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Research Article

Knockout of Noxa with CRISPR/Cas9 Increases Host Resistance to Influenza Virus Infection

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The influenza virus induces cellular apoptosis during viral propagation, and controlling this virus-induced apoptosis process has been shown to have significant antiviral effects. The proapoptotic BH3-only protein Noxa is a strong inducer of apoptosis that can be activated by this virus, suggesting that Noxa has the potential as an anti-influenza target. To assess the value of Noxa as an antiviral target, we utilized CRISPR/Cas9 technology to produce a Noxa-knockout cell line. We found that the knockout of Noxa resulted in a dramatic reduction in the cytopathic effect induced by the influenza virus. Moreover, Noxa knockout decreased the expression of influenza viral proteins (NP, M2, HA, and NS2). In addition, Noxa deficiency triggered a complete autophagic flux to weaken influenza virus-induced autophagosome accumulation, indicating that Noxa may be a promising antiviral target for controlling influenza virus infections.

1. Introduction

The influenza virus is a member of the Orthomyxoviridae family and contains an envelope and eight negative-sense RNA segments that encode ten viral proteins, including hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), matrix protein (M), proton channel protein M2, and two nonstructural proteins, NS1 and NS2 [1]. Influenza, caused by the influenza virus, is a highly infectious respiratory disease that may result in pneumonia and is considered a threat to public safety [2]. Controlling influenza virus infections remains the most urgent issue at present. Due to the constant evolution of the influenza virus with antigenic drift and shift, vaccination as the main method against specific influenza viruses often fails to achieve the desired effect [3]. In addition, synthetic drugs based on viral genomes have resulted in the production of drug-resistant strains [4]. Therefore, discovery of novel antiviral drugs is essential.

It is known that the influenza virus closely relies on the host, including host factors and cellular processes [5–7]. The influenza virus needs to bind to its sialic acid receptor for entry [8]. Cellular clathrin- and caveolin-mediated endocytosis is important for viral uptake [9, 10]. Host factor glucosylceramidase (GBA) knockout significantly reduces the infection by transforming influenza virus trafficking to late endosomes [11]. Further, acidic nuclear phosphoprotein 32 (ANP32A) is required for influenza A viral RNA (vRNA) and complementary positive-sense RNA (cRNA) synthesis [12], suggesting that host factors and cellular processes have potential as anti-influenza targets.

Apoptosis is an innate cellular response to viruses [13]; however, the influenza virus has evolved special mechanisms to utilize host cellular apoptosis for survival and spread [7]. The influenza virus can utilize both intrinsic and extrinsic apoptosis signaling pathways to mediate apoptosis [7]. Therefore, understanding how the host controls the apoptotic process upon influenza virus infection is critical for the discovery of novel antiviral strategies. TGF-β1 treatment inhibits apoptosis and activation of influenza virus-induced caspase-1 and IL-1β [14]. Bax knockout not only blocks

