we induced RNAi by co-transfection of siRNAs with the luciferase and VP3 expression

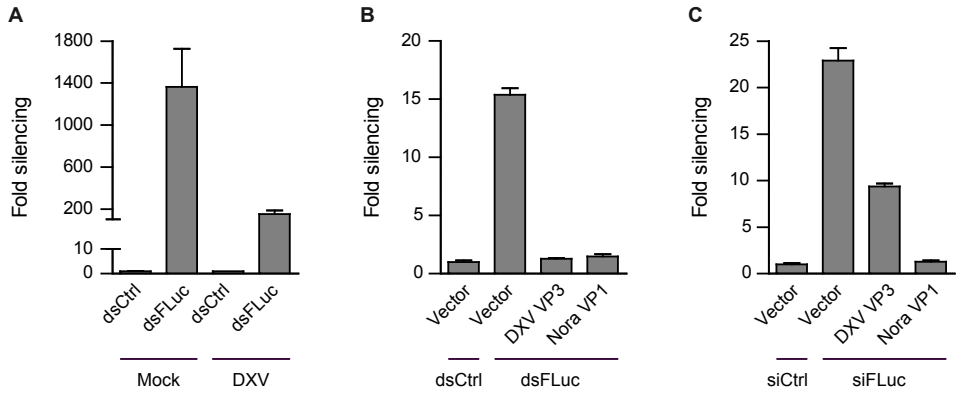

Figure 4: RNAi is suppressed by DXV infection and DXV VP3 expression. (**A**) dsRNA-induced RNAi sensor assay in infected S2 cells. Mock- and DXV-infected S2 cells were transfected with firefly (FLuc) and *Renilla* luciferase (RLuc) reporter plasmids, together with either non-silencing control dsRNA (dsCtrl) or FLuc dsRNA (dsFLuc). Two days after transfection, luciferase activities were measured. FLuc counts were normalized to RLuc counts and presented as fold silencing relative to dsCtrl. (**B**) dsRNA-induced, and (**C**) siRNA-induced RNAi sensor assays in S2 cells, as described in the legend to Figure 2, with plasmids encoding DXV VP3 and, as a positive control, Nora virus VP1 (Nora VP1). Bars in all figures represent averages and standard deviations of three independent samples.

plasmids (Fig. 4C). These results indicate that the VSR activity of VP3 is conserved in DXV.

CYV and DXV VP3 inhibit dicing of dsRNA

VSRs can target different aspects of the RNAi machinery, such as Dcr-2-mediated cleavage of dsRNA and slicing of target RNAs by AGO2. To characterize the VSR activity of CYV and DXV VP3 in more detail, we performed a series of biochemical assays using maltose-binding protein (MBP)-tagged recombinant proteins purified from *Escherichia coli*.

We first tested whether the recombinant VP3 proteins interfere with dicing of dsRNA, the initiation phase of the RNAi pathway. We incubated radioactively-labelled 126-nt dsRNA in *Drosophila melanogaster* S2 and *Aedes albopictus* U4.4 cell extracts and monitored its processing into 21-nt siRNAs on denaturing polyacrylamide gels. The dsRNA was efficiently processed into siRNAs in extracts from both cell types (Fig. 5A, lanes 10 and 15). Processing of the dsRNA was, however, inhibited in a dose-dependent manner in the presence of increasing concentrations of both CYV and DXV VP3 (Fig. 5A, lanes 3-8 and 11-13), and in the presence of DCV 1A, a VSR that is known to interact with dsRNA [13] (Fig. 5A, lane 1). As expected, MBP alone did not inhibit dsRNA processing (Fig. 5A, lanes 9 and 14). These data indicate that the entomobirnavirus VP3 proteins interfere with siRNA production by Dcr-2. Importantly, inhibition of dsRNA cleavage into siRNAs was also observed in cells infected with CYV. The dsRNA was almost completely processed in extracts from mock-infected cells (Fig. 5B, lane 5), but no dsRNA processing was observed in extracts from CYV-infected cells (Fig. 5B, lane 1). Titration of CYV-infected cell extracts into mock-infected cell extracts abolished dsRNA processing (Fig. 5B, lanes 2-4), which confirms the presence of a dsRNA processing inhibitor in CYV-infected cells.

We next tested whether the recombinant VP3 proteins are capable of interfering with slicing of target RNAs, the effector phase of the RNAi pathway. For this purpose, a radioactively 5′ cap-labelled target RNA, consisting of 492-nt of the FLuc coding sequence, was incubated in *Drosophila* embryo extracts in the presence of an FLuc-specific siRNA that triggers its cleavage. Cleavage of the target RNA results in the production of a 164-nt 5′ cleavage product that can be visualized on a denaturing polyacrylamide gel. As expected, cleavage of the target RNA was induced by the FLuc-specific siRNA, but not by a non-specific control siRNA (Fig. 5C, lanes 1 and 2). Neither MBP alone (Fig. 5C, lanes 3,4, and 14), nor CYV or DXV VP3 (Fig. 5C, lanes 6-11), inhibited target RNA cleavage. Complete inhibition of target RNA cleavage was, however, seen in the presence of the positive control Nora virus VP1, an established VSR that interferes with the Slicer activity of AGO2 [8] (Fig. 5C, lane 13). These results demonstrate that the entomobirnavirus VP3 proteins do not interfere with target RNA cleavage by AGO2.

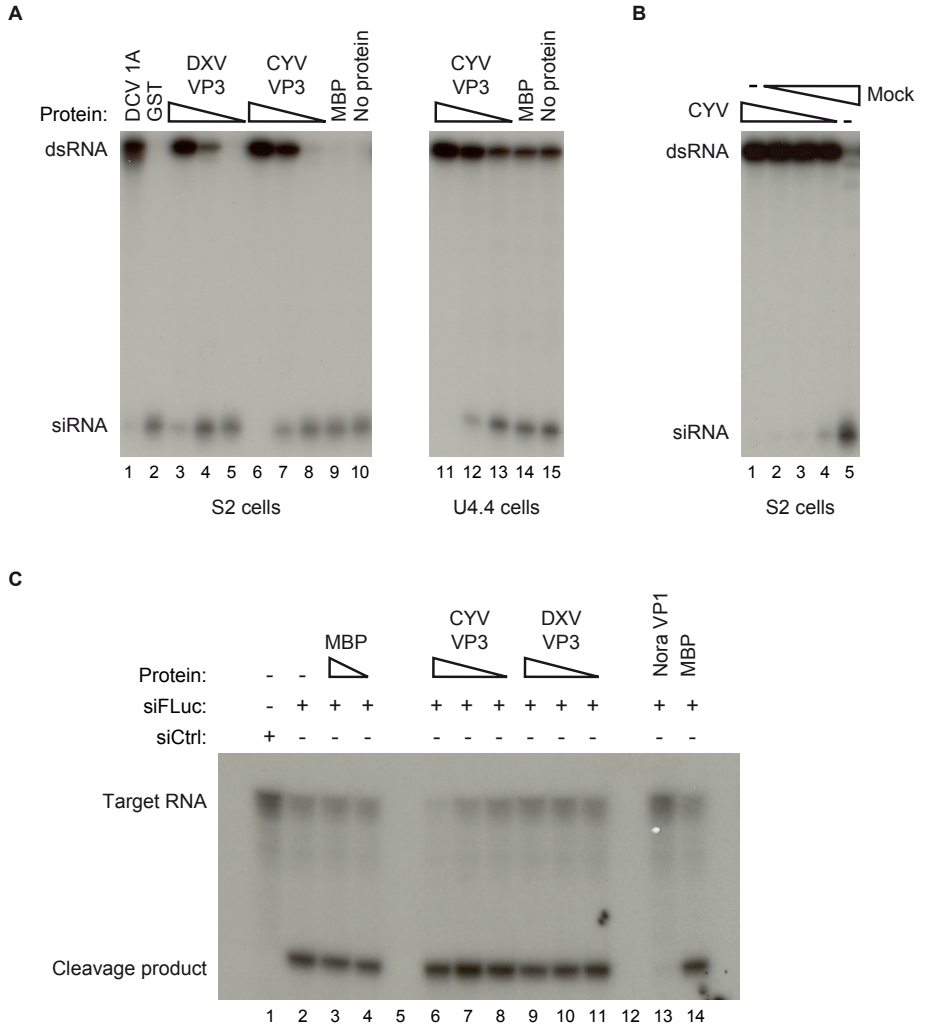

Figure 5: DXV and CYV inhibit Dicer, but not Slicer activity. (**A**) Processing of dsRNA in cell extracts from *Drosophila melanogaster* S2 (left panel) and *Aedes albopictus* U4.4 cells (right panel). Uniformly radiolabelled 126-nt dsRNA was incubated in S2 or U4.4 cell extracts together with the indicated recombinant proteins. Processing of the dsRNA into 21-nt siRNAs was monitored by denaturing polyacrylamide gel electrophoresis followed by autoradiography. Protein concentrations of maltose-binding protein (MBP)-tagged DXV and CYV VP3 proteins were 3 μM (lanes 3,6,11), 0.3 μM (lanes 4,7,12), and 0.03 μM (lanes 5,8,13). Glutathione S-transferase (GST)-tagged DCV 1A (lane 1; 1.5 μM) was used as a positive control; GST (lane 2; 1.5 μM) and MBP (lanes 9,14; 3 μM) were used as negative controls. (**B**) Dicer assay in mock- and CYV-infected S2 cell extracts. Radiolabelled 126-nt blunt dsRNA was incubated in mixtures of CYV- and mock-infected S2 cell extracts and siRNA production was monitored as in (A). (**C**) In vitro RNA cleavage (Slicer) assay in *Drosophila melanogaster* embryo lysate. Embryo lysates were first incubated with firefly luciferase siRNAs (siFLuc) or non-specific control siRNAs (siCtrl) for 30 minutes, after which no protein (lanes 1,2) or the indicated recombinant proteins (lanes 3-14) were added. Finally, a radioactive 5′ cap labelled FLuc target RNA was added 30 minutes after the recombinant proteins. The following recombinant protein concentrations were used: 1.2 μM (lanes 3,6,9), 0.6 μM (lanes 7,10), 0.3 μM (lanes 4,8,11,13,14). The FLuc target RNA and cleavage product were separated on a denaturing polyacrylamide gel and visualized by autoradiography.

CYV and DXV VP3 possess siRNA and dsRNA-binding activity

Many VSRs employ dsRNA binding as a mechanism to suppress RNAi [13,16,18,45]. The ability of the entomobirnavirus VP3 proteins to inhibit Dcr-2-mediated cleavage of dsRNA into siRNAs suggests a similar strategy of RNAi suppression. To study whether these proteins indeed possess dsRNA-binding activity, we performed EMSAs using the recombinant VP3 proteins and different radioactively labelled probes.

