Supporting Information

Mueller et al. 10.1073/pnas.1014378107

SI Materials and Methods

Tissue Culture and Virus Production. Vero cells were grown as monolayers in DMEM (Gibco) supplemented with 10% FCS (Biowest). *Drosophila* S2 and Kc167 cells were cultured in Schneider's medium (Biowest) supplemented with 10% FCS. Propagation and titration by plaque assay of vesicular stomatitis virus (VSV) (strain Indiana isolate PI10/Lab-strain), Sindbis virus (SINV), and Semliki Forest virus (SFV) was performed on Vero cells. Propagation and titration by $TCID_{50}$ of *Drosophila* C virus (DCV) and flock house virus (FHV) was done on S2 cells. $TCID_{50}$ was determined by immunostaining with anti-DCV and anti-FHV antisera.

Fly Culture and Infection. *yw*, w^{1118}, $Dcr\text{-}2^{R416X}$, $Dcr\text{-}2^{L811fsX}$, $AGO2^{414}$, $R2D2^{1}$, and UAS > B2 flies were previously described (1–4). Hsp-Gal4 lines were obtained from the Bloomington stock center. The fly stocks were raised on standard cornmeal-agar medium at 25 °C. Adult flies 4 to 6 d of age for Dataset 1 (Table S1) and adult females 3 to 5 d of age for Dataset 2 (Table S1) were used in infection experiments. Transgene expression was induced by heat-shock (20 min at 37 °C, 30 min at 18 °C, 20 min at 37 °C, 6 h at 25 °C). Infections were done by intrathoracic injection (Nanoject II apparatus; Drummond Scientific) of a viral suspension in 10 mM Tris-HCl, pH 7.5 (VSV 1×10^9 PFU/mL, DCV 1×10^8 PFU/mL, and SINV 2×10^8 PFU/mL). Injection of the same volume of 10 mM Tris-HCl, pH 7.5, was used as a control. Infected flies were then incubated at 22 °C (Dataset 1) or 25 °C (Dataset 2).

Poly(I:C) Transfection. For transfection of Vero cells with synthetic dsRNA, 10 μg of poly(I:C) (Amersham) was prepared with 10 μL of X-tremeGENE reagent (Roche) in 100 μL of serum-free DMEM according to the manufacturer's instructions. After 15 min of incubation, the dsRNA-liposome mixture was dropped onto cells using the same medium. Immunostaining was performed 16 h after transfection.

Immunostaining. Cells were grown on eight-well coverslips (LabTex) to 80% confluence. Infection with VSV, SFV, or SINV [multiplicity of infection (MOI) 0.01] was performed for 18 h. Cells were fixed with 4% paraformaldehyde and permeabilized using 0.05% Triton X-100 in PBS. Immunostaining was performed with the mouse monoclonal antibodies J2 or K1 diluted 1:200 in blocking solution (TSA cyanine 3 system; Perkin Elmer). SFV and SINV viral E-proteins were detected with mouse anti-eastern equine encephalitis virus antibody (1:100) (MAB8754; Chemicon). After incubation at room temperature for 2 h, the coverslips were washed three times in PBS and then incubated for 1 h with secondary goat anti-mouse antibodies coupled to Alexa 546 for detection of dsRNA or Alexa 488 for detection of viral antigens (Invitrogen). VSV G Protein was detected directly with a FITC labeled anti-VSV-G antibody (1:100) (GTX23863; GeneTex). Cells were washed three times after secondary antibody incubation and mounted in Vectashield medium (Vector Laboratories). Cells were then examined using an Axioskope2 Microscope (Zeiss) equipped with a Nikon digital camera. The same microscope settings and exposure times were used within each set of experiments.

Sensor Plasmid Construction. Sensor plasmids were constructed on the backbone of the copper-inducible expression vector pMT/V5-HisB (Invitrogen). First, firefly (*Photinus pyralis*) luciferase sequence from the plasmids pGL3 (Promega) were cloned in the expression vector generating pMT-Luc. Second, VSV and DCV sequences were amplified from viral genomic RNA by RT-PCR (sequences of the PCR primers provided upon request). The PCR fragments were cloned into the SacI and ApaI restriction sites of the pMT-Luc plasmid 3′ of the firefly luciferase coding sequence. The transfection control plasmid used to normalize for transfection efficiency was constructed by cloning the Renilla (*Renilla reniformis*) luciferase sequence from pRL-CMV into pMT, creating the plasmid pMT-Ren.

