Design of synthetic riboswitches that modulate mRNA stability

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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 evolutionarly 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].

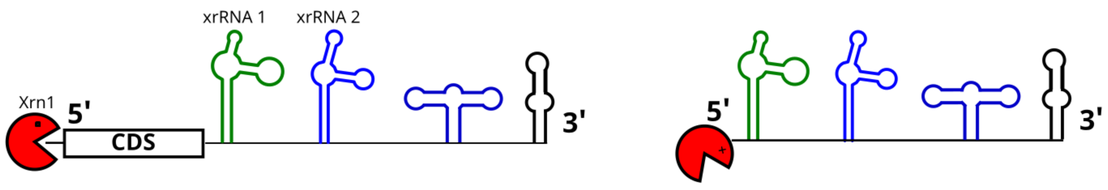
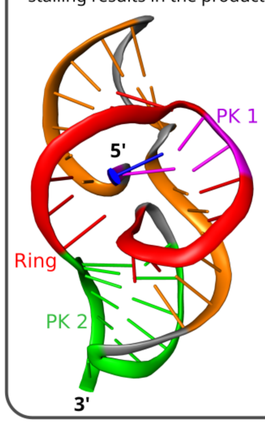


Fig 1: Schematic representation of the mechanism by which flaviviruses hijack the host mRNA degradation pathway. Conserved xrRNA elements, found in single or tandem arrangement within 3'UTRs, act as mechanical blocks of host exonuclease Xrn1 (red pac-man). This stalling results in the production of subgenomic 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 structures of xrRNAs within the genus Orthoflavivirus are highly conserved, the underlying sequences are not. Architecturally, xrRNAs fold into a three-way junction (3WJ), encompassing stems P1-3, and two pseudoknots (PK1 and PK2) which play a pivitol role in ensuring the stability of the ring-like structure. We tested the effect of weakening pseudoknot interactions on the xrRNAs stalling capacity.

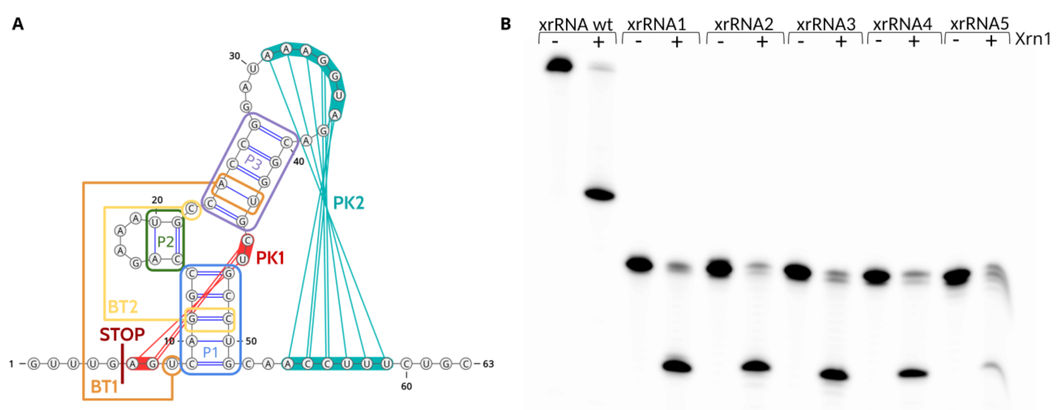


Fig 2: **A** Conserved secondary structure elements of xrRNA in the genus Flavivirus; **B** Degradation assay of xrRNAs with varying stability to further understand the influence of the individual structure elements on the function. If the xrRNA is able to stop degradation, there is a shift in the band when Xrn1 is added (+) compared to the sample without the enzyme (-). The presence of PK1 did not appear necessary for the ability to stall Xrn1, whereas the deletion of PK2 leads to a drastic decrease in resistance (xrRNA 5). Wt: wildtype xrRNA from Aroa virus (AROAV); xrRNA1: AROAV xrRNA with no PK 1; xrRNA2-4: weakening the PK2 of the AROAV xrRNA by stepwise removal of base pairs from the pseudoknot interaction; xrRNA5: AROAV xrRNA with no PK 2.

3. De-novo design of artificial xrRNAs

Designing fully synthetic xrRNAs allows us to adjust the stability of the fold, enabling their integration into larger and more complex RNA structures. We use an innovative method for efficient Bolzmann Sampling [4] to generate numerous sequences that fulfill a set of constraints defined by the secondary structure requirements extracted from biological xrRNAs. These constraints encompass the length, energy, and GC-content of the xrRNA, in addition to specific structural traits such as the base composition of a helix.

