Supplementary Information

Deciphering ligand and metal ion dependent intricate folding landscape of Vc2 c-di-GMP riboswitch aptamer

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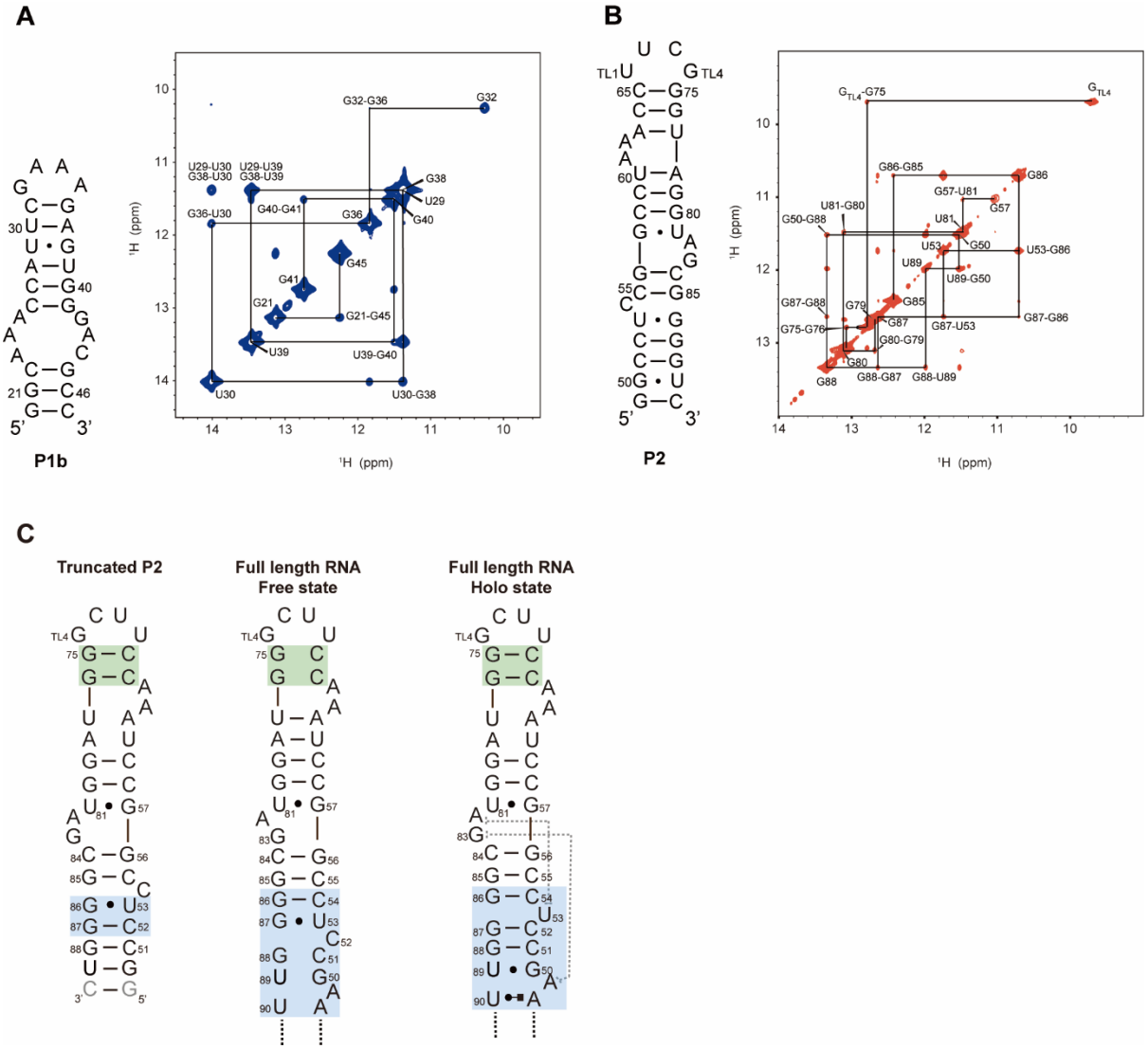
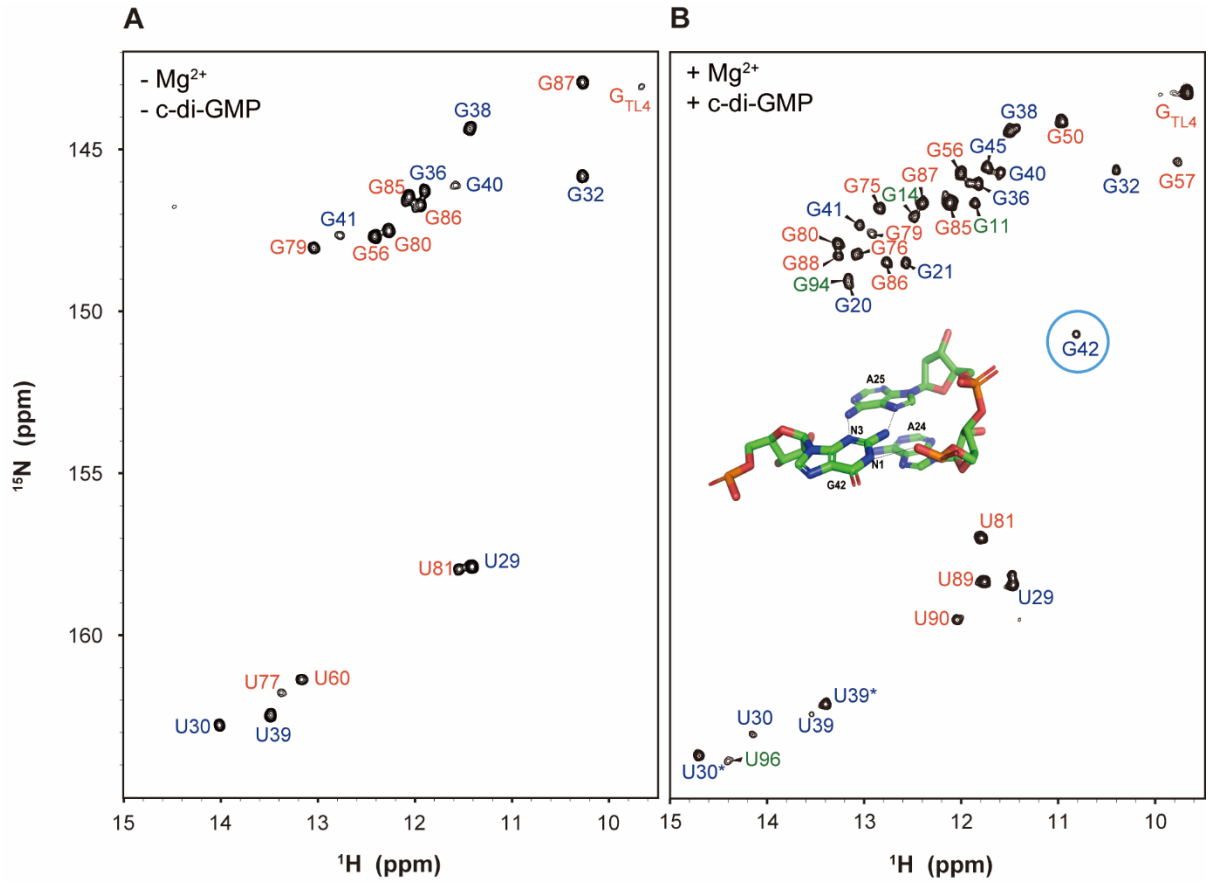
Keywords

