piRNA pathway is not required for antiviral defense in *Drosophila melanogaster*

Marine Petit[a,b], Vanesa Mongelli[a,1], Lionel Frangeul[a], Hervé Blanc[a], Francis Jiggins[c], and Maria-Carla Saleh[a,1]

[a]Viruses and RNA Interference, Institut Pasteur, CNRS Unité Mixte de Recherche 3569, 75724 Paris Cedex 15, France; [b]Sorbonne Universités, Université Pierre et Marie Curie, Institut de Formation Doctorale, 75252 Paris Cedex 05, France; and [c]Department of Genetics, University of Cambridge, Cambridge CB2 3EH, United Kingdom

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Since its discovery, RNA interference has been identified as involved in many different cellular processes, and as a natural antiviral response in plants, nematodes, and insects. In insects, the small interfering RNA (siRNA) pathway is the major antiviral response. In recent years, the Piwi-interacting RNA (piRNA) pathway also has been implicated in antiviral defense in mosquitoes infected with arboviruses. Using *Drosophila melanogaster* and an array of viruses that infect the fruit fly acutely or persistently or are vertically transmitted through the germ line, we investigated in detail the extent to which the piRNA pathway contributes to antiviral defense in adult flies. Following virus infection, the survival and viral titers of *Piwi*, *Aubergine*, *Argonaute-3*, and *Zucchini* mutant flies were similar to those of wild type flies. Using next-generation sequencing of small RNAs from wild type and siRNA mutant flies, we showed that no viral-derived piRNAs were produced in fruit flies during different types of viral infection. Our study provides the first evidence, to our knowledge, that the piRNA pathway does not play a major role in antiviral defense in adult *Drosophila* and demonstrates that viral-derived piRNA production depends on the biology of the host–virus combination rather than being part of a general antiviral process in insects.

antiviral RNAi | insect | virus | small RNA | piRNA

Three main small RNA-based silencing pathways have been described in animals: the microRNA (miRNA), small interfering RNA (siRNA), and Piwi-interacting RNA (piRNA) pathways. These pathways are involved in the regulation of different key biological processes, including organism development (1), defense against viral pathogens (2), and genome protection from transposable element (TE) activity (3). Despite their different biological functions, all three pathways use small RNAs (from 21 to 30 nt) to guide the sequence-specific recognition of target sequences by an Argonaute protein family member.

The siRNA pathway is a major antiviral defense mechanism in insects (4–10). This pathway is triggered in host cells by the presence of viral double-stranded RNA (vdsRNA) derived from viral replication intermediates, genomes of dsRNA viruses, overlapping transcripts of DNA viruses, or secondary viral genome structures. In *Drosophila melanogaster*, vdsRNA is recognized and processed into 21-nt-long viral siRNAs (vsiRNAs) by Dicer-2 protein (Dcr-2), a type III RNA endonuclease. Once diced, double-stranded vsiRNAs are first loaded into the RNA-induced silencing complex (RISC), then unwound, and one strand is ejected from the complex. Single-stranded vsiRNAs are finally methylated in their 3′ end nucleotide 2'-OH group by Hen1 methyltransferase (11). Through the activity of its main catalytic component, the RNase H type nuclease Argonaute-2 protein (Ago-2), RISC guides the sequence-specific recognition and cleavage of viral target RNAs (12), leading to viral genome degradation and, consequently, restriction of viral replication.

The piRNA pathway has been identified as the main protection mechanism against the activity of TEs in animal genomes. The biogenesis of piRNAs involves two steps, the primary processing mechanism and the secondary amplification mechanism. Production of piRNAs is Dicer-independent and relies mainly on the activity of Piwi proteins, a subclass of the Argonaute family (13). Primary piRNAs are processed from single-stranded RNA precursors, which are transcribed mostly from chromosomal loci consisting mainly of remnants of TE sequences, termed piRNA clusters (14). In *D. melanogaster*, the cleavage of primary piRNA precursors and generation of 5′ end of mature piRNAs were recently linked to Zucchini endonuclease (Zuc) activity (15–18). The cleaved precursor is loaded into Piwi family Argonaute proteins Piwi or Aubergine (Aub) and then trimmed by a still-unknown nuclease to reach its final length, which can vary from 24 to 30 nt. For example, piRNAs have a size centered around 25 nt in the fruit fly, but centered around 28 nt in *Aedes* mosquitoes. After trimming, piRNAs undergo a final 3′ end 2′-O-methyl nucleotide modification catalyzed by the methyltransferase Hen1 (11, 19) to become mature piRNAs. Primary piRNAs harbor a 5′ uridine bias (U1) and are usually antisense to TE transcripts (20). The cleavage of complementary active transposon RNA by primary piRNAs loaded into Aub proteins initiates the second biogenesis round and leads to the production of secondary piRNAs that are loaded in Argonaute-3 protein (Ago-3). During this ping-pong or amplification cycle, Aub and Ago-3 proteins loaded with secondary piRNAs mediate the cleavage of complementary RNA to generate new secondary piRNAs identical in sequence to the piRNA that initiated the cycle. Because target slicing by Piwi proteins occurs

Significance

In animals, one of the main forms of RNA interference involves Piwi-interacting RNAs (piRNAs), which protect genomes against the activity of transposable elements. Several groups have recently described piRNAs from viruses in mosquitoes and suggested their involvement in antiviral defense. To understand the extent to which the piRNA pathway contributes to antiviral defense in insects, we used *Drosophila melanogaster* and different viruses. Using high-throughput sequencing, we were unable to find any evidence of piRNAs from viruses in flies. Furthermore, flies lacking components of the piRNA pathway were not unusually susceptible to viral infection. Taken together, our results indicate that fundamental differences have arisen between the antiviral defenses of flies and mosquitoes since they last shared a common ancestor >200 Mya.

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[1]To whom correspondence may be addressed. Email: vanesa.mongelli@pasteur.fr or carla.saleh@pasteur.fr.

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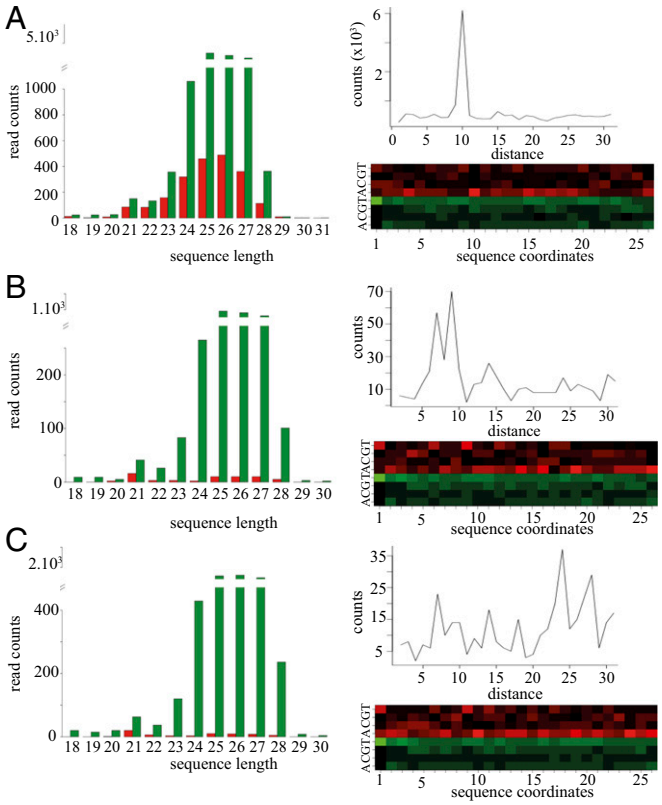

