Self-capping of nucleoprotein filaments protects Newcastle Disease Virus genome

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Abstract

Non-segmented negative-strand RNA viruses, such as Measles, Ebola and Newcastle disease viruses (NDV), encapsidate viral genomic RNAs into helical nucleocapsids which serve as the template for viral replication and transcription. Here, the clam-shaped nucleocapsid structure, where the NDV viral genome is sequestered, was determined at 4.8 Å resolution by cryo-electron microscopy. The clam-shaped structure is composed of two single-turn spirals packed in a back-to-back mode, and the tightly packed structure functions as a seed for nucleocapsid to assemble from both directions and grows into double-headed filaments with two separate RNA strings inside. Disruption of this structure by mutations on its loop interface yielded a single-headed unfunctional filament.

Research organism: Newcastle disease virus

Introduction

Members of the order Mononegavirales encompass some of the most lethal human and animal pathogens, including Ebola, rabies virus, measles, nipah virus and the human respiratory syncytial virus (RSV) (Amarasinghe et al., 2017; Kuhn et al., 2010). Mononegaviruses commonly contain a non-segmented, linear, negative-strand RNA genome, and its replication is vital for virus survival and pathogenicity (Ruigrok et al., 2011). One remarkable character of negative-strand RNA viruses is their genomes are enwrapped by the nucleoprotein (N), which results in the formation of helical nucleocapsids (Finch and Gibbs, 1970; Heggeness et al., 1980; Longhi, 2009). During viral RNA synthesis, the assembled nucleocapsid, rather than the naked RNA genome, is opened and unveiled for the recognition of the viral RNA-dependent RNA polymerase (RdRp) and serves as the template for both replication and transcription (Dochez et al., 2012; Emerson and Wagner, 1972; Emerson and Yu, 1975; Fearnets et al., 1997; Perlman and Huang, 1973; Severin et al., 2016). In the cases of Paramyxoviridae or Rhabdoviridae viruses, the viral Phosphoprotein (P) mediates the access of RdRp to nucleoprotein, and the RdRp moves across the nucleocapsid for viral transcription (Blanchard et al., 2004; Bourhis et al., 2006; Kingston et al., 2004). Since RNA is susceptible to nuclease in vivo, the virus has evolved a complicated mechanism to protect its viral genome, in which its nucleoprotein (N) plays a major role in enwrapping nascent RNA thereby preventing possible damage (Dortmans et al., 2010; Ruigrok et al., 2011).

A great deal of effort has been spent on understanding the assembly mechanism of N to protect the genome of the Mononegavirales. N has two domains, the N-terminal domain (NTD) and the C-terminal domain (CTD), with a positively charged cleft in between, which is suitable for RNA binding. In the presence of RNA, each N can bind 6, 7 or 9 nucleotides and clamp RNA into the cleft, forming a ribonucleoprotein complex (RNP) (Albertini et al., 2006; Gutsch et al., 2015; Tawar et al., 2009). RNP can further assemble into either a helical or ring structure with 10, 11 or 13 protomers per turn (Albertini et al., 2006; Green et al., 2006; Gutsch et al., 2015; Tawar et al., 2009). In RNP oligomers, NTD and CTD interact successively with adjacent N proteins, forming
long helical filaments to efficiently protect the viral genome, and serving as the template for viral RNA transcription and replication for new virions (Ge et al., 2010; Zhou et al., 2013).

Detailed structural analyses have shown that measles RNP filaments exhibited more rigid and regular single-headed, herringbone-like characteristics after trypsin treatment, which were seemingly not sufficient for protecting RNA genome at the tips of the filaments (Schoehn et al., 2004). The mechanism for viral RNP to protect its tips from digestion by proteases remains to be discovered. Here, the Newcastle Disease Virus (NDV), a member of the genus Avulavirus, family Paramyxoviridae, which is relatively safe for handling, was selected as the model to discover new ways on how NDV RNP protects its viral genome and may provide new insight into the development of nucleocapsid based antivirus therapies.

Results

Clam-Shaped NDV Nucleocapsid
Following previous reports (Guryanov et al., 2015; Peng et al., 2016), the NDV N was expressed in Escherichia coli system and pure protein was obtained after a tandem affinity and gel-filtration chromatography. N is of high purity in SDS-PAGE, with an absorbance of A260/A280 ~1.1 suggesting the presence of RNA bounded N (Figure 1A). Under negative stain EM, purified N exhibited round-shaped structures with a small portion of double-headed filaments of different lengths (Figure 1B), which is similar to the expression of nucleocapsids of measles in Sf21 insect cells (Jensen et al., 2011), sendai virus in mammalian cells (Buchholz et al., 1993) as well as hendra virus in E. coli (Communie et al., 2013). Those two kinds of assemblies were further separated with continuous sucrose gradient ultracentrifugation. The separated round-shaped sample was quite homogenous with a diameter of 200 Å and was used for subsequent structure determination (Figure 1A).

The cryo-electron microscopy (cryo-EM) images for the round-shaped samples were collected and the single particle analysis were carried out. Two-dimensional (2D) and three-dimensional (3D) classification results showed a clam-shaped rigid body with some flexible extensions (Figure 1C). Further 3D refinement resolved the clam-shaped structure to 6.4 Å resolution showing obvious C2 symmetry in the rigid body. The C2 symmetry was then applied to improve resolution, yielding an overall 4.8 Å resolution of the core structure (Figure 1B and Figure 1D). Each protomer was easily recognized from the reconstruction. Those protomers furthest from the seam were better resolved while those closer to the seam were of lower resolution (Figure 1E). However, an atomic resolution structure of NDV N was still missing. Homologue modeling on NDV N based on 40% sequence identity of N between NDV and Parainfluenza virus 5 (PIV5) (Alayyoubi et al., 2015) resulted the subunit N model and the model was flexibly docked into the EM density map (Figure 1C, Figure 1D). The docked model fits the EM density well with minor modification, and resulted in a reliable initial model of NDV N.

