Evidence For Long-Lasting Transgenerational Antiviral Immunity in Insects

Graphical Abstract

Highlights

- *Drosophila* and mosquitoes transmit antiviral immunological memory to their progeny

- Protection is virus and sequence specific and lasts throughout generations

- Progeny inherit a viral DNA form that is a partial copy of the RNA virus genome

- Progeny display enriched expression of genes related to chromatin and DNA binding

Authors

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In Brief

Mondotte et al. determine that antiviral transgenerational immune priming in *Drosophila* and mosquitoes occurs after parental priming with different single-stranded RNA viruses. The progeny are protected from infection with the same virus for several generations.

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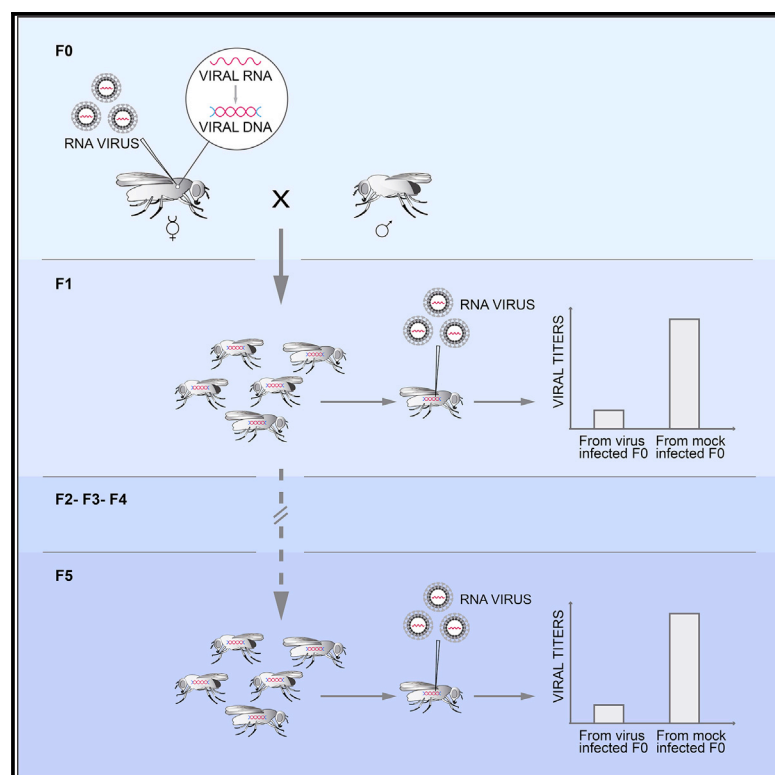# Evidence For Long-Lasting Transgenerational Antiviral Immunity in Insects

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https://doi.org/10.1016/j.celrep.2020.108506

SUMMARY

Transgenerational immune priming (TGIP) allows memory-like immune responses to be transmitted from parents to offspring in many invertebrates. Despite increasing evidence for TGIP in insects, the mechanisms involved in the transfer of information remain largely unknown. Here, we show that *Drosophila melanogaster* and *Aedes aegypti* transmit antiviral immunological memory to their progeny that lasts throughout generations. We observe that TGIP, which is virus and sequence specific but RNAi independent, is initiated by a single exposure to disparate RNA viruses and also by inoculation of a fragment of viral double-stranded RNA. The progeny, which inherit a viral DNA that is only a fragment of the viral RNA used to infect the parents, display enriched expression of genes related to chromatin and DNA binding. These findings represent a demonstration of TGIP for RNA viruses in invertebrates, broadly increasing our understanding of the immune response, host genome plasticity, and antiviral memory of the germline.

INTRODUCTION

The immune response has traditionally been divided into the innate and adaptive arms. Adaptive immunity, which is based on lymphocytes and antibodies, is specific, has memory, and is generally restricted to jawed vertebrates. Given that insects lack antibodies—the carriers of immunological memory in vertebrates—they are thought to be deprived of adaptive immunity. They are thus considered to rely only on non-pathogen-specific innate defense mechanisms, to lack immunological memory, and to be incapable of transgenerational transfer of immune factors (Hoffmann et al., 1999; Janeway and Medzhitov, 2002). Over the past years, however, this view has been increasingly challenged. In a seminal paper from Kurtz and Franz (2003), it was revealed that the invertebrate copepod *Macrocyclops albidus* develops memory against infection by a natural parasite, the tapeworm *Schistocephalus solidus*. Likewise, a growing number of studies across a vast range of invertebrates support the notion that invertebrates that have previously encountered a parasite appear to exhibit increased protection upon secondary exposure through a phenomenon known as "immune priming" (IP) (Contreras-Garduño et al., 2016; Little and Kraaijeveld, 2004; Milutinović and Kurtz, 2016; Sadd et al., 2005; Tetreau et al., 2019). IP has also been documented against viruses; shrimps inoculated with the envelope protein of white spot syndrome virus (WSSV), a double-stranded DNA virus that infects the shrimp *Penaeus monodon*, display increased survival upon subsequent exposure to the same virus (Witteveldt et al., 2004). Similarly, using *Plodia interpuctella* (Lepidoptera) and its natural

DNA virus, *Plodia interpunctella* granulosis virus (*PiGV*), it has been shown that individuals previously exposed to the virus are less susceptible to viral challenges (Tidbury et al., 2011). In this context, we have previously shown that adult *Drosophila melanogaster* flies emerging from larvae primed with *Drosophila* C virus (DCV) display increased tolerance to a subsequent challenge with the same virus compared to flies emerging from unprimed larvae, in which the DCV challenge is lethal. In this case, IP was found to be RNA interference (RNAi) dependent and virus specific (Mondotte et al., 2018).

The immune experience of insects exposed to pathogens has also been demonstrated to increase resistance to infection in their progeny, leading to enhanced immunocompetence and increased survival during infection in the offspring, a phenomenon known as transgenerational IP (TGIP) (Moret, 2006). A pioneering study showed increased survival of the progeny of the greater wax moth, *Galleria mellanoella*, upon bacterial challenge when the parents were exposed to bacteria (Ishimori and Metalnikov, 1924). More recently, antibacterial defense in bumble bee offspring was observed (Moret and Schmid-Hempel, 2001) followed by work of Little et al. (2003) that documented TGIP-mediated resistance to bacterial infection in the offspring of the crustacean *Daphnia magna*. This general pattern of enhanced immunity or increased survival upon pathogen challenge in offspring following maternal pathogen exposure has been described in a wide range of host taxa including coleopteran, crustacean, hymenopteran, orthopteran, and mollusk species (for a comprehensive review, see Tetreau et al., 2019).
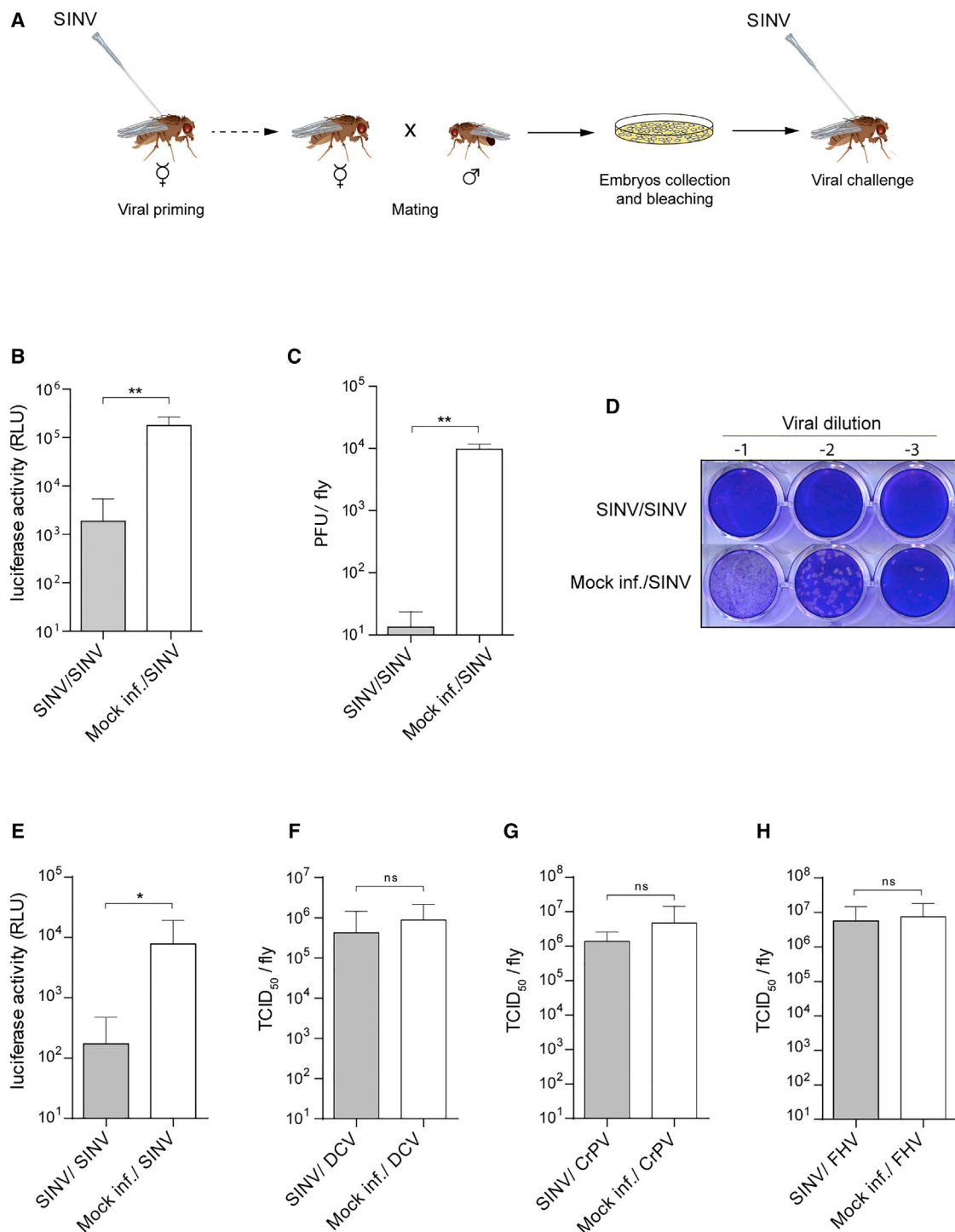
Figure 1. *Drosophila melanogaster* Flies Primed with SINV Transmit Antiviral Immunity to Their Progeny

(A) Schematic of the protocol. w^{1118} virgin female flies were infected with 100 PFU of SINV or mock infected and then crossed with non-infected males 2 days after priming. The F1 embryos were collected and treated with 50% bleach to eliminate the virus coming from the mother that could be present on the surface of the embryo. The F1 adult flies were recovered 3 to 4 days after emergence and challenged with 3,000 PFU of SINV-R.

