Supporting Information:

Shortening the HIV-1 TAR RNA Bulge by a Single Nucleotide Preserves Motional Modes Over a Broad Range of Timescales

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Supporting Discussion 1. G28U-wtTAR and G28U-ΔC24-wtTAR ES2 trap mutants.

In a prior study[1], nine resonances belonging to residues U31 (C6H6 and C1′H1′), C30 (C6H6 and C1′H1′), A22 (C8H8 and C1′H1′), G32 (C8H8 and C1′H1′), and U23-C1′H1′ could not be unambiguously assigned in G28U-wtTAR. Based on the present study, and assignments of the $ES2^{uucg}$ mutant, six of the ambiguous assignments have been resolved and three assignments previously labeled as unambiguous (U23-C6H6, A35-C8H8 and C1′H1′) have been corrected. In total, 14 resonances have been corrected or added to the assignments of G28U-TAR: U23-C6H6, C24-C6H6, A22-C8H8 and C1′H1′, A35-C8H8 and C1′H1′, U40-C6H6 and C1′H1′, C39-C6H6, C41-C6H6 and C1′H1′, G32-N1H1, U31-N1H1, and G43-N1H1.

Among these resonances U23-C6H6 and A35-C8H8 and C1′H1′ were previously not deemed ambiguous, but were updated by switching with ambiguous or unassigned peaks: A22*-C8H8 → A35-C8H8, A22*-C1′H1′→ A35-C1′H1′ and U31*-C6H6 → U23-C6H6. Because new assignments retain very similar carbon chemical shifts, they do not impact the prior agreement reported between the ES2 chemical shifts and those measured in wtTAR-G28U mutants or any of the conclusions in the prior TAR ES2 study.

Resonances previously assigned as U38-N3H3 and G36-N1H1 could not be reconciled with additional data shown here on G28U-ΔC24-wtTAR and the $ES2^{uucg}$ traps. This led us to deduce that G28U-TAR is prone to forming kissing dimers or duplexes resulting in a GU resonance in both wtTAR and ΔC24-wtTAR (updated assignments: G36-N1H1 → G32-N1H1, U38-N3H3 → U31-N3H3). Once again, this correction does not affect the prior RD analysis since similar ^{15}N chemical shifts were maintained for all residues affected.

To expand, NOE connectivity data measured on G28U-ΔC24-wtTAR yielded a tri-guanine walk in G28U-ΔC24-wtTAR (Fig S8, highlighted in orange). This walk could not be explained based on the monomeric G28U-ΔC24-wtTAR secondary structure, but could easily be explained by a kissing dimer or duplex. These NOE connectivities were not observed previously in G28U-wtTAR, possibly due to the lower RNA concentration or poorer resolution in the NOESY spectrum. Indeed, a non-denaturing gel on aliquots of NMR samples used in measuring NOESY spectra (data not shown) shows two well-separated populations for both G28U-wtTAR and G28U-ΔC24-wtTAR. Therefore, at the high concentrations used to collect unlabeled NMR data (≈ 2-3 mM RNA), G28U-wtTAR forms a kissing dimer or duplex in solution. Indeed, lowering the RNA concentration to ≈ 50 μM resulted in the disappearance of the imino assigned to the GU mispair. The formation of a GU mispair associated with the kissing dimer or duplex formation also helps explain the unusual chemical shift of the peak previously assigned as U28, which appears in a region of in a 2D NH spectrum typical of GU mispairs and not WC AU bps[2]. It should be noted that there are still unassigned and ambiguous assignments in G28U-TAR due to severe spectral overlap in the non-exchangeable region of the NOESY spectra.

Supplementary Information Figure Legends

Figure 1: Secondary structure and assignments of ΔC24-wtTAR and ΔC24-TAR^{uucg}.

(A) Overlay of 2D HSQC spectra of wtTAR (black) and ΔC24-wtTAR (red) where large chemical shift perturbations are highlighted with black lines. (B) Secondary structure (left) and 2D HSQC overlays of ΔC24-wtTAR (red) and ΔC24-TAR^{uucg} (blue). Asterisks highlight apical loop residues that differ between RNA molecules.

Figure 2: Comparison of chemical shifts for wtTAR and ΔC24-wtTAR with and without Mg^{2+}. Shown is the secondary structure of wtTAR, highlighting the deleted bulge nucleotide in ΔC24-wtTAR with a red X, and 2D HSQC overlays of wtTAR (black), ΔC24-wtTAR (red), wtTAR in the presence of 4 mM Mg^{2+} (orange)[3]. Black lines highlight significant chemical shift perturbations from wtTAR at low salt conditions to Mg^{2+} bound wtTAR, and ΔC24-wtTAR at low salt conditions.

Figure 3: 2D HSQCs of wtTAR and ΔC24-wtTAR in the presence of Mg^{2+}. (A) Shown are 2D HSQC overlays of magnesium titrations with wtTAR (top) and ΔC24-wtTAR (bottom) at 0 mM (black), 2 mM (blue) and 4 mM (green) Mg^{2+}. (B) 2D HSQC overlays of wtTAR (black) and ΔC24-wtTAR (red) at the 4 mM Mg^{2+} titration point to highlight the similarities of the spectra in comparison to free wtTAR and ΔC24-wtTAR (main Fig 2A, Fig S1A).

Figure 4: ^{13}C Spin Relaxation comparison. Shown are correlation plots of ^{13}C R_1 (A) and R_2 (B) relaxation rates where residues are colored according to helix 1 (red), helix 2 (blue), bulge (orange), and apical loop (green). See insert for symbols depicting the different spins measured.

Figure 5: Residual dipolar couplings measured in ΔC24-wtTAR. (A) Comparison of RDCs measured based on splittings encoded in ^{13}C and ^{1}H dimensions. The unity line is also shown. RMSD was calculated over all RDCs measured and final values utilized for further analyses were obtained from averaging the two sets of RDC measurements (B) 2D overlay of the ^{1}H downfield and ^{13}C upfield TROSY component for ΔC24-wtTAR free

(red) and in the presence of Pf1 phage (blue). (C) Comparison of the measured RDCs in helix 1 (red) and helix 2 (blue) and values back predicted when best fitted to an idealized A-form geometry (see methods).

Figure 6: RD profiles. In all cases, RD profiles show the dependence of $R_2 + R_{ex}$ on spin lock power ($\omega_{eff}/2\pi$) and offset ($\Omega/2\pi$) with global fits (solid lines) to the two-state Laguerre equation (Eq 2)[4]. Error bars represent experimental uncertainty (1 s.d., see methods). (A) RD profiles measured in ΔC24-wtTAR (B) RD profiles measured in wtTAR and ΔC24-wtTAR in 5 mM Mg^{2+}. Shown are representative off-resonance (A35-C8) and on resonance (U38-N3) RD profiles measured in wtTAR and ΔC24-wtTAR. (C) ES3 RD profiles measured in wtTAR and ΔC24-wtTAR.

Figure 7: MC-Fold results for ΔC24-wtTAR. Shown are the top four results from MC-Fold[5] using standard input options exploring the best 15% sub-optimal structures. Structures are in order as they were ranked from MC-Fold and labeled as the ground state (GS) and excited states (ES) as determined by NMR.

Figure 8: NMR Spectra of G28U-ΔC24-wtTAR. (A) Secondary structure of G28U-ΔC24-wtTAR with the mutated G to U highlighted in green. (B) Example imino proton NOE connectives that support a kissing or duplex dimer in G28U-ΔC24-wtTAR. NOE connectivities are colored according to the G28U-ΔC24-wtTAR secondary structure, with the lower helix formation (black), uridine mispair formation (orange) and upper helix walk (red). (C) Exchangable NOE proton walk for the lower helix of G28U-ΔC24-wtTAR from residues G17 to U23, and highlighting the strong NOE for G33 H1'-H8 consistent with

G28U-wtTAR and the *syn* conformation of residue G33. (D) 2D HSQC overlay of ∆C24-wtTAR (blue) and G28U-∆C24-wtTAR (red).

