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Cell fusing agent virus and dengue virus mutually interact in *Aedes aegypti* cell lines

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The genus *Flavivirus* contains more than 70 single-stranded, positive-sense arthropod-borne RNA viruses. Some flaviviruses are particularly medically important to humans and other vertebrates including dengue virus (DENV), West Nile virus, and yellow fever virus. These viruses are transmitted to vertebrates by mosquitoes and other arthropod species. Mosquitoes are also infected by insect-specific flaviviruses (ISFs) that do not appear to be infective to vertebrates. Cell fusing agent virus (CFAV) was the first described ISF, which was discovered in an *Aedes aegypti* cell culture. We found that while CFAV infection could be significantly reduced by application of RNAi against the NS5 gene, removal of the treatment led to quick restoration of CFAV replication. Interestingly, we found that CFAV infection significantly enhanced replication of DENV, and vice versa, DENV infection significantly enhanced replication of CFAV in mosquito cells. We have shown that CFAV infection leads to increase in the expression of ribonuclease kappa (RNASEK), which is known to promote infection of viruses that rely on endocytosis and pH-dependent entry. Knockdown of RNASEK by dsRNA resulted in reduced DENV replication. Thus, increased expression of RNASEK induced by CFAV is likely to contribute to enhanced DENV replication in CFAV-infected cells.

Flaviviruses have single-stranded positive sense RNA genomes and are transmitted to vertebrate species mostly by mosquitoes and other arthropods¹. A number of these viruses cause serious diseases leading to considerable morbidity and mortality around the world. Among mosquito-borne flaviviruses are dengue virus (DENV), West Nile virus, Japanese encephalitis virus and yellow fever virus. Because of poor vector control and lack of effective vaccines or drugs, the resurgence and expansion of mosquito-borne diseases has been an important global health concern in recent decades; for example dengue and Zika, which are most commonly transmitted by the mosquito *Aedes aegypti*².

Mosquitoes can also become infected by insect-specific flaviviruses (ISFs) that do not infect vertebrates. Cell fusing agent virus (CFAV) was the first described ISF discovered from an *Ae. aegypti* cell line³. It was later reported from *Ae. aegypti* mosquitoes in Puerto Rico⁴. It is believed that this virus is transmitted vertically⁴ and therefore the embryos used to initiate the original cell line must have been infected. CFAV has similar genome size, structure and gene order to other flaviviruses. For example, there is over 40% identity of the amino acid sequence of the NS5 protein between CFAV and other flaviviruses⁵. It has been reported that segments of the CFAV genome have integrated into *Ae. aegypti* and *Ae. albopictus* genomes⁶, which suggests that CFAV has been persistently infecting the mosquitoes for a long time. However, it is unclear what functional role the CFAV plays in mosquitoes.

While coinfections or superinfections (sequential infections) of a variety of homologous or heterologous arboviruses has been tested in different insect cell lines (mostly in C6/36 cells) and mosquitoes (reviewed in refs 7 and 8), none of these studies included CFAV. In these studies, the outcomes of coinfections or superinfections were either negative or no interference. In this study, we investigated infection of *Ae. aegypti* cell lines, Aag2 and Aa20, with CFAV and explored the interaction of CFAV with DENV.

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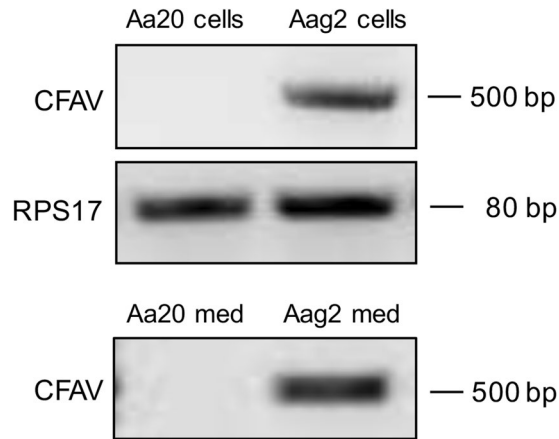


Figure 1. CFAV is found in *Ae. aegypti* Aag2 cell line. RT-PCR analysis of RNA extracted from *Ae. aegypti* Aa20 and Aag2 cells, and their corresponding media (med). *RPS17* gene was used as control. Full-length gel images are displayed in the Supplementary Information file.

