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A salivary protein of *Aedes aegypti* promotes dengue-2 virus replication and transmission

Chalida Sri-in[a], Shih-Che Weng[b], Wen-Yu Chen[c], Betty A. Wu-Hsieh[c], Wu-Chun Tu[a,**], Shin-Hong Shiao[b,*]

[a] Department of Entomology, College of Agriculture and Natural Resources, National Chung Hsing University, Taichung, Taiwan
[b] Department of Tropical Medicine and Parasitology, College of Medicine, National Taiwan University, Taipei, Taiwan
[c] Graduate Institute of Immunology, College of Medicine, National Taiwan University, Taipei, Taiwan

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ABSTRACT

Although dengue is the most prevalent arthropod-borne viral disease in humans, no effective medication or vaccine is presently available. Previous studies suggested that mosquito salivary proteins influence infection by the dengue virus (DENV) in the mammalian host. However, the effects of salivary proteins on DENV replication within the *Aedes aegypti* mosquito remain largely unknown. In this study, we investigated the effect of a specific salivary protein (named AaSG34) on DENV serotype 2 (DENV2) replication and transmission. We showed that transcripts of AaSG34 were upregulated in the salivary glands of *Aedes aegypti* mosquitoes after a meal of blood infected with DENV2. Transcripts of the dengue viral genome and envelop protein in the salivary glands were significantly diminished after an infectious blood meal when AaSG34 was silenced. The effect of AaSG34 on DENV2 transmission was investigated in *Stat1*-deficient mice. The intradermal inoculation of infectious mosquito saliva induced hemorrhaging in the *Stat1*-deficient mice; however, saliva from the AaSG34-silenced mosquitoes did not induce hemorrhaging, suggesting that AaSG34 enhances DENV2 transmission. This is the first report to demonstrate that the protein AaSG34 promotes DENV2 replication in mosquito salivary glands and enhances the transmission of the virus to the mammalian host.

1. Introduction

Dengue is the most prevalent arthropod-borne viral disease in humans. It occurs primarily in tropical areas, infecting 50–200 million people a year worldwide (Murray et al., 2013). At present, there is no effective vaccine or medicine to protect against or treat it, although mosquito vector control remains an effective strategy for controlling virus transmission. Another possible way to control dengue in communities is to reduce the viral load or inhibit viral growth in the mosquito vector, an approach that by-passes the problems of drug resistance and insecticide pollution. To this end, it is important to understand the interaction between the virus and the mosquito vectors.

The dengue virus (DENV) is a positive-stranded RNA virus of the family *Flaviviridae* with four known serotypes. The virus is spread by *Aedes* mosquitoes, the most important being *Aedes aegypti* (Gubler, 2002). The mosquito vector becomes infected with the virus via a blood meal from a DENV-infected human during the viremia phase (Guzman et al., 2016). When a biting mosquito is filled with infected blood, the virus passes directly to the midgut, where the virus replicates. Eventually the virus disseminates to the mosquito's salivary glands, where it replicates and enters the saliva (Cheng et al., 2016; Guzman et al., 2016; Sánchez-Vargas et al., 2009). The virus can then be transmitted to a human via the infected mosquito's saliva during a subsequent blood meal (Patramool et al., 2012). Thus, the salivary gland is important for blood feeding and for DENV transmission.

For a mosquito to successfully obtain a blood meal, it must prevent blood coagulation and combat the host's immune response. It does this by injecting into the host's skin saliva that contains a complex mixture of salivary proteins (Ribeiro et al., 1984). More than 20 proteins have been identified in the saliva of *Ae. aegypti* (Conway et al., 2016; Racioppi and Spielman, 1987). The salivary proteins have diverse functions, including odorant binding, protease inhibition, the cleavage of phosphodiester and peptide bonds, thrombin inhibition, lipid transport, collagen binding, triggering of the host's innate immune response, countering blood coagulation, preventing platelet aggregation, and dilating the host's blood vessels (Oktarianti et al., 2015; Ribeiro et al.,

* Corresponding author.
** Corresponding author.
E-mail addresses: wctu@dragon.nchu.edu.tw (W.-C. Tu), shshiao@ntu.edu.tw (S.-H. Shiao).

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2007; Sun et al., 2006; Wichit et al., 2016). It has been shown that certain salivary gland proteins were highly expressed in blood-fed mosquitoes (Wasinpiyamongkol et al., 2010), and that DENV infection induced changes in salivary proteins in *Ae. aegypti*, which affected the subsequent viral replication (Chisenhall et al., 2014). Infection of *Ae. aegypti* with DENV was shown to induce proteins with anti-hemostatic and pain inhibitory properties, which helped to increase biting rates and transmission success (Chisenhall et al., 2014). These findings indicate that mosquito saliva not only assists the mosquito in obtaining a blood meal, but also facilitates transmission of the virus.

Specific saliva components have been shown to affect arbovirus infection or transmission. A 15-kDa *Ae. aegypti* salivary protein, LTRIN, which interferes with signaling via the lymphotoxin-β receptor, has been shown to facilitate the transmission of Zika virus and to exacerbate its pathogenicity (Jin et al., 2018), and a 34-kDa recombinant *Ae. aegypti* salivary protein has been shown to enhance DENV replication in human keratinocytes by reducing antimicrobial peptides and type I IFN activity (Surasombatpattana et al., 2014). Conversely, D7L2, another recombinant *Ae. aegypti* salivary protein, inhibits DENV serotype 2 (DENV2) replication by binding to DENV2 virions and E protein; it has been shown that adding D7L2 inhibited DENV2 replication in U937 cells and in mice deficient in receptors for type I and type II IFNs (Conway et al., 2016).

Although studies have revealed various roles of mosquito salivary proteins in arbovirus replication within the mammalian host, their roles in DENV replication within the mosquito's own salivary gland and the effect of this on viral transmission remain largely unknown. The aim of this study was to investigate the potential roles of six salivary proteins on DENV2 replication and transmission within the salivary glands of *Ae. aegypti* mosquitoes. We examined the regulation of the proteins in the presence of DENV2 and the effects of the proteins on DENV2 replication, and we confirmed the findings with *Stat1*-deficient mice.

We examined six mosquito salivary gland proteins and found that transcription levels of Apyrase, D7L2, and a 34-kDa protein were significantly upregulated in the salivary glands after DENV2 infection. Silencing the 34-kDa protein reduced DENV2 replication in the salivary glands; silencing the other two proteins did not have this effect. We therefore named this protein "*Ae. aegypti* salivary gland protein of 34 kDa" (AaSG34). Remarkably, mosquitoes with AaSG34 gene knockdown failed to transmit DENV2 to *Stat1*-deficient mice. Our results demonstrated that AaSG34 is critical for DENV2 replication in mosquito salivary glands and for DENV2 transmission to the mammalian host.

