

# Exoribonuclease-resistant RNAs in tick-borne flaviviruses

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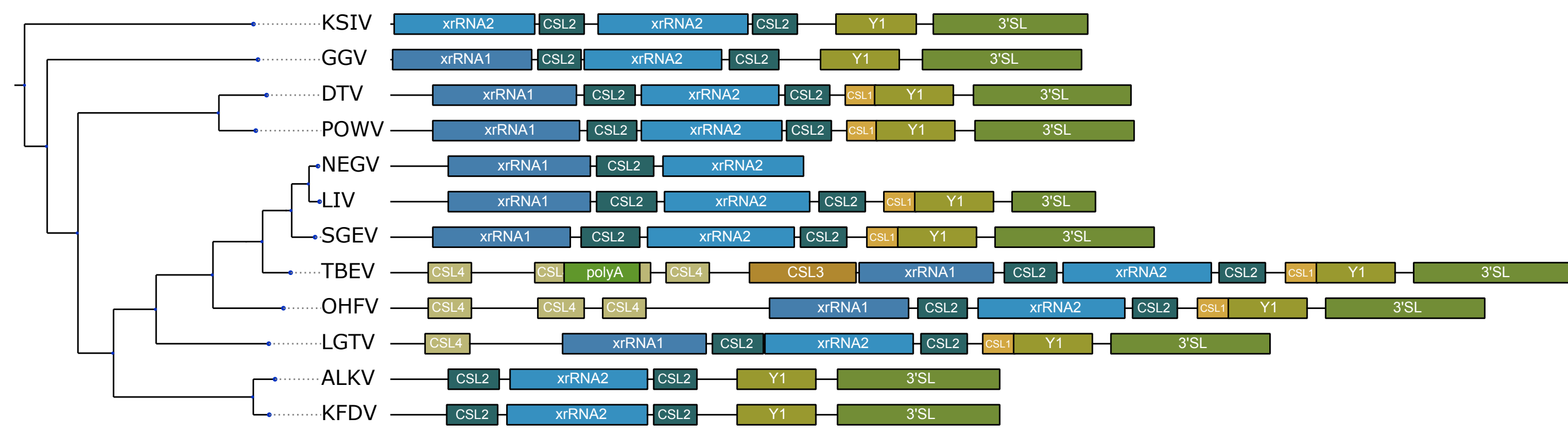
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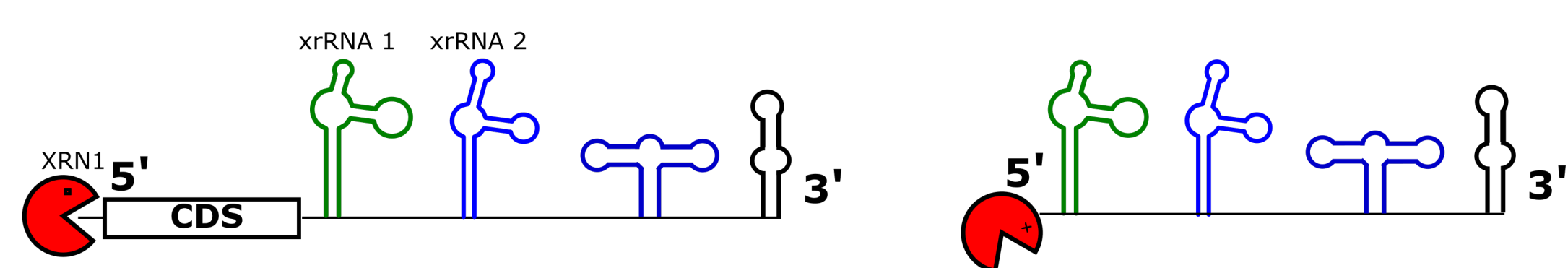
## 1. RNA structure conservation in TBF 3'UTRs

Tick-borne flaviviruses (TBF) are a group of emerging and re-emerging pathogens that affect large geographic regions, posing a major public health threat. A critical aspect of their molecular biology and pathology is the presence of **evolutionarily conserved functional non-coding RNAs** (ncRNAs) in the 3' untranslated region (3'UTR).



**Fig 1** Top: Annotated 3' UTRs of selected TBF (plotted to scale), highlighting variable length and architectural organization. Colored boxes represent structurally homologous, evolutionarily conserved RNAs. Maximum likelihood phylogeny on the left has been computed from full genome nucleotide sequences. Bottom: RNA secondary structure prediction of the TBEV Neudoerfl 3'UTR [1]. Structured non-coding RNAs are shown in the same colors as in the overview plot above.

