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Influenza virus NS1 protein binds cellular DNA to block transcription of antiviral genes

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ABSTRACT

Influenza NS1 protein is an important virulence factor that is capable of binding double-stranded (ds) RNA and inhibiting dsRNA-mediated host innate immune responses. Here we show that NS1 can also bind cellular dsDNA. This interaction prevents loading of transcriptional machinery to the DNA, thereby attenuating IAV-mediated expression of antiviral genes. Thus, we identified a previously undescribed strategy, by which RNA virus inhibits cellular transcription to escape antiviral response and secure its replication.

1. Introduction

Influenza A viruses (IAVs) are important human pathogens that cause global epidemics and pandemics (www.who.int). It is estimated that IAVs are responsible for up to 500,000 deaths a year [1]. The successful recovery from viral infection largely depends on efficient activation of innate and adaptive immune responses [2–4]. Innate immune responses are triggered by cellular Toll-like receptors (TLR3 and TLR7), which recognize viral patterns upon IAV entry in the cell [4–6]. These pattern recognition receptors (PRRs) activate transcription of interferon genes (*IFNB1*, *IL28A*, *IL29*, *IL28B*, *IFNG*, *IFNA1*, *IFNA2*, and *IFNW1*), whose products mediate expression of interferon stimulated genes (ISGs) [7–10]. Following transcription of ISGs, their protein products, such as RIG-I (*DDX58*), MDA5 (*IFIH1*), and PKR (*EIF2AK2*) recognize viral RNA and its replication intermediates to trigger activation of innate immune responses and apoptosis, as well as to inhibit protein synthesis [11–13]. In addition, the ribonucleases encoded by other ISGs (*OASL*, *OAS1*, *ISG20*) degrade viral RNA [14–16]. Moreover, E3-ligases and ubiquitin-like molecules encoded by *HERC5*, Trim25, and *ISG15* ISGs modify influenza and cellular proteins to alter their cellular functions [17,18]. Simultaneously, cytokines produced from *IL1B*, *IL8*, *IL6*, *CXCL10*, *CCL5* and some other ISGs are secreted from infected cells to alarm bystander uninfected cells of a viral infection as well as to attract immune cells to the site of infection [2,4,19]. Some of these cytokines are processed into secretory forms by the inflammasome, which is activated by IFN-inducible GTPases, such as GBP1, GBP4, GBP5, MX1 and MX2 [20–22]. Moreover, COX2, IDO and 25HC encoded by *PTGS2*, *IDO*, and *CH25H* ISGs catalyse the production of prostaglandin H2, kynurenine, and oxysterol 25-hydroxycholesterol, respectively, which act as immuno- and neuromediators [19,23–25]. Thus, antiviral response consist of transcriptional, post-transcriptional, translational and post-translational events resulting in the clearance of infection.

To counteract the cellular defence and secure viral replication, IAV utilizes its non-structural NS1 protein, which is synthesized by infected cells only few hours after infection. NS1 interacts with replication intermediates of viral RNA to hinder these molecules from recognition by cellular PRRs [26]. It also binds RIGI, PKR, TRIM25, ISG15, GBP1 and other ISG products, to inhibit their functions [27,28]. However, NS1 interactions with these host antiviral proteins are virus- or host cell-specific [27–30]. We hypothesized that NS1 could also block the

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transcription of innate antiviral genes by binding cellular DNA to prevent the loading of cellular transcriptional machinery (Fig. 1A and B). Indeed, we demonstrate that NS1 binds cellular dsDNA, antagonizes RNA polymerase II (Pol II) recruitment to the DNA and, consequently, inhibits the transcription of IFNs and ISGs. Thus, our study offers a previously undescribed mechanism, by which RNA virus manipulates cellular transcription to downregulate the antiviral responses.

2. Materials and methods

2.1. Viruses and cells

Influenza A/WSN/33(H1N1) viruses expressing wild type (WSN^{WT}) or R38A/K41A mutant NS1 ($WSN^{RK/AA}$) were generated using WSN eight-plasmid-based reverse genetics system in HEK and Vero cells as described previously [31]. We sequenced the viral NS1 genes of $WSN^{RK/AA}$ and WSN^{WT} viruses to verify the authenticity of the mutations. Viruses were titrated in Madin-Darby canine kidney epithelial (MDCK) cells using plaque assay as described [32]. We obtained smaller plaques and approximately 100 times lower titres for $WSN^{RK/AA}$ virus than for WSN^{WT} (Fig. S1). The viruses were stored at −80 °C.

MDCK, human embryonic epithelial cells (HEK293T) and African green monkey kidney epithelial cells (Vero) were grown in Dulbecco modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2 mM L-glutamine (Lonza; Basel, Switzerland), 50 U/ml penicillin-streptomycin mix (PenStrep, Lonza) and 10% fetal bovine serum (FBS; Gibco, Paisley, UK). Human telomerase reverse transcriptase-immortalized retinal pigment (RPE) cells were grown in DMEM-F12 medium supplemented with 50 U/ml PenStrep, 2 mM L-glutamine, 10% FBS, and 0,25% sodium bicarbonate (Sigma-Aldrich). The cells were propagated at 37 °C in 5% CO_2.

2.2. Transfection of RPE cells with siRNA

RPE cells were cultured to 80% confluency in 24 well plates and transfected with 100 nM siGenome SMARTpool or ON-TARGETplus non-targeting control siRNA (Table S1; Dharmacon, Lafayette, CO, USA) using Lipofectamine® RNAiMAX Reagent (Thermo Fisher Scientific). Importantly, some of these siRNA have been validated in previous [33,34] and in the present study (Fig. S2). 24 h after transfections, the cells were infected with WSN^{WT}, $WSN^{RK/AA}$ viruses or mock infected. 8 h after infection, the levels of genes and proteins of interest were analysed using RT-qPCRs and immunoblotting, respectively.

2.3. Infection of RPE cells with WSN^{WT} and $WSN^{RK/AA}$ viruses

The growth medium of RPE cells was changed to the virus growth medium (VGM) containing 0.2% BSA (Sigma-Aldrich), 2 mM L-glutamine, 0.348% $NaHCO_3$ and 1 μg/ml l-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin (TPCK)-trypsin (Sigma-Aldrich) in DMEM-F12. The cells were infected with WSN^{WT}, $WSN^{RK/AA}$ viruses or mock.

2.4. Gene expression profiling

RNA was extracted from WSN^{WT}-, $WSN^{RK/AA}$- or mock-infected RPE cells at 8 h.p.i. using RNeasy Plus mini kit (Qiagen, Germany). Gene expression profiling was done using Illumina Human HT-12 v4 Expression BeadChip Kit according to manufacturer's recommendation as described previously [35]. Raw microarray data were normalized using the BeadArray and Limma packages from Bioconductor suite for R. Normalized data were further processed using a variance and intensity filter. Genes differentially expressed between samples and controls were determined using the Limma package. Benjamini-Hocberg multiple testing correction testing method was used to filter out differentially expressed genes based on a q-value threshold ($q < 0.05$). Filtered data were sorted by logarithmic fold change (log_2Fc). The gene-expression data was deposited to Gene Expression Omnibus (GEO accessory number: GSE65699). Gene set enrichment analysis was performed using open-source software (www.broadinstitute.org/gsea).

2.5. Quantitative PCR

Quantitative PCRs were done on the Lightcycler 480 using Fast SYBR Green Master Mix (Roche, USA). The following sets of primers were used for detection of specific genes or cDNA: EML4 (forward: 5′-TGGC TTCAGTGCAACTCTT-3′, reverse: 5′-AATCTCCATCACTGCCCATC-3′), IFNB1 promotor (forward: 5′-GTCAGTAGAATCCACGGATACAG-3′ and reverse: 5′-CTTGGGAGAAAGCAAAGGAAAG-3′) and exon (forward: 5′-GCCGCATTGACCATCTATGA-3′ and reverse: 5′-GCCAGGAGGTTCTCAA CAATAG-3′), IFNA1 (forward: 5′-ATGGCAACCAGTTCCAGAAG-3′, reverse: 5′-CATCCCAAGCAGCAGATGAA-3′), IFNA16 (forward: 5′-GACT CACTTCTATAACCACCACAA-3′, reverse: 5′-TAGTGCCTGCACAGGTAAAC-3′), IL6 (forward: 5′-TCATCACTGGTCTTTTGG-3′, reverse: 5′-CTCTGG CTTGTTCCTCAC-3′), CXCL1 (forward: 5′-TGAGCATCGCTTAGGAGA-3′, reverse: 5′-AGGACAGTGTGCAGGTAG-3′), and IL29 (forward: 5′-AGGC TGAGCTGGCCCTGA-3′, reverse: 5′-GGTGTGAAGGGGCTGGTC-3′). The relative gene expression differences were calculated as described