Figure 9: NMR assignments of wtTAR-ES2^{uucg} and ∆C24-wtTAR- ES2^{uucg}. Shown are the non-exchangeable NOE walk for wtTAR-ES2^{uucg} (A) and ∆C24-wtTAR- ES2^{uucg} (B) taken at 10˚C (left) and exchangeable NOE walks taken at 25˚C (right). The imino walks completed are highlighted on the secondary structure inserts**.** (C) 2D HSQCs of ES2^{uucg} traps overlaid on the corresponding spectra of wtTAR and ∆C24-wtTAR. Ambiguous assignments in are marked with an asterix.

Figure 1

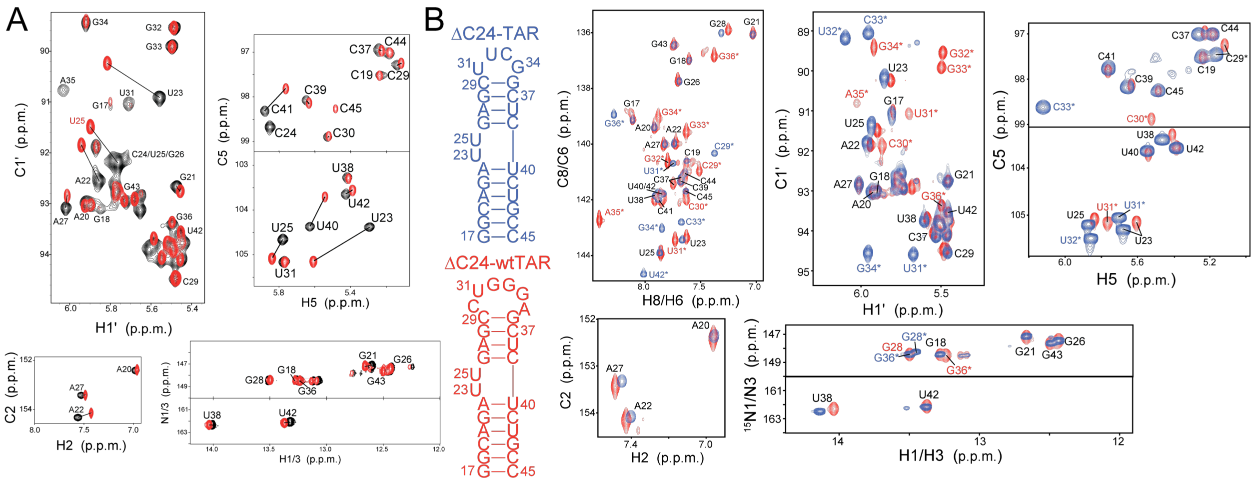


Figure 2

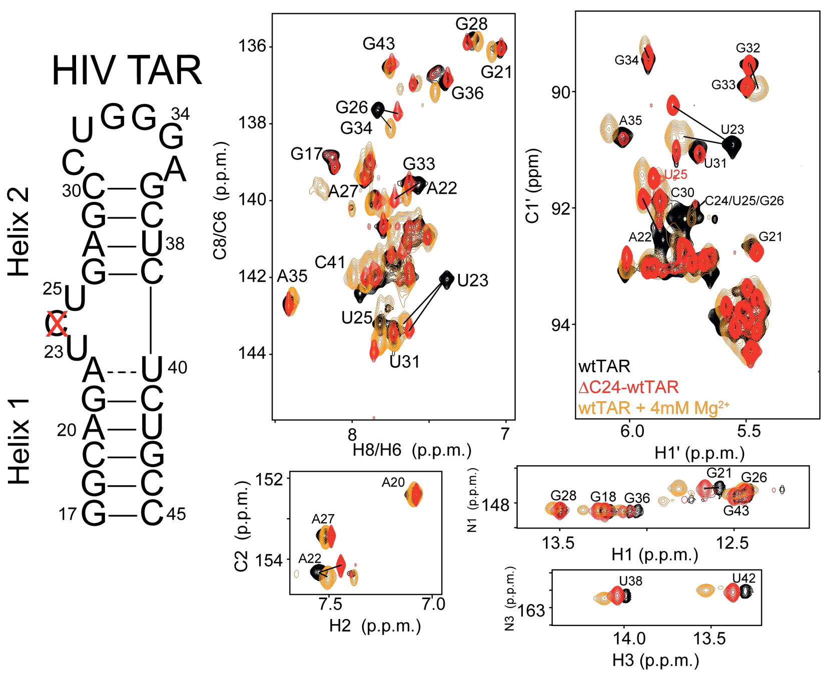


Figure 3

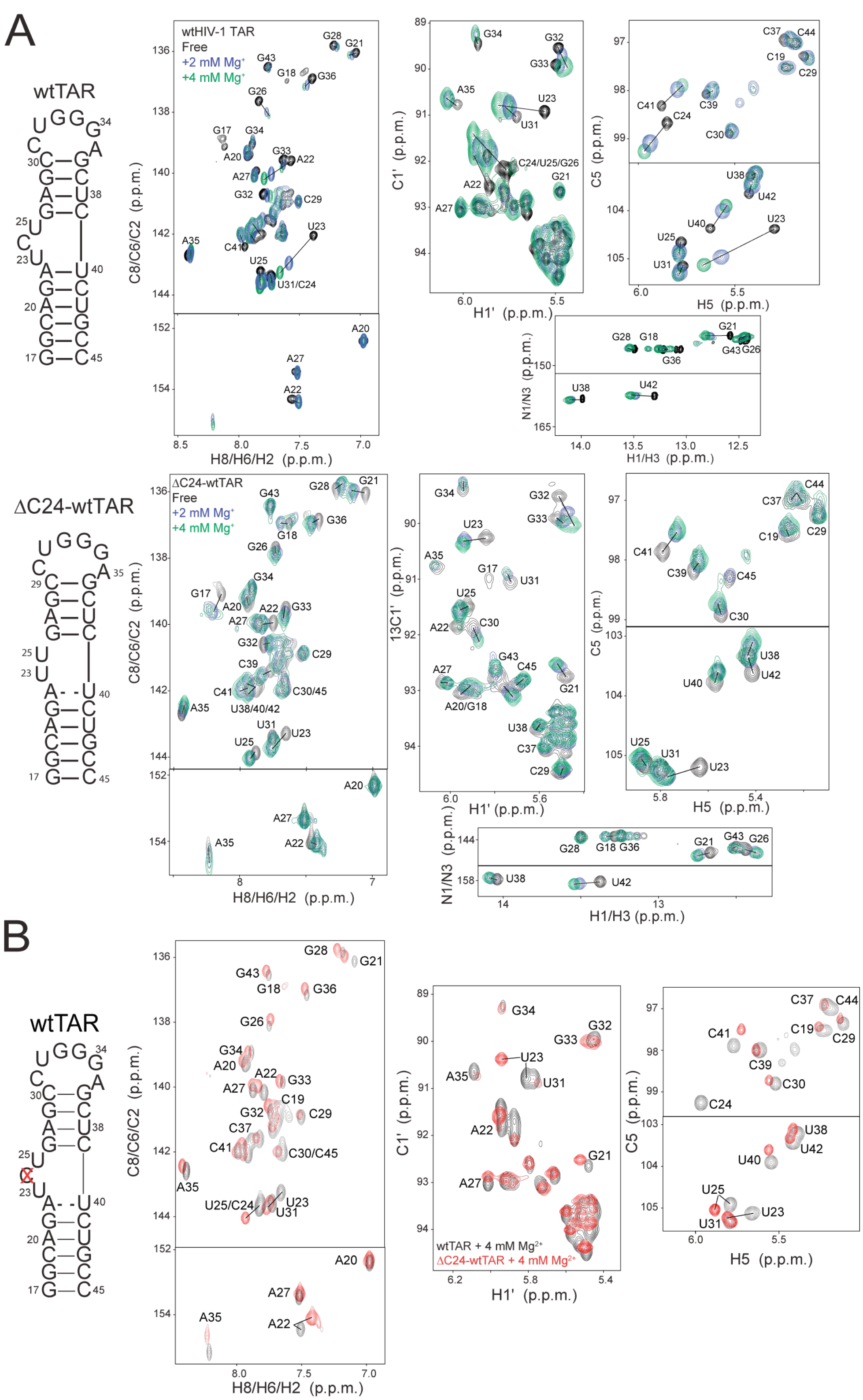

A
wtTAR
ΔC24-wtTAR
wtHIV-1 TAR
Free
+2 mM Mg+
+4 mM Mg+
ΔC24-wtTAR
Free
+2 mM Mg+
+4 mM Mg+
C8/C6/C2 (p.p.m.)
H8/H6/H2 (p.p.m.)
C1' (p.p.m.)
13C1' (p.p.m.)
H1' (p.p.m.)
C5 (p.p.m.)
H5 (p.p.m.)
N1/N3 (p.p.m.)
H1/H3 (p.p.m.)
B
wtTAR
wtTAR + 4 mM Mg2+
ΔC24-wtTAR + 4 mM Mg2+

