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# Dicer-2 regulates resistance and maintains homeostasis against Zika virus infection in *Drosophila*

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## Abstract

Zika virus (ZIKV) outbreaks pose a massive public health threat in several countries. We have developed an *in vivo* model to investigate the host-ZIKV interaction in *Drosophila*. We have found that a strain of ZIKV replicates in wild-type flies without reducing their survival ability. We have shown that ZIKV infection triggers RNA interference, and that mutating *Dicer-2*, results in enhanced ZIKV load and increased susceptibility to ZIKV infection. Using a flavivirus-specific antibody, we have found that ZIKV is localized in the gut and fat body cells of the infected wild-type flies and results in their perturbed homeostasis. In addition, *Dicer-2* mutants display severely reduced insulin activity, which could contribute towards the increased mortality of these flies. Our work establishes the suitability of *Drosophila* as the model system to study host-ZIKV dynamics, which is expected to greatly advance our understanding of the molecular and physiological processes that determine the outcome of this disease.

## Introduction

Zika virus (ZIKV) is a hitherto understudied member of the *Flaviviridae*, a viral family that also includes yellow fever, West Nile virus (WNV) and Dengue virus (DENV) (1). In recent years, due to the wide geographical distribution of the mosquito vector, ZIKV suddenly expanded its range dramatically and severe outbreaks appeared in the Americas and other parts of the world (2). Being a vector-borne virus, ZIKV-induced outbreaks are difficult to control. As vector control is the only viable alternative for alleviating the disease, a thorough understanding of host-ZIKV interaction is critical. Thus, there is an urgent need to develop an *in vivo* model for identifying and characterizing the number and types of molecular components that directly or indirectly participate in the host immune response against ZIKV. Since host innate immune responses are evolutionary conserved across many phyla (3), investigating the effect of ZIKV infection on the immune signaling and function of animal

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models will be particularly insightful because it could potentially lead to the identification of anti-ZIKV immune mechanisms in humans.

The use of the fruit fly *Drosophila melanogaster* has led to significant advances in the characterization of the molecular events leading to the activation of immune responses against infectious microorganisms, including viral pathogens (4, 5). Apart from those viruses that naturally infect *Drosophila* (6), previous work indicates that the fly is also a suitable model for dissecting host interactions with human pathogenic viruses including ZIKV (7, 8). *Drosophila* for instance was instrumental in deciphering antiviral immune mechanisms against Sindbis virus (SINV), Vesicular stomatitis virus (VSV) and WNV and in particular the importance of RNA interference (RNAi) against these viruses (9–11). Apart from unraveling the cellular and molecular basis of antiviral immunity, *Drosophila* is also a suitable model for understanding host-virus interaction and the associated pathology (12, 13). Few compelling evidences further indicate that *Drosophila* can be a reliable model to analyze virus tropism (14). The insect-specific viruses Drosophila C Virus (DCV) and Flock house virus (FHV) for example, have been shown to infect the fat body, digestive tract, trachea and egg chamber, which results in infection-induced pathologies (12, 15, 16). These findings are of paramount importance to elucidate the physiological mechanisms that regulate the complex interactions between insects and viral pathogens. Comparative genomics studies have addressed the conservation between *Drosophila* and mosquitoes and shown that *Drosophila* developmental genes are largely conserved in three vector mosquito species (17). Deciphering the complete genome sequences of the mosquito vectors *Anopheles gambiae* and *A. aegypti* has enabled the identification and comparison of antiviral immune genes like *Dicer-2* and *Ago-2* (18–20). In the context of host pathology, forward genetic screens in *Drosophila* have identified genes regulating *Plasmodium* growth in *A. gambiae* (21). Therefore, molecular and functional characterization of innate immune factors acting against ZIKV and the consequent ZIKV-induced pathogenesis will potentially lead to a comprehensive understanding of the host-ZIKV interactions, which in turn will potentially lead to novel strategies for blocking ZIKV transmission.

In the absence of a classical adaptive immune system, *Drosophila* relies on innate defenses for immunity against viral infections. For instance, the Toll, Immune deficiency (Imd) and Janus kinase/signal transduction and activators of transcription (JAK/STAT) signaling pathways in *Drosophila* participate in antiviral responses; however, each of those pathways confers antiviral effects against certain viruses (22–24). The central antiviral immune response in the fly involves the RNAi mechanism, a conserved sequence-specific nucleic-acid-based immune defense that is induced by double stranded RNA (dsRNA). In *Drosophila*, RNAi involves the ribonuclease Dicer-2 that recognizes and cleaves dsRNA to generate viral small interfering RNAs (siRNAs) (25, 26). These siRNAs are loaded onto Argonaute-2 (Ago-2) and guide the RNA-induced silencing complex (RISC) to complementary RNAs in the cell, leading to their sequence-specific degradation (27, 28). RNAi mediates a strong antiviral response against a range of viruses in *Drosophila* (5, 7, 23, 29–32). Upon infection with RNA viruses, *Drosophila Dicer-2* and *Ago-2* null mutants display a substantial increase in viral replication and rapid decrease in survival (11, 30–33). The major importance of RNAi in host antiviral defense is further reinforced by the identification of viral proteins that act *in vivo* as suppressors of this mechanism (31, 33–35).

We have developed an *in vivo* model for studying the molecular basis of the host immune response to ZIKV infection and the occurring pathophysiological defects. We show that *Drosophila* flies are able to support ZIKV replication, which leads to the activation of stress-induced genes *Turandot* and *Diedel* and induction of the RNAi pathway. We find that the two central mediators in RNAi, Ago-2 and Dicer-2 have differential function in the context of ZIKV infection. While Ago-2 is dispensable, Dicer-2 regulates ZIKV replication and renders resistance to infection. In addition, we find that ZIKV exhibits tissue tropism by infecting the fat body, crop and gut of the adult fly. The tissue-specific infiltration of ZIKV results in local pathologies marked by perturbed homeostasis of the gut and the fat body lipid droplets. Furthermore, we find that the ZIKV mediated perturbed homeostasis is aggravated in *Dicer-2* mutants along with severely reduced insulin signaling resulting in significantly increased sensitivity to the infection. These are important findings because they demonstrate that using the *Drosophila*-ZIKV model enables the identification of host factors with anti-ZIKV immune activity and allows the characterization of tissue-specific effects that occur in the host during the infection process.

## Materials and methods

### Fly stocks

The following fly lines were used: $w^{1118}$ (wild-type and background control), $Ago\text{-}2^{414}$, the null allele $Ago\text{-}2^{321}$ in trans to *Df[BSC558]*(36) , $Dicer\text{-}2^{R461X}$ (37), $Dicer\text{-}2^{L811fsX}$ (37) compared to rescue with a *Dicer-2* genomic transgene (BL33053), *esg-Gal4* (NP5130, Drosophila Genomics Resource Center). Flies were reared on standard medium at 25°C. All fly lines were tested for *Wolbachia* infection and cured whenever necessary (38).

### Zika virus stock preparation

Vero cells (ATCC, Manassas, VA, USA) were grown in EMEM (ATCC) supplemented with 10% fetal bovine serum (Gemini Bio-Products), penicillin/streptomycin (VWR), gentamicin (Sigma Aldrich), and amphotericin B (Quality Biological). ZIKV strain MR766 was added to Vero cells at MOI of 0.1 and incubated for 4–6 days. The supernatants were centrifuged at 1,500 rpm for 5 min and filtered (0.45 μm) before being concentrated via SnakeSkin dialysis tubing 3.5K MWCO (Thermo Scientific) in polyethylene glycol 8000 powder (Alfa Aesar) until all liquid was drawn out. The tubing was then placed in PBS overnight at 4°C. The reconstituted ZIKV was then aliquoted and stored at −80°C. ZIKV titers were determined using plaque assays on Vero cells as previously described (39). Briefly, ZIKV stocks were serially diluted and adsorbed to confluent monolayers of Vero cells. After 3 hours, the inoculum was removed, and cells were overlaid with semisolid medium containing 1% carboxymethyl cellulose (Sigma Aldrich). Cells were further incubated for 5 days, fixed with 4% paraformaldehyde (Electron Microscopy Sciences), and stained with 0.5% aqueous crystal violet solution (Sigma Aldrich) for plaque visualization. Titers were expressed as plaque forming units (PFU) per milliliter.

### Fly infection

To avoid heterogeneity in ZIKV load, only adult female flies were used in these experiments. Injections were performed by anesthetizing the flies with $CO_2$. For each

experiment, two to five-day old adult female flies were injected with ZIKV suspensions in PBS (pH 7.5) using a nanoinjector (Nanoject III, Drummond Scientific). ZIKV stocks were prepared in PBS, pH 7.5. Heat-inactivated ZIKV stocks were generated by exposing the virus inoculum to 56$^{o}$C for 1 hour in a water bath. Heat-inactivated or live ZIKV solution (11,000 PFU/fly) (100 nl) was injected into the thorax of flies and injection of the same volume of PBS acted as negative control. Injected flies were then maintained at 25°C and transferred to fresh vials every third day throughout the experiment. They were collected at the indicated time points and directly processed for RNA analysis. Flies that died right after injection were not considered for further analysis. Statistical analyses of the differences in ZIKV titers between fly strains and experimental conditions were conducted with data from three independent experiments.

