Regulated mRNA decay plays a vital role in determining both the level and quality of cellular gene expression. Viral RNAs must successfully evade this host RNA decay machinery to establish a productive infection. One way for RNA viruses to accomplish this is to target the cellular exoribonuclease XRN1, because this enzyme is accessible in the cytoplasm and plays a major role in mRNA decay. Members of the Flaviviridae use RNA structures in their 5′- or 3′-untranslated regions to stall and repress XRN1, effectively stabilizing viral RNAs while also causing significant dysregulation of host cell mRNA stability. Here, we use a series of biochemical assays to demonstrate that the 3′-terminal portion of the nucleocapsid (N) mRNA of Rift Valley fever virus, a phlebovirus of the Bunyaviridae family, can also effectively stall and repress XRN1. The region responsible for impeding XRN1 includes a G-rich portion that likely forms a G-quadruplex structure. The 3′-terminal portions of ambisense-derived transcripts of multiple arenaviruses also stalled XRN1. Therefore, we conclude that RNAs from two additional families of mammalian RNA viruses stall and repress XRN1. This observation emphasizes the importance and commonality of this viral strategy to interfere with the 5′-to-3′-exoribonuclease component of the cytoplasmic RNA decay machinery.

Eukaryotic cells possess a well-characterized, multifaceted RNA decay machinery that plays a major role in determining both the quantity and quality of mRNAs (1). When transcripts from RNA viruses are released into the cytoplasm of infected cells, they are likely recognized as “non-self” by this RNA decay machinery due to characteristics such as the presence of non-standard 5′ modifications (2, 3), lack of a poly(A) tail (4, 5), the absence of appropriate ribonucleoprotein signatures (6), or by open reading frame (ORF) positioning that elicits nonsense-mediated decay (7). The observation that viral transcripts are not rapidly degraded, but rather persist to establish high levels of viral gene expression and replication, is consistent with the notion that they evolved effective strategies to avoid or manipulate the cellular RNA decay machinery (8). Conversely, an inability to effectively evade the cellular RNA decay machinery results in dramatically reduced yields of viral RNAs (9). Thus, in many ways, the cellular RNA decay machinery represents part of the first line arsenal of innate cellular antiviral defense.

Several strategies by which RNA viruses protect their transcripts from the cellular RNA decay machinery have been uncovered to date. Alphaviruses (e.g. Venezuelan equine encephalitis virus and Sindbis virus) possess high-affinity binding sites for HuR protein, a well-characterized cellular RNA stabilizer that protects transcripts from premature deadenylation and decay (9–12). Polioviruses target several RNA decay factors for proteolytic cleavage, sponge several RNA-binding proteins, and possess an RNA element that inhibits endoribonuclease RNase L (13). Flaviviruses (e.g. Dengue virus, West Nile virus, and Zika virus), however, target XRN1, a highly processive 5′-to-3′-exoribonuclease that degrades the body of transcripts in the major pathway of cytoplasmic RNA decay (14). XRN1 stalls on a stabilized knot-like three-helix junction motif located in the 3′ untranslated region (UTR) of many if not all arthropod-borne flaviviruses (15, 16). In addition, XRN1 stalls on internal ribosome entry site-associated structures in the 5′ UTR of the non-insect-associated hepacivirus and pestiviruses of the Flaviviridae (17). Interestingly, these RNA structures also serve as reversible inhibitors of XRN1 activity as the stalled enzyme is likely released relatively slowly when stalled on the transcript (17, 18).