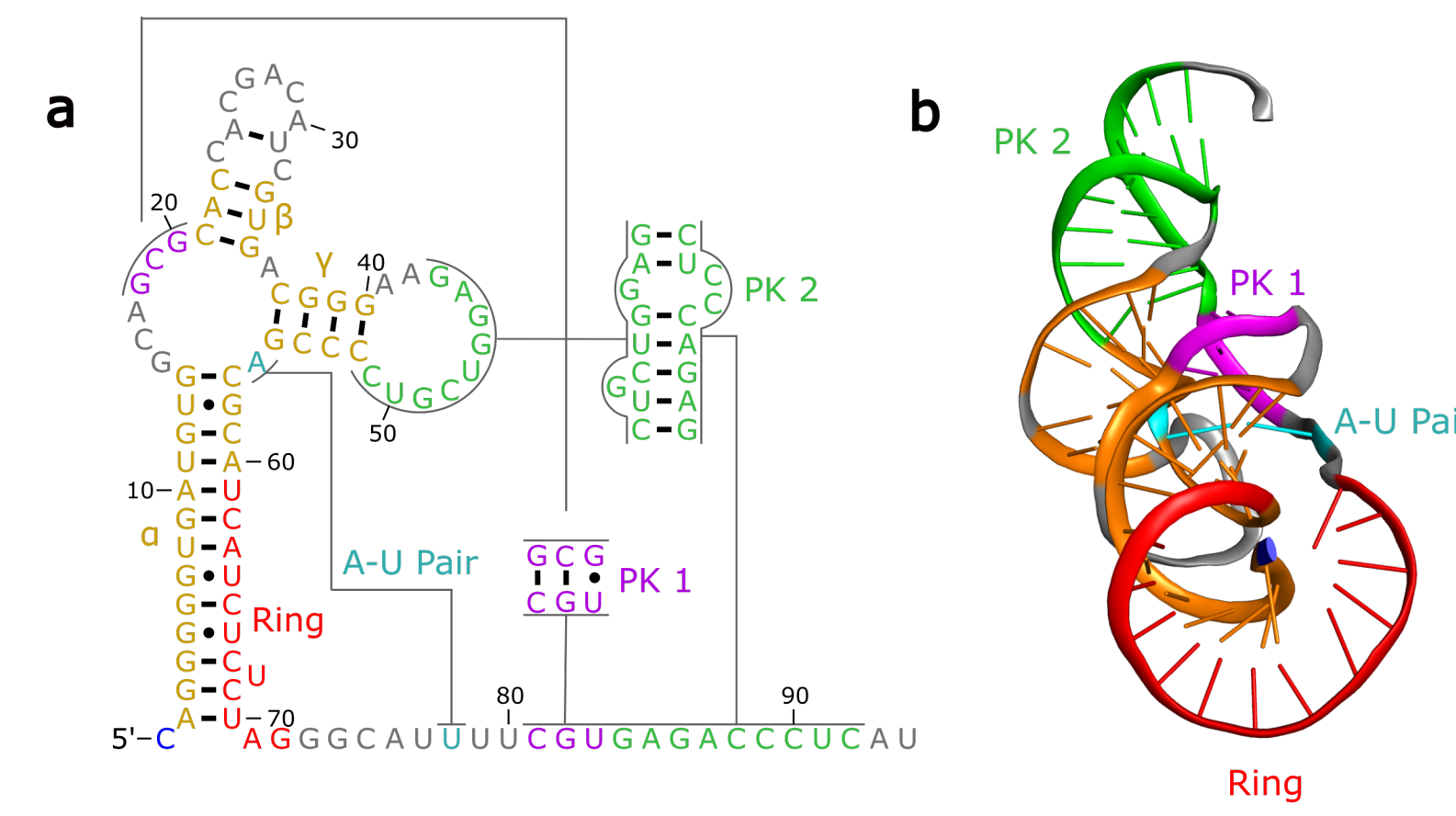
Among these ncRNAs are **exoribonuclease-resistant RNAs (xrRNAs)** that can resist degradation by host exoribonucleases such as XRN1. xrRNAs typically occur in multiple, structurally homologous yet distinguishable copies in the viral 3'UTR. Flaviviruses exploit xrRNAs to shield downstream regions of their genomes from degradation, resulting in the formation and accumulation of so-called **short flavivirus RNA (sfRNA)** fragments that promote viral pathogenicity by active dysregulation of infected cells [2].