The whole reconstruction revealed a clam shaped structure with the symmetry axis perpendicular to the spiral axis, where two single-turn spirals pack in a back-to-back manner (Figure 1B,C). In each single-turn spiral, there are around 13 N per turn and each N uses its N-arm (residues 2-34) and C-arm (residues 370-398) to horizontally interact with neighboring N for domain exchange contact (Figure 1C and Figure 1D), as reported in previous ring structures (Alayyoubi et al., 2015; Albertini et al., 2006; Green et al., 2006; Tawar et al., 2009).
More specifically, in a NDV clam-shaped structure, N_i uses the N-arm to interact with N_{i-1} CTD and the C-arm to make extensive contact with the N_{i+1} CTD tip to form an exceedingly stable structure (Figure 1—figure supplement 3C-E). Different from the ring structure of PIV5 N (Alayyoubi et al., 2015), the add-on N shifts upward by ~ 4.6 Å, which drives NDV N to form a single-turn spiral instead (Video 2).

Endogenous RNA from *E. coli* can be traced in the EM map. Limited by resolution, poly-Uracil was modeled into the EM map to mimic cellular RNA. In the clam-shaped structure, the RNA follows a relaxed helical pattern, orients outside the N molecule, more similar to RSV nucleocapsid than that of rhabdovirus or vesicular stomatitis virus nucleocapsids (Figure 1—figure supplement 3E,Video 3) (Albertini. et al., 2006; Green et al., 2006; Tawar et al., 2009). The external RNA is deeply buried in the interdomain cleft between NTD and CTD following the “rule of six” with alternating three-base-in and three-base out conformation (Figure 1—figure supplement 3F) (Calain and Roux, 1993; Kolakofsky et al., 1998). Six nucleotides are covered by one N, and there will be 78 nucleotides per single-turn spiral (Figure 1—figure supplement 3F).

Based on nucleocapsid structural similarity between NDV and the measles virus, the RNA in NDV is estimated to be left-handed and the 5' end of RNA lying inside as labeled in Figure 1B and C (Gutsche et al., 2015), which would be first replicated and enwrapped by N immediately after synthesis.

Of particular note is an obvious seam between the two single-turn spirals, which disconnects two RNA molecules (Figure 1B,C). The separation between the two 5’ ends of RNAs is ~ 6 nm and the bending angle of which is approximately 120°, which blocks the continuity of RNA, since it is impossible for one RNA to span two back-to-back spirals. Thus, the clam-shaped structure is not an integrated helix at all, but rather is composed of two spirals self-capping each other in a back-to-back mode. To confirm whether the NDV nucleocapsid is packed using this mode *in vivo*, the negative stain EM images of highly polymeric RNPs extracted from Newcastle disease virus were obtained. Interestingly, the images showed a filamentous assembly of the genomic RNA with the clam-like structure similar to what was observed in the resolved structure (Figure 1—figure supplement 4).

**Double-Headed Filament Derived from Clam-Shaped Nucleocapsid**

Importantly, decreasing threshold values to show more EM densities with or without C2 symmetry revealed that each single-turn spiral had the potential to grow further into a longer filament following a helical trajectory (Figure 1—figure supplement 2A,B). The pseudo-model of N could be docked into extra densities following the helical trajectory without any structural conflicts. Iteratively adding N protein in such manner to both single-turn spirals would yield a longer helix with double heads (Video 4).

To verify this, the filaments fraction after ultracentrifugation was examined with cryo-EM. Almost every filament had double heads derived from one clam-shaped structure (Figure 2A). Due to the heterogeneity of the double-headed filaments, so their structures could not be directly resolved via the single particle reconstruction approach. Alternatively, the filament was split into two parts for structural analysis: the clam-shaped core and helical part (Figure 2A,B). For the former, 2,608 clam-shaped particles truncated from double-headed filaments yielded a 14.0 Å resolution structure. The overall shape of the core fitted very well with the 4.8 Å clam-shaped structure (Figure 2B and Figure 2—figure supplement 1B). The distinctive back-to-back packing mode and
the seam between two single-turn spirals were clearly recognizable, suggesting the clam-shaped core acts as the seed for filament growth (Figure 2D).

The helical part of the filament was reconstructed at 15.0 Å resolution (Figure 2B and Figure 2—figure supplement 1A). Similar to the clam-shaped structure, the helix was composed of 13 protomers per turn, with an outer diameter of 200 Å, in agreement with the pseudo-atomic model. The helical pitch varied by ~ 60 Å, which provided flexibility for the helical nucleocapsids to fit into the crowded virus. Thus, the clam-shaped structure was perfectly compatible with the helical filament and could further grow into helical filament (Figure 2C,D). Following the direction of RNAs in the clam-shaped structure, the 5’ ends of the RNAs of the double-headed filament were depicted similar to that of the clam-shaped structure (Figure 1C and Figure 2C,D).

Interestingly, the lengths of the two helixes in around 90% of the back-to-back spirals were not equal and one helix was obviously longer than the other one observed in the raw images (Figure 2A and Figure 2—figure supplement 1C). The statistics showed that the shorter helix had an average length of ~14 nm with less than two helical turns, while the average length for the longer one was doubled to ~34 nm (Figure 2E), although the factors that determine the length difference are uncertain.