(B–D) Viral replication in F1 flies produced by primed (SINV/SINV) or unprimed (Mock inf/SINV) mothers was quantified 2 days after infection as Renilla luciferase counts (B) and by plaque assays (C and D).

(E–H) F1 flies whose mothers were primed with 100 PFU of SINV or mock infected were challenged with a set of different viruses: 3,000 PFU of SINV-R (E), 5 $TCID_{50}$ of DCV (F), 5 $TCID_{50}$ of CrPV (G), or 5 $TCID_{50}$ of FHV (H).

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TGIP against viruses has also been demonstrated. In the shrimp *P. monodon*, the progeny of mothers exposed to glucans from *S. cerevisae* were protected from infection with WSSV (Huang and Song, 1999). Similarly, in the lepidopteran insect *P. interpuctella*, maternal exposure to *PiGV* led to protection of the offspring against infection with the same virus (Tidbury et al., 2011). In *Caenorhabditis elegans*, ectopic expression of flock house virus (FHV) results in the production of virus-derived small RNAs (vsRNAs). These vsRNAs are transgenerationally transmitted and silence the expression of the viral genome in subsequent generations (Rechavi et al., 2011). These responses, transmitted across generations via sRNAs and associated with histone modifications of the endogenous targeted genes, can lead to drastic transgenerational epigenetic effects by controlling the overall potency and duration of sRNA inheritance (Lev et al., 2017, 2019). Using the negative-sense single-stranded RNA virus, vesicular stomatitis virus (VSV), it was demonstrated that the antiviral RNAi response inhibits vertical VSV transmission in *C. elegans* while promoting transgenerational inheritance of antiviral immunity (Gammon et al., 2017). Similarly, a heritable RNAi response was demonstrated to be an important antiviral mechanism for conveying resistance to Orsay virus in the offspring of parents exposed to the virus (Sterken et al., 2014). Of note, it was demonstrated in a different study that the antiviral RNAi response to Orsay virus does not spread systemically throughout the organism and cannot be passed between generations (Ashe et al., 2015), highlighting the controversy as to the role(s) that the RNAi pathway plays in TGIP.

The mechanisms involved in TGIP remain partially uncharacterized. TGIP appears to be mediated through factors inside the eggs in bumblebees (Sadd and Schmid-Hempel, 2007), and lepidoptera exposed to bacteria have been shown to transfer fragments of pathogens to their eggs (Freitak et al., 2014). Likewise, in honeybees, the egg-yolk precursor protein, vitellogenin, acts as a carrier for fragments of bacteria into eggs (Salmela et al., 2015). Gene expression studies have shown that TGIP may increase basal expression levels of immune effectors in the offspring (Barribeau et al., 2016) and may induce differential expression of immunity-related genes (Tate et al., 2017; Trauer-Kizilelma and Hilker, 2015). Furthermore, changes in histone acetylation and DNA methylation have been observed in the F1 generation of the tobacco hornworm (*Manduca sexta*) when the parents were infected with *Escherichia coli* or *Serratia entomophila* (Gegner et al., 2019). The nematode, *C. elegans*, has been shown to learn to avoid pathogenic bacteria, and this behavior is transmitted to their progeny through transforming growth factor β signaling in sensory neurons and the Piwi-Argonaute sRNA pathway (Moore et al., 2019).

Here, we explore the occurrence of TGIP in the fruit fly, *D. melanogaster*, following infection with different RNA viruses. We demonstrate the existence of antiviral TGIP across many generations after a single viral encounter in the F0 generation. This TGIP is RNA dependent and sequence specific but RNAi in-

dependent, and it can also be triggered by viral double-stranded RNA (dsRNA). The progeny of primed flies display enriched expression of genes related to chromatin and DNA binding. We also found that TGIP occurs in *Aedes aegypti* mosquitoes infected with Chikungunya virus (CHIKV), highlighting that TGIP could be a pervasive antiviral mechanism among insects.

RESULTS

Maternally Transmitted Antiviral TGIP in *Drosophila melanogaster*

To test the hypothesis of the occurrence of antiviral TGIP in insects, we used a reporter system based on Sindbis virus (SINV) expressing Renilla luciferase (SINV-R). SINV constitutes an ideal model to test TGIP, as it produces persistent infection in *D. melanogaster* with no fitness costs for the host despite high viral titers (Figures S1A and S1B), and is not vertically transmitted to the progeny (Figures S1C–S1F).

The protocol used to detect viral TGIP is described in Figure 1A. Briefly, virgin female flies were primed with 100 plaque-forming units (PFU) of SINV and crossed with non-infected males 2 days later. The F1 embryos were collected and treated with 50% bleach. This treatment eliminates all infectious viruses on the surface of the embryo's chorion and is routinely used in the laboratory to eliminate persistent viral infections from fly stocks. The F1 adult flies were recovered 3 to 4 days after emergence and challenged with 3,000 PFU of SINV-R. Viral replication was monitored 48 h later as Renilla counts. For ease of understanding, the priming received by the F0 (mother fly) is indicated before the backslash, and the challenge received by the progeny is indicated after the backslash. For example, SINV/SINV-R denotes an F0 primed with SINV and an F1 challenged with SINV-R.

F1 progeny from SINV-primed mothers (SINV/SINV-R) showed a significant decrease in luciferase activity (100-fold-lower Renilla luciferase counts) compared to the mock-primed control (Mock inf/SINV-R) (Figure 1B). To rule out the possibility that Renilla luciferase counts do not reflect viral replication due to potential loss of the Renilla gene insert in the recombinant SINV-R, viral replication was followed by measuring PFU/fly. Using this method, a 10,000-fold decrease in viral replication in the SINV/SINV-R flies was also observed when compared to Mock inf/SINV-R control flies (Figures 1C and 1D).

We then sought to test whether viral TGIP was virus specific, or if priming by one virus could protect the F1 against challenge with another unrelated virus. To do so, we challenged the F1 from mothers primed with SINV with SINV-R, DCV, cricket paralysis virus (CrPV), or FHV (Figures 1E–1H). Only the F1 progeny from SINV-primed mothers (SINV/SINV-R) showed a significant decrease in viral replication compared to the control (Mock inf/SINV-R), indicating that TGIP is virus specific.

To address whether males could also transmit TGIP, we applied the same protocol depicted in Figure 1A, but we primed adult males (instead of females) before crossing with uninfected

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For (A), two independent experiments with three biological replicates consisting of n = 3 pools of three flies each per condition were analyzed. For (B), three independent experiments with three biological replicates consisting of n = 3 pools of three flies each per condition were analyzed. For (E)–(H), two independent experiments with at least three biological replicates of n = 3 pools of three flies each per condition were analyzed. Asterisks indicate statistical significance of pairwise comparisons by Mann-Whitney test (**p ≤ 0.01). The absence of asterisks indicates lack of statistical significance (p > 0.05). Error bars indicate SEM.
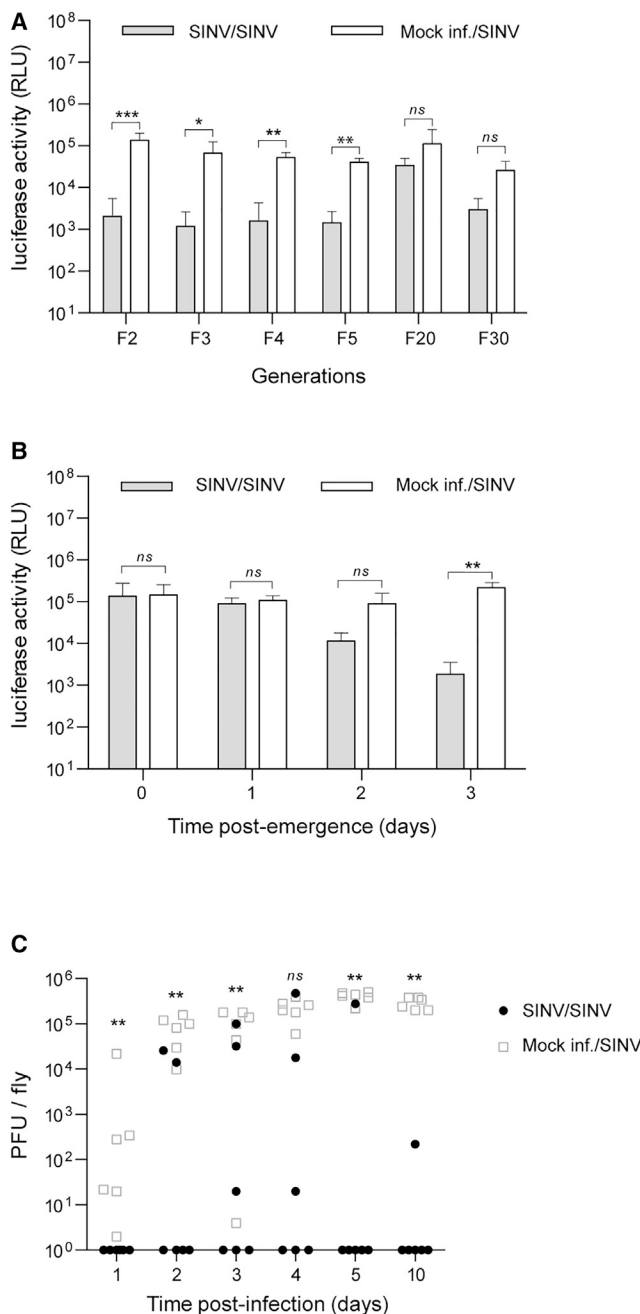
Figure 2. Inherited Antiviral Immunity Persists Across Generations and Is Long Lasting

(A) To obtain the F2 and the subsequent generations, F1 females (produced by SINV-primed F0 mothers) were crossed with their male siblings. One half of the F2 flies were used for virus challenge experiments and viral titer quantification. The other half was used to obtain the F3 and so on. Antiviral immunity was inherited for 5 generations (F5).

