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Short Communication

The nsp1α and nsp1β papain-like autoproteinases are essential for porcine reproductive and respiratory syndrome virus RNA synthesis

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The two N-terminal cleavage products, nsp1α and nsp1β, of the replicase polyproteins of porcine reproductive and respiratory syndrome virus (PRRSV) each contain a papain-like autoproteinase domain, which have been named PCPα and PCPβ, respectively. To assess their role in the PRRSV life cycle, substitutions and deletions of the presumed catalytic cysteine and histidine residues of PCPα and PCPβ were introduced into a PRRSV infectious cDNA clone. Mutations that inactivated PCPα activity completely blocked subgenomic mRNA synthesis, but did not affect genome replication. In contrast, mutants in which PCPβ activity was blocked proved to be non-viable and no sign of viral RNA synthesis could be detected, indicating that the correct processing of the nsp1β/nsp2 cleavage site is essential for PRRSV genome replication. In conclusion, the data presented here show that a productive PRRSV life cycle depends on the correct processing of both the nsp1α/nsp1β and nsp1β/nsp2 junctions.

RNA synthesis of the positive-stranded RNA viruses that belong to the order *Nidovirales* involves the replication of genome-length RNA (RNA1) and the synthesis of subgenomic mRNAs (sg mRNAs). Both processes are mediated by a 'replication/transcription complex' (RTC) composed of virus-encoded nonstructural proteins (nsps) and presumably also host factors. The order *Nidovirales* consists of the families *Arteriviridae*, *Coronaviridae* and *Roniviridae*. The family *Arteriviridae* comprises the species *Porcine reproductive and respiratory syndrome virus* (PRRSV), *Equine arteritis virus* (EAV), (murine) *Lactate dehydrogenase-elevating virus* (LDV) and *Simian hemorrhagic fever virus* (SHFV) (reviewed by Gorbalenya *et al.*, 2006).

Following virus entry and release of the genome into the cytoplasm, the nidovirus life cycle starts with the expression of the large replicase gene that consists of open reading frame (ORF) 1a and ORF1b. Genome translation yields two multidomain replicase polyproteins, named pp1a and pp1ab, with the latter being a C-terminally extended version of the former due to a ribosomal frameshift mechanism (Fig. 1). Polyproteins pp1a and pp1ab are co- and post-translationally processed by the viral 'main' proteinase and one to three 'accessory' autoproteinases (reviewed by Ziebuhr *et al.*, 2000). The resulting mature nsps direct viral RNA synthesis, presumably after forming a RTC that is associated with endoplasmic reticulum-derived paired membranes and double membrane vesicles (Gosert *et al.*, 2002; Pedersen *et al.*, 1999). The sg mRNAs of arteri- and coronaviruses consist of a 5′-terminal common 'leader' sequence, derived from the 5′ nontranslated region (NTR) of RNA1, that is fused to different 3′-proximal regions of the genome, the 'mRNA bodies'. The 5′-proximal cistron of each sg mRNA is translated to produce the viral structural proteins. The subgenome-length templates for sg mRNA synthesis are thought to be generated by discontinuous minus-strand RNA synthesis during which sequences that are non-contiguous in the genome are joined (reviewed by Pasternak *et al.*, 2006). This leader-to-body fusion event is mediated by transcription-regulating sequences that are

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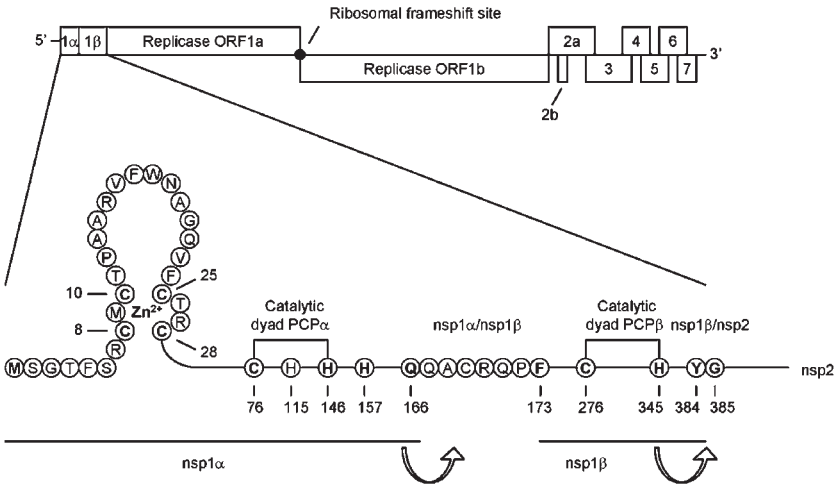