First, we incubated 126-nt blunt dsRNA with serial dilutions of the recombinant VP3 proteins and resolved dsRNA-protein complexes on native polyacrylamide gels. As expected, incubation of dsRNA with MBP alone (Fig. 6A, lane 17) did not alter its mobility when compared to the control reaction without recombinant protein (Fig. 6A, lane 18). However, the mobility of the dsRNA was inhibited in a dose-dependent manner by the VP3 proteins of both DXV (Fig. 6A, lanes 1-7) and CYV (Fig. 6A, lanes 9-15). DXV and CYV VP3 displayed similar affinities for dsRNA, with dissociation constants of 166.1 +/- 33.2 nM and 115.6 +/- 24.5 nM, respectively (Fig. 6A).

The dsRNA-binding activity of the entomobirnavirus VP3 proteins most likely suppresses RNAi by inhibiting dsRNA processing by Dcr-2. However, inhibition of siRNA production cannot fully explain the RNAi suppressive activity in entomobirnavirus infection, since RNAi was suppressed in CYV-infected cells when RNAi was induced with synthetic siRNAs (Fig. 1B). This observation indicates that CYV targets additional steps of the RNAi pathway, such as loading of siRNAs into RISC or slicing of target RNAs by AGO2. Since our biochemical assays indicate that the VP3 proteins do not interfere with the AGO2 Slicer activity (Fig. 5C), we tested whether these proteins have the potential to scavenge siRNAs to prevent their incorporation into RISC. To this end, we tested serial dilutions of the recombinant VP3 proteins in EMSAs with 21-nt siRNA duplexes containing 2-nt 3' overhangs. When compared to the control reaction without recombinant protein (Fig. 6B, lane 18), the mobility of the siRNAs was inhibited in a dose-dependent manner after incubation with the VP3 proteins of CYV (Fig. 6B, lanes 9-15) and DXV (Fig. 6B, lanes 1-7), but not after incubation with MBP alone (Fig. 6B, lane 17). As for long dsRNA, CYV and DXV VP3 showed similar affinities for siRNAs, with dissociation constants of 2.6 +/- 1.4 μM and 5.9 +/- 1.6 μM, respectively (Fig. 6B). Interestingly, both proteins also bound 21- and 19-nt blunt dsRNA, but only showed weak binding to a 23-nt miRNA duplex (Fig. 6C), consistent with the observation that VP3 does not inhibit the miRNA pathway (Fig. 3B). Binding was RNA-specific, as neither VP3 protein was able to interact with 21-nt dsDNA (Fig. 6C). Taken together, our data indicate that the entomobirnavirus VP3 proteins are RNAi suppressors that bind both long dsRNA as well as siRNAs. The dsRNA-binding activity of the VP3 proteins inhibits dsRNA processing into siRNAs and, presumably, loading of siRNAs into RISC.

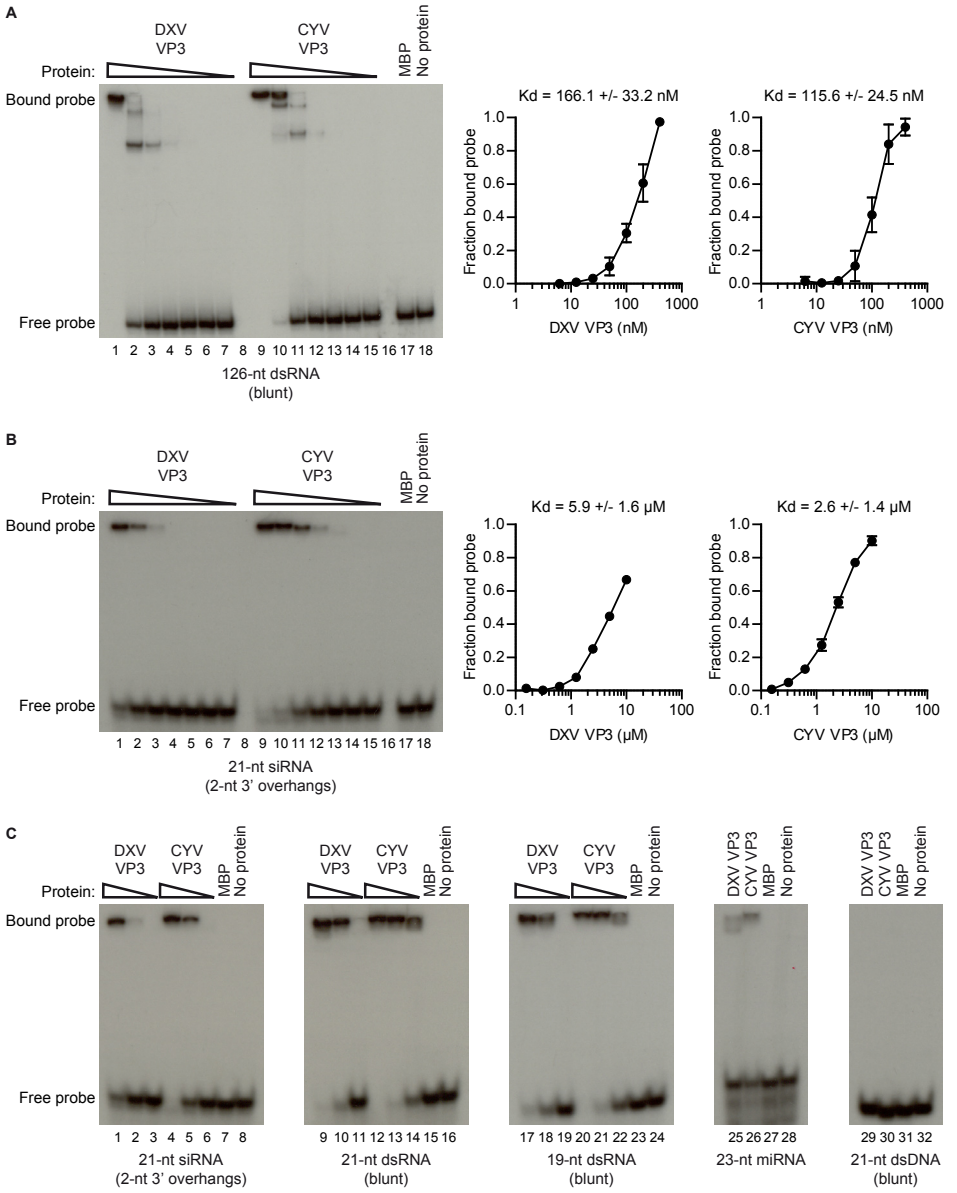

Figure 6: DXV and CYV VP3 bind dsRNA independent of length. (**A**) Electrophoretic mobility shift assay (EMSA) for binding of MBP-tagged DXV and CYV VP3 proteins to radiolabelled 126-nt blunt dsRNA (left panel). Two-fold dilutions, starting at 400 nM, were used for DXV VP3 (lanes 1-7), and CYV VP3 (lanes 9-15). In addition, buffer (No protein, lane 18) or 400 nM of MBP (lane 17) were used as negative controls. The shift assay is representative for three independent experiments. The fraction of bound probe was quantified for the different protein concentrations (right panels). Data points represent average and standard deviation of samples from three independent experiments. (**B**) EMSA for binding of MBP-tagged DXV and CYV VP3 proteins to radiolabelled 21-nt siRNAs with 2-nt 3' overhangs. The assay was performed as in (A), except that two-fold protein dilutions of DXV (lanes 1-7) and CYV VP3 (lanes 9-15), starting at 10 µM, were used. The MBP concentration was 10 µM (lane 17). (**C**) EMSAs using

different RNA and DNA probes. Each probe was incubated with the MBP-tagged VP3 proteins of DXV and CYV or, as negative controls, with MBP or buffer (No protein). In the EMSAs with 21-nt siRNAs (2-nt 3' overhangs), 21-nt dsRNAs (blunt), and 19-nt dsRNAs (blunt), four-fold dilutions of DXV and CYV VP3 were used starting at a concentration of 10 μM. In the 23-nt miRNA and 21-nt dsDNA EMSAs, DXV VP3 and CYV VP3 were analyzed at a concentration of 10 μM. MBP was used as a negative control at 10 μM in all EMSAs.

Discussion

Viruses employ many different strategies to suppress or evade the innate and adaptive immune responses of their hosts. In arthropods, RNAi has antiviral activity against all major classes of insect viruses, including (+) and (-) strand RNA, dsRNA and DNA viruses [3,6,12-15,18,46]. Nevertheless, RNAi suppression has thus far only been identified in (+) strand RNA virus infections [13,18,19]. How widespread viral RNAi antagonism is, and, more specifically, whether other classes of viruses encode RNAi suppressors remains unclear. Here, we show that the antiviral RNAi pathway is inhibited during infections with mosquito and *Drosophila* dsRNA viruses from the *Entomobirnavirus* genus (*Birnaviridae* family).

We mapped the VSR activity to VP3, a multifunctional protein that is involved in many aspects of the viral replication cycle. Most of our knowledge on the role of VP3 in birnavirus replication is derived from experiments with infectious bursal disease virus (IBDV) and infectious pancreatic necrosis virus (IPNV), members from the genera *Avibirnavirus* and *Aquabirnavirus*, respectively. In these viruses, the VP3 proteins act as scaffolds during capsid assembly by interacting with the viral genome as well as with the viral VP1 (RNA-dependent RNA polymerase), pVP2 (capsid) and other VP3 proteins [47-51]. Our data reveal yet another function of VP3. We demonstrate that the entomobirnavirus VP3 proteins possess dsRNA- and siRNA-binding activity and inhibit Dicer-mediated siRNA production.

