Responsive fluorescent nucleotides serve as efficient substrates to probe terminal uridylyl transferase

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Electronic Supplementary Information (ESI)

1. Materials: Nucleotides BFUTP (**1**) and SeUTP (**2**) were synthesized as previously reported.[S1,S2] 5′-FAM-labeled and unlabeled RNA oligonucleotides (ONs) **3** and **4** were purchased from Dharmacon Inc, deprotected according to the manufacturer's protocol and purified by denaturing polyacrylamide gel electrophoresis (PAGE). The ONs were desalted employing Sep-Pak Classic C18 cartridges (Waters Corporation). Ribonuclease inhibitor (RiboLock) and UTP were obtained from Thermo Fischer Scientific. Stains-All and reagents for buffers were acquired from Sigma Aldrich (Bio Ultra Grade). SpCID1 enzyme was expressed as per our previously described report.[S3]

2. Instrumentation: RNA was quantified by measuring absorbance in UV-2600 Shimadzu or NanoDrop™ 2000c spectrophotometer. ESI-MS mass analysis of RNA was performed using Waters SYNAPT G2-Si Mass Spectrometry instrument in negative mode. HPLC analysis was performed on an Agilent Technologies 1260 Infinity HPLC. Fluorescent RNA was resolved by polyacrylamide gel electrophoresis on OWL S4S sequencing gel electrophoresis instrument and was imaged using Typhoon FLA 9500 Variable mode Imager. Fluorescence spectra were recorded in a micro fluorescence cuvette (Hellma make path length 1.0 cm) on Horiba Fluoromax 4 spectrofluorometer.

3. Terminal uridylation of RNA ONs

3′ RNA labeling with BFUTP and SeUTP using SpCID1: In order to label RNA ONs at the 3′-end with microenvironment responsive nucleotides, 5′-FAM-labeled RNA ON **3** (10 μM) was incubated with SeUTP **1** or BFUTP **2** (500 μM) in Tris-HCl buffer (10 mM, pH 7.9 at 25 °C) containing NaCl (50 mM), $MgCl_2$ (10 mM), DTT (2 mM), Ribonuclease inhibitor (1 U/μL) and 1 μL of SpCID1 enzyme (10.25 pmol) in a final volume of 20 μL. After 5, 15 and 30 min, 5 μL aliquots of reaction mixture (50 pmol of RNA ON) were mixed with 15 μL of denaturing loading buffer (7 M urea in 10 mM Tris-HCl, 100 mM EDTA, 0.05% bromophenol blue, pH 8) and heat-denatured at 75 °C for 3 min. 5 μL of the sample was loaded on to 20% denaturing polyacrylamide gel, electrophoresed and imaged using Typhoon gel scanner at FAM wavelength.

Optimization of single modification on 5′-FAM-labeled RNA 3 with BFUTP (2): Terminal uridylation of RNA with a single BFU at the 3′-end was optimized by incubating 5′-FAM-labeled RNA ON **3** (10 μM) with varying concentrations of **2** (10–50 μM) in Tris-HCl buffer (10 mM, pH 7.9 at 25 °C) containing NaCl (50 mM), $MgCl_2$ (10 mM), DTT (2 mM), Ribonuclease inhibitor (1 U/μL) and SpCID1 (1.71 pmol) in a final volume of 20 μL. After 5, 15 and 30 min, 5 μL aliquots of reaction mixture (50 pmol of RNA ON) were mixed with 15 μL of denaturing loading buffer and resolved by PAGE and imaged as mentioned above. See Fig. S1a.

