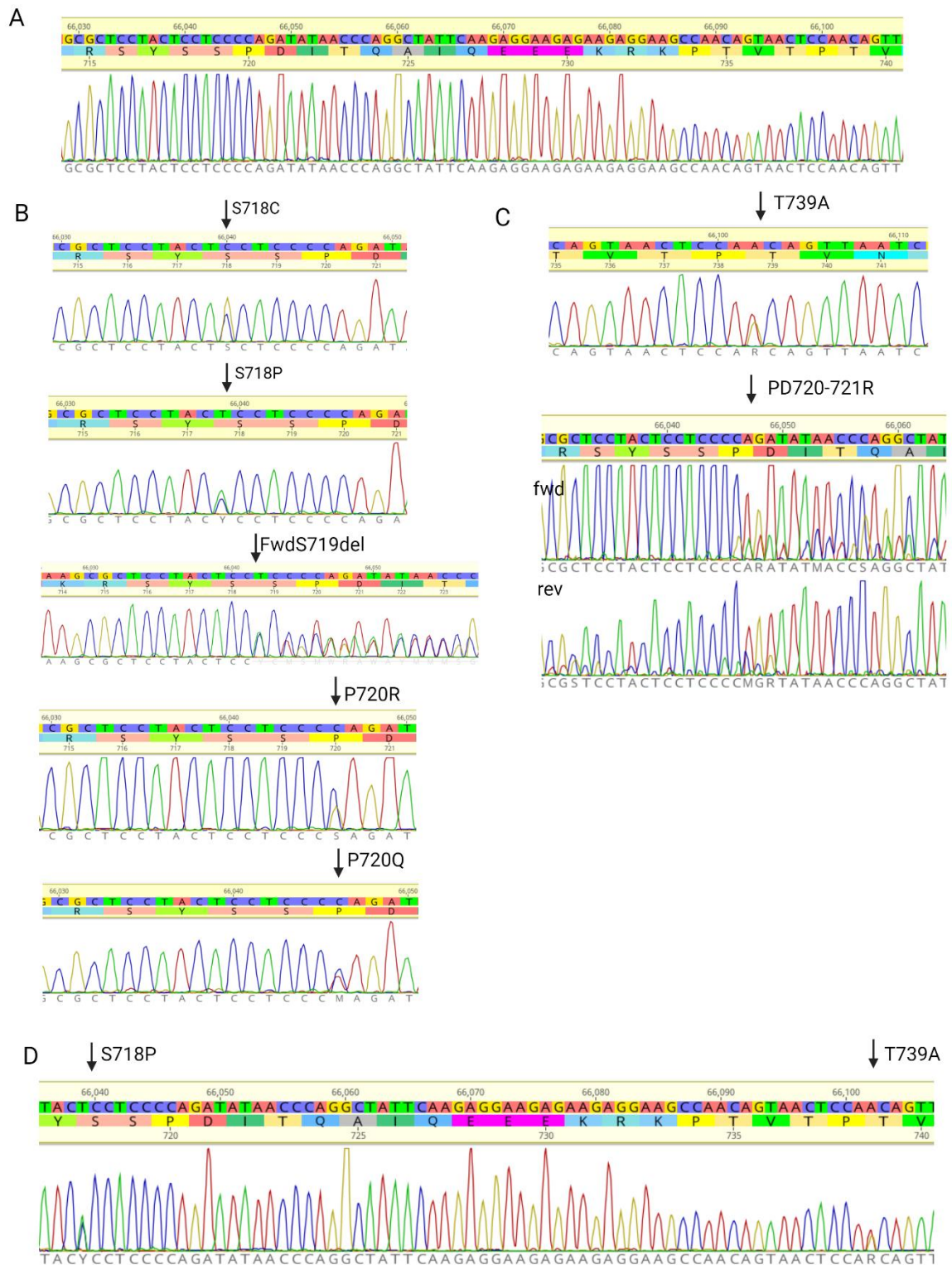


Supplementary Material

***USP8* mutations associated with Cushing's disease alter protein structure dynamics**

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Supplemental Figure 1: Chromatograms of 14 exon USP8 Sanger sequencing showing fragments with mutations for patients. (A) Fragment of 14 exon USP8 gene with wild-type; (B) Known mutations for patients with Cushing's disease (CD); (C) Mutations were not previously described for patients with CD; (D) The patient carrying two kinds of mutation.

