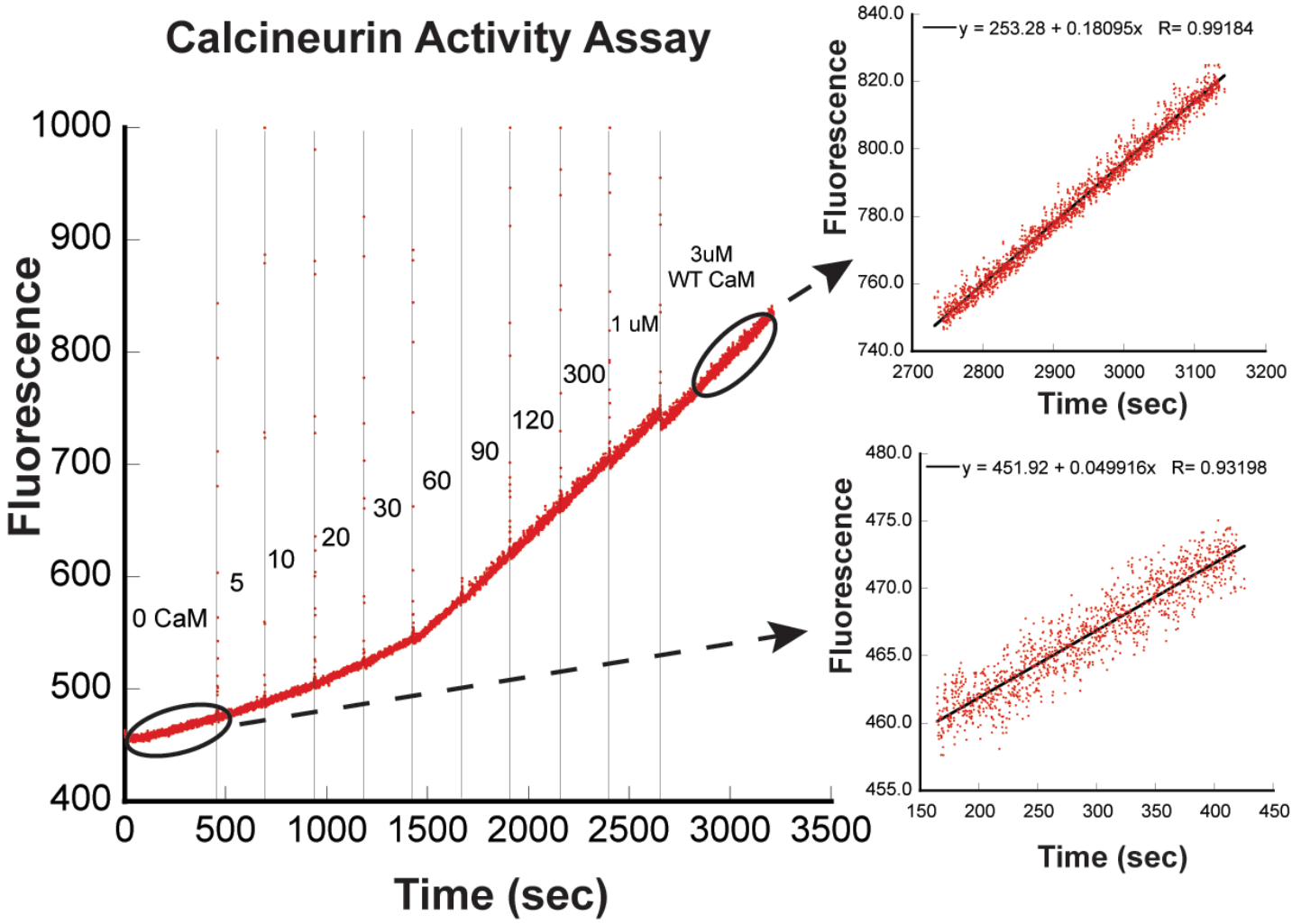


Figure S1. Representative data set of CaN activity measurements. Fluorescence intensity of MUPP substrate monitored during addition of CaM in the presence of 100 µM Ca^{2+} and 150 nM CaN. Zoomed in regions at the start and end of the titration demonstrate linearity which is consistent with each condition being monitored at steady state. Each steady state measurement is fit to a linear line where the slope corresponds to CaN activity.

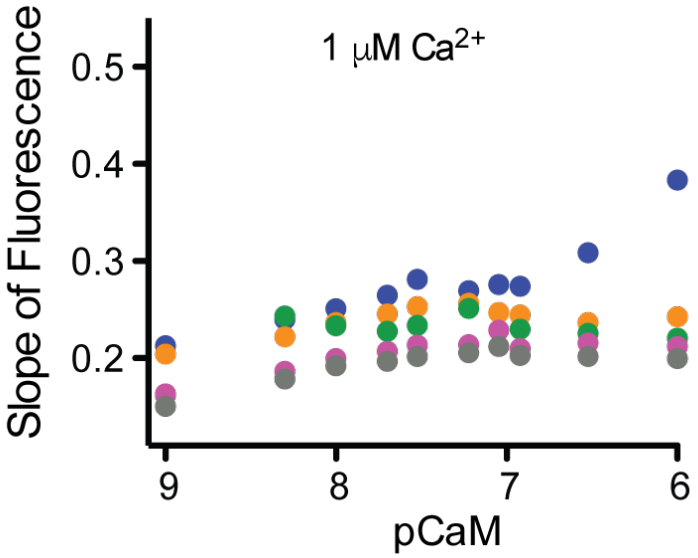


Figure S2. Activation of CaN by CaM at 1 μM [Ca^{2+}]. Data are color coded by CaM mutation identify (WT, N53I, F89L, D129G, and F141L).

