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Search for host factors involved in flavivirus life cycle

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Abstract

Several arthropod species in Japan are capable of carrying and transmitting flaviviruses, posing a potential risk of flaviviral disease outbreaks following the introduction of these viruses into the country. Despite this threat, effective treatments for many flaviviral infections remain unavailable. A comprehensive understanding of host factors involved in the flavivirus life cycle is essential for elucidating the detailed mechanisms of viral replication and developing novel antiviral therapies. In this study, we employed two complementary approaches to identify host factors involved in flavivirus replication. First, we conducted a genome-wide CRISPR screen using Zika virus and Japanese encephalitis virus. This screen led to the identification of several candidate host factors, including OR11H12 and PDE6H. Second, we investigated the impact of microRNAs previously identified in Indonesia on dengue virus replication. Our findings revealed that miR-663b and miR-7848 exhibit proviral effects, particularly during the early stages of viral replication in Huh-7 cells. Further studies are necessary to elucidate the molecular mechanisms by which these candidate host factors regulate flavivirus replication.

Keywords

Flavivirus, dengue virus, Japanese encephalitis virus, zika virus, host factor, CRISPR screen, microRNA

Running title

Host factors involved in flavivirus replication

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Introduction

Flaviviruses, including Japanese encephalitis virus (JEV), dengue virus (DENV), Zika virus (ZIKV), and West Nile virus (WNV), are significant human pathogens that require concrete measures in public health, accounting for millions of mortalities worldwide [1]. There are many mosquito vectors such as *Culex tritaeniorhynchus* and *Aedes albopictus* that transmit flaviviruses in Japan, and an outbreak of dengue occurred in Tokyo in 2014 [2]. However, there are no antivirals available for the vast majority of flaviviral infections [3-4]. To develop novel therapeutics, it is important to reveal the regulatory mechanisms of flaviviral replication. To this end, it is necessary to identify host factors involved in the flavivirus life cycle.

Flaviviruses enter host cells using a number of receptors-mediated endocytosis. Endosomal acidification alters virus particles, causing it to release viral genomic RNA into the cytoplasm. The positive sense RNA is translated to a large single polyprotein, cleaved by host and viral proteases, and then produces the structural proteins and non-structural proteins. Later, RNA replication and particle assembly start, and eventually, virus particles mature. Matured viruses are released from the host cell by exocytosis. In the flavivirus life cycle described above, flaviviruses use numerous host factors since they encode the minimum essential proteins required for their replication in the viral RNA genome. Therefore, identifying host factors may lead to finding novel targets for developing new therapeutics.

Several host factors have been identified to be involved in the replication of flaviviruses. Dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN, CD209), a type of C-type lectin, has been reported to be important as viral receptors in flavivirus replication during DENV, WNV, and JEV infection [5-7]. In addition, heat shock proteins have been reported as a candidate receptor for ZIKV, JEV, and DENV [8-10]. Glycosaminoglycans (GAGs) are also involved in the initial interaction of various flaviviruses with the cell membrane [11]. Binding of DENV to C-type lectin domain family 5, member A [CLEC5A, also known as myeloid DAP12-associating lectin (MDL-1)] triggers cytokine release, functioning as a signaling receptor [12]. However, host factor analysis on flaviviruses is still insufficient, and a more detailed analysis is needed.

MicroRNAs (miRNAs) are small, 21-25 base noncoding RNAs that bind to target mRNAs and inhibit protein production through translational repression or degradation [13]. miRNAs are involved in a variety of biological processes such as cell growth, apoptosis, or metabolism [14]. miRNAs also play an important role in viral infection, where host miRNAs may directly target the viral genome or regulate host factors to promote or inhibit viral replication. It has also been shown that overexpression of miR-378 in DENV-infected mice suppresses the expression of serine esterase granzyme B (Grzb), which can induce target cell death through multiple pathways, and promotes DENV replication [15].

CRISPR Cas9 system is an engineered genome editing tool based on a prokaryotic immune defense system[16]. Gene knockout (KO) is generated by the endonuclease Cas9 that is directed to the specific genomic region by a single-guide RNA (sgRNA), and makes a double-stand break (DSB) at the target sequence. Then, the DSB site is repaired by non-homologous end joining. This causes frameshift mutation and loss of gene functions [17]. Pooled CRISPR loss-of-function screen can identify host factors genome-widely by disrupting a number of genes simultaneously using the CRISPR Cas9 sgRNA library. It had been employed to identify flaviviral host factors in previous studies [18-20].

In this study, we attempted to identify host factors involved in flavivirus replication by 2 strategies. One is CRISPR screens for identifying host factors involved in ZIKV and JEV replication, while the other is the analysis of the role of miRNAs in the DENV replication. We ~~had~~ identified miRNAs that are differentially expressed in peripheral white blood cells between severe and mild dengue cases in our Indonesian dengue study (unpublished data).

Materials and Methods

Cell lines and viruses

C6/36 cells derived from *Aedes albopictus* were cultured in Dulbecco's modified Eagle medium (DMEM) (NACALAI TESQUE, INC., Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS) (NICHIREI BIOSCIENCE INC., Tokyo, Japan) and 1% non-essential amino acid solution (NEAA) (NACALAI TESQUE, INC.) at 28 ℃ with 5% CO_2. Huh-7 cells, a human hepatocellular carcinoma cell line, were cultured in DMEM supplemented with 10% FBS and 1% NEAA at 37℃ with 5% CO_2. A549 cells, a human lung adenocarcinoma cell line, and Lenti-X 293T cells (Sigma Aldrich, St. Louis, Missouri, USA) were maintained in DMEM supplemented with 10% FBS at 37℃ with 5% CO_2. Vero cells, an African green monkey kidney cell line, were cultured in Eagle's Minimum Essential Medium (MEM) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% FBS and 1% L-glutamine (Vero medium) at 37℃ with 5% CO_2. ZIKV PRVABC59 strain, JEV Nakayama strain, and DENV serotype 2 New Guinea C strain were used in this study. These viruses were propagated in C6/36 cells, as essentially described previously [21]. Seventy-two hours later, viral supernatant was harvested, clarified by centrifugation, and stored in aliquots at -80℃. Viral titer was determined by focus forming assay described below.