No	ID	USP8 mutation status	Sex Female Male	Age, years	The largest adenoma's diameter, mm	Volume, ml	ACTH, pg/ml	Morning serum cortisol, nmol/l	24-h urine cortisol excretion, nmol/l	Ki 67, %	SST2	SST5	Remission of hypercortisolism after TSS, yes/no	Follow-up period, years	Remission of hypercortisolism, follow up, yes/no	Adenoma growth, follow up, yes/no
1	P1	S718P	F	66	15	1.41	38.57	696.1	137.88	0.3	>80%	>80%	Yes	3	Yes	No
2	P31	S718P	F	30	18	1.45	99.7	749	420.12	1.4	>60%	>90%	Yes	1	Yes	No
3	C112	S718P	F	34	12	0.41	97.6	613.8	662.4	3.29	30%	>80%	No	2	No	Yes
4	C61	S718P	F	37	15	0.3	83.45	594.2	1540.9	0.4	NA	NA	Yes	2	No	Yes
5	c81	T739A	F	67	3	0.003	41.81	735	485	-	NA	NA	Yes	4	Yes	No
6	P122	T739A	F	56	4.6	0.016	71.73	896.6	244.26	-	NA	NA	Yes	9	No	Yes
7	C120	T739A	F	58	6	0.07	58.8	582.2	156.6	0.4	NA	NA	Yes	8	Yes	No
8	c73	S718P, T739A	M	20	8	0.25	78.96	778	630	0.8	NA	NA	Yes	1	Yes	No
9	P20	FwdS719del	F	60	12	0.28	23.54	901.1	382	1	5%	>80%	Yes	3	Yes	No
10	c126	FwdS719del	F	25	18	1.03	54.71	1094	500	2.84	3%	>60%	No	3	No	Yes
11	P3	FwdS719del	F	18	16.7	1.56	41.81	758.6	-	0.6	>60%	>90%	Yes	2	Yes	No
12	P28	P720Q	F	39	8.5	0.11	53.47	-	149	5.3	>80%	>60%	Yes	3	No	Yes
13	P22	P720Q	F	31	10.5	0.26	120	743.2	1752	-	>50%	>90%	Yes	1	Yes	No
14	P25	P720Q	F	26	12	0.31	128.3	448	352.58	4.5	50%	>30%	Yes	1	No	Yes
15	P27	P720R	F	36	8.5	0.12	81.2	471.4	892	-	NA	>80%	Yes	1	Yes	No
16	c150	P720R	F	37	14	0.43	55	734	708	3.24	60%	>60%	Yes	1	Yes	No
17	29	S718C	F	36	5	0.06	78.69	1066	-	-	NA	NA	Yes	2	No	Yes
18	c117	PD720-721R	F	38	9	0.1	64.34	535	648.25	0.1	15%	>80%	Yes	8	No	Yes

ACTH – adrenocorticotrophic hormone, TSS-transsphenoidal surgery, NA- not applicable

Supplemental Table 1: Clinical data and USP8 status in patients enrolled into the study

SNP	PredictSNP	MAPP	PhD-SNP	PolyPhen-2	SIFT	SNAP	Alpha Missense
p.S718C	Pathogenic	Neutral	Pathogenic	Pathogenic	Pathogenic	Neutral	Pathogenic
p.S718P	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Neutral	Pathogenic
p.P720R	Pathogenic	Neutral	Neutral	Pathogenic	Pathogenic	Pathogenic	Pathogenic
p.P720Q	Neutral	Neutral	Neutral	Pathogenic	Pathogenic	Pathogenic	Pathogenic
p.T739A	Neutral	Neutral	Neutral	Neutral	Neutral	Neutral	Neutral

(a)

SNP	MUpro	mCSM	DUET	SDM	DynaMut	PremPS
p.S718C	Pathogenic	Pathogenic	Pathogenic	Neutral	Neutral	Pathogenic
p.S718P	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Pathogenic
p.P720R	Pathogenic	Neutral	Neutral	Neutral	Neutral	Pathogenic
p.P720Q	Pathogenic	Pathogenic	Neutral	Neutral	Neutral	Pathogenic
p.T739A	Pathogenic	Pathogenic	Neutral	Neutral	Neutral	Pathogenic

(b)

Supplemental Figure 2: Preliminary evaluation using automated tools. "Pathogenic" and "Neutral" effects are indicated.