Sensor Plasmid Transfection, Infection of Cells, and Luciferase Assays. For transfection of S2 cells with the sensor plasmids, 25 ng of sensor plasmid (VSV-N, VSV-Ni, VSV-G, VSV-Gi, VSV-L, VSV-Li, or DCV), and 6.3 ng of normalization plasmid (pMT-Ren) were diluted in 10 μL EC buffer. This DNA solution was mixed with 20 μL EC buffer containing 2.5 μL of Effectene reagent (Qiagen), incubated for 10 min at 25 °C, and added to the cells. VSV infection (MOI 10) was done 2 h after transfection. Luciferase expression was induced 24 h after transfection by addition of 500 μM $CuSO_4$ to the cells. Luciferase expression was measured 3 d after infection using the Dual Luciferase reporter system (Promega), according to the manufacturer's instructions.

PCR for Detection of Viral Genomic and Antigenomic RNAs. The total RNA of virus infected cells or the RNA immunoprecipitated with J2 and K1 antibodies were used for PCR detection of genomic and antigenomic viral RNA. Either 1 μg of total RNA or 2 μL of immunoprecipitated RNA were reverse transcribed using the SuperScript II Kit (Invitrogen), according to the manufacturer's instructions, and VSV primers specific for the genome (5′-attatgctacatatgaaaaaaactaacagatat-3′) or the antigenome (5′-cgatctctgttagttttttttcatagggatag-3′). Two microliters of the 20-μL reverse-transcriptase reaction were then used for the PCR using GoTaq polymerase (Promega) according to the manufacturer's instructions, and the primer pair VSV genome forward and reverse (5′-gtagactatgaaaaaaagtaacagatatcacg-3′ and 5′-cgatctctgttagttttttttcatagggatag-3′, respectively), or VSV antigenome forward and reverse (5′-attatgctacatatgaaaaaaactaacagatat-3′ and 5′-cgtgatatctgttacttttttttcatagtctac-3′, respectively). In the case of DCV and FHV, the sequences of the primers used were: DCV_fwd 5′-aagatcctatattcaacgtgactgttatgaa-3′; DCV_rev 5′-aaaaagctagcgcttcctcatatgttaaaatgcg-3′; FHV_fwd 5′-agtgcgactacgtttaacccc-3′; FHV_rev 5′-cgattgttcggatacttgccc-3′. RNase A, RNase T1, RNaseIII and RNase V1 were purchased from Ambion and used according to the manufacturer's recommendation.

Knock-Down of *AGO2* by dsRNA. DsRNA for the knock-down of *AGO2* was produced from a T7 promoter containing PCR product using the MEGAScript T7 Kit (Ambion) according to the manufacturer's instructions. The PCR product was amplified from cDNA clones of *AGO2* using the primers *AGO2*-fwd (5′-TTTTTAATACGACTCACTATAGGGAGAtgacagatcgtaatggtcgtac-3′; capital letters correspond to the T7 promoter, small letters correspond to *AGO2* sequences) and *AGO2*-rev (5′-TTTTTAATACGACTCACTATAGGGAGAcctgaccttcctcgatgctg-3′). For dsRNA knock-down, 200 ng sensor plasmid, 10 ng pMT-Ren and 1.5 μg dsRNA were transfected into 1×10^6 cells in a 24-well plate using Effectene reagent (Qiagen), as described above. VSV was added to the cells 2 h later and luciferase expression was measured 72 h after infection.

Small RNA Clustering. For a better representativity of the genome coverage by VSV-derived small RNAs (vsiRNAs), the small

RNAs were clustered according to their position in the VSV genome. Sequences were clustered together according to the following conditions: two consecutive aligned sequences are not separated by more then 4 nt and are matching the viral RNA on the same strand. A cluster is considered if at least one small RNA has an occurrence higher then the SD of the small RNA distribution over the whole genome. The cluster occurrence equals to the summation of the occurrences of the sequences clustered together.

