Computational and Structural Biotechnology Journal 19 (2021) 4373–4380

COMPUTATIONAL AND STRUCTURAL BIOTECHNOLOGY JOURNAL

journal homepage: www.elsevier.com/locate/csbj

Shared properties and singularities of exoribonuclease-resistant RNAs in viruses

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ARTICLE INFO

Article history:
Received 29 April 2021
Received in revised form 21 July 2021
Accepted 23 July 2021
Available online 26 July 2021

Keywords:
Evolution
Exoribonuclease
Structured viral RNA element
Virus
xrRNA
Xrn1

ABSTRACT

What viral RNA genomes lack in size, they make up for in intricacy. Elaborate RNA structures embedded in viral genomes can hijack essential cellular mechanisms aiding virus propagation. Exoribonuclease-resistant RNAs (xrRNAs) are an emerging class of viral elements, which resist degradation by host cellular exoribonucleases to produce viral RNAs with diverse roles during infection. Detailed three-dimensional structural studies of xrRNAs from flaviviruses and a subset of plant viruses led to a mechanistic model in which xrRNAs block enzymatic digestion using a ring-like structure that encircles the 5′ end of the resistant structure. In this mini-review, we describe the state of our understanding of the phylogenetic distribution of xrRNAs, their structures, and their conformational dynamics. Because xrRNAs have now been found in several major superfamilies of RNA viruses, they may represent a more widely used strategy than currently appreciated. Could xrRNAs represent a 'molecular clock' that would help us understand virus evolution and pathogenicity? The more we study xrRNAs in viruses, the closer we get to finding xrRNAs within cellular RNAs.

Contents

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https://doi.org/10.1016/j.csbj.2021.07.024

1. Introduction: xrRNAs as generic mechanical blocks to exoribonucleases

The compact genomes of RNA viruses (max. 30 kb) are littered with structured elements [1,2]. Structured RNA elements are key contributors to all stages of viral infection and usually to pathogenicity. They are often used by viruses to manipulate transcription, translation, antiviral responses, and other cellular mechanisms [3]. Such elements comprise for example hairpin structures, which protect the 3′ end and may affect translation [4], and pseudoknotted folds, which may start translation or alter the translation reading frame [3,5].

An emerging class of structured viral RNAs are the exoribonuclease-resistant RNAs (xrRNAs), which were first discovered in the 3′ untranslated region (UTR) of flaviviruses and plant viruses [6–8]. xrRNAs resist degradation from the 5′ direction by the host cellular exoribonuclease Xrn1 (or its homologs, like Xrn4 in plants [9]) and more generally by any 5′-to-3′ exoribonuclease (e.g., bacterial RNase J1 and yeast decapping and exoribonuclease protein 1) [10–12]. Viral xrRNAs can therefore be viewed as a generic roadblock to directional degradation.

