

Influenza virus targets the mRNA export machinery and the nuclear pore complex

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The NS1 protein of influenza A virus is a major virulence factor that is essential for pathogenesis. NS1 functions to impair innate and adaptive immunity by inhibiting host signal transduction and gene expression, but its mechanisms of action remain to be fully elucidated. We show here that NS1 forms an inhibitory complex with NXF1/TAP, p15/NXT, Rae1/mrnp41, and E1B-AP5, which are key constituents of the mRNA export machinery that interact with both mRNAs and nucleoporins to direct mRNAs through the nuclear pore complex. Increased levels of NXF1, p15, or Rae1 revert the mRNA export blockage induced by NS1. Furthermore, influenza virus down-regulates Nup98, a nucleoporin that is a docking site for mRNA export factors. Reduced expression of these mRNA export factors renders cells highly permissive to influenza virus replication, demonstrating that proper levels of key constituents of the mRNA export machinery protect against influenza virus replication. Because Nup98 and Rae1 are induced by interferons, down-regulation of this pathway is likely a viral strategy to promote viral replication. These findings demonstrate previously undescribed influenza-mediated viral–host interactions and provide insights into potential molecular therapies that may interfere with influenza infection.

NS1 | nucleoporin | nuclear transport | mRNA nuclear export

Influenza viruses cause $\approx 500,000$ deaths worldwide per year (1). Strains that are extremely pathogenic pose an even greater health threat. The 1918 influenza pandemic, for example, resulted in ≈ 30 million deaths around the world (2). The nonstructural 1 (NS1) protein of influenza A virus is a major virulence factor that inhibits host immune responses by disrupting host signal transduction (3–11) and gene expression (12, 13) pathways. Genetic studies have shown that abrogation of NS1 function by mutation results in highly attenuated viruses, except in animals lacking innate antiviral mechanisms (12, 14). These observations define NS1 as an essential element of viral virulence and suggest that its function represents a point of vulnerability for inhibiting influenza viral pathogenesis.

NS1 is a multifunctional protein that, in infected cells, is localized in both the cytoplasm and in the nucleus (15). In the cytoplasm, NS1 inhibits innate immune response by forming a complex with the pathogen sensor RIG-I (4, 7, 9, 10) and by targeting PKR and the RNase L pathway (6, 8). In addition, NS1 has been shown to activate phosphatidylinositol-3-kinase signaling, which may be important for promoting viral replication (16). In the nucleus, NS1 inhibits host gene expression. This effect includes mRNA processing, which is mediated by interaction of NS1 with polyadenylation factors (CPSF and PABII) (12, 13) and a putative splicing factor (NS1-BP) (17). The binding of NS1 to CPSF and PABII inhibits polyadenylation of host mRNAs, contributing to nuclear retention of these messages (12, 13). However, the potent ability of NS1 to inhibit host mRNA nuclear export led us to explore a potential interaction of NS1 with the

mRNA nuclear export machinery that could explain further the mechanism of mRNA export inhibition induced by NS1.

The mRNA export receptors NXF1-p15 (TAP-NXT) are key constituents of the mRNA export machinery that is responsible for nuclear exit of $\approx 70\%$ of cellular mRNAs (18). This heterodimer interacts with both messenger ribonucleoprotein particles and nuclear pore complex proteins [nucleoporins (Nups)] to direct mRNAs through the nuclear pore complex (NPC) (18). Another factor termed E1B-AP5, identified as a cellular protein that interacts with the adenovirus protein E1B-55 and implicated in mRNA export (19), is an hnRNP-like protein that likely mediates the interaction of NXF1 with mRNAs (20). In addition, other RNA-binding proteins such as the REF family (21) and SR splicing factors (22) also are known as adapters for the interaction between NXF1 and mRNAs. Furthermore, the mRNA export factor Rae1/mrnp41/Gle2 (23–25), which shuttles between the nucleus and the cytoplasm, forms a complex with RNPs (24, 26), NXF1 (27), and the nucleoporin Nup98 (26). It has been proposed that Rae1 may recruit TAP to Nup98 to mediate transport through the NPC (27).

Nuclear export of mRNAs has been shown to be targeted by viruses. For instance, the VSV matrix (M) protein is an inhibitor of bulk mRNA nuclear export (28–30), and we showed that it interacts with Rae1 (31), which is in complex with Nup98 (30, 31). However, this mRNA export block can be fully reverted by increasing the intracellular levels of Rae1 or partially reverted by inducing higher levels of Nup98-Nup96 (31, 32). Furthermore, treatment of cells with interferons, which up-regulates Nup98-Nup96 and Rae1, also can revert this mRNA export blockage mediated by VSV M protein (32). Recently, by targeting the Nup96 gene in mice to produce a strain that expresses low levels of Nup96 protein, we demonstrated a role for Nup96 in preferentially facilitating expression of IFN-regulated proteins (33). Thus, these findings showed a complex role for the mRNA export machinery in both viral-mediated cytotoxicity and antiviral response.

