The RNA chaperone StpA enables fast RNA refolding by destabilization of mutually exclusive base pairs within competing secondary structure elements

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1 Chemical Synthesis

1.1 Chemicals and conditions

All reactions were performed under argon atmosphere using dry solvents purchased from Acros Organics or Merck KGaA. Reagents were purchased from Acros Organics, Merck KGaA, ChemPur, TCI, Alfa Aesar or ChemGenes and used without further purification. For flash chromatography the used silica gel was purchased from Macherey-Nagel (particle size: 40-63 µm), solvents were of technical grade. NMR spectra were recorded on Bruker DPX250, AV400 and DRX600 instruments at ambient temperature.

1.2 Synthesis of the (S)-NPE caged guanosine (G\textsuperscript{(S)-NPE}) phosphoramidite

The synthesis of the (S)-NPE protected guanosine phosphoramidite was performed according to literature procedure (1).

![Scheme S1 Overview of the synthesis of (S)-NPE G phosphoramidite 6. a) 1 (purchased from ChemGenes), (R)-1-(2-nitrophenyl)ethanol (prepared according to the literature procedure (2)), PPh\textsubscript{3}, DIAD, THF, 0°C to rt, 76%; b) 12M MeNH\textsubscript{2} in H\textsubscript{2}O, THF, rt, 93% (crude product); c) (4-isopropylphenoxy)acetyl chloride, DMAP, pyridine, 0°C to rt, 91%; d) NH\textsubscript{3}/MeOH/THF 1:1:1 (v/v/v), rt, 83%; e) 2-cyanoethyl-\textit{N,N}-diisopropylchlorophosphoramidite, DIPEA, CH\textsubscript{2}Cl\textsubscript{2}, rt, 73%.](image-url)
1.2.1 Synthesis of 5'-O-(4,4'-Dimethoxytrityl)-2'-O-triisopropylsilyloxymethyl-N^2-acetyl-O^6-[(S)-1-(2-nitrophenyl)ethyl] guanosine (2)

1.00 g 5'-O-(4,4'-dimethoxytrityl)-2'-O-triisopropylsilyloxymethyl-N^2-acetyl guanosine (1) (1.23 mmol, 1.0 eq, purchased from ChemGenes) was dissolved in 5 mL dry THF. (R)-1-(2-nitrophenyl)ethanol (1) (205 mg, 1.23 mmol, 1.0 eq) and PPh₃ (483 mg, 1.84 mmol, 1.5 eq) were added and the resulting solution was cooled with an icebath to 0°C. 363 µL diisopropylazodicarboxylate (DIAD) (1.84 mmol, 1.5 eq) were added dropwise. The reaction mixture was stirred at room temperature for 9 h and concentrated under reduced pressure. The crude product was purified by column chromatography (SiO₂, cyclohexane/EtOAc 4:1+ 1% (v/v) Et₃N → cyclohexane/EtOAc 1:3). Product 2 was isolated as a pale yellowish foam.

Yield: 908 mg (76%)

TLC (cyclohexane/EtOAc 1:1): R_f=0.36

1H-NMR (400 MHz, DMSO-d₆): δ= 10.02 (s, 1H, N-H-Ac), 8.31 (s, 1H, H8), 8.06-8.04 (m, 1H, H_ar, NPE), 7.82-7.80 (m, 1H, H_ar, NPE), 7.74-7.70 (m, 1H, H_ar, DMTr), 7.32-7.30 (m, 2H, H_ar, DMTr), 6.87 (q, 3J(H,H)=6.5 Hz and 6.3 Hz, 1H, O-C₆H₄-NPE), 6.81-6.76 (m, 4H, H_ar, DMTr), 6.02 (d, 3J(H,H)=5.6 Hz, 1H, 1'-H), 5.16 (d, 3J(H,H)=5.7 Hz, 1H, 3'-OH), 4.93-4.92 (m, 1H, O-C₆H₄-O and 2'-H), 4.35-4.34 (m, 1H, 3'-H), 4.03-4.02 (m, 1H, 4'-H), 3.70-3.69 (m, 6H, 2x DMTr-OMe), 3.31-3.29 (m, 1H, 5'-H), 3.21-3.18 (m, 1H, 5'-H), 2.08 (s, 3H, NH-Ac), 1.80 (d, 3J(H,H)=5.6 Hz, 3H, CH₃-NPE), 0.80-0.76 (m, 21H, TOM)ppm.

1.2.2 Synthesis of 5'-O-(4,4'-Dimethoxytrityl)-2'-O-triisopropylsilyloxymethyl-N^2,3'-O-bis(4-isopropylphenoxyacetyl)-2'-O-triisopropylsilyloxymethyl-O^6-[(S)-1-(2-nitrophenyl)ethyl] guanosine (3)

850 mg of 2 (881 µmol, 1.0 eq) were dissolved in 8 mL dry THF and treated with 13 mL of 12M MeNH₂ in H₂O. After stirring at room temperature for 2 h, the solvent was removed under reduced pressure. The crude product was obtained as a colorless foam and used without further purification in the next synthesis step.

Yield: 758 mg (93%, crude product)

TLC (CH₂Cl₂/acetone 19:1): R_f=0.66

1.2.3 Synthesis of 5'-O-(4,4'-Dimethoxytrityl)-N^2,3'-O-bis(4-isopropylphenoxyacetyl)-2'-O-triisopropylsilyloxymethyl-O^6-[(S)-1-(2-nitrophenyl)ethyl] guanosine (4)
A solution of crude 3 (800 mg, 869 µmol, 1.0 eq) and 4-(dimethylamino)pyridine (53 mg, 434 µmol, 0.5 eq) in 4.5 mL pyridine was cooled to 0°C before 751 µL (4-isopropylphenoxy)acetyl chloride (4.34 mmol, 5.0 eq) were added dropwise. The reaction mixture was stirred at room temperature for 18 h. After quenching the reaction by the addition of 40 mL MeOH the solvent was removed under reduced pressure. The crude product was purified by column chromatography (SiO₂, cyclohexane/EtOAc 4:1+ 1% (v/v) Et₂N → cyclohexane/EtOAc 2:3) to give 4 as a pale yellow solid.

