Porcine complement regulatory protein CD46 is a major receptor for atypical porcine pestivirus but not for classical swine fever virus

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Abstract

Pestiviruses such as bovine viral diarrhea virus (BVDV) and classical swine fever virus (CSFV) belong to the family *Flaviviridae* and represent pathogens of outstanding veterinary relevance. Pestiviruses enter cells via receptor-mediated endocytosis. For entry in bovine cells, complement regulatory protein CD46\textsubscript{bov} serves as a cellular receptor for BVDV. In this study, the role of porcine CD46\textsubscript{pig} in cellular entry was investigated for the recently discovered atypical porcine pestivirus (APPV), CSFV, and Bungowannah virus (BuPV) in order to elucidate the observed differences in host cell tropism. A cell culture adapted APPV variant, which shows enhanced viral replication in vitro, was generated and demonstrated a strict tropism of APPV for porcine cells. One of the porcine cell lines displayed areas of CD46\textsubscript{pig} expressing and areas of non-expressing cells and one single cell line revealed not to express any CD46\textsubscript{pig}, respectively. The CD46\textsubscript{pig} deficient porcine lymphoma cells, known to facilitate CSFV replication, was the only porcine cell line non-permissive to APPV, indicating a significant difference in the entry mechanism of APPV and CSFV. Infection experiments with a set of genetically engineered CD46\textsubscript{pig} knockout cells confirmed that CD46\textsubscript{pig} is a major receptor of APPV as CD46\textsubscript{bov} is for BVDV. In contrast, it is apparently not an essential determinant in host cell entry of other porcine pestiviruses such as CSFV and BuPV. Existence of a CD46\textsubscript{pig} independent entry mechanism illustrates that the pestiviral entry process is more diverse than previously recognized.

IMPORTANCE

Pestiviruses comprise animal pathogens such as classical swine fever virus (CSFV) and bovine viral diarrhea virus (BVDV) that cause notifiable diseases with great economic impact. Several additional pestivirus species affecting animal health were recently identified, including atypical porcine pestivirus (APPV). APPV is associated with health problems in piglets and highly abundant in pig populations worldwide. Complement control protein CD46 serves as a receptor for diverse bacterial and viral pathogens, including particular adenoviruses, herpesviruses, measles virus (MeV), and BVDV. Porcine CD46 (CD46\textsubscript{pig}) was suggested to be a major receptor for CSFV. Here, we identified...
remarkable differences in relevance of CD46\textsubscript{pig} during entry of porcine pestiviruses. Resembling BVDV, efficient APPV infection in cell culture depends on CD46\textsubscript{pig}, while other porcine pestiviruses can efficiently enter and infect cells in absence of CD46\textsubscript{pig}. Thus, the study provides insights into the entry process of these pathogens and may help to understand differences in their biology.

Introduction

The genus \textit{Pestivirus} belongs to the family \textit{Flaviviridae} and currently comprises 11 different species termed \textit{Pestivirus A–K} and a growing number of putative new species (1). Pestiviruses such as bovine viral diarrhea virus (BVDV) and classical swine fever virus (CSFV) are RNA viruses of outstanding veterinary and economic relevance being causative agents of notifiable diseases (2, 3). Porcine host species are significantly implicated in pestivirus biology. CSFV (\textit{Pestivirus C}) is the only established pestivirus selectively infecting porcine hosts. Moreover, pigs are susceptible for several pestivirus species including ruminant pestiviruses like BVDV type 1 and 2 (\textit{Pestivirus A} and \textit{B}) and Border disease virus (BDV, \textit{Pestivirus D}). In addition to CSFV, three additional porcine pestivirus species were discovered, comprising atypical porcine pestivirus (APPV, \textit{Pestivirus K}), Bungowannah virus (BuPV, \textit{Pestivirus F}), and LINDA virus (LindaV, species not yet approved). These three pestivirus species can cause severe diseases in young piglets (4-6). It appears likely that infections of pigs with LindaV and BuPV were results of rare or even unique spill over infections from so far unknown reservoir hosts since subsequent attempts to detect these pathogens in domestic pig and wild boar populations failed (5-8). In contrast to BuPV and LindaV, APPV was found to be highly abundant in domestic pigs from many countries worldwide and wild boar must be considered as a natural reservoir host (9, 10). Pestivirus genomes that are most closely related to APPV were detected in bats, which together with rodents, represent the first non-ungulate hosts harboring novel pestivirus species (11-13). So far, it is not known whether pigs are the only host for APPV and investigation of \textit{in vitro} cell tropism was restricted due to lack of an APPV cell culture isolate. In contrast to BuPV and...
LindaV, APPV isolation and propagation is highly inefficient on established porcine cell lines and virus isolation was only reported on embryonic porcine kidney epithelial cells (SPEV) with very low infectious titers (14, 15).

Although being a crucial step in the viral replication cycle, the entry process of pestiviruses is still poorly understood. Pestiviruses enter the host cell by receptor-mediated endocytosis, likely via a multistep process using different host cell factors (16, 17). Historically, bovine complement control protein CD46 (CD46_{bov}) was identified as a receptor for BVDV by a set of neutralizing monoclonal antibodies (mabs) that were not directed against the viral antigen, but the cellular protein (18, 19). CD46 was previously named a “pathogen’s magnet” as it is used as a receptor by diverse bacterial and viral pathogens, including measles virus (MeV) vaccine strains, certain human adenoviruses and herpesviruses such as human cytomegalovirus (20, 21). Expression of CD46_{bov} on porcine cells increases the susceptibility to BVDV (18). Nevertheless, BVDV was not able to infect non-permissive cells substituted with CD46_{bov}. As CD46_{bov} is an almost ubiquitously expressed molecule, it remained unclear what determines tropism of BVDV for certain tissues. One possible explanation would be the use of co-receptors or different CD46_{bov} variants by the virus (20, 22). Evidence for an additional BVDV receptor was provided by a study, which demonstrated cellular entry independent from binding of E2 to CD46_{bov} (23). Subsequently, porcine CD46 (CD46_{pig}) was suggested to play a role in the entry process of CSFV as CD46_{pig} specific monoclonal antibodies (mabs) diminished infectivity to some degree (24).

In the present study, we systematically investigated the role of CD46_{pig} for entry of the porcine pestivirus species APPV, CSFV, and BuPV. The results of our study show that CD46_{pig} is essential for efficient entry of APPV, while CSFV and BuPV rely on different host cell factors for cellular entry.
Material and methods

Cells and viruses

The porcine kidney cell lines SPEV and PK15 were maintained as a monolayer in Earle’s minimal essential medium (EMEM) containing 5% fetal bovine serum (FBS) for the infection experiments and generation of the knockout cell lines. Other cell lines were maintained as a monolayer in Dulbecco’s Modified Eagle Medium (DMEM) containing either 10% FBS or horse serum (MDBK and CRFK) (Table 1).

Selected CSFV strains include representatives of the most relevant genotypes 1 and 2. CSFV strains Alfort-Tübingen (AlfT, CSF0904, genotype 2.3), Diepholz (CSF0104, genotype 2.3), Paderborn (CSF0277, genotype 2.1), Koslov (CSF0382, genotype 1.1), Riems (CSF0913, vaccine strain, genotype 1.1) were obtained from the virus collection (CSF catalogue numbers are given in brackets) of the Institute of Virology, University of Veterinary Medicine Hannover, Germany (25). These CSFV strains show differences in virulence properties as including the attenuated vaccine strain Riems, a variant of the live attenuated C-strain, and the extraordinary virulent strain Koslov, which is often used in vaccination-challenge studies. Glycoprotein E\textsuperscript{capsid} coding regions were monitored for a point mutation (Ser\textsuperscript{476} to Arg\textsuperscript{476}), which was previously reported to be responsible for cell culture adaptation via heparan sulphate (HS) usage (26). RT-PCR and subsequent Sanger sequencing were performed using primer pair 1539fw/2222rev (Table 2).

