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Article

Cellular DDX21 RNA Helicase Inhibits Influenza A Virus Replication but Is Counteracted by the Viral NS1 Protein

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SUMMARY

Influenza A virus RNA synthesis is catalyzed by the viral polymerase comprised of the PA, PB1, and PB2 proteins. We show that the host DDX21 RNA helicase restricts influenza A virus by binding PB1 and inhibiting polymerase assembly, resulting in reduced viral RNA and protein synthesis. Later during infection, the viral NS1 protein overcomes this restriction by binding to DDX21 and displacing PB1. DDX21 binds to a region of the NS1 N-terminal domain that also participates in other critical functions. A virus mutant whose NS1 protein is unable to bind DDX21 exhibits reduced viral protein synthesis at both late and early times of infection, a phenotype converted to wild-type upon DDX21 knockdown. As sequential interaction of PB1 and NS1 with DDX21 leads to temporal regulation of viral gene expression, influenza A virus likely uses the DDX21-NS1 interaction not only to overcome restriction, but also to regulate the viral life cycle.

INTRODUCTION

Influenza A viruses, which cause an annual highly contagious respiratory disease in humans, infect many avian and mammalian species and are responsible for periodic human pandemics that can result in high mortality rates (Wright et al., 2013). The primary defense against influenza A has been vaccination, but antiviral drugs also play an important role, particularly in fast-spreading pandemics (Ferguson et al., 2006). Currently, two classes of influenza antiviral drugs are available, targeting either the viral neuraminidase (oseltamivir, zanamivir) or the viral M2 ion channel protein (amantadine/rimantadine) (Das et al., 2010). However, most circulating influenza A viruses are resistant to amantadine/rimantadine (Deyde et al., 2007), and oseltamivir resistance is developing among influenza A virus strains (Bloom et al., 2010; Le et al., 2005), highlighting the need for developing new antivirals. One approach for developing new antivirals is to identify host proteins that play critical roles in influenza A virus replication and to target these host proteins and/or their interactions with specific viral proteins.

Influenza A viruses are enveloped negative-strand RNA viruses that contain 8 genome segments encoding 13 or 14 proteins (Krug and Fodor, 2013; Wise et al., 2012). Replication of influenza viral RNA (vRNA), which occurs in the nucleus, is catalyzed by the three polymerase proteins (PA, PB1, and PB2) in association with the nucleoprotein (NP) (Krug and Fodor, 2013; Resa-Infante et al., 2011). Efficient replication of influenza A virus requires the functions of the NS1 protein, a nonstructural protein that employs several strategies to inhibit the host antiviral response and regulates other cell and virus functions (Krug and Garcia-Sastre, 2013). One important function of the NS1 protein is the inhibition of host mRNA synthesis by binding a cellular 3′ end-processing factor, the 30 kDa subunit of the cleavage and polyadenylation specificity factor (CPSF30) (Nemeroff et al., 1998). Interestingly, some of the viral polymerases in the nucleus are associated with NS1-CPSF30 complexes that were purified by affinity selection of CPSF30 (Kuo and Krug, 2009).

Here, we purify CPSF30-NS1 complexes by sequential affinity selection of CPS30 and NS1 and identify the associated host proteins by mass spectrometry. We demonstrate that one of the associated proteins, the DDX21 RNA helicase, is a host restriction factor that regulates influenza A virus gene expression. Our results show that DDX21 suppresses viral RNA synthesis and hence viral protein synthesis at early times after infection. DDX21 binds the PB1 protein and, as a result, inhibits assembly of the tripartite viral RNA polymerase. Further, we show that DDX21-mediated antiviral activity is countered by the NS1 protein, which binds DDX21 and displaces PB1 from DDX21. The NS1 protein is comprised of two functional domains that are connected by a short linker: N-terminal RNA-binding domain (RBD) (amino acids 1–73), and C-terminal effector domain (ED) (amino acids 85–end) (Krug and Garcia-Sastre, 2013). We identify the binding site for DDX21 on the NS1 protein, which is in the RBD, and demonstrate that a recombinant virus encoding a NS1 protein with a mutated DDX21 binding site does not counteract endogenous DDX21-induced inhibition of viral protein synthesis at later times of infection. These results establish that the NS1 protein is responsible for countering endogenous DDX21 antiviral activity at later times in infected cells.

Our results strongly support a mechanism by which the sequential interaction of PB1 and NS1 with endogenous DDX21 regulates viral gene expression in infected cells. In this mechanism, influenza A virus transforms the DDX21 host

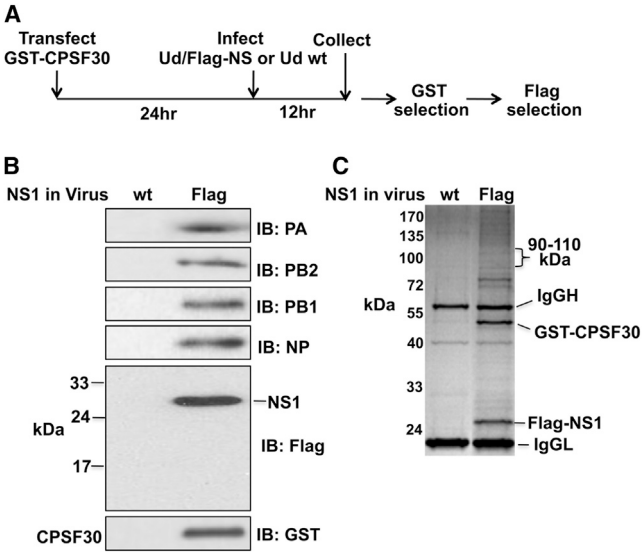

Figure 1. Purification of Infected Cell CPSF30-NS1 Complexes from Influenza A Virus-Infected Cells

(A) Purification procedure.

(B) Immunoblots (IBs) for the viral PA, PB1, PB2, NP, and NS1 proteins and for CPSF30 in the purified CPSF30-NS1 complexes (Flag-NS1 sample) and in the control (WT NS1) sample.

(C) Gel electrophoresis, followed by Coomassie blue staining, of the proteins in the purified CPSF30-NS1 complexes and the control sample. See also Table S1.

restriction factor into a host regulator of viral gene expression via the binding of DDX21 by the NS1 protein.

RESULTS

Purification of Infected Cell CPSF30-NS1 Complexes

To purify these infected-cell CPSF30-NS1 complexes, 293T cells were transfected for 24 hr with a plasmid expressing glutathione S-transferase (GST)-tagged CPSF30 and then infected for 12 hr with 2 plaque-forming units (pfu)/cell of a recombinant influenza A/Udorn/72 (Ud) virus that expresses a NS1 protein with an N-terminal Flag tag (Figure 1A). As a control, cells were infected with wild-type (WT) Ud virus that expresses an untagged NS1 protein. The CPSF30-NS1 complexes were purified from infected cell extracts by sequential GST and Flag selections. Immunoblots showed that the purified complexes contain the three polymerase subunits and NP (Figure 1B), confirming our previous results (Kuo and Krug, 2009). GST-CPSF30 and Flag-NS1 (27 kDa) were also identified, but Flag-tagged NS2/NEP protein (15 kDa) was not detected, presumably because it does not bind to CPSF30 and hence was removed by GST selection. The proteins associated with the purified NS1-CPSF30 complexes (Flag-NS1 sample) and the control preparation (WT NS1 sample) were resolved by denaturing gel electrophoresis, followed by Coomassie blue staining (Figure 1C). The Flag-NS1 sample contains proteins larger than 65 kDa that were largely absent from the WT NS1 sample. Mass spectrometry of the proteins of 70–90 kDa molecular weights in the Flag-NS1 sample primarily identified the viral polymerase and NP proteins. In contrast, mass spectrometry of the 90–110 kDa molecular proteins identified a substantial number of peptides of DDX21 (Table S1).