2. Materials and methods

2.1. Ethics statement

The research plan for the experiments involving animals was approved by the Laboratory Animal Center at National Taiwan University (approval ID, #20100268). All procedures and care were performed according to the Standard Operating Procedures of the Laboratory Animal Center. Everyone involved in the animal work had successfully completed Animal Care Training at National Taiwan University and was specifically trained in the protocols used in the research plan.

2.2. Mosquitoes and virus stock

Ae. aegypti UGAL/Rockefeller strain mosquitoes were maintained as previously described (Chang et al., 2018) until adult at 28 °C in 80% relative humidity with a 12:12 h light:dark photoperiod. The experiments used mosquitoes 3–5 days after emergence. *Ae. albopictus* C6/36 cells were cultured in DMEM/MM (1:1) containing 2% heat-inactivated fetal bovine serum (FBS) and 1 × penicillin–streptomycin solution. These were infected with DENV2 strain 16681 at a multiplicity of infection of 0.01. The culture supernatant was harvested 5 days after infection and subjected to a plaque assay to determine the viral titer. DENV2 at approximately 1.0×10^7 PFU/ml was used to infect the mosquitoes.

2.3. Double-stranded RNA synthesis

Six *Ae. aegypti* salivary proteins were initially considered in the analysis: Aegyptin, SRPN23, SRPN26, Apyrase, D7L2, and AaSG34 (a 34-kDa protein we named as an abbreviation of "*Ae. aegypti* salivary gland protein of 34-kDa"). However, it was found that Apyrase, D7L2, and AaSG34 were upregulated following infection with DENV2, whereas Aegyptin, SRPN23, and SRPN26 were not. Double-stranded RNA (dsRNA) was therefore generated for the genes that transcribe Apyrase, D7L2, and AaSG34, using a method described previously (Chang et al., 2018). Briefly, whole bodies of *Ae. aegypti* mosquitoes were used for RNA extraction using Trizol Reagent (Invitrogen, CA, USA), following the manufacturer's protocol. Complementary DNA (cDNA) synthesis was performed with a high capacity cDNA reverse transcription kit using 2 μg of total RNA with Random Hexamers primer (Applied Biosystems, USA). The cDNA served as a template for polymerase chain reaction (PCR) amplification of fragments of the target genes by ExTaq DNA polymerase (Takara, Japan), using the specific primers listed in Table 1. Fragments were cloned into a pCR2.1 TOPO vector (Invitrogen, USA), and the constructed plasmid was transformed into *Escherichia coli* HIT-DH5α competent cells. The competent cells were incubated for 16–18 h on an LB/ampicillin agar plate for blue–white screening. The positive colonies were amplified in the culture tubes with 3 ml LB broth and 3 μl ampicillin. High copy number plasmids from the positive colonies were isolated using FavorPrep Plasmid DNA Extraction Mini Kit (Favorgen, Taiwan). A restriction enzyme was used to digest the plasmids and the fragments were separated using 1% agarose gel. The target fragments were isolated by agarose gel electrophoresis, then excised from the gel, and purified with a FavorPrep Gel/PCR Purification Kit (Favorgen, Taiwan). The fragments were amplified by ExTaq DNA Polymerase (Takara, Japan) and purified with the FavorPrep Gel/PCR Purification Kit (Favorgen). These preparations were used as the templates for dsRNA synthesis using an AmpliScribe T7 Transcription Kit (Epicenter Technologies). The dsRNA was purified and dissolved in DEPC-H_2O. The primers used for the dsRNA preparation are shown as Table 1.

Table 1
Primers used for dsRNA synthesis.

Primer Name	5′-sequence-3′
T7-Apyrase Forward primer	TAA TAC GAC TCA CTA TAG GGG CTG GAA GAC CGG GAT ACA G
T7-Apyrase Reverse primer	TAA TAC GAC TCA CTA TAG GGG CCG TCA CAT TCC ACC TTA G
T7-D7L2 Forward primer	TAA TAC GAC TCA CTA TAG GGG TAG ACA GCC CCG CAA CTC
T7-D7L2 Reverse primer	TAA TAC GAC TCA CTA TAG GGA TTG CTT CTT CTG GCG AAT G
T7-AaSG34 Forward primer	TAA TAC GAC TCA CTA TAG GGC GAA GAG AAG CTG AAC GAC C
T7- AaSG34 Reverse primer	TAA TAC GAC TCA CTA TAG GGC ACA GCA CCA ACC GAT CC
T7-LacZ Forward primer	TAA TAC GAC TCA CTA TAG GGT TTC CCC GTC AAG CTC TAA A
T7-LacZ Reverse primer	TAA TAC GAC TCA CTA TAG GGA ATC ATG CGA AAC GAT CCT C

2.4. RNA interference

Female mosquitoes were injected with dsRNA to silence the individual genes that express Apyrase, D7L2, or AaSG34 in the salivary glands. As a control, mosquitoes were injected with dsRNA for LacZ (dsLacZ). LacZ is an operon in *E. coli* and many other enteric bacteria (Joung et al., 2000). The mosquitoes were immobilized with CO_2 and administered an intrathoracic microinjection via the lateral side of the thorax of 1 μg of dsRNA (5 μg/μl) using a Nanoinject II Injector (Drummond Scientific, USA). After injection, the mosquitoes were transferred into cylindrical containers fitted with a nylon mesh on the top and supplied with 10% sucrose solution. They were maintained at 28 °C and 80% humidity according to the standard rearing conditions (Weng and Shiao, 2015).

2.5. Infection of the mosquitoes with DENV2

Infection was achieved either through intrathoracic injection or with a blood meal. For the injection, female mosquitoes were immobilized with CO_2 and intrathoracically inoculated with 69 nl of DENV2 16681 (6.9×10^2 PFU in 69 nl) using a Nanoinject II Injector (Drummond Scientific, USA). They were then transferred into cylindrical containers fitted with a nylon mesh on the top. The infected mosquitoes were dissected on days 0–4 after infection to analyze the levels of Apyrase, D7L2, and AaSG34 in different tissues by quantitative reverse transcription PCR (qRT-PCR). Infectious saliva was collected on days 3, 5, and 7 after infection for Western blot analysis to determine the expression level of dengue virus E protein.