BuPV was kindly provided by Peter Kirkland (Elizabeth Macarthur Agriculture Institute, Menangle, Australia). Virus stock from 17\textsuperscript{th} passage of the APPV isolate Ger-NRW_L277 (GenBank MF167291) represents the non-cell culture adapted APPV stock (APPV\textsubscript{P17}). Virus stock from the 100\textsuperscript{th} passage of APPV (APPV\textsubscript{P100}) was obtained as previously described, showing a viral titer of approx. 8 x 10\textsuperscript{4} TCID\textsubscript{50}/ml on SPEV cells (7).

Increasing numbers of APPV positive cells were observed during continuous passaging of persistently infected SPEV cells. For characterization of the cell culture adaptation naïve SPEV cells...
were infected with 1 ml APPV cell culture supernatant from 25\textsuperscript{th}, 45\textsuperscript{th}, 55\textsuperscript{th} and 100\textsuperscript{th} passage in six-well plates at the time point of seeding. Cells were heat fixed 72 h p.i. for immunofluorescence staining as described below. Viral titers were determined from the supernatants of 25\textsuperscript{th}, 55\textsuperscript{th} and 100\textsuperscript{th} passages of APPV. Genomic alterations within the predicted glycoprotein encoding regions were monitored after the 17\textsuperscript{th}, 25\textsuperscript{th}, 35\textsuperscript{th}, 45\textsuperscript{th}, 55\textsuperscript{th} and 100\textsuperscript{th} passages of APPV by RT-PCR and subsequent Sanger sequencing, using primer pairs 1114fw/1594rev, 1459fw/2002rev, 1721fw/2251rev, 2109fw/2554rev, 2353fw/2912rev, 2749fw/3324rev (Table 2). Moreover, at the 100\textsuperscript{th} passage (APPV\textsubscript{P100}), the complete polyprotein coding sequence was determined using next generation sequencing as previously described (27).

**Permissivity of different cell lines to atypical porcine pestivirus (APPV)**

One day prior to infection, porcine cell lines SPEV, PK15, SK6, NPTr, IPECJ2, ST, 38A\textsubscript{D}, and non-porcine cell lines MDBK, CRFK, RK13, Vero76, Lovo, HypNi/1.1, EidNi/41 were seeded in 24 well-plates. On the day of infection, cells from two wells of each plate were trypsinized and counted. All cells were infected with a multiplicity of infection (MOI) of 1 with APPV\textsubscript{P100}. Cells were incubated at 37°C for 72 hours (h), heat fixed at 80°C (4 h) and analyzed by APPV-specific immunofluorescence. Immunofluorescence staining was performed using porcine APPV-specific antiserum (dilution 1:2000) in combination with secondary mab Alexa fluor® 594 goat anti-swine IgG (111-585-003, Dianova, 1:1000 dilution).

**Generation of CD46\textsubscript{pig} knockout cell lines**

Generation of knockout cells by CRISPR/Cas9 technology was performed as described previously (28). Briefly, oligonucleotides containing the guide sequences (primers gCD46-2 and gCD46-7) targeting the region encoding the N-terminal part of complement control protein 1 (ccp1) within CD46\textsubscript{pig} were designed (Table 2) and cloned into the plentiCRISPR-v2 plasmid to generate plentiCRISPR-v2-CD46-2 and plentiCRISPR-v2-CD46-7 (29, 30). For production of lentiviral particles, the recombinant plasmids were co-transfected with a packaging vector (pCMVΔR8.91) and a plasmid encoding for the glycoprotein of vesicular stomatitis virus (VSV-G-pMD.G) into HEK293T cells, using
Polyethylenimine transfection reagent (24765-1, Polysciences, Inc.). Harvested lentiviral particles were used for transduction of SPEV and PK15 cells, respectively, followed by a subsequent puromycin selection (P8833, Sigma-Aldrich) with 5 µg/ml puromycin-supplemented medium for two weeks. At least three rounds of biological cloning of SPEVΔCD46 and PK15ΔCD46 cell lines were performed by single cell expansion. Purity of the obtained knockout cell lines was confirmed by immunofluorescence analysis and genetic characterization as described below.

**Phenotypic and genetic characterization of wild type porcine cell lines and CD46\textsubscript{pig} knockout cells**

Presence of CD46\textsubscript{pig} in wild type (WT) porcine cell lines as well as individual knockout cell clones was evaluated by immunofluorescence staining. Cells were grown for three days and heat fixed at 80°C (4 h). Staining was performed using commercially available mabs against CD46\textsubscript{pig} (MCA2310GA and MCA2262GA, Bio-Rad, 1:500 dilution) and a secondary mab Alexa fluor® 488 goat anti-mouse IgG (A11029, Invitrogen, 1:1000 dilution). Additionally, cell nuclei were stained with DAPI (D3571, Thermofisher Scientific, 1:500 dilution).

For genetic characterization of CD46\textsubscript{pig} expressed by parental SPEV and PK15 cells, RNA preparations were used for reverse transcription by SuperScript III Reverse Transcriptase (18080093, Invitrogen) and random hexamers (N8080127, Invitrogen). Amplification of a partial CD46\textsubscript{pig} coding sequence was performed using ALLin™ HS Red Tag Mastermix (HSM0305, highQu) with two primer pairs (101fw/710rev and 604fw/1192rev, **Table 2**) for subsequent generation of a consensus sequence (1092 bp). The following thermal profile was applied: 2 min at 95°C, 40 cycles: 15 s at 95°C, 15 s at 54°C, 15 s at 72°C, and final extension for 5 min at 72°C. Sanger sequencing was conducted by LGC genomics (Berlin, Germany). Attempts to amplify the CD46\textsubscript{pig} coding sequence from 38A\textsubscript{D} cells with these primers were not successful. Thus, four additional primer pairs were designed, which target genetic regions representing the individual domains ccp1 to ccp4 of CD46\textsubscript{pig} (192fw/353rev, 387fw/543rev, 571fw/710rev, 766fw/937rev; **Table 2**).

To characterize the CRISPR/Cas9 induced genome alterations in the engineered CD46\textsubscript{pig} knockout cells, primers (101fw/710rev) flanking the target site of the guide RNAs were applied.
These primers were specific for regions coding for the signal peptide and the ccp3 domain, respectively (Table 2). Gel purified amplicons (wild type genome: 610 bp) were cloned into TOPO vector (450030, Thermofisher Scientific) and propagated in TOP10 E. coli bacteria. Individual colonies were subjected to conventional PCR using vector specific primers T7fw and M13rev (Table 2). Subsequently, amplicons of at least ten individual colonies were subjected to Sanger sequencing (LGC genomics, Berlin, Germany) to determine genetic variation within the individual cell clones. Obtained sequences were analyzed using the GENtle software (version 1.9.4.0) and compared to the CD46 pig coding sequences of SPEV and PK15 WT cells.

Infection of wild type and CD46 pig knockout cell lines

Titers of virus stocks of APPV P100, BuPV and different CSFV strains were determined and virus stocks were stored at -80°C until use for infection experiments. Titer of virus stock APPV P17 could not be determined due to inefficient viral replication. Titers were determined by three independent endpoint dilution assays each performed in quadruplicates. Visualization of viral infection was done by immunofluorescence staining.

