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Heterogeneous Nuclear Ribonucleoprotein K Supports Vesicular Stomatitis Virus Replication by Regulating Cell Survival and Cellular Gene Expression

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Heterogeneous Nuclear Ribonucleoprotein K Supports Vesicular Stomatitis Virus Replication by Regulating Cell Survival and Cellular Gene Expression

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The heterogeneous nuclear ribonucleoprotein K (hnRNP K) is a member of the family of hnRNPs and was recently shown in a genome-wide small interfering RNA (siRNA) screen to support vesicular stomatitis virus (VSV) growth. To decipher the role of hnRNP K in VSV infection, we conducted studies which suggest that the protein is required for VSV spreading. Virus binding to cells, entry, and nucleocapsid uncoating steps were not adversely affected in the absence of hnRNP K, whereas viral genome transcription and replication were reduced slightly. These results indicate that hnRNP K is likely involved in virus assembly and/or release from infected cells. Further studies showed that hnRNP K suppresses apoptosis of virus-infected cells, resulting in increased cell survival during VSV infection. The increased survival of the infected cells was found to be due to the suppression of proapoptotic proteins such as Bcl-X_S and Bik in a cell-type-dependent manner. Additionally, depletion of hnRNP K resulted in not only significantly increased levels of T-cell-restricted intracellular antigen 1 (TIA1) but also switching of the expression of the two isoforms of the protein (TIA1a and TIA1b), both of which inhibited VSV replication. hnRNP K was also found to support expression of several cellular proteins known to be required for VSV infection. Overall, our studies demonstrate hnRNP K to be a multifunctional protein that supports VSV infection via its role(s) in suppressing apoptosis of infected cells, inhibiting the expression of antiviral proteins, and maintaining the expression of proteins required for the virus.

The K homology (KH) domain-containing subfamily of heterogeneous nuclear ribonucleoproteins (hnRNPs) has five members, namely, the hnRNP K (the prototypic member of the subfamily), the poly(C) binding protein 1 (PCBP1, also known as hnRNP E1), PCBP2 (hnRNP E2), PCBP3, and PCBP4. All members of this subfamily carry three KH domains, which are responsible for binding to C- or U-rich regions in RNA and/or DNA (1). These proteins, and in particular, hnRNP K, are known to participate in various cellular processes such as chromatin organization, mRNA translation, regulation of transcription and splicing, RNA shuttling, mRNA and/or protein stability, and cell survival (2, 3). hnRNP K interacts with numerous cellular partners, including oncogenic proteins such as tyrosine kinases (Lck and c-Src) (4) and serine/threonine kinases (extracellular signal-regulated kinase/mitogen-activated protein kinase [ERK/MAPK]) (5), and plays critical roles in cell growth, tissue development and differentiation including red blood cell differentiation (6), ovary development (7), and neuronal development (8). The observation that hnRNP K is highly expressed in multiple cancerous tissues (9–12) suggests its possible roles in cancer development and tumorigenesis. On the other hand, its sequestration, deficiency, or degradation marks the initial step for apoptotic progression (13–15).

hnRNP K has also been demonstrated to play key roles in many viral infections. While interacting with the 5′ untranslated region (UTR), it supports replication of enterovirus 71 (16, 17); its interaction with the hepatitis B virus (HBV) genome leads to increased viral DNA synthesis (18, 19). Dengue virus and herpes simplex virus 1 (HSV-1) also have been shown to require the functions of hnRNP K in progeny virus production (20, 21). hnRNP K not only serves as a splicing factor for Tat/Rev exon 3 of HIV-1 (22) but also interacts with viral components of Sindbis virus, chikungunya virus, hepatitis C virus, African swine fever virus, human cytomegalovirus (CMV), and Epstein-Barr virus (23–28) to support virus growth.

Vesicular stomatitis virus (VSV) is an enveloped, nonsegmented, negative-stranded RNA virus in the *Rhabdoviridae* family and replicates exclusively in the cytoplasm of infected cells. Recently, we demonstrated that PCBP2 and PCBP1 (PCBP1/2), two members of the KH-domain-containing subfamily of hnRNPs, inhibit VSV growth by negatively regulating viral gene expression (29). Although the mechanism by which the PCBPs inhibit viral gene expression and virus growth is unknown at this time, further studies have revealed that the infected cells induce formation of stress granule (SG)-like structures that contain not only PCBP2 but also other cellular RNA-binding proteins such as the T-cell-restricted intracellular antigen 1 (TIA1) and TIA1-related (TIAR) proteins, which have been shown to inhibit VSV replication (30).

hnRNP K resides predominantly in the nucleus (31); however, studies have shown that in VSV-infected cells, it is translocated into the cytoplasm (32). The reason(s) for this altered subcellular localization in infected cells is unclear, but it is possible that hnRNP K might be directly or indirectly involved in VSV replication and growth. This contention is further strengthened by the identification of hnRNP K as one of the host factors required for VSV infection in a genome-wide small interfering RNA (siRNA) screen (33). Since both PCBP2 and hnRNP K proteins are in the same subfamily with similar domain organizations and functions, it is

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surprising to observe opposite effects of these two proteins on VSV infection. In this communication, we conducted studies to examine the role of hnRNP K in supporting VSV infection. Our studies show that hnRNP K supports virus growth and infection by enhancing the cell viability of infected cells. Further studies reveal that the enhanced cell viability is due to increased levels of several antiapoptotic factors such as Bcl-X_L, Bcl-2, and Bag1 and/or decreased levels of proapoptotic factors such as Bcl-X_S and Bik in a cell-type-dependent manner. In addition, our studies reveal that hnRNP K modulates expression of certain cellular proteins that have been found to be required for VSV replication, while also downregulating expression of certain cellular proteins previously shown to inhibit virus replication. The key findings reported here suggest that hnRNP K supports VSV growth by prolonging survival of the virus-infected cells and modulating expression of cellular factors influencing virus growth.

MATERIALS AND METHODS

Cell culture and reagents. Monolayer cultures of HeLa (ATCC CCL2), HEK293, Huh7, and MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal bovine serum (FBS) and the antibiotics penicillin (100 units/ml), kanamycin (20 units/ml), and streptomycin (20 units/ml) (PKS). Baby hamster kidney (BHK-21) cells were maintained in minimal essential medium (MEM) supplemented with 5% fetal bovine serum (FBS) and the antibiotics (PKS). Immortalized murine embryonic fibroblasts (MEFs) from wild-type (wt) and TIA1-knockout mice (34) were obtained from P. Anderson (Harvard University) and were maintained as described previously (30).

Viruses and VSV RNP or nucleocapsid (NC) preparation. Stocks of wt VSV, VSV expressing enhanced green fluorescent protein (eGFP) and eGFP fused to the VSV phosphoprotein (VSV-eGFP and VSV-PeGFP, respectively) were prepared as described earlier (35, 36). VSVΔG (VSV lacking the G protein) virus encoding PeGFP and VSV ribonucleocapsids (RNPs) were prepared as previously described (29). VSV-PLuc was constructed by replacing the eGFP coding sequence in the VSV-PeGFP backbone (35) with the coding sequence of the luciferase (Luc) protein of *Renilla reniformis*. First, the coding sequence of *Renilla* luciferase was amplified from plasmid pRL-TK (Promega) by primers containing an NotI site. After digestion with NotI, the fragment was inserted into a VSV-PeGFP plasmid (35), which was digested with NotI to remove the eGFP sequences. This resulted in the insertion of the entire coding sequences of luciferase in frame following amino acid 196 of the P protein. Subsequently, VSV-PLuc was recovered according to a protocol described previously (36).

