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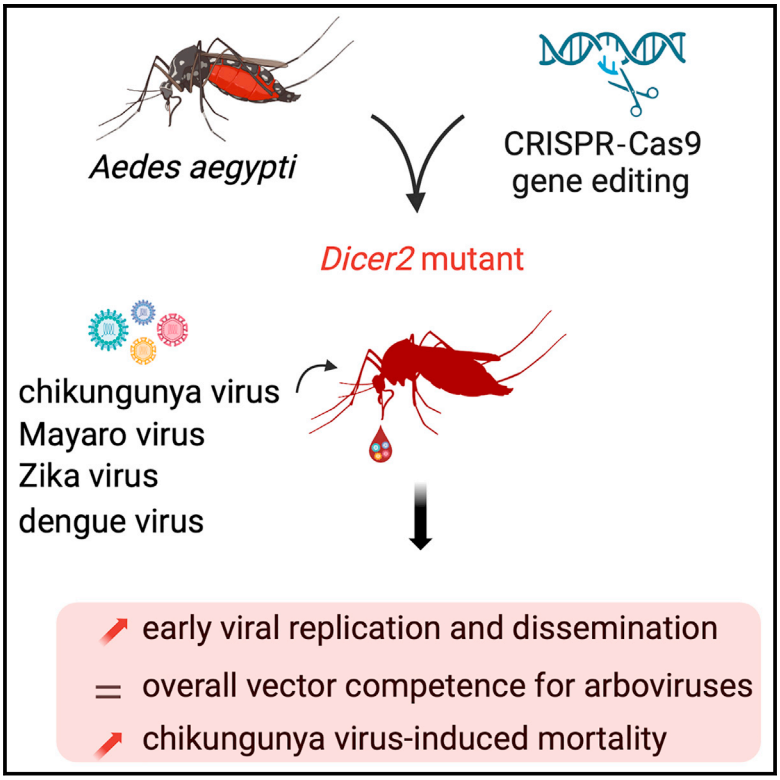
Multifaceted contributions of *Dicer2* to arbovirus transmission by *Aedes aegypti*

Graphical abstract



Highlights

- *Dicer2* limits early replication of chikungunya, Mayaro, dengue, and Zika viruses
- *Dicer2* hinders systemic viral dissemination of the aforementioned arboviruses
- *Dicer2* mutants and wild-type mosquitoes display similar levels of vector competence
- *Dicer2* mutants are sensitive to chikungunya virus, but not to dengue virus infection

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In brief

In this study, Merkling et al. find that genetically disabling the antiviral factor *Dicer2* has a limited impact on the ability of the mosquito *Aedes aegypti* to acquire, replicate, and subsequently transmit medically relevant arboviruses such as chikungunya, Mayaro, dengue, and Zika viruses.

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Multifaceted contributions of *Dicer2* to arbovirus transmission by *Aedes aegypti*

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SUMMARY

Arthropod-borne viruses (arboviruses) transmitted by *Aedes aegypti* mosquitoes are an increasing threat to global health. The small interfering RNA (siRNA) pathway is considered the main antiviral immune pathway of insects, but its effective impact on arbovirus transmission is surprisingly poorly understood. Here, we use CRISPR-Cas9-mediated gene editing *in vivo* to mutate *Dicer2*, a gene encoding the RNA sensor and key component of the siRNA pathway. The loss of *Dicer2* enhances early viral replication and systemic viral dissemination of four medically significant arboviruses (chikungunya, Mayaro, dengue, and Zika viruses) representing two viral families. However, *Dicer2* mutants and wild-type mosquitoes display overall similar levels of vector competence. In addition, *Dicer2* mutants undergo significant virus-induced mortality during infection with chikungunya virus. Together, our results define a multifaceted role for *Dicer2* in the transmission of arboviruses by *Ae. aegypti* mosquitoes and pave the way for further mechanistic investigations.

INTRODUCTION

Mosquitoes are vectors of several medically significant arthropod-borne viruses (arboviruses) that have been emerging or reemerging over the past decades.[1] Despite significant efforts to control mosquito populations, develop specific antiviral drugs, or deploy effective vaccines, arboviral diseases constitute a growing threat for global health.[2] The main arbovirus vectors are the mosquito species *Aedes aegypti* and *Aedes albopictus*, which are distributed extensively on all continents and extend their geographical range rapidly.[3] A mosquito acquires an arbovirus through ingestion of a blood meal taken from a viremic vertebrate host. In the digestive tract, the virus crosses the epithelial barrier and infects the midgut cells.[4] After a few days, the virus exits the midgut and disseminates to the hemocoel, infecting other organs and cells, such as the fat body and hemocytes.[4] Eventually, the virus reaches the salivary glands, where it replicates,[5] before being retransmitted to another vertebrate host upon the next infectious bite.[4]

At every step of the mosquito infection, arboviruses encounter tissue barriers and innate immune responses. Major immune signaling pathways such as the Toll, immune deficiency (IMD), and Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathways have been implicated in mosquito antiviral defense.[6–8] The small interfering RNA (siRNA) pathway is considered the primary antiviral defense mechanism in dipteran insects.[7,9–12] The siRNA pathway relies on RNA interference (RNAi), an overarching mechanism of RNA silencing mediated by small RNAs (sRNAs). Viral double-stranded RNA (dsRNA) is recognized by Dicer2 (Dcr2), an endonuclease that cleaves viral dsRNA into siRNAs that are 21 bp in length. Next, siRNA duplexes are loaded through a process that involves the dsRNA-binding protein, R2D2,[13] into the Argonaute2 (Ago2)-containing RNA-induced silencing complex (RISC), which subsequently targets viral RNA for cleavage. Dcr2 belongs to the family of DExD/H-box helicases, similar to RIG-I-like receptors in vertebrates.[14]

The antiviral role of the siRNA pathway in *Ae. aegypti* has been established by several earlier studies. In the *Ae. aegypti* cell line Aag2, *Dcr2* knockout resulted in higher Semliki Forest virus replication, and *Dcr2* transient reexpression rescued the antiviral phenotype.[15] In adult *Ae. aegypti* mosquitoes, *Dcr2* knockdown increased Sindbis virus (SINV) and dengue virus (DENV) titers after an infectious blood meal[16,17] and shortened the extrinsic incubation period (i.e., the time required for an infected mosquito to become infectious) before DENV transmission.[17] However, a few studies indicated that the antiviral activity of the siRNA pathway in *Ae. aegypti* could not always be generalized. Antiviral activity of *Dcr2* upon DENV infection was observed in different laboratory strains[17] but not in a field-derived mosquito strain.[18]

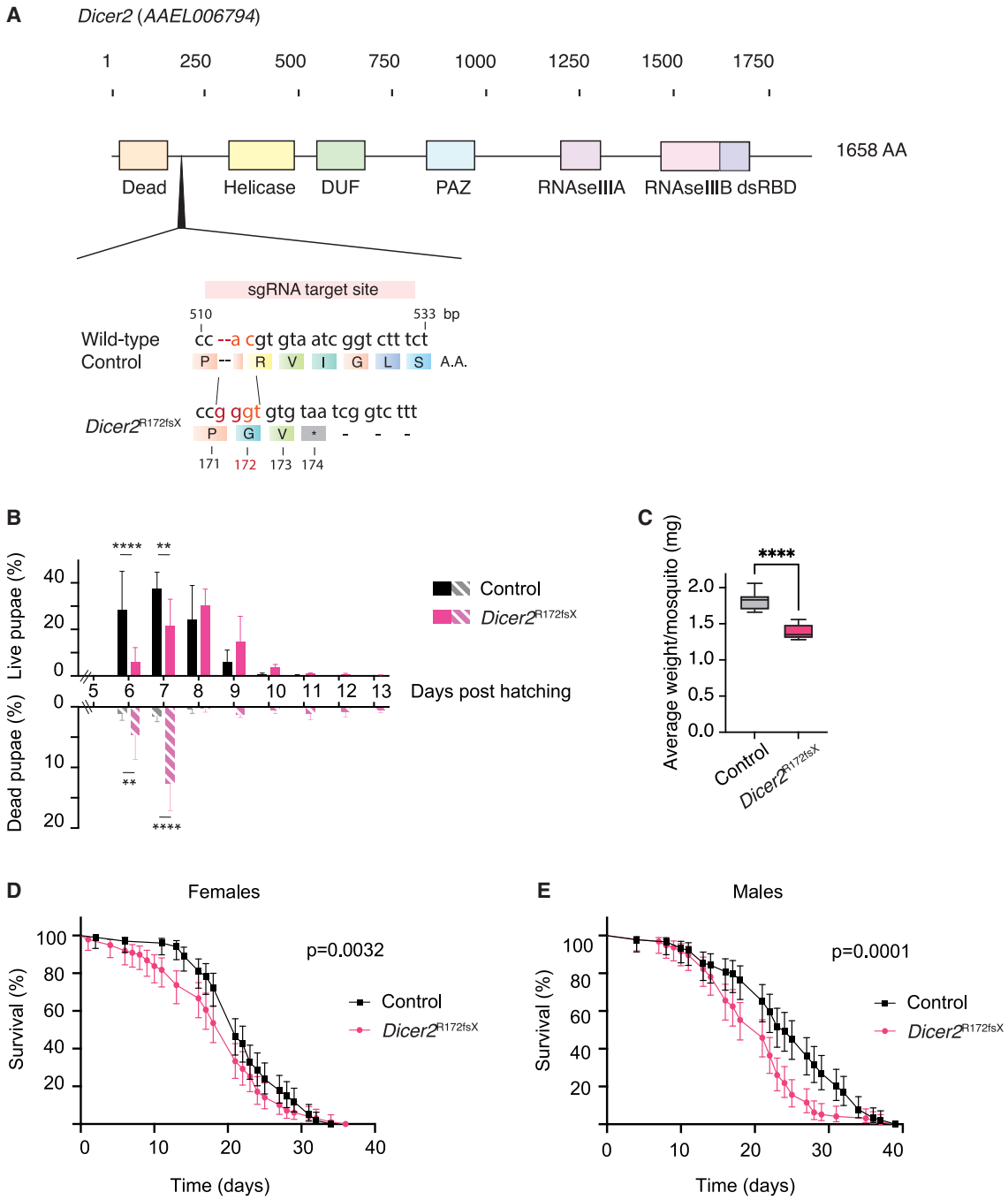

Figure 1. *Dicer2*^{R172fsX} mutants display modest fitness defects

(A) Structure of the *Dicer2* locus (*AAEL006794*) with functional domains highlighted in colored boxes and size shown in amino acid (AA) length. Nucleotide and AA sequences of the region targeted by the single-guide RNA (sgRNA) are shown for *Dicer2*^{R172fsX} mutants and wild-type mosquitoes.

