Virus Research 263 (2019) 207–216

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

W protein expression by Newcastle disease virus

Julia Karsunke[a], Sandra Heiden[a], Magdalena Murr[a], Axel Karger[a], Kati Franzke[b], Thomas C. Mettenleiter[a], Angela Römer-Oberdörfer[a,*]

[a] Institute of Molecular Virology and Cell Biology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Südufer 10, D-17493 Greifswald-Insel Riems, Germany

[b] Institute of Infectology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Südufer 10, D-17493 Greifswald-Insel Riems, Germany

ARTICLE INFO

Keywords:
Newcastle disease virus
W protein

ABSTRACT

Differential editing of transcripts from the Newcastle disease virus (NDV) phosphoprotein gene results in mRNAs capable of encoding the phosphoprotein (P), the V protein, and the W protein which share a common N-terminus but specify different C-termini. Whereas the expression and viral incorporation of the P - and V proteins by NDV has been documented, evidence for the existence of a W protein was lacking. To analyze expression of the NDV W protein, two peptides encompassing predicted antigenic sites of the unique C-terminal W protein amino acid sequence of NDV Clone 30 were used for the generation of W-specific rabbit antisera. One of them detected plasmid-expressed W protein and identified W protein after infection by indirect immunofluorescence and Western blot analyses. W protein was absent in cells infected by a newly generated recombinant NDV lacking W protein expression. Furthermore, Western blot and mass spectrometric analyses indicated the incorporation of W protein into viral particles. Confocal microscopic analyses of infected cells revealed nuclear accumulation of W protein that could be attributed to a bipartite nuclear localization sequence (NLS) within its unique C-terminal part. Redistribution of the W protein to the cytoplasm within transfected cells confirmed functionality of the NLS after mutation of its two basic clusters. This finding was additionally corroborated in cells infected with a recombinant virus expressing the mutated W protein.

1. Introduction

Newcastle disease virus (NDV) causes one of the most fatal infectious diseases of poultry that can result in high economic losses. NDV is a virus of the genus Avian *Avulavirus*-1 (AAvV-1) within the family *Paramyxoviridae* of the order *Mononegavirales* (Afonso et al., 2016, ICTV online.org) which has been under investigation since its first description in 1927 (Doyle, 1927). The nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and the RNA-dependent RNA polymerase or large protein (L) are encoded by the NDV genome. Like other paramyxoviruses, NDV produces by co-transcriptional mRNA editing (Steward et al., 1993) from the P gene besides the P mRNA two further mRNAs, encoding potential V and W proteins. Editing occurs by insertion of one or two non-templated guanine residues (G) within the conserved editing site (3′-UUUUUCC-5′) during transcription, resulting in a shift of the respective open reading frame. Therefore, P, V, and W proteins have a common N-terminal sequence (amino acid (aa) 1 to 133) but a unique C-terminus varying in length and amino acid composition (Steward et al., 1993). Transcript frequencies from the P gene have been determined for several members of the family *Paramyxoviridae* like measles virus, Sendai virus, and NDV ranging from 60 to 70 % for unedited P encoding transcripts, 25–35 % for edited V transcripts (1 G insertion) and 2–8.5 % for edited W (2 G insertions) mRNAs (Steward et al., 1993; Selenda et al., 1998; Mebatsion et al., 2001; Bankamp et al., 2008). Occasionally, more than two G residues have been found inserted which leads to an additional amino acid (glycine) within the respective protein (Steward et al., 1993; Locke et al., 2000; Kulkarni et al., 2009).

P is a non-catalytic subunit of the RNA polymerase of paramyxoviruses and acts as a chaperone for NP to prevent uncontrolled encapsidation of non-viral RNA (Yabukarski et al., 2014; Guryanov et al., 2016). V protein, which is expressed by insertion of one non-templated guanine residue (+1 G) at the editing site, is characterized by a conserved cysteine-rich C-terminus (Cattaneo et al., 1989; Baron et al., 1993; Steward et al., 1993) that enables the binding of two zinc atoms ions (Steward et al., 1993; Liston and Briedis, 1994; Paterson et al., 1995; Steward et al., 1995; Huang et al., 2000). Furthermore, V

* Corresponding author at: Südufer 10, D- 17493, Greifswald-Insel Riems, Germany.
E-mail address: angela.roemer-oberdoerfer@fli.de (A. Römer-Oberdörfer).

https://doi.org/10.1016/j.virusres.2019.02.003
Received 25 September 2018; Received in revised form 23 January 2019; Accepted 11 February 2019
Available online 12 February 2019
0168-1702/ © 2019 Elsevier B.V. All rights reserved.

counteracts the cellular interferon response, explaining its role in NDV virulence (reviewed in Alamares et al., 2010; Samal and Samal, 2011). Steward et al. (Steward et al., 1993) detected an additional mRNA which arises from the P gene with two extra G residues inserted at the editing site and hypothesized it to encode a W protein (W) by analogy with the previously described Sendai virus W protein (Curran et al., 1991a). However, the NDV W could not be detected so far. In contrast, the existence of W was shown for Nipah virus and it was found that it plays a role in modulating immune responses (Shaw et al., 2005; Satterfield et al., 2015). Here, we show the existence of NDV W and demonstrate its cellular distribution as well as the properties of a newly generated W-deficient NDV.

2. Material and methods

2.1. Viruses and cells

Lentogenic vaccine strain NDV Clone 30 (Cl30) (Genbank Acc. No. Y18898) was obtained from MSD Animal Health (Boxmeer, The Netherlands). Recombinant NDVGu (rNDVGu) based on Cl30 has already been described (Ramp et al., 2012). QM9 cells (CCLV-RIE999, quail muscle cells clone 9) and CEF (chicken embryo fibroblasts) prepared from 10-day-old specific-pathogen-free (SPF) chicken embryos were used for virus characterization and grown or maintained in minimum essential medium supplemented with $NaHCO_3$, Na-Pyruvate, non-essential amino acids and 10% FBS. Embryonated SPF chicken eggs for CEF preparation and virus propagation were purchased from Lohmann (Cuxhaven, Germany) and incubated at 37 °C with 55% humidity. BSR-T7 cells (CCLV-RIE 582, BHK 21, clone BSR-T7/5) (Buchholz et al., 1999), which stably express phage T7 RNA polymerase were used for transfection and virus recovery. They were grown in Glasgow minimal essential medium supplemented with $NaHCO_3$, caseine peptone, meat peptone, yeast extract, essential amino acids and 10% FBS.

2.2. Preparation of polyclonal P-, V- and W-specific antisera

Antigenic regions within the specific C-terminal part of NDV Cl30 W protein were predicted based on the algorithm of Kolaskar and Tongaonkar (1990) using Geneious software (Created by the Biomatters development team, © 2005–2016 Biomatters Ltd.). Two peptides, containing two predicted antigenic regions each, were synthesized and linked *via* the SH group of cysteine to keyhole limpet hemocyanin (KLH) that was modified with maleimido groups (EMC microcollections, Tübingen, Germany).

Peptides used for preparation of αNDV_P and αNDV_V sera encode the specific C-terminal parts of NDV LaSota P and V proteins (which correspond to Clone 30) and were generated as described (Zhao et al., 2018). Briefly, the sequences encoding the unique C-terminal parts were cloned into pET19b downstream of the T7 promoter. The open reading frames (ORFs) were N-terminally linked by a SGS sequence to a heptamerization fragment of the human C4 binding protein (C4BP), and connected to a hexa-histidine- and Avi-tag in-frame. The expression plasmids were co-transfected with a pBirCam plasmid into *E. coli* Rosetta-gami, and protein expression was induced after addition of IPTG and Biotin to THY Medium for 4 h. The recombinant proteins were purified and refolded after ultrasonic disruption and solubilization of inclusion bodies.

Rabbits were immunized with the specific peptides, mixed with complete Freund's adjuvant, and boosted by peptides in incomplete Freund´s adjuvant four and eight weeks later. Sera were collected prior to every boost, and 12 weeks after the first immunization.

2.3. Construction of expression plasmids

Different genes of NDV Cl30 were cloned into the pCAGGS expression vector under control of the human cytomegalovirus immediate-early promotor/enhancer using the restriction sites *Eco*RI and *Nhe*I. Primers with *Eco*RI (5´-end, forward primer) and *Nhe*I restriction sites (3′ ends, reverse primer) were used to amplify the ORF coding for the NP protein (5´- CACGAATTCATGTCTTCCGTATTTGATGAG -3´, 5´- CACGCTAGCTCAATACCCCCAGTCGGTGTC -3´), the P protein (5´− CCTAGAATTCATGGCCACCTTTACAGATGC-3´, 5´- CAAGCTAGCTTAG CCATTTAGAGCAAGGCGC-3)´, the V protein (5´− CCTAGAATTCATG GCCACCTTTACAGATGC-3´, 5´- CAAGCTAGCTTACTTACTCTCTGTGA TATC-3´), the W protein (5´− CCTAGAATTCATGGCCACCTTTACAGA TGC-3´, 5´- CACGCTAGCTCACGTCTGTGCCCTGGTTTCCA-3´) or the N-terminal part (aa 1–133) of the P protein (5´− CCTAGAATTCATGGCC ACCTTTACAGATGC-3´, 5´- CACGCTAGCCTTTTTAGCATTGGACGATT TATTGC-3´). The ORFs of the V and W proteins were firstly subcloned into the vector pGEM-Teasy for mutagenesis using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA) and the primers MPVCl30 F (forward, 5´- CGTCCAATGC TAAgAAGGGgCCCATGGTCGAGC-3´) or MPWF (5´- CGTCCAATGC TAAgAAGGGggCCCATGGTCGAGCC-3´). Non-templated nucleotide insertions or mutations are represented by lowercase letters.

