Title

Identification and Analysis of Host Proteins that Interact with the 3′-Untranslated Region of Tick-Borne Encephalitis Virus Genomic RNA

Running title: Identification of the host proteins interact with the variable region of 3'-UTR of TBEV

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Abstract

Tick-borne encephalitis virus (TBEV) causes severe neurological disease, but the pathogenetic mechanism is unclear. The conformational structure of the 3′-untranslated region (UTR) of TBEV is associated with its virulence. We tried to identify host proteins interacting with the 3′ -UTR of TBEV. Cellular proteins of HEK293T cells were co-precipitated with biotinylated RNAs of the 3′-UTR of low- and high-virulence TBEV strains and subjected to mass spectrometry analysis. Fifteen host proteins were found to bind to the 3′-UTR of TBEV, four of which— cold shock domain containing-E1 (CSDE1), spermatid perinuclear RNA binding protein (STRBP), fragile X mental retardation protein (FMRP), and interleukin enhancer binding factor 3 (ILF3)—bound specifically to that of the low-virulence strain. An RNA immunoprecipitation and pull-down assay confirmed the interactions of the complete 3′-UTRs of TBEV genomic RNA with CSDE1, FMRP, and ILF3. Partial deletion of the stem loop (SL) 3 to SL 5 structure of the variable region of the 3′-UTR did not affect interactions with the host proteins, but the interactions were markedly suppressed by deletion of the complete SL 3, 4, and 5 structures, as in the high-virulence TBEV strain. Further analysis of the roles of host proteins in the neurologic pathogenicity of TBEV is warranted.

Key words

Tick-borne encephalitis virus, 3′-untranslated region, host factor, pathogenicity

Text

Tick-borne encephalitis (TBE) virus (TBEV), a member of the genus Flavivirus in the family *Flaviviridae*, is a major arbovirus that causes thousands of cases of severe

neurological illness annually (WHO Publication, 2011). Humans are accidental hosts, and become infected via a tick bite, and unpasteurized goat-milk also can be a source of infection. TBE is a huge public health problem in endemic areas in European and Asian countries (Carletti et al., 2017; Lindquist and Vapalahti, 2008; Mansfield et al., 2009; Yoshii et al., 2017). Mortality rates vary from about 0.5 to 30%, and neurological sequelae occur in 30 to 60% of survivors (Grard et al., 2007; Gritsun et al., 2003; Heinz and Kunz, 2004).

TBEV has a single-stranded RNA genome of positive polarity that encodes a long polyprotein in a single open reading frame, flanked by 5′- and 3′-untranslated regions (UTRs). The 5′- and 3′-UTRs are believed to be associated with viral genome replication (Khromykh et al., 2001; Kofler et al., 2006). The 3′-UTR of TBEV is divided into two domains: the 5′-terminal variable region and the 3′-terminal core element. The core element is highly conserved among TBEV strains and contains a sequence that is essential for viral genome replication (Kofler et al., 2006). In recent studies, it was shown that subgenomic flavivirus RNA (sfRNA) is produced by the 3'-UTR of viral genomic RNA, and it was suggested that sfRNA is involved in innate immunity response in mosquito-borne flavivirus infection (Chang et al., 2013; Pijlman et al., 2008; Rouha et al., 2010; Sakai et al., 2015; Schnettler et al., 2014) .

The variable region of the 3'-UTR is considered to be essential for the natural transmission cycle of TBEV, but was previously considered not to be involved in viral replication and virulence in mammals (Mandl et al., 1998). The sequence and length of the variable region vary among TBEV strains (Wallner et al., 1995). Notably, few strains contain polyA sequences in the variable region isolated from ticks (Mandl et al., 1998; Růzek et al., 2008), and deletions of sequences in the variable region were identified in strains passaged in mammalian cell culture and in clinical isolates (Formanova et al., 2015; Leonova et al., 2013; Mandl et al., 1998). Those reports suggested that the viral