Large-scale uridylation reaction on 5′-FAM-labeled RNA ON 3 or unlabeled RNA ON 4 with BFUTP (2): Single incorporation of BFU at the 3′-end of RNA was performed by incubating 5′-FAM-labeled RNA **3** or unlabeled RNA **4** (10 μM) with BFUTP **2** (20 μM) in Tris-HCl buffer (10 mM, pH 7.9 at 25 °C) containing NaCl (50 mM), $MgCl_2$ (10 mM), DTT (2 mM), Ribonuclease inhibitor (1 U/μL) and SpCID1 (1.71 pmol) in a final volume of 20 μL for 30 min. Reaction with **4** (100 pmol of RNA ON) was analyzed by 20% denaturing polyacrylamide gel and stained with Stains-All gel staining reagent. A 5-set reaction (1 nmol) yielded 21%, and 42% of the 3′-BFU-labeled RNA products $\mathbf{3_{BFU}}$ and $\mathbf{4_{BFU}}$, respectively. ESI-MS analysis was performed in negative mode by injecting RNA ON products (100 pmol) dissolved in 50% acetonitrile in an aqueous solution of 10 mM triethylamine and 100 mM hexafluoro-2-propanol. See Table S1 and Fig. S2 for yield and mass data.

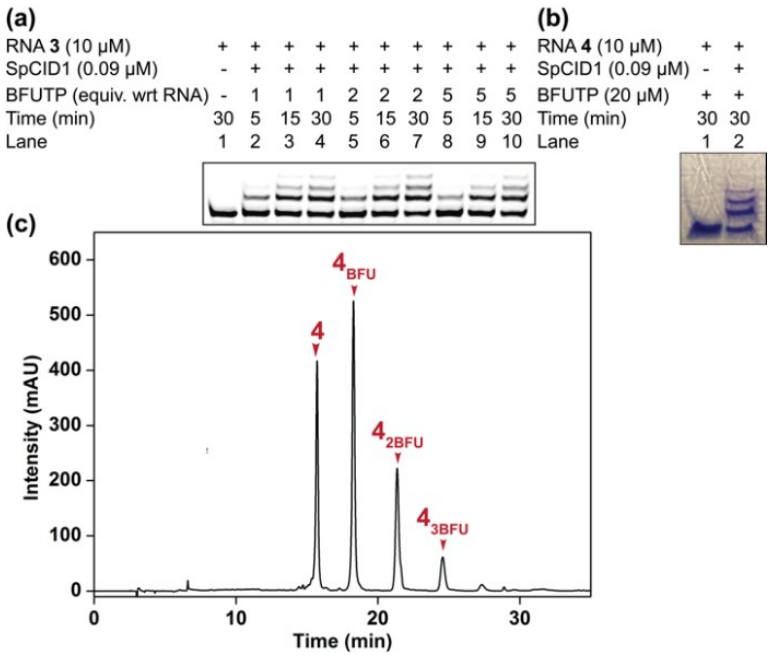

Fig. S1 (**a**) Gel picture showing reaction conditions tested to synthesize RNA ONs containing a single BFU label at the 30-end ($\mathbf{3_{BFU}}$). (**b**) Products of a large-scale terminal uridylation reaction with unlabeled RNA ON 4. Products were stained using Stains-All reagent. (**c**) Products of these reactions were resolved using RP-HPLC. A representative chromatogram at 260 nm depicting products corresponding to single ($\mathbf{4_{BFU}}$), double ($\mathbf{4_{2BFU}}$) and triple ($\mathbf{4_{3BFU}}$) incorporation is shown.

