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**Investigation of the life cycles of novel viruses
in arthropods and molecular analyses of
the intracellular proliferation mechanisms of
Chikungunya virus**

節足動物から検出された新規ウイルスの生活環の解析と
蚊媒介性チクングニアウイルスの細胞内増殖の分子生物学的解析

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List of abbreviations

Arbovirus	Arthropod-borne virus	EEE	Eastern equine encephallitis
ARF	ADP ribosylation factors	EILV	Eilat virus
ATP	Adenosine triphosphate	ER	Endoplasmic reticulum
AURAV	Aura virus	ESCRT	Endosomal sorting complexes
B	Base		required for transport
bp	base pair	FBS	Fetal bovine serum
BHK-21 cell	Baby hamster kidney cell	FITC	Fluorescein isothiocyanate
BSA	Bovine serum albumin	GAPDH	Human
C	Capsid		glyceraldehyde-3-phosphate
CHIKF	Chikungunya fever		dehydrogenase
CHIKV	Chikungunya virus	<i>H. flava</i>	<i>Haemaphysalis flava</i>
CHIKV-VLP	CHIKV-like particle	HCV	Hepatitis C virus
Co-IP	Co-immunoprecipitation	HEK	Human Embryonic Kidney
CPE	Cytopathic effect	HGS	Hepatocyte growth
CPV-I	Type I cytopathic vacuoles		factor-regulated tyrosine
CSE	Conserved sequence element		kinase substrate
DAPI	4',6-diamidino-2-phenylindole	HTRV	Heartland virus
DENV	Dengue virus	HIV	Human immunodeficiency
DMEM	Dulbecco's modified Eagle's		virus
	medium	hpi	hours post-infection
DMSO	Dimethyl sulfoxide	hpt	hours post-transfection
dpi	days post-inoculation	HRP	Horseradish peroxidase
EDTA	Ethylenediaminetetraacetic	HSV	Herpes simplex virus
	acid	<i>I. persulcatus</i>	<i>Ixodes persulcatus</i>

IFA	Immunofluorescence assay	ORF	Open reading frame
IgG	Immunoglobulin G	pAb	polyclonal antibody
IIF assay	Indirect immune fluorescence assay	PBS	Phosphate buffered saline
IP	Immunoprecipitation	PCR	Polymerase chain reaction
ISE6 cell	<i>Ixodes scapularis</i> embryo cell	PFA	Paraformaldehyde
		PFU	Plaque-forming unit
		qPCR	quantitative PCR
JEV	Japanese encephalitis virus	RT-PCR	Reverse transcription
KD	Knockdown		polymerase chain reaction
kDa	kilodalton	RACE	Rapid amplification of cDNA
KO	Knockout		ends
KURV	Kuriyama virus	RdRp	RNA-dependent RNA
L domain	Late assembly domain		polymerase
LD	Liner discriminant	SAR	Secretion associated Ras
mAb	monoclonal antibody		related GTPase
MEM	Minimum Essential Medium	SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel
MKWV	Mukawa virus		electrophoresis
MOI	Multiplicity of infection	SFTSV	Severe fever with
MT	Mutant type		thrombocytopenia virus
MVB	Multivesicular body	SFV	Semliki Forest virus
MWAV	Mwinilunga alphavirus	SINV	Sindbis virus
NGS	Next generation sequencing	siRNA	small interfering RNA
		sP	structural protein
nsP	nonstructural protein	TALV	Tai Forest alphavirus

TBPV	Tick-borne phlebovirus
TCID ₅₀	50% tissue culture infectious dose
TGN	Trans-Golgi network
TPB	Tryptose phosphate broth
TROV	Trocar virus
UIM	Ubiquitin-interacting motif
UTR	Untranslated region
VEE	Venezuelan equine encephalitis
VPS4	Vacuolar protein sorting 4
VSV	Vesicular stomatitis virus
WEE	Western equine encephalitis
WT	Wild type
ZIKV	Zika virus

General Introduction

A variety of viruses have been detected in arthropods and these viruses maintain different life cycles. While arthropod-specific viruses have life cycles within arthropods, arthropod-borne viruses (arboviruses) can infect both arthropod vectors and vertebrate animals. Since arthropods are involved in multiple ecological systems interacting with other eukaryotes, including animals, plants, and fungi, some arboviruses might adapt to their surrounded environment and obtain infectivity and pathogenicity to vertebrates in evolutionary processes.

Recently, arboviruses have geographically expanded their habitats and caused the public health concerns. The arboviruses are mainly consistent of three virus groups: the genus *Flavivirus* and *Alphaavirus*, and the order *Bunyavirales*. Arboviruses are spread through bites of virus-infected arthropods such as ticks and mosquitoes to vertebrate animals. Dengue virus (DENV), Japanese encephalitis virus (JEV), Zika virus (ZIKV) and Chikungunya virus (CHIKV) can be transmitted by mosquitoes. Severe fever with thrombocytopenia virus (SFTSV) and Heartland virus (HTRV) can be transmitted by ticks. These arboviruses are also transported from one place to another by infected travelers. Outbreak of transboundary arboviral diseases seriously affected public health worldwide including Europe and the Americas. For instance, it is estimated that 390 million people are infected by DENV annually and 96 million people have clinical symptoms. However, there are no available vaccines nor effective treatments for some arboviral diseases (i.e. DENV, ZIKV, CHIKV, SFTSV and HTRV infection), as the detailed virulence and intracellular proliferation mechanisms of these viruses are still unknown. To establish the effective measurements for arboviral diseases, epidemiological studies and basic experiments for the viruses are necessary.

In chapter 1, the genetic surveillance of phleboviruses was conducted in ticks in Hokkaido, to determine the existence of tick-borne phleboviruses (TBPVs), and discovered genetically unique phleboviruses, Mukawa virus (MKWV) and Kuriyama virus (KURV). In addition, serological screening of animals in Hokkaido district and experimental infection were performed to understand their ecological life cycles in nature and the pathogenicity in mammals.

In chapter 2, comprehensive detection of alphaviruses in mosquitoes was conducted in Zambia. Although human diseases caused by alphaviruses have been reported in Africa, the prevalence of alphaviruses has not been previously investigated in Zambia. A novel alphavirus, Mwinilunga alphavirus (MWAV), was characterized to know its life cycle in this study.

In chapter 3, the host machinery required for intracellular CHIKV propagation was investigated. While Chikungunya fever (CHIKF) cases have been identified in over 60 countries to date, no effective control measures, including vaccines nor specific anti-viral drugs, for CHIKF are available currently. Thus, better understanding for molecular mechanisms underlying CHIKV infection is demanded. I have attempted to identify intracellular trafficking factors potentially regulating CHIKV infection and examined their roles in CHIKV infection.

In this thesis, I discussed how to control arboviruses by performing epidemiological research and basic experiments for establishment of the effective measures for them.

Chapter 1

Infection of newly identified phleboviruses in ticks and wild animals in Hokkaido, Japan indicating tick-borne life cycles

Introduction

The recent discoveries of human-pathogenic tick-borne viruses have impacted public health by revealing the silent risks of emerging pathogens transmitted by ticks (Yu et al. 2011, McMullan et al. 2012, Kosoy et al. 2015). Ticks hardly spread disease outside their local habitats (Eisen and Eisen 2018, Eisen et al. 2017, Jongejan and Uilenberg 2004), and thus, early detection and characterization of novel viruses in local ticks is important to highlight the endemic threats of unrecognized pathogens. In the *Huaiyangshan banyangvirus* species, genus *Banyangvirus*, family *Phenuiviridae* [previously known as genus *Phlebovirus*, family *Bunyaviridae*] (King et al. 2018), two human-pathogenic TBPVs have been identified in the 2010s; SFTS virus emerged in east Asian countries (Yu et al. 2011, Kim et al. 2013, Takahashi et al. 2014) and HTRV in the United States (McMullan et al. 2012), followed by several discoveries of RNAs from novel TBPVs in ticks using pan-phlebovirus reverse transcription polymerase chain reaction (RT-PCR) (Matsuno et al. 2015, Papa et al. 2016, Papa et al. 2017, Prinz et al. 2017, Pereira et al. 2017) and/or next generation sequencing (NGS) (Tokarz et al. 2014, Li et al. 2015). Since epidemiological information of these novel viruses that have been identified only in ticks are limited, their potential risks of spillover into the human population have not been fully understood.

Screening of novel TBPVs in ticks is required especially in endemic countries of tick-borne diseases for differentiating novel TBPV infections from known diseases, as replication of a novel TBPV may cause nonspecific febrile illness. In Japan, SFTS and

tick-borne encephalitis (Lindquist and Vapalahti 2008, Takashima 1998, Yoshii, Kojima and Nishiura 2017) are reported as viral tick-borne diseases, and tick-borne diseases caused by bacteria or parasites such as Lyme disease (Takano et al. 2014, Sato et al. 2014, Saito et al. 2007), rickettsiosis (NIID 2017, Ando et al. 2010, Imaoka et al. 2011), and human granulocytic anaplasmosis (Ohashi et al. 2013), are also recognized. These diseases present non-specific febrile illness (Suttinont et al. 2006, Parola and Raoult 2001, Bakken and Dumler 2006). To identify the potential causes of febrile illness by novel TBPVs, clarification of TBPV's distribution and pathogenicity will be beneficial in case of their future emergence.

In the present study, the ecological characteristic of a previously reported TBPV, MKWV was examined (Matsuno et al. 2018). The previous study discussed the unique genetic and biological characteristics of MKWV, a tick-derived virus genetically similar to mosquito/sandfly-borne phleboviruses rather than other TBPVs. Even though the potential of MKWV to adapt to a human cell line has been shown, the life cycle of MKWV and its infectivity and pathogenicity in mammals is unknown. Here, serological screening and experimental infection was performed to understand the ecological life cycle as well as the pathogenicity of MKWV in nature.

The current epidemiological landscape of tick-borne diseases is complicated, as studies of known and novel pathogens in ticks as well as their correlations with each other in nature are limited. Thus, potential pathogens in ticks should be discovered using a comprehensive method in addition to studying each pathogen specifically. The present study also aimed to reveal a complete picture of TBPV distribution in a limited area in Japan (i.e. Hokkaido) by genetic screening of ticks using pan-phlebovirus RT-PCR (Matsuno et al. 2015).

Materials and Methods

Study site and sample collection

Only adult ticks (n = 1,481) were collected from various locations in Hokkaido, the northernmost island in Japan from 2013 to 2015 and used for genetic surveillance of TBPVs. Ticks (n = 67) collected in Mukawa area in the previous study were also included. Questing ticks (n = 1,217) were captured by the flagging method, and blood-sucking ticks were removed from wild Yezo-deer (*Cervus nippon yesoensis*) captured by hunters in 2013 (n = 54) and from wild raccoons (*Procyon lotor*) captured by local veterinary offices in 2015 (n = 210). Tick species were identified under a stereomicroscope based on morphologic features (Sasa and Aoki 1977).

Serum samples of Yezo-deer and raccoons were used for serological testing for MKWV. Serum samples of Yezo-deer (n = 50) hunted in Hidaka area from 2010 to 2011 (Fig. 1) were a part of the samples previously used for a serological study targeting hepatitis E virus (Tomiyama et al. 2009). Serum samples were also obtained from raccoons (n = 64) captured in Mukawa, Kuriyama, Atsuma, and Ebetsu areas from 2007 to 2010 as a part of raccoon population control programs implemented by the Hokkaido Government (Fig. 1). These samples were stored at -80°C until use.

RNA extraction from ticks

Each tick was washed once in 70% ethanol and soaked twice in distilled sterile water for 10 min. The samples were then homogenized with 100 μl of Dulbecco's modified Eagle's medium (DMEM) (Nissui) using a homogenizer (Tomy Seiko) twice at 3,000 rpm. Total RNA was extracted from 50 μl of the homogenate using blackPREP Tick DNA/RNA Kit (Analytic Jena) according to the manufacturer's protocol and the remaining homogenized samples were stored at -80°C until use for virus isolation.



Fig. 1. Map of the present study sites.

Hokkaido is the second largest island in Japan and is geographically isolated from other prefectures. The areas where MKWV or KURV-positive ticks were captured from 2013 to 2015 (Mukawa, Kuriyama, Obihiro, and Shimizu) are indicated by black stars, areas where Yezo-deer (*Cervus nippon yesoensis*) were captured from 2010 to 2011 (Hidaka) are colored in dark gray, and areas where raccoons (*Procyon lotor*) were captured from 2007 to 2010 (Mukawa, Kuriyama, Atsuma, and Ebetsu) are colored in light gray.

Detection of TBPV RNAs

A one-step RT-PCR system reported previously (Matsuno et al. 2015) was employed to detect a wide range of TBPVs in the extracted tick RNAs. Briefly, around a 500-base pair (bp) fragment of the L segment RNA was amplified using a PrimeScript One step RT-PCR Kit Ver. 2 (Dye Plus) (TAKARA) from the tick RNA using the primer set; HRT-GOUL2759F (5'-CAGCATGGIGGIYTIAGRGAATYATGT-3') and HRT-GOUL3276R (5'-GAWGTRWARTGCAGGATCCYTGCATCAT-3'). The amplified products were sequenced using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit on a 3500 Genetic Analyzer (ABI3500) (Thermo Fisher Scientific) using the HRT-GOUL2759F or HRT-GOUL3276R primer. The nucleotide sequences were determined using GENETYX version 13 (GENETYX).

Virus isolation

The remaining homogenates of RT-PCR-positive ticks were subjected to virus isolation. The *Ixodes scapularis* embryo-derived ISE6 cells were cultured in modified L-15B medium supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific), 5% tryptose phosphate broth (TPB) (Sigma), 0.1% bovine lipoprotein concentrate (MP Biomedicals), and 2% penicillin-streptomycin (Thermo Fisher Scientific) at 34°C as reported previously (Munderloh and Kurtti 1989). After ISE6 cells were cultured in 12-well plates for one day, the media were removed and 20 µl of tick homogenates were inoculated into cultured cells in 1 ml of the modified L-15B medium. One hour after inoculation, the media were changed, and the cells were cultured for seven days at 34°C under 5% CO₂. Following three blind passages (i.e. four weeks after the inoculation), viral production in the supernatant was confirmed using the RT-PCR described above.

Electron microscopy

Supernatants of virus-inoculated ISE6 cells were subjected to ultracentrifugation (27,000 rpm, 90 min) through 25% sucrose in phosphate buffered saline (PBS), following low-speed centrifugation to remove debris. The ultracentrifuged sample was adsorbed to collodion-carbon-coated copper grids and negatively stained with 2% phosphotungstic acid solution (pH 5.8). Virus particles were observed under an H-7650 transmission electron microscope (Hitachi) at 80 kV.

Determination of nucleotide sequences of full-length viral genome

Viral RNA was extracted from the supernatants of ISE6 cells using QIAamp Viral RNA Mini Kit (QIAGEN) and the viral genome was amplified using PrimeScript One Step RT-PCR kit with specific primer pairs, which were designed based on the nucleotide sequence of MKWV (primer sequences available upon request). The 5'- and 3'- termini of each segment RNA were respectively amplified using the rapid amplification of cDNA ends (RACE) method (Li et al. 2005) with specific primers for MKWV. Amplified products were directly sequenced in both directions on the ABI3500. The full-genome sequence of each segment RNA was determined, and the amino acid sequences were predicted using GENETYX version 13.

Phylogenetic analysis

The determined full-length nucleotide sequences of the isolated viruses and those of other known phleboviruses available at GenBank, were aligned using MUSCLE implemented in MEGA version 6.0 (Tamura et al. 2013). Multiple sequence alignments were modified manually. Phylogenetic trees based on three segment RNAs were

constructed by the maximum-likelihood method using MEGA version 6.0. The robustness of the nodes was tested using 1,000 bootstrap replications.

Detection of viral antigens by immunofluorescence assay

Monolayers of ISE6 cells infected with TBPVs were fixed in ice cold acetone at three days post infection. After fixation, the cells were incubated with 1:1,000 diluted MKWV-infected mouse serum in the antibody dilution buffer: PBS containing 1.0% bovine serum albumin (BSA) fraction V (Roche Diagnostics), and 0.05% Tween 20 (Nacalai Tesque), for 1 hour at room temperature (approximately 24°C), followed by washing three times in PBS. The viral antigens were visualized by incubation with 1:1,000 diluted Fluorescein isothiocyanate (FITC)-conjugated Goat Immunoglobulin G (IgG) Fraction to Mouse IgG (MP Biomedicals) in the antibody dilution buffer for 1 hour at room temperature, followed by staining with 4',6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific) (1:2,000 dilution) for 10 minutes. The cells were examined for staining under a fluorescence microscope (Olympus) with an appropriate barrier and excitation filters for visualizing FITC and DAPI (Chroma).

Detection of antibodies to MKWV by indirect immunofluorescence (IIF) assay

MKWV-infected and mock-infected Huh-7 cells (human liver carcinoma cells) were prepared for the IIF assay. Huh-7 cells were cultured in DMEM with 2% FBS, 2 mM L-glutamine (Sigma), and 2% penicillin-streptomycin at 37°C with 5% CO₂. Three days after MKWV infection, cells were fixed with 4% Paraformaldehyde (PFA) at room temperature for 10 minutes, followed by permeabilization with PBS containing 0.1% Triton X-100 (Nacalai Tesque) for 10 minutes. Serum samples of deer and raccoons were serially diluted 2-fold from 1:20 to 1:2,560 and added to both the fixed

MKWV-infected and mock-infected cells. The cells were incubated at 4°C overnight. After washing with PBS, the antigens on the slides were treated with Protein A/G-FITC (BioVision) at a dilution of 1:5,000 for 1 hour at room temperature. After another wash with PBS, the cells were examined under a fluorescence microscope with appropriate barrier and excitation filters for FITC. The titers of tested samples were recorded as the reciprocals of the highest dilutions showing positive staining.

Neutralization assay

IIF-positive serum samples were then used to detect neutralizing antibodies against MKWV. Serum samples were serially diluted 2-fold from 1:40 to 1:5,120 and mixed with an equal volume of MKWV at 500 50% tissue culture infectious dose (TCID₅₀). The mixtures were incubated at 37°C for one hour, and then added to cultured Huh-7 cells. Cells were incubated at 37°C with 5% CO₂ for four days, as inhibition of viral infection by MKWV-infected mouse serum used for positive serum control was clearly observed by the immunofluorescence assay. The titers were recorded as the reciprocals of the highest dilutions of serum samples that prevent infection. Titers of 1:40 or greater were considered positive results.

Experimental inoculation of MKWV into eight-week-old C57BL/6J mice

For the animal experiment, MKWV passaged four times in ISE6 cells (ISE6 cell-passaged) and MKWV passaged eight times in Huh-7 cells after the four passages in ISE6 cells (Huh-7 cell-passaged) were used. Huh-7 cell-passaged MKWV was inoculated intramuscularly (Group 1) or subcutaneously (Group 2), and ISE6 cell-passaged MKWV was inoculated subcutaneously (Group 3) at 10⁵ TCID₅₀/mouse in a group of fifteen eight-week-old female C57BL/6J mice (Japan SLC). In total, 18

mice were inoculated with supernatants of Mock ISE6 cells or Huh-7 cells as negative controls. To analyze viral distribution in the blood and tissues of MKWV-inoculated mice, five mice from each group (three mice of each negative-control group) were sacrificed at 3, 7, and 14 days post-inoculation (dpi). The remaining mice were observed for their clinical signs, and their body weights were recorded for 14 days.

Detection of viral RNA and infectious titers in blood and tissues

Tissues (liver, spleen, and kidney) were collected individually from the mice and homogenized in DMEM supplemented with 2% FBS. Homogenates and whole blood of the three representative individuals in each group were serially diluted 10-fold with DMEM containing 2% FBS and inoculated into Huh-7 cells for titration. The remaining homogenates and whole blood were also subjected to RNA extraction with TRIzol LS (Thermo Fisher Scientific). After generation of cDNAs from the extracted RNAs with Superscript IV Reverse Transcriptase (Thermo Fisher Scientific), a quantitative PCR (qPCR) assay targeting MKWV was performed with the generated cDNA and a MKWV-specific primer set; Mukawa-L-6314F (5'-AGATCTTGTTGGGAAACACC-3') and Mukawa-L6414R (5'-ACACAAAGTCCGCCCATACCAATGAGATG-3') using THUNDERBIRD SYBR qPCR Mix (TOYOBO). The reaction was performed on a Bio-Rad CFX96 system (Bio-Rad) and the MKWV titers were calculated as the TCID₅₀/g-equivalents of tissues or TCID₅₀/ml-equivalents of whole blood.

Results

Genetic surveillance of TBPVs in ticks collected in Hokkaido

To reveal the distribution of TBPVs in Hokkaido, ticks were collected from various locations and examined for TBPV genes by one-step RT-PCR. MKWV was identified in

a part of this screening (Matsuno et al. 2018). Based on morphological identification, ticks (n = 625) collected in 2013 comprised 9 species in 2 genera, ticks (n = 109) collected in 2014 comprised 5 species in 2 genera, and ticks (n = 747) collected in 2015 comprised 7 species in 2 genera (Table 1). TBPV genes were detected in *I. persulcatus* in 2013 (17/183); *I. persulcatus* in 2014 (3/41); and *Haemaphysalis flava* (1/14), *H. megaspinosa* (1/121), and *I. persulcatus* (10/219) in 2015. Only 1 TBPV was detected in an engorged *H. flava* tick collected from a raccoon in 2015 among engorged ticks. Although *I. ovatus* was dominant in Hokkaido throughout all years, no TBPV genes were detected. The homogenates of TBPV-positive ticks were further investigated based on the nucleotide sequences amplified by one-step RT-PCR. The homology values between nucleotide sequences were calculated with the corresponding partial sequences of MKWV L segment RNA. No SFTS virus-positive ticks were identified, and the nucleotide sequences derived from five *I. persulcatus* samples collected in Mukawa and Shimizu areas in 2013 [including MKW73 from which MKWV has been isolated (Matsuno et al. 2018)] were 100% matched to the corresponding partial sequences of MKWV MKW73 (Table 2). Interestingly, the nucleotide sequences in ticks collected from Kuriyama and Obihiro areas in 2015 demonstrated an 84% identity to the MKWV sequence and 100% identity with each other (Table 2). The putative MKWV-like virus has been named Kuriyama virus (KURV). MKWV and KURV RNAs were not detected in *I. persulcatus* collected from Honshu island, the largest island of Japan (Table 1).

