TNK2/ACK1 Strengthen Influenza A Virus Infection by Blocking Viral Matrix 2 Protein(M2) into Lysosome to Degradation

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Research

Keywords: TNK2, CRISPR, autophagy, lysosome, influenza virus

Posted Date: January 29th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-153964/v1

Abstract

Background

TNK2/ACK1, a non-receptor tyrosine kinase, plays critical roles in signalling transduces and trafficking. Our previous genome-wide CRISPR/CAS9 knockout screen revealed that mutant of TNK2 produced more restrict to influenza virus infection. In this study, we aim to illustrate the role of TNK2 for influenza A virus (IAV) replication in human cells.

Results

CRISPR/Cas9-mediated mutant of TNK2 resulted in a significant reduction in viral proteins expression and viral titres for multiple influenza strains, and furthermore, a decrease of nuclear import of IAV in the infected TNK2 mutant cells was observed in 3h post-infection. Interestingly, TNK2 mutation enhanced the colocalization of LC3 with autophagic receptor p62 and led to the attenuation of influenza virus-caused accumulation of autophagosomes in TNK2 mutant cells. Further, confocal microscopy visualization result showed that influenza viral matrix 2 (M2) was colocalized with Lamp1 in the infected TNK2 mutant cells in early infection, while almost no colocalization between M2 and Lamp1 was observed in IAV-infected wild-type cells. Moreover, TNK2 depletion also affected the trafficking of early endosome and the movement of influenza viral NP and M2.

Conclusions

Our results identified TNK2 as a critical host factor for influenza viral M2 protein trafficking, suggesting that TNK2 will be an attractive target for the development of antivirals therapeutics.

Background

Influenza caused by IAV is a severe respiratory disease capable of causing epidemics and public health security as well as economic loss. IAV is an enveloped RNA virus that contains negative-stranded RNA genome. IAV entry into host cells via receptor-mediated endocytosis. Endocytosed viruses are then trafficked into endosomes, where the low pH environment triggers the fusion of the viral and endosomal membranes, leading to viral genomes release[1,2,3,4]. In addition, during the viral life cycle, various of host factors are required by the virus to complete these processes[5]. Because IAV infection causes substantial morbidity and mortality, threatening public health as well as significant economic losses [6,7], it is very important to find efficient strategies to control virus infection and novel viral strain production.

The 2009 influenza pandemic gave us some warnings that novel antiviral strategy needs to be created to improve viral clearance and the prevalence of pneumonia, reduce secondary bacterial infections. Current vaccines and antivirals directed targeting influenza virus proteins have been developed and available to prevent annual epidemics[8,9]. However, IAV with genomic instability can rapidly develop resistance to these vaccines or antiviral drugs such as adamantanes, leading to inefficient protection against virus

infection[10]. Combing with the limited number of viral drug directly targeting viral proteins and viral similar entry routes for replication, the development of new influenza therapies targeting cellular factors required for viral replication will be of great attractive[9,11,12]. Multiple studies have been reported toward identifying host factors instead of virus proteins as drug targets by genome-wide screening approaches, including overexpression, arrayed or pooled RNAi screen, proteomic and CRISPR/Cas9 knockout or activated screen [13,14,15,16,17].

TNK2 (activated Cdc42-associated kinase 1 or ACK1) is a multi-domain structural non-receptor tyrosine kinase, consisting of the Sterile alpha motif (SAM) domain, tyrosine kinase catalytic domain, a SH3 domain, GTPase binding domain (also known as Cdc42-binding domain), Clathrin interacting region, EGFR binding domain and an ubiquitin-association domain, leading to its functional complexity[18,19,20,21,22]. With its multi-structures, TNK2 is activated by multiple cellular signals and exploit various biological function though switching to different modes of kinase activation, resulting in adapt rapidly to cellular requirements[23]. In addition, TNK2 acts as an intermediary kinase that bridges the receptor tyrosine kinases (RTKs) and effecter proteins to control host cellular signalling transduces[24]. Recently, Sylwia Jones reported that TNK2 interacts and colocalises with autophagic receptors p62/SQSTM1, leading to activated EGFR into autophagic degradative pathway, whereas silencing of TNK2 resulted in an increased location of EGFR in lysosome[25]. Previous studies showed that inhibition of tyrosine kinase activity or Receptor tyrosine kinase inhibitors leads to reduced virus uptake and progeny virus titers[26,27], indicating that TNK2 may involve in virus replication by regulating the trafficking of receptor tyrosine kinase. Although there are no direct evidence illustrating the role of TNK2 in virus infection, a forward genetic screen showed that the *Caenorhabditis elegans* ortholog of TNK2, sid-3 has been identify as host factors critical for Orsay virus infection[28]. Moreover, multiple genome-wide RNAi screens also revealed that TNK2 can act as a potential candidate involved in virus infection, including influenza A virus (IAV), hepatitis C virus (HCV), and vesicular stomatitis virus (VSV)[13,29,30,31,32,33]. Collectively, these data indicate that TNK2 may participate in IAV infection, although the function of TNK2 remains unanswered.

In this study, we analysed the role of TNK2 for IAV infection. We found that CRISPR/Cas9-meidiated mutant of TNK2 reduced the viral replication and destroyed IAV infection-induced accumulation of autophagosomes. Further studies demonstrated that the mechanism by which TNK2 mutation enhanced the fusion of autophagosome with lysosomes was by mediating influenza matrix protein 2 (M2) trafficking into the classical lysosomal pathway.

Results

Generation of TNK2 mutant single-colony by CRISPR/Cas9 technology

In order to produce the indel mutant of TNK2, dual gRNAs were designed to delete the exon 2 (181bp) of TNK2 (ENSG00000061938) (Fig. 1A). By co-transfecting cells with Cas9 and dual TNK2 gRNAs vectors, drug-selectable A549 cells were established. BFP-positive cells were screened by supplementing the culture medium with 1.5 ug/ml puromycin for 10 days. after puromycin selection, the single colonies were picked and detected with molecular biology technology. PCR and Sanger sequencing revealed that TNK2-A549#15 and TNK2-A549#12 monoclonal is a homozygous mutation with an almost 660bp nucleotide deletion, respectively (Fig. 1A). In addition, Western blotting and qPCR analysis of these two mutant cells (TNK2-A549#15 and TNK2-A549#12) showed a significate reduced in mRNA and protein levels of TNK2 compared with wild-type (WT) (Fig. 1B and 1C). qPCR results showed that TNK2 was almost undetectable in TNK2-A549#15 (TNK2-KO) cells. Taken together, TNK2 homozygous mutation clone was generated via CRISPR/Cas9 technology.

TNK2 deficiency inhibits different species of influenza virus replication

To verify whether TNK2 is required for influenza A virus infection, immunofluorescence staining for influenza virus proteins NP as a marker of viral ribonucleoprotein (vRNP) localization and M2 as an ion channel protein were used to assess virus replication. TNK2-A549#15, TNK2-A549#12 and wild-type A549 cells were infected with human influenza virus (MOI 10). After 3h post-infection, a strong NP signal was detected in the nuclei of infected cells, while in TNK2 knockout cells, barely any nuclear NP was detectable and most of NP seemed to attach with membrane (Fig. 2A). In addition, knockout of TNK2 also reduced NP and M2 expression levels (Fig. 2A, 2B), indicating that TNK2 knockout interferes dissociation of the viral RNA and blocks nuclear import of influenza virus vRNPs. Moreover, the virus titer was strongly reduced in TNK2-KO cells compared to those for wild-type cells. Additionally, swine influenza virus (HB, H1N1) and avian influenza virus (CK, H9N2-GFP) also displayed reduced growth in A549 cells upon TNK2 knockout (Fig. 2C, 2D, 2E), suggesting that the function of TNK2 in influenza virus replication may be conserved.

