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Changes in Cellular mRNA Stability, Splicing, and Polyadenylation through HuR Protein Sequestration by a Cytoplasmic RNA Virus

Michael D. Barnhart,[1,3] Stephanie L. Moon,[2,3] Alexander W. Emch,[2] Carol J. Wilusz,[1,2] and Jeffrey Wilusz[1,2,*]
[1]Cell and Molecular Biology Program, Colorado State University, Fort Collins, CO 80525, USA
[2]Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO 80525, USA
[3]These authors contributed equally to this work
*Correspondence: jeffrey.wilusz@colostate.edu
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SUMMARY

The impact of RNA viruses on the posttranscriptional regulation of cellular gene expression is unclear. Sindbis virus causes a dramatic relocalization of the cellular HuR protein from the nucleus to the cytoplasm in infected cells. This is to the result of the expression of large amounts of viral RNAs that contain high-affinity HuR binding sites in their 3′ UTRs effectively serving as a sponge for the HuR protein. Sequestration of HuR by Sindbis virus is associated with destabilization of cellular mRNAs that normally bind HuR and rely on it to regulate their expression. Furthermore, significant changes can be observed in nuclear alternative polyadenylation and splicing events on cellular pre-mRNAs as a result of sequestration of HuR protein by the 3′ UTR of transcripts of this cytoplasmic RNA virus. These studies suggest a molecular mechanism of virus-host interaction that probably has a significant impact on virus replication, cytopathology, and pathogenesis.

INTRODUCTION

Posttranscriptional processes such as splicing, polyadenylation, and mRNA decay play a major role in regulating cellular gene expression and shaping the transcriptome and proteome. On average, human genes contain approximately eight introns, and approximately 70% of genes are alternatively spliced. This generates significant diversity in the proteome and can influence regulatory signals present in transcripts (Kornblihtt et al., 2013). Polyadenylation is also a dynamically regulated process with more than 50% of pre-mRNAs containing alternative poly(A) signals that can dramatically alter the composition of regulatory elements in the 3′ UTR as well as influence mRNA coding capacity (Shi, 2012). Finally, in concert with transcription, regulated mRNA stability plays a major role in determining mRNA abundance and quality control of gene expression (Schoenberg and Maquat, 2012). All three of these posttranscriptional processes are regulated by RNA binding proteins (RBPs). The HuR protein, for example, is a ubiquitously expressed RNA binding protein that is predominantly nuclear but shuttles between the nucleus and cytoplasm and is differentially phosphorylated. HuR has a well-established role in the posttranscriptional regulation of gene expression and is the best-characterized mRNA stabilizing factor (Abdelmohsen and Gorospe, 2010) to date. In addition, HuR has been shown to influence translation as well as play a role in the regulation of alternative splicing and alternative polyadenylation in the nucleus (Zhu et al., 2007; Lebedeva et al., 2011). Thus, the protein appears to be pivotal to achieving the coordination of changes in cellular gene expression in response to various stimuli (von Roretz et al., 2011).

A key question is how the regulators of posttranscriptional processes are themselves regulated. Important routes include changes in expression levels and posttranslational modifications, which can lead to altered RNA binding affinity or localization. Interestingly, both miRNAs and RBPs can also be modulated through nonfunctional interaction with RNA molecules bearing their target sequences. These RNA decoys or sponges can have dramatic effects on gene expression. Long noncoding RNAs (Wang et al., 2013), pseudogenes (Johnsson et al., 2013), and circular RNAs (Hansen et al., 2013) have been shown to effectively sequester miRNAs in cells and significantly influence gene expression. Harnessing RNA sponge technologies has also been proposed as a way of directing the regulation of posttranscriptional gene expression (Kong et al., 2006). In addition to sequestering miRNAs, it is also possible that RNA sponges can sequester RNA binding proteins. Although there is little solid evidence for this occurring naturally, artificial decoy RNAs have been successful in interfering with RBP function in cell culture (Bevilacqua et al., 2007; Soundararajan et al., 2008; Eiring et al., 2010; Bolognani et al., 2012). We hypothesized that RNA viruses, through their ability to produce copious amounts of viral RNAs, may use this sponge approach as an integral part of their strategy to usurp cellular gene expression and interfere with targeted cellular processes. To date, only DNA herpesviruses have been shown to encode small RNAs that act as miRNA sponges (Cazalla et al., 2010; Libri et al., 2012). However

it has not been investigated whether RNA viruses can act as sponges to sequester cellular RNA binding proteins and have an important, directed effect on cellular gene expression.

Alphaviruses (e.g., Sindbis virus [SinV], Chikungunya virus, and Venezuelan equine encephalitis virus) are capped and polyadenylated positive-sense RNA viruses that encode a single subgenomic RNA (Schwartz and Albert, 2010). Their replication occurs exclusively in the cytoplasm. Alphavirus infection has dramatic effects on many aspects of cellular physiology. However, in most cases the direct cause of these effects has not been elucidated. In particular, the influence of alphavirus infections on the cellular posttranscriptional processes of splicing, polyadenylation, and mRNA stability has not been extensively investigated. We have previously demonstrated that alphavirus transcripts avidly bind to the cellular HuR protein through conserved high-affinity binding sites in their 3′ UTR (Garneau et al., 2008; Sokoloski et al., 2010; Dickson et al., 2012). Furthermore, this interaction between viral RNAs and the cellular HuR protein is important for efficient viral gene expression/replication. We now wish to explore the key question of the impact of interactions between alphavirus RNAs and the HuR protein on cellular posttranscriptional processes.

In this study, we demonstrate that the 3′ UTR of SinV RNAs serves as an effective sponge that sequesters HuR protein in the cytoplasm during infection. Furthermore, sequestration of HuR by viral RNAs results in the dramatic destabilization of cellular mRNAs that normally rely on the protein to regulate their stability. In addition, viral RNA sequestration of HuR also significantly alters alternative polyadenylation and splicing of select cellular transcripts that normally rely on HuR binding to regulate processing site choice. Collectively, these data demonstrate an important role for the concept of "sponging," or sequestration of a cellular RNA binding protein by the transcripts of RNA viruses, and mechanistically show how a cytoplasmic RNA virus can directly influence nuclear posttranscriptional processes.

