

Article

Allosteric modulation of *F. prausnitzii* HDV-like ribozyme by glucosamine 6-phosphate — the substrate of the adjacent gene product

Luiz F. M. Passalacqua, Randi M. Jimenez, Jennifer Y Fong, and Andrej Luptak

Biochemistry, Just Accepted Manuscript • DOI: 10.1021/acs.biochem.7b00879 • Publication Date (Web): 18 Oct 2017

Downloaded from <http://pubs.acs.org> on October 19, 2017

Just Accepted

“Just Accepted” manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides “Just Accepted” as a free service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. “Just Accepted” manuscripts appear in full in PDF format accompanied by an HTML abstract. “Just Accepted” manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are accessible to all readers and citable by the Digital Object Identifier (DOI®). “Just Accepted” is an optional service offered to authors. Therefore, the “Just Accepted” Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the “Just Accepted” Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these “Just Accepted” manuscripts.



ACS Publications

Biochemistry is published by the American Chemical Society, 1155 Sixteenth Street N.W., Washington, DC 20036

Published by American Chemical Society. Copyright © American Chemical Society. However, no copyright claim is made to original U.S. Government works, or works produced by employees of any Commonwealth realm Crown government in the course of their duties.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Allosteric modulation of *F. prausnitzii* HDV-like ribozyme by glucosamine 6-phosphate — the substrate of the adjacent gene product

Luiz F. M. Passalacqua^{‡1}, Randi M. Jimenez^{‡2}, Jennifer Y. Fong¹, Andrej Lupták^{*1,2,3}

¹Department of Pharmaceutical Sciences, University of California, Irvine, California 92697, USA

²Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92697, USA

³Department of Chemistry, University of California, Irvine, California 92697, USA

ABSTRACT: Self-cleaving ribozymes were discovered 30 years ago and have been found throughout nature, from bacteria to animals, but little is known about their biological functions and regulation, particularly how cofactors and metabolites alter their activity. An HDV-like self-cleaving ribozyme maps upstream of a phosphoglucosamine mutase (*glmM*) open reading frame (ORF) in the genome of the human gut bacterium *Faecalibacterium prausnitzii*. The presence of a ribozyme in the untranslated region of *glmM* suggests a regulation mechanism of gene expression. In the bacterial hexosamine biosynthesis pathway, the enzyme *glmM* catalyzes the isomerization of glucosamine 6-phosphate into glucosamine 1-phosphate. In this study, we investigated the effect of these metabolites on the co-transcriptional self-cleavage rate of the ribozyme. Our results suggest that glucosamine 6-phosphate, but not glucosamine 1-phosphate, is an allosteric ligand that increases the self-cleavage rate of *drz-Fpra-1*, providing the first known example of an allosteric modulation of a self-cleaving ribozyme by the substrate of the adjacent gene product. Given that the ribozyme is activated by the *glmM* substrate, but not the product, this allosteric modulation may represent a potential feed-forward mechanism of gene expression regulation in bacteria.

INTRODUCTION

Ribozymes are RNA molecules that can catalyze a chemical transformation in the absence of a protein cofactor¹⁻³. Self-cleaving ribozymes comprise a group of RNA molecules that promote a site-specific self-scission reaction. In all known self-cleaving ribozymes, the cleavage reaction is a transesterification that involves a nucleophilic attack by a 2' oxygen on the adjacent phosphodiester bond, producing a 2'-3' cyclic phosphate and a 5'-hydroxyl products⁴⁻¹⁴. To date, nine self-cleaving ribozyme families have been discovered, comprising the hairpin¹⁰, hammerhead^{8,9}, hepatitis delta virus (HDV)^{6,7}, glucosamine-6-phosphate synthase (*glmS*)¹¹, *Neurospora* Varkud satellite (VS)¹², twister¹³, twister sister (TS), pistol, and hatchet motifs¹⁴. First characterized 30 years ago^{6,7,15}, the HDV self-cleaving ribozyme has been extensively studied, with elucidated crystal structures

and mechanism of self-scission¹⁶⁻²¹. HDV-like self-cleaving ribozymes exhibit great sequence diversity, but fold into a conserved secondary structure that includes a nested double-pseudoknot²². HDV-like ribozyme have been found in many eukaryotes, including humans, as well as in *Chilo* iridescent virus, several bacteria, and most recently in several microbial metagenomic datasets^{14, 22-27}. These self-cleaving RNAs likely play a number of distinct roles in biology²²⁻²⁷, but little is known about their regulation, particularly with regard to the role ligands or metabolites may have in modulating HDV-like ribozyme self-cleavage as either allosteric effectors or cofactors.

One particular case of interest is the *drz-Fpra-1* HDV-like ribozyme, found in the human gut bacterium *Faecalibacterium prausnitzii*²². *F. prausnitzii* is a Gram positive Firmicute that represents more than 5 % of the total bacterial population in the fecal microbiota of a healthy human and is suggested to be negatively correlated with certain pathologies, such as Crohn's disease and ulcerative colitis²⁸⁻³⁰. The ribozyme cleavage site maps 106 nucleotides upstream of phosphoglucosamine mutase (*glmM*) open reading frame (ORF)²² (Figure 1A). The enzyme *glmM* is a component of the hexosamine biosynthesis pathway, catalyzing the transformation of glucosamine 6-phosphate (GlcN6P) into glucosamine 1-phosphate (GlcN1P)³¹ (Figure 1B). The final product of the hexosamine biosynthesis pathway is uridine diphosphate N-acetyl-glucosamine (UDP-GlcNAc), a key substrate used for cell wall biosynthesis³². The secondary structure of the ribozyme is shown in Figure 1C.

In bacteria, a common mode of regulation of gene expression involves sensing a metabolite related to the adjacent gene product through an RNA regulatory element called a riboswitch³³. In the case of the *glmS*, a GlcN6P-sensing riboswitch³⁴, the RNA is also a self-cleaving ribozyme that resides at the 5' untranslated region (UTR) of the transcript encoding glutamine fructose-6-phosphate amidotransferase, which catalyzes the transformation of fructose-6-phosphate into GlcN6P¹¹. The metabolite GlcN6P acts as a co-factor of the *glmS* ribozyme, accelerating its self-scission nearly one-million-fold^{11, 35-39}, and promoting the degradation of the adjacent ORF through an RNase J-dependent mechanism⁴⁰. Thus, this feedback system senses the amount of GlcN6P in the cell and represses gene expression through the ribozyme-dependent activity. This metabolite-responsive regulation system has been well-characterized, by structural, bio-

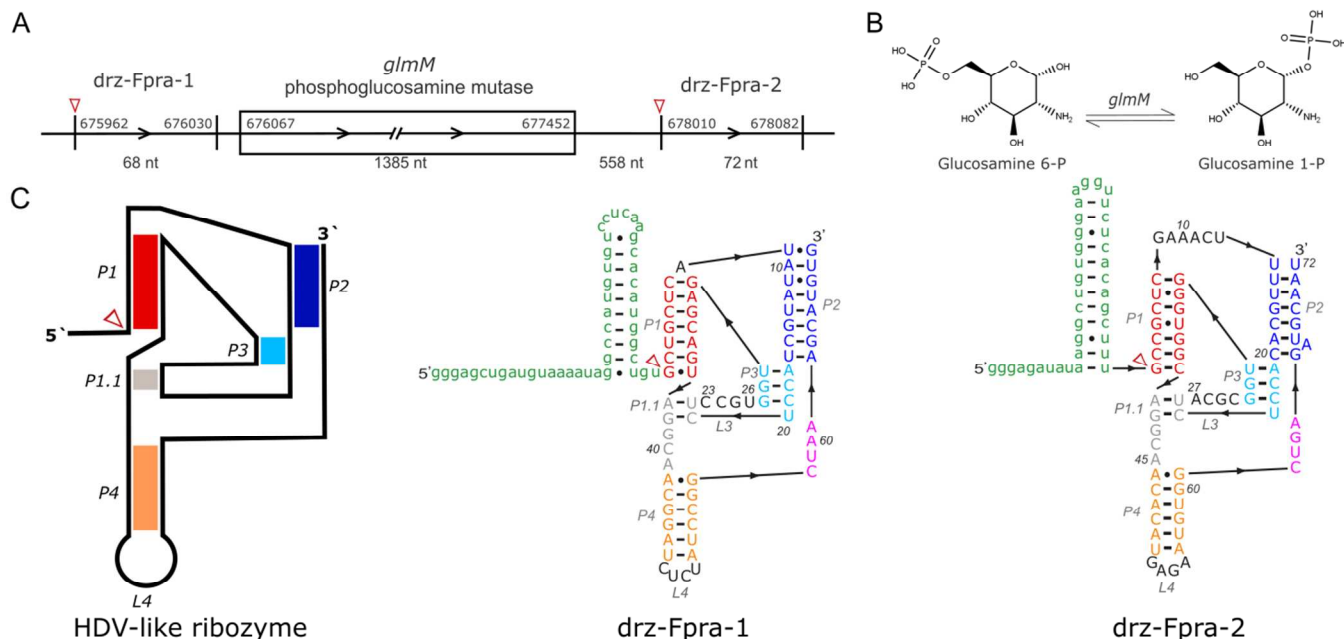


Figure 1. HDV-like ribozymes in *F. prausnitzii*. (A) Genome locus of *F. prausnitzii* showing the *glmM* ORF, the upstream drz-Fpra-1 ribozyme and the newly discovered drz-Fpra-2 ribozyme. (B) The isomerization reaction promoted by the *glmM* enzyme. (C) A schematic representation of the secondary structure of HDV-like ribozymes (left), and predicted secondary structures of drz-Fpra-1 and drz-Fpra-2 ribozymes, including hairpins in the 5' leader sequence. Red arrowheads mark the cleavage sites.