Figure 4

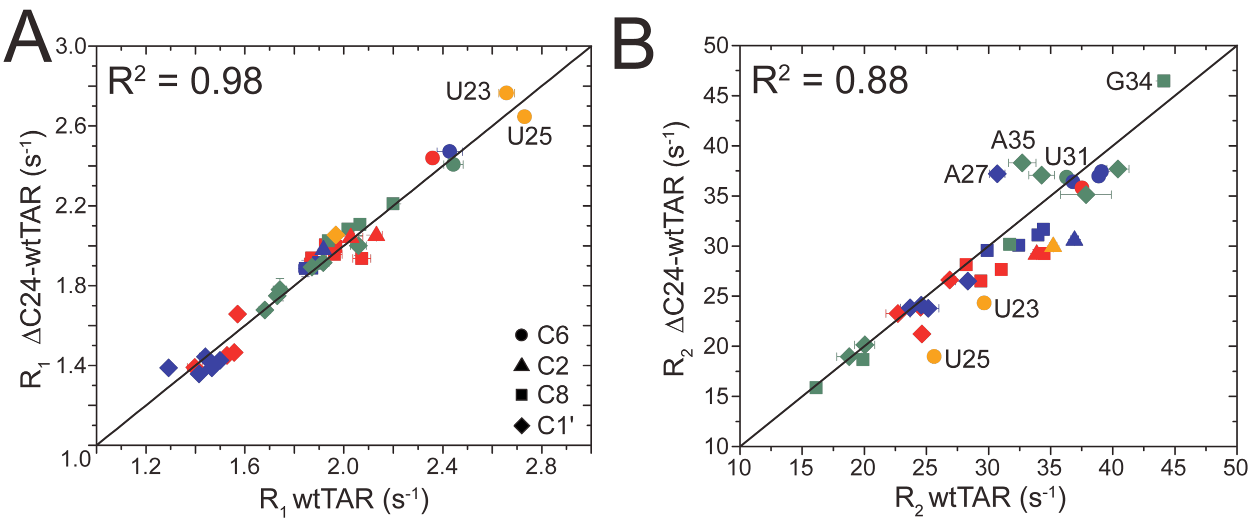


Figure 5

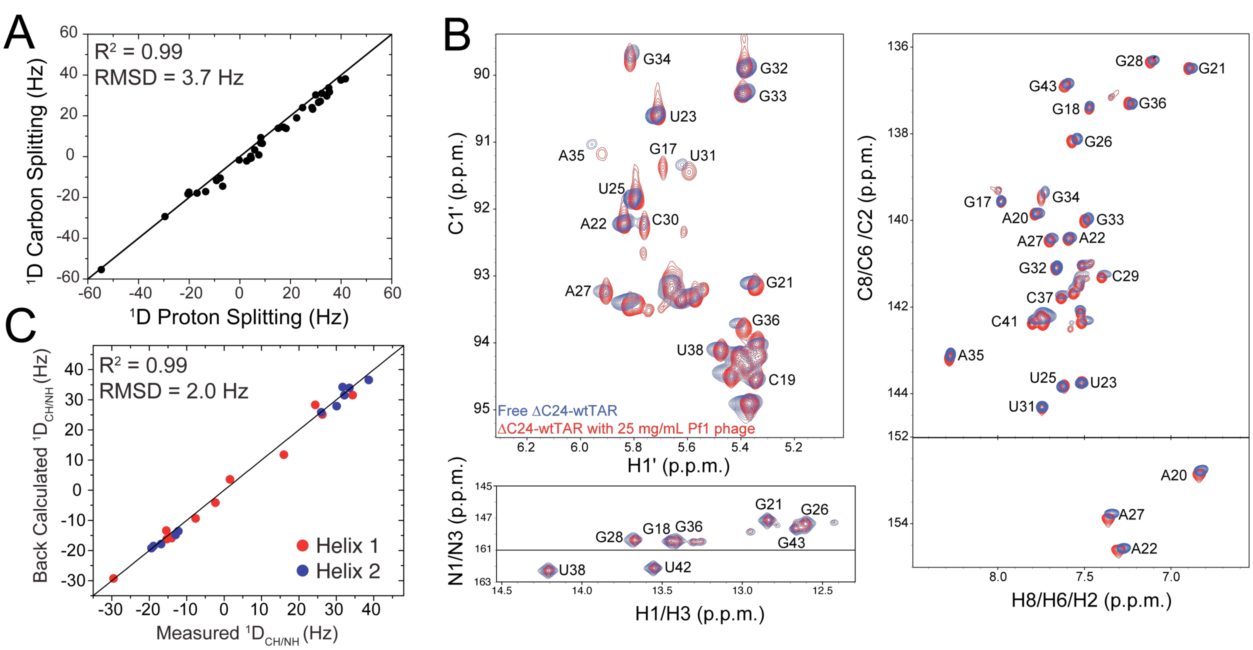
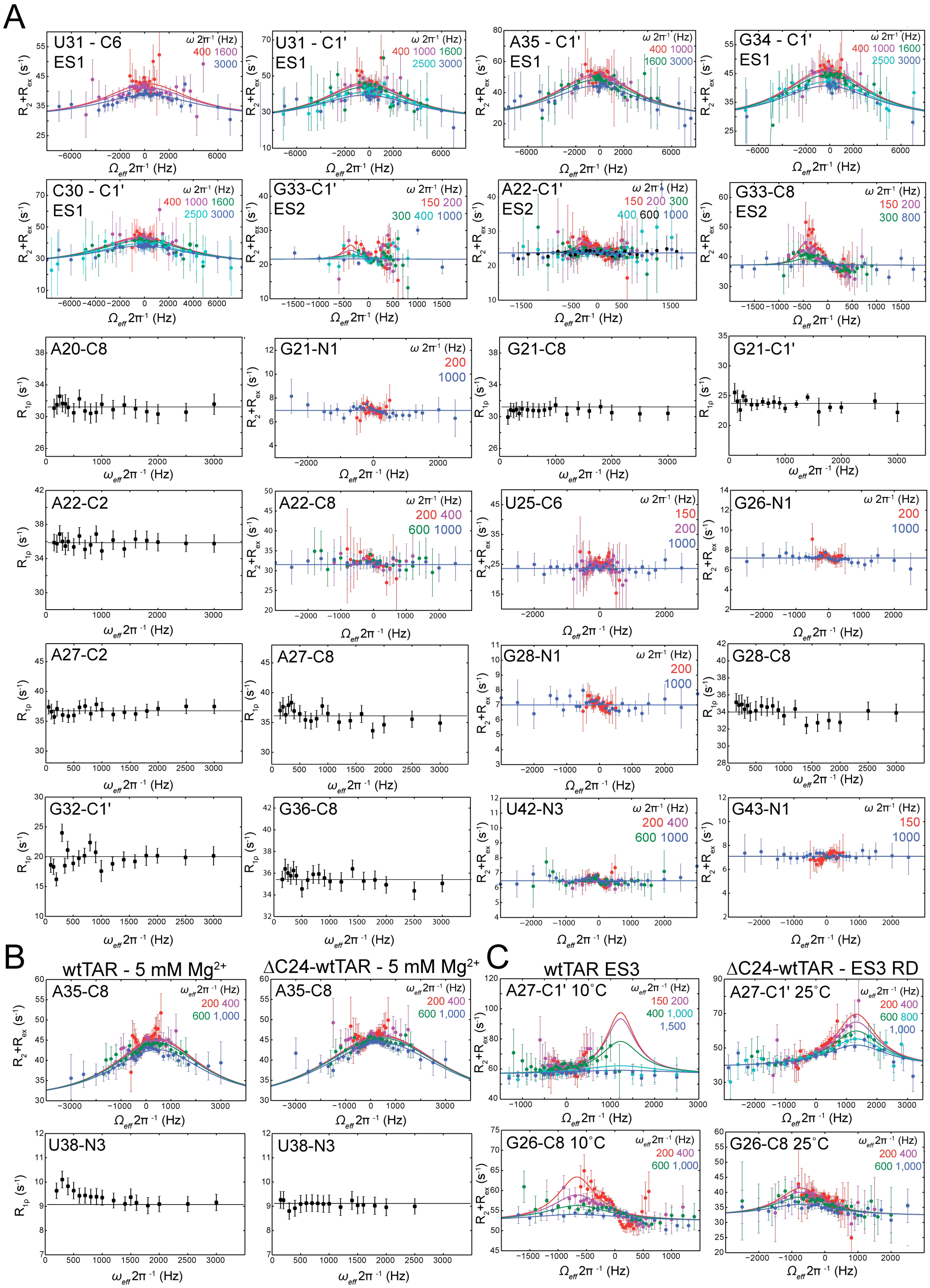


