The glutamic residue at position 402 in the C-terminus of Newcastle disease virus nucleoprotein is critical for the virus

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The nucleocapsid proteins (NPs) of Newcastle disease virus (NDV) and other paramyxoviruses play an important functional role during genomic RNA replication. Our previous study showed that the NP-encoding gene significantly influenced viral replication. Here, we investigated the roles of certain amino acid residues in the NP C-terminus in viral replication and virulence. Results showed that the glutamic acid residue at position 402 (E402) in the C-terminus of the NP is critical for RNA synthesis in the NDV mini-genome system. Mutation of E402 resulted in larger viral plaques that appeared more quickly, and increased the virulence of NDV. Further study indicated that the mutant virus had increased RNA levels during the early stages of virus infection, but that RNA replication was inhibited at later time points. These findings increase our knowledge of viral replication and contribute to a more comprehensive understanding of the virulence factors associated with NDV.

Newcastle disease (ND), caused by Newcastle disease virus (NDV), is a severe infectious disease that affects over 250 bird species1. NDV is a member of the genus Avulavirus, within the family Paramyxoviridae2. The NDV genome encodes six structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin- neuraminidase (HN), and large polymerase protein (L)3. The M, F, and HN proteins are associated with the viral envelope. The M protein is involved in morphogenesis and budding. The F and HN proteins mediate entry and release4,5. The viral RNA genome is always found tightly associated with NP, and the NP-RNA complex associates with the viral RNA-dependent RNA polymerase (RdRp) complex, which consists of P and L proteins, to form an active ribonucleoprotein (RNP) complex that is necessary for transcription and replication6–10.

NP is the most abundant protein in virus particles, and plays an important role in viral transcription and replication. It consists of two major domains: a highly conserved structured N-terminal region (N_CORE), which forms the globular body, and a C-terminal region (N_TAIL), which extends from the N-terminal body11–13. The N_CORE contains all of the components necessary for NP self-assembly and RNA-binding to form the NP-RNA complex, while the C-terminal tail is mainly responsible for the interaction of the NP-RNA complex with the P protein14,15. Thus, binding of the NP-RNA template to the RdRp complex for viral RNA synthesis mainly occurs via the C-terminus of NP16,17.

Several studies have investigated the specific domains responsible for the different functions of NP in non-segmented negative-stranded RNA viruses, but there are discrepancies between the different studies. Several studies indicated that the N-terminus, but not the C-terminus, of NP is required for NP-RNA complex formation and self-assembly, while another study showed that the C-terminus of NP is an important determinant of encapsidation and RNA synthesis18–22. Both the N_CORE and N_TAIL regions of the NP are likely to play key roles in binding to P protein14,23,24. However, there are very few studies on this aspect of NP that are specific to NDV, and the functional domains of NP require further exploration. Furthermore, our previous study showed that exchanging the NP gene between NDV strains LaSota (genotype II) and SG10 (genotype VII) had a significant effect on the replication kinetics of the resulting chimeric viruses25.

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Here, we investigated the role of the NDV NP C-terminus in viral replication by generating a series of truncated and alanine-scanning mutants, and testing the associated activity of the mutant proteins. Our results suggested that the glutamic acid residue at position 402 (E402) in the C-terminus is critical for RNA synthesis. Mutation of this critical residue led to increased levels of mRNA transcription during the early stages of virus infection, but inhibited RNA replication at later time points. Experiments using the mutant strains also revealed that the E402 residue negatively regulates the virulence of NDV. Therefore, this study identifies a specific site that plays a key role in maintaining NDV NP function.

Results
Prediction of the functional sites in the NDV NP.
A bioinformatics analysis of the potential function sites in the NDV NP was performed using three separate online programs. Previous studies indicated that interactions between the NP and P proteins are regulated by an IDR26. The PONDR prediction program was used to predict the structural disorder within NDV NP. The disorder prediction value for a given residue is plotted against the residue number. The significance threshold, above which residues are considered to be disordered, was set to 0.5. The bold black line indicates the main structurally disordered region. (B) COILS prediction of the potential coiled-coil motif of NDV NP. The graph displays the probability of coiled-coil formation as a function of residue number using 14-amino-acid, 21-amino-acid, and 28-amino-acid scan windows. (C) The MegAlign program was used to analyze the amino acid sequence differences among genotypes.

Here, we investigated the role of the NDV NP C-terminus in viral replication by generating a series of truncated and alanine-scanning mutants, and testing the associated activity of the mutant proteins. Our results suggested that the glutamic acid residue at position 402 (E402) in the C-terminus is critical for RNA synthesis. Mutation of this critical residue led to increased levels of mRNA transcription during the early stages of virus infection, but inhibited RNA replication at later time points. Experiments using the mutant strains also revealed that the E402 residue negatively regulates the virulence of NDV. Therefore, this study identifies a specific site that plays a key role in maintaining NDV NP function.