The DDX21 Helicase Inhibits Virus Replication by Suppressing Viral RNA Synthesis at Early Times after Infection

To determine whether DDX21 plays a role in influenza A virus replication, we transfected a DDX21-specific siRNA into HeLa cells, efficiently knocking down DDX21 (Figure 2A). The knockdown cells and cells transfected with a control siRNA were infected with H3N2 influenza A/Udorn/72 (Ud) or H1N1 influenza A/WSN/33 (WSN) at a low multiplicity of infection (0.001 pfu/cell) (Figure 2B). Replication of these two influenza A viruses was substantially enhanced in DDX21 knockdown cells, approximately 30-fold compared to control cells, demonstrating that DDX21 inhibits influenza A virus replication. To verify these results, we used a second siRNA and a different human cell line, Calu3 cells, which supports robust replication of Ud and other influenza A viruses (Figure S1 available online). Knockdown of DDX21 with either siRNA enhanced replication of Ud virus approximately 10-fold in Calu3 cells, establishing that DDX21 inhibits influenza A virus replication in human cells.

To identify the step(s) at which suppression occurs, DDX21 knockdown and control HeLa cells were infected at a high multiplicity (2 pfu/cell) with the Ud virus, and the synthesis of viral proteins was assayed using immunoblots (Figure 2C). The time course of viral protein production in control cells exhibits the temporal regulation of gene expression characteristic of influenza A virus replication (Shapiro et al., 1987; Skehel, 1973): early synthesis of the NS1 protein that increases in the 6–9 hr period of infection, followed by high-level synthesis of viral structural proteins, HA (hemagglutinin), NP, and M1 (matrix protein) at 12 hr postinfection. This temporal regulation was largely absent in the DDX21 knockdown cells. The HA, NP, and M1 proteins were produced in the DDX21 knockdown cells at substantial levels during the 6–9 hr time period, greatly exceeding the levels produced in the control cells. At 12 hr after infection, synthesis of all the viral proteins in control cells increased substantially, and as a consequence, the levels of all the viral proteins in the DDX21 knockdown and control cells were similar. Hence, the synthesis of late proteins occurred at much earlier times in the DDX21 knockdown cells, demonstrating that DDX21 delays the synthesis of these viral proteins. A similar stimulation of the early synthesis of late viral proteins resulting from DDX21 knockdown was observed in another human cell line, HEL-299 cells (Figure S2).

To determine whether DDX21 acts at the level of viral RNA synthesis, we measured the levels of the mRNAs and vRNAs that encode two late proteins (M1 and neuraminidase [NA]) using quantitative RT-PCR (Figure 2D). The levels of these viral RNAs were substantially higher at early times (at 1–3 hr postinfection) in the DDX21 knockdown cells, accounting for the increased early synthesis of late viral proteins in these cells. We conclude that DDX21 inhibits viral RNA synthesis at early times after infection and, as a result, delays the onset of the rapid synthesis of late vRNAs and mRNAs. At 6 hr after infection, the levels of these mRNAs and vRNAs were similar in knockdown and control cells, indicating that the inhibition of viral RNA synthesis caused by the DDX21 protein had largely ceased.

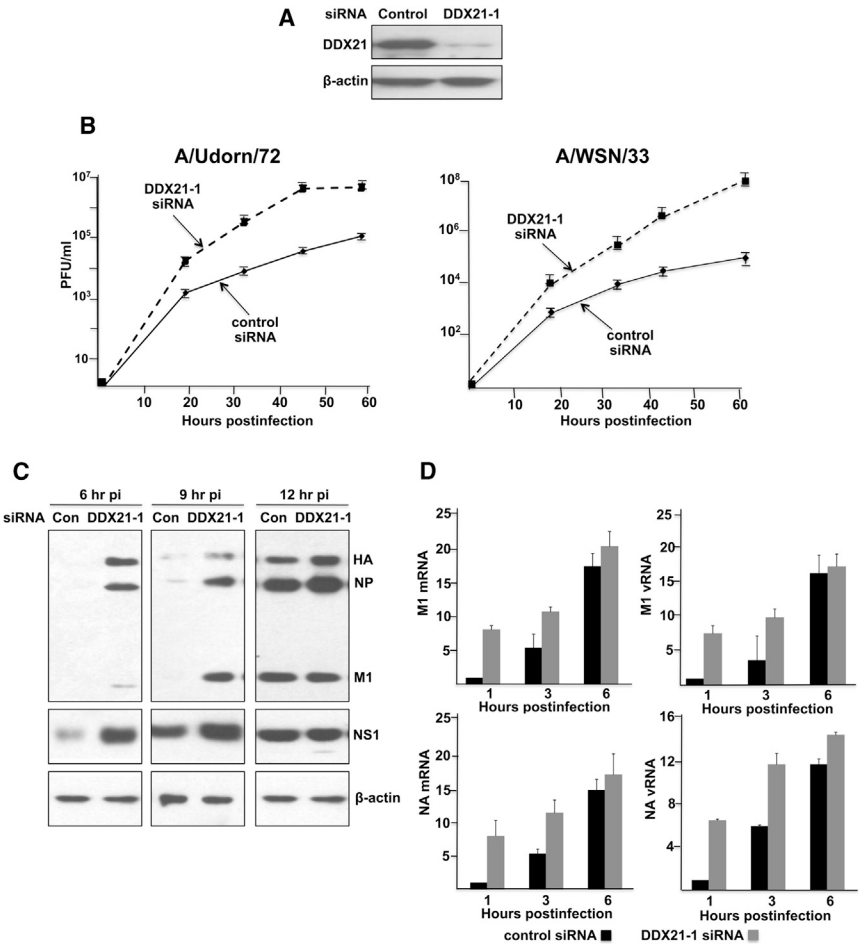

Figure 2. The DDX21 Helicase Inhibits Virus Replication by Suppressing Viral RNA Synthesis at Early Times of Infection

(A) Efficient knockdown of endogenous DDX21 in HeLa cells using DDX21-1 siRNA. Cell extracts were immunoblotted with an antibody (Ab) against DDX21 (Abcam).

(B) DDX21 inhibits replication of Ud and WSN virus in HeLa cells. HeLa cells were transfected with DDX21-1 siRNA or control siRNA for 36 hr, followed by infection with 0.001 pfu/cell of Ud or WSN virus. Virus production at the indicated times after infection was determined by plaque assays in MDCK cells. The bars show the SD of triplicate measurements of virus production.