Figure 6



A
U31 - C6
ES1
U31 - C1'
ES1
A35 - C1'
ES1
G34 - C1'
ES1
C30 - C1'
ES1
G33-C1'
ES2
A22-C1'
ES2
G33-C8
ES2
A20-C8
G21-N1
G21-C8
G21-C1'
A22-C2
A22-C8
U25-C6
G26-N1
A27-C2
A27-C8
G28-N1
G28-C8
G32-C1'
G36-C8
U42-N3
G43-N1
B
wtTAR - 5 mM Mg2+
ΔC24-wtTAR - 5 mM Mg2+
A35-C8
A35-C8
U38-N3
U38-N3
C
wtTAR ES3
ΔC24-wtTAR - ES3 RD
A27-C1' 10°C
A27-C1' 25°C
G26-C8 10°C
G26-C8 25°C

Figure 7

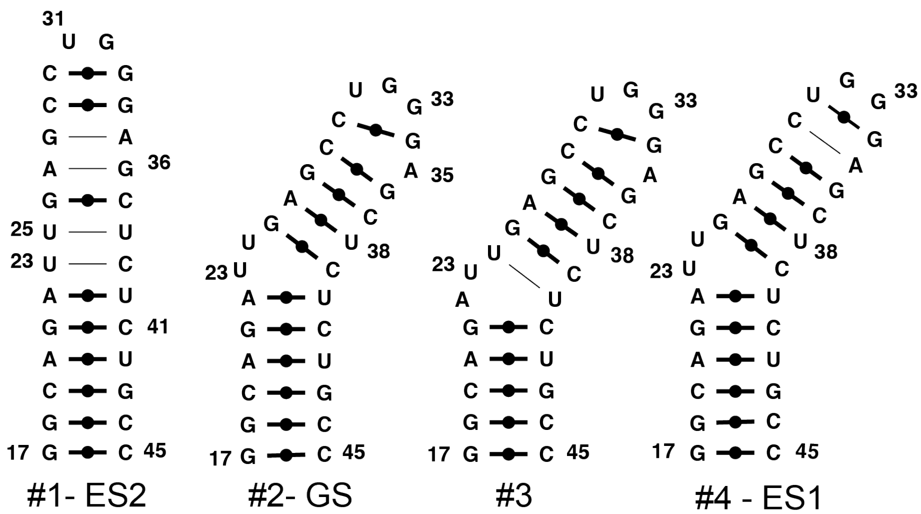


Figure 8

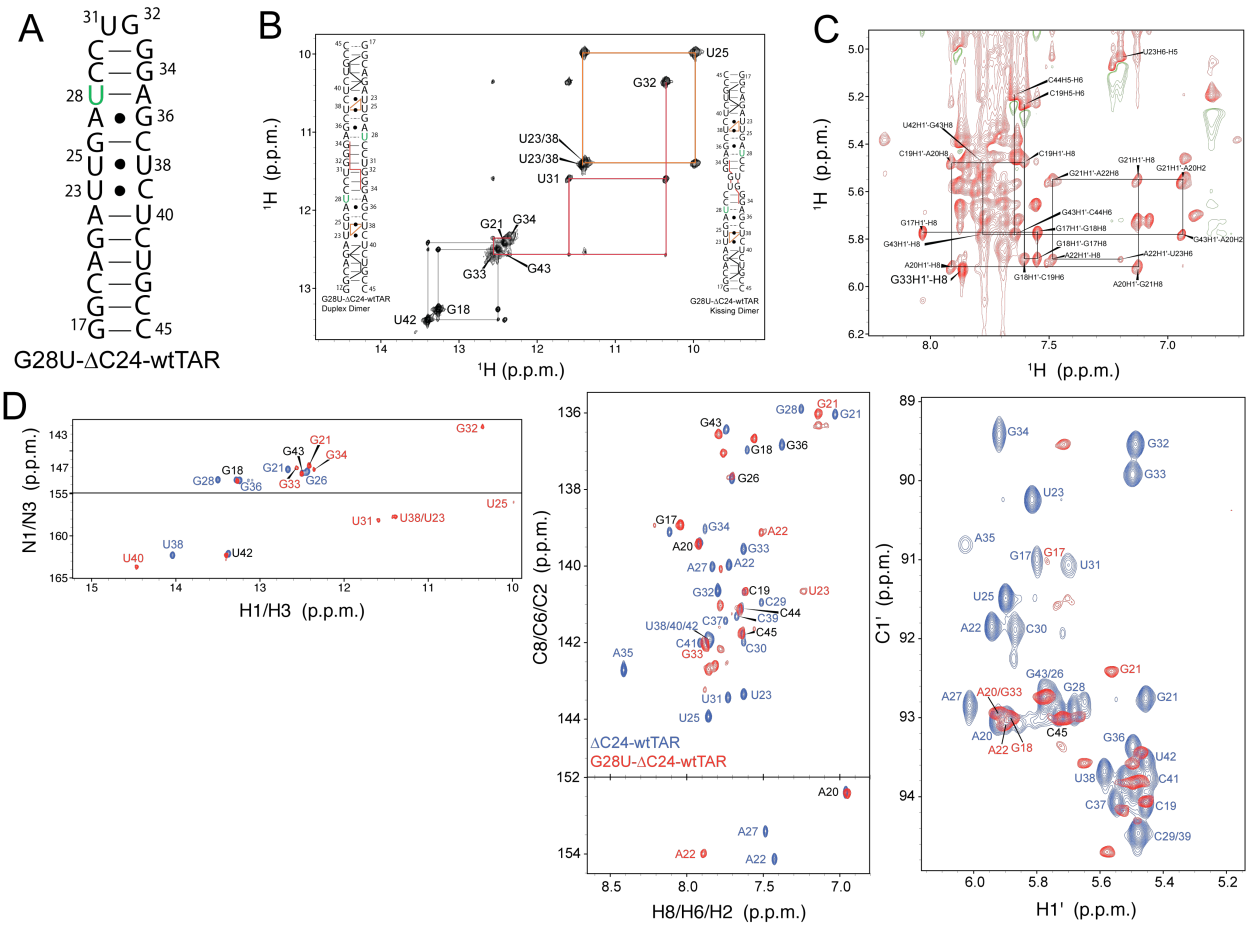
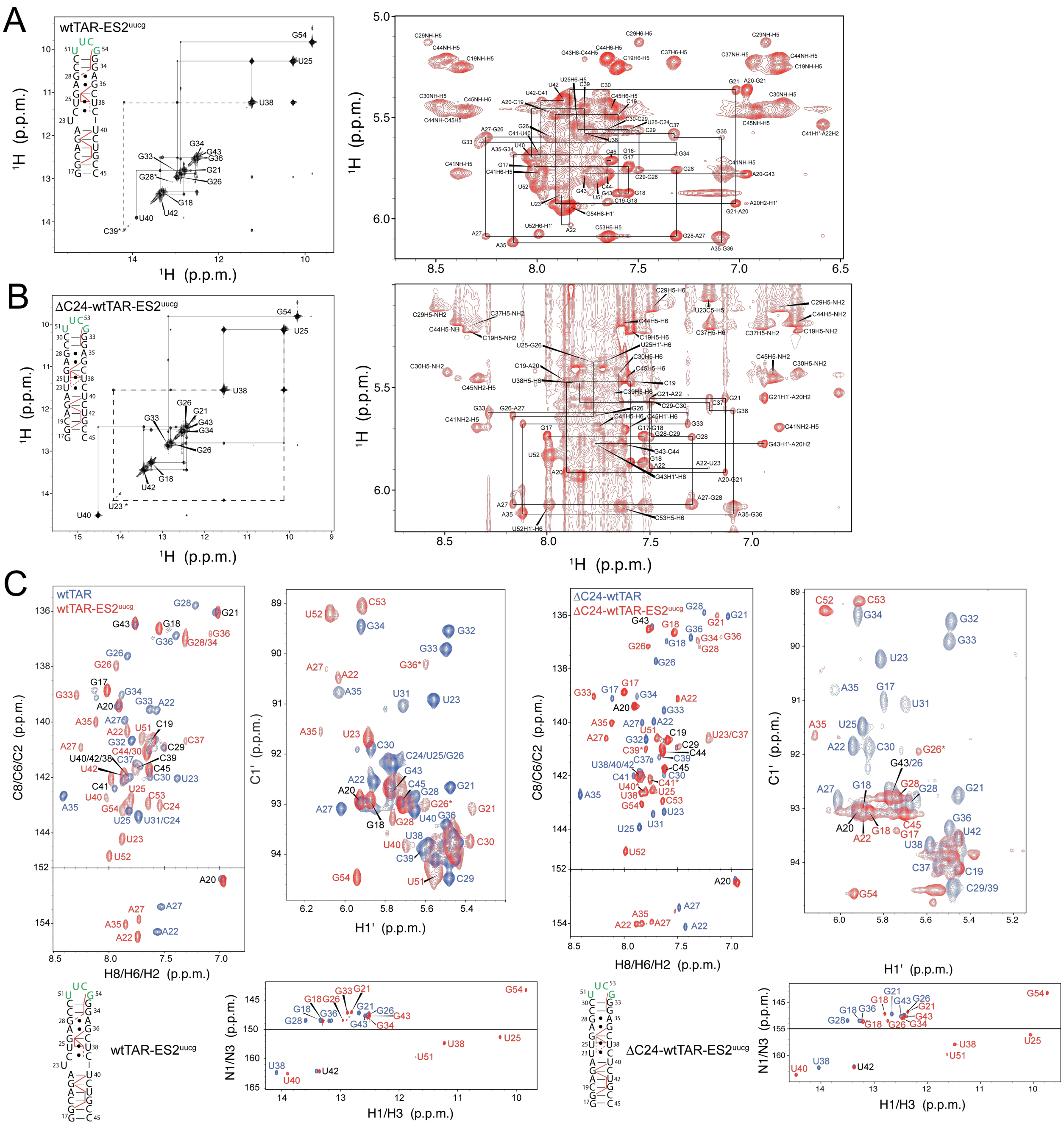