Expression constructs for N-terminally mCherry tagged W proteins were cloned by restriction sites *Msc*I (extreme N-terminus of mCherry and W ORF) and *Pas*I (N-terminus W ORF) from a precursor plasmid. The N-terminus of the Cl30 W ORF was substituted by the mCherry tagged insert within the pCAGGS expression vector to obtain plasmid pCAGGS_mCherryW_{Cl30}. The W ORF harboring a mutated nuclear localization signal (NLS) was produced synthetically (Invitrogen GeneArt Gene Synthesis, Regensburg, Germany), it is characterized by the following nucleotide exchanges: AG421/422GC, G485C, G496C as well as 5´-*Eco*RI and 3´-*Nhe*I restriction sites. The GC-content of the sequence was adjusted by silent mutations within the N-terminus to enable *in vitro* synthesis. The synthetic gene was inserted in a pCAGGS vector prior to linkage with the mCherry tag as described above. The resulting expression vector was named pCAGGS_mCherryW-NLS_{mut}.

2.4. Construction of full-length plasmids and recovery of recombinant viruses

The full-length plasmids pNDVPdedVded and pNDVW-NLS_{mut} were cloned on the basis of the lentogenic NDV Cl30, clone rNDVGu, and inserted into plasmid X8δT (Schnell et al., 1994). The full length plasmid of rNDVGu (Ramp et al., 2012) was digested with *Rsr*II and religated for mutation of the editing site sequence of the P gene using the primer MPded2PF (5´- CAGCAATAAATCGTCCAATGCGGGCCCAT GGTCGAGC-3´) and the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA) to delete nucleotide (nt) 2279–2284 (nt positions refer to Y18898). To generate plasmid pNDVPded, the *Rsr*II-shortened precursor plasmid was cleaved with *Aat*II and *Apa*I, and the corresponding region in rNDVGu was replaced by the resulting insert. The V gene of NDV Cl30 flanked by the non-coding regions of the P gene was amplified with *Mlu*I restriction site at the extreme 5´- and 3´-end and subcloned into pGEM-Teasy (Promega, Madison, USA) for the following deletion of 6 nt within the editing site by QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA) using the primer MPdedVF (5´- CAGCAATAAAT CGTCCAATGCGGGGCCCATGGTCGAGC-3´). The mutated V gene sequence was finally inserted in pNDVPded at an artificial *Mlu*I restriction site at position 3239 (nt position refers to Y18898) to obtain the full-length plasmid pNDVPdedVded. To generate a full-length plasmid with a mutated NLS within the W ORF the full-length pNDVGu was cleaved with *Apa*I and *Not*I to subclone the resulting fragment spanning from the P to the F gene. The fragment was inserted into a pUC18 vector with an altered multi cloning site to generate plasmid pUCPedF. An *Apa*I-*Ale*I fragment of the expression plasmid pCAGGS_mCherryW-NLS_{mut} carrying the NLS mutation but not changes in the GC-content was used to substitute the corresponding region in the previously assembled pUCPedF plasmid. The *Apa*I-*Not*I fragment of the resulting plasmid, named

pUCPW-NLSmutF was reinserted in the rNDVGu full-length plasmid to obtain the full length plasmid pNDVW-NLS$_{mut}$. The presence of the introduced mutations was confirmed by sequencing of the respective regions within the full-length plasmids.

To recover infectious NDV, full-length plasmids were transfected into BSR-T7 cells. Briefly, BSR-T7 cells were grown to ˜ 80% confluency in 6-well plates and transfected with 5 µg pNDVPdedVded or pNDVW-NLS$_{mut}$, and the helper plasmids coding for Cl30 NP, P and L (3,0 µg pCiteNP, 1,5 µg pCiteP, 0,5 µg pCiteL) at a DNA : lipofectamine ratio of 1 µg : 1,5 µl using Lipofectamine®3000 (Invitrogen, Karlsruhe, Germany) following the manufacturer's instructions. Cell monolayers were split 1:2 24 h post transfection (pt) and supernatants and one cell monolayer were harvested 72 h pt. To test the formation of infectious virus, the other monolayer was fixed with acetone-methanol (1:1) for indirect immunofluorescence assay using monoclonal antibody HN10 (Werner et al., 1999) directed against HN protein of NDV and a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG F (ab)$_2$ (Dako, Glostrup, Denmark). Virus propagation in embryonated SPF chicken eggs was performed as described (Römer-Oberdörfer et al., 1999). Shortly, 400 µl transfection supernatant was inoculated into the allantoic cavity of 10-day-old embryonated SPF chicken eggs, and allantoic fluids were harvested 5 days post inoculation. Hemagglutinating activity (CEC, 1992) was determined, and QM9 cells were infected with probably virus containing allantoic fluids and fixed with acetone-methanol (1:1) 18–20 h pi for indirect immunofluorescence assay (IFA) with NDV hyperimmune serum (HIS) and Alexa Fluor® 488 conjugated goat anti-rabbit IgG secondary antibody (Life technologies, Carlsbad, USA).

2.5. Sequence analysis of recovered recombinant viruses

Viral RNA was isolated from allantoic fluid after two passages in embryonated SPF chicken eggs using QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The nucleotide sequences of the mutated regions were amplified by RT-PCR using suitable primers and OneStep RT-PCR Kit (Qiagen, Hilden, Germany), eluated from a 1% agarose gel with the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced (Sequencer 3130Genetic Analyzer, Applied Biosystems, Foster City, USA).

2.6. Replication kinetics and virus titration

Replication kinetics in CEF cells were determined as described (Heiden et al., 2014). Briefly, CEF were infected with the respective virus at a multiplicity of infection (MOI) of 0.01. The inoculum was removed 40 min post infection (pi), and the monolayers were washed twice with medium prior to further incubation at 37 °C and 3% CO_2. Supernatants were harvested and frozen at indicated time points. After thawing, virus titers ($TCID_{50}$/ml) were determined on QM9 cells by indirect immunofluorescence using NDV HIS and Alexa Fluor® 488 conjugated goat anti-rabbit IgG secondary antibody (Life technologies, Carlsbad, USA).

2.7. Virus purification

NDV Cl30, rNDVPdedVded, and rNDVW-NLS$_{mut}$ were propagated in 10-days-old SPF embryonated chicken eggs, infected allantoic fluids were harvested 100 h pi, and clarified by centrifugation for 10 min at 3000 rpm. Virions were purified and concentrated on a sucrose cushion (65% (W/V) in TEN buffer (20 mM Tris, 150 mM NaCl, 0.1 mM EDTA, pH 7.4)) at 25,000 rpm for 2 h in a SW32.Ti rotor (Beckman Coulter, Optima LE-80 K ultracentrifuge) at 4 °C, followed by centrifugation through a preformed sucrose density gradient (20%, 30%, 40%, 50%, and 60% W/V) or CsCl density gradient (20%, 25%, 30%, 35%, 40%, 45% W/V) in TEN buffer for 1 h at 27,000 rpm and 4 °C in a SW40.Ti rotor (Optima LE-80 K). The purified virus was collected and transferred to a sterile dialysis-tubing (Thermo Scientific SnakeSkin, 7 K MWCO Dialysis Tubing), stored in TEN buffer for at least 9 h with twice buffer changes to remove remaining sucrose or CsCl. Total protein content was determined (NanoDrop™ Lite Spectrophotometer), and purified virions were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for further Western blot analyses.

2.8. Transfection

QM9 cells were transfected with 100 ng (96 well) or 2.5 µg (6 well) of respective expression plasmids using Lipofectamine®2000 according to manufacturer's instructions (Invitrogen) at a DNA : lipofectamine ratio of 1.0 µg : 2.0 µl. Cells were fixed with acetone-methanol for IFA, with 3.7% formaldehyde for confocal microscopy, or lysed in 1x Roti®-Load 1 (Roth) for Western blotting.

2.9. Western blotting

QM9 cells seeded in 6 well cell culture plates were transfected with respective expression plasmids or infected with respective viruses at an MOI of 1.0 and lysed at indicated time points in 1x Roti®-Load 1 (Roth, Karlsruhe, Germany). Purified virion solutions were mixed 1:1 with 1x Roti®-Load buffer. Samples were incubated 5 min at 95 °C prior to separation of the proteins by 10.4% SDS-PAGE. Gels were used subsequently for transfer of the proteins to a nitrocellulose membrane (Protran BA83, 0.2 µm, GE healthcare, Little Chalfont, UK). After incubation with appropriate primary antibodies and peroxidase-linked secondary antibodies (Dianova, Hamburg, Germany), proteins were detected using a chemiluminescence detection system (SuperSignal West Pico Chemiluminescent Substrate, Thermo Fischer, Waltham, USA) and a ChemiDoc XRS + (Bio-Rad, Hercules, USA).

2.10. Immunofluorescence and confocal microscopy

For IFA, QM9 cells were seeded in 96-well cell culture plates and transfected with respective expression plasmids or infected with NDV Cl30 or rNDVPdedVded at an MOI of 1.0. Cells were fixed by acetone-methanol 30 h pt or 24 h pi, and incubated with αNDV_W$_{Cl30}$, αNDV_P or αNDV_V, followed by incubation with secondary Alexa Fluor® 488-goat –α-rabbit-antibody, AlexaFluor®568-goat-α-rabbit-IgG or AlexaFlour®488-goat-α-mouse-IgG (Life technologies, Carlsbad, USA).