In this study, we investigated the mechanisms used by influenza A virus to disrupt the mRNA nuclear export machinery, leading to a block in mRNA export. In addition, we showed a previously undescribed role for the mRNA export machinery in regulating influenza virus replication and cytotoxicity.

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Abbreviations: MOI, multiplicity of infection; Nup, nucleoporin; NS1, nonstructural 1.

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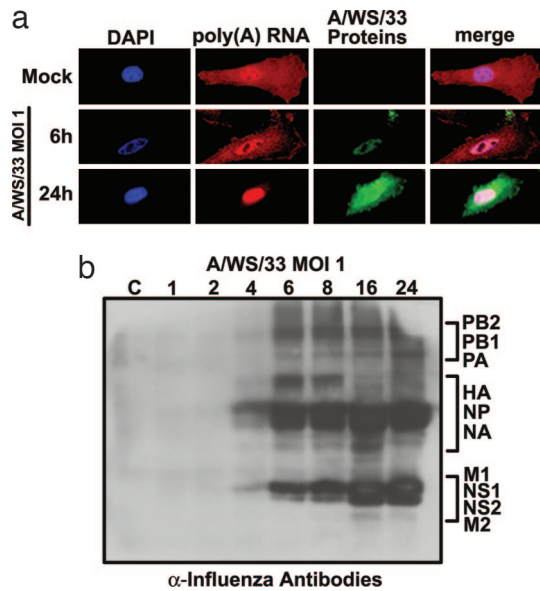


Fig. 1. Influenza virus inhibits poly(A) RNA nuclear export. (a) MDCK cells were mock infected or infected with A/WS/33 influenza virus at an MOI of 1 for 6 and 24 h. Immunofluorescence using antibodies against influenza proteins (green) and oligo(dT) *in situ* hybridization (red) was performed. (b) Expression of influenza proteins in MDCKs. Cell extracts from MDCK cells infected with A/WS/33 at an MOI 1 for the indicated time points were subjected to immunoblot analysis with anti-influenza protein antibodies.

Results and Discussion

NS1 Interacts with Key Constituents of the mRNA Export Machinery.

To examine the importance of regulated bulk mRNA export in the context of viral infection, we determined the distribution of

host poly(A) RNA in influenza virus-infected cells. We detected inhibition of bulk host poly(A) RNA nuclear export in MDCK cells infected with the influenza virus strain A/WS/33 at a multiplicity of infection (MOI) of 1 (Fig. 1a). Expression of influenza proteins clearly was detected by immunoblot analysis, starting at 4 h after infection (Fig. 1b). Inhibition of mRNA export was observed as early as 6 h after infection (Fig. 1a), which was a time point at which we could easily detect infected cells by using polyclonal antibodies against all influenza proteins. However, inhibition of mRNA export may begin even earlier, because a key constituent of the mRNA export machinery is degraded at early stages of infection (see below). The inhibition of mRNA export was enhanced by 24 h of infection. Inhibition of export appeared to be selective for host mRNAs; viral proteins were expressed throughout the course of infection, presumably because viral RNAs are exported through the Crm1 pathway (34), which does not involve NXF1, p15, or Rae1.

We then investigated whether NS1 interacts with the mRNA nuclear export machinery. Purified GST-NS1 or GST alone was incubated with cell extracts from 293T cells, which expressed zz-p15. As shown in Fig. 2a, NS1 interacted with endogenous NXF1, p15, Rae1, E1B-AP5, and very weakly with Nup98. In contrast, we did not detect any interaction of NS1 with other constituents of the nuclear transport machinery including Nup96, Nup62, Nup153, and Nup214 (Fig. 2b and c). These results indicate that NS1 binds specifically to the mRNA export factors NXF1, p15, Rae1, and E1B-AP5, which are known to form a complex (27).

