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Role of TGF-β-activated kinase 1 (TAK1) activation in H5N1 influenza A virus-induced c-Jun terminal kinase activation and virus replication

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ABSTRACT

Activation of c-Jun terminal kinase (JNK) by the nonstructural protein 1 (NS1) of the H5N1 subtype of influenza A virus (IAV) plays an important role in inducing autophagy and virus replication. However, the mechanisms of NS1-induced JNK activation remain elusive. Here we first confirmed the ability of H5N1 (A/mallard/Huadong/S/2005) to activate JNK and to induce autophagy in 293T cells, a human embryonic kidney cell line. We further showed that TAK1, MAP kinase kinase 4 (MKK4), and JNK were activated in 293T cells transfected with the NS1 gene of the H5N1 virus. JNK activation by the NS1 protein or by H5N1 virus was blocked by 5Z-7-Oxozeaenol (5Z), a TAK1-specific inhibitor, and by TAK1 siRNA. Further study showed that 5Z and TAK1 siRNA suppressed H5N1 virus-induced autophagy and inhibited virus replication. Our study unveiled a previously unrecognized role of TAK1 in IAV replication, IAV-induced JNK activation, and autophagy.

1. Introduction

Influenza A virus (IAV) is an important pathogen that causes respiratory tract and lung infection (Paules and Subbarao, 2017). IAV replication and synthesis of several viral proteins activate a variety of signaling pathways such as the Toll-like receptor (TLR) pathway, the MAP kinase pathway, and the PI-3 kinase pathway (Gaur et al., 2011; Klemm et al., 2018). The non-structural protein 1 (NS1) of different IAV strains can differentially activate c-Jun terminal kinase (JNK) and the PI-3 kinase pathway (Nacken et al., 2014). Our recent study showed that the H5N1 and H1N1 subtypes reciprocally activate JNK and the PI-3 kinase pathway in chicken embryonic fibroblasts (CEF); whereas the H9N2 subtype activates both JNK and the PI-3 kinase pathway in CEF (Zhang et al., 2019). The molecular mechanisms of IAV-induced JNK activation remain obscure.

Autophagy is a highly conserved intracellular lysosome-dependent self-digesting process that removes misfolded proteins and damaged cytoplasmic organelle, and to eliminate invading intracellular microbes (Galluzzi et al., 2014; Russell et al., 2014). Autophagy also functions to maintain intracellular energy balance. JNK is a stress-activated protein kinase in the MAP kinase pathway that regulates autophagy indirectly. JNK phosphorylates Bcl-2 and disrupts its interaction with Beclin-1 (Wei et al., 2008a; Wei et al., 2008b). Once Beclin-1 is released, it joins the preinitiation complex to initiate autophagy (Wei et al., 2008a; Wei et al., 2008b). JNK is activated by MKK4 and MKK7, two immediate upstream MAP kinase kinases that are regulated by their top tier MAP3Ks, including MEKK1/2/4, MLK1-4, DLK, ASK1, TAK1, TAO1/2, ZAK, and LZK (Bogoyevitch et al., 2010). These MAP3Ks are activated by a variety of extracellular and intracellular stimuli. For example, apoptosis signal-regulating kinase 1 (ASK1), a MAP3K, is activated by multiple apoptosis signals such as TNF-α, reactive oxygen species, endoplasmic stress, and lipopolysaccharide (LPS) (Obsil and Obsilova, 2017). TNF-α and LPS activates ASK1 through TNF receptor-associated factor (TRAF) 2 and 6, respectively (Obsil and Obsilova, 2017). TAK1, another member of the MAP3K family capable of activating MKK4/7, is also activated by a wide spectrum of stimuli, including cytokines, TLRs, and virus infections (Aashaq et al., 2019). Our present study showed that TAK1 is activated by the H5N1 virus and is required for JNK

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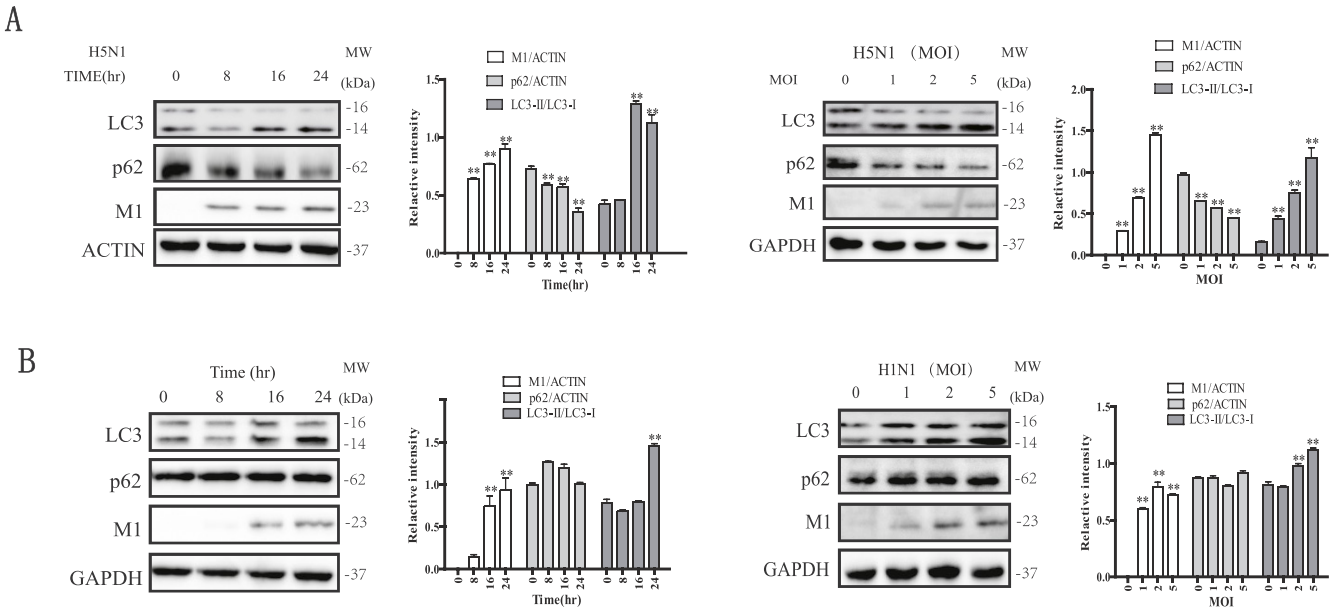

