Designing Artificial xrRNA Structures for Regulation of Gene Expression

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1. Xrn1 resistant RNA structures in flaviviruses

Flaviviruses are a group of 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 RNA structures in the 3' untranslated region (3' UTR). One of these functional RNAs are exoribonuclease-resistant RNAs (**xrRNA**), which stall the 5'-3'-exoribonuclease Xrn1, thereby preventing degradation of downstream located RNA regions [1,2].

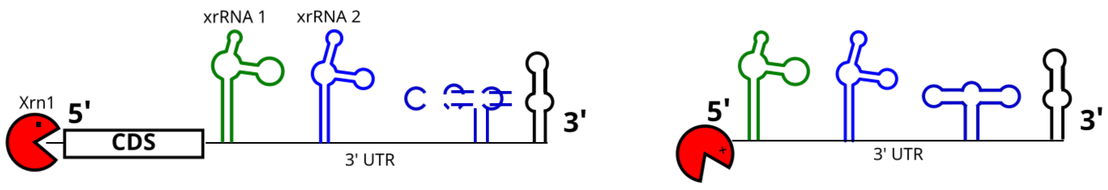
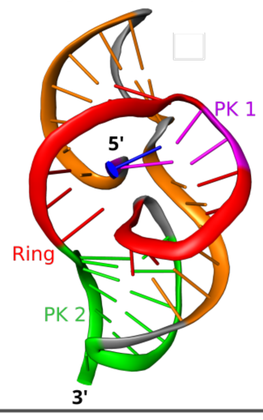


Fig 1: Schematic representation of the mechanism by which flaviviruses hijack the host mRNA degradation pathway. Conserved xrRNA elements, found in single or tandem arrangement within 3'UTRs, act as mechanical blocks of host exonuclease Xrn1 (red pac-man). This stalling results in the production of subgenomic flaviviral RNA (sfRNA), which promotes viral pathogenicity.



The most characteristic 3D feature of xrRNA is a **ring-like structure** that threads around the 5' end of the RNA. When Xrn1 approaches the xrRNA from the 5' side, it braces against the ring, stopping the degradation process. This resistance is not mediated by a specific sequence and therefore not limited to Xrn1, but also other 5'-3'-exoribonucleases and potentially unrelated enzymes moving along the RNA in the same direction [3].

2. Analyzing xrRNA architecture and function

While the overall structures of xrRNAs are highly conserved, the underlying sequences are not. Architecturally, xrRNAs fold into a three-way junction (3WJ), encompassing stems P1-P3, their haripins H2-H3, and two pseudoknots (PK 1 and PK 2) which play a pivotal role in ensuring the stability of the ring-like structure. We characterized sequence conservation, essential structure elements and correlated their lengths.

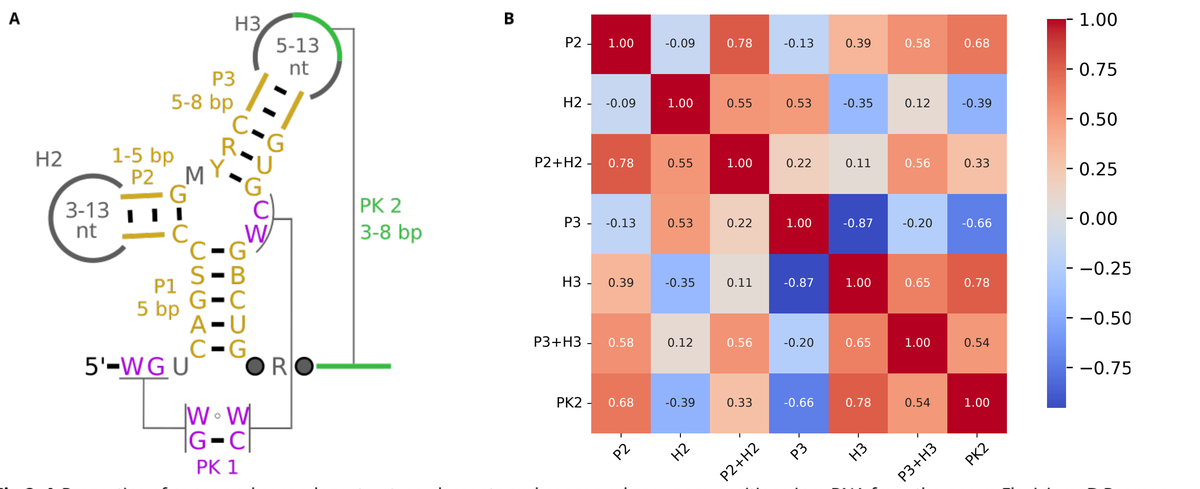


Fig 2: A Properties of conserved secondary structure elements and conserved sequence positions in xrRNA from the genus Flavivirus. **B** Pearson Correlation Coefficients for the lengths of conserved xrRNA elements. The length of PK2 corelates strongly with P3 and H3.