(a) Initial assessment of functional impact. The tools in this table are sourced from the PredictSNP web resource and include PredictSNP, MAPP, PhD-SNP, PolyPhen-2, SIFT and SNAP, representing consensus predictions or specific threshold values. The results for Alpha Missense are sourced from the Alpha Missense prediction dataset.

(b) Initial evaluation of effects on thermodynamic stability using tools MUpro, mCSM, DUET, SDM, DynaMut, and PremPS. DUET combines results from mCSM and SDM.

All interactions calculated over a 100 ns simulation (10,000 frames) :

	DUB domain interaction with region (402–644)				DUB domain interaction with WW-Like region (645–684)				DUB domain interaction with interdomain region (685–776)				DUB domain (intradomain interactions) (777 – 1088)				DUB domain interaction with post-DUB region (1089 – 1118)			
	N	F			N	F			N	F			N	F			N	F		
WT	132	0	68 326	0	26	0	1550	0	97	0	133 092	0	5852	0	21 576 263	0	114	0	310 218	0
S718C	331	+150.76%	105 312	+54.13%	35	+34.62%	10 356	+568.13%	92	-5.15 %	142 796	+7.29%	5998	+2.49	21 515 218	-0.28%	119	+4.39%	290 656	-6.31%
P720R	266	+101.52%	100 715	+47.40%	97	+273.08%	30 806	+1887.48%	96	-1.03 %	154 648	+16.20%	6173	+5.49%	21 714 144	+0.64%	114	0.0%	326 208	+5.15%
P720Q	200	+51.52%	72 389	+5.95%	19	-26.92%	8 938	+476.65%	94	-3.09%	151 139	+13.56%	6242	+6.66%	21 530 820	-0.21%	117	+2.63%	296 197	-4.52%
S719del	377	+185.61%	148 440	+117.25%	34	+30.77%	2 456	+58.45%	88	-9.28%	148 730	+11.75%	6188	+5.74%	21 488 159	-0.41%	109	-4.39%	310 823	+0.20%
PD720-721R	212	+60.61%	95 516	+39.79%	51	+96.15%	35 836	+2212.00%	86	-11.34%	148 263	+11.40%	6030	+3.04%	21 799 267	+1.03%	126	+10.53%	272 282	-12.23%
T739A	196	+48.48%	80 787	+18.24%	18	-30.77%	3 747	+141.74%	104	+7.22%	151 238	+13.63%	6058	+3.52%	21 638 556	+0.29%	105	-7.89%	307 644	-0.83%
Mutants (mean)	264	+100.0%	100 526	+47.13%	42	+61.54%	15 356	+890.71%	93	-4.12%	149 469	+12.31%	6115	+4.49%	21 614 361	+0.18%	115	+0.88%	300 635	-3.09%