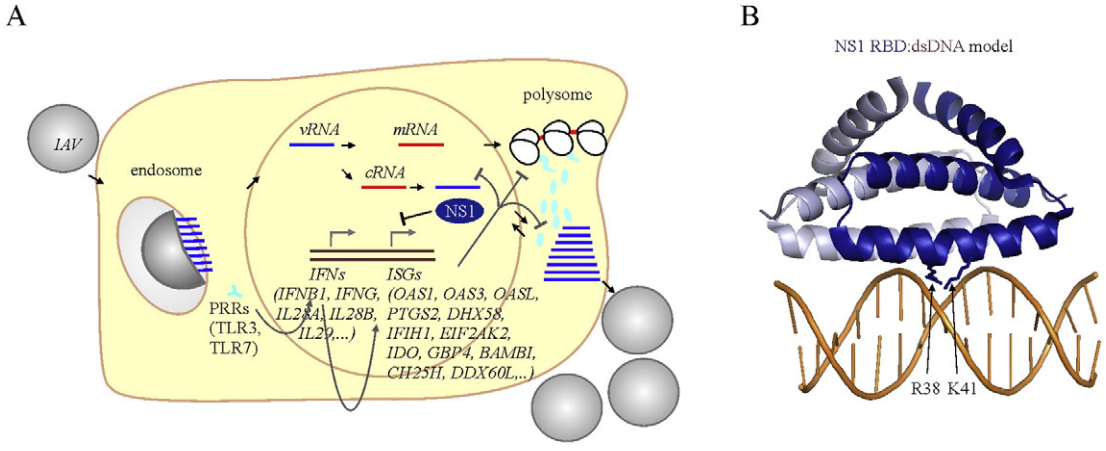

Fig. 1. Influenza NS1 through R38 and K41 may bind dsDNA to inhibit transcription of antiviral genes. A. Novel hypothetical mechanism of action of influenza NS1 against antiviral responses in influenza A virus-infected cells. IAV infection stimulates the transcription of IFNs and ISGs, which inhibit virus replication. Newly synthetized influenza NS1 protein blocks transcription of IFNs and ISGs by binding to cellular dsDNA. Representative PRRs, IFNs and ISGs are shown. IAV - influenza A virus, PRRs - pattern recognition receptors, IFNs- interferon genes, ISGs- interferon stimulated genes. B. Hypothetical model of NS1 RBD-dsDNA complex. The structures of NS1 RBD (PDBID: 2ZKO) and B-form dsDNA (4W9M) were used to build this model.

previously [36] and the results were represented as relative units (RU). Technical triplicates of each sample were performed on the same qPCR plate and non-templates and non-reverse transcriptase samples were analysed as negative controls. Statistical significance ($p < 0.05$) of the quantitation results was evaluated with *t*-test. Benjamini-Hochberg method was used to adjust the *p*-values.

2.6. Immuno-fluorescence analysis (IFA)

RPE cells were infected with WSN^{WT} (moi 1) or $WSN^{RK/AA}$ (moi 1) viruses or they were mock-infected. After 10 h of infection the cells were fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), then permeabilized and blocked in the BP buffer (10% Bovine serum albumin (BSA) and 0.1% Triton X-100 in PBS) supplemented with 5% goat serum (Life Technologies, USA). Primary rabbit *anti*-NS1 antibodies (32) were added followed by secondary goat anti-rabbit antibodies with an Alexa488 fluorophore (Life Technologies, USA) in BP buffer, nuclei were counterstained with DAPI, and the slides were mounted with Prolong Gold anti-fade reagent (Life Technologies, USA). Images were captured with Nikon 90i microscope and processed with NIS elements AR software.

2.7. Chromatin immunoprecipitation

ChIP experiments were performed on RPE cells infected with WSN^{WT} (moi 1), $WSN^{RK/AA}$ (moi 1) or mock as previously described (34). Briefly, after cross-linking with formaldehyde at room temperature, chromatin was prepared, sonicated on ice using Bioruptor (Diagenode, Philadelphia, PA, USA) and pre-cleared. Samples were incubated with the antibody and then pulled down using protein G Sepharose beads. After extensive washes the protein-DNA complexes were eluted, the cross-linking was heat-reverted. DNA was purified with QIAquick PCR purification kit (Qiagen) and quantified by PCR with primers targeting promotor or exon region of IFNB1 gene was performed.

2.8. Protein electrophoresis and immunoblotting

Cells were lysed with a 2× Laemmli loading buffer (4% sodium dodecyl sulphate, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl, pH 6.8). Proteins were resolved in 4–20% gradient SDS-polyacrylamide gel (Biorad, Hercules, USA) at 150 V for 50 min. The gels were stained using Coomassie blue or immunoblotted. For immunoblotting, proteins were transferred from SDS-PAGE onto Immobilon-P membranes (Millipore, MA, USA). The membranes were blocked with 5% non-fat milk or 5% BSA (Sigma-Aldrich) in TBST, stained with different primary antibodies overnight, followed by secondary HRP-conjugated antibody labelling and detection by chemiluminescence. The primary antibodies used in this study were *anti*-RNA polymerase II CTD repeat YSPTSPS antibody (1:1000 dilution in 5% BSA-TBST; Abcam, 8WG16), goat *anti*-TLR3 (1:1000 dilution in 5% BSA-TBST; sc8692, SantaCruz), rabbit *anti*-MAVS (1:1000 dilution in 5% BSA-TBST; from I. J. laboratory), guinea pig anti-MDA5 (1:250 dilution in 5% milk-TBST; from I. J. laboratory), and rabbit anti-IAV NS1 antibody (1:5000 dilution in 5% BSA-TBST; from I. J. laboratory). The secondary HRP antibodies (anti-guinea pig, anti-goat and anti-rabbit) were from Dako and used as 1:1000 dilution. To confirm equal sample loading the membranes were stripped and labelled with anti-GAPDH antibody (1:1000 dilution in 5% milk-PBST, Santa Cruz, sc-47724).

2.9. Enzyme-linked immunosorbent assay (ELISA)

The levels of CXCL10, TNFα, and IFNλ in the cell supernatants were assayed with ELISA (PBL Interferon Source) as described previously [37].

2.10. Mass spectrometry

Five 175 cm^2 plates of RPE cells were infected with WSN^{WT} (moi 1) or $WSN^{RK/AA}$ (moi 3) viruses or were left mock-infected. 10 h post infection the media was removed. 400 μl of lysis buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl and 1% Tryton-X100) was added to each plate. The lysates were collected and centrifuged at 14,000 rpm at 4C for 10 min. Insoluble fractions were resuspended in 2 ml buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl and 0.1 mg/ml RNAse A (Qiagene) and incubated on ice for 1 h. The mixtures were centrifuged at 14,000 rpm at 4C for 10 min. Insoluble fractions were resuspended in 2 ml lyses buffer and centrifuged again to remove remaining RNAse and cleaved RNA. Insoluble fractions were resuspended in 2 ml buffer containing 20 mM Tris-HCl, pH 7.5 and 600 mM NaCl. Insoluble fractions were obtained by centrifugation, resuspended in 2 ml buffer containing 20 mM Tris-HCl, pH 7.5 and 100 mM NaCl and sonicated for 20 min (2 s pulse/2 s pause; amplitude 25%, 20 kHz, 750 W). Proteins were resolved on SDS page.

Identification and quantification of proteins of "insoluble fraction" was done using quadri-plex iTRAQ (isobaric tag for relative and absolute quantitation) labelling combined with liquid chromatography-tandem mass spectrometry (LC–MS/MS) analysis as described previously [38]. In brief, protein alkylation, trypsin digestion and labelling of the resulting peptides were done according to manufacturer's instructions (AB Sciex). Labelled peptides were fractionated by strong cation exchange chromatography and each fraction containing labelled peptides was analysed twice with nano-LC-ESI-MS/MS using Ultimate 3000 nano-LC (Dionex) and QSTAR Elite hybrid quadrupole time-of-flight-MS (AB Sciex). MS data were acquired automatically using Analyst QS 2.0 software. Protein identification and relative quantitation was performed using ProteinPilot 4.0 software (AB Sciex). Data files from both technical replicates of an iTRAQ sample set were processed together. The search database was a self-built combination of Uniprot Human protein sequences and Uniprot ssRNA negative-strand virus sequences (both form the release 55.0, 02/08). The search criteria were: cysteine alkylation with MMTS, trypsin digestion, biological modifications allowed, thorough search and detected protein threshold of 95% confidence (Unused ProtScore >1.3). Additionally, automatic bias correction was used. False discovery rates were calculated using a concatenated normal and reversed sequence database.

2.11. In vitro assays

Wild type ($NS1^{WT}$) and R38A, K41A mutant ($NS1^{RK/AA}$) of NS1 of influenza A/chicken/Nigeria/OG10/2007(H5N1) virus were produced in *E. coli* BL21(DE3) cells and purified to homogeneity as described previously [39]. Importantly, we also attempted but did not succeed to purify wild type proteins of A/WSN/1933, A/Udorn/1972, and many other IAV strains because they were insoluble when overexpressed in *E. coli* BL21(DE3) cells.

Run-off transcription assay was performed using highly-purified TFIIB, TFIIE, TFIIF, TFIIH, TBP, RNA polymerase II and $NS1^{WT}$ or $NS1^{RK/AA}$ protein as described previously [40].

