Title

A unique amino acid substitution in NS2A protein of Japanese encephalitis virus affects virus propagation in vitro but not in vivo

Running title

A unique amino acid substitution in NS2A of JEV

Authors

Yuki Takamatsu, Kouichi Morita and Daisuke Hayasaka*

Affiliations

Department of Virology, Institute of Tropical Medicine, GCOE program, Leading Graduate School Program, Nagasaki University, Nagasaki, Nagasaki, Japan

*Corresponding author: Daisuke Hayasaka

Address: 1-12-4, Sakamoto, Nagasaki, Nagasaki 852-8524, Japan

Phone: +95-819-7828, Fax; +95-819-7830

E-mail: hayasaka@nagasaki-u.ac.jp

Abstract

We identified a unique amino acid of $NS2A_{113}$ phenylalanine that affects the efficient propagation of two Japanese encephalitis virus strains, JaTH160 and JaOArS982 in neuroblastoma Neuro-2a cells but not in cell lines of extraneural origin. This amino acid did not affect viral loads in the brain nor survival curves in mice. These findings suggest that virus propagation in vitro may not reflect the level of virus neuroinvasiveness in vivo.

Japanese encephalitis (JE) virus (JEV) causes approximately 30,000 to 50,000 cases and 10,000-15,000 deaths in Asian countries annually (1, 2). JEV belongs to the family *Flaviviridae*, genus *Flavivirus* (3, 4), whose genomic RNA encodes one polyprotein, cleaved into three structural (C, prM, and E) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins (5). The clinical symptoms of JE vary from mild to severe and include a non-specific febrile illness, meningitis, encephalitis and meningoencephalitis (6, 7). The mechanism of severe central nervous system (CNS) disease is not fully understood.

To evaluate disease pathogenesis and virulence, mice have been employed as an infection model. Several viral and host factors affect disease severity during JEV infection. We previously suggested that the host response, resulting in immunopathological effects, contributes to fatal infections (8). Furthermore, we also demonstrated that the JaOArS982 and JaTH160 strains of JEV exhibited different virulence in mice (8). Therefore, a genetic-based comparison between these strains may provide an effective approach to identify viral factors contributing to severe disease.

Our previous results showed that following subcutaneous infection with 10^4 PFU of JaTH160, mice showed 100% mortality, whereas JaOArS982 caused approximately 30% mortality in mice (8). We first constructed infectious cDNA clones harboring full

45 length genes of JaOArS982 and JaTH160, and produced infectious viruses of S982-IC
46 and JaTH-IC from each cDNA, respectively (9). In the present study, subcutaneous
47 infection with 10^4 PFU of S982-IC and JaTH-IC viruses caused 40% and 100%
48 mortality, respectively, in C57BL/6j (B6) mice (Japan SLC Cooperation, Japan CLEA
49 Cooperation) (Figure 1A), indicating that both JaTH-IC and S982-IC viruses possessed
50 virulence potentials similar to their parent JaOArS982 and JaTH160 viruses. Our animal
51 experimental protocols were approved by the Animal Care and Use Committee,
52 Nagasaki University (approval number: 091130-2-7 /0912080807-9, 100723-1-3 /
53 1008050873-3).

54 Our previous data showed that viral loads in the CNS of JaTH160-infected mice
55 were significantly higher than those of JaOArS982-infected mice (8). This raised the
56 possibility that virus propagation in neuronal cells is different between JaTH160 and
57 JaOArS982. Thus, we next compared virus propagations in murine neuroblastoma
58 Neuro-2a (N2a) cell lines.

59 N2a cells were infected with each JEV strain at an m.o.i. of 0.1 and supernatants
60 were harvested at 0, 24, 48 and 72 hours post-infection (pi). Virus titers were
61 determined by plaque forming assays on Baby Hamster Kidney (BHK) cells (10). In
62 N2a cells, JaTH-IC and the parent JaTH160 viruses exhibited significantly higher virus

yields compared with S982-IC and the parent JaOArS982 viruses (Figure 1B). However, there were no significant differences of virus yields between the four viruses in BHK cells (Figure 1C), Vero (rhesus monkey kidney), PS (porcine kidney) and HeLa (human epithelial) cells (data not shown). These results suggest that replication in neuronal cells is different between JaTH160 and JaOArS982 viruses.

To determine the specific region of the viral gene affecting virus propagation in N2a cells, we constructed four chimeric JEV clones, S982_J1-IC, S982_J2-IC, S982_J3-IC and S982_J4-IC, as shown in Figure 2A. S982_J2-IC exhibited a similar growth curve to JaTH-IC and produced significantly higher virus titers than S982-IC, S982_J1-IC, S982_J3-IC and S982_J4-IC (Figure 3A). Thus, the viral genome sequence in the region of $NS1_{322}$ to $NS3_{35}$ affects virus propagation in N2a cells.

