The Cricket Paralysis Virus Suppressor Inhibits microRNA Silencing Mediated by the Drosophila Argonaute-2 Protein

Corinne Besnard-Guérin, Caroline Jacquier*, Josette Pidoux, Safia Deddouche, Christophe Antoniewsk*

Drosophila Genetics and Epigenetics, Institut de Biologie Paris Seine, CNRS UMR7622 & Université Pierre et Marie Curie, 9 quai Saint-Bernard, F75005, Paris, France

* Current address: Chromatin and Cell Biology, Institut de Génétique Humaine, CNRS UPR 1142, 141 rue de la Cardonille, F34396, Montpellier, France
* christophe.antoniewski@upmc.fr

Abstract

Small RNAs are potent regulators of gene expression. They also act in defense pathways against invading nucleic acids such as transposable elements or viruses. To counteract these defenses, viruses have evolved viral suppressors of RNA silencing (VSRs). Plant viruses encoded VSRs interfere with siRNAs or miRNAs by targeting common mediators of these two pathways. In contrast, VSRs identified in insect viruses to date only interfere with the siRNA pathway whose effector Argonaute protein is Argonaute-2 (Ago-2). Although a majority of Drosophila miRNAs exerts their silencing activity through their loading into the Argonaute-1 protein, recent studies highlighted that a fraction of miRNAs can be loaded into Ago-2, thus acting as siRNAs. In light of these recent findings, we re-examined the role of insect VSRs on Ago-2-mediated miRNA silencing in Drosophila melanogaster. Using specific reporter systems in cultured Schneider-2 cells and transgenic flies, we showed here that the Cricket Paralysis virus VSR CrPV1-A but not the Flock House virus B2 VSR abolishes silencing by miRNAs loaded into the Ago-2 protein. Thus, our results provide the first evidence that insect VSR have the potential to directly interfere with the miRNA silencing pathway.

Introduction

RNA interference (RNAi) provides one of the main lines of defense against RNA viruses in plants and invertebrates [1–4]. Recently, RNAi was also shown to play an antiviral role in mouse embryonic stem cells and suckling mice, suggesting that it could also participate in early antiviral defenses in mammals [5,6].

In Drosophila melanogaster, viral infection triggers the production of 21 nt small interfering RNAs duplexes (siRNAs) from the processing of viral double-stranded RNA (dsRNA) intermediates by the RNase III enzyme Dicer-2 (Dcr-2). SiRNA duplexes are loaded into the
Argonaute-2 (Ago-2) protein complexes, unwounded and one of the strand guides the cleavage of target RNAs by sequence complementarity [7–9]. The importance of RNAi in Drosophila antiviral defense is illustrated by the dramatic increase in sensitivity of dcr-2 and ago-2 mutants upon viral infections [1,2,4].

To counterattack, viruses encode viral suppressors of RNAi (VSRs) that interfere with host antiviral silencing [3,10,11]. Several plant viruses express VSRs that use a variety of tactics to inhibit both siRNAs and miRNAs silencing pathways by targeting common processing factors [12–14]. In contrast, VSRs identified in insect viruses are described to specifically suppress the siRNA pathway through diverse evolutionarily convergent strategies. For example, the Flock House virus (FHV) B2 binds long dsRNAs to inhibit their processing by Dicer proteins [15–17], and sequesters siRNA duplexes to prevent their loading to Argonaute complexes [18–20]. In contrast, the Cricket Paralysis virus (CrPV) CrPV-1A directly binds to the Drosophila Ago-2 protein and inhibits both siRNA loading and Ago-2 slicing activity [21].

In animals, microRNAs (miRNAs) regulate most cellular processes including development, cell proliferation, differentiation and immune responses [22]. They mediate gene silencing through imperfect sequence complementarity with their target mRNAs [23]. Duplexes of ~22nt long miRNA/miRNA* molecules derive from stem-loop precursor transcripts through sequential processing by the Drosha/Pasha microprocessor and the Dicer-1/Loqs complex [24]. In Drosophila melanogaster, most miRNA duplexes are loaded into a RNA-induced silencing complex (RISC) containing Argonaute-1 (Ago-1). Following elimination of the miRNA* passenger strand, the mature miRNA strand guides Ago-1 for translational repression and/or destabilization of mRNA targets [23]. Pairing between the 5’ seed regions of miRNAs and the 3’UTR of their mRNA targets is generally sufficient to ensure silencing [25–27]. In Drosophila, the Ago-2-dependent siRNA and Ago1-dependent miRNA pathways are compartmentalized. Nevertheless, a subset of miRNAs do not conform to this compartmentalization: they preferentially associate with the RNAi component Ago-2 and, similarly to siRNA, can guide potent mRNA target slicing, provided that they have extensive sequence complementarity with the target [9,26,28–30]. The Drosophila community largely exploits this miRNA property for gene knockdown experiments using Valium transgenes that express artificial miRNAs reprogrammed against specific gene sequences [31–33]. Likewise, we have recently shown that artificial miRNAs with perfect complementarity to GFP sequences interact with Ago-2 for potent GFP reporter silencing [34].

