

Supporting Information for:

Characterizing the conformational free-energy landscape of RNA stem-loops using single-molecule field-effect transistors

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IV. References

I. Materials and Methods

All reagents and solvents were purchased from Sigma-Aldrich, Fisher Scientific, or VWR unless otherwise stated. Buffers were prepared with distilled water that was further purified using a Barnstead™ Genpure™ Pro Water Purification System. RNA and DNA oligos (Tables S1 and S2) were purchased from Integrated DNA Technologies (IDT).

A. *smFET device fabrication and characterization*

smFET devices were fabricated according to previously described methods.¹⁻² Carbon nanotubes (CNTs) were grown *via* chemical vapor deposition on $1 \times 1 \text{ cm}^2$ silicon (Si)/silicon dioxide (SiO_2) substrates. Si wafers covered with a 285 nm-high layer of SiO_2 were purchased from Nova Electronic Materials. Pure anhydrous ethyl alcohol with a purity of $\geq 99.5\%$ was used as the carbon source and iron particles obtained from commercially purchased horse-spleen ferritin were used as a growth catalyst. CNT growths were verified with a Zeiss Sigma VP Scanning Electron Microscope (SEM). Each Si/ SiO_2 substrate was patterned with sixty-four pairs of titanium source-drain electrodes, where each electrode was 15 μm wide with a source-drain separation of 2 μm . Patterns were written using Nanobeam nB4 Electron Beam Lithography on a spin-coated bilayer of poly(methyl methacrylate) (PMMA) purchased from MicroChem (molecular weight 495K with 4% anisole, followed by 950K with 6% anisole). Metal deposition was then achieved *via* Angstrom UHV E-beam Deposition. An additional lithography and deposition cycle were used to deposit a platinum pseudo-reference electrode on the top and bottom of each substrate and an additional layer of platinum on the end of each titanium electrode (serving as the bond pad for later wire-bonding). The locations of the CNTs were then mapped with respect to the deposited metal using SEM (Figure S1a) and characterized with AFM (Bruker Dimension Icon AFM) (Figure S1b) and Raman microscopy (Renishaw InVia Micro-Raman). One CNT per substrate was selected according to its length, location, and Raman characteristics, prioritizing SWCNTs with no noticeable defects or visible residue under AFM. SWCNTs were confirmed by the characteristics of the tangential mode G-band peaks as well as the number of radial breathing mode (RBM) peaks, and defects were identified by the presence of D-band peaks (Figure S1c-d). The removal of the remaining CNTs, as well as the isolation of the selected SWCNT into sixty-four individual devices, was achieved using oxygen (O_2) plasma reactive-ion etching (RIE, Plasma Etch) of CNTs exposed *via* electron beam lithography patterning on a single layer of PMMA. Isolation was also confirmed by SEM. The isolated devices were then screened by measuring the current-voltage (I-V) characteristics in air using a semiconductor parameter analyzer (Agilent 4155C). For each device, the I-V curve was obtained by applying a constant source-drain voltages (V_{ds}) and a sweeping back-gate voltage (V_{bg}) from -10 V to $+10 \text{ V}$ to the Si/ SiO_2 substrate (Figure S1e).

B. *Characterizing and optimizing the number of nucleic acid molecules tethered to an smFET device*

The general framework for the non-covalent tethering protocols were adapted based on previously described methods.³⁻⁵

Validating the specific tethering of pyrene onto SWCNTs

To test the specificity of pyrene tethering to the SWCNT, amine-functionalized, gold nanoparticles purchased from Cytodiagnostics were reacted with pyrene-NHS and tethered to the SWCNT (Figure S3). For the control experiment, the same steps were followed with pyrene-COOH in place of pyrene-NHS. To achieve tethering, the devices were immersed for one hour in a solution of 1 mM pyrene-NHS prepared in methanol. After the incubation, the devices were rinsed with 0.1 %

v/v Tween-20 in methanol to remove excess pyrene in solution and from the chip surface. Immediately following the rinsing steps, the devices were immersed in 200 nM amine-functionalized 10 nm gold nanoparticles in Functionalization Buffer (10 mM sodium hydride (NaH₂)/disodium phosphate (Na₂HPO₄) and 100 mM sodium chloride (NaCl), pH = 8.4, and 0.025 % v/v Tween-20) for 90 minutes. The devices were then thoroughly rinsed in water and dried for AFM imaging. AFM measurements for these experiments were carried out in scanAsyst mode using a Bruker Dimension Icon AFM and commercially available scanAsyst-air tips (70 KHz resonance frequency, Bruker).

Purification and characterization of EcoRI^{E111Q}

Constructs encoding the hydrolytically defective EcoRI^{E111Q} were purchased from Addgene (Dr. Paul Modrich, HHMI & Duke University).⁶⁻⁷ For overexpression and purification, the gene encoding EcoRI^{E111Q} was sub-cloned into the pET28a(+) vector, which introduces a six-histidine (6×His) affinity tag followed by a highly specific tobacco etch virus (TEV) protease cleavage site at the amino (N) terminus of the EcoRI^{E111Q}. The plasmid was transformed into BL21 RIPL cells. 4.5 L of a cell culture was grown to an optical density at 600 nm (OD₆₀₀) of ~0.6 in the presence of 50 µg/ml kanamycin and cells were induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM, collected 4 hours after induction, resuspended in 30 mL of Resuspension Buffer (50 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl), pH at 25 °C (pH_{25°C}) = 7.5; 1 cComplete™, EDTA-free Protease Inhibitor Cocktail Tablet (Sigma-Aldrich); and 10% sucrose), and frozen in liquid nitrogen. The frozen cells were then lysed using a SPEX SamplePrep 6875D Freezer/Mill Dual Chamber Cryogenic Grinder and the resulting lysate was diluted by the addition of 100 mL of Wash Buffer 1 (20 mM Tris-HCl, pH_{25°C} = 7.5, and 0.5 M NaCl). The lysate was clarified by high-speed centrifugation in a Beckman Avanti J-E Centrifuge using a JA-17 rotor spinning at 16500 rpm for 30 min and subsequently loaded onto a column containing 4 mL of nickel (Ni²⁺)-nitrilotriacetic acid (NTA) agarose resin. The column was washed with 20 column volumes of Wash Buffer 1 supplemented with 20 mM imidazole. Column-bound EcoRI^{E111Q} was subsequently eluted in 15 mL of Washing Buffer 1 that had been supplemented with 300 mM imidazole. Subsequently, the N-terminal 6×His tag was proteolytically cleaved by adding TEV protease to a final concentration of 0.75 µM to the eluted EcoRI^{E111Q} and dialyzing the cleavage reaction against 2 L of Cleavage Buffer (20 mM Tris-HCl, pH_{25°C} = 7.5; 0.3 M NaCl; and 2 mM βME) at 4°C. After overnight dialysis, TEV-protease-cleaved EcoRI^{E111Q} was flowed through the nickel column once more supplemented with 20 mM imidazole. Further purification of the TEV-protease-cleaved EcoRI^{E111Q} lacking the 6×His tag was achieved by loading it onto a HiTrap Q HP anion exchange column (Cytiva) and eluting it using Anion Exchange Buffer (20 mM Tris-HCl, pH_{25°C} = 7.5, and 0.15 M NaCl) at 4°C. The resulting 15 mL of anion exchange-purified EcoRI^{E111Q} was dialyzed against 2 L of Storage Buffer (40 mM Tris-HCl, pH_{25°C} = 7.5; 300 mM NaCl; 10 mM βME; 0.1 mM ethylenediaminetetraacetic acid (EDTA); 0.15% Triton-X; and 15% glycerol) overnight at 4°C. The resulting 20 mL of dialyzed EcoRI^{E111Q} was then pipetted into 500 µL aliquots and stored at -80 °C until further use. The monomeric concentration of the final, purified EcoRI^{E111Q} was 1.8 µM, as quantified by generating a bovine serum albumin (BSA) concentration standard curve on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and comparing the total intensity of the EcoRI^{E111Q} gel band to the total intensities of the BSA standards using ImageJ software. Finally, the ability of our purified EcoRI^{E111Q} to actively bind to its recognition site on double-stranded DNA was characterized using a previously described EcoRI digestion protection assay.⁸⁻⁹ The results of this assay demonstrate that binding of our purified EcoRI^{E111Q} to λ-DNA protects the 5 EcoRI recognition sites located in the λ-DNA from being recognized, bound, and nucleolytically cleaved into 6 λ-DNA fragments of

