Arbovirus-vector protein interactomics identifies Loquacious as a co-factor for dengue virus replication in *Aedes* mosquitoes

Short title: Loquacious is a dengue virus co-factor in mosquitoes

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ABSTRACT

Efficient virus replication in *Aedes* vector mosquitoes is essential for the transmission of arboviral diseases such as dengue virus (DENV) in human populations. Like in vertebrates, virus-host protein-protein interactions are essential for viral replication and immune evasion in the mosquito vector. Here, 79 mosquito host proteins interacting with DENV non-structural proteins NS1 and NS5 were identified by label-free mass spectrometry, followed by a functional screening. We confirmed interactions with host factors previously observed in mammals, such as the oligosaccharyltransferase complex, and we identified protein-protein interactions that seem to be specific for mosquitoes. Among the interactors, the double-stranded RNA (dsRNA) binding protein Loquacious (Loqs), an RNA interference (RNAi) cofactor, was found to be essential for efficient replication of DENV and Zika virus (ZIKV) in mosquito cells. Loqs did not affect viral RNA stability or translation of a DENV replicon and its proviral activity was independent of its RNAi regulatory activity. Interestingly, Loqs colocalized with DENV dsRNA in viral replication organelles in infected cells and directly interacted with high affinity with DENV RNA in the 3' untranslated region *in vitro* ($K_D = 100\text{-}200\text{ nM}$). Our study provides an interactome for DENV NS1 and NS5 and identifies Loqs as a key proviral host factor in mosquitoes. We propose that DENV hijacks a factor of the RNAi mechanism for replication of its own RNA.

AUTHOR SUMMARY

Dengue virus is a mosquito-transmitted virus endemic to the tropics and subtropics, affecting an estimated 390 million people yearly. While the mechanisms of infection, pathogenesis and immune evasion have been extensively studied in humans, replication in *Aedes* mosquitoes has received much less attention, despite being a critical step in the arbovirus transmission cycle. Here,

we used a proteomic approach to identify *Aedes* mosquito proteins recruited by dengue virus non-structural proteins NS1 and NS5. In addition to previously established host proteins that interact with DENV in mammals, we identified Loquacious, a double-stranded RNA binding protein involved in the antiviral RNAi immune response of mosquitoes. Unexpectedly, our data showed Loquacious functions as a proviral factor that is recruited to replication organelles to facilitate viral RNA replication. We propose that DENV exploits host immune components, such as Loquacious, for its own benefit.

INTRODUCTION

Mosquito-borne flaviviruses such as dengue virus (DENV) and Zika virus (ZIKV) are transmitted between humans by *Aedes* mosquitoes, causing major public health and economic burden in the tropics and subtropics [1]. Whereas *Aedes aegypti* is considered the most common vector for DENV transmission, vector competence of *Ae. albopictus* and its ability to adapt to colder climates raise concerns of a shift of arbovirus endemic regions toward the Northern hemisphere, further enhanced by global warming [2–5]. As no specific treatment against DENV is available and vaccine development faces many obstacles [6], there is a need to understand the mechanisms of flavivirus replication in mosquitoes to develop transmission blocking strategies.

Flaviviruses have a single-stranded positive-sense RNA genome, which is translated upon release into the host cell and replicated at the membrane of the endoplasmic reticulum (ER) where viral proteins hijack the host cell machinery to form membrane invaginations functioning as viral replication organelles [7]. While structural proteins are essential for viral entry, assembly and release of viral particles, non-structural (NS) proteins together with host factors are required for viral RNA replication and modulation of host cell functions. Expressed in the lumen of the ER and released outside of the host cell, NS1 is an important pathogenicity factor in mammals [8,9]. In addition, NS1 contributes to the formation of viral replication organelles and recruits the oligosaccharyltransferase (OST) complex, as well as mRNA translation and protein folding factors [10–12]. As the viral RNA-dependent RNA polymerase, NS5 cooperates with other viral and host proteins to replicate the viral RNA, but also interacts with host proteins to suppress Jak-STAT signaling and modulate the spliceosome [13].

For transmission to a mammalian host, arboviruses need to efficiently replicate in their mosquito vector, which, like in mammals, requires extensive virus-host protein-protein interactions. Flavivirus host factors have been extensively studied in mammalian models using proteomics [10,13–15] as well as other high-throughput approaches such as RNAi and CRISPR screens [11,16–19]. In comparison, few studies have addressed host factors in mosquitoes. While it is likely that flaviviruses use homologous host proteins and cellular processes in mosquitoes and mammals, this has only been confirmed for few proteins, such as SEC61 [14,19]. In addition, given the evolutionary distance, the different physiologies and diverse immune systems of mosquitoes and mammals, it is to be expected that flavivirus proteins additionally interact with specific sets of proteins in mosquito vector and vertebrate host.

A key element differentiating mosquito and mammalian immunity is the crucial role of RNAi in antiviral defense in insects [20–22]. Double-stranded (ds)RNA formed during viral replication is cleaved by the nuclease Dicer-2 into small interfering (si)RNA duplexes that are loaded into Argonaute 2 (Ago2) to guide the recognition and cleavage of complementary viral RNA. Loading of siRNA duplexes into Argonaute proteins is facilitated by the paralogous dsRNA-binding proteins R2D2, Loquacious (Loqs) and Loqs2, the latter of which is unique to *Aedes* mosquitoes [23–27]. The *Ae. aegypti* *Loqs* gene encodes multiple, non-redundant splice isoforms [24], of which Loqs-PB along with R2D2 facilitates siRNA processing, whereas Loqs-PA is required for microRNA (miRNA) processing [24]. In line with a function in siRNA processing, it was found that silencing *R2D2*, *Dicer-2* and *Ago2* increased DENV replication [28]. In addition, the mosquito specific Loqs2 interacts with Loqs and R2D2 and is essential to control systemic flavivirus infections *in vivo* [23]. Note that *Loqs* transcript annotation of the reference genome AaegL5 does not correspond to the annotation used in [24]. The transcript referred to as *Loqs-RA* by Haac et al.

[24] corresponds to *Loqs-RB* in *AaegL5* and *vice versa*. The transcript referred to as *Loqs-RC* by Haac et al. does not exist in *AaegL5*. In the current manuscript, we use the *AaegL5* annotation.

To identify host proteins interacting with DENV proteins in mosquitoes, we purified FLAG-tagged DENV NS1 and NS5 expressed in the context of all DENV non-structural proteins, followed by quantitative mass spectrometry. We identified Loqs as an essential co-factor for DENV and ZIKV RNA replication, independent of its function in RNAi. We showed that Loqs interacts with the 3' untranslated region of DENV RNA and localizes to viral replication organelles during infection of *Aedes* cells. Our data provide new insights into flavivirus replication in mosquitoes and illustrate a case in which a potential antiviral protein, Loqs, is recruited by a virus for its own benefit.

