Epigenetic Modification Is Regulated by the Interaction of Influenza A Virus Nonstructural Protein 1 with the *De Novo* DNA Methyltransferase DNMT3B and Subsequent Transport to the Cytoplasm for K48-Linked Polyubiquitination

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ABSTRACT The influenza virus nonstructural protein 1 (NS1) is a nonstructural protein that plays a major role in antagonizing host interferon responses during infection. However, a clear role for the NS1 protein in epigenetic modification has not been established. In this study, NS1 was found to regulate the expression of some key regulators of JAK-STAT signaling by inhibiting the DNA methylation of their promoters. Furthermore, DNA methyltransferase 3B (DNMT3B) is responsible for this process. Upon investigating the mechanisms underlying this event, NS1 was found to interact with DNMT3B but not DNMT3A, leading to the dissociation of DNMT3B from the promoters of the corresponding genes. In addition, the interaction between NS1 and DNMT3B changed the localization of DNMT3B from the nucleus to the cytosol, resulting in K48-linked ubiquitination and degradation of DNMT3B in the cytosol. We conclude that NS1 interacts with DNMT3B and changes its localization to mediate K48-linked polyubiquitination, subsequently contributing to the modulation of the expression of JAK-STAT signaling suppressors.

IMPORTANCE The nonstructural protein 1 (NS1) of the influenza A virus (IAV) is a multifunctional protein that counters cellular antiviral activities and is a virulence factor. However, the involvement of NS1 in DNA methylation during IAV infection has not been established. Here, we reveal that the NS1 protein binds the cellular DNMT3B DNA methyltransferase, thereby inhibiting the methylation of the promoters of genes encoding suppressors of JAK-STAT signaling. As a result, these suppressor genes are induced, and JAK-STAT signaling is inhibited. Furthermore, we demonstrate that the NS1 protein transports DNMT3B to the cytoplasm for ubiquitination and degradation. Thus, we identify the NS1 protein as a potential trigger of the epigenetic deregulation of JAK-STAT signaling suppressors and illustrate a novel mechanism underlying the regulation of host immunity during IAV infection.

KEYWORDS DNA methyltransferase 3B, influenza A virus, nonstructural protein 1

Influenza A virus (IAV) is a negative-sense single-stranded RNA virus that causes annual global epidemics of respiratory illnesses (1). The IAV genome consists of 8 RNA segments that encode 10 to 11 proteins, depending on the viral strain (2). Among these proteins, nonstructural protein 1 (NS1), which is encoded by the eighth segment of IAV

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genome, is a major virulence factor that is required for pathogenesis (3). A major function of NS1 is as an interferon (IFN) antagonist (4). NS1 has consistently been shown to inhibit host innate IFN-mediated antiviral responses at multiple levels. NS1 functionally interacts with some important host factors to perform these functions. For example, NS1 specifically interacts with TRIM25, thereby preventing RIG-I CARD ubiquitination, which is required to induce interferon beta (IFN- β) expression (5). NS1 interacts with the 30-kDa subunit of cleavage and polyadenylation specificity factor (CPSF30), inhibiting cellular pre-mRNA processing (6). NS1 inhibits IFN- β expression by preventing the activation of the IRF-3, NF- κ B, and ATF-2/c-Jun transcription factors (7, 8). NS1 also blocks the activation of OAS and PKR, which are two important IFN-inducible antiviral proteins (9, 10).

Based on recent advances, cytokines play a key role in the control of immune responses (11). The biological functions of cytokines mainly depend on cytokine-mediated gene activation or repression (11). In the universal and essential cytokine receptor signaling network, the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway is one of the best understood signal transduction cascades (12). The JAK-STAT pathway has been shown to be a common signaling pathway used by almost 40 cytokine receptors (13). Although this pathway has been well illuminated in the past few decades, major questions remain concerning how the cascades are regulated. JAK-STAT signaling has recently been shown to be regulated at many steps through distinct mechanisms. To date, at least three key regulator families have been discovered, including the suppressor of cytokine signaling (SOCS) family, protein inhibitor of activated STAT (PIAS) family, and protein tyrosine phosphatase (PTP) family (11). The SOCS family of proteins comprises eight members: CIS (cytokine-inducible SH2 domain protein) and SOCS1 to SOCS7 (14). The mammalian PIAS family comprises four members: PIAS1, PIASX (PIAS2), PIAS3, and PIASY (PIAS4) (15). The PTP family comprises at least five members: SHP1, SHP2, CD45, PTP1B, and T-cell PTP (TCPTP) (16). Interestingly, IAV infection strongly induces SOCS1 and SOCS3 expression (17). However, researchers have not yet clearly determined how IAV regulates JAK-STAT signaling through those regulators and whether IAV regulates the expression of PIAS and PTP family members.

The term epigenetics has been used to describe the wide range of heritable changes in gene expression without any change in gene sequence (18). Epigenetic modifications occur during development and cell proliferation to regulate transcriptional activity in normal tissues (19). Epigenetic changes include reversible DNA methylation, histone acetylation or methylation, and nucleosome positioning (20). DNA methylation is a common epigenetic modification that involves the addition of a methyl group to the 5' position of the cytosine ring in the CpG dinucleotide and contributes to gene silencing, thus regulating gene expression (20). Mammalian DNA methylation is catalyzed by DNA methyltransferases (DNMTs), including DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L, but only DNMT1, DNMT3A, and DNMT3B possess methyltransferase activity (21). In general, DNMT1 is responsible for copying and maintaining methylation patterns after DNA replication, whereas DNMT3A and DNMT3B function as *de novo* methyltransferases (21).

To date, two studies have reported epigenetic changes during IAV infection. One study found that the NS1 protein suppresses antiviral responses by acting as a histone mimic that suppresses transcriptional elongation (22). The other study showed that IAV infection causes changes in the methylation of promoter DNA of genes encoding inflammatory proteins (23). One study by our group found that interleukin 32 (IL-32) is upregulated by aberrant DNA methylation modifications during IAV infection (24). Another study by our group found that IAV infection inhibits DNMT3B expression, leading to cyclooxygenase 2 and lambda-1 interferon production (25). Based on these previous findings, a study on the effect of NS1 on DNA methylation was conducted. As shown in the present study, NS1 interacts with DNMT3B and induces its dissociation from the regulatory regions of promoters of some key regulators of JAK-STAT signaling, epigenetically triggering their transcriptional activity. Furthermore, NS1 transports

DNMT3B into the cytoplasm, where it undergoes ubiquitination and degradation. Our results provide new insights into the IFN antagonist functions of NS1.

RESULTS

NS1 regulates the expression of some key regulators of JAK-STAT signaling via DNA methylation of their promoters. To study the importance of NS1 in DNA methylation during IAV infection, a series of known recombinant IAV viruses were generated, and their replication was tested in cell and mouse models. Results showed that, compared with that in H1N1 influenza A/PR/8/34 (PR8), the replication of the PR8/delNS1 virus (in which the NS1 gene is deleted) (26) and the R38A/K41A NS1 mutant virus (in which the NS1 gene is deficient in double-stranded RNA [dsRNA] binding capacity) (27) was reduced in A549 cells (see Fig. S1A in the supplemental material). In addition, the high dose (1 PFU per cell) of PR8/delNS1 virus and of the R38A/K41A NS1 mutant virus (1 PFU per cell) yielded similar titers that the low dose of the PR8 wild-type (WT) virus (0.001 PFU per cell) yielded in A549 cells (Fig. S1A). Because Vero cells constitute an IFN-deficient cell line (26, 28), the replication of PR8/delNS1 virus and the R38A/K41A NS1 mutant virus was similar to that of the WT virus in Vero cells (Fig. S1A). Similar results were obtained using the H1N1 influenza A/WSN/33 (WSN) virus and the mutant WSN (L144A+L146A) virus, in which the nuclear export signal (NES) of NS1 has been mutated (29) (Fig. S1B).