Isolation and characterization of KURV

Homogenates of TBPV-positive samples were inoculated into ISE6 cells for virus isolation. Successful isolation of KURV was confirmed after 3 passages in ISE6 cells,

Table 1.
Screening of ticks for tick-borne phleboviruses in Hokkaido, Japan.

Place	Year	Tick species	Collection methods	Total	TBPV-positive	MKWV-positive ^{*1}
Hokkaido	2013	<i>Haemaphysalis concinna</i>	Flagging ^{*2}	7	0	0
		<i>H. flava</i>	Yezo-deer	8	0	0
		<i>H. japonica</i>	Flagging	27	0	0
			Yezo-deer	32	0	0
		<i>H. longicornis</i>	Flagging	4	0	0
		<i>H. megaspinosa</i>	Flagging	24	0	0
			Yezo-deer	3	0	0
		<i>Ixodes ovatus</i>	Flagging	323	0	0
			Yezo-deer	11	0	0
		<i>I. pavlovskyi</i>	Flagging	2	0	0
		<i>I. persulcatus</i>	Flagging	183	17	5
		<i>I. tanuki</i>	Flagging	1	0	0
		Total		625	17	5
	2014	<i>H. japonica</i>	Flagging	1	0	0
		<i>H. megaspinosa</i>	Flagging	1	0	0
		<i>I. ovatus</i>	Flagging	53	0	0
		<i>I. pavlovskyi</i>	Flagging	13	0	0
		<i>I. persulcatus</i>	Flagging	41	3	0
		Total		109	3	0
	2015	<i>H. flava</i>	Flagging	10	0	0
			Raccoons ^{*4}	4	1	0
		<i>H. japonica</i>	Flagging	33	0	0
		<i>H. megaspinosa</i>	Flagging	111	1	0
			Raccoons	10	0	0
		<i>I. ovatus</i>	Flagging	144	0	0
			Raccoons	136	0	0
		<i>I. pavlovskyi</i>	Flagging	34	0	0
			Raccoons	6	0	0
		<i>I. persulcatus</i>	Flagging	204	10	2
			Raccoons	15	0	0
		<i>I. tanuki</i>	Flagging	1	0	0
			Raccoons	39	0	0
		Total		747	12	2
Honshu	2013	<i>I. persulcatus</i>	Flagging	12	1	0
island	2014	<i>I. persulcatus</i>	Flagging	61	3	0

^{*1} TBPV-positive samples with >80% nucleotide sequence identity to MKWV strain MKW73.

^{*2} Questing ticks captured by the flagging method.

^{*3} Blood-sucking ticks removed from wild Yezo-deer (*Cervus nippon yezoensis*).

^{*4} Blood-sucking ticks removed from wild raccoons (*Procyon lotor*).

Table 2.
Breakdown of MKWV-positive ticks.

Sample ID	Tick species	Year	Area	% Homology to MKWV ^{*1}
CZC69	<i>I. persulcatus</i>	2013	Mukawa	100
CZC73	<i>I. persulcatus</i>	2013	Mukawa	100
CZC74	<i>I. persulcatus</i>	2013	Mukawa	100
CZC209	<i>I. persulcatus</i>	2013	Shimizu	100
CZC215	<i>I. persulcatus</i>	2013	Shimizu	100
CZC80Q	<i>I. persulcatus</i>	2015	Kuriyama	84
CZC1957	<i>I. persulcatus</i>	2015	Obihiro	84

^{*1}MKWV: The corresponding partial sequences of MKWV strain MKW73

without any cytopathic effects (CPE). No other putative TBPVs apart from KURV and MKWV were isolated. The KURV isolate KUR80Q originated from the tick CZC80Q collected in Kuriyama and was used in the following experiments. The virions of KURV KUR80Q were enveloped spherical particles (100-nm average in diameter) with surface spike proteins (Fig. 2A), indicating morphological similarity to MKWV and other bunyaviruses (Plyusnin et al. 2012).

Full-genome sequences of KURV RNA segments were determined by MKWV-specific primer sets followed by primer walking and the RACE method. KURV genome was consisting of 3 RNA segments: L segment, 6,444 base (b); M segment, 3,328 b; and S segment, 1,908 b. Each L or M segment RNA encoded 1 open reading frame (ORF) in the negative sense and the S segment RNA encoded 2 ORFs in the ambisense orientation. BLAST search indicated that the deduced amino acid sequences of KURV ORFs were related to those of proteins encoded in each segment from phleboviruses: RNA-dependent RNA polymerase (RdRp) on L segment, glycoproteins (Gn and Gc) on M segment, and nonstructural and nucleocapsid proteins (NSs and N, respectively) on S segment RNAs, respectively. The pairwise amino acid identities of KURV were 95% (L), 93% (glycoprotein precursor), 93% (N), and 74% (NSs) with MKWV. In the phylogenetic trees constructed based on all 3 segment RNAs, KURV was classified into the same phylogroup as MKWV (Fig. 2B). In all phylogenetic trees, the phylogroup of MKWV and KURV was branched together with the mosquito/sandfly-borne phlebovirus group and was closely related to mosquito/sandfly-borne phleboviruses rather than TBPVs. Despite their phylogenetically unique positions, MKWV and KURV were isolated from ticks and they replicated in ISE6 cells but not in *Aedes albopictus* mosquito-derived C6/36 cells like the tick-borne Uukuniemi virus (Matsuno et al. 2018) (data not shown for KURV in C6/36 cells). These results support the idea that the

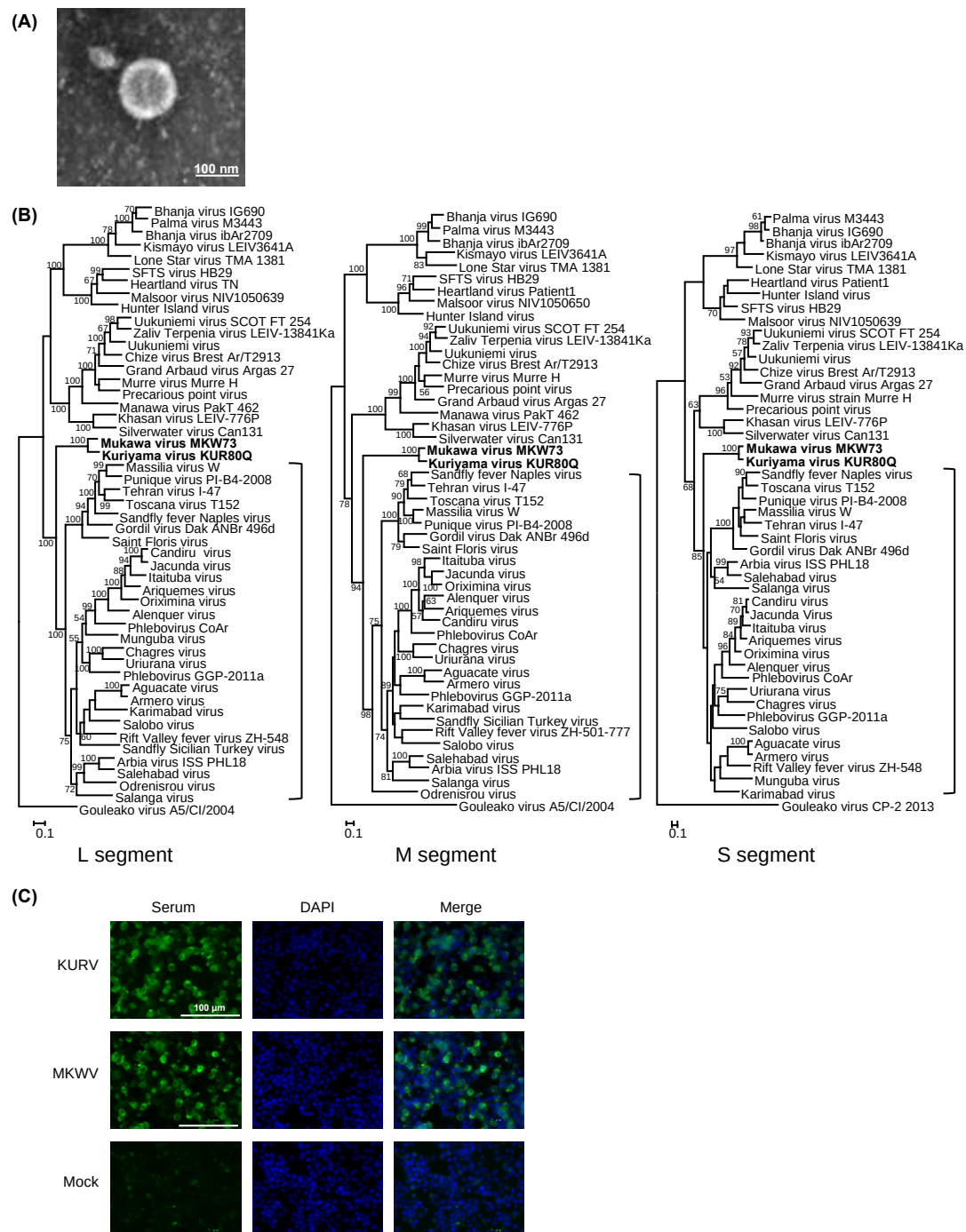


Fig. 2. Isolation and characterization of KURV.

(A) Electron micrograph of a KURV particle. (B) Phylogenetic trees constructed based on the full-length nucleotide sequences of 3 RNA segments of phleboviruses using the maximum-likelihood method in MEGA version 6.0. Bootstrap values greater than 50 are shown near the branch nodes and the scale bar indicates the number of substitutions per site. A square bracket on the right side of each tree indicates mosquito/sandfly-borne phleboviruses. (C) The antigens of KURV and MKWV were detected in tick cell monolayer at 7 dpi using MKWV-infected mouse serum with indirect fluorescence-labelled antibodies. The antigens reacting with the serum were visualized in green; the blue color indicates nuclei stained with DAPI.

ancestor of MKWV and KURV evolved into mosquito/sandfly-borne phleboviruses from tick-borne viruses as discussed previously (Matsuno et al. 2018).

To compare the serological reactivity of KURV and MKWV, an immunofluorescence assay was conducted using MKWV-infected mouse serum. Immunofluorescence signals were mainly detected in the cytoplasm of both KURV- and MKWV-infected cells and the intensity of immunofluorescence signals was nearly identical to each other (Fig. 2C). The results indicated that the anti-MKWV serum cross-reacted with KURV antigens.

Serological survey

IIF assay was performed using serum samples from Yezo-deer and raccoons to investigate MKWV infections in wild animals in Hokkaido. Antibodies recognizing MKWV antigens were detected in 12% (6/50) of Yezo-deer and 27% (17/64) of raccoons, respectively (Table 3). Titers of positive sera ranged from 20 to 2,560 (Table 4). IIF-positive serum samples were then used for the neutralization assay to confirm MKWV infection. Serum samples of Yezo-deer (2/6) and raccoons (16/17) could neutralize MKWV; the seropositivity of anti-MKWV neutralizing antibodies was 4% (2/50) in Yezo-deer and 25% (16/64) in raccoons (Table 3). As described in the Materials and Methods section, titers of 1:40 or greater were considered positive results. Neutralizing titers ranged from 40 to 640 (Table 4). The neutralizing antibody-positive animals were found at least in 4 different areas in Hokkaido during the four-year period.

Evaluation of MKWV pathogenicity in eight-week-old C57BL/6J mice

Table 3.
Seropositivity against MKWV in wildlife captured in Hokkaido.

Animal species	Area	Year	IIF ^{*1}	NT ^{*2}
Yezo-deer (<i>Cervus nippon yezoensis</i>)	Hidaka	2010	5/27	1/5
		2011	1/23	1/1
		Total	6/50	2/6
Raccoons (<i>Procyon lotor</i>)	Mukawa	2007	1/7	1/1
		2010	0/6	-/-
	Kuriyama	2007	3/8	3/3
		2008	1/10	1/1
	Atsuma	2008	1/4	1/1
	Ebetsu	2007	5/10	5/5
		2008	3/10	2/3
		2010	3/9	3/3
		Total	17/64	16/17

^{*1}IIF: Indirect immunofluorescence assay (Positive / Tested)
Titers of 1:20 or greater were considered as positive results.

^{*2}NT: Neutralization assay (Positive / Tested)
Titers of 1:40 or greater were considered as positive results.

Table 4.
Detection of Anti-MKWV antibodies in wildlife in Hokkaido.

Sample ID	Animal species	Area	Year	IIF ^{*1} titers	NT ^{*2} titers
52863	Yezo-deer	Hidaka	2010	40	<40
52881	Yezo-deer	Hidaka	2010	40	160
52890	Yezo-deer	Hidaka	2010	20	<40
52900	Yezo-deer	Hidaka	2010	160	<40
53151	Yezo-deer	Hidaka	2010	80	<40
53195	Yezo-deer	Hidaka	2011	80	160
NP07-1	Raccoon	Ebetsu	2007	20	40
NP07-2	Raccoon	Ebetsu	2007	20	160
NP07-3	Raccoon	Ebetsu	2007	640	640
NP07-4	Raccoon	Ebetsu	2007	320	320
NP07-8	Raccoon	Ebetsu	2007	80	80
NP08-23	Raccoon	Ebetsu	2008	80	160
NP08-32	Raccoon	Ebetsu	2008	40	80
NP08-33	Raccoon	Ebetsu	2008	40	<40
NP10-7	Raccoon	Ebetsu	2010	20	40
NP10-10	Raccoon	Ebetsu	2010	80	160
NP10-11	Raccoon	Ebetsu	2010	2560	320
ATM08-30	Raccoon	Atsuma	2008	80	320
KUR07-4	Raccoon	Kuriyama	2007	40	40
KUR07-27	Raccoon	Kuriyama	2007	20	160
KUR07-28	Raccoon	Kuriyama	2007	40	160
KUR08-2	Raccoon	Kuriyama	2008	80	160
MKW07-4	Raccoon	Mukawa	2007	160	40

^{*1}IIF: Indirect immunofluorescence assay

^{*2}NT: Neutralization assay

To explore MKWV pathogenesis in adult mice, the MKWV isolate MKW73 was inoculated into groups of eight-week-old C57BL/6J mice, and their clinical signs were monitored until 14 dpi. None of the mice inoculated with MKWV demonstrated any clinical signs such as severe weight loss for 14 days. No infectious MKWVs were recovered from whole blood or tissues collected on 3, 7, and 14 dpi (data not shown), and MKWV RNA genomes were not detected in some tissues but not in whole blood (Fig. 3). On 7 and 14 dpi, MKWV RNA was detected in a limited number of organs that were inconsistent between animals (Fig. 3).

Discussion

Recently, an increasing number of viruses has been identified in ticks (Paules et al. 2018, Mansfield et al. 2017), and their potential to cause diseases in humans as well as animals is seriously discussed to disclose the impact on our public health. However, the ecological feature of these tick viruses has not fully addressed due to lack of comprehensive tick screening. The comprehensive screening of TBPVs in ticks in the present study was conducted on ticks collected in a limited area, i.e., Hokkaido, Japan, and discovery of novel viral RNAs suggested that a variety of TBPVs are still undiscovered in ticks worldwide. In this study, the genes of 2 putative TBPVs, MKWV and KURV, were detected from *I. persulcatus* ticks, and these viruses were isolated using the tick-derived ISE6 cell line. Though MKWV and KURV are genetically close to mosquito-borne viruses, replication of them in ISE6 cells indicated the major vectors of these viruses may be ticks. Infection of MKWV in mammals was also suggested by serological tests in wildlife and detection of viral RNA in animal experiments, while cross reactivity of antisera against MKWV to other endemic TBPVs may need to investigate carefully. Taken together, a tick-borne life cycle is indicated for MKWV.

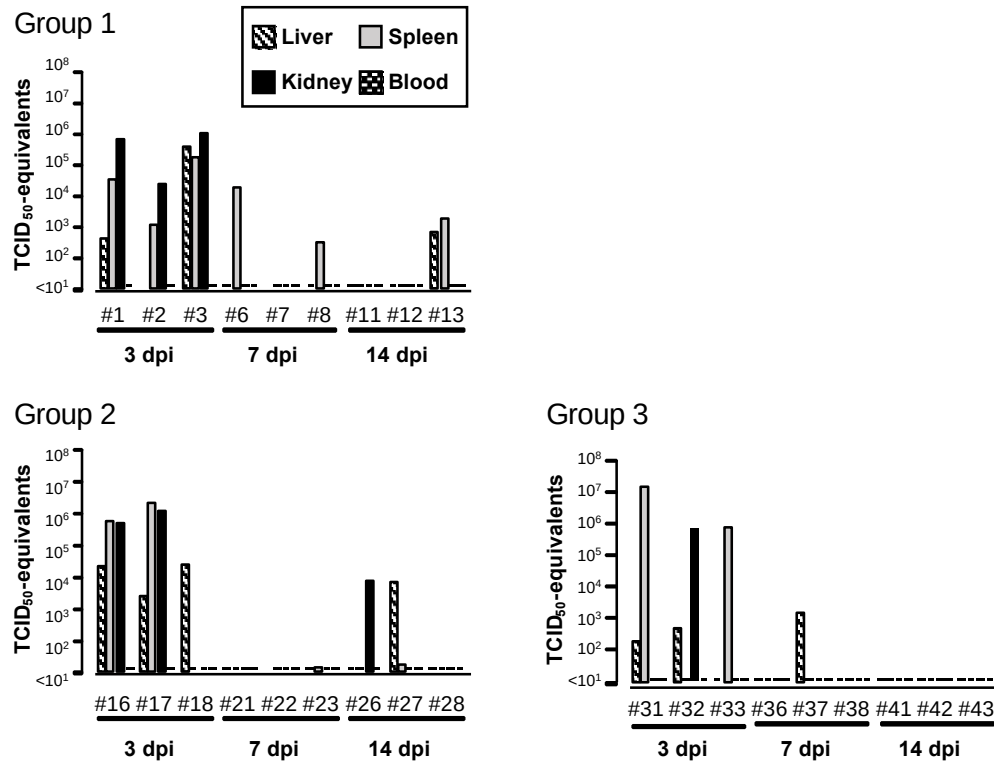


Fig. 3. Viral RNA loads in the MKWV-infected C57BL/6J mice.

Nine mice in each group were inoculated with 10^5 TCID₅₀ of Mukawa virus as follows: intramuscular inoculation with Huh-7 cell-passaged Mukawa virus (group 1), subcutaneous inoculation (group 2), or inoculation with ISE6 cell-passaged MKWV (group 3). At 3, 7, and 14 dpi, tissues (liver, spleen, and kidney) and whole blood collected from three individuals were subjected to reverse transcription followed by qPCR to quantify the viral RNA load. The load of MKWV RNA was indicated as the TCID₅₀/g-equivalents in tissues or TCID₅₀/ml-equivalents in whole blood. The IDs of mice are indicated as #number (1–63) below each graph.

Further experiments are demanded to confirm transmission of MKWV between ticks and mammals. However, the distribution of MKWV and KURV in ticks seemed sporadic and was not identical to the areas where antibody-positive animals were captured. Further investigation of *I. persulcatus* ticks using more sensitive detection methods for MKWV and KURV may be necessary to fill the gaps between genetic screening in ticks and serological studies in wild animals.

So far, both MKWV and its close relative KURV were detected only from *I. persulcatus* collected in Hokkaido, but not in Honshu island. While the number of *I. persulcatus* ticks collected in Honshu island was insufficient to prove the absence of MKWV and KURV using the pan-phlebovirus RT-PCR, the sporadic discoveries of MKWV and KURV in only a single tick species in limited areas suggested that their distribution could be restricted by host ticks as well as host animals. Testing on individual ticks allowed us to determine the detailed spatiotemporal distribution of MKWV and KURV rather than testing on pooled samples because a number of ticks collected in a single site is limited. So far, the detailed mechanism(s) underlying the emergence of similar viruses such as SFTS virus and HTRV, which are genetically related but discovered in different continents has not been clarified. Furthermore, selection pressure(s) to produce divergence in each TBPV species has also not been revealed yet. Since MKWV and KURV are closely related and were identified in a limited island, a spatiotemporal approach to reveal the evolutionary pressures defining and maintaining the divergence of MKWV and KURV may be crucial to understand process of TBPV evolution.

To assess the potential risk of MKWV, understanding its virulence in humans and livestock animals is essential. Although serological evidences indicate that MKWV infection occurred in wild animals, no infectious virus was recovered upon experimental

infection in adult C57BL/6 mice, which show no visible clinical signs. However, as the virulence of a TBPV may vary depending on the host animal species, it is necessary to confirm the virulence of MKWV using other models. An immunocompromised mouse model such as interferon- α receptor knockout mice (Liu et al. 2014, Müller et al. 1994), may be a useful fatal model for assessing the potential of these viruses to replicate in an animal model. Infection of MKWV in mammals as well as arthropods suggested the potential risk of spillover to humans in the future. Thus, the seroprevalence of MKWV in humans and/or domestic animals is also interesting to assess the public health concerns.

In the present study, I revealed that divergent TBPVs have been maintained in ticks in Hokkaido, and demonstrated possible infection of one of the TBPVs in wildlife animals. Virus isolation is essential to conduct downstream experiments such as serological assays, experimental infections to laboratory animals, and biological characterization. Since most of recently reported novel TBPVs have not been isolated, establishment of isolation methods for these TBPVs is essential for comprehensively understanding their potential risks to public health.

Accession numbers

The reported nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under accession numbers LC133180.1, LC133178.1 and LC133179.1.