TNK2 deficiency induced the formation of autolysosome during influenza virus infection

Previous study has illustrated that influenza virus infection inhibits autophagosome maturation to enhance viral budding and virion stability, and TNK2 is implicated in receptor endocytic trafficking and lysosome degradative pathway. In order to investigate whether the accumulation of autophagosomes caused by influenza virus infection was altered under deletion of TNK2, western blotting analysis showed that the amount of endogenous lapidated Atg8/LC3- II form in influenza-infected wild-type cell was significantly accumulated than those in influenza-infected TNK2 knockout cells. Moreover, the expression of p62 protein, which is used to monitor autophagic flux, was strongly reduced in influenza-infected TNK2 mutant cells compared to influenza-infected wild-type cell (Fig. 3A), demonstrating that deletion of TNK2 is able to weaken the accumulation of autophagosomes induced by influenza infection. In addition, to

confirm this promotion of autophagosome fusion with lysosomes in TNK2 mutant cells infected with influenza virus, we investigated the colocalization of LC3 with p62 and Lamp1 visualized by confocal microscopy, respectively (Fig. 3B, 3C). The results showed that p62 was overlapped with autophagosomes upon influenza infection in both wild-type and TNK2-KO cells, but the greater number of overlapped dots were found in TNK2-KO cells. Additionally, LC3 was not found to co-localize with Lamp1 and the expression of Lamp1 was much lower in wild-type cells with influenza virus infection, while this autophagosome marker LC3 was partially overlapped with Lamp1 in TNK2-KO cells with influenza virus infection. These data suggested that TNK2 deficiency can promote the fuse of autophagosomes with lysosomes during influenza virus infection.

TNK2 regulates the trafficking of influenza virus M2 protein into lysosome degradative pathway

To illustrate more precisely how TNK2 regulates autolysosome formation during influenza virus infection, we firstly detected intracellular dynamic location of influenza virus M2 in TNK2 knockout cells. The results showed that silencing of TNK2 resulted in the different localization of influenza virus NP and M2 protein in early infection (Fig. 4). Then we investigated whether the trafficking of influenza virus M2 is impaired in the absence of TNK2. As shown in Fig. 5A, influenza virus M2 did colocalise with Lamp1 in influenza virus infected TNK2-KO cells, while barely colocation singling was detected in wild-type cells. Reversely, the colocation of M2 with p62 was accumulated in wild-type cells, although there were more punctate p62-positive structures that did not localize with M2 in TNK2-KO cells (Fig. 5C). In addition, given that TNK2 partially localises to EEA1-positive compartment, where early endosomes are required for autophagosomal maturation, we also analysed whether the fusion of early endosomes with autophagosomes is affected in cells depleted of TNK2. After 1h post-infection, when compared with the wild-type cells, knockout of TNK2 triggered the colocalization of EEA1-postive early endosome with Lamp1-postive lysosome (Fig. 5B). Interestingly). Altogether, these data show that TNK2 associates with influenza virus M2 trafficking and is involved in influenza virus-induced autophagosome accumulation.

Overexpression of TNK2 rescues influenza virus infection and blocks the degradation of EEA1-postitive early endosome in TNK2-knockout cells

To assess whether TNK2 expression can rescue influenza virus infection in TNK2-knockout cells, rescues assay was adopted to evaluate the biological effects of TNK2 overexpression on influenza virus infection in TNK2-knockout cells. FLAG-tagged TNK2 vector was transfected into TNK2-knockoout cells, followed by infection with influenza A/WSN/33 virus. Weston blotting assay showed that TNK2 overexpression increased the expression of viral protein NP, HA and M2 in TNK2-knockout cells (Fig. 6A), and Immunofluorescence assay also showed that TNK2 overexpression enhanced influenza virus propagation in TNK2-knockout cells (Fig. 6B), determining that TNK2 restored proviral activity in the TNK2 knockout cells. Furthermore, the confocal microscopy showed that early endosome maker EEA1 no long colocalized with lysosome maker Lamp1 in TNK2 knockout cells (Fig. 6C), suggesting that TNK2 is critical for localization of early endosomes and escape of influenza virus from early endosomes.

TNK2 inhibitors decrease influenza virus replication

To investigated the antiviral activity of TNK2 inhibitor, two inhibitors (XMD8-87 and AIM-100) were used to assess the replication of influenza virus. Weston blotting data showed that total TNK2 and phosphorylated TNK expressions were decreased after both XMD8-87 and AIM-100 treatment (Fig. 7A). Subsequently, compared with control, western blotting results showed that both XMD8-87 and AIM-100 efficiently reduced influenza viral M2 expression levels in dose dependant manner (Fig. 7B), suggesting that lower levels of TNK2 can efficiently protect host against influenza virus infection.

Discussion

In the present study, we demonstrate a novel role for TNK2 in influenza virus infection. Our data showed that TNK2 served as a pivotal regulator of influenza virus and was required for the trafficking of virus protein and the accumulation of influenza virus-induced autophagosomes. CRISPR/Cas9 mediated TNK2 deficiency appears to impair endosomal maturation, as supported by directly delivering early endosomes to Lamp1-positive lysosomes. Furthermore, we found that knockout of TNK2 triggered autophagic flux that was impaired in influenza virus infection by degrading influenza viral protein M2 by lysosome pathway. Moreover, we found that TNK2 inhibitors were also able to inhibit virus replication. This suggested that TNK2 played important roles in influenza virus infection.

Early endosomes have a low pH that is very critical for the fusion of the viral and endosomal membranes and the opening of influenza viral M2 involved in viral genomic release [36, 37]. Our data showed that influenza viral M2 exhibited a different localization pattern in TNK2 knockout cells comparing with wild type cells in influenza virus infection. Notably, a large number of early endosome and viral M2 were found in Lamp1-positive lysosome in TNK2 knockout cells. Thus, we conclude that TNK2 may control early endosome-lysosome trafficking in viral infection. Interestingly, influenza virus M2 protein could block autophagosome fusion with lysosomes by directly interacting with LCE and driving LC3 relocalization to plasma membrane[38, 39]. Additionally, previous studies suggest that TNK2 localized to early endosomes and autophagosomes upon stimulation with EGF[25]. Therefore, we propose that TNK2 may be critical for influenza virus M2 protein-induced autophagosome accumulation.

In addition, TNK2 acts as a major integrator in ligand-induced degradation of EGFR and suppression of the ACK1 expression by siRNA resulted in inhibition of EGF-induced degradation of EGFR[40]. Accordingly, EGFR knockdown also impaired efficient uptake of influenza virus into A549 cells and influenza virus. In addition, when influenza virus infected host cells, the attachment of influenza virus induced EGFP endocytosis and EGFR kinase activity required for influenza virus internalization[41]. Because ACK1 contained a clathrin-binding motif, interacted with clathrin, and participated in clathrin-mediated endocytosis[42]. Thus, we hypothesize that ACK1 may control EGFR internalization and clathrin-mediated endocytosis to block the uptake of influenza virus.

Conclusions

In summary, our studies identify a novel role of TNK2 in regulating influenza virus infection. Our data indicate that TNK2 regulates influenza virus M2 transport to effect autophagy flux and highlight TNK2 may be as a potential antiviral target against influenza virus infection.

Methods

Cells and viruses

A549 cells were maintained in DMEM F-12 medium (Gibco). MDCK and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) with high glucose. All of the media was supplemented with 2 mM L-glutamine, 100 U ml penicillin/streptomycin and 10% foetal bovine serum (FBS, Life Technologies). All cells were cultured in a humidified incubator with 5% CO_2 at 37°C.

Human influenza A/WSN/33 (WSN) virus, swine influenza virus (HB, H1N1) and avian influenza virus (CK, H9N2-GFP) were used in this study and propagated in MDCK cells in DMEM supplemented with 1 μg/ml TPCK-trypsin per ml in the absence of FBS. The stocks of influenza A/WSN/33 (WSN) virus were titrated by standard plaque assay on MDCK cells using a 2.5% Avicel overlay medium[34]. For swine influenza virus (HB, H1N1) and avian influenza virus (CK, H9N2-GFP), The titres of the virus were determined as the tissue culture infective dose 50 (TCID50) per millilitre by using the Reed-Muench method[35].