Virus titration and infection. Virus titers were determined by plaque assay on BHK-21 and HeLa cells. Virus infection in each experiment was performed at a multiplicity of infection (MOI) of 1 or 0.01 PFU per cell, except where indicated otherwise.

Antibodies. Anti-M (23H12) monoclonal antibodies were kindly provided by D. Lyles. Antibodies for actin (mouse monoclonal; sc-47778), PCBP1 (goat polyclonal; sc-16504), PCBP2 (mouse monoclonal; sc-101136), hnRNP K (mouse monoclonal; sc-28380), TIA1 (goat polyclonal; sc-1751), GS28 (rabbit polyclonal; sc-15270), NF90 (rabbit polyclonal; sc-22530), procaspase 3 (mouse monoclonal; sc-166589), alanine deaminase-like (ADAL) (rabbit polyclonal; sc-138086), FLICE-inhibitory protein (FLIP) (mouse monoclonal; sc-5276), Bad (mouse monoclonal; sc-8044), Bak (rabbit polyclonal; sc-832), and Noxa (rabbit polyclonal; sc-30209) were purchased from Santa Cruz Biotechnology, Inc. Anti-hemagglutinin (HA) monoclonal antibody HA-7 (H3663), goat anti-rabbit immunoglobulin (IgG)-horseradish peroxidase (A6154), goat anti-mouse IgG-horseradish peroxidase (A4416), and rabbit anti-goat IgG-horseradish peroxidase (A4174) were obtained from Sigma-Aldrich. Antibody for cleaved caspase 3 (p17) (rabbit polyclonal; 9661) was purchased from Cell Signaling.

Plasmid constructs. The plasmid encoding the full-length TIA1a (variant 2, NM_022173.2) has been described before (30). TIA1b (variant 1, NM_022037.2) and hnRNP K (variant 1, NM_002140.3) were amplified from total RNA of HeLa cells using the specific primers shown in Table 1. The PCR products were digested with KpnI and EcoRI and cloned into pcDHA as described previously (29). These plasmids were named pcDHA-TIA1b and pcDHA-hnRNP K. These constructs express the inserted gene with the HA epitope at the N terminus of the protein. Expression of the proteins can be driven by either a CMV promoter or T7 promoter located upstream of the coding sequences.

siRNA-mediated silencing. For depletion of PCBP1, PCBP2, TIA1, and hnRNP K in HeLa, HEK293, Huh7, MCF-7, or BHK-21 cells, siRNAs (pool of four different siRNAs) targeting PCBP1, PCBP2, TIA1 (catalog numbers M-012243-01-0005, M-012002-01-0005, and L-013042-00-0005, respectively; Dharmacon), and hnRNP K (catalog numbers J-011692-05, -06, -07, and -08, respectively, for duplexes 1, 2, 3, and 4; Dharmacon) were transfected at a final concentration of 10 to 20 nM (except where indicated otherwise), according to the protocol of reverse transfection, with Lipofectamine RNAiMax (Invitrogen) as recommended by the manufacturer. At 24 h posttransfection (hpt), the transfection mix was replaced with DMEM containing 10% FBS and PKS, and the cells were incubated further for 40 to 48 h to allow knockdown of the gene. A nontargeting (NT) siRNA (catalog number 1027281; Qiagen) which does not target any of the known mammalian genes was used as a control siRNA.

Plasmid DNA and RNP (or NC) transfection. Transfection of plasmids was performed using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions. At 4 hpt, the transfection mix was replaced with complete growth medium and incubated for 40 to 44 h before being used for assays. Transfection of viral RNPs was also performed using the same procedure as used for plasmid transfection and as described previously (29).

WB. Western blotting (WB) and quantification of the protein bands were performed as described before (29). Concentrations of antibodies used were as follows: M, 1:1,000 to 1:3,000; actin, 1:3,000 to 1:5,000; procaspase 3 and PCBP2, 1:3,000; hnRNP K, 1:15,000; HA, 1:10,000; PCBP1, TIA1, FLIP, Bad, Bak, and Noxa, 1:250 to 1:500 each; GS28, NF90, ADAL, and cleaved caspase 3 (p17), 1:1,000 each. Secondary antibodies including goat anti-rabbit immunoglobulin (IgG)-horseradish peroxidase, goat anti-mouse IgG-horseradish peroxidase, and rabbit anti-goat IgG-horseradish peroxidase were used at dilutions of 1:500 to 1:10,000.

qRT-PCR. The primers, probes, and the methods used for quantification of VSV P mRNA and antigenome RNA by quantitative real-time PCR (qRT-PCR) were as described previously (33).

Semiquantitative RT-PCR. Total RNA from cells was extracted with TRIzol (Invitrogen). First-strand cDNA was synthesized using total RNA and oligo(dT) primer (Roche), followed by PCR amplification with specific primers (Table 1) to determine the mRNA levels of anti- and proapoptotic proteins. For an internal control, oligo(dT)-derived cDNA was used to amplify ribosomal protein L32 (L32) mRNA using specific primers as described before (37). PCR cycling conditions used were 20 s at 94°C, 30 s at 54°C, and 30 s at 72°C for 28 to 40 cycles.

Cell viability assay and VSV entry assay based on luciferase activity. A total of 2×10^4 HeLa cells/well were reverse transfected with 10 nM NT or hnRNP K siRNA in opaque-walled, round-bottom, black 96-well plates using Lipofectamine RNAiMax (Invitrogen). For cell viability assays, at 60 hpt, cells were washed with 1× phosphate-buffered saline (PBS) and then mock infected or infected with VSV-eGFP at an MOI of 0.1 in a volume of 40 μl/well. After 1 h of incubation at 37°C, 60 μl of complete medium was added to each well, and the incubation continued for time points indicated in the figures. Cell viability was determined by directly adding 100 μl of CellTiter-Glo Reagent (Promega) to each well as per the manufacturer's instructions. Luciferase activity was recorded in a Veritas Microplate Luminometer (Turner Biosystems). For a VSV entry assay based on luciferase activity, at 60 hpt, the cells were infected with VSV-PLuc at an MOI of

