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Proteomic Studies of the Influenza Virus-human Cell Interactions: the Responses of Host Cell Protein Expression to Viral Infection and the Novel Host Proteins That Interact with Virus Protein NS1

Proteomic Studies of the Influenza Virus-human Cell Interactions: the Responses of Host Cell Protein Expression to Viral Infection and the Novel Host Proteins That Interact with Virus Protein NS1

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Cell and Molecular Biology

By

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May 2013
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ABSTRACT

Influenza A viruses (IAVs) continue to be a threat to human health. Despite extensive studies, the mechanisms underlying the IAVs-host interactions during IAV infection remain elusive. We employed quantitative proteomic methods to systematically explore the host cell protein expression responses to IAV infection and examine the function of a critical IAV protein called NS1 by identifying its host binding partners. Specifically, we used a 2-dimentional gel electrophoresis (2-DE) based proteomic method to screen host proteins whose expression was substantially altered by IAV. One critical protein named IκB kinase-gamma (IKKγ) was found to be significantly down-regulated during IAV infection. Functional studies indicated that IKKγ and IAVs were mutually inhibitory and IKKγ might be the target for virus to inhibit IFN production.

IAV protein NS1 is known to play critical roles in viral pathogenesis and host immune responses. Through 2-DE proteomic approach and mass spectrometry, we identified several novel host cellular proteins that were associated with NS1. First, we found that heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP A2/B1) interacted with NS1, affected replication, transcription, expression and nucleo-cytoplasmic translocation of NS1 mRNA, and the eventual whole virus replication. Second, two ATPase proteins, RUVBL1 and RUVBL2, were identified to associate with NS1 for regulation of cell apoptosis in the absence of IFNs. Third, based on previous finding of the interaction between a DEAD family protein designated as DDX100 and NS1 through a more sensitive proteomic approach called SILAC (stable isotope labeling with amino acids in cell culture), we found this interaction promoted virus replication through enhancing viral NS1 gene replication, transcription, and dsRNA unwinding.

In summary, through quantitative proteomic, molecular and cell biology studies, we generated the global picture of host cell protein expression responses to IAV infection. For IAV NS1, several host cellular proteins were found to interact with NS1 to regulate the host cell action and virus proliferation.

This dissertation is approved for recommendation
to the Graduate Council.

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PREVIOUS PUBLICATIONS

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ABBREVIATIONS

%	Percentage
℃	Degrees Celsius
2-D	Two-dimensional
AAA+	ATPase associated with diverse cellular activities
AS160	Akt substrate of 160 kDa
ATPases	ATPase associated with diverse cellular activities
BAD	Bcl-2-associated death promoter
Bcl-2	B cell lymphoma 2
Bid	Bcl-2 interacting protein
BSA	Bovine serum albumin
cFLIP	Cellular FLICE inhibitory protein
cIAP-1	Cellular inhibitor of apoptosis 1
CPSF	Cleavage and polyadenylation specificity factor
CREB	cAMP response element-binding protein
DAPI	DNA-binding fluorochrome 4', 6-diamidino-2-phenylindole
DDX	DEAD box protein
DMEM	Dulbecco modified eagle medium
DMSO	Dimethyl sulphoxide
ds	Double-stranded
DFF	DNA fragmentation factor
DPBS	Phosphate buffered saline without Mg^{2+} and Ca^{2+}
DTT	Dithiothreito
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
eIF	Eukaryotic initiation factor
F	Flag
FADD	Fas-associating protein with death domain
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
Flu	Influenza
g	Gram
h	Hour(s)
HA	Haemagglutinin
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
hnRNP	Heterogeneous nuclear ribonucleoprotein
IAV	Influenza A virus
IEF	Isoelectric focusing
IFN	Interferon
IFNAR	IFN-α/β receptor
IgG	Immunoglobulin G
IL	Interleukin
IKBKG	Inhibitor of kappa light polypeptide gene enhancer in B-cells
IκB	Inhibitor of NF-κB
IKK	IκB kinase
IP	Immunoprecipitation
IRAK	IL-1 receptor-associated kinase
IRF-3	Interferon regulatory factor 3
kDa	Kilo Dalton
LB	Luria-Bertani
LPS	Lipopolysaccharides
M	Molar
MOI	Multiplicity of infection

MS	Mass spectrometry
Mda-5	Melanoma differentiation-associated gene-5
Min	Minute(s)
mRNA	Message RNA
MyD88	Myeloid differentiation primary-response protein 88
NA	Neuraminidase
NEMO	NF-kappa-B essential modulator
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	Nuclear localization signals
NP	Nucleocapsid protein
NS1	Non-structural protein 1
NTC	Non-template control
PAMP	Pathogen-associated molecular pattern
PEA	Palmitoylethanolamide
PDK1	Protein serine/threonine kinase 3′-phosphoinositide-dependent klinase1
PI3K	Phosphotidylinositol 3-kinase
PIP2	Phosphatidylinositol-4, 5-bisphosphate
PIP3	Phosphatidylinositol-3, 4,5-triphosphate
PKR	Protein kinase R
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative real time- polymerase chain reaction
RIG-I	Retinoic acid-inducible gene product I
RIP1	Receptor interacting protein-1
RNAi	RNA interference
RT-PCR	Reverse transcriptase-polymerase chain reaction
RUVBL	RuvB-like protein
S	Second
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
shRNA	Small hairpin RNA
siRNA	Small inhibitory RNA
snoRNP	Small nucleolar ribonucleoprotein
ss	Single-stranded
TBK1	TKK binding kinase 1
TCF	T-cell factor
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TNF-R	Tumor necrosis factor receptor
TPCK	Tosylsulfonyl phenylalanyl chloromethyl ketone
TRADD	TNF receptor type 1-associated DEATH domain protein
TRAF	TNF receptor-associated factor
TRIF	TIR domain-containing adaptor inducing IFN-β
Tris	Tris-hydroxymethyl-aminomethane
UV	Ultraviolet
V	Volts
vRNA	Viral RNA

Background

1. Seasonal, Pandemic Influenza, and Influenza Virus

Influenza (flu) is a public health threat and causes a long-term economic burden. Since the beginning of the 20th century, there have been four flu pandemics: 1. 1918 Spanish flu killed approximately 50-100 million people (Johnson and Mueller, 2002); 2. 1957 Asian flu was responsible for 70 thousand deaths; 3. 1968 Hong Kong flu was much milder, but still caused about 40 thousand deaths (Poland et al., 2007); 4. 2009 swine flu caused 8,829 infections with 74 deaths in 40 countries in the first month of infection (Garten et al., 2009). Additionally, other outbreaks of flu were significant, for example, the 1997 Hong Kong avian influenza, which was the first recorded avian influenza virus to break the species barrier with transmission to human beings, resulted in 358 deaths in 607 reported cases by July, 2012 (WHO, 2012). Besides the destructive pandemics, the seasonal flu circulates worldwide and causes annual epidemics with more than 200,000 hospitalizations and up to 49,000 deaths every year in the United States (CDC, 2011). Variant virus strains cause seasonal flu outbreaks every year. Due to the influenza virus variance, people may not have specific antibodies to protect themselves from each type of strain. So CDC recommends that people are vaccinated with seasonal flu vaccines at the beginning of each flu season.

The main transmission vehicle for influenza between humans is respiratory contents from coughs and sneezes. Flu symptoms include a sudden onset of high fever or chills, uncomfortable nose and throat, dry cough, headache, joint aches and some people, especially children may experience complications such as vomiting and diarrhea. The flu infection increases the possibility of other viral or bacterial infections, some resulting in death. So it is vital for people to receive immunizations of the seasonal influenza

vaccines, in particularly for older and younger people, and those who are pregnant or have a chronic medical condition, since they are at higher risk of influenza infection and flu-related diseases.

Both seasonal and pandemic influenza are caused by influenza viruses, which are classified into three types: A, B and C. Influenza A viruses (IAVs) are the most virulent and can be further divided into different subtypes based on two influenza virus proteins: hemagglutinin (HA) and neuraminidase (NA). Seventeen H antigens (H1-H17) and nine N antigens (N1-N9) have been reported. IAV is a member of the family *Orthomyxoviridae* containing a single-stranded, negative sense, eight-segmented RNA genome encoding 11 proteins (Hale et al., 2008). The total genome size is 13,588 bases. HA gene encodes hemagglutinin, responsible for virus intracellular entry through binding sialic acid on the surface of epithelial cells. NA gene encodes neuraminidase, which assists newly formed virion release by catalyzing the hydrolysis of sialic acid residues. M gene encodes matrix proteins 1 and 2 (M1 and M2) mediated by RNA splicing, which are responsible for formation of viral coat and ion channel. NS gene encodes distinct non-structural proteins 1 and 2 (NS1 and NS2) mediated also by RNA splicing, responsible for counteracting host immune responses and blocking cellular mRNA nuclear export, etc. NP gene encodes nucleoprotein (NP) responsible for encapsulating the virus genome for viral RNA replication, transcription and packaging. PB1, PB2 and PA genes encode three subunits of viral RNA polymerase, which can associate with NP and viral RNA to form the viral ribonucleoproteins (vRNPs) as the viral core for virus genome replication, mRNA transcription and translation.

The IAV replicative cycle starts as HA binds to sialic acid sugars on the surface of epithelial cells with the battlefield being in the nose, throat or lungs of mammals and intestines of birds. The sialic acid receptor mediates the endocytosis of the virus internalization. HA then undergoes a conformational

change to assist virus fusion with the cellular membranes (Carr and Kim, 1993). Once inside the cell, virus is disassembled by the acidic cellular environment. The vRNA molecules, accessory proteins and RNA polymerase are then released into the host cell cytoplasm (Lakadamyali et al., 2003) and migrate to the cell nucleus, where the vRNA can be replicated by RNA-dependent RNA polymerase through a transient complementary positive-sense RNA (cRNA) (Kash et al., 2006). The vRNA can be packaged as genome or processed to become mRNA, followed by nuclear-export to the cytoplasm for translation. Influenza viral HA and NA proteins are segregated immediately after synthesis by the ribosomes in the ER, further sorted in the Golgi complex and exclusively transported to apical plasma membranes via secretory vesicles. Other viral proteins are translated on free ribosomes, and then transported back to the nucleus to form new viral particles with negative-sense vRNA genome. NP and NS1 proteins bind to nascent RNAs to suppress viral RNA synthesis. HA and NA proteins budding from the Golgi cluster into a bulge in the cell membrane thereby prepare to coat viral particles, and then vRNA and viral core proteins enter this membrane protrusion with HA and NA to complete assembly and bud off through the plasma membrane. After the bridge between viral NA and sialic acid residues is cleaved, the mature virions will be released to infect neighboring cells. The influenza viral replicative cycle is shown in Fig. 1.

IAV protein NS1 has been shown to play important roles in viral pathogenesis and host immune responses during IAV infection. These include: (i) modulation of viral RNA synthesis and viral mRNA splicing (Fortes et al., 1994); (ii) inhibition of cellular translation and RNA process but assisting viral mRNA translation (Aragon et al., 2000); (iii) regulation of virus particle morphogenesis (Garaigorta et al., 2005); (iv) inhibition of apoptosis by activating phosphoinositide 3-kinase (PI3K) pathway (Ehrhardt et al., 2007b; Zhirnov et al., 2002) or promoting host cell apoptosis (Zhang et al., 2011); (v) regulation of strain-

dependent viral pathogenesis; and (vi) inhibition of dendritic cell action (Fernandez-Sesma et al., 2006). The effect of NS1 on host cell apoptosis has been controversial. On one hand, NS1 has been reported to play a role in pro-apoptosis (Zhang et al., 2011). On the other hand, NS1 has been reported to bind to and activate PI3K and its effector AKT (Ehrhardt et al., 2007b), which subsequently phosphorylates and inactivates the pro-apoptotic factors (Igney and Krammer, 2002).

The biggest challenge to eliminate influenza is the variance of virus strains resulting from frequent mutation. There are two ways for influenza virus to mutate: antigenic drift and antigenic shift. Antigenic drift is much more common and involves point mutations in the HA and NA genes. Since viral RNA polymerase lacks the RNA proofreading function, viral genome replication induces higher error rates. So the mutants frequently appear in viral genes. This mutation may cause the existing antibodies to fail to recognize the virus infection, so people have to be immunized with new influenza seasonal vaccines. Antigenic shift results from vRNA reassortment of two or more virus gene fragments. Antigenic shift may happen when more than one type of influenza virus strain infects one cell, and one strain can mistakenly coat another virus strain gene(s) like HA; then a new type of virus strain will be generated. This change is sudden, large and more destructive, since the new type of virus can not be counteracted by existing immunity. Consequently, the emergence of pandemics may be triggered if the new virus can spread easily from person to person in a sustained manner.

There are two classes of commercial anti-flu drugs approved by the FDA (Jackson et al., 2011). The first class of drugs includes M2 inhibitors, amantadine and rimantadine, which can block ion channels formed by M2 and then inhibit the nucleo-cytoplasmic translocation of viral genome (Pinto and Lamb, 2007). However, virus-based drug-resistance to this type of drugs has been observed. It is known that

recent virus strains, such as the 2009 pandemic A/H1N1 strain are resistant to M2 inhibitors (Garten et al., 2009). So this class of drug for influenza treatment is not highly recommended. The second class of anti-flu drugs includes NA inhibitors, oseltamivir and zanamivir. This class mainly functions in inhibiting the release of newly synthesized viruses from the infected cells by inhibiting NA enzymatic activity (Fig. 2). NA inhibitor drugs are quite promising, especially as the only class of antiviral drug for human influenza virus treatment. Most influenza viruses are sensitive to this class of drugs, like novel pandemic A/H1N1, but the virus resistance to this class of anti-flu drugs is still evident during the flu season (Renaud et al., 2011). Another drawback of this class of drugs is that patients need to apply drugs as early after virus infection as possibly, but most patients can not recognize the flu infection at the beginning. So new anti-flu drug development is necessary to control the potential influenza pandemics and many new antiviral strategies are undergoing development, including targeting different viral or cellular proteins, interference with the virus-required critical interactions or boosting immune-modulating drugs.

2. Type I IFN Responses

Innate immune responses are the first line of defense for the host against pathogens, like bacteria and viruses. The responses triggered by the innate immune system are antigen-nonspecific and relatively short-term. Innate immune system can recognize “foreign” by targeting pathogens with distinguished characters, like virus double-stranded (ds) RNA, CpG and gram-negative bacterial wall LPS. Those unique characters from pathogens are called pathogen-associated molecular patterns (PAMPs), and pattern-recognition receptors, like TLRs on the plasma membrane of host cells, are responsible for

recognizing PAMPs of the invading pathogen with consequence of defense initiation. One main function of the innate immune system is to secret cytokines to block pathogen invasion. As signaling molecules, cytokines are produced by many kinds of cells, especially by T-helper lymphocytes, and circulate to deliver the “message”. They are small, soluble and can bind to specific cytokine receptors. More than 80 known cytokines are secreted by virus-infected cells, and the first secreted ones after the virus infection include type I IFNs, type II IFN (IFN-gamma), interleukin-6 (IL-6), IL-12, and tumor necrosis factor alpha (TNF-α). Type I IFNs (IFN α, β, ε, κ and ω) as multifunctional cytokines are essential for host innate immune responses to viruses, bacteria, protozoa, and cancer. Three main pathways can trigger the production of type I IFNs: RIG, TLR7 and TLR3/4 (Fig. 3). (i) Retinoic acid-inducible gene product I (RIG-I) and melanoma differentiation-associated gene-5 (Mda-5) as two TLR-independent viral nucleic acid detectors can be activated by viral nucleic acids (Andrejeva et al., 2004). The binding of viral nuclei acid to the receptors activates IKK epsilon and TKK binding kinase 1 (TBK1), two serine/threonine kinases that phosphorylate and further promote IRF3 and IRF7 to transfer to the nucleus resulting in initiation of type I IFN transcription. (ii) TLR7 and TLR9 in endosomal compartments of plasmacytoid Dendritic Cells (pDCs) are responsible for recognition of ssRNA (Heil et al., 2004) and unmethylated CpG motifs (Hemmi et al., 2000) for type I IFN production. The activated TLR7 and TLR9 cause the adaptor myeloid differentiation primary-response protein 88 (MyD88) to recruit a protein complex consisting of IL-1 receptor-associated kinase 1 (IRAK1), IRAK4, IRF7 and TNF receptor-associated factor 6 (TRAF6) (Krug et al., 2004). IRF7 is the predominant IRF activated protein early after pathogen infection in pDCs (Prakash et al., 2005) and becomes ubiquitinated by TRAF6 E3 ligase with consequence of IRF7 translocation to the nucleus to stimulate transcription of type I IFN. (iii) In TLR3/4 pathway, TLR3 and

TLR4 can identify viral dsRNA or bacterial LPS and transmit signal through TRIF adaptor protein and TRAF members (Yamamoto et al., 2002). After receptor interacting protein-1 (RIP1) is recruited (Wertz and Dixit, 2010), TRAF6 catalyzes the Lys63 polyubiquitination on itself. The activated TRAF6 in turn activates IκB kinase (IKK), which is composed by two catalytic IKK subunits (IKK-α and IKK-β) and one regulatory subunit (IKK-γ, also termed NEMO or IKBKG). The activated IKK further activates NF-κB through releasing its inhibitors (IκBs) and then activated NF-κB exposes its nuclear localization signal (NLS) to translocate to the nucleus to initiate the transcription of various genes, like type I IFNs.

Type I IFNs bind to a common receptor consisting of IFNAR1 and IFNAR2 chains to regulate immune responses or cell activities (like apoptosis) through several downstream pathways. One pathway induced by type I IFNs is PI3K signaling pathway, which plays an important role in the IFN responses. PI3K contains catalytic subunits (p110 α, β, γ, and δ) and regulatory subunits (p85 α and β) (Guiducci et al., 2008). The activated PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4,5-triphosphate (PIP3), which further regulates an array of substrates, such as Akt or kinase 3′-phosphoinositide-dependent klinase 1 (PDK1) through a pleckstrin-homology (PH) domain on substrates. The binding of Akt PH domain to PIP3 phosphorylates and activates Akt, which in turn activates its numerous substrates to modulate cell activities, like cell survival and growth. Taking pro-apoptotic factors as an example, Bcl-2-associated death promoter (Bad) and Forkhead family member FKHR can be phosphorylated and inactivated by Akt to inhibit apoptosis (Datta et al., 1997; Fresno Vara et al., 2004). Other substrates, cAMP response element-binding protein (CREB) and IKK can also be phosphorylated by Akt to induce the activation of the Bcl 2 promoter, leading to up-regulation of Bcl 2 for apoptosis inhibition (Wang et al., 1999); (Fresno Vara et al., 2004). Besides apoptosis, Akt regulates cell

proliferation and metabolism. For example, Akt phosphorylates p21 to inhibit its anti-proliferative effects through restricting it within the cytoplasm (Zhou et al., 2001); AS160 (Akt substrate of 160 kDa) can also be phosphorylated by Akt in response to insulin (Kane et al., 2002). AS160 is reported to play roles on cellular metabolism, like glucose translocation (Manning and Cantley, 2007). For effect of type I IFNs on anti-virus, type I IFNs promote virus-infected cells to be recognized by cytotoxic T cells or killed by NK cells, or promote cell apoptosis to limit virus replication.

3. Apoptosis

In order to respond to a wide range of stimuli like virus, cells induce cell-programmed death, called apoptosis. Apoptosis can be triggered by both internal and external signals, such as virus infection, heat, growth factors, or cytokines. The characteristic changes induced by apoptosis include chromosomal DNA fragmentation, chromosome condensation, membrane blebbing, and collapse of potential between outside and inside of mitochondrion. Two signal pathways in the receptor-activated apoptosis have been extensively studied: Fas-Fas ligand-mediated and TNF-induced pathways. Fas-induced apoptosis starts at the Fas ligand binding to Fas receptor, which processes to trimerizate, followed by recruiting adaptor molecules such as Fas-associating protein with death domain (FADD). The recruited FADD further collects procaspase 8 to the receptor complex for self-cleavage; and then caspase 8 is released and activated, which in turn activates Bcl-2 interacting protein (Bid) with consequence of cytochrome c release (Chawla-Sarkar et al., 2003). Release of cytochrome c by the mitochondria is a hallmark for apoptosis. In the cytoplasm, cytochrome c binds to apoptotic protease activating factor 1 (APAF1) to form apoptosome that activates caspase 9. The activated caspase 9 further activates caspase 3. Alternatively, FADD-

activated caspase 8 directly cleaves procaspase 3 to release and activate caspase 3 with consequence of the cleavage of DNA fragmentation factor 45 (DFF 45). The activation of DFF45 induces DFF40 to translocate to the nucleus, resulting in internucleosomal DNA fragmentation, as another hallmark of apoptosis.

The second pathway is the TNF-induced pathway. TNF-α is secreted by activated T cells and macrophages in response to bacterial infection. The interaction between TNF-α and its receptor TNF-R1 causes the receptor conformational change with consequence of recruiting the downstream molecules, like RIP1 and TRAF2. TRAF2 binds to cellular inhibitor of apoptosis 1 (cIAP1) to form TNF-R1 signaling complex. TRAF2 also ubiquitinates RIP1, resulting in IKK-gamma recruiting, followed by the activation of NF-κB. The activated NF-κB promotes the transcription of anti-apoptotic genes, like cFLIP (cellular FLICE inhibitory protein), cIAP-1, cIAP-2 and TRAF 1. Alternatively, after receptor activation, TRADD, RIP1 and TRAF2 dissociate from TNF-R1 and recruit FADD to cleave procaspase 8 to produce caspase 8 for apoptosis induction as mentioned above. NF-κB signals are central for the life and death of cells: if NF-κB signals are inhibited, the anti-apoptotic proteins are down-regulated, leading to cell death; if NF-κB signals are activated, anti-apoptotic proteins are up-regulated, resulting in cell survival.

Besides receptor-based regulation of apoptosis, some factors, such as the Bcl-2 family, Bax family and IFNs, also utilize distinct pathways to regulate apoptosis. Type I and type II IFNs induce apoptosis in a wide range of cell lines. One mechanism by which IFN induces apoptosis is similar to the TNF-induced pathway as mentioned above (Thyrell et al., 2002; Xu et al., 1998). IFN strongly induces TRAIL and/or Fas/FasL to recruit and activate FADD, which in turn activates caspase 8. The activated caspase 8 further cleaves Bid, resulting in disruption of mitochondrial potential with the consequence of cytochrome c

release as mentioned above. The IFN-induced apoptosis is important during the early stages of virus infection, since apoptosis suppresses virus replication through killing virus-infected cells. However, numerous viruses have evolved some mechanisms to counteract the IFNs-inducted apoptosis for the completion of, at least, the progeny virus replication. Vaccinia viral protein inhibits apoptosis mediated by Fas or TNF-α (Dobbelstein and Shenk, 1996). Adenovirus encodes one protein called E1B-19K to block apoptosis mediated by TNF (Teodoro and Branton, 1997). As mentioned above, one of IFN-induced pathway, PI3K pathway, results in apoptosis inhibition, and influenza virus NS1 protein was reported to inhibit apoptosis by binding PI3K (Ehrhardt et al., 2007a). Besides targeting downstream factors of IFNs, viruses also inhibit the secretion of IFNs for apoptosis inhibition. Hepatitis C virus (HCV) encodes viral nonstructural 5A protein to suppress the secretion of IFNs by inactivating PKR (Gale et al., 1997). Influenza virus NS1 protein also suppresses PKR activation by competitively binding to viral dsRNA (Wang et al., 2000).

4. Objective of This Project

There are two specific aims in the present study: First, to analyze the host-influenza virus interactions at the proteome level by examining the global protein expression alteration using quantitative proteomic methods. Second, to assess the role of NS1, a vital IAV protein, by identifying host cellular proteins that are associated with NS1; and to characterize the functions of the identified critical interactions between NS1 and NS1-associated cellular proteins using biochemical, molecular and cell biology methods.

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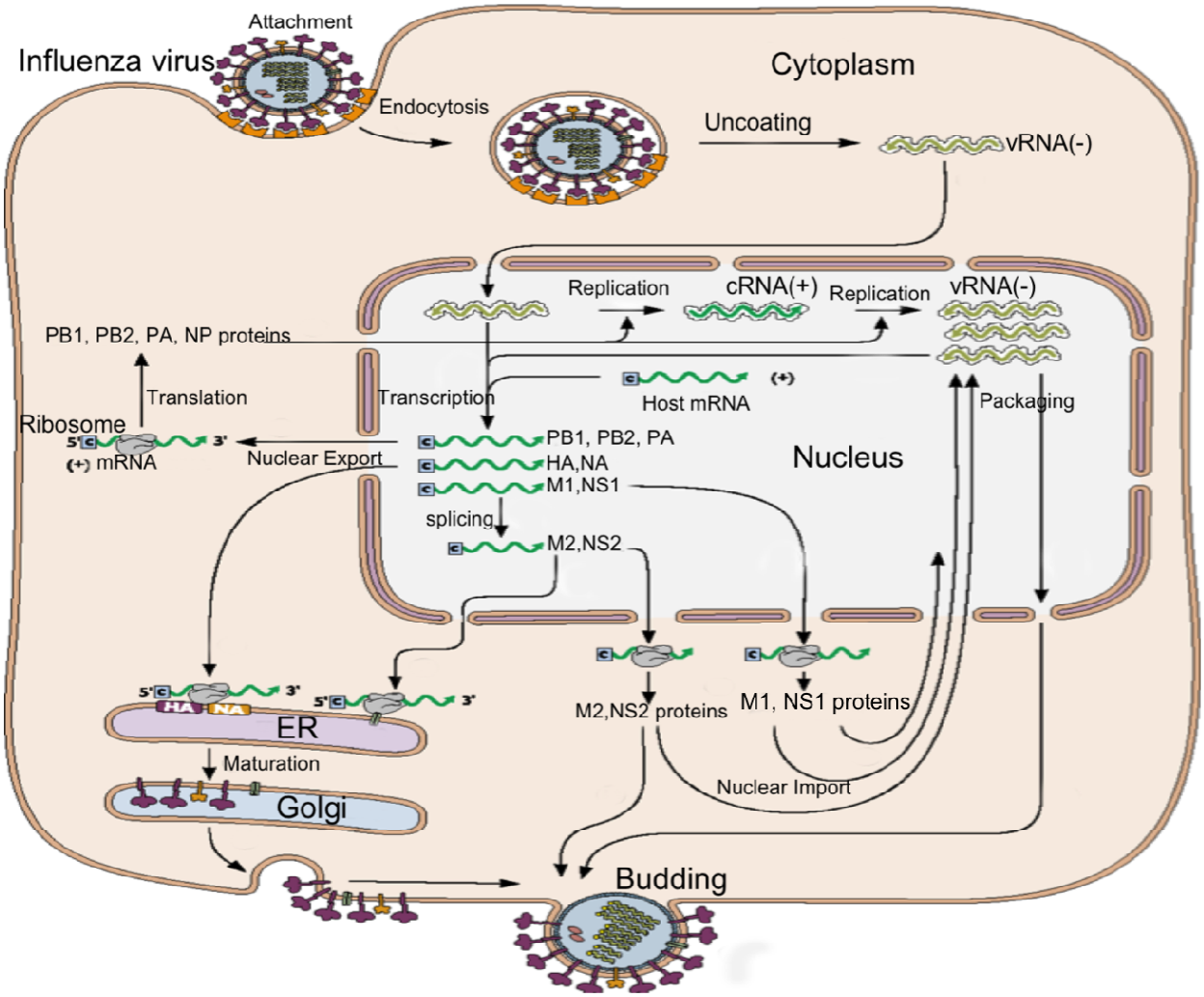

Figure 1. The replicative cycle of influenza virus in host cell (modified from textbook) (Flint et al., 1999). The detailed process is discussed in the text.

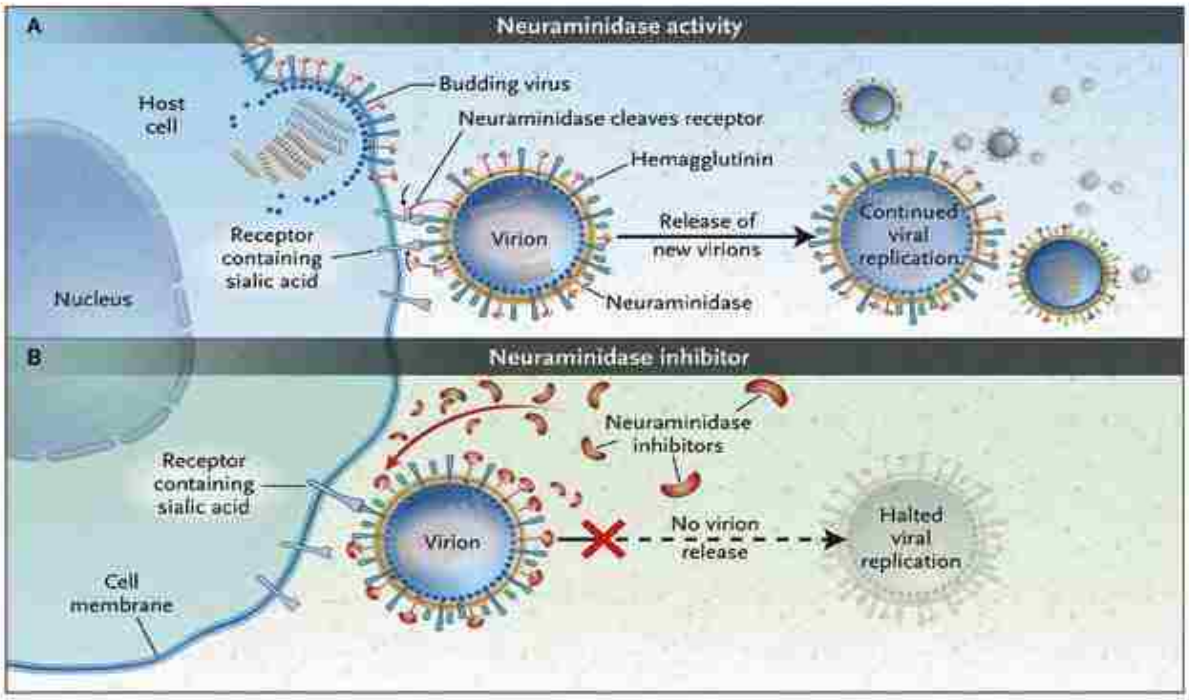

Figure 2. NA inhibitors suppress virion release (Moscona, 2005). NA inhibitors suppress NA enzymatic activity to hold new virions on the plasma membrane.