For infection by blood meal, female mosquitoes were transferred into cylindrical containers fitted with a nylon mesh on the top and starved for 12 h by sugar deprivation. They were then offered an infectious blood meal comprising 250 μl of blood mixed with 250 μl of DENV2 16681 (2.5×10^6 PFU in 250 μl) and 10 μl of 1 mM adenosine triphosphate (ATP). For a normal blood meal, mosquitoes were offered a blood meal comprising 250 μl of blood mixed with 250 μl of DMEM containing 2% heat-inactivated FBS and 10 μl of 1 mM ATP. The artificial blood meal was wrapped in a stretched Parafilm-M membrane. The mosquitoes took the meal for 1 h. Each mosquito was then examined on a stereomicroscope to confirm it had taken a full meal and was transferred into a cylindrical container with the nylon mesh. The infected mosquitoes were dissected on days 1, 3, and 5 after the blood meal to measure the transcription levels of AaSG34 in various tissues using qRT-PCR. The salivary glands were dissected on days 0, 1, 3, 5, and 7 after the blood meal to measure the transcription levels of AaSG34 and dengue viral RNA (vRNA) by qRT-PCR, or on days 7, 10, and 13 after the blood meal to determine the amount of dengue viral E protein. Infectious saliva was collected on days 7, 10, and 13 after the blood meal for Western blot analysis to measure dengue viral E protein and for a plaque assay to determine the viral titer.

2.6. Quantitative real-time PCR analysis

The total RNA in each sample was isolated and transcribed into cDNA. The cDNA sample was quantified by using a KAPA SYBR FAST Universal qPCR Kit (Sigma-Aldrich, USA). The transcription levels of salivary proteins and DENV2 were measured using qRT-PCR. The primers for this were designed by using ABI Primer Expression Software (Applied Biosystems instruments, USA); they are listed in Table 2. The signals of the target genes were analyzed with an ABI 7900HT Fast Real-Time PCR System, and the relative quantification results were normalized using the ribosomal protein gene S7 as an internal control. The signal strength was calculated from the cycle threshold values of the target gene and determined using the $2^{-\Delta\Delta}$Ct method (the relative mRNA expression level), based on two or three independent experiments for each individual day post-infection.

Table 2
Primers used for qPCR analysis.

Primer Name	5′-sequence-3′
Apyrase Forward primer	AGC CCA AAC GGG AAA GGT TA
Apyrase Reverse primer	ACA TCG ATA TCG TCA CCG GC
D7L2 Forward primer	GTA TAT AAA ATG CAT AAG GCC TGT C
D7L2 Reverse primer	CCA CGG AAA AGG TTG TAA CT
AaSG34 Forward primer	TGA AAG CGG CCA TCT CAA GT
AaSG34 Reverse primer	TTG CGT TTT CAG GAC CTC CA
S7 Forward primer	TCA GTG TAC AAG AAG CTG ACC GGA
S7 Reverse primer	TTC CGC GCG CGC TCA CTT ATT AGA TT
3′ UTR DENV-Forward primer	TTG AGT AAA CYR TGC TGC CTG TAG CTC
3′ UTR DENV-Reverse primer	GGG TCT CCT CTA ACC TCT AGT CCT

2.7. Protein extraction and western blot analysis

The mosquito salivary gland proteins were extracted by placing the salivary glands in 100 μl protein lysis buffer as previously described (Weng and Shiao, 2015). The homogenized suspensions were centrifuged at 18,928 × *g* for 30 min. The supernatant was transferred to a QIAshredder spin column (Qiagen, USA) and centrifuged at 18,928 × *g* for 30 min. The eluted samples were collected and transferred to new tubes. Saliva was collected by exposing the mosquitoes for 1 h to a feeding solution containing 100 μl of 1 × phosphate-buffered saline (PBS) and 10 μl of 1 mM ATP (Brackney et al., 2008; Chisenhall et al., 2014) via a stretched Parafilm-M membrane. After probing, the feeding solutions were transferred to tubes and stored at −80 °C.

The mosquito saliva or salivary gland extract proteins were fractionated by protein electrophoresis on a 12% polyacrylamide gel and transferred to a 0.45 μm polyvinylidene fluoride membrane (Millipore). The membranes were then blocked with 5% skim milk in PBST (1 × PBS, 0.4% Tween20) for 30 min at room temperature and then incubated with the primary antibody against dengue viral E protein (GTX127277; GeneTex) at 1:10,000 dilution in PBST. The membranes were washed in PBST and incubated for 1 h at 4 °C with the secondary antibody (Anti-mouse horseradish peroxidase IgG) at 1:5000 dilution in PBST. The membranes were washed in PBST and developed in the presence of WesternBright ECL (Advansta Inc.) as a chemiluminescent substrate for the enzyme horseradish peroxidase. The intensity of the band with positive signals was quantified by ImageJ software.

2.8. Plaque assay

Infected saliva was collected from the AaSG34-silenced mosquitoes and the dsLacZ-treated control mosquitoes as described above. C6/36 cells were seeded in a 24-well tissue culture plate and left overnight. The cell monolayers were rinsed with PBS, and 200 μl of 10-fold serial dilutions of the infectious saliva were added to cell monolayers for 2 h. After absorption, 500 μl of 1% methyl cellulose cell media was added, and the plates were kept in an incubator at 28 °C for 5 days (Das et al., 2008; de Wispelaere et al., 2017). After this, the plates were fixed with 4% formaldehyde for 1 h at room temperature. The methyl cellulose overlays were then removed, and the plates were stained with 1% crystal violet for 30 min. Plaques were quantified by manual counting.

2.9. Transmission testing in a hemorrhage mouse model

An *in vivo* infectivity analysis was performed using a hemorrhage mouse model. Mosquitoes were given dsRNA injections to silence either AaSG34 or LacZ (as a control). Three days later, they were administered 69 nl of DENV2 16681 (6.9×10^2 PFU in 69 nl) via an intrathoracic inoculation. At 7 days after infection, saliva was collected by exposing the mosquitoes to a feeding solution containing 490 μl RPMI medium (with no phenol red) and 10 μl of 2 mM ATP. Titers of DENV2 in the saliva were determined by plaque assays and protein concentrations by