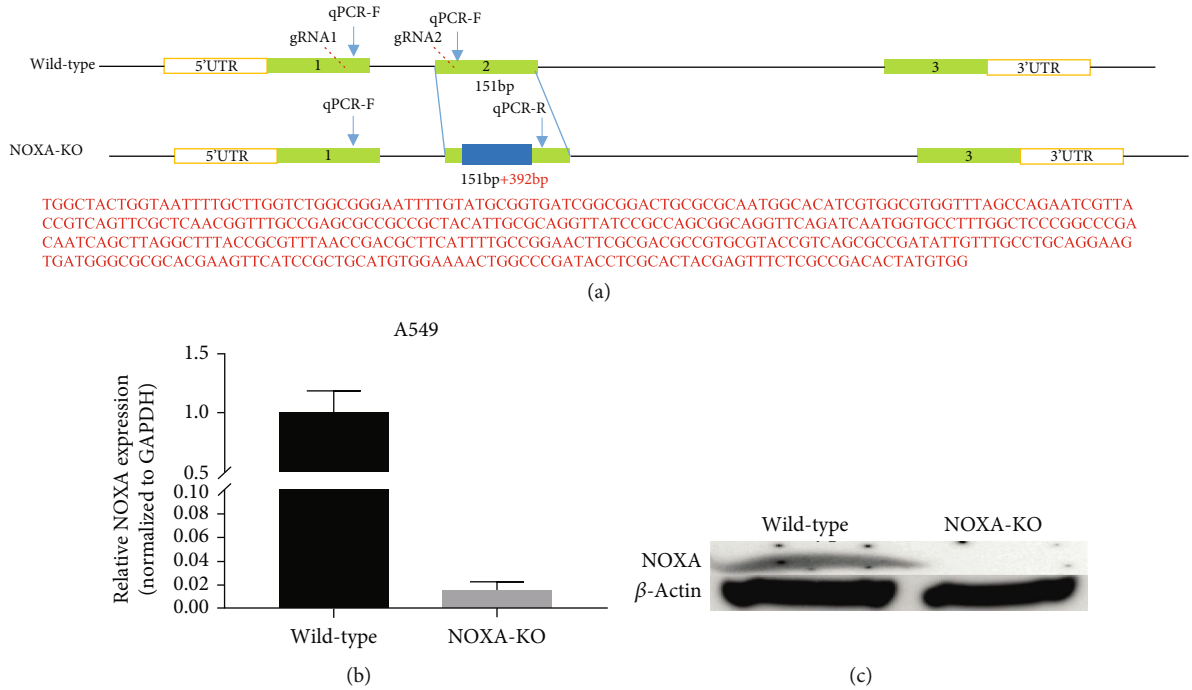

Figure 1: CRISPR/Cas9-mediated Noxa-knockout cells were generated and identified. (a) Schematic of gRNAs targeting Noxa. Sanger sequence showed a 392 bp inset in exon2. (b) Noxa mRNA expression was detected via qPCR analysis. The location of qPCR primers is shown. (c) Western blotting results showing that there is no expression in mutant A549 cells with Noxa gRNAs, comparing with wild-type cells.

influenza virus nucleoprotein (NP) trafficking from the nucleus to the cytomembrane but also interferes with virus propagation by apoptosis inhibition [15]. Overexpression of Musashi RNA-binding protein 1 (MSI1) inhibits the release of the Newcastle disease virus in DF-1 cells [16]. In addition, some natural compounds possess antiviral properties by repressing cellular apoptosis. Chrysin treatment reduces caspase-9 and caspase-3 activation, leading to the inhibition of influenza virus-triggered mitochondrial apoptosis [17]. Ebselen, a synthetic organoselenium compound, inhibits influenza virus-induced apoptosis and inflammatory responses to viral infection [18]. Taken together, these results suggest that apoptosis inhibition is an important antiviral strategy against influenza virus infections.

BH3-only protein Noxa is a proapoptotic mitochondrial protein that plays an important role in the apoptotic process [19]. Noxa can directly activate Bax and Bak to affect the interaction between BH3-only sensitizer and activator proteins [20]. Some reports have revealed that Noxa is involved in apoptosis related to pathogen infection [21, 22]. Knockout of Noxa reduced the cytopathic effect induced by viral infections, and Noxa-null cells showed high resistance to vesicular stomatitis virus- (VSV-) or encephalomyocarditis virus- (EMCV-) induced cytopathicity compared with wild-type and Noxa-complemented cells [21, 22]. In this study, we explored the anti-influenza virus effects of Noxa knockout and revealed its potential as a drug target. We found that Noxa knockout suppressed influenza virus replication and viral protein expression. Our results further demonstrate that Noxa deficiency weakens influenza virus-induced apoptosis and induces complete autophagic flux.

2. Materials and Methods

2.1. Cells and Virus. Human lung epithelial cells (A549) and Madin–Darby canine kidney cells (MDCK) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 2 mM L-glutamine, 100 U ml penicillin/streptomycin, and 10% fetal bovine serum (FBS, Life Technologies) and incubated at 37°C with 5% CO_2.

Influenza virus A/WSN/33 (WSN, H1N1) obtained from Huazhong Agricultural University was propagated in MDCK cells with 1 μg/ml TPCK-trypsin. Titrated Virus was detected with TCID50. Viruses were stored at -80°C until they were used in subsequent experiments.