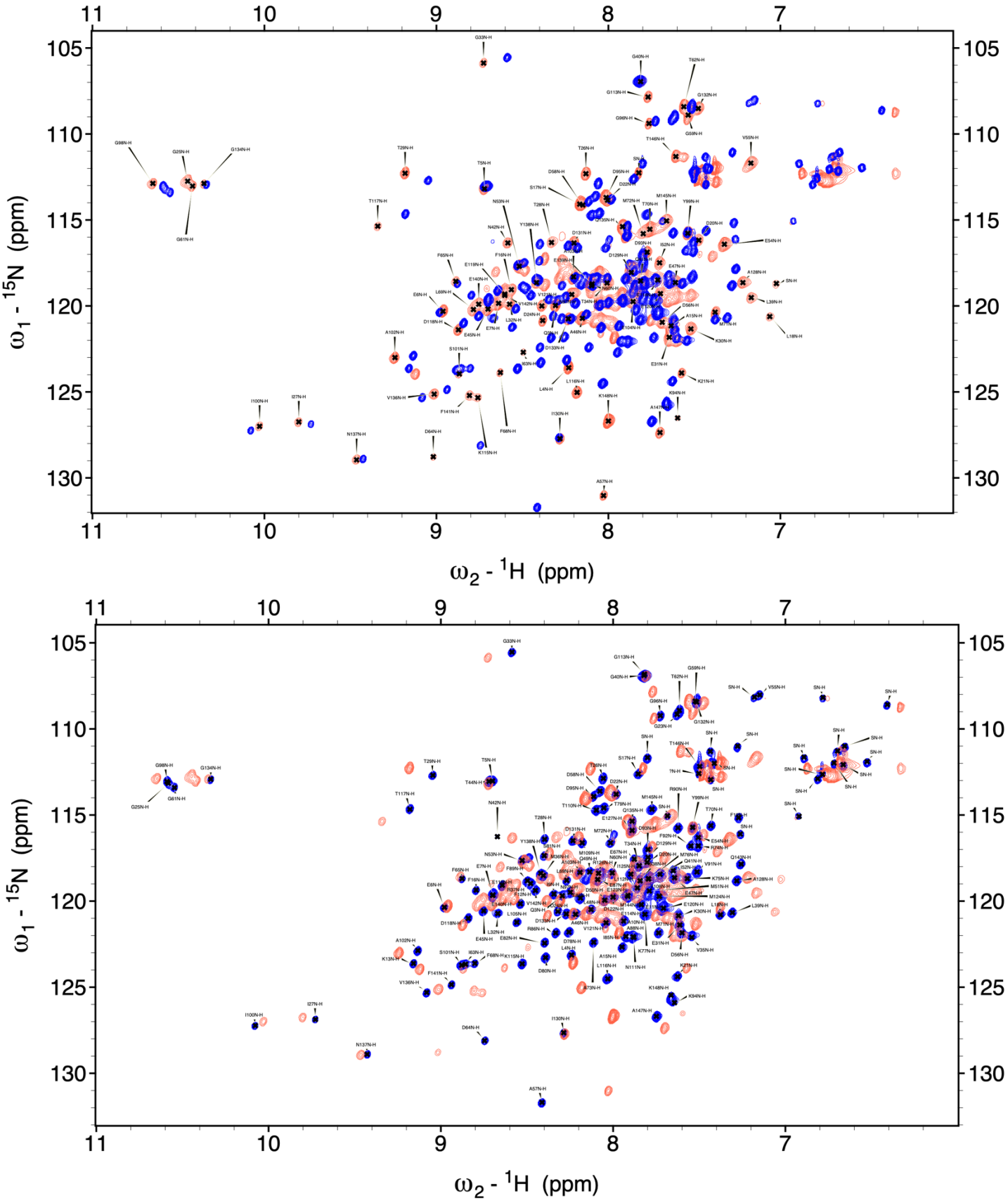
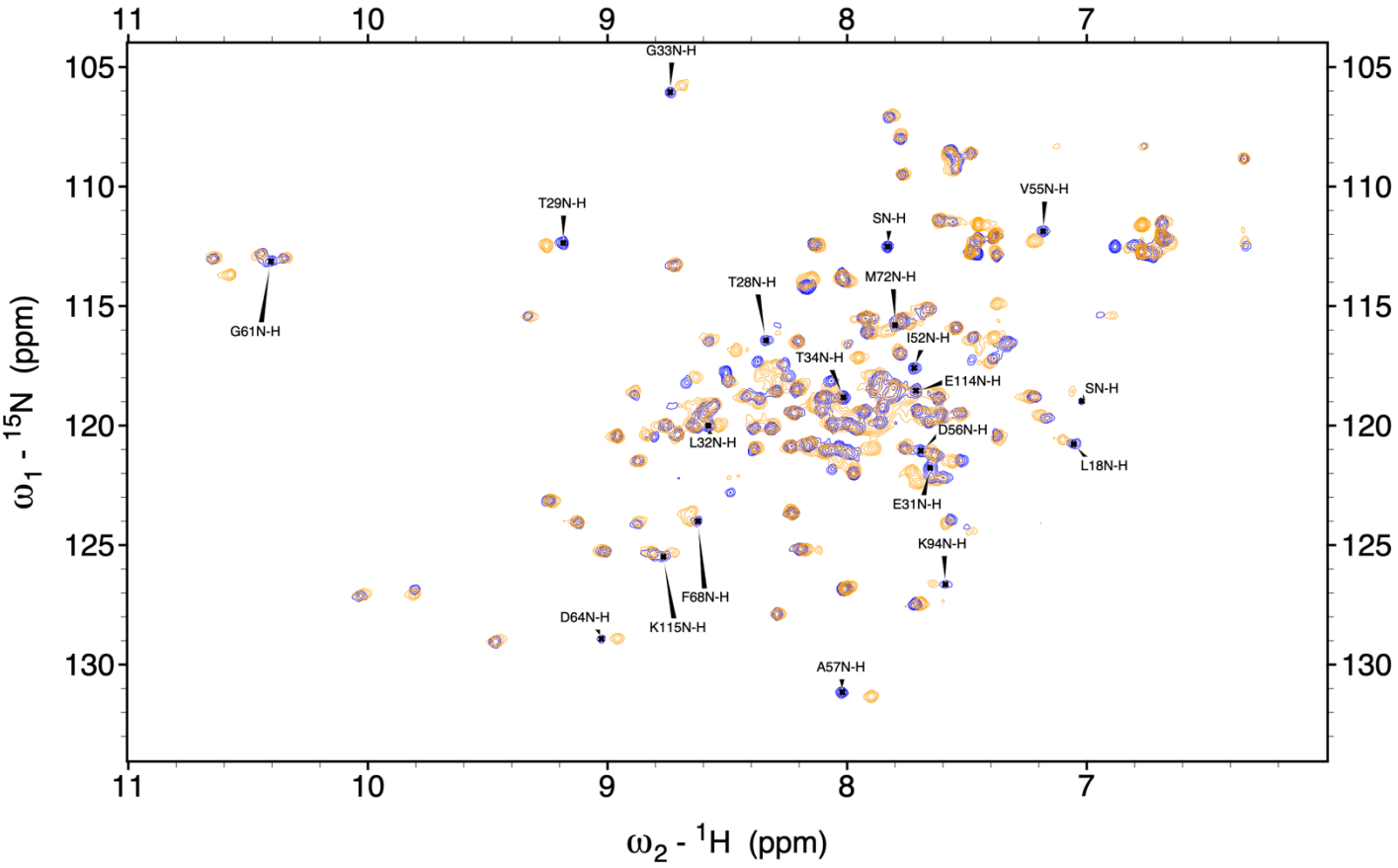
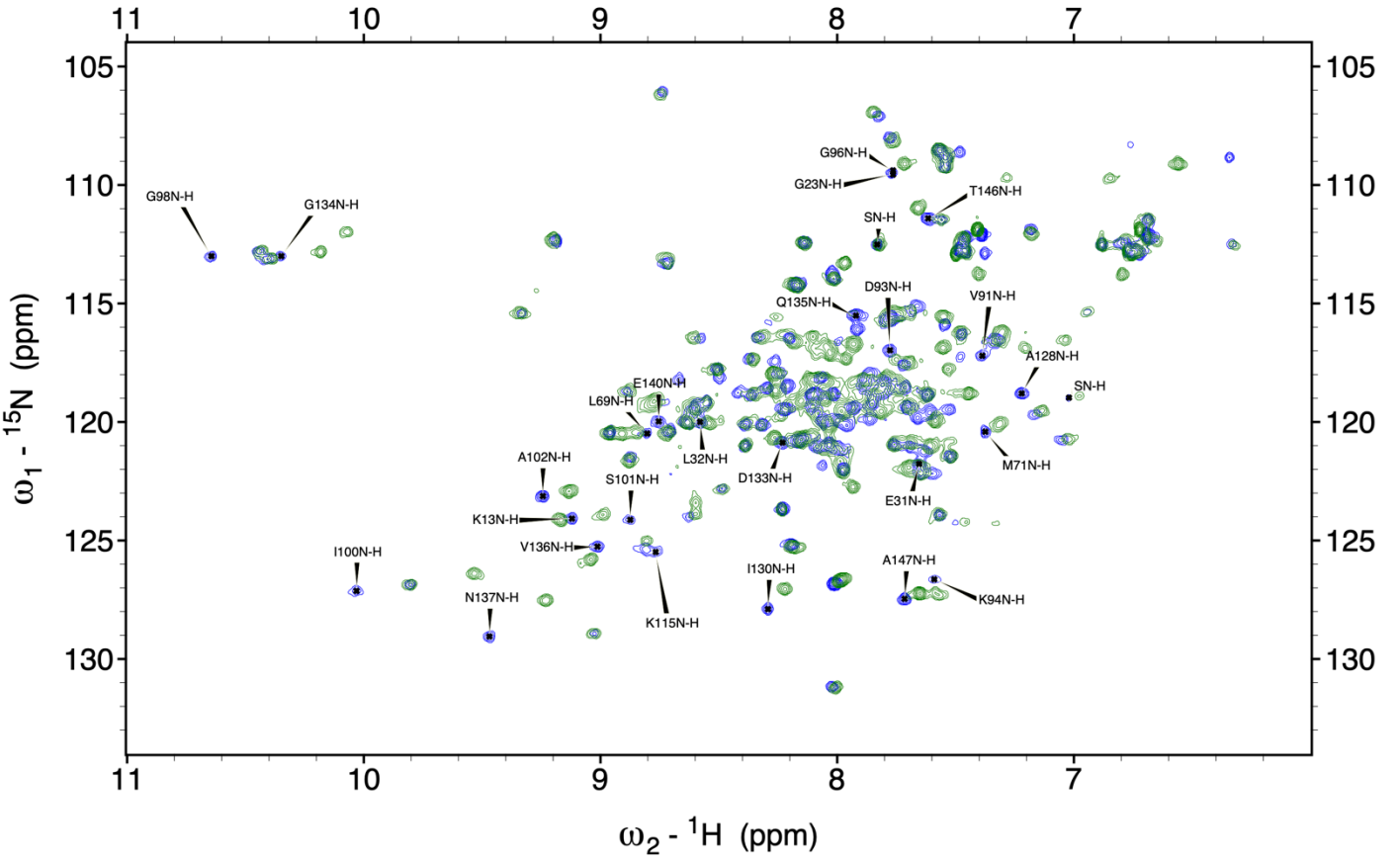


