Supporting Information

Conditional Deoxyribozyme-Nanoparticle Conjugates for miRNA-Triggered Gene Regulation

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Note 1. Customized algorithm for prediction of mouse TNFα DNAzymes

1. **Selection of DNAzyme binding sites:** Binding sites were selected using a software package custom written in MATLAB 2017a or later (Supplementary Software 1). The algorithm is illustrated in Figure S1a. First, the RNA sequence of interest (in this case, NM_000594.3 (human) or NM_001278601.1 (mouse)) was scanned from 5' to 3' for AU or GU junctions. At each AU or GU junction, the "target base" was defined as either the A or the G. Next, the free energy of hybridization (ΔG_{hyb}) of a 5-base sequence immediately 5' to the target base (but not including the target base) was calculated according to the nearest-neighbor model presented by Sugimoto et al.[1] using MATLAB's oligprops() function and T=37 °C and [salt]=0.015 M. The sequence length was increased incrementally until the calculated ΔG was below -10 kcal/mol, and all sequence lengths with $-8\,kcal/mol > \Delta G_{hyb} > -10\,kcal/mol$ were stored as potential "left arm" DNAzyme binding candidates. A similar process was then repeated to determine "right arm" binding candidates by evaluating the sequence immediately 3' to the target base. If both left arm and right arm binding candidates were found, then all combinations of left and right arms were concatenated (with the target base included between the two arms) and stored as potential DNAzyme target sequences.
2. **Ranking of DNAzyme binding sites:** DNAzyme target sequences were ranked by the free energy of their most stable secondary structure (ΔG_{2° calculated using the rnafold() function in MATLAB with all default settings), which serves as an estimate for the target sequence's expected availability to DNAzyme binding. Target sequences with the same ΔG_{2° were then ranked based on the location of the target base as measured from the 5' end. Each target sequence was then converted to a DNAzyme sequence by taking the complement and replacing the target base with the sequence of the DNAzyme core. A ranked list of all DNAzyme sequences including human and mouse gene target base locations, left and right arm lengths, and ΔG_{2° was then stored to a .xlsx file (Supplementary Spreadsheet 1) for distribution.

