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Supplementary Materials for

The structure of the RCAN1:CN complex explains the inhibition of and substrate recruitment by calcineurin

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Table S1: Surface plasmon resonance (SPR) spectroscopy

Ligand	Analyte	K_D (nM)	k_{on} ($M^{-1}s^{-1} \times 10^6$)	k_{off} ($s^{-1} \times 10^{-3}$)
$RCAN1_{89-197}$	CN_{391}	0.5 ± 0.1	2.5 ± 0.3	1.4 ± 0.3
$RCAN1_{89-197}$ $LxVP_{dead}$	CN_{391}	0.8 ± 0.2	2.7 ± 0.1	2.0 ± 0.4
$RCAN1_{89-197}$ $TxxP_{dead}$	CN_{391}	3.0 ± 0.1	1.7 ± 0.4	5.3 ± 1.3

Table S2. Data collection and refinement statistics for the CNA:RCAN1$_{core}$ complex

[a]Data was collected from a single crystal
*Values in parentheses are for highest-resolution shell.

	CNA:RCAN1$_{core}$
PDBID	6UUQ
Beamline	APS 23ID-B (GM/CA)
Wavelength (Å)	1.03320
Data collection	
Space group	$P2_1\,2_1\,2_1$
Cell dimensions	
a, *b*, *c* (Å)	57.8, 71.2, 92.0
α, β, γ (°)	90, 90, 90
Resolution (Å)	50.00-1.85 (1.88-1.85)
R_{sym} or R_{merge}	0.175 (0.936)
$I / \sigma I$	24.4 (2.5)
CC 1/2	0.996 (0.749)
Completeness (%)	99.9 (99.9)
Redundancy	4.9 (4.8)
Refinement	
Resolution (Å)	29.87-2.70 (2.77-2.70)
No. reflections	40847
R_{work} / R_{free}	0.20 (0.33)/0.25 (0.37)
No. atoms	
Protein	9351
Ligand/ion	77
Water	52
B-factors	
Protein	61.79
Ligand/ion	77.12
Water	46.16
R.m.s. deviations	
Bond lengths (Å)	0.006
Bond angles (°)	0.771
Ramachandran	
Outliers (%)	0.35
Allowed (%)	8.36
Favored (%)	91.30
Rotamer Outliers	2.48
Clashscore	5.39

Table S3: Catalytic efficiency of CN

RCAN1 variant	k_{cat} ($x10^{-2}s^{-1}$)	k_{cat}/K_m ($M^{-1}s^{-1}$)
none	38.5 ± 0.2	37.1 ± 0.9
WT	1.5 ± 0.1	2.7 ± 0.8
$LxVP_{dead}$	1.8 ± 0.1	5.5 + 1.3
$PxIxIT_{dead}$	44.8 ± 0.1	11.1 ± 1.0
$^{108}SPP_{dead}$	6.0 ± 0.8	1.1 ± 0.3
$^{108}SPP_{dead}/TxxP_{dead}$	38.0 ± 0.3	31.4 + 0.8
$^{112}SPP_{dead}$	4.8 ± 0.4	0.9 ± 0.2
$^{112}SPP_{dead}/TxxP_{dead}$	29.6 ± 0.2	32.6 ± 0.9
$TxxP_{mut3}$ (TRAP)	20.2 ± 0.2	12.8 ± 0.5
$TxxP_{mut3}$ (TAAP)	20.9 ± 0.2	21.6 ± 0.7
$TxxP_{mut3}$ (TAAA)	21.0 ± 0.1	24.6 ± 0.6
$TxxP_{dead}$ (AAAA)	22.2 ± 0.1	30.0 ± 0.8