Construction of TNK2-KO-gRNA Plasmids

Human TNK2 sequence was obtained from the ENSEMBL database and then designed two different gRNAs by using an online CRISPR Design Tool (http://crispr.mit.edu/), named as gRNA1 (TGGCAGGAATAGGGGACGT) and gRNA2 (CTCATCATTCTGACTACCG) to delete the entire exon2 (181bp) of TNK2 (ENSG00000061938). By synthesizing the oligo-DNAs of these gRNAs and annealing them to a U6 promoter-driven gRNA vector (BbsI-digested pKLV-U6gRNA_CCDB_PB_BbsI_PGKpuro2ABFP), two gRNA-expressing plasmids, TNK2-KO-gRNA1 and TNK2-KO-gRNA2 were formed. The construction of the established plasmids was then confirmed by sequence analysis.

Generation of TNK2-KO-A549 Cell Line

A549 cells were co-transfected with TNK2-KO-gRNA1 and TNK2-KO-gRNA2 using the Lipofectamine® 3000 reagent (Invitrogen) according to the manufacturer's instructions. At 24 h after transfection, cells were selected with 1.5 μg/mL of puromycin (Invitrogen) which was diluted in DMEM with 10% FBS. Seven days later, the positive clones were isolated, trypsinized and diluted in 96-well plates. The single cell clones were then further used to extract DNA by DNeasy Blood & Tissue Kit (Qiagen) and amplificated by PCR with primers (Forward: TCCGTCACATCTAAGGAGCC and Reverse: GAGCACGAATCAGCAAACCA) and the PCR products were performed DNA sequencing analysis.

Virus infection

Cells were washed with PBS and then infected with influenza at the indicated multiplicity of infection (MOI) in the infection buffer (PBS supplemented with 0.3% bovine serum albumin) for 60 min on ice. Cells were next washed twice with PBS and incubated in DMEM supplemented with 0.3% bovine serum albumin, with 2 mM L-glutamine, 100 U ml penicillin/streptomycin and 1 µg/ml TPCK-trypsin at 37°C. The samples were harvested at indicated postinfection for further analysis

Immunofluorescence microscopy

The colocalization between influenza viruses and cellular early endosomes was assessed using confocal microscopy. Cells were grown on glass coverslips. The next day, cells were infected with A/WSN/33 virus at a MOI of 10 on ice for 1h and then were incubated for the indicated times at 37℃. Cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100 and blocked with 2% bovine albumin. Cells were then incubated with primary antibodies overnight at 4℃. After the cells were incubated with primary antibodies, they were washed three times with PBS before fluorescently labelled secondary antibodies (Abcam) were added for 1 to 2h at room temperature. Nuclei were stained with DAPI, and slides were mounted using ProLong Antifade (Invitrogen). All the specimens were analysed by a confocal laser-scanning microscope (Leica SP8) and images were acquired using the LAS X software.

For the immunofluorescence analysis of the endosome-to-TGN retrograde trafficking, wild type and TNK2-Knockout cells were grown on coverslips and were firstly incubated on ice for 30min before moving to the 37℃ incubators at the indicated times. Cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100 and blocked with 2% bovine albumin. Cells were then incubated with primary antibodies overnight at 4℃. After the cells were incubated with primary antibodies, they were washed three times with PBS before fluorescently labelled secondary antibodies were added for 1 to 2h at room temperature. Nuclei were stained with DAPI, and slides were mounted using ProLong Antifade (Invitrogen). All the specimens were analysed by a confocal laser-scanning microscope (Leica SP8) and images were acquired using the LAS X software.

Primary antibodies used for this experiment included; Mouse anti-influenza A virus nucleoprotein [C43] (Abcam), rabbit anti-EEA1 antibody (Abcam), Mouse anti-Golgi 58k (Novus Biologicals), Rabbit anti-TGN36 (Novus Biologicals), mouse anti-TGN36 (Abcam), rabit anti-TGN(Abcam), rabbit anti-GM130 (GeneTex) and rabbit anti-M2 (GeneTex).

Secondary antibodies used for this experiment included: Goat anti-Mouse IgG (DyLight488) (Invitrogen), Goat anti-Rabbit IgG (DyLight550) (Invitrogen).

Western blots

$3x10^6$ cells were trypsinized and pelleted at 500g for 5 minutes. Cells were lysed with a RIPA buffer (25mM Tris•HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail (Invitrogen) at ice for 20 minutes. Protein concentration was measured using the Bradford assay (Pierce). 20µg protein per sample was loaded onto a 4–20% TGX Stain-Free Gel (Bio-Rad). Protein was transferred to a PVDF membrane at 200mA for 120 minutes. Blots were blocked

overnight with 5% milk powder in PBS + 0.1% Tween20 before incubation with the primary antibodies at a 1:500 dilution in PBS + 0.1% Tween 20 for overnight. Blots were washed three times with PBS + 0.1% Tween 20, and probed with the HRP conjugated secondary antibodies at 1:5,000 dilution for 1 hour, then washed again for 3 times. The western blots were developed using the Clarity Western ECL Substrate (Bio-rad) and imaged with the Amersham Hyperfilm ECL system (GE Healthcare). Primary antibodies used for this experiment included: Rabbit anti-COG8(GeneTex) antibody, Rabbit anti-GAPDH antibody (GeneTex), Rabbit anti-M2 antibody (GeneTex)., mouse anti-actin antibody (CST), Rabbit anti-HA antibody (GeneTex), rabbit anti-NS2 antibody (GeneTex), and mouse anti-NP antibody (Abcam). Goat anti-rabbit and goat anti-mouse secondary antibody linking with HRP were purchased from Invitrogen. All bands of western blots were detected within the linear range.

qRT-PCR

Total RNA extracts from each sample were obtained using the Arcturus Picopure RNA Isolation Kit (Invitrogen) following the manufacturer's instructions, and then complementary DNA was synthesized using the Superscript III reverse transcriptase kit (Invitrogen). Real-time RT-PCR was performed using the SYBR Green Real Time PCR Master Mix (Toyobo Biologics) in the LightCycler 480 (Roche Molecular Biochemicals). Individual transcripts in each sample were assayed three times. The PCR conditions were as follows: initial denaturation for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 15 s at 60°C and 40 s at 72°C. Relative expression levels of gene expression was determined by evaluating the threshold cycle (Ct) of target gene after normalization against the Ct value of GAPDH (housekeeping gene) dependent on the delta delta cycles to threshold (ΔΔCT) method. Primers used for the qRT-PCR assays were: TNK2 (Forward: GCAAGAGGCGAATTGGCTG and Reverse: CCTTCCCGTTCAGGTAGGTT) and GAPDH (Forward: ACAACTTTGGTATCGTGGAA GG and Reverse: GCCATCACGCCACAGTTTC).

Statistical analyses

Data were expressed as means ± standard errors of the means (SEM). Statistical analysis was performed by paired two-tailed Student's t test. P value equal or lower to 0.05 was considered significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Abbreviations

IAV: Influenza A virus; TNK2: activated Cdc42-associated kinase 1; EGFR: epidermal growth factor receptor; EEA1: early endosome antigen 1; CRISPR: Clustered regularly interspaced short palindromic repeats; CAS9: CRISPR-associated protein 9; LAMP1: lysosome-associated membrane protein1.

Declarations

Acknowledgments

This work was financially sponsored by Key Lab of Process Analysis and Control of Sichuan Universities (No.2018001), The Project-sponsored by Sichuan Province for ROCS (0903/00021728) and NSFC

(81902073), Newton international fellow and Southwest Medical University (0903/00030699, 0903/00030827).

Author contributions

All authors contributed to this manuscript and approved the final manuscript.

A. Zhou designed this project and did most of the experiments, analysed and interpreted data, and contributed to the writing of the manuscript; X. Dong analysed and interpreted data; A. Zhou made immunofluorescences; B. Tang and A. Zhou provided financial support; A. Zhou and B. Tang designed the experiments, supervised the research and wrote the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable

Consent of publication

All authors agree to publish this paper.