For confocal laser scan microscopy, QM9 cells were seeded onto sterile glass slides within 24-well plates, infected with NDV Cl30 or rNDVPdedVded at an MOI of 5.0 or transfected with pCAGGS expression vectors coding for NDV Cl30 P, V, W proteins or mCherry-tagged proteins. Cells were incubated at 37 °C and 3% CO_2 and fixed with 3.7% formaldehyde 24 h pi or 5–24 h pt. Following permeabilization using 0.1% Triton X-100, slides were blocked with 5% BSA in 1x PBS and incubated with a P-specific monoclonal antibody (mabNDVP, (Werner et al., 1999)), a P-specific antiserum (αNDV_P), a V-specific antiserum (αNDV_V) or αNDV_W$_{Cl30}$. After three washing steps with 5% BSA, samples were incubated with respective Alexa Fluor®488-goat-α-mouse-IgG (Life technologies, Carlsbad, USA) and AlexaFluor®568-goat-α-rabbit-IgG (Life technologies, Carlsbad, USA) and mounted with Mowiol following further washing steps. Images were taken on a Leica SP5 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) with an oil immersion objective (HCX PL APO 63x/1.40-0.60 objective). Sequential z-sections of stained cells were acquired for maximum projection and images were processed using ImageJ software.

2.11. Fluorescence intensity histograms

Fluorescence intensities of a representative z-section of the

maximum projections were analyzed with ImageJ software. A straight line of 70 µm length was drawn along the maximal cell expansion for generation of a plot profile. Every sixth value of the detected signal intensities in the direction of the arrow was graphed within a histogram.

2.12. Electron microscopy

Purified virions were adsorbed to Formvar coated nickel grids for 10 min. Grids were then washed once with 1x PBS and sterile distilled water and incubated with glutaraldehyde at a final concentration of 2.5%. After negative staining with 1% phosphotungstic acid (pH 6.0), the grid was dried and analyzed with a Tecnai-Spirit (FEI, Eindhoven, The Netherlands) transmission electron microscope at an acceleration voltage of 80 kV.

2.13. Mass spectrometry

Mass spectrometric analysis was carried out using a nano-LC MALDI-TOF/TOF platform (Bruker, Bremen, Germany). Regions of interest were excised from a SDS-PAGE gel and subjected to standard trypsin in-gel digestion (Shevchenko et al., 2006). Proteins were identified essentially as described elsewhere (Henning et al., 2014). In short, peptides were separated by nano-LC (easy-nLC, Bruker), spotted to a MALDI target with a proteineer fc-II robot and analysed by MALDI-TOF/TOF MS on an UltrafleXtreme instrument (Bruker). Spectra were processed with Flexanalysis software and proteins were identified using a Mascot server (version 2.4, Matrixscience, UK). Carbamidomethylation of cysteine was set as fixed modification, oxidation of methionine and acetylation of protein N-termini as variable modifications. One missed trypsin cleavage was tolerated. Mass tolerances were set to 25 ppm and 0.7 Da for peptide and fragment spectra respectively, a significance level of 95% was used. The sequence database was compiled from the *Gallus gallus* proteome (The UniProt, 2017) and the proteins specified by the used mutants. Results were drawn from the Mascot server by Proteinscape software (Bruker) for further evaluation.

3. Results

3.1. Generation of a W-specific antiserum

Specific antisera were generated against the C-terminal part of the W protein of NDV Clone 30. Antigenic regions were predicted based on the algorithm of Kolaskar and Tongaonkar (Kolaskar and Tongaonkar, 1990) using Geneious software (Biomatters Ltd. © 2005–2016). Two peptides harboring two antigenic regions each (Fig. 1A) were synthetically produced and used for immunization of rabbits. Subsequently, collected sera were tested for reactivity and specificity. To this end, QM9 cells were transfected with several expression plasmids, and cells were fixed or lysed for subsequent IFA or Western blotting. Only peptide serum 2, henceforth referred to as αNDV_W_{Cl30}, specifically reacted in the IFA with cells expressing the putative W protein (Fig. 1B). A protein of approximately 30 kDa could be identified by Western blot analysis, thus confirming the specificity of serum 2 (Fig. 1C). Although the molecular mass of the NDV Cl30 W protein is calculated as 19.2 kDa, the slower migration is a known phenomenon of P proteins of negative-strand RNA viruses and their editing products as a result of N-terminal stretches rich in negatively charged amino acids and phosphorylation (Emerson and Schubert, 1987; Curran et al., 1991b; Huber, Cattaneo et al. 1991; Pickar et al., 2014). Possible unspecific binding of this serum to other P gene products like P_{Cl30}, V_{Cl30} or the common C-terminal 133 aa of P, V and W ($P_{Cl30\ aa1\text{-}133}$) was not observed after transfection (Fig. 1B, C). Possible cross-reactivity with the NP protein of NDV Cl30 was investigated after transfection of pCAGGS_NP_{Cl30}, resulting in no signal in Western blot and IF analyses, indicating the suitability of this serum for the detection of NDV Cl30 W protein (Fig. 1B, C). NDV hyperimmune serum (HIS) confirmed the expression of the corresponding proteins. Subsequently, W protein expression was analyzed during infection. QM9 cells were infected at an MOI of 1.0 for IFA. Whereas no W protein was detected in non-infected cells, W protein was clearly demonstrated in NDV Cl30 infected cells, identifying W protein expression in NDV infected cells for the first time (Fig. 1D).

3.2. Generation of a W deficient recombinant virus and W protein detection in infected cells

To study W protein function during *in vitro* replication, the recombinant rNDVPdedVded was generated which lacks W protein expression. rNDVPdedVded is derived from the recombinant virus rNDVGu that corresponds to NDV Clone 30 (Ramp et al., 2012). It was altered by a 6 nt deletion within the P gene editing site (Fig. 2A). Since this deletion results in elimination of any editing, it would not only prevent expression of W but also of V. Therefore, a complete V gene, flanked by the non-coding regions of the P gene, was inserted at an artificial *Mlu*I restriction site, resulting in a virus genome consisting of an additionally inserted V gene between P and M genes. In addition, the V gene possesses a deletion in the editing site sequence, and its sequence is characterized by an extra guanine residue to secure the necessary frameshift of V ORF. Virus was rescued by reverse genetics using the generated full-length plasmid.

QM9 cells were infected with rNDVGu or rNDVPdedVded at an MOI of 1.0. Cell lysates were harvested at the indicated time points and subjected to SDS-PAGE, Western blotting and staining by αNDV_W_{Cl30}, and a peroxidase-linked secondary antibody. In agreement with the previous transfection study (Fig. 1C), a ~30 kDa W protein could be identified after infection of QM9 cells with NDV Cl30, whereas the recombinant rNDVPdedVded did not show W protein expression (Fig. 2B), as expected.

For a more detailed analysis by confocal laser scanning microscopy, QM9 cells were infected at an MOI of 5.0, fixed 24 h pi, and incubated with a P protein specific monoclonal antibody (mabNDV_P (Werner et al., 1999)), and serum αNDV_W_{Cl30}, as well as corresponding secondary antibodies coupled to AlexaFluor®488 (P, green) or AlexaFluor®568 (W, red) fluorophores. For NDV Cl30 infected cells, a specific intranuclear staining was shown with the W-specific antiserum, demonstrating W protein accumulation in the nucleus. Again, rNDVPdedVded infected cells lack any W protein reactivity (Fig. 2C).

To gain an insight whether W protein expression influences *in vitro* replication, growth kinetics of rNDVPdedVded were analyzed in CEF as described, and titrated on QM9 cells for calculation of the $TCID_{50}$/ml. As reference virus, rNDVGu was included. Replication patterns and final titers at 72 h pi were found to be similar for both viruses (Fig. 2D).

3.3. Analysis of purified Cl30 and rNDVPdedVded virions

Virions of Cl30 and rNDVPdedVded were purified from allantoic fluids by density gradient centrifugation and purity of the sample preparation was demonstrated by electron microscopic analyses (Fig. 3A). First evidence for the existence of W came from mass spectrometric analysis of SDS-PAGE separated proteins. After in-gel digestion of a gel slice corresponding to a molecular weight of approximately 30 kDa and analysis by nano-LC MALDI-TOF/TOF mass spectrometry, two peptides of the specific C-terminus of W (aa 135–179) were identified (Fig. 3C). This finding was confirmed by Western blot analyses of purified virions with αNDV_W_{Cl30} demonstrating that W was present in NDV Cl30 virions whereas no reactivity was observed with purified rNDVPdedVded virion preparations (Fig. 3B).

