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**Intracellular membrane association of the N-terminal domain of classical swine fever virus
NS4B determines viral genome replication and virulence**

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27 SUMMARY

28 Classical swine fever virus (CSFV) causes a highly contagious disease in pigs that can range
29 from a severe hemorrhagic fever to a nearly unapparent disease, depending on the virulence of the
30 virus strain. Little is known on the viral molecular determinants of CSFV virulence. The
31 nonstructural protein NS4B is essential for viral replication. However, the roles of CSFV NS4B for
32 viral genome replication and pathogenesis have not yet been elucidated. NS4B of the GPE⁻
33 vaccine strain and of the highly virulent Eystrup strain differ by a total of seven amino acid residues,
34 two of which are located in the predicted trans-membrane domains of NS4B and were described
35 previously to relate to virulence, and five residues clustering in its N-terminal part. In the present
36 study, we examined the potential role of these five amino acids in modulating genome replication and
37 determining pathogenicity in pigs. A chimeric low virulent GPE⁻-derived virus carrying the complete
38 Eystrup NS4B showed enhanced pathogenicity in pigs. The *in vitro* replication efficiency of the
39 NS4B chimeric GPE⁻ replicon was significantly higher than that of the replicon carrying only the two
40 Eystrup-specific amino acids in NS4B. *In silico* and *in vitro* data suggest that the N-terminal part of
41 NS4B forms an amphipathic α -helix structure. The N-terminal NS4B with these five amino acid
42 residues associated the intracellular membranes. Taken together, this is the first gain-of-function
43 study showing that the N-terminal domain of NS4B can determine CSFV genome replication in cell
44 culture and viral pathogenicity in pigs.

45

INTRODUCTION

Classical swine fever is an economically important and highly contagious disease of pigs caused by classical swine fever virus (CSFV) (Lindenbach *et al.*, 2013). The disease can range from a severe hemorrhagic fever to a nearly unapparent disease, depending on the virulence of the virus strain. CSFV belongs to the genus *Pestivirus* of the family *Flaviviridae* together with bovine viral diarrhoea virus (BVDV) and border disease virus. CSFV possesses a single-stranded positive-sense RNA genome of approximately 12.3 kb with one large open reading frame flanked by 5' and 3' untranslated regions (UTRs). The genome encodes approximately 4000 amino acids that yield at least 12 cleavage products N^{pro}, C, E^{ns}, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B through co- and post-translational processing of the polyprotein by cellular and viral proteases (Lamp *et al.*, 2013; Lindenbach *et al.*, 2013). Little is known on the role of the different viral proteins in determining CSFV virulence. Nonstructural proteins NS3–NS5B are essential for pestivirus RNA replication (Behrens *et al.*, 1998). Therefore, these nonstructural proteins at least consist of the viral replication complex together with unknown host factors, the replicating viral RNA and intracellular membranes.

The nonstructural protein NS4B is a 38-kDa intracellular membrane-associated protein of the viral replicase, which is analogous to HCV NS4B and other related members of the family *Flaviviridae* (Hugle *et al.*, 2001; Miller *et al.*, 2006; Weiskircher *et al.*, 2009). However, NS4B is a poorly characterised protein and its roles in CSFV replication and pathogenesis are not well understood. A putative Toll/interleukin-1 receptor-like domain was identified in the C-terminal region of NS4B (Fernandez-Sainz *et al.*, 2010). Mutations in this domain of NS4B in the highly virulent Brescia strain resulted in an attenuated phenotype along with enhanced activation of TLR-7-induced genes. In addition, NS4B harbours an NTPase motif (Gladue *et al.*, 2011), but there is no evidence that modulating the NTPase activity may affect virulence. In BVDV, NS4B can act as a modulator of virus cytopathogenicity (Qu *et al.*, 2001). Our previous study demonstrated that two amino acid residues within the predicted trans-membrane domains of NS4B are involved in viral genome

72 replication and that they contribute to the pathogenicity of CSFV in pigs (Tamura *et al.*, 2012).
73 These two residues are a part of the seven amino acids that differ in NS4B between the highly
74 virulent Eystrup strain and the GPE⁻ vaccine strain.

75 Here, we examined whether the five additional amino acid differences in the N-terminal domain
76 of NS4B would further determine viral genome replication *in vitro* and the pathogenicity of CSFV in
77 pigs. To this end, a chimeric virus and corresponding replicons carrying the complete NS4B of the
78 highly virulent Eystrup strain in a modified GPE⁻ vaccine strain backbone were generated and
79 analysed for pathogenicity *in vivo* and for replication efficiency in cell culture, respectively. An *in*
80 *silico* approach was used to predict the localisations of these amino acid residues in the N-terminal
81 domain of NS4B. Intracellular membrane localisation and the association mediated by the
82 N-terminal domain of NS4B were examined.

83

84 RESULTS

85 **The N-terminal domain of NS4B of the highly virulent CSFV strain Eystrup confers enhanced** 86 **pathogenicity to a low virulent GPE⁻-derived virus.**

87 Our previous studies demonstrated the combination of four amino acid residues in N^{pro} (D₁₃₆), E2
88 (A₈₃₀), and NS4B (A₂₄₇₅/V₂₅₆₃) can confer pathogenicity to the CSFV GPE⁻ vaccine strain in pigs
89 (Tamura *et al.*, 2014; Tamura *et al.*, 2012) (Fig. 1). These four amino acid residues are conserved in
90 the highly virulent Eystrup strain, although a total of 62 amino acids differ between GPE⁻ and Eystrup
91 strains. The two amino acid residues in the central part of NS4B (A₂₄₇₅/V₂₅₆₃) are part of a total of
92 seven amino acid differences in NS4B. The remaining five amino acid differences (positions at
93 2377, 2391, 2398, 2399 and 2414) were observed in the N-terminal domain of NS4B. In order to
94 assess whether the five amino acids differing from the GPE⁻ strain in the N-terminal domain of
95 Eystrup NS4B contribute to pathogenicity in pigs, NS4B in the low virulent GPE⁻-derived virus
96 (vGPE⁻/N136D; T830A; V2475A; A2563V) was replaced with NS4B from the highly virulent Eystrup
97 strain. This chimeric virus was termed vGPE⁻/N136D; T830A; Eystrup NS4B. Groups of six

10-week-old to 13-week-old pigs were inoculated intranasally with $10^{6.0}$ TCID₅₀ of these two GPE⁻-derived viruses. As a control, six pigs were inoculated with the highly virulent Eystrup strain. Infection with the parental virus (vGPE⁻/N136D; T830A; V2475A; A2563V) did not result in any clinical symptoms in this trial [Fig. 2(a), left panel]. Nevertheless, the body temperature of these pigs was slightly elevated between days 3 and 7 after infection, and low-level viremia was detected during 2 days [Fig. 2(b) and 2(c), left panels]. In contrast, all six pigs inoculated with the vGPE⁻/N136D; T830A; Eystrup NS4B virus developed overt clinical manifestations between days 4 and 7 pi, with five out of six pigs showing fever [Fig. 2(a) and 2(b), middle panels]. Viremia was detected during 4 days [Fig. 2(c), middle panel]. Infection with the highly virulent Eystrup strain resulted in severe clinical signs with high and prolonged fever and viremia in all six pigs. Four pigs were euthanized when they reached a clinical score above 18, between days 6 and 7 pi. One pig died unexpectedly on day 8 pi, and one pig recovered by day 14 pi. These data demonstrate that the N-terminal domain of NS4B contributes to pathogenicity of CSFV in pigs. Nevertheless, NS4B of the highly virulent Eystrup virus is not solely responsible for the high pathogenicity of this virus.

The N-terminal domain of NS4B of the highly virulent CSFV strain Eystrup contributes to enhanced RNA replication.

