SUPPLEMENTARY DATA

MCPIP1 ribonuclease exhibits broad-spectrum antiviral effects through viral RNA binding and degradation

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SUPLEMENTARY MATERIALS & METHODS

Lactate dehydrogenase (LDH) assay. Cell cytotoxicity was assessed by the release of the cytoplasmic enzyme LDH using a commercial kit (Cytotoxicity Detection Kit; Roche) according to the manufacturer's instructions.

Plasmids construction. MCPIP1-C306R mutant was generated by single-primer PCR method (31) by using primer annealing to MCPIP1 cDNA with the mutated codons underlined:

5'-GAGCACAGGAAGCAGCCGCGTCCCTATGGAAGGAAATGCAC-3'. For *in vitro* synthesis of truncated JEV RNAs, JEV cDNA plasmids (Δ2520-7116, Δ290-5863, Δ6965-10910, and Δ2811-10044) were obtained from SP6-driven JEV RP-9 infectious clone plasmid by restriction enzymes digestion. All of the constructs were generated using standard molecular cloning techniques and verified by DNA sequencing.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Total cellular RNA was isolated from A549 cells by using RNeasy Total RNA kit according to the manufacturer's protocol (Qiagen). RT-PCR was carried out with 1~2 μg of RNA by using the ThermoScript RT-PCR System (Invitrogen) using oligo$(dT)_{20}$ as a primer. Gene expression was determined by using the following TaqMan primers/probes mixes (Applied Biosystems) with TaqMan Gene Expression Assay ID: MCPIP1:

Hs00962356_m1, IL-1β: Hs02758991_g1, IL-6: Hs00985639_m1, TNF-α: Hs01113624_g1, MCP-1: Hs00234140_m1 and GAPDH: Hs02758991_g1. The real-time RT-PCR was performed using an ABI-Prism 7500 Sequence Detector system (Applied Biosystems). The relative mRNA levels were normalized to GAPDH internal control. For Stat1 and IRF-9 expression, total RNA was extracted and examined by RT-PCR using the specific primers for human Stat1 (5′-ATGTCTCAGTGGTACGAACTTCA-3′ and 5′-ACTCTTTGCCACACCATTG-3′), IRF-9 (5′-CGGAGTGTGCTGGGATGAT-3′ and 5′-TCAGCAACATCCATGCGGC-3′), and β-actin (5′-TCCTGTGGCATCCACGAAACT-3′ and 5′-GAAGCATTTGCGGTGGACGAT-3′), respectively. Amplification reactions were run in a total volume of 25 μl for 25 cycles by using a T3000 Thermal Cycler (Biometra), and then the amplified PCR products were analyzed by 2% agarose gel electrophoresis.

SUPLEMENTARY FIGURE LEGENDS

Figure S1. No cytotoxicity was induced in T-REx-293 cells with MCPIP1 over-expression. T-REx-293 cells were seeded at a high density of 8 x 10^5 cells per well of 12-well plate and cultured in medium without (-) or with (+) Dox (1 μg/ml)

for the indicated times. **(A)** The degree of cytotoxicity was measured by LDH release by using a commercial kit. **(B)** Cell survival rate was measured by trypan blue exclusion using Countess® Automated Cell Counter (Invitrogen). Values are shown as mean ± SD for three independent experiments.

Figure S2. RNase activity of human MCPIP1 is essential for its antiviral effect against JEV and DEN-2 infection. Human T-REx-293 cells with inducible expression of vector, MCPIP1, or MCPIP1-D141N mutant were cultured in medium without (-) or with (+) Dox (1 μg/ml) for 12 h, then infected with JEV or DEN-2 (MOI 5). At 24 h post-infection, cells were fixed and permeabilized for immunofluorescence assay of viral protein NS1 (green) expression for JEV **(A)** and DEN-2 **(B)**. Nuclei were stained with DAPI (blue). **(C)** The representative JEV plaque forming images were stained with crystal violet.

Figure S3. Human MCPIP1 blocks the reporter expression of JEV and DEN-2 replicons. Human T-REx-293 cells with WT or MCPIP1-D141N mutant were cultured in the absence (-) or presence (+) of Dox (1 μg/ml) for 12 h, and then transfected with JEV or DEN-2 replicon constructs as outlined. The cell lysates were harvested at 24 h post-transfection and the luciferase activities of JEV **(A)** or DEN-2 **(B)** replicons were determined using a Luciferase Assay System Kit (Promega). The luciferase activities derived from JEV and DEN-2 replicons are shown as means and standard deviations

of three independent experiments and were compared by two-tailed Student's *t*-tests. * $P \leq 0.05$; ** $P \leq 0.01$; NS: not significant.