In principle, any dsRNA-binding protein has the potential to inhibit RNAi when overexpressed. Even a dsRNA-binding protein from *E. coli*, a species that is not targeted by an RNAi response, suppressed RNAi under overexpression conditions [52]. It has been previously shown that the IBDV, IPNV and DXV VP3 proteins have the capacity to suppress RNAi [53]. However, these studies were done in a heterologous system and the *in vivo* relevance in a relevant host remained unclear. Our study now provides evidence that both CYV and DXV infections suppress the RNAi response in insect cells.

dsRNA is an important activator of innate immune pathways, such as the interferon response in vertebrates and antiviral RNAi in invertebrates. To avoid recognition by the host's immune system, viruses have evolved different mechanisms to shield their dsRNA. The genomes of most dsRNA viruses, for example, are replicated in specialized viral cores that encapsulate the dsRNA genome throughout the viral replication cycle [54]. These viral cores are very common among dsRNA viruses, but

they are absent from birnaviruses. Instead, studies with IBDV and IPNV have shown that birnaviruses form ribonucleoprotein (RNP) complexes consisting of the dsRNA genome and the VP1 and VP3 proteins [55,56]. Since VP3 binds homogenously along the dsRNA genome, the RNP complexes are thought to prevent the activation of cellular immune pathways. Our observation that the VP3 proteins of CYV and DXV inhibit RNAi by dsRNA-binding supports this hypothesis. Entomobirnaviruses most likely form similar RNP complexes that shield the viral genome from the antiviral activity of the RNAi pathway. Nevertheless, the detection of vsiRNAs in cells infected with DXV, Drosophila birnavirus and Mosquito X virus implies that some viral dsRNA is available for Dcr-2 [11,22]. By RNP complex formation, VP3 probably limits the accessibility of the viral genome for Dcr-2, but it cannot prevent that some viral dsRNA is processed into siRNAs. These siRNAs may be loaded into RISC, where they guide cleavage of single-stranded viral transcripts, thereby adding another level of antiviral activity. We observed that the CYV and DXV VP3 proteins do not only bind long dsRNA, but also siRNA duplexes. These data suggest that VP3 does not merely protect the viral genome against Dcr-2, but that it also scavenges siRNAs to prevent their incorporation into RISC. Thus, entomobirnavirus VP3 proteins counteract multiple aspects of the antiviral RNAi machinery. The identification of a VSR in CYV indicates that the mosquito antiviral RNAi pathway has the potency to drive the evolution of RNAi antagonists in mosquito viruses.

Materials and methods

Cells and viruses

Drosophila melanogaster S2, and *Aedes albopictus* U4.4 and C6/36 cells were cultured as described previously [8,30,57]. CYV [23] and DXV (kindly provided by JL Imler) were propagated in S2 cells and titered by end-point dilution in C6/36 and S2 cells, respectively.

Plasmids

The following plasmids were described previously: pAWH-CrPV 1A [19], pAc5-V5-His-VP1$^{\Delta N351}$, pMal-C2X-VP1$^{\Delta N284}$ [8], pGEX-DCV 1A, pMT-FLuc, pMT-RLuc [13], pMT-FLuc-miR1, pMT-pri-miR1, pMT-pri-miR12 [58].

To clone the individual CYV and DXV open reading frames (ORFs) into insect expression plasmids, RNA was isolated from CYV- and DXV-infected S2 cells using Isol-RNA Lysis Reagent (5 PRIME). The isolated RNA was then used in a cDNA synthesis reaction with the Taqman Reverse Transcription Reagents (Life Technologies) and random hexamers. The CYV VP1 to VP5 and DXV VP3 ORFs were PCR amplified from the cDNA with primers that introduce either BsiWI and NotI (CYV VP1 to VP5) or Acc65I

Table 1: Sequences of the DNA and RNA oligonucleotides

Primer/probe name	Primer/probe sequence
CYV-VP1-F	5′-ACGTCGTACGCAAAATGAGTGACATATTCAACCAGCAGG-3′
CYV-VP1-R	5′-ACGTGCGGCCGCCAGTTCAACCTTTGCACGACGAAC-3′
CYV-VP2-F	5′-ACGTCGTACGCAAAATGAATACCTCAAACGAATACCTCA-3′
CYV-VP2-R	5′-ACGTGCGGCCGCCATGAGTGTGCACTCTTGATCCTC-3′
CYV-VP3-F*	5′-ACGTCGTACGCAAAATGGCCAGCGTGCCTTTCTCAAACA-3′
CYV-VP3-R*	5′-ACGTGCGGCCGCCAGATCATCTCTGATTCTCCACCG-3′
CYV-VP4-F	5′-ACGTCGTACGCAAAATGGCTGACACCCCAATAGGTGATA-3′
CYV-VP4-R	5′-ACGTGCGGCCGCCAGGAGCTTGCAGTCCAGTGGTTT-3′
CYV-VP5-F	5′-ACGTCGTACGCAAAATGCTATCAACTATTCGACGGAAAG-3′
CYV-VP5-R	5′-ACGTGCGGCCGCCAAGTACAATCCCTTGCCTTGCAA-3′
CYV-VP3-F1†	5′-AGTGGATCCGCCAGCGTGCCTTTCTCA-3′
CYV-VP3-R1†	5′-GGTAAGCTTTCAGATCATCTCTGATTCTCCACC-3′
DXV-VP3-F1*	5′-ACGTGGTACCCAAAATGAACCCATTCATGAACACG-3′
DXV-VP3-R1*	5′-ACGTGCGGCCGCCATACAATGTCATTGTCCTCTCC-3′
DXV-VP3-F2†	5′-AGTGGATCCGCGAGCATGAACCCATTCATG-3′
DXV-VP3-R2†	5′-GGTAAGCTTTTATACAATGTCATTGTCCTCTCCACG-3′
miR-2b probe	5′-GCTCCTCAAAGCTGGCTGTGATA-3′
miR-252 probe	5′-CTCCTGCGGCACTAGTACTTA-3′
FLuc-F1	5′-TAATACGACTCACTATAGGGAGATATGAAGAGATACGCCCTGGTT-3′
FLuc-F2	5′-GGGAGATATGAAGAGATACG-3′
FLuc-R1	5′-GGGAGAATAGCTTCTGCCAACC-3′
FLuc-R2	5′-TAATACGACTCACTATAGGGAGAATAGCTTCTGCCAACCGAAC-3′
Blunt-19nt-F	5′-UGAGGUAGUAGGUUGUAUA-3′
Blunt-19nt-R	5′-UAUACAACCUACUACCUCA-3′
Blunt-21nt-R	5′-ACUAUACAACCUACUACCUCA-3′
siRNA guide	5′-UGAGGUAGUAGGUUGUAUAGU-3′
siRNA passenger	5′-UAUACAACCUACUACCUCUCU-3′
miR-2b-3p	5′-UAUCACAGCCAGCUUUGAGGAGC-3′
miR-2b-2-5p	5′-UUCUUCAAAGUGGUUGUGAAAUG-3′
Blunt-dsDNA-F	5′-TGAGGTAGTAGGTTGTATAGT-3′
Blunt-dsDNA-R	5′-ACTATACAACCTACTACCTCA-3′

*Primers used for cloning into insect expression vector
†Primers used for cloning into bacterial expression vector

and NotI (DXV VP3) restriction site into the amplified fragment (Table 1). The PCR fragments were digested with the appropriate enzymes and cloned into the Acc65I and NotI restriction sites of the pAc5.1-V5-His-Ntag plasmid [8].

To generate bacterial expression constructs, the CYV and DXV VP3 ORFs were amplified from the cDNA with primers that introduce BamHI and HindIII restriction sites into the amplified fragment (Table 1). The PCR fragments were digested with BamHI and HindIII and cloned into the corresponding restriction sites of pMAL-C2X (New England Biolabs).

Western blot analysis

To analyze the protein products of the CYV insect-expression plasmids, S2 cells were seeded at a density of $2.5x10^5$ cells per well in a 24-well plate. The seeded cells were transfected with 400 ng of CYV expression plasmid or an empty control plasmid, using Effectene Transfection Reagent (Qiagen). Four days after transfection, the cells were lysed in 100 µL Laemli buffer and incubated at 95 ºC for 10 minutes. Subsequently, 40 µL of lysate was separated on a 12.5% SDS-PAGE gel and transferred to a 0.2 µm nitrocellulose membrane (Bio-Rad). The membrane was then incubated in blocking buffer (5% (wt/vol) non-fat dry milk in PBS/0.1% tween) for 1 hour followed by incubation with anti-V5 antibodies (Invitrogen, 1:5000 dilution in blocking buffer) for 1 hour. After three washes of 5 minutes with PBS/0.1% tween, the membrane was incubated with Goat anti-Mouse antibodies conjugated to Alexa Fluor 680 (LI-COR, 1:15000 dilution in PBS/0.1% tween). The bound antibodies were visualized using an Odyssey infrared imager (LI-COR).

RNAi sensor assays

The RNAi and miRNA sensor assays with the insect CYV and DXV protein expression constructs were performed as described previously [8,44]. To assess RNAi suppressor activity during infection, CYV- and DXV-infected S2 cells were co-transfected with 13.5 ng pMT-FLuc, 3 ng pMT-RLuc, and either 10 ng of dsRNA or 2 pmol of siRNAs using the Effectene Transfection Reagent (Qiagen). Expression of the luciferase reporter plasmids was induced the following day by the addition of 0.5 mM $CuSO_4$ to the culture medium. The next day, the cells were lysed and FLuc and RLuc activities were measured using the Dual-Luciferase Reporter Assay System (Promega).

Small RNA Northern blots

For the detection of miRNAs in infected cells, RNA was isolated from mock- and CYV-infected S2 cells using Isol-RNA Lysis Reagent. Subsequently, 10 µg of total RNA was separated on a 12% denaturing polyacrylamide gel and transferred to a Hybond NX nylon membrane (GE Healthcare). The RNA was cross-linked to the membrane using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) [59]. Next, the blots were hybridized in ULTRAhyb-oligo hybridization buffer (Ambion) containing ^{32}P end-labelled DNA oligonucleotide probes (miR-2b probe or miR252 probe, Table 1). The blots were washed and the radioactive signal was visualized using autoradiography.

Protein purification

The bacterial CYV and DXV VP3 expression constructs and the parental pMAL-C2X plasmid were transformed into *E. coli* BL21 (DE3) cells. When the bacterial cultures had reached an OD_{600} of 1.0, expression of the recombinant proteins was induced by the addition of 0.2 mM IPTG, followed by overnight incubation at 18ºC. Recombinant proteins were purified on amylose resin columns (New England Biolabs) according to manufacturer's protocol. Recombinant proteins were dialyzed and stored as described previously [8]. Protein concentrations were determined by a Bradford assay (Bio-Rad).

Radioactively labelled probes

For the production of uniformly labelled 126-nt blunt dsRNA, two FLuc DNA fragments were amplified by PCR using primers that introduce a T7 promoter sequence on either the 5' or 3' end of the amplified fragment (Table 1). The PCR products were used in *in vitro* transcription reactions in the presence of α-[^{32}P]-UTP and T7 RNA polymerase. The RNA strands were annealed and unincorporated nucleotides were removed using a G-25 Sephadex column (Roche). The dsRNA was then purified from an 8% native polyacrylamide gel. For EMSAs with synthetic small RNA or DNA duplexes, oligonucleotides were annealed, end-labelled using γ-[^{32}P]-ATP and polynucleotide kinase (Roche), and purified using a G-25 Sephadex column. For sequences, see Table 1. Oligonucleotides siRNA guide and Blunt-21nt-R were annealed to generate 21-nt blunt dsRNA.

EMSAs, Dicer and Slicer assays

EMSAs were performed as described previously [8,13]. Gel shifts of 126-nt blunt dsRNA were analyzed on 6% native polyacrylamide gels and those of small dsRNA and dsDNA duplexes on 12% native polyacrylamide gels. Radioactive signals were visualized using the Bio-Rad FX molecular imager and quantified with ImageJ, version 1.47K [60].