Focus Forming Assay

The level of progeny virus production from infected cells was evaluated by infecting Vero cells followed by the immunostaining of viral antigen. Briefly, culture supernatants of virus-infected cells were serially diluted in Vero medium and incubated with Vero cells seeded in 96-well plates

at 24 hours before infection. After 1 hour of incubation, fresh medium was added to the cell culture. Twenty-four hours later, the infected cells were then fixed with 4%-Paraformaldehyde Phosphate Buffer Solution (NACALAI TESQUE, INC.) for 20 min, and treated then with 0.5% Triton X-100 in Phosphate-buffered saline (PBS). Samples were then incubated with PBS containing 1% of normal horse serum. The cells were then stained with mouse anti-flavivirus envelope protein antibody, 4G2 (at 1:1000 dilution), followed by incubating with biotinylated horse anti-mouse IgG (H+L) secondary antibody (BA-2000; Vector Laboratories, Burlingame, California, USA). Immunocomplex was visualized by an avidin-biotin complex method using the VECTASTAIN Elite ABC Kit, Peroxidase (PK-6100; Vector Laboratories) and the Vector VIP Substrate Kit, Peroxidase (SK-4600; Vector Laboratories). The samples were photographed using All-in-One Fluorescence Microscope (BZ-X810; KEYENCE, Osaka, Japan). Focus forming units (FFUs) were counted using BZ-X810 Analyzer (KEYENCE). Focus was also manually counted and corrected the FFU values adequately.

Genome-wide CRISPR screen

We used ready-to-use, lentiviral vector (LV)-based human CRISPR knockout pooled library (Brunello) containing 76,441 different sgRNAs targeting 19,114 human genes (Addgene #73179-LV) for the genome wide CRISPR screen. First, in order to optimize the transduction condition of the LV-based CRISPR knockout pooled library, Huh-7 cells was infected with 25, 50, 100, 200, 400 or 800 ng (p24 antigen) of LV expressing green fluorescent protein (GFP) (GFP-LV) (described below), and determined optimal amount of LV to infect Huh-7 cells at the multiplicity of infection (MOI) of 1. After determine the optimal condition, Huh-7 cells were transduced with the LV-based CRISPR knockout pooled library. Twenty-four hours later, CRISPR knockout pooled library-transduced Huh-7 cells were re-seeded to 6 T75 flasks for ZIKV, JEV and MOCK-infection. Twenty-four hours later, Huh-7 cells in 2 T75 flasks were MOCK-infected or infected with ZIKV or JEV at MOI of 0.5. In the infection condition, all Huh-7 cells were killed by the cytopathic effects (CPE) of ZIKV and JEV (data not shown). Culture medium was changed every 3-4 days until surviving cells were appeared. Cells were harvested at 21 days after infection, and total cellular DNA was extracted using the QIAamp DNA Blood Maxi Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. The sgRNA library was then amplified by polymerase chain reaction (PCR) from total cellular DNA with the condition of thermal cycling, as follows. Initial denaturation at 95 ℃ for 1 min, then 28 cycles of 95℃ for 30 sec, 53℃ for 30 sec and 72 ℃ for 30 sec, followed by the final extension at 72℃ for 10 min. After PCR, amplicons were purified using AMPure XP-PCR purification (Beckman Coulter, California, USA). The amplified fragments containing sgRNA sequences were subjected to next-generation sequencing by a contract analysis at the Department of Infection Metagenomics, Research Institute for Microbial Diseases, Osaka University.

sgRNA design and preparation of LV

Target gene fragments of host factor candidates and sgRNA sequences were identified *in silico* using the CRISPOR (http://crispor.tefor.net) web design tool. Synthetic sgRNA oligos for genes encoding host factor candidates were cloned into a Cas9- and sgRNA-expressing lentiviral vector plasmid, lentiCRISPRv2 (Addgene #52961), as previously described [22]. The sequences of synthetic sgRNA oligos are available upon request. LV for gene knockout experiments (KO-LV) were prepared by co-transfecting Lenti-X 293T cells with the sgRNA-cloned lentiCRISPRv2, psPAX2-IN/HiBiT, a HiBiT-tagged Gag-Pol expression plasmid for the packaging of LV [23], and pHiT/G, a vesicular stomatitis virus envelope glycoprotein (VSV-G) expression plasmid for the VSVG-pseudotyping of LV [24], at the rate of 4:3:1 using FuGENE HD Transfection Reagent (Promega, Madison, Wisconsin, USA). In addition, GFP-LV was prepared by co-transfecting Lenti-X 293T cells with pWPT-GFP (Addgene #12255), psPAX2-IN/HiBiT and pHiT/G. Forty-eight hours after transfection, LV released into cell culture supernatant was harvested, clarified by centrifugation and stored in aliquots at -80℃. The concentration of LV was determined by measuring human immunodeficiency virus type 1 (HIV-1) capsid p24 antigen using HIV-1 p24 Antigen Assay Kit (RIMCO CORPORATION, Okinawa, Japan) as well as by measuring HiBiT chemiluminescence using the Nano Glo HiBiT Lytic Detection System (Promega).

Gene knockout of a host factor candidate and viral infection

Gene knockout of a host factor candidate was performed using 2 sets of KO-LV containing sgRNA targeting different sites in a target gene. Huh-7 or A549 cells were infected with 2 sets of KO-LV at MOI of 10. Forty-eight hours later, cells were infected again with equivalent KO-LV at MOI of 10 to achieve efficient gene knockout. Twenty-four hours later, cells were trypsinized and re-seeded at 5.0×10^4 cells per 500 µl for viral infection. Twenty-four hours later, cells were infected with ZIKV at MOI of 0.01 or with JEV at MOI of 0.001. Cells and culture supernatant were collected at 48 hours after infection. Total cellular RNA was extracted from cells using NucleoSpin RNA (TaKaRa Bio Inc., Shiga, Japan), and was subjected to the evaluation of ZIKV and JEV RNA replication by the quantitative real-time reverse transcription PCR (qRT-PCR) described below, while cell supernatant was subjected for evaluating the level of viral production from infected cells by focus forming assay.

