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Standing your ground to exoribonucleases: Function of *Flavivirus* long non-coding RNAs

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ABSTRACT

Members of the *Flaviviridae* (e.g., Dengue virus, West Nile virus, and Hepatitis C virus) contain a positive-sense RNA genome that encodes a large polyprotein. It is now also clear most if not all of these viruses also produce an abundant subgenomic long non-coding RNA. These non-coding RNAs, which are called subgenomic flavivirus RNAs (sfRNAs) or Xrn1-resistant RNAs (xrRNAs), are stable decay intermediates generated from the viral genomic RNA through the stalling of the cellular exoribonuclease Xrn1 at highly structured regions. Several functions of these flavivirus long non-coding RNAs have been revealed in recent years. The generation of these sfRNAs/xrRNAs from viral transcripts results in the repression of Xrn1 and the dysregulation of cellular mRNA stability. The abundant sfRNAs also serve directly as a decoy for important cellular protein regulators of the interferon and RNA interference antiviral pathways. Thus the generation of long non-coding RNAs from flaviviruses, hepaciviruses and pestiviruses likely disrupts aspects of innate immunity and may directly contribute to viral replication, cytopathology and pathogenesis.

1. Introduction

The transcripts of RNA viruses serve numerous functions in addition to directing protein synthesis and serving as genomes/genomic templates. An examination of the various roles played by untranslated regions present at the 3′ ends of viral transcripts illustrates the interesting breadth of non-coding activities of these RNAs. First, the 3′ untranslated region (UTR) of viral transcripts often contains identifiable domains that play key roles in facilitating both local and long range structural interactions of the RNA. Local structures in the 3' UTR can serve a variety of functions, including influencing the formation of binding sites for regulatory proteins (Paingankar and Arankalle, 2015), ribosome/translation factor recruitment (Bai et al., 2013; Sharma et al., 2015) and RNA stabilization (Weil et al., 2009). Long-range 3′ UTR structural interactions, particularly with the 5' UTR (Fricke et al., 2015), can play important roles in viral replication and translation (Nicholson and White, 2014; de Borba et al., 2015). The primary sequence of the viral 3′ UTR often contains target elements for cellular RNA binding proteins (Oakland et al., 2013; Dong et al., 2015). These protein-RNA interactions can play key roles in virus replication, gene expression and host-virus interactions. Given the huge amount (up to ~10^5 copies or more) of viral RNA present in a cell during infection, it is also possible that viral UTRs can serve as sponges for cellular RNA binding proteins and micro-RNAs (miRNA) s that can disrupt cellular post-transcriptional regulation of gene expression (Barnhart et al., 2013). The 3′ UTR also has the capacity to contain rather novel regulatory features that are either naturally present or engineered. MiRNA binding sites present in the 3′ UTR, for example, can have a major effect on virus biology in terms of affecting tissue-specificity of infection and pathogenesis (Bogerd et al., 2014; Trobaugh et al., 2014). MiRNA sites can also be engineered into 3′ UTRs to regulate virus gene expression on a cell-specific basis and perhaps increase the safety/efficacy of vaccine vectors (Langlois et al., 2013; Tsetsarkin et al., 2015). Riboswitches, RNA structures that can signal through an effector module to alter transcript function upon the binding of small molecules (Mellin and Cossart, 2015), can also be effectively engineered into a viral 3′ UTR (Bell et al., 2015). Finally, it should not be overlooked that the 3′ UTR also contains important and often highly conserved promoter elements that aid in viral polymerase binding and replication (Gebhard et al., 2011). Thus it should not be surprising that viral 3′ UTRs often contain conserved evolutionary signatures within a virus family (Gritsun et al., 2014) and have a demonstrable association with viral virulence (Chen et al., 2013; Sakai et al., 2015; Manokaran et al., 2015). It is now coming to light that the non-coding regions of a viral RNA may have additional unforeseen functions beyond conventional UTR-related activities.

Cellular long non-coding RNAs (lncRNAs), which are arbitrarily defined as non-coding transcripts >200 nucleotides in length,