The NP amino acids 402–408 hardly support NDV mini-genome expression. To examine the function of the NP C-terminus, a series of plasmids encoding progressively truncated C-terminal mutants of NP with a Myc tag were constructed based on the predicted results (Fig. 2A), and a NDV mini-genome system was produced25.
We first generated a plasmid encoding wild-type NP with a Myc tag to confirm that the luciferase expression of the Myc-tagged NP was similar to that of untagged NP, suggesting that NP-Myc has a similar function to the untagged NP (data not shown). Next, we measured the RNA synthesis activities of the progressively truncated C-terminal NP mutants in the mini-genome system. As shown in Fig. 2B, mutants NP(1–480)-Myc, NP(1-460)-Myc, and NP(1-440)-Myc had luciferase expression that was higher than or similar to that of NP-Myc, while the luciferase expression of NP(1-420)-Myc, NP(1-412)-Myc, NP(1-408)-Myc, NP(1-401)-Myc, and NP(1-398)-Myc was lower than that of NP-Myc. The luciferase expression of NP(1-401)-Myc and NP(1-398)-Myc was particularly low, with levels that were almost undetectable.

To further confirm the main functional sites within amino acid region 399–440, we evaluated the RNA synthesis activity of NP(Δ421-440)-Myc, NP(Δ409-420)-Myc, NP(Δ402-408)-Myc and NP(Δ399-401)-Myc (Fig. 2C). Similar to the above results, all of these mutants caused demonstrated reduced luciferase expression compared with NP-Myc in the mini-genome system, with the lowest levels of luciferase recorded for the NP(Δ402-408)-Myc mutant (Fig. 2D). This suggested that amino acids 402–408 play an important role in the function of the NDV NP protein. The different Myc-tagged NP protein mutants were also analyzed by western blotting using a monoclonal anti-Myc antibody, which confirmed that the correct mutant NP proteins were present, and that the expression levels were comparable (Fig. 2B and D, bottom panel).

**Effects of mutating E402 to alanine in the NDV mini-genome system.** To identify the precise amino acid residue that is indispensable for the RNA synthesis function of NP, we generated a further seven plasmids encoding Myc-tagged NP mutants with unique alanine (A) substitutions in amino acid region 402–408 (Fig. 3A). We then evaluated the resulting luciferase expression in BSR T7 cells expressing P and L proteins, mini-genome, and NP-Myc, or one of the individual alanine-scanning NP mutants. Mutants NP_{la402A}-Myc, NP_{la403A}-Myc, NP_{la404A}-Myc, NP_{la405A}-Myc, NP_{la406A}-Myc, and NP_{la407A}-Myc had luciferase expression levels similar (75–110%) to those of NP-Myc, while the luciferase expression of mutant NP_{la408A}-Myc was only 25% of that of NP-Myc. However, the relative luciferase expression of NP_{E402A}-Myc was only about 8% of that of NP-Myc (Fig. 3B, upper panel), although the expression levels of all the mutant NP proteins were similar (Fig. 3B, bottom panel). These findings indicate that NP_{E402A} showed very little activity in the mini-genome assay.
Recovery of recombinant chimeric virus with a NP E402A mutation. To further determine the role of amino acid residue E402 in the virus, we constructed a recombinant NDV strain harboring an E402A mutation based on the cDNA of NDV strain SG10 (rNDV-WT). The recombinant virus, named rNDV-NP E402A, was viable, and could be rescued by taking advantage of reverse genetics. To ensure the genetic stability of the rescued virus, it was serially passaged 10 times in 9-day-old specific-pathogen-free (SPF) chicken eggs. The mutant virus was amplified by reverse-transcription PCR (RT-PCR) and then sequenced, with the results confirming the presence of the introduced mutations and the absence of adventitious mutations (data not shown).

Biological characteristics of the recombinant virus. The biological characteristics of the mutant and wild-type NDVs were then compared (Table 1). The results revealed that the rNDV-NP E402A mutant had similar viral titers to those of the wild-type (rNDV-WT) in both embryos and cells (Log 10 9.37 and 8.64 EID 50/ml; Log 10 8.57 and 9.0 TCID 50/ml, respectively). In addition, the pathogenicity of the two viruses in chicken embryos and 1-day-old chickens was comparable. We further evaluated the cytopathic effects by measuring the numbers and sizes of the plaques formed by the chimeric mutated virus rNDV-NP E402A and rNDV-WT on BSR T7 cell monolayers. In plaque assays, there was a more rapid and extensive cytopathic effect in cells infected with rNDV-NP E402A (average plaque diameter: 0.70 ± 0.06 mm) compared with the cells infected with rNDV-WT (average plaque diameter: 0.26 ± 0.03 mm), although the numbers of the plaques were similar for the two strains (Fig. 4A and B).

