Co-degradation of interferon signaling factor DDX3 by PB1-F2 as a basis for high virulence of 1918 pandemic influenza

Eun-Sook Park^{1,2}, Young Ho Byun^{3,4} , Soree Park¹, Yo Han Jang^{3,4}, Woo-Ry Han¹, Juhee Won¹ , Kyung Cho Cho⁵ , Doo Hyun Kim¹, Ah Ram Lee¹, Gu-Choul Shin¹ , Yong Kwang Park¹, Hong Seok Kang¹, Heewoo Sim¹, Yea Na Ha¹, Byeongjune Jae¹, Ahyun Son³, Paul Kim^{3,4}, Jieun Yu³, Hye-Min Lee³, Sun-Bin Kwon³, Kwang Pyo Kim⁵, Seung-Hyun Lee⁶, Yeong-Min Park⁷, Baik L Seong^{3,4,*} & Kyun-Hwan Kim^{1,2,8,**}

Abstract

The multifunctional influenza virus protein PB1-F2 plays several roles in deregulation of host innate immune responses and is a known immunopathology enhancer of the 1918 influenza pandemic. Here, we show that the 1918 PB1-F2 protein not only interferes with the mitochondria-dependent pathway of type I interferon (IFN) signaling, but also acquired a novel IFN antagonist function by targeting the DEAD-box helicase DDX3, a key downstream mediator in antiviral interferon signaling, toward proteasome-dependent degradation. Interactome analysis revealed that 1918 PB1-F2, but not PR8 PB1-F2, binds to DDX3 and causes its co-degradation. Consistent with intrinsic protein instability as basis for this gain-of-function, internal structural disorder is associated with the unique cytotoxic sequences of the 1918 PB1-F2 protein. Infusing mice with recombinant DDX3 protein completely rescued them from lethal infection with the 1918 PB1-F2-producing virus. Alongside NS1 protein, 1918 PB1-F2 therefore constitutes a potent IFN antagonist causative for the severe pathogenicity of the 1918 influenza strain. Our identification of molecular determinants of pathogenesis should be useful for the future design of new antiviral strategies against influenza pandemics.

Keywords 1918 pandemic; DDX3; influenza PB1-F2; type I interferon signaling

Subject Categories Immunology; Microbiology, Virology & Host Pathogen Interaction

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Introduction

The Spanish influenza of 1918 remains the deadliest infectious disease in human history that claimed up to 50 million victims worldwide (Beveridge, 1991; Tumpey *et al.*, 2005). As compared against the 1957 and 1968 pandemic strains, which were reassortants of avian viruses, the genetic origin of the 1918 strain appears to be unique, calling for a different scope and focus of surveillance and preventive efforts (Reid *et al.*, 2004). The extremely high pathogenicity among humans has been ascribed to the host tropism by hemagglutinin (HA) (Tumpey *et al.*, 2007; Zambon, 2007). The non-structural PB1-F2, initially identified as a+1 open reading frame (ORF) of PB1 gene segment (Chen *et al.*, 2001), plays a multifunctional role in the deregulation of innate immune responses, including the inhibition of type I interferon (IFN) induction by targeting mitochondrial MAVS (Varga *et al.*, 2011, 2012). Amino acid substitutions in the PB1-F2 protein contribute to its stability and functions (Alymova *et al.*, 2014; Cheng *et al.*, 2017). Notably, 1918 PB1-F2 is involved in the unique immunopathology of secondary bacterial infections (Lamb & Takeda, 2001; Conenello & Palese, 2007; Kobasa *et al.*, 2007; McAuley *et al.*, 2007, 2010), which contributes significantly to pathogenesis (Zamarin *et al.*, 2006).

Being part of the first line of defense against infection, type I interferons are key components of the host antiviral innate immune response and modulators of adaptive immune responses (García-Sastre & Biron, 2006). To foil the host defense system, influenza viruses developed strategies to attenuate IFN responses. The NS1 protein of highly pathogenic influenza viruses, such as the H5N1

1 Department of Pharmacology, Center for Cancer Research and Diagnostic Medicine, IBST, School of Medicine, Konkuk University, Seoul, Korea

2 KU Open Innovation Center, Konkuk University, Seoul, Korea

3 Department of Biotechnology, College of Life Science and Biotechnology, Yonsei University, Seoul, Korea

4 Vaccine Translational Research Center, Yonsei University, Seoul, Korea

5 Department of Applied Chemistry, Kyung Hee University, Yongin, Korea

6 Department of Microbiology, School of Medicine, Konkuk University, Seoul, Korea

7 Laboratory of Dendritic Cell Differentiation and Regulation, Department of Immunology, School of Medicine, Konkuk University, Seoul, Korea

8 Research Institute of Medical Science, Konkuk University, Seoul, Korea

*Corresponding author. Tel: +82 2 2123-2885; E-mail: blseong@yonsei.ac.kr

**Corresponding author. Tel: +82 2 2030 7833; Fax: +82 2 2049 6192; E-mail: khkim10@kku.ac.kr

avian influenza virus and the 1918 pandemic virus, strongly suppresses type I IFNs (Geiss *et al*, 2002; Seo *et al*, 2002). These viruses also lead to the acute recruitment of neutrophils, severe lung injury, and aggressive inflammatory cytokine production in IFN α / β receptor 1-deficient (*Ifnar1*^{-/-}) mice (Kobasa *et al*, 2007; Perrone *et al*, 2008; Cillóniz *et al*, 2009). Compared to the PB1-F2 proteins of influenza epidemic viruses, that of the 1918 pandemic (1918 PB1-F2) enhances the pathogenesis of viral infection to a much greater extent (McAuley *et al*, 2007). However, the host target(s) and the molecular mechanism underlying this high pathogenicity remain elusive (Conenello & Palese, 2007). The reconstruction of the 1918 virus (Reid *et al*, 2004; Tumpey *et al*, 2005, 2007) and subsequent landmark discoveries on PB1-F2 as a virulence factor (Zamarin *et al*, 2006; Conenello & Palese, 2007; McAuley *et al*, 2007, 2010) prompted us to address this unresolved issue associated with the most devastating infection in human history.

Through comparative interactome analysis between the two isogenic viruses differing only in PB1-F2, we showed that 1918 PB1-F2 hijacks the key mediator of IFN signaling, acquiring a new gain-of-function of shutting off the antiviral response (García-Sastre & Biron, 2006). Our results reveal a novel molecular mechanism behind the unparalleled high mortality of the 1918 pandemic and may help to design novel antiviral strategies.

