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Article

**Tudor-SN Promotes Early Replication of Dengue Virus in the Aedes aegypti Midgut**

HIGHLIGHTS

- Tudor-SN is upregulated in the Ae. aegypti midgut early upon dengue virus infection
- Tudor-SN promotes viral replication in vitro and in vivo
- Tudor-SN localizes to the nucleolus in mosquito cells
- Tudor-SN is not required for RNAi function in vivo

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Tudor-SN Promotes Early Replication of Dengue Virus in the Aedes aegypti Midgut

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SUMMARY
Diseases caused by mosquito-borne viruses have been on the rise for the last decades, and novel methods aiming to use laboratory-engineered mosquitoes that are incapable of carrying viruses have been developed to reduce pathogen transmission. This has stimulated efforts to identify optimal target genes that are naturally involved in mosquito antiviral defenses or required for viral replication. Here, we investigated the role of a member of the Tudor protein family, Tudor-SN, upon dengue virus infection in the mosquito Aedes aegypti. Tudor-SN knockdown reduced dengue virus replication in the midgut of Ae. aegypti females. In immunofluorescence assays, Tudor-SN localized to the nucleolus in both Ae. aegypti and Aedes albopictus cells. A reporter assay and small RNA profiling demonstrated that Tudor-SN was not required for RNA interference function in vivo. Collectively, these results defined a novel proviral role for Tudor-SN upon early dengue virus infection of the Ae. aegypti midgut.

INTRODUCTION
The mosquito Aedes aegypti transmits a wide range of pathogens to humans, many with severe consequences on public health, including dengue, Zika, and chikungunya viruses (Gould et al., 2017). For instance, dengue virus (DENV) infects 390 million people annually (Bhatt et al., 2013) and 50% of the world’s population is at risk for infection (Brady et al., 2012). DENV belongs to the Flaviviridae family and has a positive-sense, single-stranded RNA genome. DENV exists as four genetic types (DENV-1, -2, -3 and 4) that are phylogenetically related and loosely antigenically distinct (Katzelnick et al., 2015). In the wild, mosquitoes acquire DENV by feeding on a viremic host. After the infectious blood meal, DENV infection is first established in the mosquito midgut before spreading systematically and reaching the salivary glands, where the virus engages in further replication (Raquin and Lambrechts, 2017) before being transmitted to the next host via the saliva released during the bite (Salazar et al., 2007; Black et al., 2002).

The primary prevention strategy against arboviral diseases relies on the control of vector populations. Current vector control methods are mainly based on insecticides. Despite having been applied for decades, the burden of arboviral diseases keeps increasing (Messina et al., 2019). Human travel, urbanization, climate change, and geographic expansion of mosquito vectors increase pathogen transmission and spread (Weaver, 2013). Over the last two decades, research efforts have led to the production of laboratory-engineered mosquitoes that either suppress wild vector populations or render them incapable of transmitting pathogens (Champer et al., 2016; Yakob et al., 2017). As the methods for genetic modification of mosquitoes develop, the need to identify optimal target genes that are naturally involved in mosquito antiviral defenses or required for viral replication also increases. Preferably, such pro- or antiviral target genes would act early during the course of an infection, and, when engineered, would permit early blocking of virus replication, at the level of the midgut cells. This would hinder viral dissemination and make further transmission of the virus impossible.

The majority of our knowledge about insect antiviral immunity originates from investigations in the model organism Drosophila melanogaster (Merkling and Van Rij, 2013; Mongelli and Saleh, 2016), whereas studies in mosquito vectors remain more limited (Bartholomay and Michel, 2018; Simeso et al., 2018; Lee et al., 2019). The Toll, IMD, and Jak-Stat pathways have been implicated in insect innate immune responses to bacteria, fungi, viruses, and parasites. Their activation triggers translocations of NF-κB-like or Stat transcription factors to the nucleus, inducing the expression of an array of immune genes encoding antimicrobial peptides and virus restriction factors, among others (Bartholomay and Michel, 2018; Simeso et al., 2018; Lee et al., 2019; Merkling and Van Rij, 2013; Mongelli and Saleh, 2016). Another major branch of insect
innate immunity is RNA interference (RNAi), which encompasses several pathways leading to the production of small RNA molecules of different characteristics, such as small interfering RNAs (siRNAs), micro-RNAs (miRNAs), and P element-induced wimpy testis (PIWI)-interacting RNAs (piRNAs) (Miesen et al., 2016). The siRNA pathway is hitherto considered as the cornerstone of antiviral immunity in insects. It is initiated with the sensing and cleavage of viral double-stranded RNA (dsRNA) into 21-nucleotide-long siRNAs by the endonuclease Dicer-2. These siRNAs are loaded in the RNA-induced silencing complex (RISC) that guides Ago2-mediated cleavage of viral target sequences (Miesen et al., 2016). Numerous studies reported that depletion of siRNA pathway components in mosquitoes resulted in increased arbovirus replication (Campbell et al., 2008; Keene et al., 2004; Myles et al., 2008; Sanchez-Vargas et al., 2009; Franz et al., 2006).

Although several pathways involved in antiviral immunity have been characterized in mosquitoes, several aspects of anti-DENV defense remain elusive. For example, the siRNA pathway was shown to be insufficiently restrict DENV replication in the Aedes aegypti midgut (Olmo et al., 2018). Besides, most of previous studies have focused on mosquito antiviral or restriction factors that antagonize DENV, but little is known about mosquito host factors with a proviral function, that is, factors enhancing DENV propagation. Several human factors required for DENV infectivity were recently discovered through genome-wide CRISPR screens (Savidis et al., 2016; Zhang et al., 2016; Marceau et al., 2016), whereas only a handful of DENV host factors have been identified in mosquitoes to date (Londono-Renteria et al., 2015; Jupatanakul et al., 2014; Sessions et al., 2009; Raquin et al., 2017). Although CRISPR screens cannot be readily carried out in live mosquitoes, transcriptome analysis by high-throughput RNA sequencing is a powerful method to identify DENV host and restriction factors in vivo (Sigle and Mcgraw, 2019). For example, novel DENV restrictions factors (DVRF-1 and -2) that depend on the Jak-Stat pathway activation have been uncovered by overlapping transcriptional profiles of mosquitoes infected with DENV and mosquitoes with a hyperactive Jak-Stat pathway (Souza-Neto et al., 2009).