Because increasing evidence suggests that NS1 is an IFN antagonist, we speculated that NS1 plays an important role in the expression of negative key regulators of JAK-STAT signaling. To test our hypothesis, A549 cells were inoculated with the PR8 WT virus and the PR8/delNS1 virus. Real-time reverse transcriptase PCR (RT-PCR) results indicated that PR8 WT virus infection induced the expression of all regulators of JAK-STAT signaling, with the exception of SOCS2 and CD45 (Fig. 1A). Interestingly, the absence of NS1 completely abolished the induction of SOCS1, SOCS3, PIAS1, and PIAS3 expression, partially repressed the induction of PTP1B, TCPTP, PIASX, and PIASY expression, and did not affect the induction of SHP-1 and SHP-2 expression (Fig. 1A, top). The analysis of virus titers, the expression of three different forms of IAV RNA (mRNA, cRNA, and vRNA), and the expression of PKR and MxA as controls for efficient PR8/delNS1 virus infection are shown in Fig. 1A (bottom). Because IAV infection has been shown to induce expression of only SOCS1 and SOCS3 among the members of the SOCS family (17, 30), we did not examine the effect of NS1 on the expression of other SOCS family members. For the purpose of comparison, SOCS2 expression was measured as a negative control. Based on the results of Western blot analysis, PR8 WT virus infection significantly increased the expression of the SOCS1, SOCS3, PIAS1, and PIAS3 proteins, whereas PR8/delNS1 virus infection did not affect the expression of those proteins (Fig. 1B). Using the demethylating agent 5-aza-2'-deoxycytidine (5-aza-CdR), demethylation was shown to increase the mRNA levels of NS1-regulated genes (*SOCS1*, *SOCS3*, *PTP1B*, *TCPTP*, *PIAS1*, *PIASX*, *PIAS3*, and *PIASY*) in a dose-dependent manner (Fig. 1C and Fig. S2). High-sensitivity mapping of the methylated cytosines was performed using bisulfite modification to obtain more precise information about the methylation status of the *SOCS1*, *SOCS3*, *PIAS1*, and *PIAS3* promoters. As shown in Fig. 1D, the percentage of methylated CpGs in the four genes was significantly lower in PR8 WT virus-infected cells than in control cells. However, infection of cells with the PR8/delNS1 virus mutant did not affect the methylation status of the promoters for the four genes, indicating that NS1 induces *de novo* demethylation in these cells (Fig. 1D). Interestingly, infection of cells with the PR8 WT virus also decreased the percentage of methylated CpGs in the *PTP1B*, *TCPTP*, *PIASX*, and *PIASY* promoters, whereas PR8/delNS1 virus infection partially eliminated the demethylation of the promoters of the four genes (Fig. S3A to D). These data may explain why the NS1 deficiency partially inhibited the induction of PTP1B, TCPTP, PIASX, and PIASY expression. The role of NS1 on these suppressors was confirmed by repeating the experiments in Vero cells, in which the replication level of PR8/delNS1 virus was comparable to that of the PR8 WT virus (see Fig. S4). These results suggest that NS1 induces the expression of genes encoding

FIG 1 Verification of the DNA methylation status of key NS1-regulated suppressors of the JAK-STAT pathway. (A) A549 cells were infected with the PR8 WT virus (0.001 PFU per cell) and the PR8/delNS1 virus (1 PFU per cell) for 18 h and then treated with MG132 (100 μ M) for 6 h prior to real-time RT-PCR analysis. (B) Experiments were performed as described for panel A, except that SOCS1, SOCS3, PIAS1, and PIAS3 expression was analyzed by Western blotting. (C) A549 cells were treated with or without the indicated concentrations of 5-aza-CdR for 24 h prior to real-time RT-PCR analysis. (D) A549 cells were infected with the PR8 WT virus (0.001 PFU per cell) and the PR8/delNS1 virus (1 PFU per cell) for 18 h and then treated with MG132 (100 μ M) for 6 h prior to bisulfite sequencing of the indicated gene promoter regions. Open circle, methylated; solid circle, methylated CpG dinucleotide. The percentages of methylated CpG dinucleotides are indicated in parentheses. In the real-time RT-PCR experiments, the control was designated 1. All experiments were repeated at least three times with similar results. Bar graphs present the means $\pm$ SDs ($n = 3$). **, $P < 0.01$; N.S., not significant.

FIG 2 DNMT3B is responsible for NS1-induced expression of key regulators of the JAK-STAT pathway. (A) A549 cells were transfected with the vector or pcDNA-DNMT1. Twenty-four hours after transfection, cells infected with or without the PR8 virus (0.001 PFU per cell) for 18 h and treated with MG132 (100 μ M) for 6 h prior to real-time RT-PCR analysis. (B and C) Experiments were performed as described for panel A, except that cells were transfected with pcDNA-DNMT3A (B) or pcDNA-DNMT3B (C). (D) A549 cells were transfected with the indicated plasmids for 42 h and treated with MG132 (100 μ M) for 6 h prior to real-time RT-PCR analysis. In the real-time RT-PCR experiments, the control was designated 1. Bar graphs present the means $\pm$ SDs ($n = 3$). **, $P < 0.01$; *, $P < 0.05$; N.S., not significant.

specific suppressors of JAK-STAT signaling by inducing the demethylation of their promoter regions.

DNMT3B is responsible for NS1-regulated expression of JAK-STAT signaling suppressors. Because the DNMT family includes at least three functional DNMTs, DNMT1, DNMT3A, and DNMT3B, we set out to determine which DNMT plays a role in the expression of genes encoding JAK-STAT signaling suppressors. In real-time RT-PCR assays, the overexpression of DNMT1 did not affect the expression of the suppressors, with the exception of *PIASX* (Fig. 2A). Similarly, DNMT3A overexpression did not regulate the expression of the suppressors (Fig. 2B). However, the suppressors were expressed at lower levels in cells overexpressing DNMT3B (Fig. 2C). *SOCS2* expression was measured as a negative control. The effect of DNMT3B on the expression of the suppressors was further evaluated using an NS1 overexpression plasmid. As shown in Fig. 2D, the NS1-PR8 overexpression plasmid promoted the suppressor mRNA expression, whereas the expression induced by the NS1 overexpression plasmid was inhibited by the DNMT3B overexpression plasmid but not by the DNMT1 and DNMT3A overex-

pression plasmids. Similar results were obtained using the H3N2 influenza A/Hong Kong/498/97 (HK) virus and H3N2/deIN51 virus (see Fig. S5). Thus, DNMT3B plays a major role in NS1-regulated expression of genes encoding JAK-STAT signaling suppressors.

The IAV NS1 protein binds to DNMT3B. Since DNMT3B is responsible for NS1-regulated gene expression, we speculated that NS1 interacts with DNMT3B. To test this hypothesis, DNMT3B or DNMT3A was fused in-frame with the GAL4 DNA-binding domain to create the plasmid pM-DNMT3B or pM-DNMT3A, respectively. The NS1 protein of H1N1 influenza A/WSN/33 (WSN) virus was fused in-frame with the VP16 transactivation domain in the pVP16-NS1 plasmid. The results of a mammalian two-hybrid analysis showed that DNMT3B, but not DNMT3A, interacted with NS1-WSN in mammalian cells (Fig. 3A). Co-immunoprecipitation and reverse co-immunoprecipitation experiments were performed to further confirm the binding of the IAV NS1 protein to DNMT3B or DNMT3A. As shown in Fig. 3B, PR8/NS1 interacted with DNMT3B but not with DNMT3A. We further performed endogenous co-immunoprecipitation experiments, and the results indicated that the NS1-DNMT3B interaction increased upon infection with the PR8 virus (Fig. 3C). Similar results were also obtained using WSN virus-infected cells (Fig. 3D). We performed co-immunoprecipitation experiments with a series of truncations of NS1-WSN and DNMT3B to identify the domains responsible for the NS1-DNMT3B interaction. The middle domain of DNMT3B (amino acids 361 to 558) and the N-terminal domain of NS1 (amino acids 1 to 73) were required for the interaction (Fig. 3E). We further investigated the role of NS1 (1-73) on DNMT3B localization and expression. As shown in Fig. 3F and G, NS1 (1-73) predominantly localized to the nucleus, and a small amount of NS1 (1-73) localized to the cytoplasm. The NS1 RNA-binding domain has little effect on DNMT3B localization and expression.