Fig. 1. H5N1 and H1N1 viruses differentially induce autophagy. 293T cells were infected with the indicated MOI of H5N1 (**A**) or H1N1 (**B**) virus for 16 hr or with 1 MOI of H5N1 (**A**) or H1N1 (**B**) virus for the indicated time. Cell lysates were analyzed for LC3-II lipidation and the levels of p62, M1, and GAPDH expression by Western blot. The band density of LC3-II lipidation, p62 and actin were analyzed by using NIH Image-J software and presented as bar graphs. Data are presented as the mean ± SD (n = 3) relative to control. $^{**}p < 0.01$, compared to untreated control.

activation by the NS1 protein of H5N1 virus. Inhibition of TAK1 activity or expression led to the suppression of H5N1 virus-induced JNK activation and autophagy; JNK1 and TAK1 inhibitors suppressed H5N1 virus replication in 293T cells. Our study suggests that TAK1 plays a critical role in H5N1 virus-induced JNK activation, autophagy, and virus replication.

2. Results

H5N1 virus induces functional autophagy in 293T cells. Our recent study showed that the H5N1 (A/mallard/Huadong/S/2005) and H1N1 (A/PR8/34, PR8) subtypes of IAV differentially activate JNK and induce autophagy in primary CEF and a DF1 chicken fibroblast cell line (Zhang et al., 2019). Here we tested if these two IAV subtypes also differentially induced autophagy and JNK activation in 293T cells, a human embryo kidney cell line. This cell line was chosen because it has a very high transfection efficiency and is ideal for studying the function of the NS1 gene in the experiments described below. As shown in Fig. 1A, H5N1 virus infection increased LC3 lipidation and decreased p62 levels in 293T cells in a time- and dose-dependent manner. An infection dose as low as 1 multiplicity of infection (MOI) was able to dramatically increase LC3 lipidation and decrease p62 levels in 293T cells 24 hr post-inoculation (hpi). Viral M1 protein synthesis was readily detectable as early 8 hpi, which appeared to be a few hours earlier than LC3 lipidation could be detected. In contrast, H1N1 virus increased LC3 lipidation in a time- and dose-dependent manner (Fig. 1B). However, H1N1 virus did not decrease but rather slightly increased p62 levels (Fig. 1B), suggesting that H1N1 virus may have blocked autophagic flux.

JNK activation by H5N1 virus. JNK is implicated in regulating autophagy by disrupting Bcl-2 and Beclin-1 interaction (Wei et al., 2008a; Wei et al., 2008b). Here we tested if JNK and TAK1 were differentially activated by H5N1 and H1N1 subtypes. As shown in Fig. 2A, H5N1 virus infection of 293T cells induced TAK1, MKK4, JNK, and Bcl-2 phosphorylation in a time-dependent manner. H5N1 virus infection with a dose of as low as 1 MOI dramatically increased TAK1, MKK4, JNK, and Bcl-2 phosphorylation. Unexpectedly, H1N1 virus increased TAK1^{T187} phosphorylation but had no or only minimal effect on MKK4, JNK, and Bcl-2 phosphorylation (Fig. 2B).

We next determined if JNK impacted H5N1-induced autophagy and virus replication. SP600125 (SP) (10 μM), a specific inhibitor of JNK, blocked H5N1 virus-induced LC3 lipidation and p62 degradation, and blocked H5N1 virus-induced JNK and Bcl-2 phosphorylation after incubation with H5N1 virus (1 MOI)-infected 293T cells (Fig. 3A). SP600125 also significantly reduced NS1 protein levels in H5N1 virus-infected cells, compared to the dimethyl sulphoxide (DMSO)-treated controls (Fig. 3A). Moreover, SP600125 significantly lowered the titer of H5N1 virus in the conditioned media of 293T cells (Fig. 3B). SP600125 did not inhibit 293T cell proliferation (Fig. 3C), indicating that inhibition of virus replication by SP600125 was not due to its effect on 293T cell growth.

TAK1 activation is required for H5N1 virus-induced JNK activation. A previous study showed that ASK1 is responsible for IAV-induced JNK activation in PR8-infected A549 cells (Lu et al., 2010). Here we assessed if MSC 2032964A (MSC) (10 μM), an ASK-specific inhibitor, could also block H5N1 virus-induced JNK activation. Unexpectedly, MSC had no effect on H5N1 virus-induced JNK and MKK4 phosphorylation (Fig. 4A). In contrast, 5Z, a TAK1-specific inhibitor, largely blocked IAV-induced TAK1, Bcl-2, MKK4, and JNK phosphorylation (Fig. 4B). 5Z also blocked TAK1, Bcl-2, MKK4, and JNK phosphorylation in 293T cells transfected with the expression vector encoding the NS1 gene of H5N1 virus (Fig. 4C). Further study showed that TAK1 siRNA suppressed TAK1 expression by approximately 70%, and blocked H5N1 virus-induced Bcl-2, JNK, and MKK4 phosphorylation (Fig. 4D).

TAK1 is required for H5N1 virus-induced autophagy and virus replication. We next determined if inhibition of TAK activity also suppressed H5N1 virus-induced autophagy and virus replication. As shown in Fig. 5A, 5Z (5 μM) significantly blocked IAV-induced LC3 lipidation and p62 degradation. Consistent with this observation, TAK1 siRNA partially blocked H5N1 virus-induced LC3 lipidation and restored p62 expression (Fig. 5B). The role of TAK1 in mediating H5N1 virus-induced autophagy was further confirmed by confocal microscopy to examine autolysosomes in Vero cells stably expressing GFP-RFP-LC3 (green fluorescence protein-red fluorescence protein-LC3). The green fluorescence of GFP is quenched in the acidic environment in