Summary

Recent discoveries of tick-borne pathogens have raised public health concerns on tick-borne infectious diseases and emphasize the need to assess potential risks of unrecognized tick-borne pathogens. First, to determine the existence of TBPVs, genetic surveillance of phleboviruses in ticks was conducted mainly in Hokkaido, the northernmost island in Japan from 2013 to 2015. Genes of 2 TBPVs, previously reported as MKWV and a newly identified relative of MKWV, KURV, were detected and the viruses were isolated from *Ixodes persulcatus* collected in Hokkaido, but not in *I. persulcatus* collected from other areas of Japan. These viruses were phylogenetically and antigenically similar to each other. Next, to investigate the infection of MKWV in mammals, serum samples from wildlife captured in Hokkaido from 2007 to 2011 were used for serological screening. Neutralizing antibodies against MKWV were detected in both Yezo-deer (*Cervus nippon yesoensis*) (2/50) and raccoons (*Procyon lotor*) (16/64). However, no infectious MKWV was recovered from laboratory mice in experimental infections, though viral RNAs were detected in their tissues. Thus, MKWV and KURV may maintain tick-mammalian life cycles in Hokkaido, suggesting their potential as causative agents of tick-borne diseases in mammals.

Chapter 2

Discovery of Mwinilunga alphavirus: a novel alphavirus in *Culex* mosquitoes in Zambia

Introduction

Alphaviruses, belonging to the genus *Alphavirus* in the family *Togaviridae*, can infect diverse vertebrate hosts, including mammals, birds, reptiles, amphibians and fish (Griffin 2013). Most alphaviruses are mosquito-borne and are transmitted by mosquitoes from at least 8 genera (Webb et al. 2008, Ortiz, Anishchenko and Weaver 2005). Restricted interactions between viruses, their invertebrate vector species and vertebrate hosts usually occur in nature; however, some alphaviruses occasionally can cause widespread epizootics (Weaver and Reisen 2010). Humans and/or animals infected by pathogenic alphaviruses exhibit febrile illnesses that may culminate either in encephalitis or arthritis, depending upon the viral etiology (Atkins 2013).

In Africa, human diseases caused by the Old World alphaviruses, such as CHIKV, o'nyong-nyong virus and Sindbis virus (SINV), have been reported (Lwande et al. 2015). While the Republic of Zambia is located in southern Africa and vector mosquitoes of alphaviruses have been found widely in the country (Gaffigan et al. , Kent 2006), human cases of Chikungunya fever (CHIKF), o'nyong-nyong fever or Sindbis fever have not been documented in prior studies (Lwande et al. 2015, Rezza, Chen and Weaver 2017, Storm et al. 2014) (<https://www.cdc.gov/chikungunya/index.html>). Moreover, comprehensive detection of alphaviruses in mosquitoes has not been conducted in Zambia to date. Therefore, screening of vector mosquitoes in Zambia is crucial to better predict future outbreaks of alphaviral diseases.

The alphavirus genome consists of a single-stranded, positive-sense RNA, encoding nonstructural proteins (nsPs; nsP1-nsP4), which are important for transcription and replication of viral RNA (Lemm and Rice 1993), and structural proteins (sPs; Capsid, E3, E2, 6k and E1), which are the main constituents of virions (Voss et al. 2010). Based on genetic and serological similarities, around 30 species of alphaviruses are classified into antigenic complexes (Powers et al. 2001, Powers et al. 2012). In addition, an unclassified alphavirus, Eilat virus (EILV), has been discovered in *Anopheles coustani* (Nasar et al. 2012) and Tai Forest alphavirus (TALV) has been recently discovered in *Culex decens* (Hermanns et al. 2017).

EILV appears to infect only mosquitoes and differs from other known mosquito-borne alphaviruses which generally maintain their life cycles between mosquitoes and vertebrate hosts, although EILV is phylogenetically grouped within the mosquito-borne clade (Nasar et al. 2012, Nasar et al. 2014). Studies of EILV have contributed to the identification of the molecular determinants responsible for host range restriction and provided new insights into the evolutionary history of alphaviruses. However, further studies on insect-only alphaviruses are required for a better understanding of the ecology and evolutionary relationships in the genus *Alphavirus* and the molecular determinants of host range restriction (Nasar et al. 2015b, Nasar et al. 2015a). In this study, I have focused on the detection of alphaviruses in mosquitoes collected in Zambia.

Materials and Methods

Study site and Mosquito collection

A total of 9,699 mosquitoes were collected from various locations (Fig. 4) between 2014 and 2017 (Table 5). Mosquito collection was carried out in protected areas with

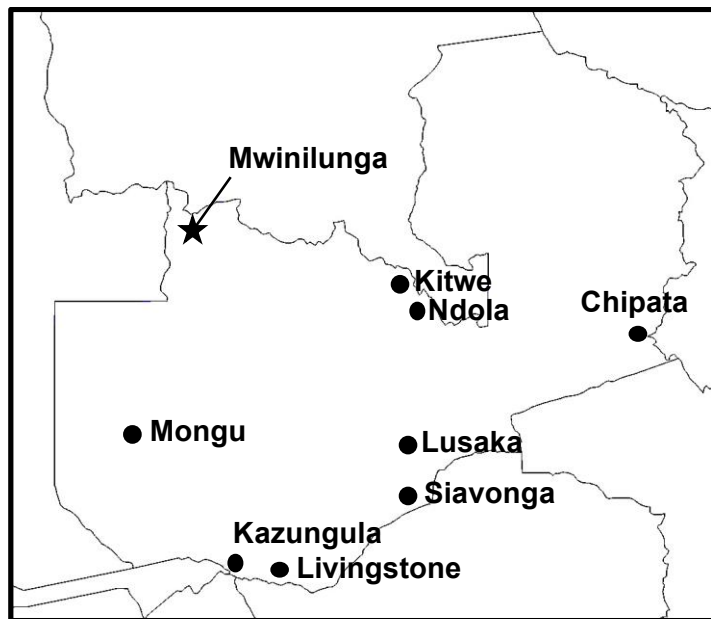


Fig. 4. The map of study sites in Zambia.

Geographic areas where mosquitoes were collected from 2014 to 2017 (black circles) are shown. The district where the alphavirus-positive mosquito pool was collected in North-Western Province is indicated by a black star.

Table 5. Information of collected mosquitoes

Year	Place	Mosquito genus	Pool no.	Mosquito no.	
2014	Lusaka	<i>Culex</i>	10	258	
	Mongu	<i>Anopheles</i>	2	19	
		<i>Coquillettidia</i>	6	19	
		<i>Culex</i>	52	1399	
		<i>Mansonia</i>	6	26	
2015	Chipata	<i>Aedes</i>	1	1	
		<i>Culex</i>	16	306	
	Kazungula	<i>Aedeomya</i>	1	1	
		<i>Aedes</i>	3	5	
		<i>Anopheles</i>	9	38	
		<i>Culex</i>	5	45	
		<i>Mansonia</i>	1	2	
	Livingstone	<i>Aedes</i>	3	12	
		<i>Anopheles</i>	3	4	
		<i>Culex</i>	27	774	
		<i>Mansonia</i>	1	8	
	Lusaka	<i>Aedes</i>	3	20	
		<i>Anopheles</i>	1	1	
		<i>Culex</i>	54	1574	
		<i>Mansonia</i>	2	2	
	2016	Kitwe	<i>Culex</i>	10	275
		Mongu	<i>Aedes</i>	3	4
<i>Anopheles</i>			25	366	
<i>Coquillettidia</i>			10	153	
<i>Culex</i>			23	413	
<i>Mansonia</i>			5	99	
Mwinilunga		<i>Uranotaenia</i>	1	2	
		<i>Aedes</i>	4	4	
		<i>Anopheles</i>	2	3	
		<i>Culex</i>	6	40	
		<i>Uranotaenia</i>	1	2	
Ndola		<i>Culex</i>	2	19	
Siavonga		<i>Aedes</i>	22	141	
		<i>Anopheles</i>	3	3	
		<i>Culex</i>	19	385	
2017		Livingstone	<i>Aedes</i>	8	12
			<i>Anopheles</i>	16	73
	<i>Culex</i>		46	812	
	<i>Mansonia</i>		2	2	
	Mongu	<i>Aedeomya</i>	2	10	
		<i>Aedes</i>	4	11	
		<i>Anopheles</i>	66	1451	
		<i>Coquillettidia</i>	9	62	
		<i>Culex</i>	49	710	
		<i>Mansonia</i>	7	132	
		<i>Uranotaenia</i>	1	1	
Total			552	9.699	

the permission of the Zambia Wildlife Authority, now the Department of National Parks and Wildlife, Ministry of Tourism and Arts of the Republic of Zambia and the Excellence in Research Ethics and Science Converge Ethics Committee IRB (No: 00005948). Species were at first identified morphologically using a stereomicroscope, and subsequently some species were genetically confirmed by PCR and sequencing of the cytochrome-oxidase subunit I gene (Folmer et al. 1994).

RNA extraction from mosquitoes

After species identification, mosquito samples were divided into 552 pools containing 1 to 40 individual mosquitoes, belonging to the same species and sampling location. Subsequently, the pools were homogenized with Minimum Essential Medium (MEM) containing 2% FBS (Sigma) using the BioMasher (Nippi). Total RNAs were extracted from 100 µl of the homogenates using the Direct-zol kit (Zymo Research) according to the manufacturer's protocol and the remaining homogenates were stored at -80°C until use for virus detection.

Detection of alphavirus genomes

I employed a one-step RT-PCR assay for detection of a wide-range of alphaviruses in the extracted mosquito RNAs. Oligonucleotide primers for the RT-PCR assay were designed based on an alignment of the conserved region of nsP4 among defined alphavirus species. One-step RT-PCR was performed with the PrimeScript One Step RT-PCR Kit, Ver. 2 with 1 µl of extracted mosquito RNA and 1 µM (final concentration) of each of the primers, pan-Alpha-nsP4-6692F (5'-CAYACRYTRTTYGAYATGTCDGC-3') and pan-Alpha-nsP4-7152R (5'-GCRTCDATKATYTTBACYTCCAT-3') in 15 µl reaction volumes, with the

following thermocycling protocol: 50°C for 30 minutes; 94°C for 2 minutes; 43 cycles of 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 30 seconds; and 72°C for 5 minutes. The PCR products (approximately 460 bp) were visualized on a 1% (w/v) agarose gel stained with ethidium bromide. CHIKV and SINV nsP4 genes were confirmed to be detected by this methodology (data not shown). The amplified products were sequenced using the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) with the pan-Alpha-nsP4-6692F primer and pan-Alpha-nsP4-7152R primer, and the nucleotide sequences were analyzed using GENETYX, version 13.

Virus isolation

The remaining homogenates of RT-PCR-positive mosquitoes were subjected to virus isolation. Homogenized samples of mosquitoes were diluted five-fold with DMEM supplemented with 2% FBS, penicillin, streptomycin, gentamycin and 2 mM L-glutamine, filtered and used to inoculate onto *Aedes albopictus* cells (C6/36), African green monkey kidney cells (Vero) and baby hamster kidney cells (BHK-21). After inoculation of the mosquito homogenates, supernatants of the cells were cultured through 3 passages for 3 weeks. Viral production into the supernatant was checked by RT-PCR with extracted RNA from supernatants of cultured cells and the pan-Alpha-nsP4 primers after 3 blind passages.

Determination of nucleotide sequences of full-length viral genome

To determine the full-length viral genome sequence, RNA extracted from the mosquitoes was subjected to NGS. A library was constructed by the Nextera XT DNA Library Prep kit (Illumina). Sequencing was performed with a MiSeq Reagent Kit v3 (600 cycles) and Illumina MiSeq System (Illumina) following the manufacturer's

protocol. After trimming low-quality reads, the resulting reads were *de novo* assembled using a CLC Genomics Workbench 10 (CLC bio) with default parameters. The sequences were confirmed by specific PCRs using specific primer pairs. The 5'- and the 3'- terminal regions were amplified using the RACE approach with the SMARTer RACE cDNA Amplification Kit (TAKARA) and specific primers. Amplified products were directly sequenced in both directions by Sanger sequencing. The full-viral genome sequence was determined and the amino acid sequences were predicted using GENETYX, version 13.

Phylogenetic analysis

The full-length nucleotide sequences encoding either the nsPs or sPs ORFs of detected virus and alphaviruses available at GenBank were aligned using the ClustalW protocol (Thompson, Higgins and Gibson 1994). Multiple sequence alignments were modified manually. For the alignments, the C terminus of nsP3 and the N terminus of capsid sequences were excluded, since these regions have numerous insertions and deletions and extensive sequence divergence in previous studies (Forrester et al. 2012, Nasar et al. 2012). Phylogenetic trees were constructed by maximum-likelihood method using MEGA, version 6.0 (Tamura et al. 2013). The robustness of the nodes was tested by 1,000 bootstrap replications.

A linear discriminant analysis

A linear discriminant analysis was performed based on the dinucleotide ratios in various virus sequences, which has been reported previously to determine host specificity based on the frequencies of dinucleotide in flavivirus sequences (Colmant et al. 2017). Complete genome sequences from 53 mosquito-borne alphaviruses with a

defined host (bird, horse, donkey, human, hamster and mosquito) and full-length sequences of nsPs and sPs from MWAV, EILV, TALV, Trocara virus (TROV) and Aura virus (AURAV) were used for the analysis (Table 6). The dinucleotide ratios were calculated using the formula $P_{XY} = f_{XY}/f_X f_Y$, in which f_X and f_Y denote the frequencies of mononucleotides X and Y, respectively, and f_{XY} denotes the frequency of dinucleotide XY (Lobo et al. 2009, Karlin and Mrázek 1997). The linear discriminant analysis was performed in the R package (Colmant et al. 2017, Di Giallonardo et al. 2017) (<https://www.R-project.org/>).

Results

Genetic surveillance of alphaviruses in mosquitoes collected in Zambia

To analyze the prevalence of alphaviruses in mosquitoes in Zambia, mosquitoes were collected from various locations and examined for alphavirus genes by one-step RT-PCR. Based on morphological identification, the largest numbers of mosquito species were within the genus *Culex*. From 552 pools, one pool, which contained 19 *Culex quinquefasciatus* mosquitoes collected in Mwinilunga (Fig. 4, North-Western Province of Zambia) in 2016, was found to be positive by RT-PCR assay. Sequence comparison of the amplified fragment in the National Center for Biotechnology Information database employing BLASTN analysis showed 76% nucleotide identity to TALV and 75% identity to EILV. The detected alphavirus was tentatively named Mwinilunga alphavirus (MWAV). Other known alphaviruses were not detected in the present study. Next, MWAV was attempted to isolate from RT-PCR-positive mosquitoes using C6/36, Vero and BHK-21 cells; however, proliferation of MWAV was not detected in any of the cell lines tested.

Table 6. Reference sequences for linear discriminant analysis

Accession number	Species	Isolation host	Accession number	Species	Isolation host
NC 018615	Eilat virus	Mosquito	NC 023812	Madariaga virus	Human
NC 032681	Tai Forest alphavirus	Mosquito	NC 003417	Mayaro virus	Human
NC 003900	Aura virus	Mosquito	KY302801	Ross River virus	Human
HM147991	Trocar virus	Mosquito	JQ771794	Sindbis virus	Human
GU001935	Eastern equine encephalitis virus	Bird	JQ771797	Sindbis virus	Human
GU001913	Eastern equine encephalitis virus	Bird	JQ771798	Sindbis virus	Human
GU001914	Eastern equine encephalitis virus	Bird	KC344505	Venezuelan equine encephalitis virus	Human
FJ827631	Highlands J virus	Bird	KP282671	Venezuelan equine encephalitis virus	Human
GU167952	Highlands J virus	Bird	GQ287640	Western equine encephalomyelitis virus	Human
KT429024	Highlands J virus	Bird	KC344491	Venezuelan equine encephalitis virus	Hamster
KJ409555	Highlands J virus	Bird	KC344515	Venezuelan equine encephalitis virus	Hamster
KT429026	Highlands J virus	Bird	GU908223	Chikungunya virus	Mosquito
GU001917	Eastern equine encephalitis virus	Horse	AY726732	Chikungunya virus	Mosquito
GU001915	Eastern equine encephalitis virus	Horse	HM045805	Chikungunya virus	Mosquito
GU001920	Eastern equine encephalitis virus	Horse	HM045818	Chikungunya virus	Mosquito
U01557	Eastern equine encephalomyelitis virus	Horse	HM045820	Chikungunya virus	Mosquito
KT429021	Highlands J virus	Horse	HM045815	Chikungunya virus	Mosquito
KT429023	Highlands J virus	Horse	HM045819	Chikungunya virus	Mosquito
AY973944	Venezuelan equine encephalitis virus	Horse	HM045784	Chikungunya virus	Mosquito
AY986475	Venezuelan equine encephalitis virus	Horse	HM045785	Chikungunya virus	Mosquito
L01442	Venezuelan equine encephalitis virus	Donkey	GU361116	Sindbis virus	Mosquito
KJ554965	Western equine encephalitis virus	Horse	JQ771793	Sindbis virus	Mosquito
GQ287645	Western equine encephalomyelitis virus	Horse	JQ771799	Sindbis virus	Mosquito
GQ287643	Western equine encephalomyelitis virus	Horse	KC344525	Venezuelan equine encephalitis virus	Mosquito
KX702402	Chikungunya virus	Human	KC344512	Venezuelan equine encephalitis virus	Mosquito
KX496989	Chikungunya virus	Human	KC344500	Venezuelan equine encephalitis virus	Mosquito
KY703990	Chikungunya virus	Human	KJ554983	Western equine encephalitis virus	Mosquito
KY704955	Chikungunya virus	Human	KJ554989	Western equine encephalitis virus	Mosquito
KX000164	Eastern equine encephalitis virus	Human			

Characterization of MWAV

Full-genome sequences of MWAV were determined by NGS and MWAV-specific primer sets followed by the RACE method. The MWAV genome consisted of 11,547 bp, coding 2 ORFs (2,439 and 1,235 amino acids) in the positive sense (Fig. 5). The full-genome sequence of MWAV was deposited in the GenBank/EMBL/DDBJ database under accession number LC361437. The pairwise nucleotide identities of MWAV were 70% to EILV, 58% to TALV and 52% to SINV. BLAST analysis indicated that the deduced amino acid sequence of a ORF (2,439 amino acids) was related to that of nsPs from alphaviruses and the highest identity was 83% to EILV. The deduced amino acid sequence of the second ORF (1,235 amino acids) was related to that of sPs from alphaviruses and the highest identity was 76% to TALV. The pairwise amino acid identities of MWAV ranged between 64% (E2 protein) and 88% (nsP1) to EILV for the corresponding proteins, and between 68% (nsP3) and 87% (nsP4) to TALV for the corresponding proteins (Fig. 5). According to the species designation criteria of the International Committee on Taxonomy of Viruses (Powers et al. 2012), the genetic analysis of MWAV suggested that this represented a novel alphavirus species, closely related to EILV and TALV.

Since conserved sequence elements (CSEs) have important roles in the lifecycle of alphaviruses (Hyde et al. 2015, Niesters and Strauss 1990, Fayzulin and Frolov 2004, Zhu, Zhao and Liang 2013), the sequence identities of CSEs were examined with other defined alphaviruses. The CSEs of MWAV within the nsP1 51 bp, subgenomic promoter sequence and the 3'-untranslated region (UTR) shared more than 90% nucleotide sequence identities with EILV and TALV (Fig. 6). The identity of the CSE within the putative 5'-UTR was 80% to that of EILV, and mFold analysis suggested that the putative 5'-UTR of MWAV might form secondary stem-loop structures, similar to

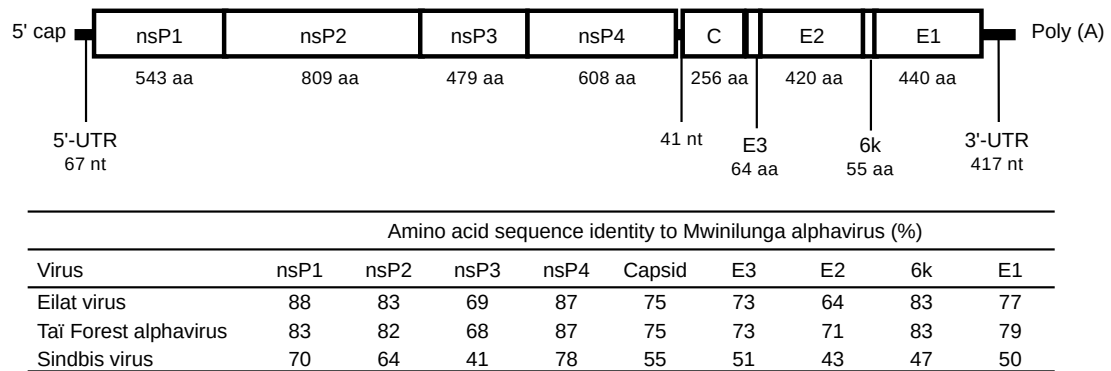


Fig. 5. Genome organization of MWAV.

The nucleotide lengths of the UTRs and amino acid sizes of each protein are displayed under the genome. Amino acid sequence identities of each protein between MWAV and closely related alphaviruses are indicated.

