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Dengue subgenomic RNA binds TRIM25 to inhibit interferon expression for epidemiological fitness

Gayathri Manokaran[1,2,3], **Esteban Finol**[1,3,4], **Chunling Wang**[5], **Jayantha Gunaratne**[3,6], **Justin Bahl**[7], **Eugenia Z. Ong**[1], **Hwee Cheng Tan**[1], **October M. Sessions**[1], **Alex M. Ward**[1], **Duane J. Gubler**[1], **Eva Harris**[5], **Mariano A. Garcia-Blanco**[1,8,9], and **Eng Eong Ooi**[1,3,10,11,*]

[1]Program in Emerging Infectious Diseases, Duke–National University of Singapore Graduate Medical School, Singapore. [2]Defence Medical and Environmental Research Institute, DSO National Laboratories, Singapore. [3]Yong Loo Lin School of Medicine, National University of Singapore, Singapore. [4]Swiss Tropical and Public Health Institute, Universität Basel, Switzerland. [5]Division of Infectious Diseases and Vaccinology, School of Public Health, University of California, Berkeley, CA, USA. [6]Institute of Molecular and Cell Biology, Singapore. [7]Center for Infectious Diseases, The University of Texas School of Public Health, Houston, TX, USA. [8]Department of Biochemistry and Molecular Biology, The University of Texas Medical Branch, Galveston, TX, USA. [9]Department of Molecular Genetics and Microbiology and Center for RNA Biology, Duke University, Durham, NC, USA. [10]Saw Swee Hock School of Public Health, National University of Singapore, Singapore. [11]Singapore–Massachusetts Institute of Technology Alliance in Research and Technology Infectious Disease Interdisciplinary Research Group, Singapore.

Abstract

The global spread of dengue virus (DENV) infections has increased viral genetic diversity, some of which appears associated with greater epidemic potential. The mechanisms governing viral fitness in epidemiological settings, however, remain poorly defined. We identified a determinant of fitness in a foreign dominant (PR-2B) DENV serotype 2 (DENV-2) clade, which emerged during the 1994 epidemic in Puerto Rico and replaced an endemic (PR-1) DENV-2 clade. The PR-2B DENV-2 produced increased levels of subgenomic flavivirus RNA (sfRNA) relative to genomic RNA during replication. PR-2B sfRNA showed sequence-dependent binding to and prevention of tripartite motif 25 (TRIM25) deubiquitylation, which is critical for sustained and amplified retinoic acid–inducible gene 1 (RIG-I)–induced type I interferon expression. Our findings demonstrate a distinctive viral RNA–host protein interaction to evade the innate immune response for increased epidemiological fitness.

[*]**Corresponding author. engeong.ooi@duke-nus.edu.sg**.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Figs. S1 to S13

Table S1

References (*28-40*)

Dengue outbreaks emerge when DENVs are introduced into populations with low herd immunity. However, several outbreaks have ensued when new DENV strains emerged and displaced endemic strains, albeit of the same serotype and genotype (1–6), suggesting that genetic variation altered viral fitness in epidemiological settings.

To gain insights into how DENV genome variation affects its epidemiological phenotype, we examined a DENV-2 clade replacement event that coincided with an epidemic of severe dengue in Puerto Rico in 1994 (1, 7, 8). Phylogenetic analysis of the complete coding sequences of DENV-2 isolated from Puerto Rico and neighboring countries showed that three distinct clades within the Asian/American genotype circulated in 1994 (fig. S1), concordant with previous findings (1). Clade PR-1 was prevalent from 1986 to 1995. Clade PR-2 contained viruses in two subclades, both first isolated in 1994: PR-2A persisted at low levels until 1998, whereas PR-2B spread and replaced PR-1 to become the dominant DENV-2 in Puerto Rico from 1995 to 2007. Immune escape is an unlikely explanation for PR-2B emergence, as both PR-1 and PR-2B belong to the Asian/American genotype. Furthermore, prM and E amino acid sequence analysis showed weak bootstrap values (fig. S2), indicating low levels of antigenic differences between the clades. Instead, PR-2B emergence and subsequent replacement of PR-1 viruses could be due to differences in epidemiological fitness.