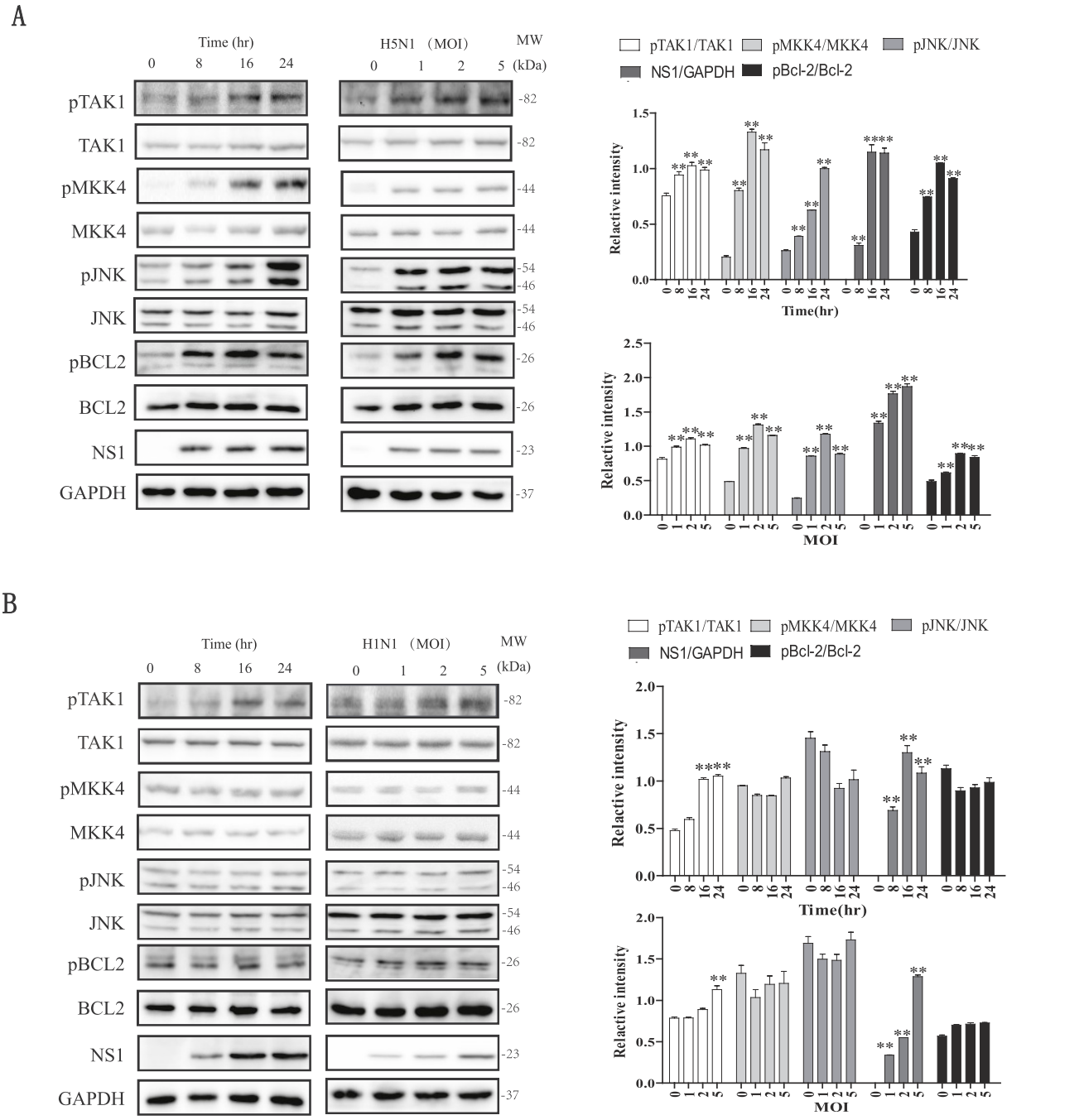

Fig. 2. H5N1 and H1N1 viruses differentially induce MKK4, JNK, and Bcl-2 phosphorylation. 293T cells were infected with the indicated MOI of H5N1 (**A**) or H1N1 (**B**) virus for 16 hr or with 1 MOI of H5N1 (**A**) or H1N1 (**B**) virus for the indicated time. Cell lysates were analyzed for TAK1, MKK4, JNK, and Bcl-2 phosphorylation and for NS1 and GAPDH levels by Western blot. The band density of phosphorylated TAK1, MKK4, JNK, and Bcl-2 and their total protein bands were quantified by using NIH Image-J software and presented as bar graphs. Data are the mean ± SD (n = 3) relative to control. $^{**}p < 0.01$, compared to untreated control.

autolysosomes, whereas the red fluorescence of RFP is illuminated in autolysosomes. Autophagosomes illuminate as the orange puncta through the combination of red and green fluorescence. As shown in Fig. 5C, there were very few puncta (8.33 ± 0.19/cell) in the uninfected cells. There were significantly more autophagosomes present perinuclearly in the cytoplasm in H5N1 virus-infected cells. The mean number of puncta per cell in H5N1-infected Vero cells was 55 ± 0.66. Among them, 21 ± 0.9 of the puncta illuminated with red RFP fluorescence (Fig. 5D), which represent autolysosomes. The number of autophagosomes in 5Z-treated Vero cells was comparable to that in DMSO-treated controls. However, 5Z significantly decreased the number of autophagosomes and autolysosomes in H5N1-infected Vero cells. These observations further suggest that TAK1 plays a critical role in H5N1 virus-induced autophagy.

Effect of 5Z on H5N1 virus replication. Finally, we determined if inhibition of autophagy by TAK1 inhibitor also led to inhibition of H5N1 virus replication. As shown in Fig. 6A, 5Z inhibited H5N1 virus-induced JNK phosphorylation (Fig. 6A) and the synthesis of two viral proteins, NS1 and M1, in a dose-dependent manner. 5Z lowered the $TCID_{50}$ values in the conditioned medium of H5N1 virus-infected 293T cells in a dose-dependent manner (Fig. 6B) but only marginally inhibited 293T cell proliferation in the absence or presence of H5N1 virus