Figure S4. The proline-rich domain of human MCPIP1 is essential for its antiviral activity against JEV and DEN-2 infection. Human T-REx-293 cells with vector, WT or MCPIP1-Δ458-536 mutant were cultured without (-) or with (+) Dox (1 μg/ml) for 12 h, then infected with JEV or DEN-2 (MOI 5) for 24 h. Virus titers of JEV **(A)** and DEN-2 **(B)** are means and standard deviations of two independent experiments. The titers were compared by two-tailed Student's *t*-tests. * $P \leq 0.05$; NS: not significant.

Figure S5. The human MCPIP1-C306R mutant partially loses its antiviral activity against JEV and DEN-2 infection. Human T-REx-293 cells with inducible expression of vector, MCPIP1, or MCPIP1-C306R mutant were cultured in medium without (-) or with (+) Dox (1 μg/ml) for 12 h, then infected with JEV or DEN-2 (MOI 5) for 24 h. **(A, B)** Western blot analysis of protein expression of HA for MCPIP1, JEV NS3, DEN-2 NS3, and actin. **(C, D)** Cells were fixed and permeabilized for immunofluorescence assay of viral protein NS1 (green) expression for JEV **(C)** and DEN-2 **(D)**. Nuclei were stained with DAPI (blue). **(E, F)** Human T-REx-293 cells with WT or MCPIP1-C306R mutant were infected with JEV or DEN-2 (MOI 5) for 18 h, then cultured in medium without (-) or with (+) Dox (1 μg/ml) for 18 h. The viral RNA bound with MCPIP1 protein was pulled down with HA-beads and

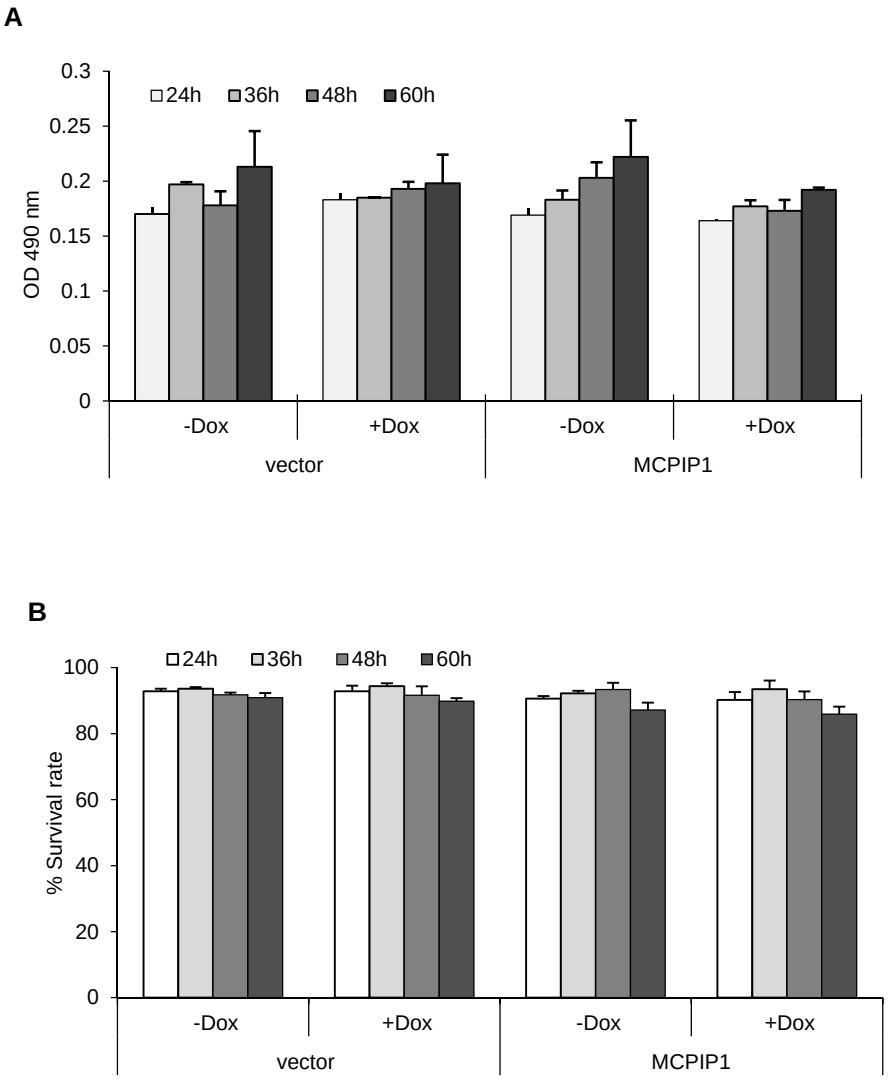
amplified by RT-PCR with JEV **(E)** or DEN-2 **(F)** 3′-UTR specific primers (middle panels). RT-PCR of mRNA levels of JEV and DEN-2 3′-UTR in virus-infected cells (bottom panels). Western blot analysis of HA-tagged MCPIP1 protein expression in cell lysates (top panels).