Western blot analyses of QM9 cells infected with NDV Cl30 or with rNDVPdedVded showed that αNDV_W_{Cl30} detected an additional band of about 55 kDa (Fig. 2B, 3B) that was not visible in cells transfected with only protein expressing plasmids (Fig. 1C). Mass spectrometric analyses showed the presence of large amounts of NP in this molecular

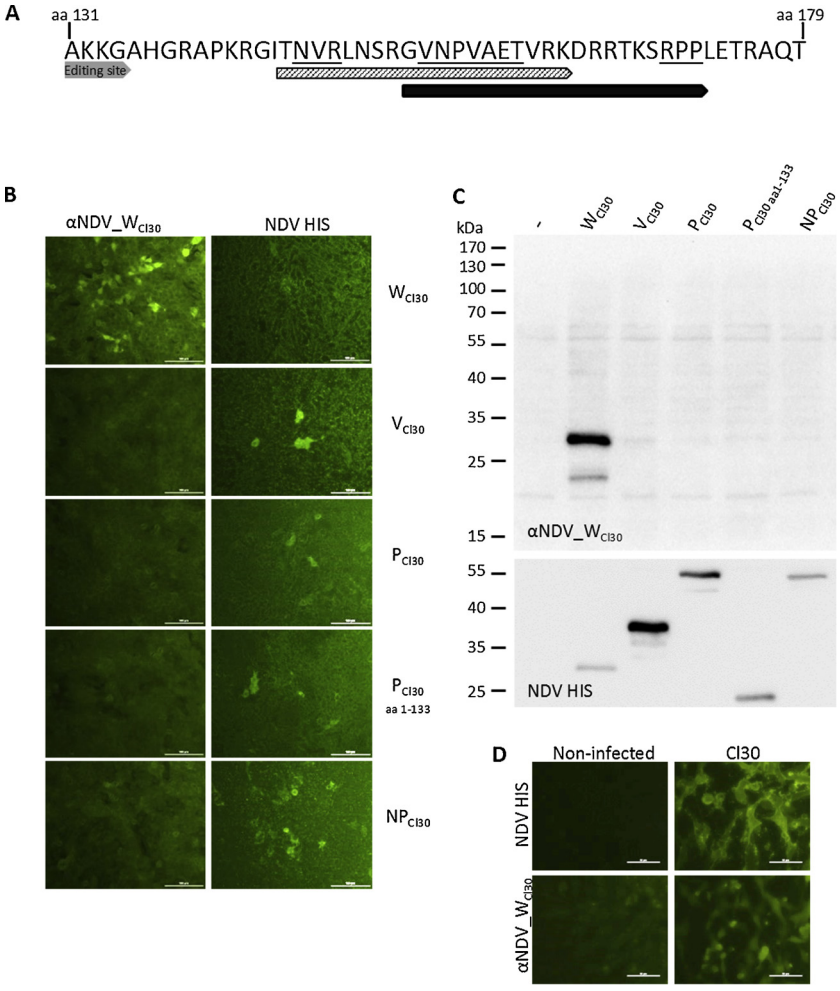

Fig. 1. Generation and specificity testing of an NDV Cl30W protein specific serum.
A) Prediction of antigenic regions (underlined) within the unique C-terminal part (aa 135–179) of the Cl30 W protein and definition of sequences for synthetic peptide 1 (dashed arrow) and 2 (black arrow) used for rabbit immunization. B) Indirect immunofluorescence assay using αNDV_W_{Cl30} after transfection of QM9 cells with expression plasmids coding for NDV Cl30 W, V P, $P_{1\text{-}133aa}$ and NP proteins. C) Reactivity and specificity test of αNDV_W_{Cl30} in Western blot analysis utilizing lysates of accordingly transfected QM9 cells. The molecular weight of marker proteins is indicated in kDa. Non-transfected cells (-) served as negative control. D) Detection of W protein in NDV Cl30 infected QM9 cells following staining with αNDV_W_{Cl30}. Non-infected cells served as negative control. Transfection and infection efficacies were controlled using NDV HIS.

weight range (data not shown) so that we assume NP to cause this unspecific antibody binding, probably by a different protein structure or expression level after infection.

3.4. Nuclear localization of the W protein and identification of an NLS within the specific C-terminus of the W protein

Since confocal microscopy pointed to a nuclear localization of the W protein in infected cells, this finding was addressed in detail after transfection of QM9 cells with pCAGGS expression constructs coding for NDV Cl30 P, V and W proteins. Monolayers were fixed 24 h after transfection and stained with antisera, specific for either P (αNDV_P), V (αNDV_V) or W (αNDV_W_{Cl30}) for subsequent confocal microscopy. In contrast to P and V that localized exclusively (P) or predominantly (V) to the cytoplasm, W displayed a pronounced nuclear accumulation after transfection (Fig. 4 A).

NDV Cl30 W possesses a calculated molecular mass of 19.2 kDa and is therefore able to pass through nuclear pores passively. However, the detected strong nuclear signal indicated the presence of a specific nuclear localization signal (NLS). The online tool cNLS mapper (Kosugi et al., 2009) was used for the prediction of an importin α-dependent NLS, and a bipartite sequence motif with two basic amino acid clusters could be identified in the specific C-terminal part of the W protein (Fig. 4B).

In order to demonstrate functionality of the predicted bipartite NLS, plasmid-based constructs for expression of N-terminally mCherry-tagged W protein with the wild type or a mutated NLS motif were engineered (Fig. 4B). For mutation of the NLS, both basic amino acid clusters were disrupted by nucleotide changes leading to amino acid alterations (Fig. 5A). Hence, αNDV_W_{Cl30} serum displayed a decreased reactivity to the mutated W protein, since the antigenic region of peptide 2 overlaps with one of the basic clusters (Fig. 4B). To avoid that problem, the mCherry-tagged proteins were used for further analyses.

QM9 cells were transfected with pCAGGS_mCherryW_{Cl30} or pCAGGS_mCherryW-NLS_{mut}, fixed at the indicated time points and analyzed by confocal microscopy (Fig. 4C). Signal intensities were measured along the drawn white arrows using ImageJ (Schneider et al., 2012) and displayed by histogram. Increasing nuclear accumulation over time could be seen for W protein with the wild type NLS sequence, whereas the mutated W protein was evenly distributed all over the cell body (Fig. 4C).

3.5. Construction of a recombinant virus lacking a functional W NLS

To study the effect of the altered cellular distribution of W protein in infected cells, a recombinant virus with a mutated NLS within the W ORF (within the P gene) was constructed (rNDVW-NLS_{mut}) (Fig. 5A). Like rNDVPdedVded, rNDVW-NLS_{mut} was derived from the recombinant virus rNDVGu that corresponds to NDV Clone 30. Introduced nucleotide changes AG421/422GC, G485C and G496C (nt numbers refer to the W ORF) resulted in three aa changes within the two basic clusters of the NLS in the same way as for the pCAGGS_mCherryW-NLS_{mut} expression construct. These alterations also result in one or two aa changes in P and V, respectively (Fig. 5A).

Western blot analyses of lysates of QM9 cells infected at an MOI of 1.0 using αNDV_W_{Cl30} demonstrated expression of the mutated ˜30 kDa W protein (Fig. 5B, stars). Decreased signal intensities are due to the lower reactivity of αNDV_W_{Cl30} with the mutated sequence.

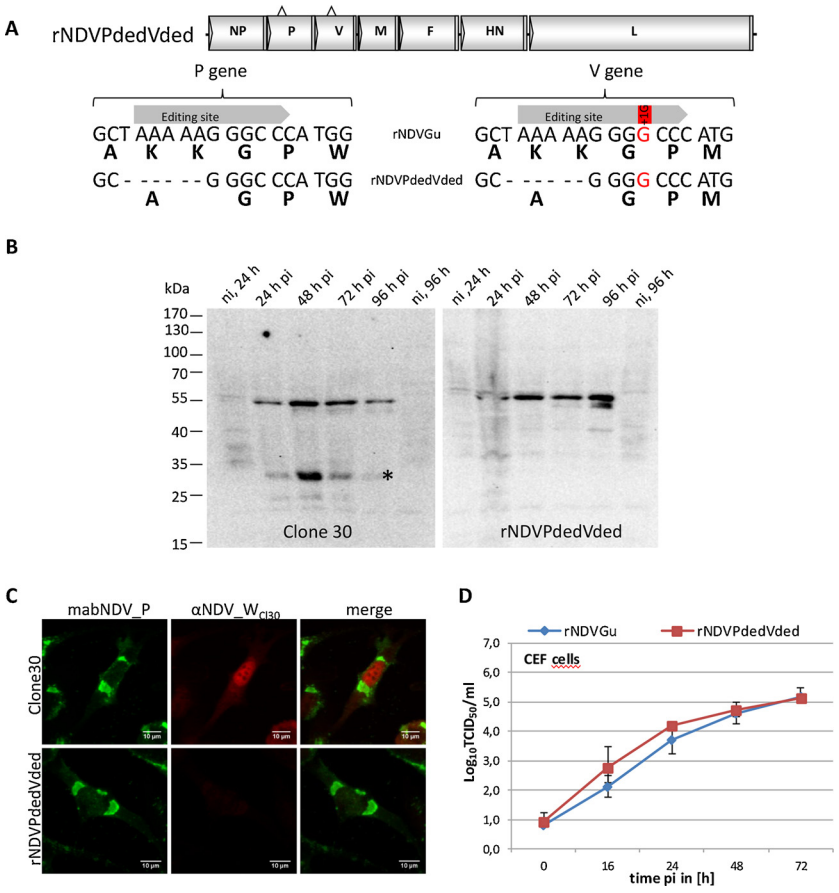

Fig. 2. Generation and characterization of recombinant virus rNDVPdedVded lacking W protein expression.
A) Schematic representation of the genome organization of the newly generated recombinant rNDVPdedVded. Deleted editing site sequences are marked by triangles. Corresponding nt and aa sequences are given in comparison to rNDVGu. B) Lysates of QM9 cells infected with NDV Cl30 or rNDVPdedVded (MOI 1.0) were harvested 24–96 h pi and subjected to SDS-PAGE and Western blotting using αNDV_W_{Cl30}. The star (*) marks W specific protein bands. Non-infected cells (ni) served as negative control. The molecular weight of marker proteins is indicated in kDa. C) Cellular distribution of P or W proteins was analyzed in Cl30 or rNDVPdedVded infected QM9 cells (MOI 5.0) after staining with αNDV_W_{Cl30} and mabNDV_P by confocal microscopy. D) Replication kinetics were determined on CEF infected at an MOI of 0.01 with the respective viruses. Cell culture supernatants were harvested at the indicated time points and $TCID_{50}$/ml was determined by titration on QM9 cells followed by immunofluorescence. Error bars represent minima and maxima.

