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Supporting Information

Two Histidines in an α-Helix: A Rigid Co^{2+}-Binding Motif for PCS Measurements by NMR Spectroscopy

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1. Construct Design

Genes of the ubiquitin double mutants E24H/A28H and A28H/D32H and the GB1 double mutant E15H/T17H were ordered as gBlocks (Integrated DNA Technologies, IDT), amplified by PCR and cloned into the T7 expression vector pETMCSI[1] between the *Nde*I and *Eco*RI sites. The amino acid sequence of the GB1 construct was preceded by the hexapeptide MASMTG at the N-terminus. The plasmids were transformed into *E. coli* BL21 (DE3) pLysS for expression.

2. Expression and purification

Cells were grown at 37 °C in LB (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) containing 100 mg/L ampicillin. Following cell growth to OD_{600} = 0.8-1, the cells were centrifuged and the pellet suspended in M9 medium (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl) supplied with 1 g/L $^{15}NH_4Cl$ for uniform ^{15}N-labelling. Following further growth for 1 h, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to final concentration of 1 mM to induce protein expression. After induction, the cells were incubated overnight at room temperature. The cell culture was centrifuged at 5000 *g* for 15 min at 4 °C and the cells were lysed in lysis buffer (10% glycerol, 1 mM PMSF, 2 mM 2-mercaptoethanol, 150 mM NaCl, 20 mM sodium phosphate, pH 6.85) using sonication. The supernatant was applied to a 5 mL Ni-NTA column (GE Healthcare, USA) and bound protein was eluted with a gradient of 10–300 mM imidazole in 150 mM NaCl and 50 mM sodium phosphate, pH 6.85. Following elution, the purified proteins were treated with EDTA in 5-fold excess to remove any divalent metal ions bound to the dHis motif and the buffer was exchanged to NMR buffer (20 mM MES, pH = 6.8 for the ubiquitin mutants and pH = 6.5 for the GB1 mutant) using an Amicon Ultra-15 centrifugal filter unit with Ultracel-3 membrane (Merck Millipore). The absence of bound divalent metal ions was confirmed by inductively coupled plasma optical emission spectrometry (ICP-OES). 10% D_2O were added to the final samples. The yields were about 12 mg of purified protein per litre of medium. SDS-PAGE indicated >95% purity.

Initially, the ubiquitin E24H/A28H mutant was constructed with an additional C-terminal His_6 tag, but binding to the Ni-NTA column proved to be so tight that the protein could not be eluted even with 500 mM imidazole. In agreement with the relative binding affinities of Co^{2+} found by ITC (Figure S1), the ubiquitin dHis mutants without His_6-tag eluted from the Ni-NTA column at 200–300 mM imidazole and the GB1 dHis mutant at 50–70 mM imidazole.

3. NMR spectroscopy

All NMR data were recorded at 25 °C on a Bruker Avance II 800 MHz NMR spectrometer equipped with a TCI cryoprobe, except for the data of ERp29-C, for which the experimental parameters are reported in Figure S5. [^{15}N,^{1}H]-HSQC spectra were recorded using t_{1max} = 40 ms, t_{2max} = 106 ms and total recording times of about 40 min. N_z exchange spectra were recorded using t_{1max} = 83 ms, t_{2max} = 68 ms, a mixing time of 6 ms, and a total recording time of about 0.5 h. I_zS_z exchange spectra were recorded using t_{1max} = 83 ms, t_{2max} = 68 ms, a mixing time of 10 ms, and a total recording time of about 14 h.

PCSs were measured as the ^{1}H chemical shift observed in [^{15}N,^{1}H]-HSQC spectra of samples with $CoCl_2$ minus the chemical shift measured for a sample containing $ZnCl_2$. In these samples, proteins and metal ion were present in equimolar ratio. The PCSs were used to fit $\Delta\chi$ tensors to published coordinates of the proteins using the program Numbat.[2] Quality factors of the fits were calculated as the root-mean-square (RMS) of the differences between experimental and back-calculated PCSs divided by the RMS of the experimental PCSs.

