High-Resolution Proteomics of Aedes Aegypti Salivary Glands Infected With DENV2, ZIKV and CHIKV Reveal Virus-Specific and Broad Antiviral Factors

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Research Article

Keywords: Arboviruses such as dengue (DENV), Zika (ZIKV), chikungunya (CHIKV), Aedes aegypti salivary glands

Posted Date: August 24th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-830517/v1

Abstract

Arboviruses such as dengue (DENV), Zika (ZIKV) and chikungunya (CHIKV) viruses infect close to half a billion people per year, and are transmitted through *Aedes aegypti* bites. Infection-induced changes in mosquito salivary glands (SG) influence transmission by inducing immunity, which restricts virus replication, and by altering saliva composition, which influences skin infection. Here, we profiled SG responses to DENV2, ZIKV and CHIKV infections by using high-resolution quantitative proteomics. We identified 218 proteins related to immunity, blood-feeding or cellular machinery. We observed that 58, 27 and 29 proteins were regulated by DENV2, ZIKV and CHIKV infections, respectively. While the regulation patterns were mostly virus-specific, we determined the function of four uncharacterized proteins that were upregulated by all three viruses. We revealed the anti-ZIKV function of gamma-interferon responsive lysosomal thiol-like (GILT-like), the anti-CHIKV function of adenosine deaminase (ADA), the pro-ZIKV function of salivary gland surface protein 1 (SGS1) and the antiviral function against all three viruses of an uncharacterized protein we called salivary gland broad-spectrum antiviral protein (SGBAP). The comprehensive description of SG responses to three global pathogenic viruses and the identification of new restriction factors improves our understanding of the molecular mechanisms influencing transmission.

Introduction

Arboviruses like dengue (DENV), Zika (ZIKV) and chikungunya (CHIKV) viruses are primarily spread through the bites of *Aedes aegypti* mosquitoes [1]. DENV and ZIKV belong to the *Flavivirus* genus (Flaviviridae family), while CHIKV is an *Alphavirus* (Togaviridae family). These viruses infect more than 400 million people yearly, mostly in tropical and sub-tropical countries where the environment is conducive to natural mosquito breeding [2]. In the absence of effective vaccine and curative drug [3], mitigation of these mosquito-borne diseases relies on vector population control. Insecticide-based controls often fail to curb outbreaks partly due to insecticide resistance [4,5]. Alternatively, novel biological strategies are being developed to limit vector populations. Population suppression or replacement by releasing *Wolbachia*-infected mosquitoes is currently being evaluated in field trials in several countries [6]. However, the ability to scale up the suppression strategy and the sustainability of the replacement strategy [7] remain to be validated. The production of pathogen-refractory mosquitoes by genetic modification offers another promising vector-targeted strategy [8,9]. In this regard, identification of pro- and anti-viral factors in mosquitoes is a prerequisite.

While biting an infected host, female mosquitoes ingest viruses that infect the mosquito midgut before disseminating throughout their whole body finally reaching the salivary glands (SG), from where the virus is secreted during a subsequent bite. The period the virus takes from entering the mosquito midgut to finally exiting through the SG into mosquito saliva is defined as extrinsic incubation period (EIP). EIP varies among virus species. EIP for flaviviruses like DENV and ZIKV is estimated between 10–14 days [10,11] and for alphaviruses like CHIKV between two to nine days [12]. *Aedes aegypti* displays pathogen-

responsive innate immune pathways throughout the above path of midgut, hemolymph and SG, which limit virus infection, and thus transmission [13,14]. Transcriptomics studies of SG showed that the induction of Toll and IMD pathways by DENV serotype 2 (DENV2) leads to the production of a cecropin-like antimicrobial peptide with anti-DENV2, anti-CHIKV and anti-*Leishmania* properties [15]. Using high-throughput RNA sequencing, we recently showed that DENV2, ZIKV and CHIKV infections trigger a broad antiviral response through the c-jun N terminal kinase (JNK) pathway, which activates complement and apoptotic effectors [16]. Alternatively, other factors unrelated to the canonical immune pathways have been implicated in the regulation of DENV2 infection in SG [17]. While multiple evidence indicates that mosquito SG response modulates infection, additional studies are required to characterize the response at the proteomic level, identify novel viral factors and determine how infection-regulated proteins influence infection.

SG infection also alters the composition of saliva, thereby influencing blood acquisition and skin infection [18–20]. Mosquito saliva contains a cocktail of biologically active molecules with functions in hemostasis, inflammation and immunity [21,22]. Among others, *A. aegypti* saliva contains a vasodilatory tachykinin decapeptide named sialokinin, a factor Xa-directed anticoagulant and an anti-platelet apyrase, all of which may facilitate blood acquisition by preventing clotting to maintain steady blood flow [23–25]. Immune-modulators such as a secreted 387 kDa protein can supress cytokine release and proliferation of T and B cells in mouse splenocytes *in vitro* [26]. A venom allergen-1 protein detected in *A. aegypti* saliva was recently found to enhance DENV2 and ZIKV infection in skin cells by augmenting autophagy [27]. Alternatively, salivary proteins can also inhibit skin infection. A 30 kDa collagen-binding protein called aegyptin [28] and a D7 protein [29] reduce DENV2 multiplication. Characterization of SG proteomic response to infection will inform about changes in saliva composition, which can affect transmission.

The earliest studies of *A. aegypti* SG proteome used uninfected mosquitoes and one- or two-dimensional gel electrophoresis (DGE) coupled with mass spectrometry (MS) to identify a few proteins [30,31]. Recently, using high-resolution MS, 1,208 proteins were detected in uninfected *A. aegypti* SGs [32], although these included proteins identified by only one unique peptide. To our knowledge, only two studies for DENV2, one for CHIKV and none for ZIKV reported SG proteomic response to infection with low resolution MS [33–35]. Here, to bridge the knowledge gap in SG proteomic response to infections, we deployed high-resolution MS with isobaric tag for relative and absolute quantitation (i-TRAQ) on DENV2-, ZIKV- and CHIKV-infected and non-infected *A. aegypti* SGs. We identified 218 proteins using a custom protein database and described those with functions related to immunity, blood feeding, digestion, metabolism and ribosome, stress and mitochondria. DENV2 infection regulated 58 proteins, ZIKV infection 27, and CHIKV infection 29. While a majority of differentially expressed proteins (DEPs) were virus-specific, we characterized the function in SG of the four proteins upregulated by all three viruses. We identified the anti-ZIKV function of gamma-interferon responsive lysosomal thiol-like (GILT-like), the anti-CHIKV function of an adenosine deaminase (ADA), the pro-ZIKV function of salivary gland surface protein 1 (SGS1) and the antiviral

function against all three viruses of a protein we named salivary gland broad-spectrum antiviral protein (SGBAP).

Methods

Mosquito rearing

Aedes aegypti mosquitoes were collected in Singapore in 2010, and ever since reared in the insectary. Eggs were hatched in MilliQ water, larvae fed on a mixture of TetraMin fish flakes (Tetra, Germany), yeast and liver powder (MP Biomedicals, France) and adults maintained on 10% sucrose (1st Base, Singapore). Mosquitoes were maintained at 28°C and 50% relative humidity with a 12h:12h light: dark cycle.

