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Autophagy Benefits the Replication of Newcastle Disease Virus in Chicken Cells and Tissues

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Newcastle disease virus (NDV) is an important avian pathogen. We previously reported that NDV triggers autophagy in U251 glioma cells, resulting in enhanced virus replication. In this study, we investigated whether NDV triggers autophagy in chicken cells and tissues to enhance virus replication. We demonstrated that NDV infection induced steady-state autophagy in chicken-derived DF-1 cells and in primary chicken embryo fibroblast (CEF) cells, evident through increased double- or single-membrane vesicles, the accumulation of green fluorescent protein (GFP)-LC3 dots, and the conversion of LC3-I to LC3-II. In addition, we measured autophagic flux by monitoring p62/SQSTM1 degradation, LC3-II turnover, and GFP-LC3 lysosomal delivery and proteolysis, to confirm that NDV infection induced the complete autophagic process. Inhibition of autophagy by pharmacological inhibitors and RNA interference reduced virus replication, indicating an important role for autophagy in NDV infection. Furthermore, we conducted *in vivo* experiments and observed the conversion of LC3-I to LC3-II in heart, liver, spleen, lung, and kidney of NDV-infected chickens. Regulation of the induction of autophagy with wortmannin, chloroquine, or starvation treatment affects NDV production and pathogenesis in tissues of both lung and intestine; however, treatment with rapamycin, an autophagy inducer of mammalian cells, showed no detectable changes in chicken cells and tissues. Moreover, administration of the autophagy inhibitor wortmannin increased the survival rate of NDV-infected chickens. Our studies provide strong evidence that NDV infection induces autophagy which benefits NDV replication in chicken cells and tissues.

Newcastle disease virus (NDV) is a single-stranded, nonsegmented, negative-sense RNA virus that belongs to the *Paramyxoviridae* family (1). Pathogenic strains of NDV are able to infect most species of birds and cause highly contagious Newcastle disease. NDV strains can be categorized as velogenic (highly virulent), mesogenic (intermediate virulence), or lentogenic (nonvirulent). Velogenic strains produce severe nervous and respiratory signs, spread rapidly, and cause up to 90% mortality. Mesogenic strains cause coughing, affect egg quality and production, and result in up to 10% mortality. Lentogenic strains produce mild signs with negligible mortality (1). Herts/33 is a velogenic NDV strain, infection with which caused high titers of virus in various tissues, including the lung, heart, kidney, spleen, etc. (2). In recent years, although some aspects of NDV pathogenesis have been investigated, the factors that affect NDV replication in its host are still poorly understood (3–5).

Autophagy is a highly conserved process that generates double-membrane vesicles that engulf and sequester portions of the cytoplasm to be delivered to the lysosome for degradation (6, 7). Autophagy is induced in response to diverse stress stimuli, including nutrient starvation, endoplasmic reticulum (ER) stress, oxidative stress, pathogen-associated molecular patterns (PAMPs), and virus infection (6). Several autophagy-related proteins have been implicated in the formation of autophagosomes. Microtubule-associated protein 1 light chain 3 (LC3), the mammalian homologue of yeast Atg8 (8), is the most widely monitored autophagy-related protein (9, 10). Accumulation of autophagosomes may be the outcome of either enhanced autophagosome biogenesis or disrupted trafficking to lysosomes. Autophagic flux is a more accurate index to judge autophagy activity (11, 12); it is a dynamic and continuous process of autophagy, referring to not the increased number of autophagosomes but rather flux through the entire system, including lysosomes or the vacuole, followed by the release of breakdown products. The mammalian target of rapamycin (mTOR) and phosphatidylinositol 3-kinase (PI3K) signaling pathways have been shown to control autophagy in mammalian cells (13, 14). The autophagy-related proteins, such as Beclin 1, are critical for the signaling pathways involved in autophagosome formation (15, 16).

Autophagy, from monocellular eukaryotic organisms to primates, is a housekeeping mechanism. It may contribute as an intrinsic host defense mechanism against invading viruses by delivering them to the lysosomal compartment (17). On the other hand, viruses have a number of mechanisms to block autophagy or even manipulate autophagy for their benefit. Autophagy can favor viral replication in a number of ways, including assisting virus biogenesis, egress, and the translation of incoming viral RNA. Viruses also utilize autophagy as a platform for replication (17). Measles virus, which, like NDV, belongs to the family *Paramyxoviridae*, induces autophagy through a pathogen receptor CD46-Cyt-1/GOPC pathway (18). Another paramyxovirus, parainfluenza virus type 5 (PIV5), requires autophagy pathways to activate human plasmacytoid dendritic cells (19). In the present study, we demonstrated that autophagy is induced upon NDV infection of chicken cells and tissues. By regulating the autophagy pathway through pharmacological inhibitors and RNA interfer-

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ence, we established the important function of autophagy in NDV infection. Additionally, inhibition of autophagy reduces virus production and ameliorates pathogenesis *in vivo*.

MATERIALS AND METHODS

Reagents and antibodies. Rapamycin (R0395), wortmannin (W1628), chloroquine (CQ) (C6628), E64d (E8640), pepstatin A (P5318), rabbit polyclonal anti-LC3B antibody (L7543), rabbit polyclonal anti-p62/SQSTM1 antibody (P0067), and mouse monoclonal anti-β-actin antibody (A1978) were purchased from Sigma-Aldrich. Rabbit polyclonal anti-Beclin 1 (3738) antibodies were purchased from Cell Signaling Technology. Rabbit polyclonal anti-lysosome-associated membrane protein 1 (anti-LAMP1) antibody (ab 24170) was purchased from Abcam. Monoclonal antibody against NDV phosphoprotein (P protein) was prepared in our laboratory. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit or -mouse secondary antibody and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit secondary antibody were purchased from Jackson ImmunoResearch. *Beclin 1* small interfering RNAs (siRNAs), consisting of three target-specific 21-nucleotide siRNAs designed to specifically knock down chicken *Beclin 1* gene expression, along with control scrambled siRNA, were designed and synthesized by GenePharma. Premixed WST-1 cell proliferation reagent (630118) was purchased from Clontech.

Cells, viruses, and plasmids. DF-1 chicken fibroblast cells were purchased from the American Type Culture Collection (ATCC). Chicken embryo fibroblast (CEF) primary cells were prepared from 9-day-old specific-pathogen-free (SPF) embryonated chicken eggs as described previously (20). DF-1 and CEF cells were maintained in RPMI medium 1640 (Gibco) with 10% fetal calf serum (FCS; Gibco). NDV strain Herts/33 was obtained from the China Institute of Veterinary Drug Control (Beijing, China). To obtain the replication-incomplete NDV Herts/33 strain, the infected allantoic fluid (3 ml) was irradiated with UV at 75 mW/cm^2 using a low-pressure mercury vapor discharge lamp. The absence of virus infectivity after UV treatment was confirmed by the lack of replication in 9-day-old SPF embryonated chicken eggs. The plasmid GFP-LC3 was purchased from Origene. The tandem fluorescent monomeric red fluorescent protein (mRFP)-GFP-LC3 (ptfLC3) was obtained from Tamotsu Yoshimori (Osaka University, Osaka, Japan) (21).

Viral infection, drug treatment, and cell viability assay. DF-1 and CEF cells were infected with NDV at a multiplicity of infection (MOI) of 1 at 37°C. Following a 1-h absorption period, unattached viruses were removed and the cells were then washed three times with phosphate-buffered saline (PBS) and cultured in complete medium at 37°C. Viral titers on DF-1 cells were determined as median tissue culture infective dose ($TCID_{50}$) as described previously (22). The replication of NDV in the cells was determined as the expression of NDV P protein with monoclonal antibody against NDV P protein using Western blotting as described previously (23).

For pharmacological experiments, the optimal concentrations of drugs used in this experiment were determined by WST-1 assay according to the manufacturer's guidelines. The concentrations tested for wortmannin were 100, 300, and 500 nM; those for CQ were 25 and 50 μM; and those for rapamycin were 50, 100, 300, and 500 μM. The drugs were dissolved in dimethyl sulfoxide (DMSO) and diluted in complete medium, respectively. DMSO was used for a control. Briefly, 5×10^4 cells grown in each well of a 96-well plate were treated with each drug at different concentrations in a 100-μl volume for 24 h. Premixed WST-1 cell proliferation reagent (10 μl; Clontech) was then added to each well and incubated for 4 h. Cell viability was determined by measuring the absorbance at 450 nm (reference wavelength, 690 nm) against the background control. DF-1 or CEF cells were pretreated with optimal concentrations of rapamycin, wortmannin, or CQ for 4 h prior to viral infection. Subsequently, the cells were infected with NDV at an MOI of 1 as described above.