There are three amino acid differences in this region between JaOArS982 and JaTH160. Thus, we inserted amino acid substitutes into S982-IC and JaTH-IC, as shown in Figure 2B. Site-directed mutagenesis was used, as described previously (11). S982_$I_{A23}\mathit{F}_{A113}D_{B81}$ showed significantly higher virus production in N2a cells compared with S982-IC, S982_$\mathit{V}_{A23}L_{A113}D_{B81}$ and S982_$I_{A23}L_{A113}\mathit{E}_{B81}$ (Figure 3B). Conversely, JaTH_$V_{A23}\mathit{L}_{A113}E_{B81}$ exhibited significantly lower virus yields than JaTH-IC, JaTH_$\mathit{I}_{A23}F_{A113}E_{B81}$ and JaTH_$V_{A23}F_{A113}\mathit{D}_{B81}$ (Figure 3C). These results indicate that an

amino acid substitution in $NS2A_{113}$, F in JaTH-IC and L in S982-IC, is responsible for the difference in propagation in N2a cells.

To examine whether the amino acid substitution in $NS2A_{113}$ contributes to the virulence and virus propagation in vivo, B6 mice were subcutaneously inoculated with S982_$I_{A23}F_{A113}D_{B81}$ and JaTH_$V_{A23}L_{A113}E_{B81}$, and their mortality was observed. Viral loads in the brain were also compared as previously shown (8, 12). Unexpectedly, S982_$I_{A23}F_{A113}D_{B81}$ showed similar survival curves to the parent S982-IC virus (Figure 4A) and there was no significant difference in viral loads in the brain between S982-IC- and S982_$I_{A23}F_{A113}D_{B81}$-infected mice (Figure 4B). JaTH_$V_{A23}L_{A113}E_{B81}$ also showed similar survival curves and similar viral loads in the brain to the parent JaTH-IC virus (Figure 4B). Thus, an amino acid substitution in $NS2A_{113}$ did not explain the different viral loads and virulence in the brain between S982-IC and JaTH-IC viruses.

Flavivirus NS2A protein is a 22-kDa hydrophobic protein (13). Previous studies have shown that NS2A protein is involved in viral assembly/release, viral RNA synthesis, regulation of NS1' expression and inhibition of type-I interferon response (14-20). These functions are affected by amino acid substitutions within NS2A, such as NS2A -G11A, -E20A, -P30A, -T33I, -L46H, -I59N, -D73H, -R84S/A/E, -E100A, M108K, D125A, -Q187A, -K188A, -K190S, and -G200A (14, 16-25).

99 The influence of the NS2A-L113F substitution identified here in JEV infection has
100 not been reported previously. NS2A has eight predicted transmembrane segments
101 (pTMS), and $NS2A_{113}$ appears to localize to pTMS4 (14). However, how $NS2A_{113}$
102 substitution affects virus propagation in N2a cells remains unclear. Further investigation
103 may provide information on the unknown function of NS2A.
104 Although a single amino acid substitution in $NS2A_{113}$ alters viral propagation in N2a
105 cells, this substitution did not affect viral loads in the brain nor survival curves in mice.
106 These findings suggest that virus propagation in vitro does not necessarily reflect virus
107 replication in vivo. Further, other amino acid and/or nucleotide substitutions may affect
108 host responses such as antiviral activity. In this regard, this study helps to elucidate the
109 mechanism of pathogenesis due to JEV infection in a mouse model.
110 Interestingly, our preliminary experiments showed that there were no significant
111 differences of mortality following intracerebral inoculation between JaOArS982 and
112 JaTH160. In our previous study of tick-borne encephalitis viruses, we suggested that the
113 mechanism of fatal infection is fundamentally different between intracerebral and
114 peripheral infection (10, 26). We further showed that immune responses were different
115 between JaOArS982- and JaTH160-infected mice (8). From these observations, we
116 assumed that different viral replications in the brains between JaOArS982 and JaTH160

117 attributes to the peripherally induced host immune responses and those immune cells
118 infiltrating in the brains. In addition, it appears that most of the volume of inoculum
119 leaked from the brain due to intracranial pressure following intracerebral inoculation.
120 Thus, we consider that intracerebral inoculation does not simply reflect virus infection
121 and replication in neurons, and it appears that it is difficult to examine the different
122 virulence mechanism between JaOArS982 and JaTH160.

