Supporting Information

Bisaria et al. 10.1073/pnas.1703507114

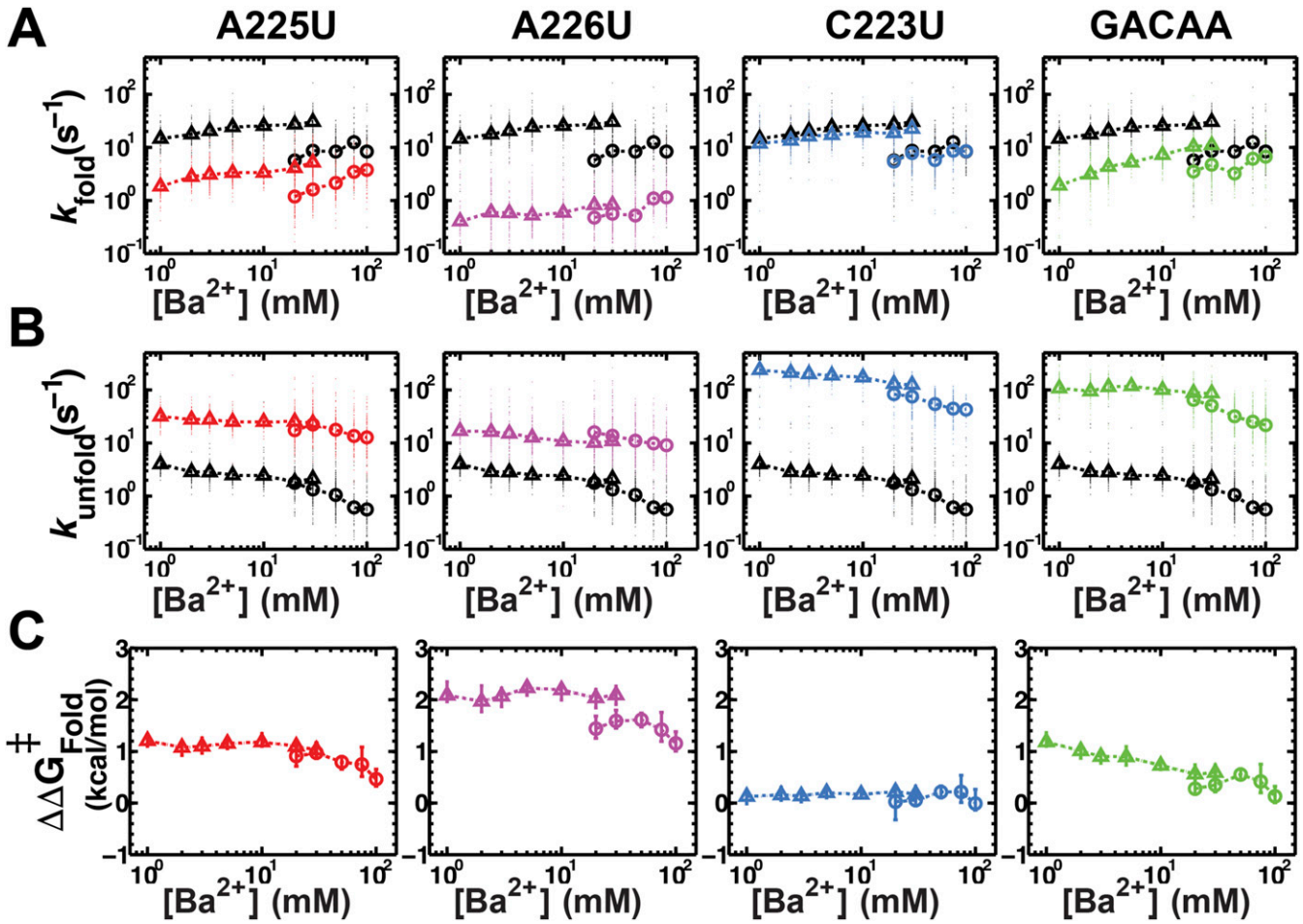

Fig. S1. Folding kinetics of TL/TLR mutations in TL/TLR$_{iso}$ and P4–P6 RNA. Folding (*A*) and unfolding (*B*) rate constants of each mutant (colors as in Fig. 1) and WT (black; repeated in each panel) RNA across Ba^{2+} concentration for TL/TLR$_{iso}$ (open triangles) and P4–P6 (open circles). The rate constant values of each individual molecule under each condition are shown in small circles. Data are presented in Tables S1 and S2. (*C*) The effect of each mutant on the folding rate constant ($\Delta\Delta G^{\ddagger}_{Fold}$; see *Methods*) relative to the WT folding rate constant across [Ba^{2+}] in the TL/TLR$_{iso}$ (open triangles) and P4–P6 (open circles). Errors represent the 95% confidence interval of the mean determined by bootstrapping (*Methods*).