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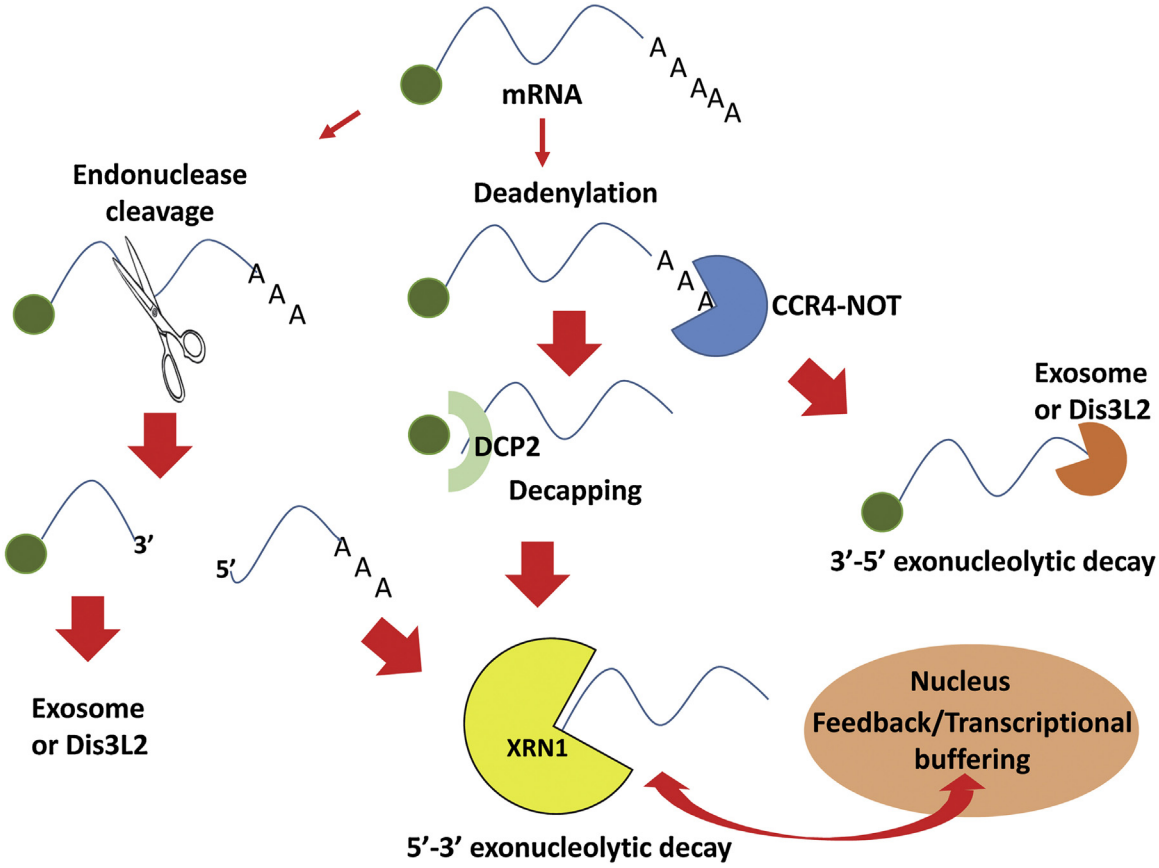

Fig. 1. An overview of the general pathways of cytoplasmic mRNA decay in eukaryotic cells.
The degradation of mRNAs is generally initiated by deadenylation via the CCR4-NOT deadenylase complex (as well as some contribution from other deadenylases). As seen in the center portion of the diagram, deadenylated mRNAs then generally undergo decapping by DCP2 to generate a 5′ monophosphate containing RNA. This decapped mRNA is then rapidly and processively degraded by the 5′-3′ exonuclease Xrn1. Xrn1-mediated mRNA decay can feedback in some fashion to the nucleus where it helps to buffer mRNA synthesis at the level of transcription to maintain cellular homeostasis. As seen on the right side of the diagram, deadenylated mRNAs can also be degraded by an alternative 3′-5′ exonucleolytic pathway mediated by the Dis3 enzyme associated with the RNA exosome or an independent exonuclease called Dis3L2. Finally (left side of the diagram), if an mRNA is subjected to endonucleolytic cleavage (e.g., by RNAi-mediated decay, RNAse L digestion, etc.), the newly formed and fully accessible 5′ monophosphate and 3' OH containing RNA fragments are rapidly degraded by the two exonucleolytic pathways as indicated.

are extensively generated from the cellular genome and have been implicated in the regulation of fundamental aspects of cell biology such as proliferation, differentiation and apoptosis (Bassett et al., 2014; Wilusz, 2015). While the study of lncRNA-mediated mechanisms is still in its infancy, the transcripts have been implicated in fundamental cellular processes such as chromatin remodeling (Kawaguchi et al., 2015), transcriptional enhancers (Pefanis et al., 2015), splicing (Gonzalez et al., 2015), mRNA stability (Gong and Maquat, 2011), translation (Ruiz-Orera et al., 2014; Essers et al., 2015), miRNA function (Dhir et al., 2015), and subcellular organization (Quinodoz and Guttman, 2014) through interactions with DNA, proteins, and other RNAs. Taking advantage of the key role played by these transcripts in the cell, viruses have developed ways to usurp cellular lncRNAs – or make some of their own (Tycowski et al., 2015) – to promote dysregulation of cellular functions to enhance viral infections.