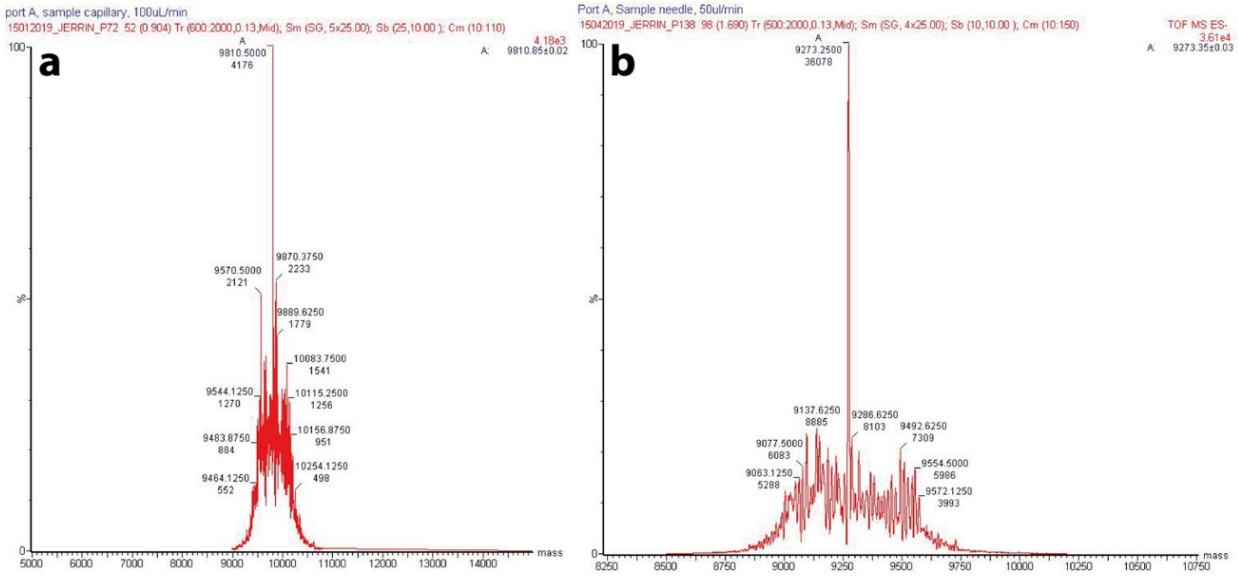

Fig. S2 ESI-MS spectra of BFU-labeled RNA ON products (**a**) $\mathbf{3_{BFU}}$ and (**b**) $\mathbf{4_{BFU}}$. See Table S1 for details.

Table S1. Mass and yield data of BFU-labeled RNA ON products.

RNA ON product	calcd. mass	found mass	isolated yield (nmol)	isolated yield (%)
$\mathbf{3_{BFU}}$	9811.1	9810.5	0.21[a]	21
$\mathbf{4_{BFU}}$	9273.7	9273.3	1.66[a]	42

Isolated yields are with respect to [a]1 and [b]4 nmol of the substrate RNA ON.

4. Steady-state fluorescence binding assay for BFU-labeled RNA/nucleotide analog and SpCID1 enzyme

A series of samples having either BFU-labeled RNA ON $\mathbf{4_{BFU}}$ or BFUTP **2** (0.2 μM) were incubated with increasing concentrations of SpCID1 (0–2 μM) in Tris-HCl buffer (10 mM, pH 7.9 at 25 °C) containing NaCl (50 mM), $MgCl_2$ (10 mM), DTT (1 mM) and Ribonuclease inhibitor (0.2 U/μL) in a final volume of 200 μL maintaining an overall ~0.9 % glycerol. The samples were incubated for 30 min at room temperature followed by fluorescence measurements. Fluorescence spectrum of individual samples were performed by exciting samples at 330 nm and fluorescence was recorded from 340 to 600 nm maintaining an excitation emission slit width of 5 and 6 nm respectively. Control samples without RNA ON $\mathbf{4_{BFU}}$ or nucleotide **2** showed no observable fluorescence and was used for background subtraction. Fluorescence binding assay was performed in duplicate. Dose-dependent quenching of fluorescence observed at 435 nm for BFU-labeled RNA ON $\mathbf{4_{BFU}}$ and 436 nm for BFUTP **2** was fit to Hill equation (Origin 8.5) by plotting normalized fluorescence intensity (F_N) versus protein concentration. A Hill coefficient (n) of nearly 2 was observed for binding of BFU-labeled RNA ON and BFUTP to SpCID1.

$$F_N = \frac{F_i - F_s}{F_0 - F_s}$$

F_i is the fluorescence emission intensity measured at each protein concentration. F_0 and F_S are the fluorescence emission intensity measured in the absence of protein SpCID1 and at saturation point where n is the Hill coefficient measuring the degree of cooperativity in binding.