We then analyzed the domains of NS1 involved in the interactions with these constituents of the mRNA nuclear export machinery. A domain within the amino terminal region of NS1, from amino acids 19 to 38, is required for NS1-mediated inhibition of mRNA nuclear export (35). Furthermore, a region at the carboxyl terminal domain of NS1, from amino acids 134

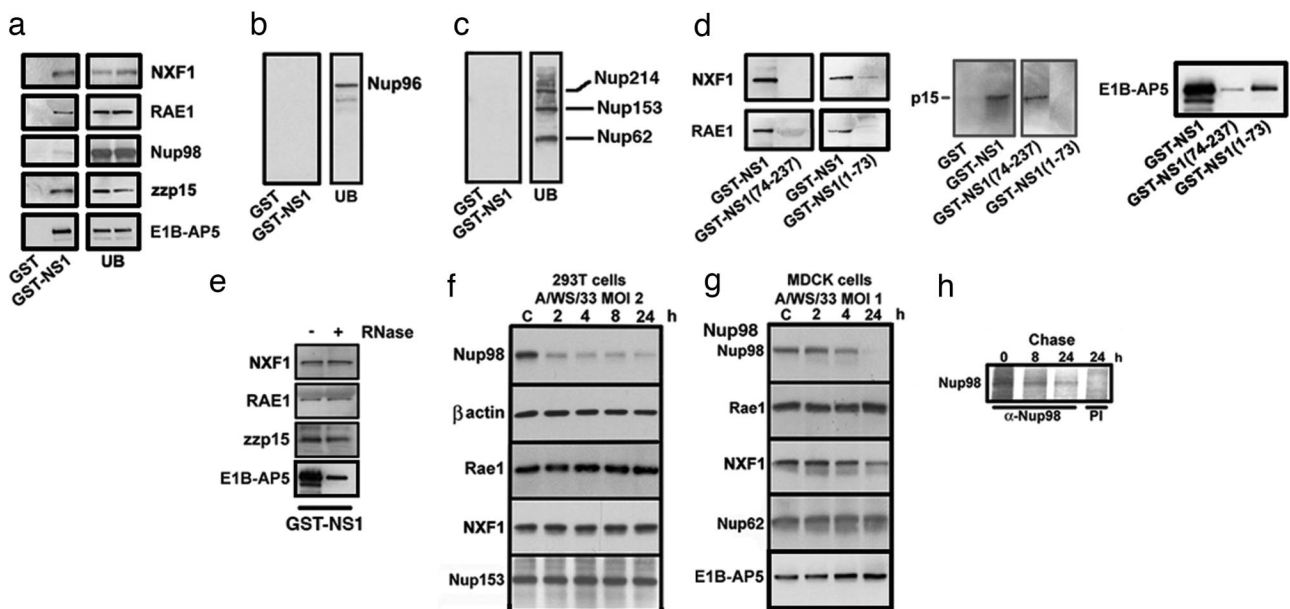


Fig. 2. The NS1 protein of influenza virus interacts with key constituents of the mRNA export pathway. (a) Cell lysates from 293T cells were incubated with immobilized recombinant GST or GST-NS1. Bound and unbound (UB) fractions were analyzed by 4–20% SDS/PAGE followed by immunoblot analysis with antibodies to NXF1, p15, Rae1, E1B-AP5, and Nup98. (b and c) Experiments were performed as in a except that antibodies against Nup96 and against Nup62 and Nup153 (mAb414) were used for immunoblot analysis. (d) GST-NS1 or the amino-terminal or carboxyl-terminal domains of NS1 fused with GST were incubated with cell lysates and processed as in a. (e) GST-NS1 was incubated with cell lysates untreated or treated with RNase A and processed as in a. (f and g) Expression levels of Nups and mRNA export factors in 293T (f) and MDCK (g) cells infected with influenza virus. Cell extracts were subjected to immunoblot analysis with antibodies against Nup98, β -actin, Rae1, NXF1, and E1B-AP5, and with mAb414 antibodies. (h) Half-life measurements of Nup98. MDCK cells were pulse-labeled for 2 h and chased for the depicted time points. Immunoprecipitations were performed with anti-Nup98 antibodies or preimmune serum (PI). Nup98 bands were analyzed by densitometry as described in *Methods*.