The Clam-Shaped Nucleocapsid Affects the Function of the Viral genome

In the clam-shaped structure or in the derived double-headed filament, the self-capping interface came from loops (residues 114-120) of vertically adjacent N in the clam-shaped core. Distance analysis of the residues in the loop suggested that hydrogen bonds may exist between two pairs of Gly391 and Arg317 (Figure 3A,B). The Loop114-120 region was only involved in the assembly of the clam-shaped core but not in the helical assembly of the double-headed filament. All the residues in Loop114-120 were mutated to Alanine to check if the mutations affected the clam-shaped assembly. The mutated N (Nloop) was purified using the same protocol as that of NWt and yielded on average of 50 nm longer filaments than those of NWt. A zoomed in view of the Nloop filaments clearly showed a single-headed, herringbone-like filament instead of a double-headed assembly from 2D classification (Figure 3C and Figure 2—figure supplement 2). Direct fast fourier transformation (FFT) analysis on one single-headed Nloop filament showed clean diffraction bands with ~1/60 Å intervals, and the 3D reconstruction of Nloop showed similar helical structure as that of the double-headed NWt. The evidence suggests that Loop114-120 has no influence on helical assembly but has a crucial role in clam-shaped structure formation (Figure 2—figure supplement 2).

To further investigate whether the Loop114-120 is functional relevant in vivo, the minigenome analyses on several N mutants (Figure 3D,E and Figure 3—figure supplement) were performed. The N-arm and C-arm were proven critical for the assembly of N previously (Buchholz et al., 1993; Kho et al., 2003) and the truncations of NARM, ANARMΔC-armΔC-tail and ANC-armΔC-tail disabled the assembly to higher ordered structure compared to NWt, Nloop and ANC-tail, shown in size exclusion chromatography and negative stain EM images (Figure 3D,E and Figure 3—figure supplement). While RNA synthesis was fully functional in the presence of wild type N (NWt), truncation mutants lacking either the N-arm (NAN-arm), N-arm or C-arm/C-tail (ANC-armΔC-tail and ANC-armΔC-tail) were all nonfunctional and lost the ability to express the GFP reporter. Surprisingly, the ANC-tail were partially functional according to the weak fluorescence signals in the minigenome assay. Although the mutation of Nloop could form longer single-headed
filaments as mentioned above, it showed negative result in the fluorescence assay (Figure 3D,E and Figure 3—figure supplement). RNA replication, transcription or translation was not successful in the minigenome assay of N_{Loop}, which indicates that the clam-shaped structure is critical for the expression of the GFP reporter gene.

**Clam-Shaped Nucleocapsid Resistant to Nuclease**

The detailed structural analysis did show that single-headed filament from N_{Loop} exposed the RNP 5’ end to the environment, while the double-headed filament from N_{WT} enabled intermolecular self-capping to cover the sensitive 5’ end (Figure 4A,B). To test the sensitivity of RNP 5’ end to protease, elastase was incubated with double-headed or single-headed filament samples. The SDS-PAGE gel showed a ∼40 kDa band with some smears from elastase digested N_{WT} (Figure 4C). Peptide mapping on 40 kDa band via Mass Spectrum results showed a residue range of 33 to 361, which suggested the cleaving site was at the loop in the C-arm (Figure 4D and Figure 4—figure supplement 1A). For the single-headed filament from N_{Loop} with the 5’ end exposed, an obvious difference was that the 40 kDa band was further digested to 30 kDa from the N-arm after elastase treatment based on the SDS-PAGE and Mass Spectrum results, which strongly indicates that there is another cleavage site in the NTD loop regions.

In addition, the influence of nuclease on RNA genome stability was tested. RNase A was added in the solutions containing N_{WT} or N_{Loop} filaments to check the digestion result of the assemblies. The results showed that N_{Loop} was more sensitive to RNase A compared to N_{WT} after 180 s exposure (Figure 4—figure supplement 1B). The statistical results showed that almost all of the N_{Loop} samples were complete disassembled while over 25% of the N_{WT} filaments remained intact (Figure 4E). Meanwhile, the N_{WT} rather than the N_{Loop} contained RNA with an absorbance of A260/A280 ∼0.9, while that of N_{Loop} was ∼0.6. The enzyme digestion analysis showed that the N_{WT}, rather than N_{Loop}, was resistant to the digestion of nuclease and protease, from which one hypothesis could be surmised that the N_{Loop} exposed its RNP 5’ end without the self-capping protection, and was exposed to protease and became accessible by nuclease. Via self-capping, N can protect the viral genome not only from side attack but also from both ends.

**Discussion**

N is the key factor for protecting the nascent RNA from degradation during RNA replication. Different from the reported ring-structure and helical spirals (Alayyoubi et al., 2015; Albertini et al., 2006; Green et al., 2006; Tawar et al., 2009; Gutsche et al., 2015), a novel clam-shaped structure of NDV N with two single-turn spirals packing in a back-to-back manner was identified and determined, corresponding to the extracted nucleocapsid assembly of NDV (Figure1—figure supplement 4). The clam-shaped structure of the NDV nucleocapsid was verified by in vivo transcription and translation experiments with minigenome analysis. The deletion of N-arm or C-arm of N disrupted the formation of ordered nucleocapsid and resulted the absence of fluorescence signals in minigenome assay. However, in similar minigenome assay with the truncation of C-tail, namely N_{AC-tail}, some weaker fluorescence signals were observed. The previous studies showed that the P protein used its NTD domain (P_{NTD}) to uncoil the nucleocapsid and allowed the RdRp to access the genomic RNA and then tethered the RdRp to the nucleocapsid with its XD domain binding to the C-tail of the N protein (Cox et al., 2014). One possible explanation is that even though the N_{AC-tail} lacks C-tail, the P protein can still mediate the formation
of N-RNA-RdRp complex in the minigenome assay due to the interaction of \( P_{\text{NTD}} \) with the nucleocapsid.

The double-headed spiral adopts a self-capping mechanism via the clam-shaped core to protect the RNA genome’s integrity. This is the possible reason why the single-headed \( N_{\text{Loop}} \) was unfunctional in minigenome assay. More interestingly, this clam-shaped structure functions as a seed for assembling into double-headed spirals with two separate RNAs inside. The illumination of the clam like nucleocapsid expands the understanding for N to assembly into the helical nucleocapsid, especially introducing the clam-like core as the starting point for N to assembly and then elongate in both sides, which keep the genome integrity in vivo. A self-capping mechanism is quite common in filaments that are involved in biological processes. For example, in DNA repair, Rad51 paralogs, RFS-1/RIP-1, induce remodeling at the tips of Rad51-ssDNA filaments to stimulate Rad51 strand exchange activity (Taylor et al., 2015; Taylor et al., 2016). In microtubule assembly, \( \gamma \)-tubulin pre-assembles into single-turn spirals as the template to nucleate \( \alpha/\beta \)-tubulin assembling sequentially (Kollman et al., 2010; Zehr et al., 2014). The previous researches have shown that self-capping is a mechanism for proteins or their homologues to easily fit into the spiral assembly and efficiently fine-tune its function.

