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How RNA catalyzes cyclization

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The long-awaited crystal structure of the VS ribozyme dimer provides atomic-level insights into how the VS ribozyme folds and catalyzes RNA circularization during rolling circle replication as well as revealing convergent evolution utilized by RNAs to catalyze an S_N2 reaction.

RNAs can fold into complex and dynamic structures and play essential roles including catalysis and gene regulation in viral and cellular life cycles. The *Neurospora* Varkud satellite (VS) RNA has a unit length of 881 nucleotides, and the VS ribozyme (covering residues 600-790 of the VS RNA) is the largest nucleolytic ribozyme, responsible for processing the linear multimeric VS RNA products into circular monomeric forms during rolling circle replication (**Fig 1a**)^{1,2}. The folding and catalytic mechanisms of the VS ribozyme have been extensively studied by solution biochemical and biophysical techniques²⁻⁴, but the absence of a high-resolution crystal structure of the complete VS ribozyme has limited the detailed mechanistic understanding of this RNA sequence at the atomic level. In this issue, Suslov *et al.* end this drought in their report of the first crystal structures of the dimeric VS ribozyme at 3.1 Å resolution (**Fig 1b**)⁵.

Although the secondary structure, or two-dimensional Watson-Crick base pairing pattern, of the VS ribozyme was largely deduced two decades ago by biochemical methods and RNA secondary structure prediction programs⁴, it has remained unclear how the RNA stems and loops were precisely oriented and projected in three dimensions. For example, due to the limited understanding of the energetics of non-Watson-Crick pairs, it is nontrivial to correctly match the potential base pairs and thus determine stacking in RNA junctions⁶⁻⁸. Three-way junctions (**Fig 1b**) are commonly found in RNAs, often using unpaired nucleobases to anchor the structure and thus organize the three-dimensional fold. Coaxial stacking of the stems is further stabilized by the minor-groove and major-groove base triple formation involving the unpaired residues around

junctions. Finally, pseudoknots involve Watson-Crick base pairing between residues in a loop and those outside of the loop. As none of these structural features can be easily deduced from the base pairing pattern alone, the newly reported structures of the VS ribozyme dimer⁵ provide a rewarding glimpse into the mechanisms by which RNA structural motifs can combine to achieve a highly coordinated and dynamic fold.

To obtain a homogeneous conformation and facilitate crystallization, Suslov *et al.* engineered the VS ribozyme by replacing the tetraloop capping stem 4 with a more flexible pentaloop, which eliminates RNA aggregation and mediates crystal packing. Two catalytically essential loop nucleobases were mutated separately to prevent cleavage. Furthermore, three stems remote from the catalytic sites were altered by truncating the A-U-rich stem 4 by 3 base pairs, and by increasing the G-C content of stems 6 and 7. The structures reveal several non-Watson-Crick pairs that define three-way junctions, which contribute to aligning the stems⁵. Furthermore, the long-range loop-loop interactions outside of the junction induce the pairing **and breaking** of Watson-Crick and non-Watson-Crick pairs around the 3-way junction^{3,9}. A 3-base-pair kissing complex – a special class of pseudoknot – formed between stem loop 1 and stem loop 5 (**Fig 1b**) in the VS ribozyme is critical for facilitating tertiary interactions to create the catalytic site. Remarkably, formation of the magnesium ion-induced kissing complex induces secondary structure rearrangement by shifting three pre-formed G-C Watson-Crick base pairs, a conformation that was captured in part by the authors' engineering efforts⁵.

An important methodological detail of the study from Suslov *et al.* is that the authors purified the VS ribozyme dimer from the *in vitro* transcription products without denaturation and renaturation, and thus the crystal structures show a functionally relevant dimer where one monomer creates a docking site for the substrate helix of the other protomer⁵. It has been hypothesized that the kissing complex can form either intramolecularly and intermolecularly, explaining how the VS ribozyme can act in *cis* or in *trans*, respectively. The dimeric VS ribozyme structure further suggests a catalytic mechanism in which the *trans* cleavage reaction is followed by a *cis* ligation reaction, resulting in the generation of intact circular RNA from the multimeric linear RNA products created during the rolling circle replication of *Neurospora*⁵. The crystal structure also provides evidence that G638 in the substrate loop in stem 1 and A756 in stem 6 serve as the general base and acid in the cleavage reaction (**Fig 1b**), with the roles reversed in the ligation reaction, consistent with the principle of microscopic reversibility². Catalysis also seems to require tertiary loop-loop interactions and other tertiary interactions to

induce the breaking of the non-Watson-Crick pairs in the substrate loop, followed by base flipping to align the residues for catalysis. Overall, the detailed architecture of the catalytic site of the VS ribozyme resembles those of hairpin and hammerhead ribozymes despite the global structural and catalytic mechanistic differences, suggesting convergent evolution in RNA⁵, which is often observed in protein enzymes.

The ultimate goal of RNA structural biology is to build up our understanding of and thus ability to rationally engineer the structure, dynamics, and function of these intriguing biomolecules, and the new data from Suslov *et al.* expand these horizons in completing the structural inventory of all known ribozymes. The questions remaining to be answered include: How does the VS ribozyme precisely employ a sequential *trans* cleavage-*cis* ligation mechanism to produce the unit length RNA circles? How does the VS ribozyme prevent self-cleavage of the circular VS RNA? Are the other parts of the 881-nucleotide VS RNA and/or protein factors such as a helicase involved in the structural dynamics and catalysis? Can we follow the folding dynamics and cleavage/ligation reactions of multimeric VS RNA in real time by time-resolved chemical mapping and single-molecule techniques? Can we engineer and chemically modify ribozymes to act on any desired RNA sequence? Both the reported folding motifs evolved in nature for VS ribozyme function and the molecular engineering strategies for facilitating crystallization may inspire other RNA researchers to design new experiments and develop RNA as scaffolds for applications in biochemistry and biotechnology.

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Figure 1. Secondary and three-dimensional structures of the VS ribozyme responsible for catalyzing the unit length VS RNA cyclization. (a) Diagram of the rolling circle replication of VS

RNA. The generation of 881-nucleotide monomeric circular VS RNA is catalyzed by the VS ribozyme (covering residues 600-790 of the VS RNA) through a proposed sequential *trans* cleavage-*cis* ligation of residues A621 and G620. **(b)** The secondary structure diagram and three-dimensional structure of the VS ribozyme. The three-way junctions are critical for organizing the three-dimensional fold. The kissing complex formed between stem loop 1 and stem loop 5 in the VS ribozyme is critical for facilitating the tertiary interaction to create the catalytic site. In the cleavage/ligation reaction, G638 (red diamond) in the substrate loop in stem 1 and A756 (red diamond) in stem 6 serve as general base/acid and general acid/base, respectively. Diagram and structure reprinted from ref. 5.

a **b**