(B) Pupation success in the *Dicer2*^{R172fsX} mutant and control lines. Numbers of live and dead pupae were scored daily and percentages were calculated relative to the total number of pupae present at the end of the experiment. Data are shown as mean and standard deviation (SD) of three independent groups of 150–200 pupae each. Statistical significance of the pairwise differences was determined with Student's t test (**$p < 0.01$; ****$p < 0.0001$).

(C) Average weight of *Dicer2*^{R172fsX} mutant and control adult female mosquitoes. Data are shown as means and SD of six replicates containing five mosquitoes that had been starved 24 h before weighing. Statistical significance of the differences was determined with Student's t test (****$p < 0.0001$).

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This could reflect strain-specific differences in the contribution of antiviral immune pathways to DENV infection or could be linked to technical variation, such as differences in gene knockdown efficiency. Elevated levels of SINV replication were observed upon midgut-specific *Dcr2* knockdown, suggesting an antiviral role of the siRNA pathway in this tissue.[19] In contrast, another study showed that the siRNA pathway failed to limit DENV replication in the mosquito midgut, although it efficiently restricted systemic DENV replication and remained effective in the midgut against both endogenous and exogenous dsRNA substrates.[20] This could be linked to the dsRNA-binding protein Loqs2 (a paralog of Loquacious and R2D2 that is not expressed in the midgut), because transgenic expression of *Loqs2* in the midgut was sufficient to decrease DENV RNA levels upon an infectious blood meal.[20]

In addition to studies using gene-silencing assays, two studies genetically engineered mosquitoes to target the siRNA pathway *in vivo*. First, during the revision process of the present paper, a study was published describing the effects of a loss-of-function mutation in the *Dcr2* locus on viral pathogenesis in *Ae. aegypti*.[21] Mutant mosquitoes inoculated intrathoracically with SINV, DENV, or yellow fever virus exhibited increased viral replication and reduced survival, demonstrating the role of the siRNA pathway in preventing virus-induced pathogenesis in *Ae. aegypti*. A similar effect was seen in *Dcr2* mutant mosquitoes that were orally infected with SINV, but infectious blood meals with medically relevant arboviruses were not tested.[21] Second, the impact of the siRNA pathway on arbovirus replication in *Ae. aegypti* was assessed using transgenic overexpression of *Dcr2* and *R2D2* in the midgut following an infectious blood meal.[22] Replication of DENV, Zika virus (ZIKV), and chikungunya virus (CHIKV) was suppressed upon overexpression of both siRNA genes, consistent with a broad-spectrum antiviral activity of the siRNA pathway in *Ae. aegypti*.[22]

Despite the well-established antiviral activity of the siRNA pathway *in vivo* in *Ae. aegypti*, the full antiviral spectrum of the siRNA pathway and its effective contribution to transmission of arboviruses from different families remain patchy. Most earlier studies on the antiviral activity of the siRNA pathway in *Ae. aegypti* focused on measuring viral replication in various tissues rather than directly assessing virus transmission potential. Arbovirus transmission is influenced not only by vector competence, defined as the intrinsic ability of the mosquito to acquire and subsequently transmit a pathogen,[23] but also by other entomological parameters such as survival rate, which has not been assessed in most previous studies on the siRNA pathway in *Ae. aegypti*. While exploiting the antiviral activity of the siRNA pathway as a novel arbovirus control strategy in the future is very promising, characterizing the full impact of manipulating key siRNA pathway components on arbovirus transmission is an essential prerequisite.

Here, we report on the generation and initial characterization of a viable and fertile *Dcr2* mutant line of *Ae. aegypti*. We monitored the infection dynamics of four medically relevant arboviruses, namely, two flaviviruses, DENV[24,25] and ZIKV,[26] and two alphaviruses, CHIKV[27] and Mayaro virus (MAYV).[28,29] The lack of *Dcr2* modestly enhanced early viral replication in the midgut and promoted early viral dissemination from the midgut to other tissues. However, the absence of *Dcr2* did not result in major changes in the overall level of vector competence. In addition, the survival of *Dcr2* mutants was strongly reduced after infection with CHIKV (an alphavirus) but not with DENV (a flavivirus). Together, our results define a multifaceted role for *Dcr2* in arbovirus transmission by *Ae. aegypti* mosquitoes.

RESULTS

Generation of a *Dicer2* mutant line of *Ae. aegypti*

To investigate the role of *Dcr2* and the siRNA pathway during arbovirus infection in *Ae. aegypti*, we generated a *Dcr2* mutant line using CRISPR-Cas9-mediated gene editing.[30] The line was generated from a field-derived *Ae. aegypti* colony originating in Gabon.[31] In brief, we injected mosquito embryos with the Cas9 endonuclease coupled to single-guide RNAs (sgRNAs) targeting exon 5 (which had previously been targeted with transcription activator-like effector nucleases[32]) and exon 7 of the *Dcr2* gene (*AAEL006794*) and obtained generation zero (G_0) adult mosquitoes with potential gene edits. We screened the *Dcr2* locus of the G_0 individuals for mutations and found, in one male mosquito, an insertion of 2 bp followed by point mutations of the next 2 bp in exon 5 (see STAR Methods for details). The sequence edit led to a frameshift at position 172 resulting in an arginine-to-glycine substitution at position 172 and a premature stop codon at position 174 of the Dcr2 protein sequence (Figure 1A). Thus, we generated a mutant line encoding a truncated version of the Dcr2 protein with only 173 of the 1,658 amino acids, which we called *Dicer2*^{R172fsX}. In parallel with generating the mutant line, we also produced a wild-type "sister" line derived from the same crossing scheme as the mutant line, which we refer to as the control line hereafter. The first 173 amino acids of Dcr2 correspond to the DEAD component of the DEAD-box helicase domain, which is responsible for unwinding dsRNA and initiating RNAi. Because the DEAD-box helicase domain was left incomplete, we hypothesized that the mutation triggered a *Dcr2* loss of function. We measured a strong decrease in *Dcr2* mRNA transcripts in the *Dicer2*^{R172fsX} mutant line compared with the control line (Figure S1). This is a hallmark of nonsense-mediated mRNA decay, a mechanism that targets mRNAs encoding truncated proteins and prevents aggregation of aberrant transcripts and abnormal proteins.[33]

Dicer2^{R172fsX} mutants display moderate fitness defects

The final step in establishing the *Dicer2*^{R172fsX} mutant and control lines consisted of crossing heterozygous individuals (carrying one copy of the mutant *Dcr2* allele and one copy of the wild-type allele) to obtain homozygous mutants (the mutant line)

(D and E) Survival curves of female (D) and male (E) adult *Dicer2*^{R172fsX} mutants and controls under standard insectary conditions. Mosquitoes were anesthetized on ice and sorted in incubation boxes by groups of 25 in four replicates for each condition. Mortality was scored daily and fresh pads containing 10% sucrose solution were renewed twice weekly. Data are shown as means and SD of survival rates. Statistical significance of the differences was determined by Gehan-Breslow-Wilcoxon tests.

and homozygous wild-type individuals (the control "sister" line). Although the *Dicer2*^{R172fsX} mutant line was viable and fertile, we detected a series of modest fitness defects relative to the control line. First, we measured daily pupal mortality over the 9-day time window during which pupation is expected to occur in our laboratory conditions. We found that pupal death rates were higher in *Dicer2*^{R172fsX} mutants than in the control line on days 6 and 7 post hatching (Figure 1B). For instance, on day 7, we observed 12.5% pupal mortality in the *Dicer2*^{R172fsX} line, compared with only 1.5% in the control line ($p < 0.0001$). The cumulative pupal death rate was 22.1% for *Dicer2*^{R172fsX} mutants compared with 3.1% for controls. Finally, we used a four-parameter logistic model to determine the median pupation time, which was 7.5 days for *Dicer2*^{R172fsX} mutants compared with 6.4 days for control mosquitoes ($p = 0.0002$). Thus, we concluded that *Dicer2*^{R172fsX} mutants had slower larval development and higher pupal mortality.

Second, we measured the weight of adult female mosquitoes 5–7 days of age. We starved the mosquitoes 24 h prior to weighing to ensure that no sugar water remained in their digestive tract. We found that *Dicer2*^{R172fsX} mutants had a lower body weight (1.3 mg/mosquito on average) than the controls (1.8 mg/mosquito; $p < 0.0001$), despite exactly the same rearing conditions (Figure 1C). Altogether, these results point to fitness defects during development in the *Dicer2*^{R172fsX} line.

Next, we tested whether adult lifespan was affected in the *Dicer2*^{R172fsX} mutant line by performing a survival analysis in male and female adult mosquitoes kept on a sugar-only diet under standard rearing conditions. We found a slight reduction in survival for *Dicer2*^{R172fsX} females (median lifespan of 21 days vs. 22 days in controls; $p = 0.02$; Figure 1D) and stronger reduction in survival for *Dicer2*^{R172fsX} males (median lifespan of 21 days vs. 24 days in controls; $p < 0.0001$; Figure 1E). Altogether, our data demonstrate moderate fitness losses in developing and adult *Dicer2*^{R172fsX} mutants.

The siRNA pathway is impaired in *Dicer2*^{R172fsX} mutants

To assess whether the siRNA pathway was impaired in *Dicer2*^{R172fsX} mutant mosquitoes, we first used an *in vivo* RNAi reporter assay.[34] In brief, adult females were injected intrathoracically with a transfection mixture containing a firefly luciferase reporter plasmid and dsRNA targeting firefly luciferase (Fluc) or green fluorescent protein (GFP) as a negative control. A reporter plasmid encoding a *Renilla* luciferase (Rluc) was also added and used as an *in vivo* transfection control. Three days later, whole mosquito bodies were homogenized, and Fluc and Rluc luminescence counts were measured (Figure 2A). Results were expressed as $\log_{10}$-transformed Fluc/Rluc ratios, and we analyzed the data from two separate experiments by accounting for the variation between experiments (see STAR Methods for details). When injected with reporter plasmids and dsRNA targeting GFP, *Dicer2*^{R172fsX} mutant and control lines displayed comparable Fluc/Rluc ratios, confirming that Fluc and Rluc were well translated and that non-specific silencing activity was not detected in the control line (Figure 2B). However, when *Dicer2*^{R172fsX} mutant and control lines were injected with reporter plasmids and dsRNA targeting Fluc, the Fluc/Rluc ratios were significantly higher in *Dicer2*^{R172fsX} mutants (Figure 2B). This indicates that Fluc expression from the plasmid was not efficiently silenced and that RNAi activity was in fact reduced in *Dicer2*^{R172fsX} mutants. We note that the Fluc/Rluc ratios were higher in the *Dicer2*^{R172fsX} mutants treated with GFP dsRNA than when they were treated with Fluc dsRNA. This residual silencing activity may reflect antisense-mediated silencing triggered by single-stranded RNA molecules possibly present among the injected dsRNAs, silencing due to the binding of the Fluc dsRNA to the plasmid DNA, and/or non-canonical RNAi-mediated silencing mediated by Dicer1.

