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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.

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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, antagonizing 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 $\mu\text{g}/\text{ml}$ 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-Hochberg 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 ($\log_2\text{Fc}$). The gene-expression data was deposited to Gene Expression Omnibus (GEO accession 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 promoter (forward: 5'-GTCAGTAGAATCCACGGATACAG-3' and reverse: 5'-CTTGGGAGAAAGCAAAGGAAAG-3') and exon (forward: 5'-GCCGATTGACCATTCTATGA-3' and reverse: 5'-GCCAGGAGGTTCTCAA CAATAG-3'), IFNA1 (forward: 5'-ATGCGAACCAGTTCAGAAAG-3', reverse: 5'-CATCCCAAGCAGCAGATGAA-3'), IFNA16 (forward: 5'-GACT CACTTCTATAACCACCACAA-3', reverse: 5'-TAGTGCCTGCACAGTAAAC-3'), IL6 (forward: 5'-TCATCACTGGTCTTTTGG-3', reverse: 5'-CTCTGG CTGTTCCTCAC-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 synthesized 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% bromophenol 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, TNF α , and IFN λ in the cell supernatants were assayed with ELISA (PBL Interferon Source) as described previously [37].

2.10. Mass spectrometry

Five 175 cm² 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 μ l 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 (Qiagen) 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 from 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, TFIIIF, 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'-GTTTCAGAGTTC 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 (log₂ 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, immunoblotting, 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 H3.2, 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, H3.2 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.

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 non-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^{RK/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^{RK/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^{RK/AA} were tested in a transcription assay. Increasing amounts of the proteins were incubated with transcription factors (TFx), pol II, nucleotides (NTPs), ³²P-labeled ATP and DNA for 45 min. Reactions were stopped and synthesized ³²P-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 $11.1 \pm 0.7 \mu\text{M}$, whereas the K_d for NS1^{RK/AA} was $> 100 \mu\text{M}$ (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 TFIIF 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.bbagr.2016.09.005>.

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