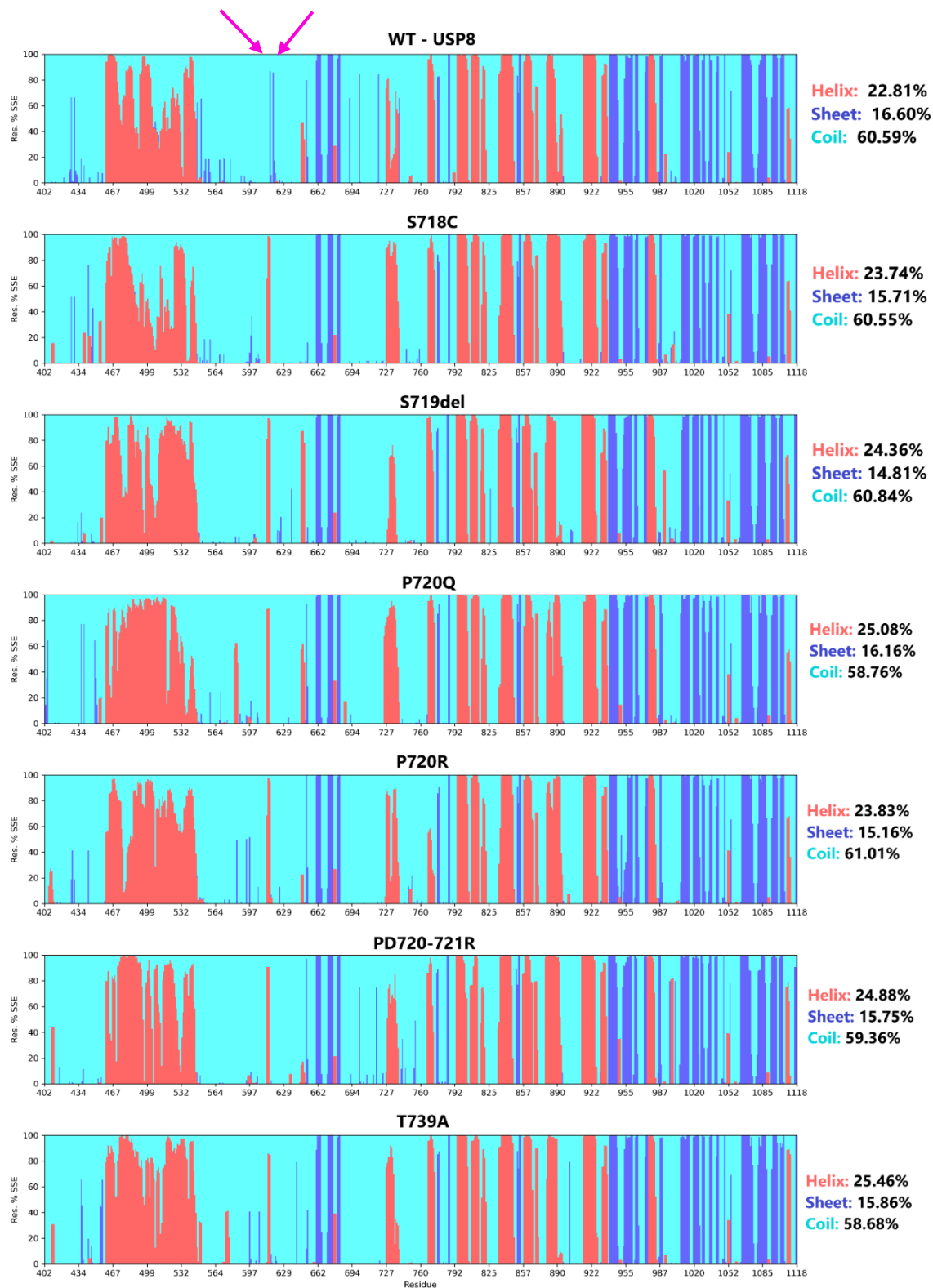
Interactions exceeding 10% of the simulation time (more than 1,000 frames) :

WT	18	0	59520	0	0	0	0	0	23	0	121028	0	3056	0	21 149 564	0	50	0	303343	0
S718C	29	+61.11%	76028	+27.74%	4	-	6769	-	26	+13.04%	134922	+11.48%	3022	-1.11%	21 044 042	-0.5%	46	-8.00%	279811	-7.76%
P720R	26	+44.44%	73912	+24.18%	11	-	16375	-	29	+26.09%	144221	+19.16%	3136	+2.62%	21 221 172	+0.34%	46	-8.00%	317227	+4.58%
P720Q	22	+22.22%	58416	-1.85%	3	-	7051	-	30	+30.43%	140391	+16.00%	3143	+2.85%	21 029 046	-0.57%	45	-10.00%	285720	-5.81%
S719del	41	+127.78%	115276	+93.68%	0	-	0	-	27	+17.39%	142359	+17.62%	3050	-0.2%	20 991 095	-0.75%	45	-10.00%	305111	+0.58%
PD720-721R	23	+27.78%	79880	+34.21%	9	-	29446	-	33	+43.48%	140933	+16.45%	3105	+1.6%	21 337 546	+0.89%	50	0.0%	260654	-14.07%
T739A	19	+5.56%	62545	+5.08%	2	-	2706	-	29	+26.09%	136026	+12.39%	3070	+0.46%	21 177 164	+0.13%	52	+4.00%	300893	-0.81%
Mutants (mean)	27	+50.00%	77676	+30.50%	5	-	10391	-	29	+26.09%	139809	+15.52%	3088	+1.05%	21 101 323	-0.23%	47	-6.00%	291569	-3.88%