The mechanisms by which other RNA viruses maintain the stability of their transcripts during infection have not been well-characterized. Interestingly, XRN1 stalling at specific sites in viral RNAs has also been noted for several segmented plant RNA viruses (19, 20). Thus, we hypothesized that segmented RNA viruses infecting mammalian systems might have also evolved RNA structure-based strategies to stall XRN1 as part of their approach for effective host interaction. To test this hypothesis, we opted to focus on three families of RNA viruses that contained structured segments in their non-coding regions. Two of these RNA virus families, the phleboviruses and arenaviruses, use an ambisense strategy of gene expression during infection, generating mRNAs from both the genome and antigenomic RNA of their segmented RNA genomes (see Figs. 1B and 6A). The other virus family, the coronavirus, contains a conserved, well-characterized pseudoknot structure in the 3′ UTR of its mRNAs (see Fig. 7A). In this study, we use reconstituted XRN1 biochemical assays to search for regions from additional RNA viruses that were capable of stalling and repressing XRN1. Notably, the 3′ UTR from the nucleocapsid-encoding S
segment of Rift Valley fever virus (a phlebovirus of the Bunyaviridae family), as well as the 3' UTRs from all four mRNAs of Junin virus, an arenavirus, contained RNA segments that stalled and repressed XRN1. The ability of 3'UTR structures to stall XRN1 was conserved in several additional phleboviruses and arenaviruses. The ability to stall XRN1, therefore, is a strategy employed by at least three mammalian RNA virus families, reflecting the importance of targeting not only the RNA decay machinery, but this enzyme in particular, in RNA virus infections.

Results

3' Terminal region of the N mRNA of Rift Valley fever virus stalls XRN1

A knot-like structure involving a highly stabilized three-helix junction in the 3' UTR of arthropod-borne flaviviruses stalls the cellular 5'-to-3'-exoribonuclease XRN1 (15, 16, 21). This surprisingly effective stalling of the highly processive XRN1 exonuclease is illustrated in Fig. 1A. Although a standard control RNA is rapidly degraded by XRN1 with no apparent decay intermediates in a reconstituted reaction using purified components, RNA substrates containing the upstream portion of the Dengue virus (DENV) type 2 3' UTR stall the enzyme, forming stable decay intermediates (Fig. 1A). Multiple decay intermediates are formed because XRN1 can occasionally degrade through the first upstream knot-like three-helix junction that it encounters, likely due to structural breathing in the RNA. XRN1 then reaches a second knot-like structure just downstream of the first (22), resulting in the smaller decay intermediate seen on the gel. Furthermore, all members of the Flaviviridae that have been tested to date, including the non-

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2 The abbreviations used are: DENV, Dengue virus; RVFV, Rift Valley fever virus; eGFP, enhanced GFP; RCNMV, Red clover necrotic mosaic virus; BNYVV, beet necrotic yellow vein virus; CoV, coronavirus; N, nucleocapsid; L, large; G, glycoprotein; NSs, non-structural small.
insect-associated hepativiruses (hepatitis C virus) and pestiviruses (bovine viral diarrhea virus), contain structures that effectively stall and repress XRN1 (17, 18, 23). Because XRN1 stalling appears to be a valuable strategy for a flavivirus to interfere with the cellular RNA decay machinery and dysregulate host gene expression (17, 18), we now wished to determine whether RNAs from other virus families were capable of stalling XRN1.

Rift Valley fever virus (RVFV) is a member of the phlebovirus family of the Bunyaviridae. RVFV contains a tripartite genome with the smallest S segment encoding mRNAs from both its genome and antigenome segments in an ambisense gene expression strategy (Fig. 1B). To determine whether any of the mRNAs encoded by RVFV possessed structures that could stall XRN1, we focused on the distal end of the transcript, including the 3′ UTR region, for two reasons. First, structural elements located in the 3′ UTR downstream of the stop codon would not interfere with ribosome translocation during protein synthesis. Second, the three-helix junction structure of the arthropod-borne flaviviruses is located in the 3′ UTR (22). To determine whether any of the mRNAs encoded by RVFV possessed structures that could stall XRN1, we cloned a 206-base fragment representing the 3′ end of the open reading frame along with the 3′ UTR of the four RVFV mRNAs into a reporter construct, generated radiolabeled RNAs containing a 5′-monophosphate to serve as substrates for XRN1, and incubated them with HeLa cytoplasmic extract that we had previously established is highly active for XRN1 activity (18). As seen in Fig. 1C, neither control RNA nor RNAs containing the large (L), glycoprotein (G), or non-structural small (NSs) 3′-terminal regions stalled XRN1 to form decay intermediates. The 3′-terminal region from the RVFV N mRNA, however, effectively stalled XRN1. Similar decay intermediates were observed using the RVFV N 3′-terminal region when the RNA was incubated with purified recombinant Kluyveromyces lactis XRN1 in the absence of other proteins (Fig. 1D). We therefore conclude that an RNA structure in the 3′-terminal region of the RVFV N mRNA is capable of stalling both human and recombinant K. lactis XRN1 in the absence of RNA-binding proteins.