RESULTS

The 3′ UTR of SinV RNA Acts as a Sponge to Sequester the Cellular HuR Protein in the Cytoplasm of Infected Cells

We have previously shown that infection by SinV, as well as several other alphaviruses, results in a redistribution of the cellular HuR protein to the cytoplasm (Sokoloski et al., 2010; Dickson et al., 2012). The underlying mechanism for this phenomenon, however, is not known. As shown in Figure 1A, HuR protein goes from being a predominantly nuclear protein in uninfected cells (14.3 ± 6.0% in the cytoplasm) to a mainly cytoplasmic protein (91.7 ± 1.5%) 24 hr post infection with SinV in 293T cells. The redistribution of HuR protein to the cytoplasm required SinV replication/gene expression and appears to be caused by a mechanism distinct from the shuttling of HuR that is observed during cellular stress responses (Dickson et al., 2012). Furthermore, the cytoplasmic redistribution of HuR was not observed during infections with other cytoplasmic RNA viruses, suggesting that it was caused by an alphavirus-specific function.

A survey of viral protein mutants failed to identify a viral open reading frame (ORF) required for HuR redistribution to the cytoplasm (data not shown). However, based on studies with SinV mutants, we noticed a correlation between the overall abundance of viral RNA and the relocalization of HuR to the cytoplasm of infected cells. Thus, we hypothesized that HuR relocalization may be an RNA-dependent phenomenon. SinV genomic and subgenomic RNAs both contain two high-affinity HuR binding sites (the U-rich element [URE] and conserved sequence element [CSE]) in their 3′ UTRs (Sokoloski et al., 2010). To assess whether the SinV 3′ UTR could mediate HuR relocalization to the cytoplasm, plasmids encoding GFP reporter mRNAs with or without the SinV 3′ UTR were transfected into 293T cells. Based on fluorescence microscopy, the two plasmid constructs produced similar overall amounts of GFP in the transfections (data not shown). As shown in Figure 1B, transfection of a plasmid that expressed only the GFP reporter mRNA had no effect on HuR localization. Interestingly, transfection of a plasmid expressing a GFP reporter mRNA containing the SinV 3′ UTR caused an ~4-fold increase in the proportion of HuR protein in the cytoplasm. Thus, we conclude that the SinV 3′ UTR on its own appears to have a propensity to affect HuR localization in the cell.

Plasmid-generated RNAs are made in the nucleus, whereas SinV transcripts are produced in the cytoplasm. Thus, a limitation of the plasmid-based transfection study described in Figure 1B is that the reporter RNAs have a nuclear experience and thus do not necessarily recapitulate the scenario of expression of viral 3′ UTR sequences during the cytoplasmic-only replication of SinV. Therefore, we transfected RNAs into cells and assayed HuR localization to directly assess the ability of the SinV 3′ UTR to induce the redistribution of HuR protein to the cytoplasm. Transfection of a control RNA had no effect on HuR localization in cells (Figure 1C, control RNA panels). However, transfection of a SinV 3′ UTR RNA caused a dramatic redistribution of HuR protein from the nucleus to the cytoplasm within 6 hr post transfection (Figure 1C, SinV 3′ UTR RNA panels). As shown in Figure 1D, transfection of RNA fragments of the SinV 3′ UTR mapped the region of the 3′ UTR required for HuR relocalization to a 60 base region that contains a U-rich element (URE) and the conserved sequence element (CSE). This 60 base region was demonstrated previously to contain high-affinity HuR binding sites (Sokoloski et al., 2010). To determine whether the amount of SinV 3′ UTR-derived RNA that was transfected into cells was biologically relevant, we used quantitative RT-PCR (qRT-PCR) and converted values to numbers of RNA molecules using a standard curve generated by titration of known amounts of linearized SinV 3′ UTR-containing plasmid DNA. Not surprisingly, the functional concentration of transfected RNA—the amount of RNA that actually gets into 293T cells and is stable at the time of analysis (6 hr post transfection)—was very much less than the overall amount of RNA that is used in the transfection experiment (1 μg, or ~3×10^{12} molecule). Although there was variation in the amount of RNA that was present inside of cells because of natural variability in transfection efficiencies, 1.54×10^9 copies/μg of total RNA (or ~75,000 copies of transfected RNA per cell) was sufficient to see the effects shown in Figure 1D. This amount of RNA was similar to the amount of SinV 3′ UTR that is produced during infection (~1.0×10^9 copies per μg of total RNA, or ~50,000 copies of SinV 3′ UTR per cell) in Figure 1A. Similar values were seen in two independent experiments. Furthermore,

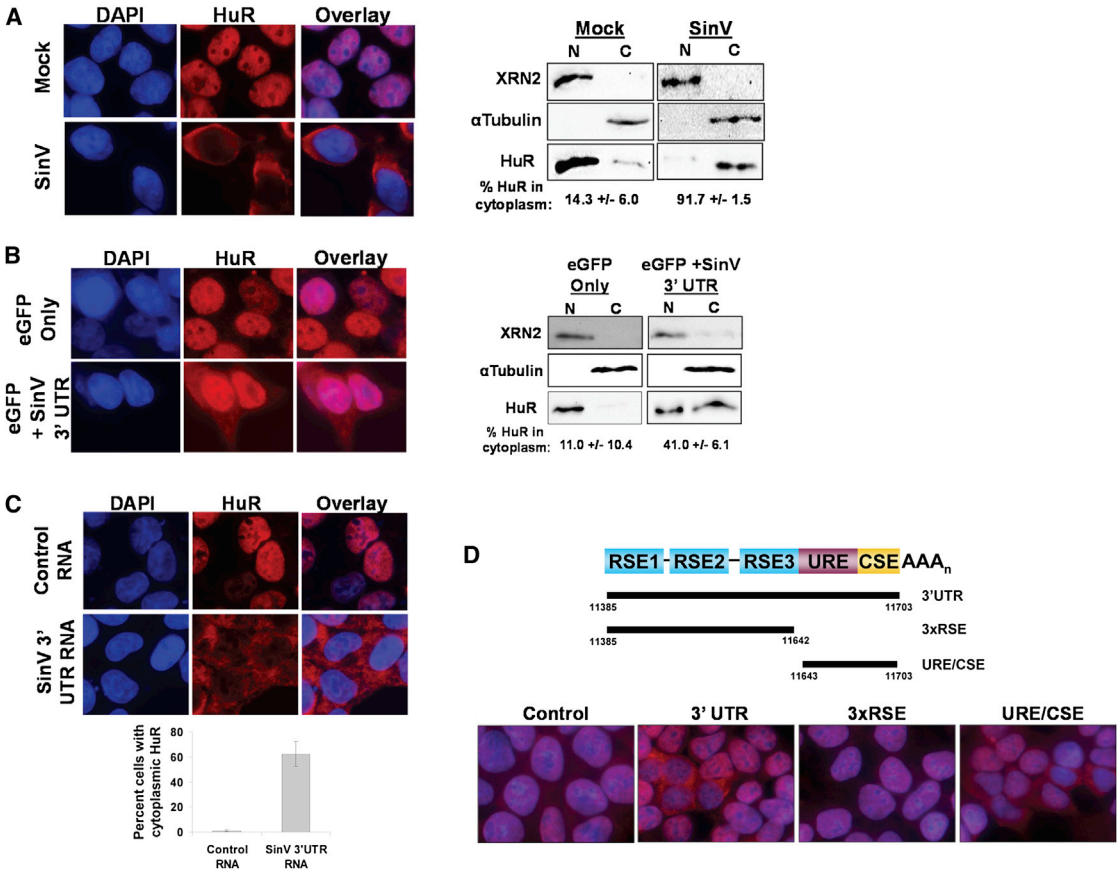

Figure 1. The SinV 3′ UTR Is Sufficient to Induce the Relocalization of HuR Protein from the Nucleus to the Cytoplasm

(A) 293T cells were mock-treated or infected with SinV. At 24 hr post treatment, cells were analyzed for HuR protein localization by immunofluorescence (left panel) or by subcellular fractionation and western blotting (right panel) using antibodies specific for the indicated proteins. DAPI was used to identify the nuclear compartment.