123 We propose that actual virus propagation in the brain in vivo reflects a combined
124 mechanism of viral replication properties in neuronal cells and the host antiviral
125 immune response. Furthermore, we believe that the disease mechanisms of JEV in vivo
126 involve a complex mechanism that includes the host immune response and neuronal
127 infection in the CNS. Further investigations in a step-by-step fashion will provide clues
128 to elucidate the precise pathogenic mechanisms of JEV infection and enable the
129 development of effective treatment strategies for JE.

130

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References

1. **Ghosh D, Basu A.** 2009. Japanese encephalitis-a pathological and clinical perspective. PLoS Negl Trop Dis **3:**e437.
2. **Solomon T.** 2004. Flavivirus encephalitis. N Engl J Med **351:**370-378.
3. **Westaway EG, Brinton MA, Gaidamovich S, Horzinek MC, Igarashi A, Kaariainen L, Lvov DK, Porterfield JS, Russell PK, Trent DW.** 1985. Flaviviridae. Intervirology **24:**183-192.
4. **Gubler DJ, Kuno G, Markoff L.** 2007. Field's Virology, vol. fifth edition. Wolters Kluwer Lippincott Williams and Wilkins, Philadelphia.
5. **Sumiyoshi H, Hoke CH, Trent DW.** 1992. Infectious Japanese encephalitis virus RNA can be synthesized from in vitro-ligated cDNA templates. J Virol **66:**5425-5431.
6. **Tsai TF.** 2000. New initiatives for the control of Japanese encephalitis by vaccination: minutes of a WHO/CVI meeting, Bangkok, Thailand, 13-15 October 1998. Vaccine **18 Suppl 2:**1-25.
7. **Misra UK, Kalita J.** 2010. Overview: Japanese encephalitis. Prog Neurobiol

91:108-120.

8. **Hayasaka D, Shirai K, Aoki K, Nagata N, Simantini DS, Kitaura K, Takamatsu Y, Gould E, Suzuki R, Morita K.** 2013. TNF-alpha acts as an immunoregulator in the mouse brain by reducing the incidence of severe disease following Japanese encephalitis virus infection. PLoS One **8:**e71643.

9. **Takamatsu Y, Okamoto K, Dinh DT, Yu F, Hayasaka D, Uchida L, Nabeshima T, Buerano CC, Morita K.** 2014. NS1' protein expression facilitates production of Japanese encephalitis virus in avian cells and embryonated chicken eggs. J Gen Virol **95:**373-383.

10. **Hayasaka D, Nagata N, Fujii Y, Hasegawa H, Sata T, Suzuki R, Gould EA, Takashima I, Koike S.** 2009. Mortality following peripheral infection with tick-borne encephalitis virus results from a combination of central nervous system pathology, systemic inflammatory and stress responses. Virology **390:**139-150.

11. **Yu F, Hasebe F, Inoue S, Mathenge EG, Morita K.** 2007. Identification and characterization of RNA-dependent RNA polymerase activity in recombinant Japanese encephalitis virus NS5 protein. Arch Virol **152:**1859-1869.

12. **Tun MM, Aoki K, Senba M, Buerano CC, Shirai K, Suzuki R, Morita K, Hayasaka D.** 2014. Protective role of TNF-alpha, IL-10 and IL-2 in mice infected with the Oshima strain of Tick-borne encephalitis virus. Sci Rep **4:**5344.

13. **Chambers TJ, McCourt DW, Rice CM.** 1989. Yellow fever virus proteins NS2A, NS2B, and NS4B: identification and partial N-terminal amino acid sequence analysis. Virology **169:**100-109.

14. **Xie X, Gayen S, Kang C, Yuan Z, Shi PY.** 2013. Membrane topology and function of dengue virus NS2A protein. J Virol 10.1128/JVI.02424-12.

15. **Firth AE, Atkins JF.** 2009. A conserved predicted pseudoknot in the NS2A-encoding sequence of West Nile and Japanese encephalitis flaviviruses suggests NS1' may derive from ribosomal frameshifting. Virol J **6:**14.

16. **Kummerer BM, Rice CM.** 2002. Mutations in the yellow fever virus nonstructural protein NS2A selectively block production of infectious particles. J Virol **76:**4773-4784.

17. **Leung JY, Pijlman GP, Kondratieva N, Hyde J, Mackenzie JM, Khromykh AA.** 2008. Role of nonstructural protein NS2A in flavivirus assembly. J Virol **82:**4731-4741.

18. **Mackenzie JM, Khromykh AA, Jones MK, Westaway EG.** 1998. Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A. Virology **245:**203-215.

