Circularly-Permuted Pistol Ribozyme: a Synthetic Ribozyme Scaffold for Mammalian Riboswitches

Kamila Mustafina[1,2], Yoko Nomura[1], Rachapun Rotrattanadumrong[1], Yohei Yokobayashi[1]

[1]Nucleic Acid Chemistry and Engineering Unit, Okinawa Institute of Science and Technology Graduate University, Okinawa, 904-0495, Japan

[2]Department of Bioengineering, Massachusetts Institute of Technology, Cambridge, MA 02141, USA

[*]Corresponding author. Email: kamilams@mit.edu

Background

Controlling gene expression in mammalian cells by addition of a small molecule is one of the major research directions in therapeutics and Synthetic Biology. Aptazymes are a promising RNA-based tool for achieving that goal. Aptazymes are engineered by fusing a self-cleaving ribozyme with an RNA aptamer that recognizes a small molecule so that the ribozyme is either activated or inhibited in the presence of the small molecule. When embedded in the untranslated region of an mRNA, aptazyme functions as a riboswitch that allows chemical regulation of gene expression in mammalian cells. However, the variety of aptamers, ribozymes, and aptazyme design strategies suitable for mammalian riboswitch applications is still limited. In this work we introduce a new synthetic ribozyme scaffold for engineering aptazymes and riboswitches that function in mammalian cells. We engineer naturally occurring pistol ribozymes to derive variants better suited to act as riboswitches.
To our knowledge, the topology of this circularly permuted pistol ribozyme (CPP) scaffold has not been observed in nature, suggesting new opportunities to explore functional RNAs beyond naturally occurring sequences and structures.

Methods

We generated new variants of the pistol ribozyme either by ordering oligos from IDT or by mutagenesis PCR and then followed the standard molecular biology techniques to clone them into a plasmid. The plasmid contained a gene encoding a reporter EGFP, and the aptazyme was embedded in its 3'UTR, so the ribozyme/aptazyme activity was negatively correlated with the fluorescence level. Upon amplification in competent *E.Coli*, the plasmids were harvested and sequence-verified by Sanger sequencing. The plasmids were subsequently transfected into HEK293 cells seeded in 96-well plates by following TransIT-293 (Mirus Bio) manufacturer's protocol. Five hours after transfection, the medium in each well was replaced with fresh medium with (250 μM) or without guanine. Aptazymes' activity was assessed by measuring fluorescence with a Tecan plate reader.

For the high throughput screening a library of 1024 variants was generated using a randomized primer from IDT and mutagenesis PCR. The library was transfected into HEK293T cells and was next processed according to the workflow from Mustafina et al, 2021. The resulting library was then sequenced with Illumina Miseq using MiSeq Reaget Kit v3 with a loading concentration of 12 pM with 15% PhiX control to increase the sequence diversity. Resulting reads were processed using a custom Python script.

Results

In our previous work we assessed activity of pistol ribozymes in mammalian cells but failed to identify variants suitable for aptazyme design (Nomura et al 2017). This time we decided to examine circularly permuted variants of the pistol ribozymes. Since the 5' and 3' termini of the pistol ribozyme motif do not form a stem as hammerhead and twister, we connected the native termini were by a 6-nt linker. The new 5' and 3' termini were generated by breaking the L3 loop (Figure 1A)

Out of the CPP variants, cp-sp51343 and cp-sp56441 strongly repressed EGFP expression suggesting strong ribozyme activity in HEK293 cells (Figure 1B). Importantly, insertion of a 10-nt anti-Rz sequence upstream of these CPP variants resulted in upregulation of EGFP expression, indicating that the anti-Rz sequence interferes with the CPP scaffold structure. anti-Rz sequence is an important component of our aptazyme design strategy previously demonstrated with a twister ribozyme (Mustafina et al. 2020). Due to its lower EGFP expression (higher ribozyme activity), cp-sp51343 was used as a CPP scaffold in the following experiments.