One-bond couplings between ^{1}H and ^{15}N spins of backbone amides were measured by the IPAP-^{15}N-HSQC experiment,[3] using t_{1max} = 80 ms, t_{2max} = 100 ms, and total recording times of about 16 h per spectrum. Peak positions were measured using the program Sparky.[4] The RDCs, $^{1}D_{HN}$, were calculated as the ^{1}H-^{15}N one-bond couplings measured for the paramagnetic Co^{2+} complex minus the corresponding values measured for the diamagnetic Zn^{2+} complex. The $^{1}D_{HN}$ RDCs were used to fit $\Delta\chi$ tensors using the program Numbat.[2] Numbat accepts PCSs rather than RDCs as input, but $^{1}D_{HN}$ RDCs can be disguised as ^{1}H PCSs, if the protein structure used in the input is first altered by a coordinate transformation, which shifts all amide nitrogens to the origin (0, 0, 0) to position all amide protons at the same distance from the origin, thereby removing the distance dependence of the equation used to fit the $\Delta\chi$ tensor. For consistency, all H-N bond lengths were set to 1.008 Å[5] in all structures used to fit RDC data. (We note that different NMR and MD structures of ubiquitin display different H-N bond lengths. In particular, the H-N bond lengths found in different conformers of the structure 2KOX[6] vary by as much as ±0.5 Å.) Prior to fitting the $\Delta\chi$ tensor, the H-N bond lengths were scaled by 8.1, so that the numerical values of the PCSs back-calculated by Numbat (reported in ppm) corresponded RDCs (in Hz). For structure ensembles, the values back-calculated by Numbat are averages of the values determined for the individual conformers in the ensemble.

4. Isothermal calorimetry

Isothermal calorimetry (ITC) measurements were carried out at 25 °C on a Nano ITC titration calorimeter (TA Instruments, USA). The titrant (8.37 mM $CoCl_2$) and sample (0.8 mM ubiquitin) solutions were made with the same stock buffer solution (20 mM MES buffer, pH 6.5) and were thoroughly degassed in vacuum for at least 20 minutes before each measurement. The solution in the cell was stirred at 300 rpm by the syringe to ensure rapid mixing. Typically, about 3 μL of titrant were injected over 25 s with intervals of 5 minutes between injections to allow complete equilibration. Titrations continued until the metal ion was present in 3-4 molar excess. The heat of dilution was accounted for by subtracting background titration data obtained with the identical titrant solution but with only buffer solution in the sample cell. The data were analyzed with the assumption of 1:1 binding using the NanoAnalyze software v3.7.5 supplied by the manufacturer.