**Fig 2**: Schematic depiction of how flaviviruses hijack the host mRNA degradation pathway. Conserved xrRNA elements, found in single or tandem arrangement within 3'UTRs, impede the progression of the host exonuclease XRN1 (red pac-man). The resulting sfRNA, an incomplete degradation product, enhances viral pathogenicity through interaction with the antiviral interferon response.

## 2. A ring is required for xrRNA functionality

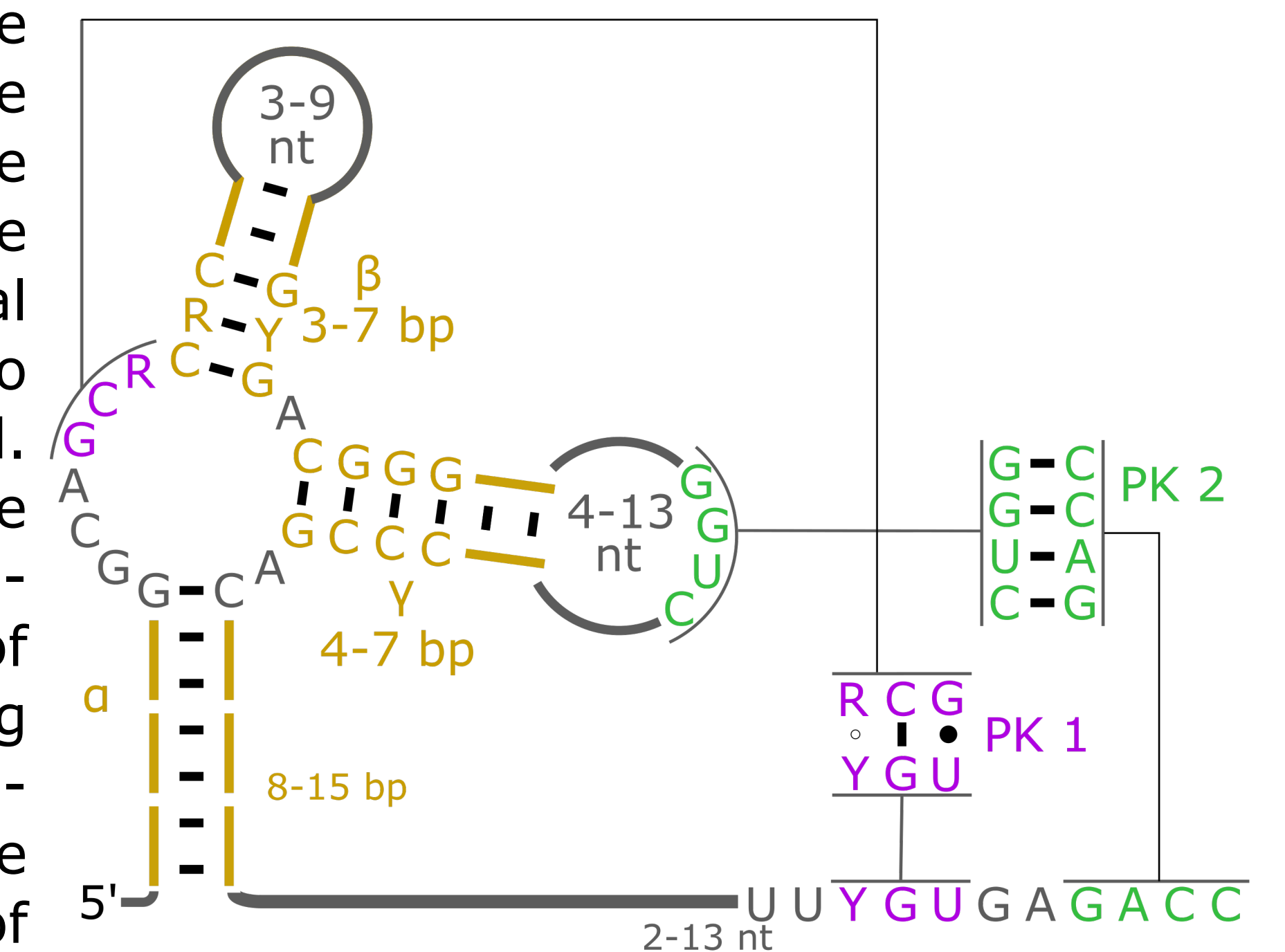
The structural configuration of an xrRNA in 2D and 3D is intricately tied to its function. A defining part of the xrRNA fold is a **ring-like structure** through which the 5' end threads. This stable element braces against an exoribonuclease that approaches the xrRNA from the 5' end, leading to a direction-dependent anisotropy that induces a mechanical block [3]. Conversely, the viral RdRP can readily traverse the element during (-)-strand synthesis when approaching from the 3' end.