Results

The 1918 PB1-F2 protein is very unstable and is degraded by the ubiquitin–proteasome pathway

We first compared the expression profiles of the PB1-F2 proteins in infected cells. A549 and U937 cells were infected with either IAV (PR8) or IAV (1918), two isogenic viruses with the PR8 backbone that differ only in their PB1-F2 and the corresponding PB1 region. IAV (PB1-F2[-]), a mutant virus that does not express PB1-F2, was used as a negative control. In both cell lines, the expression level of 1918 PB1-F2 was very low compared to that of PR8 PB1-F2 (Fig 1A). However, the level of the 1918 PB1-F2 transcript was similar to that of PR8 PB1-F2 (Fig 1B). When treated with the proteasome inhibitor MG132, the level of the 1918 PB1-F2 protein recovered significantly. We obtained similar results using an immunofluorescence assay (Fig 1C). Both 1918 and PR8 PB1-F2 proteins were heavily ubiquitinated in infected cells (Fig 1D), demonstrating that the very low steady-state level of the 1918 PB1-F2 protein in cells was due to its degradation by the ubiquitin–proteasome pathway.

Amino acid residues 68 and 69 determine the characteristics of the 1918 PB1-F2 protein

The 1918 PB1-F2 protein is highly divergent in comparison with PR8 PB1-F2 (12 amino acid substitutions; Fig 2A). To narrow down the molecular determinants of its instability, we constructed various mutants containing the sequences of PR8 or 1918 PB1-F2 (Fig 2A). Comparing the chimeric PB1-F2 mutants, the C-terminus of 1918 PB1-F2 (1918-C) was found responsible for the low stability (Fig 2B). Site-specific mutations within the C-terminus showed that both positions 68 and 69 of the 1918 strain crucially reduced the stability of PB1-F2 (Fig 2C). Conversely, if a PR8 amino acid was

introduced into the 1918 backbone at the corresponding positions, the stability was restored (Fig 2D). Moreover, interesting changes in cellular localization was noted; PR8 PB1-F2 was localized predominantly in mitochondria as punctate structures, whereas 1918 PB1-F2 was detected both in the cytoplasm and the nucleus as diffused expression pattern (Figs 2E and EV1; summarized in Table EV1). The changes in subcellular localizations by mutations are consistent with recent studies on H5N1 and further extend to 1918 PB1-F2 with relevance to enhanced virulence (Cheng *et al*, 2017). Thus, biochemical and functional characteristics of 1918 PB1-F2-stability, cellular localization, expression pattern, and inhibition of IFN induction (see below) all correlated with the I68T and L69P mutations (Table EV1).

The 1918 PB1-F2 protein strongly inhibits IFN β induction

We then examined potential effects of PB1-F2 on key molecules in the signaling pathways of IFN β . We observed only marginal effects on NF- κ B, pro-inflammatory cytokines (Fig 3A), and IFN α expression (Fig EV2). However, the induction of IFN β , a critical element of the innate immune system, was strongly inhibited by the expression of 1918 PB1-F2 in two different cell lines; in contrast, PR8 PB1-F2 had no effect (Fig 3B). Furthermore, the expression of 1918 PB1-F2 but not that of PR8 PB1-F2 strongly suppressed polyI:C-induced IFN β activation in the reporter assay (Fig 3C). Consistent with the reporter system *in vitro*, infection of mice with IAV (1918) resulted in a strong suppression of IFN β , whereas infection with either IAV (PR8) or IAV (PB1-F2[-]) did not (Fig 3D). The suppression of IFN β was accompanied by a low level of PB1-F2 expression (Fig 3D). Of note, IFN β inhibition by 1918 PB1-F2 was more prominent than by NS1 (Fig 3C), a previously established IFN antagonist in influenza viruses (Geiss *et al*, 2002; García-Sastre & Biron, 2006). These data suggest that 1918 virus acquired an ability to inhibit the type I IFN response uniquely associated with the in-cell stability of PB1-F2.

Proteasome-dependent degradation of 1918 PB1-F2 is linked to the potent inhibition of type I IFN induction

The inhibition of IFN β induction by 1918 PB1-F2 was abrogated by MG132 treatment (Fig 3E). Thus, a strong association between the inhibition of IFN β induction (Fig 3B) and the instability of PB1-F2 (Fig 2) was confirmed in two independent cell lines. When the 1918-specific mutations (I68T and L69P) were introduced into PR8 PB1-F2, IFN β induction was dramatically inhibited, and the inhibition was associated with the instability of PB1-F2 (Fig 3F). Likewise, when the PR8 sequence (T68I and P69L) was introduced back into 1918 PB1-F2, IFN β induction was restored, along with a concomitant increase in PB1-F2 stability. These results confirm that the instability of 1918 PB1-F2 is invariably associated with a potent inhibition of IFN β .

Amino acids 68 and 69 of 1918 PB1-F2 contribute to viral pathogenicity

We next compared the pathogenicity of recombinant viruses in mice. Higher mortality was observed in mice infected with IAV (1918) than in mice infected with IAV (PR8), suggesting that 1918 PB1-F2 is indeed responsible for the increased virulence (Fig 4A).

Figure 1. The stability of the 1918 PB1-F2 protein is very low, and it is degraded by the Ub-dependent proteasome pathway.

A A549 and U937 cells were infected with each IAV at MOI 1 or 5 for 24 h, and lysates were analyzed by Western blotting using polyclonal anti-PB1-F2 antibody.

B A549 cells were transfected with a plasmid encoding Flag-tagged PB1-F2 derived from the PR8 or 1918 strain and treated with the proteasome inhibitor MG132 (25 μ M) or vehicle for 6 h before harvesting. PB1-F2 transcripts were analyzed by semi-quantitative RT-PCR (top panel), and the PB1-F2 protein was analyzed by Western blotting with monoclonal anti-Flag antibody (bottom panel).

C Representative immunofluorescence images showing expression of PB1-F2 (red). A549 cells were transfected with plasmids encoding indicated PB1-F2. At 18 h after transfection, cells were treated with MG132 for 6 h and subjected to immunofluorescence analysis. Magnification, $\times 100$; scale bar, 100 μ m.

D Each HA-tagged PB1-F2 was transfected into 293T cells with or without Flag-ubiquitin. At 18 h after transfection, cells were treated with MG132 for 6 h. The lysates were immunoprecipitated with anti-HA antibody and analyzed by Western blotting with anti-Flag antibody. PR8, A/Puerto Rico/8/34 (H1N1); 1918, A/Brevig Mission/1/1918 (H1N1).