Next, we aimed to assess siRNA production levels by performing high-throughput sRNA sequencing of *Dicer2*^{R172fsX} mutant and control lines exposed to DENV type 1 (DENV-1) and CHIKV via an infectious blood meal. Five days post virus exposure, we extracted RNA from mosquito carcasses (i.e., abdomen without the midgut) and confirmed their infected status by qRT-PCR before preparing sRNA sequencing libraries. Although we found abundant positive (sense orientation) sRNAs mapping across the CHIKV or DENV-1 genomes in both the *Dicer2*^{R172fsX} and the control lines, negative (antisense orientation) viral sRNA reads were virtually absent from the mutant line and predominantly 21 bp in size in the control line (Figures S2 and S3). Accordingly, the percentage of negative 21-bp RNAs mapping on the CHIKV genome (Figure 2C) or the DENV-1 genome (Figure 2D) was strongly reduced in *Dicer2*^{R172fsX} mutants compared with controls. Finally, we assessed the production of endogenous siRNAs in the same mosquitoes infected with DENV-1 or CHIKV. We found that the percentage of negative 21-bp RNAs mapping on a cluster of histone genes was strongly reduced in *Dicer2*^{R172fsX} mutants (Figure 2E). Taken together, our results demonstrate that both exogenous and endogenous siRNAs are undetectable in *Dicer2*^{R172fsX} mutants.

Dicer2 affects viral infection dynamics upon alphavirus infection *in vivo*

We performed a series of experimental infections aimed at analyzing the impact of *Dcr2* on viral infection dynamics *in vivo*. We exposed *Dicer2*^{R172fsX} mutant and control lines to blood meals containing alphaviruses (CHIKV and MAYV; Figure 3) or flaviviruses (DENV and ZIKV; Figure 4) and dissected the midgut, carcass, and head at two time points post exposure chosen to represent early and late stages of the infection process. At the late time point, we also collected mosquito saliva to assess the impact of *Dcr2* on virus transmission potential, a direct measure of vector competence. For every time point and body part, we assessed infection prevalence and viral loads, by qRT-PCR (DENV) or infectious titration assay (CHIKV, MAYV, and ZIKV). Midgut prevalence was calculated as the number of virus-positive midguts over the total number of exposed mosquitoes. Carcass prevalence was calculated as the number of virus-positive carcasses over the number of virus-positive midguts. Head prevalence was calculated as the number of virus-positive heads over the number of virus-positive carcasses. Finally, saliva prevalence was calculated as the number of virus-positive saliva samples over the number of virus-positive heads. To quantify overall vector competence, we also calculated the proportion of virus-exposed mosquitoes with virus-positive saliva, which we refer to as transmission efficiency (Figure 5). When saliva

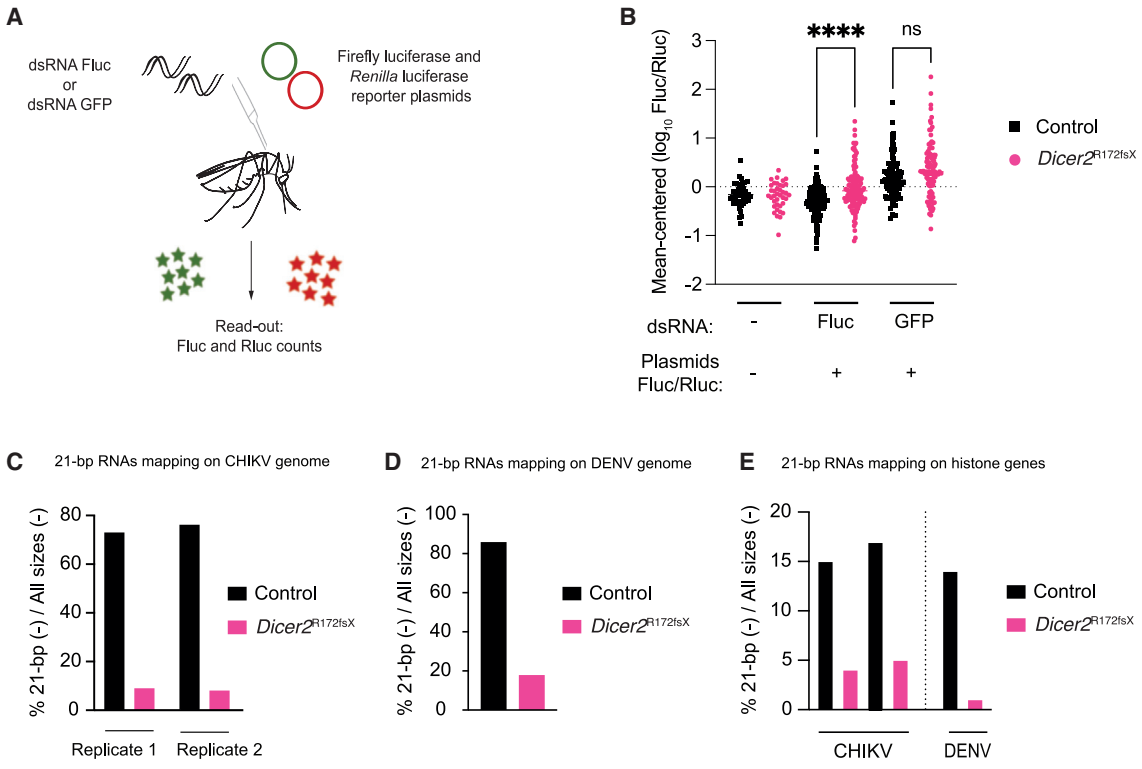


Figure 2. The siRNA pathway is impaired in *Dicer2*^{R172fsX} mutants

(A) Experimental scheme of the *in vivo* RNAi reporter assay. Adult *Dicer2*^{R172fsX} mutants and control mosquitoes were co-injected with firefly luciferase (Fluc) and *Renilla* luciferase (Rluc) reporter plasmids and dsRNA targeting Fluc or GFP. Luminescence was measured 3 days post injection in individual mosquitoes.

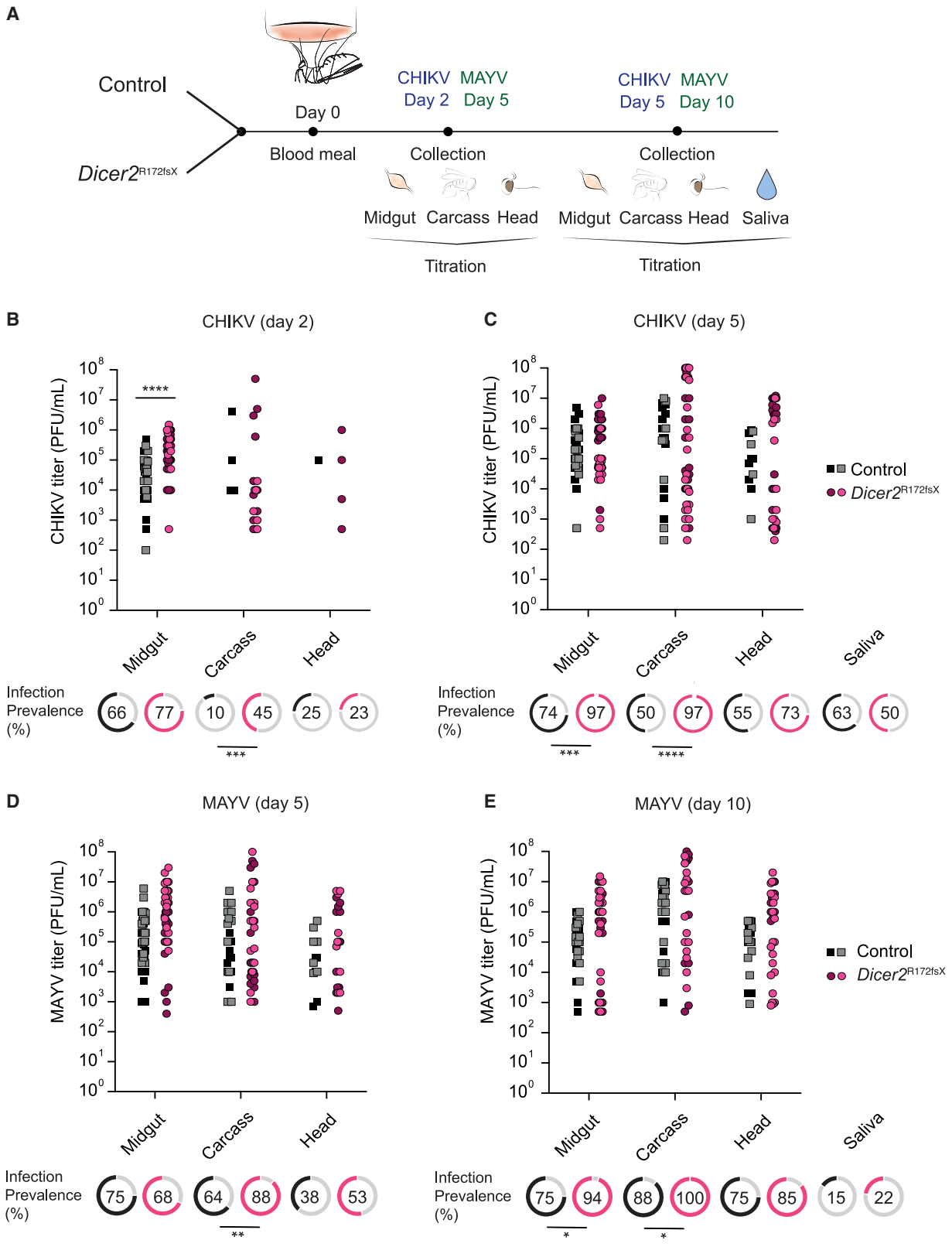
(B) *In vivo* RNAi activity in the *Dicer2*^{R172fsX} mutant and control lines. Fluc luminescence counts were normalized to Rluc counts and $\log_{10}$ transformed. Mean-centered values represent the raw data corrected for differences between two experimental replicates for visual purposes. Mean-centered values were compared by one-way ANOVA (****$p < 0.0001$; ns, non-significant).

(C–E) Production of siRNAs in the *Dicer2*^{R172fsX} mutant and control lines. Female *Dicer2*^{R172fsX} mutants and control mosquitoes were exposed to CHIKV or DENV-1, and mosquito carcasses were harvested on day 5 (CHIKV, in duplicate) or day 6 (DENV-1) post infection. Virus infection was confirmed by qRT-PCR prior to sRNA sequencing. Negative 21-bp RNA reads were mapped to CHIKV (C), DENV-1 (D), or a histone transcript cluster located between positions 161,180,000 and 161,236,000 of chromosome 3 (E), and mapped reads were expressed as a percentage relative to negative reads of all sizes.

testing could not be performed due to technical issues, we used the proportion of virus-exposed mosquitoes with a virus-positive head as a proxy for transmission efficiency.