Since the R38A/K41A NS1 mutant is known to be deficient in dsRNA-binding activity and in IFN antagonism (5), we examined whether the R38A/K41A NS1 mutant plays a role in the NS1-DNMT3B interaction. As shown in Fig. S6A and B, R38A/K41A NS1 mutants of the WSN and PR8 viruses showed a complete loss of DNMT3B binding ability in co-immunoprecipitation assays. Furthermore, the R38A/K41A NS1 mutant, in contrast to the PR8 WT virus, did not regulate DNMT3B expression (Fig. S6C). We next investigated the ability of the PR8 WT virus and mutant R38A/K41A to regulate JAK-STAT signaling suppressors, STAT phosphorylation, and interferon-stimulated gene (ISG) expression. As shown in Fig. S6D, the PR8 WT virus potently induced the expression of JAK-STAT signaling suppressors, whereas the R38A/K41A NS1 mutant had no effect on the expression of the suppressors. In line with this observation, the R38A/K41A NS1 mutant virus, compared with the PR8 WT virus, did not inhibit IFN- α -induced STAT1 and STAT3 phosphorylation and ISG expression (Fig. S6E and F). Thus, IAV NS1 interacts with DNMT3B both *in vivo* and *in vitro*, and amino acids R38 and K41 of NS1 play an important role in the interaction.

NS1 regulates the translocation of DNMT3B from the nucleus to the cytosol. As IAV NS1 interacts with DNMT3B, we next detected the subcellular localization of DNMT3B and NS1 after IAV infection. As shown in Fig. 4A, compared to cells infected with the PR8/deIN51 virus, the PR8 WT virus inhibited the nuclear localization of DNMT3B. Endogenous DNMT3A was distributed throughout the cells before and after H3N2 influenza A/Hong Kong/498/97 (HK) virus infection (Fig. 4B). However, endogenous DNMT3B was distributed throughout the cells before the HK virus infection, similar to DNMT3A, but after the HK virus infection took place, the nuclear DNMT3B was translocated from the nucleus to the cytosol (Fig. 4B). Similar results were also obtained in A549 cells (Fig. 4C). IAV NS1 has different functions in different cellular compartments. When expressed in mammalian cells without infection, the NS1 proteins of many influenza A virus strains, such as the NS1 protein of H3N2 influenza A/Sydney/5/97 (Syn) virus, are localized in the nucleus, but the NS1 protein of the PR8 virus primarily displays a cytoplasmic distribution pattern (31). We next generated an NS1 nuclear export signal (NES) mutant (L144A+L146A) of the WSN virus (WSN L144A+L146A) and infected

FIG 3 NS1 interacts with DNMT3B. (A) 293T cells were cotransfected with control plasmids, pG5-luc (a luciferase reporter plasmid), pVP16-NS1, pM-DNMT3A (left), or pM-DNMT3B (right) for 48 h prior to the mammalian two-hybrid analysis. A *Renilla* luciferase reporter vector pRL-TK was used as the internal control. (B) 293T cells were transfected with Flag-tagged NS1 (Flag-NS1) and Myc-tagged DNMT3A (Myc-DNMT3A) (left) or Myc-DNMT3B (right) for 42 h and treated with MG132 (100 μ M) for 6 h. Co-immunoprecipitation and immunoblot analyses were performed with the indicated antibodies. (C) A549 cells were infected with the PR8 virus for the indicated times or left uninfected and treated with MG132 (100 μ M) for 6 h. Immunoprecipitation and immunoblot analyses were performed with the indicated antibodies. (D) A549 cells were infected with the WSN WT virus for the indicated times. Immunoprecipitation and immunoblot analyses were performed with the indicated antibodies. (E) Domain mapping of the NS1 and DNMT3B interaction. 293T cells were transfected with the indicated truncations for 42 h and treated with MG132 (100 μ M) for 6 h. Co-immunoprecipitation and immunoblotting were performed with the indicated antibodies. The schematic representations of NS1 and DNMT3B truncations are shown at the top. (F) A549 cells were transfected with the indicated plasmid for 36 h. Cytosolic and nuclear extracts were subjected to Western blot analysis. Lamin A and β -actin were used as markers for nuclear and cytosolic fractions, respectively. (G) A549 cells were transfected with the indicated plasmid for 36 h prior to Western blot analysis. All experiments were repeated at least three times with consistent results. In immunoprecipitation and immunoblot experiments, cell extracts were treated with 10 mg/ml of RNase A for 4 h. Bar graphs present the means $\pm$ SDs ($n = 3$). **, $P < 0.01$; *, $P < 0.05$; N.S., not significant.

primary ATII cells to validate the observation that the cytosolic localization of NS1 is required for DNMT3B translocation during viral infection. Compared to cells infected with the WSN WT virus, the NS1 NES mutant inhibited the cytosolic localization of NS1, although the NES mutation did not completely restrict the NS1 protein within the nucleus of the host cells (Fig. 4D). The translocation of DNMT3B also changed in the NES mutant virus-infected cells compared with that in the WSN WT virus-infected cells (Fig. 4D). Three residues (148R, 152E, and 153E) of NS1-Syn are key sites for the nuclear export inhibitory activity, and residue 221 of NS1-Syn was identified to be a key residue in the nuclear localization signal (NLS) (31). We further examined whether the subcel-

FIG 4 Detection of the subcellular distribution and colocalization of DNMT3A or DNMT3B with IAV NS1. (A) A549 cells were infected with the PR8 WT virus (0.001 PFU per cell) and the PR8/delNS1 virus (1 PFU per cell) for 18 h and then treated with MG132 (100 μ M) for 6 h. Cytosolic and nuclear extracts were subjected to Western blot analysis. A549 cells (B) or A11 cells (C) were infected with the HK virus (0.001 PFU per cell) for 18 h and treated with MG132 (100 μ M) for 6 h. Cytosolic and nuclear extracts were subjected to Western blot analysis. (D) A11 cells were infected with the indicated virus for 18 h and treated with MG132 (100 μ M) for 6 h. Cytosolic and nuclear extracts were subjected to Western blot analysis. (E) 293T cells were transfected with pmCherry-NS1-Syn (R148A+E152A+E153A) or NLS mutant pmCherry-NS1-Syn (K221E) for 18 h and treated with MG132 (100 μ M) for 6 h. Cytosolic and nuclear extracts were subjected to Western blot analysis. Lamin A and β -actin were used as markers for nuclear and cytosolic fractions, respectively. (F) A549 cells were infected with the HK virus for 24 h or left uninfected and treated with MG132 (100 μ M) for 6 h. Immunoprecipitation and immunoblot analyses were performed with the indicated antibodies. (G) Experiments were performed as described for panel F, except the WSN WT virus or the mutant WSN (L144A+L146A) virus was used. (H) A549 cells were transfected with pmCherry-NS1-Syn (R148A+E152A+E153A) or NLS mutant pmCherry-NS1-Syn (K221E) for 36 h and treated with MG132 (100 μ M) for 6 h. Immunoprecipitation and immunoblot analyses were performed with the indicated antibodies. In immunoprecipitation and immunoblot experiments, cell extracts were treated with 10 mg/ml of RNase A for 4 h. All experiments were repeated at least three times with similar results.

ular localization of DNMT3B was affected by mutations at these sites. In cells transfected with the NS1-Syn triple mutant (R148A+E152A+E153A) or the mutant NS1-Syn (K221E), DNMT3B was translocated from the nucleus to the cytosol (Fig. 4E). The effect of NS1 on the translocation of DNMT3B was also confirmed using immunofluorescence assays (see Fig. S7). Co-immunoprecipitation experiments were performed to further confirm the binding of those recombinant IAV virus NS1 proteins to DNMT3B or

DNMT3A. As shown in Fig. 4F, NS1 was associated with DNMT3B in cells infected with the H3N2 influenza A/Hong Kong/498/97 (HK) virus. Similar results were also obtained using WSN virus-infected cells or NS1-Syn transfected cells (Fig. 4G and H).

We also investigated the effect of the subcellular localization of NS1 on the expression of key regulators of JAK-STAT signaling. Results from the real-time RT-PCR experiments confirmed that WSN WT virus infection strongly induced the expression of the genes encoding these regulators (see Fig. S8A). However, due to the incomplete localization of the NS1 protein within the nuclei of cells infected with the mutant WSN (L144A+L146A) virus, the virus still induced a low level of expression of these genes (Fig. S8A). Similarly, because the NS1-Syn (R148A+E152A+E153A) and NS1-Syn (K221E) mutants were located in the cytosol, both mutants increased the NS1-regulated expression of key regulators of JAK-STAT signaling (Fig. S8B). Thus, we concluded that IAV infection induces DNMT3B translocation from the nucleus to the cytosol. Furthermore, the stimulation depends on the NES and cytosolic localization of NS1.

