Supplementary Information for

Host-Guest Recognition-Mediated Reversible and Orthogonal Regulation of DNAzyme Activity

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Materials and Methods

The synthesis of all guest-modified compounds and C_8bim was conducted based on previous literature procedures.[1-4] Unmodified Dz 10-23 and the FAM/TMR-labeled chimeric substrate strands used in this study were purchased from Sangon Biotech. The DNA phosphoramidites and CPG were purchased from DNA Chem. Enzyme cleavage experiments were carried out in 15% denaturing polyacrylamide gels, and gel shifts were visualized using (Tanon 2500R) gel imaging system. The sequences of the ODNs used in this study are listed in Table S1.

Synthesis of guest-modified Dz 10-23

The guest-modified Dz utilized in this study were synthesized on a K&A H-8 DNA synthesizer, with the modifying monomers obtained through the phosphoramidite method, and subsequently universal CPG was employed as the solid-phase support to obtain. Following standard DNA synthesis procedures, the DNA products were separated from the CPG and treated with a 28% ammonium hydroxide solution at 55°C overnight to remove the protecting groups. Ethanol precipitation was then conducted, followed by the purification of crude nucleotides using high-performance liquid chromatography (HPLC). The HPLC purification products were subsequently lyophilized and desalted using the Bio-Spin 6 column (Bio-Rad). The resulting product was tested for purity by mass spectrometry and stored frozen at -20°C for subsequent experiments.

The introduction of a guest molecule through the core region of Dz 10-23 regulates its cleavage function via host-guest recognition.

The guest-modified Dz1 was introduced at the core region position, and the enzymatic cleavage activity was assessed in the presence or absence of CB[7]. Diverse site-modified Dz1 (1 μM) and substrate R1 (FAM-labeled, 100 nM) were mixed and divided into two parallel groups. One group was treated with CB[7] (200 μM), while the other group was subjected to an equivalent volume of water under the same buffer conditions (pH= 7.2, 20 mM Tris-HCl, 100 mM NaCl, 100 μM EDTA) for 45 minutes. Enzymatic cleavage was initiated by the addition of 10 mM Mg^{2+}, and aliquots of the reaction mixture were quenched at 8, 32, and 64 minutes with a buffer solution containing 100 mM EDTA. The migration bands reflecting enzyme cleavage were visualized using 15% denaturing acrylamide gel imaging.

Reversible and orthogonal manipulation of the cleavage activity of Dz 10-23

For the reversible manipulation of guest-containing Dz1, the guest (AD/FC) modified the DNAzyme (1 μM) at the C_{10} position (Dz1-C_{10}^{AD} and Dz1-C_{10}^{FC}) mixed with the substrate R1 (FAM-labeled, 100 nM) in a buffer solution (pH= 7.2, 20 mM Tris–HCl, 100 mM NaCl, and 100 μM EDTA) into three parallel groups. The control group was treated without CB[7] and the guest molecule, the inhibited enzyme activity group had only CB[7] added (200 μM), and the reactivated DNAzyme group was treated with CB[7] (200 μM) for 45 minutes, followed by the addition of the competing guest molecule FC (400 μM) and then incubated for 2 hours. Enzymatic digestion commenced upon the addition of 10 mM Mg^{2+} in each of the three groups. Equal volumes of the reaction system were sampled at 0, 2, 4, 8, 16, 24, 32, 40, 52, and 64 minutes and mixed with a quenching buffer solution containing 100 mM EDTA. The migration bands reflecting enzyme cleavage were visualized using 15% denaturing acrylamide gel imaging.

To quantify the cleavage rate, a single-exponential equation was employed to fit the percentage of cleaved product (Eq. 1):

$$[Product]=A[1-\exp(-kt)]+\text{Constant}(1)$$

Where A represents the amplitude of the exponential phase and k is the observed rate constant. The rate constant was analyzed using GraphPad Prism 9.

To monitor the cleavage activity of DNAzyme in real-time, the guest (AD/FC) was introduced to modify the A_{11} site of Dz1-C_{11}^{AD} / Dz1-C_{11}^{FC} (1 μM), followed by incubation with the substrate R1 (FAM-labeled, 100 nM) in a buffer solution across two parallel groups. Cleavage was initiated after the addition of 10 mM Mg^{2+}. In the experimental group, 200 μM CB[7] was introduced at specified time points to inhibit the enzyme activity, while 400 μM FC was added to reactivate the enzyme activity. In the control group, an equivalent volume of water was added at the same time points. Simultaneously, equal volumes of samples were obtained from the control and experimental groups at the specified time points and mixed with quenching buffer containing 100 mM EDTA. The migration bands reflecting enzyme cleavage were visualized using 15% denaturing acrylamide gel imaging.

For orthogonality of inhibitory regulation of DNAzyme cleavage activity, parallel grouping after the substrates R1 (FAM-labeled with rG-U, 100 nM) corresponding to Dz1-C_{11}^{AD} and the substrates R2 (TMR-labeled with rG-U and 4 T are extended at one end of the fluorescent group, 100 nM) corresponding to Dz2-C_{11}^{FC} were mixed. Then add different concentrations of CB[7] (0.02, 0.04, 0.08, 0.2, 0.4 0.8, 2, 4, 8, 20, 40, 80, and 200 μM) were incubated with 2 h. The reaction system was initiated upon adding 10 mM Mg^{2+} and equal amounts of reaction system were taken into quenching buffer solution mixed with 100 mM EDTA after two hours of reaction. The migration changes of the enzyme cleavage were

demonstrated on 15% denaturing acrylamide gel imaging.

To achieve orthogonal activation of DNAzyme cleavage activity, Dz1-C_{11}^{AD} and Dz2-C_{11}^{FC} were pre-incubated with 40 μM CB[7] to form complexes Dz1-C_{11}^{AD}-CB[7] and Dz2-C_{11}^{FC}-CB[7]. Substrates R1 and R2 were then mixed and processed in parallel. Then add different concentrations of C_8bim (0.5, 1, 2, 5, 10,20, 50, 100, 200, 500, 1000, 2000, and 5000 μM) were incubated with 2 h. The reaction system was initiated upon adding 10 mM Mg^{2+} and equal amounts of the reaction system were taken into quenching buffer solution mixed with 100 mM EDTA after two hours of reaction. The migration changes of the enzyme cleavage were demonstrated on 15% denaturing acrylamide gel imaging.

Expressional regulation of cellular green fluorescent protein by DNAzyme

HEK 293T-dsGFP cells with a good growth trend were seeded in 6-well plates separately containing 3.5×10^5 cells per well and 1 ml of complete medium (DMEM plus 10% FBS). After 6 hours of cell adherence, change the fresh complete medium and cells were then transfected with DNAzyme (400 nM) using a standard transfection protocol. For complexed Dz3^{PS}-C_{10}^{AD}-CB[7], Dz3^{PS}-C_{10}^{AD} was incubated with CB[7] (100 μM) before transfection to form a host-guest complex. Each strand was incubated in 100 μL DMEM with 5 μL Lipofectamine 3000 for 15 min, and the mixture was subsequently added dropwise to the cells. After 4 h of incubation, the transfection medium was removed and replaced with fresh complete medium or medium containing CB[7] (200 μM) or guest AM (100 μM). And each DNAzyme was tested as biological replicates in two or three separate wells and treated independently. Cells were then further incubated at 37°C and 5% CO_2 for 20 h.

Cultured cells were assayed for dsGFP expression by flow cytometry. Briefly, the medium was aspirated, and each well was rinsed twice with 1× PBS and then digested with 0.25% trypsin-EDTA. Allow complete digestion of the complete medium to terminate the digestion, centrifuge at 1000 rpm for 10 min at 4℃, discard the upper layer, and precipitate the cells by washing the suspension with 1×PBS and then filtering with a 35-μm cell strainer tube. The dsGFP level of each well was measured using the flow cytometer. Data was collected using a BD FACSCanto II flow cytometer (BD Biosciences). Data were analyzed with FlowJo software (version 7.2).

Supplementary Tables

Table S1. Sequences of ODNs used in this study.

* indicate phosphorothioate (PS). The italic bases indicate the guest modification.

Name		Sequence (from 5' to 3')	Cal	Found	
Dz1		GTCATGA$_0$G$_1$G$_2$C$_3$T$_4$A$_5$G$_6$C$_7$T$_8$A$_9$C$_{10}$A$_{11}$A$_{12}$C$_{13}$G$_{14}$A$_{15}$GGTTAGG	—	—	
R1		CCTAACC/rG//U/CATGAC(**FAM**)	—	—	
Dz1^{AD}	**Dz1-C$_N^{AD}$**	GTCATGAGG*C$_3$*TAG*C$_7$*TA*C$_{10}$*AA*C$_{13}$*GAGGTTAGG	9155.1	9153.9	
	Dz1-A$_N^{AD}$	GTCATG*A$_0$*GGCT*A$_5$*GCT*A$_9$C$A_{11}A_{12}$*CG*A$_{15}$*GGTTAGG	9155.1	9154.7	
Dz1^{FC}	**Dz1-C$_N^{FC}$**	GTCATGAGG*C$_3$*TAG*C$_7$*TA*C$_{10}$*AA*C$_{13}$*GAGGTTAGG	9205.0	9205.0	In test tubes
	Dz1-A$_N^{FC}$	GTCATG*A$_0$*GGCT*A$_5$*GCT*A$_9$C$A_{11}A_{12}$*CG*A$_{15}$*GGTTAGG	9205.0	9206.8	
Dz1-A$_N^{BA}$		GTCATG*A$_0$*GGCT*A$_5$*GCT*A$_9$CA_{11}*ACGAGGTTAGG	9126.1	9127.8	
Dz2-C$_{10}^{FC}$		CTCTCCAGGCTAGCTA***C$_{10}^{FC}$***AACGACGACACT	8990.0	8990.5	
R2		AGTGTCG/rG//U/GGAGAGTTTT(**TMR**)	—	—	
Dz3^{PS}		G∗T∗C∗A∗C∗G∗A∗AGGCTAGCTACAACGAT∗C∗C∗A∗G∗C	—	—	
Dz4^{PS}		C∗C∗A∗G∗C∗T∗C∗G∗AGGCTAGCTACAACGAC∗A∗G∗G∗A∗T∗G∗G∗G	—	—	Cellular studies
Dz5^{PS}		G∗C∗T∗G∗C∗AGGCTAGCTACAACGAG∗C∗T∗G∗C∗C	—	—	
Dz3^{PS}-C$_{10}^{AD}$		G∗T∗C∗A∗C∗G∗A∗AGGCTAGCTA***C$_{10}^{AD}$***AACGAT∗C∗C∗A∗G∗C	9221.8	9223.0	

Table S2. HPLC elution gradient used for ODN purification.

Time(min)	Flow (mL/ min)	%A	%B
0	1.00	95	5
20	1.00	50	50
35	1.00	0	100
50	1.00	0	100

Solvent A was 0.1 M TEAA (aqueous) in 5% acetonitrile, and Solvent B was 100% acetonitrile.