RESULTS

Interactome of DENV NS1 and NS5 proteins in *Aedes mosquito* cells

To identify host factors that interact with DENV proteins in an RNA-independent manner in mosquitoes, we established a system to express all non-structural proteins in *Ae. albopictus* cells. Expression of non-structural proteins in the absence of viral RNA is sufficient to induce membrane rearrangements, reminiscent of those induced during viral infections [7,29]. All non-structural genes were cloned under the control of an *Ae. aegypti* poly-ubiquitin promoter (PUB), introducing a 3xFLAG tag at the N-terminus of NS1 after the ER localization signal and conserving the signal peptidase cleavage site, or at the C-terminus of NS5, according to previous work [10,13] (Fig. 1A). Expression of the recombinant proteins was confirmed by western blot, and both FLAG-tagged NS1 and NS5 were efficiently concentrated by FLAG-immunoprecipitation (Fig. 1B). As expected, NS1 and NS5 were expressed in distinct cell compartments in *Ae. albopictus* C6/36 cells (Fig. 1C).

Using a plasmid expressing untagged DENV non-structural proteins as a control, we characterized the interactome of FLAG-tagged NS1 and NS5 in C6/36 cells by label-free mass spectrometry (Fig. 1D-E and Table S1). NS1 interacted with 55 proteins, including the viral NS2A, NS3, NS4A and NS4B proteins, whereas NS5 interacted with 45 proteins, including NS1, NS2A, NS3 and NS4A, and a total of 15 host proteins interacted with both NS1 and NS5 (Fig. 1D-E, 2A and Table S1). NS2B peptides were not detected, likely due to limitations of detection. However, given that NS2B is a cofactor for the NS3 protease and polyprotein processing was efficient (Fig. 1B), NS2B is likely expressed at physiologically relevant levels in our system. The identification of viral non-structural proteins in NS1 and NS5 immunoprecipitations confirmed the formation of

macromolecular complexes in the absence of structural proteins and viral RNA in our experimental system.

Biological processes and functional protein network analysis identified several functional protein clusters in association with NS1 and NS5 (Fig. 2B-C). Among the cellular interactors enriched at least two-fold in NS1 immunoprecipitations, we identified 11 proteasome subunits and five proteins of the OST complex (Fig. 1D-E, 2 and Table S1): Oligosaccharide transferase Δ subunit (Ost Δ , ortholog of human ribophorin II, RPN2), Oligosaccharide transferase γ subunit (OST γ , ortholog of human MAGT1), UDP-glucose-glycoprotein glucosyltransferase (UGGT, ortholog of human RPN1), Oligosaccharyltransferase 48kD subunit (OST48, ortholog of human dolichyl-diphosphooligosaccharide-protein glucosyltransferase non-catalytic subunit, DDOST) and catalytic subunit 3A of the oligosaccharyltransferase complex (STT3B). Amongst the cellular interactors enriched in NS5 immunoprecipitations, we identified three elements of the survival of motor neuron (SMN) complex involved in the spliceosome and small nuclear ribonucleoprotein (snRNP) assembly (Gemini 2, Gemini 3 and Smn) as well as the E3 ligase HYD (ortholog of human UBR5). The OST complex and proteasome have previously been described as proviral host factors interacting with DENV NS1 in mammals [10,30–32], NS5 was shown to hijack the snRNP and human ortholog UBR5 was purified with NS5 in mammals [13], illustrating virus-host interactions that occur both in the mosquito vector and human host. Together, these data validate our experimental model to identify physiologically relevant protein-protein interactions between virus and vector.

Identification of DENV proviral and antiviral host factors

From the 79 host proteins interacting with NS1 and/or NS5, we selected 22 hits for a functional knockdown screen in the RNAi competent *Ae. albopictus* U4.4 cell line (Fig. 3A). Cells were transfected with dsRNA, infected with DENV, and viral RNA was quantified by RT-qPCR. From the initial screen (Fig. 3B), 10 hits were selected for a confirmation screen with a second set of dsRNA, targeting a different region of the gene to avoid off-target effect (Fig. 3C). We classified genes as high or low-confidence hits, based on selective criteria for gene knockdown efficiency, effect on gene knockdown on DENV RNA levels, and consistency between both dsRNA data sets. One NS1 interactor was identified as proviral factor as silencing consistently resulted in a decrease in viral RNA (Fig. 3B-C), Loquacious (Loqs, ortholog of human TARBP2) as well as two NS5 interactors: the E3 ubiquitin-protein ligase HYD (ortholog of human UBR5) and Muskelin. In addition, four NS1 interactors were found to be antiviral as silencing consistently resulted in an increase in viral RNA (Fig. 3B-C): OSTA (RPN2), RNA polymerase I subunit H or RPL12 (ortholog of human RPA12), DISCO-interacting protein 1 (DIP1, ortholog of human ADARs), ER retention protein RER1 in addition to one common NS1/NS5 interactor: HSP60A.

Among the hits, the RNAi cofactor *Loqs* had the strongest phenotype with up to 70-90% inhibition of DENV RNA levels upon its knockdown (Fig. 3B-C). Two potential Loqs host interactors were also included in the functional screen (Fig. 2). *Pancreatic eIF2A-α kinase (PEK)*, distant ortholog of human PRKRA known to interact with the human *Loqs* ortholog TARBP2, of was not efficiently silenced, and knockdown of RNA helicase *Gemini 3* (ortholog of human DEAD box helicases like DDX20) [33–36] did not modulate DENV replication (Fig. 3C). The finding that *Loqs* was a proviral host factor is unexpected, given that *Loqs* is an essential cofactor of RNAi, a pathway with antiviral activity in mosquitoes. For this reason and because of the strong NS1 association (Fig. 1D-E) and consistent phenotype (Fig. 3B-C), we focused the remainder of the

study on Loqs, using *Ae. aegypti* as a model, for which a better genome annotation is available than for *Ae. albopictus*.

Loqs is a flavivirus proviral factor in *Ae. aegypti*

The *Loqs* gene encodes three splice isoforms, which differ from each other by the presence or absence of short exons. Loqs-RA and Loqs-RB are very similar to each other, except that the 144 nt-long exon 5 is retained in Loqs-RA. Loqs-RC is a shorter isoform, in which exon 6 is retained, but exon 7 is skipped (Fig. 4A). As a consequence, the gene products Loqs-PA and Loqs-PB contain two dsRNA-binding domains (dsRBD, IPR014720) as well as a Staufen, C terminal domain (IPR032478), which may be involved in protein-protein interactions. Loqs-PC contains only the two N-terminal dsRBDs.

From the mass spectrometry data, we identified one peptide unique to Loqs-PA in NS1 immunoprecipitations, whereas other peptides were shared between the three isoforms. Using primers flanking exon5 (Fig. 4C, Table S3) as well as unique primers to distinguish the isoforms (Fig. 4D, Table S3), we showed that Loqs-RA and Loqs-RB are expressed at similar levels in *Ae. aegypti* Aag2 cells, whereas Loqs-RC is only lowly expressed, in line with its low expression *in vivo* (Fig. S1A). Together, these results indicate that NS1 interacts with Loqs-PA, although we cannot rule out interactions with Loqs-PB and Loqs-PC (Fig. 4B).