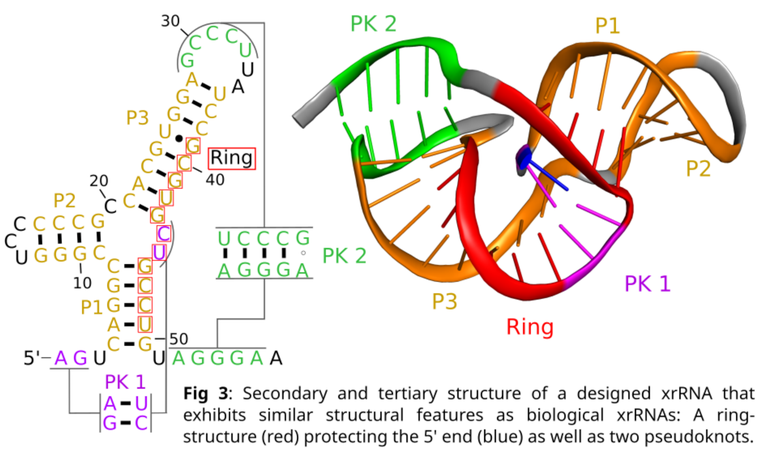


Fig 3: Secondary and tertiary structure of a designed xrRNA that exhibits similar structural features as biological xrRNAs: A ring-structure (red) protecting the 5' end (blue) as well as two pseudoknots.

This enables us to modulate the PK strength, thereby fine-tuning the exoribonuclease stalling capability. A 3D structure can then be calculated to ensure the formation of a ring-like structure and ensuring the overall fold of the xrRNA.

4. Designing xrRNA riboswitches

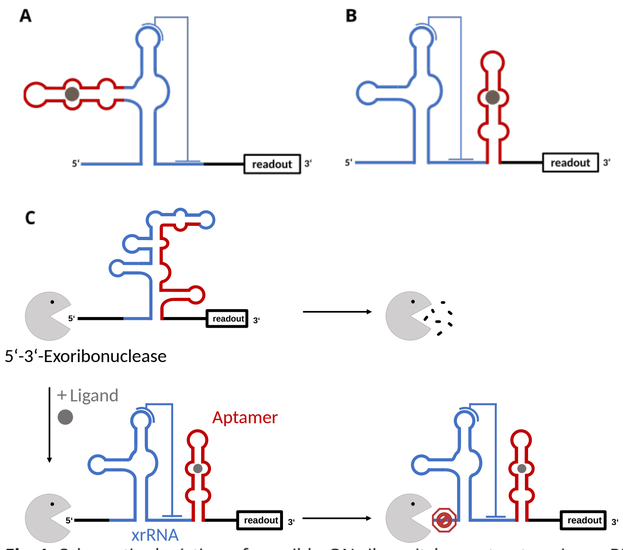


Fig 4: Schematic depiction of possible ON-riboswitch-constructs using xrRNA (blue) as an expression platform with the ligand bound to the aptamer (red). **A** The aptamer can be incorporated into the xrRNA instead of P2, as it serves no apparent function or **B** xrRNA and aptamer can be placed in sequence. **C** Competing fold of the xrRNA riboswitch without and with the ligand present.

Riboswitches are externally inducible molecular devices with a modular structure that undergo conformational change in the presence or absence of a ligand. They encompass an expression plattform and an aptamer. We use a rational approach to design novel riboswitches, that can control the half-life of downstream RNA through ligand-induced structural rearrangement. xrRNA riboswitches can be designed as ON or OFF-switches under kinetic or thermodynamic control. This technology for targeted RNA degradation holds the potential for widespread application beyond synthetic biology: It can be used to fine-tune mRNA vaccines or regulate gene expression in various therapeutic contexts, paving the way for more precise and effective treatments.

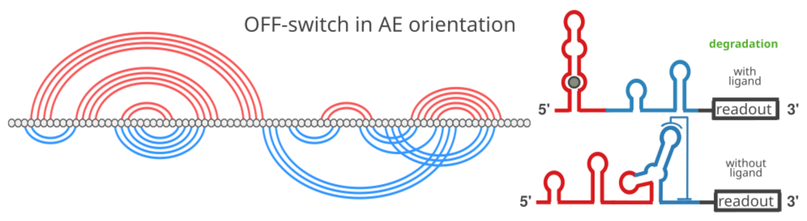


Fig 5: Left: Alternative base paring of a synthetic riboswitch which can either fold into an unprotective (top) or protective (bottom) structure. Right: Schematic secondary structure of the two possible folds. An alternative structure is formed in the presence of a ligand (top), while the native xrRNA structure forms if the ligand is not present.