Yield: 1.01 g (91%)

TLC (cyclohexane/EtOAc 2:3): Rₓ=0.73

1H-NMR (400 MHz, DMSO-d₆): δ= 10.09 (s, 1H, NH), 8.37 (s, 1H, H8), 8.05-8.03 (m, 1H, H₉, NPE), 7.86-7.83 (m, 1H, H₂, NPE), 7.77-7.72 (m, 1H, H₈, NPE), 7.58-7.54 (m, 1H, H₉, NPE), 7.30-7.28 (m, 2H, H₉, DMTr), 7.20-7.13 (m, 7H, H₉, DMTr), 7.10-7.07 (m, 4H, H₉, PrPac), 6.89-6.87 (m, 1H, O-CH₃-NPE), 6.85-6.82 (m, 4H, H₉, DMTr), 6.80-6.72 (m, 4H, H₉, PrPac), 6.05 (d, 3J(H,H)=6.8 Hz, 1H, 1'-H), 5.41-5.35 (m, 2H, 2'-H and 3'-H), 4.86-4.63 (m, 6H, O-CH₂-O and 2x CH₂-OPrPac), 4.24-4.22 (m, 1H, 4'-H), 3.68-3.67 (m, 6H, 2x DMTr-OMe), 3.60-3.56 (m, 1H, 5'-H), 3.26-3.22 (m, 1H, 5'-H), 2.86-2.77 (m, 2H, 2x C-PrPac), 1.82 (d, 3J(H,H)=6.5 Hz, 3H, CH₃-PrPac), 1.17-1.12 (m, 12H, CH₂-PrPac), 0.72-0.67 (m, 21H, TOM)ppm.

1.2.4 Synthesis of 5'-O-(4,4'-Dimethoxytrityl)-N²-(4-isopropylphenoxyacetyl)-2'-O-trisopropylsilyloxyethyl-O[[(S)-1-(2-nitrophenyl)ethyl] guanosine (5)

1.00 g of 4 (785 µmol, 1.0 eq) were dissolved in 15 mL aq. NH₃/MeOH/THF (1:1:1, v/v) and stirred at room temperature for 2 h. The solvent was removed under reduced pressure and the crude product was purified by column chromatography (SiO₂, cyclohexane/EtOAc 1:1+ 1% (v/v) Et₂N → cyclohexane/EtOAc 1:1) to give 5 as a yellow solid.

Yield: 713 mg (83%)

TLC (cyclohexane/EtOAc 1:1): Rₓ=0.58

1H-NMR (400 MHz, DMSO-d₆): δ= 10.11 (s, 1H, NH), 8.35 (s, 1H, H8), 8.04-8.02 (m, 1H, H₉, NPE), 7.83-7.81 (m, 1H, H₂, NPE), 7.74-7.70 (m, 1H, H₈, NPE), 7.56-7.52 (m, 1H, H₉, NPE), 7.31-7.29 (m, 2H, H₉, DMTr), 7.21-7.10 (m, 9H, 7H, DMTr and 2H, 2x H₉, PrPac), 6.89-6.84 (m, 1H, O-CH₃-NPE), 6.82-6.74 (m, 6H, 4H, 4H, DMTr and 2H, 2x H₉, PrPac), 6.05 (d, 3J(H,H)=5.8 Hz, 1H, 1'-H), 5.17 (d, 3J(H,H)=5.7 Hz, 1H, 3'-OH), 4.92-4.89 (m, 2H, 2'-H and O-CH₂-O), 4.84-4.83 (m, 1H, O-CH₃-O), 4.77 (brs, 2H, CH₂-OPrPac), 4.32-4.28 (m, 1H, 1'-H), 4.06-4.03 (m, 1H, 4'-H), 3.69-3.68 (m, 6H, 2x DMTr-OMe), 3.38-3.34 (m, 1H, 5'-H), 3.18-3.15 (m, 1H, 5'-H), 2.83 (q, 3J=5.9 Hz, 1H, C-PrPac), 1.81 (d, 3J(H,H)=6.4 Hz, 3H, CH₃-PrPac), 1.17-1.16 (m, 6H, CH₃-PrPac), 0.76-0.73 (m, 21H, TOM)ppm.

1.2.5 Synthesis of 5'-O-(4,4'-Dimethoxytrityl)-N²-(4-isopropylphenoxyacetyl)-2'-O-trisopropylsilyloxyethyl-O[[[(S)-1-(2-nitrophenyl)ethyl] phosphoramidite (6)
500 mg of 5 (456 µmol, 1.0 eq) were dissolved in dry CH₂Cl₂ and treated with 397 µL N,N-diisopropylethylamine (2.28 mmol, 5.0 eq). 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (204 µL, 911 µmol, 2.0 eq) was added and the mixture was stirred at room temperature for 6 h. The solution was diluted with 50 mL dry CH₂Cl₂ and washed with saturated aqueous NaHCO₃ solution. The organic layer was dried over Na₂SO₄ and the solvent removed under reduced pressure. The crude product was purified by column chromatography (SiO₂, hexane/EtOAc 4:1+ 1% (v/v) Et₂N → hexane/EtOAc 1:3) to give 5 as a slight yellow foam.