Fig. 1. Overview of the PRRSV genome organization and nsp1 domains and residues relevant to this study (den Boon *et al.*, 1995; Tijms *et al.*, 2001). PCPα and PCPβ are responsible for the autoproteolytic release of nsp1α and nsp1β, respectively. Predicted catalytic dyads of both PCP domains, the estimated position of the nsp1α/nsp1β cleavage site and predicted nsp1β/nsp2 junction (den Boon *et al.*, 1995), and the four cysteine residues forming the predicted nsp1 zinc finger domain (Tijms *et al.*, 2001) are indicated. Residues conserved in the replicase sequences of 23 full-length PRRSV isolates are shown in bold. Numbers correspond to the amino acid positions in the pp1ab polyprotein of PRRSV (GenBank accession no. M96262).

located at the 3′-end of the leader and upstream of all genes encoding the structural proteins. Additional factors such as viral nsps, presumably host proteins, and higher order RNA structures that direct leader-to-body fusion are thought to be involved (van den Born *et al.*, 2004).

The replicase polyproteins of the porcine arterivirus PRRSV are predicted to be cleaved into 14 nsps by the nsp4 main proteinase and three accessory proteinases residing in nsp1α, nsp1β and nsp2 (van Aken *et al.*, 2006; Ziebuhr *et al.*, 2000). Papain-like proteinase α (PCPα) directs the release of nsp1α, whereas the liberation of nsp1β depends on the activities of both PCPα and a second proteinase, PCPβ (Fig. 1). A third cysteine proteinase, residing in the N-terminal domain of nsp2, is responsible for the cleavage of the nsp2/nsp3 site (den Boon *et al.*, 1995; Snijder *et al.*, 1995). Viral papain-like proteinases are characterized by a catalytic dyad that consists of a nucleophilic cysteine residue and a downstream histidine. Based on comparative sequence analysis and *in vitro* activity assays, Cys-276 and His-345 were proposed to form the catalytic dyad of PRRSV PCPβ. Cys-76 and His-146 form the putative catalytic dyad of PRRSV PCPα. Mutagenesis of two other PCPα His residues (His-115 and His-157) partially inhibited proteolytic activity *in vitro*. EAV PCPα has lost its proteolytic activity since the equivalent of PRRSV Cys-76 is no longer present (den Boon *et al.*, 1995). Comparative sequence analysis among arteriviruses identified a putative zinc finger domain near the N terminus of EAV nsp1 (Tijms *et al.*, 2001, 2007) and a C-terminal PCP domain (PCPβ) (den Boon *et al.*, 1995; Snijder *et al.*, 1994).

To investigate the importance of the PCPα and PCPβ autoproteinase domains in the PRRSV life cycle, their putative active-site residues were probed by site-directed mutagenesis in the context of a PRRSV reverse genetics system (Meulenberg *et al.*, 1998). The predicted catalytic residues of both PCP domains were substituted by residues that completely inactivated proteolytic activity *in vitro*. Also PCPα residues His-115 and His-157 were probed by substituting them with residues that still allowed partial PCPα activity *in vitro* (den Boon *et al.*, 1995). These mutations (Table 1) were cloned into pABV437, an infectious cDNA clone of a European PRRSV strain (Meulenberg *et al.*, 1998). *In vitro*-transcribed full-length PRRSV RNA was electroporated into baby hamster kidney-21 (BHK-21) cells, which support replication and yield progeny virus, but are not permissive to infection with PRRSV. Consequently, virus harvested from transfected BHK-21 cells needs to be amplified in permissive cells, like porcine alveolar macrophages (PAMs).