A
B
C
D
G28U-ΔC24-wtTAR
G28U-ΔC24-wtTAR Duplex Dimer
G28U-ΔC24-wtTAR Kissing Dimer
1H (p.p.m.)
H1/H3 (p.p.m.)
N1/N3 (p.p.m.)
C8/C6/C2 (p.p.m.)
H8/H6/H2 (p.p.m.)
C1' (p.p.m.)
H1' (p.p.m.)
ΔC24-wtTAR
G28U-ΔC24-wtTAR

Figure 9



		wtTAR	ΔC24-wtTAR
Aromatic	R_1	10 (2x), 100, 200, 300, 580 (2x)	10, 50 (2x), 150, 350 (2x), 500
	R_2	4, 9, 14, 24, 29, 34, 39, 44, 50	4, 8 (2x), 16, 20, 24, 28, 32, 36, 40 (2x)
Aliphatic	R_1	10 (2x), 90, 190, 300, 600 (2x)	10 (2x), 90, 190, 300, 440, 600 (2x)
	R_2	0.4 (2x), 4, 10, 20, 40, 42 (2x)	0.4 (2x), 4, 10, 30, 42 (2x)

Table 1: Delays (ms) used in R_1 and R_2 relaxation experiments. 2x refers to repeated delays to assist in estimating experimental error.

	Atom	**R_1**	**Error**	**R_2**	**Error**
G17	C8	1.87	0.02	29.39	0.08
G18	C1'	1.40	0.03	26.9	0.5
A20	C8	1.97	0.02	28.2	0.1
	C2	2.13	0.02	33.9	0.3
	C1'	1.47	0.01	24.5	0.6
G21	C8	1.87	0.01	34.5	0.2
	C1'	1.53	0.01	23	1
A22	C8	1.93	0.01	31.03	0.09
	C2	2.03	0.05	35.2	0.2
	C1'	1.57	0.01	24.6	0.3
U23	C6	2.66	0.03	29.67	0.19
	C1'	1.97	0.01	17.1	0.9
U25	C6	2.73	0.01	25.64	0.07
G26	C8	1.84	0.03	32.4	0.2
A27	C8	1.90	0.02	29.9	0.2
	C2	1.92	0.01	36.9	0.3
	C1'	1.29	0.02	30.7	0.6
G28	C8	1.87	0.02	34.0	0.1
	C1'	1.47	0.04	23.9	0.7
C29	C6	2.43	0.05	39.1	0.3
	C1'	1.44	0.02	25.1	0.9
C30	C6	2.44	0.04	36.3	0.5
	C1'	1.68	0.02	38	2
U31	C1'	1.92	0.02	34	1
G32	C8	2.20	0.02	19.9	0.1
	C1'	1.87	0.02	19	1
G33	C8	2.02	0.02	31.71	0.08
	C1'	1.73	0.01	20.0	0.8
G34	C8	1.94	0.01	44.1	0.3
	C1'	1.74	0.03	40.4	0.9
A35	C8	2.07	0.01	16.13	0.03

	C1'	2.06	0.03	33	1
G36	C8	1.90	0.01	34.42	0.06
	C1'	1.41	0.01	28	1
C37	C6	2.40	0.03	38.9	0.1
	C1'	1.47	0.01	24.6	0.2
U38	C1'	1.50	0.01	23.7	0.8
C39	C6	2.39	0.03	36.8	0.3
	C1'	1.52	0.01	23.9	0.6
U40	C1'	1.63	0.03	23	1
C41	C6	2.36	0.02	37.5	0.1
U42	C1'	1.56	0.02	24.7	0.8
G43	C8	1.96	0.03	24.7	0.8
C45	C1'	1.63	0.04	19.2	0.3

Table 2: Carbon R_1 and R_2 relaxation rates (s^{-1}) of wtTAR

	Atom	**R_1**	**Error**	**R_2**	**Error**
G17	C8	1.91	0.01	26.50	0.19
G18	C8	1.94	0.01	29.31	0.19
	C1'	1.39	0.01	29.31	0.19
C19	C6	2.43	0.02	35.07	0.16
A20	C8	2.00	0.02	28.14	0.10
	C2	2.05	0.00	29.25	0.42
	C1'	1.41	0.02	23.91	0.09
G21	C8	1.94	0.00	29.25	0.18
	C1'	1.45	0.02	23.26	0.15
A22	C8	2.00	0.02	27.65	0.19
	C2	2.04	0.02	30.02	0.32
	C1'	1.66	0.03	21.22	0.06
U23	C6	2.76	0.00	24.31	0.07
	C1'	2.05	0.03	17.98	0.11
U25	C6	2.65	0.01	18.97	0.13
	C1'	2.12	0.01	16.38	0.09
G26	C8	1.89	0.04	30.09	0.12
A27	C8	1.92	0.03	29.55	0.10
	C2	1.98	0.02	30.65	0.23
	C1'	1.39	0.02	37.22	0.24
G28	C8	1.89	0.01	31.09	0.18
C29	C6	2.47	0.02	37.41	0.19