Mono-cistronic and bi-cistronic replicons [Fig. 3(a) and 3(b)] were used to determine the effect on viral RNA replication of the amino acids of NS4B differing between the two strains. In SK-6 cells at 24 h post electroporation, the bi-cistronic replicon carrying NS4B of the highly virulent Eystrup strain (rGPE⁻-N^{pro}-Luc-IRES-NS3/Eystrup NS4B) resulted in 2-fold higher luciferase activity than the replicon carrying only the two amino acid substitutions in the predicted trans-membrane domains of NS4B (rGPE⁻-N^{pro}-Luc-IRES-NS3/V2475A; A2563V), and in 5-fold higher luciferase activity than the parental GPE⁻ virus-derived replicon [Fig. 3(c)]. Consistent with the results obtained with the bi-cistronic replicons, Eystrup NS4B conferred significantly higher *Gaussia* luciferase production to the GPE⁻-derived mono-cistronic replicon compared with the replicon harbouring only the two

124 V2475A and A2563V substitutions in GPE⁻ backbone that was also more efficient at expressing the
125 reporter protein than the replicon representing the parental GPE⁻ vaccine strain [Fig. 3(b) and 3(d)].
126 These data indicate that the additional five amino acid differences in the N-terminal domain of
127 Eystруп NS4B contribute to enhanced RNA replication efficiency in cell culture.

128

129 **The N-terminal domain of NS4B forms a predicted amphipathic α -helix and mediates**
130 **intracellular membrane association.**

131 Secondary-structure prediction was carried out with the NS4B polyprotein sequence of the GPE⁻
132 and Eystруп strains by using several different software. The consensus structures are shown in Fig.
133 4(a). CSFV NS4B comprises an N-terminal domain (amino acids [aa] of NS4B 1–81), a central
134 part that harbours four predicted trans-membrane segments (TM) (aa 82–245) and a C-terminal
135 domain (aa 246–347). The N-terminal domain contains two predicted α -helices from aa 5 to 30 (α 1)
136 and from aa 40 to 81 (α 2), respectively. Interestingly, five out of the seven amino acid differences
137 between NS4B of the GPE⁻ vaccine strain and of the Eystруп strain are located within the 2nd α -helix,
138 α 2. This α -helix (aa 40–81) is predicted to have an amphipathic structure [Fig. 4(b)]. In order to
139 elaborate on this, the plasmids were constructed for cytomegalovirus promoter-driven expression of
140 the N-terminal α 1-helix (aa 1–39), of the helix α 2 (aa 40–81), of the two α -helices together (aa 1–81)
141 and of the full-length NS4B protein, all tagged with the green fluorescent protein (GFP) at the C
142 terminus [Fig. 5(a)]. Then, the hydrophobic face of the helix was genetically disrupted by
143 substitution of charged amino acids from the nonpolar amino acids [Fig. 5(b)]. When these
144 different GPE⁻-derived GFP-tagged NS4B variants were expressed in SK-6 cells, the fluorescence
145 signal of NS4B(40-81)-GFP, NS4B(1-81)-GFP and NS4B-GFP was located essentially in the
146 cytoplasm, with a tendency towards “dot-like” granular structures, which was clearly different from
147 the pattern observed with NS4B(1-39)-GFP and with GFP alone, showing diffuse distribution in the
148 cytoplasm and nucleus at the late stage of transfection. The fluorescence of helix mutants (HMT) of
149 NS4B(40-81)-GFP and NS4B(1-81)-GFP was diffuse distribution and did not observe the “dot-like”

150 granular structures at all [Fig. 5(c)]. These results indicate that the putative amphipathic α 2-helix
151 (aa 40–81) is an essential segment for alternation of the fluorescent pattern.

152 A membrane flotation assay was used to further characterise the predicted membrane
153 association of the α 2-helix of NS4B. A representative Western blot from a membrane flotation
154 experiment with extracts from SK-6 cells expressing NS4B(40-81)-GFP from the GPE⁻ strain is
155 shown in Fig. 5(d). Low-density membrane fractions are raised towards the top of the gradient,
156 whereas soluble proteins remain with the denser fractions at the bottom. Accordingly, GFP was
157 retained at the bottom of the gradient (fractions 7 and 8) similar to the cytoplasmic soluble protein
158 glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The GFP-tagged α 2-helix of NS4B came
159 up in the low-density fractions 3 and 4 (from the top) of the flotation gradient. This was consistent
160 with the accumulation of the membrane-associated calnexin in the gradient fractions 2 and 3 [Fig.
161 5(d), bottom panel]. In contrast, the disrupted amphipathic helix mutant of the GFP-tagged α 2-helix
162 of GPE⁻ NS4B [GPE⁻ NS4B(40-81HMT)-GFP] detected at the soluble fraction. Next, to analyse the
163 membrane association of this segment in more details, membrane flotation was examined after
164 treatments that discriminate between peripheral and integral membrane association. To this end,
165 the postnuclear supernatants from cells expressing NS4B(40-81)-GFP were centrifuged at 100000 ×
166 g, and the pelleted membranes were subjected to different extraction methods. The GFP-tagged
167 NS4B(40-81) remained in the membrane fraction under physiological conditions and after high-salt
168 and alkaline extractions, while detergent extraction resulted in accumulation of NS4B(40-81)-GFP in
169 the higher density soluble fractions. Taken together, the flotation assays confirmed strong
170 intracellular membrane association of the N-terminal amphipathic α 2-helix of NS4B (aa 40–81).

171

172 **Intracellular membrane association mediated by the N-terminal domain of NS4B is a**
173 **determinant of CSFV RNA replication in cell culture.**

174 In order to clarify the roles of intracellular membrane association mediated by the N-terminal
175 amphipathic domain of NS4B for RNA replication, the both cistronic replicons carrying the same

176 mutations for disruption of the amphipathic α -helix were generated. The luciferase activity of the
177 helix mutant (rGPE⁻-N^{pro}-Luc-IRES-NS3/NS4B HMT) was significantly low compared with the
178 luciferase activity of the parental GPE⁻ replicon [Fig. 6(a)]. This was similar to that of the
179 replication-deficient replicon, indicating that the replication capability was completely abolished by
180 the mutations. In the mono-cistronic replicon assay, the helix mutant behaved similarly as the
181 replication-deficient replicon [Fig. 6(b)]. Altogether, these data indicate that intracellular membrane
182 association mediated by the N-terminal domain of NS4B is critical for RNA replication in cell culture.

183

184 **The N-terminal domain of NS4B modulates subcellular localisation.**

185 In order to determine the subcellular distribution of the N-terminal domain of NS4B,
186 co-localisation experiments were performed in SK-6 cells expressing the GFP-tagged fusion proteins.
187 As shown in Fig. 7(a), the fluorescence pattern of the GFP-tagged N-terminal domain of NS4B,
188 NS4B(40-81)-GFP and NS4B(1-81)-GFP, appeared to partially overlap with the endoplasmic
189 reticulum (ER). The co-localisation efficient was the same level of the entire NS4B [Fig. 7(b)].
190 These data indicate that the N-terminal amphipathic helix determines the subcellular localisation of
191 NS4B.