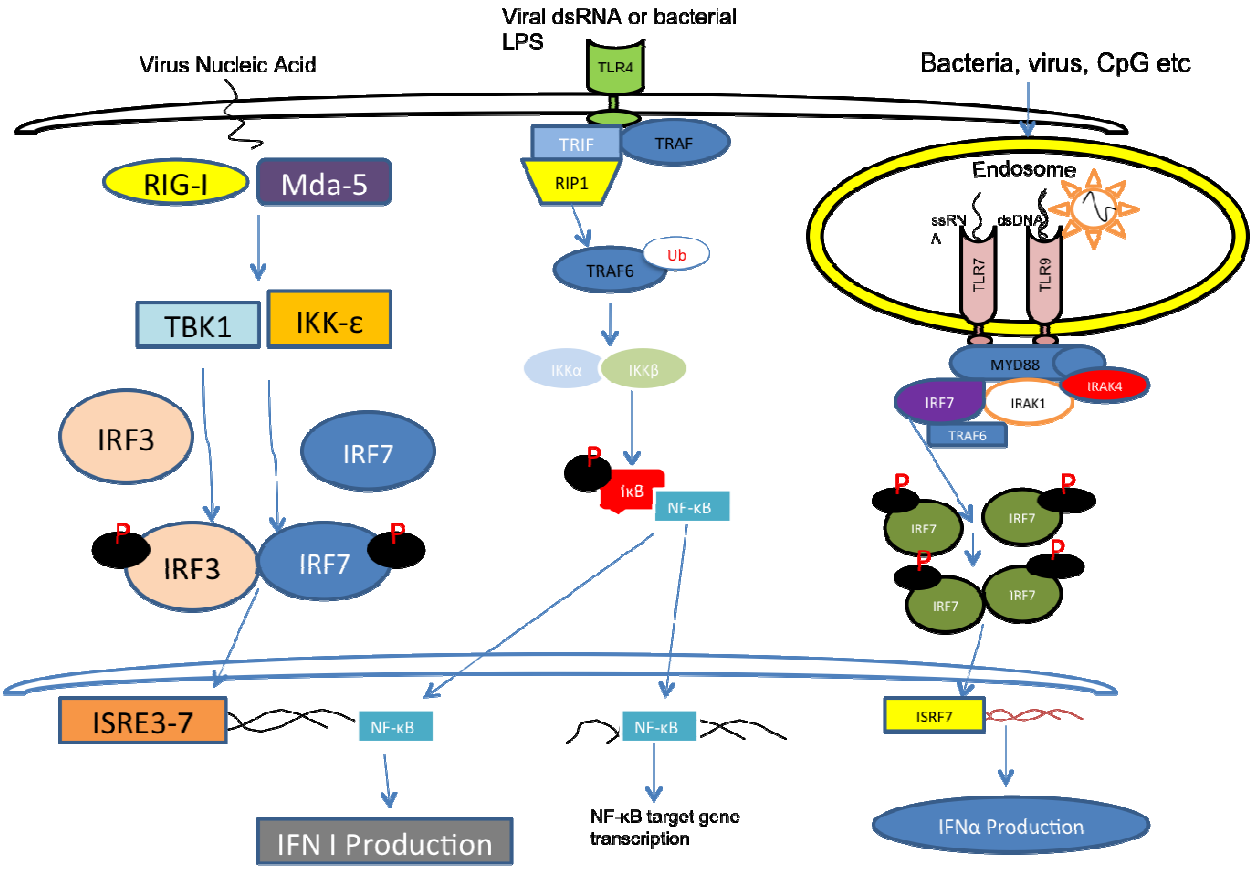

Figure 3. Three main pathways induced by pathogen to trigger the production of type I IFN. The pathways induced by RIG, TLR7 and TLR3/4 are discussed in the text.

Chapter 1. Inhibition of Type I Interferon Production via Suppressing IKK-Gamma Expression: A New Strategy for Counteracting Host Antiviral Defense by Influenza A Viruses?

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Abstract

Blockage of the induction of type I interferons (IFNs) is essential for the success of influenza virus proliferation in host cells. Several molecular mechanisms by which influenza viruses inhibit IFN induction have been characterized. Here we report a potentially new strategy influenza viruses employ to inhibit IFN production during viral infection. Through a two-dimensional gel electrophoresis based proteomic approach, we found that the expression of IκB kinase-gamma (IKKγ) was suppressed by influenza A virus infection in human lung epithelial A549 cells. Silencing of cellular IKKγ by small interfering RNA led to enhanced replication of influenza viruses. Concomitantly, overexpression of IKKγ resulted in increased production of IFNα/β, whereas influenza virus infection completely eliminated the IKKγ-overexpression-induced production of IFNα/β. Our results suggest that IKKγ and influenza virus are mutually inhibitory, and influenza viruses may inhibit IFN production through suppressing the expression of IKKγ during viral infection.

Keywords: Influenza virus, H1N1, interferon, IKKγ, NF-κB, 2-DE, proteomics, protein expression, LC-MS/MS

Introduction

NF-κB is an important transcription factor and plays a critical role in antiviral defense (Balachandran et al., 2011; Munir et al., 2011; Santoro et al., 2003). NF-κB normally binds to its inhibitor, IκB and is localized in the cytosol in its inactivated form. Upon virus infection, the virus-activated IκB kinase (IKK) phosphorylates IκB, resulting in its degradation through the ubiquitin-dependent pathway. The freed NF-κB then translocates to the nucleus and initiates the transcription of antiviral cytokines including type I interferons (IFNs), which are major components of host innate antiviral defense (Bernasconi et al., 2005; Bonnet et al., 2000). IKK is a trimeric protein complex consisting of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ. IKKγ (also termed NEMO or IKBKG) regulates the kinase activity of IKKα/β (Rothwarf et al., 1998). IKKγ-deficient cells lack the ability to activate NF-κB in response to multiple stimuli (Yamaoka et al., 1998).

Influenza A viruses, belonging to the *Orthomyxoviridae* family with 8 segmented genes (Bouvier and Palese, 2008), continue to be a threat to human health. It has been well established that influenza viral protein NS1 plays a vital role in suppressing IFN production (Garcia-Sastre, 2001; Geiss et al., 2002; Jia et al., 2010). In this regard, one important host antiviral factor is protein kinase R (PKR), which is a serine/threonine protein kinase functioning upstream of IKK in activating NF-κB. PKR is activated by binding to dsRNA, and the activated PKR in turn activates the IKK complex through physically binding to IKKβ (Bonnet et al., 2000). Viral protein NS1 is known to suppress the activation of NF-κB through either competitively binding to dsRNA (Wang et al., 2000), or directly interacting with PKR to block its activation (Krug et al., 2007; Sen et al., 2006; Tan and Katze, 1998). Another important antiviral factor is interferon

regulatory factor 3 (IRF-3), which is a key regulator of IFN gene expression (Au et al., 1995). dsRNA-bound NS1 was reported to prevent retinoic acid-inducible gene I (RIG-I)-mediated activation of IRF-3 (Garcia-Sastre et al., 2007). Furthermore, NS1 protein can bind to a 30-kDa subunit of the cleavage and polyadenylation specificity factor (CPSF) to mediate the inhibition of posttranscriptional processing of cellular mRNAs, resulting in blockage of the nuclear export of newly synthesized cellular mRNAs including IFNs and IFN-stimulated genes (Das et al., 2008; Kim et al., 2002; Krug et al., 2003). However, NS1 from some influenza virus strains, including the A/PR/8/34 (H1N1) strain, may have lost the CPSF binding capability (Krug and Kuo, 2009; Martinez-Sobrido et al., 2007).

In the present study, through a two-dimensional gel electrophoresis (2-DE) based comparative proteomic approach, we found that the expression of IKKγ was suppressed by influenza virus during viral infection. Functional validation experiments demonstrated that IKKγ and influenza virus were mutually inhibitory. Our results suggest that influenza viruses may inhibit IFN production via suppressing the expression of IKKγ during viral infection.

Experimental Procedures

Cell Culture and Virus Infection. Human embryonic kidney 293T cells, human lung epithelial A549 cells and Madin-Darby canine kidney (MDCK) cells (ATCC, Manassas, VA) were cultivated in Dulbecco modified eagle medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT) and 1% penicillin and streptomycin. Influenza A/PR/8/34 H1N1 viruses (ATCC, Manassas, VA) were propagated and titrated in MDCK cells as described (Coico, 2006). For virus infection, cells at 90-95% confluency were washed twice with phosphate buffered saline without Mg^{2+} and Ca^{2+} (DPBS) followed by incubation with viruses at the indicated multiplicity of infection (MOI) for 1 hour in a humidified incubator at 37℃ with 5 % CO_2. The virus solution was then aspirated, and cells were incubated with virus growth medium [DMEM with 0.2% BSA, 25 mM HEPES, 2 mM L-glutamine, sodium pyruvate, 2 μg/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin and antibiotics] at 37 ℃ in a 5% CO_2 incubator. For control, the same amount of virus growth medium was used in place of virus solution. Other procedures were the same as the procedures for the virus infection.

2-DE. Ten hours postinfection, the mock- and virus-infected A549 cells were harvested, washed twice with isotonic buffer (10 mM Tris-HCL, pH 7.5 and 250 mM sucrose), and lysed with rehydration buffer (8 M urea, 2% w/v CHAPS, 50 mM DTT, 0.2% w/v Bio-Lyte and 0.002% w/v bromophenol blue). After centrifugation at 50,000 g for 30 minutes at room temperature, the supernatant was collected, and the protein concentration was determined using a RC DC protein assay kit (BioRad, Hercules, CA) for 2-DE fractionation. Briefly, 450 μg of protein was loaded onto a 17 cm ReadyStrip IPG strip (pH 3-10 or 4-7), which was in turn kept at room temperature overnight. Isoelectric focusing was carried out with a Protean

IEF Cell using the following conditions: 250 V for 20 minutes with a linear ramp, 10,000 V for 1 hour with a linear ramp, and 10,000 V for a total of 50,000 V/h with a rapid ramp. Other procedures were performed according to the manufacturer's instructions (BioRad, Hercules, CA).

Mass Spectrometry (MS) Analysis and Database Search. In-gel digestion was performed as described previously (Du et al., 2009; Du et al., 2006; Gu et al., 2004), and liquid chromatography – tandem mass spectrometry (LC-MS/MS) analysis was carried out using a LTQ-XL mass spectrometer (Thermo, San Jose, CA) in the Proteomic Facility at the University of Arkansas for Medical Sciences (Little Rock, AR). Briefly, proteins were in-gel-digested with trypsin (Promega, Madison, WI) overnight at 37 ℃, and the resulting peptides were dissolved in 20 µl 0.1% formic acid for LC-MS/MS analysis. In the MS analysis, peptides were separated by an IntegraFrit column (10 cm × 50 µm ID; New Objective, Woburn, MA). Solvent A was 0.5% acetonitrile and 0.1% formic acid, and solvent B was 75% acetonitrile and 0.1% formic acid. The gradient started with a mixing of A:B = 95:5 and increased to A:B = 60:40 over 30 min. The flow rate was 500 nl/min. The LTQ-XL was operated in ESI positive-ion mode with the following settings: collision-induced dissociation (CID) fragmentation, data-dependent acquisition, and centroid mode for both MS and MS/MS spectrum recordings. MASCOT (Version 2.2; Matrix Science, Boston, MA) was used to search against a target-decoy (Elias et al., 2005) International Protein Index (IPI) human protein database (version 3.68) or Swiss-Prot database taxonomic field for virus (version 51.6) using LC-MS/MS data as described (Du et al., 2009; Du et al., 2006; Gu et al., 2004). The parameters for database searching were as follows: (i) 2.0 Da mass error tolerance for MS and 0.65 Da for MS/MS, (ii) a maximum of one missed cleavage, and (iii) variable modifications: acetylation at peptide N terminus, phosphorylation on tyrosine/serine/threonine and oxidation on methionine. Proteins with two or more

peptides with a score of more than 44 ($p < 0.05$) were considered as positive identification. Search results were further processed by Scaffold software (version 2_06_00; Proteome Software, Portland, OR) for viewing protein and peptide identification information. In the Scaffold analysis, protein identification probability with at least two peptides was set to 99% and the peptide identification probability was set to 95%. For the target-decoy database search, the false-positive rate for peptide identification was <5%.

Plasmid DNA Construction and Transfection. For the generation of NS1 expression plasmid, NS1 cDNA (GenBank accession no: CY021961) was inserted into the *BamH* I and *Xho* I sites of pcDNA3.1 vector (Invitrogen, Carlsbad, CA) to generate pcDNA3.1-NS1. IKKγ (GenBank accession no: NM_003639.3) was amplified from a human cDNA library using primers with *BamH* I and *Xho* I sites (forward: 5'-GGATCCACCATGAATAGGCACCTCTGGAAG-3' and reverse: 5'-CTCGAGCTACTCAATGCACTCCATGAC-3') and inserted into a pcDNA3.1 vector. All expression plasmids were verified by DNA sequencing. Expression plasmid was transiently transfected into 293T cells with the standard calcium phosphate method [basic protocol, (Kingston et al., 2001)].

Western Blotting. Western blotting was performed as described previously (Du et al., 2009; Du et al., 2006; Liu et al., 2005). Mouse monoclonal anti-NS1 antibody was a gift from Dr. Stephan Ludwig at the University of Muenster (Muenster, Germany). Rabbit polyclonal anti-IKKγ antibody was purchased from Santa Cruz Biotech (Santa Cruz, CA).

RNA Interference (RNAi). siRNA fragment (5'-GAGAAUCAAGAGCUCCGAGAUGCUU-3') targeting IKKγ was designed using a tool from the Whitehead Institute (http://www.whitehead.mit.edu/index.html). A randomized siRNA sequence (Shanghai GenePharma Co., Ltd., Shanghai, China) was used as the control. The siRNA as well as control oligos were transfected into A549 cells with Lipofectamine™

RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, A549 cells were seeded in a 6-well plate (3×10^5 cells/well) in DMEM with 10% FBS one day before transfection. Five µl Lipofectamine™ RNAiMAX reagent and 120 pmol siRNA were diluted in 250 µl Opti-MEM I reduced serum medium (Invitrogen, Carlsbad, CA) respectively, followed by mixing the diluted siRNA with the diluted reagent. After incubation at room temperature for 20 minutes, the mixture was added to the cells with a pipette. The cells were then incubated at 37 ℃ in an incubator with 5% CO_2 for 48 hours. The IKKγ-silenced as well as control cells were either harvested for analysis or infected by viruses for further treatments.

Plaque Assay. For influenza A/PR/8/34 virus plaque assay, 95% confluent MDCK cells in each well of the six-well plate were washed twice with warm DPBS and then incubated with 200 µl 10-fold serially diluted viruses for 1 hour at a 35 ℃ incubator with 5% CO_2. The plates were swirled every 15 minutes. Cells were then overlaid with 3 ml overlay medium containing 1% agarose and 2 µg/ml TPCK-trypsin in diluted DMEM (diluted by 30% with DPBS). After incubation in a 35 ºC incubator with 5% CO_2 for 96 hours, the agarose was removed and 2 ml of 70% ethanol was added to each well, followed by incubation of the plates at room temperature for 20 minutes. Cells were then stained with 0.3% crystal violet solution for 10 minutes for visualization of the plaques. Virus titer was expressed as PFU/ml determined by dilution factors and plaque numbers.

Quantitative Real Time PCR (qRT-PCR). RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA), and the first strand of cDNA was synthesized from 1 µg of RNA using the iScript cDNA synthesis kit (BioRad, Hercules, CA), according to the manufacturer's instructions. The IFNα/β and reference (actin) transcripts were amplified with BioRad CFX detection system as described (Spann et al.,

2004). mRNA abundance was measured using SYBR Green Supermix (Invitrogen, Carlsbad, CA) from three independent sample preparations. Relative gene expression of IFNα/β was calculated in the traditional $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical Analysis. Statistical analysis was performed using an independent-sample T test by Systat 13 (SPSS 13). A *p*-value of <0.05 was considered significant.

Results and Discussion

Identification of the Proteins Whose Expression is Affected by Influenza Viral Infection. In order to identify the proteins whose expression was affected by influenza viral infection, we infected human lung epithelial A549 cells with influenza A/PR/8/34 (H1N1) viruses at an MOI of 1. Ten hours postinfection, mock- and virus-infected cells were harvested and analyzed by 2-DE. We analyzed the proteins using 2-DE with IPG strips of both pH 4-7 and 3-10 to increase the chance of protein identification (Fig. 1). After 2-DE, the differences in protein spot intensity between the control gel (the gel that resolved the proteins from mock-treated cells) and the "virus" gel (the gel that resolved the proteins from virus-infected cells) were quantified by PDquest (BioRad, Hercules, CA). Protein spots with a more than 2-fold change in intensity were excised for LC-MS/MS analysis. Table 1 lists the identified proteins, which can be classified into several different biologically functional areas. Most of the identified proteins were in the expected size and pH ranges on the 2D-PAGE gel. However, a few of the proteins were identified in unexpected locations on the 2D-PAGE gel. For example, the full length heat shock cognate 71 kDa protein **(**HSPA8) has a theoretical molecular weight of 71 kDa, but the protein was identified in spot 3, which was close to the molecular weight marker of 25 kDa (Fig. 1). When we examined the peptides identified by MS, we found that the 15 unique peptides detected by MS all matched to the first 236 residues at the N-terminal end of HSPA8 (Supplemental Fig. S1), suggesting that the HSPA8 in spot 3 was a truncated but not the full-length version of the protein. One issue in protein identification that has some uncertainties is that several proteins were identified from same protein spot (Table 1). Further expression validation tests are

needed to examine the actual levels of changes in the expression of those proteins in the IAV infected cells.

Among the identified proteins, some have been previously reported to be related to influenza virus infections. For example, the expressions of enoyl-CoA hidratase (mitochondrial precursor) and glutathione S-transferase were reported to be altered by avian influenza H9N2 and H5N1 viruses, respectively (Liu et al., 2008; Zhang et al., 2008). The expression of vimentin was found to be up-regulated by influenza H1N1 viruses (Coombs et al., 2010). Identification of those proteins that have been reported from other research groups to be related to influenza viruses serves as a good validation of our current experimental approach. In addition to the proteins that have been reported previously, we also identified multiple proteins that have not been reported previously, such as eukaryotic translation initiation factor (EIF) (different isoforms), eukaryotic elongation factor (different isoforms) and IKKγ. EIFs are involved in initiating protein synthesis. It is known that host cellular protein synthesis in influenza virus infected cells is shutdown by viral elements, leaving the exclusive translation of viral mRNAs (Inglis, 1982; Lyles, 2000). Specifically, viral protein NS1 recruits EIF4GI (the large subunit of the cap-binding complex EIF4F) to the 5' untranslated region of the viral mRNA to facilitate the preferential translation of the viral mRNA (Aragon et al., 2000; Burgui et al., 2003). The highly confident identification of EIF4H (5 unique peptides and 21% sequence coverage) in the present study suggests that EIF4H may also play an important role in regulating viral and host protein expression in virus infected cells.

IKKγ Expression Is Suppressed by Influenza Viruses. One more protein that was identified in this study but has not been reported to be related to influenza viral infection was IKKγ, whose expression was found to be suppressed by viral infection (Fig. 1). The protein was identified by LC-MS/MS with high

confidence, having 5 unique peptides and a 12% protein sequence coverage (Fig. 2). We decided to choose this protein for further analysis because IKKγ is known to play an important role in regulating the NF-κB pathway (Rothwarf et al., 1998), a pathway that determines the production of IFNs (Bonnet et al., 2000; Wang et al., 2000). Fig. 3 shows results of a Western blot analysis of mock- and virus-infected A549 cells. Consistent with the 2-DE results (Table 1 and Fig. 1), Western blot analysis demonstrated that IKKγ expression was indeed suppressed by influenza viral infection (Fig. 3).

IKKγ Inhibits Influenza Virus Replication. In order to test whether the alteration in IKKγ expression affects influenza viral replication, we used an RNAi technique to suppress the expression of endogenous IKKγ in A549 cells and examined its effect on viral replication. We first transfected the A549 cells with siRNA oligos targeting the IKKγ sequence. Western blot analysis demonstrated that the expression of IKKγ was suppressed by 80% (judged by image analysis with ImageJ) by the siRNA (Fig. 4A; upper panel). We then infected the mock-treated (non-silenced) and the IKKγ-silenced A549 cells with influenza A/PR/8/34 at an MOI of 0.5, followed by 30 hours of incubation. Western blot analysis indicated that IKKγ silencing enhanced viral replication, as more viral protein NS1 was produced in the IKKγ-silenced cells than the mock-treated cells (Fig. 4A; middle panel). We also harvested the viruses in the supernatants of the control and the IKKγ-silenced cells for plaque assay. Consistent with the Western blot results (Fig. 4A; middle panel), the plaque assay demonstrated that silencing of endogenous IKKγ significantly raised virus titers ($p < 0.05$) (Fig. 4B and C). Results from this and previous sections (Figs. 1, 3 and 4) suggest that endogenous IKKγ plays an inhibitory role in influenza viral replication in the infected host cells, and influenza viruses counteract the inhibitory effect of IKKγ by suppressing its expression.

Influenza Viruses may Inhibit IFNα/β Production through Suppressing IKKγ Expression. It is well-known that IKKγ is an important immune regulatory factor in activating NF-κB, which can promote the transcription of anti-apoptosis factors and immune cytokines, such as IFNα/β (Bernasconi et al., 2005; Bonnet et al., 2000). Previous studies have established that influenza viruses can inhibit IFNα/β production in viral infected cells through several molecular mechanisms as described in the previous sections, but none of those reported mechanisms involves direct action on IKKγ. Results from our 2-DE based proteomic analysis (Table 1, Figs. 1 and 3) implied that the expression of IKKγ may be under direct influence of influenza viral infection. To further confirm our proteomic results, we examined the effect of overexpression of IKKγ on IFNα/β production. Since IKKγ can indirectly initiate the transcription of IFNs via activating NF-κB, we expected that overexpression of IKKγ can lead to the increased expression of IFNα/β. As expected, when IKKγ was overexpressed in human 293T cells (Fig. 5A), qRT-PCR analysis demonstrated that the levels of IFNα/β mRNA increased more than three-fold (Fig. 5B). We then challenged the control cells that expressed an endogenous IKKγ and the IKKγ overexpressing cells with influenza viruses and compared IFNα/β production between the two types of cells that differed only in IKKγ expression. As shown in Fig. 5C, after cells were infected with influenza viruses, the cells overexpressing IKKγ had slightly but significantly reduced levels of IFNα/β mRNA compared with the control cells expressing an endogenous IKKγ. This result contrasted sharply with what was observed in non-virus-infected cells shown in Fig. 5B, which demonstrated that overexpression of IKKγ increased IFNα/β mRNA levels. In other words, the results in Fig. 5B and C demonstrated that influenza viral infection completely eliminated the IKKγ-overexpression-induced increases in production of IFNα/β in the

infected cells, suggesting that influenza viruses may block IFNα/β production by affecting the expression of IKKγ.

The NF-κB pathway is one of the most important mechanisms underlying the suppression of IFNα/β production by influenza viruses (Santoro et al., 2003). IKKγ is the only regulatory factor in the IKK complex and is located upstream of the NF-κB signaling pathway. IKKγ is essential for NF-κB activation (Rothwarf et al., 1998; Yamaoka et al., 1998). The influenza virus must have evolved certain mechanism(s) to block IFNα/β production through directly or indirectly regulating this essential component. Viral protein NS1 has been shown to play a vital role in suppressing IFNα/β production via inhibiting NF-κB activation. Because NS1 exerts its effect on NF-κB activation in several previously reported mechanisms largely through physically binding to the target molecules such as dsRNA and PKR (Krug et al., 2007; Sen et al., 2006; Tan and Katze, 1998), we performed coimmunoprecipitation to examine whether NS1 was also associated with IKKγ during influenza viral infection. The result was negative, suggesting that NS1 may not be directly involved in the suppression of IKKγ expression during influenza viral infection (data not shown). However, the result does not exclude the possibility that NS1 may contribute to the influenza virus infection-induced suppression of IKKγ expression in an indirect way. On the other hand, because the NS1 protein in the A/PR/8/34 virus strain cannot inhibit host cellular gene expression through binding to CPSF (Martinez-Sobrido et al., 2007), it was unlikely that the decreased expression of IKKγ in the virus infected cells resulted from influenza viral NS1-mediated host cellular gene expression inhibition. Therefore, one possibility is that the reduced expression of IKKγ in the influenza virus infected cells resulted from NS1-induced inhibition of NF-κB or IRF-3. Alternatively, it is also possible that Influenza viruses have evolved a novel mechanism that has yet to be characterized to

suppress the IKKγ expression during viral infection. The exact molecular mechanism underlying the suppression of IKKγ during influenza viral infection remains to be investigated.

ACKNOWLEDGMENT. We thank Dr. Stephan Ludwig (University of Muenster, Muenster, Germany) for kindly providing the mouse monoclonal anti-NS1 antibody. This work was supported by part of NIH P20RR015569/3P20RR015569-10S2 (Y. Du).

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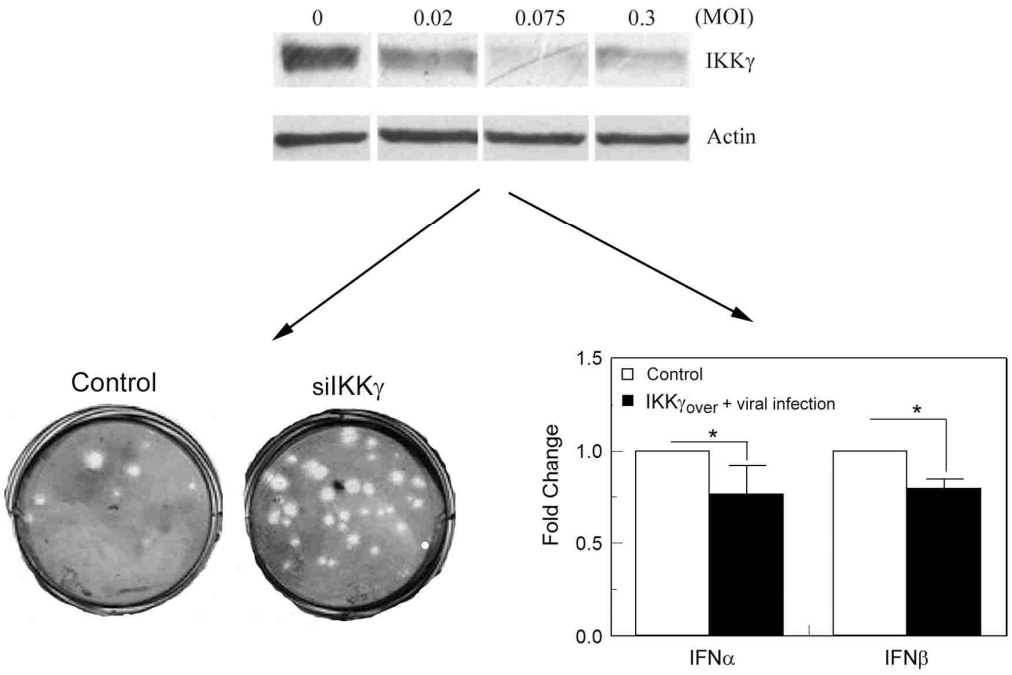
Table of Contents Synopsis and Graphic

Through a two-dimensional gel electrophoresis based comparative proteomic approach, we found that the expression of IKKγ was suppressed by influenza virus during viral infection. Functional validation experiments demonstrated that IKKγ and influenza virus were mutually inhibitory. Our results support a potentially new mechanism by which influenza viruses suppress IFNα/β production — via inhibiting the expression of IKKγ.



