The N-terminal domain of N\textsuperscript{pro} of classical swine fever virus determines its stability and regulates type I IFN production

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The viral protein N\textsuperscript{pro} is unique to the genus Pestivirus within the family Flaviviridae. After autocatalytic cleavage from the nascent polypeptide, N\textsuperscript{pro} suppresses type I IFN (IFN-\alpha/\beta) induction by mediating proteasomal degradation of IFN regulatory factor 3 (IRF-3). Previous studies found that the N\textsuperscript{pro}-mediated IRF-3 degradation was dependent of a TRASH domain in the C-terminal half of N\textsuperscript{pro} coordinating zinc by means of the amino acid residues C112, C134, D136 and C138. Interestingly, four classical swine fever virus (CSFV) isolates obtained from diseased pigs in Thailand in 1993 and 1998 did not suppress IFN-\alpha/\beta induction despite the presence of an intact TRASH domain. Through systematic analyses, it was found that an amino acid mutation at position 40 or mutations at positions 17 and 61 in the N-terminal half of N\textsuperscript{pro} of these four isolates were related to the lack of IRF-3-degrading activity. Restoring a histidine at position 40 or both a proline at position 17 and a lysine at position 61 based on the sequence of a functional N\textsuperscript{pro} contributed to higher stability of the reconstructed N\textsuperscript{pro} compared with the N\textsuperscript{pro} from the Thai isolate. This led to enhanced interaction of N\textsuperscript{pro} with IRF-3 along with its degradation by the proteasome. The results of the present study revealed that amino acid residues in the N-terminal domain of N\textsuperscript{pro} are involved in the stability of N\textsuperscript{pro}, in interaction of N\textsuperscript{pro} with IRF-3 and subsequent degradation of IRF-3, leading to downregulation of IFN-\alpha/\beta production.

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\textbf{INTRODUCTION}

Viral infection triggers complex cellular antiviral defence mechanisms. dsRNA triggers the type I IFN (IFN-\alpha/\beta) pathway, leading to antiviral responses such as the destruction of viral RNA, inhibition of cellular transcription and translation, and promotion of apoptosis (Randall & Goodbourn, 2008). IFN-\alpha/\beta induction depends on a family of transcription factors – the IFN regulatory factors (IRFs) (Taniguchi et al., 2001). IRF-3 is ubiquitously expressed in the cytoplasm and activated in response to viral infection (Au et al., 1995). The activation of the pathway leads to phosphorylation, dimerization and translocation of IRF-3 into the nucleus, and to formation of the enhanceosome that binds to the IFN-\alpha/\beta promoters (Honda et al., 2006; Saitoh et al., 2006). Previous studies demonstrated that several viruses employ various strategies to counter this antiviral response. For instance, classical swine fever virus (CSFV) promotes IRF-3 degradation, hepatitis C virus inhibits IRF-3 phosphorylation, Thogoto virus inhibits transcription complex assembly and influenza virus inhibits IRF-3 translocation into the nucleus (Talon et al., 2000; Jennings et al., 2005; La Rocca et al., 2005; Haller et al., 2006; Hilton et al., 2006).

The GenBank/EMBL/DDBJ accession number for the KPP/93 sequence is LC016722.

Three supplementary figures are available with the online Supplementary Material.
CSFV belongs to the genus Pestivirus of the family Flaviviridae together with Bovine viral diarrhea virus and Border disease virus. CSFV possesses a positive-sense ssRNA genome of ~12.3 kb with one large ORF flanked by 5’ and 3’ UTRs. It yields 12 cleavage products (Npro, C, E*, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) through co- and post-translational processing of the polyprotein by cellular and viral proteases (Lindenbach et al., 2007; Lamp et al., 2013). Npro is a protein unique to pestiviruses and is generated autocatalytically by cleaving its own C terminus through its protease activity. The amino acid residues H49 and C69 in the N-terminal domain of Npro form a catalytic diad responsible for the autoprotease activity (Gottipati et al., 2013; Zögg et al., 2013). Npro is not essential for viral replication (Tratschin et al., 1998), but is involved in pathogenicity by suppressing IFN-α/β induction through IRF-3 degradation in host cells (Mayer et al., 2004; Hilton et al., 2006; Bauhofer et al., 2007; Ruggli et al., 2009; Tamura et al., 2014). A TRASH zinc-binding domain located in the C-terminal half of Npro, and involving the amino acid residues at positions 112, 134, 136 and 138, is required for mediating IRF-3 degradation (Ruggli et al., 2009; Szymanski et al., 2009; Tamura et al., 2014).