chemical and mechanistic studies^{11, 35-39, 41-44}. Other metabolites, such as glucose 6-phosphate (Glc6P), may compete with GlcN6P and thus upregulate the gene expression by inhibiting the self-cleavage of the RNA^{39, 41}. Surprisingly, only 3 mutations in the active site are necessary to convert the ribozyme into a coenzyme-independent self-cleaving ribozyme, revealed by an *in vitro* selection of a GlcN6P-insensitive *glmS* ribozyme that used a divalent cation for catalysis without changing the overall fold of the ribozyme⁴².

Due to the proximity of the HDV-like ribozyme and the *glmM* gene, we hypothesized that the ribozyme drz-Fpra-1 and the metabolites involved in the hexosamine biosynthesis pathway may contribute to gene expression regulation through modulation of the ribozyme activity. During the course of this work, we found another HDV-like ribozyme, named drz-Fpra-2 (Figure 1C), downstream of the *glmM* gene and we chose to study the two ribozymes in parallel. To explore the mechanism of this putative regulation, we studied the *in vitro* ribozyme self-cleavage kinetics in the presence of several metabolites. The kinetic and structural probing of the drz-Fpra-1 in the presence of the metabolites shows an allosteric modulation of the ribozyme by the substrate, but not the product, of the adjacent gene product. The effect on drz-Fpra-2 was largely the opposite.

MATERIALS AND METHODS

***In vitro* RNA transcription.** RNA was transcribed at 37 °C for one hour in a 20 µL volume containing 10 mM DTT, 2 mM spermidine, 1.25 mM each rNTP, 7.5 mM MgCl₂, 1 unit of T7 RNA polymerase, and 0.5 pmole of DNA template. The transcripts were purified by 10 % polyacrylamide gel electrophoresis (PAGE) under denaturing conditions (7M urea). RNA was eluted from the gel into 300 µL of 300 mM KCl and precipitated by adding 700 µL of 100% ethanol at -20 °C.

***In vitro* co-transcriptional cleavage kinetics.** *In vitro* transcription was performed similarly to the above-described RNA tran-

scription assay with the following modifications: 4.5 mM MgCl₂; 1.25 mM of each GTP, UTP, CTP; 250 µM ATP; 4.5 µCi [α -³²P]-ATP (Perkin Elmer); and 40 mM HEPES pH 7.4. A 10 µL transcription reaction was initiated by the addition of DNA and incubated at 24 °C for 10 min. A 1.0 µL aliquot of the reaction was withdrawn and its transcription and self-scission terminated by the addition of urea loading buffer. The remaining 4.0 µL volume was diluted 25-fold (100 µL final volume) into a physiological-like buffer: 50 mM HEPES pH 7.4, 10 mM NaCl, 140 mM KCl, and the desired concentration of MgCl₂ and metabolite. Control experiments showed that this dilution efficiently prevented any new RNA synthesis; therefore, the kinetics of transcription did not need to be accounted for in our kinetic analysis, contrasting with the previously described analysis of co-transcriptional cleavage by Long and Uhlenbeck⁴⁵. For conditions requiring consistent ionic strength, the buffer and metabolite stocks were pH-adjusted by the addition of KOH and the contribution of K⁺ and Na⁺ from the metabolite stocks was tracked. The concentration of K⁺ was adjusted by the addition of KCl for a final reaction concentration of 140 mM. 5 µL aliquots were collected at the indicated times following the dilution of the transcription reaction into the physiological-like buffer at 37 °C and self-scission was terminated by adding 5 µL volume of stop buffer containing 20 mM EDTA, 5 mM Tris pH 7.4, 8 M urea, with xylene cyanol and bromophenol blue loading dyes. The denaturing PAGE gel of cleavage products was exposed to phosphorimage screens and analyzed using Typhoon phosphorimager and ImageQuant software (GE Healthcare). The band intensities were analyzed by creating line profiles of each lane using ImageQuant, exporting the data to Microsoft Excel. Self-cleavage data were fit to a mono-exponential decay function (Equation 1)

$$k_{obs} = A \cdot e^{(-kt)} + C \quad (\text{Equation 1})$$

where A and C represent the relative fraction of the ribozyme

1 population cleaving with a rate constant k or remain uncleaved, respectively. The model was fit to the data using a linear least-squares analysis and the Solver module of Microsoft Excel.

2
3 **RNA 3'-terminus labeling.** RNA was *in vitro* transcribed and PAGE-purified. The appropriate RNA species was excised, precipitated, and re-suspended in water. RNA was then ligated at 37 °C for 3 hours in a volume of 10 μ L, in RNA ligase buffer containing 50 mM Tris-HCl, 10 mM MgCl₂, 1 mM DTT and pH 7.5 (NEB), 2 μ M [5'-³²P] cytidine 3', 5'- bisphosphate (Perkin Elmer) and one unit of T4 RNA ligase (NEB) and PAGE purified again.

4
5
6 **In-line probing.** The 3'-end labeled RNA was incubated with varying amounts of ligand for up to 2 days at 37 °C in a buffer containing 140 mM KCl, 10 mM NaCl, 20 mM Tris chloride, pH 8.5, 1 mM MgCl₂, and 1 mM spermidine, based on the in-line probing technique of Soukup and Breaker⁴⁶. The partially hydrolyzed RNAs were resolved using denaturing PAGE, exposed to phosphorimage screens (Molecular Dynamics/GE Healthcare), and scanned by GE Typhoon phosphorimager. The sequences in the degradation pattern were assigned by running α -phosphorothioate nucleotide modified RNA cleaved by treatment with iodoethanol in parallel lanes^{47,48}. The band intensities were analyzed by creating line profiles of each lane using ImageQuant and exporting the data to Microsoft Excel. The areas of the fitted curves were used to measure intensity changes related to the binding, divided by intensities of a control band. The resulting ratios were plotted in Excel as a function of ligand concentration and modeled with a dissociation constant equation for a single ligand:

7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

$$\text{Fraction Bound} = \frac{\left(\frac{[\text{ligand}]}{([\text{ligand}] + K_{Dd})} - \text{Baseline} \right)}{\text{Range}} \quad (\text{Equation 2})$$

The model was fit to the data using a linear least-squares analysis and the Solver module of Microsoft Excel.

Metabolites. All the metabolites used in this study were purchased from Sigma Aldrich. To measure the concentration of free phosphate, which may affect the ribozyme kinetics by forming an insoluble complex with the Mg²⁺ ions, we used a fluorescent phosphate sensor based on the bacterial phosphate-sensing protein (Thermo Fisher). The molar ratio of free phosphate in the GlcN1P, GlcN6P, and GlcP was found to be 0.0001, 0.002, and 0.0004.

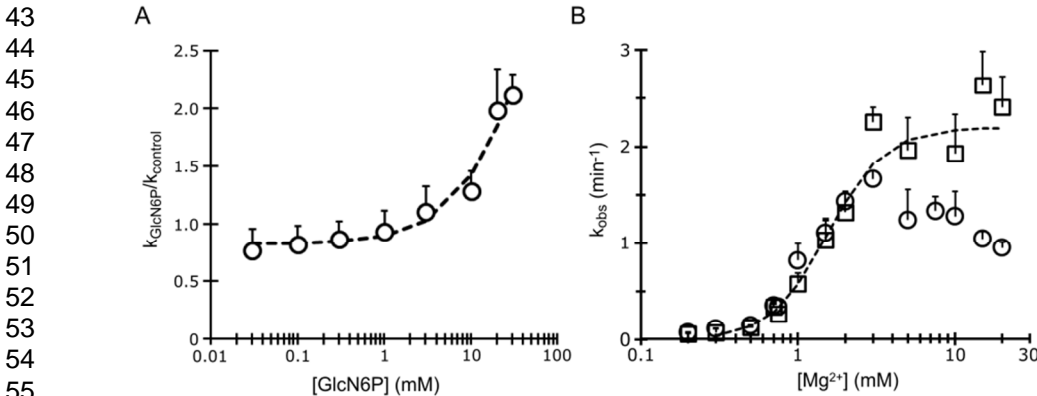


Figure 3. Effect of GlcN6P and Mg²⁺ on the drz-Fpra ribozymes. (A) Drz-Fpra-1 self-scission dose-response to GlcN6P at constant (5 mM) Mg²⁺, normalized to the no-metabolite control. (B) Mg²⁺ dependence of drz-Fpra-1 self-scission in presence of 20 mM GlcN6P (open squares) or no metabolite (open circles). (C) Mg²⁺ dependence of drz-Fpra-1 (open circles) and drz-Fpra-2 (open triangles) self-scission in 20 mM GlcN6P normalized to a no-metabolite control.