192

193 **DISCUSSION**

194 In CSFV, the nonstructural protein NS4B is essential for viral replication (Behrens *et al.*, 1998).
195 Our previous study demonstrated that the two amino acid residues that differ between the vaccine
196 strain GPE⁻ and its parental strain ALD in the predicated trans-membrane domains of NS4B can
197 modulate viral genome replication and influence the pathogenicity of the virus in pigs (Tamura *et al.*,
198 2012). Here, we generated GPE⁻-derived virus carrying the complete NS4B of the Eystrup strain, in
199 order to determine whether the additional five amino acid differences in the N-terminal domain of
200 NS4B may contribute to the different pathogenicity in pigs. The GPE⁻-derived virus carrying NS4B
201 of the highly virulent Eystrup strain resulted in more severe clinical symptoms, earlier and prolonged

202 viremia compared with the virus carrying only the two amino acid substitutions in the predicted
203 trans-membrane domains. These data indicated that the N-terminal domain of NS4B may
204 represent a virulence determinant *in vivo*. However, as expected, NS4B from the Eystруп strain did
205 not confer a full high virulent phenotype to the GPE⁻-derived virus, demonstrating that additional viral
206 proteins and amino acid residues are involved in determining the virulence of the Eystруп strain. In
207 addition, the specific residues identified in this study as critical for determining virulence in pigs are
208 not general virulence determinants in CSFV (Fig. 8). This suggests that the virulence of CSFV is
209 more likely a multi-genic trait determined by a complex interplay of several viral proteins or genes
210 acting in concert as already postulated in previous studies (Leifer *et al.*, 2013; Tamura *et al.*, 2012).
211 To the best of our knowledge, this is the first study to show that the N-terminal domain of CSFV NS4B
212 can confer enhanced pathogenicity to a low virulent virus, which was related to an enhanced viral
213 replication.

214 The five amino acid residues differing between the GPE⁻ and Eystруп strains in the N-terminal
215 domain of NS4B clearly determine the RNA replication efficiency of replicons *in vitro*. In previous
216 studies, two types of replicons were utilized for studying RNA replication: Mono-cistronic (Risager *et al.*
217 *et al.*, 2013) and bi-cistronic (Behrens *et al.*, 1998). The minimal viral elements required for RNA
218 replication by pestiviruses including CSFV are NS3–NS5B, together with the 5' and 3' UTRs
219 (Behrens *et al.*, 1998). In addition to these minimal elements, the bi-cistronic replicon carries an
220 artificial internal ribosomal entry site (IRES) derived from the encephalomyocarditis virus (EMCV), a
221 luciferase reporter gene, and the viral auto-protease N^{pro} for optimal translation and processing of the
222 reporter protein. The replicative properties of this replicon may however be modified by the EMCV
223 IRES. For these reasons, a mono-cistronic replicon was included. Comparable results of
224 NS4B-dependent differences in RNA replication were obtained with the two types of replicons,
225 strengthening the data.

226 The granular fluorescent signal derived from the N-terminal domain of NS4B was partially
227 co-localised with the ER by confocal fluorescence microscopy. In the membrane flotation assay, the

228 N-terminal domain of NS4B was also bound to the ER in part, suggesting that the ER could be a
229 candidate site of the replication complex in infected cells. However, the major part of the N-terminal
230 domain of NS4B detected the different fractions of the ER marker, calnexin. Thus, further studies
231 will be needed to identify the exact subcellular localisation of the replication complex including the
232 natural NS4B in CSFV infected cells. It was described that BVDV NS4B alone can rearrange the
233 host membrane (Weiskircher *et al.*, 2009) as well as HCV NS4B (Egger *et al.*, 2002), suggesting that
234 pestivirus NS4B might act as a trigger for building replication complex. So far, however, pestivirus
235 replication is considered not to remodel cytoplasmic host cell membranes (Schmeiser *et al.*, 2014) as
236 opposed to HCV which induces organelle-like structures designated as the membranous web where
237 the formation of the viral replication complex is triggered (Romero-Brey *et al.*, 2012) and to other
238 positive-strand RNA viruses (Knoops *et al.*, 2008; Kopek *et al.*, 2007; Spuul *et al.*, 2007; Welsch *et al.*,
239 2009). Therefore, we hypothesize that the role of pestivirus NS4B may be partially different from
240 that in other members of the *Flaviviridae*. Further studies on the functions of the pestivirus NS4B
241 will allow to clarify how the NS4B-dependent modulation of RNA replication may relate to differences
242 in viral replication, morphogenesis and virulence.

243 Intracellular membrane association mediated by the N-terminal amphipathic domain of NS4B is
244 essential for RNA replication. This amphipathic structure is conserved among CSFV strains (Fig. 8).
245 Interestingly, the amino acid differences in the N-terminal part of NS4B between the prototype
246 vaccine virus and the highly virulent strain studied here are located on the predicated hydrophilic
247 face of the amphipathic α 2-helix. These residues are not conserved with respect to virulence of
248 CSFV, however, the amino acid differences among CSFV strains were found mostly on the
249 hydrophilic face of the amphipathic α 2-helix, suggesting that the functional differences observed may
250 depend on subtle structure-related differences in association with host proteins and/or other viral
251 nonstructural proteins, modulating replication complex formation, RNA replication and eventually
252 pathogenicity of the virus in pigs. For HCV, the N-terminal amphipathic α 2-helix of NS4B is critical
253 for its polymerization, and thus for its accumulation in organelles (Gouttenoire *et al.*, 2010; Yu *et al.*,

254 2006). A recent study of HCV demonstrated that hydrophilic amino acids in the N-terminal
255 cytoplasmic NS4B are responsible for virus production (Gouttenoire *et al.*, 2014).

256 In summary, this is the first study showing the roles of intracellular membrane association
257 mediated by the N-terminal domain of CSFV NS4B in viral RNA replication and pathogenicity in pigs.
258 Further studies are required in order to understand the functionality of the pestivirus replication
259 complex at the molecular level, and how this affects pathogenicity in pigs. Understanding the
260 molecular mechanisms involved in viral replication and pathogenesis will permit designing effective
261 tools for controlling the disease.

262

263 **METHODS**

264 **Ethical statement**

265 The animal experiments described here were approved by the Animal Welfare Committee of the
266 Canton of Berne with the license number BE94/12, and conducted in compliance with the Swiss
267 animal protection law and with the national and international animal experimentation guidelines.

268

269 **Viruses and cells**

270 The CSFV strains, (vGPE⁻/N136D; T830A; V2475A; A2563V), (vGPE⁻/N136D; T830A; Eystrup
271 NS4B) and (vEy-37) which is identical to Eystrup were derived from the full-length cDNA clones, *i.e.*,
272 pGPE⁻/N136D; T830A; V2475A; A2563V (Tamura *et al.*, 2014), pGPE⁻/N136D; T830A; Eystrup
273 NS4B and pEy-37 (Mayer *et al.*, 2003), respectively. All of the cDNA-derived viruses were rescued
274 as aforementioned (Moser *et al.*, 1999). The complete genomes of the rescued viruses were
275 verified by nucleotide sequencing to exclude any accidental mutations. SK-6 cells were propagated
276 at 37°C in the presence of 5% CO₂ in Eagle's minimum essential medium (Nissui Pharmaceutical)
277 supplemented with 0.3 mg/ml L-glutamine and 7% horse serum (Life Technologies).

