Table S1. Dimensions and composition of the simulated systems

		100 ns (Ca^{2+}-bound)	PMF Simulations	100 ns ($TnC+Ca^{2+}+TnI_{SW}$)	1 microsecond (Ca^{2+} Bound)
WT	**Box Dimensions (nm)**	6 x 6 x 6	15 x 6 x 6	7.65 x 7.65 x 7.65	6 x 6 x 6
	K^+ Ions	14	13	11	14
	Cl^- Ions	1	0	0	1
	Ca^{2+} Ions	1	1	1	1
	Water Atoms	20077	52044	39891	20077
A8V	**Box Dimensions (nm)**	6 x 6 x 6	15 x 6 x 6	7.65 x 7.65 x 7.65	6 x 6 x 6
	K^+ Ions	14	13	11	14
	Cl^- Ions	1	0	0	1
	Ca^{2+} Ions	1	1	1	1
	Water Atoms	20031	52023	39882	20031
L29Q	**Box Dimensions (nm)**	6 x 6 x 6	15 x 6 x 6	7.65 x 7.65 x 7.65	6 x 6 x 6
	K^+ Ions	14	13	11	14
	Cl^- Ions	1	0	0	1
	Ca^{2+} Ions	1	1	1	1
	Water Atoms	20043	52038	39885	20043
A31S	**Box Dimensions (nm)**	6 x 6 x 6	15 x 6 x 6	7.65 x 7.65 x 7.65	6 x 6 x 6
	K^+ Ions	14	13	11	14
	Cl^- Ions	1	0	0	1
	Ca^{2+} Ions	1	1	1	1
	Water Atoms	20034	52029	39894	20034
L48Q	**Box Dimensions (nm)**	6 x 6 x 6	15 x 6 x 6	7.65 x 7.65 x 7.65	6 x 6 x 6
	K^+ Ions	14	13	11	14
	Cl^- Ions	1	0	0	1
	Ca^{2+} Ions	1	1	1	1
	Water Atoms	20034	52041	39894	20034
Q50R	**Box Dimensions (nm)**	6 x 6 x 6	15 x 6 x 6	7.65 x 7.65 x 7.65	6 x 6 x 6
	K^+ Ions	14	13	11	14
	Cl^- Ions	1	0	0	1
	$Ca2^+$ Ions	1	1	1	1
	Water Atoms	20040	52032	39891	20040
C84Y	**Box Dimensions (nm)**	6 x 6 x 6	15 x 6 x 6	7.65 x 7.65 x 7.65	6 x 6 x 6
	K^+ Ions	14	13	11	14
	Cl^- Ions	1	0	0	1
	Ca^{2+} Ions	1	1	1	1
	Water Atoms	20022	52020	39876	20022

Table S2. Melting Temperatures of APO TnC constructs

Construct	**WT**	**A8V**	**L29Q**	**A31S**	**L48Q**	**Q50R**	**C84Y**
Melt Temp (ºC)	64	58.5	65	65	42.5	65.5	64.5

Table S3. MD derived Ca^{2+} Coordination Distances (Å). Distances are the average of 5 replicated 100 ns simulations.