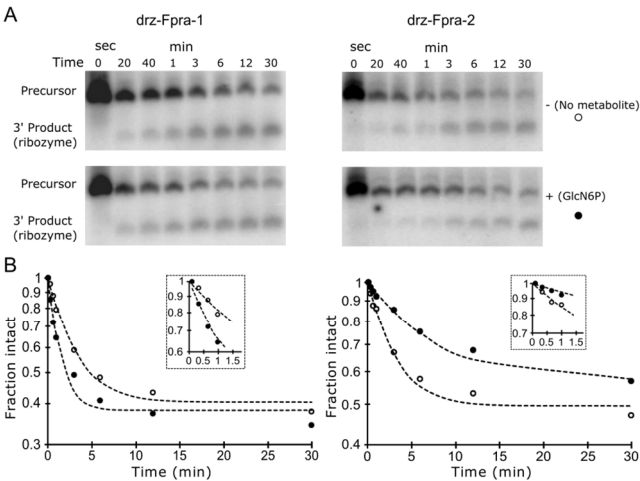


Figure 2. Influence of GlcN6P on the co-transcriptional self-scission of drz-Fpra-1 and drz-Fpra-2. To study the effect of the metabolite on the cleavage rate of the ribozymes, *in vitro* co-transcriptional cleavage kinetics were performed in presence and absence of GlcN6P. (A) PAGE analysis of the co-transcriptional self-scission of the two ribozymes in the absence (top) and presence (bottom) of 20 mM GlcN6P. (B) Log-linear graphs of the ribozyme self-scission. GlcN6P accelerates the self-cleavage rate of drz-Fpra-1 (left; no GlcN6P, open circles; 20 mM GlcN6P, solid circles) but inhibits drz-Fpra-2 (right). Early time points are shown in insets.

RESULTS

Discovery of a second HDV-like ribozyme in the same locus of *F. prausnitzii* genome. We decided to analyze the *F. prausnitzii* genome in order to verify the mapping of the drz-Fpra-1 ribozyme near the *glmM* gene. To our surprise, we found another HDV-like ribozyme, drz-Fpra-2, with high sequence similarity to drz-Fpra-1 in the same locus. This second ribozyme was found 558 nucleotides downstream of the *glmM* gene (Figure 1A). Although similar in structure and sequence, some differences near the active site were observed in the proposed secondary structures (Figure 1C), as discussed below. One unique feature found in both drz-Fpra ribozymes is an A-U base-pair in the top of the P1.1 region, where a G-C base-pair is usually found in other HDV-like ribozymes⁴⁹.

Self-scission kinetics of drz-Fpra ribozymes in the presence of metabolites. To study the effect of the metabolite on the cleavage rate of the ribozymes, we started with an *in vitro* co-transcriptional cleavage kinetics performed in the presence or absence of 20 mM GlcN6P at 5 mM Mg²⁺. Surprisingly, the effect

of the metabolite on each ribozyme was different. GlcN6P accelerates the self-scission of the drz-Fpra-1 ribozyme, whereas it decreases the self-cleavage rate of the drz-Fpra-2 ribozyme (Figure 2). The low amplitude of the effect of the metabolite on the two ribozymes indicated that GlcN6P is an allosteric effector (modulator) and not a co-factor or co-enzyme that directly participates in catalysis. To further investigate the ligand effect on the activity of drz-Fpra-1, we performed a titration of GlcN6P, keeping the ionic strength of the solution constant and $[Mg^{2+}]$ fixed at 5 mM. Self-cleavage kinetics under conditions approximating co-transcriptional self-scission showed a dose-response between the cleavage rate constant and the metabolite, increasing the k_{GlcN6P} more than 2-fold at higher concentrations of GlcN6P, when compared to no-metabolite $k_{control}$ (Figure 3A). At low concentrations of GlcN6P, the cleavage rate was similar to the no-metabolite control, indicating that the metabolite may not be affecting the catalytic mechanism of the ribozyme.

To investigate the origin of this dose-response, we varied the concentration of Mg^{2+} , the divalent metal ion used by the ribozyme for catalysis^{17,18,50-52}, in the presence of 20 mM GlcN6P. At low concentrations of Mg^{2+} , both k_{GlcN6P} and $k_{control}$ behave similarly, with a strong overlap between both data sets (Figure 3B). This trend changes at about physiological Mg^{2+} , where the cleavage rate increases in the presence of GlcN6P. At higher concentrations of the divalent metal ion, $k_{control}$ decreases, possibly due to misfolding. In contrast, this effect is not seen for k_{GlcN6P} , which continues to gradually increase. This result is consistent with a model, in which the metabolite interacts with the ribozyme, but does not act directly in the catalytic step of the reaction (Figure 3B). We also performed the same titration of Mg^{2+} in the presence of 20 mM of GlcN6P for drz-Fpra-2. As expected, an opposite effect was observed, where a decrease in the cleavage rate was found in the same conditions when compared to a no-metabolite control (Figure 3C).

To investigate the specificity of the ribozyme-metabolite interaction, we decided to test compounds related to GlcN6P. The ribozyme self-cleavage kinetics were tested in the presence of the following molecules: GlcN6P, the precursor of the reaction promoted by the glmM enzyme; GlcN1P, the isomer of GlcN6P and the product of the reaction; glucose (Glc); Glc6P and glucosamine (GlcN) – the last two were chosen due to their chemical similarity to the metabolites, because each carries a different chemical group present in both metabolites of the enzymatic reaction (the amino and the phosphate groups). The kinetic rate constants were normalized to no-metabolite cleavage kinetics at the same conditions, and showed that GlcN1P, the product of the reaction catalyzed by glmM, activated only the Fpra-2 ribozyme – albeit with low statistical significance, whereas Glc slightly increased the activity of both ribozymes (Figure S1). GlcN and Glc6P increased the cleavage rate of both drz-Fpra-1 and drz-Fpra-2 ribozymes to a similar extent (Figure S1). This is an unexpected result for drz-Fpra-2, because it was not inhibited by GlcN and Glc6P, as it was in the presence of GlcN6P. These results suggest that both the amino and the phosphate groups are important in modulating the self-scission rate of the ribozymes, and their relative positioning around the sugar ring leads to the differential effect on the two ribozymes. Finally, we decided to probe the ribozymes in the presence of UDP-GlcNAc, the final product of the hexosamine biosynthesis pathway, but no significant effect was observed (Figure S2).

Another way to compare the effects of GlcN6P and GlcN1P is to calculate the ratio of the self-scission rate constants at the

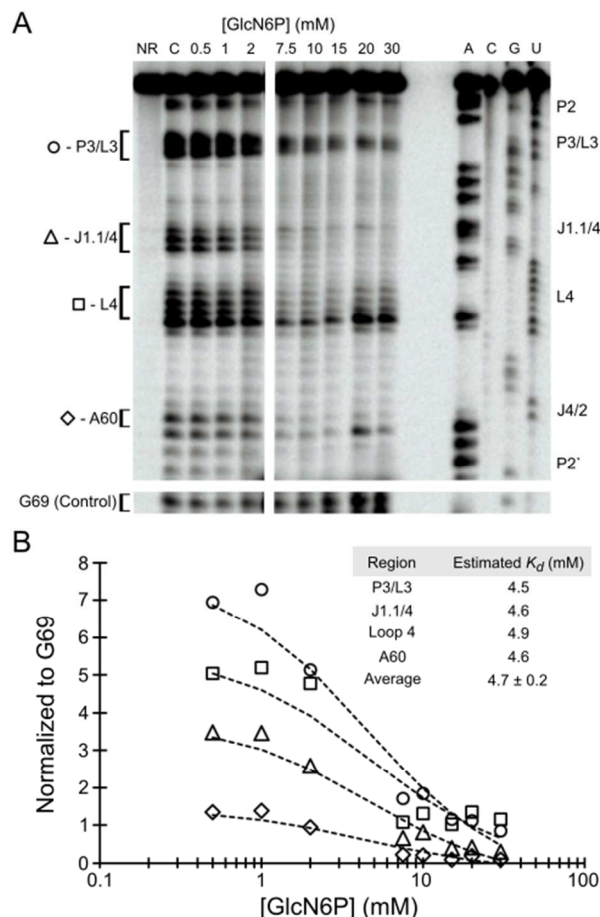


Figure 4. Structural probing of GlcN6P binding of the drz-Fpra-1 ribozyme. (A) In-line probing of the 3'-labeled drz-Fpra-1 ribozyme in the presence of GlcN6P. The band intensities that change with GlcN6P concentration are indicated in the figure by the nucleotide identity. The sequence of the RNA was determined using iodoethanol cleavage of ribozymes with phosphorothioate-modified backbone at positions indicated above each lane. The intensities of the control band (G69) from the same experiment are shown below the rest of the gel. (B) Graph of band intensities for the regions P3/L3 (open circles), J1.1/4 (open triangles), L4 (open squares), and A60 (open diamonds), normalized to a control band at G69. The data were fit to a model based on Equation 2. The average dissociation constant is estimated in 4.7 ± 0.2 mM. The positions where the band intensities respond to metabolite concentration are also indicated left of the gel in (A).