Redistribution of rNDVW-NLS_{mut} W protein was addressed after infection of QM9 cells at an MOI of 5.0 for 24 h and staining with αNDV-P (Werner et al., 1999), and αNDV_W_{Cl30}, as well as corresponding secondary antibodies coupled to AlexaFluor®488 (P, green) or AlexaFluor®568 (W, red) fluorophores. While nuclear accumulation was confirmed for Cl30 W, mutated W of rNDVW-NLS_{mut} was found evenly distributed within cytoplasm and nucleus (Fig. 5C) but also incorporated into the virion as again demonstrated for NDV Cl30 (Fig. 5E). To analyze whether the altered intracellular distribution of NDV W has an impact on viral replication, *in vitro* growth kinetics were performed. In comparison with rNDVGu, no replication defects of rNDVW-NLS_{mut} could be detected in CEF (Fig. 5D).

4. Discussion

Here, we demonstrate for the first time the expression of the hitherto hypothetical ~ 30 kDa NDV W protein using the NDV Cl30 W protein specific peptide antiserum αNDV_W_{Cl30} which can be used for

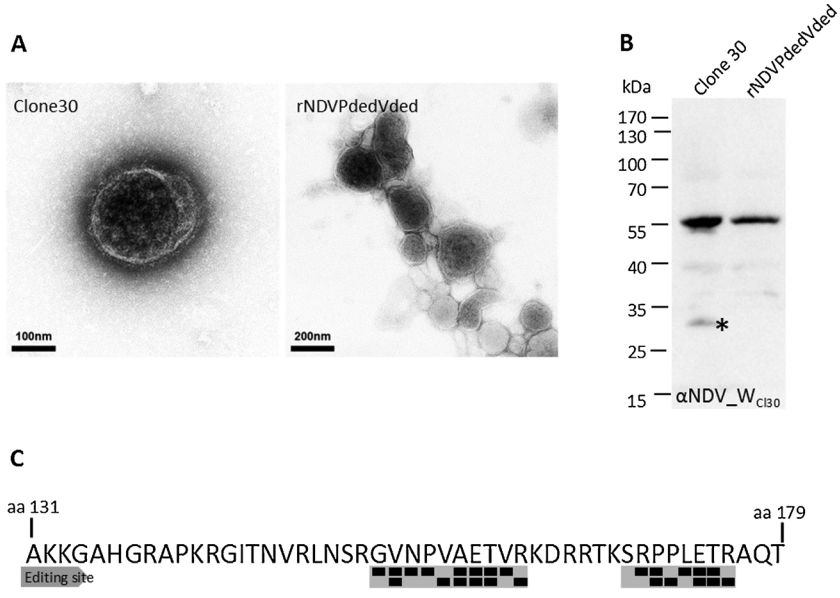

Fig. 3. Characterization of purified Cl30 and rNDVPdedVded virions.
A) Purified virions of NDV Cl30 and rNDVPdedVded adsorbed to Formvar coated nickel grids were subjected to negative staining with phosphotungstic acid prior to analysis by electron microscopy. B) Incorporation of W proteins into viral particles was tested with lysates of purified Cl30 or rNDVPdedVded virions from allantoic fluids by Western blotting using αNDV_W_{Cl30}. The star (*) marks the W specific protein band. C) Following SDS-PAGE, region of interest was excised from the gel, subjected to trypsin in-gel-digestion and mass spectrometric analysis using a nano-LC MALDI-TOF/TOF platform. The amino acid sequence of the specific C-terminal part of NDV Cl30 W protein is given as reference from aa 131 to aa 179. Grey bars indicate the sequence regions covered by the two peptides identified by mass spectrometry. Black boxes inside grey bars indicate the amino acids that could be identified by fragmentation of the peptides in the TOF/TOF mass spectrometry mode.

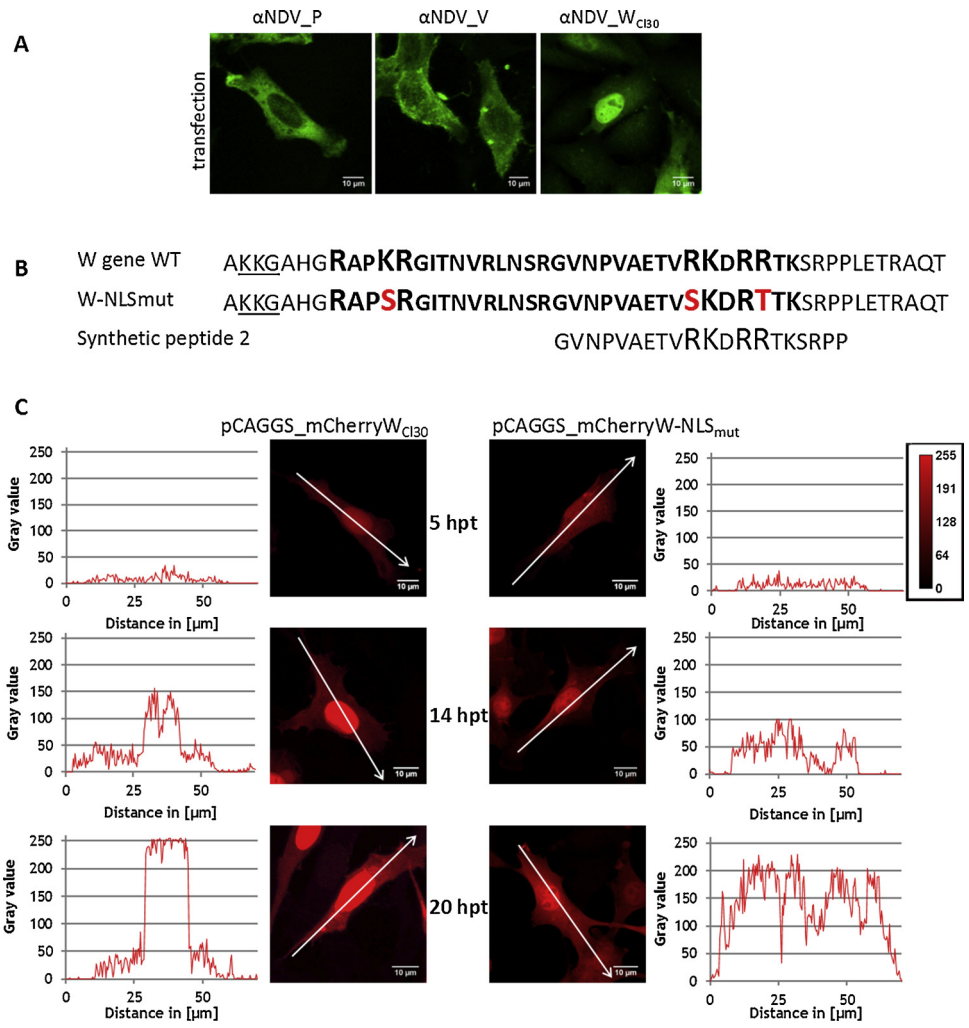

Fig. 4. Identification and functionality testing of a bipartite nuclear localization sequence within the unique C-terminal part of the W protein.
A) Cellular distribution of P, V or W proteins was analyzed 24 h pt in transfected QM9 cells after staining with αNDV_W$_{Cl30}$ (W), αNDV_V (V) or αNDV_P (P) by confocal microscopy. B) Amino acid sequences of the wild type NLS predicted by cNLSmapper (in bold) with two basic clusters (larger letters) following the editing site (underlined), the mutated NLS (amino acid changes in red) and the synthetic peptide 2 used for immunization. C) Confocal images and fluorescence intensity histograms of QM9 cells along the indicated arrows after transfection with mCherry-tagged W protein expression constructs fixed 5–20 h pt. The calibration bar indicates which color corresponds to each gray-scale value.

W detection in Western blot, classical IFA and confocal microscopy. Although W-specific mRNAs had been detected already in 1993 (Steward et al., 1993), NDV W has not been described so far in contrast to W proteins of other paramyxoviruses like Sendai virus, measles virus, human and bovine parainfluenza viruses or Nipah virus (Pelet et al., 1991; Galinski et al., 1992; Delenda et al., 1998; Bankamp et al., 2008; Kulkarni et al., 2009). To date, only the W protein of Nipah virus was studied more extensively with respect to its cellular distribution or potential interferon antagonistic functions.

A specific intranuclear staining with the W-specific antiserum revealed NDV W protein accumulation in the nucleus, as demonstrated for Nipah virus W (Shaw et al., 2004; Lo et al., 2009). In accordance with findings for Nipah virus, an importin α-dependent bipartite nuclear NLS was predicted within the specific C-terminal part of NDV Cl30 W protein and proven as functional after mutation of the two respective basic amino acid clusters.