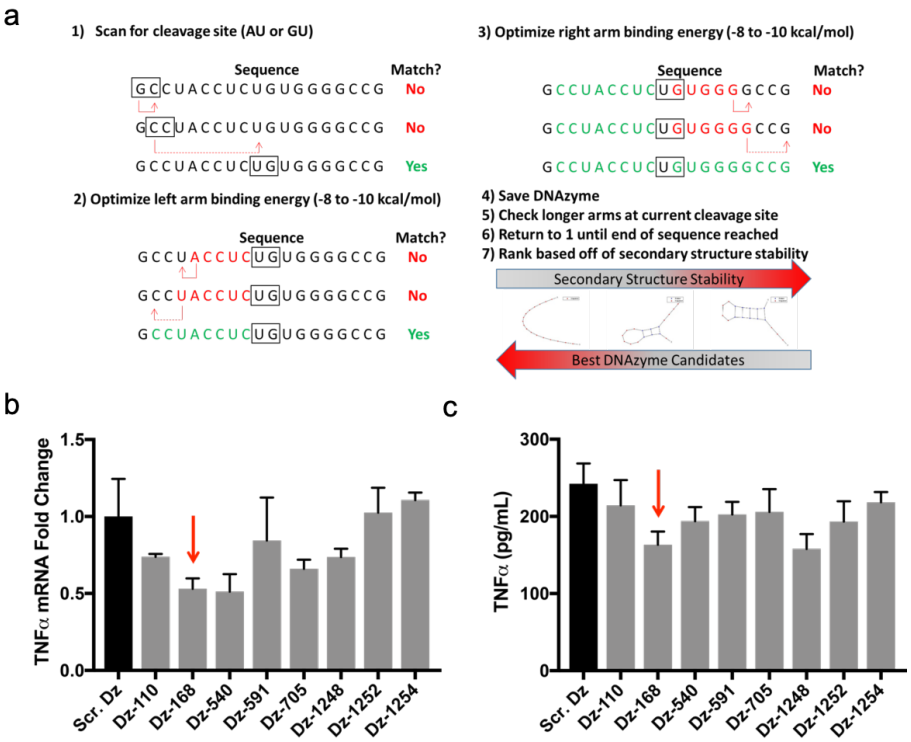

Figure S1. Screen of a small library of DNAzymes that target mouse TNFα in RAW264.7 cells. (a) Schematic describing the computational DNAzyme selection process. First, the mRNA sequence is scanned for target sites and, at each target site, arm length is tuned until each arm has a free energy of hybridization within a specified range. Target sequences are then ranked by secondary structure free energy and stored. This process is repeated for the entire mRNA sequence and multiple DNAzymes can be obtained for a given target site. (b, c) RAW264.7 cells were transfected with 200 nM of each DNAzyme using Oligofectamine, and incubated for 24 h. A non-specific DNAzyme (NS Dz) was included as a negative control. The standard manufacturer recommended concentration of Oligofectamine was used for this screen (2 μL/well for 24 well plate). Each well was plated with ~100,000 cells. (b) RNA was isolated using RNease kit (QIAGEN #74104), and TNFα mRNA levels were quantified by qRT-PCR. The primers are listed in Table S3. (c) The cell medium was collected for ELISA analysis of secreted TNFα using a commercial ELISA kit (Invitrogen #88-7324-22). Sequences for each DNAzyme are included in Table S2. The error bars represent SEM of triplicate samples. The red arrow corresponds to the DNAzyme used in this work.

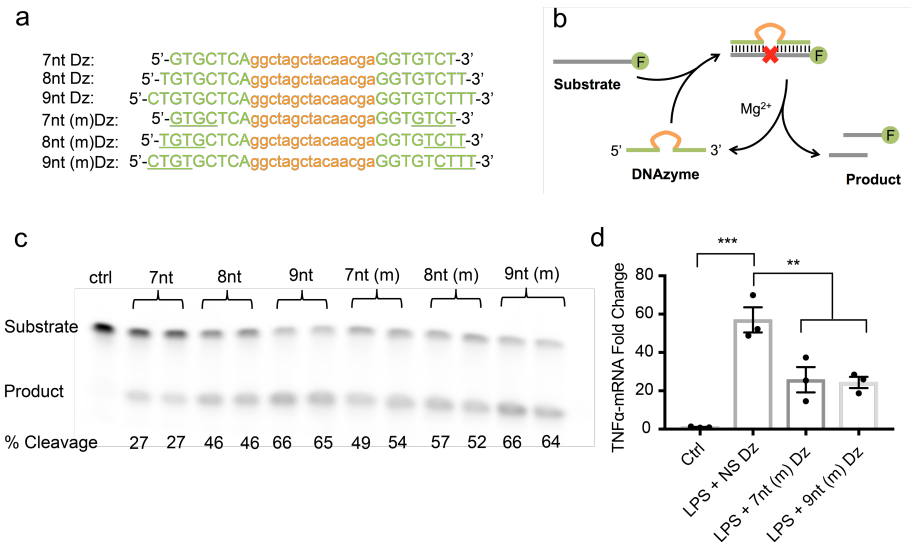

Figure S2. Optimization of TNFα DNAzyme Dz-168. (a) Sequences of mouse TNFα DNAzyme (Dz-168) with different arm lengths and with or without 2'-OMe modifications. The green color corresponds to the left and right arms of the Dz that are complementary to the start codon region of mouse TNFα transcript. The lowercase orange sequence is the 10-23 catalytic domain of the Dz. The underlined nucleotides are 2'-OMe modified. (b) Schematic of substrate cleavage assay used to quantify catalytic activity of DNAzymes. (c) DNAzyme catalyzed multiple turnover of substrate cleavage. 200 nM of DNAzymes were incubated with 1 μM FAM-labeled substrates in 50 mM Tris-HCl supplemented with 150 mM NaCl and 2 mM $MgCl_2$ with pH 7.4. After incubation in a water bath at 37 °C for 2 h 20 min, the reaction mixture was mixed with the same volume of gel loading buffer and subjected to 15% Mini-PROTEAN® TBE-Urea Gel. The gel was run with 170 V in 1× TBE buffer and imaged with an Amersham Typhoon Biomolecular Imager using the FITC channel. The % cleavage values were determined using ImageJ analysis after background subtraction. (d) Plot showing TNFα levels in peritoneal macrophages transfected with non-specific (NS), 7nt(m) and 9nt(m) DNAzyme at a concentration of 1 μM. 24 h after transfection, 0.5 ng/mL lipopolysaccharide (LPS) was added, and cells were incubated for another 4 h before RNA isolation and qPCR analysis of TNFα mRNA level. The error bars represent SEM and each dot represents a biological replicate (**p<0.01, ***p<0.001, one-way ANOVA with Tukey's multiple comparison).