Table S1. Folding parameters of P4–P6 obtained from smFRET

Construct	$[Mg^{2+}]$, mM	$[Ba^{2+}]$, mM	k_{Fold},* s^{-1}	k_{Unfold},* s^{-1}	K_{fold}*,†	ΔG,*,‡ kcal/mol	−ΔΔG,*,§ kcal/mol	*N*
WT	0.0	20.0	5.68 ± 0.8	1.78 ± 0.15	3.2 ± 0.57	−0.68 ± 0.1	—	106
WT	0.0	30.0	8.65 ± 0.97	1.34 ± 0.08	6.48 ± 1.1	−1.09 ± 0.1	—	145
WT	0.0	50.0	8.35 ± 0.8	1.05 ± 0.11	7.94 ± 0.8	−1.21 ± 0.06	—	234
WT	0.0	75.0	12.55 ± 3.94	0.61 ± 0.14	20.46 ± 7.04	−1.76 ± 0.18	—	55
WT	0.0	100.0	8.37 ± 1.43	0.56 ± 0.1	14.89 ± 3.44	−1.57 ± 0.14	—	104
WT	1.0	0.0	0.44 ± 0.04	1.51 ± 0.1	0.29 ± 0.04	0.72 ± 0.07	—	272
WT	2.0	0.0	1.92 ± 0.14	0.52 ± 0.03	3.71 ± 0.6	−0.76 ± 0.1	—	351
WT	3.0	0.0	3.37 ± 0.89	0.4 ± 0.02	8.34 ± 2.41	−1.23 ± 0.17	—	234
WT	5.0	0.0	6.3 ± 0.51	0.23 ± 0.02	26.82 ± 3.87	−1.91 ± 0.09	—	368
A225U	0.0	20.0	1.19 ± 0.15	17.42 ± 2.78	0.07 ± 0.01	1.56 ± 0.11	2.24 ± 0.15	131
A225U	0.0	30.0	1.62 ± 0.24	22.28 ± 2.72	0.07 ± 0.01	1.53 ± 0.07	2.61 ± 0.12	145
A225U	0.0	50.0	2.14 ± 0.12	17.76 ± 2.5	0.12 ± 0.02	1.23 ± 0.07	2.44 ± 0.09	140
A225U	0.0	75.0	3.47 ± 0.42	13.57 ± 2.13	0.26 ± 0.04	0.79 ± 0.07	2.55 ± 0.19	115
A225U	0.0	100.0	3.77 ± 0.32	12.78 ± 1.7	0.29 ± 0.04	0.71 ± 0.08	2.28 ± 0.16	133
A225U	2.0	0.0	0.32 ± 0.04	5.36 ± 0.61	0.06 ± 0.01	1.64 ± 0.08	2.41 ± 0.12	117
A225U	3.0	0.0	0.93 ± 0.05	4.39 ± 0.45	0.21 ± 0.02	0.9 ± 0.06	2.14 ± 0.18	79
A225U	5.0	0.0	1.79 ± 0.12	3.25 ± 0.14	0.55 ± 0.09	0.35 ± 0.1	2.26 ± 0.13	96
A226U	0.0	20.0	0.48 ± 0.09	15.94 ± 5.73	0.03 ± 0.01	2.04 ± 0.16	2.72 ± 0.18	62
A226U	0.0	30.0	0.56 ± 0.1	13.73 ± 1.39	0.04 ± 0.01	1.86 ± 0.07	2.94 ± 0.12	180
A226U	0.0	50.0	0.52 ± 0.05	11.06 ± 1.76	0.05 ± 0.01	1.78 ± 0.06	2.98 ± 0.08	183