Materials and Methods

Insect cell lines. *Ae. aegypti* Aag2 cells were maintained in a medium with a 1:1 mixture of Mitsuhashi-Maramorosch and Schneider's insect media (Invitrogen) supplemented with 10% FBS and in the presence of penicillin (100 U ml⁻¹) and streptomycin (100 µg ml⁻¹). Aa20 cells established from *Ae. aegypti* larvae were kindly provided by the late Prof Richard Elliott. The cells were maintained in Leibovitz's L15 medium supplemented with 10% FBS and 10% Tryptose phosphate broth⁹. To infect Aa20 cells with CFAV, Aag2 culture medium containing the virus (Fig. 1) was collected, centrifuged at 2150 × g for 5 min to remove cells and debris, and used as a CFAV inoculation source. The titre of CFAV was determined using tissue culture infectious dose₅₀ (TCID₅₀) endpoint fixed cell-enzyme-linked immunosorbent assay method as previously described¹⁰.

RNA extraction, cDNA synthesis and polymerase chain reaction (PCR). Total RNA from mosquito cells was isolated using Tri-Reagent (Ambion Inc., USA) after removal of medium and washing cells three times with PBS. RNA was incubated with DNase I at 37 °C for 10 min and then inactivated at 75 °C for 10 min. The first strand cDNA was synthesized by reverse transcription (RT) with CFAV-specific or poly(dT) primers (for ribosomal protein S17, *RPS17*, housekeeping gene detection as control). In each RT reaction, approximately 2 µg of total RNA was used as template in a volume of 20 µl. Following cDNA synthesis, 2 µl of RT products was used for each PCR in a total reaction volume of 25 µl with CFAV NS5 gene-specific primers (Forward: 5'-GCCCCACATCTGGGCRTRNGCCTTNGC-3'; Reverse: 5'-GGGCAAGTARBMACTTATGCVTTGAACAC-3'). These are referred to as CFAV-specific detection primers. Amplification was performed at 95 °C for 1 min, followed by 35 cycles of 95 °C for 30 sec, 56 °C for 30 sec, 68 °C for 1 min, and a final extension at 68 °C for 5 min. PCR products were run on agarose gels, stained by ethidium bromide, and bands were visualized in a gel documentation system (Red, Proteinsimple) using UV light. Images were recorded and shown in negative.

RT-qPCR. Total RNA was extracted from mosquito cells and treated with DNase I. The synthesis of first strand cDNA was carried out using a specific reverse primer to DENV or CFAV (CFAV-qR 5'-CACAACGGTAGCGAGAGACA-3'). Following the RT, qPCRs with DENV (forward: 5'-GTGGTGGTGACTGAGGACTG-3'; reverse: 5'-CCATCCCGTACCAGCATCCG-3') and CFAV specific primers (CFAV-qF 5'-CTGATGTGCGTGCAGTTCTT-3' and CFAV-qR) were carried out to determine the DENV and CFAV genomic RNA levels in cells. Platinum SYBR Green Mix (Invitrogen) was used for qPCR with 1 µl of RT products in a Rotor-Gene thermal cycler (QIAGEN) as described above. The *RPS17* gene was used for normalizing data as described previously¹¹. Each reaction was run with 3 biological replicates, each with 3 technical replicates. The relative abundance of viral RNA to the host reference gene was determined by qGENE software and analyzed using GraphPad Prism.

RNAi-mediated silencing. For RNAi-based experiments, dsRNAs were synthesized *in vitro* using the T7 MEGAscript transcription kit according to the manufacturer's instruction (Ambion Inc., USA). T7 promoter sequences (TAATACGACTCACTATAGGG) were incorporated in both forward and reverse primers designed to amplify an around 500 bp fragment of the CFAV NS5 (forward: 5'-GAGGAGGATCTGGAGGATGA-3'; reverse: 5'-CCCTCGCCACCTGTACCTTA-3'). These are referred to as CFAV RNAi primers, which were different to the CFAV-specific detection primers. For dsRNA synthesis, 200–500 ng of PCR product were used for each reaction. Reaction was incubated at 37 °C overnight, DNase-treated and precipitated by the lithium chloride method following the manufacturer's instruction. A total of 4 µg of dsRNA was used to transfect Aag2 cells with 5 µl of Cellfectin transfection reagent (Invitrogen). The cells were transfected again with the same reagent at 48 h intervals after the first transfection. Cells were collected for RNA extraction as required for further analysis at 24 h after the second transfection. Gene silencing was confirmed by RT-PCR using CFAV-specific detection primers.