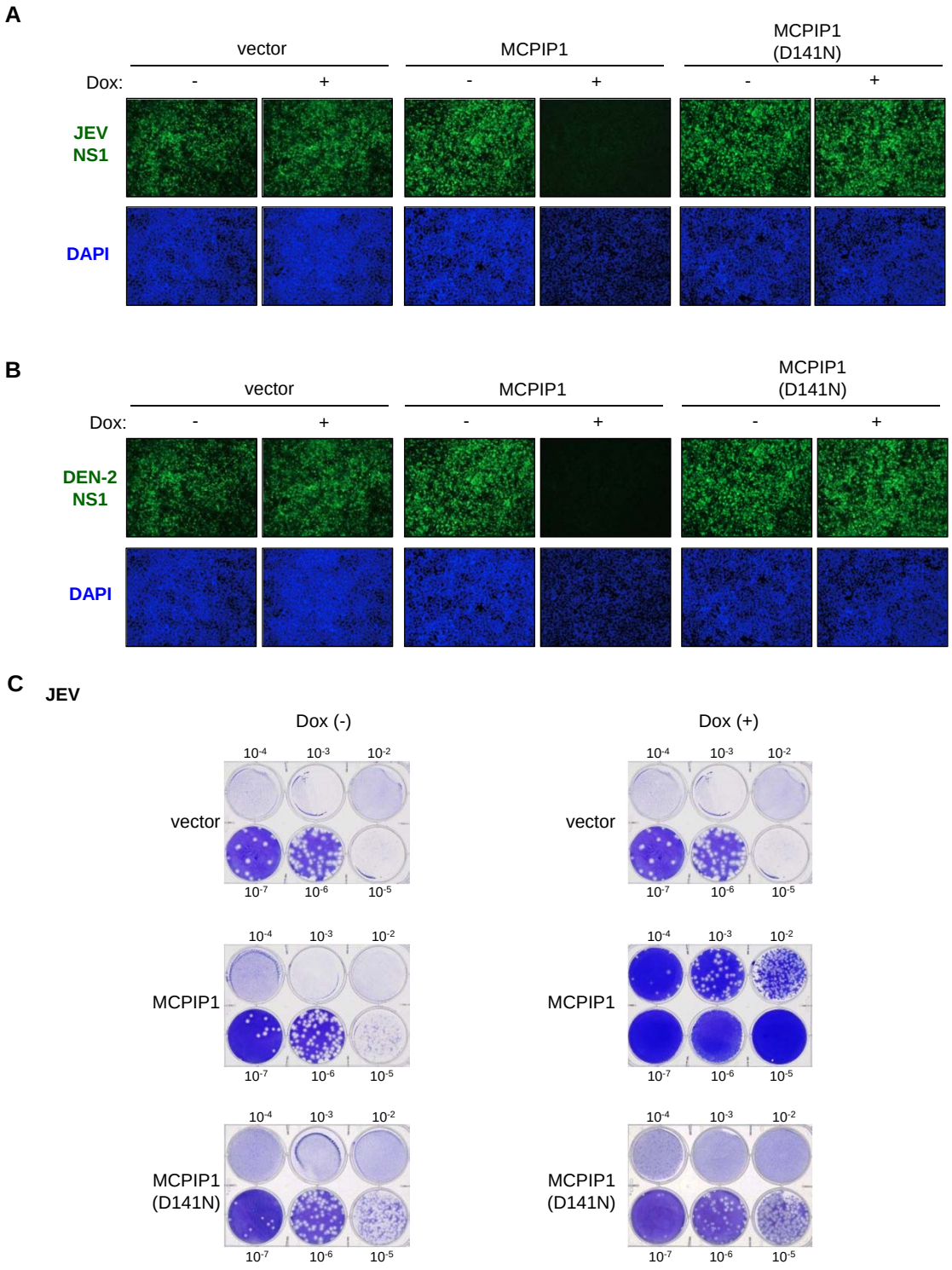
Figure S6. Subfragments of JEV RNA are degraded by human MCPIP1. *In vitro* transcribed full-length and various truncated JEV RNAs (1.5 μg) as indicated in the upper panel were incubated with HA-tagged WT or MCPIP1-D141N mutant proteins (~ 100 ng) in a reaction buffer with 5 mM Mg^{2+} at 30 ^{o}C for 1 h. RNAs separated by electrophoresis in 0.8% agarose gel were detected by ethidium bromide staining. The photos are shown as inverse images.