Supplementary Figures

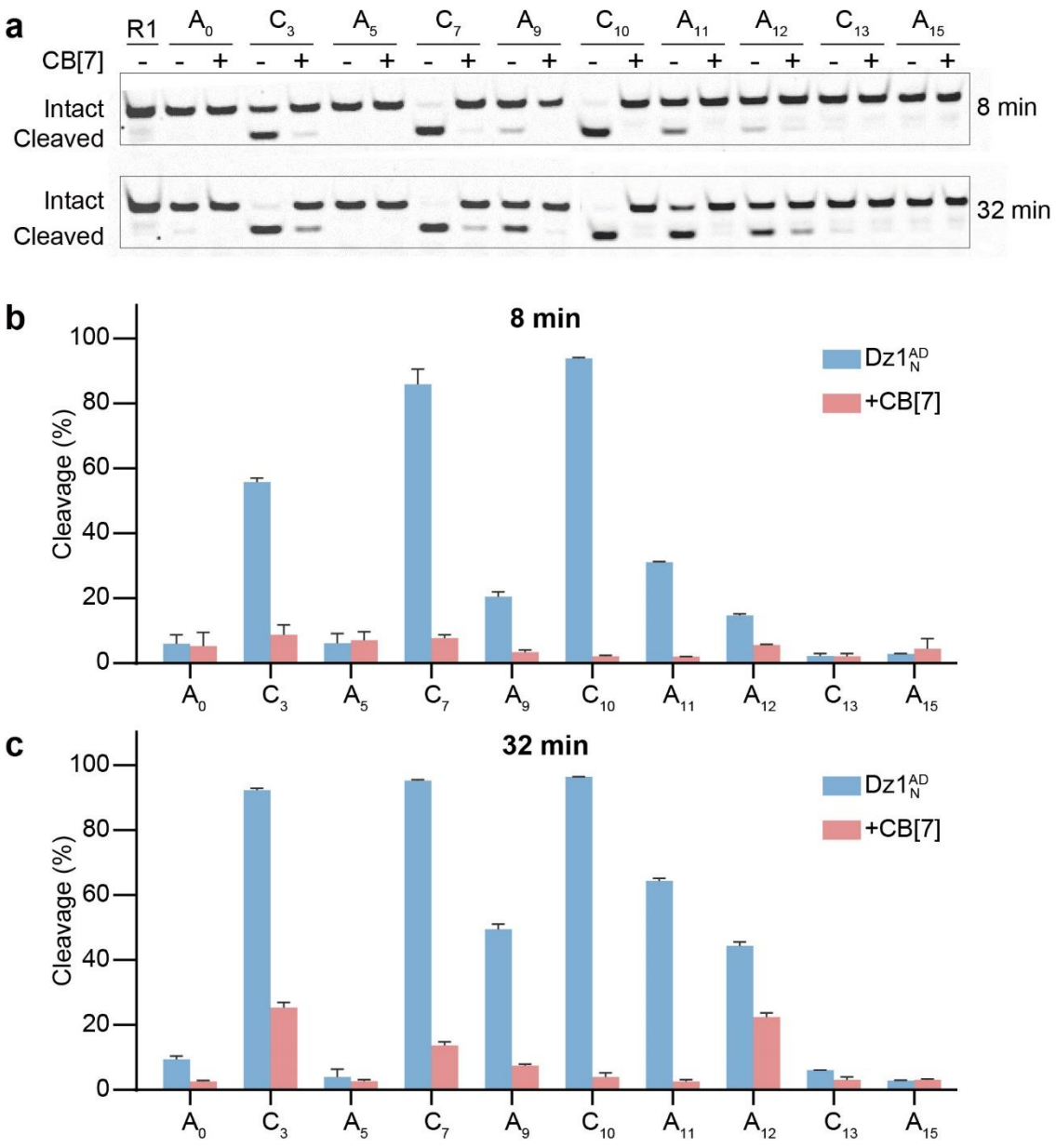


Fig. S1. Functional inhibition analysis of AD-containing DNAzyme 1 ($Dz1_N^{AD}$) by CB[7]. (a) Photographs of acrylamide gels and statistical results showing cleavage efficiency of the $Dz1_N^{AD}$, incubated with and without 200 μM CB[7] for (b) 8 and (c) 32 minutes, respectively. R1 represents the substrate R1, and base subscript numbers indicate base positions. Error bars represent means ±SD (n = 2).

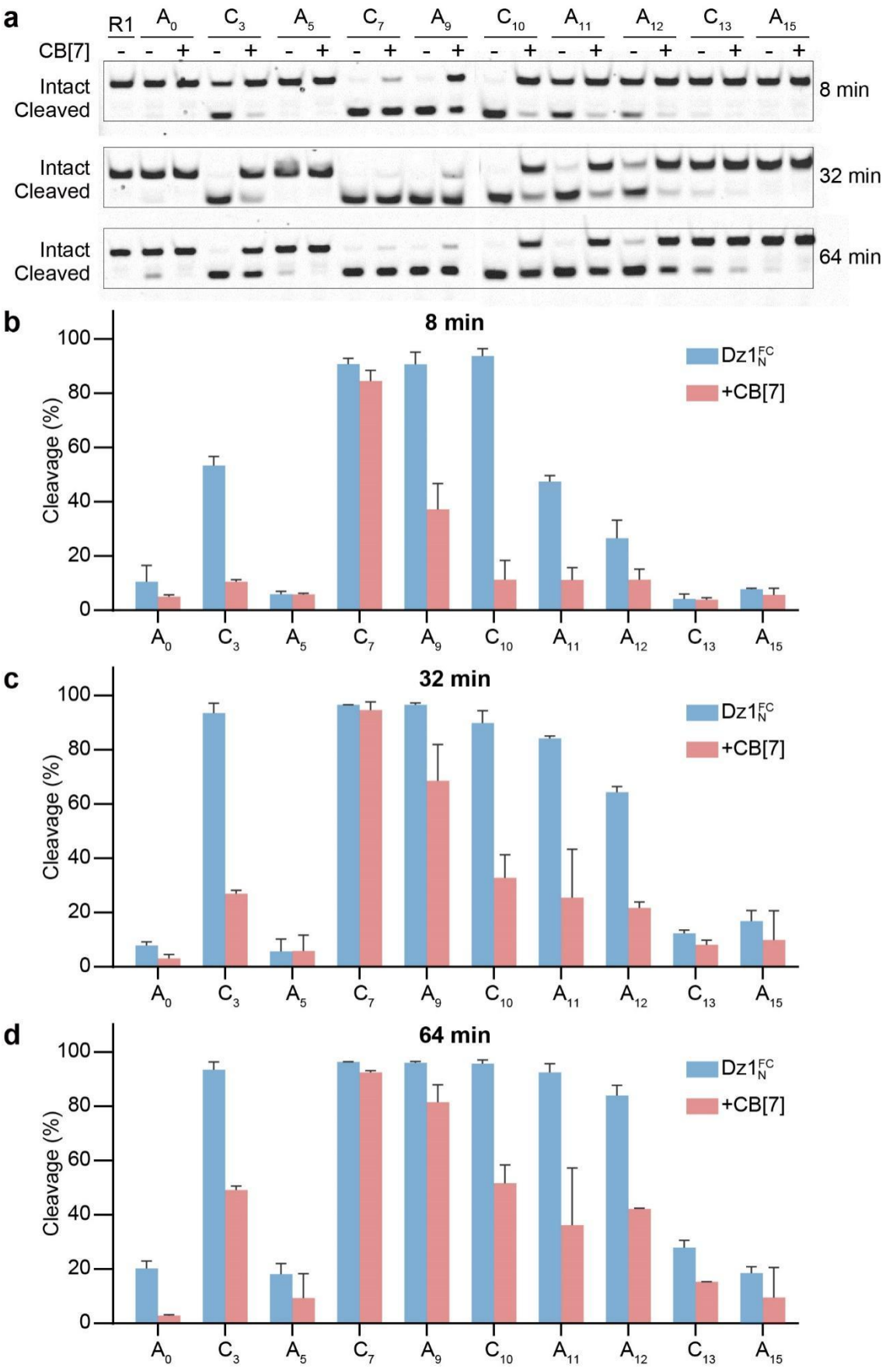


Fig. S2. Functional inhibition analysis of FC-containing DNAzyme 1 ($Dz1_N^{FC}$) by the CB[7]. (a) Photographs of acrylamide

gels (a) and statistical results showing DNA cleavage efficiency (b), (c), and (d) of the $Dz1_N^{FC}$, incubated with and without 200 μM CB[7] for 8, 32, and 64 minutes, respectively. R1 represents the substrate R1, and base subscript numbers indicate base positions. Error bars represent means ±SD (n = 2).

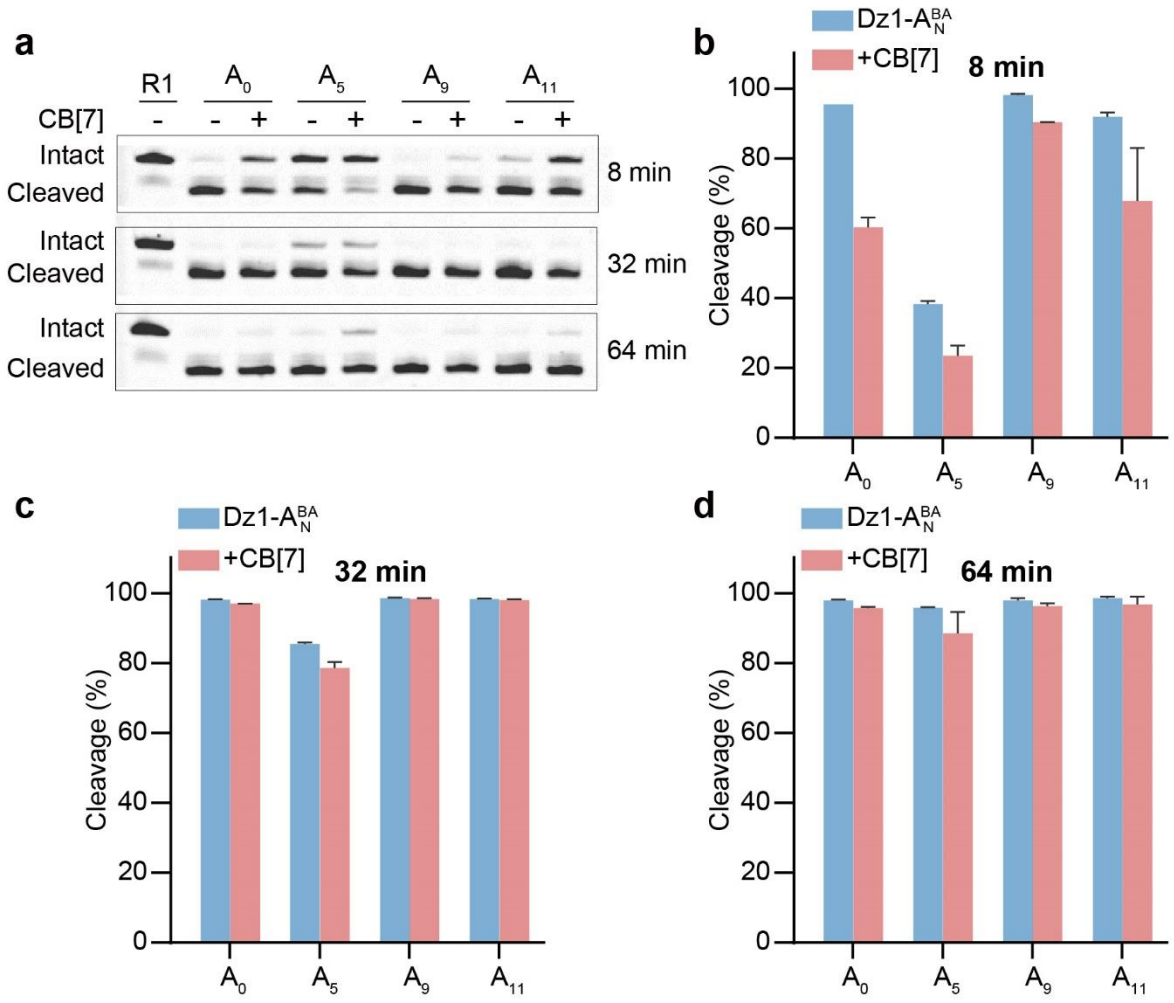


Fig. S3. Functional inhibition analysis of BA-containing DNAzyme 1 (Dz1-A_N^{BA}) by the CB[7]. Photographs of acrylamide gels (a) and statistical results showing DNA cleavage efficiency (b), (c), and (d) of the Dz1-A_N^{BA}, incubated with and without 200 μM CB[7] for 8, 32, and 64 minutes, respectively. R1 represents the substrate R1, and base subscript numbers indicate base positions. Error bars represent means ±SD (n = 2)

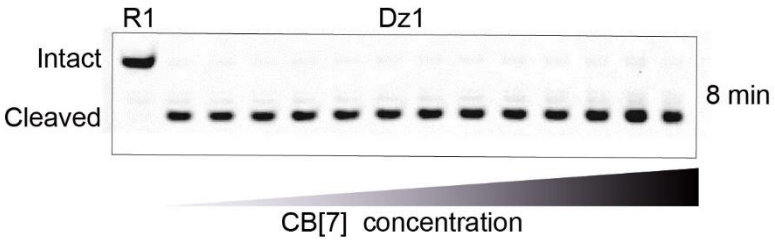


Fig. S4. Photographs of acrylamide gels after 8-minute incubation of the DNAzyme 1 (Dz1) in solutions containing varying concentrations (0.02, 0.04, 0.08, 0.2, 0.4, 0.8, 2, 4, 8, 20, 40, 80, and 200 μM) of CB[7]. R1 refers to the substrate R1.