Table S4. RCAN1$_{core}$ NOE restraints

HN – HN NOEs	Tyr 133 HN – Ser 136 HN	Ile 158 HN – Val 157 Hγ
Tyr 129 HN – Leu 131 HN	His 147 HN – Val 156 HN	Thr 159 HN – Val 160 Hγ
Asp 130 HN – Leu 131 HN	Leu 132 HN – Ile 135 HN	Thr 159 HN – Val 157 Hγ1
Tyr 133 HN – Ala 134 HN	Leu 131 HN – Ala 7 HN	Thr 159 HN – Ile 158 Hδ
Ser 136 HN – Lys 137 HN	Thr 150 HN – Thr 153 HN	Cys 161 HN – Val 160 Hγ1
Ser 136 HN – Leu 138 HN	Val 156 HN – Val 157 HN	Cys 161 HN – Val 160 Hγ2
Leu 138 HN – Gly 139 HN	Val 157 HN – Ile 158 HN	Glu 162 HN – Val 160 Hγ1
Gly 141 HN – Glu 142 HN	Glu 145 HN – Leu 146 HN	**CH_3 – CH_3 NOEs**
Glu 142 HN – Lys 143 HN	**HN – CH_3 NOEs**	Leu 131 Hδ1– Ile 135 Hδ
Tyr 144 HN – Glu 145 HN	Leu 131 HN – Val 156 Hγ	Leu 131 Hδ2– Ile 135 Hδ
Leu 146 HN – His 147 HN	Tyr 133 HN – Val 156 Hγ1	Leu 132 Hδ1– Ile 135 Hδ
Ala 148 HN – Ala 149 HN	Tyr 133 HN – Val 156 Hγ2	Leu 132 Hδ1– Leu 138 Hδ1
Ala 149 HN – Thr 150 HN	Lys 137 HN – Leu 138 Hδ1	Ile 135 Hδ – Ile 158 Hδ
Thr 150 HN – Asp 151 HN	Gly 139 HN – Leu 138 Hδ1	Leu 146 Hδ2– Leu 131 Hδ1
Asp 151 HN – Thr 152 HN	Gly 139 HN – Leu 138 Hδ2	Ile 158 Hδ – Val 160 Hγ1
Asp 151 HN – Thr 153 HN	Gly 139 HN – Val 156 Hγ1	Ile 158 Hδ – Val 160 Hγ2
Thr 152 HN – Thr 153 HN	Gly 141 HN – Val 160 Hγ1	Ile 158 Hδ – Val 157 Hγ1
Ser 155 HN – Val 156 HN	Gly 141 HN – Val 160 Hγ2	Ile 158 Hδ – Val 156 Hγ1
Ile 158 HN – Thr 159 HN	Glu 142 HN – Val 160 Hγ1	Ile 158 Hδ – Val 156 Hγ2
Thr 159 HN – Val 160 HN	Glu 142 HN – Val 160 Hγ2	
Val 160 HN – Cys 161 HN	Lys 143 HN – Leu 138 Hδ1	
Cys 161 HN – Glu 162 HN	Lys 143 HN – Leu 138 Hδ2	
Glu 162 HN – Ser 163 HN	Tyr 144 HN – Leu 138 Hδ1	
Ser 163 HN – Asp 164 HN	Tyr 144 HN – Leu 138 Hδ2	
Leu 131 HN – Thr 150 HN	Tyr 144 HN – Ile 158 Hδ	
Asp 130 HN – Ala 148 HN	Glu 145 HN – Leu 146 Hδ1	
Ile 135 HN – Ala 148 HN	His 147 HN – Leu 146 Hδ1	
Gly 139 HN – Glu 142 HN	His 147 HN – Leu 146 Hδ2	
Glu 142 HN – Val 160 HN	His 147 HN – Val 156 Hγ1	
Lys 143 HN – Val 160 HN	Ser 155 HN – Val 157 Hγ1	
Tyr 144 HN – Ile 158 HN	Ser 155 HN – Val 157 Hγ2	
Glu 145 HN – Ile 158 HN	Ser 155 HN – Val 156 Hγ1	
Leu 146 HN – Val 156 HN	Ser 155 HN – Val 156 Hγ2	
His 147 HN – Asp 151 HN	Val 157 HN – Val 156 Hγ	