Figure S7. Expression levels of TNF-α, IL-1β, MCP-1 and MCPIP1 in JEV- and DEN-2-infected cells. A549 cells were mock-infected, or infected with JEV (MOI 5) **(A)** or DEN-2 (MOI 5) **(B)** for the indicated times. The relative mRNA levels of the indicated genes were analyzed by quantitative real-time RT-PCR and normalized to that of GAPDH mRNA.

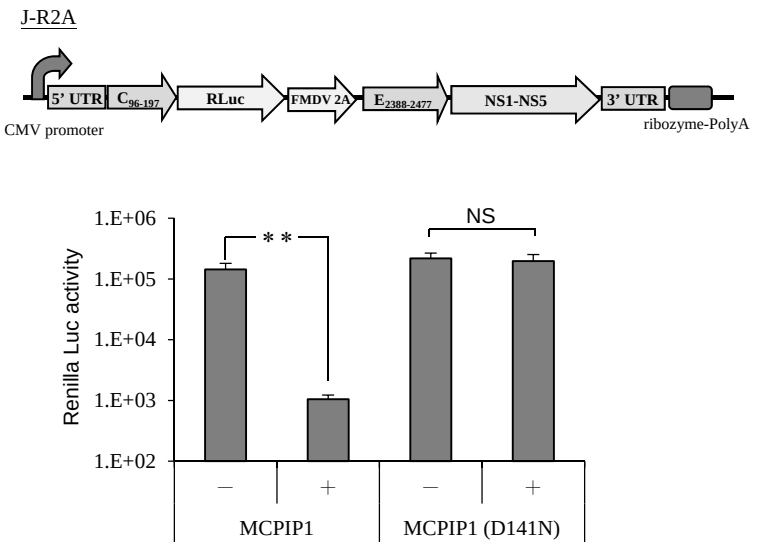
Figure S8. The human MCPIP1 is not inducible by type-I IFN. **(A)** Human A549 cells were treated with IL-1β (25 ng/ml) or IFN-α (1000 U/ml) for 3 h. The relative MCPIP1 mRNA levels were analyzed by quantitative real-time RT-PCR and normalized to that of GAPDH mRNA. **(B)** A549 cells were cultured in the presence

or absence of IFN-α (1000 U/ml) for 3 h. The induction of Stat1 and IRF-9 mRNA were analyzed by RT-PCR as described in Materials and Methods. The expected sizes of PCR fragments for each gene are shown on the right sides of the panels.