The clam shaped nucleocapsid also provides the possible explanations for the pleomorphism and polyplody of the Mononegavirales. The mononegavirus morphology appears to vary considerably, especially the Paramyxoviruses and Filoviruses, ranging from about 110-540 nm in diameter for Sendai virus(Loney et al., 2009), 100-250 nm for spherical NDV particles(Battisti et al., 2012), and 50-510 nm in the case of measles virus (MeV) (Cox and Plenmer, 2017). This character of flexible volume of virus could accommodate variation in the copy number of the genome. It is a common observation for mononegavirus to contain more than one genome, such as NDV(Dahlberg and Simon, 1969; Goff et al., 2012; Kingsbury and Darlington, 1968), Hemagglutinating virus of Japan (HVJ)(Hosaka et al., 1966), Sendai virus(Loney et al., 2009; Lynch and Kolakofsky, 1978), measles virus(Liljeroos et al., 2011a; Rager et al., 2002), and Ebola virus(Beniac et al., 2012; Booth et al., 2013). Multiple genomes in a virion is essential for their infections, for example, the genetically complement two types of genomes analyses of MeV infections have provided independent evidence of multi-genome MeV transmission(Rager et al., 2002; Shirogane et al., 2012). In addition, the multi-genome in one virion seems to be packaged in continuous mode in Ebola virus(Beniac et al., 2012; Booth et al., 2013), and even in a “end to end” mode in HVJ(Hosaka et al., 1966). One interesting aspect on self-capping assemblies is that it provides the possibility of accommodating two copies of viral genomes with different lengths in one virion. So, the double-headed mode provides a possible organizing pattern for the multiple genomes of the polyplody viruses.

In addition, the C-tail of N may exist inside rather than outside of the nucleocapsids of Mononegavirales. Even though full-length N was purified and used for structural analysis, the C-tail (residues 399-489) is hard to be recognized in EM map due to the long, intrinsically flexible domain as reported in other structures (Houben et al., 2007; Longhi et al., 2003). Compared to ring structures, the extra cone-like densities in the center (117 nm\(^3\) at the threshold of 0.0054) of the density map of the clam shaped structure were shown, which are apparently from the C-tail after density assignment (Figure 4—figure supplement 2). The C-tail is located inside the clam-shaped structure, and is only accessible from either end by P or other proteins in order to form the
N-RNA-RdRp complex for the replication and transcription of the genomic RNA, which differs from previous reports concerning outside orientated C-tails (Jensen et al., 2011; Krumm et al., 2013).

NDV infects many domestic and wild avian species, severely impacting on the poultry industries in many countries. The structure of NDV N significantly improves the understanding on how NDV protects itself and infects hosts. It is important to highlight that NDV shares many common features with other members of the order *Mononegavirales*. For example, measles virus nucleocapsid was reported to assemble into herringbone-shaped structures (Gutsche et al., 2015), similar to the NDV N_{Loop} single-headed spiral. Therefore, it is reasonable to predict that nucleocapsid of measles virus as well as other mononegaviruses, might adopt a similar self-capping mechanism to keep its genome secure. N is a most conserved viral protein and the vital building block for nucleocapsid assembly, which makes it an ideal target for the antivirus development (Cox and Plemper, 2016). The positive charged clefts between the CTD/NTD lobes of N and especially the interaction loop between the vertically adjacent N in the clam-shaped structure are the possible druggable sites for further structure-based design of small-molecule drugs. The structural study of the NDV provides new insights into the negative sense RNA virus field and represents the starting point for inspiring new antiviral drug design for mononegavirus diseases.

**Methods**

### Key Resources Table

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Plasmid Construction

The nucleoprotein (N) gene of the Newcastle disease virus (NDV) (GenBank ID: HM063424.1) was synthesized via Sangon Biotech company (China). The gene was cloned into pMCSG7 vector with N-terminal 6×His-tag and C-terminal 8×His-tag and C-terminal 8×His-tag (Stols et al., 2002). The transition mutation of amino acids from 114 to Ala and the truncations by deleting N-arm (residues 1-33), C-tail (residues 399-489), C-arm and C-tail (371-489) as well as the combination of N-arm, C-arm and C-tail (1-33 and 371-489) of N gene were also cloned into pMCSG7 vector and designed as N_{Loop}, N_{AN-arm}, N_{AC-tail}, N_{AC-armAC-tail} and N_{AN-armAC-armAC-tail}, respectively. All the recombinant plasmids with target genes were sequenced to verify their sequences.