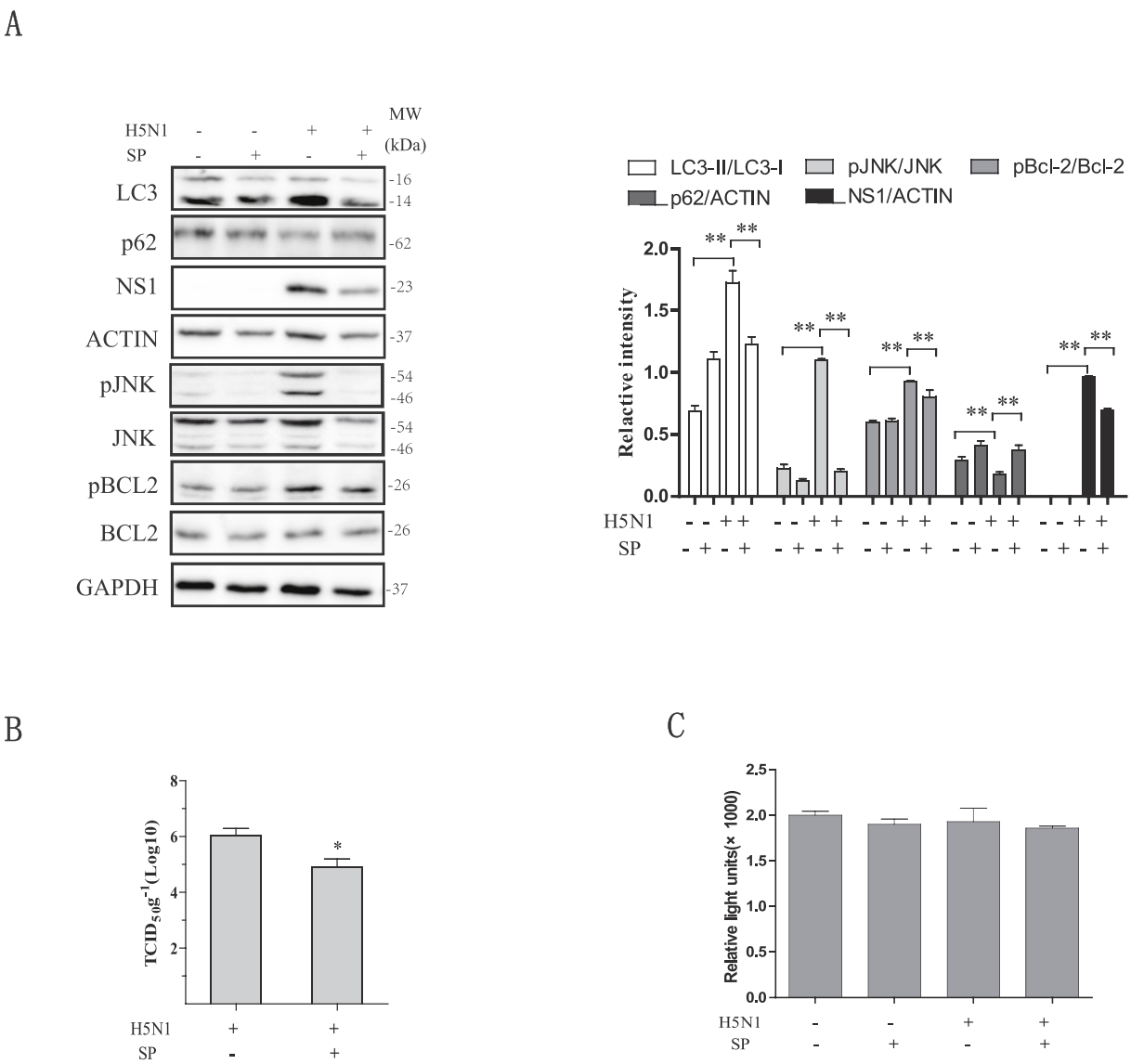

Fig. 3. Effect of JNK inhibition on IAV-induced autophagy and virus replication. (**A**) Effect of SP600125 on IAV-induced autophagy. 293T cells were inoculated with the H5N1 virus (1 MOI) and then incubated in the presence of DMSO (0.5%) or SP600125 (10 μM) for 24 hr. The cell lysates were analyzed for LC3-II lipidation, p62, NS1, and GAPDH levels and for JNK and Bcl-2 phosphorylation. The band density of LC3-II lipidation, p62 and β-actin levels and for phosphorylated JNK and Bcl-2 as well as their total proteins were semi-quantified using NIH Image-J software and presented as bar graphs. Data are presented as the mean ± SD (n = 3) relative to control. (**B**) Effect of SP600125 on IAV replication. 293T cells were inoculated with the H5N1 virus (1 MOI) and then incubated in the presence of DMSO (0.5%) or SP600125 (10 μM) for 24 hr. Viral titers in the conditioned media were analyzed for the $TCID_{50}$ values. *$p < 0.05$, compared to untreated control. (**C**) Effect of SP600125 on 293T cell proliferation. 293T cells seeded in 96-well plates were left uninfected or infected with H5N1 virus (1 MOI) and incubated in the presence of DMSO (0.5%) or SP600125 (10 μM) for 24 hr. Cell proliferation was analyzed as described in Materials and Methods. Data are the mean ± SD of three independent experiments. There was no significant difference in cell proliferation rates between untreated controls and SP600125-treated cells in the absence or presence of virus infection.

replication after incubation for 24 hr (Fig. 6C).

3. Discussion

Our present study focuses on the mechanisms of H5N1 virus-induced JNK activation and its role in IAV-induced autophagy and virus replication. We provide evidence that JNK was activated by H5N1 virus in 293T cells through its NS1 protein, and that activation of JNK was required for IAV-induced autophagy and promoted virus replication. Further studies showed that TAK1 inhibition by 5Z or by siRNA significantly suppressed H5N1 virus-induced JNK activation and autophagosome formation, and that inhibition of TAK1 activity by 5Z led to decreased virus replication. Our study is the first one to implicate TAK1 being involved in IAV-induced JNK activation and autophagy, and that TAK1 is required for efficient virus replication.

Accumulating evidence suggests that a variety of viruses can activate TAK1. For example, the Vpr protein and glycoprotein gp41 of HIV-1 binds TAK1 and induces TAK1 phosphorylation and activation (Postler and Desrosiers, 2012; Liu et al., 2014). TAK1 activation by HIV-1 is required for NF-κB activation and efficient virus replication (Postler and Desrosiers, 2012). The A52 protein of poxvirus interacts with TRAF6 and induces TRAF6 oligomerization (Stack et al., 2013). Interestingly, TAK1 activation leads to p38 MAP kinase but not NF-κB activation. Whether A52 leads to increased TAK1 phosphorylation is unclear (Stack et al., 2013). Consistently, TAK1 is required for Epstein–Barr viral protein Latent Membrane Protein 1 (LMP1)-induced JNK activation but is dispensable for NF-κB activation (Uemura et al., 2006). Our present study showed that H5N1 virus increased $TAK1^{S187}$