Competing Interest

The authors declare that they have no competing interests

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References

1. Skehel JJ, Wiley DC. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Rev. Biochem. 2000; 69:531–569.
2. Luo M1. Influenza virus entry. Adv Exp Med Biol. 2012; 726:201-21. doi: 10.1007/978-1-4614-0980-9_9.
3. Lakadamyali M, Rust MJ, Zhuang X. Endocytosis of influenza viruses. Microbes Infect. 2004 Aug;6(10):929-36.

4. Rust MJ1, Lakadamyali M, Zhang F, Zhuang X. Assembly of endocytic machinery around individual influenza viruses during viral entry. Nat Struct Mol Biol. 2004 Jun;11(6):567-73. Epub 2004 May 2.
5. Watanabe T, Watanabe S, Kawaoka Y. Cellular networks involved in the influenza virus life cycle. Cell Host Microbe. 2010 Jun 25;7(6):427-39. doi: 10.1016/j.chom.2010.05.008.
6. Taubenberger JK, Kash JC. Influenza virus evolution, host adaptation, and pandemic formation. Cell Host Microbe. 2010 Jun 25;7(6):440-51. doi: 10.1016/j.chom.2010.05.009.
7. Reperant LA, Kuiken T, Osterhaus AD.Adaptive pathways of zoonotic influenza viruses: from exposure to establishment in humans.Vaccine. 2012 Jun 22;30(30):4419-34. doi: 10.1016/j.vaccine.2012.04.049. Epub 2012 Apr 24.
8. Vanderlinden E, Naesens L. Emerging antiviral strategies to interfere with influenza virus entry. Med Res Rev. 2014 Mar;34(2):301-39. doi: 10.1002/med.21289. Epub 2013 Jun 25.
9. Edinger TO, Pohl MO, Stertz S. Entry of influenza A virus: host factors and antiviral targets. J Gen Virol. 2014 Feb;95(Pt 2):263-77. doi: 10.1099/vir.0.059477-0. Epub 2013 Nov 13.
10. Ison MG. Antivirals and resistance: influenza virus. Curr Opin Virol. 2011 Dec; 1(6):563-73.
11. Villalón-Letelier F, Brooks AG, Saunders PM, Londrigan SL, Reading PC. Host Cell Restriction Factors that Limit Influenza A Infection. Viruses. 2017 Dec 7;9(12). pii: E376. doi: 10.3390/v9120376.
12. Lee SM, Yen HL. Targeting the host or the virus: current and novel concepts for antiviral approaches against influenza virus infection. Antiviral Res. 2012 Dec;96(3):391-404. doi: 10.1016/j.antiviral.2012.09.013. Epub 2012 Sep 26.
13. Karlas A, Machuy N, Shin Y, Pleissner KP, Artarini A, Heuer D, Becker D, Khalil H, Ogilvie LA, Hess S, et al. 2010. Genome-wide RNAi screen identifies human host factors crucial for influenza virus replication. Nature463:818–822. 10.1038/nature08760
14. Shapira SD, Gat-Viks I, Shum BO, Dricot A, de Grace MM, Wu L, Gupta PB, Hao T, Silver SJ, Root DE, et al.A physical and regulatory map of host-influenza interactions reveals pathways in H1N1 infection. Cell. 2009 Dec 24;139(7):1255-67. doi: 10.1016/j.cell.2009.12.018.
15. Su WC, Chen YC, Tseng CH, Hsu PW, Tung KF, Jeng KS, Lai MM. 2013.Pooled RNAi screen identifies ubiquitin ligase Itch as crucial for influenza A virus release from the endosome during virus entry. Natl. Acad. Sci. U. S. A. 110:17516–17521. 10.1073/pnas.1312374110
16. Heaton BE, Kennedy EM, Dumm RE, Harding AT, Sacco MT, Sachs D, Heaton NS. A CRISPR Activation Screen Identifies a Pan-avian Influenza Virus Inhibitory Host Factor. Cell Rep. 2017 Aug 15;20(7):1503-1512. doi: 10.1016/j.celrep.2017.07.060.
17. Han J, Perez JT, Chen C, Li Y, Benitez A, Kandasamy M, Lee Y, Andrade J, tenOever B, Manicassamy B. Genome-wide CRISPR/Cas9 Screen Identifies Host Factors Essential for Influenza Virus Replication. Cell Rep. 2018 Apr 10;23(2):596-607. doi: 10.1016/j.celrep.2018.03.045.
18. Yokoyama N, Miller WT. Biochemical properties of the Cdc42-associated tyrosine kinase ACK1. Substrate specificity, authphosphorylation, and interaction with Hck. J Biol Chem. 2003 Nov 28; 278(48):47713-23.

19. Galisteo ML, Yang Y, Ureña J, Schlessinger J. Activation of the nonreceptor protein tyrosine kinase Ack by multiple extracellular stimuli. Proc Natl Acad Sci U S A. 2006 Jun 27; 103(26):9796-801.
20. Teo M, Tan L, Lim L, Manser E. The tyrosine kinase ACK1 associates with clathrin-coated vesicles through a binding motif shared by arrestin and other adaptors. J Biol Chem. 2001 May 25; 276(21):18392-8.
21. Shen F, Lin Q, Gu Y, Childress C, Yang W. Activated Cdc42-associated kinase 1 is a component of EGF receptor signaling complex and regulates EGF receptor degradation. Mol Biol Cell. 2007 Mar; 18(3):732-42.
22. Chan W, Tian R, Lee YF, Sit ST, Lim L, Manser E. Down-regulation of active ACK1 is mediated by association with the E3 ubiquitin ligase Nedd4-2. J Biol Chem. 2009 Mar 20; 284(12):8185-94.
23. Prieto-Echagüe V, Gucwa A, Brown DA, Miller WT. Regulation of Ack1 localization and activity by the amino-terminal SAM domain. BMC Biochem. 2010 Oct 27; 11():42.
24. Mahajan K, Mahajan NP1. ACK1/TNK2 tyrosine kinase: molecular signaling and evolving role in cancers. Oncogene. 2015 Aug 6;34(32):4162-7. doi: 10.1038/onc.2014.350. Epub 2014 Oct 27.
25. Jones S1, Cunningham DL, Rappoport JZ, Heath JK. The non-receptor tyrosine kinase Ack1 regulates the fate of activated EGFR by inducing trafficking to the p62/NBR1 pre-autophagosome. J Cell Sci. 2014 Mar 1;127(Pt 5):994-1006. doi: 10.1242/jcs.136895. Epub 2014 Jan 10.
26. Eierhoff T, Hrincius ER, Rescher U, Ludwig S, Ehrhardt C. The epidermal growth factor receptor (EGFR) promotes uptake of influenza A viruses (IAV) into host cells. PLoS Pathog. 2010 Sep 9;6(9):e1001099. doi: 10.1371/journal.ppat.1001099.
27. Kumar N, Liang Y, Parslow TG, Liang Y. Receptor tyrosine kinase inhibitors block multiple steps of influenza a virus replication. J Virol. 2011 Mar;85(6):2818-27. doi: 10.1128/JVI.01969-10. Epub 2011 Jan 5.
28. Jiang H, Chen K, Sandoval LE, Leung C, Wang D. An Evolutionarily Conserved Pathway Essential for Orsay Virus Infection of Caenorhabditis elegans. MBio. 2017 Sep 5;8(5). pii: e00940-17. doi: 10.1128/mBio.00940-17.
29. Johannsdottir HK, Mancini R, Kartenbeck J, Amato L, Helenius A. Host cell factors and functions involved in vesicular stomatitis virus entry.J Virol. 2009 Jan; 83(1):440-53.
30. Lupberger J, Zeisel MB, Xiao F, Thumann C, Fofana I, Zona L, Davis C, Mee CJ, Turek M, Gorke S, et al. EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy. Nat Med. 2011 May; 17(5):589-95.
31. Hao L, Sakurai A, Watanabe T, Sorensen E, Nidom CA, Newton MA, Ahlquist P, Kawaoka Y. Drosophila RNAi screen identifies host genes important for influenza virus replication. Nature. 2008 Aug 14; 454(7206):890-3.
32. Fujimoto Y, Ochi H, Maekawa T, Abe H, Hayes CN, Kumada H, Nakamura Y, Chayama K. A single nucleotide polymorphism in activated Cdc42 associated tyrosine kinase 1 influences the interferon therapy in hepatitis C patients. J Hepatol. 2011 Apr; 54(4):629-39.