1. Berry B, Deddouche S, Kirschner D, Imler JL, Antoniewski C (2009) Viral suppressors of RNA silencing hinder exogenous and endogenous small RNA pathways in *Drosophila*. *PLoS ONE* 4:e5866.
2. Lee YS, et al. (2004) Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell* 117:69–81.
3. Liu X, Jiang F, Kalidas S, Smith D, Liu Q (2006) Dicer-2 and R2D2 coordinately bind siRNA to promote assembly of the siRISC complexes. *RNA* 12:1514–1520.
4. Okamura K, Ishizuka A, Siomi H, Siomi MC (2004) Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. *Genes Dev* 18:1655–1666.

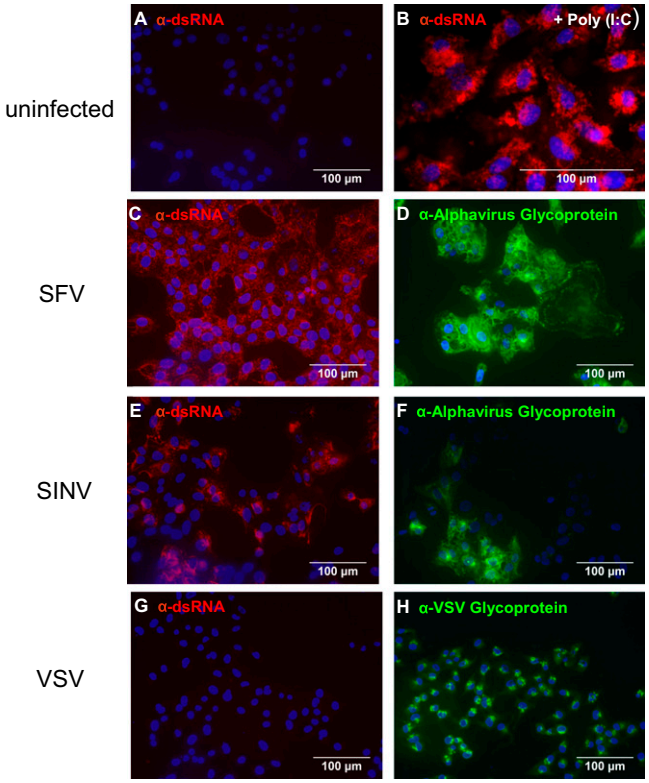


Fig. S1. VSV does not produce detectable amounts of dsRNA in infected mammalian cells. *(A–H)* Immunostaining of Vero cells infected with Semliki Forest virus (SFV) (*C* and *D*), SINV (*E* and *F*), or VSV (*G* and *H*). Infections at MOI 0.01 for each virus were performed for 18 h. Uninfected cells (*A*) and uninfected cells transfected with poly(I:C) (*B*) were used as controls. Double-stranded (ds)RNA (red) was detected with the dsRNA-specific monoclonal antibody J2 (*A–C, E,* and *G*). Viral production (green) was detected with an anti-α-virus E glycoprotein monoclonal antibody for SFV (*D*) and SINV (*F*), or with the VSV anti-G glycoprotein polyclonal antibody for VSV (*H*). Nuclei were visualized by DAPI staining.