In this study, we exploited a unique transcriptomic dataset that we previously generated by performing RNA sequencing on individual midguts in a field-derived Ae. aegypti population during early DENV-1 infection (Raquin et al., 2017). In addition to a conventional pairwise comparison of gene expression between DENV-infected and uninfected controls, we also used an approach to detect correlations between viral RNA load and gene expression. Of 269 candidate genes identified by either method, only four were differentially expressed upon DENV-1 infection and had expression levels that correlated with viral RNA load in infected mosquitoes (Raquin et al., 2017). Among the four candidate genes identified by both methods was a gene encoding a member of the Tudor protein family, Tudor Staphylococcal Nuclease (abbreviated Tudor-SN or TSN), which we selected for further investigation in the present study. Using RNAi-mediated gene knockdown in vivo, we found that reduced TSN expression resulted in lower viral loads in vitro and in vivo. Immunofluorescence assays revealed that TSN localized to the nucleolus and did not colocalize to DENV replication sites in DENV-infected cells. Finally, we used a reporter assay and small RNA profiling to show that TSN was not involved in RNAi function in the midgut of adult mosquitoes. Altogether, our results demonstrate that TSN has an early proviral effect on DENV replication in the midgut and could be considered as a target to develop genetically modified mosquitoes that are refractory to DENV infection.

RESULTS

TSN Expression Is Upregulated upon DENV-1 Infection and Positively Correlates with Viral Loads

Our previous transcriptomic analysis revealed that TSN (AAEL000293) expression was significantly upregulated upon DENV-1 infection relative to mock controls 1 day after exposure to the infectious blood meal (Figure 1A) but not 4 days post blood meal (Figure 1B). Inversely, we found that TSN expression was not significantly correlated with DENV-1 viral loads 1 day post blood meal (Figure 1C) but was positively correlated with DENV-1 RNA loads 4 days post blood meal (Figure 1D). Thus, we concluded that TSN expression was induced by DENV-1 infection within 24 h after the infectious blood meal and that subsequently, its expression was positively correlated with DENV-1 replication. The positive correlation was suggestive of a proviral role for TSN upon DENV-1 infection.

TSN Is a DENV Proviral Factor In Vitro

First, we sought to test the proviral role of TSN in vitro by using Ae. aegypti Aag2 cells in culture. We transfected Aag2 cells with dsRNA to trigger RNAi-mediated knockdown of TSN or an exogenous green
fluorescent protein (GFP) sequence (Table 1) and subsequently inoculated them with DENV-1 at a multiplicity of infection of 1. We measured TSN expression levels by reverse transcription quantitative PCR (RT-qPCR) at 0, 12, 24, 36, 48, 72 and 96 h post infection and found that TSN knockdown efficiency ranged from ~50% to 80% and was statistically significant at most of the time points (Figure 2A). We visualized TSN protein levels by western blotting using an antibody directed against the human ortholog of TSN named SND1, which also reacted against the Ae. aegypti TSN. We confirmed that TSN knockdown reduced TSN protein levels by 70%–80% in Aag2 cells at 24 and 48 h post DENV-1 infection, compared with the GFP control (Figure 2B). To determine whether TSN also augmented viral infection in vitro, we measured both DENV-1 RNA levels (Figure 2C) and DENV-1 infectious titers by focus-forming assay (Figure 2D) over the course of infection. We found that DENV-1 RNA levels were significantly reduced upon TSN knockdown relative to control levels at 24 h post infection (Figure 2C, p < 0.01). Moreover, DENV-1 RNA levels were consistently lower in TSN-depleted cells from 24 to 96 h post infection. DENV-1 infectious titers were also significantly reduced upon TSN knockdown at 24 and 48 h post infection (Figure 2D, p < 0.05 and p < 0.001, respectively). Overall, these data demonstrated a proviral role of TSN in vitro.

**Figure 1. TSN Is Upregulated upon DENV-1 Infection and Correlates Positively with Midgut Viral Loads**

(A and B) TSN midgut expression levels on day 1 and day 4 post DENV-1 exposure. Log2-transformed TSN normalized RNA-seq counts are shown in mock-infected (n = 6) and DENV-1-infected (n = 16) midguts. p values of the pairwise t tests are indicated.

(C and D) Correlation of TSN expression level and viral load in DENV-1-infected midguts on day 1 and day 4 post virus exposure. Log2-transformed TSN normalized RNA-seq counts are shown as a function of the log10-transformed midgut viral load. Black lines represent the linear regression and light purple shaded areas represent the 95% confidence intervals of the regression. Pearson’s coefficients of determination (r) and p values of the linear regression coefficient are indicated.

fluorescent protein (GFP) sequence (Table 1) and subsequently inoculated them with DENV-1 at a multiplicity of infection of 1. We measured TSN expression levels by reverse transcription quantitative PCR (RT-qPCR) at 0, 12, 24, 36, 48, 72 and 96 h post infection and found that TSN knockdown efficiency ranged from ~50% to 80% and was statistically significant at most of the time points (Figure 2A). We visualized TSN protein levels by western blotting using an antibody directed against the human ortholog of TSN named SND1, which also reacted against the Ae. aegypti TSN. We confirmed that TSN knockdown reduced TSN protein levels by 70%–80% in Aag2 cells at 24 and 48 h post DENV-1 infection, compared with the GFP control (Figure 2B). To determine whether TSN also augmented viral infection in vitro, we measured both DENV-1 RNA levels (Figure 2C) and DENV-1 infectious titers by focus-forming assay (Figure 2D) over the course of infection. We found that DENV-1 RNA levels were significantly reduced upon TSN knockdown relative to control levels at 24 h post infection (Figure 2C, p < 0.01). Moreover, DENV-1 RNA levels were consistently lower in TSN-depleted cells from 24 to 96 h post infection. DENV-1 infectious titers were also significantly reduced upon TSN knockdown at 24 and 48 h post infection (Figure 2D, p < 0.05 and p < 0.001, respectively). Overall, these data demonstrated a proviral role of TSN in vitro.