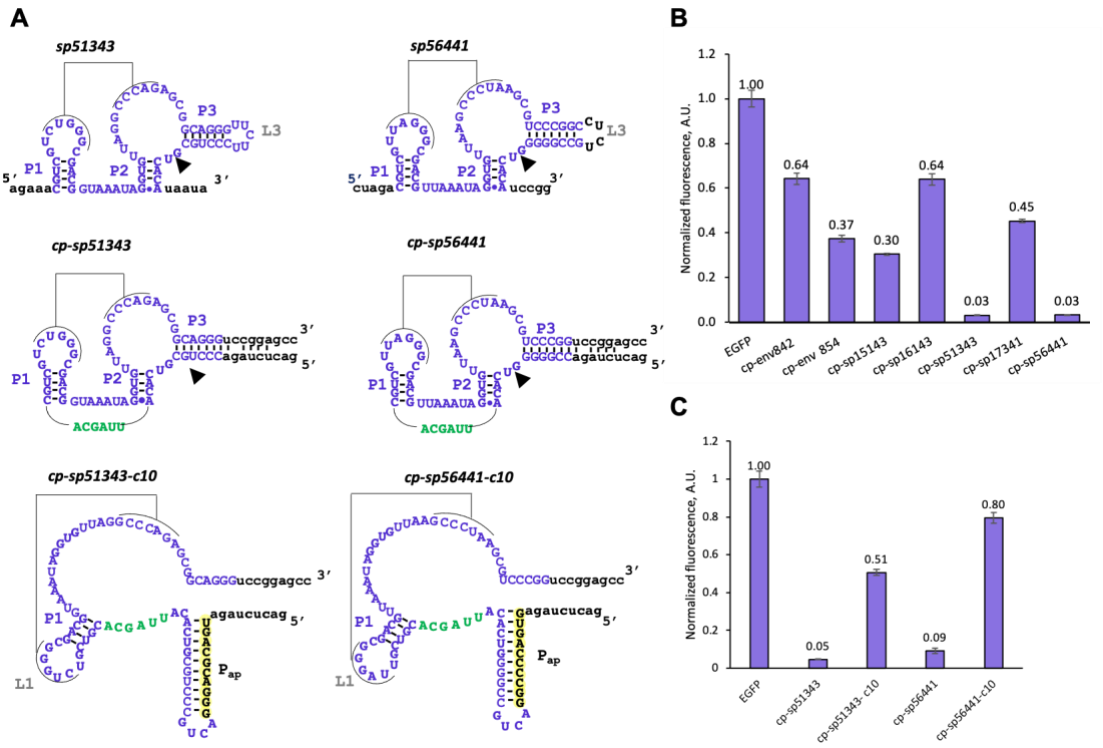

Figure 1. Circular permutation of pistol ribozymes. Seven highly active pistol variants from Nomura et al 2017 (env842, env854, sp14343, sp16143, sp51343, sp17341, sp56441) were circularized by the introduction of flexible circular junction (green) connecting stems P1 and P2. A) Predicted secondary structures of two most active circularized variants cp-sp51343 and cp-sp56441 (middle), their precursors sp51343 and sp56441 (top), and variants with 10-nt complementary insert cp-sp51343-c10 and cp-sp56441-c10 (bottom). Ribozyme sequence is shown in purple, circular junction in green, complementary insertion (10nt) is in blue, and added spacer loop is shown in black. Surrounding plasmid sequence is shown in lowercase letters. Cleavage site is indicated with a black triangle. B),C) Normalized EGFP expression of circularized ribozymes in HEK293 cells. EGFP: empty control plasmid without the ribozyme. B) sp51343 and sp56441 variants remained highly active after circular permutation (cp-sp51343, cp-sp56441). C) Cp-sp51343-c10 and cp-sp56441-c10 variants have reduced ribozyme activity (increased EGFP expression) compared to their precursors with no complementary inserts.

Next we designed aptazymes using the cp-sp41343 variant and guanine aptamer (Figure 2), and yielded switches with on-off ratios over 8.