EMSA assay was performed with recombinant purified $NS1^{WT}$ or $NS1^{RK/AA}$ proteins and dsDNA fragments as described previously [41]. Briefly, the synthetic 199 bp-long dsDNA (DNA-199) was produced by PCR using two oligonucleotides (forward 5′-ATGGATCCAAACACTGTGTCA-3′, reverse 5′-CTCCACTATTTGCTTTCCA-3′) and pHW188-NS plasmid as a template (29). The synthetic 76 bp-long dsDNA (DNA-SELEX) was produced as described in [42]. Hundred ng of dsDNA was incubated with purified recombinant proteins for 15 min on ice. 10× loading buffer (20 mM Tris-HCl pH 8.6, 50 mM NaCl, 10% glycerol) was added to the samples and the samples were resolved in 1% agarose gel containing ethidium bromide in a TAE buffer (40 mM Tris-base, 20 mM acetic acid, 1 mM EDTA). Protein-DNA-SELEX complex were excised from the gel, and DNA was purified using Qiaquick gene extraction kit

(Qiagen). The DNA was sequenced with SR3 primer (5′-GTTCAGAGTTC TACAGTC-3′) using ABI3730xl DNA Analyzer and standard Sanger method, adjusted for sequencing of short fragments.

Microscale thermophoresis assay was performed with recombinant highly-purified $NS1^{WT}$ or $NS1^{RK/AA}$ proteins and fluorescently labeled synthetic 199 bp-long dsDNA (DNA-199). DNA-199 was produced by PCR using oligonucleotides containing a cyanine fluorophore covalently linked to the 5′ ends and pHW188-NS plasmid as a template. 10 pM of Cy5-labeled synthetic dsDNA was incubated with different concentrations of purified proteins for 5 min on ice. Differences in thermophoretic properties of free and protein-bound dsDNA were determined using Monolith NT.115 instrument (NanoTemper Technologies, Munich, Germany).

3. Results

3.1. NS1 inhibits transcription of antiviral genes in virus-infected cells

It has been previously shown that NS1 through R38 and K41 residues binds dsRNA to sequester it from recognition by PRRs [15,43,44]. We hypothesized that *via* the same residues, NS1 could also bind dsDNA to inhibit transcription of IFNs and ISGs (Fig. 1B). To test this hypothesis, we used wild-type influenza A/WSN/33(H1N1) virus (WSN^{WT}) and its variant ($WSN^{RK/AA}$), which expresses NS1 protein with R38A and K31A [31]. We infected human RPE cells with WSN^{WT} or $WSN^{RK/AA}$ viruses, and used the uninfected cells (mock) as a control. 8 h post infection we analysed the expression of cellular genes using DNA microarray. We found that infection with WSN^{WT} virus activated the expression of 33 genes more than eight-fold, whereas $WSN^{RK/AA}$ virus induced expression of 88 genes over the same fold, including 31 transcripts that were the same as those up-regulated by WSN^{WT} (Fig. 2A). We validated our microarray results using RT-qPCR and ELISA (Fig. 2B and C). These results suggested that WSN virus *via* R38A and K41 residues of NS1 was able to down-regulate the transcription of antiviral genes in infected human RPE cells.

Importantly, we obtained similar results using human macrophages previously [31]. In particular, infection of human macrophages with WSN^{WT} virus up-regulated the expression of 57 IFNs and ISGs more than eight-fold, whereas $WSN^{RK/AA}$ virus infection activated the expression of 93 genes (>8-fold), including 32 of WSN^{WT}-up-regulated genes. These data suggested that WSN virus NS1 protein, *via* R38A and K41 residues was able to control the transcription of antiviral genes in both infected human macrophages and RPE cells. However, human macrophages derived from donors with different genetic and epigenetic background possessed slightly different responses to infection with WSN virus (Fig. S3) [29,31,45]. To exclude these variations, we used only RPE cells in our next experiments.

Gene set enrichment analysis showed that the majority of differentially expressed genes were involved in antiviral responses. To confirm this, we suppressed the expression of some of these genes using specific siRNAs before infection. We demonstrated that *IFNB1*, *EIF2AK2* (PKR), *IDO*, *BAMBI*, *CH25H*, *DDX60L*, *OAS1*, *OAS3*, *PTGS2* (COX2), and *IFIH1* (MDA5) as well as *TLR3* and *MAVS* (used as controls) were necessary

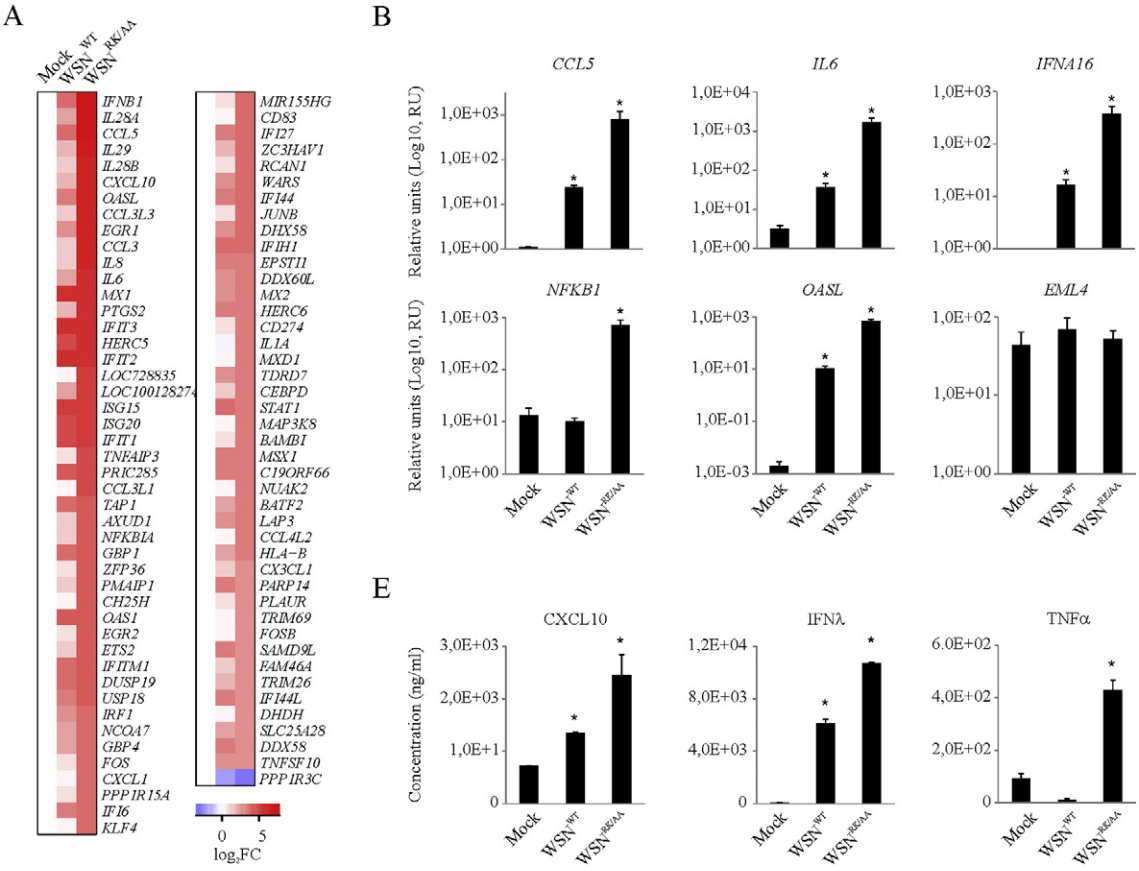

Fig. 2. Influenza NS1 through R38 and K41 inhibits transcription of antiviral genes. A. RPE cells were mock-, WSN^{WT}-, or $WSN^{RK/AA}$- infected. 10 h post infection cells were collected, total RNA was isolated and subjected to genome-wide transcription profiling. Three independent experiments were performed. Differentially expressed genes were selected ($p < 0.05$) and shown on a heatmap. Each cell is colored according to the average of the log2-transformed and quantile-normalized expression values (log_2 fold change >3 and <−3) of the triplicate samples with the average of mock controls subtracted. B. RPE cells were treated as for panel A, total RNA was isolated at 10 hpi, and the expression of 5 antiviral (CCL5, IL6, IFNA16, NFKB1 and OASL) and one housekeeping (EML4) gene was analysed using RT-qPCRs. The data points are mean values and error bars represent the SD from three independent experiments. Statistically significant ($p < 0.05$) differences in gene expression between virus- and mock-infected cells are indicated with asterisks. C. Cells were treated as for panel A, cell culture supernatants were collected at 24 h post-infection, and cytokine levels were determined using ELISA.

to limit transcription and replication of viral M1 RNAs upon WSN^{WT} and WSN$^{RK/AA}$ virus infections (Fig. 3A). Interestingly, *TLR3* but not *TLR7* was required for transcription of *IFNB1*, which triggered the expression of a set of ISGs in response to IAV infection (Fig. 3B). Thus, IAV *via* R38 and K41 of NS1 attenuated transcription of antiviral IFNs and ISGs.