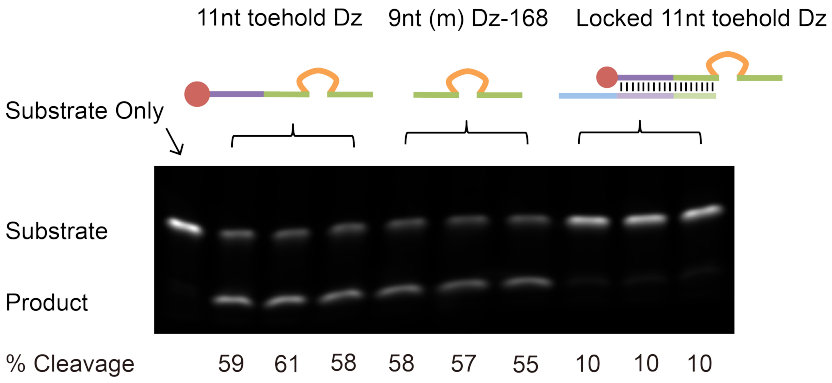

Figure S3. Activity of Dz strand and locked Dz compared to parental Dz-168 (9nt modified arms). To quantify Dz activity, 200 nM 11nt toehold Dz, parental Dz-168, and locked 11 nt toehold Dz were incubated with 1 μM FAM-labeled substrate in 50 mM Tris-HCl supplemented with 150 mM NaCl and 2 mM $MgCl_2$ with pH 7.4 at 37 °C for 3 h. The reaction mixture was then subjected to 15% Mini-PROTEAN® TBE-Urea Gel. The first lane only includes substrate. The next three lanes were replicates of the 11 nt toehold Dz, followed by three replicates of the parental Dz-168, and finally by the 11 nt toehold Dz hybridized to the lock strand. The % cleavage values were determined using ImageJ analysis after background subtraction. The sequences are shown in Table S2.

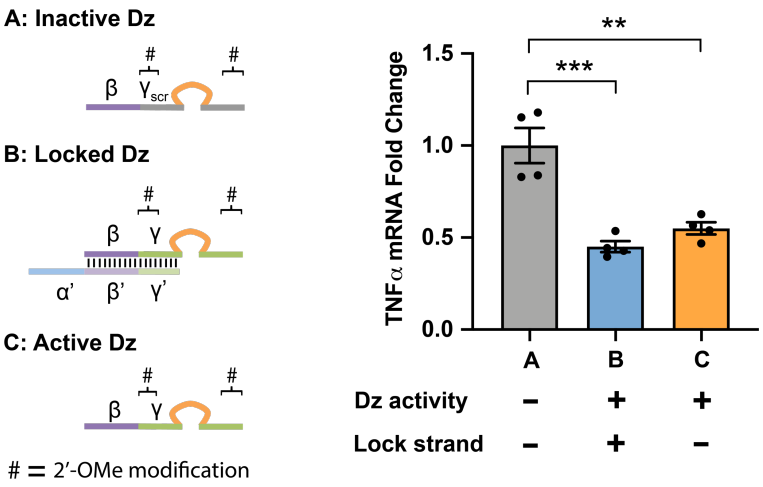

Figure S4. Dz locking is inefficient with unmodified toehold and branch migration domains *in vitro*. RAW264.7 cells were transfected with inactive Dz (A), locked Dz with toehold (B) and unlocked Dz (C) using Oligofectamine and incubated for 24 h. TNFα mRNA level was quantified by qRT-PCR. Dz activity: "-" indicates Dz with scrambled Dz binding arms, "+" indicates Dz with TNFα mRNA complementary binding arms; Lock strand: "-" indicates that the Dz is not hybridized to the lock strand, "+" indicates that the Dz is hybridized to the lock strand. The error bars represent SEM of biological replicates (**p <0.01, ***p <0.001, one-way ANOVA compared to A with Tukey's multiple comparison).

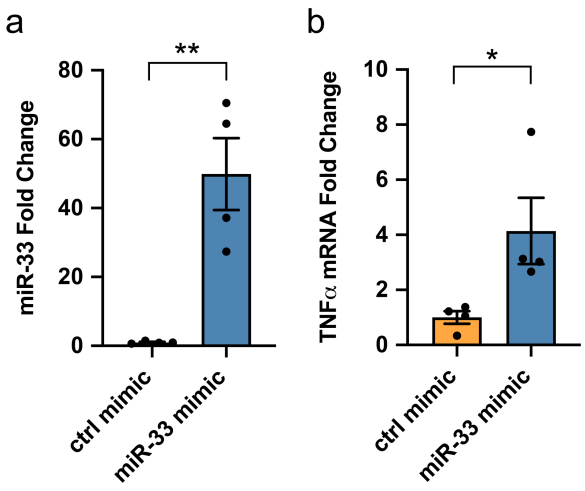

Figure S5. Exogenously transfected miR-33 upregulates TNFα expression. RAW264.7 cells were transfected with 200 nM *mir*Vana™ miR-33 mimic (#4464066) or *mir*Vana™ negative ctrl mimic (#4464058) using Oligofectamine. 24 h later, cells were transfected with 200 nM locked Dz, and incubated for another 24h before RNA isolation and qRT-PCR analysis of (a) miR-33 and (b) TNFα mRNA. The error bars represent SEM for biological replicates (*p <0.05, **p <0.01, two-tailed t test).