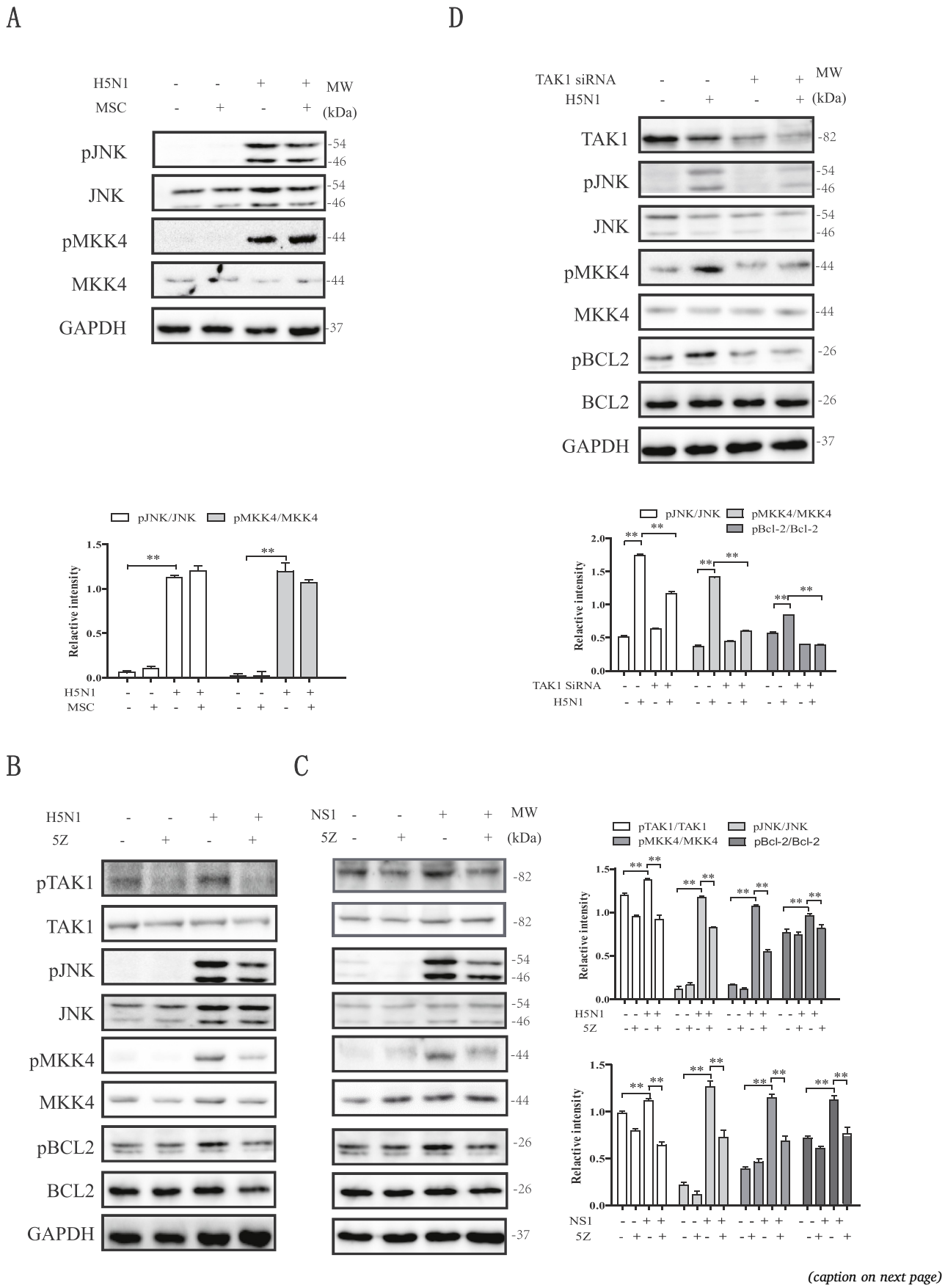

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Fig. 4. TAK1 is required for IAV-induced JNK activation. 293T cells were left uninfected or infected with H5N1 (1 MOI). After incubation for 16 hr in the presence of DMSO (0.5%) or MSC (10 μM) (**A**) or 5Z (5 μM) (**C**), the cells were harvested and prepared for cell lysates. (**B**) 293T cells were transfected with PHW2000 or the vector encoding the NS1 gene of the H5N1 virus. After incubation for 24 hr, the cells were treated with DMSO or 5Z (5 μM) for another 16 hr. (**D**) 293T cells were transfected with a scrambled control or TAK1 siRNA. After incubation for 24 hr, the cells were infected with H5N1 virus with H5N1 (1 MOI). After incubation for 16 hr, cell lysates were prepared and analyzed for TAK1, JNK, MKK4, and Bcl-2 phosphorylation by Western blot. After stripping, the membranes were re-probed with antibodies against total proteins. GAPDH was detected as a loading control. The band density of proteins was analyzed by using NIH Image-J software and presented as bar graphs. Data are the mean ± SD (n = 3) relative to control. $^{**}p < 0.01$, compared to untreated control.

phosphorylation, and that 5Z and TAK1 siRNA significantly inhibited H5N1 virus- and NS1-induced JNK phosphorylation, and inhibited H5N1-virus induced autolysosome formation. These results collectively suggest that TAK1 plays a critical role in IAV-induced JNK activation and autophagy. TLR3 and TLR7 are required for enterovirus-induced TAK1 and JNK activation (Lei et al., 2014). A very recent study by Wang et al. showed that TLR3 activation by poly(I:C) or by enteroviruses such as CVA16 and EV71 induces TAK1^{T187} and TAK1^{S192} phosphorylation (Wang et al., 2019a,b). It is likely that NS1 and/or TLR3 and TLR7 activation in IAV -infected cells can mediate virus-induced TAK1 activation. Of note, the PR8 virus increased TAK1 but did not increase the phosphorylation of its downstream molecules, MKK4, JNK, and Bcl-2 (Fig. 2B). This puzzle is solved by an earlier study showing that the NS1 protein of PR8 virus is able to block JNK activation induced by virus infection itself or by dsRNA (Ludwig et al., 2002).