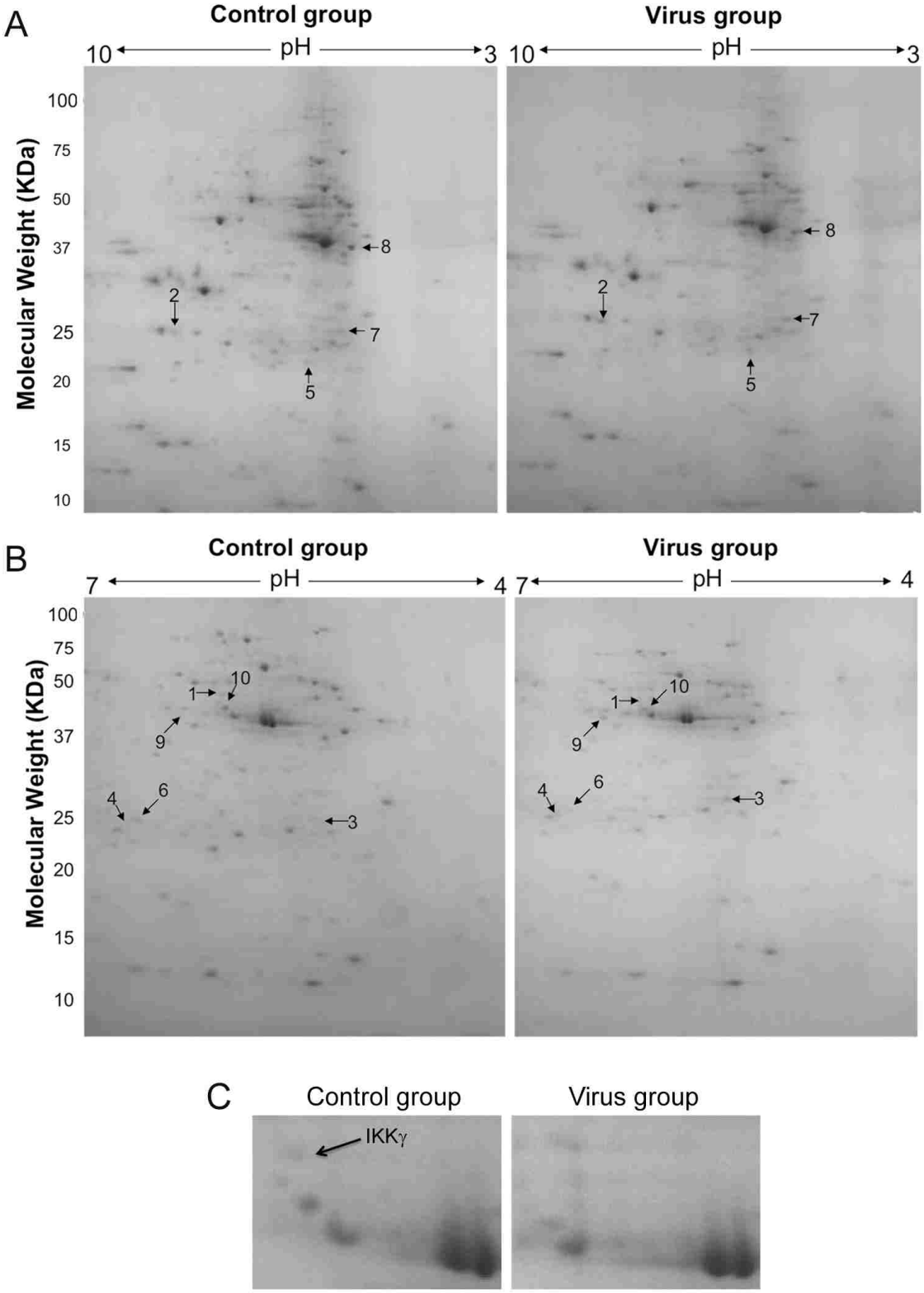


Fig. 1. Comparative 2-DE of cellular proteins extracted from mock- (left panels) and influenza virus-

infected cells (right panels). A549 cells at 90-95% confluency were mock-infected or infected by influenza A/PR/8/34 H1N1 viruses at an MOI of 1. At 10 hours postinfection, cells were harvested, lysed and separated on a pH 3–10 (A) or 4–7 (B) linear IPG strip, followed by an 8-16% gradient SDS-PAGE fractionation and coomassie blue staining. Protein spots with a more than 2-fold change in intensity were marked. Proteins identified by MS analysis from the marked spots are listed in Table 1. (C) An enlarged image of gel regions around spot 1, which contains IKKγ. When the wet gels were visually inspected, the IKKγ protein spot in the control gel was clearly visible but was very faint in the “virus” gel.

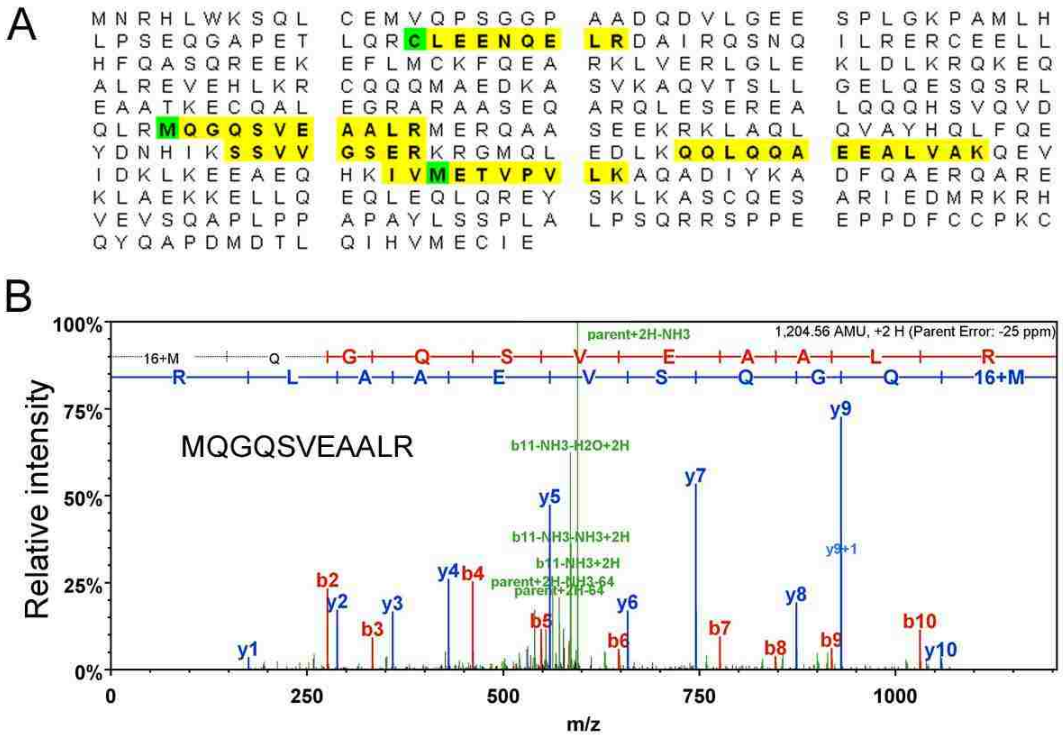


Fig. 2. Identification of IKKγ by MS. (A) IKKγ was identified by LC-MS/MS with 5 unique peptides (highlighted). (B) MS spectrum of a representative peptide.

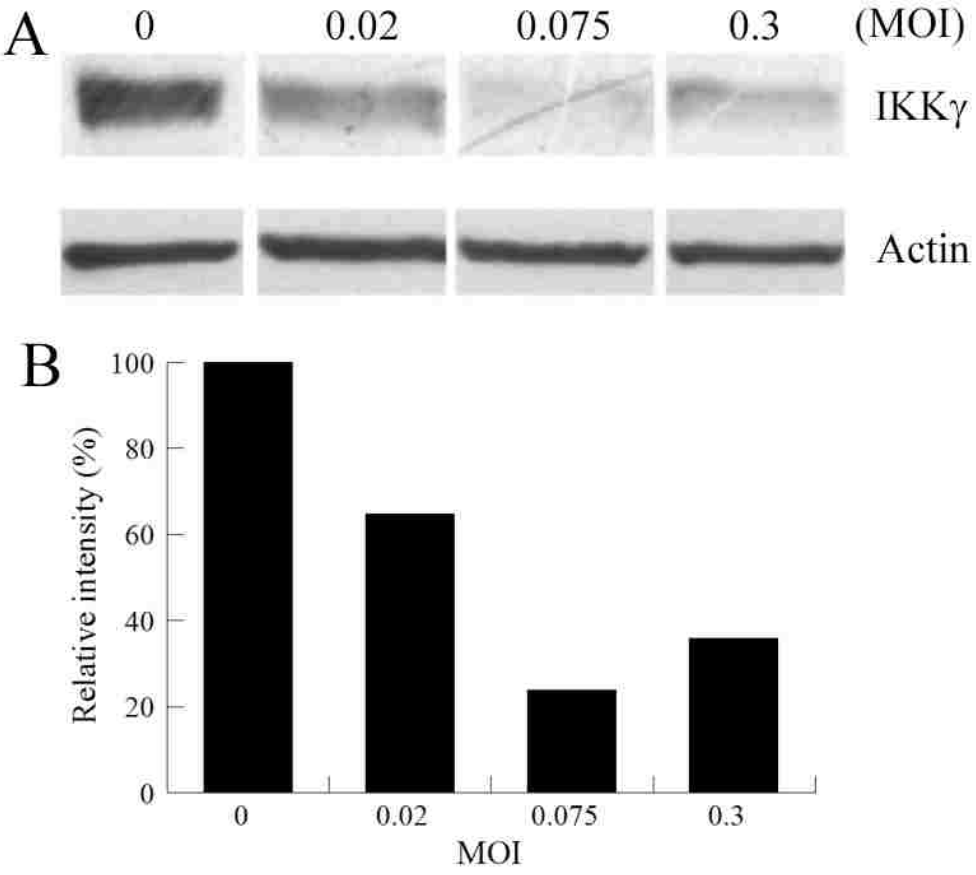


Fig. 3. Validation of the suppression of IKKγ expression in the influenza virus infected cells with Western blotting. (A) A549 cells were infected by influenza A/PR/8/34 at an MOI of 0, 0.02, 0.075 and 0.3, followed by 24 hours of incubation. Total protein extracted from cells was analyzed by Western blotting with anti-IKKγ. β-actin was used as a loading control. (B) Quantitation of the intensity of IKKγ protein bands shown in (A). The quantitation was performed using software ImageJ.

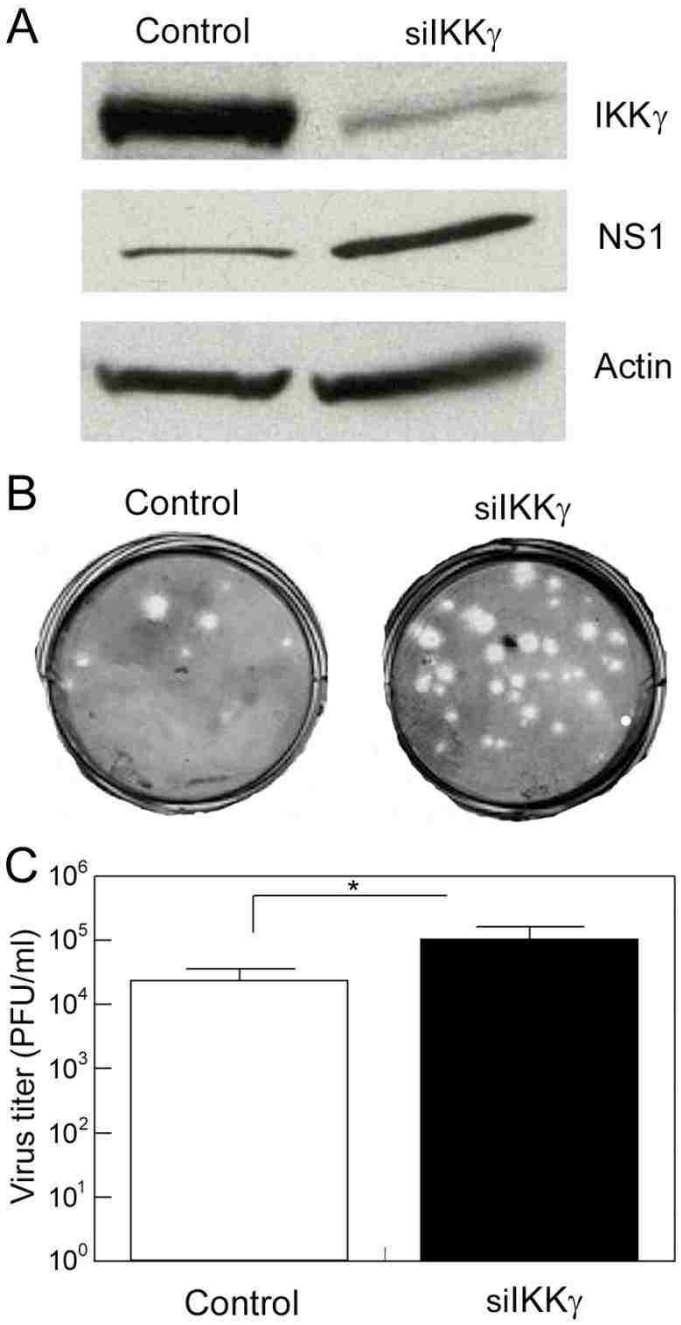
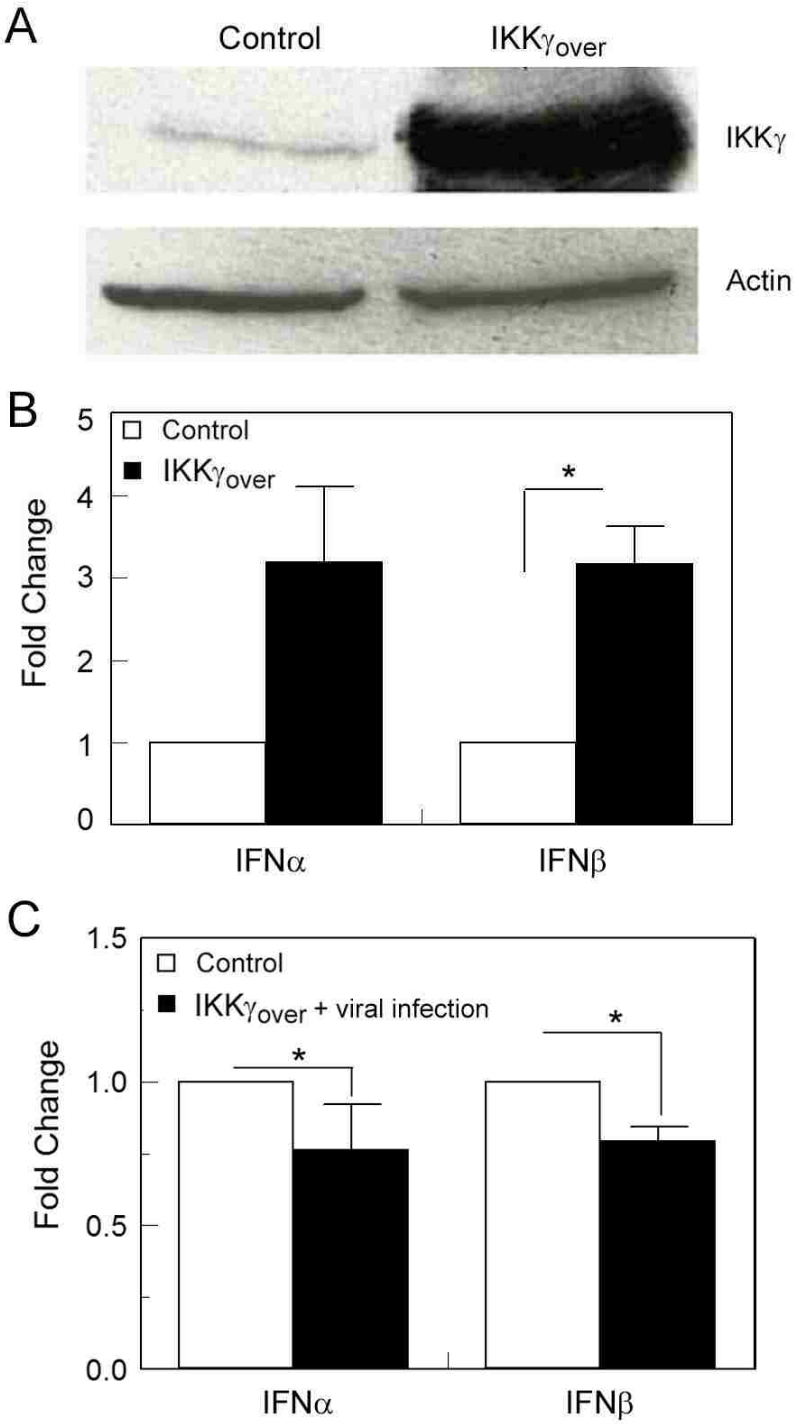


Fig. 4. Silencing of endogenous IKKγ enhances influenza virus replication. A549 cells (3×10^5) were mock-treated or transfected with siRNA targeting IKKγ, followed by 48 hours of incubation. The cells were then infected with influenza A/PR/8/34 at an MOI of 0.5 and incubated for 30 hours. The resulting cells were harvested for Western blot analysis and the supernatants for virus plaque assay. (A) Silencing of

endogenous IKKγ leads to increased NS1 expression. (B) Silencing of endogenous IKKγ results in more viral plaques. A representative plaque assay for control as well as siIKKγ-treated cells is shown. (C) Silencing of endogenous IKKγ results in higher virus titers. Values are the means ± standard errors of five independent titrations. * denotes $p < 0.05$. siIKKγ, IKKγ silencing by siRNA.


A
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B
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IFNα
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C
Control
IKKγover + viral infection
Fold Change
1.5
1.0
0.5
0.0
*
*
IFNα
IFNβ

Fig. 5. Overexpression of IKKγ enhances IFNα/β production and influenza viral infection eliminates IKKγ-overexpression-induced increases in IFNα/β production. (A) Overexpression of IKKγ in 293T cells. Whole cell lysates from 293T cells transiently transfected with IKKγ plasmid were analyzed by Western blotting with anti-IKKγ. (B) Overexpression of IKKγ results in higher levels of IFNα/β mRNA. IFNα/β mRNA extracted from IKKγ overexpression and control cells were determined by qRT-PCR. (C) Influenza viral infection eliminates IKKγ-overexpression-induced increased IFNα/β production. The cells prepared in the same way as in (B) were challenged with influenza viruses at an MOI of 0.5. Thirty hours postinfection, IFNα/β mRNA levels were analyzed by qRT-PCR. Actin was used as an endogenous control. Values are the means ± standard errors of three separate sample preparations. * denotes $p < 0.05$. $IKK\gamma_{over}$, IKKγ overexpression.

Table 1. List of proteins whose expression is altered by the infection of H1N1 virus in A549 cells

Category	Protein name	Spot no.[a]	Access no.	No. unique peptide	No. assigned spectra	Sequence coverage (%) [b]	Mascot score	Fold change (Infected/ control)
Immune response	IκB kinase-gamma	1	IPI00002411	5	5	12	122	< 0.2
ATPase	RuvB-like 2	1	IPI00009104	6	6	15	105	< 0.2
Translation factors	Eukaryotic translation initiation factor 4H	2	IPI00014263	5	5	21	119	2.5
	Elongation factor 1-delta	3	IPI00023048	7	8	35	179	2.2
Carbohydrate and energy metabolisms	Isoform 1 of Triosephosphate isomerase	4	IPI00465028	10	19	42	420	> 6.6
	Aldo-keto reductase family 1 member C1	2	IPI00029733	10	18	35	389	2.5
	Isoform alpha-enolase of Alpha-enolase	2	IPI00465248	4	6	12	185	2.5
	Glutathione S-transferase P	5	IPI00219757	10	35	61	797	2
	Enoyl-CoA hydratase, mitochondrial precursor	6	IPI00024993	10	28	37	591	< 0.2
Signal transduction	14-3-3 protein zeta/delta	3	IPI00021263	9	13	42	188	2.2
	14-3-3 protein beta/alpha	7	IPI00216318	4	7	32	174	2
Structure and chaperone proteins	Vimentin	8	IPI00418471	12	14	29	204	3.5
	Tubulin beta chain	9	IPI00011654	17	73	43	980	3.4
	Ezrin	10	P15311	12	24	17	645	0.2
	Heat shock cognate 71 kDa protein	3	IPI00003865	15	65	28	652	2.2
Viral protein	Non-structural protein 1 (NS1)	4	P03496	11	49	55	1143	> 6.6

[a] Spot numbers correspond to those on Fig. 1.
[b] Coverage of all peptide sequences matched to the identified protein sequence (%).

Supplementary Figure S1. Identification of the truncated HSPA8 by liquid chromatography tandem mass spectrometry (LC-MS/MS). Partial HSPA8 was identified by LC-MS/MS with 15 unique peptides (highlighted), which all locate at the N-terminal end.

Supplemental explanations:

1, **Potential mechanism underlying the regulation of IKKα/β by IKKγ.** The two catalytic subunits of the IKK complex, IKKα and IKKβ, need to be phosphorylated to activate NF-κB. As the regulatory subunit, IKKγ forms homodimer to interact with either an IKKα/β heterodimer or an IKKβ homodimer (Miller and Zandi, 2001). IKKγ interacts with several different signaling molecules, by which it links upstream activators to phosphorylate IKKα and IKKβ. IKKγ recruits the TAK1 complex to phosphorylate IKKβ, leading to its activation with consequence of NF-κB activation (Israel, 2010).

Two references:

Israel, A., 2010. The IKK complex, a central regulator of NF-kappaB activation. Cold Spring Harb Perspect Biol 2, a000158.

Miller, B.S., Zandi, E., 2001. Complete reconstitution of human I kappa B kinase (IKK) complex in yeast - Assessment of its stoichiometry and the role of IKK gamma on the complex activity in the absence of stimulation. J Biol Chem 276, 36320-36326.

2, **The identification of IKKγ by MS**. In the present study, the IKKγ was identified by MS with 5 unique peptides and a 12% protein sequence coverage. In MS protein analysis, a protein can be unambiguously identified by one unique peptide, and additional unique peptides identified from the protein serve as validation of the identification. Based on this, we viewed the identification of IKKγ by MS with 5 unique peptides as high confidence identification. A 12% protein sequence coverage in MS protein identification is not very high, but is acceptable. The number of peptide matching to a protein and sequence coverage

in MS analysis are affected by many factors, such as protein abundance, sample complexity, protein sequence, MS instrument setting, and variations from batch to batch in MS analysis, etc. Therefore, it is not so meaningful to compare the number of peptide identified and sequence coverage for one protein in one analysis with those for another protein in a separate analysis.

3, **Statistic analysis for figure 5B/C**. The data were from three separate, independent sample preparations. Specifically, we prepared 3 treated and 1 control samples for each analysis and repeated the analysis 3 times. So, the values for the treated samples were the averages of 9 separate sample preparations, and the values for the control were the averages of three separate sample preparations. In the calculation for fold change induced by treatment, the value for control sample was set to 1 for each analysis. Therefore, there were no variations for the values (hence error bars) for the controls. The values in Figure B for treated samples were 3.19 ± 0.91 for IFN-α and 3.16 ± 0.46 for IFN-β and in Figure C were 0.76 ± 0.16 for IFN-α and 0.79 ± 0.05 for IFN-β.

Appendix

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Title: Inhibition of Type I Interferon Production via Suppressing IKK-Gamma Expression: A New Strategy for Counteracting Host Antiviral Defense by Influenza A Viruses?

Author: Yimeng Wang, Jianhong Zhou, Chuanmin Ruan, and Yuchun Du

Publication: Journal of Proteome Research

Publisher: American Chemical Society

Date: Jan 1, 2012

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Co-authors agreement:

As the co-authors for the publication (J Proteome Res. 2012;11(1):217-23), we agree to incorporate this publication into Yimeng Wang's dissertation.

Chuanmin Ruan — Date

Jianhong Zhou — 1/7/13 Date

Yuchun Du — 1/7/13 Date

Chapter 2. hnRNP A2/B1 Interacts with the Influenza A Viral Protein NS1 and Inhibits Its Expression and Viral Replication

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Department of Biological Sciences, University of Arkansas, Fayetteville, Arkansas, United States

This chapter has been submitted for journal publication.

Abstract

The NS1 protein of influenza virus is a major virulence factor and contributes significantly to virus pathogenesis. The multifunctional NS1 protein exerts its function through interacting with viral/cellular RNAs and proteins. In this study, we identified heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP A2/B1) as an interacting partner of NS1 protein by a two-dimensional gel electrophoresis-based proteomic approach and mass spectrometry. We verified the interaction between hnRNP A2/B1 and NS1 by reciprocal coimmunoprecipitations and observed that the two proteins colocalize to each other in the nucleus at the early stage of infection by confocal microscopy. Knockdown of hnRNP A2/B1 by small interfering RNA (siRNA) resulted in increased synthesis of NS1 viral RNA and NS1 mRNA in the virus-infected cells. In addition, we demonstrated that hnRNP A2/B1 is associated with NS1 and NS2 mRNAs, and siRNA-mediated knockdown of hnRNP A2/B1 promotes transport of NS1 mRNA from the nucleus to the cytoplasm in the infected cells. Lastly, we showed that siRNA-mediated knockdown of hnRNP A2/B1 leads to enhanced expression of NS1 protein and virus replication. Our results suggest that hnRNP A2/B1 plays an important inhibitory role in the replication of influenza A virus in host cells potentially through inhibiting NS1 gene replication, transcription, and NS1 mRNA nucleocytoplasmic translocation.

Introduction

Influenza A viruses cause contagious respiratory illness and are responsible for more than 200,000 hospitalizations and up to 49,000 deaths each year in the United States (CDC, 2011a). Influenza A viruses belong to the family *Orthomyxoviridae* and harbor an eight segmented, single-stranded, negative-sense RNA genome, which codes for 11 viral proteins (Hale et al., 2008). Different from most other RNA viruses, influenza viruses replicate in the nucleus of the infected cells (Herz et al., 1981). In the nucleus, the negative-sense virion RNAs (vRNAs) from the input viruses are synthesized into full-length complementary RNAs (cRNAs), which in turn serve as templates for the synthesis of more vRNAs. The resulting vRNAs are either used as templates for producing more viral mRNAs or encapsidated into ribonucleoprotein structures to be exported to the cytoplasm for virion assembly at the plasma membrane.

Influenza viral genome segment 8 codes for NS1 protein from unspliced primary mRNA transcript and NS2 protein from spliced mRNA (Lamb and Lai, 1980; Robb et al., 2010). NS1 protein is localized in both the cytoplasm and nucleus and plays multiple roles in viral replication cycle (Hale et al., 2008; Li et al., 1998). In the cytoplasm of infected cells, NS1 antagonizes host interferon (IFN) system through targeting protein kinase R (PKR) (Min et al., 2007; Wang et al., 2000b), interferon regulatory factor 3 (IRF-3) (Mibayashi et al., 2007), and potentially also IKKγ (Wang et al., 2012). In the nucleus, NS1 inhibits pre-mRNA splicing and mRNA nuclear export through targeting a 30-kDa subunit of the cleavage and polyadenylation specificity factor (CPSF)(Das et al., 2008; Krug et al., 2003; Nemeroff et al., 1998), poly (A)-binding protein II (PABII) (Chen et al., 1999), and/or components of the mRNA export machinery (Satterly et al., 2007; Wolff et al., 1998).

After being transcribed, the pre-mRNAs are known to associate with nuclear proteins to form heterogeneous nuclear ribonucleoprotein (hnRNP) complexes, which function to affect the structure or nucleocytoplasmic transport of mRNAs (Dreyfuss et al., 1993). The hnRNP family includes approximately 20 proteins, ranging from hnRNPs A1 to U, and each hnRNP protein contains RNA binding motifs and auxiliary domains for protein-protein or protein-nucleic acid interactions (Krecic and Swanson, 1999; Pinolroma et al., 1988). Multiple influenza viral proteins have been reported to interact with different

hnRNP members, such as hnRNP M, H1 (Jorba et al., 2008) and A1(Mayer et al., 2007), to modulate influenza virus replication in virus-infected cells.

Like other viruses, influenza viruses depend on host cellular components, proteins in particular, to complete most (if not all) steps in the viral proliferation cycle, including viral gene replication/ transcription/translation, intracellular trafficking, and virion assembly. This kind of dependence and the intracellular warfare between influenza viruses and host cells create a vast plethora of interactions between viral components and host cellular components in virus-infected cells. Identification of the host cellular factors that play critical roles in viral replication cycle through interactions with viral factors may provide valuable information for designing novel antiviral therapy. In this study, through a two-dimensional gel electrophoresis (2-DE)-based proteomic method, we identified hnRNP A2/B1 as an interacting partner of the influenza viral protein NS1 and found that hnRNP A2/B1 affects influenza A virus replication via inhibiting NS1 gene replication, transcription, and NS1 mRNA nucleocytoplasmic export.

Materials and Methods

Cell Culture and Virus Infection. Human embryonic kidney (HEK) 293T cells, human lung epithelial A549 cells, and Madin-Darby canine kidney (MDCK) cells (ATCC, Manassas, VA) were cultivated in Dulbecco modified eagle medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT) and 1% penicillin and streptomycin. Influenza A/PR/8/34 H1N1 viruses (ATCC, Manassas, VA) were propagated and titrated in MDCK cells as described previously (Wang et al., 2012).

Plasmid Construction and Cell Transfection. Flag tagged NS1 gene and Flag alone were cloned into pcDNA3.1 as described previously (Wang et al., 2012). For mammalian two hybrid assay, NS1 and hnRNP A2/B1 cDNAs (GenBank accession no: NM_031243) were inserted into *EcoR* I and *Sal* I sites of pM vector with GAL4 DNA binding domain (BD) and pVP16 vector with transcriptional activation domain (AD) (BD Biosciences, San Jose, CA), respectively. hnRNP A2/B1 gene was also cloned into pCruz HA vector (Santa Cruz Biotech, Santa Cruz, CA) by inserting the cDNA into the *Not I* and *Bgl II* sties of the vector. All expression plasmids were verified by DNA sequencing. Cell transfection was performed as described previously (Wang et al., 2012).

Affinity Purification and 2-DE. Human 293T cells transiently transfected with plasmids expressing Flag alone or Flag-NS1 (approximately 1×10^9 each) were harvested and washed twice with cold phosphate-buffered saline (PBS) 48 h after the transfection. The cells were lysed in 5 packed cell pellet volumes of a lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 10 mM NaF, 10 mM β-glycerophosphate, and 1 mM Na_3VO_4) supplemented with protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) by douncing with a glass dounce homogenizer (Kontes Glass Co., Vineland, NJ). After centrifugation at 20,000 g for 15 min at 4 ℃, the pellets were further extracted once with the lysis buffer and sonication. The combined supernatants from the cells that express Flag-NS1 or the control cells that express Flag alone were incubated separately with 200 µl pre-washed anti-Flag M2 resin (Sigma, St. Louis, MO) for 5 h at 4℃. After washing (4 x 1 ml) , the bound proteins were eluted with an elution buffer containing 50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 250 mM Flag peptide (Sigma, St. Louis, MO) and then concentrated by trichloroacetic acid precipitation. The precipitated proteins from the two groups of cells were separately fractionated by two identical 2-DE gels, as described previously (12).

MS Analysis and Database Search. After 2-DE fractionations, the two 2-DE gels that resolved the affinity-purified proteins from control cells (express Flag alone) or the cells that express Flag-NS1 were visually inspected. The protein spots uniquely appearing in the gel that resolved the proteins purified from the cells that express Flag-NS1 (but not in the control gel) were excised, in-gel digested, and the resulting peptides analyzed by mass spectrometry. In-gel digestion, MS analysis, and database search were carried out as described previously (Wang et al., 2012).