## RNA analysis

Total RNA was extracted from 10 adult female flies, using Trizol according to manufacturer's protocol. Total RNA (500 ng - 1 μg) was used to synthesize cDNA using the High Capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative RT-PCR (qRT-PCR) experiments were performed with technical triplicates and gene-specific primers (Table I) using iQ SYBR Green Supermix (Bio-Rad) and a CFX96 Real-Time PCR detection system (Bio-Rad). Quantification was performed from three biological replicates for both test and control treatments. ZIKV copy numbers were estimated by using previously described primers (ZIKV F9027; ZIKV R9197c) (40). Fold changes were calculated with the delta delta Ct method using *RpL32* as a housekeeping gene. Absolute copy numbers of ZIKV were extrapolated by a standard curve constructed out of 6-point dilution series of viral cDNA.

## Fly survival

For each fly strain, three groups of 20 female flies were injected with ZIKV and one group was injected with PBS for control. Following injection, flies were maintained at a constant temperature of 25°C with a 12 h light/dark cycle and mortality was recorded daily. Fly deaths occurring within one day of injection were attributed to injury and they were not included in the results. Log-rank (Mantel-Cox) was used to analyze the survival curves.

## Nile Red staining of neutral lipids

Fat body tissues from $w^{1118}$ untreated, PBS or ZIKV injected adult female flies were dissected and fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. Fixed tissues were then rinsed twice in PBS, incubated for 30 min in 1:1000 dilution of 0.05% Nile Red prepared in 1 mg/ml of methanol, and finally mounted with Vectashield (Vector labs, H1200). Images were taken using Zeiss LSM 510 confocal microscope. To quantify LD size, the area of the three largest LDs per cell from 25 fat body cells was measured using ImageJ. This experiment was repeated three times with three samples for each experiment.

## Immunostaining and antibodies

Anti-Prospero antibody (1:30), Anti-PH3 (1:500) and Anti-4G2 (1:100) antibody were purchased from DSHB and Abcam, respectively. Secondary antibodies included AlexaFluor

488, 555 and 633 (Invitrogen). DAPI was used for nuclear marking (Invitrogen). Phalloidin-FITC and Phalloidin TRITC were used for actin staining (Sigma). Standard procedures were followed for immunostaining. Briefly, fly tissues were dissected and fixed in PBS containing 4% formaldehyde for 30 min. Following double rinsing in PBS containing 0.1% Triton X-100, the samples were incubated overnight at 4°C with the primary antibody. The samples were then blocked with 1% BSA for 2 hours followed by 2-hour incubation with secondary antibody at room temperature. Finally, the samples were mounted with Vectashield medium (Vector Laboratories). Images were acquired with Zeiss LSM 510 confocal microscope and processed using Adobe Photoshop CS6. Fluorescence intensity plots were generated from a single slice of 40X confocal images using ImageJ software. A line segment was drawn across the two points of expression and the plot profile function was used to generate a fluorescence intensity plot for the desired channel. The raw data file generated by these plot profiles were analyzed in Excel with each plot value corresponding to the peak value creating intensity plots.

### Fly climbing ability

Climbing assays were carried out as previously described (41, 42). Groups of 10 adult female flies were transferred into empty vials and incubated for 1 hour at room temperature for acclimatization. The flies were gently tapped down to the bottom of the vials and then the number of flies reaching an 8 cm mark was counted after 18 sec of climbing. The experiment was repeated three times.

### Statistical analysis

An unpaired two-tailed Student's t-test was used for statistical analysis of data using GraphPad Prism (GraphPad Software). Log-rank (Mantel-Cox) within GraphPad Prism program was used to analyze the survival curves. The p values $< 0.05$ were considered statistically significant.

## Results

### *Drosophila* flies support ZIKV replication without succumbing to the infection

We first investigated whether *Drosophila* flies can support replication of ZIKV. For this, we injected 11,000 PFU/fly of the strain MR766 into the thorax of $w^{1118}$ adult flies and estimated viral copy numbers using ZIKV gene-specific primers. ZIKV belongs to the Flaviviridae family of viruses with an 11 kb single-stranded positive-stranded RNA genome (43, 44). The positive- stranded RNA serves as the mRNA for translation of a large polyprotein, which in turn codes for three structural and seven non-structural (NS) proteins (44). Among the latter, NS5 is the largest and most crucial NS protein in the viral replication complex because it performs both methyltransferase and polymerase functions, and therefore it forms an important therapeutic target for interfering with viral RNA production (45, 46). We estimated ZIKV copy numbers in the infected flies compared to PBS control treated flies at three time-points post infection using primer sequences against NS5, as previously described (40). We found strongly elevated levels of ZIKV copies at 8 days post infection (dpi) (200-fold increase compared to 4 dpi), which declined subsequently at 12 dpi (Fig. 1A) and did not change at 20 dpi (Fig. 1A). To test whether infection of *Drosophila*

with ZIKV affects the survival of wild-type flies, we injected $w^{1118}$ flies with MR766 and estimated survival rates over time. We found that infection with this ZIKV strain failed to reduce fly survival, which was similar to the survival of PBS injected controls (Fig. 1B). These results indicate that although ZIKV does not kill wild-type *Drosophila* through direct delivery into the hemolymph, it can replicate efficiently in the infected flies.

### Adult flies injected with ZIKV activate RNAi and stress-induced *Turandot* genes

*Drosophila* lacks an adaptive immune system and solely relies on innate immune mechanisms (4). At the molecular level, sensing of viral infection results in induction of signaling pathways and the production of antiviral effectors. To examine whether ZIKV infection stimulates innate immunity in *Drosophila*, we injected $w^{1118}$ adult flies with the MR766 strain and estimated the time-course expression of genes regulated by immune signaling pathways in the fly. We analyzed the activation of the RNAi pathway that forms a potent antiviral defense in *Drosophila* (5, 31, 32), the JAK/STAT pathway that is required but not sufficient for the inducible antiviral response in the fly (47), and the Toll and Imd pathways that regulate the activation of the nuclear factor κB (NF-κB) transcription factors DIF/Dorsal and Relish, respectively (24, 48, 49). We found that *Dicer-2*, encoding the sole siRNA-producing Dicer-2 protein that acts as a pattern recognition receptor and as a component of the RISC in *Drosophila* (29), was significantly upregulated at 4 and 8 dpi with ZIKV, whereas *Ago-2*, encoding the Argonaute protein in the RISC (31), was significantly induced at 4 dpi only compared to control injections with PBS (Fig. 1C). JAK/STAT is a mammalian antiviral signaling pathway and is a crucial component of the interferon response (50). While insects do not possess interferon activity, JAK/STAT has been instrumental in providing immunity against several viruses in *Drosophila* and mosquitoes (22, 51, 52). In corroboration with previous findings, we found a strong (up to 500-fold) upregulation of the antiviral cytokine *Diedel* and the JAK/STAT regulated genes *Turandot* (*Tot*), *TotA* and *TotM* in ZIKV injected flies (53, 54). We also found a moderate upregulation of *Thioester-containing protein 1* (*Tep1*) at 8 dpi (24), and slight upregulation of the STAT regulated antiviral genes *Vago, Vir-1* and *Listericin* mainly at 12 dpi (22, 55, 56) (Fig. 1D). In case of bacterial challenge, *Tot* genes are regulated through JAK/STAT signaling (57). To examine whether the ZIKV-induced *Tot* genes are also regulated by JAK/STAT signaling, we estimated *TotA* and *TotM* transcript levels in *hop* mutants infected with ZIKV. Unlike bacterial infection, mutating *hop* did not affect *TotA* and *TotM* induction upon ZIKV infection (Fig. S1A). We also found no significant upregulation of *Upd-3* (Fig. S1B), the ligand necessary for JAK/STAT mediated *TotA* activation (57), further emphasizing on JAK/STAT independent regulation of *Tot* genes in case of ZIKV infection. Also, there was no significant change in the mRNA levels of the Toll regulated antimicrobial peptide (AMP) gene *Drosomycin* and a slight decrease of *Metchnikowin* at 4 dpi (Fig. 1E); however, we noticed reduced transcript levels of the Imd regulated AMP genes *Diptericin* at 4 dpi and *Cecropin-A1* at 4 and 8 dpi, and increase of *Diptericin* at 12 dpi (4) (Fig. 1F). We then asked whether injection with inactivated ZIKV alters immune signaling regulation in *Drosophila*. Inactivated viruses are incapable of replication, but are still capable of binding cells and performing endocytosis and entry to the cells (35, 58). Similar to the effect of live ZIKV, we found robust induction of RNAi and upregulation of *TotM* in flies injected with the heat-inactivated ZIKV (Fig. S1C and D). These results demonstrate that *Drosophila* can sense the

presence of different ZIKV components and respond by activating certain antiviral immune mechanisms controlled by RNAi and the stress induced *Tot* genes. Interestingly, the latter are regulated independently of JAK/STAT signaling.