NS1 dissociates DNMT3B from activated gene promoters. We performed a chromatin immunoprecipitation (ChIP) assay on the NS1-activated genes using a DNMT3B antibody and primers designed to bind specific gene promoters to determine whether NS1 upregulates the transcription of target genes by repositioning DNMT3B and altering promoter methylation. As shown in Fig. 5A, DNMT3B binding to respective promoter elements was alleviated in the PR8 WT virus-infected cells but was completely restored in the PR8/delNS1 virus-infected cells. SOCS2 was used as a negative control. We further examined the effect of the NS1 subcellular localization on the association of DNMT3B and gene promoters. Results from ChIP experiments confirmed that the WSN WT virus infection also decreased DNMT3B binding to the activated gene promoters, whereas infection with the mutant WSN (L144A+L146A) virus increased DNMT3B binding to gene promoters compared to infection with the WT virus (Fig. 5B). Moreover, because the NES mutations did not completely inhibit the nuclear localization of the NS1 protein, the mutant WSN (L144A+L146A) virus partially restored DNMT3B binding to the respective promoter elements (Fig. 5B). The effect of NS1 on the dissociation of DNMT3B from promoters was further evaluated by transfecting NS1-Syn WT plasmid and its mutant. As shown in Fig. 5C, because NS1-Syn WT was localized in the nucleus and the NS1-Syn (R148A+E152A+E153A) and (K221E) mutants were localized in the cytosol, NS1 WT did not influence the binding of DNMT3B to the respective promoter elements, and both NS1 mutants dissociated DNMT3B from the respective elements of gene promoters. In addition, the effects of NS1 and its subcellular localization on the association of DNMT3B with the *PTP1B*, *TCPTP*, *PIASX*, and *PIASY* promoters were evaluated, and similar results were obtained (see Fig. S9). In addition, the percentages of methylated CpGs in SOCS1 and SOCS3 were significantly lower in the WSN WT virus-infected cells than in control cells. However, infection of cells with the mutant WSN (L144A+L146A) virus did not affect the methylation status of the promoters (see Fig. S10A). Interestingly, NS1-Syn WT was unable to affect the percentages of methylated CpGs in the SOCS1 and SOCS3 promoters, whereas the NS1-Syn (R148A+E152A+E153A) and (K221E) mutants decreased the percentages of methylated CpGs of the promoters of SOCS1 and SOCS3 (Fig. S10B). Based on these results, IAV NS1 may induce the epigenetic modification of these promoters by relieving the interaction between DNMT3B and the promoters.

NS1 regulates the ubiquitination of DNMT3B. As shown in our previous report, DNMT3B expression is downregulated during IAV infection (24); thus, we determined whether NS1 regulated DNMT3B protein levels. As shown in the left panel of Fig. 6A, DNMT3B protein levels were markedly upregulated in the PR8/delNS1-infected cells compared to levels in PR8 WT virus-infected cells. We next investigated the effect of the NS1 subcellular localization on DNMT3B protein levels. According to a Western blot analysis, the mutant WSN (L144A+L146A) virus induced higher DNMT3B protein levels than the WSN WT virus (Fig. 6A, middle). Similarly, both the NS1-Syn (R148A+E152A+E153A) and (K221E) mutants inhibited DNMT3B protein levels in A549

FIG 5 NS1 dissociates DNMT3B from the *SOCS1*, *SOCS3*, *PIAS1*, and *PIAS3* promoters. (A) A549 cells were infected with the PR8 WT (0.001 PFU per cell) or the PR8/delNS1 (1 PFU per cell) virus for 18 h and then treated with MG132 (100 μ M) for 6 h. ChIP assays were performed with anti-DNMT3B- or IgG-conjugated agarose. Promoter sequences in the input DNA and the DNA recovered from antibody-bound chromatin segments were detected using real-time RT-PCR. (B) Experiments were performed as described for panel A, except that A549 cells were infected with the WSN WT virus (0.001 PFU per cell) or the mutant WSN (L144A+L146A) virus (1 PFU per cell). (C) Experiments were performed as described for panel A, except that A549 cells were transfected with the indicated plasmids for 42 h. Enrichment was determined relative to input controls. Bar graphs present the means $\pm$ SDs ($n = 3$). **, $P < 0.01$; *, $P < 0.05$.

cells and PBMCs (Fig. 6A, right panel). Moreover, the effects of NS1 and its subcellular localization on the DNMT activity were investigated. Notably, both the PR8/delNS1 and mutant WSN (L144A+L146A) viruses reversed the repression of DNMT activity by the corresponding WT virus in A549 cells and peripheral blood mononuclear cells (PBMCs) (see Fig. S11A and B). Similarly, the NS1-Syn (R148A+E152A+E153A) and (K221E) mutants also restrained DNMT activity (Fig. S9C). We next investigated the degradation of DNMT3B by using the protein synthesis inhibitor cycloheximide (CHX). Results indicated that the protein levels of DNMT3B were reduced within 24 h after CHX treatment, whereas its levels were reduced within 12 h in the presence of IAV infection (Fig. 6B). We treated A549 cells with various inhibitors of protein degradation pathways to investigate the mechanisms by which NS1 regulated the stability of DNMT3B. The proteasome inhibitor MG132, but not the lysosome inhibitor ammonium chloride (NH_4Cl) nor the autophagosome inhibitor 3-methyladenine (3MA), markedly inhibited the degradation of DNMT3B, suggesting that NS1 regulates DNMT3B degradation through a proteasome-dependent pathway (Fig. 6C). In an overexpression system, NS1-HK enhanced K48-linked, but not K63-linked, polyubiquitination of DNMT3B (Fig. 6D). Furthermore, K48-linked ubiquitination of DNMT3B was decreased in the

FIG 6 NS1 regulates the stability of DNMT3B. (A) A549 cells were infected with the indicated virus for indicated times (left and middle) or transfected with the indicated plasmid for 36 h (right). Levels of the DNMT3B protein were detected by Western blotting. Normalized densitometric values (in relative arbitrary units) are reported below each band and represent the average from three independent experiments. (B) A549 cells were infected with the indicated virus for 12 h in the presence of cycloheximide (50 μ g/ml) for the indicated times. DNMT3B protein levels were determined by Western blotting (top) and densitometry (bottom) with β -actin as an internal control. (C) A549 cells were infected with the indicated virus for the indicated times and then treated with MG132 (100 μ M), NH_4Cl (20 mM), or 3MA (400 ng/ml) for 6 h. Levels of the DNMT3B protein were detected by Western blotting. (D) 293T cells were transfected with Myc-DNMT3B or/and pmCherry-NS1-PR8 and the indicated ubiquitin plasmids. Twenty-four hours after transfection, cells were treated with MG132 (100 μ M) for 6 h before co-immunoprecipitation and immunoblot analyses were performed with the indicated antibodies. (E) A549 cells were infected with the PR8 WT (0.001 PFU per cell) or the PR8/delNS1 (1 PFU per cell) virus for the indicated times and then treated with or without MG132 (100 μ M) for 6 h before co-immunoprecipitation and immunoblot analyses were performed with the indicated antibodies. (F) Experiments were performed as described for panel D, except that A549 cells were infected with the WSN WT virus (0.001 PFU per cell) or the mutant WSN (L144A+L146A) virus (1 PFU per cell). (G) Experiments were performed as described for panel D, except that A549 cells were transfected with the indicated plasmids for 36 h. In immunoprecipitation and immunoblot experiments, cell extracts were treated with 10 mg/ml of RNase A for 4 h. All experiments were repeated at least three times with consistent results.