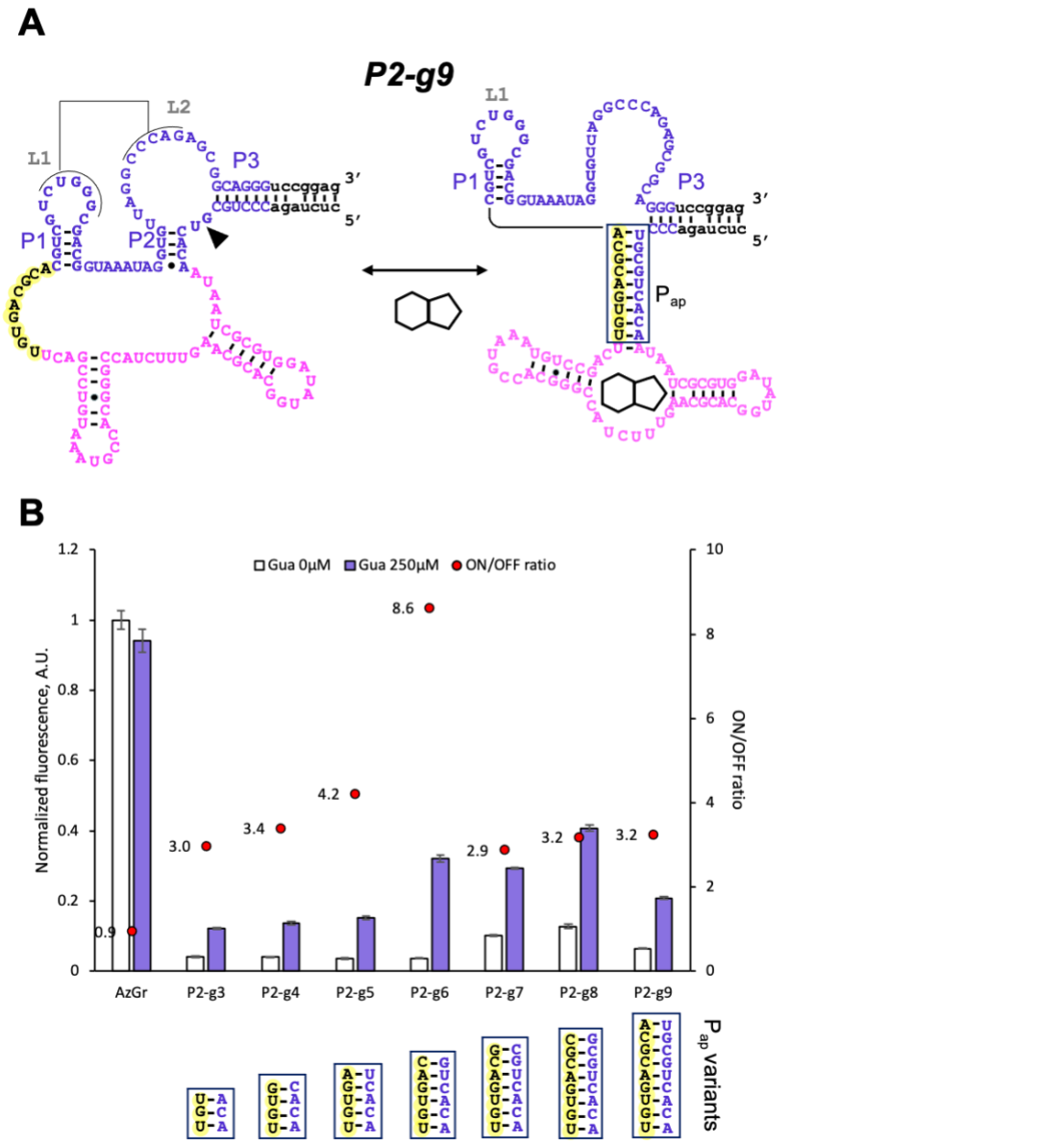

Figure 2. Size optimization of P_{ap} competing with P2 stem yields CPP-guanine on-switches. A) *predicted secondary structure of the aptazyme with 9-nt communication module in the absence (left) and in the presence (right) of guanine. Formation of the P_{ap} is expected to interfere with P2 and partially P3 stem. Ribozyme is marked in purple, guanine aptamer in pink and communication module in blue. P_{ap} is boxed. B) Activity of the aptazyme variants in HEK293 cells. Boxes indicate P_{ap} composition of each variant. AzGr: empty vector with no aptazyme.*

We further characterized this promising design by randomizing positions in the anti-Rz sequence and we ran RNA-seq-based screening of this library in HEK293T cells (Figure 3) (Xiang et al 2019, Strobel et al 2020). Although we did not find variants better than the original switch P2-g6, we confirmed sequence motifs in the anti-Rz that were conserved for the best switches.

