Supplementary Information

Structure and mechanism of a molecular rheostat, an RNA thermometer that modulates immune evasion by *Neisseria meningitidis*

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RNA preparation

All transcriptions were optimized for Mg^{2+}, NTPs and DNA template concentrations as well as T7 RNA polymerase to provide maximum yields. Typically, 5-20 mls of RNA transcription reactions for unlabeled, $^{13}C/^{15}N$-uniformly labeled, $^{13}C/^{15}N$-AU labeled and partially deuterated RNAs were performed. Unlabeled RNAs were prepared using rNTPs (Carbosynth Inc) whereas ($^{13}C/^{15}N$; AGCU or AU)-labeled and partially deuterated (H6/H8, H1′, H2′, D3′, D4′, D5′/D5″ and D5) RNAs were synthesized with (^{13}C,^{15}N)-labeled ribonucleotide triphosphates (rNTPs, Silantes Inc.) and partially deuterated rNTPs, respectively (Supplementary Table S1). RNAs eluted from chromatography were dialyzed directly into NMR buffer (10 mM NaCl, 10 mM Phosphate pH 6.0, 0.1 μM EDTA) and concentrated to 0.5-1 mM.

Secondary structure

All secondary structures drawing used in this study were generated either by VARNA (1) or mfold (2).

SHAPE

Chemical acylation - Four microliters of 1μM RNA were diluted to 36μL with final buffer content of 100mM HEPES pH 8.0 and 100mM NaCl. Three 9μL aliquots of the RNA solution were distributed to individual Eppendorf tubes and combined with either 1μL DMSO (control), 1μL 65mM NMIA (dissolved in DMSO), or 1μL of 130mM NMIA. Reactions were incubated at the desired temperature for 5 half-lives of NMIA hydrolysis according to the equation (3):

$$\text{half-life (minutes)}: 360e^{(-0.102x°C)}$$

Following chemical modification, the RNA solution was diluted to 100μL, adjusted to 0.2M NaCl, 20μg glycogen, and 2mM EDTA and finally precipitated with 3.5x ice cold absolute ethanol. Precipitation was carried out on dry ice for 15 minutes and RNA was finally pelleted at 4°C and 16,000g for 30 minutes. The RNA pellet was allowed to air dry before resuspension in 10μL MilliQ water.

Reverse Transcription and analysis - One picomole of DNA primer was 5′-end labeled with 100μCi γ-[^{32}P]-ATP and 10 units of T4 polynucleotide kinase (PNK) in 50μL reaction adjusted to 1x PNK buffer. The labeling reaction was incubated at 37°C for 30 minutes. In order to separate the labeled RNA from unincorporated nucleotides, the RNA solution was diluted to 100μL and applied to a 1.0mL G50 resin spin column. The ^{32}P-labeled DNA was filtered through the resin at 2,773xg at room temperature for 2 minutes. Five microliters of modified RNA (~0.5pmol) were combined with 1.5pmol ^{32}P-labeled DNA primer and annealed by heating at 95°C for 5 minutes followed by 5 minutes on ice. Reverse transcription was initiated upon addition of the enzyme mixture (100U Superscript III Reverse Transcriptase (RT), 1.5x RT buffer, 12.5mM DTT, 1.25mM of each dNTPs (dATP, dCTP, dGTP, dITP) followed by incubation at 52°C for 10 minutes. Similarly, sequencing reactions were prepared using 1pmol of unmodified RNA per each reaction. Each sequencing reaction included either 0.5mM of ddATP or ddCTP or ddTTP or 12.5μM ddGTP. The reaction was stopped by degrading the RNA with 200mM NaOH at 95°C for 5 minutes. The cDNA mixture was neutralized with 14.5μL acid solution (80mM unbuffered Tris in 2x Novex TBE urea loading dye) at 95°C for 5 minutes. Four μl of

each sequencing and modification reaction were loaded onto a pre-warmed 8% denaturing polyacrylamide gel and separated by electrophoresis at 70 W for 2 hours. The resulting gel was fixed with a solution of 40 % methanol and 10% acetic acid and then transferred to Whatman paper. The fixed gel was dried at 80°C for 30 minutes and exposed overnight onto a storage phosphor screen.

Autoradiographs were imaged with a Typhoon laser scanner and manipulated with Semi-Automated Footprinting Analysis (SAFA) software (4). Secondary structure analysis was conducted with RNAstructure (5), a software program that generates a pseudo Gibbs free energy term from the SHAPE reactivities (i.e. band intensities) to guide the secondary structure prediction.

Generation of modified plasmids

NEBuilder® assembly tool was used to design primers using pEGFP-N2_S3_GFP plasmid as a template per the manufacturer's instructions. Overlap primers were designed to include the mutated residues (Supplementary Tables S6 and S7). Upstream and downstream fragments for each modification were ligated into pEGFP plasmid digested with Not-1 and Nde-1 using the Gibson Assembly® master mix (New England Biolabs, UK) as per manufacturer's instructions. Assembled products were transformed into *E. coli* DH5α.