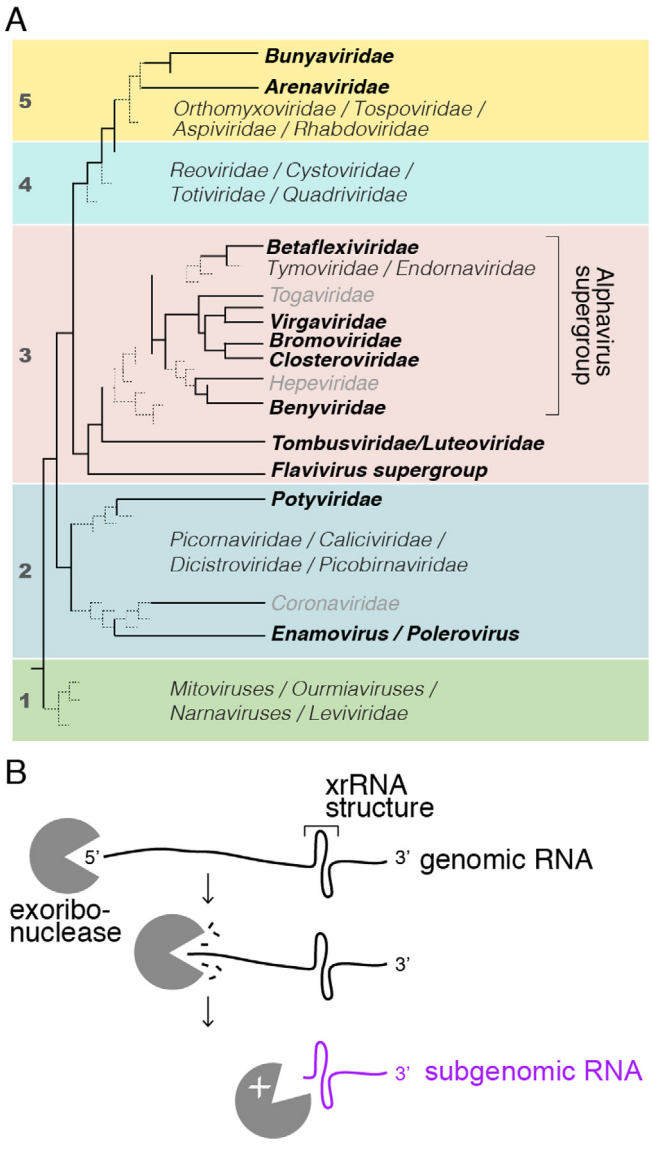

Fig. 1. xrRNAs across the viral phylogeny. (A) Relationship between viruses based on RNA-dependent RNA polymerase sequences (adapted from [13]). The five main branches stemming from a putative common ancestor are shown by colored boxes. Only relevant viral families are shown, the rest are symbolized as dashed lines. Color coding for the viral families: bold & black, contains an xrRNA; black, representative families within each branch in which xrRNAs have not (yet?) been found; grey, human pathogen closely related to viruses containing xrRNAs. (B) Schematic exoribonuclease-mediated degradation pathway. The xrRNA element blocks the exoribonuclease.

Altogether, xrRNAs have now been reported in at least three of the five major virus branches (Fig. 1A) [13]. Outside of *Flaviviridae*, but still within branch 3, xrRNAs have been identified in *Tombusviridae* and *Luteoviridae* [7,12,14] as well as in the Alphavirus supergroup [15,16]. Putative xrRNAs have also been proposed within branch 2 (e.g., *Potyviridae* [17]) and branch 5 (e.g., *Bunyaviridae* and *Arenaviridae* [18]). Overall, these findings highlight that xrRNAs are more widespread than originally thought.

Generally, xrRNAs are responsible for the production of certain subgenomic RNAs (sgRNAs), through partial degradation of the genomic RNA (Fig. 1B; these are distinguished from sgRNAs resulting from internal transcription initiation [19]). For example, in flaviviruses —where the sgRNAs are referred to as sfRNAs for 'subgenomic flaviviral RNAs'— sfRNAs act as noncoding RNAs that interact with the cellular proteome to alter antiviral responses [20,21], but the details of this remain poorly understood [11]. For some plant-infecting viruses, xrRNAs are located within subgenomic RNAs (sgRNAs) that encode proteins and these sgRNAs may therefore be translated [14,22]. In *Tombusviridae*, an xrRNA is similarly responsible for the formation of a non-coding RNA with translation regulation properties [7,12,23]. In *Benyviridae*, *Betaflexiviridae* and *Virgaviridae*, an xrRNA is sufficient to block Xrn4, leading in *Benyviridae* to the formation of non-coding RNA3 (ncRNA3) and RNA5 (ncRNA5), which are essential for viral long-distance movement within the infected plant [15–17,24]. Hence, xrRNAs may be important for producing or protecting a variety of viral RNAs with diverse functions; they are emerging as an important mode of viral RNA maturation and protection.

2. Encircling the 5′ end: Different strategies for a shared feature

The three-dimensional crystal structures of xrRNAs from several viruses within the flavivirus supergroup (Murray Valley Encephalitis Virus, MVEV; Zika Virus, ZIKV; Tamana Bat Virus, TABV) offer detailed insights into the molecular basis of exoribonuclease resistance [25–27]. They reveal that xrRNAs adopt a ring-like structure through which the 5′ end threads (Fig. 2A). In all cases observed thus far, the ring comprises 15–16 nucleotides. Complex tertiary interactions including pseudoknots, non-canonical base-pairs, bound metal ions, and intricate stacking and hydrogen bonding schemes stabilize the ring feature. Some of these interactions form patterns that are specific to different xrRNA classes (Fig. 3; see next section). The resulting compact fold braces against the surface of the enzyme approaching from the 5′ side, preventing further progression of the enzyme through the xrRNA [25].

The importance of a ring structure for blocking the enzyme is further supported by structures from xrRNAs outside the flaviviruses. Specifically, plant-infecting viruses from *Tombusviridae* and *Luteoviridae* (Fig. 1A), have xrRNAs with sequences and secondary structures that differ from flaviviral xrRNAs [12,25,26]. Although the crystal structures of xrRNAs from Sweet Clover Necrotic Mosaic Virus (SCNMV; *Tombusviridae*) and Potato Leaf Roll Virus (PLRV; *Luteoviridae*) did not capture the RNA in its active ring-supported conformation, a ring-like structure could be modeled that also contained 15 nucleotides encircling the 5′ end (Fig. 2B). As in flaviviruses, this ring depends on the formation of a pseudoknot whose importance was verified by site-directed mutagenesis and via an infection system [12].