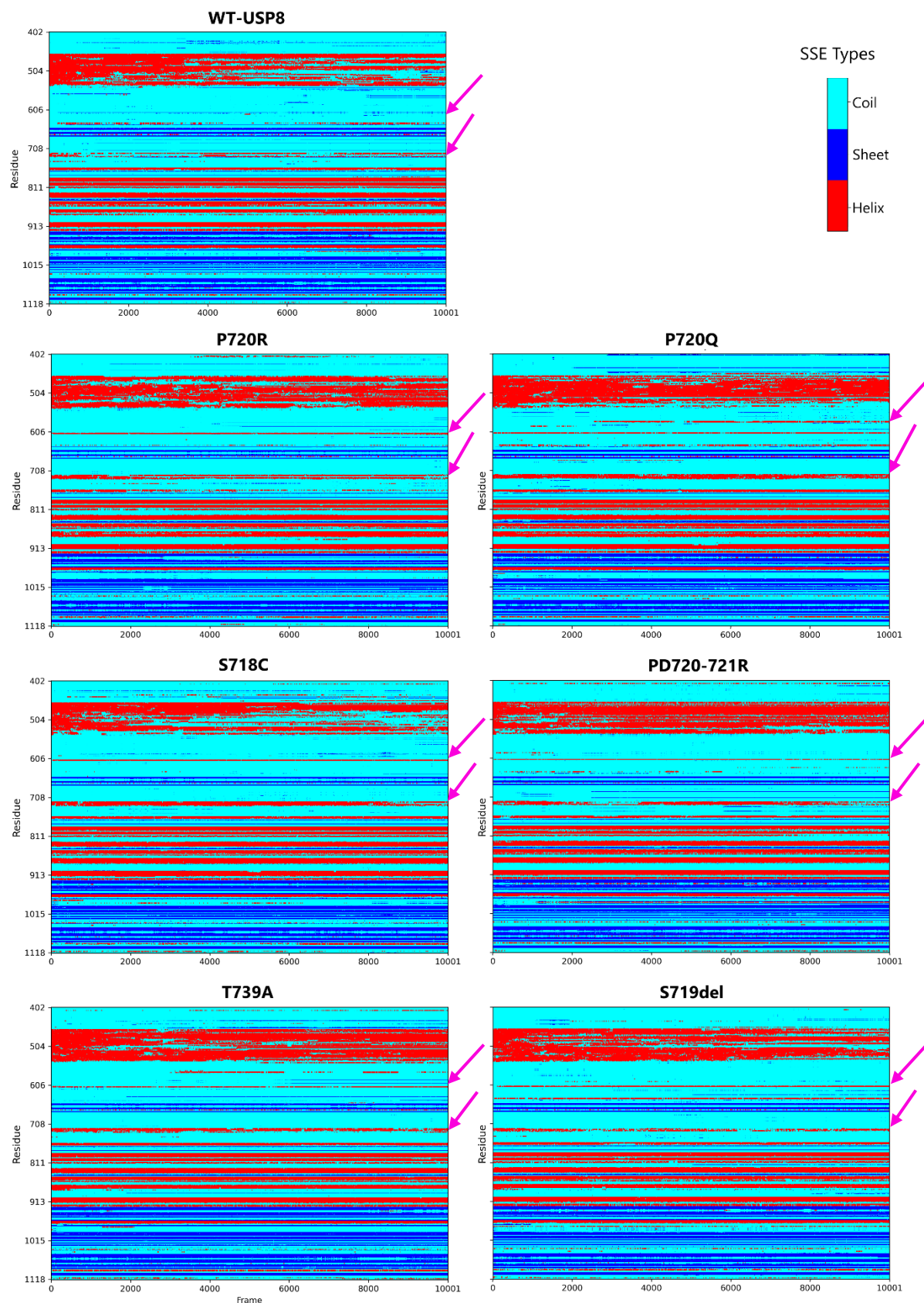
Interactions exceeding 30% of the simulation time (more than 3,000 frames) :