(C) Time course of viral protein synthesis in HeLa cells treated with DDX21-1 siRNA or control siRNA. After 36 hr of siRNA treatment, cells were infected with 2 pfu/cell of Ud virus. Cells collected at the indicated times after infection were lysed in RIPA buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) plus complete protease inhibitor (Roche). An aliquot was analyzed by IBs probed with Abs against the NS1 protein, β-actin (Cell Signaling), or a goat Ab against the major structural proteins of the Ud virus (HA, NP, and M1) provided by Robert A. Lamb (designated as Ud Ab) (Chen et al., 2007).

(D) Time course of synthesis of M1 and NA mRNAs and vRNAs in HeLa cells treated with DDX21 siRNA or control siRNA. Cells were infected with 2 pfu/cell of Ud virus, and total RNA was isolated at the indicated times after infection using the TRIzol reagent. The amounts of the mRNAs and vRNAs were determined by quantitative RT-PCR using the primers shown in Supplemental Information. The reactions were carried out in triplicate and normalized to the levels of β-actin mRNA amplified with β-actin-specific primers. The numbers on the y axis correspond to the levels of the mRNA or vRNA relative to the level of the mRNA or vRNA at 1 hr postinfection in cells treated with the control siRNA. The bars show the SD of the triplicate determinations. See also Figures S1 and S2.

DDX21 Inhibits Viral RNA Synthesis by Binding PB1

To elucidate the mechanisms of these DDX21-mediated effects on viral RNA synthesis, we identified the viral protein(s) that bind DDX21 during infection. Human 293T cells were transfected for 24 hr with a plasmid expressing GST-DDX21 and, as control, a plasmid expressing GST, followed by infection with 2 pfu/cell of Ud virus. At 12 hr after infection, cell extracts were GST selected, followed by immunoblots to detect viral proteins (Figure 3A). Both the NS1 and PB1 proteins were specifically selected by GST-DDX21, and these associations were ribonuclease A (RNase A)-resistant, demonstrating that both NS1 and PB1 bind DDX21 in an RNA-independent manner in infected cells. Binding of DDX21 to the other two polymerase subunits, PB2 and PA, or to other viral proteins was not detected. To determine whether DDX21 interacts with the PB1 protein in uninfected cells, we carried out a cotransfection experiment. As shown in Figure 3B, Ud PB1 binds DDX21 independently of RNA in cotransfection assays. In contrast, no detectable binding of PB2 or PA to DDX21 was observed in cotransfection assays (Figure S3), as is also the case in infected cells (Figure 3A). The PB1 proteins of H1N1 A/WSN/33 (WSN) and H5N1 A/Hong Kong/483/97 (HK97) influenza A viruses also bind DDX21 in transfection assays (Figure 3B), showing that DDX21 binding is shared by the PB1 proteins of other influenza A virus strains. As analyzed by cell fractionation, whereas plasmid-expressed PB1 protein is localized in the cytoplasm, as shown previously by others (Fodor and Smith, 2004), cotransfection of PB1 and DDX21 plasmids results in a significant portion of PB1 entering the nucleus (Figure S4).

To establish whether DDX21 inhibits viral RNA synthesis via its binding of PB1, we tested the effect of DDX21 on viral RNA synthesis as measured in the dual-luciferase minigenome assay, which was carried out as previously described (Marklund et al., 2012). Polymerase II-driven pcDNA3 plasmids expressing PB1, PB2, PA, and NP were cotransfected into 293T cells. The vRNA template was provided by transfecting a polymerase I-driven pHH21 plasmid that expresses in the negative sense the firefly luciferase containing the 5′- and 3′-terminal regions of one of the Ud vRNAs. In addition, a polymerase II plasmid expressing the *Renilla* luciferase was transfected to correct for differences in transfection efficiencies. The firefly/*Renilla* luciferase ratio measures the level of luciferase mRNA synthesis (Figure 4A). Increasing amounts of a pcDNA3 plasmid expressing DDX21 inhibited viral RNA synthesis, demonstrating that

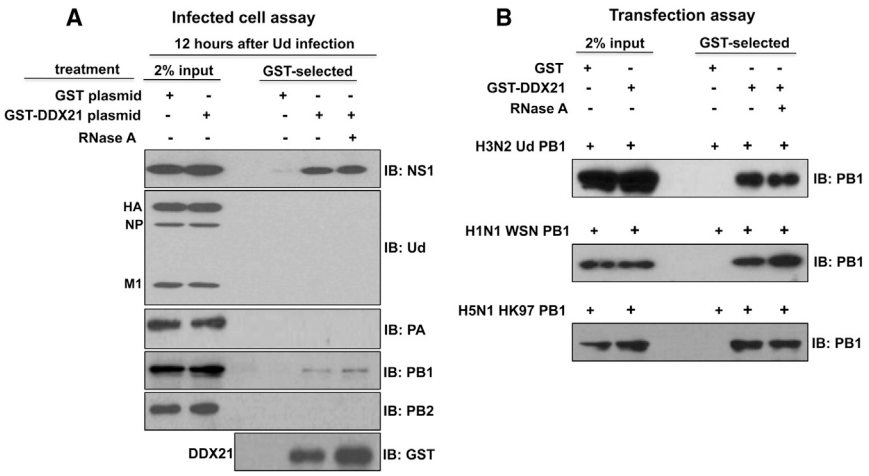

Figure 3. Identification of the Viral Proteins that Bind DDX21

(A) DDX21 binds NS1 and PB1 in infected cells. 293T cells were transfected for 24 hr with a plasmid expressing GST-DDX21 or GST. The cells were infected with 2 pfu/cell of Ud virus for 12 hr, and cell extracts with or without treatment with 10 μg/ml of RNase A for 4 hr were GST selected. Aliquots of the eluates were immunoblotted using the indicated Abs. Rabbit Ab against PB1 was provided by Krister Melen and Ikka Julkunen. Monoclonal Abs against PA and PB2 were provided by Juan Ortin (Ochoa et al., 1995).

(B) DDX21 binds the PB1 protein of several influenza A virus strains in uninfected cells. 293T cells were transfected for 48 hr with a plasmid expressing the PB1 protein from the indicated influenza A virus strain and a plasmid expressing GST-DDX21 or GST. Cell extracts were prepared by treating cells with a buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.5% Nonidet P-40, plus complete protease inhibitor, followed by rotation of the extracts for 30 min at 4°C. After clarification by low-speed centrifugation at 4°C, the extracts with and without RNase A treatment (10 μg/ml) were GST selected using glutathione magnet beads (Pierce), and the eluates were immunoblotted with PB1 Ab. Also see Figures S3 and S4.

DDX21 inhibits viral RNA synthesis. This inhibition was verified by directly measuring luciferase mRNA using quantitative RT-PCR (lower right panel of Figure 4A).

To determine whether the inhibition of viral RNA synthesis by DDX21 requires its helicase activity as well as its binding of PB1, we generated a mutant DDX21 protein in which the DEAD sequence required for helicase activity was changed to AAAD. This mutant DDX21 protein inhibited viral RNA synthesis as effectively as WT DDX21 (Figure 4B), demonstrating that inhibition does not require DDX21 helicase activity and is most likely solely due to DDX21 binding of PB1. Based on this result, we then tested whether one of the three major DDX21 domains is sufficient for inhibiting viral RNA synthesis (Figure S5). This was not the case: the N-terminal domain, the core helicase domain, and the C-terminal domain were poorly, if at all, active in inhibiting viral RNA synthesis.