First, we exposed *Dicer2*^{R172fsX} mutants and control mosquitoes to an infectious blood meal containing 10^6 plaque-forming units (PFU)/mL of CHIKV and collected body parts (midgut, body, head) 2 and 5 days post exposure, as well as saliva at the later time point (Figure 3A). Two days post exposure, we found a significantly higher midgut viral titer in *Dicer2*^{R172fsX} mutants, but no difference in infection prevalence (Figure 3B). We found a significantly higher carcass prevalence in *Dicer2*^{R172fsX} mutants compared with controls (45% vs. 10%; $p = 0.0008$), but no difference in infectious titers in the virus-positive carcasses. Five days after the infectious blood meal, we did not detect any differences in viral loads in any body part (Figure 3C). However, we observed a higher midgut prevalence (97% vs. 74%; $p = 0.0007$) and higher carcass prevalence (97% vs. 50%; $p < 0.0001$) in *Dicer2*^{R172fsX} mutants relative to controls. Infection prevalence in heads and saliva samples did not significantly differ between *Dicer2*^{R172fsX} mutants and controls on day 5 post exposure. To determine the overall impact of *Dcr2* on vector competence, we compared the transmission efficiency of the *Dicer2*^{R172fsX} mutant and control lines on day 5 (Figure 5A). *Dicer2*^{R172fsX} mutants had a marginally significantly higher transmission efficiency in the first experiment (50% vs. 17%; $p = 0.0305$) but not in the second experiment (21% vs. 10%; $p = 0.4429$). These results demonstrate that the loss of *Dcr2* enhances early CHIKV replication in the midgut, promotes early viral dissemination from the midgut to the abdomen, and results in a higher midgut infection rate and higher dissemination rate from midguts to abdomens on day 5 post exposure. However, these differences do not lead to a major increase in vector competence on day 5 post virus exposure.

To test whether such observations could be extended to another virus of the *Alphavirus* genus, we exposed mosquitoes to an infectious blood meal containing 10^6 PFU/mL of MAYV and collected samples 5 and 10 days post exposure (Figure 3A). On day 5, the viral loads in midguts, carcasses, and heads did not differ between *Dicer2*^{R172fsX} and control mosquitoes (Figure 3D). Infection prevalence was not altered in the mosquito midguts or heads, but it increased significantly in carcasses of *Dicer2*^{R172fsX} mutants relative to controls (88% vs. 64%; $p = 0.0069$). On day 10 post



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exposure, we did not detect differences in viral loads between mosquito lines in any body part but observed a moderate increase in infection prevalence in midguts (from 75% to 94%; p = 0.0157) and carcasses (from 88% to 100%; p = 0.0424) in *Dicer2*^{R172fsX} mutants relative to controls (Figure 3E). We found no difference in infection prevalence in heads and saliva samples on day 10 post exposure. Our estimates of transmission efficiency were based on virus-positive heads in the first experiment (Figure 5B) and virus-positive saliva samples in the second experiment (Figure 5A). Transmission efficiency estimates on day 10 did not significantly differ between *Dicer2*^{R172fsX} mutants and controls in the first experiment (57% vs. 45%; p = 0.7374) or in the second experiment (22% vs. 8%; p = 0.2448). These results demonstrate that *Dcr2* does not have an impact on MAYV viral loads 5 and 10 days post exposure in the mosquito midgut, carcass, or head. Yet, loss of *Dcr2* increased MAYV carcass prevalence on days 5 and 10, and midgut prevalence on day 10, similar to our observation with CHIKV. Thus, we conclude that *Dcr2* does not seem to play a major role in the replication of the alphaviruses we tested but contributes to controlling viral dissemination from the midgut to the carcass. Overall, these differences do not result in a major change in vector competence on day 10 post virus exposure.

Dicer2 affects viral infection dynamics upon flavivirus infection *in vivo*

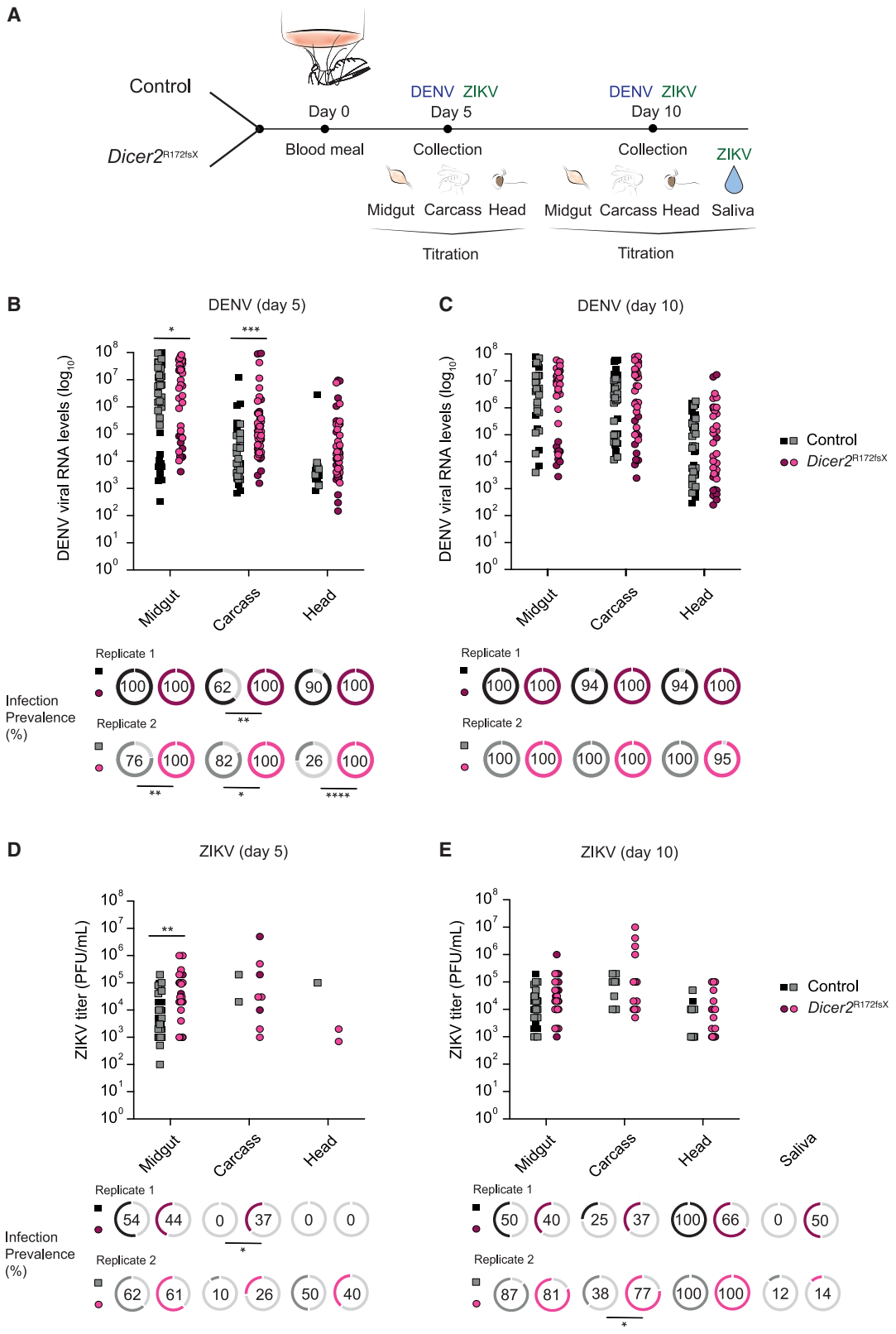
To evaluate the impact of *Dcr2* on flavivirus infection dynamics, we next exposed mosquitoes to an infectious blood meal containing 5 × 10^6 focus-forming units (FFU)/mL of DENV type 3 (DENV-3) and collected samples 5 and 10 days post exposure (Figure 4A). Overall, infection prevalence was higher in the first experimental replicate, and we accounted for this effect in the statistical model and by plotting infection prevalence separately. Five days after exposure, infection prevalence was significantly higher in *Dicer2*^{R172fsX} mutants for all body parts (midgut, carcass, and head) in the second experimental replicate. In the first replicate, the difference in infection prevalence was significantly higher only in the carcass of *Dicer2*^{R172fsX} mutants (100% vs. 62%; p = 0.0052), but our ability to detect differences was limited by the higher overall infection prevalence in this experiment (Figure 4B). We concluded that DENV-3 dissemination from the midgut to the abdomen, and from the abdomen to the head, was enhanced in *Dicer2*^{R172fsX} mutants. This was associated with an increase in viral RNA loads in the midguts (p = 0.0230) and carcasses (p = 0.0006) of *Dicer2*^{R172fsX} mutants on day 5 post exposure (Figure 4B). Ten days post exposure, infection prevalence was almost 100% in both experimental replicates and all body parts, indicating that DENV-3 disseminated throughout the mosquito body (Figure 4C). We did not observe any differences in viral RNA levels between *Dicer2*^{R172fsX} mutants and control mosquitoes in any body part on day 10, when viral levels are high and most likely reaching a plateau. To compare transmission efficiency between the *Dicer2*^{R172fsX} mutant and control lines, we used the proportion of virus-exposed mosquitoes with a virus-positive head because saliva results were unavailable (Figure 5B). *Dicer2*^{R172fsX} mutants did not exhibit significantly higher transmission efficiency on day 10 in the first experiment (100% vs. 89%; p = 0.4891) or in the second experiment (96% vs. 100%; p = 0.9999). Overall, our analysis of DENV-3 infection dynamics demonstrates that *Dcr2* restricts early viral dissemination from the midgut to the abdomen, and from the abdomen to the head, probably by controlling early viral replication in the midgut and carcass.

Finally, we proceeded to analyze the impact of *Dcr2* on infection dynamics of another member of the *Flavivirus* genus, ZIKV. We collected samples on days 5 and 10 after exposure to an infectious blood meal containing 10^6 PFU/mL of ZIKV (Figure 4A). Overall infection prevalence differed between the two experimental replicates, and we accounted for this effect in the statistical model and by displaying the data separately. We found only subtle differences in ZIKV infection dynamics between *Dicer2*^{R172fsX} mutants and control mosquitoes. Infectious titers were higher in the midguts of *Dicer2*^{R172fsX} mutants on day 5 (Figure 4D). Carcass prevalence was higher in *Dicer2*^{R172fsX} mutants than in control mosquitoes only on day 5 in the first experimental replicate (37% vs. 0%; p = 0.0171; Figure 4D) and on day 10 in the second experimental replicate (77% vs. 38%; p = 0.0127; Figure 4E). We determined the overall impact of *Dcr2* on vector competence by estimating transmission efficiency on day 10 (Figure 5A). Transmission efficiency did not significantly differ between the *Dicer2*^{R172fsX} mutant and the control lines in the first experiment (5% vs. 0%; p = 0.4545) or in the second experiment (9% vs. 4%; p = 0.6). We concluded that the loss of *Dcr2* enhanced early ZIKV replication in the midgut and facilitated viral dissemination to the abdomen, but this did not result in a detectable change in vector competence on day 10 post virus exposure.