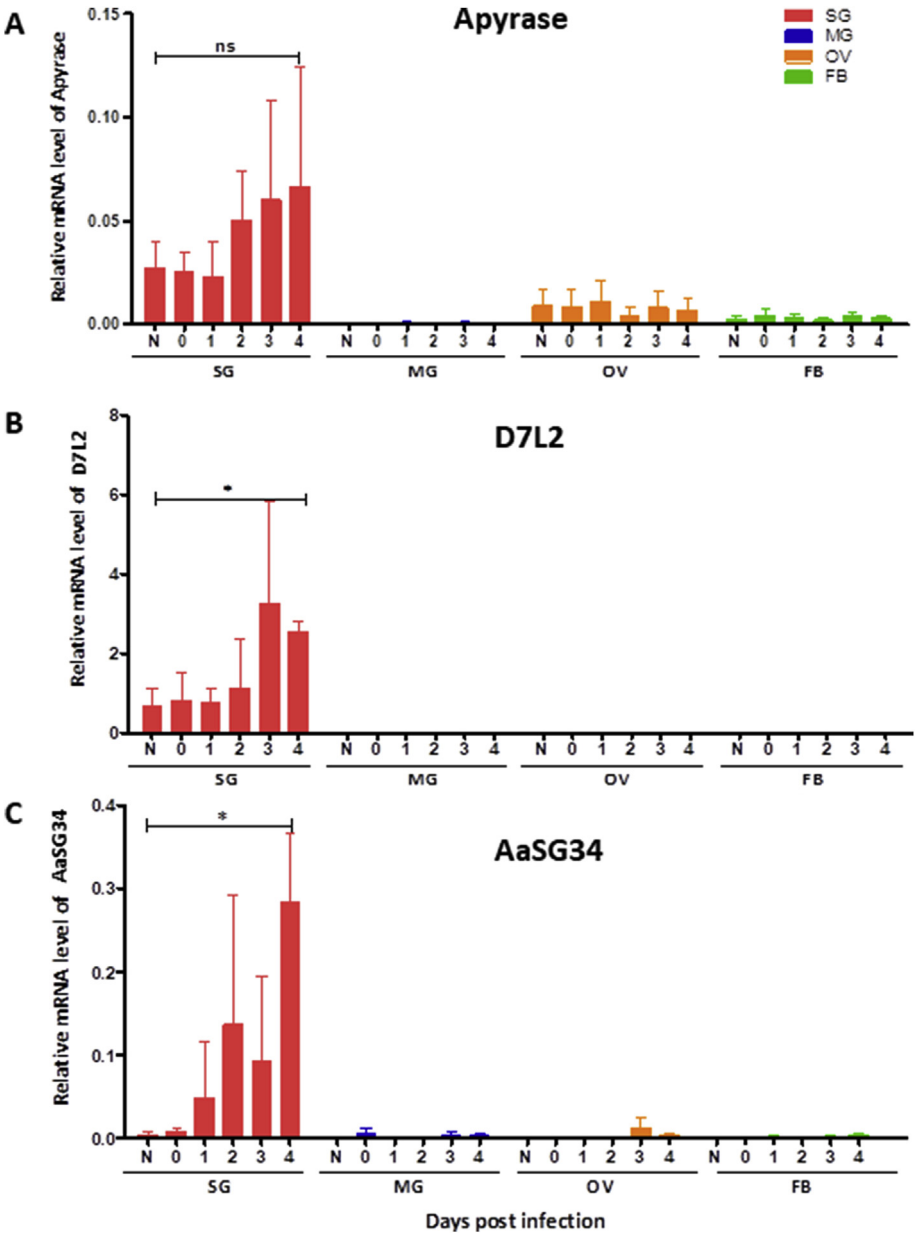

Fig. 1. Intrathoracic DENV2 infection induces *D7L2*, and *AaSG34* mRNA levels in mosquito salivary glands.
Total RNA was extracted from naïve mosquito (N) and infected mosquito salivary gland (SG), midgut (MG), fat body (FB), and ovary (OV) on days 0–4 after intrathoracic injection with DENV2. Transcripts of Apyrase **(A)**, D7L2 **(B)**, and AaSG34 **(C)** were quantified by qRT-PCR. Relative values of Apyrase **(A)**, D7L2 **(B)**, and AaSG34 **(C)** were normalized against S7. Data were pooled from two independent experiments and shown as mean ± SD. Each experiment included 20 mosquitoes. ns, not significant; *p < 0.05 (Unpaired *t*-test).

Bradford protein assays. The viral titer in saliva from the infected control mosquitoes was approximately 1.125×10^4 PFU/ml, whereas that from the infected AaSG34-silenced mosquitoes was below the detection limit. The protein concentrations for the infected control and AaSG34-silenced mosquitoes were 254.4 μg/ml and 310.0 μg/ml, respectively.

STAT1-deficient (*Stat1*$^{-/-}$) mice (on a C57BL/6 (AB6) background) were intradermally inoculated at four sites on the upper back with saliva from the infected control or AaSG34-silenced mosquitoes. Saliva from the infected control mosquitoes was adjusted so that each mouse received a total of 6×10^3 PFU in 400 μl. Saliva from the AaSG34-silenced mosquitoes was adjusted such that each mouse received an equivalent protein concentration (1.35 μg/mouse) to that in the saliva from the infected control mosquitoes. On day 6 after infection, the dorsal and abdominal skin of the mice was examined for the development of hemorrhaging (Chen et al., 2007). The hemorrhaged areas were quantified by ImageJ software, and the percentage of hemorrhaging in a selected region was obtained by dividing the hemorrhaged area by the total area of the selected region (Fig. S2).

2.10. Statistical analysis

The statistical analyses were performed using GraphPad Prism 5 software. Differences between groups were evaluated with unpaired t tests or two-way analysis of variance (ANOVA). A P value < 0.05 was considered to be statistically significant.