To determine whether DDX21 inhibits assembly of the viral polymerase or binds to the formed polymerase complex, we cotransfected plasmids expressing PB2, PA, and C-TAP-tagged PB1, followed by TAP selection to determine which polymerase proteins were associated with PB1 in the absence and presence of DDX21 (1 μg) (Figure 4C). DDX21, which was TAP selected, and hence bound to PB1, substantially reduced the amounts of PB2 and PA that were TAP selected. In the experiment shown in Figure 4C, the amounts of TAP-selected PB2 and PA were reduced by approximately 65% and 75%, respectively. The reductions observed in two other experiments were 60%–70% and 70%–80% for PB2 and PA, respectively. These results demonstrate that DDX21, via its binding of PB1, inhibits assembly of the viral polymerase.

The NS1 Protein Binds DDX21 and Displaces PB1 from DDX21

The Ud NS1 protein, like PB1, binds DDX21 independently of RNA in cotransfection assays (Figure 5A), demonstrating that the NS1 protein can bind DDX21 in uninfected cells as well as in infected cells. To determine whether the NS1 protein competes with the PB1 protein for binding DDX21, we cotransfected 1 μg each of plasmids expressing GST-DDX21 and PB1, plus increasing amounts (0.2–1.0 μg) of a plasmid encoding 3×Flag-tagged WSN NS1. The amounts of PB1 and NS1 associated with DDX21 were assayed by GST selection of DDX21 followed by PB1 and Flag immunoblots, respectively (Figure 5B). Transfection of 1 μg of the WSN NS1 plasmid led to the displacement of PB1 from DDX21 coupled with the binding of NS1 to DDX21 (lane 10), demonstrating that NS1 competes with PB1 for binding to DDX21.

DDX21 Binds to the N-Terminal RNA-Binding Domain of the NS1 Protein

The NS1 protein is comprised of two major domains: (1) N-terminal RBD (amino acids 1–73) that binds dsRNA and (2) ED (amino acids 85–C terminus) (Krug and Garcia-Sastre, 2013). To determine which of these two NS1 domains bind DDX21, we expressed the following GST-tagged NS1 regions in bacteria: amino acids 1–215, representing the full-length protein; amino acids 1–73, representing the RBD; and amino acids 74–215, representing the ED. These GST-tagged proteins, and also the GST tag alone, were affinity selected on glutathione magnetic beads, which were then mixed with 293T cell extracts containing plasmid-expressed Flag-DDX21. After RNase A treatment, the amount of DDX21 that was bound to the immobilized GST species on the glutathione beads was assayed using a Flag immunoblot. As shown in Figure 6A, DDX21 binds the RBD, but not the ED, of the NS1 protein.

To determine which amino acids in the NS1 RBD are required for binding DDX21, we first focused on surface-exposed amino acids in one region of the RBD (R37, R38, K41, R44) and, as a control, a surface-exposed amino acid in another region of the RBD (K70) (Figure 6B). We expressed GST-tagged RBDs in which one of these amino acids was changed to alanine (A) (Figure 6C). We also expressed a GST-tagged RBD in which both K41 and R44 were changed to As. The RBDs containing K41A, R44A, or both K41A and R44A bound little or no DDX21, and the RBDs containing R37A or R38A bound only low amounts of DDX21. In contrast,

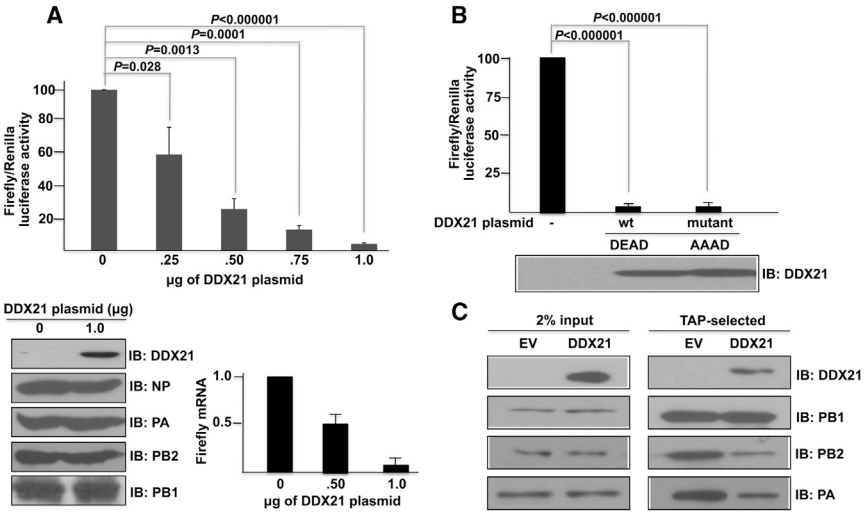

Figure 4. DDX21 Inhibits Viral RNA Synthesis by Binding PB1 and Inhibiting Formation of the Viral Polymerase

(A) Effect of DDX21 on viral RNA synthesis measured in dual-luciferase reporter assays carried out as described in the text. The firefly/*Renilla* luciferase ratio, which measures luciferase mRNA synthesis, was determined in the absence and presence of the indicated amounts of the transfected DDX21 plasmid. The ratios in the presence of the DDX21 plasmid were normalized to the ratio in the absence of DDX21. The assays were carried out in triplicate. The bars show the SD of the triplicate determinations. Quantitative RT-PCR was carried out in triplicate to verify that this ratio accurately reflects the amount of luciferase mRNA produced (lower right panel). The firefly mRNA levels in cells transfected with the DDX21 plasmid were normalized to the firefly mRNA level in cells transfected with the empty plasmid (0 μg DDX21). The bars show the range of the triplicate results. The levels of the viral proteins in the absence and presence (1 μg) of the DDX21 plasmid were measured by IBs to establish that DDX21 did not affect the synthesis of the viral proteins (lower left panel).

(B) DDX21 lacking helicase activity maintains the ability to inhibit viral RNA synthesis. The DDX21 plasmid (1 μg) that was cotransfected in the luciferase reporter assay expressed either WT DDX21 or DDX21 in which the DEAD sequence was changed to AAAD.

(C) DDX21 inhibits the formation of the viral polymerase. 293T cells were cotransfected for 48 hr with plasmids expressing TAP-tagged PB1, PA, and PB2 and with a plasmid expressing DDX21 or an empty vector (EV). Cell extracts prepared as described in the legend of Figure 2B were TAP selected, and the eluates were immunoblotted with the indicated Abs. ImageJ software (NIH) was used to quantitate the PB1, PA, and PB2 IBs. p values calculated via a one-tailed Student's t test are shown. See also Figure S5.

the RBD containing K70A bound DDX21 almost as well as the WT RBD. Consequently, we conclude that the region comprised of R37, R38, K41, and R44 is required for the binding of the NS1 RBD to DDX21.