Viruses

Dengue virus serotype 2 PVP110 was isolated from an EDEN cohort patient in Singapore in 2008 [36]. Zika virus Paraiba_01/2015 was isolated from a febrile female in the state of Paraiba, Brazil in 2015 [37]. Chikungunya virus SGP011 was isolated from a patient at the National University Hospital in Singapore in 2008 [38]. DENV2 and ZIKV isolates were propagated in C6/36 (CRL-1660) and CHIKV in Vero (CCL-81) cell lines. Virus stocks were titered with BHK-21 cell plaque assay as previously described [39], aliquoted and stored at -80 °C.

Mosquito infection

Three-to-five day old female mosquitoes were starved for 24 h and fed on an infectious blood meal containing 40% volume of washed erythrocytes from specific pathogen free (SPF) pig's blood (Prestige BioResearch, Singapore), 5% 10 mM ATP (Sigma-Aldrich, USA), 5% human serum (Corning human AB serum, Thermo Fisher Scientific, USA) and 50% virus solution in RPMI media (Gibco, Thermo Fisher Scientific, USA), using Hemotek membrane feeder system (Discovery Workshops, UK). The virus titers in blood meals were 2 x 10^7 pfu/ml for DENV2, 6 x 10^6 pfu/ml for ZIKV, and 1.5 x 10^8 pfu/ml for CHIKV which resulted in 100% SG infection for each virus [16]. Bloodmeal titers were validated in plaque assay using BHK-21 cells. Control mosquitoes were fed with the same blood meal composition except for virus solution, which was replaced by RPMI media. Following oral feeding, fully engorged females were selected and kept in a cage with *ad libitum* access to a 10% sucrose solution in an incubation chamber with conditions similar to insect rearing.

For inoculation, mosquitoes were cold-anesthetized and intrathoracically injected with 0.5 pfu of either DENV2, ZIKV or CHIKV using Nanoject-II (Drummond scientific company, USA). The same volume of RPMI media was injected as control. Virus inoculation was conducted four days post dsRNA injection.

Sample preparation and i-TRAQ labeling

Mosquito SGs were dissected and collected in 1X phosphate buffer saline (pH 7.4, Cytiva HyClone, Thermo Fisher Scientific, USA) at 14 days post oral infection (dpi) for DENV2 and ZIKV, and seven dpi for CHIKV. Ninety SGs were pooled together for each condition, and freeze-thawed twice. The samples were finally homogenized using mini beadbeater-96 (Biospec Products, USA) and centrifuged to collect the supernatant as salivary gland extract (SGE). The protein content of each sample was normalized based on their concentration as measured by Pierce BCA protein assay kit (Thermo Fisher Scientific, USA). SGEs were denatured, alkylated, trypsin (Promega) digested, and labeled using i-TRAQ 8plex Protein quantitation kit (AB SCIEX, Singapore) following manufacture's protocol. Each condition was conducted in triplicate.

LC-MS/MS analysis

The 1st dimension of peptide separation was conducted using an Eksigent nanoLC Ultra and ChiPLC-nanoflex (USA) in TrapElute configuration. Subsequently, the samples were loaded on a 200 µm x 0.5 mm column and eluted on an analytical 75 µm x 15 cm column (ChromXP C18-CL, 3 µm). A gradient formed by mobile phase A (2% acetonitrile, 0.1% formic acid) and mobile phase B (98% acetonitrile, 0.1% formic acid) was used to separate 2 and 5 µl of the sample at a 0.3 µl/min flow rate. The following gradient elution was used for peptide separation: 0 to 5% of mobile phase B in 1 min, 5 to 12% of mobile phase B in 15 min, 12 to 30% of mobile phase B in 114 min, 30 to 90% of mobile phase B in 2 min, 90% for 7min, 90 to 5% in 3 min and finally held at 5% of mobile phase B for 13 min. The tandem MS analysis was performed using a 5600 TripleTOF system (AB SCIEX, USA) under Information Dependent Mode. The mass range of 400-1800 m/z and accumulation times of 250 ms per spectrum were chosen for precursor ion selection. MS/MS analysis was performed on the 20 most abundant precursors (accumulation time: 100 ms) per cycle with 15 s dynamic exclusion. Recording of MS/MS was acquired under high sensitivity mode with rolling collision energy and adjusted capillary electrophoresis (CE) when using i-TRAQ reagent was selected.

Protein identification and quantification

Peptide identification and quantification was carried out on the ProteinPilot 5.0 software Revision 4769 (AB SCIEX, Singapore) using the Paragon database search algorithm (5.0.0.0.4767) and the integrated false discovery rate (FDR) analysis function. The data were searched against a custom protein database including *Aedes* VectorBase (VB) (AAegL3.3 on July 2017 and AAegL5.1 on February 2019), *Aedes* UniProt (February 2019) and a *de novo A. aegypti* SG transcriptome (unpublished) based on our previous work [16]. The MS/MS spectra obtained were searched using the following user-defined search parameters: Sample Type: iTRAQ 8-plex (Peptide Labeled); Cysteine Alkylation: methyl methanethiosulfonate; Digestion: Trypsin; Instrument: TripleTOF5600; Special Factors: None; Species: None; ID Focus: Biological

Modification; Database for *Aedes* VB Search Effort: Thorough; and FDR Analysis: Yes. The MS/MS spectra were searched against a decoy database to estimate the false discovery rate (FDR) for peptide identification. The decoy database consisted of reversed protein sequences from the same custom protein database as mentioned earlier. Different modification states of the same peptide sequences were considered distinct by the software. Peptides with confidence score ≥ 95% were considered identified, and proteins with at least two unique identified peptides were quantified. Proteins were identified as upregulated when they had the i-TRAQ ratio above 1.5 (p-value < 0.05) and downregulated when they had the ratio below 0.67 (p-value < 0.05). Proteins with ratio from 1.5 to 0.67 were considered not regulated. Functional annotation of the proteins was done using Blast2go software with mosquito database from VB, and Diamond search algorithm with *Drosophila melanogaster* homologs from FlyBase[40,41].

Phylogenetic analysis of SGBAP in different mosquito species

A phylogenetic tree for SGBAP and its homologs was inferred by using the maximum likelihood method and general time reversible model [42]. Twenty-one homologs in *Aedes aegypti*, *Aedes albopictus* and *Culex quinquefasciatus* were identified using paralogs and orthologs from VB. We did not find homologs in other mosquito species or *D. melanogaster*. Trees for heuristic search were obtained by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach on cDNA sequences. The topology with the superior log likelihood value (-15281.44) was selected. A discrete Gamma distribution was used to model evolutionary rates (5 categories (+*G*, parameter = 6.7916)) with some sites allowed to be evolutionarily invariable ([+*I*], 1.98% sites). Evolutionary analyses were conducted in MEGA X [43,44].