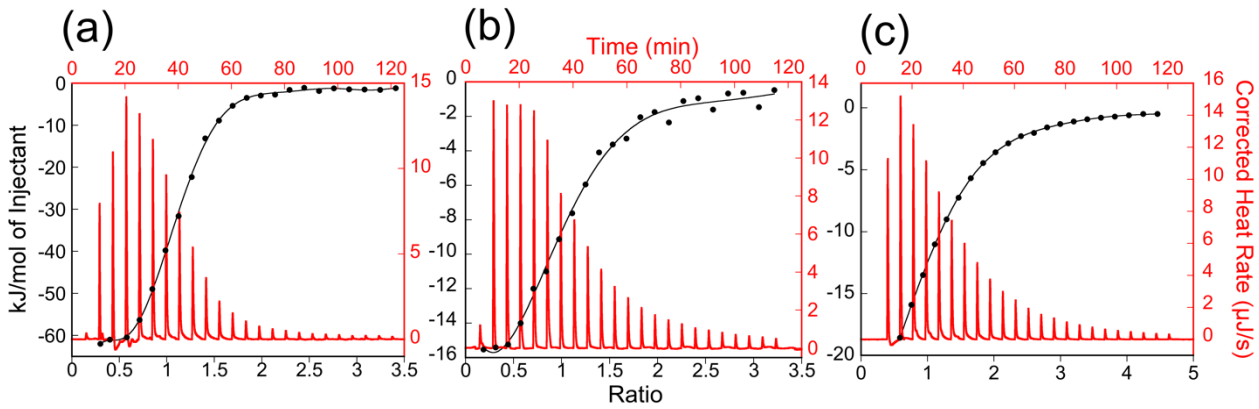

Figure S1. Isothermal calorimetry (ITC) data of Co^{2+} ions binding to ubiquitin and GB1 with dHis motif. Thermograms are shown in red and binding isotherms in black. Solutions of $CoCl_2$ and proteins were in 20 mM MES, pH 6.5. Measurements were performed at 25 °C. The solid black lines represent the best fits assuming 1:1 binding. (a) Titration of 8.3 mM $CoCl_2$ into 0.8 mM ubiquitin E24H/A28H. The best fit yielded a dissociation constant K_d of 40 μM. (b) Titration of 14.5 mM $CoCl_2$ into 1 mM ubiquitin A28H/D32H. The fit corresponds to K_d = 85 μM. (c) Titration of 34 mM $CoCl_2$ into 2.3 mM GB1 E15H/T17H. The fit corresponds to K_d = 180 μM.

5. Mass Spectrometry

To confirm the stoichiometric ratio of Co^{2+} ions binding to the dHis motif, we performed native mass spectrometry on the ubiquitin mutant E24H/A28H, using a syringe pump for direct infusion into an Orbitrap Elite mass spectrometer. A 50 µM solution in a buffer of 100 mM ammonium acetate was infused at a rate of 10 µl min^{-1} into a HESI-II electrospray ionization (ESI) source and full-scan mass spectra were acquired in the m/z range of 200 to 4000 using the Orbitrap mass analyzer. The data were analyzed using the Thermo Xcalibur Qual Browser software (version 3.0.63). For each data set, the signal intensities of the two species were normalized to that of the species with the highest signal intensity, thereby giving relative signal intensities.

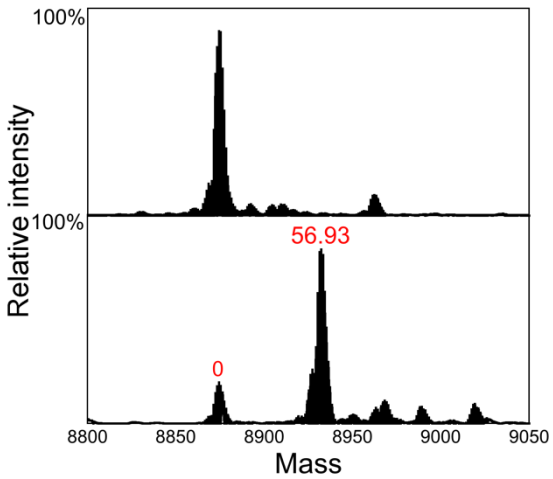

Figure S2. ESI-MS analysis of native ubiquitin E24H/A28H and its complex with a Co^{2+} ion. The buffer was 100 mM aqueous ammonium acetate, pH 6.7. Top panel: spectrum of the free protein. Bottom panel: spectrum of the complex with cobalt. The numbers in red indicate the mass with respect to the mass of the protein peak. Observation of the cobalt complex demonstrates formation of the 1:1 complex and maintenance of the tertiary structure in the gas phase.