To identify the genomic variations that segregated these DENVs into different clades and modulated epidemiological fitness, we applied the Shimodaira-Hasegawa (SH) test (9) that compares the topology of region-specific with full-genome trees. This approach identified NS1, NS3, NS5, and the 3′ untranslated region (3′UTR) sequence variations as potentially critical in segregating DENVs into PR-1 and PR-2B (figs. S3 to S5).

NS1, NS3, and NS5 are DENV replication complex components that also regulate the innate immune response (10). Less is known about the 3′UTR. During flaviviral replication, the cellular 5′-to-3′ exoribonuclease digests uncapped genomes but stalls at pseudoknot RNA structures in the 3′UTR. The resultant 0.3- to 0.5-kb subgenomic flavivirus RNA (sfRNA) regulates pathogenicity in both mammalian and mosquito cells, likely through mechanisms and structural elements within its noncoding RNA that remain to be fully defined (11–15). Recently, we showed that sfRNA could interact with proteins to inhibit translation of interferon-stimulated genes (16). Because two substitutions identified in the PR-2B 3′UTR were located where pseudoknots could form (fig. S6) (17–19), we tested for a possible role for sfRNA in modulating fitness in our viruses.

To measure viral fitness in vitro, we performed DENV infection assays in human hepatoma (HuH-7) cells. We expected PR-2B DENVs to replicate more efficiently than PR-1 viruses. Paradoxically, however, more viral progeny (Fig. 1A) and genomic RNA (gRNA) (Fig. 1B) were produced by PR-1 than PR-2B viruses at 24 hours postinfection (hpi). The difference in sfRNA levels between PR-1 and PR-2B viruses was smaller, although statistically significant (Fig. 1C), resulting in higher sfRNA:gRNA ratios in PR-2B as compared to PR-1 viruses (Fig. 1D). While the absolute values of the sfRNA:gRNA ratios should be interpreted with caution, our data clearly show that these ratios are markedly higher for the epidemic PR-2B viruses.

Increased production of sfRNA relative to gRNA in an epidemic clade of viruses is not specific to the PR-2B viruses. In Nicaragua, NI-2B DENV-2 emerged abruptly in 2005, displaced the endemic NI-1 DENV-2, and caused increased rates of severe disease across several epidemic seasons (5). SH test on these Asian/American genotype DENV-2 isolates similarly identified variations in NS1, NS5, and 3′UTR as important in segregating these viruses into NI-1 and NI-2B clades (fig. S7 and S8). In this case, two compensatory 3′UTR substitutions in NI-2B may produce energetically more stable secondary RNA structures (fig. S9). Further analysis indicated three- to fourfold higher sfRNA:gRNA ratios upon NI-2B as compared to NI-1 DENV-2 infection (Fig. 1E). Collectively, these data suggest that increased sfRNA production and possibly the sequence or structure of sfRNA may contribute to DENV-2 fitness in distinct epidemiological settings.

To understand how sfRNA production and/or sequence could lead to greater epidemiological fitness, we compared the replication kinetics of selected PR-1 and PR-2B viruses and their effect on interferon expression. Although significant differences in plaque titers and gRNA levels exist at 24 hpi, PR-2B viruses grew at a faster rate than PR-1 such that no difference in either parameter was observed at 96 hpi (Fig. 2, A and B). In addition, interferon-beta (IFN-b) expression was consistently lower in PR-2B– than in PR-1–infected cells, despite diminished differences in DENV gRNA levels at later time points (Fig. 2, C to F). This suggests that higher sfRNA:gRNA ratios produced during PR-2B infection suppressed IFN-β induction. Indeed, silencing interferon regulatory factor–3 (IRF-3), a transcription factor for IFN-β, increased PR-1 but not PR-2B replication (Fig. 2G).

The trends observed in infection of HuH-7 cells were recapitulated in primary monocytes, which are target cells for DENV in humans. Antibody-enhanced infection, which is associated with greater risk of severe dengue, produced less gRNA, greater sfRNA:gRNA ratios, and reduced IFN-β expression at 24 hpi when PR-2B instead of PR-1 viruses were used (Fig. 2, H to J). Likewise, at 72 hpi, differences between the plaque titers of PR-1 and PR-2B isolates were reduced or even reversed (Fig. 2K).