same concentrations of the metabolite, while varying the Mg^{2+} concentration. At 20 mM metabolite concentration, the k_{GlcN6P}/k_{GlcN1P} ratios for drz-Fpra-1 were 1.4 ± 0.1 , 2.2 ± 0.4 , and 2.5 ± 0.5 at 1, 5, and 10 mM Mg^{2+} . Interestingly, when both GlcN1P and GlcN6P were included in the drz-Fpra-1 reaction, the effect of GlcN6P was enhanced (Figure S3).

The structure of the ribozyme drz-Fpra-1 is stabilized by GlcN6P. In order to investigate the effect of the metabolite on the ribozyme structure, we performed an in-line probing experiment in the presence of different concentrations of GlcN6P. This technique profiles the natural degradation of an RNA molecule at different conditions, providing information on the relative stability of individual phosphodiester⁴⁶. More flexible, solvent-exposed regions (such as single-strand regions) and specific conformations are more likely to promote a 2'-OH attack on the scissile phosphate, cleaving the molecule. The pattern of the

cleavage at different conditions can indicate structural changes, suggesting differences in the flexibility and protection promoted by a ligand. Our results showed that GlcN6P promotes an apparent stabilization of the ribozyme at specific sites, suggesting that the metabolite directly interacts with the RNA (Figure 4A). The regions showing the most prominent change in degradation at higher GlcN6P concentration are P3/L3, J1.1/4, L4 and A60 (J4/2). Three of those regions (P3/L3, J1.1/4 and A60) surround the active site of the ribozyme, with A60 only 2 nucleotides away from the catalytic cytosine residue (C58). No region of increased degradation was observed in this experiment.

The data obtained for each concentration of metabolite, including the no-metabolite control, were normalized to G69 near the 3' terminus of the ribozyme, used as a control position with ligand-independent degradation. The data were modeled by a dissociation constant equation (Equation 2) for a single ligand and a K_d was calculated independently for each affected region (Figure 4B). The K_d estimations for all 4 regions were consistent, resulting in an average K_d of 4.7 ± 0.2 mM. Performing the same experiment with the metabolite GlcN1P, the isomer of GlcN6P and product of the enzymatic reaction that does not affect the cleavage rate of the ribozyme, we did not observe any region with significant degradation pattern changes (Figure S4A and B). We measured the degradation for the regions P3/L3, L4 and A60 (J4/2), and used 3 different control bands for normalization. No apparent K_d was revealed for any of the regions, demonstrating that GlcN1P does not affect the degradation pattern of the ribozyme.

Metabolite effect is ribozyme- and position-specific. To verify that the effects promoted by GlcN6P in both drz-Fpra-1 and drz-Fpra-2 were specific, we decided to test the ligand influence on different ribozymes of the same family, starting with two inactive drz-Fpra-1 mutants, C58A and C58U. Based on previous mutagenesis of HDV ribozymes^{16, 17, 19, 22, 23, 51, 53}, we expected a very slow self-cleavage of C58A and an abolishment of catalysis for C58U. In two-hour-long experiments, neither mutant of drz-Fpra-1 showed self-scission in the presence of GlcN6P (data not shown). These results demonstrate that GlcN6P does not rescue the activity of the ribozyme, further supporting a role as an allosteric modulator and not a catalytic co-factor for the ligand. To further investigate the specificity of GlcN6P, we probed two control ribozymes with the metabolite, the genomic HDV (gHDV) and the antigenomic HDV (aHDV), the two distinct HDV ribozymes

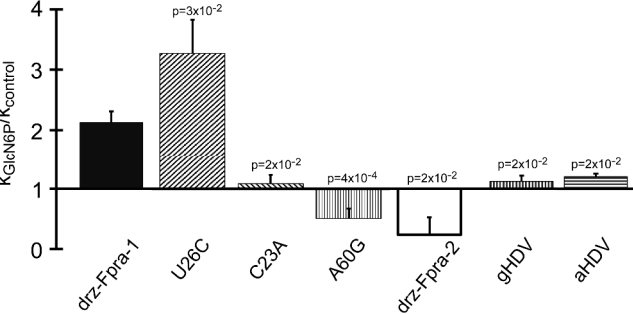


Figure 5. Effect of GlcN6P on self-scission of drz-Fpra-1/2 hybrids and HDV ribozymes. Comparison of modulation of the ribozyme activity by 20 mM GlcN6P normalized to the no-metabolite control at 5mM Mg^{2+} . U26C, C23A and A60G are drz-Fpra-1/drz-Fpra-2 hybrids referenced to the drz-Fpra-1 positions. gHDV and aHDV are the genomic and antigenomic HDV ribozymes, respectively. All p-values were calculated with respect to the wild-type Fpra-1 ribozyme and show statistical significance of the results.

from the hepatitis delta virus^{6, 7}. No significant change in the cleavage rate was observed for these ribozymes (Figure 5), suggesting that the kinetic modulation by the metabolite is specific to the drz-Fpra ribozymes.

To narrow down the cause of the opposite effect promoted by GlcN6P on drz-Fpra-1 and drz-Fpra-2, we decided to study the discrepant nucleotides near the active sites of the two otherwise similar ribozymes. The catalytic cores of the two ribozymes differ by only three nucleotides. We tested three hybrids C23A and U26C in the L3, and A60G in the J4/2 regions of the drz-Fpra-1 sequence. We found that U26C retains, and even slightly increases the effect of GlcN6P on the cleavage rate constant; C23A seems to abrogate the metabolite effect; and A60G reverts the metabolite effect, decreasing the cleavage rate of the ribozyme when compared to the k_{WT} (Figure 5), to a level similar to the drz-Fpra-2. These results indicate that the nucleotides C23 and A60 are involved in the allosteric modulation of drz-Fpra-1.

DISCUSSION

One of the nine natural families of self-cleaving ribozymes, the HDV-like ribozymes are found in several bacteria, many eukaryotes (including humans), *Chilo* iridescent virus, and in microbial metagenomic datasets^{14, 22-27}. Little is known about their biological functions and regulation, particularly any roles that cofactors and metabolites have in altering their activity. Drz-Fpra-1²² and drz-Fpra-2 HDV-like ribozymes were found in the human gut bacterium *F. prausnitzii* genome, surrounding the phosphoglucosamine mutase (*glmM*) open reading frame (ORF)⁴⁹. The enzyme *glmM* catalyzes the transformation of glucosamine 6-phosphate (GlcN6P) into glucosamine 1-phosphate (GlcN1P) in the hexosamine biosynthesis pathway³¹. We have studied the effect of the metabolites from this pathway on the self-cleavage rate of the drz-Fpra ribozymes. Whereas GlcN6P increases the cleavage rate of the drz-Fpra-1 ribozyme and appears to stabilize its structure, GlcN1P, the product of the *glmM* enzymatic reaction, does not significantly affect the ribozyme.

The GlcN6P effect on drz-Fpra-1 showed that the ligand modestly increases the self-cleavage rate of the ribozyme when compared to the no-metabolite control. Titration of Mg^{2+} at constant GlcN6P or titration of ligand at constant Mg^{2+} showed similar results, increasing the self-cleavage rate of the ribozyme. When we examined the influence of GlcN6P on the Mg^{2+} dependence of the ribozymes, we observed that the ligand effect is not seen at low concentrations of the divalent cation, behaving similarly to the no-metabolite control. Mg^{2+} coordinated with a hydroxide anion likely acts as the general base in the self-cleavage reaction of HDV ribozymes, deprotonating the 2'-OH of the base that promotes the attack in the scissile phosphate¹⁷, while the essential cytosine in J4/2 (C58 in drz-Fpra-1) acts as a general acid, mediating a proton transfer to the 5'-oxygen of G1, the leaving group of the self-scission reaction¹⁷. If the effect promoted by the metabolite directly influenced the role of Mg^{2+} in the mechanism of the reaction, the k_{GlcN6P} would be expected to increase the cleavage rate in all instances throughout the ligand titration, including at low concentrations of Mg^{2+} . This result suggests that the effect caused by the ligand does not directly influence the catalytic role of the metal ion in the self-cleavage reaction.