6. PCSs by dHis-Co^{2+} motifs in ubiquitin, GB1, and ERp29-C

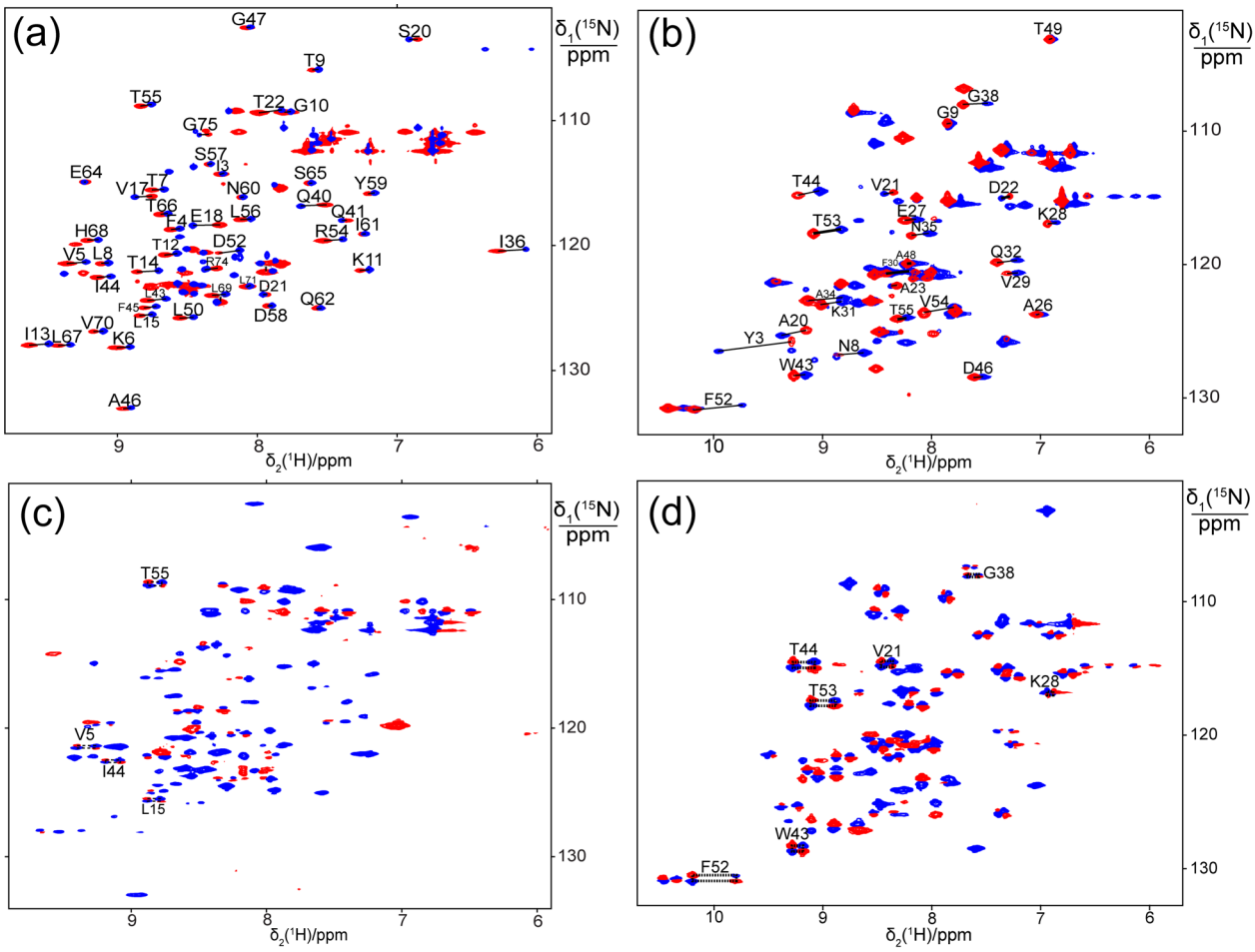

Figure S3. PCS measurements in ubiquitin A28H/D32H and GB1 E15H/T17H. (a) Superimposition of [^{15}N,^{1}H]-HSQC spectra of 0.8 mM solutions of uniformly ^{15}N-labeled ubiquitin A28H/D32H with $CoCl_2$ (blue spectrum) or $ZnCl_2$ (red spectrum). (b) Superimposition of [^{15}N,^{1}H]-HSQC spectra of 1.5 mM solutions of uniformly ^{15}N-labeled GB1 E15H/T17H with $CoCl_2$ (blue spectrum) or $ZnCl_2$ (red spectrum). (c) I_zS_z exchange spectrum recorded of ubiquitin A28H/D32H in the presence of 0.5 equivalents $CoCl_2$. Exchange and auto peaks have opposite sign and are colored red and blue, respectively. Rectangles highlight the pattern of exchange peaks and auto peaks for selected residues. (d) Same as (c), but for GB1 E15H/T17H.