Furthermore, purified virion preparations of NDV Cl30 indicated the incorporation of W protein into the viral particle. Incorporation of V has been observed for several paramyxoviruses (Thomas et al., 1988; Takeuchi et al., 1990; Paterson et al., 1995; Mebatsion et al., 2001; Samal and Samal, 2011), whereas virion localization of W has been reported for Nipah virus only (Lo et al., 2009). Incorporation of accessory proteins could potentially be attributed to the shared amino-terminus, since P, V and W of different paramyxoviruses are known to interact N-terminally with monomeric NP (Curran et al., 1991a,b; Precious et al., 1995; Horikami et al., 1996; Randall and Bermingham, 1996; Watanabe et al., 1996; Tober et al., 1998; Witko et al., 2006). However, for NDV, the N-terminal region of P, and therefore V and W, as well, appears not to be involved in NP binding (Jahanshiri et al., 2005). Another possibility is the RNA binding ability of accessory proteins like published for the simian virus 5 (Lin et al., 1997), hPIV2 (Nishio et al., 2006) or measles virus V proteins (Parks et al., 2006). However, the extent of incorporation of V and W proteins into NDV virion particles requires further structural and functional studies.

In this study, recombinant viruses either completely lacking expression of W protein (rNDVPdedVded) or expressing a W protein redistributed to the cytoplasm due to an NLS mutation (rNDVW-NLS$_{mut}$) were successfully rescued. The absence of W protein expression from rNDVPdedVded in infected cells, as well as in purified virions was confirmed by Western blotting and confocal microscopy. An even cellular distribution of the NLS-mutated W protein was validated by confocal microscopic analysis. Both viruses showed no differences in *in vitro* replication studies, indicating no essential functions of W protein expression itself or W protein localization within infected cells. Despite the mutation of the NLS, W was incorporated into the virion (Fig. 5E) as shown for the parental NDV Cl30 (Fig. 3B, 5E). Therefore, it seems not to be important, whether W was mainly localized in the nucleus or in the cytoplasm. Although the function of the nonessential NDV W protein remains unclear, a possible role in virus-host interaction is conceivable. The size and sequence variability of NDV W proteins may play a role within infected cells by impacting on the cellular machinery of different hosts in different ways. Some NDV W proteins lack a long C-terminus, because translation is stopped already after few aa, resulting in absence of any NLS or presence of only the first basic cluster. Even W

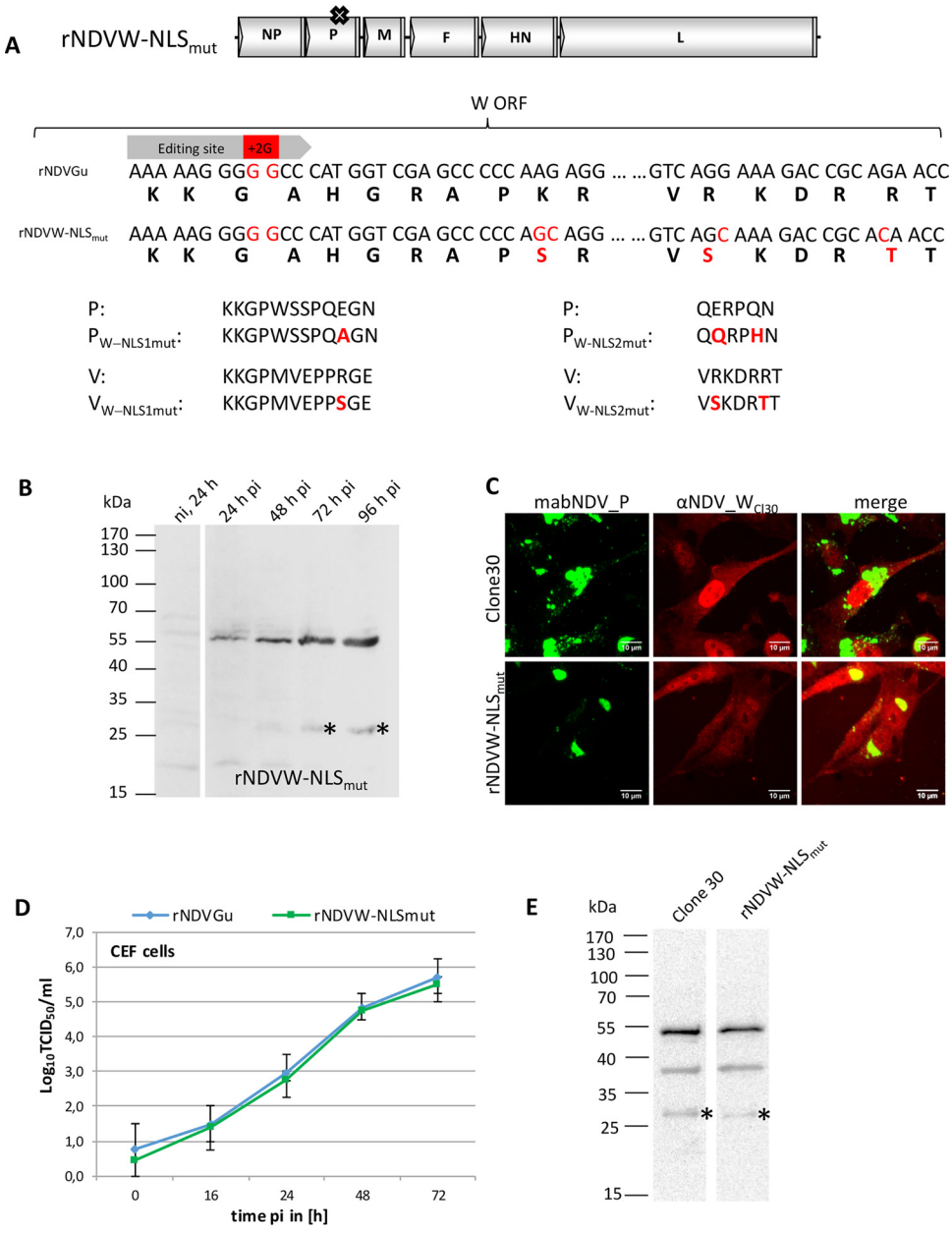

Fig. 5. Generation and characterization of recombinant virus rNDVW-NLSmut expressing a W protein with mutated NLS motif.
A) Schematic representation of genome organization of new generated recombinant rNDVW-NLS$_{mut}$. The mutated NLS motif within the P gene is marked by a cross. Corresponding nt and aa sequences of the W ORF are given in comparison to rNDVGu. Consequential alterations in P and V aa sequence are given below. B) Lysates of QM9 cells infected with rNDVPdedVded (MOI 1.0) were harvested 24–96 h pi and subjected to SDS-PAGE and Western blotting using αNDV_W$_{Cl30}$. Non-infected cells (ni) served as negative control. Stars (*) mark W specific protein bands. The molecular weight of marker proteins is indicated in kDa. C) Cellular distribution of P or W proteins was analyzed in NDV Cl30 or rNDVW-NLS$_{mut}$ infected QM9 cells after staining with αNDV_W$_{Cl30}$ and mabNDV_P by confocal microscopy. D) Replication kinetics was performed on CEF infected at an MOI of 0.01 with the respective viruses. Cell culture supernatants were harvested at the time points indicated, and TCID$_{50}$/ml was determined by titration on QM9 cells followed by immunofluorescence. Error bars represent minima and maxima. E) Incorporation of W proteins into viral particles was tested with lysates of purified Cl30 or rNDVW-NLS$_{mut}$ virions from allantoic fluids by Western blotting using αNDV_W$_{Cl30}$. The star (*) marks the W specific protein band.

proteins with a size encompassing both basic clusters do not always specify two functional motives. Most of them specify the first basic cluster. Interestingly, virulent NDV like Herts 33/56 (AY741404) and Italy (EU293914), are characterized by a W protein sequence that codes for a bipartite NLS with slight differences. However, there are also velogenic isolates like ZJ1 (AF431744) that have a different sequence in the second basic cluster.

The amino acid sequence at the F protein cleavage site represents the major virulence determinant of NDV (Nagai et al., 1976; Peeters et al., 1999). However, the V protein, derived from co-transcriptionally edited mRNAs of the P gene was found to play a role in modulating and inhibiting the interferon response of the host (Mebatsion et al., 2001; Huang et al., 2003), which qualifies it as a critical virulence factor as well. Interactions of NDV V with MDA5, prevention of IRF3 phosphorylation or induction of degradation of phosphorylated STAT molecules are some of the so far studied antagonizing functions (Childs et al., 2009; Irie et al., 2012; Qiu et al., 2016), and several of these mechanisms are conserved for all members of the *Paramyxoviridae* (Andrejeva et al., 2004; Yoneyama et al., 2005). Since not only V expression and functions are conserved among paramyxoviruses but also the expression of W proteins, this could indicate that W represents also a potential virulence factor *in vivo*. Studies on Nipah virus W protein pointed to an interferon antagonistic effect that parallels its nuclear accumulation, resulting from the C-terminal importin α- dependent NLS (Audsley et al., 2016). Among others, Nipah virus W protein was found to interact with the nuclear complex PR19 (Martinez-Gil et al., 2017) or functions as a complexing agent for STAT molecules within the nucleus (Ciancanelli et al., 2009). In addition, inhibition of IRF3 phosphorylation and hence IRF3 activation has been described (Shaw et al., 2004, 2005) and infection with a recombinant Nipah virus lacking W extended the survival time in comparison to infection with wild type virus, while increasing neurological symptoms in a ferret infection model (Satterfield et al., 2015). With the detection of NDV W for the first time here, it can now be studied which functions this protein during virus infection performs and whether functions depend on virus strain.