Taken together, our data on the infection dynamics of CHIKV, MAYV, DENV, and ZIKV reveal a role for *Dcr2* in controlling early viral replication and dissemination, when viruses are infecting the midgut and starting to disseminate systemically in the abdomen. We found that the loss of *Dcr2* increased viral loads in the midgut (except for MAYV) and promoted viral dissemination from the midgut to the carcass (except for ZIKV) at early infection time points. Conversely, our data showed that *Dcr2* was not required

Figure 3. *Dcr2* hinders early infection dynamics of alphaviruses

(A–E) Experimental scheme (A) for the analysis of CHIKV and MAYV infection dynamics. Adult *Dicer2*^{R172fsX} mutants and control female mosquitoes were exposed to an infectious blood meal containing 10^6 PFU/mL CHIKV or MAYV. Midguts, carcasses, and heads from the same mosquitoes were collected on days 2 and 5 post exposure (CHIKV) or days 5 and 10 post exposure (MAYV). Saliva was collected at only 5 (CHIKV) or 10 (MAYV) days post exposure. Infection prevalence and CHIKV titers were determined by plaque assay 2 (B) and 5 (C) days post exposure. Infection prevalence and MAYV titers were determined by plaque assay 5 (D) and 10 (E) days post exposure. The viral loads from two experimental replicates were combined and depicted using two shades of the same color (gray/black for control mosquitoes, pink/red for *Dicer2*^{R172fsX} mutants). Viral loads are plotted only for virus-positive mosquitoes. The data are merged for infection prevalence because the experiment effect was non-significant. Each experimental condition is represented by 48–56 mosquitoes (B and C, CHIKV) or 37–64 mosquitoes (D and E, MAYV). The statistical significance of pairwise comparisons shown (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001) was obtained with Student's t test applied to the raw values after correction for the variation between experiments (viral loads) or a chi-squared test (prevalence). The full statistical analyses are provided in Figure S4A (CHIKV) and Figure S4B (MAYV).



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for controlling viral replication at later infection stages, at least in the body parts that we analyzed. The overall levels of vector competence for the four arboviruses were similar between the *Dicer2*^{R172fsX} mutant line and the control line (Figure 5), except for one CHIKV experiment.

Dicer2 affects viral replication of CFAV *in vivo*

In addition to arboviruses, we asked whether *Dcr2* could have an impact on replication levels of cell-fusing agent virus (CFAV), an insect-specific flavivirus.[35] We inoculated mosquitoes intrathoracically with 50 tissue-culture infectious dose 50% ($TCID_{50}$) units of CFAV and measured viral RNA levels by qRT-PCR in whole mosquito bodies 2, 4, 6, and 8 days post infection (Figure S6A). We found that *Dcr2* had a very limited impact on CFAV loads. We observed only slightly increased viral loads on days 2 and 6 post inoculation in *Dicer2*^{R172fsX} mutants compared with control mosquitoes (Figure S6B), indicating a limited influence of *Dcr2* on the systemic replication of the insect-specific flavivirus CFAV. This is consistent with our observations above demonstrating a limited role for *Dcr2* in controlling systemic replication of arboviruses.

Dicer2 prevents virus-induced mortality upon CHIKV, but not DENV, infection

Finally, we asked whether *Dcr2* could have an impact on mosquito survival after exposure to arboviruses via an infectious blood meal. We monitored the survival of *Dicer2*^{R172fsX} mutants and control mosquitoes after oral exposure to 10^6 PFU/mL of CHIKV or 5×10^6 FFU/mL of DENV-3 and included a non-infectious blood meal as a negative control. We verified on day 7 post blood meal that most of the mosquitoes (>94% across conditions) were virus positive (Figure S7). The survival rates of control mosquitoes did not differ after CHIKV exposure or mock exposure ($p = 0.09$; Figure 6A). In contrast, the survival rate of *Dicer2*^{R172fsX} mutants was significantly reduced after CHIKV exposure relative to the mock exposure control ($p < 0.0001$; Figure 6B). Median survival time was 10 days upon CHIKV infection, compared with 18.5 days upon mock infection. Thus, we concluded that *Dcr2* was required to prevent virus-induced mortality during CHIKV infection. For DENV-3, the survival rates of control mosquitoes slightly increased after virus exposure relative to mock exposure ($p = 0.01$; Figure 6C). However, we did not detect any difference in the survival rates of *Dicer2*^{R172fsX} mutants after DENV-3 exposure or mock infection ($p = 0.63$; Figure 6D). These data indicate that *Dcr2* does not influence mosquito survival during infection with DENV-3. Taken together, our survival analyses reveal different consequences of *Dcr2* absence on mosquito survival upon DENV-3 or CHIKV infection. Whereas *Dcr2* is required to prevent virus-induced mortality upon CHIKV infection, this is not the case upon DENV-3 infection.

DISCUSSION

The siRNA pathway is considered a major, broad-spectrum antiviral pathway in insects.[36] The antiviral activity of the siRNA pathway is well established in mosquito vectors such as *Ae. aegypti* and thus represents a potential key target in the development of novel arbovirus control strategies aimed at developing lab-engineered mosquitoes incapable of transmitting viral pathogens. Several earlier studies have characterized the molecular components and function of the siRNA pathway in *Ae. aegypti*, but surprisingly little is known of its effective impact on arbovirus transmission. Here, we assessed the effects of genetically inactivating the siRNA pathway on infection dynamics and transmission potential of several arboviruses of medical significance.

Our *Dicer2*^{R172fsX} mutant line is viable and fertile, demonstrating that *Dcr2* is not an essential gene in *Ae. aegypti*, at least not in the genetic background of our Gabonese mosquito colony. The *Dicer2*^{R172fsX} mutant line does exhibit modest fitness defects, such as slower development, increased pupal mortality, and smaller adult body size. How exactly *Dcr2* relates to these fitness defects is currently unknown and deserves further investigation. In *Drosophila*, *Dcr2* plays a key role in the somatic control of transposable elements (TEs).[37,38] A recent study demonstrated that TE activity increases mortality and accelerates aging phenotypes in *Drosophila*.[39] It will be interesting in future studies to characterize TE and gene expression patterns in the *Ae. aegypti Dicer2*^{R172fsX} line.

Although we confirmed that the *Dicer2*^{R172fsX} mutant line does not produce siRNAs and displays impaired dsRNA-triggered silencing activity, we found that the loss of *Dcr2* had a limited impact on viral replication and infection dynamics of arboviruses acquired via an infectious blood meal. In fact, the antiviral effect of *Dcr2* could be detected only at the early stage of midgut infection and dissemination out of the midgut. *Dcr2* was not required for controlling viral replication during the later stage of infection when the virus has already disseminated throughout the mosquito body. Accordingly, the absence of *Dcr2* did not significantly affect systemic replication of the insect-specific virus CFAV following intrathoracic inoculation. We did not detect major changes in vector competence, estimated as the overall proportion of virus-exposed mosquitoes that eventually became infectious (transmission efficiency). Note that some of our

Figure 4. *Dcr2* hinders early infection dynamics of flaviviruses

(A–E) Experimental scheme (A) for the analysis of DENV-3 and ZIKV infection dynamics. Adult *Dicer2*^{R172fsX} mutants and control female mosquitoes were exposed to an infectious blood meal containing 5×10^6 FFU/mL of DENV-3 or 10^6 PFU/mL of ZIKV. Midguts, carcasses, and heads from the same mosquitoes were collected on days 5 and 10 post exposure. Saliva was collected only 10 days post exposure with ZIKV. Infection prevalence and DENV-3 titers were determined by qRT-PCR 5 (B) and 10 (C) days post exposure. Infection prevalence and ZIKV titers were determined by plaque assay 5 (D) and 10 (E) days post exposure. Data from two experimental replicates were combined and depicted on the graph using two shades of the same color (gray/black for control mosquitoes, pink/red for *Dicer2*^{R172fsX} mutants). Viral loads are plotted only for virus-positive mosquitoes. Each experimental condition is represented by 40–50 mosquitoes (B and C, DENV-3) or 42–56 mosquitoes (D and E, ZIKV). The statistical significance of pairwise comparisons shown (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$) was obtained with Student's t test after correction for the variation between experiments (viral loads) or a chi-squared test (prevalence). The full statistical analyses are provided in Figure S5A (DENV-3) and Figure S5B (ZIKV).

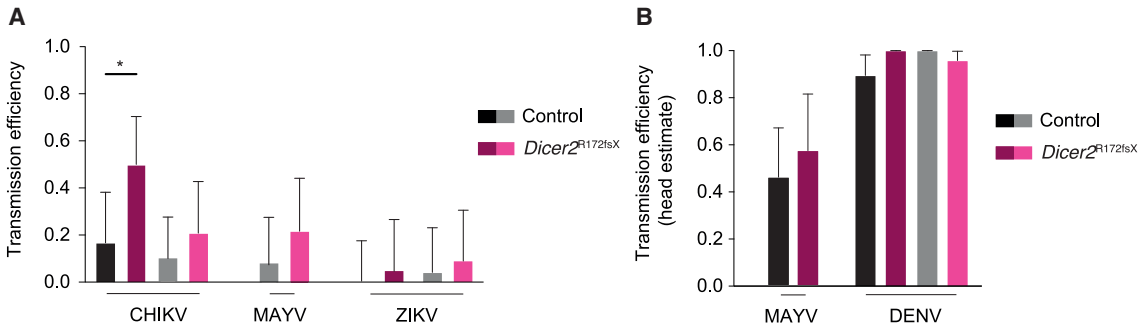

Figure 5. *Dcr2* does not significantly affect overall vector competence for arboviruses

(A) Transmission efficiency (the overall proportion of virus-exposed females with virus-positive saliva) is shown for each experimental replicate of CHIKV (Figure 3) and ZIKV (Figure 4), and the second replicate of MAYV infection (Figure 3).