3.2. NS1 locates to chromatin to suppress transcription of antiviral genes

Next we asked how does NS1 control the transcription of antiviral genes. We first investigated the cellular localization of NS1^{WT} and NS1$^{RK/AA}$. We infected RPE cells with WSN^{WT} or WSN$^{RK/AA}$ viruses and after 10 h post infection examined the distribution of NS1^{WT} and NS1$^{RK/AA}$ by immunofluorescence. These experiments showed that both NS1^{WT} and NS1$^{RK/AA}$ were located mainly in the nucleus of infected cells (Fig. 4A).

We then purified NS1-associated factors from mock-, WSN^{WT}-, and WSN$^{RK/AA}$-infected RPE cells. Cells were lysed with Triton X-100, and cell extracts were subjected to fractionations, high-RNAse A and high-salt treatments followed by SDS-PAGE, immunobloting, and agarose gel electrophoresis analysis. Surprisingly, the majority of NS1^{WT} and NS1$^{RK/AA}$ were found in insoluble fraction together with cellular DNA, RNA Pol II and histones (Fig. 4B). This indicates that NS1 can be co-purified with chromatin factors from virus-infected cells.

We next extracted proteins from the insoluble fractions and analysed them by quantitative mass spectrometry (iTRAQ LC–MS/MS). We found that histones *H*3.2, H1.2, H1.5, H2A.1D and other chromatin-associated proteins were enriched in NS1^{WT} and NS1$^{RK/AA}$-containing insoluble fractions in comparison to mock (Fig. 4C; Table S2). Of note, some differences in the levels of H2A1D, YBOX-1, *H*3.2 and other proteins were seen between NS1^{WT}- and NS1$^{RK/AA}$-containing fractions. These data suggest that WSN^{WT} and WSN$^{RK/AA}$ infections alter protein composition of chromatin fractions.

Next we investigated the consequences of the altered histone composition on NS1 interaction with specific gene regions during IAV infection. For this, RPE cells were mock-infected or infected with either WSN^{WT} or WSN$^{RK/AA}$ viruses for 8 h. NS1- and Pol II-associated DNA was extracted from chromatin fractions of infected and non-infected cells. The promoter and exon regions of *IFNB1* were analysed using q-PCR. We found that NS1^{WT} was enriched on the promoter and exon region of *IFNB1* in WSN^{WT} infected cells, whereas Pol II was enriched on the same *IFNB1* regions in WSN$^{RK/AA}$ infected cells (Fig. 4D). These results suggest that wild type NS1 can prevent the association of Pol II with *IFNB1* gene during virus infection. Altogether, these results may indicate that upon IAV infection PRRs mediated chromatin remodelling to activate the expression of antiviral genes and suppress general transcription, and NS1 upon expression suppresses transcription of some antiviral genes, depending on chromatin context.

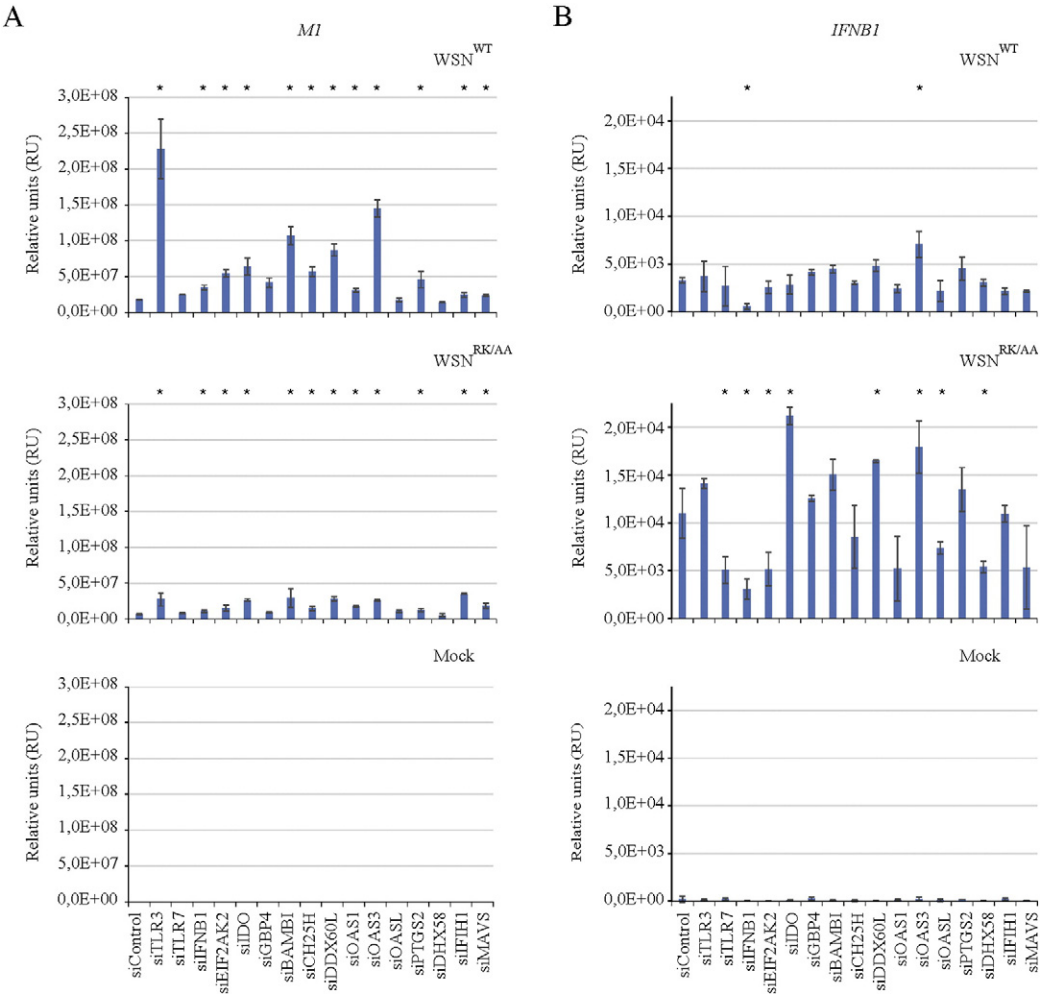

Fig. 3. SiRNA experiment revealed several cellular factors, which restrict both WSN^{WT} and WSN$^{RK/AA}$ virus replication in RPE cells. A, B. RPE cells were transfected with specific or control siRNA. 24 h later cells were infected with WSN^{WT} (moi 1) or WSN$^{RK/AA}$ (moi 1) viruses or mock-infected. Expression levels of (A) viral M1 and (B) cellular IFNB1 gene were analysed 10 h later by RT-qPCRs. Statistically significant ($p < 0.05$) differences in gene expression between non-targeted siRNA control and targeted siRNA condition are indicated with asterisks.