(B) Virgin flies of different ages (0, 1, 2, or 3 days after emergence) were primed with SINV and treated with the TGIP protocol depicted in Figure 1A. F0 mothers transmit antiviral immunity to their progeny when they are primed at 3 days old, suggesting that the maturity of the F0 immune response is relevant for TGIP.

(C) F1 flies produced by primed or unprimed mothers were challenged with SINV (SINV/SINV and Mock inf/SINV, respectively), and viral load was measured at different time points (1, 2, 3, 4, 5, and 10 dpi).

virgin females. Out of four independent experiments, we observed that the F1 progeny from SINV-primed fathers (SINV/SINV-R) showed a decrease in luciferase activity with respect to the control (Mock inf/SINV-R) in only two experiments, with no difference observed between primed or mock-primed fathers in the other two experiments (Figures S2A–S2D). We conclude that in contrast to maternal transmission of TGIP, antiviral immunity is not consistently transmitted by male flies.

TGIP Persists Across Generations and Is Long-Lasting

Next, we tested over how many generations the antiviral TGIP lasted. To do so, we collected F1 virgin females (from F0 primed mothers) and crossed them with sibling F1 males to obtain the F2. Some F2 individuals were retained for virus infection experiments, and the remainder were used to obtain the F3. This process was repeated for 30 generations. We observed significant protection against virus challenge until the F5 generation. Beyond F5, we observed a decrease in protection with each subsequent generation, and no significant protection was observed in the F20 through F30 generations (Figure 2A).

To determine if the age of the mother had an effect on TGIP, F0 virgin flies were primed at different times (0, 1, 2, or 3 days) after fly emergence and treated as described in Figure 1A. The progeny of flies from young primed mothers (F0 primed 0, 1, or 2 days post-emergence) were not protected, but F1 flies produced by F0 flies that had been primed 3 days post-emergence were protected (Figure 2B). The fact that protection becomes more efficient as the mother flies grow older indicates that there is a factor that requires maturation in order for mother flies to transmit antiviral IP to the progeny.

We also tested how long TGIP lasts during the lifespan of the progeny. We challenged F1 flies produced by primed or not-primed mothers and measured PFU/fly at different time points (1, 2, 3, 4, 5, and 10 days post-challenge) (Figure 2C). We observed that protection is long-lasting, with flies being protected for as long as 10 days post-challenge. Of note, although protection was always present, an important variability in the degree of protection among individual flies was apparent.

TGIP Is Widespread Among Positive-Sense Single-Stranded RNA Viruses

To test whether the transmission of antiviral memory was an inherent feature of SINV or a more general phenomenon, we applied the same protocol (Figure 1A) to a set of different viruses, none of which are vertically transmitted. When the F1 progeny produced by virgin females primed with DCV, CrPV, or FHV were challenged with the same virus that had been used to prime their mother, an inhibition in viral replication was observed (Figures 3A–3C). All of these viruses have a positive-sense single-stranded RNA genome and belong to different families with
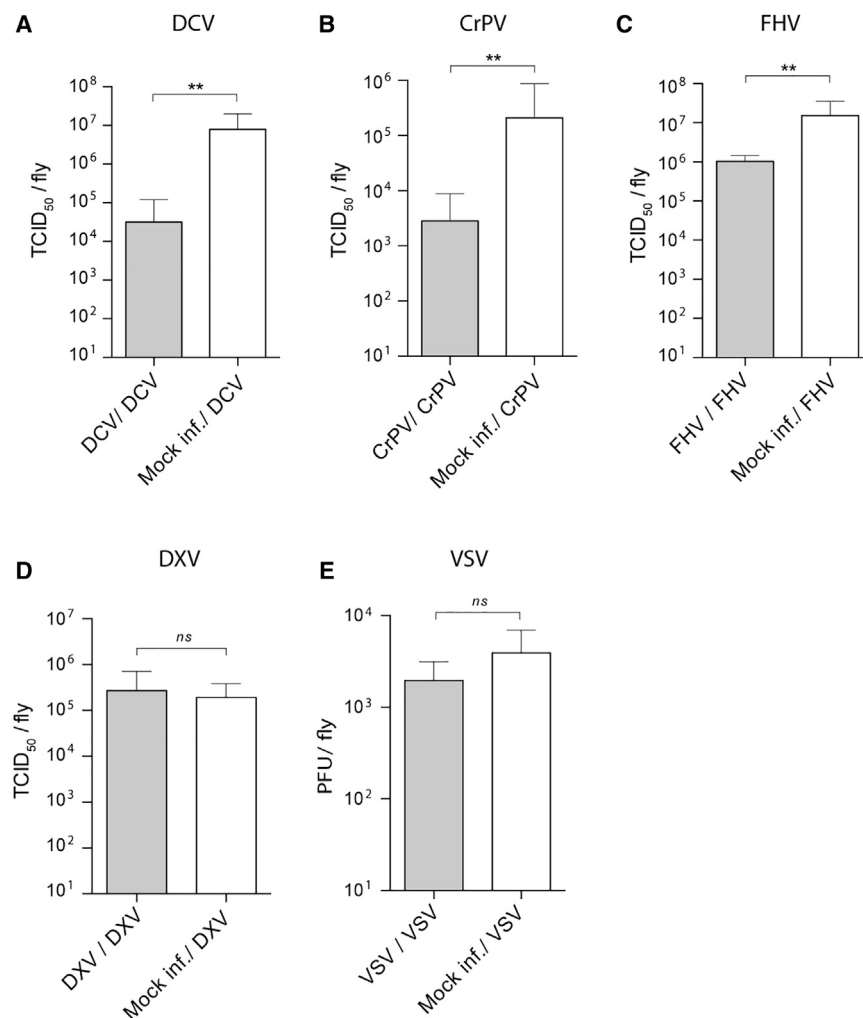
Figure 3. Antiviral Immunity Is Inherited Following Priming With Different Positive-Sense Single-Stranded RNA Viruses

w^{1118} virgin female flies were primed or mock infected with different RNA viruses. The F1 embryos were collected and treated with 50% bleach to eliminate the viruses coming from the mother that could be present on the surface of the embryo.

(A) F0 flies were primed with 1 $TCID_{50}$/fly or mock infected, and the F1 flies were challenged with 5 $TCID_{50}$/fly (DCV/DCV and Mock inf/DCV, respectively). Three independent experiments with three biological replicates of n = 3 pools of three flies each per condition were analyzed.

(B) F0 flies were primed with 1 $TCID_{50}$/fly or mock infected, and the F1 flies were challenged with 5 $TCID_{50}$/fly (CrPV/CrPV and Mock inf/CrPV, respectively). Four independent experiments with three biological replicates of n = 3 pools of three flies each per condition were analyzed.

(C) F0 flies were primed with 1 $TCID_{50}$/fly or mock infected, and the F1 flies were challenged with 5 $TCID_{50}$/fly (FHV/FHV and Mock inf/FHV, respectively). Two independent experiments with three biological replicates of n = 3 pools of three flies each per condition were analyzed.

(D) F0 flies were primed with 200 PFU/fly or mock infected, and the F1 flies were challenged with 200 PFU/fly (DXV/DXV and Mock inf/DXV, respectively). Three independent experiments with three biological replicates of n = 3 pools of three flies each per condition were analyzed.

(E) F0 flies were primed with 100 PFU/fly or mock infected, and the F1 flies were challenged with 100 PFU /fly (VSV/VSV and Mock inf/VSV, respectively). Three independent experiments with three biological replicates of n = 3 pools of three flies each per condition were analyzed.

Asterisks indicate statistical significance of pairwise comparisons by Mann-Whitney test (**p $\leq$ 0.01). The absence of an asterisk indicates lack of statistical significance (p > 0.05). Error bars indicate SEM.

different replication strategies. While DCV and FHV can produce acute or persistent infections in *D. melanogaster*, CrPV infection is always acute. We also tested the presence of TGIP using a dsRNA virus (*Drosophila* X virus [DXV]) and a negative-sense stranded RNA virus (VSV), but in both cases, protection was not observed (Figures 3D and 3E). These results indicate that the phenomenon of TGIP is widespread among positive-sense single-stranded RNA viruses.

TGIP Reduces Viral Loads and Increases Survival in the Progeny

We previously showed that larval exposure to DCV protects adult flies against subsequent reinfection, a concept known as IP (Mondotte et al., 2018). When adult flies derived from larvae orally infected with DCV were challenged with DCV by injection, viral loads were not significantly different than viral loads in DCV-challenged flies derived from uninfected larvae. However, the DCV-challenged flies derived from DCV-infected larvae exhibited increased survival compared to DCV-challenged flies derived from uninfected larvae, suggesting a change in tolerance. To explore if

TGIP has an effect on survival in addition to the observed effect on viral titers (Figures 1B, 1C, and 3A–3C), we performed the TGIP protocol using DCV and CrPV. We measured viral loads and survival for the F1 and observed that viral loads were significantly reduced in the F1 progeny of primed mothers compared to the F1 progeny of unprimed mothers (Figures S3A and S3C). We observed significantly increased survival of CrPV-challenged F1 flies from primed mothers compared to F1 flies from unprimed mothers. While the survival of DCV-challenged F1 flies was not significantly different for flies produced by primed or unprimed mothers, we observed that at least 5% of the DCV-challenged flies from DCV primed mothers were alive at 15 days post-infection (dpi) (Figures S3B and S3D). The results indicate that a resistance mechanism, defined as a limitation of the pathogen burden, is engaged by TGIP to confer increased protection to the progeny.