The importance of individual secondary structure motifs for the stalling capacity of the xrRNA was evaluated experimentally by gradually weakening (xrRNA 2-4) or completely deleting the two essential pseudoknots (xrRNA 1&5). The results of Xrn1 degradation assays were then used for quantification of the xrRNA stability.

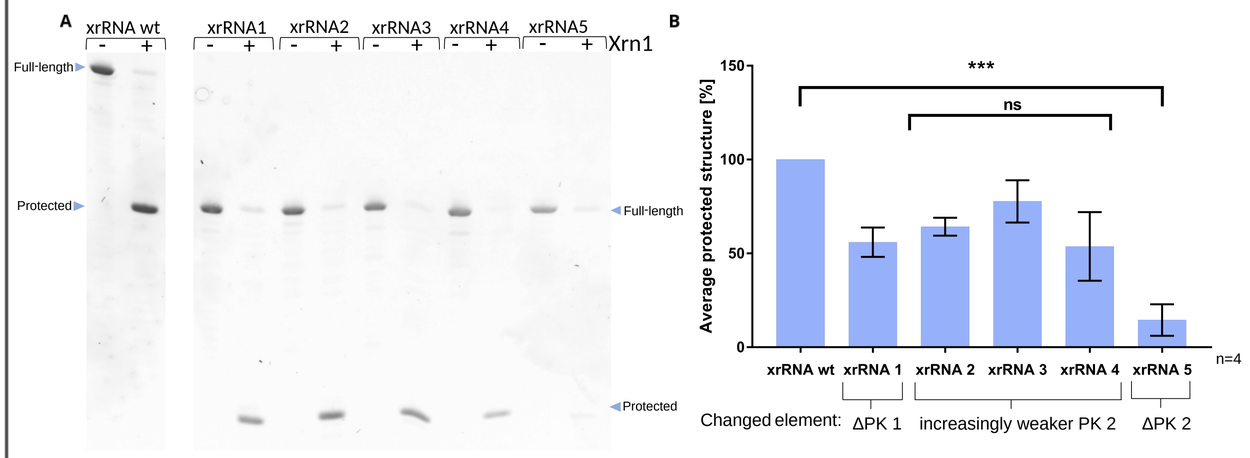


Fig 3: **A** Xrn1 degradation assay of xrRNAs with varying stability of the two pseudoknots PK 1 and PK 2. The Aroa virus xrRNA (xrRNA wt) was used as a starting point to create constructs in which PK 1 (xrRNA1) and PK 2 (xrRNA5) were deleted, respectively. Additionally, in the constructs xrRNA 2 to 4, PK 2 was gradually weakened by deleting one base pair at a time from the interaction. **B** Quantification results of the degradation assay. The graph illustrates the ratio of band intensity of RNA constructs treated and not treated with Xrn1, normalized to the xrRNA wt. PK 1 does not appear to be necessary for the ability to stall Xrn1. Deleting PK 2 led to a drastic decrease in resistance. However, the presence of at least one base pair to create the PK 2 (xrRNA4) was sufficient to partially restore the ability to stall Xrn1. The results were tested for significance (P value <0,05) using Welch's t-test and one-way ANOVA, respectively.

3. Design and *in silico* validation of xrRNAs

We designed functional xrRNAs by sampling sequences which fit into the framework of biological xrRNAs shown in Figure 2 [4]. This approach allows us to fine-tune stability and similarity to biological examples. As a first validation step, we predicted the 3D structures of new designs to confirm the presence of a ring-like structure.