(B) Cells were transfected with plasmids encoding an eGFP reporter bearing either the default 3′ UTR (eGFP only) or the SinV 3′ UTR (eGFP + SinV 3′ UTR) and analyzed for HuR protein subcellular localization by immunofluorescence (left panel) or by subcellular fractionation and western blotting (right panel).

(C) 293T cells were transfected with a control RNA or with a SinV 3′ UTR RNA. At 6 hr post transfection, cells were analyzed for HuR protein subcellular localization by immunofluorescence. Quantification is shown with SD.

(D) 293T cells were transfected with a control RNA or with the indicated fragment of the SinV 3′ UTR (diagrammed at the top of the panel). At 6 hr post transfection, cells were analyzed for HuR protein subcellular localization by immunofluorescence.

these transcript numbers per cell are similar to values reported previously (160,000 genomes per infected cell) for SinV at late times post infection (Wang et al., 1991). Therefore, we conclude that cytoplasmic expression of the SinV 3′ UTR RNA, in the absence of any viral proteins, is sufficient to induce the relocalization of the cellular HuR protein. These observations suggest that the large amount of viral transcripts produced during an infection act like a sponge to sequester HuR protein in the cytoplasm.

The Sequestration of HuR by SinV Has Dramatic Effects on the Relative Stability of a Subset of Cellular mRNAs

We hypothesized that the dramatic relocalization and sequestration of HuR protein by SinV RNAs would have major effects on the posttranscriptional regulation of cellular gene expression in infected cells. One of the best-described roles of the cellular HuR protein is to regulate and mediate the stability of cellular mRNAs by direct binding through U-rich elements (von Roretz et al., 2011). To assess whether SinV infection influenced the stability of cellular mRNAs, the relative half-lives of a set of transcripts were compared between infected and mock-infected cells. 293T cells were either mock-treated or infected at a multiplicity of infection (moi) of 10 with SinV. At 24 hr post infection, cells were treated with actinomycin D to halt further cellular transcription. Total RNA samples were obtained at designated times post shut-off of cellular transcription and the relative abundances of selected mRNAs assessed. As shown in Figure 2A, numerous cellular mRNAs such as *DDX58 (RIG I)*, *TUT1*, *LEPROTL1*, and *RSPRY1* were significantly destabilized during SinV infection, with mRNA half-lives reduced between 2- and 4-fold. Furthermore, the transfection of RNA containing only the 3′ UTR of SinV was sufficient to cause a significant decrease in half-lives of these mRNAs (Figure 2B). However, as shown in Figures 2C and 2D, not all cellular mRNAs were destabilized

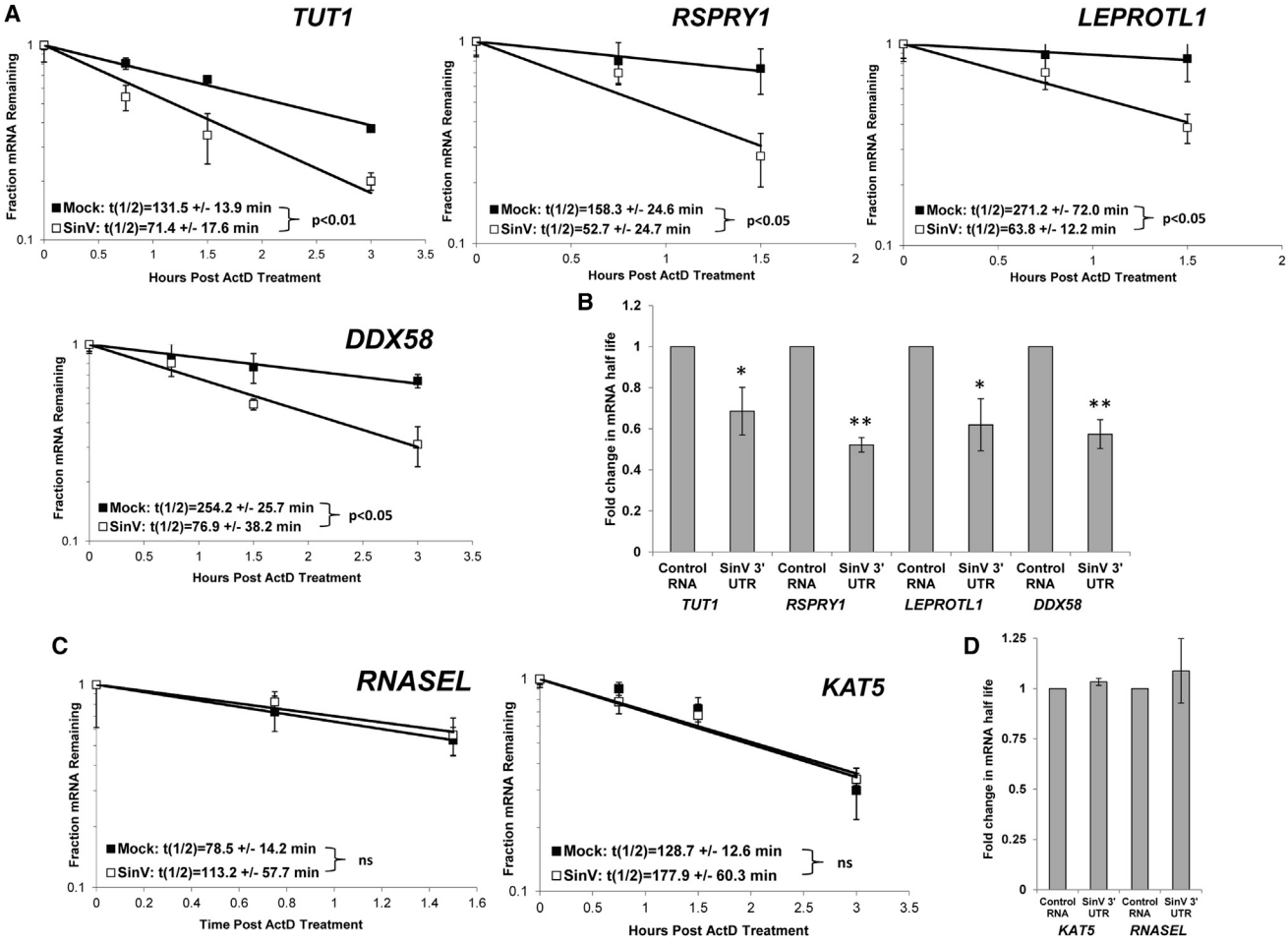

Figure 2. SinV Infection Influences the Stability of Some but Not All Cellular mRNAs