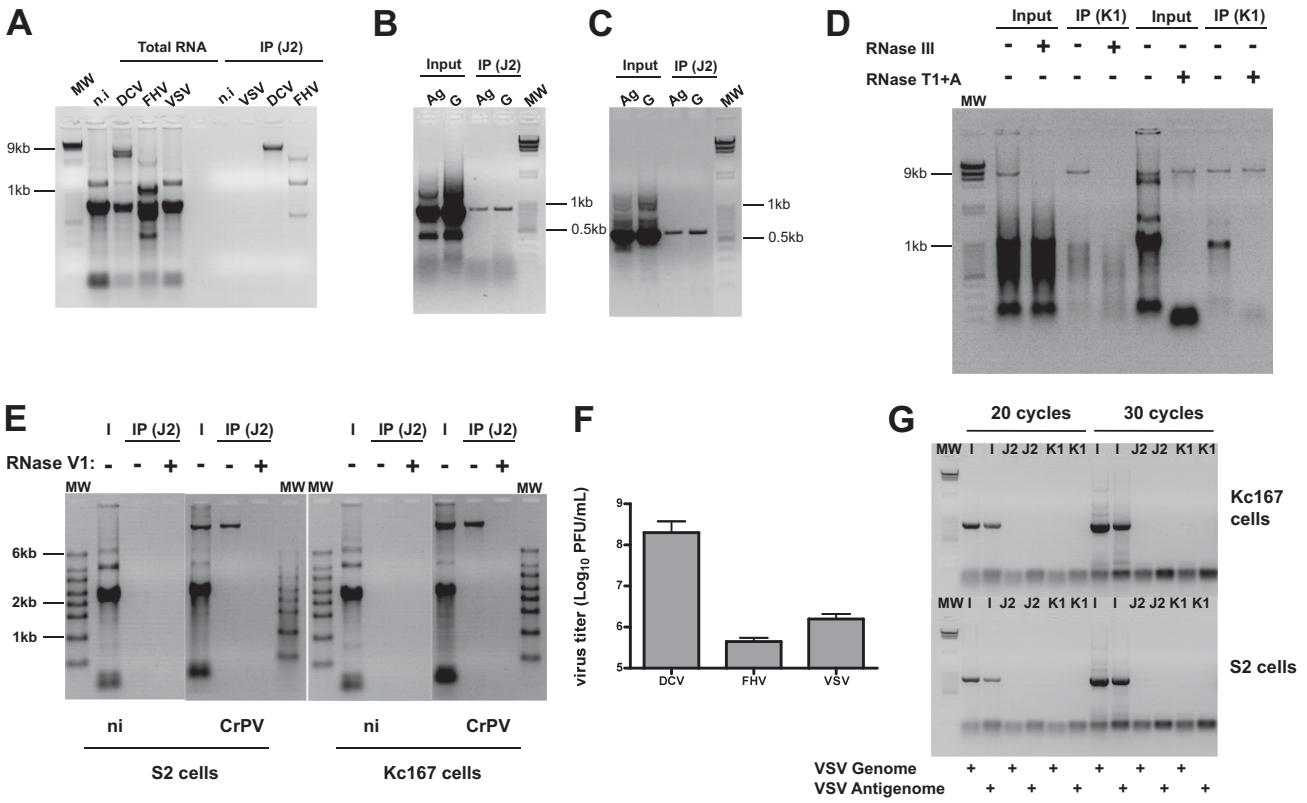


Fig. S2. VSV does not produce detectable amounts of dsRNA in *Drosophila*-infected cells. (*A*) Immunoprecipitation with dsRNA-specific antibodies (J2) of total RNA (*Left*) extracted from *Drosophila* S2 cells infected with DCV (MOI 10), FHV (MOI 0.1), or VSV (MOI 10) at 4 d postinfection (dpi). Immunoprecipitates were subjected to agarose gel electrophoresis. MW, molecular weight marker (DNA); n.i., non infected control. (*B* and *C*) Immunoprecipitation of FHV and DCV dsRNA with the monoclonal antibody J2. Genome- and antigenome-specific PCR was used to detect viral RNA in S2 cells infected with FHV (*B*) and DCV (*C*). IP (J2), RNA immunoprecipitated with the J2 monoclonal antibody. Input total RNA was used as control. MW, molecular weight marker (DNA); Ag, reverse-transcribed antigenome RNA; G, reverse-transcribed genome RNA. Primers complementary to the genome or antigenome were used for the reverse transcription of each sample. The expected size for the PCR products is 863 bp for FHV and 595 bp for DCV. The identity of the PCR products was confirmed by sequencing. (*D*) The RNA immunoprecipitated from DCV-infected cells is sensitive to RNaseIII digestion. Total RNA from DCV infected S2 cells (MOI 10) was immunoprecipitated with the K1 monoclonal antibody. The immunoprecipitated RNA was then digested with either the dsRNA cleaving enzyme RNaseIII or a mix of the ssRNA cleaving enzymes T1 and A. Total RNA from DCV infected cells before immunoprecipitation (input) was used as a control. MW, molecular weight marker (DNA). (*E*) Detection of dsRNA in Cricket-paralysis virus (CrPV)-infected *Drosophila* cells. Total RNA was extracted from *Drosophila* S2 or Kc167 cells infected with CrPV (MOI 0.01) at 3 d postinfection and immunoprecipitated with the dsRNA-specific monoclonal antibody J2. Input total RNA (I) and immunoprecipitated RNA [IP (J2)] were separated by agarose gel electrophoresis. The RNA immunoprecipitated by J2 was sensitive to digestion with the dsRNA degrading enzyme RNase V1. MW, molecular weight marker (RNA); ni, noninfected. (*F*) DCV, FHV, and VSV titers measured from supernatant of the infected S2 culture described in *A* before RNA extraction. The values represent the mean and SD of three independent experiments. (*G*) Genome-specific and antigenome-specific PCR failed to detect immunoprecipitated dsRNA in Kc167 (*Upper*) and S2 (*Lower*) cells infected with VSV (MOI 10). Input total RNA was used as control. MW, molecular weight marker; I, input; J2, immunoprecipitation with the J2 dsRNA-specific antibody; K1, immunoprecipitation with the K1 dsRNA-specific antibody. Primers complementary to the genome or antigenome were used for the reverse transcription of each sample.