TEM. Transmission electron microscopy (TEM) was used for observation of autophagy as described previously (23). Specifically, DF-1 or CEF cells were infected with NDV at an MOI of 1, collected at 12 h postinfection, and subjected to preparation of samples for TEM observation. Images of the ultrathin sections were acquired using a CM-120 transmission electron microscope (Philips) at 80 kV. Autophagosome-like vesicles were defined as double- or single-membrane vesicles measuring 0.3 to 2.0 μm in diameter.

Confocal fluorescence microscopy. Confocal fluorescence microscopy was used for analysis of LC3 expression after NDV infection or rapamycin treatment. Specifically, DF-1 or CEF cells grown to 60 to 70% confluence on coverslips were transfected with plasmid GFP-LC3 (Origene) according to the manufacturer's guidelines. The cells were treated with virus infection or rapamycin as described above at 12 h posttransfection, and the localization of LC3 was visualized at 18 h posttreatment using a Nikon C1-si confocal fluorescence microscope (Nikon Instruments, Inc.).

Western blotting. Western blotting was performed to determine the conversion of endogenous LC3-I to LC3-II as described previously (24). Briefly, after NDV infection or drug treatment, cells were washed thoroughly and lysed in cell lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton X-100, sodium pyrophosphate, β-glycerophosphate, EDTA, Na_3VO_4, leupeptin). The lysates were briefly sonicated and cleared by centrifugation for 5 min at 12,000 × *g* at 4°C. The lysates were further denatured by incubation for 5 min at 95°C in sample buffer (2% SDS, 10% glycerol, 60 mM Tris [pH 6.8], 5% β-mercaptoethanol, 0.01% bromophenol blue). The samples were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Whatman International, Ltd.). After incubation in blocking buffer (5% nonfat milk powder in Tris-buffered saline containing 0.2% Tween 20 [TBS-T]) for 1 h at room temperature, the membrane was reacted with primary antibodies overnight at 4°C and HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) for 1 h at room temperature. The antibody-antigen complex was visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech) with a Kodak imager (Carestream Health, Inc.). Quantification of protein blots was performed using the Quantity One 1-D software (version 4.4.0) (Bio-Rad Laboratories, Inc.) on images acquired from an EU-88 image scanner (Seiko Epson Corp.).

Autophagic flux measurements. p62/SQSTM1 (p62) degradation was analyzed by Western blotting for measurement of the autophagic flux as described previously (12). DF-1 cells were infected with NDV Herts/33 at an MOI of 1 at 37°C, and cells were harvested and detected at 6, 12, 18, 24, and 36 h postinfection (hpi) using anti-p62 antibody as primary antibody.

Turnover of LC3-II in the presence or absence of lysosomal protease inhibitors E64d and pepstatin A was investigated as described previously (12). DF-1 cells were subjected to a 1-h absorption period of NDV Herts/33 and further cultured in fresh medium in the absence or presence of E64d and pepstatin A at 10 μg/ml each. Cells were harvested at indicated time points and detected with anti-LC3B and -p62 antibodies.

GFP-LC3 lysosomal delivery and proteolysis have been used to monitor autophagic flux (25). DF-1 cells were transfected with GFP-LC3 followed by treatment at 24 h posttransfection with infection by NDV Herts/33 at an MOI of 1 or mock treatment as a negative control. Cells were harvested at indicated time points and detected with anti-green fluorescent protein (anti-GFP) and anti-P antibody.

GFP-LC3 colocalization with the lysosome-associated membrane protein 1 (LAMP1) was also investigated as described previously (12). Briefly, DF-1 cells grown to 60 to 70% confluence on coverslips were transfected with GFP-LC3 and infected with NDV Herts/33 at an MOI of 1 for 24 h. Cells were fixed and probed with rabbit antibody against LAMP1 (Abcam). The antibody-antigen complexes were detected with TRITC-conjugated goat anti-rabbit secondary antibody (Jackson) and examined with a Nikon C1-si confocal fluorescence microscope (Nikon

Instruments, Inc.). A tandem reporter construct, mRFP-GFP-LC3 (ptfLC3), was used as an additional plasmid to measure the autophagic flux (12, 21). DF-1 cells grown to 60 to 70% confluence on coverslips were transfected with ptfLC3 and infected with NDV Herts/33 at an MOI of 1. At 24 and 30 hpi, cells were fixed and visualized by confocal microscopy (Nikon).

RNA interference. RNA interference was used to knock down *Beclin 1*, a key gene for autophage formation. DF-1 cells grown to 60 to 70% confluence in 6-well plates were transfected with *Beclin 1* and scrambled siRNA with Lipofectamine 2000 (Invitrogen) as described previously (24). Briefly, 100 pmol siRNA was diluted in 250 μl serum-free Opti-MEM medium (Invitrogen) and 5 μl Lipofectamine 2000 was diluted in 250 μl of Opti-MEM, respectively. After incubation for 5 min at room temperature, the diluted siRNA and Lipofectamine 2000 were mixed and incubated for 20 min at room temperature. The mixtures were then added dropwise to each well. The plates were incubated at 37°C for 6 h, and the supernatants were removed. Cells were washed three times with PBS and incubated for an additional 48 h before the samples were collected for silencing efficiency tests.

Animal experiments. One-day-old SPF chickens were obtained from Merial (Merial Vital Laboratory Animal Technology Company, Beijing, China) and kept under a controlled temperature (28 to 30°C). The chickens were housed in cages under biosafety conditions and a 12-h light/dark cycle with free access to food and water during the study. Care and maintenance of all animals were in accordance with the Institutional Animal Care and Use Committee (IACUS) guidelines set by the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Science (CAAS). Experiment 1 (groups 1 to 3) was designed to measure the autophagy induction in chickens infected with NDV strain Herts/33. Nine 7-day-old SPF chickens were randomly divided into 3 groups of 3 and treated with PBS, starvation, and NDV infection, respectively. At 48 h posttreatment, chicken tissues were subjected to Western blotting to measure the conversion of LC3-I to LC3-II. The mRNA level of the NDV P gene in the tissue was used for evaluation of the quantity of NDV and measured by real-time quantitative reverse transcription-PCR (qRT-PCR) as described previously (26). The β-actin gene was used as an endogenous control. Experiment 2 (groups 4 to 9) was designed to measure the roles of drugs and starvation in the pathogenesis of NDV infection. Thirty 7-day-old SPF chickens were randomly divided into 6 groups of 5. *In vivo* drug experiments were carried out as described previously (27). Briefly, the chickens in groups 5, 6, 7, 8, and 9 were treated with PBS, rapamycin (2 mg/kg of body weight), starvation, wortmannin (2 mg/kg), or CQ (20 mg/kg) and infected with NDV strain Herts/33, respectively. The chickens in group 4 were used as nontreatment controls. At 48 h posttreatment, the virus levels in chicken tissues were measured and the tissues were subjected to histopathological observation as described previously (27). Experiment 3 (groups 10 to 15) was designed to measure the roles of drugs and starvation on the survival of chickens after NDV infection. Sixty 7-day-old SPF chickens were randomly divided into 6 groups of 10. The treatment for chickens in groups 10 to 15 was the same as that for chickens in groups 4 to 9, respectively. The survival rates of chickens in each group were recorded for 6 days. Chickens in group 14 were fed every other day but had free access to drinking water.

Statistical analysis. Data were expressed as means ± standard deviations. Significance was determined with the two-tailed independent Student *t* test ($P < 0.05$).

RESULTS

Infection of replication-competent NDV triggers steady-state autophagy in chicken cells. TEM was used to monitor autophagy induction on DF-1 or CEF cells post-NDV infection. The results showed that double-membrane vesicles, in which cytosolic components or organelles were sequestered, were significantly increased in DF-1 cells postinfection compared with mock-infected cells (Fig. 1A).

To further verify that NDV infection can activate the autophagy machinery, GFP-LC3 dot formation during NDV infection was investigated. Compared with mock-infected DF-1 or CEF cells, large amounts of punctate GFP-LC3 proteins were observed in virus-induced syncytia, which are the characteristic of NDV-infected permissive chicken cells. Furthermore, most of the GFP-LC3 dots were detected as cup- and ring-shaped structures (Fig. 1B, panels c and d).