microRNA (miRNA) transfection and virus infection

Synthetic, double stranded miRNA mimics and inhibitors for miR-545, -545-3p (inhibitor), -574, -574-3p (inhibitor), -663b, -1303, -1307, -1307-3p (inhibitor), -3133, -5194, -6892, -6892-3p (inhibitor), -7848, -7848-3p (inhibitor) and -8086, were purchased from Bioneer Corporation (Daejeon, Republic of Korea). Huh-7 or A549 cells were transfected with miRNA mimic or inhibitor

at the concentration of 20 nM using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Forty-eight hours later, cells were infected with DENV at MOI of 0.1. Cells and culture supernatant were collected at 48 hours after infection for the evaluation of the levels of DENV RNA expression and progeny viral production.

qRT-PCR

RNA extracted from ZIKV-, JEV- or DENV-infected cells were subjected to qRT-PCR using THUNDERBIRD Probe One-step qRT-PCR Kit (TOYOBO CO., Osaka, Japan) and the CFX Connect qPCR Detection System (Bio-Rad Laboratories, Inc., Hercules, California, USA). The primers and probes for the detection and quantify of ZIKV, JEV and DENV RNA were as follows: ZIKV-forward primer (5’-CCGCTGCCCAACACAAG-3’), ZIKV-reverse primer (5’-CCACTAA-CGTTCTTTTGCAGACAT-3’), JEV-forward primer (5’-GGTGTAAGGACTAGAGGTTAGAGG-3’), JEV-reverse primer (5’-ATTCCCAGGTGTCAATATGCTGTT-3’), DENV-2-forward primer (5’-CATGGCCCTKGTGGCG-3’), DENV-2-reverse primer (5’-CCCCATCTYTTCAGTATCCCT-G-3’), ZIKV-probe (FAM-5’-AGCCTACCTTGACAAGCAATCAGACACTCAA-3’-TAMRA), JEV-probe (FAM-5’-CCCGTGGAAACAACATCATGCGGC-3’-TAMRA), DENV-2-probe (FAM-5’-TCCTTCGTTTCCTAACAATCC-3’-TAMRA) [25-27]. In addition, one-step qRT-PCR reaction was performed as follows: reverse transcription at 50°C for 10 min followed by denaturation at 95℃ for 1 min. After that, 40 cycles of denaturation at 95°C for 15 seconds and extension at 60°C for 45 seconds. The relative expression level of the viral RNA was normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, an internal housekeeping gene of human cells. For the detection and quantify of GAPDH mRNA, qRT-PCR was conducted using iTaq Universal SYBR Green One-Step Kit (Bio-Rad Laboratories) and the CFX Connect qPCR Detection System. GAPDH forward primer (5’-GCACCGTCAAGGCTGAGAAC-3’) and GAPDH reverse primer (5’-TGGTGAAGACGCCAGTGGA-3’) were used for the assay. One-step qRT-PCR reaction was carried out as follows: reverse transcription at 50°C for 10 min followed by denaturation at 95℃ for 1 min. After that, 40 cycles of denaturation at 95°C for 10 seconds and annealing/extension/detection at 60°C for 30 seconds.

Statistics

All calculations were performed using GraphPad Prism 8 software (GraphPad Software Inc., Boston, MA) with a one-way analysis of variance (ANOVA).

Results

Genome-wide CRISPR screens identified 45 genes likely to encode host factor candidates involved in flavivirus replication

We conducted genome-wide CRISPR screens to identify host factors involved in flavivirus replication as summarized in Figure 1. ZIKV and JEV were used as viral inoculums because they showed strong CPE and killed Huh-7 cells. The genome-wide CRISPR screens were designed as follows. First, we determined the MOI for ZIKV and JEV that killed 100% of Huh-7 cells seeded in the T75 culture flask within 72 hours, and decided it as MOI of 0.5. Second, Huh-7 cells were transduced with the LV-based genome-wide, CRISPR-knockout pooled library, and the cells were then infected with JEV or ZIKV at the determined MOI. After viral infection, most cells were killed, while some cells survived even after viral infection in the condition that 100% of cells were supposed to be killed. It was assumed that host cellular genes knocked out in surviving cells were involved in viral replication. The sgRNA transduced in surviving cells were then subjected to PCR amplification followed by next-generation sequencing. We utilized our conditions using the values of sgRNA counts to select candidate host genes. The CRISPR-knockout pooled library (Brunello) consisted of an average of 4 sgRNAs per host cellular gene, and we selected the genes satisfying the conditions that two or more sgRNA count values exceeded 500 and one of them had a 5,000 or more sgRNA count value. Previous studies have also identified flavivirus host factors playing a role in lipid metabolism, protein translation, or cell cycle control [10, 28-29]. As the results, a total of 45 candidate genes likely to encode host factor candidates were selected from the CRISPR screens, followed by the analyzed sgRNA abundance and gene ranking by MAGeCK score [30] (Table 1).

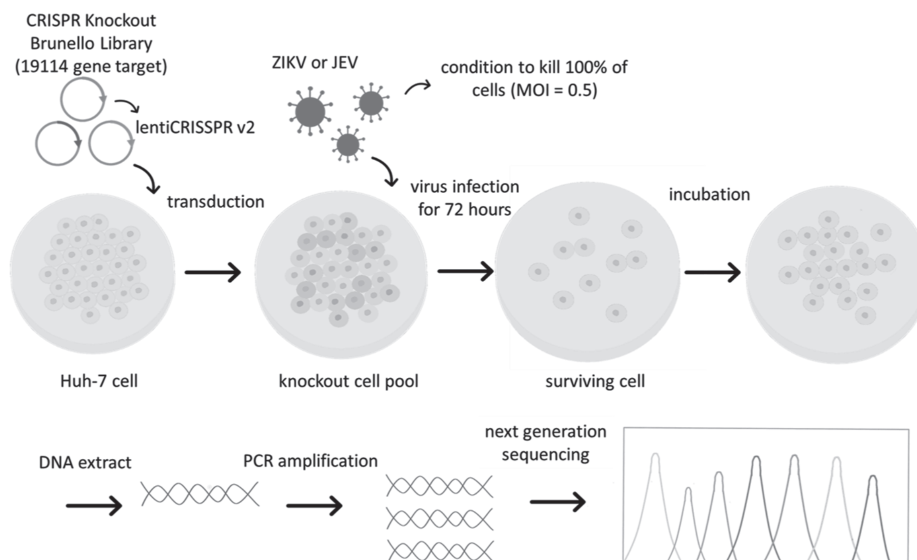

Fig 1. Genome-wide CRISPR screen

Schematic illustration of genome-wide CRISPR screens using ZIKV and JEV.