One day prior to infection, 3 x 10^4 SPEV, PK15 and CD46 pig knockout cells (SPEVΔCD46 #2, #7, and PK15ΔCD46 #2) were seeded into 24-well plates. Cells were infected with 500µl of APPV P17 since the viral titer is unknown. Infections with APPV P100, BuPV and the different CSFV strains were performed at a MOI of 1 for 2 h at 37°C, respectively. Subsequently, inoculum was removed, infected cells were washed three times with PBS and incubated for 72 h at 37°C. Supernatant was harvested to determine the virus titer at 72 h post infection. Cells were washed three times with PBS and subsequently analyzed by immunofluorescence staining or RT-PCR analysis. Additionally, infections of WT and CD46 pig knockout cell lines with CSFV and BuPV (MOI of 1, described above) as well as with APPV P100 (MOI of 0.5) were analyzed 16 h post infection (p.i.) by either immunofluorescence staining (CSFV and BuPV) or fluorescence in situ hybridization (FISH) assay (APPV). For the FISH assay, one day prior to the APPV infection, 4 x 10^5 SPEV, PK15 and knockout SPEVΔCD46 #2 and PK15ΔCD46 #2 cells
were seeded on Nunc™ Lab-Tek™ II CC2™ chamber slides (S6690, Merck). All infection experiments were repeated three times.

Detection of viral replication by immunofluorescence or fluorescence in situ hybridization assay

For immunofluorescence staining, cells were heat fixed at 80°C (4 h). Immunofluorescence staining was performed using a porcine APPV specific antiserum (dilution 1:2000) or a porcine BuPV specific antiserum (dilution 1:12000) in combination with secondary antibody Alexa fluor® 594 goat anti-swine IgG (111-585-003, Dianova, 1:1000 dilution), respectively. For visualization of CSFV infection, pestivirus specific mab C16 (dilution 1:50) was used together with secondary antibody Cy3-AffiniPure goat anti-mouse IgG (115-165-146, Dianova, 1:800 dilution) (31).

As immunofluorescence staining was not suitable to detect APPV infection at an early time point of infection (16 h p.i.), FISH assay was performed. The assay was optimized for the cell lines used in this study based on the manufacturer’s protocol (QVC0001, Thermofisher Scientific). Formaldehyde fixation was applied for 30 minutes. Cy3 labelled APPV specific probes were based on the sequence of APPV isolate L277 (GenBank MF167291) and located within the NS3 to NS4B region (3958-7236 base sequence, ViewRNA Type1 probe set, Thermofisher Scientific). FITC labelled pig β-actin-specific probes were used as an internal reference (ViewRNA Type4 probe set, Thermofisher Scientific).

Quantitative analyses of the images, which are generated from tree independent FISH assays were carried out using ImageJ software (version 1.51.0). The APPV specific staining image is converted to a mask, by implementing the Renyi Entropy thresholding method. APPV specific signal is determined using the pixel count tool in histogram.

Determination of viral replication by RT-PCR assay

For RT-PCR analysis, cells were lysed and collected using a RA1 buffer (740961.500, Macherey Nagel). RNA extraction was performed with KingFisher Duo Prime instrument (Thermofisher Scientific).
Scientific) using IndiMag Pathogen Kit (SP947257, Indical Bioscience). RNA amounts were measured using NanoDrop™ 2000 (Thermofisher Scientific) and adjusted to 30 ng per reaction. TaqMan based qRT-PCR assays were used for detection of CSFV and APPV genomes (27, 32). For detection of BuPV genomes, previously established SYBR Green based real-time RT-PCR using a primer pair (LinBu) targeting part of the NS5B coding region was performed (9). All samples were tested in duplicates using the Mx3005P QPCR System (Agilent Technologies, Santa Clara, USA) and the QuantiTect® SYBR® Green RT-PCR Kit or QuantiTect® Probe RT-PCR Kit (204245, 204445, QIAGEN) according to the manufacturer’s protocols. The housekeeping gene coding for GAPDH was used in all real-time RT-PCR assays for normalization. Statistical analyses were carried out using an unpaired t test implemented in GraphPad Prism software (version 8.4.3).

Results

Adaptation of atypical porcine pestivirus (APPV) to cell culture conditions

Ongoing passages of SPEV cells persistently infected with APPV isolate L277, which was obtained from the liquor cerebrospinalis of a piglet affected by congenital tremor, resulted in increasingly higher numbers of APPV infected cells (Fig 1A). APPV stocks obtained from 17th, 25th, 55th and 100th passages of persistently infected SPEV cells were used for determination of viral titers. Due to inefficient viral replication, quantification of infectious virus harvested at 17th and 25th passage was not possible, whereas increasing viral titers of approximately $3 \times 10^4$ TCID$_{50}$/ml and $8 \times 10^4$ TCID$_{50}$/ml were determined for passages 55 and 100, respectively.

Determination of the complete polyprotein coding sequence of APPV from the 100th passage (APPV$_{100}$) using next generation sequencing revealed a total of 23 nucleotide exchanges compared to the original sequence obtained directly from the sample material (GenBank MF167291). These genome alterations consist of seven synonymous and 16 non-synonymous mutations (Fig 1B). Three synonymous and three non-synonymous substitutions, namely H330Q (E$^\text{ns}$), N751K (E2) and D752N (E2), were located within the predicted glycoprotein coding regions (Fig 1B). Sanger sequencing of viral genomes obtained from different APPV passages confirmed that these mutations were acquired.
during passaging, between the 45th and 100th passages. APPV stock from 17th passage (APPV_{P17}) did not show any mutations within the predicted glycoprotein coding regions. In five independent repetitions of passaging, the same synonymous and non-synonymous mutations occurred within the predicted glycoprotein coding regions and correlated with an increased number of infected SPEV cells, suggesting their importance for the adaptation of APPV to cell culture conditions.

**Permissivity of different cell lines to atypical porcine pestivirus (APPV)**

We investigated the permissivity of different porcine and non-porcine cell lines to APPV infection using the newly established cell culture adapted APPV strain (APPV_{P100}). All porcine cell lines except 38A1D (lymphoma) were susceptible to APPV_{P100} infections, however, to various extent (Fig 1C). This is in line with the situation in vivo, as APPV can be detected in cells of diverse tissues and different cell types of infected piglets (27). APPV_{P100} replicated most efficiently in SPEV (kidney) cells, resulting in an almost completely infected monolayer of SPEV cells at 72 h p.i. after infection with a MOI of 1. In contrast, on PK15 (kidney) cells, which are routinely used for propagation of CSFV, only few APPV_{P100} infected foci were observed under equal conditions. Similar to PK15 cells, inefficient and limited viral replication of APPV_{P100} was observed on porcine SK6 (kidney), NPT (neonatal trachea), IPECJ2 (jejunum) and ST (testis) cells. These results demonstrate so far unknown differences in the permissivity of different porcine cell lines to APPV infection (Fig 1C).

Several non-porcine cells (MDBK, CRFK, RK13, Vero76, Lovo, HypNi/1.1, EidNi/41, Table 1) were also tested for their permissivity to APPV, since BuPV was shown to have a broad cell tropism in vitro and sequences of pestiviruses closely related to APPV were detected in diverse rodent and bat species (11-13). None of the investigated non-porcine cell lines was permissive to APPV_{P100} (data not shown). Thus, the data provide no evidence that APPV can efficiently infect and replicate in non-porcine cell lines.

**Characterization of CD46\textsubscript{pig} expression patterns in porcine cell lines**
The observed differences in permissivity of porcine cell lines to APPV infection prompted us to characterize the CD46\textsubscript{pig} surface expression level of these cells, as CD46\textsubscript{bov} was previously shown to represent a major receptor for the related ruminant pestivirus BVDV (18). In addition, CD46\textsubscript{pig} was suggested to be a major entry factor of CSFV (24). With the exception of two cell lines, immunofluorescence staining of CD46\textsubscript{pig} using commercially available CD46\textsubscript{pig} specific mabs revealed that most of the porcine cell lines including SPEV and PK15 showed similar surface expression of CD46\textsubscript{pig} (Fig 2A). The only porcine cell line (38A\textsubscript{1D}) non-permissive to APPV\textsubscript{P100} was also the only one not expressing any CD46\textsubscript{pig}. A neonatal porcine tracheal cell line NPT\textsubscript{r} revealed expression of CD46\textsubscript{pig} only by some clusters of cells (Fig 2A). CD46\textsubscript{pig} positive NPT\textsubscript{r} cells were particularly located in the upper layers of cells that grew in multilayer clusters. Co-staining of APPV\textsubscript{P100} and CD46\textsubscript{pig} showed that APPV\textsubscript{P100} positive cells were located only on the CD46\textsubscript{pig} positive cell clusters (Fig 2B).