Fig. 1. Loss of secondary piRNAs for *Idefix* TE in the *Aubergine* mutant strains. (*Left*) Size distribution of β-eliminated small RNAs extracted from w^{1118} (*A*), *Aub* parental mutant strain (*B*), and backcrossed *Aub* (*C*) mutant flies. (*Right*) Frequency map of the distance between 24–26 nt small RNAs that mapped to opposite strands of the *Idefix* sequence. A peak is observed at position 10 for w^{1118}, but not for either of the *Aub* mutant strains. The relative nucleotide frequencies per position of the 24–26 nt small RNAs that map the sense and antisense of the genome are shown in red and green, respectively. The intensity varies in correlation with frequency. A nucleotide bias (U1 and A10) is observed for w^{1118}, but not for the *Aub* mutant strains.

between nucleotides 10 and 11, the complementary secondary piRNAs typically have a 10-nt overlap and contain an adenine at position 10 (A10) (14, 21).

Unlike the siRNA pathway that seems to be ubiquitously expressed in insect tissues, most experimental data indicate that the piRNA pathway is active mainly in germ-line tissues. Nevertheless, endogenous piRNAs also have been identified in various somatic tissues from fly, mouse, and macaque, as well as in mosquito head and thorax (22, 23).

The piRNA pathway was recently implicated in antiviral defense in insects. The antiviral activity of the piRNA pathway was first suggested in 2010, when viral small RNAs with the length of piRNAs were detected in *Drosophila* ovarian somatic sheet (OSS) cells (24). Since then, work on the subject has centered exclusively on mosquito-arbovirus experimental systems. In *Aedes* mosquitoes and cell lines, an expanded family of Piwi proteins is expressed in somatic tissues, and viral-derived piRNAs (vpiRNAs) are produced from the genomes of several arboviruses (23, 25–29). Functional links among the piRNA pathway, arbovirus replication, and vpiRNA production have been described as well. Depletion of Piwi-4 protein was found to enhance replication of Semliki Forest virus [SFV; (+)ssRNA, *Togaviridae*] without interfering with vpiRNA production in Aag2 cells (30), whereas both Piwi-5 and Ago-3 were shown to be required for the biogenesis of piRNAs from Sindbis virus [SINV; (+)ssRNA, *Togaviridae*] in the same cell line (31). Nevertheless, functional in vivo experimental data are scarce, and more work is needed to fully understand the extent to which the piRNA pathway contributes to antiviral defense not only in mosquitoes, but also in the context of other insect–virus interactions.

D. melanogaster is a powerful insect model for studying virus–host interactions (32, 33). Mutants for virtually all genes encoded by the genome of the fruit fly are publicly available, and *Drosophila* viruses from several families have been isolated, their genomes sequenced, and their biological characteristics described. These include *Drosophila* C virus [DCV; (+)ssRNA *Dicistroviridae*], *Drosophila* X virus [DXV; bisegmented dsRNA, *Birnaviridae*], *Drosophila* A virus [DAV; (+)ssRNA, unclassified, related to *Permutotetraviridae*], Nora virus [NoraV; (+)ssRNA, unclassified, related to *Picornaviridae*], and *D. melanogaster* sigma virus [DMelSV, (−)ssRNA, *Rhabdoviridae*] (34). Infection of flies with viruses from other insect hosts, such as SINV, a mosquito-infecting arbovirus; Flock house virus [FHV; bisegmented (+)ssRNA, *Nodaviridae*], originally isolated from the grass grub *Costelytra zealandica* (35), and the rice stem borer larvae-isolated Invertebrate iridescent virus 6 [IIV-6; dsDNA, *Iridoviridae*] (36), among others, is also possible under laboratory conditions.

Despite the numerous molecular tools available to decipher antiviral responses in flies, and the fact that viral small RNAs with the length of piRNAs were first reported in fly tissues (24), no further work addressing the antiviral role of piRNAs has been performed in *Drosophila*. Only two studies published before the discovery of vpiRNA highlighted a functional link between piRNA pathway and antiviral defense in flies. Piwi mutant flies were found to be more susceptible to both DXV and West Nile virus [WNV; (+)ssRNA, *Flaviviridae*] infections, and Aub mutant flies were found to be more susceptible to DXV (10, 37).

In the present work, we aimed to characterize the impact of the piRNA pathway on the fly antiviral response. We sought to understand whether the viral-derived piRNAs are part of the general antiviral process, or whether their production depends on the biology of the host–virus combination. Our results indicate that the piRNA pathway does not play a major direct role in antiviral defense in *Drosophila*, and that vpiRNAs are not produced in the fruit fly during different types of viral infection. We speculate that during speciation and diversification of the piRNA pathway proteins in insects, the piRNA pathway evolved solely to repress transposon activity in the fruit fly while expanding to an antiviral role in mosquitoes.

Results

Isogenization and Characterization of piRNA Mutant Flies on the w^{1118} Isogenic Background. To reduce genetic background effects when studying the impact of the piRNA pathway mutants on antiviral response, we backcrossed *Zuc*, *Aub*, and *Ago-3* mutants to wild type (WT) flies (w^{1118}) so they were in similar genetic backgrounds (*SI Appendix*, Fig. S1*A*). For each generation, we genotyped the flies by PCR to retain only those carrying the mutant allele (*SI Appendix*, Fig. S1). After 10 backcrosses, we compared the phenotypes from the new *Aub* (Fig. 1 and *SI Appendix*, Figs. S3 and S4), *Ago-3*, and *Zuc* (*SI Appendix*, Figs. S2–S4) backcrossed lines with the parental lines by performing small RNA deep sequencing and analyzing piRNA production. Small RNAs ranging from 19 to 30 nt were recovered and sequenced in mutant and WT flies, as well as in the parental mutant lines. Data analysis was centered on the following features: (*i*) overall small RNA production, (*ii*) production of piRNAs from the TE *Idefix* (Fig. 1 and *SI Appendix*, Fig. S2), (*iii*) production of piRNAs from the germ-line piRNA cluster *42AB* (*SI Appendix*, Fig. S3), and (*iv*) production of piRNAs from the somatic piRNA cluster *Flamenco* (*SI Appendix*, Fig. S4). Fig. 1 *B* and *C* show that loss of Aub strongly impacted the secondary piRNA population from the *Idefix* TE sequence. In addition, loss of Aub interrupted the piRNA ping-pong cycle on the germ-line *42AB* cluster (*SI Appendix*, Fig. S3*C*; loss of A10 bias). In contrast, the loss of Ago-3, which participates in the ping-pong amplification cycle, produced piRNAs with a ping-pong signature and the U1-A10 bias (*SI Appendix*, Figs. S3*D* and S4*D*) due