TABLE 1 Primers used in this study

Primer name	Primer sequence (5′ to 3′)[a]	Purpose
TIA1-KpnI-F	ATATGGTACCATGGAGGACGAGATGCCCAAG	Amplification and cloning of TIA1a and TIA1b
TIA1-EcoRI-R	ATATGAATTCTCACTGGGTTTCATACCCTGCC	
TIA1-KpnI-F	ATATGGTACCATGGAGGACGAGATGCCCAAG	Differentiation of TIA1a from TIA1b
TIA1-spliced-R	GATCACCAACAAAGACATGGAAATG	
hnRNPK-KpnI-F	ATATGGTACCATGGAAACTGAACAGCCAG	Amplification and cloning of hnRNP K
hnRNPK-EcoRI-R	ATATGAATTCTTAGAAAAACTTTCCAGAATACTG	
BclX-F	CATATCAGAGCTTTGAACAG	Differentiation of Bcl-X_L from Bcl-X_S
BclX-R	TCATTTCCGACTGAAGAGTG	
Bax-F	CAGCTCTGAGCAGATCATG	Amplification of Bax
Bax-R	CACAGGGCCTTGAGCAC	
Bik-F	TCTCCAGAGACATCTTGATG	Amplification of Bik
Bik-R	GTTCGCAGGACACCCAG	
Bid-F	TGGACCGTAGCATCCCTC	Amplification of Bid
Bid-R	CAGTCCATCCCATTTCTGG	
Mcl-1-F	CGGGAGGGCGACTTTTG	Amplification of Mcl-1
Mcl-1-R	GAACTCCACAAACCCATCC	
Bcl2-A1-F	CAGTGCGTCCTACAGATAC	Amplification of Bcl2-A1
Bcl2-A1-R	GCCTTATCCATTCTCCTGTG	
Bag1-F	CAGCAATGAGAAGCACGAC	Amplification of Bag1
Bag1-R	TGCAGAGAGCTTCAGCTTG	
Bcl-2-F	CGATAACCGGGAGATAGTG	Amplification of Bcl-2
Bcl-2-R	CACCGTGGCAAAGCGTC	
Bcl-W-F	CTGGTGGCAGACTTTGTAG	Amplification of Bcl-W
Bcl-W-R	CATCCACTCCTGCACTTG	
XIAP-F	TGGCACGAGCAGGGTTTC	Amplification of XIAP
XIAP-R	CTCGGGTATATGGTGTCTG	

[a] Underlined sequences indicate restriction sites.

300 for 1 h on ice in a volume of 40 μl/well, washed, and incubated at 37°C. At the time points indicated in the figures, luciferase assays were performed by using a dual-luciferase assay kit (Promega).

Statistical analysis. Statistical analyses were performed using the MIXED procedure of SAS (SAS Institute, Inc., Cary, NC). The statistical model included "treatment" as a fixed effect. Data are presented as means ± standard errors of means. A P value of <0.05 was considered statistically significant.

RESULTS

hnRNP K is required for VSV infection. In a previous high-throughput siRNA screening assay (33), we identified hnRNP K as a cellular factor required for VSV infection. To understand further the role of hnRNP K in VSV infection, we examined how depletion of hnRNP K affects virus growth in cultured HeLa cells. Treating the cells with a pool of siRNAs specific for hnRNP K resulted in a greater than 90% reduction in the levels of hnRNP K compared to those seen in cells treated with a nontargeting (NT) siRNA or in mock-treated cells (Fig. 1A). Infection of hnRNP K-depleted cells with VSV resulted in a 5- to 6-fold reduction in viral protein expression, as examined by monitoring the levels of the viral M protein, compared to those in NT siRNA-transfected cells (Fig. 1A and B). Evaluation of viral growth kinetics revealed that the virus growth was significantly attenuated in cells depleted of hnRNP K at all time points examined. At a low multiplicity of infection (MOI of 0.01), the virus yield at 24 h postinfection (hpi) was reduced over 15-fold in hnRNP K-depleted cells compared to the yield from control NT siRNA-treated cells (Fig. 1C). A similar difference in growth kinetics and virus yield was also observed with the use of a higher MOI (1.0), and the overall virus yield was diminished by about 5-fold (data not shown). The requirement of hnRNP K for VSV infection was also observed in MCF-7, Huh-7, and HEK293 (data not shown) cells as well as in MEFs (see Fig. 4). While depletion of hnRNP K resulted in significant inhibition of VSV growth, ectopic expression of HA-tagged hnRNP K in cells yielded reproducibly a 2-fold increase in viral protein expression, as indicated by the M protein levels (Fig. 1D), and a similar 2-fold increase in overall virus growth was observed (data not shown). Taken together, the results from depletion and overexpression studies suggest that hnRNP K is required for VSV growth and infection.

The requirement for hnRNP K is primarily at the level of virus propagation. To determine the step(s) at which hnRNP K is required for VSV infection, we next examined the relative levels of viral mRNA and full-length antigenomic RNAs synthesized in infected cells depleted of hnRNP K. In VSV infection at a low MOI (0.01), we observed that the levels of M mRNAs and antigenomic RNAs in hnRNP K-depleted cells were significantly decreased, representing approximately 20% of those in NT siRNA-treated cells (Fig. 2A). This was consistently seen in infected cells at early (6 h) (data not shown) and late times (12 h) (Fig. 2A) postinfection. However, at a high MOI (1.0), the reduction in viral mRNA and antigenomic RNA levels in hnRNP K-depleted cells was less pronounced. The levels of these RNAs were about 70% of those seen in NT siRNA-treated cells (Fig. 2B). The observations that viral RNA synthesis was less affected by hnRNP K depletion at a high MOI (a condition in which most of the cells in the culture were infected) but that it was significantly reduced at a low MOI (a condition in which majority of the cells in the culture were uninfected at early times of infection but could potentially be infected with recently produced virions) suggest that although hnRNP K

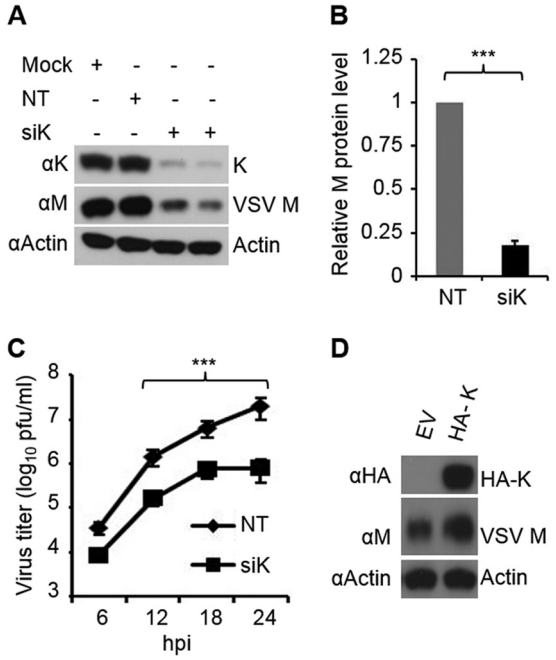

FIG 1 hnRNP K is required for VSV infection. (A) Suppression of VSV replication in cells depleted of hnRNP K. HeLa cells were transfected with 10 nM siRNA for NT (lane 2) or 10 nM and 20 nM hnRNP K (siK) (lanes 3 and 4, respectively) for 60 h. Cells were then infected with VSV at an MOI of 0.01 for 12 h. Equal amounts of cell lysates were analyzed by WB with anti-hnRNP K and anti-M antibodies. Actin served as the loading control. (B) Quantification of M protein levels (arbitrary units) from three independent experiments as described in panel A with error bars representing the standard errors of means. The level of M expression in the NT siRNA-treated sample was set at 1. ***, $P < 0.001$. (C) Multicycle growth of VSV in cells depleted of hnRNP K. siRNA (10 nM) treatment and virus infections were performed as described in panel A; virus titers in supernatants collected at indicated time points were determined by plaque assay and are expressed as $\log_{10}$ PFU/ml. Statistical significance was determined for virus titers at 12, 18, and 24 hpi. ***, $P < 0.001$. (D) VSV infection is enhanced in hnRNP K-overexpressing cells. HeLa cells transfected with 1.5 μg of empty vector (EV) or an HA-hnRNP K (HA-K)-encoding plasmid for 48 h were infected with VSV at an MOI of 0.01 for 12 h, and cell lysates were analyzed by WB using anti-M or anti-HA antibodies. Actin served as the loading control. α, anti; siK, siRNA targeting hnRNP K.