Replication and pathogenicity in 4-week-old chickens. The replication and pathogenicity of rNDV-WT and rNDV-NP E402A was evaluated in 4-week-old SPF white Leghorn chickens by inoculating each bird with 10^6 EID 50/100 μl of viral particles via the eye drop/intranasal (ED/IN) route. The resulting survival curves are shown in Fig. 5A. Birds inoculated with rNDV-WT exhibited a slight depression at 2 dpi, severe depression (2/10), wing drop/incoordination (4/10), prostration (3/10), and death (1/10) at 3 dpi, and 100% mortality by 4 dpi. At necropsy, all euthanized chickens showed severe hemorrhage in the throat and trachea, duodenum mucosa, and proventriculus, necrosis in the caecum, and white, necrotic, pinpoint foci in the spleen at 3–4 dpi. In comparison, the birds inoculated with rNDV-NP E402A developed moderate illness by 3 dpi, severe illness by 4 dpi, and 100% mortality by 5 dpi (Fig. 5A). Virus titration assays (Fig. 5B, C, and D) showed that both rNDV-WT and rNDV-NP E402A could replicate in all organs, and that viral titers in the sampled tissues gradually increased over time. In general, the titers in most of the sampled tissues were similar between the two treatment groups, except for in the brain at 2 dpi (Fig. 5B) and in the duodenum at 3 dpi (Fig. 5C) and 4 dpi (Fig. 5D), where the replication...
was significantly different (P < 0.05). These results indicated that rNDV-NP_E402A had a similar tissue tropism to the wild-type virus, but had a slightly different replication level in certain tissues.

Based on the histopathological analysis, both rNDV-WT and rNDV-NP_E402A caused the following moderate to severe histopathological changes in all of the sampled tissues (Fig. 6): loose brain tissue structure (empty arrows) with increased accumulation of microglial cells in the brain (black arrows); interstitial broadening and edema (black arrows) and slight mucosal epithelium shedding of the tracheal mucosa (empty arrows); lymphocyte necrosis (black arrows), multifocal confluent coagulative necrosis, and an increased inflammatory cell infiltration (empty arrows) in the spleen; extreme expansion in the bronchi of the lung (black arrows); mucosal epithelium shedding (black arrows) and inflammatory cell infiltration of the mucosal lamina propria in the duodenum (empty arrows); and mucosal epithelium shedding (black arrows) and increased goblet cells in the mucosal epithelium of the caecum. Overall, the two viruses caused similar histopathological changes in most tissues, except for in the spleen and brain. In the spleen, the tissue from the rNDV-NP_E402A-inoculated group had particularly severe histopathological changes compared with that of the rNDV-WT-inoculated group (Fig. 6). As expected, no apparent histopathological changes were observed in any of the tissues from the control group, which was treated with 0.9% NaCl.

Figure 4. Plaque morphologies of the wild-type and mutant viruses in BSR T7 cells. (A) The shape and size of plaques formed by these viruses. (B) Summary of the average plaque number and size for each virus. P-values were calculated based on a two-tailed, unpaired t-test (95% confidence levels). *P = 0.01–0.05; **P = 0.001–0.01; ***P = 0.0001–0.001. n = 3.

Figure 5. Survival curves and replication of rNDV-NP_E402A and rNDV-WT in 4-week-old chickens. (A) Survival curves of 4-week-old specific pathogen-free chickens (n = 10). Birds were infected with rNDV-WT or rNDV-NP_E402A. (B–D) Replication of the two viruses in 4-week-old chickens. Birds were inoculated with 10^6 EID_50/100 μl of rNDV-NP_E402A or rNDV-WT through the eye drop/intranasal (ED/IN) route, and sacrificed at 2 (B), 3 (C), or 4 (D) days post-inoculation (n = 2). The indicated tissues were collected, and viral titers were determined in DF-1 cells. Asterisks indicate a statistically significant difference between the titers of the chimeric virus and the parental virus. P-values were calculated based on a two-tailed, unpaired t-test (95% confidence levels). *P = 0.01–0.05; **P = 0.001–0.01.
Pathogenicity of wild-type and mutant NDV viruses in 1-day-old chicks. To compare the pathogenicity of the two viruses in 1-day-old chicks, an assay similar to the ICPI test was conducted. Birds were inoculated via the intracerebral route with different titers of rNDV-WT or rNDV-NPE402A (10^1 EID_{50}/μl, 10^2 EID_{50}/μl, or 10^3 EID_{50}/μl per bird). The resulting mean scores are shown in Table 2. There was no difference between the two viruses at dose 10^3 EID_{50}/μl. However, obvious differences were observed in the pathogenicity of the two viruses at doses 10^2 EID_{50}/μl and 10^1 EID_{50}/μl. This finding reveals that the rNDV-NPE402A mutant virus is more virulent than the wild-type in 1-day-old chickens at lower viral titers.