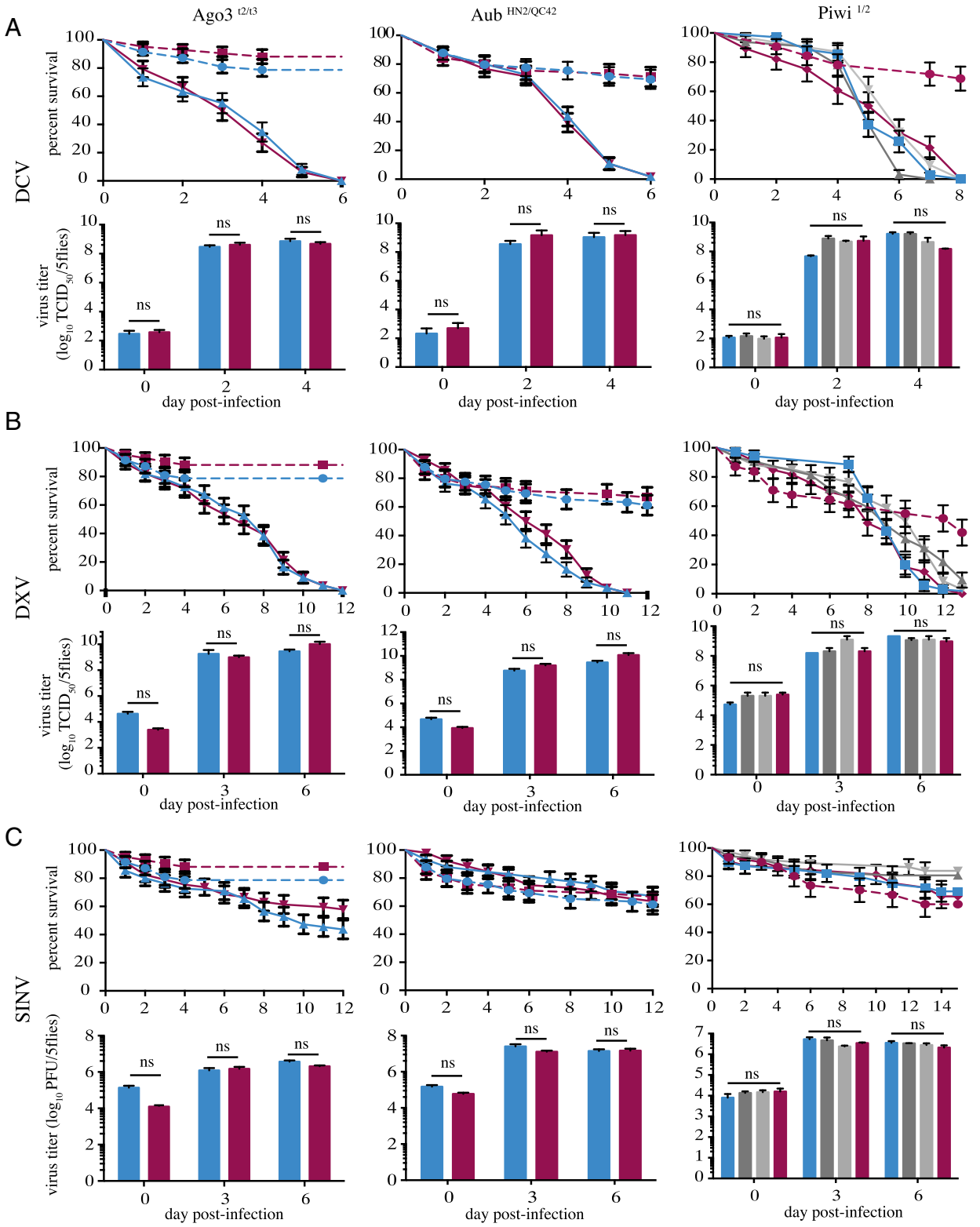

Fig. 2. piRNA mutant flies are no more sensitive to viral infection than WT flies. Group of 20 flies control or piRNA mutants, *Ago-3*$^{t2/t3}$, *Aub*$^{HN2/QC42}$ and *Piwi*$^{1/2}$ were infected by injection with three different viruses: DCV (2 $TCID_{50}$) (*A*), DXV (100 $TCID_{50}$) (*B*), and SINV (5,000 PFU) (*C*). (*Upper*) Survival was monitored daily. Each experiment was repeated three times. Data shown are mean ± SD. Dashed lines correspond to Tris injection to control the effect of injection on fly survival. Control (*w*^{1118}) flies are shown in blue; mutant flies, in red. For *Piwi*$^{1/2}$ flies, the dark-gray/light-gray lines correspond to the heterozygote control. Differences in survival between control and piRNA mutant flies were not statistically significant [log-rank (Mantel–Cox) test]. (*Lower*) Viral titer was determined for a group of five flies in triplicate for each genotype. The viral titration was done at 0, 2, and 4 dpi for DCV (*A*) and at 0, 3, and 6 dpi for DXV (*B*) and SINV (*C*). No significant difference (two-way ANOVA) between the different genotypes was observed.