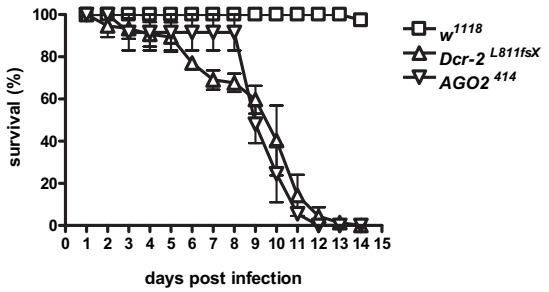


Fig. S3. RNAi controls VSV infection in *Drosophila*. *Dcr-2*^{L811fsX} and *AGO2*^{414} mutant flies infected with 5,000 pfu VSV display a strong susceptibility to VSV challenge and died within 12 dpi, in contrast to the *w*^{1118} control flies. The values represent the mean and SD of three independent groups of 25 flies each for *Dcr-2*^{L811fsX} and WT (*w*^{1118}) and two independent groups of 25 flies each for *AGO2*^{414}.

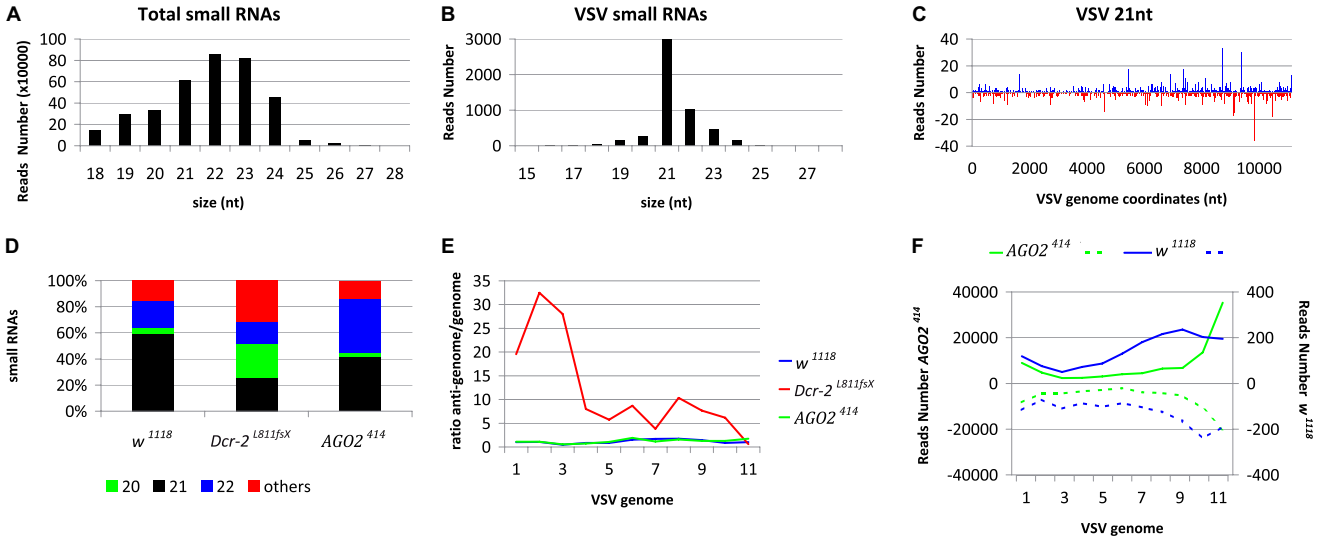


Fig. S4. Virus-derived siRNAs in VSV-infected flies in Dataset 2 (Table S1). (*A*) Size distribution of total small RNAs in VSV-infected w^{1118} flies. (*B*) Size distribution of VSV-specific small RNAs in VSV-infected w^{1118} flies. (*C*) Profile of 21 nt VSV-derived reads along the VSV genome. To comply with the orientation of the VSV reference sequence (NC_001560) used for all of the analyses, the genome is represented under its antigenomic polarity. Each vsiRNA is represented by the position of its first nucleotide. The vsiRNAs matching the antigenome and the genome are shown in blue and red, respectively. Horizontal axis represents the antigenome coordinates. (*D*) Relative abundance of VSV-derived small RNAs of 20, 21, 22 nt and other sizes (19, 23–28 nt) in w^{1118}, *Dcr-2*^{L811fsX}, and *AGO2*^{414} flies. (*E*) Relative strand representation of the 21 nt reads expressed as the ratio between the number of reads matching the antigenome over the number of reads matching the genome using 1,000-bp contiguous windows (1–11). (*F*) Representation of VSV-derived vsiRNAs from w^{1118} (blue) and *AGO2*^{414} (green) flies expressed as the sum of the reads over 1,000-bp contiguous windows. The vsiRNAs matching the antigenome and genome are showed as solid and dotted lines, respectively. The sum of the reads corresponding to each window is represented in the vertical axis.