33. König R, Stertz S, Zhou Y, Inoue A, Hoffmann HH, Bhattacharyya S, Alamares JG, Tscherne DM, Ortigoza MB, Liang Y, et al. Human host factors required for influenza virus replication. Nature. 2010 Feb 11; 463(7282):813-7.
34. Matrosovich M, Matrosovich T, Garten W, Klenk HD. New low-viscosity overlay medium for viral plaque assays. Virol J. 2006 Aug 31; 3: 63.
35. Ramakrishnan MA. Determination of 50% endpoint titer using a simple formula. World J Virol. 2016 May 12;5(2):85-6. doi: 10.5501/wjv.v5.i2.85.
36. Skehel JJ, Wiley DC. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. 2000. Annu Rev Biochem 69:531–569. http://dx.doi.org/10.1146/annurev.biochem.69.1.531.
37. Pinto LH, Lamb RA. The M2 proton channels of influenza A and B viruses. J Biol Chem. 2006 Apr 7;281(14):8997-9000. Epub 2005 Dec 30.
38. Gannagé M, Dormann D, Albrecht R, Dengjel J, Torossi T, Rämer PC, Lee M, Strowig T, Arrey F, Conenello G, et al. Matrix protein 2 of influenza A virus blocks autophagosome fusion with lysosomes. Cell Host Microbe. 2009 Oct 22;6(4):367-80. doi: 10.1016/j.chom.2009.09.005.
39. Beale R, Wise H, Stuart A, Ravenhill BJ, Digard P, Randow F. A LC3-interacting motif in the influenza A virus M2 protein is required to subvert autophagy and maintain virion stability. Cell Host Microbe. 2014 Feb 12;15(2):239-47. doi: 10.1016/j.chom.2014.01.006.
40. Shen F, Lin Q, Gu Y, Childress C, Yang W . Activated Cdc42-associated kinase 1 is a component of EGF receptor signaling complex and regulates EGF receptor degradation. Mol Biol Cell. 2007 Mar; 18(3):732-42.
41. Eierhoff T, Hrincius ER, Rescher U, Ludwig S, Ehrhardt C. The epidermal growth factor receptor (EGFR) promotes uptake of influenza A viruses (IAV) into host cells. PLoS Pathog. 2010 Sep 9;6(9):e1001099. doi: 10.1371/journal.ppat.1001099.
42. Teo M, Tan L, Lim L, Manser E. The tyrosine kinase ACK1 associates with clathrin-coated vesicles through a binding motif shared by arrestin and other adaptors.J Biol Chem. 2001 May 25; 276(21):18392-8.

Figures

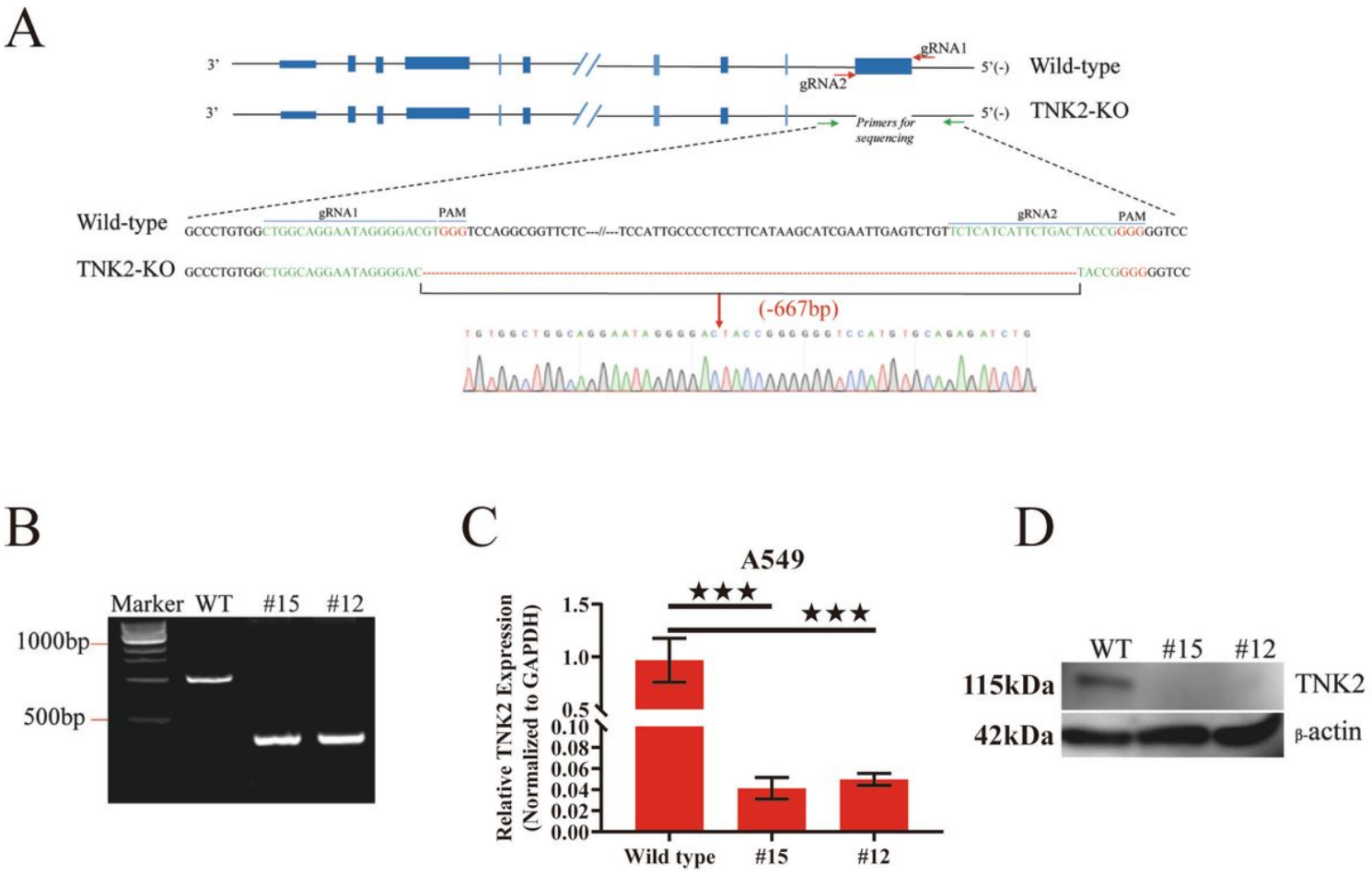

Figure 1

CRISPR/Cas9 induced the mutant of TNK2 and single colony of TNK2 deletion was produced. (A) Schematic of gRNAs targeting TNK2. Positions of gRNAs-guided cleavage sites (red array) are indicated; Sanger sequencing results showing that mutant A549 cells with TNK2 gRNAs have almost 660bp deletion by PCR primers (green array); (B) PCR analysis to targeted locus showing almost 200bp band corresponding the genomic deletion in cells transfected with both gRNAs; (C) TNK2 mRNA expression was detected by qPCR analysis; ★★★ $p<0.001$. (D) Western blotting results showing that there is no expression in mutant A549 cells with TNK2 gRNAs, comparing with wild-type cells.