To analyze which of the isoforms is responsible for the observed proviral phenotype, we compared DENV replication after RNAi-mediated knockdown of specific *Loqs* isoforms as well as other components of the miRNA and siRNA pathways in Aag2 cells (Fig. 4E). Silencing of *Ago2* resulted in an increase in viral replication, in agreement with an antiviral function of the RNAi pathway, whereas *R2D2* and *Loqs2* silencing did not significantly affect DENV replication in cells.

Ago1 silencing was also associated with an increase in viral replication, suggesting an important function of miRNA regulated genes in viral replication. In contrast, silencing of all *Loqs* isoforms combined resulted in a 60% reduction in DENV RNA levels (Fig. 4E), confirming our observations *Ae. albopictus* U4.4 cells (Fig. 4B-C). Furthermore, silencing of the Loqs-RA isoform specifically was associated with a 43% reduction in DENV replication, whereas Loqs-RC silencing did not affect DENV replication, in line its low expression (Fig. 4E). Due to the *Loqs* gene structure, it was impossible to design dsRNA that specifically targets Loqs-RB but not the other isoforms. These results indicate that Loqs-PA and perhaps Loqs-PB are proviral host factors for DENV replication in *Ae. aegypti*.

To further characterize the role of Loqs in flavivirus replication, we compared the effect of *Loqs* silencing on DENV and ZIKV replication over time. *Loqs* silencing reduced viral RNA levels for both viruses at all time points analyzed (Fig. 4F). Furthermore, the effect of *Loqs* silencing on viral replication was consistently stronger for ZIKV (up to 90% inhibition at 48 h). In contrast and as expected, *Ago2* silencing resulted in an increase in DENV and ZIKV RNA levels (Fig. 4F). These results suggest that Loqs is a pan-flavivirus proviral host factor in *Aedes* mosquitoes.

Loquacious proviral activity is independent of its RNAi regulatory functions

Having established the importance of Loqs for flavivirus replication, we further characterized its role during viral replication. To analyze the effect of Loqs on viral RNA stability and translation, we monitored the expression of *Renilla* luciferase from a DENV subgenomic replicon in cells in which *Loqs* was silenced [37]. To this end, luciferase expression was monitored after transfection of replicon RNA into *Ae. albopictus* U4.4 cells and *Ae. aegypti* Aag2 cells (Fig. 4G). Luciferase expression from direct translation of the transfected replicon RNA was similar in cells treated with

Loqs dsRNA or control dsRNA, whereas luciferase expression was blocked upon treatment with the translation inhibitor cycloheximide, as expected. These results suggest that *Loqs* does not affect viral RNA stability and translation efficiency, but that it is required for viral RNA replication. As *Loqs* has been described as a co-factor of the siRNA pathway, it is unlikely that its proviral phenotype is dependent on RNAi. To directly test this, we next sequenced small RNAs produced in *Loqs*-depleted Aag2 cells infected with DENV or ZIKV. To account for differences in virus accumulation upon *Loqs* silencing, virus-derived siRNA (vsiRNA) levels were normalized to virus RNA levels in the same RNA used for small RNA sequencing. Despite efficient silencing of *Loqs* expression (Fig. S1D) and consequent reduction of viral RNA levels, no differences were observed in DENV or ZIKV-derived vsiRNA (Fig. 4H-I). Moreover, we did not observe major differences in miRNA levels (Fig. S1E-F), as also observed previously when both isoforms were depleted [24]. These results indicate that the proviral activity of *Loqs* is independent of its function in the RNAi pathway.

Loquacious colocalizes with dsRNA in viral replication organelles

Considering that *Loqs* contains multiple dsRBD domains, we explored the potential role of *Loqs* as a cofactor of viral RNA replication. We analyzed subcellular expression of *Loqs*-PA and *Loqs*-PB in mock- and DENV infected Aag2 cells (Fig. 5A). In non-infected cells, GFP-tagged *Loqs*-PA or *Loqs*-PB showed a discrete, punctate staining across the cytoplasm as well as lower, diffuse staining in the cytoplasm. In DENV-infected cells, both *Loqs* isoforms likewise showed a punctate pattern, but the signal was strongly focused in perinuclear punctae and this relocalization seemed to be more apparent for *Loqs*-PA. Importantly, for both isoforms a strong colocalization with viral

dsRNA was observed, suggesting that Loqs relocates to viral replication organelles in infected cells (Fig. 5B).

We next investigated whether Loqs directly interacts with DENV RNA. To this end, we purified recombinant Loqs-PA and Loqs-PB as a fusion protein with maltose binding protein (Fig. S2A) and performed electrophoretic mobility shift assays (EMSA). As expected, Loqs-PA and Loqs-PB bound with high affinity (5-40 nM) to control dsRNA in gel mobility shift assays (Fig. S2B). We next incubated Loqs with *in vitro* transcribed RNA corresponding to the 5' untranslated region (UTR), the 3'UTR, as well as coding sequences (in the NS1 and NS5 genes) and complexes were resolved on native polyacrylamide gels. We found that both Loqs isoforms bound with high affinity (95-191 nM) to the DENV 3'UTR, whereas binding to the other RNAs was much less efficient (Fig. 5). Interestingly, multiple Loqs-3'UTR complexes were formed at higher Loqs concentrations, suggesting that Loqs may bind to multiple RNA structures in the 3' UTR. Altogether, we propose that DENV non-structural proteins recruit Loqs to viral replication organelles, where it interacts through its dsRNA-binding motifs with viral RNA to facilitate viral RNA replication.

DISCUSSION

Arboviruses replicate efficiently in their invertebrate vector as well as in their vertebrate hosts, and viral RNA and proteins thus interact with cellular proteins from these evolutionary diverse hosts. In this study, we used a proteomic approach to identify proteins interacting with DENV NS1 and NS5 in *Aedes* mosquitoes. Among the interactors, we identified the dsRNA binding protein Loquacious as a proviral factor for DENV and ZIKV that interacts with viral RNA at the 3' UTR. We propose that Loquacious is recruited to replication organelles to facilitate viral RNA replication. Loquacious is a cofactor of RNA silencing pathways [24] and our data thus suggest that DENV exploits proteins of an antiviral immune response for its own benefit. The human Loqs ortholog TARBP2 (also known as TRBP), a cofactor for HIV-1 replication that binds to the structured TAR RNA element [38], has been found to weakly interact with DENV NS1 [10], suggesting that the interaction may play a role in both mammals and mosquitoes. Like in *Drosophila* [25–27], there seems to be functional specialization among the three *Loqs* paralogs in *Aedes* mosquitoes. Loqs-PA was proposed to be essential for miRNA function, whereas R2D2 and Loqs-PB are involved in siRNA production, although their relative importance could not be fully resolved [24]. Later, it was found that *Aedes* encodes another paralog, *Loqs2*, the product of which interacts with both R2D2 and Loqs, and is essential for antiviral defence [23]. Interestingly, *Loqs2* has a more restricted expression than *R2D2* and *Loqs* and it is not expressed in the midgut, the site of initial replication of arboviruses in the mosquito, whereas other RNAi pathway genes, including *Loqs* and *R2D2* are. Three *Loqs* isoforms are annotated in the current *Ae. aegypti* reference genome (AaegL5), of which the isoform with only two dsRBD, *Loqs-RC*, is only lowly expressed and did not affect DENV replication in our experiments. We recovered one peptide specific to isoform Loqs-PA in the NS1 interactome and *Loqs-RA* silencing reduced DENV

replication. However, we expect Loqs-PB to be proviral as well, as it bound DENV RNA and localized to replication organelles to a similar extent as Loqs-PA.