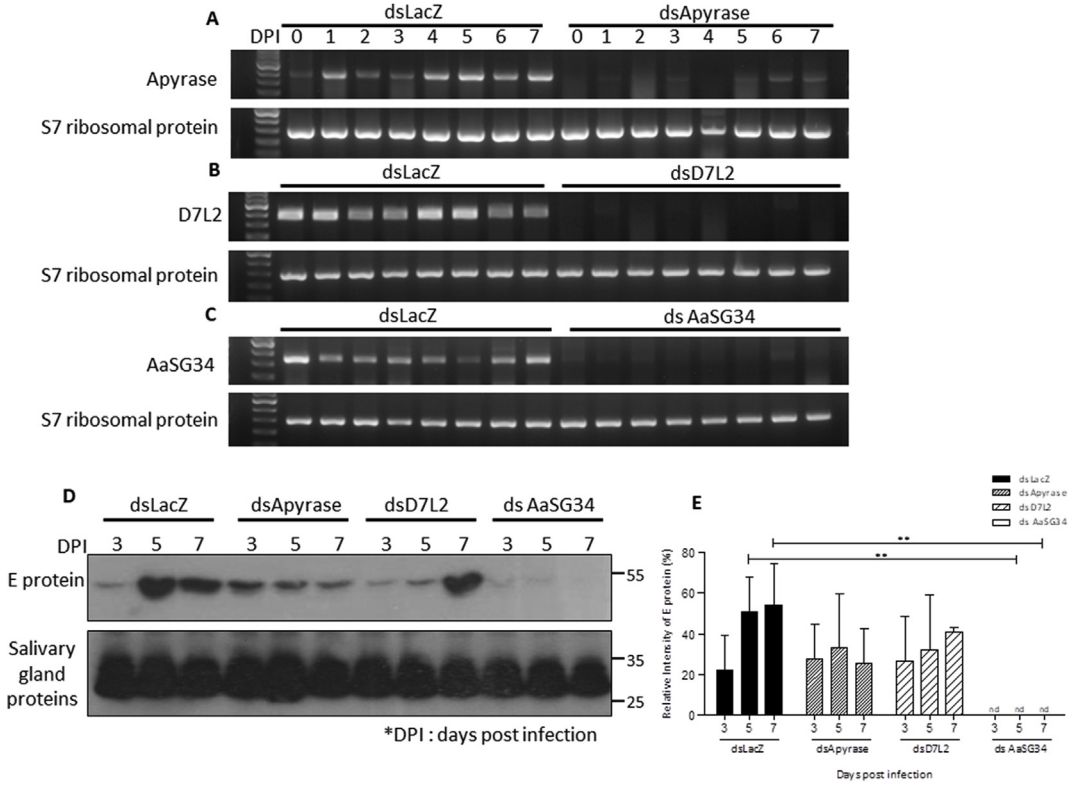

Fig. 2. Silencing AaSG34 but not Apyrase or D7L2 suppresses DENV2 replication in mosquito salivary glands.
Mosquitoes were intrathoracically injected with dsRNA of LacZ, Apyrase, D7L2, or AaSG34. Three days later, mosquitoes were intrathoracically infected with DENV2. Total RNA was extracted from mosquito whole body on days 0–
7 after DENV injection. Transcripts of Apyrase **(A)**, D7L2 **(B)**, and AaSG34 **(C)** were amplified by PCR and analyzed genes expression by gel electrophoresis. S7 ribosomal protein was used for internal control. Salivary glands (**D,** n = 50 for each experiment) were extracted from Apyrase, D7L2, AaSG34-silenced and LacZ control mosquitoes on days 3, 5 and 7 after DENV2 infection and subject to Western blot analysis for dengue viral E protein. Serum from mouse bitten by naïve mosquitos was used as a control to blot total salivary gland extracts. Bar graph in **(E)** shows the relative E protein value against total salivary gland proteins. Data were pooled from two independent experiments **(E)**. nd, not detected; **p < 0.01 (2-way ANOVA).

3. Results

3.1. The roles of Apyrase, D7L2, and AaSG34 proteins following DENV2 infection via intrathoracic injection

To investigate the interactions between *Ae. aegypti* salivary proteins and DENV2 infection, we injected mosquitoes with the virus intrathoracically and assessed changes in the transcription levels of six selected salivary proteins. Apyrase, D7L2, and AaSG34, but not Aegyptin, SRPN23, or SRPN26, were upregulated from day 1–10 after infection. Apyrase, D7L2, and AaSG34 were found to be expressed in the salivary glands of naïve mosquitoes; on day 4 after infection, D7L2 and AaSG34 but not Apyrase transcripts were significantly upregulated in the salivary glands but not in other tissues (Fig. 1A–C). We therefore examined the effects of Apyrase, D7L2, and AaSG34 on DENV2 replication by silencing each gene before DENV2 infection. The dsRNA efficiently silenced *Apyrase*, *D7L2*, and *AaSG34* mRNA compared to the control mosquitoes treated with dsLacZ (Fig. 2A–C); however, DENV2 growth in the mosquito salivary glands was affected (for up to day 7 after infection) only by silencing AaSG34 and not by silencing D7L2 or Apyrase (Fig. 2D and E).

3.2. Expression of AaSG34 after an infectious blood meal

We investigated how oral infection with DENV2 affected the expression level of AaSG34 in the salivary glands. After a normal blood meal, AaSG34 was preferentially expressed in the mosquitoes' salivary glands and marginally in their ovaries (Fig. 3A). The infectious blood meal induced significant upregulation of AaSG34 in the salivary glands, but not in other tissues, on day 5 after infection (Fig. 3B). From day 1 after the infectious blood meal, the viral genome was detectable and increased in copy number in the salivary glands, midgut, ovary, fat body, and carcass, becoming significantly higher in the salivary glands and midgut on day 5 after infection (Fig. 3C). The expression of AaSG34, however, was upregulated only in the salivary glands and not in the midgut (Fig. 3B). Thus, there may be an association between AaSG34 expression levels and DENV2 replication in the salivary glands.

3.3. Silencing AaSG34 inhibited DENV2 replication after an infectious blood meal

A previous study showed that a recombinant 34 kDa salivary protein enhanced DENV replication in human keratinocytes (Surasombatpattana et al., 2014). In the present study, we investigated whether AaSG34 affected DENV2 replication in mosquito salivary glands. From day 5 after infection with an infectious blood meal, AaSG34 expression was upregulated in control mosquitoes (Fig. 4A). Silencing of AaSG34 significantly inhibited the increase in vRNA up to day 7 after infection (Fig. 4B). As with the vRNA levels, dengue viral E protein expression was almost undetectable on day 7 after infection, although there was then a gradual increase (Fig. 4C and D), possibly because of the short half-life (7 days) of the dsRNA. These findings suggest that AaSG34 facilitates DENV2 replication in the salivary gland. In its absence, DENV2 growth is retarded but the virus remains viable. Once the expression of AaSG34 is restored, viral growth resumes.