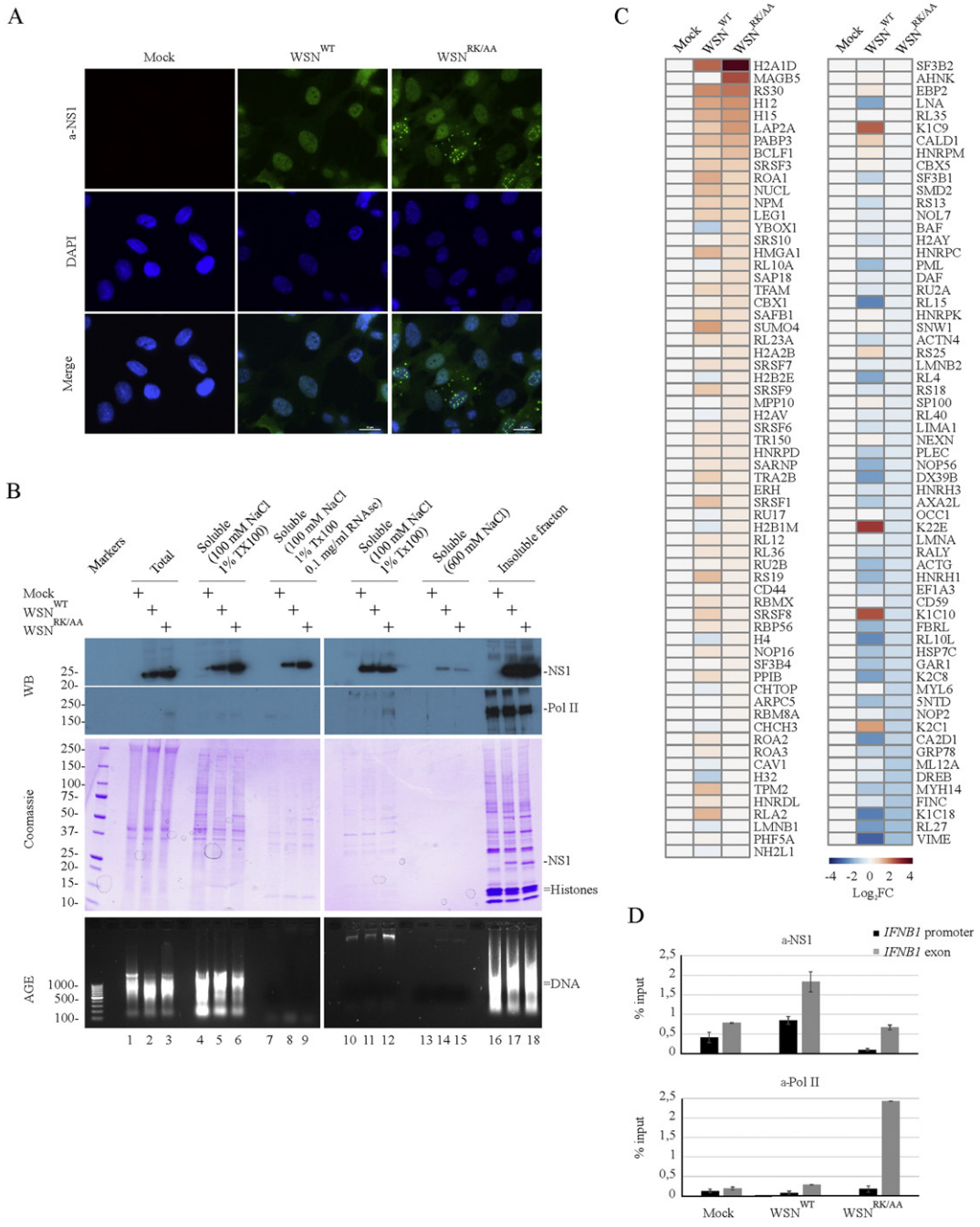

Fig. 4. Functional influenza A virus NS1 protein associates with chromatin and inhibits the transcription of cellular IFNB1 gene in IAV-infected RPE cells. A. Immunostaining of NS1 in RPE cells infected with mock, WSN^{WT}, or $WSN^{RK/AA}$ viruses. Cells were infected with mock, WSN^{WT} (moi 1), or $WSN^{RK/AA}$ (moi 3) viruses, fixed at 10 h post-infection, and stained with *anti*-NS1 antibody and DAPI. Scale bars, 10 μm. B. Purification of NS1-enreached fractions from WSN^{WT} or $WSN^{RK/AA}$ infected RPE cells. Cells were infected as for panel A. 10 h post infection cells were lysed with Triton X-100 and NS1-interacting cellular and viral factors were purified and analysed by western blotting (WB), SDS-PAGE (Comassee) and agarose gel electrophoreses (AGE). C. Quantitative mass-spectrometry analysis of cellular proteins in NS1-enriched fractions. Cells were infected and NS1-enriched fractions were purified as for panel (B). The proteins were identified and quantified using iTRAQ labelling combined with LC–MS/MS. The heatmap with fold change in concentrations of selected cellular proteins in infected *versus* non-infected cells are shown. Each cell is coloured according to the average of the log2-transformed and quantile-normalized expression values of the triplicate samples with the average of mock controls subtracted. D. NS1 co-precipitation with the promoter and exon regions of cellular IFNB1 gene. RPE cells were mock-, WSN^{WT}-, or $WSN^{RK/AA}$- infected. Cells were collected at 8 h post infection, and chromatin was prepared for immuno-precipitation. ChIP was performed with anti-NS1 and anti-pol II antibodies. qPCRs were performed with primers targeting promotor and exon regions of IFNB1. The percentage of input DNA associated with NS1 or with pol II was quantified. The data points are mean values, and error bars represent the SD.

3.3. Purified recombinant NS1 through R38 and K41 binds synthetic dsDNA and inhibits Pol II transcription in vitro

To further analyse the mechanisms of NS1 binding to DNA, we performed EMSA experiment with purified $NS1^{WT}$ and $NS1^{RK/AA}$ proteins (Fig. 5A). For this experiment, we produced linear synthetic dsDNA (DNA-SELEX), which contained central random 16 N base pair (bp) region flanked by defined 30 bp sequences. 16 N bp region was chosen because NS1 RBD dimer was shown to occupy 16 bp of A-form dsRNA [46]. EMSA showed that $NS1^{WT}$ but not $NS1^{RK/AA}$ retarded the migration of dsDNA in EMSA analysis (Fig. 5B). We analysed the possible sequence-specificity of NS1 interaction with its target DNA using Sanger sequencing, *i.e.* we sequenced NS1-interacting DNA. We found that NS1 bind DNA independently of

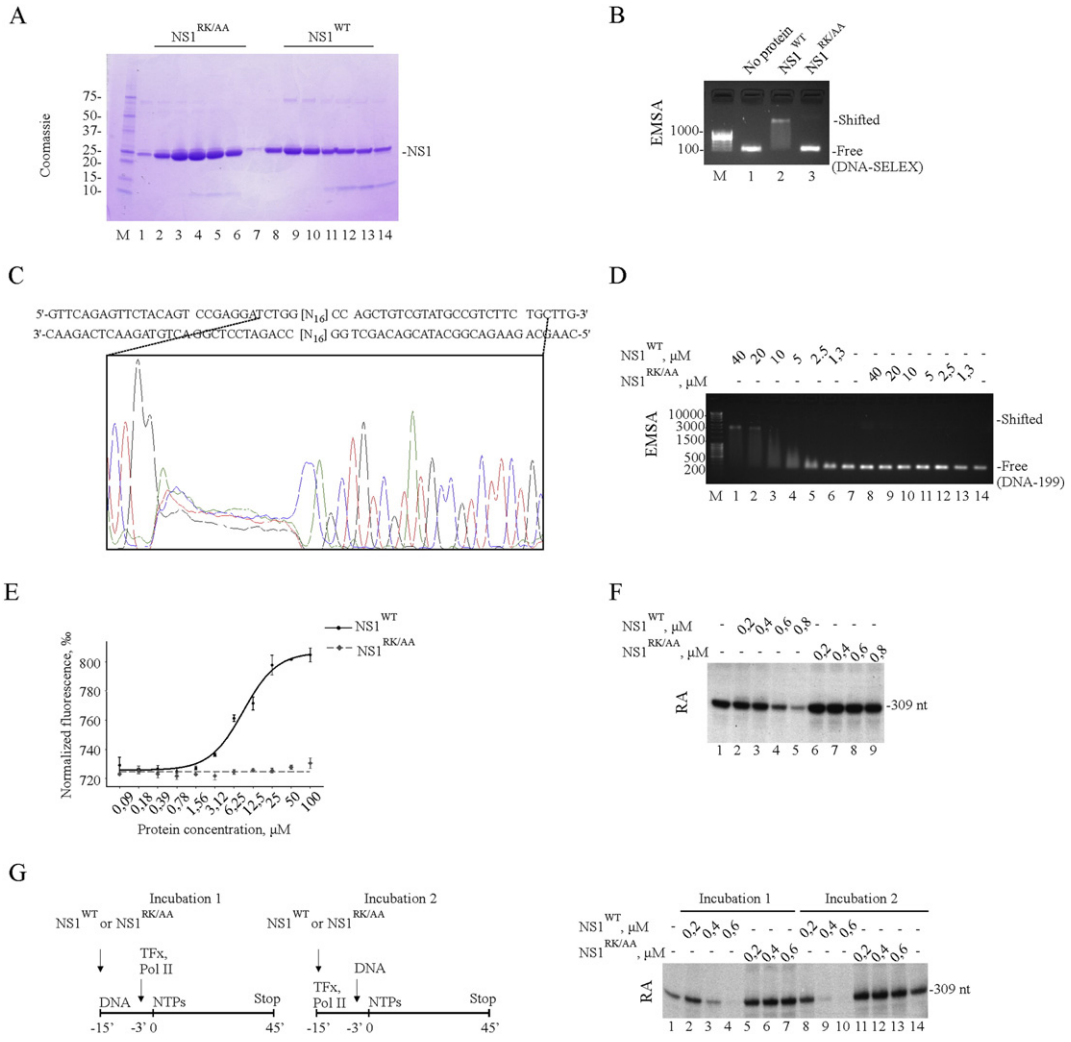

Fig. 5. Purified recombinant NS1 binds synthetic dsDNA in a sequence no-specific manner and inhibits pre-initiation complex formation and pol II loading on DNA *in vitro*. A. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of purified recombinant NS1^{WT} and NS1$^{RK/AA}$ proteins. Pick fractions from size-exclusion chromatography (Superdex-200) of purified NS1 proteins were resolved on SDS-PAGE. The gels were stained using Coomassie blue. B. Electrophoretic mobility shift assay (EMSA) monitoring binding of recombinant purified NS1^{WT} and NS1$^{KK/AA}$ proteins to synthetic dsDNA fragment DNA-SELEX. C. The sequence of DNA-SELEX probe (top) and DNA fraction retarded by NS1^{WT} in EMSA. D. EMSA monitoring binding of recombinant purified NS1 (NS1^{WT}) and its R38A/K41A mutant (NS1$^{KK/AA}$) to synthetic dsDNA fragment DNA-199. Two-fold dilutions of NS1^{WT} and NS1$^{RK/AA}$ were pre-incubated with dsDNA for 15 min at room temperature prior to analysis on agarose gel. E. Microscale thermophoresis assay monitoring thermophoretic mobility of dsDNA upon its binding to NS1^{WT} or NS1$^{RK/AA}$ mutant. Normalized fluorescence of Cy5-labeled synthetic dsDNA after its incubation with indicated concentrations of NS1^{WT} or NS1$^{RK/AA}$ is shown. An increase in normalized fluorescence is observed upon dsDNA interaction with NS1. The data points are mean values and error bars represent the SD from three independent experiments. F. Purified recombinant NS1^{WT} or NS1$^{KK/AA}$ were tested in a transcription assay. Increasing amounts of the proteins were incubated with transcription factors (TFx), pol II, nucleotides (NTPs), ^{32}P-labeled ATP and DNA for 45 min. Reactions were stopped and synthesized ^{32}P-labeled RNAs were resolved on denaturing PAGE. RA – radio-autograph. G. NS1^{WT} or NS1$^{RK/AA}$ was pre-incubated with DNA, or TFx and pol II. The remaining components of the transcription reaction were added and transcription reaction was initiated by addition of NTPs. Reactions were stopped at the indicated times and the products were analysed as in panel F. Schematic representations of the experiments and the autoradiographs (RA) are shown.

sequence of central region, suggesting that the interaction was not sequence-specific (Fig. 5C).