Interestingly, it is now clear that in addition to conventional protein-encoding transcripts, all members of the *Flaviviridae* likely produce a long non-coding RNA (lncRNA) that impacts viral biology and host-virus interactions (Roby et al., 2014). These RNAs have been referred to as sfRNAs (subgenomic flavivirus RNAs) or xrRNAs (Xrn1–resistant RNAs) (Pijlman et al., 2008; Chapman et al., 2014a). Arthropod-borne flaviviruses produce large amounts of a ~300-500 base sfRNA which corresponds to the 3′ UTR region of the genomic RNA (Wengler et al., 1978; Lin et al., 2004; Pijlman et al., 2008). Hepaciviruses (e.g., Hepatitis C virus (HCV)) and pestiviruses (e.g. bovine viral diarrhea virus (BVDV)) produce a long subgenomic RNA with a 5' end that maps 30–120 bases from the original 5′ end of the genomic RNA (Moon et al., 2015). The goal of this review is to provide an overview of our understanding of the generation and functions of these flaviviral lncRNAs as well as speculate upon additional roles for these long non-coding transcripts in virus biology and pathogenesis.

2. Flaviviral lncRNAs are generated via stalling of a cellular RNA decay enzyme

Transcription is not the sole way to regulate gene expression in eukaryotic cells. The degradation of cellular RNAs plays a significant role in regulating both the level of mRNAs (Braun and Young, 2014) as well as quality-controlling gene expression to remove unwanted or deleterious transcripts (Miller and Pearce, 2014). Since viral mRNAs in the cytoplasm clearly fall into the 'unwanted' category of transcripts, the cellular RNA decay machinery also actively acts upon virus RNAs during infection (Moon and Wilusz, 2013). We envision this attack on viral RNAs early in infection by the cellular RNA decay machinery as an aspect of innate immunity that attempts to control the infection prior to activation of classical innate pathways such as interferon. Cellular mRNAs are generally degraded in a two-step process (Schoenberg and Maquat, 2012) (Fig. 1). First, the poly(A) tail is shortened/removed by regulated deadenylase enzymes followed by decapping mediated by

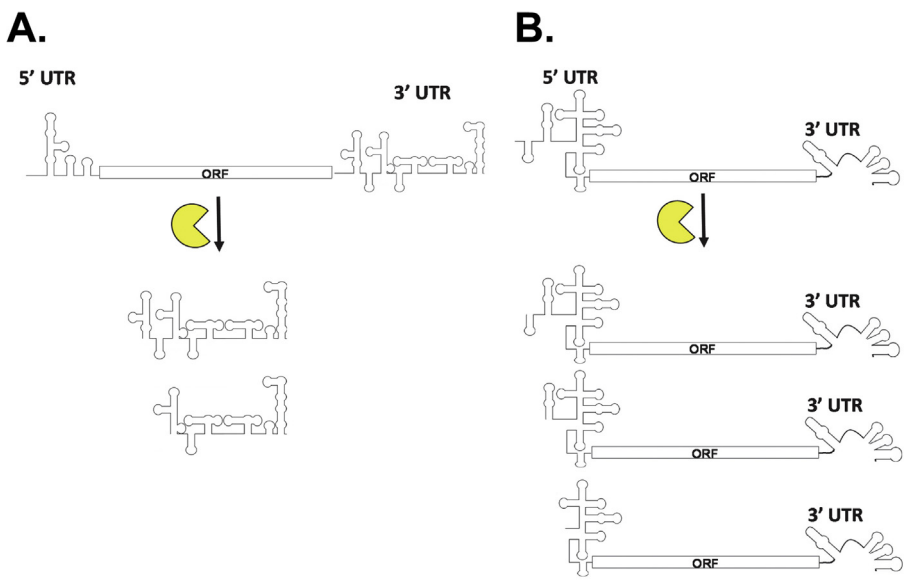

Fig. 2. LncRNAs formed by Xrn1 stalling on flavivirus transcripts.
Panel A. Xrn1-mediated exonucleolytic decay of the positive sense genomic RNA of arthropod-borne flaviviruses (e.g., Dengue virus or West Nile virus) generates a set of sfRNAs from the 3′ UTR. The 5′ end of each of the depicted RNA products represent Xrn1 stalling just 5′ to knot-like tertiary structures formed by higher order folding of the depicted stem loops (Roby et al., 2014). Panel B. Xrn1-mediated exonucleolytic decay of the positive sense genomic RNA of HCV generates a set of sfRNAs from the 5′ UTR. Reconstitution assays using recombinant Xrn1 generate the three RNA products depicted. The 5′ end of each of the RNA products represent Xrn1 stalling just 5′ to the indicated secondary structure landmarks (Moon et al., 2015).

DCP2 or one of a number of alternative decapping enzymes recently described (Song et al., 2013). The body of the RNA is then degraded in a 5′-3′ fashion by the exoribonuclease Xrn1 (Nagarajan et al., 2013). Alternatively, deadenylated transcripts can be removed by 3′-5′ decay mediated by the cytoplasmic exosome (Robinson et al., 2015) or Dis3L2 (Reis et al., 2013).