Table 1. MAGeCK analysis of genome-wide CRISPR screen for host factor candidates involved in ZIKV and JEV infection

ZIKV			JEV		
Gene	pos|score	pos|rank	Gene	pos|score	pos|rank
LMAN2	3.40E-06	1	BRINP1	3.50E-06	1
DAP	2.62E-05	3	DHX29	2.62E-05	2
FANCL	5.30E-05	4	P4HTM	8.03E-05	6
CTAG1A	9.89E-05	8	GSTA4	1.77E-04	8
OR11H12	1.31E+04	11	PDE4B	1.85E-04	10
PRADC1	2.36E-04	17	FGF11	2.02E-04	11
ADIPOR2	2.88E-04	24	SLCO3A1	3.40E-04	16
LYRM1	3.40E-04	28	MYH7B	3.82E-04	19
LYAR	3.68E-04	31	HMGN3	4.19E-04	21
IER3IP	6.02E-04	48	ANKRD16	4.61E-04	23
SEC23IP	6.54E-04	51	NOC4L	4.63E-04	24
GRAMD1B	6.59E-04	52	TOR2A	5.05E-04	27
KIF15	7.07E-04	56	HNRNPUL1	5.10E-04	28
RNASE10	7.59E-04	58	FIZ1	5.40E-04	29
OR7C2	8.11E-04	60	PIGO	5.34E-04	30
CTHRC1	1.18E-03	77	OR5V1	5.40E-04	31
ZFP14	1.75E-03	104	IL20	5.52E-04	33
PRSS37	1.87E-03	112	PALMD	1.39E-03	61
CNDP2	2.07E-03	126	NELFB	3.06E-03	128
C16orf74	4.16E-03	221	AAGAB	4.05E-03	164
ATPAF2	4.94E-03	253	UBE2L6	8.16E-03	296
			LHFPL2	1.02E-02	365
			C16orf74	1.25E-02	454
			PDE6H	1.39E-02	499
			KCNJ2	1.10E-01	3415

Optimization of viral infection conditions in Huh-7 and A549 cells

To validate the results of CRISPR screens, it is necessary to knock out a host gene candidate individually and examine the impact on viral replication. To this end, viral infection conditions were optimized to clearly observe the effects of the loss of gene function. Huh-7 and A549 cells were infected with ZIKV, JEV, or DENV for 48 hours, and viral replication was evaluated by the level of progeny virus production from infected cells by focus forming assay. The results showed that the levels of progeny virus production were elevated according to the initial MOIs of virus inoculums in certain ranges. Namely, the levels of progeny virus production were constantly elevated within the range of indicated initial MOIs for ZIKV infection in Huh-7 cells (Figure 2A), for ZIKV infection in A549 cells (Figure 2D), for JEV infection in A549 cells (Figure 2E) and for DENV infection in A549 cells (Figure 2F). In contrast, if the initial MOIs for JEV were more than 0.04 in

Huh-7 cells (Figure 2B), and for DENV more than 0.4 in Huh-7 cells (Figure 2C), the levels of progeny virus productions from infected cells were not correlated to the initial MOIs; therefore, there was a possibility that the levels of viral replication were beyond the saturation conditions. Thus, we decided to perform viral infection assay at MOI of 0.01 for ZIKV, at MOI of 0.001 for JEV, and at MOI of 0.1 for DENV in Huh-7 cells, as well as at MOI of 0.1 for ZIKV, at MOI of 0.025 for JEV, at MOI of 0.05 for DENV in A549 cells.

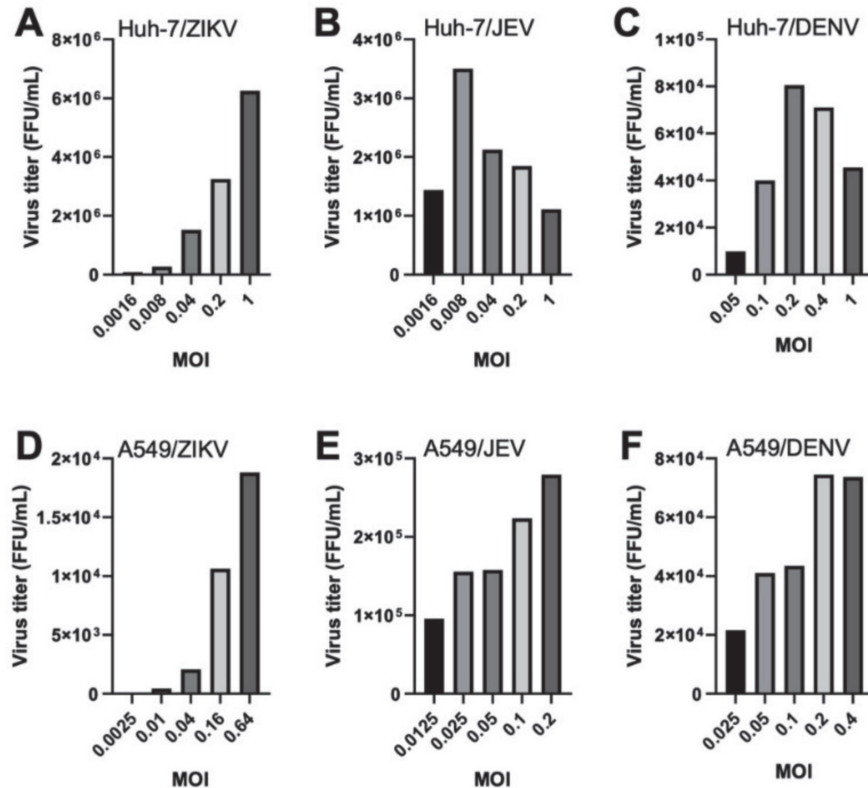

Fig 2. The levels of progeny virus productions from infected cells at different initial MOIs.
Huh-7 (A-C) or A549 cells (D-F) were infected with ZIKV (A and D), JEV (B and E), or DENV (C and F) at various MOIs as indicated below panels. Forty-eight hours after infection, the levels of progeny virus productions from infected cells (viral titer) were evaluated by focus forming assay, as described in Materials and Methods.