Protein Expression and Purification

NDV N and its derived mutants were expressed in Escherichia coli BL21(DE3) cells and purified using a tandem affinity and gel filtration columns. In detail, the cells were grown in LB media at 37°C until the OD600 nm reached 0.6. The target protein was induced at 16 °C for an extra 20 h with the final concentration of IPTG (isopropyl-B-D-1-thiogalactopyranoside) at 0.1 mM. The cells were harvested by centrifugation at 4,680 g for 20 min to obtain the sediments. The pellets were resuspended in PBS buffer (137 mM NaCl, 2.7 mM KCl, 50 mM Na_{2}HPO_{4}, and 10 mM KH_{2}PO_{4} (pH 7.4)) and disrupted with microfluidizer. Cell debris was removed by centrifugation at 38,900 g for 50 min. The clarified supernatant was loaded onto a 5 ml nickel-nitrilotriacetic acid (Ni-NTA) resin gravity column (Qiagen), which was preequilibrated with PBS buffer. The column was washed with 50 ml PBS buffer containing 20 mM imidazole followed by a 100 mM imidazole wash. Finally, the protein was eluted using PBS buffer containing 500 mM imidazole. The proteins with His-tags were concentrated and loaded on Superdex G200 size-exclusion chromatography column (120 ml, Amersham, USA) preequilibrated with TRIS buffer at pH 8.0 (20 mM Tris-HCl, 150 mM NaCl and 2 mM DTT). The target proteins with endogenous RNA were collected for the following experiments. The samples obtained above were loaded onto the top of a continuous 10 to 30% (w/v) sucrose gradient in the same TRIS buffer and centrifuged for 6 h at 16 × 10^4 g and 4 °C with an SW40 rotor (Beckman). The samples were collected by puncturing the tube and dialyzing in the TRIS buffer.

Negative Stain EM

Grids of N or its mutants for negative stain EM were prepared as described previously (Ohi et al., 2004). Specifically, 4 μl of samples (0.15 mg/ml) were applied to glow-discharged EM grids covered by a thin layer of continuous carbon film and stained with 2% (w/v) uranyl acetate. Negatively stained grids were imaged on a Tecnai Spirit 120 microscope (FEI, USA) operated at 120 kV. Images were recorded at a magnification of ×43,000 and a defocus set to -2 μm, using a 4K×4K scintillator-based charge-coupled device camera (UltraScan 4000, Gatan, USA).
Cryo-EM Data Collection

To prevent sample aggregation, the N-RNA sample was diluted to 0.65 mg/ml containing 0.018 mg/ml Qβ virus-like particles. A 4 µl sample was applied to a glow-discharged holey carbon grid (Quantifoil, R1.2/1.3, Ted Pella) with a thin layer of continuous carbon film. The grids were blotted using a Vitrobot Mark IV (FEI, USA) with 5 s blotting time, force level of 2 at 100% humidity and 4 °C and then immediately plunged into liquid ethane cooled by liquid nitrogen.

The micrographs of the clam-shaped structure samples were recorded on a 300 kV Titan Krios G² electron microscope equipped with Cs corrector (FEI, USA) and a K2 Summit direct electron detector (Gatan, USA) and in counting mode with a pixel size of 1.35 Å. Each movie was exposed for 7.6 s and dose-fractioned into 38 frames with 0.2 s for each frame, generating a total dose of ~ 41 e/A² on the samples. Defocus values during data collection varied from -1.5 to -3 µm. All the images were collected under the SerialEM automated data collection software package (Mastronarde, 2005). The micrographs of the filament samples were collected on a 200 kV Talos F200C electron microscope (FEI, USA) equipped with a DE20 Summit direct electron detector (DE, USA) in counting mode with a pixel size of 1.582 Å. Each movie was exposed for 40 s and contained 32 frames, generating a total dose of ~ 41 e/A² on the samples. Defocus values for the date collection varied from -1.5 to -3 µm. All the images were collected by utilizing the SerialEM automated data collection software package (Mastronarde, 2005).

Cryo-EM Data Processing and 3D Reconstruction.

A total of 3,200 micrographs were used for the clam-shaped structure determination. Before further image processing, the images were aligned and summed with MotionCorr software (Li et al., 2013) and the CTF parameters of each image were determined by CTFFIND3 (Mindell and Grigorieff, 2003). The single particle analysis and reconstruction was mainly executed in Relion1.4 (Scheres, 2012) and Relion 2.0 (Kimanius et al., 2016). First of all, the particles were picked automatically by Gautomatch and bad particles were excluded by manual selection and reference-free two-dimensional (2D) classification, with 167,588 particles selected for further processing. The initial model was produced by EMAN2 using typical 2D classes with different orientations (Tang et al., 2007). The initial model was lowpass-filtered to 60 Å to limit reference bias during three-dimensional (3D) classification and later refinement. No symmetry was applied in the 3D classification process and one of the three classes with a better structure feature was selected for further 3D auto-refinement. A 3D map with an overall resolution of 6.4 Å was obtained without symmetry by 3D refinement of the cleaned-up 75,290 particles. Then a soft mask was applied to avoid the influence of the spreading map for the alignment meanwhile the C2 symmetry was also applied and the final resolution was improved to 4.8 Å with the gold-standard Fourier Shell correlation (FSC) 0.143 criteria. The map was filtered and sharpened with Relion post-processing session and the local resolution was estimated with Resmap (Kucukelbir et al., 2014).

Double-headed filaments were divided into two parts for structure determination: helical filament and clam-shaped junction. Both helix and joint parts of the filament were picked manually, 2D classified and reconstructed with Relion2.0 (Kimanius et al., 2016). For helical reconstruction, 4,909 good segments were selected with 75% overlap. The 3D refinement using a cylinder as the initial map yielded a 15 Å-resolution helical map with the helical twist of -27.30° and a helical rise of 4.78 Å. For single particle reconstruction of the clam-shaped junction, 2,608 particles were manually picked, and the same cylinder in helical reconstruction was used as the
initial model. 3D refinement without symmetry yielded a structure, which was used as reference for the next refinement with C2 symmetry. All the reference structures were pre-filtered to 60 Å to avoid reference bias during the reconstruction. The C2 refinement yielded a map at the resolution of 14 Å. Both the helical map and C2 symmetric map were filtered and b-factor sharpened with Relion post-processing session.

The direct FFT analysis on single-headed filament was performed with EMAN2 software package (Tang et al., 2007). In total, 6,333 segments of the single-headed filament samples were manually picked and the helical reconstruction was done in Relion 2.0 (Kimanius et al., 2016), similar to the above.