$$F_N = F_0 + (Fs - F_0)\left(\frac{[SpCID1]^n}{[K_d]^n + [SpCID1]^n}\right)$$

5. Electrophoretic mobility shift assay for the binding of BFU-labeled RNA ON with SpCID1. A series of samples having 5′-FAM,3′-BFU-labeled RNA ON $\mathbf{3_{BFU}}$ (0.2 μM) was titrated with SpCID1 (0–12 μM) in Tris-HCl buffer (10 mM, pH 7.9 at 25 °C) containing NaCl (50 mM), $MgCl_2$ (10 mM), DTT (1 mM) and Ribonuclease inhibitor (0.2 U/μL) in a final volume of 20 μL maintaining overall 5.6% glycerol. The samples (2 pmol of RNA ON $\mathbf{3_{BFU}}$) were incubated for 30 min at room temperature followed by addition of 20 μL of native loading buffer. The samples were loaded on a native 12% polyacrylamide gel maintaining a 10 mM $MgCl_2$ concentration. The gel was run in 1X TBE supplemented with 10 mM $MgCl_2$. The gel was resolved at 4 °C and was imaged using Typhoon gel scanner at FAM wavelength. Since the bound fraction was stuck in the well, binding curve was generated by calculating relative band intensity corresponding to unbound RNA ON $\mathbf{3_{BFU}}$ on addition of SpCID1 compared to the sample without SpCID1 in Fiji Image analysis software and was fit to Hill equation (Origin 8.5) using the equation mentioned above.

F_i is the fluorescence band intensity measured at each protein concentration. F_0 and F_S are the fluorescence band intensity measured in the absence of protein SpCID1 and at saturation point where n is the Hill coefficient measuring the degree of cooperativity in binding. A Hill coefficient (n) of 3.9 was observed for the binding of RNA ON $\mathbf{3_{BFU}}$ to SpCID1. See Fig. S3.

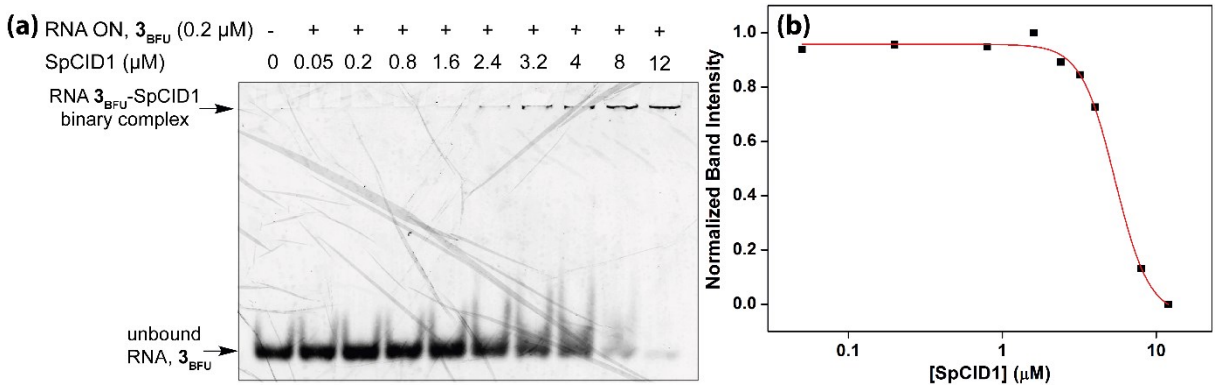

Fig. S3 EMSA for RNA ON titrated with increasing SpCID1. (**a**) RNA ON $\mathbf{3_{BFU}}$ (0.2 μM) and SpCID1 (0–12 μM) were incubated and resolved by 12% native PAGE. The protein-RNA ON binary complex remained at the well. (**b**) Curve-fit for normalized band intensity measured upon increasing SpCID1 concentration. The binding curve was generated by calculating relative band intensity corresponding to the unbound RNA ON $\mathbf{3_{BFU}}$ on addition of SpCID1.

