

Supporting Information

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SI Materials and Methods

DNA Constructs. Plasmids for expression of full-length or partial porcine reproductive and respiratory syndrome virus (PRRSV) ORF1a were constructed by RT-PCR amplification of corresponding regions from genomic RNA (nucleotides 191–7702 of the SD95-21 genome; nucleotides 222–7361 of the SD01-08 genome). The PCR product was digested with NcoI and NotI restriction enzymes and ligated into a pL1a backbone digested with the same enzymes. The design of pL1a was described previously (1). Except for the KO2 (Fig. S1) and nsp1 β cc-2 (Fig. S3) constructs, which were generated by gene synthesis, all mutations were introduced by using the Quick-Change site-directed mutagenesis kit (Stratagene) following the manufacturer's instructions.

Plasmid pLnsp1 β cc-2 was constructed by extensively mutating the SD01-08 PRRSV nsp1 β -coding region with synonymous replacements (Fig. S3), while keeping the encoded amino acid sequence intact and avoiding rare codons. The modified sequence (nsp1 β cc) was produced as a synthetic gene and fused back with the nsp2-coding region to generate the nsp1 β cc-2 construct. To construct plasmids for the dual-luciferase assay, a 79-nt oligonucleotide (nucleotides 3506–3584 of SD01-08 genome) containing the wild-type (WT) sequence or mutations (Fig. S3, IFC and KO2) at the ribosomal frameshifting region was synthesized and cloned into the dual luciferase vector pDluc as described previously (2, 3).

The plasmid expressing FLAG-tagged nsp1 β (pFLAG-nsp1 β) was generated by PCR amplification of the nsp1 β -coding region (nucleotides 762–1376 of the SD01-08 genome) and cloned into the plasmid vector p3xFLAG-CMV-24 (Sigma). Plasmids expressing the EGFP-tagged PRF sequence (pR79WT-EGFP, pR79KO2-EGFP, and pR79CC2-EGFP) were generated by cloning the PRF region (nucleotides 3506–3584 of SD01-08) into the plasmid vector pEGFP-N1 (Clontech).

In Vitro Transcription and Radioimmunoprecipitation Analysis of SD01-08 WT and Mutants. Full length SD01-08-WT, KO2, or 1 β KO RNA was transcribed from 1 μ g linearized plasmid DNA using the mMESSAGE mMACHINE T7 kit (Ambion) following the manufacturer's instructions. BHK-21 cells (4×10^6) were electroporated with 8 μ g in vitro-transcribed RNA using program T-020 of the Amaxa Nucleofector and kit T (Lonza). Newly synthesized proteins were labeled from 16.5 to 18.5 h posttransfection in cysteine- and methionine-free medium containing 200 μ Ci/mL [³⁵S]-methionine and [³⁵S]-cysteine (Perkin-Elmer). Cell lysis and immunoprecipitation analysis were performed as described previously (4). The monoclonal antibodies (mAbs) α -EU-nsp1 β and α -EU-nsp2 were used to immunoprecipitate nsp1 β and nsp2/nsp2TF/nsp2N, and precipitated proteins were separated on a 12% (wt/vol) SDS/PAGE gel. Protein bands were visualized using phosphorimaging and a Typhoon Variable Mode Imager (GE Healthcare). Image analysis was performed with the ImageQuant TL software (GE Healthcare).

Pulse-Chase Analysis of nsp1 β Expression by SD01-08 WT and Mutants KO2 and 1 β KO. Following electroporation with in vitro-transcribed SD01-08 WT, KO2, 1 β KO RNA, or a 1:1 mixture of KO2 and 1 β KO RNA, 0.3×10^6 BHK-21 cells were labeled at 16.5 h posttransfection for 15 min in cysteine- and methionine-free medium containing 500 μ Ci/mL [³⁵S]-methionine and [³⁵S]-

cysteine (Perkin-Elmer). After removal of the label medium, cells were either lysed immediately or chased for 1 h in the presence of an excess of unlabeled methionine and cysteine. Immunoprecipitation was performed as described previously (4) with mAb α -EU-nsp1 β . Precipitated proteins were separated on a 12% (wt/vol) SDS/PAGE gel and phosphorimaging was performed as described above.

Immunofluorescence Microscopy of Transfected BHK-21 Cells. Following electroporation with in vitro-transcribed SD01-08 WT, KO2, or 1 β KO RNA, 0.15×10^6 BHK-21 cells were seeded on glass coverslips. At 18 h posttransfection, cells were fixed in 3% (wt/vol) paraformaldehyde in PBS. Cells were double labeled with a mAb recognizing dsRNA and Hoechst 33342 to stain nuclear DNA.