Salivary gland gene silencing using double stranded RNA

Aedes aegypti salivary gland cDNA was used to amplify dsRNA targets with T7-tagged primers (Table S2). The PCR products were *in vitro* transcribed using T7 Scribe kit (Cellscript, USA). dsRNA was annealed by heating to 95 °C and slow cooling in a thermocycler. Three to five-day-old adult female mosquitoes were cold-anesthetized and intra-thoracically injected with 2 µg of dsRNA using Nanoject II (Drummond Scientific Company, USA). The same quantity of dsRNA against the bacterial gene *LacZ* was injected as control (dsCtrl). Four days post dsRNA injection, gene depletion was validated in SGs by RT-qPCR.

Gene expression quantification using real-time quantitative polymerase chain reaction

Total RNA was extracted from 10 SGs using E.Z.N.A. Total RNA kit I (Omega Bio-Tek, USA), DNAse treated using Turbo DNA-free kit (Thermo Fisher Scientific, USA), and reverse transcribed using iScript cDNA

synthesis kit (Bio-Rad, USA). Gene expression was quantified using qPCR with SensiFast Sybr no-rox kit (Bioline, USA) and gene specific primers (Table S2). *Actin* expression was used for normalization. The reactions were performed using the following conditions: 95°C for 10 min, 40 cycles of 95°C for 5 s, 60°C for 20s and melting curve analysis. The 2^{-DDCq} method was used to calculate relative fold changes.

Quantification of viral genomic RNA (gRNA) copies using RT-qPCR

At 8 days post inoculation, individual pairs of SGs were collected in 350 µl of TRK lysis buffer (E.Z.N.A Total RNA kit I, Omega Bio-Tek, USA) and homogenized with the mini beadbeater-96 (Biospec Products, USA) before RNA extraction with E.Z.N.A Total RNA kit I (Omega, Bio-Tek, USA). DENV2 gRNA copies were quantified by RT-qPCR using i-Taq universal probes one-step kit (Bio-Rad, USA), and ZIKV and CHIKV gRNA copies with i-Taq Universal SYBR green one-step kit (Bio-Rad, USA) with specific primers (Table S2). CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Singapore) was used for amplification with the following thermal profile: 50°C for 10 min, 95°C for 1 min, 40 cycles at 95 °C for 10s, 60 °C for 15 s. A melt-curve analysis was added for the SYBR-based quantification.

To quantify gRNA copies, a standard curve for each qPCR target was generated. qPCR targets were amplified from viral cDNA with the qPCR primers and forward primer tagged with T7. RNA fragments were generated with T7-Scribe kit (Cell Script, USA). RNA target copies were estimated based on Nanodrop quantification and used to generate an absolute standard equation. Three standard dilutions per plate were then added to adjust for inter-plate variation.

Statistical analyses

Differences in gene expression and gRNA copies were tested with unpaired T-test (in Microsoft excel) after log2 and log10 transformation, respectively, to meet normality.

Results

High-resolution proteomics of salivary glands

A total of 218 proteins with at least two unique peptides (95% confidence per peptide) were identified in uninfected *A. aegypti* SG (Table S1). To identify proteins, we used a custom protein database including annotated proteome database from VB and UniProt as well as *de novo* proteome data (unpublished) generated from our previous work on *A. aegypti* SG transcriptome collected at the same time point from the same mosquito colony [16]. This custom protein database is unique to our study conditions with the *A. aegypti* Singapore strain. We identified five newly annotated proteins which included one putative-C-type lectin, one 18.6 kDa secreted protein, one uncharacterized (no homolog in *Drosophila melanogaster*) protein, one aggrecan core-like protein and one 34 kDa salivary protein. A large majority (175 out of 218)

of the proteins that we detected were also found in the only other high-resolution SG proteomic analysis [32] (Fig. S1a). Discrepancies between the studies may stem from different starting protein amounts, mosquito colonies and mosquito age at collection. Signal peptides (SP), which directs proteins to the secretory pathways [45], may indicate secretion into saliva. We detected 71 SP-proteins (32.57% of all proteins), among which 39 were previously found in *A. aegypti* saliva [27] (Fig. S1b). Of note, proteins could also be secreted by non-classical pathways [45,46] as exemplified by SGS1 that do not have an SP but is detected in saliva [27]. Hereafter, proteins with a SP (including the secreted SGS1) were categorized as secretory proteins, whereas non-SP proteins were considered as cellular proteins.

Among the annotated proteins, 31 had a putative function in ribosome, stress and mitochondria (RSM), 30 in metabolism (MET), 23 in replication, transcription, translation (RTT) and 19 in cytoskeleton (CS). Most of these (97 out of 103) did not have a SP (Fig. 1a, b). There were also seven proteins related to proteolysis (PROT), two related to polysaccharide digestion (DIG), seven related to transport (TRP), 30 related to diverse functions (DIV) and 51 had unknown functions (UNK) (Fig. 1a, 1b; Table S1). Interestingly, there were 11 proteins related to immunity, including three serpins (SRPNs), one serine protease, four C-type lectins (CTLs), two fibrinogen related protein (FREPs) and one lysozyme (LYS) (Table S1). We did not detect proteins related to signaling of the canonical immune pathways. Seven proteins were related to blood-feeding (BF) and included two D7 proteins, two apyrases, one ADA, a prosialokinin precursor and one odorant binding protein (OBP) (Fig. 1a, 1b; Table S1). All immunity- and BF-related proteins had a SP (Fig. 1a, 1b; Table S1).

Salivary gland proteome response to DENV2, ZIKV or CHIKV infections

Owing to the different EIP for flaviviruses and alphaviruses [10,12], SG were dissected at 14 days post oral infection (dpi) for DENV2 and ZIKV, and at seven dpi for CHIKV. Blood inocula resulted in 100 % of infected SG at the collection time, as determined previously [16]. Controls for DENV2 and ZIKV, and for CHIKV were dissected at the corresponding times post uninfectious blood feeding. Using iTRAQ-based quantitative proteomics, we found 35, 17 or 16 upregulated, and 23, 10 or 13 downregulated proteins by DENV2, ZIKV or CHIKV infection, respectively (Fig. 2a, 2b; Fig. S2, Table S2). Detection of the corresponding viral proteins in the SG proteome further confirmed infection.

Seven proteins were commonly regulated by all infections (Fig. 2a, 2b; Table S2). Among them, immunity-related GILT-like (AAEL004873), BF-related ADA (AAEL026165), two proteins without conserved functional domains named SGS1 (AAEL09993) and SGBAP (AAegL5.3 AAEL019996/ NCBI GenBank Accession No. EAT45119.1 [47], ABF18177.1 [48]) were upregulated by all three virus infections (Fig. 2a, 2b; Table S2). The BF-related D7 protein (AAEL006424) and immunity-related CTL25 (AAEL000556) were upregulated by ZIKV infection and downregulated by DENV2 and CHIKV infections. Finally, the immunity-related SRPN23

(AAEL002704) was increased by CHIKV infection and decreased by both flaviviral infections (Table S2). All regulated proteins except SGS1 have a SP.