TGIP in Mosquitoes

To explore whether TGIP is a widespread phenomenon among insects, we next infected female *Aedes aegypti* mosquitoes with CHIKV, a positive-sense single-stranded RNA arbovirus
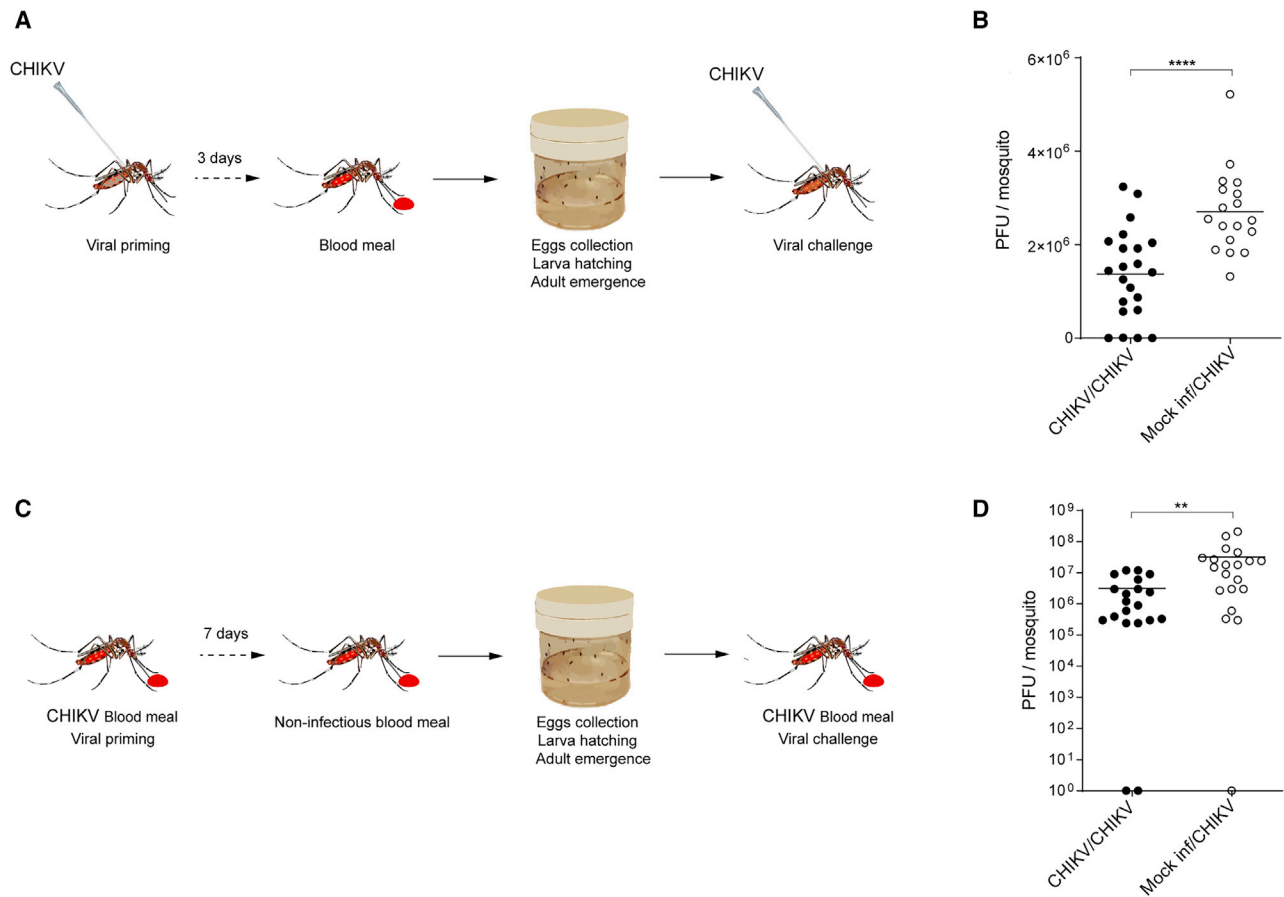
Figure 4. *Aedes aegypti* **Mosquitoes Infected With CHIKV Transmit Antiviral Protection to Their Progeny**

(A) Schematic of the protocol. Female *Aedes aegypti* mosquitoes were infected with 100 PFU of CHIKV or mock infected by injection. Three days later, mosquitoes were fed with a non-infectious blood meal to provide the nutrients for egg production. Eggs were hatched and F1 females were challenged with 100 PFU of CHIKV between 2 and 5 days after mosquito emergence.

(B) Viral load was measured for individual mosquitoes by plaque assays 2 days after the viral challenge for mosquitoes produced by primed F0 mosquitoes (CHIKV/CHIKV) or by mock-infected F0 mosquitoes (Mock inf/CHIKV).

(C) Schematic of the protocol. Female *Aedes aegypti* mosquitoes were infected with a blood meal containing 10^6 PFU/mL of CHIKV or carrier buffer. Seven days after the infection, the mosquitoes were fed with a non-infectious blood meal to provide the nutrients for egg production. Eggs were hatched and F1 females were challenged with a blood meal containing 10^6 PFU/mL of CHIKV.

(D) Viral load was measured for individual mosquitoes by plaque assays 3 days after the viral challenge for mosquitoes produced by primed F0 mosquitoes (CHIKV/CHIKV) or by mock-infected F0 mosquitoes (Mock inf/CHIKV).

For (B) and (D), filled black circles (CHIKV/CHIKV) and open black circles (Mock inf/CHIKV) represent individual mosquitoes. Between 18 and 22 mosquitoes per condition were analyzed. Asterisks indicate statistical significance of pairwise comparisons by Mann-Whitney test (**p $\leq$ 0.01, ****p $\leq$ 0.0001). Bars indicate the mean.

with a recent history of outbreaks (Levi and Vignuzzi, 2019). Briefly, adult female mosquitoes were intrathoracically injected with CHIKV, and 3 days later, the injected mosquitoes were fed with a non-infectious blood meal to provide the nutrients for egg production from stored sperm (Figure 4A). The experiment was carried out under conditions in which vertical transmission for CHIKV was undetectable (Mourya, 1987; Vazeille et al., 2009). Eggs were hatched and F1 females were challenged by injection of CHIKV after mosquito emergence. Viral load was measured in individual F1 mosquitoes by plaque assays 2 days after the viral challenge. A decrease in viral titer in mosquitoes

from primed mothers (CHIKV/CHIKV) was observed when compared to the control (Mock inf/CHIKV) (Figure 4B).

To test the presence of TGIP in mosquitoes under more natural conditions using the oral route of infection, we primed F0 female *Ae. aegypti* mosquitoes with an infectious blood meal containing CHIKV and subsequently challenged the F1 progeny via an infectious blood meal containing CHIKV (Figure 4C). Viral titers in the F1 mosquitoes were measured by plaque assays 3 days after the viral challenge. We found that F1 mosquitoes produced by primed mothers (CHIKV/CHIKV) showed decreased viral load when compared to the controls (Mock inf/CHIKV) (Figure 4D).
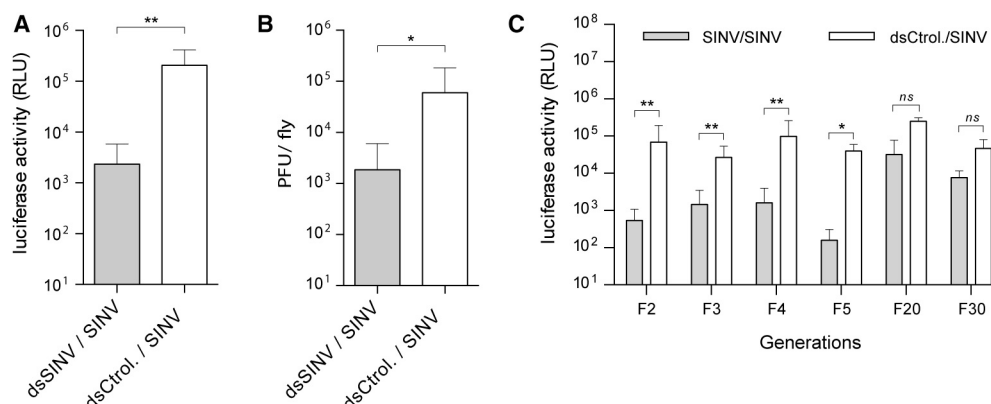
Figure 5. TG Antiviral Immunity Is Viral RNA Dependent and Sequence Specific
(A and B) w^{1118} virgin female flies were primed with 700 bp dsRNAs corresponding to two different regions of the SINV virus genome (dsSINV) or with control dsRNAs (dsCtrl). Flies were crossed with non-treated males, and the F1 flies were challenged with 3,000 PFU of SINV-R (dsSINV/SINV and dsCtrl/SINV, respectively). Viral replication was estimated at 2 dpi by measuring Renilla luciferase activity (A) or by plaque assays (B).
(C) F1 females (produced by F0 dsSINV-primed flies) were crossed with their male siblings in order to obtain the F2 generation. One half of the F2 generation was used for virus challenge experiments and viral titer quantification. The other half was used to obtain the F3 and the following generations. Antiviral immunity was inherited by the first 5 generations (F5). See also Figure S3.
Two independent experiments with three biological replicates of n = 3 pools of three flies each per condition were analyzed. Asterisks indicate statistical significance of pairwise comparisons by Mann-Whitney test (*p ≤ 0.05, **p ≤ 0.01). The absence of an asterisk indicates lack of statistical significance (p > 0.05). Error bars indicate SEM.

Altogether, these results indicate that TGIP is not a unique characteristic of *Drosophila* but is a general inherited antiviral mechanism among insects.