(A)	
Virus	5'-UTR
Mwinilunga alphavirus	ACAUGGGGAUAGGCUAUAUAACACACAAU-UAAACCCAGUACCAAAUAGCCUCCACUUUCAUCGAAU
Eilat virus	-----GC.C.UU....U.AG...U.----GUA.CG.....U.....C
Tai Forest alphavirus	-----CG.U....C.U....U.GU.GAU.....U.....
(B)	
Virus	nsP1 51 b CSE
Mwinilunga alphavirus	ACAGGUCACUCCGAAUGACCACGCCAAUGCGAGGGCGUUCUCGCAUUGCGC
Eilat virus	CAU.....C.....A..C.....C.....
Tai Forest alphavirus	C.U.....C.....U..A..U.....
Trocar virus	G.....G.C.....U..U.....C..A.....CUG..
Aura virus	G.....U..U.....C..A..U..U.....CUG..
Western equine encephalitis virus	G.....GAC.....U.....C..A.....U.....GUG..
Sindbis virus	G.....A.....U..U.....C..A..A..U.....CUG..
Eastern equine encephalitis virus	G.....GAC.....U..U.....U.....U.....CCUA..
Venezuelan equine encephalitis virus	G.....GAU.....U..U.....C..A.....U.....CUG..
Ross River virus	G.....A..C.....U..U.....C..A..U..U.....CUG..
Semliki forest virus	G.....A..A.....U..A.....C..A..A..U.....CCUG..
Una virus	G.....A.....U..G..C.....U.....CCU..
Chikungunya virus	G.....A.....U..U.....U..A.....CUA..
Barmah Forest virus	G..AC..A..A..C..U..U..AC.C.....U.....CCUU..
Southern elephant seal virus	G.....A..U.....U.....C..A..U..U.....CUG..
Salmon pancreas disease virus	CA.UAGGU.GU.U..C.....U...GCC..C..A..U.....C..C.UG..
(C)	
Virus	Subgenomic promoter
Mwinilunga alphavirus	CCCUCUACAACUGACCUGAAAUAUAGU
Eilat virus	A.....
Tai Forest alphavirus	A...G...GGUG.....
Trocar virus	A.....G..A...U...C..
Aura virus	A.....GGUG.U.....A
Western equine encephalitis virus	GG.....G
Sindbis virus	AU.....GGUG.U.....
Eastern equine encephalitis virus	GG.....G
Venezuelan equine encephalitis virus	.U.....GG..A...G...G..A
Ross River virus	A.....GG..G..U.....A
Semliki forest virus	A.....GG..G..U...G..U..G
Chikungunya virus	.UU..G...GG..G..U.....G
Barmah Forest virus	AU.....GGUG.U.....
Southern elephant seal virus	.G.....GG...U.....A
Salmon pancreas disease virus	GU..A...U...U.
(D)	
Virus	3'-UTR
Mwinilunga alphavirus	AUUUUGUUUUUAUAUUUC
Eilat virus	.A.....C
Tai Forest alphavirus	
Western equine encephalitis virus	A.....
Sindbis virus	C.....
Eastern equine encephalitis virus	
Venezuelan equine encephalitis virus	
Ross River virus	U..G..U.....UAC
Semliki forest virus	.A..G.....C
Una virus	...GGU.....C
Barmah Forest virus	...GU.....UAC
Salmon pancreas disease virus	C..A...G.....A...UCAAUAC

Fig. 6. Comparison of deduced CSEs of alphaviruses.

Alignment of the deduced nucleotide sequences of CSEs were analyzed with the MWAV and selected alphavirus sequences using MEGA, version 6.0. Alignment of CSE (A) in the 5'-UTR region, (B) in nsP1 51 nucleotide ORF, (C) in subgenomic promoter and (D) in the 3'-UTR region. Nucleotides identical to MWAV sequence are shown with black dots.

EILV (data not shown) (Zuker 2003). In addition, the conserved amino acid motifs, such as the ribosomal binding site, conserved RdRp motifs of MWAV were similar to those of other alphaviruses (data not shown) and the putative protease cleavage sites resembled those of EILV (Fig. 7). These sequence similarities suggest that MWAV may possess similar biological properties to EILV, which also displays a narrow vector range in mosquitoes (Nasar et al. 2014).

In both phylogenies based on nucleotide sequences of nsPs and sPs, MWAV was classified into the same phylogroup with EILV and TALV (Fig. 8), suggesting that MWAV belongs to the same antigenic complex with these 2 viruses in agreement with the genome analysis (Fig. 5). In both the nsPs and sPs trees, Eilat-like viruses (EILV, TALV and MWAV) have the same ancestral virus within the Western equine encephalitis (WEE) complex, placed between TROV and AURAV, as has been reported previously (Nasar et al. 2012, Hermanns et al. 2017).

Prediction of the host range of MWAV

The host range of MWAV were predicted by linear discriminant analysis based the dinucleotide ratios in various virus sequences as previously reported. The analysis showed correlations of dinucleotide usage patterns between alphaviruses and their host ranges (Fig. 9). Mosquito-borne alphaviruses showed similar dinucleotide usage patterns regardless of their phylogenetic relationships and hosts. On the other hands, the dinucleotide usage pattern of Eilat-like viruses was also similar to each other, indicating that MWAV may be a mosquito-specific alphavirus. Interestingly, TROV and AURAV, who have no known vertebrate hosts showed a different pattern from those of mosquito-borne alphaviruses and Eilat-like viruses.

Virus	nsP1/nsP2	nsP2/nsP3	nsP3/nsP4	Capsid/E3	E3/E2
Mwiniunga alphavirus	DVGG/ALVE	EIGA/APSY	GAGG/YIFS	TVEW/SAIV	RSKR/SAPG
Eilat virus	.I../....	GV../....	/....	/....	.AR../AVAP
Tai Forest alphavirus	/....	GV../....	/....	/....	.IR../.TQP
Trocar virus	.I.A/...D	G..C/....	.V../....	..K../.AT	.P../.TEL
Aura virus	.A.A/....	GS../....	.V../....	/.RAI	.HV../.T.T
Western equine encephalitis virus	EA.A/GS..	EA.R/..A.	E..A/....	SES../.LVT	.Q../.ITD
Sindbis virus	.I.A/....	GV../....	.V../....	.E../.AP	/.VID
Eastern equine encephalitis virus	EA.A/GS..	EA.R/..A.	E..A/....	SEP../.LAT	.TR../DLDT
Venezuelan equine encephalitis virus	EA.A/GS..	EA.C/....	D..A/....	CEQ../.LVT	.KR../.TEE
Ross River virus	RA.A/GV..	TA.C/....	R..A/....	.E../.AL	.HR../.VTE
Semliki forest virus	HA.A/GV..	TA.C/....	R..A/....	SE../.PL	.HR../.VS.
Una virus	RA.A/GV..	TA.C/..A.	R.../....	/.PL	.HR../.VT.
Chikungunya virus	RA.A/GII.	RA.C/....	R.../....	AE../.LAI	.QR../.IKD
Barmah Forest virus	RA.E/GV..	PA.S/..A.	R.../....	S.../..AA	.PK../.VAH
Southern elephant seal virus	RA.A/GV..	PA.T/..N.	R..A/....	/.LT	.GK../.VAS
Salmon pancreas disease virus	GA.A/TIID	MV../..G.	.L../....	AIP../TRAP	.RK../AVST

Fig. 7. Comparison of the protease cleavage sites of alphaviruses.

Alignment of the amino acid sequences of protease cleavage sites were analyzed with MWAV and selected alphavirus sequences using MEGA, version 6.0. Conserved residues are indicated with black dots.

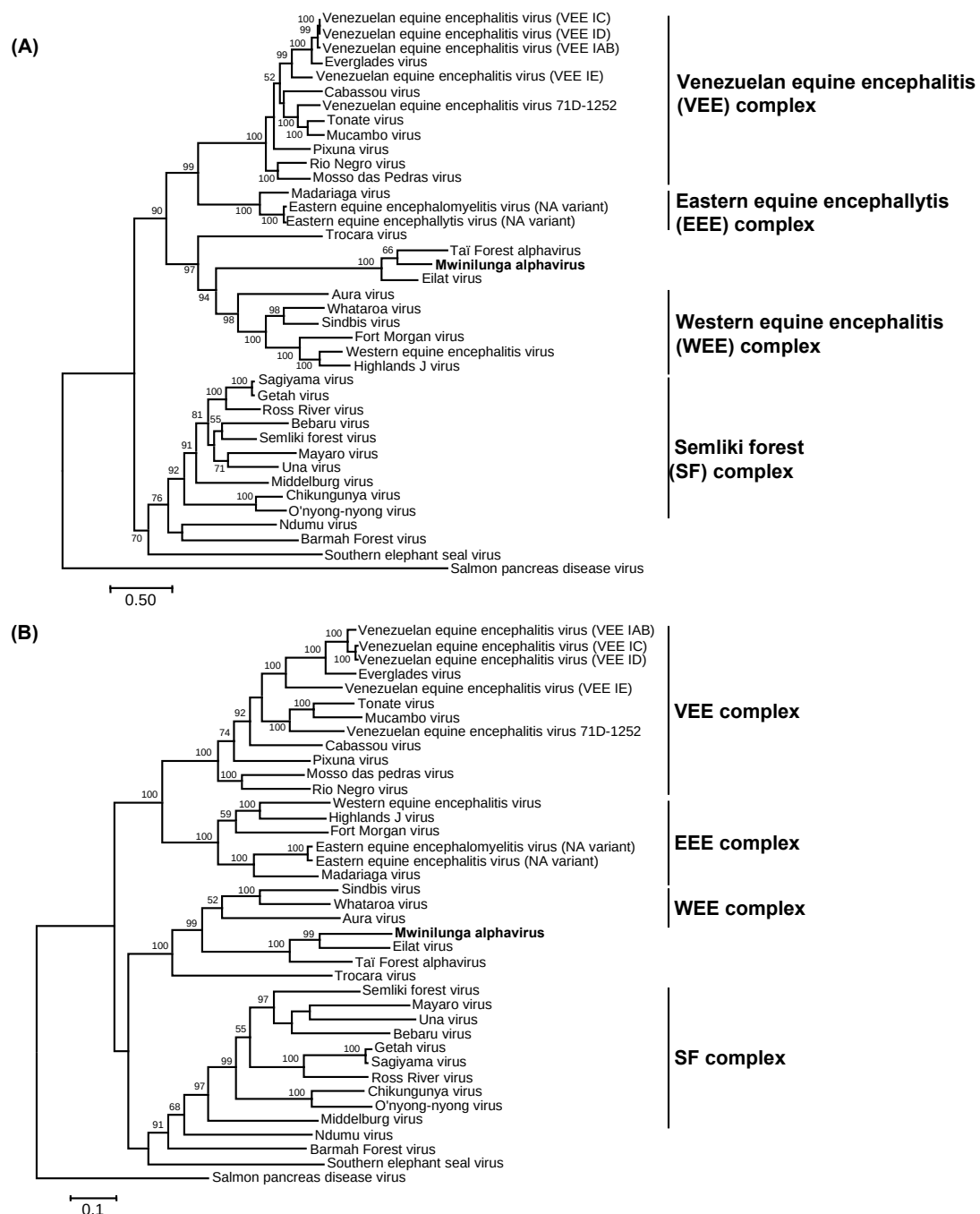


Fig. 8. Phylogenetic analysis of MWAV with previously described alphaviruses. Phylogenetic trees were constructed using the maximum-likelihood method in MEGA, version 6.0 with 1,000 bootstrap replicates based on multiple alignment of nucleotide sequences of the (A) sPs and (B) nsPs. Bootstrap values greater than 50 are shown near the branch nodes and the scale bar indicates the number of substitutions per site.

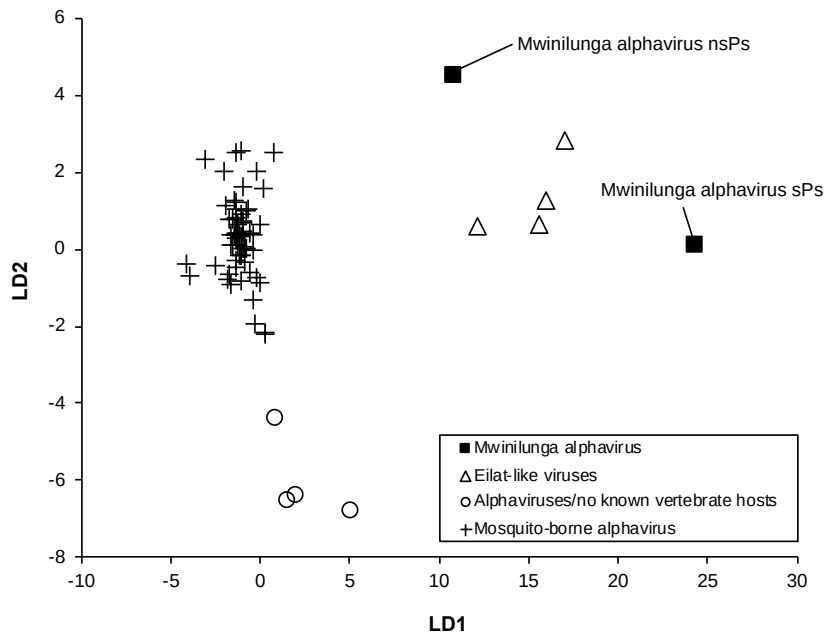


Fig. 9. Score plot of the linear discriminant analysis.

The figure shows a scatterplot of the two discriminant scores that explain the largest amount of the components from the linear discriminant analysis [72.3% and 16.2% for Linear discriminant 1 (LD1) and LD2, respectively]. Markers represent values for individual sequences as follows: complete genome sequences from mosquito-borne alphaviruses (crosses), full length of nsPs and sPs sequences from MWAV (black squares), Eilat-like viruses (triangles) or alphaviruses with no known vertebrate hosts (circles).

Discussion

Mosquito-borne alphaviral diseases have been reported largely in African countries, however the distribution of alphaviruses has not been examined in mosquitoes in Zambia to date. Here, my comprehensive and specific screening for alphaviruses has identified a novel alphavirus, tentatively named Mwinilunga alphavirus from *Culex quinquefasciatus* mosquitoes in Zambia. Complete genome sequence analyses revealed that MWAV is a novel alphavirus species highly similar to EILV, a mosquito-specific alphavirus, which is notably distinct from other known mosquito-borne alphaviruses due to its inability to replicate in vertebrate cell lines. Here, I report the existence of unique alphavirus, which will be a key to the better understanding of the evolution of alphaviruses.

Eilat-like viruses, including EILV, TALV and MWAV, were detected from different mosquito species including *Anopheles coustani* (Nasar et al. 2012), *Culex decens* (Hermanns et al. 2017) and *Culex quinquefasciatus*, respectively. And EILV were reported to display a narrow vector range in mosquitoes (Nasar et al. 2014). If MWAV can infect to only some mosquito species as same as EILV, this may potentially explain why MWAV could not be isolated using *Aedes*-derived and mammalian-derived cells. To acquire MWAV for the further analyses, different isolation methods using *Culex* mosquito and/or *Culex* mosquito-derived cells should be desired. Otherwise, to generate MWAV and chimeric viruses with other mosquito-borne alphavirus seems to be useful similar to the analyses of life cycle of insect-specific flaviviruses (Piyasena et al. 2017, Piyasena et al. 2019).

Although Eilat-like viruses have been detected in the Old World, TROV and AURAV have also been isolated from mosquitoes in South American countries (Rümenapf, Strauss and Strauss 1995, Travassos da Rosa et al. 2001). Similar to

Eilat-like viruses, vertebrate hosts of both TROV and AURAV have not been identified (Forrester et al. 2012). If Eilat-like viruses and phylogenetically related viruses have adapted to specific mosquito hosts and relied on vertical and/or venereal transmission, it is possible that these viruses have not been spread *via* vertebrate hosts to other mosquito species. Therefore, 1) Eilat-like viruses, and phylogenetically related viruses, may exist in different parts of the world and have lifecycles involving vertical and/or venereal transmission exclusively in invertebrate mosquito hosts, or 2) Eilat-like viruses might be sporadically transported from other areas and based on a previous report, geographic introductions of alphaviruses may have repeatedly occurred (Forrester et al. 2012). It is possible that Eilat-like viruses and/or their ancestral viruses might have lost their ability to infect vertebrates after being spread to other mosquito species in the Old World. Analyses of the biological properties of MWAV and TALV after isolation will greatly help to clarify the factors that determine the host range and the evolutionary histories of the Eilat-like alphaviruses.

This study revealed that unrecognized alphaviruses still exist in mosquitoes. Further epidemiological and functional studies investigating arthropod and animal host species will help to better characterize future alphaviral disease risk and also provide new insights into their ecology.

Summary

Mosquito-borne alphaviruses are disseminated globally and cause febrile illness in humans and animals. Since the prevalence and diversity of alphaviruses has not been previously investigated in Zambia, RT-PCR was employed as a broad-spectrum approach for the detection of alphaviruses in mosquitoes. From 552 mosquito pools, a novel alphavirus, tentatively named MWAV, was discovered from a single *Culex quinquefasciatus* mosquito pool. The full genome of MWAV was subsequently determined, and pairwise comparisons demonstrated that MWAV represented a new alphavirus species. Phylogenetic analyses and a linear discriminant analysis based on the dinucleotide ratios in various virus sequences indicated that MWAV is related to a mosquito-specific alphavirus distinct from other known mosquito-borne alphaviruses. Further analyses of these novel alphaviruses will help to facilitate a greater understanding of the molecular determinants of host range restriction and the evolutionary relationships of alphaviruses.

Chapter 3

Chikungunya virus uniquely recruits the endosomal sorting complexes required for transport (ESCRT) trafficking machinery at multiple steps during the intracellular replication cycle

Introduction

CHIKF is a re-emerging zoonotic disease caused by CHIKV, a member of the genus *Alphavirus*, family *Togaviridae* (Chen et al. 2018). The major symptoms of CHIKF are an acute febrile illness accompanied by arthralgia and rash (Pialoux et al. 2007, Couderc and Lecuit 2015, Borgherini et al. 2007). Arthralgias may persist, and relapse, for weeks to months and even years, consequently reducing the quality of life of CHIKF patients (Borgherini et al. 2008, Schilte et al. 2013). During the major outbreaks of CHIKF in the Indian Ocean islands in 2005, following the re-emergence of CHIKF in Kenya (Chretien et al. 2007), CHIKF became disseminated worldwide *via* thousands of infected travelers (Weaver and Forrester 2015). To date, CHIKF cases have been identified in over 60 countries, including outbreaks in Europe and the Americas (Rezza et al. 2007, Grandadam et al. 2011, Leparac-Goffart et al. 2014). Effective control measures for CHIKF are desired; however, no commercial vaccines nor effective antiviral treatments currently exist. To develop new treatment strategies for CHIKV infection, a better understanding of the molecular mechanisms underlying CHIKV infection is required.

The genome of CHIKV is a positive sense, single-stranded RNA composed of two open reading frames, encoding both nonstructural and structural polyproteins. The nsPs consisting of nsP1-4 are required for transcription and replication of viral RNA

(Solignat et al. 2009, Rupp et al. 2015). The sPs, comprising the capsid (C) and envelope glycoproteins (E2 and E1), are the main constituents of virus particles and facilitate receptor binding and cellular entry (Voss et al. 2010, Solignat et al. 2009). The CHIKV replication cycle begins with cellular attachment and entry *via* endocytosis (van Duijl-Richter et al. 2015). The post-entry steps of CHIKV have not been fully characterized but genome replication may take place both at the plasma membrane and type I cytopathic vacuoles (CPV-I), based on data obtained with other alphaviruses [e.g. Semliki Forest virus (SFV)] (Spuul et al. 2010, Froshauer, Kartenbeck and Helenius 1988). Colocalization of SFV nsPs and newly synthesized viral RNAs is initially observed in spherule structures near the plasma membrane (Spuul et al. 2010). SFV replication complexes are then thought to be transported to CPV-I originating from late endosomes and lysosomes (Spuul et al. 2010, Froshauer et al. 1988). At the late stages of the viral infection, the newly synthesized E1/E2 glycoproteins are transported to the endoplasmic reticulum (ER), trans-Golgi network (TGN), TGN-derived CPV-II and finally the plasma membrane (de Curtis and Simons 1988, Griffiths, Quinn and Warren 1983, Griffin 2013). Following the formation of nucleocapsid by binding of the alphaviral C protein and RNA (Geigenmüller-Gnirke, Nitschko and Schlesinger 1993, Perera et al. 2001), virus particles are assembled and released from the plasma membrane (Griffin 2013). Both CPV-I and -II have been observed in CHIKV-infected cells and CHIKV has been shown to bud from the plasma membrane (Chen et al. 2013, Solignat et al. 2009). Although alphaviruses seem to be transported by a dynamic and unique subcellular trafficking machinery, little is known about the host factors involved in the intracellular CHIKV trafficking processes (Bernard et al. 2010, Thomas et al. 2013).

A variety of enveloped viruses can exploit the cellular ESCRT in their infection steps

(Votteler and Sundquist 2013). In the ESCRT pathway, which is highly conserved across Eukarya, ubiquitinated proteins are sorted into the multivesicular bodies (MVBs) by deforming the membrane inwards. A series of four distinct complexes (ESCRTs -0, -I, -II and -III) and vacuolar protein sorting 4 (VPS4) proteins are sequentially recruited for membrane deformation (Henne, Buchkovich and Emr 2011). Since human immunodeficiency virus type 1 (HIV-1) was initially reported to engage the ESCRT pathway to acquire its envelope and pinch off viral particles during the budding step, other enveloped viruses have been shown to employ this pathway for their budding steps (Votteler and Sundquist 2013). While VPS4 is required for the budding of a variety of viruses, SFV was shown to bud from cells independent of both VPS4 and ubiquitin, suggesting a possible ESCRT-independent budding process (Taylor, Hanson and Kielian 2007). However, the putative roles of ESCRT factors in infection of other alphaviruses have not been examined.

The molecular mechanisms of viral trafficking by ESCRT factors remain to be elucidated. Herpes simplex virus (HSV), hepatitis C virus (HCV), JEV and DENV utilize ESCRT factors, whereas their late assembly domains (L domains) known to serve as a viral motif to recruit ESCRT factors are still undiscovered (Barouch-Bentov et al. 2016, Kumar et al. 2019, Pattanakitsakul et al. 2010, Chiou et al. 2003, Tabata et al. 2016, Crump, Yates and Minson 2007, Calistri et al. 2007, Pawliczek and Crump 2009). VPS4-independent ESCRT pathways were described in DENV and JEV infection (Tabata et al. 2016). Among viruses, even within the same families, interactions between viruses and ESCRT factors appear different (Votteler and Sundquist 2013). These studies suggest that the role of ESCRT factors in viral infection differ from virus to virus.