21.2, 7.4, 5.8, 5.6, 4.9, and 3.5 kilobases (kbs) by wild-type EcoRI (wtEcoRI) purchased from New England Biolabs (NEB).

Tethering DNA_T to the pyrene-derivatized, SWCNT surface of smFET devices

Prior to tethering DNA_T to the smFET devices, a solution of 6 mM pyrene-NHS was prepared in *N,N*-dimethyl formamide (DMF) and a 25 µL aliquot of the pyrene-NHS solution was pulled through the flow cell to soak the devices for one hour. After the incubation, the flow cell was rinsed with two separate 25 µL aliquots of DMF, followed by 150 µL of 0.1% v/v Tween-20 in ethanol to remove the excess pyrene-NHS solution from the flow cell. Immediately following the rinsing steps, the devices were soaked in 50 µM DNA_T in Functionalization Buffer for 90 minutes. From 5' to 3', DNA_T contains a primary amine that is capable of forming an amide bond to pyrene-NHS via an NHS ester-activated, cross-linking reaction; a six-carbon linker; a five-nucleotide spacer; three consecutive EcoRI^{E111Q} recognition sites, each separated from the other by 10 nucleotides; and a final, five-nucleotide 3'-terminal sequence (Table S2).

The devices were then rinsed with two separate volumes of 150 µL of Wash Buffer 2 (10 mM NaH₂PO₄/Na₂HPO₄ and 100 mM NaCl, pH = 7.0, and 0.05% v/v Tween-20). After conjugation of DNA_T to the pyrene-derivatized SWCNT surface, the devices were soaked in 100 nM DNA_C in Recording Buffer (10 mM NaH₂PO₄/Na₂HPO₄ and 100 mM NaCl, pH = 7.0) for 30 min to preassemble a DNA duplex. The devices were then incubated in 10 nM DNA_C with 40 nM EcoRI^{E111Q} dimer for 30 minutes in Binding Buffer (10 mM Tris-HCl, pH_{25°C} = 8.0; 150 mM NaCl; and 10 mM MgCl₂) to form EcoRI^{E111Q}-dimer-bound DNA complexes, with up to three EcoRI^{E111Q} dimers binding to each DNA duplex. Finally, the surface of the SWCNT was characterized using an AFM to monitor the formation of EcoRI^{E111Q}-dimer-bound DNA complexes tethered to the SWCNTs. AFM measurements were carried out in the presence of 10 nM DNA_C and 40 nM EcoRI^{E111Q} dimer in Binding Buffer. A Bruker Dimension Fastscan AFM was used in scanAsyst mode with commercially available Fastscan-C tips (300 KHz resonance frequency, Bruker).

Poisson distribution analysis of DNA_T tethering

Each of 32 AFM images was collected by scanning a 4 × 4 µm² area. The majority of the images included either one or zero triple-EcoRI^{E111Q}-dimer-bound DNA complex per 2 µm length of CNT, presumably reflecting the tethering of one or zero DNA_Ts to the pyrene-derivatized, SWCNT surface of an smFET device. To calculate the probability of tethering any of various numbers of DNA_Ts to an smFET device, the Poisson distribution of DNA_T tethering to the smFET device was defined as

$$P(N = k) = \frac{\lambda^k e^{-\lambda}}{k!}, \quad \text{Eq. 1}$$

where N is the discrete random variable, in this case it is the number of tethered DNA_Ts per smFET device; k is the number of tethered DNA_Ts per smFET device whose probability of occurring is being calculated; λ is the expected value of N , given by dividing the length of the smFET device by the observed average distance between tethered DNA_Ts ($\lambda = 2 \mu\text{m}/5.79 \mu\text{m} = 0.345$); and e is Euler's number, a constant equal to 2.71828.

C. Design rationale of stem-loop constructs

Originally, we selected a pair of RNA stem-loops extensively studied by Tinoco and co-workers¹⁰⁻¹² as the target constructs for our smFET studies. The structural and thermodynamic

properties of these two stem-loops, which were composed of the same four base-pair stem and either a UUCG or a UACG tetraloop sequence, have been very well-characterized. We were ultimately unable to use these original constructs by Tinoco and co-workers, however, as the results of our initial smFET experiments and technical limitations on the highest temperatures we could achieve on our laboratory-built smFET instrument necessitated a change in the design of our constructs so that they would have T_m s within the temperature range accessible to our instrument. At this point in our study, we designed a new pair of stem-loop constructs based on the UUCG stem-loop investigated by Tinoco and co-workers in which we introduced a mismatched base-pair at one of two positions in the four base-pair stem. As predicted, ensemble UV melting experiments demonstrated that these new constructs had T_m s within the range of temperatures accessible to our smFET instrument. An alternative approach would have been to use a pair of constructs selected from a set of stem-loops that have been very well-studied by Bevilacqua, Gruebele, and co-workers¹³⁻¹⁴ and that are lower- T_m variants of the UUCG stem-loops originally investigated by Tinoco and co-workers. Unfortunately, we did not become aware of the constructs studied by Bevilacqua, Gruebele, and co-workers until much later in our study, once we had already collected and analyzed all of the data, obtained the thermodynamic and kinetic parameters, and were ready to make comparisons to the thermodynamic and kinetic parameters reported in previous studies of stem-loop folding, particularly those reported in T-jump experiments reporting multiphasic kinetics (i.e., the studies by Bevilacqua, Gruebele, and co-workers¹³⁻¹⁶)."