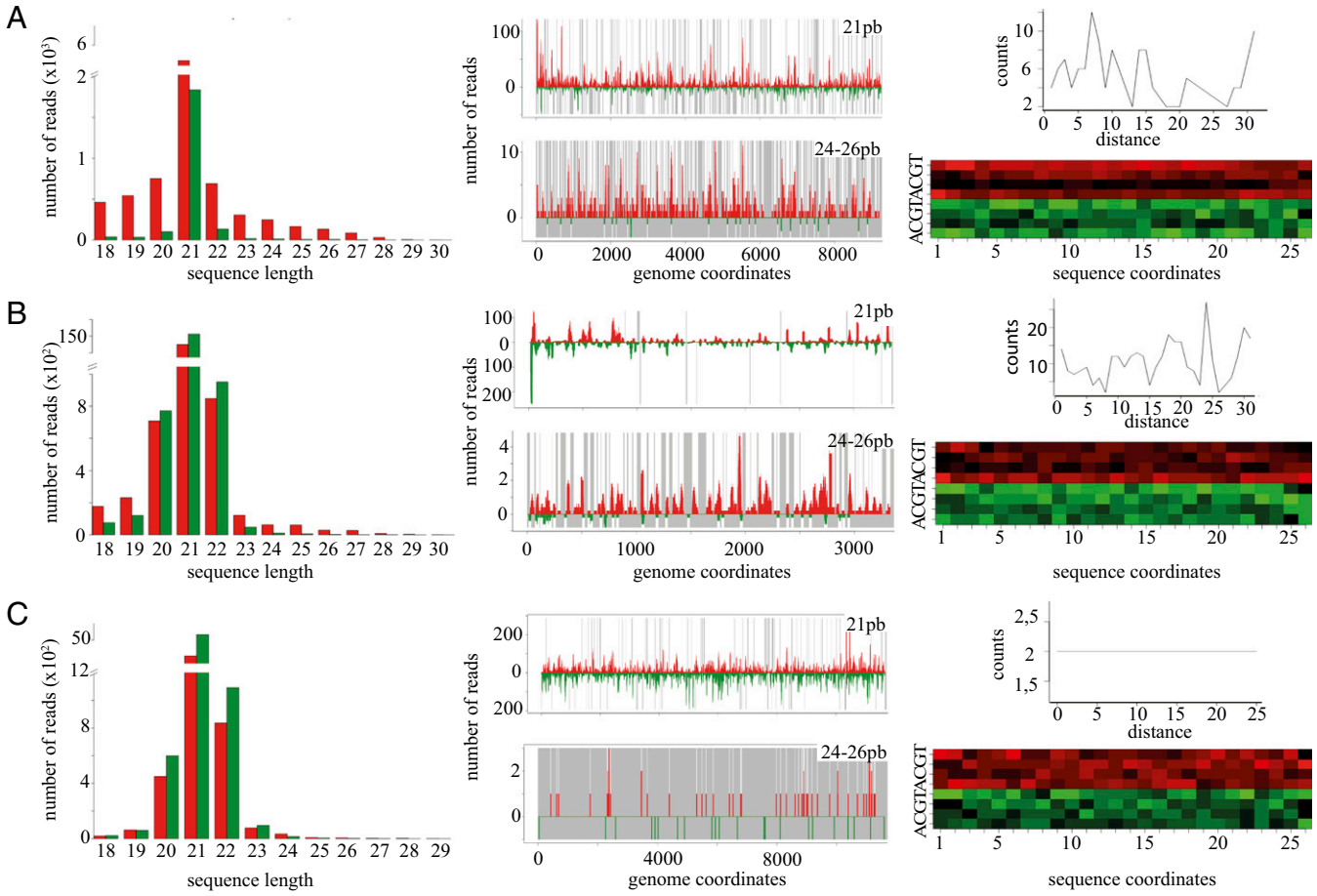

Fig. 3. No viral-derived piRNAs are produced during acute infection of flies. (*Left*) Size distribution of β-eliminated small RNAs extracted from w^{1118} *Drosophila* infected with DCV (*A*), DXV segment A (*B*), and SINV (*C*). (*Center*) Profile of 21 nt and 24–26 nt small viral RNAs that mapped along the viral genome of interest. The sense and antisense small RNAs are shown in red and green, respectively. Uncovered regions are represented as gray lines. (*Right*) Frequency map of the distance between 24–26 nt small RNAs that mapped to opposite strands of the viral genome. No peak is observed at position 10. The relative nucleotide frequencies per position of the 24–26 nt viral small RNAs that map the sense and antisense of the viral genome are shown in red and green, respectively. The intensity varies in correlation with the frequency. No nucleotide bias (U1 and A10) is observed.

to the Aub-Aub ping-pong that is still active in the absence of Ago-3 (38, 39). All mutants produced similar profiles when mapped to *Flamenco* (*SI Appendix*, Fig. S4), a specific piRNA cluster of somatic cells (40). Finally, the loss of Zucchini protein led to the absence of primary piRNAs for *Idefix*, *42AB*, and *Flamenco* (*SI Appendix*, Figs. S2*B*, S3*B*, and S4*B*). It is noteworthy that in the absence of Zucchini, the ping-pong signature and the U1-A10 bias were still present, because the loss of primary piRNAs increased detection of the secondaries.

We also compared primary and secondary piRNAs in whole flies versus ovaries. Germ line-dominant piRNA clusters, as well as somatic dominant piRNA clusters and TEs, were readily detectable in both conditions (*SI Appendix*, Fig. S5).

Through the foregoing analyses, we established not only the behavior of the backcrossed mutant flies as the parental lines regarding piRNA production, but also the capacity of our bioinformatic pipelines to detect somatic and germ-line piRNAs. All experiments with *Aub*, *Ago-3*, and *Zuc* mutant flies were performed in backcrossed flies, whereas the *Piwi* mutant was used in its original genetic background.

piRNA Mutant Flies Are No More Susceptible to Viral Infections than WT Flies. To determine the impact of the piRNA pathway on antiviral immunity, we studied the effect of acute viral infections on fly mutants for key components of both primary (Zuc and Piwi) and secondary (Aub and Ago-3) piRNA biogenesis pathways. We inoculated control, *Piwi*, *Aub*, and *Ago-3* mutant flies with two *Drosophila* viruses, DCV and DXV, and one mosquito-infecting arbovirus, SINV. We assessed fly survival and virus accumulation after inoculation. We did not find any difference in mortality of control and mutant flies following infection; all flies died between 6 and 10 d postinfection (dpi) with DCV and DXV (Fig. 2 *A* and *B*, *Upper*).

Even in the absence of lethality, it is possible that piRNA mutant flies cannot successfully control viral loads, which would indicate a role of the piRNA pathway in viral defense. To test this, we collected fly samples at 0, 2 and 4 dpi with DCV and at 0, 3 and 6 dpi with DXV. We measured viral load by 50% tissue culture infective dose ($TCID_{50}$). We did not find differences in DCV or DXV accumulation between control and piRNA mutant flies at any time point analyzed (Fig. 2 *A* and *B*, *Lower*). When inoculating *Zuc* mutant flies with DCV and DXV we observed a significant resistance to virus infection in these flies that is not associated to the *Pastrel* gene allele (41) because flies were backcrossed. Similar to the others piRNA mutants, no difference in viral load was observed (*SI Appendix*, Fig. S6 *A* and *B*). In the case of SINV inoculations, neither control nor piRNA mutant flies succumbed after infection, as SINV developed a persistent infection (Fig. 2*C*, *Upper*). We assessed SINV accumulation by plaque assay in fly samples collected at 0, 3, and 6 dpi, and again found no differences