Mammalian Two-hybrid Analysis. Mammalian two-hybrid analysis of protein-protein interactions was performed according to our previous protocol (Du et al., 2006b; Wang et al., 2005). Briefly, the coding sequences of NS1 and hnRNP A2/B1 were inserted into the vectors encoding BD and AD (BD Biosciences, San Jose, CA), respectively, and the two constructs were co-transfected into 293T cells with a Gal4 GFP reporter plasmid (2 μg of each plasmid in a 60-mm plate). The negative control was performed by co-transfection of the 293T cells with Gal4 GFP reporter plasmid and the two expression vectors in which the BD and AD were fused with two proteins that are known to not interact. The expression of GFP was detected by Western blotting using an anti-GFP antibody.

Co-immunoprecipitation (IP). Vectors expressing HA-hnRNP A2/B1 or Flag-NS1 were co-transfected into 293T cells (~1×10^8 cells). For control, the vector expressing Flag alone was used to replace the vector that expresses Flag-NS1 in the co-transfection. Forty-eight h after the transfection, cells were lysed and immunoprecipitated with anti-Flag M2 resin as described above. The NS1-associated proteins were detected by Western blotting using an anti-HA antibody (Santa Cruz Biotech, Santa Cruz, CA). In a reciprocal co-IP, cell transfection, IP, and Western blotting were performed as described above except that 1) for control, the vector expressing HA alone was used to replace the vector that expresses HA-hnRNP A2/B1 in the co-transfection; 2) the IP was performed using immobilized anti-HA antibody (Santa Cruz Biotech, Santa Cruz, CA) (or Protein A-Sepharose beads (Sigma, St. Louis, MO) pre-incubated with anti-HA antibody overnight); 3) the hnRNP A2/B1-associated proteins were detected by Western blotting using an anti-Flag antibody (Sigma, St. Louis, MO).

Immunofluorescence Staining and Confocal Microscopy. The immunofluorescence staining and image acquisition were performed as described in our previous papers (Zhou et al., 2012a, b). Specifically, A549 cells at 90% confluency on a coverslip were infected with A/PR/8/34 viruses at an MOI

of 1. At 6 hpi, cells were washed with PBS, fixed by 4% formaldehyde in PBS for 15 min, and permeabilized by 0.2% Triton X-100 in PBS for 10 min. The cells were then washed and blocked with 10% normal goat serum in PBS for 1 h, followed by incubation with mouse anti-NS1 antibody (1:1,000) in 5% normal goat serum in PBS overnight at 4℃. After washing, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotech, Santa Cruz, CA) for 1 h at room temperature. The cells were then washed and incubated with rabbit anti-hnRNP A2/B1 antibody (1:2000), followed by incubation with rhodamine-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotech, Santa Cruz, CA). The images were acquired by a NIKON Eclipse 90i confocal fluorescence microscope (Nikon, Tokyo, Japan).

RNA Interference (RNAi). Three siRNA oligos specifically targeting hnRNP A2/B1 [5'-AAGCUUUGAAACCACAGAAGA-3' (Patry et al., 2003), 5'-AAAGAUCAAGAGGAUUUGGUU-3' and 5'-GGAACAGUUCCGUAAGCUC-3' (Iwanaga et al., 2005)] were co-transfected into A549 cells using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) as described previously (Wang et al., 2012).

Quantitative Real-Time PCR (qRT-PCR). The analysis was performed according to our previous protocol (Liu et al., 2012; Wang et al., 2012) with the following specifications. A549 cells treated with siRNAs oligos targeting hnRNP A2/B1 or nontargeting siRNAs (negative control) were infected with the A/PR/8/34 viruses. The cells were harvested and total RNA extracted using an RNeasy Mini kit (Qiagen, Valencia, CA) at appropriate hpi. Viral RNAs (vRNAs) and mRNA were reverse-transcribed (Improm-II Reverse Transcriptase kit; Promega, Madison, WI) with the Uni-12 primers (Hoffmann et al., 2001) and the oligo (dT) primers (Promega, Madison, WI), respectively. The levels of cDNAs reverse-transcribed from vRNAs and mRNAs were determined by qRT-PCR with primers specific for the NS1 gene (Forward: 5'-GACCGGCTGGAGACTCTAAT-3' and reverse: 5'-CTGGAAGAGAAGGCAATGGT-3'). The concentrations of mRNAs were determined using actin mRNA as an internal control and calculated using the traditional $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001b). The relative levels of vRNA were determined based on a standard curve generated by serial dilutions of the NS1 expression plasmids used in cell transfection (Shin et al., 2007). Each time, we prepared three independent samples treated with siRNAs oligos targeting hnRNP A2/B1 and one sample treated with nontargeting siRNAs for the calculation of fold change induced by hnRNP A2/B1 knockdown. We repeated the analysis three times.

The Analysis of the Interaction between NS1/2 mRNAs and hnRNP A2/B1 Proteins. The vectors expressing HA-hnRNP A2/B1 or HA tag alone as control were transiently transfected into 293T cells. Forty-eight h after the transfection, the cells were infected with A/PR/8/34 viruses at an MOI of 3. Ten h after the infection, the cells were harvested and lysed for IP with immobilized anti-HA antibody as described above. The immunoprecipitated complexes were treated with 150 μg/ml proteinase K for 90 min at 37 ℃. After extraction of the total RNA fro m the immunoprecipitated complexes, oligo (dT) primer was used to reverse-transcribe mRNAs into cDNAs, and the following primers were then used to PCR-amplify DNA (25 cycles) from the reverse-transcribed cDNAs: NS1 primers (Forward: 5'-ATGGATCCAAACACTGTGTC-3' and reverse: 5'-TCAAACTTCTGACCTAATTGTTCC-3'); GAPDH primers (Forward: 5'-CGGAGTCAACGGATTTGGCC-3' and reverse: 5'-GTGGCAGAGATGGCATGGAC-3'). The amplified DNAs were detected with a 1.3% agarose gel and further confirmed by DNA sequencing.

Subcellular Fractionation. Nuclear and cytoplasmic fractionations were performed according to a published protocol (Wang et al., 2006b). Briefly, cells were harvested and swelled on ice in a hypertonic buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, and 3 mM $MgCl_2$) for 3 min, followed by centrifugation at 1,500 *g* for 3 min. The cell pellet was lysed in four packed cell pellet volumes of buffer A [10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 10% (v/v) glycerol, 0.5% (v/v) NP-40, 0.5 mM DTT, and 100 U/ml RNasin or 1x protease inhibitor]. After centrifugation at 4,500 *g* for 3 min at 4 ℃, supernatant was saved as cytoplasmic fraction. The pellet was resuspended in buffer A supplemented with detergents [3.3% (w/v) sodium deoxycholate and 6.6% (v/v) Tween 20] and incubated on ice for 5 min. The insoluble materials were designated as nuclei and collected by centrifugation at 10,000 *g* for 5 min and washed with buffer A once to remove the possible residue of cytoplasmic fraction. The integrity of the isolated nuclei was examined by microscopy after staining with trypan blue. The total RNAs were extracted from the cytoplasmic and nuclear fractions and the isolated RNAs were treated with RNase-free DNase I (Invitrogen, Carlsbad, CA) at room temperature for 15 min, followed by incubation at 37 ℃ for 30 min to remove genomic DNAs. Three micrograms of the resulting RNAs were reverse transcribed into cDNAs as described above. 18S-rRNA was used as an internal control in the qRT-PCR (Zhu and Altmann, 2005).

Plaque Assay. Plaque assay was carried out as described previously (Wang et al., 2012).

Statistical Analysis. Statistical analysis was performed using an independent-sample T test by Systat 13 (SPSS 13).

Results

Identification of hnRNP A2/B1 as an Influenza Virus NS1 Interacting Protein. We used a 2-DE-based proteomic method to identify the proteins that were associated with influenza viral protein NS1 (Zhou et al., 2012a). Two populations of 293T cells were transiently transfected with plasmids that express Flag alone (control) and Flag-NS1, respectively. After affinity purification of the whole cell lysates from the two populations of cells, the bound proteins eluted from the affinity beads were fractionated with two identical 2-DE gels. The protein spots uniquely appearing in the gel that resolved the proteins purified from the cells that expressed Flag-NS1, were excised, in-gel digested, and the resulting peptides analyzed by MS. One of the proteins that were identified by MS was hnRNP A2/B1, which is produced by alternative splicing from a single gene and has been shown to play important roles in RNA processing like RNA transport, translation, stabilization, splicing and trafficking (Kamma et al., 1999). hnRNP A2/B1 was identified with high confidence by MS with 18 unique peptides to the proteins and a 58% sequence coverage (Supplemental Fig. S1). Several other proteins were also identified to potentially interact with NS1, such as RUVBL1 and RUVBL2, which will be reported in a separate paper.

Validation of the Interaction between hnRNP A2/B1 and NS1 by Co-IPs and Mammalian Two-hybrid Analysis. We used co-IPs to validate the interaction between NS1 and hnRNP A2/B1. As shown in Fig. 1A, immobilized anti-Flag antibodies precipitated large amount of HA-hnRNP A2/B1 from the cells co-transfected with the plasmids that express Flag-NS1 and HA-hnRNP A2/B1, but precipitated much less amount of the proteins from the cells co-transfected with the plasmids that express Flag alone and HA-hnRNP A2/B1 (upper row). Similarly, in a reciprocal IP, immobilized anti-HA antibodies precipitated large amount of Flag-NS1 from the cells co-transfected with the plasmids that express Flag-NS1 and HA-hnRNP A2/B1, but failed to do so from the cells co-transfected with the plasmids that express HA tag alone and Flag-NS1 (lower row). These results strongly suggest that NS1 is specifically associated with hnRNP A2/B1. hnRNP A2/B1 is an RNA binding protein (Han et al., 2010b). In order to test whether the NS1-hnRNP A2/B1 interaction is mediated by RNAs, we used RNase (Sigma, St Louis, MO) to treat the cell lysates before the IPs. The results demonstrated that the RNase treatment did not change the association of NS1 with hnRNP A2/B1 in the reciprocal co-IPs (data not shown), suggesting the interaction between proteins of NS1 and hnRNP A2/B1 is not mediated by RNAs. To further test whether

NS1 and hnRNP A2/B1 interact *in vivo* in cells, we performed mammalian two-hybrid assays. In this assay, the interaction between hnRNP A2/B1 and NS1 brings the association of Gal4 BD and AD, which in turn initiates the transcription of a reporter gene GFP (Du et al., 2006a). Co-transfection of 293T cells with the Gal4 GFP reporter plasmid and the constructs encoding fusion proteins of BD-NS1 and AD-hnRNP A2/B1 resulted in higher expression of GFP than the negative control, in which BD and AD were fused with two proteins that are known to not interact (Fig. 1B, compare the right lane with the left lane). These results suggest that NS1 interacts with hnRNP A2/B1 in human cells *in vivo.*

hnRNP A2/B1 Colocalizes with NS1 in Human Lung A549 Cells. We then performed immunostaining and confocal microscopy analysis using human lung A549 cells to determine whether viral NS1 and hnRNP A2/B1 physically colocalize to each other in cells. A549 cells were infected with A/PR/8/34 viruses and fixed at 6 hpi, followed by incubation with anti-NS1 and anti-hnRNP A2/B1 antibodies and appropriate fluorescence-labeled secondary antibodies. Confocal microscopy analysis of the stained cells demonstrated that hnRNP A2/B1 was predominantly located in the nucleus; while the majority of NS1 protein in the infected cells was located in the nucleus, a small portion of it was located in the cytoplasm at 6 hpi, which was also observed by other research groups (Li et al., 1998; Wolff et al., 1998). Merge of the hnRNP A2/B1- and NS1-stained images strongly suggest that hnRNP A2/B1 and NS1 colocalize to each other in the nucleus of the infected cells at 6 hpi (Fig. 2).

hnRNP A2/B1 Suppresses NS1 Protein Expression in the Virus-infected Cells. To determine the potential roles of the interaction between hnRNP A2/B1 and NS1, we silenced the expression of endogenous hnRNP A2/B1 by siRNAs in A549 cells and then examined the effect of the hnRNP A2/B1 silencing on NS1 protein expression in the virus-infected cells. The results demonstrated that when the expression of hnRNP A2/B1 was silenced by siRNAs (Fig. 3; upper row), viral NS1 protein expression increased (Fig. 3; middle row), suggesting that hnRNP A2/B1 plays an inhibitory role in the protein expression of NS1 in the virus-infected cells.

hnRNP A2/B1 Inhibits NS1 vRNA Replication and mRNA Transcription in the Virus-infected Cells. To elucidate the mechanism by which hnRNP A2/B1 inhibits viral NS1 protein expression in the virus-infected cells, we examined the effect of knockdown of hnRNP A2/B1 expression on NS1 gene replication (vRNA) and transcription (mRNA) in the virus-infected A549 cells using qRT-PCR. We found knockdown

of hnRNP A2/B1 increased NS1 vRNA levels by 1.4-fold (1.4 ± 0.05) and 2.1-fold (2.1 ± 0.1) (Fig. 4A) and NS1 mRNA levels by 1.2-fold (1.2 ± 0.04) and 1.7-fold (1.7 ± 0.07) at 24 hpi and 36 hpi (Fig. 4B), respectively. These results suggest that hnRNP A2/B1 inhibits both viral NS1 gene replication and transcription in the virus-infected cells.

hnRNP A2/B1 Binds to NS1 and NS2 mRNAs, Does Not Affect NS mRNA Splicing, but Inhibits NS1 mRNA Nuclear Export. hnRNP proteins are known to regulate splicing (Caputi et al., 1999; Han et al., 2010b) and nuclear export of mRNAs (Reed and Hurt, 2002; Schneider and Wolff, 2009). Thus, we tested whether hnRNP A2/B1 plays a role in regulating the splicing and nuclear export of NS1 mRNA in the virus-infected cells. For this purpose, we first examined whether hnRNP A2/B1 proteins are associated with NS1 or NS2 mRNAs. 293T cells were transfected with the plasmids that express HA-hnRNP A2/B1 or HA tag alone for control, and then infected with A/PR/8/34 viruses at an MOI of 3. Ten h after the infection, the cells were harvested for IPs with immobilized anti-HA antibodies to pull down the complexes that were associated with hnRNP A2/B1. After the IPs, proteinase K was used to release the hnRNP A2/B1-associated RNAs from the precipitated complexes, and total RNA was purified, followed by reverse-transcription with oligo (dT) primer to reverse-transcribe the mRNAs [leaving vRNA and cRNA un-affected (Robb et al., 2010)], and PCR amplification of the reverse-transcribed cDNAs. Because the primers we used in the PCR amplification were specific for the cDNAs of both NS1 and NS2 mRNAs (Fig. 5A), the amounts (or presence/absence) of both NS1 and NS2 mRNAs in the precipitated complexes could be detected simultaneously. As shown in Fig. 5B, transfection of the cells with the plasmid that expresses HA-hnRNP A2/B1 did not affect the transcription of either NS1 or NS2 (left panel). The immobilized anti-HA antibody precipitated substantially more NS1 and NS2 mRNAs from the cells that express HA-hnRNP A2/B1 than from the control cells. These results suggest that both NS1 and NS2 mRNAs are specifically associated with hnRNP A2/B1 proteins in the virus-infected cells.

After confirming that NS1 and NS2 mRNAs are associated with hnRNP A2/B1 proteins in the virus-infected cells, we examined whether hnRNP A2/B1 proteins affect NS pre-mRNA splicing. To test this possibility, we compared the ratios of NS2 to NS1 mRNAs in the infected cells with a normal level of endogenous hnRNP A2/B1 to those with a depleted hnRNP A2/B1 using reverse transcriptions as

described above. The results demonstrated knockdown of hnRNP A2/B1 by siRNAs does not affect the NS1 and NS2 splicing in the virus-infected A549 cells (data not shown).

We then examined the potential role of hnRNP A2/B1 in nucleocytoplasmic translocation of NS1 mRNAs. We used a differential-centrifugation-based method (Wang et al., 2006b) to fractionate cell lysates into cytoplasmic and nuclear parts. Western blot analysis using marker proteins of the cytoplasm and nucleus demonstrated that there were no noticeable cross-contaminations in our cytoplasmic and nuclear fractionations (Fig. 6A). We then transfected the A549 cells with the siRNAs oligos targeting hnRNP A2/B1 or with a negative control siRNA, and infected the transfected cells with A/PR/8/34 viruses. The transfected and infected cells were harvested and fractionated, and NS1 mRNAs in the cytoplasmic and nuclear factions quantified by qRT-PCR. The results demonstrated that when hnRNP A2/B1 expression was silenced by siRNAs, the ratios of the cytosolic to nuclear NS1 mRNAs at 6 and 10 hpi increased (Fig. 6B). These results suggest that hnRNP A2/B1 proteins inhibit the nuclear export of viral NS1 mRNAs in the virus-infected cells.

hnRNP A2/B1 Inhibits Influenza Virus Replication. Since hnRNP A2/B1 affects influenza viral NS1 gene replication, transcription (Fig. 4), expression (Fig. 3) and NS1 mRNA nuclear export (Fig. 6), we speculated hnRNP A2/B1 may affect virus replication. Indeed, when the expression of hnRNP A2/B1 was silenced by siRNAs in the A549 cells, the virus titers increased (Fig. 7), suggesting that hnRNP A2/B1 inhibits influenza virus replication in the infected cells.

Discussion

Although NS1 protein is not part of the influenza virion, it is expressed at very high level in the virus-infected cells (Lazarowitz et al., 1971; Liu et al., 2012). NS1 is a multifunctional protein (Hale et al., 2008). One major function of NS1 is to inhibit host immune responses through suppressing the induction of type I IFNs and IFN-mediated proteins (Krug et al., 2003). In addition to its role in inhibiting host immune responses, NS1 also plays important roles in other aspects in the virus-infected cells (Hale et al., 2008). For example, NS1 has been shown to affect mRNA splicing and nuclear export (Garaigorta and Ortin, 2007), protein synthesis (de la Luna et al., 1995; Enami et al., 1994), and cell apoptosis (Ehrhardt et al., 2007a). Most, if not all, of the reported functions of NS1 are realized through the physical interactions with cellular/viral proteins or RNAs (Hale et al., 2008). In the present study, through a 2-DE-based proteomic approach, we found that NS1 is associated with hnRNP A2/B1, a member of a large family of proteins that are highly divergent in structure and function (Han et al., 2010b; Shyu and Wilkinson, 2000). hnRNP A2 and B1 proteins are produced by alternative splicing of a single gene with the difference of a 12-amino-acid insertion in N-terminal RNA-binding motif in B1 (Burd et al., 1989; Kozu et al., 1995). hnRNP A2/B1 has been shown to regulate RNA alternative splicing (Bilodeau et al., 2001; Mayeda et al., 1994), RNA trafficking (Munro et al., 1999; Pinol-Roma, 1992; Shan et al., 2003), and telomere maintenance (Ford et al., 2002).

Most cellular mRNAs are transported from the nucleus to the cytoplasm through the mRNA export receptors Tap/NXF1 that interact with both mRNAs and components of the nuclear pore complex to direct mRNAs through the nuclear pore complex (Stutz and Izaurralde, 2003). The interaction between mRNA and Tap/NXF1 is mediated by adapter proteins such as Aly (Strasser and Hurt, 2000; Stutz et al., 2000), which is recruited to the mRNA during splicing by export factor UAP56 (Gatfield et al., 2001; Luo et al., 2001). Several lines of evidence suggest that viral mRNA export in the virus-infected host cells is mediated by the Tap/NXF1 export pathway (Hao et al., 2008; Wang et al., 2008), and viruses have evolved strategies to introduce viral mRNAs into the Tap/NXF1 export system through recruiting cellular export factors, such as export factors Aly and UAP56 (Chen et al., 2005; Koffa et al., 2001; Lischka et al., 2006). In the case of influenza viral mRNA nuclear export, it seems that NS1 protein plays an important role in directing viral mRNAs into the nuclear mRNA export machinery via recruiting essential export

factors (Schneider and Wolff, 2009). In supporting this notion, it has been shown that NS1 proteins from influenza A and B viruses interact with mRNA export factors Tap/NXF1 and UAP56, respectively (Satterly et al., 2007; Schneider et al., 2009), to facilitate nuclear export of viral mRNAs (Schneider and Wolff, 2009). In the present study, we found that nuclear protein hnRNP A2/B1 interacts with both NS1 proteins (Fig. 1) and NS1 mRNAs (Fig. 5), and inhibits the nuclear export of NS1 mRNAs in the virus-infected cells (Fig. 6). A potential mechanism underlying the inhibitory effect of hnRNP A2/B1 on viral NS1 mRNA nuclear export is that the interaction of hnRNP A2/B1 with NS1 protein interferes NS1 protein's ability to recruit mRNA export factors such as Tap/NXF1 or other factors to the NS1 mRNAs (Satterly et al., 2007; Schneider and Wolff, 2009). It has been shown that influenza viral NS1 protein is associated with influenza viral mRNAs of NA, M1, and PB1 genes (Wang et al., 2008). If the hypothesis described above is correct, it is highly likely that hnRNP A2/B1 may also play an important role in regulating the nuclear export of other influenza viral mRNAs including (but not limited to) those of NA, M1, and PB1 (Wang et al., 2008).

We also found in the present study that knockdown of hnRNP A2/B1 expression by siRNA resulted in increased synthesis of viral NS1 vRNA and mRNA (Fig. 4), suggesting that endogenous hnRNP A2/B1 inhibits NS1 gene replication and transcription in the virus-infected cells. Because the viral mRNAs exported from the nucleus are translated into viral proteins in the cytoplasm, and some of the resulting viral proteins are transported back to the nucleus to facilitate the new viral particles assembly with vRNAs, it is highly likely that the observed increases in viral RNA replication and transcription of NS1 gene (Fig. 4) were a consequence of the increased nuclear export of NS1 mRNA (Fig. 6) when the expression of hnRNP A2/B1 was silenced (Fig. 3). Alternatively, since hnRNP proteins are formed by the association of nuclear proteins and pre-mRNAs that are transcribed by RNA polymerase II, pre-mRNAs may bridge the association between hnRNP proteins and RNA polymerase II, which is also required for influenza viral RNA transcription. So it is also theoretically possible that the NS1-hnRNP A2/B1 complex affects the catalytic activity of RNA polymerase II with consequence of affecting both viral RNA transcription and nuclear export. This interpretation is consistent with the results obtained from the RNA polymerase inhibitor-based studies (Amorim et al., 2007). Future studies will be needed to examine how hnRNP A2/B1 proteins inhibit viral mRNA nuclear export and whether the protein directly affects polymerase-

mediated transcription of NS1 [and potentially other viral genes (Wang et al., 2008)] in the virus-infected cells.

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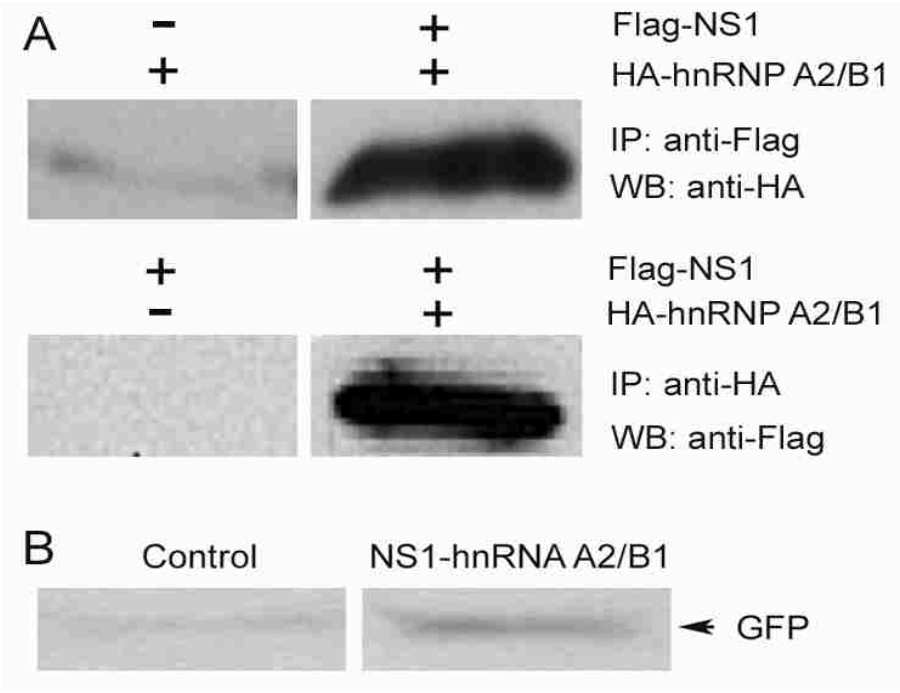

Fig. 1. Validation of the interaction between hnRNP A2/B1 and NS1. A, co-IPs. Cell lysates from the cells expressing Flag-NS1 and HA-hnRNP A2/B1, or the cells expressing Flag alone and HA-hnRNP A2/B1 (control), were immunoprecipitated with anti-Flag M2 resin, and the immunoprecipitated proteins were probed with anti-HA antibody in Western blotting. As shown, the anti-Flag antibody precipitated substantially more HA-hnRNP A2/B1 from the cells that express Flag-NS1 and HA-hnRNP A2/B1 than from the control cells (top panel). In a reciprocal co-IP, cell lysates from the cells expressing Flag-NS1 and HA-hnRNP A2/B1, or the cells expressing HA alone and Flag-NS1 (control), were precipitated with immobilized anti-HA antibody, and the immunoprecipitated proteins were probed with anti-Flag antibody in Western blotting. As shown, Flag-NS1 was immunoprecipitated with HA-hnRNP A2/B1 by immobilized anti-HA antibody, but not from the control cells (bottom panel). B, mammalian two-hybrid analysis. 293T cells were co-transfected with plasmids expressing BD-NS1, AD-hnRNP A2/B1, and a Gal4 GFP reporter, and the induction of reporter GFP expression was detected by Western blotting with an anti-GFP antibody. The negative control was performed by co-transfection of 293T cells with Gal4 GFP reporter plasmids and constructs in which the BD and AD were fused with two proteins that are known to not interact. The induction of Gal4 GFP reporter by the expression of BD-NS1 and AD-hnRNP A2/B1 fusion proteins is shown.

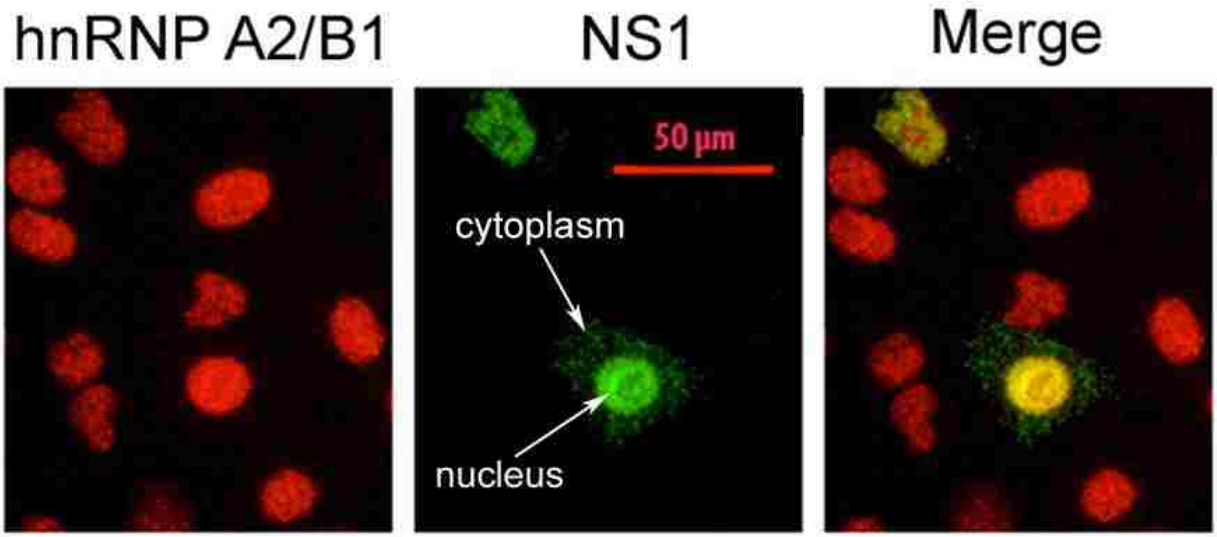

Fig. 2. Colocalization of hnRNP A2/B1 with NS1 in the nucleus of the virus-infected cells. A549 cells were infected with A/PR/8/34 viruses at an MOI of 1, stained with antibodies directed against hnRNP A2/B1 and NS1 at 6 hpi, and the images were acquired by using a confocal laser-scanning microscope. Merged image of the hnRNP A2/B1-staining (in red) and NS1-staining (in green) shows the overlap of hnRNP A2/B1 and NS1 in the nucleus of the infected cells at 6 hpi.

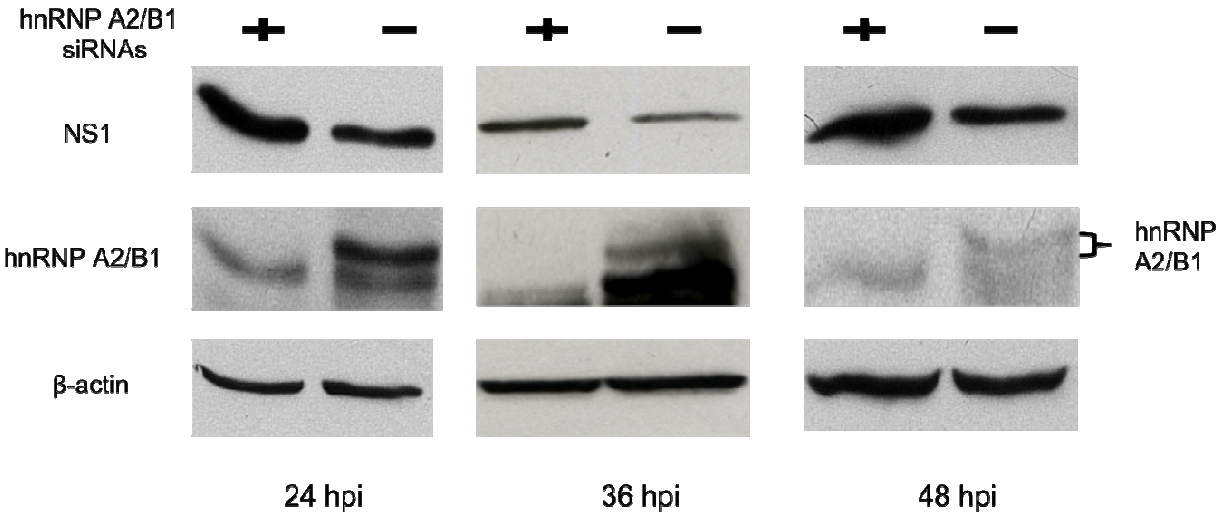

Fig. 3. Silencing of endogenous hnRNP A2/B1 enhances NS1 protein expression. A549 cells were transfected with a randomized siRNA sequence (control) or a siRNA sequence targeting hnRNP A2/B1. Forty-eight h after the transfection, the cells were infected with A/PR/8/34 viruses at an MOI of 0.5, and harvested at the indicated times for examination of the expression of NS1 and hnRNP A2/B1 by Western blotting. Actin was used as a loading control.