For all PCPα mutants (Table 1; Fig. 2a), immunostaining of transfected cells revealed abundant expression of replicase subunit nsp3, which was used as a marker for genome replication. At 24 h post-transfection (h p.t.), the number of positive BHK-21 cells and their staining pattern were comparable to that of the wild-type control, indicating that RNA1 replication was not dramatically affected by the PCPα mutations. As a negative control, we used a control cDNA clone that lacked an essential signal for RNA synthesis in its 3′-proximal domain (data not shown; the construct lacks nt 11786–14581). The fact that no positive cells were observed following transfection of this replication-incompetent RNA indicated that translation of the input RNA in itself did not yield detectable levels of nsp3 expression, since the replicase gene of the deletion mutant was intact and available for translation. Thus, the detection of nsp3 by immunostaining could indeed be used as a read-out for successful RNA1 replication. In addition, intracellular RNA isolated from BHK-21 cells that had been transfected with a selection of mutants was used for RT-PCR analysis. Antisense oligonucleotide LV388 (nt 15 067–15 098; 5′-AATTTCGGTCACATGGTTCCTGCCTGATT-AAG-3′) or LV385 (nt 224–250; 5′-CACATGCACCGG-GAGAACGTCCCAGAC-3′) was used in the reverse

Table 1. Summary of transfection and infection experiments with PRRSV nsp1 mutants

Nsp1 mutant (wt and mutant codons)*	p0-BHK-21 cells†		p1-PAMs†		p5-PAMs†		
	Immunostaining‡		Immunostaining‡	Codon at site of mutation*	Immunostaining‡	Titre§	Codon at site of mutation*
	α-nsp3	α-N	α-N		α-N		
Positive control‖	+++	+++	+++	ND	+++	8.7	ND
Negative control‖	−	−	−	ND	ND	ND	ND
nsp1α mutants							
Cys-76 (UGU)→Ser (AGC)	+++	−/+	−/+	Cys-76 (UGC)	ND	ND	ND
His-115 (CAC)→Tyr (UAC)	+++	+	−/+	ND	+++	7.6	Asn-115 (AAC)
ΔHis-115 (ΔCAC)	+++	−	−	ND	ND	ND	ND
His-146 (CAC)→Tyr (UAC)	+++	−/+	−/+	His-146 (CAC)	ND	ND	ND
His-157 (CAU)→Asp (GAC)	+++	−/+	−/+	ND	+++	8.4	Tyr-157 (UAC)
His-157 (CAU)→Asn (AAU)	+++	+	+	ND	+++	8.1	His-157 (CAU)
His-157 (CAU)→Ile (AUU)	+++	−/+	−/+	ND	+++	8.5	Phe-157 (UUU)
ΔHis-157 (ΔCAU)	+++	−	−	ND	ND	ND	ND
nsp1β mutants							
Cys-276 (UGC)→Leu (CUU)	−	−	−	ND	ND	ND	ND
Cys-276 (UGC)→Ser (UCC)	−	−	−	ND	ND	ND	ND
His-345 (CAC)→Tyr (UAC)	−	−	−	ND	ND	ND	ND

*Numbers correspond to the amino acid residues in replicase pp1ab of strain Lelystad virus of PRRSV (GenBank accession no. M96262).

†BHK-21 cells were transfected with *in vitro* transcribed RNA and supernatants were harvested at 24 h p.t. (p0-BHK-21 cells). PAMs were inoculated with p0-supernatants once (p1-PAMs) and/or used to serially passage virus populations in PAMs to a maximum of five times (p5-PAMs).

‡Immunostainings were performed with an anti-nsp3 serum (α-nsp3) and an anti-nucleocapsid mAb (α-N). Explanations of symbols for p0-BHK-21 cells: +++, positive staining similar to the wild-type control (3–5 % N-positive BHK-21 cells); +, less than 0.005 % N-positive cells. Explanations of symbols for p1/p5-PAMs, +++, positive staining similar to the wild-type control (60–80 % N-positive PAMs); +, positive staining less than 10 % of the wild-type; −/+, depending on the emergence of transcription-competent revertants (see text), a negative or positive staining was observed; −, negative staining; ND, not determined.

§Titres were determined by end-point dilution on PAMs 5 days post-inoculation and expressed as log 50 % tissue culture infective dose per ml (TCID50 ml^{-1}).

‖Transcripts from the wild-type cDNA clone served as the positive control, whereas a full-length cDNA clone lacking an essential RNA replication element was used as a negative control (see text).

transcription reaction, followed by a PCR in which the leader-specific (sense) oligonucleotide LV383 (nt 1–29; 5′-ATGATGTGTAGGGTATTCCCCCTACATAC-3′) was added to produce either a 252 bp fragment from RNA1 or a 733 bp fragment from sg mRNA7 (Fig. 2b). For all mutants, RNA1 amounts were detected similar to those of the wild-type control (Fig. 2c), confirming that replication was indeed not affected.