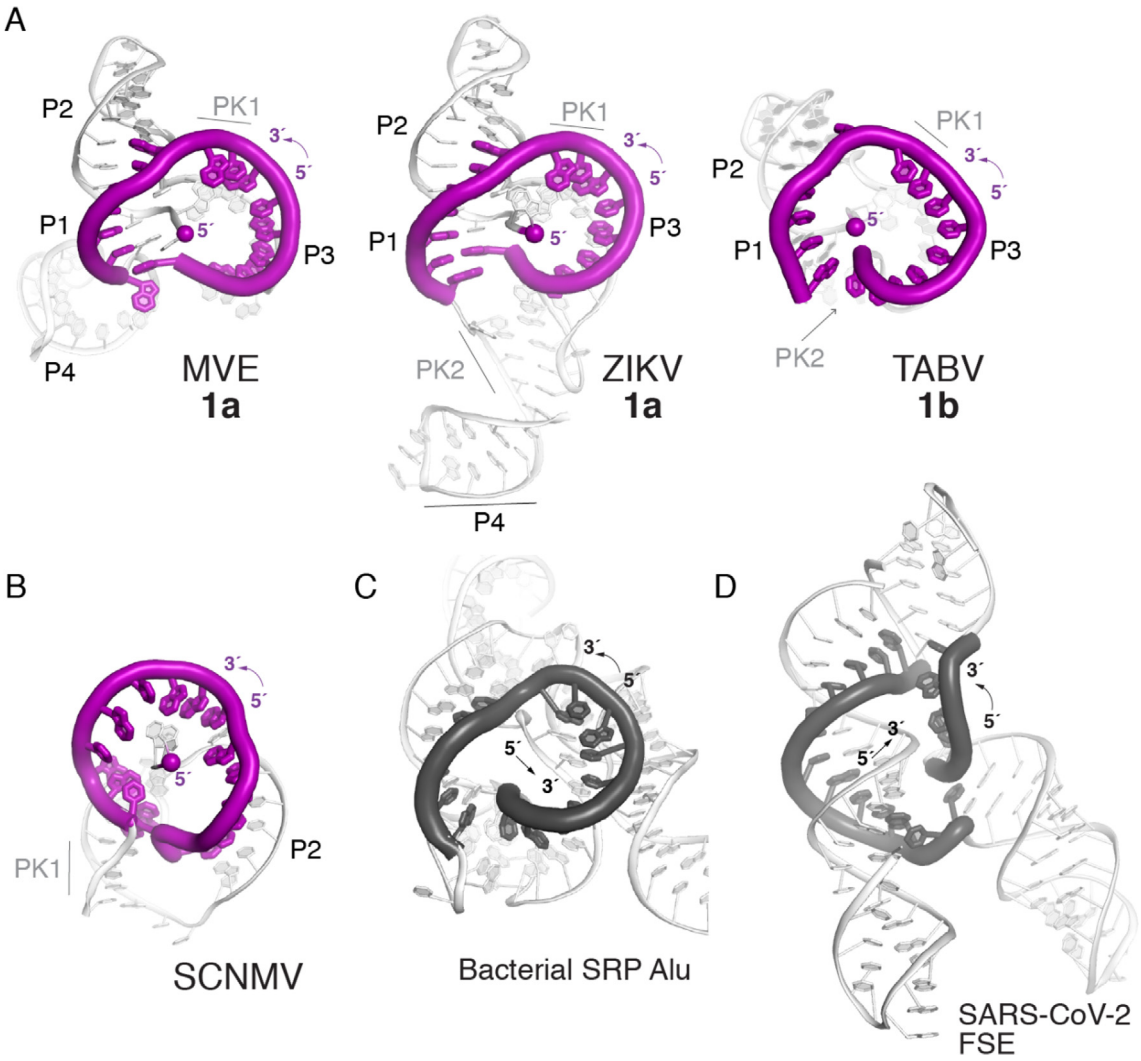

Fig. 2. Ring-like structures in RNA. (A) The ~15 nucleotides forming a ring around the 5′ end are highlighted in bold and purple on the three-dimensional structure of xrRNAs from MVE, ZIKV and TABV (PDB ID: 4PQV, 5TPY, 7K16). See Fig. 3B for corresponding secondary structure cartoons. (B) 3D model computed for the SCNMV xrRNA. (C) Ring-like topology in the bacterial SRP Alu RNA (PDB ID 4WFL). (D) Helical packing leading to a ring-like arrangement within the SARS-CoV-2 frameshift stimulation element (PDB ID 6XRZ). The 5′ end and strand directionality around the ring structure are indicated in all panels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The ring-like architecture in both flavivirus and plant virus xrRNAs requires at least one pseudoknot. In the flavivirus xrRNAs, the ring topology appears to be formed by interactions between a three-way junction and the 5′ end, forming the PK1 pseudoknot (Figs. 3, 4). A second pseudoknot forms to "latch" the structure closed [28] (Figs. 3, 4). In plant virus xrRNAs, formation of PK1 (Figs. 3, 4) and the ring follows degradation by Xrn1 through the P1 stem, leading to the exoribonuclease resistant fold, as shown by Förster resonance energy transfer (FRET; [12]). A similar phenomenon of structural remodeling could occur within other xrRNA families, particularly when alternative pairing schemes are proposed, which may result in the Xrn1 halt site being located within a predicted stem (compare for example secondary structure predictions in [29–31]. In short, just like 'co-transcription folding' can occur as an RNA chain is progressively extended [32], progressive degradation of an RNA in the 5' to 3' direction can create 'co-degradational' stabilization of a specific structure. While these proposed folding pathways remain to be fully tested, they may present two different ways to solve the topological challenge of 'threading' a single strand of RNA through a ring.