Western blotting and immunoprecipitation. We next investigated whether the E402A substitution impacted on the interaction between the NP and P proteins, thereby affecting the pathogenicity of rNDV-NPE402A. We immunoprecipitated Flag-tagged NP and NPE402A from 293T cells overexpressing these proteins, and probed the precipitates from HA-tagged P protein (Fig. 7). The results showed that both NP and NPE402A associated
with P protein, and that the E402A substitution had no obvious effect on the interaction of NP with P protein. This revealed that the observed change in the pathogenicity of rNDV-NP E402A was not related to the interaction between NP and P proteins.

Viral replication in DF-1 cells. The growth kinetics of the two viruses were compared using multicycle growth curves in DF-1 cells (Fig. 8A). The rNDV-NP E402A mutant showed a lower level of replication compared with the rNDV-WT. While rNDV-WT underwent exponential replication until 36 hpi, after which the replication level plateaued, exponential replication was only observed for the rNDV-NP E402A mutant until 24 hpi. In addition, the rNDV-WT virus titer was significantly higher than that of rNDV-NP E402A from 36 hpi to the end of the observation period (P < 0.01). Together, these results demonstrate that the E402A mutation in the C-terminus of the NP protein had a significant effect on the cytopathogenicity and replication capability of NDV.

Effect of the NP E402 mutation on viral RNA synthesis. We next investigated RNA levels in the rNDV-WT and rNDV-NP E402A strains using qRT-PCR. Individually, the levels of genomic RNA corresponding to the NP and P segments were similar across the two treatment groups for each of the time points (Fig. 8B and C). However, there were statistically significant differences in the relative RNA levels between cells infected with rNDV-WT and rNDV-NP E402A, at 12 hpi (P < 0.05), suggesting the faster replication by rNDV-NP E402A during the early stages of infection. Despite this, the relative levels of viral RNA in the rNDV-NP E402A-infected samples were lower than those in rNDV-WT-infected cells at 24 hpi, and significantly lower at 36 hpi (P < 0.001). The data suggested that the E402A mutation specifically increased the levels of RNA expression in the earlier stages of infection, but reduced the RNA expression levels at later time points.

Discussion
For NDV, vesicular stomatitis virus, and other non-segmented negative-stranded RNA viruses, the NP-RNA complex, consisting of the viral RNA genome and the NP protein, is necessary for transcription and replication. Here, we examined the effects of substitutions in the amino acid residues located in the NDV NP C-terminal region on RNA synthesis regulation and viral virulence.

We first used three different online programs to identify possible functional sites. The intrinsically disordered region (IDR) has been shown to act as an anchor point for binding with the polymerase complex in the NP C-terminus in several paramyxoviruses, including Nipah virus, Hendra virus, measles viruses, and human parainfluenza virus type 3 (HPIV3) [41,42]. However, little is known regarding this aspect of the NDV NP.

| Virus titers | Virus | WT | NP E402A |
|-------------|-------|----|---------|
| 10^3 EID<sub>50</sub> | 1.66 | 1.65 |
| 10^2 EID<sub>50</sub> | 1.52 | 1.66 |
| 10 EID<sub>50</sub> | 0.45 | 1.56 |

Table 2. Pathogenicity of the wild-type and mutant viruses in 1-day-old chicks. *The maximum score for velogenic strains is 2.0.

Figure 7. Nucleoprotein (NP) interacts with phosphoprotein (P) independent of the E402 site. 293 T cells were co-transfected with 2.5 μg of plasmids expressing HA-tagged P protein, together with 2.5 μg of plasmids expressing Flag-tagged NP or NP E402A. At 48 h post-transfection, cells were harvested and immunoprecipitated with anti-Flag M2 beads. The precipitates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and P as well as NP proteins were detected in immunoblots using anti-HA or anti-Flag antibodies. The gels are uncropped images.
Our analyses showed that the NP C-terminus contains two IDRs (amino acids 460–489 and amino acids 360–430) that were especially highly disordered. These regions were also identified by COILS program and MegAlign program analyses. We then explored the role of these IDRs in the RNA synthesis and virulence of NDV. Using in vivo mini-genome RNA synthesis assays, we found that amino acids 399–440 in the NP C-terminus are important for RNA synthesis, especially amino acids 402–408. Interestingly, the NP mutant with a deletion of the 49 C-terminal amino acids had an ~160% increase in RNA synthesis activity compared with the wild-type NP. Similar results were obtained in other studies. It is possible that the interaction between the NP-RNA template and the RdRp became relatively weak in these truncated proteins, allowing the polymerase greater access to the NP-RNA template during RNA synthesis. Thus, the full-length NP may result in a tight association between the P protein and the NP-RNA complex, which may not be beneficial for transcription and replication.