	WT	**A8V**	**L29Q**	**A31S**	**L48Q**	**Q50R**	**C84Y**
Ca-65ASP-OD1	3.4 ± 0.3	2.26 ± 0.2	2.99 ± 0.4	2.92 ± 0.3	3.07 ± 0.3	3.94 ± 0.5	2.89 ± 0.3
Ca-65ASP-OD2	2.96 ± 0.3	3.03 ± 0.2	3.13 ± 0.2	3.58 ± 0.3	3.25 ± 0.3	3.94 ± 0.4	3.86 ± 0.3
Ca-66GLU-OE1	8.58 ± 0.5	6.86 ± 0.3	8.56 ± 0.8	8.97 ± 0.5	8.67 ± 0.4	8.19 ± 0.5	8.09 ± 0.5
Ca-66GLU-OE2	8.59 ± 0.4	6.86 ± 0.3	8.57 ± 0.8	8.95 ± 0.5	8.67 ± 0.4	8.1 ± 0.6	8.08 ± 0.5
Ca-67ASP-OD1	2.8 ± 0.2	2.44 ± 0.2	2.81 ± 0.3	2.98 ± 0.3	3.17 ± 0.2	3.33 ± 0.3	4.15 ± 0.3
Ca-67ASP-OD2	2.94 ± 0.2	2.3 ± 0.2	2.63 ± 0.3	2.72 ± 0.1	2.7 ± 0.1	3.45 ± 0.5	4.14 ± 0.3
Ca-69SER-OG	4.42 ± 0.3	3.29 ± 0.3	3.98 ± 0.5	4.35 ± 0.7	4.74 ± 0.4	4.91 ± 0.5	5.8 ± 0.6
Ca-69SER-O	6.96 ± 0.3	5.44 ± 0.2	6.71 ± 0.6	7.05 ± 0.4	7.23 ± 0.2	7.49 ± 0.3	7.59 ± 0.3
Ca-71THR-OG1	5.57 ± 0.3	3.96 ± 0.2	4.8 ± 0.4	5.07 ± 0.3	5.47 ± 0.3	5.11 ± 0.3	4.95 ± 0.2
Ca-71THR-O	3.36 ± 0.3	2.2 ± 0.1	2.75 ± 0.2	2.85 ± 0.2	3.18 ± 0.1	3.14 ± 0.2	2.79 ± 0.2
Ca-73ASP-OD1	4.73 ± 0.5	3.46 ± 0.3	4.63 ± 0.7	4.7 ± 0.7	5.43 ± 0.6	4.89 ± 1.0	3.65 ± 0.5
Ca-73ASP-OD2	4.19 ± 0.5	3.36 ± 0.7	4.6 ± 0.6	4.19 ± 0.3	5.85 ± 0.5	4.59 ± 1.1	3.77 ± 0.4
Ca-76GLU-OE1	2.68 ± 0.1	2.14 ± 0.1	2.59 ± 0.2	2.7 ± 0.1	2.67 ± 0.1	2.67 ± 0.1	2.63 ± 0.1
Ca-76GLU-OE2	2.7 ± 0.1	2.12 ± 0.1	2.54 ± 0.2	2.7 ± 0.1	2.67 ± 0.1	2.71 ± 0.1	2.68 ± 0.1

Table S4. Simulation-derived properties of WT cTnC and mutant constructs

	WT	A8V	L29Q	A31S	L48Q	Q50R	C84Y
Average h-sasa TnC+Ca (Closed) (nm^2)	22.6 ± 0.3	22.6 ± 0.4	22.9 ± 0.4	22.3 ± 0.5	22.7 ± 0.6	22.5 ± 0.2	22.5 ± 0.4
Average h-sasa TnC+Ca (Open) (nm^2)	25.3 ± 0.7	24.8 ± 0.5	25.4±0.6	25.0 ± 0.6	25.6 ± 0.3	25.4 ± 0.3	25.5 ± 0.4
Number of H-bonds (Closed)	58.5±0.9	59.5 ± 1.7	58.9 ± 1.5	59.5 ± 1.4	59.7 ± 0.9	60.6 ± 2.0	60.6 ± 1.6

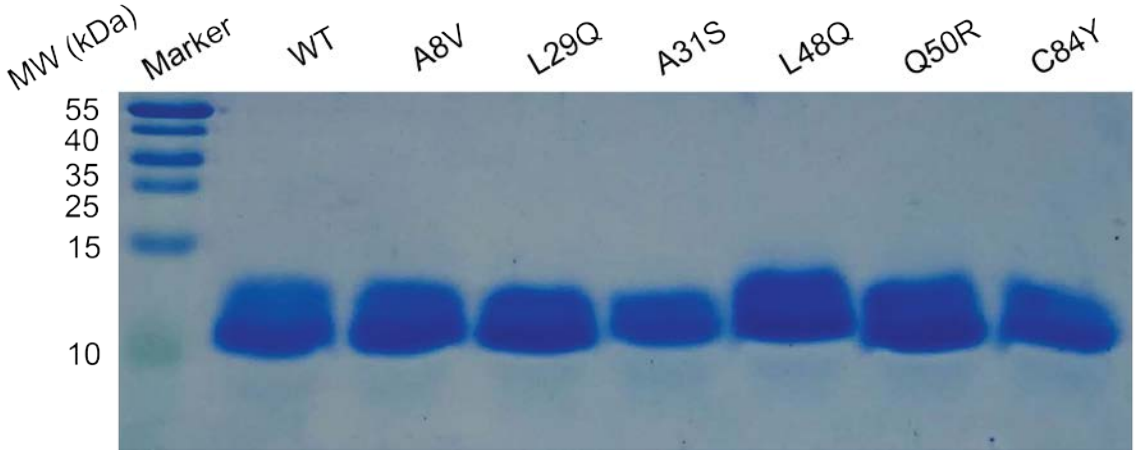

Figure S1. SDS PAGE of the N-cTnC constructs used in ITC. Each sample of WT and mutant N-cTnC samples was pure, free of degraded protein and ran at ~10.1 kDa.