Bacteria and growth conditions

Escherichia coli was grown in Luria-Bertani (LB) broth (2% w/v in dH_2O, Oxoid, UK) or on LB agar (1% w/v) plates. All liquid bacterial cultures were grown in 5 ml of media inoculated from a single colony overnight at 37 ˚C with shaking (180 r.p.m.). Bacteria grown overnight were diluted 1/100 in media and grown to an abs_{600} of ~0.4 in the presence of Kanamycin (50µg/ml).

SDS-PAGE and Western blotting

Proteins were separated on polyacrylamide gels alongside Precision Plus All Blue markers (Bio-Rad, USA) and transferred to immobolin-P polyvinylidine fluoride (PVDF) membranes (Millipore, USA) using the semi-dry transfer system (Trans-Blot Turbo system, Bio-Rad, USA). For Western blot analysis, membranes were washed three times in 0.05% (w/v) dry milk/PBS with 0.05% (v/v) Tween-20 for 10 minutes, then incubated with the primary antibody for one hour. Membranes were washed again three times and incubated for a further hour with a secondary, HRP-conjugated antibody. Binding was detected with an Amersham ECL Western Blotting Detection kit and exposed to ECL Hyperfilm (GE Healthcare). An α-GFP mouse antibody (JL-8, Clontech) was used at a final dilution of 1:16,000, α-RecA rabbit antibody (Abcam, UK) was used at a final dilution of 1:5,000. Secondary Goat α-rabbit IgG HRP-conjugated antibody (Santa Cruz, UK) and Goat α-mouse IgG HRP-conjugated antibody (Dako, UK) were used at a final dilution of 1:3,000.

NMR spectral assignments

Base paired residues and exchangeable protons were identified based on imino-imino and Cytidine NH_2-Guanine NH1 and Adenine H2-Uridine H3 patterns in 2D ^1H-^1H-NOESYs (120 ms mixing time) acquired in H_2O at both 7°C and 15°C. Sequential assignments of non-exchangeable protons followed typical H1′-H6/H8 NOE patterns observed in 2D ^1H-^1H NOESYs (120ms mixing time) acquired in D_2O at 7°C and 25°C. Further validation and structural restraints were obtained from H2′-H6/H8 and H1′-H2′ NOEs of samples incorporating rNTPs deuterated at the ribose H3′, H4′, and

H5′/5″ and pyrimidine base H5. Pyrimidine H5/H6 resonances were identified from ^{1}H-^{1}H TOCSYs (30 and 70ms) acquired in D_2O. Correlated ^{15}N and ^{13}C chemical shifts were determined from ^{1}H-^{15}N HSQC, ^{1}H-^{13}C HSQC, and ^{1}H-^{13}C edited NOESY-HSQC experiments. Additional assignments for the UUCG tetraloop were correlated with chemical shift values from previously reported structures (BMRB 5705).

Spectral assignments for CssA2 were guided by the assignments of the three smaller fragments (Supplementary Figure S1). Imino patterns for the base pairs were verified by comparison of the H_2O ^{1}H-^{1}H NOESY for CssA3, CssA4 and CssA5 acquired on Bruker Avance 800 MHz and 7°C. The ^{1}H-^{1}H NOESY of CssA was done on deuterated nucleotides acquired in the same conditions. The H1′-H6/H8 walk was validated in a similar way by comparing the D_2O ^{1}H-^{1}H NOESYs. Non-exchangeable protons at the positions H1′, H2″, H5, H6, H8, and H2; the exchangeable imino protons and the corresponding nitrogen atoms, the exchangeable NH_2 protons, and H3′ proton when resonances were obtained, but carbon resonances were not fully assigned for CssA2 because increased relaxation broadened the peaks too significantly.

Specific challenges and solutions:

CssA3: Assignments for CssA3 were somewhat challenging due to inherent dynamics near the internal bulge (A5 and G6) and the upper bulge formed by U14, U15, U28, C29, and C30. In the lower helix, weak base pairing of U4-A39 was apparent from A39 (H2)-U4 (H3) NOEs at 7°C and the typical cross-strand and sequential NOEs to A4H1′, A5H1′, and U40H1′, but base-pairing could not be established for A7-U37 despite the typical cross strand and sequential NOEs from the A7H2 to U37H1′, U38H1′ and G8H1′. Neither of the G3-U40 or G6-U38 wobble pairs could be identified. A13-U31 showed typical cross-strand and sequential AH2 to H1′ NOEs but no observable H2 to H3 NOE or HNN-COSY(6) transfer. At low temperature (7°C), U15 and U28 form a U-U base pair which could be established from the cross-strand NOEs from U15 (H3) to U28(H3) and U27(H3) (Supplementary Figure S2). Assignments of CssA3 resulted in the identification of 56.4% of nonexchangeable protons (all H1′, H2″, H5, H6, H8, H2, and 35% of H3′) and 55% of the exchangeable protons excluding the H2′ hydroxyl (70% of imino protons), 8.5% of the ribose carbons (43 % of the C1′), 64% of the base carbons (C5, C6, C8, and C2) and 54% of the imino nitrogens.