After identifying NP C-terminus amino acids 402–408 as being functionally important, we investigated which precise sites in this region are indispensable for RNA synthesis. Using individual alanine-scanning mutagenesis and the NDV mini-genome system, we found that the NPE402A-Myc mutant had the lowest levels of mini-replicon reporter gene expression, and that the reporter gene expression remained low despite gradual increases in the expression in related NP mutants. These findings confirm that amino acid E402 in the NP C-terminus is important for the RNA synthesis function of NP. These results are in agreement with previous studies on other non-segmented negative-stranded RNA viruses.

To further investigate if the E402A mutation effects virus growth, a recombinant NDV harboring the E402A mutation was generated using reverse genetics. The two viruses showed comparable results in the EID₅₀, TCID₅₀, MDT, and ICPI assays (Table 1), but the rNDV-NP₄₀₂₂ₐ mutant developed faster and had 2.7-fold larger plaques.
compared with the rNDV-WT. Based on previous reports, we propose that NP_{E402A} from recombinant virus
rNDV-NP_{E402A} had an increased ability to interact with the M protein. This could influence the association
between the M protein and the F and HN proteins, which are more efficient at inducing cell-cell fusion than the
wild-type NP, resulting in the enlarged plaque. The increased plaque size may also be associated with differences
in the innate immune response of the host cell to the different strains.

We further determined the pathogenicity of the mutant virus rNDV-NP_{E402A} and its WT counterpart
in 4-week-old SPF chickens inoculated via a natural route of infection. All infected chickens developed severe
signs of illness, including severe lesions, and the mortality rate was 100% for both virus strains. A high viral
titration was obtained from all harvested tissues, and except for the spleen, tissue samples from the
rNDV-NP_{E402A}-inoculated group showed severe histopathological changes that were comparable with those in
the tissues from the rNDV-WT-inoculated group. As our earlier results suggested differences in the virulence
between the two strains, we conducted intracerebral tests, which are similar to ICPI tests, to accurately compare
the pathogenicity in 1-day-old chickens. The results showed that the rNDV-NP_{E402A} mutant was more virulent
in 1-day-old chickens than the wild-type virus when lower virus titers were used for infection. We speculate that
when hosts are infected with virulent viruses at a relatively high viral titer, the host dies too quickly to detect
subtle differences in virulence.

Our analyses showed that the glutamic acid residue at position 402 (E402) in the NP C-terminus plays an
important role in regulating viral virulence. To exploit the inherent mechanism responsible for the changes in
virulence, we examined the interaction between NP and P proteins. The results showed that both NP and NP_{E402A}
associated with P protein, and that the E402A mutation had no obvious effect on the interaction of NP with
the P protein. This revealed that the observed change in the pathogenicity of rNDV-NP_{E402A} was not related to
the interaction between NP and P proteins. We then investigated the replication ability of the two viruses in
DF-1 cells using multicycle growth curves. Interestingly, the results showed significantly decreased replication by
rNDV-NP_{E402A} compared with rNDV-WT from 36 hpi to the end of the observation period (P < 0.01 or 0.001).

Based on the differences in replication ability between the rNDV-NP_{E402A} and rNDV-WT strains, we next
investigated the levels of viral RNA in infected DF-1 cells using qRT-PCR. The results showed that the E402A
mutation specifically increased the levels of viral RNA expression at 12 hpi, but that expression was reduced at 36
hpi in the virus-infected cells. Previous studies also showed a differential effect on RNA synthetic processes.
The reason for the differential activity of replication and transcription of the same RNP template may be that
two distinct complexes are involved, and that the different viral transcription and replication machinery rec-
ognize distinct regions of the RNP template. The decreased replication of rNDV-NP_{E402A} compared with
rNDV-WT may be the result of the enhanced cytopathic ability of strain rNDV-NP_{E402A}. Because rNDV-NP_{E402A}
developed faster and had a greater cytopathogenic effect, it caused serious damage to the host cells by 24 hpi, and
the injured cells could not support effective viral RNA synthesis. Another reason may also be associated with the
M protein. Some studies have shown that the M protein regulates viral RNA synthesis by interacting with the
NP protein, which can stop viral RNA synthesis and promote viral particle production. The close interaction
between NP_{E402A} and M protein makes the incorporation of RNP complexes into progeny virions more efficiently. At the same time, more RNA synthesis is needed to allow transcription to occur, followed by replication. These findings also explain the increased levels of mRNA transcription in the early stages of virus infection.