Dicer assays in S2 and U4.4 cell lysates and Slicer assays in embryo extracts were done as described previously [8,30]. In the Slicer assays, the recombinant proteins were added to the embryo lysate 30 minutes after the addition of siRNA duplexes, to allow assembly of mature RISC.

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Chapter 7

General discussion

The discovery of small RNA-mediated regulatory networks had a major impact on many aspects of biology, including virology and immunology. More specific, the identification of RNA interference (RNAi) as an antiviral defense mechanism in plants, insects, nematodes and fungi led to a better understanding of virus-host interactions. In the following sections, the results from this thesis will be discussed in relation to the current literature on antiviral RNAi in insects.

Small RNAs during virus infection in *Drosophila*

An important prerequisite for an effective immune system is the ability to distinguish self from non-self. Since double-stranded RNA (dsRNA) is produced by viruses with positive (+) strand RNA, dsRNA, or DNA genomes, but absent from non-infected cells, it may act as an important danger signal [1]. Antiviral RNAi in insects is triggered by viral dsRNA that is sensed and processed by Dicer-2 (Dcr-2) into viral small interfering RNAs (vsiRNAs). Therefore, viruses have evolved mechanisms to protect their dsRNA from cleavage by Dcr-2. First, (+) strand RNA viruses shield their dsRNA replication intermediates in membranous vesicles, which may physically exclude Dcr-2 from the site of dsRNA production [2,3]. Second, some viruses, like Drosophila C virus (DCV) and Flock House virus (FHV), express dsRNA-binding proteins that may bind the replication intermediates and shield them from Dcr-2 cleavage [4-7]. In addition, the genomic RNA of most dsRNA viruses is protected by the viral capsid throughout the life cycle of the virus [8]. Nevertheless, we and others have detected vsiRNAs by deep sequencing, suggesting that viral dsRNA is accessible for Dcr-2 cleavage (Chapters 1 and 3). These vsiRNAs may be derived from dsRNA genomes, dsRNA structures in viral single-stranded RNA (ssRNA) molecules, dsRNA replication intermediates, or dsRNA produced by convergent transcription of DNA viruses. As discussed below, the polarity and distribution of vsiRNAs across the viral genome provides important insight into the nature of the dsRNA precursor.

Small RNA profiles of (+) strand RNA viruses

In chapter 3, we identified Nora virus 21-nt RNAs in persistently infected flies. These vsiRNAs exhibited signatures of Dcr-2 cleavage products, mapped in equal proportions to the genomic (+) and antigenomic (-) RNA strand, and showed a uniform distribution along the viral genome. Therefore, we concluded that the Nora virus replication intermediates are targets of Dcr-2. This finding is in agreement with a profile of Nora virus-derived small RNAs that were detected in a persistently infected cell line [9]. In contrast, a bias towards the genomic (+) strand was found for DCV-derived vsiRNAs in two independent reports [10,11]. Since Nora virus and DCV employ different mechanisms to suppress the antiviral RNAi pathway (Argonaute-2

antagonism and dsRNA binding, respectively), we hypothesize that the suppression mechanism might influence the small RNA profiles. We speculate that dsRNA replication intermediates are the preferred substrates for Dcr-2, whereas dsRNA structures within viral ssRNA molecules are inefficiently processed into vsiRNAs and contribute to a minor fraction of the total vsiRNA pool. However, since the 1A protein of DCV binds long dsRNA molecules, it might protect the replication intermediates from cleavage by Dcr-2. As a result, vsiRNAs that derive from dsRNA structures in ssRNA molecules may become the major fraction of vsiRNAs. This skews the vsiRNA population towards the (+) strand, as viral genomic RNAs of (+) polarity greatly outnumber the viral antigenomic (-) strand RNAs.

The hypothesis that viral suppressors of RNAi (VSRs) modulate vsiRNA profiles is supported by studies with FHV, which encodes the VSR protein B2 that binds replication intermediates [4,12]. While vsiRNAs were skewed towards the (+) strand in infections with wildtype FHV, they mapped in an equal ratio to the (+) and (-) RNA strand in infections with a FHV mutant that lacks the B2 protein [12]. Nora virus viral protein 1 (VP1), on the other hand, counteracts the slicer activity of Argonaute-2 (AGO2), but does not inhibit Dcr-2 (Chapter 3), which leaves the replication intermediates accessible for Dcr-2. The observation that vsiRNAs map equally to both the (+) and (-) RNA strand in Nora virus infections is consistent with the proposed model. Whether the correlation between VSR mechanism and vsiRNA profile is generally applicable to RNA viruses remains to be established.

The uniform distribution of Nora virus vsiRNAs along the genome indicates that all regions of the viral genome are equally accessible for Dcr-2 cleavage. This is in contrast to FHV, for which prominent vsiRNA hotspots were observed in the central region and in the 5' and 3' untranslated regions (UTRs) of the viral genome [13-15]. Since these hotspots mainly correspond to genomic regions that are retained in defective interfering (DI) RNAs, DI RNAs were proposed to be a major source of the FHV vsiRNAs [9,14]. DI RNAs contain large deletions of viral genes needed for replication and encapsidation, yet retain cis-acting RNA elements that allow them to be replicated by the RNA-dependent RNA polymerase (RdRP) of a parental helper virus. Due to their smaller genome size, FHV DI RNAs replicate more efficiently than wildtype genomic RNAs. This leads to an abundant production of DI dsRNAs, and therefore to an overrepresentation of vsiRNAs in these regions of the viral genome. The lack of hotspots in the Nora virus small RNA profile suggests that there is no prominent production of Nora virus DI RNAs during infection.

Factors influencing small RNA profiles

Small RNA profiles from virus-infected insects give important insight into the biogenesis of virus-derived siRNAs. Nevertheless, caution should be taken with the analysis of the small RNA profiles since several factors can influence the observed

profile. First, to enable massive parallel sequencing, adapter sequences need to be ligated to the 5' and 3' ends of the small RNAs. Recently, it was shown that base pairing between adapter and small RNA molecule increased the ligation efficiency, which leads to a sequencing bias [16]. Introduction of four random nucleotides at the end of the adapter resolved part of the cloning bias, as these modified adapters base pair with a more diverse population of siRNAs. To assess the effect of the cloning bias on vsiRNA profiles, a side-by-side comparison of conventional and modified adapters for viral small RNA cloning is needed. Second, the viral template sequence to which the small RNAs are mapped is a factor that influences the small RNA profile. Due to their error prone RdRP, RNA viruses accumulate mutations at a high frequency, which may lead to changes in the genome sequence compared to previously published sequences. Using the published genome sequence as a template to map the viral small RNAs could therefore lead to an inaccurate profile. Recently, a method was described to accurately reconstruct viral genomes from the sequenced small RNAs [11]. By using reconstituted viral genomes as a reference sequence, more accurate small RNA profiles can be acquired (Chapter 3) that give better insight into the dsRNA precursors of vsiRNAs.

Drosophila AGO2 in antiviral defense

Viral siRNAs are mainly derived from replication intermediates for most (+) and (-) strand RNA viruses and from viral genomes of dsRNA viruses (viral small RNA profiles are discussed in chapter 1). Given their crucial roles during virus infection, dicing of viral dsRNA replication intermediates or dsRNA genomes could in theory be sufficient to control virus infections. This hypothesis, however, contradicts the observation that *AGO2* and *r2d2* knockout flies, in which Dcr-2 is fully functional, are hypersensitive to infections with viruses from various families [7,17-21]. The important role of AGO2 in antiviral defense is further supported by the identification of Nora virus VP1 and Cricket paralysis virus 1A (CrPV 1A) as RNAi suppressors that antagonize the catalytic activity of AGO2 (Chapter 3 and [22]).

Interactions between VSRs and AGO2

Given the lack of amino acid sequence similarity between Nora virus VP1 and CrPV 1A, these VSRs likely evolved through convergent evolution. Since both proteins interact with AGO2 to inhibit its catalytic activity, the question rises whether they bind to the same site. Interestingly, several eukaryotic proteins utilize Glycine/Tryptophan (GW) and/or Tryptophan/Glycine (WG) motifs for their interaction with AGO proteins [23-26]. Presumably, the tryptophan residues dock into two hydrophobic pockets that reside in the PIWI domain of AGO proteins [27]. Similar to cellular proteins,

the viral RNAi suppressor from a plant virus, the P38 protein of turnip crinkle virus, depends on two GW motifs that mediate binding to the *Arabidopsis* AGO1 protein [28]. However, GW/WG motifs cannot account for the AGO2 interaction of Nora virus VP1 and CrPV 1A, since these proteins do not contain such motifs. Moreover, the residues that form the tryptophan-binding hydrophobic pockets in AGO proteins are not conserved in *Drosophila* AGO2 [27]. In contrast, these pockets are present in *D. melanogaster* AGO1, which interacts with the GW motif-containing protein GW182 [23]. The lack of a tryptophan-binding pocket in *Drosophila* AGO2 suggests that this protein developed alternative mechanisms to interact with constituents of the RNA-induced silencing complex (RISC). Nora virus VP1 and CrPV 1A may exploit these alternative mechanisms, thereby leaving the AGO1-dependent miRNA pathway undisturbed (Chapter 3 and [22]). Elucidation of the mechanisms that mediate the interaction of Nora virus VP1 and CrPV 1A with *Drosophila* AGO2 may, therefore, identify docking sites on AGO2 for interaction partners.

Species-specific RNAi suppression

AGO2 and other components of the RNAi pathway (R2D2 and Dcr-2) are amongst the 3% fastest evolving genes in *Drosophila* species. The rapid evolution of AGO2 resulted in a high divergence in amino acid sequence between the AGO2 proteins of different *Drosophila* species [29]. The persistent nature of Nora virus infections suggests that Nora virus VP1 co-evolves with and adapts to the AGO2 protein of its host. This may lead to effiecient, but host-specific suppression of AGO2, which might come at the cost of losing suppressor activity against AGO2 proteins from non-host *Drosophila* species. Indeed, we showed that the suppressor activity of the VP1 protein from a Nora-like virus detected in *Drosophila immigrans* (DimmNV), is host-specific and depends on its ability to interact with Dimm AGO2 (Chapter 5). The inability of DimmNV VP1 to bind and suppress *D. melanogaster* (Dmel) AGO2 raises the question which VP1 residues are responsible for the host-specificity of the VP1 VSR activity. As the suppressor activity of both VP1 proteins is confined to the C-terminus, exchanging C-terminal amino acid motifs between DmelNV VP1 and DimmNV VP1 might give an answer to this question. At the same time, functional domains of Dmel and Dimm AGO2 could be exchanged to map the AGO2 amino acids required for an efficient VP1-AGO2 interaction. These approaches may not only reveal the VP1-AGO2 interaction domains, they could also provide a better mechanistic insight into how VP1 inhibits AGO2 slicer activity.