Conversion from LC3-I to LC3-II was monitored at 6 h, 12 h, 18 h, 24 h, and 36 h post-NDV infection. As shown in Fig. 1C, DF-1 cells presented significant LC3-I-to-LC3-II conversion at the late stages of infection. The densitometry ratio of LC3-II to β-actin was notably increased by 18 h postinfection compared with the mock-infected DF-1 cells (Fig. 1D). Meanwhile, a monoclonal antibody that specifically recognizes NDV P protein was applied to track the progression of NDV infection. The results showed that the LC3-II level correlates well with P protein expression, indicating that steady-state autophagy was indeed induced by NDV infection. Likewise, by monitoring LC3-I-to-LC3-II conversion, we found that the Herts/33 strain also induced autophagy in CEF cells (data not shown).

To our surprise, rapamycin treatment induced LC3-I-to-LC3-II conversion at 18 hpi only, not at other time points that we investigated (Fig. 1C). In addition, just a small quantity of punctate GFP-LC3 was observed in rapamycin-treated DF-1 cells (Fig. 1B, panel b). Moreover, single-membrane vacuoles were found in magnified rapamycin-treated cells (Fig. 1A, panels b to h), whereas the typical double-membrane vesicle was absent there. Therefore, we concluded that rapamycin did not induce obvious autophagy in chicken DF-1 cells as it did in mammalian cells.

To investigate whether viral replication is required in NDV-induced autophagy, UV-inactivated NDV Herts/33 was used for the experiment. The results showed neither detectable conversion from LC3-I to LC3-II nor expression of P protein at all time points postinfection, while native Herts/33 triggered autophagy normally (Fig. 1E). The results indicate that steady-state autophagy is induced by NDV infection and not by interaction of chicken cells with noninfecting virus particles.

Increased level of autophagic flux in NDV-infected chicken cells. p62 serves as a link between LC3 and ubiquitinated substrates and accumulates with the inhibition of autophagy, and so its degradation is considered an indicator for assessing autophagic flux. As shown in Fig. 2A and B, Herts/33 infection increased degradation of p62 in DF-1 cells over time. No detectable p62 was shown at 36 hpi (Fig. 2A and B). There was no significant change of p62 between 6 h and 36 h in the mock-infected control. Similar results were obtained in Herts/33-infected CEF cells (data not shown).

To further confirm that NDV infection can enhance autophagic flux, we measured turnover of LC3-II in the presence or absence of lysosomal protease inhibitors E64d and pepstatin A. E64d is a membrane-permeative inhibitor of cathepsins B, H, and L, and pepstatin A is an inhibitor of cathepsins D and E. The upregulation of LC3-II in the presence of lysosomal protease inhibitors represents the increase of autophagic flux. As demonstrated in Fig. 2C, D, and E, employment of both E64d and pepstatin significantly inhibited LC3-II and p62 degradation in NDV-infected cells. Similar results were obtained in Herts/33-infected CEF cells (data not shown).

The GFP-LC3 chimera is sensitive to degradation when delivered to a lysosome, whereas GFP is relatively resistant to hydroly-

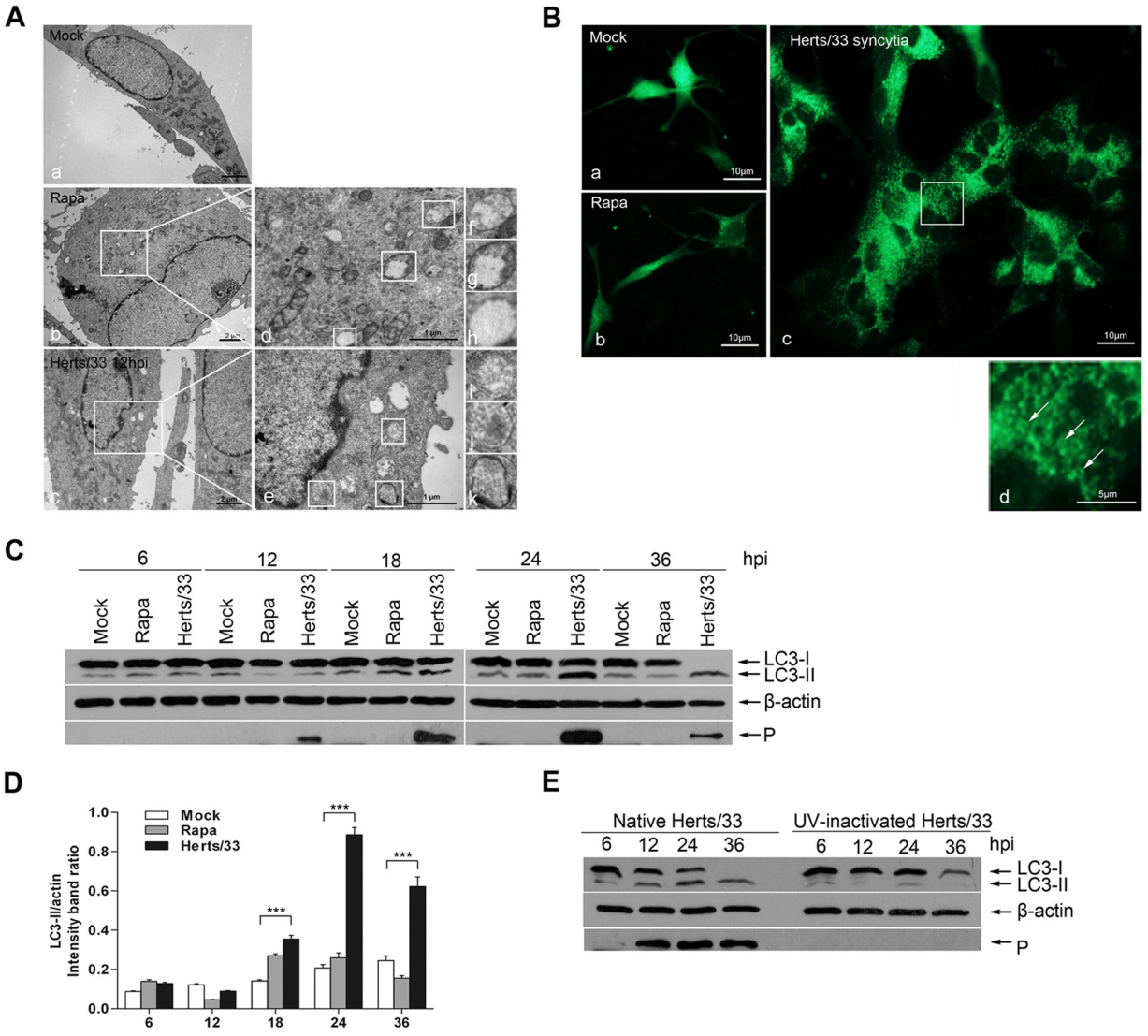

FIG 1 NDV infection triggers autophagy in DF-1 cells. (A) TEM observation. (a) DF-1 cells were mock treated as a negative control; (b) DF-1 cells were treated with rapamycin as a control; (c) DF-1 cells were infected with NDV Herts/33 at an MOI of 1 for 12 h; (d and e) higher-magnification views of panels b and c, respectively; (f, g, h, i, j, and k) further enlargement of vacuole or autophagosome-like structure. Bars, 2 μm (a, b, and c) and 1 μm (d and e). (B) (a to c) Confocal microscopy. DF-1 cells were transfected with GFP-LC3 followed by treatment at 24 h posttransfection with mock treatment as a negative control (a); rapamycin treatment as a control (b); or infection of NDV Herts/33 at an MOI of 1 for 24 h, indicating a distribution of GFP-LC3 in the syncytia induced by NDV infection (c). (d) Higher-magnification view of panel c. Bars, 10 μm (a, b, and c) and 5 μm (d). (C) Western blotting. The turnovers of LC3-I to LC3-II were detected for mock-treated DF-1 cells, rapamycin-treated DF-1 cells, or DF-1 cells infected with NDV Herts/33 at an MOI of 1. Cells were harvested at indicated time points and detected with anti-LC3B antibody. β-Actin was used as a protein loading control. P indicates the expression of NDV P protein, which was detected with anti-P monoclonal antibody. (D) The intensity band ratio of LC3-II to β-actin. Representative results are shown with graphs representing the ratio of LC3-II to β-actin normalized to the control condition. Data are presented as means from three independent experiments. Significance was analyzed with two-tailed Student's *t* test. ***, $P \leq 0.001$. (E) Comparison of LC3-I-to-LC3-II turnovers and NDV P expression levels in DF-1 cells inoculated with replication-competent or UV-inactivated NDV Herts/33 at an MOI of 5.

sis. As shown in Fig. 2F, free GFP was detected at 36 hpi, and it was much more abundant in NDV-infected DF-1 cells than in mock-infected cells. In addition, no detectable GFP-LC3-II was shown in NDV-infected DF-1 cells.