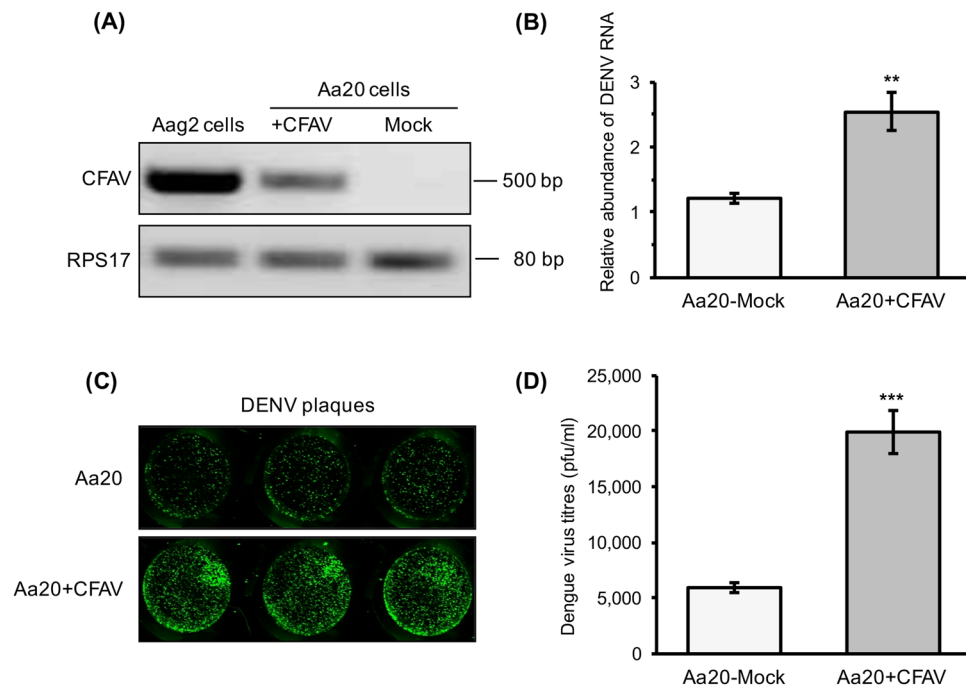


Figure 3. DENV replication is enhanced by CFAV in Aa20 cells. **(A)** RT-PCR analysis of CFAV was performed using RNA extracted from Aa20 mock and Aa20 cells infected with CFAV. *RPS17* gene was used as control to show the integrity of RNA. Full-length gel images are displayed in the Supplementary Information file. **(B)** RT-qPCR analysis of DENV was performed using RNA extracted from Aa20 and Aa20 + CFAV cells infected for 3 days with 1 MOI of DENV. Three biological replicates with three technical replicates were carried out for each transfection (* $P < 0.05$; t test). **(C)** Plaque assay of media collected from the experiment described in **(B)** without dilution using anti-DENV2-Envelope protein antibody. Each well represents a biological replicate, and only one representative replicate of the plaque assay is shown here. **(D)** DENV titration of medium from the experiment described in **(B)** (*** $P < 0.001$; t test).

DENV enhances replication of CFAV in mosquito cells. The above results showed that CFAV promoted the replication of DENV in cells. To investigate whether DENV reversely influences the replication of CFAV, Aag2 cells and Aa20 + CFAV cells were inoculated with DENV. Total RNA at 72 hpi was extracted and analysed by RT-PCR with CFAV-specific detection primers. RT-PCR results showed that the CFAV RNA levels were higher in Aag2 cells infected with DENV as compared with mock-infected Aag2 cells (Fig. 4A). Similarly, in Aa20 + CFAV cells that were used at 7 days after CFAV infection, the CFAV RNA levels were found to be higher at 3 days following DENV infection as compared with DENV non-infected Aa20 + CFAV cells (Fig. 4B). The results suggest that DENV mutually enhances replication of CFAV.