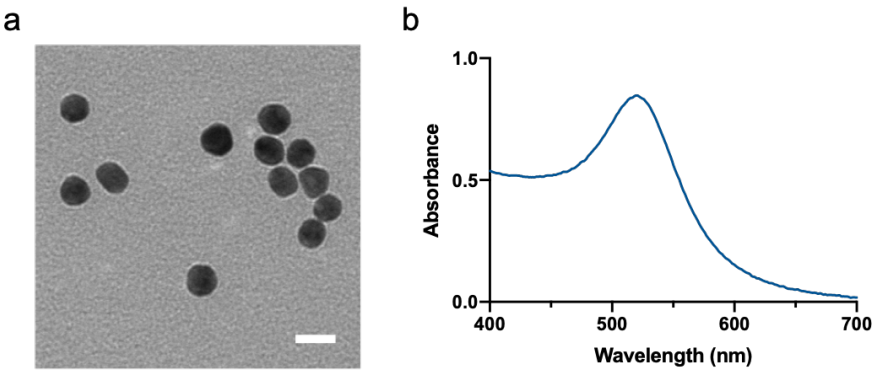

Figure S6. Characterization of AuNPs. (a) Representative TEM image (scale bar=20 nm), and (b) absorption spectrum of AuNPs used in this work.