In light of these recent developments, we decided to re-visit the effects of VSRs on silencing mediated by miRNA associated with Ago-2. Here we show that CrPV-1A but not B2 strongly interfere with the Ago-2-dependent miRNA silencing. Altogether our results demonstrate that VSRs targeting Ago-2 processing complexes have the potential to directly interfere with part of the miRNA regulatory network in Drosophila.

Results

The CrPV-1A but not the B2 VSR inhibits the Ago-2-dependent Drosophila miRNA pathway

In order to analyze the effect of VSRs on miRNAs loaded into Ago-2, we took advantage of the automiG reporter system recently developed in our laboratory [34]. As previously described, this reporter is sensitive to Ago-2-silencing activity mediated by the artificial miRNAs miG-1 and miG-2 targeting GFP sequences. Here, we set up an automiG-derivate system combining a miG-1-miR-6.1-mRFP silencing vector and a pUbi-GFP target plasmid (Fig. 1A). The ubiquitin promoter in the miG-1-miR-6.1-mRFP construct drives the expression of both the monomeric Red Fluorescent Protein and the stem-loop precursor sequences of miG-1 and miR-6.1.
miRNAs inserted in an Rpl17 intron (Fig. 1A, right panel). It is noteworthy that since mRFP and miG-1 expressions rely on the same ubiquitin promoter, the level of mRFP protein directly reflects the expression level of miG-1. As a negative control, we used the miR-5-miR-6.1-mRFP construct that is devoid of GFP-targeting miRNA (Fig. 1A, left panel). The GFP protein was readily detected in western blot experiments upon co-transfection of Drosophila S2 cells with the miR-5-miR-6.1-mRFP control plasmid and the pUbi-GFP target plasmid (Fig. 1B, lane 1). In contrast, the GFP protein was barely detectable in the presence of the miG-1-miR-6.1-mRFP silencing plasmid (Fig. 1B, lanes 5 and 8), indicating that miG-1

**Fig 1.** CrPV-1A suppresses the Ago-2-dependent miRNA silencing in S2 cells. (A) Schematic representation of the reporter system for miRNA silencing. Pre-miRNAs inserted in an Rpl17 intron and the mRFP gene are expressed from the same unspliced transcript under the control of the ubiquitin promoter. The miR-5-miR-6.1-mRFP construct (left) expresses miR-5 and miR-6.1 without complementarity to the GFP and was used as a control. The miG-1-miR-6.1-mRFP construct (right) expresses miR-6.1 as well as miG-1 which targets the GFP mRNA expressed from pUbi-GFP with perfect complementarity. (B) Western blot analysis of Drosophila S2 cell lysates, co-transfected with miRNA expression constructs described in (A), the pUbi-GFP sensor plasmid, and the C-terminal HA-tagged CrPV-1A or B2 expression vectors. Control plasmid with non-cognate miR-5 and miR-6 was used as a control for miRNA target specificity and α-tubulin is the loading control. One representative experiment out of five is shown.
efficiently silences GFP expression. The mRFP protein was detected at similar level across samples, which reflected similar levels of miRNA expression (Fig. 1B).

The GFP protein was also undetectable upon co-transfections of miG-1-miR-6.1-mRFP, pUbi-GFP with a vector expressing the HA-tagged B2 VSR (Fig. 1B, compare lane 4 with lane 5). As the HA-tagged B2 protein was shown to efficiently suppress RNAi triggered by long dsRNA in S2 cells (CA, unpublished results), this result indicates that active B2 is unable to suppress miG-1 silencing activity. In striking contrast, strong expression of the GFP protein was restored in the presence of a vector expressing the HA-tagged CrPV-1A VSR (Fig. 1B, lane 7), which was previously characterized as a 148 amino acids (aa) polypeptide able to bind Ago-2 and efficiently suppress its silencing activity [21]. A C-terminal shortened 108 aa CrPV-1Am polypeptide fails to bind Ago-2 and to suppress siRNA silencing [21]. Consistently, a construct expressing this HA-tagged CrPV-1Am mutant form failed to suppress GFP silencing (Fig. 1B, lane 6) indicating that direct interaction with Ago-2 is required. Together, these data indicate that the CrPV-1A VSR efficiently suppress Ago-2-mediated miG-1 silencing.

The CrPV-1A VSR can prevent the silencing of miRNAs with perfect or imperfect mRNA binding sites

Most endogenous miRNAs imperfectly match multiple sites in their mRNA targets [25]. We thus examined the effect of CrPV-1A on miRNAs imperfectly matching their complementary sequences. To this end, we set up two additional reporter systems based on mRFP silencing by the artificial miG-2 miRNA (Fig. 2A), that can be loaded into either Ago-1 or Ago-2 complexes [34]. mRFP target constructs in these systems were generated by inserting 4 target sites with perfect (pMTmRFP-PM) or imperfect (pMTmRFP-IM) matches to miG-2 in the 3'UTR of an mRFP reporter gene driven by the copper-inducible metallothionein promoter (Fig. 2A).