Data information: Results shown are representative of three independent experiments. Source data are available online for this figure.

All mice infected with 500 pfu of IAV (PR8) or IAV (PB1-F2[-]) recovered from weight loss and survived after 2 weeks post-infection (Fig 4B). However, the virulence of IAV (I68T), IAV (L69P), or IAV (I68T/L69P) was much higher and comparable to that of IAV (1918) (Fig 4B). The virulence at different infection doses showed that both I68T and L69P mutations, in particular, among sequence variations between PR8 and 1918 PB1-F2 (Fig 2A), contributed to the high pathogenicity of IAV (1918) (Figs EV3A and B, and 4C), consistent with the definition of cytotoxic sequences (Conenello & Palese, 2007; Alymova *et al*, 2014; Cheng *et al*, 2017). Structural prediction *in silico* (Obradovic *et al*, 2005; Xue *et al*, 2010) revealed that PB1-F2 belongs to a family of intrinsically disordered proteins (IDPs) and has a high propensity

for disorder at the N-terminus and extreme C-terminus (Fig EV3C). Interestingly, in contrast to PR8, 1918 PB1-F2 had an additional disordered region which was affected by substitutions at positions 68 and 69. Of note, the elements identified in 1918 PB1-F2 that are crucial for increased pathogenicity mapped within the internal intrinsically disordered region (IDR) that encompasses the positions previously identified as cytotoxic sequences (Conenello & Palese, 2007; Alymova *et al*, 2014; Cheng *et al*, 2017). The results suggest that the biochemical instabilities that lead to vulnerability to proteasomal degradation (Fig 2) and the consequent suppression of IFN responses (Figs 3 and 4) are associated with the unique structural perturbation within the 1918 PB1-F2 protein.

Figure 2. Ile68 and Leu69 in the C-terminal part of PB1-F2 determine its stability.

A Amino acid sequences of PR8 PB1-F2, 1918 PB1-F2, and the cloned PB1-F2 mutants.
B–D A549 cells were transfected with the indicated plasmids encoding Flag-tagged PB1-F2 derived from the PR8 or 1918 strain and chimeric PB1-F2 mutants. At 18 h after transfection, cells were treated with or without MG132 for 6 h. The expression of PB1-F2 and control genes was detected by semi-quantitative RT-PCR (upper panel) or Western blotting (lower panel).
E Representative immunofluorescence images of PB1-F2 expression in A549 cells. Cells transfected with the Flag-tagged PB1-F2 plasmid were stained with anti-Flag (red) antibody and analyzed by immunofluorescence assay. Magnification, $\times 400$; scale bar, 50 μ m.
 Data information: Results shown are representative of three independent experiments.

The 1918 PB1-F2 protein inhibits the induction of IFN β in mice

To further address whether 1918 PB1-F2 inhibits the induction of IFN β , we assessed the levels of viral proteins in mouse lung tissues 2 days post-infection (500 pfu). The expression levels of viral proteins including HA and NP were higher in the IAV (1918) group than in the IAV (PR8) or IAV (PB1-F2[-]) group, suggesting a much more robust infection with IAV (1918) (Fig 4D). Indeed, the peak lung viral titer was significantly higher with IAV (1918) than with the two other viruses (Fig 4E). Despite higher replication, the expression level of PB1-F2 was remarkably lower with IAV (1918) (Fig 4D). The level of IFN β , both in mRNA transcript and protein in the lung of infected mice, was significantly lower with IAV (1918) than with IAV (PR8) or IAV (PB1-F2[-]) (Fig 4F and G). Collectively, these results suggest that 1918 PB1-F2 interferes with the induction of IFN β , which may lead to greater viral replication and delayed clearance of the virus in mice.

1918 PB1-F2 inhibits IFN β induction via interaction with DDX3

To elucidate the mechanism by which 1918 PB1-F2 inhibits IFN β induction, we analyzed its interactome in A549 cells overexpressing 1918 PB1-F2 (Fig 5A). Using LC-MS/MS, we identified a total of 134 proteins as 1918 PB1-F2 binding partners (Fig 5B). Based on the Ingenuity Pathway Analysis (IPA) to identify the biological

processes enriched in the 1918 PB1-F2 interactome (Fig 5C), we identified 22 PB1-F2-interacting proteins associated with “viral infection” (Table EV2). Focusing on this category, we performed network analysis (Fig 5D) and found several proteins known to interact with the IFN-signaling network (green circles in Fig 5D). Among them, DDX3X (DDX3), which is essential for the induction of antiviral type I IFNs (Fullam & Schröder, 2013; Gu *et al*, 2013), was identified at the top of the list of proteins differentially enriched in the 1918 PB1-F2 interactome (Table EV2).

Co-immunoprecipitation analysis showed that 1918 PB1-F2 bound DDX3 (Fig 5E, left panel). The interaction between endogenous DDX3 and virus-encoded 1918 PB1-F2 was also observed in infected cells. However, other DDX3-interacting proteins such as TBK1 and IKK ϵ did not associate with the 1918 PB1-F2–DDX3 complex in our experimental condition (Fig 5E, middle panel). To examine the direct interaction between DDX3 and 1918 PB1-F2, we purified recombinant proteins from transformed *Escherichia coli* (Fig EV4) and performed *in vitro* pulldown assay. Consistent with the co-immunoprecipitation data, 1918 PB1-F2 directly bound DDX3 (Fig 5E, right panel), suggesting that DDX3 is a real target of 1918 PB1-F2. Although PR8 PB1-F2 failed to bind DDX3, the binding was restored by the introduction of the 1918-specific mutations (Fig 5F). The level of DDX3 was upregulated by IFN β (Fig 5G). Since IAV (PR8) infection induced IFN β expression (Fig 3D), IAV (PR8) infection upregulated DDX3 expression, whereas IAV (1918) infection

Figure 3. Inhibition of IFN β induction and its association with proteasomal degradation of 1918 PB1-F2.

A Relative activity of NF- κ B luciferase reporter was measured in A549 cells transfected with plasmids encoding PB1-F2. The mRNA levels of pro-inflammatory cytokines were determined by semi-quantitative RT-PCR.

B Cells were transfected with PB1-F2-expressing plasmids. Relative IFN β mRNA levels were determined by semi-quantitative RT-PCR (top) and qPCR (bottom).

C U937 cells were co-transfected with the IFN β luciferase reporter plasmid and the PB1-F2 or NS1 expressing plasmid and treated with poly(I:C) for 12 h before harvesting. IFN β promoter activity was determined by luciferase reporter assay.