We then determined the dissociation constant (K_d) of NS1^{WT} and dsDNA fragment (DNA-199). EMSA showed that NS1^{WT} retarded the migration of DNA-199 probe in a concentration-dependent manner with micromolar K_d (Fig. 5D). Microscale thermophoresis assay revealed that the K_d for NS1^{WT} was 11.1 ± 0.7 μM, whereas the K_d for NS1$^{RK/AA}$ was >100 μM (Fig. 5E). Interestingly, the K_d for NS1^{WT}-dsDNA complex was comparable to that of NS1^{WT}-dsRNA complex reported previously [39,46–48]. These results suggested that *in vitro* NS1 binds dsDNA non-specifically with micromolar affinity, and that the residues R38 and K41 of NS1 are essential for the binding.

To address whether NS1 through R38 and K41 can inhibit transcription *in vitro*, we purified recombinant NS1^{WT} or NS1$^{RK/AA}$ proteins (Fig. 4A) and added them to *in vitro* run-off transcription assays containing naked AdMLP DNA template. We found that NS1^{WT} but not NS1$^{RK/AA}$ inhibited the *in vitro* synthesis of RNA in a concentration-dependent manner (Fig. 4F). To further understand how NS1 was able to block transcription, we set up two different experimental conditions. We first pre-incubated NS1 with dsDNA, and then added pre-initiation complex (PIC) and Pol II before the reaction started (Fig. 4G, incubation 1). Alternatively, we pre-incubated NS1 with PIC and Pol II, and then added dsDNA (Fig. 4G, incubation 2). In both experimental settings, NS1^{WT} but not NS1$^{RK/AA}$ inhibited the *in vitro* RNA synthesis. We concluded that NS1 inhibits *in vitro* transcription by binding to DNA, which prevents the loading of PIC and Pol II.

4. Discussion

Influenza NS1 protein is an important virulence factor and deciphering the mechanism by which NS1 antagonizes antiviral responses is critical for understanding of disease progression. Here we

demonstrated that influenza NS1 binds synthetic dsDNA in a sequence non-specific manner. Furthermore, we showed that this interaction inhibited the loading of transcriptional machinery on the synthetic DNA and thereby prevented the transcription reaction *in vitro*. In infected cells, NS1 inhibited Pol II recruitment to the exon and promoter regions of *IFNB1*. This observation could be potentially expanded to other IFN genes and ISGs whose transcription was up-regulated in response to $WSN^{RK/AA}$ in comparison to WSN^{WT} and mock infections. In addition, our results indicate that IAV infection promoted chromatin remodelling, which could be associated with inhibition of general transcription and activation of expression of certain IFNs and ISGs. Influenza NS1 protein could, therefore, bind DNA of transcriptionally active genes and attenuate their expression. This may potentially lead to reduced expression of IFNs and ISGs, leading to compromised antiviral responses of infected cells.

Importantly, R38 and K41 residues of NS1 mediate an interaction of IAV NS1 with non-specific dsDNA and dsRNA, and the dissociation constants of these interactions are very similar [39,46–48]. This suggests that NS1 can bind both dsDNA/RNA *via* the phosphate backbone. Binding of NS1 to dsDNA/RNA can hinder them from loading of cellular transcription machinery and recognition by cellular PRRs, respectively.

Interestingly, our attempt to purify NS1 from infected cells revealed that the majority of NS1 was in insoluble fraction together with histones, transcription machinery and DNA. High salt and RNAse treatments of these fraction, as well as R38A and K41A mutations in NS1, did not increase NS1 solubility. This indicates that additional transcriptional regulation mechanisms involving NS1 interactions with cellular factors may take place, which is in agreement with previous findings showing that NS1 non-conserved residues outside dsDNA/RNA binding site may interact with chromatin-associated factors [49,50].

The RNA/DNA-binding residues R38 and K41 of NS1 protein are evolutionary conserved among IAVs. Moreover, the corresponding NS1 residues are also conserved in influenza B viruses [51–53]. Thus, our findings point to a general strategy, by which influenza viruses can antagonize antiviral responses in infected cells to secure its replications. Other RNA viruses are also able to inhibit cellular transcription to secure their replication. For example, bunyamwera virus NS-S protein inhibits the phosphorylation of Pol II C-terminal domain, while Rift Valley Fever Virus NSs protein targets TFIIH to inhibit the transcription of cellular genes including those of antiviral genes [54,55]. However, to our knowledge, there are no data available except this study, that negative- or positive-sense RNA viruses can inhibit cellular transcription by direct binding of viral proteins to cellular DNA. Thus, our work provides a first example of such a mechanism that could be potentially exploited by other RNA virus families.

Finally, the influenza NS1-dsDNA interaction can be potentially exploited for treatment of IAV infection. In particular, small-molecular inhibitors of this interaction can potentially restore innate immune responses and inhibit virus replication. In addition, viruses expressing dsDNA binding deficient NS1 may display characteristics desirable for potential live-attenuated viral vaccines.

5. Conclusions

Host cell activates transcription of a set of antiviral genes in response to IAV infection. Our results suggest that the viral NS1 protein can inhibit transcription of some of these genes by binding to dsDNA and preventing the loading of cellular transcription machinery. Thus, IAV can exploit its NS1 protein to attenuate antiviral responses at transcriptional level to secure its replication.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagrm.2016.09.005.