As expected, Loqs interacted efficiently with dsRNA, but we found it to also interact with high affinity with single-stranded RNA corresponding to the DENV 3' UTR. The 3' UTR is extensively structured in diverse stem-loop structures, including pseudoknots (PK1 and PK2), dumbbell-like structures (DB1 and DB2), as well as a long 3' stem-loop (3' SL) [39,40]. The 3' SL is essential for viral RNA replication by mediating RNA-RNA interactions between the 5' and 3' ends of the genome. Specifically, interaction of a cyclization sequence upstream of the initiator AUG, called the upstream AUG region (5' UAR), with a complementary sequence in the stem of the 3' SL (3' UAR) and mediates genome circularization that is required for initiation of negative strand RNA synthesis [41,42]. It is likely that interaction of Loqs with the 3' UTR affects accessibility or stability of RNA structures or the transition between linear and circularized states of the DENV genome akin to the role of eukaryotic translation elongation factor (eIF1A) that interacts with the 3'SL to mediate flavivirus negative strand synthesis in mammals [43].

NS1 interacts with other viral non-structural proteins, is essential for early events in viral RNA replication and replication organelle formation, and colocalizes with viral dsRNA [12,44,45]. We identified Loqs as an interactor of NS1, expressed as part of a polyprotein with other non-structural proteins flanked by non-viral UTRs, suggesting that Loqs was recruited to NS1 via protein-protein interactions. In contrast, Loqs was not identified in the interactome of NS1 expressed individually in mosquito cells [32]. Given that NS1 is an ER resident protein that is secreted through the secretory pathway [46,47] and Loqs is a cytoplasmic protein, the interaction of Loqs with NS1 is likely to be an indirect one. We suggest that the interaction is mediated by other viral non-structural proteins interacting with NS1.

300

301 In addition to Loqs, we found multiple other interactors of NS1 and NS5 that merit further

302 investigation, including the putative pro- and antiviral factors identified in the functional screen.

303 Proviral E3 ubiquitin-protein ligase HYD (UBR5) was recently identified as a proviral host factor

304 of ZIKV in a capsid interactome in mosquitoes [48], and the human ortholog UBR5 was also found

305 to interact with DENV NS5 [13], suggesting HYD/UBR5 to be a broad cofactor for flaviviruses.

306 The independent pull-down of HYD with the capsid and NS5 proteins in, respectively, the

307 Gestuevo *et al.* study [13] and the present study suggests a prominent role of ubiquitin metabolism

308 in flavivirus replication in mosquitoes. The identification of several proteins associated with

309 ubiquitin metabolism (proteasome subunits $\beta 1$, $\beta 2$, $\alpha 5$ and $\alpha 6$, piccolo, mahjong and proviral

310 muskelin) in NS5 immunoprecipitations agrees with previous reports suggesting that NS5

311 functions as an adaptor for the ubiquitination system, as it interacts with several E3-ligases in

312 humans, including UBR4 to promote the degradation of STAT2 [13,49].

313 Among the potential antiviral factors from our screen are RNA binding proteins DIP1, an ortholog

314 of human ADAR proteins to which pro- and antiviral activities have been attributed [50,51], and

315 RPL12 (RPA12), which could modulate viral replication through its dsRNA cleavage and

316 transcriptional termination activities [52]. In addition, we found the ER retention protein RER1

317 involved in protein degradation via the proteasome [53] and the chaperone protein HSP60A [54]

318 to be antiviral factors, possibly related to a role of the stress response in antiviral immunity [55].

319 Yet, these observations need further investigation, especially since chaperone proteins, including

320 HSP60, were proposed as flavivirus proviral cofactors in mammals [56,57]. We found a subunit

321 of the OST complex, Ost Δ (RPN2), to be antiviral activity in mosquito cells, which is in stark

322 contrast to the proviral activity of the OST complex in mammals [10,58,59]. The OST complex

has previously been associated with the immune response [60,61] and could represent a host factor with different activities in vertebrate and invertebrate hosts. Viruses recruit host factors for viral replication and several dsRNA binding proteins have been shown to interact with the 3'UTR of flaviviruses [62,63]. Despite its role as a regulator of RNAi pathways central to antiviral immunity, our work establishes that the RNA binding protein Loqs is hijacked through protein-protein and protein-RNA interactions to promote flavivirus replication in mosquitoes. Our study provides novel insights into the mechanisms of replication of pathogenic flaviviruses and identifies Loqs as a potential target to develop strategies to block flavivirus transmission by vector mosquitoes.

METHODS

Cells and viruses

Aedes albopictus C6/36 (ECACC General Cell Collection, #89051705), U4.4 (kindly provided by Gorben Pijlman, Wageningen University, the Netherlands) and *Aedes aegypti* Aag2 (kindly provided by Raul Andino, University of California San Francisco) cells were maintained at 27°C in Leibovitz L15 medium (Gibco) supplemented with 10% heat inactivated fetal calf serum (FCS, PAA), 2% tryptose phosphate broth solution (Sigma), 1x MEM non-essential amino acids (Gibco), and 50 U/ml penicillin and 50 µg/ml streptomycin (Gibco).

DENV serotype 2 (DENV2, strain 16681) was provided by Beate Kümmerer (University of Bonn). ZIKV (strain H/PF/2013) was obtained from the European Virus Archive (EVAg, catalog number 001v-EVA1545). Virus stocks were prepared on C6/36 cells and titrated by end-point dilution on Vero FM cells. All viral infections were performed at an MOI of 0.1 in L15 medium without FCS and the medium was refreshed at 1 h post-infection with supplemented medium, containing 2% FCS.

Plasmids

DENV2 (strain NGC) non-structural genes were PCR amplified in three fragments from the pRepDVRluc plasmid [37] and all fragments were inserted into the PUB-MCS-2A-Puro plasmid [64] using In-Fusion cloning kit (Takara) to generate the PUB-NS(Ø) plasmid. PUB-NS(Ø) was subsequently mutated by site-directed mutagenesis to insert a cassette containing AgeI and SphI restriction sites. A 3xFLAG tag was then inserted by In-Fusion ligation of annealed oligos either at the N-terminus of NS1 to generate plasmid PUB-NS(1F) or at the C-terminus of NS5 to generate PUB-NS(5F). A PUB-GFP plasmid was generated by removing the Gateway cassette in the pUGW

vector [64], followed by the introduction of restriction sites for SphI, AvrII, AflIII and NheI included in primers. *Ae. aegypti* *Loqs-RA* and *Loqs-RB* sequences (AaegL5) were cloned from the plasmids PUB-HA-Loqs-PB and PUB-HA-Loqs-PA (AaeL3), kindly provided by Zach Adelman (Texas A&M University) [24]. Loqs isoforms were inserted using the In-Fusion cloning kit into the PUB-GFP plasmid to express recombinant proteins with a GFP tag at the C-terminus and generate plasmids PUB-Loqs-PA-GFP and PUB-Loqs-PB-GFP, or into the pMal-C2X (NEB) to express recombinant proteins with a maltose-binding protein (MBP) tag at the N-terminus and generate pMal-Loqs-PA and pMal-Loqs-PB.