CssA4: The assignments of CssA4 were straightforward with a clear imino-imino walk throughout the RNA. Strong NOEs from 9 AU and 2 GU base pairs in the helix observed at 25 °C indicate the molecule is fully base paired. Additionally, the unusual AC mismatch is also apparent even at 25 °C (Supplementary Figure S3). Assignment of CssA4 resulted in identification of 61.5% of nonexchangeable protons (all H1′, H2″, H5, H6, H8, H2, and 72% of H3′) and 60% of the exchangeable protons excluding H2′ (85% of H1 and H3 iminos), 13% of the ribose carbons (entirely from assignment of 64% of the C1′), 81% of the base carbons (C5, C6, C8, and C2) and 85% of the imino nitrogen.

CssA5: CssA5 iminos could be assigned for the upper and lower helices flanking C9-G13 and U32-A36; however the A-C mismatch could not be established, as observed instead for CssA4 (Supplementary Figure S4). NMR assignment of CssA5 resulted in the identification of 51.3% of nonexchangeable ribose and base protons (all H1′, H2″, H5, H6, H8, H2) and 41% of exchangeable protons excluding

H2′ (79% of iminos), 43% of the base carbons (C5, C6, C8, and C2) and 86% of the imino nitrogens. The ribose carbons (C1′) were not assigned for CssA5.

Altogether, transfer of the CssA3, CssA4, and CssA5 assignments to CssA2 resulted in the identification of 56% of nonexchangeable protons (all H1′, H2″, H5, H6, H8, H2, and 41% of H3′) and 46% of the exchangeable protons excluding H2′ hydroxyls (82% of imino protons), and 68% of the imino nitrogens.

Structure determination

Experimental constraints

We generated a restraint table for CssA2 by combining distance restraint and torsion angle restraints from the segments CssA3 and CssA4, while the CssA5 segment was used only for validation. Restraints from CssA3 spanned resonances G16-U53; those for CssA4 spanned the resonances G1-A14 and U55-C68, while CssA5 contributed resonances around the A-C mismatch, namely A14-U17 and A52-U55. Structural calculations were performed first for CssA3 and CssA4, as described in below, and for CssA5, which was used only for the AC mismatch and the GA bulge, which were incorporated in the calculation of CssA2 (Supplementary Figure S4).

The NMR experimental constraints are summarized in Supplementary Table S4. We obtained a total of 920 NOEs to calculate the structure of CssA2, which includes 212 intra-residue, 531 sequential and 177 non-sequential NOE restraints derived from NOESY spectra (mixing times of 50, 120, and 250ms). Distance constraints were classified as strong (1.8-3.0 Å), medium (1.8-4.0 Å), weak (1.8-5.0 Å) and very weak (3.5-6.5 Å for NOEs observed in H_2O spectra and 1.8-6.5 Å for D_2O NOEs) based on their peak intensities and comparison with peaks corresponding to known interproton distances.

A total of 110 H-bond constraints were also used. The observation of J_{NN} couplings from HNN-COSY experiments were the predominant means of validating hydrogen bonding restraints; however, restraints for weak base pairs (A-U, A-C, and U-U base pairs) were also incorporated based on characteristic NOE patterns observed in 2D ^{1}H-^{1}H NOESYs (120ms mixing time recorded at 7°C and 25°C). Potential A-U base pairs showing typical cross-strand H1′-H2, sequential H1′-H2, and strong AH2-UH3 imino NOEs were considered base paired despite the lack of HNN-COSY transfer.

Residual dipolar couplings (RDCs) were measured from modulation of the resonance intensity between ^{1}H-^{13}C or ^{1}H-^{15}N couplings acquired for isotropic and partially aligned samples using the ARTSY pulse sequence. RDCs were obtained for the following internuclear vectors: H8-C8, H6-C6, H5-C5, H2-C2 (Adenine), H1′-C1′ (ribose), and ^{1}H-^{15}N (base imino resonances).

Several less clearly defined base pairs could be established confidently from the analysis of the smaller RNA segments due to more favorable spectral characteristics. In the lower helix, base-pairing of U4-A39 was apparent from U4(H3)-A39(H2) NOEs but the A7-U37 and A13-U31 base pair could not be confirmed through inter-strand imino NOEs or HNN-COSY transfer, despite the typical cross strand and sequential NOEs from the A7H2 to U37H1′, U38H1′ and G8H1′. Neither the G3-U40 nor G6-U38 could be identified conclusively, but the U15-U28 base pair could be established from the unusual cross-strand NOEs from U15 (H3) to U28(H3) and U27(H3) (Figure 5E). However, base pairs found within

CssA4 could be easily assigned in the full RNA, identifying 9 AU and 2 GU base pairs, and an AC mismatch apparent even at 25 °C (Supplementary Figure S3).