3. How unique are xrRNA ring-like structures?

Ring-like structures in xrRNAs beg the question as to how common these folds are. In fact, ring-like topologies have been previously observed in RNA structures. For example, double-stranded regions may form a ring-shaped super-structure, as in the packaging motor prohead RNA [33]. Ring structures are also appealing for synthetic biology [34], which focuses on the design RNA nanoparticle for disease diagnosis and drug delivery [35]. Such RNA rings combine several molecules, which is different from the situation in xrRNAs, where a single continuous section of an RNA strand encircles a single-stranded element. The resulting structure is "knot-like" and thus far seems unique to xrRNAs (Fig. 2A,B). The Alu region from the bacterial single recognition particle (SRP) shows a stunningly similar ring architecture to that of xrRNAs, but no RNA threads through the ring [36] (Fig. 2C). In the case of the SARS-CoV-2 frameshift stimulation element, a ring was proposed to represent a critical structural feature, but that ring is assembled from several parts of the RNA [37] (Fig. 2D). Additional evidence may be required to support a ring-like status for features

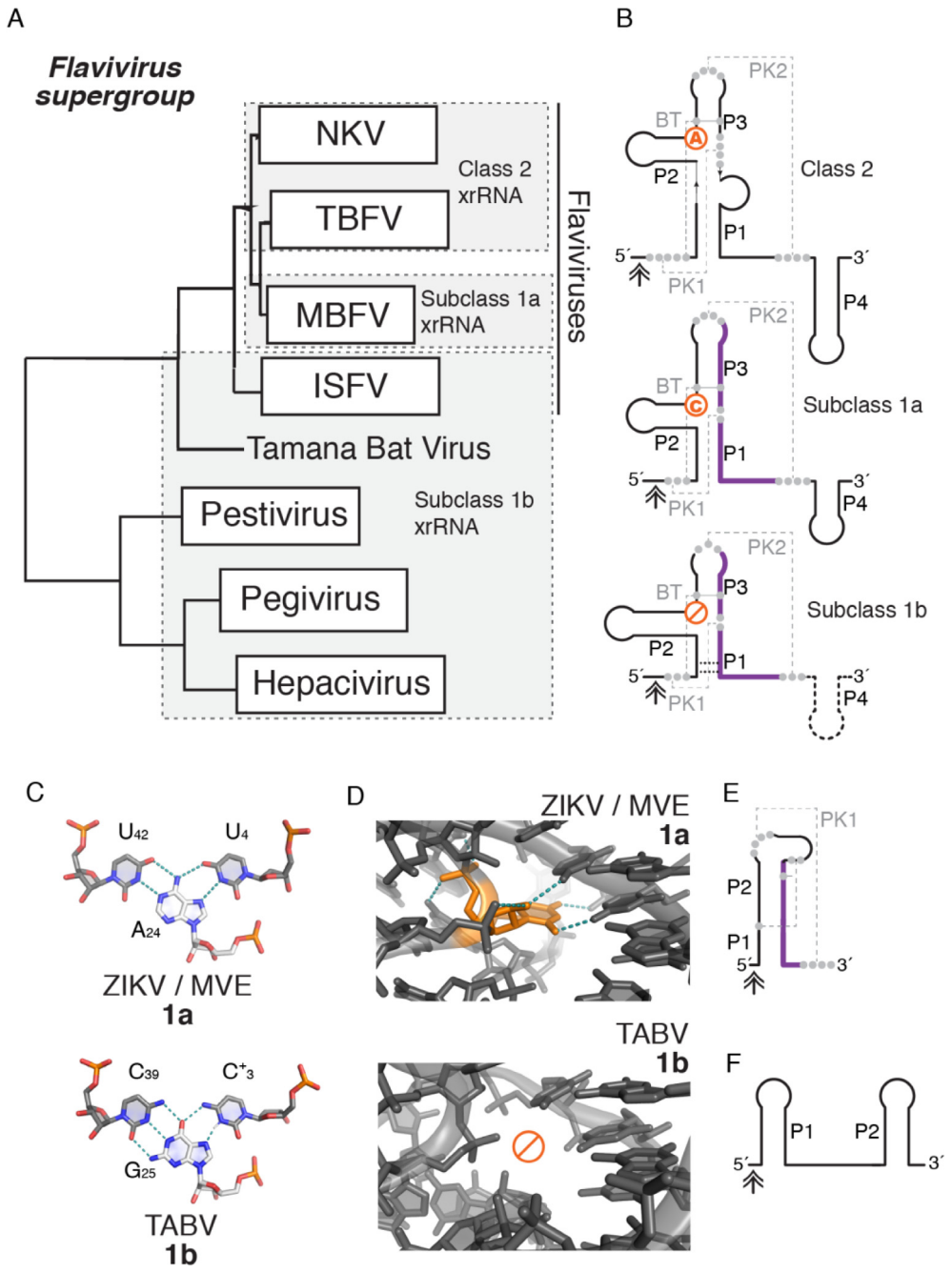

Fig. 3. Towards a structure-based taxonomy of xrRNAs. (A) Phylogeny within the Flavivirus supergroup, based on the NS5 sequence (adapted from [69]). Genera belong to one of three (sub)classes: **1a**, **1b**, or **2**. The position between P2 and P3 (orange circle) is a key discriminator between xrRNA (sub)classes of xrRNAs. NKV, no known vector flavivirus; TBFV, tick-borne flavivirus; MBFV, mosquito-borne flavivirus; ISFV, insect-specific flavivirus. (B) Cartoon representation