Primers used for cloning are listed in Table S3.

Immunoprecipitation

For standard immunoprecipitation, C6/36 cells were seeded in a 60 mm culture dish and incubated for 24 h. Cells were then transfected with 10 mg of PUB-NS(Ø), PUB-NS(1F) or PUB-NS(5F) and 20 µl of X-tremeGENE HP (Roche) and medium was refreshed after 3 hours. At 24 h after transfection, cells were harvested and washed in ice-cold PBS using 5 min centrifugations at 900 x g. Cell pellets were resuspended in 400 µL lysis buffer (10 mM Tris/Cl pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40, 1x protease inhibitor cOmplete (Roche)). Samples were incubated with end-over-end rotation for 1 h at 4°C and followed by centrifugation at 18,000 x g for 30 min at 4°C. Total protein extracts were collected, diluted with 600 µL of dilution buffer (lysis buffer without NP-40) and incubated with 25 µL of pre-washed Anti-FLAG M2 Magnetic Beads (Sigma) on a rotor overnight at 4°C. The beads were washed three times in ice-cold dilution buffer, eluted in 50 µL of 2X SDS containing 5% β-mercaptoethanol at 95 °C for 10 min and analysed by western blot.

For mass spectrometry, immunoprecipitation was performed as described above, with the following modifications. C6/36 cells were seeded in 150 mm culture dishes (four dishes per condition) and transfected with 30 µg of plasmid and 60 µl of X-tremeGENE HP per dish. Cells were harvested at 24 h after transfection and washed in 5 mL of ice-cold PBS and cell pellets were resuspended in 2 mL of lysis buffer. Total protein extracts were diluted with 3 mL of dilution buffer and protein concentrations were estimated using the BCA Protein Assay (Pierce). 6 mg of total protein extract was incubated with 40 µL of Anti-FLAG M2 Agarose Beads (Sigma) and beads were washed a total of five times (three times in ice-cold dilution buffer and twice in ice-cold PBS), followed by on-bead trypsin digestion prior to MS analysis, as previously described [65]. For each condition, three biological replicates were used.

Western blotting

Protein samples were separated at 120 mV in 7.5% polyacrylamide gels for 90 min and transferred at 80 mA to PVDF membranes overnight at 4 °C using Bio-Rad systems. Proteins were labeled with primary antibodies diluted at 1:1000, mouse anti-FLAG M2 (Sigma, F1804) and rat anti-tubulin-α (MCA78G), and secondary antibodies diluted at 1:10,000, IRDye 800 goat anti-rat (Li-Cor, 926-32219) and IRDye 680RDye goat anti-mouse (Li-Cor, 926-32220). Western blots were imaged on the Odyssey CLx System (ThermoFischer).

Sample preparation and mass spectrometry

Samples were subjected to on-bead digestion [66], as follows: 50 µl of Elution buffer (EB: 2M urea, 100 mM Tris-pH 8.0 and 10 mM DTT) was added to each sample and incubated for 20 min at room temperature. Cysteines were alkylated using 50 mM iodoacetamide for 10 min, after which

0.25 µg of MS-grade trypsin (Promega) was added per sample. After 2 h incubation in a thermoshaker at room temperature, the supernatants were collected. Beads were washed once with 50 µl EB to collect as many peptides as possible, and this supernatant was combined with the first, after which 0.1 µg trypsin was added and samples were incubated overnight. The next day, samples were subjected to STAGE-tipping [67]. Tiny discs of C18 material were fixated in p200 tips. The C18 material was activated using methanol, and then thoroughly washed 1x with buffer B (80% acetonitrile, 0.1% TFA) and 2x with buffer A (0.1% TFA), after which the samples were loaded. Salts from the digestion buffer were washed away by an additional buffer A wash. Samples were eluted using buffer B for measurements.

Flag-immunoprecipitated samples were analyzed using reverse phase chromatography on an EASY-nLC1000 instrument coupled online to a Thermo Exploris 480 mass spectrometer. A 60 min gradient of buffer B (80% acetonitrile, 0.1% TFA) was applied to gradually release peptides from the C18 column into the mass spectrometer, which was ran at Top20 mode. A dynamic exclusion list was enabled for 30 proteins for 45 seconds after first occurrence. Only peptide ions with a charge between 2 and 6 were selected for fragmentation.

Mass spectrometry data analysis

The raw mass spectrometry data were analyzed using MaxQuant version 1.6.0.1 [68] and a database for *Ae. albopictus* (Aalbo_primary.1, RefSeq assembly: GCF_006496715.1) and DENV 16681 proteins). In addition to default settings, Deamidation (NQ) was used as a variable modification, and LFQ and iBAQ were enabled. Perseus [69] was used for filtering. Contaminants, reverse hits and hits with less than one peptide were removed. LFQ-values were subsequently log2-transformed and samples were divided into triplicates and filtered to have at least 3 valid

values in one group of replicates. The missing data were imputed using default settings. Students *t*-tests were performed for each of the baits compared to the control. R was used to visualize the data in volcano plots and a heatmap. When available, names of the closest ortholog from *D. melanogaster* as indicated in VectorBase were used to refer to mosquito proteins. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository [70] with the dataset identifier PXD031112. In the PRIDE accession, samples are referred to as “control”, “1_flagip” and “5_flagip” for NS(Ø), NS(1F) and NS(5F), respectively.

Immunofluorescence assay

C6/36 cells were seeded on coverslips in 24-well plates 24 h before introducing transgenes. Cells were transfected with 1 µg of Pub-NS(Ø), Pub-NS(1F) or Pub-NS(5F) using 1 µL of XtremeGENE HP (Roche) and incubated for 3 h before refreshing the medium. For infection experiments, Aag2 cells were infected 24 h after plating with DENV2 or mock for 72 h before transfection with Pub-Loqs-PA-GFP, Pub-Loqs-PB-GFP. At 24 h after transfection, cells were washed with PBS and fixed in 2% paraformaldehyde for 15 min at 4°C. Samples were further treated at room temperature and washed in PBS containing 0.05% Tween-20 between each of the following step. Cells were permeabilized with 0.1% Triton-X100 in PBS and treated with blocking buffer (2% BSA, 2% normal goat serum, 0.1% Triton, 0.05% Tween-20, 100 mM glycine in PBS) for 30 min. Samples were stained with primary mouse antibodies anti-FLAG M2 (Sigma, F1804) or anti-dsRNA J2 (Jena Bioscience, RNT-SCI-10010500), followed by secondary goat anti-mouse antibodies conjugated with Alexa Fluor 488 (ThermoFisher, A11001) or Alexa Fluor 594 (ThermoFisher, A11005). All antibodies were diluted 1:200 in blocking buffer. Nuclei were

stained with hoechst (5 µg/ml) for 15 min. Slides were mounted in Mowiol and stored at 4°C before imaging.