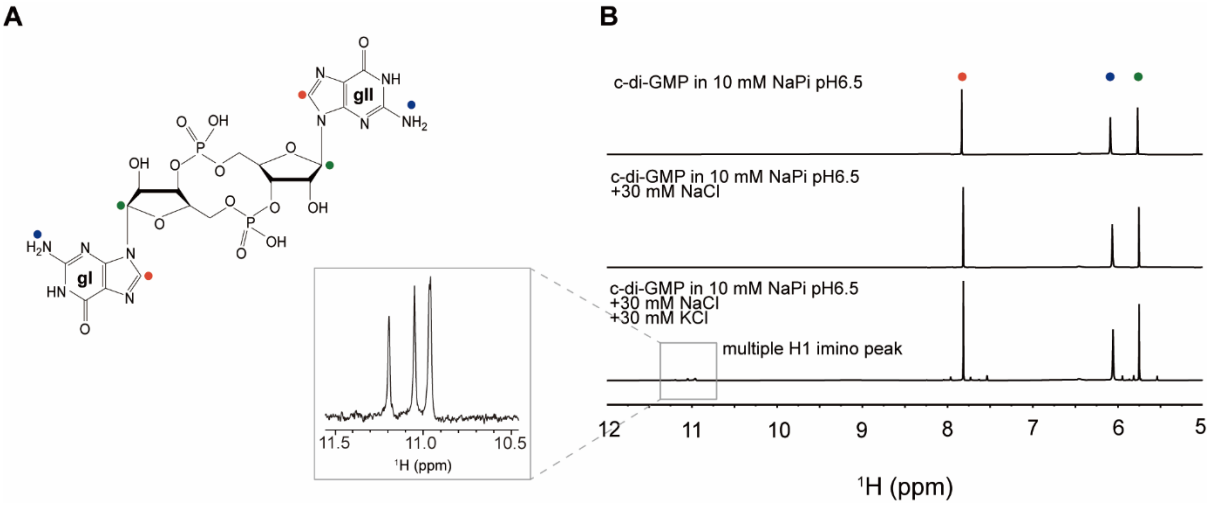
Riboswitch, NMR, structure, cyclic-di-GMP, RNA folding