Figure S3. ^{1}H ^{15}N HSQC spectra of isotopically enriched 75 μM ^{15}N **CaM-CaN peptide complex** (red) overlaid with 75 μM ^{15}N **WT CaM** (blue). Solution conditions were 50 mM HEPES, 100 mM KCl, 6 mM $CaCl_2$, pH 7.4 at 298K. Resonance frequency assignments shown for clearly resolved cross peaks of **a)** CaM-CaN peptide complex and **b)** WT CaM. Complete list of assigned data from analysis of mutant complexes shown in **Figure S4**.


11
10
9
8
7
105
110
115
120
125
130
G33N-H
T29N-H
V55N-H
SN-H
M72N-H
T28N-H
G61N-H
I52N-H
T34N-H
E114N-H
SN-H
L32N-H
D56N-H
L18N-H
E31N-H
K94N-H
F68N-H
D64N-H
K115N-H
A57N-H
ω1 - 15N (ppm)
ω2 - 1H (ppm)


11
10
9
8
7
105
110
115
120
125
130
G96N-H
G23N-H
G98N-H
G134N-H
T146N-H
SN-H
D93N-H
Q135N-H
V91N-H
A128N-H
SN-H
E140N-H
L69N-H
L32N-H
M71N-H
A102N-H
D133N-H
S101N-H
E31N-H
K13N-H
V136N-H
I100N-H
I130N-H
A147N-H
K94N-H
N137N-H
K115N-H
ω1 - 15N (ppm)
ω2 - 1H (ppm)