The sugar conformation and population of the major C2′-endo and C3′-endo conformers were estimated from the observed peak intensity in DQF-COSY and ^{1}H-^{1}H TOCSY (30ms and 70ms) of unlabeled samples in D_2O. A-form torsion angles for β, γ, and ε were assumed for all nucleotides that produced typical A-form NOE patterns; nucleotides with atypical NOE patterns or that appeared to experience conformational exchange were left unrestrained. The χ angle of unpaired bases were constrained in the anti or syn conformation on the basis of the intensity of H1′-H5/H6/H8 NOEs. Residues within the UUCG tetraloop were restrained with the known UUCG tetraloop torsion angles for β, γ, δ, ε, and χ, obtained from the analysis of structure deposited in the PDB.

Structure refinement

CssA2 structure refinement

CssA2 refinement was performed in two steps. First, local RDCs for the upper and lower helices were introduced from CssA3 and CssA4, respectively, to regularize the local structure. Second, global RDCs measured on CssA2 NH's were introduced to orient the upper and lower stems relative to each other. Both refinement procedures were similar to that described below for CssA3 and CssA4, with the exception of the initial bath temperature for torsion angle dynamics. The first refinement protocol began with starting coordinates read from the lowest energy structure following the initial CssA2 simulated annealing protocol. The first round of RDCs refinement incorporated $^{1}D_{CH}$ and $^{1}D_{NH}$ of CssA3 and CssA4, with a final force constant of 0.25 kcal mol^{-1} Hz^{-1}, allowing Da and R to vary. High temperature dynamics at 2500K was done to adjust the local environment of the upper and lower helices, then the bath was cooled to 298K over 200 steps in 0.4ps, followed by Powell minimizations in torsion angle and Cartesian space. The top 10 structures from the initial refinement were then subjected to additional rounds of refinement to generate 10 structures each with different seeds, for a total of 100 final structures.

The final round of CssA2 refinement used CssA3 and CssA4 $^{1}D_{CH}$ for retaining the local geometry, while $^{1}D_{NH}$ measured on CssA2 were used to globally orient the upper and lower helices. The tensors for all RDCs were allowed to vary; high temperature dynamics was conducted at 1500K to allow for reorientation of the stems and cooled to 298K. The top 10 structures without violations in distance (> 0.3Å) or torsion angle (>5°) restraints were retained and used for further analysis (Supplementary Table S4).

CssA3 and CssA4 refinement

Structure refinement for the segments also makes use of both torsion angle dynamics and Cartesian coordinate minimization. The refinement protocol uses a number of potential terms found in the simulated annealing protocol: NOEs and Hydrogen bonds (final force constant = 50 kcal mol^{-1} Å^{2}), dihedral torsion angles (final force constant = 200 kcal mol^{-1} rad^{-2}), van der Waals repulsion (final force constant of 4 kcal mol^{-1} Å^{2} and with a van der Waals radius scale factor of 0.8), angles (final scale factor of 1; with a maximum deviation of 5°), impropers (final scale factor of 1; with a maximum deviation of 5°), and RAMA (with final weighting of 1). Additionally, the refinement protocol includes further potential terms for correcting both local and overall geometry: planarity restraints for base pair interactions (weight factor = 50), the ORIE database for relative positioning of base

pairs (final scale factor = 0.2), and residual dipolar couplings for orienting the entire molecule (final force constant = 0.75 kcal mol^{-1} Hz^{-1}, with force constants for $^1D_{CH}$ scaled to that for $^1D_{NH}$). The starting coordinates are read from the lowest energy structure without violations following the initial simulated annealing protocol. During the first rounds of refinement, the RDC tensor was allowed to vary in order to find the optimum values of D_a and R; during the final structure calculations, these values were fixed.

The procedure for refinement followed a simple protocol: initial high temperature dynamics using only the phosphate-phosphate non-bonded terms, followed by high temperature torsion angle dynamics (10,000 steps at 3,000K). The bath temperature was gradually reduced from 3,000 to 298 K over 200 steps with increases in a number of force constants (angles, impropers, dihedral angles, NOEs, van der Waals repulsion, RAMA, ORIE, and RDCs). Cooling occurs over 200 steps and, at each step, torsion angle dynamics is ran for 0.4ps followed by Powell minimizations in torsional angle and Cartesian space. The calculation was repeated for 100-200 structures and the 10 lowest energy structures without violations in distance (> 0.3Å) or torsion angle (>5°) restraints were retained for analysis (Supplementary Table S5).

CssA5 Refinement:

Refinement of CssA5 was limited to distance and torsion angle restraints. Refinement followed the same protocol as described for CssA3 and CssA4 but omits incorporation of RDCs, the RDC force constant, and evaluation of the tensor variables D_a and R. A full refinement was not undertaken because the flexibility of the molecule reduced the usefulness of RDCs. The calculations were repeated for 100-200 structures and the 10 lowest energy structures were retained for analysis.