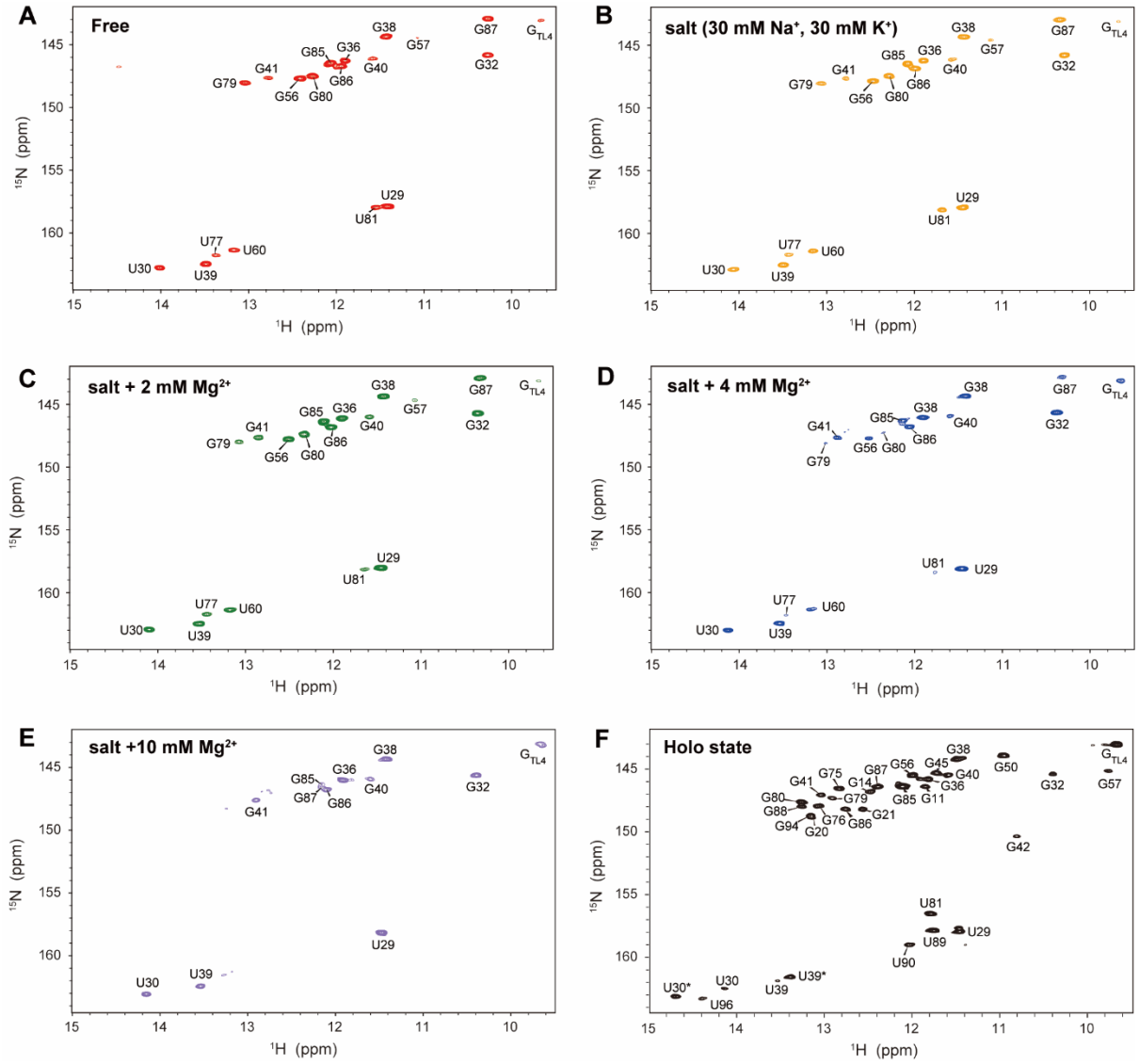
Supplementary Figure S1. Predicted secondary structures and 2D ^{1}H-^{1}H NOESY spectra in the imino proton resonance region of (A) truncated P1b and (B) P2. Nucleotides were added to the terminus of each RNA to increase transcription efficiency. The RNA was prepared in 10 mM sodium phosphate buffer at pH 6.5, and experiments were conducted at 283 K. (C) The secondary structures of truncated P2 and the full-length P2 aptamer in free and holo states, respectively. The changes of base pairs are colored in green and cyan for clarity.



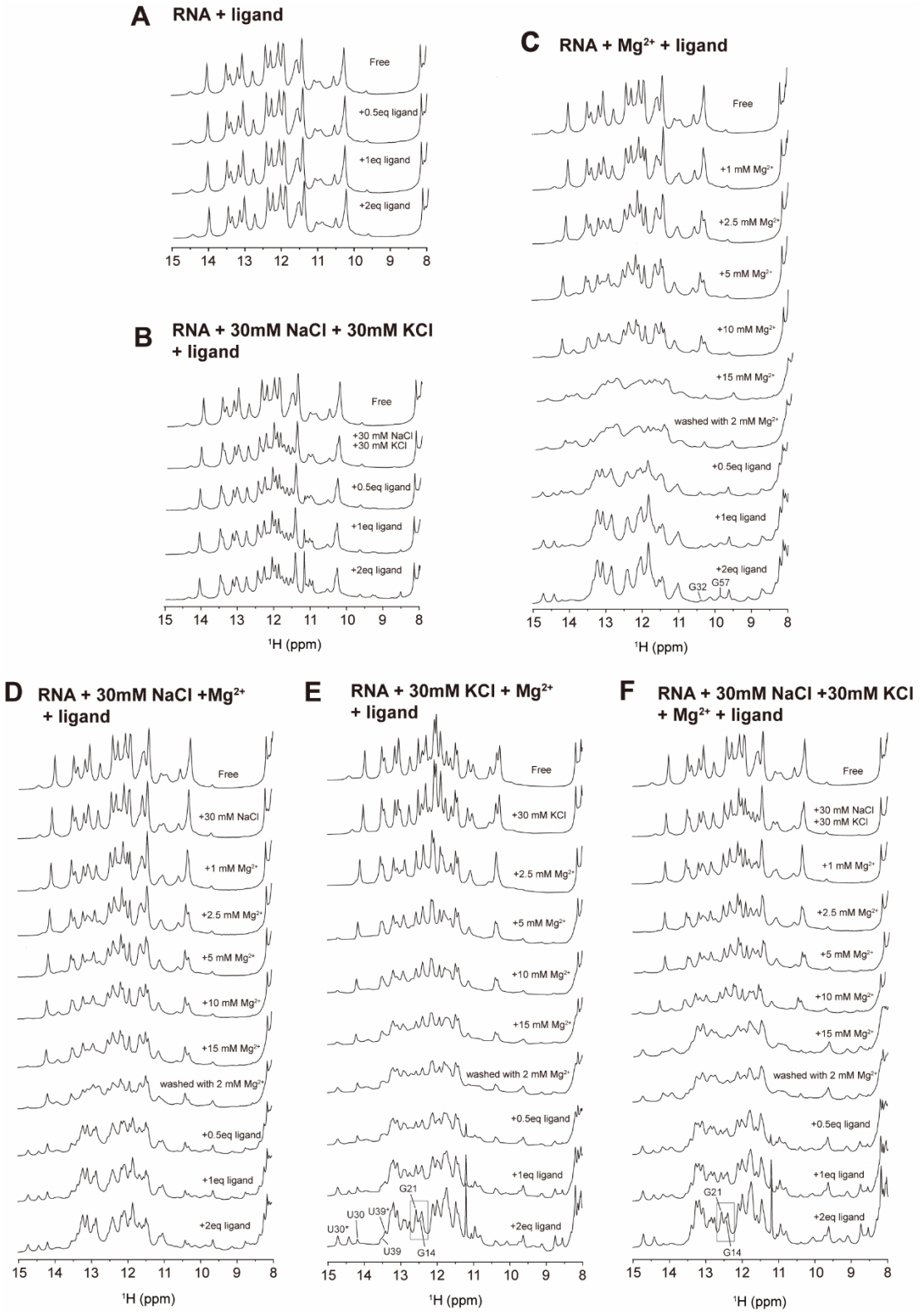
Supplementary Figure S2. Imino region of a 2D ^{1}H-^{15}N HSQC spectrum of the c-di-GMP riboswitch. Complete imino resonance assignment in the absence (A) and presence (B) of Mg^{2+} and ligand at 288 K. The downfield shifted ^{15}N of G42 is highlighted with a circle. The crystal structure shows a Hoogsteen-sugar-edge hydrogen bonding between A25 and G42 as well as the proximity between G42 imino nitrogen and A24 phosphate oxygen. Residue numbers are colored as indicated in Figure 1. The residues in P1a, P1b, and P2 are colored in green, blue, and red, respectively.