RVFV is transmitted to mammalian hosts via a mosquito vector (24). Thus, we next extended the observation of RVFV N 3′-terminal region-mediated 5′-to-3′-exoribonuclease stalling to the mosquito XRN1 enzyme. Previous studies have shown strong 5′-to-3′-exoribonuclease activity in cytoplasmic extracts of cultured mosquito cells (18, 25). When 5′-monophosphate-containing RNAs possessing the RVFV N 3′-terminal region were incubated in extracts from C6/36 Aedes albopictus cells, clear RNA decay intermediates accumulated, indicating stalling of XRN1 (Fig. 2A). As seen in Fig. 2B, the stable decay intermediate generated by XRN1 stalling is approximately the same size regardless of the source of enzyme. Note that faint but reproducible smaller bands representing decay intermediates from XRN1 stalling at a site further downstream than the primary stall site can be seen in both Figs. 1 and 2A. This is reminiscent of multiple XRN1-stalling sites that have been previously observed in flavivirus UTRs (17, 18, 23). Finally, to test whether the RVFV N 3′ UTR is capable of stalling XRN1 in living cells, the RVFV N 3′-terminal region was inserted downstream of GFP in a reporter construct and transfected into HEK 293T cells along with a control lacking the RVFV 3′ UTR. To enhance the decay of the GFP transcripts, we also transfected the cells with an shRNA targeting a site within the GFP ORF. We then looked for accumulation of a decay intermediate by Northern blotting. As seen in Fig. 2C, an apparent decay intermediate is observed for the reporter construct carrying the RVFV N 3′ UTR but not for the GFP control. In summary, we conclude that a 206-base fragment from the 3′-terminal region of the N mRNA of RVFV possesses the ability to stall the highly processive cellular 5′-to-3′-exonuclease XRN1.

**Ability of the N mRNA to stall XRN1 is conserved among the Phlebovirus family of the Bunyaviridae**

A striking feature of the structural element that stalls XRN1 in DENV is that it is conserved throughout all of the insect-borne flaviviruses (22). To determine whether the ability to stall XRN1 that we detected in the N 3′-terminal region of RVFV is a conserved property among other members of the phleboviruses, we cloned portions of the S segment (representing a similar portion of the 3′-terminal region of the N mRNA as was tested for RVFV) from two additional phleboviruses, Sandfly Fever Naples virus (26) and Heartland virus (27), into a reporter construct. RNAs containing a 5′-monophosphate were incubated with either purified recombinant XRN1 or mosquito cell-derived C6/36 cytoplasmic extracts. As seen in Fig. 3, RNAs containing the portion of the N 3′ UTR encoded by the intergenic regions of the S segment from both Sandfly Naples and Heartland viruses were capable of stalling XRN1. Although the portion of the N 3′ UTR tested from Sandfly Naples virus was more efficient at stalling XRN1 than the Heartland virus RNA segment, XRN1 stalling by the latter RNA segment was still highly reproducible. Therefore, we conclude that the ability of the 3′ UTR of the N mRNA appears to be a conserved property among members of the phlebovirus family.

**XRN1 stalling at the RVFV N 3′-terminal region also can repress enzymatic activity**

XRN1 stalling at the DENV 3′ UTR also results in repression of enzymatic activity (18). The repression of XRN1 by flavivirus 3′ UTRs is reversible and is presumably due to the slow release of the stalled enzyme from the RNA substrate (17). To determine whether the RVFV N 3′-terminal region can also repress XRN1 activity, control, DENV 3′ UTR, and RVFV N 3′-terminal region-containing cold competitor RNAs were generated and incubated along with a radiolabeled reporter RNA and recombinant XRN1. RNA reaction products were analyzed on an acrylamide gel at various time points post-XRN1 incubation to assess relative exonuclease activity in the presence of each competitor RNA. As seen in Fig. 4, the inclusion of a 20-fold molar excess of monophosphorylated unlabeled DENV competitor RNA relative to radiolabeled RNA substrate effectively reduced XRN1 activity during the time course, consistent with previously published results (18). In this experiment, 1,800 fmol of competitor RNA was used to repress 266 fmol of recombinant XRN1. Interestingly, the RVFV N 3′-terminal region competitor RNA repressed XRN1 to a similar level as DENV (Fig. 4).
We therefore conclude that RVFV N 3’-terminal region, similar to flavivirus 3’ UTRs, can both stall and repress the cellular XRN1 enzyme.