References

[1] GBD 2013, Mortality and Causes of Death Collaborators. Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990–2013: a systematic analysis for the global burden of disease study 2013, Lancet 385 (9963) (2015) 117–171, http://dx.doi.org/10.1016/S0140–6736(14)61682–2 (PubMed PMID: 25530442; PubMed Central PMCID: PMCPMC4340604).
[2] A. Iwasaki, P.S. Pillai, Innate immunity to influenza virus infection, Nat. Rev. Immunol. 14 (5) (2014) 315–328, http://dx.doi.org/10.1038/nri3665 (PubMed PMID: 24762827; PubMed Central PMCID: PMCPMC4104278).
[3] M.J. Ciancanelli, S.X. Huang, P. Luthra, H. Garner, Y. Itan, S. Volpi, et al., Infectious disease. Life-threatening influenza and impaired interferon amplification in human IRF7 deficiency, Science 348 (6233) (2015) 448–453, http://dx.doi.org/10.1126/science.aaa1578 (PubMed PMID: 25814066; PubMed Central PMCID: PMCPMC4431581).
[4] P.S. Pillai, R.D. Molony, K. Martinod, H. Dong, I.K. Pang, M.C. Tal, et al., Mx1 reveals innate pathways to antiviral resistance and lethal influenza disease, Science 352 (6284) (2016) 463–466, http://dx.doi.org/10.1126/science.aaf3926 (PubMed PMID: 27102485).
[5] M. Miettinen, T. Sareneva, I. Julkunen, S. Matikainen, IFNs activate toll-like receptor gene expression in viral infections, Genes Immun. 2 (6) (2001) 349–355, http://dx.doi.org/10.1038/sj.gene.6363791 (PubMed PMID: 11607792).
[6] S.M. Makela, P. Osterlund, V. Westenius, S. Latvala, M.S. Diamond, M. Gale Jr., et al., RIG-I signaling is essential for influenza B virus-induced rapid interferon gene expression, J. Virol. 89 (23) (2015) 12014–12025, http://dx.doi.org/10.1128/JVI.01576–15 (PubMed PMID: 26378160; PubMed Central PMCID: PMCPMC4645339).
[7] A.G. Bowie, L. Unterholzner, Viral evasion and subversion of pattern-recognition receptor signalling, Nat. Rev. Immunol. 8 (12) (2008) 911–922, http://dx.doi.org/10.1038/nri2436 (PubMed PMID: 18989317).
[8] S.Y. Liu, D.J. Sanchez, R. Aliyari, S. Lu, G. Cheng, Systematic identification of type I and type II interferon-induced antiviral factors, Proc. Natl. Acad. Sci. U. S. A. 109 (11) (2012) 4239–4244, http://dx.doi.org/10.1073/pnas.1114981109 (PubMed PMID: 22371602; PubMed Central PMCID: PMCPMC3306696).
[9] J.W. Schoggins, S.J. Wilson, M. Panis, M.Y. Murphy, C.T. Jones, P. Bieniasz, et al., A diverse range of gene products are effectors of the type I interferon antiviral response, Nature 472 (7344) (2011) 481–485, http://dx.doi.org/10.1038/nature09907 (PubMed PMID: 21478870; PubMed Central PMCID: PMCPMC3409588).
[10] H.M. Lazear, T.J. Nice, M.S. Diamond, Interferon-lambda: immune functions at barrier surfaces and beyond, Immunity 43 (1) (2015) 15–28, http://dx.doi.org/10.1016/j.immuni.2015.07.001 (PubMed PMID: 26200010; PubMed Central PMCID: PMCPMC4527169).
[11] D. Goubau, M. Schlee, S. Deddouche, A.J. Pruijssers, T. Zillinger, M. Goldeck, et al., Antiviral immunity via RIG-I-mediated recognition of RNA bearing 5′-diphosphates, Nature 514 (7522) (2014) 372–375, http://dx.doi.org/10.1038/nature13590 (PubMed PMID: 25119032; PubMed Central PMCID: PMCPMC4201573).
[12] A.A. Benitez, M. Panis, J. Xue, A. Varble, J.V. Shim, A.L. Frick, et al., In vivo RNAi screening identifies MDA5 as a significant contributor to the cellular defense against influenza A virus, Cell Rep. 11 (11) (2015) 1714–1726, http://dx.doi.org/10.1016/j.celrep.2015.05.032 (PubMed PMID: 26074083; PubMed Central PMCID: PMCPMC4586153).
[13] A.M. Pham, F.G. Santa Maria, T. Lahiri, E. Friedman, I.J. Marie, D.E. Levy, PKR transduces MDA5-dependent signals for type I IFN induction, PLoS Pathog. 12 (3) (2016), e1005489 http://dx.doi.org/10.1371/journal.ppat.1005489 (PubMed PMID: 26939124; PubMed Central PMCID: PMCPMC4777437).
[14] J. Melchjorsen, H. Kristiansen, R. Christiansen, J. Rintahaka, S. Matikainen, S.R. Paludan, et al., Differential regulation of the OASL and OAS1 genes in response to viral infections, J. Interf. Cytokine Res. 29 (4) (2009) 199–207, http://dx.doi.org/10.1089/jir.2008.0050 (PubMed PMID: 19203244).
[15] J.Y. Min, R.M. Krug, The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: inhibiting the 2′-5′ oligo (A) synthetase/RNase L pathway, Proc. Natl. Acad. Sci. U. S. A. 103 (18) (2006) 7100–7105, http://dx.doi.org/10.1073/pnas.0602184103 (PubMed PMID: 16627618; PubMed Central PMCID: PMC1459024).
[16] U.Y. Choi, J.S. Kang, Y.S. Hwang, Y.J. Kim, Oligoadenylate synthase-like (OASL) proteins: dual functions and associations with diseases, Exp. Mol. Med. 47 (2015),