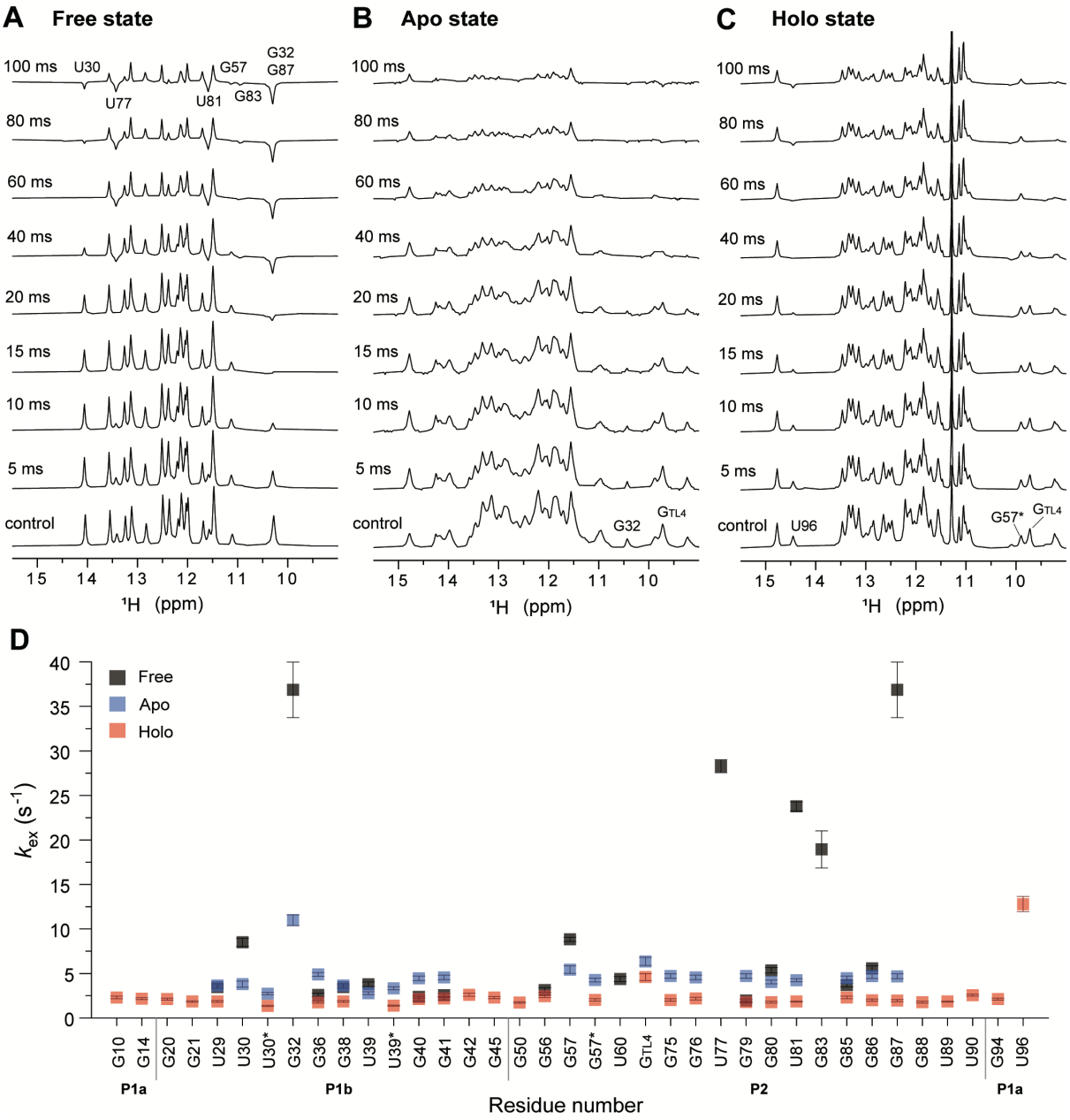
Supplementary Figure S3. (A) The molecular structure and (B) 1D ^{1}H NMR spectra of c-di-GMP. The proton resonances of H8, NH2, and H1' are shown in red, blue, and green dots, respectively. The monomer c-di-GMP (top and middle) forms an oligomeric structure in the presence of K^{+} ions (bottom). The multiple H1 imino peaks appear at ~11 ppm. The experiments were conducted at 288 K.