RNA Isolation, Denaturing Formaldehyde Gel Electrophoreses, and In-Gel Hybridization. BHK cells (0.75×10^6) were electroporated with in vitro-transcribed RNA of SD01-08 WT, KO2, and 1 β KO. At 18 h posttransfection, cells were lysed in 20 mM Tris-HCl (pH 7.4), 100 mM LiCl, 2 mM EDTA, 5 mM DTT, 5% (wt/vol) lithium dodecyl sulfate, and 100 μ g/mL proteinase K. Total RNA was extracted and separated on a denaturing formaldehyde gel. Positive-stranded viral RNA was visualized by gel drying and hybridization with a ³²P-labeled oligonucleotide probe (PRRSV-hyb1 5'-TCGCCCTAATTGAATAGGTG-3') complementary to the 3' end of the viral genome and therefore recognizes all viral mRNAs; 18S ribosomal RNA was used as a loading control and was detected with probe 5'-ATGCCCCCGGCCGTCCCTCT-3'. Hybridized gels were analyzed by phosphorimaging as described above. Correction for loading variations was performed using the amount of 18S RNA in the same lane. The sum of the signal for all viral mRNAs in each lane was used to calculate the relative abundance of each individual mRNA.

RNA-Binding Protein Immunoprecipitation and RNA Pull-Down Assay. RNA-binding protein immunoprecipitation was performed using a Magna RIP kit (Millipore) according to the manufacturer's instructions. Briefly, HEK-293T cells seeded in 10-cm Petri dishes cotransfected with plasmids expressing the RNA bait (R79WT-EGFP, R79KO2-EGFP, R79CC2-EGFP, or pEGFP; 8 μ g) and the nsp1 β bait (nsp1 β -WT, nsp1 β -KO, or pFLAG; 2 μ g). At 24 h posttransfection, cell lysates were prepared for coimmunoprecipitation. FLAG-tagged nsp1 β was immunoprecipitated using an α -FLAG mAb, and coprecipitating target RNA was quantified by quantitative RT-PCR using a TaqMan Gene Expression Assay kit (Life Technologies) targeting the EGFP RNA sequence. The expression of nsp1 β in all cotransfected samples was determined by Western blot analysis using an nsp1 β -specific mAb. A RiboTrap kit (Medical & Biological Laboratories) was used to further confirm the interaction between nsp1 β and the 79-nt RNA sequence from the PRRSV PRF region. The R79WT, R79KO2, or R79CC2 RNA were labeled with 5-bromo-U and in vitro synthesized using the MEGAscript T7 Kit (Life Technologies). The 5-bromo-U-labeled RNA transcripts were bound to magnetic beads conjugated with anti-BrU mAb. Subsequently, these magnetic beads were incubated with lysates of HEK-293T cell expressing FLAG-tagged 1 β KO or WT of nsp1 β . The amount of nsp1 β pulled down with the RNA bait was determined by Western blot analysis using an nsp1 β -specific mAb.

1. Fang Y, et al. (2012) Efficient -2 frameshifting by mammalian ribosomes to synthesize an additional arterivirus protein. *Proc Natl Acad Sci USA* 109(43):E2920–E2928.

2. Grentzmann G, Ingram JA, Kelly PJ, Gesteland RF, Atkins JF (1998) A dual-luciferase reporter system for studying recoding signals. *RNA* 4(4):479–486.

3. Fixsen SM, Howard MT (2010) Processive selenocysteine incorporation during synthesis of eukaryotic selenoproteins. *J Mol Biol* 399(3):385–396.

4. Snijder EJ, Wassenaar AL, Spaan WJ (1994) Proteolytic processing of the replicase ORF1a protein of equine arteritis virus. *J Virol* 68(9):5755–5764.