To examine the sg mRNAs synthesis of the PCPα mutants, the synthesis of the nucleocapsid protein (N), which is expressed from the smallest and most abundant sg mRNA (sg mRNA7), was analysed by immunostaining at 24 h p.t. (Table 1; Fig. 2a). Only a small number of BHK-21 cells transfected with any of the PCPα mutants were positive for N, and in these cells the intensity of the N-staining was comparable to that in cells transfected with the wild-type control. When comparing the signal for nsp3 and N in double staining experiments, only a few of the nsp3-positive cells were also positive for N. Mutants His-115→Tyr and His-157→Asn produced N-positive cells in all experiments, whereas transfections with the Cys-76→Ser, His-146→Tyr, His-157→Asp and His-157→Ile mutants resulted in the occasional appearance of a few double-positive cells, which was likely due to reversion (see below). Intracellular RNA from cells transfected with selected mutants was analysed for the synthesis of sg mRNA7 by means of RT-PCR (Fig. 2b), which confirmed that sg mRNA synthesis was severely affected in these mutants, since no signal for mRNA7 could be detected (Fig. 2c), not even when an increased number of cycles was used for the PCR. The same was true for samples that were harvested after an additional 24 h incubation (data not shown).

To test for the production of progeny virus, the supernatants of transfected BHK-21 cells were transferred to PAMs (p1). Some N-positive cells were present at 24 h post-inoculation in PAM cultures infected with the harvest from cells transfected with mutants Cys-76→Ser and His-146→Tyr (Table 1). The nsp1 region containing the original mutation was amplified from genomic RNA and