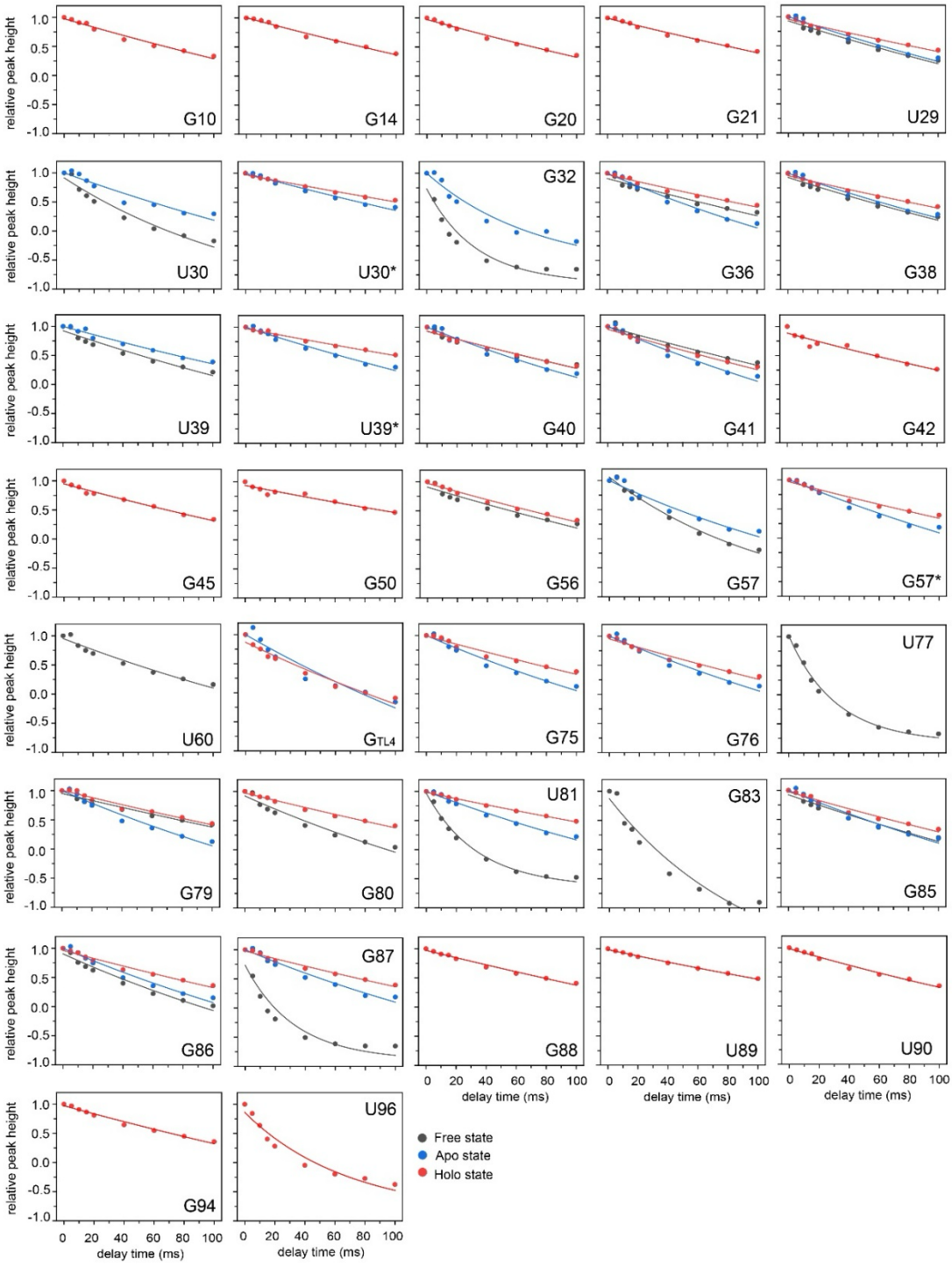
Supplementary Figure S4. 2D ^{1}H-^{15}N HSQC titration results of the c-di-GMP riboswitch aptamer (0.2 mM) in 10 mM NaPi, pH 6.5, at different concentrations of Na^{+}, K^{+}, Mg^{2+}, and ligand. The experiments were performed at 288 K.