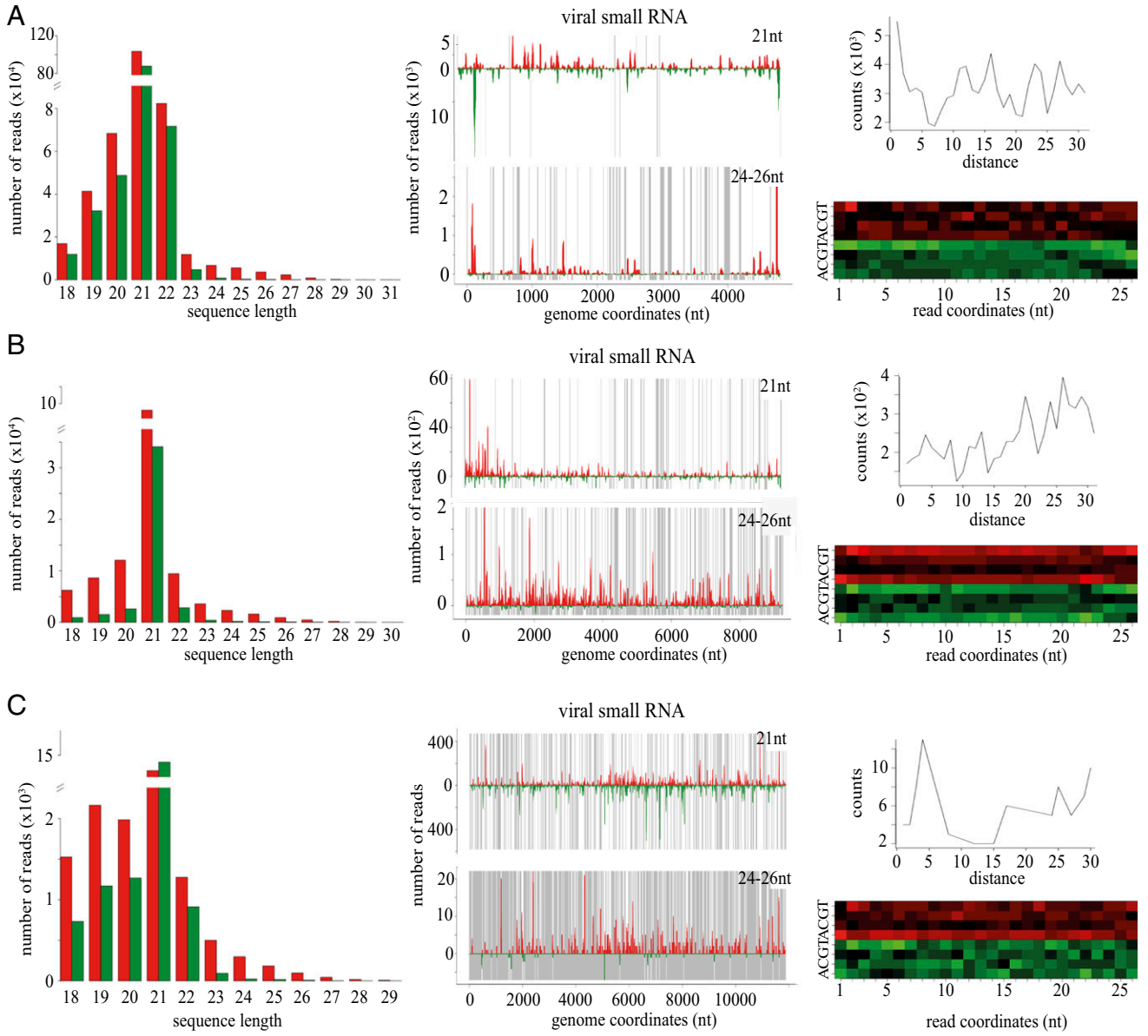

Fig. 4. *Drosophila* persistently infected with viruses do not produce viral-derived piRNAs. (*Left*) Size distribution of β-eliminated small RNAs extracted from *Drosophila* persistently infected with DCV (*A*), DAV (*B*), and NoraV (*C*). (*Center*) Profile of 21 nt and 24–26 nt small RNA reads that mapped along the viral genome of interest. The sense and antisense small RNAs are shown in red and green, respectively. Uncovered regions are represented as gray lines. (*Right*) Frequency map of the distance between 24–26 nt small RNAs that mapped to opposite strands of the viral genome. No peak is observed at position 10. The relative nucleotide frequencies per position of the 24–26 nt viral small RNAs that map the sense and antisense of the viral genome are shown in red and green, respectively. The intensity varies in correlation with the frequency. No nucleotide bias (U1 and A10) is observed.

in SINV load among the different genotypes (Fig. 2*C*, *Lower* and *SI Appendix*, Fig. S6*C*). Taken together, these results show that piRNA *Zuc*, *Piwi*, *Aub*, and *Ago-3* mutants are no more susceptible to DCV, DXV, or SINV infections than control flies, suggesting that neither primary nor secondary piRNA biogenesis pathways play a major antiviral role for these viruses in adult *Drosophila*.

No Viral-Derived piRNAs Are Produced in Flies During Acute Viral Infection. The potential of the piRNA pathway to recognize and process viral RNAs in *Drosophila* has been demonstrated in the OSS cell line (24). Because OSS cells do not express Aub and Ago-3 proteins, only the primary piRNA biogenesis step is active in these cells (42), and consequently, only primary 25–30 nt vpiRNAs with a U1 bias were observed (24). The implication of secondary piRNA biogenesis pathway in vpiRNAs production in flies has not yet been addressed, nor has the production of vpiRNAs in vivo. To investigate this, we inoculated WT (w^{1118}) flies with DCV, DXV, or SINV. Infected flies were sampled at 2 dpi for DCV and at 3 dpi for DXV and SINV after inoculation, and small RNAs ranging from 19 to 30 nt were recovered and subjected to β-elimination to enrich small RNA molecules harboring a 3′ end 2′-O-methyl nucleotide modification, such as mature siRNAs or piRNAs (43). Following high-throughput sequencing, the analysis of size distribution for DCV-, DXV-, and SINV-derived small RNAs showed a sharp peak of 21 nt enrichment, characteristic of vsiRNAs produced by Dcr-2 activity during the RNAi antiviral response (Fig. 3 *A*–*C*, *Left*). vsiRNAs represented 63%, 88%, and 90% of all viral reads for DCV, DXV, and SINV respectively, and were distributed across the entire viral genomes and derived from both negative and positive strands of viral replication intermediates