Given the observed association between increased sfRNA:gRNA ratios and reduced IFN-β expression, we next examined if the three nucleotide substitutions in the 3′UTR could functionally account for the increased sfRNA:gRNA ratios. To eliminate any contributions from NS1, NS3, and NS5, we performed mutagenesis studies on a standardized genomic backbone using a previously characterized DENV-2 replicon reporter, pDENrep-FH (20). At 72 hours after electroporation, replicons with PR-2B residues produced less gRNA but higher sfRNA:gRNA ratios compared to that bearing PR-1 residues (Fig. 3A). Likewise, luciferase signals were lower in replicons with PR-2B instead of PR-1 residues (Fig. 3B), indicating that substitutions in these three nucleotide positions can account for the observed differences in PR-1 and PR-2B sfRNA:gRNA ratios.

To investigate whether sfRNA plays a functional role in modulating IFN-β expression, we co-electroporated in vitro–transcribed sfRNA from PR-1 or PR-2B or a size-matched RNA control with pDENrep-FH into HuH-7 cells. As compared to nuclear factor κB (NF-κB) expression, where no difference was seen, IFN-β expression was significantly reduced with PR-2B but not PR-1 sfRNA, at 96 hours after electroporation (Fig. 3C). Correspondingly,

pDENrep-FH replication was significantly increased when co-electroporated with PR-2B sfRNA compared to either PR-1 sfRNA or control RNA (Fig. 3D). Furthermore, sfRNA but not size-matched control RNA inhibited polyinosinic-polycytidylic acid (polyIC)–induced IFN-β expression, with a greater difference observed for PR-2B than PR-1 sfRNA (fig. S10 and Fig. 3E). Collectively, these data indicate that both the amount and sequence of sfRNA are important in attenuating type I interferon expression for increased DENV replication.

The sequence specificity observed suggests that IFN-β suppression is likely to be due to specific sfRNA-protein interactions (21). Using stable isotope labeling by amino acids in cell culture coupled with quantitative mass spectrometry (SILAC-qMS), we detected 198 proteins that were enriched (at least 1.5-fold change) with DENV 3′UTR compared to control RNA (table S1 and fig. S11, A and B) (22). Notably, tripartite motif containing 25 (TRIM25) and mitochondrial antiviral signaling (MAVS) proteins (fig. S11C), which are known retinoic acid–inducible gene 1 (RIG-I) signaling intermediates for type I interferon expression (23–25), were significantly enriched with PR-2B compared to PR-1 3′UTR.

To validate our SILAC-qMS findings, we immunoprecipitated TRIM25 and MAVS separately from DENV-2–infected cells and quantified the protein-bound sfRNA and gRNA. Significantly higher sfRNA than gRNA levels were detected with TRIM25 immunoprecipitation as compared to isotype immunoglobulin G (IgG) control, with the difference being more pronounced when PR-2B DENV was used (Fig. 4A). Conversely, neither sfRNA nor gRNA was enriched with MAVS immunoprecipitation (fig. S12). In addition, incubating PR-2B sfRNA with TRIM25 yielded more RNA-protein complex than PR-1 sfRNA as measured on an electrophoretic mobility shift assay (EMSA) (Fig. 4B), indicating sequence-specific differences in TRIM25 binding.

Besides being an RNA-binding protein (26), TRIM25 is an E3 ligase that, upon deubiquitylation by ubiquitin-specific peptidase 15 (USP15) (27), polyubiquitylates RIG-I for sustained and amplified signal transduction (24). To examine if sfRNA interferes with these processes, we immunoprecipitated TRIM25 from DENV-2–infected cell lysates and probed for ubiquitin. Results showed a band identical in size to TRIM25 in infected cell lysates (Fig. 4C), although the intensity of the ubiquitylated TRIM25 band is stronger with PR-2B compared to PR-1 DENV (Fig. 4D). Furthermore, RIG-IimmunoprecipitationfromsfRNA-transfected cell lysates followed by TRIM25 immunoblotting showed that sfRNA did not prevent TRIM25 from binding RIG-I (Fig. 4E). Instead, higher levels of ubiquitylated TRIM25 were coimmunoprecipitated with RIG-I upon transfection of PR-2B relative to PR-1 sfRNA (Fig. 4E). Consistently, silencing TRIM25 did not augment PR-2B DENV-2 replication, whereas PR-1 DENV-2 replication was significantly increased (Fig. 4F). These findings collectively indicate that PR-2B sfRNA binds TRIM25 more efficiently to interfere with its deubiquitylation, thus preventing amplified and sustained RIG-I signaling for type I IFN induction.