(B) A proxy for transmission efficiency (the overall proportion of virus-exposed females with virus-positive heads) is shown for both experimental replicates of DENV-3 infection (Figure 4) and the first replicate of MAYV infection (Figure 3) because saliva samples were not available. Error bars represent the 95% confidence intervals of the proportions. Statistical significance of the difference in proportions was determined with Fisher's exact test (*$p < 0.05$).

transmission efficiency estimates could have been overestimated because they relied on virus detection in head tissues, not in saliva; however, our comparisons between the mutant and the control lines were always based on the same measurement. In addition, the loss of *Dcr2* significantly increased virus-induced mortality during CHIKV infection, but not DENV-3 infection. Reduced survival of infected mosquitoes is predicted to strongly decrease arbovirus transmission, because only long-lived mosquitoes can effectively contribute to it.[23]

Our study demonstrated a modest but detectable impact of *Dcr2* on the control of arbovirus replication *in vivo* in *Ae. aegypti* mosquitoes. Most previously published studies relied on the use of gene knockdown assays to assess the contribution of siRNA pathway components to virus replication control.[16–18] The gene knockdown approach is limited by its intrinsic variability and efficiency in targeting gene expression. Thus, some of the previous studies were inconclusive because they could not disambiguate insufficient knockdown efficiency from a true lack of biological effect. Our *Dicer2*^{R172fsX} mutant line allowed us to rule out such technical limitations and assess the effective contribution of *Dcr2* to the control of viral replication. Our results conclusively show that the impact of *Dcr2* on viral replication is transient and mainly limited to the midgut tissue. A previous study reported that the siRNA pathway was not required for controlling DENV replication in the midgut,[20] based on gene knockdown of *Ago2*. It is possible that gene knockdown did not provide sufficient sensitivity to detect the minor contribution of the siRNA pathway to control DENV replication in the midgut. Alternatively, it is possible that the antiviral role we observed for *Dcr2* in the midgut is independent of the downstream factors of the siRNA pathway. Dcr2 is an RNA sensor that acts upstream of Ago2 and is possibly involved in other branches of antiviral immunity that affect viral replication in the midgut.[18,22,40–42]

Despite its modest impact on viral titers, we found that *Dcr2* had a significant role in controlling the rate of viral dissemination from the midgut to the rest of the mosquito body. We found that the absence of *Dcr2* promoted viral spread from the midgut to the mosquito hemocoel, as indicated by a higher proportion of virus-positive carcasses during the early stage of infection. This is consistent with previous studies reporting that silencing *Dcr2* and *Ago2* accelerated systemic dissemination of DENV.[17,20] The mechanistic basis of this observation is still unclear, but we speculate that, for some viruses, such as CHIKV, the slight increase in midgut viral titers we observed in the *Dicer2*^{R172fsX} mutant line may allow an earlier viral exit of the midgut tissue. However, higher rates of early dissemination were also observed in the case of MAYV infection, in the absence of increased midgut viral titers. The mechanisms of virus spread from the midgut to the hemocoel remain unknown and might involve active virus replication in circulating cell populations such as hemocytes.[43] The putative role of *Dcr2* in controlling virus replication in these cells, in particular phagocytes, remains to be investigated.

Together with a recent study,[21] we provide evidence for a protective role for *Dcr2* against virus-induced mortality during arbovirus infection of *Ae. aegypti*. Virus-induced mortality is generally modest during arbovirus infection of mosquitoes[44,45] and, indeed, we found that CHIKV or DENV infection had no major effect on the survival of control mosquitoes. In the absence of *Dcr2*, however, the median lifespan of mosquitoes was almost halved following CHIKV infection. It is worth noting that this protective effect benefits both the host and the virus at an epidemiological level. Indeed, viral fitness is positively correlated with mosquito lifespan because the amount of transmission depends on (1) the probability that the mosquito survives through the extrinsic incubation period and (2) the life expectancy of the mosquito after becoming infectious.[46] We did not observe a similar protective effect of *Dcr2* against virus-induced mortality for DENV, and whether this discrepancy is virus-strain specific or genus specific remains to be determined. In the case of CHIKV, the increased viral loads observed in the midgut of mutant mosquitoes during the early stage of infection could be directly responsible for the higher virus-induced mortality, due to increased tissue damage, for example. Alternatively, *Dcr2* could be necessary to prevent virus-induced mortality without directly acting on viral replication,

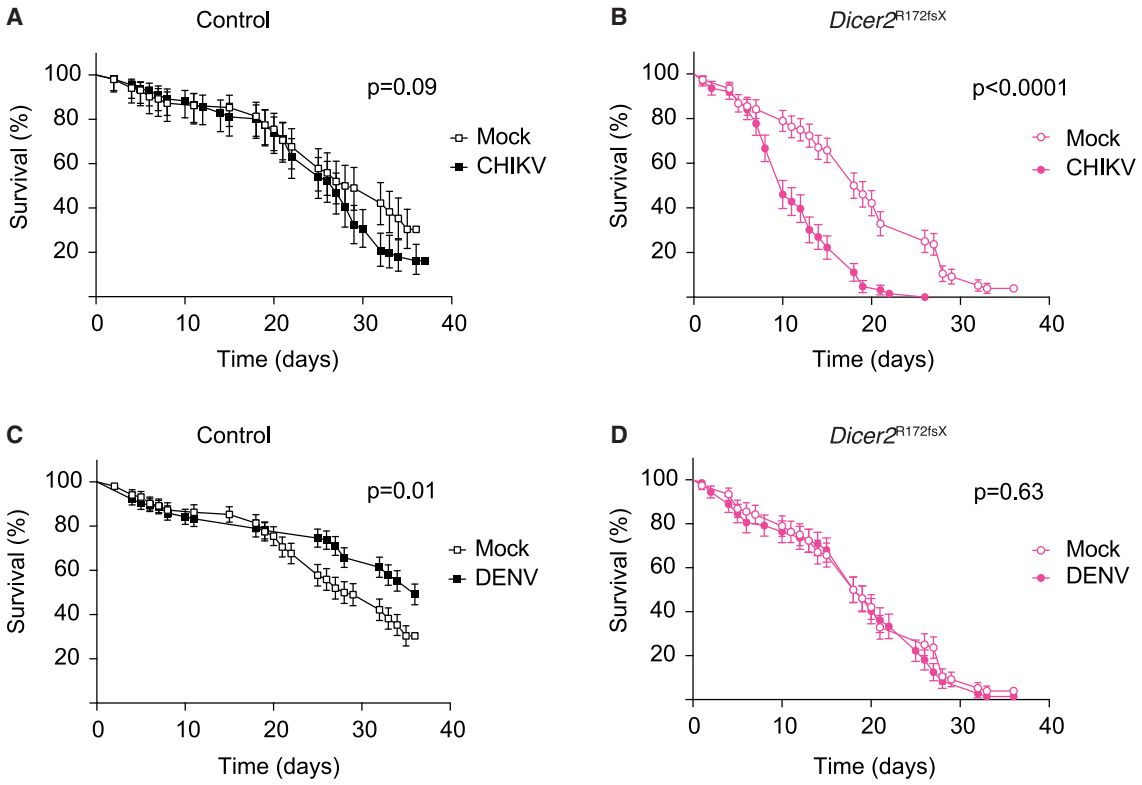

Figure 6. *Dcr2* prevents virus-induced mortality during CHIKV infection but not DENV-3 infection

(A–D) Adult female mosquitoes of the control line (A and C) or *Dicer2*^{R172fsX} mutant line (B and D) were exposed to an infectious blood meal containing 10^6 PFU/mL of CHIKV (A and B), 5×10^6 FFU/mL of DENV-3 (C and D), or a non-infectious blood meal (mock). Data are shown as the mean and SD of three replicates represented by 21–38 mosquitoes each. Mortality was scored daily, and fresh pads containing 10% sucrose solution were renewed biweekly. Statistical significance of the differences was determined by Kaplan-Meier analysis and Gehan-Breslow-Wilcoxon tests. See also Figure S7 for corresponding mosquito infection levels.

a defense mechanism often referred to as tolerance.[47] This could be through activating signaling pathways that enhance tissue repair[48] or limiting tissue damage upon infection, such as apoptosis.[49] The contrasted outcome observed with DENV could reflect differences in the physiopathology of DENV infection in mosquito tissues or differences in the immune or tissue repair pathways activated by viral infection. More broadly, we observed that the loss of *Dcr2* affected each arbovirus slightly differently. This could be linked to different tissue tropisms of arboviruses, which remain poorly characterized to date, or to differential activation of other antiviral immune pathways that have virus-specific effects in *Ae. aegypti*.[50]

In conclusion, our study sheds new light on the effective contribution of *Dcr2* to arbovirus transmission by *Ae. aegypti* mosquitoes. Using a panel of arboviruses, we demonstrated the role of *Dcr2* in controlling early viral replication and in restricting early systemic viral spread for both flaviviruses and alphaviruses. Overall, however, *Dcr2* was not a major determinant of vector competence based on our estimates of transmission efficiency. We also discovered that *Dcr2* was involved in preventing virus-induced mortality during CHIKV infection, but not DENV infection. Finally, we anticipate that our *Dicer2*^{R172fsX} mutant line will be instrumental in advancing basic research on the role of sRNA pathways in mosquito biology. It will be an important tool to investigate the mechanisms underlying essential processes such as somatic TE control, antiviral defense in the germline, and possibly immune priming and memory.

Limitations of the study

The *Dicer2*^{R172fsX} mutant line carries CRISPR-Cas9-induced DNA mutations causing an early stop codon in the *Dcr2* sequence. Several lines of evidence support the conclusion that this line is a *bona fide* loss-of-function mutant of the siRNA pathway. The mutant *Dcr2* allele is associated with reduced transcript levels consistent with nonsense-mediated decay of the aberrant mRNA. Analysis of sRNA production revealed a lack of both exogenous and endogenous siRNAs, and the *in vivo* RNAi reporter assay detected impaired silencing. Finally, mosquito survival was severely diminished upon CHIKV infection. However, we note that we cannot fully rule out the possibility that the *Dicer2*^{R172fsX} allele is hypomorphic (i.e., causing only a partial loss of function) due to some residual activity of the truncated protein. Further studies investigating Dcr2 activity at the protein level will be necessary to conclusively exclude this unlikely possibility.

The focus of our study was to evaluate the impact of the siRNA pathway on arbovirus transmission by *Ae. aegypti*. We confirmed that our *Dicer2*^{R172fsX} mutant line was incapable of producing siRNAs, but could not determine whether the production of viral piwi-interacting RNAs (piRNAs) was affected, because they were not detectable at the early time points we analyzed (days 5 and 6 post infection by CHIKV and DENV, respectively).[20,51] Given that the *Aedes* piRNA pathway processes non-canonical substrates such as viral RNA,[52–54] additional investigations are required to assess whether increased viral piRNA production compensates for the loss of siRNA production and to examine the interplay between the siRNA and the piRNA pathways.

We genetically modified the *Dcr2* locus in mosquitoes that were originally collected in Bakoumba, Gabon, and belong to the African subspecies *Ae. aegypti formosus (Aaf)*. *Aaf* mosquitoes are known to be poorly susceptible to flavivirus infection, especially ZIKV infection.[55] This might explain why the loss of *Dcr2* activity in this mosquito population caused only subtle changes in viral infection phenotypes. To assess the influence of the mosquito genetic background on the antiviral function of the siRNA pathway, it will be interesting in future studies to introduce the *Dicer2*^{R172fsX} mutation into a more susceptible mosquito genotype.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Cells
  - Virus strains
  - Mosquito rearing
- METHOD DETAILS
  - Ethics statement
  - Mosquito genetic modification
  - *Dicer2* expression measurement
  - Mosquito exposure to infectious blood meals
  - Virus quantification
  - Mosquito fitness assays
  - RNAi reporter assay
  - Small RNA analysis
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2023.112977.