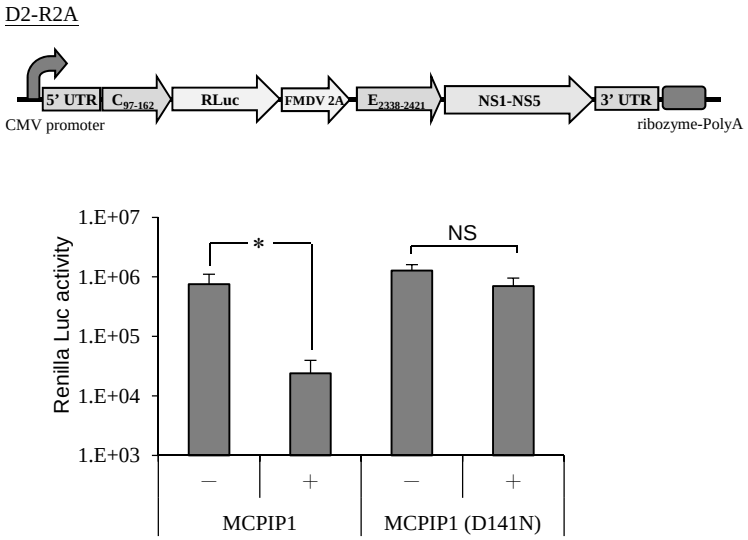

A
0.3
0.25
0.2
0.15
0.1
0.05
0
OD 490 nm
24h
36h
48h
60h
-Dox
+Dox
-Dox
+Dox
vector
MCPIP1
B
100
80
60
40
20
0
% Survival rate
24h
36h
48h
60h
-Dox
+Dox
-Dox
+Dox
vector
MCPIP1

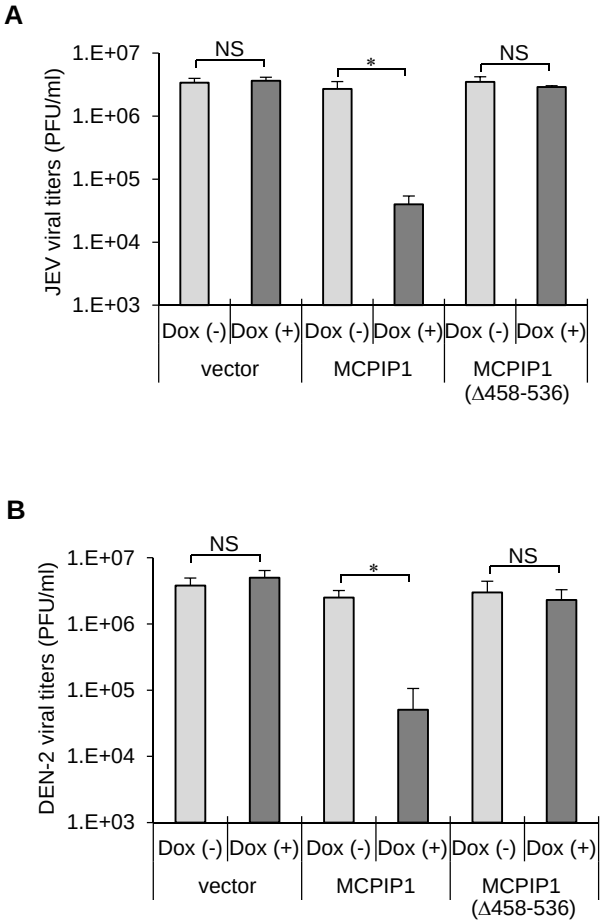

A
vector
MCPIP1
MCPIP1 (D141N)
Dox: - + - + - +
JEV NS1
DAPI
B
vector
MCPIP1
MCPIP1 (D141N)
Dox: - + - + - +
DEN-2 NS1
DAPI
C
JEV
Dox (-)
Dox (+)
vector
MCPIP1
MCPIP1 (D141N)
10-4 10-3 10-2
10-7 10-6 10-5