D Cells were infected for 24 h with each virus at MOI 1. The mRNA level of IFN β was determined by semi-quantitative RT-PCR, and PB1-F2 protein expression was assessed by Western blotting.

E Cells were transfected with each plasmid encoding PB1-F2. 18 h after transfection, cells were treated with MG132 for 6 h and the level of IFN β mRNA was determined by semi-quantitative RT-PCR (left) and qPCR (right). PB1-F2 protein expression was assessed by Western blotting.

F A549 cells were transfected with PB1-F2 clones mutated at positions 68 and 69; 24 h after transfection, the IFN β mRNA level and PB1-F2 protein expression were determined by semi-quantitative RT-PCR and Western blotting, respectively (left). The relative expression level of IFN β mRNA was also analyzed by qPCR (right).

Data information: All data are shown as mean ($\pm$ SEM) from at least three independent experiments (** P < 0.01, *** P < 0.001).

Source data are available online for this figure.

reduced it (Fig 5G). To clearly demonstrate that 1918 PB1-F2 leads to DDX3 degradation, we further examined the expression level of DDX3 after MG132 proteasome inhibitor treatment. The level of DDX3 was rescued by MG132 treatment in IAV(1918)-infected cells (Fig 5G). Overexpression of 1918 PB1-F2 also reduced the GFP-DDX3 level which was partially restored by MG132 treatment; however, overexpression of PR8 PB1-F2 showed no effect on its level (Fig 5H). Consistent with the A549 cell infection *in vitro*, only mice infected with IAV (1918) showed decreased levels of endogenous DDX3 in their lung tissues (Fig 5I). Considering that 1918 PB1-F2 is unstable and subject to proteasomal degradation (Fig 1), these results suggest that DDX3 is hijacked by PB1-F2 and is co-degraded via the proteasomal pathway.

Phosphorylation followed by nuclear translocation of IRF3, which is required for IFN β induction by external stimuli (Takeuchi & Akira, 2010), was reduced by the expression of 1918 PB1-F2

(Fig 5J). Of note, the phosphorylation and nuclear translocation of IRF3 was restored by the supplementation of DDX3 (Fig 5J). Furthermore, the inhibition of IFN β induction and its promoter activity by 1918 PB1-F2 was alleviated by the supplementation of DDX3 (Fig 5K). Taken together, these results suggest that 1918 PB1-F2 inhibits IFN β induction through interaction-mediated co-degradation of DDX3.

Recombinant DDX3 rescues mice from lethal IAV (1918) infection

We generated a recombinant DDX3 carrying an HIV-Tat-derived protein transduction domain (PTD). DDX3 is refractory to soluble expression in *E. coli*, probably due to its propensity to form amyloid fibers (Chevalier *et al.*, 2010; Vidic *et al.*, 2016; data not shown). The problem was circumvented by fusion to LysRS as a novel RNA interaction-mediated folding vehicle (Choi *et al.*, 2008,

Figure 4. IAV (1918) PB1-F2 increases viral pathogenicity in Balb/c mice and its amino acid residues 68 and 69 contribute to virulence.

- A Mice ($n = 5$ or 6 per group) were infected with IAV with indicated pfu. Survival was monitored daily for 2 weeks.
- B Mice ($n = 6$ – 9 per group) were infected with each IAV as indicated. Body weight was recorded daily after infection for 2 weeks.
- C Mice ($n = 5$ or 6 per group) were infected with viruses (1,000 pfu) carrying PR8 PB1-F2 with the I68T, L69P, or I68T+L69P mutation. Body weight changes and survival rate were monitored daily. Detailed analyses on the virulence necessitated the monitoring of body weight below the standard level (80%) of mortality.
- D Three cohorts of mice (5 or 6 per group) were infected with 500 pfu of one of the three IAVs. Viral protein expression in mouse lung was determined by Western blotting 2 days after infection.
- E Mice (6 per group) were infected with 500 pfu of one of the three IAVs. Viral titration by plaque assay was performed at 2 days post-infection using lung homogenates.
- F Mice (5–9 per group) were infected with 500 pfu of each virus. At 2 days post-infection, IFN β mRNA levels were determined by qPCR.
- G Mice (5–9 per group) were infected with 1×10^5 pfu of each virus. At 2 days post-infection, IFN β protein levels in lung homogenates were measured by ELISA.

2009) (Fig EV4B). LysRS-DDX3 was expressed predominantly as soluble form and purified (Figs EV4B and 5C). The ability of the protein to transduce into cultured cells was confirmed by Western blot and immunofluorescence assay (Figs EV4C and 4D).

The infusion with recombinant DDX3 rescued the mice completely from lethal IAV (1918) infection (10^5 pfu) (Fig 6B) and significantly reduced the lung viral titer (Fig 6C). Furthermore, IFN β induction was significantly increased in the lung tissues of infected mice by treatment with recombinant DDX3 (Fig 6D). Consistently, a similar treatment significantly inhibited viral growth as examined by plaque formation in MDCK cells *in vitro* (Figs EV5E and 5F). None of control viruses tested—PR8, avian virus (H5N2), or H3N2—were susceptible to DDX3 (Fig EV5G), confirming that the rescue from viral virulence is 1918-specific. Independent mouse experiments with different infection doses and dosing schedule confirmed these results (Figs EV5A and 5D). In agreement with these results, transduction of DDX3 in cell culture greatly reduced the viral titer after infection (Figs EV5E and 5F). Taken together, these data indicate that recombinant DDX3 rescues mice from lethal infection with IAV (1918) and that this rescue is associated with the restoration of the IFN β antiviral response.

Discussion

The causes of excess mortality during the 1918 pandemic, especially among healthy young adults, are poorly understood. Unique features of the 1918 H1N1 pandemic have been ascribed to the dysfunction in host inflammatory and immunological responses shaped by past influenza infections in the H1N1-naïve population (McAuley *et al*, 2015; Gagnon *et al*, 2018). The lack of an adaptive immune response in this age group (unlike in older adults), and consequent reliance on the innate antiviral response, could explain the detrimental effects of innate-type I IFN suppression as reported here.