In the absence of pAct-miG-2, expression of the mRFP protein was readily detected in transfected cells after induction with copper sulfate of pMTmRFP-IM or pMTmRFP-PM target constructs (Fig. 2B, lanes 1 and 6). As expected, mRFP expression from both constructs was strongly reduced in the presence of the miG-2 expressing vector pAct-miG-2 (Fig. 2B, lanes 2 and 7). To further characterize the miG-2 silencing activity in our reporter system, we carried out knockdown experiments of Ago-1 and Ago-2 as previously described [34]. Thus, Ago-1 or Ago-2 dsRNAs were co-transfected with pMTmRFP-IM or pMTmRFP-PM. The reporter constructs were copper-induced 24 hours post-transfection and mRFP expression was monitored by western blot 48 hours later.

Silencing of pMTmRFP-PM was released upon Ago-2 knockdown but not upon Ago-1 knockdown (Fig. 2B, lanes 9 and 10), indicating that pMTmRFP-PM exclusively reports for miG-2 silencing through Ago-2. In contrast, silencing of pMTmRFP-IM was strongly released upon Ago-1 knockdown and, to a lesser extent, upon Ago-2 knockdown (Fig. 2B, compare lanes 5 and 4 with the luciferase dsRNA control lane 3). Altogether (one representative experiment out of four is shown in Fig. 2B) these results indicate that pMTmRFP-IM is repressed by Ago-1 associated with miG-2, agreeing with the notion that Ago-1 accommodates well imperfectly matched target sites. Nevertheless, our results also indicates that part of the pMTmRFP-IM silencing involves Ago-2 which is consistent with previous reports showing that Ago-2 can mediate silencing through imperfectly matched target sites, although to a lesser extent than through perfectly matched target sites [27].

We then tested the ability of CrPV-1A to suppress the silencing of the target constructs pMTmRFP-PM and pMTmRFP-IM by miG-2 in S2 cells (Fig. 3, one representative experiment out of four is shown). In the presence of the CrPV-1A-HA expression vector, the silencing of pMTmRFP-PM by miG-2 (compare lanes 4 and 5 in Fig. 3) was completely released (Fig. 3,
In contrast, the silencing of pMTmRFP-IM was released to a lesser extent (Fig. 3, lanes 1 to 3). These results further support that CrPV-1A suppresses miRNA silencing mediated by the Ago-2 argonaute protein and has little if any effect on Ago-1-mediated miRNA silencing.

Fig 2. Silencing of mRFP reporters bearing four imperfect or perfect miG-2 binding sites in Drosophila S2 cells. (A) Schematic representation of miG-2 silencing sensors. The copper inducible metallothionein promoter drive the expression of the mRFP transcript bearing perfectly matched (pMTmRFP-PM) or imperfectly matched (pMTmRFP-IM) miG-2 target sites. Expression of miG-2 is provided in trans by the pAct-miG-2 construct. (B) Western blot analysis of Drosophila S2 cells co-transfected with pAct-miG-2, pMTmRFP-IM or pMTmRFP-PM and the indicated dsRNAs targeting Ago-2 (A2), Ago-1 (A1) or the control luciferase dsRNA (L). α-tubulin is used as loading control. One representative experiment out of four is shown.

doi:10.1371/journal.pone.0120205.g002
The CrPV-1A VSR interferes with the Ago-2-dependent miRNA pathway in adult flies

To further investigate the effect of CrPV-1A on Ago-2 miRNA silencing in flies, we set up a transgenic reporter system. Thus, we established a *Drosophila* transgenic line expressing an automiW construct containing a UAS promoter driving the expression of the GFP protein together with two artificial miRNAs miW-1 and miW-2 embedded in the *Rpl17* intron (Fig. 4A). The sequences of the mature miW-1 and miW-2 miRNAs were designed to be perfectly complementary to sequences of the *white* gene marker in the construct, whose product is involved in red pigment deposition in adult eyes.

In the absence of GAL4 driver, homozygous automiW transgenic flies harbored a dark red eye color (Fig. 4B, panel 1). In contrast, flies homozygous for both the eye-specific GMR-GAL4 [w-] driver which does not bear a functional *white* gene (see http://flybase.org/reports/FBti0072862.html) and the automiW transgenes harbored a white eye color (Fig. 4B, panel 3), similar to that observed in homozygous GMR-GAL4 [w-] flies (Fig. 4B, panel 2). This result indicated that the artificial miW-1 and miW-2 miRNAs silence mini-white expression in vivo. Although to a lesser extent, this silencing was still efficient in flies heterozygous for both the GMR-GAL4 [w-] driver and the automiW transgene (compare the white and orange pale colors in Fig. 4B, panels 3 and 4, respectively). This data indicates that the level of white silencing depends on the relative gene dosage of the GAL4 driver and automiW transgene. RNAi knockdowns of Drosha or Ago-2 by inverted repeats (IR) strongly inhibited the *white* gene silencing in these heterozygous GMR-GAL4 [w-], automiW flies (compare Fig. 4B, panels 6 and 7 with the control GFP RNAi knockdown in panel 5). These data strongly suggest that the automiW system is a sensitive biosensor for Ago-2 mediated silencing of the *white* gene by the artificial miW-1 and miW-2 miRNAs.