A couple of prior studies suggest that ASK1 is responsible for IAV-induced JNK activation (Lu et al., 2010; Shiizaki et al., 2013). ASK1 and JNK phosphorylation is increased in a human NC-H292 bronchial epithelial cell line infected with A/Udorn/307/72 (H3N2) virus, and that JNK is poorly phosphorylated in ASK$^{-/-}$ murine embryonic fibroblast (MEF) (Maruoka et al., 2003). Intriguingly, it is not clear why increased ASK1 activation precedes JNK phosphorylation for several hours (Maruoka et al., 2003). Lu et al. (Lu et al., 2010) reported that inhibition of ASK1 activity by thioredoxin, an ASK1 inhibitor, suppresses JNK activation in PR8-infected A549 cells. It was proposed that ASK1 is an upstream kinase responsible for PR8 virus-induced JNK activation (Lu et al., 2010). In contrast to these observations, we found that MSC 2032964A, an ASK1 inhibitor, was unable to inhibit H5N1 virus-induced JNK activation. This discrepancy is likely due to the fact that the strong JNK activation mediated by H5N1 virus and the weak JNK activation by PR8 virus are mediated by different mechanisms. PR8 virus has a serine residue at the 103 position in its NS1 protein (Nacken et al., 2014). This unique NS1 protein not only does not activate JNK (Ludwig et al., 2001) but rather suppress viral RNA-induced JNK activation (Ludwig et al., 2002). In contrast, H5N1 virus and the majority of other IAV subtypes harbor a phenylalanine at position 103 of their amino acid sequence of the NS1 protein. The NS1 protein of H5N1 virus alone strongly activates JNK activation (Ludwig et al., 2001) (Nacken et al., 2014). The H5N1 virus used in our study contains a F103 in the NS1 gene (He et al., 2013) and can strongly induce JNK phosphorylation and activation in CEF (Zhang et al., 2019) and in 293T cells as shown in this study. Therefore, we speculate that in the study by Lu et al. (Lu et al., 2010), JNK activation by PR8 is likely mediated by viral RNA through ASK1, whereas JNK activation in H5N1 virus-infected 293T cells is largely mediated by the NS1 protein through TAK1.

In the past decade, the role of JNK in inducing autophagy has been increasingly appreciated. For example, under nutrient deprivation, Bcl-2 is phosphorylated by JNK1 at multiple sites, including T69, S70, and S87 (Wei et al., 2008a; Wei et al., 2008b). Phosphorylated Bcl-2 disrupts its interaction with Beclin1 and makes it available for the pre-initiation and induction of autophagy (Wei et al., 2008a; Wei et al., 2008b). JNK activation is required for autophagy initiation triggered by hepatitis B virus (Zhong et al., 2017), Sendai virus (Siddiqui and Malathi, 2012), oncolytic adenovirus (Klein et al., 2015), and cytosine e-phosphate-guanine (CpG) (Wu et al., 2016). Our recent and current study showed that JNK activation is required for H5N1 virus-induced autophagy in chicken fibroblast cells (Zhang et al., 2019) and in a mammalian cell line, respectively, and that the JNK inhibitor is able to inhibit H5N1 virus-induced autophagy. These observations collectively suggest that JNK activation plays a critical role in inducing complete autophagy. We further demonstrated that TAK1 inhibition also led to the blockade of autophagy in H5N1-infected 293T cells, as evidenced by the restoration of LC3 lipidation and p62 levels. Since TAK1 was required for JNK activation, inhibition of autophagy by 5Z is likely mediated by inhibition of JNK.

Autophagy can promote or suppress virus replication (Dong and Levine, 2013). Whether autophagy is beneficial or detrimental to IAV replication remains a controversial issue (Dumit and Dengjel, 2012). For example, an H3N2 virus (A/Aichi/68) replicates at an equivalent rate in wild-type or ATG5-deficient MEF (Gannage et al., 2009). Inhibition of autophagy by ATG5 siRNA or 3-methylademine (3-MA) does not lower the titers of H5N1 virus (A/Jilin/9/2004) in A549 cells or in the lungs of mice (Sun et al., 2012). In contrast, H1N1 viruses (A/WSN/33 & A/PR8/34) replicate much slower in ATG7-deficient cells than in wild-type MEF cells (Liu et al., 2016). The HKx31 strain of the H3N2 virus replicates slower in ATG5$^{+/-}$ alveolar epithelial cells (Hahn et al., 2014). We recently reported that bafilomycin A significantly decreases viral protein levels and lowers the titer of H5N1 virus in CEF but suppresses H1N1 replication at a much lower magnitude (Zhang et al., 2019). More recently, Wang et al. reported that H5N1 virus-induced autophagy promotes virus replication (Wang et al., 2019b). In the present study, we showed that TAK1 was required for efficient IAV replication in 293T cells. Since TAK1 plays an important role in JNK activation and autophagy, suppression of H5N1 virus replication by TAK1 inhibition is likely mediated in part by inhibition of autophagy. Kang et al. (Kang et al., 2013) reported that ginsenoside Rg3 inhibits hepatitis B virus replication by degrading TRAF6 and TAK1 and by blocking JNK activation in HepG2 cells. TAK1 plays an important role in aiding HIV-1 replication by activating NF-κB (Postler and Desrosiers, 2012). NF-κB has been shown to be beneficial to IAV replication (Pauli et al., 2008), it is not clear if TAK1 may also regulate IAV replication by modulating NF-κB activity.

In conclusion, our study highlights the role of TAK1 in IAV-induced JNK activation and autophagy, and provides evidence that TAK1 is required for efficient virus replication in 293T cells. Our study for the first time suggests that TAK1 controls IAV replication by regulating autophagy.