D. UV melting experiments on stem-loop constructs

UV melting experiments were carried out using a Chirascan V100 Spectrometer. For each UV melting experiment, a 10 μ M sample of the stem-loop was prepared in Recording Buffer. Each sample was pre-heated to 95 °C, followed by gradual cooling to 5 °C before each experiment. UV absorbance was recorded at 280 nm from 5 °C to 95 °C with a ramp rate of 1 °C min⁻¹, and data collected every 0.2 °C. Thermodynamic parameters were determined by nonlinear curve fitting a set of parametric equations for a two-state, unimolecular transition¹⁴ using *cftool* in Matlab.

E. smFET experiments on stem-loop constructs

Our smFET experimental platform, including the details of electrical measurements and temperature controls, has been described in several previous articles.^{1-2, 17} For each smFET experiment, the flow cell was initially flushed with Recording Buffer and a current-voltage characteristic (I-V) curve was collected from -0.4 V to +0.3 V along with a real-time recording at a gate voltage of -0.3 V and a source-drain voltage of either 25 mV or 100 mV. After these electrical measurements, the gate bias and source-drain voltage were set to zero and the flow cell was rinsed with water followed by DMF. To tether the C4MM or C2MM stem-loop constructs (Table S1) to the smFET devices under conditions in which an smFET device containing a tethered stem-loop had an ~84 % probability of having a single tethered stem-loop, we used our optimized DNA_T tethering conditions, determined as described in Section I.B., to tether the stem-loops to the smFET devices. RNA oligos for smFET experiments were not exposed to UV light or thermal treatment to prevent covalent modifications/crosslinking of the RNA that can manifest as kinetic heterogeneity¹⁸. Disparate levels of such heterogeneities across different studies could differentially affect kinetic measurements and lead to discrepancies in reported rates of RNA (un)folding.

After the stem-loop construct to be studied was tethered and the flow cell was rinsed with Wash Buffer 2, the flow cell was further rinsed in Recording Buffer and maintained in this buffer during

recordings. Current versus time trajectories were recorded at T_{low} (i.e., room temperature, or 23–24 °C) at a time resolution of 200 μs for 10 minutes at a gate voltage of -0.3 V and a source-drain voltage of either 25 mV or 100 mV. Following the recordings obtained at T_{low} under these conditions, additional 10-minute recordings at T_{high} (44–45 °C), but otherwise under the same conditions, were obtained by turning on the fan inside the heating chamber and adjusting the voltage of the heat capacitor until achieving T_{high} (Figures S2, 3).

F. smFET data analysis

The low-frequency ' $1/f$ ' noise inherent to smFET devices results in a 'wandering' baseline current^{2, 17} that makes it generally difficult to apply a thresholding algorithm or a hidden Markov model to the raw current versus time trajectories¹⁹. Additionally, the existence of a wandering baseline and large number of transitions between various current states in each of the trajectories makes it difficult to calculate an accurate rolling baseline for the trajectory that might facilitate the use of a thresholding algorithm or hidden Markov model¹⁹. To overcome these challenges, the trajectories were analyzed using a two-state, drift-corrected, thresholding algorithm that has been previously developed and used to analyze current versus time trajectories obtained using solid-state nanopore devices¹⁹ as well as those obtained using smFET devices^{2, 17}. Briefly, this iterative detection algorithm first performs a rolling average over the entire current versus time trajectory by using a 2,000-time-point window to determine a local baseline current value and subsequently rolls the 2,000-time-point window forward in time by 1 timepoint in order to calculate ~ 1.5 million local baseline current values for the entire trajectory. Because the two stem-loop constructs used here predominantly sampled the higher current state under all conditions studied, the local baseline current values for all of the smFET experiments performed here were generally closer to the higher current state. Thus, in order to assign each timepoint into either the higher- or lower current state, we used the local baseline current value corresponding to each timepoint as a threshold for the higher current state and set a threshold for the lower current state as follows: (i) we created a histogram of the standard deviations of the current values within each of the ~ 1.5 million 2,000-time-point windows; (ii) we took the standard deviation at the peak of this histogram as the representative standard deviation of the entire trajectory; (iii) we multiplied this representative standard deviation by a factor of 3 or more, depending on the construct and the smFET device used (a factor of at least 3 was used in order to minimize mis-assignment of timepoints due to noise); and (iv) we used the product of this multiplication as a negative offset from the local baseline current value at each given timepoint to set a local threshold for the lower current state. Transitions into the lower current state were defined as those in which a timepoint assigned to the higher current state crossed below the local threshold for the lower current state in the next timepoint. Analogously, transitions into the higher current state were defined as those in which a timepoint assigned to the lower current state crossed above the local threshold for the higher current state in the next timepoint. Finally, a new current versus time trajectory was generated by replacing the timepoints assigned to the lower current state with the local baseline current values at those timepoints. This new trajectory is then used to calculate new rolling averages and local baseline current values. Because the current values assigned to the lower current state in the previous trajectory were replaced with the previously calculated local baseline current values in the new trajectory, the new local baseline current values begin to trend toward the current values of the higher current state. These new local baseline current values are then once again used to set the local thresholds for the higher current state and, likewise, negative offsets from these new local baseline current values calculated as described above are used to set the local thresholds for the lower current state. These new local thresholds are then applied to the original trajectory in order to assign each timepoint to either the higher or lower current state. The algorithm then continues to be iterated until the number of transitions converges.

For each raw current versus time trajectory, the current state assignments made as described above were used to generate an idealized current state versus time trajectory. The idealized trajectories were then used to conduct the thermodynamic and kinetic analyses of the stem-loop constructs described in the main text of this article. Briefly, the fractional occupancy of each current state in each idealized trajectory was calculated by quantifying the total number of timepoints spent in each of the current states. The fractional occupancy of each current state was then used to calculate the K s and ΔG s as described in the main text of this article. To obtain the k_{off} s and k_{on} s, dwell times in each of the current states were compiled and used as input for the *ecdf()* MATLAB function to generate a survival probability distribution and the resulting distribution was fit to either a single-exponential decay function of the form

$$P(t) = e^{-kt}, \quad \text{Eq. 2}$$

where t is time, and k is the rate constant, or a double exponential decay function of the form

$$P(t) = Ae^{-k_1 t} + (1 - A)e^{-k_2 t}, \quad \text{Eq. 3}$$

where t is time, k_1 and k_2 are rate constants, and A is the amplitude, using the built-in *curve fitting tool(cftool)* in MATLAB.

G. Generating the conformational free-energy landscape figure

The conformational free-energy landscape shown in Figure 5 was generated using a custom-written Python script, generously provided by Prof. Colin Kinz-Thompson (Rutgers University at Newark) and Mr. Korak Kumar Ray (Columbia University), and is freely available at https://github.com/GonzalezBiophysicsLab/energy_landscape/blob/main/2022_JACS_smFET.ipynb.

H. Calculating the rates of stem-loop (un)folding based on a previously described global fit of T-jump relaxation data to a sequential 4-state model.

In Sarkar *et al.*, the authors globally fit their T-jump data to a sequential 4-state model (Eq. 4) in order to describe the (un)folding of two different tetraloops¹⁵⁻¹⁶. The four states are simply denoted as 1-4 in this Supplementary Information section. For a detailed description of the model and the structural conformations assigned to states 1-4, please refer to Sarkar *et al.*¹⁵⁻¹⁶.