Our report provides a mechanistic explanation for increased DENV fitness in an epidemiological setting. It adds to the general theme by which *Flaviviruses* use their abundant noncoding RNA to bind and inactivate RNA-binding proteins critical for innate immunity (21). TRIM25 is also targeted by influenza virus to inhibit RIG-I signaling,

although in this instance, inhibition is mediated by NS1 protein binding to TRIM25 (23). Reducing sustained and amplified IFN expression could thus be important to many viruses, as IFN renders uninfected cells resistant to viral infection.

Based on our findings, we propose a model to explain the 1994 dengue outbreak in Puerto Rico (fig. S13). The high sfRNA:gRNA ratios produced by PR-2B viruses during early phases of the infection constitute a “one-two punch” against host response; greater levels of sfRNA inhibit TRIM25 in a sequence-dependent manner, whereas reduced gRNA results in lower stimulation of RIG-I/melanoma differentiation–associated protein 5 (MDA5) RNA sensors. Reduced IFN responses in the early stages of infection would ensure availability of susceptible cells for viral spread in a human host to reach viremia levels sufficient for further mosquito-borne transmission.

In conclusion, our study provides unique molecular insights into the epidemiological fitness of DENV. It also suggests that by combining epidemiologic studies with molecular investigations, viral phylogenetic information can be informative not only with respect to virus evolution but also as a predictor of its epidemic potential.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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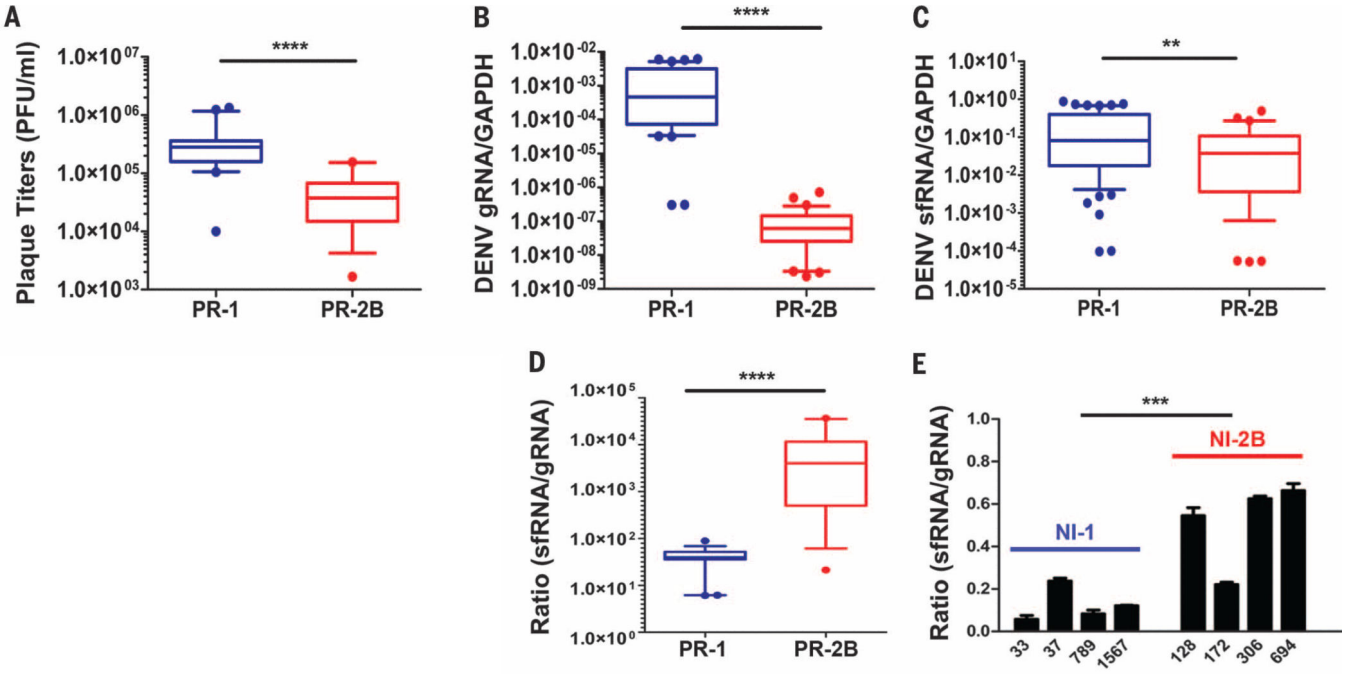