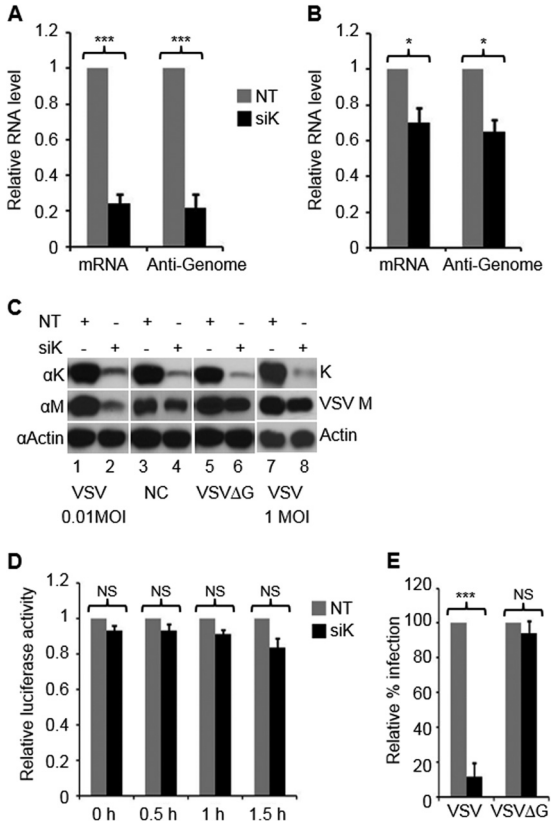

FIG 2 hnRNP K is required for VSV propagation. (A and B) VSV mRNA and antigenomic RNA levels in cells depleted of hnRNP K and infected with the virus at a low (0.01) or high (1.0) MOI (panels A and B, respectively) were determined by qRT-PCR. Experimental conditions were as described in the legend of Fig. 1A. Error bars represent the standard errors of means from three independent experiments. ***, $P < 0.001$; *, $P < 0.05$. (C) hnRNP K is not required for early stages of the VSV life cycle. HeLa cells were transfected with 10 nM siRNA for NT or targeting hnRNP K for 60 h. Cells were then treated as follows: infected with VSV at an MOI of 0.01 for 12 h (lanes 1 and 2), supertransfected for 6 h with viral NC prepared from VSV (lanes 3 and 4), infected with VSVΔG at an MOI of 0.5 for 8 h (lanes 5 and 6), or infected with VSV at an MOI of 1 for 4 h (lane 7 and 8). Cell lysates corresponding to equal amounts of total proteins were analyzed by WB with anti-hnRNP K and anti-M antibodies. Actin served as the loading control. (D) HeLa cells were transfected with siRNA as described in the legend of Fig. 1A. At 60 hpt, cells were infected with VSV-PLuc at an MOI of 300. After 1 h of virus adsorption on ice, cells were washed with PBS, and cell extracts at various times postincubation were analyzed for luciferase activity. The luciferase activity in NT siRNA-treated cells at various times postinfection was set at 1. Data show relative luciferase activity and are expressed as the averages of three independent experiments, with error bars representing the standard errors of means. NS, nonsignificant. (E) HeLa cells were transfected with siRNA as described in the legend of Fig. 1A. At 60 hpt, cells were infected with VSV-PeGFP at an MOI of 0.05 or VSVΔG at an MOI of 0.5 for 12 h. The percentages of infected cells were determined by counting the number of cells expressing green fluorescence from PeGFP. Data show relative percent infection after normalizing the value of cells treated with siRNA targeting hnRNP K to that of NT siRNA-treated cells, which is set at 100, and are expressed as average of three independent experiments with error bars representing the standards error of means. ***, $P < 0.001$; NS, nonsignificant.

may play a role in viral gene expression to some extent, it likely plays a more prominent role(s) at another step (or other steps) in the virus replication cycle.

To further elucidate the role of hnRNP K in the VSV life cycle, we investigated the requirement of hnRNP K at VSV entry/uncoating and assembly/budding steps. Transfection of VSV nucleocapsids (NCs) directly into cells bypasses the viral entry and uncoating steps, resulting in expression of the viral genes (29, 33). Thus, we examined the effect of depletion of hnRNP K on viral gene expression in cells transfected with the viral NCs. Results show that NC transfection led to only a slight decrease (approximately 30%) in the levels of the viral M protein in cells treated with siRNA for hnRNP K compared to levels in NT siRNA-transfected cells (Fig. 2C, lanes 3 and 4). In contrast, an 8- to 10-fold reduction in M protein levels was observed in cells infected at a low MOI (0.01) with VSV (Fig. 2C, lanes 1 and 2). Infection of cells with VSVΔG virus (a virus that lacks the G gene and cannot produce infectious particles, thereby limiting the infection with this virus, which is incompetent in budding, to a single cycle only) also led to a slight decrease (approximately 30%) in the levels of the viral M protein in hnRNP K siRNA-treated cells compared to the control NT siRNA-treated cells (Fig. 2C, lanes 5 and 6). Furthermore, using a high MOI (1.0) of VSV, we observed viral gene expression at 4 hpi (a time at which viral gene expression is readily detectable