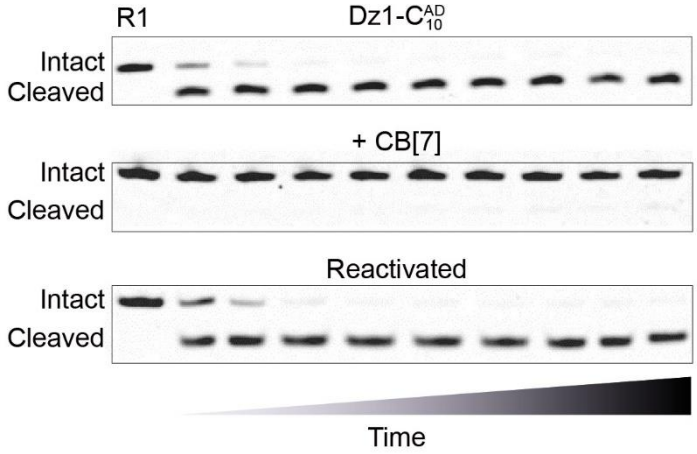


Fig. S5. Acrylamide gel photographs of Dz1-C_{10}^{AD} incubated for 0, 2, 4, 8, 16, 24, 32, 40, 52, and 64 minutes under various treatment conditions. The Dz1-C_{10}^{AD} group indicates incubation in a blank solution, while the +CB[7] group represents Dz1-C_{10}^{AD} in CB[7]-containing solutions. The Reactivated group refers to the CB[7]-containing DNAzyme (Dz1-C_{10}^{AD}-CB[7]) incubated in a competitive guest solution containing FC. R1 denotes the substrate R1.

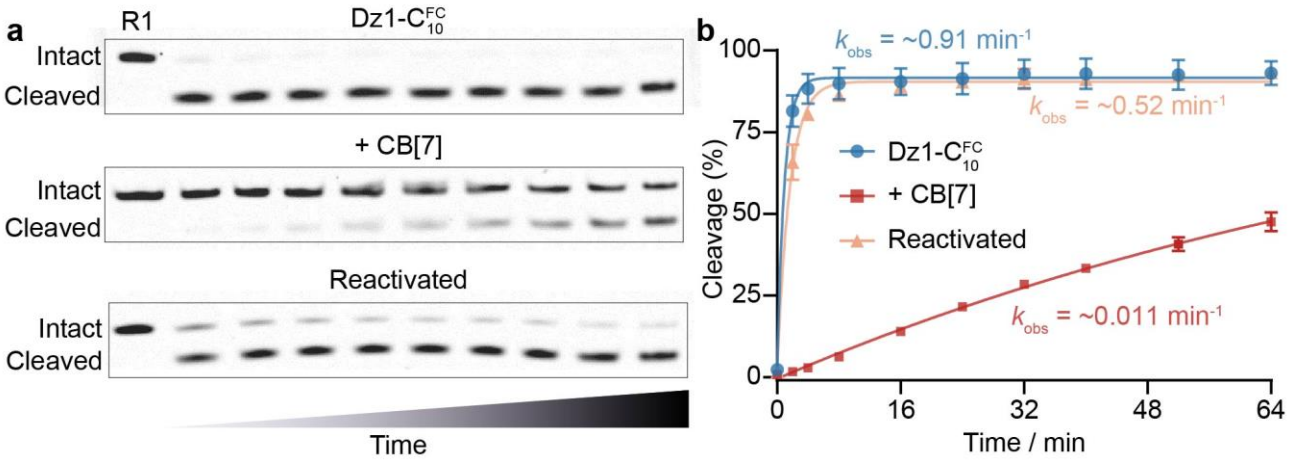


Fig. S6. (a) Acrylamide gel photographs of Dz1-C_{10}^{FC} incubated for 0, 2, 4, 8, 16, 24, 32, 40, 52, and 64 minutes under various treatment conditions. The Dz1-C_{10}^{FC} group indicates incubation in a blank solution, while the +CB[7] group represents Dz1-C_{10}^{FC} in CB[7]-containing solutions. The Reactivated group refers to the CB[7]-containing DNAzyme (Dz1-C_{10}^{FC}-CB[7]) incubated in a competitive guest solution containing FC. (b) Quantitative analysis of the kinetic behavior of substrate cleavage by Dz1-C_{10}^{FC} under various treatment conditions. To quantify the cleavage rate, a single-exponential equation was employed to fit the percentage of cleaved product as described in Materials and Methods. The observed rate constants (k_{obs}) are presented alongside the corresponding kinetic curves. The reactions were performed at 37°C in a buffer solution (pH 7.2) consisting of 20 mM Tris-HCl, 100 mM NaCl, 100 μM EDTA, and 10 mM $MgCl_2$. The concentration of Dz1-C_{10}^{FC} was 1 μM, and the substrate concentration was 0.1 μM.R1 denotes the substrate R1. Error bars represent means ±SD (n = 3).

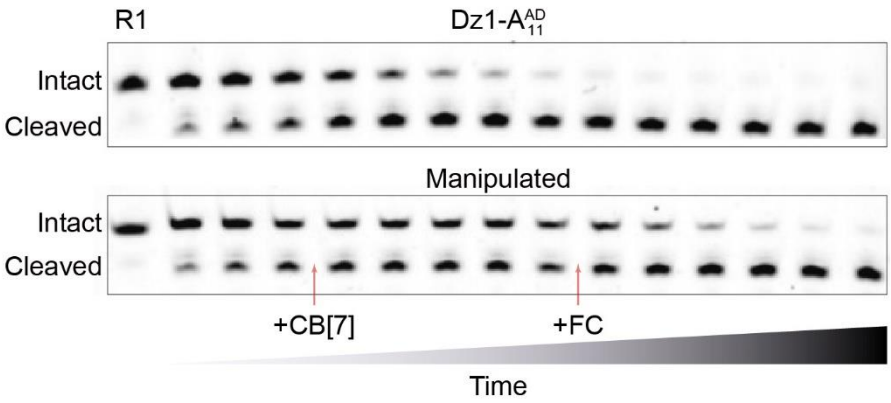


Fig. S7. Acrylamide gel photographs of real-time regulatory analysis of the Dz1-A_{11}^{AD} activity. Control group Dz1-A_{11}^{AD} had been incubated in blank solution, Manipulated group was added 200 μM CB[7] and 400 μM competitive guest FC at 12 and 60 min of incubation, respectively. Sample incubation times from left to right are 0, 4, 8, 12, 20, 30, 40, 50, 60, 80, 100, 120, 140, 160 and 180 minutes. R1 refers to the substrate R1.

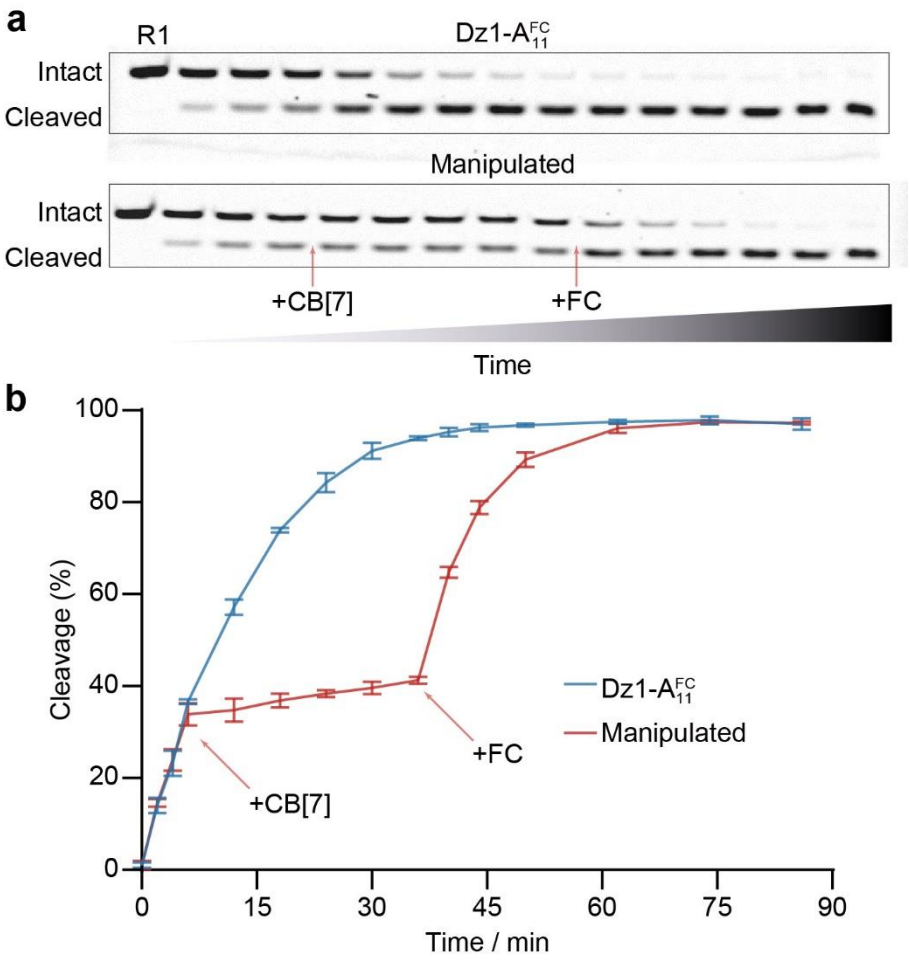


Fig. S8. Real-time regulatory analysis of the Dz1-A_{11}^{FC} activity. Photographs of acrylamide gels (a) and statistical results of DNA cleavage rates (b) after incubation with DNAzyme Dz1-A_{11}^{FC} for 0, 3, 6, 9, 15, 21, 27, 33, 39, 51, 63, 75, and 87 min. Control group Dz1-A_{11}^{FC} had been incubated in blank solution, Manipulated group was added 200 μM CB[7] and 400 μM competitive guest FC at 9 and 39 min of incubation, respectively. R1 refers to the substrate R1. Error bars represent means ±SD (n = 3).

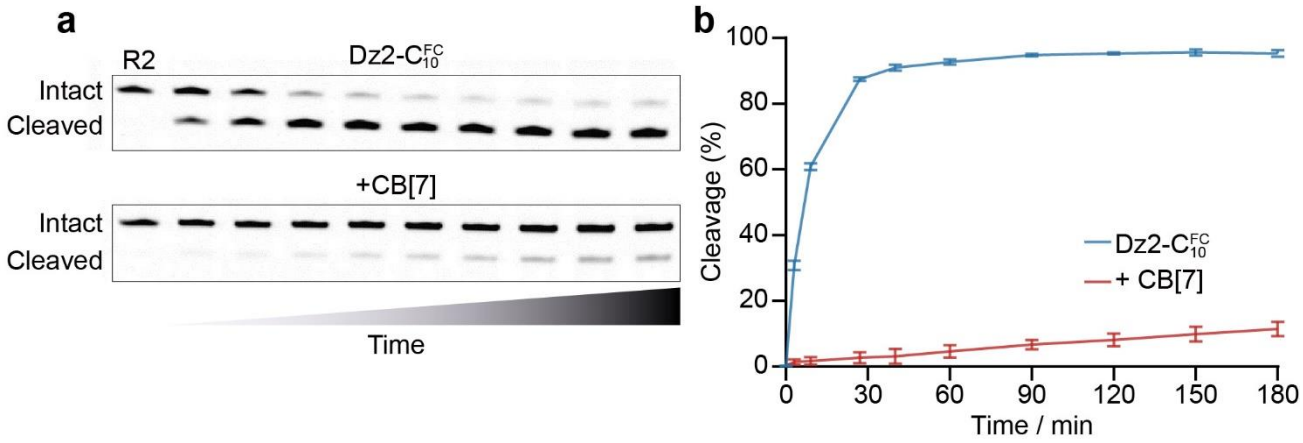


Fig. S9. Inhibition curve of CB[7] on the guest FC-containing DNAzyme 2 (Dz2-C_{10}^{FC}). Photographs of acrylamide gels (a) and statistical analysis (b) of DNA cleavage rates at various incubation times for DNAzyme Dz2-C_{10}^{FC} in blank (Dz2-C_{10}^{FC} group) and in a solution containing 200 μM CB[7] (+CB[7] group), respectively. R2 refers to the substrate R2. Error bars represent means ±SD (n = 3).