2.2. Construction of Noxa-Knockout Cells. Dual gRNAs were designed based on the human Noxa sequences from the ENSEMBL database by using an online CRISPR Design Tool (http://crispr.mit.edu/), named as gRNA1 (GGTTGGCAT CTCCGCGCGTC) and gRNA2 (GGGTACGGCGGGTA CGGCGA) targeting exon1 and exon2, respectively. The oligo-DNAs were synthesized and annealed to a U6 promoter-driven gRNA vector (BbsI-digested pKLV-U6gRNA_CCDB_PB_BbsI_PGKpuro2ABFP); two gRNA-expressing plasmids, Noxa-gRNA1 and Noxa-gRNA2, were successfully constructed according to the result from Sanger sequence. Then, A549 cells stably expressing Cas9 protein were cotransfected with Noxa-KO-gRNA1 and Noxa-KO-gRNA2 with Lipofectamine® 3000 reagent (Invitrogen) according to the manufacturer's instructions. At 24 h after transfection, cells were trypsinized and replated into 10 cm culture plats in DMEM with 10% FBS containing 1.5 μg/ml

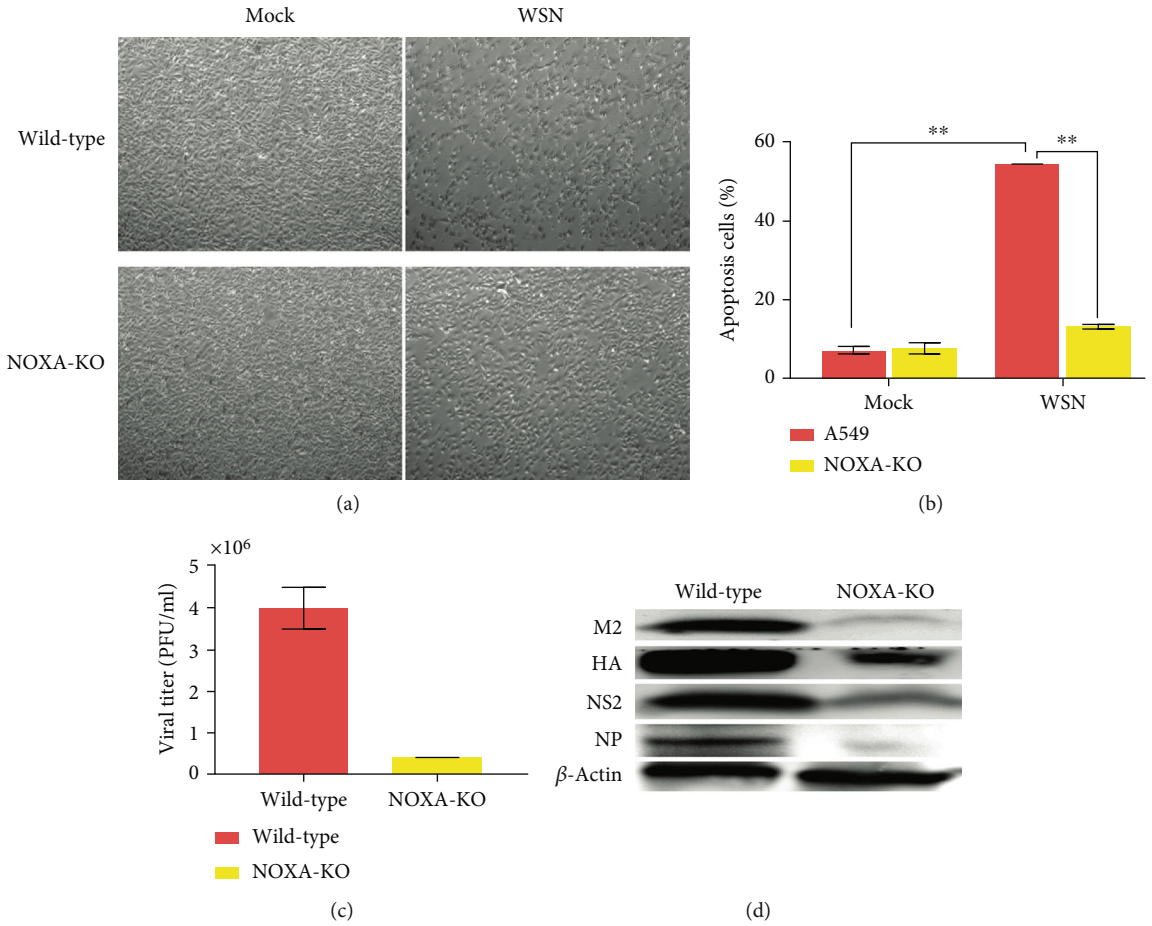

FIGURE 2: Noxa knockout inhibits influenza virus replication. (a) Microscopy observations of virus-induced cytopathic effect reduction in Noxa-knockout cells 24 h postinfection. Phase contrast microscopy was used to observe and photograph cells. Scale bar, 100 μm. (b) Flow cytometry showed the apoptotic cell death in Noxa-knockout cells 24 h postinfection. (c) Viral titer in the supernatant of the indicated cell lines was detected with a TCID50 assay 24 h postinfection. (d) Influenza viral protein expression was analyzed via western blotting in Noxa-knockout cells 24 h postinfection.

of puromycin (Invitrogen). After several days' selection, the positive clones were isolated into 96-well plates and sequenced to identify the mutation clone.