Among the secretory proteins, immunity and BF related proteins were the most regulated ones (Fig. 2c; Table S2). Among the immunity proteins, SRPN25 (AAEL007420) increased upon DENV2 and CHIKV infections, while SRPN26 (AAEL003182) was only increased by DENV2 infection. CTL16 (AAEL000533) and CTL25 (AAEL00556) decreased with DENV2 infection and increased with ZIKV infection, whereas CTL21 (AAEL011408) only increased upon ZIKV infection. C-type lysozyme (LYSP, AAEL009670) and FREP20 (AAEL000726) were reduced by DENV2 and ZIKV infections, while FREP22 (AAEL000749) was reduced upon DENV2 infection only. BF-related proteins included two apyrases (AAEL006347, AAEL006333) upregulated by DENV2 and CHIKV infections, one D7 protein (AAEL007394) downregulated by DENV2 and CHIKV infections, another D7 protein (AAEL006417) downregulated by CHIKV infection, a prosialokinin precursor (AAEL000229) downregulated by ZIKV infection, and a 34 kDa salivary protein (AAEL003600) downregulated by both DENV2 and CHIKV infections.

Among the cellular proteins, MET-related proteins were the most regulated (Fig. 2d; Table S2). Within the glycolytic pathway, seven proteins (glucose-6-phosphate isomerase, AAEL012994; glyceraldehyde-3-phosphate dehydrogenase, AAEL016984; enolase, AAEL024228; triosephosphate isomerase, AAEL002542; phosphoglycerate kinase, AAEL004988; fructose-bisphosphate aldolase, AAEL005766; and pyruvate kinase, AAEL014913) were upregulated by DENV2 infection and two (AAEL016984, AAEL014913) by CHIKV infection. Within the TCA cycle, three proteins (aconitase, AAEL012897; two malate dehydrogenases, AAEL007707, AAEL008166) were upregulated by DENV2 infection, while another malate dehydrogenase (AAEL008166) was upregulated by CHIKV infection. Two proteins related to fatty acid metabolism (pyruvate carboxylase, AAEL009691; saposin, AAEL003046), one protein related to energy metabolism (arginine kinase, AAEL009185) and one related to amino acid metabolism (aspartate amino transferase, AAEL002399) were upregulated by DENV2 infection. Another protein related to amino acid metabolism (pyrroline-5-carboxylate dehydrogenase, AAEL005422) was downregulated by ZIKV infection (Table S2).

Among RSM-related proteins, protein disulfide isomerase (PDI, AAEL002501) was commonly upregulated by both flaviviral infections. However, another PDI (AAEL000641) was uniquely downregulated by DENV2 infection alone. Thioredoxin reductase (AAEL002886) and 3-ketoacyl-CoA thiolase (AAEL0010697) were commonly upregulated by DENV2 and CHIKV infections. Few other RSM-related proteins were uniquely regulated by all three infections (Table S2). The weak overlap between infections indicates an overall virus-specific response at the protein level in SGs.

Functional evaluation of virus-induced proteins in SGs

To determine the function of the four upregulated proteins (i.e., SGBAP, SGS1, ADA and GILT-like) on DENV2, ZIKV or CHIKV infections, we depleted these proteins in SGs by RNAi-mediated gene silencing (silencing efficiency ranged from 48.7–73.9%; Fig. S3). DsRNA (dsCtrl) targeting the bacterial gene LacZ

was injected as control. To study the impact of gene depletion in SG only, we bypassed the midgut barrier by infecting mosquitoes through intra-thoracic inoculation with an inoculum enabling an increase or a decrease in infection [16]. At 8 days post inoculation (dpin) with DENV2 and ZIKV, and 4 dpin with CHIKV, we quantified gRNA in SGs and calculated infection prevalence (defined as percentage of infected SG) and infection intensity (measured as viral gRNA copies per infected SG). We used different mosquito batches to test the different genes, and because we observed that infection in control mosquitoes varied between batches (Fig. 3–5), infection outputs were compared within batches.

For all three viruses, infection prevalence was not altered by any gene silencing (Fig. 3–5). Of note, infection prevalence was 100% for ZIKV and CHIKV, thereby preventing observation of a pro-viral effect with this parameter. Interestingly, gene silencing altered infection intensity in a virus-specific manner. DENV2 infection intensity was increased by SGBAP depletion (Fig. 3). ZIKV infection intensity increased upon SGBAP and GILT-like depletions, and decreased upon SGS1 depletion (Fig. 4). CHIKV infection intensity was higher when SGBAP and ADA were depleted (Fig. 5). By studying SG proteins with uncharacterized impact on infection, we identified the virus-specific function of GILT-like, SGS1 and ADA, and the broad antiviral function of SGBAP (Table 1).

Table 1
Functions of commonly regulated proteins on DENV2, ZIKV and CHIKV infection in salivary glands

Gene name	Gene ID	Virus	Fold change regulation	Function
Salivary gland broad antiviral protein (SGBAP)	AAEL019996	DENV2	3.9253	Antiviral
		ZIKV	1.6492	Antiviral
		CHIKV	2.7667	Antiviral
Salivary gland surface protein 1 (SGS1)	AAEL009993	DENV2	2.0232	No effect
		ZIKV	1.7684	Proviral
		CHIKV	3.1224	No effect
Adenosine deaminase (ADA)	AAEL026165	DENV2	5.0684	No effect
		ZIKV	1.4967	No effect
		CHIKV	3.3300	Antiviral
Gamma interferon responsive lysosomal thiol-like (GILT-like)	AAEL004873	DENV2	2.2093	No effect
		ZIKV	4.6737	Antiviral
		CHIKV	1.7219	No effect

Discussion

SG response to infection regulates viral transmission (i) by modulating the mosquito antiviral response, which reduces virus amount in SG and saliva, and (ii) by altering the production of salivary components, which influences skin infection. Despite the relevance of SG in transmission, there is a dearth of knowledge about its response to infection at the proteome level. Leveraging cutting-edge proteomics technology, this study bridges this knowledge gap by describing the SG response to DENV2, ZIKV and CHIKV infections in *A. aegypti* at the global proteome level. Using high-resolution MS, we identified 218 proteins expressed in SG with putative functions in immunity, blood-feeding and cellular machinery. Using isobaric-based quantitative proteomics, we detected 58 proteins that were regulated by DENV2 infection, 27 by ZIKV infection and 29 by CHIKV infection. While a majority of proteins were not commonly regulated by all three viruses, four proteins were significantly upregulated in SG by DENV2, ZIKV and CHIKV infections. Hypothesizing that their upregulation was related to an antiviral response, we separately tested their functions in SG. The results revealed the antiviral function of GILT-like against

ZIKV, ADA against CHIKV and the proviral function of SGS1 for ZIKV. Most interestingly, we showed that SGBAP reduced DENV2, ZIKV and CHIKV infections in SG, thereby identifying a potential target to generate arbovirus-refractory mosquitoes.