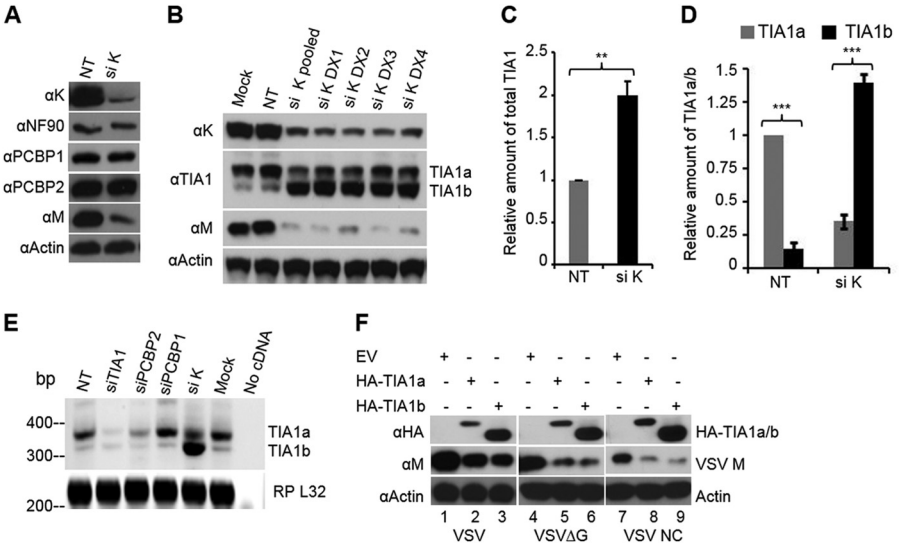

FIG 3 hnRNP K regulates the expression of the two isoforms of TIA1 but not PCBP1/2. (A) Depletion of hnRNP K does not affect the expression of PCBP1/2. HeLa cells were transfected with NT or hnRNP K siRNA at a concentration of 10 nM, and at 60 hpt, cells were infected with VSV at an MOI of 0.01 for 12 h. Cell lysates were used for WB to detect the indicated proteins. (B to D) hnRNP K depletion results in increased levels of total TIA1 and the TIA1b isoform. HeLa cells were mock transfected or transfected with NT or hnRNP K siRNA as a pool of four duplexes (pooled) or as individual duplexes (si DX1 to si DX4) at a concentration of 10 nM, and at 60 hpt, the cells were infected with VSV at an MOI of 0.01 for 12 h. Cell lysates were used for WB to detect the indicated proteins (B). Quantification of total TIA1 protein levels (arbitrary units) from three independent experiments is shown in panel C while the levels of TIA1a and TIA1b isoforms are shown in panel D. The level of TIA1a expression in the NT siRNA-treated sample was set at 1. ***, $P < 0.001$; **, $P < 0.01$. (E) Switching of TIA1 isoform expression is regulated by hnRNP K protein at the mRNA level. The experiment was done as described in panel B. Total RNA was subjected to semiquantitative RT-PCR to detect the two isoforms of TIA1. Ribosomal protein (RP) L32 mRNA served as an internal control. (F) Overexpression of TIA1a and TIA1b inhibits VSV gene expression. HeLa cells transfected with 1.5 μg of empty vector (EV) or HA-TIA1a- or HA-TIA1b-encoding plasmids for 48 h were infected with VSV at an MOI of 0.1 for 12 h (lanes 1 to 3) or with VSVΔG at an MOI of 0.5 for 8 h (lanes 4 to 6) or supertransfected for 6 h with viral NC prepared from VSV (lanes 7 to 9), and cell lysates were analyzed by WB using anti-M or anti-HA antibodies. Actin served as the loading control. siPCBP1, siRNA targeting PCBP1; siPCBP2, siRNA targeting PCBP2; siTIA1, siRNA targeting TIA1.

but virus assembly/budding is minimal) to be reduced by only about 30% in cells treated with an siRNA for hnRNP K (Fig. 2C, lanes 7 and 8) compared to that in NT siRNA-transfected cells. These results show that bypassing entry/uncoating steps (NC transfection), using assembly/budding-incompetent (VSVΔG) virus, or using high MOIs did not adversely affect overall viral gene expression. On the other hand, a significant reduction in viral gene expression under conditions of VSV infection at a low MOI suggests that the supportive roles of hnRNP K in VSV infection are manifested when the infection undergoes multiple cycles, indicating that this cellular protein may be involved in virus entry/uncoating or assembly/budding steps.

To determine if virus entry and uncoating steps are affected by depletion of hnRNP K, we used a recombinant virus (VSV-PLuc) which encodes a P-luciferase (P-Luc) fusion protein, in which the entire coding region of luciferase is fused in frame following amino acid 196 of the P protein. The VSV-PLuc virus is similar to VSV-PeGFP described previously (35) and incorporates P-Luc within virus particles. Luciferase activity can be readily detected in extracellular virions and in infected cells (data not shown). Cells transfected with NT or hnRNP K siRNA were infected with VSV-PLuc at an MOI of 300 to allow readily detectable levels of luciferase. Following virus adsorption on ice, cells were washed, and luciferase activity was determined in extracts of cells at 0, 0.5, 1, and 1.5 h postincubation at 37°C to enable virus entry and uncoating. Results (Fig. 2D) show that luciferase activity in these cells was not significantly affected in the presence or absence of hnRNP K, indicating that this protein does not play any discernible role in virus entry and uncoating steps. Furthermore, using a low MOI (0.01), a multicycle virus growth condition, we observed that the percentage of cells infected with wt VSV increased with time in the presence of hnRNP K, while in cells depleted of hnRNP K, the relative percentage of infected cells remained low, reaching up to about 15% of that of NT siRNA-treated cells at 16 hpi (Fig. 2E). On the other hand, we found no differences in the percent infection when these cells were infected with the budding-incompetent VSVΔG (Fig. 2E). Taken together, the results suggest that hnRNP K is required for virus egress in infected cells and that its depletion may negatively affect assembly and/or release of virus required for further rounds of infection.

hnRNP K alters the expression of TIA1, a known negative regulator of VSV replication. Since hnRNP K is known to influence expression of many cellular genes, we wondered whether its depletion alters expression of cellular genes that have been shown to be involved in VSV replication. Previous studies from our laboratory showed that PCBP2 and PCBP1 as well as TIA1 inhibit VSV replication (29, 30). Thus, we examined the levels of these proteins in cells depleted of hnRNP K by siRNA transfection. Figure 3A shows that knockdown of hnRNP K had no measurable effect on the levels of PCBP1/2. Expression levels of another cellular protein, NF90, which is known to inhibit replication of several other viruses such as HIV-1 (38), Ebola virus (39), and influenza virus (40), was also not affected by hnRNP K depletion (Fig. 3A). Interestingly, under this condition, we observed a reproducibly greater than 2-fold increase in the total amount of TIA1 protein (Fig. 3B and C). Strikingly, hnRNP K depletion resulted in a

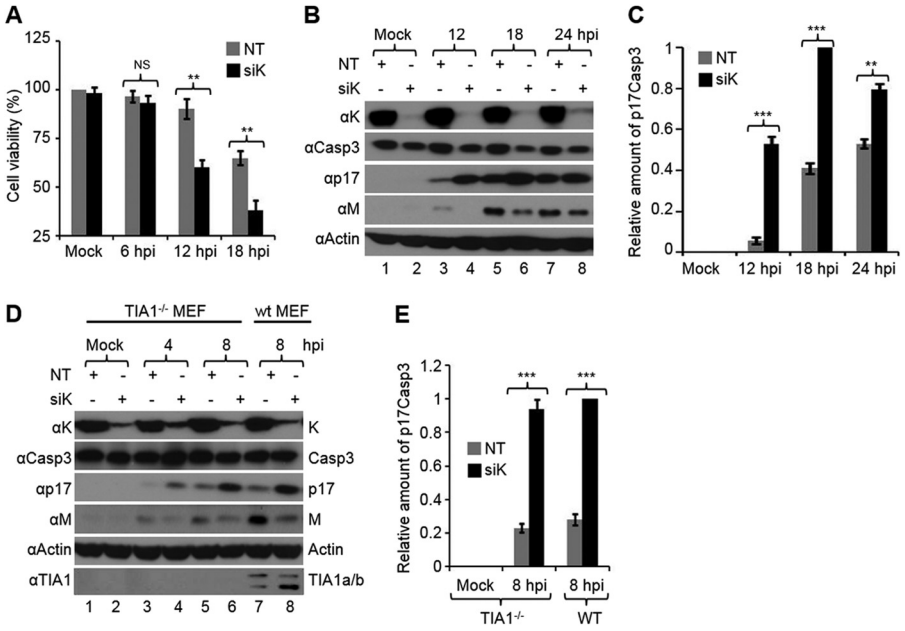

FIG 4 hnRNP K is required for survival of VSV-infected cells. (A) Knockdown of hnRNP K reduces cell survival during VSV infection. HeLa cells were transfected with NT or hnRNP K siRNA at concentration of 10 nM for 60 h. Cells were mock infected or infected with VSV at an MOI of 0.01. At 6, 12, and 18 hpi, cell survival was determined by a CellTiter-Glo Kit. The percentage of cell viability in NT siRNA-transfected and mock-infected culture at the beginning of infection was set at 100%. Data from three independent experiments are shown with error bars representing the standard errors of means. **, $P < 0.01$; NS, nonsignificant. (B) Caspase 3 activation is more pronounced in infected cells lacking hnRNP K. The experiment was conducted as described in panel A. Infected cells were lysed and analyzed at the indicated time points to detect procaspase 3, activated caspase 3 (p17), hnRNP K, and VSV M protein using specific antibodies. Actin served as a loading control. (C) Quantification of p17 protein levels (arbitrary units) from three independent experiments as described in panel B. The level of activated caspase 3 (p17) in cells treated with siRNA targeting hnRNP K at 18 hpi was set arbitrarily at 1, and the relative levels of p17 in other samples were then determined. Error bars represent the standard errors of means from three independent experiments. ***, < 0.001; **, $P < 0.01$. (D) wt MEF or TIA1$^{-/-}$ MEF cells were transfected with NT or hnRNP K siRNA at a concentration of 20 nM for 60 h. Cells were mock infected (mock) or infected with VSV at an MOI of 1. At 4 and 8 hpi, cells were lysed and subjected to WB with the indicated antibodies. (E) Quantification of p17 levels (arbitrary units) from three independent experiments, with error bars representing the standard errors of means. The level of p17 in wt MEFs treated with siRNA targeting hnRNP K was set arbitrarily at 1, and the relative levels of p17 in other samples were then determined. ***, $P < 0.001$.