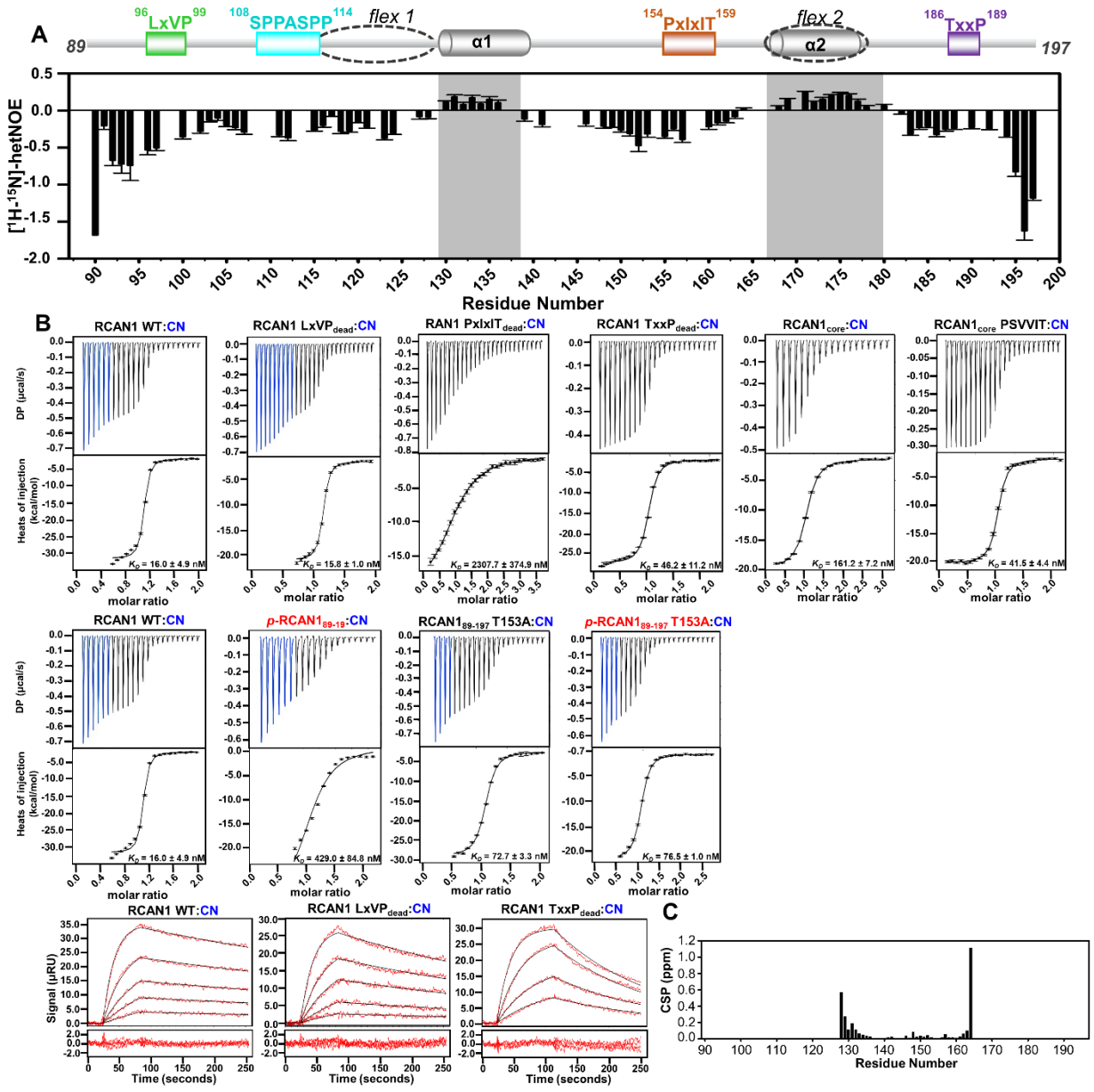

***Figure S1*. Properties of free RCAN1 and the RCAN1 interaction with CN. A.** ^{1}H-^{15}N heteronuclear NOE (hetNOE) of RCAN1. Gray boxes highlight the two partially populated helices. **B.** Top: ITC data for CN titrated with RCAN1 WT (n=3), $LxVP_{dead}$ (n=3), $PxIxIT_{dead}$ (n=3), $TxxP_{dead}$ (n=2), $RCAN1_{core}$ (n=3), $RCAN1_{core}$ PSVVIT (n=3), phosphorylated RCAN1 (n=2), T153A (n=2) and phosphorylated T153A (n=2). RCAN1 constructs (especially those including the TxxP motif) have atypical thermograms; black injections points were used to calculate K_D; blue injections were not used. Bottom: SPR measurements of immobilized CN with 1.25, 2.5, 5.0, 10, 20 µM of RCAN1 (n=6), $LxVP_{dead}$ (n=6), and 2.5, 5.0, 10, 20 µM of $TxxP_{dead}$ (n=6); Global fits are shown. **C.** CSPs between free $RCAN1_{core}$ and free RCAN1.