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AUTHOR CONTRIBUTIONS

S.H.M., A.B.C., A.H.-L., E.C., A. Bergman, O.R., V.G., A. Baidaliuk, H.B., L.F., M.-C.S., and L.L. designed the experiments; S.H.M., A.B.C., A.H.-L., E.C., A. Bergman, O.R., V.G., A. Baidaliuk, and H.B. performed the experiments; S.H.M., A.B.C., A.H.-L., E.C., A. Bergman, O.R., V.G., A. Baidaliuk, H.B., L.F., L.L., and M.-C.S. analyzed the data; S.H.M. and L.L. wrote the paper with input from the co-authors. All authors reviewed and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Dengue Virus Complex Antibody, clone D3-2H2-9-21	Merck-Milipore	MAB8705; RRID: AB_95338
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488	Life Technologies	A-11029; RRID: AB_2534088
Bacterial and virus strains		
DENV-1 KDH0026A	Ref. #56	GenBank: HG316481
DENV-3 GA28-7	Ref. #57	GenBank: JX080299.1
ZIKV FSS13025	Ref. #58	N/A
CHIKV M105	Ref. #59	GenBank: LN898104.1
MAYV TRVL 4675	Ref. #60	N/A
CFAV-KPP	Ref. #61	ENA: LR596014
Chemicals, peptides, and recombinant proteins		
Cas9 Nuclease, *S. pyogenes*	New England Biolabs	Cat#M0646
Critical commercial assays		
DNAzol DIRECT	Molecular Research Center, Inc.	Cat#DN131
TRIzol Reagent	Thermo Fisher Scientific	Cat#15596026
DreamTaq Green DNA Polymerase	Thermo Fisher Scientific	Cat#EP0714
MEGAscript T7 *in vitro* transcription kit	Life Technologies	Cat#AM1333
M-MLV Reverse Transcriptase	Thermo Fisher Scientific	Cat#28025013
RNaseOUT Recombinant Ribonuclease Inhibitor	Thermo Fisher Scientific	Cat#10777019
GoTaq Probe 1-Step RT-qPCR System	Promega	Cat#A6002
MEGAscript RNAi kit	Life Technologies	Cat#AM1626M
NEBNext Multiplex Small RNA Library Prep Set for Illumina	New England Biolabs	Cat#E7300L
Universal miRNA Cloning Linker	New England Biolabs	Cat#S1315S
Dual-Luciferase Reporter Assay System	Promega	Cat#E1910
MinElute PCR Purification Kit	Qiagen	Cat#2552893
Cellfectin II	Thermo Fisher Scientific	Cat#10362100
DNAseI	Thermo Fisher Scientific	Cat#AM1906
GoTaq 1-Step RT-qPCR System	Promega	Cat#A6020
Deposited data		
Small RNA sequencing datasets	This paper	SRA: PRJNA907782
Experimental models: Cell lines		
Aedes albopictus C6/36	ATCC	CRL-1660
Vero E6	ATCC	CRL-1586
Experimental models: Organisms/strains		
Aedes aegypti outbred colony from Bakoumba	Ref. #31	N/A
Aedes aegypti Dicer2 (−/−) mutant line derived from outbred colony	This study	N/A
Aedes aegypti Dicer2 (+/+) sister line derived from outbred colony	This study	N/A
Oligonucleotides		
All oligonucleotide sequences are listed in Table S1		N/A
Recombinant DNA		
pUb-GL3 plasmid	Ref. #15	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
pCMV-Rluc plasmid	Ref. #15	N/A
Software and algorithms		
FastQC	http://www.bioinformatics.babraham.ac.uk/projects/fastqc/	N/A
Cutadapt	Ref. #68	N/A
Bowtie1	Ref. #69	N/A
Rsamtools v1.20.4 (R package)	Ref. #71	N/A
ShortRead v1.26.0 (R package)	Ref. #72	N/A
Graphpad Prism v.9.3.1	www.graphpad.com	N/A
JMP v14.0	www.jmp.com	N/A
CRISPOR	Ref. #62	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, Louis Lambrechts (louis.lambrechts@pasteur.fr).

Materials availability

Research materials generated in this study are available upon request.

Data and code availability

- All sRNA sequencing data are available at SRA: PRJNA907782.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cells

C6/36 cells (derived from *Aedes albopictus*) were cultured in Leibovitz's L-15 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 1% non-essential amino acids (Life Technologies), 2% Tryptose Phosphate Broth (Life Technologies) and 1% Penicillin/Streptomycin (P/S, Life Technologies) at 28°C without additional CO_2. Vero E6 cells (ATCC CRL-1586) were cultured in Dulbecco's modified Eagle's medium (DMEM) GlutaMAX® (Life Technologies) containing 10% FBS (Invitrogen) and 1% P/S (Life Technologies) at 37°C with 5% CO_2.

Virus strains

DENV-1 strain KDH0026A was originally isolated in 2010 from the serum of a patient in Kamphaeng Phet, Thailand.[56] DENV-3 strain GA28-7 was originally derived in 2010 from the serum of a patient in Moanda, Gabon.[57] ZIKV strain FSS13025 was originally isolated in 2010 from the serum of a patient in Cambodia.[58] CHIKV isolate M105 was originally derived in 2014 from the serum of a patient in Martinique.[59] MAYV strain TRVL 4675 was derived from a previously described infectious clone.[60] Viral stocks were prepared in C6/36 cells for DENV and ZIKV and Vero E6 cells for CHIKV and MAYV. The wild-type CFAV strain CFAV-KPP was isolated from mosquito homogenates and produced from an RNA template diluted in Opti-MEM™ Reduced Serum Medium (Thermo Fisher Scientific) as previously described.[61]

Mosquito rearing

Experiments were conducted with *Ae. aegypti* mosquitoes derived from a wild-type colony originally from Bakoumba, Gabon[31]. Mosquitoes were reared under controlled conditions (28°C, 12-hour light/12-hour dark cycle and 70% relative humidity). Prior to performing the experiments, their eggs were hatched synchronously in a SpeedVac vacuum device (Thermo Fisher Scientific) for one hour. Their larvae were reared in plastic trays containing 1.5 L of tap water and supplemented with Tetramin (Tetra) fish food at a density of 200 larvae per tray. After emergence, adults were kept in BugDorm-1 insect cages (BugDorm) with permanent access to 10% sucrose solution.

METHOD DETAILS

Ethics statement

Mosquito artificial infectious blood meals were prepared with human blood. Blood samples were supplied by healthy adult volunteers at the ICAReB biobanking platform (BB-0033-00062/ICAReB platform/Institut Pasteur, Paris/BBMRI AO203/[BIORESOURCE]) of the Institut Pasteur in the CoSImmGen and Diagmicoll protocols, which had been approved by the French Ethical Committee Ile-de-France I. The Diagmicoll protocol was declared to the French Research Ministry under reference 343 DC 2008-68 COL 1. All adult subjects provided written informed consent. Genetic modification of *Ae. aegypti* was performed under authorization number 7614 from the French Ministry of Higher Education, Research and Innovation.

Mosquito genetic modification

The *Dicer2* gene (*AAEL006794*) was targeted using CRISPR-Cas9-mediated gene editing, using a protocol adapted from a previously described method.[30] Gene-targeting reagents were injected into the eggs of a laboratory *Ae. aegypti* colony originally from Bakoumba, Gabon[31]. Single-guide RNAs (sgRNAs) were designed using CRISPOR[62] by searching for sgRNAs of 20 bp in length with the NGG protospacer-adjacent-motif (PAM). To reduce chances of off-target mutations, and give preference to highly specific sgRNAs, only guides with off-targets that contained 3 or more mismatches were selected. sgRNAs were produced exactly as previously described.[30] The final injection mix contained 300 ng/μL spCas9 protein (M0386, New England Biolabs), 40 ng/μL of each sgRNA (3 targeting exon 5 and 7 of *Dicer2* and 4 targeting the *kmo* gene, *AAEL008879*; sequences provided in Table S1). Embryos were injected 30-60 min after egg laying using a FemtoJet (Eppendorf) equipped with microinjection quartz needles (QF100-70-10, Sutter Instrument), and handled according to a standard protocol.[63] Embryos were hatched 3 days post injection and reared to adulthood according to standard rearing procedures. When disrupted, *kmo* produces a mosaic red-eye phenotype in G_0 adults. We used this phenotype to easily verify CRISPR-Cas9 activity and screen for *Dicer2* mutations in red-eyes G_0 mosquitoes preferentially. Genomic DNA was extracted from single legs of individual mosquitos using NucleoSpin DNA Insect Kits (Machery-Nagel) or DNAzol DIRECT (Molecular Research Center, Inc.), according to manufacturer's instructions. *Dicer2* mutations were first tracked by PCR using DreamTaq DNA Polymerase (Thermo Fisher Scientific) and subsequent restriction enzyme assay of exon 5 (with BSaaI, NEB) and exon 7 (BSErI, NEB). PCR amplicons were also purified with MinElute PCR Purification Kit (Qiagen) and subjected to Sanger sequencing for sequence verification (sequences provided in Table S1). The initial *Dicer2* mutant mosquito (G_0) that founded the *Dicer2*^{R172fsX} mutant line was a male individual with mosaic red eyes (indicating mutation in the *kmo* gene), and mutations in both exon 5 and 7 of the *Dicer2* gene. Only the mutation in exon 5 that led to an early stop codon was tracked throughout subsequent crosses. The G_0 founder mutant male was outcrossed to 8 wild-type females from the Bakoumba colony. The first generation (G_1) inherited the *Dicer2* mutation, but did not display the red-eye phenotype, indicating non-inheritance of the *kmo* mutation. A single G_1 male heterozygous for the *Dicer2* mutation was outcrossed to 10 wild-type females. For the subsequent cross, a brother-sister mating was performed using 13 and 10 heterozygous G_2 males and females, respectively. This cross was intended to be the final cross to establish two G_3 lines: (1) a "sister line" composed of individuals homozygous for the wild-type allele to be used as a control for the crossing scheme, and (2) a homozygous *Dicer2* mutant line. At this point, the "sister line" was successfully established with 26 males and 43 females homozygous for the wild-type allele in exon 5 of *Dicer2* and maintained separately. Due to low numbers of viable homozygous *Dicer2* mutant mosquitoes, an additional "mutant-enriched" G_3 cross (6 homozygous mutant males mated with 3 homozygous mutant females, and 4 heterozygous females) was set up. The eggs from this cross were combined and hatched along with eggs from a pure heterozygous G_3 cross (35 males and 33 females) to produce the G_4 generation. Next, 7 males and 9 females homozygous for the *Dicer2* exon 5 mutation were crossed, establishing the homozygous mutant line. To ensure lack of contamination of the *Dicer2* mutant homozygous and control "sister" lines, at least 20 individuals from each generation have been routinely collected for PCR and Sanger sequencing surrounding the mutation site on exon 5 of the *Dicer2* gene. Throughout this article, the "sister line" is referred to as the control line, and the *Dicer2* mutant homozygous line is referred to as *Dicer2*^{R172fsX} line.