5. Conclusions

Here, we demonstrate for the first time the expression in infected cells of the hitherto hypothetical NDV W protein by Western blot, indirect immunofluorescence and confocal microscopy using a specifically generated NDV W specific peptide antiserum, and by mass spectrometry. NDV Cl30 W localized to the nucleus and could be detected in purified virions. Recombinant viruses lacking expression of W protein (rNDVPdedVded) or expressing a W protein with disrupted NLS (rNDVW-NLS$_{mut}$) displayed no replication defects *in vitro*, indicating no essential functions in this process, at least for lentogenic NDV Cl30. Thus, our studies serve as a basis for future functional analyses of NDV W *in vitro* and *in vivo*.

Funding information

These studies were funded by the Deutsche Forschungsgemeinschaft (DFG grant RO 4012/3-1).

Authors' statement

ARO, TCM, and JK designed the study, JK, AK, KF, SH, and MM performed experiments and analyzed data, JK, ARO, and TCM wrote the manuscript. All authors reviewed, and modified the manuscript and agreed to submit this manuscript to the journal Virus Research.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgement

We like to thank Martina Lange, Barbara Bettin, Petra Meyer and Mandy Jörn for excellent technical assistance, we are grateful to Dr. Stefan Finke for kindly providing the pCAGGS vector. Furthermore, we thank all colleagues from the animal core facility (ATB) for the immunization experiments and conscientiously taking care of the animals.

References

Afonso, C.L., Amarasinghe, G.K., Banyai, K., Bao, Y., Basler, C.F., Bavari, S., Bejerman, N., Blasdell, K.R., Briand, F.X., Briese, T., Bukreyev, A., Calisher, C.H., Chandran, K., Cheng, J., Clawson, A.N., Collins, P.L., Dietzgen, R.G., Dolnik, O., Domier, L.L., Durrwald, R., Dye, J.M., Easton, A.J., Ebihara, H., Farkas, S.L., Freitas-Astua, J., Formenty, P., Fouchier, R.A., Fu, Y., Ghedin, E., Goodin, M.M., Hewson, R., Horie, M., Hyndman, T.H., Jiang, D., Kitajima, E.W., Kobinger, G.P., Kondo, H., Kurath, G., Lamb, R.A., Lenardon, S., Leroy, E.M., Li, C.X., Lin, X.D., Liu, L., Longdon, B., Marton, S., Maisner, A., Muhlberger, E., Netesov, S.V., Nowotny, N., Patterson, J.L., Payne, S.L., Paweska, J.T., Randall, R.E., Rima, B.K., Rota, P., Rubbenstroth, D., Schwemmle, M., Shi, M., Smither, S.J., Stenglein, M.D., Stone, D.M., Takada, A., Terregino, C., Tesh, R.B., Tian, J.H., Tomonaga, K., Tordo, N., Towner, J.S., Vasilakis, N., Verbeek, M., Volchkov, V.E., Wahl-Jensen, V., Walsh, J.A., Walker, P.J., Wang, D., Wang, L.F., Wetzel, T., Whitfield, A.E., Xie, J.T., Yuen, K.Y., Zhang, Y.Z., Kuhn, J.H., 2016. Taxonomy of the order Mononegavirales: update 2016. Arch. Virol. 161 (8), 2351–2360.

Alamares, J.G., Elankumaran, S., Samal, S.K., Iorio, A.R.M., 2010. The interferon antagonistic activities of the V proteins from two strains of Newcastle disease virus correlate with their known virulence properties. Virus Res. 147 (1), 153–157.

Andrejeva, J., Childs, K.S., Young, D.F., Carlos, T.S., Stock, N., Goodbourn, S., Randall, R.E., 2004. The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. Proc Natl Acad Sci U S A 101 (49), 17264–17269.

Audsley, M.D., Jans, D.A., Moseley, G.W., 2016. Nucleocytoplasmic trafficking of Nipah virus W protein involves multiple discrete interactions with the nuclear import and export machinery. Biochem. Biophys. Res. Commun. 479 (3), 429–433.

Bankamp, B., Lopareva, E.N., Kremer, J.R., Tian, Y., Clemens, M.S., Patel, R., Fowlkes, A.L., Kessler, J.R., Muller, C.P., Bellini, W.J., Rota, P.A., 2008. Genetic variability and mRNA editing frequencies of the phosphoprotein genes of wild-type measles viruses. Virus Res. 135 (2), 298–306.

Baron, M.D., Shaila, M.S., Barrett, T., 1993. Cloning and sequence analysis of the phosphoprotein gene of rinderpest virus. J. Gen. Virol. 74 (Pt 2), 299–304.

Buchholz, U.J., Finke, S., Conzelmann, K.K., 1999. Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. J. Virol. 73 (1), 251–259.

Cattaneo, R., Kaelin, K., Baczko, K., Billeter, M.A., 1989. Measles virus editing provides an additional cysteine-rich protein. Cell 56 (5), 759–764.

CEC, 1992. Council Directive 92/66/EEC introducing Community measures for the control of Newcastle disease. Off. J. Eur. Commun. L 260, 1–20.

Childs, K.S., Andrejeva, J., Randall, R.E., Goodbourn, S., 2009. Mechanism of mda-5 inhibition by paramyxovirus V proteins. J. Virol. 83 (3), 1465–1473.

Ciancanelli, M.J., Volchkova, V.A., Shaw, M.L., Volchkov, V.E., Basler, C.F., 2009. Nipah virus sequesters inactive STAT1 in the nucleus via a P gene-encoded mechanism. J. Virol. 83 (16), 7828–7841.

Curran, J., Boeck, R., Kolakofsky, D., 1991a. The Sendai virus P gene expresses both an essential protein and an inhibitor of RNA synthesis by shuffling modules via mRNA editing. EMBO J. 10 (10), 3079–3085.

Curran, J., de Melo, M., Moyer, S., Kolakofsky, D., 1991b. Characterization of the Sendai virus V protein with an anti-peptide antiserum. Virology 184 (1), 108–116.

Delenda, C., Taylor, G., Hausmann, S., Garcin, D., Kolakofsky, D., 1998. Sendai viruses with altered P, V, and W protein expression. Virology 242 (2), 327–337.

Doyle, T.M., 1927. A hitherto unrecorded disease of fowls due to a filter-passing virus. J. Comp. Pathol. Ther. 40, 144–169.

Emerson, S.U., Schubert, M., 1987. Location of the binding domains for the RNA polymerase L and the ribonucleocapsid template within different halves of the NS phosphoprotein of vesicular stomatitis virus. Proc Natl Acad Sci U S A 84 (16), 5655–5659.

Galinski, M.S., Troy, R.M., Banerjee, A.K., 1992. RNA editing in the phosphoprotein gene of the human parainfluenza virus type 3. Virology 186 (2), 543–550.

Guryanov, S.G., Liljeroos, L., Kasaragod, P., Kajander, T., Butcher, S.J., 2016. Crystal structure of the measles virus nucleoprotein core in complex with an N-Terminal region of phosphoprotein. J. Virol. 90 (6), 2849–2857.

Heiden, S., Grund, C., Röder, A., Granzow, H., Kühnel, D., Mettenleiter, T.C., Römer-Oberdörfer, A., 2014. Different regions of the Newcastle disease virus fusion protein modulate pathogenicity. PLoS One 9 (12), e113344.

Henning, A.K., Groschup, M.H., Mettenleiter, T.C., Karger, A., 2014. Analysis of the bovine plasma proteome by matrix-assisted laser desorption/ionisation time-of-flight tandem mass spectrometry. Vet. J. 199 (1), 175–180.

Horikami, S.M., Smallwood, S., Moyer, S.A., 1996. The Sendai virus V protein interacts with the NP protein to regulate viral genome RNA replication. Virology 222 (2), 383–390.

Huang, C., Kiyotani, K., Fujii, Y., Fukuhara, N., Kato, A., Nagai, Y., Yoshida, T., Sakaguchi, T., 2000. Involvement of the zinc-binding capacity of Sendai virus V protein in viral pathogenesis. J. Virol. 74 (17), 7834–7841.

Huang, Z., Krishnamurthy, S., Panda, A., Samal, S.K., 2003. Newcastle disease virus V protein is associated with viral pathogenesis and functions as an alpha interferon antagonist. J. Virol. 77 (16), 8676–8685.

Huber, M., Cattaneo, R., Spielhofer, P., Orvell, C., Norrby, E., Messerli, M., Perriard, J.C., Billeter, M.A., 1991. Measles virus phosphoprotein retains the nucleocapsid protein in the cytoplasm. Virology 185 (1), 299–308.

Irie, T., Kiyotani, K., Igarashi, T., Yoshida, A., Sakaguchi, T., 2012. Inhibition of interferon regulatory factor 3 activation by paramyxovirus V protein. J. Virol. 86 (13), 7136–7145.

Jahanshiri, F., Eshaghi, M., Yusoff, K., 2005. Identification of phosphoprotein:phosphoprotein and phosphoprotein:nucleocapsid protein interaction domains of the Newcastle disease virus. Arch. Virol. 150 (3), 611–618.

Kolaskar, A.S., Tongaonkar, P.C., 1990. A semi-empirical method for prediction of antigenic determinants on protein antigens. FEBS Lett. 276 (1-2), 172–174.