Confocal images were acquired using a Zeiss LSM900 microscope and analysed with Icy ICY Imaging [71]. Uncropped images are shown in Fig. S4.

Bioinformatic analysis

The interaction network for proteins of interest was predicted with STRING v10 [72], considering a medium confidence (0.4) and a false discovery rate stringency of 5%, using the following four sources: *text mining*, *experiments*, *databases* or *co-expression*. The network was visualized with Cytoscape [73] with thickness of the edges relative to the number of sources supporting the information. The genes enriched with NS1 and NS5 were separately analyzed with DAVID 6.8 [74] to identify enrichments for specific GO terms from the “biological process” (BP) category, using *p*-value < 0.1 as cutoff and containing at least 3 members per group. The complete GO term analysis is presented in Table S2.

Sequences of Loqs isoforms and orthologs were aligned using MUSCLE [75], alignments were curated with Gblocks [76] and a maximum likelihood phylogenetic tree was built with PhyML and aLRT [77] using phylogeny.fr [78]. The phylogenetic tree was then visualized on iTOL [79]. Percentage identity were determined with Mview [80]. Reference sequences used are listed in Table S4.

***In vitro* transcription of replicon, dsRNA and EMSA probes**

The pRepDV2Rluc plasmid [37] was linearized with XbaI and used as template for *in vitro* transcription of replicon RNA using the T7 RiboMAX Large Scale RNA Production System

(Promega) in the presence of Ribo m7G Cap Analog (Promega) at a cap analog to GTP ratio of 2.5. Replicon RNA was purified with the Rneasy Mini Kit (QIAGEN).

Templates for *in vitro* transcription of gene-specific 300-500 nt dsRNA and DENV2 EMSA probes were generated by PCR introducing a T7 promoter sequence or a universal tag at both 5' and 3' ends using the GoTaq Flexi DNA Polymerase (Promega). If present, the universal tag was then used in a second PCR to add T7 promoter sequences at both ends of the amplicon. T7 PCR products were used as a template for *in vitro* transcription by T7 RNA polymerase for 4 h at 37 °C. RNA for dsRNA was denatured at 95 °C for 10 min and gradually cooled to room temperature for annealing. Annealed dsRNA and EMSA probed were purified with the GenElute Mammalian Total RNA Miniprep Kit (Sigma) and quantified with the Nanodrop-1000 Spectrophotometer (ThermoFisher).

Primers used for *in vitro* transcription are listed in Table S3.

dsRNA-mediated gene silencing

Aag2 or U4.4 cells were plated in 48-well plates and transfected with 100 ng of dsRNA per well using 0.4 µL of X-tremeGENE HD (Roche). The medium was refreshed after 3 h and cells were incubated for 48 h. For infections, cells were transfected again with dsRNA as described above, incubated for 3 h, and then infected with DENV. Cells were harvested at the indicated time points for total RNA isolation using RNA-Solv (Omega Bio-tek). For replicon assays, Aag2 and U4.4 cells were transfected with 100 ng dsRNA and 250 ng RepDV2Rluc RNA per well using the TransIT-mRNA transfection kit (Mirus). As a positive control, cells transfected with RepDV2Rluc RNA in the absence of dsRNA were treated with 50 µM cycloheximide (CHX) at 1 h post-transfection. Proteins were harvested in passive lysis buffer 1x (Promega) at indicated times and

Renilla luciferase activity was measured using the Renilla-Glo Luciferase Assay system (Promega).

Reverse transcription and quantitative PCR

Transcript annotation according to the reference genome AegL5.2 was used to generate PCR primers. For RT-qPCR, 200-500 ng of total RNA were treated with DNaseI (Ambion) for 45 min at 37°C and then incubated with 2.5 mM EDTA for 10 min at 75°C. Total RNA was reverse transcribed using the Taqman reverse transcription kit (Applied Biosystems). Relative quantitative PCR analysis was performed using the GoTaq qPCR SYBR mastermix (Promega) on a LightCycler 480 instrument (Roche). Target gene expression levels were normalized to the expression of the housekeeping gene, *lysosomal aspartic protease* (LAP) for *Ae. aegypti* or *ribosomal protein L5* (RPL5) for *Ae. albopictus*, and fold changes were calculated using the using the 2^(-ΔΔCT) method [81].

Primers used for in vitro transcription are listed in Table S3.

Preparation of small RNA libraries

Small RNA deep sequencing libraries were generated using the NEBNext Small RNA Library Prep Set for Illumina (E7560, New England Biolabs), using 1 μg RNA as input. Libraries were prepared in accordance with the manufacturer's instructions and sequenced on an Illumina HiSeq4000 by the GenomEast Platform (Strasbourg, France).

Small RNA sequence analysis

The initial quality control was performed using FastQC and 3' adapters were trimmed using cutadapt [82]. Small RNA sequences in the size range of 21-23 bp were considered as siRNA and 25-32 bp considered as piRNA. Subsequently, reads were uniquely mapped to the corresponding virus genome, DENV2 (NCBI Reference Sequence: NC_001474.2) or ZIKV (GenBank: KJ776791.2), allowing a maximum 1 mismatch. Samtools were used to quantify piRNA mapped to the tapiR1 locus. To compare the vsiRNA changes upon *Loqs* silencing, uniquely mapped siRNA reads were normalized to piRNAs mapped to the tapiR1 locus [83] to account for differences in library size, and then normalized to DENV or ZIKV RNA copies based on RT-qPCR performed on the same RNA as was used for small RNA sequencing. Small RNA sequences in the size range of 19-25 nt were mapped to *Aedes* mature miRNA and pre-miRNA sequences using Bowtie [84], allowing a maximum of 1 mismatch. miRNA sequences and accession numbers were from the miRBase repository [85]. To compare total miRNA changes upon dsLoqs, miRNA reads were normalized to piRNA mapped to the tapiR1 locus or total small RNA library size. Samtools were used to quantify miRNA counts per sample [86]. DEseq2 was used for differential analysis of miRNA between samples [87].