As a molecular basis of high pathogenicity of 1918 pandemic, here we showed that the 1918 PB1-F2 protein uncouples the type I IFN response from viral infection by hijacking a key mediator protein DDX3 into proteasomal degradation (Fig 3). As an integral part of the type I IFN response during influenza virus infection, RIG-I-dependent signal transduction requires the multi-protein complexes comprising DDX3, I κ B kinase ϵ (IKK ϵ), and TANK-binding kinase 1 (TBK1) for the phosphorylation and subsequent nuclear translocation of IRF3, which in turn induces type I IFNs (Takeuchi & Akira, 2010). Previously, DDX3 was shown to interact

with the influenza NS1 and NP, exerting antiviral function through regulation of stress granule formation (Thulasi Raman *et al*, 2016). Here, the depletion of DDX3 via proteasomal degradation inhibiting IRF3 phosphorylation and effectively uncoupling viral infection from the IFN β response is suggested to contribute to the enhanced virulence of 1918 pandemic (Fig 6E). The overexpression of DDX3

in human cells does not appreciably enhance the IFN β induction (Fig 5K), suggesting that the level of DDX3 in cells is tightly regulated by the stoichiometric balance among complex components. Therefore, any small changes in the level of the DDX3 protein caused by 1918 PB1-F2 may lead to a significant impact on IFN signaling (Fig 5G–K).

Figure 5.

Figure 5. 1918 PB1-F2 inhibits IFN β production via an interaction with DDX3.

- A SDS-PAGE analysis of proteins immunoprecipitated from A549 cell lysates using HA antibody. Bands were stained by Coomassie Blue.
- B Venn diagrams classifying the proteins identified in immunoprecipitated samples. A total of 318 proteins were identified by LC-MS/MS analysis.
- C The list of top ten networks obtained from Ingenuity Pathway Analysis of the 1918 PB1-F2 interactome.
- D The “viral infection” network of interactome proteins. Proteins shaded in gray directly interact with 1918 PB1-F2. Interferon signaling pathway is shown in yellow. Solid lines, protein–protein interactions; solid arrows, phosphorylation; dotted arrows, induction.
- E Analysis of interaction between DDX3 and 1918 PB1-F2. Cells were transfected with indicated plasmids; 18 h later, cells were treated with MG132 for 6 h. Cell lysates were immunoprecipitated with anti-HA antibody. Proteins were detected by immunoblotting with the indicated antibodies (left panel). IAV-infected cell lysates were immunoprecipitated with anti-PB1-F2 antibody and immunoblotted with the indicated antibodies (middle panel). Purified recombinant 1918 PB1-F2 protein was incubated with recombinant LysRS or LysRS-DDX3, and the interaction was analyzed by immunoblotting (right panel).
- F Analysis of interaction between DDX3 and PB1-F2 mutants. A549 cells were transfected with the indicated clones; 18 h after transfection, cells were treated with MG132 for 6 h and analyzed by immunoprecipitation.
- G A549 cells were treated with IFN β for the indicated time periods and subjected to Western blotting (top). A549 cells were infected with indicated IAV. At 24 h post-infection, cell lysates were analyzed by Western blotting (bottom).
- H Representative fluorescence images showing expression level of GFP-DDX3 (green). A549 cells were co-transfected with plasmids encoding the GFP-DDX3 and Flag-tagged PR8 or 1918 PB1-F2. At 18 h after transfection, cells were treated with MG132 for 6 h and subjected to fluorescence analysis.
- I Mice were infected with 1×10^5 pfu of each virus, and lung homogenates were examined for endogenous Ddx3 expression.
- J A549 cells were transfected with the indicated plasmids or treated with poly I:C. Cytoplasmic and nuclear fractions were separated and subjected to Western blotting.
- K A549 cells were transfected with the indicated plasmids, and IFN β mRNA levels were determined by semi-quantitative RT-PCR (top) and qPCR (middle). IFN β promoter activity was determined by luciferase reporter assay (bottom).

Data information: Results were confirmed in three independent experiments, and representative data are shown.
Source data are available online for this figure.

Figure 6. DDX3 rescues mice from 1918 PB1-F2 virus infection.

- A–D Groups of five mice were intranasally infected with IAV (1918), and recombinant DDX3 protein was administered intranasally. (A) Scheme of the experiments. (B) Survival was monitored daily for 2 weeks. (C) Two days post-infection, virus titers in lung homogenates were determined by plaque assay. (D) Two days post-infection, the expression level of IFN β in the lungs was analyzed by semi-quantitative RT-PCR (left panel) and qPCR (right panel). ** $P < 0.01$, *** $P < 0.001$.
- E Proposed model for innate immune evasion by the 1918 pandemic virus.

Source data are available online for this figure.

The acquisition of a novel unique function by 1918 PB1-F2 is intriguing. While consistent with an intrinsic instability of the protein (Cheng *et al*, 2017), the natively unstructured and dynamic

nature of the protein is associated with a new gain-of-function as relevant to foiling the host antiviral responses by viral infection (García-Sastre & Biron, 2006). Of note, the new function is indirectly

reflected in the changes in both expression pattern and its subcellular localization (Table EV1). PB1-F2 from PR8 virus was shown to inhibit the induction of type I interferon by binding to MAVS and decreasing mitochondrial membrane potential (Varga *et al*, 2011, 2012), probably due to its intrinsic properties to target mitochondrial membrane (Gibbs *et al*, 2003). While the predominant localization of PR8 PB1-F2 in mitochondria (Table EV1) is consistent with this, the increase in the cytoplasmic location of 1918 PB1-F2 could be associated with a new gain-of-function targeting cytoplasmic components. The high solubility of 1918 PB1-F2 as reflected by its diffuse expression may render it vulnerable to proteasomal degradation as compared to the punctate form of its PR8 counterpart (Table EV1). The PB1-F2 protein is intrinsically disordered (Vidic *et al*, 2016), and the changes in morphology and subcellular localization of PB1-F2 (Table EV1 and Fig EV1) may be associated with additional structural disorder conferred by mutations unique to 1918 (Fig EV3C). Thus, a two-pronged attack on type I IFN responses, targeting both mitochondrial and cytoplasmic resident components, contributed to the high mortality of 1918 influenza virus.