TGIP Is Viral RNA Dependent but RNAi Independent

Given that a strong TGIP effect was observed when both flies and mosquitoes were primed with replicative viruses, we wondered if the protection detected in the progeny needed the intact virus or could be recapitulated by priming flies with dsRNAs, mimics of viral replication intermediates. Indeed, we have previously observed that inoculation of naked dsRNA corresponding to regions of SINV efficiently protects the same fly against SINV infection (Saleh et al., 2009). Following this reasoning, we inoculated virgin female flies with 700 bp dsRNAs corresponding to two different regions of SINV (dsSINV) or with control dsRNAs (dsCtrl). These flies were crossed with non-treated males, and the F1 progeny were challenged with 3,000 PFU of SINV-R. Viral replication was estimated at 2 dpi by measuring Renilla luciferase activity (Figure 5A) or by plaque assays (Figure 5B). In both cases, we observed a decrease in viral replication in the F1 progeny produced by mothers primed with dsRNAs corresponding to SINV (dsSINV/SINV) compared to control flies (dsCtrl/SINV).

To determine how many generations this protection initiated by dsRNA lasts, we collected F1 virgin female flies (produced by the F0 primed mothers) and crossed them with their male siblings to obtain the F2. A portion of the virgin female flies were challenged with SINV, while another portion was crossed to obtain the F3 and so on. Statistically significant protection against a viral challenge was observed up to the F5 generation (Figure 5C). A degree of protection was found to persist beyond the F5 generation, but the trend was not statistically significant,

mostly due to the variation in the degree of protection among individual flies.

Given that TGIP is viral RNA dependent, we wondered if the RNAi pathway, the main antiviral system in insects, could be involved in TGIP. We thus primed $Dcr-2^{-/-}$ mutant (null protein; Lee et al., 2004) and w^{1118} virgin female flies with 100 PFU of SINV and crossed them with uninfected males 2 days later. The resulting F1 flies were challenged with 3,000 PFU of SINV-R. The F1 progeny produced by $Dcr-2^{-/-}$ mutant females displayed a similar amount of protection as the F1 progeny produced by w^{1118} females when compared to mock-primed controls (Mock inf/SINV) (Figure S4A). We also tested for the presence of mono- and tri-phosphate viral small interfering RNAs (siRNAs) in unchallenged F1 flies produced by SINV-primed females, but they were undetectable (Figure S4B). Collectively, these results reveal that TGIP is viral RNA dependent and sequence specific but is not mediated by the RNAi pathway.

A vDNA Form is Transmitted From Infected Flies to the Progeny

To uncover the mechanism that facilitates TGIP, we next sought to determine if viral DNA (vDNA) is present in the F1 progeny produced by primed mothers. We and others have already shown that a vDNA form is necessary to establish a persistent infection in flies and mosquitoes (Goic et al., 2013, 2016; Nag et al., 2016; Tassetto et al., 2017). Moreover, a vDNA form is retained in adult flies following their maturation from virus-infected larvae, and these adults are protected from lethality during reinfection with the same virus (Mondotte et al., 2018). Virgin w^{1118} female flies were primed with 100 PFU of SINV and crossed 2 days later with uninfected males. The F1 embryos were collected and bleached. The F1 adult flies were collected after emergence
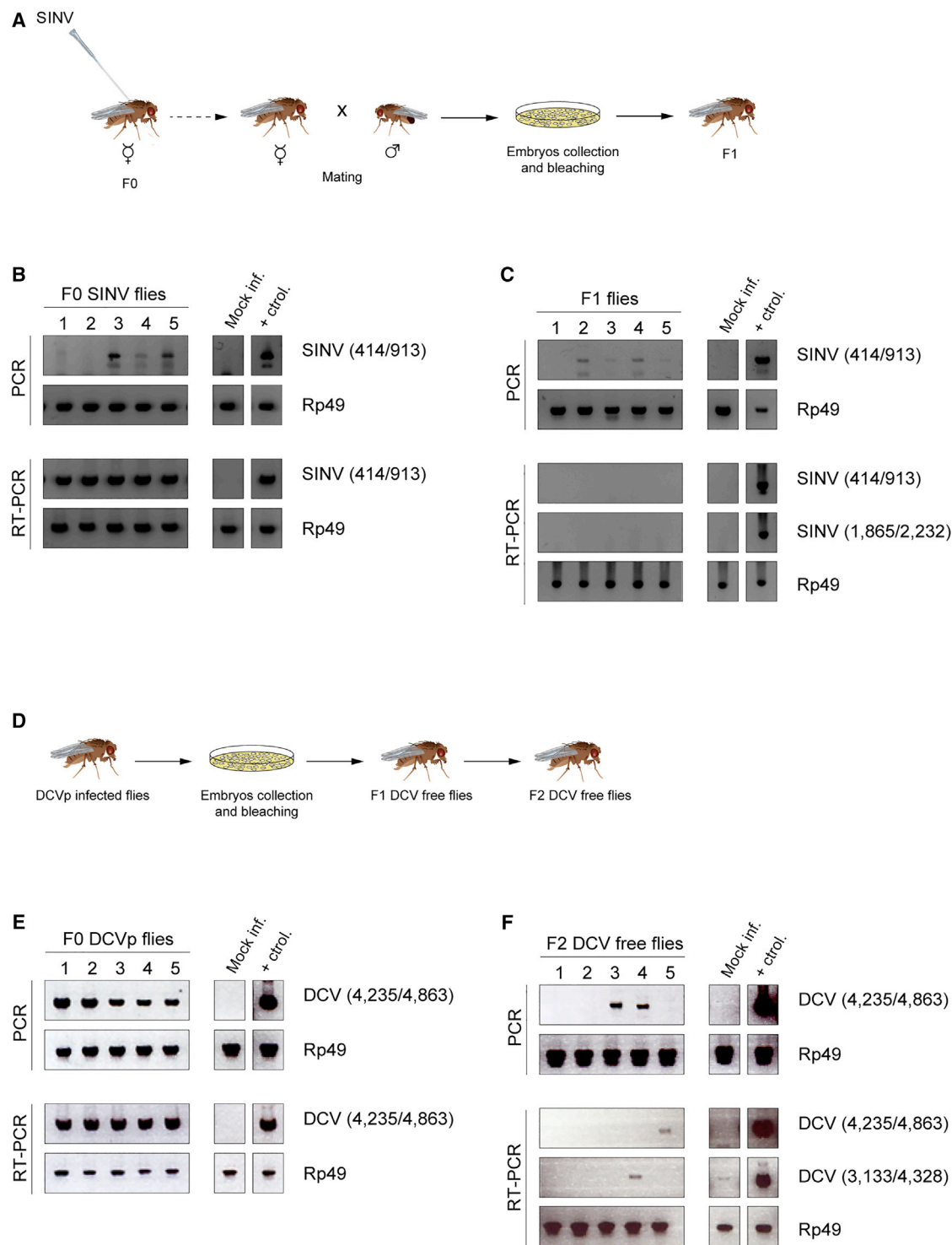
Figure 6. A vDNA Form Is Transmitted From Infected Flies to Their Progeny

(A) Schematic of the protocol. w^{1118} virgin female flies were primed with 100 PFU of SINV or mock infected and crossed with non-infected males 2 days later. The F1 embryos were collected and treated with 50% bleach to eliminate the viruses coming from the mother that could be present on the surface of the embryo.
(B and C) SINV-infected flies (B) and the F1 flies (C) were recovered, and vDNA (top) or viral RNA (bottom) was amplified by PCR or RT-PCR, respectively, from individual flies.

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from these embryos (Figure 6A) and checked for the presence of virus (by RT-PCR) and vDNA (by PCR). A vDNA was detected in infected F0 flies (Figures 6B, top panel, and S5) in the presence of viral replication (Figures 6B, bottom panel, and S5). The vDNA was also detected in almost all F1 flies tested (Figures 6C, top panel, and S6). Viral RNA, however, was not detected in any of the F1 flies (Figures 6C, bottom panel, and S6).

To determine whether vDNA transmission occurs during a natural infection, we took advantage of a fly stock persistently infected with DCV. We collected and bleached embryos from this fly stock to obtain virus-free F1 and F2 generations (Figure 6D). The vDNA was detected in all of the persistently infected F0 flies (Figures 6E, top panel, and S7) in the presence of viral replication (Figures 6E, bottom panel, and S7). The vDNA was also observed in 30% of the F2 flies analyzed (Figures 6F, top panel, and S8) in the absence of viral replication (Figures 6F, bottom panel, and S8). Together, these results show that vDNA is TG transmitted during a natural infection as well as following virus inoculation by injection, thus supporting the notion that this vDNA might be involved in TGIP.

Chromatin and DNA Binding Genes Expression Is Upregulated in Immune-Primed Flies

To dive deeper into a possible mechanism to explain TGIP, we used RNA sequencing (RNA-seq) to delineate the potential roles of some genes in TGIP. This was performed on the F2 generation obtained from SINV-infected or mock-infected F0 female flies. We chose the F2 to be sure that the results were specifically related to the TGIP phenomenon. The F2 flies were challenged with SINV or mock infected prior to RNA-seq. Principal-component analysis (PCA) of the RNA-seq profiles shows that a first component explained 41.7% of the variance separating transcripts of infected from uninfected F2 flies, regardless of whether the F2 flies were produced by primed or unprimed F0 flies. A second component representing 20.6% of the variance distinguished the transcripts of F2 flies produced by primed F0 flies (red circles) from the transcripts of F2 flies produced by unprimed F0 flies (blue circles) (Figure 7A). To avoid the effect of different levels of infection in the transcripts of F2 flies produced by primed (red circles) or unprimed (blue circles) F0 flies, only the transcripts from uninfected (unchallenged) F2 flies were further analyzed. Figure 7B shows that a first component explaining 50.7% of the variance is sufficient to differentiate the uninfected F2 progeny produced by primed (red circles) or unprimed (blue circles) F0 flies, indicating that transcripts unrelated to the infection per se are differentially expressed between the two groups of flies (Tables S1 and S2).

To better understand the nature of the transcripts that are upregulated in the SINV/Mock F2 flies compared to the Mock/Mock F2 flies, we performed a Gene Ontology enrichment analysis. Most of the upregulated transcripts were enriched in the molecular term "chromatin DNA binding" (Figure 7C; Table S1). The

Kyoto encyclopedia of gene and genomes (KEGG) enrichment analysis confirmed that the overexpressed transcripts mostly belong to the DNA replication, nucleotide excision repair, and mismatch repair pathways, with 87 hits corresponding to chromosome and associated proteins and 44 hits corresponding to DNA repair and recombination proteins (Table S3).