We found that a large majority of the SG proteins related to immunity were regulated by the infections. Among the four CTLs detected in SG, CTL16, CTL21, CTL25 were upregulated by ZIKV infection, while CTL16 and CTL25 were downregulated by DENV2 infection and CTL25 downregulated by CHIKV infection. CTL16 and CTL25 were previously reported in SG [49,50]. CTLs are soluble proteins with carbohydrate binding activity and have multiple functions in regulating pathogen infection [51]. Studies have shown that CTLs can facilitate arbovirus attachment and entry into cells, as well as enhance infection. For example, galactose-binding CTL1 (mos-*GCTL1*, AAEL000563) recruitment by protein tyrosine phosphatase-1 facilitates West Nile Virus attachment and cell entry [52], CTL3 (mos-*GCTL3*, AAEL029058) interacts with DENV2 envelop to enhance viral infection [53] and mosquito CTL4 (AGAP005335) and CTLMA2 (AGAP005335) are required for clearance of *Escherichia coli*, suggesting their roles in immunity [54]. Both FREP20 and FREP22 expressed in SGs were downregulated by DENV2 infection, while only FREP20 was downregulated by ZIKV. FREPs, also called immunolectins, are pattern recognition receptors, which activate innate immune pathways [55]. Among the three SRPNs identified in SG, SRPN 23 was downregulated by both DENV2 and ZIKV infections, but upregulated by CHIKV infection. SRPN 25 was upregulated by both DENV2 and CHIKV infections, and SRPN 26 was upregulated by DENV2 infection alone. SRPNs regulate innate immunity by inhibiting protease signaling cascade [56]. For this, they bind trypsin-like targets through an arginine or lysine residue at P1 position [57]. Of note, the three SG SRPNs lack the characteristic inhibitory sequence and could therefore be non-inhibitory or act in a non-classical way as protease inhibitor [48,57]. The one LYS (LYSC9) expressed in SG was downregulated by both DENV2 and ZIKV infections. Its closest *D. melanogaster* homolog (i.e., LYSP) is specifically expressed in SG [58], while another LYSC in *A. aegypti* was upregulated in midgut by DENV2 infection [59]. LYS have functions in both digestion and immunity [60]. Overall, we identified immunity-related proteins regulated by DENV2, ZIKV or CHIKV infections in SG. Determination of their roles in SG immune response will require functional characterization.

We also observed that SG infection influenced the expression of proteins expectorated in saliva. ADA was upregulated by all three viruses. Saliva ADA can convert adenosine into inosine at the bite site, thereby inhibiting inflammatory cytokines to prevent the peripheral pain signalling [61]. ADA also enhances DENV2 infection *in vitro* by inhibiting type I IFN response in human keratinocytes [62]. Both apyrases detected in SG were upregulated by DENV2 and CHIKV. Apyrases are SG specific proteins that when secreted in saliva can prevent clot formation at the bite site by inhibiting ATP and ADP-mediated platelet aggregation [63,64]. Accordingly, apyrase content in mosquito SG is inversely proportional to probing time [65]. D7 proteins are highly abundant in mosquito saliva [49,66,67] and function as scavengers of biogenic amines [68], which induce vasoconstriction, platelet-aggregation and pain signaling [68]. Among the five D7 proteins identified in SG, three were regulated by at least one of the virus infections. A 37kDa D7 long protein (AAEL006424)

was downregulated by both DENV2 and CHIKV infections and upregulated by ZIKV infection. This D7 long form interacts with DENV2 envelope to inhibit infection in vertebrates [29]. Another D7 long protein (AEL006417) was downregulated by both DENV2 and CHIKV infection, while a D7 short protein (AAEL007394) was only downregulated by CHIKV infection alone. Prosialokinin that was downregulated by ZIKV infection is the precursor of secreted Sialokinin I and II, which have vasodilatory properties [23]. While OBP22 was identified in SG, its expression was not altered by any infection. OBPs are soluble ligand binding proteins with high affinity towards hydrophobic odorants and pheromones. They are involved in perception of odor and chemosensory signals, which regulate host-seeking behavior [69]. OBP22 was previously found to be a ligand for fatty acids [70] and required for efficient biting [17]. Our data suggests that SG infection can modulate transmission by altering saliva composition. Moreover, proteins related to digestion, metabolism and redox are discussed in supplemental (S1 Text).

A large proportion of SG proteins that were regulated by the infections remains uncharacterized. In this study, we determined the function of four uncharacterized infection-responsive proteins in SG infection, i.e., ADA, SGS1, GILT-like and SGBAP (Table 1). Tissue expression analysis based on available *A. aegypti* transcriptome literature [49,71–74] showed that SGBAP and SGS1 are specifically expressed in SG (Fig. S4). ADA is highly expressed in SG but is also present at lower levels in female abdominal tips. GILT-like protein is expressed in a wide range of tissues. While all four proteins were upregulated by DENV2, ZIKV and CHIKV in SG in the current study, we observed that ADA and GILT-like had virus-specific antiviral properties against CHIKV and ZIKV, respectively, that SGS1 had virus-specific proviral function for ZIKV, and that SGBAP had broad-spectrum antiviral properties by inhibiting all three viruses. ADA is indirectly involved in immune regulation, as it degrades adenosine, which suppresses immune response [75]. Accordingly, ADA enhances DENV2 infection in keratinocyte cells by inhibiting type I interferon response [75]. ADA levels in SG may thus regulate the balance between immune activation and repression. GILT-like was originally discovered as an interferon-inducible gene in mammals and subsequent characterization revealed its function in antigen presentation, bacterial infection and production of reactive oxygen species [76], which provides a rational for its antiviral function. In mosquitoes, GILT-like interacts with *Plasmodium* parasites and limits the parasite motility in skin when expectorated during biting [77]. The SGS1 is secreted in saliva through a non-classical pathway [78] and is a potential receptor for cell entry of avian malaria sporozoites in *A. aegypti* SG [79]. Its proviral effect for ZIKV might be related to a similar mechanism.

Factors with broad antiviral properties are of particular interest in designing transmission blocking interventions. We revealed the antiviral function of SGBAP against two flaviviruses and one alphavirus in SG of *A. aegypti.* SGBAP does not contain conserved functional domains as determined with NCBI conserved domain search, InterPro - EMBL-EBI or PROSITE-Expasy and has no homolog in *D. melanogaster*, making it difficult to speculate on its structure or mechanism of action. SGBAP is a small protein of 130 amino acid residues (mature protein) (we suggest a re-annotation of the gene in S1 text and Fig. S5) and is secreted in *A. aegypti* saliva [27]. An earlier transcriptomic study suggested it originated

from a truncation of a gene from the 34 kDa protein family [48]. To determine whether SGBAP function is conserved in other arbovirus mosquito vectors, we built a phylogenetic tree (Fig. S6). Among all mosquito species, SGBAP showed 21 putative homologs in *A. aegypti*, *Aedes albopictus* and *Culex quinquefasciatus*. *SGBAP* sequence did not cluster with other genes, thereby not supporting the existence of SGBAP antiviral homologs. Similarly, the closest homolog protein (i.e., *A. albopictus* gene - AALF004420) had only 56% identity. Functional homology among SGBAP orthologues should be experimentally tested. In *A. aegypti*, SGBAP broad antiviral function warrants further studies to understand its mechanism in SG and its function in saliva, where it could inhibit virus propagation.

In conclusion, we expanded our understanding of SG response to DENV2, ZIKV and CHIKV infections by using high-resolution quantitative proteomics for the first time in mosquito SG. We also identified new antiviral factors in SG, shedding new light on the antiviral response, which can be used to promote transmission blocking interventions.