Effect of gene knockout of candidate genes on ZIKV and JEV replication

From the 45 candidate genes selected based on MAGeCK score in the genome-wide CRISPR screens (Table 1), we refined the selection to 10 genes each for ZIKV and JEV by using the following approach. We needed this selection due to the limitations in the research scale of this study. For ZIKV, we selected the top 10 genes based on the highest average sgRNA count values, although the values were in part not consistent with the MAGeCK scores. For JEV, we prioritized genes with both high average sgRNA count values and minimal variation among the individual

sgRNA count values. Ten genes each for ZIKV and JEV were analyzed here as follows: Protease Associated Domain Containing 1 (PRADC1), LYR Motif-Containing 1 (LYRM1), Immediate Early Response 3 Interacting Protein 1 (IER3IP), Adiponectin Receptor 2 (ADIPOR2), Death-Associated protein (DAP), Olfactory Receptor Family 11 Subfamily H Member 12 (OR11H12), Ribonuclease A Family Member 10 (Inactive) (RNASE10), Olfactory Receptor Family 7 Subfamily C Member 2 (OR7C2), ZFP14 Zinc Finger Protein (ZFP14) and Kinesin Family Member 15 (KIF15) for ZIKV, while Alpha And Gamma Adaptin Binding Protein (AAGAB), DExH-Box Helicase 29 (DHX29), Palmdelphin (PALMD), Potassium Inwardly Rectifying Channel Subfamily J Member 2 (KCNJ2), Phosphodiesterase 6H (PDE6H), Solute Carrier Organic Transporter Family Member 3A1 (SLCO3A1), High Mobility Group Nucleosomal Binding Domain 3 (HMGN3), BMP/Retinoic Acid Inducible Neural Specific 1 (BRINP1), LHFPL Tetraspan Subfamily Member 2 (LHFPL2) and Negative Elongation Factor Complex Member B (NELFB) for JEV.

Gene knockout of an individual gene was carried out by transducing Huh-7 cells with 2 sgRNAs targeting different sites in a gene candidate together with Cas9 nuclease by using 2 sets of KO-LV for each candidate host gene, followed by infecting the cells with a virus at a determined MOI described above. Viral replication was evaluated by the levels of progeny virus productions. When Huh-7 cells individually knocked out a candidate gene were infected with ZIKV, viral replication in OR11H12- and PRADC1-knockout cells tended to be decreased compared to these in negative control, while the knockout of remaining candidate genes did not show notable effects on ZIKV replication (Figure 3A). In addition, when Huh-7 cells individually knocked out a candidate gene were infected with JEV, viral replication in PDE6H-, SLCO3A1-, LHFPL2-, and NELFB-knockout cells tended to be decreased compared to these in negative control, while the knockout of remaining candidate genes did not show notable effects on JEV replication (Figure 3B). The changes in the levels of progeny virus productions were not statistically significant; however, the results were consistent and reproducible among independent, triplicate experiments (Figures 3A and 3B).

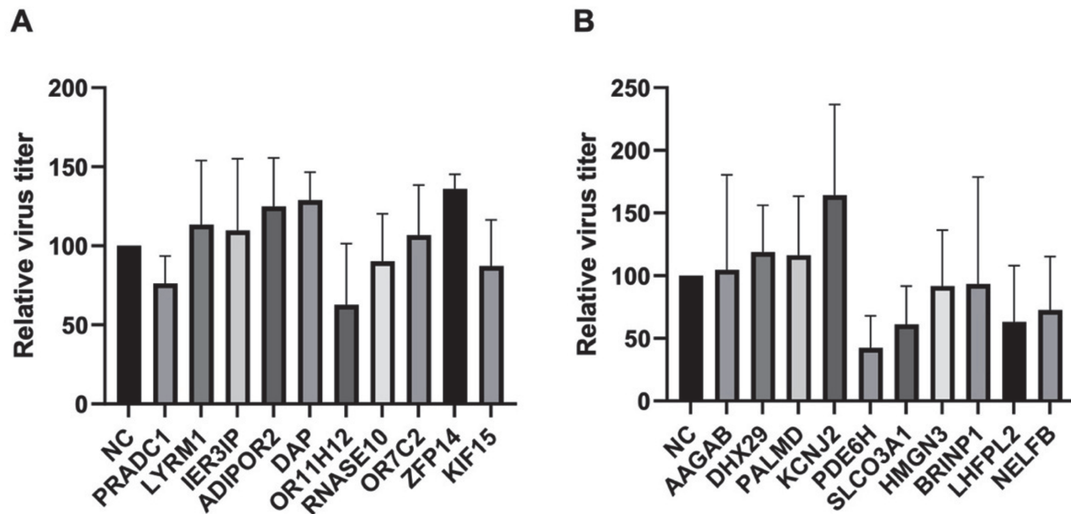

Fig 3. The levels of progeny virus productions from infected, host factor candidate-knocked out Huh-7 cells

Indicated host gene candidate was individually knocked out in Huh-7 cells. The cells were then infected with ZIKV (A) or JEV (B) at MOI of 0.01 or 0.001, respectively. Progeny virus production was evaluated by focus forming assay, as described in Materials and Methods. The levels of viral productions from host gene candidate-knockout Huh-7 cells at 48 hours after infection were normalized to the level in the corresponding negative control cells (NC), which was set to 100, and were shown as relative virus titers. For a negative control, Huh-7 cells were transduced with LV expressing Cas9 and control sgRNA. Means and standard deviations of independent, triplicate experiments are shown. Statistical significance was analyzed using one-way ANOVA; however, all were not significant ($P > 0.05$) for the comparison between NC and host factor candidate-knocked out cells.