PR8/delNS1-infected cells compared to that in PR8 WT virus-infected cells (Fig. 6E). Interestingly, due to differences in NS1 subcellular localization, the mutant WSN (L144A+L146A) virus decreased the K48-linked ubiquitination of DNMT3B, whereas the NS1-Syn (R148A+E152A+E153A) and (K221E) mutants increased the K48-linked ubiquitination of DNMT3B (Fig. 6F and G). Thus, NS1, which interacts with DNMT3B and transports it to the cytosol, promotes the K48-linked ubiquitination and degradation of DNMT3B.

DISCUSSION

As shown in our previous report, IAV infection reduces DNMT3B expression (24), but the mechanism underlying the phenomenon was not clear. This study provides evidence supporting the hypothesis that IAV regulates epigenetic modification through an interaction between the IAV NS1 protein and DNMT3B, which transports DNMT3B to the cytoplasm and promotes its K48-linked ubiquitination and degradation. Host gene

dysregulation is mediated by epigenetic modification after IAV infection. Our findings provide new insights that improve our understanding of the molecular mechanism underlying virus infections and the interactions between the host and virus.

The function of NS1 localization is very intriguing. NS1 translocates to both the nucleus and cytoplasm of infected cells; thus, NS1 engages in many functions (32). In the cytoplasm, NS1 blocks viral RNA detection by RIG-I signaling and inhibits the IFN-stimulated expression of antiviral proteins by targeting PKR and the RNase L pathway (5). In addition, the NS1 protein binds p85 β and activates phosphatidylinositol 3-kinase signaling, which is important for efficient virus replication (33). In the nucleus, NS1 binds to CPSF and PABII, which has been reported to block host gene expression (34). Despite our substantial knowledge of this amazing and fascinating protein, we still have much to learn regarding its roles in NS1 nucleocytoplasmic shuttling. Here, for the first time, we revealed that nuclear NS1 binds to DNMT3B and translocates it to cytoplasm, resulting in DNMT3B degradation. Our work expands the knowledge of NS1 and host protein interactions and reveals a new dynamic function of NS1.

IAV infection has been reported to induce SOCS1 and SOCS3 expression (17); however, little is known about the underlying mechanism. In addition, a clear role for IAV infection in the expression of other JAK-STAT signaling suppressors has not been established. In this study, NS1 was found to dissociate DNMT3B from gene promoters, contributing to the overexpression of at least eight JAK-STAT signaling suppressors. Our work raises an important question. Why does NS1 need to induce the expression of so many suppressors of JAK-STAT signaling? To our knowledge, this phenomenon seems to provide multiple forms of security for the virus to escape the immune system by inhibiting some signaling pathways that play key roles in the immune response. Moreover, IAV may have developed different strategies to counteract IFN signaling in different stages of the virus life cycle.

Epigenetic changes induced by viral infection have been reported. For example, the hepatitis C virus (HCV) core protein upregulates DNMT1 and DNMT3B (35), human papillomavirus (HPV) E7 and adenovirus E1A upregulate DNMT1 (36), and hepatitis B virus (HBV) X protein upregulates DNMT1 and DNMT3A but downregulates DNMT3B (37, 38). In addition, an acute infection of cells with human immunodeficiency virus type 1 (HIV-1) results in an increase in DNMT1 expression and a decrease in interferon gamma (IFN- γ) expression (39). Although additional studies have examined the epigenetic changes induced by several kinds of viral infections, they only focused on oncogenic viruses and retrovirus. Limited reports are available for other viruses, particularly viruses that induce acute infections and host immune responses. Our data explain the mechanism by which IAV downregulates DNMT3B expression, through the interaction between NS1 and DNMT3B and subsequent transport of DNMT3B to the cytoplasm from the nucleus. Thus, the decrease in nuclear DNMT3B expression and activity alter DNA methylation. The results of this study advance our knowledge of the mechanisms by which an IAV infection evades the immune response and reveals that epigenetic modification plays roles in the development of the immune system and in the host immune response to viral infection.

Finally, although we have reported a new mechanism for IAV immune escape, some questions still remain. (i) NS1 interacted with DNMT3B and conditioned it for ubiquitination and degradation, but we did not provide evidence showing that NS1 is an E3 ubiquitin ligase. Does NS1 recruit E3 ubiquitin ligases to DNMT3B in the cytoplasm? (ii) Epigenetic changes should regulate the expression of many genes. In this study, we only focused on regulators of JAK-STAT signaling. Are other genes regulated by the NS1-induced epigenetic changes? (iii) Although we have used representative strains from different hosts (H1N1 influenza A/PR/8/34, H3N2 influenza A/Sydney/5/97, etc.), we cannot exclude that not all strains from a specific host exhibit the observed specificity of antagonism. (iv) In Fig. 2A, DNMT1 appeared to play a role in IAV-induced PIAX expression. Do other IAV proteins control the expression of host factors via DNMT1? (v) Why are epigenetic changes induced by NS1 of concern if the infected cell will die as a result of infection? There are observations that Clara cells can be infected,

FIG 7 Model of the biological effect of the interaction between DNMT3B and IAV NS1. (A) Before IAV infection, the expression of key regulators of JAK-STAT signaling is strictly regulated, and DNA methylation is one of the mechanisms adopted by the host immune system. DNMT3B binds to the methylated DNA to inhibit gene expression. (B) However, after IAV infection, the newly expressed NS1 protein is translocated to the nucleus, where it interacts with DNMT3B. Directed by the nuclear export signal (NES) of NS1, the NS1-DNMT3B complex is translocated from the nucleus to the cytosol, where it undergoes the K48-linked polyubiquitination of DNMT3B. Soon afterwards, the DNMT3B translocation leads to demethylation of the methylated promoters of antiviral genes, controlling their expression. Thus, NS1 increases the expression of key regulators of JAK-STAT signaling to reduce the response of the host immune system by autocrine or paracrine pathways.

but do not support productive infection. (vi) Prior studies indicate that SOCS genes are upregulated by the activation of the RIG-I-MAVS pathway and IFN- α receptor (IFNAR) signaling during IAV infection. Does NS1 regulate SOCS genes through the RIG-I-MAVS pathway and IFNAR signaling or are the RIG-I-MAVS pathway and IFNAR signaling controlled by other IAV components? When considering the next step, studies exploring these questions would be of great help in further clarifying the role of NS1 in the IAV infection.

We propose a working model describing the role of NS1 in epigenetic modification regulated by influenza A virus (Fig. 7). In this model, before IAV infection, DNMT3B and other DNA methyltransferases have already been recruited to promoters of JAK-STAT signaling suppressors, such as SOCS1, SOCS3, PIAS1, etc. Promoter methylation inhibits the expression of the suppressors (Fig. 7A). Once infected with IAV, the host immune system responds to the viral infection, and DNMT3B and IAV NS1 interact. The interaction leads to the transport of DNMT3B to the cytoplasm from the nucleus and its subsequent degradation. Rapid demethylation then occurs in the methylated promoter regions of the suppressors; thus, the expression of suppressors is quickly upregulated. As a result, those suppressors inhibit interferon signaling by autocrine or paracrine pathways (Fig. 7B). In conclusion, the results of this study advance our knowledge of the host immune response to IAV infection and reveal the broad-spectrum connection between epigenetics and human diseases, indicating the potential use of epigenetics to treat acute viral infections.

MATERIALS AND METHODS

Ethics statement. Human PBMCs were collection from blood samples for research according to the principles of the Declaration of Helsinki, and this procedure was approved by the Institutional Review Board of the College of Life Sciences, Wuhan University, in accordance with guidelines for the protection of human subjects. All study participants provided written informed consent for the collection of samples and subsequent analyses.