dramatic shift in the ratio of the two isoforms (TIA1a and TIA1b) of the protein (Fig. 3B and D). In mock-treated or NT siRNA-treated cells, the amount of TIA1b was approximately 12% of TIA1a, whereas in hnRNP K-depleted cells, TIA1b was detected at significantly higher levels (about 3-fold) than TIA1a (Fig. 3B). These results were observed when individual siRNAs specific for hnRNP K or pooled siRNAs were used (Fig. 3B), indicating that the effect of hnRNP K protein on altered expression of TIA1 isoforms was not due to off-target effects. The altered expression of the two isoforms was detected at the mRNA level (Fig. 3E), indicating that hnRNP K regulates the expression of these two isoforms at the transcription and/or splicing level. Similar results were also obtained in HEK293 cells (data not shown) and wt MEFs (see Fig. 4D).

Since depletion of hnRNP K led to increased levels of the TIA1b isoform and inhibition of virus replication, we examined whether TIA1b inhibits VSV replication, as was demonstrated for the TIA1a isoform previously (30). In VSV-infected cells overexpressing HA-tagged TIA1b, the viral M protein levels were reduced (Fig. 3F). The reduction in M protein levels was observed in TIA1b-expressing cells infected with VSV (Fig. 3F, lanes 1 to 3) or VSVΔG (lanes 4 to 6) or in NC-transfected cells (lanes 7 to 9), suggesting that TIA1b inhibits VSV replication. Thus, it appears that hnRNP K depletion results in increased accumulation of TIA1b, which is also responsible for inhibition of VSV replication.

hnRNP K is required for survival of VSV-infected cells. hnRNP K plays essential roles in cell survival. Its cleavage by different granzymes initiates cell death processes, and its depletion primes the cells to undergo programmed cell death under various apoptotic stimuli (9, 13, 41, 42). While performing our studies, we observed that VSV-infected cells depleted of hnRNP K exhibited enhanced cytopathic effects and cell death compared to the infected cells not depleted of the protein. Therefore, we examined if hnRNP K plays any role in cell survival during VSV infection. Depletion of hnRNP K did not affect the viability of mock-infected cells significantly; however, the viability of VSV-infected cells was significantly reduced with time postinfection (Fig. 4A). The results show that viability of VSV-infected cells depleted of hnRNP K was reduced to approximately 63% and 25% at 12 and 18 hpi, respectively, whereas the viability of NT siRNA-treated cells infected with VSV was reduced only to 92% and 60%, respectively, at the corresponding times postinfection (Fig. 4A). Consistent with the increase in cell death, the amount of cleaved caspase 3 (p17) was found to be 7-fold higher in cells lacking hnRNP K at 12 hpi (Fig. 4B and C).

Since TIA1 is a proapoptotic protein (43, 44), we wondered if the upregulation of TIA1 in hnRNP-depleted cells is responsible for the enhanced apoptosis observed in these cells infected with VSV. To this end, we examined the apoptosis occurring in wt and TIA1$^{-/-}$ MEFs (34) when hnRNP K is silenced by siRNA and

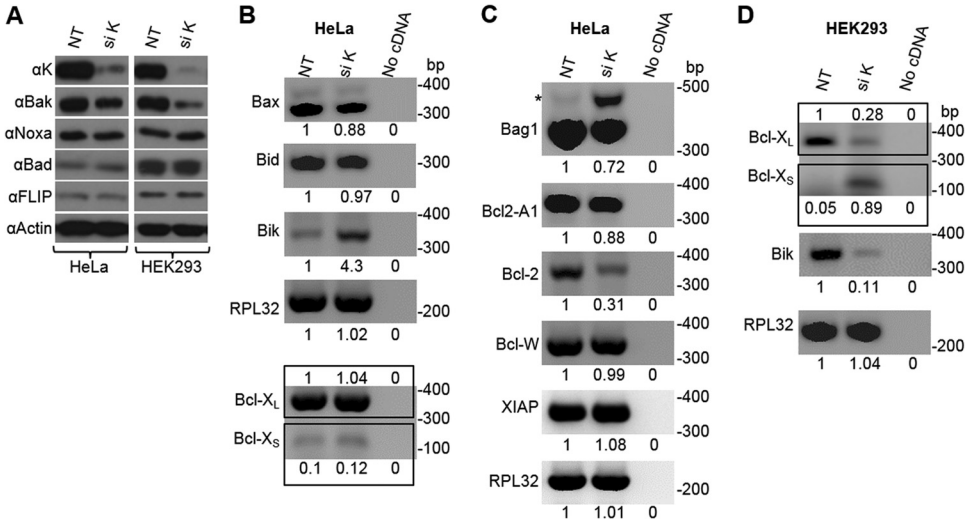

FIG 5 VSV-mediated cell death in hnRNP K-depleted cells is associated with increased levels of proapoptotic proteins Bcl-X_S and Bik. (A) HeLa or HEK293 cells transfected with siRNAs for 60 h were infected with VSV at an MOI of 1 for 6 h. The levels of proapoptotic proteins Bak, Bad, Noxa, and antiapoptotic factor FLIP were detected by WB using specific antibodies. (B and C) mRNA levels of proapoptotic proteins (B) or antiapoptotic proteins (C) in hnRNP K-depleted HeLa cells infected with VSV. siRNA treatment and virus infections were performed as described in panel A. Total RNA was converted to cDNA using oligo(dT) primers followed by PCR with specific primers. The mRNA level of cellular ribosomal protein L32 was used as an internal control. A DNA ladder (bp) is shown on the right. Values at the top or bottom of the lanes represent relative levels of mRNAs, using levels in the NT lane as 1. The Bcl-X_S level is relative to the Bcl-X_L level in the NT lane. The asterisk indicates the DNA product of an undocumented isoform of Bag1 or a nonspecific product. (D) mRNA levels of antiapoptotic or proapoptotic proteins in hnRNP K-depleted HEK293 cells infected with VSV. siRNA treatment, virus infection, and mRNA detection were as described above.

subsequently infected with VSV. Results (Fig. 4D and E) show that activation of caspase 3 (as measured by the levels of p17) occurred to similar extents in VSV-infected wt and TIA1$^{-/-}$ MEFs depleted of hnRNP K, indicating that VSV-induced cell death in hnRNP K-depleted cells is independent of TIA1.