Sequence analysis of CD46\textsubscript{pig} obtained from SPEV and PK15 cells revealed no differences in the deduced amino acid sequences of the four ccp domains. In 38A\textsubscript{1D} cells, no mRNAs coding for the individual ccp domains (ccp1 to ccp4) were detectable by different RT-PCRs (Fig 2C), demonstrating the absence of CD46\textsubscript{pig} coding transcripts. This confirms the negative immunofluorescence results when staining for CD46\textsubscript{pig}. Taken together, these data provide strong evidence that 38A\textsubscript{1D} cells are deficient for CD46\textsubscript{pig} expression and highlight the importance of CD46\textsubscript{pig} for the APPV entry process.

**Generation and characterization of CD46\textsubscript{pig} knockout cell lines**

To investigate the CD46\textsubscript{pig} dependency in the entry process of different porcine pestiviruses, CD46\textsubscript{pig} deficient cell lines were generated by CRISPR/CAS9 technology and lentiviral transduction of CD46\textsubscript{pig} specific guide RNAs. Two different guide RNAs (gRNAs #2 and #7) were designed to avoid misinterpretation due to off-target effects (Figs 2C and 3B, Table 2). Both oligonucleotides were located very close to each other targeting the genomic region encoding for the ccp1 domain of CD46\textsubscript{pig}, the receptor binding site of CD46\textsubscript{bov} for the ruminant pestivirus BVDV (33). Two different CD46\textsubscript{pig} knockout SPEV cell lines (SPEV\textsubscript{\textDelta}CD46) were obtained with gRNA #2 (SPEV\textsubscript{\textDelta}CD46 #2) and
gRNA #7 (SPEVΔCD46 #7), respectively (Fig 3). Additionally, one CD46<sub>pig</sub> knockout PK15 cell line was generated with gRNA #2 (PK15ΔCD46 #2, Fig 3) prompted by the observed differences in permissivity of WT SPEV and PK15 cells. All CD46<sub>pig</sub> knockout cell lines were cloned biologically to obtain clonal cell lines. Immunofluorescence staining using mabs against CD46<sub>pig</sub> confirmed all generated knockout cell lines were phenotypically CD46<sub>pig</sub> deficient (Fig 3A). Sequence analysis of plasmids containing cloned PCR products of the N-terminal region of CD46<sub>pig</sub> ccp1 domain confirmed different genetic alterations in this region, due to random repair mechanisms of the cell. Genetic characterization of at least ten independent CD46<sub>pig</sub> PCR plasmid clones demonstrated the presence of altered CD46<sub>pig</sub> coding sequences in a diploid set of chromosomes for each cell clone. Each one of the altered alleles revealed different frameshifts within the N-terminal region of the ccp1 domain (Fig 3B). The SPEVΔCD46 clone #2 revealed to have a small deletion of five nucleotides on one allele resulting in a stop codon and codes for only three unchanged amino acids at the N terminus of ccp1. In the other allele, an extended large deletion of 289 nucleotides was found, affecting the parts coding for the SP and the complete ccp1 sequence (195 nucleotides) as well as the N-terminal half of ccp2 (94 of 180 nucleotides, Fig 3B). Both alleles of SPEVΔCD46 clone #7 revealed to have deletions of seven and eight nucleotides, respectively, resulting in frameshifts and stop codons (Fig 3B). In consequence, only the first six amino acids following the signal peptide (SP) of CD46<sub>pig</sub> remained unchanged in cell clone SPEVΔCD46 #7 (Fig 3B). In the PK15ΔCD46 clone #2, in one allele, a single nucleotide insertion is resulting in an immediate stop codon. The other allele contains the same extended deletion as observed in SPEVΔCD46 clone #2 affecting the SP, ccp1 and ccp2 sequences.

In consequence, in addition to the 38A<sub>1</sub>D cell line that was identified to be naturally deficient for CD46<sub>pig</sub>, three genetically engineered CD46<sub>pig</sub> knockout porcine cell lines were successfully generated with the aim to determine the relevance of CD46<sub>pig</sub> in the entry of porcine pestiviruses.

Relevance of CD46<sub>pig</sub> for the entry of porcine pestiviruses
A comparative analysis regarding the permissivity of WT and CD46 pig knockout cell lines was performed using APPV, a set of CSFV strains, and BuPV. Early (non-cell culture adapted APPV\textsubscript{P17}) and late (cell culture adapted APPV\textsubscript{P100}) passages of the APPV isolate were used. Different CSFV isolates that are representatives of genotypes 1 and 2 with different virulence properties were selected, including the attenuated vaccine strain Riems (gt. 1.1), the highly virulent strain Koslov (gt. 1.1), the strains Paderborn (gt. 2.1), Diepholz (gt. 2.3), as well as Alft (gt. 2.3) rescued from a reverse genetic system (34, 35). Sequence analysis of glycoprotein E\textsuperscript{\textsubscript{rns}} coding regions revealed that selected CSFV isolates does not possess a point mutation (Ser\textsuperscript{476} to Arg\textsuperscript{476}), which was previously reported to be responsible for cell culture adaptation (26). CSFV isolate Paderborn is naturally possessing Arg\textsuperscript{476} as also described earlier (GenBank ADI58615; AAL68894).

Infection of WT SPEV and SPEV\textDeltaCD46 cells revealed a strong impact of CD46\textsubscript{pig} expression on permissivity to APPV\textsubscript{P100} at 16 or 72 h p.i. (Figs 4, 5 and 6B). This effect was observed on both SPEV\textDeltaCD46 cell lines and confirmed the results observed with the naturally CD46\textsubscript{pig} deficient 38A\textsubscript{1D} lymphoma cell line. Nevertheless, single small foci of APPV\textsubscript{P100} infected cells were evident on both SPEV\textDeltaCD46 cell lines. Viral titers were determined 72 h p.i. (Fig 5A). High titers were detected after de novo infections with APPV\textsubscript{P100} reaching approx. 1 \times 10^6 TCID\textsubscript{50}/ml in the supernatant of WT SPEV cell line. In contrast, no infectious titers could be determined in the supernatants of the two different CD46\textsubscript{pig} knockout cell lines. Determination of APPV\textsubscript{P100} genome equivalents revealed 11 to 22 times less viral RNA in the SPEV\textDeltaCD46 cells compared to WT SPEV at 72 h p.i. (Fig 5B). In the case of PK15 cells, the dependency on CD46\textsubscript{pig} for APPV\textsubscript{P100} permissivity were less pronounced since the WT PK15 cell line already displayed a low permissivity comparable to SPEV\DeltaCD46 cells (Figs 4 and 5). Quantification of APPV\textsubscript{P100} genome equivalents in three independent experiments revealed in average 90 times lower genome equivalents in PK15 cells compared to SPEV cells (Fig 5B). Accordingly, no infectious titers could be determined in the supernatants of the PK15 WT and knockout cell lines due to the low viral load. Interestingly, WT PK15 cells contained approximately ten
times lower amount of APPV\textsubscript{P100} genomes than the two SPEV\textDelta CD46 cells, suggesting a less efficient RNA replication in PK15 cells.