Genome-wide screens using CHIKV-infected cells have identified a variety of host

factors required for CHIKV infection; however, host trafficking factors have not been fully characterized (Zhang et al. 2018, Karlas et al. 2016). In this study, components of the ESCRT machinery have been identified to be required for CHIKV infection through small interfering RNA (siRNA) screens. Subsequently, the interaction of the identified ESCRT factors with CHIKV and a role for ESCRT factors in CHIKV infection have been characterized. These findings clearly demonstrated the importance of the host ESCRT trafficking machinery in the CHIKV replication cycle.

Materials and Methods

Cells and viruses

Human Embryonic Kidney (HEK) 293T cells were maintained in high-glucose DMEM (Sigma), containing 10% FBS (Sigma), 2 mM L-glutamine, 100 unit/ml penicillin and 100 µg/ml streptomycin (Sigma). For VPS4B-expressing HEK293T cells, Tet system approved FBS (Clontech) was used. Vero cells, Huh-7 cells, Huh-7 cells stably expressing HGS (HGS-Huh-7 cells) and TSG101 knockout (KO)-Huh-7 cells were grown in DMEM (Nissui) supplemented with 10% FBS, 2 mM L-glutamine, 100 unit/ml penicillin and 100 µg/ml streptomycin. All cells were cultured at 37°C under 5% CO₂.

CHIKV-SL10571 (AB455494) was kindly provided by Dr. Tomohiko Takasaki (National Institute of Infectious Diseases) (Lim et al. 2009). SFV (Original strain) was kindly provided by Dr. Masahiro Hayashi (National Institute of Infectious Diseases). All experiments involving CHIKV and SFV were performed in biosafety level-3 laboratories, following standard biosafety protocols approved by the Research Center for Zoonosis Control, Hokkaido University. SINV strain AR339 was obtained from the American Type Culture Collection, and vesicular stomatitis virus (VSV) (Indiana strain)

from our repository was used.

All viruses were initially amplified in Vero cells and supernatants from the virus-infected cells were harvested and stored at -80°C until use.

Antibodies

To detect CHIKV antigen, mouse anti-CHIKV monoclonal antibodies (mAbs) 5.5G9 and 5.2B2 recognizing CHIKV C and E2 protein in each were employed as previously described (Goh et al. 2013, Goh et al. 2015). The mAbs were able to detect CHIKV antigens by immunoblotting, immunofluorescence assay (IFA) and Enzyme-linked immunosorbent assay. Other antibodies were purchased as follows: rabbit anti-CHIKV 181/25 polyclonal antibody (pAb) (04-0008, IBT Bioservices); mouse anti-dsRNA mAb J2 (10010200, English & Scientific Consulting); rabbit anti-CHMP4B, anti-VPS4B pAbs (ab105767, ab224736, Abcam); rabbit anti-HGS pAb (PA5-27491, Thermo Fisher Scientific); rabbit anti-TSG101 pAb (14497-1-AP, Proteintech); mouse anti-FLAG M2, anti-HA-7, mAbs and mouse horseradish peroxidase (HRP)-conjugated anti-FLAG M2 mAb (F3165, H9658, A8592, Sigma); mouse HRP-conjugated anti-HA-tag mAb, rabbit HRP-conjugated anti- β -Actin pAb (M180-7, PM053-7, MBL).

Plasmids

FLAG-tagged CHIKV protein-expressing plasmids were generated by PCR-amplified CHIKV genome from CHIKV SL10571 into a pCXSN expression vector, which was created by removing the myc-tag from pCMV-myc (Clontech Laboratories) and adding Xho I, Sal I, and Not I recognition sites (Sasaki et al. 2011). HA-tagged ESCRT protein-expressing plasmids were also generated by PCR-amplified ESCRT genome from total RNAs of HEK293T cells into a pCXSN expression vector.

The plasmid expressing the HA-tagged HGS truncation mutants were derived from the wild type HGS backbone by deleting amino acids either 1-277, 278-527 or 528-777 by PCR amplification.

CHIKV-like particle (CHIKV-VLP)-expressing plasmid (pCXSN-CHIKV-VLP) was constructed as described previously (Akahata et al. 2010). The only CHIKV sP genes (C-E3-E2-6K-E1) from SL10571 strain were inserted into a pCXSN expression vector. CHIKV-VLPs were released in culture supernatants of pCXSN-CHIKV-VLP plasmid-transfected cells and were unable to produce progeny particles because of a lack of nsP genes.

All plasmids driven by pCXSN expression vector were transfected to the HEK293T or Huh-7 cells using Lipofectamine 3000 (Thermo Fisher Scientific).

CHIKV replicon-expressing plasmid [pSMART-CHIKV-rep-wild type (WT)] and RdRp-inactivated replicon-expressing plasmid [pSMART-CHIKV-rep-mutant type (MT)], which has no RdRp activity as negative control, were used as previously described (Wada et al. 2017). SINV replicon-expressing plasmid (pSMART-SINV-rep-WT) and RdRp-inactivated replicon-expressing plasmid (pSMART-SINV-rep-MT) were also constructed in a similar fashion to the CHIKV replicon-expressing plasmids. Briefly, the full-length genome sequence of SINV AR339 strain was inserted into the pSMART-LCKan vector (Lucigen), thereafter the sequences encoding the structural polyprotein were replaced by a NanoLuc luciferase sequence derived from pNL2.1 vector (Promega). The pSMART-SINV-rep-MT had introduced amino acid mutations from GDD to GAA in the motif C of pSMART-SINV-rep-WT, which is the catalytic core of the RdRp (the GDD motif) and therefore luciferase activity represented genome replication efficiency.

DENV subgenomic reporter replicon and an RdRp-inactivated DENV replicon,

which has no detectable RdRp activity were used as previously described (Nobori et al. 2018). The DENV replicon plasmid was transfected into HEK293T cells using Lipofectamine 3000. The sequences of all the above plasmids were confirmed by Sanger sequencing (ABI 3100).

Establishment of a Huh-7 cell line overexpressing HGS

The HGS-Huh-7 cell line was obtained by an retrovirus vector pseudotyped with the VSV-G, using Platinum-E packaging cells (Morita, Kojima and Kitamura 2000). Briefly, HGS-expressing plasmid (pMX-HGS) was constructed by inserting HGS gene into pMX-IRES-bd vector. The Platinum-E packaging cell was then transfected with pMX-HGS and pCMV-VSV-G by FuGENE HD transfection reagent (Promega). The supernatants containing retrovirus vector were collected after incubation of the cells at 37°C for 96 hours. The HGS-Huh-7 cell was established by infecting Huh-7 cells with the collected retroviral vector and cultured in the presence of 10 µg/mL blasticidin S HCl (Invitrogen). The HGS-Huh-7 cells were maintained by the same methods as those for Huh-7 cells.

RNA interference

Silencer select siRNAs targeting 73 trafficking genes, and control siRNA were purchased from Thermo Fisher Scientific. For imaging-based siRNA screen, a set of three different siRNAs targeting the same genes were prepared to rule out potential off-target effects of individual siRNAs. HEK293T cells were used for the RNA interference assays because of their permissiveness to CHIKV infection and to high-efficiency of siRNA transfection. In all RNA interference assays, siRNAs (at 10 nM final concentration) were reverse-transfected into the HEK293T cells using

Lipofectamine RNAiMAX (Thermo Fisher Scientific) 48 hours before virus infection or plasmid transfection.

IFA

CHIKV-infected cells were fixed with 4% PFA. After fixation, the cells were permeabilized with PBS containing 0.1% Triton X-100 for 10 minutes, blocked with 1% BSA fraction V (Sigma) in PBS, and then reacted with the indicated primary antibodies in PBS overnight at 4°C. After washing with PBS three times, cells were incubated with a 1: 1,000 dilution of goat anti-mouse IgG Alexa Fluor 488 or 594-conjugated, or anti-rabbit IgG Alexa Fluor 488-conjugated secondary antibodies (Thermo Fisher Scientific) in PBS for 1 hour at room temperature. The cells were then incubated with DAPI (Thermo Fisher Scientific) (1: 2,000 dilution) for 10 minutes. Immunopositive signals were confirmed under a fluorescent microscope with appropriate barrier and excitation filters.

Imaging-based siRNA screen

siRNAs were reverse-transfected into HEK293T cells in 96-well plates. Control siRNA reverse-transfected cells were prepared in all screening plates. At 24 hours post-infection (hpi) of CHIKV [Multiplicity of infection (MOI=0.1)], IFA was performed with mAb 5.5G9 recognizing C protein and DAPI. Quantitative imaging data for the screen was obtained by an IN Cell Analyzer 2000 (GE Healthcare), and the obtained images were analyzed using IN Cell developer Toolbox software (GE Healthcare). Relative infection rates were then calculated by dividing the number of CHIKV-positive cells recognized with mAb 5.5G9 by total number of cell nuclei stained with DAPI.

co-immunoprecipitation (co-IP)

HEK293T cells were co-transfected with an HA-tagged ESCRT protein-expressing plasmid and a FLAG-tagged CHIKV protein-expressing plasmid. As negative control, only CHIKV protein-expressing plasmid was transfected into HEK293T cells. HEK293T cells were also transfected with HA-tagged ESCRT protein-expressing plasmid and pCXSN-CHIKV-VLP. The cells were lysed in either high NP-40 cell lysis buffer [1% NP-40, 250 mM NaCl and 50 mM Tris-HCl (pH 7.4)] or low NP-40 cell lysis buffer [0.125% NP-40, 250 mM NaCl and 50 mM Tris-HCl (pH 7.4)] for CHIKV sP or nsP-expressing cells respectively. Both lysis buffer contained complete Protease Inhibitor Mixture (Roche Diagnostics). The lysates were incubated at 4°C overnight with Dynabeads Protein G (Thermo Fisher Scientific) and the indicated antibodies. Cell lysate immunoprecipitates were analyzed by SDS-PAGE and immunoblotting using HRP-conjugated mAbs.

Immunoblotting

Samples were lysed with an indicated lysis buffer, mixed with the equal amount of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer [0.5 M Tris-HCl (pH 6.8), 10% SDS and 11% glycerol] and fractionated by SDS-PAGE. Then, separated proteins were transferred to polyvinylidene difluoride filter (Merck Millipore) using a semidry system (Bio-Rad).

The membrane was blocked with 5% skimmed milk (Megmilk Snow Brand) in Tris-buffered saline [50 mM Tris-HCl (pH 7.5) and 150 mM NaCl] containing 0.1% Tween-20 (TBST) for 1 hour at room temperature. The membrane was then washed with TBST and subsequently incubated for 1 hour at room temperature with the indicated primary antibody in TBST containing 5% skimmed milk. The membrane was

then washed three times with TBST and treated with TBST containing goat anti-rabbit or mouse IgG HRP-conjugated secondary antibody (Cell Signaling Technology Japan) (1:1,000 dilution) for 1 hour at room temperature. After washing three times with TBST, the proteins were detected using Immobilon Western HRP-Substrate (Merck Millipore), according to the manufacturer's instructions. The chemiluminescence signals were visualized with the VersaDoc 5000MP (Bio-Rad), and obtained images were analyzed using Quantity One software (Bio-Rad).

Confocal microscopy

To detect localization of HGS, HGS-Huh-7 cells were cultured on chambered coverglasses. IFA was performed with CHIKV-infected cells using the indicated antibodies. Confocal images of CHIKV-infected cells were acquired by a Zeiss 780 LSM confocal microscope (Carl Zeiss), and colocalization was analyzed using ZEN 2011 software (Carl Zeiss).

Plaque assay

CHIKV (MOI=1.0 or 0.1) or SINV (MOI=0.05) was infected to siRNA reverse-transfected HEK293T cells in 24-well plates. Culture supernatants from virus-infected cells were inoculated onto Vero cells in 24-well plates after ten-fold serial dilution with DMEM containing 2% FBS. Then, the cells were incubated for 1 hour to allow virus attachment. After removal of virus-containing supernatants, cells were added with overlay medium [Eagle's MEM containing 5% FBS and 1.25% methyl cellulose] and incubated for three days. To count plaque numbers, cells were fixed by buffered-formalin and stained by 1% crystal violet solution in 70% ethanol. The titers [plaque-forming unit (PFU)/ml] were determined by taking the plaque numbers for a

dilution and the inverse of the total dilution factor. The relative CHIKV titers were normalized against control siRNA-reverse-transfected cells.

Validation of RNA interference

The expression levels of targeted genes of siRNA-treated cells were examined by immunoblotting. At 48 hours after reverse-transfection of siRNAs, HEK293T cells in 24-well plates were collected. Collected cells were then lysed with TNE lysis buffer [1% NP-40, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 20 mM Tris-HCl (pH 7.5) and 10% glycerol] supplemented with complete Protease inhibitor Mixture (Roche Diagnostics). Then, cell lysate immunoprecipitates were subjected to SDS-PAGE followed by immunoblotting. The expression levels of VPS37D were analyzed by qRT-PCR with the collected cell samples. Cell viabilities of the gene knockdown cells were assessed by the CellTiter-Glo assay (Promega), according to the manufacturer's instructions. In brief, at 48 and 72 hours after reverse-transfection of siRNAs (at 10 nM final concentration) into HEK293T cells in 96-well plates, CellTiter-Glo reagent (100 μ l) was added to the cells. After a ten-minute incubation at room temperature, luminescence indicating the amount of adenosine triphosphate (ATP) present was recorded by using a GloMax 96 microplate luminometer (Promega). The ATP levels were normalized against control siRNA-reverse-transfected cells.

RNA extraction and qRT-PCR

For evaluation of the intracellular CHIKV RNA levels, CHIKV-infected cells were collected and treated with 0.25% trypsin EDTA (Wako) at 37°C for 5 minutes to detach extra CHIKV on the cell surface. After trypsinized cells were collected to microcentrifuge tubes, the cells were washed three times by pelleting. For pelleting, we

repeated centrifugation 1,500 x *g* for 2 minutes, carefully removing the PBS and resuspending pellets in 400 µl of PBS. Following the final PBS wash and removing PBS, the cell pellets were subjected to RNA extraction using TRIzol (Thermo Fisher Scientific) according to the manufacturer's protocol. CHIKV RNA levels in CHIKV-infected cells were then determined by qRT-PCR for CHIKV with extracted RNA, a primer set (CHIKV UBIV F and CHIKV UBIV R) and a CHIK probe as previously described (Andriamandimby et al. 2013) using TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific), following the manufacturer's protocol.

Expression levels of VPS37D in siRNA-treated cells were also determined by qRT-PCR. Total RNAs were extracted from the siRNA-treated HEK293T cells using TRIzol, according to the manufacturer's protocol. qRT-PCR was performed using the One Step TB Green PrimeScript PLUS RT-PCR Kit (TAKARA) with extracted RNA and the following primer set; vps37d-386F (5'-TTCGTGAGGTGGCCGAGAAC-3') and vps37d-524R (5'-TCCATCTGCTCCTCTTGCCT-3').

The relative expression levels of target mRNA were normalized against control siRNA-reverse-transfected cells with human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) employed as an internal standard. The mRNA levels of GAPDH gene were analyzed using Human GAPD (GAPDH) Endogenous Control (FAM Dye/MGB Probe, Non-Primer Limited) (Thermo Fisher Scientific).

Evaluation of the early phase of CHIKV infection

CHIKV was allowed to bind to HEK293T cells in 24-well plates at 4°C for 1 hour. Subsequently, cells were shifted to 37°C for 3 hours to allow endocytosis in new media. As control, we prepared cells incubated in the new media containing NH₄Cl (at 30 mM final concentration). After removing the culture supernatants and washing with PBS,

cell samples were collected and subjected to RNA extraction followed by trypsin treatment. Then, qRT-PCR for CHIKV was performed with the extracted RNAs to determine intracellular CHIKV RNA levels.

Time course assay

CHIKV was inoculated into HEK293T cells in 24-well plates. After incubation at 4°C for 1 hour to allow CHIKV to attach to the cells, the cells were incubated at 37°C in new media. The cell samples were collected at 0, 3, 6, 9 and 12 hpi. Trypsin treatment followed by RNA extraction was performed with the collected samples. Amplification of CHIKV RNA was determined by qRT-PCR for CHIKV with extracted RNA.

Reverse transcription for replicon RNA and replicon assay

Either CHIKV replicon plasmids (pSMART-CHIKV-rep-WT and pSMART-CHIKV-rep-MT) or SINV replicon plasmids (pSMART-SINV-rep-WT and pSMART-SINV-rep-MT) were linearized with either Not I or Xba I, respectively. Then, linearized plasmids were *in vitro* transcribed with the mMESSAGE mMACHINE T7 transcription kit (Thermo Fisher Scientific) according to the manufacturer's protocol. HEK293T cells in 96-well plates were reverse-transfected with siRNA, incubated for 48 hours and transfected with reverse-transcribed RNAs (100 ng) from replicon plasmids by the Lipofectamine Messenger MAX (Thermo Fisher Scientific). At the same time point, DENV replicon plasmids (20 ng) were also transfected into HEK293T cells. At the indicated time points, NanoLuc activities were measured using the Nano-Glo luciferase assay kit (Promega) and the GloMax 96 microplate luminometer with cell lysates, following the manufacturer's instructions.

CHIKV-VLP release assay

HEK293T cells were prepared in 24-well plates. The cells were then transfected with the pCXS_N-CHIKV-VLP (200 ng). At 48 hours post-transfection (hpt), the culture supernatants were collected and mixed with SDS-PAGE sample buffers. The plasmid-transfected cells were lysed with TNE lysis buffer and mixed with SDS-PAGE sample buffer. Both lysate and supernatant samples were then analyzed by immunoblotting using mAb 5.5G9. The amount of CHIKV-VLP was estimated as the intensity of specific C protein band. Expression of sPs in the cells was confirmed by the relative intensity of C protein in lysates and the release efficiency of CHIKV-VLP was evaluated in supernatants.

Density gradient sedimentation analysis

Release of CHIKV-VLPs in the supernatant of the pCXS_N-CHIKV-VLP-transfected cells was confirmed by density gradient sedimentation analysis. HEK293T cell in 100 mm TC-Treated Culture Dish transfected with 20 µg of pCXS_N-CHIKV-VLP plasmid was harvested at 37°C for 96 hours. Then, the culture supernatant was collected and filtered through a 0.45-µm pore-sized filter. Thereafter the sample was centrifuged at 1,500 × *g* for 3 minutes to remove cells and debris followed by the concentration using ultracentrifugation at 30,000 rpm for 2 hours in a SW32 Ti rotor (Beckman Coulter). The CHIKV-VLP pellet was resuspended with 1 ml of PBS and placed at 4°C for 30 minutes. For sedimentation, 10, 20, 30, 40 and 50% sucrose in PBS was prepared. Sucrose-buffer solution (50%, 2.2 mL) was added to the bottom of centrifugation tube. Then, the same volume of 40% sucrose-buffer solution was layered. This procedure was repeated with the same volumes of the 30%, 20% and 10% sucrose-buffer solutions. After placing the tubes at 4°C for 30 minutes at 4°C, the resuspended CHIKV-VLP

sample was layered carefully on the top of the gradient. This gradient was centrifuged at 38,000 rpm in a SW41 rotor (Beckman Coulter) at 4°C for 14 hours and then fractionated from the top into 24 fractions (500 µl each). All fractions (20 µl) were analyzed by SDS-PAGE and immunoblotting using anti-CHIKV pAb or mAb 5.5G9.

MG-132 treatment

HEK293T cells in 24-well plates were infected with CHIKV, SFV or VSV (MOI=0.1). After incubation at 4°C for 1 hour, the cells were incubated for 2 hours at 37°C in new media. MG-132 (at 50 µM final concentration) in dimethyl sulfoxide (DMSO) was then added into the virus-infected cells. Cellular lysate and supernatant samples were collected at the indicated time points. The viral titers in supernatant samples were determined by a plaque assay. Amplification of CHIKV RNA was determined by qRT-PCR for CHIKV with extracted RNA from cell lysates.

Generation and infection of Tetracycline-inducible VPS4B-expressing cells.

The ATP hydrolysis-deficient VPS4B E235Q mutant (VPS4BEQ) was derived from the VPS4B WT backbone by PCR mutagenesis. Tetracycline-inducible stable cell lines expressing WT or mutant HA-tagged VPS4B with Tet-On 3G transactivator protein were generated using Retro-X Tet-On 3G inducible expression system (Clontech). Briefly, HA-tagged VPS4B-expressing plasmids (pRetroX-TRE3G-VPS4B WT and pRetroX-TRE3G-VPS4BEQ) were constructed by inserting VPS4B genes into RetroX-TRE3G vector. The above plasmids and pCMV-VSV-G were transfected into GP2-293 packaging cells by FuGENE HD transfection reagent (Promega). TRE3G-VPS4B retroviruses were collected from the culture media 48 hours after transfection. HEK293T cells were then co-infected with TRE3G-VPS4B retrovirus and

Tet-On 3G retrovirus, expressing Tet-On 3G transactivator protein. Tetracycline-inducible VPS4B-stable expressing HEK293T cell lines were selected under G418 and puromycin treatments.

Tetracycline-inducible VPS4B-expressing HEK293T cells in 24-well plates were cultured with or without doxycycline hydrochloride (Sigma) for 24 hours, and then infected with CHIKV, SFV or VSV (MOI=0.1). After incubation at 4°C for 1 hour, the cells were incubated at 37°C in new media. The culture supernatants were collected from the virus-infected cells at the indicated time points. The viral titers in the supernatant samples were determined by a plaque assay.

Generation and infection of TSG101-KO Huh-7 cells.

TSG101-KO cells were obtained by CRISPR-Cas9 system. The guided RNA targeting TSG101 was inserted into the plasmid pX330, which encodes hCas9 and sgRNA (Addgene). Huh-7 cells were then transfected with the generated plasmid by Trans IT LT-1 (Mirus), following the single cell isolation. For the screening of TSG101-KO cells, protein expression levels were examined by immunoblotting using rabbit anti-TSG101 pAb. Then, the KO of TSG101 was confirmed by direct sequencing of the target site and immunoblotting.

CHIKV (MOI=1.0), SINV and SFV (MOI=0.1) were infected to the TSG101-KO cells. After incubation at 4°C for 1 hour, the cells were incubated at 37°C in new media. Then the virus titers were determined by plaque assay using the culture supernatants collected at 24 hpi.