	C1'	1.44	0.02	23.79	0.14
C30	C6	2.41	0.02	36.85	0.13
	C1'	1.68	0.02	35.13	0.27
U31	C6	2.75	0.01	28.89	0.06
	C1'	1.92	0.02	37.08	0.24
G32	C8	2.21	0.01	18.66	0.07
	C1'	1.89	0.02	18.97	0.11
G33	C8	2.08	0.01	30.16	0.06
	C1'	1.75	0.02	20.14	0.19
G34	C8	2.02	0.01	46.46	0.25
	C1'	1.78	0.06	37.68	0.24
A35	C8	2.11	0.01	15.86	0.13
	C1'	2.00	0.05	38.30	0.29
G36	C8	1.91	0.01	31.66	0.12
	C1'	1.36	0.03	26.50	0.12
C37	C6	2.45	0.03	36.96	0.38
	C1'	1.39	0.02	24.11	0.06
U38	C1'	1.43	0.02	23.83	0.11
C39	C6	2.44	0.03	36.39	0.08
C41	C6	2.44	0.01	35.78	0.25
G43	C8	1.96	0.01	29.22	0.06
	C1'	1.46	0.02	27.05	0.23
C44	C6	2.45	0.03	35.69	0.12
C45	C6	2.44	0.01	32.98	0.22

Table 3: Carbon R_1 and R_2 relaxation rates (s^{-1}) of ΔC24-wtTAR

Residue	Bond Vector	RDC (Hz)
G17	C8H8	4.2
	C1'H1'	-15.3
G18	C8H8	1.6
	N1H1	-2.3
C19	C1'H1'	-1.8
A20	C8H8	26.3
	C2H2	16.0
G21	C8H8	34.4
	C1'H1'	-29.5
	N1H1	-15.4
A22	C8H8	28.8
	C2H2	29.5
	C1'H1'	8.8
U23	C6H6	13.8
	C1'H1'	7.5
U25	C6H6	1.8
	C1'H1'	-9.1
G26	C8H8	32.2
	N1H1	-12.9
A27	C8H8	26.0
	C2H2	33.5
	C1'H1'	-19.4
G28	C8H8	30.1
	N1H1	-16.8
C29	C6H6	38.7
C30	C1'H1'	7.7
U31	C6H6	-9.7
	C1'H1'	-10.5
G32	C8H8	0.3
	C1'H1'	4.6
G33	C8H8	20.7
	C1'H1'	-1.0
G34	C8H8	32.0
	C1'H1'	14.5
A35	C8H8	2.2
	C1'H1'	-17.4
G36	C8H8	39.9

	C1'H1'	-55.0
	N1H1	-40.7
C37	C6H6	31.7
U38	C1'H1'	-18.8
	N3H3	-12.2
U42	N3H3	-14.0
G43	C8H8	24.4
	N1H1	-7.6

Table 4: RDCs measured on ΔC24-wtTAR at 25˚C in 15 mM sodium phosphate 25 mM sodium chloride and 0.1 mM EDTA at pH 6.4. Estimated error is 3.7 Hz (see methods).

Table 5: List of spin-lock powers and offsets used for $R_{1\rho}$ experiments

ΔC24-wtTAR at 25˚C	**On-resonance spinlock power (Hz)/Off-resonance spinlock power (Hz) with [offsets]**
A20 – C8	150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000
G21 – C8	150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000
G21 – C1'	150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000
G21 – N1	100, 150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000
	200 & ± [20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 225, 250, 300, 350, 400, 500]
	1000 & ± [100, 200, 300, 400, 500, 600, 700, 900, 1100, 1300, 1500, 2000, 2500, 3000, 3500, 4000]
G21 – N1	100, 150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000
	200 & ± [20, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 225, 250, 300, 350, 400, 500]
	1000 & ± [100, 200, 300, 400, 500, 600, 700, 900, 1100, 1300, 1500, 2000, 2500, 3000, 3500, 4000]
A22 – C8	50, 100, 150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000, 3500
	100 & ± [50, 100, 150, 200, 250, 300, 350, 400, 450], [-500]
	200 & [-800] ±[50, 100, 150, 200, 300, 400, 500, 600, 700]
	400 &± [100, 200, 300, 400, 500, 600, 700, 800, 1000, 1200]
	600 & [100, 200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800]
	1000 & [-3000] ± [200, 400, 600, 800, 1200, 1600, 2000, 2500]
A22 – C1'	150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000, 3500
	150 & ± [20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240, 260, 280, 300, 320, 400, 500, 600]
	200 & ± [30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390, 420, 480, 540, 600, 700, 800]
	300 & ± [40, 80, 120, 160, 200, 240, 280, 320, 360, 400, 440, 480, 520, 560, 600, 700, 800, 1000, 1200]
	400 & ± [50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 700, 800, 900, 1000, 1200, 1500]

	600 & ± [75, 150, 225, 300, 375, 450, 550, 650, 800, 1000, 1200, 1400, 1600, 2000, 2500, 3000, 4000, 5000]
	1000 & [1300] ± [100, 200, 300, 400, 500, 700, 900, 1100, 1500, 1700, 2000, 2500, 3000, 3500, 4000, 5000, 7500]
A22 – C2	150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000, 3500
U23 – C6	250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000, 3500
	200 & [-30] ± [60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390, 420, 480, 540, 600, 700, 800]
	400 & ± [50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 700, 800, 900, 1000, 1200, 1500]
	600 & [375, 450, 550, 650, 800, 1000, 1200, 1400, 1600, 2000, 2500, 3000, 4000, 5000]
	1000 & ± [100, 200, 300, 400, 500, 700, 900, 1100, 1300, 1500, 1700, 2000, 2500, 3000, 3500, 4000, 5000, 7500]
U25 – C6	150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000, 3500
	150 & ± [20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240, 240, 260, 280, 300, 320, 400, 500, 600]
	200 & ± [30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390, 420, 480, 540, 600, 700, 800]
	1000 & ± [100, 200, 300, 400, 500, 700, 900, 1100, 1300, 1500, 1700, 2000, 2500, 3000, 3500, 4000, 5000, 7500]
G26 – N1	100, 150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000
	200 & ± [20, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 225, 250, 300, 350, 400, 500]
	1000 & ± [100, 200, 300, 400, 500, 600, 700, 900, 1100, 1300, 1500, 2000, 2500, 3000, 3500, 4000]
G26 – C8	150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000, 3500
	200 & ± [30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390, 420, 480, 540, 600, 700, 800]
	400 & ± [50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 700, 800, 900, 1000, 1200, 1500]
	600 & ± [75, 150, 225, 300, 375, 450, 550, 650, 800, 1000, 1200, 1400, 1600, 2000, 2500, 3000, 4000, 5000]
	1000 & ± [100, 200, 300, 400, 500, 700, 900, 1100, 1300, 1500, 1700, 2000, 2500, 3000, 3500, 4000, 5000, 7500]
A27 – C2	150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000, 3500
A27 – C8	150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000, 3500
A27 – C1'	150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000, 3500
	200 & ± [10, 64, 128, 192, 256, 320, 384, 448, 512, 576, 640, 700]
	400 & ± [10, 127, 254, 381, 508, 635, 762, 889, 1016, 1143, 1270, 1400]
	600 & [1528] ± [10, 191, 382, 573, 764, 955, 1146, 1719, 1910, 2100]
	800 & ± [10, 255, 510, 765, 1020, 1275, 1785, 2040, 2295, 2550, 2800]
	1000 & [1590] ± [10, 318, 636, 954, 1272, 1590, 1908, 2226, 2544, 2862, 3180]
G28 – C8	150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000, 3500
G28 – N1	100, 150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000
C30 – C1'	100, 150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000, 3500