A226U	0.0	75.0	1.09 ± 0.13	9.87 ± 2.63	0.11 ± 0.02	1.28 ± 0.08	3.04 ± 0.2	92
A226U	0.0	100.0	1.15 ± 0.17	9.07 ± 1.33	0.13 ± 0.02	1.2 ± 0.09	2.77 ± 0.16	118
A226U	2.0	0.0	0.12 ± 0.02	2.57 ± 0.55	0.05 ± 0.01	1.77 ± 0.13	2.53 ± 0.16	82
A226U	3.0	0.0	0.33 ± 0.03	2.53 ± 0.25	0.13 ± 0.02	1.19 ± 0.1	2.43 ± 0.19	101
A226U	5.0	0.0	0.71 ± 0.04	1.87 ± 0.09	0.38 ± 0.05	0.56 ± 0.08	2.47 ± 0.12	107
C223U	0.0	20.0	5.43 ± 1.45	83.3 ± 22.1	0.07 ± 0.02	1.59 ± 0.15	2.27 ± 0.18	37
C223U	0.0	30.0	7.83 ± 0.69	75.05 ± 3.24	0.1 ± 0.01	1.32 ± 0.06	2.40 ± 0.11	193
C223U	0.0	50.0	5.78 ± 0.4	54.06 ± 4.39	0.11 ± 0.01	1.3 ± 0.03	2.51 ± 0.07	218
C223U	0.0	75.0	8.67 ± 0.6	44.32 ± 4.35	0.2 ± 0.02	0.95 ± 0.05	2.71 ± 0.19	126
C223U	0.0	100.0	8.5 ± 1.83	42.57 ± 6.99	0.2 ± 0.05	0.94 ± 0.12	2.51 ± 0.17	31
C223U	2.0	0.0	0.62 ± 0.15	12.02 ± 2.06	0.05 ± 0.02	1.73 ± 0.21	2.49 ± 0.23	90
C223U	3.0	0.0	2.99 ± 0.29	12.19 ± 0.55	0.25 ± 0.02	0.82 ± 0.05	2.05 ± 0.18	213
C223U	5.0	0.0	4.35 ± 0.41	6.76 ± 0.73	0.64 ± 0.08	0.26 ± 0.07	2.17 ± 0.11	77
GACAA	0.0	20.0	3.52 ± 0.91	64.84 ± 7.7	0.05 ± 0.01	1.69 ± 0.07	2.37 ± 0.12	102
GACAA	0.0	30.0	4.73 ± 1.00	50.65 ± 6.82	0.09 ± 0.01	1.38 ± 0.07	2.47 ± 0.11	153
GACAA	0.0	50.0	3.22 ± 0.16	31.92 ± 4.03	0.1 ± 0.01	1.33 ± 0.05	2.54 ± 0.07	165
GACAA	0.0	75.0	6.12 ± 0.71	25.27 ± 2.86	0.24 ± 0.03	0.83 ± 0.07	2.58 ± 0.19	94
GACAA	0.0	100.0	6.68 ± 0.6	21.75 ± 3.28	0.31 ± 0.04	0.69 ± 0.07	2.26 ± 0.15	158
GACAA	2.0	0.0	1.34 ± 0.16	22.22 ± 2.97	0.06 ± 0.01	1.64 ± 0.09	2.40 ± 0.13	140
GACAA	3.0	0.0	2.02 ± 0.23	20.13 ± 3.03	0.1 ± 0.01	1.34 ± 0.06	2.57 ± 0.18	90