LAMP1 is a marker of endosomes and lysosomes that colocalizes with LC3 during the autolysosome maturation. GFP-LC3 colocalization with LAMP1 was investigated to confirm NDV-induced autophagic flux. As shown in Fig. 2G, colocalization of LAMP1 and LC3 was detected in virus-infected DF-1 cells, whereas mock-infected cells showed a diffuse GFP-LC3 pattern with no colocalization with LAMP1 (Fig. 2G). GFP is sensitive to lysosomal proteolysis and may diminish quickly in acidic pH, while red fluorescent protein (RFP) retains fluorescence even at acidic pH; therefore, a tandem reporter construct, ptfLC3, was used to measure the NDV-induced autophagic flux. The results showed that NDV infection of ptfLC3-transfected DF-1 cells resulted in a shift from yellow to partially red fluorescence from 24 to 30 hpi, indicating the increased level of autophagic flux (Fig. 2H). Taken together, these data clearly suggest that NDV infection enhances autophagic flux in DF-1 and CEF cells.

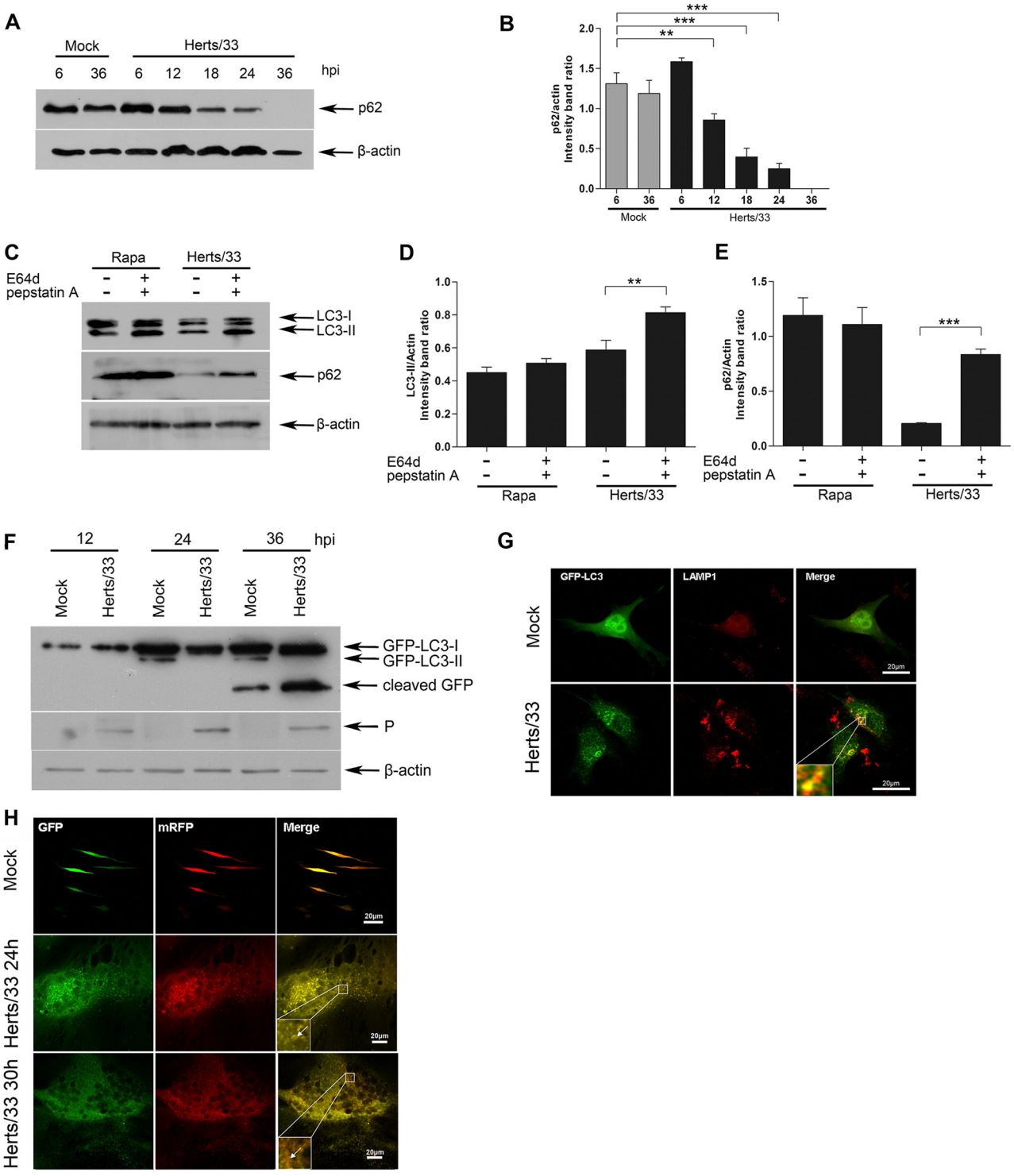

FIG 2 Measurement of the autophagic flux in DF-1 cells infected with NDV. (A) Western blotting of the degradation of p62. p62 in DF-1 cells infected with NDV Herts/33 at an MOI of 1 was monitored using anti-p62 antibody. Mock-infected DF-1 cells were used as negative controls. β-Actin was used as a protein loading control. (B) Representative results are shown with graphs representing the ratio of LC3-II to β-actin normalized to the control condition. Data are presented as means from three independent experiments. Significance is analyzed with two-tailed Student's *t* test (**, $P \leq 0.01$; ***, $P \leq 0.001$), compared to the control group. (C) DF-1 cells were subjected to a 1-h absorption period of NDV Herts/33 and further cultured in fresh medium in the absence (−) or presence (+) of E64d and pepstatin A at 10 μg/ml each. Western blotting was performed using anti-LC3B and anti-p62 antibodies. Rapamycin-treated DF-1 cells were used as controls. β-Actin was used as a protein loading control. (D and E) Representative results are shown with graphs representing the ratio of LC3-II to β-actin (D) or p62 to β-actin (E) normalized to the control condition. Data are presented as means from three independent experiments. Significance is analyzed with two-tailed Student's *t* test (**, $P \leq 0.01$; ***, $P \leq 0.001$), compared to the control group. (F) DF-1 cells were transfected with GFP-LC3 followed by treatment at 24 h posttransfection with infection of NDV Herts/33 at an MOI of 1 or mock treatment as a negative control. Cells were harvested at indicated time points and detected with anti-GFP and anti-P antibody. β-Actin was used as a protein loading control. (G) DF-1 cells transfected with GFP-LC3 were mock infected or infected with NDV Herts/33 at an MOI of 1. Cells were fixed and stained with anti-LAMP1 antibody and then visualized by confocal microscopy. Bars, 20 μm. (H) DF-1 cells transfected with ptfLC3 were mock infected or infected with NDV Herts/33 at an MOI of 1. The cells were collected, fixed, and visualized at 24 h and 30 h postinfection, respectively. Bars, 20 μm.