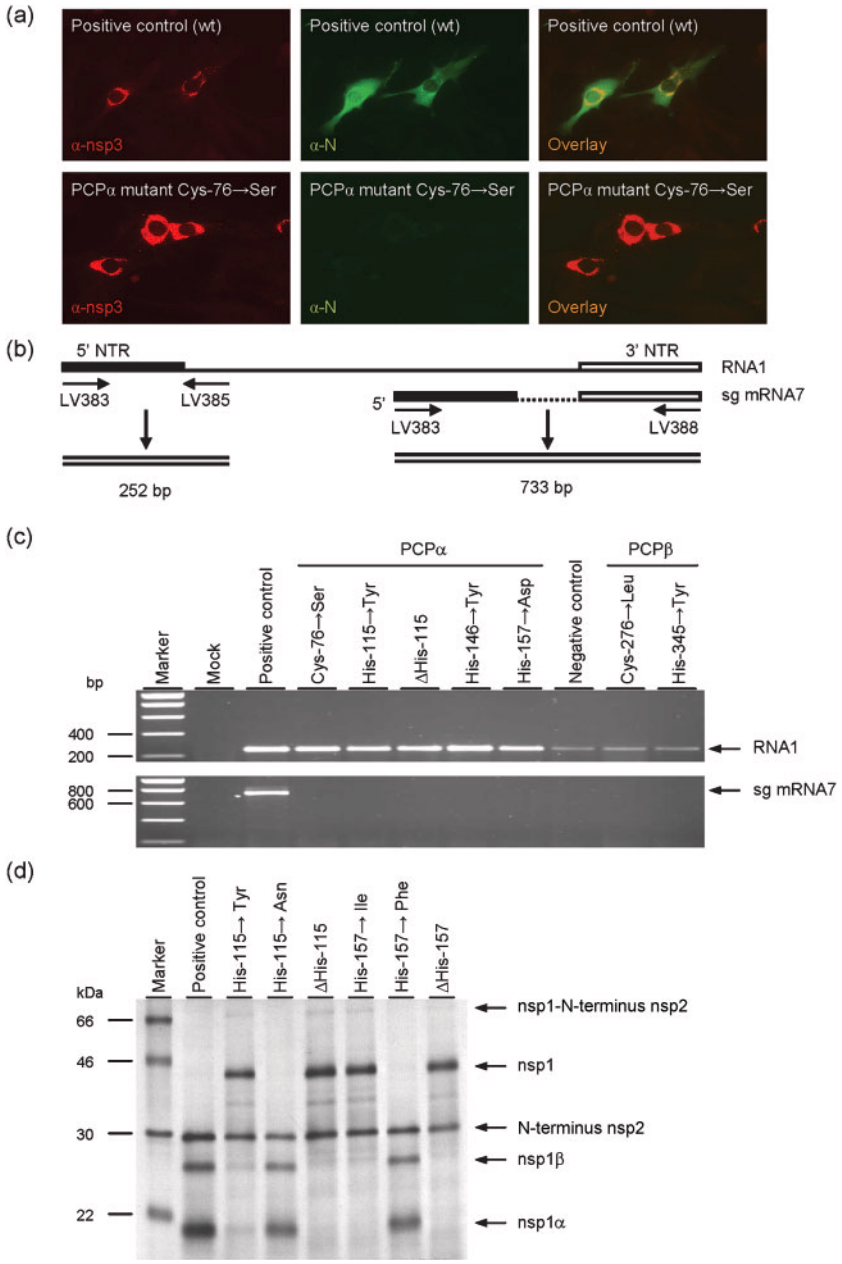

Fig. 2. *In vivo* analysis of PRRSV PCPα and PCPβ mutants. (a) Immunofluorescence microscopy of transfected BHK-21 cells fixed at 24 h p.t. (p0) and immunostained using a rabbit anti-nsp3 peptide serum (α-nsp3, J. C. Zevenhoven-Dobbe and E. J. Snijder, unpublished data; left panels) and a mouse monoclonal antibody directed against the nucleocapsid protein (α-N, van Nieuwstadt *et al.*, 1996; central panels), which were used to monitor RNA1 replication and sg mRNA7 synthesis, respectively. Upon reversion, a small number of cells transfected with PCPα mutants showed staining similar to that in the wild-type control (top row). The bottom row shows an example of a replication-competent PCPα mutant that was transcription-negative (no N signal). See Table 1 for a complete overview of the immunostaining results for all mutants. (b) Schematic representation of the RT-PCR analysis used to monitor genome replication and sg mRNA7 synthesis. The genomic 5′ NTR is represented by a black box, the 3′ NTR by an open bar. (c) RT-PCR analysis of RNA1 and sg mRNA7 production. Transfected BHK-21 cells were lysed at 24 h p.t. and intracellular RNA was screened for the presence of PRRSV-specific RNAs using a RNA1-specific (upper panel) or a sg mRNA7-specific (lower panel) RT-PCR. Transcripts from the wild-type cDNA clone served as the positive control, whereas a full-length cDNA clone lacking an essential RNA replication element (see text) was used as a negative control. Size markers and PCR products are indicated. (d) *In vitro* analysis of the proteolytic activity of PCPα and PCPβ mutants/revertants. Size markers and cleavage products are indicated.

codons 76 and 146 were found to differ by one nucleotide from the sequence in the original mutant cDNA clones. In both cases, the codon specifying the wild-type amino acid at that position had been restored (Table 1), strongly supporting the importance of Cys-76 and His-146 for PRRSV viability. Mutant His-157→Asn produced N-positive cells in all passaging experiments, whereas the other substitution mutants occasionally yielded positive cells, but to a much lesser extent than the wild-type control and His-157→Asn (Table 1). To assess the genetic stability of substitution mutants giving partial cleavage of the nsp1α/nsp1β junction (His-115→Tyr, His-157→Asp, His-157→Asn and His-157→Ile), the p1 supernatants were used to passage these mutant viruses four more times. Subsequently, the genome sequence of the p5 viruses at the site of mutation was determined. Revertants of the His-157→Asn mutant contained the wild-type codon (true revertant), whereas mutants His-115→Tyr, His-157→Asp and His-157→Ile yielded pseudo-revertants. Mutant His-115→Tyr changed to Asn, His-157→Asp to Tyr, and His-157→Ile reverted to Phe (Table 1). Titres of the p5 viruses were similar to that of the wild-type control (Table 1). As an alternative approach to probe the importance of His-115 and His-157, the corresponding codons were deleted (mutants ΔHis-115 and ΔHis-157). RNA derived from these two mutant clones did replicate in BHK-21 cells, but did not produce detectable levels of sg mRNA7, as established by immunostaining and RT-PCR analysis