Due to the lack of highly specific APPV mabs and low amounts of viral antigen early after infection, immunofluorescence staining of APPV was not possible as early as 16 h p.i. In order to perceive the impact of CD46\textsubscript{pig} at an early time point of the infection, a more sensitive FISH assay was performed on APPV\textsubscript{P100} infected WT and CD46\textsubscript{pig} knockout cell lines (SPEV\textDelta CD46 #2 and PK15\textDelta CD46 #2) at 16 h p.i. (Fig 6B). Results of the FISH assay were in line with the immunofluorescence staining of infected cells at later time points (72 h p.i.). APPV\textsubscript{P100} genomes were detected only in very few CD46\textsubscript{pig} knockout cells (SPEV\textDelta CD46 #2 and PK15\textDelta CD46 #2), whereas most of the CD46\textsubscript{pig} positive WT SPEV and PK15 cells were highly positive for APPV\textsubscript{P100} genome (Fig 6B).

Quantitative analyses of the images revealed equal amount of positive signal in both CD46\textsubscript{pig} knockout cell lines in average (SPEV\textDelta CD46 #2 and PK15\textDelta CD46 #2). Positive signal detected in WT SPEV cells were 1.9 fold higher than in WT PK15 cells and 5.9 fold higher than in both CD46\textsubscript{pig} knockout cell lines. Difference between the WT PK15 cells and the PK15\textDelta CD46 #2 cells was 3.1 fold.

APPV harvested from early passage 17 (APPV\textsubscript{P17}) showed only inefficient replication on WT SPEV and PK15 cells. Nevertheless, the impact of CD46\textsubscript{pig} expression on the permissivity to APPV\textsubscript{P17} was obvious, when WT and CD46\textsubscript{pig} knockout cells were compared (Fig 4). Multiple infected foci were detected on WT SPEV and PK15 cells, whereas only few single infected cells were present on CD46\textsubscript{pig} knockout cell lines at 72 h p.i. (Fig 4). Due to inefficient replication of APPV, infectious titers could not be determined in the supernatants of neither WT nor CD46\textsubscript{pig} knockout cell lines.

In contrast to APPV, no visible differences between WT and CD46\textsubscript{pig} knockout cell lines were observed with regard to the permissivity to CSFV and BuPV at 16 h and 72 h p.i. (Figs 4 and 6A). Viral titers of CSFV AlfT determined at 72 h p.i. from cell culture supernatants of infected PK15 WT and CD46\textsubscript{pig} knockout cell lines were comparable (Fig 5). Titers obtained from the infected CD46\textsubscript{pig} knockout cell lines were ranging between $1 \times 10^6$ and $5 \times 10^6$ TCID\textsubscript{50}/ml, while titers of $5 \times 10^6$
TCID\textsubscript{50}/ml were determined from PK15 WT cells. Interestingly, approximately ten times lower infectious titers of CSFV AlfT were detected in supernatants of SPEV WT cells ($6 \times 10^5$ TCID\textsubscript{50}/ml) when compared to PK15 WT cells (Fig 5A). Similar to CSFV, BuPV titers produced in WT SPEV and PK15 cell lines ($5 \times 10^6$ and $6 \times 10^6$ TCID\textsubscript{50}/ml) were comparable to the viral titers produced in CD46\textsubscript{pig} knockout cell lines ($4 \times 10^6$ to $6 \times 10^6$ TCID\textsubscript{50}/ml). In addition, no CD46\textsubscript{pig} dependent differences were observed when analyzing the amounts of viral genomes obtained from different WT and CD46\textsubscript{pig} knockout cell lines infected with CSFV or BuPV at 72 h p.i., respectively (Fig 5).
Although pestiviruses are of outstanding importance in veterinary medicine, molecular determinants of pestiviral entry are still only poorly understood. The entry process of pestiviruses is likely to be a multi-step process, as it is known for the related human Hepatitis C virus (HCV), which uses at least four different cellular factors (36). The cellular uptake of BVDV particles is mediated by Clathrin-dependent endocytosis and it was shown to be dependent on interaction between viral E2 and cellular CD46bov (14, 16, 33). For BVDV, CD46bov was identified to function as the main receptor, but there are several lines of evidence indicating that an additional receptor may exist (23, 33, 37, 38).

Entry mechanism of genetically distinct pestiviruses such as APPV as well as rodent and bat pestiviruses, so far could not be investigated due to lack of virus isolates and established cell culture systems. Generation of a cell culture adapted APPV isolate (APPVp100) allowed us for the first time to characterize this novel pestivirus species biologically (7). In this study, reproducible genomic alterations within the predicted E_{rms} and E2 regions were identified in the culture adapted APPV_{p100}. Their appearance correlated with an increased number of infected SPEV cells (Fig 1A). Additional alterations were also identified in the non-structural proteins p7, NS2, NS3, NS5A and NS5B (Fig 1B).

To clarify the relevance of observed mutations throughout the APPV genome for cell culture adaptation and to elucidate the underlying mechanisms of improved in vitro replication, further experiments e.g. by reverse genetics or viral pseudotypes will be necessary.

Recently established, genetically distinct pestivirus species, including bat and rodent viruses, are apparently not restricted to ungulate hosts. Pestiviruses identified in different bat species are most closely related to APPV (Fig 7A). The BuPV, another pestivirus species identified in pigs, shows a broad host cell tropism and is able to efficiently replicate in cells of bat and even in cells of human origin (39). Previous work showed that in vivo APPV has differences in tissue tropism when compared to CSFV (27). So far, host cell tropism of APPV could not be investigated due to inefficient and limited
viral replication *in vitro*. The presented data revealed that APPV\textsubscript{P100} obviously has a narrow host
tropism and (within the cell lines used in this study) selectively infects cells of porcine origin (*Fig 1C*).
Different non-porcine cell lines permissive to BuPV (bat and human cells) were not permissive to
APPV\textsubscript{P100} (data not shown).

Investigated cell lines of porcine origin showed major differences in their permissivity to
APPV\textsubscript{P100} (*Fig 1C*). The embryonic porcine kidney epithelial cell line SPEV allowed most efficient
replication of APPV\textsubscript{P100}, which may explain reports of successful virus isolation only on this cell line
(14, 15). Differences in permissivity to APPV\textsubscript{P100} prompted us to investigate the expression of CD46\textsubscript{pig}.
SPEV and PK15 cells, displaying major differences in permissivity to APPV\textsubscript{P100}, did not differ in their
CD46\textsubscript{pig} expression pattern. Moreover, sequence analysis did not identify differences in the regions
coding for the ectodomain of CD46\textsubscript{pig}. Thus, the major differences in PK15 and SPEV cell permissivity
to APPV\textsubscript{P100} cannot be explained by genetic differences in the ccp1 domain, which was previously
reported to bind glycoprotein E2 of BVDV (33). The molecular basis for restricted growth of APPV\textsubscript{P100}
in established porcine cell lines and differences in permissivity of CD46\textsubscript{pig} expressing porcine cells
should be in the focus of future studies with the aim to establish a fully permissive cell culture
system. All porcine cell lines investigated in this study expressed CD46\textsubscript{pig} in uniformly distributed
pattern with two exceptions (NPT\textsubscript{r}, 38A\textsubscript{1}D, *Fig 2A*). Remarkably, neonatal porcine tracheal cell line
NPT\textsubscript{r}) revealed to express CD46\textsubscript{pig} only in the upper layers of cell clusters, putatively in dependence
on the differentiation status. Co-staining of APPV\textsubscript{P100} and CD46\textsubscript{pig} in these cells indicated that CD46\textsubscript{pig}
expression is a prerequisite for APPV\textsubscript{P100} infection (*Fig 2B*). Along this line, it is an interesting finding
that the porcine lymphoma cell line 38A\textsubscript{1}D is naturally deficient for CD46\textsubscript{pig}. 38A\textsubscript{1}D was the only
porcine cell line tested in this study that was non-permissive to APPV\textsubscript{P100} and the absence of CD46\textsubscript{pig}
in 38A\textsubscript{1}D cells might be responsible for this phenotype (*Fig 1C*). Oddly, previous results showed that a
CSFV isolate could be grown on 38A\textsubscript{1}D cells to titers ten times higher than on established PK15 cells
(40). Thus, the finding of 38A\textsubscript{1}D cells to be deficient for CD46\textsubscript{pig} is remarkable since CD46\textsubscript{pig} was
proposed to be a major receptor of CSFV entry (24).
To elucidate the role of CD46 pig in the entry of porcine pestiviruses, porcine knockout cell lines incapable of expressing CD46 pig were generated by CRISPR-Cas9, based on SPEV and PK15 cell lines. All cell lines were characterized genetically and phenotypically to be deficient for CD46 pig (Fig 3). Differences in permissivity of WT and CD46 pig knockout cells to APPV P17 were observed at 72 h p.i. (Fig 4). Moreover, with the use of culture adapted APPV P100, the high impact of CD46 pig expression on permissivity to APPV could be observed as early as 16 h p.i. and also at 72 h p.i. (Figs 4 and 6B). These results demonstrate that entry of APPV is highly dependent on CD46 pig. A similar strategy based on culture adaptation was followed for other viruses that were difficult to be isolated and to be propagated in vitro, e.g. the related HCV and its culture adapted JFH-1 clone (41). Adaptive mutations in glycoprotein regions can result in improved binding affinity of the viral ligands with attachment factors or with receptors and sometimes might even result in receptor switch. In the glycoprotein of MeV only a single exchanged amino acid in the viral receptor ligand can be sufficient to result in a switch between usage of SLAM or nectin-4 receptors and entering the cell via human CD46 (e.g. as known for strain Edmonston). Detailed studies demonstrated that the interaction sites for the cellular receptors on the viral glycoprotein at least partially overlap and alteration of a single amino acid can be sufficient to change receptor preference (42, 43). However, the mutations observed in the APPV glycoproteins after cell culture adaptation apparently do not result in a receptor switch since both APPV harvested from early passage (APPV P17) and cell culture adapted APPV P100 showed comparable dependency on CD46 pig. An increase in the affinity for CD46 pig in the cell culture adapted virus is one plausible explanation. As known for HS usage by BVDV and CSFV, improved attachment to the host cell might also help to bind to the receptor and thus result in more efficient entry. Consequently, the cell culture adapted APPV P100 established in this study represents a precious tool to identify molecular determinants of entering the host cell.