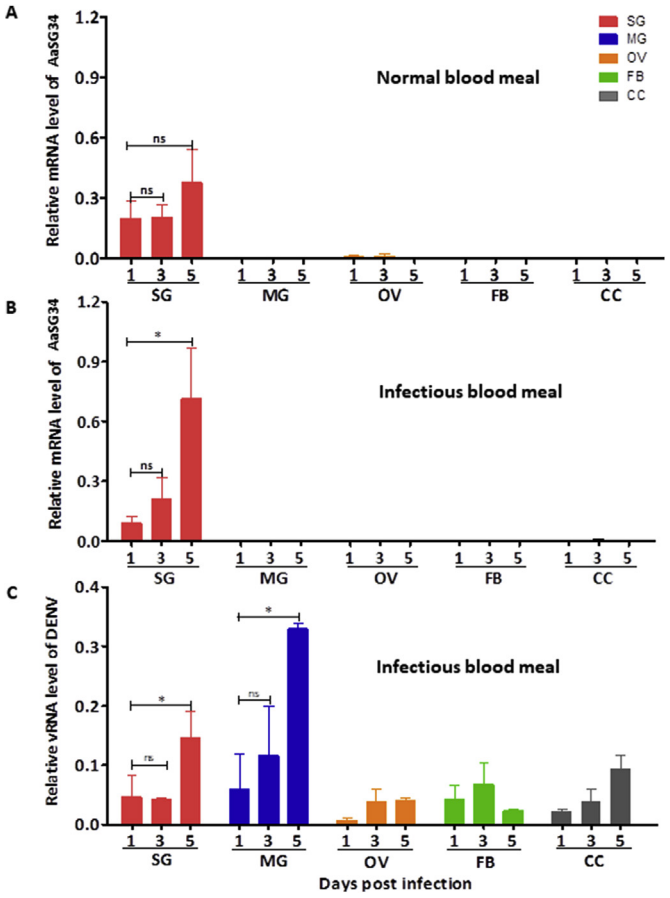

Fig. 3. DENV2 infection induces *AaSG34* mRNA in mosquito salivary glands. Total RNA was extracted from mosquito salivary gland (SG), midgut (MG), fat body (FB), ovary (OV), and carcass (CC, whole body after removal of SG, MG, FB and OV) on days 1, 3 and 5 after a normal blood meal **(A)** or an infectious blood meal **(B and C)**. Transcripts of AaSG34 **(A and B)** and dengue viral RNA (vRNA) copies **(C)** were quantified by qRT-PCR. Relative values of AaSG34 **(A and B)** and vRNA copies were normalized against S7. Data were pooled from three independent experiments. Each experiment included 50 mosquitoes. ns, not significant; *p < 0.05 (Unpaired *t*-test).

3.4. Silencing AaSG34 blocked the dissemination of DENV2 into saliva

Infected mosquitoes transmit DENV2 by injecting infectious saliva into human skin through a proboscis. We therefore examined how AaSG34 expression in the salivary glands affected DENV2 secretion into the mosquitoes' saliva. Silencing AaSG34 in the salivary glands completely abrogated the release of DENV2 into saliva, not only on day 7, but also days 10 and 13 after infection, as shown by Western blot analyses of dengue viral E protein in the saliva (Fig. 5A and B) as well as by plaque assays (Fig. 5C). These findings indicate that silencing AaSG34 not only inhibits DENV2 replication in the salivary glands but also prevents its release into saliva.

3.5. Silencing AaSG34 inhibited the transmission of DENV2 into mice

Stat1$^{-/-}$ mice were intradermally inoculated on the upper back with saliva collected from control (treated with dsLacZ) or AaSG34-silenced mosquitoes infected with DENV2. Hemorrhage development was assessed on day 6 after inoculation. Severe hemorrhaging developed in both the dorsal and abdominal skin of the mice inoculated with the saliva from the infected control mosquitoes, whereas the mice inoculated with the saliva from infected AaSG34-silenced mosquitoes did not develop hemorrhaging in either skin region (Fig. 6A–D). The percentage of hemorrhaging in both dorsal skin (Fig. 6B) and abdominal skin (Fig. 6D) was significantly lower for the mice injected with the infected AaSG34-silenced mosquito saliva than for the mice injected with the infected control mosquito saliva ($P < 0.05$ and $P < 0.01$, respectively). The spleens of the mice inoculated with the saliva from infected control mosquitoes were > 200 mg in weight, whereas those of the mice inoculated with the saliva from infected AaSG34-silenced mosquitoes remained at about 70 mg. This indicated that the mice exhibited an active immune response to the saliva from the infected control mosquitoes but almost no immune response to the saliva from the infected AaSG34-silenced mosquitoes. These results demonstrate that silencing AaSG34 inhibited the transmission of DENV2 from mosquitoes to the mammalian host. They suggest the importance of AaSG34 in the virus–vector–vertebrate interrelationship.

4. Discussion

DENV is known to take advantage of salivary proteins of the *Ae.*

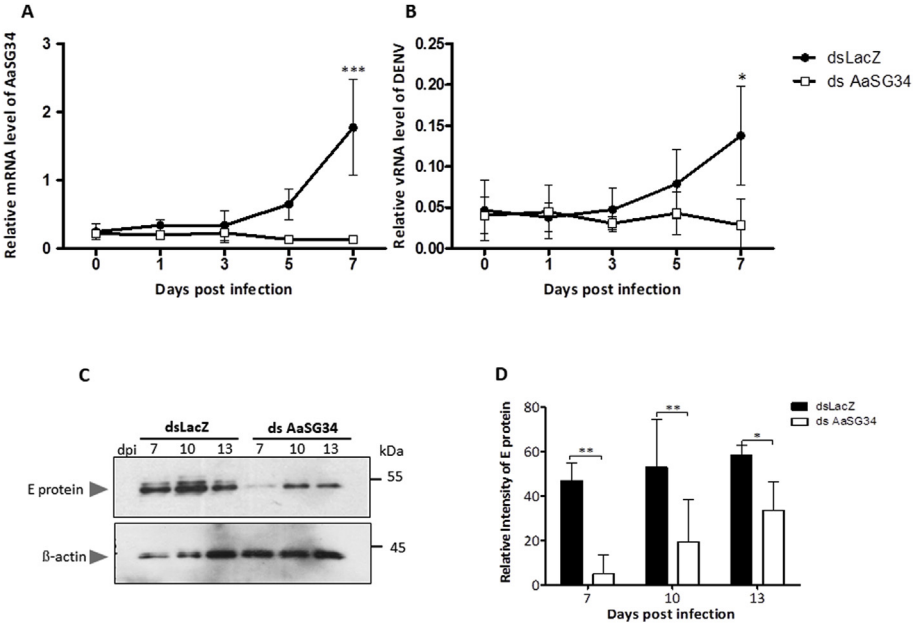

Fig. 4. Silencing AaSG34 suppresses DENV2 replication in mosquito salivary glands. Mosquitoes were intrathoracically injected with dsRNA of LacZ or AaSG34. Three days later, mosquitoes were given infectious blood meal. Total RNA of salivary gland **(A and B)** were collected from AaSG34-silenced (**empty square**, n = 50 for each experiment) or LacZ-silenced control (**filled circle**, n = 50 for each experiment) mosquitoes on days 0, 1, 3, 5, and 7 after an infectious blood meal. Transcripts of AaSG34 **(A)** and dengue viral RNA (vRNA) copies **(B)** were quantified by qRT-PCR. Relative values of AaSG34 **(A)** vRNA **(B)** were normalized against S7. Salivary gland proteins were extracted from AaSG34-silenced and LacZ control mosquitoes **(C,** n = 100 for each lane) on days 7, 10 and 13 after infectious blood meal and subjected to Western blot analysis for dengue viral E protein. Experiment was performed three times. One representative experiment is shown. Bar graphs in **(D)** show the relative E protein value against β-actin. Data were pooled from three independent experiments **(A, B, and D)**. *p < 0.05, **p < 0.01, ***p < 0.001 (2-way ANOVA).