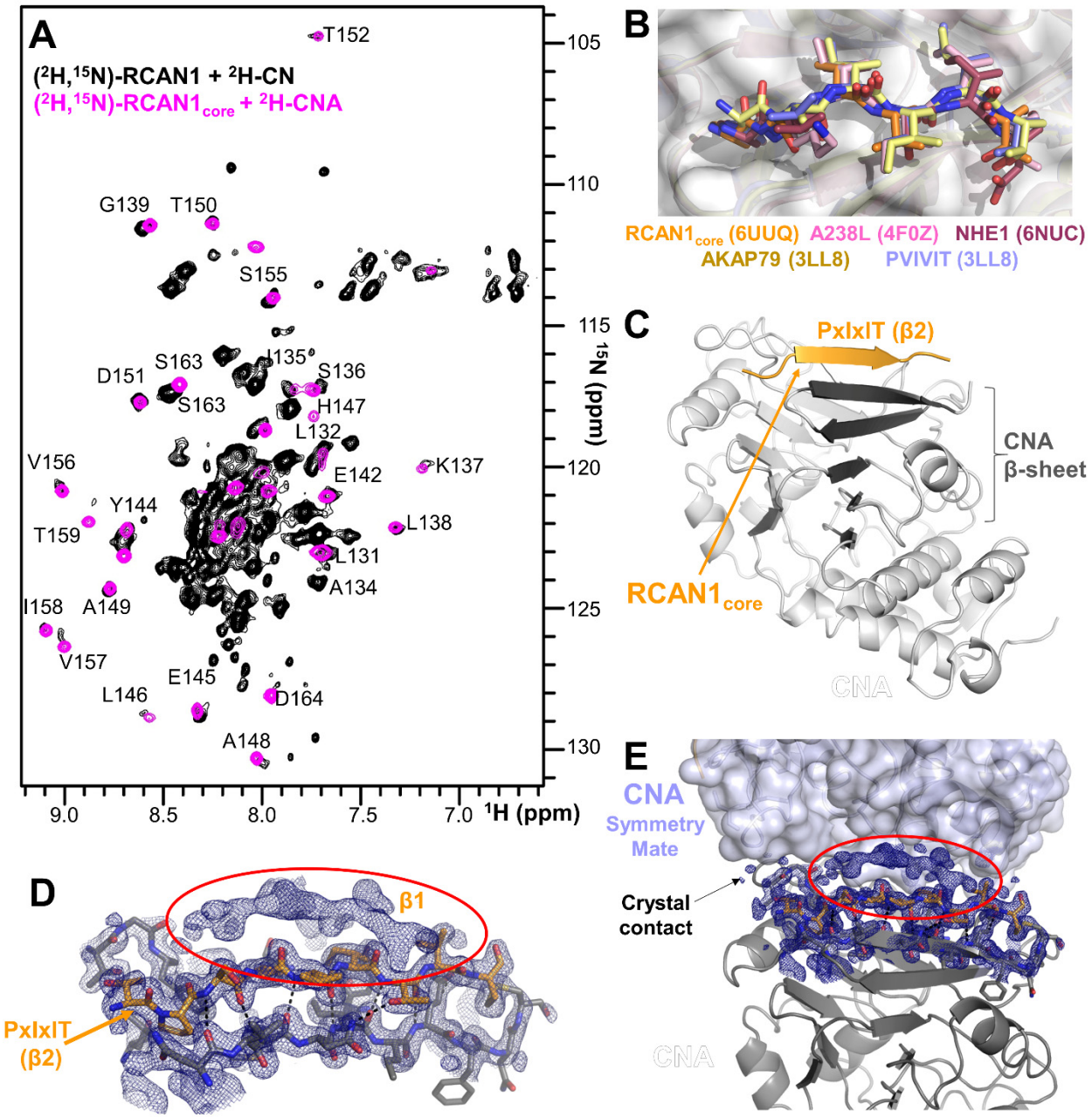

***Figure S2*. The structure of the CNA:RCAN1$_{core}$ complex. A.** Overlay of [^{1}H, ^{15}N] HSQC spectra of 1:1 complexes of RCAN1:CN (black) and RCAN1$_{core}$:CNA (pink). RCAN1$_{core}$ residues are indicated. **B.** CN PxIxIT motif binding pocket (gray) with PxIxIT motifs from RCAN1$_{core}$ (orange), A238L (pink), NHE1 (maroon), AKAP79 (dark yellow) and PVIVIT peptide (lavender). **C.** RCAN1 PxIxIT motif (orange) interacts with CNA β-strand β14 and extends the CNA β-sheet (black). **D.