quasispecies with deletions or polyA insertions in the variable region are selected during adaptation from the tick vector to mammalian host environment (Asghar et al., 2014). Our previous studies reported that the deletion in the variable region of the 3′-UTR was involved in the pathogenicity of the strains from the Far-Eastern subtype of TBEV. In a mouse model, Sofjin-HO (accession no. AB062064) showed higher virulence than Oshima 5-10 strain (accession no. AB062064) (Chiba et al., 1999) . The Oshima strain putatively forms seven Stem Loop (SL) structures in the variable region (Fig. 1A), while the SL 3 to 5 structures were deleted in the Sofjin strain. By reverse genetics analysis, introduction of the deletion into the Oshima strain drastically increased the virulence (Sakai et al., 2015). However, the role of this region in virus pathogenicity remains unclear.

Host factors that bind to the viral 3′-UTR RNA including sfRNA play important roles in infections by mosquito-borne flaviviruses, such as Dengue virus (DENV), Japanese encephalitis virus (JEV), and West Nile virus (WNV) (Bidet et al., 2014; Manokaran et al., 2015). However, few study has focused on TBEV, and prion-like T-cell-restricted intracellular antigen 1 (TIA-1) and TIA-1-related protein (TIAR) were the only identified proteins interacting the TBEV RNA (Albornoz et al., 2014). In this study, we hypothesized that differences in the variable region of the 3′-UTR of TBEV affect the interaction of this virus with host factors. We thus identified and characterized host proteins that bind to the 3′-UTR of TBEV.

To identify host proteins that bind to the variable region of the 3′-UTR of TBEV, biotinylated RNAs for 100 nt of the 3′ end of the coding sequence and the 3′-UTR of the Oshima (Full) or Sofjin (Δ_SL3-5) strain, and the coding sequence for EGFP as a control, were synthesized *in vitro* using a Biotin RNA Labeling Kit (Roche, Basel, Switzerland). One μg of the RNAs were mixed with 200 μg of proteins in lysate of HEK293T cells and precipitated using streptavidin magnetic beads (GE Healthcare, Buckinghamshire, UK) (Fig.

100 1A). The co-precipitated proteins were subjected to sodium dodecyl sulfate-polyacrylamide
101 gel electrophoresis and silver stained using a SilverQuest Staining Kit (Invitrogen, Carlsbad,
102 CA, USA) (Fig. 1B). Proteins bound to the viral RNA were detected at 72–90 kDa. The
103 bands were excised from the gels, and each was trypsinized and subjected to liquid
104 chromatography-electrospray tandem mass spectrometry (LC-ESI-MS/MS) (LTQ Orbitrap
105 Velos + ETD, Thermo Fisher Scientific) to identify co-precipitated proteins. All of the
106 proteins were analyzed with Mascot Server2.5 (MATRIX SCIENCE, Tokyo, Japan), and
107 those from cells containing the 3′-UTR RNA of TBEV but not in those containing EGFP
108 RNA were regarded as candidate binding partners of the 3′-UTR of TBEV. The proteins
109 with a spectral and ion score of > 200 are listed in Table 2. Cold shock domain
110 containing-E1 (CSDE1), interleukin enhancer binding factor 3 (ILF3), spermatid
111 perinuclear RNA binding protein (STRBP) which is a paralogue of ILF3 (Schumacher et al.,
112 1995), and fragile X mental retardation protein (FMRP) bound specifically to the 3′-UTR of
113 the Oshima strain. Neither of these 4 proteins interacted with the 3′-UTR of the Sofjin strain.
114 CSDE1, FMRP, and ILF3 were confirmed to co-precipitate with the 3′-UTR of the Oshima
115 strain, but not that of the Sofjin strain or the EGFP coding sequence, by western blot
116 analysis using rabbit anti-CSDE1 (AB176584, Abcam, Cambridge, UK), mouse
117 monoclonal anti-FMRP (MAB2160, Merck KGaA, Darmstadt, Germany), and mouse
118 anti-ILF3 antibodies (H00003609-B01P, Abnova, Taipei) (Fig. 1C). Similar results were
119 obtained for CSDE1 and ILF3 in human neuroblastoma SHSY5Y cells while the binding of
120 FMRP could not be confirmed due to the low expression of endogenous FMRP in the cell
121 line (Data not shown).