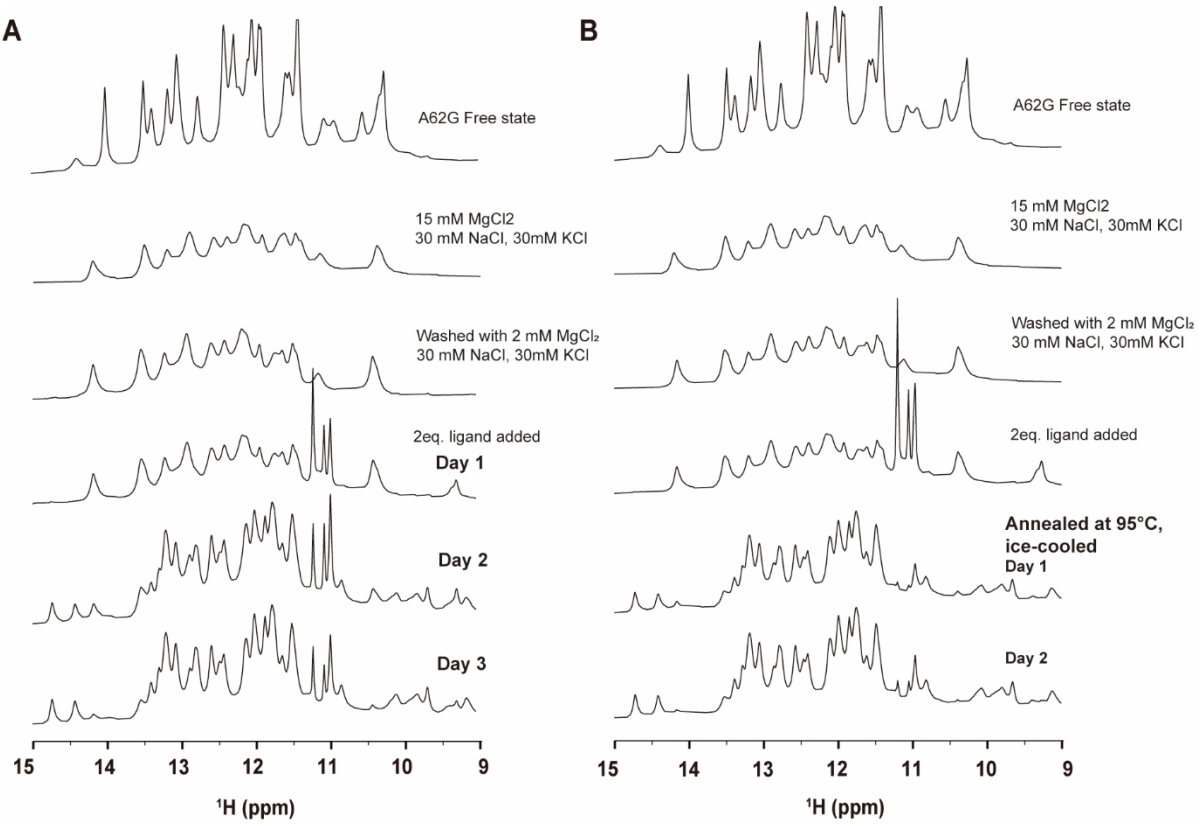
Supplementary Figure S5. Imino proton region of the 1D ^{1}H NMR spectra of c-di-GMP riboswitch aptamer at 288 K. The spectra show that both salt (30 mM Na^{+} and 30 mM K^{+}) and Mg^{2+} are required to drive the proper folding of aptamer. The buffer for spectra (A, B, C, D, and F) is 10 mM NaPi, pH 6.5, while spectrum (E) uses 10 mM KPi, pH 6.5.



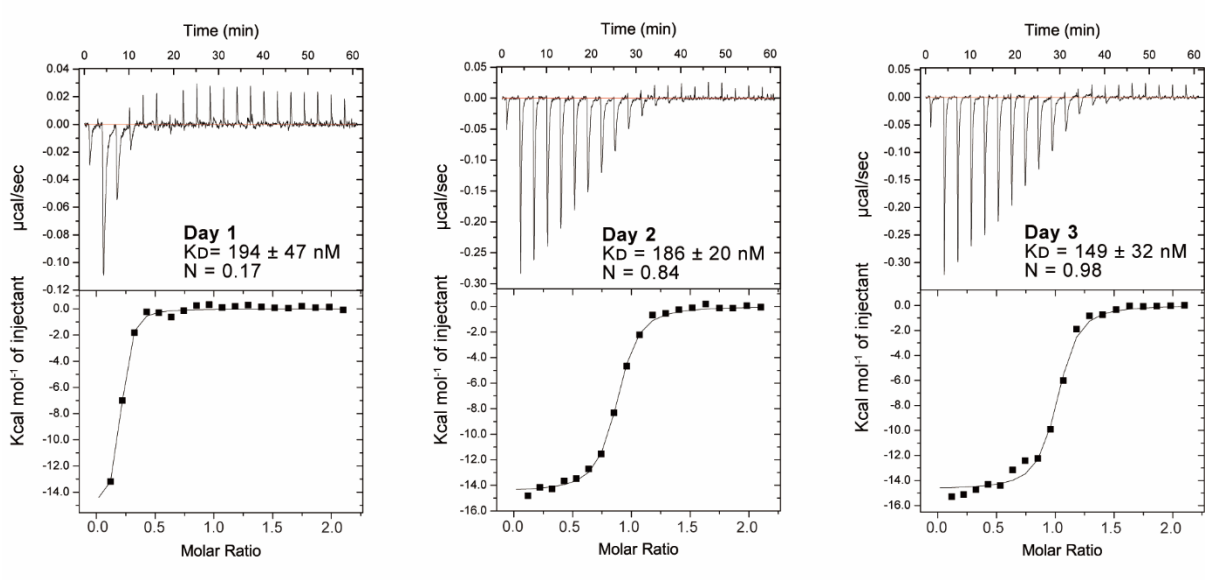
Supplementary Figure S6. Hydrogen exchange data of riboswitch in free, apo, and holo states. 1D imino proton spectra from water magnetization transfer experiments for the riboswitch at the (A) Free, (B) Apo, and (C) Holo state, acquired by varying the delay time (5 to 100 ms) after water inversion at 298 K. (D) Exchange rate constants (k_{ex}) of the imino protons for each residue in the free, apo, and holo states are represented by black, blue, and red squares, respectively. Error bars indicate uncertainties associated with the curve fitting results.