of the secondary structure of xrRNAs from the Flavivirus supergroup. P, paired region; PK, pseudoknot; BT, base triple. (C) BT interactions visualized in the crystal structures of the xrRNAs from ZIKV (PDB ID 5TPY) and TABV (PDB ID 7K16). (D) Close up on the discriminating position (orange) in the ZIKV and TABV structures. (E) Cartoon representation of the secondary structure of an xrRNA from *Luteoviridae*. (F) Cartoon representation of the secondary structure of an xrRNA from *Benyviridae*. Double arrow in panels B, E, F: Xrn1 halt site. Regions forming the ring as highlighted in Fig. 2 are shown in purple in panels B, E. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that essentially constitute tightly packed RNA. Whether the topology seen in xrRNAs represents a distinct and defining characteristic of xrRNAs remains to be determined as more xrRNA structures are solved, or perhaps similar features are identified in other RNAs.

4. Flavivirus xrRNA structures in context

xrRNAs form discrete folded structures, which are often found in series in the 3′ UTRs of many flaviviruses [14,17,38,39]. While these 'tandem' xrRNAs might initially seem independent and redundant, evidence suggests that the structural integrity of one can affect the function of the other [25,40,41]. This may be an important virological feature, as studies of the Dengue virus (DENV) and Zika show tandem xrRNA1 and xrRNA2 allow for a higher fitness as the virus cycles between hosts (such as mosquito and human) [38], where sfRNAs may play different roles. When two xrRNA folds are present in tandem, the pressure to maintain one of them may be relaxed to accommodate mutations that would be beneficial for that particular environment.