VSV-mediated cell death in hnRNP K-depleted cells is associated with increased levels of proapoptotic proteins and decreased levels of antiapoptotic proteins. VSV infection upregulates the expression of proapoptotic proteins such as Noxa and Bak that are involved in virus-mediated apoptosis in cell culture (45–47). On the other hand, hnRNP K is known to support the expression of antiapoptotic proteins such as the caspase inhibitor protein XIAP (X-linked inhibitor of apoptosis protein) (12), FLICE-inhibitory protein (FLIP) (9), and Bcl-X_L but suppresses the expression of proapoptotic Bcl-X_S (42). Therefore, we examined if depletion of hnRNP K affects the expression of anti- and/or proapoptotic proteins that may result in altered survival of VSV-infected cells. Our results show that the levels of Noxa, Bad, and FLIP were unchanged while the expression of Bak decreased in both HeLa and HEK293 cells depleted of hnRNP K and infected with VSV (Fig. 5A). Through semiquantitative RT-PCR, we found that the mRNA levels of proapoptotic factors Bax and Bid were not altered significantly in the absence of hnRNP K, whereas the level of another proapoptotic factor, Bik, was upregulated over 4-fold (Fig. 5B). The mRNA levels of antiapoptotic factors Bag1 and Bcl-2A1 but not Bcl-W or XIAP under similar experimental conditions were slightly reduced, whereas the level of Bcl-2 was downregulated greater than 3-fold (Fig. 5C).

A previous study reported that depletion of hnRNP K by siRNA treatment led to a significant decrease in the level of mRNA for the antiapoptotic protein Bcl-X_L and an increase in the proapoptotic Bcl-X_S mRNAs in PC-3 cells as well as several other cell lines, but no significant differences were observed in HeLa cells (42), which was suggested to be due to less efficient depletion of the protein in this cell line. Therefore, it was of interest to examine the mRNA levels of Bcl-X_L and Bcl-X_S in HeLa cells depleted of greater than 90% of hnRNP K using the pool of four siRNAs described in our studies above (Fig. 1A). Results from such an experiment show (Fig. 5B) that the mRNA levels of Bcl-X_L and Bcl-X_S remained unchanged in VSV-infected HeLa cells depleted of hnRNP K compared to levels in the infected cells not depleted of the protein. In contrast to the results in HeLa cells, we observed a significant decrease of Bcl-X_L and a concomitant increase in Bcl-X_S mRNA levels in HEK293 cells under similar experimental conditions (Fig. 5D). Interestingly, the level of Bik mRNA was also reduced significantly (Fig. 5D). These results indicate that the VSV-mediated apoptosis in hnRNP K-depleted cells is associated with alterations in the balance of pro-/antiapoptotic Bcl-2 proteins (decrease of antiapoptotic proteins such as Bcl-X_L, Bag1, and Bcl-2 and an increase of proapoptotic factors including Bcl-X_S and Bik) though the identity of these proteins might differ in different cell lines.

hnRNP K maintains expression of cellular proteins required for VSV infection. Since hnRNP K is involved in regulating expression of many cellular genes (1), it is possible that depletion of hnRNP K might influence levels of cellular proteins that are known to be required for VSV replication, leading to inhibition of virus replication. Recently, we reported through a genome-wide siRNA screen the requirement of several host factors for VSV replication at various stages of the virus infection cycle (33). Among these, alanine deaminase-like (ADAL) protein was shown to be required for the VSV assembly/budding step, whereas GBF1, ARF1, and the proteins of the coatomer I (COPI) complex were required for VSV genome transcription and replication (33, 48). Therefore, we examined if hnRNP K silencing affects the expression of some of these cellular factors. Results show that hnRNP K

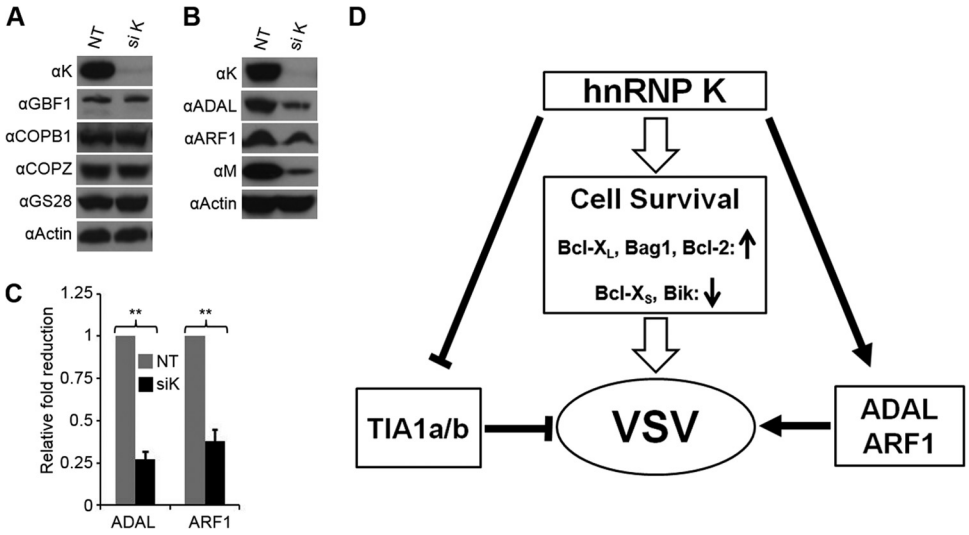

FIG 6 hnRNP K regulates expression of cellular proteins required for VSV infection. (A and B) HeLa cells transfected with siRNA for 60 h were infected with VSV at an MOI of 0.01 for 12 h. The levels of the proteins were detected by WB using specific antibodies. (C) Quantification of ADAL and ARF1 protein levels (arbitrary units) from three independent experiments, with error bars showing the standard errors of means. The level of ADAL or ARF1 in the NT lane was arbitrarily set at 1. **, $P < 0.01$. (D) Involvement of hnRNP K in regulating expression of cellular proteins that affect VSV infection. hnRNP K inhibits TIA1a/b expression, which is known to inhibit VSV replication. Increased levels of antiapoptotic proteins such as Bcl-X_L, Bag1, and Bcl-2 and/or reduction in the levels of apoptotic proteins Bcl-X_S and Bik promotes cell survival for increased VSV replication, while maintenance of factors such as ADAL and ARF1 is required for VSV replication.

depletion by siRNA did not alter the levels of COPI proteins such as COPB1 and COPZ or GBF1 (Fig. 6A). Furthermore, under the same conditions, the level of GS28, a putative protein of the *cis*-Golgi compartment that is involved in endoplasmic reticulum (ER)-Golgi transport (49) and apoptosis (50, 51), was not affected either (Fig. 6A). On the other hand, depletion of hnRNP K resulted in a 3- to 4-fold reduction in the levels of ARF1 and ADAL proteins (Fig. 6B and C). These results suggest that hnRNP K regulates expression of certain cellular proteins required for VSV replication.