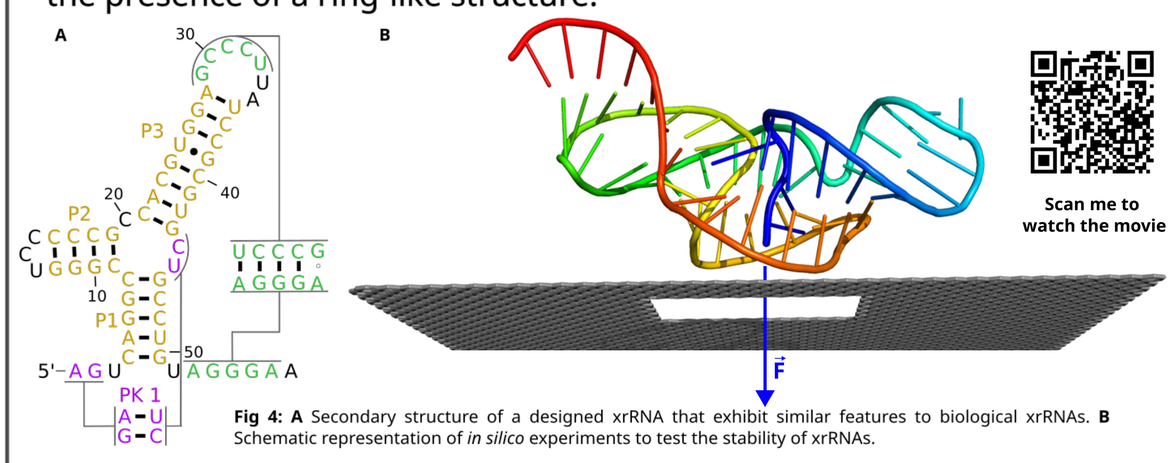


Fig 4: A Secondary structure of a designed xrRNA that exhibit similar features to biological xrRNAs. **B** Schematic representation of *in silico* experiments to test the stability of xrRNAs.

New designs were further tested using molecular dynamics (MD) simulations that mimic the force applied by Xrn1 during degradation. The xrRNA is positioned as shown in Figure 4 and a force gradient is applied to the 5' end. The force at which the RNA unfolds can be used to approximate the stability of the ring-like fold and resistance to degradation by Xrn1.

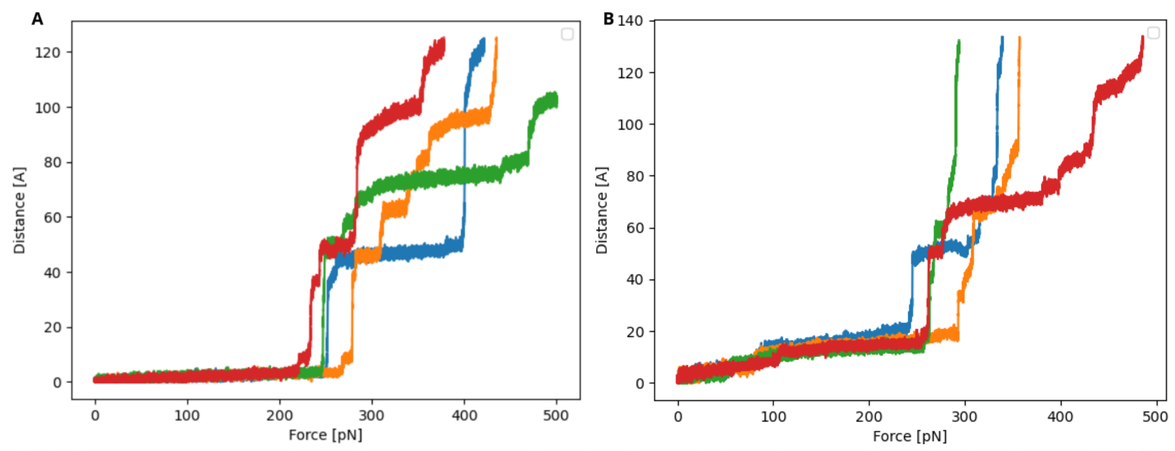


Fig 5: Distance between the starting position and current position of the 5' end with increasing force F for a biological (**A**) and artificial (**B**) xrRNA. A sudden increase of that distance is characteristic for the opening of the ring-like structure. The artificial xrRNA shows similar stability to biological examples.

4. Experimental validation of designs

We validated the artificially designed xrRNA arti1 *in vitro* using degradation assays. This construct exhibited a low resistance towards Xrn1. In-line probing (ILP) was peformed to further investigate its structure. The results were then used to further improve the xrRNA design (arti1.2).