Virus and cell culture. The influenza virus strain A/Hong Kong/498/97 (H3N2) used in this study was provided by the China Center for Type Culture Collection (Wuhan, China). The virus stock was propagated in Madin-Darby canine kidney cells (MDCK). The recombinant human influenza A virus A/WSN/33 (H1N1) and NS1 nuclear export signal (NES) mutant A/WSN/33 (H1N1) (mt-NS1 L144A+L146A) virus were

generated by transfecting MDCK cells with the eight plasmid transfection system to generate influenza A virus, as previously described (40). The eight-plasmid transfection system was a gift from R. G. Webster (Department of Infectious Diseases, St. Jude's Children's Research Hospital, Memphis, TN, USA). The reassortants of influenza A/PR/8/34 viruses and the A/PR/8/34 delNS1 virus, in which the NS1 gene is deleted, were a gift from Adolfo García-Sastre (Global Health and Emerging Pathogens Institute, Icahn School of Medicine at Mount Sinai) (41). Human lung epithelial cells (A549) were cultured in F12K medium (Gibco-BRL, Gaithersburg, MD, USA). Human embryonic kidney cells (293T) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL). The Raw264.7 murine macrophage cell line (ATCC TIB-71) was cultured in RPMI 1640 medium (Gibco) supplemented with 1 mM sodium pyruvate, GlutaMAX-I, and 10% fetal bovine serum (FBS). African green monkey kidney Vero (ATCC CCL-81) cells were cultured in DMEM (Gibco) supplemented with 1% penicillin-streptomycin (Gibco) and 5% FBS (HyClone). Primary human alveolar type II (ATII) cells were purchased from Wuhan Pricells Company (Wuhan, China). All cell cultures were maintained at 37°C in a 5% CO₂ incubator.

Plasmids, antibodies, and biochemical reagents. The pmCherry-NS1 (A/PR/8/34) (accession number [NC_002020](#)), pmCherry-NS1 (A/Sydney/5/97) (accession number [F1494955](#)), pmCherry-mtNS1 (A/Sydney/5/97 K221E) (NS1 nuclear localization signal mutant [NLS mutant]) and pmCherry-mtNS1 (A/Sydney/5/97 R148A+E152A+E153A) (NS1 nuclear export signal inhibitory sequence mutant [NES-inhibit sequence mutant]) plasmids were provided by Zongqiang Cui (Wuhan Institute of Virology, Chinese Academy of Sciences). The pCMV-Tag2B-NS1 (HB) (accession number [AF256183](#)), pcDNA-DNMT3B, and pcDNA-DNMT3A plasmids were used as described in a previous study (24). The pM-DNMT3B, pEGFP-DNMT3B, and pM-DNMT3A plasmids were subcloned from pcDNA-DNMT3B and pcDNA-DNMT3A plasmids. The pVP16-NS1 and pCMV-Tag2B-NS1 (accession number [GT687152](#)) plasmids were cloned from A/WSN/33 H1N1. Antibodies (Abs) against human β -actin (cw0096A), β -tubulin (CW0098), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; cw0100A) were purchased from ComWin Biotech (Beijing, China). Abs against human DNMT3B (D7O7O), PIAS1 (D33A7), and PIAS3 (D5F9) were purchased from Cell Signaling Technology, and Abs against His (H1029), Flag (F3165), Myc (M4439), and hemagglutination (HA; H9658) were purchased from Sigma-Aldrich. Abs against human DNMT1 (sc-20701), DNMT3A (sc-20703), HDAC1 (sc-7872), SOCS1 (sc-9021), SOCS3 (sc-9023), and Ubi (sc-8017) were purchased from Santa Cruz Biotechnology. Abs against human DNMT3B (ChIP, ab2851) and DNMT3A (ChIP, ab2850) were purchased from Abcam. Fluorescein isothiocyanate (FITC)-labeled rabbit anti-mouse and phycoerythrin (PE)-labeled donkey anti-goat secondary antibodies were purchased from Proteintech Group Inc. (PTG, IL, USA). The Lipofectamine 2000 transfection reagent was purchased from Invitrogen. Specific DNMT1, DNMT3A, DNMT3B, and control siRNAs were used as described in a previous study (24). Specific TRIM25 siRNAs were used as described in a previous study (42). Dimethyl sulfoxide (DMSO; W387520), MG132 (M7449), NH₄Cl (A9434), and 3MA (M9281) were purchased from Sigma-Aldrich.

Virus quantitation. Viral titers were determined using a modified version of a previously described 50% tissue culture infective dose (TCID₅₀) assay (1). Briefly, octuplicate samples of confluent monolayers of Madin-Darby canine kidney cells on 96-well plates were incubated with 100 μ l of 10-fold serially diluted lung homogenates. After a 3-day incubation at 37°C, wells exhibiting a cytopathic effect were observed and counted. The TCID₅₀ was calculated using the Reed-Muench method.

Mammalian two-hybrid analysis. 293T cells were transfected with the luciferase reporter plasmid pG5-luc (Promega), test plasmids, negative-control plasmids, and positive-control plasmids. Forty-eight hours posttransfection, cells were harvested, and luciferase activities were assayed using the luciferase reporter assay system (Promega) according to the manufacturer's recommendations.

Quantitative RT-PCR analysis. Quantitative RT-PCR analyses were performed as previously described (43, 44). Briefly, total RNA was isolated with TRIzol (Invitrogen, Basel, Switzerland). Cellular RNA samples were reverse-transcribed using random primers. Quantitative real-time RT-PCR was performed using a LightCycler 480 (Roche) and the SYBR green system (Applied Biosystems). GAPDH was amplified as an internal control. Primers used in this study are listed in Table S1 in the supplemental material.

Western blot analysis. Western blot analyses were performed using a previously described method (45). Briefly, whole-cell lysates were prepared by lysing cells with phosphate-buffered saline (PBS; pH 7.4) containing 0.01% Triton X-100, 0.01% EDTA, and 10% protease inhibitor cocktail (Roche). Protein concentrations were determined using the Bradford assay (Bio-Rad). Polypeptides from cell lysates were separated on SDS-12% polyacrylamide gels cross-linked with *N,N*-methylenebisacrylamide and electrophoretically transferred to nitrocellulose membranes. Nonspecific sites were blocked with 5% nonfat dried milk before being incubated with a specific antibody targeting the proteins assessed in this study. Blots were developed using SuperSignal chemiluminescent reagent (Pierce, Rockford, IL, USA), and the stained membranes were analyzed with a LAS-4000 image document instrument (FujiFilm, Tokyo, Japan).

Bisulfite modification and sequencing analysis. The bisulfite treatment was performed after influenza virus infection. Briefly, 2 mg of genomic DNA extracted from cells was denatured in 0.2 M NaOH. Sodium bisulfite and hydroquinone were added to final concentrations of 3.1 M and 0.5 mM, respectively, and the samples were incubated at 55°C for 16 h. After purification with a DNA Clean-Up system (Promega, Madison, WI), the DNA samples were desulfonated in 0.3 M NaOH, precipitated with ethanol, and dissolved in 30 ml of Tris-EDTA buffer. The modified DNA (20 ng) was amplified by PCR using primers specific for specific promoter regions (see Table S2). The resulting PCR products were cloned into the pGEM-T Easy vector (Promega) and subjected to sequencing analysis.

Chromatin immunoprecipitation. Formaldehyde was added to the culture medium to a final concentration of 1%. The cells were then washed twice with PBS, scraped, and lysed in lysis buffer (1% SDS, 10 mM Tris-HCl [pH 8.0], 10% protease inhibitor cocktail, 50 mg/ml each of aprotinin and leupeptin) for 10 min at 4°C. The lysates were sonicated on ice, and the debris was removed by centrifugation at

12,000 rpm for 15 min at 4°C. One-fourth of the supernatant was used as the DNA input control. The remaining sample was diluted 10-fold with dilution buffer (0.01% SDS, 1% Triton X-100, 1 mM EDTA, 10 mM Tris-HCl [pH 8.0], and 150 mM NaCl) followed by incubation with antibodies overnight at 4°C. Immunoprecipitated complexes were collected using protein A/G-Sepharose. The pellets were washed four times with dialysis buffer containing 2 mM EDTA and 50 mM Tris-HCl, pH 8.0. After washing, the precipitates were incubated with an elution buffer (1% SDS and 0.1 M NaHCO₃) at room temperature. Supernatants were transferred to clean tubes, and RNase A was added to destroy the bound RNA in the sample. Samples were incubated at 65°C for 5 h to reverse the formaldehyde cross-links, and DNA was precipitated with ethanol and extracted two times with phenol-chloroform. Finally, pellets were resuspended in Tris-EDTA (TE) buffer and subjected to PCR amplification. Primers used in this study are listed in Table S3.