In naturally infected pigs, APPV can be detected in many different tissues as reported earlier (27, 44). This is in line with the finding of this study that APPV uses the ubiquitously expressed CD46 pig molecule for cell entry. Nevertheless, other cellular factors are apparently implicated in the
entry process and will be required to complete the viral replication cycle successfully. Therefore, it is not surprising that APPV and BVDV show some differences in tissue tropism although both are highly depending on CD46 during entry process. It remains obscure why BVDV preferentially infects certain cell types, e.g. epithelial and immune cells. One explanation might be interaction with additional (co-localized) surface proteins (20). Very recent data demonstrated that in polarized bovine respiratory epithelial cells, CD46\textsubscript{bov} is a major BVDV receptor on the apical but not the basolateral cell membranes despite the observation that basolateral infection was more efficient than apical infection (38). In consequence, the entry process of BVDV is much more complex than previously recognized. Although CD46\textsubscript{pig} represents a crucial factor in APPV entry, it remains unknown which additional factors are required for efficient replication. The future identification of such host factors may also explain differences in tissue tropism of BVDV and APPV. Nevertheless, the loss-of-function experiments presented in this study clearly demonstrate an important role of CD46\textsubscript{pig} during cell entry of APPV. Diverse genetic and splice variations of CD46\textsubscript{bov} have been reported to shift permissivity of bovine cells to BVDV (22). Future studies need to figure out whether CD46\textsubscript{pig} complementation of CD46 knockout cells \textit{in trans} can restore the permissive phenotype of wild type cells. Successful complementation might depend on a specific CD46\textsubscript{pig} variant or availability of specific co-factors and thus can provide novel insights into molecular interaction between CD46\textsubscript{pig} and APPV during the entry process.

Several cellular factors were suggested to be involved in the entry process of CSFV, including CD46\textsubscript{pig}, heparan sulphate (HS), laminin receptor (LamR/ RPSA), the low density lipoprotein receptor (LDL), beta-actin, and vinculin (24, 45, 46). As antibody mediated blocking of CD46\textsubscript{pig} resulted in a reduction of CSFV infection, CD46\textsubscript{pig} was proposed to be a main receptor of CSFV, along with the attachment factor HS and possibly other unknown receptors (24). However, the inhibitory effect of anti-CD46\textsubscript{pig} antibodies may rely on steric side effects interfering with virus/receptor interaction. In a different study, blocking of the CD46\textsubscript{bov} receptor with an anti-serum provided no evidence for involvement in entry of CSFV, BDV or Giraffe pestivirus (33). Importantly, amino acid composition of...
the BVDV E2 binding motif (E66QIV69 and G82QVLAL87) of CD46bov and the analogous region in CD46pig of porcine cells display major differences (33). The data obtained from infecting the well-defined porcine CD46pig knockout cells clearly show that – in contrast to APPV - CD46pig is not essential for cell entry of the porcine pestiviruses CSFV and BuPV (Figs 4, 5 and 6A). Surprisingly, CSFV and BuPV seem to use an alternative route of cell entry. Cell-to-cell spread may result in overcoming the CD46pig receptor usage via engagement of so far unknown alternate receptors on the target cell (47).

Nevertheless, even as early as 16 h.p.i., avoiding prominent effects of cell-to-cell transmission, no impact of CD46pig on CSFV or BuPV infection became evident (Fig 6A). A specific mutation in the E\textsuperscript{cm} protein of CSFV (Ser\textsuperscript{476} to Arg\textsuperscript{476}) was previously reported to result in cell culture adaptation via improved attachment to the cell by increasing the affinity to HS (26, 48). CSFV isolates used in this study including the recombinant CSFV strain AlfT as well as isolates Riems, Koslov and Diepholz do not possess this specific mutation and thus are not adapted to efficient HS attachment. All isolates were able to infect the CD46pig deficient knockout cells very efficiently. This strongly suggests that HS usage is not implicated in efficient entry of these CSFV strains (Fig 4, 6A). In consequence, it is likely that a so far unknown receptor is used by CSFV to enter the cells. The representative selection of CSFV isolates (different genotypes and virulence properties) used in this study provide evidence that CD46pig independent entry of CSFV is not restricted to a particular CSFV isolate.

Glycoprotein E2 of classical pestivirus species is the only known viral determinant possessing receptor-binding properties. However, simple transfer of BuPV E2 protein was not sufficient to expand host cell range of BVDV to human cells which are susceptible to BuPV (39). Interestingly, amino acid composition and predicted size of E2 glycoproteins of classical and recently discovered atypical pestiviruses display major differences (Fig 7A). These differences can be expected to result in altered protein structure and different protein-protein interactions and may correlate with different mechanisms of cellular entry (49). There is strong evidence that a stretch of amino acids in domain II of pestivirus E2 functions as the receptor ligand (amino acids 834 to 863 in BVDV strain NADL, PDB #4JNT). This motif is exposed as a hairpin in the crystal structure of BVDV E2 (50). The distinct E2
amino acid composition of APPV did not allow identifying a corresponding receptor-binding motif (Fig 7B). Against this background, it is not surprising that the distantly related porcine pestiviruses APPV and CSFV use different strategies of cell entry. Remarkably, BVDV and APPV obviously share the same receptor, although being only distantly related and infecting different host species (ruminant and porcine, respectively).