Supplementary Table S1: **Sequences of the RNA molecules used in the present study.**

S.No.	Name of the RNA	Length (nt)	RNA Sequence
1	CssA	110	GGCCUAAUUAUAGCACUUAAUCGAAAUAAAUUUAUGAGUACGU AGAGUAUAAUUAGUAUUCUUCUUUCCAACUUCCUUAUACUUAU AUACUUAUAGAUUCUAAAAUCAUG
2	CssA-Δ8bp	102	GGCCUAAUUAUAGCACUUAAUCGAAAUAAAUUUAUGAGUACGU AGAGUAUAAUUAGUAUUCUUCUUUCCAACUUCCUUAUACUUAU AGAUUCUAAAAUCAUG
3	CssA1	75	GGAAUUUAUGAGUACGUAGAGUAUAAUUAGUAUUCUUCUUUCC AACUUCCUUAUACUUAUAUACUUAUAGAUUCC
4	CssA2	68	GGAAUUUAUGAGUACGUAGAGUAUAAUUAGUCUUCGGACUUCC UUAUACUUAUAUACUUAUAGAUUCC
5	CssA3	42	GGGUAGAGUAUAAUUAGUCUUCGGACUUCCUUAUACUUAUCC
6	CssA4	36	GGAAUUUAUGAGUACCUUCGGAUACUUAUAGAUUCC
7	CssA5	43	GGUGAGUACGUAGAGUAUACUUCGGUAUACUUAUAUACUUACC
8	CssA6	73	GGAAUUUAUGAGUACGUAGAGUAUAAUUAGUAUUCUUCUUUCC AACUUCCUUAUACUUAUAUACUUAUAGAUU
9	CssA7	66	GGAAUUUAUGAGUACGUAGAGUAUAAUUAGUCUUCGGACUUCC UUAUACUUAUAUACUUAUAGAUU
10	CssA71	72	GGAAUUUAUGAGUACGUGAGUAUAAUUAGUAUUCUUCUUUCCA ACUUCCUUAUACUUAUAUACUUAUAGAUU
11	CssA72	72	GGAAUUUAUGAGUACGUAAGUAUAAUUAGUAUUCUUCUUUCCA ACUUCCUUAUACUUAUAUACUUAUAGAUU
12	CssA73	70	GGAAUUUAUGAGUACGUAGUAUAAUUAGUAUUCUUCUUUCCAA CUUCCUUAUACUAUAUACUUAUAGAUU
13	CssA74	73	GGAAUUUAUGAGUAUGUAGAGUAUAAUUAGUAUUCUUCUUUCC AACUUCCUUAUACUUAUAUACUUAUAGAUU
14	CssA75	72	GGAAUUUAUGAGUAUGUAAGUAUAAUUAGUAUUCUUCUUUCCA ACUUCCUUAUACUUAUAUACUUAUAGAUU
15	CssA76	73	GGAAUUUAAUUCAUAUUAGAGUAUAAUUAGUAUUCUUCUUUCC AACUUCCUUAUACUUAGCAUGAGUUAGAUU
16	CssA77	73	GGAAUUUAUGAGUACGAUUCAUAUAUUAGUAUUCUUCUUUCCA ACUUCCUAUAUGAGAUUAUACUUAUAGAUU
17	CssA78	73	GGAAUUUAAUUCAUAUAUUCAUAUAUUAGUAUUCUUCUUUCCA ACUUCCUAUAUGAGAUGCAUGAGUUAGAUU
18	CssA6-Δ8bp	65	GGAAUUUAUGAGUACGUAGAGUAUAAUUAGUAUUCUUCUUUCC AACUUCCUUAUACUUAUAGAUU
19	CssA6-Δ8bp (AU/GC)	65	GGAAUUUAUGAGUACGUAGAGUAUAAUUAGUAUUCUUCUUUCC AACUUCCUUAUGCCUAUAGAUU
20	CssA6-Δ8bp (A/G)	65	GGAAUUUAUGAGUACGUAGAGUAUAAUUAGUAUUCUUCUUUCC AACUUCCUUAUGCUUAUAGAUU
21	CssA6-Δ8bp (U/C)	65	GGAAUUUAUGAGUACGUAGAGUAUAAUUAGUAUUCUUCUUUCC AACUUCCUUAUACCUAUAGAUU

Supplementary Table S2: Thermodynamic properties and translational activity of mutations introduced to alter the stability of the CssA RNA thermometer.