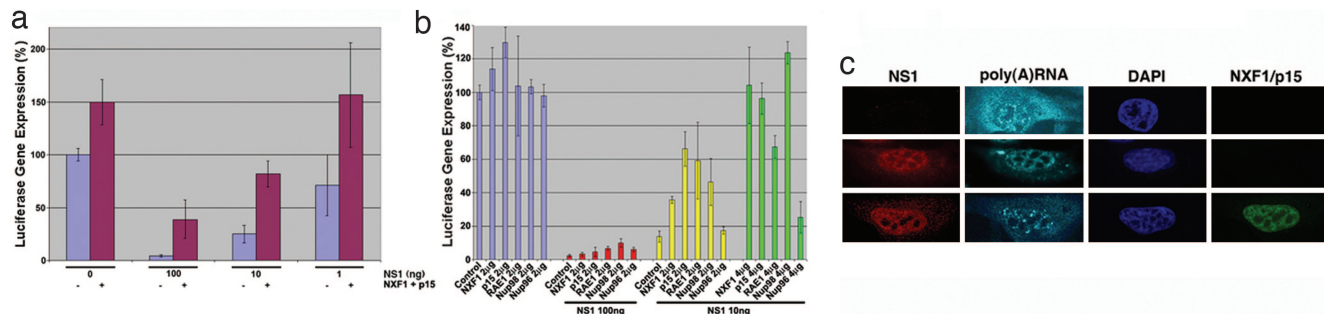


Fig. 3. The mRNA export inhibition induced by NS1 is reverted by increased levels of mRNA export factors. (a and b) Luciferase reporter gene expression assays were performed with 293T cells by cotransfection of reporter plasmids and plasmids encoding NXF1, p15, Rae1, Nup98, and Nup96, as indicated. (c) HeLa cells were transfected with a plasmid encoding myc-NS1 alone or cotransfected with plasmids encoding myc-NS1, GFP-NXF1, and GFP-p15. Cells were subjected to immunofluorescence with anti-myc antibody (red) followed by oligo(dT) *in situ* hybridization (blue). Green shows GFP-NXF1 and GFP-p15.

to 161, also was shown to be required for the inhibitory effect of NS1 on mRNA export (35). Deletion of the first 73 aa of NS1 inhibited its interaction with NXF1 and Rae1 and decreased considerably its interaction with E1B-AP5 (Fig. 2*d*). However, lack of the first 48 or 73 residues of NS1 retained significant interaction with endogenous p15 (Fig. 2*d*) or ectopically expressed zz-p15 [supporting information (SI) Fig. 6]. On the other hand, NXF1, Rae1, and E1B-AP5 bound poorly to the amino-terminal domain of NS1 (amino acids 1–73), and p15 showed no significant interaction with this domain. These results demonstrate that p15 interacts with the carboxyl-terminal domain of NS1 whereas NXF1, Rae1, and E1B-AP5 binding requires residues within the amino- and carboxyl-terminal domains of NS1. Interaction of NS1 with NXF1, p15, and Rae1 is not dependent on RNA, because incubation of cell extracts with RNase A did not affect these interactions (Fig. 2*e*). However, interaction of E1B-AP5 with NS1 was diminished in the presence of RNase A (Fig. 2*e*), indicating partial dependence on RNA. These findings demonstrate that NS1 is able to interact with constituents of the mRNA nuclear export machinery and that NS1 may cause a rearrangement of the NXF1/p15/E1B-AP5/Rae1 complex, resulting in inhibition of mRNA nuclear export. Alternatively, NS1 may mask binding sites of this mRNA export

complex, preventing its proper interaction with other constituents of the mRNA export pathway.

Down-Regulation of Nup98 in Cells Infected with Influenza Virus. To investigate additional effects of influenza virus on the mRNA nuclear export machinery, we determined the levels of constituents of this machinery after infection of 293T and MDCK cells. We observed that Nup98 levels were depleted markedly at ≈ 2 –4 h after infection of 293T cells and by 24 h in MDCK cells (Fig. 2*f* and *g*). We did not detect any major differences in the levels of Rae1, NXF1, E1B-AP5, Nup153, and Nup62 (Fig. 2*f* and *g*). We then tested whether the observed changes in Nup98 levels are a consequence of general inhibition of protein synthesis by influenza virus. Fig. 2*h* shows that Nup98 has a long half-life of ≈ 26 h, which indicates that it is actively degraded during influenza virus infection. This degradation likely contributes to the inhibition of mRNA nuclear export observed upon influenza infection.

Increased Expression of mRNA Export Factors Maintains Nuclear Export of mRNA in the Presence of NS1. To determine whether blocking mRNA nuclear export is critical for influenza virus mediated-inhibition of host gene expression, we tested whether