Table S1. $\Delta\chi$ tensor parameters of a Co^{2+} ion bound to the double mutants E24H/A28H and A28H/D32H of ubiquitin and E15H/T17H of GB1.[a]

		$\Delta\chi_{ax}$	$\Delta\chi_{rh}$	Euler angles			Metal coordinates			
		10^{-32} m^3	10^{-32} m^3	α	β	γ	x	y	z	*Q*-factor
Ubiquitin E24H/A28H[b]	1UBQ	-4.41	-2.31	120	74	48	41.44	21.20	18.04	0.03
	1D3Z	-4.10	-2.13	152	92	179	61.84	-85.68	-9.90	0.03
	2K39	-4.27	-2.25	39	19	137	30.91	22.22	7.94	0.03
	2KOX	-4.15	-2.25	39	160	52	-4.07	4.36	-11.76	0.03
	2MJB	-4.13	-2.16	169	46	148	2.40	3.26	-11.49	0.03
	<x-ray>	-4.29	-2.37	122	137	15	2.41	0.43	13.20	0.03
Ubiquitin A28H/D32H[b]	1UBQ	-3.07	-0.24	36	33	144	43.21	24.56	10.93	0.10
	1D3Z	-3.38	-0.39	95	158	178	58.77	-93.01	-7.91	0.12
	2K39	-3.60	-0.38	164	95	151	29.49	30.05	6.16	0.13
	2KOX	-3.23	-0.30	155	82	37	-3.71	-2.62	-14.06	0.11
	2MJB	-3.46	-0.38	120	106	22	-0.24	-3.92	-13.90	0.11
	<x-ray>[c]	-3.23	-0.28	120	65	49	9.59	1.47	11.08	0.11
GB1 E15H/T17H	1PGA	-1.45	-0.84	170	135	149	33.83	22.47	30.97	0.12
ERp29-C A163H/Q167H	2M66	-2.4	-0.11	166	116	131	4.71	15.33	12.58	0.12

[a] Tensor parameters determined from backbone amide PCSs (Tables S5–S7). The corresponding complex with a Zn^{2+} ion served as the diamagnetic reference. Tensors reported in the unique tensor representation of Numbat.[2]

[b] Only residues 1-70 were used to obtain $\Delta\chi$ tensor parameters.

[c] <x-ray> denotes a weighted average of ubiquitin crystal structures with the weighting factors given in Table S9 by Maltsev et al.[7]

Table S2. Quality factors comparing experimental backbone amide RDCs with RDCs back-calculated from the $\Delta\chi$ tensors of Table S3 for the ubiquitin mutants E24H/A28H and A28H/D32H.[a]

	Reference	Size of ensemble	Low rmsd residues[b]		High rmsd residues[c]	
			E24H/A28H	A28H/D32H	E24H/A28H	A28H/D32H
1UBQ	8	1	0.36	0.29	0.86	**0.33**
1D3Z	9	10	0.26	0.20	**0.52**	0.40
2K39	10	116	0.22	**0.21**	0.58	**0.33**
2KOX	6	640	**0.21**	0.22	0.63	0.34
2MJB	7	20	0.26	0.22	0.66	0.42
<x-ray>[d]	7	15	0.31	0.25	0.72	0.34

[a] Best *Q*-factors highlighted in bold.

[b] Residues 2-6, 12-31, 36-45, 48-51 and 53-69.

[c] Residues 7-11, 32-35, 46, 47, 52 and 70.