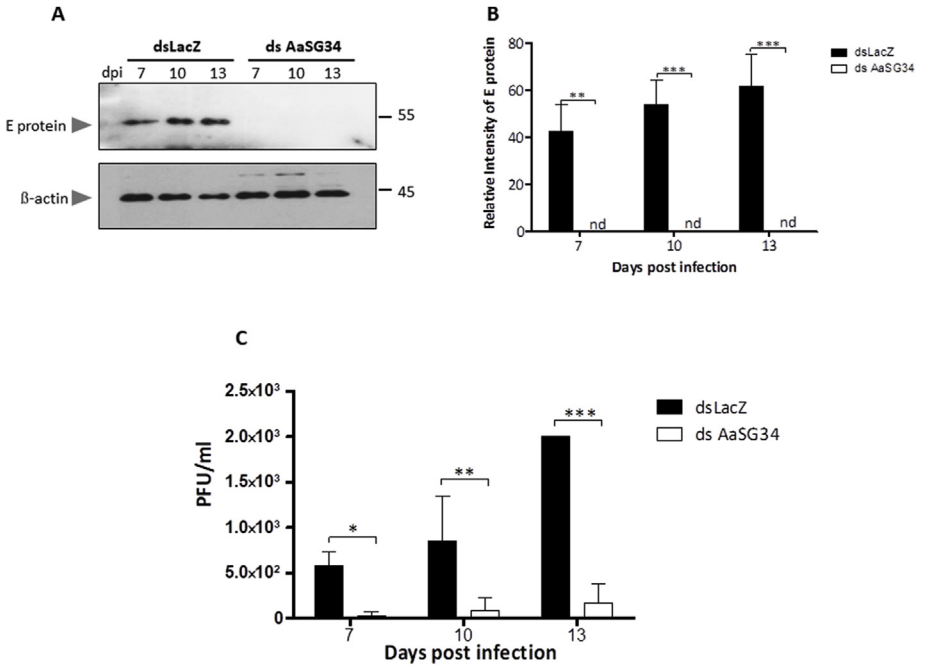

Fig. 5. Silencing AaSG34 reduces viral burden in saliva.
Mosquitoes were intrathoracically injected with dsRNA of LacZ or AaSG34. Three days later, mosquitoes were given infectious blood meal. Saliva was collected from AaSG34-silenced and LacZ control mosquitoes on days 7, 10 and 13 after infectious blood meal and subjected to W
estern blot analysis **(A)** for dengue viral E protein. Experiment was performed three times. One representative experiment is shown. Saliva collected from at least 100 mosquitoes was pooled for each lane. Bar graphs in **(B)** show the relative E protein value against β-actin in Western blot as quantified by ImageJ. Data were pooled from three independent experiments. Mosquito saliva (200 μg/ml) was quantified for viable virus by plaque assay on C6/36 cells **(C)**. Data were pooled from three independent experiments with > 100 mosquitoes in each experiment. nd, not detected; *p < 0.05, **p < 0.01, ***p < 0.001 (2-way ANOVA).

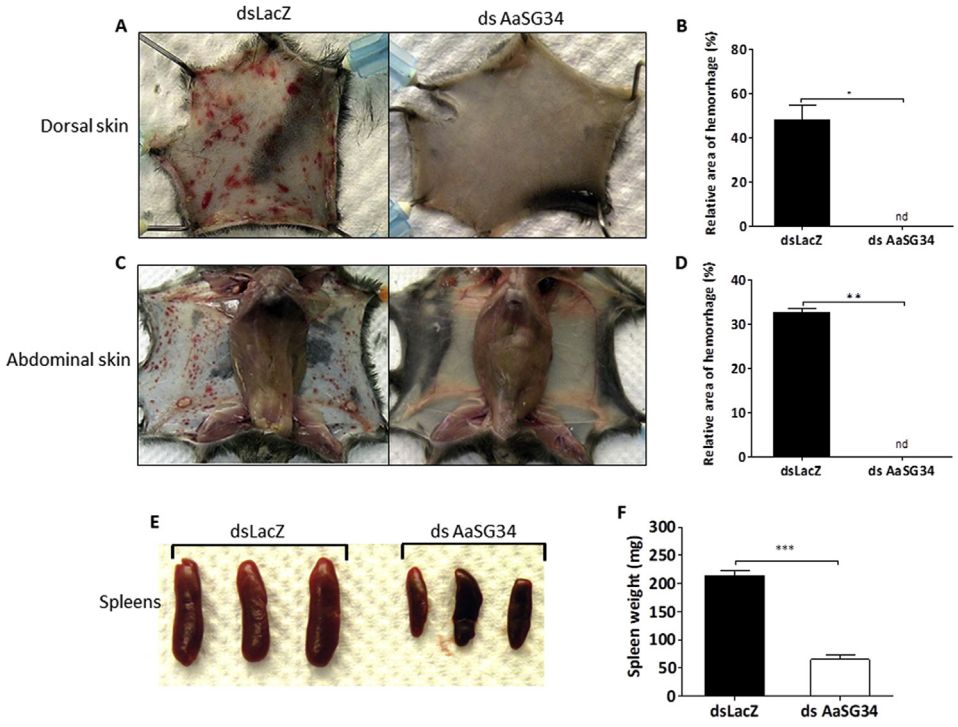

Fig. 6. Saliva from DENV2-infected AaSG34-silenced mosquitoes does not induce hemorrhage in mice.
Mosquitoes were intrathoracically injected with dsRNA of LacZ or AaSG34. Three days later, mosquitoes were given intrathoracic inoculation of DENV2 (at 6.9 × 10^2 PFU in 69 nl). Saliva was collected from mosquitoes from AaSG34-silenced and LacZ control mosquitoes on day 7 after DENV2 inoculation. *Stat1*$^{-/-}$ mice were intradermally inoculated with saliva from DENV2-infected LacZ control mosquito (dsLacZ) or DENV2-infected AaSG34-silenced mosquito saliva (ds AaSG34). On day 6 after infection, mice were killed and their dorsal skin (**A, B**), and abdominal skin (**C, D**) were observed for hemorrhage development and spleens were dissected (**E**). Hemorrhage area in dorsal skin (**B**) and abdominal skin (**D**) were quantified by ImageJ. Percentage of hemorrhage area was obtained from dividing the hemorrhage area in selected area by the total area selected. Mouse spleens weights were recorded (**F**). Images in (**A, C**) are representative of three independent experiments. Data in **B, D** and **F** were pooled from three independent experiments. nd, not detected; *p < 0.05, **p < 0.01, ***p < 0.001 (Unpaired *t*-test).

aegypti mosquito for its infectivity and transmission from the mosquito to the mammalian host. It has been shown that mice that had undergone mosquito probing prior to an intradermal injection of DENV had significantly higher viral titers compared to mice that had not received mosquito probing (McCracken et al., 2014a,b). However, how salivary proteins affect DENV replication in the mosquito vector remains poorly understood. In this study, we sought to determine whether any individual salivary protein modulated DENV2 replication in mosquitoes.