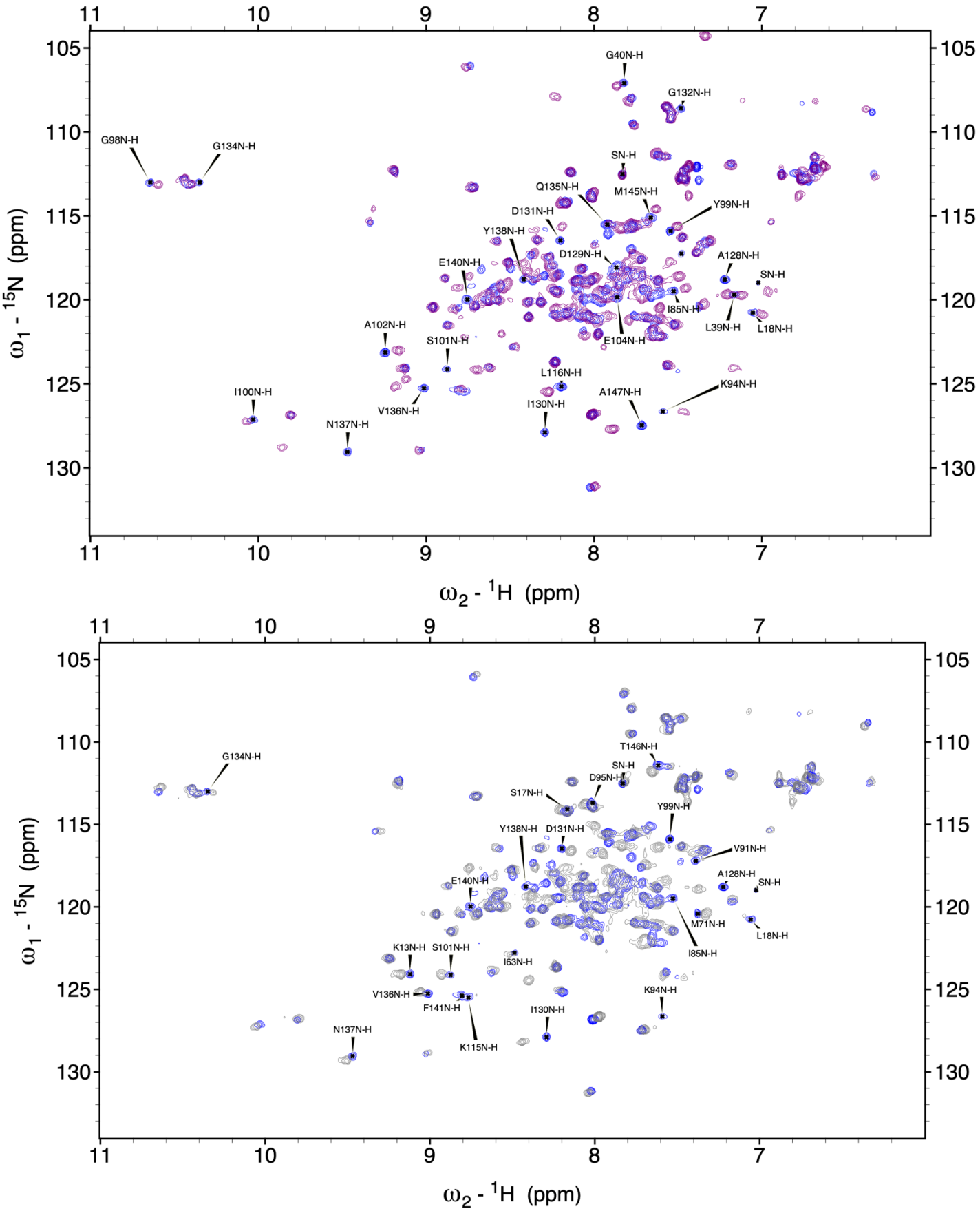


Figure S4. Overlays of ^{1}H-^{15}N HSQC NMR spectra of ^{15}N WT CaM -CaN peptide complex compared to spectra of mutant CaM complexes at 80 :1 [Ca^{2+}]:[protein]. Labels are shown on each overlay for cross-peaks of amino acids where data could be clearly attribute to differences imparted each disease-associated mutation. SN-H indicates side chain. Spectra are color coded by CaM mutation identify (**WT**, **N53I**, **F89L**, **D129G**, and **F141L**).

CaM	N53I	F89L	D129G	F141L
A1	A1	A1	A1	A1
D2	D2	D2	D2	D2
Q3	Q3	Q3	Q3	Q3
L4	L4	L4	L4	L4
T5	T5	T5	T5	T5
E6	E6	E6	E6	E6
E7	E7	E7	E7	E7
Q8	Q8	Q8	Q8	Q8
I9	I9	I9	I9	I9
A10	A10	A10	A10	A10
E11	E11	E11	E11	E11
F12	F12	F12	F12	F12
K13	K13	K13	K13	K13
E14	E14	E14	E14	E14
A15	A15	A15	A15	A15
F16	F16	F16	F16	F16
S17	S17	S17	S17	S17
L18	L18	L18	L18	L18
F19	F19	F19	F19	F19
D20	D20	D20	D20	D20
K21	K21	K21	K21	K21
D22	D22	D22	D22	D22
G23	G23	G23	G23	G23
D24	D24	D24	D24	D24
G25	G25	G25	G25	G25
T26	T26	T26	T26	T26
I27	I27	I27	I27	I27
T28	T28	T28	T28	T28
T29	T29	T29	T29	T29
K30	K30	K30	K30	K30
E31	E31	E31	E31	E31
L32	L32	L32	L32	L32
G33	G33	G33	G33	G33
T34	T34	T34	T34	T34
V35	V35	V35	V35	V35
M36	M36	M36	M36	M36
R37	R37	R37	R37	R37
S38	S38	S38	S38	S38
L39	L39	L39	L39	L39
G40	G40	G40	G40	G40
Q41	Q41	Q41	Q41	Q41
N42	N42	N42	N42	N42
P43	P43	P43	P43	P43
T44	T44	T44	T44	T44
E45	E45	E45	E45	E45
A46	A46	A46	A46	A46
E47	E47	E47	E47	E47
L48	L48	L48	L48	L48
Q49	Q49	Q49	Q49	Q49
D50	D50	D50	D50	D50
M51	M51	M51	M51	M51
I52	I52	I52	I52	I52
N53	I53	N53	N53	N53
E54	E54	E54	E54	E54
V55	V55	V55	V55	V55
D56	D56	D56	D56	D56
A57	A57	A57	A57	A57
D58	D58	D58	D58	D58
G59	G59	G59	G59	G59
N60	N60	N60	N60	N60
G61	G61	G61	G61	G61
T62	T62	T62	T62	T62
I63	I63	I63	I63	I63
D64	D64	D64	D64	D64
F65	F65	F65	F65	F65
P66	P66	P66	P66	P66
E67	E67	E67	E67	E67
F68	F68	F68	F68	F68
L69	L69	L69	L69	L69
T70	T70	T70	T70	T70
M71	M71	M71	M71	M71
M72	M72	M72	M72	M72
A73	A73	A73	A73	A73
R74	R74	R74	R74	R74