In conclusions, we evaluated the roles of the specific amino acid residues in the C-terminus of the NDV NP
in viral RNA synthesis and pathogenicity in the present study. Our findings reveal that the glutamic acid residue
at position 402 (E402) in the NP C-terminus plays an important role in viral RNA synthesis. Mutation of this
critical residue resulted in increased levels of RNA during the early stages of infection, but inhibited replication
at later time points as a result of more severe cellular damage. The findings of the current study will be used to
further investigate the distinction between transcription and replication, and will also contribute to a more com-
prehensive understanding of the virulence factors of NDV. However, it is not known if the NP E402 residue affects
the encapsidation of RNA, the interaction of NP with the M protein, the formation of the NP-RNA template, or
structural changes, and further studies are needed to elucidate this.

Materials and Methods

Cells and viruses. Chicken embryonic fibroblasts (DF-1) and 293 T cells were grown in Dulbecco’s modified
Eagle’s medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum
(FBS) (Gibco), and maintained in DMEM containing 2% FBS. Baby hamster kidney cells stably expressing T7
RNA polymerase (BSR T7 cells) were grown in DMEM containing 10% FBS and 1 mg/ml G418 (Invitrogen,
Carlsbad, CA, USA). All cell lines were maintained at 37 °C in a 5% CO₂ incubator (Thermo Forma, Marietta,
OH, USA). NDV strain SG10 is a velogenic genotype VII virus, which was propagated in 9-day-old SPF embry-
nated chicken eggs via the allantoic cavity route.

Animals and ethics statement. SPF chicken eggs and chickens were purchased from Beijing Merial Vital Laboratory Animal Technology Co., Ltd. (Beijing, China). All chickens were housed in isolators at China Agricultural University (Beijing, China), and the rearing facilities were approved by the Beijing Administration Committee of Laboratory Animals under the auspices of the Beijing Association for Science and Technology (approval ID SYXK (Jing) 2013–0013). All of the animals used in this study were cared for in accordance with established guidelines, and the experimental protocols were specifically considered and approved by the Animal Welfare and Ethical Censor Committee of China Agricultural University. All experiments were conducted in a Biosafety Level 2 (BSL-2) laboratory.
Prediction of the functional sites in the NDV NP protein. The amino acid sequences of the NDV NP genes were analyzed using three different online programs to identify possible functional sites. PONDR (http://www.pondr.com/) analysis of intrinsically disordered regions (IDRs) was performed to predict the natural structural disorder within the NP protein, while coiled-coil regions of NP were predicted using the COILS program (http://www.ch.embnet.org/software/COILS_form.html)46,47. The complete coding sequences of NP from strains belonging to different NDV genotypes were retrieved from the GenBank database (Table S1). The amino acid sequences were analyzed using the ClustalW multiple alignment algorithm in the MegAlign program of the DNASTAR Lasergene package, version 7.1 (DNASTAR Inc. Madison, WI, USA).

Plasmid construction. The full-length antigenomic cDNA of NDV strain SG10 (rNDV-WT), three helper plasmids encoding the NP, P, and L proteins (pCI-NP, pCI-P, and pCI-L), and the NDV mini-genome plasmid pNDV-MG, containing the firefly luciferase (F-Luc) gene driven by the T7 promoter, were constructed previously25,45. pCI-NP cDNA was used as template to PCR-amplify cDNA encoding full-length wild-type NP (489 amino acids) with a Myc tag at its C-terminus, which was cloned into PCI-neo, generating PCI-NP (1-489)-Myc. To generate the mutant constructs, cDNA encoding different mutants with a Myc tag at their C-terminus were cloned into PCI-neo based on the PCI-NP (1-489)-Myc cDNA.

To construct a recombinant cDNA clone containing the E402A mutation within the NP gene, the full-length antigenomic cDNA rNDV-WT was used as the backbone. Briefly, the NP was mutated using overlapping PCR, resulting in the amino acid change E402A. The fragment containing the mutated site was used to replace the corresponding fragment in the full-length cDNA using two unique restriction enzymes, XbaI and SalI. The mutated cDNA clone containing the E402A substitution was designated rNDV-NP_E402A. To analyze the interaction between NP and P protein, plasmids expressing HA-tagged P protein and Flag-tagged NP or NP_E402A proteins were constructed by inserting the P or NP/NP_E402A genes into the HA-tagged pRK5 vector or Flag-tagged pRK5 vector between the EcoRI and XhoI sites. All of the constructs were confirmed by nucleotide sequencing.