## Dicer-2 controls ZIKV replication and resistance to the infection

RNAi plays a major role in the insect immune response to certain viral infections (5). To test whether activation of RNAi confers resistance to ZIKV infection in *Drosophila*, we estimated the ZIKV load and survival ability of flies carrying loss-of-function mutations in *Ago-2* or *Dicer-2*. Ago-2 plays a pivotal immune role against viral infections in the fly (11, 31, 33). Time-course infection revealed significant increase in ZIKV load at the early time-point (4 dpi) in *Ago-2* mutant flies ($Ago\text{-}2^{414}$) (Fig. 2A). However, ZIKV load in *Ago-2* mutants for the later time-points remained unaffected compared to the $w^{1118}$ background controls (Fig. 2A). In line with the ZIKV load results, *Ago-2* mutant flies were able to survive infection with ZIKV at similar levels compared to their background controls (Fig. 2B). We further confirmed the survival ability of ZIKV infected $Ago\text{-}2^{414}$ in another trans-heterozygous null allelic combination $Ago\text{-}2^{321}/Df$ (Fig. S2A). In contrast, *Dicer-2* mutants ($Dicer\text{-}2^{L811fsX}$) contained higher ZIKV load at both 4 and 8 dpi. ZIKV load increased up to 5 and 11 times, respectively (Fig. 2C). *Dicer-2* mutant flies infected with ZIKV succumbed at a much faster rate with 50% of the infected mutants dying after 6.5 days post injection compared to 100% survival of their background controls (Fig. 2D). The survival defect of ZIKV infected $Dicer\text{-}2^{L811fsX}$ null mutant flies was also validated in another null mutant of *Dicer-2* ($Dicer\text{-}2^{R416X}$) (Fig. S2B). Similar to $Dicer\text{-}2^{L811fsX}$ mutants, ZIKV infected $Dicer\text{-}2^{R416X}$ flies succumbed at a faster rate as compared to the wild-type ($w^{1118}$) flies (Fig. S2B). We further observed that the survival defect of ZIKV infected *Dicer-2* ($Dicer\text{-}2^{L811fsX}$) mutant flies was rescued by the introduction of a genomic copy of *Dicer-2* (Fig. S2C). Of note, WNV, an arbovirus of the Flaviviridae family that can readily infect *Drosophila*, shows an opposite replication pattern in RNAi mutants, as it amplifies at higher titers in *Ago-2* (but not in *Dicer-2*) deficient flies than in controls (11). It has also been shown that Dicer-2 can regulate Toll signaling and the expression of Vago, a secreted protein with antiviral activity (55). However, unlike previous studies, here we found increased expression of *Vago* in *Dicer-2* mutants infected with ZIKV. *Vago* mRNA levels were 1.5-fold higher in ZIKV infected *Dicer-2* mutant flies compared to the background controls (Fig. S2D). These findings indicate that Dicer-2 is crucial in regulating ZIKV replication, while Ago-2 regulates the replication at a modest level. In particular, Ago-2 confers a tolerant phenotype during the early stages of ZIKV infection, while Dicer-2 is important for conferring resistance to ZIKV.

## Characterization of ZIKV tropism reveals replication in the midgut, fat body and crop of the infected flies

Once inside the host, viruses need to propagate for their own survival, and thereby they interact with certain cell types or host tissues, also known as viral tropism, which determines the outcome of infection (59). In *Drosophila*, there have been compelling evidences depicting the tissue tropism of DCV, Nora virus, FHV, VSV and Bluetongue virus (BTV) in the infected flies. (14). In order to examine ZIKV tissue tropism, we used the flavivirus-specific antibody 4G2, which detects the viral envelope protein (60–62). We noticed strong

4G2 positive signal in the midgut of the ZIKV-infected flies (Fig. 3A), while the midgut of the uninfected flies was devoid of 4G2 expression (Fig. 3A). We also examined 4G2 expression in the midgut of both uninfected controls and ZIKV-infected wild-type flies. We generated fluorescence intensity plots across two linear points of 4G2 expression (yellow line in Fig. 3A) to precisely analyze 4G2 expression. Intensity plot showed two peaks of 4G2 fluorescence corresponding to the yellow line in ZIKV-infected gut tissue (magenta line) (Fig. 3B), while negligible 4G2 intensity was observed for the expression in uninfected gut tissue (green line) (Fig. 3B). Similar to midgut, the crop (digestive organ) of the ZIKV-infected flies also showed numerous 4G2 positive ZIKV particles (Fig. 3C) as compared to the uninfected flies, in which no expression of 4G2 was detected (Fig. 3C). However, unlike DCV infection (12), we were not able to detect any morphological defect in the crop of ZIKV infected flies as compared to the uninfected controls (Fig. 3C). Similar to the midgut, the intensity plot for 4G2 expression in the crop revealed sharp peak for ZIKV-infected crop tissue while diminished 4G2 intensity was found for the uninfected crop (Fig. 3D). Furthermore, closer examination revealed 4G2 positive signal in the primary metabolic/immune organ, the fat body of the infected flies, and no signal was observed in the fat body of uninfected flies (Fig. 3E). Measurement of intensity plots showed two strong peaks corresponding to 4G2 expression in ZIKV-infected fat body while a flat-line expression was observed for 4G2 expression in uninfected fat body tissue (Fig. 3F). We also examined 4G2 expression in the reproductive organ and noticed that similar to the uninfected flies, there was no expression of 4G2 in the egg chambers of the infected flies (Fig. S3). To further validate the expression of 4G2 in these different tissues of the infected wild-type flies, we next estimated ZIKV load in these tissues. Analysis through qRT-PCR revealed elevated level of ZIKV copies in fat body, gut and crop of the infected flies (Fig. 3G). These findings suggest that ZIKV depict a broad tissue tropism in *Drosophila* by infecting the midgut, crop and fat body of the infected flies.

## Midgut from ZIKV-infected wild-type and *Dicer-2* mutant flies differ in their ability to induce intestinal stem cell proliferation

Characterizing the physiological events occurring during the course of a microbial infection is essential for a better understanding of host-microbe dynamics. Infection with certain microbial pathogens induces pathophysiological responses in *Drosophila* (63). For instance, adult flies infected with *Salmonella typhimurium*, *Listeria monocytogenes* and *Mycobacterium marinum* demonstrate various infection-induced pathologies (64, 65). However, virus-induced pathologies in *Drosophila* are still poorly understood (12, 13, 66). In correlation with tissue tropism of ZIKV, we next aimed at investigating whether viral infection of the midgut affects the overall gut homeostasis. *Drosophila* gut homeostasis is primarily maintained by epithelial renewal, where the stress or infection-induced damage results in enterocyte (EC, the main gut cell type) loss that is compensated by over-proliferation of intestinal stem cells (ISCs)(67). Interestingly, flies compromised in the ability to renew the gut epithelium succumb to infection, demonstrating the importance of ISC-induced epithelium renewal in anti-bacterial immunity (68, 69).

In order to investigate the gut-related pathologies upon ZIKV infection, ZIKV was injected in flies carrying an ISC-specific reporter construct (e*sgGal4UAS-GFP*)(70), where Escargot

(Esg) is a transcription factor that maintains stemness in ISCs (71). In the absence of infection, the expression of *Escargot* was restricted to a few dispersed subsets of ISCs (Fig. 4A). In contrast to the midgut of the uninfected flies, the midgut from ZIKV-infected individuals displayed a strong increase in ISC proliferation marked by enhanced expression of *Escargot* (Fig. 4A). The increased number of ISCs was further validated by quantification showing that up to 60% of the total midgut cells were comprised of *Escargot*-positive ISCs (Fig. 4B). In agreement with the enhanced expression of *esg-GFP*, we noticed significant enrichment of *escargot* mRNA in the midgut of ZIKV-infected wild-type flies as compared to the uninfected controls (Fig. 4C). In order to understand the causative mechanism for the increased susceptibility, we next examined the midgut-related defects in ZIKV-infected *Dicer-2* (*Dicer-2*$^{L811fsX}$) mutant flies. We found that unlike wild-type flies, ZIKV infected *Dicer-2* mutants showed a significant reduction in the mRNA expression of *escargot* (Fig. 4C).

We then investigated the signaling pathways regulating ZIKV-induced ISC proliferation. Infection-induced ISC proliferation is regulated by several signaling pathways (67). However, EGFR signaling is the core regulatory mechanism for ISC proliferation. Activation of EGFR signaling is sufficient to induce ISC proliferation while its inactivation in ISCs renders them unable to proliferate upon infection (67). We next analyzed the transcriptional activation of EGFR signaling components in wild-type and *Dicer-2* infected flies. qRT-PCR analysis showed that mRNA level of EGFR ligands, *Spitz, Keren* and *Vein* were significantly upregulated in the ZIKV-infected wild-type flies as compared to the uninfected individuals (Fig. 4D). However, in case of *Dicer-2*, although there was a significant reduction in level of *Vein*, there was no change in expression of *Keren* and *Spitz* (Fig. 4D).

For further characterization of ISCs, we next examined the overall rate of proliferation in the midgut cells using antibodies against mitosis marker, phospho-histone 3 (PH3). We found that even in uninfected condition, the midgut of *Dicer-2* mutant flies showed a markedly reduced rate of proliferation as compared to the uninfected wild-type flies (Fig. 4E). Quantification analysis further showed that the number of mitotically active PH3-marked cells was reduced 10 times in the midgut of *Dicer-2* mutant flies as compared to the uninfected wild-type flies (Fig. 4F). Upon ZIKV infection, the midgut of wild-type flies showed a significantly increased number of mitotic cells, but the midgut of *Dicer-2* mutants failed to trigger proliferation and was devoid of any mitotically active PH3-positive cells (Fig. 4E and F).