**Fig 3.** CrPV-1A interferes with miRNA silencing mediated by perfect or imperfect target sites in S2 cells. Western blot analysis of S2 cells co-transfected with pMTmRFP-IM or pMTmRFP-PM, pAct-miG-2 and CrPV-1A-HA or no VSR expression constructs. α-tubulin is shown as loading control. One representative experiment out of four is shown.

doi:10.1371/journal.pone.0120205.g003
We then examined the effect of CrPV-1A on the automiW silencing using a UAS-CrPV-1A-Flag transgene [21]. When co-expressed under the control of the eye-specific GMR-GAL4 [w-] driver with the automiW transgene, CrPV-1A but not its mutant version UASp-CrPV-1Am efficiently suppressed the white silencing by miW-1 and miW-2 (Fig. 4C, compare panels 1 and 2 with flies expressing a control GFP protein in panel 4). As expected from our previous observations in S2 cells, expression of the B2 VSR did not suppress the silencing of the automiW system (Fig. 4C, panel 3). Altogether, our data indicate that, in addition to its antiviral function, the CrPV-1A VSR can inhibit Ago-2-mediated miRNA silencing in adult flies.

Discussion

So far, insect VSRs were only shown to interfere with the RNAi pathway. The Flock House virus B2 as well as the Drosophila C Virus 1A VSRs interfere with the Dicer-2 processing of long double-stranded RNAs into siRNAs. Several evidence also suggest than B2 sequesters siRNA duplexes, preventing their loading into Ago-2. In contrast, both these VSRs failed to inhibit the silencing of reporter systems by the endogenous miRNAs miR-2b, bantam or miR-7 [35–37]. These observations support the notion that B2 and DCV-1A interfere with siRNA biogenesis but not with miRNA biogenesis.

Recent studies revealed that a fraction of miRNAs mediate silencing of perfectly complementary targets through their loading into Ago-2 [9,26,28–30]. As the Cricket Paralysis virus VSR CrPV-1A was shown to specifically interfere with Ago-2 [21], we decided to analyze its effect on Ago-2-mediated miRNA silencing. Indeed, we have shown here that CrPV-1A is a potent inhibitor of miRNA silencing in both S2 cells and transgenic flies, when this silencing is mediated by Ago-2. In agreement with a previous report [21], CrPV-1A had no noticeable effect on Ago-1-mediated miRNA silencing (Fig. 4). In contrast, we did not detect any effect of the B2 VSR on Ago-2-mediated miRNA silencing in S2 cells (Fig. 1) or in adult flies (Fig. 4). These results are in agreement with previous findings that B2 interfere with siRNA biogenesis but not with the biogenesis or the silencing activity of miRNAs [15–17,36,38]. Previously, only one report had suggested a potential role of an insect virus VSR on the miRNA pathway in insect cells [39]. Using hairpin construct targeting GFP, Kakumani et al. showed that the Dengue virus NSB4 was able to inhibit miRNA dependent silencing. This is in concordance with our data showing that most of the perfectly complementary miRNA are mediating silencing through Ago-2. It is also interesting to speculate that Dengue NS4B could be interfering at the level of Ago-2 explaining why similarly to CrPV-1A, it inhibits both RNAi and the Ago-2-dependent miRNA pathway.

Transgenic Drosophila lines expressing the CrPV-1A suppressor do not exhibit noticeable developmental defects and are viable and fertile. These observations are in agreement with the findings that essential functions fulfilled by miRNAs in development, differentiation and homeostasis are mediated by Ago-1 whose loss-of-function mutations are lethal during early embryogenesis [7]. In contrast, Ago-2 is dispensable under normal laboratory conditions [7] and
the biological function of Ago-2 miRNA mediated silencing remains to be established. Bioinformatics analyses have failed to reveal perfectly complementary targets of Ago-2 loaded miRNAs or miRNA∗’s in the transcriptome [28]. However, the clear biochemical evidence for Ago-2 loading by endogenous miRNA and miRNA∗’s suggest that this feature is under selection pressure. It is possible that some yet to be identified RNAs with more limited complementarity to miRNAs and miRNA∗’s are indeed targeted by Ago-2 silencing, either through cleavage or translational repression [27]. The CrPV-1A suppressor would likely affect the regulation of such putative mRNA targets. Interestingly, a recent work suggests a potential role of the miRNA loaded into Ago-2 complexes in aging. It was shown in Drosophila that partitioning of miRNAs between Ago-1 and Ago-2 is modulated with age. This shift of miRNAs into Ago-2 complexes is associated with a potential effect on targets downregulation. Furthermore, disruption of this process in ago-2 mutant is associated with neurodegeneration and reduced life span [40]. Thus, it will be of high interest to test in further studies whether expression of CrPV-1A induces similar phenotypes and has impact on Drosophila aging.