S. No.	Name of the RNA*	Melting point (T_m, °C)	Activity
1	CssA	39.0, 69.0	+
2	CssA6	40.6, 71.0	+
3	CssA71	41.6/69.8	+
4	CssA72	43.6/69.4	+
5	CssA73	43.4/69.8	ND
6	CssA74	40.1/70.0	ND
7	CssA75	44.9/71.3	+
8	CssA76	35.7/67.8	ND
9	CssA77	41.2/70.0	ND
10	CssA78	34.6/66.7	ND
11	CssA6- Δ8bp	37.2/69.8	Loh E. Nature (2013)(7)
12	CssA6- Δ8bp (AU/GC)	40.5, 69.6	Loh E. Nature (2013)(7)
13	CssA6- Δ8bp (A/G)	40.4, 69.3	Loh E. Nature (2013)(7)
14	CssA6- Δ8bp (U/C)	35.5/69.4	Loh E. Nature (2013)(7)

*****Complete RNA sequences can be found in Supplementary Table S1
**ND- not determined

Supplementary Table S3: **Thermodynamic melting points of the different RNAs used in this study**

.	Name of the RNA*	Melting point (T_m, °C)
1	CssA	39.0, 69.0
2	CssA-Δ8bp	35.0, 64.6
3	CssA1	45.2, 70.1
4	CssA2**	50.5
5	CssA3**	46.3
6	CssA4**	59.2
7	CssA5**	59.1
8	CssA6$^{\#}$	40.6, 71.0
9	CssA7**,$^{\#}$	46.0
10	CssA71$^{\#}$	41.6/69.8
11	CssA72$^{\#}$	43.6/69.4
12	CssA73$^{\#}$	43.4/69.8
13	CssA74$^{\#}$	40.1/70.0
14	CssA75$^{\#}$	44.9/71.3
15	CssA76$^{\#}$	35.7/67.8
16	CssA77$^{\#}$	41.2, 70.3
17	CssA78$^{\#}$	34.6/66.7
18	CssA6- Δ8bp	37.2/69.8
19	CssA6- Δ8bp (AU/GC)	40.5, 69.6
20	CssA6- Δ8bp (A/G)	40.4, 69.3
21	CssA6- Δ8bp (U/C)	35.5/69.4

*****Complete RNA sequences can be found in Supplementary Table S1
Note: **- with UUCG tetraloop, $^{\#}$- with two additional GG nucleotides at the 5′-end

Supplementary Table S4: **NMR experimental restraints and structural statistics for CssA2.**

	CssA2
NMR experimental restraints	
Distance restraints	
Total NOEs	920
Intra-residue	212
Inter-residue	708
Sequential (|i-j| =1)	531
Non-sequential (|i-j| >1)	177
Hydrogen bond restraints	110
Dihedral angle restraints	
Total dihedral angles	499
Sugar pucker	272
Based on A-form geometry	199
Based on UUCG tetraloop	28
Residual dipolar coupling	
Total RDCs	135
CH (upper stem) (R-factor)	61 (1.28)
CH (lower stem) (R-factor)	53 (0.81)
NH (full RNA) (R-factor)	21 (1.32)
Da (upper stem)	-50.000±0.000
Rh (upper stem)	0.029±0.006
Da (upper stem)	-50.000±0.000
Rh (upper stem	0.110±0.010
Da (full RNA)	-48.834±1.311
Rh (full RNA)	0.103±0.030
Structure statistics	
Violations (mean and s.d)	
Distance constraints (>0.3 Å)	0
Dihedral angle constraints (> 5°)	0
RDC constraints (> 4 Hz)	0
Max. RDC violation (Hz)	3.4
Deviation from idealized geometry	
Bond lengths (Å)	0
Bong angles (°)	0
Average pairwise r.m.s.d. (Å)	
All RNA heavy (1-68, Å)	2.04
All RNA backbone (1-68; Å)	2.02
Upper stem heavy (20-50, Å)	1.09
Upper stem heavy (20-50, Å)	1.15
Lower stem heavy (1-14+55+68, Å)	0.68
Lower stem backbone (1-14+55+68, Å)	0.69
Middle heavy (10-20+50-59, Å)	1.14
Middle backbone (10-20+50-59, Å)	0.94
RMSD before RDCs	
All RNA heavy (1-68, Å)	8.20
All RNA backbone (1-68; Å)	8.13
χ^2 of NMR structure with SAXS scattering profile	2.34