19. **Tu YC, Yu CY, Liang JJ, Lin E, Liao CL, Lin YL.** 2012. Blocking double-stranded RNA-activated protein kinase PKR by Japanese encephalitis virus nonstructural protein 2A. J Virol **86:**10347-10358.
20. **Xie X, Zou J, Puttikhunt C, Yuan Z, Shi P.** 2015. Two Distinct Sets of NS2A Molecules Are Responsible for Dengue Virus RNA Synthesis and Virion Assembly. J Virol **89:**1298-1313.
21. **Liu WJ, Chen HB, Wang XJ, Huang H, Khromykh AA.** 2004. Analysis of adaptive mutations in Kunjin virus replicon RNA reveals a novel role for the flavivirus nonstructural protein NS2A in inhibition of beta interferon promoter-driven transcription. J Virol **78:**12225-12235.
22. **Liu WJ, Wang XJ, Clark DC, Lobigs M, Hall RA, Khromykh AA.** 2006. A single amino acid substitution in the West Nile virus nonstructural protein NS2A disables its ability to inhibit alpha/beta interferon induction and attenuates virus virulence in mice. J Virol **80:**2396-2404.
23. **Yoshii K, Igarashi M, Ito K, Kariwa H, Holbrook MR, Takashima I.** 2011. Construction of an infectious cDNA clone for Omsk hemorrhagic fever virus, and characterization of mutations in NS2A and NS5. Virus Res **155:**61-68.
24. **Rossi SL, Fayzulin R, Dewsbury N, Bourne N, Mason PW.** 2007. Mutations in West Nile virus nonstructural proteins that facilitate replicon persistence in vitro attenuate virus replication in vitro and in vivo. Virology **364:**184-195.
25. **Liu WJ, Chen HB, Khromykh AA.** 2003. Molecular and functional analyses of Kunjin virus infectious cDNA clones demonstrate the essential roles for NS2A in virus assembly and for a nonconservative residue in NS3 in RNA replication. J Virol **77:**7804-7813.
26. **Hayasaka D, Nagata N, Hasegawa H, Sata T, Takashima I, Koike S.** 2010. Early mortality following intracerebral infection with the Oshima strain of tick-borne encephalitis virus in a mouse model. J Vet Med Sci **72:**391-396.

Figure Legends

FIG 1 Virulence in mice and viral yields in cultured cells infected with the S982-IC and JaTH-IC viruses. (A) Survival curves and (B) Weight ratios of mice subcutaneously

228 infected with 10^4 PFU of each virus (n=10). P: Log-rank (Mantel-Cox) Test.
229 Propagation of JaOArS982 (original virus) and S982-IC (derived from infectious cDNA
230 clone of JaOArS982), JaTH160 (original virus) and JaTH-IC (derived from infectious
231 cDNA clone of JaTH160) viruses in N2a (C) and BHK cells (D) at 0, 24, 48 and 72
232 hours post-infection. Error bars represent standard deviations. p: One-way analysis of
233 variance.
234
235 **FIG 2** Schematic representation of full-length chimeric and amino acid
236 substituted-viruses derived from S982-IC and JaTH-IC. (A) A genome representations
237 of S982_J1-IC, S982_J2-IC, S982_J3-IC and S982_J4-IC showing the replacement of
238 5'UTR-$NS1_{322}$, $NS1_{323}$-$NS3_{35}$, $NS3_{36}$-$NS5_{566}$, $NS5_{567}$-3'UTR of S982-IC, respectively,
239 with the corresponding region of JaTH-IC. (B) A genome representation of a single
240 amino acid substituted-S982-IC and JaTH-IC. The white and black arrowheads indicate
241 amino acids derived from S982-IC and JaTH-IC, respectively. S982-IC and JaTH-IC
242 are also named S982_$I_{A23}L_{A113}D_{B81}$ and JaTH_$V_{A23}F_{A113}E_{B81}$, respectively.
243
244 **FIG 3** Growth curves for virus propagation of the chimeric and amino acid
245 substituted-viruses in N2a cells at 0, 24, 48 and 72 hours post-infection. (A) Viral yields

of S982_J1-IC, S982_J2-IC, S982_J3-IC and S982_J4-IC compared with S982-IC and JaTH-IC viruses. Viral yields of JaTH-IC and S982-IC are the same data as FIG 1C. Viral yields of amino acid substituted-S982-IC (B) and JaTH-IC (C) viruses. p: One-way analysis of variance. The white and black arrowheads indicate amino acids derived from S982-IC and JaTH-IC, respectively.

FIG 4 Virulence in mice and viral loads in the brains of mice following subcutaneous infection with 10^4 PFU of S982_$I_{A23}L_{A113}D_{B81}$ (S982-IC), S982_$I_{A23}F_{A113}D_{B81}$, JaTH_$V_{A23}F_{A113}E_{B81}$ (JaTH-IC), and JaTH_$V_{A23}L_{A113}E_{B81}$. (A) Survival curves (n=10) P: Gehan-Breslow-Wilcoxon Test. (B) Viral loads in the brain (n=6). P: Kruskal-Wallis test, *: $p<0.05$ by Dunn's Multiple Comparison Test.