122 To examine their specific interactions with viral 3′-UTR RNA, total RNA was
123 extracted from HEK293T cells and subjected to RT-PCR using gene specific primer sets
124 (Table 1). The Flag-tagged candidate proteins were overexpressed in HEK293T cells and

125 lysates were mixed with *in vitro*-synthesized RNA and subjected to immunoprecipitation
126 (IP) using an anti-Flag antibody (F3165; Sigma, St. Louis, MO, USA). Co-precipitated
127 RNA was amplified by reverse transcription-polymerase chain reaction (RT-PCR). The
128 full-length 3′-UTR RNA of the Oshima strain (Full) co-precipitated with all of the
129 RNA-binding proteins, but that of the Sofjin strain (Δ_SL3-5) did not (Fig. 2). Therefore,
130 several host proteins interact specifically with the 3′-UTR RNA of the Oshima strain but
131 not the Sofjin strain.

132 Next, we examined the effects of deletions in the 3′-UTR on the differential binding
133 to host proteins of the Oshima and Sofjin strains. Biotinylated Δ_SL3-4, Δ_SL5, and
134 Δ_SL3-5 RNAs were prepared by deleting the SL 3 and 4 (nt 10,443–10,567), SL 5 (nt
135 10,568–10,649) and SL 3 to 5 (nt 10,443-10,649) regions in the Oshima RNA, respectively.
136 A pull-down assay using streptavidin beads showed that all three proteins interacted with
137 the Full, Δ_SL3-4, and Δ_SL5 RNA, but not with the Δ_SL3-5 RNA (Fig. 3). These results
138 indicate that complete deletion of SL3, SL4, and SL5, but not their partial deletion, affects
139 the interactions of the 3′-UTR with RNA-binding proteins.

140 To investigate the role of the host proteins in viral replication, gene silencing was
141 performed in SH-SY5Y cells (Fig. 4). For knockdown of the CSDE1 and ILF3 gene,
142 siRNA for CSDE1 (HSS111760, Thermo Fisher Scientific) and for ILF3 (HSS105413,
143 Thermo Fisher Scientific) were transfected using Lipofectamine RNAiMax (Invitrogen).
144 For knockdown of the FMRP gene, a lentiviral vector that expresses a short hairpin RNA
145 (shRNA) for FMRP
146 (5’-CCGGGCGTTTGGAGAGATTACAAATCTCGAGATTTGTAATCTCTCCAAACGC
147 TTTTTG-3’) was generated by using pFU6-pGK puro vector. Pseudotyped lentivirus was
148 infected to SH-SY5Y cells and selected with 6 μg/ml of Puromycin. The treatment of
149 siRNA for ILF3 did not reduced the ILF3 expression significantly. The CSDE1

knock-down cells showed no significant differences in the viral titers as compared to mock cells (Fig. 4A). In contrast, knockdown of FMRP reduced the viral titer of all virus strains, but the viral titer of Oshima-IC was slightly recovered by the deletion of SL3-5 (Δ_SL3-5) to the same extent as that of the Sofjin ($p < 0.01$) (Fig. 4B).

CSDE1, FMRP, and ILF3 bound specifically to the 3′-UTR RNA of the low-virulence Oshima strain, and higher MS scores of ILF3 were observed in the host proteins co-precipitated with the 3′-UTR RNA of the Oshima strain than that of the highly virulent Sofjin strain. The SL 3–5 structures, which are recognized by host proteins, are deleted in the Sofjin strain (Fig. 3). As described previously, the interaction of host factors with the viral RNA via the SL structures of the 3′-UTR might affect the pathogenicity of TBEV, possibly by enabling escape from host antiviral responses. The variable region may act as a spacer separating the folded 3′-UTR from the rest of the genome, which could facilitate binding of the viral RNA polymerase and cellular factors involved in transcription. Alternatively, binding of the host proteins identified in this study may compete with that of viral or host proteins involved in transcription.