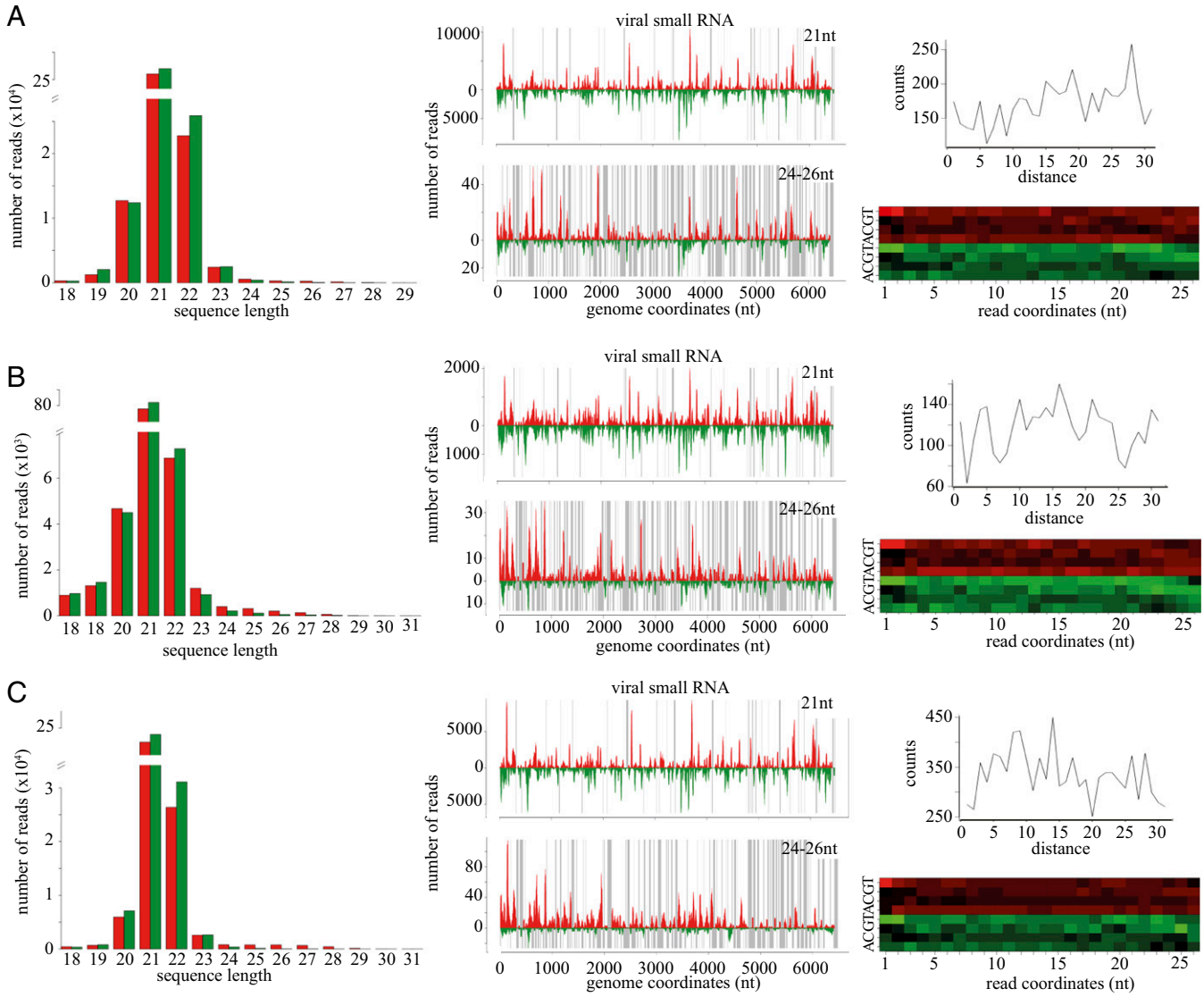

Fig. 5. There is no production of viral-derived piRNAs during Sigma virus infection. (*Left*) Size distribution of β-eliminated small RNAs extracted from *Drosophila* injected with DmelSV. RNA was extracted at 12 dpi for the w^{1118} (*A*) and *22a* (*B*) strains of *Drosophila*. *E320* corresponds to a fly strain persistently infected with DmelSV (*C*). (*Center*) Profile of 21 nt and 24–26 nt small viral RNA that mapped along the viral genome of interest. The sense and antisense small RNAs are shown in red and green, respectively. Uncovered regions are represented as gray lines. (*Right*) Frequency map of the distance between 24–26 nt small RNAs that mapped to opposite strands of the viral genome. No peak is observed at position 10. The relative nucleotide frequencies per position of the 24–26 nt viral small RNAs that map the sense and antisense of the viral genome are shown in red and green, respectively. The intensity varies in correlation with the frequency. No nucleotide bias (U1 and A10) is observed.

(Fig. 3 *A*–*C*, *Center* and *SI Appendix*, Table S9). Only a small proportion of virus-derived small RNAs corresponded to the expected size of vpiRNAs of 24–26 nt: 5.7%, 0.4%, and 0.2% of all small RNA reads for DCV, DXV, and SINV, respectively (Fig. 3 *A*–*C*, *Center* and *SI Appendix*, Table S9). These reads, distributed along complete viral genomes, were derived mainly from the viral positive strand and did not exhibit any of the characteristic biochemical biases described for piRNA: uridine at the 5′ end position (U1), adenosine at the tenth position (A10), and 10 nt overlaps between sense and antisense sequences (Fig. 3 *A*–*C*, *Right*).

We next analyzed the production of vpiRNAs in flies deficient for the siRNA pathway—*Dcr-2* and *Ago-2* null mutants—to test whether the absence of the main antiviral mechanism could reveal a contribution of the piRNA pathway to the antiviral response. We inoculated *Dcr-2* and *Ago-2* mutant flies with DCV, DXV, or SINV. As for WT flies, small RNAs ranging from 19 to 30 nt were recovered, samples were subjected to β-elimination and deep-sequenced. During SINV infection, as expected, the amount of viral small RNAs in *Dcr-2* mutant flies was close to zero (*SI Appendix*, Fig. S7*A*), whereas *Ago-2* mutant flies accumulated vsiRNAs of 21 nt from both polarities to a greater extent (*SI Appendix*, Fig. S7*B*). In no case were vpiRNAs detectable, and the few small RNAs in the size range of piRNAs did not exhibit a piRNA signature. In DCV and DXV infection, there was a significant accumulation of small RNAs of positive polarity in both *Dcr-2* and *Ago-2* mutant flies (*SI Appendix*, Figs. S7 *C* and *D* and S8). Analysis of the small RNA reads corresponding to the size of piRNAS did not reveal any vpiRNAs, and the few reads that accounted for piRNA size did not display a ping-pong signature or U1-A10 bias (*SI Appendix*, Figs. S7 *C* and *D* and S8). Taken together, and considering the capability of our sequencing and bioinformatics pipeline to detect either somatic or germ-line piRNAs, these data suggest that vpiRNAs are not produced in adult WT flies during DCV, DXV, and SINV acute infections, and that in the absence of a fully functional antiviral

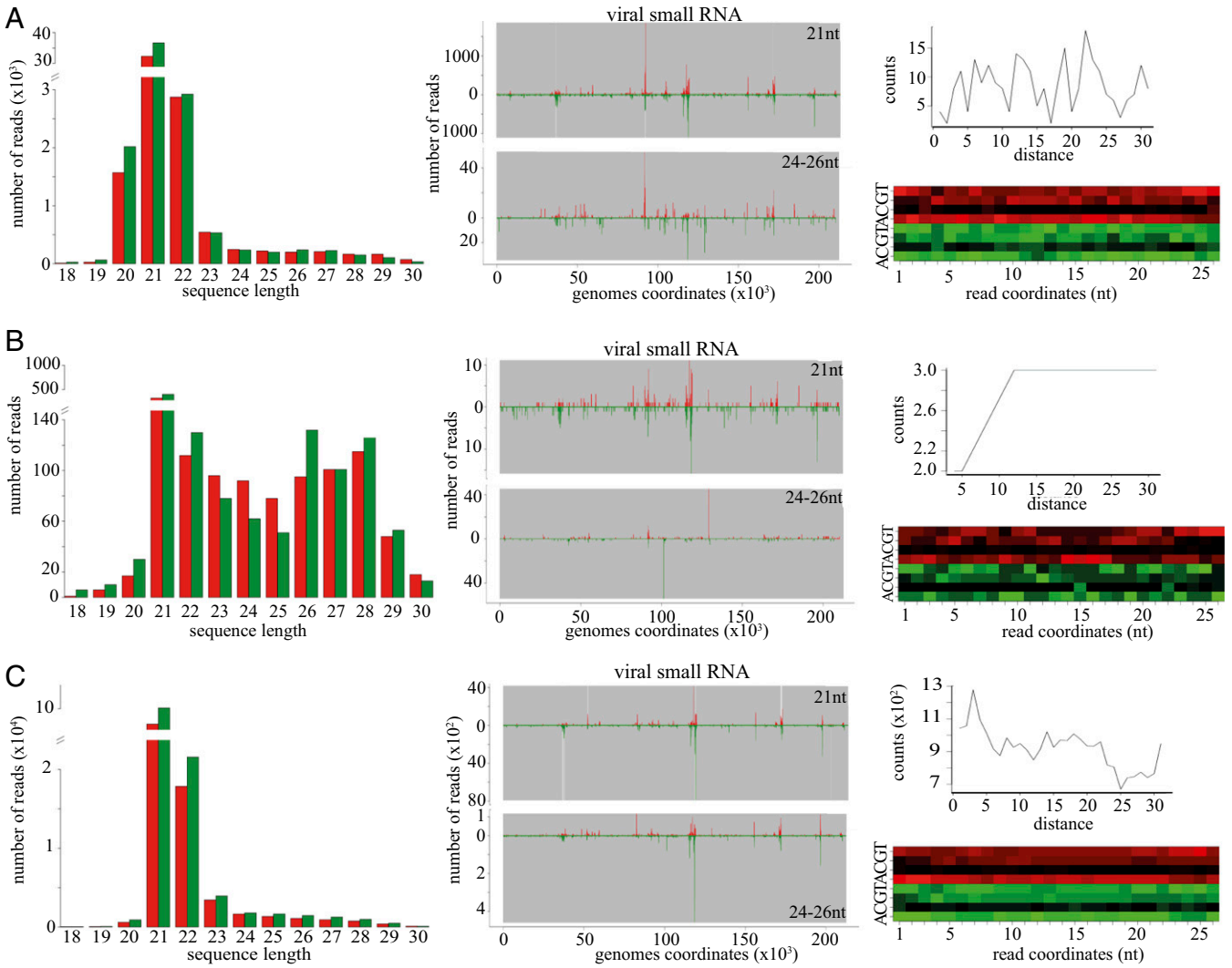

Fig. 6. The infection of *Drosophila* with a DNA virus does not lead to viral-derived piRNA production. (*Left*) Size distribution of small RNAs extracted from *Drosophila* injected with IIV6 (28). w^{1118} (*A*), $Dcr\text{-}2^{L811fsX}$ (*B*), and $Ago\text{-}2^{414}$ (*C*) mutant strains of *Drosophila*. (*Center*) Profile of 21 nt and 24–26 nt small viral RNAs that mapped along the viral genome of interest. The sense and antisense small RNAs are shown in red and green, respectively. Uncovered regions are represented as gray lines. (*Right*) Frequency map of the distance between 24–26 nt small RNAs that mapped to opposite strands of the viral genome. No peak is observed at position 10. The relative nucleotide frequencies per position of the 24–26 nt viral small RNAs that map the sense and antisense of the viral genome are shown in red and green, respectively. The intensity varies in correlation with the frequency. No nucleotide bias (U1 and A10) is observed.