Effect of host factor candidate gene-knock out on the RNA expression of ZIKV and JEV in infected Huh-7 cells

We next evaluated the impact of a host factor candidate gene-knock out on the RNA expression of ZIKV and JEV in Huh-7 cells. The results showed that the ZIKV RNA level in OR11H12-knocked out cells was reduced to 0.35 times of the negative control ($P < 0.05$). Similarly, ZIKV RNA levels in PRADC1-knocked out cells were also reduced; however, the reduction was not statistically significant (Figure 4A). On the other hand, there were no marked differences in JEV RNA levels in all of the host factor candidate gene-knock out cells, suggesting that the candidate genes affect the process after RNA replication in the JEV life cycle (Figure 4B).

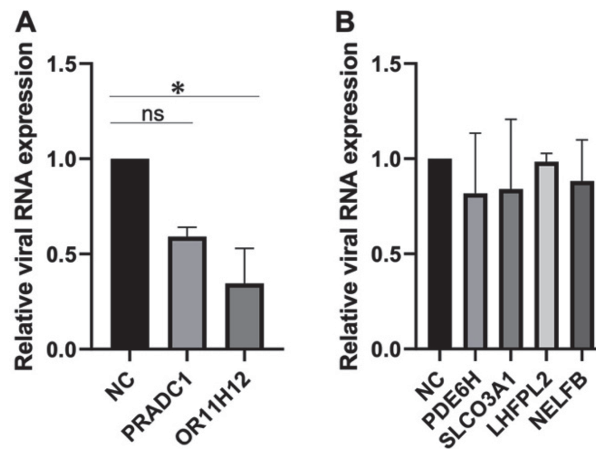

Fig 4. Viral RNA expression in host factor candidate-knocked out Huh-7 cells

Indicated host factor candidate-knocked out cells as well as negative control cells (NC) were infected with ZIKV at MOI of 0.01 (A) or JEV at MOI of 0.001 (B). Forty-eight hours after infection, total cellular RNA was extracted and subjected to qRT-PCR, as described in Materials and methods. Viral RNA levels in host factor candidate-knocked out cells were normalized to that in corresponding negative control cells (NC), which was set to 1, providing the relative viral RNA expression levels in knocked out cells. GAPDH mRNA was measured as an endogenous control and used to normalize viral RNA levels. Statistical significance was analyzed using one-way ANOVA with $p<0.05$ considered to be significant (*). ns: not significant.

Effect of host factor candidate gene-knock out on ZIKV and JEV replication in A549 cells

To verify whether the host factor candidates identified above are involved in viral replication in human cultured cell lines other than Huh-7 cells, we performed similar experiments using A549 cells. The results showed that there were no marked differences in the levels of progeny virus production (Figure 5A) and ZIKV RNA expression among PRADC1-, OR11H12- and control (NC)-knocked out cells (Figure 5C), suggesting that PRADC1 and OR11H12 were not involved in ZIKV replication in A549 cells. In contrast, JEV replication in PDE6H-knocked out cells was reduced to 0.34-fold of control cells (NC) ($P<0.05$) (Figure 5B), while JEV replication in SLCO3A1-, LHFPL2- and NELFB-knocked out cells was also reduced, although there were not significantly different (Figure 5B). However, there were no marked differences in the levels of JEV RNA expression in all of the host factor candidate gene- and control-knock out cells (Figure 5D). These results suggest that PDE6H, SLCO3A1, LHFPL2, and NELFB affect the process after RNA replication in the JEV life cycle both in Huh-7 and A549 cells.

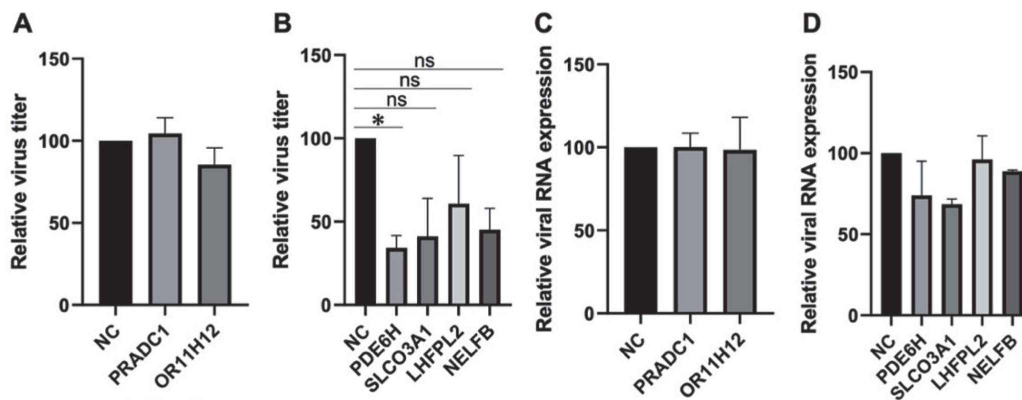

Fig 5. The levels of viral replication and RNA expression in infected, host factor candidate-knocked out A549 cells

Indicated host gene candidate was individually knocked out in A549 cells. The cells were then infected with ZIKV (A and C) or JEV (B and D) at MOI of 0.1 or 0.025, respectively. Progeny virus production (A and B) and viral RNA expression (C and D) were evaluated by focus forming assay and qRT-PCR, respectively, as described in Materials and Methods. The levels of viral production from host gene candidate-knockout cells and of viral RNA expression at 48 hours after infection were normalized to the level in the corresponding negative control cells (NC), which was set to 100, and were shown as relative virus titers. As a negative control, A549 cells were transduced with LV expressing Cas9 and control sgRNA. Means and standard deviations of independent, duplicate experiments are shown. Statistical significance was analyzed using one-way ANOVA with $p<0.05$ considered to be significant. ns: not significant.