Taken together, the presented results demonstrate that CD46<sub>pig</sub> is a major cellular factor for efficient entry of APPV. With regard to its role in viral entry, the function of CD46<sub>pig</sub> for APPV infection resembles the function of CD46<sub>bov</sub> for BVDV entry. Additionally, our study demonstrates that (in contrast to BVDV and APPV) other porcine pestiviruses like CSFV and BuPV apparently use a CD46<sub>pig</sub> independent way to enter the host cell. This indicates that CD46 is not a general receptor of pestiviruses and different pestivirus species apparently use diverse mechanisms for host cell entry.
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Fig 1. Adaptation of APPV to cell culture conditions and permissivity of different porcine cell lines to APPV (A) SPEV cells were infected with supernatant from the indicated passages (P25, P45, P55 and P100) of SPEV cells persistently infected with APPV isolate L277. Scale bars indicate 200 µm. (B) Schematic representation of the 100th passage of APPV isolate L277 (APPV<sub>P100</sub>) genome including locations of synonymous (blue) and non-synonymous mutations (red). (C) The cell culture adapted APPV<sub>P100</sub> was used to infect different porcine cell lines with a MOI of 1. All porcine cell lines except 38A1D were permissive to APPV, showing either nearly complete APPV infected monolayer (SPEV) or only infected foci (PK15, SK6, NPTr, IPECJ2 and ST). The respective non-infected cells (NIC) served as a control. Immunofluorescence staining was performed at 72 h p.i. using porcine APPV specific antiserum (red) and DAPI (blue).

Fig 2. Characterization of porcine cell lines with regard to their CD46<sub>pig</sub> expression. (A) Phenotypical characterization of porcine cell lines by immunofluorescence staining using a CD46<sub>pig</sub>-specific mab (green, MCA2310GA) and DAPI (blue). Asterisks (*) indicate cell lines subjected to conventional RT-PCR for subsequent sequencing. (B) Immunofluorescence staining of APPV<sub>P100</sub> (porcine APPV specific antiserum, red), CD46<sub>pig</sub> (green, MCA2310GA) and DAPI (blue) at 72 h after infection of NPTr cells. (C) Strategy used for genetic characterization and manipulation of the CD46<sub>pig</sub> gene locus. The CD46<sub>pig</sub>-encoding mRNA was amplified by two RT-PCRs (101/710 and 604/1192) for subsequent cloning and sequencing. Absence of a CD46<sub>pig</sub>-encoding mRNA in porcine lymphoma cell line 38A1D was confirmed by RT-PCRs targeting the individual CD46<sub>pig</sub> domains (primer pairs: 192/353, 387/543, 571/710, 766/937). Positions of signal peptide (SP), complement control proteins 1 to 4 (ccp1-4), serine, threonine, and proline rich region (STP), and transmembrane domain (TM) encoded by the messenger RNA (mRNA) are depicted. In addition, positions of guide RNAs (gRNA#2, #7) used for construction of CD46<sub>pig</sub> knockout cells are indicated (for details see Fig 3B).
Fig 3. Characterization of genetically engineered CD46\textsubscript{pig} knockout cells. (A) Phenotypical characterization of CD46\textsubscript{pig} knockout cells by immunofluorescence staining using a mab against CD46\textsubscript{pig} (green, MCA2310GA) and DAPI (blue). Immunofluorescence staining of CD46\textsubscript{pig} (green) from wild type (WT) cell lines served as a control and are shown in Fig 1. (B) CRISPR/Cas9 induced genome alterations on both alleles characterized by sequencing of plasmids containing PCR amplicons flanking target sites of the guide RNAs (primers 101fw/710rev). Consensus nucleotide sequences and deduced amino acid sequences of the regions encoding for C-terminus of SP and N-terminus of ccp1 are shown. For comparison, nucleotide and deduced CD46\textsubscript{pig} amino acid sequences of WT as determined for SPEV and PK15 cells are given in top row. The border between SP/ccp1 and position of gRNAs including respective protospacer adjacent motifs (PAM, boxed) are indicated. For selected engineered CD46\textsubscript{pig} knockout cell lines (∆CD46) the corresponding sequences including deletions (\(\Delta\) nt) and insertions (+ nt) are shown below the WT CD46 sequence.

Fig 4. Relevance of CD46\textsubscript{pig} for the entry of porcine pestiviruses. Wild type (WT) SPEV and PK15 as well as CD46\textsubscript{pig} knockout cell lines (SPEV∆CD46 #2, #7 and PK15∆CD46 #2) were infected with APPV\textsubscript{P17}, APPV\textsubscript{P100}, BuPV and CSFV strains Alfort-Tübingen (AlfT), Diepholz, Riems, Koslov and Paderborn at an MOI of 1, respectively. Immunofluorescence staining was performed at 72 h p.i. using porcine APPV specific antiserum, a porcine BuPV specific antiserum, and a mab against CSFV, respectively. A strong reduction of APPV infection is evident on all SPEV∆CD46 cell lines in comparison to SPEV cells. PK15 cells display significantly lower permissivity to APPV\textsubscript{P100} when compared to SPEV cells. Non-culture adapted APPV\textsubscript{P17} obtained from early passage revealed the same CD46\textsubscript{pig} dependency as the culture adapted variant (APPV\textsubscript{P100}). With regard to infections with CSFV and BuPV, there are no differences in permissivity between the WT and the CD46\textsubscript{pig} knockout cell lines.
Fig. 5. Production of infectious particles and RNA replication of porcine pestiviruses in dependence on CD46<sub>pig</sub>. Wild type (WT) SPEV and PK15 as well as CD46<sub>pig</sub> knockout cell lines (SPEVΔCD46 #2, #7 and PK15ΔCD46 #2) were infected with APPV<sub>P100</sub>, CSFV Alfort-Tübingen (AlfT) and BuPV at an MOI of 1, respectively. (A) Supernatants were harvested 72 h p.i. to determine virus titers by using endpoint dilution assays in quadruplicates and in three repetitions. (B) Cells were collected at 72 h p.i. for RNA preparation and subsequent RT-PCR analysis. TaqMan based qRT-PCR assays were used for detection of CSFV and APPV genomes, whereas a SYBR Green based real-time RT-PCR was performed for detection of BuPV genomes. 30 ng total RNA was used per reaction. Samples collected from three individual experiments were tested in duplicates. Mean values with standard deviations are shown. APPV genome copy numbers obtained from WT cells are significantly higher when compared to CD46<sub>pig</sub> knockout cells (*** p < 0.0001, highly significant; * p < 0.01, significant). CSFV and BuPV genome levels obtained from WT cells did not show significant differences compared to genome loads detected in infected knockout cells.