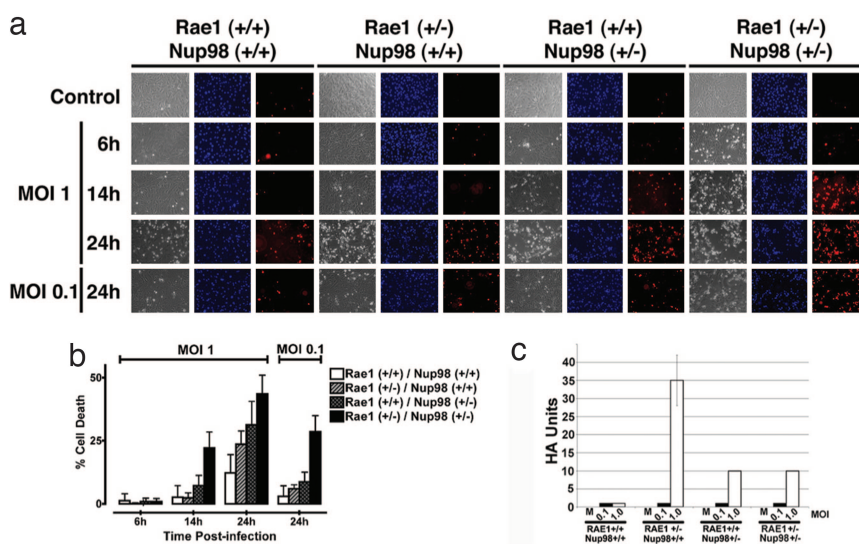


Fig. 4. Low levels of Rae1 and Nup98 induce higher susceptibility to influenza virus-mediated cell death and an increase in viral replication. (a and b) Rae1^{+/+} Nup98^{+/+}, Rae1^{+/-} Nup98^{+/+}, Rae1^{+/+} Nup98^{+/-}, and Rae1^{+/-} Nup98^{+/-} MEFs were infected with A/WS/33 influenza virus, and cell viability was determined by comparing and quantifying bright-field microscopy (gray), DAPI (blue), and exclusion of 2 mM ethidium homodimer-1 (red). (c) The number of influenza viral particles was measured in the supernatants of the cells in a by using the hemagglutinin assay.

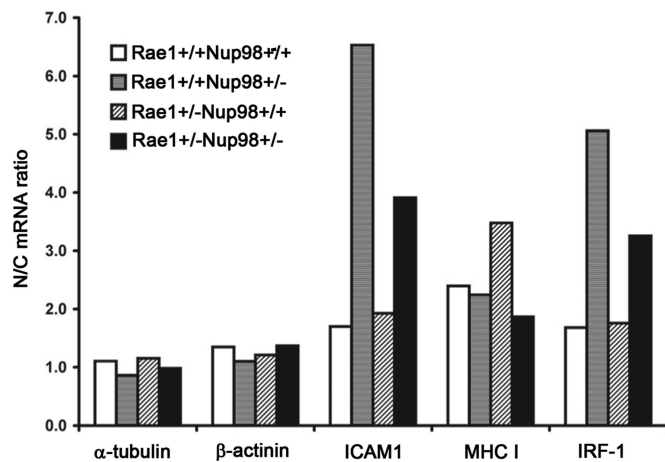


Fig. 5. Selective impairment of mRNA nuclear export in Rae1 and Nup98 mutant cells. Nuclear (N) and cytoplasmic (C) RNA was isolated from Rae1^{+/+} Nup98^{+/+}, Rae1^{+/+} Nup98^{+/-}, Rae1^{+/-} Nup98^{+/+}, and Rae1^{+/-} Nup98^{+/-} MEFs. Relative mRNA levels were quantified by real-time RT-PCR using gene-specific primers and normalized to a set of five housekeeping genes.

increasing expression of mRNA export factors could prevent this inhibition. As shown in Fig. 3*a*, NXF1-p15 reversed the dose-dependent inhibition of NS1. Inhibition of gene expression also was reversed when Rae1, Nup98, NXF1, or p15 were overexpressed individually, but it was not altered by overexpression of Nup96 (Fig. 3*b*). Nup96 does not form a complex with NXF1-p15 (Fig. 2*b*), but it has a role in mRNA export (33, 36). Thus, reversal of NS1 function is specific for constituents of the NXF1-p15 complex. Then, to demonstrate that both the inhibition of gene expression by NS1 and its reversal by increased levels of mRNA export factors occurred at the mRNA export level, we performed immunofluorescence and oligo(dT) *in situ* hybridization in cells expressing NS1 alone or in cells coexpressing NXF1 and p15. As shown in Fig. 3*c*, expression of NS1 caused nuclear retention of poly(A) RNA, and this effect was significantly reverted in cells that coexpressed NS1 and NXF1-p15. These results show that mRNA export factors can antagonize the inhibitory effects of the NS1 protein on mRNA nuclear export. One possibility is that NS1 may bind to more than one constituent of the NXF1 mRNA export complex by using its different domains, and this would explain the reversal of the NS1 effect by more than one constituent of this machinery.

Influenza Virus Virulence Correlates with Impaired mRNA Export Function. To demonstrate the role of the mRNA nuclear export machinery in influenza virus-mediated cytotoxicity, we used cells from mice that express low levels of two key mRNA export factors to determine their susceptibility to influenza infection. Cells from Rae1^{+/+} and/or Nup98^{+/-} mice express low levels of Rae1 or Nup98, respectively, and normal levels of other nuclear export factors (31, 37). We found that Rae1^{+/+} or Nup98^{+/-} cells are more susceptible to influenza virus-mediated cell death than wild-type cells, whereas cells that are heterozygous for both Rae1 and Nup98 show enhanced susceptibility to cell death induced by influenza infection (Fig. 4*a* and *b*). Mutant cells also produced more viral particles compared with wild-type cells (Fig. 4*c*). These results demonstrate a key role for the mRNA export machinery components in antiviral response. Interestingly, we observed a higher number of viral particles in the supernatants of Rae1^{+/+} Nup98^{+/+} cells as compared with Nup98^{+/-} Rae1^{+/+} or Rae1^{+/-} Nup98^{+/-} cells, despite the fact that the later cell types showed more cell death than the Rae1^{+/-} cells (Fig. 4*a* and *b*). This may indicate that viral replication as

observed in Rae1^{+/+} cells requires wild-type levels of Nup98 or that the enhanced cytopathology due to impaired mRNA export prevented efficient viral replication.