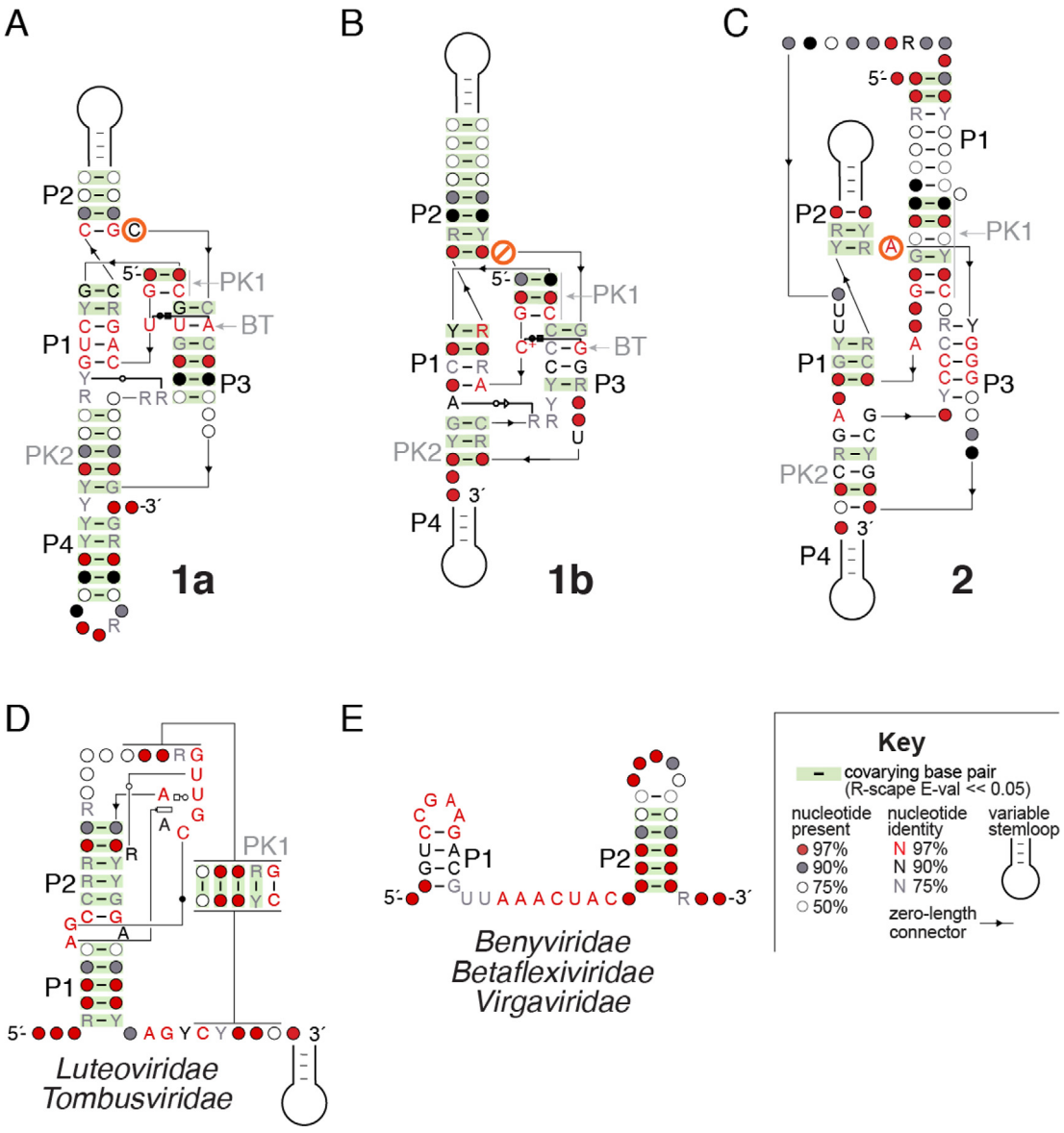

Fig. 4. Covariation of xrRNAs. (A) Secondary structure of subclass **1a** xrRNAs emphasizing the three-dimensional architecture [25,26] (32 sequences and models from [28,43]). Core features (P1, P3, PK1, BT and the discriminating position (in orange)) are horizontally aligned between panels A–C. (B) 3-D based secondary structure of subclass **1b** xrRNAs [27] (87 sequences from [43]). (C) Predicted secondary structure of class **2** xrRNAs (28 sequences from [29,31], with automatic improvement of covariation patterns using R-scape [70,71]). (D) 3-D based secondary structure of xrRNAs from *Luteoviridae* and *Tombusviridae* (55 sequences from [12,72]). (E) Predicted secondary structure of xrRNAs from *Benyviridae*, *Betaflexiviridae* and *Virgaviridae* (original 10–12 sequence alignment [15,17] expanded to 47 sequences from searching the viral sequence database [73] using a published methodology [14,74,75]). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

How do two tandem xrRNAs communicate to couple their function? The most straightforward hypothesis is through a direct physical interaction. Consistent with this, small-angle X-ray scattering (SAXS) revealed that tandem xrRNAs from ZIKV, DENV2 and WNV have a defined envelope and thus may have a preferred relative orientation, but that they do not appear to be in intimate contact [42]. However, because SAXS envelopes reflect the populationally weighted average of the structural ensemble, more data are needed at higher resolution to fully assess the presence and nature of likely physical contacts between individual xrRNAs in tandem. Overall, these current data suggest that flavivirus 3′UTRs and sfRNAs are best described as a structural ensemble rather than by a single conformation [42]. This characteristic may be important for the virus, allowing alternative folds to be adopted in different environments or at different stages of the viral life cycle to coordinate different processes important for infection.

5. A budding structure-based taxonomy of xrRNAs

Within the flavivirus supergroup where they were originally found, xrRNAs have now been identified and characterized in all genera of *Flaviviridae* (Fig. 3A) [11,30,39,43]. Initial characterization of these xrRNAs based on proposed secondary structures, conserved sequences, and the halt point of the enzyme suggested two major genera-specific classes [29], both organized around a three-way junction (3WJ [44]) and at least one pseudoknot [45] (Fig. 3B). The high-resolution structures of class **1** (from MVE and ZIKV) revealed additional tertiary features, including a conserved base-triple interaction (BT; Fig. 3B,C) supplemented with additional non-canonical interactions necessary to support such intricate folds. A three-dimensional structure of a class **2** xrRNA (e.g., within Tick-Borne Encephalitis Virus, Langat Virus and Powassan Virus) has yet to be solved, but alignments of the conserved xrRNA fea-

tures in both classes have been harnessed by a search algorithm for automatic identification of xrRNAs [46]. Overall, a taxonomy of xrRNAs emerges from the knowledge of their features at the secondary and tertiary structure levels [30,31,39,47,48]. By definition, the current classification for xrRNAs will be amended as more xrRNAs are discovered.