Some of these proteins have previously been shown to function in innate immune signaling in *Drosophila*. This is the case for the DNA methyltransferase-1 associated protein 1 that has been proposed to act at the level of chromatin remodeling during microbial infection (Goto et al., 2014). Brahma (Dmel_CG4303 Bap60), a member of the switching defective (SWI)/sucrose nonfermenting (SNF) complex, is an evolutionarily conserved chromatin remodeling complex originally identified in yeast that uses the energy from ATP hydrolysis to modulate chromatin structure, and it has been shown to modulate innate immunity genes (Valanne et al., 2020). We also identified Nup98. This nucleoporin is known for its role in nuclear-cytoplasmic transport, but it displays a strong antiviral function in *Drosophila* (independently of the nuclear pore function) by restricting SINV and DCV infection via the regulation of a subset of virus-induced antiviral genes (Panda et al., 2014). Altogether, the analysis of upregulated transcripts indicates an interaction between TGIP and pathways of DNA alteration and/or modification.

DISCUSSION

In this study, we discovered the existence of viral TGIP in insects after parental priming with several different RNA viruses. The progeny are protected from an infection with the same virus for several generations in a long-lasting manner. We observed TGIP in response to parental priming with four different positive-sense single-strand RNA viruses and in two different model insects: *D. melanogaster* and *Ae. aegypti*. These results clearly indicate that viral TGIP may be a general antiviral mechanism in insects.

We detected viral TGIP in *Ae. aegypti* mosquitoes that were infected by injection or by feeding on an infectious blood meal, which mimics an infection in the natural setting. This is relevant because the immune response of the host can vary greatly depending on the infection route used for the pathogen (Mondotte and Saleh, 2018). Furthermore, reports of TGIP in other invertebrate models suggest that protection might depend on the pathogen used for priming or on the procedure used for infection (Tetreau et al., 2019).

TGIP was recapitulated following parental priming with dsRNA corresponding to a portion of the SINV genome, and the degree of protection was similar to what was observed following parental priming with replicative SINV. This result implies that TGIP is viral RNA dependent and that dsRNA replication intermediates may be recognized to initiate the phenomenon. In contrast to observations in *C. elegans*, we showed that TGIP is RNAi independent. These results are consistent with recent studies

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(D) Schematic protocol. Embryos from *w1118* flies naturally harboring persistent DCV infection were treated with 50% bleach in order to produce DCV-free F1 and F2 generations.

(E and F) F0 flies persistently infected with DCV (E) and F2 flies (F) were recovered, and vDNA (top) or viral RNA (bottom) was amplified by PCR or RT-PCR, respectively, from individual flies. To confirm the absence of viral replication in the F2 flies, two different pairs of primers were used for RT-PCR. Rp49 was used as housekeeping control.
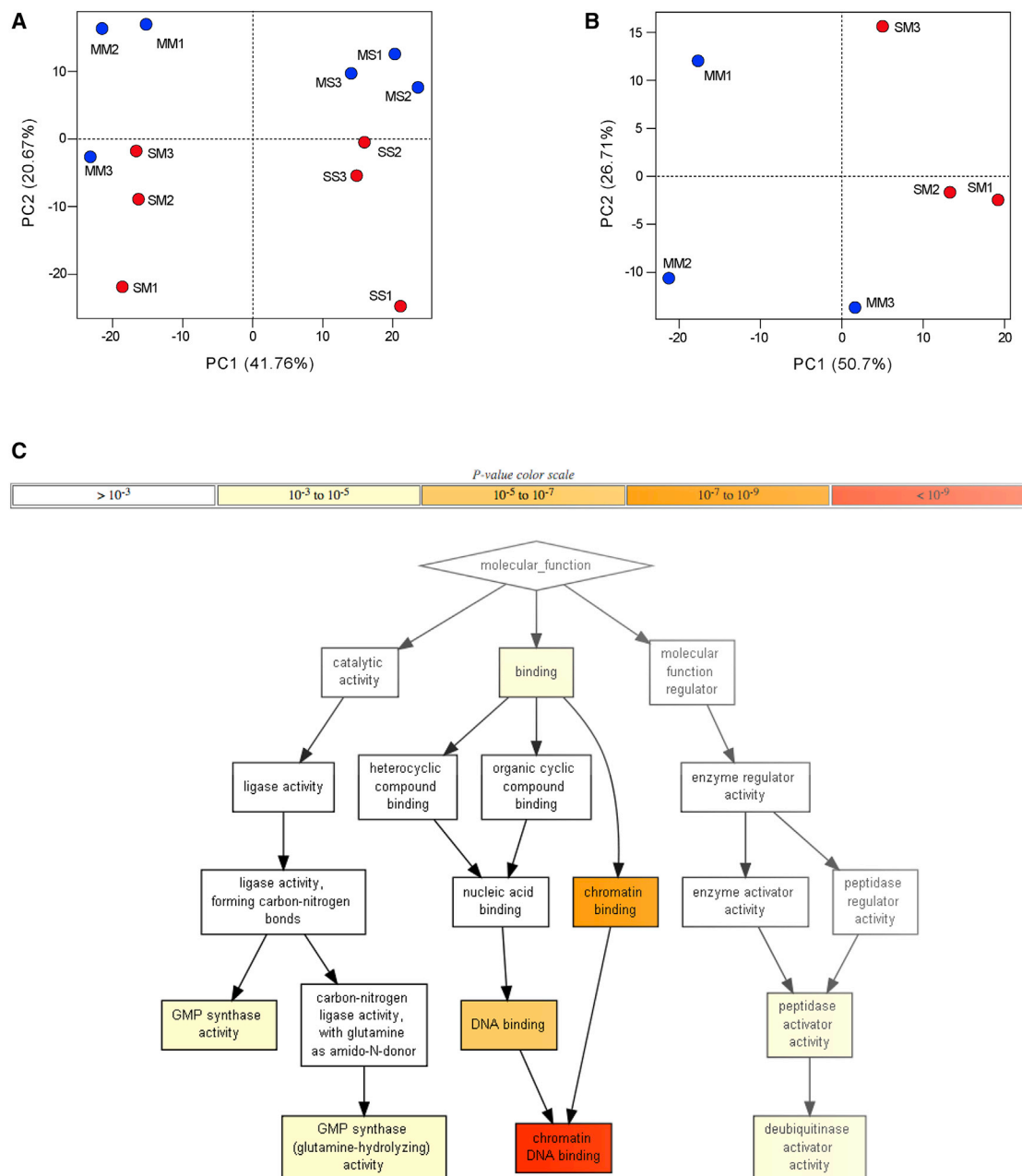
Figure 7. The Transcriptome of the F2 Progeny Produced by Primed F0 Parents Differs from the Transcriptome of the F2 Progeny Produced by Unprimed F0 Parents

F1 females (from SINV- or mock-infected F0 female flies) were crossed with their male siblings to obtain the F2 generation. The F2 flies were infected with SINV or mock infected. Two days later, RNA was extracted from three pools of four flies per condition to perform RNA-seq to produce a transcriptome for Gene Ontology enrichment analysis. Conditions: SM (F0 SINV/F2 mock infected), SS (F0 SINV/F2 SINV), MS (F0 mock infected/F2 SINV), MM (F0 mock infected/ F2 mock infected). In (A) and (B), the first two components of principal-component analysis (PCA) with percentages of variance associated with each axis are shown.

(A) The PC1 separates infected from non-infected flies regardless of the priming status of their F0 mothers, while the PC2 separates the F2 progeny of primed F0 mothers (red circles) from the F2 progeny of unprimed F0 mothers (blue circles).

(B) To avoid the effect of different levels of infection between the F2 flies produced by primed F0 mothers (red circles) and unprimed F0 mothers (blue circles), only the non-infected F2 flies were analyzed. The PC1 and PC2 are sufficient to differentiate the progeny produced by primed or non-primed F0 flies.

(C) Gene Ontology enrichment analysis of upregulated genes in the SM progeny compared to the MM progeny shows an enrichment in the expression of genes related to chromatin and DNA binding.

showing the existence of other mechanisms besides the siRNA pathway that control viral infection in flies and mosquitoes (Mondotte et al., 2018; Olmo et al., 2018). Although we did not detect the production of vsRNAs in unchallenged F1 flies produced by primed parents, it is possible that production of vsRNAs could be induced in the progeny in the presence of the virus. Such a mechanism would not be surprising because the costs associated with TGIP in the offspring have strong negative implications for their fitness (Dhinaut et al., 2018; Roth et al., 2010; Trauer and Hilker, 2013; Zanchi et al., 2012).

Following parental priming in the F0 generation, we observed significant protection against viral challenge until the F5 generation. This result was consistent regardless of whether the F0 flies were primed with a replicative virus or with viral dsRNAs. However, substantial inter-individual variability in the degree of protection was observed. A similar pattern of heritable silencing effects was observed in populations of isogenic worms where a fraction of individuals in each generation lost heritable silencing, and the RNAi silencing response drastically diminished after three to five generations (Vastenhouw et al., 2006).

Using RNA-seq, we compared the F2 generation obtained from primed or unprimed parental flies. We observed that gene expression profiles are sufficient to differentiate the ancestry of the F2 flies according to the priming status of the F0 parental flies. We observed that most of the upregulated transcripts in the progeny are enriched in the molecular term "chromatin DNA binding." This result suggests a possible relationship between viral TGIP and host DNA modifications, and we propose here, as seen in other organisms (Beemelmanns and Roth, 2017; Lev et al., 2017; Schulz et al., 2018), that chromatin modifications could be responsible for viral TGIP. Only a few studies have used unbiased transcriptomic approaches based on RNA-seq to study the potential roles of genes in TGIP by comparing primed and unprimed individuals. These studies found a metabolic shift in *Tribolium castaneum* primed with *Bacillus thuringiensis* (Tate et al., 2017), an upregulation of the antimicrobial peptides in bumblebee workers primed with heat-inactivated bacteria (Barribeau et al., 2016), and no changes in the transcriptomic profiles of larvae of the Pacific oyster primed with Ostreid herpesvirus 1 (Lafont et al., 2019). Future studies should adopt these global approaches to identify new candidates that could be specific to TGIP.