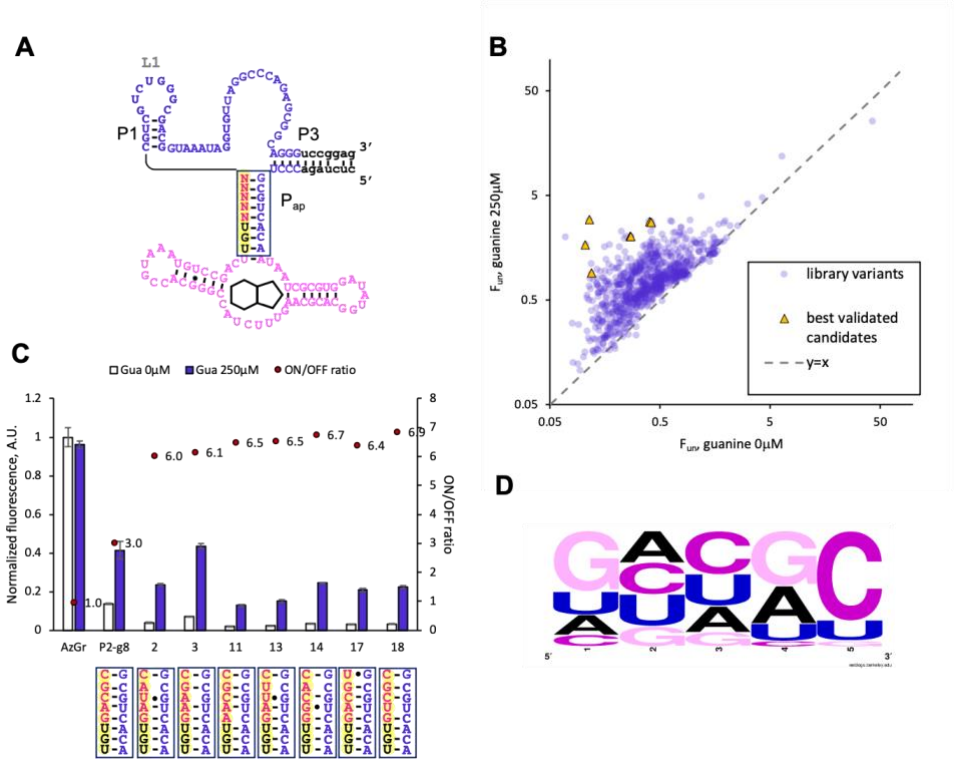

Figure 3. Summary of RNA-seq assay for the CPP-guanine P2 library. A) Schematic of the library design. Ribozyme is marked in purple, guanine aptamer in pink, communication module in blue, and randomized nucleotides (N) in red. P_{ap} is boxed. Cleavage site is sequestered in P_{ap}. B) Distribution of fractions uncleaved for each variant with and without guanine. Each dot represents a variant with unique sequence of N5 randomized region. Variants with higher fractions uncleaved in the presence of guanine are potential on-switches. The best 7 switches identified by screening and validated in cells in (C) are marked in yellow triangles. Names of variants correspond to their ranking based on ON/OFF ratio from replicate 1 of RNA-seq assay. C) Normalized Azami Green expression of selected 7 switches with highest ON/OFF ratios in cells identified by screening. AzGr: empty vector with no aptazyme. N5: CPP-guanine P2 library containing all randomized variants. G8: variant with fully complementary P_{ap}. D) Sequence logo (Crooks et al, 2004) of the 40 candidates with highest ON/OFF ratio based on RNA-seq assay.

Conclusion

CPP is the fourth self-cleaving ribozyme scaffold demonstrated to function as riboswitches after the hammerhead, HDV, and twister ribozymes. Notably, it is the first nonnatural ribozyme scaffold to be used as a riboswitch in living cells. We adapted the previously reported aptazyme design strategy to this new scaffold extended it further by inserting an aptamer and an anti-Rz sequence in other parts of the ribozyme, such as within the flexible linker region. We also characterized and improved the aptazyme performance by high-throughput screening in mammalian cells directly. Thus, this work corroborates our previously reported aptazyme design

principles and introduces a novel ribozyme scaffold along with aptazymes based on it for Synthetic Biology needs.

Funding

Okinawa Institute of Science and Technology Graduate University

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