Declarations

Acknowledgements

We thank Professor Eng Eong Ooi from Duke-NUS Medical School, and Professor Lisa Ng from Singapore Immunology Network (SIgN,A-STAR, Singapore) for providing the DENV2, ZIKV and CHIKV stocks, respectively. We thank Teck Kwang Lim from Protein and Proteomics Centre in NUS for his support in the mosquito SGs mass spectrometric analysis. We thank all staff in the core insectary facility at Duke-NUS Medical School where the mosquito experiments were conducted. Finally, we thank the Ministry of Education, Singapore (MOE 2015-T3-1-003) and the French Agence National pour la Recherche (ANR-20-CE15-006) for funding this work.

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Figures

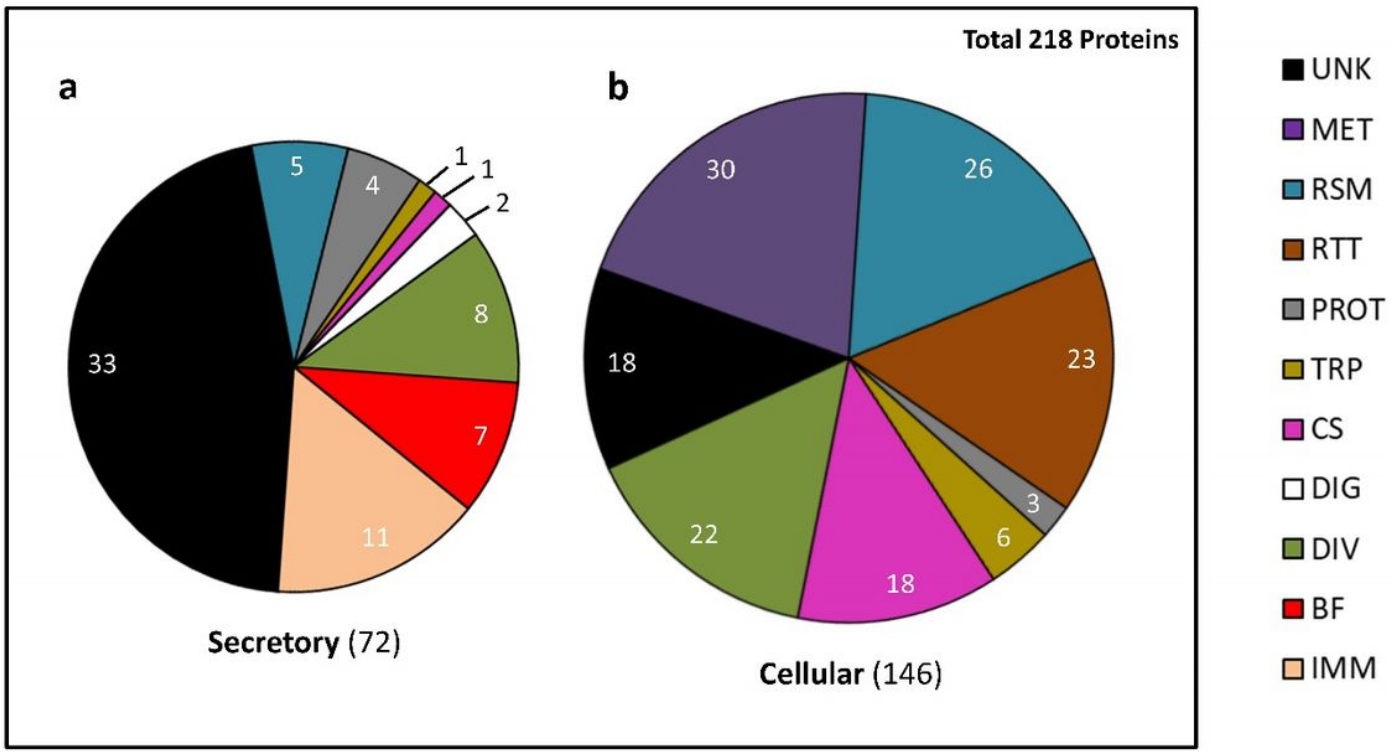

Figure 1

Proteome of uninfected A. aegypti SG. Proportions of secretory (identified by a SP, except for SGS1) (a) and cellular (b) proteins in each functional groups. Numbers of proteins per group are indicated. UNK,

unknown functions; MET, metabolism; RSM, redox, stress and mitochondria; RTT, replication, transcription and translation; PROT, proteolysis; TRP, transport; CS, cytoskeleton and structure; DIG, digestion; DIV, diverse functions; BF, blood feeding and IMM, immunity.