Statistical analysis and normalizing

All assays were performed in triplicate and independently at least 2 times. The data

were expressed as means $\pm$ S.D. Statistical significance was determined by the two-tailed Student's *t*-test or one-way ANOVA with Dunnett's test. *P*-values <0.05 or <0.001 were considered significant and indicated by a single asterisk (*) or double asterisks (**), respectively.

The relative data were normalized against control siRNA-reverse-transfected cells.

Results

Imaging-based siRNA screens demonstrate that ESCRT factors are required for CHIKV infection

To identify the trafficking genes required for CHIKV infection, imaging-based siRNA screens was performed. siRNAs against 73 trafficking genes belonging to RAB, RHO, ADP ribosylation factors (ARF)/ secretion associated Ras related GTPase (SAR), GOLGIN and ESCRT families were selected for the screen. HEK293T cells were reverse-transfected with siRNA, cultured 48 hours and then infected with CHIKV. The CHIKV infection rate was determined by dividing the number of CHIKV-positive cells by total cells. Three different siRNAs targeting the same gene product were examined independently. The heatmap was obtained by the relative cell count and relative infection rate in the presence of each siRNA (Fig. 10).

For the siRNA screen, siRNA treatment was considered as a positive effect if the relative CHIKV infection rate of each siRNA treatment was less than 60% of control siRNA treatments, and the relative cell count numbers of each siRNA treatment was more than 80% of control siRNA treatments. Then, the gene was selected as a hit if at least two of the three siRNAs targeting the same gene product had positive effects, in order to exclude potential off-target effects of each siRNA. As a result of the siRNA

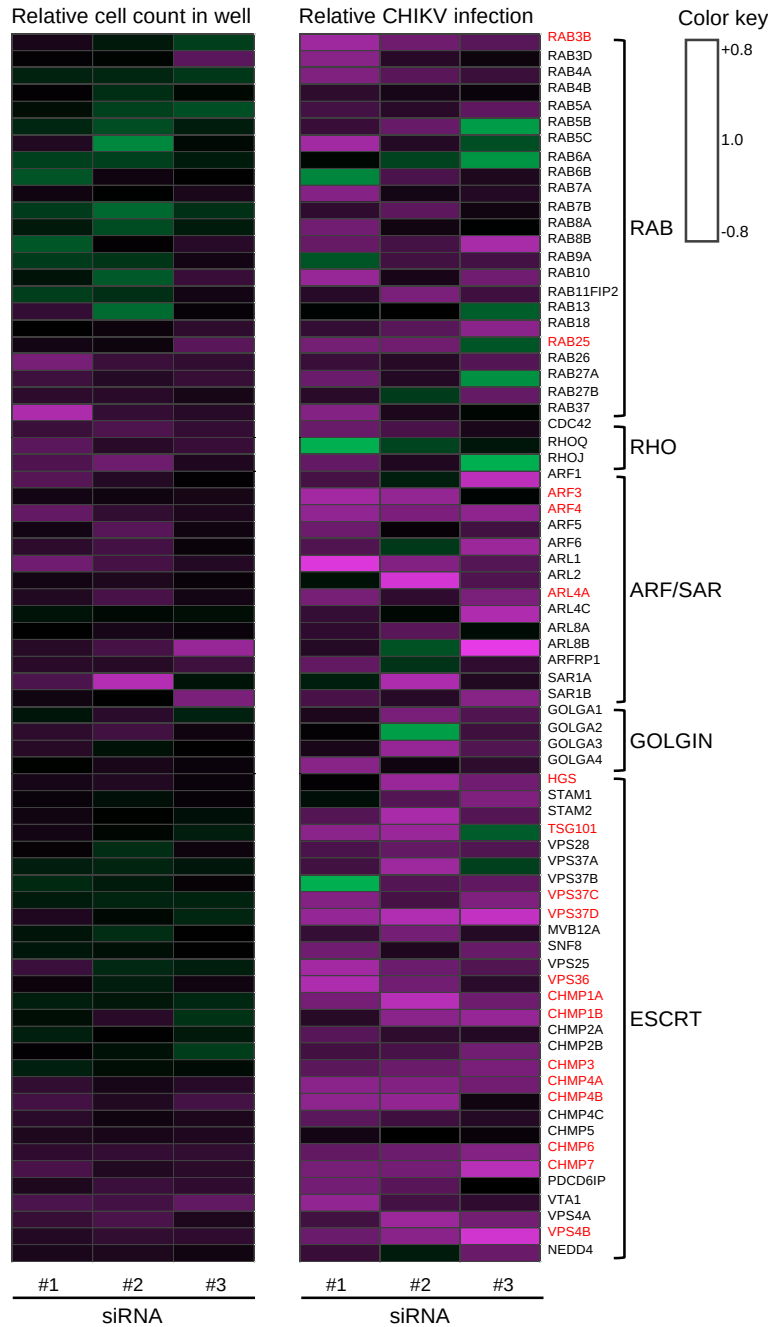


Fig. 10. Heatmap for the quantitative imaging-based siRNA screen of CHIKV infection.

HEK293T cells were individually reverse transfected with three different siRNAs targeting 73 host trafficking factors, and inoculated with CHIKV at a MOI of 0.1. Infection rates were determined by dividing the number of CHIKV-positive cells by total numbers of cells following IFA performed at 24 hpi. The ratio is normalized against control siRNA-reverse-transfected cells in the same plates and demonstrated according to the color key. The genes validated as hits are highlighted in red.

screen, 18 hits were found including RAB (2/23), ARF/SAR (3/13) and ESCRT (13/29) genes, highlighted in red (Fig. 10). ARF3 and ARF4 in ARF/SAR family were validated as hits consistent with a previous report demonstrating the requirement of ARFs in CHIKV replication (Zhang and Zhang 2017). Strikingly, 13/29 of the investigated ESCRT genes were related to the CHIKV infection and those genes are belonged to multiple ESCRT complexes including ESCRTs -0, -I, -II, -III and VPS4. Moreover, siRNAs against some ESCRT genes such as VPS37D and VPS4B had great effects on CHIKV infection without the reduction of cell numbers. These results suggested that a series of ESCRT factors are involved in CHIKV infection. Since budding of SFV was considered independent of the ESCRT pathway, interactions between ESCRT factors and CHIKV infection have not been previously examined (Taylor et al. 2007). Therefore, ESCRT factors were picked up for further examination of specific roles in CHIKV infection.

The ESCRT factor HGS interacts with CHIKV sPs and nsPs proteins

To identify the ESCRT partners physically interacting with CHIKV proteins, HA-tagged host factors and FLAG-tagged CHIKV proteins were introduced into mammalian cells, and thereafter anti-HA co-IP was performed. HA-tagged host proteins binding CHIKV proteins were detected with anti-FLAG antibody. Eight ESCRT-relating factors were selected in these studies. HGS, also known as Hrs, and STAM1 belong to ESCRT-0 complex and act as a sorting machinery that recognize ubiquitinated receptors (Henne et al. 2011). ALIX and NEDD4 (accessory proteins) and TSG101 (ESCRT-I) are reported to bind to viral L domains (Votteler and Sundquist 2013). VPS37D (ESCRT-I), CHMP4B (ESCRT-III) and VPS4B (VPS4 proteins) were considered as potential interactors with CHIKV proteins, since siRNA treatments against them markedly

reduced CHIKV infection rates with little reduction of the relative cell count in the siRNA screen (Fig. 10). In addition, the arrestin domain-containing protein ARRDC1, which can recruit ESCRT factors at the plasma membrane in an L domain-dependent viral budding process, was also examined (Rauch and Martin-Serrano 2011). A total of seven CHIKV proteins were examined (C, E1, E2 and nsP1-4).

Immunoblot analyses of immunoprecipitated fractions (IP) showed that HGS strongly interacted with E1, E2 and nsP4 (Fig. 11A). The co-IP results of nsP3 fractions were excluded, since exogenously expressed FLAG-nsP3 was non-specifically precipitated with anti-HA antibody (data not shown). The obtained results were confirmed by reciprocal immunoprecipitation with FLAG-tagged CHIKV proteins and detection of HA-tagged ESCRT proteins with anti-HA antibody. Co-IP results showed that HGS interacted with E1, E2 and nsP4 (Fig. 11B). In addition, specific bands for TSG101 were detected in the nsP3 and nsP4 fractions (Fig. 11B). The results of VPS37D and CHMP4B fractions were excluded, since exogenously expressed HA-VPS37D and CHMP4B were non-specifically precipitated with anti-FLAG antibody (data not shown).

Interaction between HGS and CHIKV sPs was also investigated in HA-tagged-ESCRT protein- and CHIKV-VLP-expressing cells. By exclusively expressing sPs, CHIKV-VLPs can be assembled and released from cells in a similar manner as whole infectious virions (Akahata et al. 2010, Noranate et al. 2014). As shown in Fig. 11C, specific signals for HGS were detected by participating with mAbs 5.5G9 for the C protein and 5.2B2 for the E2 protein in CHIKV-VLP-expressing cells, while no specific signals for STAM1 were detected in CHIKV-VLP-expressing cells with the above antibodies. These results showed that HGS interacted with both sPs and nsPs of CHIKV, suggesting that HGS may have a critical role in multiple steps of

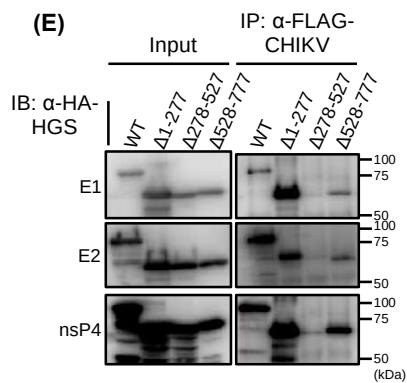
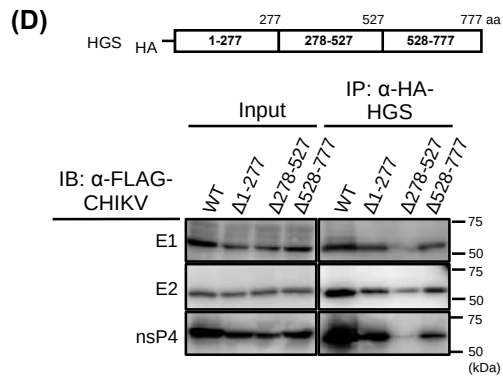
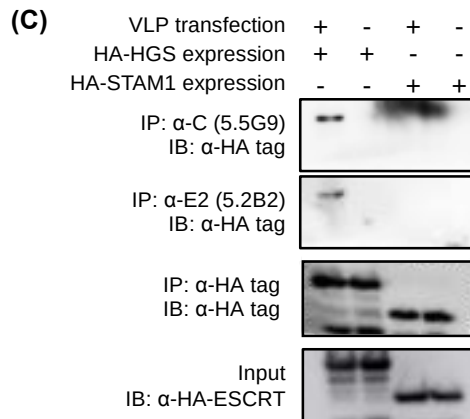
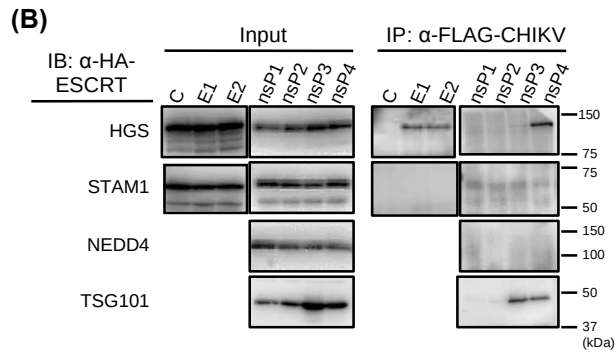
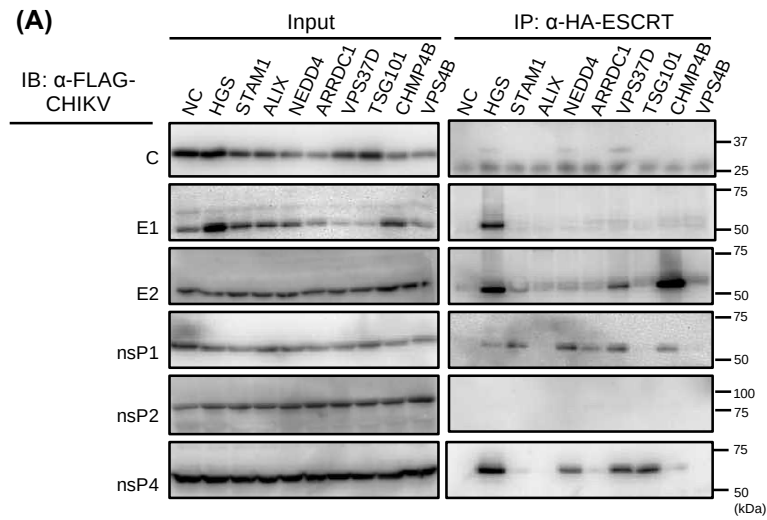


Fig. 11. Interactions between CHIKV and ESCRT proteins analyzed by co-IP.

(A) HA-tagged host factors and FLAG-tagged CHIKV proteins were exogenously introduced into HEK293T cells. Following anti-HA IP, individual FLAG-tagged CHIKV proteins in each fraction were detected by immunoblotting using the cell lysates. In the leftmost fraction, only FLAG-tagged CHIKV protein-expressing plasmid was transfected into the cells as a negative control (NC). (B) Reciprocally, anti-FLAG mAb was utilized for IP and anti-HA mAb was utilized for detection of HA-tagged ESCRT proteins. (C) HA-tagged host factors and CHIKV-VLP were exogenously introduced into HEK293T cells. Following IP with anti-CHIKV C (upper panel) or E2 (middle panel) antibodies, HA-tagged ESCRT proteins were detected in each fraction. (D) Genome organization of HGS is shown in the above schematic. HGS truncated mutants were lacking amino acid residues either 1-277, 278-527 or 528-777 from WT HA-tagged HGS, designated as Δ 1-277, Δ 278-527 or Δ 528-777, respectively. Interactions between CHIKV and truncated HGS mutants analyzed by co-IP. HA-tagged truncated HGS mutants or full-length HGS, and FLAG-tagged CHIKV proteins were exogenously introduced into HEK293T cells. Following anti-HA IP, individual FLAG-tagged CHIKV proteins in each fraction were detected by immunoblotting using the cell lysates. (E) Reciprocally, anti-FLAG mAb was utilized for IP and anti-HA mAb was utilized for detection of HA-tagged HGS mutants.

CHIKV infection.

To further characterize the interactions of HGS with CHIKV proteins, the HGS region responsible for binding to CHIKV proteins was examined using truncated HGS mutants lacking amino acid residues 1-277, 278-527 or 528-777 (Δ 1-277, Δ 278-527 or Δ 528-777, respectively). HA-tagged truncated HGS mutants were immunoprecipitated followed by detection of CHIKV proteins. The IP fractions indicated that HGS Δ 278-527 had markedly reduced binding ability to CHIKV E1, E2 and nsP4 compared with full-length HGS protein (WT), Δ 1-277 and Δ 528-777 (Fig. 11D). The reciprocal experiments confirmed that HGS Δ 278-527 also failed to bind to CHIKV E1, E2 and nsP4 (Fig. 11E). These results suggested that the amino acid residues 278-527 of HGS play an important role in binding to CHIKV sPs and nsPs.

CHIKV E2 and dsRNA colocalize with HGS in CHIKV-infected cells

Next, the interactions of HGS and CHIKV in CHIKV-infected cells was examined by confocal microscopy. As commercially available antibodies failed to visualize endogenous HGS in Huh-7 cells, probably due to the low level of endogenous HGS protein which has been previously reported (Chou et al. 2015), HGS-Huh-7 cells were generated (see Materials and Methods). The distribution of HGS was confirmed to be similar to the distribution of endogenous HGS in HeLa cells as shown in the previous report (Komada et al. 1997); the fluorescence signals were detected diffusely in the cytoplasm of mock-infected HGS-Huh-7 cells (Fig. 12A). The dot-like signals were also detected in the cytoplasm of mock-infected HGS-Huh-7 cells in a similar fashion to a previous report (Komada et al. 1997). In addition, the morphology and growth activity of HGS-Huh-7 cells was similar to the parent HGS cells.

Using HGS-Huh-7 cells, the localization of HGS and CHIKV E2 was firstly

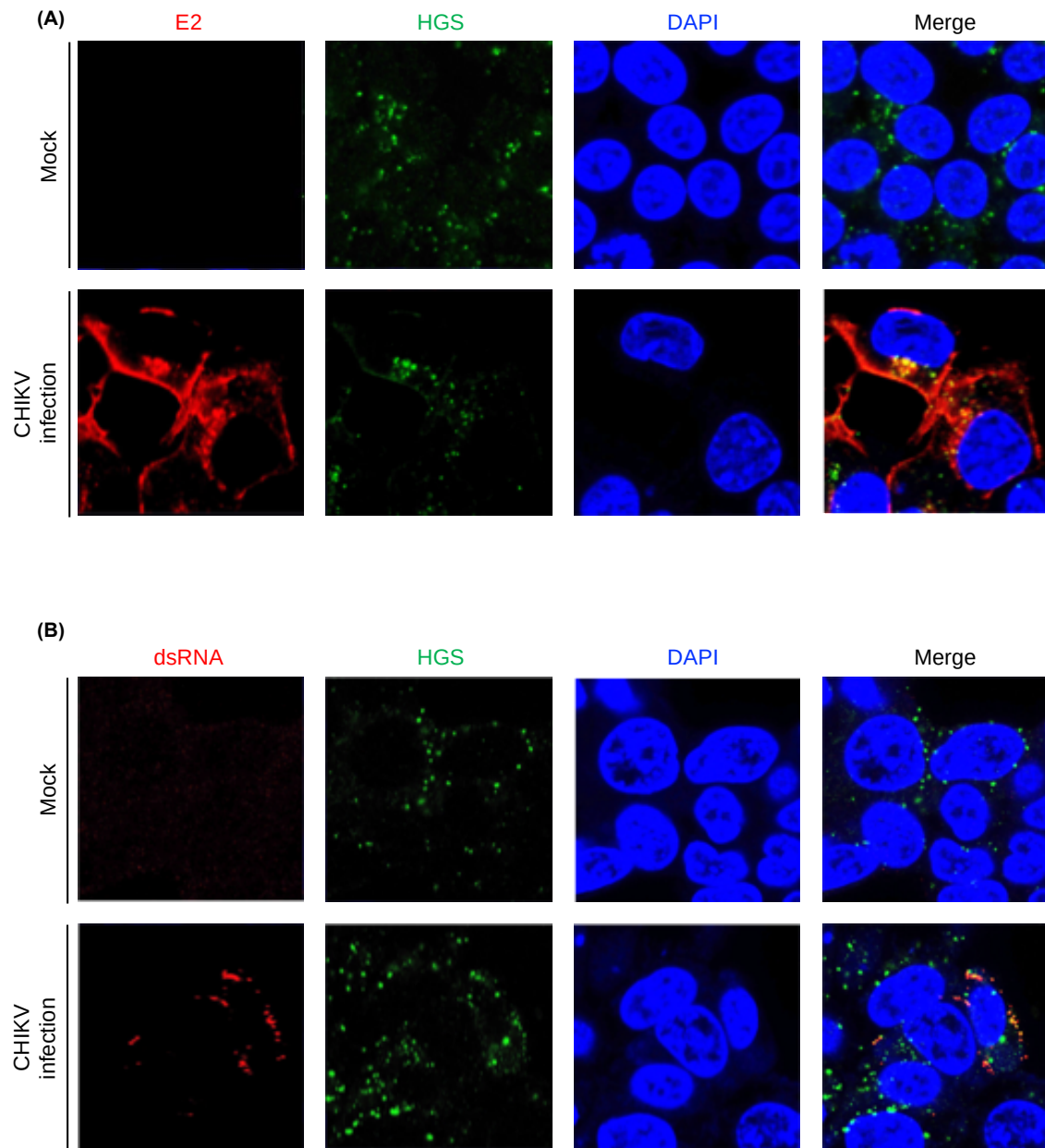


Fig. 12. HGS colocalizes with CHIKV E2 and dsRNA.

(A) CHIKV (MOI=10) was inoculated into HGS-Huh-7 cells. At 12 hpi, CHIKV- or mock-infected HGS-Huh-7 cells were fixed, costained for anti-CHIKV E2 (red), anti-HGS (green) and DAPI (blue). (B) CHIKV (MOI=1.0) was inoculated into HGS-Huh-7 cells. At 24 hpi, CHIKV- or mock-infected HGS-Huh-7 cells were fixed, costained for anti-dsRNA (red), anti-HGS (green) and DAPI (blue). The cells were observed by confocal microscopy (Zeiss 780 LSM confocal microscope).

examined at 12 hpi of CHIKV. As shown in the lower panels of Fig. 12A, colocalization of HGS and CHIKV E2 was observed in the cytoplasm of CHIKV-infected HGS-Huh-7 cells. Next, the colocalization of the HGS and CHIKV replication site was investigated. To achieve this, I applied anti-dsRNA antibody, as dsRNA is exclusively expressed in the CHIKV replication site in CHIKV-infected cells (Remenyi et al. 2018). Colocalization of HGS and dsRNA in the cytoplasm of CHIKV-infected cells was confirmed (lower panels, Fig. 12B). Taken together, the ESCRT protein, HGS colocalized with CHIKV structural protein and replication sites in CHIKV-infected cells. Thus, CHIKV infection efficiency in HGS-knockdown (HGS-KD) cells was also examined.