**TSN Is a DENV Proviral Factor In Vivo**

To confirm the proviral role of TSN in vivo, we experimentally reduced TSN expression in adult female mosquitoes by intrathoracic injection of dsRNA and subsequently exposed them to an infectious blood
meal containing 10⁷ focus-forming units (FFU)/mL of DENV-1 (Figure 3A). First, we monitored TSN expression levels in individual mosquitoes by RT-qPCR on days 0, 1, and 4 after the infectious blood meal. On day 0, which corresponds to 3 days after injection of dsTSN, TSN expression was significantly knocked down relative to mosquitoes injected with a control dsRNA targeting GFP (Figure 3B, p < 0.001). Reduced TSN expression persisted over time through day 1 (Figure 3C, p < 0.0001) and day 4 (Figure 3D, p = 0.01) after exposure to the infectious blood meal. Importantly, reduced TSN expression did not significantly impact the survival of mosquitoes during the seven days following injection compared with the dsGFP control (Figure 3E, p = 0.54). We also measured TSN expression in head, thorax, abdomen, ovary, and midgut tissues in sugar-fed or blood-fed mosquitoes and found that TSN expression was significantly upregulated in midguts 1 day after a blood meal, suggesting a tissue-specific role within the first day after a blood meal (Figures S1A and S1B, related to Figure 3). Next, we measured DENV-1 RNA loads by RT-qPCR and found an ~50% reduction of viral loads in mosquito midguts depleted for TSN, compared with the GFP control, 4 days after the infectious blood meal (Figure 3F, p < 0.0001). We also measured DENV-1 RNA loads one day after the infectious blood meal and did not observe a decrease of viral loads. However, this is most likely due to the presence of viral RNA in the undigested blood still present in the midgut at this time point, as shown in a previous report (Raquin et al., 2017). These results confirmed the proviral role of TSN during DENV-1 infection in the mosquito midgut 4 days after the infectious blood meal. However, we found no evidence that TSN knockdown had an impact on infection prevalence after DENV-1 exposure. Among the mosquito midguts analyzed by RT-PCR on day 4, we found that 88% and 90% were positive for DENV-1 RNA in the TSN knockdown and the dsGFP control groups, respectively (Figure 3G, p = 0.59). In addition to DENV-1, we assessed the proviral role of TSN upon infection by another DENV serotype, DENV-3, and the alphavirus chikungunya virus (CHIKV). We injected adult mosquitoes with dsRNA against TSN or luciferase as a negative control and offered them an infectious blood meal 3 days later. We confirmed TSN knockdown (Figures S2A, related to Figure 3) and observed a significant reduction in DENV-3 RNA levels in individual mosquito midguts (Figures S2B, related to Figure 3) 4 days post DENV-3 exposure. We confirmed TSN knockdown 2 and 4 days post CHIKV exposure (Figures S2C and S2E).

| Organism | Primer/Probe^a | Sequence (5’-3’) | Product Size (bp) | Reference |
|----------|----------------|------------------|-------------------|-----------|
| Ae. aegypti | rp49-F | ACAAGCTTGCCCCCAACT | 97 | (Gentile et al., 2005) |
|           | rp49-R | CGTAAACCGATGTTGGC | 97 |           |
|           | TSN-F  | CTGCAGATGACAGTGAGTA | 100 | This study |
|           | TSN-R  | CTCGCTGACCAGTCCTT | 100 |           |
|           | dsTSN-F | taatagacctactatagggAAAGGCAAATGGAGCGACT | 312 | This study |
|           | dsTSN-R | taatagacctactatagggGACGTCACGTGCAGCAG | 312 |           |
|           | dsGFP-F | taatagacctactatagggATGGTGAGCAAGGCGGAG | 501 | This study |
|           | dsGFP-R | taatagacctactatagggTACTTGACAGTCGTC | 501 |           |
|           | dsLuc-F | taatagacctactatagggGCGCTGTCCCTGGAAC | 556 | This study |
|           | dsLuc-R | taatagacctactatagggAGAATCTCACGCAGAGTTC | 556 |           |
| DENV-1    | NSS-F  | GGAAGGAGAAGAGACTCCCAC | 105 | (Fontaine et al., 2016) |
|           | NSS-R  | ATCCCTGTATCCCTACCGGCT | 105 |           |
|           | NSS-Probe | CTCAGAGACATCATCAAAGATTCCAGGG | 105 |           |
| DENV-3    | NSS-F  | AGAAGGAGAAGAGACTGACA | 105 | This study |
|           | NSS-R  | ATCCCTGTATCCCTACCGGCT | 105 |           |
| CHIKV     | CHIK_10366_F | AAAGTCCCGGTYCCTTTACCAAAG | 208 | (Modified from Pastorino et al., 2005) |
|           | CHIK_10574_R | CCAAATTGTCCYGGTCTTCTC | 208 |           |

Table 1. List of Oligonucleotide Primers and Molecular Probes Used in This Study

^aF stands for forward and R stands for reverse.
^bT7 sequences are written in bold.
related to Figure 3), but despite a slight reduction of CHIKV RNA levels at both time points the difference with controls was not statistically significant (Figures S2D and S2F, related to Figure 3). Therefore, we found that TSN acted as a proviral factor for two DENV types, DENV-1 and DENV-3, but not for the alphavirus CHIKV. Overall, our data demonstrated that, although TSN does not influence the probability of DENV infection, it promotes early DENV replication in the mosquito midgut.

**TSN Localizes to the Nucleolus in Mosquito Cells**

It was previously shown that TSN could interact with DENV RNA in mammalian cells (Lei et al., 2011), which led us to ask whether TSN co-localized with DENV-derived RNA in mosquito cells. Double-stranded RNA is produced during the replication of single-stranded RNA viruses like DENV and is a hallmark of RNA virus infection (Weber et al., 2006). Previous reports demonstrated that antibodies directed against dsRNA did not cross-react with cellular rRNA or tRNA and could be used to identify flavivirus replication complexes in infected cells (Emara and Brinton, 2007). To determine the subcellular localization of TSN, we performed immunofluorescence assays in mosquito cells derived from *Ae. albopictus* (C6/36, Figure 4A) or *Ae. aegypti* (Aag2, Figure 4B) using the anti-SDN1 antibody previously validated by western blotting (Figure 3B) and a monoclonal antibody targeting dsRNA (called αK1). In both cell types, we found that TSN was expressed and localized to the nucleolus. Indeed, it localized to the nucleus region but did not overlap with DAPI staining, which is reported to exclude the nucleolus (Sirri et al., 2008). Moreover, the staining was more intense at the nucleus-nucleolus interface where it formed a “ring.” TSN localization did not change upon DENV-1 infection, nor did its expression level. Six days after DENV-1 infection of C6/36 and Aag2 cells, dsRNA staining was readily detectable and mainly localized to cytoplasmic regions of infected cells, likely corresponding to viral replication sites. Since TSN localized to the nucleolus, and the dsRNA to the cytoplasm, we did not observe overlapping signals between both stainings. Thus, we conclude that TSN...
Figure 3. TSN Promotes DENV-1 Infection in the Mosquito Midgut

(A) Experimental scheme of the gene-silencing assays in vivo.