An essential requirement for successful TGIP is the TG transmission of information from the parents to their progeny. Several different potential TGIP mechanisms have been proposed: transfer of active immune components, transfer of pathogen-associated molecular patterns (PAMPs), transfer of sRNAs, and epigenetic modifications that might influence gene expression in the progeny (Roth et al., 2018; Tetreau et al., 2019). We found that vDNA is present in the uninfected progeny of primed flies. The inheritance of a vDNA form by the offspring represents a new potential TGIP mechanism. We have previously found that adult flies emerging from DCV-infected larvae harbored a vDNA that persisted even after viral clearance, and these flies were protected from future reinfections with the same virus (Mondotte et al., 2018). It was also previously described that generation of vDNA in response to virus infection in flies and mosquitoes is associated with retotransposons and that episomal vDNA can be present in a linear or circular form (Poirier et al., 2018). For this reason, it is tempting to speculate that this vDNA could be the agent involved in the transmission of TGIP and may protect the progeny flies from future reinfections. The vDNA could be passed across generations because it could be capable of being replicated and transmitted. This vDNA might be capable of being transcribed to actively inhibit viral replication, as was shown for adult flies and mosquitoes infected with FHV or CHIKV, respectively (Goic et al., 2016; Poirier et al., 2018). The existence of viral TGIP and vDNA in mosquitoes opens the possibility that natural mosquito populations harbor vDNA sequences from different arboviruses or insect-specific viruses. This vDNA could affect vector competence and could be related to seasonal outbreaks.

Several questions remain to be answered, including the reasons underlying inconsistent transmission of TGIP by males, the role of vDNA in the transmission of protection, the inheritance patterns of TGIP, how a fragment of viral dsRNA is able to initiate a TGIP response, and the downstream effects that might result from the use of dsRNA in biotechnological applications. We cannot address most of these questions at the time being, but we are certain that our results will fuel the TGIP field. Altogether, our work increases our understanding of the antiviral immune response, host genome plasticity, and antiviral memory of the germline in insects.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.108506.

AUTHOR CONTRIBUTIONS

J.A.M. and M.-C.S. conceptualized the study. J.A.M. coordinated and performed infection experiments, analyzed and visualized the data, wrote the first draft, and edited the manuscript. Y.S., V.G., M.V., and V.M. participated in the infection experiments. H.B. performed sRNA sequencing. L.F. analyzed and visualized NGS data. A.-B.F. participated in the interpretation of the results. M.-C.S. supervised the study, provided resources, and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
Drosohila C Virus (DCV)	Obtained from Jean-Luc Imler (University of Strasbourg)	ENA: GCA_000850085
Drosophila X Virus (DXV)	Obtained from Peter Dobos (University of Guelph)	N/A
Sindbis virus (SINV)	DOI: 10.1128/JVI.72.9.7357-7366.1998	Virus stocks were generated by *in vitro* transcription of pTR339 plamid.
Cricket paralysis virus (CrPV)	Obtained from Peter Sarnow (University of Stanford)	N/A
Flock house virus (FHV)	Obtained from Annette Schneemann (The Scripps Research Institute)	N/A
Vesicular stomatitis virus (VSV)	DOI: 10.1073/pnas.1014378107	strain Indiana isolate PI10/Lab-strain
Chemicals, Peptides, and Recombinant Proteins		
mMESSAGE mMACHINE SP6 Transcription Kit	Thermo Fisher Scientific/Ambion	Cat# AM1340
Renilla Luciferase Assay System	Promega	Cat# E2820
TURBO DNase	Thermo Fisher ScientificAmbion	Cat# AM2238
MEGAscript T7 *in vitro* transcription kit	Thermo Fisher Scientific/Ambion	Cat# AM1333
TRIzol Reagent	Thermo Fisher Scientific/Invitrogen	Cat# 15596026
DNase I	Roche	Cat# 10104159001
SuperScript II Reverse Transcriptase	Thermo Fisher Scientific/Invitrogen	Cat# 18064022
DreamTaq Green DNA Polymerase	Thermo Fisher Scientific	Cat# EP0714
NEBNext Multiplex Small RNA Library Prep Set for Illumina (Set 1)	New England Biolabs	Cat# E7300
RNA 5′-polyphosphatase	Epicenter	Cat# RP8092H
Acid-Phenol:Chloroform, pH 4.5	Thermo Fisher Scientific/Ambion	Cat# AM9722
TruSeq RNA Sample Prep Kit	Illumina	Cat# FC-122-1001
Deposited Data		
All Sequencing data	This paper (Sequence Read Archive repository)	PRJNA646880
Experimental Models: Cell Lines		
Baby hamster kidney cells (BHK)	Andrea Gamarnik (Fundación Instituto Leloir)	N/A
Schneider 2 cells (S2)	Invitrogen	Cat# R69007
Experimental Models: Organisms/Strains		
Aedes aegypti	https://dx.plos.org/10.1371/journal.pgen.1006111	N/A
Aedes aegypti	DOI: 10.4269/ajtmh.1999.60.292	N/A
Drosophila melanogaster: w[1118]: w[1118] +; +	Bloomington Drosophila Stock Center	RRID: BDSC_3605
Drosophila melanogaster: Dcr-2 −/−:w[1118] ;Dcr-2 L811fsX ;+	http://www.pnas.org/lookup/doi/10.1073/pnas.1607952113	N/A
Oligonucleotides		
All primer sequences used in this paper	See Table S1	N/A
Recombinant DNA		
Recombinant Sindbis virus expressing Renilla luciferase (SINV-R)	This paper	pTR339-RLuc2A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and Algorithms		
Cutadapt v1.18	http://journal.embnet.org/index.php/embnetjournal/article/view/200	N/A
Bowtie1 v1.1.2	http://genomebiology.biomedcentral.com/articles/10.1186/gb-2009-10-3-r25	N/A
Rsamtools v1.20.4 (R package)	https://bioconductor.org/packages/Rsamtools	N/A
R v3.5.2	http://www.r-project.org/	N/A
DESeq2	https://doi.org/10.1186/s13059-014-0550-8	N/A
GOrilla	https://bmcbioinformatics.biomedcentral.com/articles/10.1186/1471-2105-10-48 https://dx.plos.org/10.1371/journal.pcbi.0030039	N/A
KEGG-search pathway	https://doi.org/10.1002/pro.3711	N/A
Graphpad Prism 6	https://www.graphpad.com/scientific-software/prism/	N/A
FastQC v0.10.1	http://www.bioinformatics.babraham.ac.uk/projects/fastqc/	N/A
Bowtie2 v2.3.4.3	http://www.nature.com/articles/nmeth.1923	N/A

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Maria-Carla Saleh (carla.saleh@pasteur.fr).

Materials availability
The sequences of the oligonucleotides used in this study are listed in the Table S4.
   The SINV-R plasmid generated in this study is fully available upon request.

Data and code availability
All data generated during this study are available in the Sequence Read Archive repository under project PRJNA646880.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Fly strains and husbandry
The following fly lines were used: w^{1118} ;+; + and w^{1118} ;Dcr-2 ^{L811fsX} ;+
   Fly stocks were on the same genetic background to that of w^{1118} flies and harbored the sensitive allele of Pastrel 3L:7350895 (Thr). Flies were maintained on a standard cornmeal diet (Bloomington) at a constant temperature of 25°C and kept under a 12:12 photoperiod. All fly lines were cleaned of possible chronic infections (viruses and Wolbachia) as described previously (Merkling and van Rij, 2015). 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, fly stocks were analyzed by RT–PCR with pairs of primers specific for CrPV, *Drosophila* A virus, *Drosophila* X virus, DCV, FHV, or Nora virus.

Mosquito strains and husbandry
Mosquitoes for TGIP by viral injection
The laboratory colony of *Ae. aegypti*, Paea, originated from Tahiti, French Polynesia, from eggs collected in 1994 (Vazeille-Falcoz et al., 1999). The mosquitoes were maintained at 28°C, 70% relative humidity and a 12:12 photoperiod. Adult mosquitoes were fed with 10% sucrose solution for their maintenance.

Mosquitoes for TGIP by viral blood meal

The laboratory colony of *Ae. aegypti* mosquitoes was originally sampled from a wild population in Thep Na Korn Village, Kamphaeng Phet Province, Thailand (2013). Experiments were performed within 15 generations after laboratory colonization. The mosquitoes were maintained at 28°C, 70% relative humidity and a 12:12 photoperiod. Adult mosquitoes were fed with 10% sucrose solution for their maintenance.

METHOD DETAILS

Virus production and titration

DCV, CrPV, DXV, and FHV 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 by 50% tissue-culture infectious dose ($TCID_{50}$) according to a published method (L.J. Reed and Muench, 1938). SINV and VSV 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, individual or pools of flies (as indicated for each experiment) were squashed in PBS (200 μl) at the indicated time points and titered.

The CHIKV stock was prepared from the 06-049 infectious clone derived from the Indian Ocean Lineage as described previously (Goic et al., 2016). Briefly, the linearized plasmid was used to produce CHIKV RNA *in vitro* with the SP6 mMessage mMachine kit (Ambion). Turbo DNase treated CHIKV RNA was purified by phenol:chloroform extraction and ethanol precipitation. BHK-21 cells were electroporated with 10 μg RNA to produce the CHIKV stock. The CHIKV stock was amplified by another passage in BHK-21 cells and titrated by plaque assay.

SINV-R production

Recombinant Sindbis virus expressing Renilla luciferase (SINV-R) was generated by cloning Rluc into the Sap I and BglII sites of the Sindbis vector pTR339-GFP2A (Thomas et al., 2003). *In vitro* transcribed RNA was transfected into BHK-21 cells. Virus titer was determined by plaque assay on BHK-21 cells and expressed in plaque forming unit (pfu)/mL.