278

279 **Plasmid constructs**

280 The cDNA clone of the mono-cistronic replicon carrying the *Gaussia* luciferase reporter gene,
281 pGPE⁻2GL, was constructed from rPad2GL (Risager *et al.*, 2013) by replacing the genome of the
282 Paderborn strain with that of the GPE⁻ strain applying the In-Fusion[®] HD Cloning (Clontech)
283 techniques as described previously (Tamura *et al.*, 2012). The cDNA clone of rPad2GL was a gift of
284 T. B. Rasmussen (DTU National Veterinary Institute, Denmark). The infectious cDNA clone
285 (pGPE⁻/N136D; T830A; Eystруп NS4B) and the replicon cDNA clones
286 (pGPE⁻-N^{pro}-Luc-IRES-NS3/Eystруп NS4B and pGPE⁻2GL/Eystруп NS4B) were constructed by
287 replacing NS4B in the backbone with the corresponding gene of the highly virulent strain Eystруп as
288 described above. The cDNA clones of the replication-deficient replicons
289 (pGPE⁻-N^{pro}-Luc-IRES-NS3/GAA and pGPE⁻2GL/GAA) encoding the amino acid sequence “GAA”
290 instead of “GDD” in the polymerase active site of the nonstructural protein NS5B (Zhong *et al.*, 1998)
291 were constructed from the replicon cDNA clones, pGPE⁻-N^{pro}-Luc-IRES-NS3 and pGPE⁻2GL using
292 the QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies) and oligonucleotide
293 primers containing the respective mutations based on the aforementioned standard techniques
294 (Tamura *et al.*, 2012). The cDNA clones of the disrupted amphipathic character in the N-terminal
295 domain of GPE⁻ NS4B (pGPE⁻-N^{pro}-Luc-IRES-NS3/NS4B HMT and pGPE⁻2GL/NS4B HMT) were
296 accordingly generated from the parental clones (pGPE⁻-N^{pro}-Luc-IRES-NS3 and pGPE⁻2GL) by
297 mutagenesis described above.

298 In order to construct the GFP, the NS4B construct with a C-terminal GFP-tag and the truncated
299 versions thereof, the cDNA fragments of the respective regions of the GPE⁻ and Eystруп strains were
300 obtained using reverse transcription and PCR, and the GFP fragments were cloned into the pCI
301 Mammalian Expression Vector (Promega) with In-Fusion[®] HD Cloning and appropriate restriction
302 enzymes. Details of the constructs are available on request.

303

304 Sequencing

305 The cDNA clones, *in vitro*-rescued viruses, and expression vectors were completely sequenced

306 as aforementioned (Tamura *et al.*, 2012). In brief, nucleotide sequencing of the cDNA clones,
307 expression vectors and PCR fragments from viral RNA was performed using a BigDye Terminator
308 v3.1 Cycle Sequencing Kit (Life Technologies) and a 3500 Genetic Analyser (Life Technologies).
309 The sequencing data were analysed using Genetyx[®]-Network version 12 (Genetyx).

310

311 **Antibodies and markers**

312 The E2-specific monoclonal antibody (mAb) HC/TC26 (Greiser-Wilke *et al.*, 1990) was kindly
313 provided by I. Greiser-Wilke (Hannover Veterinary School, Hannover, Germany). The mAb against
314 viral NS3, 46/1 was generated previously (Kameyama *et al.*, 2006a). The antibodies against
315 cellular organelles, GAPDH and calnexin were purchased from Cell Signaling Technology and Enzo
316 Life Sciences, respectively. The mAb against GFP was obtained from Santa Cruz Biotechnology.
317 A secondary antibody, FITC-conjugated goat anti-mouse IgG, was purchased from MP Biomedicals,
318 and Alexa Fluor 488 F(ab')₂ Fragment of Goat Anti-Mouse IgG (H+L) was obtained from Life
319 Technologies. Fluorescent probes for detecting the ER (ER-Tracker) was purchased from Life
320 Technologies. For counterstaining of the cell nuclei, 4', 6-diamino-2-phenylindole dihydrochloride
321 (DAPI) was obtained from Dojindo Molecular Technologies.

322

323 **Virus titration**

324 The virus titres were determined by end-point dilution with SK-6 cells and immunoperoxidase
325 staining using anti-E2 mAb HC/TC 26 or anti-NS3 mAb 46/1 as described earlier (Mittelholzer *et al.*,
326 1997; Sakoda *et al.*, 1998). The titres were calculated using the Reed and Muench formula and
327 expressed as the 50% tissue culture infective dose (TCID₅₀) per ml (Reed & Muench, 1938).

328

329 **Experimental infection of pigs**

330 In order to assess the pathogenicity of the cDNA-derived viruses, groups of six 10-week-old to
331 13-week-old Large White specific-pathogen-free (SPF) pigs obtained from the breeding unit of The

332 Institute of Virology and Immunology (IVI, Switzerland) were inoculated intranasally with $10^{6.0}$ TCID₅₀
333 of the respective viruses. All pigs were kept in separate isolation units in the BSL-4 facility of the IVI.
334 The body temperature and clinical scores were monitored daily according to a defined scoring
335 system as described previously (Mittelholzer *et al.*, 2000). Whole blood was collected for serum
336 preparation at 3 days before infection and on days 3, 4, 5, 6, 7, 10 and 12 post-inoculation (pi).
337 The pigs that survived the infection were euthanized on day 14 pi. The virus titres in the serum
338 samples were expressed as the TCID₅₀ ml⁻¹.

339

340 **Luciferase assay**

341 Luciferase assays using bi-cistronic replicons were conducted as previously described (Tamura
342 *et al.*, 2012). In brief, $10^{6.0}$ SK-6 cells were electroporated with 1 µg of replicon RNA.
343 Electroporation was performed at 200 V and 500 µF. At different times after transfection, cell
344 extracts were prepared with 200 µl of passive lysis buffer, and the firefly luciferase activity was
345 measured using a Luciferase Assay System (Promega) and a Lumat LB9507 luminometer
346 (Berthold).

347 Luciferase assays with mono-cistronic replicons were performed as aforementioned, with some
348 modifications (Risager *et al.*, 2013). Electroporation was conducted under the conditions described
349 above. At the time indicated, 50 µl of cell medium was harvested from the supernatants of cells
350 transfected with replicons, which was then frozen at -20°C. After thawing, 20 µl of the cell medium
351 was analysed to determine the *Gaussia* luciferase activity using *Renilla* luciferase substrate and a
352 Lumat LB9507 luminometer.

353 The results representing the luciferase activities that were compared with the luciferase activity
354 of the replicon devoid of polymerase activity at the initial timepoints were measured on the basis of
355 three independent experiments.

356

357 **Immunofluorescence assay**

SK-6 cells were transfected with the expression vectors using Lipofectamine 3000 (Life Technologies) and Opti-MEM (Life Technologies), according to the manufacturer's protocol. At the time indicated, SK-6 cells grown on an eight-well chamber slide (Matsunami) were fixed and stained as aforementioned (Sharma *et al.*, 2012; Yamasaki *et al.*, 2012). Anti-GFP mAb as the primary antibody was used at a concentration of 1 mg ml⁻¹. As the secondary antibodies, FITC-conjugated goat anti-mouse IgG (1: 2500) or Alexa-Fluor-conjugated IgG (1: 1000) were used. In order to counterstain the cell nuclei, the cells were incubated for 30 min with 2 µg DAPI ml⁻¹ in PBS at room temperature. For labeling the cell organelle, the cells were grown on 35-mm glass-based dishes (IWAKI) and washed with PBS, before staining for 30 min with 100 mM ER-Tracker in complete medium at 37°C with 5% CO₂. The cells were then washed twice with PBS and pre-warmed PBS was added. The fluorescent cells were analysed with a BZ-9000 (KEYENCE) microscope and a BZ-II Analyzer (KEYENCE). The confocal fluorescent images were acquired using Zeiss LSM700 (Carl Zeiss) microscopes (upright and inverted) and ZEN 2012 (Carl Zeiss). Quantitative co-localisation analysis was performed as described elsewhere (Dunn *et al.*, 2011). The co-localisation ratio of the GFP signals with the organelle marker that represents a co-localisation coefficient was quantified using ZEN 2012 software.