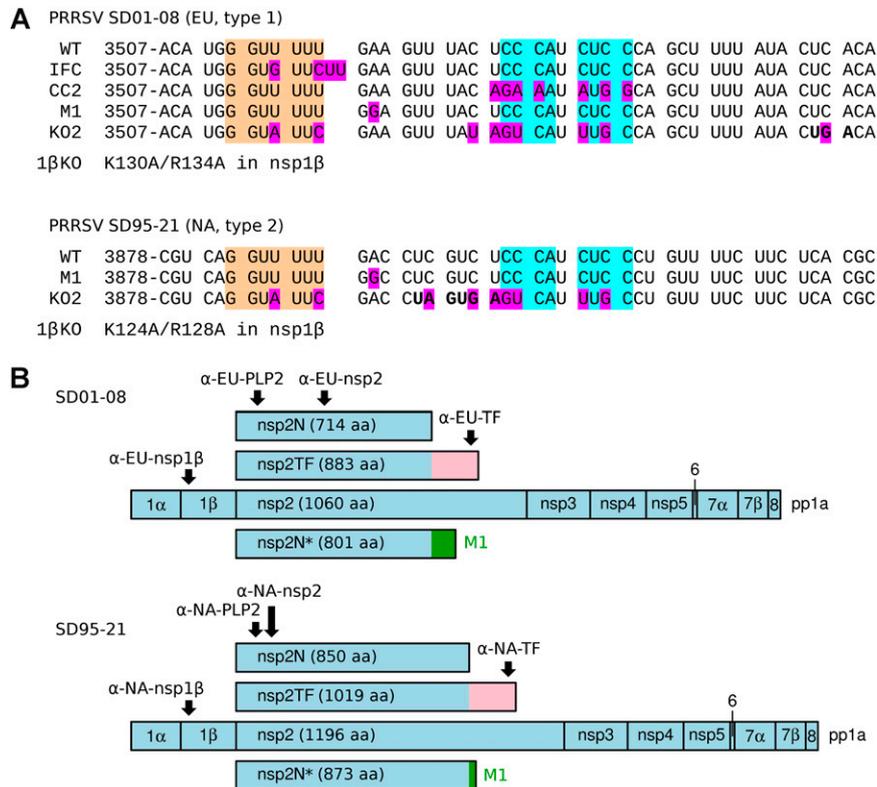


Fig. S1. Overview of mutants and antibodies used in this study. (A) List of WT and mutant sequences of the PRRSV PRF region (GGUUUUU shift site and conserved CCCANCUCC motif indicated with orange and cyan boxes, respectively). Mutated nucleotides are highlighted in magenta. Coordinates of starting nucleotides refer to PRRSV sequences DQ489311 (type 1 PRRSV) and KC469618 (type 2 PRRSV). CC2, disrupted CCCANCUCC motif; IFC, in-frame control; KO2, knockout mutant 2 (premature –2 frame termination codon and disrupted frameshift cassette); M1, mutated –1 frame termination codon to C-terminally extend nsp2N; 1βKO, nsp1β knockout mutant (double mutation introduced into the nsp1β GKYLQRRLQ motif). (B) Sizes of nsp2-related polypeptides described in this study and location of epitopes recognized by the PRRSV-specific antibodies in the nsp1β-2 region. Nsp2N* refers to the C-terminally extended version of nsp2N that is produced by mutant M1, due to removal of the stop codon (A). The origin and original name of each antibody are provided in *Materials and Methods*. Sizes (in number of amino acids) for nsp2-related products are shown for GenBank sequences DQ489311 (SD01-08, PRRSV type 1) and KC469618 (SD95-21, PRRSV type 2).