In the case of titration of GlcN6P, the ribozyme responds to the ligand, cleaving faster than the no-metabolite control. Again, this effect is not seen at low concentrations of the metabolite,

indicating that the effect is not directly related to the catalysis in the active site, otherwise we would expect to see an appreciably higher k_{GlcN6P} even at lower concentrations of GlcN6P. Moreover, testing the effect of the metabolite in the two inactive mutants of drz-Fpra-1, C58A and C58U, we found that the ligand does not rescue their activity, indicating that GlcN6P does not act as the general acid. This finding contrasts with the role of GlcN6P in the *glmS* ribozyme, where the cofactor actively participates in the active site, mediating the protonation of the 5'-oxyanion leaving group^{36-39, 42-44, 54}. The *glmS* cofactor has been implicated in other roles in catalysis, including helping to align the active site, stabilizing the developing charge during the self-cleavage reaction, and participating in a set of competing hydrogen bonds to ensure potent activation and regulation of the catalysis^{54, 55}. In contrast, our results suggest that the effect produced by GlcN6P on drz-Fpra-1 is not directly in the catalytic step of the self-cleavage reaction, suggesting an allosteric modulation of the ribozyme activity.

The structural probing of the drz-Fpra-1 in the presence of GlcN6P and GlcN1P showed decreased conformational flexibility of the RNA solely in the presence of GlcN6P (Figures 4 and S4). The active site of HDV ribozymes is formed by the P3/L3, P1.1 and J4/2 elements^{16, 18, 19}, and our probing data show that the ligand decreases the degradation of P3/L3, J1.1/4, L4 and A60 in J4/2. Even though GlcN6P clearly affects the active site stability and promotes faster self-scission, the in-line probing experiment does not pinpoint the site of interaction between the metabolite and the ribozyme, because the changes in degradation pattern may result from a conformational change promoted by the intermolecular interaction, and additional studies would be needed to map the exact position of interaction.

For all regions of intensity change of the in-line probing bands, the obtained estimated K_d was similar, with an average of 4.7 ± 0.2 mM. The intracellular concentration of GlcN6P in *F. prausnitzii* is not known, but the reported steady-state concentration in *E. coli* cells is 1.2 mM⁵⁶. Although it is around 4 times lower than the estimated K_d for drz-Fpra-1 ribozyme, subcellular localization and spikes in metabolic activity may result in a higher concentration of the metabolite than the cell culture average. Thus, the metabolite concentration may reach intracellular levels that significantly alter the activity of the ribozyme.

The drz-Fpra-2 ribozyme, located 558 nucleotides downstream of the *glmM* gene, was found by sequence similarity when searching for drz-Fpra-1. Interestingly, GlcN6P promoted an opposite effect on drz-Fpra-2 than on drz-Fpra-1, decreasing the self-cleavage rate when compared to the k_{control} . This effect was unexpected and intriguing, bringing to our attention those nucleotides of the active site, where the ribozymes differ. We tested the effect of GlcN6P on the activity of the hybrids of drz-Fpra-1 and drz-Fpra-2, with the following mutations made to drz-Fpra-1: C23A and U26C in the L3, and A60G in the J4/2 regions. The results demonstrate that U26C retains the effect of GlcN6P on the cleavage rate, maintaining the elevated k_{GlcN6P} and suggesting that the metabolite does not interact with the ribozyme at this nucleotide. On the other hand, the C23A mutation abrogates the metabolite effect, bringing the self-cleavage rate to the same level as the no-metabolite control. The third mutation, A60G, reverts the metabolite effect, decreasing the cleavage rate of the ribozyme below the k_{control} , similarly to what is observed in drz-Fpra-2. Thus, our data suggest that C23A and A60G are the discrepant nucleotides from the catalytic core that play a role in the opposite effect caused by GlcN6P in the two

ribozymes. C23 is part of L3 and is important to maintain the active site structure, stacking on C24 and possibly hydrogen bonding with neighboring residues^{16, 18, 19}. C23 may also be important for the stacking with U-1, similarly to the role of U23 in the HDV ribozyme^{16, 18, 19}. A mutation at C23 may promote a conformational change that prevents the interaction of GlcN6P with the ribozyme. A60 is part of the trefoil turn of the ribozyme structure and makes an A-minor interaction with P3, participating in a network of hydrogen bonding between P3/L3 and J4/2 regions^{16, 18, 19}. Thus, the A60G mutation would sterically hinder the interaction of the nucleobase, causing the J4/2 to move away from P3 and potentially disrupting the interactions of the A60 ribose as well. This change may be sufficient to abrogate the already weak interaction between the ribozyme and the metabolite.

Testing ribozyme kinetics in the presence of other metabolites elucidated the functional moieties critical in GlcN6P sensitivity in drz-Fpra-1. The aforementioned GlcN1P, the product of the enzymatic reaction of *glmM*, showed no significant effect on drz-Fpra-1 and a small effect on drz-Fpra-2. Similarly, Glc and UDP-GlcNAc, had little to no effect on the ribozymes. On the other hand, GlcN and Glc6P increased the cleavage rate for both ribozymes, leading us to speculate that both the amino and phosphate groups are relevant for the effect of the ligand in the ribozyme, as long as they are not at adjacent carbons of the sugar ring, as seen in GlcN1P. The concentration of Glc6P in the cytosol is likely very low and a previous report on the concentration of metabolites in *E. coli* with a detection limit of 130 nM did detect Glc6P⁵⁶. Thus, despite an *in vitro* effect of Glc6P on the ribozymes, it is unlikely that it affects their activity *in vivo*.

The cleavage rate constants obtained for gHDV and aHDV ribozymes in the presence of GlcN6P were similar to those of the no-metabolite control. These results suggest that the interaction between the metabolite and drz-Fpra-1 is specific. Concerning the structure of the ribozymes, to our knowledge, the first P1.1 base pair is found to be A-U only in bacteria^{22, 24, 25, 27, 49}, and the drz-Fpra-1 was the only case of a bacterial HDV-like ribozyme upstream of a neighboring gene. However, a recent discovery of several ribozymes using comparative genomic analysis¹⁴ also revealed HDV-like ribozymes in environmental samples, of which 4 showed the *glmM* gene 34-to-42 nts downstream of the ribozymes, similarly to drz-Fpra-1 in *F. prausnitzii* (37 nucleotides). Seven other HDV-like ribozymes showed the *glmM* gene upstream of the ribozymes. All of these ribozymes have an A-U base pair in P1.1, suggesting that they originate from closely related bacteria. The alignment of the drz-Fpra-1, drz-Fpra-2 and other microbial HDV-like ribozymes to the secondary structure is shown in Figure S5. The alignment, combined with the results presented here, suggests that env-26 HDV-like ribozyme would respond to GlcN6P, because it is the only sequence with a cytosine in the same position of L3 as in drz-Fpra-1 (C23), whereas all other ribozymes bear an adenosine at this position, like in drz-Fpra-2, where we found no effect of the metabolite. Regarding the J4/2 strand, only drz-Fpra-2 contains a guanosine residue at the position equivalent to A60 in drz-Fpra-1, here reported to be responsible for the inhibitory effect of GlcN6P on the activity of the ribozyme.

We show that the metabolite GlcN6P interacts with the drz-Fpra-1 ribozyme and increases its self-cleavage activity; however, we do not know the biological significance of these findings, and future studies will include biological assays addressing this question. In contrast to the *glmS* riboswitch-ribozyme, which is

part of a feedback regulatory loop utilizing the product of the downstream metabolic step to induce the ribozyme self-scission and mRNA degradation, the activity of drz-Fpra-1 increases with the concentration of the substrate, providing an example of a putative feed-forward mechanism. The downstream RNA product, which starts with the cleaved drz-Fpra-1 ribozyme and is followed by the GlmM open reading frame, is terminated with a 5'-OH that is sequestered by the structure of the cleaved ribozyme^{16, 18, 19}. This structure may protect the 5' terminus against endonucleases, thus increasing the mRNA stability. Moreover, RNAs with 5'-OH termini have an extended half-life when compared with 5'-phosphorylated mRNAs, because 5'-hydroxyls are inferior substrates for the endonucleolytic degradation by RNase E^{57, 58}. The extended half-life of mRNAs with 5'-OH termini over phosphorylated 5' termini has been used in bacterial metabolic engineering to design a number of aptazymes that increased stability of the downstream transcripts upon ligand-dependent self-scission⁵⁹, and similar mechanism may be acting in the case of the *F. prausnitzii* ribozyme-terminated *glmM* mRNA. To the best of our knowledge, the drz-Fpra-1 activation by GlcN6P is the first example of an allosteric modulation of a natural self-cleaving ribozyme by a metabolite. We believe that this is not a unique case and that other examples of natural allosterically-regulated self-cleaving ribozymes exist, providing another example of gene expression regulation at the RNA level.