CaM	N53I	F89L	D129G	F141L
Q75	Q75	Q75	Q75	Q75
M76	M76	M76	M76	M76
K77	K77	K77	K77	K77
D78	D78	D78	D78	D78
T79	T79	T79	T79	T79
D80	D80	D80	D80	D80
S81	S81	S81	S81	S81
E82	E82	E82	E82	E82
E83	E83	E83	E83	E83
E84	E84	E84	E84	E84
I85	I85	I85	I85	I85
R86	R86	R86	R86	R86
E87	E87	E87	E87	E87
A88	A88	A88	A88	A88
F89	F89	L89	F89	F89
R90	R90	R90	R90	R90
V91	V91	V91	V91	V91
F92	F92	F92	F92	F92
D93	D93	D93	D93	D93
K94	K94	K94	K94	K94
D95	D95	D95	D95	D95
G96	G96	G96	G96	G96
N97	N97	N97	N97	N97
G98	G98	G98	G98	G98
Y99	Y99	Y99	Y99	Y99
I100	I100	I100	I100	I100
S101	S101	S101	S101	S101
A102	A102	A102	A102	A102
A103	A103	A103	A103	A103
E104	E104	E104	E104	E104
L105	L105	L105	L105	L105
R106	R106	R106	R106	R106
H107	H107	H107	H107	H107
V108	V108	V108	V108	V108
M109	M109	M109	M109	M109
T110	T110	T110	T110	T110
N111	N111	N111	N111	N111
L112	L112	L112	L112	L112
G113	G113	G113	G113	G113
E114	E114	E114	E114	E114
K115	K115	K115	K115	K115
L116	L116	L116	L116	L116
T117	T117	T117	T117	T117
D118	D118	D118	D118	D118
E119	E119	E119	E119	E119
E120	E120	E120	E120	E120
V121	V121	V121	V121	V121
D122	D122	D122	D122	D122
E123	E123	E123	E123	E123
M124	M124	M124	M124	M124
I125	I125	I125	I125	I125
R126	R126	R126	R126	R126
E127	E127	E127	E127	E127
A128	A128	A128	A128	A128
D129	D129	D129	G129	D129
I130	I130	I130	I130	I130
D131	D131	D131	D131	D131
G132	G132	G132	G132	G132
D133	D133	D133	D133	D133
G134	G134	G134	G134	G134
Q135	Q135	Q135	Q135	Q135
V136	V136	V136	V136	V136
N137	N137	N137	N137	N137
Y138	Y138	Y138	Y138	Y138
E139	E139	E139	E139	E139
E140	E140	E140	E140	E140
F141	F141	F141	F141	L141
V142	V142	V142	V142	V142
Q143	Q143	Q143	Q143	Q143
M144	M144	M144	M144	M144
M145	M145	M145	M145	M145
T146	T146	T146	T146	T146
A147	A147	A147	A147	A147
K148	K148	K148	K148	K148

KEY
Clearly Resolved Cross-Peaks
< 0.5 peak width deviation
> 0.5, < 1 peak width deviation
> 1 peak width deviation or peak missing
unable to resolve overlapped peaks

Figure S5. CaM sequence color coded by chemical shift perturbation relative to WT-CaM-CaN peptide complex at 80:1 [Ca^{2+}]:[protein]. Disease associated point mutations highlighted in yellow. Amino acids shaded green denote cross-peaks where cross-peaks could be clearly attributed to a specific amino acid. Grey amino acids indicate cross-peaks overlapped to an extent where it was not possible to discern which amino-acid was altered. We note

were we were not overly surprised to observe overlapped cross peaks for amino acids that connected the CaM -N and -C domains, as others have indicated this helix a propensity for unwinding in solution (1,2). Unwound or disordered amino acids often have resonance frequencies that are similar and often overlapped.

1. Fiorin G, Biekofsky RR, Pastore A, Carloni P. Unwinding the helical linker of calcium-loaded calmodulin: A molecular dynamics study. Proteins Struct Funct Genet. 2005;61(4):829–39.
2. Wriggers W, Mehler E, Pitici F, Weinstein H, Schulten K. Structure and dynamics of calmodulin in solution. Biophys J [Internet]. 1998;74(4):1622–39.