Yield: 430 mg (73%)

TLC (cyclohexane/EtOAc 2:1): Rf=0.36

¹H-NMR (400 MHz, DMSO-d₆): δ= 10.09-10.6 (m, 1H, NH), 8.36 (s, 1H, H8), 8.05-8.03 (m, 1H, Har, NPE), 7.84-7.82 (m, 1H, Har, NPE), 7.75-7.71 (m, 1H, Har, NPE), 7.57-7.53 (m, 1H, Har, NPE), 7.33-7.28 (m, 2H, Har, DMTr), 7.23-7.09 (m, 9H, 7Har, DMTr and 2Har, tPrPac), 6.88-6.86 (m, 1H, O-CH-NPE), 6.80-6.72 (m, 6H, 4Har, DMTr and 2Har, tPrPac), 6.07-6.02 (m, 1H, 1'-H), 5.14-5.07 (m, 1H, 2'-H), 4.89-4.73 (m, 4H, O-CH₂-O and CH₂-O tPrPac), 4.41-4.34 (m, 1H, 3'-H), 4.18-4.14 (m, 1H, 4'-H), 3.76-3.68 (m, 8H, OCH₂CH₂CN and 2x DMTr-OMe), 3.44-3.42 (m, 1H, 5'-H), 3.21-3.18 (m, 1H, 5'-H), 2.83 (m, 1H, COCH₂CH₂CN), 1.81 (d, 3J(H,H)=6.4 Hz, 3H, CH₃-NPE), 1.17-1.16 (m, 6H, CH₂-tPr), 1.10-1.05 (m, 9H, CH₃-tPr), 0.96-0.94 (m, 3H, CH₂-tPr), 0.73 (m, 21H, TOM)ppm.

³¹P-NMR (DMSO-d₆, 162 MHz): δ= 149.55, 149.44ppm.

1.3 NMR spectra

Figure S 1. ¹H-NMR spectrum of 2 in DMSO-d₆ (400 MHz, 298 K). Impurities of ethyl acetate and cyclohexane were not assigned.
Figure S 2. $^1$H-NMR spectrum of 4 in DMSO-$_6$ (400 MHz, 298 K). Impurities of cyclohexane were not assigned.

Figure S 3. $^1$H-NMR spectrum of 5 in DMSO-$_6$ (400 MHz, 298 K).
Figure S 4. $^1$H-NMR spectrum of (S)-NPE$_{rG}$ phosphoramidite 6 in DMSO-d$_6$ (400 MHz, 298 K).

Figure S 5. $^{31}$P-NMR spectrum of (S)-NPE$_{rG}$ phosphoramidite 6 in DMSO-d$_6$ (162 MHz, 298 K).
2 Oligonucleotide synthesis

Table S 1. RP-HPLC conditions used for the RNA purification

| time [min] | % solvent A | % solvent B |
|------------|-------------|-------------|
| 0          | 95          | 5           |
| 13         | 76.3        | 23.7        |
| 15         | 0           | 100         |
| 20         | 0           | 100         |

Table S 2. Sequence and ESI-MS results of the synthesized oligonucleotide

| Sequence | Calculated Mass [Da] | Measured Mass [Da] |
|----------|----------------------|--------------------|
| RNA: 5’-r[GAC CGG(S-NPE) AAG GUC CGC CUU CC]-3' | 6502.9 | 6503.0 |

2.1 Mass spectra

Figure S 6. Mass spectra of photocaged RNA recorded on a Bruker micrOTOF-Q device (ESI). Top: BPC and UV chromatogram, middle: full spectrum, bottom: deconvoluted molecular peak. The calculated mass is 6502.9.
3 StpA-CTD expression - Sequences

Table S 3. StpA-CTD DNA Sequence that was cloned into the SUMO vector. Primer sequences used for cloning strategy.

| StpA-CTD DNA Sequence | 1 5’-CGCCAGCCGC GTCCGGCGAA ATATAAATTCA CGATGTTA |
|-----------------------|-------------------------------------------------|
|                       | 51 ACGGTGAAAC TAAAACCTGG ACCGGTCAGG GCCGTACACC |
|                       | 91 GAAGCCGAATT GCTCAGGCGC TGGCAGAAGG TAAATCTCTC |
|                       | 131 GACGATTTC TGATC-3’ |
| forward primer        | 5’-CCGGTCTCGAGGTCGCCAGCCCGCGTC-3’ |
| reverse primer        | 5’-CCGGTCTCTCTCTAGATTAGATCGAGCAGGAAATCGTAGCAGAG-3’ |

Figure S 7. MALDI Mass spectra of purified StpA-CTD recorded on a Applied Biosystems Voyager-DE STR device. The calculated mass is 5031.71 Da.
Figure S 8: Amide and aliphatic proton region of $^1$H NMR spectra of StpA-CTD via expression with SUMOstar vector and of reference StpA-CTD at 25°C, recorded at 800 MHz/ 600 MHz Bruker NMR spectrometers. The expression of StpA-CTD yields correctly folded protein. Concentrations $c$ (expression with SUMO StpA-CTD) = 860 µM, $c$ (reference StpA) = 1000 µM.
Figure S9: (top) Amide $^1$H NMR spectra of StpA-CTD via expression with SUMOstar vector and of reference StpA-CTD at 25°C. (below) $[^1]$H, $^{15}$N]-HSQC spectra of reference StpA-CTD for the purpose of better resolution of the backbone and side-chain HN resonances, confirming the folded state of StpA-CTD in both cases. Spectra recorded at 800 MHz/ 600 MHz Bruker NMR spectrometers. Concentrations $c$(expression with SUMO StpA-CTD)= 860 µM, $c$(reference StpA)=1000 µM.