Specifically, class **1** xrRNAs are distinguished from class **2** xrRNAs on the basis of (1) their 3WJ configuration, (2) a longer distance between the regions forming the PK2 pseudoknot in **2**, and (3) a longer P4 in **2** [29,31,49]. xrRNAs originally described as belonging to class **1** are now further divided into subclasses **1a** (32 sequences, from e.g., MVEV, ZIKV, and DENV [28,43]) and **1b** (87 sequences, from e.g., Culex Flavivirus, Cell Fusing Agent Virus and TABV [43]): (1) in **1b**, the P1 and P3 stems are shorter than in **1a**, (2) characteristic non-Watson-Crick pairs in P1 are found in **1b** but not **1a**, (3) a P4 stem may not always be present in **1b**, and (4) the BT interaction tends to be U-A-U for **1a** but C-G-C^+ for **1b** [43] (Fig. 3B,C and 4A–C). Whether the BT interaction is present in **2** remains an open question. (Sub)classes **1a**, **1b** and **2** are distinct on the basis of bioinformatic and structural analysis; in the case of subclasses **1a** and **1b** this extends to distinct tertiary interactions beyond Watson-Crick base-pairing.

In addition, the region of the 3WJ between stems P2 and P3 (which comprises either a single nucleotide or no nucleotide) acts as a convenient discriminator between classes and subclasses (orange on Fig. 3B,D and 4A-C). A ~90% conserved C in **1a** forms tertiary contacts at the core of the fold (Figs. 3D, 4A); replacing it with a G or deleting it abrogates exoribonuclease resistance [25,26]. Conversely, no nucleotide is present at the equivalent position in **1b** (Figs. 3D, 4B) and introducing one leads to loss of resistance [27]. Hence, subclasses **1a** and **1b** achieve a similar fold and architecture with different sets of tertiary interactions. In class **2**, a highly conserved A is found at what is proposed to be the analogous location (Fig. 4C), which can be hypothesized to similarly support the architecture of the 3WJ, although this has not yet been visualized in a structure.

Overall, using the solved crystal structures (for **1a** and **1b** representatives), comparative sequence alignments, site-directed mutagenesis, and chemical mapping data (for all classes), we can hypothesize that all xrRNAs from the Flavivirus supergroup fold around a generally similar core (Fig. 4A–C). Locally, key interactions differ, leading to the classification into classes and subclasses, but globally, core ring structures are similar. **1b** is more compact than **1a**, while **2** presents a longer PK1, with additional pairing upstream of PK1 as well as 10–15 nucleotides within P1 (Fig. 4C) [29,31]. The hypothesis that xrRNAs fold around a common core remains to be validated through structural analysis. In particular, the three-dimensional structure of a class **2** representative would illustrate the role of the discriminating A between P2 and P3 and would indicate whether the conserved but covarying BT interaction in other classes is present in **2**.

The xrRNAs from *Luteoviridae* and *Tombusviridae* adopt distinct secondary structures from the flavivirus xrRNAs, with no central 3WJ (Fig. 3E). The crystal structures of *Tombusviridae* xrRNAs in an inactive conformation revealed a stem-loop structure with appendages so the apical loop 'falls back' to make contacts with an internal loop region [12] (Fig. 4D). These xrRNAs nonetheless form a functionally important and hence conserved pseudoknot (Fig. 4D). In contrast, the *Benyviridae* xrRNAs contain neither a 3WJ, nor a pseudoknot (Fig. 3E). The currently accepted secondary structure model for the *Benyviridae* xrRNA has two stem loops separated by a 10 nucleotide-long linker [15,17] (Fig. 4E). The simplicity of that arrangement does not account for the complex fold needed to block an exoribonuclease. In fact, the strong conservation of the first ~20 nucleotides neither supports nor disproves this secondary structure. More xrRNAs have been identified with even more distinct predicted structures [18,50]. Whether these more 'exotic' xrRNAs form more distinct classes remains uncertain until their tertiary folds have been visualized.

6. Footprints of evolution?

The presence of diverse xrRNAs in divergent RNA virus clades raises the question of their origins and evolutionary relationships. It seems likely that within the Flavivirus supergroup, xrRNA classes **1** and **2** evolved from a common fold, then the basic core structure supporting the ring-like structure diversified as new lineages arose. This idea is highlighted by the structures of class **1** xrRNAs, which showed variations within a similar overall fold [27]. Furthermore, these variations led to distinct subclasses based on diverged tertiary interactions [30,39,43]. However, except for class **1** flavivirus xrRNAs, we do not know what the active fold of xrRNAs looks like, which limits fully understanding xrRNA evolution. Solving the structures of xrRNAs from distinct viral families and lineages remains a key to determining how these RNAs evolved.