Mitotically active ISCs divide to generate the enteroendocrine cells (EE) in the *Drosophila* midgut. Primarily involved in regulating intestinal physiology, their immune role has been recently characterized (71–73). Similar to the rate of proliferation, we noticed a strikingly reduced number of Prosper-marked EE cells in the midgut of uninfected *Dicer-2* mutant flies as compared to the uninfected wild-type flies (Fig. 4G and H). Upon ZIKV infection, although there was no difference in the proportion of EE cells between infected and uninfected *Dicer-2* mutants, there was significant increase in the number of Prospero-marked EE cells in the midgut of the wild-type flies (Fig. 4G). Quantification analysis revealed that in case of ZIKV-infected wild-type flies, the number of midgut-specific Prospero positive EE cells increased 3 times as compared to the uninfected wild-type

controls (Fig. 4H). These findings indicate that the midgut of resistant wild-type flies is naturally more proliferative than the midgut of susceptible *Dicer-2* mutant flies. Upon ZIKV infection, the midgut of wild-type cells responds promptly triggering enhanced proliferation while the midgut of *Dicer-2* mutant flies refrains from undergoing any proliferation

## Fat body from ZIKV-infected flies shows perturbed lipid metabolism with *Dicer-2* mutants suffering from lipodystrophy and severely reduced insulin activity

To further characterize the ZIKV induced-pathology in *Drosophila*, we next examined the effect of ZIKV infection on fat body homeostasis. Being an adipose tissue, fat body acts as the reservoir for lipids, which in turn are stored in specialized organelles, called lipid droplets (LDs). Emerging evidence indicates that except for lipid metabolism, LDs might participate in host immune activities against microbial infections by acting as a source of antibacterial and antiviral proteins (74). Here we found that the fat body from ZIKV-infected $w^{1118}$ flies consistently displayed accumulation of enlarged lipid droplets (Fig. 5A). Upon ZIKV infection, the size of Nile Red marked fat body LDs increased 3–4-fold as compared to those in uninfected flies (Fig. 5B). Because lipid forms the primary source of energy reserve, we asked whether perturbed lipid homeostasis could contribute to the susceptibility of ZIKV-infected *Dicer-2* mutant flies. In contrast to the increased size of LDs in ZIKV-infected $w^{1118}$ flies, ZIKV-infected *Dicer-2* mutants displayed a dispersed set of LDs with a strikingly diminished size (Fig. 5A). Reduced size of LDs is a sign of lipodystrophy, which in turn could lead to faster death and has also been shown to be a morphological characteristic of the fat body in FHV-infected flies (29, 75). Quantification analysis showed that the size of fat body LDs in ZIKV-infected *Dicer-2* mutants was reduced to one-third compared to those in ZIKV-infected $w^{1118}$ flies (Fig. 5B).

We next assessed the expression status of lipid metabolism genes in ZIKV-infected $w^{1118}$ and *Dicer-2* mutant flies. In *Drosophila*, Perilipin-like domain containing proteins (76), DmPLIN1 (Lsd-1) and DmPLIN2 (Lsd-2) modulate the rate of lipolysis (77–79). Lsd-1 is broadly expressed in LDs of fat body cells and promotes lipolysis (78, 80). Lsd-2 functions in opposite fashion to Lsd-1 and protects triacylgycerides stores in a dose-dependent manner (80). We therefore analyzed the transcript levels of *Lsd-1* and *Lsd-2* in ZIKV-infected $w^{1118}$ and *Dicer-2* mutant flies. We found that mRNA levels of *Lsd-2* were significantly upregulated in ZIKV-infected $w^{1118}$ flies while ZIKV-infected *Dicer-2* mutant flies showed reduced expression of *Lsd-2* (Fig. 5C). However, mRNA levels of *Lsd-1* were not altered in any of the infected strains (Fig. 5C).

We further examined the expression of genes regulating lipogenesis in the ZIKV-infected flies. Lipin and mdy are major regulators of lipid biogenesis in *Drosophila*; knockdown of *lipin* and *mdy* in the fly results in reduced lipid storage and increased lethality (75, 81). While ZIKV-infected $w^{1118}$ flies showed upregulation of *lipin* and *mdy*, ZIKV-infected *Dicer-2* mutants showed reduced expression of *lipin* only (Fig 5D).

One of the pathways regulating lipid metabolism is the insulin signaling pathway (82, 83), which is indispensable for the growth of an organism and forms an important regulator of survival in *Drosophila* responding to microbial infection (65). Here we found that expression of *4E-BP*, a negative regulator of insulin signaling (84), significantly increased in ZIKV-

infected $w^{1118}$ flies (Fig. 5E), suggesting reduced insulin activity and further emphasizing the possibility that these flies suffered from metabolic defects. However, ZIKV-infected $w^{1118}$ flies showed normal level of *Impl2* (Fig. 5E), which is a known antagonist of insulin signaling that has been implicated recently in wasting phenotypes (84–86). Interestingly, ZIKV-infected *Dicer-2* mutants exhibited significant reduction in insulin signaling, as indicated by 3.5-fold increase in *4E-BP* mRNA levels and markedly increased mRNA levels of *Impl2* (Fig. 5E), a condition that leads to wasting syndrome and inevitably death of the infected flies (65, 84, 85). In addition, ZIKV-infected $w^{1118}$ flies were less active and showed mild defect in climbing ability (Fig. 5F and G); however, the climbing ability and speed were severely affected in the *Dicer-2* mutants with only 10% of these flies being able to climb compared to 50% of the $w^{1118}$ individuals (Fig. 5F and G).

We next wanted to determine whether the observed pathologies in ZIKV-infected *Dicer-2* mutants are linked to the *Dicer-2* mutation *per se* or they are resulting from the enhanced ZIKV load. To address this issue, we investigated the occurrence of these pathologies in uninfected *Dicer-2* mutant flies. The fat body LDs in uninfected *Dicer-2* mutant flies were similar in size to those in background control uninfected flies (Fig. S4A and B). Although uninfected *Dicer-2* mutant flies showed a mild defect in climbing (Fig. S4C and D), unlike the ZIKV-infected *Dicer-2* mutants, there was no change in the activation of insulin signaling as shown by the normal levels of *4E-BP* and *Impl2* (Fig. S4E). In order to further ascertain whether the LD phenotype is indeed a genuine phenotype owing to *Dicer-2* mutation induced ZIKV enrichment, we next used a fly strain carrying *Dicer-2* genomic construct in the background of *Dicer-2* mutation. We found that the LDs of ZIKV-infected *Dicer-2* rescue carrying flies were similar in size to those of the wild-type controls (Fig. S4F and G). Further, we examined the level of insulin signaling and found no change in mRNA level of *4E-BP* as compared to the ZIKV-infected wild-type flies (Fig. S4H). Collectively these findings indicate that *Drosophila* infection with ZIKV induces perturbed lipid metabolism followed by reduced insulin activity. These pathologies deteriorate severely in *Dicer-2* mutants, which sustain enhanced ZIKV burden, defects that result in increased mortality upon ZIKV infection.

## Discussion

Our results suggest that *Drosophila* is a suitable model for dissecting the molecular and pathophysiological basis of host-ZIKV dynamics. Our findings indicate that *Drosophila* flies subjected to intrathoracic injection of the ZIKV African strain MR766 are capable of sustaining replication of this virus. Using gene-specific primers designed against ZIKV NS5, a crucial molecule for ZIKV replication, we show that ZIKV copy number increases steadily up to 8 dpi before reaching a peak, and subsequently declines at 12 dpi and remains constant thereafter. These findings indicate that NS5 is a reliable indicator for estimating *in vivo* ZIKV gene expression and replication. Interestingly, enhanced amplification of ZIKV does not compromise fly survival, signifying that *Drosophila* wild-type flies are resistant to ZIKV infection.

One of the primary innate immune defense mechanisms in *Drosophila* is the induction of antimicrobial peptides (AMP) regulated by the Toll and Imd signaling pathways, which have

been shown to protect against certain viruses, although the mechanistic details are still poorly understood. *Drosophila* Toll is induced by and regulates Drosophila X virus (DXV) and VSV infection, while in *Aedes* mosquitoes it is critical against DENV infection (6, 24, 57, 87, 88). Previous studies also indicate that Imd signaling participates in the control of SINV and Cricket paralysis virus (CrPV) infection in *Drosophila*, but the level of its involvement in the overall antiviral response is currently unclear (48, 89). Here we have found that ZIKV infected flies fail to trigger significant activation of Toll and Imd regulated AMP encoding genes. This finding denotes that the antiviral activities of these pathways are probably virus-specific and they are not directed against ZIKV infection. Unlike Toll and Imd, JAK/STAT is a canonical mammalian antiviral pathway and is a crucial component of the interferon response (50). Though its antiviral role is increasingly perceived in *Drosophila* and mosquitoes, accumulating evidence indicates that its contribution might also be virus-specific. While flies mutant for JAK are more sensitive to DCV and CrPV, these mutant flies display a rather weak phenotype to SINV, VSV and DXV (22, 23). While activation of JAK/STAT restricts DENV infection, it has no effect on resistance of *Aedes aegypti* to ZIKV or Chikungunya virus (52, 90). A recent transcriptomic analysis further showed that components of JAK/STAT signaling are not induced in ZIKV-infected mosquitoes (91). In line with these results, our data show that the viral regulated JAK/STAT targets *Vago, Vir-1 and Listericin*, which are known to be induced by DCV, show only moderate upregulation upon ZIKV infection. Also, the *Tot* genes, which are activated by bacterial infection and regulated by JAK/STAT signaling, are markedly upregulated in wild-type flies upon ZIKV infection. Primarily induced upon stress (54, 92), the role of *Tot* genes in the fly antiviral immune response is still unclear. Here, we provide evidence that *Tot* gene regulation through JAK/STAT signaling in *Drosophila* might be restricted to bacterial infections only and is probably not a general feature of viral infections. *Tot* gene induction upon ZIKV infection indicates a virus-specific mechanism of activation for certain genes in the fly, which could imply a general stress response rather than an antiviral immune mechanism. Predominantly upregulated upon septic injury, Diedel is a negative regulator of JAK/STAT signaling although its exact molecular function is still unknown (53, 93). ZIKV injection in *Drosophila* results in significant upregulation of *Diedel* throughout the course of infection. This finding suggests that ZIKV infection results in negative regulation of JAK/STAT signaling, which could form a potential strategy of ZIKV to evade the host immune response. The NS5 protein of flaviviruses, which is critically involved in replication, is also able to mediate antagonism of JAK/STAT signaling. DENV infection has been associated with loss of STAT2 expression (94), while WNV infection results in failed JAK activation (95). In line with these findings, our data further stress that the interaction of flaviviruses with JAK/STAT signaling is particularly complex and requires future thorough examination.