(Table 1; Fig. 2c). Subsequently, the mutations recovered from the p5 populations were investigated for their effect on cleavage of the nsp1α/nsp1β junction. To this end, mutations specifying His-115→Asn and His-157→Phe, as well as the deletions of both codons, were introduced individually into expression vector pIP627 and used to translate the PRRSV nsp1 region *in vitro* (rabbit reticulocyte lysate) in the presence of [^{35}S]methionine (den Boon *et al.*, 1995). The polyprotein consisted of nsp1α (20 kDa), nsp1β (27 kDa) and the N-terminal part of nsp2 (30 kDa). Translation products were resolved in a 15 % gel by SDS-PAGE and visualized by autoradiography (Fig. 2d). The His-157→Tyr mutant was not included, as den Boon *et al.* (1995) had already shown that this mutation did not affect the nsp1α/nsp1β cleavage. Whereas mutant His-115→Tyr produced only trace amounts of cleavage products, the His-115→Asn mutant retained wild-type proteolytic activity. In addition, mutant His-157→Ile showed no cleavage of the nsp1α/nsp1β junction, whereas pseudo-revertant His-157→Phe had wild-type activity. In accordance with the results obtained in the *in vivo* analysis, deletion of His-115 or His-157 completely abolished processing of the nsp1α/nsp1β junction.

As for PCPα, substitutions targeting either residue of the presumed PCPβ catalytic dyad were tested as well. Den Boon *et al.* (1995) had already described the complete inhibition of PCPβ proteolytic activity *in vitro* due to the substitutions Cys-276→Leu, Cys-276→Ser and His-345→Tyr. *In vivo*, none of these mutants showed any sign of replication, as evidenced by (i) the lack of signal in immunostaining using the anti-nsp3 serum, (ii) the absence of RT-PCR signal for genome RNA (Table 1; Fig. 2c), and (iii) the absence of infectious progeny in the medium harvested from transfected BHK-21 cells. The small amounts of 252 bp RNA1-derived RT-PCR product seen in the upper panel of Fig. 2(c) (RNA1) should be attributed to amplification from remaining transfected input RNA and not to newly synthesized RNA1, as evidenced by RT-PCR analysis of RNA isolated from the supernatants of BHK-21 cells transfected with RNase-free DNase I-treated RNA transcripts using the same wild-type and negative control as well as a transcript lacking the 3′ NTR (data not shown).

The data presented above indicate that PCPα activity is required for PRRSV sg mRNA synthesis, most likely because the function of nsp1α, which contains the putative zinc finger domain that was implicated in EAV sg RNA synthesis, depends on the release of this subunit from the replicase polyproteins. However, genome replication can proceed normally when PCPα is completely inhibited, a phenotype also previously observed for EAV mutants lacking nsp1, leading to the conclusion that the protein is critical for sg mRNA synthesis, but completely dispensable for genome replication (Tijms *et al.*, 2001, 2007). Theoretically, the effect of PCPα inactivation could also be explained by a negative effect on PCPβ activity towards the nsp1β/nsp2 site, which was here shown to be absolutely required for genome replication (Table 1). However, in previous studies, den Boon *et al.* (1995) did not observe any effect of PCPα mutations on processing of the nsp1β/nsp2 junction *in vitro*. An effect of the PCPα mutations on the functionality of the nsp1α zinc finger domain could also explain the transcription-negative phenotype, but this is deemed less likely since the corresponding domain in EAV nsp1 that does not contain an active PCPα proteinase is functional.

The PCPβ knockout mutants tested in this study, which were completely blocked in processing of the nsp1β/nsp2 junction *in vitro* (den Boon *et al.*, 1995), showed no sign of RNA synthesis at all. These results, together with data obtained for EAV (Tijms *et al.*, 2007) showing that the nsp1/nsp2 cleavage is required for virus viability, suggest that the liberation of the nsp2 N-terminus is essential for arterivirus replication. EAV nsp2 has been shown to be involved in at least three processes; (i) cleavage of the nsp2/nsp3 junction *in cis* by the cysteine proteinase in its N-terminal domain (Snijder *et al.*, 1995), (ii) acting as cofactor for the nsp4 proteinase (Wassenaar *et al.*, 1997), and (iii) formation of the double-membrane structures with which the RTC appears to be associated (Snijder *et al.*, 2001). It remains to be established whether one or more of these functions can be extrapolated to PRRSV and how inactivation of PCPβ may affect these functions. Apart from containing the proteinase performing the important nsp1β/nsp2 cleavage, nsp1β likely has an additional role in the PRRSV life cycle, but no indications about the nature of this function have been obtained so far.

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