In their studies, the free energies of the four states (ΔG_i , where i denotes a specific state) and the average free energy barriers connecting them ($\Delta G_{i \leftrightarrow j}^\ddagger$ where i and j denote neighboring states) were fit to the following linear models:

$$\Delta G_i(T) = \Delta G_i^{(0)} + \Delta G_i^{(1)}(T - T_0), \quad \text{Eq. 5}$$

$$\Delta G_{i \leftrightarrow j}^\ddagger(T) = \Delta G_{i \leftrightarrow j}^{\ddagger(0)} + \Delta G_{i \leftrightarrow j}^{\ddagger(1)}(T - T_0), \quad \text{Eq. 6}$$

where T is the temperature at which the free energies and average free energy barriers are specified (for the current study, $T = 328.15$ K), T_0 is the reference temperature, $\Delta G^{(0)}$ is the free energy at the reference temperature, and $\Delta G^{(1)}$ is the first derivative of the free energy with respect to temperature. The values of T_0 , $\Delta G^{(0)}$, and $\Delta G^{(1)}$ are provided in Sarkar *et al.*¹⁵⁻¹⁶.

Using the ΔG_i s and $\Delta G_{i \leftrightarrow j}^\dagger$ s provided by Sarkar *et al.*¹⁵⁻¹⁶, we then calculated the directional free energy barriers using the following relationships:

$$\Delta G_{ij}^\dagger = \Delta G_{i \leftrightarrow j}^\dagger + \Delta G_{ij}/2 \quad \text{Eq. 7}$$

$$\Delta G_{ji}^\dagger = \Delta G_{i \leftrightarrow j}^\dagger - \Delta G_{ij}/2 \quad \text{Eq. 8}$$

Where ΔG_{ij} is the free energy difference between states i and j .

Subsequently, we calculated the rate constant from state i to j (k_{ij}) by plugging each directional free energy barrier at a given temperature into the following equation:

$$k_{ij} = k_m \exp \left[\frac{\Delta G_{ij}^\dagger(T)}{RT} \right], \quad \text{Eq. 9}$$

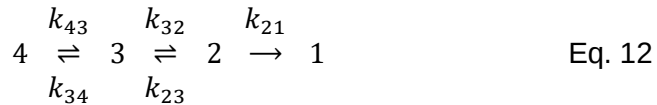
where k_m is the pre-exponential factor calculated at T using the previously described temperature dependence of k_m ^{13, 15} and R is the universal gas constant ($8.314 \text{ J K}^{-1} \text{ mol}^{-1}$).

To determine the rate of unfolding from the fully folded state (State 1) to the fully unfolded state (State 4), we calculated the mean first passage time from State 1 to 4 based on an equation derived in Kinz-Thompson *et al.*²⁰,

$$\begin{array}{ccccccc} & k_{12} & & k_{23} & & k_{34} & \\ 1 & \rightleftharpoons & 2 & \rightleftharpoons & 3 & \rightarrow & 4 \\ & k_{21} & & k_{32} & & & \end{array} \quad \text{Eq. 10}$$

$$\langle \tau_f \rangle = \frac{1}{k_{12}} \left[1 + \frac{k_{21}}{k_{23}} + \frac{k_{21}k_{32}}{k_{23}k_{34}} + \frac{1}{k_{23}} \left[1 + \frac{k_{32}}{k_{34}} \right] + \frac{1}{k_{34}} \right] \quad \text{Eq. 11}$$

Similarly, to calculate the rate of folding, the mean first passage time from the fully unfolded state (State 4) to the fully folded state (State 1) was calculated based on the analogous equation derived in Kinz-Thompson *et al.*²⁰:



$$\langle \tau_u \rangle = \frac{1}{k_{43}} \left[1 + \frac{k_{34}}{k_{32}} + \frac{k_{34}k_{23}}{k_{32}k_{21}} + \frac{1}{k_{32}} \left[1 + \frac{k_{23}}{k_{21}} \right] + \frac{1}{k_{21}} \right] \quad \text{Eq. 13}$$

Below we provide the MATLAB script we used to perform the calculations described above and obtain the rates reported in Table S5. This script includes the numbers that were used to

calculate the overall rate of unfolding from State 1 to State 4 and the overall rate of folding from State 4 to State 1 for the gacUUCGguc construct studied by Sarkar, et al.¹⁵.

```
%% gacUUCGguc at 55C
R = 8.314e-3;

% preexponential factor (Ref 13)
T=25+273.1;
n_T_25=(146+1090*exp(-0.2085*(T- 273.1))+557*exp(-0.07*(T - 273.1)))/890;
T=55+273.1;
n_T=(146+1090*exp(-0.2085*(T- 273.1))+557*exp(-0.07*(T - 273.1)))/890;
k_m = 1*n_T_25/n_T;

% Equilibrium thermodynamics parameters
T0_u = [326.67 326.85 327 345.21]; % Reference temperatures, Ref(15)

dG_0= [0 0 0 0];
dG_1= [0.4297 0.3678 0 -0.77]; % Ref(15)
dG_i = dG_0+dG_1.*(T-T0_u);
dG_ij=dG_i(2:4)-dG_i(1:3);

% Activation energy parameters
dG_i_j_0 = [7.44 11.42 19.1]; % Ref(15)
dG_i_j_1 = [0.183 -0.0513 0.274]; % Ref(15)

AE_i_j = dG_i_j_0+dG_i_j_1.*(T-T0_u(1:3));
AE_ij=AE_i_j+dG_ij/2;
AE_ji=AE_i_j-dG_ij/2;
k_ij= k_m*exp(-AE_ij./R./T)*1e6;
k_ji= k_m*exp(-AE_ji./R./T)*1e6;

% first passage time (Ref 20)
a43 = k_ji(3);
a34 = k_ij(3);
a32 = k_ji(2);
a23 = k_ij(2);
a21 = k_ji(1);
a12 = k_ij(1);

a = a43*a32*a21;
b = a12*a43*a32;
c = a12*a23*a43;
d = a12*a23*a34;

p(1) = a/(a+b+c+d);
p(2) = b/(a+b+c+d);
p(3) = c/(a+b+c+d);
p(4) = d/(a+b+c+d);

t_1(1) = 1/a12*(1+a21/a23+(a21*a32)/(a23*a34))+1/a23*(1+a32/a34)+1/a34;
t_1(2) = 1/a43*(1+a34/a32+(a34*a23)/(a32*a21))+1/a32*(1+a23/a21)+1/a21;

k(1) = 1/t_1(1);
k(2) = 1/t_1(2);
```

A script analogous to the one above was used to calculate the corresponding rates for the gacUACGguc stem-loop construct studied by Sarkar et al¹⁶ with the only difference being that the equilibrium thermodynamic parameters and the activation energy parameters were changed from those corresponding to the gacUUCGguc stem-loop to those corresponding to the gacUACGguc stem-loop.