4 RNA Sequences, Abbreviations, sample concentration

| RNA | Sequence | Modification |
|-----|----------|--------------|
| 20mer | 5'- GAC CGG AAG GUC CGC CUU CC -3' | (S)-NPE O6-nitrophenyl-ethyl |
| 20mer caged | 5'- GAC CGG AAG GUC CGC CUU CC -3' | m1G N1-methylguanosine |
| G6m1G | 5'- GAC CGG AAG GUC CGC CUU CC -3' | m1G N6-dimethyladenosin |
| A2-DMA | 5'- GAC CGG AAG GUC CGC CUU CC -3' | DMA N6-dimethyladenosin |
| 5'-HP | 5'- GAC CGG AAG GUC C -3' |  |
| 3'-HP | 5'- CGG AAG GUC CGC CUU CC -3' |  |
| 5'-SS-OV | 5'- GC CUU CC -3' |  |
5 NMR spectroscopy

NMR experiments were performed on Bruker NMR spectrometers with different probe heads listed in Table S 4. NMR experiments were performed with standard Bruker pulse sequences and spectra were recorded and analyzed with TopSpin 3.5. All samples containing 10% D_2O and the same buffer: 50 mM BisTris, 25 mM NaCl, pH 6.4. 1D ^1H imino proton spectra were recorded using a jump return echo pulse sequence. Thermal equilibration for all samples at each temperature was done for at least 20 minutes before the experiments were recorded.

Table S 5. Spectrometers

| Spectrometer | Probe Head |
|---------------|------------|
| 800 MHz       | 5 mm TCI cryo ^1H,^15N,^13C Z-GRD |
|               | 5 mm TXO cryo ^13C,^1H,^15N, Z-GRD |
| 700 MHz       | 5 mm QC cryo ^1H,^15N,^13C,^31P Z-GRD |
| 599 MHz       | 5 mm TCI cryo ^1H,^15N,^13C Z-GRD |
| 600 MHz       | CryoProbe Prodigy 5 mm TCI ^1H/^19F,^15N,^13C Z-GRD |

Table S 6: RNA sample concentrations for NMR

| RNA          | conc. RNA alone | conc. RNA in complex |
|--------------|----------------|----------------------|
| 20mer        | 444 µM         | 73.34 µM             |
| 20mer caged  | 100 µM         | 100 µM               |
| G6m1G        | 689 µM         |                      |
| A2-DMA       | 800 µM         | 200 µM               |
| 5'-HP        | 483 µM         |                      |
| 3'-HP        | 1068 µM        |                      |
6 Data collection and analysis of thermodynamic parameters

6.1 Titration 20 nt bistable

**Figure S 10.** ^1^H-NMR Imino proton region of the 20 nt RNA (Figure 1e in the text) titrated with StpA-CTD until a ratio of 1:5 at 25°C. Buffer conditions: 50 mM BisTris, 25 mM NaCl, pH 6.4. Color-coded assignment of the resonances (5'-fold blue, 3'-fold red). Spectrometer: 599 MHz Bruker, $c_{RNA}=100 \mu M - 73.34 \mu M$, 4k ns.

**Table S 7.** Experimental values for different complex ratios of RNA and StpA-CTD. Obtained by division of the integral of imino resonances U17 from the 3'-fold and U11 from the 5'-fold.

| RNA:StpA-CTD | $K = \text{int(U17)/int(U11)}$ |
|-------------|-------------------|
| 1:0         | 2.94 ± 0.07       |
| 1:1         | 3.39 ± 0.11       |
| 1:2         | 4.02 ± 0.11       |
| 1:3         | 4.28 ± 0.14       |
| 1:4         | 4.28 ± 0.18       |
| 1:5         | 4.23 ± 0.12       |
6.2 Temperature Rows and free energy analysis

Figure S 11. $^1$H-NMR Imino proton region of the 20 nt RNA (left) and the RNA in complex with 5 eq. of StpA-CTD (right). Buffer conditions: 50 mM BisTris, 25 mM NaCl, pH 6.4. Color-coded assignment of the resonances (5'-fold blue, 3'-fold red). Spectrometer: 900/599 MHz Bruker. cRNA=477 µM/73.94 µM, 1k/6k ns.

Table S 8. Experimental values of equilibrium constant K for the RNA and the RNA in complex with 5 eq. StpA-CTD at different temperatures. Obtained by division of the integral of imino resonances U17 from the 3'-fold and U11 from the 5'-fold. Also ΔG according to ΔG=−RTln(K) for RNA and complex at different temperatures.

| Temperature [°C] | K = Int(U17(3'-fold))/Int(U11(5'-fold)) | ΔG [kJ/mol] |
|------------------|----------------------------------------|-------------|
|                  | RNA | Complex 1:5    | RNA   | Complex 1:5  |
| 40               | 1.46 ± 0.04 | 3.9 ± 0.4    | -0.99 ± 0.08 | -3.5 ± 0.3 |
| 35               | 1.90 ± 0.04 | 4.00 ± 0.17  | -1.64 ± 0.05 | -3.55 ± 0.12 |
| 30               | 2.48 ± 0.04 | 4.04 ± 0.19  | -2.29 ± 0.04 | -3.52 ± 0.12 |
| 25               | 3.09 ± 0.04 | 4.23 ± 0.15  | -2.80 ± 0.03 | -3.57 ± 0.09 |
| 20               | 3.60 ± 0.05 | 4.7 ± 0.2    | -3.12 ± 0.03 | -3.77 ± 0.11 |
| 15               | 4.37 ± 0.07 | 5.2 ± 0.4    | -3.53 ± 0.04 | -3.96 ± 0.15 |
| 10               | 5.22 ± 0.09 | 6.2 ± 0.4    | -3.89 ± 0.04 | -4.30 ± 0.16 |
| 5                | 6.64 ± 0.14 | 6.7 ± 0.4    | -4.38 ± 0.05 | -4.38 ± 0.13 |
### 7 ITC isothermal titration calorimetry