CrPV-1A is not the only VSR able to target Ago-2 function. Recently the ORF1 of Nora virus was identified as a potent inhibitor of RNAi by targeting Ago-2 [35]. Importantly, Nora virus is a persistent virus shown to be naturally present in many laboratory and wild-type Drosophila strains [41]. If our observation on the effect of CrPV-1A holds true for Nora virus ORF1 suppressor, it is most likely that Nora virus ORF1 will interfere with the Ago-2 dependent miRNA pathway in infected tissues. This is particularly important for in vivo gene knockdown experiments that rely on the use of Drosophila hairpins to silence perfectly complementary target genes which most likely exert their silencing activity through Ago-2. In light of our results, it is possible that conditions such as persistent Nora virus infections will impair gene knockdown experiments using these tools.

Materials and Methods

Plasmid constructs

VSR plasmids. VSR cDNAs were cloned into pENTRY-TOPO vector (Life Technologies) as described previously [21,36] and were subsequently transferred using the Gateway system (Life Technologies) into the pAWH destination vector which allows expression of the insert fused to a C-terminal hemagglutinin tag (3xHA) under the control of the constitutive Actin 5C promoter.

miRNA silencing reporter systems. The pENTRY-miR-5-miR-6.1 and pENTRY-miG-1-miR-6.1 plasmids [34] were recombined with the Gateway pUWR destination vector (a kind gift from Clara Moch and Jean-Rene Huynh) described at the Drosophila Genome Ressource Center (https://dgrc.cgb.indiana.edu/) and carrying an ubiquitin promoter and a mRFP gene reporter to generate the miR-5-miR-6.1-mRFP and miG-1-miR-6.1-mRFP plasmids respectively (Fig. 1A).

To generate the pMTmRFP-PM and pMTmRFP-IM sensor plasmids, we first PCR-amplified a fragment carrying mRFP coding sequences using the primers NotI- ATG-mRFP and XhoI-Stop-mRFP (S1 Table). The resulting mRFP DNA fragment was inserted between the NotI and XhoI restriction sites in place of the GFP sequences in the pENTRY-miG-1-miG-2-GFP plasmid [34] to generate the pENTRY-miG-1-miG-2-mRFP plasmid. DNA oligonucleotides (S1 Table) containing four sites complementary to the miG-2 mature sequence without (PM) or with (IM) mismatches at positions 9–11 relative to the 5’ end of miG-2 were annealed and cloned into pENTRY/D-TOPO and next subcloned into the pENTRY-miG-1-miG-2-mRFP-stop plasmid using XhoI and XbaI sites. The pENTRY-miG-1-miG-2-mRFP-PM and pENTRY-miG-1-miG-2-mRFP-IM plasmids were then deleted of the miG-1 and miG-2
sequences by digestion/re-ligation with EcoRI/SphI and HindIII/ClaI, respectively. Finally, the resulting pENTRY-mRFP-PM and mRFP-IM constructs were recombined with the pMT-DEST48-V5-His vector (Life Technologies), generating the pMTmRFP-PM and pMTmRFP-IM biosensors inducible by copper.

To generate the pAct-miG-2 plasmid, we deleted the pre-miG-1 sequences from the pENTRY-3C_miG-1_miG-2 plasmid [34] by EcoRI/SphI digestion and re-ligation. The resulting pENTRY-3C_miG-2 construct was then recombined with pAWH destination vector to give pAct-miG-2.

To generate the automiW construct, DNA oligonucleotides containing the backbone sequences of pre-miR-5 and pre-miR6-1 [34] and 22 nt perfectly complementary to exons 6 and 5 of the white gene, respectively, in place of the mature miR-5 and miR-6.1 sequences (bolded in S1 Table) were annealed and cloned in place of miG-1 and miG-2, respectively, in the pENTRY-3C_miG-1_miG-2 construct [34]. The resulting pENTRY-3C_miW-1_miW-2-GFP was then transferred using the Gateway technology (Life Technologies) into pTGW (Drosophila Gateway Vector collection, Carnegie Institution) to generate the automiW construct which contains an attB site pour PhiC31 mediated transgenesis as well as a mini-white gene transformation marker (Fig. 4A).