The ability of a virus to establish a productive infection in a given host species depends on multiple factors. One of the barriers that can determine host tropism is the host's immune response. In vertebrates, the interferon response provides powerful antiviral activity that controls virus infections. The inability to suppress the interferon response in a non-host species restricts host tropism for several vertebrate-infecting

viruses, including Measles virus, Polio virus, Parainfluenza virus, and Respiratory Syncytial virus [30]. Similarly, the host-specific RNAi suppressor activity of DimmNV VP1 may limit DimmNV infections to *D. immigrans*. The recently reported infectious clone of DmelNV, which allows swapping the DmelNV VP1 sequence for the DimmNV VP1 sequence, provides an excellent tool to test this hypothesis. A similar approach could be used with regard to the host. As Dimm AGO2 is fully functional in a *D. melanogaster* background (Chapter 5), a Dimm AGO2 rescue construct could be introduced into *D. melanogaster AGO2* knockout mutants. Successful infection of these transgenic flies, but not wildtype *D. melanogaster*, by a DmelNV-DimmNV VP1 chimeric virus, would establish VP1 as a host-specificity determinant.

Role of RNAi in Nora virus infections

The detection of vsiRNAs and the identification of virus-encoded RNAi suppression strongly suggest that Nora virus is a target of the antiviral RNAi pathway in flies (Chapters 1 and 3). Nevertheless, Nora virus titers were reported not to be affected in flies with defects in the RNAi pathway, suggesting that RNAi does not control Nora virus replication [31]. Although this study seems to contradict the data presented in this thesis, these findings may not be mutually exclusive. As hypothesized by Habayeb *et al.* [31], a potent VSR that completely blocks RNAi activity might explain the apparent insensitivity of the virus to the antiviral activity of RNAi. Hence, although vsiRNAs are produced during Nora virus infection, their function might be efficiently blocked by VP1. Although plausible, this is not observed for FHV, DCV and CrPV. These viruses encode potent VSR proteins, yet they replicate to higher levels and induce increased mortality in RNAi pathway mutants [6,7,20]. To reconcile the results in this thesis with the results of Habayeb *et al.*, and to obtain insight into the role of the RNAi suppressor *in vivo*, it is essential to study Nora virus infection in the absence of VP1 expression. The aforementioned infectious clone of Nora virus should allow the production of a Nora virus mutant that is deficient in VP1 VSR activity [32]. Subsequent infection of wildtype and RNAi mutant flies with the VP1-deficient virus may resolve the (in)sensitivity of Nora virus to RNAi and the role of the VP1 suppressor therein.

Identification of viral RNAi antagonists in RNAi sensor assays

In chapter 2, we describe an *in vitro* RNAi reporter assay in S2 cells for the identification of potential VSRs. Established VSRs, like DCV 1A and CrPV 1A, efficiently suppress RNAi-mediated silencing of the reporter (Chapters 2 and 3). More importantly, using the RNAi reporter assay, we have identified the novel VSRs Nora virus VP1 and Culex Y virus (CYV) viral protein 3 (VP3) (Chapters 3 and 6). In addition, we made an attempt

to identify VSRs in two natural pathogens of *Drosophila*, Drosophila A virus (DAV) and Sigma virus [33,34]. DAV is a (+) strand RNA virus that contains two large open reading frames (ORFs), ORF1 and ORF2, which encode the RdRP and capsid proteins, respectively. We analyzed DAV ORF1 and ORF2, as well as a smaller ORF downstream of ORF1 in the -1 reading frame, in our reporter assay. Similarly, we tested the N (nucleocapsid), P (phosphoprotein), X (unknown function), M (matrix protein), and G (glycoprotein) proteins encoded by the (-) strand RNA genome of Sigma virus for VSR activity. Despite robust expression of the DAV and Sigma virus proteins in S2 cells, none of these viral proteins showed clear suppression of dsRNA-induced RNAi (data not shown). These data suggest that DAV and Sigma virus do not encode a VSR protein. Nevertheless, DAV-derived vsiRNAs were detected in a persistently infected cell line [9], indicating that DAV is a target of the antiviral RNAi pathway. In addition, even though small RNA profiles are not yet reported for Sigma virus, vsiRNAs are detected in infections with vesicular stomatitis virus, a family member of Sigma virus [10,19]. Thus, although DAV and Sigma virus are both likely targets of the antiviral RNAi response, we were unable to detect VSR activity for these viruses using the current set-up of the RNAi sensor assay.

Although failure to detect RNAi suppressor activity in the sensor assay may suggest that a virus does not encode a VSR, it might also be caused by several other factors. First, the expression level of the viral proteins might be insufficient to detect RNAi suppressor activity. Second, a viral protein might depend on other viral proteins or processing from a polyprotein precursor for its functionality. Reporter assays with individual mature viral proteins, therefore, may fail to identify potential RNAi suppressors. Dependence on polyprotein processing was, for example, reported for inhibition of the interferon response in mammalian cells by Dengue virus (DENV). It was shown that the DENV non-structural (NS) proteins induce degradation of the immune signalling protein STAT2 when expressed from a replicon, but not when expressed as individual proteins [35]. Maturation of DENV NS5 through proteolytic processing was demonstrated to be essential for the STAT2 degrading activity [36]. Third, VSR activity is not limited to viral proteins, as non-coding viral RNAs can also mediate RNAi suppression. For example, high levels of adenovirus virus-associated (VA) RNAI and RNAII can saturate Dicer and thereby compete with other Dicer substrates [37]. Similarly, the subgenomic flavivirus RNAs (sfRNAs) of West Nile virus and DENV were shown to act as nucleic acid-based VSRs [38]. To overcome these limitations, RNAi sensor assays with infectious viruses can be performed, in which all viral proteins as well as potential non-coding RNAs are expressed at physiological levels.

Our results expose a fourth limitation of the RNAi sensor assay in S2 cells, since we found that viruses may encode RNAi suppressors whose activity is restricted to their natural host species. As S2 cells are derived from *D. melanogaster*, VSRs

from viruses that have a host species other than *D. melanogaster* may fail to show suppressor activity in our reporter assay (Chapter 5). Finally, spread of RNAi beyond the site of infection (systemic spread) was suggested to be essential for an effective antiviral immune response in *Drosophila* (discussed in the next section). Therefore, it is possible that some VSRs specifically inhibit systemic spread of an antiviral RNAi signal. An example of such a suppressor is the 2b protein from the plant virus Asparagus virus 2 (AV-2), which suppresses systemic silencing, but is unable to suppress local silencing [39]. Similarly, it was suggested that the P19 protein of Cymbidium Ringspot Tombusvirus specifically blocks systemic silencing but not local silencing [40]. As the RNAi sensor assay in S2 cells solely monitors cell-autonomous RNAi, it will not detect a VSR that only inhibits systemic spread of RNAi.

Systemic spread of RNAi in *Drosophila*?

Nora virus is an enteric virus that is transmitted via the fecal-oral route [41]. Despite the absence of an apparent pathogenic effect, the intestine of Nora virus-infected flies is abnormally fragile, and shows enlarged extracellular space between epithelial cells [41]. This suggests that the gut epithelial barrier is compromised during Nora virus persistent infections, which could allow the virus to disseminate into the hemolymph and to more distal sites. Indeed, we found Nora virus vsiRNAs in the head of infected flies as well as in the body (abdomen and thorax) and thorax (Chapter 3). The vsiRNAs in the head showed a uniform distribution along the viral genome and mapped in an equal ratio to the viral (+) and (-) RNA strands. Relative levels of Nora virus vsiRNAs were similar in head, body, and thorax, which may suggest active Nora virus RNA replication at these sites. Nevertheless, Habayeb and colleagues detected Nora virus RNA almost exclusively in the intestine of persistently infected flies, with only low levels of viral RNA in other tissues [41]. Therefore, it seems that the levels of small RNAs do not reflect virus titers. One explanation for the discrepancy between the reported tissue tropism and vsiRNA levels could be that the Nora virus small RNAs in the head originated from a putative spread of the RNAi response.

Systemic spread of RNAi is characterized by dissemination of the silencing signal beyond its site of initiation [42]. In plants, systemic RNAi consists of a local short-range spread as well as a systemic component that depends on a cellular RdRP for amplification of the silencing signal [43,44]. Both local and systemic spread of silencing in plants is mediated by siRNA duplexes, either protein-bound or not [43,44]. Extracellular small RNAs can also be detected in cell-free serum of mammals. These small RNAs, mainly microRNAs, are incorporated into exosomes and are suggested to provide a mechanism of intercellular communication [45]. Although the canonical antiviral RNAi pathway in insects seems to be a cell-autonomous

defense mechanism, a systemic RNAi response was suggested to occur during viral infections in *Drosophila* [46]. This systemic RNAi response seems to depend on the uptake of dsRNA, since dsRNA-uptake deficient flies are hypersensitive to DCV and Sindbis virus infection. Moreover, a sequence-specific silencing signal was shown to move ahead of a Sindbis virus infection. The authors suggested that through cell death, lysis, or a virus-induced shedding mechanism, viral dsRNA is released and taken up by non-infected cells that subsequently process the dsRNA. Similarly, Nora virus dsRNA molecules could be released during infection. Uptake of these dsRNA molecules by cells at distal sites would lead to the production of Nora virus vsiRNAs that bear the same characteristics as vsiRNAs derived from a dsRNA replication intermediate. In *Drosophila*, dicing of dsRNA typically results in 21-nt duplex siRNAs in which the two strands form a 19-nt base-paired duplex with 2-nt single-stranded 3' overhangs. Indeed, Nora virus 21-nt RNA reads from the (+) and (-) RNA strands tend to overlap by 19 nt in the body and thorax. Strikingly, viral small RNAs in the head failed to show this signature (Chapter 3). A speculative hypothesis for the absence of this siRNA signature is that vsiRNAs in the head result from transport of single-stranded siRNAs bound by AGO2 or another transporter. Regardless, the Nora virus small RNAs in the heads of infected flies may form the basis for future investigations of systemic RNAi in *Drosophila*.