Kosugi, S., Hasebe, M., Tomita, M., Yanagawa, H., 2009. Systematic identification of cell cycle-dependent yeast nucleocytoplasmic shuttling proteins by prediction of composite motifs. Proc. Natl. Acad. Sci. U. S. A. 106 (25), 10171–10176.

Kulkarni, S., Volchkova, V., Basler, C.F., Palese, P., Volchkov, V.E., Shaw, M.L., 2009. Nipah virus edits its P gene at high frequency to express the V and W proteins. J. Virol. 83 (8), 3982–3987.

Lin, G.Y., Paterson, R.G., Lamb, R.A., 1997. The RNA binding region of the paramyxovirus SV5 V and P proteins. Virology 238 (2), 460–469.

Liston, P., Briedis, D.J., 1994. Measles virus V protein binds zinc. Virology 198 (1), 399–404.

Lo, M.K., Harcourt, B.H., Mungall, B.A., Tamin, A., Peeples, M.E., Bellini, W.J., Rota, P.A., 2009. Determination of the henipavirus phosphoprotein gene mRNA editing frequencies and detection of the C, V and W proteins of Nipah virus in virus-infected cells. J. Gen. Virol. 90 (2), 398–404.

Locke, D.P., Sellers, H.S., Crawford, J.M., Schultz-Cherry, S., King, D.J., Meinersmann, R.J., Seal, B.S., 2000. Newcastle disease virus phosphoprotein gene analysis and transcriptional editing in avian cells. Virus Res. 69 (1), 55–68.

Martinez-Gil, L., Vera-Velasco, N.M., Mingarro, I., 2017. Exploring the human-nipah virus protein-protein interactome. J. Virol. 91 (23).

Mebatsion, T., Verstegen, S., De Vaan, L.T., Römer-Oberdörfer, A., Schrier, C.C., 2001. A recombinant newcastle disease virus with low-level V protein expression is immunogenic and lacks pathogenicity for chicken embryos. J. Virol. 75 (1), 420–428.

Nagai, Y., Klenk, H.D., Rott, R., 1976. Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle disease virus. Virology 72 (2), 494–508.

Nishio, M., Tsurudome, M., Ito, M., Ito, Y., 2006. Identification of RNA-binding regions on the P and V proteins of human parainfluenza virus type 2. Med. Microbiol. Immunol. 195 (1), 29–36.

Parks, C.L., Witko, S.E., Kotash, C., Lin, S.L., Sidhu, M.S., Udem, S.A., 2006. Role of V protein RNA binding in inhibition of measles virus minigenome replication. Virology 348 (1), 96–106.

Paterson, R.G., Leser, G.P., Shaughnessy, M.A., Lamb, R.A., 1995. The paramyxovirus SV5

V protein binds two atoms of zinc and is a structural component of virions. Virology 208 (1), 121–131.
Peeters, B.P., de Leeuw, O.S., Koch, G., Gielkens, A.L., 1999. Rescue of Newcastle disease virus from cloned cDNA: evidence that cleavability of the fusion protein is a major determinant for virulence. J. Virol. 73 (6), 5001–5009.
Pelet, T., Curran, J., Kolakofsky, D., 1991. The P gene of bovine parainfluenza virus 3 expresses all three reading frames from a single mRNA editing site. EMBO J. 10 (2), 443–448.
Pickar, A., Xu, P., Elson, A., Li, Z., Zengel, J., He, B., 2014. Roles of serine and threonine residues of mumps virus P protein in viral transcription and replication. J. Virol. 88 (8), 4414–4422.
Precious, B., Young, D.F., Bermingham, A., Fearns, R., Ryan, M., Randall, R.E., 1995. Inducible expression of the P, V, and NP genes of the paramyxovirus simian virus 5 in cell lines and an examination of NP-P and NP-V interactions. J. Virol. 69 (12), 8001–8010.
Qiu, X., Fu, Q., Meng, C., Yu, S., Zhan, Y., Dong, L., Song, C., Sun, Y., Tan, L., Hu, S., Wang, X., Liu, X., Peng, D., Liu, X., Ding, C., 2016. Newcastle disease virus V protein targets phosphorylated STAT1 to block IFN-I signaling. PLoS One 11 (2), e0148560.
Ramp, K., Topfstedt, E., Wäckerlin, R., Höper, D., Ziller, M., Mettenleiter, T.C., Grund, C., Römer-Oberdörfer, A., 2012. Pathogenicity and immunogenicity of different recombinant Newcastle disease virus clone 30 variants after in ovo vaccination. Avian Dis. 56 (1), 208–217.
Randall, R.E., Bermingham, A., 1996. NP:P and NP:V interactions of the paramyxovirus simian virus 5 examined using a novel protein:protein capture assay. Virology 224 (1), 121–129.
Römer-Oberdörfer, A., Mundt, E., Mebatsion, T., Buchholz, U.J., Mettenleiter, T.C., 1999. Generation of recombinant lentogenic Newcastle disease virus from cDNA. J.Gen.Virol. 80 (Pt 11), 2987–2995.
Samal, S.K., 2011. Newcastle disease and related avian paramyxoviruses. In: Samal, S.K. (Ed.), The Biology of Paramyxoviruses. Caister Academic Press, Norfolk, UK, pp. 69–114.
Satterfield, B.A., Cross, R.W., Fenton, K.A., Agans, K.N., Basler, C.F., Geisbert, T.W., Mire, C.E., 2015. The immunomodulating V and W proteins of Nipah virus determine disease course. Nat. Commun. 6, 7483.
Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9 (7), 671–675.
Schnell, M.J., Mebatsion, T., Conzelmann, K.K., 1994. Infectious rabies viruses from cloned cDNA. EMBO J. 13 (18), 4195–4203.
Shaw, M.L., Garcia-Sastre, A., Palese, P., Basler, C.F., 2004. Nipah virus V and W proteins have a common STAT1-binding domain yet inhibit STAT1 activation from the cytoplasmic and nuclear compartments, respectively. J. Virol. 78 (11), 5633–5641.
Shaw, M.L., Cardenas, W.B., Zamarin, D., Palese, P., Basler, C.F., 2005. Nuclear localization of the Nipah virus W protein allows for inhibition of both virus- and toll-like receptor 3-triggered signaling pathways. J. Virol. 79 (10), 6078–6088.
Shevchenko, A., Tomas, H., Havlis, J., Olsen, J.V., Mann, M., 2006. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat. Protoc. 1 (6), 2856–2860.
Steward, M., Vipond, I.B., Millar, N.S., Emmerson, P.T., 1993. RNA editing in Newcastle disease virus. J.Gen.Virol. 74 (Pt. 12), 2539–2547.
Steward, M., Samson, A.C., Errington, W., Emmerson, P.T., 1995. The Newcastle disease virus V protein binds zinc. Arch.Virol. 140 (7), 1321–1328.
Takeuchi, K., Tanabayashi, K., Hishiyama, M., Yamada, Y.K., Yamada, A., Sugiura, A., 1990. Detection and characterization of mumps virus V protein. Virology 178 (1), 247–253.
The UniProt C, 2017. UniProt: the universal protein knowledgebase. Nucleic Acids Res. 45 (D1), D158–D169.
Thomas, S.M., Lamb, R.A., Paterson, R.G., 1988. Two mRNAs that differ by two non-templated nucleotides encode the amino coterminal proteins P and V of the paramyxovirus SV5. Cell 54 (6), 891–902.
Tober, C., Seufert, M., Schneider, H., Billeter, M.A., Johnston, I.C., Niewiesk, S., ter Meulen, V., Schneider-Schaulies, S., 1998. Expression of measles virus V protein is associated with pathogenicity and control of viral RNA synthesis. J. Virol. 72 (10), 8124–8132.
Watanabe, N., Kawano, M., Tsurudome, M., Nishio, M., Ito, M., Ohgimoto, S., Suga, S., Komada, H., Ito, Y., 1996. Binding of the V proteins to the nucleocapsid proteins of human parainfluenza type 2 virus. Med. Microbiol. Immunol. 185 (2), 89–94.
Werner, O., Römer-Oberdörfer, A., Köllner, B., Manvell, R.J., Alexander, D.J., 1999. Characterization of avian paramyxovirus type 1 strains isolated in Germany during 1992 to 1996. Avian Pathol. 28, 79–88.
Witko, S.E., Kotash, C., Sidhu, M.S., Udem, S.A., Parks, C.L., 2006. Inhibition of measles virus minireplicon-encoded reporter gene expression by V protein. Virology 348 (1), 107–119.
Yabukarski, F., Lawrence, P., Tarbouriech, N., Bourhis, J.M., Delaforge, E., Jensen, M.R., Ruigrok, R.W., Blackledge, M., Volchkov, V., Jamin, M., 2014. Structure of Nipah virus unassembled nucleoprotein in complex with its viral chaperone. Nat. Struct. Mol. Biol. 21 (9), 754–759.
Yoneyama, M., Kikuchi, M., Matsumoto, K., Imaizumi, T., Miyagishi, M., Taira, K., Foy, E., Loo, Y.M., Gale Jr, M., Akira, S., Yonehara, S., Kato, A., Fujita, T., 2005. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. J. Immunol. 175 (5), 2851–2858.
Zhao, N., Grund, C., Beer, M., Harder, T.C., 2018. Engineered recombinant protein products of the avian paramyxovirus type-1 nucleocapsid and phosphoprotein genes for serological diagnosis. Virol. J. 15 (1), 8.