Viral subgenomic RNAs are classically generated by internal promoters located on the viral genome. However flaviviruses generate subgenomic RNAs in a rather novel way using the cellular RNA decay machinery. To generate subgenomic flavivirus RNAs, the Xrn1 5′-3′ exoribonuclease stalls on RNA structures and creates stable RNA decay intermediates that accumulate to high levels in infected cells. It is very rare to observe intermediates in RNA decay pathways, particularly outside of *Saccharomyces cerevisiae* where even engineered transcripts containing poly(G) structures fail to afford reproducible stalling of exonucleases to generate decay intermediates (Muhlrad et al., 1994).

Xrn1 is an essential, 1706 amino acid (in humans) cytoplasmic 5′-3′ exoribonuclease. Defects in Xrn1 are associated with developmental abnormalities in a variety of organisms (Jones et al., 2012). A related 5′-3′ exonuclease, Xrn2, is predominantly localized in the nucleus and has a very similar enzymatic domain (Miki and Großhans, 2013). The catalytic N-terminal domain of *Drosophila* Xrn1 has been crystallized (Chang et al., 2011; Jinek et al., 2011) and, given the high sequence conservation of this region, the structure is likely to be similar to that of Xrn1 from other organisms (Nagarajan et al., 2013). RNAs must move through a narrow channel to reach the active site, requiring at least four single-stranded bases at the 5' end to reach the enzymatic core. Basic residues of the core bind and position the 5′ monophosphate of the RNA into the active site for Mg^{2+}-mediated cleavage, followed by processive ratcheting of the RNA through the core to provide rapid 5′-3′ decay. The large C-terminal portion of Xrn1 is likely rather flexible and has not yet been crystallized. The mechanistic role of the C-terminal portion of Xrn1 in RNA decay is currently unclear, although it is nonetheless important as mutations/deletions in this region do influence Xrn1 activity (Bashkirov et al., 1995). Curiously, Xrn1 has also been recently implicated in the yeast *S. cerevisiae* as a key player in feeding back information on RNA decay to the nucleus to modulate transcriptional output and buffer gene expression (Haimovich et al., 2013; Sun et al., 2013). Data from several analyses of global gene expression suggest that such buffering between transcription and RNA decay could exist in mammalian cells (Lee et al., 2012; Neff et al., 2012), although it may not be as dramatic as in the yeast system (Maekawa et al., 2015).

Xrn1 plays a central role in both a major pathway of mRNA decay as well as the networking between mRNA decay and other cellular processes. Based on work in the flavivirus system, Xrn1 can also be used to generate novel 5′ ends of transcripts to increase the breadth of viral gene expression through the generation of flaviviral subgenomic (or sf/xr) RNAs (Fig. 2). For all arthropod-borne flaviviruses investigated to date, Xrn1 stalling occurs near the beginning of the 3′ UTR downstream of the translation stop codon. This generates an sfRNA that represents most of the 3′ UTR of the flavivirus genomic RNA (Pijlman et al., 2008; Silva et al., 2010; Funk et al., 2010; Moon et al., 2012). Hepaciviruses (HCV) and pestiviruses (BVDV) of the *Flaviviridae* cause stalling of the Xrn1 enzyme in the 5′ UTR either at or upstream of the core portion of the Internal Ribosome Entry Site (IRES) element (Moon et al., 2015). Therefore lncRNAs made by the arthropod-borne flaviviruses are in the 300–500 base range while the HCV/BVDV lncRNAs are much longer, only 15 −~130 bases shorter than the full length ~9.6–12.5 Kb genomes. While the two types of lncRNAs have a common mechanism for their generation, the differences in size imply that they may have distinct roles in the flaviviral life cycle.

3. How do flavivirus RNAs stall the cellular Xrn1 enzyme?

Xrn1 is a highly processive exoribonuclease that appears to rarely, if ever, stall while degrading cellular mRNAs. The addition of poly(G) tracts to reporter RNAs was instrumental in trapping mRNA decay intermediates to map decay pathways in *S. cerevisiae* (Muhlrad et al., 1994) and $G_{30}C_{30}$ sequences generated decay intermediates in trypanosomes (Haile et al., 2003). However, the insertion of poly(G) tracts or similar sequences into mammalian mRNAs to generate strong secondary structures

has not been reported to trap any reproducible exonucleolytic decay intermediates to date. In addition, recombinant Xrn1 exonuclease is routinely used to degrade uncapped RNAs such as rRNAs in RNA-seq protocols, indicating that typical RNA secondary/tertiary structures such as those found in Pol I transcripts do not present a barrier for the enzyme. Flaviviruses, therefore, must have evolved a rather special RNA structure to stop Xrn1 in its tracks.