II. Supplementary Figures

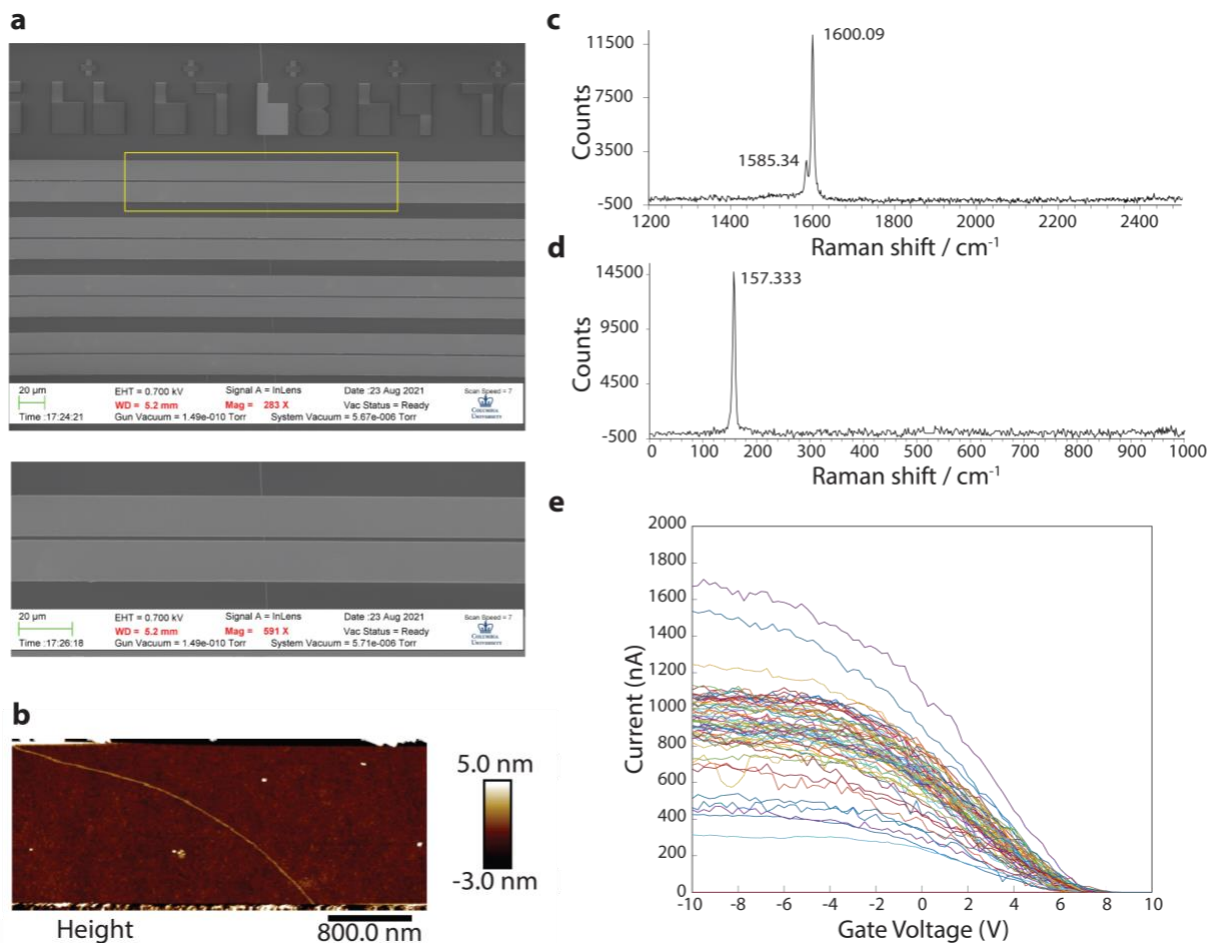


Figure S1. Data from the fabrication and characterization of various, representative smFET devices. (a) SEM image of four representative smFET devices, with source and drain electrodes deposited on a SWCNT. One of these smFET devices is shown in a magnified view. (b) AFM image of a single, representative smFET device between source and drain electrodes. (c) Raman spectrum of a representative CNT showing the G-band peaks. (d) Raman spectrum of a representative CNT showing the RBM peak. (e) Superimposed I-V curves for all smFET devices on a single Si/SiO₂ substrate, after isolation.

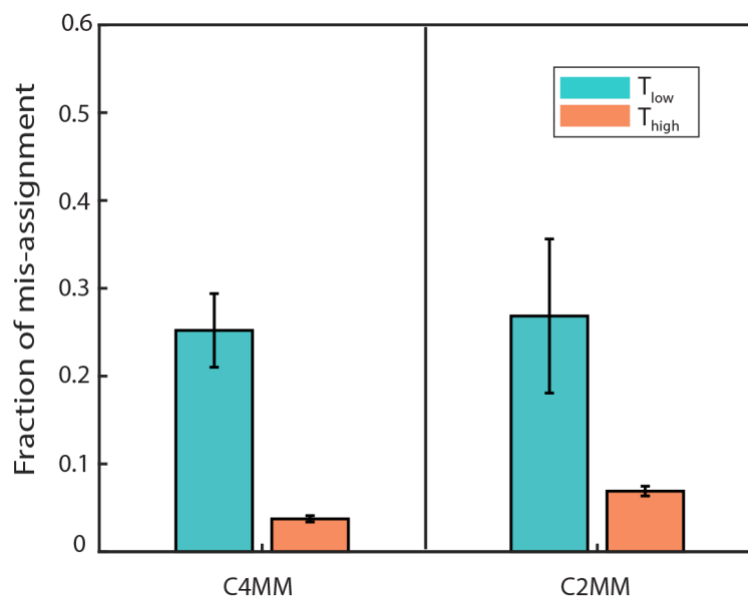


Figure S2. Quantitative comparison of smFET signal before and after stem-loop tethering. Fraction of mis-assignments were calculated by comparing the number of transitions observed in a 60s current trajectory prior to tethering a stem-loop at T_{low} (23–24 °C) to the number of transitions observed in a 60s current trajectory after tethering a stem-loop at T_{low} (23–24 °C) or T_{high} (44–45 °C). Error bars represent the standard deviations of three fraction of mis-assignments, each calculated from a pair of 60-s trajectories before and after stem-loop tethering.

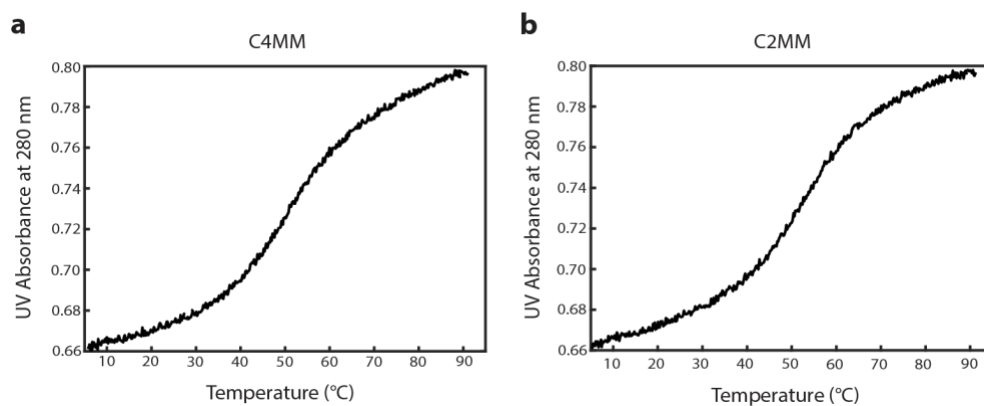


Figure S3. UV melting experiments for C4MM and C2MM. UV melting curves for **(a)** C4MM and **(b)** C2MM were collected in 100 mM NaCl, 10 mM NaH₂PO₄/Na₂HPO₄, pH = 7.0.