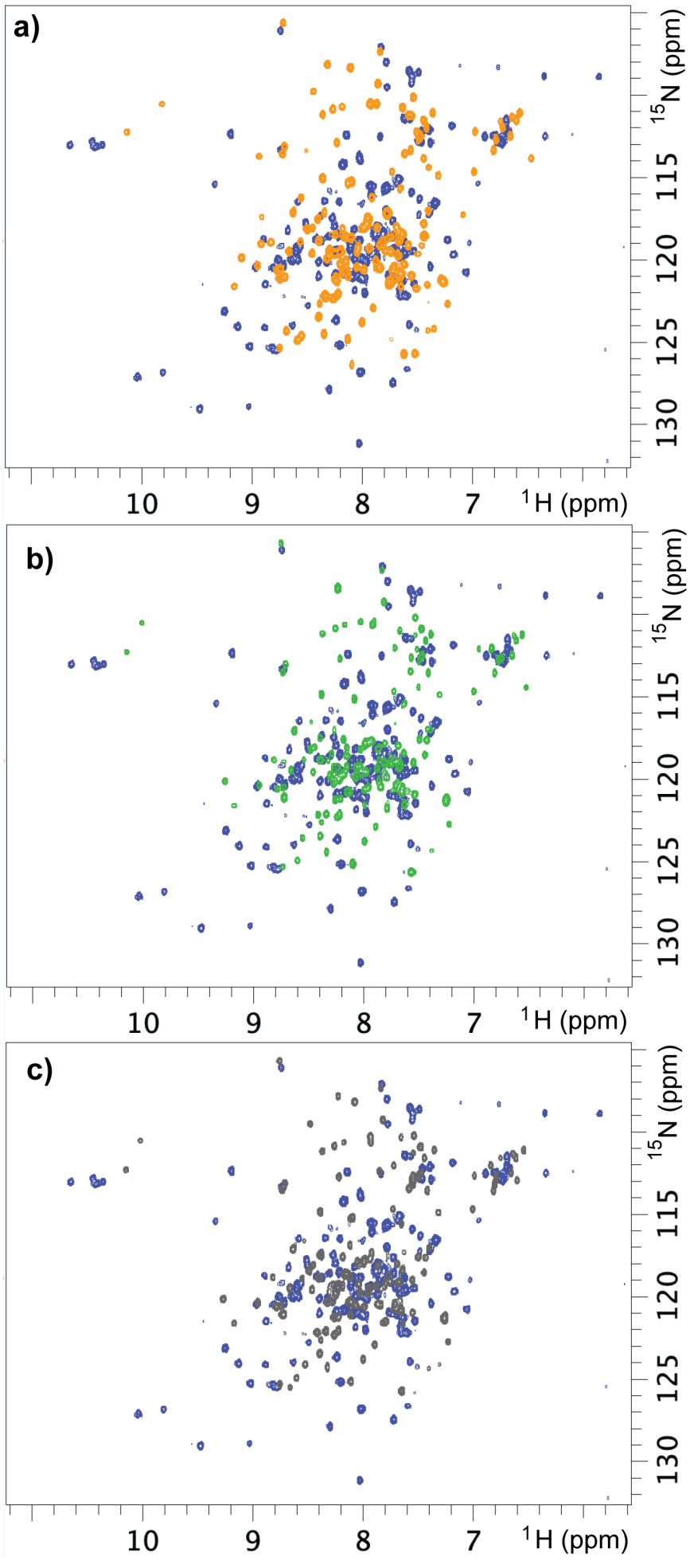
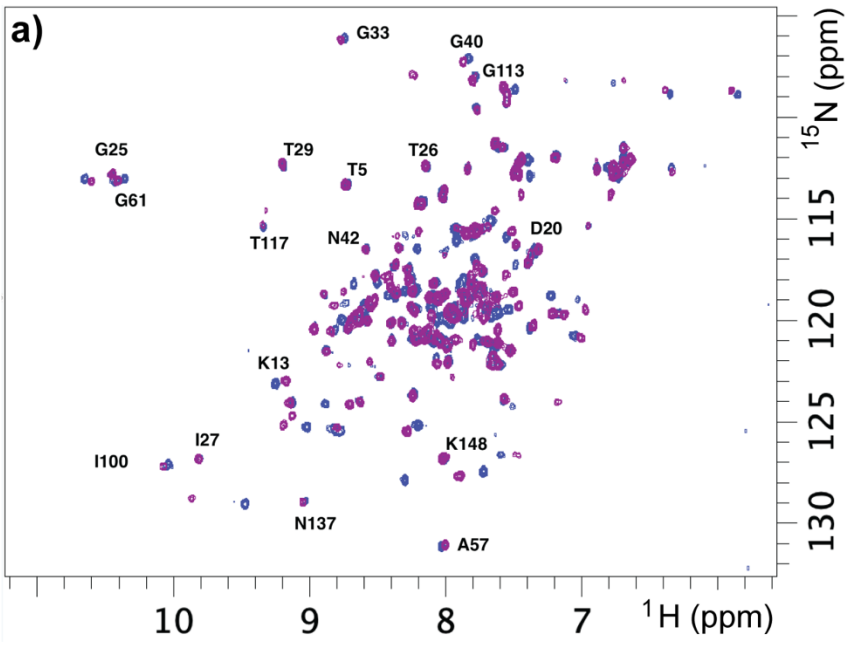


Figure S6. 2D ^{1}H ^{15}N HSQC NMR spectra of Ca^{2+} free ^{15}N enriched mutant CaM (**N53I**, **F89L** and **F141L**) in the presence of CaN peptide overlaid on spectra of **WT** Ca^{2+} CaM-CaN. The lack of similarity in the spectra confirm that the individual point mutations do not facilitate interaction between CaM and the traditional CaM binding region of CaN in the absence of Ca^{2+}.



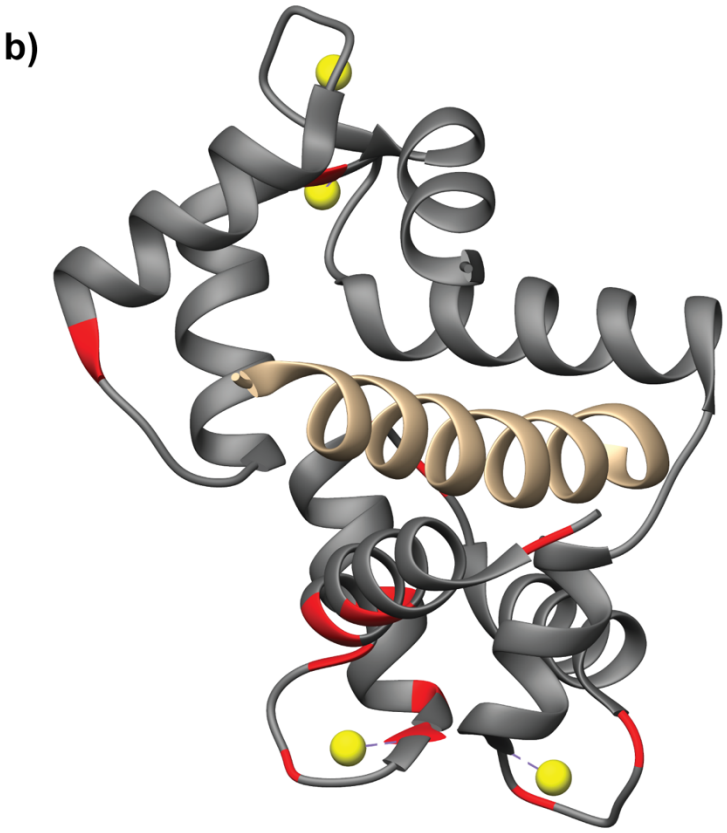


Figure S7. a) 2D ^{1}H ^{15}N HSQC NMR spectra of Ca^{2+} free ^{15}N enriched **D129G** CaM in the presence of CaN peptide overlaid on spectra of **WT** Ca^{2+} CaM-CaN. **b)** Overlapped chemical shifts mapped onto structure of CaM-CaN peptide complex in red (PDB ID 4Q5U) reveal that the spectral similarities are for amino acids not contained at the CaM-CaN binding interface. CaM shown in grey, CaN peptide shown in tan.