With a potential conformational repertoire characteristic of IDPs (Xue & Uversky, 2014), 1918 PB1-F2 can probably interact with multiple host proteins as reflected in our differential interactome analysis (Fig 5A–D), interrupting or dysregulating host responses. Residues 68 and 69 of 1918 PB1-F2, which we identified as crucial for the increased pathogenicity, have a particular propensity to increase structural disorder (Fig EV3C). This likely enables the protein to indiscriminately bind host proteins, in particular DDX3 (Fig 5 and Table EV2), hijacking it into proteasomal degradation, and shutting off the IFN responses (Fig 6E) as a prime example of the function of IDPs in antiviral immunity (Xue & Uversky, 2014). Interestingly, an internal IDR in 1918 PB1-F2 is likely to encompass positions 66 and 70 (Fig EV3C), previously identified as cytotoxic sequences (Conenello *et al*, 2011; Chakrabarti & Pasricha, 2013; Cheng *et al*, 2017). PR8 PB1-F2 is more cytotoxic than all other multiple mutants tested (Alymova *et al*, 2014), probably suggesting that the effects of individual mutations could be variable in different combinations and in a sequence-context manner. It should be noted that previous analyses did not report the 69P mutation either alone or in combination with other mutations. Our present results showed that 69P, which was not tested before, was most pronounced in destabilization (Fig 2D) and enhancing the virulence (Fig 4), probably due to intrinsic ability of proline residue as helix breaker leading to local structural perturbation. It is intriguing, therefore, that the 1918 virus acquired unique mutations and consequent structural disorder within its PB1-F2, establishing a new interactome network with host proteins (Table EV2), rendering a key component of antiviral IFN responses vulnerable to co-degradation (Fig 5), and consequently leading to the shutoff of IFN responses (Fig 6E).

The complete rescue by recombinant DDX3 from lethal infection across a wide range of infection doses (Fig EV5G) suggests that DDX3 effectively titrates out 1918 PB1-F2 in infected animals and prevents it from engaging in signaling pathways with pathogenic consequences (Table EV2). Thus, the 1918 PB1-F2, as an independent weapon to evasion the host antiviral defense, could synergize with pre-existing NS1-dependent IFN antagonist pathways to further increase pathogenicity (Geiss *et al*, 2002; Hale *et al*, 2008). Here, a two-pronged attack on type I IFN responses, targeting both mitochondrial and cytoplasmic resident components

(García-Sastre & Biron, 2006; Varga *et al*, 2011, 2012), synergistically contributed to the high mortality of 1918 influenza virus. These conclusions are mainly based on the designed isogenic recombinant viruses with the PR8 backbone and therefore require further confirmation in the reconstituted 1918 virus (Tumpey *et al*, 2005).

This observation led us to examine the sequence of PB1-F2 at the genetic level (updated until Feb 2017); the results are summarized in Table EV3. In most (> 80%) human-infecting H1N1 IAV isolates, PB1-F2 underwent truncation, whereas the majority of avian H1N1 isolates retain its full ORF (Table EV3B) (Chakrabarti & Pasricha, 2013). All human IAVs isolated during the last decade that have maintained the entire PB1-F2 have CAG (Q) or CTG (L) instead of the CCG codon encoding P at position 69 of 1918 PB1-F2 (Table EV3A). A question now arises whether a single base mutation at this particular position might occur among current IAVs, and potential IAV candidates could be suggested with caution (Table EV3C). With respect to the crucial role of 69P in part of cytotoxic sequences, we note that 69P that has been unique to 1918 virus up until 2016 (Table EV4) are now present in the most recent circulating IAVs (DB updated until Apr 2018) (Table EV3D). Remarkably, A/Netherlands/3315/2016 (H1N1) of swine origin was shown to cause a severe respiratory infection in humans in 2016 (Fraaij *et al*, 2016). The molecular basis for the severity of infection remains to be elucidated, but it is tempting to speculate if DDX3 may rescue from the virulence. The other virus, A/Tennessee/29/2017 of H3N2 subtype, has not yet been characterized. The present analysis warrants close monitoring of the PB1-F2 as part of global pandemic planning (WHO, 2009). As a molecular basis of the severe pathogenicity of 1918 pandemic, the present report will help guide future design of novel antiviral strategies against influenza pandemics.

Materials and Methods

Cell culture

A549 and 293T cells were grown in Dulbecco's modified Eagle's medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco-BRL) and 1% penicillin/streptomycin solution (Gibco-BRL). Cells were cultured at 37°C in a humidified atmosphere with 5% CO₂. U937 cells were grown in RPMI medium (Gibco-BRL) under the same conditions. Cells were transfected by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols.

Plasmid construction

The expression plasmids for wild-type PR8 PB1-F2 and 1918 PB1-F2 were generated by PCR in the pcDNA3.1 (+) vector (Invitrogen) using EcoRI and XhoI restriction enzyme sites. Two chimeric PB1-F2 mutants were also generated by PCR. One had the N-terminal domain from the PR8 strain (see below) and the C-terminal domain from the 1918 strain; the other one had the N-terminal domain from the 1918 strain and the C-terminal domain from the PR8 strain. Additionally, site-directed mutants (Fig 2A) of PR8 and 1918 PB1-F2 were generated. Primer sets used for cloning PB1-F2 are shown in Table EV5.

The DDX3 expression plasmid was cloned by PCR into pcDNA3.1 (+) vector using HindIII and XhoI restriction enzyme sites. The GFP-DDX3 plasmid was cloned into pEGFP-C1 vector using EcoRI site. Plasmids for IRF3 and TLR3 expression were donated by Dr. Wang-Shick Ryu (Yonsei University, Seoul, Korea).

The protein expression plasmid pGE-LysRS-R9-DDX3 encoding LysRS-R9-DDX3 was constructed from the pGE-LysRS-4 vector, which contains a T7 promoter-LysRS-tobacco etch virus (TEV) protease recognition sequence-multicloning sites (KpnI-BamHI-EcoRV-SalI-HindIII), and a histidine tag. The LysRS-R9-DDX3 gene was amplified by PCR using oligonucleotide primers 5'-GTC ACG GGT ACC CGT CGC CGT CGC CGT CGC CGT ATG AGT CAT GTG GCA GTG-3' (forward) and 5'-GTC ACG GTC GAC GTT ACC CCA GTC AAC CCC CTG GGA GTT A-3' (reverse). The obtained PCR product was cut with KpnI/SalI and inserted into the KpnI/SalI sites of pGE-LysRS-4, yielding pGE-LysRS-R9-DDX3. The identity of this clone was confirmed by sequencing.

The protein expression plasmid pGE-hRID-TEVsite-6xhis-PB1-F2 (hRID-1918 PB1-F2) was constructed using the pGE-hRID vector (RNA-interaction domain of human LysRS) (Kwon *et al*, 2018). The PCR products were digested with KpnI/HindIII. We used hRID as a fusion partner to enhance the solubility of the target protein because the expression level and the solubility of 1918 PB1-F2 were very poor in *E. coli*.