Supplementary Figure S7. Relative imino peak intensities of residues in the water magnetization transfer across various states are plotted as a function of delay time. Each panel displays the decay of relative peak intensity with increasing delay times (ms) for individual imino resonances, labeled in each panel, across the free, apo, and holo states. Data for free, apo and holo states are represented as black, blue, and red circles, respectively. Solid lines represent the best-fit curves based on the equation described in the MATERIALS AND METHODS section.



Supplementary Figure S8. Imino proton region of the 1D NMR spectra of the A62G mutant c-di-GMP riboswitch aptamer at 288 K, observed to monitor folding over time. (A) Shows the spectra for the A62G mutant, where folding progression is tracked daily after adding 2 equivalents of ligand. (B) Shows spectra after adding 2 equivalents of ligand followed by an annealing step at 95°C and ice cooling, with spectra recorded over consecutive days. The buffer conditions are indicated on the spectrum.



Supplementary Figure S9. Isothermal Titration Calorimetry (ITC) data for the A62G mutant riboswitch. Experiments were conducted in a buffer containing 10 mM NaPi (pH 6.5), 30 mM NaCl, 30 mM KCl, and 10 mM $MgCl_2$ at 298K. Measurements were taken immediately after buffer addition (Day 1), after 24 hours (Day 2), and after 48 hours (Day 3), with samples stored at room temperature between experiments. Calculated dissociation constants (K_D) and stoichiometry (N) values are indicated in each panel.

Supplementary Table S1. Chemical shift values for ^{15}N and ^{1}H of the wild-type aptamer and the c-di-GMP ligand. Residues marked with stars indicate those that are predominant in the aptamer when it is in the holo state.

	Free State					Holo State					
	H1	**H2**	**H3**	**N1**	**N3**	**H1**	**H2**	**H3**	**N1**	**N3**	**N7**
gI	-	-	-	-	-	11.96	-	-	-	-	-
G10	-	-	-	-	-	12.09	-	-	-	-	-
G11	-	-	-	-	-	11.84	-	-	146.7	-	-
A12	-	-	-	-	-	-	7.388	-	221.8	-	-
C13	-	-	-	-	-	-	-	-	-	198.3	-
G14	-	-	-	-	-	12.47	-	-	147.1	-	-
G20	-	-	-	-	-	13.16	-	-	149.1	-	-
G21	-	-	-	-	-	12.57	-	-	148.6	-	-
C22	-	-	-	-	-	-	-	-	-	197.4	-
C26	-	-	-	-	197.6	-	-	-	-	196.5	-
C27	-	-	-	-	195.7	-	-	-	-	195.8	-
A28	-	7.153	-	220.8	-	-	7.114	-	220.7	-	-
U29	-	-	11.43	-	157.9	-	-	11.47	-	158.5	-
U30	-	-	14.06	-	162.9	-	-	14.16	-	163	-
U30*	-	-	-	-	-	-	-	14.71	-	163.8	-
C31	-	-	-	-	196	-	-	-	-	195.6	-
G32	10.29	-	-	145.8	-	10.4	-	-	145.6	-	-
G36	11.9	-	-	146.2	-	11.82	-	-	146	-	-
A37	-	7.142	-	220.6	-	-	6.879	-	220.5	-	-
G38	11.44	-	-	144.3	-	11.47	-	-	144.4	-	-
U39*	-	-	-	-	-	-	-	13.39	-	162.1	-
U39	-	-	13.49	-	162.5	-	-	13.54	-	162.4	-
G40	11.57	-	-	146.1	-	11.6	-	-	145.7	-	-
G41	12.78	-	-	147.6	-	13.06	-	-	147.4	-	-
G42	-	-	-	-	-	10.81	-	-	150.7	-	-
G45	-	-	-	-	-	11.72	-	-	145.6	-	-
C46	-	-	-	-	-	-	-	-	-	195.9	-
A48	-	-	-	-	-	-	-	-	-	-	233.3
G50	-	-	-	-	-	10.96	-	-	144.1	-	-
C51	-	-	-	-	-	-	-	-	-	197.9	-
C52	-	-	-	-	-	-	-	-	-	196.7	-
C54	-	-	-	-	198.3	-	-	-	-	197.8	-
C55	-	-	-	-	196.4	-	-	-	-	196.3	-
G56	12.46	-	-	147.9	-	12	-	-	145.7	-	-
G57	11.13	-	-	144.6	-	9.75	-	-	145.4	-	-
C58	-	-	-	-	195.3	-	-	-	-	197.1	-
C59	-	-	-	-	197.5	-	-	-	-	-	-
U60	-	-	13.17	-	161.4	-	-	-	-	-	-
A61	-	-	-	221.1	-	-	-	-	-	-	-
C64	-	-	-	-	-	-	-	-	-	198.3	-
C65	-	-	-	-	-	-	-	-	-	194.9	-
GTL4	9.669	-	-	143.1	-	9.636	-	-	143.3	-	-
G75	-	-	-	-	-	12.84	-	-	146.8	-	-
G76	-	-	-	-	-	13.08	-	-	148.2	-	-
U77	-	-	13.41	-	161.7	-	-	-	-	-	-
A78	-	6.873	-	222.6	-	-	-	-	-	-	-
G79	13.06	-	-	148.1	-	12.92	-	-	147.5	-	-
G80	12.28	-	-	147.4	-	13.27	-	-	147.8	-	-
U81	-	-	11.66	-	158.1	-	-	11.77	-	157	-