By means of Npro, CSFV interferes with IFN-α/β induction, which can be measured with IFN-α/β indicators such as Newcastle disease virus (NDV). This is termed ‘exaltation of NDV’ (END) (Kumagai et al., 1958; Tamura et al., 2014). Four CSFV strains termed KPP/93, RBR/93, NKRS/98 and NKS/98 were isolated from diseased pigs in Thailand in 1993 and 1998. Surprisingly, these isolates were END+. Ruggli et al. (2009) demonstrated that the amino acid residues C112, C134, D136 and C138 of CSFV Npro form a TRASH zinc-binding domain and are essential for the suppression of IFN-α/β induction (Ruggli et al., 2009; Szymanski et al., 2009). In the present study, we identified amino acid residues responsible for the suppression of IFN-α/β induction and elucidated the molecular mechanisms underlying this activity by Npro of CSFVs isolated in Thailand.

RESULTS

Characterization of CSFVs isolated in Thailand

To assess the involvement of Npro of the Thai isolates in pathogenicity in pigs, three 4-week-old pigs were inoculated intramuscularly with 10⁷.⁰ TCID₅₀ of the KPP/93 strain and observed for 14 days. None of the inoculated pigs showed clinical symptoms. Although a small amount of virus was isolated from the tissue of one pig, no virus was isolated from other tissues and blood (Table 1). These data indicate that the pathogenicity of the KPP/93 strain in pigs is very low. Following this, we analysed the characteristics of the four Thai isolates in porcine cells. To clarify whether these isolates prevented IFN-α/β induction in vitro, porcine SK-L cells were infected at m.o.i. 1.0 with the vGPE⁻ and vGPE⁻/N136D viruses as control, and with the four Thai isolates KPP/93, RBR/93, NKRS/98 or NKS/98. As expected, the vGPE⁻ virus did not suppress IFN-α/β induction, as opposed to the vGPE⁻/N136D virus, in which the zinc-binding domain important for IRF-3 degradation was restored with an aspartic acid at position 136, which mediated IRF-3 degradation in accordance with the findings of previous reports (Ruggli et al., 2009; Tamura et al., 2014). All four Thai isolates induced IFN-α/β in SK-L cells, as observed with vGPE⁻ (Fig. 1a). In addition, they did not induce IRF-3 degradation as observed with vGPE⁻, whilst IRF-3 was not detected in SK-L cells inoculated with vGPE⁻/N136D (Fig. 1b). These data showed that the four Thai isolates KPP/93, RBR/93, NKRS/98 and NKS/98 did not suppress IFN-α/β induction.

Comparison of the amino acid sequences of Npro of END⁺ and END⁻ CSFV strains

Previous studies demonstrated that the amino acid residues C112, C134, D136 and C138 of CSFV Npro form a TRASH domain and are essential for the suppression of IFN-α/β induction (Ruggli et al., 2009; Szymanski et al., 2009). Four CSFV isolates in Thailand were classified into genotype 1.1 based on the E2 gene sequence (Fig. S1, available in the online Supplementary Material). These isolates shared 99 and 100% nucleotide sequence identity in E2 and Npro, respectively (data not shown). The sequence of 11677 nt of the genome between the 5’ terminal domain and NS5B of the KPP/93 strain was deposited in GenBank under accession number LC016722. The amino acid sequences of Npro of two out of the four strains isolated in Thailand, of three laboratory END⁻ strains (ALD-END⁺, Ames-END⁻ and GPE⁻) and of seven END⁺ strains (Alfort/187, Alfort/Tübingen, ALD, Brescia, C-strain, CAP and Eystrup) were

Table 1. Virus recovery and neutralization titres from pigs inoculated with the KPP/93 strain