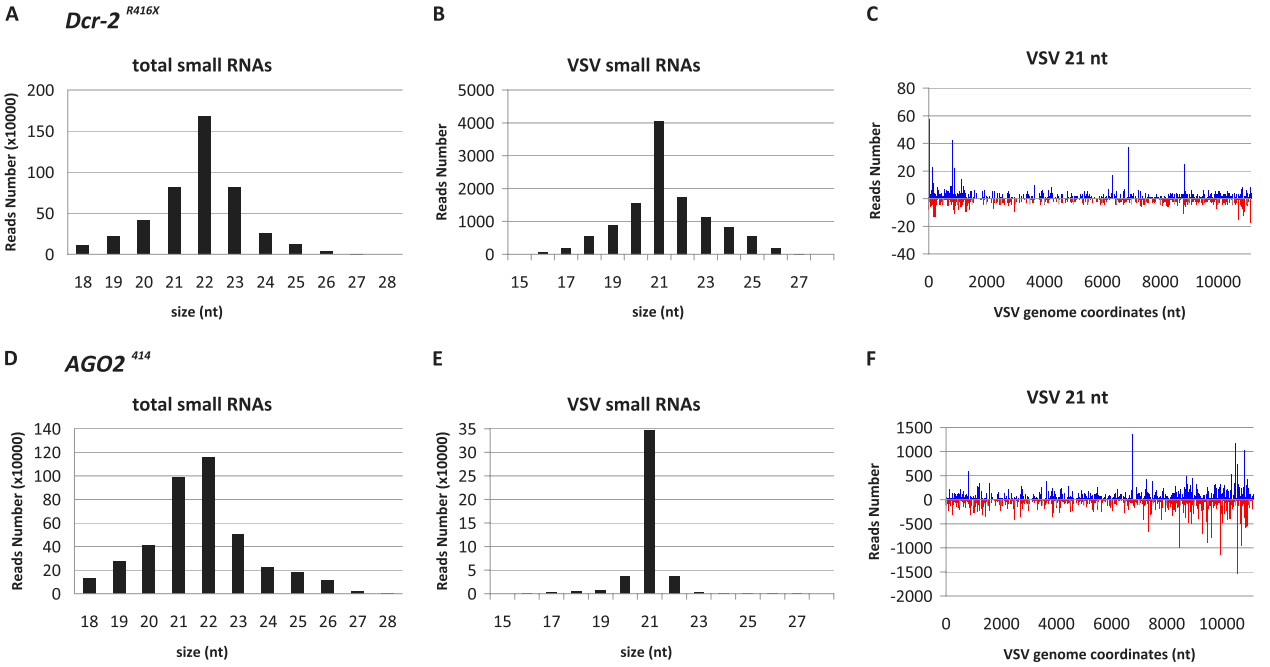


Fig. S5. Virus-derived siRNAs in VSV *Dcr-2*^{R416X} and *AGO2*^{414} infected flies in Dataset 1 (Table S1). (*A*) Size distribution of total small RNAs in VSV-infected *Dcr-2*^{R416X} flies. (*B*) Size distribution of VSV-specific small RNAs aligned against the viral genome in VSV-infected *Dcr-2*^{R416X} flies. (*C*) Profile of 21 nt VSV-derived reads recovered from *Dcr-2*^{R416X} infected flies along the VSV genome. To comply with the orientation of the VSV reference sequence (NC_001560) used for all of the analyses, the genome is represented under its antigenomic