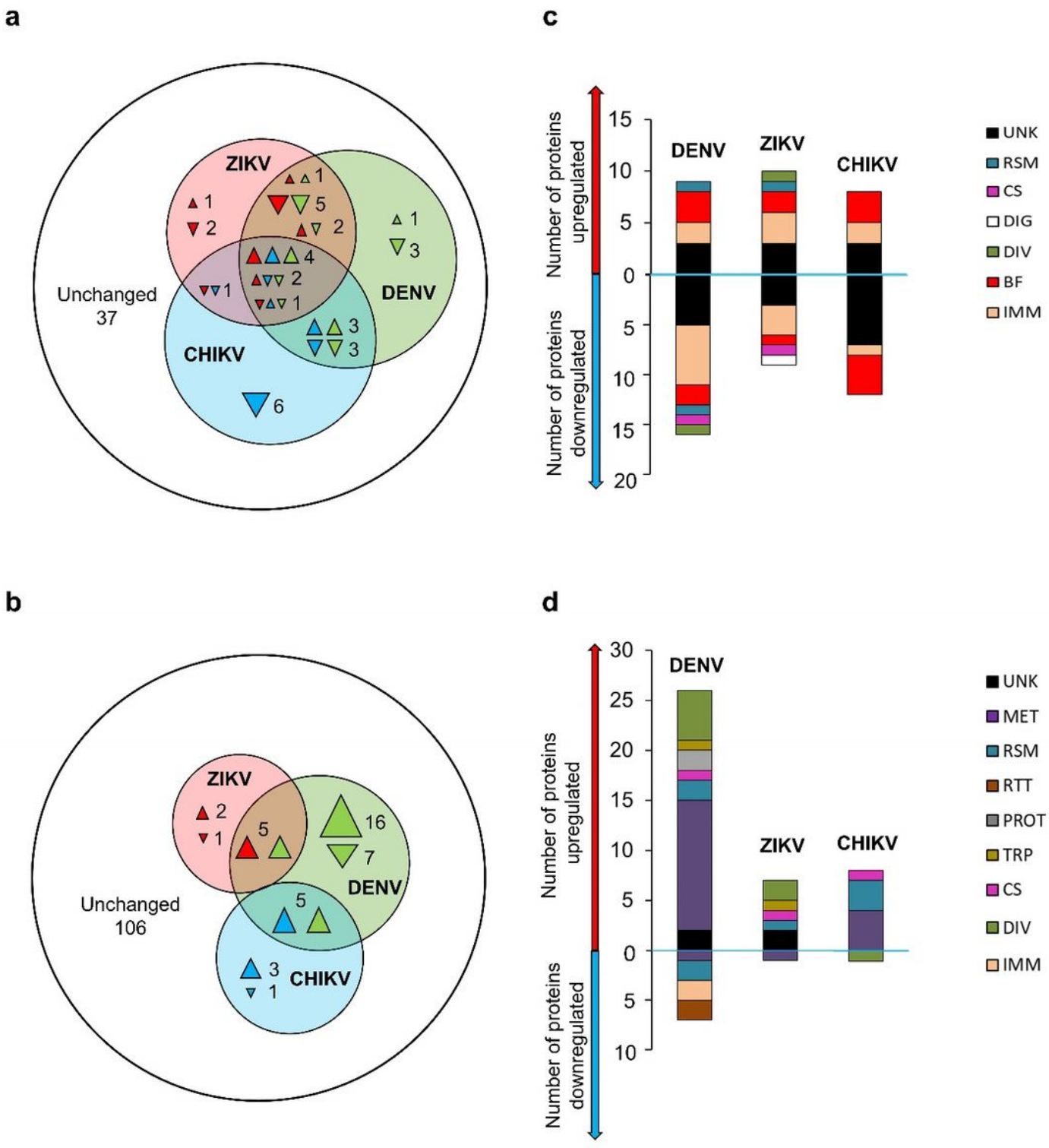

Figure 2

Differentially expressed proteins (DEPs) in A. aegypti SG upon infection with DENV2, ZIKV or CHIKV. Overlapping expression of secretory (identified by the SP, except SGS1) (a) and cellular (b) proteins

regulated by DENV2 (green), ZIKV (red) and CHIKV (blue). Colorless area shows number of unregulated proteins. Arrows indicate the direction of regulation for the corresponding color code. Functional annotation of secretory (c) and cellular (d) DEPs in DENV2, ZIKV and CHIKV infected SG. UNK, unknown functions; MET, metabolism; RSM, redox, stress and mitochondria; RTT, replication, transcription and translation; PROT, proteolysis; TRP, transport; CS, cytoskeleton and structure; DIG, digestion; DIV, diverse functions; BF, blood feeding and IMM, immunity.

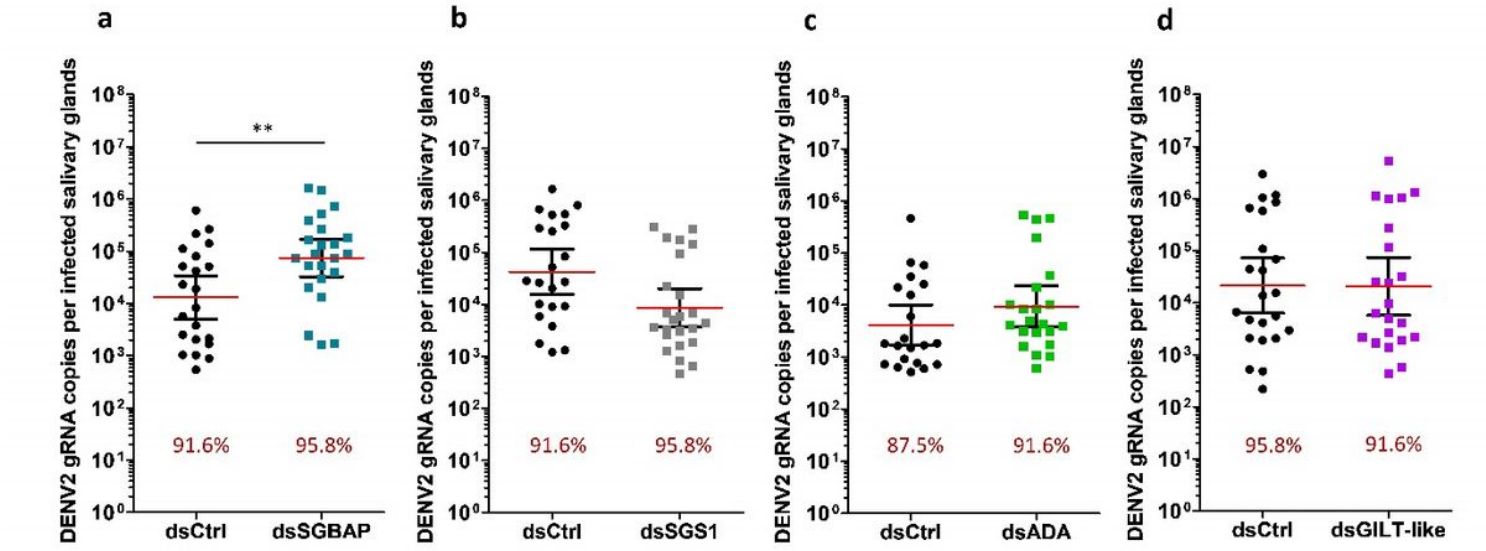

Figure 3

Effect of gene silencing on DENV2 infection in SG. DENV2 infection in SG upon silencing for SGBAP (a), SGS1 (b), ADA (c), and GILT-like (d). Infection intensity (defined as gRNA copies per infected SG) is shown as plots with lines showing geometric mean ± 95% C.I. from 24 individual pairs of SG. Each dot represents one sample. Infection prevalence is indicated in red numbers. dsCtrl, dsRNA against LacZ; dsSGBAP, dsRNA against salivary gland broad spectrum antiviral protein; dsSGS1, dsRNA against salivary gland surface protein 1; dsADA, dsRNA against adenosine deaminase protein; dsGILT-like, dsRNA against gamma interferon responsive lysosomal thiol protein-like. *, $p < 0.05$; **, $p < 0.01$ as determined by unpaired t-test. Each graph combines results from the same mosquito batch.