The structure in the RVFV N 3’ UTR that stalls XRN1 contains a G-rich domain

The flavivirus structure that stalls XRN1 is ~70 bases long with a three-helix junction at its core (15, 16). To map the XRN1-stalling site in the RVFV N 3’ UTR, we determined both the size of the decay intermediate based on migration in an acrylamide gel relative to size markers, as well as determined the stall site. The site of XRN1 stalling was approached by purifying the RNA decay intermediate, circularizing it, converting it to cDNA using reverse transcriptase, and cloning and sequencing the junction fragment. As seen in Fig. 5A, XRN1 is stalling at a series of adjacent sites within a highly G-rich region. The multiple G-tracts in this segment are reminiscent of a G-quadruplex-based structure (28). Interestingly, poly(G) tracts have been previously used successfully in yeast transcripts to stall XRN1 and map RNA decay pathways (29). To determine whether G-quadruplexes indeed play an important role in the formation or stabilization of the RNA structure in the RVFV N 3’-terminal region that stalls XRN1, we altered the monovalent cations in the reaction to either favor (KCl) or disfavor (LiCl) G-quadruplex formation. As seen in Fig. 5B (left panel), incubation of the RVFV N 3’-terminal region-containing RNA with XRN1 in the presence of LiCl as the source of monovalent cations in the reaction diminished but did not totally abrogate the ability of the RNA structure to stall XRN1 compared with when the RNA is incubated in the presence of KCl. In the presence of LiCl, the decay intermediate was reduced to 35.5 ± 7.8 of the level to which it accumulates in the presence of KCl. Stalling of XRN1 by the DENV 3’ UTR, which does not contain a G-rich tract, was not affected by LiCl (Fig. 5B, right). Quantitative analysis of the overall decay indicated that the inclusion of LiCl in the reaction had no effect on the ability of XRN1 to degrade RNAs (as indicated by the percent of input radiolabel lost over time). Therefore, we conclude that the RNA structure in the
The N mRNA is a major transcript made from the ambisense S segment of the RVFV/phyloivirus genome. Because viral ambisense RNA segments often contain large intergenic structures that presumably aid in the generation of mRNAs (30, 31), we wished to determine whether RNAs from other viruses that use the ambisense approach to gene expression also stall XRN1 as part of their strategy for host interaction. Arenaviruses have a bipartite genome and use an ambisense approach to generate mRNAs from both the genomic and anti-genomic version of both segments (Fig. 6A). To determine whether the 3′ UTRs of these mRNAs could stall XRN1, we inserted these non-coding segments from Junin virus into a reporter construct, generated RNA substrates with 5′-monophosphate ends, and incubated them with recombinant XRN1. As seen in Fig. 6B, the 3′ UTR segments of all four Junin virus mRNAs stalled XRN1 and formed decay intermediates. In the case of the GPC, NP, and Z transcripts, multiple decay intermediates were formed, reminiscent of the multiple sfRNAs formed by XRN1 action on flavivirus 3′ UTRs (23). To generalize this observation to the 3′ UTRs of other arenaviruses, we isolated the L or Z 3′ UTR from two novel snake arenaviruses (32). As seen in Fig. 6C, both 3′ UTR segments from the SN-68 and SN-90 boa-derived arenavirus transcripts were capable of stalling XRN1. Finally, we added arenavirus 3′ UTR segments as an unlabeled competitor RNA to XRN1 RNA decay reactions to determine whether they were capable of repressing enzymatic activity like the flavivirus and RVFV RNA segments. As seen in Fig. 6D, the Junin virus L and SN90 virus Z 3′ UTR segments were both capable of significantly repressing XRN1 activity. Therefore, we conclude that the ability to stall and repress XRN1 extends to transcripts of the Arenaviridae. Interestingly, the primary sequence of the 3′ UTRs of the arenaviruses are not G-rich, and thus likely possess a structure different from the ambisense phlebovirus RNAs that can stall the 5′-to-3′ exoribonuclease.