e144 http://dx.doi.org/10.1038/emm.2014.110 (PubMed PMID: 25744296; PubMed Central PMCID: PMCPMC4351405).
[17] M. Moriyama, I.Y. Chen, A. Kawaguchi, T. Koshiba, K. Nagata, H. Takeyama, et al., The RNA- and TRIM25-binding domains of influenza virus NS1 protein are essential for suppression of NLRP3 inflammasome-mediated interleukin-1beta secretion, J. Virol. 90 (8) (2016) 4105–4114, http://dx.doi.org/10.1128/JVI.00120–16 (PubMed PMID: 26865721; PubMed Central PMCID: PMCPMC4810543).
[18] S. Ludwig, T. Wolff, Influenza A virus TRIMs the type I interferon response, Cell Host Microbe 5 (5) (2009) 420–421, http://dx.doi.org/10.1016/j.chom.2009.05.004 (PubMed PMID: 19454344).
[19] E.S. Gold, A.H. Diercks, I. Podolsky, R.L. Podyminogin, P.S. Askovich, P.M. Treuting, et al., 25-Hydroxycholesterol acts as an amplifier of inflammatory signaling, Proc. Natl. Acad. Sci. U. S. A. 111 (29) (2014) 10666–10671, http://dx.doi.org/10.1073/pnas.1404271111 (PubMed PMID: 24994901; PubMed Central PMCID: PMCPMC4115544).
[20] D. Pilla-Moffett, M.F. Barber, G.A. Taylor, J. Coers, Interferon-inducible GTPases in host resistance, Inflamm. Dis. J. Mol. Biol. (2016) http://dx.doi.org/10.1016/j.jmb.2016.04.032 (PubMed PMID: 27181197).
[21] E. Meunier, P. Broz, Interferon-inducible GTPases in cell autonomous and innate immunity, Cell. Microbiol. 18 (2) (2016) 168–180, http://dx.doi.org/10.1111/cmi.12546 (PubMed PMID: 26572694).
[22] B.H. Kim, A.R. Shenoy, P. Kumar, C.J. Bradfield, J.D. MacMicking, IFN-inducible GTPases in host cell defense, Cell Host Microbe 12 (4) (2012) 432–444, http://dx.doi.org/10.1016/j.chom.2012.09.007 (PubMed PMID: 23084913; PubMed Central PMCID: PMCPMC3490204).
[23] S.E. Dudek, K. Nitzsche, S. Ludwig, E. C., Influenza A viruses suppress cyclooxygenase-2 expression by affecting its mRNA stability, Sci. Rep. 6 (2016), 27275 http://dx.doi.org/10.1038/srep27275 (PubMed PMID: 27265729; PubMed Central PMCID: PMCPMC4893666).
[24] L. Huang, R. Ou, G. Rabelo de Souza, C. TM, H. Lemos, E. Mohamed, et al., Virus infections incite pain hypersensitivity by inducing indoleamine 2,3 dioxygenase, PLoS Pathog. 12 (5) (2016), e1005615 http://dx.doi.org/10.1371/journal.ppat.1005615 (PubMed PMID: 27168185; PubMed Central PMCID: PMCPMC4863962).
[25] J.M. Fox, J.M. Crabtree, L.K. Sage, S.M. Tompkins, R.A. Tripp, Interferon lambda upregulates IDO1 expression in respiratory epithelial cells after influenza virus infection, J. Interf. Cytokine Res. 35 (7) (2015) 554–562, http://dx.doi.org/10.1089/jir.2014.0052 (PubMed PMID: 25756191; PubMed Central PMCID: PMCPMC4507134).
[26] Z.A. Bornholdt, B.V. Prasad, X-ray structure of NS1 from a highly pathogenic H5N1 influenza virus, Nature 456 (7224) (2008) 985–988, http://dx.doi.org/10.1038/nature07444 (PubMed PMID: 18987632; PubMed Central PMCID: PMC2798118).
[27] J. Ayllon, A. Garcia-Sastre, The NS1 protein: a multitasking virulence factor, Curr. Top. Microbiol. Immunol. 386 (2015) 73–107, http://dx.doi.org/10.1007/82_2014_400 (PubMed PMID: 25007846).
[28] Z. Zhu, Z. Shi, W. Yan, J. Wei, D. Shao, X. Deng, et al., Nonstructural protein 1 of influenza A virus interacts with human guanylate-binding protein 1 to antagonize antiviral activity, PLoS ONE 8 (2) (2013), e55920 http://dx.doi.org/10.1371/journal.pone.0055920 (PubMed PMID: 23405236; PubMed Central PMCID: PMCPMC3566120).
[29] M. Anastasina, B. Schepens, S. Soderholm, T.A. Nyman, S. Matikainen, K. Saksela, et al., The C terminus of NS1 protein of influenza A/WSN/1933(H1N1) virus modulates antiviral responses in infected human macrophages and mice, J. Gen. Virol. 96 (8) (2015) 2086–2091, http://dx.doi.org/10.1099/vir.0.000171 (PubMed PMID: 25934792).
[30] I. Marazzi, J.S. Ho, J. Kim, B. Manicassamy, S. Dewell, R.A. Albrecht, et al., Suppression of the antiviral response by an influenza histone mimic, Nature 483 (7390) (2012) 428–433, http://dx.doi.org/10.1038/nature10892 (PubMed PMID: 22419161; PubMed Central PMCID: PMC3598589).
[31] S. Soderholm, M. Anastasina, M.M. Islam, J. Tynell, M.M. Poranen, D.H. Bamford, et al., Immuno-modulating properties of saliphenylhalamide, SNS-032, obatoclax, and gemcitabine, Antivir. Res. 126 (2016) 69–80, http://dx.doi.org/10.1016/j.antiviral.2015.12.011 (PubMed PMID: 26738783).
[32] O.V. Denisova, L. Kakkola, L. Feng, J. Stenman, A. Nagaraj, J. Lampe, et al., Obatoclax, saliphenylhalamide, and gemcitabine inhibit influenza a virus infection, J. Biol. Chem. 287 (42) (2012) 35324–35332, http://dx.doi.org/10.1074/jbc.M112.392142 (PubMed PMID: 22910914; PubMed Central PMCID: PMCPMC3471742).
[33] A.H. Broquet, Y. Hirata, C.S. McAllister, M.F. Kagnoff, RIG-I/MDA5/MAVS are required to signal a protective IFN response in rotavirus-infected intestinal epithelium, J. Immunol. 186 (3) (2011) 1618–1626, http://dx.doi.org/10.4049/jimmunol.1002862 (PubMed PMID: 21187438).
[34] L. Slater, N.W. Bartlett, J.J. Haas, J. Zhu, S.D. Message, R.P. Walton, et al., Co-ordinated role of TLR3, RIG-I and MDA5 in the innate response to rhinovirus in bronchial epithelium, PLoS Pathog. 6 (11) (2010), e1001178 http://dx.doi.org/10.1371/journal.ppat.1001178 (PubMed PMID: 21079690; PubMed Central PMCID: PMCPMC2973831).
[35] O.V. Denisova, S. Soderholm, S. Virtanen, C. Von Schantz, D. Bychkov, E. Vashchinkina, et al., Akt inhibitor MK2206 prevents influenza pH1N1 virus infection in vitro, Antimicrob. Agents Chemother. 58 (7) (2014) 3689–3696, http://dx.doi.org/10.1128/AAC.02798–13 (PubMed PMID: 24752266; PubMed Central PMCID: PMCPMC4068572).
[36] T. Ohman, N. Lietzen, E. Valimaki, J. Melchjorsen, S. Matikainen, T.A. Nyman, Cytosolic RNA recognition pathway activates 14-3-3 protein mediated signaling and caspase-dependent disruption of cytokeratin network in human keratinocytes, J. Proteome Res. 9 (3) (2010) 1549–1564, http://dx.doi.org/10.1021/pr901040u (PubMed PMID: 20070120).
[37] L. Kakkola, O.V. Denisova, J. Tynell, J. Viiliainen, T. Ysenbaert, R.C. Matos, et al., Anticancer compound ABT-263 accelerates apoptosis in virus-infected cells and imbalances cytokine production and lowers survival rates of infected mice, Cell Death Dis. 4 (2013), e742 http://dx.doi.org/10.1038/cddis.2013.267 (PubMed PMID: 23887633; PubMed Central PMCID: PMC3730437).
[38] N. Lietzen, T. Ohman, J. Rintahaka, I. Julkunen, T. Aittokallio, S. Matikainen, et al., Quantitative subcellular proteome and secretome profiling of influenza A virus-infected human primary macrophages, PLoS Pathog. 7 (5) (2011), e1001340 http://dx.doi.org/10.1371/journal.ppat.1001340 (PubMed PMID: 21589892; PubMed Central PMCID: PMCPMC3093355).
[39] D.E. Kainov, K.H. Muller, L.L. Theisen, M. Anastasina, M. Kaloinen, C.P. Muller, Differential effects of NS1 proteins of human pandemic H1N1/2009, avian highly pathogenic H5N1, and low pathogenic H5N2 influenza A viruses on cellular pre-mRNA polyadenylation and mRNA translation, J. Biol. Chem. 286 (9) (2011) 7239–7247, http://dx.doi.org/10.1074/jbc.M110.203489 (PubMed PMID: 21163951; PubMed Central PMCID: PMCPMC3044980).
[40] M. Gerard, L. Fischer, V. Moncollin, J.M. Chipoulet, P. Chambon, J.M. Egly, Purification and interaction properties of the human RNA polymerase B(II) general transcription factor BTF2, J. Biol. Chem. 266 (31) (1991) 20940–20945 (PubMed PMID: 1939143).
[41] D.E. Kainov, L.A. Selth, J.Q. Svejstrup, J.M. Egly, A. Poterzsman, Interacting partners of the Tfb2 subunit from yeast TFIIH, DNA Repair (Amst) 9 (1) (2010) 33–39, http://dx.doi.org/10.1016/j.dnarep.2009.10.002 (PubMed PMID: 19897425).
[42] T.R. Riley, M. Slattery, N. Abe, C. Rastogi, D. Liu, R.S. Mann, et al., SELEX-seq: a method for characterizing the complete repertoire of binding site preferences for transcription factor complexes, Methods Mol. Biol. 1196 (2014) 255–278, http://dx.doi.org/10.1007/978-1-4939-1242-1_16 (PubMed PMID: 25151169; PubMed Central PMCID: PMCPMC4265583).
[43] N.R. Donelan, C.F. Basler, A. Garcia-Sastre, A recombinant influenza A virus expressing an RNA-binding-defective NS1 protein induces high levels of beta interferon and is attenuated in mice, J. Virol. 77 (24) (2003) 13257–13266 (PubMed PMID: 14645582; PubMed Central PMCID: PMC296096).
[44] C.M. Newby, L. Sabin, A. Pekosz, The RNA binding domain of influenza A virus NS1 protein affects secretion of tumor necrosis factor alpha, interleukin-6, and interferon in primary murine tracheal epithelial cells, J. Virol. 81 (17) (2007) 9469–9480, http://dx.doi.org/10.1128/JVI.00989-07 (PubMed PMID: 17596305; PubMed Central PMCID: PMC1951395).
[45] Y. Fu, L. Gaelings, S. Söderholm, S. Belanov, J. Nandania, T.A. Nyman, et al., JNJ872 inhibits influenza A virus replication without altering cellular antiviral responses, Antivir. Res. (2016) http://dx.doi.org/10.1016/j.antiviral.2016.07.008 (in press).
[46] A. Cheng, S.M. Wong, Y.A. Yuan, Structural basis for dsRNA recognition by NS1 protein of influenza A virus, Cell Res. 19 (2) (2009) 187–195, http://dx.doi.org/10.1038/cr.2008.288 (PubMed PMID: 18813227).
[47] W. Wang, K. Riedel, P. Lynch, C.Y. Chien, G.T. Montelione, R.M. Krug, RNA binding by the novel helical domain of the influenza virus NS1 protein requires its dimer structure and a small number of specific basic amino acids, RNA 5 (2) (1999) 195–205 (PubMed PMID: 10024172; PubMed Central PMCID: PMC1369752).
[48] C.Y. Chien, Y. Xu, R. Xiao, J.M. Aramini, P.V. Sahasrabudhe, R.M. Krug, et al., Biophysical characterization of the complex between double-stranded RNA and the N-terminal domain of the NS1 protein from influenza A virus: evidence for a novel RNA-binding mode, Biochemistry 43 (7) (2004) 1950–1962, http://dx.doi.org/10.1021/bi030176o (PubMed PMID: 14967035).
[49] S.N. Thulasi Raman, Y. Zhou, Networks of host factors that interact with NS1 protein of influenza A virus, Front. Microbiol. 7 (2016) 654, http://dx.doi.org/10.3389/fmicb.2016.00654 (PubMed PMID: 27199973; PubMed Central PMCID: PMCPMC4855030).
[50] S. Qin, Y. Liu, W. Tempel, M.S. Eram, C. Bian, K. Liu, et al., Structural basis for histone mimicry and hijacking of host proteins by influenza virus protein NS1, Nat. Commun. 5 (2014) 3952, http://dx.doi.org/10.1038/ncomms4952 (PubMed PMID: 24853335).
[51] C. Yin, J.A. Khan, G.V. Swapna, A. Ertekin, R.M. Krug, L. Tong, et al., Conserved surface features form the double-stranded RNA binding site of non-structural protein 1 (NS1) from influenza A and B viruses, J. Biol. Chem. 282 (28) (2007) 20584–20592, http://dx.doi.org/10.1074/jbc.M611619200 (PubMed PMID: 17475623).
[52] B. Dauber, J. Schneider, W. T., Double-stranded RNA binding of influenza B virus nonstructural NS1 protein inhibits protein kinase R but is not essential to antagonize production of alpha/beta interferon, J. Virol. 80 (23) (2006) 11667–11677, http://dx.doi.org/10.1128/JVI.01142–06 (PubMed PMID: 16987984; PubMed Central PMCID: PMCPMC1642593).
[53] W. Wang, R.M. Krug, The RNA-binding and effector domains of the viral NS1 protein are conserved to different extents among influenza A and B viruses, Virology 223 (1) (1996) 41–50, http://dx.doi.org/10.1006/viro.1996.0453 (PubMed PMID: 8806538).
[54] N. Le May, S. Dubaele, P. De Santis L, A. Billecocq, M. Bouloy, E. JM, TFIIH transcription factor, a target for the Rift Valley hemorrhagic fever virus, Cell 116 (4) (2004) 541–550 (PubMed PMID: 14980221).
[55] D. Thomas, G. Blakqori, V. Wagner, M. Banholzer, N. Kessler, R.M. Elliott, et al., Inhibition of RNA polymerase II phosphorylation by a viral interferon antagonist, J. Biol. Chem. 279 (30) (2004) 31471–31477, http://dx.doi.org/10.1074/jbc.M400938200 (PubMed PMID: 15150262).