To determine whether this region of the RBD is sufficient for the binding of full-length NS1 protein to DDX21 and displacing PB1 from DDX21, we cotransfected plasmids expressing GST-DDX21, PB1, and either WT NS1 protein or a NS1 protein with K41A and R44A mutations (41/44 NS1 protein). GST-DDX21 was affinity selected on glutathione magnetic beads, and the amounts of the PB1 and NS1 protein bound to the immobilized DDX21 after RNase A treatment were assayed by immunoblots (Figure 6D). The WT NS1 protein binds DDX21 and largely displaces PB1 from binding DDX21. In contrast, the 41/44 NS1 protein does not bind DDX21 and does not displace PB1 from binding DDX21. We also compared the abilities of the WT and 41/44 NS1 proteins to reverse DDX21-induced inhibition of viral RNA synthesis as determined using the minigenome assay (Figure 6E). The addition of 1 μg of a plasmid expressing the 41/44 mutant NS1 protein did not relieve DDX21-induced inhibition of viral RNA synthesis. In contrast, addition of 1 μg of a plasmid expressing WT NS1 protein restored approximately 20%–25% of the viral RNA synthesis observed in the absence of DDX21. Larger amounts of the WT NS1 plasmid did not increase the extent of restoration of viral RNA synthesis. Complete restoration of viral RNA synthesis did not occur, presumably because the NS1 protein inhibits the activity of one or more components of the viral RNA polymerase. For example, the NS1 protein has been shown to bind NP (Robb et al., 2011), and such binding could inhibit viral polymerase activity.

A Recombinant Influenza A Virus Encoding a 41/44 Mutant NS1 Protein Establishes that the NS1 Protein Counteracts Endogenous DDX21 Antiviral Activity in Infected Cells

The region of the NS1 RBD required for binding DDX21 (Figure 6C) also participates in binding dsRNA, α-importin, TRIM25, and NP (Cheng et al., 2009; Chien et al., 2004; Gack et al., 2009; Melén et al., 2007; Robb et al., 2011). Consequently, substitution of any of the amino acids in this RBD region is expected to affect functions in addition to DDX21 binding. For this reason, it was not feasible to generate a mutant virus encoding a NS1 protein that is solely defective in binding DDX21 and inhibiting its antiviral activity. Our approach was to determine whether we could generate a recombinant virus encoding a mutant NS1 protein whose defects include an inability to combat DDX21-mediated inhibition of virus gene expression. We generated a Ud recombinant virus that expresses a NS1 protein with both the K41A and R44A mutations (41/44 virus). The 41/44 virus is attenuated during multiple-cycle growth in human Calu3 cells (0.001 pfu/cell). It replicates slower than the WT virus, yielding virus approximately 30-fold less at 30–60 hr after infection (Figure 7A). The 41/44 virus is also attenuated during single-cycle growth in Calu3 cells (5 pfu/cell), producing virus approximately 5-fold less than the WT virus at 10 and 12 hr after infection (Figure 7B). Attenuation of the 41/44 virus could be caused by several defects of the 41/44 NS1 protein, including not only a defect in countering DDX21 antiviral activity, but also defects in binding dsRNA, α-importin, and NP (Cheng et al., 2009; Chien et al., 2004; Gack et al., 2009; Melén et al., 2007; Robb et al., 2011).

To establish that one of the defects of the 41/44 virus stems from the inability of its NS1 protein to counteract DDX21 antiviral

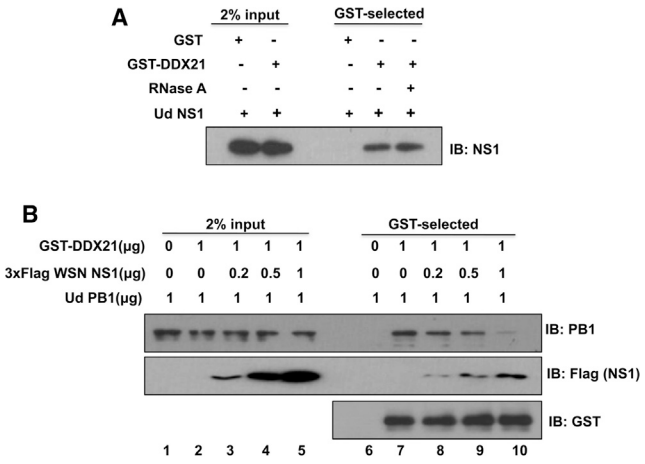

Figure 5. NS1 Protein Binds DDX21 and Displaces PB1 from DDX21

(A) DDX21 binds the NS1 protein in uninfected cells. 293T cells were transfected for 48 hr with a plasmid expressing the Ud NS1 protein and a plasmid expressing GST-DDX21 or GST. Cell extracts prepared as described in the legend of Figure 3B, with and without RNase A treatment, were GST selected, and the eluates were immunoblotted with NS1 Ab.

(B) NS1 displaces PB1 from binding DDX21. 293T cells were transfected for 48 hr with the indicated amounts of plasmids expressing GST-DDX21, 3×Flag-WSN NS1, and Ud PB1. Cell extracts treated with RNase A were GST selected, and the amounts of PB1 and NS1 in the eluates were measured by IBs probed with PB1 or Flag Ab, respectively.

activity in infected cells, DDX21 knockdown and control HeLa cells were infected with 2 pfu/cell of the WT or 41/44 virus, and the levels of viral proteins at 9 and 12 hr after infection were determined using immunoblots (Figure 7C). In both WT- and 41/44-infected cells, DDX21 knockdown increased the levels of viral proteins at 9 hr after infection (lanes 1, 2, 5, and 6), resulting from the inhibition of viral RNA synthesis by DDX21 (Figure 2D). In WT-infected cells at 12 hr after infection, essentially the same levels of viral proteins were produced in control and DDX21 knockdown cells (Figure 7C, lanes 3 and 4), indicating that DDX21-mediated inhibition of viral protein synthesis ceased between 9 and 12 hr after infection. This was not the case in 41/44 virus-infected cells: at 12 hr after infection, the levels of viral proteins in control cells remained substantially lower than the amounts in DDX21 knockdown cells (Figure 7C, lanes 7 and 8), indicating that DDX21-mediated inhibition of viral protein synthesis continued from 9 to 12 hr after infection. We conclude that the WT NS1 protein displaces PB1 from binding endogenous DDX21, whereas the 41/44 mutant NS1 protein, which does not bind DDX21, does not displace PB1 from binding DDX21 during infection. Consistent with this conclusion, DDX21 knockdown increased the replication of the 41/44 virus in Calu3 cells significantly more than the replication of the WT virus (Figure S6), showing that the interaction of DDX21 with PB1 is more deleterious to the 41/44 virus.