Fig. 6. Impact of CD46<sub>pig</sub> at early time points of porcine pestivirus infections. (A) Immunofluorescence analysis of CSFV and BuPV infected cells. Wild type (WT) PK15 and PK15ΔCD46 #2 cells were infected with CSFV strains Alfort-Tübingen (AlfT), Diepholz, Riems, Koslov and Paderborn and BuPV at an MOI of 1. Infections with different CSFV strains and BuPV showed no dependency on CD46<sub>pig</sub> even very early after infection (16 h p.i.). (B) Fluorescence in situ hybridization (FISH) analysis of APPV infected cells. WT SPEV and PK15 as well as CD46<sub>pig</sub> knockout cell lines (SPEVΔCD46 #2 and PK15ΔCD46 #2) were infected with cell culture adapted APPV<sub>P100</sub> at a MOI of 0.5. Scale bars indicate 100 µm for lower magnification and 50 µm for higher magnification. A strong reduction of APPV<sub>P100</sub> infection is evident on both CD46<sub>pig</sub> knockout cell lines in comparison to WT cells at early time point of infection (16 h p.i.). APPV<sub>P100</sub> genomes were observed only on single CD46<sub>pig</sub> knockout cells within the infected wells. APPV<sub>P100</sub> infection of CD46<sub>pig</sub>-expressing WT SPEV cells at a later time point (72 h p.i.) and non-infected SPEV cells (NIC) served as controls.
Fig. 7. Comparison of E2 envelope protein sequences of pestiviruses. (A) Phylogenetic tree based on E2 amino acid sequences of known pestivirus species (APPV: AUL76967; bat: AKF85014, AYV99177; rodent: ATP66856, ATP66857, YP009109567; Pangolin: QIE06437; LindaV: YP009407716; whale: MK910228; BuPV: YP008992092; BDV: AAC16444; Aydin: YP006860588; ovine Italy: MG770617; Giraffe: NP620053; Pronghorn: YP009026415; BVDV-1: Q01499; BVDV-2: YP009513240; BVDV-3: AB871953; CSFV: YP009508222). APPV and CSFV sequence (bold) are the same as shown in the alignment. (B) Alignment (ClustalW) of APPV (isolate L277) and CSFV (Alfort 187) E2 amino acid sequences. Highlighted is the CSFV sequence analogous to the motif in the E2 of BVDV folding into a hairpin that might serve as ligand to the CD46 bov receptor (50). The positions of two non-synonymous mutations (N751K and D752N) which occurred during cell culture adaptation of APPV are highlighted by a box.
| Species       | Name   | Tissue                | Source/Reference       |
|--------------|--------|-----------------------|------------------------|
| pig          | SPEV   | embryonic kidney      | cell line 0008, FLI, Germany |
| pig          | PK15   | kidney                | cell line 5-1, FLI, Germany |
| pig          | SK6    | kidney                | Institute’s collection |
| pig          | 38A,D  | lymphoma              | (25)                   |
| pig          | NPTr   | neonatal trachea      | (26)                   |
| pig          | ST     | testis                | Institute’s collection |
| pig          | IPECJ2 | jejunum               | ACC-701, DSMZ           |
| cattle       | MDBK   | kidney                | ATCC: CCL-22           |
| cat          | CRFK   | kidney                | Institute’s collection |
| rabbit       | RK13   | kidney                | Institute’s collection |
| African green monkey | Vero76 | kidney                | Institute’s collection |
| hammer-headed fruit bat | HypNi/1.1 | kidney                  | (27) |
| straw-coloured fruit bat | EidNi/41 | kidney                  | (28) |
| human        | Lovo   | colon adenocarcinoma  | ATCC: CCL-229          |
| human        | HEK293T | embryonic kidney     | ACC-635, DSMZ           |
Table 2. Primers used in this study.

| Primer | Sequence (5' ->3') | Target | Purpose                        |
|--------|--------------------|--------|--------------------------------|
| 1539fw | CAACGTGGTCACCCAGGC | CSFV E                          | PCR, seq. of CSFV              |
| 2222rev| CCACCATGTGCTGTAACAC | CSFV E                          | PCR, seq. of CSFV              |
| 1114fw | GGTCTATAAGTATCAGGAG | APPV E                          | PCR, seq. of APPV              |
| 1594rev| TTAATCTCTTCTAGCTGC | APPV E                          | PCR, seq. of APPV              |
| 1459fw | TGGCTGCTGTAACATTTAC| APPV E                          | PCR, seq. of APPV              |
| 2002rev| TACCTGACCAAGCAGAGC | APPV E                          | PCR, seq. of APPV              |
| 1721fw | ATGGAGCAAGCATAAGGC | APPV E1                         | PCR, seq. of APPV              |
| 2251rev| TCCAAATCTGTGTAAGCCAC| APPV E1                         | PCR, seq. of APPV              |
| 2109fw | GATCTGAGTGTTAGAACAC | APPV E1                         | PCR, seq. of APPV              |
| 2554rev| TTTGCTTCTACCTTTCTT | APPV E1                         | PCR, seq. of APPV              |
| 2353fw | CCTGAAATAGTCTACCTTC | APPV E2                         | PCR, seq. of APPV              |
| 2912rev| GTAACTGGACCCATGCTTTC | APPV E2                         | PCR, seq. of APPV              |
| 2749fw | TTACTGAGTGAAAGCAGAC | APPV E2                         | PCR, seq. of APPV              |
| 3244rev| AAAGCTCAAGGCTACGGAC | APPV E2                         | PCR, seq. of APPV              |
| gCD46-2fw| CACGGCAGATTTCATGCCC | ccp1 K.O. CD46                  | PCR, seq. of CD46              |
| gCD46-2rev| AAACCGAAGTGTAGGATGGG | CCP1 K.O. CD46                  | PCR, seq. of CD46              |
| gCD46-7fw| CACGGCAGTTTCATGCCC | CCP1 K.O. CD46                  | PCR, seq. of CD46              |
| gCD46-7rev| AAGCCAGTTTCATGCCC | CCP1 K.O. CD46                  | PCR, seq. of CD46              |
| pigCD46_101fw| CCCCCATGGACCCACACTTCC | SP PCR                           | PCR, seq. of CD46              |
| pigCD46_192fw| GTTGAAGGTTCAAGGAGGTC | CCP1 PCR                         | PCR, seq. of CD46              |
| pigCD46_353rev| GGGTGTGACAGATGATATCG | CCP1 PCR                         | PCR, seq. of CD46              |
| pigCD46_387fw| ATCTACCGAGGCCTAATGCC | CCP2 PCR                         | PCR, seq. of CD46              |
| pigCD46_543rev| GGGCTAATTCAGGCTACAC | CCP2 PCR                         | PCR, seq. of CD46              |
| pigCD46_571fw| TAAACACGCTGCGGAGGATCC | CCP3 PCR                         | PCR, seq. of CD46              |
| pigCD46_604fw| CACAACTAGAAGGATGTTAG | CCP3 PCR                         | PCR, seq. of CD46              |
| pigCD46_710rev| AAGGCTGCTCTCTCAACGA | CCP3 PCR                         | PCR, seq. of CD46              |
| pigCD46_766fw| ATGCCATATCCAGTATCCAC | CCP4 PCR                         | PCR, seq. of CD46              |
| pigCD46_937rev| TATACCTGCTGCTGAGTTATC | CCP4 PCR                         | PCR, seq. of CD46              |
| pigCD46_1192rev| TTACACGCTACAGATTGATCG | CCP4 PCR                         | PCR, seq. of CD46              |
| T7fw | TAATAGCTGCTACATTAAG | TOPO Vector                     | PCR, seq. of CD46              |
| M13rev | AAGCTATAGACCTGTCATG | TOPO Vector                     | PCR, seq. of CD46              |

Underlined sequences indicate the target site sequences (20 bp). ccp: complement control protein, SP: signal peptide, K.O.: knockout, NTR: non-translated region.
|                | WT   | SPEVΔCD46 | PK15ΔCD46 |
|----------------|------|-----------|-----------|
| APPV<sub>p17</sub> |      |           |           |
| APPV<sub>p100</sub> |      |           |           |
| BuPV            |      |           |           |
| CSFV            |      |           |           |
| AlfT            |      |           |           |
| CSFV Diepholz   |      |           |           |
| CSFV Riems      |      |           |           |
| CSFV Kozlov     |      |           |           |
| CSFV Paderborn  |      |           |           |
### A

|          | WT PK15 | PK15ΔCD46 #2 |
|----------|---------|--------------|
| BuPV     | ![BuPV](image1) | ![BuPV](image2) |
| CSFV AlfT| ![CSFV AlfT](image3) | ![CSFV AlfT](image4) |
| CSFV Diepholz | ![CSFV Diepholz](image5) | ![CSFV Diepholz](image6) |
| CSFV Riems | ![CSFV Riems](image7) | ![CSFV Riems](image8) |
| CSFV Kozlov | ![CSFV Kozlov](image9) | ![CSFV Kozlov](image10) |
| CSFV Paderborn | ![CSFV Paderborn](image11) | ![CSFV Paderborn](image12) |

### B

|          | APPV 16hpi | APPV 72hpi |
|----------|------------|------------|
|          | SPEV | PK15 | SPEV |
| WT       | ![WT SPEV](image13) | ![WT PK15](image14) | ![WT SPEV](image15) |
| ΔCD46    | ![ΔCD46 SPEV](image16) | ![ΔCD46 PK15](image17) | ![ΔCD46 SPEV](image18) |

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*(Images representing the images shown in the figures are not provided in the natural text representation.)*