We also examined the dynamics of the effect of DENV infection on CFAV and vice versa. For this, Aag2 cells that are already infected with CFAV were infected with MOI 0.1 of DENV. RNA was extracted from cells at 1, 3 and 5 dpi of DENV and analysed by RT-qPCR using specific primers to CFAV. While in mock-infected Aag2 cells CFAV levels remained constant, in those infected with DENV, there was an increase in CFAV levels, although this was only statistically significant at 3 dpi (Fig. 4C), consistent with Fig. 4A. To examine the effect of CFAV on DENV infection, Aa20 cells were infected either with DENV alone or DENV together with CFAV (both at 0.1 MOI). RT-qPCR results showed significant but modest increase in the levels of DENV RNA in Aa20 cells when co-infected with CFAV at 1 and 3 dpi, while it decreased at 5 days post-infection (Fig. 4D). It appears that when Aa20 cells were already infected with CFAV prior to DENV infection, the effect on DENV was more prominent (Fig. 3) as compared to when they were co-infected with both viruses at the same time. Similarly, co-infection of Aa20 cells with CFAV and DENV enhanced CFAV replication as compared to CFAV infection alone (Fig. 4E). In an independent experiment, in which Aa20 cells were infected either with DENV alone or co-infected with CFAV, more DENV virions were found in the media collected from cells with CFAV-DENV co-infection at 4 dpi (Fig. 5).

Increase in RNASEK by CFAV and its effect on DENV. In testing a number of genes (five genes cecropin, defensin, attacin, domeless and RNASEK) reported in the literature to be involved in insect virus-host interactions and immunity^{13–18}, we noticed a significant increase in the transcript levels of RNASEK in Aa20 + CFAV cells as compared to Aa20 cells (Fig. 6A). The genes were assessed based on their transcriptional changes in response to DENV infection analysed by RT-qPCR. RNASEK was also highly expressed in Aag2 cells as compared to Aa20 cells that are devoid of CFAV (Fig. 6B). Ribonuclease kappa (RNASEK) was shown to promote infection of a range of viruses that rely on endocytosis and pH-dependent entry, including DENV¹⁶. The role of RNASEK in replication of these viruses was examined in *Drosophila* S2 cells and human osteosarcoma cells (U2OS) but not in mosquito cells. To confirm that RNASEK promotes DENV replication in mosquito cells, we silenced RNASEK in