Model Building and Validation

The homology model of N and RNA were generated by Modeller (Eswar et al., 2008) using the crystal structure of the parainfluenza virus 5 (PDB accession number 4XJN) as the template. Then the pseudo-atomic model of N was flexibly docked into the protomer furthest from the seam in the EM density map with Rosetta software (Das and Baker, 2008). The extra density excluding N was assigned as RNA enwrapped between NTD and CTD and docked using poly-Uracils due to the uncertain sequence of RNA in Coot (Emsley et al., 2010). The model refinement on a N with six Uracils was carried out using secondary structure restraints to maintain proper stereochemistry in Phenix.refine (v1.12) (Afonine et al., 2012). The model was further optimized manually for better local density fitting using Coot (Emsley et al., 2010). To prevent overfitting, TLS refinement and weight optimization were used to improve overfitting across a wide range of resolutions. Ramachandran outliers were corrected semi-automatically in Coot, and MolProbity statistics were computed to ensure proper stereochemistry. The model of the N was validated by computing a Fourier shell correlation (FSC) slush with the density map. The revised atomic NDV N and poly-Uracils were duplicated and docked as a rigid body to the other protomers using UCSF Chimera software (Pettersen et al., 2004).

Elatase and RNase A Enzymatic Assay

Elatase and RNase A were selected to test the susceptibility of NWT and NLoop samples. A mixture of 40 μl Tris buffer at pH 8.0 (20 mM Tris-HCl, 150 mM NaCl and 2 mM DTT) containing NWT or NLoop (0.15 mg/ml) and 0.275 mg/ml of RNase A was incubated at 37 °C and sampled every 90 s for negative stain EM. Forty five images were captured at ×49,000 magnification for each grid and the number of either clam-shaped structures or filaments was counted at different digestion timepoints.

NWT or NLoop (0.15mg/ml) was incubated with 0.1 mg/ml Chymotrypsin-like Elatase at 37°C and sampled every 30 min for negative stain EM.

Statistical Analysis

For double-headed filaments, the distance between the helix tip and the clam-shaped core were measured via ImageJ software. A total of 1371 filaments from 169 raw micrographs were statistically counted to calculate the averaged length of the filaments and the percentage of filaments with unequal length of single spiral.

In nuclease and elatase cleavage assay, the number of particles of both clam-shaped structures and filaments of NWT and NLoop were counted at different timepoints such as 0 s, 90 s and 180 s. A total of 120 micrographs were statistically counted.
MALDI-TOF-MS Analysis

The samples of N<sub>Loop</sub> and N<sub>WT</sub> after Chymotrypsin-like Elastase digestion were resolved by SDS PAGE and the gel bands were reduced with 10 mM dithiothreitol in 25 mM NH<sub>4</sub>HCO<sub>3</sub> at 56 °C for 60 min and alkylated by 55 mM iodacetamide in 25 mM NH<sub>4</sub>HCO<sub>3</sub> in the dark for 45 min at room temperature. The gel pieces were washed with 40 μl of 25 mM NH<sub>4</sub>HCO<sub>3</sub> for 5 min following the addition of 40 μl acetonitrile and incubated for 15 min. After gel pieces were dried in Speedvac for 15 min, proteins were digested with trypsin (100 ng for each band) in 25 mM NH<sub>4</sub>HCO<sub>3</sub> overnight at 37 °C. The samples of N<sub>Loop</sub> and N<sub>WT</sub> after trypsin treatment were excised for Ultraflexextreme matrix-assisted laser desorption ionization time-of-flight/time-of-flight mass spectrometer (MALDI-TOF/TOF-MS) assay. MALDI data processing was performed by the Peptide Mass Fingerprint method of the website www.matrixscience.com using the SwissProt database.

NDV Minigenome Assay for the Assembly Mechanism of N-RNA Complex in vivo

NDV minigenome p-LGT and three helper plasmids pCI-N, pCI-P and pCI-L from the NDV strain ZJ1 were constructed by Zhang et al (Zhang et al., 2005). BSR-T7/5 cells, stably expressing the phage T7 RNA polymerase, developed by Buchholz et al (Buchholz et al., 1999), was donated by Dr. Zhigao Bu (Harbin Veterinary Institute, China). The cells were maintained in DMEM (Gibco) supplemented with 10% fetal calf serum (FCS) and 1 mg/ml G418, as previously reported (Peeters et al., 2000).

Different mutant and truncations of N were cloned into the pCI-neo plasmid (Promega) and designed as N<sub>Loop</sub>, N<sub>AN-arm</sub>- N<sub>AC-tail</sub>, N<sub>AC-arm</sub>-N<sub>tail</sub> and N<sub>AN-arm</sub>-N<sub>arm</sub>-N<sub>tail</sub>, respectively. The co-transfection was performed with minigenome and helper plasmids as reported previously (Peeters et al., 2000; Zhang et al., 2005). Briefly, the minigenome p-LGT (3μg), pCI-P (1.5μg), pCI-L (1.5μg) each with different N expression plasmid (3μg), pCI-Np and pCI-NP mutants, were cotransfected into BSR-T7/5 cells expressing T7 RNA polymerase. One co-transfection, in which the N expression plasmid was replaced by vector pCI-neo was also conducted as the negative control. The transfection reagent was PolyJet™ reagent and the transfection procedure was carried out according to the manufacturer’s protocol. At 96 h posttransfection, the GFP fluorescence of different samples was observed by fluorescence microscopy.

Ribonucleoprotein complex isolation from NDV strain LaSota

NDV strain LaSota was propagated in 9-day-old specific-pathogen-free (SPF) embryonated chicken eggs at 37°C for 96 h. The infected allantoic fluid was collected and centrifuged at 4,320 g for 30 min to remove the cell debris. The supernatants were then subjected to pelleting through a 20% sucrose cushion at 38,900 g for 2 h at 4 °C. The pellets were resuspended in PBS buffer (pH 7.4) in the presence of the protease inhibitor cocktail complete™-EDTA free from Roche Diagnostics, and lysed by five cycles of freezing and thawing (in liquid nitrogen and at 37 °C, respectively)(Schoehn et al., 2004). The NDV lysate was loaded onto the top of a continuous 10% to 30% (w/v) sucrose gradient in PBS buffer (pH 7.4) and centrifuged for 6 h at 16 × 10⁴ g and 4 °C with the SW40 rotor (Beckman). The samples were collected by puncturing the tube and dialyzing in PBS buffer. The presence of the N-RNA complex was verified by negative stain EM.