Fig. 1. sfRNA is a critical determinant of the epidemic potential of DENV-2

(**A**) Plaque titers of PR-1 and PR-2B DENV-2 viruses at 24 hpi in HuH-7 cells infected at a multiplicity of infection (MOI) of 0.1. PFU, plaque-forming units. (**B**) PR-1 and PR-2B gRNA in infected HuH-7 cells (MOI = 0.1), 24 hpi. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (**C**) PR-1 and PR-2B sfRNA in infected HuH-7 cells (MOI = 0.1), 24 hpi. (**D**) Ratio of sfRNA:gRNA in HuH-7 cells infected with PR-1 and PR-2B viruses at MOI 0.1, 24 hpi. (**E**) Quantification of sfRNA:gRNA ratios in HuH-7 cells at 48 hpi with clinical isolates from Nicaragua. Numbers below the bars refer to the names of the viral isolates. Data are expressed as mean ± SD from three independent experiments. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$, as determined by *t* test.

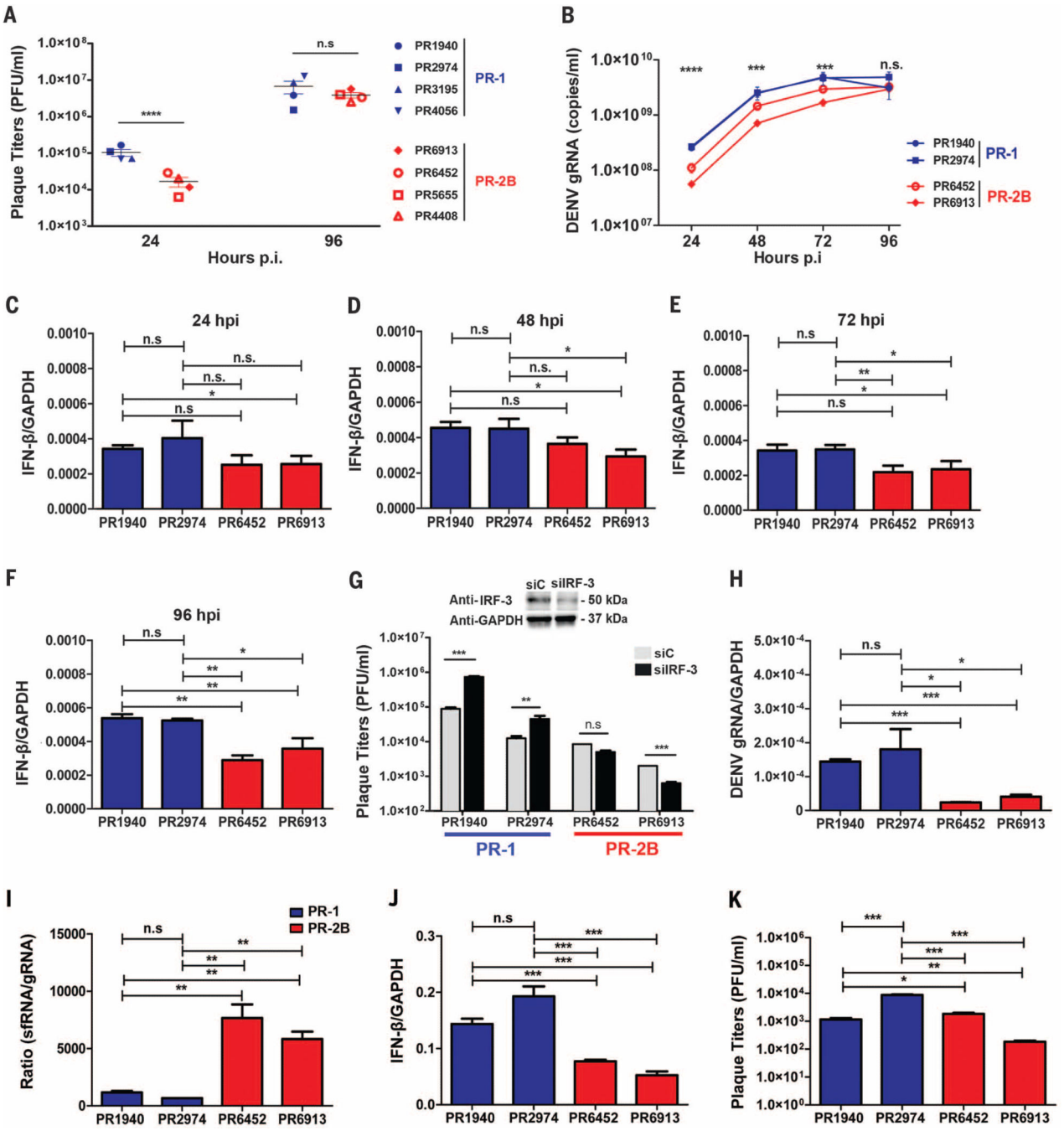

Fig. 2. PR-2B DENV-2 replication is associated with reduced expression of IFN-β relative to PR-1 DENV-2