FIG 1

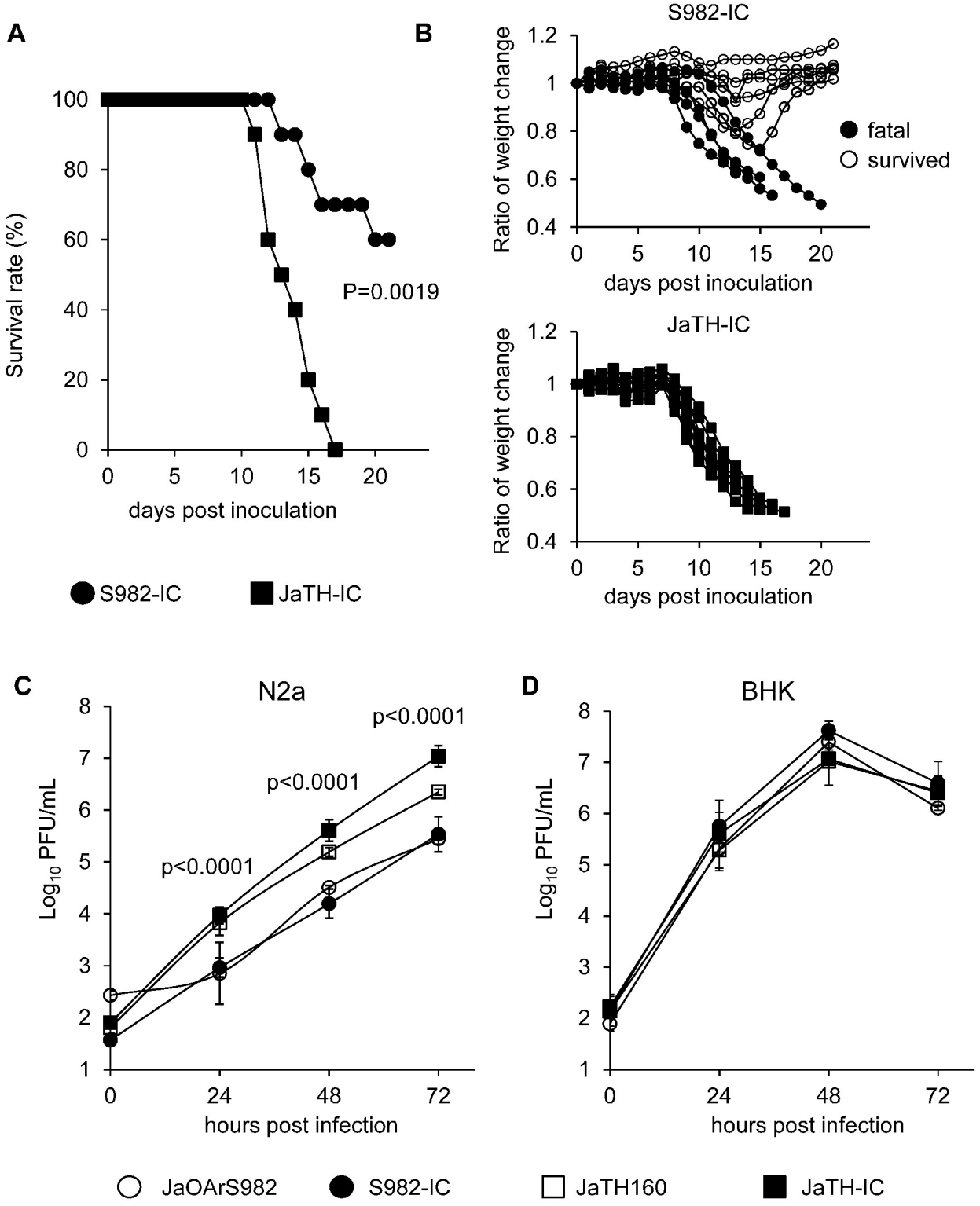

A
B
Survival rate (%)
100
80
60
40
20
0
0
5
10
15
20
days post inoculation
P=0.0019
S982-IC
JaTH-IC
S982-IC
Ratio of weight change
1.2
1
0.8
0.6
0.4
fatal
survived
days post inoculation
JaTH-IC
Ratio of weight change
days post inoculation
C
N2a
p<0.0001
p<0.0001
p<0.0001
Log10 PFU/mL
8
7
6
5
4
3
2
1
0
24
48
72
hours post infection
D
BHK
Log10 PFU/mL
hours post infection
JaOArS982
S982-IC
JaTH160
JaTH-IC