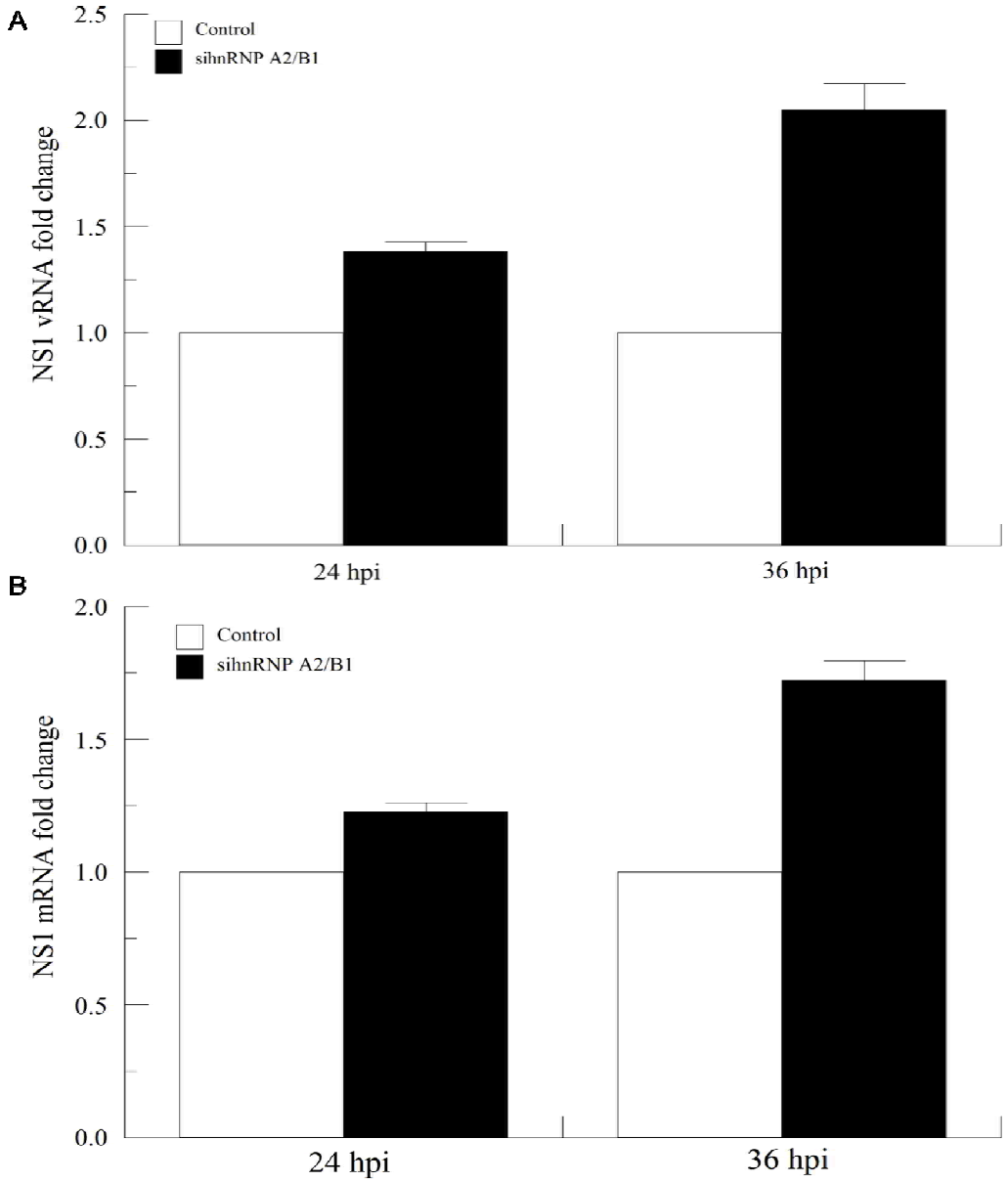

Fig. 4. hnRNP A2/B1 inhibits both replication and transcription of NS1 gene. A549 cells were transfected with a randomized siRNA sequence (control) or a siRNA sequence targeting hnRNP A2/B1. Forty-eight h after the transfection, the cells were infected with A/PR/8/34 viruses at an MOI of 0.5 and harvested at 24 and 36 hpi for examination of NS1 gene replication (A) and transcription (B) by qRT-PCR. Values are the means ± S.E. of at least three separate sample preparations. The values for controls in both (A) and (B) were set to 1. The values for hnRNP A2/B1-silenced samples in (A) were 1.4 (1.4 ± 0.05) and 2.1 (2.1 ± 0.1), and in (B) were 1.2 (1.2 ± 0.04) and 1.7 (1.7 ± 0.07) at 24 hpi and 36 hpi, respectively.

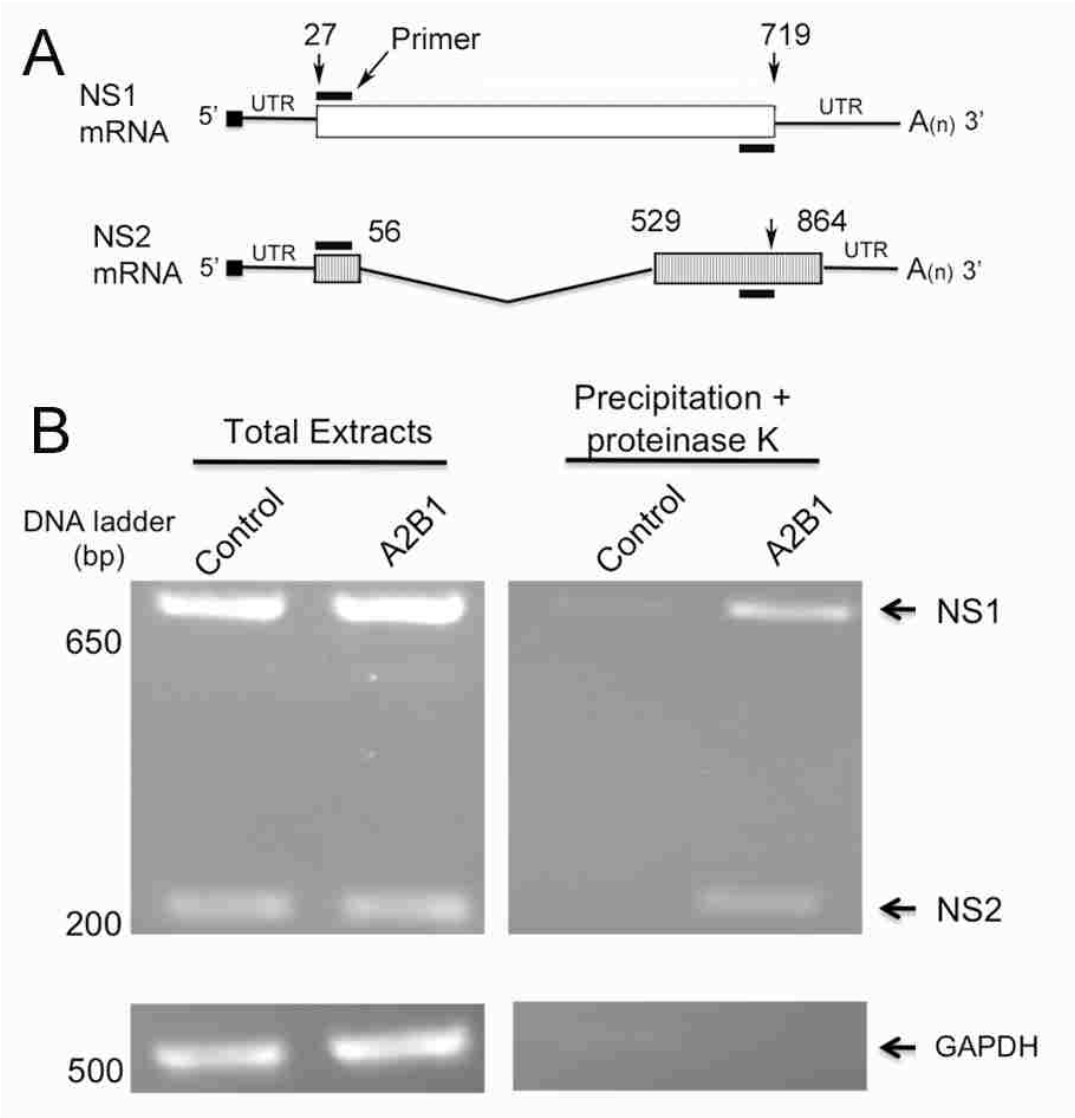

Fig. 5. hnRNP A2/B1 proteins are associated with NS1 and NS2 mRNAs. A, schematic representation of NS1 mRNA, NS2 mRNA, and the positions of the primers used to amplify NS1 and NS2 mRNAs. The coding regions of NS1 and NS2 mRNAs are shown as white and hatched boxes, respectively. The numbers above the coding regions indicate the start and end nucleotide positions in the NS1 mRNA and NS2 mRNA. The NS2 mRNA is alternatively spliced from NS1 mRNA, and the V-shaped line denotes the region that is removed in splicing. Using the primers (black bar) indicated in the diagram, the amplicon size for NS1 and NS2 was calculated to be 693 and 221 bps, respectively. B, hnRNP A2/B1 proteins are associated with NS1 and NS2 mRNAs. 293T cells transiently transfected with the plasmids that express

HA-hnRNP A2/B1 or HA tag alone (control) were infected with A/PR/8/34 viruses at an MOI of 3. At 10 hpi, the cells were harvested, lysed, and the resulting whole cell lysates immunoprecipitated with immobilized anti-HA antibody. The immunoprecipitated RNAs were released from the complexes by incubating with proteinase K, reverse-transcribed, PCR-amplified with the primers indicated in (A), and the resulting DNAs examined by a 1.3% agarose gel. GAPDH was used as an internal reference.

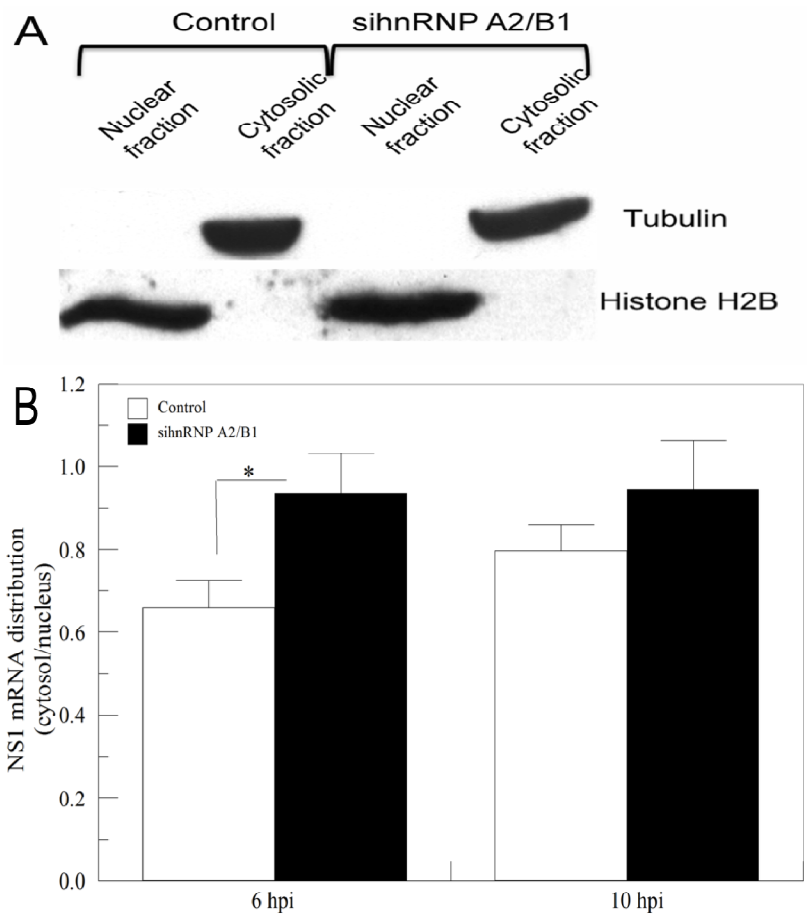

Fig. 6. hnRNP A2/B1 inhibits NS1 mRNA nuclear export. A, cytoplasmic and nuclear fractionation. A549 cells transfected with a randomized siRNA sequence (control) or a siRNA sequence targeting hnRNP A2/B1 were lysed and fractionated into nuclear and cytoplasmic parts, and the purity of each part was examined by Western blotting using antibodies against the nuclear and cytoplasmic markers (histone H2B and tubulin, respectively). B, hnRNP A2/B1 inhibits NS1 mRNA nucleocytoplasmic translocation. A549 cells transfected with randomized siRNA sequence (control) or siRNA sequence targeting hnRNP A2/B1 were infected with A/PR/8/34 viruses at an MOI of 1. The cells were harvested at the indicated times, fractionated into the nuclear and cytoplasmic parts, and the NS1 mRNA in each part was quantified by qRT-PCR. 18s-rRNA was used as an internal control. Values are the means ± S.E. of three separate sample preparations. * denotes $p < 0.05$.

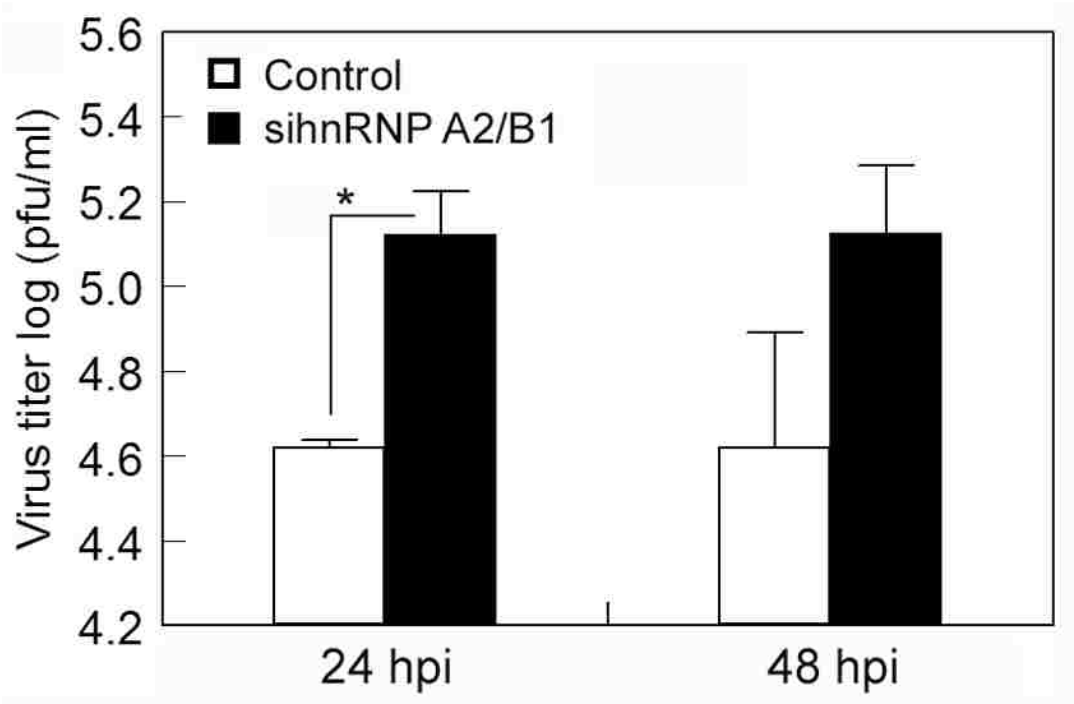

Fig. 7. Silencing of endogenous hnRNP A2/B1 enhances virus replication. A549 cells transfected with a randomized siRNA sequence (control) or a siRNA sequence targeting hnRNP A2/B1 were infected by A/PR/8/34 viruses at an MOI of 0.5. The supernatants were harvested at the indicated times for examinations of virus titers. Values are the means ± S.E. of three separate sample preparations. * denotes $p < 0.05$.

MEKTLETVPLERKKREKEQFRKLFIGGLSFETTEESLRNYYEQWGKLTDCVVMRDPASKRSRGFGFVT
FSSMAEVDAAMAARPHSIDGRVVEPKRAVAREESGKPGAHVTVKKLFVGGIKEDTEEHHLRDYFEEYGKI
DTIEIITDRQSGKKRGFGFVTFDDHDPVDKIVLQKYHTINGHNAEVRKALSRQEMQEVQSSRSGRGGNFG
FGDSRGGGGNFGPGPGSNFRGGSDGYGSGRGFGDGYNGYGGGPGGGNFGGSPGYGGGRGGYGGGGPGYGN
QGGGYGGGYDNYGGGNYGSGNYNDFGNYNQQPSNYGPMKSGNFGGSRNMGGPYGGGNYGPGGSGGSGGYG
GRSRY

Supplementary Figure S1. Identification of hnRNP A2/B1 by liquid chromatography tandem mass spectrometry (LC-MS/MS). hnRNP A2/B1 was identified by LC-MS/MS with 18 unique peptides (highlighted) and a 58% sequence coverage.

Chapter 3. Ruvb-like Proteins Interact with Influenza A Virus Protein NS1 and Affect Apoptosis of the Virus-infected Cells in the Absence of Interferons

Abstract

RuvB-like proteins 1 and 2 (RUVBL1 and RUVBL2) were identified as the binding partners of NS1 through a 2-DE based proteomic approach and mass spectrometry. In order to explore the biological functions of the interactions, we examined the expression of RUVBL1 and RUVBL2 in influenza virus-infected cells. Infection of IFN-competent A549 cells or IFN-deficient Vero cells with wild-type (WT) or NS1-deleted (delNS1) influenza A/PR/8/34 (H1N1) viruses reduced the expression of RUVBL1 and RUVBL2 in Vero cells, but only RUVBL2 in A549 cells. Infection of Vero cells with both kinds of viruses induced earlier expression reduction of RUVBL2 than that of RUVBL1, and delNS1 virus infection led to more pronounced expression reduction of RUVBL2 and cell apoptosis (evidenced by increased PARP cleavage and caspase 3/7 activities) than WT virus. These results suggest that (1) RUVBL2 is affected by virus infection prior to RUVBL1, and (2) the interaction between NS1 and RUVBL2 increases RUVBL2 level by inhibiting virus-induced RUVBL2 reduction during virus infection. We then showed that knockdown of endogenous RUVBL2 in Vero cells by siRNAs induced cell apoptosis, suggesting that virus-induced expression reduction of RUVBL2 may promote apoptosis of the virus-infected host cells. To ensure RUVBL2 plays a significant role in regulating cell apoptosis, we overexpressed RUVBL2 in Vero cells followed by virus infection and found that overexpression of RUVBL2 resulted in increased cell resistance to virus-induced cell apoptosis. However, we found that the virus reduced RUVBL2 expression in IFN-competent A549 cells but not affect apoptosis, suggesting IFNs play a critical role in the RUVBL2-induced apoptosis. To confirm the role of IFNs, we pre-treated the Vero cells with IFN-alpha and infected cells with viruses. The results were the same as the observation in A549 cells, suggesting that IFNs can block the apoptosis induced by the virus-induced RUVBL2 reduction. Taken together, our data suggest that influenza viruses reduce RUVBL2 expression to induce apoptosis of the virus-infected cells in the absence of IFNs and NS1 down-regulates apoptosis potentially through interacting with RUVBL2 to raise its level, which is a novel mechanism for NS1 to ensure efficient virus replication.

Introduction

RUVBL1 and RUVBL2 are putative ATPases and belong to the family of AAA+ (ATPase associated with diverse cellular activities) (Neuwald et al., 1999). RUVBL1 and RUVBL2 proteins consist of 456 and 463 amino acids, respectively, and share 43% identity (Gorynia et al., 2008). RUVBL1 can form a hexamer, which can further form a dodecamer with RUVBL2. These two proteins were initially identified by different methods and therefore have different names: TIP49 and TIP48 (Wood et al., 2000), TIP49a and TIP49b (Kanemaki et al., 1999), Pontin52 and Reptin52 (Bauer et al., 1998) and Rvb1 and Rvb2 (Jonsson et al., 2001). RUVBL1 and RUVBL2 are relatively conserved in evolution, suggesting that they mediate important cellular functions. The first role of RUVBL proteins is to couple the energy of ATP hydrolysis to unwind the DNA double helix, which is important for any processes requiring single-stranded DNA, such as DNA replication, transcription, and DNA repair. RUVBL1 contains two ATP binding sites by which hydrogen bonds between complementary base pairs can be destabilized (Patel and Picha, 2000). The second role is involved in chromatin remodeling. RUVBL proteins have been found to regulate the chromatin structure to promote access of proteins to DNA (Gorynia et al., 2008). They are also found to assist the chromatin remodeling by catalyzing ATP-dependent replacement of H2A-H2B histone polymers in nucleosomes (Jin et al., 2005). The third role is transcription regulation. RNA polymerase II holoenzyme complex and many transcription factors are found to interact with RUVBL1 and RUVBL2 for transcription regulation of many genes, like β-catenin (Bauer et al., 2000), c-Myc (Wood et al., 2000), and ATF2 [RUVBL2 only, (Cho et al., 2001)]. RUVBL1 and RUVBL2 regulate T-cell factor (TCF)-mediated transcription through β-catenin in Wnt signal pathway (Bauer et al., 2000). RUVBL proteins are also found to be related to cancer development through affecting the activity of transcription factors, such as β-catenin and c-Myc (Cole, 1986). In addition, RUVBL proteins can also regulate cell apoptosis through Myc and E2F1. Lastly, RUVBL proteins contribute to the assembly and maturation of small nucleolar ribonucleoprotein (snoRNP) in the nucleoplasm. RUVBL2 is found to be present in the precursor complex, while RUVBL1 is associated with the maturing snoRNP complex (Watkins et al., 2004b).

In this present study, we identified RUVBL2 as the interacting partner of NS1 and demonstrated that influenza viruses reduced RUVBL2 expression to induce cell apoptosis in the absence of IFNs, while NS1 down-regulated cell apoptosis potentially through interacting with RUVBL2 to raise its level.

Materials and Methods

Cell Culture and Virus Infection. A549 cells, 293T cells and African green monkey kidney Vero cells were purchased from ATCC (Manassas, VA) and cultivated in DMEM medium supplemented with 10% FBS and 1% penicillin and streptomycin. NS1-deleted (delNS1) influenza A/PR/8/34 H1N1 virus was kindly provided by Adolfo Garcia-Sastre (Mount Sinai School of Medicine, NY), propagated and titrated in Vero cells as described previously (Wang et al., 2012). Wide-type (WT) influenza A/PR/8/34 H1N1 virus was cultured and titrated as described in Chapter 1.

Two-dimensional Gel Electrophoresis (2-DE) and MS Analysis. As described in Chapter 2, we used 2-DE based proteomic approach and mass spectrometry to identify cellular proteins that interact with NS1. RUVBL1 or RUVBL2 were among of those identified cellular proteins.

Mammalian Two-hybrid Analysis and Reciprocal Co-IPs. RUVBL1 gene (GenBank accession no. NM_003707.2) and RUVBL2 gene (GenBank access no. CR533507) was amplified from a human genome library and inserted into an AD contained pVP16 vector for mammalian two-hybrid assay as described in Chapter 2. The cDNAs of RUVBL1 and RUVBL2 were also inserted into HA tagged pCruz vector for reciprocal co-IPs as described in Chapter 2.

RNA interference (RNAi) and Overexpression. Two RUVBL2 specific siRNAs, 5'-GAGACCAUCUACGACCUGGGCAC-3' and 5'-GAGAGUGACAUGGCGCCUGUCCU-3' (Watkins et al., 2004a), were co-transfected into Vero cells for RNAi as described previously (Wang et al., 2012). RUVBL2 cDNA was inserted into pcDNA3.1 vector to generate pcDNA3.1-RUVBL2. For RUVBL2 overexpression, the plasmid of pcDNA3.1-RUVBL2 was trans-transfected into Vero cells using Lipofectamine LTX with PLUS (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Western Blotting. Western blotting was conducted as described previously (Wang et al., 2012). Antibodies of anti-RUVBL1, anti-β-actin and anti-Annexin I were purchased from Santa Cruz Biotech (Santa Cruz, CA); antibodies of anti-RUVBL2 and anti-PARP were from BD (San Jose, CA).

Caspase 3/7 Assay. Cell apoptosis was examined by Caspase-Glo 3/7 kit (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, the treated Vero or A549 cells (3 treated and 1 control samples in each analysis) in 96-well plates were incubated with caspase reagent (supplied in the

kit) at room temperature for 1 h, followed by luminescence detection in a spectrofluorometer (SPECTRAmax GEMINI XS, Molecular Devices). We repeated this analysis three times.

Pre-treatment of Vero cells with IFN-α. After Vero cells were seeded into 6-well plates for overnight, the culture medium was replaced with fresh DMEM supplemented with 10% FBS and recombinant human IFN-αA/D (rHuIFN-αA/D; PBL Biomedical Labs, Piscataway, NJ) at 1,000 units/ml (Carlos et al., 2005; Yi et al., 2011). After 6 h incubation, cells were infected by delNS1 or WT viruses at an MOI of 1 and harvested at 36 and 48 hpi.

Results

Identification of RUVBL1 and RUVBL2 as NS1 Interacting Proteins. Through a 2-DE-based proteomic approach described in Chapter 2, we identified the RUVBL1 and RUVBL2 proteins as the NS1 binding partners. To validate the interactions between NS1 and RUVBL1 or RUVBL2, reciprocal co-IPs and mammalian two-hybrid screening were conducted as described in Chapter 2. As shown in Fig. 1A, immobilized anti-Flag antibodies precipitated greater amount of RUVBL1 (right) or RUVBL2 (left) from the cells co-transfected with the plasmids that express Flag-NS1 and HA-RUVBL1 or HA-RUVBL2, respectively, but precipitated much less amount of proteins from the cells co-transfected with the plasmids that express Flag alone and HA-RUVBL1 or HA-RUVBL2. Similarly, in a reciprocal IP, immobilized anti-HA antibodies precipitated greater amount of Flag-NS1 from the cells co-transfected with the plasmids that express Flag-NS1 and HA-RUVBL1 or HA-RUVBL2 (RUVBL1 on the right and RUVBL2 on the left in Fig. 1B), but failed to do so from the cells co-transfected with the plasmids that express HA tag alone and Flag-NS1 (Fig. 1B). To further test whether the interactions occur *in vivo* in cells, we performed mammalian two-hybrid assay. We observed that co-transfection of 293T cells with the Gal4 GFP reporter plasmid and the constructs encoding fusion proteins of BD-NS1 and AD-RUVBL1 or AD-RUVBL2 resulted in higher expression of GFP than the negative control, in which BD and AD were fused with two proteins that are known to not interact, and protein pair of NS1-RUVBL2 induced higher expression of GFP than protein pair of NS1-RUVBL1 (Fig. 1C). These results suggest that NS1 is associated with RUVBL complex, especially RUVBL2 in human cells.

Influenza Viruses Reduce RUVBL2 Expression While NS1 Inhibits Virus-induced Expression Reduction of RUVBL2. In order to study the role of the interactions between NS1 and RUVBL1 or RUVBL2, we first mock-infected or infected A549 cells with WT influenza A/PR/8/34 H1N1 virus at MOIs of 0.02, 0.075 and 0.3. We found RUVBL2 expression was dramatically reduced by virus infection (Fig. 2A), but RUVBL1 expression was barely changed (data not shown). We further infected Vero cells with either WT or delNS1 influenza A/PR/8/34 H1N1 virus strains at the same initial amount. NS1 deletion in delNS1 virus-infected cells was confirmed by Western blotting with anti-NS1 antibody (4th row in Fig. 2B). We found RUVBL2 expression was reduced by both types of viruses in Vero cells from the early stage (DelNS1 at 24 hpi) and delNS1 virus led to more pronounced expression reduction of RUVBL2 than WT

virus at 24, 36, and 48 hpi (Fig. 2B). Fig. 2C showed RUVBL1 expression was also reduced by both types of viruses at the late stage (48 hpi), but not 24 (data not shown) or 36 hpi, and its expression level in delNS1 virus-infected cells was lower than that in WT virus-infected cells at 48 hpi. These data suggest that (1) the virus induces an earlier expression reduction of RUVBL2 than RUVBL1; (2) other viral element(s), but not NS1, causes RUVBL2 expression reduction, while NS1 inhibits this virus-induced RUVBL2 reduction through the interaction.