(B–D) TSN expression levels following gene knockdown on day 0 (B), day 1 (C), and day 4 (D) after exposure to DENV-1 infectious blood meal. Mean percentage of gene expression knockdown on day 0 (B), day 1 (C), and day 4 (D) after DENV-1 exposure are indicated. Boxplots show TSN expression normalized by rp49 and expressed as $2^{-\Delta\Delta Ct}$ values in n = 12–24 individual mosquito midguts per group. Individuals with less than 50% gene expression knockdown are shown as empty dots. Data are representative of three separate experiments. p values above the graph indicate statistical significance assessed with a Wilcoxon test.

(E) Percentage of survival following dsRNA injection and/or DENV-1 exposure. Mosquitoes were injected with dsRNA targeting TSN (n = 127), targeting GFP (dsGFP, n = 110) 3 days prior to DENV-1 exposure. Non-injected mosquitoes that fed on an infectious (n = 71) or a non-infectious blood meal (n = 62) were used as controls. No significant difference in mortality was detected between dsGFP and dsTSN mosquitoes according to a Cox model (p = 0.54).

(F) DENV-1 RNA levels in mosquito midguts dissected from mosquitoes previously injected with dsGFP (n = 53) or dsTSN (n = 55). Boxplots represent the viral load measured by RT-qPCR on day 4 post exposure. Data represent three separate experiments combined. The negative effect of TSN knockdown on viral load was statistically significant in each of the three experiments. Viral loads are adjusted for differences between experiments and expressed in mean-centered DENV-1 RNA loads. The p value above the graph indicates statistical significance of the treatment effect assessed with an analysis of variance accounting for the experiment effect.

(G) DENV-1 infection prevalence in mosquito midguts measured by RT-qPCR on day 4 post virus exposure following injection with dsGFP (n = 53) and dsTSN (n = 43). Data from three separate experiments were combined after verifying the lack of a detectable experiment effect. Error bars represent 95% confidence intervals of the percentages. The p value above the graph indicates statistical significance of the treatment effect assessed with a logistic regression.

See also Figures S1 and S2.
does not interact with DENV-1 RNA at its replication site. However, it remains possible that interactions occur with other forms of DENV-1 RNA (positive or negative single-stranded RNA) or viral proteins.

**RNAi Is Functional in TSN-Depleted Mosquitoes**

Proteins containing Tudor motifs have been implicated in multiple aspects of RNA metabolism such as RNA splicing or small RNA pathways (Lasko, 2010; Siomi et al., 2010). TSN was shown to be a component of the RISC in Caenorhabditis elegans, Drosophila, and mammals (Caudy et al., 2003) and was suggested to participate in RNAi function in the tick Ixodes scapularis (Ayllon et al., 2015). Therefore, we asked whether TSN was involved in RNAi function in Ae. aegypti. We adapted a luciferase-based RNAi sensor assay developed in Drosophila to mosquitoes (Merkling et al., 2015a, 2015b, Van Cleef et al., 2011). Adult females were intrathoracically injected with a mix of lipofectant along with Firefly luciferase reporter plasmid with Firefly luciferase-specific dsRNA and dsRNA targeting GFP (as a negative control), Ago2 (as a positive control), or TSN (Figure 5A). A reporter plasmid encoding a Renilla luciferase was used as an in vivo transfection control. Three days after injection, the efficiency of Firefly luciferase silencing was measured in whole-mosquito homogenates (Figure 5B). When reporter plasmids were injected together with control dsGFP, we observed a wide range of luminescence counts (likely due to variable in vivo transfection efficiency), but the average luciferase activity was about 100-fold higher than when dsRNA targeting Firefly luciferase (dsLuc) was co-transfected with the reporter plasmids and control dsGFP. The silencing of Firefly luciferase was partially restored upon knockdown of Ago2, a key gene of the RNAi pathway, demonstrating the
validity of the reporter assay. Finally, we observed that the silencing of Firefly luciferase was maintained upon co-transfection with the dsRNA targeting TSN, suggesting that TSN does not enhance RNAi function in A. aegypti (Figure 5B). We measured expression levels of TSN and Ago2 upon co-transfection with reporter plasmids and dsRNA and verified that TSN and Ago2 expression levels were significantly reduced upon knockdown with their specific dsRNA (Figures 5C and 5D). Although we cannot exclude that residual TSN expression could suffice to maintain its activity, the knockdown efficiency was similar to that of Ago2. Overall, these results supported the conclusion that TSN is not a positive regulator of RNAi in A. aegypti. One caveat of this RNAi reporter assay is that we could only reliably assess a positive effect of TSN on RNAi activity (i.e., measure higher luminescence counts). Indeed, the efficiency of luciferase silencing was very high in the presence of dsRNA, which may have prevented our ability to detect a negative effect of TSN knockdown on RNAi activity (i.e., lower luminescence counts than the dsLuc + dsGFP control).

Small RNA Profiling in TSN-Depleted Mosquitoes

To overcome the limitations inherent to the RNAi reporter assay, and further assess the impact of TSN depletion on RNAi activity, we deep sequenced small RNA populations in TSN-depleted mosquitoes infected with DENV-1. We first injected adult mosquitoes with dsRNA targeting TSN or luciferase as a control. Two days later, we exposed mosquitoes to DENV-1 via an infectious blood meal. Four days after exposure to the virus, we performed a second injection of dsRNA against TSN and luciferase to prolong gene silencing (Figure 6A). We verified TSN knockdown by measuring TSN expression levels on days 4 and 10 post infection (Figures S3A, related to Figure 6). We then selected mosquitoes that were infected with
DENV-1 and displayed low TSN expression for sequencing, as well as control mosquitoes injected with dsRNA targeting luciferase or TSN 2 days before and 4 days after a DENV-1 infectious blood meal. Whole bodies were harvested on day 10 post infection for RNA extraction and deep sequencing of small RNAs.