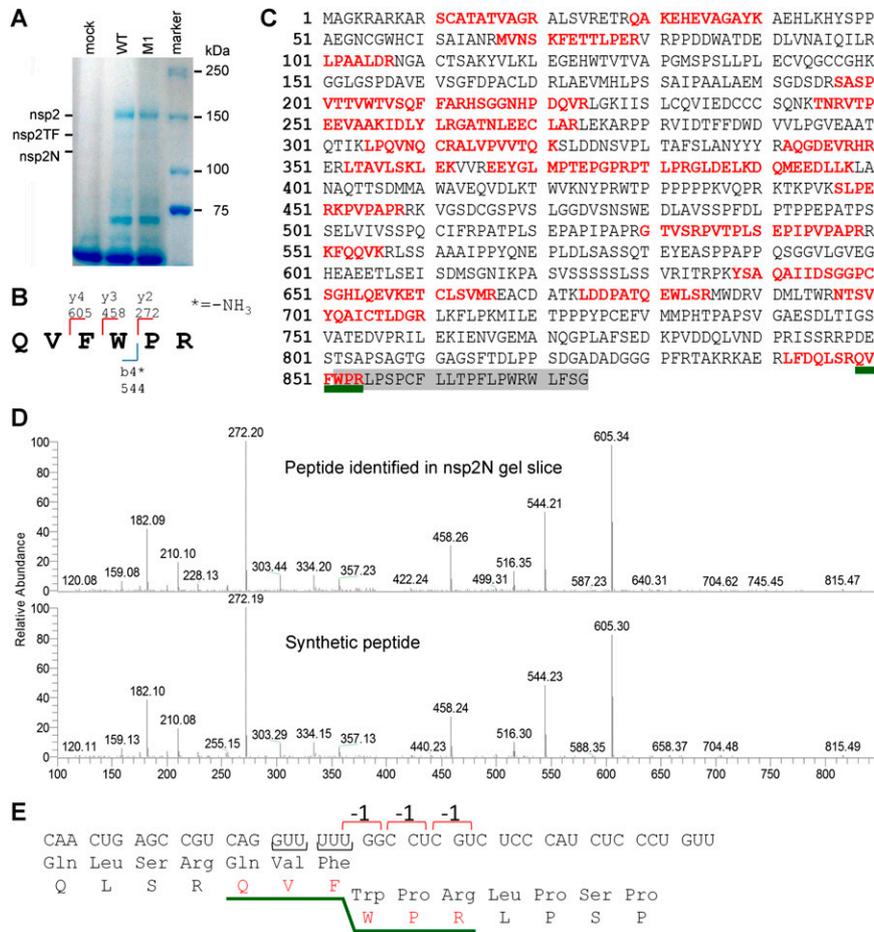


Fig. S2. Mass spectrometric (MS) analysis of nsp2N* (a C-terminally extended version of nsp2N) purified from cells infected with mutant SD95-21-M1. (A) PRRSV-infected or mock-infected MARC-145 cell lysates were immunoprecipitated with nsp2-specific α -NA-PLP2. Immunoprecipitated proteins were separated by SDS/PAGE and stained with Coomassie Blue. The positions of nsp2, nsp2TF, and nsp2N are indicated. (B) Peptide sequence of the nsp2N* -1 frameshift-specific peptide. The fragment ions that were identified in the liquid chromatography tandem MS analysis of the gel slice are indicated. (C) Complete amino acid sequence of nsp2N* comprising nsp2N and a 23-amino acid C-terminal extension (highlighted in gray). Peptides identified by MS are depicted in red. The peptide spanning the -1 frameshift site is underlined in green. (D) Fragmentation spectrum of the -1 frameshift-specific peptide QVFWPR. (E) Nucleotide sequence and -1 PRF-directed translation of nsp2N* at the frameshift site.

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1 TCTGACGTTTACAGGTGGAAGAAATTTGTGATTTTTACGGACTCCTCTCCCAACGGTCGATTTCGC
1 TCCGATGTGTATAGATAGAAAGTTTCGTCATCTTACCGGATAGCAGCCCTAATGGCAGATCAGA
  S D V Y R W K K E V I F T D S S P N G R F R
67 ATGATGTGGACGCCGAATCCGATGACTCAGCCGCCCTGGAGGTGCTGCCGCCAGTTAGAACGT
67 ATGATGTGGACCCCGAGAGCGACGATAGCGCTGCTCTCGAAGTCTCCCCCTGAACTGGAGAGA
  M M W T P E S D D S A A L E V L P P E L E R
133 CAGGTCCGATCTCACTCGGAGTTTCCCGCTCATCACCTATCAACCTAGCTGACTGGGAGCTC
133 CAAGTGGAAATCTGACCGAAGCTTCCCTGCCACCATCCCATTAATCTGGCCGATGGGAATCTG
  Q V E I L T R S F P A H H P I N L A D W E L
199 ACTGAGTCCCCTGAGAACGGTTTTTCTTTCGGCAGTCCCATTTCTGCGCCACATCGTCCAGAAC
199 ACCGAAAGCCCGAAATGGCTTTCAGCTTGGGACAGCCACAGCTGTGGGCATTTGTGCAAAAT
  T E S P E N G F S F G T S H S C G H I V Q N
265 CCCAACGTGTTTACGGCAAGTGTGGCTCACCTGCTTTTTGGGCCAATCGGCTGAAGTGTGCTAC
265 CCTAATGTCTTCGATGGGAAATGTGGCTGACATGTTTCTGGGCGAGGCGCGAGGTCTGTATT
  P N V F D G K C W L T C F L G Q S A E V C Y
331 CACGAGGAACATCTAGCTAACGCCCTCGGTTACCAAACCAAGTGGGGCGTGCATGGTAAAGTACCTC
331 CATGAGAGCACCTGGCCAAATGCTCTGGGCTATCAGACAAAATGGGGGCTCCACGGCAAATATCTG
  H E E H L A N A L G Y Q T K W G V H G K Y L
397 CAACGCAGGCTTCAAGTCCGCGGCATGCGTGTGTGGTTCGATCCTGACGCCCTATTCACGTTGAA
397 CAGAGAAGACTGCAGGTGAGAGGATGAGAGCCGCTGTGGACCCGATGGGCCATCCATGTGGAG
  Q R R L Q V R G M R A V V D P D G P I H V E
463 GCGCTGTCTTGTCCAGTCTTGGGTCAGGCACCTGACTCTGAATAATGATGTACCCACAGGATTC
463 GCCCTCAGCTTAGCCAAGCTGGGTGAGACATCTCACCTCAACACGACGTGACACCCGGCTTT
  A L S C S Q S W V R H L T L N N D V T P G F
529 GTTCGCCTGACATCCATCCGATTGTGTCCAACACAGAACCCACCGCTTTCGGGATCTTTTCGGTTT
529 GTGAGACTCACAGCATTAGAATCGTCAGCAATACCGGCTACAGCCTTAGAATTTTTCAGATTC
  V R L T S I R I V S N T E P T A F R I F R F
595 GGAGCACATAAGTGGTATGGC
595 GGCGCCCAAAATGGTACGGG
  G A H K W Y G