ASSOCIATED CONTENT

Supporting Information

Effect of multiple ligands in drz-Fpra-1 and drz-Fpra2 (Figure S1), UDP-GlcNAc effect on both ribozymes (Figure S2), activity of drz-Fpra-1 in mixtures of GlcN6P and GlcN1P (Figure S3), in-line probing of drz-Fpra-1 in the presence of GlcN1P (Figure S4), secondary structure alignment of ribozymes (Figure S5), sequences of constructs used in this study (Table S1). The Supporting Information is available free of charge on the ACS Publications website – <http://pubs.acs.org>.

AUTHOR INFORMATION

Corresponding Author

*Phone: (949) 824-9132; E-mail: aluptak.uci.edu.

Author Contributions

L.F.M.P., R.M.J. and A.L. designed the study; L.F.M.P., R.M.J. and J.Y.F performed the experiments; all authors analyzed the data; and L.F.M.P., R.M.J. and A.L. wrote the manuscript.
‡ These authors contributed equally.

Funding Sources

This work was supported by Science Without Borders Program – CAPES Foundation, Ministry of Education of Brazil – Process 99999.013571/2013-03 (L.F.M.P.), NIH 5F31GM103241-02 (R.M.J.), NSF MCB 1330606 and a grant from the John Templeton Foundation (to A.L.). This project/publication was made possible through the support of a grant from the John Templeton Foundation. The opinions expressed in this publication are those of the author(s) and do not necessarily reflect the views of the John Templeton Foundation.

Notes

The authors declare no competing financial interests.

ACKNOWLEDGMENT

We thank all members of the Lupták laboratory for helpful discussions.

ABBREVIATIONS

HDV, hepatitis delta virus; glmS, glucosamine-6-phosphate synthase; glmM, phosphoglucosamine mutase; ORF, open reading frame; GlcN6P, glucosamine-6-phosphate; GlcN1P, glucosamine-1-phosphate; UDP-GlcNAc, uridine diphosphate N-acetylglucosamine; Glc, glucose; Glc6P, glucose-6-phosphate; GlcN, glucosamine; PAGE, polyacrylamide gel electrophoresis; nt, nucleotide.

REFERENCES

[1] Doudna, J. A., and Cech, T. R. (2002) The chemical repertoire of natural ribozymes. *Nature* 418, 222-228.

[2] Kruger, K., Grabowski, P. J., Zaug, A. J., Sands, J., Gottschling, D. E., and Cech, T. R. (1982) Self-splicing RNA: autoexcision and autocyclization of the ribosomal RNA intervening sequence of Tetrahymena. *Cell* 31, 147-157.

[3] Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N., and Altman, S. (1983) The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. *Cell* 35, 849-857.

[4] Jimenez, R. M., Polanco, J. A., and Luptak, A. (2015) Chemistry and biology of self-cleaving ribozymes. *Trends Biochem Sci* 40, 648-661.

[5] Fedor, M. J. (2009) Comparative enzymology and structural biology of RNA self-cleavage. *Annu Rev Biophys* 38, 271-299.

[6] Wu, H. N., Lin, Y. J., Lin, F. P., Makino, S., Chang, M. F., and Lai, M. M. (1989) Human hepatitis delta virus RNA subfragments contain an autocleavage activity. *Proc Natl Acad Sci U S A* 86, 1831-1835.

[7] Sharmeen, L., Kuo, M. Y., Dinter-Gottlieb, G., and Taylor, J. (1988) Antigenomic RNA of human hepatitis delta virus can undergo self-cleavage. *J. Virol.* 62, 2674-2679.

[8] Prody, G. A., Bakos, J. T., Buzayan, J. M., Schneider, I. R., and Bruening, G. (1986) Autolytic processing of dimeric plant virus satellite RNA. *Science* 231, 1577-1580.

[9] Hutchins, C. J., Rathjen, P. D., Forster, A. C., and Symons, R. H. (1986) Self-cleavage of plus and minus RNA transcripts of avocado sunblotch viroid. *Nucleic Acids Res* 14, 3627-3640.

[10] Buzayan, J. M., Gerlach, W. L., and Bruening, G. (1986) Non-enzymatic cleavage and ligation of RNAs complementary to a plant virus satellite RNA. *Nature* 232, 349-353.

[11] Winkler, W. C., Nahvi, A., Roth, A., Collins, J. A., and Breaker, R. R. (2004) Control of gene expression by a natural metabolite-responsive ribozyme. *Nature* 428, 281-286.

[12] Saville, B. J., and Collins, R. A. (1990) A site-specific self-cleavage reaction performed by a novel RNA in Neurospora mitochondria. *Cell* 61, 685-696.

[13] Roth, A., Weinberg, Z., Chen, A. G. Y., Kim, P. B., Ames, T. D., and Breaker, R. R. (2014) A widespread self-cleaving ribozyme class is revealed by bioinformatics. *Nat Chem Biol* 10, 56-U92.

[14] Weinberg, Z., Kim, P. B., Chen, T. H., Li, S., Harris, K. A., Lunse, C. E., and Breaker, R. R. (2015) New classes of self-cleaving ribozymes revealed by comparative genomics analysis. *Nat Chem Biol* 11, 606-610.

[15] Kuo, M. Y., Sharmeen, L., Dinter-Gottlieb, G., and Taylor, J. (1988) Characterization of self-cleaving RNA sequences on the genome and antigenome of human hepatitis delta virus. *J Virol* 62, 4439-4444.

[16] Ferre-D'Amare, A. R., Zhou, K., and Doudna, J. A. (1998) Crystal structure of a hepatitis delta virus ribozyme. *Nature* 395, 567-574.

[17] Das, S. R., and Piccirilli, J. A. (2005) General acid catalysis by the hepatitis delta virus ribozyme. *Nat Chem Biol* 1, 45-52.