FIG 2

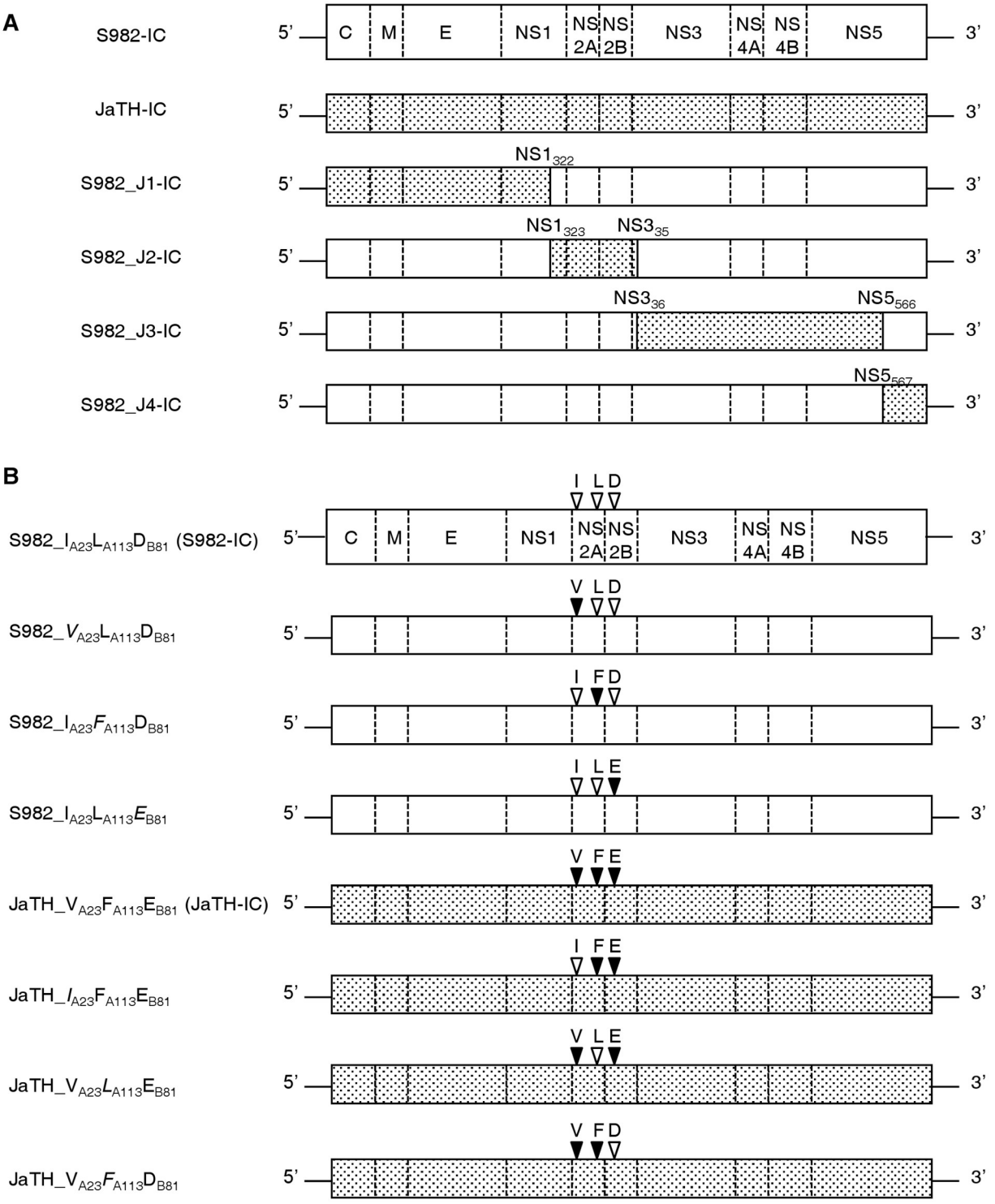

A
S982-IC
JaTH-IC
S982_J1-IC
S982_J2-IC
S982_J3-IC
S982_J4-IC
5'
3'
C
M
E
NS1
NS 2A
NS 2B
NS3
NS 4A
NS 4B
NS5
NS1322
NS1323
NS335
NS336
NS5566
NS5567
B
S982_IA23LA113DB81 (S982-IC)
S982_VA23LA113DB81
S982_IA23FA113DB81
S982_IA23LA113EB81
JaTH_VA23FA113EB81 (JaTH-IC)
JaTH_IA23FA113EB81
JaTH_VA23LA113EB81
JaTH_VA23FA113DB81
I L D
V L D
I F D
I L E
V F E
I F E
V L E
V F D