Table S9: Overview if all ITC-experiments measured with StpA (c(StpA)=800/920 µM) and all RNA constructs (c(RNA=40µM)) at 5°C and 25°C in Buffer (50 mM BisTRIS, 25 mM NaCl, pH 6.4) and the derived binding parameters. Errors represent the standard deviation of 3 independent measurements.

| RNA     | T   | $K_D$ [µM] | N  | $\Delta H$ [kJ/mol] | $\Delta S$ [J/mol/deg] | $\Delta G$ [kJ/mol] |
|---------|-----|------------|----|---------------------|-------------------------|--------------------|
| 20mer   | 5°C | 9.4 ± 1.3  | 1.84 ± 0.02 | -18.7 ± 1.9 | 29.2 ± 7.6 | -26.8 ± 0.4 |
|         | 25°C| 12.31 ± 1.7| 1.93 ± 0.05 | -30.4 ± 0.6 | -8.0 ± 3.0 | -28.0 ± 0.4 |
| G6-m1G  | 5°C | 16.26 ± 0.7| 1.93 ± 0.07 | -37.4 ± 0.4 | -42.75 ± 1.75 | -25.54 ± 0.08 |
|         | 25°C| 17.52 ± 1.3| 1.80 ± 0.11 | -43.1 ± 0.8 | -53.6 ± 1.9 | -27.1 ± 0.3 |
| A2-DMA  | 5°C | 8.31 ± 0.8 | 2.21 ± 0.26 | -17.10 ± 0.12 | 35.9 ± 0.7 | -27.1 ± 0.3 |
|         | 25°C| 13.6 ± 1.8 | 2.33 ± 0.17 | -24.3 ± 0.8 | 12.1 ± 2.6 | -27.9 ± 0.3 |
| 5'-HP   | 5°C | 13.3 ± 3.7 | 1.74 ± 0.06 | -29.6 ± 0.5 | -12.5 ± 3.1 | -25.8 ± 0.7 |
|         | 25°C| 24.7 ± 0.6 | 1.69 ± 0.05 | -36.6 ± 0.8 | -34.5 ± 2.4 | -26.30 ± 0.06 |
| 3'-HP   | 5°C | 11.3 ± 1.4 | 1.78 ± 0.18 | -18.6 ± 0.7 | 28.0 ± 2.0 | -26.4 ± 0.3 |
|         | 25°C| 16.15 ± 2.6| 1.74 ± 0.05 | -28.2 ± 0.3 | -2.7 ± 1.2 | -27.4 ± 0.5 |

Figure S12: Binding affinity expressed as $K_D$ and the free energy of the binding process of StpA to all RNA constructs at 5°C and 25°C. Error bars represent the standard deviation of 3 independent measurements.

Figure S13. (A) ITC thermogram for StpA-CTD (920 µM) titrated to buffer (50 mM BisTRIS, 25 mM NaCl, pH 6.4) at 25°C, resulting in a baseline. (B) ITC thermograms for StpA-CTD (400 µM) titrated to 5'-SS-OV (40 µM) at 5°C and 25°C.
Figure S 14: Thermodynamic signatures of StpA binding to different RNA constructs at 5°C and 25°C. ΔH is represented by green bars, -TΔS by red bars and ΔG by black bars. Error bars represent the standard deviation of 3 independent measurements.

8 Data collection and analysis real-time NMR

8.1 Degree of photolysis

Table S 10. Degree of photolysis p [%] after the first laser-pulse was determined from the appearing signals, by comparing the signals after the first laser pulse of 355 nm, 1 s and 4 W with signals of the unmodified RNA or complex at the same temperature. The RNA concentration was 100 µM.

| Temperature [°C] | K (1s) | % 5' (unmodified) | K (1s) | % 5' (unmodified) | K (1s) | % 5' (unmodified) | % p | % 5' (unmodified) | K (1s) | % 5' (unmodified) | % p | % 5' (unmodified) | % p |
|-----------------|--------|-------------------|--------|-------------------|--------|-------------------|-----|-------------------|--------|-------------------|-----|-------------------|-----|
| 5               | 3.4    | 22.8              | 6.9    | 12.7              | 89.9   | 2.6               | 27.9| 6.5               | 13.3   | 85.4              |
| 10              | 1.0    | 49.1              | 5.8    | 14.6              | 65.5   | 1.9               | 34.3| 6.0               | 14.4   | 80.0              |
| 17              | 1.2    | 45.7              | 4.3    | 18.8              | 73.0   | 0.7               | 59.9| 5.1               | 16.4   | 56.5              |
| 25              | 0.5    | 65.3              | 3.4    | 22.7              | 57.4   | 1.2               | 46.4| 4.5               | 18.1   | 71.7              |
8.2 Analysis of uncaged RNA sample

An analytical denaturing 15% PAGE (polyacrylamide) was performed to ensure that laser light, StpA-CTD or PEG-8000 do not degrade uncaged RNA. Uncaged RNAs do not show degradation, also in complex with StpA-CTD or in presence of PEG-8000. After uncaging the RNA is at the same horizontal position as unmodified RNA.

Figure S 15. Quality control of uncaged RNA, uncaged RNA in complex with StpA and in presence of PEG-8000 at different temperatures by analytical denaturing 15% PAGE.