In *Drosophila*, the main antiviral RNAi pathway relies on siRNAs and involves *Ago-2* and *Dicer-2* as the central operating genes (5, 29, 31, 32). Our findings show that *Ago-2* and *Dicer-2* are significantly upregulated in *Drosophila* flies infected with ZIKV implying their anti-ZIKV role. Our finding implicating that Dicer-2 regulate ZIKV infection and provide resistance, is in contrast to a recent work reporting that Dicer-2 is dispensable in regulating ZIKV replication in *Drosophila* (8). This discrepancy could be attributed to the genetic nature of the strains used in the recent study. Here we further show that while Dicer-2 is

instrumental in regulating ZIKV replication, the effect of Ago-2 is only marginal. This distinction in the level of surveillance between the two RNAi components is surprising but not unusual. The distinct functions of Ago-2 and Dicer-2 in the context of viral infection have been documented in *Drosophila* in case of WNV, where Ago-2 substantially regulates viral replication while Dicer-2 controls viral load at a modest level (11). Several studies in mosquitoes have implicated the siRNA, and in particular, Dicer-2 based immunity in case of ZIKV infection (96, 97). In addition, there have been instances where knockdown of Ago-2 in mosquito cells has only a modest/marginal effect on replication of Dengue and Orthobunyaviruses (98, 99). In a recent report, knockdown of Ago-2 did not lead to increased ZIKV replication, while knockdown of Dicer-2 increased ZIKV replication in mosquito cells (97). It is likely that direct dicing of RNA is more relevant in these cases than RISC-mediated antiviral response. Previous evidence also suggests that independent of RNAi, Dicer-2 can regulate Toll signaling and expression of antiviral gene, *Vago*. (55, 100). In the context of ZIKV infection, *Dicer-2* loss results in enhanced expression of *Vago* suggesting that the induction of Dicer-2 by *Vago* might be DCV-specific and not a general antiviral response. How exactly RNAi effectors regulate viral replication is thus an intriguing question and our findings further highlight the specificity of certain RNAi signaling components against different viral pathogens. Identification of putative ZIKV dsRNA targets recognized by Dicer-2 may provide mechanistic insight into how it regulates ZIKV replication in *Drosophila*.

Our study indicates the importance of siRNAs in anti-ZIKV immunity. It is only recently that other forms of small RNAs, miRNAs and piRNAs, have been implicated in mosquito antiviral immunity. Deep sequencing has revealed accumulation of piRNAs during infection of mosquitoes with Dengue and Chikungunya virus (101, 102). Infection of *A. aegypti* with a Mexican strain of ZIKV results in modulation of miRNAs and dramatic increase of siRNAs and piRNAs (103). The role of piRNAs and miRNAs in *Drosophila* antiviral immunity has also been demonstrated recently. Although infection with DXV, DCV or SINV failed to trigger any viral-derived piRNAs and mutation in piRNAs including Ago3, Aub and Piwi had no effect on *Drosophila* survival (104), flies and cells infected with DCV showed reduced expression of miR-8–3p, a known antagonist of viral replication in *Bombyx mori* (105). Further experiments would provide an insight into the contribution of these RNAi pathways in the regulation of ZIKV infection.

For successful infection, a virus should target certain cell types or tissues in the host. The ability to colonize and replicate in a specific host tissue also determines the nature and extent of viral pathogenesis (12, 15, 16, 106). Interestingly, the available information of tissue tropism is restricted to insect-specific viruses and the tissue tropism in the case of human pathogenic viruses has not been investigated in detail. Unlike the insect-specific viruses, infection with flaviviruses is asymptomatic and no defect in survival has been reported. This could be one of the reasons for the lack of tissue tropism related studies in the case of human pathogenic viruses. Our findings indicate that firstly, 4G2, a flavivirus specific antigen is a reliable marker for *in vivo* detection of ZIKV. Using expression of 4G2 as indicator of ZIKV infection, our study provides the first demonstration of a human pathogenic virus infecting the immune/metabolic tissues of the fly. Infection of these organs

further implies the relevance of the tissue tropism in dictating the level of viral pathogenesis that can be local, and not always demonstrated at the organismal level (defect in survival).

Although significant advances on the cellular and genetic basis of antiviral immunity in *Drosophila* have been made, the effects of viral infection on fly physiology have not been widely explored (13, 66). For instance, DCV infection triggers intestinal obstruction marked by crop enlargement due to the disrupted movement of the ingested food in the fly midgut (12). In *Drosophila*, cytotoxic agents such as bleomycin or paraquat or pathogenic bacterial infection can trigger gut remodeling and in the process increase ISC proliferation (73, 107, 108). The importance of cell renewal in regulating viral infection has been recently demonstrated in mosquitoes and has been proposed to be one of the primary determinants for the susceptibility to Dengue infection. (109). Our finding that ZIKV-infected *Dicer-2* mutant flies fail to trigger midgut proliferation as compared to the ZIKV-infected wild-type flies which display considerable increase in ISC proliferation, suggests that in manner similar to Dengue infection, ISC proliferation might also be acting as a determinant for fly susceptibility to ZIKV. Apart from gut-related perturbations (shown in this study), mutation in *Dicer-2* has been linked with a large number of perturbations including stress, perturbed metabolism (110), and thus could be considered as the host factor dictating the sensitivity or predisposition to infection. Ago-2 in contrast, has not been shown to be involved in any tissue-specific perturbations, which might in turn explain why *Ago-2* mutant flies are refractory to ZIKV infection as compared to *Dicer-2* mutant flies. Future investigations will explore the extent to which the restricted proliferation of the midgut contributes to the mortality of these susceptible flies. In particular, future efforts could focus on examining the contribution of other pathways (such as Wingless, Hippo or Notch) to ZIKV induced ISC proliferation, and whether or how the latter could interfere with ZIKV replication.

Emerging evidence indicates that viral infection can modulate lipid metabolism. Hepatitis C virus (HCV) and DENV infection in the hepatoma and kidney cell lines has been linked to enhanced lipogenesis and sharp increase in LD numbers (111, 112). In addition, liver steatosis or fatty liver (the accumulation LDs) is a commonly observed pathology in HCV infected patients (113, 114). Although the mechanism of lipid accumulation is not known, it has been proposed that certain viruses might reside in LDs to promote their own assembly and replication (115). A recent finding has implicated the role of mosquito gut LDs in regulating replication of DENV (116). The perturbed lipid metabolism has also been observed in *Drosophila* S2 cells where infection with FHV results in increased transcription of genes required for lipid biogenesis (117). Although further studies are required, these previous results suggest that modulation in lipid metabolism could be a general response upon viral infection. Our findings reveal that ZIKV infection in *Drosophila* flies results in multiple pathologies, most of which are closely associated with lipid metabolic processes. To our knowledge, this is the first *in vivo* demonstration of perturbed lipid metabolism upon viral infection in *Drosophila.* While a moderate ZIKV load results in enlarged LDs in wild-type flies, an enhanced load in *Dicer-2* mutants results in reduced accumulation of LDs. Reduced size of LDs is a sign of lipodystrophy, which in turn could lead to accelerated death (75, 118).

The reduced energy storage could further be attributed to strikingly decreased insulin signaling in ZIKV-infected *Dicer-2* mutant flies. Insulin signaling is one of the main metabolic signaling pathways and previous evidence indicates that reduction in insulin signaling results in depletion of energy reserves, which can lead to infection-induced wasting (65). Impl2, a secreted antagonist of insulin signaling (86), has been implicated recently in robust wasting phenotypes (84, 85). Transcriptional activation of *Impl2* in ZIKV-infected *Dicer-2* mutant flies further confirms that enhanced ZIKV load could induce host wasting, which points out a potential interplay between host metabolism and viral infection. A fascinating future question pertains to the mechanism by which ZIKV regulates lipid metabolism of the host and how this regulation affects the propagation of the virus. It would also be interesting to investigate the participation of Dicer-2 in ZIKV-induced perturbed lipid metabolism.

Infection-induced behavioral changes are not uncommon. These can be attributed to the pathogen infecting certain tissues and organs in the host or they can be caused by the host *per se* in an attempt to store limited resources (119). Of note, locomotion of an organism is strongly correlated with its state of metabolism (120, 121). Mosquitoes infected with DENV or *Wolbachia* (wMelPop) have been proved more active than uninfected individuals (122, 123). Virus infection-induced behavioral changes are also evident in our study showing that ZIKV-infected *Dicer-2* mutant flies (which have been metabolically impaired) suffer from lethargy and display impaired locomotion. Whether this behavioral defect is the result of muscular or nervous dysfunction remains unknown and it will be further explored to yield more insights into the mechanistic processes governing host-ZIKV interactions.