2.3. Apoptotic Cell Assay Using Flow Cytometry. Wild-type and Noxa-KO cells were infected by the influenza virus for 24 h. The apoptotic cell assay was analyzed via flow cytometry using an Annexin V-FITC/propidium iodide (PI) apoptosis detection kit according to the manufacturer's instructions.

2.4. Real-Time Quantitative PCR (qPCR) Analysis. Total cellular RNA was extracted using the Arcturus PicoPure RNA Isolation Kit (Invitrogen) following the manufacturer's instructions, and the complementary DNA was synthesized using the SuperScript III reverse transcriptase kit (Invitrogen) with a gRNA Eraser according to the manufacturer's protocol. Real-time PCR was carried out in the SYBR Green PCR Master Mix (Toyobo Biologics) on a LightCycler 480 (Roche). Cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 15 s at 60°C, and 72°C for 40 s. GAPDH was used as a housekeeping gene. The relative expression level of gene expression normalized to GAPDH was determined using the comparative Ct method ($2^{-\Delta Ct}$). Primers used for the qPCR assays were as follows: Noxa (forward: GCAAGAACGCTCAACCGA and reverse: CATCCCAATCGCAAATCCGG) and GAPDH (forward: ACAACTTTGGTATCGTGGAAGG and reverse: GCCATCACGCCACAGTTTC).

2.5. Western Blotting Analysis. Cells were lysed in protein lysis buffer (25 mM Tris·HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) supplemented with a protease inhibitor cocktail (Invitrogen) on ice for 20 minutes and centrifuged 12,000 × g for 30 min. Protein concentration in the supernatant was measured using the Bradford assay (Pierce). 20 μg of proteins was fractioned on a 4-20% TGX Stain-Free Gel (Bio-Rad) and subsequently transferred to a PVDF membrane. After blocking with 5% milk powder in PBS+0.1% Tween20, the membranes were incubated with primary antibodies for overnight at 4°C. The HRP-conjugated secondary antibodies were added and incubated for 1 h after washing the membranes three times. The western blots were imaged with the