FIG 3

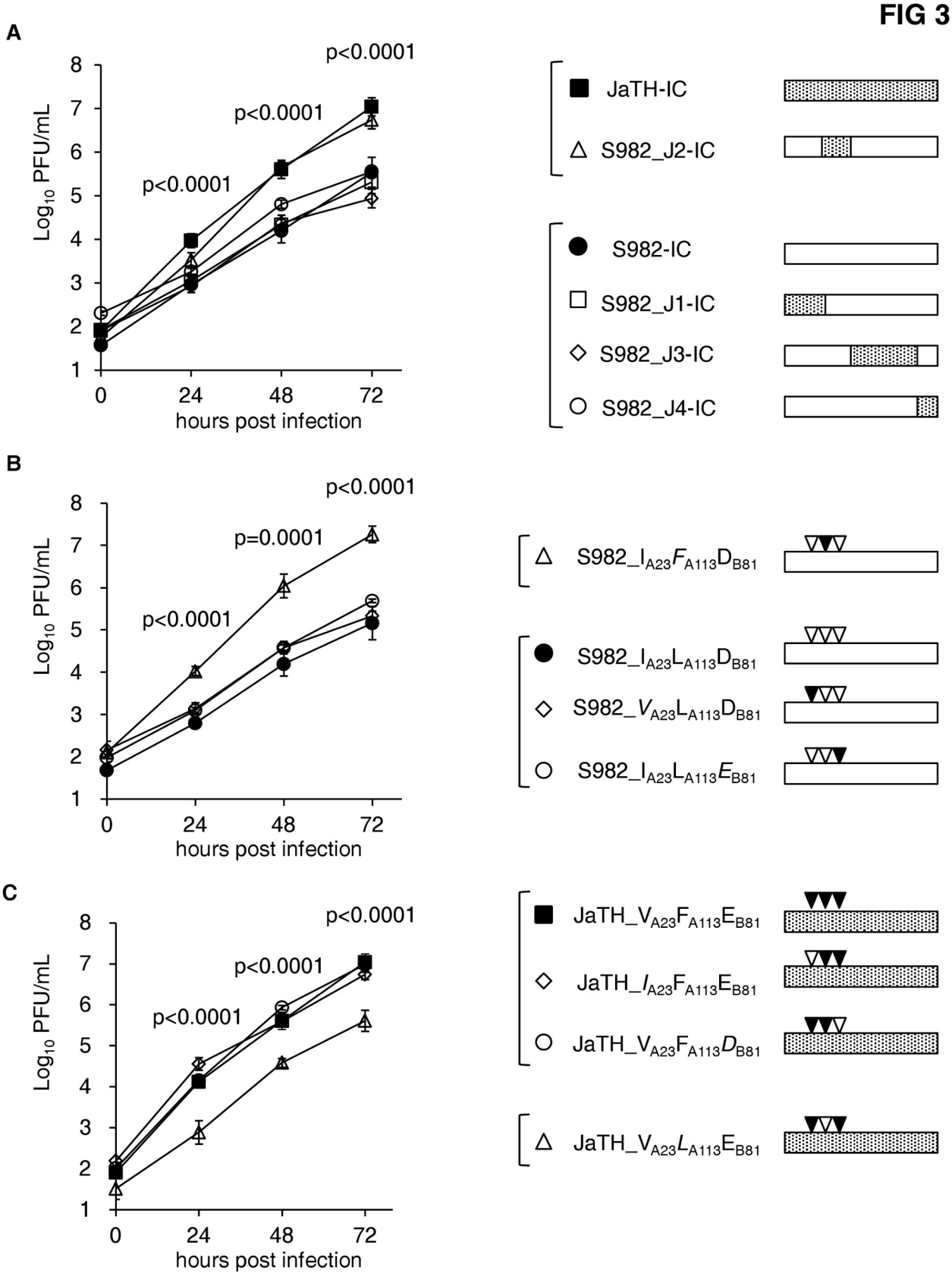
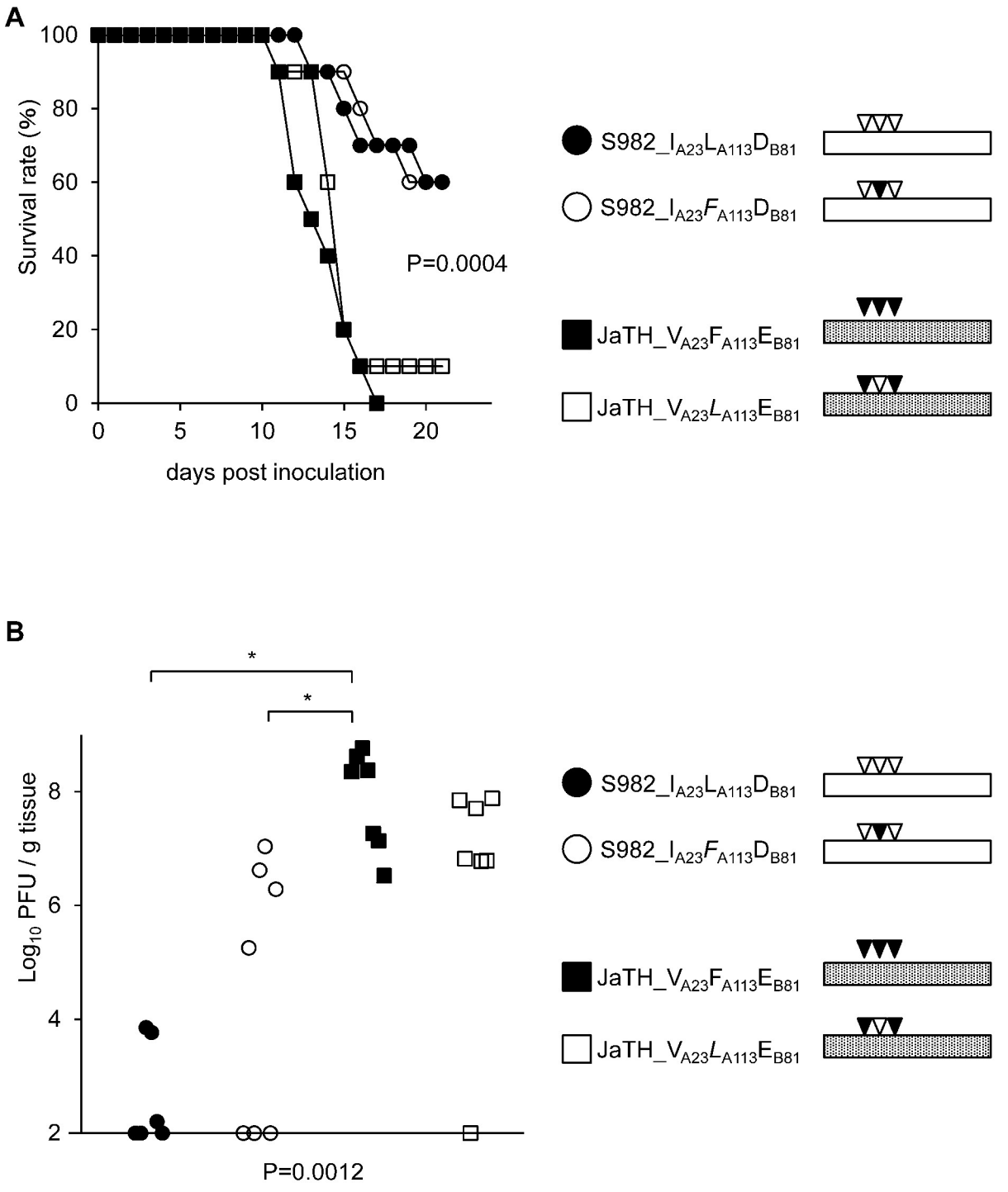