Effect of miRNA transfection on the DENV replication

It was revealed in our Indonesian dengue study that the expression levels of miR-545, -663b, -1307, and -6892 were elevated in peripheral white blood cells among 10 patients with severe dengue, while the levels of miR-574, -1303, -3133, -5194, -7848 and 8086 were elevated in the cells among 10 patients with mild dengue (unpublished results). To study the possible involvement of these miRNAs in the regulation of flavivirus replication, we evaluated the DENV replication in Huh-7 cells transfected with synthetic miRNA mimics or inhibitors. We employed synthetic, double-stranded miRNA mimics and inhibitors that mimic and inhibit endogenous miRNAs, respectively, to evaluate the roles of corresponding miRNAs on viral replication. The miRNA-transfected Huh-7 cells were infected with DENV and the levels of progeny virus productions were examined by focus forming assay. The results showed that the levels of progeny virus productions were more than 50% lower in miR-574, -663b, -1303, -7848, and -8086 inhibitor-transfected cells than in the corresponding miRNA mimic-transfected cells (Figure 6A). In contrast, there were no significant differences in the levels of progeny virus production among miR-545, -1307, -3133, -5194, and -6893 inhibitors and mimics-transfected cells (Figure 6A). Since experiments using miR-574, -663b,

-1303, -7848, and -8086 inhibitors and mimics showed reproducible results in all four replicates, viral RNA levels in infected cells were also measured. As the results, viral RNA levels were approximately 2.34 times higher in miR-663b mimic-transfected cells than in miR-663b inhibitor-transfected cells, and approximately 3.17 times higher in miR-7848 mimic-transfected cells than in miR-7848 inhibitor-transfected cells (Figure 6B). In contrast, there were no significant differences in viral RNA levels among miR-574, -1303, and -8086 inhibitors and mimics-transfected cells (Figure 6B).

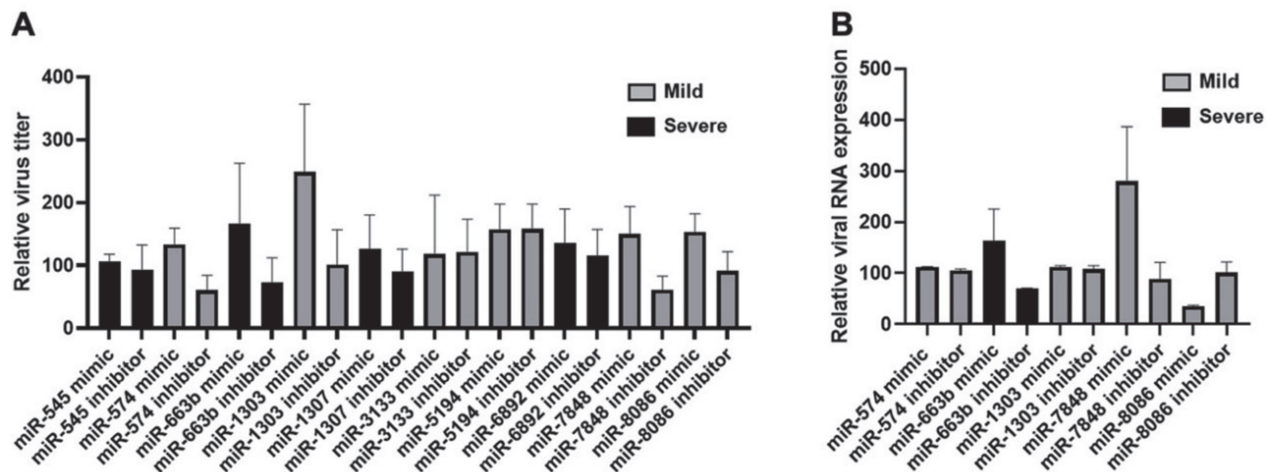

Fig 6. The levels of viral replication and viral RNA expression in miRNA transfected and DENV-infected Huh-7 cells

Huh-7 cells were transfected with indicated miRNA mimics or inhibitors and were then infected with DENV at an MOI of 0.1. The levels of progeny virus productions and viral RNA expressions were evaluated, as described in Materials and Methods. The levels of progeny virus productions (A) and viral RNA expressions (B) in miRNA mimic or inhibitor-transfected, and DENV-infected cells were normalized to that from DENV-infected, miRNA-non-transfected cells, which was set to 100, giving the relative virus titers or viral RNA expression levels. In Figure B, GAPDH mRNA was measured as an endogenous control and used to normalize viral RNA levels.

Effect of miRNA transfection on DENV Replication in A549 Cells

To determine whether the miRNAs identified above are involved in viral replication in human cell lines other than Huh-7 cells, we conducted similar experiments using A549 cells. The results showed no significant differences in the levels of progeny virus production and DENV RNA expression across all miRNA-transfected cells (Figures 7A and 7B), suggesting that these miRNAs are not involved in DENV replication in A549 cells.

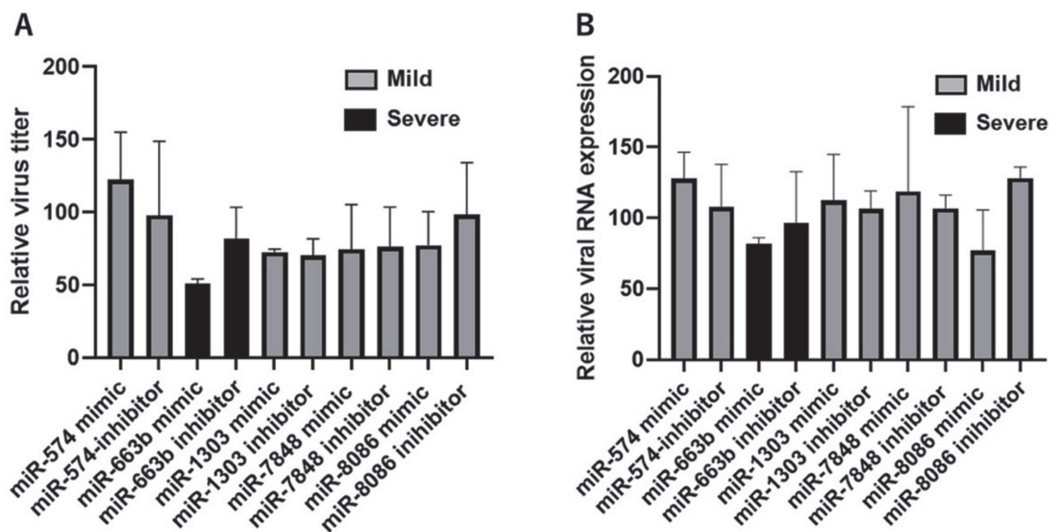

Fig 7. The levels of virus replication and viral RNA expression in miRNA-transfected and DENV-infected A549 cells

A549 cells were transfected with indicated miRNA mimics or inhibitors and were then infected with DENV at an MOI of 0.05. The levels of progeny virus productions and viral RNA expressions were evaluated, as described in Materials and Methods. The levels of progeny virus productions (A) and viral RNA expressions (B) in miRNA mimic or inhibitor-transfected, and DENV-infected cells were normalized to that from DENV-infected, miRNA-non-transfected cells, which was set to 100, giving the relative virus titer or viral RNA expression. In Figure B, GAPDH mRNA was measured as an endogenous control and used to normalize viral RNA levels.