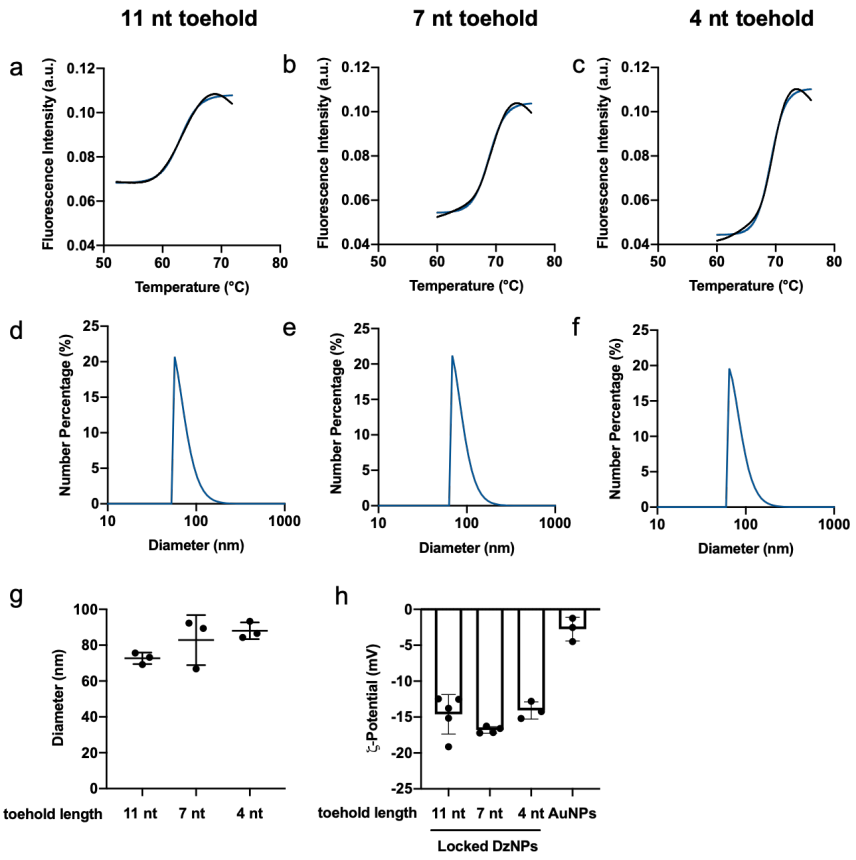

Figure S7. Characterization of locked DzNPs with different toehold length. (a-c) Representative melting curves of locked DzNPs with (a) 11nt, (b) 7nt and (c) 4nt toehold. The fluorescence intensity of 5 nM Cy5-labeled locked DzNPs with different toehold length in PBS was measured with a LightCycler® 96 instrument as a function of temperature. The temperature was ramped from 45°C to 95 °C at the rate of 0.04 °C/s, and 25 measurements were performed per °C with an interval of 0.04 °C. The black lines indicate measured data, and the blue lines indicate fitted curves. T_m was determined as the temperature that generates a half-maximal fluorescence increase in the fitted curves. The data shown in Figure 4d was compiled from three independent melts collected for each locked DzNPs. (d-f) Size distribution of locked DzNPs with (d) 11nt, (e) 7nt and (f) 4nt toehold measured by dynamic light scattering (NanoPlus zeta/nano particle analyzer, Particulate Systems). (g) Hydrodynamic diameters of locked DzNPs with different toehold length. (h) ζ-potentials of locked DzNPs with different toehold length, as well as citrate-stabilized AuNPs, measured with the NanoPlus zeta/nano particle analyzer. The error bars represent SD.

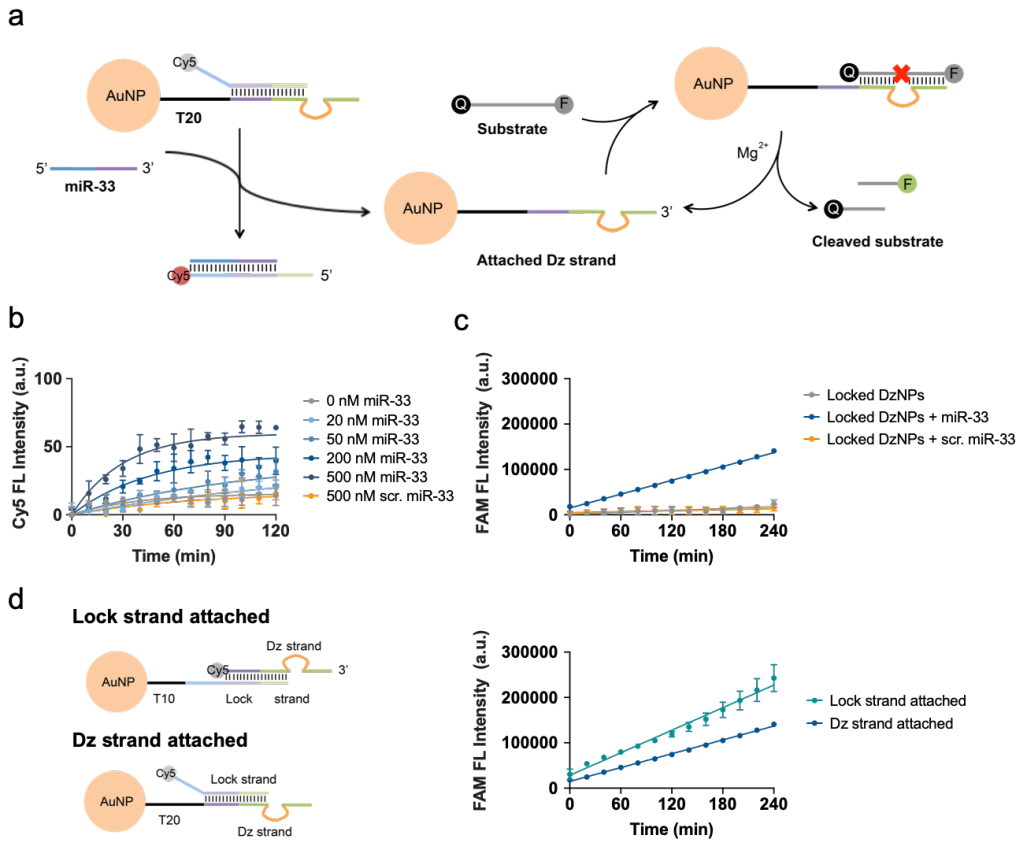

Figure S8. Characterization of DzNPs engineered with an anchored Dz strand. (a) Scheme for Dz strand anchored locked DzNPs. (b) Cy5 fluorescence intensity of 0.5 nM locked DzNPs incubated with different concentrations (0, 5, 20, 50, 200, 500 nM) of miR-33 trigger and 500 nM scrambled miR-33 for 2h. The Cy5 fluorescence intensity quantifies the efficiency of toehold exchange as a function of time. The error bars represent SD of three replicates. (c) 0.5 nM locked DzNPs was pre-incubated with 500 nM miR-33 trigger or scrambled miR-33. After a 1h incubation, 300 nM of fluorophore-quencher tagged substrate was added and the fluorescence of FAM was measured for 4h. (d) Comparison of activities of 0.5 nM locked DzNPs of two configurations incubated with 500 nM miR-33 trigger. The plot shows the average intensity from three replicates, and the error bar represent SD and some of them were too small to show on the plot.