**Not every conserved viral 3′ UTR structure can stall XRN1**

Finally, we tested one additional conserved viral RNA structure that is found in 3′-untranslated regions for the ability to stall XRN1. Members of the Coronaviridae contain a conserved pseudoknot structure in their 3′ UTR (Fig. 7A) that has previously been shown to be required for efficient viral gene expression (33, 34). We inserted the sequence containing the conserved 3′ UTR from MERS coronavirus (MERS-CoV) into a reporter RNA and incubated it with XRN1. As seen in Fig. 7B, the RNA containing the MERS-CoV 3′ UTR was effectively degraded by recombinant XRN1 enzyme similar to the control RNA with no detectable intermediates. In addition, we failed to identify any RNA decay intermediates accumulating in total RNA from MERS-CoV-infected cells (data not shown). Therefore, we conclude that the role of the conserved 3′ UTR pseudoknot structure in MERS-CoV (and likely in other coronaviruses) does not involve stalling of the cellular XRN1 enzyme, suggesting alternative strategies for this family of viruses to evade the cellular RNA decay machinery.

**Discussion**

This study demonstrates that the 3′ UTRs of several phleboviruses and arenaviruses contain RNA regions that effectively stall and repress XRN1. This significantly extends the Flaviviridae strategy of using RNA structures to target XRN1 (17, 18, 23) to two other major families of mammalian viruses, the Bunyaviridae and Arenaviridae. Notably, two segmented RNA plant viruses Red clover necrotic mosaic virus (RCNMV) of the dianthoviruses of the Tombusviridae (19) and beet necrotic yellow vein virus (BNYVV) of the Benyviridae (20) also produce RNAs with the capability of stalling XRN1. RCNMV contains a 58-base sequence in the proximal portion of the 3′ UTR of its RNA 1 segment that stalls XRN1. BNYVV contains a conserved 20 nucleotide “coremin” motif that is part of a larger domain involved in stalling exonucleases and producing a non-coding RNA. This clearly emphasizes the importance and generality of RNA viruses having developed strategies to interface with the 5′-to-3′-exoribonuclease component of the cytoplasmic RNA decay machinery.

There are numerous reasons why XRN1 appears to be an attractive target for stalling and repression by viral RNAs. First, the enzyme plays a major role in a key pathway of RNA decay that is responsible for regulating 20–50% of gene expression (35–37). Therefore, stalling this enzyme will significantly affect host cell gene expression along with its ability to actively respond to the virus infection. Second, XRN1 has been implicated in mediating the apparent cross-talk between transcription rates and decay rates to maintain homeostasis of gene expression in cells (38, 39). Thus by targeting XRN1 an RNA virus may not only influence RNA decay but also may influence transcriptional responses by short-circuiting this gene expression buffering activity. Third, the XRN1 enzyme is cytoplasmic and thus readily accessible to RNA viruses that replicate in the...
XRN1 repression by segmented cytoplasmic RNA viruses

Figure 4. 3′-Terminal portion of the RVFV N mRNA also represses XRN1 activity. A 61-base radiolabeled reporter RNA derived from pGEM4 was incubated with recombinant XRN1 in the presence of a 20-fold excess of a cold competitor RNA derived from either pGEM4 (control RNA lanes), the DENV-2 UTR (DENV 3′ UTR lanes), or the 3′-terminal portion of the RVFV N mRNA (RVFV 3′ UTR lanes) for the times indicated. Radiolabeled reaction products derived from XRN1 acting on the reporter RNA were resolved on a 5% acrylamide gel containing urea and visualized by phosphorimaging. Gels from three independent experiments were quantified, and results are shown graphically in the bottom panel. The asterisk represents a p value of < 0.001 at both time points for viral 3′ UTR/fragments compared with the control as determined using Tukey’s multiple comparisons test as a post hoc test. Graphical data are derived from three independent experiments. The gel in the top portion is representative data from these same experiments.