** 2mF$_o$-DF$_c$ electron density map (blue; 1σ) of the CNA β-strand β14, RCAN1 PxIxIT motif and weak electron density corresponding to RCAN1 β-strand β1. The RCAN1 PxIxIT motif β-strand β2 forms 6

hydrogen bonds with CNA β-strand β14 (black dotted lines). **E.** CNA's RCAN1 binding pocket is located at a crystal contact.

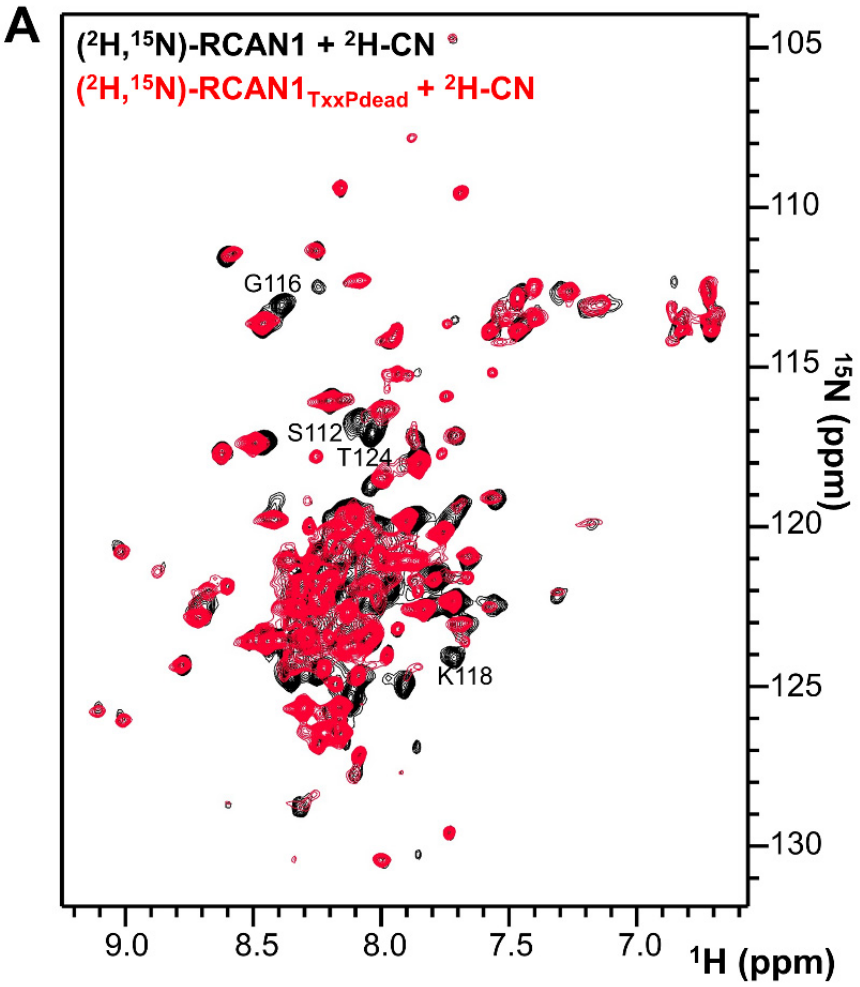

***Figure S3*. The RCAN1 TxxP and ^{108}SPPASPP^{114} motifs compete for the CN active site. A.** Overlay of the 2D [^{1}H,^{15}N] TROSY spectra of RCAN1:CN (black) and RCAN1$_{TxxPdead}$:CN (red).