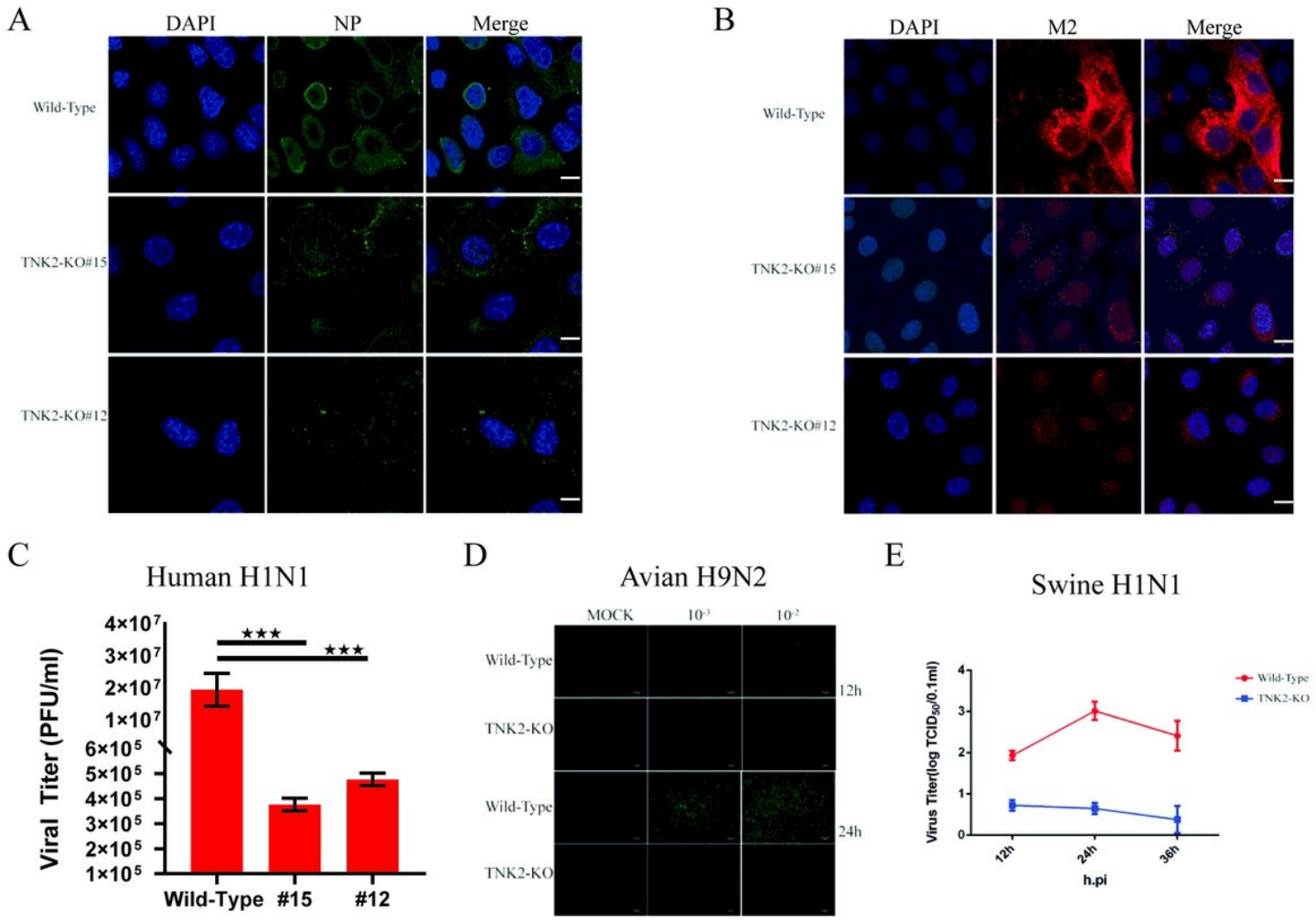

Figure 2

TNK2 mutant inhibited replication of influenza A virus. (A)and (B) Wild-type, TNK2-A549#15 and TNK2-A549#12 were infected with influenza A/WSN/33 (WSN) virus (MOI 5) for 3 h. Cells were stained for nuclei (blue), NP (green) and M2 (red) and then analysed by confocal microscopy. Scale bars: 10 μm. (C) Wild-type, TNK2-A549#15 and TNK2-A549#12 cells were infected with influenza A/WSN/33 (WSN) virus at an MOI of 0.01. The supernatants were collected at 24 h post-infection and used for determining viral titers by plaque-forming unit assay in MDCK cells. ▯▯▯ $p<0.001$. (D)Wild-type and TNK2-KO (TNK2-A549#15) cells were infected with avian influenza CK (H9N2-GFP) with difference concentration. After 12h and 24h post-infection, GFP signal was detected via immunofluorescence microscopy. (E)Wild-type and TNK2-KO (TNK2-A549#15) cells were infected with swine influenza HB (H1N1). The infected cells were collected at the indicated time points for TCID50 assay to determine viral titers.

Figure 3

Knockout of TNK2 sensitized influenza virus-induced autophagosomes to fuse with lysosomes. (A) Wild-type A549 and TNK2-A549#15(TNK2-KO) cells were infected with human influenza A/WSN/33 (WSN) virus (MOI 5) for 6h, harvested, and then subjected to Western blotting by using the indicated antibodies. Error bars, mean ± SD of 3 independent experiments (***p<0.001; ** p<0.01; * p<0.05). (B) and (C) Wild-type A549 and TNK2-A549#15(TNK2-KO) cells were infected with human influenza A/WSN/33 (WSN) virus (MOI 5) for 1h on ice. The inoculum was washed off, and the temperature was shifted to 37°C. Samples were fixed and permeabilized at 6h post-infection. Cells were stained with the indicated antibodies. Scale bars: 20 μm.