Production and purification of recombinant protein

E. coli strain XL10 Gold were transformed with plasmids encoding MBP-Loqs-PA or MBP-Loqs-PB and cultured until midlog phase (OD₆₀₀ of 0.6). Expression of recombinant proteins was then induced with 1 mM isopropylβ-D-1-thiogalactopyranoside (IPTG) and cells were cultured overnight at 27 °C. Cells were pelleted (13,000 x g, 15 min) and resuspended in *E. coli* Lysis Buffer (PBS, 0.5% (w/v) Tween-20, 1 mM EDTA, 1x protease inhibitor cOmplete). Cells were subjected to three freeze (-80 °C) / thaw (37 °C) cycles and sonicated (Branson Sonifier 250, 10

seconds, 3 x 5 cycles) before clearing debris by centrifugation at 13,000 x g for 30 min. Recombinant proteins were affinity-purified using amylose resin using the manufacturer's protocol (NEB) and eluted with 20 mM maltose. Protein concentration was measured in eluate fractions by a Bradford assay (Bio-Rad) and the fractions with the highest concentration were transferred in a Slide-A-Lyzer dialysis cassette (Thermo Fisher) and dialyzed to buffer (20 mM Tris-HCl pH 7.4, 0.5 mM EDTA, 5 mM MgCl₂, 1 mM DTT, 140 mM NaCl, 2.7 mM KCl). Protein concentration was determined by a Bradford assay. Recombinant proteins were snap-frozen in liquid nitrogen and stored at -80°C in dialysis buffer with 30% glycerol.

Electrophoretic mobility shift assays (EMSA)

The synthesized probes were treated with DNaseI (Promega), dephosphorylated (Roche) and end-labeled using T4 polynucleotide kinase (NEB) with [γ -³²P] ATP (Perkin Elmer). Unincorporated nucleotides were removed with MicroSpin G-50 columns (Illustra). Samples were then heated for 5 min at 85°C, cooled to room temperature and incubated for 20 min in RNA folding buffer (111 mM HEPES, 6.7 mM MgCl₂, 111 mM NaCl). Purified proteins were diluted in dialysis buffer and incubated for 30 min at room temperature with 1-10 ng of the labeled RNA in binding buffer (5 mM HEPES, 25 mM KCl, 2 mM MgCl₂, 3.8% glycerol) in the presence of 0.625 mg/mL yeast tRNA (Sigma). The reactions were loaded on a 6% native acrylamide gel and run at 4 °C. The radioactive signal was quantified using a phosphor screen and a Typhoon FLA 7000 biomolecular imager. RNA_{total} and RNA_{free} were quantified using Fiji [88] to determine the fraction bound = 1 - (RNA_{free}/RNA_{total}). Binding isotherms were fitted using specific binding with Hill slope in GraphPad Prism7.

562 **Statistical analysis**

563 Graphical representation and statistical analyses were performed using GraphPad Prism7 software.

564 Differences were tested for statistical significance using unpaired two-tailed t-tests.

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LEGENDS

Figure 1. Interactome of NS1 and NS5 in *Aedes* mosquito cells

A. Schematic representation of constructs used to express DENV non-structural proteins in mosquito cells. Constructs were generated with a 3xFLAG tag at the N-terminus of NS1 (NS(1F)), the C-terminus of NS5 (NS(5F)). A construct without a tag (NS(ØF)) was included as a control. 2A, self-cleaving peptide from foot-and-mouth disease virus; PAC, Puromycin N-acetyltransferase. **B.** Western blot of input and FLAG immunoprecipitation samples of C6/36 cells expressing NS(ØF), NS(1F) and NS(5F), stained with FLAG and α -tubulin (Tub) antibodies. See uncropped gel in Fig. S3. **C.** Confocal microscopy image of FLAG-tagged NS1 and NS5 in C6/36 cells at 24 h after transgene transfection. Cells were stained with anti-FLAG M2 antibody (green) and Hoechst to stain nuclei (blue). **D.** Volcano plot of proteins interacting with 3xFLAG-tagged NS1 (top) or NS5 (bottom) in C6/36 cell lysates as determined by label-free quantitative mass spectrometry. The X-axis shows the log₂ fold enrichment over untagged NS(ØF) (control), and the Y-axis shows -log₁₀(*p*-value). Proteins in the top right are identified as significantly enriched proteins. Colored dots indicate proteins of interest. Each condition was performed in triplicate. **E.** Heatmap of the relative enrichment (red) and depletion (blue) of proteins in each sample, based on row-mean subtraction and K-means clustering. Statistical enrichment in the volcano plot analysis is indicated. Ost48 and Stt3A were included, although not significantly enriched. ‘Low’ indicates an enrichment between 2 and 2.5-fold, below the threshold of the volcano plot. **D-E** Colored dots indicate proteins of interest.

Figure 2. Characterization of DENV NS1 and NS5 interactomes

A. Venn diagram of highlighting 85 interactors of NS1 and/or NS5 identified by mass spectrometry. **B.** GO term analyses of interactors of NS1 (top panel) and NS5 (lower panel). Enrichment of biological processes was based on *D. melanogaster* ortholog annotation. Numbers indicate $-\log_{10} p$ values. See complete list of GO terms in Table S2. **C.** Functional STRING networks based on *D. melanogaster* ortholog annotation. Hits were classified and colored according to their enrichment in ≥ 2 out of 3 NS(1F) or NS(5F) samples in the heatmap of Fig. 1E. Node sizes represent the fold enrichment in NS(1F) or NS(5F) immunoprecipitation, keeping the highest value if the interactor was present in both. Edges are representative of the number of sources (solid or dashed) and the confidence (color) supporting the interaction as defined by STRING. Font indicates hits confirmed (bold) or not (italic) as modulators of DENV in the functional screening (Fig. 3).

Figure 3. Functional screen identifies Loqs as a DENV proviral host factor in *Aedes* mosquitoes

A. Schematic outline of the functional RNAi screen. **B-C.** Relative quantification of target gene expression (top panel) and DENV RNA levels (lower panel) in U4.4 cells upon silencing of the indicated genes. Selected genes from the initial screen (B) were tested in an independent validation screen (C) using dsRNA targeting a different region of the gene. Expression was quantified by RT-qPCR, normalized to the house-keeping gene *ribosomal protein L5*, and expressed relative to expression in cells treated with dsRNA targeting firefly luciferase (CTRL). *Ago2* was used as a positive control. Data represent means and standard deviation of three replicates. Color coding represents classification of hits based on gene knockdown efficiency, phenotype, and consistency between screens. Light grey, inefficient knockdown (< 0.5 -fold); dark grey, no phenotype despite

efficient knockdown (> 0.5 -fold); dark red, strong hit with efficient knockdown, and DENV RNA levels < 0.66 -fold or > 1.5 -fold in both dsRNA sets; light red, weak hits for which one of the criteria was not met. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