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Competing interests
The authors declare no competing interests.

Data availability
The cryo-EM density map of clam-shaped structure was deposited in the Electron Microscopy Data Bank (EMDB) with the accession number EMD-9793. The atom coordinates of single N subunit with six uracils were deposited in the Protein Data Bank (PDB) with the PDB ID code 6JC3.

References
on in virology.


Figure 1. NDV N assembles into clam-shaped structures with two single-strand spirals packing in a back-to-back manner. (A) The images show negative stain EM micrographs of the round-shaped structures (top image, upper fraction) and filaments (bottom image, lower fraction) after sucrose gradient centrifugation with close-ups of the box areas shown on the right. (B) Various views of the 3D reconstruction of the clam-shaped structure of N from the upper fraction. The C2 symmetry axis enforced during reconstruction is indicated in the center view (middle). NTD, CTD and RNA are colored in pink, green and gold, respectively. (C) Atomic model of clam-shaped structure of N shown from the same view as in B and using the same color code. Two 5' ends of enwrapped RNA and the seam between them are labeled in the middle view.
**Figure 2.** Double-headed filament derived from clam-shaped structure. (A) Representative cryo-EM micrograph of N filament from the lower fraction. One typical double-headed filament is selected, magnified and colored in blue (helical structure) and red (clam-shaped core). (B) 3D reconstructions of helical structure (Upper) and clam-shaped core (Lower). The 4.8 Å clam-shaped structure is docked into clam-shaped core in filament. (C) Combination of both helical filaments and clam-shaped core yields the whole double-headed filament. The position of the clam-shaped core in the composite structure is delineated by the dashed line. (D) Atomic model of double-headed filament shows the position of clam-shaped core with the dashed line. Corresponding 5’ and 3’ ends from the same RNA are labeled in red and blue. (E) Two helixes in one double-headed filament are of unequal length. The length of each helix is defined as the distance between the helix tip and the center of clam-shaped core in the cartoon. The length measurement and RNA direction from 5’ to 3’ are given.
Figure 3. The clam-shaped nucleocapsid is important for the function of the Viral genome. (A) Loop pairs from the vertically adjacent Nform the self-capping interface in the clam-shaped structure. Five loop pairs furthest from the seam are shown. Colors as in Fig. 1. (B) View of one loop pair of the clam-shaped structure. Seven residues (114-120) in the upper loop are labeled and the lower loop is docked into the EM density. (C) Raw micrograph of single-headed helix from NLoop and the 2D classification on the filament tip (circled). Zoomed in view of selected raw filaments (examples in dashed boxes) with two typical 2D classes on the tip are shown. (D) NWT could form double-headed filaments and functioned well in the minigenome assay. The retention volume of NWT in gel filtration chromatography was ~47 ml (Left) and the negative stain EM of this fraction consisted of clam-shaped structure and filaments (Middle). NWT exhibited strong fluorescence signals in minigenome assay in BSR-T7/5 cells (Right). (E) NLoop formed filaments but was unfunctional in minigenome assay. The retention volume of NLoop was ~47 ml, close to NWT (Left). Negative stain EM showed more filaments than NWT (Middle). However, there was no fluorescence signal in the minigenome assay (Right).
**Figure 4.** Clam-shaped nucleocapsid is resistant to Elastase and RNase A. (A) Atomic model of N\textsubscript{WT} double-headed filament from different views shows reciprocal capping between two single-headed spirals. Colors as in Fig. 1. One single-headed spiral is marked in line and labelled by “capping”. (B) Atomic model of N\textsubscript{Loop} single-headed filament from different views with no cap and its 5’ end of RNA exposed. The supposed capping spiral marked by the dashed line is missing in the single-headed filament. (C) SDS-PAGE gel of N\textsubscript{WT} and N\textsubscript{Loop} after elastase digestion. There was a ~40 kDa main band with some smears in elastase digesting N\textsubscript{WT} assay (Left), while elastase cuts N\textsubscript{Loop} into ~30 kDa band (Right). (D) Mass spectrum results identified peptides drawn on the atomic structure of N, which indicates one additional cutting site on N\textsubscript{Loop} (gold scissors). The common regions mapped by Mass Spectrum in N\textsubscript{WT} and N\textsubscript{Loop} are colored in blue, and the unique region checked in N\textsubscript{WT} is shown in red. Five peptides were identified from the 40 kDa band of N\textsubscript{WT} and marked on N atomic structure, leaving CTD loops as the cutting site. Only four peptides were identified from the 30 kDa of N\textsubscript{Loop} and marked on N atomic structure. Given the reduced molecular weight and the missing NTD peptide, another cutting site should exist within NTD. (E) The comparison of RNase A digesting N\textsubscript{WT} and N\textsubscript{Loop} at different timepoints. Both clam-shaped structures and filaments are counted. The numbers of oligomers in N\textsubscript{WT} and N\textsubscript{Loop} at 0 s are
normalized to 100%. At 180 s, almost 100% disassembly of nucleocapsid was seen in N<sub>Loop</sub> compared to over 25% of filaments remained in N<sub>WT</sub>. (F) A hypothetical cartoon depicts the full protection of N<sub>WT</sub> to the viral RNA genome via the self-capping clam-shaped structure. When the clam-shaped structure is broken, nuclease is able to access RNA 5' end and digest the whole RNA strand.