DISCUSSION

A major goal of influenza A virus research is to identify specific host proteins that play critical roles in virus replication via their interactions with viral proteins. Our current approach for identifying such host proteins has been to purify infected cell macromolecular complexes that contain the NS1 protein bound to the cellular CPSF30 3′ end-processing factor, and to identify cellular proteins that are consistently associated with these complexes. The only proteins that should be present are CPSF30, NS1, and cellular and viral proteins that CPSF30 and NS1 interact with, either directly or indirectly (most likely via specific host proteins). We then determine whether these cellular proteins have specific functions during influenza A virus infection. Here, we show that the DDX21 RNA helicase that is associated with these NS1-CPSF30 complexes is a host factor that regulates viral RNA synthesis during infection via sequential interaction with the viral PB1 and NS1 proteins. The binding of DDX21 to NS1 explains why it is associated with NS1-CPSF30 complexes. Interestingly, DDX21 was not identified in any of the RNAi-based genome-wide screens for host proteins involved in influenza A virus replication (Watanabe et al., 2010). In addition, a recent bioinformatics approach to identify host proteins that interact with influenza virus proteins did not predict an interaction of DDX21 with either NS1 or PB1 (de Chassey et al., 2013). It was reported previously that DDX21 is associated with a PA-PB1 complex that was generated in uninfected cells using plasmids to overexpress these two proteins and that this association was eliminated by RNase A treatment (Bradel-Tretheway et al., 2011). Our results clearly show that this is not the case in virus-infected cells, where DDX21 binds the PB1 protein in the absence of PA in a RNase A-resistant manner, highlighting the importance of identifying specific interactions between viral and host proteins in virus-infected cells.

Many cellular proteins have previously been reported to bind to the NS1 protein, and some of these interactions with NS1 may be mutually exclusive (Krug and Garcia-Sastre, 2013). In addition, at least some of the cellular proteins other than DDX21 that are associated with the purified NS1-CPSF30 complexes (Table S1) may also bind to NS1. It is therefore likely that there are multiple NS1-CPSF30 complexes that differ with respect to the cellular protein(s) that are bound to the NS1 protein. These complexes also probably differ with respect to the presence of the tripartite viral polymerase with its associated NP protein. Our results indicate that the NS1-CPSF30 complex that contains DDX21 most likely does not also contain the viral polymerase with its associated NP, particularly because DDX21 binds only the PB1 subunit of the viral polymerase in infected cells. It is not known how the viral polymerase and its associated NP are brought into the NS1-CPSF30 complexes. One possibility is that the reported interaction of NS1 with NP (Robb et al., 2011) is responsible for bringing the viral polymerase with its associated NP into a NS1-CPSF30 complex. If this were the case, DDX21 and the viral polymerase with its associated NP would be in different NS1-CPSF30 complexes because DDX21 and NP bind to essentially the same site in the NS1 RBD (Robb et al., 2011; present study). Alternatively, other cellular proteins that we identified in the purified NS1-CPSF30 complexes could be responsible for bringing the polymerase with its associated NP into NS1-CPSF30 complexes. It will be of interest to determine whether these other cellular proteins have previously unidentified roles during influenza A virus infection.

In the present study, we used several approaches to demonstrate that endogenous DDX21 sequentially interacts with the

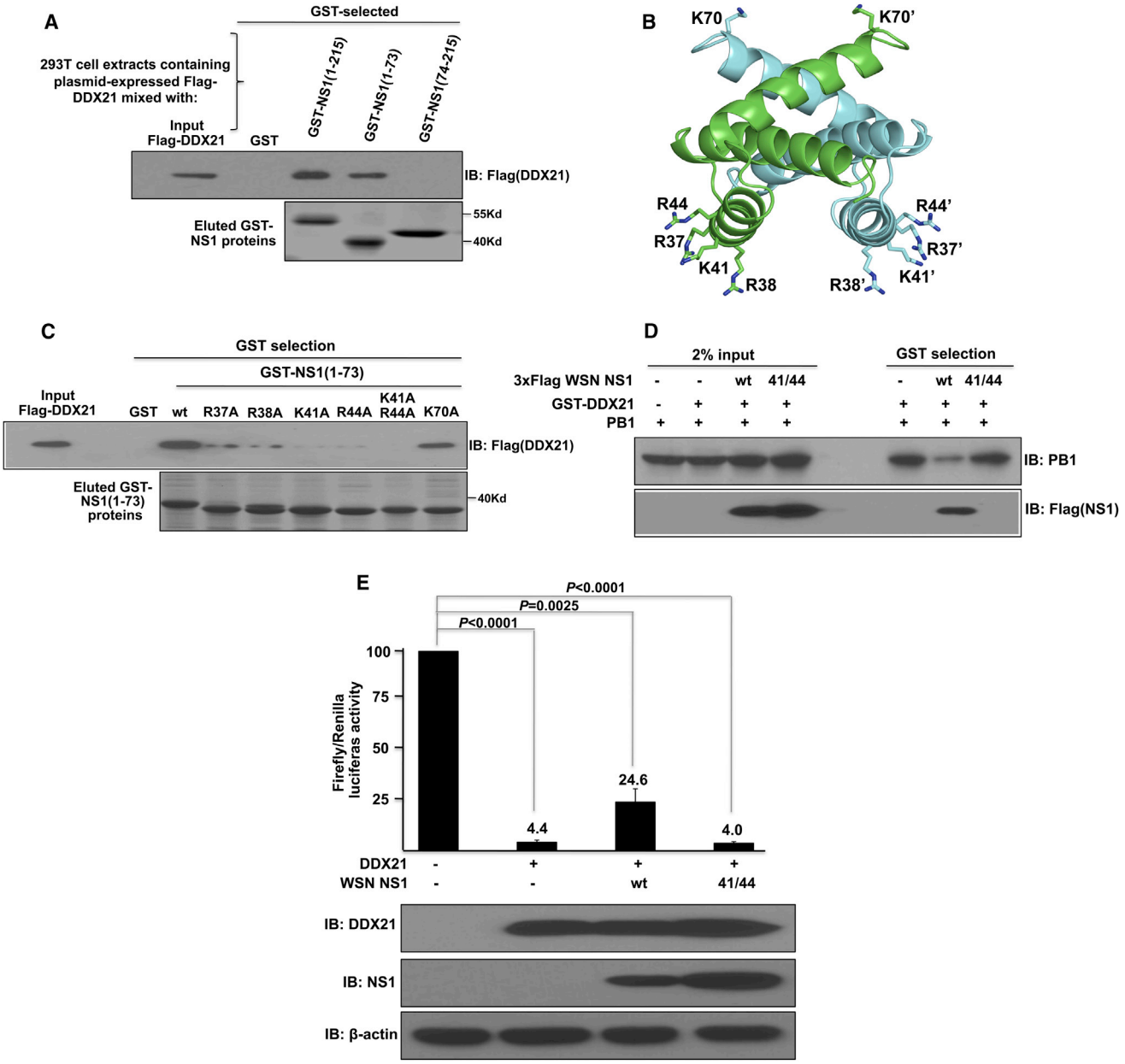

Figure 6. Identification of the Amino Acids in the NS1 RBD that Are Required for Binding DDX21

(A) Bacteria-expressed GST and the indicated GST-tagged NS1 proteins were bound to glutathione magnetic beads, which were then incubated in the presence of RNase A for 4 hr with 293T cell extracts containing plasmid-expressed Flag-DDX21. The proteins eluted from the beads were analyzed by an IB probed with Flag Ab (top panel) and by Coomassie blue staining (bottom panel).

(B) The structure of the RBD showing the amino acids (R37/R37′, R38/R38′, K41/K41′, R44/R44′, K70/K70′) that were replaced with alanines. The RBD structure was generated from Protein Data Bank (PBD) ID 1NS1.