Influenza Virus Modulates Host Cell Apoptosis through RUVBL2 in Vero Cells. To examine the biological consequence of virus-induced RUVBL2 reduction, we used RNAi technique to knockdown endogenous RUVBL2 expression and examine the effect of reduced expression of RUVBL2 on viral NS1 protein expression, virus replication and host cell apoptosis. The results demonstrated that silencing of RUVBL2 by siRNAs had no significant effects on viral NS1 protein expression and virus replication (data not shown). However, silencing of RUVBL2 by siRNAs induced cell apoptosis as evidenced by PARP cleavage (Fig. 3A), which is consistent with the report of Rousseau etc (Rousseau et al., 2007). We then performed the same experiments as Fig. 2B and examined cell apoptosis. We found that virus infection reduced RUVBL2 expression and induced apoptosis, evidenced by PARP cleavage (Fig. 3B) and caspase 3/7 activities (Fig. 3C) in Vero cells (the values of fold change for WT virus-infected cells were 2.86 ± 0.24, 5.17 ± 0.19 and 5.54 ± 1.07, and for delNS1 virus-infected cells were 5.37 ± 0.04, 6.49 ± 1.63 and 5.1 ± 0.92 at 24, 36 and 48 hpi, respectively). Fig. 3B,C also indicated that the greater expression reduction of RUVBL2 induced by delNS1 virus generally led to greater PARP cleavage and caspase 3/7 activities than the less reduction of RUVBL2 induced by WT virus, which suggests that influenza viruses may induce cell apoptosis by reducing RUVBL2 expression in Vero cells. In order to verify this, we overexpressed RUVBL2 expression followed by virus infection and observed that when RUVBL2 was overexpressed in Vero cells, RUVBL2 expression was still reduced to a lower level by viruses, but cells with RUVBL2 overexpression had higher resistance to virus-induced apoptosis than cells without RUVBL2 overexpression infected with same kind of virus (the luminescence value reflects caspase 3/7 activities in Fig. 3D). The data suggest that influenza virus reduces RUVBL2 expression to induce Vero cell apoptosis.

IFNs Inhibit Apoptosis Regulated by the Virus-induced RUVBL2 Reduction. Even RUVBL2 expression was reduced by virus in A549 cells (Fig. 2A), but when we performed the same experiments as Fig. 3B and C in A549 cells, the apoptosis (evidenced by PARP cleavage and caspase activities) was insignificant altered by both types of viruses (Fig. 4A,B). The different apoptosis response to virus infection in A549 cells suggested that the type I IFNs might inhibit apoptosis induced by the virus-induced RUVBL2 reduction. In order to verify the role of IFNs, we pre-treated Vero cells with recombinant universal human IFN-alpha, followed by delNS1 or WT virus infection for different time. We found that RUVBL2 expression was still reduced by both delNS1 and WT viruses at 36 and 48 hpi (Fig. 4C), but PARP cleavage (data not shown) and caspase 3/7 activities (Fig. 4D) were barely affected in virus-infected Vero cells with pre-treatment of IFN, which was consistent to the observation in A549 cells (Fig. 4A,B). This result contrasted sharply with what was observed in Vero cells without IFN pre-treatment shown in Fig. 3B and C, which demonstrated that PARP cleavage and caspase 3/7 activities were significantly increased when RUVBL2 expression was reduced by virus. In other words, the different results between Fig. 3C and 4D demonstrated that type I IFNs counteract cell apoptosis induced by the virus-induced RUVBL2 reduction.

Discussion

In this present study, we found that RUVBL1 and RUVBL2, RUVBL2 in particular, are important interaction partners of influenza viral protein NS1. We verified the interactions by reciprocal co-IPs (Fig. 1A,B) and mammalian two-hybrid assay (Fig. 1C). Mammalian two-hybrid assay showed the greater expression of reporter GFP induced by the interaction of RUVBL2 and NS1 than that induced by RUVBL1 and NS1 interaction, and the further function studies demonstrated that virus-induced change in expression of RUVBL2 occurred prior to RUVBL1, suggesting RUVBL2 is more directly relative to both influenza virus and viral NS1, and the interaction between RUVBL1 and NS1 might be bridged by RUVBL2 (Fig. 5).

The fact that delNS1 viruses induced more pronounced expression reduction of RUVBL2 than WT viruses suggests there is viral element(s) responsible for the RUVBL2 reduction while NS1 increases RUVBL2 level by inhibiting the virus-induced expression reduction of RUVBL2 through the interaction. Three RNA polymerase subunits encoded by influenza virus genes, PB1, PB2 and PA, together with nucleoprotein (NP) and viral RNAs form the viral ribonucleoproteins (vRNPs). Since RUVBL2 was reported to interact with vRNPs (Kakugawa et al., 2009; Mayer et al., 2007) to inhibit virus replication (Kakugawa et al., 2009) and NS1 protein was reported to interact with vRNPs gene (Wang et al., 2008), we hypothesized that the influenza vRNPs may be responsible to reduce RUVBL2 expression for eliminating the virus replication inhibitors (Fig. 5). Consider the effect on cell apoptosis, viral vRNPs may play a pro-apoptotic role by reducing RUVBL2 expression while viral NS1 may play an anti-apoptotic role by increasing RUVBL2 level. The opposite roles of different influenza viral components on apoptosis regulation may explain why current findings about NS1 effects on apoptosis conflict (Ehrhardt et al., 2007; Zhang et al., 2011a).

For the pathway utilized by RUVBL2 to regulate apoptosis, it was reported that the expression of pro-apoptotic factor, BAD, was increased significantly when RUVBL2 was silenced by RNAi (Rousseau et al., 2007). PI3K pathway is found to inhibit apoptosis by activating PI3K effector AKT, which in turn phosphorylates and inactivates pro-apoptotic proteins like BAD (Igney and Krammer, 2002). AKT has one substrate named AS160, which was reported to interact with RUVBL2 (Xie et al., 2009). So based on these findings, we hypothesized that RUVBL2 affects apoptosis possibly through the PI3K/AKT pathway

via the interaction with AS160. Moreover, influenza NS1 was reported to inhibit apoptosis by binding to PI3K (Ehrhardt et al., 2007a) and the activated AKT (Matsuda et al., 2010). Taken together, NS1 plays anti-apoptotic roles through the PI3K/AKT pathway by either activating survival signal AKT (Matsuda et al., 2010) or increasing RUVBL2 level via inhibiting vRNPs-induced expression reduction (Fig. 5).

Type I IFNs (IFN α, β, ε, κ and ω) as multifunctional cytokines are essential for host innate immune responses against viruses, bacteria, protozoal, and cancer. They bind to a common receptor consisting of IFNAR1 and IFNAR2 chains to activate several downstream pathways and one of them is PI3K/AKT pathway. IFNs can activate PI3K/AKT to protect cells against pro-apoptotic signals by recruiting the regulatory subunit p85 of PI3K to IFNAR complex and then tyrosine-phosphorylating it (Ruuth et al., 2001; Yang et al., 2001). So the regulation of apoptosis by IFNs also through PI3K/AKT pathway supplies the possibility to explain that the apoptosis induced by the RUVBL2 reduction only occur in the absence of IFNs. The reduction of RUVBL2 expression decreases AKT expression or reduces AKT activity with consequence of apoptosis induction, while the survival signals from IFNs increase AKT expression or enhance AKT activity with consequence of apoptosis inhibition (Fig. 5), and the modulation on apoptosis by IFNs overwhelms the regulation induced by the RUVBL2 reduction. For the real virus infection surroundings with IFNs presence in infected cells, it is also possibly that influenza virus may regulate cell apoptosis through RUVBL2, since the virus has evolved to employ several strategies to suppress IFN production as described in Background section and Chapter 1.

We also found that knockdown of RUVBL2 by siRNA increased apoptosis in A549 cells (data not shown), which is consistent with the reported results (Rousseau et al., 2007). But it does not conflict to our present finding that virus-induced RUVBL2 reduction only induces apoptosis in absence of IFNs. Virus infection brings pathogen characters, like viral dsRNA, which are immediately recognized by host immune system to trigger immune defenses, such as IFN secretion in IFN-competent cells, while siRNAs for RNAi usually do not trigger much immune response. So this difference might explain that siRNA-induced RUVBL2 reduction can increase apoptosis in IFN-competent cells while virus-induced RUVBL2 reduction can not, which is consistent with the our explanation mentioned in last paragraph.

Besides RUVBL2 effect on host cell apoptosis, RUVBL1 is also reported to be involved in apoptosis pathways. RUVBL1 has been reported to bind to E2F1 to modulate c-Myc-mediated apoptosis (Dugan et

al., 2002), which mainly depends on the activity of p53 (Hermeking and Eick, 1994), Fas/FasL (Hueber et al., 1997) or Bax (Eischen et al., 2001), as illustrated in Fig. 5. It is possibly that RUVBL1 is an alternative target for influenza virus to regulate host cell apoptosis.

Based on our findings and other reports, we hypothetically constructed a pathway model to explain the relationships of influenza virus protein NS1, vRNPs, cellular RUVBL1/RUVBL2 and IFNs with protein complex effect on regulation of cell apoptosis (Fig. 5). However, some hypotheses among this pathway need verification in the future, such as vRNPs reducing RUVBL2 expression.

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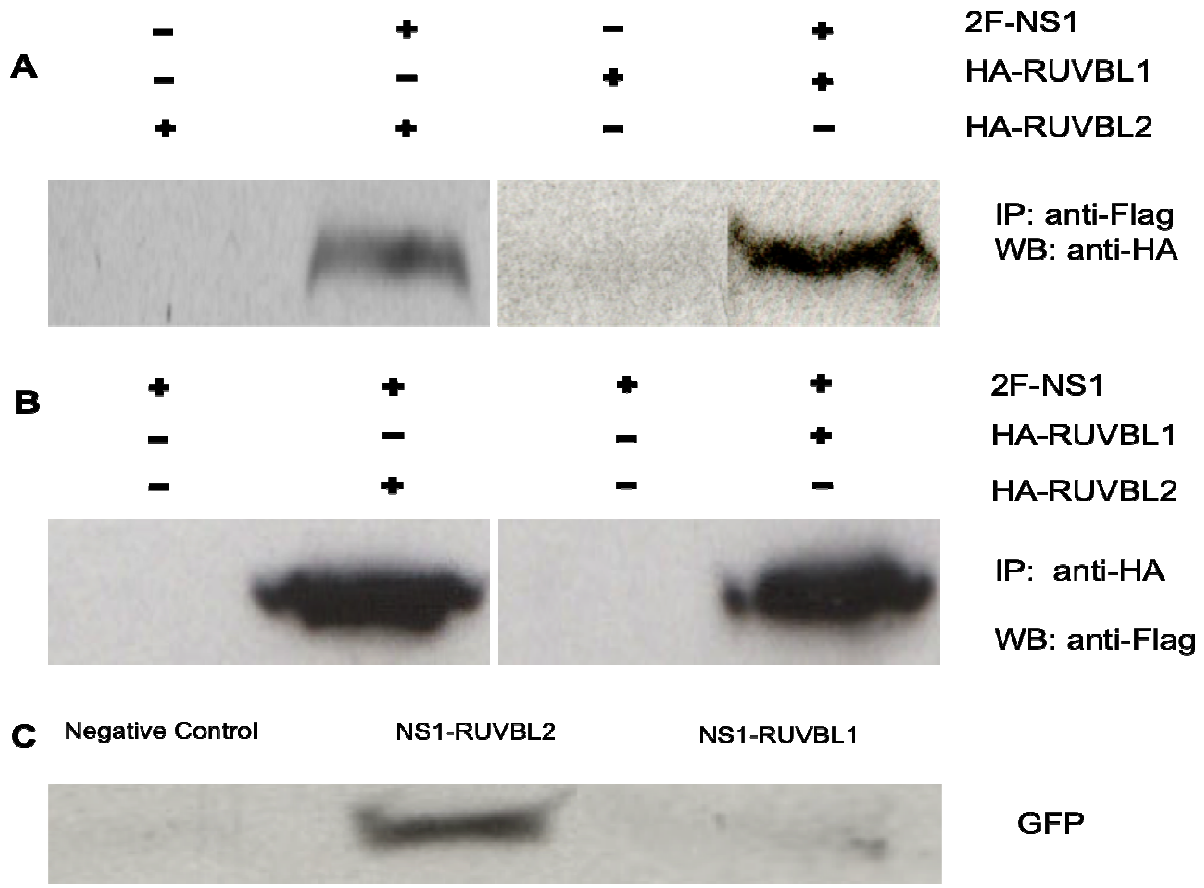

Fig. 1. Validation of the interactions between RUVBL proteins and NS1. A, co-IPs. Cell lysates from the cells expressing Flag-NS1 and HA-RUVBL2 (left) or HA-RUVBL1 (right), or the cells expressing Flag alone and HA-RUVBL2 or HA-RUVBL1 (control) were immunoprecipitated with anti-Flag M2 resin, and the immunoprecipitated proteins were probed with anti-HA antibody in Western blotting. B, reciprocal co-IP, cell lysates from the cells expressing Flag-NS1 and HA-RUVBL2 (left) or HA-RUVBL1 (right), or the cells expressing HA alone and Flag-NS1 (control) were precipitated with immobilized anti-HA antibody, and the immunoprecipitated proteins were probed with anti-Flag antibody in Western blotting. C, mammalian two-hybrid analysis. 293T cells were co-transfected with plasmids expressing BD-NS1, AD-RUVBL2 (middle) or AD-RUVBL1 (right) and a Gal4 GFP reporter; and the induction of reporter GFP expression was detected by Western blotting with an anti-GFP antibody. The negative control was performed by co-transfection of 293T cells with Gal4 GFP reporter plasmid and the constructs in which the BD and AD were fused with two proteins that are known to not interact.

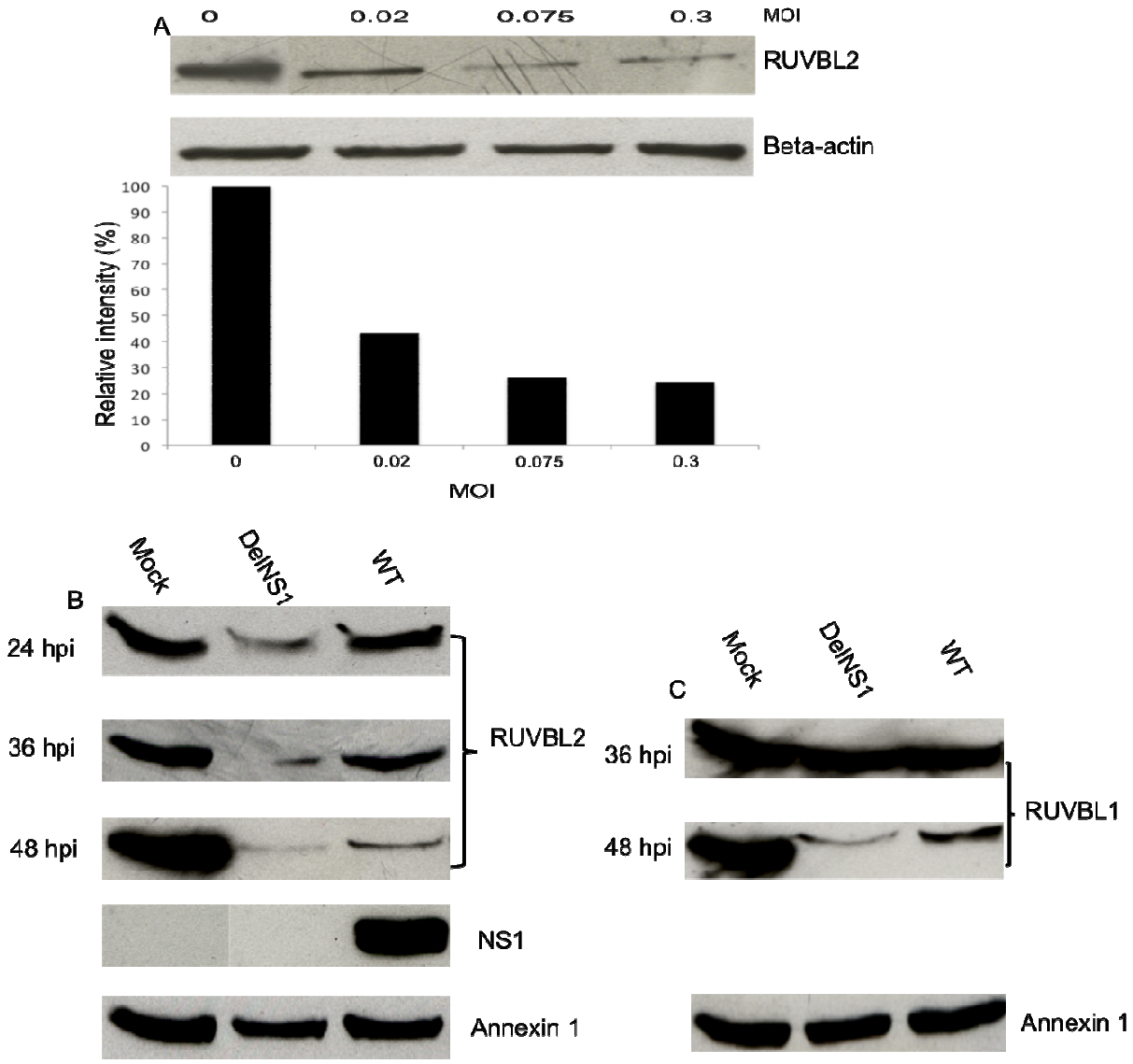

Fig. 2. Influenza virus reduces RUVBL1 and RUVBL2 expression, especially RUVBL2, and NS1 increases RUVBL2 level by inhibiting the virus-induced RUVBL2 reduction. (A) Influenza virus reduces RUVBL2 expression in A549 cells. Cell lysates from A549 cells infected with WT virus at an MOI of 0, 0.02, 0.075 and 0.3 for 36 h were analyzed by Western blotting with anti-RUVBL2 antibody. β-actin was used as a loading control. The relative intensities of RUVBL2 bands in Western blotting were quantified using ImageJ software to generate column diagram. (B, C) Influenza virus reduces RUVBL1 and RUVBL2 expression in Vero cells and NS1 raises RUVBL2 level by inhibiting the virus-induced RUVBL2 reduction. Cell lysates from Vero cells mock-infected or infected by WT or delNS1 viruses at an MOI of 1 for

appropriate time were analyzed by Western blotting with antibodies to anti-RUVBL2 and anti-NS1 (B) or anti-RUVBL1 (C). Annexin-1 was used as a loading control. This experiment has been independently repeated at least three times.

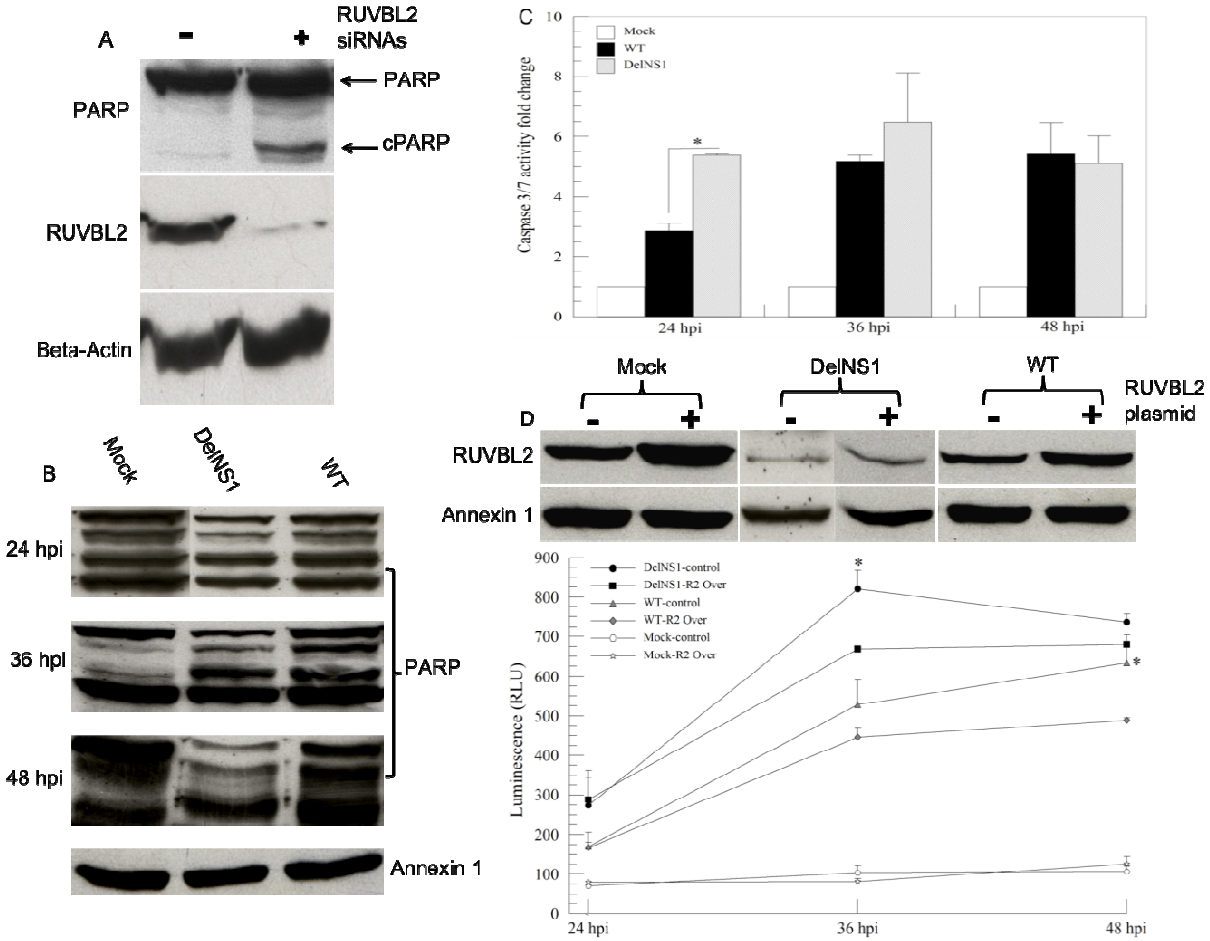

Fig. 3. Influenza virus induces apoptosis through reducing RUVBL2 in Vero cells. (A) Silencing of endogenous RUVBL2 leads to increased PARP cleavage. Cell lysates from Vero cells transfected with a randomized siRNA sequence (control) or a siRNA sequence targeting RUVBL2 were analyzed by Western blotting with anti-RUVBL2 and anti-PARP antibodies. (B) Influenza virus infection induces PARP cleavage. Cell lysates from Vero cells mock-infected or infected by WT or delNS1 viruses at an MOI of 1 for 24, 36 and 48 h were analyzed by Western blotting with anti-PARP antibody. (C) Influenza virus increases caspase 3/7 activities. Vero cells were mock-infected or infected by WT or delNS1 viruses at an MOI of 1 for 24, 36 and 48h, followed by incubation with caspase 3/7 substrates for luminescent signal detection. Caspase 3/7 activities were determined as the ratios of virus-infected cell luminescence to mock-infected cell luminescence. Values are the means + S.E. of three separate sample preparations. Values for control samples were set to 1 at each time point. The values of fold change for WT virus-

infected cells were 2.86 ± 0.24, 5.17 ± 0.19 and 5.54 ± 1.07, and for delNS1 virus-infected cells were 5.37 ± 0.04, 6.49 ± 1.63 and 5.1 ± 0.92 at 24, 36 and 48 hpi, respectively. (D) RUVBL2 overexpression increases cell resistance to virus-induced apoptosis. Vero cells were introduced with RUVBL2 expression plasmid or empty vector as control by Lipofectamine LTX with PLUS, followed by mock-infected or infected with WT or delNS1 viruses at an MOI of 1 for the appropriate time. Cells were either harvested for Western blotting with anti-RUVBL2 antibody (36 hpi) or incubated with caspase 3/7 substrates to detect caspase 3/7 luminescence. Values are the means + S.E. of three separate sample preparations. * denotes $p < 0.05$, comparison in same type virus infection group; R2 Over, RUVBL2 overexpression.

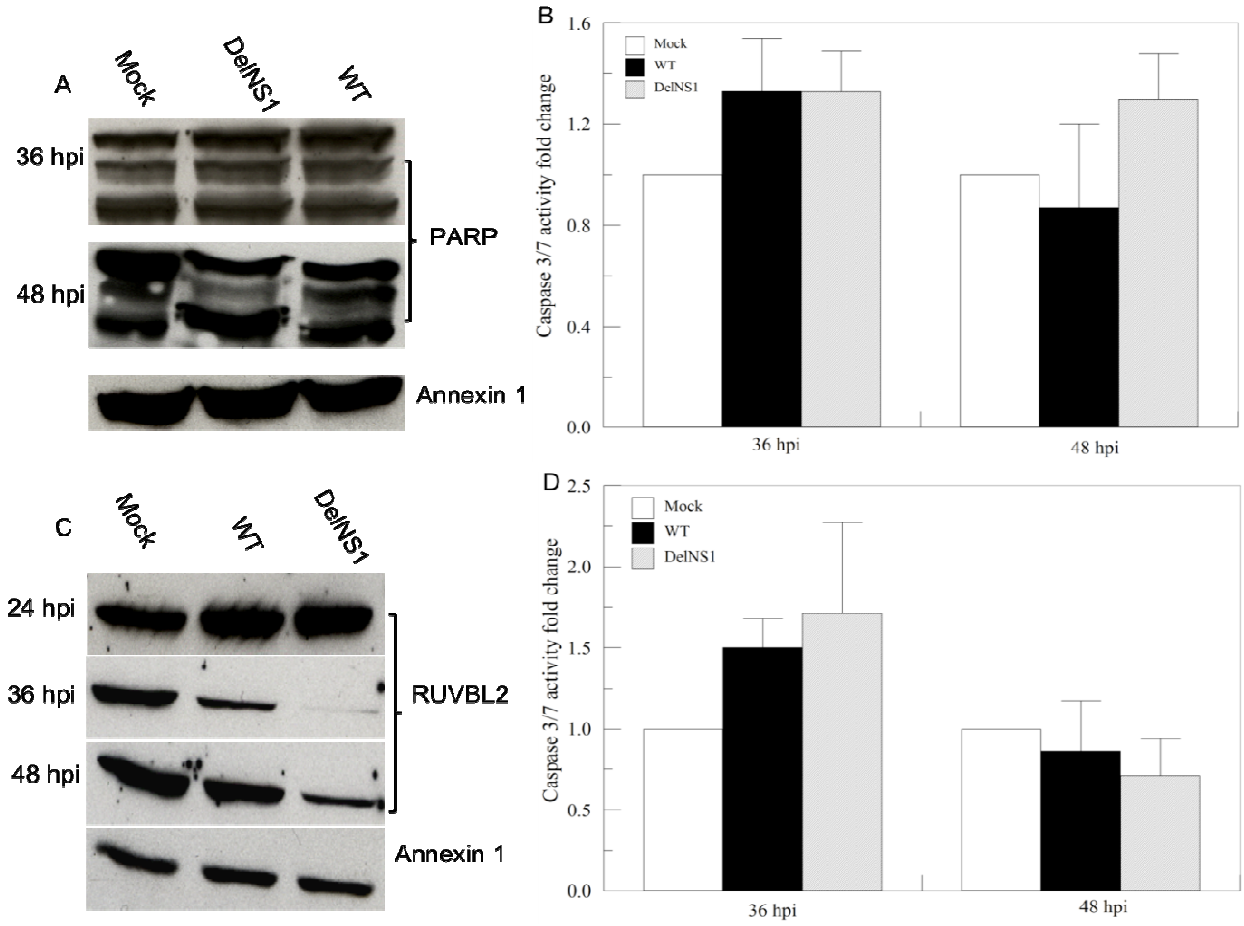

Fig. 4. IFNs inhibit cell apoptosis induced by the virus-induced expression reduction of RUVBL2. (A,B) Influenza virus insignificantly affects A549 cells apoptosis. A549 cells were mock-infected or infected with WT or delNS1 viruses at an MOI of 1 for 36 and 48 h, followed by either harvest for Western blotting with anti-PARP antibody (A) or incubation with caspase 3/7 substrates to detect luminescence (B). (C, D) Influenza virus reduces RUVBL2 expression but does not affect apoptosis in Vero cells pre-treated with IFN-α. Vero cells were pre-treated with universal IFN-α at 1,000 U/ml for 6 h, followed by mock-infection or infection with WT or delNS1 viruses at an MOI of 1 for the appropriate time. Cells were either harvested for Western blotting with anti-RUVBL2 antibody (C) or incubated with caspase 3/7 substrates to detect luminescence (D). Values are the means + standard errors of three separate sample preparations.