KD of ESCRT factor, HGS reduces CHIKV titers

To validate the involvement of HGS in CHIKV infection, virus titers were measured in HGS-KD HEK293T cultures. From the set of three different siRNAs targeting HGS, a single siRNA (siRNA#3 in Fig. 10) was selected. The plaque assays were performed to determine CHIKV titers with cell supernatants collected at 12 and 24 hpi. The KD of HGS significantly reduced CHIKV titers at 12 hpi, and showed greater than a ten-fold reduction of CHIKV titers at 24 hpi (Fig. 13A). Furthermore, to examine the requirement of HGS in other alphavirus infection, titers of SINV were also measured at 24 hpi in HGS-KD cells by plaque assay. The KD of HGS also inhibited infection of SINV more than ten-fold (Fig. 13B). The KD of HGS was confirmed by immunoblotting with siRNA-treated cell lysates (Fig. 13C). The KD of HGS did not affect cell viabilities during gene knockdown analyses was confirmed by measuring cellular ATP levels of HGS-KD mock and CHIKV-infected cells 48 and 72 hours after

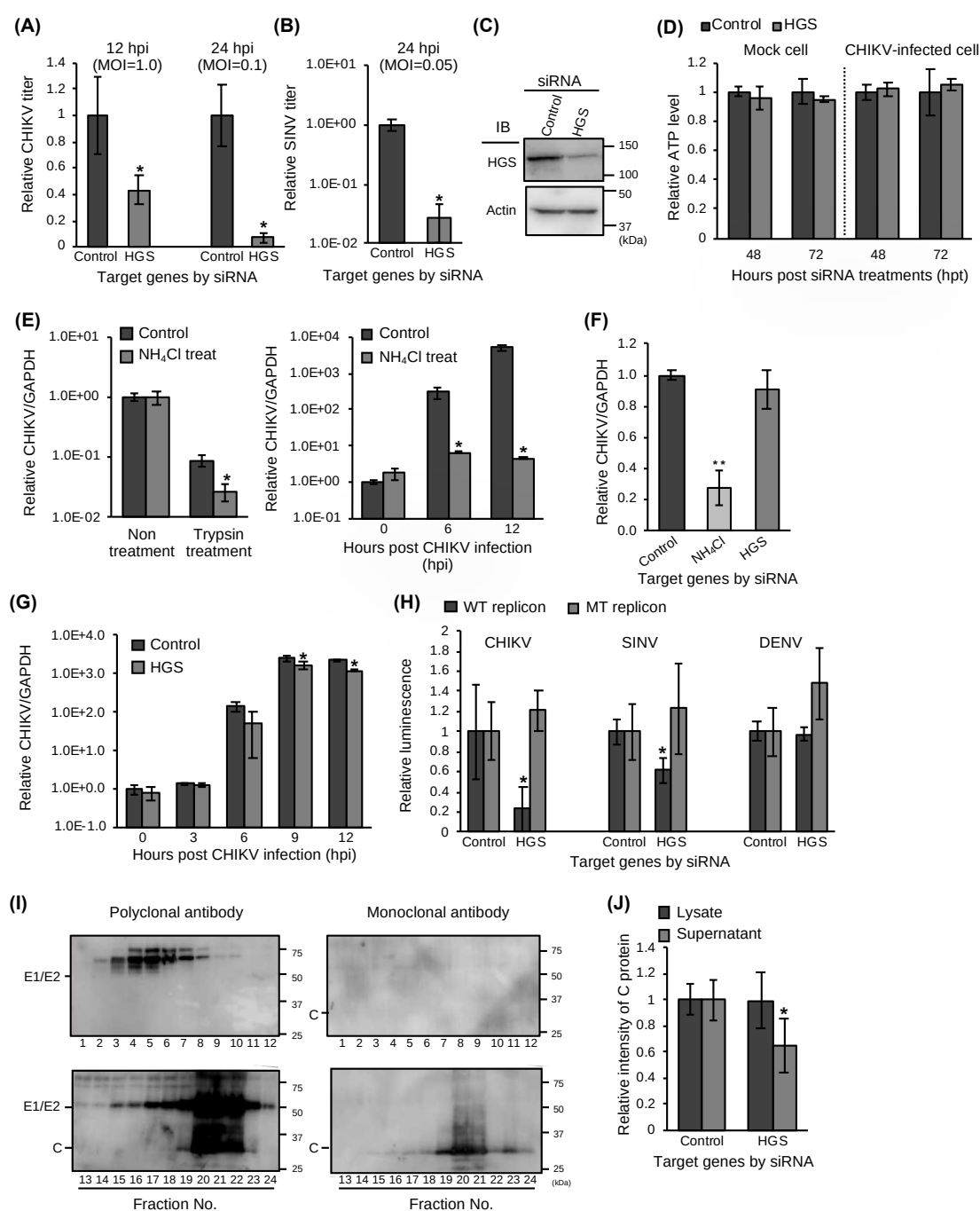


Fig. 13. Significant inhibition of genome replication and post-translational steps of CHIKV infection by KD of HGS.

(A and B) Following the reverse transfection of siRNAs against HGS, HEK293T cells were inoculated with CHIKV (MOI=1.0 or 0.1) (A) or SINV (MOI=0.05) (B). Virus titers were determined by plaque assay using cell supernatants collected at the indicated time points and normalized with control siRNA-treated cells. (C) Protein expression levels of HGS and β -Actin (control) in siRNA-treated cells were determined by immunoblotting. (D) Relative viability of the siRNA-treated cell was evaluated by measuring cellular ATP levels 48 and 72 hours after siRNA treatments and normalizing with control siRNA-treated cells. The results of mock cells were shown in the left and

CHIKV-infected cells were shown in the right. (E) Left bar graph showed the intracellular viral RNA levels of control and NH₄Cl-treated cells at 3 hpi of CHIKV infection (MOI=0.1) with or without trypsin treatment before RNA extraction. Right bar graph showed the intracellular viral RNA levels of control and NH₄Cl-treated cells at 0, 6 and 12 hpi of CHIKV infection (MOI=0.1). All of the cells were treated with trypsin before RNA extraction. (F) Intracellular viral RNA levels were examined at 3 hpi of CHIKV infection (MOI=0.1) into siRNA-treated HEK293T cells. NH₄Cl-treated cells were used as control. (G) Time course of CHIKV genome expression in siRNA-treated HEK293T cells inoculated with CHIKV (MOI=0.1) for 12 hours after CHIKV infection. Intracellular viral RNA levels were measured at the indicated time points. (E, F and G) Expression of viral RNA and GAPDH mRNA was determined by qRT-PCR using total RNAs extracted from trypsinized CHIKV-infected cells. (H) HEK293T cells were transfected with siRNAs, incubated 48 hours and transfected with replicon-expressing plasmids (WT CHIKV, SINV or DENV replicon- or RdRp-inactivated MT CHIKV, SINV or DENV replicon-expressing plasmids). The luciferase activities, that represent replication efficiency, were measured at 24 hpt of replicon-expressing plasmids. (I) The cells were transfected with the pCXS-CHIKV-VLP plasmid and the supernatants were collected at 96 hpt, following the sucrose density gradient sedimentation. The supernatants were fractionated through sucrose gradients (0-50%) into 24 fractions and lysed with TNE buffers. The expression of structural protein in each fraction was confirmed by immunoblotting for structural proteins using anti-CHIKV pAb or mAb 5.5G9, respectively. (J) HEK293T cells were transfected with siRNAs, incubated for 48 hours and CHIKV-VLP-expressing plasmids. At 48 hpt of CHIKV-VLP-expressing plasmids, the culture supernatants and cells were collected and lysed with TNE lysis buffers. The amount of C protein, representing the amount of CHIKV-VLP in both lysate and supernatant samples was then analyzed by the intensity of specific bands observed by immunoblotting. All relative data were normalized against control cells and represented as means $\pm$ S.D., collected in triplicate and independently at least 2 times. Statistical significance was assessed using Student's *t*-test (A, B, D, E, G, H and J) or one-way ANOVA with Dunnett's test (F) and indicated by asterisks (*, $p < 0.05$ and **, $p < 0.001$ compared between control cells).

siRNA treatments (Fig. 13D). In these experiments, the KD of HGS was significantly inhibited infection of both CHIKV and SINV.

HGS is essential for CHIKV genome replication and post-translational steps

Next, which phase(s) of CHIKV replication cycle is dependent on HGS with HGS-KD cells was examined. Firstly, intracellular CHIKV RNA levels at 3 hpi were measured by qRT-PCR, to focus on the early phase of CHIKV replication cycle (from 0-3 hpi). NH_4Cl -treated cells, which inhibit the endosomal acidification, were employed for the assay control. The expression levels of intracellular CHIKV RNA after trypsin treatments were confirmed to be decreased significantly under the NH_4Cl treatment at 3, 6 and 12 hpi, indicating that NH_4Cl treatment inhibited amplification of CHIKV RNA (Fig. 13E). Unlike NH_4Cl treatment, the KD of HGS had no significant effect on intracellular viral RNA level at 3 hpi (Fig. 13F).

Secondly, intracellular viral RNA levels in HGS-KD cells were measured at various time points from 0 to 12 hpi to examine the viral genome replication steps. An increase in intracellular viral RNA levels was found from 6 hpi (Fig. 13G). While there was no difference of intracellular viral RNA levels between HGS-KD cells and control siRNA treated cells until 6 hpi, the KD of HGS significantly reduced viral RNA levels at 9 and 12 hpi (Fig. 13G). These results demonstrate that HGS plays a role in the replication step of CHIKV infection cycle. Therefore, CHIKV replication efficiency was examined using CHIKV replicon system, encoding the luciferase gene (Wada et al. 2017).

The relative luciferase intensity of a WT CHIKV replicon was significantly inhibited by KD of HGS at 24 hpt of the CHIKV replicon RNA (Fig. 13H). Meanwhile, an RdRp-inactivated MT CHIKV replicon prepared by the introduction of a GAA substitution at nsP4 was not affected by HGS-KD (Fig. 13H). These results support that

HGS is involved in CHIKV genome replication. The role of HGS in the replication efficiency of SINV was further examined using a newly-established SINV replicon system, which consists of a WT and a RdRp-inactivated MT SINV replicon, encoding the luciferase gene. The luminescence of WT SINV replicon was significantly higher than the RdRp-inactivated MT SINV replicon from 12 to 96 hpt (data not shown). At the same time, the replication efficiency of the flavivirus DENV was also investigated using a previously established DENV replicon system, which also consists of WT and RdRp-inactivated MT replicons, encoding luciferase gene (Nobori et al. 2018). As shown in Fig. 13H, the KD of HGS reduced relative luminescence from WT SINV replicon, while the KD of HGS did not affect those of MT SINV replicon or DENV replicons compared to control cells. Taken together, HGS specifically regulated replication activities of CHIKV and SINV.

Finally, the post-translational steps of CHIKV infection was examined using the CHIKV-VLP system. To examine whether the detection of sPs in the supernatants of CHIKV sPs-expressing cells reflect the release of particles, the supernatants from CHIKV sPs-expressing cells were fractionated through sucrose gradients (0-50%) into 24 fractions (Fig. 13I). Subsequently, each fraction was applied to immunoblotting using an anti-CHIKV 181/25 pAb for CHIKV sPs and an mAb 5.5G9 for the C protein. Both CHIKV E1 and E2 at 50 kilodalton (kDa) and C at 30 kDa were clearly detected in fractions 19-23, demonstrating that CHIKV-VLP existed in the supernatants from the CHIKV sPs-expressing cells (Fig. 13I). The detection of CHIKV C protein in the supernatants reflected the extracellular release of CHIKV-VLP. Using this CHIKV-VLP system, release efficiency of CHIKV-VLP from HGS-KD cells was examined. The relative intensity of C protein expression in these cells was similar to that in the cellular lysates from control siRNA-treated and HGS-KD cells; however, the

levels of C protein detected in supernatants from HGS-KD cells was significantly decreased (Fig. 13J). These results indicate that HGS contributes to efficient release of CHIKV-VLP from cells.

Taken together, HGS, a member of ESCRT proteins, plays an important role in CHIKV and SINV infection. Next, the effects of other ESCRT factors in CHIKV infection was investigated.

Selected ESCRT factors are essential for the replication and post-translational steps in CHIKV infection

To further examine the ESCRT machinery engagement in CHIKV infection, the role and requirement of other ESCRT factors were examined. four ESCRT factors [VPS37D (ESCRT-I), TSG101 (ESCRT-I), CHMP4B (ESCRT-III) and VPS4B (VPS)], which were considered to be involved in CHIKV infection as siRNA treatment against them inhibited CHIKV infection in the imaging-based siRNA screen (Fig. 10), were selected.

Firstly, the four selected ESCRT factors were confirmed to be involved in CHIKV as well as SINV infection by titration with ESCRT factor-KD cells. CHIKV and SINV titers in the supernatants from virus-infected cells were significantly reduced by KD of the ESCRT factors (Fig. 14A). The KD of TSG101, CHMP4B and VPS4B was validated by immunoblotting with siRNA-treated cell lysates and the KD of VPS37D by qRT-PCR with RNA extracted from siRNA-treated cells (Fig. 14B). The KD of all four factors did not induce severe cytotoxicity in mock nor CHIKV-infected cells at 48 and 72 hours post siRNA treatments (Fig. 14C). These results suggested that these four selected factors are required for infection of both CHIKV and SINV.

Next, it was examined which phase(s) of CHIKV infection are related to these four ESCRT factors. At first, the involvement of the ESCRT factors in the early phases of

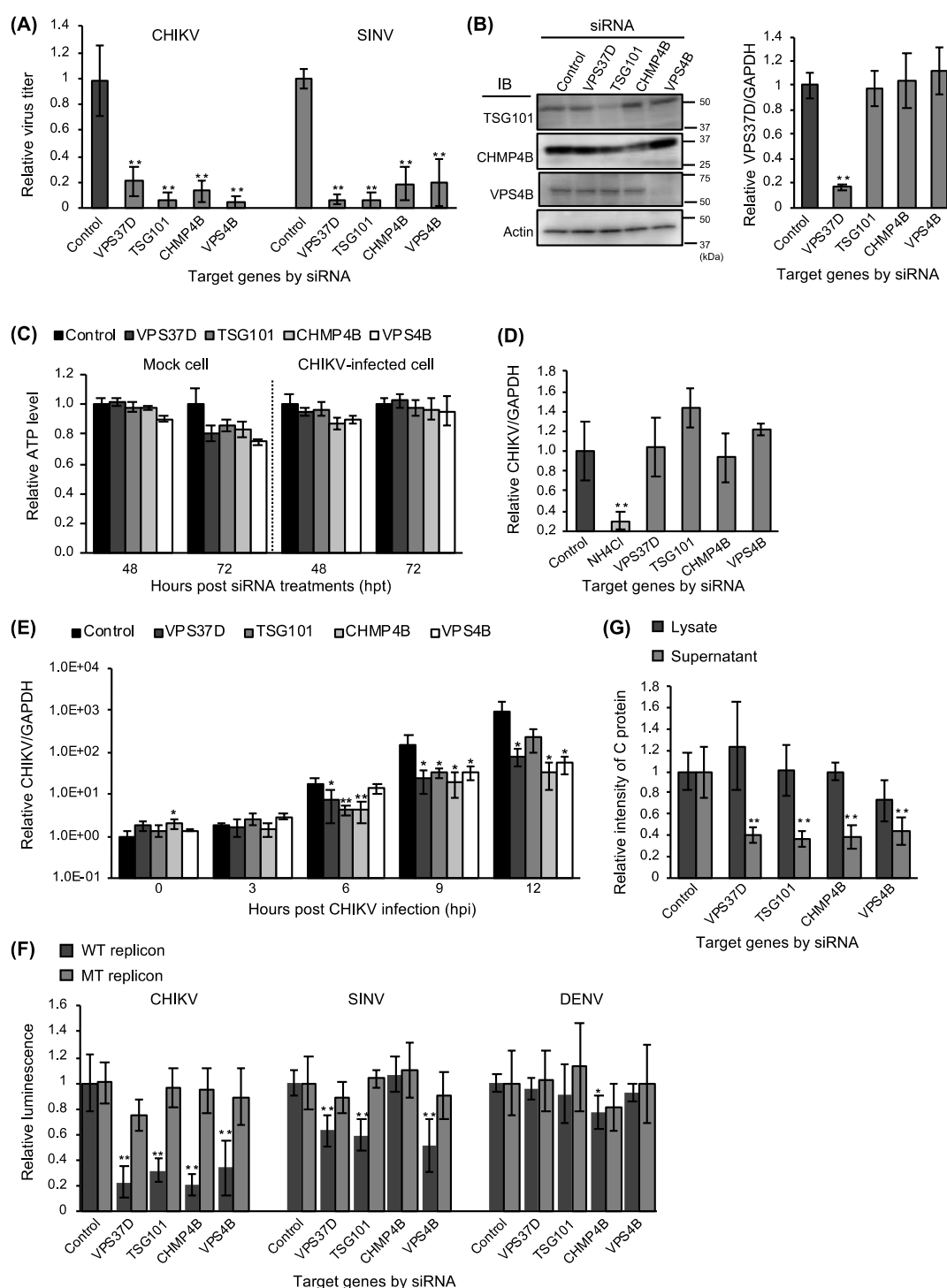


Fig. 14. KD of ESCRT factors inhibits genome replication and release steps of CHIKV infection.

(A) Following the reverse transfection of siRNAs against 4 ESCRT factors, HEK293T cells were infected with CHIKV (MOI=0.1) or SINV (MOI=0.05) for 24 hours. CHIKV titers were then determined by plaque assay using the cell supernatants and normalized with control siRNA-treated cells. (B) Protein expression levels of TSG101, CHMP4B, VPS4B and β -Actin (control) in siRNA-treated cells were determined by

immunoblotting. The relative expression levels of VPS37D to GAPDH mRNA were determined by qRT-PCR using the total RNAs extracted from siRNA-treated cells. (C) Relative viability of siRNA-treated cells was evaluated by measuring cellular ATP levels from 48 and 72 hours after siRNA treatments and normalizing with control siRNA-treated cells. (D) Intracellular viral RNA levels were examined at 2 hpi of CHIKV (MOI=1.0) into siRNA-treated HEK293T cells. NH₄Cl-treated cells were used as control. (E) Time course of CHIKV genome expression in siRNA-treated HEK293T cells inoculated with CHIKV (MOI=0.1) for 12 hours. Intracellular viral RNA levels were measured at the indicated time points. (D) and (E) Expression of viral RNA and GAPDH mRNA was determined by qRT-PCR using the total RNAs extracted from trypsinized CHIKV-infected cells. (F) HEK293T cells were transfected with siRNAs and CHIKV, SINV or DENV replicon-expressing plasmids. The luciferase activities were measured at 24 hpt of replicon-expressing plasmids. (G) HEK293T cells were transfected with siRNAs and CHIKV-VLP-expressing plasmids. At 48 hpt of CHIKV-VLP-expressing plasmids, the culture supernatants and cells were collected and lysed with TNE lysis buffer. The amount of C proteins in both lysate and supernatant samples was then analyzed by immunoblotting. The all relative data were normalized against control siRNA-reverse-transfected cells and represented as means $\pm$ S.D., collected in triplicate and independently at least 2 times (A, B, D, E, F and G). Statistical significance was assessed using one-way ANOVA with Dunnett's test and indicated by asterisks (*, $p < 0.05$ and **, $p < 0.001$ compared between control siRNA-treated cells).

CHIKV replication was investigated. The KD of each ESCRT factor did not reduce intracellular viral RNA level at 2 hpi, while NH₄Cl treatment significantly reduced the level of intracellular CHIKV RNA (Fig. 14D).

A time course assay was also performed in the presence of siRNAs targeting these four ESCRT factors. At 0 and 3 hpi, these four ESCRT factor-KD cells did not decrease intracellular viral RNA levels (Fig. 14E), similar to HGS. Increases in intracellular viral RNA levels were observed in control siRNA-treated cells from 3 to 12 hpi, suggesting that CHIKV genome replication started prior to this time point, and inhibition of CHIKV increases in RNA levels by the KD of these four ESCRT factors were observed at 6, 9 and/or 12 hpi, demonstrating that these four ESCRT factors play a role in CHIKV replication (Fig. 14E). Therefore, replication activities of CHIKV as well as SINV and DENV in ESCRT factor-KD cells were examined. The results of CHIKV replicon assays demonstrated that the KD of these four ESCRT factors significantly reduced the relative luminescence of the WT CHIKV replicon, while there was no effect on RdRp-inactivated MT CHIKV replicon (Fig. 14F). The KD of VPS37D, TSG101 and VPS4B also decreased the relative luminescence of WT SINV replicon, but did not decrease WT DENV replicon activities (Fig. 14F). These results demonstrated that VPS37D, TSG101 and VPS4B regulate genome replication steps of CHIKV and SINV specifically. In addition, CHMP4B may be involved in replication of CHIKV, but not SINV.

Next, the post-translational steps of CHIKV infection in ESCRT factor-KD cells using the CHIKV-VLP system was examined. The KD of the examined ESCRT factors apparently reduced the relative intensity of C protein in supernatants but not in lysates (Fig. 14G). Taken together, ESCRT factors, including VPS37D, TSG101, CHMP4B and VPS4B have a pivotal role in the replication and the post-translational step of CHIKV

as well as HGS (ESCRT-0). In addition, HGS, VPS37D, TSG101 and VPS4 are involved in replication of SINV, but not of DENV.

ESCRT factors are required for CHIKV infection as well as SFV

Since the previous study demonstrated ubiquitin depletion and dominant-negative VPS4 inhibited budding of VSV but not SFV (Taylor et al. 2007), whether CHIKV infection is dependent on ubiquitin and/or VPS4B was examined.

A role for ubiquitination in CHIKV infection was assessed by using the proteasome inhibitor, MG-132, according to the previous report (Taylor et al. 2007). HEK293T cells were infected with CHIKV for 1 hour, cultured for 2 hours, and then incubated for 22 hours in the presence of MG-132. HEK293T cells were also infected with VSV and SFV as the assay controls. Virus titers were determined by plaque assays with culture supernatants of virus-infected cells. The treatment of MG-132 significantly reduced VSV titers but not SFV titers at 7 hpi (Fig. 15A) in agreement with the previous report (Taylor et al. 2007). However, significant reduction of VSV and SFV titers was observed by MG-132 treatment at 12 hpi. The treatment of MG-132 also inhibited CHIKV infection at 12 and 24 hpi, indicating that the treatment of MG-132 affected CHIKV and SFV infection as same as VSV. The effect of MG-132 on intracellular CHIKV RNA levels was also examined. HEK293T cells were infected with CHIKV for 1 hour, cultured for 2 hours, and then incubated for 10 hours in the presence of MG-132. CHIKV RNA levels were measured by qRT-PCR. MG-132 treatment significantly reduced intracellular CHIKV RNA levels at 12 hpi (Fig. 15B), suggesting that the presence of MG-132 affected CHIKV replication step.