Initial structural predictions demonstrated a conserved pseudoknot structure in the area of Xrn1 stalling in the 3′ UTR of arthropod-borne flaviviruses (Funk et al., 2010; Silva et al., 2010). Deletion of key 3′ UTR segments to prevent pseudoknot formation also prevented Xrn1 stalling and sfRNA generation. It was not, however, clear whether the pseudoknot alone was sufficient to stall Xrn1 as such RNA structural elements are not uncommon (Brierley et al., 2007). Recent RNA crystallization studies of a 68 base fragment of the 3′ UTR region of Murray Valley Encephalitis virus provided a clear picture of the novel structure involved in Xrn1 stalling (Chapman et al., 2014b). The RNA forms a three-helix junction comprised of interwoven pseudoknots with the 5′ end of the RNA passing through the ring-like structure that is formed. This knot-like structure would be largely impervious to unwinding by an enzyme moving in the 5′-3′ direction (like Xrn1), but would be fully passable by an enzyme moving in the opposite 3′-5′ direction (like viral RNA-directed RNA polymerase). A detailed structural model for how Xrn1 is stalled by this structure can be found in Chapman et al. (2014b). The pseudoknots are not essential for the structure, but clearly enhance its stability. Many arthropod-borne flaviviruses contain back-to-back duplicated versions of this three-helix junction structure at the start of their 3′ UTR (Fig. 2), thus providing a back-up to stall Xrn1 if it can get through the first knot-like domain due to structural breathing, or altered conformation caused by RNA binding proteins, etc.

On HCV and BVDV RNAs, Xrn1 stalls in the 5′ UTR at and around secondary structural elements that precede the core of the IRES element (Moon et al., 2015) (Fig. 2). These Xrn1 decay intermediates can be observed in cell-free reconstituted systems as well as in analyses of viral RNAs in infected cells (Li et al., 2013; Moon et al., 2015). Notably, the HCV IRES has been shown to contain a conserved pseudoknot involving the IIIf stem along with domain IV that contains the AUG start codon (Lavender et al., 2010). While the global structure of the pseudoknot is important for translation, mutational analyses using compensatory mutations produced conflicting results. This could imply that the structure is more complex than a simple pseudoknot (Kieft et al., 2001). Modeling of the BVDV IRES element also indicates a conserved pseudoknot structure in Helix 3 (Burks et al., 2011). Whether or not these pseudoknot-containing structures actually play a role in stalling Xrn1 in the 5′ UTR of HCV and BVDV awaits further experimentation. Finally, while Xrn1 clearly stalls in the 5′ UTR of HCV (Moon et al., 2015), there has been a report that the related Xrn2 5′-3′ exonuclease may also play a role in HCV RNA decay (Sedano and Sarnow, 2014). Although a recent study suggests that the effects of Xrn2 that are observed on HCV RNAs could be indirect (Li et al., 2015), it will be interesting to see if this alternative 5′-3′ exoribonuclease can also be stalled by 5′ UTR structure.

4. Xrn1 repression as a result of flavivirus-mediated stalling on RNA structures

In addition to stalling Xrn1-mediated degradation of flavivirus transcripts, the embedded RNA structures also appear to repress the enzyme. Several lines of evidence support this conclusion. First, competition assays using reconstituted systems demonstrate a specific reduction of Xrn1 activity in the presence of a cold competitor RNA containing the three-helix junction structure (Moon et al., 2012, 2015). This repression required that the competitor RNA possess a 5' monophosphate and thus be susceptible to Xrn1-mediated decay. Capped RNAs containing the three-helix junction structure failed to affect Xrn1 activity in these assays (Moon et al., 2012). Second, an increase in the level of uncapped mRNA was detected in a number of flavivirus-infected cells (Moon et al., 2012, 2015). Since decapped RNAs are normally rapidly degraded by Xrn1, this provides *in cellulo* evidence for Xrn1 repression. Finally, the results of biochemical experiments in which Xrn1 was titered into a reaction containing a fixed amount of competitor RNA were consistent with the three-helix junction structure-containing RNA acting as a reversible inhibitor (Moon et al., 2015). Thus the simplest model for Xrn1 repression is that the enzyme is released rather slowly when it stalls on a flavivirus RNA that contains a repressive structure (Fig. 3). Slow release could be mediated by additional interactions of the RNA structure with portions of Xrn1 outside of the enzymatic channel (e.g., the C-terminal domain), although this remains to be experimentally assessed.

Based on current models of mRNA decay, repression of Xrn1 activity in cells would be expected to result in accumulation of decapped mRNAs as cells have few other means to remove cytoplasmic RNA with 5′ monophosphates (Jinek et al., 2011; Chang et al., 2011). However, in Dengue virus type 2 (DENV2), Kunjin virus (KUNV) or HCV-infected cells it appears that not only is Xrn1 activity down, but the entire 5′-3′ decay pathway is also repressed resulting in the stabilization of numerous cellular mRNAs and the increased accumulation of capped and polyadenylated cellular mRNAs that appear to be fully translatable. The underlying mechanism for this complete shut-down of a major RNA decay pathway in infected cells is unclear, but could be related to protein-protein interactions between Xrn1 and other RNA decay factors (e.g. Xrn1 can interact with Dcp1) (Braun et al., 2012) and/or the co-localization of RNA decay factors in P bodies (Chen and Shyu, 2013).