Cells

Drosophila S2 cells (Invitrogen) were cultured at 25°C in Schneider’s Drosophila medium (GIBCO) supplemented with 5% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. S2 cells were seeded at 2.5 x10⁴ cells per well of 24-well plates and grown for 24 hours before transfection. Transient transfections were performed using Effectene reagent (QIAGEN) according to manufacturer’s instructions. Ago-1, Ago-2 and luciferase double-stranded RNAs were generated as described in Carré et al., 2013. For RNAi knockdown experiments, 3 μg of dsRNA were co-transfected with the appropriate DNA plasmids using Effectene reagent (QIAGEN) and expression of the reporter plasmids was triggered 24 hours later by addition of 500 μM CuSO₄. After 48 additional hours, cells were harvested for western blot analyses.

Western-blot analysis

For protein analysis, equal amounts of proteins extracted from transfected S2 cells were denatured in Laemmli sample buffer, submitted to 12% SDS-PAGE and transferred onto nitrocellulose membranes. After blocking for one hour in PBS supplemented with 0.1% Tween 20 (TPBS) and 5% fat-free milk, membranes were incubated in TPBS overnight at 4°C in the presence of appropriate primary antibodies. Antibodies were obtained from the following sources: mouse anti-GFP (Roche), rabbit anti-mRFP polyclonal rabbit (Clontech), mouse anti-tubulin (Tebu santa cruz), rat anti-HA (Roche), mouse anti-luciferase (Sigma). After three washes in TPBS, the membranes were incubated for 2H at room temperature in TPBS supplemented with 5% fat-free milk and HRP-conjugated secondary antibodies from Amersham. After three washes in TPBS, detection was performed using ECL Western Blotting (Pierce).

Transgenic lines

Drosophila transgenic lines for the UASp-CrPV-1A, UASp-CrPV-1Am, UASp-B2 and UASp-GFP constructs were established previously [36]. The transgenic automiW was established using PhiC31 integrase mediated transgenesis [42] of the strain 24483 (Bestgene Inc) which contains an attP docking site at 51D9 in the right arm of chromosome 2.
Eye pigment dosage

Assays were performed as previously described [36] on one day old virgin females. Five heads per genotype were manually collected and homogenized in 50 μl of a freshly prepared solution of acidified methanol (0.1% HCl). Pigment was extracted by rocking tubes for 36 hours at 4°C. Homogenates were then incubated at 50°C for 5 min, clarified by centrifugation and optical density of each sample was read at 480 nm. Three independent extractions were performed for each genotype and the mean values of the absorption per extraction were calculated.

Supporting Information

S1 Table. Primers and oligonucleotide sequences. Sequences of mature miRNAs are bolded. (DOCX)

Acknowledgments

We thank Anne-Laure Bougé and Hélène Thomassin for technical help and Clément Carré and Marius van den Beek for critical reading of the manuscript.

Author Contributions

Conceived and designed the experiments: CBG CJ CA. Performed the experiments: CBG CJ JP. Analyzed the data: CBG CJ SD CA. Contributed reagents/materials/analysis tools: CBG CJ JP. Wrote the paper: CBG SD CA.