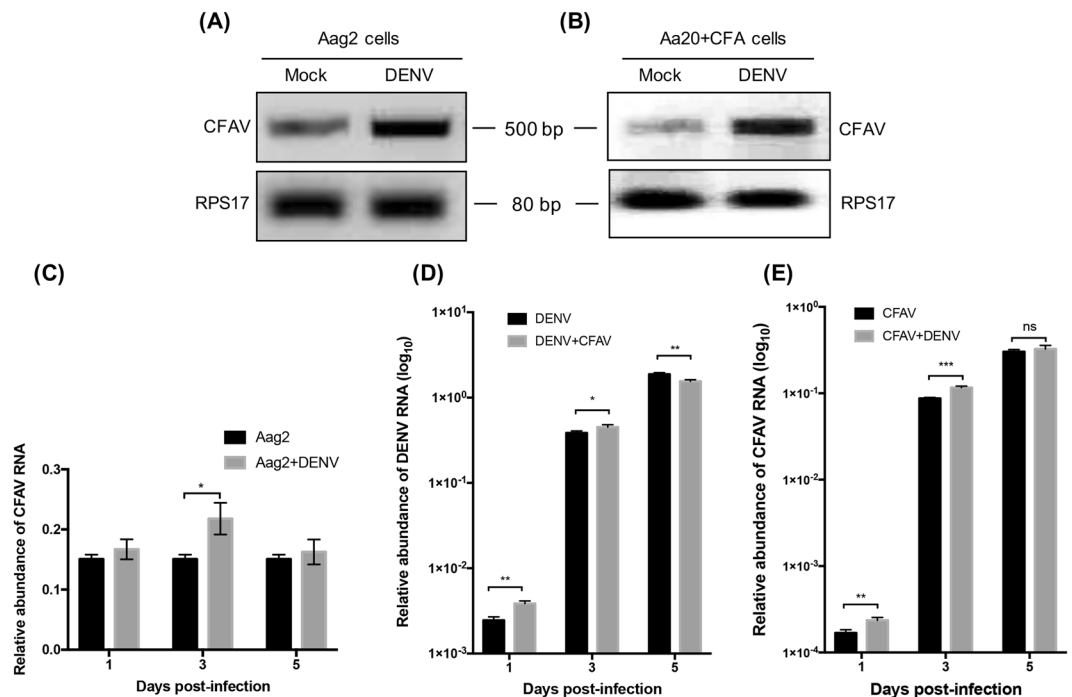


Figure 4. CFAV replication is increased by DENV in CFAV-infected Aa20 and Aag2 cells. **(A)** RT-PCR analysis of CFAV was performed using RNA extracted from Aag2 (Mock) and Aag2 cells infected with 1 MOI DENV for 72 h. **(B)** RT-PCR analysis of CFAV was performed by using RNA extracted from Aa20 + CFAV cells (infected with CFAV for 7 days prior to DENV infection) extracted at 72 h after infection with 1 MOI DENV. Mock represents RNA from Aa20 + CFAV cells that were not infected with DENV and collected at the same time as DENV-infected cells. *RPS17* gene was used as control to show the integrity of RNA. Full-length gel images are displayed in the Supplementary Information file. **(C)** RT-qPCR analysis of RNA from Aag2 cells infected with 0.1 MOI of DENV-2 and collected at three time points following infection. **(D)** and **(E)** RT-qPCR analysis of RNA from Aa20 cells either singly infected or co-infected with 0.1 MOI of DENV and CFAV and collected at three times points following infection, which were analysed by DENV and CFAV specific qPCR primers, respectively. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ANOVA test with Tukey *post hoc* comparison).

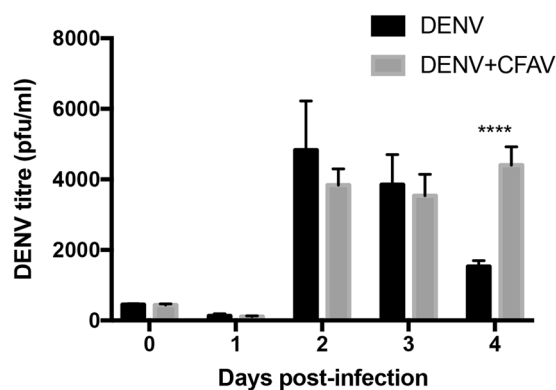


Figure 5. DENV virion production is enhanced by CFAV co-infection. Aa20 cells were infected with 0.1 MOI DENV only or co-infected with 0.1 MOI DENV + CFAV. Media were collected from day 0 to 4 days post-infection and analysed for virion production by plaque assay (**** $P < 0.0001$; ANOVA test with Tukey *post hoc* comparison).

Aa20 cells by transfection of dsRNASEK into the cells (Fig. 7A) followed by their infection with 1 MOI of DENV-2. After 72 h, RT-qPCR analysis revealed significantly less DENV gRNA present in dsRNASEK-transfected cells as compared with mock or dsGFP transfected cells (Fig. 7B). This result was further confirmed by plaque assay using media collected from cells in the silencing experiment, which clearly showed substantially less infectious DENV in the media from cells transfected with dsRNASEK as compared with the controls (Fig. 7C and D). The