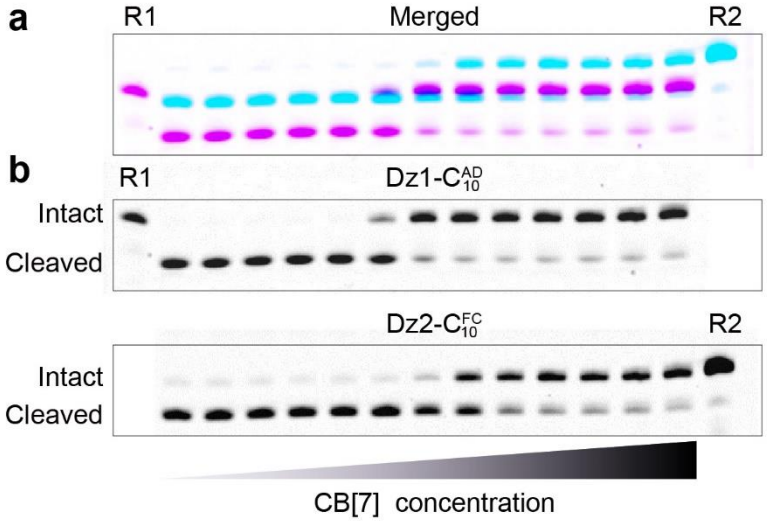


Fig. S10. Orthogonality analysis of CB[7] inhibition on the activities of AD- and FC-containing DNAzymes (Dz1-C_{10}^{AD} and Dz2-C_{10}^{FC}). (a) Bifluorescent acrylamide gel images after incubating the two DNAzymes for 2 h in solutions containing 0-200 μM CB[7]. The prune band represents the FAM-labeled DNAzyme Dz1-C_{10}^{AD}, and the blue band represents the TMR-labeled DNAzyme Dz2-C_{10}^{FC}. (b) Unique analyses of the DNAzymes Dz1-C_{10}^{AD} (top) and Dz2-C_{10}^{FC} (bottom) from panel (a), with R1 and R2 indicating the respective substrates.

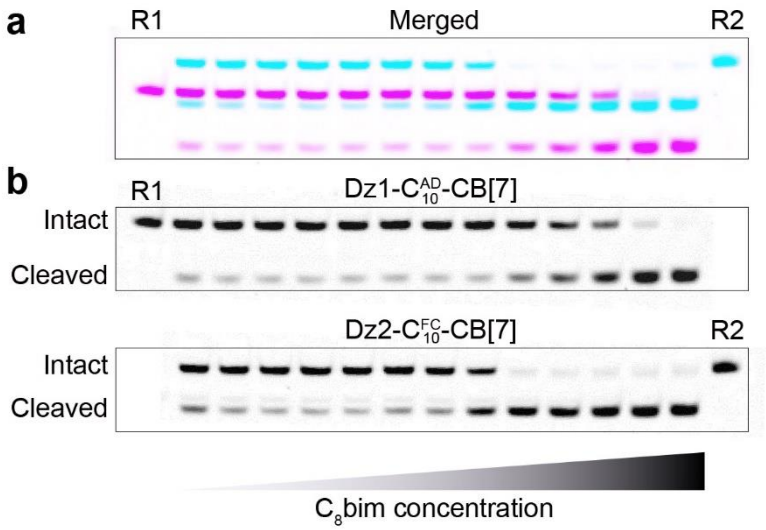


Fig. S11. Orthogonality analysis of the competitive guest C_8bim on the activation of Dz1-C_{10}^{AD}-CB[7] and Dz2-C_{10}^{FC}-CB[7]**.** (a) Photographs of bifluorescent acrylamide gels after incubation of the two DNAzymes complexes for 2 h in the solution containing 0.5-5000 μM C_8bim, respectively. The prune band is the FAM-labeled DNAzyme Dz1-C_{10}^{AD}-CB[7], and the blue is the TMR-labeled DNAzyme Dz2-C_{10}^{FC}-CB[7]. (b). Photographs of unique analyses of the DNA enzymes Dz1-C_{10}^{AD}-CB[7] (top) and Dz2-C_{10}^{FC}-CB[7] (bottom) from panel (a), with R1 and R2 indicating the respective substrates.

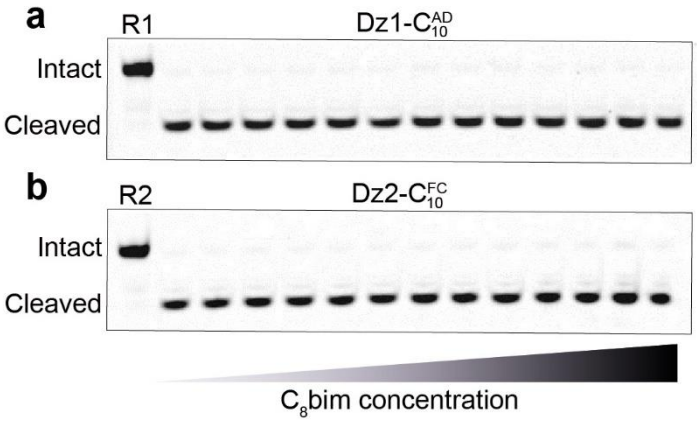


Fig. S12. Analysis of the effect of the competitive guest C_8bim on the activities of the Dz1-C_{10}^{AD} and Dz2-C_{10}^{FC}. Photographs of acrylamide gels after incubation of DNAzymes Dz1-C_{10}^{AD} (a) Dz2-C_{10}^{FC} (b) in solutions containing 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, 2000, and 5000 μM of competitive guest C_8bim, respectively, for 2 hours. R1 and R2 refer to the substrate R1 and R2.

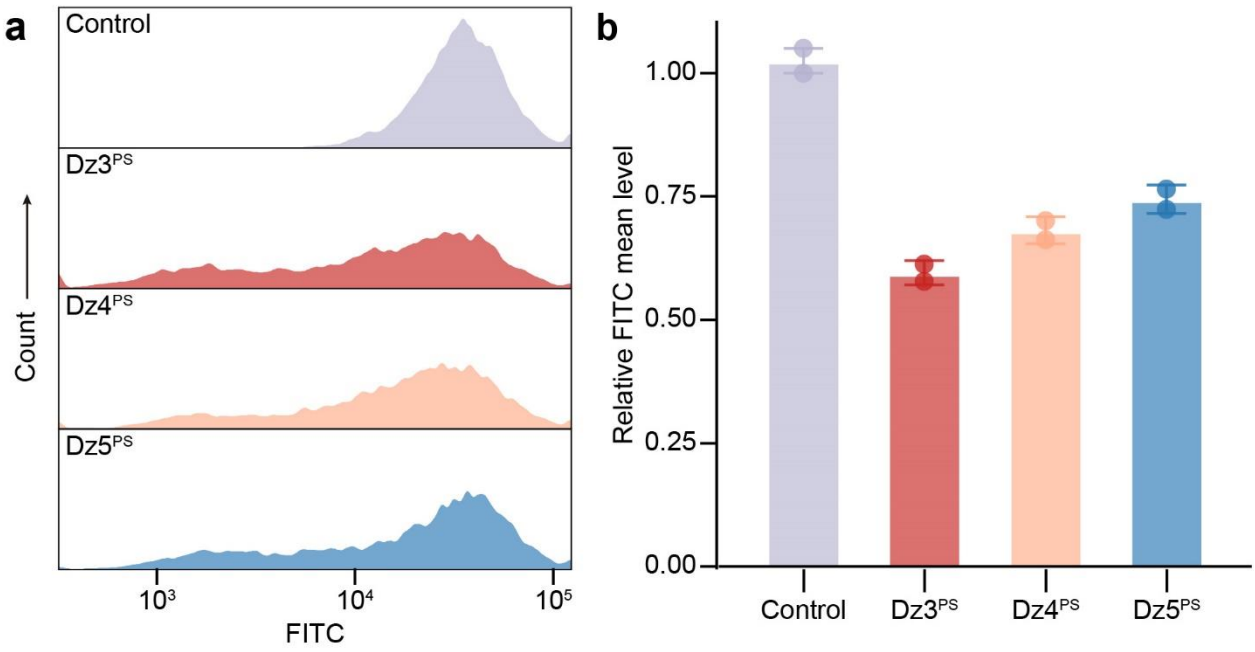


Fig. S13. Screening for the optimal DNAzyme binding site in dsGFP-HEK293T cells. (a) Flow cytometry analysis of dsGFP expression in test samples, with a nonsensical oligonucleotide as a control. Analysis of fluorescent dsGFP intensity in cells transfected with three thio-modified oligonucleotides ($Dz3^{PS}$, $Dz4^{PS}$, and $Dz5^{PS}$). (b) Quantitative analysis of average fluorescence normalized to control samples. Error bars represent means ±SD (n = 2).

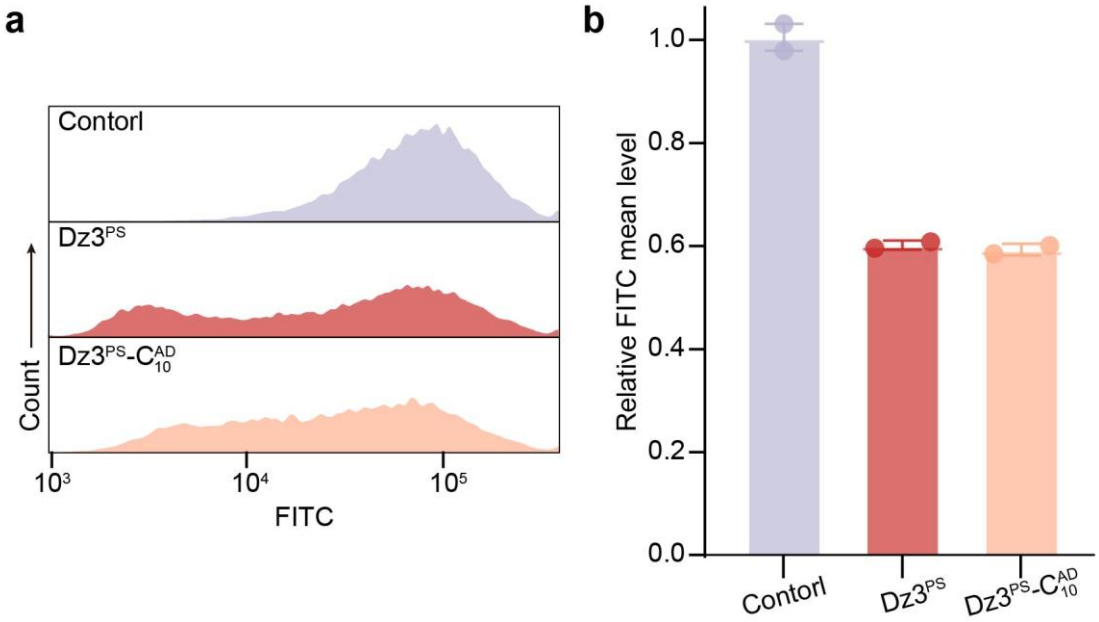


Fig. S14. Analyzing the effect of $Dz3^{PS}$ modified with AD ($Dz3^{PS}$-C_{10}^{AD}) on intracellular fluorescence expression. (a) Flow cytometry analysis of dsGFP expression in test samples, using a nonspecific oligonucleotide as a control. Fluorescent

intensity of dsGFP in cells transfected with $Dz3^{PS}$-C_{10}^{AD} and $Dz3^{PS}$ was assessed. (b) Quantitative analysis of average fluorescence normalized to control samples. Error bars represent means ± SD (n = 2).