The repression of the major pathway of RNA decay in flavivirus-infected cells results in a dramatic dysregulation of cellular gene expression (Moon et al., 2012, 2015). Cellular mRNAs that are normally regulated by degradation now accumulate in cells – and the impact on normally short-lived mRNAs is, as expected, the greatest and most significant. Since many cytokines, growth factors, cell cycle regulators and oncogenes are encoded by short-lived mRNAs (Rigby and Rehwinkel, 2015), the generation of sf/xrRNAs may be a major factor in pathogenesis. Since immune regulation relies on tight control of cytokine/chemokine expression, dysregulation of the decay of these mRNAs could perhaps contribute to the cytokine storm seen during some flavivirus infections. Along the same line, the prolonged expression of oncogenes during persistent HCV infection due to the stabilization of their normally very short-lived mRNAs could contribute to the development of hepatocellular carcinoma. Finally, since Xrn1 may be involved in maintaining the homeostasis of cellular gene expression by being a key factor in buffering relative levels of transcription and RNA decay, virus-induced repression of Xrn1 may render the cell less effective at responding to the infection, thus enhancing overall pathogenesis. KUNV variants that cannot generate full-length, abundant sfRNA do indeed shown reduced levels of pathogenesis and cytopathology (Pijlman et al., 2008).

5. Functions of Xrn1-generated sfRNAs and HCV xrRNA

The sfRNA products of Xrn1 stalling accumulate to relatively high levels in flavivirus infections (Pijlman et al., 2008; Manokaran et al., 2015). In addition, HCV RNA with a shortened 5' UTR

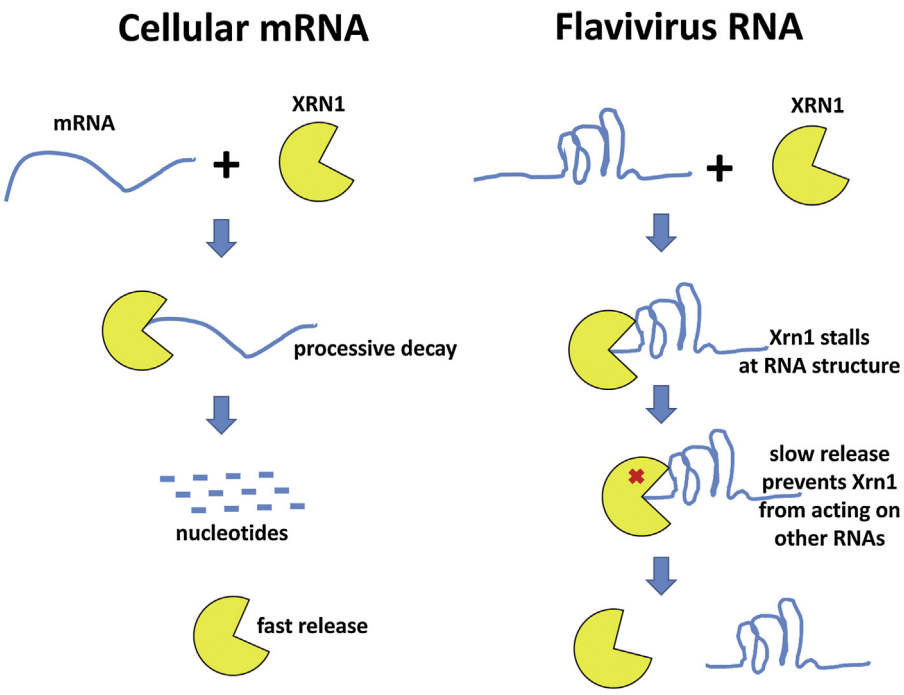

Fig. 3. Xrn1 stalling at flaviviral RNA structures represses enzymatic activity in a reversible fashion.
Left panel: cellular mRNAs are degraded rapidly in a processive fashion by Xrn1. The enzyme is naturally released when it is finished degrading the RNA and can rapidly begin degrading other RNA substrates. Right panel. Xrn1 stalls at knot-like RNA structures while degrading flavivirus transcripts and is only slowly released from the RNA substrate. This results in reversible inhibition of Xrn1 while the enzyme remains associated with the flaviviral RNA decay intermediate. Once Xrn1 is released, the enzyme can go onto degrade other RNAs and the long non-coding flavivirus RNA can perform its function(s) (e.g., as a decoy for cellular RNA binding proteins in the interferon pathway).

due to Xrn1 stalling also accumulates in infected Huh7 cells (Moon et al., 2015). Given their abundance, these transcripts might play key roles as viral-derived lncRNAs in the cell. While this is currently an active area of investigation, several indications of functional relevance of these transcripts have been reported.