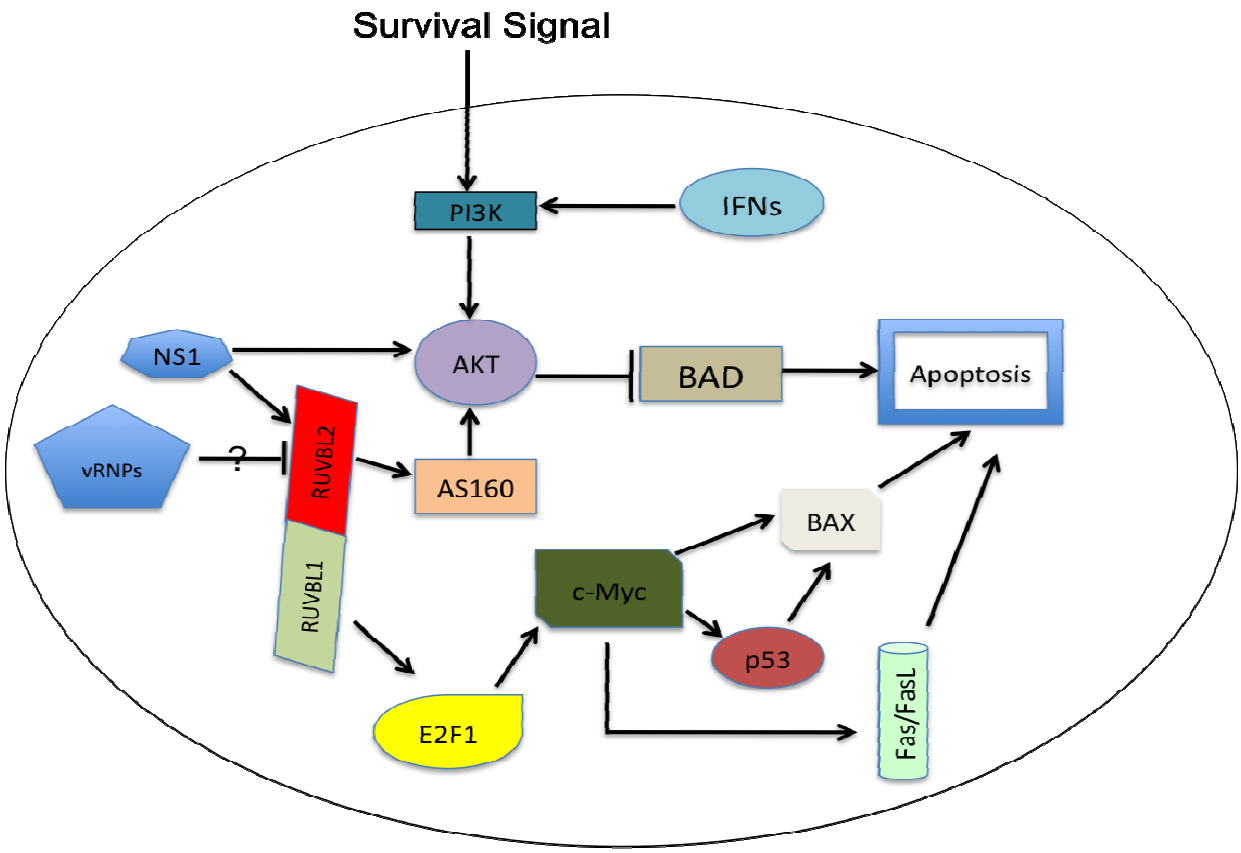

Fig. 5. A hypothesized signal pathway utilized by influenza virus to regulate host cell apoptosis through RUVBL proteins. The present study suggests that influenza virus element(s), not NS1, reduces RUVBL2 expression while NS1 increases RUVBL2 level by interacting with RUVBL2. Based on the report that RUVBL2 interacts with influenza vRNPs and inhibits virus replication (Kakugawa et al., 2009), we hypothesized that vRNPs may be responsible to reduce RUVBL2 expression. RUVBL2 was reported to interact with AS160 (Xie et al., 2009), a substrate of AKT, and to induce cell apoptosis through pro-apoptotic factor, BAD (Rousseau et al., 2007), so we hypothesized that RUVBL2 regulates apoptosis through PI3K/AKT pathway. IFNs were reported to use PI3K/AKT pathway to protect cells against pro-apoptotic signals (Ruuth et al., 2001; Yang et al., 2001), which may explain our finding that virus-induced expression reduction of RUVBL2 promotes cell apoptosis only in the absence of IFNs. In addition, RUVBL1 was reported to bind to E2F1 to modulate c-Myc-mediated apoptosis (Dugan et al., 2002) through p53 (Hermeking and Eick, 1994), Fas/FasL (Hueber et al., 1997) or Bax (Eischen et al., 2001).

Chapter 4. The Interaction between Influenza A Virus Protein NS1 and DDX100 Is Required for Virus Replication

Abstract

The NS1 protein of influenza virus plays various roles through interacting with cellular proteins. In this study, we used two-step affinity precipitations to verify an interaction between a DDX isoform (we designated it here as DDX100) and influenza virus protein NS1, which was previously identified through a more sensitive proteomic method called SILAC (stable isotope labeling with amino acids in cell culture). Knockdown of DDX100 by small interfering RNA (siRNA) resulted in decreased synthesis of NS1 viral RNA and NS1 mRNA in the virus-infected cells. Concomitantly, we performed *in vivo* and *in vitro* experiments to demonstrate that DDX100 plays a helicase role in influenza viral NS1 dsRNA and NS1 recruits DDX100 to assist viral NS1 dsRNA unwinding. In addition, we showed that siRNA-mediated knockdown of DDX100 leads to reduced expression of NS1 protein and virus replication. Lastly, we screened other cellular proteins in NS1-DDX100 complex by a two-step affinity precipitation-based proteomic approach and mass spectrometry, and found that most of identified proteins shared the same function in mRNA maturation, suggesting the interaction between NS1 and DDX100 may affect mRNA maturation. Our results suggest that the interaction between NS1 and DDX100 contributes to the replication of influenza virus in host cells potentially through facilitating NS1 gene replication, transcription, and viral dsRNA unwinding, which provides valuable information for designing novel antiviral therapy in the future.

Introduction

The DEAD-box family was named based on the nine conserved motifs with the second motif containing four amino acids sequence Asp-Glu-Ala-Asp (D-E-A-D). Many different protein members of DEAD-box family exist in eukaryotes but very few in prokaryotes (Linder, 2006). More than 500 proteins are found to carry the DEAD-box characterizes (Silverman et al., 2003). Twenty-five DEAD-box proteins are identified in yeast *Saccharomyces cerevisiae* (de la Cruz et al., 1999*)* and thirty-eight DEAD-box proteins with subfamilies (DDX1-6, 10, 17-21, 23-25, 27-28, 31, 39, 41-43, 46-56, 59 and BAT 1) identified in human (Linder, 2006). In contrast, only a few of DEAD-box proteins is found in bacteria and some bacterial genomes even do not encode DEAD-box proteins at all (Iost and Dreyfus, 2006). DEAD-box family is often referred to DExD/H proteins together with its two relative families, DEAH and the Ski (de la Cruz et al., 1999), since they share eight conserved motifs (Tanner and Linder, 2001). But they also can be distinguished by variations with their unique conserved motifs (Cordin et al., 2006).

DEAD-box family, the biggest family among DExD/H proteins, play roles in nearly every aspect of RNA processing, like RNA-binding, unwinding, annealing, translation initiation, maturation and decay (Cordin et al., 2006; Fuller-Pace, 2006; de la Cruz et al., 1999). One of the common functions of the DEAD-box proteins is ATP-dependent RNA helicase since the typical DEAD-box protein contains an essential helicase core distributed between N and C terminals. The protein-binding domain of DEAD-box protein mediates the helicase role by nonspecifically binding to dsRNA and then inducing RNA conformational changes in an ATP-dependent manner. DDX100 belongs to the DEAD-box family and expresses in all mammalian cell types in either nucleus or cytoplasm depending on the cell type (Edgcomb et al., 2012). DDX100 functions in several host cell processes, like RNA metabolism (Godbout et al., 1998), RNA transcription (Ishaq et al., 2009), mRNA translation (Kanai et al., 2004), protein interaction and enzymatic activity. DDX100 was reported to contribute to the HIV viral replication (Edgcomb et al., 2012).

In this present study, we showed that the interaction between DDX100 and influenza virus protein NS1 was required for viral NS1 gene replication, transcription, translation, viral dsRNA unwinding and whole virus replication. In addition, identifying other cellular proteins in the NS1-DDX100 complex suggested that the interaction between NS1 and DDX100 might affect viral or cellular mRNA maturation.

Materials and Methods

Cell Culture, Virus Infection and Proteome Labeling. A549 cells, 293T cells, Vero cells and MDCK cells were cultivated in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Influenza A/PR/8/34 H1N1 viruses were propagated and titrated as described (Wang et al., 2012).

Plasmid DNA Construction and Transfection. Flag tagged NS1 plasmid was constructed as described previously (Wang et al., 2012). NS1 cDNA was also amplified and inserted into pcDNA3.1 vector without tags. DDX100 cDNA was amplified from a human genome library and inserted into *BamH I* and *Xho I* sites of pcDNA3.1 vector with a TAP tag at N terminal (NTAP) to generate the plasmid of pcDNA3.1-NTAP-DDX100 for two-step affinity precipitations. DDX100 cDNA was also inserted into pcDNA3.1 vector with Flag tag to generate the plasmid of pcDNA3.1-2F-DDX100 for dsRNA unwinding experiments. Expression plasmids were confirmed by DNA sequencing and then transiently transfected into 293T cells with the standard calcium phosphate method.

Western Blotting. Western blotting was conducted as described (Wang et al., 2012) and rabbit anti-DDX100 polyclonal antibody was purchased from Santa Cruz Biotech (Santa Cruz, CA).

Two-step Affinity Precipitations. NTAP tagged plasmid of pcDNA3.1-NTAP-DDX100 and Flag tagged plasmid of pcDNA3.1-2F-NS1 were co-transfected into 2 billion of 293T cells. Cells were lysed and precipitated by M2 resin to enrich Flag-NS1 in first step affinity precipitation as described in Chapter 2. NaCl concentration in precipitated Flag-NS1 complex was diluted by water to 150 mM. In the second step affinity precipitation, IgG beads that specially bind to TAP tag were used to precipitate NTAP-DDX100 from the first step precipitated Flag-NS1 complex for 4 h at 4℃. After being washed for 4-8 times by TEV buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 0.1% NP-40, 1 mM DTT), the precipitated IgG beads were incubated with acTEV enzyme in 16 ℃ water bath for 2 h to cleave NTAP, followed by rotation for overnight at 4 ℃. The supernatant con taining cleaved NTAP-DDX100 and its bound proteins was collected by centrifugations. After two-step immunoprecipitations, the NS1-DDX100 protein complex was either purified by sucrose gradient ultracentrifugation (13 ml) or detected by Western blotting with antibodies of anti-DDX100 and anti-Flag to validate the interaction between NS1 and DDX100. For sucrose gradient ultracentrifugation, each layer was collected after ultracentrifugation and further

detected by Western blotting with antibodies of anti-DDX100 and anti-Flag. The layers containing NS1 and DDX100 were subjected to a desalted process by NAT-25 column (GE, Pittsburgh, PA), followed by the separation on a SDS-PAGE gel and protein identification by MS as described (Wang et al., 2012).

RNAi. One siRNA fragment (5'-CAAGCCCUCUUUCCUGCCUGUUU-3') targeting DDX100 was designed. A randomized siRNA sequence was used as a control. The siRNA transfection into cells was performed with Lipofectamine ™ RNAiMAX as described (Wang et al., 2012).

Plaque Assay. Plaque assay to detect virus replication titer was conducted as described (Wang et al., 2012).

qRT-PCR. The levels of IFN-α/β mRNA in cells, NS1 mRNA and NS1 vRNA were detected by qRT-PCR as described in Chapters 1 and 2.

Viral dsRNA Synthesis and Detection by Immunoblotting. Viral NS1 dsRNA was synthesized using Ambion MEGAscript RNAi kit (Grand Island, NY) according to manufacture's instruction. Briefly, NS1 cDNA was amplified using a pair of primers with T7 promoter at each end (forward: 5'-TAATACGACTCACTATAGGGATGGATCCAAACACTG-3' and reverse: 5'-CCCTATAGTGAGTCGTATTATCAAACTTCTGACC-3'). When PCR was used to amplify NS1 with T7 promoters, the annealing temperature in first five cycles was set up based on NS1 specific primer Tm, and the rest cycles based on whole primer Tm. Agarose electrophoresis was used to identify and purify the target band. Then four ribonuclotide solutions (ATG, CTP, GTP, UTP), reaction buffer and T7 RNA polymerase (all supplied in the kit) were mixed together with template (total 20 μl) and incubated at 37 °C for overnight. Since the NS1 fragment is short (<800bp), two ssRNA can anneal together to generate dsRNA after synthesis without extra annealing process. On the next day, both DNase and RNase (all supplied in the kit) were added to dsRNA mixture to cleave DNA and unannealed RNA (dsRNA can not be cleaved by the supplied RNase), followed by purification on the supplied column. UV spectrometer was used to detect the dsRNA absorbance at 260nm and dsRNA concentration was calculated by A260 × 10 (dilution fold) × 40 (μg/ml). dsRNA was confirmed by agarose electrophoresis with a dsRNA size marker and the following immunoblotting with anti-dsRNA J2 antibody.

The synthesized dsRNA transfection into Vero cells was carried out by the standard calcium phosphate method or Lipofectamine LTX with PLUS described in Chapter 3. Eight h after transfection, total RNA was

isolated from cells and then loaded onto 5% native gel (Non-denature PAGE gel) with a dsRNA marker. Electrophoresis was run at 120V for 1 h (6 × 9 cm gel), followed by semi-dry transfer to positively charged Nylon membrane (Nytran SPC, Whatman) (Polvino et al., 1983) at 250 mA for 1 h. Then the membrane was blocked by 5% non-fat dry milk in TBS for 30 min, followed by incubation with mouse anti-dsRNA J2 antibody (Engscicons, Hungary) diluted by 500 fold in 5% milk with 0.1% (v/v) Triton X-100 diluted in TBS for overnight at 4℃. Mouse J2 monoclonal antibody can specially recognize the secondary structure of dsRNA with greater than 40 bp. After being washed, membrane was further incubated with rabbit anti-mouse second antibody conjugated with HRP for 1 h. The target dsRNA bands were developed with SuperSignal West Femto Chemiluminescent Substrate (Thermo, San Jose, CA). After development, the membrane was further stained with methylene blue solution [0.02% (wt/vol) in 0.3 M sodium acetate, pH 5.5] to observe 18S ribosomal subunit as the convenient size marker (1.9 kb) and loading reference.

Biotin UTP Labeled dsRNA Synthesis and Detection. NS1 cDNA was inserted into *Xba I* and *Sac I* sites of pSP64/poly (A) vector (Promega, Madison, WI), which contains SP6 promoter at the 5' terminal and a 33 nt poly (A) tail at the 3' terminal (Chen et al., 2002). The plasmid was then linearized with EcoR *I* and transcribed by SP6 RNA polymerase to generate one NS1 ssRNA. NS1 cDNA was also amplified with a pair of primer with T7 promoter on the reverse primer (forward: 5'-ATGGATCCAAACACTG-3' and reverse: 5'- CCCTATAGTGAGTCGTATTATCAAACTTCTGACC-3'). The amplified NS1 cDNA fragment with T7 promoter at the 3' terminal was transcribed by T7 RNA polymerase with substrate of Biotin-11-UTP (Ambion) to generate an complementary NS1 ssRNA labeled with biotin UTP. Two ssRNAs were annealed to generate biotin UTP labeled NS1 dsRNA by heating two ssRNA mixture to 75°C for 5 min and cooling down to room temperature for overnight. After treated with DNase and RNase as described above, the synthesized biotin UTP labeled dsRNA was confirmed by immunoblotting with anti-dsRNA J2 antibody and streptavidin described in the following.

dsRNA unwinding assay was performed as described (Chen et al., 2002). Briefly, DDX100 was enriched by anti-Flag M2 resin from 293T cells transfected with Flag-DDX100 plasmid or co-transfected with plasmids of Flag-DDX100 and non-tagged NS1. Biotin UTP labeled NS1 dsRNA (500 ng) was incubated in a 10 μl reaction containing 17 mM HEPES, pH 7.5, 150 mM KCl, 2 mM DTT, 1 mM $MgCl_2$, 5% glycerol, 0.3% PEG 8,000, 1 mM ATP, 40 units of RNasin, 1 μg of tRNA, and the appropriate amount

of precipitated DDX100 (attached on M2 resin, not eluted) at 37 ℃ for 20 min. Two μl of termination buffer (1 mg/ml proteinase K, 5% SDS) was then added to terminate the reaction and further incubated for 15 min. The unwinding reaction mixture was loaded onto a 5% native gel, followed by semi-dry transfer as described above. The membrane was blocked by Odyssey Blocking Buffer (LI-COR, Lincoln, NE) with 1% SDS for 30 min, followed by incubation with IRDye 800CW Streptavidin (LI-COR) diluted by 10,000 fold in the same blocking buffer for another 30 min. After being washed by PBS, the membrane was scanned with Odyssey Imager.

Results

Identification and Confirmation of Interaction between DDX100 and NS1. A more sensitive quantitative proteomic method, SILAC, was used to identify cellular proteins that interact with NS1 and DDX100 was one of identified proteins (Lin Liu's work). To confirm the interaction between DDX100 and NS1, we used two-step affinity precipitations: immobilized anti-Flag M2 resin was first used to precipitate Flag-NS1 complex from 293T cells co-transfected with NTAP-DDX100 and Flag-NS1 plasmids; and immobilized anti-NTAP IgG beads were then used to precipitate NTAP-DDX100 from the first step precipitated Flag-NS1 complex. The precipitated protein complexes from sequential precipitations were subjected to Western blotting with antibodies to anti-DDX100 and anti-Flag. Fig. 1 showed that immobilized anti-Flag M2 resin with Flag-NS1 complex (upper left) precipitated large amount of NTAP-DDX100 (lower left) from cells co-transfected with NTAP-DDX100 and Flag-NS1 plasmids in the first step precipitation. In the second step precipitation, immobilized anti-NTAP IgG beads with DDX100 complex (lower right) precipitated large amount of Flag-NS1 (upper right) from the first step precipitated Flag-NS1 complex. Besides, IPs and immunofluorescence to detect the colocalization were also conducted to validate the interaction between NS1 and DDX100 (Lin Liu's results). These data suggest that NS1 interacts with DDX100 in human cells.

DDX100 Promotes NS1 Protein Expression in the Virus-Infected cells. To determine the potential roles of the interaction between DDX100 and NS1, we silenced the expression of endogenous DDX100 by siRNAs in A549 cells and then examined the effect of the DDX100 silencing on NS1 protein expression in the virus-infected cells. The results demonstrated that when the expression of DDX100 was silenced by siRNA (Fig. 2; upper row), viral NS1 protein expression decreased (Fig. 2; middle row), suggesting that DDX100 is required for viral NS1 protein expression in the virus-infected cells.

DDX100 Promotes NS1 vRNA Replication and mRNA Transcription, but does not Affect NS1 mRNA Nuclear Export. To elucidate the mechanism by which DDX100 promotes viral NS1 protein expression in the virus-infected cells, we examined the effect of knockdown of DDX100 expression on NS1 gene replication (vRNA) and transcription (mRNA) in the virus-infected A549 cells using qRT-PCR as described in Chapter 2. We found the knockdown of DDX100 decreased NS1 vRNA levels by 66% (0.34 ± 0.08) and 27% (0.73 ± 0.09) at 8 hpi and 12 hpi, respectively compared with those non-silenced cells (Fig. 3A).

Similarly, knockdown of DDX100 decreased NS1 mRNA levels by 40% (0.6 ± 0.08) and 66 % (0.34±0.02) at 8 hpi and 12 hpi, respectively compared with those levels in non-silenced cells (Fig. 3B).

These results suggest that DDX100 promotes both viral NS1 gene replication and transcription in the virus-infected cells. Nucleo-cytoplasmic translocation of mRNA affects mRNA transcription. To examine whether the effect of DDX100 on NS1 mRNA transcription results from the effect of DDX100 on NS1 mRNA nucleo-cytoplasmic translocation, we used a differential-centrifugation-based method to fractionate the lysates of DDX100 silenced or non-silenced cells into cytoplasmic and nuclear parts, followed by measuring NS1 mRNA distribution in cytoplasmic and nuclear parts by qRT-PCR as described in Chapter 2. We found that when DDX100 expression was knocked down, the ratio of NS1 mRNA distribution in nucleus to cytoplasm altered insignificantly (data not shown), suggesting that DDX100 does not affect viral NS1 mRNA nuclear/cytoplasmic trafficking.

DDX100 Unwinds Viral NS1 dsRNA. Helicase activity is the major function of DEAD family. Influenza virus genome does not encode helicase but the virus requires viral dsRNA to be unwound (Wisskirchen et al., 2011). So influenza virus must utilize host machinery to unwind viral dsRNA and we hypothesized DDX100 may be one of host machinery for viral dsRNA unwinding. To prove that, we first synthesized one viral dsRNA *in vitro* based on NS1 sequence, since the native influenza viral dsRNA is undetectable by the immunoblotting with anti-dsRNA J2 antibody (data not shown) or immunofluorescence (Weber et al., 2006). The synthesized NS1 dsRNA contains biotin UTP on one strand of RNA and a poly (A) tail on another strand of RNA, which is required for DDX100 unwinding *in vitro* (Chen et al., 2002). We first used the immobilized anti-Flag M2 resin to enrich DDX100 protein from 293T cells transfected with Flag-DDX100 plasmid, and then incubated the increased amount of precipitated DDX100 with biotin UTP labeled NS1 dsRNA *in vitro*. We found that as the increase in DDX100 amount, more dsRNA was unwound to ssRNA *in vitro* (Fig. 4A). To verify the helicase role of DDX100 *in vivo*, we mock-treated or treated Vero cells with DDX100 specific siRNAs, followed by introduction of NS1 dsRNA [no biotin UTP and poly (A) tail] into cells. NS1 dsRNA was examined by immunoblotting with anti-dsRNA J2 antibody and the intensity of 18s rRNA was considered as the loading control. As shown in Fig. 4B, the band intensity of NS1 dsRNA isolated from DDX100 silenced cells was significantly higher than that from control cells, suggesting the silencing of DDX100 increases dsRNA amount, indicating of the potential

helicase role of DDX100 *in vivo*. Viral dsRNA is known to activate host protein kinase R (PKR) and further NF-κB, which initiates the transcription of IFN-β (Lu et al., 1995; Wang et al., 2000). To further validate the helicase role of DDX100 in viral dsRNA *in vivo*, IFN-α/β transcriptions were examined. A549 cells were mock-treated or treated with DDX100 specific siRNA, followed by infection of influenza virus at an MOI of 2 for 8 and 12 h. qRT-PCR showed the silencing of DDX100 increased IFN-β mRNA levels by 1-3 folds (2.88 ± 0.33 and 1.75 ± 0.23 at 8 hpi and 12 hpi, respectively) (Fig. 5) but not IFN-α (data not shown), suggesting that higher level of dsRNA resulted from DDX100 knockdown triggers more IFN-β secretion. Our data suggest that DDX100 plays a helicase role in viral NS1 dsRNA unwinding.

NS1 Recruits DDX100 for Viral dsRNA Unwinding. Viral dsRNA is a pathogen character that can easily be recognized by the host immune system to trigger immune defenses, so the virus evolves mechanisms to eliminate the effect of dsRNA on host cell immune response. Influenza virus NS1 was reported to competitively bind to viral dsRNA against PKR activation (Wang et al., 2000). So after we knew the helicase role of DDX100, we hypothesized that the interaction between NS1 and DDX100 may promote viral dsRNA unwinding. To verify this, we first precipitated DDX100 from cells co-transfected with plasmids of Flag-DDX100 and non-tagged NS1 or plasmid of Flag-DDX100 alone as control by immobilized anti-Flag M2 resin. After quantify and equal the concentration of DDX100 precipitated from cells with or without NS1 expression, we incubated the increased amount of precipitated DDX100 with biotin UTP labeled NS1 dsRNA substrate and found the DDX100 precipitated from control cells required more than 7 fold higher amount to completely unwind dsRNA substrate than DDX100 precipitated from cells with NS1 expression (Fig. 6A), suggesting NS1 promotes DDX100 to unwind viral NS1 dsRNA *in vitro*. To verify the effect of NS1 on DDX100 helicase activity *in vivo*, we repeated the experiment as Fig. 4B except the introduction of NS1 plasmid rather than viral infection into DDX100 silenced cells. As shown in Fig. 6B, compared to DDX100 silence alone, the addition of NS1 dramatically reduced dsRNA level, suggesting NS1 promotes DDX100 to unwind viral dsRNA *in vivo*. Our data suggest that NS1 promotes DDX100 helicase role in viral dsRNA unwinding.

DDX100 Promotes Influenza Virus Replication. Since DDX100 affects influenza viral NS1 gene replication, transcription (Fig. 3), expression (Fig. 2) and viral dsRNA unwinding (Fig. 4), we speculated DDX100 may affect virus replication. Indeed, when the expression of DDX100 was silenced by siRNA in

the A549 cells, the virus titers decreased significantly (Fig. 7), suggesting that DDX100 promotes influenza virus replication in the infected cells.

Cellular Proteins in NS1-DDX100 Complex. In order to further explore the biological function of the interaction between NS1 and DDX100, we screened cellular proteins in the NS1-DDX100 complex by a two-step affinity precipitation-based proteomic approach coupled with MS. The plasmids of 2F-NS1 and NTAP-DDX100 were co-transfected into 2 billion of 293T cells, followed by NS1 and DDX100 enrichment as described above. The protein complex precipitated by both NS1 and DDX100 was further purified by gradient sucrose ultracentrifugation. After each layer of sucrose was examined by Western blotting with antibodies of anti-DDX100 and anti-Flag, the target layers were further separated by SDS-PAGE. Through MS analysis, we identified cellular proteins in NS1-DDX100 complex. Most of identified proteins shared the function in pre-mRNA maturation, a progress including splicing and addition of 5'-cap and 3'-poly (A) tail onto pre-mRNA. hnRNP K is the major pre-mRNA binding protein and reported to interact with DDX100 (Chen et al., 2002). DDX5 plays the regulative role on pre-mRNA splicing (Kar et al., 2011). Polypyrimidine tract-binding protein 1 (PTB/hnRNP I) as the splicing regulator antagonizes exon definition with preferentially binding to pyrimindine-rich intronic silencers (Lin and Tarn, 2005; Wagner and Garcia-Blanco, 2001). Polypyrimidine tract-binding protein-associated-splicing factor (PSF) was also identified. PSF is essential for the pre-mRNA splicing steps of intron release and exon ligation by regulating splice site selection (Gooding et al., 1998). TAR DNA-binding protein (TDP43) stabilizes mRNA through directly interacting with the 3'-untranslated region (3'UTR) (Strong et al., 2007). ELAV-like protein 1 (ELAVL1/HuR) as the only ARE (AU rich-rich element)-binding protein functions in stabilizing mRNA containing ARE (Tran et al., 2003). In addition of proteins with function in pre-mRNA maturation, a tRNA ligase complex was identified to associate with NS1-DDX1 complex. UPF0027 protein C22orf28 (HSPC117) as the only catalytic subunit together with FAM98, UPF0568 and C14orf166 in tRNA ligase complex (Popow et al., 2011) were all identified to associate with NS1-DDX100 complex, which increases the confidence of their genuine partnership.

Discussion

In this present study, the DEAD-box protein DDX100 was verified to interact with influenza virus protein NS1 and this interaction was required for influenza viral NS1 gene replication, transcription, translation, dsRNA unwinding and eventual whole virus replication. We found that NS1 recruited DDX100 for viral dsRNA unwinding, which not only promotes viral RNA replication, but also counteracts dsRNA-induced host cell immune defenses. Consider the significance of this interaction, we further screened other cellular proteins in NS1-DDX100 complex and found most of identified proteins shared the same function in pre-mRNA maturation, suggesting the interaction between NS1 and DDX100 may affect viral or cellular mRNA maturation.

The DEAD-box proteins were reported to contribute to virus replication in various viral systems through interactions with various viral proteins. HIV-1 Rev protein required for viral mRNAs nuclear/cytoplasmic trafficking and the generation of infectious HIV-1 virion was reported to associate with DDX100 to maintain the proper subcellular distribution of lentiviral regulatory protein (Fang et al., 2004). NS proteins of severe acute respiratory syndrome coronavirus (SARS-Cov) and infectious bronchitis virus (IBV) were reported to interact with DDX100 for the efficient virus replication in host cells (Xu et al., 2010). Besides DDX100, DDX3 was reported to assist hepatitis C virus (HCV) RNA replication by binding to HCV core protein (Ariumi et al., 2007) and play an enhancement role on HIV-1 RNA nuclear export (Yedavalli et al., 2004). Thus, the beneficial roles of DEAD-box proteins, especially DDX100, in other viral systems boost the confidence of our findings in influenza viral system.

DDX100-induced increase in NS1 gene replication and transcription may result from the helicase effect of DDX100 on viral NS1 dsRNA unwinding. During influenza viral genome replication, the (-) strand genome RNAs serve as templates to synthesize the complementary intermediate RNA (cRNA) with a transient phase of dsRNA existence. Once dsRNA is broken down to one (-) strand and one (+) strand RNA, (-) strand vRNAs are assembled into new virions and (+) strand cRNAs are either served as the templates to replicate more (-) strand vRNAs or proceeded to generate viral mRNA with addition of 5' capped primer and 3' poly (A) tail. Our results about significant decrease in NS1 vRNA and mRNA in DDX100-silenced cells suggested that the insufficient unwinding of NS1 dsRNA, rather than insufficient

NS1 mRNA nuclear export, resulted from DDX100 depletion caused less release of both (-) and (+) strand RNAs.

There might be more than one pathway utilized by DDX100-dsRNA to induce type I IFNs. DDX100 was reported to sense dsRNA to activate type I IFN response through TRIF pathway (Zhang et al., 2011). PKR, also known as dsRNA-activated protein kinase, was reported to detect viral dsRNA to initiate IFNs transcription through NF-κB (Lu et al., 1995; Wang et al., 2000). Two pathways used by DDX100-dsRNA to induce type I IFNs may explain our result that the level of IFN-β not IFN-α was increased by virus infection when DDX100 expression was knocked down. Silencing of DDX100 expression increased freer viral dsRNA, which induces transcription of both IFN-β and IFN-α by PKR-NF-κB pathway, while, according to Zhang etc (Zhang et al., 2011), silencing of DDX100 should reduce DDX100 capability to sense dsRNA with the consequence of TRIF pathway inactivation. Since TRIF pathway is mainly utilized by TLR3/4 for IFN-α production (Richez et al., 2009), the inactivated TRIF pathway may lead to decrease in IFN-α transcription, which compromises the IFN-α level induced by the activated PKR pathway.

Viral dsRNA unwinding benefits both virus genome replication and virus escape from host immune recognition. The loss of dsRNA unwinding would expose dsRNA to be recognized by host immune system to trigger immune responses and one of triggered immune responses is IFN secretion. IFNs induce murine Mx1 protein, which inhibits influenza virus RNA replication and transcription (Broni et al., 1990; Krug et al., 1985). IFNs also promote cytotoxic T cells to recognize virus by enhancing MHC I expression, activate NK cells to kill virus-infected cells or induce virus-infected cell apoptosis to limit virus infection. The loss of dsRNA unwinding would also decrease virus replication suggested by our results about significant decrease in NS1 gene replication and transcription induced by DDX100 silence (Fig. 3). So virus must evolve mechanism(s) to unwind viral dsRNA and DEAD-box family is likely one of the most potential candidates to take responsibility to unwind viral dsRNA. Besides the DDX100 present here, another DEAD-box family protein, UAP56, was reported to unwind influenza viral dsRNA through viral NP protein (Wisskirchen et al., 2011).