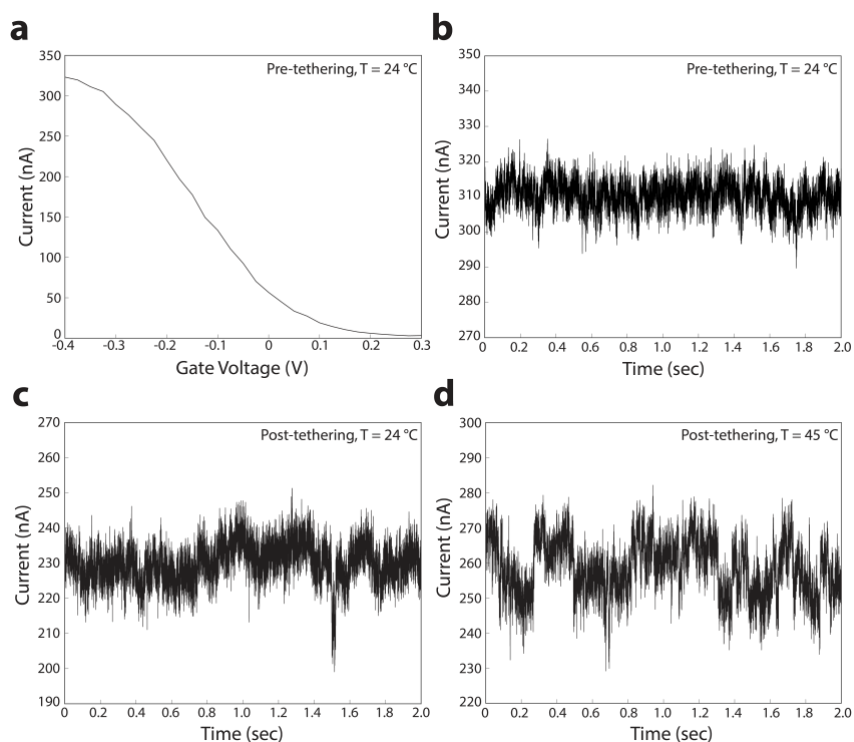


Figure S4. Temperature dependence of current versus time trajectories recorded using a single, representative smFET device containing a tethered RNA. (a) I-V curve for this smFET device. Trajectories were recorded: (b) at T_{low} , prior to the introduction of pyrene-NHS into the flow cell; (c) at T_{low} , after the incubation of the flow cell with pyrene-NHS and the introduction of a 5'-primary-amine-modified, stem-loop construct; and (d) at T_{high} after the recording in (b).

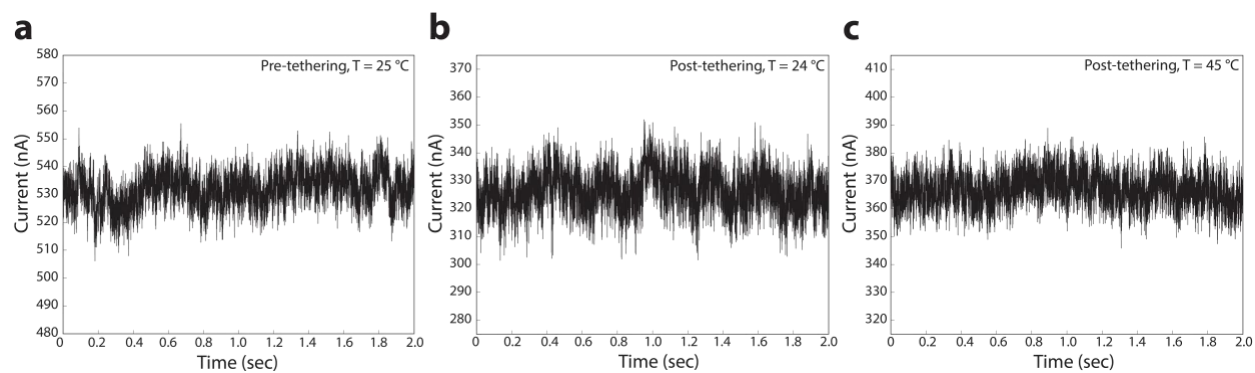


Figure S5. Temperature dependence of current versus time trajectories recorded using a single, representative smFET device lacking a tethered RNA. Trajectories were recorded: **(a)** at T_{low} , prior to the introduction of pyrene-NHS into the flow cell; **(b)** at T_{low} , after the incubation of the flow cell with pyrene-NHS and the introduction of a 5'-primary-amine-modified, stem-loop construct; and **(c)** at T_{high} after the recording in (b).

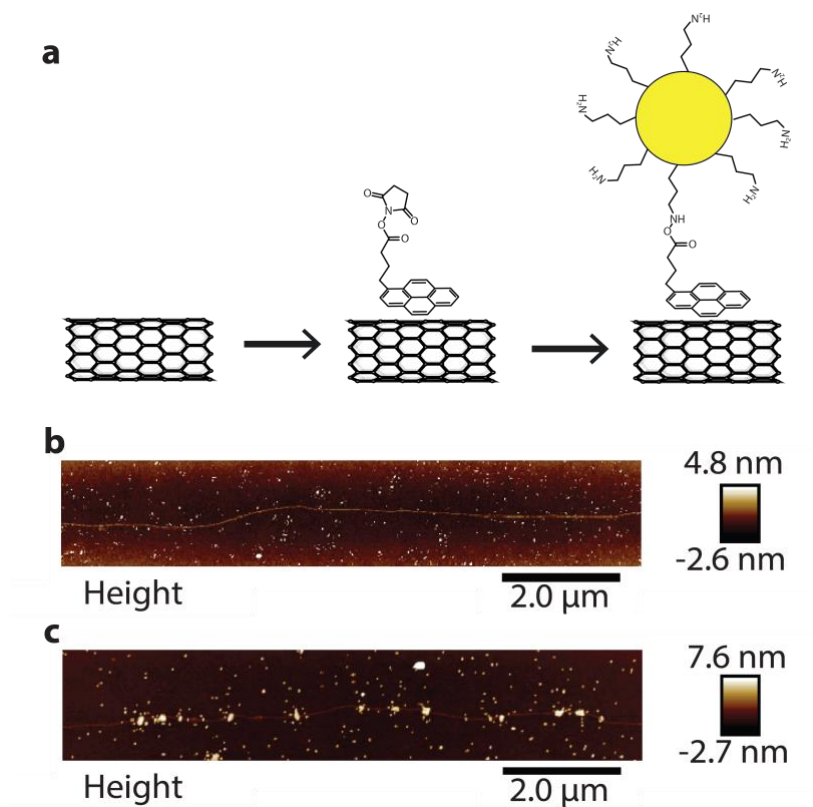


Figure S6. Validating the specific tethering of pyrene onto SWCNTs. (a) Schematic cartoon of the non-covalent stacking of the pyrene moiety of pyrene-NHS on the SWCNT surface of an smFET device and the subsequent reaction of the NHS moiety with a primary-amine-functionalized gold nanoparticle. (b-c) AFM images of smFET devices and gold nanoparticles after running the reaction depicted in (a) using (b) pyrene-COOH or (c) pyrene-NHS.