Since sfRNAs are only generated from the 3′ UTR of arthropod-borne flaviviruses, they may play a role in viral-vector interactions. RNA interference is a major antiviral mechanism in insects as these viral vectors lack conventional mammalian-style immune responses (Blair, 2011). Pijlman and colleagues have demonstrated that sfRNA serves as a mild repressor of RNAi responses in both insect and mammalian cells (Schnettler et al., 2012; Schnettler et al., 2014). While not a very efficient RNAi repressor, sfRNA may shut down the RNAi response to infection just enough for the virus to replicate to sufficient levels in the insect without significantly compromising the health of the vector host to allow effective transmission. The underlying mechanism may involve sequestration of Dicer due to the large amount of secondary structure in the sfRNA. Interestingly, the inclusion of strong RNAi repressors into an arbovirus results in increased virus yields, but also leads to rapid killing of the mosquito with no subsequent chance for virus transmission (Cirimotich et al., 2009). Thus sfRNA may help generate a balance between the RNAi machinery, viral replication, tissue dispersion, and the overall health/activity of the insect vector that is optimal for virus transmission.

The interferon pathway has also been shown to be antagonized by sfRNA. A variant West Nile virus that could not produce full-length, abundant sfRNA was significantly more sensitive to type I interferon responses (Schuessler et al., 2012). Interferon-sensitive Semliki Forest virus replication could also be rescued in this study by the transfection of sfRNA. Next, a recent study of a DENV-2 clade variant (PR-2B) with significantly increased fitness contained three nucleotide variations in and around the pseudoknot structures that stall and repress Xrn1 (Manokaran et al., 2015). These mutations result in an apparent enhancement of sfRNA expression relative to genomic RNA accumulation during infection. Interestingly, sfRNA was shown to interact with tripartite motif 25 (TRIM25), a key protein involved in regulating RIG-I-induced type-I interferon expression. TRIM25 is an E3 ligase that normally polyubiquitinylates RIG-I to amplify its effects on gene expression. TRIM25 activity itself is regulated by ubiquitinylation and becomes activated when it is deubiquitinylated by ubiquitin-specific peptidase 15 (USP15). Binding of TRIM25 by PR-2B sfRNA prevented its deubiquitinylation and thus resulted in a dramatic decrease in the interferon response to infection by the cell. Finally, a previous study also implicated sfRNA in binding G3BP1, G3BP2 and CAPRIN, factors required for the effective translation of interferon stimulated genes (Bidet et al., 2014).

Given their recent discovery, the role(s) of the nearly full length Xrn1 decay intermediates generated from the HCV or BVDV 5′ UTR remain to be explored. One possibility is that some of the decay intermediates (particularly the species whose 5' end is only missing the terminal-most stem loop structure of the genome) may still be translatable given that the core of the IRES element is largely intact. If the large HCV/BVDV decay intermediates are instead acting as *bona fide* lncRNAs, they could serve in a similar role as sfRNA by acting as decoys/sinks to trap cellular proteins. Given the large amount of viral RNAs in a cell (e.g., $\sim 10^5$ or higher molecules per cell compared to $\sim 10^3$ molecules of an abundant mRNA), the idea of these decay intermediates acting as molecular sponges is particularly attractive (Barnhart et al., 2013). Finally, mirroring the novel roles of cellular lncRNAs, these decay intermediates may have additional functions that would essentially make the HCV and BVDV viral RNAs a 'triple threat' *per se*. The transcripts can not only serve as an mRNA and a genome, but also a regulatory effector in the cell.

6. Possible relationship between miR122 and Xrn1 stalling on the HCV 5' UTR

MiR-122 is highly expressed small RNA in liver cells and naturally regulates cellular genes at the post-transcriptional level, including many mRNAs involved in cholesterol and lipid metabolism (Horie et al., 2014). Interestingly, miR-122 binding to two sites at the 5′ end of HCV genomic RNA is very important (but not absolutely essential) for viral replication (Jopling et al., 2005; Wilson and Huys, 2013). Miravirsen, a Locked Nucleic Acid (LNA)-containing antagomir that sequesters miR-122, is currently in Phase II clinical trials as a novel HCV therapeutic (Janssen et al., 2013). Instead of targeting the HCV genome for degradation or silencing, miR-122 binding to HCV is associated with translational regulation and increased HCV RNA stability. In terms of translation, miR-122 interaction appears to compete for binding of the cellular protein PCBP2 to viral genomic RNA and reduce the amount of RNA available for translation, concomitantly increasing the number of viral RNAs available for replication (Masaki et al., 2015). One possible model is that miR-122 binding prevents access of the cellular Xrn1 decay enzyme to the RNA (and/or a cellular pyrophosphatase to reduce the terminal triphosphate to a 5′ mono-phosphate for exonuclease accessibility). The two independent miR-122 binding sites contribute to protection from Xrn1, albeit in an unequal fashion (Thibault et al., 2015). Finally, Ago2 is also loaded onto the HCV genome via miR-122 interactions and in some fashion assists in the stimulation of HCV replication (Shimakami et al., 2012). Other members of the hepaciviruses (e.g., GBV) also appear to use miR-122 to stabilize their genomic RNA and promote efficient replication (Sagan et al., 2013). Thus the strategy to usurp a cellular miRNA appears to be conserved throughout the family.