<table>
<thead>
<tr>
<th>Pig no.</th>
<th>Blood (log TCID₅₀ ml⁻¹) on day p.i.</th>
<th>Tissue (log TCID₅₀ g⁻¹) on day 14 p.i.</th>
<th>Neutralization titre on day 14 p.i.</th>
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*−, Not isolated.
Identification of amino acid residues critical for the suppression of IFN-α/β induction

To identify amino acid residues of Npro involved in the suppression of IFN-α/β induction, vA187-Npro(KPP)-derived mutant viruses with substitutions in Npro were constructed as described in Fig. 3. The backbone virus vA187-Npro(KPP) was a chimeric virus obtained by replacing the Npro gene of vA187-1 (Ruggli et al., 1996) with the Npro gene of the KPP/93 strain. The original vA187-1 virus downregulates IFN-α/β production and is an established END+ virus in vitro (Ruggli et al., 2009). The nucleotide sequence identity of Npro of the four CSFV isolates in Thailand was 100%, as described above; therefore, Npro of the KPP/93 strain was considered as a prototype for the four Thai isolates. IFN-α/β bioactivity was measured in the supernatant of cells inoculated with the different mutant viruses (Fig. 3). The vA187-Npro(KPP)/D113E; S151T virus in which the vA187-1 sequence was restored in the C-terminal part of KPP/93 Npro did not suppress IFN-α/β production. Therefore, the five N-terminal amino acid residues at positions 5, 8, 17, 40 and 61 were suspected to be involved in this function. The histidine at position 40 of vA187-1 was close to the TRASH domain according to the three-dimensional structure of Npro (Fig. S2). The histidine at position 5 and the phenylalanine at position 8 were not plotted because of the lack of the N-terminal 16 amino acids in the three-dimensional structure of Npro. Interestingly, the L40H substitution in the KPP/93 Npro backbone sequence was sufficient to confer the END+ phenotype as demonstrated with complete suppression of IFN-α/β production in SK-L cells infected with vA187-Npro(KPP)/L40H. Interestingly, vA187-Npro(KPP)/Y5H; F8L; S17P; N61K also suppressed IFN-α/β production, suggesting that the remaining four amino acid residues were involved in this function too, independently of the residue at position 40. Through systematic analyses of mutant viruses carrying substitutions of either of these four residues alone or combination, we found that the two S17P and N61K substitutions together in the KPP/93 Npro backbone sequence were sufficient to restore functional Npro as measured by suppression of IFN-α/β production in SK-L cells inoculated with vA187-Npro(KPP)/S17P; N61K (Fig. 3). In addition, IFR-3 protein was down-regulated in SK-L cells inoculated with vA187-Npro(KPP)/L40H and vA187-Npro(KPP)/S17P; N61K (Fig. 4). These data indicated that H40 or both P17 and K61 were critical for the suppression of IFN-α/β induction by vA187-Npro(KPP).