[d] <x-ray> denotes a weighted average of ubiquitin crystal structures with the weighting factors given in Table S9 by Maltsev et al.[7]

Table S3. Dihedral angles found by modeling α-helical dHis-Co^{2+} motifs onto 3D structures of ubiquitin and ERp29-C.[a]

position	*i*		*i*+4	
dihedral angle	χ_1/°	χ_2/°	χ_1/°	χ_2/°
ubiquitin E24H/A28H	-173	77	-69	57
ubiquitin A28H/D32H	178	65	-70	25
ERp29-C A163H/Q167H	-173	80	-66	78

[a] The modeling was performed using the first conformer of the structure 2MJB of ubiquitin,[7] and the PCS-Rosetta structure of ERp29-C.[11]

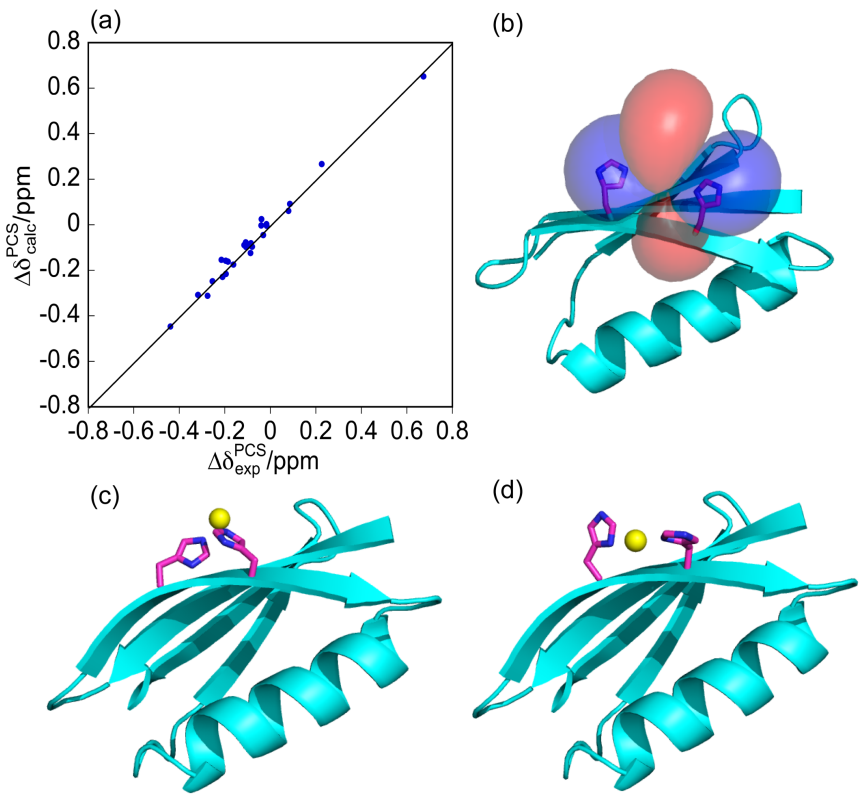

Figure S4. $\Delta\chi$ tensor observed for GB1 E15H/T17H. (a) Correlation between back-calculated and experimental PCSs. (b) PCS isosurfaces (blue: +1 ppm, red: -1 ppm) plotted on the crystal structure of wild-type GB1 (PDB ID: 1PGA[12]). (c) Model of the dHis-Co^{2+} motif produced with the constraints that the metal ion (yellow ball) is positioned in the planes of the imidazole rings with a nitrogen–cobalt distance of 2 Å. (d) Metal position indicated by the $\Delta\chi$ tensor fit. The side chain conformations of the histidine residues were manually adjusted to explain the position of the metal ion, which differs by almost 6 Å from the position in (c). Dihedral angles are reported in Table S4.

Table S4. Comparison of dHis-Co^{2+} motifs modeled on GB1 E15H/T17H[a]

Histidine residue in position	*i*		*i*+4	
Side chain dihedral angle	χ_1/°	χ_2/°	χ_1/°	χ_2/°
Model 1[b]	170	-13	-59	-86
Model 2[c]	166	-22	48	-85

[a] Modeling performed using the crystal structure GB1 (PDB ID: 1PGA[12]).