Figure 5. XRN1 stalls within a G-rich portion of the RVFV N 3′-terminal fragment. A, top, 5 μg of unlabeled RNA containing the RVFV 3′-terminal segment was incubated with recombinant XRN1 for 15 min, and reaction products were resolved on a 5% acrylamide gel containing urea and visualized by SYBR Green staining. The arrow at right indicates the size of the stable decay intermediate as calculated by migration relative to size markers from multiple gels. nts, nucleotides. Bottom, RNA decay intermediates from the top panel were excised, circularized, and reverse-transcribed, and the 5′–3′-junction fragment was cloned, and nine were sequenced. Sequence of the RVFV 3′-terminal fragment is presented with the position of the 5′ end of the decay intermediates (i.e., the XRN1 stall site) indicated by the arrows. The larger arrows indicate two clones out of the nine sequenced indicated that Xrn1 stalled at that site. The underlined nucleotide is the approximate stall site indicated by sizing of decay intermediates on acrylamide gels as described in the top panel. B, radiolabeled RNA containing the RVFV 3′-terminal segment (left panel) or the 3′ UTR of DENV (right panel) was incubated with recombinant XRN1 in the presence of 100 mM of the designated monovalent cation for the times indicated. Radiolabeled reaction products derived from XRN1 acting on the reporter RNA were resolved on a 5% acrylamide gel containing urea and visualized by phosphorimaging. The values below the lanes indicate the amount of overall complete RNA degradation observed at the various time points as a quantitative measure of the ability of XRN1 to degrade through any structural elements. All data shown are representative of three independent experiments. Nucleotide size markers are indicated to the left of the gels.
Transporting RNAs and proteins, particularly ones of substantial size, into the nucleus requires the assembly of specialized complexes (40), and thus it greatly simplifies the dynamics of the infection to avoid a need for nuclear import. Next, XRN1 is an essential protein, and thus targeting it can significantly influence cytopathology and pathogenesis (41, 42). Finally, 5'-to-3' exoribonucleases of the XRN family have been shown to directly impact virus replication and RNA evolution of plant viruses. XRN4 has been implicated in causing the degradation of tombusvirus RNAs as well as suppressing recombination of these plant RNA viruses (43, 44). Thus targeting XRN enzymes has a direct impact on the dynamics of virus RNA production.

Stalling and repression of XRN1 by viral RNA structures can have significant biological implications for the dynamics of phlebovirus and arenavirus infections. First, stalling of XRN1 has been associated with flavivirus pathogenesis/cytopathology (18, 23). Thus, it is likely that disruptions in cellular regulation of mRNA stability associated with XRN1 stalling may also have implications in the pathology of arenavirus and phlebovirus infections as well. Next, both phleboviruses and arenaviruses rely on cap snatching from host cell mRNAs in the cytoplasm to initiate transcription (45). Therefore, by repressing XRN1, this may feedback and co-repress decapping as it does in flavivirus infections (17, 18), allowing a sufficient population of capped cellular mRNAs to be maintained throughout infection for effective initiation of viral transcription. Hence, stalling XRN1 may be part of the molecular arms race between host and virus. It is important to note, however, that assessing the direct contribution stalling XRN1 has on cellular gene expression in phlebovirus- or arenavirus-infected cells is not straightforward due to the fact that both viruses naturally steal caps from cellular mRNAs to initiate transcription of their own genes. Thus, the interplay between cap snatching, which effectively decaps mRNAs and should lead to decreased RNA stability, and repression of XRN1, which would lead to increased RNA...
stability, makes a mechanistic interpretation of changes in mRNA half-lives in phlebovirus- or arenavirus-infected cells very challenging. Future studies using targeted knockdowns/knock-outs of specific mRNA decay enzymes in conjunction with virus RNA replicons should be able to make headway in this regard.

XRN1 stalling may lead to the generation and accumulation of small non-coding RNA decay intermediates in phlebovirus- and arenavirus-infected cells (Fig. 2B). Alternatively, these RNA decay intermediates may fail to accumulate as they could be co-targeted by other decay pathways, including 3-to-5’ decay by the exosome/DIS3L2 or by endonucleases. If they accumulate to significant levels in infected cells, these small RNAs, like flavivirus sfRNAs, could have interesting functions in virus biology. They could, for example, play a role in combating the RNAi (46, 47) and/or interferon responses (48, 49) by accumulating to high amounts and serving as a sponge for key factors that have a propensity for binding to double-stranded RNAs (50). Thus, future studies in this area should be particularly interesting.