(A and C) At 24 hpi with SinV, 293T cells were treated with actinomycin D, and the relative levels of the indicated mRNAs were assessed at the designated time points following shutoff of transcription using qRT-PCR to determine mRNA half-lives. (A) depicts mRNAs that were destabilized during SinV infection, whereas (C) contains mRNAs whose stability was not affected. Representative decay curves are shown with SD of experimental measurements, and the average half-lives are reported with SD from three independent experiments.

(B and D) 293T cells were transfected with equimolar amounts of either a control RNA (GemA60) or the SinV 3′UTR RNA. At 4.5 hr post transfection, cells were treated with actinomycin D, and the relative levels of the indicated mRNAs were assessed by qRT-PCR. (B) depicts mRNAs that were destabilized during SinV infection, whereas (D) contains mRNAs whose stability was not affected. Average fold change in half-lives is reported with SD from two independent experiments. *$p < 0.05$; **$p < 0.01$.

during viral infection or upon transfection of the SinV 3′ UTR RNA. *KAT5* and *RNASEL* mRNAs showed no significant change in half-life. Thus, we conclude that there was a selective destabilization of cellular mRNAs during SinV infection or RNA transfection of the 3′ UTR of SinV.

To elucidate the underlying mechanism for the selective destabilization of cellular mRNAs, we hypothesized that differential binding of the cellular HuR protein may be involved because of sequestration of the protein by viral RNAs. To assess this hypothesis, formaldehyde-stabilized HuR-containing ribonucleoprotein complexes were immunoprecipitated from mock-infected and SinV-infected cells, and coprecipitating mRNAs were assessed by RT-PCR and qRT-PCR. As shown in Figure 3A, HuR protein normally associated with four of the mRNAs tested (*TUT1*, *RSPRY1*, *DDX58*, and *LEPROTL1*), but HuR interactions with these transcripts were dramatically reduced in SinV-infected cells. Interestingly, all four of these mRNAs were also destabilized in SinV infection (Figure 2). Both the *KAT5* and *RNASEL* mRNAs failed to substantially interact with HuR protein under any condition tested—and notably neither of these mRNAs experienced a change in stability during SinV infection. Figure 3B demonstrates an independent replicate of the experiment in Figure 3A but analyzed by qRT-PCR with samples normalized to the immunoglobulin G (IgG) control immunoprecipitation to allow a quantitative analysis. In addition, SinV RNAs were effectively coprecipitated with the HuR-specific antisera from infected samples (Figure 3A), consistent with the notion that they are largely bound by HuR protein. Finally, we note that *RNASEL* has been previously shown to be regulated by HuR protein and that endogenous *RNASEL* mRNA was

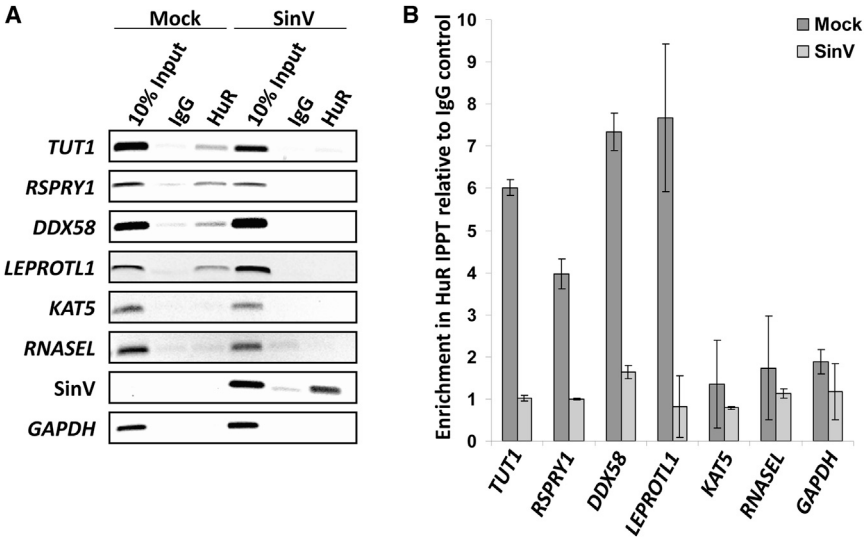

Figure 3. SinV Infection Significantly Reduced the Association of HuR with Cellular mRNAs

293T cells were either mock-infected or infected with SinV. At 24 hr post treatment, HuR protein-RNA complexes were isolated by immunoprecipitation using HuR-specific antibodies or a control normal mouse IgG.

(A–B) Coprecipitating mRNAs were analyzed by RT-PCR (A) or qRT-PCR (B). (A) and (B) depict results from independent infections. Quantification is shown with SD.

shown to bind HuR in murine C2C12 cells (Li et al., 2007). We assume that differences in cell type used may account for the observations reported here.

Therefore, we conclude that the destabilization of cellular mRNAs during SinV infection is associated with a reduction of HuR binding resulting from sequestration of the cellular stability factor by viral RNAs. Given the importance of several of these proteins in innate immunity (e.g., RIG I), cellular gene expression (e.g., TUT1), and overall cellular biology, it is likely that SinV RNA-induced alterations in cellular mRNA stability will have important biological consequences on cytopathology, host response to the virus, and overall viral replication.