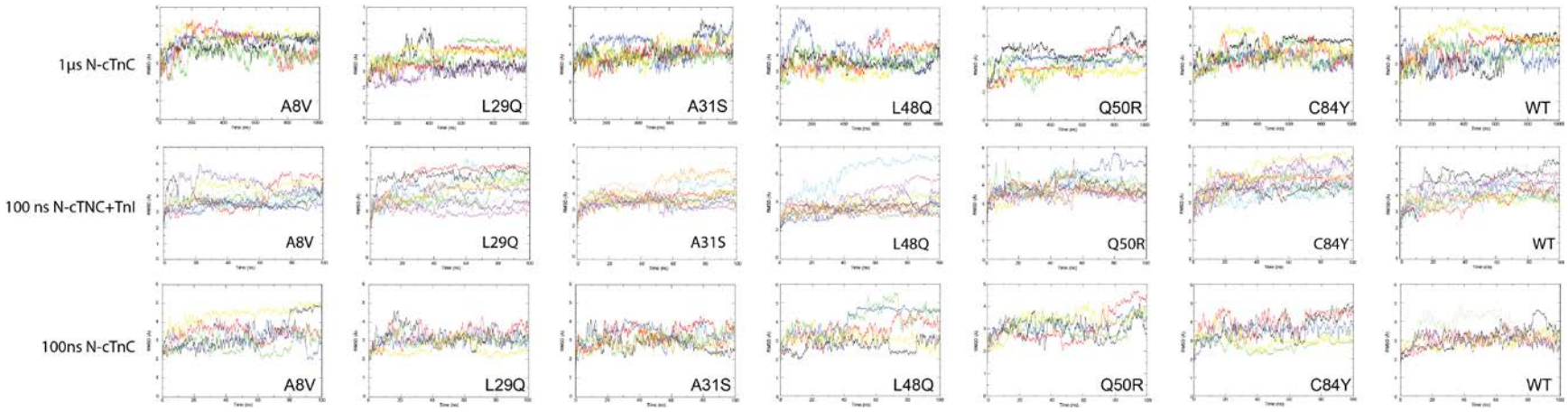

Figure S2. RMSD as a function of time for 1 μs and 100 ns simulations of TnC+Ca^{2+}, and 100 ns of TnC+Ca^{2+}+TnI_{SW} for each mutant construct. These indicate that each of the simulations has diverged substantially from the starting coordinates. Plots are a running average over 0.5% of the total number of data points.

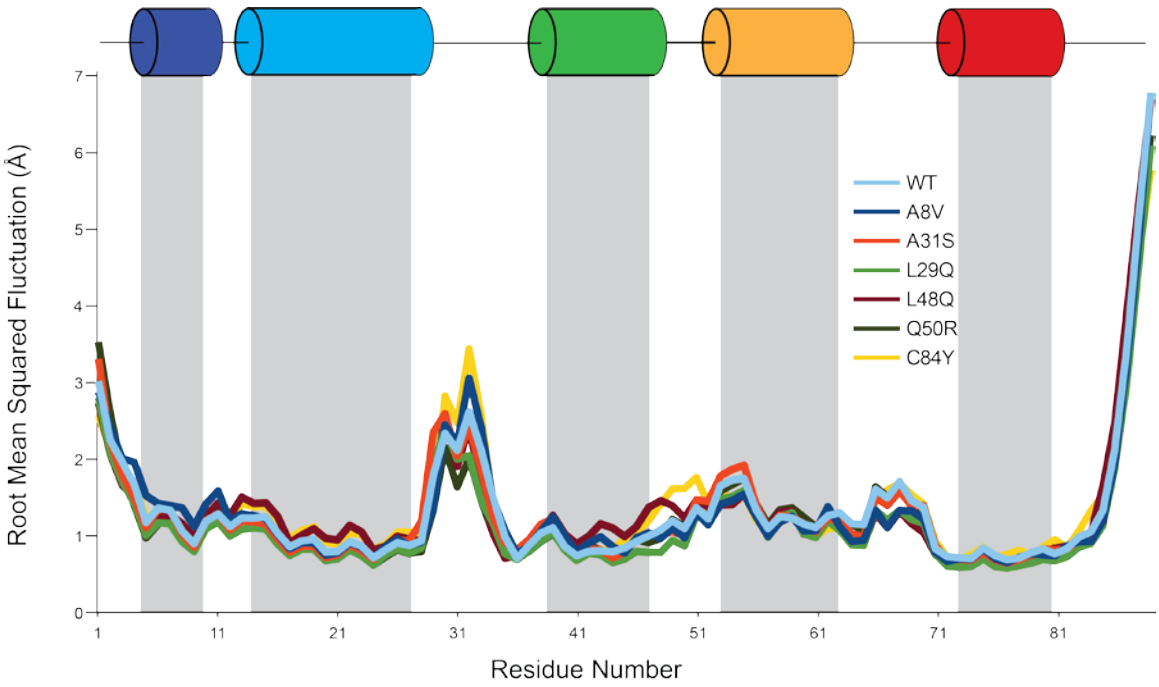

Figure S3. Root mean squared fluctuations (RMSF) plotted by residue for the models of WT NcTnC and each of the mutated constructs from 100 ns simulations. The local flexibility is similar for each for these constructs and is highest at the termini and loop regions between helices A and B.