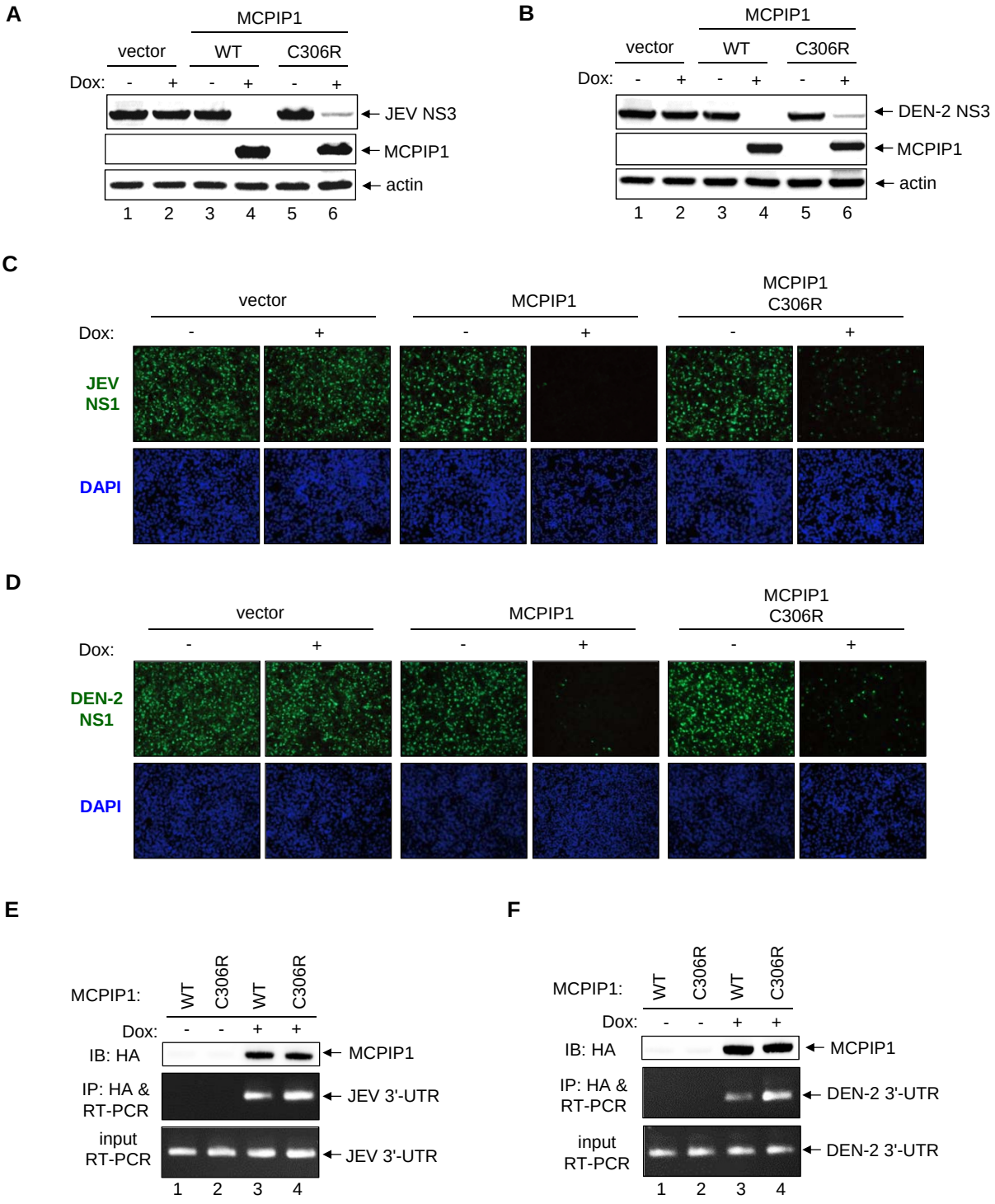
A.

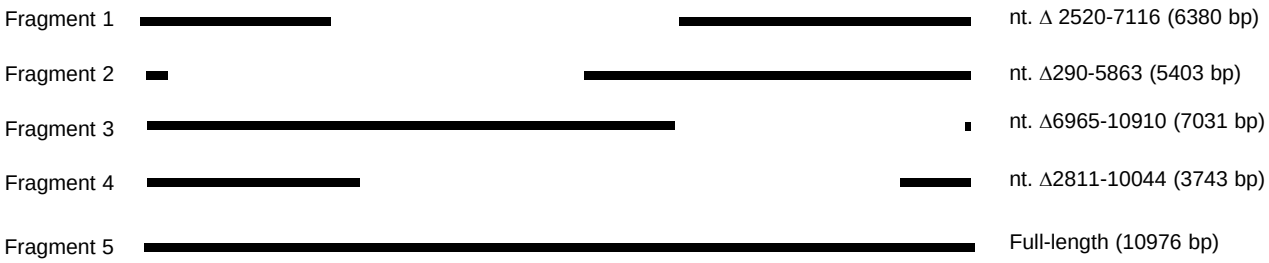

J-R2A
5' UTR
C96-197
RLuc
FMDV 2A
E2388-2477
NS1-NS5
3' UTR
CMV promoter
ribozyme-PolyA
Renilla Luc activity
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
* *
NS
–
+
–
+
MCPIP1
MCPIP1 (D141N)