When designing such a system, the sampled sequences need to be able to fold into two different secondary structures. Our pipeline uses a well-tested thermodynamic energy model [5] to ensure the two structures have an energy difference that allows switching between alternative conformations. We evaluate the functionality of our designs using Molecular Dynamics simulations, approximating the progression of Xrn1 along the RNA. To achieve this, the 5' end of the xrRNA is positioned in front of a pore with the same diameter as the active site of Xrn1. A force, which increases linearly over time, is applied to the 5' end, pulling the RNA through the pore.

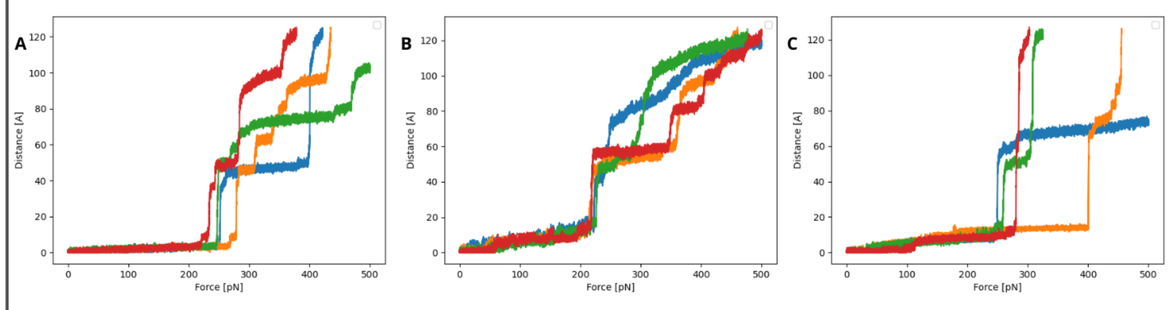


Fig 6: Results of computational pulling experiments on biological and designed xrRNA structures. The force applied to the 5' end is defined by a gradient of 1000 pN/ns. Depicted is the distance between the initial and current position of the 5' end during the simulations. **A** Biological xrRNA from Aroa virus. **B** Artificially designed riboswitch with stem P2 replaced by a theophylline aptamer. **C** designed xrRNA as shown in Fig3.

5. Destroying xrRNAs with antisense oligos

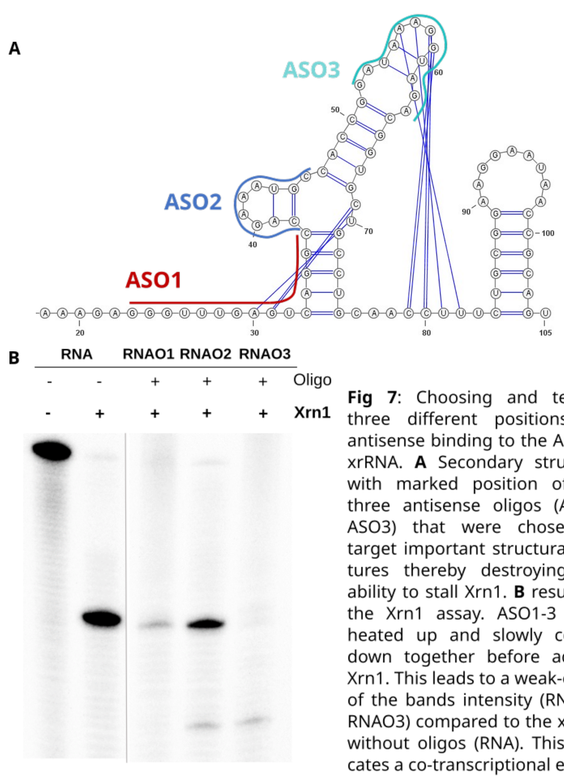


Fig 7: Choosing and testing three different positions for antisense binding to the AROAV xrRNA. **A** Secondary structure with marked position of the three antisense oligos (ASO1-ASO3) that were chosen to target important structural features thereby destroying the ability to stall Xrn1. **B** results of the Xrn1 assay. ASO1-3 were heated up and slowly cooled down together before adding Xrn1. This leads to a weak-ening of the bands intensity (RNAO1-RNAO3) compared to the xrRNA without oligos (RNA). This indicates a co-transcriptional effect.

Another way to regulate mRNA stability is to destroy the xrRNA structure using antisense oligos (ASO) as a toehold. Based on the knowledge gained from our experiments on the structure-function relationship, we chose three binding sites (ASO1-3). ASO1 and ASO3 had the ability to destroy xrRNA resistance. The inability of ASO2 to disrupt resistance supports our assumption that P2 does not play a role in the stability of the fold. The goal is to use ASOs in vivo, which means they are required to displace the xrRNA strands at 37 °C.

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