The requirement of functional VPS4B in CHIKV infection by using dominant-negative VPS4B-expressing HEK293T cells was also examined. The

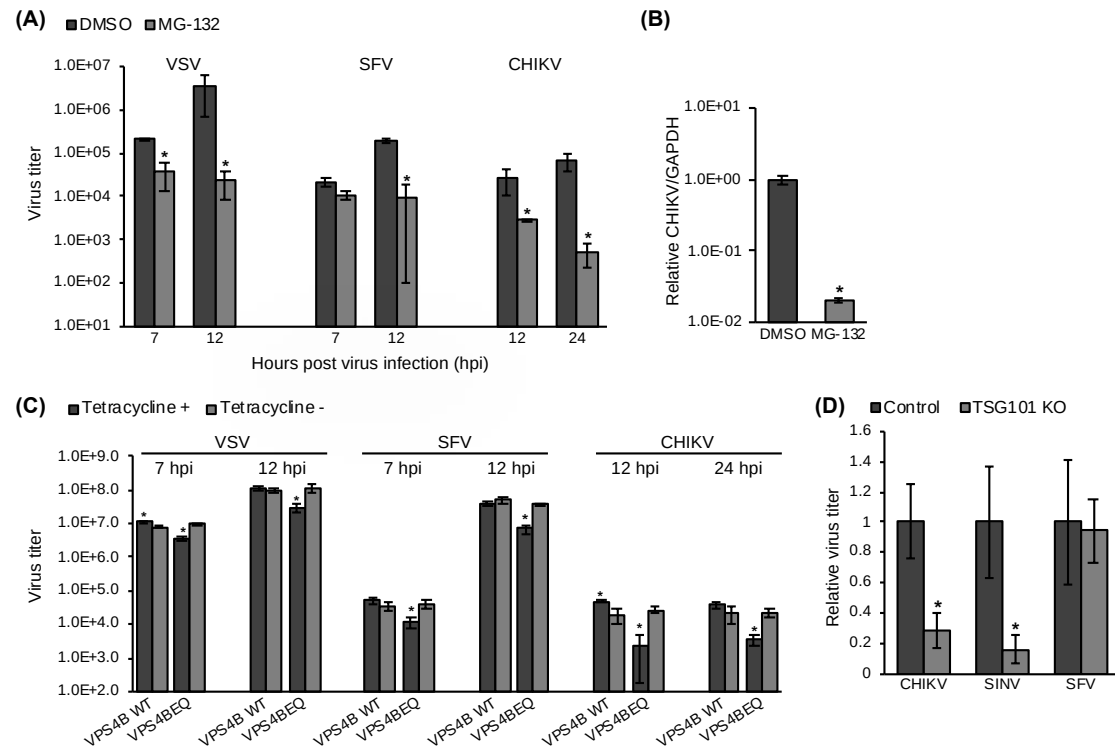


Fig. 15. Treatment of MG-132 and expression of dominant-negative VPS4B inhibited infection of some alphaviruses.

(A) HEK293T cells were infected with VSV, SFV or CHIKV (MOI=0.1). After incubation of the cells for 2 hours, the virus-infected cells were treated with MG-132. The viral titers in the supernatant samples collected at the indicated time points were determined by a plaque assay. (B) Intracellular CHIKV RNA levels were determined by qRT-PCR using extracted RNAs from cell lysates collected at 12 hpi of CHIKV infection (MOI=0.1). (C) The expression of VPS4B WT or VPS4BEQ were induced with doxycycline for 24 hours, following infection of VSV, SFV or CHIKV (MOI=0.1). Virus titers in the supernatants at the indicated time points were determined by plaque assay. (D) TSG101-KO Huh-7 cells or control Huh-7 cells were infected with CHIKV (MOI=1.0), SINV (MOI=0.1) or SFV (MOI=0.1). At 24 hpi, the culture supernatants were collected and subjected to the plaque assay. All relative data were normalized against control cells and represented as means $\pm$ S.D., collected in triplicate and independently at least 2 times. Statistical significance was assessed using Student's *t*-test. and indicated by asterisks (*, $p < 0.05$ compared between control cells).

exogenous expression of VPS4B WT or ATP hydrolysis-deficient VPS4BEQ were induced by doxycycline-inducible gene expression system (see Materials and Methods for details). After doxycycline induction for 24 hours, VPS4B-expressing HEK293T cells were infected with viruses the supernatants were collected from the virus-infected cells at the indicated time points. The virus titers were determined by plaque assay. In agreement with the previous study (Taylor et al. 2007), induction of VPS4BEQ expression decreased VSV titers at 7 and 12 hpi, while induction of VPS4B WT expression did not decrease titers (Fig. 15C). Similarly, expression of VPS4BEQ reduced titers of SFV at 7 and 12 hpi, and CHIKV at 12 and 24 hpi, while expression of VPS4B WT did not reduce them. These results indicated that functional VPS4B is involved in SFV and CHIKV infection.

Finally, the requirement of TSG101 in the ESCRT-III complex among CHIKV, SINV and SFV infection was examined by using TSG101 KO-Huh-7 cells. The KO of TSG101 gene by the CRISPR-Cas9 system was confirmed by genome sequence of the target site and protein expression level (data not shown). CHIKV, SINV and SFV were infected to the TSG101-KO cells and then the virus titers were determined by plaque assay using the culture supernatants collected at 24 hpi. The KO of TSG101 significantly reduced CHIKV and SINV titers, while deletion of TSG101 had no effect on SFV infection (Fig. 15D). These results suggested that some alphaviruses utilize ESCRT factors, and usage patterns may differ among alphaviruses.

In this study, it was clearly demonstrated that these ESCRT factors regulate propagation of some alphaviruses including CHIKV (Fig. 16).

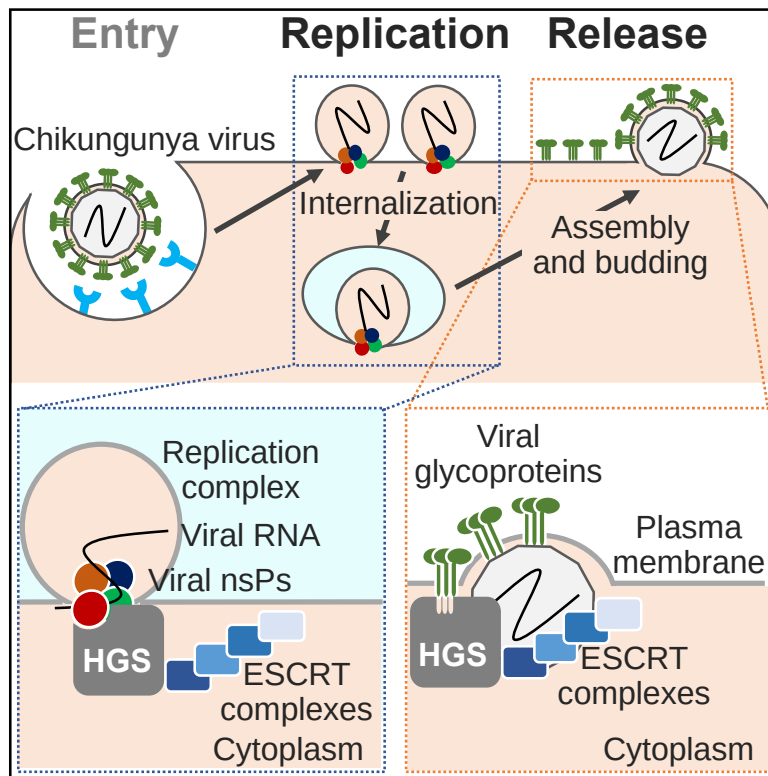


Fig. 16. Schematic image of ESCRT trafficking machinery in CHIKV infection. ESCRT factors regulate both the CHIKV replication and post-translational steps. CHIKV nsPs interact with HGS, a component of the ESCRT-0 complex, and recruit other ESCRT complexes in genome replication steps. CHIKV glycoproteins also interact with HGS and recruit other ESCRT complexes in post-translational steps.

Discussion

Viruses are known to hijack the host cellular machinery for their efficient propagation in infected target cells. Although CHIKV seems to utilize host membrane trafficking factors for its intracellular transportation in infected cells, host factors related to trafficking are still largely uncharacterized, due to the limited studies focusing on CHIKV.

A variety of enveloped viruses exploit the ESCRT machinery to promote budding of their infectious particles from cell membranes (Votteler and Sundquist 2013); however similar roles have not been reported for alphaviruses, including CHIKV. In this study, specific siRNA screen targeting trafficking genes demonstrated that ESCRT factors are indeed required for efficient CHIKV infection. By co-IP analyses, HGS in ESCRT-0 was found to be bound to CHIKV E1, E2 and nsP4. Confocal analyses showed that HGS colocalized with E2 and dsRNA in CHIKV-infected cells. Functional analyses indicated that the KD of ESCRT factors inhibited replication and post-translational steps of CHIKV infection, though ESCRT machinery was reported to be utilized mainly for budding of viruses. Here, it was proposed that CHIKV requires ESCRT factors during the intracellular replication cycle (Fig. 16).

Since there are no known L domains in CHIKV proteins, the nsPs and sPs of CHIKV would appear to interact with HGS by an L domain-independent manner. Prior studies suggest that HGS recognizes ubiquitinated cargo by its ubiquitin-interacting motif (UIM) for sorting into the MVBs (Hirano et al. 2006). For instance, polyubiquitination of HCV NS2 has been reported to mediate binding to the HGS UIM and access into the ESCRT network without viral L domains (Barouch-Bentov et al. 2016, Kumar et al. 2019). In contrast, the ubiquitin-independent association with HGS was reported at dual steps of hepatitis B virus infection, though the detailed binding

mechanisms remain to be elucidated (Chou et al. 2015). Since little is known about polyubiquitination of CHIKV (Karpe, Pingale and Kanade 2016) and the interaction mechanisms between HGS and viruses appeared variable, the UIM domain was not focused on, but the binding ability of truncated HGS mutants to CHIKV proteins was investigated by co-IP experiments, which determined that amino acid residues 278-527 of HGS were critical for binding to the CHIKV E1, E2 and nsP4 (Figs. 11D and E). The putative HGS UIM domain is between residues 258-277 (Hirano et al. 2006) and immediately adjacent to residues 278-527; therefore, it is possible that deletion of residues 278-527 affected the UIM domain of HGS and the interactions of HGS with CHIKV may depend upon ubiquitination. Further investigation using HGS mutants lacking several amino acids within 258-277 is needed to determine HGS binding sites to CHIKV proteins.

As well as HGS, another ESCRT factor, TSG101 was shown to bind nsP4 (Fig. 11B), indicating that nsP4 may have multiple binding sites for ESCRT factors. It has been reported that retroviruses and filoviruses have more than one binding domain for ESCRT factors (Liu and Hartly 2010, Gordon et al. 2019, Fujii, Hurley and Freed 2007). For instance, a decreased efficiency of HIV release was associated by mutation of an L domain interacting with TSG101 which was rescued by expression of ALIX in trans (Usami, Popov and Göttinger 2007, Fisher et al. 2007). In that context, it is important to investigate the detailed binding mechanisms between TSG101 and CHIKV nsPs to identify the key factor(s) necessary for recruiting the ESCRT machinery. To date, most ESCRT studies have focused on ubiquitin and certain other factors such as L domain-binding proteins and VPS4 at the budding steps of virus particles; however, it was demonstrated that unique interaction mechanisms of ESCRT factors were independent of viral L domains in CHIKV infection, indicating the importance to

examine detailed mechanisms in individual viruses.

Initial siRNA screens demonstrated that multiple ESCRT factors were related to CHIKV infection (Fig. 10); moreover at least five ESCRT factors in different ESCRT complexes would be demonstrated to regulate the same CHIKV propagation steps (Figs. 13 and 14). Hence, it is possible that a series of ESCRT components may be consequently recruited after recognition of CHIKV by HGS, and they play a role in the invagination of the host membranes and biogenesis of vacuoles during the viral replication cycle (Fig. 16). Interaction of HGS with structural proteins and inhibition of CHIKV-VLP release by KD of HGS indicates that ESCRT factors may mediate assembly and budding of CHIKV similar to the budding of HIV. Interestingly, this study also implicates the ESCRT machinery in regulation of the CHIKV replication. This finding is not entirely surprising since brome mosaic virus, belonging to the alphavirus-like superfamily, as well as tomato bushy stunt virus in the family *Tombusviridae*, have been reported to require particular ESCRT factors for formation of their RNA replication compartments (Barajas, Jiang and Nagy 2009, Diaz et al. 2015, Barajas et al. 2014). Therefore, it was speculated that the KD of ESCRT factors inhibited proper formation of plasma membrane-derived replication complex spherules and/or CPV-I, in which genome replication of alphaviruses occurs, resulting in a decrease of the replication efficiency of CHIKV. Colocalization of dsRNA and HGS in the cytoplasm in the CHIKV-infected cells provides evidence in support of this hypothesis (Fig. 12). Since the biogenesis of the unique spherules in CHIKV replication remains poorly characterized, high-resolution electron microscopy-based approaches will be needed to resolve this. Generally, ESCRT factors play a role in invagination of the membrane from the cytosol. It has been reported that ESCRT factors regulate various virus propagation steps relating to membrane compartments: STAM1 in

ESCRT-0 interacts with TIM-1, an authentic DENV entry receptor (Dejarnac et al. 2018), and ESCRT-III factors are recruited for the nuclear export of HSV-1 (Arii et al. 2018). These findings indicate that comprehensive study of intracellular trafficking of viruses will be essential for understanding of precise roles of ESCRT factors in virus infection.

In consideration of the profound impact of CHIKV infection on public health, a deeper understanding of the intracellular CHIKV replication cycle is demanded. Currently, there is little information describing CHIKV replication, assembly and budding steps, therefore the knowledge of CHIKV replication cycle has largely been deduced from data obtained with other alphaviruses (Griffin 2013). However, mechanism(s) of viral replication in association with host factors seem to be different even in the same genus. In fact, it was demonstrated that the KO of TSG101 had different effects on CHIKV, SINV and SFV, which belongs to the same genus *Alphavirus*. While it has been reported that SFV bud from infected cells independent of both ubiquitin and the activity of VPS4 (Taylor et al. 2007), treatment of MG-132 and expression of a dominant-negative VPS4B mutant inhibited the infection of CHIKV as well as SINV and SFV in this study. The differences in results might be derived from the growth efficiency of SFV, virus strains, cell types and virus titers. Time points of the experiment on the effect of MG-132 treatment and expression of dominant-negative VPS4B on SFV titers are also different from the previous study (Taylor et al. 2007). Follow up studies to clarify the detailed mechanisms of inhibition of alphavirus infection by MG-132 treatment and VPS4B is necessary. In the present study, it was proposed that ESCRT components the key factors regulating at least two different steps of CHIKV propagation. These findings will open molecular studies of CHIKV to broaden our knowledge regarding the pathogenesis and proliferation mechanisms of

CHIKV and it was considered that further analysis of CHIKV-ESCRT interactions will provide a base for the rational design of effective antiviral strategies.

Summary

Few studies exist describing host factors necessary for the intracellular trafficking of CHIKV. Thus, siRNA screen was conducted to identify intracellular trafficking factors potentially regulating CHIKV infection, examined their interaction properties with CHIKV proteins, and investigated which infection steps of CHIKV were affected by the identified molecules. The siRNA screen showed that ESCRT proteins were involved in CHIKV infection. Co-immunoprecipitation analyses between ESCRT proteins and CHIKV proteins revealed that both structural and non-structural proteins of CHIKV interacted with HGS, a component of ESCRT-0 complex. Colocalization of HGS with CHIKV E2 and dsRNA, a marker for the replicated CHIKV genome, was also observed. Gene knockdown analyses demonstrated that HGS facilitates both genome replication and post-translational steps of CHIKV infection, in addition to other identified ESCRT factors. These results demonstrate unique recruitment of ESCRT factors *via* HGS and involvement of a series of ESCRT factors in replication and post-translational steps of CHIKV.

General Conclusion

Viruses in arthropods possess a variety of life cycles. In this study, novel viruses were discovered from arthropods by RT-PCR screening and the molecular characteristics of novel viruses were examined. In chapter 1, two TBPVs, MKWV and KURV, were detected from *I. persulcatus* ticks. Importantly, infection of MKWV in wildlife was demonstrated by serological tests, while the sporadic discoveries of MKWV and KURV in only a single tick species in Hokkaido suggested that distribution of MKWV and KURV could be restricted in limited areas. In chapter 2, the existence of an alphavirus in *Culex quinquefasciatus* mosquitoes in Zambia was firstly reported by comprehensive and specific screening for alphaviruses. The detected virus is a novel alphavirus species, tentatively named Mwinilunga alphavirus, which seemed to have a mosquito-specific life cycle, distinct from other known mosquito-borne alphaviruses. Recent large-scale sequence analyses have revealed the existence of novel viral genomes; however, their ecological features have been left unknown in many cases, since most studies have accidentally discovered only partial viral genome fragments. By using tick-derived cell lines, two viruses (MKWV and KURV) were successfully isolated. Thus, downstream experiments could be conducted to know the infectivity and pathogenicity of these viruses in mammals. Establishment of efficient isolation methods is demanded for viruses in arthropods. On the other hands, MKWV was not isolated, since MWAV may possess a narrow host range. Therefore, generation of MWAV by reverse genetics method will be performed similar to that for insect-specific flaviviruses in the following study. Identification of the biological characters of novel viruses is essential for comprehensively understanding their potential risks to public health.

Effective control measures for infection of arboviruses such as DENV and CHIKV are desired; however, no commercial vaccines nor effective antiviral treatments

currently exist. Since little is known about the molecular mechanisms underlying CHIKV infection, siRNA screen targeting host trafficking factors was performed using CHIKV and identified that ESCRT factors were required for CHIKV infection in chapter 3. Following functional analyses demonstrated that ESCRT factors regulated two different steps of CHIKV propagation. Mechanism(s) of viral replication in association with host factors seem to be different even in the same genus, suggesting the importance to examine individual viruses, not to deduce from data obtained with other viruses. The follow-up study will help to understand pathogenesis and propagation mechanisms of CHIKV and develop antiviral strategies to control CHIKV infection.

In consideration of the current impact of arbovirus infection on public health, a deeper understanding of the ecological features of arboviruses is demanded. There are still uncharacterized viruses in arthropods. To draw the whole maps of network among viruses in arthropod, sensitive virus detection tool is necessary. In addition to that, following specific epidemiological and biological study is important to know their ecological feature. This study provides a base for both epidemiological and molecular analyses of arthropod viruses.

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Summary in Japanese

節足動物には様々なウイルスが存在し、それぞれ異なる生活環を形成している。節足動物に特異的に感染するウイルスが存在する一方で、節足動物媒介性ウイルス（アルボウイルス）は哺乳動物と節足動物の両者に感染する。近年、ジカ熱やデング熱といった蚊媒介性アルボウイルス感染症は、物質輸送のボーダーレス化に伴い、発展途上国を中心に世界規模で蔓延し、経済上の影響を与えてきた。加えてチクングニアウイルスなどのアルボウイルスは、アメリカやヨーロッパでも感染が認められ、日本でも毎年、輸入症例が報告されている。これら越境性のアルボウイルス感染症は公衆衛生上大きな問題となっているが、チクングニアウイルスやデング熱ウイルスなどのアルボウイルスは、細胞内増殖機構や病原性発現機序において不明点が多いことから、未だ有効な治療法は確立されていない。今後、治療薬の有用なターゲットを見出し、アルボウイルス感染症対策を構築するためには、ウイルスの分布を把握する疫学調査及びウイルスの性状を明らかにする分子生物学的研究が必要とされる。

そこで本研究では節足動物に存在するウイルスの分布状況を国内外で調査し、公衆衛生学上対策が必須であるアルボウイルスの細胞内増殖機構を解析した。

第1章では、北海道のマダニを対象に網羅的なフレボウイルスの調査を実施した。RT-PCR によるフレボウイルス遺伝子調査の結果、ムカワウイルス及びクリヤマウイルスの2種のフレボウイルスをシュルツェマダニから検出し、さらにマダニ由来細胞を用いてクリヤマウイルスを分離した。また、北海道の野生動物を対象とした血清疫学調査及びマウスを用いた感染実験の結果、ムカワウイルスは哺乳動物に感染し得ることが明らかとなった。

第2章では、これまでアルファウイルスの検出が報告されていないアフリカのザンビア国で、蚊を対象に網羅的にアルファウイルスを探索した。その結果、

ネッタイシマカから新規アルファウイルスであるムウニルンガアルファウイルスを検出した。決定したウイルス遺伝子全長配列を元に解析を実施した結果、ムウニルンガアルファウイルスは、他の蚊媒介性アルファウイルスと異なり、蚊特異的に感染するウイルスであることが明らかとなった。

第 3 章では、未だ特異的治療法及び予防法が確立されていないチクングニアウイルスの細胞内の増殖機構を解析した。siRNA を用いてチクングニアウイルスの感染を制御する宿主因子を探索した結果、ESCRT 蛋白質群が同定された。更に、チクングニアウイルスのレプリコンやウイルス様粒子を用いたアッセイにより、ESCRT 蛋白質群がチクングニアウイルスのゲノム複製及び粒子形成・放出過程を制御することを明らかにした。

近年のデングウイルス感染症やジカ熱感染症などの世界規模でのアルボウイルス感染症症例の増加を鑑みると、自然界におけるアルボウイルスの生活環、存続機構を考慮した対策が必須である。本研究では、アルボウイルス感染症対策に向けて節足動物に存在するウイルスの疫学調査及び分子生物学的性状解析の基盤を構築することができた。