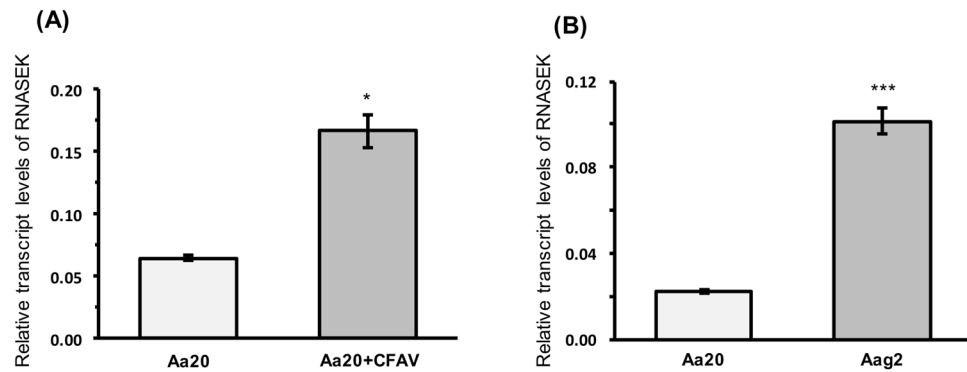


Figure 6. RNASEK transcript levels increased by CFAV. **(A)** RT-qPCR analysis of RNASEK was performed using RNA extracted from Aa20 and Aa20 + CFAV cells ($*P < 0.0453$; Wilcoxon non-parametric test). **(B)** Relative transcript levels of RNASEK in Aa20 (without CFAV) and Aag2 (infected with CFAV) cells. Three biological replicates with three technical replicates were carried out for each treatment ($***P < 0.001$; t test).

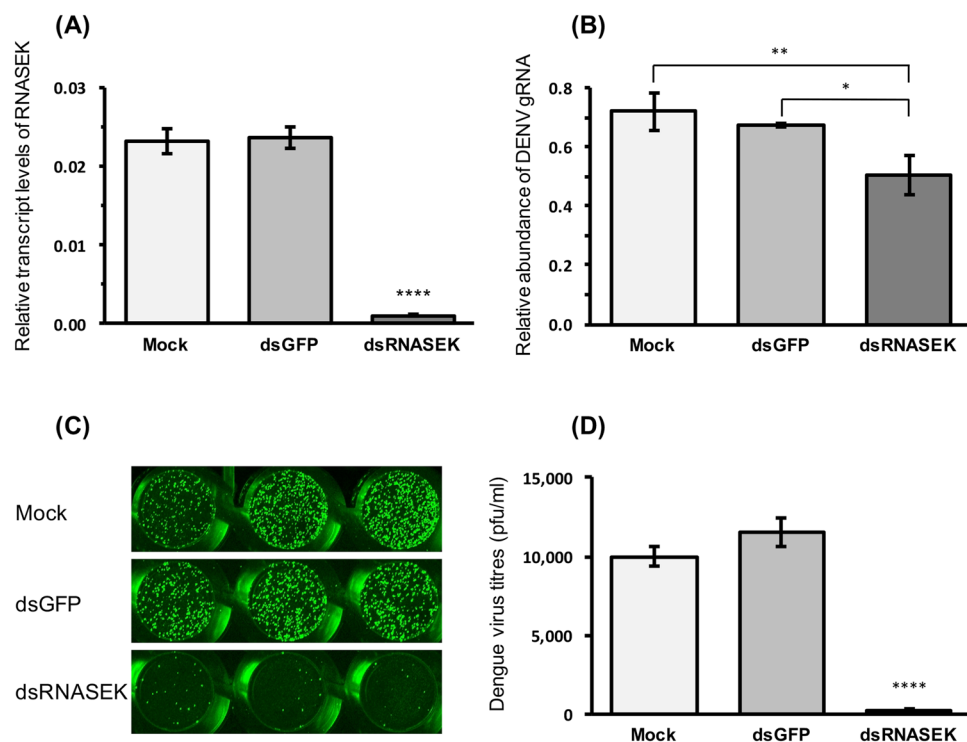


Figure 7. Silencing RNASEK reduces DENV replication. **(A)** Confirmation of RNASEK silencing in Aa20 cells by RT-qPCR ($****P < 0.00001$; ANOVA test). **(B)** Relative abundance of DENV gRNA was assessed in Aa20 cells transfected with mock, dsGFP and dsRNASEK by RT-qPCR. Three biological replicates with three technical replicates were carried out for each treatment ($**P < 0.001$; $*P < 0.05$; Wilcoxon non-parametric test with Tukey *post hoc* comparison). **(C)** Plaque assay of media collected from the experiment described in **(B)** without dilution using anti-DENV2-Envelope protein antibody. Each well represents a biological replicate, and only one representative replicate of the plaque assay is shown here. **(D)** DENV titration of medium from the experiment described in **(B)** ($****P < 0.0001$; ANOVA test).

data show that RNASEK knockdown results in reduction of DENV RNA in infected cells and of infectious virus in the culture fluid.