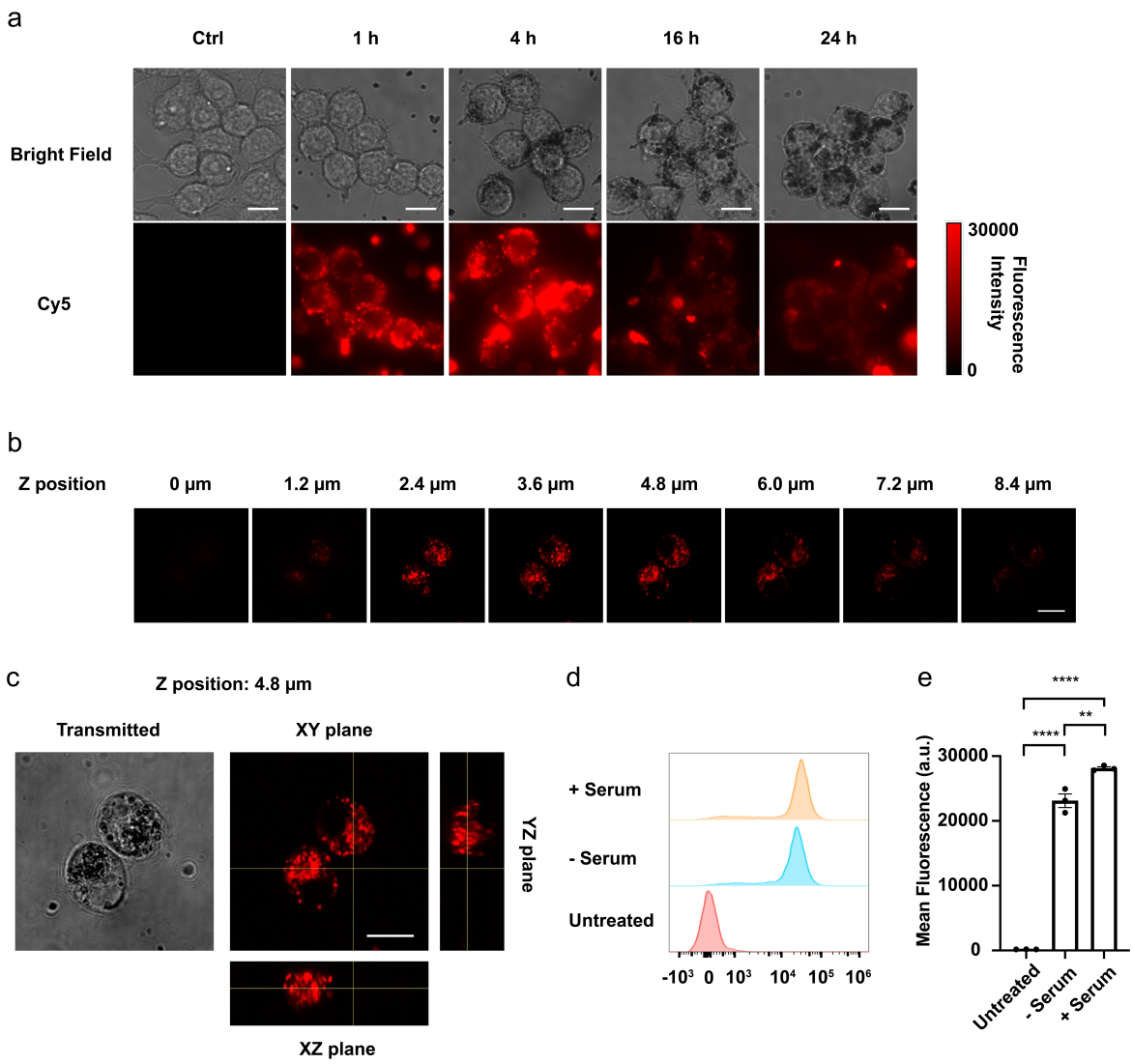

Figure S9. Uptake of locked DzNPs by RAW264.7 cells. (a) 5 nM of 11nt toehold locked DzNPs (with Cy-5 labeled Dz strands) were incubated with RAW264.7 cells for 1h, 4h, 16h and 24h. Cell uptake was assessed with widefield fluorescence imaging in the Cy5 channel. Scale bar represents 10 µm. (b, c) Confocal microscopy images of RAW264.7 cells incubated for 4h with 5 nM 11nt toehold locked DzNPs (with Cy-5 labeled Dz strands). (b) Confocal images at different Z positions and (c) orthogonal view showing XY, YZ, and YZ planes when Z position was set to 4.8 µm. Scale bar represents 10 µm. (d,e) Flow cytometry analysis of RAW264.7 cells incubated for 4h with 5 nM of the 11nt toehold locked DzNPs (with Cy-5 labeled Dz strands) in the presence or absence of serum. Flow cytometry was performed to quantify DzNP uptake. (d) Representative histograms of cell-associated fluorescence and (e) mean fluorescence intensity, for untreated cells and cells that were incubated with DzNPs without (-) or with (+) serum. The error bars represent SEM for biological replicates. Each data point represents the mean fluorescence intensity of 4000 to 13000 cells in one replicate. (**p<0.01, ****p<0.0001, one-way ANOVA with Tukey's multiple comparison).