References

1. Galiana-Arnoux D, Dostert C, Schneemann A, Hoffmann JA, Imler J-L. Essential function in vivo for Dicer-2 in host defense against RNA viruses in drosophila. Nat Immunol. 2006; 7: 590–597. doi:10.1038/ni1335 PMID: 16554838
2. van Rij RP, Saleh M-C, Berry B, Foo C, Houk A, Antoniewski C, et al. The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in Drosophila melanogaster. Genes Dev. Cold Spring Harbor Lab; 2006; 20: 2985–2995. doi:10.1101/gad.1482006 PMID: 17079687
3. Ding S-W, Voinnet O. Antiviral immunity directed by small RNAs. Cell. 2007; 130: 413–426. doi:10.1016/j.cell.2007.07.039 PMID: 17693253
4. Wang X-H, Aliyari R, Li W-X, Li H-W, Kim K, Carthew R, et al. RNA interference directs innate immunity against viruses in adult Drosophila. Science, American Association for the Advancement of Science; 2006; 312: 452–454. doi:10.1126/science.1125694 PMID: 16556799
5. Maillard PV, Ciaudo C, Marchais A, Li Y, Jay F, Ding SW, et al. Antiviral RNA interference in mammalian cells. Science. 2013; 342: 235–238. doi:10.1126/science.1241930 PMID: 24115438
6. Li Y, Lu J, Han Y, Fan X, Ding S-W. RNA interference functions as an antiviral immunity mechanism in mammals. Science. American Association for the Advancement of Science; 2013; 342: 231–234. doi:10.1126/science.1241911 PMID: 24115437
7. Okamura K, Ishizuka A, Siomi H, Siomi MC. Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. Genes Dev. Cold Spring Harbor Lab; 2004; 18: 1655–1666. doi:10.1101/gad.1210204 PMID: 15231716
8. Miyoshi K, Tsukumo H, Nagami T, Siomi H, Siomi MC. Slicer function of Drosophila Argonautes and its involvement in RISC formation. Genes Dev. Cold Spring Harbor Lab; 2005; 19: 2837–2948. doi:10.1101/gad.1370605 PMID: 16287716
9. Tomari Y, Du T, Zamore PD. Sorting of Drosophila Small Silencing RNAs. Cell. 2007; 130: 299–308. doi:10.1016/j.cell.2007.05.057 PMID: 17662944
10. Alvarado V, Scholthof HB. Plant responses against invasive nucleic acids: RNA silencing and its suppression by plant viral pathogens. Semin Cell Dev Biol. 2009; 20: 1032–1040. doi:10.1016/j.semcdb.2009.06.001 PMID: 19524057
11. Vijayendran D, Airts PM, Dolezal K, Bonning BC. Arthropod viruses and small RNAs. J Invertebr Pathol. 2013; 114: 186–195. doi:10.1016/j.jip.2013.07.006 PMID: 23932976
12. Morel J-B, Godon C, Mounir A, Béclin C, Boutet S, Feuerbach F, et al. Fertile hypomorphic ARGO-
N AUTE (ago1) mutants impaired in post-transcriptional gene silencing and virus resistance. Plant Cell. 2002; 14: 629–639. PMID: 1210010
13. Zhang X, Yuan Y-R, Pei Y, Lin S-S, Tuschi T, Patel DJ, et al. Cucumber mosaic virus-encoded 2b sup-
pressor inhibits Arabidopsis Argonaute1 cleavage activity to counter plant defense. Genes Dev. 2006; 20: 3255–3268. doi: 10.1101/gad.1495506 PMID: 17158744
14. Voinnet O. Origin, biogenesis, and activity of plant microRNAs. Cell. 2009; 136: 669
15. Sullivan CS, Ganem D. A virus-encoded inhibitor that blocks RNA interference in mammalian cells. J Virol. American Society for Microbiology; 2005; 79: 7371–7379. doi: 10.1128/JVI.79.12.7371-7379. 2005 PMID: 15919982
16. Lu R, Maduro M, Li F, Li HW, Broitman-Maduro G, Li WX, et al. Animal virus replication and RNAi-medi-
ated antiviral silencing in Caenorhabditis elegans. Nature. 2005; 436: 1040–1043. doi: 10.1038/
nature03870 PMID: 16107851
17. Chao JA, Lee JH, Chapados BR, Debler EW, Schneemann A, Williamson JR. Dual modes of RNA-si-
encing suppression by Flock House virus protein B2. Nat Struct Mol Biol. 2005; 12: 952–957. doi: 10.1038/nsmb1005 PMID: 16228003
18. Vargas JM, Sztitya G, Burgýán J, Hall TMT. Size selective recognition of siRNA by an RNA silencing suppressor. Cell. 2003; 115: 799–811. PMID: 14697199
19. Ye K, Malinina L, Patel DJ. Recognition of small interfering RNA by a viral suppressor of RNA silencing. Nature. 2003; 426: 874–878. doi: 10.1038/nature02213 PMID: 14661029
20. Lakatos L, Csorba T, Pantaleo V, Chapman EJ, Carrington JC, Liu Y-P, et al. Small RNA binding is a
common strategy to suppress RNA silencing by several viral suppressors. EMBO J. 2006; 25: 2768–
2780. doi: 10.1038/sj.emboj.7601164 PMID: 16724105
21. Nayak A, Berry B, Tassetto M, Kunitomi M, Acevedo A, Deng C, et al. Cricket paralysis virus antago-
nizes Argonaute 2 to modulate antiviral defense in Drosophila. Nat Struct Mol Biol. 2010; 17: 547–554. doi: 10.1038/nsmb.1810 PMID: 20400949
22. Till E, Michaille J-J, Croce CM. MicroRNAs play a central role in molecular dysfunctions linking inflam-
mation with cancer. Immunol Rev. 2013; 253: 167–184. doi: 10.1111/imr.12050 PMID: 23550646
23. Ameres SL, Zamore PD. Diversifying microRNA sequence and function. Nat Rev Mol Cell Biol. 2013; 14: 475–488. doi: 10.1038/nrm3611 PMID: 23800994
24. Leuschner PJF, Obermoster G, Martinez J. MicroRNAs: Laquacious speaks out. Current Biology. 2005; 15: R603–5. doi: 10.1016/j.cub.2005.07.044 PMID: 16085484
25. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009; 136: 215–233. doi: 10.1016/j.cell.2009.01.002 PMID: 19167326
26. Förstemann K, Horwich MD, Wee L, Tomari Y, Zamore PD. Drosophila microRNAs Are Sorted into Functionally Distinct Argonauta Complexes after Production by Dicer-1. Cell. 2007; 130: 287–297. doi:10.1016/j.cell.2007.05.056 PMID: 17862943
27. Iwasaki S, Kawamata T, Tomari Y. Drosophila argonaute1 and argonaute2 employ distinct mecha-
nisms for translational repression. Mol Cell. Elsevier; 2009; 34: 58–67. doi: 10.1016/j.molcel.2009.09.027 PMID: 19917251
28. Okamura K, Liu N, Lai EC. Distinct mechanisms for microRNA strand selection by Drosophila Argonautes. Mol Cell. 2009; 36: 431–444. doi: 10.1016/j.molcel.2009.09.027 PMID: 19917251
29. Czech B, Zhou R, Erlich Y, Brennecke J, Binari R, Villalta C, et al. Hierarchical rules for Argonaute load-
ing in Drosophila. Mol Cell. 2009; 36: 445–456. doi: 10.1016/j.molcel.2009.09.028 PMID: 19917252
30. Ghildiyal M, Xu J, Seitz H, Weng Z, Zamore PD. Sorting of Drosophila small silencing RNAs partitions microRNA+ strands into the RNA interference pathway. RNA. Cold Spring Harbor Lab; 2010; 16: 43–56. doi: 10.1261/ma.1972910 PMID: 19917635
31. Haley B, Hendrix D, Trang V, Levine M. A simplified miRNA-based gene silencing method for Drosophila melanogaster. Dev Biol. 2008; 321: 482–490. doi: 10.1016/j.ydbio.2008.06.015 PMID: 18598689
32. Haley B, Foys B, Levine M. Vectors and parameters that enhance the efficacy of RNAi-mediated gene disruption in transgenic Drosophila. Proc Natl Acad Sci USA. National Acad Sciences; 2010; 107: 11435–11440. doi: 10.1073/pnas.1006689107 PMID: 20534445
33. Ni J-Q, Zhou R, Czech B, Liu L-P, Holderbaum L, Yang-Zhou D, et al. A genome-scale shRNA resource for transgenic RNAi in Drosophila. Nat Methods. 2011; 8: 405–407. doi: 10.1038/nmeth.1592 PMID: 21460824
34. Carré C, Jacquier C, Bougé A-L, de Chaumont F, Besnard-Guerin C, Thomassin H, et al. AutomiG, a biosensor to detect alterations in miRNA biogenesis and in small RNA silencing guided by perfect target
complementarity. White-Cooper H, editor. PLoS ONE. Public Library of Science; 2013; 8: e74296. doi:10.1371/journal.pone.0074296 PMID: 24019960