Transgenerational immune priming protocol

Flies. w^1118 virgin female flies were mock infected or infected (primed) with 100 PFU of SINV/fly three days after emergence, 100 PFU /fly of VSV, 200 $TCID_{50}$ /fly of DXV, 1 $TCID_{50}$ /fly of DCV, 1 $TCID_{50}$ /fly of CrPV or 1 $TCID_{50}$ of FHV. One (flies infected with DCV, CrPV or FHV) or two days later (flies infected with SINV, VSV or DXV), the infected flies were crossed with non-infected males, and transferred to egg-laying cages made of grape juice plates with yeast paste on top. The next two or three days, embryos were collected and treated with 50% bleach to eliminate all traces of viruses on the surface of the embryo's chorion. The F1 adult flies were recovered three to four days after emergence and challenged with 3000 PFU of SINV-R, 100 PFU /fly of VSV, 200 $TCID_{50}$ /fly of DXV, 5 $TCID_{50}$ /fly of DCV, 5 $TCID_{50}$ /fly of CrPV or 5 $TCID_{50}$ of FHV, respectively. Two days later, the infected (challenged) flies were collected and viral replication was measured by calculating renilla luciferese counts (SINV-R), by plaque assay (SINV and VSV) or by $TCID_{50}$ (DXV, DCV, CrPV and FHV).

Mosquitoes
TGIP by viral injection

5- to 7-day-old *Ae.aegypti* female mosquitoes were intrathoracically injected using a nanoject (Nanoject II apparatus; Drummond Scientific) with 100 pfu of CHIKV or with an equivalent volume of culture cell media (50nL). Three days after injection, mosquitoes were subjected to a non-infectious blood feeding with whole rabbit blood supplemented with 10 mM ATP. The blood-fed mosquitoes were incubated for one week to allow them to lay eggs. These eggs were hatched and the F1 adult females were challenged with 100 pfu of CHIKV by intra-thoracic injection. Injected mosquitoes were collected three days after the infection and viral load was determined by plaque assay.

TGIP by viral blood-meal

4- to 7-day-old *Ae.aegypti* female mosquitoes were infected with 10^6 PFU/mL of CHIKV or carrier medium (DMEM). The infectious blood-meal was prepared by mixing PBS-washed human blood and CHIKV stock (or DMEM) at a 2:1 ratio and supplemented with 10 mM ATP, 0.075% sodium bicarbonate. One week after the infection, the mosquitoes were subjected to non-infectious blood feeding with whole rabbit blood supplemented with 10 mM ATP. The blood-fed mosquitoes were incubated for one week to let them lay eggs. These eggs were hatched and the F1 adult females were infected with 10^6 PFU/mL of CHIKV by blood feeding in the same manner as the F0 mosquitoes. Infected mosquitoes were collected three days after the infection and the viral load was titered by plaque assay.

SINV-Rluc Renilla luciferase assay

Renilla luciferase was measured using the Renilla luciferase assay system (Promega, Madison, WI). Flies previously infected with SINV-Rluc were collected in eppendorf tubes and euthanized in dry ice. 200 μL of Renilla luciferase assay lysis buffer was added to each sample and then the samples were homogenized using pellet pestles. Homogenized samples were incubated at room

temperature for 10 min and then centrifuged for 5 min at 14,000 × g at 4°C. 20 μL of supernatant of each sample were then transferred to a white-walled 96-well microplate. Then, 25 μL of Renilla luciferase assay reagent was added to each sample and luminescence was immediately measured on a Centro XS 3 LB 960 microplate reader (Berthold Technologies) (0.1 s integration time).

dsRNA preparation and injection into adult flies

dsRNA was generated by *in vitro* transcription using T7 RNA polymerase using as template PCR products corresponding to nucleotides 1,211–2,112 (NSP1/2) and 5,487–6436 (NSP3/4) of the Sindbis virus genome, or nucleotides 5,589–6,008 of the DCV genome (control dsRNA). See sequences in the DNA oligonucleotides list provided in the supplementary table. Three-day-old female flies were anaesthetized with CO2 and intrathoracically injected with 50 nL of the appropriate dsRNA (2 mg/ml) using a nanoinjector (Nanoject II, Drummond Scientific).

Fly viral infections and survival assays

The infection experiments were conducted with flies three days post-emergence. Infections were done by intrathoracic injection (Nanoject II apparatus; Drummond Scientific) of 50 nL of a viral suspension in 10 mM Tris, pH 8. Infected flies were kept at 25°C and changed to fresh vials every 2 days. Survival of SINV infected flies was measured daily by counting the number of dead flies in each test tube. Fly mortality at day one was attributed to damage induced by the injection and/or manipulation procedure and was excluded from further analyses.

RNA extractions and RT–PCR

Virus-containing samples were extracted with TRIzol (Invitrogen). Before RT–PCR analysis, samples were treated with Dnase I (Roche). Complementary DNA molecules were produced with SuperScript II Reverse Transcriptase (Invitrogen) with random hexamer primers. PCR assays were performed using DreamTaq DNA Polymerase.

DNA extraction for vDNA PCR analysis

DNA was simultaneously isolated with total RNA from individual flies according to the manufacturer's instructions using TRIzol. PCR analysis for the detection of DCV vDNA was performed using DreamTaq DNA polymerase with the primers indicated in each figure (see sequences in the DNA oligonucleotides list provided in the supplementary table). PCR products were sequenced to confirm that the products being amplified corresponded to the targeted sequence.

RNA extraction and library production

For each time point of infection analyzed, total RNA was extracted from 15 flies. For each sample, 19-31 nt long RNAs were cut and extracted from a 15% acrylamide/bis-acrylamide (37.5:1), 7M urea gel and the purified RNAs were subjected to small RNA library preparation using the NEBNext Multiplex Small RNA Library Prep (New England Biolabs) kit with a 3′ adaptor from Integrated DNA Technologies (IDT) and in-house designed indexed primers. Libraries diluted to 4 nM were sequenced with the NextSeq 500 High Output Kit v2 using a NextSeq 500. Reads were analyzed using in-house Perl scripts.

5′-tripRNA sequencing

Total RNA (2 μg) extracted with TRIzol was treated with 20 units of RNA 5′-polyphosphatase enzyme (Epicenter) for 30 min at 37°C. Treated samples were extracted with acid phenol:chloroform, pH 4.5 (Ambion) and precipitated with Glycoblue (15 μg; ThermoFisher Scientific), 0.1 volume of 3 M sodium acetate and 2.5 volumes of ice-cold 100% ethanol overnight at −80°C. After centrifugation (30 min, 4°C) and a 70% ethanol wash, RNA pellets were resuspended in water (20 μl) and used to produce the small RNA libraries.

Bioinformatics analysis of small RNA libraries

The quality of the fastq files was assessed with FastQC software (www.bioinformatics.babraham.ac.uk/projects/fastqc/). Low-quality bases and adaptors were trimmed from each read using the Cutadapt program and only reads showing an acceptable quality (Phred score, 20) were retained. A second set of graphics was generated by the FastQC software using the trimmed fastq files. Reads were mapped to the genome sequences of SINV (GenBank: NC_001547.1) using Bowtie (https://sourceforge.net/projects/bowtie-bio/files/bowtie/1.0.0/). The default parameters for small RNAs were used, with the exception that one mismatch was allowed between each read and the target (the −v 1 parameter). Bowtie generates results in the Sequence Alignment/Map (SAM) format. All SAM files were analyzed using the SAMtools package to produce bam indexed files. Homemade R scripts with Rsamtools and Short-reads in the Bioconductor software were used for the analysis of the bam files to create graphs. The number of small RNA reads mapping to the virus was normalized by the total number of reads in each small RNA library.

RNA transcriptome sequencing and analysis

F1 females (from SINV or mock primed F0 female flies, *see Transgenerational immune priming protocol*) were crossed with their sibling males to obtain the F2 generation. The F2 flies were infected with 100 PFU of SINV/fly or mock infected. Two days later, RNA was extracted from 3 pools of 4 flies per condition for each sample. Libraries were prepared with the Illumina TruSeq RNA Library Prep kit. Libraries were diluted to 4 nM and sequenced using a NextSeq 500 High-Output Kit v.2 on a NextSeq 500 sequencer. The quality of

the fastq files was assessed with FastQC software (www.bioinformatics.babraham.ac.uk/projects/fastqc/). Sequencing reads with a quality score < 20 were trimmed using Cutadapt. Filter-passing reads were mapped to *D. melanogaster* transcripts (Dmel6, http://flybase.org) using Bowtie2 with the "sensitive" option. The output was processed with the Samtools suite to create of a matrix of raw counts used for gene expression analysis. All analyses of transcript expression were performed with Sartool using R (http://www.r-project.org/) and DESeq2 v.1.8.0. Following normalization of raw read counts by the relative log expression method implemented in DESeq2. All genes with an adjusted probability lower than 0.05 were considered as differentially expressed (Dillies et al., 2013; Langmead and Salzberg, 2012; Love et al., 2014; Martin, 2011; Varet et al., 2016).

Gene Ontology enrichment analysis
All the genes differentially expressed were listed and submitted to the web form of GOrilla (Eden et al., 2009) and KEGG-search pathway (Kanehisa and Sato, 2020) tools using *Drosophila melanogaster* as the organism option.

Human blood and ethics statement
Human blood used to feed mosquitoes was obtained from healthy volunteer donors. Healthy donor recruitment was organized by the local investigator assessment using medical history, laboratory results, and clinical examinations. Biological samples were supplied through participation of healthy volunteers at the ICAReB biobanking platform (BB-0033-00062/ICAReB platform/Institut Pasteur, Paris/BBMRI AO203/[BIORESOURCE]) of the Institut Pasteur to the CoSImmGen and Diagmicoll protocols which have been approved by the French Ethical Committee (CPP) Ile-de-France I. The Diagmicoll protocol was declared to the French Research Ministry under the reference: DC 2008–68 COL 1.

QUANTIFICATION AND STATISTICAL ANALYSIS

Differences in infection between treated (primed) and not-treated (not-primed) flies were assessed by a *Mann–Whitney U* test. The comparison of survival curves was performed using a log-rank (Mantel–Cox) test. All statistical analyses were performed in Prism v.6.00 (www.graphpad.com:443/). *The statistical details of experiments can be found in the figure legends.*