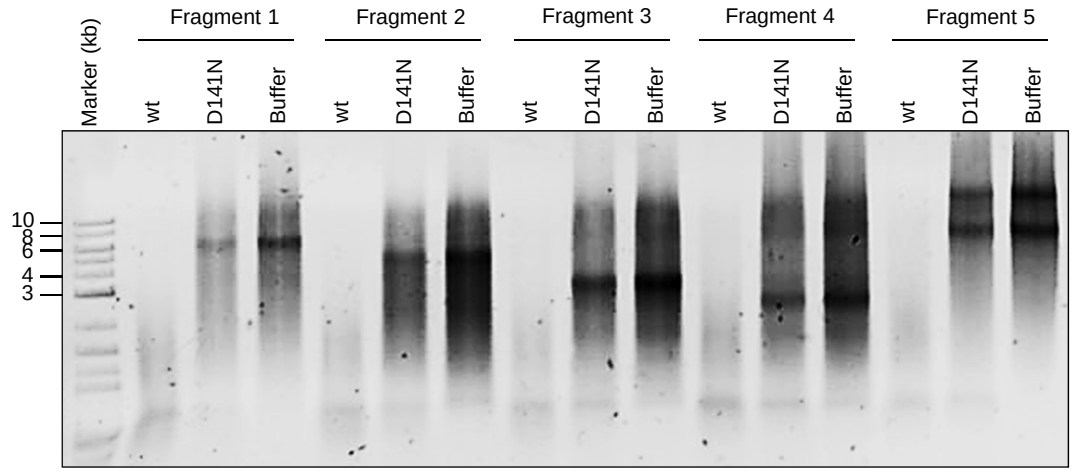
B.


D2-R2A
5' UTR
C97-162
RLuc
FMDV 2A
E2338-2421
NS1-NS5
3' UTR
CMV promoter
ribozyme-PolyA
Renilla Luc activity
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
*
NS
–
+
–
+
MCPIP1
MCPIP1 (D141N)