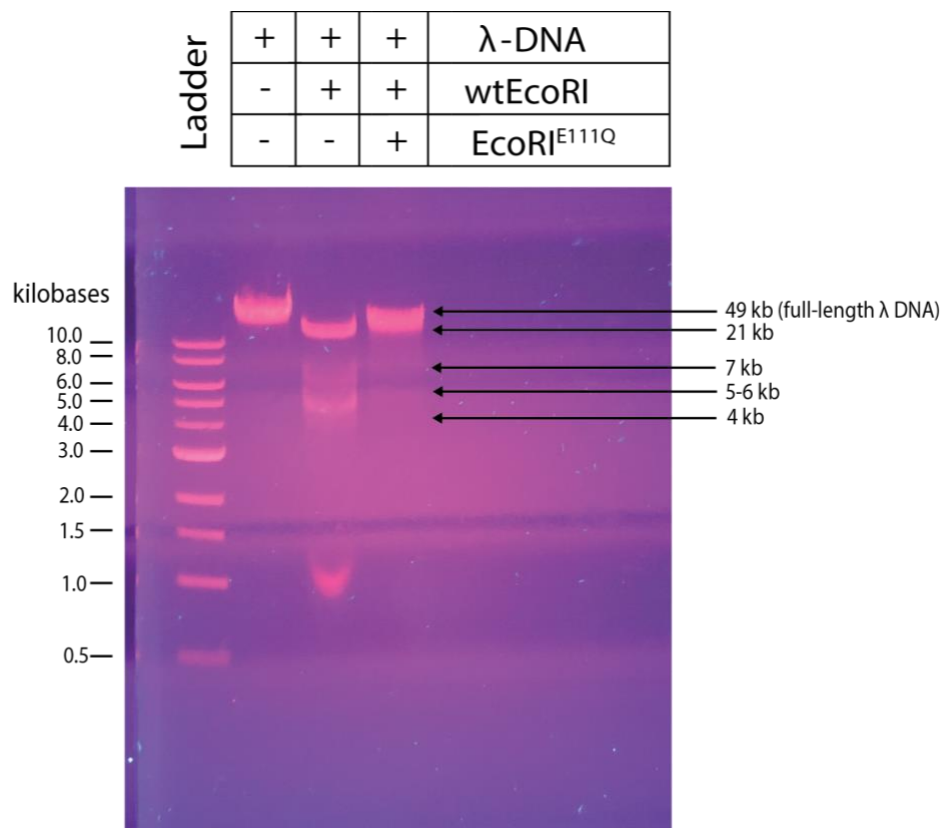


Figure S7. Characterization of purified EcoRI^{E111Q}. An 0.6 % agarose gel showing the analysis of DNA fragments produced by wtEcoRI-mediated nucleolytic cleavage and protected from wtEcoRI-mediated nucleolytic cleavage by EcoRI^{E111Q} as part of an EcoRI digestion protection assay.

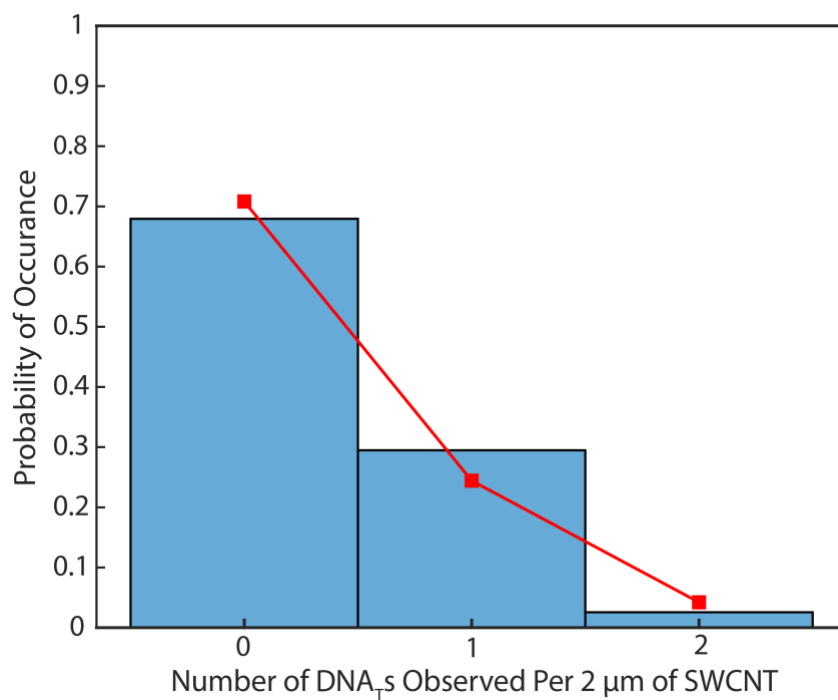


Figure S8. Distribution of the number of tethered DNA_Ts per 2μm of SWCNT. The probabilities of observing 0, 1, or 2 DNA_Ts, detected using AFM imaging of triple-EcoRI^{E111Q}-dimer-bound DNA complexes, tethered per 2μm of SWCNT is plotted as a bar graph. These probabilities are well described by the Poisson distribution function reported in Section I-B and shown as a red datapoints and curve overlaid on the bar graph.