WT	11	0	48827	0	0	0	0	0	16	0	106809	0	2396	0	19 895 204	0	33	0	269182	0
S718C	10	-9.09%	41009	-16.01%	0	-	0	-	19	+18.75%	122309	+14.51%	2378	-0.75%	19 809 616	-0.43%	33	0.0 %	257499	-4.34%
P720R	10	-9.09%	43429	-11.06%	0	-	0	-	16	0.0 %	118264	+10.72%	2389	-0.25%	19 790 563	-0.53%	38	+15.15%	301926	+12.16%
P720Q	6	-45.45%	30170	-38.21%	0	-	0	-	20	+25.00%	124970	+17.00%	2426	+1.25%	19 670 064	-1.13%	35	+6.06%	268724	-0.17%
S719del	17	+54.56%	66538	+36.27%	0	-	0	-	19	+18.75%	128938	+20.72%	2392	-0.17%	19 758 723	-0.69%	35	+6.06%	287340	+6.75%
PD720-721R	12	+9.09%	61285	+25.51%	5	-	21737	-	17	+6.25%	111694	+4.83%	2424	+1.17%	20 056 467	+0.81%	30	-9.09%	223021	-17.15%
T739A	8	-27.27%	43316	-11.29%	0	-	0	-	17	+6.25%	112597	+5.42%	2414	+0.75%	19 945 540	+0.25%	36	+9.09%	269308	+0.05%
Mutants (mean)	10	-9.09%	47624	-2.46%	0.8	-	3623	-	18	+12.50%	119795	+12.16%	2404	+0.33%	19 838 496	-0.29%	34	+3.03%	267970	-0.45%

Supplemental Table 2: Time-resolved dynamics of DUB domain interactions with structural regions. "N" shows the number of interactions, and "F" indicates their frequency, with percentages showing the difference compared to the wild type. The table summarizes all interactions over a 100 ns simulation, including those exceeding 10% and 30% of the simulation time, highlighting the stability and prevalence of interactions across different regions.



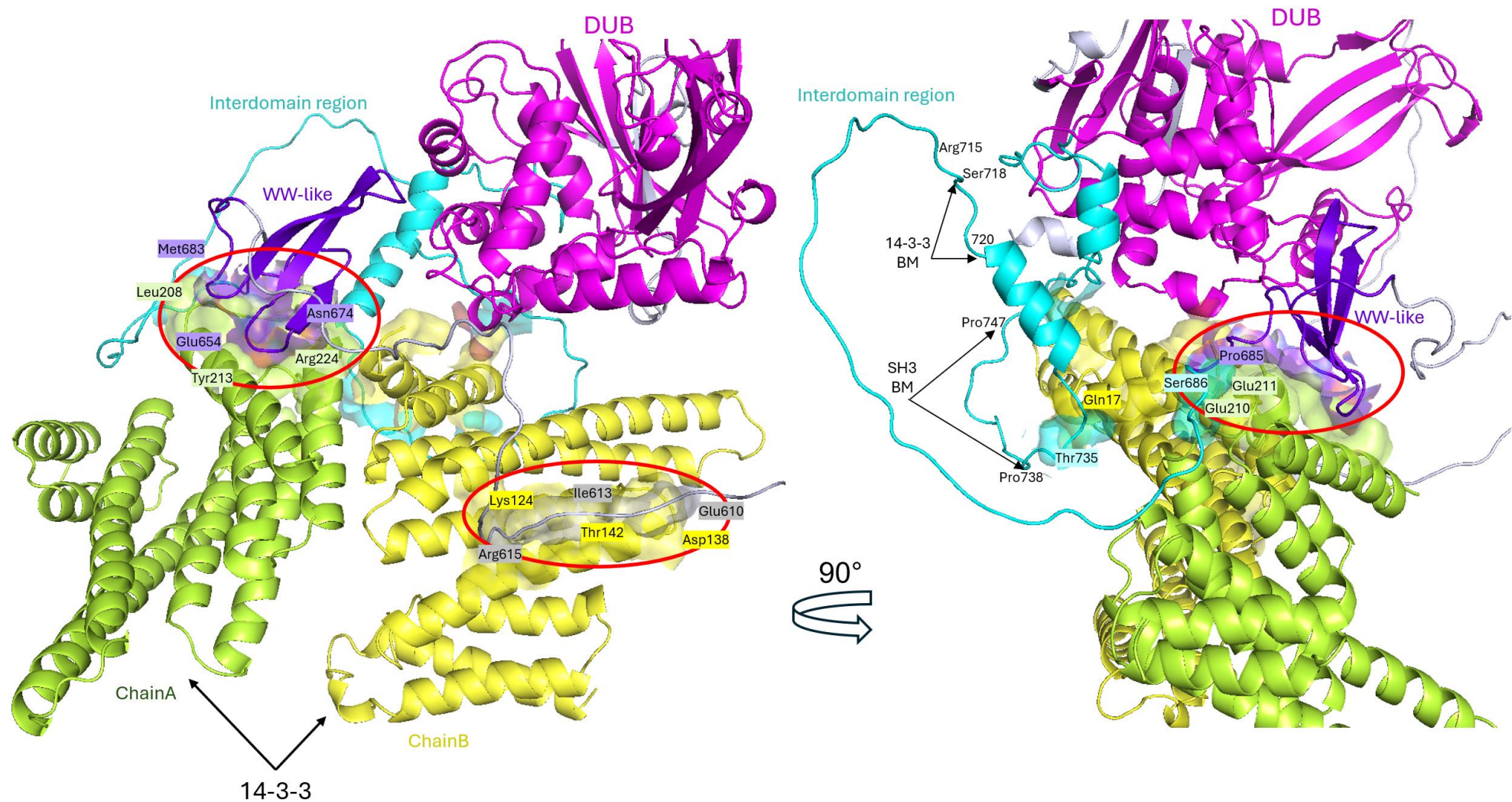
Supplemental Figure 3: Secondary Structure Composition Comparison: Pink arrows highlight the 610-615 region, showing a sheet in the wild type, replaced by a helix in all mutants