	400 & ± [50, 100, 200, 300, 400, 500, 600, 700, 800, 1000]
	1000 & [1250] ± [50, -100, 250, 500, 750, 1000, 1500, 2000, 2300, 2600]
	1600 & [1000, 1400] ± [50, 100, 200, 300, 400, 500, 800, 900, 1000, 1400, 1550, 1750, 2000, 2600, 3200, 3740, 3200, 3740, 4270, 4800, 3740, 4270]
	2500 & [800, 900, 1750] ± [50, 100, 200, 300, 400, 500, 2000, 1550, 2600, 3200, 3740, 4270, 4800, 5000, 7100]
	3000 & [750, 1000, 1500] ± [50, 150, 200, 300, 450, 500, 1900, 2000, 2400, 3000, 4000, 6000, 7000, 8000, 9000]
U31 – C1'	100, 150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000, 3500
	400 & ± [50, 100, 200, 300, 400, 500, 600, 700, 800, 1000]
	1000 & [1000, 1250] ± [50, 100, 250, 500, 750, 900, 1500, 1400, 2000, 2300, 2600]
	1600 & [1000, 1200, 1400] ± [50, 100, 200, 300, 400, 500, 800, 900, 1550, 1750, 2000, 2600, 3200, 3740, 4270, 4800]
	2500 & [625, 900, 1250, 1000] ± [50, 100, 125, 250, 375, 500, 1875, 1550, 2000, 2500, 3125, 3740, 4270, 4800]
	3000 & [450, 500, 750, 1000] ± [50, 100, 200, 300, 2000, 2400, 3000, 4000, 6000, 7000, 9000]
U31 – C6	250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000, 3500
	400 & ± [50, 100, 200, 300, 400, 500, 600, 700, 800, 1000]
	1600 & ± [50, 100, 200, 300, 400, 600, 800, 1000, 1200, 1400, 1750, 2000, 2100, 2600, 3200, 3740, 4270, 4800]
	3000 & ± [50, 150, 300, 450, 600, 750, 1000, 1250, 1500, 1900, 2250, 2625, 3000, 3300, 3750, 6000, 7000, 8000, 9000]
G32 – C1'	150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000, 3500
G33 – C1'	50, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000, 3500
	150 & [275, 325, 350, 375, 425,450, 550, 600, -100, -500] ± [50, 150, 200, 250, 300, 400]
	200 & [150, 250, 275, 325, 350, 375, 425, 450, 500, 550, 700] ± [50, 100, 200, 300, 400, 600]
	300 & [150, 250, 275, 300, 325, 350, 375, 400, 425, 450, 500, 550, 600, 700, 800] ± [50, 100, 200]
	400 & [-100] ± [50, 200, 300, 400, 500, 600, 800]
	1000 & [-500, -2500] ± [200, 1000, 1500, 2000, 3000]
G33 – C8	150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000, 3500
	150 & ± [50, 100, 150, 200, 250, 275, 300, 325, 350, 375, 400, 420, 450, 500]
	200 & [-800] ± [50, 100, 150, 200, 250, 275, 300, 325, 350, 375, 400, 425, 450, 500, 550, 600, 700]
	300 & ± [50, 100, 200, 250, 275, 300, 325, 350, 375, 400, 425, 450, 500, 550, 600, 700, 800, 900
	800 & ± [50, 100, 250, 500, 750, 1000, 1250, 1500, 1750, 2000]
G34 – C1'	150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000, 3500
	400 & [1000] ± [50, 100, 200, 300, 400, 500, 600, 700, 800]
	1000 & [1000] ± [50, 100, 250, 500, 750, 800, 1250, 1500, 1400, 2000, 2300, 2600]
	1600 & [700, 800, 900, 1200, 1300, 1400] ± [50, 100, 200, 300, 400, 600, 1300, 1750, 2000, 2600, 3200, 3740, 4270, 4800]
	2500 [375, 500] & ± [50, 100, 125, 250, 1600, 1875, 2500, 3125, 3750, 4250, 4800, 5000, 7100]
	3000 & [300, 450, 500, 750] ± [50, 100, 200, 2000, 3000, 4000, 7000]
G34 – C8	150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000, 3500
	400 & ± [50, 100, 200, 300, 400, 500, 600, 700, 800, 1000]
	1600 & ± [50, 100, 200, 300, 400, 600, 800, 1000, 1200, 1400, 1750, 2000, 2600, 3200, 3740,

	4270]
	2300 & ± [50, 125, 250, 375, 500, 650, 825, 1150, 1800, 2200, 3000, 4000, 4500, 5000
	3000 & ± [50, 150, 300, 450, 600, 750, 900, 1000, 1300, 1500, 1900, 2250, 2625, 3000, 3300, 3750, 6000, 7000, 8000, 9000]
A35 – C1'	150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000, 3500
	400 & ± [50, 100, 200, 300, 400, 500, 600, 700, 800, 1000]
	1000 & [1000, 1250] ± [50, 100, 250, 500, 750, 900, 1500, 1400, 2000, 2300, 2600
	1600 & [800, 900, 1000, 1200, 1400] ±[50, 100, 200, 300, 400, 500, 1550, 1750, 2100, 2600, 3200, 3740, 4270, 4800]
	2500 & [625, 1250, 1000,] ± [50, 100, 250, 500, 1875, 2000, 3125, 3750, 4250, 4800, 5000, 6600, 7100, 7600]
A35 – C8	100, 150, 200, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000, 3500
	150 & [-500, -550] ± [50, 100, 150, 200, 250, 300, 350, 400, 450]
	200 & [-800] ± [50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 700]
	300 & ± [50, 100, 200, 300, 350, 400, 450, 500, 600, 700, 800, 1000]
	400 & [600, 800, 1000, 1200]
	1000 & ± [200, 500, 1000, 1500, 2000, 2500]
G36 – C8	150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000, 3500
U38 – N3	50, 100, 150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800
	50 & [25, 70, 90, 110, 130] ± [50, 100, 150]
	100 & [25, 70, 90, 110, 130, 170, 190, 210, 230, 270, 290, 310, -100] ± [50, 150, 250]
	150 & [25, 70, 90, 110, 130, , 170, 190, 210, 230, 270, 290, 310, 330, 350, 370, 390, 410, 430, 450, -100] ± [50, 150, 250]
	200 & [25, 70, 90, 110, 130, 170, 190, 210, 230, 270, 290, 310, 330, 350, 370, 390, 410, 430, 450, 475, 500, 550, 600, -100] ±[50, 150, 250]
	300 & ± [30, 60, 90, 120, 150, 180, 210, 250, 300, 350, 400, 500, 600, 700, 800]
	600 & ± [40, 80, 120, 160, 200, 240, 300, 400, 500, 600, 800, 1000, 1200, 1400, 1600]
U42 – N3	100, 150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000
	200 & ± [40, 60, 80, 100, 120, 140, 160, 180, 200, 225, 250, 300, 350, 400, 500]
	400 & ± [30, 60, 90, 120, 150, 180, 210, 250, 300, 350, 400, 500, 600, 700, 800]
	600 & ± [40, 80, 120, 160, 200, 240, 300, 400, 500, 600, 800, 1000, 1200, 1400, 1600, 2000]
	1000 & ± [100, 200, 300, 400, 500, 600, 700, 900, 1100, 1300, 1500, 2000, 2500, 3000, 3500, 4000]
G43 – N1	100, 150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000
	200 & ± [20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 225, 250, 300, 350, 400, 500]
	1000 & ± [100, 200, 300, 400, 500, 600, 700, 900, 1100, 1300, 1500, 2000, 2500, 3000, 3500, 4000]

wtTAR at 10˚C	**On-resonance spinlock power (Hz)/Off-resonance spinlock power (Hz) with [offsets]**
A27 – C1'	100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000
	150 & [450] ± [50, 100, 150, 200, 250, 300, 350, 400]
	200 & ± [30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390, 420, 480, 540, 600]
	300 & [750, 850] ± [50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 700, 800, 900]
	400 & ± [50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 700, 800, 900, 1000, 1200]
	1000 & [-1300, -2500, -3000] ± [100, 200, 300, 400, 500, 700, 900, 1100, 1500, 1700, 2000]
	1500 & ± [100, 200, 300, 400, 500, 700, 900, 1100, 1500, 1700, 2000, 2500, 3000, 3500, 4000, 5000, 7500]