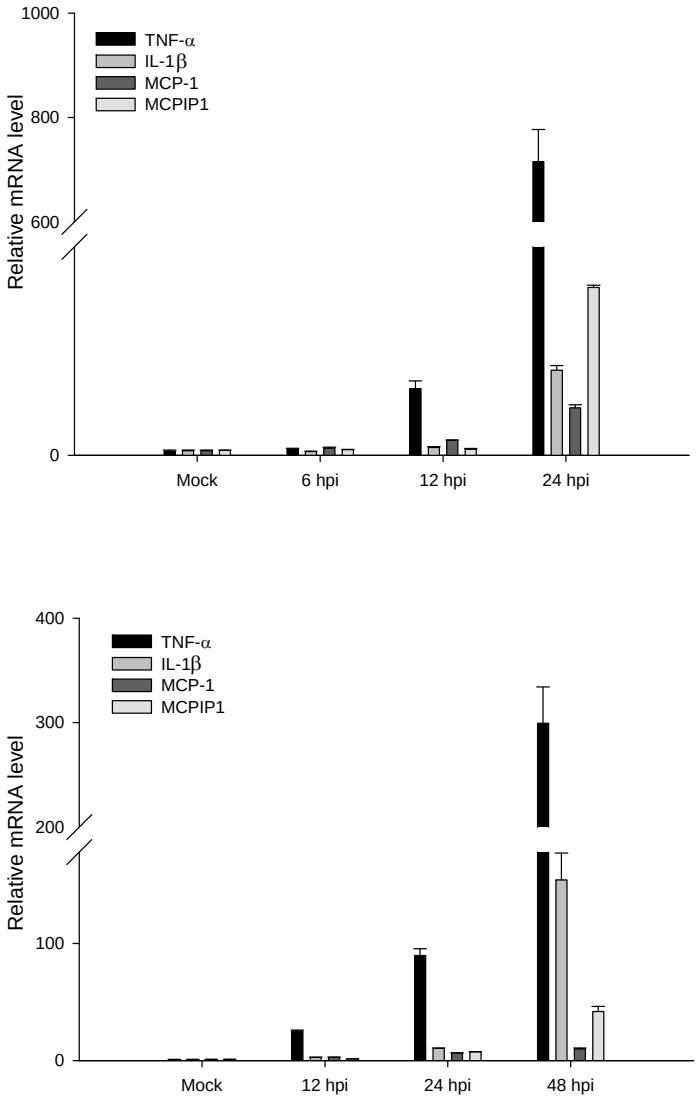

A
JEV viral titers (PFU/ml)
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
NS
*
NS
Dox (-)
Dox (+)
Dox (-)
Dox (+)
Dox (-)
Dox (+)
vector
MCPIP1
MCPIP1
(Δ458-536)
B
DEN-2 viral titers (PFU/ml)
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
NS
*
NS
Dox (-)
Dox (+)
Dox (-)
Dox (+)
Dox (-)
Dox (+)
vector
MCPIP1
MCPIP1
(Δ458-536)

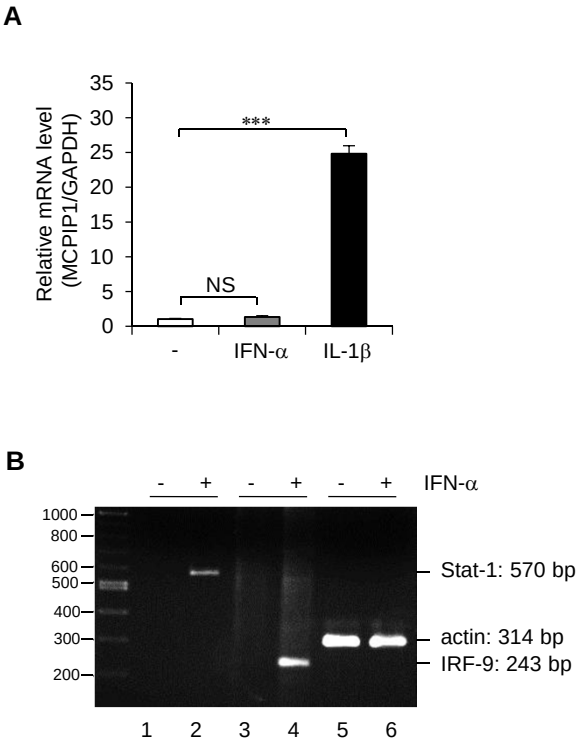

A
MCPIP1
vector
WT
C306R
Dox: - + - + - +
JEV NS3
MCPIP1
actin
1 2 3 4 5 6
B
MCPIP1
vector
WT
C306R
Dox: - + - + - +
DEN-2 NS3
MCPIP1
actin
1 2 3 4 5 6
C
vector
MCPIP1
MCPIP1 C306R
Dox: - + - + - +
JEV NS1
DAPI
D
vector
MCPIP1
MCPIP1 C306R
Dox: - + - + - +
DEN-2 NS1
DAPI
E
MCPIP1: WT C306R WT C306R
Dox: - - + +
IB: HA
MCPIP1
IP: HA & RT-PCR
JEV 3'-UTR
input RT-PCR
JEV 3'-UTR
1 2 3 4
F
MCPIP1: WT C306R WT C306R
Dox: - - + +
IB: HA
MCPIP1
IP: HA & RT-PCR
DEN-2 3'-UTR
input RT-PCR
DEN-2 3'-UTR
1 2 3 4


Fragment 1
Fragment 2
Fragment 3
Fragment 4
Fragment 5
nt. Δ 2520-7116 (6380 bp)
nt. Δ290-5863 (5403 bp)
nt. Δ6965-10910 (7031 bp)
nt. Δ2811-10044 (3743 bp)
Full-length (10976 bp)


Marker (kb)
Fragment 1
Fragment 2
Fragment 3
Fragment 4
Fragment 5
wt
D141N
Buffer
10
8
6
4
3

A


1000
800
600
0
Relative mRNA level
TNF-α
IL-1β
MCP-1
MCPIP1
Mock
6 hpi
12 hpi
24 hpi
400
300
200
100
0
Relative mRNA level
TNF-α
IL-1β
MCP-1
MCPIP1
Mock
12 hpi
24 hpi
48 hpi


A
Relative mRNA level (MCPIP1/GAPDH)
35
30
25
20
15
10
5
0
***
NS
-
IFN-α
IL-1β
B
- + - + - + IFN-α
1000
800
600
500
400
300
200
Stat-1: 570 bp
actin: 314 bp
IRF-9: 243 bp
1 2 3 4 5 6