Supplemental Figure 4: Secondary Structure Dynamics Comparison Between WT and Mutant Variants Over Time Frames. Pink arrows indicate regions that differ between WT and are consistently observed across all six mutants.

	<i>S718C</i>	<i>P720R</i>	<i>P720Q</i>	<i>S719del</i>	<i>PD720_721R</i>	<i>T739A</i>
LEU779 MET788	Hydrophobic - 10	Hydrophobic - 1	Hydrophobic - 11	Hydrophobic - 1	Hydrophobic - 36	Hydrophobic - 1
ASN818 ASN885	VdWContact - 42	VdWContact - 3	HBDonor - 1 VdWContact - 78 HBAcceptor - 1	HBDonor - 1650 VdWContact - 2062 HBAcceptor - 1650	VdWContact - 690	HBDonor - 5112 VdWContact - 5797 HBAcceptor - 5112
HIS822 THR853	Hydrophobic - 318 VdWContact - 144 HBDonor - 104 HBAcceptor - 104	Hydrophobic - 1854 VdWContact - 254 HBDonor - 1996 HBAcceptor - 1996	Hydrophobic - 1316 HBDonor - 379 VdWContact - 637 HBAcceptor - 379	Hydrophobic - 560 HBDonor - 27 VdWContact - 122 HBAcceptor - 27	Hydrophobic - 1000 HBDonor - 96 VdWContact - 229 HBAcceptor - 96	Hydrophobic - 433 HBDonor - 30 VdWContact - 93 HBAcceptor - 30
ASP883 GLU825	HBDonor - 693 VdWContact - 1599 HBAcceptor - 693	HBDonor - 2390 VdWContact - 3944 HBAcceptor - 2390	HBDonor - 228 VdWContact - 778 HBAcceptor - 228	HBDonor - 8 VdWContact - 63 HBAcceptor - 8	HBDonor - 560 VdWContact - 1470 HBAcceptor - 560	HBDonor - 2 VdWContact - 38 HBAcceptor - 2
GLN866 GLY1062	HBDonor - 4710 HBAcceptor - 4710 VdWContact - 5358	HBDonor - 5094 HBAcceptor - 5094 VdWContact - 6135	HBDonor - 18 VdWContact - 55 HBAcceptor - 18	HBDonor - 68 VdWContact - 71 HBAcceptor - 68	HBDonor - 1571 VdWContact - 1751 HBAcceptor - 1571	HBDonor - 4423 VdWContact - 5093 HBAcceptor - 4423
ASP888 GLN916	HBDonor - 14 VdWContact - 22 HBAcceptor - 14	HBAcceptor - 1 HBDonor - 8 VdWContact - 10	HBDonor - 19 VdWContact - 36 HBAcceptor - 19	HBDonor - 24 VdWContact - 24 HBAcceptor - 24	HBDonor - 3 VdWContact - 3 HBAcceptor - 3	HBDonor - 14 VdWContact - 19 HBAcceptor - 14
ASN981 SER994	HBDonor - 4304 HBAcceptor - 4304 VdWContact - 7179	HBAcceptor - 39 VdWContact - 863 HBDonor - 39	HBAcceptor - 4744 VdWContact - 6288 HBDonor - 4744	HBAcceptor - 941 VdWContact - 1611 HBDonor - 941	HBDonor - 2714 HBAcceptor - 2714 VdWContact - 4116	HBAcceptor - 1588 VdWContact - 4205 HBDonor - 1588
ASP993 PHE983	HBDonor - 1137 VdWContact - 9599 HBAcceptor - 1137	VdWContact - 798	HBDonor - 2 VdWContact - 7944 HBAcceptor - 2	VdWContact - 9955	VdWContact - 9970	VdWContact - 7979