RNAi pathway, the piRNA pathway is not able to engage in an antiviral response based on the production of viral small RNAs.

No Viral-Derived piRNAs Are Detected in Persistently Infected Flies. Insects develop viral persistent infections as a common outcome. During a persistent infection, the virus replicates but has relatively little effect on host fitness. It has been proposed that the siRNA pathway is boosted during persistent infection by the production of more abundant and distinct vsiRNAs (44). Those vsiRNAs are generated from a viral DNA form. Because endogenous piRNAs sources are host genome-encoded DNA clusters, we thought that this viral-derived DNA could constitute the source of vpiRNAs. Thus, we decided to analyze the presence of vpiRNAs in fly stocks persistently infected with DCV, NoraV, or DAV. Small RNAs ranging from 19 to 30 nt were recovered from persistently infected flies, and the samples were subjected to β-elimination and deep-sequenced. DCV-, NoraV-, and DAV-derived small RNA profiles displayed the expected 21-nt vsiRNAs accumulation peak, a product of Dcr-2 activity during RNAi antiviral response (Fig. 4, *Left*). vsiRNAs were distributed across the complete viral genomes, matched both positive and negative viral strands, and represented 69.97% of the short RNA reads for DCV, 67.40% of those for NoraV, and 82.33% of those for DAV (Fig. 4, *Center* and *SI Appendix*, Table S9). In contrast, viral-derived small RNAs of the expected size for vpiRNAs (24–26 nt) represented only 2.89%, 1.66%, and 0.73% of the reads for DCV, NoraV, and DAV respectively (Fig. 4 and *SI Appendix*, Table S9). Reads were derived mainly from the viral positive strand and did not display any of the signatures of piRNAs (Fig. 4, *Center* and *Right*). Taken together, our data show that during persistent viral infections, *D. melanogaster* does not produce vpiRNAs.

No Viral-Derived piRNAs Are Detected in Flies Infected with a Vertically Transmitted *Drosophila* Virus. Although one study has reported the presence of endogenous piRNA-like small RNAs in fly heads and imaginal discs (22), piRNA pathway components are expressed and piRNAs produced predominantly in the fly

germ-line tissues and surrounding somatic cells (45). DMelSV is a vertically transmitted natural *Drosophila* virus that is able to replicate in fly germ-line tissues (46, 47). To study whether vpiRNA production is restricted to viruses able to replicate in germ-line tissues, we analyzed the presence of these small RNA species in flies infected with DMelSV. We used three *Drosophila* strains: a line (*22a*) naturally susceptible to DMelSV (48), a line (*E320*) persistently infected with the same virus (46), and w^{1118} WT flies. We inoculated w^{1118} and *22a* flies with DMelSV. Small RNA populations present in WT and *22a* line-infected flies were analyzed at 12 dpi. Small RNAs from the *E320* line were analyzed in 4- to 6-d-old flies. In all cases, small RNAs ranging from 19 to 30 nt were recovered, and the samples were subjected to β-elimination (except for *22a* flies) and deep-sequenced. As shown in Fig. 5, DMelSV infection produced abundant vsiRNAs distributed across the complete viral genomes, matching both positive and negative viral strands. For the small RNAs of 24–26 nt, no *vpiRNAs* were detected. This finding indicates that viruses that specifically infect the germ line as well as somatic tissues are also controlled by the siRNA antiviral response and do not trigger a piRNA-mediated antiviral response in adult flies.

No Viral-Derived piRNAs Are Detected in Flies Infected with Other Model Viruses. IIV-6 and FHV are not natural *Drosophila* viruses. Nevertheless, both are able to replicate in *D. melanogaster* under experimental conditions (49–51). IIV-6 is commonly used as a DNA virus model, and we thought that vpiRNAs could originate from a DNA source, as is the case for endogenous piRNAs. In contrast, FHV is a single-stranded positive-sense RNA virus that has been extensively used to study antiviral responses in flies. Work from our laboratory has demonstrated that during FHV infection of both flies and the S2 *Drosophila* cell line, an FHV-derived DNA form was produced that was implicated in the establishment of persistent infections (44). Based on the same rationale that we used for IIV-6, we hypothesized that the FHV DNA form could constitute a vpiRNAs source. We analyzed published small RNA libraries (SRA 048623 and SRA 045427) for the presence of vpiRNAs. As in all of the previous conditions and as shown in Fig. 6 for IIV-6, we were not able to detect viral small RNAs with the characteristic signature of piRNAs in *D. melanogaster*.

Discussion

The piRNA pathway was recently implicated in antiviral defense in insects. This pathway is based on small RNAs and was first identified as the main mechanism controlling TE activity in animal genomes. Despite the fact that viral-derived piRNAs were first suggested to be present in *Drosophila* OSS cells (24), and that *Aubergine* (10) and *Piwi* (37) mutant flies appeared to be more sensitive to viral infection, none of these studies actively sought to prove or disprove the existence of viral piRNAs in *Drosophila*. The reports that followed used arboviral infections of mosquitoes or mosquito cells in culture as model systems. We decided to carry out a comprehensive study on the role of the piRNA pathway during viral infection in *D. melanogaster*. Using an array of RNAi mutants, an array of viruses corresponding to different families with (+)ssRNA, dsRNA, and dsDNA genomes that, naturally or not, infect the fly, our phenotypic observations of the impact of viral infection on piRNA mutants via measures of fly survival and viral loads, as well as high-throughput sequencing, we demonstrate that (*i*) mutant adult flies for key components of the piRNA pathway (*Zuc*, *Piwi*, *Aub*, and *Ago-3*) are no more susceptible than WT flies to two *Drosophila* viruses (DCV and DXV) and an arbovirus (SINV); (*ii*) no vpiRNAs are produced during acute infections with DCV, DXV, SINV, DMelSV, IIV6, and FHV; (*iii*) no vpiRNAs are produced during persistent infections with DCV, NoraV, DAV, or DMelSV; (*iv*) in the absence of the siRNA pathway (*Ago-2* and *Dcr-2* mutants), no vpiRNAs are produced during DCV, DXV, and SINV infection; and (*v*) no vpiRNAs are detected during infection with DmelSV, DXV, and FHV, shown to infect ovarian cells (51, 52). Taken together, these results indicate that the piRNA pathway is not involved in the antiviral response mediated by vpiRNAs. In addition, our capacity to detect somatic piRNAs from ovarian somatic sheet cells when performing deep sequencing of ovaries indicates that we are not missing a very low number of vpiRNAs that could have been generated by this cell type. Indeed, *Flamenco* has been proposed as an ovarian somatic sheet cells-only piRNA cluster, and as such validates the sensitivity of our deep-sequencing methodology.