G26 – C8	100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000
	200 & ± [30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390, 420, 480, 540, 600
	400 & ± [50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 700, 800, 900, 1000, 1200]
	600 & ± [75, 150, 225, 300, 375, 450, 550, 650, 800, 1000, 1200, 1400, 1600]
	1000 & ± [100, 200, 300, 400, 500, 700, 900, 1100, 1300, 1500, 1700, 2000, 2500, 3000, 3500, 4000, 5000, 7500]

wtTAR in 5 mM Mg^{2+}	**On-resonance spinlock power (Hz)/Off-resonance spinlock power (Hz) with [offsets]**
A35 – C8	100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000
	200 & ± [30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390, 420, 480, 540, 600]
	400 & ± [50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 700, 800, 900, 1000, 1200]
	600 & ± [75, 150, 225, 300, 375, 450, 550, 650, 800, 1000, 1200, 1400, 1600]
	1000 & ± [100, 200, 300, 400, 500, 700, 900, 1100, 1300, 1500, 1700, 2000, 2500, 3000]
U38 – N3	200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000, 3500

ΔC24-wtTAR in 5 mM Mg^{2+}	**On-resonance spinlock power (Hz)/Off-resonance spinlock power (Hz) with [offsets]**
A35 – C8	100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000
	200 & ± [30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390, 420, 480, 540, 600, 700]
	400 & ± [50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 700, 800, 900, 1000, 1200, 1500]
	600 & ± [75, 150, 225, 300, 375, 450, 550, 650, 800, 1000, 1200, 1400, 1600]
	1000 & ± [100, 200, 300, 400, 500, 700, 900, 1100, 1300, 1500, 1700, 2000, 2500, 3000]
U38 – N3	150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2500, 3000

Table 6: Parameters for $R_{1\rho}$ data fitting

ES1 of ΔC24-wtTAR

ΔC24-wtTAR	Temp.	R_1 (Hz)	R_2 (Hz)	k_{ex} (s^{-1})	k_1 (s^{-1})	k_{-1} (s^{-1})	Population and Lifetime	$\Delta\omega_{ES}$ (p.p.m.)
C30 – C1'	25˚C	2.0 ± 0.2	26 ± 1	27016 ± 1425	4267 ± 1080	22749 ± 5760	16 ± 4 %	2.0 ± 0.2
U31 – C1'	25˚C	1.4 ± 0.2	25 ± 1					2.2 ± 0.2
U31 – C6	25˚C	2.1 ± 0.1	31 ± 1					-1.4 ± 0.1
G34 – C1'	25˚C	2.2 ± 0.1	28 ± 1				44 ± 11 μs	2.0 ± 0.2
G34 – C8	25˚C	1.4 ± 0.1	35 ± 2					2.6 ± 0.2
A35 – C1'	25˚C	2.0 ± 0.3	22 ± 3					2.6 ± 0.3

ES2 of ΔC24-wtTAR

ΔC24-wtTAR	Temp.	R_1 (Hz)	R_2 (Hz)	k_{ex} (s^{-1})	k_1 (s^{-1})	k_{-1} (s^{-1})	Population Lifetime	$\Delta\omega_{ES}$
A22 – C1'	25°C	1.07 ± 0.07	23.7 ± 0.1	541 ± 93	0.9 ± 0.2	540 ± 115	0.17 ± 0.02 % 1.8 ± 0.4 ms	1.7 ± 0.3
U23 – C6	25°C	2.1 ± 0.1	31.2 ± 0.2					-3.5 ± 0.6
G33 – C8	25°C	1.4 ± 0.1	37.2 ± 0.2					2.7 ± 0.5
G33 – C1'	25°C	0.53 ± 0.08	21.6 ± 0.1					2.4 ± 0.5
A35 – C8	25°C	1.86 ± 0.03	20.55 ± 0.05					-2.3 ± 0.1
U38 –N3	25°C	1.52 ± 0.03	6.52 ± 0.03					-4.5 ± 0.1

Individual Fit for U23-C6 in ΔC24-wtTAR

ΔC24-wtTAR	Temp.	R_1 (Hz)	R_2 (Hz)	k_{ex} (s^{-1})	k_1 (s^{-1})	k_{-1} (s^{-1})	Population	$\Delta\omega_{ES}$
U23 – C6	25°C	2.09 ± 0.07	30.5 ± 0.2	761 ± 399	2.3 ± 0.9	759 ± 494	0.3 ± 0.1 %	-3.0 ± 0.2

ES3 of ΔC24-wtTAR

ΔC24-wtTAR	Temp.	R_1 (Hz)	R_2 (Hz)	k_{ex} (s^{-1})	k_1 (s^{-1})	k_{-1} (s^{-1})	Population Lifetime	$\Delta\omega_{ES}$
G26 – C8	25°C	1.12 ± 0.05	32.2 ± 0.2	5043 ± 327	12 ± 7	5031 ± 372	0.24 ± 0.01 %	4.1 ± 0.2
A27 – C1'	25°C	-0.12 ± 0.07	38.9 ± 0.4				19 ± 15 μs	-7.5 ± 0.2

ES3 of wtTAR

wtTAR	Temp.	R_1 (Hz)	R_2 (Hz)	k_{ex} (s^{-1})	k_1 (s^{-1})	k_{-1} (s^{-1})	Population Lifetime	$\Delta\omega_{ES}$
G26 – C8	10°C	1.29 ± 0.08	52.4 ± 0.2	2303 ± 387	4.3 ± 0.8	2299 ± 425	0.18 ± 0.01 %	4.5 ± 0.3
A27 – C1'	10°C	1.19 ± 0.2	56.82 ± 0.4				434 ± 80 μs	-6.5 ± 0.9

ES of ΔC24-wtTAR in the presence of 5 mM Mg^{2+}

ΔC24-wtTAR	Temp.	R_1 (Hz)	R_2 (Hz)	k_{ex} (s^{-1})	k_1 (s^{-1})	k_{-1} (s^{-1})	Population	$\Delta\omega_{ES}$
A35-C8	25°C	0.06 ± 0.06	28 ± 2	18583 ± 1643	750 ± 200	17833 ± 4730	4 ± 1 %	-3.1 ± 0.3

ES of wtTAR in the presence of 5 mM Mg^{2+}

wtTAR	Temp.	R_1 (Hz)	R_2 (Hz)	k_{ex} (s^{-1})	k_1 (s^{-1})	k_{-1} (s^{-1})	Population	$\Delta\omega_{ES}$
A35-C8	25˚C	-0.52 ± 0.07	30 ± 1	13303 ± 930	348 ± 86	12954 ± 2829	2.6 ± 0.5%	-3.0 ± 0.3

Table 7: Comparison of the individual fitted parameters from $R_{1\rho}$ using and 2- and 3-state exchange with no minor exchange in a linear topology, for ΔC24-wtTAR A27 – C1' at 25˚C

ΔC24-wtTAR	Parameters	2-state (GS, ES3)	3-state (GS, ES2, ES3)
A27 – C1'	R_1 (Hz)	-0.14 ± 0.08	-0.21 ± 0.8
	R_2 (Hz)	39.2 ± 0.4	39.8 ± 0.4
	k_{ex} (s^{-1}), ES2	-	2014 ± 1263
	Pop. ES2	-	0.15 ± 0.06%
	$\Delta\omega_{ES2}$	-	-2.1 ± 0.8
	k_{ex} (s^{-1}), ES3	4879 ± 456	4012 ± 560
	Pop. ES3	0.229 ± 0.009%	0.20 ± 0.01%
	$\Delta\omega_{ES3}$	-7.6 ± 0.2	-8.3 ± 0.3
	Reduced χ^2	0.28	0.26
	R^2	0.999	0.998
	AIC Test	3-State	

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