374

375 **Membrane flotation assay**

SK-6 cells were grown on 10-cm dishes overnight and transfected with the GFP-tagged NS4B and truncated vectors or with the control GFP constructs according to the conditions described above. At 30 h post-transfection (pt), the cells were re-suspended in hypotonic buffer (10 mM Tris-HCl [pH 7.5], 10 mM KCl, 5 mM MgCl₂) containing a protease inhibitor (Complete Mini, Roche). The cells were then disrupted by 20 passages through a 25G needle with 1ml syringe, ensuring approximately 90% disruption. The samples were spun at 1000 × g for 5 min at 4°C to pellet the cellular debris and nuclei, thereby obtaining postnuclear supernatants. A discontinuous iodixanol (OptiPrep, Alere Technologies AS) gradient (5%, 25% and 30%) was layered on top of the samples,

384 followed by centrifugation at $230140 \times g$ for 24 h at 4°C in a P40ST Rotor (Hitachi Koki). In total,
385 eight equal fractions were collected from the top to bottom. Each fraction was precipitated with
386 methanol-chloroform and analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis
387 followed by immunoblotting using anti-GFP, anti-GAPDH and anti-calnexin as described previously
388 (Kameyama *et al.*, 2006b). For the membrane dissociation experiments, the postnuclear
389 supernatants were adjusted to 0.25 M sucrose and centrifuged at $100000 \times g$ for 45 min at 4°C.
390 The pellet containing the membranes and their associated proteins was re-suspended in either high
391 salt buffer (1 M NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA), 100 mM sodium carbonate (pH 11.5) or
392 1% Triton X-100. After incubation at 4°C for 30 min, the samples were subjected to membrane
393 flotation followed by immunoblotting, as described above. Typically, the membrane-bound proteins
394 were associated with fractions 1–4 whereas the soluble proteins were generally present in fractions
395 5–8, which were followed by the respective protein markers.

396

397 **Structure predictions and sequence analyses**

398 Multiple sequence alignment and amino acid conservation analyses were performed with the
399 CLUSTALW2 program (Larkin *et al.*, 2007) using the default parameters. The protein secondary
400 structure predictions were generated using DSC (King & Sternberg, 1996), HNN (Guermeur, 1997),
401 MLRC (Guermeur *et al.*, 1999), PHD (Rost, 1996), Jpred 3 (Cole *et al.*, 2008), Porter (Pollastri &
402 McLysaght, 2005), PSIPRED (Jones, 1999) and APSSP2 (Raghava, 2002). The trans-membrane
403 domains were predicted using TopPred II (Claros & von Heijne, 1994). HeliQuest (Gautier *et al.*,
404 2008) was used for α -helix projections.

405

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549

550

551

552 **Figure legends**

553 **Figure 1. Schematic representation of the viral genome and of the amino acids of the viral** 554 **proteins relevant for this study**

555 The CSFV genome and the viral proteins N^{pro}, E2 and NS4B of the GPE⁻ vaccine strain and of
556 the highly virulent Eystrup strain are depicted schematically. The amino acid differences related to
557 virulence reported previously (Tamura *et al.*, 2014; Tamura *et al.*, 2012) and the five additional
558 residues in the N-terminal half of NS4B (2377, 2391, 2398, 2399 and 2414) evaluated in the present
559 study (#) are shown. Numbering starts with the methionine encoded by the AUG start codon.

560

561 **Figure 2. Evaluation of the contribution of NS4B to CSFV pathogenicity**

562 Three groups of six 10-week-old to 13-week-old SPF pigs were inoculated intranasally with 10^{6.0}
563 TCID₅₀ of the parental vGPE⁻/N136D; T830A; V2475A; A2563V virus, the mutant vGPE⁻/N136D;
564 T830A; Eystrup NS4B virus and the highly virulent Eystrup strain, respectively. Clinical scores (a),
565 body temperatures (b) and virus titre in the serum (c) were monitored. A body temperature of above
566 40°C was considered fever. At terminal stages of the disease, the animals were euthanized
567 according to defined endpoint criteria (‡) or died unexpectedly (†). The statistical significance of the
568 differences was calculated using the Student's *t*-test. An asterisk indicates significance (*p*<0.05)
569 between the GPE⁻-derived mutant viruses (vGPE⁻/N136D; T830A; V2475A; A2563V and
570 vGPE⁻/N136D; T830A; Eystrup NS4B).

571

572 **Figure 3. Comparison of the effect of NS4B from low and highly virulent CSFV on the** 573 **replication efficiency of GPE⁻-derived replicons**

574 GPE⁻-derived bi-cistronic (a) and mono-cistronic (b) luciferase reporter replicons carrying the
575 GPE⁻ NS4B or its NS4B with the two mutations V2475A and A2563V or the complete NS4B of the
576 highly virulent Eystrup strain were utilized. “#” indicates the locations of the five amino acid
577 differences (2377, 2391, 2398, 2399 and 2414) in the N-terminal domain of these strains.

578 Corresponding bi-cistronic (rGPE⁻-N^{pro}-Luc-IRES-NS3/GAA) and mono-cistronic (rGPE⁻-2GL/GAA)
 579 replicons encoding an inactive NS5B polymerase were used as controls. After the indicated
 580 incubation periods, the firefly luciferase activity from cell lysates (c) and the *Gaussia* luciferase
 581 activity from cell culture supernatants (d) was measured as described in METHODS. Results that
 582 represented the mean values of the luciferase activity compared with the luciferase activity of the
 583 replicons devoid of NS5B polymerase activity at the initial timepoints were obtained from three
 584 independent experiments. The error bars represent the standard error. The statistical significance
 585 of the differences was calculated using the Student's *t*-test. Significance ($p < 0.05$) is indicated with
 586 an asterisk.

587

588 **Figure 4. Comparison of the NS4B amino acid sequences of the low virulent GPE⁻ and the**
 589 **highly virulent Eystrup strains**

590 (a) Based on the secondary structure predictions and their consensus, the α -helices and
 591 β -strands are depicted by white columns and arrows, respectively. The trans-membrane domains
 592 (TM) are shown with black rectangles. Amino acid numbering started with the beginning of NS4B.
 593 The amino acid differences are indicated based on their positions relative to the translation start site
 594 of the polyprotein, and they are highlighted in gray. (b) Amphipathic helix wheels in the N-terminal
 595 2nd α -helix ($\alpha 2$, amino acid [aa] of NS4B 40–81) of two strains are illustrated, with the amino acid
 596 differences indicated by an asterisk. White and black colours indicate hydrophobic and hydrophilic
 597 amino acids, respectively. The clustering of hydrophobic, nonpolar residues on one face of the helix
 598 suggests an amphipathic configuration. The broken lines separate the two faces of the helix.

599

600 **Figure 5. Role of the predicted N-terminal amphipathic helix domain for protein localisation in**
 601 **porcine cells**

602 (a) Schematic representation of the NS4B-GFP chimeric proteins and of the truncated versions
 603 expressed from the pCI vector under the control of a cytomegalovirus promoter. The 2nd α -helix

domain (aa 40–81) is shown in black. (b) Amino acid sequences and a helical wheel plot of N-terminal domain of NS4B amino acids showing the localisation of the amino acid substitutions for disruption of its amphipathic character. (c) SK-6 cells were transfected with pCI constructs for expression of the indicated NS4B-GFP proteins from the GPE⁻ strain. GFP fluorescence (green) was analysed by confocal microscopy after 30 h of culture. A plasmid expressing GFP served as control. Nuclei were stained with DAPI (blue). Bars = 10 µm. (d) Membrane flotation analysis of SK-6 cells expressing the N-terminal domain (aa 40–81) of NS4B. SK-6 cells transfected with the pCI vectors, NS4B(40-81)-GFP and its helix mutant, were disrupted mechanically at 36 h after transfection. Equal amounts of postnuclear supernatants were used for membrane flotation and Western blot analysis with an antibody to GFP, GAPDH or calnexin. For the membrane extraction experiments, the postnuclear supernatants were treated with 100 mM Na₂CO₃ (pH 11.5), with 1 M NaCl or with 1% Triton X-100 and subjected to membrane flotation followed by Western blot analysis.