Co-immunoprecipitation assays. Co-immunoprecipitation assays were performed using a previously described method (46). Briefly, 293T cells were cotransfected with the pCMV-Tag2B-NS1 plasmid for expression of Flag-tagged NS1 and pcDNA-DNMT3B plasmid (for expression of DNMT3B protein) or pcDNA-DNMT3A plasmid (for expression of DNMT3A protein). Forty-eight hours posttransfection, cells were collected and co-immunoprecipitations were performed as previously described (47). For the endogenous co-immunoprecipitation assay, A549 cells were infected with IAV at a multiplicity of infection (MOI) of 1, and cells were collected for the co-immunoprecipitation assay 24 h postinfection.

Laser scanning confocal microscopy analysis. For the double immunofluorescence staining assay, A549 cells or human ATII cells were infected with IAV at an MOI of 1. After 24 h, cells were fixed, permeabilized, and incubated with the NS1 antibody and DNMT3B antibody for 2 h. Then, cells were washed with wash buffer 4 times and incubated with a FITC-labeled secondary antibody and PE-labeled secondary antibody for 1 h. After 5 washes, cells were incubated with DAPI (4',6-diamidino-2-phenylindole) to label the nucleus. Then, fluorescence was detected using a confocal laser scanning microscope.

For direct fluorescence assays, 293T cells were cotransfected with the recombinant pEGFP-DNMT3B plasmid and pmCherry-NS1 (A/PR/8/34), pmCherry-NS1 (A/Sydney/5/97), pmCherry-mtNS1 (A/Sydney/5/97 R148A+E152A+E153A), or pmCherry-mtNS1 (A/Sydney/5/97 K221E) plasmids. Twenty-four to thirty-six hours posttransfection, cells were fixed with 4% paraformaldehyde for 15 min, washed three times with PBS, counterstained with DAPI for 15 min, and then washed three times with PBS. Fluorescence was detected using a confocal laser scanning microscope.

DNMT3B expression and activity assay. A549 cells were infected with the recombinant A/WSN/33 (H1N1) or NS1 NES mutant A/WSN/33 (H1N1). Twenty-four hours postinfection, nuclear proteins were fractionated using a nuclear extraction kit (Chemicon, Billerica, MA) and utilized in the DNMT3B activity assay performed with an EpiQuick DNA methyltransferase 3B activity/inhibitor screening assay core kit (Epigentek, Brooklyn, NY) according to the manufacturer's protocol. For the nuclear DNMT3B expression assay, nuclear protein fractions were prepared 24 h after infection by using a nuclear extraction kit (Chemicon), and Western blot analyses were performed as previously described (24). Immunoblots were visualized with the ECL detection system (Pierce, Rockford, IL, USA) and quantified by densitometry to calculate fold changes.

Statistical analysis. Statistical analyses were performed using Origin 7.5 software. The results are shown as means $\pm$ standard deviations (SDs) and statistical analysis was performed using Student's *t* tests. Where more than two groups were compared, one-way analysis of variance (ANOVA) with Bonferroni's test was performed. *P* values of <0.05 were considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JVI.01587-18>.

SUPPLEMENTAL FILE 1, PDF file, 1.6 MB.