Figure 4. Loquacious is an essential co-factor for flavivirus replication

A. Structure of *Loquacious* splice variants in *Ae. aegypti* (DSRM, dsRNA-binding motif) and maximum likelihood phylogenetic tree based on the protein sequence of Loqs-PA and its orthologs and paralogs. ce, *Caenorhabditis elegans*; dm, *Drosophila melanogaster*; h, *Homo sapiens*; aae, *Ae. aegypti*; aal, *Ae. albopictus*. Percentages indicates the identity between protein sequences compared to *Ae. aegypti* Loqs-PA. Branch lengths are proportional to the number of substitutions per site. *Loqs* transcript annotation is according to the reference genome AaegL5, which differs from the annotation used in [24]. **B.** Loqs peptides identified by mass spectrometry in NS1 immunoprecipitations. The peptide unique to Loqs-RB is indicated in blue; other peptides are shared between *Loqs* isoforms. **C.** Loqs-RA and Loqs-RB-specific amplicons from PCR using primers spanning exon 5 on cDNA from Aag2 cells. **D.** PCR amplification of *Loqs* splice variants with various set of primers on cDNA or genomic DNA isolated from Aag2 cells. Numbers indicate expected sizes. **E.** Relative quantification of DENV RNA at 72 h infection of Aag2 cells in which siRNA and miRNA pathway genes were silenced. **F.** Relative quantification of DENV RNA (top panel) or ZIKV RNA (lower panel) at the indicated time after infection in *Ago2* or *Loqs*-depleted Aag2 cells. **G.** *Renilla* luciferase activity in Aag2 cells (left) and U4.4 cells (right) transfected with DENV2 subgenomic replicon RNA and dsRNA targeting *Loqs* (dsLoqs) or firefly luciferase (dsGL3) as a control. Luciferase activity was assessed at the indicated time points and normalized to dsGL3-treated cells at 3 h after transfection. Cells treated with cycloheximide (CHX) were

included as control. **H-I.** Relative quantification of DENV (H) and ZIKV (I) RNA (left) and vsiRNA (right) 48h after infection at a MOI of 0.1 in *Loqs*-depleted or control (GL3 dsRNA treated) Aag2 cells. vsiRNA were normalized to cellular tapiR1 piRNA and viral RNA. **E-F, H-I.** Viral RNA levels were quantified by RT-qPCR, normalized to the housekeeping gene *lysosomal aspartic protease* and expressed relative to the expression in cells treated using dsRNA targeting luciferase (GL3) as negative control. See relative knockdown-target quantification in Supplementary Fig. S2. Bars represent mean and standard deviation from at least three biological replicates: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Figure 5. Loquacious colocalizes with DENV replication organelles

A. Confocal microscopy images of mock or DENV infected Aag2 cells. Cells were transfected at 72 h post infection with plasmids encoding the indicated transgenes or mock transfected (Ø) and processed for microscopy 24 h later. Cells were stained with anti-dsRNA J2 antibody (red) and Hoechst to stain nuclei (blue). Scale bar corresponds to 5 µm and is the same for all panels. In mock infected cells, a nuclear background signal is detectable that is distinct from the cytoplasmic viral dsRNA signal in DENV infected cells. **B.** Three-dimensional visualizations of two DENV infected Aag2 cells transfected with *Loqs*-PA-GFP indicated with a dashed square in panel A. The dsRNA and GFP signals were optimized for visualization in the three-dimensional projection. The grid is scaled with 2 µm xyz units.

Figure 6. Loquacious interacts directly with DENV 3'UTR

A. Electrophoretic mobility assay of *Ae. aegypti* *Loqs*-PA (left panels) and *Loqs*-PB (right panels) with the indicated probes corresponding to the DENV2 5' UTR, NS1, NS5 and 3' UTR sequences.

978 Top panel indicates a schematic representation of the position of the probes on the DENV genome
 979 (not to scale). Probes were incubated with 2-fold dilutions of recombinant MBP-Loqs and
 980 complexes were resolved on native polyacrylamide gels. The images of the free probe and the
 981 Loqs-RNA complex for the 5' UTR are cropped from the same gel. **B.** Quantification of (A) with
 982 dissociation constants for the indicated probes.

Supplementary data legends

Figure S1. Expression of Loquacious isoforms in *Ae. aegypti* mosquitoes and depleted cells.

A. Density plot of *Loqs* RNA seq reads in *Ae. aegypti*. Each line represents a unique sequence library from three separate VectorBase datasets: DS_dcde6b4ec9, DS_24f2db6f66 and DS_ded344cb5e [89–91]. **B–D.** Relative quantification of targeted gene mRNA expression from Fig. 3E (S1B), 3F (S1C) and 3H–I (S1D). Gene expression was normalized to the housekeeping gene *lysosomal aspartic protease* and expressed relative to expression in cells treated with control dsRNA targeting luciferase (GL3, dashed horizontal lines). **E.** Total miRNAs at 48 h after infection at a MOI of 0.1 in *Loqs*-depleted or control (GL3 luciferase dsRNA treated) Aag2 cells. Total miRNA reads were normalized to tapiR1. **F.** Correlation of log₂-transformed read per million levels of known *Ae. aegypti* miRNAs in GL3 (x-axis) and *Loqs* (y-axis) dsRNA treated Aag2 cells infected with DENV (left panel) or ZIKV (right panel). The sum of raw read counts of miRNAs across three small RNA library replicates were calculated via Samtools. Highlighted are miRNAs with > 2-fold differential expression with an adjusted p-value < 0.05 using DEseq2.

Figure S2. Purification of recombinant Loqs-PA, -PB and interaction with dsRNA

A. Coomassie blue stained polyacrylamide gel containing lysates from the indicated purification steps of *Ae. aegypti* MBP-tagged Loqs-PA and Loqs-PB. **B.** Electrophoretic mobility assay of *Ae. aegypti* Loqs-PA (left panel) and Loqs-PB (right panel) with a 117 bp dsRNA corresponding to the firefly luciferase sequence. Probes were incubated with 5-fold dilutions of recombinant MBP-Loqs and complexes were resolved on native polyacrylamide gels. **C.** Quantification of (B) with dissociation constants for the indicated probes.

1006

1007 **Figure S3. Uncropped gel images**

1008 **A.** Uncropped images of the western blots from Fig. 1A. **B.** Uncropped images of the EMSAs from
1009 Fig. 5A.

1010

1011 **Figure S4. Uncropped IFA images**

1012 Uncropped images of IFAs from Fig. 4.

1013

1014 **Table S1. Mass spectrometry hit summary**

1015 List of proteins enriched at least 2.5-fold in NS1 or NS5 immunoprecipitations with their $-\log_{10}(p$ -
1016 value) and $\log_2(\text{fold change})$ values plotted on the volcano plot (Fig. 1D), the LFQ after row-mean
1017 subtraction plotted on the heatmap (Fig. 1E) as well as protein and gene identifiers in *Ae.*
1018 *albopictus* and references to orthologs in *Ae. aegypti*, *D. melanogaster* and *H. sapiens*. Individual
1019 values plotted on the heatmap were labeled when below (blue) or above (red) the row average.
1020 Proteins associated with both NS1 and NS5 are highlighted in yellow. Hits included in the
1021 STRING network (Fig. 2) and GO terms are indicated. Ost48 and Stt3A were included after
1022 literature review.

1023

1024 **Table S2. GO term analysis data**

1025 Complete GO term analysis of Fig. 2A, including GO terms for cellular component (CC) and
1026 molecular function (MF).

1027

1028 **Table S3. List of primers used for cloning, dsRNA production, qPCR, PCR and EMSA**
 1029 **probes**

1030

1031 **Table S4. Reference sequences**

1032 List of protein sequences used as reference for sequence alignment illustrated in Fig. 4A.

1033

Fig. 1

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Fig. 2

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Fig. 3

Fig. 4

Fig. 5

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Fig. 6