Discussion

Flaviviral infections are a serious public health concern worldwide, with epidemics causing many deaths each year; however, few effective antiviral drugs are available for most flaviviral diseases, and the treatments are primarily symptomatic. We performed genome-wide CRISPR screens using ZIKV and JEV to identify host factors involved in flavivirus replication. Host factor candidates were knocked out using lentiviral knockout vectors. In Huh-7 cells, the knockout of PRADC1 and OR11H12 significantly reduced ZIKV replication. However, validation experiments using A549 cells revealed no substantial reduction, indicating that this phenomenon is specific to Huh-7 cells. Viral RNA expression in ZIKV-infected Huh-7 cells was decreased, suggesting that the early phase of viral replication, such as viral adsorption to and incorporation into host cells, uncoating of viral nucleocapsid and RNA replication, was impaired. For JEV, knockout of PDE6H, SLCO3A1, LHFPL2, and NELFB in both Huh-7 and A549 cells resulted in reduced viral replication. Notably, viral RNA expression in these cells showed no significant decrease, implying that the late

stages of viral replication, such as viral protein synthesis, virion assembly and maturation, and viral release from infected cells, were affected.

Comparison of the candidate host factors identified in this study with other genome-wide CRISPR screening studies revealed that these genes were ranked lower as candidates, despite being included in the target gene libraries [18, 20]. Alternatively, they were not listed among the top candidates in those screenings [31-32]. This discrepancy may be attributed to variations in the libraries and cell types used, which could have led to the selection of different host factors.

The candidate host factors identified in this study exhibit diverse functions and may be implicated in flavivirus replication. PRADC1, a ~30-kDa N-glycosylated secretory protein with a protease-associated domain, regulates physical activity and adiposity [33]. SLCO3A1, a part of the solute carrier organic anion transporter family, acts as a bile acid efflux transporter to maintain bile acid homeostasis [34]. LHFPL2, a transmembrane protein, is essential for distal reproductive tract development in both sexes [35]. NELFB, a subunit of the negative elongation factor (NELF) complex, mediates RNA polymerase II pausing at promoter-proximal regions [36]. Additionally, OR11H12, an olfactory receptor protein, may facilitate ZIKV entry into the central nervous system (CNS) via the olfactory nerve, similar to flaviviruses like JEV and West Nile virus. Reduced viral replication in OR11H12-knocked out cells supports this hypothesis [37]. PDE6H encodes the inhibitory PDE6g subunit of cGMP-specific phosphodiesterase 6 in cone photoreceptor cells [38]. While no direct relationship between JEV and PDE6H has been reported, inhibition of PDE12, another member of the phosphodiesterase family, has shown antiviral activity against RNA viruses, including DENV and WNV [39].

Additionally, miRNAs that were differentially expressed in peripheral white blood cells between severe and mild dengue cases in our Indonesian dengue study were transfected into Huh-7 cells to assess their effects on DENV replication. Transfection with a mimic of miR-663b, which was upregulated in peripheral white blood cells of patients with severe dengue, led to an increase in DENV replication, whereas transfection with its inhibitor resulted in decreased replication. The upregulation of miR-663b in severe dengue suggests that this miRNA may enhance viral replication, contributing to higher viral loads and progression to severe disease. Similarly, transfection with a mimic of miR-7848, which was upregulated in peripheral white blood cells of patients with mild dengue, also resulted in increased DENV replication, while its inhibitor reduced replication. The upregulation of miR-7848 in mild dengue cases suggests that it may be associated with other cellular regulatory processes rather than directly promoting viral replication. Furthermore, transfection of miR-663b and miR-7848 mimics into Huh-7 cells led to an increase in viral RNA expression, indicating that these miRNAs may be involved in the early stages of viral replication. However, validation experiments using A549 cells showed no substantial changes in either DENV replication or viral RNA expression, suggesting that these effects may be specific to Huh-7 cells. We consider

that it is necessary to study further the impact of these miRNAs, as well as of other host factor candidates, on the replication of flaviviruses using primary monocyte-derived macrophages which are more physiologically relevant as natural DENV hosts, or using a mouse animal model for DENV infection in future studies.

To date, several miRNAs involved in DENV replication have been identified. miR-548g-3p binds to the Stem Loop A (SLA) promoter in the 5' untranslated region of the viral genome, a critical component of DENV RNA synthesis and replication, and downregulates DENV replication [40]. In addition, overexpression of miR-Let-7c reduces DENV RNA and NS1 levels, modulating DENV infection by directly targeting the virus and regulating the immune response [41]. Conversely, overexpression of miR-146a enhances DENV replication, a proviral effect facilitated by targeting TRAF6 and suppressing IFN-β production [42]. The miRNAs identified in this study, miR-663b and miR-7848, may directly regulate viral gene expression and promote flavivirus replication. Alternatively, these miRNAs may indirectly regulate viral replication by participating in the regulation of gene expression of the host factor candidates, including OR11H12 and PDE6H, identified in this study. Further analysis is required to clarify these possibilities.

In conclusion, we identified several host factor candidates, including miRNAs, that are involved in the regulatory mechanisms of flavivirus replication *in vitro*. We believe that the results of our study will help to clarify the flavivirus replication mechanism. However, it is still unclear how each host factor candidate is involved in the regulation of viral replication. It is important to investigate the mechanism of action of the candidate host factors in future studies.

Acknowledgments

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