Discussion

With the utilization of advanced next generation sequencing approaches for viral detection/discovery, there has been a significant increase in the isolation and characterization of ISFs recently^{19–27}. The existence of ISFs in mosquitoes may affect their vector competence in transmission of pathogenic arboviruses to vertebrate hosts. For instance, in *Culex pipiens*, *Culex flavivirus* (CxFV) suppressed dissemination of West Nile virus (WNV) at early stages of infection (7 days), which may consequently affect the severity of enzootic transmission of WNV by the

mosquito^{28,29}. In addition, it was reported that CxFV from Guatemala could significantly enhance the transmission rate of WNV in *Culex quinquefasciatus* from Honduras³⁰. Similarly, another ISF, Palm Creek virus, repressed replication of WNV and Murray Valley encephalitis virus in mosquito cells, but did not repress replication of the alphavirus Ross River virus²⁷. Further, Eilat virus, reduced replication of several mosquito-borne viruses such as Sindbis virus, eastern, western, and Venezuelan equine encephalitis viruses, and Chikungunya virus²⁶. While the majority of examples indicate that ISFs may have a negative effect on the replication of pathogenic flaviviruses, we found rather positive interaction of CFAV and DENV; although as mentioned above in the case of CxFV and WNV in *Cx. quinquefasciatus*, our observation is not the first instance of a positive interaction between an ISF and a pathogenic flavivirus.

Cell fusing agent virus (CFAV) was the first described ISF, which was discovered in an *Ae. aegypti* cell culture named Peleg³. Different strains of the virus have also been detected from field-collected *Ae. aegypti* and other mosquito species^{4,21,31,32}. CFAV has similar genome size, structure and gene order to other flaviviruses³³. In the present study, CFAV was detected in *Ae. aegypti* Aag2 cell line, but not in another *Ae. aegypti* cell line, Aa20. Because Aag2 cells are one of the commonly used mosquito cells, and a persistent infection with CFAV could affect the cells' biology and response to other infections, we attempted to remove it from Aag2 cells by RNAi using CFAV NS5-specific dsRNA. After continuous transfection of dsRNA into the cells for five passages, CFAV RNA was hardly detectable in the treated cells. Unfortunately, once the dsRNA application was stopped, CFAV came back as soon as one passage and was completely restored to its original levels in three passages. We also found that CFAV significantly promoted the replication of DENV in mosquito cells, particularly if cells were already infected with CFAV prior to DENV infection. Conversely, DENV was also found to moderately enhance the replication of CFAV in *Ae. aegypti* cells.

RNASEK is a protein with two putative transmembrane regions that is highly conserved across vertebrates and invertebrates but its function is largely unknown^{34,35}. A recent study demonstrated that RNASEK promotes the uptake of viruses that enter host cell via clathrin-mediated endocytic route (e.g. flaviviruses, alphaviruses, bunyaviruses, and orthomyxoviruses) but not those that fuse at the cell membrane (e.g. picornavirus and paramyxovirus)¹⁶. Furthermore, RNASEK was shown to be associated with vATPase and required for the function of vATPase throughout the cells¹⁷, and depletion of either of the genes led to increase in the pH of endosomes. We also found that silencing RNASEK resulted in reduction in DENV replication in mosquito cells, which corroborates with previous studies with other viruses. Interestingly, our results showed that RNASEK expression was significantly increased in CFAV-infected mosquito cells, which may explain the enhancement of DENV infection in these cells.

With more deep sequencing data becoming available from insects and cell lines showing the presence of a variety of undetected viruses that have established persistent infections, it might be rare that we find a cell line or an insect lab colony that is completely free of any virus. While these cell lines and insect colonies continue to be useful as tools in laboratories, depending on the experimental design and aims of the study, caution should perhaps be undertaken in interpretation of data.

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Author Contributions

G.Z. designed experiments, carried out experiments, analysed data and drafted the manuscript. SAsa carried out the plaque assays for CFAV and DENV time trials. A.A.K. designed experiments and edited the manuscript. SAsg conceived the study, designed experiments and edited the manuscript. All authors read and approved the final manuscript.

Additional Information

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