The novel RNA elements that can stall XRN1 uncovered in this study raise the question as to whether these RNAs represent variants of the same three-helix-containing knot-like structure possessed by flavivirus 3’ UTRS (15, 16) or whether RVFV and arenaviruses use novel structural strategies for stalling XRN1. The observation that the coronavirus 3’ UTR pseudoknot structure does not stall XRN1 reinforces the idea that structures capable of stalling XRN1 are highly specialized. Based on the results shown in Fig. 5, stalling of XRN1 by the RVFV N 3’-terminal region appears to be mediated by G-rich motifs. Although the G-rich motif is maintained in the N 3’-terminal regions of the related Sandfly Fever Naples phlebovirus (Fig. 3), it is not found in the N 3’ UTR of the Heartland virus that can stall XRN1 (albeit much less efficiently). The contribution of G-rich tracts observed in RVFV is reminiscent of poly(G) tracts that have been previously successfully used in yeast system to stall XRN1 (29). Poly(G) stretches, however, fail to effectively stall XRN1 in most other eukaryotic systems to date (25). Thus, the RVFV G-rich region likely is highly specialized and/or works in conjunction with another structural element to effectively impede XRN1 movement toward the 3’ end of these viral transcripts. Future structural analyses will be needed to shed additional insight into the exact contribution of the G-rich motifs to XRN1 stalling.

In summary, this study indicates that several mammalian RNA virus families beyond the Flaviridae have evolved what
appear to be independent RNA structural elements that are capable of stalling and transiently repressing the cellular XRN1 exoribonuclease. This suggests one or more of the following intriguing broad implications. First, XRN1 could be a key cytoplasmic restriction factor that RNA viruses must overcome to establish a productive infection. Second, these RNA structures could be a major virulence factor of these virus families as XRN1 repression causes dramatic changes in cellular mRNA half-lives and subsequent ability of the cell to respond to infection (8). Finally, the creation of stable decay intermediates by stalling of XRN1 can also generate novel non-coding RNA species from viral genomes in the absence of sub-genomic promoters, thereby increasing the diversity of gene expression from viral RNA genomes of limited size. Collectively, these broad implications of XRN1 stalling on viral RNA biology uncovered in this study uncover interesting implications on virus–host interactions as well as strategies of virus gene expression.

**Experimental procedures**

### Plasmids, PCR templates, and RNAs

DNA templates to generate RNAs by *in vitro* transcription were generated by cloning PCR products into pGEM4 (Promega) or pGEM60 (9) vectors or, alternatively, generated directly from the PCR product by incorporating an SP6 promoter into the DNA oligonucleotide primers used. The plasmid to produce the RNA containing the upstream portion of the DENV 3’ UTR was described previously (17). PCR products from the 3’-terminal regions of the RVFV L, G, NSs, and N mRNAs, as well as the 3’-terminal regions from the Junin Virus GPC, NP, Z, and L mRNAs, were obtained from Brian Gowen (Utah State University). PCR products from the arenavirus SN90 Z and SN68 L 3’ UTRs were generated from plasmids received from Dr. Mark Stenglein (Colorado State University) (32). The MERS-CoV 3’ UTR was obtained by RT-PCR from total infected cell RNA using the primers 5’-GCCTGGTCCACATGATGGGTCA and 5’-TTTGCACATCTATA-TaggcatatcTa. DNA oligonucleotides containing the 3’-terminal fragments of the Sandfly Naples and Heartland virus N mRNA were obtained from IDT. The full sequences of these oligonucleotides and PCR products generated are listed in Table S1. Except for the RVFV G fragment, PCR products were inserted into the pGEM4 or pGEM60 at the PstI and HindIII sites. Because of incompatibility with the HindIII restriction site, the RVFV G fragment was inserted into pGEM60 at the PstI and SphI sites. PCR products were purified using the Wizard SV gel and PCR clean-up system (Promega) prior to use as transcription templates. Finally, the RVFV N 3’-terminal fragment PCR products were also subcloned into the NotI site of peGFP-N1 (Clontech). A portion of the eGFP 3’ UTR (1402–1520 nucleotides, GenBank™ U55762.1) was subcloned from peGFP-N1 into the EcoRI and HindIII sites of pGEM4 to generate peGFP-UTR. This plasmid was used to generate a probe for Northern blotting. Plasmids were isolated using the Zymopure Plasmid Maxiprep Kit per the manufacturer’s instructions (Zymo Research).