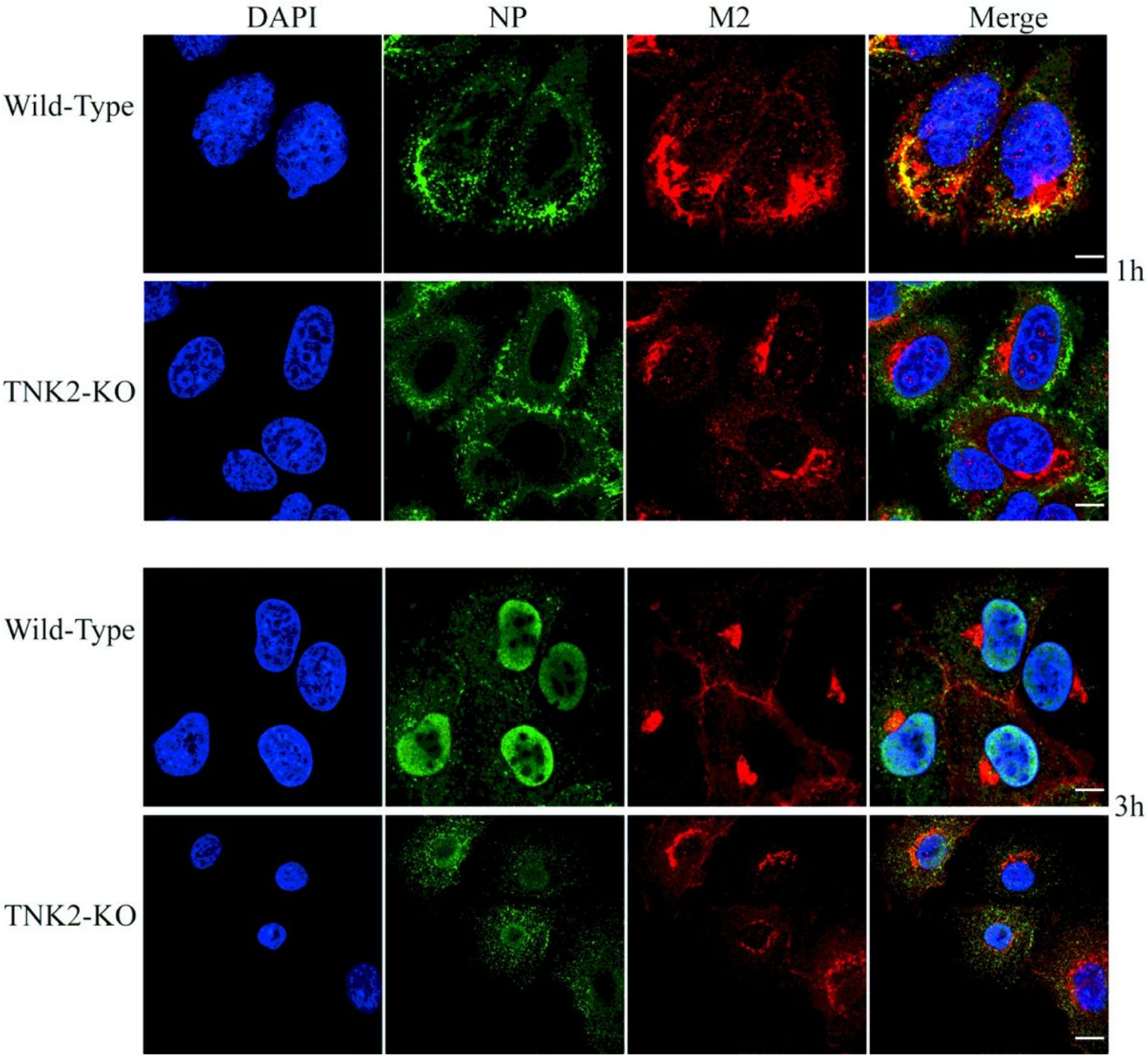

Figure 4

TNK2 modulated the trafficking of influenza M2. Wild-type A549 and TNK2-A549#15 (TNK2-KO) cells were infected with human influenza A/WSN/33 (WSN) virus (MOI 5). After 1h post-infection, cells were fixed, permeabilized and stained with rabbit anti-influenza M2 (red), mouse anti-NP (green) and DAPI (blue) followed confocal microscopy analysis. Scale bars: 20μm.

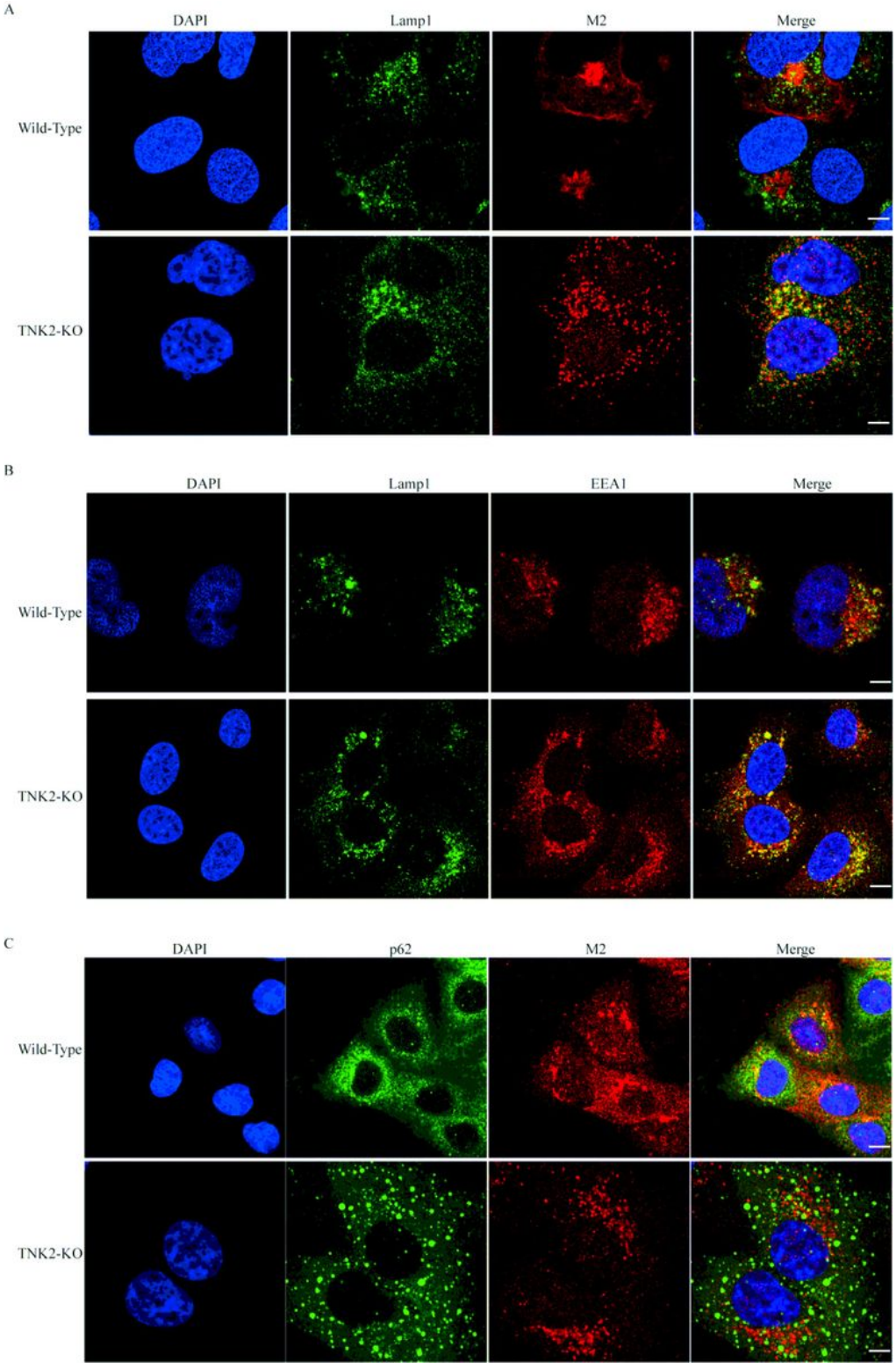

Figure 5

TNK2 mutant caused directly the fusion of EEA1-positive early endosome with lysosome (A) Wild-type A549 and TNK2-A549#15(TNK2-KO) cells were infected with human influenza A/WSN/33 (WSN) virus (MOI 5). After 1h, cells were fixed, permeabilized and stained with rabbit anti-influenza M2 (red), mouse anti-Lamp1 (green) and DAPI (blue) followed confocal microscopy analysis. Scale bars: 20μm. (B) Wild-type A549 and TNK2-A549#15(TNK2-KO) cells were infected with human influenza A/WSN/33 (WSN)

virus (MOI 5). After 1h, cells were fixed, permeabilized and stained with rabbit anti-EEA1 (red), mouse anti-Lamp1(green) and DAPI (blue) followed confocal microscopy analysis. Scale bars: 20μm. (C) Wild-type A549 and TNK2-A549#15(TNK2-KO) cells were infected with human influenza A/WSN/33 (WSN) virus (MOI 5). After 1h, cells were fixed, permeabilized and stained with rabbit anti-influenza M2 (red), mouse anti-p62 (green) and DAPI (blue) followed confocal microscopy analysis. Scale bars: 20μm.