Figure 6. Small RNA Populations Are Unchanged Following TSN Knockdown

(A) Experimental scheme. Adult female mosquitoes were injected with dsRNA targeting luciferase or TSN 2 days before and 4 days after a DENV-1 infectious blood meal. Whole bodies were harvested on day 10 post infection for RNA extraction and deep sequencing of small RNAs.

(B and C) Size distribution of the total number of DENV-1-specific small RNA reads normalized to the total number of reads upon (B) TSN knockdown or (C) luciferase control.

(D–F) Linear relationship between the amount of viral and cellular small RNAs in TSN knockdown versus luciferase control mosquitoes 10 days post DENV-1 infection. Data points represent the normalized number of reads (coverage per 1M reads) corresponding to (D) miRNAs, (E) endo-siRNAs mapping on histone coding sequences, and (F) endo-piRNAs mapping on histone coding sequences. For miRNAs, each dot represents one miRNA. For siRNAs and piRNAs mapping on histone genes, each dot represents a histone gene. For siRNAs and piRNAs mapping on the DENV-1 sequence, each dot represents a 500-bp region of the viral genome. Lines represent the linear regression of each set of values. The equation and R² value of each regression are shown next to the line.

See also Figure S3.
mapping on the mosquito genome. We found that TSN depletion did not affect the miRNA machinery, as miRNA abundance was very similar between both conditions tested (Figure 6D). Likewise, we found that TSN knockdown did not influence siRNA and piRNA biogenesis. The abundance of both histone-derived siRNAs (Figure 6E) and histone-derived piRNAs (Figure 6F) was similar between the TSN-depleted mosquitoes and controls.

**DISCUSSION**

Antiviral immunity in Ae. aegypti mosquitoes remains poorly understood. Using a novel approach of transcriptomic analysis, we previously uncovered four genes that not only responded to DENV infection in the mosquito midgut but also had expression levels that correlated with viral loads in infected mosquitoes (Raquin et al., 2017). Here, we focused on one of these four genes, Tudor-SN, encoding a member of the Tudor protein family. TSN was induced upon DENV-1 infection, and its expression correlated positively with viral RNA load (Raquin et al., 2017). Using RNAi-mediated knockdown assays in vivo and in vitro, we demonstrated that TSN promotes both DENV-1 and DENV-3 replication. TSN knockdown also resulted in a slight decrease of CHIKV replication, but it was not statistically significant. We performed localization studies and discovered that TSN localizes to the nucleolus of Ae. aegypti and Ae. albopictus cells and does not colocalize with DENV-1 replication sites. Moreover, we found that, despite belonging to the Tudor family, TSN was not essential for RNAi function in adult mosquitoes.

TSN is a known component of the RISC, the RNAi protein complex that carries siRNAs and directs cleavage of complementary viral sequences in *Caenorhabditis elegans*, *Drosophila*, and mammals (Caudy et al., 2003). Additionally, previous work in *Drosophila* and other model organisms found essential functions for Tudor domain-containing proteins in piRNA biogenesis. A recent study describing a functional knockdown screen of all predicted *Ae. aegypti* Tudor proteins did not reveal a role for Tudor-SN in piRNA biogenesis (Joosten et al., 2019). This finding is consistent with our observations that Tudor-SN is not necessary for the piRNA pathway function in vivo in *Ae. aegypti*. Finally, Tudor-SN was also shown to be a conserved component of the basic RNAi machinery in *Ixodes* ticks (Ayllon et al., 2015). This study reported an effect of Tudor-SN on dsRNA-mediated gene silencing, which possibly involves the siRNA pathway. However, no evidence was obtained for a role of Tudor-SN in the response to microbial infection (Ayllon et al., 2015). Taken together, the data described in this study and discussed above did not find strong links between RNAi function and Tudor-SN in arthropods. Further studies using knockout mutants might be necessary to confirm these findings.

The mammalian ortholog of Tudor-SN is generally referred to as p100 and was identified as a host factor interacting with the 3’ untranslated region of the DENV genome (Lei et al., 2011). Moreover, p100 knockdown led to reduced levels of viral RNA and protein in mammalian cells, providing evidence that p100 was required for efficient DENV replication. Although these results are consistent with those reported here, in mammalian cells p100 was shown to interact with DENV genomic RNA and dsRNA replication intermediates, which we did not observe. Importantly, the subcellular localization of p100 in mammalian cells was perinuclear, whereas it was nucleolar in mosquito cells. This discrepancy in localization hints to a divergence in function between mammals, in which p100 interacts directly with viral RNA, and insects, for which evidence is lacking. Microscopy-based localization studies being limited in sensitivity and resolution, elucidating Tudor-SN function in mosquitoes will require further experiments to more definitely exclude interactions between viral RNA and Tudor-SN, such as protein immunoprecipitation and sequencing of associated RNA (RIP-seq).

The predicted structure of *Ae. aegypti* Tudor-SN includes four Staphylococcal Nuclease (SN)-like domains and a Tudor domain embedded in a fifth SN domain. Tudor-SN homologs are found in diverse eukaryotic species such as plants, humans (Staphylococcal Nuclease and Tudor domain containing 1), and insects (*Drosophila* Tudor-SN). The very similar structure of eukaryotic Tudor-SN homologs is consistent with potentially conserved functions (Figure 7). However, Tudor-SN subcellular localization is variable in other species, which also hints toward species-specific function(s) of Tudor-SN proteins.