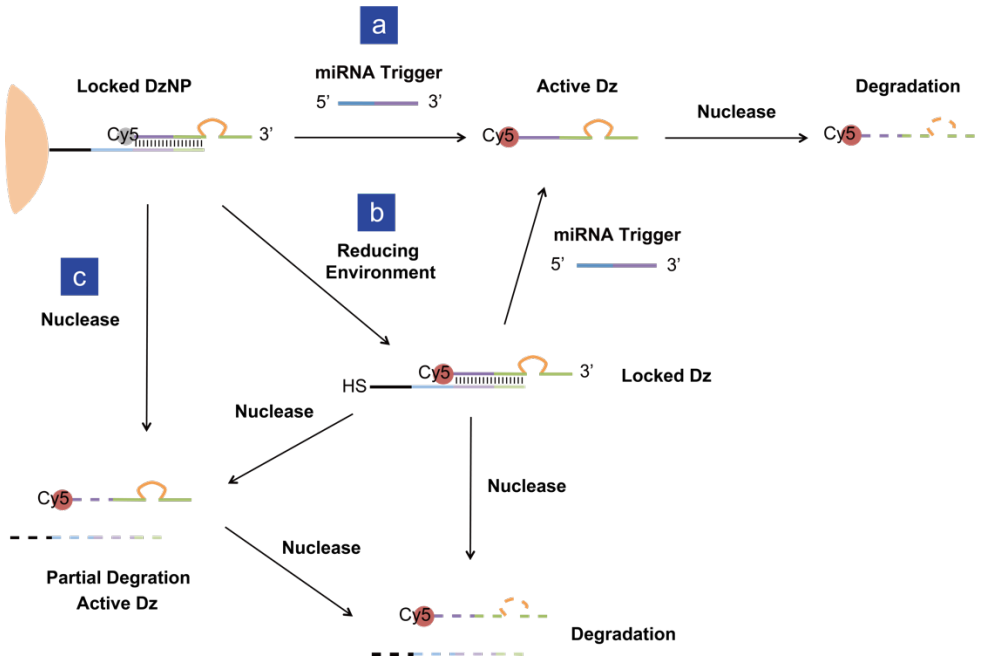

Figure S10. Potential pathways of Dz activation and DNA degradation. (a) Dz strand is displaced from the lock strand while the lock strand is attached on the AuNPs; (b) DNA duplex detachment from the AuNP surface due to thiol-exchange, followed by Dz strand displacement and activation; and (c) Locked Dz is partially degraded and released from AuNP surface, which causes background activation if the Dz is not completely degraded. Eventually, all DNA is degraded and Dz activity is eliminated due to the activity of celluar nucleases.

Table S1. Oligonucleotide sequences

BHQ = Black Hole Quencher; FAM =6-Carboxyfluorescein; m=2'-O-Methyl modification; r=RNA base; /5AmMC6/=5' Amino Modifier C6; /3ThioMC3-D/=3' Thiol Modifier C3 SS; /5ThioMC6-D/=5' Thiol Modifier C6 SS; /3AmMO/=5' Amino Modifier