Time-course of IRF-3 expression in cells infected with parent and mutant CSFVs

To elaborate on the contribution of residues 17, 40 and 61 to the degradation of IRF-3 by Npro, SK-L cells were infected with the END- strain vA187-Npro(KPP) carrying Npro of the END- KPP/93 in the vA187-1 backbone, and with the END+ strains vA187-1, vA187-Npro(KPP)/L40H carrying the mutation at amino acid position 40 or vA187-Npro(KPP)/S17P; N61K carrying the mutations of amino acid residues 17 and 61. Cells were lysed at 0, 12, 24, 36, 48, 60, 72, 84, 96, 108 and 120 h post-infection (p.i.) and the extracts were analysed for IRF-3 expression. IRF-3 remained unchanged for the 5 days of the experiment in cells infected with vA187-Npro(KPP), whilst it was clearly detectable for 24 h p.i. and then rapidly decreased to 3% of the initial IRF-3 level by 36 h p.i. in cells infected with vA187-1 (Fig. 4). In cells infected with vA187-Npro(KPP)/L40H, IRF-3 was detected at 0, 12, 24...
**Fig. 2.** Amino acid sequence alignment of N\(^{pro}\) of selected END\(^{+}\) and END\(^{-}\) CSFV strains. The N\(^{pro}\) amino acid sequences of selected END\(^{+}\) CSFV strains that suppressed IFN-\(\alpha/\beta\) induction (Alfort/187, Alfort/Tübingen, ALD, Brescia, C-strain, CAP and Eystrup) and of selected END\(^{-}\) strains that suppressed IFN-\(\alpha/\beta\) induction (KPP/93, NKS/98, ALD-END\(^{-}\), Ames-END\(^{-}\) and GPE\(^{-}\)) are aligned. The GenBank accession numbers for Alfort/187, Alfort/Tübingen, Eystrup, Brescia, C-strain, CAP, ALD and GPE\(^{-}\) are X87939, J04358, AF326963, AF091661, Z46258, X96550, D49532 and D49533, respectively. The N\(^{pro}\) sequences of the ALD-END\(^{-}\) and Ames-END\(^{-}\) strains were published previously (Ruggli et al., 2009). The amino acid numbering corresponds to the Alfort/187 sequence. The grey boxes highlight the amino acids unique to KPP/93 and NKS/98. The dotted boxes indicate the amino acids at positions 112, 134, 136 and 138, located in the TRASH domain.
and 36 h p.i., and then gradually decreased to <9% of the original IRF-3 level by 48 h p.i. A similar decrease of IRF-3 expression was observed in cells infected with the KPP/93 strain carrying the double mutations at positions 17 and 61 [vA187-Npro(KPP)/S17P; N61K] from 12 to 36 h p.i., with 27% of the original IRF-3 level at 36 h p.i. IRF-3 degradation in cells infected with these latter viruses was dependent on proteasomal activity as shown with the proteasome inhibitor MG-132 (Fig. S3). These data suggested that histidine at position 40 or both proline at position 17 and lysine at position 61 were critical for Npro to mediate proteasomal degradation of IRF-3 in infected cells.

Stability of Npro of the KPP/93 strain and mutant viruses in cell culture

In a previous study, Npro of the END+ strain Alfort/187 carrying a single mutation at amino acid positions 112 or

Fig. 3. Production of IFN-α/β in supernatants of SK-L cells inoculated with vA187-1, vA187-Npro(KPP) or mutant viruses. The swine SK-L cells were inoculated at m.o.i. 1.0 with vA187-1, vA187-Npro(KPP) or 12 mutant viruses. The vA187-Npro(KPP) virus was generated by replacing the Npro gene in the END+ vA187-1 backbone with the Npro gene of the END- KPP/93 strain. Twelve mutant viruses were constructed by selected amino acid mutagenesis in Npro of the vA187-Npro(KPP) virus. SK-L cells infected with the different viruses were incubated at 37°C in the presence of 5% CO2 for 5 days. IFN-α/β bioactivity in the cell supernatants was measured in duplicate using the SK6-MxLuc reporter gene assay. The data represent the mean ± SEM from three independent experiments. The significance of the differences was calculated using Student’s t-test; *P<0.05.
136, which are located in the TRASH domain, was less stable than WT Npro in vitro (Seago et al., 2010). To determine whether the amino acid residues at positions 17, 40 and 61 were responsible for the stability of Npro of the vA187-1 strain, KPP/93 strain and different amino acid mutants of Npro of the KPP/93 virus were expressed in HEK 293T cells and analysed for stability over time after treatment with the translation inhibitor cycloheximide (CHX) (Fig. 5). vA187-1-Npro carrying a single mutation at position 136 [Npro(A187-1)/D136N] was mostly degraded after 12 h in comparison with vA187-1-Npro. This was in accordance with the findings of a previous report by Seago et al. (2010).

KPP/93 Npro became undetectable within the first 4 h after CHX treatment. The KPP/93 Npro carrying the residues of the vA187-1 virus at positions 17 and 61 [Npro(KPP)/S17P; N61K] was detected at 4 h after CHX treatment, but became undetectable at 8 h. KPP/93 Npro carrying the histidine of vA187-1 at position 40 [Npro(KPP)/L40H] or the three residues of vA187-1 at positions 17, 40 and 61 [Npro(KPP)/S17P; L40H; N61K] was detected for 12 h after CHX treatment, similarly to vA187-1-Npro. These data demonstrated that the amino acids of END+ vA187-1 at positions 40 or 17 and 61 enhanced the stability of KPP/93 Npro.