Alternative Pre-mRNA Polyadenylation and Splicing in the Nucleus Is Also Influenced by SinV Infection

In addition to its well-studied role in mRNA stability, the cellular HuR protein also influences mRNA processing events in the nucleus. Through competition for binding sites on pre-mRNAs, HuR has been described to affect both alternative polyadenylation and alternative splicing (Zhu et al., 2007; Lebedeva et al., 2011). Interestingly, HuR also autoregulates the polyadenylation site choice of its own mRNA in certain cell/tissue types. The differential use of polyadenylation sites changes the length of the 3′ UTR of the HuR mRNA, which influences the stability/translatability of the mRNA isoform that is produced (Dai et al., 2012). Thus, we hypothesized that SinV may influence nuclear mRNA processing events via the sequestration of HuR in the cytoplasm. To test this hypothesis, we assessed alternative polyadenylation or splicing for a number of mRNAs known to be influenced by HuR protein. As shown in Figure 4A, the HuR mRNA is normally present in an ~50/50 ratio of a long (6 Kb) isoform that uses the downstream poly(A) site versus a set of shorter (1.5–2.7 Kb) isoforms that use an upstream poly(A) site. There are HuR binding sites in the vicinity of the upstream poly(A) site that regulate its usage (Dai et al., 2012). Interestingly, during a SinV infection, the relative usage of the upstream poly(A) sites on the HuR pre-mRNA increases dramatically. By 72 hr post infection (hpi), more than 80% of the HuR transcripts that are made represent the more translatable forms that utilize the upstream poly(A) site. A similar alteration in the regulation of alternative polyadenylation in the calcitonin mRNA during SinV infection was also observed (Figure 4B). In this case, calcitonin pre-mRNA polyadenylation site usage is normally influenced by HuR regulation of alternative splicing of exon 4, which determines the inclusion of the upstream poly(A) site in the transcript (Lebedeva et al., 2011). As shown in Figure 4B, SinV infection promotes the accumulation of the longer isoform that is processed at the downstream poly(A) site—likely by sequestering HuR protein and preventing its function in the combinatorial regulation that promotes usage of the upstream polyadenylation site. Finally, HuR is also known to be a regulator of alternative splicing in the mammalian cell nucleus. As shown in Figure 4C, SinV infection caused significant increases in the splicing of exon 10 of PCBP2 (1.28 ± 0.09-fold; $p < 0.05$) and exon 49 of DST (2.1 ± 0.46-fold; $p < 0.05$), both of which are known to be regulated by HuR interactions (Lebedeva et al., 2011). Notably, the effect of SinV infection on nuclear RNA processing could also be observed in transfections of RNA containing the SinV 3′ UTR. As shown in Figure 4D, transfection of RNAs containing the SinV 3′ UTR caused an ~3-fold difference in the relative abundance of isoforms of the calcitonin mRNA compared to cells transfected with a control RNA. Therefore, we conclude that SinV infection can influence regulated pre-mRNA processing events in the nucleus, presumably through the sequestration of HuR protein in the cytoplasm. Given the importance of posttranscriptional RNA processing in determining the breadth of the cellular proteome, these SinV-induced alterations can have a dramatic effect on cellular biology. Furthermore, a cytoplasmic positive-sense RNA virus is shown here to selectively influence alternative mRNA processing events in the nucleus.

DISCUSSION

This study demonstrates that the 3′ UTR of SinV on its own is capable of causing the relocalization of the normally predominately nuclear HuR protein into the cytoplasm. This sequestration of HuR protein by viral RNAs has several dramatic effects of posttranscriptional regulation of cellular mRNAs. Cellular

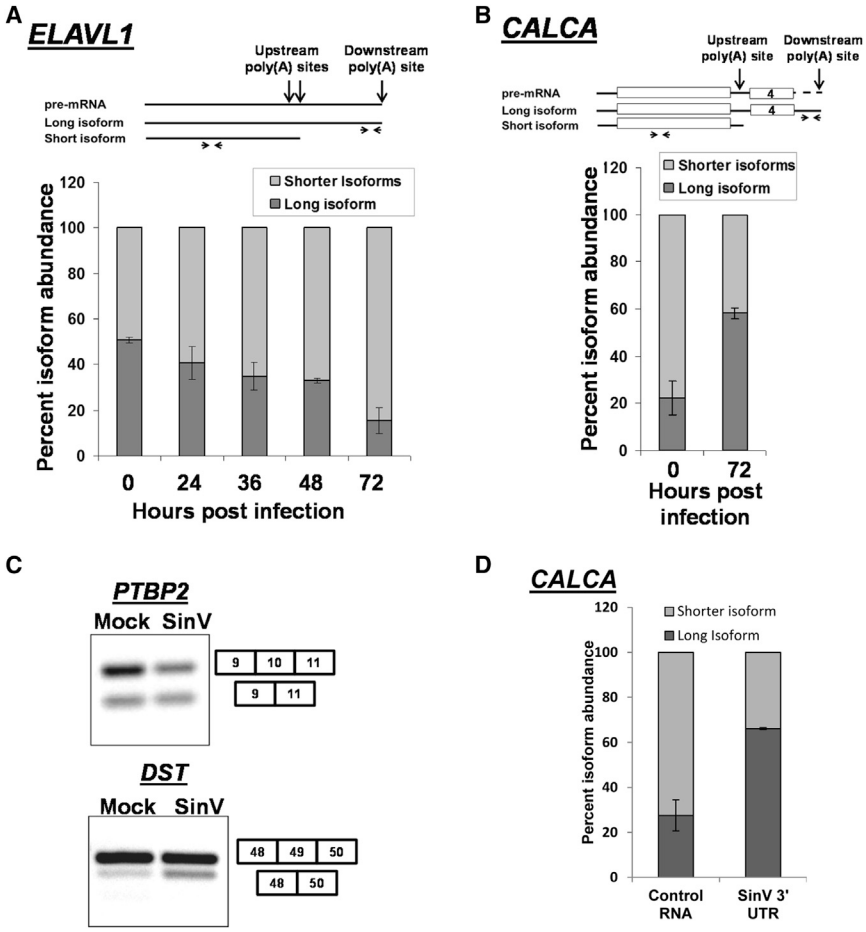

Figure 4. SinV Infection Influences Alternative Polyadenylation and Splicing of Cellular Pre-mRNAs

293T cells were infected with SinV for the indicated times.

(A and B) Short or long isoforms of the *ELAVL1* (HuR) mRNA formed by alternative polyadenylation (A) or the indicated isoforms of CALCA (calcitonin) (B) were quantified by qRT-PCR using the primers illustrated at the top of the panels. Note that the level of "shorter isoforms" was determined by subtraction of the amount of the longer isoform from the level of total RNA detected by the upstream primer. Quantification is shown with SD calculated from three independent experiments.

(C) Total RNA was isolated 24 hpi, probed for the presence of the splicing isoforms of the indicated genes by RT-PCR, and analyzed on a 2% agarose gel.

(D) 293T cells were transfected with equimolar amounts of either a control RNA (GemA60) or the SinV 3′ UTR RNA. Total RNA was isolated at 6 hr post transfection and analyzed for CALCA isoforms as described in (B). Quantification is shown with SD calculated from two independent experiments.

mRNAs that are normally stabilized by HuR protein have significantly reduced half-lives in virus-infected cells. In addition, changes can be observed in alternative polyadenylation and splicing patterns of pre-mRNAs normally regulated by nuclear HuR protein. These findings highlight aspects of virus-host interactions that are likely to be highly significant to viral pathogenesis and cytopathology.