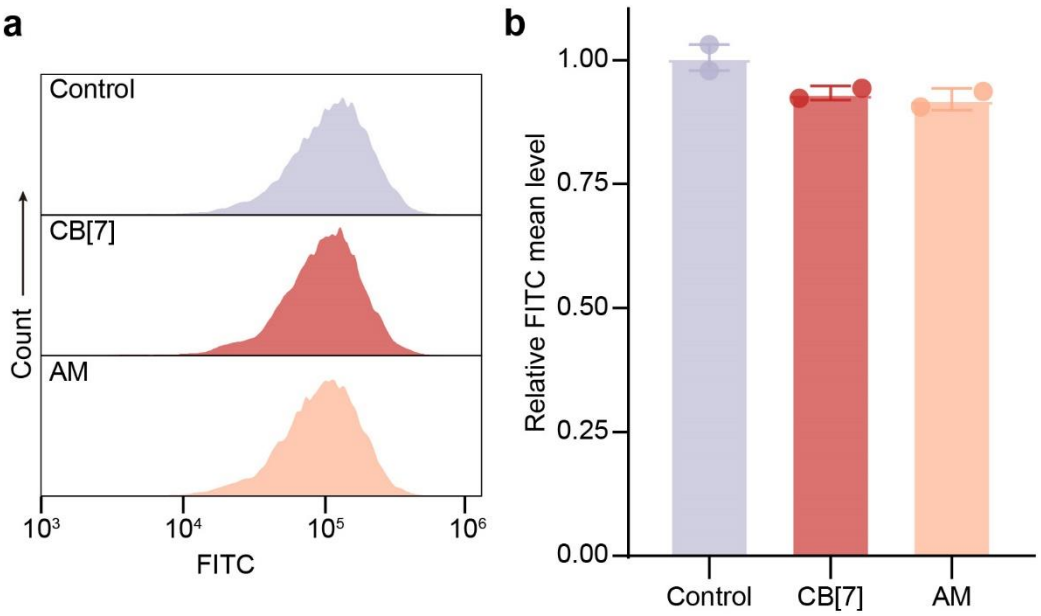
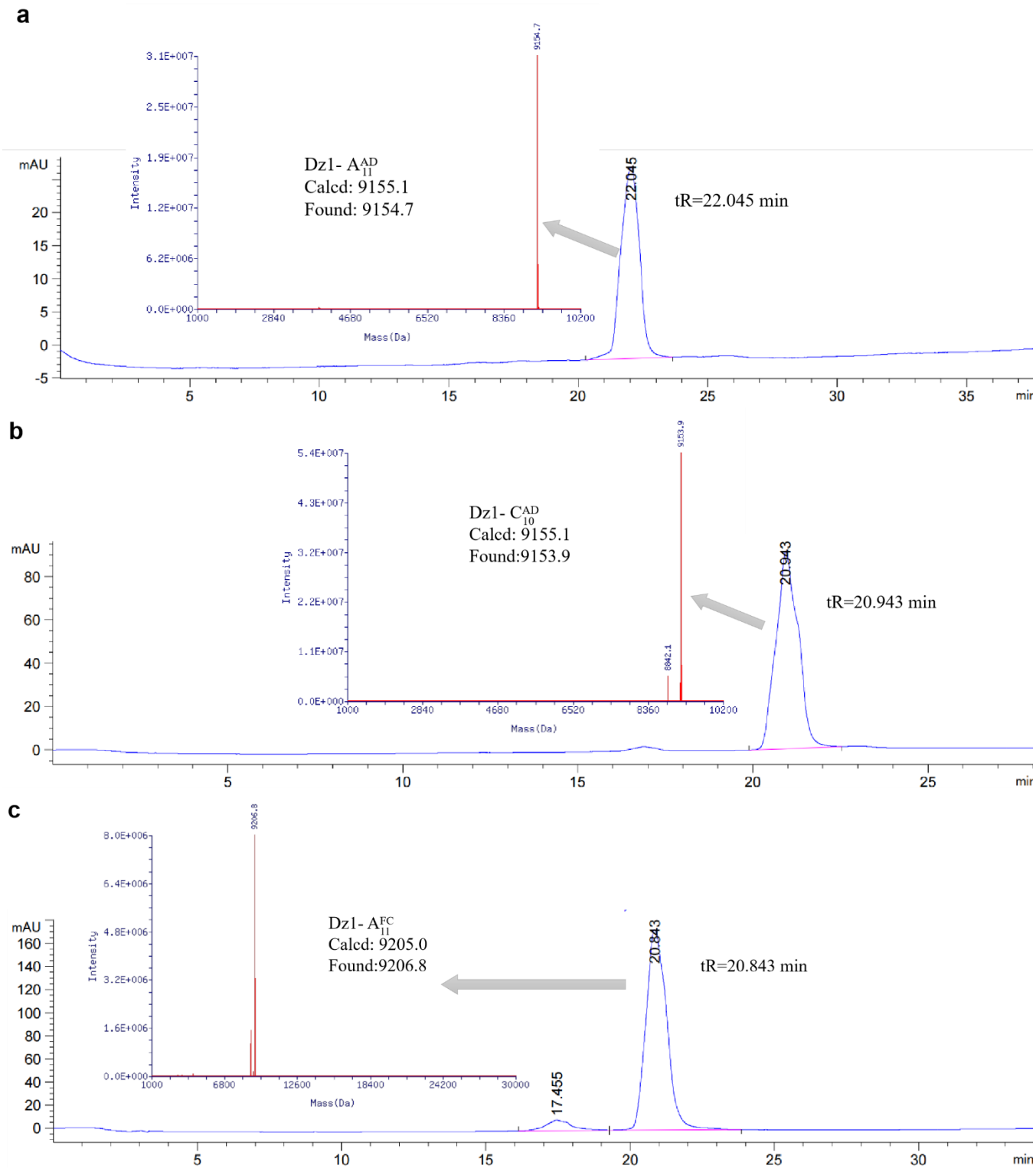


Fig. S15. Analysis of the effects of CB[7] and guest AM on dsGFP expression in HEK293T cells. (a) Flow cytometric analysis of fluorescence intensity in test samples. (b) Quantitative analysis of average fluorescence normalized to control samples. The control group consists of untreated blank samples, while the CB[7] and AM groups represent samples incubated with 100 μM CB[7] and AM, respectively. Error bars represent means ±SD (n = 2).

HPLC chromatograms and ESI spectra of the purified guest-containing Dz



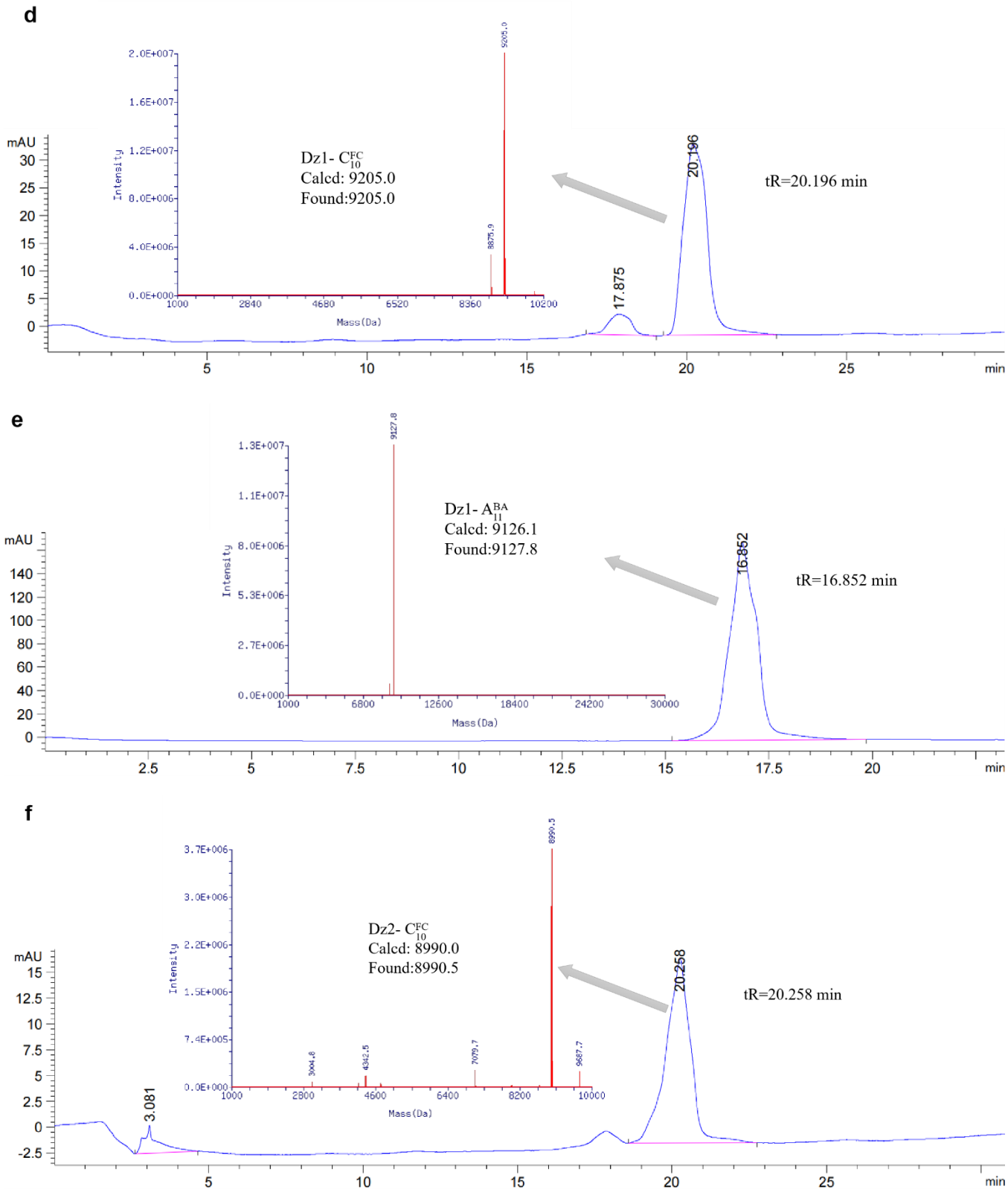


Fig. S16. HPLC chromatographic analysis (retention time, tR) and ESI mass spectrometry of the guest-modified DNAzymes for in vitro analysis.