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Fig. S3. Nucleotide sequences of the WT and synonymously mutated nsp1 β -coding region. For each block, the top, middle, and bottom lines give the WT nsp1 β -coding sequence (black), the synonymously mutated sequence (mutations present in pLnsnsp1 β cc-nsp2 given in red), and the (unchanged) translation into amino acids (blue), respectively.

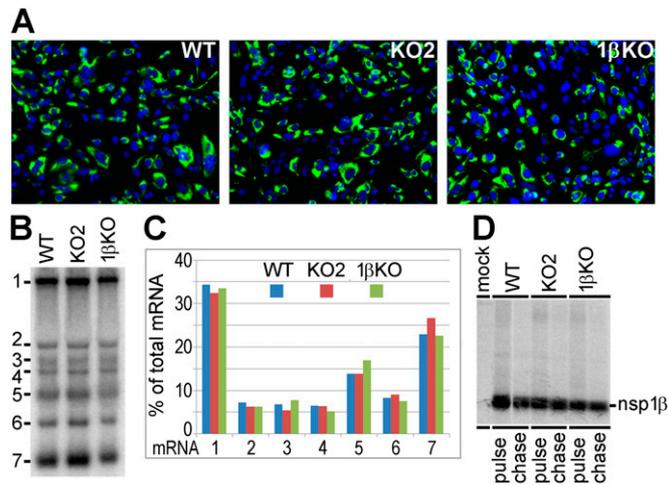


Fig. S4. First-cycle analysis of RNA synthesis and nsp1 β stability of WT and mutant (KO2 or 1 β KO) SD01-08 virus in BHK-21 cells. (A) Immunofluorescence microscopy analysis of transfection rate in BHK-21 cells electroporated with in vitro-transcribed full-length PRRSV RNA. Transfected cells were double labeled with a mAb specific for viral dsRNA (green) and Hoechst 33342 (blue) for staining of DNA in cell nuclei. (B and C) Gel hybridization analysis and quantification of PRRSV-specific mRNA accumulation in cells transfected with mutants KO2 and 1 β KO or a WT control. (B) Total intracellular RNA was isolated at 18 h posttransfection and resolved by denaturing formaldehyde agarose gel electrophoresis. PRRSV-specific mRNAs were detected by hybridization of the dried gel with a 32 P-labeled probe complementary to the 3'-end of the viral genome and subsequent phosphorimaging. The positions of the PRRSV genome (RNA1) and the six subgenomic mRNAs (RNA2 to RNA7) are indicated. (C) The volume of the bands corresponding to each of the viral mRNAs was quantified by phosphorimaging and adjusted for the control 18S ribosomal RNA band in the same lane. The sum of the signals for all viral mRNA bands in each lane was used to calculate the relative abundance of each individual mRNA. (D) Pulse-chase analysis of nsp1 β expression. BHK-21 cells were transfected with RNA transcribed from WT or mutants of PRRSV full-length cDNA clones. At 16.5 h posttransfection, protein synthesis was labeled for 15 min and chased for 1 h. Cells were lysed and, following immunoprecipitation with mAb α -EU-nsp1 β , the production and turnover of nsp1 β were analyzed using SDS/PAGE and autoradiography.