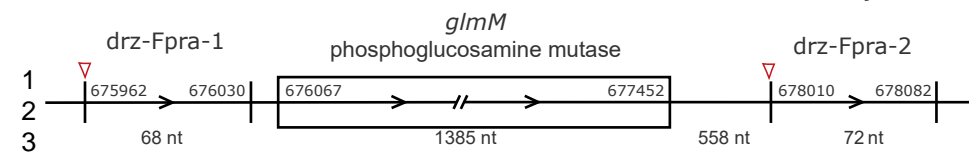
- [18] Chen, J. H., Yajima, R., Chadalavada, D. M., Chase, E., Bevilacqua, P. C., and Golden, B. L. (2010) A 1.9 Å crystal structure of the HDV ribozyme precleavage suggests both Lewis acid and general acid mechanisms contribute to phosphodiester cleavage. *Biochemistry* 49, 6508-6518.
- [19] Ke, A., Zhou, K., Ding, F., Cate, J. H., and Doudna, J. A. (2004) A conformational switch controls hepatitis delta virus ribozyme catalysis. *Nature* 429, 201-205.
- [20] Koo, S. C., Lu, J., Li, N. S., Leung, E., Das, S. R., Harris, M. E., and Piccirilli, J. A. (2015) Transition state features in the hepatitis delta virus ribozyme reaction revealed by atomic perturbations. *J Am Chem Soc* 137, 8973-8982.
- [21] Golden, B. L., Hammes-Schiffer, S., Carey, P. R., and Bevilacqua, P. C. (2013) An integrated picture of HDV ribozyme catalysis. In *Biophysics of RNA Folding* (Russell, R., Ed.), pp 135-167, Springer New York, New York, NY.
- [22] Webb, C. H., Riccitelli, N. J., Ruminski, D. J., and Lupták, A. (2009) Widespread occurrence of self-cleaving ribozymes. *Science* 326, 953.
- [23] Salehi-Ashtiani, K., Lupták, A., Litovchick, A., and Szostak, J. W. (2006) A genomewide search for ribozymes reveals an HDV-like sequence in the human CPEB3 gene. *Science* 313, 1788-1792.
- [24] Ruminski, D. J., Webb, C. H., Riccitelli, N. J., and Lupták, A. (2011) Processing and translation initiation of non-long terminal repeat retrotransposons by hepatitis delta virus (HDV)-like self-cleaving ribozymes. *J Biol Chem* 286, 41286-41295.
- [25] Eickbush, D. G., and Eickbush, T. H. (2010) R2 retrotransposons encode a self-cleaving ribozyme for processing from an rRNA cotranscript. *Mol Cell Biol* 30, 3142-3150.
- [26] Riccitelli, N. J., Delwart, E., and Luptak, A. (2014) Identification of minimal HDV-like ribozymes with unique divalent metal ion dependence in the human microbiome. *Biochemistry* 53, 1616-1626.
- [27] Sanchez-Luque, F. J., Lopez, M. C., Macias, F., Alonso, C., and Thomas, M. C. (2011) Identification of an hepatitis delta virus-like ribozyme at the mRNA 5'-end of the L1Tc retrotransposon from *Trypanosoma cruzi*. *Nucleic Acids Res* 39, 8065-8077.
- [28] Miquel, S., Martin, R., Rossi, O., Bermudez-Humaran, L. G., Chatel, J. M., Sokol, H., Thomas, M., Wells, J. M., and Langella, P. (2013) *Faecalibacterium prausnitzii* and human intestinal health. *Curr Opin Microbiol* 16, 255-261.
- [29] Heinken, A., Khan, M. T., Paglia, G., Rodionov, D. A., Harmsen, H. J., and Thiele, I. (2014) Functional metabolic map of *Faecalibacterium prausnitzii*, a beneficial human gut microbe. *J Bacteriol* 196, 3289-3302.
- [30] Sokol, H., Pigneur, B., Watterlot, L., Lakhdari, O., Bermudez-Humaran, L. G., Gratadon, J. J., Blugeon, S., Bridonneau, C., Furet, J. P., Corthier, G., Grangette, C., Vasquez, N., Pochart, P., Trugnan, G., Thomas, G., Blottiere, H. M., Dore, J., Marteau, P., Seksik, P., and Langella, P. (2008) *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci U S A* 105, 16731-16736.
- [31] Mengin-Lecreulx, D., and van Heijenoort, J. (1996) Characterization of the essential gene *glmM* encoding phosphoglucosamine mutase in *Escherichia coli*. *J Biol Chem* 271, 32-39.
- [32] Raetz, C. R. (1986) Molecular genetics of membrane phospholipid synthesis. *Annu Rev Genet* 20, 253-295.
- [33] Mandal, M., and Breaker, R. R. (2004) Gene regulation by riboswitches. *Nat Rev Mol Cell Biol* 5, 451-463.
- [34] McCown, P. J., Corbino, K. A., Stav, S., Sherlock, M. E., and Breaker, R. R. (2017) Riboswitch diversity and distribution. *RNA* 23, 995-1011.
- [35] Klein, D. J., and Ferre-D'Amare, A. R. (2006) Structural basis of *glmS* ribozyme activation by glucosamine-6-phosphate. *Science* 313, 1752-1756.
- [36] Cochrane, J. C., Lipchock, S. V., and Strobel, S. A. (2007) Structural investigation of the *GlmS* ribozyme bound to its catalytic cofactor. *Chem Biol* 14, 97-105.
- [37] Viladoms, J., and Fedor, M. J. (2012) The *glmS* ribozyme cofactor is a general acid-base catalyst. *J Am Chem Soc* 134, 19043-19049.
- [38] Cochrane, J. C., Lipchock, S. V., Smith, K. D., and Strobel, S. A. (2009) Structural and chemical basis for glucosamine 6-phosphate binding and activation of the *glmS* ribozyme. *Biochemistry* 48, 3239-3246.
- [39] McCarthy, T. J., Plog, M. A., Floy, S. A., Jansen, J. A., Soukup, J. K., and Soukup, G. A. (2005) Ligand requirements for *glmS* ribozyme self-cleavage. *Chem Biol* 12, 1221-1226.
- [40] Collins, J. A., Irnov, I., Baker, S., and Winkler, W. C. (2007) Mechanism of mRNA destabilization by the *glmS* ribozyme. *Genes Dev* 21, 3356-3368.
- [41] Watson, P. Y., and Fedor, M. J. (2011) The *glmS* riboswitch integrates signals from activating and inhibitory metabolites in vivo. *Nat Struct Mol Biol* 18, 359-363.
- [42] Lau, M. W., and Ferre-D'Amare, A. R. (2013) An in vitro evolved *glmS* ribozyme has the wild-type fold but loses coenzyme dependence. *Nat Chem Biol* 9, 805-810.
- [43] Soukup, G. A. (2006) Core requirements for *glmS* ribozyme self-cleavage reveal a putative pseudoknot structure. *Nucleic Acids Res* 34, 968-975.
- [44] Jansen, J. A., McCarthy, T. J., Soukup, G. A., and Soukup, J. K. (2006) Backbone and nucleobase contacts to glucosamine-6-phosphate in the *glmS* ribozyme. *Nat Struct Mol Biol* 13, 517-523.
- [45] Long, D. M., and Uhlenbeck, O. C. (1994) Kinetic characterization of intramolecular and intermolecular hammerhead RNAs with stem II deletions. *Proc Natl Acad Sci U S A* 91, 6977-6981.
- [46] Soukup, G. A., and Breaker, R. R. (1999) Relationship between internucleotide linkage geometry and the stability of RNA. *RNA* 5, 1308-1325.
- [47] Ryder, S. P., and Strobel, S. A. (1999) Nucleotide analog interference mapping. *Methods* 18, 38-50.
- [48] Vu, M. M., Jameson, N. E., Masuda, S. J., Lin, D., Larralde-Ridaura, R., and Luptak, A. (2012) Convergent Evolution of Adenosine Aptamers Spanning Bacterial, Human, and Random Sequences Revealed by Structure-Based Bioinformatics and Genomic SELEX. *Chem Biol* 19, 1247-1254.
- [49] Webb, C. H., and Lupták, A. (2011) HDV-like self-cleaving ribozymes. *RNA Biol* 8, 719 - 727.
- [50] Cerrone-Szakal, A. L., Siegfried, N. A., and Bevilacqua, P. C. (2008) Mechanistic characterization of the HDV genomic ribozyme: solvent isotope effects and proton inventories in the absence of divalent metal ions support C75 as the general acid. *J Am Chem Soc* 130, 14504-14520.
- [51] Nakano, S., Chadalavada, D. M., and Bevilacqua, P. C. (2000) General acid-base catalysis in the mechanism of a hepatitis delta virus ribozyme. *Science* 287, 1493-1497.
- [52] Chen, J., Ganguly, A., Miswan, Z., Hammes-Schiffer, S., Bevilacqua, P. C., and Golden, B. L. (2013) Identification of the catalytic Mg(2)(+) ion in the hepatitis delta virus ribozyme. *Biochemistry* 52, 557-567.
- [53] Perrotta, A. T., Shih, I., and Been, M. D. (1999) Imidazole rescue of a cytosine mutation in a self-cleaving ribozyme. *Science* 286, 123-126.
- [54] Bingaman, J. L., Zhang, S., Stevens, D. R., Yennawar, N. H., Hammes-Schiffer, S., and Bevilacqua, P. C. (2017) The GlcN6P cofactor plays multiple catalytic roles in the *glmS* ribozyme. *Nat Chem Biol* 13, 439-445.
- [55] Bingaman, J. L., Gonzalez, I. Y., Wang, B., and Bevilacqua, P. C. (2017) Activation of the *glmS* ribozyme Nucleophile via overdetermined hydrogen bonding. *Biochemistry* 56, 4313-4317.
- [56] Bennett, B. D., Kimball, E. H., Gao, M., Osterhout, R., Van Dien, S. J., and Rabinowitz, J. D. (2009) Absolute metabolite concentrations and implied enzyme active site occupancy in *Escherichia coli*. *Nat Chem Biol* 5, 593-599.
- [57] Celesnik, H., Deana, A., and Belasco, J. G. (2007) Initiation of RNA decay in *Escherichia coli* by 5' pyrophosphate removal. *Mol Cell* 27, 79-90.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

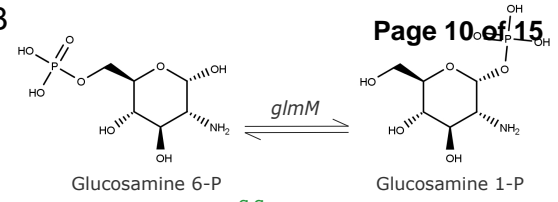
[58] Deana, A., Celesnik, H., and Belasco, J. G. (2008) The bacterial enzyme RppH triggers messenger RNA degradation by 5' pyrophosphate removal. *Nature* 451, 355-358.

[59] Carothers, J. M., Goler, J. A., Juminaga, D., and Keasling, J. D. (2011) Model-driven engineering of RNA devices to quantitatively program gene expression. *Science* 334, 1716-1719.