CSDE1 is mostly localized in cytoplasm and is involved in the regulation of mRNA stability and translation (Mihailovich et al., 2010). CSDE1 reportedly binds the 3′-UTR of DENV, and knockdown of CSDE1 suppresses DENV replication (Phillips et al., 2016). Moreover, CSDE1 is involved in neural differentiation (Ju Lee et al., 2017). TBEV infection affects neuronal functions, such as neurite development (Hirano et al., 2017; Yoshii et al., 2014) , in a manner possibly involving the interaction with CSDE1.

The transcript variants of ILF3 form a complex involved in neural transport (Larcher et al., 2004). ILF3 and interleukin enhancer-binding factor 2 (ILF2), also known as nuclear factor 90 (NF90) and nuclear factor 45 (NF45), respectively, form a protein complex that is involved in the post-transcriptional control of gene expression in vertebrates (Barber, 2009).

175 Within cytoplasmic viral replication foci, the ILF3/ILF2 complex associates with the
176 genomic RNA of HCV and DENV through regulatory structures in the 5′- and 3′-UTR
177 (Gomila et al., 2011; Isken et al., 2007). Thus, protein complexes consisting of ILF3 and
178 ILF2 may affect viral replication and neuropathogenicity.

179 FMRP is highly expressed in neurons, forms part of a complex responsible for
180 intracellular mRNA transport, and plays an important role in neuronal diseases in humans.
181 FMRP binds the 5′-UTR of TBEV genomic RNA, which results in the development of
182 neurological disease (Hirano et al., 2017). FMRP and ILF3 are involved in the formation of
183 RNA granules (El Fatimy et al., 2012; Shiina and Nakayama, 2014), which are large
184 messenger ribonucleoprotein complexes that control translation and mRNA translocation
185 (Anderson and Kedersha, 2006). Neuronal RNA granule also plays important roles in
186 transport and local translation of mRNA in dendrites (Bramham and Wells, 2007). RNA
187 granules recognized viral RNAs, which induces translation of interferon-stimulated
188 mRNAs, and antiviral responses are affected by RNAs derived from 3′-UTR of DENV
189 (Bidet et al., 2014). It was also reported that TIA-1 and TIAR are components of stress
190 granules, and bound to the RNA of TBEV (Albornoz et al., 2014) .

191 In this study, silencing of FMRP significantly reduced viral replication (Fig. 4B),
192 indicating that the FMRP played important roles in TBEV replication. On the other hand,
193 the reduction of viral replication by FMRP-knockdown was relatively lower in Sofjin-IC
194 infection, and the deletion of the complete SL 3-5 structure increased the viral replication of
195 Oshima-IC in the FMRP-knockdown cells. These results suggested that the deletion of the
196 complete SL 3-5 structures compensated the TBEV replication without the binding of
197 FMRP. However, the role of FMRP during viral infection *in vivo* was not investigate in this
198 study, therefore, further research is warranted.

199 In summary, we identified host proteins that bind to the 3′-UTR of TBEV genomic

RNA. Some of the proteins interacted specifically with the RNA of the low-virulence strain, and the SL structures in the 3′-UTR were involved in these interactions. Further analysis of the mechanism of interaction between the host proteins and viral RNA will contribute to our understanding of the pathogenicity of TBEV, and facilitate development of therapies for TBE.