Supplementary Table S5: **NMR Structural statistics for CssA3, CssA4 and CssA5**

	CssA3
Distance restraints	
Total NOEs	475
Intra-residue	108
Inter-residue	367
Sequential (|i-j| =1)	279
Non-sequential (|i-j| >1)	88
Hydrogen bonds	64
Dihedral angle restraints	
Total dihedral angles	310
Sugar pucker	168
Based on A-form geometry	114
Based on UUCG tetraloop	28
Residual dipolar coupling	
Total RDCs	64
CH (R-factor)	55 (0.29)
NH (R-factor)	9 (1.52)
Da	28.150±0.027
Rh	0.093±0.000
Structure statistics	
Violations (mean and s.d)	
Distance constraints (>0.3 Å)	0
Dihedral angle constraints (> 5°)	0
RDC constraints (> 4 Hz)	0
Max. RDC violation (Hz)	3.6
Deviation from idealized geometry	
Bond lengths (Å)	0
Bong angles (°)	0
Average pairwise r.m.s.d. (Å)	
All RNA heavy (1-42, Å)	1.72
All RNA backbone (1-42; Å)	1.64
RNA upper stem heavy (15-28, Å)	0.74
RNA upper stem backbone (15-28, Å)	0.76
RNA lower stem heavy (1-12+32-42, Å)	1.29
RNA lower stem backbone (1-12+32-42, Å)	1.13
RMSD before RDC	
All RNA heavy (1-42, Å)	6.11
All RNA backbone (1-42; Å)	5.72
RNA upper stem heavy (15-28, Å)	0.48
RNA upper stem backbone (15-28, Å)	0.48
RNA lower stem heavy (1-12+32-42, Å)	3.68
RNA lower stem backbone (1-12+32-42, Å)	3.56
χ^2 of NMR structure with SAXS scattering profile	5.16

	CssA4
Distance restraints	
Total NOEs	544
Intra-residue	129
Inter-residue	415
Sequential (|i-j| =1)	297
Non-sequential (|i-j| >1)	118
Hydrogen bonds	74
Dihedral angle restraints	
Total dihedral angles	255
Sugar pucker	144
Based on A-form geometry	88
Based on UUCG tetraloop	23
Residual dipolar coupling	
Total RDCs	90
CH (R-factor)	73 (0.28)
NH (R-factor)	17 (1.18)
Da	25.324±0.469
Rh	0.025±0.000
Structure statistics	
Violations (mean and s.d)	
Distance constraints (>0.3 Å)	0
Dihedral angle constraints (> 5°)	0
RDC constraints (> 4 Hz)	0
Max. RDC violation (Hz)	1.2
Deviation from idealized geometry	
Bond lengths (Å)	0
Bong angles (°)	0
Average pairwise r.m.s.d. (Å)	
All RNA heavy (1-36, Å)	0.70
All RNA backbone (1-36; Å)	0.56
RMSD before RDC	
All RNA heavy (1-36, Å)	2.50
All RNA backbone (1-36; Å)	2.29
χ^2 of NMR structure with SAXS scattering profile	3.02

	CssA5
Distance restraints	
Total NOEs	456
Intra-residue	102
Inter-residue	354
Sequential (|i-j| =1)	265
Non-sequential (|i-j| >1)	89
Hydrogen bonds	72
Dihedral angle restraints	
Total dihedral angles	299
Sugar pucker	172
Based on A-form geometry	109
Based on UUCG tetraloop	18
Residual dipolar coupling	
Total RDCs	0
CH	0
NH	0
Da	Not Applicable
Rh	Not Applicable
Structure statistics	
Violations (mean and s.d)	
Distance constraints (>0.3 Å)	0
Dihedral angle constraints (> 5°)	0
RDC constraints (> 4 Hz)	Not Applicable
Max. RDC violation (Hz)	Not Applicable
Deviation from idealized geometry	
Bond lengths (Å)	0
Bong angles (°)	0
Average pairwise r.m.s.d. (Å)	
All RNA heavy (1-43, Å)	2.57
All RNA backbone (1-43; Å)	2.47
RNA upper stem heavy (15-30, Å)	0.49
RNA upper stem backbone (15-30, Å)	0.45
RNA lower stem heavy (1-7+37-43, Å)	0.47
RNA lower stem backbone (1-7+37-43, Å)	0.44
RMSD before RDCs	Not Applicable
χ^2 of NMR structure with SAXS scattering profile	Not Applicable

Supplementary Table S6: Primers used in this study.

Name	Mod	Sequence 5'- 3'
Upstream_fwd (Nde-1)		agtacatcaagtgtatcaTATGCCAAGTACGCCCCC
Downstream_rev (Not-1)		ttatgatctagagtcgcggccGCTTTACTTGTACAGCTCG
Upstream_rev_GGins	CssA6	ctacgtactcataaattccTATTTCGATTAAGTGCTATAATTAGGCC
Downstream_fwd_GGins	CssA6	aatcgaaataggaatttatgagtacgtagAGTATAATTAGTATTCTTCTTTCCAAC
Upstream_rev_GGins_Adel	CssA71	cacgtactcataaattccTATTTCGATTAAGTGCTATAATTAGGCC
Downstream_fwd_GGins_Adel	CssA71	aatcgaaataggaatttatgagtacgtgAGTATAATTAGTATTCTTCTTTCCAAC
Upstream_rev_GGins_Gdel	CssA72	ttacgtactcataaattccTATTTCGATTAAGTGCTATAATTAGGCC
Downstream_fwd_GGins_Gdel	CssA72	aatcgaaataggaatttatgagtacgtaAGTATAATTAGTATTCTTCTTTCCAAC
Upstream_rev_GGins_Gdel_CUsub	CssA75	ttacatactcataaattccTATTTCGATTAAGTGCTATAATTAGGCC
Downstream_fwd_GGins_Gdel_CUsub	CssA75	aatcgaaataggaatttatgagtatgtaAGTATAATTAGTATTCTTCTTTCCAAC