A
p<0.0001
p<0.0001
p<0.0001
Log10 PFU/mL
hours post infection
JaTH-IC
S982_J2-IC
S982-IC
S982_J1-IC
S982_J3-IC
S982_J4-IC
B
p<0.0001
p=0.0001
p<0.0001
Log10 PFU/mL
hours post infection
S982_IA23FA113DB81
S982_IA23LA113DB81
S982_VA23LA113DB81
S982_IA23LA113EB81
C
p<0.0001
p<0.0001
p<0.0001
Log10 PFU/mL
hours post infection
JaTH_VA23FA113EB81
JaTH_IA23FA113EB81
JaTH_VA23FA113DB81
JaTH_VA23LA113EB81

FIG 4


A
100
80
60
40
20
0
Survival rate (%)
0
5
10
15
20
days post inoculation
P=0.0004
S982_I$_{A23}$L$_{A113}$D$_{B81}$
S982_I$_{A23}$F$_{A113}$D$_{B81}$
JaTH_V$_{A23}$F$_{A113}$E$_{B81}$
JaTH_V$_{A23}$L$_{A113}$E$_{B81}$
B
*
*
8
6
4
2
Log$_{10}$ PFU / g tissue
P=0.0012
S982_I$_{A23}$L$_{A113}$D$_{B81}$
S982_I$_{A23}$F$_{A113}$D$_{B81}$
JaTH_V$_{A23}$F$_{A113}$E$_{B81}$
JaTH_V$_{A23}$L$_{A113}$E$_{B81}$