6. Fluorescence displacement assay

Competition between BFU-labeled RNA ON 4_{BFU} and unlabeled RNA ON 4 for SpCID1. Samples made of $\mathbf{4_{BFU}}$ (0.2 μM) and SpCID1 (1.6 μM) were incubated with increasing concentration of RNA ON **4** (0–2 μM) in Tris-HCl buffer (10 mM, pH 7.9 at 25 °C) containing NaCl (50 mM), $MgCl_2$ (10 mM), DTT (1 mM) and Ribonuclease inhibitor (0.2 U/μL) in a final volume of 200 μL maintaining an overall ~0.9% glycerol. The samples were incubated for 30 min at room temperature followed by steady-state fluorescence measurements. Fluorescence of individual samples was recorded by exciting at 330 nm wavelength with an excitation emission slit width of 5 and 6 nm respectively. A control sample without RNA ON $\mathbf{4_{BFU}}$ was used for background subtraction. See Fig. S4a for fluorescence spectra.

Competition between BFUTP and UTP for SpCID1. Samples made of BFUTP **2** (0.2 μM) and SpCID1 (0.8 μM) were incubated with increasing concentration of UTP (0–40 μM) in Tris-HCl buffer (10 mM, pH 7.9 at 25 °C) and fluorescence was recorded as mentioned above. A control sample without **2** was used for background subtraction. See Fig. S4b for fluorescence spectra.

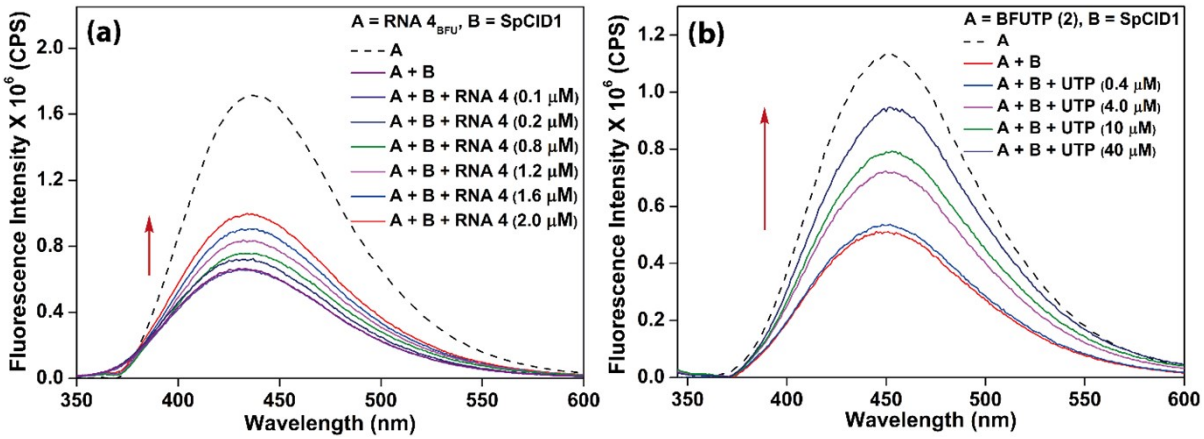

Fig. S4 (**a**) Fluorescence spectra of RNA ON $\mathbf{4_{BFU}}$ (0.2 μM) and SpCID1 (1.6 μM) complex incubated with increasing concentration of RNA, **4** (0–2 μM). Arrow denotes recovery of fluorescence when unlabeled RNA ON **4** competes with labeled ON $\mathbf{4_{BFU}}$ for SpCID1. (**b**) Fluorescence spectra of BFUTP (0.2 μM) and SpCID1 (0.8 μM) complex incubated with increasing concentration of UTP (0–40 μM). Arrow denotes recovery of fluorescence when UTP competes with **2** for SpCID1. See section 6 for details.

7. References

S1. A. A. Tanpure and S. G. Srivatsan, *Chemistry – A European Journal*, 2011, **17**, 12820–12827.

S2. M. G. Pawar, A. Nuthanakanti and S. G. Srivatsan, *Bioconjugate Chem.*, 2013, **24**, 1367–1377.

S3. J. T. George, M. Azhar, M. Aich, D. Sinha, U. B. Ambi, S. Maiti, D. Chakraborty and S. G. Srivatsan, *J. Am. Chem. Soc.*, 2020, **142**, 13954–13965.