4. Materials and Methods

Reagents. MSC 2032964A was purchased from Toris Biotechnology (Bio-Techne China Co. Ltd, Shanghai, China). 5Z-7-oxozeaenol (5Z) was purchased from Sigma (St. Louis, MO). SP600125 was purchased from Cell Signaling Technology (Danvers, MA). Anti-β-actin and anti-GAPDH mAbs were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against p62, LC3, Bcl-2, JNK, MKK4, TAK1 and their corresponding phosphorylation antibodies Bcl-2^{S10}, JNK$^{T183/Y185}$, MKK4$^{S257/T261}$, TAK1^{T187} were purchased from Cell Signaling Technology (Danvers, MA). 293T and Vero cell lines were obtained from the American Tissue Culture Collection (Manassas, VA) and cultured in complete DMEM medium supplemented with 10% fetal bovine serum, streptomycin and penicillin, and L-glutamine. Monoclonal or polyclonal antibodies against M1 and NS1 proteins were prepared by

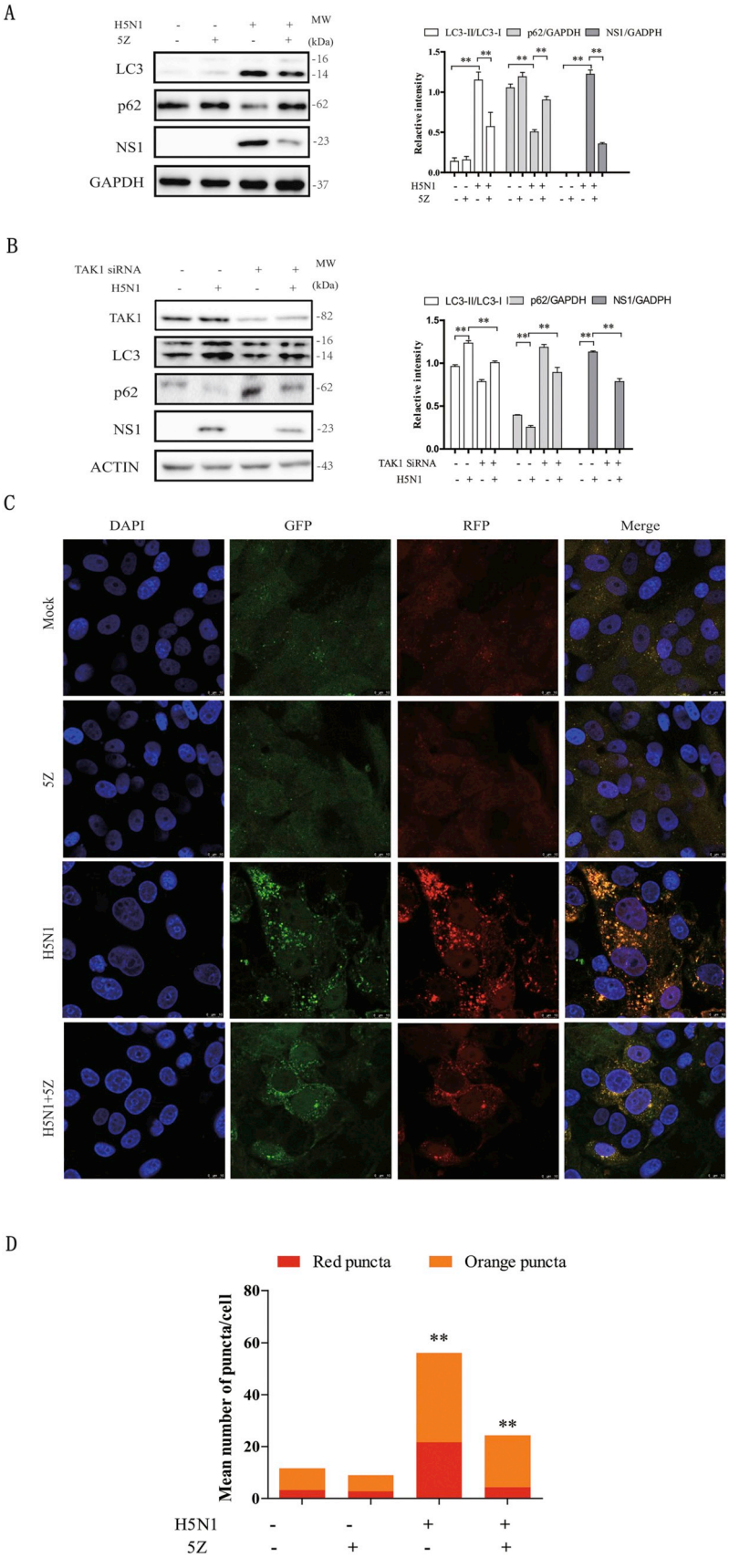

Fig. 5. Effect of 5Z on IAV-induced autophagy. (**A**) 293T cells were left uninfected or infected with H5N1 (1 MOI). After incubation for 16 hr in the absence or presence of 5Z (5 μM), cell lysates were prepared and analyzed for p62, LC3, and GAPDH expression by Western blot. (**B**) 293 cells were transfected with control or TAK1 siRNA. After incubation for 24 hr, the cells were left uninfected or infected with H5N1 virus with H5N1 (1 MOI). After incubation for another 16 hr, cell lysates were prepared and analyzed for p62, LC3, and GAPDH expression by Western blot. (**C**) Vero cells stably expressing the GFP-RFP-LC3 gene were left uninfected or infected with H5N1 (1 MOI). The cells were incubated in the presence of DMSO (0.5%) or 5Z (2 μM) for 16 hr. The cells were then fixed and stained with DAPI. Autophagosomes which presented as the orange puncta and autolysosomes which presented as the red puncta were visualized under a confocal microscope. Scale bar, 10 μm. (**D**) The numbers of red and orange puncta per cell were plotted in a bar graph. $^{**}p < 0.01$, the number of orange and red puncta in IAV-infected Vero cells compared to that in a mock-infected control.