Given the potentially large number of RNA binding proteins in a cell, an RNA must be expressed in large amounts to act as an effective sponge for a specific protein with biological consequences. Alphaviral genomic/subgenomic RNAs are obviously very highly expressed in infected cells. Furthermore, RNA sponges may interact with more than one molecule of an RNA binding protein (RBP) at a time. Along these lines, the SinV 3′ UTR contains multiple binding sites for HuR (Sokoloski et al., 2010), and the protein itself can oligomerize on RNA substrates (Benoit et al., 2010), thus increasing the potential effectiveness of such a sponge. The sponging phenomenon can also be observed when the SinV 3′ UTR is expressed as part of a GFP reporter mRNA construct from transfected plasmid DNA (Figure 1B). Thus, it is possible that the sponging of RBPs by overexpressed mRNAs in standard transfection experiments may need to be routinely considered when interpreting results (Westmark et al., 2006). Finally, this RNA sponge phenomenon may also need to be taken into account when using alphavirus vectors as a platform strategy for vaccine delivery (White et al., 2013).

In addition to HuR, alphavirus RNAs might also effectively sequester other RNA binding proteins in the cell during infection. The viral subgenomic RNA, for instance, is known to interact with hnRNP K (Burnham et al., 2007), and unknown cellular factors interact with the translational enhancer in the 5′ UTR (Patel et al., 2013). In addition to viral RNAs, viral nonstructural proteins could also serve as de facto sponges for cellular factors. Pull-downs with tagged nonstructural proteins have isolated a number of host factors (Atasheva et al., 2007; Frolova et al., 2006, Cristea et al., 2006). The viral nsp3 protein interacts with G3BP, 14-3-3 proteins, YBX1, HSC70, amphiphysin-1, and amphiphysin-2 (Cristea et al., 2006, 2010; Neuvonen et al., 2011; Gorchakov et al., 2008). Finally, it may be important to consider that transcripts from other RNA viruses may also act as sponges of cellular RBPs. Thus, it is tempting to speculate that the sequestration of cellular RNA binding proteins may be a very important aspect of virus-host interactions/viral pathogenesis that needs additional investigation.

The alteration of cellular RNA stability/posttranscriptional control through the sequestration of HuR protein by SinV may be a very significant factor in viral pathogenesis. HuR protein has been implicated in the regulation of a variety of cellular responses including stress, differentiation, apoptosis, cell cycle progression, immune responses, and coordination of inflammatory reactions (Yiakouvaki et al., 2012; Srikantan and Gorospe, 2012; Khabar, 2010). HuR affects these processes through

regulating mRNA stability and translation and through its interplay with miRNAs (Srikantan et al., 2012). Previous work has also shown that every alphavirus we have tested to date interacts with HuR protein through high-affinity sites in its 3′ UTR, indicating that this is a highly conserved host interaction of this family of viruses (Dickson et al., 2012). We are currently performing global analyses to assess the full impact of SinV infection and HuR sequestration on cellular mRNA abundance, stability, and translation. The studies reported here also highlight the potential value of HuR protein-viral RNA interactions as a therapeutic target to reduce alphavirus replication as well as perhaps pathological sequelae of infection.

The data reported here document changes in alternative polyadenylation and splicing of cellular pre-mRNAs in a cytoplasmic RNA virus infection. Some changes in pre-mRNA processing may simply be an effect of HuR sequestration and may not have a direct positive impact on viral infection. However, the SinV-induced changes in HuR pre-mRNA polyadenylation that we have observed favor the production of the shorter HuR isoforms that are inherently more translatable than the longer isoforms are (Dai et al., 2012). Thus, SinV is not only commandeering the available HuR protein in the cell, but also possibly dysregulating its synthesis to perhaps make more HuR protein for use in its own replication (Sokoloski et al., 2010).

In closing, this study highlights a set of effects of a cytoplasmic RNA virus on cellular posttranscriptional regulation of gene expression by viral RNAs acting to sequester a specific host RNA binding protein. Thus, further study of mechanisms and implications of the interference of RNA virus transcripts with the combinatorial regulation of host cell gene expression afforded by cellular RNA binding proteins should yield interesting insights into viral replication and cellular pathogenesis.

EXPERIMENTAL PROCEDURES

Cells and Viruses

293T and BHK-21 cells were grown in 5% CO_2 at 37°C and maintained in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum. Sindbis viral stocks were created by the electroporation of infectious viral RNA (transcribed from the clone pMRE16) into BHK-21 cells. All infections were performed at an moi of 10.

DNA and RNA Transfections

The 3′ UTR of SinV was inserted between the BsrG1 and Not1 sites of pEGFP-n1 (Clontech). Capped and polyadenylated RNAs for the RNA transfections were generated by in vitro SP6 transcription of linearized pGEMA60 (Control) or pMREA60 (SinV 3′ UTR) or plasmids containing the 3×RSE and URE/CSE fragments of the SinV 3′ UTR (Garneau et al., 2008) as previously described (Wilusz and Shenk, 1988). Transfections of plasmids or equimolar amounts of purified RNAs were done using Opti-MEM I Reduced Serum Medium and Lipofectamine 2000 or TransIT mRNA transfection reagent (Mirus). Cells were incubated for 6 hr for RNA transfections or 72 hr for plasmid transfections prior to analysis.

Immunofluorescence Assays

293T cells grown on glass coverslips were either mock-infected or infected with SinV. At 24 hpi, the coverslips were rinsed with PBS and fixed with 4% paraformaldehyde in PBS. Coverslips were then sequentially treated with methanol and ethanol prior to blocking with a 6% solution of bovine serum albumin in PBS. HuR primary antibody (Santa Cruz Biotechnology 3A2) was added followed by anti-mouse Alexa Fluor 594 as secondary antibody. Prolong Gold with DAPI was then added, and coverslips were cured overnight in the dark. Samples were analyzed via fluorescence microscopy using an Olympus IX71 inverted microscope equipped with a Q imaging retiga 2000R digital camera.