(**A**) Plaque titers after infection of HuH-7 cells with selected viruses from each PR clade at 24 and 96 hpi. (**B**) DENV-2 gRNA levels in infected HuH-7 cells after infection (MOI = 0.5) with selected viruses from each clade at various time points postinfection. (**C** to **F**) Expression levels of IFN-β in infected HuH-7 cells at 24 hpi (C), 48 hpi (D), 72 hpi (E), and 96 hpi (F). PR-1: blue bars; PR-2B: red bars. (**G**) Plaque titers of supernatant quantified at 24 hpi with representative viruses from each clade, following knockdown of IRF-3 in HuH-7 cells by small interfering RNA (siRNA). siC: control RNA; siIRF-3: siRNA against IRF3.

(**H**) Quantification of DENV-2 gRNA in infected primary monocytes at 24 hpi. Infection was performed with selected PR-1 and PR-2B DENV-2 viruses opsonized with enhancing concentrations of humanized 3H5 monoclonal antibody against DENV-2 E protein. (**I**) Quantification of sfRNA:gRNA ratios in primary monocytes, 24 hpi. (**J**) IFN-β expression in primary monocytes at 24 hpi. (**K**) Plaque titers from supernatant of infected primary monocytes at 72 hpi. Data are expressed as mean ± SD from three independent experiments. n.s.: not significant; *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$, as determined by *t* test.