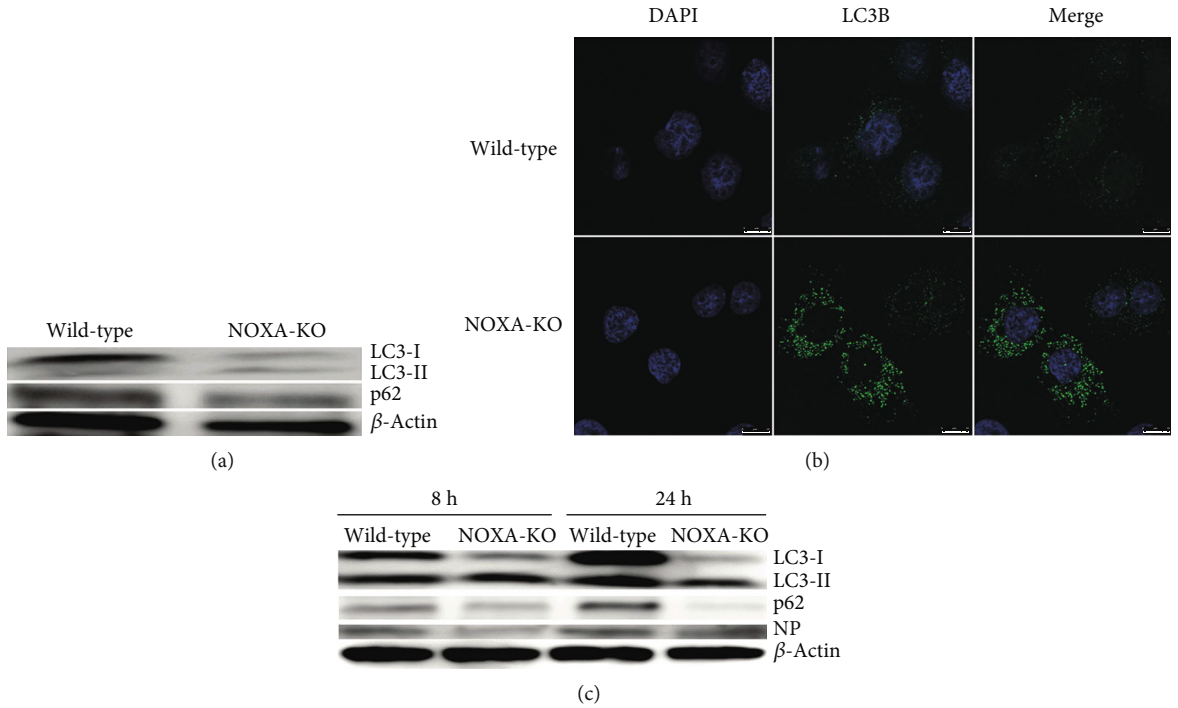

Figure 3: Loss of Noxa increases LC3 accumulation and reduces p62 expression. (a) Immunoblot analysis of LC3 and p62 expression in wild-type and Noxa-deficient A549 cells. β-Actin was used as the loading control. (b) Wild-type and Noxa-deficient A549 cells were transfected with a plasmid encoding GFP-LC3 for 24 h and then fixed and stained with DAPI (blue). Scale bars, 10 μm. (c) Immunoblot analysis of LC3 and p62 expression in wild-type and Noxa-deficient A549 cells 8 h and 24 h postinfection. β-Actin was used as the loading control.

Amersham Hyperfilm ECL system (GE Healthcare). Primary antibodies used for this experiment included rabbit anti-Noxa antibody (ab13654, Abcam), rabbit anti-LC3B (ab229327, Abcam,), mouse anti-p62 (ab56416, Abcam), rabbit anti-M2 antibody (GTX125951, GeneTex), mouse anti-actin antibody (#3700, CST), rabbit anti-HA antibody (GTX127357, GeneTex), rabbit anti-NS2 antibody (GTX125953, GeneTex), and mouse anti-NP antibody (ab128193, Abcam). Goat anti-rabbit and goat anti-mouse secondary antibodies linked with HRP were purchased from Invitrogen.

2.6. Immunofluorescence Analysis. Wild-type and Noxa-KO cells grown on glass coverslips were infected with A/WSN/33 virus at a MOI of 5 for 1 h and then were incubated for the indicated times. Cells were fixed with 4% paraformaldehyde at room temperature for 20 min and permeabilized with 0.2% Triton X-100. After blocking with 2% bovine albumin, cells were incubated with primary antibodies overnight at 4°C. After washing, fluorescently labelled secondary antibodies (Abcam) were added and incubated for 1 to 2 h at room temperature. Nuclei were stained with DAPI, and slides were mounted using ProLong Antifade (Invitrogen). Image analysis was performed with a confocal laser-scanning microscope (Leica SP8) using the LAS X software.