(C) Bacteria extracts containing GST, GST-NS1 (1–73), or GST-NS1 (1–73) containing the indicated amino acid substitutions were bound to glutathione magnetic beads, which were then incubated in the presence of RNase A with 293T containing plasmid-expressed Flag-DDX21. The eluted proteins were analyzed by an IB probed with Flag Ab and by Coomassie blue staining.

(D) 293T cells were cotransfected for 48 hr with plasmids expressing GST-DDX21, PB1, and either WT or 41/44 NS1 protein. Cell extracts were RNase A treated and GST selected, and the eluates were immunoblotted with PB1 or Flag Ab.

(E) The dual-luciferase assay for viral RNA synthesis was carried out in triplicate as described in the text. Where indicated, 1 μg of a plasmid expressing DDX21, and 1 μg of a plasmid expressing either WT or 41/44 NS1 protein, was added. The firefly/*Renilla* ratio was normalized to the ratio in the absence of DDX21. The levels of the WT and 41/44 NS1 proteins, DDX21, and β-actin were determined by IBs.

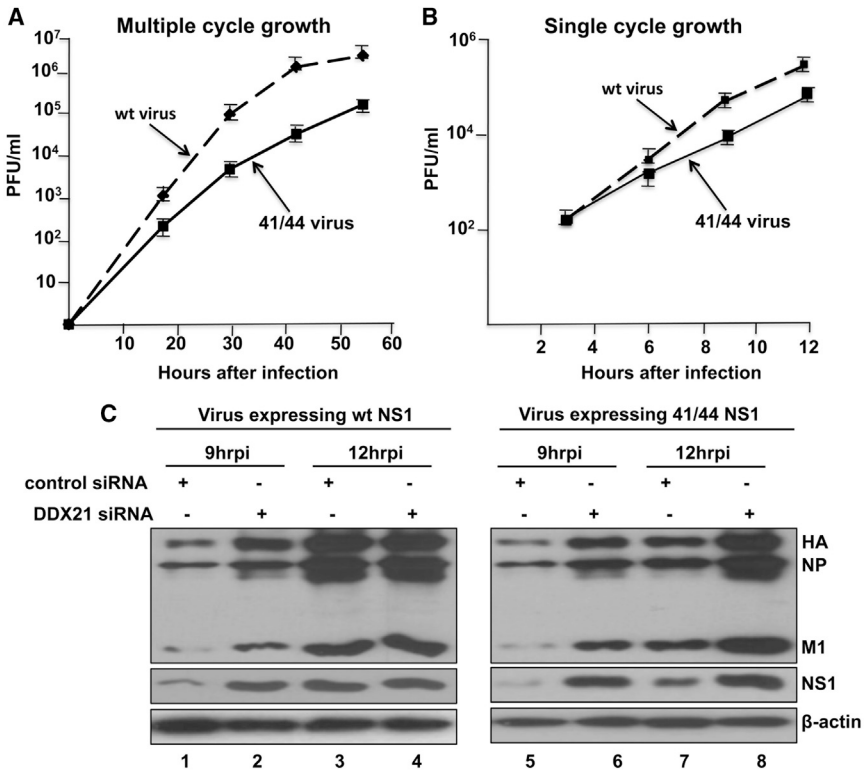

Figure 7. The NS1 Protein Counteracts Endogenous DDX21 Antiviral Activity during Virus Infection

(A) Calu3 cells were infected with 0.001 pfu/cell of WT or 41/44 virus. Virus production at the indicated times after infection was determined by plaque assays. The bars show the SD of triplicate measurements of virus production.

(B) Calu3 cells were infected with 5 pfu/cell of WT or 41/44 virus. The bars show the SD of triplicate measurements of virus production.

(C) HeLa cells were transfected with a DDX21 siRNA or a control siRNA for 36 hr, followed by infection with 2 pfu/cell of WT or 41/44 virus. At 9 and 12 hr after infection, cell extracts were immunoblotted with Ud, NS1, or β-actin Ab. See also Figure S6.

viral PB1 and NS1 proteins during influenza A virus infection, resulting in the regulation of viral RNA synthesis. DDX21 knockdown established that endogenous DDX21 inhibits viral RNA synthesis at early times after infection, even as early as 1–3 hr after infection (Figure 2D), and a GST pull-down showed that DDX21 binds PB1 as well as NS1 in infected cells. The interaction of DDX21 with PB1 would be expected to inhibit viral RNA synthesis, and this expectation was verified by minigenome assays. To establish the role of the NS1 protein, we identified the binding site of DDX21 on the NS1 protein and showed that a recombinant virus that expresses a NS1 protein with a mutated DDX21 binding site does not counteract the inhibition of viral gene expression mediated by endogenous DDX21. This result established that the binding of endogenous DDX21 by the NS1 protein in infected cells suppresses DDX21 antiviral activity. Further, this result also confirms that endogenous DDX21 is responsible for the inhibition of viral gene expression. Additional evidence for this mechanism might come from the identification of the binding site of DDX21 on PB1, possibly enabling the generation of a recombinant virus that encodes PB1 with a mutated DDX21 binding site. However, this is a formidable project that may not provide clear results. First, it will be difficult to identify the DDX21 binding site on the large (~90 kDa) PB1 protein because there is little or no structural information for PB1 (Boivin et al., 2010; Resa-Infante et al., 2011). In addition, the identified PB1 amino acids may have other required PB1 functions, e.g., viral RNA synthesis, binding to the PA and PB2 subunits of the polymerase (Boivin et al., 2010; Resa-Infante et al., 2011). Consequently, such a mutant PB1 protein may have several defects that would make it difficult to identify a specific defect caused by the lack of DDX21 binding. Future research will determine whether it is feasible to generate a recombinant virus that encodes a PB1 protein whose primary defect is in DDX21 binding.

Our results thus provide strong support for a mechanism by which influenza A viral RNA and protein synthesis are regulated in infected cells. At early times of infection, DDX21 binds the small amount of the PB1 protein that is initially synthesized and thus inhibits the association of PB1 with PA and PB2 to form a functional viral polymerase. However, the small amounts of the PA and PB2 proteins that are initially synthesized compete with DDX21 for binding PB1, albeit inefficiently, thereby allowing a low level of synthesis of viral mRNAs and proteins to occur. The NS1 protein that is synthesized binds DDX21 and displaces the PB1 protein, suggesting that the binding sites of NS1 and PB1 on DDX21 may be overlapping. The displacement of PB1 from DDX21 leads to an increase in the formation of the tripartite viral polymerase and hence to an increase in the synthesis of vRNAs, viral mRNAs, and proteins. When sufficient amounts of the NS1 protein are synthesized, essentially all of the PB1 protein is dissociated from DDX21 and combines with PA and PB2 to form the tripartite viral polymerase. Consequently, the synthesis of viral RNAs and proteins is no longer impeded. Because the amount of the NS1 protein that is synthesized in infected cells greatly exceeds the amount of the PB1 protein that is synthesized, the NS1 protein would readily outcompete the PB1 protein for binding DDX21. Thus, by virtue of the binding of DDX21 by the NS1 protein, influenza A virus transforms a host restriction factor into a host regulator of viral gene expression.