A study of infection with Semliki Forest and Bunamwera viruses showed that infection was facilitated by inflammation at the mosquito bite site, which resulted in greater retention of the virus and the recruitment of inflammatory neutrophils and virus-permissive myeloid cells (Pingen et al., 2016). Human keratinocytes are permissive to DENV infection, and it has been shown that the infection of keratinocytes with DENV in the presence of *Ae. aegypti* salivary gland extract reduced the production of antimicrobial peptides and type I interferon (IFN) and enhanced the replication of the virus (Surasombatpattana et al., 2011, 2012).

We screened six *Ae. aegypti* salivary proteins, Apyrase (Peng et al., 2001; Sun et al., 2006), D7L2 (Conway et al., 2016; Peng et al., 2006), AaSG34 (Surasombatpattana et al., 2014), Aegyptin (McCracken et al., 2014a; Peng et al., 2016), SRPN23 (Ribeiro et al., 2007), and SRPN26 (Wasinpiyamongkol et al., 2010). Of these, Apyrase, D7L2, and AaSG34 were found to be specifically expressed in mosquito salivary glands and

upregulated following DENV2 infection. Silencing AaSG34 (but not D7L2 or Apyrase) inhibited DENV2 replication in mosquito salivary glands.

The D7 protein family has several members, of which one is known to inhibit DENV2 infection. Conway et al. (2016) showed that U937 cells pretreated with a recombinant D7L2 protein inhibited DENV2 infection and that the D7L2 bound to DENV2 virions. The present study showed that, although D7L2 is upregulated in mosquito salivary glands after DENV2 infection, its silencing did not affect DENV2 infection in the salivary glands. It is possible that D7L2 may interact differently with DENV2 in mosquito salivary gland cells than in macrophage-like mammalian cells. It is also possible that the intracellular and extracellular functions of D7L2 differ. The D7 protein family includes members other than D7L2, such as D7L1 and D7s2 (Wichit et al., 2016). It is possible that, in the absence of D7L2, these other members may compensate for its function.

The gene that encodes AaSG34 protein belongs to the 34-kDa protein family (Surasombatpattana et al., 2014). Proteins of the 34-kDa protein family are exclusively expressed by the *Aedes* genus (Arcà et al., 2007) and are specifically enriched in the salivary glands of the adult female mosquitoes (Arcà et al., 2007; Ribeiro et al., 2007). Because AaSG34 proteins are secreted and specific to the *Aedes* genus, the human IgG antibody to the Nterm-peptide of the AaSG34 protein has been used as a biomarker of exposure to *Ae. aegypti* bites (Elanga Ndille et al., 2012). It has subsequently been used as an epidemiology tool to assess the efficacy of vector control interventions against *Aedes* species in chikungunya virus and DENV transmission areas (Elanga Ndille et al., 2016). The present study showed that a normal blood meal did not increase AaSG34 expression in *Ae. aegypti* salivary glands whereas an infectious blood meal had significantly upregulated AaSG34 expression by day 5 after DENV2 infection. This finding may be of value when considering the use of the anti-Nterm-AaSG34 antibody as an epidemiological tool.

The salivary glands are the critical site in mosquitoes of DENV2 infection prior to transmission to humans (Luplertlop et al., 2011). It is known that some mosquito salivary proteins have benefit for the transmission of arboviruses that cause diseases in humans (Conway et al., 2014; Wichit et al., 2016). Our study showed that silencing AaSG34 inhibited DENV2 replication, suggesting that AaSG34 provides benefit for DENV2 infection in mosquito salivary glands. The infection of primary human keratinocytes with DENV in the presence of the 34-kDa recombinant protein produced with a baculovirus expression system significantly increased the expression of DENV transcripts compared to infection in the absence of the protein (Surasombatpattana et al., 2014). Treatment with the recombinant 34-kDa protein reduced the transcription of IFN-α, IFN-β, IRF3, LL37, S100A7, and RNase7 in keratinocytes after DENV infection (Surasombatpattana et al., 2014). These studies demonstrated that the 34-kDa protein functions to suppress the innate immune response of the mammalian cell. Because DENV2 infection induces the upregulation of AaSG34 in mosquito salivary glands, we speculate that AaSG34 may suppress the antiviral innate immune response, facilitating replication of the virus and its dissemination to saliva.

Notably, silencing of AaSG34 completely inhibited DENV2 replication in the mosquitoes' salivary glands up to day 7 after infection but E protein gradually became detectable from day 10. The reason why E protein became detectable at a later time point may be because of the limited half-life of the dsRNA used to silence AaSG34. However, when E protein was detectable in the salivary glands on days 10 and 13, the virus remained completely null in the saliva. These results suggest the possibility that AaSG34 not only facilitates the replication of DENV2 in the salivary glands but also plays a role in the dissemination of the virus from the salivary glands into the saliva.

Conway et al. (2016) reported that vRNA transcription was significantly reduced in both the footpads and draining lymph nodes of *Ifnagr*$^{-/-}$ mice that had been subcutaneously inoculated on the footpad with DENV2 mixed with a recombinant D7L2 protein. In the present study, we showed that *Stat1*$^{-/-}$ mice intradermally inoculated with saliva from DENV2-infected dsLacZ-treated mosquitoes developed severe hemorrhaging and splenomegaly. Remarkably, the mice inoculated with saliva from the AaSG34-silenced mosquitoes infected with DENV2 did not develop hemorrhaging and their spleens remained a normal size. These results demonstrate that the AaSG34 protein is involved not only in the regulation of DENV2 replication in the salivary glands but also in DENV2 transmission. It was previously shown that an extract of the *Ae. aegypti* salivary gland mixed with DENV2 enhanced the dissemination of DENV2 into the mammalian host (Conway et al., 2014). The findings of the present study add a further example of the importance of specific salivary proteins in the transmission of DENV2 from mosquitoes into the mammalian hosts.

In summary, we screened six mosquito salivary proteins and discovered that AaSG34 is upregulated following DENV2 infection and that its presence is important for the replication of DENV2 in the salivary glands of mosquitoes. The presence of AaSG34 is also critical for the dissemination of DENV2 from the mosquito's salivary glands to saliva as well as for the transmission of the virus to the mammalian host. These findings suggest an alternative approach for controlling the spread of DENV2: rather than trying to eradicate the mosquito vectors with chemical sprays or bacterial infection, it may be possible instead to regulate mosquito gene expression.

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Appendix A. Supplementary data

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