Primary antibodies used for this experiment included mouse anti-influenza A virus nucleoprotein (NP) (ab128193, Abcam), rabbit anti-LC3B (ab229327, Abcam), and mouse anti-Lamp2 (ab25630, Abcam). Secondary antibodies used for this experiment included goat anti-mouse IgG (DyLight488) (SA241239, Invitrogen) and goat anti-rabbit IgG (DyLight550) (SA5-100033, Invitrogen).

2.7. Statistical Analyses. All experiments were performed with at least three independent repeats giving consistent results. A one-way analysis of variance (ANOVA) with Tukey test was used to compare differences between two groups. P value equal to or lower than 0.05 was considered statistically significant.

3. Results

3.1. CRISPR/Cas9-Mediated Noxa Knockout in A549 Cells. To produce an indel mutant of the Noxa gene, the CRISPR/Cas9 system was transfected into A549 cells. After puromycin selection for 10 days, the surviving colonies were picked and detected. Sanger sequence analysis revealed that one Noxa-knockout clone was a homozygous mutation with a 392 bp nucleotide inset, resulting in a frameshift mutation (red sequence, Figure 1(a)), while the other clones were heterozygous (data not shown). Furthermore, we found that the mRNA and protein expression of Noxa in the homozygous mutant cell lines (Noxa-KO) was not detected compared with that in the wild-type by qPCR and western blotting (Figures 1(b) and 1(c)).