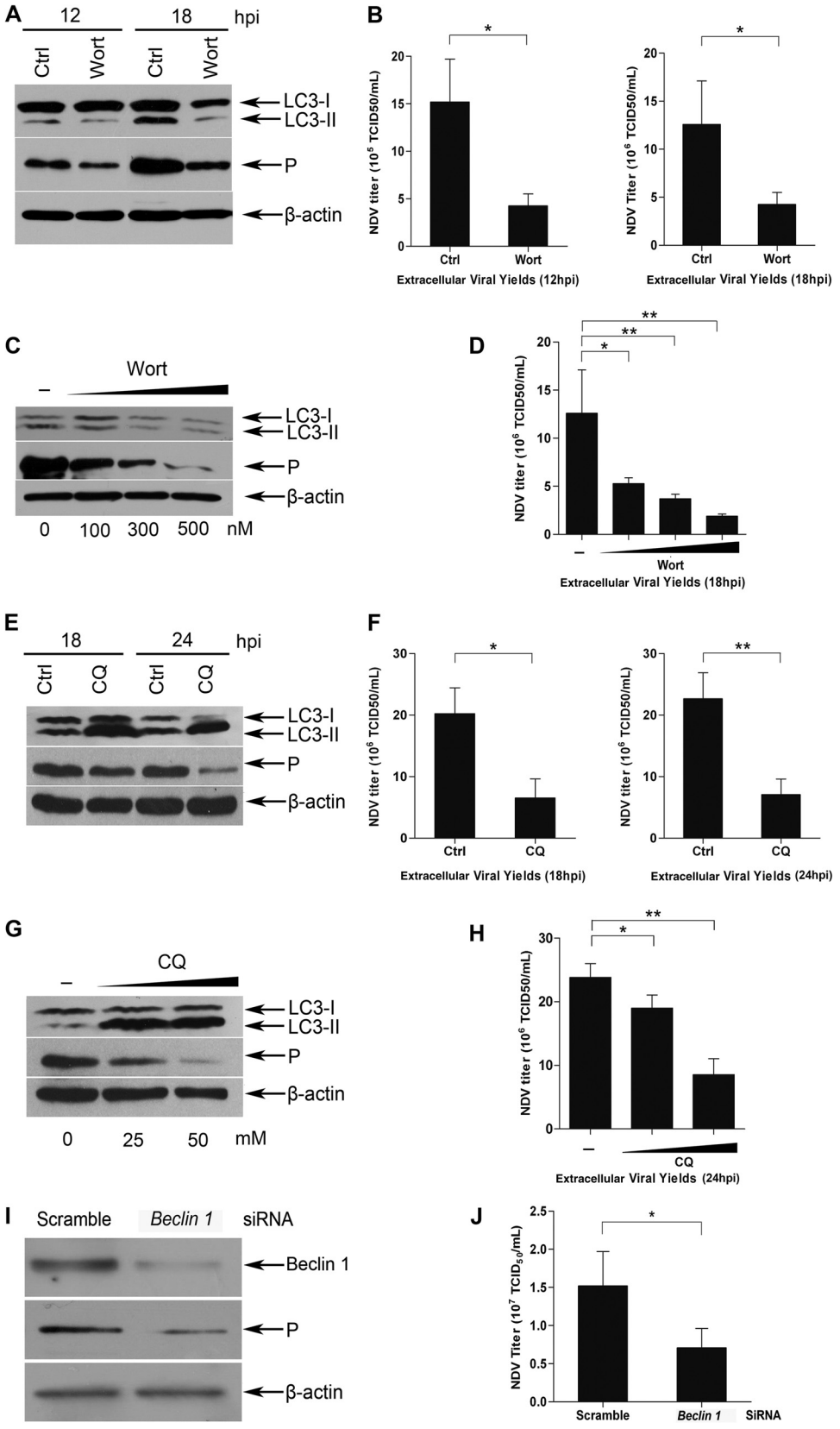

FIG 3 Inhibition of autophagy reduced NDV replication on DF-1 cells. (A) Western blotting of the autophagy inhibited with wortmannin. DF-1 cells were pretreated with wortmannin (100 nM) for 4 h followed by infection of NDV Herts/33 at an MOI of 1. DMSO was used as a control. After 1 h of virus absorption at 37°C, the cells were further cultured in fresh medium in the absence or presence of wortmannin. At 12 and 18 h after infection with NDV, the cells were

Pharmacological inhibition of autophagy reduced NDV replication in DF-1 cells. To analyze the effect of autophagy on NDV replication in chicken cells, wortmannin, which inhibits autophagy at the early stage by inhibiting the phosphatidylinositol 3-kinase (PI3K) pathway, was applied. As demonstrated in Fig. 3A1, conversion from LC3-I to LC3-II, as well as expression of P protein, was reduced in wortmannin-treated DF-1 cells compared with those in control cells. Meanwhile, viral progeny titers were significantly reduced in the cells with wortmannin treatment. There were a 3.6-fold reduction at 12 hpi and a 3-fold reduction at 18 hpi, respectively (Fig. 3B). CQ, which acts at postsequestration and causes the accumulation of sequestered material in either autophagosomes or autolysosomes, was used for inhibition of autophagy at the late stage. CQ treatment increased the conversion of LC3-I to LC3-II in NDV-infected DF-1 cells but decreased the expression of P protein and the yields of extracellular virus at 18 and 24 hpi, indicating that inhibition of the late stage of autophagy also reduced NDV replication (Fig. 3E and F). Moreover, both wortmannin and CQ treatment reduced the levels of P protein and the yields of NDV progeny in a dose-dependent manner (Fig. 3C, D, G, and H).

High concentrations of drugs might influence cell viability and thus affect viral yields in host cells. The WST-1 assay showed that the viability of DF-1 cells was not significantly affected by drug treatment at the indicated doses (data not shown). The results of these pharmacological experiments demonstrated the positive function of autophagy upon NDV infection.

Knockdown of autophagy-related genes (ATGs) reduced NDV replication. Beclin 1 is critical for the signaling pathways involved in autophagosome formation. As shown in Fig. 3I, DF-1 cells transfected with small interfering RNAs (siRNAs) specific to Beclin 1 exhibited an obvious decrease of endogenous Beclin 1 protein and viral P protein compared with cells transfected with scrambled siRNAs. The reduced Beclin 1 protein level resulted in a 2.9-fold decrease in extracellular virus yields (Fig. 3J). The results indicate that autophagy machinery is required for effective infection of NDV, which is in line with the results of pharmacological experiments.

Rapamycin does not promote NDV infection in DF-1 cells. Inhibition of autophagy by either drugs or siRNA reduced NDV infection. Next, we used rapamycin as an autophagy inducer to verify the role of autophagy in NDV infection. To our surprise, no detectable changes of viral protein synthesis or the yields of NDV progeny were observed after rapamycin treatment (Fig. 4A to D). One possibility is that chicken TOR is not sensitive to rapamycin. This could be inferred from the minimal LC3 conversion upon rapamycin treatment in Fig. 1. However, the concrete mechanism needs to be further investigated.

NDV triggers autophagy *in vivo*. Animal experiment 1 showed that the conversion from LC3-I to LC3-II in heart, liver, and lung in both group 2 and group 3 and in spleen in group 3 was apparent. However, no detectable conversion from LC3-I to LC3-II was found in intestine, kidney, bursa, and brain (Fig. 5A). The densitometry ratio of LC3-II/β-actin was increased in heart, liver, and lung of chickens in groups 2 and 3 and spleen of chickens in group 3, compared with mock-infected chickens in group 1 (Fig. 5B). Investigation of the mRNA levels of viral P protein in chickens in group 3 showed 4.6-, 3.4-, 2.1-, 2.7-, 1.9-, 1.8-, and 1.4-fold increases in heart, liver, spleen, lung, intestine, kidney, and bursa, respectively, compared with that in brain (Fig. 5C), which was in accord with the autophagic level of those tissues to some extent (Fig. 5B). These results indicated that NDV triggers tissue-dependent autophagy *in vivo*.