DISCUSSION

hnRNP K is a multifunctional protein in the subfamily of hnRNPs which carry three KH domains and is involved in gene expression and mRNA metabolism. Previously, our genome-wide screening study identified hnRNP K as a factor required for VSV infection (33). In this communication, we conducted further studies to decipher the role of hnRNP K in supporting VSV infection. Our results reveal that hnRNP K primarily supports VSV infection at the level of virus propagation, but it is also involved in viral replication and transcription to some extent (Fig. 1 and 2). Further studies showed that hnRNP K is required for suppressing apoptosis induction and promoting survival of infected cells, which in turn supports VSV growth. We found that hnRNP K helps maintain the low levels of expression of proapoptotic proteins such as BiK and Bcl-X_S and the high levels of expression of antiapoptotic proteins such as Bcl-X_L, Bag1, and Bcl-2. In addition to the role of hnRNP K in cell death suppression, our study found that hnRNP K supports expression of several cellular factors required for VSV replication and downregulates expression of some of the cellular factors known to inhibit VSV replication. Thus, the studies presented here reveal critical roles of hnRNP K in VSV infection through multiple mechanisms. The supportive role of hnRNP K in VSV infection is observed in all cell lines examined in this study, whereas its role in suppression of apoptosis is mediated by a balance between several pro- and antiapoptotic factors and is cell type dependent.

hnRNP K is reported to play diverse roles in many virus infections. Its interactions with viral components and/or cellular factors support the replication of viruses, including enterovirus 71, Sindbis virus, dengue virus, chikungunya virus, hepatitis C virus, HBV, HIV-1, HSV-1, African swine fever virus, human cytomegalovirus, and Epstein-Barr virus (16, 18–28). Interestingly, in human cytomegalovirus-infected cells, hnRNP K is essential for virus replication as it promotes the viability of infected cells (28). Although our studies reveal such a role for hnRNP K in VSV replication, it may also be involved in virus assembly and/or release. So, how then does hnRNP K facilitate VSV assembly and/or release from infected cells? The answer to this critical question requires further investigation, including examination of interactions of viral proteins with hnRNP K. It is possible that hnRNP K may directly or indirectly interact with the viral components to mediate virus assembly or egress. Since hnRNP K is known to be involved in cellular gene expression and signal transduction (3), it is also possible that it modulates expression of certain cellular proteins that are important for VSV egress, such as ADAL (Fig. 6B and C), which was shown before to be required for VSV assembly and budding (33). Alternatively, hnRNP K may recruit factors necessary for virus egress through its K protein-interacting (KI) domain.

Although interaction of VSV proteins with hnRNP K is being examined currently, one required role for hnRNP K in VSV infection is found to be at the level of maintaining the viability of infected cells. VSV-infected cells exhibited significantly reduced cytopathic effects and did not undergo apoptosis as fast in the presence of hnRNP K as in cells lacking this protein. Thus, hnRNP K suppresses VSV-induced apoptosis. Examination of the levels of

several factors involved in apoptosis suggested that increased apoptosis of VSV-infected cells lacking hnRNP K does not involve Noxa and Bak, two proapoptotic proteins previously shown to mediate VSV-induced cell death (45–47). Rather, significantly increased levels of Bik or Bcl-X_S appear to regulate apoptosis in hnRNP K-depleted cells by regulating the balance of the pro-/antiapoptotic Bcl-2 proteins. In HeLa cells, the levels of Bcl-X_S remained unchanged in the presence or absence of hnRNP K, but Bik was upregulated over 4-fold, which presumably was responsible for increased cell death. In contrast, in HEK293 cells, Bcl-X_S levels were increased significantly in the absence of hnRNP K along with decreased levels of Bcl-X_L while the level of Bik was significantly reduced, suggesting that in this cell type, the decrease in the Bcl-X_L/Bcl-X_S ratio may primarily regulate apoptosis of VSV-infected cells. Thus, it appears that hnRNP K regulates the balance between pro- and antiapoptotic factors in a cell-type-dependent manner so as to allow infected cells to survive longer for increased virus growth. It should be noted, however, that considering the multiple roles played by hnRNP K in many cellular processes, it is possible that suppression of apoptosis may not be solely responsible for increased growth of VSV in infected cells. Further studies would have to be conducted to delineate additional roles of hnRNP K, if any, in supporting VSV growth.

hnRNP K is known to function closely with p53, a well-known mediator of apoptosis, at multiple levels to coregulate cell fate. Together with p53, hnRNP K binds to promoters to upregulate a number of genes encoding DNA damage response factors (52), and in combination with large intergenic noncoding RNA p21 (lincRNA-p21), it regulates global gene repression downstream of a p53-mediated response (53). Evidence also suggests that hnRNP K acts as a cell survival enhancer independent of p53 by maintaining a high level of antiapoptotic proteins, including FLIP and XIAP (9, 12). Studies presented here reveal that the role of hnRNP K in survival of VSV-infected cells is independent of its interaction with p53. This interpretation is consistent with the observations that VSV-induced apoptosis in the absence of hnRNP K occurs with similar kinetics in both p53-inactive cells (HeLa and HEK293) and wild-type p53-active cells such as MCF-7 and MEF cells. Additionally, apoptosis induced by other factors such as cycloheximide treatment or other virus infections (lymphocytic choriomeningitis virus [LCMV] and human parainfluenza virus type 3 [HPIV3]) was also enhanced in hnRNP K-depleted cells (data not shown). These observations highlight the essential role of hnRNP K independent of p53 in protecting cells from apoptosis triggered by different stimuli.

VSV is being used as an oncolytic agent for various types of tumors (55–58). One of the hallmark features of most cancer cells is the defective type I interferon response and signaling (59), which allows viruses such as VSV to grow rapidly and kill the cells. hnRNP K is upregulated in many types of cancerous cells (10, 11, 60) and is considered a prognostic marker and a target for tumor therapy (11, 61). The results showing that hnRNP K supports VSV propagation further add to the understanding of why tumors provide an appropriate microenvironment for VSV growth.

Our studies also reveal a novel role of hnRNP K in suppressing not only the expression of the TIA1b isoform (a differentially spliced isoform of TIA1a) but also the overall levels of TIA1. The function of these two isoforms in cells is poorly understood, except that TIA1b displays greater splicing-stimulatory activity than TIA1a (62). Previously, we along with others demonstrated that TIA1 inhibited VSV replication (30, 63) although the contribution of the TIA1b isoform was not investigated. Our present results demonstrate that TIA1b is inhibitory to VSV replication. Since expression of TIA1 is mediated by many cellular factors including miRNAs (64, 65), it would be of interest to examine how hnRNP K regulates or is regulated by these factors, which may provide further understanding of the role of cellular factors involved in VSV replication.

The results presented here do not exclude the involvement of hnRNP K in supporting VSV assembly and budding. Although not much is known about the direct role(s) of hnRNP K in lipid metabolism, in protein trafficking, in the growth of plasma membrane, or in secretory pathways, which are directly involved in virus assembly and budding, hnRNP K regulates the expression of 15-lipoxygenase, a key enzyme in the metabolism of phospholipids and internal membrane (66). Studies of the hnRNP K interactome revealed more than 100 interacting partners and determined that hnRNP K protein is present in the nucleus, cytoplasm, mitochondria, and the vicinity of the plasma membrane (31). Thus, it is possible that hnRNP K exerts its positive impact on VSV growth through one or more of its interacting partners.

In summary, we demonstrate here a role(s) of hnRNP K in VSV infection (Fig. 6D). The mechanisms by which hnRNP K promotes VSV infection involve the following: (i) promoting cell survival via suppression of proapoptotic proteins (Bik and Bcl-X_S) and upregulation of antiapoptotic proteins (Bcl-X_L, Bag1, and Bcl-2); (ii) suppression of expression of VSV-inhibitory protein TIA1; and (iii) upregulation of cellular factors favoring VSV infection such as ARF1 and ADAL. Our data also provide a better understanding of why tumor cells provide a more conducive environment for VSV replication. Overall, our studies presented here suggest a multifunctional role of hnRNP K in VSV infection and prompt us to undertake additional research to uncover its involvement in virus-host interactions.

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