ARG989 ARG991	HBAcceptor - 324 VdWContact - 622 HBDonor - 324	HBAcceptor - 4108 VdWContact - 5187 HBDonor - 4108 Hydrophobic - 12	VdWContact - 1	VdWContact - 1	HBAcceptor - 107 VdWContact - 111 HBDonor - 107	HBAcceptor - 665 VdWContact - 1085 HBDonor - 665
LEU792 MET834	Hydrophobic - 102 VdWContact - 33	Hydrophobic - 81 VdWContact - 21	Hydrophobic - 11 VdWContact - 3	Hydrophobic - 2 VdWContact - 2	Hydrophobic - 2909 VdWContact - 851	Hydrophobic - 12 VdWContact - 1
ASP979 LEU977	VdWContact - 1	VdWContact - 58	Hydrophobic - 10 HBAcceptor - 1 VdWContact - 114 HBDonor - 1	VdWContact - 3	VdWContact - 5	VdWContact - 13
THR978 TYR1016	Hydrophobic - 198 VdWContact - 33	Hydrophobic - 66 VdWContact - 9	Hydrophobic - 318 VdWContact - 306 HBDonor - 92 HBAcceptor - 92	Hydrophobic - 3 VdWContact - 1	Hydrophobic - 1825 VdWContact - 690	Hydrophobic - 67 VdWContact - 75 HBDonor - 2 HBAcceptor - 2
ASP979 TYR1016	HBAcceptor - 110 VdWContact - 201 HBDonor - 110 Hydrophobic - 232	HBAcceptor - 1 VdWContact - 2 HBDonor - 1	Hydrophobic - 1173 VdWContact - 675 HBAcceptor - 158 HBDonor - 158	VdWContact - 1	Hydrophobic - 8 HBAcceptor - 12 HBDonor - 12 VdWContact - 40	Hydrophobic - 3414 VdWContact - 1203
ASN897 GLU895	HBAcceptor - 7 VdWContact - 9 HBDonor - 7	VdWContact - 4	VdWContact - 24 HBAcceptor - 1 HBDonor - 1	HBAcceptor - 667 VdWContact - 1069 HBDonor - 667 Hydrophobic - 33	Hydrophobic - 2 VdWContact - 3	HBAcceptor - 1 VdWContact - 1 HBDonor - 1
SER1099 VAL1028	HBDonor - 14 VdWContact - 90	HBDonor - 6 VdWContact - 19	HBDonor - 1 VdWContact - 5	HBDonor - 33 VdWContact - 51	HBDonor - 755 VdWContact - 800	HBDonor - 1 VdWContact - 1

Supplemental Table 3: Amino acid interaction pairs consistently found in all six mutant models but absent in the wild-type model. The types of interactions and their frequencies are presented as Interaction type - frequency (e.g., VdWContact - 800). The four most stable pairs are highlighted in red.



Supplemental Figure 5: Additional Protein-Protein Docking Analysis. Final frames from the simulations of all models were examined for potential binding to the 14-3-3 protein. The figure shows one of the complexes (mutant P720R + 14-3-3 β), illustrating a possible altered interaction between the unphosphorylated USP8 protein and the regulatory protein 14-3-3 β .