Taken altogether, our results illustrate the efficacy of *Drosophila* as model for understanding host-ZIKV interactions. Our findings reveal that ZIKV can replicate in *Drosophila* and the resulting infection can cause severe pathophysiological defects. The key insights into identifying the genetic basis of the interplay between *Drosophila* and ZIKV will undoubtedly assist in unraveling conserved anti-ZIKV immune mechanisms in mammals, perhaps even humans, and will contribute towards developing efficient strategies for blocking the transmission of ZIKV and other flaviviruses by mosquito vectors.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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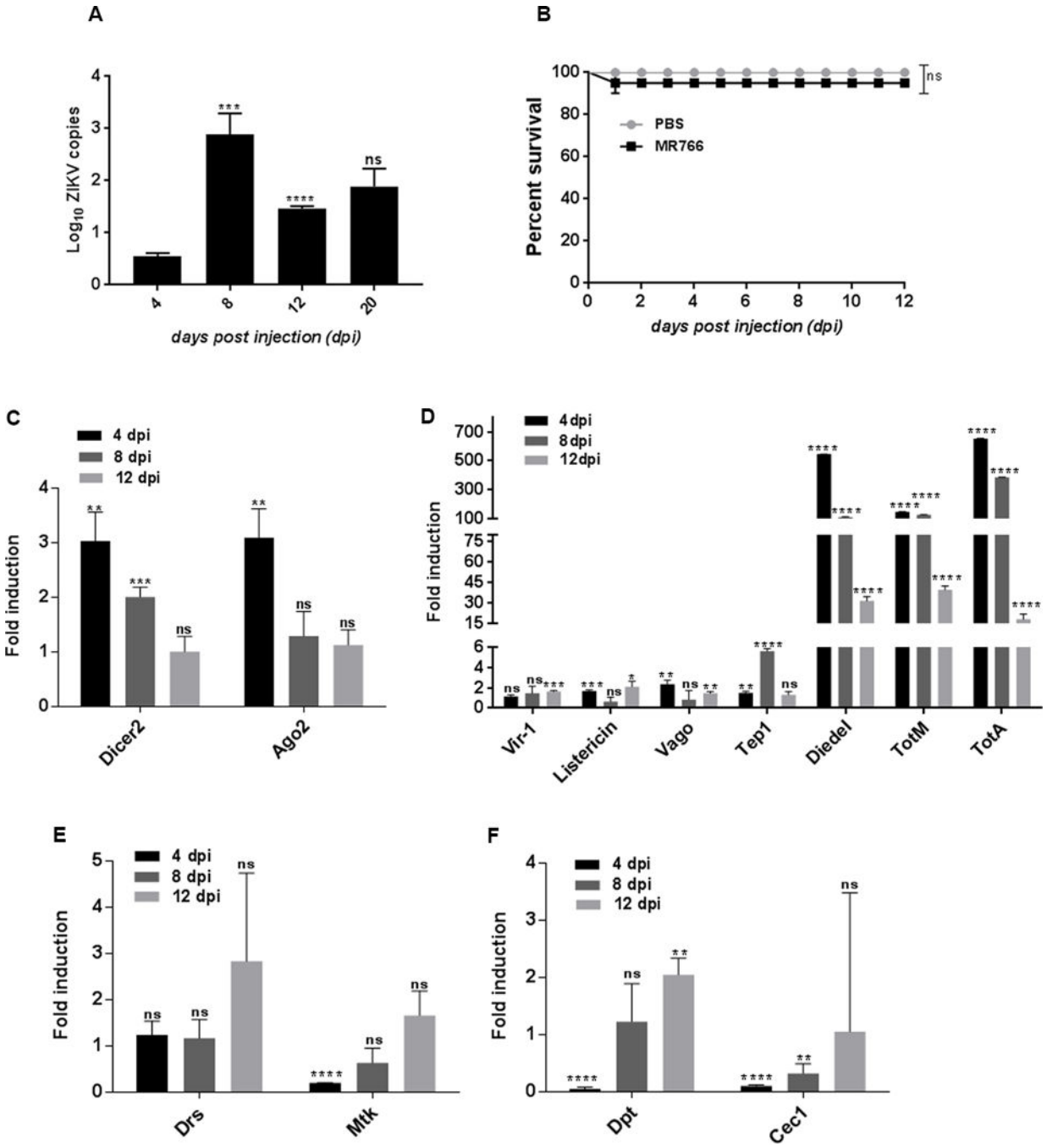


**Figure 1. ZIKV replicates in Drosophila adult flies and triggers RNAi and the expression of Turandot genes.**

(A) *Drosophila* female $w^{1118}$ adult flies were injected with ZIKV (African strain MR766; 110 million PFUs/ml) and ZIKV load was estimated at several days post injection (dpi). Absolute ZIKV copy numbers were quantified via qRT-PCR. (B) Survival of $w^{1118}$ adult flies after intrathoracic injection with ZIKV was monitored at 24-hour intervals for 12 days. Injections with PBS served as negative controls. Grey and black lines depicting the survival of PBS and ZIKV-injected flies are superimposed, but for clarity, they are shown in parallel. Data represent the mean ± SD of three biological replicates of at least 20 female flies. Log-

rank (Mantel-Cox) was used for statistical analysis; ns denotes no significant differences between experimental treatments. (C-F) ZIKV-infected flies were processed for RNA analysis and gene expression levels were determined by quantitative RT-PCR at 4, 8 and 12dpi. (C) Transcript levels of RNAi machinery, *Ago-2* and *Dicer-2.* (D) Transcript levels of JAK/STAT gene targets including the *Turandot* (*Tot*) genes *TotA* and *TotM*, the antiviral cytokine *Diedel,* the thioester-containing protein *Tep1*, and the antiviral STAT regulated target genes *Vago, Vir-1* and *Listericin*. (E) Transcript levels of Toll signaling gene readouts *Drosomycin (Drs)* and *Metchnikowin* (*Mtk)* throughout the stages of infection. (F) Transcript levels of Imd signaling gene readouts *Diptericin* (*Dpt)* and *Cecropin (Cec)* in flies responding to ZIKV infection. All data were normalized to the housekeeping gene *RpL32* shown relative to wild-type flies injected with PBS (sterile control). Three independent experiments were carried out with 10 flies per sample in triplicates (*p=0.0215, **p=0.002, ***p=0.0006, ****p<0.0001, 0.0002). Bars represent the mean ± SD and ns denotes no significant differences between experimental treatments. Statistical analysis was performed using Student's t-test.

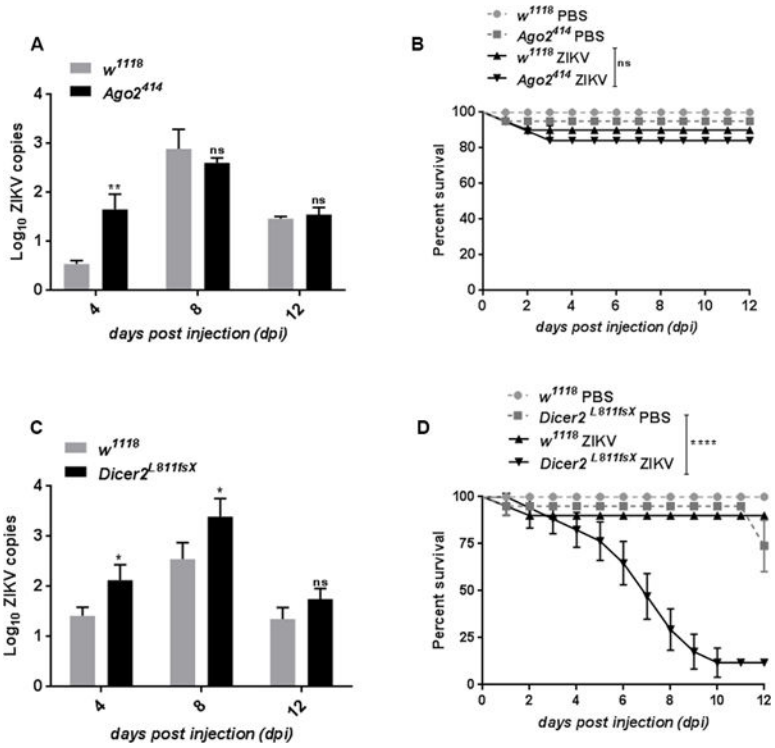


**Figure 2. Inactivating Dicer-2 results in enhanced ZIKV load and compromised fly survival.**

ZIKV load and survival in *Ago2*$^{414}$ and *Dicer2*$^{L811fsX}$ loss-of-function mutant flies and $w^{1118}$ background controls. (A) Quantitative RT-PCR analysis depicting ZIKV load in *Ago-2* mutant flies compared to the $w^{1118}$ controls. (B) Survival analysis of $w^{1118}$ and *Ago-2* mutant flies injected with PBS and ZIKV. (C) ZIKV load in *Dicer-2* mutant compared to the wild-type control flies. (D) Survival analysis of $w^{1118}$ and *Dicer-2* mutant flies injected with PBS and ZIKV. All ZIKV load data represent the mean and standard deviation of three independent experiments in triplicates (*p=0.05, **p=0.04). For survival experiments, three groups of 20 female flies from each fly strain were injected with ZIKV and fly survival was monitored at 24-hour intervals and up to 12 dpi. Log-rank (Mantel-Cox) was used for survival data analysis (****p<0.0001; ns denotes no significant differences between experimental treatments).