Our observation that *Ae. aegypti* Tudor-SN localizes primarily to the nucleolus of mosquito cells makes it unlikely that its proviral effect on DENV relies on a direct action on viral genome stability or replication. The nucleolus is a multifunctional nuclear domain involved in ribosome biogenesis and several other cellular functions, such as cell cycle regulation, telomere metabolism, or DNA damage sensing and repair (Lam and Trinkle-Mulcahy, 2015). Various nucleolar alterations during viral infection have been documented (Salvetti and Greco,
Interestingly, DENV non-structural protein 5 (NS5), which encodes the virus RNA-dependent RNA polymerase, was recently shown to localize to the nucleolus of infected mammalian cells (Fraser et al., 2016), where it interferes with precursor messenger RNAs (pre-mRNA) splicing to limit host antiviral response (De Maio et al., 2016). Human Tudor-SN was implicated in spliceosome assembly and, therefore, may influence splicing of pre-mRNAs and/or interact with DENV NS5 to facilitate viral RNA accumulation (Gao et al., 2012). More generally, Tudor-SN could promote viral replication through regulation of gene expression. For example, the Jak-Stat pathway protects *Ae. aegypti* against DENV infection (Souza-Neto et al., 2009) and Tudor-SN was shown to bind Stat proteins to modulate host gene transcription (Paukku and Silvennoinen, 2004). Also, Tudor-SN is a component of stress granules (Gao et al., 2014) and could interfere with their formation to facilitate DENV infection (Miller, 2011). Particularly interesting is the early proviral effect of TSN in the mosquito midgut, which might suggest a role for TSN in viral sensing, or early antiviral responses. For instance, TSN might sense the infection and, in the nucleus, alter the spliceosome to increase the availability of cellular resources that the virus requires to replicate. Its presence in the nucleolus and at the nucleus-nucleolus interface might enhance ribosome biogenesis and subsequently increase production of viral proteins.

Although several pathways involved in antiviral immunity have been characterized in mosquitoes, several aspects of anti-DENV defense remain to be elucidated, particularly during the early phase of infection. For example, the siRNA pathway was recently shown to inefficiently restrict DENV replication in the *Ae. aegypti* midgut (Olmo et al., 2018). The present work adds to the small number of studies that identified DENV proviral factors in mosquitoes (Londoño-Rentería et al., 2015; Jupatanakul et al., 2014; Sessions et al., 2009; Raquin et al., 2017). Such host factors have been proposed as new targets for antiviral therapy in humans (Savidis et al., 2016; Zhang et al., 2016; Marceau et al., 2016). Although the development of novel vector control methods has focused on viral restriction factors so far (Flores and O’neill, 2018), targeting essential host factors could complement antiviral strategies in mosquitoes. We showed that Tudor-SN is such a host factor for DENV in the mosquito *Ae. aegypti*. Further studies will be necessary to elucidate the specific mechanisms underlying the role of Tudor-SN in DENV replication.
Limitations of the Study
We demonstrated a proviral role of TSN upon DENV infection in the mosquito midgut. Our study primarily relied on gene knockdown to diminish TSN expression in vitro and in vivo, and it will be useful in the future to confirm the results using knockout mosquitoes. Second, this study used a strain of Ae. aegypti from Thailand, and it remains to be determined whether the proviral role of TSN extends to other mosquito strains. Finally, additional in-depth functional studies are required to elucidate the exact role of TSN.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

DATA AND CODE AVAILABILITY
The accession number for the RNA-seq data set reported in this paper is SRA: PRJNA386455.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.100870.

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AUTHOR CONTRIBUTIONS
S.H.M., V.R., S.D., M.-C.S., and L.L. designed the experiments; S.H.M., V.R., S.D., I.M.-C., A.H.-L., and H.B. performed the experiments; S.H.M., V.R., H.V., L.F., and L.L. analyzed the data; S.H.M., V.R., M.-C.S., and L.L. wrote the paper.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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Supplemental Information

*Tudor-SN Promotes Early Replication of Dengue Virus in the *Aedes aegypti* Midgut*

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Supplementary figure legends

Supplementary Figure 1. TSN is transiently upregulated in the midgut following a blood meal, related to Figure 3. TSN expression levels on day 1 (A) and day 4 (B) in the midgut, head, ovary, abdomen, and thorax in sugar-fed mosquitoes (control) or following a non-infectious blood meal. TSN expression was normalized by rp49 and expressed as 2^{-dCt} value. Organs were collected in pools of 10 and tested in triplicates. P values above the graph indicate statistical significance assessed with a Wilcoxon test.

Supplementary Figure 2. TSN promotes DENV-3 infection but not CHIKV infection in the mosquito midgut, related to Figure 3. (A, C, E) TSN expression levels and (B) DENV-3 or (D, F) CHIKV RNA levels following gene knockdown on day 2 (C, D), and day 4 (A, B, E, F) after an infectious blood meal. Mean percentages of gene expression knockdown are indicated. TSN expression was normalized by rp49 and expressed as 2^{-dCt} values in n = 20-24 individual mosquito midguts per group. P values above the graph indicate statistical significance assessed with a Wilcoxon test.

Supplementary Figure 3. TSN knockdown does not significantly affect the coverage of siRNAs mapping on the DENV-1 genome, related to Figure 6. TSN expression levels (A) and DENV-1 viral RNA levels (B) on day 4 and 10 post blood meal in whole adult mosquitoes injected with dsRNA targeting luciferase or TSN. TSN expression and viral levels were normalized by rp49 and expressed as 2^{-dCt} values in n = 17-32 individual mosquitoes per group. P values above the graph indicate statistical significance assessed with a Wilcoxon test. Colored dots represent mosquitoes selected for the small RNA sequencing. The normalized coverage of 21-nt reads mapping on the positive and negative viral strands is shown across the DENV-1 genome for the (C) luciferase control and (D) TSN knockdown conditions.
Transparent methods

Ethics
The Institut Pasteur animal facility has received accreditation from the French Ministry of Agriculture to perform experiments on live animals in compliance with the French and European regulations on care and protection of laboratory animals. This study was approved by the Institutional Animal Care and Use Committee at Institut Pasteur under protocol number 2015–0032.

Cells and virus

C6/36 cells (derived from Ae. albopictus) and Aag2 cells (derived from Ae. aegypti) were cultured in Leibovitz's L-15 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 1% non-essential amino acids (Life Technologies) and 0.1% Penicillin-Streptomycin (Life Technologies) at 28°C. DENV type 1 (DENV-1) isolate KDH0030A was originally derived in 2010 from the serum of a dengue patient at the Kamphaeng Phet Provincial Hospital, Thailand (Fansiri et al., 2013). The full-length consensus genome sequence is available from GenBank under accession number GenBank: HG316482. DENV-3 isolate GA28-7 was originally derived in 2010 from the serum of a dengue patient in Moanda, Gabon (Caron et al., 2013). CHIKV isolate M105 (Caribbean strain) was described previously (Stapleford et al., 2016) and its genome sequence is available from GenBank under accession number GenBank: LN898104.1. Viral stocks were prepared in C6/36 cells for DENV and Vero cells (ATCC CRL-1586) for CHIKV. Infectious titers of DENV were measured on C6/36 cells using a standard focus-forming assay (FFA) (Fontaine et al., 2016), and infectious titers of CHIKV were measured on Vero cells using a standard plaque assay (Goic et al., 2016).