**Fig 3**: 2D and 3D structure of an xrRNA from Deer tick virus (DTV).

## 3. Structural determinants of TBF xrRNAs

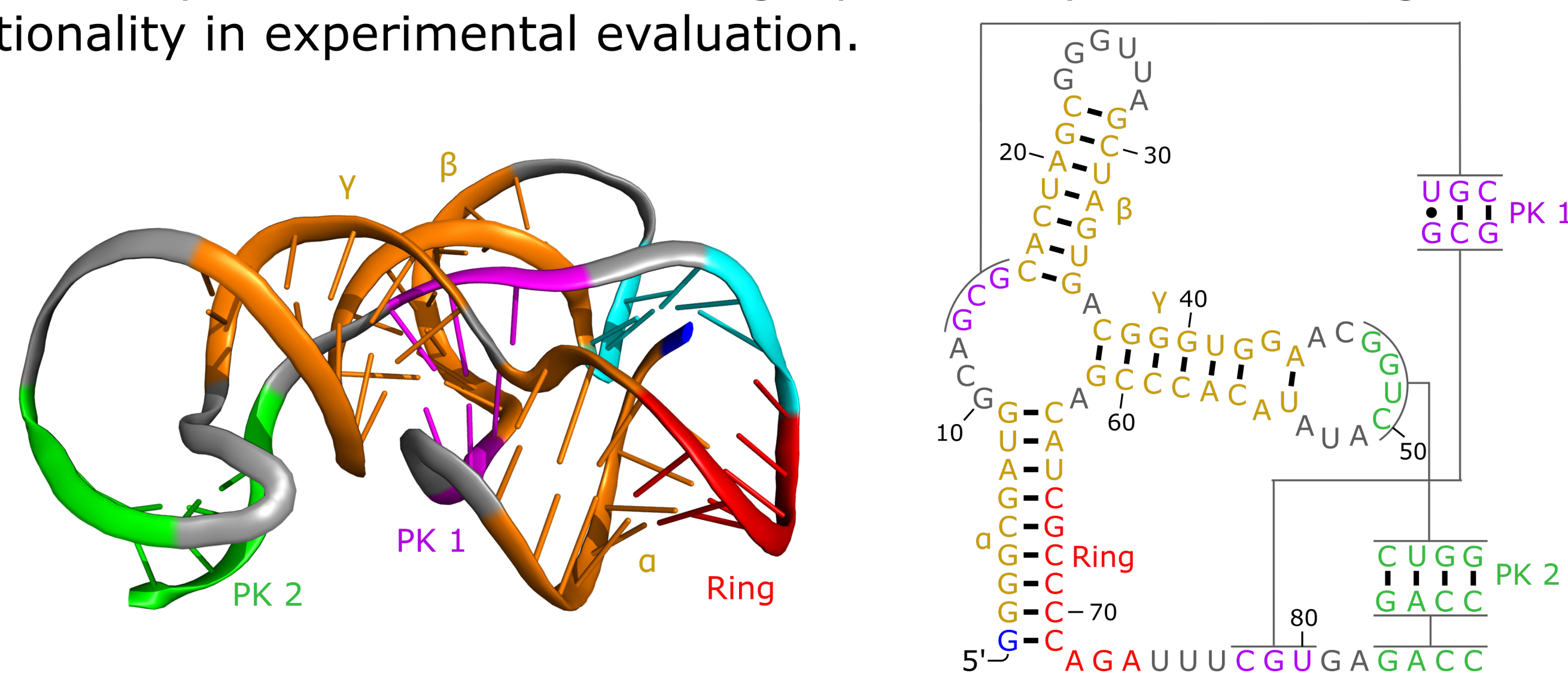
Comparing various xrRNA structures reveals notable commonalities. A central three-way junction (3WJ), consisting of stems  $\alpha$ - $\gamma$ , and two **pseudoknots** (PK1 and PK2), plays a pivotal role in ensuring the stability of the ring-like architecture. A high base conservation within the 3WJ (>95%) implies the importance of additional tertiary interactions to achieve a correct fold. Conversely, a considerable sequence variability is observed in other regions of the xrRNA, suggesting that a correct spatial positioning of the ring-like structure may be of greater importance.



**Fig 4**: Symbolic representation of structural features in TBF xrRNAs. Variability data has been extracted from a comparison of >400 viral sequences.