polarity. Each vsiRNA is represented by the position of its first nucleotide. The vsiRNAs matching the antigenome and the genome are shown in blue and red, respectively. The horizontal axis represents the antigenome coordinates. (*D*) Size distribution of total small RNAs in VSV-infected *AGO2*^{414} flies. (*E*) Size distribution of VSV-specific small RNAs in VSV-infected *AGO2*^{414} flies. (*F*) Profile of 21 nt VSV-derived reads recovered from *AGO2*^{414}-infected flies along the VSV genome. VSV-derived reads are depicted as in *C*.

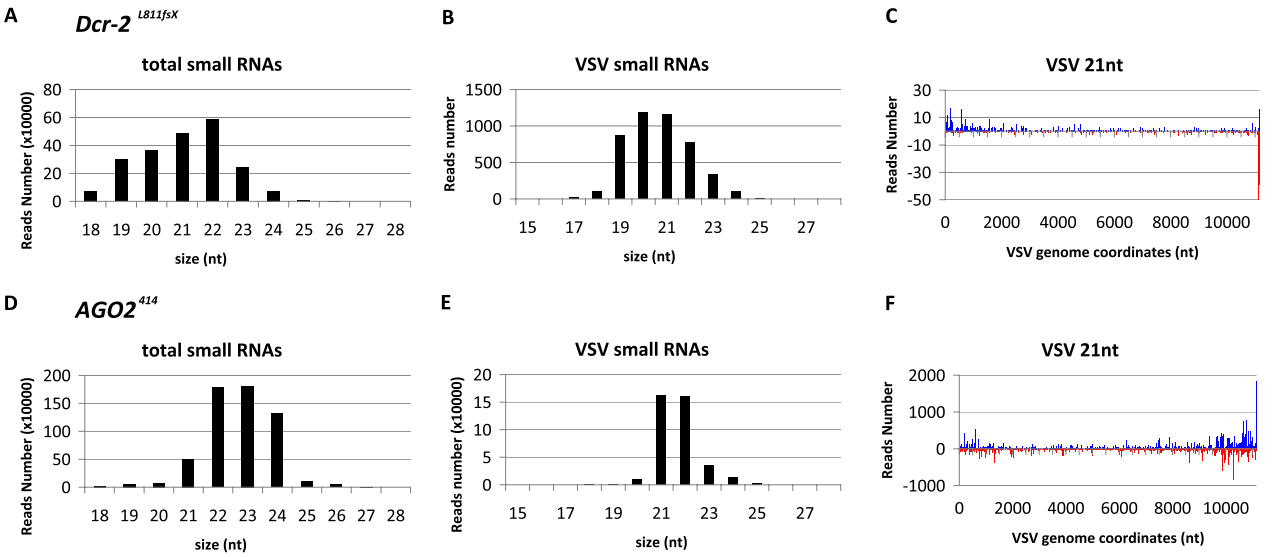
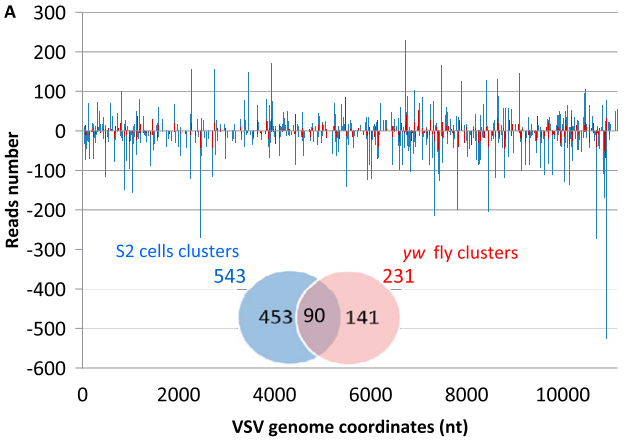