**Figure 1—figure supplement 1.** Data analysis of NDV N clam-shaped structure.

(A) The size exclusion chromatography and SDS-PAGE profile of NDV N. The retention volume of N was ~ 47 ml and the A260/A280 of N was around 1.1. N was of high purity from the SDS-PAGE gel. (B) Negative stain EM of N after gel filtration. Round-shaped structures are visible with a small portion of filaments of different lengths. (C) A typical cryo-EM micrograph and its Fast Fourier Transform (FFT). The micrograph was taken under a Titan Krios G<sub>2</sub> microscope equipped with Gatan K<sub>2</sub> summit camera and was motion corrected. Representative particles in different orientations are indicated with dashed black squares. Qβ virus-like particles for improving orientation distribution are shown in the red circle. The direct FFT of the micrograph shows the highest signal frequency at 4.6 Å or higher. (D) Eight typical 2D classes shows a wide range of angular orientations. (E) 3D classification of N.
Figure 1—figure supplement 2. 3D reconstruction of clam-shaped structure of N and resolution estimations.

(A) and (B) 3D structures after refinement without C2 (A) or with C2 (B) symmetry. Pseudoatomic structure of N and poly-U are docked into the EM map. Two views are shown and the seam is indicated using dashed lines. Dust has not been removed. (C) Angular distribution of clam-shaped structure with C2 symmetry. The major angular distribution peak and the respective structure are shown. (D) Fourier shell correlation (FSC) curves of clam-shaped structures with or without C2 symmetry, unmasked or masked (masking-effect-corrected FSC) based on 0.143 criteria. Structure with masked C2 symmetry is selected for the following analysis. (E) Local resolution analysis with Resmap shows an overall resolution of ~5 Å. The seam position is indicated with dashed line in the middle view. The resolution gradually declines from protomers furthest away from the seam to protomers closest to the seam (Right).
Figure 1—figure supplement 3. Structural analysis on clam-shaped structure of NDV N. 

(A) The domain organization of NDV N. NTD and CTD are illustrated in pink and green, respectively. C-tail is unresolved in the 3D structure and boxed by a dashed line. (B) Homology modelling of NDV N using subunit of PIV5 nucleocapsid (grey) as the template. (C) Docking of one pair of N and poly-U into the clam-shaped EM map with RNA shown in gold. (D) Comparison between NDV N in active state and Nipah N in inactive state (grey). The transition is of a rotation of CTD by 24°. (E) Horizontal packing among neighboring N. Each N interacts with neighboring N with its N-arm and C-arm (boxed in red). (F) RNA enwrapped in clam-shaped structure and the zoomed-in protomer and RNA. (G) Electrostatic potential distribution of one protomer at pH 7.4. The cleft between NTD and CTD is positively charged to bind negative charged RNA.
Figure 1—figure supplement 4. The negative stain EM of N-RNA complex isolated from NDV. The N-RNA complexes are double-headed and the clam-like cores are labeled in arrows.
Figure 2—figure supplement 1. Structural analysis on double-headed filament.

(A) FSC curves of helical part and clam-shaped core based on 0.143 criteria. The final resolutions for helical part and clam-shaped core are estimated at 15 Å and 14 Å, respectively. (B) Matching between typical 2D classes (Upper) and the respective projections (Lower, low-pass filtered to 10 Å) of atomic model of double-headed filaments. (C) Length statistics of two single spirals in one double-headed filament. The length is defined as the distance between the core center and the tip of single spiral.
Figure 2—figure supplement 2. Structural analysis on single-headed filament of N_loop.

(A) Typical cryo-EM micrograph of N_loop. Two representative round-shaped particles were selected (circled) and one 2D averaged class is shown at the upright corner, with 13 protomers recognized. One single-headed filament is marked in rectangular box and used in further analysis.

(B) Power spectrum and 2D classification analysis of single-headed filament. One single-headed filament is divided into head, middle and tail parts (Left) and 2D classes of which are shown (Middle). Power spectrum analysis on selected filament indicts clearly 1/60 Å layer line (Right).

(C) 3D reconstruction of single-headed filament and the respective pseudo-atomic model. (D) 3D projection of pseudo-atomic model of (C) after 15 Å low-pass filter with sharp herringbone-shape.
**Figure 3—figure supplement.** Comparison of N and the derived mutants in the nucleocapsid assembly and their function in minigenome assay.

Six N proteins including N<sub>WT</sub>, N<sub>∆C-arm∆C-tail</sub>, N<sub>∆C-tail</sub>, N<sub>∆N-arm∆C-arm∆C-tail</sub>, N<sub>∆N-arm</sub> and N<sub>Loop</sub> were subjected to gel filtration chromatography (Left), negative stain EM (Middle) and minigenome analyses (Right).
Figure 4—figure supplement 1. The diagram of peptide mapping and RNase A digestion of N_{WT} and N_{Loop}.

(A) The diagrams of identified peptides via Mass Spectrum are marked. Similar identified peptides including residues 207-222, 229-237, 240-260, 353-361 in both N_{WT} and N_{Loop} are colored in blue. The peculiar identified peptide 33-85 in N_{WT} is colored in red. The gold scissors indicate the possible proteolytic sites. (B) Negative stain EM images of RNase A digestion on N_{WT} and N_{Loop}. Typical micrographs of N_{WT} and N_{Loop} at different timepoints are presented.
Figure 4—figure supplement 2. C-tail maybe located inside of clam-shaped structure.

(A) C-tail density is boosted by lowering the density threshold of 3D structure without C2 symmetry. Two bulks of cone-like density (violet) emerge near the end of docked CTD (green). Both side view (Left) and top view (Right) are provided for better visualization of C-tail. (B) A typical 2D averaged class (Left) shows the extra density assigned to C-tail in 3D structure. And this 2D averaged class is colored in violet and yellow (Right).

Videocaptions:

Video 1 3D reconstruction of clam-shaped structure and the fitting of pseudoatomic model.
Video 2 Morphing of ring structure to single-turn spiral.
Video 3 RNA enwrapped between NTD and CTD.
Video 4 Double-headed spiral derived from clam-shaped structure.