Internally radiolabeled, 5’-monophosphorylated RNAs were generated using SP6 polymerase as described previously (17, 51). All pGEM4 or pGEM60 constructs containing viral 3’-terminal fragments were linearized with HindIII to create transcription templates except for the RVFV G 3’-terminal fragment template that was linearized with SphI. pGEM4 control RNAs were linearized with EarI (to generate 168-base cold competitor RNAs) or radiolabeled control RNA) or with HindIII (to generate 61-base radiolabeled reporter RNA in Fig. 4). The DENV 3’ UTR plasmid was linearized with EarI. All RNAs were purified on denaturing acrylamide gels prior to use.

### In vitro XRN1 1 decay assays and competitions

*In vitro* 5’-to-3’ decay assays and competitions were performed essentially as described in Moon et al. (18) using HeLa S100 cytoplasmic extracts (52) under conditions that favor 5’-to-3’ decay (53) or with recombinant *K. lactis* Xrn1 (residues 1–1245) (54) purified from in *Escherichia coli* BL21 cells. For XRN1 decay assays performed to search for decay intermediates, ~85 fmol of each reporter RNA was used. For RNA competition assays, ~85 fmol of a pGEM4 reporter RNA was incubated with a 20-fold molar excess of the pGEM-4 control competitor or the virus-derived DENV, RVFV N, SN90 Z, or JUNV L 3’-terminal fragment RNAs. RNA reaction products were separated on 5% denaturing polyacrylamide gels and visualized by phosphorimaging. The average of the percent RNA remaining in each time point from three independent experiments is reported. Significance was determined using a two-way analysis of variance, and *p* values were generated using Tukey’s multiple comparisons test as a post hoc test. The asterisk represents a *p* value < 0.001 at both time points for viral 3’ UTR compared with the control.

For mapping the 5’ end of decay intermediates, *in vitro* transcription were performed using the MEGAscript® SP6 kit (Ambion) with the RVFV-N 3’-terminal fragment construct described above and a 10-fold excess of GMpT createmonomethylphosphorylated transcripts. Following gel purification to remove excess unincorporated nucleotides, 5 μg of each RNA was incubated with recombinant *K. lactis* XRN1 for 15 min at 37 °C. Reactions were stopped by addition of 400 μl of HSCB (25 mM Tris-HCl, pH 7.6, 400 mM NaCl, 0.1% SDS) followed by phenol/chloroform extraction and ethanol precipitation. Remaining RNA was then separated on a 5% denaturing polyacrylamide gel and stained for 30 min with SYBR™ Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific). The indicated decay intermediate band was purified from the gel and circularized using T4 RNA ligase. Ligation products were reverse-transcribed using a reverse primer (5’-AGCATGATGGGGAGAAGA), and products were then amplified using Pfu Ultra II Fusion HS DNA polymerase (Agilent) and cloned into the pGEM T-Easy vector (Promega) for transformation of DH5α cells. Colony screens were performed by PCR with PfuUltra II Fusion HS DNA polymerase using RVFV-N-specific primers 5’-AGCCTTAACCTAGCCTTAACCT-3’ to amplify the junction between the 3’ and 5’ RNA ends. Ten positive clones were sequenced. The resulting mapped 5’ ends of the sequenced RNAs were aligned to the RVFV-N 3’-terminal fragment.
XRN1 repression by segmented cytoplasmic RNA viruses

Cell-based assays: transfection and Northern blotting

For transfection experiments, 293T cells were grown in DMEM with 10% newborn calf serum (Peak Serum). For transfection, equal quantities of peGFP-N1 or peGFP-N1 containing RENV N 3’ UTR were co-transfected with an shRNA (5’-UAC-AACAGCCCAACAGCUCAU; Sigma SHC005) targeting the GFP open reading frame into 293T cells using jetPRIME (Polyplus) according to the manufacturer’s recommendations. Total cellular RNA was collected at 48 h post-transfection using the Zymo Quick-RNA Miniprep kit (Zymo Research). Northern blotting was performed using an antisense probe to the eGFP sequence formed using a nick translation of T7 RNA polymerase from EcoRI-linearized peGFP-UTR. Hybridized RNA fragments were visualized by phosphorimaging. The depicted image is representative of at least three independent experiments.

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