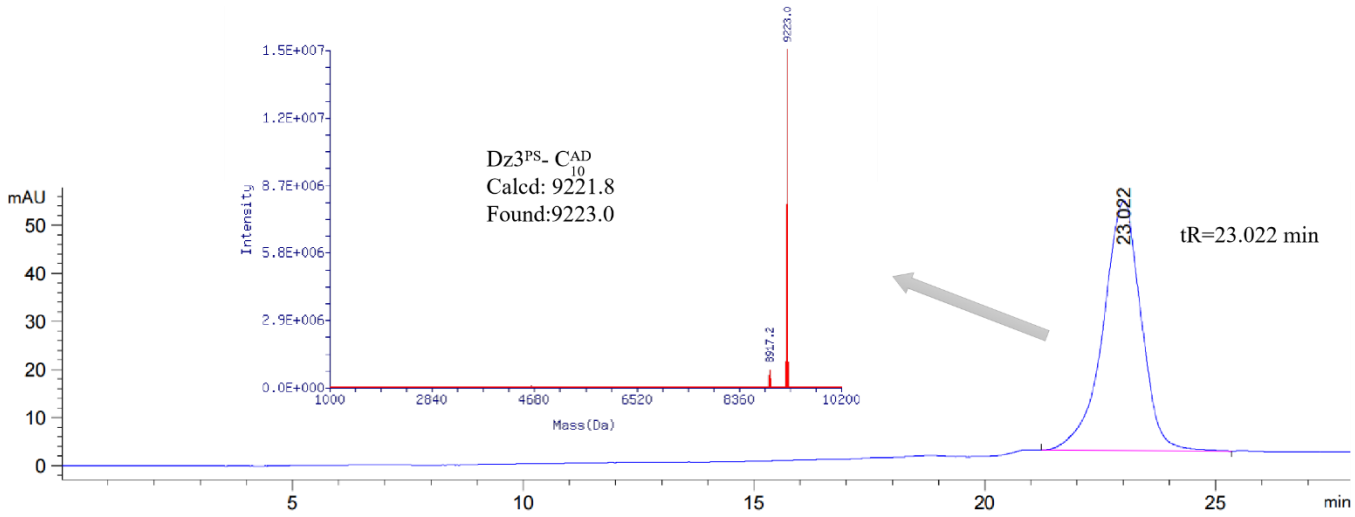
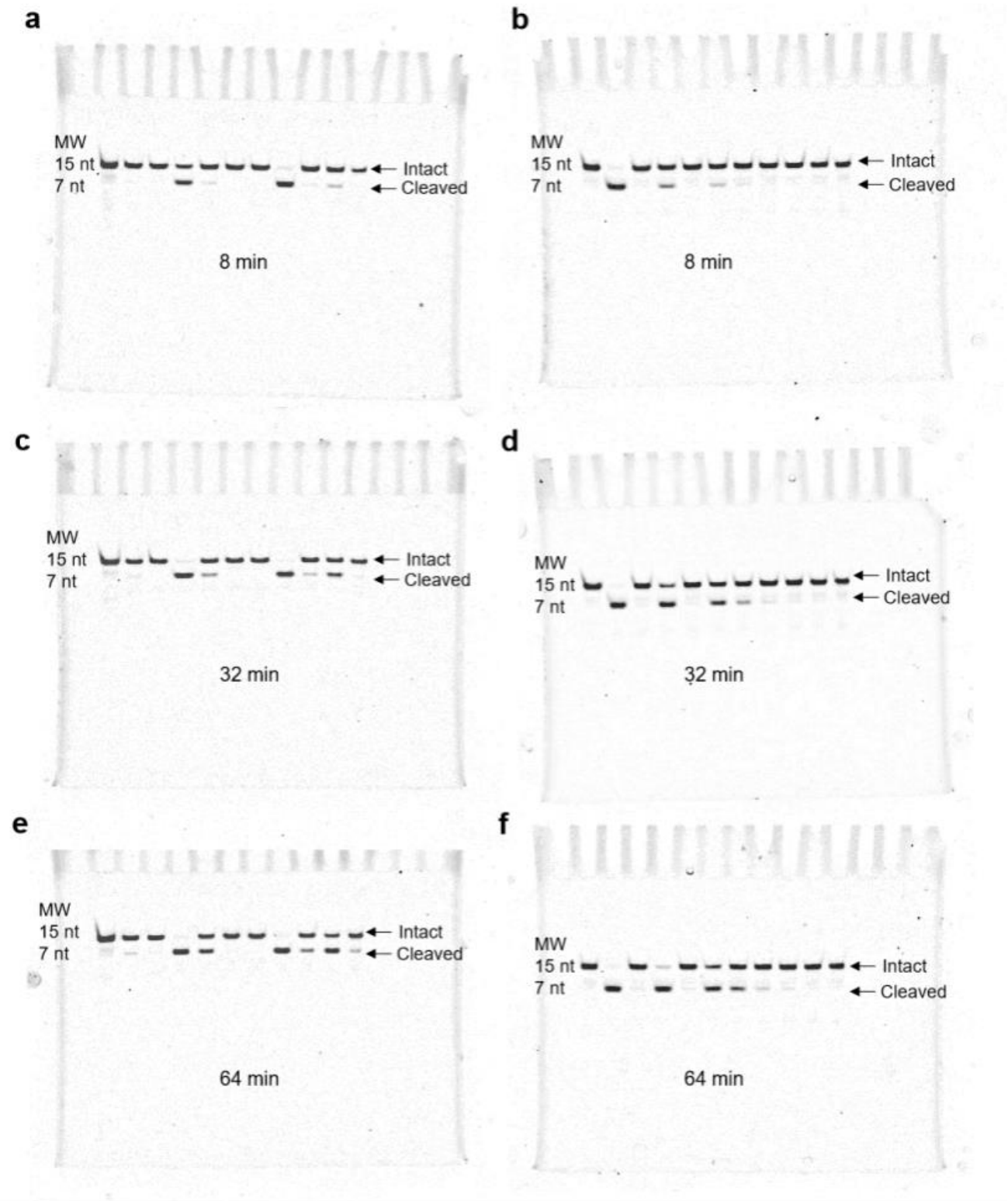
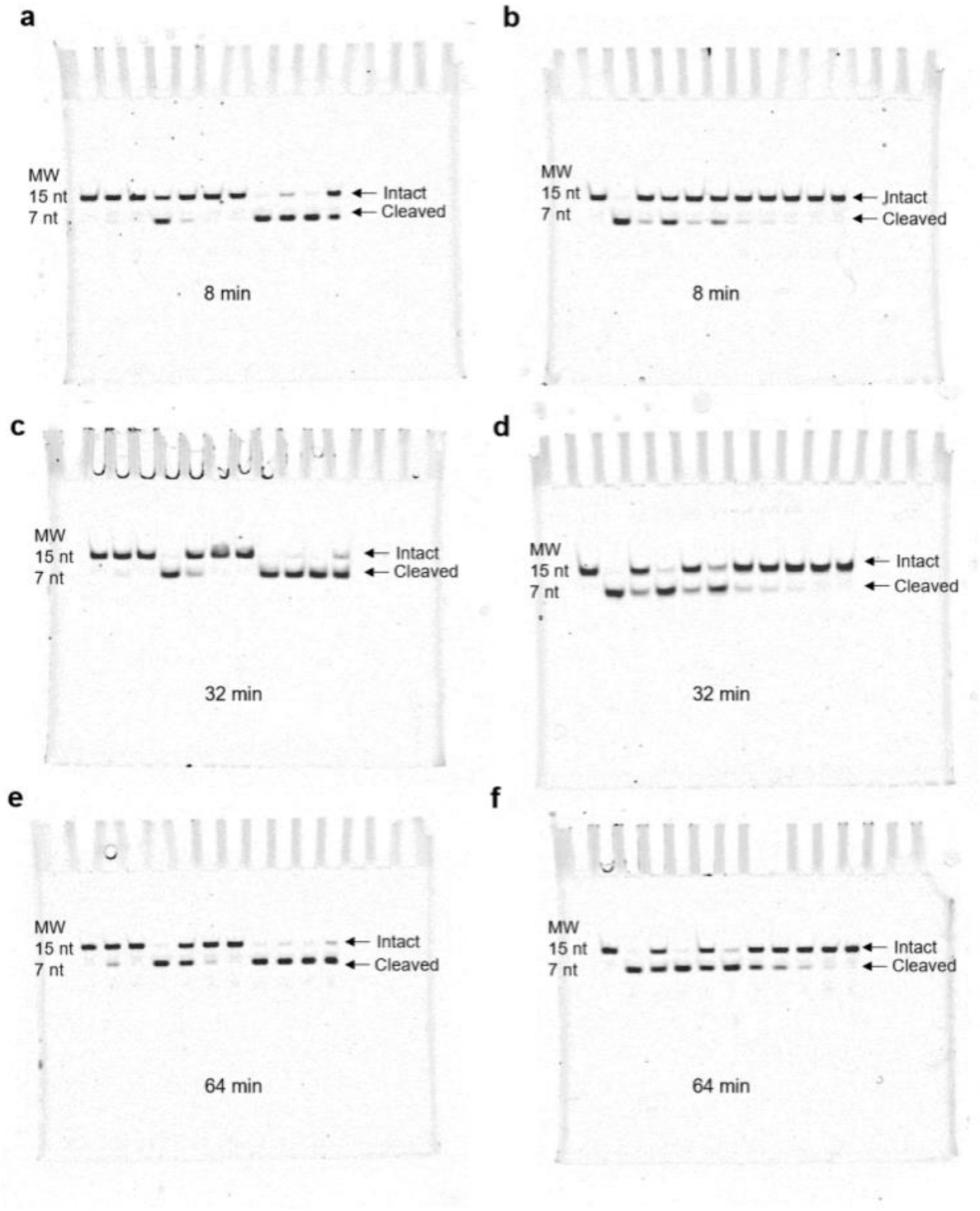


Fig. S17. HPLC chromatographic analysis (retention time, tR) and ESI mass spectrometry of the guest-modified DNAzymes for cellular experiments.

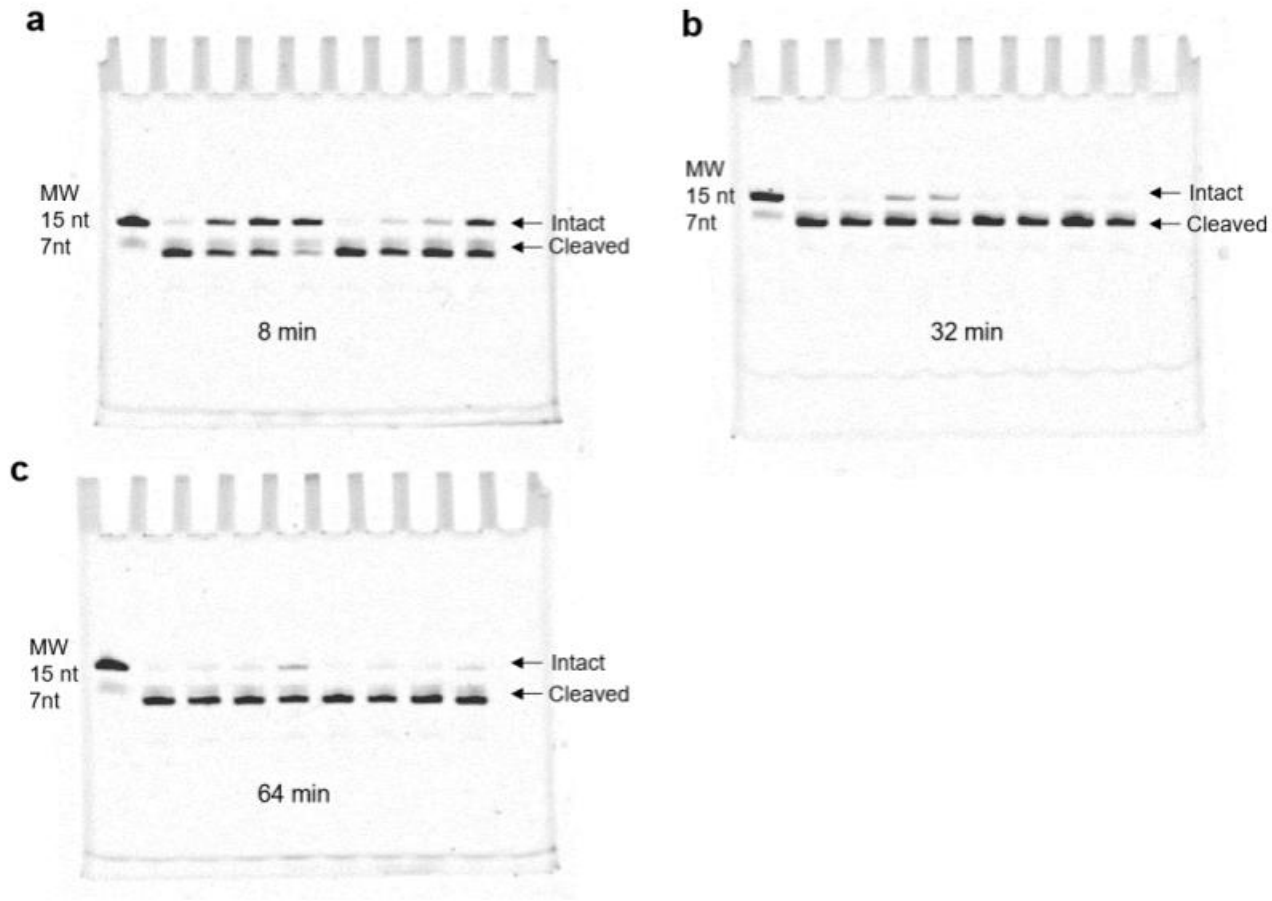
Supplementary Figures (Uncropped gel images)



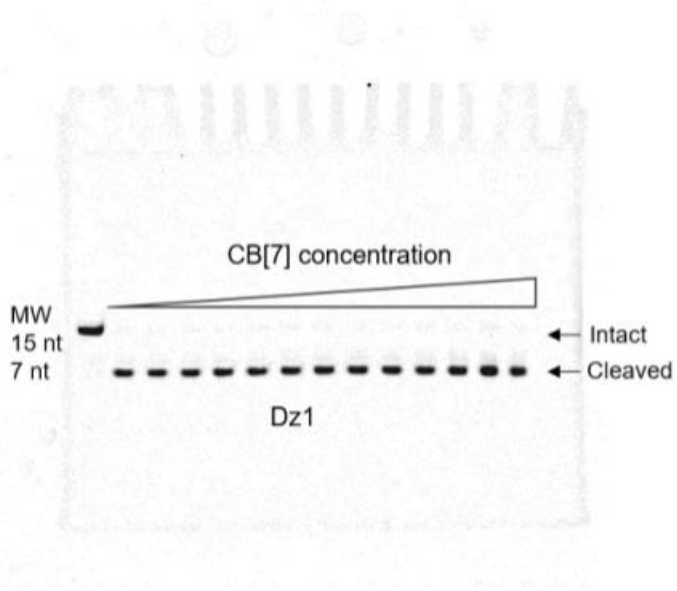
Supplementary Fig. S18. Uncropped gel images for Fig. 2 and Fig. S1.