N, number of molecules.

*Mean and error values indicate the bootstrapped 95% confidence interval of the mean.

†$K_{fold} = k_{Fold}/k_{Unfold}$.

‡$\Delta G = -RT\ln(K_{fold})$.

§$\Delta\Delta G = \Delta G_{WT} - \Delta G_{Mut}$.

Table S2. Folding parameters of TL/TLR$_{Iso}$ obtained from smFRET

Construct	[Ba^{2+}], mM	k_{Fold},* s^{-1}	k_{Unfold},* s^{-1}	K_{fold},*,†	ΔG,*,‡ kcal/mol	−ΔΔG,*,§ kcal/mol	*N*
WT	1.0	14.72 ± 0.81	4.02 ± 0.35	3.66 ± 0.6	−0.76 ± 0.1	—	108
WT	2.0	17.7 ± 1.59	2.86 ± 0.12	6.2 ± 0.97	−1.06 ± 0.1	—	104
WT	3.0	20.48 ± 1.58	2.83 ± 0.18	7.22 ± 1.06	−1.15 ± 0.09	—	112
WT	5.0	24.25 ± 1.27	2.48 ± 0.11	9.77 ± 0.94	−1.33 ± 0.06	—	103
WT	10.0	25.52 ± 1.47	2.44 ± 0.15	10.47 ± 1.5	−1.37 ± 0.09	—	126
WT	20.0	27.18 ± 2.55	1.87 ± 0.28	14.55 ± 2.54	−1.56 ± 0.11	—	66
WT	30.0	30.44 ± 3.89	2.13 ± 0.14	14.26 ± 2.12	−1.55 ± 0.09	—	85
A225U	1.0	1.85 ± 0.21	31.8 ± 1.43	0.06 ± 0.01	1.66 ± 0.06	2.41 ± 0.12	108
A225U	2.0	2.79 ± 0.14	27.75 ± 1.35	0.1 ± 0.01	1.34 ± 0.06	2.4 ± 0.11	114
A225U	3.0	3.09 ± 0.43	27.79 ± 1.92	0.11 ± 0.01	1.28 ± 0.05	2.43 ± 0.1	121
A225U	5.0	3.33 ± 0.2	25.17 ± 0.72	0.13 ± 0.02	1.18 ± 0.08	2.5 ± 0.09	118
A225U	10.0	3.34 ± 0.51	25.2 ± 2.47	0.13 ± 0.02	1.18 ± 0.07	2.54 ± 0.11	69
A225U	20.0	4.1 ± 0.29	25.38 ± 1.89	0.16 ± 0.02	1.06 ± 0.06	2.62 ± 0.12	129
A225U	30.0	5.22 ± 0.37	23.78 ± 0.68	0.22 ± 0.02	0.88 ± 0.05	2.43 ± 0.1	178
A226U	1.0	0.41 ± 0.1	16.93 ± 3.55	0.02 ± 0.01	2.17 ± 0.15	2.92 ± 0.17	51
A226U	2.0	0.6 ± 0.17	16.25 ± 2.88	0.04 ± 0.01	1.92 ± 0.09	2.98 ± 0.13	122
A226U	3.0	0.58 ± 0.08	15.13 ± 2.52	0.04 ± 0.01	1.9 ± 0.16	3.05 ± 0.17	69
A226U	5.0	0.53 ± 0.06	12.7 ± 1.08	0.04 ± 0.01	1.85 ± 0.07	3.18 ± 0.09	149
A226U	10.0	0.59 ± 0.06	10.85 ± 1.62	0.05 ± 0.01	1.69 ± 0.09	3.06 ± 0.12	90
A226U	20.0	0.82 ± 0.11	10.12 ± 0.74	0.08 ± 0.02	1.46 ± 0.11	3.02 ± 0.14	86
A226U	30.0	0.83 ± 0.09	10.87 ± 1.42	0.08 ± 0.02	1.49 ± 0.12	3.04 ± 0.14	73
C223U	1.0	11.82 ± 1.36	237.63 ± 17.73	0.05 ± 0.01	1.75 ± 0.08	2.5 ± 0.13	84
C223U	2.0	13.48 ± 1.58	210.38 ± 11.35	0.06 ± 0.01	1.6 ± 0.06	2.66 ± 0.11	114
C223U	3.0	16.14 ± 0.9	199.04 ± 4.55	0.08 ± 0.01	1.46 ± 0.06	2.61 ± 0.1	122
C223U	5.0	17.16 ± 0.97	187.83 ± 12.82	0.09 ± 0.01	1.39 ± 0.04	2.72 ± 0.06	162
C223U	10.0	19.05 ± 1.07	172.48 ± 10.92	0.11 ± 0.01	1.28 ± 0.04	2.65 ± 0.09	98
C223U	20.0	18.65 ± 1.07	131.21 ± 6.12	0.14 ± 0.01	1.14 ± 0.05	2.69 ± 0.12	109
C223U	30.0	22.47 ± 1.72	126.11 ± 3.04	0.18 ± 0.02	1.4 ± 0.05	2.55 ± 0.1	114
GACAA	1.0	1.92 ± 0.33	107.29 ± 14.67	0.02 ± 0.01	2.34 ± 0.25	3.1 ± 0.26	92
GACAA	2.0	3.11 ± 0.25	94.38 ± 6.8	0.03 ± 0.02	1.99 ± 0.28	3.05 ± 0.28	143
GACAA	3.0	4.35 ± 0.37	113.62 ± 3.55	0.04 ± 0.01	1.9 ± 0.07	3.05 ± 0.1	160
GACAA	5.0	5.21 ± 1.1	117.95 ± 10.19	0.04 ± 0.01	1.82 ± 0.11	3.14 ± 0.13	20
GACAA	10.0	7.27 ± 0.35	100.29 ± 4.34	0.07 ± 0.01	1.53 ± 0.05	2.89 ± 0.1	152
GACAA	20.0	10.35 ± 1.58	88.11 ± 2.74	0.12 ± 0.02	1.25 ± 0.07	2.8 ± 0.13	99
GACAA	30.0	11.02 ± 0.89	86.68 ± 4.03	0.13 ± 0.01	1.2 ± 0.06	2.75 ± 0.11	108

N, number of molecules.