Immunoprecipitation and Western Blotting

At 24 hpi, mock-infected or SinV-infected 293T cells were washed with PBS, and protein-RNA interactions were stabilized by adding 1% formaldehyde in PBS. The crosslinking reaction was quenched by the addition of glycine, and cells were lysed by sonication after suspension in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-Cl [pH 7.5]/1% v/v NP-40/0.5% w/v sodium deoxycholate/0.05% w/v sodium dodecyl sulfate [SDS]/1 mM EDTA/150 mM NaCl) with 1 U of ribonuclease (RNase) inhibitor. Clarified lysates were incubated with anti-HuR (Santa Cruz Biotechnology 3A2) or normal mouse IgG (Santa Cruz Biotechnology sc-2025) and 1 U of RNase inhibitor at 4°C. Complexes were bound to Protein G Sepharose beads and washed extensively with RIPA buffer containing 1 M urea. Precipitated complexes were resuspended in TEDS Buffer (50 mM Tris-Cl [pH 7.0]/5 mM EDTA/1% v/v SDS), and crosslinks were reversed by incubation at 70°C. Coprecipitated RNAs were extracted using TRIzol and analyzed by PCR using the primers indicated in Table S1.

Western blots were performed using a Trans-Blot Semi-Dry Transfer Cell and nitrocellulose membranes. Primary antibodies used were tubulin (Sigma-Aldrich T9026), XRN2 (Novus Biologicals NB100-57541), and HuR (Santa Cruz Biotechnology 3A2). Secondary antibodies were conjugated to horseradish peroxidase. Proteins were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and imaged with VersaDoc/Image Lab software (Bio-Rad).

Biochemical Subcellular Fractionation

293T cells were rinsed with 1 × PBS, swollen in EBKL Buffer (25 mM HEPES [pH 7.6]/5 mM $MgCl_2$/1.5 mM KCl/2 mM DTT/0.1% v/v NP-40) for 15 min, and the cytoplasmic membrane was selectively lysed using a dounce homogenizer. Nuclei and cytoplasm were separated via centrifugation. The nuclear pellet was washed with EMBK Buffer (25 mM HEPES [pH 7.6]/5 mM $MgCl_2$/1.5 mM KCl/75 mM NaCl/175 mM sucrose/2 mM DTT), resuspended in 0.5% NP-40, and genomic DNA was sheared via brief sonication.

mRNA Half-Life Analysis

293T cells were either mock-infected or infected with SinV. Twenty-four hours post infection, actinomycin D (5 μg/μl) was added. For mRNA half-life analysis in RNA-transfected cells, actinomycin D was added at 4.5 hr post transfection. Following a 30 min incubation to ensure transcriptional shutoff, samples were collected at the indicated times using TRIzol. Total RNA from these samples was analyzed via qRT-PCR using the standardized primers indicated in Table S1. Relative transcript abundances were determined using the ΔΔCt method and *GAPDH* as a reference. The average half-life of each transcript from three independent experiments is reported ± SD.

Analysis of Alternative Splicing/Polyadenylation

Alternative splicing of PTBP2, ZNF207, and DST was assessed as described in Lebedeva et al. (2011), and RT-PCR products were separated on 2% agarose gels. Products were visualized using ethidium bromide staining. Isoform abundances of HuR and calcitonin were assessed using primers upstream and downstream of the predicted polyadenylation sites and qRT-PCR.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.10.012.

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REFERENCES

Abdelmohsen, K., and Gorospe, M. (2010). Posttranscriptional regulation of cancer traits by HuR. Wiley Interdiscip. Rev. RNA *1*, 214–229.

Atasheva, S., Gorchakov, R., English, R., Frolov, I., and Frolova, E. (2007). Development of Sindbis viruses encoding nsP2/GFP chimeric proteins and their application for studying nsP2 functioning. J. Virol. *81*, 5046–5057.

Benoit, R.M., Meisner, N.C., Kallen, J., Graff, P., Hemmig, R., Cèbe, R., Ostermeier, C., Widmer, H., and Auer, M. (2010). The x-ray crystal structure of the first RNA recognition motif and site-directed mutagenesis suggest a possible HuR redox sensing mechanism. J. Mol. Biol. *397*, 1231–1244.

Bevilacqua, A., Ghisolfi, L., Franzi, S., Maresca, G., Gherzi, R., Capaccioli, S., Nicolin, A., and Canti, G. (2007). Stabilization of cellular mRNAs and up-regulation of proteins by oligoribonucleotides homologous to the Bcl2 adenine-uridine rich element motif. Mol. Pharmacol. *71*, 531–538.

Bolognani, F., Gallani, A.I., Sokol, L., Baskin, D.S., and Meisner-Kober, N. (2012). mRNA stability alterations mediated by HuR are necessary to sustain the fast growth of glioma cells. J. Neurooncol. *106*, 531–542.

Burnham, A.J., Gong, L., and Hardy, R.W. (2007). Heterogeneous nuclear ribonuclear protein K interacts with Sindbis virus nonstructural proteins and viral subgenomic mRNA. Virology *367*, 212–221.

Cazalla, D., Yario, T., and Steitz, J.A. (2010). Down-regulation of a host microRNA by a Herpesvirus saimiri noncoding RNA. Science *328*, 1563–1566.

Cristea, I.M., Carroll, J.W., Rout, M.P., Rice, C.M., Chait, B.T., and MacDonald, M.R. (2006). Tracking and elucidating alphavirus-host protein interactions. J. Biol. Chem. *281*, 30269–30278.

Cristea, I.M., Rozjabek, H., Molloy, K.R., Karki, S., White, L.L., Rice, C.M., Rout, M.P., Chait, B.T., and MacDonald, M.R. (2010). Host factors associated with the Sindbis virus RNA-dependent RNA polymerase: role for G3BP1 and G3BP2 in virus replication. J. Virol. *84*, 6720–6732.

Dai, W., Zhang, G., and Makeyev, E.V. (2012). RNA-binding protein HuR autoregulates its expression by promoting alternative polyadenylation site usage. Nucleic Acids Res. *40*, 787–800.

Dickson, A.M., Anderson, J.R., Barnhart, M.D., Sokoloski, K.J., Oko, L., Opyrchal, M., Galanis, E., Wilusz, C.J., Morrison, T.E., and Wilusz, J. (2012). Dephosphorylation of HuR protein during alphavirus infection is associated with HuR relocalization to the cytoplasm. J. Biol. Chem. *287*, 36229–36238.

Eiring, A.M., Harb, J.G., Neviani, P., Garton, C., Oaks, J.J., Spizzo, R., Liu, S., Schwind, S., Santhanam, R., Hickey, C.J., et al. (2010). miR-328 functions as an RNA decoy to modulate hnRNP E2 regulation of mRNA translation in leukemic blasts. Cell *140*, 652–665.

Frolova, E., Gorchakov, R., Garmashova, N., Atasheva, S., Vergara, L.A., and Frolov, I. (2006). Formation of nsP3-specific protein complexes during Sindbis virus replication. J. Virol. *80*, 4122–4134.

Garneau, N.L., Sokoloski, K.J., Opyrchal, M., Neff, C.P., Wilusz, C.J., and Wilusz, J. (2008). The 3′ untranslated region of sindbis virus represses deadenylation of viral transcripts in mosquito and Mammalian cells. J. Virol. *82*, 880–892.