8.3 Fit of kinetics rates

The individual kinetic traces of the imino proton signals were extracted from the pseudo 2D kinetic spectra. Fitting of the traces was done according to Wenter et al. (1) dependent on the corresponding equilibrium constant K (see SI table S8) for a reversible unimolecular reaction:

\[
\text{normalized signal}(3') = \frac{K}{K+1} \left( 1 - e^{-k(3'-5')t/(1+1/K)} \right)
\]

\[
\text{normalized signal}(5') = \frac{1}{K+1} \left( 1 + K e^{-k(5'-3')t/(1+1/K)} \right)
\]

\[
k_{3'-5'} = \frac{k_{5'-3'}}{K}
\]

Table S 11. Refolding kinetics data for the RNA at different temperatures. Rate constants \(k_{3'-5'}\) and calculated from these rate constants \(k_{5'-3'}\) according to \(K = k_{5'-3'}/k_{3'-5'}\), both given with fit error. Error of mean values is the standard deviation of all corresponding rates.

| RNA | U11 | U17 | G9 | G10 | mean values all bases |
|-----|-----|-----|----|-----|-----------------------|
| Temperature [°C] | \(k_{5'-3'} \times 10^2 \text{s}^{-1}\) | \(k_{5'-3'} \times 10^2 \text{s}^{-1}\) | \(k_{5'-3'} \times 10^2 \text{s}^{-1}\) | \(k_{5'-3'} \times 10^2 \text{s}^{-1}\) | \(k_{5'-3'} \times 10^2 \text{s}^{-1}\) |
| 5   | 1.0±0.3 | 13.8±0.6 | 11.0±0.5 | 9.8±0.4 | 11±3 |
| 10  | 17.1±1.2 | 14.6±1.1 | 14±2 | 23.9±2 | 16±8 |
| 17  | 41±2 | 52±5 | 56±7 | 58±7 | 55±8 |
| 25  | 300±5 | 280±50 | 200±60 | 280±50 | 240±40 |

| Temperature [°C] | \(k_{5'-3'} \times 10^2 \text{s}^{-1}\) | \(k_{5'-3'} \times 10^2 \text{s}^{-1}\) | \(k_{5'-3'} \times 10^2 \text{s}^{-1}\) | \(k_{5'-3'} \times 10^2 \text{s}^{-1}\) |
| 5   | 1.55±0.05 | 2.08±0.10 | 1.48±0.07 | 1.65±0.08 | 1.7±0.3 |
| 10  | 3.3±0.3 | 2.8±0.3 | 2.6±0.5 | 4.6±0.6 | 3.1±1.2 |
| 17  | 10.3±0.6 | 13.1±1.2 | 14.1±1.7 | 14.6±1.8 | 14±2 |
| 25  | 96±19 | 91±19 | 64±9 | 90±20 | 84±15 |
Table S 12. Refolding kinetics data for the RNA in complex with three eq. of StpA-CTD at different temperatures. Rate constants k_{5'-3'} and calculated from these rate constants k_{3'-5'} according to K= k_{5'-3'}/k_{3'-5'}, both given with fit error. Error of mean values is the standard deviation of all corresponding rates.

| Temperature [°C] | k_{5'-3'} [10^{-3} s^{-1}] | k_{0'-5'} [10^{-3} s^{-1}] | k_{0'-3'} [10^{-3} s^{-1}] | k_{5'-3'} [10^{-3} s^{-1}] |
|------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| 5                | 27.7±3                      | 20.4±1.8                    | 24.2±3                      | 24.9±2                      | 25±3                       |
| 10               | 34.4±3                      | 23.3±2                      | 29.6±4                      | 37.6±4                      | 30.4±5                     |
| 17               | 86±13                       | 140±40                      | 180±170                     | 90±30                       | 100±30                     |
| 25               | 180±30                      | 200±40                      | 220±130                     | 150±50                      | 190±40                     |

| Temperature [°C] | k_{0'-5'} [10^{-3} s^{-1}] | k_{0'-3'} [10^{-3} s^{-1}] | k_{0'-3'} [10^{-3} s^{-1}] | k_{5'-3'} [10^{-3} s^{-1}] |
|------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| 5                | 4.2±0.3                     | 3.1±0.3                     | 3.6±0.5                     | 3.7±0.3                     | 3.7±0.6                    |
| 10               | 5.5±0.5                     | 3.8±0.3                     | 4.8±0.7                     | 6.1±0.7                     | 4.9±0.9                    |
| 17               | 17±2                        | 29±9                        | 40±30                       | 19±7                        | 21±7                       |
| 25               | 42±7                        | 48±11                       | 50±30                       | 56±13                       | 46±10                      |

8.4 Kinetics under molecular Crowding conditions with PEG-8000

For these experiments at 278 K and 298 K RNA and RNA with 8 % (w/v) PEG-800 was uncaged as described previous. The kinetic traces were evaluated as described in SI 7.3. The kinetic traces for PEG-8000 and for the RNA alone, both were fitted with the corresponding equilibrium constant for the RNA at the corresponding temperature (see SI Table S 8).