## 4. Synthetic biology application: Designing artificial xrRNAs and xrRNA riboswitches

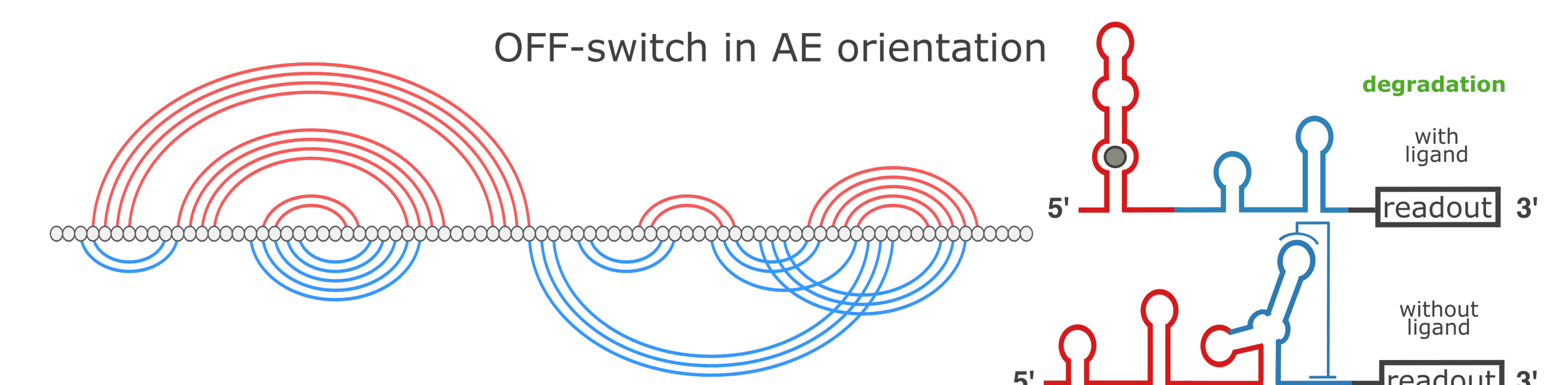
Thorough understanding of native xrRNA structures enables us to engineer synthetic RNA sequences that mimic the folding patterns of natural xrRNAs and possess the ability to stall exoribonucleases. Using RNA 3D folding simulations we could show that it is possible to design artificial sequences that have a high probability of exhibiting xrRNA functionality in experimental evaluation.



**Fig 5**: Tertiary and secondary structure of a computationally designed, artificial xrRNA that follows the folding characteristics of natural xrRNAs. A ring-like structure (red) wraps around the 5' end (blue), while both pseudoknots ensure a correct geometry.

Our RNA design pipeline leverages a well-established thermodynamic model for RNA folding [4] and an innovative method for efficient Boltzmann sampling [5]. This pipeline is capable of producing artificial sequences tailored to exhibit characteristic xrRNA properties, including total length, frequency of specific base pairs, and overall energy of the secondary structure. **Synthetic xrRNAs** offer the advantage of adjusting the overall stability of the fold, for instance, by modulating the strength of the two pseudoknots or other tertiary interactions, thereby fine-tuning the exoribonuclease stalling capability.

A profound understanding of the sequences attainable through artificial RNA design also enables the incorporation of xrRNAs into more **complex RNA structures**. One example of such functional elements is riboswitches, which undergo a conformational change in response to the presence or absence of a ligand. Consisting of an artificial xrRNA and an aptamer that selectively binds a small molecule like theophylline, an **xrRNA riboswitch** serves as an externally inducible molecular device, exhibiting exoribonuclease-stalling activity, that acts as a programmable molecular roadblock.



**Fig 6**: Left: Alternative base pairing of a synthetic RNA device that enables either the formation of a degradation-competent structure (top), or a protective xrRNA (bottom). Right: Schematic representation of a co-transcriptionally controlled xrRNA OFF-switch, comprised of an aptamer (A, red), and an xrRNA expression platform (E, blue). Top: An alternative structure is formed in presence of a ligand, which allows degradation of the entire construct by XRN1. Bottom: Without stabilizing effect of the ligand, formation of the xrRNA is favored, resulting in protection of the readout gene.

xrRNA riboswitches can be designed in different flavors, such as kinetically regulated co-transcriptional OFF-switches or thermodynamically controlled ON-switches. This **technology for targeted RNA degradation** holds the potential for wide-spread application beyond synthetic biology: It can be used to fine-tune **mRNA-based vaccines** or regulate gene expression in various therapeutic contexts, paving the way for more precise and effective treatments.

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