NDV mini-genome system and dual luciferase assay. The mini-genome assay was performed as described previously25. Briefly, BSR T7 cells were seeded in 24-well culture plates. At 90% confluence, the cells were co-transfected using Lipofectamine 2000 (Invitrogen) with the NDV mini-genome pNDV-MG plasmid carrying the FLuc reporter gene, its helper plasmids, and plasmids encoding untagged NP (PCI-NP), Myc-tagged NP (PCI-NP-Myc), Myc-tagged NP mutants, PCI-P, and PCI-L, as well as a Renilla luciferase reporter gene (RLuc). At 24 h post-transfection, the cells were washed twice in phosphate-buffered saline (PBS), and then lysed in 100 µl of passive lysis buffer (Promega, Madison, WI, USA). Cells were vigorously mixed for 15 min, and 20 µl of lysate were tested using a dual luciferase assay kit (Promega) according to the manufacturer’s instructions to determine the luciferase activity. The relative luciferase activity was defined as the ratio of the FLuc to RLuc activities. The expression of the PCI-NP-Myc and Myc-tagged NP mutant proteins was detected by western blotting with monoclonal anti-Myc antibody sc-40 (Santa Cruz, Dallas, TX, USA). Three separate experiments were performed, and luciferase expression was measured in triplicate in each experiment.

Recovery of virus from cDNA. The recombinant virus rNDV-NP_E402A was recovered by co-transfection of the NDV chimeric full-length cDNA plasmid, along with helper plasmids PCI-NP_E402A, PCI-P, and PCI-L, into BSR T7/5 cells, as previously described48. At 4 days post-transfection, the cells were frozen and thawed three times, and the supernatant was harvested. The supernatant was then injected into the allantoic cavities of 9-day-old SPF embryonated chicken eggs to recover the recombinant NDV. Recovery of the virus was confirmed using a hemagglutination (HA) assay, and the total RNA from NDV-positive allantoic fluid was extracted using TRIRsol reagent (Invitrogen) according to the manufacturer’s instructions. The rNDV-NP_E402A recombinant was sequenced, and no adventitious mutations were detected.

Mean death time and intracerebral pathogenicity index of the recombinant viruses. Viral titers of rNDV-WT and rNDV-NP_E402A were determined using the Reed and Muench end-point method, and expressed as the 50% embryo infectious dose (EID₅₀)/ml and 50% tissue culture infective dose (TCID₅₀)/ml25. The pathogenicity of the recombinant viruses was determined using the standard pathogenicity tests for NDV: the mean death time (MDT) test in 9-day-old embryonated SPF chicken eggs, and the intracerebral pathogenicity index (ICPI) test in 1-day-old SPF chicks48.

Plaque formation. BSR T7 cells were infected with viruses at a multiplicity of infection (MOI) of 0.01 in six-well plates. After 1 h of adsorption, the inoculum was removed and replaced with an overlay medium containing 2% FBS and 1% agar. Following incubation at 37°C in a 5% CO₂ incubator for 36 h, overlay medium supplemented with 0.1% neutral red was added. Plaques were observed after a further 48 h of incubation48. Plaque sizes were measured using the GNU Image Manipulation Program, version 2.8 (https://www.gimp.org/), 28.

Pathogenicity assessment in 4-week-old chickens. The pathogenicity of rNDV-WT and rNDV-NP_E402A was then determined in chickens. Four-week-old SPF while Leghorn chickens were assigned randomly into three groups of 16 birds each (6 for sampling and 10 for clinical observation). Chickens were inoculated via the eye drop/intranasal (ED/IN) route with one of the two recombinant viruses at a dose of 10⁵ EID₅₀/100 µl per bird, or with 0.9% NaCl as the negative control. The birds were monitored for clinical signs daily for 14 days post-infection (dpi). Two birds were euthanized daily from 2–5 dpi for gross lesion observation, and samples of the trachea, lung, brain, caecum, spleen, and duodenum were collected and then separated into two parts. One part was used for virus titration in DF-1 cells at 2–4 dpi. These tissue samples were homogenized in phosphate-buffered saline containing a final concentration of 10,000 units of penicillin G and streptomycin,
and the cleared tissue homogenates were serially diluted 10-fold and used to inoculate DF-1 cells. The virus titers were determined as the TCID_{50} per gram using the endpoint method of Reed and Muench. The second part of each tissue sample was fixed in 10% buffered formalin for 48 h for use in histopathology. All of the fixed tissue samples were routinely sectioned and stained with hematoxylin and eosin (H&E), and then examined for lesions using light microscopy (CX31, Olympus, Tokyo, Japan).

Pathogenicity assessment in 1-day-old chickens. To further compare the pathogenicity of the recombinant viruses, 70 1-day-old SPF chickens were assigned randomly into seven groups of 10 birds each. Each group was separately inoculated via the intracerebral route with rNDV-WT or rNDV-NP_{E402A} at doses of 10^3 EID_{50}/50 μl, 10^2 EID_{50}/50 μl, or 10 EID_{50}/50 μl per bird, or with 0.9% NaCl as the negative control. The birds were monitored for clinical symptoms and mortality once every 12 h for 8 days. At each observation, the birds were scored as follows: 0 = normal, 1 = sick, and 2 = deceased. As per the ICPI assay, results were presented as the mean of the score per bird per observation over the eight-day period.