Inhibition of autophagy induction reduced NDV titers in the lung of chicken. Animal experiment 2 was performed to investigate the role of autophagy on NDV replication *in vivo*. Wortmannin and CQ, which decreased NDV viral yields *in vitro*, were employed for autophagy inhibition *in vivo*. Rapamycin was also chosen for the experiment to identify whether it functions *in vivo*. Lung and intestine, two important target organs of NDV infection, were chosen for histopathological analysis and virus titration. As shown in Fig. 6B and D, wortmannin and CQ treatment dramatically decreased the virus titer in lung, while no change of viral progeny titer was observed in intestine. On the other hand, rapamycin treatment has no significant effect on viral progeny titers in both lung and intestine. As shown by histopathological analysis, NDV infection alone (group 5) induced local pulmonary hemorrhage in lung, but no pathological changes was observed in either wortmannin- or CQ-treated chickens in group 8 or 9 (Fig. 6A). We also observed intestinal wall ulcers in chickens infected with NDV. Treatments with wortmannin or CQ reduced the extent of intestinal ulcers caused by NDV infection (Fig. 6C). However, treatment with rapamycin has no apparent effect on tissue pathological change (group 6). Starvation treatment (group 7),

subjected to Western blotting using anti-LC3B and -P antibodies. β-Actin was used as a protein loading control. (B) Determination of the NDV replication on wortmannin-treated DF-1 cells. The extracellular virus yields were determined at 12 and 18 hpi and expressed as $TCID_{50}$/ml. Data are presented as means from three independent experiments. Significance is analyzed with two-tailed Student's *t* test. *, $P \leq 0.05$. (C) Western blotting of the autophagy and NDV P expression on DF-1 cells inhibited with wortmannin at the concentrations of 100, 300, and 500 nM. The samples were collected at 18 h after infection with NDV at an MOI of 1. (D) NDV titers on DF-1 cells treated with wortmannin at different concentrations. The extracellular virus yields were determined at 18 hpi and expressed as $TCID_{50}$/ml. Data are presented as means from three independent experiments. Significance is analyzed with two-tailed Student's *t* test. *, $P \leq 0.05$; **, $P \leq 0.01$. (E) Western blotting of the autophagy inhibited with CQ. DF-1 cells were pretreated with CQ (50 μM) for 4 h followed by infection with NDV Herts/33 at an MOI of 1. Double-distilled water was used as a control. After 1 h of virus absorption at 37°C, the cells were further cultured in fresh medium in the absence or presence of CQ. At 18 and 24 h after infection with NDV, the cells were subjected to Western blotting using anti-LC3B and -P antibodies. β-Actin was used as a protein loading control. (F) Determination of the NDV replication on CQ-treated DF-1 cells. The extracellular virus yields were determined at 18 and 24 hpi and expressed as $TCID_{50}$/ml. Data are presented as means from three independent experiments. Significance is analyzed with two-tailed Student's *t* test. *, $P \leq 0.05$; **, $P \leq 0.01$. (G) Western blotting of the autophagy and NDV P expression on DF-1 cells inhibited with CQ at the concentrations of 25 and 50 μM. The samples were collected at 24 h after infection with NDV at an MOI of 1. (H) NDV titers on DF-1 cells treated with CQ at different concentrations. The extracellular virus yields were determined at 24 hpi and expressed as $TCID_{50}$/ml. Data are presented as means from three independent experiments. Significance is analyzed with two-tailed Student's *t* test. *, $P \leq 0.05$; **, $P \leq 0.01$. (I) DF-1 cells were transfected with either specific siRNA targeting *Beclin 1* or scrambled siRNA. At 48 h after transfection, cells were infected with NDV Herts/33 at an MOI of 1. Samples were collected at 18 h after infection with NDV and subjected to Western blotting of Beclin 1 and NDV P expression levels using anti-Beclin 1 and -P antibodies. β-Actin was used as a protein loading control. (J) The extracellular virus yields were determined at 18 h postinfection and expressed as $TCID_{50}$/ml. Data are presented as means from three independent experiments. Significance is analyzed with two-tailed Student's *t* test. *, $P \leq 0.05$.

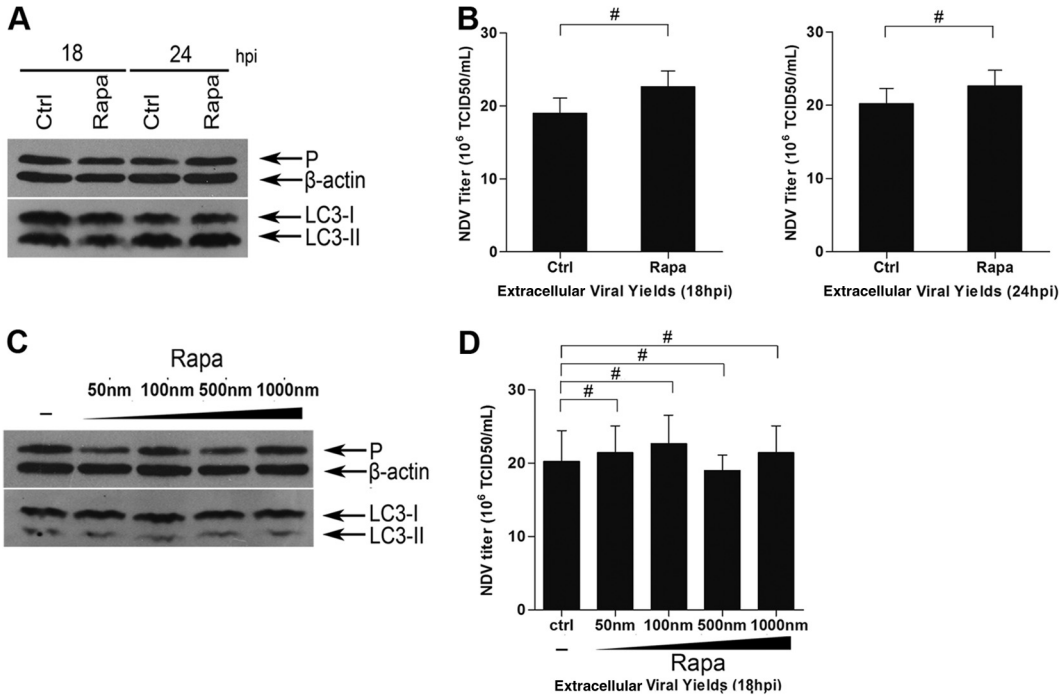

FIG 4 Administration of rapamycin has no effect on NDV infection in DF-1 cells. (A) DF-1 cells were pretreated with rapamycin (100 nm) or DMSO (control) in complete medium for 4 h and then processed as described for Fig. 3A. (B) At 18 and 24 hpi, extracellular virus yields were determined by $TCID_{50}$ in DF-1 cells and expressed as $TCID_{50}$/ml. (C) DF-1 cells were pretreated with rapamycin or DMSO (control) and then processed as described for panel A, except that the concentration of drug varied (50, 100, 500, and 1,000 μM; wedge). (D) At 18 hpi, extracellular virus yields were determined by $TCID_{50}$ in DF-1 cells and expressed as $TCID_{50}$/ml. Data are presented as means from three independent experiments. Significance is analyzed with two-tailed Student's *t* test (#, $P > 0.05$), compared to the control group.

a traditional autophagy inducer, enhanced the extent of pulmonary hemorrhage and intestinal ulcer (Fig. 6A and C) and promoted NDV replication effectively in both lung and intestine (Fig. 6B and D).

Animal experiment 3 showed that the survival rates of chickens were increased by treatment with wortmannin. CQ treatment did not raise the chicken survival rates but did delay the time of death for 12 h. In contrast, starvation severely accelerated the death of chickens (Fig. 6E). In consideration of the animals' welfare, chickens in the starvation group were fed every other day, instead of being starved constantly, in this long-duration experiment. Treatment of rapamycin has no obvious effect on the survival rates of chickens.

Together, these *in vivo* results further confirm the studies *in vitro*, which concluded that autophagy is beneficial for NDV infection of chickens.

DISCUSSION

Many strains of NDV cause serious disease in almost all birds. Knowledge of the molecular mechanism for its pathogenesis, especially the interaction between the virus and host, is limited. Recent years have seen a growing list of viruses whose replication is related to autophagy of host cells (23, 28–30). For instance, autophagy-related proteins, including Beclin 1, Atg4B, Atg5, and Atg12, are required for initiation of hepatitis C virus (HCV) replication (28). In addition, avian influenza A H5N1 virus and avian reovirus trigger autophagy through suppression of mTOR signaling (31, 32).