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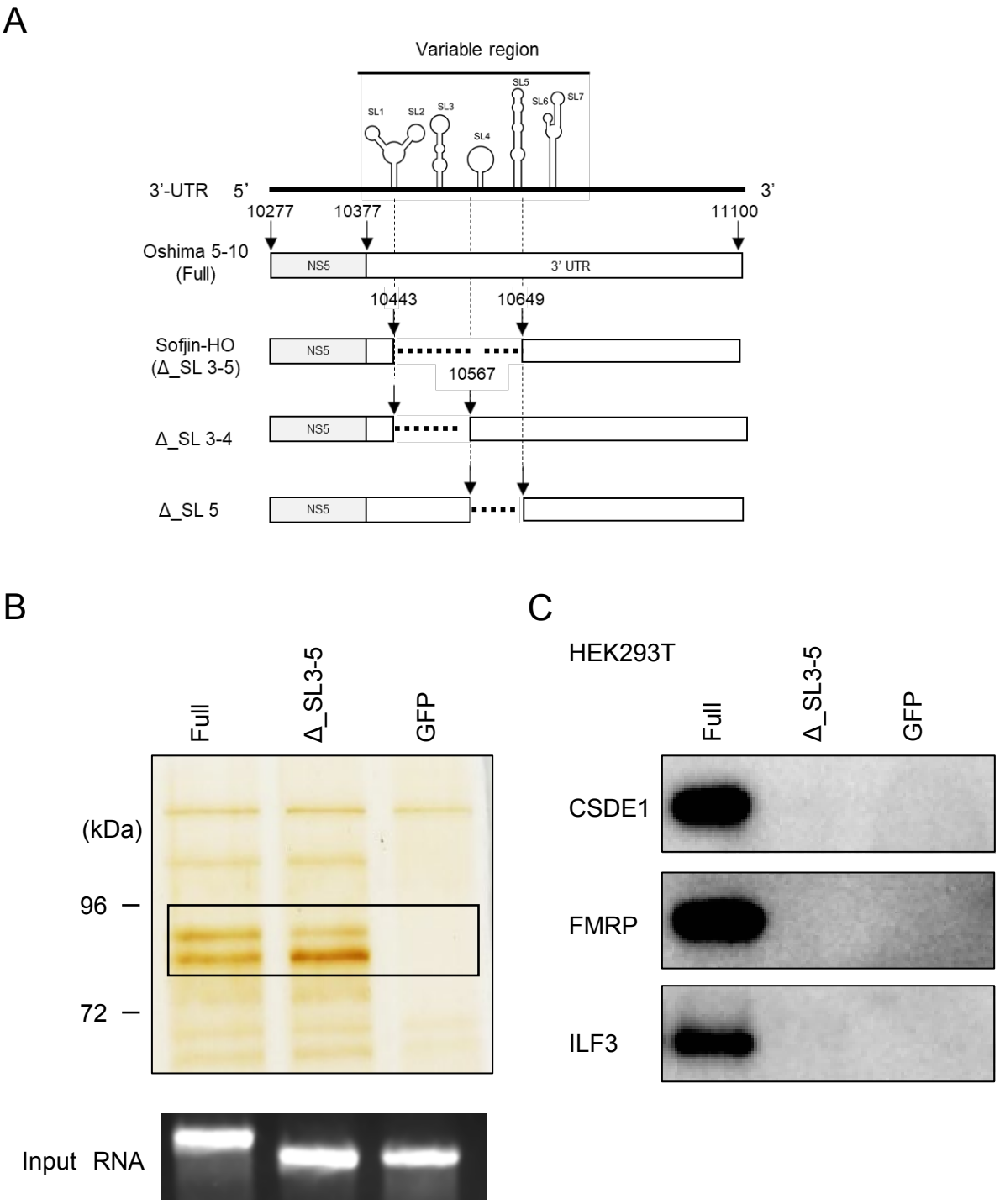
Figure Legends

Fig. 1. (A) Schematic of the 3′-untranslated region (UTR) of tick-borne encephalitis virus (TBEV) genomic RNA. The SL 3, 4, and 5 (nt 10,443–10,649) were deleted in the Sofjin-HO strain and Δ_SL3-5. The SL 3 and 4 (nt 10,443–10,567) were deleted in Δ_SL3-4. The SL 5 (nt 10,568–10,649) was deleted in Δ_SL5. (B) Cellular proteins that interact with the 3′-UTR of TBEV. HEK293T cell lysates were mixed with the biotinylated 3′-UTR of TBEV, and precipitated using streptavidin beads. The proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and silver stained. Black square indicates the range of proteins subjected to mass spectrometry analysis. The bottom panel shows input biotinylated RNA for the pull down assay. (C) Proteins that co-precipitated with the 3′-UTR of TBEV were analyzed by western blotting.