Figure 6. Role of the predicted N-terminal amphipathic helix domain for RNA replication

GPE⁻-derived bi- and mono-cistronic luciferase reporter replicons carrying the mutations for disruption of its amphipathic character were utilized. Respective firefly (a) and *Gaussia* (b) luciferase activities were analysed as described above. Significance ($p < 0.05$) is indicated with an asterisk.

Figure 7. Subcellular distribution of the N-terminal domain of NS4B in porcine cells

(a) SK-6 cells were transfected with pCI constructs expressing the NS4B-GFP chimeric proteins. At 36 h after transfection, the cells were processed for live-imaging fluorescence microscopy. The fusion proteins were detected by GFP fluorescence (Green). The ER-Tracker (red) was used as markers for the ER. (b) Co-localisation coefficient with the ER was calculated. Mean and standard deviation of the values acquired from 15 fields of view are depicted.

630 **Figure 8. Amino acid alignment of the N-terminal domain (aa 1–81) of CSFV NS4B**

631 The amino acid sequences in the N-terminal domain (aa 1–81) of selected CSFV strains of low,
632 moderate and high virulence were aligned. The hydrophilic amino acids are highlighted in gray.
633 The amino acid differences between the GPE[−] and Eystrup strains are marked with an asterisk. A
634 dot indicates the same amino acid as for the GPE[−] strain.