Gorchakov, R., Garmashova, N., Frolova, E., and Frolov, I. (2008). Different types of nsP3-containing protein complexes in Sindbis virus-infected cells. J. Virol. *82*, 10088–10101.

Hansen, T.B., Jensen, T.I., Clausen, B.H., Bramsen, J.B., Finsen, B., Damgaard, C.K., and Kjems, J. (2013). Natural RNA circles function as efficient microRNA sponges. Nature *495*, 384–388.

Johnsson, P., Ackley, A., Vidarsdottir, L., Lui, W.O., Corcoran, M., Grandér, D., and Morris, K.V. (2013). A pseudogene long-noncoding-RNA network regulates PTEN transcription and translation in human cells. Nat. Struct. Mol. Biol. *20*, 440–446.

Khabar, K.S. (2010). Post-transcriptional control during chronic inflammation and cancer: a focus on AU-rich elements. Cell. Mol. Life Sci. *67*, 2937–2955.

Kornblihtt, A.R., Schor, I.E., Alló, M., Dujardin, G., Petrillo, E., and Muñoz, M.J. (2013). Alternative splicing: a pivotal step between eukaryotic transcription and translation. Nat. Rev. Mol. Cell Biol. *14*, 153–165.

Kong, J., Sumaroka, M., Eastmond, D.L., and Liebhaber, S.A. (2006). Shared stabilization functions of pyrimidine-rich determinants in the erythroid 15-lipoxygenase and alpha-globin mRNAs. Mol. Cell. Biol. *26*, 5603–5614.

Lebedeva, S., Jens, M., Theil, K., Schwanhäusser, B., Selbach, M., Landthaler, M., and Rajewsky, N. (2011). Transcriptome-wide analysis of regulatory interactions of the RNA-binding protein HuR. Mol. Cell *43*, 340–352.

Li, X.L., Andersen, J.B., Ezelle, H.J., Wilson, G.M., and Hassel, B.A. (2007). Post-transcriptional regulation of RNase-L expression is mediated by the 3′-untranslated region of its mRNA. J. Biol. Chem. *282*, 7950–7960.

Libri, V., Helwak, A., Miesen, P., Santhakumar, D., Borger, J.G., Kudla, G., Grey, F., Tollervey, D., and Buck, A.H. (2012). Murine cytomegalovirus encodes a miR-27 inhibitor disguised as a target. Proc. Natl. Acad. Sci. USA *109*, 279–284.

Neuvonen, M., Kazlauskas, A., Martikainen, M., Hinkkanen, A., Ahola, T., and Saksela, K. (2011). SH3 domain-mediated recruitment of host cell amphiphysins by alphavirus nsP3 promotes viral RNA replication. PLoS Pathog. *7*, e1002383.

Patel, R.K., Burnham, A.J., Gebhart, N.N., Sokoloski, K.J., and Hardy, R.W. (2013). Role for subgenomic mRNA in host translation inhibition during Sindbis virus infection of mammalian cells. Virology *441*, 171–181.

Schoenberg, D.R., and Maquat, L.E. (2012). Regulation of cytoplasmic mRNA decay. Nat. Rev. Genet. *13*, 246–259.

Schwartz, O., and Albert, M.L. (2010). Biology and pathogenesis of chikungunya virus. Nat. Rev. Microbiol. *8*, 491–500.

Shi, Y. (2012). Alternative polyadenylation: new insights from global analyses. RNA *18*, 2105–2117.

Sokoloski, K.J., Dickson, A.M., Chaskey, E.L., Garneau, N.L., Wilusz, C.J., and Wilusz, J. (2010). Sindbis virus usurps the cellular HuR protein to stabilize its transcripts and promote productive infections in mammalian and mosquito cells. Cell Host Microbe *8*, 196–207.

Soundararajan, S., Chen, W., Spicer, E.K., Courtenay-Luck, N., and Fernandes, D.J. (2008). The nucleolin targeting aptamer AS1411 destabilizes Bcl-2 messenger RNA in human breast cancer cells. Cancer Res. *68*, 2358–2365.

Srikantan, S., and Gorospe, M. (2012). HuR function in disease. Front Biosci (Landmark Ed) *17*, 189–205.

Srikantan, S., Tominaga, K., and Gorospe, M. (2012). Functional interplay between RNA-binding protein HuR and microRNAs. Curr. Protein Pept. Sci. *13*, 372–379.

von Roretz, C., Di Marco, S., Mazroui, R., and Gallouzi, I.E. (2011). Turnover of AU-rich-containing mRNAs during stress: a matter of survival. Wiley Interdiscip. Rev. RNA *2*, 336–347.

Wang, Y.-F., Sawicki, S.G., and Sawicki, D.L. (1991). Sindbis virus nsP1 functions in negative-strand RNA synthesis. J. Virol. *65*, 985–988.

Wang, Y., Xu, Z., Jiang, J., Xu, C., Kang, J., Xiao, L., Wu, M., Xiong, J., Guo, X., and Liu, H. (2013). Endogenous miRNA sponge lincRNA-RoR regulates

Oct4, Nanog, and Sox2 in human embryonic stem cell self-renewal. Dev. Cell *25*, 69–80.

Westmark, P.R., Shin, H.C., Westmark, C.J., Soltaninassab, S.R., Reinke, E.K., and Malter, J.S. (2006). Decoy mRNAs reduce beta-amyloid precursor protein mRNA in neuronal cells. Neurobiol. Aging *27*, 787–796.

White, L.J., Sariol, C.A., Mattocks, M.D., Wahala M P B, W., Yingsiwaphat, V., Collier, M.L., Whitley, J., Mikkelsen, R., Rodriguez, I.V., Martinez, M.I., et al. (2013). An alphavirus vector-based tetravalent dengue vaccine induces a rapid and protective immune response in macaques that differs qualitatively from immunity induced by live virus infection. J. Virol. *87*, 3409–3424.

Wilusz, J., and Shenk, T. (1988). A 64 kd nuclear protein binds to RNA segments that include the AAUAAA polyadenylation motif. Cell *52*, 221–228.

Yiakouvaki, A., Dimitriou, M., Karakasiliotis, I., Eftychi, C., Theocharis, S., and Kontoyiannis, D.L. (2012). Myeloid cell expression of the RNA-binding protein HuR protects mice from pathologic inflammation and colorectal carcinogenesis. J. Clin. Invest. *122*, 48–61.

Zhu, H., Zhou, H.L., Hasman, R.A., and Lou, H. (2007). Hu proteins regulate polyadenylation by blocking sites containing U-rich sequences. J. Biol. Chem. *282*, 2203–2210.