To determine whether mRNA export was altered in Nup98 and Rae1 cells that express reduced levels of these mRNA export factors, we compared the nuclear and cytoplasmic abundance of several mRNA species. RNA was isolated from nuclear and cytoplasmic fractions and quantified by real-time RT-PCR to measure the number of various mRNA species, as we described in refs. 33 and 38. Although these cells did not present nuclear retention of bulk poly(A) RNA (39, 40), they showed selective nuclear retention of certain mRNAs, which encode immune-related proteins, but not of mRNAs that encode housekeeping proteins, which displayed similar nucleocytoplasmic distribution in both wild-type and mutant cells (Fig. 5). Among the mRNAs we analyzed here, IRF-1, MHC I, and ICAM-1, which have roles in antiviral response (41–43), were significantly retained in the nucleus of Nup98 or Rae1 mutant cells (Fig. 5), resulting in reduced cytoplasmic accumulation that may contribute to the increase in viral replication observed in some of these cells (Fig. 4). It is also likely that additional classes of mRNAs that were not analyzed here may be subject to impaired transport and contribute to the high viral titers presented by the Nup98 and Rae1 mutant cells. Interestingly, reduction in Rae1 and Nup98 levels did not affect mRNA export identically; rather, each factor appeared to be differentially required for individual mRNA species. We have observed a similar phenomenon of selective mRNA retention in Nup96^{+/-} cells in which a subset of immune-related mRNAs were preferentially retained in the nucleus, contributing to impaired immunity in mutant cells and animals (33). In this case as well, the set of genes differentially affected by impaired Nup96 was not identical to those affected by Nup98 or Rae1. Differential regulation of mRNA export has been observed in yeast, where a single mRNA can be exported by different pathways depending on the cellular conditions, in this case, before or after heat shock (43). In addition, preferential interaction of mRNAs with certain RNA-binding proteins may dictate the fate of certain classes of mRNAs. In fact, it has been shown that different classes of mRNAs preferentially bind specific subsets of RNA-binding proteins (44), which could contribute to differential mRNA export. Thus, the selective nuclear retention of mRNAs encoding antiviral proteins in Nup98 and Rae1 mutant cells further indicate a role for these proteins in regulating innate and adaptive immunity and demonstrate the underlying complexity of the selective export of distinct mRNA species.

We have shown that the mRNA export machinery is regulated by IFN, and induced expression of mRNA export factors can revert the mRNA export inhibition mediated by a different virus, the vesicular stomatitis virus, through the action of its M protein (31, 32). However, the consequences of this export release on viral pathogenesis had not been determined. Here, we showed that proper levels of mRNA nuclear export factors are essential for protection against influenza virus replication and influenza virus-mediated cell death. NS1 is a multifunctional protein that inhibits host gene expression at multiple levels. It inhibits mRNA processing, which involves interactions with polyadenylation factors (CPSF and PABII) (12, 13) and with a putative splicing factor (NS1-BP) (17). The effect of NS1 on polyadenylation contributes to the mRNA export inhibition of host mRNAs and may destabilize certain pool of mRNAs, targeting them to degradation. As we showed here, not all polyadenylated RNAs are degraded, and a considerable number of these RNAs can be detected in the nucleus of cells either transfected with NS1 protein or infected with influenza virus (Figs. 1 and 3). The interactions of NS1 with constituents of the mRNA export machinery shown here demonstrate an additional and direct effect of NS1 on mRNA export and contribute to nuclear

retention of poly(A) RNA. Taken together with previous findings, our results provide further mechanistic insight into the inhibition of mRNA nuclear export by NS1. Furthermore, our infection studies in cells from mice deficient in key constituents of the mRNA export machinery show a role for this pathway in regulating viral replication and nuclear export of mRNAs encoding constituents of antiviral response pathways. In summary, the combined effects of inhibition of cytoplasmic signaling pathways and nuclear inhibition of RNA processing and export targeted by NS1 result in impaired immune responses and enhanced viral virulence. NS1 is a key contributor to the virulence of avian influenza viruses (45), and as avian flu becomes a major threat to public health, our findings further emphasize the importance of NS1 as therapeutic target.