Persistent vs. lethal infections in *Drosophila*

An acute virus infection may progress into a full-blown infection that causes disease and kills the host. Alternatively, the infection may be cleared from the host, resolved into a latent infection or result in a delicately balanced persistent infection. Although poorly understood, persistent infections without any pathogenic signs are commonly observed in insects and insect cell lines. For example, several *Drosophila* cell lines are persistently infected with multiple viruses, like FHV, DCV, or Nora virus [9,14,15]. At least for DCV and Nora virus, these persistent infections mimic the natural infections *in vivo*, as these viruses also establish persistent infections in flies [47,48]. Strikingly, opposed to DCV infection, high mortality is induced by infections with the closely related CrPV [49]. Since these viruses are close relatives, the question arises what determines the difference in their virulence. One study hypothesized that the potency and mechanism of the VSR is a key determinant for pathogenic outcome. [22]. This hypothesis was based on the observation that DCV has a mild VSR that shields long dsRNA from Dcr-2 cleavage, whereas CrPV encodes a potent VSR that inhibits AGO2-mediated target RNA cleavage. However, the identification of Nora virus VP1 contradicts this hypothesis, as Nora virus causes persistent infections, yet encodes a potent VSR that employs a similar mechanism as CrPV 1A (Chapter 3). This

suggests that the course of virus infections is at least partially determined by factors other than the mechanism of RNAi suppression.

Dicing and slicing during persistent infections

An alternative view on the establishment and maintenance of persistent infections came from the analysis of vsiRNAs in a cell line persistently infected with FHV [13]. Although FHV-derived vsiRNAs were abundantly produced, the bulk of these small RNAs were not associated with AGO2. Moreover, vsiRNAs failed to efficiently silence reporter constructs harbouring complementary target sequences. These results suggest that dicing of viral dsRNA, rather than target RNA cleavage by RISC, contributes to the persistent state of FHV infection. In analogy, we show that Nora virus small RNAs are produced in persistently infected flies (Chapter 3). Although we did not demonstrate AGO2 association of the vsiRNAs, the slicer antagonism of Nora virus VP1 implies that target RNA cleavage by AGO2 is essential for the antiviral activity of the RNAi pathway, also in persistent infections. Moreover, AGO2 knockdown in persistently infected cells increased the levels of FHV RNA, demonstrating that persistent FHV infections are limited, at least in part, by AGO2 [4,13,50].

Synthesis of viral cDNA in persistent infections

The production of DI RNAs may contribute to viral persistence of FHV. Due to their smaller size, DI RNAs have a replication advantage over the full-length genome, which results in a competition for viral and host factors needed for replication. Interestingly, a recent study showed that parts of the FHV RNA genome are reverse transcribed into cDNA forms during persistent infections in *Drosophila* cells and flies [51]. Sequencing of the FHV cDNA forms revealed FHV DNA fragments fused to long terminal repeat (LTR) retrotransposons, suggesting that retrotransposons provide the reverse transcriptase activity that is needed to produce the FHV cDNA fragments. Furthermore, the FHV cDNA fragments showed similar sequence rearrangements as FHV DI RNAs, which suggests that DI RNAs are either the template for or the product of the FHV cDNA forms. Mapping of FHV-derived siRNAs during persistent infections indicated that the FHV cDNA forms are transcribed and processed into vsiRNAs. Inhibition of reverse transcriptase activity during FHV infection reduced vsiRNA production and increased viral titers and cell death. This suggests that the formation of a FHV cDNA form contributes to the establishment of a persistent infection. Viral cDNA forms were also detected by PCR in cell lines persistently infected with DCV, Drosophila X virus, or Sindbis virus [51]. Strikingly, a Nora virus cDNA form could not be detected by southern blot in persistently infected *Drosophila* [47]. However, since the southern blot probe only covered a 371-nt region of ORF2, it remains possible that Nora virus cDNA forms of other genomic regions are produced during infection. As the virus-derived cDNA forms seem to contribute to the establishment

or maintenance of persistent infections, it would be of interest to determine if such cDNA forms are formed during pathogenic infections with CrPV. In addition, it would be worth investigating whether viruses have anti-reverse-transcriptase activity to counteract the production of virus-derived cDNA forms [52].

Tissue tropism and pathogenicity

The tissue tropism of a virus and the extent of tissue damage it inflicts are important factors that determine viral pathogenicity. The capacity to tolerate damage varies between tissues and organs, and depends on several intrinsic tissue properties [53]. Although the cell types in which Nora virus and DCV replicate during a natural infection remain elusive, it is suggested that these viruses replicate in the digestive tract [41,54]. Since the intestinal epithelium has a fast renewal rate, this tissue might have a high tolerance for tissue damage inflicted by DCV and Nora virus. This property might contribute to the non-pathogenic, persistent nature of Nora virus and DCV infections. Furthermore, the intestinal epithelium might form a barrier that prevents Nora virus and DCV from spreading to other tissues with a lower tolerance towards virus infections. Indeed, DCV infections can become pathogenic if the epithelial barrier of the intestine is overcome by injection of the virus in the body cavity (Chapter 4 and [18,55]). Specifically, pathogenesis might be caused by infection of the fat body, since suppression of RNAi in the fat body leads to an increased mortality after DCV infection (Chapter 4). In contrast, injection of Nora virus into the body cavity of flies fails to induce pathogenesis, but causes a persistent infection [41]. Whether this is caused by a difference in tissue tropism between Nora virus and DCV or other host and/or viral factors remains to be determined.

Small RNA-mediated antiviral defense and viral counter-defense in mosquitoes

In analogy to Nora virus and DCV, clinically important arthropod-borne (arbo-) viruses are known to cause persistent, non-pathogenic infections in their mosquito vector. Mosquitoes can become infected with arboviruses after ingestion of an infected blood meal. The ingested virus escapes the midgut to eventually end up in the salivary glands where it needs to accumulate to sufficiently high titers for successful transmission to a new vertebrate host during blood-feeding. Depending on environmental factors, such as temperature, accumulation of an adequate level of virus in the salivary glands is estimated to take a few days to a few weeks, a period in which fitness of the mosquito needs to be maintained for successful virus transmission [56,57]. Therefore, it is believed that arboviruses need to establish a non-pathogenic infection in their mosquito vector. In addition, mosquitoes can be

infected with mosquito-restricted viruses that are not transmitted to a vertebrate host. Detection of mosquito-restricted viruses in asymptomatic adult mosquitoes suggests that these viruses may also cause non-pathogenic persistent infections [58,59].

Viral suppression of RNAi in mosquitoes

Arboviruses and mosquito-restricted viruses are targets of the mosquito's RNAi pathway, since vsiRNAs are detected during virus infections [60-66]. Furthermore, an increase in sensitivity to arbovirus infections is observed upon knockdown of RNAi pathway components ([67] and discussed in chapter 1). Therefore, it might be expected that these viruses encode VSRs to counteract the mosquito's antiviral RNAi response. Indeed, in chapter 6 we found that the VP3 protein of the mosquito-restricted entomobirnavirus CYV, which was recently isolated from *Culex pipiens* mosquitoes [68], suppresses RNAi. We showed that CYV VP3 is able to bind long dsRNA as well as siRNAs *in vitro*, which is likely responsible for the potent RNAi suppression observed during CYV infection. These results show that the antiviral activity of the mosquito's RNAi pathway is potent enough to drive the evolution of VSR activity in mosquito-restricted viruses.

Suppression of the RNAi pathway was for a long time not believed to be a common feature of arboviruses. Due to their dependence on non-pathogenic infections for successful transmission, it was assumed that evolution of a potent VSR in arboviruses is selected against in nature. Hence, expression of a potent VSR may lead to pathogenesis and death of the arthropod vector, jeopardizing the spread to vertebrate hosts. Indeed, as opposed to wildtype virus, recombinant Sindbis virus that expresses the FHV B2 protein proved to be pathogenic to mosquitoes following an infectious blood meal as well as after injection [69,70]. Nevertheless, in recent years, RNAi suppressive activities have been identified in a number of arboviruses. The first arbovirus shown to express an RNAi suppressor protein is Nodamura virus (NoV) (*Nodaviridae*), a family member of FHV [71,72]. NoV was first isolated from *Culex tritaeniorhynchus* mosquitoes near the Japanese village of Nodamura [73]. Later, neutralizing antibodies were detected in plasma from pigs in areas near Tokyo, thereby classifying NoV as an arbovirus [74]. Similar to FHV, NoV expresses a B2 protein that inhibits RNAi by shielding dsRNA from Dicer cleavage, and by binding of siRNAs to prevent RISC loading.

Recently, RNAi suppressor activity was reported for the non-coding subgenomic flavivirus RNA (sfRNA) of West Nile virus and DENV [38]. As sfRNA is a substrate for Dicer and abundantly produced during flavivirus infections, it was suggested that the sfRNAs saturate Dicer in a concentration-dependent manner. The nucleic acid-based VSR activity of the sfRNAs is remarkable, as VSR activity of plant and insect viruses is almost exclusively confined to viral proteins. Recently, the DENV non-structural

protein 4B (NS4B) was also reported to contain VSR activity [75]. Results from this study indicate that NS4B inhibits Dcr-2 activity independent of dsRNA binding. How the RNAi suppressor activities of sfRNA and NS4B affect DENV infection *in vivo* remains to be established. Altogether, these data show that both arboviruses and mosquito-restricted viruses counteract the mosquito's antiviral RNAi pathway.

Antiviral piRNAs in mosquitoes

Piwi-interacting RNAs (piRNAs) are endogenously encoded 24-30-nt small RNAs that form one of the three major classes of small silencing RNAs [76]. In *Drosophila*, piRNAs are abundantly produced in gonadal tissues, where they protect the integrity of the germline by repressing transposable elements. In addition, virus-derived piRNA-like small RNAs were detected in a *Drosophila* ovarian somatic sheet cell line, suggesting an antiviral function of the insect piRNA pathway [9]. Indeed, virus-derived piRNAs can be detected during arbovirus infections in mosquitoes and mosquito cell lines. In contrast to *Drosophila*, these piRNA-like viral small RNAs were also found in somatic tissues of mosquitoes [11,62,64,66,77]. This suggests that, in addition to the RNAi pathway, the mosquito piRNA pathway exerts antiviral activity in somatic tissues. The antiviral potential of the mosquito's piRNA pathway was demonstrated in a recent report, which showed that knockdown of PIWI proteins in cell culture enhances Semliki Forest virus infection [77]. As these data indicate that mosquito viruses are inhibited by an antiviral piRNA response, it would be interesting to study whether mosquito viruses developed counter-defense mechanisms against the mosquito's piRNA pathway. Interestingly, a recombinant Chikungunya virus expressing the FHV B2 protein (CHIKV-B2) was more cytopathic than the parental virus in cell lines that are defective in siRNA biogenesis [64]. Furthermore, the increase in pathogenicity of CHIKV-B2 infection in mosquitoes was associated with a 2-fold decrease in viral piRNAs. Therefore, the authors suggested that FHV B2 inhibits the biogenesis of viral piRNAs in mosquito cell lines. Taken together, these results suggest a complex interaction between viruses and the antiviral siRNA and piRNA pathways in mosquitoes.