**Interaction of IRF-3 with Npro of the KPP/93 virus and mutants thereof**

A previous study demonstrated that IRF-3 was not degraded in porcine cells inoculated with the vA187-1 D136N virus which carried a mutation at position 136 of Npro in the TRASH domain, abolishing zinc binding. In addition, the results of a mammalian two-hybrid assay showed that vA187-D136N Npro did not interact with IRF-3, whilst vA187-1-Npro did (Ruggli et al., 2009). According to the results of the present study, histidine at position 40 or both proline at position 17 and lysine at position 61 are required by Npro for the suppression of IFN-α/β induction. Therefore, the importance of these amino acid residues of Npro for the interaction with IRF-3 was explored using the KPP/93 Npro backbone and a mammalian two-hybrid assay. Co-expression of the VP16 transactivator fused to IRF-3 and of the GAL4 DNA-binding domain fused to vA187-1-Npro resulted in luciferase expression from the reporter plasmid due to the interaction of IRF-3 and Npro (Fig. 6). As expected, vA187-1-Npro carrying the D136N mutation [Npro(A187-1)/D136N] did not interact with IRF-3, in accordance with previous findings (Ruggli et al., 2009). KPP/93 Npro did not interact with IRF-3.
either, similarly to vA187-1-Npro. The mutant KPP/93 Npro carrying a histidine at position 40 [Npro(KPP)/L40H] resulted in significantly higher luciferase activity than KPP/93 Npro; however, the activity was not as high as with vA187-1-Npro. The mutant KPP/93 Npro carrying the two residues of vA187-1 at positions 17 and 61 [Npro(KPP)/S17P; N61K] showed low luciferase activity, which was comparable with that of KPP/93 Npro. Finally, the triple-mutant KPP/93 Npro carrying the residues of vA187-1 at positions 17, 40 and 61 [Npro(KPP)/S17P; L40H; N61K] showed significantly higher luciferase activity than KPP/93 Npro, comparable with the luciferase activity obtained with vA187-1-Npro. Taken together, these data indicated that, in addition to an intact TRASH domain, the amino acid residue at position 40 of Npro was critical for the interaction of Npro with IRF-3, and that the amino acid residues at positions 17 and 61 acted in synergy with the residue at position 40 to mediate the interaction of Npro with IRF-3.

**DISCUSSION**

From 1993 to 1998, four END− CSFs (KPP/93, RBR/93, NKRS/98 and NKS/98 strains) were isolated from diseased pigs in Thailand, whilst other CSFV strains previously isolated in nature were all END+ strains. The KPP/93 strain showed low pathogenicity in pigs. In previous studies, the suppression of IFN-α/β induction by Npro was related to pathogenicity in pigs (Mayer et al., 2004; Ruggli et al., 2009; Tamura et al., 2014), suggesting that inability of KPP/93 Npro to suppress IFN-α/β induction may contribute to the low pathogenicity of the KPP/93 strain in pigs. In the present study, we identified amino acid residues of Npro responsible for the suppression of IFN-α/β induction. We found that either the amino acid residue at position 40 or the combination of the amino acid residues at positions 17 and 61 of Npro were responsible for the suppression of IFN-α/β induction. These three amino acid residues are located outside of the TRASH domain considering the crystal structure of Npro revealed by Gottipati et al. (2013), suggesting that in addition to the C-terminal half of Npro, the N-terminal half of Npro is also important for the suppression of IFN-α/β induction.