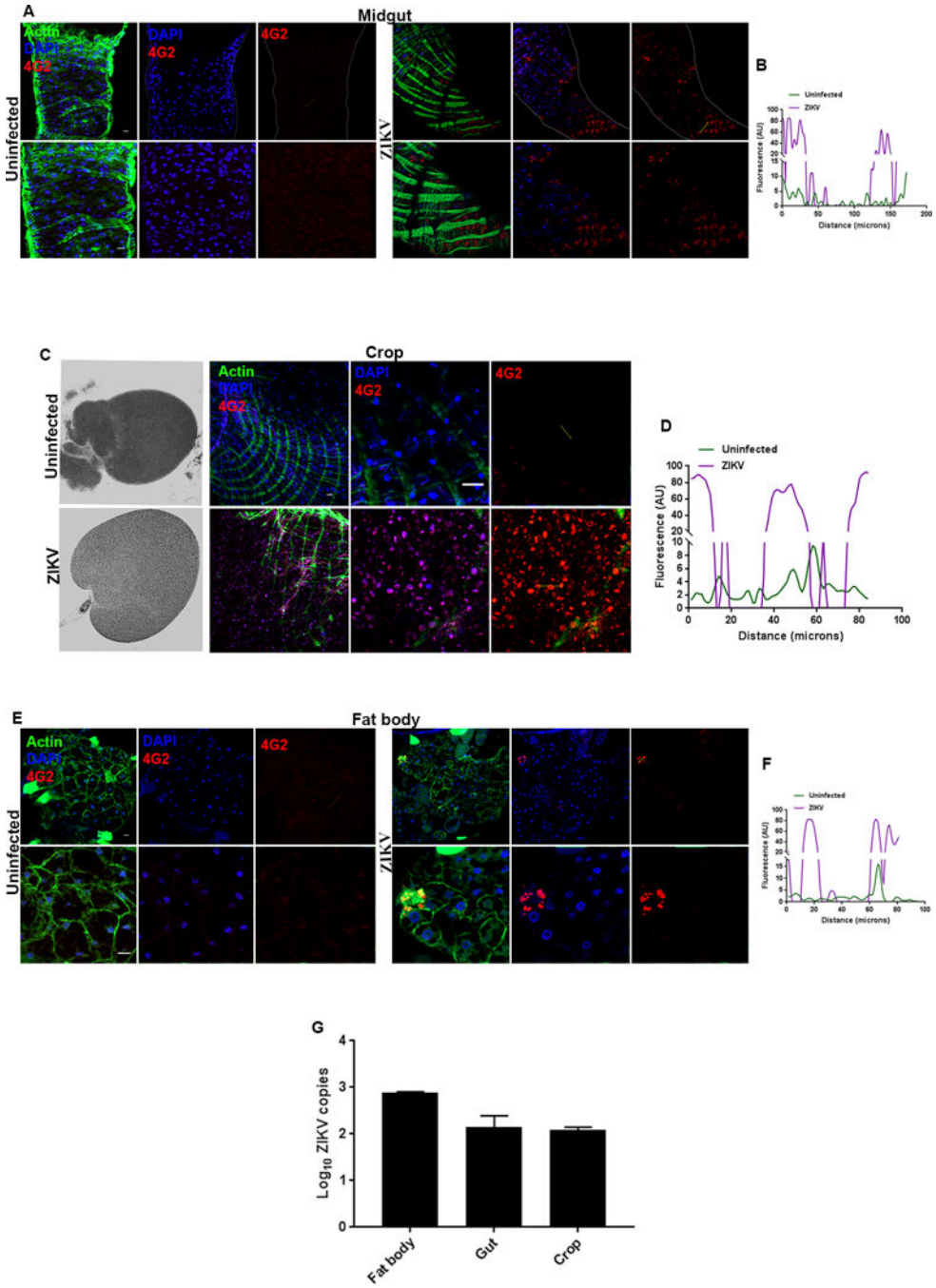


**Figure 3. Midgut, crop and fat body of ZIKV infected flies shows flavivirus-specific 4G2 expression.**

Representative midgut, crop and fat body images from uninfected $w^{1118}$ adult flies and flies infected with ZIKV at 8 days post injection (8 dpi). The expression of ZIKV was detected through anti-flavivirus antibody, 4G2. (A) The first panel shows dissected midgut from uninfected $w^{1118}$ flies along with the enlarged images in the lower panel, while the second panel shows dissected midgut from ZIKV infected adult flies. (B) Quantification of the 4G2 fluorescence intensity in midgut in uninfected and ZIKV-infected flies. The intensity plot corresponds to a line connecting two points (marked by yellow line in the images).

Fluorescence intensity is represented in pseudocolors where magenta marks the intensity for ZIKV infection and green for uninfected control (AU, arbitrary unit). (C) Expression of 4G2 in the crop (organ in the digestive tract) in uninfected controls and ZIKV-infected adult flies. (D) Quantification of the 4G2 fluorescence intensity in crop from uninfected and ZIKV-infected flies. The intensity plot corresponds to a line connecting two points (marked by yellow line in the images; AU, arbitrary unit). (E) Expression of 4G2 in the fat body tissue from uninfected controls and ZIKV-injected adult flies. (F) Measurement of fluorescence intensity of 4G2 expression in the fat body from uninfected controls and ZIKV-injected flies (AU, arbitrary unit). (G) ZIKV load in the fat body, gut and crop tissue from ZIKV-injected flies (8 dpi). In all images, 4G2 was marked in red, Actin in green and nuclei were marked in blue. Scale bars: 100 microns.

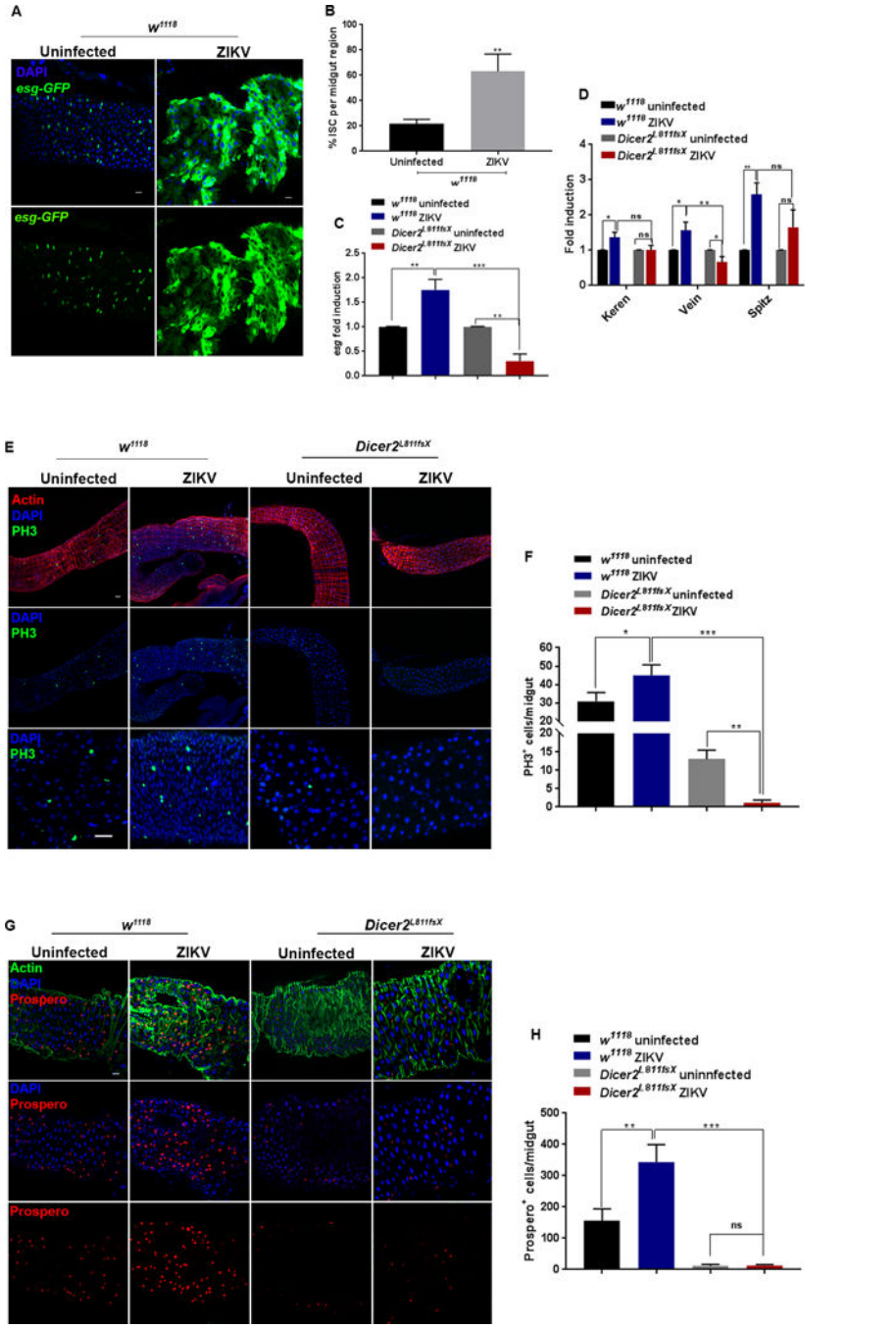


**Figure 4. ZIKV-infected Dicer-2 mutant flies fail to trigger infection-induced intestinal stem cell proliferation.**

(A) Representative images of gut in ZIKV infected wild-type ($w^{1118}$) flies at 8 days post injection (8 dpi) and in uninfected controls. Intestinal stem cells (ISC) were marked by the expression of an *esgGal4UAS-GFP* reporter. (B) Percentage of *esg* positive cells or ISC per midgut region. (C) Quantitative RT-PCR analysis showing the mRNA level of *escargot* in the midgut of uninfected and ZIKV-infected wild-type and *Dicer-2* mutant flies. (D) Quantitative RT-PCR analysis of genes encoding EGFR signaling ligands, *Keren, Vein and Spitz* in midgut of the uninfected controls and ZIKV-infected wild-type and *Dicer-2* mutant