3.2. Knockout of Noxa Inhibited Influenza Virus-Induced Apoptosis. Influenza virus infections cause severe cytopathic

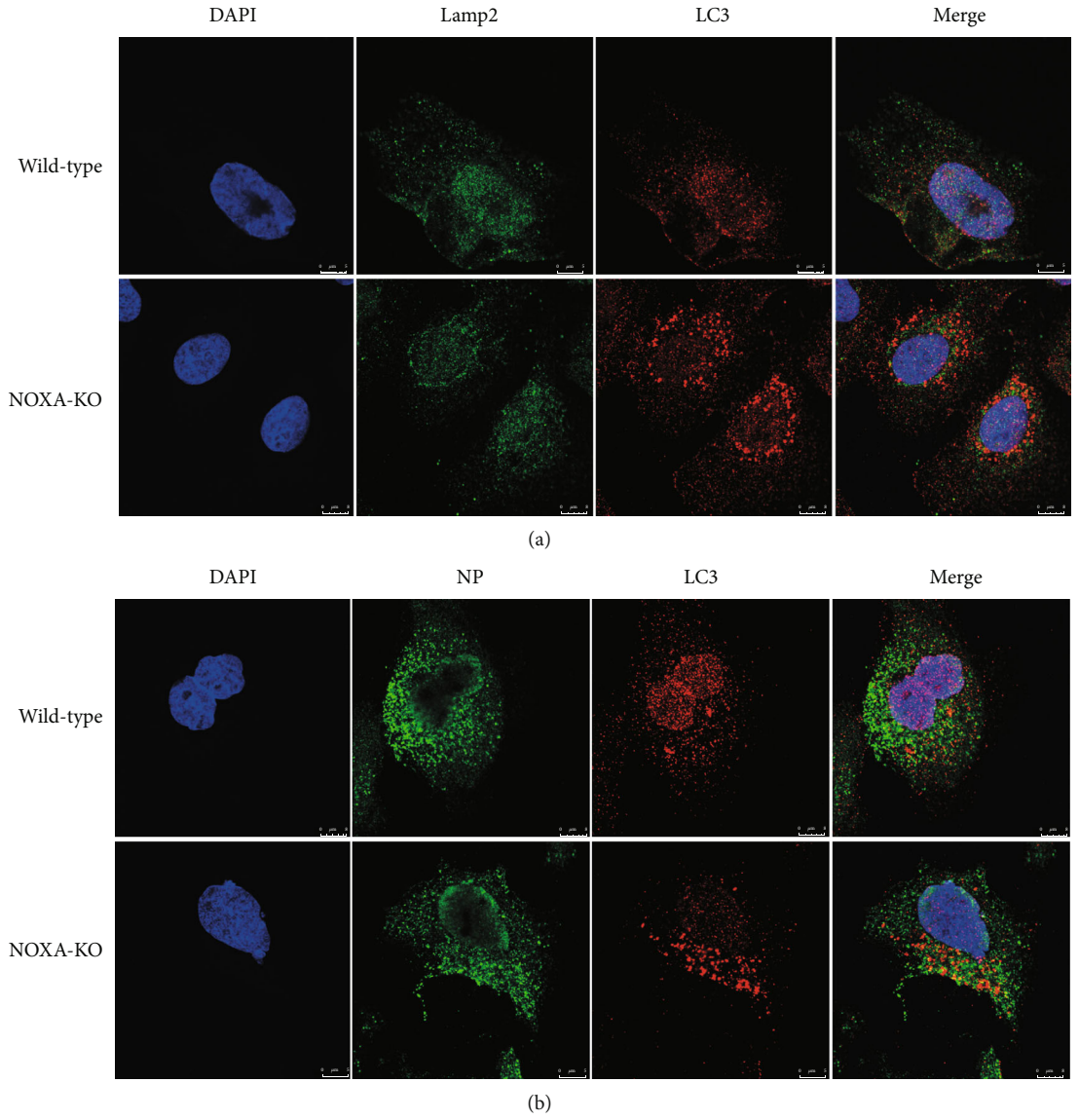

Figure 4: Noxa-deficient results in the autophagic degradation of influenza virus particles. (a) Wild-type and Noxa-deficient A549 cells were infected with the A/WSN/33 (H1N1) virus for 8 h. Representative images of immunofluorescence staining in cells with antibodies against Lamp2 (green) and LC3 (red). Blue: positive staining of DAPI. Scale bars, 10 μm. (b) Wild-type and Noxa-deficient A549 cells were infected with the A/WSN/33 (H1N1) virus for 8 h. Representative images of immunofluorescence staining in cells with antibodies against NP (green) and LC3 (red). Blue: positive staining of DAPI. Scale bars, 10 μm.

effects and apoptotic cell death [7]. Here, our results showed that knockout of Noxa resulted in a dramatic reduction in the cytopathic effect induced by the influenza virus compared to the wild-type (Figure 2(a)). Moreover, apoptotic cell death and viral titers in Noxa-knockout cells were significantly decreased (Figures 2(b) and 2(c)). Additionally, results from western blotting showed that the expression of influenza viral proteins NP, M2, HA, and NS2 was reduced in Noxa-knockout cell lines in comparison with levels in wild-type cells infected with the influenza virus (Figure 2(d)). These results revealed that the loss of Noxa inhibited influenza virus-induced apoptotic cell death and viral replication.

3.3. Involvement of Autophagy in Noxa Regulation during Influenza Virus Infection. Autophagy is an important cellular regulatory mechanism involved in influenza virus-induced apoptosis [23]. We investigated the link between reduced cytopathic effect and autophagy in Noxa-null cells. Noxa knockout increased the ratio of LC3-II to LC3-I (Figure 3(a)). Moreover, immunofluorescence staining showed punctate accumulation of LC3 in Noxa-null cells, whereas few punctate accumulations were observed in the wild-type (Figure 3(b)). In addition, the expression of p62 protein decreased, suggesting that autophagic flux was activated in Noxa-deficient cells (Figure 3(a)). Subsequently, we found that the influenza virus induced the accumulation of LC3-I and p62 in wild-type cells, whereas there was a significant decrease in p62 expression in Noxa-deficient cells (Figure 3(c)). Collectively, these findings support the proposition that Noxa deficiency promotes autophagic flux and consequently decreases influenza virus replication.

3.4. Autophagic Degradation of Influenza Virus Particles in Noxa-Deficient Cells. Influenza virus infections induce incomplete autophagy to block the formation of autolysosomes, which can degrade viral particles [24]. Our data showed the colocation of lysosomes with autophagosomes by detecting the localization of Lamp2 and LC3 (Figure 4(a)). In addition, we found that influenza virus NPs colocalized with autophagosomes in Noxa-deficient cells (Figure 4(b)). This suggests that Noxa deficiency may result in the autophagic degradation of influenza virus particles.