*Mean and error values indicate the bootstrapped 95% confidence interval of the mean.

†$K_{fold} = k_{Fold}/k_{Unfold}$.

‡$\Delta G = -RT\ln(K_{fold})$.

§$\Delta\Delta G = \Delta G_{WT} - \Delta G_{Mut}$.

Table S3. DNA and RNA sequences for constructing smP4–P6 and TL/TLR$_{iso}$ constructs

Construct	Variant	Name	Sequence*
P4–P6	WT	5′ piece (104–148)[†]	rGrGrArArUrUrGrCrGrGrGrArArArGrGrGrUrCrArArCrArGrCrCrGrUrUrCrArGrUrArCrCrArArGrUrCrUrCrArGrG
	WT	Middle piece (163–234)[‡]	AGTAATACGACTCACTATAGGCCTTGCAAAGGGTATGGTAATAAGCTGACGGACATGGTCCTAACCACGCAGCCAAGTCCTAAGTCAACAGGGGTGCGAG
	A225U	Middle piece (163–234)[‡]	AGTAATACGACTCACTATAGGCCTTGCAAAGGGTATGGTAATAAGCTGACGGACATGGTCCTAACCACGCAGCCAAGTCCTTAGTCAACAGGGGTGCGAG
	A226U	Middle piece (163–234)[‡]	AGTAATACGACTCACTATAGGCCTTGCAAAGGGTATGGTAATAAGCTGACGGACATGGTCCTAACCACGCAGCCAAGTCCTATGTCAACAGGGGTGCGAG
	C223U	Middle piece (163–234)[‡]	AGTAATACGACTCACTATAGGCCTTGCAAAGGGTATGGTAATAAGCTGACGGACATGGTCCTAACCACGCAGCCAAGTCTTAAGTCAACAGGGGTGCGAG
	WT	3′ piece (250–261 + tail)[†]	**/5Phos/**rGrGrA rUrGrC rArGrU rUrCrA rArCrC rArArA rArUrC rArArC rCrUrA rArArA rCrUrU rArCrA rCrArU
	WT	Cy3 oligo (149–162)[§]	GGAAAC(5-*N*-U)UUGAGAU
	GACAA	Cy3 oligo (149–162)[§]	GGACAAC(5-*N*-U)UUGAGAU
	WT	Cy5 oligo (235–249)[§]	AUCUUC(5-*N*-U)GUUGAUAU
	WT	Surface attachment[‡]	Biotin-TGTGTAAGTTTTAGGTTGATTTTGGT
TL/TLR$_{Iso}$	WT	Loop[†]	5AmMC6/rGrGrCrGrArArArGrCrCrArArArArArArArCrGrUrGrUrCrGrUrCrCrUrArArGrUrCrGrGrCrGrC
	A225U	Loop[†]	5AmMC6/rGrGrCrGrArArArGrCrCrArArArArArArArCrGrUrGrUrCrGrUrCrCrUrUrArGrUrCrGrGrCrGrC
	A226U	Loop[†]	5AmMC6/rGrGrCrGrArArArGrCrCrArArArArArArArCrGrUrGrUrCrGrUrCrCrUrArUrGrUrCrGrGrCrGrC
	C223U	Loop[†]	5AmMC6/rGrGrCrGrArArArGrCrCrArArArArArArArCrGrUrGrUrCrGrUrCrUrUrArArGrUrCrGrGrCrGrC
	GACAA	Loop[†]	5AmMC6/rGrGrCrGrArCrArArGrCrCrArArArArArArArCrGrUrGrUrCrGrUrCrCrUrArArGrUrCrGrGrCrGrC
	WT	Base[†]	5AmMC6/rGrCrGrCrCrGrArUrArUrGrGrArCrGrArCrArCrGrCrCACCAAAATCAACCTAAAACTTACACA

*Underlined residues indicate mutations.
[†]Ordered as a synthetic RNA (IDT) and gel purified. Presented in IDT order nomenclature.
[‡]Ordered as a synthetic DNA (IDT) and transcribed.
[§]Ordered as a synthetic RNA (Dharmacon), where (5-*N*-U) designates the 5-amino allyl-uridine.

Other Supporting Information Files

Dataset S1 (PDF)