Mosquito rearing and experimental infections

All experiments were performed with adult Ae. aegypti mosquitoes derived from a field population originally sampled in 2013 in Thep Na Korn, Kamphaeng Phet Province, Thailand. Experiments took place within 16 generations of laboratory colonization. Mosquitoes were reared in standard insectary conditions, as previously reported (Fontaine et al., 2016). Experimental virus infections were performed in a level-3 containment facility, as previously described (Fontaine et al., 2016). Shortly, 5- to 7-day-old female mosquitoes were deprived of
10% sugar solution 24h before oral exposure to viruses. The infectious blood meal consisted of a 2:1 mix of washed rabbit erythrocytes and viral suspension (to reach an infectious titer of $10^7$ FFU/mL) supplemented with 10 mM ATP (Sigma). Mosquitoes were fed for 30 min through a pig-intestine membrane using an artificial feeder (Hemotek Ltd) set at 37°C. Fully engorged females were incubated at 28°C, 70% relative humidity and under a 12-hour light-dark cycle with permanent access to 10% sucrose till further use.

**RNA isolation from mosquito midguts**

Midguts were dissected in 1x PBS, and immediately transferred to a tube containing 800 µL of Trizol (Life Technologies) and ~20 1-mm glass beads (BioSpec). Samples were homogenized for 30 sec at 6,000 rpm in a Precellys 24 grinder (Bertin Technologies). RNA was extracted as previously described (Raquin et al., 2017), and stored at -80°C till further use.

**Reverse transcription and quantitative PCR**

Viral RNA was reverse transcribed and quantified using a TaqMan based qPCR assay, using NS5-specific primers and 6-FAM/BHQ-1 double-labeled probe (sequences provided in Table 1). Reactions were performed with the Superscript III Platinum One-Step qRT-PCR kit (Life technologies) following the manufacturer’s instructions and as previously described (Fontaine et al., 2016). The limit of detection of the assay was 10 copies of viral RNA per µL. *Tudor-SN* and *Ago2* expression levels were measured using a SybrGreen based qPCR assay, using gene-specific primers (sequences provided in Table 1). First, total RNA from individual midguts or adults was reverse transcribed into cDNA using MMLV reverse transcriptase (Invitrogen), according to manufacturer’s instructions. Quantitative PCR was performed on a LightCycler 96 real-time thermocycler (Roche) using SYBR Green MasterMix from Roche (Figure 3) or Promega (Figures 2, 5, S1, S2, S3). qPCR efficiency and Ct values were unaffected by change of SYBR Green reagent. The qPCR programs was as follows: an initial denaturation step of 5 min at 95°C, followed by 40 cycles of 10 sec at 95°C, 20 sec at 60°C and 10 sec at 72°C. A melting curve was generated to confirm the absence of non-specific PCR amplicons using the following program: 5 sec at 95°C, 60 sec at 65°C and continuous fluorescence acquisition up to 97°C with a ramp rate 0.2°C/sec. Relative expression was calculated as $2^{-\Delta\Delta Cq}$, using the *Ae. aegypti* ribosomal protein-coding gene *rp49* (AAEL003396) for normalization.
**Double-stranded RNA synthesis**

Design and synthesis of dsRNA used in knockdown assays has been described previously (Raquin et al., 2017). Briefly, dsRNA was synthesized from a GFP-containing plasmid or from a cDNA template produced by RT-PCR on RNA isolated from a pool of *Ae. aegypti* mosquitoes. T7 promoter sequences were incorporated by PCR to the amplicon that was used as a template for the synthesis using the MEGAscript RNAi kit (Life Technologies).

**Gene silencing assays in vivo**

RNAi-mediated knockdown of target genes was performed as previously described (Raquin et al., 2017). Briefly, 500 ng of dsRNA targeting Firefly *luciferase* (*FLuc*), and 1 µg of dsRNA targeting *TSN*, *Ago2* or *GFP* was injected intra-thoracically using Nanoject II or III (Drummond). Injection volume was 140 nL except for the RNAi reporter assay described below. Control mosquitoes were injected with a dsRNA targeting Green Fluorescent Protein (GFP). After injection, mosquitoes were incubated for 3 days at 28°C before exposure to an infectious blood meal.

**RNAi reporter assay**

RNAi competency of adult mosquitoes was assed using a reporter assay adapted from previously published methods in *Drosophila* (Merkling et al., 2015a; Merkling et al., 2015b; van Cleef et al., 2011). *In vivo* plasmid transfection method was optimized from protocols previously published for *Ae. aegypti* (Colpitts et al., 2011; Isoe et al., 2007). Five to seven-day-old adult mosquitoes were injected in the thorax using a Nanoject III (Drummond) with a suspension of ≈ 300 nL containing a 1:1 mixture of unsupplemented Leibovitz's L-15 medium (Life technologies) and Cellfectin II (Thermo Fisher Scientific) complexed with 50 ng pUb-GL3 (encoding Firefly *luciferase*, *FLuc*), 50 ng pCMV-RLuc (encoding Renilla *luciferase*) described previously (Anderson et al., 2010; Varjak et al., 2017), and 500 ng FLuc-specific and 1 µg *GFP*-, *TSN*- or *Ago2*-specific dsRNA. After incubation for 3 days at 28°C, mosquitoes were homogenized in passive lysis buffer (Promega) using the Precellys 24 grinder (Bertin Technologies) for 30 sec at 6,000 rpm. Samples were transferred to a 96-well plate and centrifugated for 5 min at 12000 × g. Fifty microliters of supernatant were transferred to a new plate, and 50 µL LARII reagent added for the first FLuc measurement. Next, 50 µL Stop&Glow
reagent was added before the second measurement of RLuc, according to the Dual Luciferase assay reporter system (Promega). Counts of RLuc were used to control for transfection efficiency, and samples with less than 1,000 counts were discarded from the analysis. Data were normalized by calculating the ratio Fluc/RLuc.