Fig. 2. Interaction of host proteins with *in vitro*-synthesized RNA. The lysates of cells overexpressing Flag-tagged proteins were mixed with *in vitro*-synthesized RNA for the 3′-UTR of TBEV, and were precipitated using an anti-Flag antibody. The RNA was extracted from immunocomplexes and TBEV RNA was detected by reverse transcription-polymerase chain reaction (RT-PCR). Upper panel, RT-PCR analysis of

336 co-precipitated RNA; lower panel, western blot analysis of proteins immunoprecipitated
337 with the anti-Flag antibody.
338 Fig. 3. Effect of deletions in the variable region on the interactions of TBEV RNA with cold
339 shock domain containing-E1 (CSDE1), fragile X mental retardation protein (FMRP), and
340 interleukin enhancer binding factor 3 (ILF3). Biotinylated 3′-UTR RNA of TBEV with or
341 without deletion of stem loop 3–5 was incubated with HEK293T cell lysates and pulled
342 down using streptavidin beads. The co-precipitated proteins were detected using an
343 anti-CSDE1, -FMRP, or -ILF3 antibody. The synthesized RNAs were subjected to RT-PCR
344 and agarose gel electrophoresis.
345 Fig. 4. Analysis of the function of the host proteins in TBEV replication.
346 CSDE1 (A) or FMRP (B) was knocked down by siRNA or shRNA in SHSY5Y cells,
347 respectively. The expression levels of CSDE1 or FMRP were analyzed by Western blotting.
348 β-actin was used as a loading control. The knockdown cells were infected at an MOI of
349 0.05 with Oshima-IC, Δ_SL3-5, or Sofjin-IC, respectively. The supernatant from each cells
350 was harvested at 18 h.p.i. and the viral titer was measured by plaque assay. Each viral titer
351 was normalized as the ratio of viral titer in mock cells. Data represent mean ±S.D. of three
352 independent experiments. Statistical significance was assessed using the Steel test, and is
353 indicated by asterisks (*, $p < 0.01$).
354

Fig. 1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Variable region
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SL2
SL3
SL4
SL5
SL6
SL7
3'-UTR 5'
3'
10277
10377
11100
Oshima 5-10
(Full)
NS5
3' UTR
10443
10649
Sofjin-HO
(Δ_SL 3-5)
10567
Δ_SL 3-4
Δ_SL 5
B
Full
Δ_SL3-5
GFP
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72
Input RNA
C
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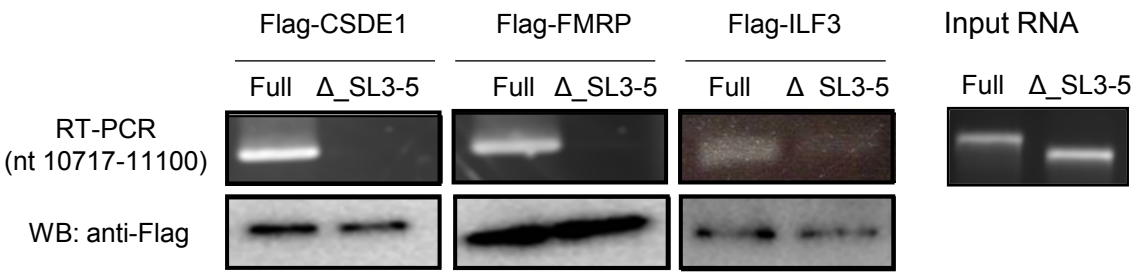
Full
Δ_SL3-5
GFP
CSDE1
FMRP
ILF3

Fig. 2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Δ_SL3-5
Full
Δ SL3-5
Full
Δ_SL3-5
RT-PCR
(nt 10717-11100)
WB: anti-Flag