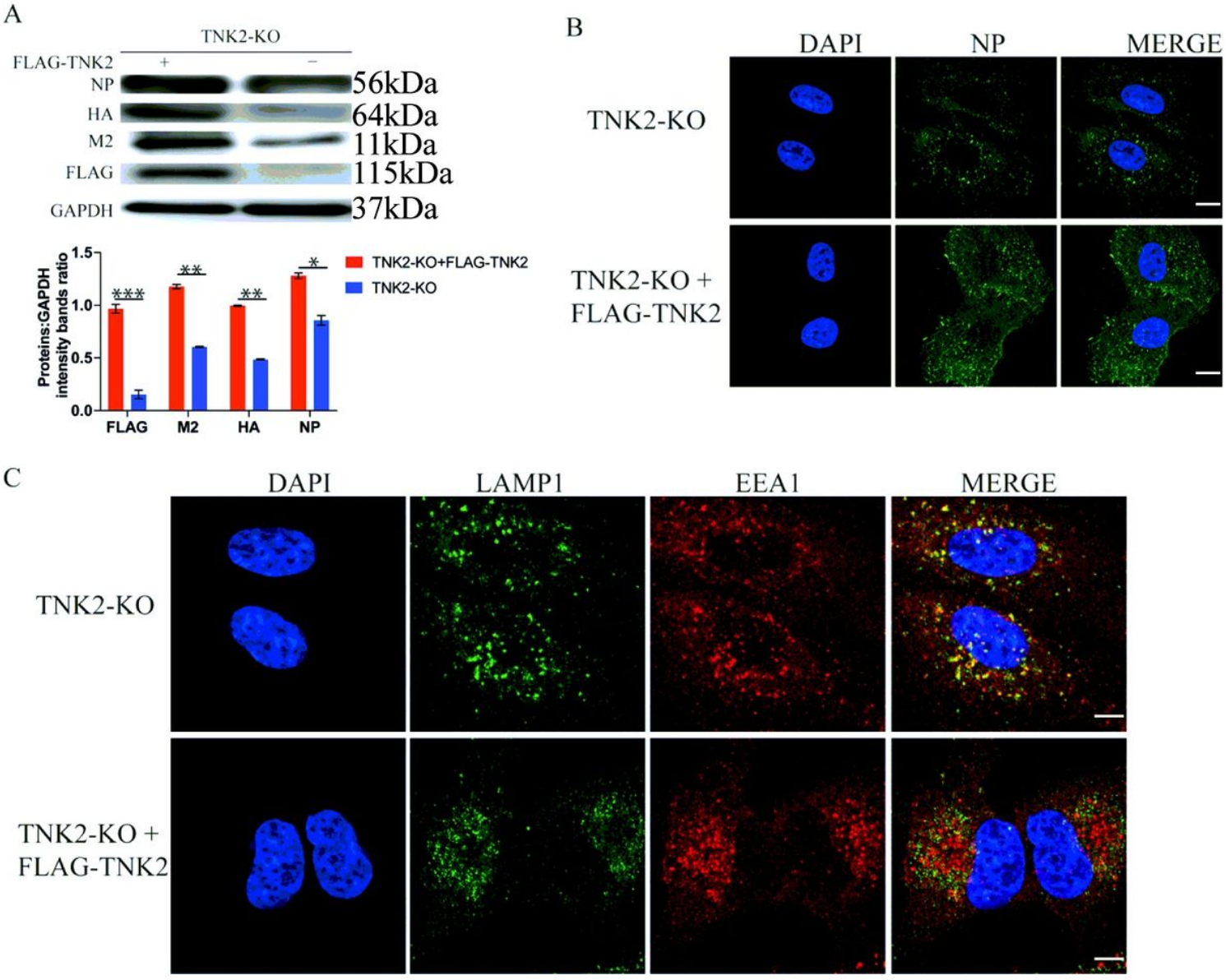

Figure 6

TNK2 overexpression rescues influenza virus infection in TNK2 knockout cells. (A) TNK2 knockout cells were transfected with FLAG-TNK2 for 24h and then infected at an MOI of 5 with influenza A/WSN/33 (WSN) virus for 3h. Samples were harvested and then blotted as indicated. Error bars, mean ± SD of 3 independent experiments (***p<0.001; ** p<0.01; * p<0.05). (B) TNK2 knockout cells were transfected with FLAG-TNK2 for 24h and then infected at an MOI of 5 with influenza A/WSN/33 (WSN) virus for 2h. Cells were fixed and Immunofluorescence assay was performed by using the indicated antibodies. Scale bars: 10μm. (C) TNK2 knockout cells were transfected with FLAG-TNK2 for 24h and then infected at an MOI of

5 with influenza A/WSN/33 (WSN) virus for 3h. Cells were fixed and confocal microscopy assay was performed by using the indicated antibodies. Scale bars: 20μm.

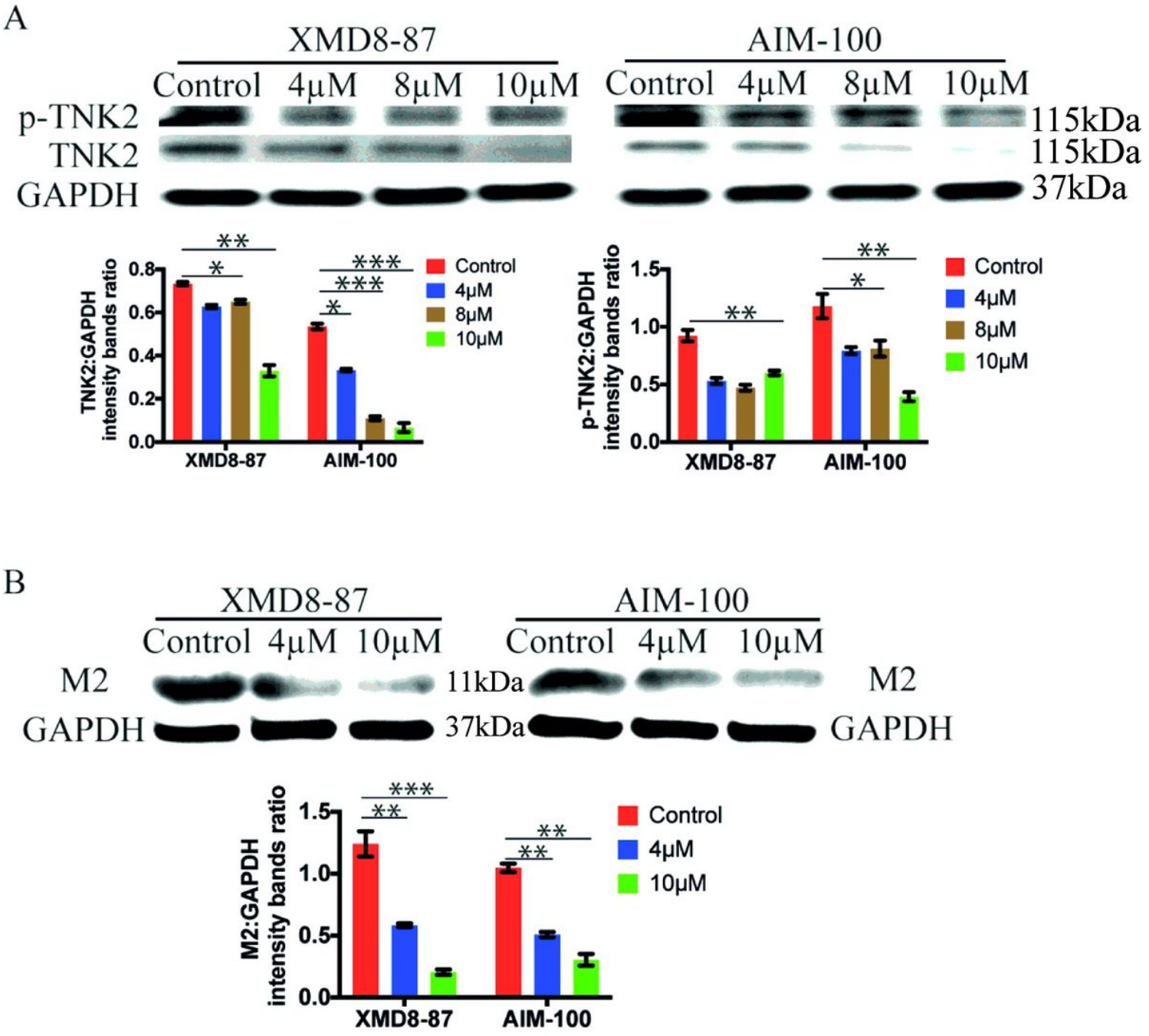

Figure 7

TNK2 inhibitor reduced influenza virus replication. (A) A549 cells were treated with XMD8-87 (0μM, 4μM, 8μM and 10μM) and AIM-100 (0μM, 4μM, 8μM and 10μM) 24h followed with influenza A/WSN/33 (WSN) an MOI of 5 for 6h. Cells were harvested and then analysed by western blotting using a rabbit anti-p-TNK2 or TNK2 antibody and an antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as loading control. Error bars, mean ± SD of 3 independent experiments (***p<0.001; ** p<0.01; * p<0.05). (B) A549 cells were treated with XMD8-87 (0μM, 4μM and 10μM) and AIM-100 (0μM, 4μM and 10μM) 24h followed with influenza A/WSN/33 (WSN) an MOI of 5 for 6h. Cells were harvested and then analysed by

western blotting using a rabbit anti-influenza M2 antibody and an antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as loading control. Error bars, mean ± SD of 3 independent experiments (*** $p<0.001$; ** $p<0.01$; * $p<0.05$).