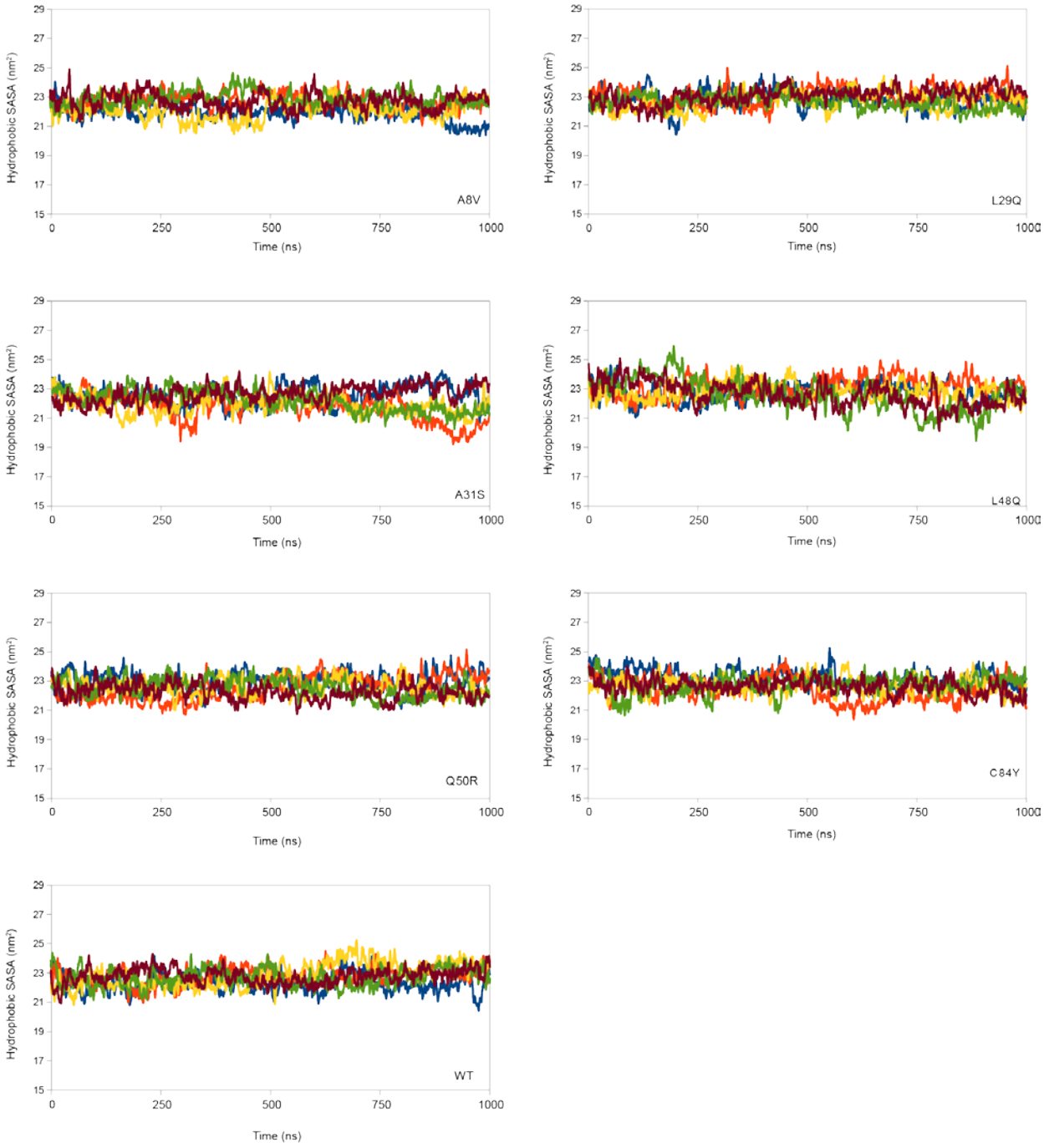

Figure S4. Hydrophobic solvent accessible surface area is plotted as a function of time for 5 replicated simulations for models of WT NcTnC and each of the mutated constructs. Plots are a rolling average of 250 ps. There is little difference between WT and most of the constructs, with the exception of L48Q and A31S.