Fig. 3

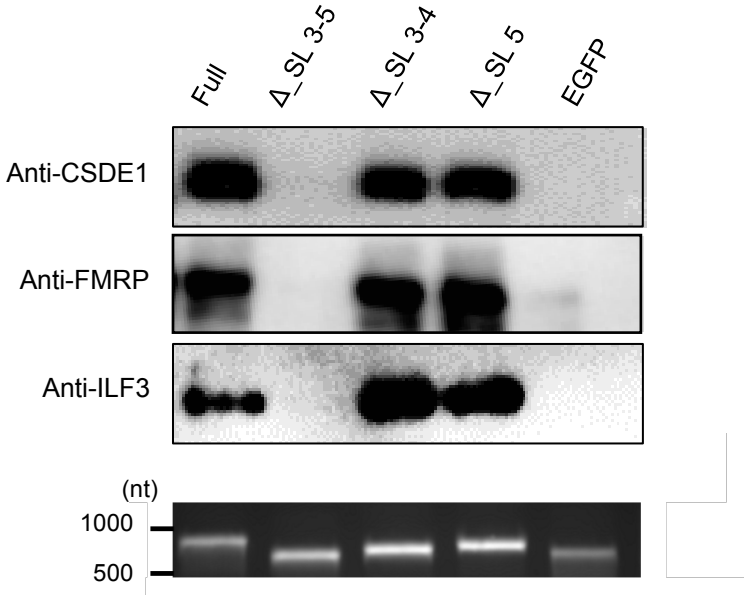
Full
Δ_SL 3-5
Δ_SL 3-4
Δ_SL 5
EGFP
Anti-CSDE1
Anti-FMRP
Anti-ILF3
(nt)
1000
500

Fig. 4

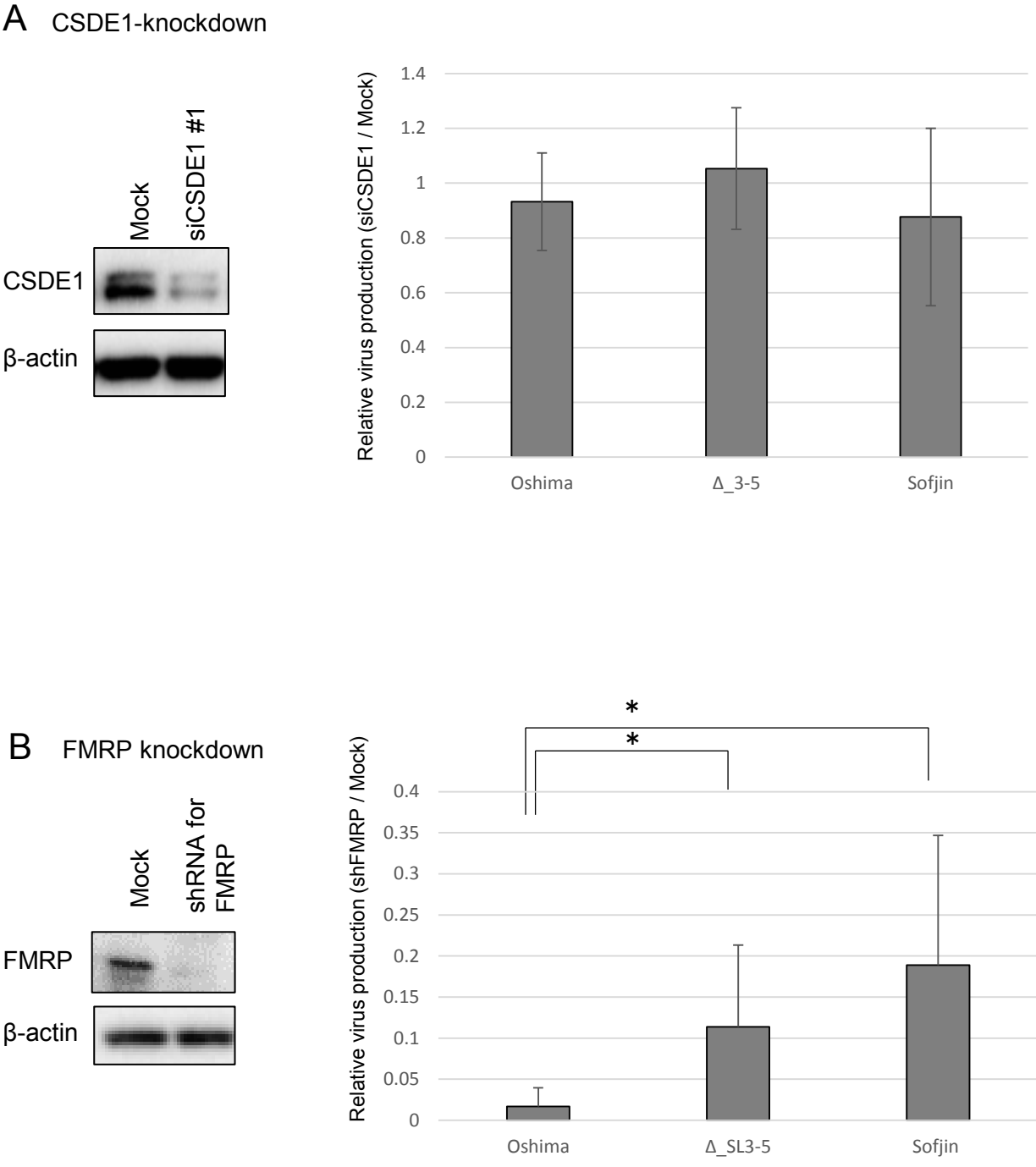
A CSDE1-knockdown
Mock
siCSDE1 #1
CSDE1
β-actin
Relative virus production (siCSDE1 / Mock)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Oshima
Δ_3-5
Sofjin
B FMRP knockdown
Mock
shRNA for FMRP
FMRP
β-actin
Relative virus production (shFMRP / Mock)
*
*
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Oshima
Δ_SL3-5
Sofjin