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REFERENCES

- Peng N, Liu S, Xia Z, Ren S, Feng J, Jing M, Gao X, Wiemer EA, Zhu Y. 2016. Inducible major vault protein plays a pivotal role in double-stranded RNA- or virus-induced proinflammatory response. *J Immunol* 196:2753–2766. <https://doi.org/10.4049/jimmunol.1501481>.
- König R, Stertz S, Zhou Y, Inoue A, Hoffmann HH, Bhattacharyya S, Alamares JG, Tscherne DM, Ortigoza MB, Liang Y, Gao Q, Andrews SE, Bandyopadhyay S, De Jesus P, Tu BP, Pache L, Shih C, Orth A, Bonamy G, Miraglia L, Ideker T, Garcia-Sastre A, Young JA, Palese P, Shaw ML, Chanda SK. 2010. Human host factors required for influenza virus replication. *Nature* 463:813–817. <https://doi.org/10.1038/nature08699>.
- Schrauwen EJ, de Graaf M, Herfst S, Rimmelzwaan GF, Osterhaus AD, Fouchier RA. 2014. Determinants of virulence of influenza A virus. *Eur J Clin Microbiol Infect Dis* 33:479–490. <https://doi.org/10.1007/s10096-013-1984-8>.
- Krug RM. 2015. Functions of the influenza A virus NS1 protein in antiviral defense. *Curr Opin Virol* 12:1–6. <https://doi.org/10.1016/j.coviro.2015.01.007>.
- Gack MU, Albrecht RA, Urano T, Inn KS, Huang IC, Carnero E, Farzan M, Inoue S, Jung JU, Garcia-Sastre A. 2009. Influenza A virus NS1 targets the ubiquitin ligase TRIM25 to evade recognition by the host viral RNA sensor RIG-I. *Cell Host Microbe* 5:439–449. <https://doi.org/10.1016/j.chom.2009.04.006>.
- Twu KY, Noah DL, Rao P, Kuo RL, Krug RM. 2006. The CPSF30 binding site on the NS1A protein of influenza A virus is a potential antiviral target. *J Virol* 80:3957–3965. <https://doi.org/10.1128/JVI.80.8.3957-3965.2006>.
- Talon J, Horvath CM, Polley R, Basler CF, Muster T, Palese P, Garcia-Sastre A. 2000. Activation of interferon regulatory factor 3 is inhibited by the influenza A virus NS1 protein. *J Virol* 74:7989–7996. <https://doi.org/10.1128/JVI.74.17.7989-7996.2000>.
- Wang X, Li M, Zheng H, Muster T, Palese P, Beg AA, Garcia-Sastre A. 2000. Influenza A virus NS1 protein prevents activation of NF-kappaB and induction of alpha/beta interferon. *J Virol* 74:11566–11573.
- Li S, Min JY, Krug RM, Sen GC. 2006. Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA. *Virology* 349:13–21. <https://doi.org/10.1016/j.virol.2006.01.005>.
- Min JY, Krug RM. 2006. 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* 103:7100–7105. <https://doi.org/10.1073/pnas.0602184103>.
- Shuai K, Liu B. 2003. Regulation of JAK-STAT signalling in the immune system. *Nat Rev Immunol* 3:900–911. <https://doi.org/10.1038/nri1226>.
- Richards KH, Macdonald A. 2011. Putting the brakes on the anti-viral response: negative regulators of type I interferon (IFN) production. *Microbes Infect* 13:291–302. <https://doi.org/10.1016/j.micinf.2010.12.007>.
- Li WX. 2008. Canonical and non-canonical JAK-STAT signaling. *Trends Cell Biol* 18:545–551. <https://doi.org/10.1016/j.tcb.2008.08.008>.
- Chen XP, Losman JA, Rothman P. 2000. SOCS proteins, regulators of intracellular signaling. *Immunity* 13:287–290.
- Shuai K. 2006. Regulation of cytokine signaling pathways by PIAS proteins. *Cell Res* 16:196–202. <https://doi.org/10.1038/sj.cr.7310027>.
- Tremblay ML. 2009. The PTP family photo album. *Cell* 136:213–214. <https://doi.org/10.1016/j.cell.2009.01.006>.
- Pothlichet J, Chignard M, Si-Tahar M. 2008. Cutting edge: innate immune response triggered by influenza A virus is negatively regulated by SOCS1 and SOCS3 through a RIG-I/IFNAR1-dependent pathway. *J Immunol* 180:2034–2038.
- Shiels PG, McGuinness D, Eriksson M, Kooman JP, Stenvinkel P. 2017. The role of epigenetics in renal ageing. *Nat Rev Nephrol* 13:471–482. <https://doi.org/10.1038/nrneph.2017.78>.
- Dawson MA, Kouzarides T. 2012. Cancer epigenetics: from mechanism to therapy. *Cell* 150:12–27. <https://doi.org/10.1016/j.cell.2012.06.013>.
- Smallwood SA, Kelsey G. 2012. De novo DNA methylation: a germ cell perspective. *Trends Genet* 28:33–42. <https://doi.org/10.1016/j.tig.2011.09.004>.
- Portela A, Esteller M. 2010. Epigenetic modifications and human disease. *Nat Biotechnol* 28:1057–1068. <https://doi.org/10.1038/nbt.1685>.
- Marazzi I, Ho JS, Kim J, Manicassamy B, Dewell S, Albrecht RA, Seibert CW, Schaefer U, Jeffrey KL, Prinjha RK, Lee K, Garcia-Sastre A, Roeder RG, Tarakhovskiy A. 2012. Suppression of the antiviral response by an influenza histone mimic. *Nature* 483:428–433. <https://doi.org/10.1038/nature10892>.
- Mukherjee S, Vipat VC, Chakrabarti AK. 2013. Infection with influenza A viruses causes changes in promoter DNA methylation of inflammatory genes. *Influenza Other Respir Viruses* 7:979–986. <https://doi.org/10.1111/irv.12127>.
- Li W, Sun W, Liu L, Yang F, Li Y, Chen Y, Fang J, Zhang W, Wu J, Zhu Y. 2010. IL-32: a host proinflammatory factor against influenza viral replication is upregulated by aberrant epigenetic modifications during influenza A virus infection. *J Immunol* 185:5056–5065. <https://doi.org/10.4049/jimmunol.0902667>.
- Fang J, Hao Q, Liu L, Li Y, Wu J, Huo X, Zhu Y. 2012. Epigenetic changes mediated by microRNA miR29 activate cyclooxygenase 2 and lambda-1 interferon production during viral infection. *J Virol* 86:1010–1020. <https://doi.org/10.1128/JVI.06169-11>.
- Garcia-Sastre A, Egorov A, Matassov D, Brandt S, Levy DE, Durbin JE, Palese P, Muster T. 1998. Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. *Virology* 252:324–330.
- Wang W, Riedel K, Lynch P, Chien CY, Montelione GT, Krug RM. 1999. 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* 5:195–205. <https://doi.org/10.1017/S1355838299981621>.
- Kramer MJ, Dennin R, Kramer C, Jones G, Connell E, Rolon N, Gruarin A, Kale R, Trown PW. 1983. Cell and virus sensitivity studies with recombinant human alpha interferons. *J Interferon Res* 3:425–435.
- Tynell J, Melen K, Julkunen I. 2014. Mutations within the conserved NS1 nuclear export signal lead to inhibition of influenza A virus replication. *Virol J* 11:128. <https://doi.org/10.1186/1743-422X-11-128>.
- Pauli EK, Schmolke M, Wolff T, Viemann D, Roth J, Bode JG, Ludwig S. 2008. Influenza A virus inhibits type I IFN signaling via NF-kappaB-dependent induction of SOCS-3 expression. *PLoS Pathog* 4:e1000196. <https://doi.org/10.1371/journal.ppat.1000196>.
- Han H, Cui ZQ, Wang W, Zhang ZP, Wei HP, Zhou YF, Zhang XE. 2010. New regulatory mechanisms for the intracellular localization and trafficking of influenza A virus NS1 protein revealed by comparative analysis of A/PR/8/34 and A/Sydney/5/97. *J Gen Virol* 91:2907–2917. <https://doi.org/10.1099/vir.0.024943-0>.
- Satterly N, Tsai PL, van Deursen J, Nussenzweig DR, Wang Y, Faria PA, Levy A, Levy DE, Fontoura BM. 2007. Influenza virus targets the mRNA export machinery and the nuclear pore complex. *Proc Natl Acad Sci U S A* 104:1853–1858. <https://doi.org/10.1073/pnas.0610977104>.
- Ehrhardt C, Wolff T, Pleschka S, Planz O, Beermann W, Bode JG, Schmolke M, Ludwig S. 2007. Influenza A virus NS1 protein activates the PI3K/Akt pathway to mediate antiapoptotic signaling responses. *J Virol* 81:3058–3067. <https://doi.org/10.1128/JVI.02082-06>.
- Krug RM, Yuan W, Noah DL, Latham AG. 2003. Intracellular warfare between human influenza viruses and human cells: the roles of the viral NS1 protein. *Virology* 309:181–189.
- Arora P, Kim EO, Jung JK, Jang KL. 2008. Hepatitis C virus core protein downregulates E-cadherin expression via activation of DNA methyltransferase 1 and 3b. *Cancer Lett* 261:244–252. <https://doi.org/10.1016/j.canlet.2007.11.033>.
- Laurson J, Khan S, Chung R, Cross K, Raj K. 2010. Epigenetic repression of E-cadherin by human papillomavirus 16 E7 protein. *Carcinogenesis* 31:918–926. <https://doi.org/10.1093/carcin/bgg027>.
- Park IY, Sohn BH, Yu E, Suh DJ, Chung YH, Lee JH, Surzycki SJ, Lee YI. 2007. Aberrant epigenetic modifications in hepatocarcinogenesis induced by hepatitis B virus X protein. *Gastroenterology* 132:1476–1494. <https://doi.org/10.1053/j.gastro.2007.01.034>.
- Zheng DL, Zhang L, Cheng N, Xu X, Deng Q, Teng XM, Wang KS, Zhang X, Huang J, Han ZG. 2009. Epigenetic modification induced by hepatitis B virus X protein via interaction with *de novo* DNA methyltransferase DNMT3A. *J Hepatol* 50:377–387. <https://doi.org/10.1016/j.jhep.2008.10.019>.
- Mikovits JA, Young HA, Vertino P, Issa JP, Pitha PM, Turcoski-Corralles S, Taub DD, Petrow CL, Baylin SB, Ruscetti FW. 1998. Infection with human immunodeficiency virus type 1 upregulates DNA methyltransferase, resulting in *de novo* methylation of the gamma interferon (IFN-gamma) promoter and subsequent downregulation of IFN-gamma production. *Mol Cell Biol* 18:5166–5177.
- Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG. 2000. A

- DNA transfection system for generation of influenza A virus from eight plasmids. *Proc Natl Acad Sci U S A* 97:6108–6113. <https://doi.org/10.1073/pnas.100133697>.
41. Talon J, Salvatore M, O'Neill RE, Nakaya Y, Zheng H, Muster T, Garcia-Sastre A, Palese P. 2000. Influenza A and B viruses expressing altered NS1 proteins: a vaccine approach. *Proc Natl Acad Sci U S A* 97:4309–4314. <https://doi.org/10.1073/pnas.070525997>.
 42. Castanier C, Zemirli N, Portier A, Garcin D, Bidere N, Vazquez A, Arnoult D. 2012. MAVS ubiquitination by the E3 ligase TRIM25 and degradation by the proteasome is involved in type I interferon production after activation of the antiviral RIG-I-like receptors. *BMC Biol* 10:44. <https://doi.org/10.1186/1741-7007-10-44>.
 43. Liu S, Hao Q, Peng N, Yue X, Wang Y, Chen Y, Wu J, Zhu Y. 2012. Major vault protein: a virus-induced host factor against viral replication through the induction of type-I interferon. *Hepatology* 56:57–66. <https://doi.org/10.1002/hep.25642>.
 44. Gui S, Chen X, Zhang M, Zhao F, Wan Y, Wang L, Xu G, Zhou L, Yue X, Zhu Y, Liu S. 2015. Mir-302c mediates influenza A virus-induced IFN β expression by targeting NF-kappaB inducing kinase. *FEBS Lett* 589: 4112–4118. <https://doi.org/10.1016/j.febslet.2015.11.011>.
 45. Jing M, Wang J, Zhu S, Ao F, Wang L, Han T, Yue X, Zhu Y, Ye L, Liu S. 2016. Development of a more efficient hepatitis B virus vaccine by targeting hepatitis B virus preS to dendritic cells. *Vaccine* 34:516–522. <https://doi.org/10.1016/j.vaccine.2015.11.069>.
 46. Liu S, Peng N, Xie J, Hao Q, Zhang M, Zhang Y, Xia Z, Xu G, Zhao F, Wang Q, Han T, Zhu Y. 2015. Human hepatitis B virus surface and e antigens inhibit major vault protein signaling in interferon induction pathways. *J Hepatol* 62:1015–1023. <https://doi.org/10.1016/j.jhep.2014.11.035>.
 47. Liu L, Cao Z, Chen J, Li R, Cao Y, Zhu C, Wu K, Wu J, Liu F, Zhu Y. 2012. Influenza A virus induces interleukin-27 through cyclooxygenase-2 and protein kinase A signaling. *J Biol Chem* 287:11899–11910. <https://doi.org/10.1074/jbc.M111.308064>.