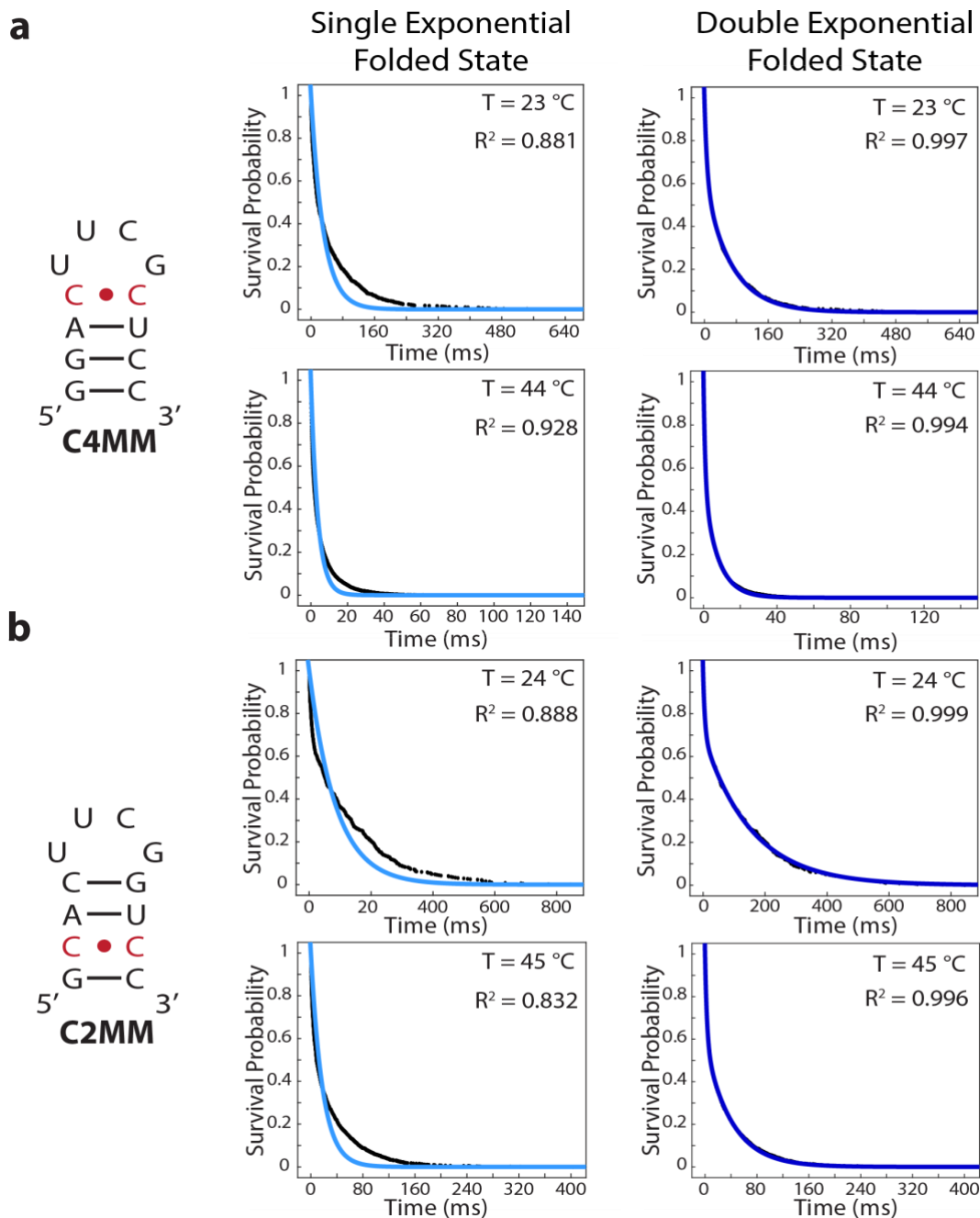


Figure S9. Single- and double-exponential fitting of survival probability plots of the ensemble of folded conformations. Secondary structure diagrams of the stem-loop constructs with the mismatched base-pairs denoted in red (first column), single-exponential fits (blue curves) to the survival probability plots of the ensemble of folded conformations (black data points) at T_{low} (second-column, top) and T_{high} (second column, bottom), and double-exponential fits (blue curves) to the survival probability plots of the ensemble of folded conformations (black data points) at T_{low} (third column, top) and T_{high} (third column, bottom) for **(a)** C4MM and **(b)** C2MM.

III. Supplementary Tables

Table S1. Thermodynamic parameters for stem-loop formation based on UV melting experiments.

Stem-loop Construct	ΔH^a (kcal mol ⁻¹)	ΔS^a (e.u)	ΔG_{37}^a (kcal mol ⁻¹)	T_m^a (°C)
C4MM	-29.2(0.7)	-90(2)	-1.22(0.04)	50.5(0.8)
C2MM	-30(2)	-92(5)	-1.47(0.09)	53.1(0.1)

^aParameters are from denaturation experiments in 100 mM NaCl, 10 mM NaH₂PO₄/Na₂HPO₄, pH = 7.0. For UV melting data, parameters are the average of 2 independently prepared samples fit to a two-state model.

Table S2. RNA sequences of the stem-loop constructs.

Stem-loop Construct	Sequence
C4MM	5'-NH ₂ (CH ₂) ₆ GGA CUU CGC UCC-3'
C2MM	5'-NH ₂ (CH ₂) ₆ GCA CUU CGG UCC-3'

Table S3. DNA sequences used for EcoRI^{E111Q}-based DNA tethering controls.

DNA Strand	Sequence ^a
DNA _T	5'–NH ₂ (CH ₂) ₆ GGT GAG <u>AAT TCG</u> GCC TGAAAT <u>GAA TTC</u> TAA GCG AAG T <u>GA ATT CAA</u> ACA–3'
DNA _C	5'–TGT TTG <u>AAT TCA</u> CTT CGC TTA <u>GAA TTC</u> ATT TCA GGC C <u>GA ATT CTC</u> ACC–3'

^aEcoRI^{E111Q} binding sites are bolded and underlined

Table S4. Comparison of thermodynamic parameters determined from smFET and UV melting experiments.

Construct	T ^a (°C)	smFET ΔG (kcal mol ⁻¹)	UV melting ΔG (kcal mol ⁻¹)	smFET f_u	UV melting f_u
C4MM	23	-1.20(0.06)	-2.48(0.01)	0.11	0.02
	44	-0.4(0.2)	-0.59(0.06)	0.33	0.28
C2MM	24	-1.8(0.2)	-2.7(0.2)	0.05	0.01
	45	-0.9(0.1)	-0.74(0.05)	0.19	0.24

^a ΔG and f_u for smFET experiments were determined at the specified temperatures. Corresponding ΔG and f_u for UV melting experiments were calculated from the standard thermodynamic parameters in Table S1.

Table S5: Overall folding and unfolding rate constants of UCG tetraloops derived from previous T-jump studies using a sequential four-state model.^a

Sequence	T ^b (°C)	k _f (s ⁻¹)	k _u (s ⁻¹)	τ _f (ms)	τ _u (ms)
gacUUCGguc ^c	55	7223	43	23.11	0.14
gacUACGguc ^d	55	1228	458	2.18	0.81

^a T-jump experiments were carried out in 10 mM sodium phosphate and 0.1 mM Na₂EDTA, pH 7.1.

^b Rate constants are calculated at the given temperature.

^c Energy parameters are from reference 15.

^d Energy parameters are from reference 16. Rate constants are calculated based on the SI Materials and Methods section G.

Table S6: Individual rate constants of UNGC tetraloops derived from previous T-jump studies using a sequential four-state model.

Sequence	T ^a (°C)	k ₁₂ (s ⁻¹)	k ₂₃ (s ⁻¹)	k ₂₃ (s ⁻¹)	k ₃₂ (s ⁻¹)	k ₃₄ (s ⁻¹)	k ₄₃ (s ⁻¹)
gacUUCGguc ^b	55	96290	90981	26674	22537	115	14366
gacUACGguc ^c	55	91167	40681	18632	12748	950	1642

^a Rate constants are calculated at the given temperature.

^b Energy parameters are from reference 15.

^c Energy parameters are from reference 16. Rate constants are calculated based on the SI Materials and Methods section H.

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