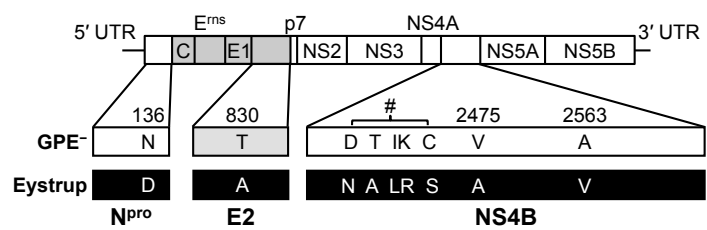


Figure 1. Tamura *et al.*

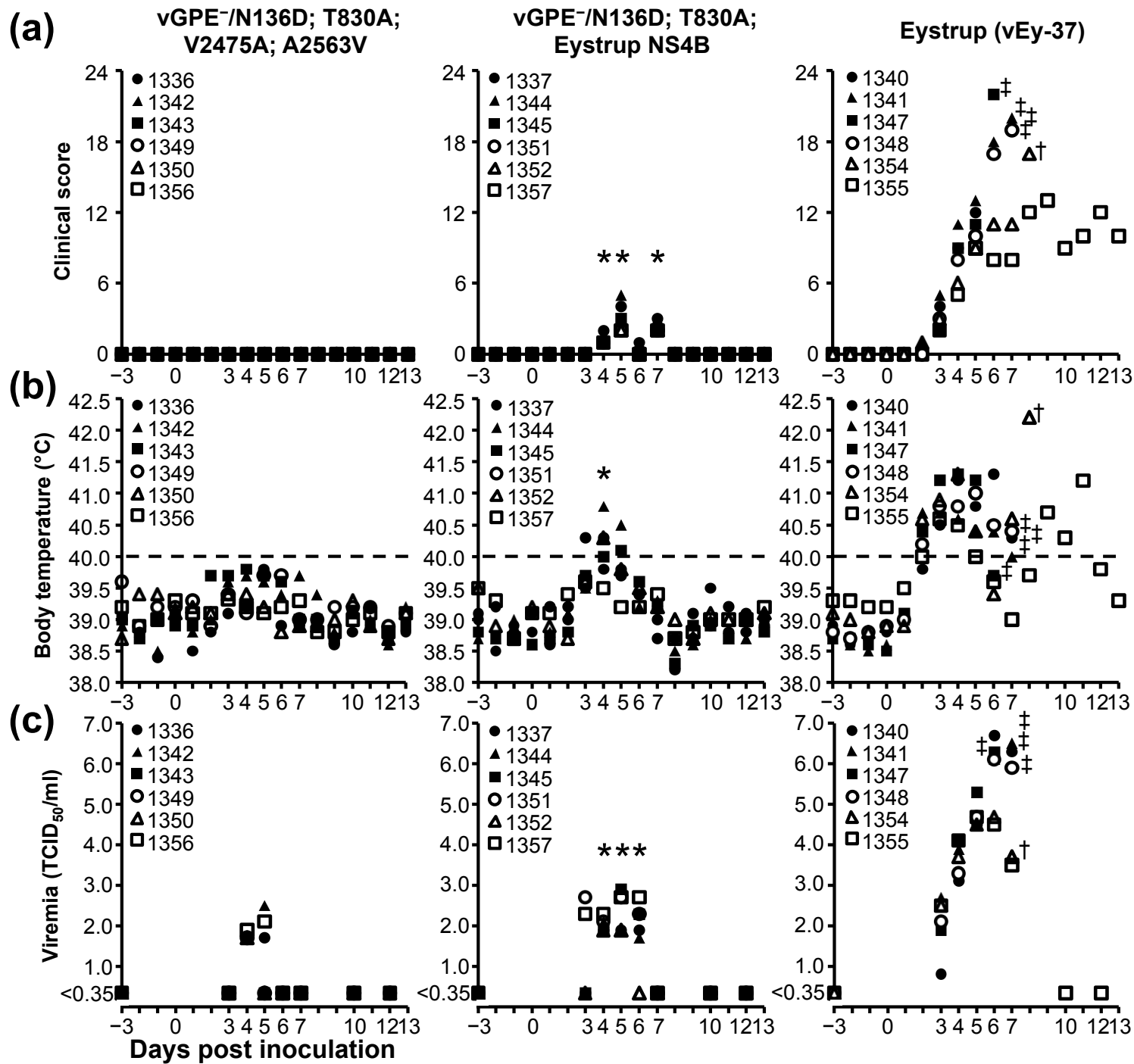


Figure 2. Tamura *et al.*

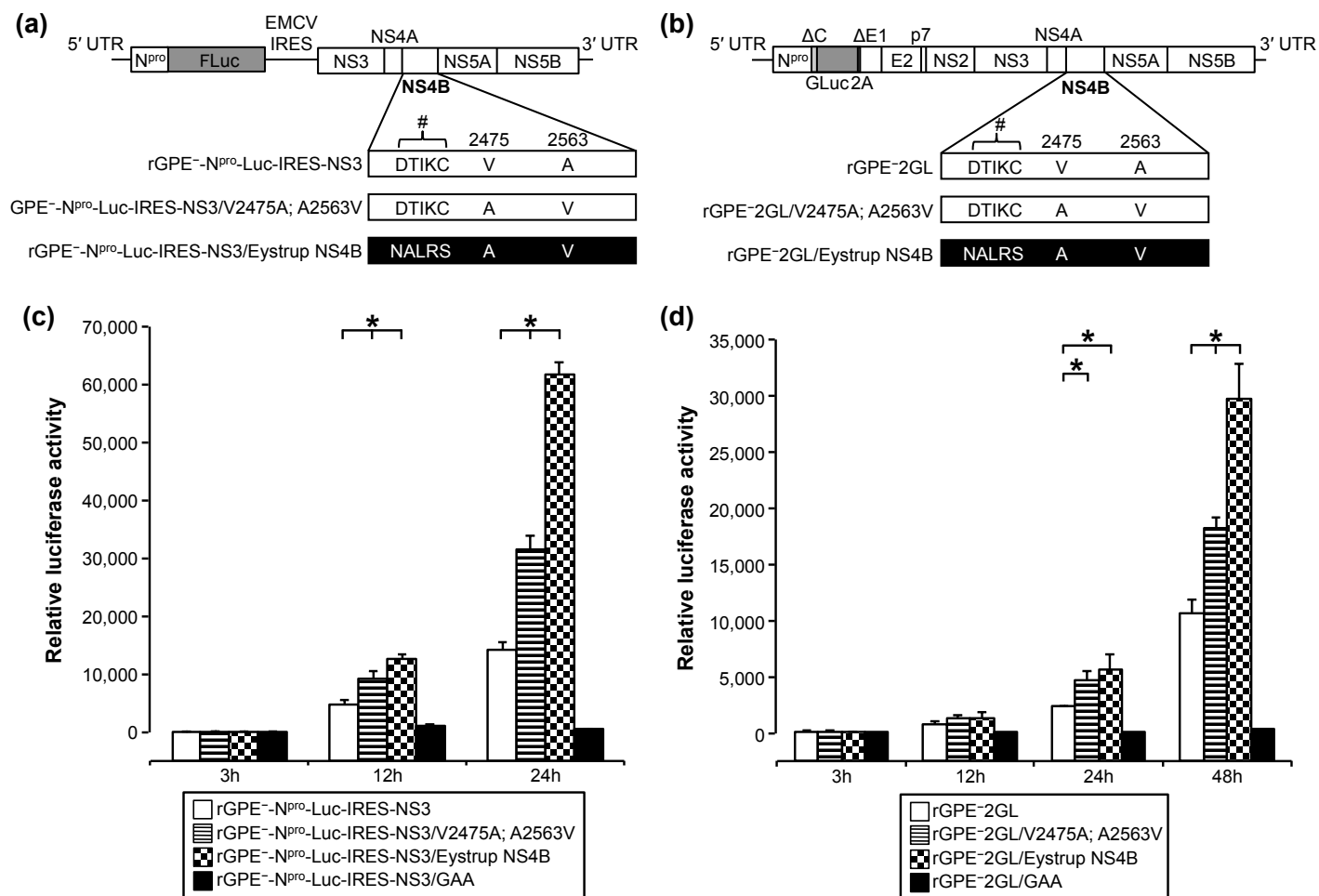
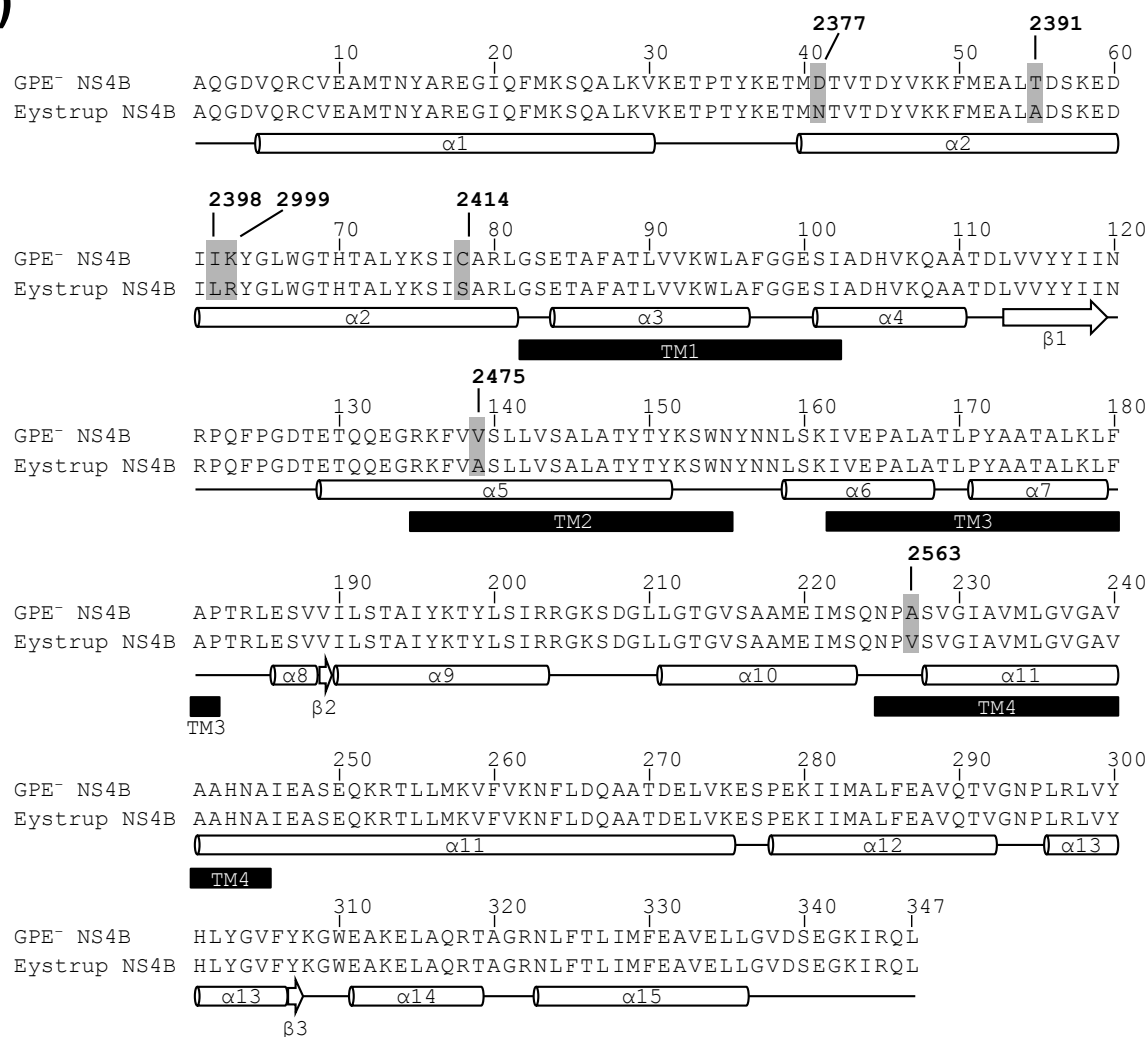


Figure 3. Tamura *et al.*

(a)



(b)

GPE⁻ NS4B(40-81)

Eystруп NS4B(40-81)