Western blotting and immunoprecipitation. To determine the role of the glutamic acid at amino acid position 402 in the interaction between NP and P proteins, 293T cells were co-transfected with 2.5 μg of DNA from plasmids expressing HA-tagged P protein, and 2.5 μg of DNA from plasmids expressing Flag-tagged NP or NP_{E402A}. At 48 h post-transfection, cells were harvested and analyzed by immunoprecipitation assay. Briefly, the cell lysates were first prepared using lysis buffer (50 mM Tris-Cl at pH 8.0, 150 mM NaCl, 1.0% Triton X-100, 10% glycerol, 20 mM NaF, 1 mM DTT, and 1 x complete protease mixture). Protein samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% skim milk in 0.1% Tween-20/PBS buffer, probed with anti-HA (Santa Cruz) or anti-Flag antibodies (Sigma-Aldrich) at dilution 1:2000, and then incubated with a dilution of 1:10000 of goat anti-mouse or anti-rabbit IgG-HRP (Santa Cruz). Detection was performed using an ECL detection kit (CwBiotech, Beijing, China). The amounts of Flag-tagged proteins were adjusted to similar levels by varying the amounts of the transfected plasmids. The cell extracts were prepared using lysis buffer as described above. Immunoprecipitation assays were then performed with anti-Flag M2 beads (Sigma-Aldrich, St. Louis, MO, USA) for 12 h at 4°C. Beads were washed three times in lysis buffer and then eluted with sample buffer. Precipitated complexes were detected by Western blotting with anti-HA (Santa Cruz) and anti-Flag antibodies (Sigma-Aldrich).

Viral replication in DF-1 cells. The growth dynamics of rNDV-WT and rNDV-NP_{E402A} were determined in DF-1 cells under multiple-cycle growth conditions. DF-1 cells in duplicate wells of six-well culture plates were infected with viruses at a MOI of 0.01. After 1 h of adsorption, the cells were washed twice with PBS and then incubated with DMEM containing 2% FBS at 37°C in a 5% CO_2 incubator. The culture supernatants were collected and replaced with an equal volume of fresh medium at 12-h intervals until 72 hpi. Virus titers in the collected supernatants were titrated via the limiting dilution method in DF-1 cells, and were expressed as TCID_{50} using the endpoint method. All experiments were performed in triplicate.

Quantification of viral RNA synthesis by quantitative RT-PCR (qRT-PCR). DF-1 cells were collected from virus infection assays at the indicated time points (12, 24, and 36 hpi), and total RNA was extracted using 1 ml of TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The resulting RNA samples (1 μg per sample) were reverse-transcribed using a previously reported method. Briefly, we used the reverse transcription primer PRT-G, which is specific for negative-sense viral RNA (5'-ACG ATA AAA GGC GA AGA AGC A-3', nucleotide positions 24–44 in the SG10 genome). Reverse transcripts were stored at -70°C until use in qRT-PCR assays. Primers qNP-F (5'-TTA CAA CTG CGT CCG GTA TG-3') and qNP-R (5'-CGA TAT AAA GGC GA AGA-3') were used to quantify the viral NP gene, and primers qP-F (5'-TGGA GAG CAA CCA GGG AAG AC-3') and qP-R (5'-GGC AGG TAG CTG GAC ACG AT-3') were used to quantify the viral P gene. qRT-PCR assays were performed using SYBR Premix Ex Taq (TaKaRa Biotechnology, Shiga, Japan) according to the manufacturer's instructions. Assays were performed using a LightCycler 96 (Roche, Basel, Switzerland) thermal cycler with the following parameters: 94°C for 30 s, followed by 40 cycles of 94°C for 5 s, 60°C for 10 s, and 60°C for 15 s. Gene expression was normalized relative to that of the housekeeping gene GAPDH. For data analysis, LightCycler 96 qRT-PCR software, version 1.1.0.1320 (Roche), was used.

Statistical analyses. All statistical analyses were performed using an unpaired t-test in GraphPad Prism Software Version 6.0 (GraphPad Software Inc., San Diego, CA, USA). Statistical differences between experimental groups were determined using the analysis of variance method. Statistical significance was set at P < 0.05 (*), P < 0.01 (**), and P < 0.001 (***).

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X.H.Y., R.Z., and G.Z.Z. conceived and designed the experiments; X.H.Y., J.L.C., Z.R.H., C.L., Y.S., J.X. and H.M.Y. performed the experiments; X.H.Y., R.Z. and G.Z.Z. analyzed the data; X.H.Y. and J.L.C. wrote the paper.

Additional Information
Competing Interests: The authors declare that they have no competing interests.

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