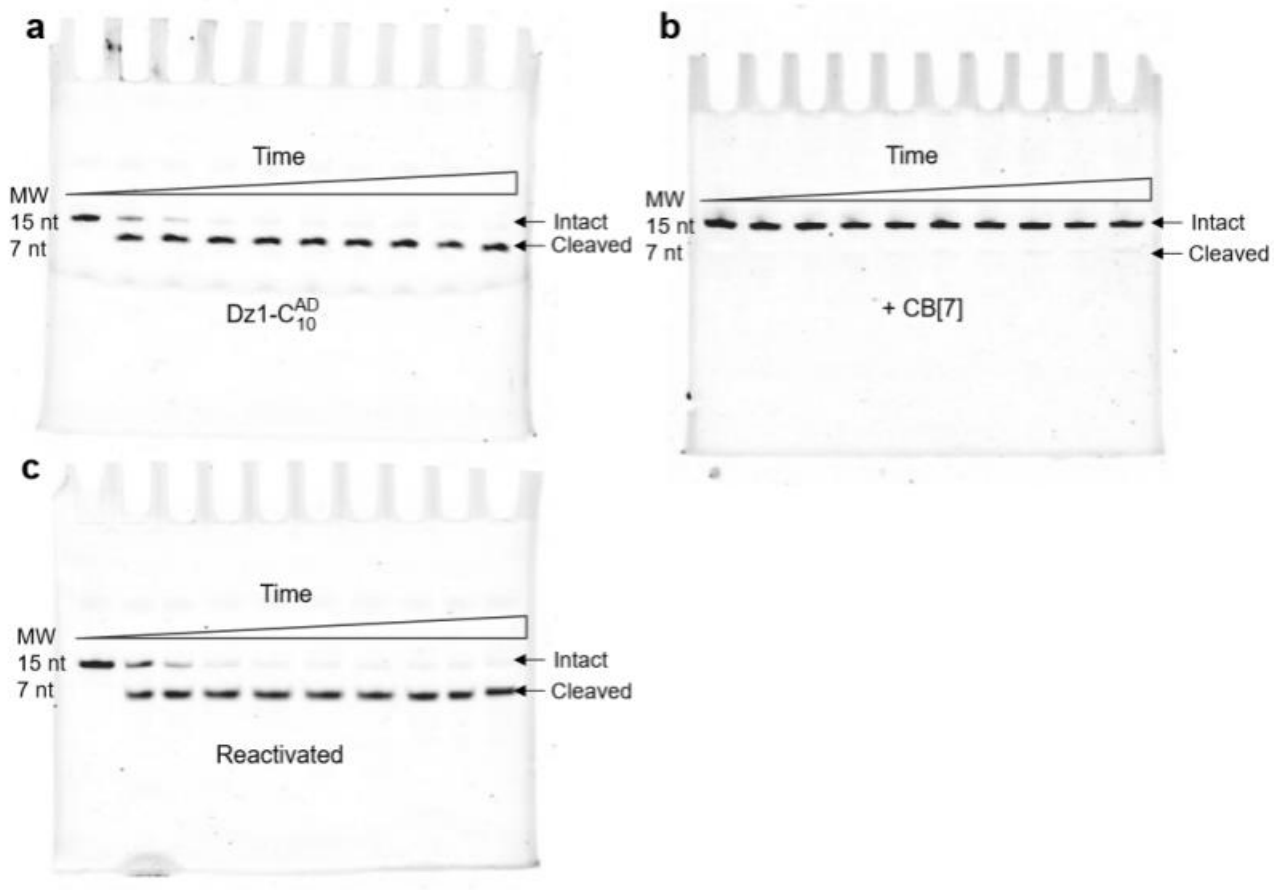
Supplementary Fig. S19. Uncropped gel images for Fig. S2.



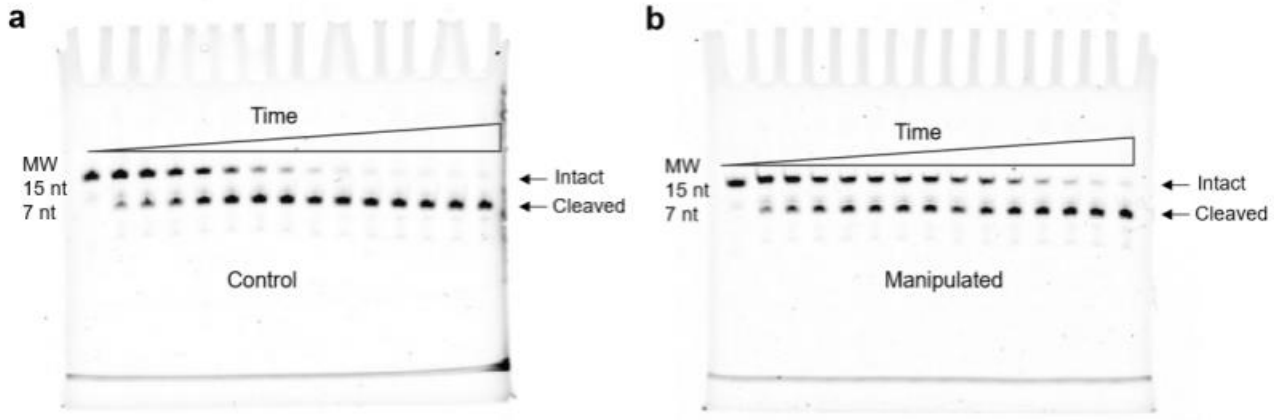
Supplementary Fig. S20. Uncropped gel images for Fig.S3.



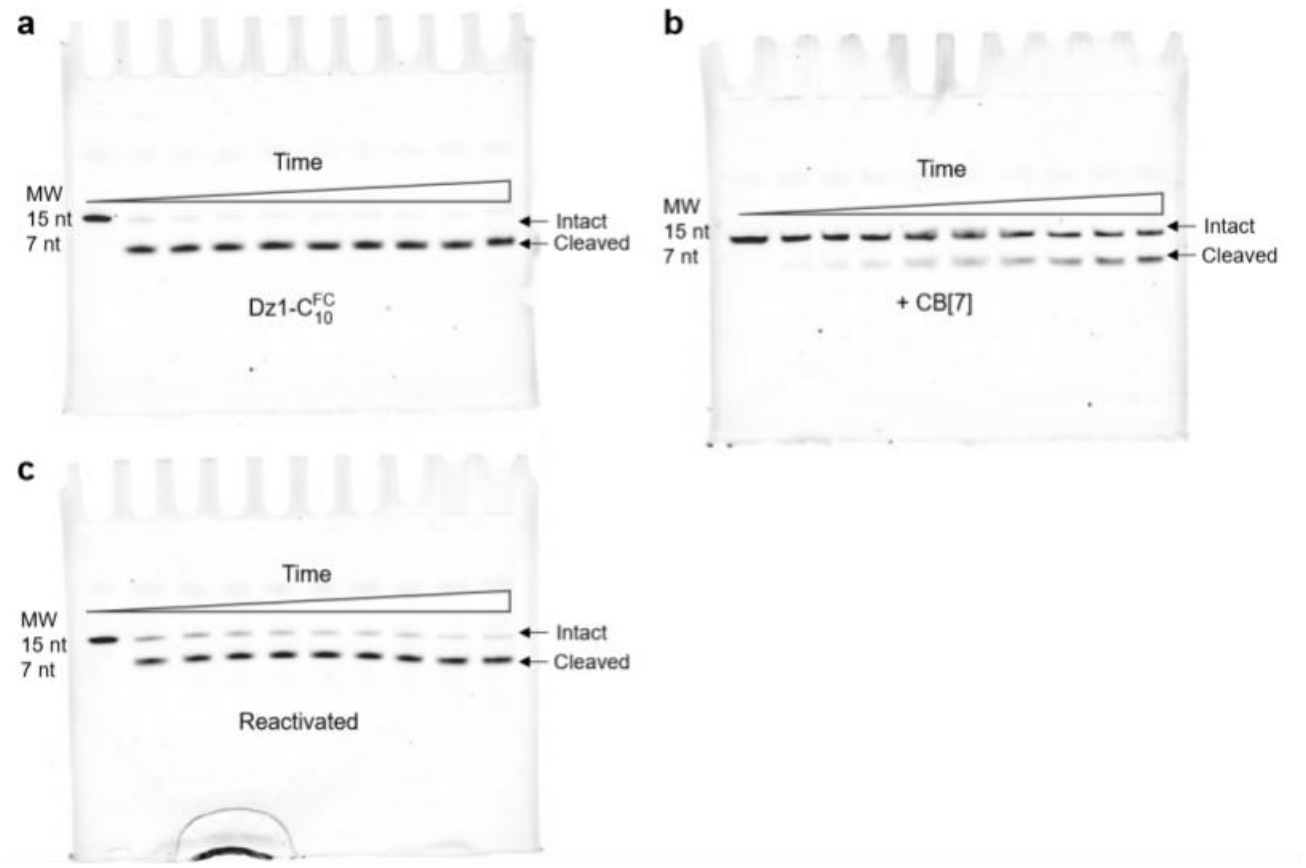
Supplementary Fig. S21. Uncropped gel images for Fig.S4.



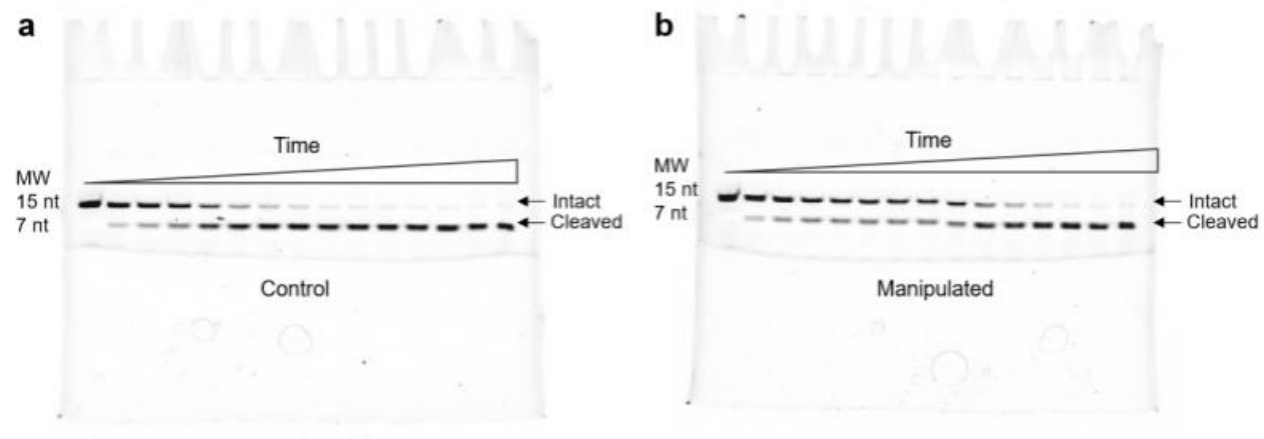
Supplementary Fig. S22. Uncropped gel images for Fig. S5.