Influenza viruses

Three different types of IAVs were used for *in vivo* and *in vitro* infections: (i) A/Puerto Rico/8/34 (H1N1) virus (PR8), (ii) engineered A/Puerto Rico/8/34 viruses either with 1918 PB1-F2 (IAV PB1-F2 (1918)), or without PB1-F2 (IAV PB1-F2[-]); gifts from Dr. McCullers, St. Jude Children's Research Hospital (1), and (iii) an engineered virus with the PR8 backbone and an amino acid change (I68T, L69P, and I68T/L69P) in the PB1-F2 protein to match the sequence from A/Brevig Mission/1/1918 (H1N1) (IAV [1918]). To generate mutant PB1-F2 viruses, the Ile68 (ATC) or Leu69 (CTG) residue of PB1-F2 of PR8 was converted into Thr (ACC) or Pro (CCG), respectively, by using site-directed mutagenesis. These mutations did not change the amino acids in the PB1 protein. To obtain a recombinant virus carrying each of the mutated PB1-F2 variants, the reverse genetics technique was used. Briefly, seven IAV cDNAs encoding wild-type gene segments and one mutated PB1 segment were cloned into pHW2000 vectors and were co-transfected into 293T cells. Three days after transfection, supernatants were collected and subjected to plaque assay (see below) for plaque purification. The purified plaques were inoculated into MDCK cells to amplify the virus. A549 and U937 cells were washed with PBS and infected with the virus at 1 MOI. Cells were harvested 24 h after infection and subjected to RT-PCR and Western blot analyses.

Mouse infection with influenza viruses

Six- to eight-week-old female Balb/c mice were used. Viruses (50 μ l) were inoculated intranasally under anesthesia. To determine viral pathogenicity, mice (3–6 per group) were inoculated with an indicated dose and monitored daily for weight loss for 14 days. Although mice that lost more than 20–25% of their initial body weight are generally euthanized, we measured up to $\geq 40\%$ body

weight loss to investigate the virulence of recombinant viruses in this study. The animal procedures were approved by the Animal Care Committee at Konkuk University.

Antibodies and reagents

The anti-Flag M2 monoclonal antibody, anti-HA antibody, and anti- β -actin antibody were obtained from Sigma (St. Louis, MO, USA). Antibodies against IRF3, p-IRF3, TBK1, and IKK ϵ were obtained from Cell Signaling Technology (Beverly, MA, USA). Mouse polyclonal antibodies for the detection of PB1-F2 were generated using full-length recombinant PB1-F2 proteins expressed in *E. coli*. Anti-DDX3 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-NP antibody was obtained from rabbits (Lab Frontier, Anyang, Korea) immunized with the NP protein. Anti-mouse and anti-rabbit IgG horseradish peroxidase-conjugated antibodies were obtained from Santa Cruz Biotechnology. MG132 was from Calbiochem (Munich, Germany) and Poly (I:C) from InvivoGen (San Diego, CA, USA).

Quantitative real-time PCR (qPCR)

Cells and mouse tissues were lysed using Trizol reagent (Invitrogen/Gibco-BRL, Carlsbad, CA, USA) according to the manufacturer's instructions, and total RNA was isolated. Reverse transcription reactions were performed using 2 μ g of total RNA in a final volume of 20 μ l with M-MLV reverse transcriptase (iNtRON, Seoul, Korea). PCR was performed under the following conditions: initial denaturation at 94°C for 5 min, followed by 25–30 cycles of 30 s at 94°C, 30 s at 55–60°C, and 30 s at 72°C, with a final extension at 72°C for 5 min. The primer sequences are listed in Table EV5. Quantitative real-time PCR (qPCR) was performed using a SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA); amplification was conducted using the Applied Biosystems (ABI7500) RT-PCR machine. Relative quantification was performed by the comparative $\Delta\Delta C_t$ method. The results are presented as an n-fold difference relative to the calibrator ($RQ = 2^{-\Delta\Delta C_t}$).

Western blot analysis

SDS-PAGE and Western blot analysis were performed as described previously (Jang *et al*, 2013). Briefly, cells were lysed in a lysis buffer (25 mM Tris-HCl, pH 7.5, 1% NP-40, and a protease inhibitor cocktail) and centrifuged. Proteins (50 μ g per lane) were separated in 12–15% gradient SDS-PAGE gels and subjected to Western blot analysis. Chemiluminescent detection was performed with the ECL Detection Reagent (GE Healthcare, Buckinghamshire, UK) and visualized with a Bio-Imaging analyzer (LAS-4000, Fuji, Tokyo, Japan).

Immunoprecipitation analysis

Cells were harvested 24 h post-transfection and lysed with RIPA buffer (Pierce, Thermo Scientific, Rockford, IL, USA) containing a protease inhibitor cocktail. The lysates were precleared with protein A agarose (Roche, Mannheim, Germany) at 4°C for 2 h and incubated with primary antibody at 4°C overnight. The immunocomplexes were incubated with protein A agarose at 4°C for 4 h,

washed three times with cold PBS and boiled. Samples were separated by SDS–PAGE and subjected to immunoblot analysis.

In vitro protein-binding assay

Purified recombinant LysRS or LysRS-DDX3 protein was incubated with recombinant 1918 PB1-F2 in TEN buffer (20 mM Tris–Cl (pH 7.5), 200 mM NaCl, 1 mM EDTA, protease inhibitor cocktail) at 4°C overnight. After incubation with LysRS antibody, protein A agarose was added and incubated for 4 h at 4°C. The beads were washed four times with cold TEN buffer and analyzed by immunoblotting with PB1-F2 antibody.

Immunofluorescence assay

To determine the intracellular localization of PB1-F2 proteins, fluorescence analysis was performed. A549 cells were transfected with 1 µg of Flag-tagged PB1-F2 expression plasmids using Lipofectamine 2000. Cells were fixed and probed with Flag-M2 monoclonal antibody (diluted 1:500) overnight at 4°C and washed three times with PBS. Cells were then labeled with Alexa Fluor 546-conjugated anti-mouse secondary antibody for 1 h at room temperature and visualized using a fluorescence deconvolution microscope (Olympus, PLACE) equipped with an image analysis system.

Luciferase reporter assay

Cells were transfected using Lipofectamine 2000. Luciferase activity was measured 48 h post-transfection using a luciferase assay system (Promega, Madison, WI, USA) according to the manufacturer's manual. The obtained values were normalized to β-galactosidase activity measured in the same lysates. Assays were performed in triplicate, and the data were expressed as mean ± SD.