Before vpiRNAs were described, Zambon et al. (10) and Chotkowski et al. (37) reported that *Piwi* and *Aub* mutant flies were more sensitive to DXV and WNV infection, displaying an accelerated death and higher viral loads compared with WT flies. The fact that we did not observe the same phenotype in flies that are backcrossed leads us to believe that their observation is based on a genetic background effect. The background effect was acknowledged in recent publications (53), and there is considerable natural genetic variation among *Drosophila* lines in their susceptibility to the viruses that we have studied. Thus, our results reinforce the importance of studying mutants in the same genetic backgrounds to avoid misleading interpretations.

Along with the genetic background, our results also allow us to rule out effects due to superinfection. Indeed, a recent study found that about 40% of fly stocks are persistently infected with different viruses (54). As described in *Materials and Methods*, our fly stocks were treated to eliminate *Wolbachia* (antibiotic treatment) and persistent viruses (bleach treatment of the embryos); therefore, previous infections and genetic background are not issues when analyzing our results.

It has been shown that genes involved in pathogen defense evolve much faster than the rest of the genome. *Ago-2* is a clear example of this observation (55). Interestingly, genes in the piRNA pathway, including *Piwi* and *Aub*, also evolve very rapidly, which has been suggested to be caused by adaptation to the ever-changing landscape of transposition activity in the fly (55, 56). The possibility that through regulation of transposons in somatic and germ-line tissues, the piRNA pathway could tune or control in a subtle manner the immune state of the host by genes involved in immunity cannot be disregarded. A curious example arises when studying the *Flamenco* locus, which is located downstream of the *DIP1* gene and is the source of piRNAs that silence various transposable elements. *Flamenco*-derived piRNAs are produced exclusively from the plus strand of the genome, indicating transcription from the *DIP1* gene toward the centromere (40). Recently, *DIP1* was reported to be involved in antiviral immunity against DCV, but not against DXV (57). Fly mutants for *DIP1* are hypersensitive to DCV infection, and the authors postulated that *DIP1* is a novel antiviral gene. The fact that *Flamenco*-derived piRNAs in somatic tissues in the ovary are produced from transcription from the *DIP1* gene (40) allows us to hypothesize that *DIP1*-mediated DCV sensitivity is dependent on the presence of piRNAs targeting the *DIP1* gene. In this way, the piRNA pathway or, more precisely, the production of piRNAs from the *Flamenco* locus, would not have a direct and general effect as an antiviral but instead would have an indirect and virus-specific effect. Alternatively, it could be presumed that the piRNA pathway exhibits an antiviral effect in *Drosophila* larvae or embryos. This hypothesis merits further study together with the antiviral response during development in flies and other model insects.

Interestingly, whereas flies encode three Piwi proteins (Piwi, Aub, and Ago-3), the Piwi family is expanded to eight members (Piwi 1–7 and Ago-3) in the mosquito *Aedes aegypti* and to seven members in *Culex pipiens* (58). It is tempting to speculate that during speciation and diversification of piRNA pathway proteins, the piRNA pathway gained additional functions in addition to the repression of transposon activity in mosquitoes, while remaining focused exclusively on the control of transposons in the fly. Alternatively, it could be proposed that the piRNA pathway has lost an ancestral antiviral

function since the last common ancestor of flies and mosquitoes. We believe that our results shed light on the complexity of the antiviral response in insects and reflect the diversity of action of the canonical RNAi pathways in invertebrates.

Materials and Methods

Fly Strains and Husbandry. Flies were maintained on a standard cornmeal diet (Bloomington) at a constant temperature of 25 °C. Fly stocks are listed in *SI Appendix*, Table S10. All fly lines were cleaned of possible chronic infections as described previously (59, 60). In brief, eggs were collected in agar/apple plates, treated with 50% bleach for 10 min, washed with water, and transferred to fresh vials. To eliminate *Wolbachia* infection, flies were treated for two generations with 0.05 mg/mL of tetracycline hydrochloride (Sigma-Aldrich) in the medium. In addition, all fly stocks were analyzed by RT-PCR with specific pairs of primers for CrPv, DAV, DXV, DCV, FHV, and NoraV.

Virus Production and Titration. DCV and DXV stocks were prepared on low-passage S2 cells, and titers were measured by end-point dilution. S2 cells (2.5×10^5 cells per well in a 96-well plate) were inoculated with 10-fold dilution of virus stocks. At 7 and 14 dpi, the cytopathic effect was analyzed. Titers were calculated as $TCID_{50}$ according to a published method (61). SINV viral stocks were produced on a BHK cell line, and virus titer (PFU/mL) was determined by a plaque assay on BHK cells.

To quantify viral load in flies, three pools of five flies each were analyzed at 0, 2, and 4 dpi for DCV infection and at 0, 3, and 6 dpi for SINV and DXV infection. DCV and DXV viral loads were measured by $TCID_{50}$, and SINV viral load was measured by a plaque assay.

Viral Infections and Survival Assays. The infection experiments were conducted using 4- to 6-d-old flies. Infections were done by intrathoracic injection (Nanoject II apparatus; Drummond Scientific) of 50 nL of a viral suspension in 10 mM Tris, pH 8. An injection of the same volume of 10 mM Tris, pH 8 served as a mock-infected control. Infected flies were kept at 25 °C and changed to fresh vials every 2 d. Survival of infected flies was measured daily by counting the number of dead flies in each test tube. Survival data were evaluated using a log-rank (Mantel–Cox) test.

RNA Extraction and Library Production. For each virus infection, small RNA was specifically extracted with the mirVana miRNA Isolation Kit (Ambion) from 150–200 flies at 2 dpi for DCV, 3 dpi for DXV and SINV, and 12 dpi for DMelSV. For each sample, 19–29 nt small RNAs were purified from a 15% acrylamide/bisacrylamide (37.5:1), 7 M urea gel as described previously (62). Purified RNAs were used for library preparation using the NEBNext Multiplex Small RNA Library Prep for Illumina (New England Biolabs) with the 3′ adapter from Integrated DNA Technologies (linker 1) and in-house–designed indexed primers. Libraries were diluted to 4 nM and sequenced using the NextSeq 500 High-Output Kit v2 (Illumina) (75 cycles) on a NextSeq 500 sequencer (Illumina). Reads were analyzed with in-house Perl scripts.

Bioinformatics Analysis of Small RNA Libraries. The quality of fastq files was assessed using graphs generated by FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/). Using cutadapt (https://cutadapt.readthedocs.io/en/stable/), low-quality bases and adaptors were trimmed from each read. Only reads with acceptable quality were retained. FastQC generated a second set of graphics on the fastq files created by cutadapt (63). Reads were mapped to genomes using bowtie1 (64) with the –v 1 (one mismatch between the read and its target). bowtie1 generates results in sam format. All sam files were analyzed by the samtools package (38) to produce bam indexed files. To analyze these bam files graphs were generated using custom R scripts and the Bioconductor Rsamtools and Shortreads libraries (65).

Statistical Analysis. Each experiment was repeated independently three times. Error bars represent SD. Statistical significance of survival data were calculated with a log-rank (Mantel–Cox) test. The statistical significance of viral load in flies was calculated by two-way ANOVA.

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