MiR-122 clearly influences HCV-Xrn1 interactions and likely influences the generation of decay intermediates from the 5' UTR. However, experiments performed to date indicate that efficient Xrn1 stalling/repression in the HCV 5' UTR can occur in the absence of miR-122. The model that we currently favor is that miR-122 serves as an effective block to Xrn1 to maintain the integrity of the 5′ end of the HCV genomic RNA for effective replication. In this scenario, HCV genomic RNAs in the absence of miR-122 get acted upon by a pyrophosphatase and becomes a substrate for Xrn1-mediated exonucleolytic decay. While the virus will eventually reduce the activity of Xrn1 via xrRNA formation, this is still insufficient to provide enough intact viral RNAs for high yield HCV replication. In the presence of miR-122, the 5′ end of the viral genomic RNA is effectively sealed by the RNA secondary structures/protein factors brought in by the binding of the miRNA to site 1 and site 2 near the 5′ end. This produces a substantial pool of intact viral genomic RNA for efficient replication. The formal testing of several aspects of this model await future experimentation.

7. Speculation on other functions of viral XRN1 decay intermediates during infection

Data obtained to date clearly establish the presence of viral lncRNAs generated as Xrn1 decay intermediates in flavivirus infected cells. These viral lncRNAs appear to serve as protein sinks to repress host defense mechanisms including interferon signaling and RNA interference. This may, however, be the tip of the iceberg for the function of these highly abundant viral non-coding transcripts. There is some evidence, for example, to suggest that sfRNA/xrRNA may serve as a sponge for numerous other cellular proteins. Recent pulldown data, for example, found 198 cellular proteins were enriched in sfRNA-associated fractions (Monokaran et al., 2015). Thus protein sponging by sfRNA/xrRNA may influence numerous cellular processes and alter the expression of RNA regulons (Blackinton and Keene, 2014) that rely on affected RNA binding proteins for their coordination. Next, while it is somewhat controversial, sfRNA/xrRNA may serve as a precursor RNA to give rise to small miRNA-like transcripts that could influence gene expression (Hussain and Asgari, 2014; Skalsky et al., 2014). Alternatively, sfRNA could serve as a structural scaffold to assemble (or perhaps promote the disassembly) of larger protein-RNA complexes. Stress granules and P-bodies, for example, can be disrupted in flavivirus infections (Courtney et al., 2012; Chahar et al., 2013). Finally, sfRNA/xrRNAs may serve as trans-regulators of viral replication directly by influencing long-range interactions of the ends of the viral genome (Fricke et al., 2015).

As our knowledge of the natural functions of the numerous cellular lncRNAs in the cytoplasm grow, so too will the possible avenues for viral sfRNA/xrRNA function. Likewise, insights into sfRNA/xrRNA function can also serve as a basis for discovery of cellular lncRNA function. Questions that have already arisen from insights into sfRNA/xrRNAs include whether these structures that stall Xrn1 are unique to viruses or are they also present on cellular RNAs to generate new 5' ends and perhaps further expand the transcriptome. In addition, do all IRES elements, including cellular IRES and IRES-like elements, have a propensity to stall Xrn1 or are the flavivirus IRESs unique in this regard.

Finally, targeting Xrn1 for repression by a cytoplasmic RNA virus appears to be a very attractive strategy to debilitate the host cell. Xrn1 is both central to the regulation of cellular RNA decay as well as an alleged important (if not the key) player in feedback between RNA decay and transcription efficiency to buffer gene expression and allow for homeostasis of gene expression. Do other viruses perhaps also target Xrn1 through RNA-mediated stalling? Arenavirus (as well as some bunyavirus) RNAs contain a well-characterized and highly stable stem structure that contributes to the ambisense strategy of gene expression (Zapata and Salvato, 2013). Coronaviruses contain a highly conserved pseudoknot structure in the 3′ UTR just downstream of the translation termination codon that influences the efficiency of replication (Williams et al., 1999). Picornaviruses contain well-characterized IRES elements in their 5′ UTRs (Niepmann, 2009). Thus many other RNA viruses could have adapted the strategy of Xrn1 stalling/repression to disrupt and overcome the initial roadblock to viral replication presented by the cellular RNA decay machinery. In addition, generation of these structured viral non-coding RNAs dysregulate the cellular response to infection by modulating the activity of this highly accessible cytoplasmic enzyme. This knowledge can open up the doors to new therapeutic targets for the development of effective broad-spectrum antivirals.

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