Measurement of the IFNβ level

Lung homogenates were centrifuged at 10,000 × g for 5 min, and the supernatants were assayed with the IFNβ ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

In-gel digestion

For interactome analysis using LC-MS/MS, immunoprecipitated samples were loaded on a 10% SDS–PAGE gel. The gel was stained with the Code Blue Stain Reagent (Pierce, Rockford, IL, USA) and cut into four slices. Each slice was treated and digested with trypsin as described (Shevchenko *et al*, 2006). Extracted peptides were desalted using a C18 spin column (Pierce), and the columns were dried using a Speed Vac.

LC-MS/MS analysis

Peptides were analyzed using a Q-Exactive mass spectrometer and an Easy-nanoLC 1000 system (both from Thermo Finnigan, San Jose, CA, USA). Peptides were separated on C18 columns (75 µm × 50 cm). UPLC was used at a flow rate 300 ml/min; peptides were eluted with a gradient from Solvent A (0.1% formic

acid in HPLC-grade water) to Solvent B (0.1% formic acid in acetonitrile). Separated peptides were ionized by electrospray at 2.3 kV, and tandem MS analysis was performed at a normalized collision energy of 27% by HCD cell. Entered ions were scanned using the Top 8 data-dependent method.

Protein identification and validation

The acquired MS/MS spectra were searched using the SEQUEST algorithm in the Proteome Discoverer program (Thermo Finnigan) against the ipi.HUMAN.v3.87 database. Search parameters were as follows: precursor and fragment ion mass tolerances of 10 ppm and ± 0.8 Da, respectively, variable modification set as Met oxidation (+16 Da), and static modification was set as Cys carbamidomethylation (+57 Da). Peptides and proteins identified by the database search were validated using Scaffold 4 (Proteome Software, Portland, OR, USA) with the following parameters: protein probability 99.9%, peptide probability 95%, minimum number of unique peptides > 2.

Biological function and protein–protein interaction network analysis of PB1-F2-interacting proteins

The biological functions of PB1-F2 interacting proteins were analyzed using the IPA software version 18488983 (Ingenuity Systems, Redwood City, CA, USA). Protein–protein interaction network was generated using STRING9.0 (Search Tool for the Retrieval of Interacting Genes) and visualized using Cytoscape. Identification of IFN pathway-related genes was based on information in the KEGG pathway database.

Expression of recombinant DDX3 and 1918 PB1-F2 proteins

pGE-LysRS-R9-DDX3 or pGE-hRID-TEVsite-6xhis-PB1-F2 vector was transformed into the *E. coli* strain ClearColi BL21(DE3) (Lucigen, Middleton, WI, USA). After the addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG, Sigma) to cells in 10 ml of culture medium, the culture was incubated at 20°C for 6 h. Cells were then harvested by centrifugation at 845 × g for 15 min, resuspended in 0.3 ml PBS (pH 7.4, 130 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄), and disrupted by sonication. The cell lysate was separated into total, soluble, and pellet fractions by centrifugation at 13,523 × g for 15 min. Each sample was mixed with the same volume of 2 × SDS gel-loading buffer (100 mM Tris–Cl, pH 6.8, 20% glycerol, 4% SDS, 200 mM dithiothreitol [DTT], 0.2% bromophenol blue). The samples were boiled at 100°C for 2 min, loaded on an SDS–PAGE gel, and stained with Coomassie Brilliant Blue. The solubility of proteins was estimated by using a gel densitometer.

Purification of recombinant proteins

Fusion proteins were purified by nickel-affinity chromatography using ÄKTA Prime (GE Healthcare Life Sciences). Cells from a 500 ml IPTG-induced culture were harvested by centrifugation at 845 × g for 15 min and lysed by sonication in 10 ml of buffer A (50 mM Tris–HCl, pH 7.5, 300 mM NaCl, 10% glycerol, 10 mM imidazole, 2 mM β-mercaptoethanol, 0.5% NP-40, 1 mM PMSF

[Sigma]). Soluble fractions of cell extracts were obtained by centrifugation at $13,523 \times g$ for 15 min and applied to a HisTrap HP column (GE Healthcare Life Sciences) equilibrated with buffer A. After washing with buffer A, target proteins were eluted with an imidazole gradient (10–300 mM) by mixing buffer A and buffer B (50 mM Tris–HCl, pH 7.5, 300 mM NaCl, 10% glycerol, 300 mM imidazole, 2 mM β -mercaptoethanol, and 0.5% NP-40). The eluted fractions were pooled, dialyzed against storage buffer (50 mM Tris–HCl, pH 7.5, 200 mM NaCl, 0.1 mM EDTA, and 1 mM DTT), and concentrated with Centriprep (Amicon, Danvers, MA, USA). Purified proteins were quantified by comparison against bovine serum albumin standards after separation by SDS–PAGE and Coomassie Brilliant Blue staining. The image of the gel was captured by an image analyzer, and the density of bands was analyzed using Bio-1D. Protein aliquots were stored at -80°C until use.

Cleavage of fusion protein by tobacco etch virus protease

Purified LysRS-R9-DDX3 protein was treated with the recombinant TEV protease (Invitrogen) in 50 mM Tris–Cl (pH 8), 0.5 mM EDTA, 1 mM DTT at 16°C for 12 h. TEV-cleaved LysRS-R9-DDX3 proteins were analyzed by SDS–PAGE. Cleaved DDX3 was purified on a HisTrap HP column.

Virus plaque assay

All virus titers were expressed as plaque forming units (pfu) calculated in a plaque assay (Jang et al, 2013).

Plaque reduction assay

Approximately 80–90% confluent monolayers of MDCK cells were cultured in a 12-well plate and infected with a mixture of influenza virus (500 pfu/well) and LysRS-DDX3 protein (1.1 $\mu\text{g}/\text{well}$). After 45 min on a rocker at room temperature, the mixture was removed and the cells were washed twice with medium and covered with overlay medium (2X DMEM containing 10 $\mu\text{g}/\text{ml}$ trypsin, 1% low melting agarose). The plates were incubated for 48 h at 37°C in 5% CO_2 , and the plaques were counted in each well.

Data analysis

All data are presented as the mean $\pm$ SD of at least three separate experiments. Differences between groups were tested for statistical significance using Student's *t*-test or analysis of variance. Statistical significance was set at $P < 0.05$.

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Author contributions

E-SP, YHB, BLS, and K-HK designed experiments; ESP, YHB, SP, YHJ, W-RH, KCC, DHK, ARL, G-CS, YKP, HSK, HS, JW, YNH, BJ, AS, PK, JY, H-ML, and S-BK performed experiments; ESP, KPK, S-HL, Y-MP, BLS, and K-HK analyzed the data; ESP, BLS, and K-HK wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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