Fig. S6. Virus-derived siRNAs in VSV *Dcr-2*^{L811fsX} and *AGO2*^{414} infected flies in Dataset 2 (Table S1). (*A*) Size distribution of total small RNAs in VSV-infected *Dcr-2*^{L811fsX} flies. (*B*) Size distribution of VSV-specific small RNAs aligned against the viral genome in VSV-infected *Dcr-2*^{L811fsX} flies. (*C*) Profile of 21 nt VSV-derived reads recovered from *Dcr-2*^{L811fsX}-infected flies along the VSV genome. To comply with the orientation of the VSV reference sequence (NC_001560) used for all of the analyses, the genome is represented under its antigenomic polarity. Each vsiRNA is represented by the position of its first nucleotide. The vsiRNAs matching the antigenome and the genome are shown in blue and red, respectively. The horizontal axis represents the antigenome coordinates. (*D*) Size distribution of total small RNAs in VSV-infected *AGO2*^{414} flies. (*E*) Size distribution of VSV-specific small RNAs in VSV-infected *AGO2*^{414} flies. (*F*) Profile of 21 nt VSV-derived reads recovered from *AGO2*^{414} infected flies along the VSV genome. VSV-derived reads are depicted as in *C*.



B

sample	Cluster overlap to WT flies
S2 cells	39%
Dcr-2^{R416X} mutant fly	6%
AGO2^{414} mutant fly	26%

Fig. S7. Cluster comparison of 21 nt VSV-derived vsiRNA profiles in infected *yw*, *Dcr-2*^{R416X}, *AGO2*^{414} flies, and S2 cells. vsiRNA sequences were clustered as described in *SI Materials and Methods*. Cluster profiles of S2 cells, *Dcr-2*^{R416X}, and *AGO2*^{414} mutant flies were compared with that of *yw* flies. (*A*) A comparison of the profiles of clusters in S2 cells (blue) and in WT flies (*yw*; red) is shown. A Venn diagram at the bottom represents the total number of clusters in S2 cells (blue) and in *yw* flies (red) and their overlap (violet). (*B*) The table summarizes the cluster overlap between *yw* flies, S2 cells and *Dcr-2*^{R416X}, and *AGO2*^{414} mutant flies.

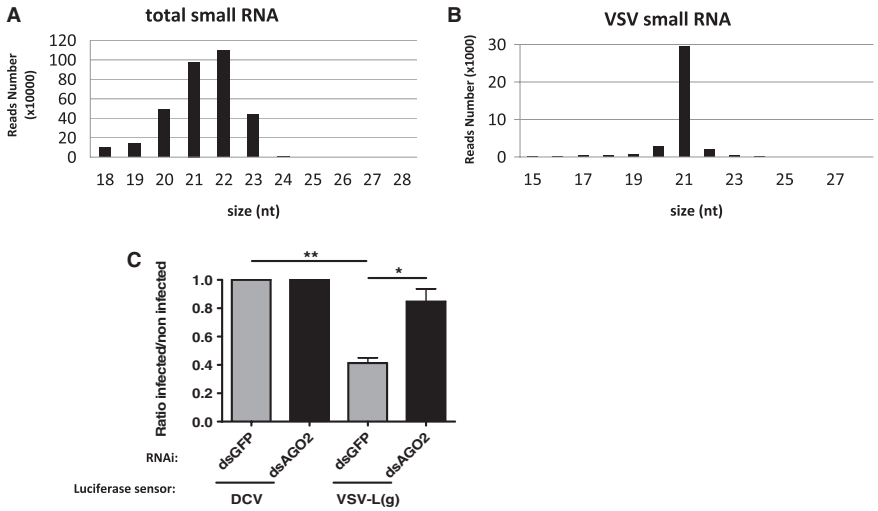


Fig. S8. Virus-derived siRNAs in VSV-infected S2 cells can silence luciferase-VSV sensors in an AGO2-dependent manner. (*A*) Size distribution of total small RNAs in VSV-infected S2 cells. (*B*) Size distribution of VSV-specific small RNAs in VSV-infected S2 cells. (*C*) VSV-derived vsiRNA silencing of a luciferase-VSV sensor is dependent on AGO2 activity. RNAi-mediated knock-down of AGO2 expression hindered luciferase-VSV silencing. Each bar represents the ratio of luciferase expression measured between infected and noninfected cells normalized to the values obtained for the DCV-GFP dsRNA control (set at 1). The values represent the mean and SD of three independent experiments. *P* values were calculated using Student's paired *t* test; $*P < 0.05$; $**P < 0.01$.

Other Supporting Information Files

Table S1 (XLS)