Future directions

Experimental work in *Drosophila* significantly contributed to our current understanding of the antiviral RNAi response in insects. Nevertheless, many questions remain. For example, how and when is the viral dsRNA recognized during virus infections? What is the nature of the systemic silencing signal? How does RNAi contribute to the establishment of persistent infections? How do arboviruses suppress the RNAi response while maintaining a persistent infection? Given the

extensive genetic toolbox in *Drosophila*, studies in flies will continue to contribute novel insights into the antiviral RNAi response in insects. This may lead to a better understanding of antiviral RNAi in other insects, including important vector mosquitoes. Nevertheless, the course of infection of arboviruses differs greatly from virus infections in *Drosophila*, since arboviruses rely on dissemination to the salivary glands for their transmission. In addition, differences between *Drosophila* and mosquito antiviral defenses are apparent, as the piRNA pathway seems to play a more prominent role in mosquitoes. These observations emphasize the need for experimental infection models and genetic tools in mosquitoes. In-depth understanding of virus-host interactions in insects might lead to improved strategies to control arbovirus transmission.

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Chapter 8

Summary/samenvatting

Summary

Viruses are microscopic particles that in their basic form consist of an RNA or DNA genome surrounded by a protective shell. As viruses are obligate intracellular parasites, they can only replicate within cells of a host organism. To enable their replication, viruses exploit cellular processes and resources of the host, which may lead to cell death and disease in the host. In turn, host organisms defend themselves by mounting an immune response that aims to restrict virus replication and spread. A productive immune response starts with the detection of the invading virus by cellular sensors that recognize pathogen associated molecular patterns (PAMPs), which are molecular structures that are conserved within classes of microbes. As double-stranded RNA (dsRNA) is produced during most virus infections, but not detected in non-infected cells, it is considered to be a major viral PAMP. In vertebrates, the detection of dsRNA induces the production of cytokines that serve as signal molecules to induce, amplify, and regulate innate and adaptive immune responses. Non-vertebrate animals, however, lack the innate and adaptive immune response of vertebrates. In insects, dsRNA triggers the RNA interference (RNAi) pathway, in which the enzyme Dicer-2 (Dcr-2) detects and cleaves the viral dsRNA molecules into small interfering RNA (siRNA) duplexes of approximately 21 nucleotides in size (**Chapter 1**). Subsequently, siRNA duplexes are loaded into an RNA-induced silencing complex (RISC), a complex of proteins that contains at least the Argonaute-2 (AGO2) protein. One strand of the siRNA duplex is retained in RISC to guide the binding of complementary viral RNA sequences, which leads to AGO2 mediated cleavage (Slicing) of the viral RNA. Since efficient AGO2 Slicer activity depends on extensive basepairing between the siRNA guide strand and the target RNA, RNAi results in sequence specific silencing of RNA transcripts.

Both Dcr-2 and AGO2-mediated cleavage of viral RNA molecules inhibits virus infection in insects. As a counter-defense, insect viruses encode viral suppressors of RNAi (VSRs) that counteract the antiviral activity of the RNAi pathway. Prior to the start of this PhD project, the Drosophila C virus (DCV) 1A protein and Flock House virus (FHV) B2 protein were the only VSRs identified in insect viruses. Both DCV 1A and FHV B2 can bind dsRNA molecules, thereby shielding them from Dcr-2 cleavage. FHV B2 is also able to prevent loading of siRNAs into RISC by binding siRNA duplexes. As only a limited number of insect VSRs were identified, we aimed in this thesis to identify and characterize new insect VSRs to gain a better understanding of the virus-host interactions in insects. In the next paragraphs the results of this thesis are summarized.

To screen for potential VSR proteins, we describe in **chapter 2** an RNAi sensor assay in *Drosophila melanogaster* S2 cells. In this assay, we induce RNAi-mediated silencing of a firefly luciferase (FLuc) reporter gene, which decreases the amount of

FLuc enzyme produced, to monitor RNAi efficiency. Hence, the more efficient the RNAi-mediated silencing of the FLuc reporter gene, the lower the FLuc production. To identify potential VSRs, we then perform this assay in the presence or absence of viral proteins. If a viral protein interferes with the RNAi-mediated silencing of the Fluc reporter, it is considered a putative VSR. Once a putative VSR is identified, additional reporter assays and biochemical experiments are needed to determine the RNAi suppressor mechanism in more detail.

Recently, Nora virus was identified as a natural pathogen of *Drosophila* that causes a non-lethal persistent infection. In **chapter 3** we detected Nora virus-derived siRNAs in persistently infected *Drosophila*, showing that the antiviral RNAi pathway targets Nora virus. Using the RNAi sensor assay described in chapter 2, we identified viral protein 1 (VP1), the protein product of open reading frame 1, as a VSR. Furthermore, we showed that Nora virus VP1 and the unrelated Cricket paralysis virus 1A (CrPV1A) protein both suppressed AGO2-mediated RNA cleavage. Convergent evolution of AGO2 slicer antagonism in these two distantly related insect RNA viruses emphasizes the important role of AGO2 slicer activity in antiviral defense.

In **chapter 4**, we explored the possibility of using the VSR activity of VP1 to study the RNAi pathway *in vivo*. Expression of a VP1 construct in transgenic flies potently suppressed RNAi-mediated gene silencing. Similar to AGO2 knock-out flies, transgenic flies ubiquitously expressing VP1 or specifically expressing VP1 in the fat body were more sensitive to virus infection. Altogether, we established the VP1 transgenic fly as a useful tool to study RNAi regulated processes *in vivo*.

Host immune responses and viral immune antagonists can co-evolve in what is called an evolutionary arms race. As a result, viral immune antagonists adapt to efficiently counteract the immune response of their host species. In turn, this may lead to the inability to suppress the antiviral response in other species, which may restrict the host range of the virus. Therefore, we investigated in **chapter 5** whether the VSR activity of VP1 can determine the host range of Nora virus. To this end, we isolated VP1 sequences from Nora-like viruses detected in the fruit fly species *Drosophila immigrans* (DimmNV VP1) and *Drosophila subobscura* (DsubNV VP1). We compared the VSR activity of these VP1 proteins to that of VP1 from Nora virus isolated from *Drosophila melanogaster* (DmelNV VP1), which we described in chapters 3 and 4. In contrast to DmelNV VP1, we were unable to detect RNAi suppressive activity of DimmNV VP1 and DusbNV VP1 in *D. melanogaster* S2 cells. Instead, DimmNV VP1 specifically interacted with, and suppressed the RNA cleavage activity of AGO2 from its natural host, *D. immigrans*. This RNAi suppressive activity was species-specific, as DimmNV VP1 did not interact with, and did not suppress the activity of AGO2 from its non-host species, *D. melanogaster*. Surprisingly, the DmelNV VP1 suppressor activity was not restricted to its host species, as it interacted with both the *D. melanogaster* and *D. immigrans AGO2* proteins and suppressed the RNA cleavage activity of both

these AGO2 proteins. Nevertheless, we suggest that the specific adaptation of Nora virus VP1 to the host AGO2 protein can result in host-specific RNAi suppressor activity. Our results imply that RNAi suppressors may be host specificity determinants.
The RNAi pathway provides antiviral activity against a broad range of insect viruses with different genome structures. Nevertheless, insect VSRs were only detected in viruses with a positive stranded RNA genome. In addition, it is unclear whether mosquito viruses generally suppress the antiviral activity of the mosquito's RNAi pathway. We therefore investigated in **chapter 6** whether two dsRNA viruses, the entomobirnaviruses Culex Y virus (CYV) and Drosophila X virus (DXV), suppress the antiviral RNAi pathway. DXV is known to persistently infect *Drosophila* cell cultures; CYV was recently isolated from wild-caught *Culex pipiens* mosquitoes. We show that both CYV and DXV suppress RNAi in infected *Drosophila* S2 cells. Furthermore, we identified viral protein 3 (VP3) of both viruses as an RNAi suppressor protein in our RNAi sensor assay. Biochemical assays showed that both CYV VP3 and DXV VP3 bind long dsRNA as well as siRNAs. In line with the ability to bind long dsRNA, we demonstrate that the VP3 proteins of CYV and DXV can inhibit Dcr-2 mediated cleavage of dsRNA. In addition, binding of siRNAs by CYV and DXV VP3 suggests that they also scavenge siRNAs to prevent their incorporation into RISC. Therefore, our results imply that entomobirnaviruses suppress two steps of the antiviral RNAi pathway, dsRNA processing and RISC loading. Furthermore, our results suggest that mosquito viruses suppress the antiviral RNAi response in their host.
In **chapter 7** the results of this thesis are discussed and suggestions for future investigations are made.

Samenvatting

Virussen zijn microscopisch klein en bestaan uit genetische informatie, in de vorm van RNA of DNA, dat wordt omringd door een beschermend omhulsel. Omdat virussen intracellulaire parasieten zijn, kunnen ze alleen repliceren in de cellen van een gastheer. Hiertoe gebruiken virussen processen en grondstoffen van de gastheer. Dit kan leiden tot celdood en ziekteverschijnselen in de gastheer. De gastheer verdedigt zichzelf op zijn beurt door middel van een immuunreactie met als doel virusreplicatie en -verspreiding tegen te gaan. Een antivirale immuunreactie begint met de detectie van het infecterende virus door cellulaire sensoren die pathogeen-geassocieerde moleculaire patronen herkennen. Dit zijn moleculaire structuren die aanwezig zijn in bepaalde groepen ziekteverwekkers. Omdat de meeste virussen dubbelstrengs RNA (dsRNA) produceren tijdens een infectie en omdat dsRNA nauwelijks aanwezig is in gezonde cellen, wordt dsRNA gezien als een belangrijk virus-geassocieerd moleculair patroon. In gewervelde dieren leidt de detectie van dsRNA tot de productie van signaalmoleculen die aspecifieke en adaptieve immuunreacties induceren en reguleren. Ongewervelde dieren beschikken daarentegen niet over de aspecifieke en adaptieve immuunmechanismen van de gewervelden en zijn afhankelijk alternatieve antivirale immuunreacties. In insecten bijvoorbeeld, wordt dsRNA gedetecteerd door het enzym Dicer-2 (Dcr-2) dat het virale dsRNA knipt in kleinere dsRNA moleculen van ongeveer 21 nucleotiden: de zogenoemde “small interfering RNAs” (siRNAs). Vervolgens worden de siRNAs ingebouwd in een “RNA-induced silencing complex” (RISC): een eiwitcomplex waar in ieder geval het Argonaute-2 (AGO2) eiwit deel van uitmaakt. Eén streng van de siRNA blijft behouden in het RISC om complementaire virale sequenties te binden, wat leidt tot een AGO2-gemedieerde knip in het virale RNA. Het complete mechanisme, van dsRNA detectie door Dcr-2 tot het knippen van het virale RNA door AGO2, wordt aangeduid als RNA-interferentie (RNAi) (**Hoofdstuk 1**). Omdat de AGO2-gemedieerde knip in het virale RNA afhangt van extensieve basenparing tussen de ingebouwde siRNA-streng en de virale RNA-sequentie, resulteert RNAi in sequentie-specifieke afbraak van het virale RNA.