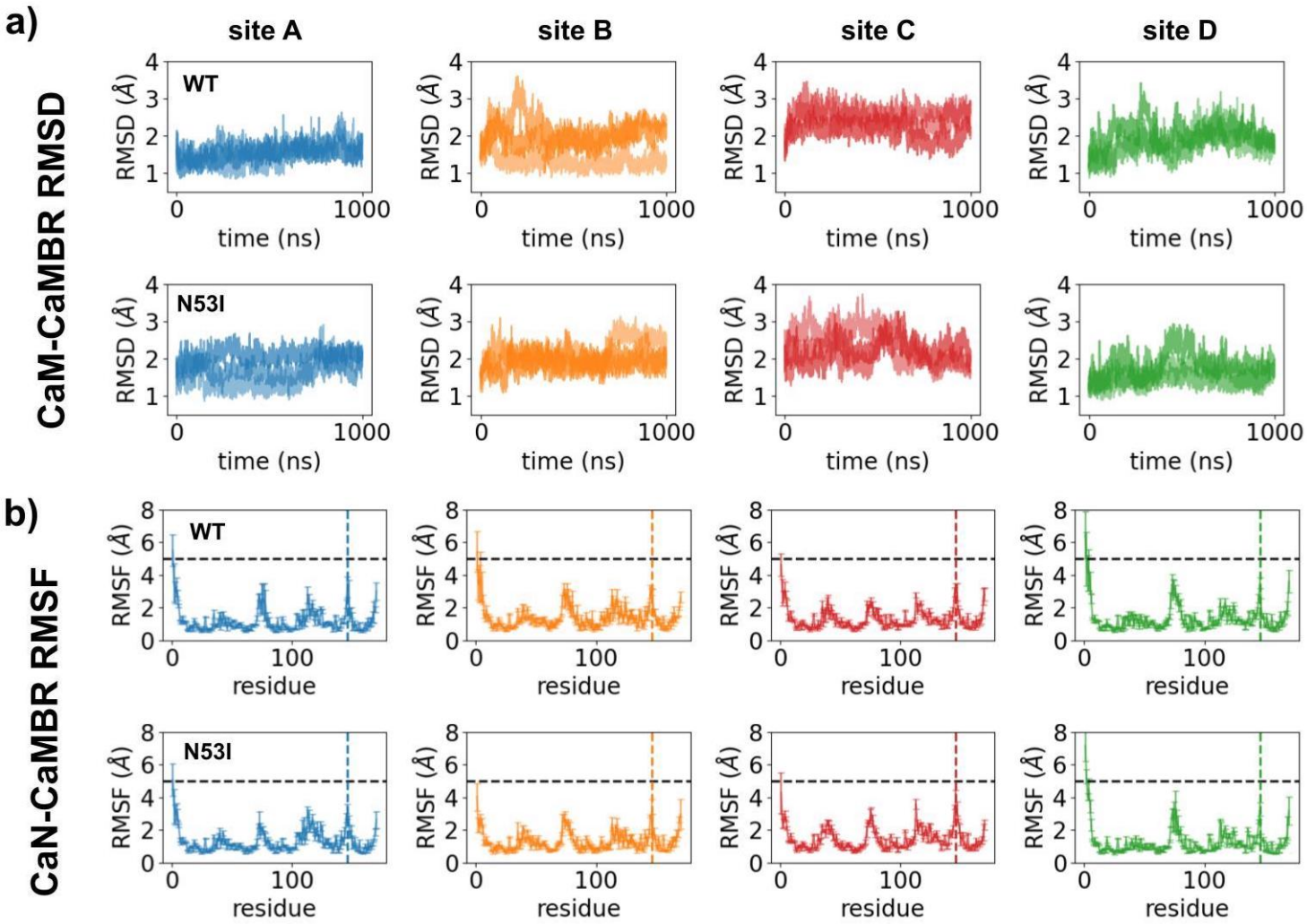


Figure S8. Molecular Dynamic Simulation Analysis. Plots of RMSD and RMSF for CaM binding region (CaMBR) of simulation contain CaMBR and distal helix for WT and N53I systems. **a)** Backbone RMSD of CaM WT (top) and N53I (bottom) at the four different distal helix binding sites. Data color-coded according to sites labeled in Figure 8 (**Site A**, **Site B**, **Site C**, **Site D**). Crystal structure of CaM-CaMBR (PDB: 4Q5U) was used as reference. **b)** RMSF of the CaM-CaN binding region of WT (top) and N53I (bottom) at the posited binding sites. Black dashed line denote 5 Å cutoff under which residues are deemed stable during the simulations.