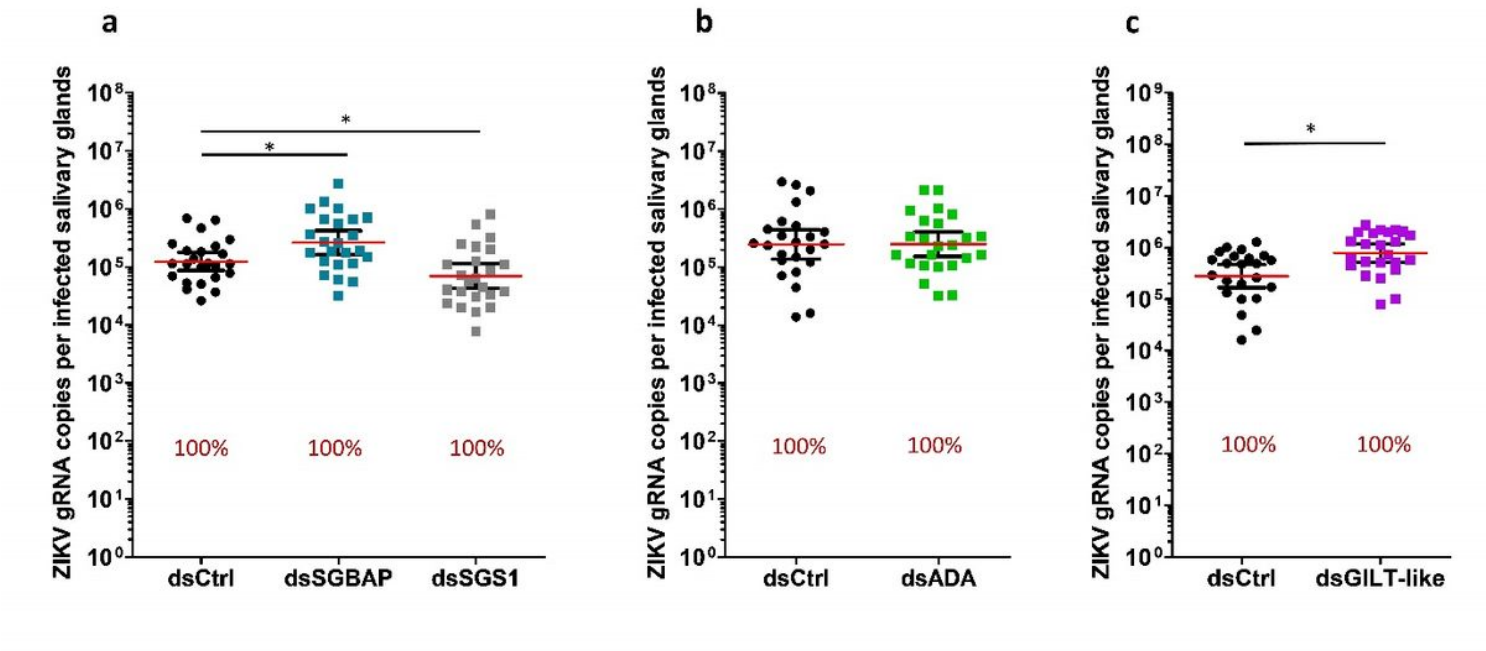

Figure 4

Effect of gene silencing on ZIKV infection in SG. ZIKV infection in SG upon silencing for SGBAP, SGS1 (a), ADA (b), and GILT-like (c). Infection intensity (defined as gRNA copies per infected SG) is shown as plots with lines showing geometric mean ± 95% C.I. from 24 individual pairs of SG. Each dot represents one sample. Infection prevalence is indicated in red numbers. dsCtrl, dsRNA against LacZ; dsSGBAP, dsRNA against salivary gland broad spectrum antiviral protein; dsSGS1, dsRNA against salivary gland surface protein 1; dsADA, dsRNA against adenosine deaminase protein; dsGILT-like, dsRNA against gamma interferon responsive lysosomal thiol protein-like. *, $p < 0.05$; **, $p < 0.01$; determined by unpaired t-test. Each graph combines results from the same mosquito batch.

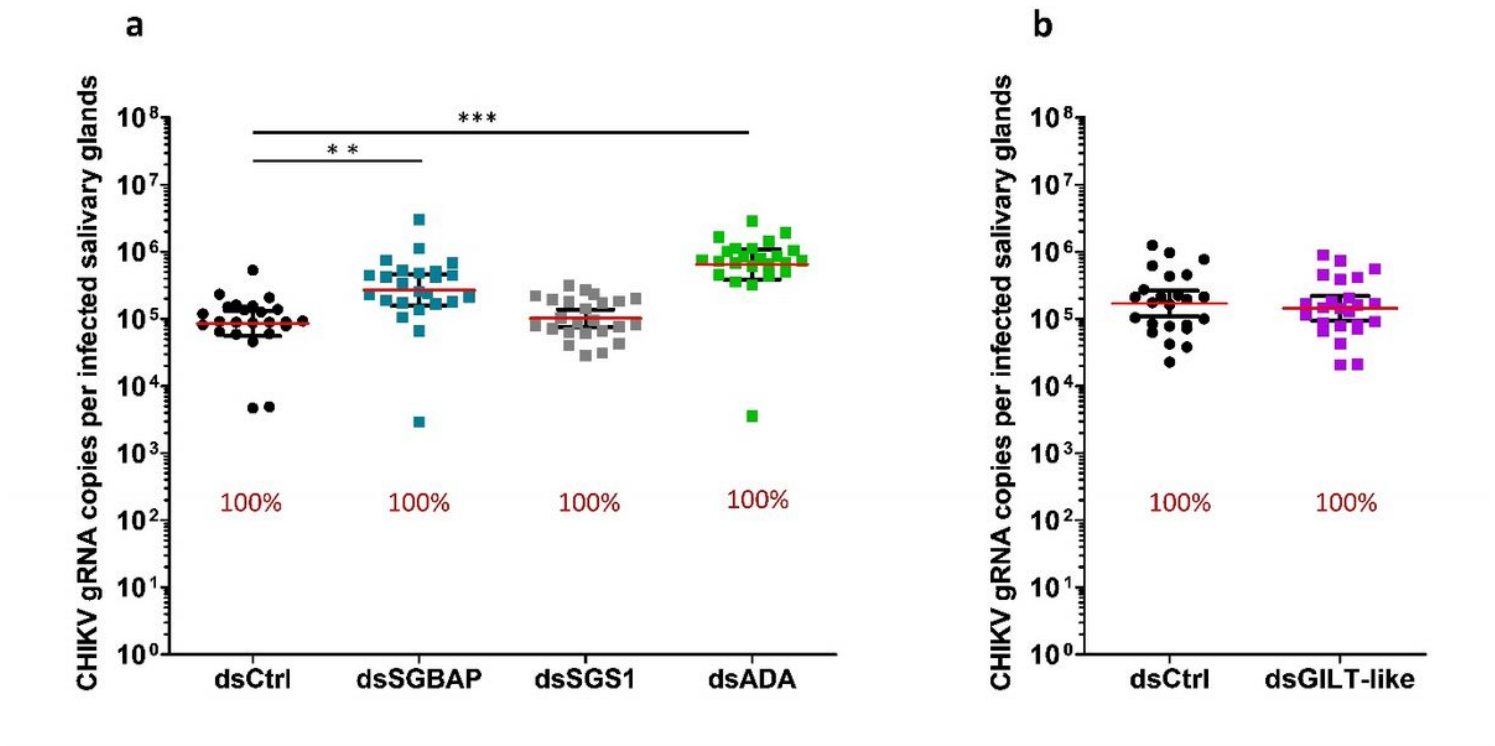

Figure 5

Effect of gene silencing on CHIKV infection in SG. CHIKV infection in SG upon silencing for SGBAP, SGS1, ADA (a), and GILT-like (b). Infection intensity (defined as gRNA copies per infected SG) is shown as plots with lines showing geometric mean ± 95% C.I. from 24 individual pairs of SG. Each dot represents one sample. Infection prevalence is indicated in red numbers. dsCtrl, dsRNA against LacZ; dsSGBAP, dsRNA against salivary gland broad spectrum antiviral protein; dsSGS1, dsRNA against salivary gland surface protein 1; dsADA, dsRNA against adenosine deaminase protein; dsGILT-like, dsRNA against gamma interferon responsive lysosomal thiol protein-like. *, $p < 0.05$; **, $p < 0.01$; determined by unpaired t-test. Each graph combines results from the same mosquito batch.

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