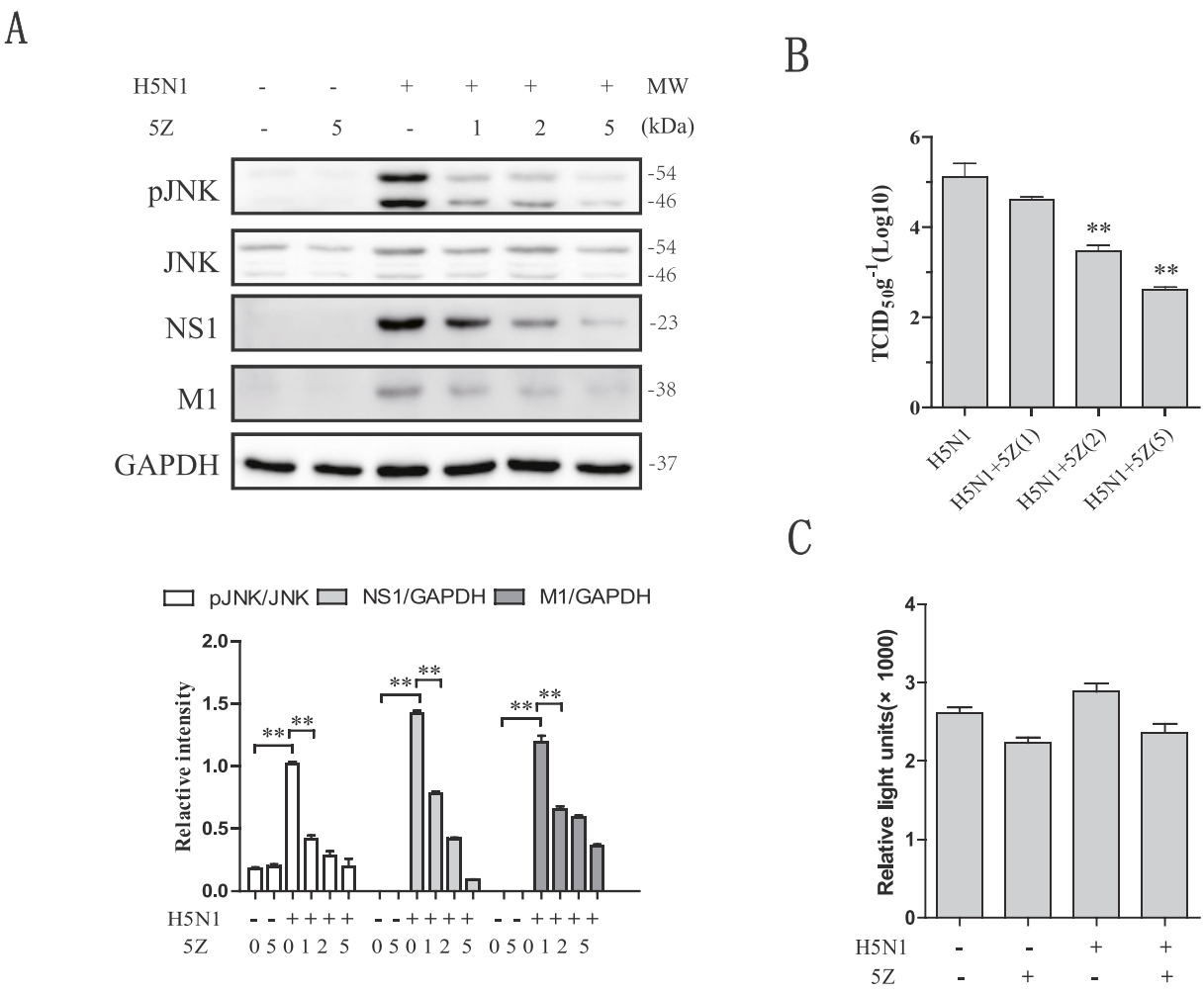

Fig. 6. Effect of 5Z on IAV replication. (**A & B**) 293T cells were infected with the H5N1 virus (1 MOI) and then incubated in the presence of DMSO (0.5%) or the indicated concentrations of 5Z for 16 hr. Cell lysates were prepared and analyzed for viral protein expression (**A**) or for viral titers in the conditioned media (**B**) and analyzed for the $TCID_{50}$ values. $^{**}p < 0.01$, compared to untreated control. (**C**) Effect of 5Z on 293 cell proliferation. 293T cells seeded in 96-well plates were left uninfected or infected with H5N1 virus (1 MOI) and incubated in the absence or presence of the indicated concentrations of 5Z for 16 hr. Cell proliferation was analyzed as described in the Materials and Methods. Data are the mean ± SD of three experiments. $^{**}p < 0.01$, compared to uninfected or H5N1 virus-infected controls.

immunizing mice with purified recombinant proteins. The expression vector PHW2000 encoding the NS1 gene of H5N1 virus has been reported previously (Hu et al., 2015).

Cell culture and virus. The A/mallard/Huadong/S/2005 (H5N1) virus was isolated from poultry and as reported previously (Zhang et al., 2008; He et al., 2013). Viruses were plaque purified three times in Madin-Darby canine kidney (MDCK) cells. A/PR8/34 (H1N1) virus was obtained from Dr. Liqian Zhu (College of Veterinary Medicine, Yangzhou University). Both IAV subtypes were prepared by inoculating 10-day-old specific-pathogen-free embryonic chicken eggs. The virus titers were determined by a 10-fold serial dilution (10^1 to 10^9, and each dilution (10^5–10^9) in CEF. The Reed and Muench method was used to determine the 50% tissue culture infection dose ($TCID_{50}$/ml).

Virus replication. 293T cells were inoculated with 1 MOI H5N1 virus and incubated in the absence or presence of SP600125 (10 μM) or the indicated concentrations of 5Z (1, 2, 5 μM) for 16 hr. The virus titers in the supernatant were determined by calculating the $TCID_{50}$ values as reported (He et al., 2013) (Zhang et al., 2008). The results represent the mean ± standard deviation (SD) of four independent experiments.

Cell proliferation assay. To exclude the possibility that the inhibitory effect of SP600125 and 5Z on virus replication was due to inhibition of cell proliferation, 293T cell proliferation was analyzed in the absence or presence of 5Z or SP600125 as recently reported (Zhang et al., 2019).

Autolysosome and autophagosome analyses. Vero cells stably transfected with GFP-RFP-LC3 were used for the analysis of autophagosome and autolysosomes according to our recent publication (Zhang et al., 2019). The red and orange puncta in the cells of ten random fields (100X) were counted in a blinded fashion. Results represent the mean puncta per cell ± SD (standard deviation) from one of three independent experiments with similar results.

Western blotting. 293T cells were infected with the indicated MOI of H5N1 or H1N1 virus for 16 hr or with 1 MOI of H5N1 or H1NI virus for the indicated length of time. Cell lysates were prepared and analyzed for the indicated proteins with their specific antibodies as previously reported (Zhang et al., 2019).

TAK1 knockdown. TAK1 knockdown in 293T cells were conducted by using siRNA purchased from Cell Signaling Technology (Danvers, MA) as previously reported (Liu et al., 2018).

Statistical analysis. Differences in the virus titers in the conditioned media of virus-infected cells, in the density of Western blot bands, and in cell proliferation were statistically analyzed by using an unpaired Student *t*-test. A *p* value of < 0.05 was considered statistically significant. All statistics were performed with SigmaPlot 11 software (Systat Software, Inc, San Jose, CA).

Conflicts of interest

All authors declare no competing interest.

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