In the present study, we first demonstrated that NDV infection significantly increased the number of double- and single-membrane vesicles in DF-1 cells by electron microscopy, indicating that autophagosomes were formed (33). Accumulation of GFP-LC3 dots in NDV-infected cells confirmed further that the observed double- and single-membrane vesicles were related to autophagy. The induction of autophagy caused by NDV infection was further confirmed by Western blotting to examine LC3 modification. We found large numbers of GFP-LC3 dots in virus-induced syncytia in NDV-infected chicken DF-1 and CEF cells in this study. It is well known that the formation of syncytia is caused by the cooperation of fusion (F) and hemagglutinin-neuraminidase (HN) proteins of paramyxoviruses (34, 35). A recent study reported that morbilliviruses, which also belong to the *Paramyxoviridae* family, rapidly induce autophagy in the host cell, which is required for cell-to-cell viral spread. In addition, only expression of the glycoprotein pairs from different members of the *Paramyxoviridae* family, including measles virus, canine distemper virus, Nipah virus, Hendra virus, and mumps virus, induces syncytia that contain LC3 puncta (36). Therefore, the HN and/or F proteins of NDV may play important roles in autophagy induction. The fact that UV-inactivated NDV could not trigger LC3 conversion suggests that viral replication is required to induce autophagy. Previous reports have indicated that some viruses, such as porcine reproductive and respiratory syndrome virus (PRRSV), HCV, and herpes simplex virus, induce an incomplete autophagic response by blocking autophagosome degradation (37–39). By several means of measuring autophagic flux, including monitoring p62 degradation, LC3-II turnover, and GFP-LC3

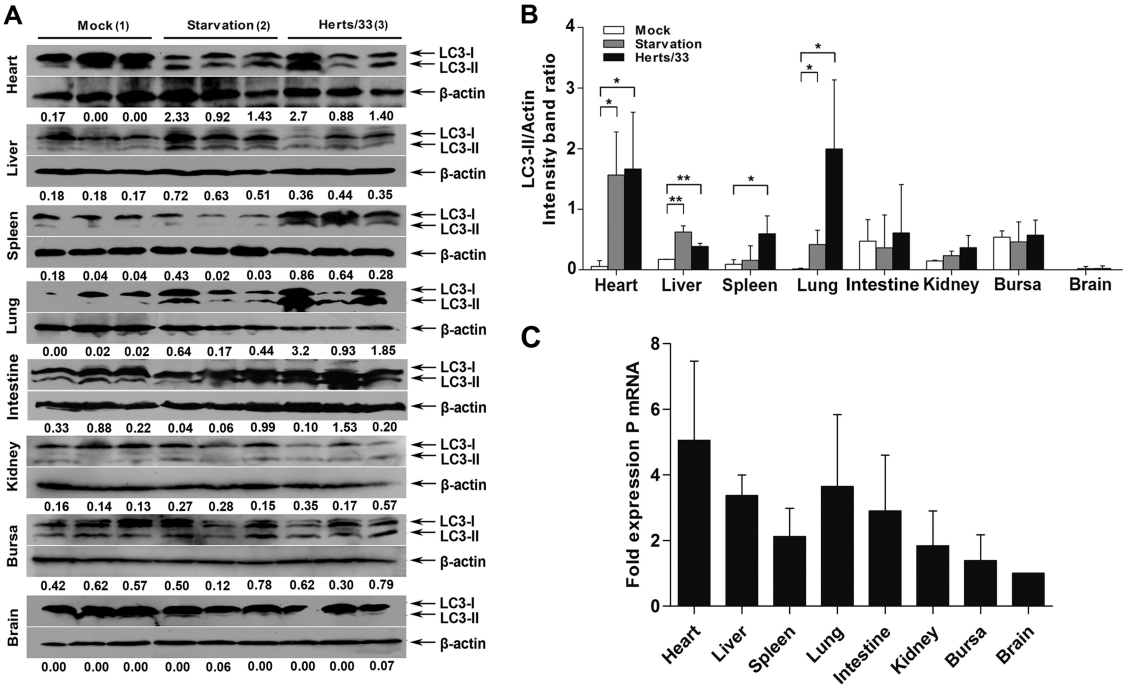

FIG 5 Western blotting of the turnovers of LC3-I to LC3-II in tissues of chickens infected with NDV. (A) Nine 7-day-old SPF chickens were randomly divided into 3 groups of 3. The chickens in the mock group were treated intranasally with PBS as a mock control. The chickens in the starvation group were deprived of food for 48 h but had free access to drinking water. The chickens in the Herts/33 group were infected intranasally with NDV strain Herts/33 at 10^6 50% egg infectious doses. Tissue homogenates prepared from three chickens in each group were subjected to Western blotting with anti-LC3B antibody. β-Actin was used as a protein loading control. Optical densities of protein bands were measured. The LC3-II/β-actin intensity band ratios are marked below each band. (B) Representative results of LC3-II/β-actin intensity are shown with graphs representing the ratio of LC3-II to β-actin normalized to the control condition. Data are presented as means from three chickens. Significance is analyzed with two-tailed Student's *t* test. *, $P \leq 0.05$; **, $P \leq 0.01$. (C) The transcription levels of NDV P gene in NDV-infected tissues were detected by real-time qRT-PCR. Fold expressions are calculated based on the level of the NDV P gene in brain.

lysosomal delivery and proteolysis, we produced strong evidence that autophagic flux was enhanced upon NDV infection.

In order to further determine the role of autophagy in NDV infection, we examined the effect of autophagy on viral replication. The inhibition of autophagy by treatments with either wortmannin or CQ decreased both viral yields and P protein synthesis in a dose-dependent manner. Given that wortmannin is not a specific inhibitor but rather inhibits both class I and class III PI3K, possibly along with protein synthesis (40, 41), we next depleted the essential endogenous Beclin 1, which is part of the class III PI3K (PI3KC3) lipid kinase complex that plays a central role in the induction of autophagy, and assessed the impact of autophagy upon NDV infection. As expected, knockdown of *beclin 1* suppressed viral yields and P protein synthesis. It has been reported that knockdown of *beclin 1* in immortalized human hepatocytes (IHHs) enhanced the innate immune response and thus inhibited hepatitis C virus (HCV) growth (42). Furthermore, the Atg5-Atg12 conjugate associates the caspase recruitment domain (CARD) of RIG-I and IPS-1 and inhibits signal transmission (43). This suggests that autophagy proteins might act indirectly on NDV viral spread by modulating the extent of the innate response against NDV, which needs further studies.

To our surprise, rapamycin, the autophagy inducer of mammalian cells, induced only a small quantity of punctate GFP-LC3 in DF-1 cells and no detectable LC3-I-to-LC3-II conversion at 6, 12, 24, and 36 h posttreatment. Moreover, the typical double-membrane vesicle was absent in rapamycin-treated cells. Therefore, we concluded that rapamycin did not induce obvious autophagy in chicken DF-1 cells, as it did in mammalian cells. Animal experiments showed no detectable changes of viral protein expression or viral titers after rapamycin treatment (Fig. 6A to D). The results indicate that rapamycin is not a good autophagy inducer of chicken cells. To confirm the unexpected results, we constructed GFP-labeled chicken LC3 for the experiment and it worked well in inducing accumulation of punctate GFP-chicken LC3 (GFP-cLC3) in NDV-infected DF-1 cells but not in rapamycin-treated DF-1 cells (data not shown).

Over the past few years, several *in vitro* studies have demonstrated the critical role of autophagy in virus infection. However, a limited number of studies have been carried out in an *in vivo* model. Lipidated LC3-II was detected by immunoblotting in mouse cervical spinal cord after enterovirus 71 (EV 71) infection (44). Our data showed that the conversion of LC3-I to LC3-II in various tissues, including heart, liver, spleen, lung, intestine, and kidney, but not bursa or brain, was observed in both starvation and virus infection groups. The changes in LC3-II levels are tissue dependent, which is in accordance with a previous report, in which the conversion of LC3-I to LC3-II was observed in mouse skeletal muscles, heart, and pancreas but not in brain after treatment by starvation (45). Regulation of autophagy induction can affect virus infection *in vivo*, which has been illustrated recently. Inhibition of autophagy through treatment with either autophagy inhibitor 3-methyladenine (3-MA) or *Atg5*-specific siRNA ameliorates acute lung injury in mice caused by influenza A H5N1