Table 1. Primers for the construction of plasmid expressing each host

Primer	**Primer sequence (5' to 3')**
CSDE1_Fw	TGACGATAAACTCGAATGGAGAACGTTTTTACT
CSDE1_Rv	TGGATCCCCGCGGCCTTAGTCAATGACACCAGC
FMRP_Fw	TGACGATAAACTCGAATGGAGGAGCTGGTGGTG
FMRP_Rv	TGGATCCCCGCGGCCTTAGGGTACTCCATTCAC
ILF3_Fw	TGACGATAAACTCGAATGCGTCCAATGCGAATT
ILF3_Rv	TGGATCCCCGCGGCCTTATCTGTACTGGTAGTTC

Table 2. Identified proteins interactiong with the 3‘-UTR of TBEV by MS analysis

	Accession No.	Score*			Protein Description	Gene
		Oshim	Sofjin	EGFP		
1	CSDE1_HUMAN	1957		14	Cold shock domain-containing protein E1	CSDE1
2	ILF3_HUMAN	1922	958	261	Interleukin enhancer-binding factor 3	ILF3
3	NUCL_HUMAN	1547	1049	827	Nucleolin	NCL
4	STRBP_HUMAN	988			Spermatid perinuclear RNA-binding protein	STRBP
5	HNRPQ_HUMAN	733	611		Heterogeneous nuclear ribonucleoprotein Q	SYNCRIP
6	HNRPR_HUMAN	771	930		Heterogeneous nuclear ribonucleoprotein R	HNRNPR
7	FMR1_HUMAN	341			Fragile X mental retardation protein 1	FMR1
8	HS90B_HUMAN	589	650	313	Heat shock protein HSP 90-beta	HSP90AB1
9	DDX21_HUMAN	440	558	53	Nucleolar RNA helicase 2	DDX21
10	HS71A_HUMAN	373	187		Heat shock 70 kDa protein 1A	HSPA1A
11	HS90A_HUMAN	431	528	208	Heat shock protein HSP 90-alpha	HSP90AA1
12	HSP7C_HUMAN	434	192		Heat shock cognate 71 kDa protein	HSPA8
13	IF2B1_HUMAN	324	404	20	Insulin-like growth factor 2 mRNA-binding protein 1	IGF2BP1
14	PABP1_HUMAN	218	229		Polyadenylate-binding protein 1	PABPC1
15	ZFR_HUMAN	205	408		Zinc finger RNA-binding protein	ZFR

* Blank indicated the score under detection limit.