Figure S 16. ^1H NMR spectra of imino and aromatic protons of the 20 nt RNA alone (light lines) and with 8% (w/v) PEG-800 (bold lines) (c(RNA)= 100 µM). Caged: O^6-(S)-NPE modified guanosine at position G6, uncaged: same samples after photolysis by a laser pulse (λ=355 nm, P=4 W, t=1 s) after 30 min of equilibration at 25°C.
Table S 13. Refolding kinetics data for the RNA in presence of 8% (w/v) PEG-8000 at different temperatures. Rate constants $k_{5'-3'}$ and calculated from these rate constants $k_{3'-5'}$ according to $K = k_{5'-3'}/k_{3'-5'}$, both given with fit error. Error of mean values is the standard deviation of all corresponding rates.

| Temperature [°C] | PEG-8000 | U11 | U17 | G9 | G10 | mean values all bases |
|------------------|----------|-----|-----|----|----|-----------------------|
| 5                |          |     |     |    |    |                       |
|                  | $k_{5'-3'}$ | $k_{3'-5'}$ | $k_{5'-3'}$ | $k_{3'-5'}$ | $k_{5'-3'}$ | $k_{3'-5'}$ |
| 16.5±0.5         | 14.6±0.6 | 10.4±0.4 | 23.4±0.4 | 16.4±4.6 |
| 25               | 350±36   | 177±14 | 271±47 | 397±57 | 261±93 |
| 5                |          |     |     |    |    |                       |
|                  | $k_{5'-3'}$ | $k_{3'-5'}$ | $k_{5'-3'}$ | $k_{3'-5'}$ | $k_{5'-3'}$ | $k_{3'-5'}$ |
| 1.54±0.03        | 2.20±0.10 | 1.57±0.10 | 3.52±0.06 | 2.5±0.7 |
| 25               | 113±12   | 58±5 | 88±16 | 129±19 | 67±44 |

Table S 14. Refolding kinetics data for the RNA at 5°C and 25°C. Rate constants $k_{5'-3'}$ and calculated from these rate constants $k_{3'-5'}$ according to $K = k_{5'-3'}/k_{3'-5'}$, both given with fit error. Error of mean values is the standard deviation of all corresponding rates.

| RNA   | Temperature [°C] | U11 | U17 | G9 | G10 | mean values all bases |
|-------|------------------|-----|-----|----|----|-----------------------|
|       |                  |     |     |    |    |                       |
|       | $k_{5'-3'}$ | $k_{3'-5'}$ | $k_{5'-3'}$ | $k_{3'-5'}$ | $k_{5'-3'}$ | $k_{3'-5'}$ |
| U11   | 10.2±0.2         | 13.4±0.4 | 9.3±0.6 | 13.4±0.04 | 11.8±3.2 |
| U17   | 284±26           | 203±17 | 224±36 | 281±34 | 239±55 |
| G9    | 1.54±0.03        | 2.02±0.06 | 1.40±0.10 | 2.02±0.06 | 1.8±0.5 |
| G10   | 92±9             | 66±6 | 72±12 | 91±11 | 70±30 |

Table S 15. Acceleration factor of refolding of 20 nt bistable RNA in presence of 8% (w/v) PEG-8000 or 3 equivalents StpA for rate constants $k_{5'-3'}$ and $k_{3'-5'}$. Error of the acceleration factor was calculated by propagation of the percentage error of the individual rates.

| acceleration factor | PEG-8000 | StpA |
|---------------------|----------|------|
|                     | 5°C | 25°C | 5°C | 25°C |
| $k_{5'-3'}$         | 1.39±0.39 | 1.07±0.37 | 2.17±0.43 | 0.8±0.10 |
| $k_{3'-5'}$         | 1.39±0.38 | 0.98±0.46 | 2.21±0.44 | 0.55±0.04 |
Data collection and analysis of base pairs stabilities by NMR

9.1 1H 1D characterization supplementary RNA constructs

Figure S17: 1H-NMR imino spectra of the different RNA constructs (left) and the different RNA constructs in complex with three eq. of StpA-CTD (right). Recorded at 600/800 MHz with a jump-return echo sequence for water suppression at 25°C. In the middle, the RNA structures are shown with corresponding spectra aside. Bottom: unmodified RNA with imino signals from both conformations color-coded. G6-m1G and 5'-HP with signals just from the 5'-fold and A2-DMA and 3'-HP with signals from the 3'-fold. All constructs in 50 mM BisTris, 25 mM NaCl, pH 6.4. c_{unmodified}=444 µM (1k ns), c_{G6-m1G}=689 µM (256 ns), c_{5'-HP}=483 µM (1k ns), c_{A2-DMA}=800 µM (1k ns), c_{3'-HP}=1068 µM (64 ns), c_{all complexes}=200 µM (1k ns). * 5% of 3'-fold present.

9.2 Water exchange rates

Pseudo 2D water exchange NMR experiments were conducted and evaluated as described in Rinnenthal et al. (3).
Figure S18. Temperature dependence of the imino proton exchange rate $k_{ex}$ for the individual imino protons of the different RNA systems based on the 3'-fold and 5'-fold. Color code: gray data points for the RNA and black for the RNA in complex with 3 eq. of StpA-CTD. Error bars represent the errors of the fit.

Table S16. $\Delta H_{diss}$, $\Delta S_{diss}$ and $\Delta G_{diss}$ (T=25°C) for the base pair opening of individual nucleobases within the truncated 3'-HP RNA alone and in complex with 3 eq. of StpA-CTD. Errors represent the standard deviation, calculated with experimental errors of $k_{ex}$.

| 3'-HP | $\Delta H_{diss}$ [kJ/mol] | $\Delta S_{diss}$ [J/mol*K] | $\Delta G_{diss}$ [kJ/mol] [T=298K] |
|-------|-----------------|-----------------|-----------------|
| U17   | 18.76±1.59      | 14.20±5.63      | 14.53±0.16      |
| U18   | 41.33±1.35      | 83.48±4.77      | 16.46±0.08      |
| G10   | 78.99±1.07      | 200.26±3.84     | 19.31±0.07      |
| G9    | 51.61±0.74      | 110.15±2.77     | 18.79±0.09      |
| G6    | 57.28±1.25      | 131.21±4.42     | 18.18±0.07      |