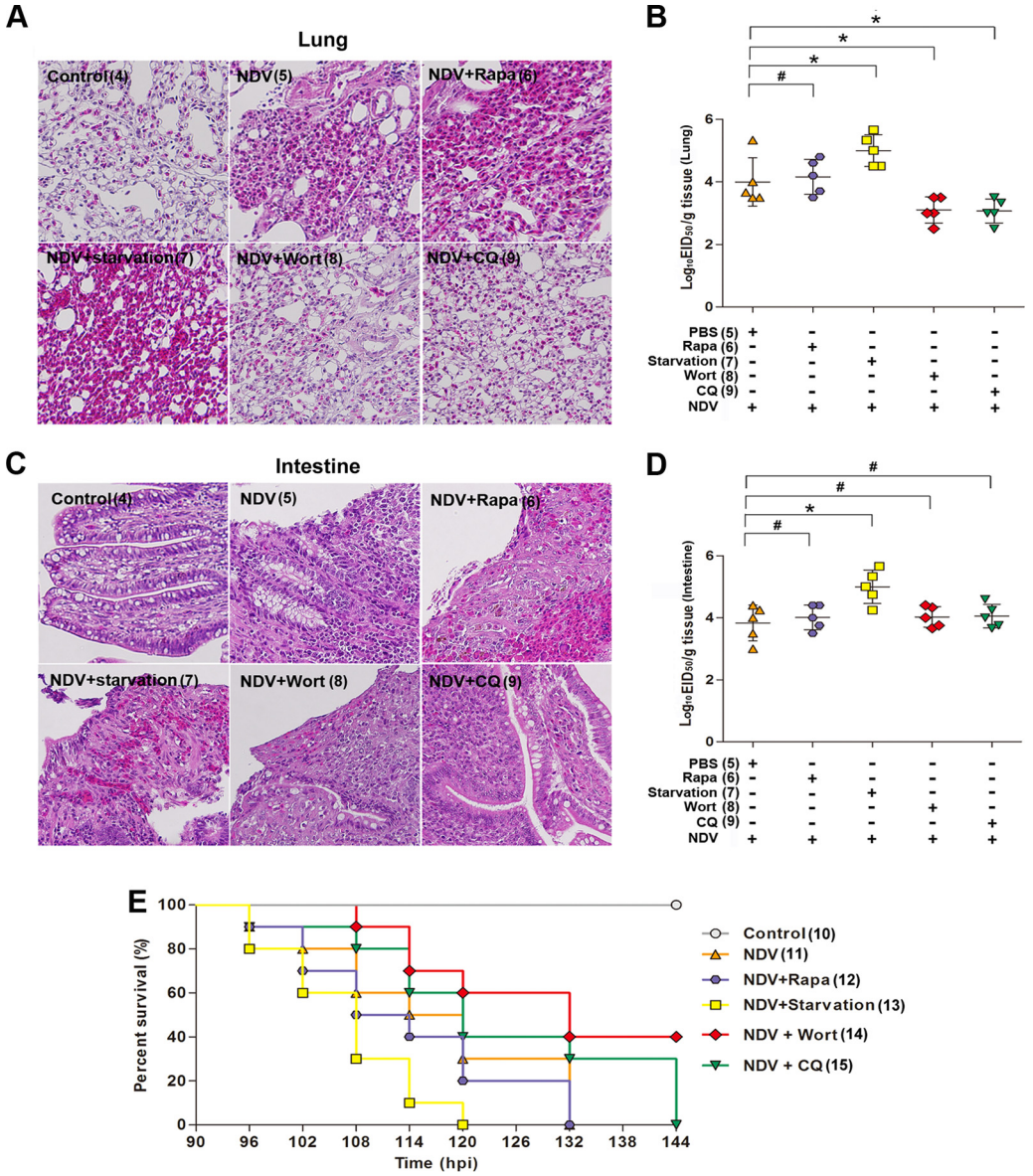

FIG 6 Regulation of autophagy affects NDV production and pathogenesis *in vivo*. Thirty 7-day-old SPF chickens were randomly divided into 6 groups of 5. *In vivo* drug experiments were carried out as described previously (27). The chickens in groups 5, 6, 8, and 9 were treated with PBS, rapamycin (2 mg/kg), wortmannin (2 mg/kg), or CQ (20 mg/kg) intramuscularly for 5 times at 2 h before virus inoculation as well as 2 h, 12 h, 24 h, and 36 h after virus inoculation, respectively. The chickens in group 7 were treated with starvation by being deprived of food for 48 h but had free access to drinking water. The chickens in groups 5 to 9 were infected intranasally with NDV strain Herts/33 at 10^6 50% egg infectious doses (EID_{50}). The chickens in group 4, as controls, received no treatment. At 48 h posttreatment, chickens were sacrificed and tissues were collected for measurement of virus titers (EID_{50}). The lungs and intestines were subjected to histopathological observation with hematoxylin and eosin staining. (A and C) Hematoxylin and eosin staining of cells in chicken lung (A) and intestine (C) infected with NDV Herts/33 in the absence or presence of either rapamycin, wortmannin, or CQ, respectively. Treatment with 48-h starvation was used as a positive control. Images are representative of three chickens. (B and D) Viral titers in chicken lung (B) and intestine (D) separated from five chickens in each group were expressed as EID_{50} per milliliter. Data are presented as the virus titers in tissues from five chickens. Significance is analyzed with two-tailed Student's *t* test. *, $P \leq 0.05$; #, $P \geq 0.05$. (E) Survival rates of chickens that were treated with rapamycin, wortmannin, or CQ before and after being infected with NDV Herts/33 ($n = 10$ chickens). Sixty 7-day-old SPF chickens were randomly divided into 6 groups of 10. The treatments for chickens in groups 10 to 15 were the same as those for chickens in groups 4 to 9, respectively. After treatment, the chickens in groups 11 to 15 were infected intranasally with NDV strain Herts/33 at 10^4 EID_{50}. The survival rates of chickens in each group were recorded for 6 days. Chickens in group 13 were fed every other day but had free access to drinking water. The numbers in parentheses represent the group numbers.

virus infection (27). A report on enteroviruses showed markedly reduced virus titers and less coxsackievirus B3-induced pancreatic pathology in *Atg5*-deficient mice (46). Similarly, our data demonstrated that inhibition of autophagy by wortmannin treatment reduced virus production and the extent of pathology in lung and intestine, thereby increasing the chicken survival rate. CQ treatment also reduced the extent of pathology but had no apparent effect on virus production and survival rate, although NDV replication was inhibited by CQ treatment *in vitro*. Starvation greatly enhanced the replication of NDV and the extent of pathology *in*

vivo. Moreover, starvation increased the death rate of chickens, confirming the general knowledge that chickens suffer from more severe NDV infection under the stress of starvation. Taken as a whole, the results of our *in vivo* experiments indicated that autophagy plays a critical role in NDV infection of chickens.

Autophagy not only is a regulated catabolic process but also plays an essential role in other signal pathways such as apoptosis and innate immunity (47–49). As NDV induced apoptosis and host immune response *in vitro* and *in vivo*, it is reasonable to conclude that autophagy might affect NDV replication through regulation of other pathways. The inhibition of autophagy significantly increases the apoptotic process in influenza virus- and Chikungunya virus (CHIKV)-infected cells (29, 50, 51). In dendritic cells infected with respiratory syncytial virus (RSV), which also belongs to the *Paramyxoviridae* family, autophagy-mediated pathways may be one of the critical mechanisms for driving innate cytokine production (52). However, in most other cell types, autophagy negatively regulates IFN-I production through the RIG-I-like receptor (RLR) pathway. The direct evidence is that Atg5-Atg12 conjugate blocks IFN-I signaling by binding to the caspase recruitment domains (CARDs) of RIG-I and IFN-promoter stimulator 1 (IPS-1) (43). Interestingly, chickens lack RIG-I and are sensitive to NDV and influenza virus infections (26, 53). These reports give us a clue that autophagy might play an important role in apoptosis or innate cytokine production after NDV infection, which needs to be further elucidated.

NDV, which can selectively replicate and can induce apoptosis in tumor cells, is being used as an experimental tumor therapy (54). We have reported that NDV infection induced autophagy in human glioma U251 cells (24). Since birds are the major host animals of NDV, we used DF-1 cells and chickens to study the autophagy induced by NDV infection in this study. It is widely known that ATGs, including LC3, are evolutionarily conserved from monocellular eukaryotic organisms to primates, although there may be some differences in their structure among eukaryotic organisms. However, the core Atg proteins are highly conserved in eukaryotes, and they act in a similar hierarchical manner (55). Our previous report has shown that human GFP-LC3 works in avian cells (31). Furthermore, the GFP-LC3 lysosomal delivery and proteolysis assay is a strong piece of evidence to confirm that GFP-LC3 does work in avian cells. A very recent report also showed that the autophagy pathway was conserved in chicken DF-1 and monkey Vero cells (56). The present study offers more data and in-depth analyses to confirm that NDV triggers autophagy in chicken DF-1 and CEF cells. Most importantly, for the first time, we identified the induction of autophagy in chicken tissues and confirmed that autophagy is critical for NDV infection *in vivo*.

In conclusion, this report demonstrates that autophagy plays an important enhancing role in NDV infection in chicken cells and tissues. Our studies provide insights into NDV-host interaction and may provide host cell targets for the development of antiviral strategies against NDV infection.

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