Supplementary Table S7: Plasmids used in this study

Name	Modification	Plasmid
S3_GFP	Wild Type	pEGFP-N2
S3_CssA6_GFP	GG insertion	pEGFP-N2
S3_CssA71_GFP	A deletion	pEGFP-N2
S3_CssA72_GFP	G deletion	pEGFP-N2
S3_CssA75_GFP	G deletion and C-U substitution	pEGFP-N2

Supplementary Figure Legends:

Supplementary Figure S1: NMR Assignments for CssA2 and superposition of its fragments CssA3, CssA4, and CssA5. H1′-H6/H8 assignment 'walk' for CssA2. Spectral assignments matched to the smaller constructs (CssA3/4/5) are shown with various colored lines. CssA3 assignments are colored in red; those for CssA4 are shown in green and assignments for CssA5 are presented in magenta color.

Supplementary Figure S2: Assignment and NMR structure ensemble of CssA3. (A) Complete H1′-H6/H8 walk in 2D ^{1}H-^{1}H NOESY recorded in 99.9 % $^{2}H_2O$ on Bruker Avance 800 MHz spectrometer at 25 °C. Ten out of 14 iminos were observed, excluding the G1-C43 terminal base pair that frays. C2′-endo sugar pucker is observed only in the tetraloop. (B) Superposition of 10 final NMR structures of CssA3 with backbone rmsd superposition of 1.6 Å, calculated with NOEs and RDCs constraints.

Supplementary Figure S3: Assignment and NMR structure ensemble of CssA4. (A) Complete H1′-H6/H8 walk in 2D ^{1}H-^{1}H NOESY recorded in 99.9 % $^{2}H_2O$ on Bruker Avance 800 MHz spectrometer at 25 °C. All imino peaks predicted from the secondary structure are observed. C2′-endo sugar pucker is observed only in the tetraloop. (B) Superposition of 10 final NMR structures of CssA4 with backbone rmsd superposition of 0.6 Å calculated with NOEs and RDCs constraints.

Supplementary Figure S4: Assignment and NMR structure ensemble of CssA5. (A) Complete H1′-H6/H8 walk in 2D ^{1}H-^{1}H NOESY recorded in 99.9 % $^{2}H_2O$ on Bruker Avance 800 MHz spectrometer at 25 °C. 15 out of 18 predicted iminos were observed, excluding the terminal G1-C43 pair. C2′-endo sugar pucker is observed only in tetraloop. (B) NMR ensemble structure of CssA5 with backbone rmsd superposition of 2.5 Å calculated with NOE constraints only but without RDC refinement.

Supplementary Figure S5: The wild type CssA thermometer forms a dimeric structure at the concentrations required for chemical physical characterization. (A) Results of small angle X-ray scattering (SAXS) analysis of the CssA1 and CssA2 variants. CssA1 has an extended shape with a long dimension of 140 Å, approximately twice what is expected from the secondary structure of the stem-loop, whereas CssA2, is around 67 Å long, the dimension expected for a monomeric RNA. (B) Thermal melting of CssA1 and CssA2. CssA1 has two transitions with melting temperatures of 45.2 and 70.1 °C, whereas a single transition with melting temperature of 50.5 °C is observed for CssA2. Even at the concentration of these experiments (about 1000-fold less than NMR), the CssA1 and CssA thermometers are partially dimeric; the higher melting transition corresponds to the breaking of the dimeric structure. (C) SHAPE studies on CssA1 and CssA2 executed in the temperature range 4-42 °C, similar to the condition used for the CssA RNA. The lower panel highlights nucleotides sensitive to SHAPE chemistry as a function of temperature for both CssA1 and CssA2. (D) An overlay of ^{1}H-^{1}H NOESYs recorded in 95% H_2O/5% $^{2}H_2O$ solution, depicting very similar imino spectra (except for the UUCG tetraloop) for CssA1 and CssA2.

Supplementary Figure S6: SHAPE analysis of CssA-Δ8bp RNA. (A) Predicted secondary structure of Δ8bp RNA; bold residues depict the single 8bp sequence followed by the red-colored RBS. A81 and U83 are labeled in blue. (B) Selective 2′-OH acylation analyzed by primer extension (SHAPE) for the Δ8bp RNA performed at different temperatures, well below and above activation of protein synthesis (4-42 °C). A81 and U83 are shown with blue colored arrow.

Supplementary Video Legends:

Supplementary Video S1: Morphing of conformational changes within the core of CssA thermometer near the apical loop and middle region during temperature elevation. The CssA RNA core is represented with blue colored oval cartoon. The RBS is shown with grey colored spheres whereas two copies of 8bp are shown with orange and forest colored spheres. This morph video was created using in-house script in pymol 1.8.0.

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