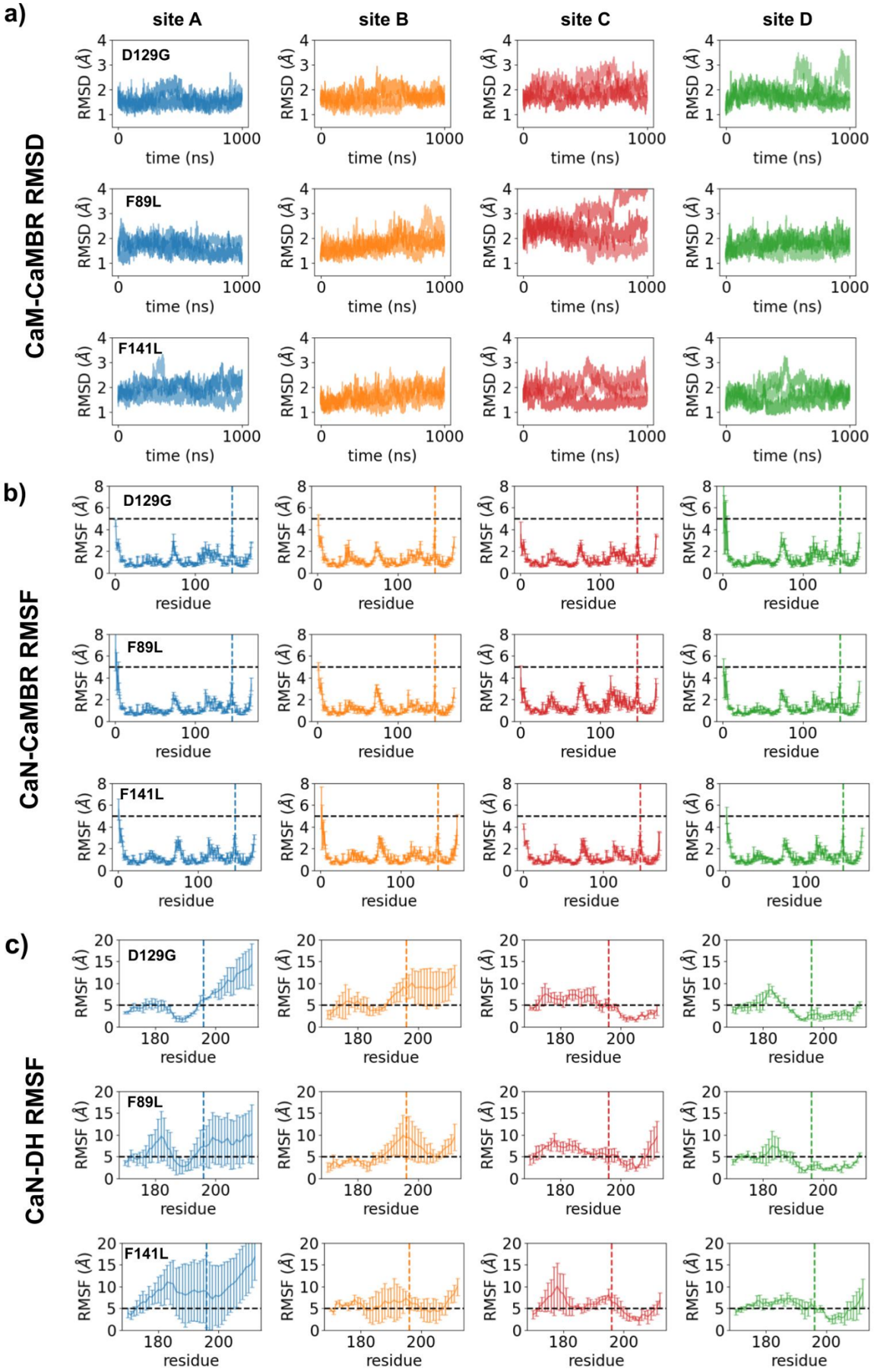


Figure S9. Analysis of molecular dynamic simulations for D129G, F89L and F141L CaM-CaN systems.

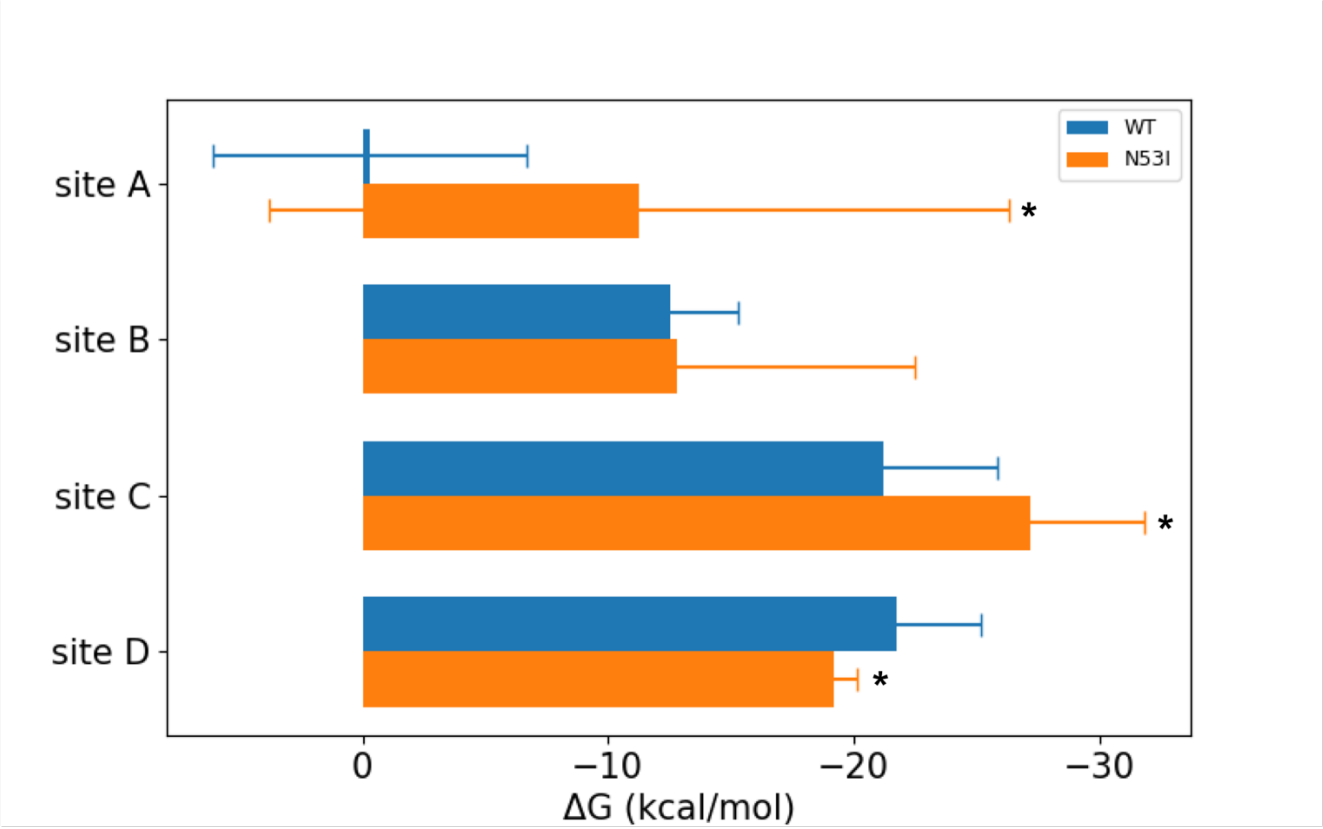


Figure S10. Approximate binding free energies between CaM and DH at four different sites computed via MM/GBSA. N53I indicates enhanced interaction between CaM and CaN-DH at sites A and C, with reduced interaction at site D. * denotes statistical significance of $p < 0.05$.