The DDX21 helicase is a member of the family of DEXD RNA helicases, which are comprised of a largely conserved helicase core containing the DEXD sequence as well as flanking N-terminal and C-terminal domains that are highly divergent and presumed to interact with various cellular proteins and/or RNAs (Fuller-Pace, 2006). In fact, some DEXD helicases carry out functions that do not require helicase activity, but rather are mediated by their N-terminal and/or C-terminal domains. We show that the inhibition of influenza viral RNA synthesis by DDX21 does not

require its helicase activity and functions solely by binding PB1 and inhibiting its association with the PA and PB2 polymerase subunits. Because neither the N-terminal domain, the core helicase domain, nor the C-terminal domain of DDX21 was sufficient for inhibiting viral RNA synthesis, the binding site for PB1 may be comprised of more than one DDX21 domain. DDX21 most likely binds PB1 in the cytoplasm, where PB1 is probably free of the other polymerase subunits, albeit transiently, before it forms a complex with PA that is imported into the nucleus (Fodor and Smith, 2004). The PB1 protein in the nucleus is predominately complexed with both PA and PB2 in the tripartite viral polymerase and is probably not available for binding to DDX21. Although DDX21 has been considered to be a nucleolar protein, it does not function in ribosome biogenesis, unlike many nucleolar proteins, indicating that DDX21 may participate in extranucleolar functions (Tafforeau et al., 2013). In fact, DDX21 has been reported to function in cytoplasmic events, namely regulating the translation of the polycistronic mRNA of Borna disease virus (Watanabe et al., 2009) and sensing dsRNA in dendritic cells as part of a complex of three helicases (Zhang et al., 2011). In addition, we have found that whereas plasmid-expressed PB1 is localized in the cytoplasm, as previously shown by others (Fodor and Smith, 2004), cotransfection of PB1 and DDX21 plasmids results in a significant portion of PB1 entering into the nucleus (Figure S4).

We showed that DDX21 binds to the RBD of NS1, specifically to a region comprised of amino acids R37, R38, K41, and R44, which also participates in binding dsRNA, α-importin, TRIM25, and NP (Cheng et al., 2009; Chien et al., 2004; Gack et al., 2009; Melén et al., 2007; Robb et al., 2011). This overlap of NS1 sequences that mediate more than one function probably reflects the fact that the NS1 protein in infected cells carries out different functions at different sites in the cell. Because this region of the NS1 RBD is crucial for multiple functions that are required for virus infection, it should be an excellent target for the development of influenza virus antivirals, particularly as mutant viruses that express NS1 proteins in which alanine is substituted for one or more of the amino acids in this region are highly attenuated (Donelan et al., 2003; Gack et al., 2009; Min and Krug, 2006). Consequently, small molecules that strongly bind to this RBD region would be expected to be extremely effective in inhibiting virus replication.

EXPERIMENTAL PROCEDURES

Cells and Viruses

HeLa, MDCK, HEL-299, 293T, and Calu3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum. WT Ud virus, Ud virus encoding NS1 and NS2/NEP proteins with a N-terminal Flag tag (Zhao et al., 2010), Ud virus expressing a mutant (R44A, K41A) NS1 protein, and WSN virus were generated by plasmid-based reverse genetics as described previously (Takeda et al., 2002; Twu et al., 2006). All eight genomic RNA segments of recombinant viruses were sequenced. Virus stocks were grown in 10-day-old fertilized eggs, and virus titers were determined by plaque assays in MDCK cells.

Purification of CPSF30-NS1 Complexes from Infected Cells

293T cells were transfected with a pcDNA3 plasmid encoding GST-CPSF30 using the Mirus TransIT-LTR Transfection Reagent, and 24 hr later were infected with 2 pfu/cell of either WT Ud virus or the Ud virus expressing a Flag-tagged NS1 protein. Cells were collected 12 hr after infection and disrupted by suspension in a solution containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 2.5 mM $MgCl_2$, 1% NP40, 10% glycerol, 1 mM PMSF, and 1× protease inhibitor (Roche), followed by passage through a 25G needle. The extracts were affinity purified using glutathione Sepharose beads as described previously. The eluate was then bound to agarose beads containing the M2 monoclonal Flag antibody, washed four times with a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.25% NP40, and Flag-containing protein complexes were eluted with 500 ng/ml of 3×Flag peptide. An aliquot of the eluate was analyzed by SDS-PAGE gel, which was stained with Coomassie blue. Several molecular weight regions were cut out and analyzed by mass spectroscopy (Harvard Taplin Mass Spectrometry Facility). The proteins identified in the 90–110 kDa molecular weight region are shown in Table S1.

DDX21 siRNAs

The siRNA duplexes against DDX21 were purchased from Invitrogen and resuspended in diethyl pyrocarbonate-treated water to a final storage concentration of 20 μM. The sequences of the two DDX21 siRNAs are as follows: CCAGCGCUCCUUGAUCAACUCAAAU (DDX21-1) and AGGCCAGAAGCG GAGUUUCAGUAAA (DDX21-2). The control siRNA, Stealth RNAi negative control (medium G/C), was obtained from Invitrogen.

Preparation of Plasmids

The coding sequence of DDX21 was RT-PCR amplified from the total RNA of 293T cells using primers specific for the 5′ and 3′ ends of the DDX21 coding sequence and containing SalI and BamH I restriction sites (shown in Supplemental Information). This DNA sequence was cloned into the expression plasmid pCMVHF treated with SalI and BglII, thereby adding a Flag tag to the 5′ end of the DDX21 coding sequence. Standard oligonucleotide mutagenesis methods were used to change the DEAD sequence in DDX21 to AAAD. The resulting DNA was sequenced to verify the substitution of the AAAD sequence. To generate a plasmid expressing GST-tagged DDX21, the GST sequence in the pGEX5X-3 plasmid was amplified using primers specific for the 5′ and 3′ ends of GST and containing Hind III and KpnI restriction sites (described in Supplemental Information), and it was cloned into pcDNA3 cut with these two restriction enzymes. Then, a DDX21 DNA sequence containing BamH I and Xho I restriction sites was generating using appropriate 5′ and 3′ primers (Supplemental Information), and this DNA was cloned into the GST-containing pcDNA3 plasmid cut with BamH I and Xho I. To generate a plasmid expressing C-terminal TAP-tagged PB1, the coding sequence of PB1 was PCR amplified using 5′- and 3′-specific primers containing BamH I and Not I restriction sites, and this DNA was cloned into pcDNA3-CTAP cut with these two restriction enzymes. The NS1 sequences in the pcDNA-Ud NS1 and the pCMV10-WSN NS1 plasmids contain a mutated 3′ splice site (Alonso-Caplen and Krug, 1991) to eliminate the production of the NS2/NEP mRNA and protein. For bacteria expression, GST and the following NS1 coding sequences were inserted into pGEX-6P1 vectors: NS1 (1–215), NS1 (74–215), WT NS1 (1–73), and NS1 (1–73) containing the indicated amino acid substitutions. The resulting GST-tagged NS1 proteins, as well as the GST tag alone, were expressed in *Escherichia coli* BL21.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2014.03.002.

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