G83	11.02	-	-	-	-	-	-	-	-	-	-
C84	-	-	-	-	197.2	-	-	-	-	196.4	-
G85	12.07	-	-	146.5	-	12.12	-	-	146.6	-	-
G86	11.99	-	-	146.8	-	12.76	-	-	148.5	-	-
G87	-	-	-	-	-	12.4	-	-	146.7	-	-
G88	-	-	-	-	-	13.27	-	-	148.3	-	-
U89	-	-	-	-	-	-	-	11.75	-	158.4	-
U90	-	-	-	-	-	-	-	12.02	-	159.5	-
C93	-	-	-	-	-	-	-	-	-	193.8	-
G94	-	-	-	-	-	13.18	-	-	149	-	-
U96	-	-	-	-	-	-	-	14.41	-	163.9	-
C97	-	-	-	-	-	-	-	-	-	198.8	-

Supplementary Table S2. Hydrogen exchange rate constants (k_{ex}) of imino protons were determined at 298 K for the free, apo, and holo states of the RNA through water magnetization transfer experiments.

Helix	Imino	k_{ex} (s^{-1})		
		Free	Apo	Holo
P1a	G10	- [1)]	-	2.30 ± 0.14 [f]
	G14	-	-	2.17 ± 0.10
	G94	-	-	2.12 ± 0.12 [g]
	U96	-	-	12.79 ± 0.86
P1b	G20	-	-	2.12 ± 0.12 [g]
	G21	-	-	1.85 ± 0.09
	U29	3.47 ± 0.20 [a]	3.66 ± 0.20 [c]	1.85 ± 0.10 [h]
	U30	8.50 ± 0.46	3.81 ± 0.34	N.D [2)]
	U30*	-	2.73 ± 0.13	1.37 ± 0.06
	G32	36.87 ± 3.12 [b]	10.98 ± 0.60	N.D.
	G36	2.58 ± 0.18	4.89 ± 0.22	1.76 ± 0.10
	G38	3.47 ± 0.20 [a]	3.66 ± 0.20 [c]	1.85 ± 0.10 [h]
	U39	3.79 ± 0.26	2.79 ± 0.15	-
	U39*	-	3.33 ± 0.16	1.38 ± 0.06
	G40	2.38 ± 0.18	4.46 ± 0.26	2.14 ± 0.13
	G41	2.56 ± 0.18	4.56 ± 0.25 [d]	2.18 ± 0.15 [i]
	G42	-	-	2.60 ± 0.17
	G45	-	-	2.29 ± 0.13
P2	G50	-	-	1.74 ± 0.08
	G56	3.13 ± 0.27	-	2.44 ± 0.12
	G57	8.84 ± 0.23	5.43 ± 0.41	-
	G57*	-	4.26 ± 0.22	2.02 ± 0.12
	U60	4.37 ± 0.27	-	-
	G_{TL4}	N.D.	6.36 ± 0.45	4.60 ± 0.39
	G75	-	4.72 ± 0.24 [e]	2.01 ± 0.13
	G76	-	4.56 ± 0.25 [d]	2.18 ± 0.15 [i]
	U77	28.28 ± 0.73	-	-
	G79	1.96 ± 0.17	4.72 ± 0.24 [e]	1.80 ± 0.10
	G80	5.35 ± 0.31	4.04 ± 0.22	1.78 ± 0.10 [j]
	U81	23.77 ± 0.50	4.24 ± 0.16	1.86 ± 0.06 [k]
	G83	18.94 ± 2.08	-	-
	G85	3.71 ± 0.21	4.47 ± 0.25	2.30 ± 0.14 [f]
	G86	5.58 ± 0.26	4.69 ± 0.25	2.00 ± 0.13
	G87	36.87 ± 3.12 [b]	4.68 ± 0.26	1.96 ± 0.12
	G88	-	-	1.78 ± 0.10 [j]
	U89	-	-	1.86 ± 0.06 [k]
	U90	-	-	2.57 ± 0.11

1) '-': Not observed

2) N.D.: Not determined

3) a,b,c,d,e,f,g,h,i,j,k : k_{ex} values are shown the same for overlapped two peaks

Supplementary Table S3. Isothermal titration calorimetry (ITC) analysis of ligand binding in the presence of 10 mM Mg^{2+} over time. The data demonstrate that the introduction of Mg^{2+} induces a pre-folded state in the A62G aptamer, leading to an increased binding affinity for the ligand as the interaction progresses.

A62G riboswitch / c-di-GMP	Day	N	K_D (nM)	ΔG (Kcal/mol)	TΔS (Kcal/mol)	ΔH (Kcal/mol)
A62G in 10 mM Mg^{2+}	1	0.17	194 ± 47	-1.95 ± 0.14	-6.17 ± 0.61	-15.32 ± 0.59
A62G in 10 mM Mg^{2+}	2	0.84	186 ± 20	-9.21 ± 0.06	-5.27 ± 0.14	-14.48 ± 0.13
A62G in 10 mM Mg^{2+}	3	0.98	149 ± 32	-9.31 ± 0.13	-5.39 ± 0.26	-14.70 ± 0.23