Methods

Plasmid Construction. Wild-type Nup98 and Nup96 were cloned into myc-pAlter-MAX as described in ref. 46. The NS1 cloned into pCAGGS-NS1(+)-SAM was kindly provided by G. Barber (University of Miami School of Medicine). The NS1A protein sequence is: [MDSNTLSSFQVDCFLWHVRKQVADQELGDAPFLDRLRRDQKSLKGRGSLGLNIETATCVGKQIVERILREESDEAFRMTMASALASRYLTDMTIEEMSRDWFMLMPKQKVAGPLCVRMDQAIMDKNIILKANFVIFDRLETLTLLRAFTEEGAI VGEISPLPLPGHTNEDVKNAIGVLIGGLEWNDNTVRVSETLQRFARWSSNENGGPPLTPTQ KRKMAGTIRSEV*].

Full-length NS1, NS1 (amino acids 1–73), and NS1 (amino acids 74–237) were subcloned into the Sall/NotI sites of the pGEX-4T3. Full-length NS1 also was cloned into Sall/NotI sites of the myc-pAlter-MAX. HA-Rae1 was cloned into pcDNA3.1 (26). pEGFC1-NXF1/TAP and pEGFN3-zzp15 were kindly provided by E. Izaurralde (Max Planck Institute, Tübingen, Germany) (47). All vectors were sequenced.

Cell Culture, Transfections, and Infections. 293T cells, HeLa cells, and the mouse embryo fibroblast (MEF) cell lines Rae1^{+/+}+Nup98^{+/+}, Rae1^{+/+}-Nup98^{+/+}, Rae1^{+/+} Nup98^{+/-}, and Rae1^{+/-} Nup98^{+/-} (48) were grown in DMEM supplemented with 10% FCS (GIBCO, Carlsbad, CA)/1% antibiotic-antimycotic (GIBCO). MEFs were grown in 3% oxygen. For transfections, infections, and immunofluorescence studies, cells were grown on coverslips placed on 35-mm dishes. Transfections were performed with Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. MDCK or 293T cells were grown in EMEM (American Type Culture Collection, Manassas, VA) containing 10% FBS in the absence of antibiotics. For infection studies, 0.75 ml of cell culture supernatants containing the A/WS/33 influenza strain (American Type Culture Collection) at the indicated MOI were added over cells cultured in 35-mm dishes. The dishes were rocked every 10 min for 1 h, and 2 ml of infecting media (EMEM; 0.125% BSA/1% Hepes/1 μ g/ml TPCK-treated Trypsin) then was added for the depicted time points.

Immunoprecipitations and Immunoblots. Immunoblot analysis was performed as described in ref. 31. Briefly, cells were lysed in SDS-sample buffer lacking reducing agent and bromophenol blue. Protein concentration was determined by the DC Protein Assay Method (Bio-Rad, Hercules, CA). Reducing agent and bromophenol blue were added to the samples, and immunoblot analysis was performed as above. In all cases, 30 μ g of total protein was added to each lane of an SDS/PAGE (NOVEX; Invitrogen, Carlsbad, CA). Immunoblot analysis was performed with anti-Nup98 (49), anti-E1B-AP5 (Proteintech Group, Chicago, IL), anti-Rae1 (26), and anti-NXF1/TAP antibodies (BD Transduction Laboratories, San Jose, CA), anti-p15/NXT antibodies (Abnova, Taipei City, Taiwan), mAb414 (50), and anti-Influenza A antibodies (virions;

Biodesign International, Saco, ME). For measuring Nup98 half-life, six 100-mm dishes of MDCK cells were pulse-labeled with 3 mCi (1 Ci = 37 GBq) of [³⁵S]methionine (PerkinElmer, Wellesley, MA) for 2 h and chased for the indicated times. Cells were lysed with the following buffer: 50 mM Tris-HCl (pH 8.0)/150 mM NaCl/15 mM MgCl₂/0.5% Nonidet P-40/0.1 mM Na₃VO₄/0.1 M NaF/complete EDTA-free protease inhibitors (Roche, Basel, Switzerland). Lysates were kept on ice for 20 min, sonicated, and then centrifuged at 14,000 \times g for 5 min. Supernatants were incubated with anti-Nup98 antibodies (49). Preimmune serum was used as control. Immunoprecipitates were washed with lysis buffer, and antigens were dissociated with SDS-sample buffer. The immunoprecipitated proteins were separated by SDS/PAGE (NOVEX) followed by autoradiography. Densitometry analysis of the Nup98 bands was performed by using the NIH Image J software. The intensity of the bands at 8 and 24 h decreased by 79.3% and 54%, respectively, in relation to the 0 time point. The half-life of Nup98 was calculated and is \approx 25.5 h.