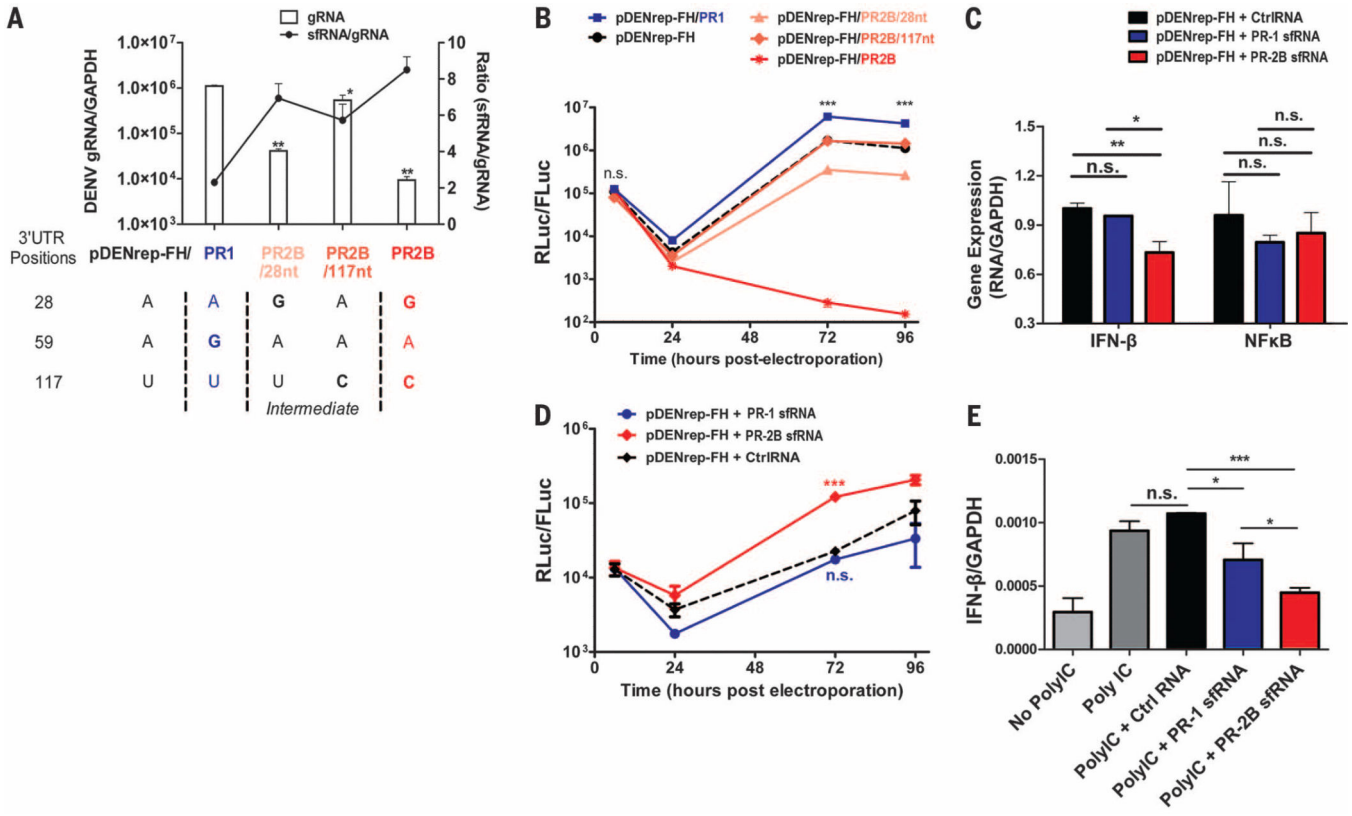

Fig. 3. sfRNA attenuates type I interferon antiviral response, resulting in increased viral replication

(**A**) Quantification of gRNA levels and sfRNA: gRNA ratios in HuH-7 cells, 72 hours after electroporation with replicons bearing nucleotide residues representative of PR-1 or PR-2B DENV-2. Bars show the gRNA levels (left axis), and lines show sfRNA:gRNA ratios (right axis). (**B**) Luciferase reporter activity for the original replicon (pDENrep-FH) and mutated replicons at various time points after electroporation. Determination of *firefly luciferase* (FLuc) levels served as internal normalization controls. (**C**) Quantification of IFN-β and NF-κB expression using quantitative reverse transcription–polymerase chain reaction (QRT-PCR) at 96 hours after electroporation and (**D**) luciferase reporter activity following co-electroporation of pDENrep-FH with control RNA or sfRNA from PR1 or PR2B. FLuc levels served as internal normalization controls. (**E**) Quantification of IFN-β expression in HuH-7 cells transfected with polyIC only, polyIC and size-matched control RNA, or sfRNA from PR-1 or PR-2B, by QRT-PCR at 24 hours after transfection. Data are expressed as mean ± SD from three independent experiments. n.s.: not significant; $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, as determined by *t* test.