Likewise, determining how widely xrRNAs are distributed across viruses would offer a valuable reference for better seizing the evolutionary relationships between viruses. If the proposition that viruses have a common ancestor is correct [13], xrRNAs may represent 'molecular clocks' that would help us understand virus evolution and pathogenicity. xrRNAs may also have emerged independently in different lineages, due to the high mutation rates and effective population size of viral particles. Again, a full accounting of xrRNAs and their structures can inform this discussion. Whether, for example, xrRNAs from the Flavivirus supergroup and from *Tombusviridae* / *Luteoviridae* are related would be worth investigating. Similarly, searching the recently reported class of Jingmenviruses for xrRNAs ought to be informative, as they are more closely related to Flaviviruses than Hepaci-, Pegi- and Pestiviruses [51,52]. Unfortunately, currently available database entries for these viruses lack the 3′UTR sequences. Continuing to expand the catalogue of xrRNAs remains a necessity to increase our chances of finding how widespread these folds are.

Tracking evolutionary relationships from comparing xrRNAs could directly inform studies of structured RNA elements in human pathogens outside flaviviruses. Extensively studying xrRNAs from plant viruses for example from *Benyviridae*, *Virgaviridae* and Poleroviruses makes sense, as these viruses are closely related to *Hepeviridae*, *Togaviridae* and *Coronaviridae* (based on RNA-dependent RNA polymerase sequences, Fig. 1A), which comprise human pathogens like hepatitis E viruses, chikungunya viruses, and coronaviruses. The chikungunya virus and coronaviruses possess structured 3WJ RNA elements with currently unknown functions [53–55], and the hepatitis E virus arose from recombinations of *Benyviridae* viruses [56]. Such putative elements could be systematically tested for exoribonuclease resistance, for example by transposing to viral sequences a genome-wide assay for Xrn1 resistance that was recently reported for a human genomic library [57].

7. xrRNAs within eukaryotic genomes?

Up to now, xrRNAs have only been characterized in viruses. However, expecting xrRNAs in eukaryotic cells makes sense, because their ability to block exoribonucleases could be a desired feature for some cellular RNAs. In addition, eukaryotic genomes are known to embed viral sequences [58,59], which would then be passed across species through horizontal gene transfer [60]. Such sequences of viral origin could comprise xrRNAs. In fact, flaviviral sequences exist in host mosquito genomes [61–64]. For example, 68% of a reported unplaced 18 kb genomic fragment from

Aedes albopictus (Foshan isolate; NCBI #KQ571998.1) are 100% identical to the complete genomic sequence of Dengue virus 1 (NCBI #AB178040.1) [64] (our own analysis using Blast [65]). Putative xrRNAs are found at positions 17807–17873 and 17880–17942 on the fragment. Whether these sequences are expressed and whether they are xrRNAs that can block exoribonucleases *in vivo* or *in vitro*, as well as their biological role, if any, are aspects that remain to be explored.

Learning more about viral xrRNA folds, their variety, and their evolutionary relationships better equips us to search for similar folds in eukaryotic genomes. Searching for xrRNAs both computationally and experimentally may lead to the discovery of folds with a quite different mechanism to block Xrn1 than their viral counterparts. Such structures could have arisen within eukaryotes without transfer from viruses. Alternatively, ring-like folds may be found within regulatory RNAs like long non-coding RNAs, similarly to folds which promote catalytic activities and which are also found in a variety of genetic contexts [57,66].

8. Summary and outlook

xrRNAs are examples of viral elements that use complex three-dimensional structures to usurp cellular mechanisms. Emerging detailed three-dimensional structural information gives deep insights into the molecular mechanism of these elements, and also allows for new interpretations of biochemical, virological and phylogenetic information. We are now in a position to better explore how widespread and diverse xrRNAs are, and how they are evolving. Doing so has the potential to illuminate the biology of diverse but evolutionarily related viruses, expand our overall knowledge of RNA structure in the viral world, and motivate futures studies into the roles of xrRNAs in virus-induced disease. Because xrRNAs are found in all major superfamilies of RNA viruses, they may represent an ancient feature that existed in the common ancestor to these superfamilies [67,68]. Active xrRNAs could also be found in cells, where they may have other functions in addition to blocking exoribonucleases.

CRediT authorship contribution statement

Quentin Vicens: Conceptualization, Funding acquisition, Writing - original draft, Writing - review & editing. **Jeffrey S. Kieft:** Conceptualization, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank David Gilmer and Matt Szucs for critical reading of the manuscript, as well as current and former Kieft Lab members for thoughtful discussions. This work was supported by NIH grants R21AI157244 (Q.V. & J.S.K.), and R01AI133348 (J.S. K.).

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