Table S17. $\Delta H_{diss}$, $\Delta S_{diss}$ and $\Delta G_{diss}$ (T=25°C) for the base pair opening of individual nucleobases within the trapped A2-DMA RNA alone and in complex with 3 eq. of StpA-CTD. Errors represent the standard deviation, calculated with experimental errors of $k_{ex}$.

| A2-DMA | $\Delta H_{diss}$ [kJ/mol] | $\Delta S_{diss}$ [J/mol*K] | $\Delta G_{diss}$ [kJ/mol] [T=298K] |
|--------|-----------------|-----------------|-----------------|
| U17    | 26.59±1.10      | 40.18±3.51      | 14.62±0.05      |
| U18    | 52.45±1.10      | 119.17±3.48     | 16.93±0.05      |
| G10    | 76.25±0.071     | 190.83±2.55     | 19.38±0.05      |
| G9     | 51.20±0.41      | 108.36±1.56     | 18.91±0.07      |
| G6     | 74.08±0.89      | 183.89±3.10     | 19.28±0.05      |

Table S18. $\Delta H_{diss}$, $\Delta S_{diss}$ and $\Delta G_{diss}$ (T=25°C) for the base pair opening of individual nucleobases within the unmodified 3'-fold RNA alone and in complex with 3 eq. of StpA-CTD. Errors represent the standard deviation, calculated with experimental errors of $k_{ex}$.

| 3'-fold | $\Delta H_{diss}$ [kJ/mol] | $\Delta S_{diss}$ [J/mol*K] | $\Delta G_{diss}$ [kJ/mol] [T=298K] |
|---------|-----------------|-----------------|-----------------|
| RNA     |                 |                 |                 |
| U17     | 36.83±0.45      | 68.28±1.82      | 16.49±0.10      |
| U18     | 58.41±0.11      | 131.73±7.01     | 19.15±0.11      |
| G10     | 56.82±2.11      | 118.41±6.39     | 21.53±0.28      |
| G9      | 46.10±2.36      | 83.05±7.19      | 21.35±0.29      |
| G6      | 70.57±0.32      | 164.11±0.65     | 21.67±0.13      |
| complex |                 |                 |                 |
| U17     | 22.52±3.16      | 29.04±11.90     | 13.87±0.27      |
| U18     | 62.44±2.41      | 154.65±9.34     | 16.35±0.36      |
| G10     | 47.86±0.51      | 107.54±3.11     | 15.82±0.39      |
| G9      | 27.48±3.86      | 22.92±11.07     | 18.86±0.60      |
| G6      | 66.50±3.92      | 168.33±14.41    | 16.33±0.36      |

Table S19. $\Delta H_{diss}$, $\Delta S_{diss}$ and $\Delta G_{diss}$ (T=25°C) for the base pair opening of individual nucleobases within the truncated 5'-HP RNA alone and in complex with 3 eq. of StpA-CTD. Errors represent the standard deviation, calculated with experimental errors of $k_{ex}$.

| 5'-HP | $\Delta H_{diss}$ [kJ/mol] | $\Delta S_{diss}$ [J/mol*K] | $\Delta G_{diss}$ [kJ/mol] [T=298K] |
|-------|-----------------|-----------------|-----------------|
| U11   | 39.44±0.94      | 82.43±3.42      | 14.88±0.09      |
| G10   | 85.21±3.60      | 206.79±11.11    | 23.59±0.29      |
| G1    | 16.22±1.04      | 8.27±3.78       | 13.76±0.09      |
| G9    | 33.59±0.31      | 57.08±1.36      | 16.58±0.10      |
Table S 20. \( \Delta H_{\text{diss}}, \Delta S_{\text{diss}} \) and \( \Delta G_{\text{diss}} \) (T=25°C) for the base pair opening of individual nucleobases within the trapped G6-m1G RNA alone and in complex with 3 eq. of StpA-CTD. Errors represent the standard deviation, calculated with experimental errors of \( k_{\text{ex}} \).

|        | \( \Delta H_{\text{diss}} \) [kJ/mol] | \( \Delta S_{\text{diss}} \) [J/mol*K] | \( \Delta G_{\text{diss}} \) [kJ/mol] |
|--------|--------------------------------------|--------------------------------------|--------------------------------------|
| G6-m1G | U11                                  | G10                                  | G9                                   | G1                                   |
|        | 20.84±3.31                           | 49.80±2.21                           | 36.75±3.06                           | 32.43±1.85                           |

Table S 21. \( \Delta H_{\text{diss}}, \Delta S_{\text{diss}} \) and \( \Delta G_{\text{diss}} \) (T=25°C) for the base pair opening of individual nucleobases within the unmodified 5'-fold RNA alone and in complex with 3 eq. of StpA-CTD. Errors represent the standard deviation, calculated with experimental errors of \( k_{\text{ex}} \).

|        | \( \Delta H_{\text{diss}} \) [kJ/mol] | \( \Delta S_{\text{diss}} \) [J/mol*K] | \( \Delta G_{\text{diss}} \) [kJ/mol] |
|--------|--------------------------------------|--------------------------------------|--------------------------------------|
| 5'-fold| RNA                                  | complex                              |                                      |
|        | U11                                  | G10                                  | G9                                   | G1                                   |
|        | 35.30±1.12                           | 93.23±1.59                           | 16.69±0.11                           | 35.34±1.18                           |

9.3 Temperature dependence of destabilization induced by StpA-CTD

Table S 22. \( \Delta \Delta G_{\text{diss}} \) difference in base pair stabilities within the RNA alone and the RNA in complex with 3 eq. of StpA-CTD at different temperatures between 5°C and 40°C.

![Table S 22](image)

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