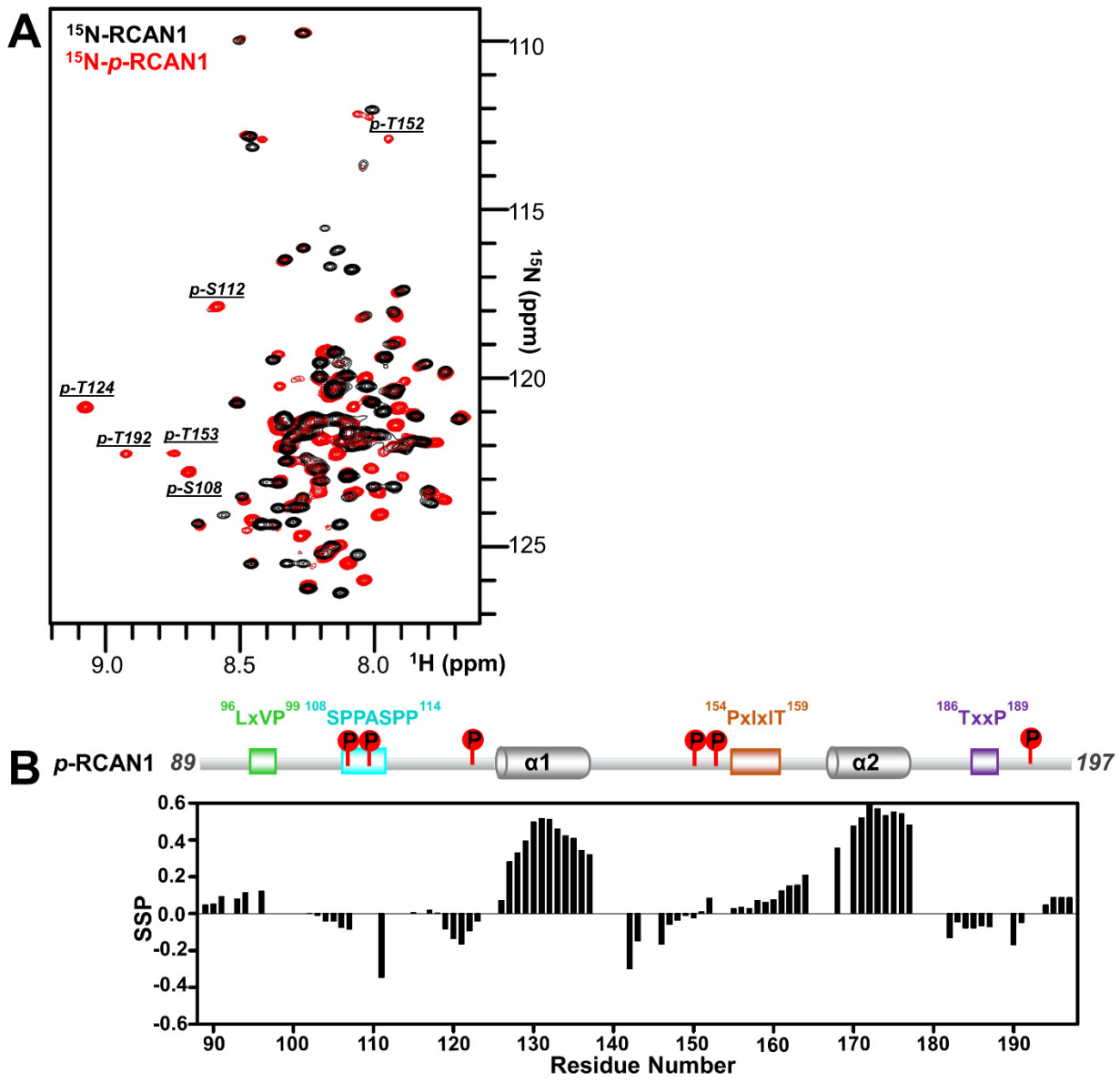

***Figure S4*. Phosphorylated RCAN1 retains its secondary structure. A.** Overlay of [^{1}H, ^{15}N] HSQC spectra of free RCAN1 (black) and p38-phosphorylated RCAN1 (*p*-RCAN1). Phosphorylated serine and threonine residues are labeled. **B.** Secondary structure propensity (SSP) calculated of *p*-RCAN1. Phosphorylated residues are marked on RCAN1 with red circles.