Dicer2 expression measurement

Mosquitoes reared in standard insectary conditions were collected 5 to 8 days post adult emergence. Three pools of 20 midguts or 20 thoraces from female mosquitoes were dissected in 1× PBS and transferred to a tube containing 500 μL of Trizol (Thermo Fisher Scientific) and ~20 1-mm glass beads (BioSpec). Samples were homogenized for 30 sec at 6,000 rpm in a Precellys 24 grinder (Bertin Technologies). RNA was extracted as previously described,[64] and treated with DNase I (Thermo Fisher Scientific) according to the manufacturer's instructions. *Dcr2* transcripts were quantified by qRT-PCR using the GoTaq® 1-Step RT-qPCR System (Promega) following the manufacturer's instructions. *Dcr2* RNA levels were normalized to the transcript abundance of the housekeeping gene *ribosomal protein subunit 17* (*RPS17*), and expressed as 2^{-dCt}, with $dCt = Ct^{Dicer2} - Ct^{RPS17}$.

Mosquito exposure to infectious blood meals

Experimental infections of mosquitoes were performed in a biosafety level-3 containment facility, as previously described.[65] Shortly, 5- to 7-day-old female mosquitoes were deprived of 10% sucrose solution 24 hours before oral exposure to viruses. The infectious blood meal consisted of a 2:1 mix of washed human erythrocytes and viral suspension supplemented with 10 mM ATP (Sigma). The infectious titers were 10^7 FFU/mL for DENV-1, 5×10^6 FFU/mL for DENV-3, 10^6 PFU/mL for ZIKV, 10^7 PFU/mL for MAYV,

and 10^6 PFU/mL for CHIKV. Mosquitoes were offered the infectious blood meal for 15 min through a desalted pig-intestine membrane using an artificial feeder (Hemotek Ltd) set at 37°C. Fully engorged females were incubated at 28°C, 70% relative humidity and under a 12-hour light-dark cycle with permanent access to 10% sucrose.

Virus quantification

CFAV intrathoracic inoculation and RNA quantification

Mosquitoes were injected intrathoracically with 50 $TCID_{50}$ units/mosquito of the CFAV-KPP strain using previously described methods.[66] Mosquitoes were collected on days 0, 2, 4, 6, 8 post inoculation and processed in 3 rounds of 4 samples per condition (inoculum × mosquito line × time point). Viral RNA was quantified by qRT-PCR as previously described.[66] CFAV RNA levels were normalized to the transcript abundance of the housekeeping gene *ribosomal protein 49* (*RP49*), and expressed as 2^{-dCt}, with $dCt = Ct^{CFAV} - Ct^{RP49}$.

DENV RNA quantification

Mosquito body parts and organs were dissected in 1× PBS, and immediately transferred to a tube containing 800 μL of Trizol (Thermo Fisher Scientific) and ~20 1-mm glass beads (BioSpec). Samples were homogenized for 30 sec at 6,000 rpm in a Precellys 24 grinder (Bertin Technologies). RNA was extracted as previously described,[64] and stored at -80°C till further use. Viral RNA was reverse transcribed and quantified using a TaqMan qPCR assay, using DENV NS5-specific primers and 6-FAM/BHQ-1 double-labeled probe (sequences provided in Table S1). Reactions were performed with the GoTaq® Probe 1-Step RT-qPCR System (Promega) following the manufacturer's instructions. The limit of detection of the assay was 10 copies of viral RNA per μL.

Virus titration

For CHIKV, MAYV and ZIKV, infectious titration was performed on confluent Vero E6 cells plated in 24-well plates, 24 hours before inoculation. Ten-fold dilutions were prepared in DMEM alone and transferred onto Vero E6 cells. After allowing infection, DMEM with 2% FCS, 1% P/S and 0.8% agarose was added to each well. Cells were fixed with 4% formalin (Sigma) after two (MAYV), three (CHIKV) or seven (ZIKV) days of incubation and plaques were manually counted after staining with 0.2% crystal violet (Sigma). DENV infectious titers were measured by standard focus-forming assay (FFA) in C6/36 cells as previously described.[65]

Salivation assays

Mosquitoes were anesthetized on ice and wings and legs were removed from each individual. Proboscis was inserted into a 20-μL tip containing 20 μL of FBS for 30 min at room temperature (20-25°C). Saliva-containing FBS was expelled into 90 μL of Leibovitz's L-15 medium (Gibco) for amplification and titration. The other tissues of interest (midgut, carcass, head) were dissected after salivations. Virus presence in saliva samples was determined by virus titration of cell culture supernatant after 5 days of amplification in C6/36 cells. Transmission potential was assessed qualitatively based on the presence or absence of infectious virus.

Mosquito fitness assays

Survival assay

Mosquito were kept in 1-pint carton boxes with permanent access to 10% sucrose solution at 28°C and 70% relative humidity and mortality was scored daily. Experiments were performed in 3 to 4 replicate boxes of 21 to 38 mosquitoes each.

Mosquito weighing

Five- to-seven-day old male and female mosquitoes were collected and starved for 24 hours prior to weighing to ensure that no sugar water remained in their digestive tracts. Mosquitoes were weighed in groups of 5 individuals of a single sex on a precision scale. Ten replicates were performed. Results are expressed as mass per individual mosquito.

Pupation rates

Mosquito eggs were hatched for 30 min in a SpeedVac vacuum device (Thermo Fisher Scientific). Two hundred larvae were counted and transferred to a standard rearing tray containing 1 L of tap water and a standard dose of fish food. Three to four separate trays were prepared for each mosquito line (control and *Dicer2* mutant). After 4-5 days of larval development, a daily count of the number of pupae (live and dead) was performed. The percentage of live and dead pupae was calculated relative to the total number of pupae recorded.

RNAi reporter assay

Double-stranded RNA synthesis

Design and synthesis of dsRNA used in RNAi reporter assays has been described previously.[64] Briefly, dsRNA was synthesized from a GFP- or luciferase-containing plasmid. T7 promotor sequences were incorporated by PCR to the amplicon that was used as a template for the synthesis using the MEGAscript RNAi kit (Life Technologies). Primer sequences are provided in Table S1.

RNAi activity read-out

RNAi activity in adult mosquitoes was tested using a reporter assay described previously.[34] In short, 5 to 7-day-old adult female mosquitoes were intrathoracically injected using a Nanoject III (Drummond) with a suspension of ~200 nL containing a 1:1 mixture of unsupplemented Leibovitz's L-15 medium (Life technologies) and Cellfectin II (Thermo Fisher Scientific) complexed with 50 ng pUb-GL3 (encoding firefly luciferase, Fluc), 50 ng pCMV-Rluc (encoding *Renilla* luciferase, Rluc), and 500 ng Fluc- or 1 μg GFP-specific specific dsRNA. After incubation for 3 days at 28°C, mosquitoes were homogenized in passive lysis buffer (Promega) using the Precellys 24 grinder (Bertin Technologies) for 30 sec at 6,000 rpm. Samples were transferred to a 96-well plate and centrifugated for

5 min at 12,000 × g. Fifty μL of supernatant were transferred to a new plate, and 50 μL LARII reagent added for the first Fluc measurement. Next, 50 μL Stop&Glow reagent was added before the second measurement of Rluc, according to the Dual Luciferase assay reporter system (Promega). Counts of Rluc were used to control for transfection efficiency, and samples with less than 1,000 counts were discarded from further analysis. Data were normalized by calculating the Fluc/Rluc ratio.

Small RNA analysis

Small RNA library preparation and sequencing

Total RNA from pools of 5 to 8 mosquitoes was isolated with TRIzol (Invitrogen). Small RNAs of 19-33 nucleotides in length were purified from a 15% acrylamide/bisacrylamide (37.5:1), 7 M urea gel as described previously.[67] Purified RNAs were used for library preparation using the NEB Next Multiplex Small RNA Library Prep kit for Illumina (E7300 L) with Universal miRNA Cloning Linker from Biolabs (S1315S) as the 3' adaptor and in-house designed indexed primers. Libraries were diluted to 4 nM and sequenced using an Illumina NextSeq 500 High Output kit v2 (75 cycles) on an Illumina NextSeq 500 platform. Sequence 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' (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Quality and adaptors were trimmed from each read using 'cutadapt' (https://cutadapt.readthedocs.io/en/stable/). Only reads with a phred score $\geq$20 were retained. A second set of graphs was generated by 'FastQC' on the fastq files created by 'cutadapt'.[68] Mapping was performed by 'Bowtie1'[69] with the '-v 1' option (one mismatch between the read and its target) using the following reference sequences: GenBank accession number HG316481 for DENV-1, GenBank accession number LN898104.1 for CHIKV, and positions 161,180,000-161,236,000 of chromosome 3 in the AaegL5 genome built[70] for the histone transcript cluster. 'Bowtie1' generates results in 'sam' format. All 'sam' files were analyzed by different tools of the package 'samtools'[71] to produce 'bam' indexed files. To analyze these 'bam' files, different kinds of graphs were generated using home-made R scripts with several Bioconductor libraries such as 'Rsamtools' and 'ShortReads'.[72]

QUANTIFICATION AND STATISTICAL ANALYSIS

Infection prevalence was analyzed as a binary variable by logistic regression and non-zero viral loads (infectious titers and RNA concentrations) were compared by analysis of variance (ANOVA) after $\log_{10}$ transformation. Each primary variable was analyzed with a full-factorial model including interactions up to the 2nd order. Effects were considered statistically significant when $p < 5\%$. The full test statistics are provided in Figures S4 and S5. Because most of experiments were repeated multiple times, uncontrolled variation between experiments was accounted for in the statistical analyses. For graphical representations of viral loads, the data from different experiments were displayed in different colors. To show the statistical significance of pairwise comparisons the figures, a Student's t-test or Mann-Whitney test was applied to the raw values after correction of the variation between experiments. This correction transforms the raw values into their deviation from the experimental mean, and the resulting values are centered around zero on the original scale. For the RNAi reporter assay, the graphical representation used the mean-centered values (after correction of the experiment effect) of the $\log_{10}$-transformed Fluc/Rluc ratios. Estimates of transmission efficiency were compared with Fisher's exact test. Pupation rates over time were analyzed with a 4-parameter model. The comparison of survival curves was performed using a Gehan-Breslow-Wilcoxon test. All statistical analyses were performed in JMP v.14.0.0 (www.jmp.com) or Prism v.9.3.1 (www.graphpad.com).