We then explored the molecular mechanisms underlying the suppression of IFN-α/β induction mediated by these amino acid residues of Npro. To this end, we analysed the kinetics of IRF-3 expression in porcine SK-L cells infected with chimeric viruses carrying Npro of the KPP/93 strain and mutants thereof in the vA187-1 backbone. No differences in the growth kinetics of CSFV vA187-1, vA187-Npro(KPP), vA187-Npro(KPP)/L40H and vA187-Npro(KPP)/S17P; N61K were observed during a period of...
120 h p.i. (data not shown). Nevertheless, IRF-3 was clearly downregulated in cells infected with vA187-1, vA187-Npro(KPP)/L40H and vA187-Npro(KPP)/S17P; N61K compared with cells infected with vA187-Npro(KPP). To clarify the reasons for these differences, the stability of Npro was examined. This was motivated by a recent study showing that Npro of the END+ strain Alfort/187 carrying a single mutation at amino acid positions 112 or 136, located in the TRASH domain, was less stable than WT Npro (Seago et al., 2010). Thus, we assessed whether the amino acid residues at positions 17, 40 and 61 also influenced the stability of Npro. Npro(KPP)/L40H and Npro(KPP)/S17P; N61K showed higher stability than the parental KPP/93 Npro, indicating that the amino acid residues at positions 17, 40 and 61 are involved in stabilizing Npro. vA187-1-Npro with the D136N mutation [Npro(A187-1)/D136N] showed reduced stability; however, the stability was higher than that of Npro(KPP)/S17P; N61K conferring an END- phenotype, whilst the vA187-1/D136N virus was END-. These results suggest that whilst Npro(KPP)/S17P; N61K can still mediate IRF-3 degradation despite slightly reduced stability, Npro(A187-1)/D136N has lost the capacity to mediate IRF-3 degradation due to the mutation destroying the TRASH domain. In a previous study, Npro of the END+ vA187-1 strain interacted with IRF-3 in cell culture as determined by a mammalian two-hybrid assay, and infection with this virus promoted IRF-3 degradation. However, Npro of the END- vA187-1/D136N virus did not interact with IRF-3 and this virus did not promote IRF-3 degradation (Ruggli et al., 2009). In the present study, a single amino acid mutation at position 40 of Npro(KPP) was sufficient to restore interaction of Npro with IRF-3, and two additional amino acid substitutions at positions 17 and 61 of Npro(KPP)/L40H further enhanced the interaction of Npro(KPP)/L40H with IRF-3. There was no interaction observed between Npro(KPP)/S17P; N61K and IRF-3 using the mammalian two-hybrid assay despite the END+ phenotype conferred by this mutant Npro, suggesting that further experiments are required to assess the interaction of Npro with IRF-3 in more detail; in addition, the interaction of Npro with other host factors also needs to be explored (Jefferson et al., 2014). Our data suggest that the differences in Npro-mediated IRF-3 degradation in SK-L cells can be attributed to the degree of Npro stability and to the strength of the Npro interaction with IRF-3.

Our results revealed that the stability of Npro may influence the interaction of Npro with IRF-3 and the subsequent downregulation of IFN-α/β production. The amino acid residue at position 40 in the N-terminal half of Npro clearly contributes to the stability of Npro. A previous study of Npro of bovine viral diarrhea virus demonstrated that this histidine at position 40 forms an ion-binding site for protein interactions together with the amino acid residues at positions 117 and 127 (Zogg et al., 2013). Therefore, the formation of this ion-binding site may act to stabilize Npro. As described in Fig. 7, stable Npro(KPP)/L40H results in a large amount of functional Npro in cells, leading to efficient degradation of IRF-3 by the proteasome. In contrast, unstable Npro(KPP) results in insufficient functional Npro for IRF-3 degradation and for inhibition of IFN-α/β induction. Restoring the vA187-1 residues at positions 17 and 61 of Npro(KPP) only slightly enhanced the stability of KPP/93 Npro, resulting in a small amount of functional Npro in cells. This small amount of Npro was nevertheless sufficient to mediate degradation of IRF-3 and subsequent downregulation of IFN-α/β production. Npro(A187-1)/D136N showed higher stability than Npro(KPP)/S17P; N61K, although it was described to be defective in mediating IRF-3 degradation (Ruggli et al., 2009). This suggests that the lack of IRF-3 degradation by this TRASH domain mutant is indeed due to the lack of interaction with IRF-3 as described by Ruggli et al. (2009) rather than to Npro instability, although a contribution by the latter cannot be excluded.