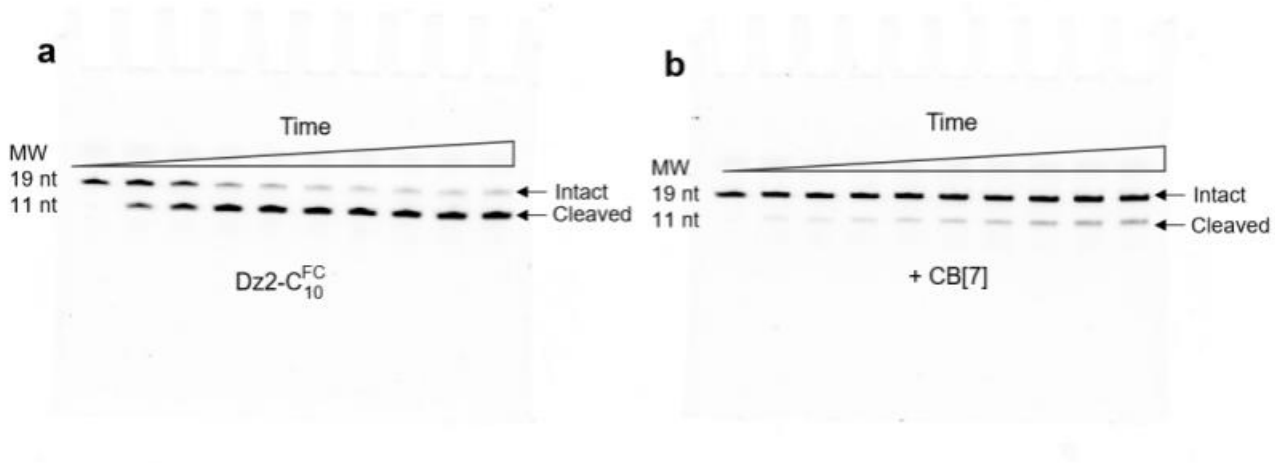
Supplementary Fig. S23. Uncropped gel images for Fig. S6



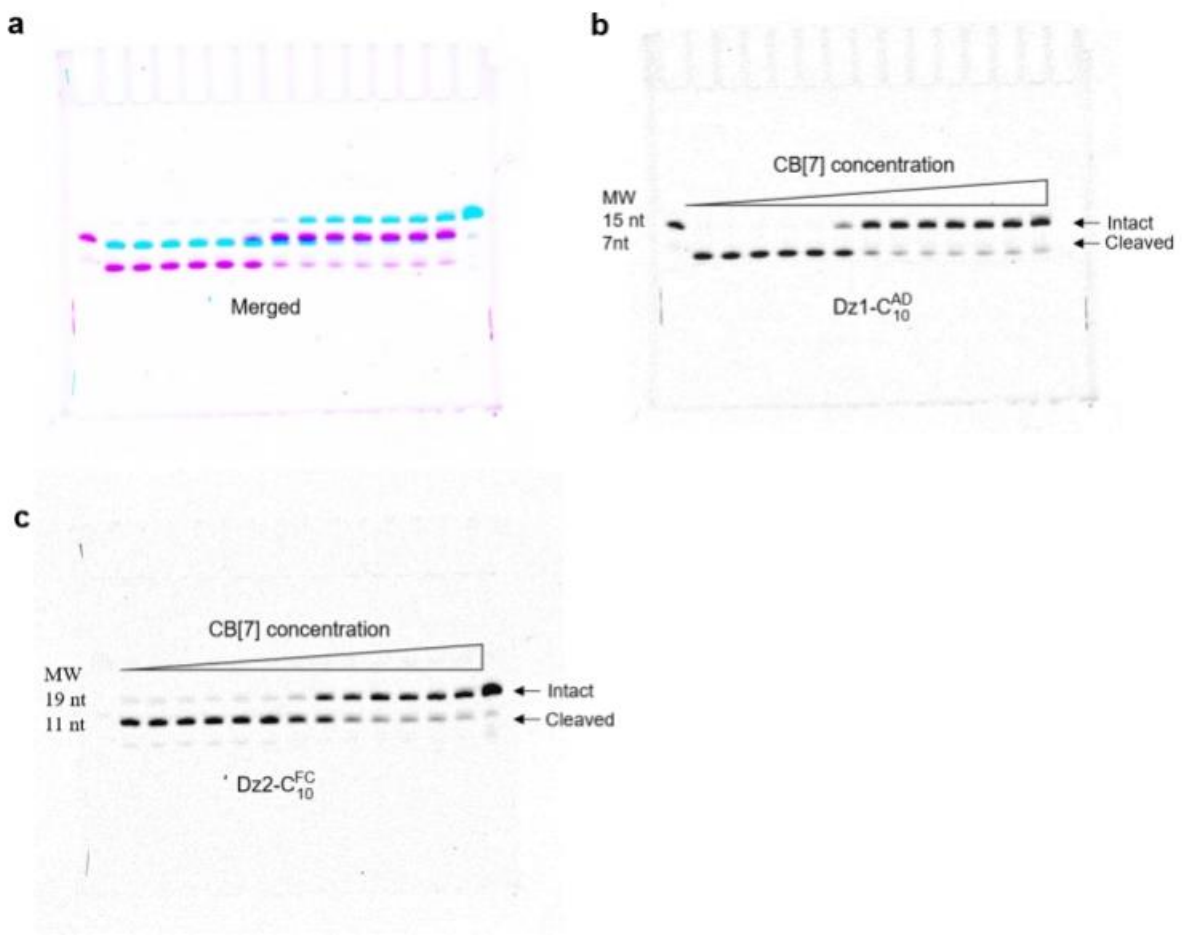
Supplementary Fig. S24. Uncropped gel images for Fig. S7.



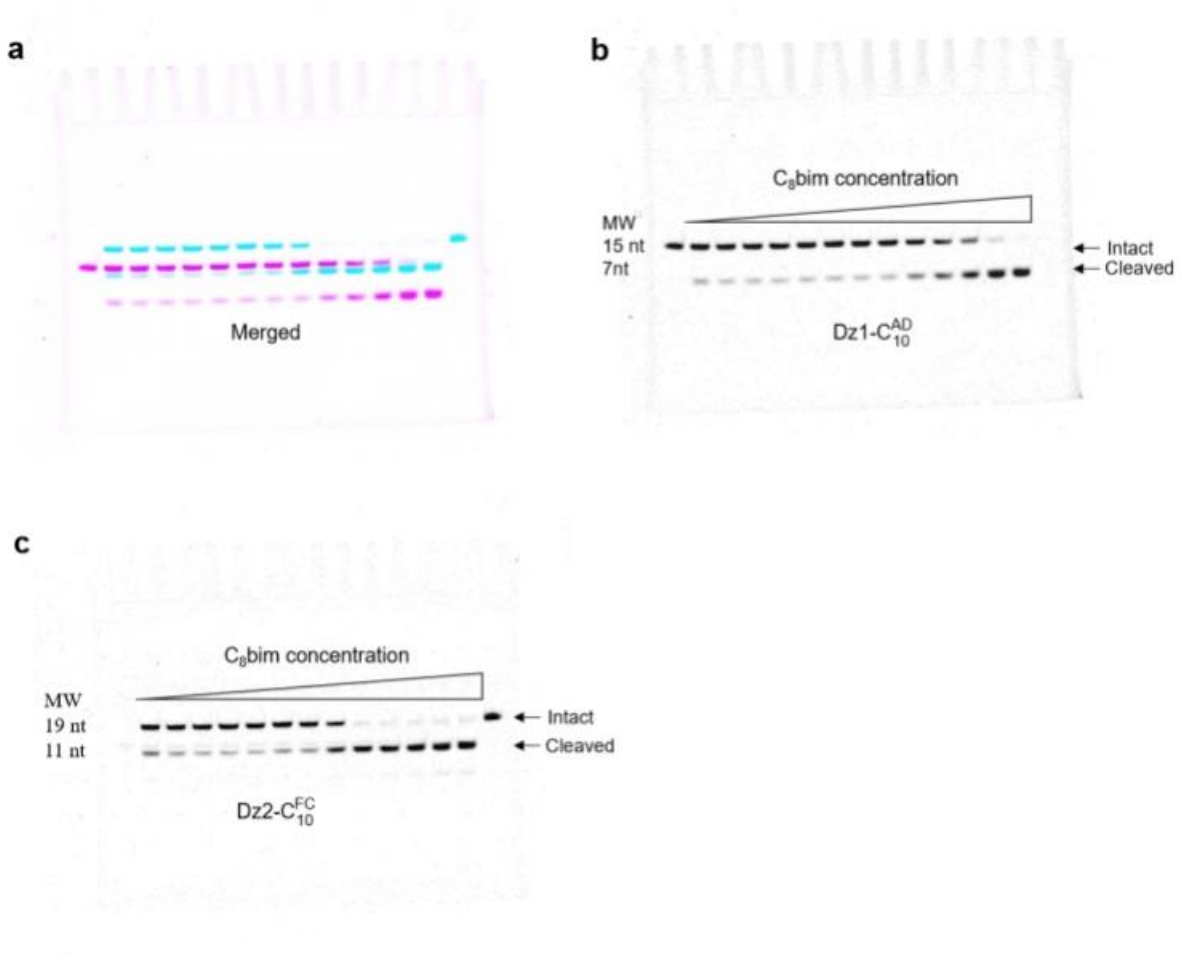
Supplementary Fig. S25. Uncropped gel images for Fig. S8.



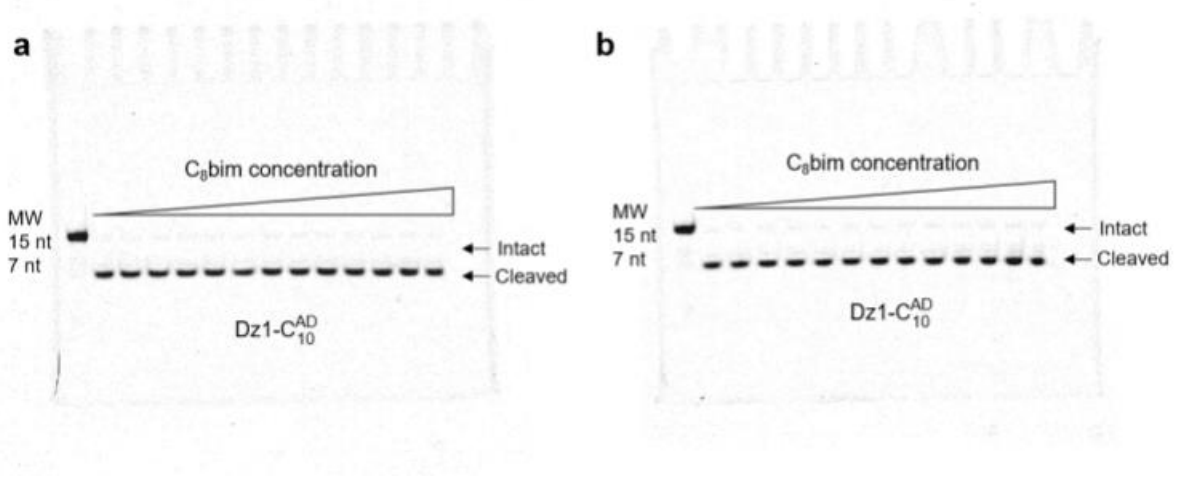
Supplementary Fig. S26. Uncropped gel images for Fig. S9.



Supplementary Fig. S27. Uncropped gel images for Fig. S10.



Supplementary Fig. S28. Uncropped gel images for Fig. S11.



Supplementary Fig. S29. Uncropped gel images for Fig. S12.

Supplementary References

1. Schäfer, F., Joshi, K. B., Fichte, M. A., Mack, T., Wachtveitl, J., & Heckel, A. , Wavelength-selective uncaging of dA and dC residues. *Organic letters* **2011,** *13* (6), 1450-1453.

2. Bae, S., & Lakshman, M. K. , O 6-(Benzotriazol-1-yl) inosine derivatives: easily synthesized, reactive nucleosides. *Journal of the American Chemical Society* **2007,** *129* (4), 782-789.

3. Xiao, L.; Wang, L. L.; Wu, C. Q.; Li, H.; Zhang, Q. L.; Wang, Y.; Xu, L., Controllable DNA hybridization by host-guest complexation-mediated ligand invasion. *Nat Commun* **2022,** *13* (1), 5936.

4. Zhao, N.; Lloyd, G. O.; Scherman, O. A., Monofunctionalised cucurbit[6]uril synthesis using imidazolium host-guest complexation. *Chem Commun (Camb)* **2012,** *48* (25), 3070-2.