In Vitro Binding Assays. 293T cells were nontransfected or transfected with pEGFN3-zzp15 where indicated. Endogenous p15 was detected with anti-p15 antibodies described above. Cell lysates were prepared as described above for immunoprecipitation. These lysates were used as the source of endogenous NXF1/TAP, p15/NXT, Rae1, and Nup98 for the binding assays shown in Fig. 2. Binding assays were performed as described in ref. 51. For the RNase treatment, cell lysates were preincubated with RNasin (1,000 units/ml) or RNase A (50 μ g/ml) (Ambion, Austin, TX) for 15 min at 37°C. Bound and unbound fractions were separated by SDS/PAGE and analyzed by immunoblot analysis with antibodies against NXF1, p15, Rae1, or Nup98. Secondary antibodies recognized zz-tagged p15 shown in Fig. 2e.

Immunofluorescence, Oligo(dT) In Situ Hybridization, and Microscopy.

To perform oligo(dT) *in situ* hybridization simultaneously with immunofluorescence, cells were fixed with formaldehyde, permeabilized with Triton X-100, and labeled with primary antibodies in a humidifying chamber as described in ref. 31. The primary antibodies were diluted in PBS containing 0.2% Triton X-100, 1 mM DTT, and 200 units/ml RNasin (Promega, Madison, WI). The anti-Myc 9E10 antibody (Roche) was diluted 1:200, and the anti-Influenza A antibodies (virions; Biodesign International) were diluted 1:100. Cells again were fixed with formaldehyde and washed with PBS. Oligo(dT) *in situ* hybridization then was performed at 42°C overnight in a humidifying chamber with biotinylated oligo(dT) probe. After hybridization, samples were washed with 2 \times SSC (1 \times SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7) and 0.5 \times SSC at 42°C. Cells then were fixed with formaldehyde, washed in PBS, and incubated with FITC-conjugated donkey anti-mouse antibodies (1:150; Jackson ImmunoResearch, West Grove, PA) or FITC-conjugated donkey anti-goat antibodies, and CY5-streptavidin (Sigma, St. Louis, MO) for 30 min at room temperature. Cells were washed again in PBS and mounted on glass slides by using the ProLong Antifade Kit (Molecular Probes, Invitrogen) with DAPI staining. Samples were examined on a Zeiss Axiovert 200 M with Deconvolution and Apotome Systems.

Reporter Gene Assays. For the experiments performed with NS1, 293T cells grown on 30-mm six-well dishes were cotransfected with plasmids encoding the luciferase reporter (2 μ g), β -galactosidase (2 μ g), and different combinations of plasmids encoding NS1, NXF1, p15, Rae1, and Nup98, as indicated in the figure legend, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. β -Galactosidase was used to determine transfection efficiency for sample normalization. After 18 h of transfection, cells

were lysed, and luciferase and β -galactosidase activities were measured as described in ref. 31.

Survival Assays and Measurement of Viral Replication. Wild-type MEFs and $Rae1^{+/-}Nup98^{+/+}$, $Rae1^{+/+}Nup98^{+/-}$, and $Rae1^{+/-}Nup98^{+/-}$ MEFs were infected with A/WS/33 influenza virus at MOIs of 0, 0.1, and 1 for 6, 14, and 24 h, and cell viability was followed by bright-field microscopy, DAPI, and exclusion of 2 mM ethidium homodimer-1 (Molecular Probes) visualized on five randomly picked $\times 100$ microscopic fields. Four hundred images, for each condition, were processed by using the NIH Image J software. Viral replication was measured by hemagglutination assays. Chicken red blood cells (0.5%) were incubated for 30 min with dilutions of supernatants from cells infected with A/WS/33 influenza virus at the indicated MOIs, and hemagglutination was scored against standardized viral concentrations. Results are the mean \pm SD.

Nuclear and Cytoplasmic mRNA Quantification by Real-Time PCR.

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Nuclear and cytoplasmic RNA was fractionated from wild-type and mutant cells, as described in ref. 38. In brief, fibroblasts were lysed in RSB containing 1.0% Nonidet P-40, 10% glycerol, and 100 units/ml rRNasin (Promega). Nuclei were further washed with 1% Tween 40 and 0.5% sodium deoxycholate, and RNA from both cytoplasmic and nuclear fractions was purified by using TRIzol (Invitrogen). One microgram of total RNA was used for cDNA synthesis by using oligo(dT)₁₈ primer, followed by real-time RT-PCR quantification. The resulting relative mRNA levels were normalized by the geometric mean of five housekeeping genes (α -tubulin, β -actin, GAPDH, L32, and RPS-11).

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