In conclusion, the present study reveals that the amino acid residues at positions 17, 40, and 61 in the N-terminal half of Npro contribute to the stability of Npro and to the interaction of Npro with IRF-3, leading to degradation of IRF-3 and subsequent downregulation of IFN-α/β production. Thus, the data show that the N-terminal half and the C-terminal TRASH domain of Npro are both involved with specific characteristics in the counteraction of type I IFN induction through mediating IRF-3 degradation.

**METHODS**

**Cells.** The porcine kidney cell line SK-L (Sakoda & Fukusho, 1998) was propagated in Eagle’s minimum essential medium (MEM) (Nissui) supplemented with 0.295% tryptose phosphate broth (TPB) (Becton Dickinson), 10 mM N,N-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid (Sigma-Aldrich) and 10% horse serum (Invitrogen). The SK6-MxLuc cell line carrying a Mx/Luc reporter gene (Ocaña-Macchi et al., 2009) was propagated in MEM supplemented with 0.295% TPB and 7% horse serum. The human embryonic kidney cell line HEK 293T was maintained in Dulbecco’s MEM (Life Technologies) and 10% FCS (Cambrex). All cells were incubated at 37 °C in the presence of 5% CO₂.

**Viruses.** The CSFV KPP/93, RBR/93, NKRS/98 and NKS/98 strains were isolated from Thai pigs in Kamphaeng Phet province in 1993, in Ratchaburi province in 1993, in Nakhon Ratchasima province in 1998 and in Nakhon Sawan province in 1998, respectively. The KPP/93 strain was isolated from diseased pigs showing clinical symptoms of classical swine fever, i.e. conjunctivitis, clustering, staggering, joint swelling and haemorrhagic skin lesions with a mortality of only 10%. After isola}
described previously (Tamura et al., 2012). All cDNA-derived viruses were rescued as described previously (Moser et al., 1999; Tamura et al., 2012). In brief, plasmid constructs were linearized at the SrfI site located at the end of the viral genomic cDNA sequence and RNA was obtained by run-off transcription using a MEGAscript T7 kit (Ambion). After DNase I treatment and purification on S-400 HR Sephadex columns (GE Healthcare), RNA was quantified using a spectrophotometer (Amersham Bioscience) and used to electroporate SK-L cells. The whole genomes of rescued viruses were verified by nucleotide sequencing to exclude any accidental mutation. Rescued viruses were stored at −20°C.

**Sequencing.** Full-length cDNA clones and in vitro rescued viruses were completely sequenced as described previously (Tamura et al., 2012). In brief, nucleotide sequencing of cDNA clones and PCR fragments of viral RNA was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) and a 3500 Genetic Analyzer (Life Technologies). Sequencing data were analysed using Network version 12 (GENETYX).

**Virus titration.** Virus titres were determined by end-point dilution on SK-L cells and immunoperoxidase staining using the anti-NS3 mAb 46/1, as described previously (Sakoda & Fukusho, 1998; Kameyama et al., 2006). The titres were calculated using the formula of Reed & Muench (1938) and expressed as TCID$_{50}$ ml$^{-1}$.

**SDS-PAGE and Western blotting.** SDS-PAGE and Western blotting were performed as described previously (Tamura et al., 2014). The concentration of SDS-polyacrylamide gels was 15%. As primary antibodies, anti-porcine IRF-3 mAb 34/1 (Bauhofer et al., 2007), anti-FLAG M2 mAb (Sigma-Aldrich) and anti-β actin antibody (Cosmo Bio) were used. Immobilon Western Detection Reagents (Millipore) and a Lumivision PRO 400EX system (Aisin Seiki) were used for signal detection.