flies. (E) Representative images of midgut in uninfected and ZIKV-infected wild-type ($w^{1118}$) and *Dicer-2* mutant flies. Midgut tissues were stained with anti-PH3 (green) to mark the mitotically active cells. Cytoarchitecture was marked with Actin (red) and nuclei were stained with DAPI (blue). (F) Quantification of PH3-positive cells per midgut tissue in uninfected and ZIKV-infected wild-type and *Dicer-2* mutant flies. (G) Proportion of enteroendocrine (EE) cells in the midgut of uninfected and ZIKV-infected wild-type and *Dicer-2* mutant flies. EE cells were stained with anti-Prospero antibody (red). Actin was marked in green while nuclei were stained with DAPI (blue). (H) Quantification of Prospero positive cells per midgut tissue. Levels of mRNA were normalized against *RpL32* and three independent experiments were performed. In all graphs, bars represent mean ± SD. Statistical analysis was performed using Student's t-test (*$p<0.05$, **$p < 0.05$, ***$p<0.05$); ns denotes no significant differences between experimental treatments. Scale bars: 100 microns.

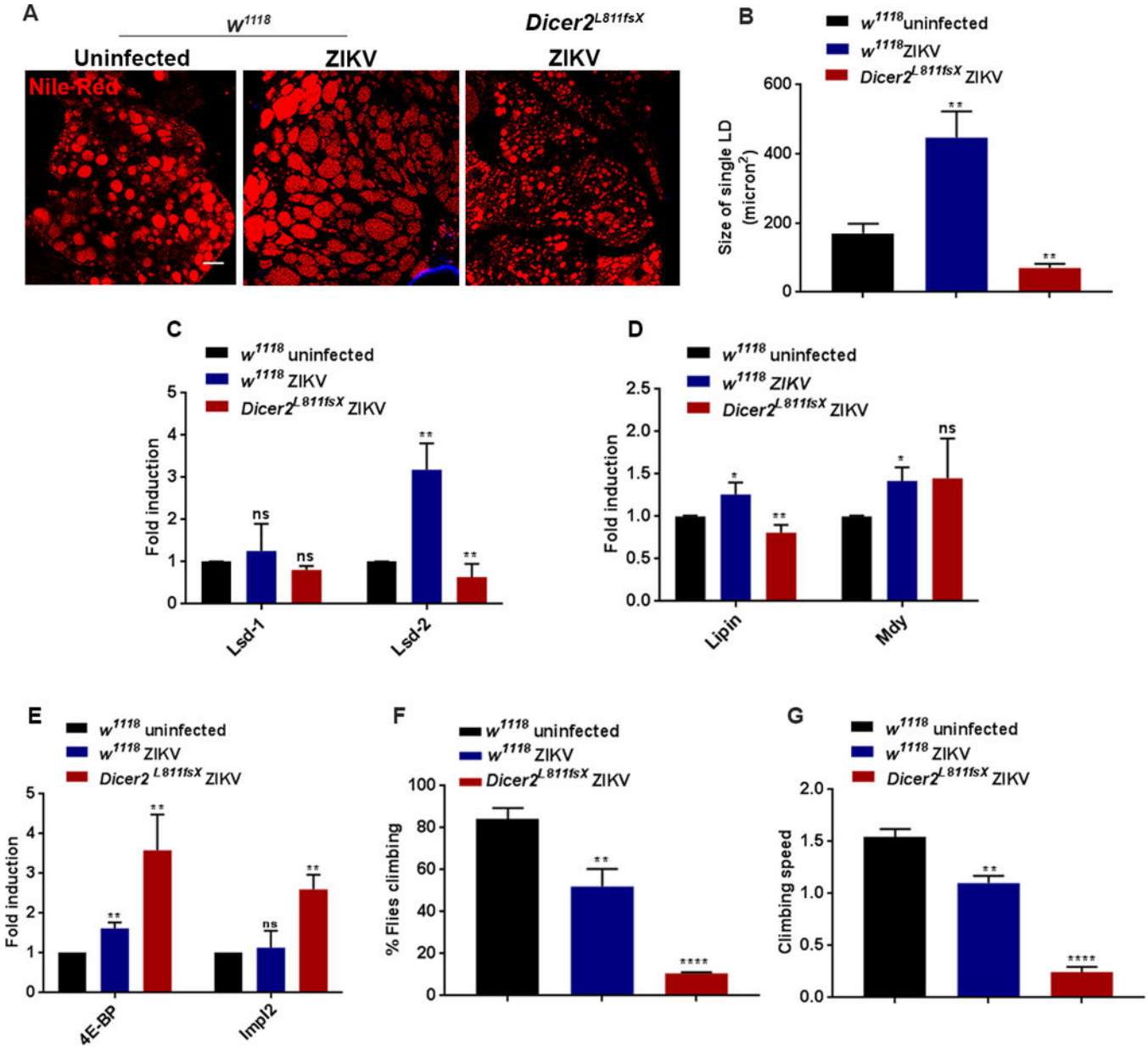


**Figure 5. The increased susceptibility of Dicer-2 mutant flies to ZIKV infection is linked tolipodystrophy and Impl2-induced wasting.**

(A) Representative images of fat body lipid droplets in ZIKV-infected wild-type ($w^{1118}$) flies, *Dicer-2* mutants and uninfected background controls at 8 days post injection (8 dpi). The neutral lipids were marked with Nile Red. (B) Quantification of lipid droplet size in ZIKV infected wild-type and *Dicer-2* mutant flies as well as in uninfected controls. (C-D) Expression analysis of lipid-metabolism related genes in ZIKV-infected $w^{1118}$ flies and *Dicer-2* mutants, and in uninfected $w^{1118}$ individuals. (C) *Lsd-1* and *Lsd-2* were used as read-outs for lipolysis, while (D) *Lipin* and *mdy* were used as read-outs for lipogenesis. (E) Quantitative RT-PCR analysis of the Foxo target gene *4E-BP* and insulin antagonist *Impl2* in ZIKV-infected wild-type ($w^{1118}$) flies, *Dicer-2* mutants and uninfected background controls. (F, G) Climbing ability and speed of climbing in uninfected wild-type controls and ZIKV-

infected wild-type and *Dicer-2* mutant flies. Levels of mRNA were normalized against *RpL32* and three independent experiments were performed. In all graphs, bars represent mean ± SD. Statistical analysis was performed using Student's t-test (*$p<0.05$, **$p < 0.05$, **** $p<0.0001$); ns denotes no significant differences between experimental treatments. Scale bars: 100 microns.

**Table I.**

List of the gene-specific primers used in the study

| Gene | Forward (5′ to 3′) | Reverse (5′ to 3′) |
|---|---|---|
| *Rpl32* | gatgaccatccgcccagca | cggaccgacagctgcttggc |
| *NS5* | ccttggattcttgaacgagga | agagcttcattctccagatcaa |
| *Drosomycin* | gacttgttcgccctcttcg | cttgcacacacgacgacag |
| *Diptericin* | gctgcgcaatcgcttctact | tggtggagttgggcttcatg |
| *Cecropin A1* | tcttcgttttcgtcgctctc | cttgttgagcgattcccagt |
| *Metchnikowin* | tcttggagcgatttttctgg | aataaattggacccggtcttg |
| *Tep1* | agtcccataaaggccgactga | cacctgcatcaaagccatattg |
| *Vago* | tgcaactctgggaggatagc | aattgccctgcgtcagttt |
| *Diedel* | gtgcgtgcaatcgaaaacta | cgtactgctggttcctcctc |
| *Turandot A* | gaagatcgtgaggctgacaac | gtcctgggcgtttttgataa |
| *Turandot M* | gctgggaaaggtaaatgctg | aggcgctgtttttctgtgac |
| *Argonaute-2* | ccggaagtgactgtgacagatcg | cctccacgcactgcattgctcg |
| *Dicer-2* | gtatggcgatagtgtgactgcgac | gcagcttgttccgcagcaatatagc |
| *Listericin* | gagttagggccgctcgtc | cctcctccacagcaatatcct |
| *Vir-1* | gatcccaattttcccatcaa | gattacagctgggtgcacaa |
| *4E-BP* | tcctggaggcaccaaacttatc | ggagccacggagattcttca |
| *Impl2* | aagagccgtggacctggta | ttggtgaacttgagccagtcg |
| *Upd-3* | gccgcgatataaagatacaaagata | actttgcttttgtaacgctgttagt |
| *Lsd-1* | tgagccggcgacagcaacagt | cgtaggcggccgaaatggtg |
| *Lsd-2* | agtgtactagccgatacg | tctgactcccggatct |
| *Lipin* | gggcatgaatgaaatcgag | tcaccaccttgtcgttgtg |
| *Mdy* | cgttctccaatatggacgtg | aaaagcagagccagcaaag |
| *Spitz* | cgcccaagaatgaaagagag | aggtatgctgctggtggaac |
| *Vein* | tcacacatttagtggtggaag | ttgtgatgcttgaattggtaa |
| *Keren* | cgtgtttggcaacaacaagt | tgtggcaatgcagtttaagg |