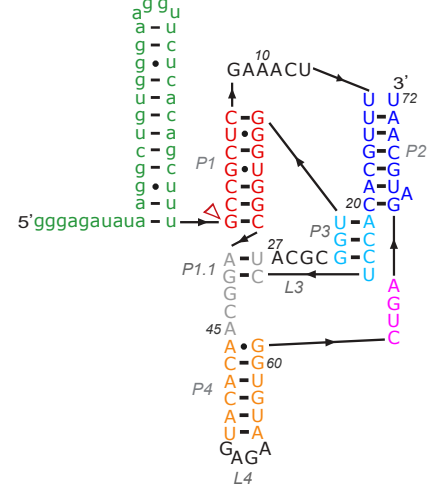
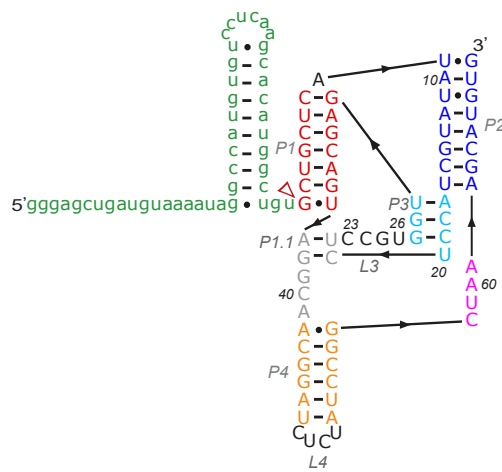
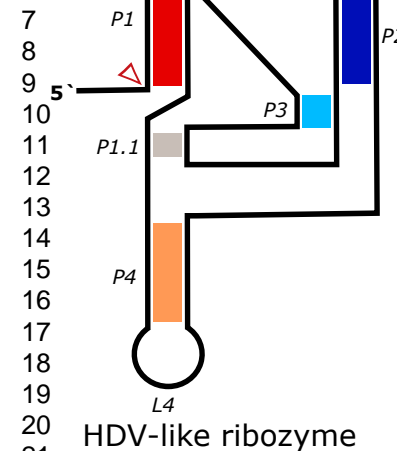
A



B



C



A

drz-Fpra-1

drz-Fpra-2

Time sec min
 0 20 40 1 3 6 12 30

Time sec min
 0 20 40 1 3 6 12 30

Precursor

3' Product
(ribozyme)

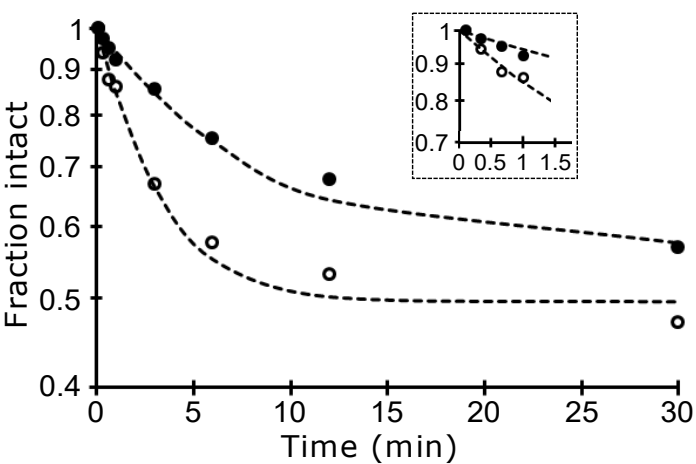
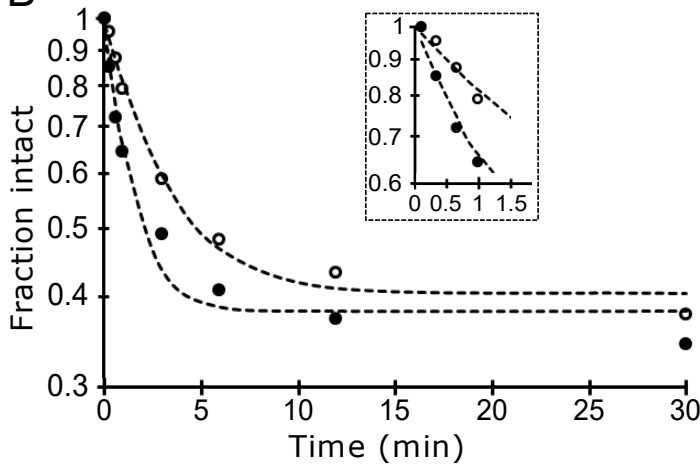
Precursor

3' Product
(ribozyme)

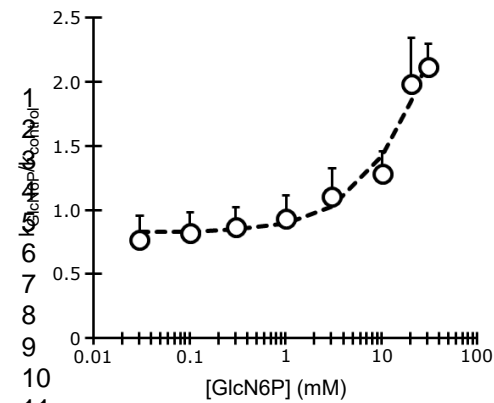
- (No metabolite)
○

+ (GlcN6P)
●

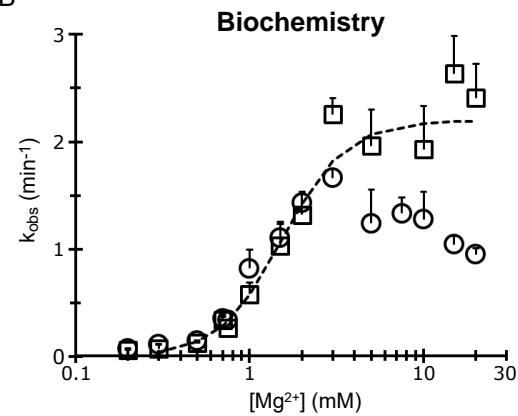
B



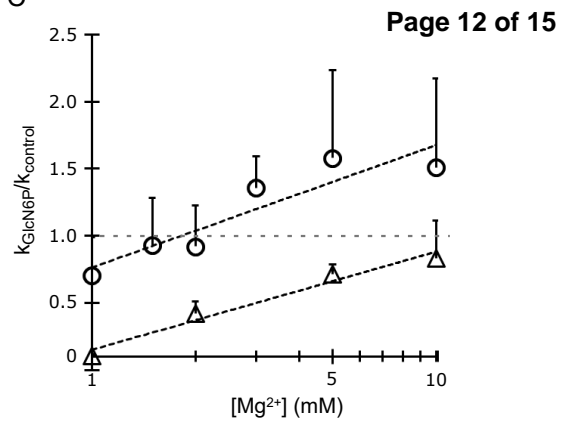
A

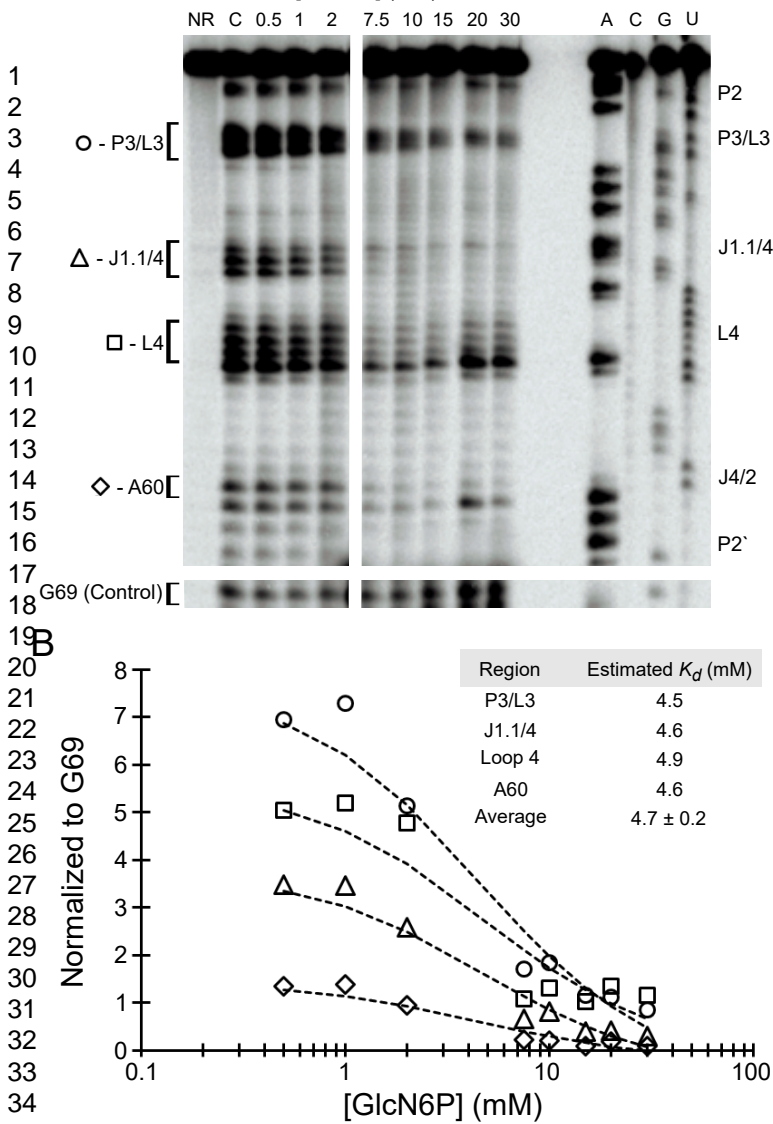


B



C





Biochemistry