In summary, we found that the interaction between NS1 and DDX100 contributes to virus replication potentially through facilitating NS1 gene replication, transcription and viral dsRNA unwinding, which supplies the strategy for antiviral therapy development in the future. The identification of other cellular

proteins in NS1-DDX100 complex suggests that the interaction between NS1 and DDX100 may affect pre-mRNA maturation, especially pre-mRNA splicing, and cellular tRNA ligation, which might be interpreted in the future.

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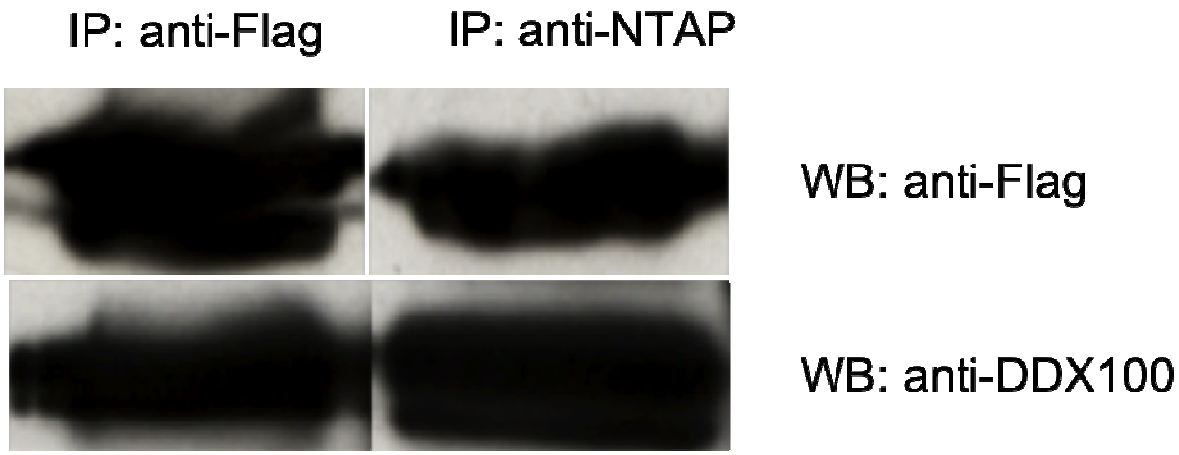

Fig. 1. Validation of interaction between NS1 and DDX100 by two-step affinity precipitations. Cell lysates from the cells co-transfected with NTAP-DDX100 and Flag-NS1 plasmids were precipitated by immobilized anti-Flag M2 resin to pull down Flag-NS1 complex in the first step affinity precipitation; in the second affinity precipitation, NTAP-DDX100 complex was pulled down by immobilized anti-NTAP IgG beads from the first step precipitated Flag-NS1 complex. Both NS1 and DDX100 precipitated complexes were subjected to Western blotting with anti-DDX100 and anti-Flag antibodies.

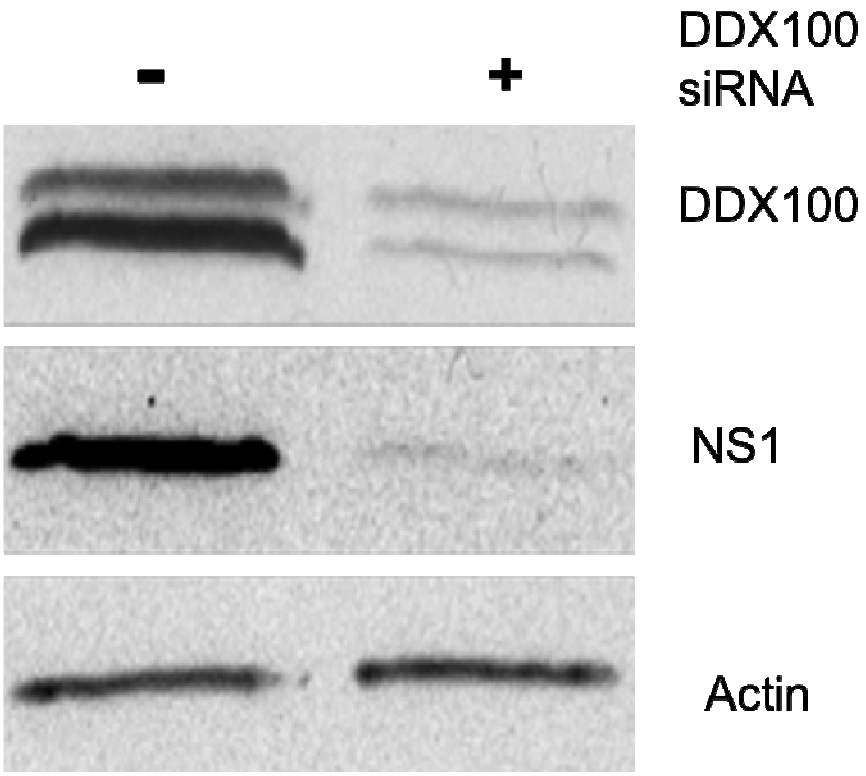

Fig. 2. Silencing of endogenous DDX100 reduces NS1 protein expression. A549 cells were transfected with a randomized siRNA sequence (control) or a siRNA sequence targeting DDX100. Forty-eight h after the transfection, the cells were infected with A/PR/8/34 viruses at an MOI of 2 and harvested at 24 hpi for examination of the expression of NS1 and DDX100 by Western blotting. Actin was used as a loading control.

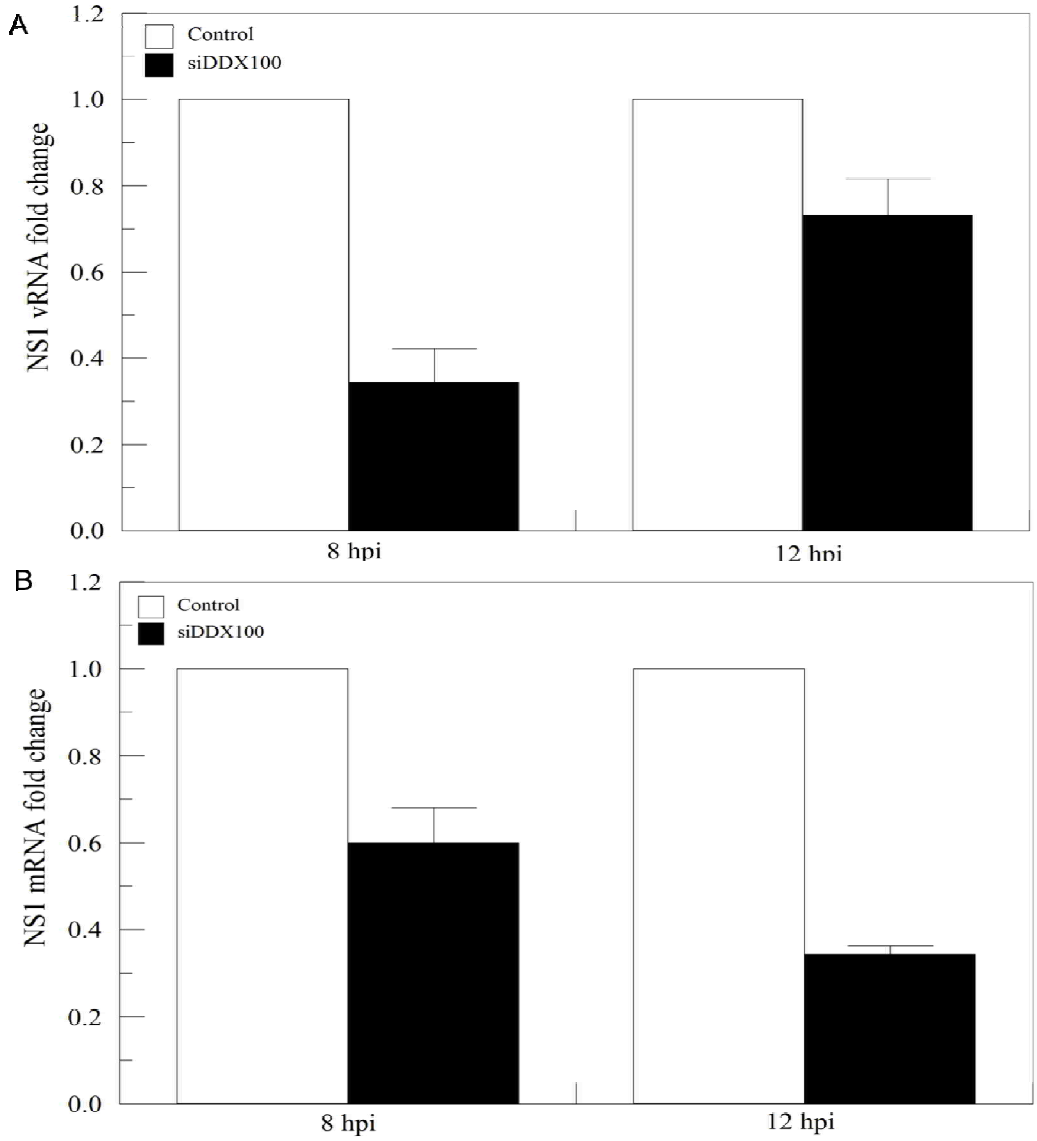

Fig. 3. DDX100 promotes both replication and transcription of NS1 gene. A549 cells were transfected with a randomized siRNA sequence (control) or a siRNA sequence targeting DDX100. Forty-eight h after the transfection, the cells were infected with A/PR/8/34 viruses at an MOI of 2 and harvested at 8 and 12 hpi for examination of NS1 gene replication (A) and transcription (B) by qRT-PCR. Values are the means + S.E. of three separate sample preparations. The values for controls in both (A) and (B) were set to 1. The values for DDX100-silenced samples in (A) were 0.34 ± 0.08 and 0.73 ± 0.09, and in (B) were 0.6 ± 0.08 and 0.34 ± 0.02 at 8 hpi and 12 hpi, respectively. siDDX100, DDX100 silencing by siRNA.

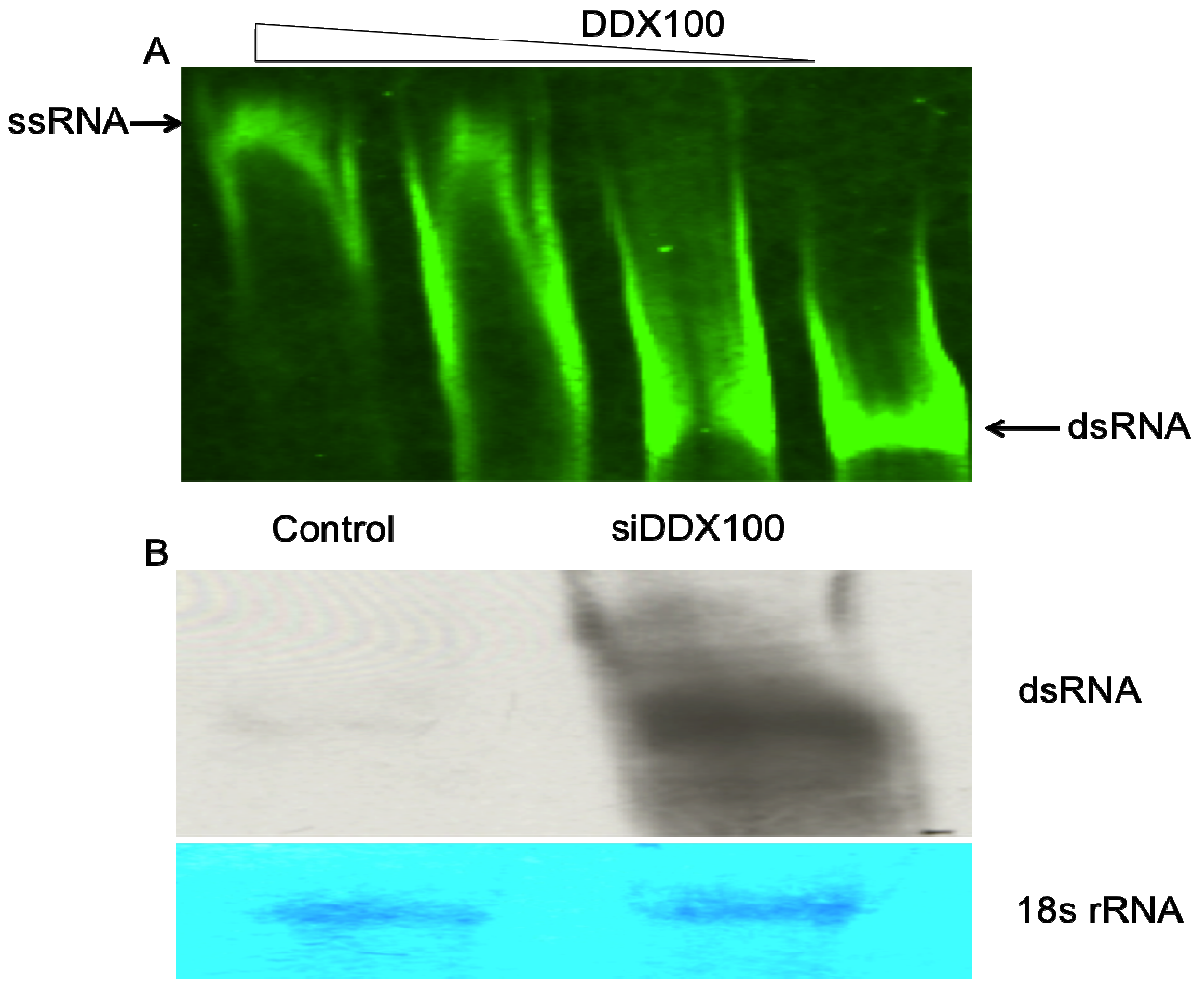

Fig. 4. DDX100 unwinds viral NS1 dsRNA. A, DDX100 unwinds viral NS1 dsRNA *in vitro*. The precipitated DDX100 on immobilized anti-Flag M2 resin with increased amount was incubated with biotin UTP labeled NS1 dsRNA, and both dsRNA and unwound ssRNA was simultaneously examined by streptavidin as described in Materials and Method section. B, DDX100 unwinds viral NS1 dsRNA *in vivo*. Vero cells were transfected with a randomized siRNA sequence (control) or a siRNA sequence targeting DDX100, followed by introduction of NS1 dsRNA (no biotin UTP labeling) and incubation for 8 h. After cells were harvested and total RNA was isolated, the introduced dsRNA was detected by immunoblotting with anti-dsRNA J2 antibody, followed by methylene blue staining on membrane. 18s rRNA was shown as loading control. This immunoblotting was repeated at least three times with different sample preparation.

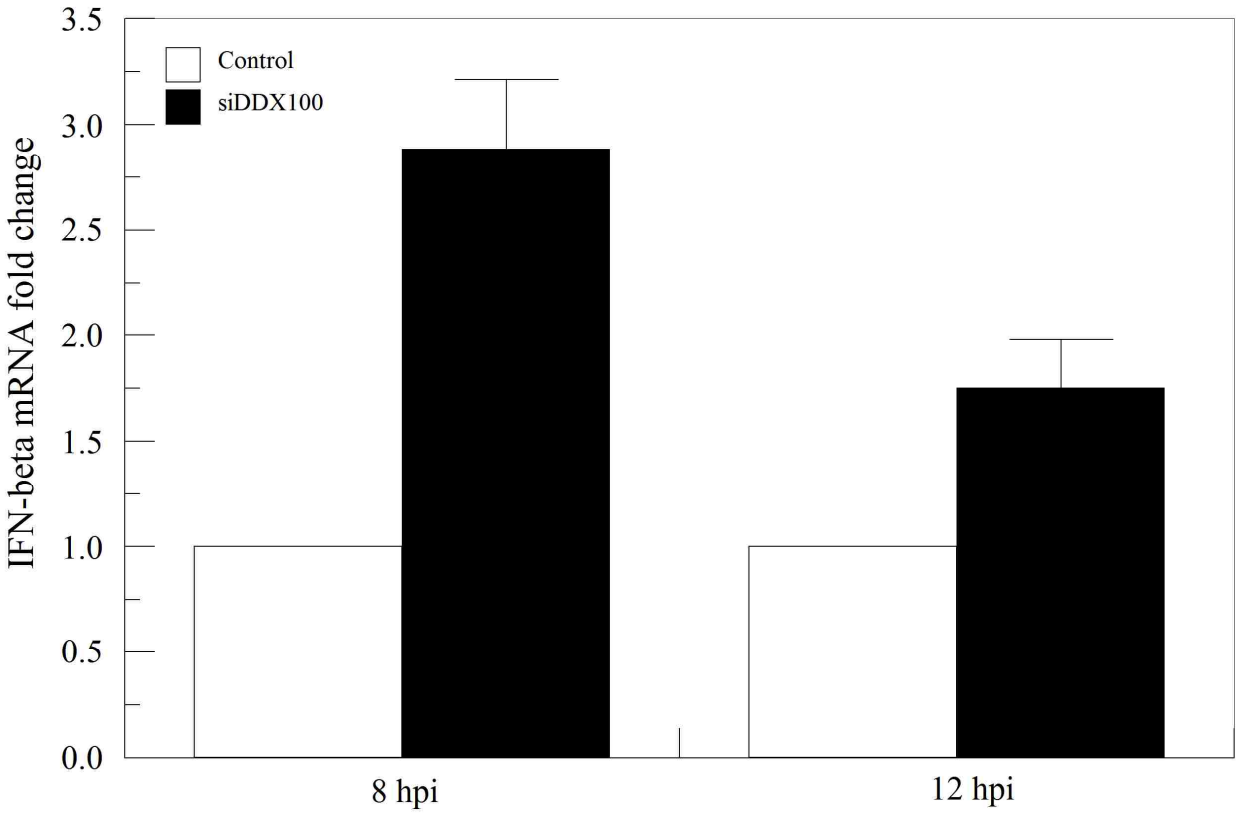

Fig. 5. Silencing of endogenous DDX100 enhances IFN-β production. A549 cells were transfected with a randomized siRNA sequence (control) or a siRNA sequence targeting DDX100, followed by infection of A/PR/8/34 viruses at an MOI of 2 for 8 or 12 h. After cells were harvested and total RNA was isolated, IFN-β mRNA level was determined by qRT-PCR. Values are the means + S.E. of three separate sample preparations. The values for controls were set to 1. The values for DDX100-silenced samples were 2.88 ± 0.33 and 1.75 ± 0.23 at 8 hpi and 12 hpi, respectively.

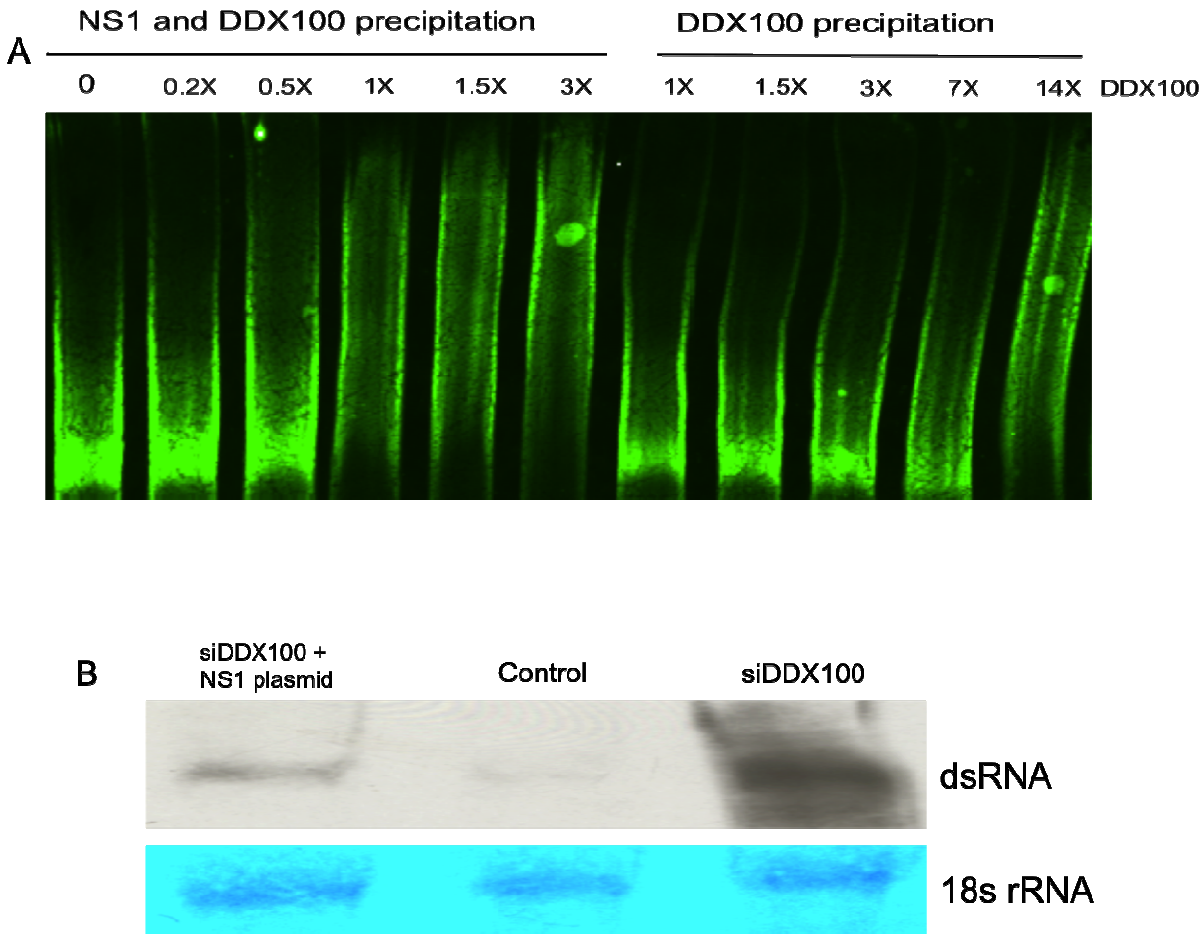

Fig. 6. NS1 recruits DDX100 to unwind viral NS1 dsRNA. A, NS1 promotes DDX100 to unwind viral NS1 dsRNA *in vitro*. Cell lysates from cells transfected with plasmids of Flag-DDX100 and non-tagged NS1 or plasmid of Flag-DDX100 alone as control were precipitated by immobilized anti-Flag M2 resin. The precipitated DDX100 in two groups of cells was quantified by Western blotting with anti-DDX100 antibody and the concentration of DDX100 in two groups was adjusted to be equal. The precipitated DDX100 with increased amount was incubated with biotin UTP labeled NS1 dsRNA *in vitro*, and both dsRNA and unwound ssRNA was simultaneously detected as Fig. 4A. B, NS1 promotes DDX100 to unwind viral NS1 dsRNA *in vivo*. Vero cells were mock-treated or treated with DDX100 specific siRNA alone or DDX100 specific siRNA plus NS1 expression plasmid, followed by introduction of NS1 dsRNA (no biotin UTP labeling) and incubation for 8 h. dsRNA immunoblotting was carried out as Fig. 4B.

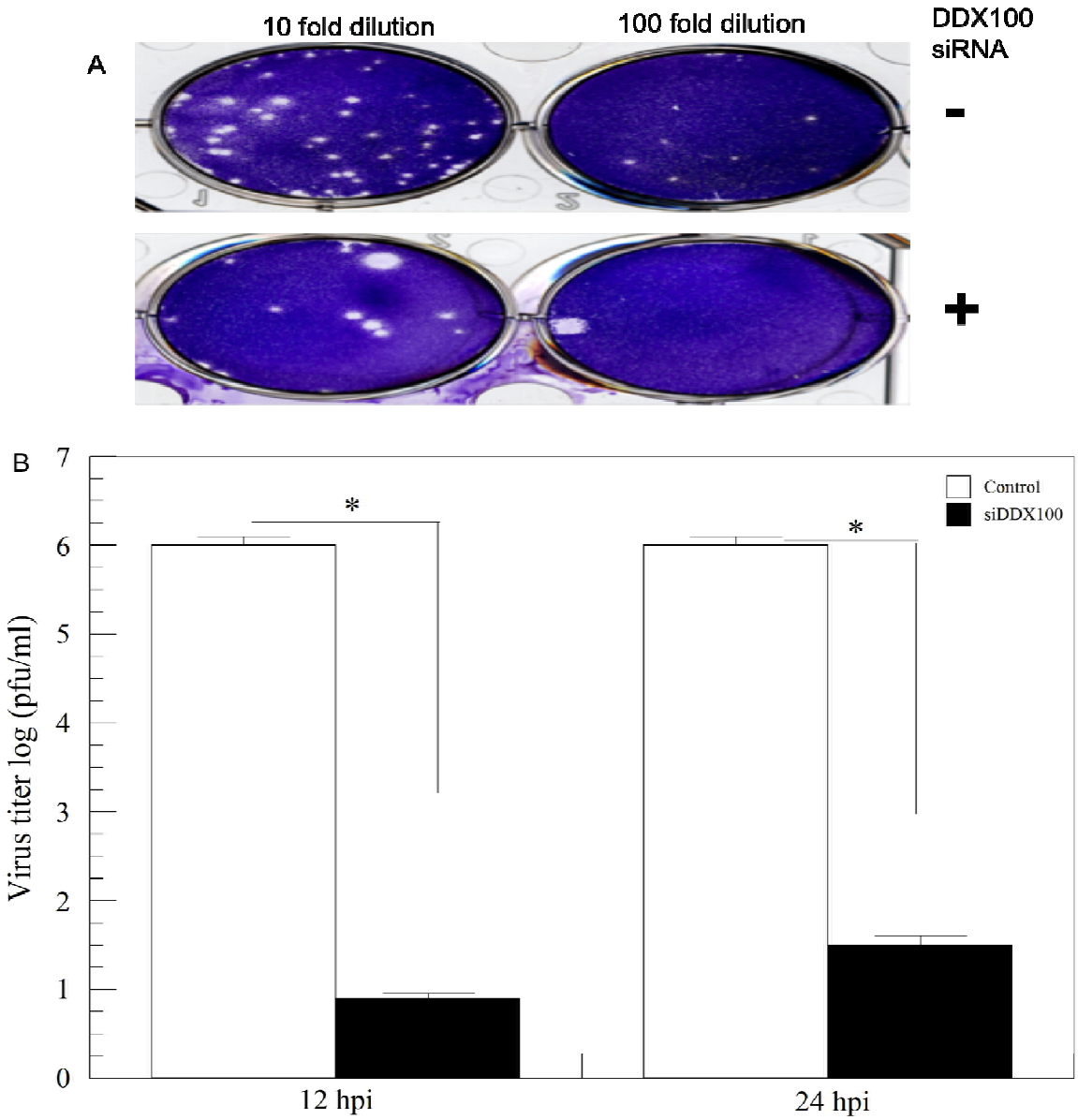

Fig. 7. Silencing of endogenous DDX100 reduces virus replication. A549 cells transfected with a randomized siRNA sequence (control) or a siRNA sequence targeting DDX100 were infected by A/PR/8/34 viruses at an MOI of 2. The supernatants were harvested at the indicated times for examinations of virus titers. A representative plaque assay for titration of virus collected from control as well as DDX100 siRNA-treated cells was shown (A) and the titers of virus in supernatants collected from control and DDX100 siRNA-treated cells were shown (B). Values are the means + S.E. of three separate sample preparations. * denotes $p < 0.05$.

General Discussion and Conclusion

In the present study, we first examined how protein expression in human lung epithelial cells responded to influenza virus infection at the proteome level by using a two-dimensional gel electrophoresis-based proteomic method. We found that the expression of IκB kinase-gamma (IKKγ) was suppressed by influenza A virus infection. Functional analyses suggest that IKKγ and influenza virus are mutually inhibitory, and influenza viruses may inhibit IFN production through suppressing the expression of IKKγ during viral infection. We then focused on identifying and characterizing novel cellular proteins that are associated with a key influenza vrial protein that is a major virulence factor and contributes significantly to pathogenesis – NS1. The first protein that was identified to interact with NS1 was heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP A2/B1). Knockdown of hnRNP A2/B1 by siRNA resulted in increased synthesis of NS1 viral RNA and NS1 mRNA in the virus-infected cells. In addition, we found that hnRNP A2/B1 is associated with NS1 and NS2 mRNAs and siRNA-mediated knockdown of hnRNP A2/B1 promotes transport of NS1 mRNA from the nucleus to the cytoplasm in the infected cells. The results suggest that hnRNP A2/B1 plays an important inhibitory role in the replication of influenza A virus in host cells potentially through inhibiting NS1 gene replication, transcription, and NS1 mRNA nucleocytoplasmic translocation. The second cellular protein that was identified to interact with NS1 was RUVBL2, and functional studies demonstrated that influenza viral protein NS1 may inhibit the apoptosis of the infected cells in the absence of IFNs through interacting, hence protecting cellular RUVBL2. The third protein that was identified to interact with NS1 was a DEAD-box family member, designated DDX100. We found that knockdown of DDX100 by siRNA resulted in decreased synthesis of NS1 viral RNA and NS1 mRNA in the virus-infected cells. More importantly, we found that NS1 inhibits IFN production through promoting the unwinding of viral dsRNAs via enhancing the helicase activity of DDX100.

The results from the present study revealed several novel mechanisms underlying the interactions between host cells and influenza viruses. At the same time, our results also raised some questions that need to be addressed in the future. First, our results showed that hnRNP A2/B1 inhibits both viral NS1 mRNA transcription and NS1 mRNA nuclear export, two molecular precesses that are closely related to each other in cells. It is not clear at the present time whether hnRNP A2/B1 independently inhibits these

two molecular processes or if hnRNP A2/B1 affects one of them and the other is a secondary change. Second, based on our results and published resutls, we hypothezied that vRNP complex may be responsible for suppressing the expression of RUVBL2 in the virus-infected cells. This postulation needs to be experimentally tested. Third, multiple cellular proteins functioning in mRNA maturation were identified to associate with the NS1-DDX100 complex, suggesting that NS1-DDX100 complex may play a vital role in viral or cellular mRNA maturation. A further investigation in this area is warranted.

Influenza continues to be a major public health burden. Viral protein NS1 is a major virulence factor and contributes significantly to the pathogenesis of influenza A viruses. Our results should be valuable for understanding the novel aspects of the host-influenza A virus interactions and may shed light on designing new drugs for preventing or treating influenza virus infection.