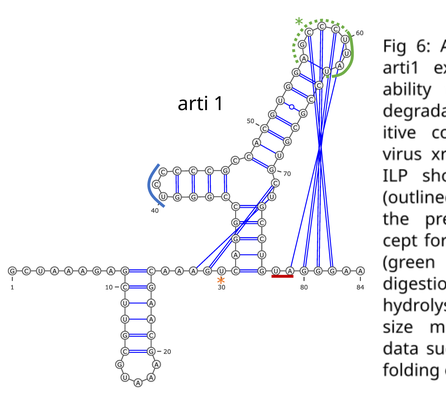
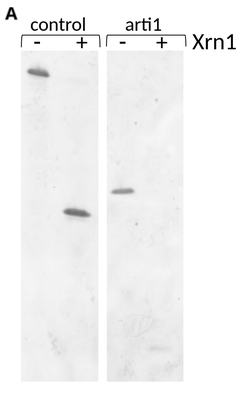
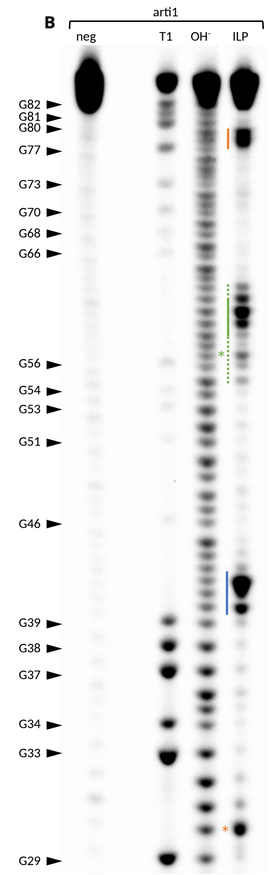


Fig 6: A The designed xrRNA arti1 exhibited only a weak ability to stall Xrn1 in the degradation assay. As a positive control, wild-type Aroa virus xrRNA was used. B The ILP shows unpaired regions (outlined) that correspond to the predicted structure, except for the formation of PK 2 (green outline). RNase T1 digestion (T1) and alkaline hydrolysis (OH-) were used as size markers. The resulting data suggested an insufficient folding of PK 2.





In arti1.2, the stability of the PK 2 was improved, which resulted in an increased resistance comparable to that of the wild-type Aroa virus xrRNA. Further-more, the Xrn1 halt position for the xrRNA arti1.2 was found to be identical to that of the wild-type.

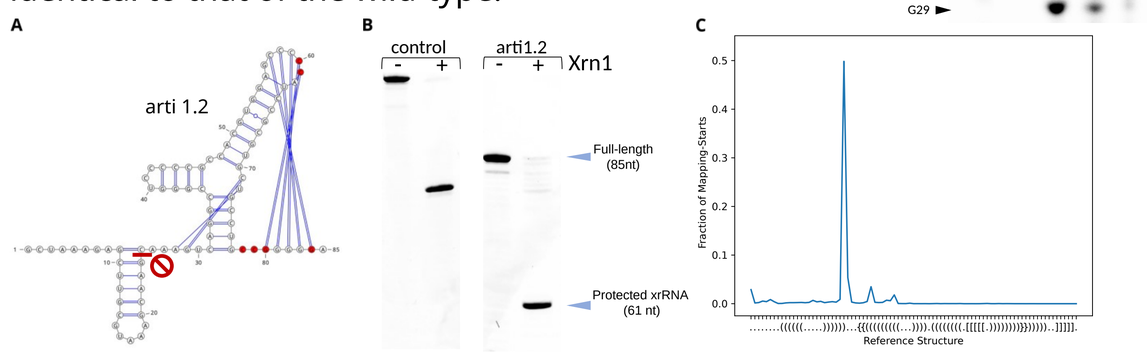


Fig 7: A Secondary structure of design arti1.2, with changes made from the previous design highlighted in red. The experimentally determined stop position is marked by a red stop symbol. **B** The improved artificial xrRNA arti1.2 showed a similar Xrn1-stalling ability to the control (Aroa virus xrRNA). **C** To determine the stop position of Xrn1 adapters were selectively ligated to Xrn1-treated xrRNA and the resulting products were sequenced using amplicon sequencing. [5]

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