4. Discussions

The influenza virus is responsible for severe morbidity and mortality worldwide. Accumulating evidence suggests that host factors and cellular processes are involved in viral infection and have potential as antiviral targets based on their broad-spectrum activity. Apoptosis is an important cellular stress response that maintains cellular homeostasis and eliminates the invading pathogens. However, influenza viruses have evolved specific mechanisms to utilize apoptosis for efficient replication and propagation. Hence, elucidating the mechanisms by which host factors affect the apoptosis pathway to control influenza virus infection can help discover novel antiviral targets. In this study, we focused on the role of the BH3-only protein Noxa—an apoptosis inducer. CRISPR-mediated Noxa-mutated cells were successfully constructed, and Noxa knockout significantly reduced influenza virus-induced cytopathic effects and apoptosis by regulating the autophagy process.

Disrupting virus-induced cell death processes and apoptosis signaling with synthetic small molecules or natural products is highly efficacious for antiviral effects. Bax inhibitor-1, a conserved apoptotic suppressor of Bax, relieved influenza virus-induced cell death and inhibited viral replication [25]. Studies have shown that knockout of Bax or Bax/Bad double in MEFs inhibits virus-induced cytopathology and virus-mediated caspase activation [23]. Inhibition of p38MAPK and CD137 by siRNA can inhibit dengue virus-(DENV-) induced apoptosis and the production of TNF-α, which regulates apoptosis during DENV infection [26]. Inhibition of calpain by both PD150606 and overexpression of calpastatin, an endogenous calpain inhibitor, attenuated myocardial apoptosis induced by CVB3 by downregulating endoplasmic reticulum (ER) stress-related proteins, including GRP78 and PERK [27].

With the development of CRISPR technology, genome-wide genetic screens have been used to identify novel host factors that are essential for virus-induced cell death. Knockout of ER-associated proteins (EMC2, EMC3, SEL1L, DERL2, UBE2G2, UBE2J1, and HRD1) in different cell lines blocks West Nile virus- (WNV-) induced cell death [28]. Genome-wide CRISPR screens indicated that depletion of HuR can weaken RNA virus-induced cell death [29]. In addition, genetic disruption of Cmas or Slc35a1 identified with a genome-wide CRISPR screen protects murine microglial cells from reovirus-induced cell death [30]. CRISPR-mediated ADAM9 mutant cells show higher resistance to encephalomyocarditis virus (EMCV) infection and virus-induced cell death [31]. Taken together, the host factors that are essential for viral infection can be targeted for antiviral treatment.

Noxa has been shown to be involved in virus-induced cell death. Loss of Noxa showed a significant protective effect in cells infected with multiple pathogens [32, 33]. Point and deletion mutations of Noxa inhibit dsRNA-, IFN-, and EMCV-induced apoptosis [34]. Our study showed that the knockout of Noxa reduced the cytopathic effect induced by influenza virus infection. In addition, we found that knockout of Noxa induced mature autophagy to promote degradation of viral genomic segments. Autophagy is an important protective mechanism for host health. Autophagy-dependent degradation can restrict viral infection. However, influenza virus infection induces accumulation and blocks the formation of autolysosomes, benefiting viral replication. Based on our data, we speculate that resistance to influenza virus infection in Noxa-deficient cells may be due to autophagy flux formation.

In summary, we demonstrated that Noxa deficiency confers resistance to influenza virus infection by blocking influenza virus-induced cell death. Noxa knockout weakened influenza virus-induced accumulation of autophagosomes, leading to autophagosomal sequestration of viral genomic segments in the lysosome. Therefore, the mechanism by which Noxa knockout regulates autophagy during viral infection can be elucidated.

Data Availability

All data utilized in this manuscript are available online from their respective databases.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

WH Zhang and BX Wang collected and analyzed the data, X Dong analyzed the data, J Zhang reviewed and edited this manuscript, HB Chen and A Zhou designed this research and drafted this manuscript, and A Zhou supported the finance. All authors contributed to the analysis and evaluation of the results. All authors contributed to the article and approved the submitted version. Ao Zhou and Wenhua Zhang contributed equally to this work.

Acknowledgments

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