35. van Mierlo JT, Bronkhorst AW, Overheul GJ, Sadanandan SA, Ekström J-O, Heestermans M, et al. Convergent evolution of argonaute-2 slicer antagonism in two distinct insect RNA viruses. Schneider DS, editor. PLoS Pathog. Public Library of Science; 2012; 8: e1002872. doi:10.1371/journal.ppat.1002872 PMID: 22916019

36. Berry B, Deddouche S, Kirschner D, Imler J-L, Antoniewski C. Viral suppressors of RNA silencing hinder exogenous and endogenous small RNA pathways in Drosophila. Preiss T, editor. PLoS ONE. Public Library of Science; 2009; 4: e5866. doi:10.1371/journal.pone.0005866 PMID: 19516905

37. Chou Y-T, Tam B, Linay F, Lai EC. Transgenic inhibitors of RNA interference in Drosophila. Fly (Austin). 2007; 1: 311–316. PMID: 18820441

38. Singh G, Popli S, Hari Y, Malhotra P, Mukherjee S, Bhatnagar RK. Suppression of RNA silencing by Flock house virus B2 protein is mediated through its interaction with the PAZ domain of Dicer. FASEB J. Federation of American Societies for Experimental Biology; 2009; 23: 1845–1857. doi:10.1096/fj.08-125120 PMID: 19193719

39. Kakumani PK, Ponia SS, S RK, Sood V, Chinnappan M, Banerjea AC, et al. Role of RNA interference (RNAi) in dengue virus replication and identification of NS4B as an RNAi suppressor. J Virol. American Society for Microbiology; 2013; 87: 8870–8883. doi:10.1128/JVI.02774-12 PMID: 23741001

40. Abe M, Naqvi A, Hendriks G-J, Feltzin V, Zhu Y, Grigoriev A, et al. Impact of age-associated increase in 2'-O-methylation of miRNAs on aging and neurodegeneration in Drosophila. Genes Dev. Cold Spring Harbor Lab; 2014; 28: 44–57. doi:10.1101/gad.226654.113 PMID: 24395246

41. Habayeb MS, Ekengren SK, Hultmark D. Nora virus, a persistent virus in Drosophila, defines a new picorna-like virus family. J Gen Virol. 2006; 87: 3045–3051. doi:10.1099/vir.0.81997-0 PMID: 16963764

42. Bischof J, Maeda RK, Hediger M, Karch F, Basler K. An optimized transgenesis system for Drosophila using germ-line-specific phiC31 integrases. Proc Natl Acad Sci USA. 2007; 104: 3312–3317. doi:10.1073/pnas.0611511104 PMID: 17360644