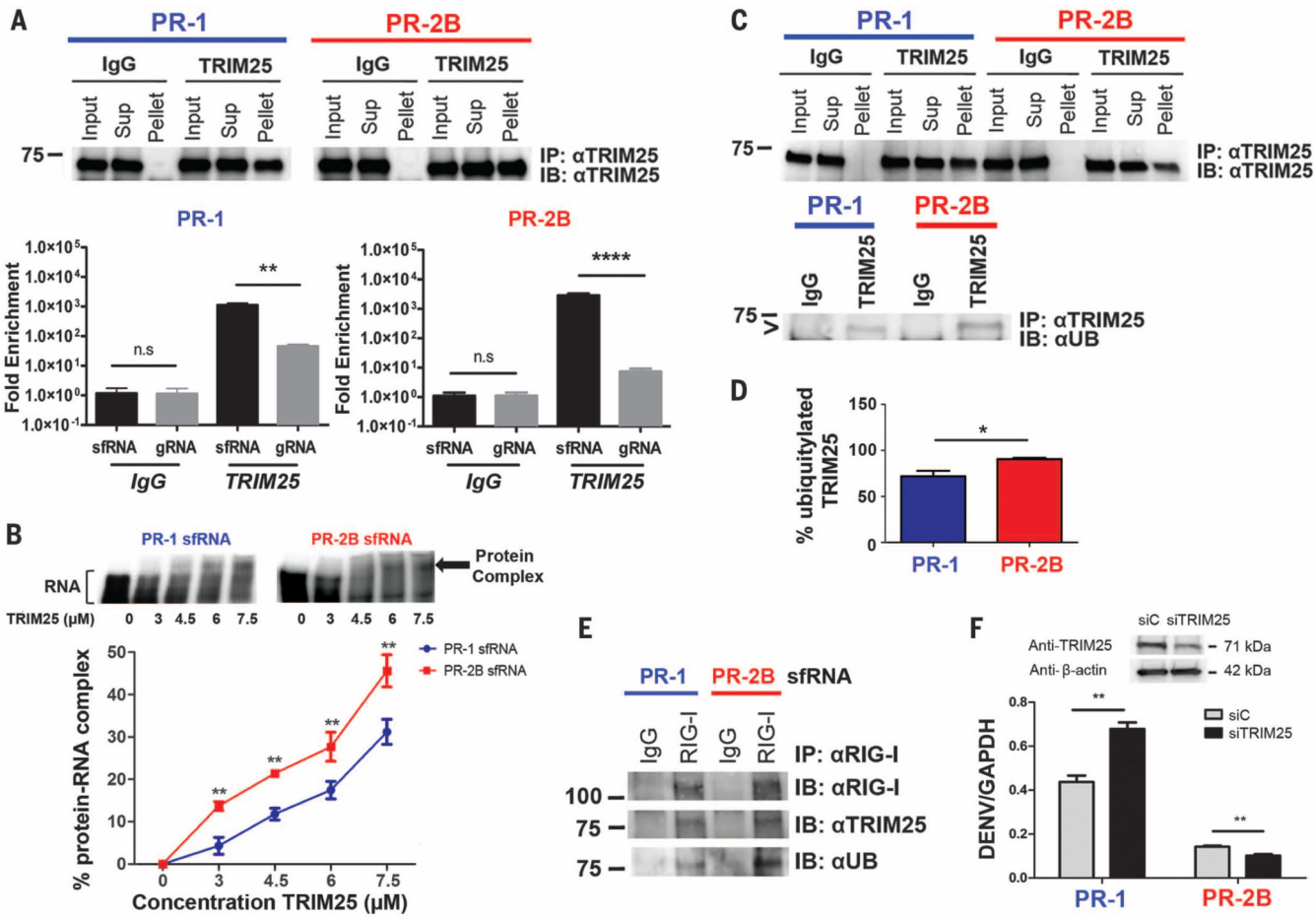

Fig. 4. sfRNA binds TRIM25 to inhibit TRIM25 deubiquitylation

(**A**) RNA-immunoprecipitation (RIP) was performed on HuH-7 cells at 24 hpi with one virus from each clade (MOI = 5). Infected cells were subjected to TRIM25 or IgG control pull-down followed by immunoblotting with TRIM25 to confirm efficacy of pull-down. Input: cell lysate before RIP; Sup: supernatant after incubation of antibody-coated beads with cell lysate; Pellet: final product after washing. Bar graphs show fold enrichment of sfRNA and gRNA upon pull-down with TRIM25, as measured by QRT-PCR. (**B**) EMSA of synthetic PR-1 and PR-2B sfRNA (30 nM) incubated with increasing concentrations of TRIM25. Arrow indicates the position of the sfRNA-protein (ribonucleoprotein) complex. Amount of ribonucleoprotein complex was quantified and expressed as a percentage of total RNA signal. (**C**) Lysates from HuH-7 cells infected with one virus from each clade (MOI = 5) were subjected to TRIM25 or IgG control pull-down followed by immunoblotting with TRIM25 and ubiquitin (UB). Arrow indicates ubiquitylated bands. (**D**) Bar chart depicts percentage of ubiquitylated TRIM25 over total TRIM25. (**E**) After transfection with PR-1 or PR-2B sfRNA, HuH-7 cell lysates were subjected to RIG-I pull-down followed by immunoblotting with RIG-I, TRIM25, and UB. (**F**) PR-1 and PR-2B DENV-2 replication at 24 hpi in TRIM25-silenced HuH-7 cells infected with one virus from each clade. siC: control RNA; siTRIM25: siRNA against TRIM25. Differences in gel electrophoresis conditions could have led to slight variations in mobility of prestained protein markers. Data

are expressed as mean ± SD from three independent experiments. n.s.: not significant; *$P \leq$ 0.05, **$P \leq$ 0.01, ****$P \leq$ 0.0001, as determined by *t* test.