Name	**Sequence (5'→3')**
miR-33 trigger	GTG CAT TGT AGT TGC ATT GCA
Scrambled miR-33	AGC TTG ATG TTC GTT AGG CAT
7nt mDz	mGmUmG mCTC AGG CTA GCT ACA ACG AGG TmGmU mC mU
8nt mDz	mUmG mUmGC TCA GGC TAG CTA CAA CGA GGT G mUmC mUmU
9nt mDz	mCmUmG mUGC TCA GGC TAG CTA CAA CGA GGT GTmC mUmUmU
Fluorogenic substrate	FAM-AA AGA CAC CrArU GAG CAC AG-BHQ
FAM-labeled substrate	FAM-AA AGA CAC CrArU GAG CAC AG
11nt toehold Dz strand	TTG CAT TGC AmCmU mGmUG CTC AGG CTA GCT ACA ACG AGG TGT mCmUmU mU
Lock strand	GAG CAC AGT GCA ATG CAA CTA CAA TGC AC
Inactive Dz strand	TTG CAT TGC AmGmC mUmGT TAT GGG CTA GCT ACA ACG ATT CCG mUmGmC mU
11nt toehold Dz strand with modified β domain	mUmUmG mCmAmU mUmGmC mAmCmU mGmUG CTC AGG CTA GCT ACA ACG AGG TGT mCmUmU mU
Inactive Dz strand with modified β domain	mUmUmG mCmAmU mUmGmC mAmGmC mUmGT TAT GGG CTA GCT ACA ACG ATT CCG mUmGmC mU
Lock strand with modified α', β' and γ' domains	GAG CmAmC mAmGmU mGmCmA mAmUmG mCmAmA mCmUmA mCmAmA mUmGmC mAmC
11nt toehold inactive Dz strand with amine (for Cy5 labeling)	/5AmMC6/TT GCA TTG CAG CTG TTA TGG GCT AGC TAC AAC GAT TCC GTG CT

Lock strand for inactive Dz strand (for locked inactive DzNPs)	ATA ACA GCT GCA ATG CAA CTA CAA TGC ACT TTT TTT TTT /3ThioMC3-D/
7nt toehold Dz strand with amine (for Cy5 labeling)	/5AmMC6/GT AGT TGC ATT GCA mCmUmG mUGC TCA GGC TAG CTA CAA CGA GGT GTmC mUmUmU
4nt toehold Dz strand with amine (for Cy5 labeling)	/5AmMC6/AT TGT AGT TGC ATT GCA mCmUmG mUGC TCA GGC TAG CTA CAA CGA GGT GTmC mUmUmU
Lock strand for 11nt, 7nt and 4nt Dz strand (for locked DzNPs with toehold)	GAG CAC AGT GCA ATG CAA CTA CAA TGC ACT TTT TTT TTT /3ThioMC3-D/
Lock strand without toehold (for locked DzNPs without toehold)	GAG CAC AGT GCA ATG CAA TTT TTT TTT T/3ThioMC3-D/
Locked strand with scrambled toehold (for locked DzNPs with scrambled toehold)	GAG CAC AGT GCA ATG CAA GTA ACA TCT TTT TTT TTT /3ThioMC3-D/
Dz strand for Dz anchored locked DzNPs	/5ThioMC6-D/TT TTT TTT TTT TTT TTT TTT TTG CAT TGC AmCmU mGmUG CTC AGG CTA GCT ACA ACG AGG TGT mCmUmU mU
Lock strand for Dz anchored locked DzNPs with amine (for Cy5 labeling)	GAG CAC AGT GCA ATG CAA CTA CAA TGC AC/3AmMO/

Table S2. Library of mouse TNFα DNAzymes screened

m=2'-O-Methyl modification; 3InvdT=3' Inverted dT

Name	**Sequence (5'→3')**
NS Dz	TCA AGG GAG GCT AGC TAC AAC GAA AGA AGC GG/3InvdT/
Dz110	mGmGmG mACA GAA GGC TAG CTA CAA CGA CTG CmCmU mGmG
Dz168	mUmGmU mGCT CAG GCT AGC TAC AAC GAG GTG TmCmU mUmU
Dz540	mUmGmA mAGA GAA GGC TAG CTA CAA CGA CTG GGmA mGmUmA
Dz591	mCmGmG mCTG AGG CTA GCT ACA ACG AGG TGmU mGmGmG
Dz705	mCmCmA mGGT ATA GGC TAG CTA CAA CGA GGG CTmC mAmUmA
Dz1248	mCmAmA mATA TAA AGG CTA GCT ACA ACG AAG AGmG mGmGmG
Dz1252	mGmUmG mCAA ATA GGC TAG CTA CAA CGA AAA TAG mAmGmG mG
Dz1254	mAmAmG mUGC AAA GGC TAG CTA CAA CGA ATA AAT AmGmA mGmG

Table S3. Primer sequences

Primer	**Sequence (5'→3')**
TNFα_Forward	CCAGAACATCTTGGAAATAGCTC
TNFα_Reverse	GGACCGATCACCCCGAAGT
18s_Forward	AGGAATTGACGGAAGGGCACCA
18s_Reverse	GTGCAGCCCCGGACATCTAAG

Reference

1. Sugimoto, N.; Nakano, S. I.; Yoneyama, M.; Honda, K. I. Improved Thermodynamic Parameters and Helix Initiation Factor to Predict Stability of DNA Duplexes. *Nucleic Acids Research* **1996,** *24* (22), 4501-4505.