Door het knippen van viraal RNA door zowel Dcr-2 als AGO2 beperken insecten de replicatie en verspreiding van virussen. Echter, insectenvirussen produceren eiwitten die de antivirale activiteit van het RNAi-mechanisme onderdrukken (“viral suppressors of RNAi”, VSRs). Voordat dit promotieonderzoek begon, waren het 1A-eiwit van Drosophila C virus (DCV) en het B2-eiwit van Flock House virus (FHV) de enige geïdentificeerde VSR-eiwitten van insectenvirussen. Zowel DCV 1A- als FHV B2-eiwitten kunnen binden aan dsRNA-moleculen waardoor deze eiwitten het dsRNA beschermen tegen het knippen door Dcr-2. Verder kan het FHV B2-eiwit siRNA moleculen binden, waardoor wordt voorkomen dat deze siRNAs ingebouwd

worden in het RISC. Omdat het aantal VSR-eiwitten van insectenvirussen beperkt was, had dit promotieonderzoek als doel nieuwe VSR-eiwitten te identificeren en te karakteriseren om daarmee meer inzicht te krijgen in de virus-gastheer interacties in insecten. In de volgende paragrafen zijn de resultaten van dit proefschrift samengevat.

Om nieuwe VSR-eiwitten te identificeren, hebben we een RNAi-sensorexperiment in *Drosophila* S2-cellen gebruikt zoals beschreven staat in **hoofdstuk 2**. In dit experiment induceren we RNAi-gemedieerde afbraak van een "Firefly luciferase" (FLuc) reporter-RNA. Dit leidt tot een lagere productie van het FLuc-enzym, waarmee de efficiëntie van de RNAi-gemedieerde afbraak wordt gemeten. Dus als de FLuc-enzymproductie lager is, is de RNAi-gemedieerde afbraak van het FLuc reporter-RNA efficiënter. Om potentiële VSR-eiwitten te identificeren doen we dit experiment in de aan- of afwezigheid van virale eiwitten. Als in de aanwezigheid van een viraal eiwit de RNAi-gemedieerde afbraak van de FLuc-reporter geremd wordt, dan beschouwen we dit eiwit als een mogelijke VSR. Als een mogelijke VSR geïdentificeerd is, dan zijn aanvullende RNAi-sensorexperimenten en biochemische experimenten nodig om het mechanisme van RNAi-suppressie te bepalen.

Nora virus is recentelijk geïdentificeerd als een natuurlijk pathogeen van *Drosophila* dat een niet-lethale persistente infectie veroorzaakt. In **hoofdstuk 3** hebben we Nora virus-specifieke siRNAs gedetecteerd in persistent geïnfecteerde *Drosophila*. Dit geeft aan dat het antivirale RNAi-mechanisme de Nora virus infectie detecteert. Door gebruik te maken van het RNAi-sensorexperiment uit hoofdstuk 2, hebben we "viral protein 1" (VP1), het eiwit dat wordt gecodeerd door het eerste open leesraam van Nora virus, kunnen identificeren als VSR. Verder hebben we aangetoond dat het VP1-eiwit van Nora virus en het 1A-eiwit van het niet-gerelateerde Cricket Paralysis virus (CrPV) beide de AGO2-gemedieerde knip van complementair RNA kunnen tegengaan. De detectie van de AGO2-antagonerende activiteit in deze niet-gerelateerde insectenvirussen benadrukt de belangrijke rol van AGO2-gemedieerde RNA-afbraak tijdens de antivirale afweer in insecten.

In **hoofdstuk 4** hebben we onderzocht of het mogelijk is om het VP1-eiwit van Nora virus te gebruiken voor het *in vivo* bestuderen van het RNAi-mechanisme. Expressie van het VP1-eiwit van Nora virus in transgene vliegen resulteerde in een efficiënte onderdrukking van het RNAi-mechanisme. Evenals vliegen die het AGO2 gen missen, waren transgene vliegen die VP1 produceerden in alle cellen of specifiek in cellen van de "fat body" gevoeliger voor een virusinfectie. In dit hoofdstuk hebben we laten zien dat transgene vliegen die het Nora virus VP1-eiwit tot expressie brengen een goed hulpmiddel zijn voor het bestuderen van RNAi-gereguleerde processen *in vivo*.

De immuunreacties van de gastheer en de virale antagonisten die deze immuunreacties tegengaan kunnen co-evolueren gedurende een zogenaamde evolutionaire wedloop. Gedurende deze tweestrijd kunnen virale immuun

antagonisten zich aanpassen aan de immuunreactie van de gastheer om deze efficiënt te onderdrukken. Dit kan leiden tot een situatie waarin het betreffende virus niet meer in staat is de antivirale immuunreactie in andere gastheersoorten te onderdrukken, waardoor het virus mogelijk gelimiteerd wordt in zijn gastheerbereik. Daarom hebben we in **hoofdstuk 5** onderzocht of de VSR-activiteit van het VP1-eiwit van Nora virus het gastheerbereik van Nora virus kan bepalen. Om deze vraag te beantwoorden, hebben we de VP1-coderende sequenties geïsoleerd van Nora virussen die gedetecteerd zijn in de fruitvliegsoorten *Drosophila immigrans* (DimmNV VP1) en *Drosophila subobscura* (DsubNV VP1). Vervolgens hebben we de VSR-activiteiten van deze VP1-eiwitten vergeleken met die van het VP1-eiwit afkomstig uit Nora virus geïsoleerd uit *Drosophila melanogaster* (DmelNV VP1). In tegenstelling tot DmelNV VP1, hebben we geen VSR-activiteit kunnen detecteren voor DimmNV VP1 en DsubNV VP1 in *D. melanogaster* S2-cellen. Wel hebben we aangetoond dat DimmNV VP1 specifiek bindt aan AGO2 van zijn natuurlijke gastheer *D. immigrans* en hierdoor de AGO2-gemedieerde knip van complementair RNA kan tegengaan. Deze RNAi-suppressie was gastheer-specifiek, aangezien DimmNV VP1 niet bindt aan het AGO2-eiwit van *D. melanogaster* en niet de knip van complementair RNA kan tegengaan in deze gastheer. Opvallend was dat de VSR-activiteit van DmelNV VP1 niet gelimiteerd was tot één gastheer aangezien dit eiwit bond aan AGO2 van zowel *D. melanogaster* als *D. immigrans* en ook de knip van complementair RNA kon tegengaan in beide gastheren. Toch veronderstellen wij dat de evolutionaire aanpassing van het VP1-eiwit van Nora virus op het AGO2-eiwit van de gastheer ertoe kan leiden dat de VSR-activiteit van VP1 gastheer-specifiek wordt. Onze resultaten impliceren dat VSR-eiwitten mogelijk het gastheerbereik van insectenvirussen bepalen.

Het RNAi-mechanisme is een belangrijke immuunreactie tegen een breed scala aan insectenvirussen met verschillende genoomstructuren. Desalniettemin zijn VSR-eiwitten van insectenvirussen alleen gedetecteerd in virussen met een plus-strengs RNA genoom. Verder is het onduidelijk of muggenvirussen in het algemeen de antivirale activiteit van het RNAi-mechanisme van de mug tegengaan. Daarom hebben we in **hoofdstuk 6** bepaald of twee insectenvirussen met een dsRNA-genoom, de entomobirnavirussen Culex Y virus (CYV) en Drosophila X virus (DXV), het antivirale RNAi-mechanisme onderdrukken. Van DXV is bekend dat het *Drosophila* cellen persistent kan infecteren en CYV is recentelijk geïsoleerd uit *Culex pipiens* muggen die afkomstig zijn uit de vrije natuur. Wij hebben in hoofdstuk 6 laten zien dat zowel CYV als DXV het RNAi-mechanisme in *Drosophila* S2 cellen kan onderdrukken. Verder hebben wij met behulp van ons RNAi-sensorexperiment een VSR-activiteit kunnen detecteren voor “viral protein 3” (VP3) van beide virussen. In biochemische experimenten binden CYV VP3 en DXV VP3 zowel lang dsRNA als siRNA-duplexen. Ook hebben we gevonden dat de VP3-eiwitten van CYV en DXV het knippen van dsRNA door Dcr-2 kunnen tegengaan, wat geheel in lijn is met het vermogen van

deze eiwitten om lang dsRNA te binden. Daarbij suggereert de binding van siRNAs door beide VP3-eiwitten dat ze ook kunnen voorkomen dat de siRNAs ingebouwd worden in het RISC. Daarom lijkt het erop dat entomobirnavirussen twee stappen in het RNAi mechanisme tegengaan, namelijk het knippen van dsRNA en het laden van het RISC. Verder suggereren onze resultaten dat ook muggenvirussen het RNAi-mechanisme in de gastheer onderdrukken.
In **hoofdstuk 7** worden de resultaten van dit promotieonderzoek bediscussieerd en worden er aanbevelingen gedaan voor vervolgonderzoek.

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About the author

Joël Thomas van Mierlo was born in Zwolle on the 24th of July 1984. He was raised in Harlingen, where he completed his high school education at the RSG Simon Vestdijk in 2002. He continued his education with a Bachelor and Master study in Biotechnology at the Wageningen University and Research Centre, for which he graduated in 2007 and 2008, respectively. As part of his study Biotechnology he joined the laboratory of Molecular Biology in Wageningen for a Master thesis of six months on chromatin remodelling factors in plants, supervised by Dr. Ludmila Mlynarova. Inspired by lectures of Prof. Rob Goldbach, he developed a major interest in virology research and decided to do a second Master thesis of six months at the laboratory of Virology in Wageningen. This thesis involved the identification of viral suppressors of RNAi in mammalian infecting viruses, supervised by Dr. Esther Schnettler. Subsequently, he moved to Scotland for an internship of five months at the School of Biology of St. Andrews University. In the lab of Prof. Richard Elliott, he worked under the supervision of Dr. Xiaohong Shi and successfully rescued and characterized recombinant Bunyamwera viruses that express fluorescently labelled viral structural proteins. After his graduation he started his PhD project in 2009 in the department of Medical Microbiology at the Radboud University in Nijmegen. In the lab of Dr. Ronald van Rij, he worked on the identification and characterization of viral suppressors of RNAi, as described in this thesis. During his PhD project, he received the best oral presentation award at the annual NCMLS PhD retreat 2012 and the best poster award at the ESF-EMBO meeting on antiviral RNAi in Poland. Currently, he is working as a senior researcher at Micreos Food Safety located in Wageningen, where he works in a team that develops natural phage products against dangerous bacteria in the food chain.

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