**Immunofluorescence assays**

Mock- and DENV-1-infected Aag2 cells were fixed on coverslips using 4% paraformaldehyde (Sigma-Aldrich) for 30 min at room temperature (20-25°C). Following permeabilization with 1X PBS, 0.1% Triton-X100, cells were incubated with mouse anti-dsRNA αK1 (English & Scientific consulting) and rabbit anti-TSN antibodies diluted 1:500 in 1X PBS, 0.1% Triton-X100, 2% Normal Goat Serum for 1 hour at room temperature. Subsequently, cells were washed three times with 1X PBS with 0.1% Triton X-100 and incubated with goat anti-mouse AlexaFluor 594 and goat anti-rabbit Alexa Fluor 488 diluted 1:1.000 in 1X PBS, 0.1% Triton-X100, 2% Normal Goat Serum (Life technologies), overnight at 4°C. After three washes in 1X PBS with 0.1% Triton X-100, cover slips were mounted on a glass slide in ~10 µL Prolong Gold anti-fade medium containing DAPI (Thermo Fisher) and imaged with a confocal microscope LSM 700 inverted (Zeiss) at 63X magnification.

**Gene silencing and DENV-1 infection in vitro**

Aag2 cells were transfected in 24-well plates with 500 ng of dsRNA using Lipofectamine LTX (Invitrogen) along with Plus reagent according to the manufacturer’s instructions. To increase knockdown efficiency, a second round of transfection with 500 ng of dsRNA was performed 48 hours after the initial transfection. Infection with DENV-1 was performed 24 hours after the last transfection. Cells were incubated for 1 hour in L-15 infection medium containing 2% FBS and DENV-1 at a multiplicity of infection of 1. After removal of the infectious inoculum, cells were refreshed with fully supplemented with L-15 medium and incubated at 28°C.

**Western blotting**

Aag2 cells were harvested, washed once in PBS and resuspended in RIPA buffer (20 mM Hepes-KOH pH 7.5, 100 mM KCl, 5% glycerol, 0.05% NP40, with freshly added 0.1M DTT and complete, EDTA-free, protease inhibitors (Roche)). Cells were lysed in Laemmelli buffer (Sigma-Aldrich) with 10% β-mercaptoethanol and incubated at 95°C for 5 min. Protein lysates were
loaded on a 4–20% precast mini-protean polyacrylamide gel (Bio-Rad) then transferred to a nitrocellulose membrane. The blot was incubated for 1 hr at room temperature with rabbit anti-TSN antibody (Abcam 65078) diluted 1:200 in blocking buffer (5% skimmed milk powder, 0.1% Triton-X100 in 1X PBS). After 3 washes with 1X PBS, the blot was incubated with a secondary antibody, HRP-conjugated polyclonal goat anti-rabbit IgG (GE Healthcare) diluted 1:5.000 in 1X PBS, 0.1% Triton-X100 for 1 hour at room temperature. Next, the blot was incubated with a primary anti-β-actin murine antibody (Sigma-Aldrich, clone AC-74) at 1:6.000 dilution in blocking buffer, and an HRP-conjugated polyclonal goat anti-mouse IgG (GE Healthcare) diluted at 1:5.000 in 1X PBS, 0.1% Triton-X100 was used as a secondary antibody. Bound antibodies were revealed by chemiluminescence with the SuperSignal West Pico Chemiluminescent Substrate (Fisher Scientific). Quantification of band intensities was performed in ImageJ (Davarinejad, 2017).

Small RNA libraries

Total RNA from pools of 2 (dsLuc) or 3 (dsTSN) whole mosquitoes was isolated with TRIzol (Invitrogen). Small RNAs of 19-33 nucleotides in length were purified from a 15% acrylamide/bisacylamide (37.5:1), 7 M urea gel as described previously (Gausson and Saleh, 2011). Purified RNAs were used for library preparation using the NEBNext Multiplex Small RNA Library Prep kit for Illumina (E7300 L) with the 3’ adaptor from IDT (linker 1) and in-house designed indexed primers. Libraries were diluted to 4 nM and sequenced using an Illumina NextSeq 500 High Output kit v2 (75 cycles) on an Illumina NextSeq 500 platform. Sequence reads were analyzed with in-house Perl scripts.

Bioinformatics analysis of small RNA libraries

The quality of fastq files was assessed using graphs generated by ‘FastQC’ (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Quality and adaptors were trimmed from each read using ‘cutadapt' (https://code.google.com/p/cutadapt/). Only reads with acceptable quality (phred score ≥20) were retained. A second set of graphs was generated by ‘FastQC' (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) on the fastq files created by ‘cutadapt' (Martin, 2011). Mapping was performed by ‘Bowtie1' (Langmead et al., 2009) with the ‘-v 1’ option (one mismatch between the read and its target). ‘Bowtie1' generates results in ‘sam' format. All ‘sam' files were analyzed by different tools of the package ‘samtools' (Li et al., 2009) to produce ‘bam' indexed files. To analyze these ‘bam'
files, different kind of graphs were generated using home-made R scripts with several Bioconductor libraries such as ‘Rsamtools’ or ‘Shortreads' (http://bioconductor.org/).

Statistical analysis

Statistical analysis methods have been described previously (Raquin et al., 2017). Additionally, statistical significance tests as implanted in GraphPad Prism version 7. $P$ values below 0.05 were considered statistically significant.

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Supplementary figure 1

A Day 1 post blood meal

B Day 4 post blood meal

TSN expression levels (relative to rp49)

- Control
- Blood fed

p = 0.019
Supplementary figure 2

DENV-3 - Day 4 post infection

A. TSN expression levels (relative to rp49)

B. DENV-3 RNA levels (relative to rp49)

CHIKV - Day 2 post infection

C. TSN expression levels (relative to rp49)

D. CHIKV RNA levels (relative to rp49)

CHIKV - Day 4 post infection

E. TSN expression levels (relative to rp49)

F. CHIKV RNA levels (relative to rp49)
Supplementary figure 3

A

TSN expression levels (relative to rp49)

DENV-1 RNA levels (relative to rp49)

B

Luciferase control

TSN knockdown

C

Coverage

D

Coverage