**IFN bioassay.** The bioactivity of porcine IFN-α/β was assessed as described previously (Tamura et al., 2014). In brief, supernatants of cells inoculated with viruses were inactivated using a UV cross-linker (ATTO) and added to SK6-MxLuc cells. Recombinant porcine IFN-α/β produced in HEK 293T cells was used as a standard. The cell

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**Fig. 7.** Model of the molecular mechanisms of suppression of IFN-α/β induction by N$_{\text{pro}}$. In cells infected with vA187-N$_{\text{pro}}$(KPP), N$_{\text{pro}}$ is rapidly degraded and dsRNA triggers the activation of the IRF-3 phosphorylation pathway according to mechanisms described by Honda et al. (2006), leading to IFN-β production. In cells infected with vA187-1 or vA187-N$_{\text{pro}}$(KPP)/L40H, N$_{\text{pro}}$ is stable, which results in a large amount of functional N$_{\text{pro}}$ leading to efficient IRF-3 degradation by the proteasome to suppress IFN-β production. In cells infected with vA187-N$_{\text{pro}}$(KPP)/S17P; N61K, despite limited stability of N$_{\text{pro}}$, there is sufficient functional N$_{\text{pro}}$ to degrade IRF-3, resulting in suppression of IFN-β production.
extracts were prepared with 100 μl passive lysis buffer, and firefly luciferase activities were measured using a Luciferase Assay System (Promega) and a PowerScan4 microplate reader (DS Pharma Biomedical). The activities were analysed using Gen5 software (DS Pharma Biomedical). Results were recorded for three independent experiments and each experiment was performed in duplicate.

Experimental infection of pigs. To assess the pathogenicity of the KPP/93 strain, three 4-week-old crossbred Landrace × Duroc × Yorkshire specific-pathogen-free pigs (Yamanaka Chikusan) were intramuscularly injected with 10⁷ TCID₅₀ KPP/93 strain and observed for 14 days. From the three pigs kept for 14 days, blood was collected in tubes containing EDTA (Terumo) on days 0, 3, 5, 7, 9, 11 and 14 p.i. for virus titration. The pigs were euthanized with pentobarbital on day 14 p.i., and tissues from tonsils, kidneys and mesenteric lymph nodes were collected aseptically. The collected samples were homogenized in XM to obtain a 10% suspension for virus titration. Virus titres were expressed as TCID₅₀ ml⁻¹ (blood) or g⁻¹ (tissue). Neutralization titres against the KPP/93 strain of sera collected on day 14 p.i. were measured. This animal experiment was conducted in the Biosafety Level 3 facility of the Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan accredited by AAALAC International. The institutional animal care and use committee of the Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan authorized the experiments and each experiment was performed in duplicate.

Time-course analysis. The SK-L cells seeded in six-well plates were inoculated with viruses at m.o.i. 5.0 and incubated at 37°C in the presence of 5% CO₂. At 0, 12, 24, 36, 48, 60, 72, 84, 96, 108 and 120 h p.i., the supernatants were collected for virus titration and IFN-α/β quantification. The cell lysates were prepared for the detection of IRF-3 protein. The intensity of the specific band of IRF-3 was quantified using ImageJ image analysis software (Schneider et al., 2012).

Stability test of Npro in HEK 293T cells. For the measurement of the stability of Npro, HEK 293T cells were seeded in 24-well plates at a density of 10⁴ cells per well. After 24 h, the cells were transfected with 1 μg pcI-M-FLAG-Npro-derived plasmids. FLAG-Npro was expressed at 37°C for 24 h. After incubation, the expression was stopped by adding 200 ng CHX protein synthesis inhibitor (Sigma-Aldrich). Cell lysates were prepared with the passive lysis buffer at 0, 4, 8 and 12 h after CHX treatment. FLAG-Npro was detected by Western blotting.

Mammalian two-hybrid assay. The mammalian two-hybrid assays were performed as described previously (Ruggli et al., 2009). In brief, HEK 293T cells were transfected with pFN10A(ACT)-IRF3 expressing porcine IRF-3 fused to the VP16 transactivator and with pFN11A (BIND)-derived plasmids expressing a fusion of the GAL4 DNA-binding domain and Npro protein of CSFVs. The empty vectors pACT and pBIND and the corresponding plasmids expressing MyoD and Id (Promega) served as controls. Cells were incubated for 24 h at 37°C in the presence of 5% CO₂ prior to extraction and luciferase activity was measured as described above. Results were recorded for three independent experiments and each experiment was performed in duplicate.

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