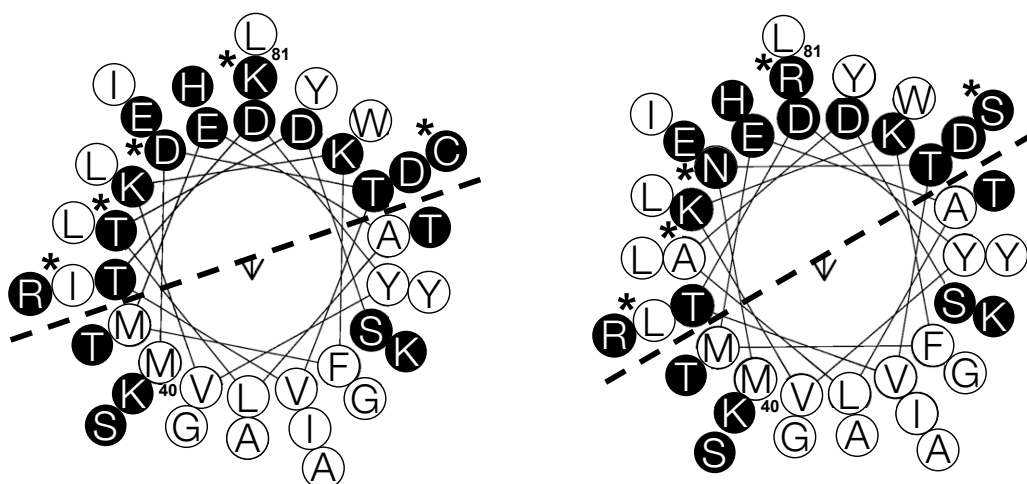


Figure 4. Tamura *et al.*

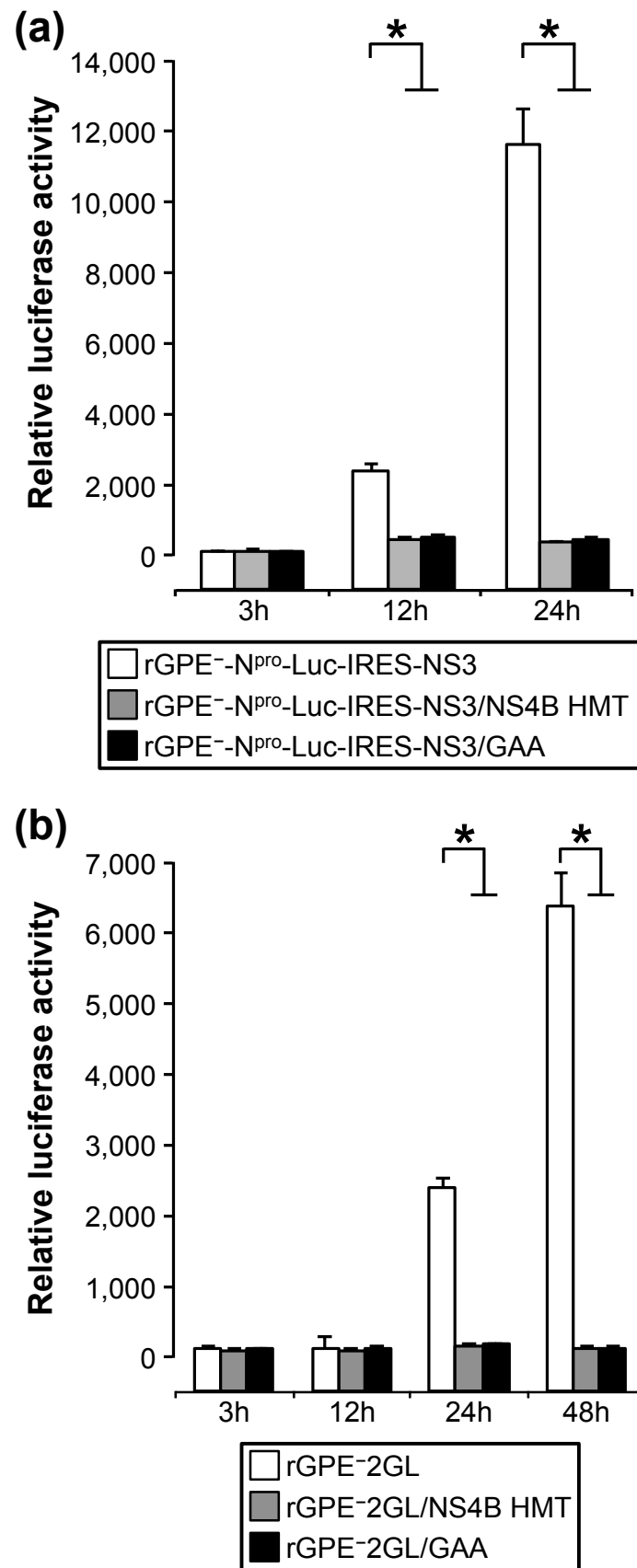


Figure 6. Tamura *et al.*

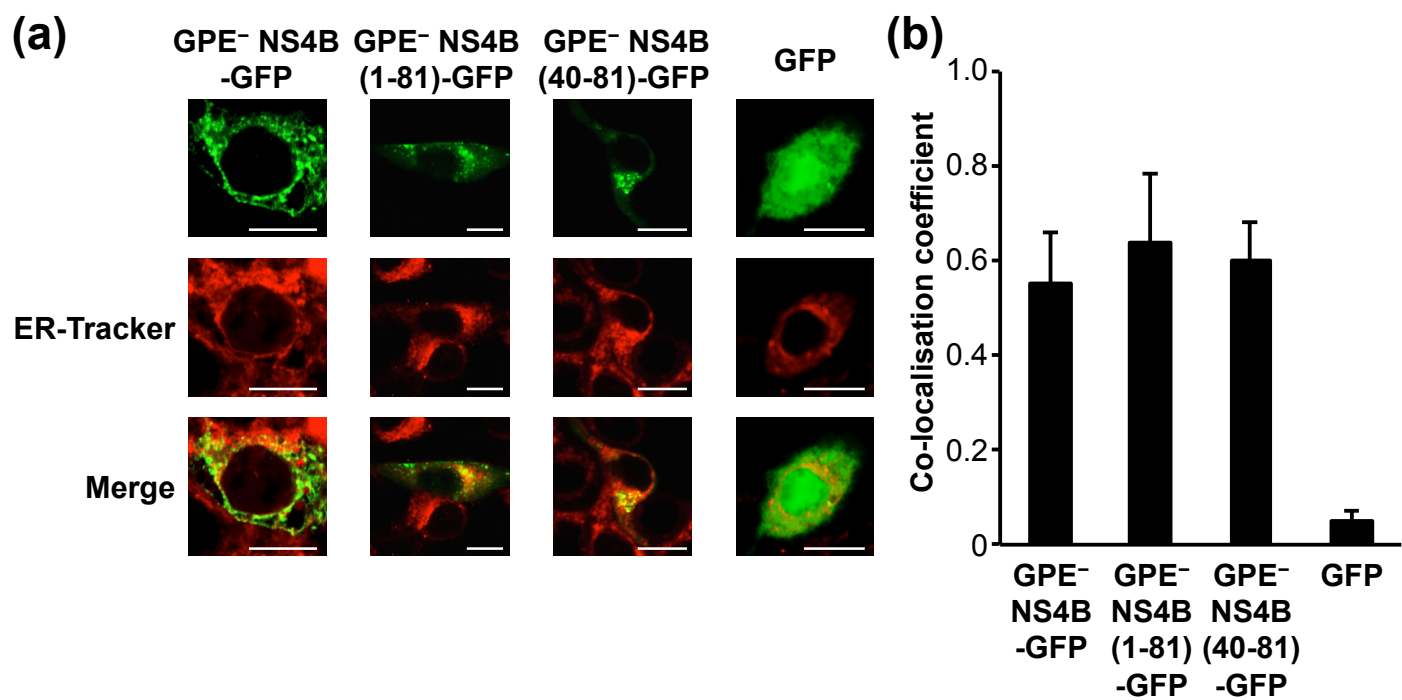


Figure 7. Tamura *et al.*

	10	20	30	40	50	60	70	80
Low virulent/vaccine strains				*		*	**	*
GPE ⁻	AQGDVQRCVEAMTNYAREGIQFMKSQALKVKETPTYKETMDTVDYVKKFMEAL	TDSKEDI	IKYGLWG	TH	TALYKS	I	CARL	
C strain						A		S
CAP		K			A			
Glentorf		K			A			
HCLV	P					A		S
India				V		A	R	S
LOM			E				R	
Thivaler	G							
Moderately virulent strains								
Alfort/187								
Borken		V		R	N	A	I	A
Euskirchen		V		R		A	V	A
GXWZ02		V	V	R		A	ER	I
Hennef		V		R		A	A	V
Paderborn		V	K	R		N	A	E
Roesrath		V		R		NA	A	V
Uelzen		V		R	A	N	A	I
Highly virulent strains								
ALD		V					A	L
Alfort/Tübingen		V		R		N	A	I
Brescia			D				A	
JL1 (06)							K	M
Koslov							A	L
Shimen							K	M
SWH						V	K	M
Eystrup				N			A	LR

Figure 8. Tamura *et al.*