[b] Model shown in Figure S4c, which was produced with the same constraints as for the α-helical dHis motif, assuming the nitrogen–cobalt bond (bond length 2 Å) to be coplanar and symmetric with respect to each of the imidazole rings. The model results in a bond angle between the two nitrogen-cobalt bonds of about 65°.

[c] Model shown in Figure S4d, where the metal position is determined by the $\Delta\chi$ tensor fit. The nitrogen-cobalt bond lengths are 2.4 Å and the bond angle between the two nitrogen–cobalt bonds is about 180°.

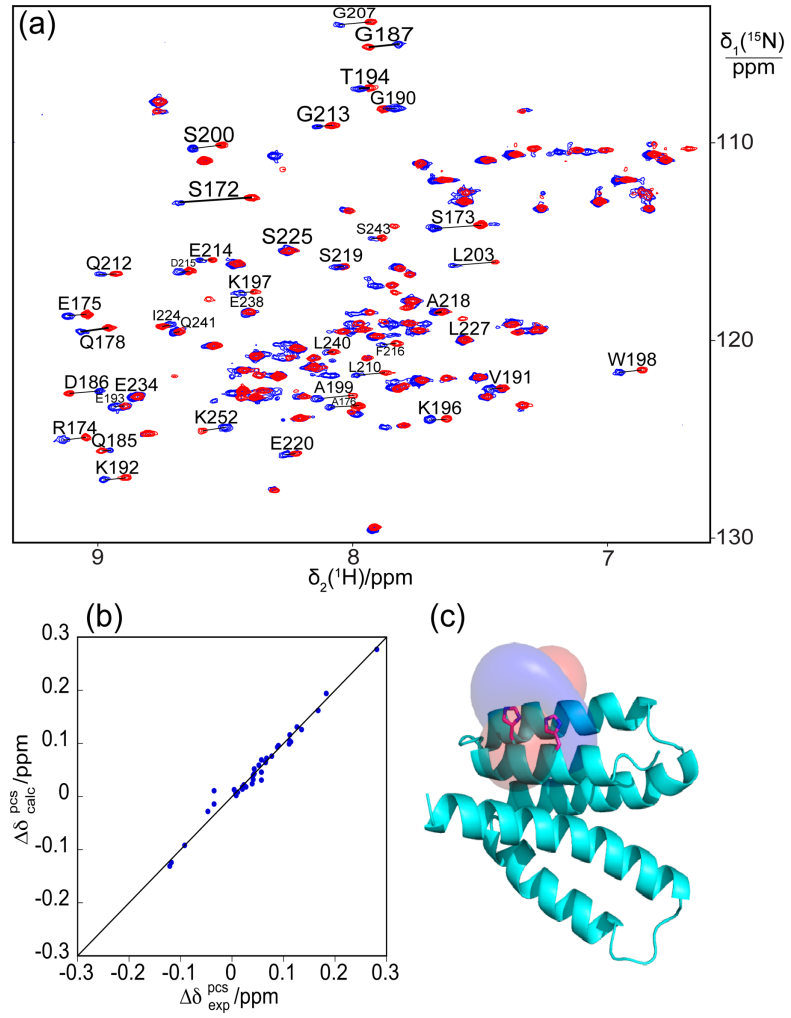

Figure S5. PCS measurements and $\Delta\chi$ tensor determination for the A163H/Q167H mutant of the C-terminal domain of rat ERp29 (ERp29-C). The protein contains a solvent exposed cysteine residue at position 157. (a) Superimposition of [^{15}N,^{1}H]-HSQC spectra of 0.4 mM solutions of uniformly ^{15}N-labeled protein with $CoCl_2$ (blue spectrum) or $ZnCl_2$ (red spectrum). The spectra were recorded at 25 °C on a Bruker 600 MHz NMR spectrometer equipped with TCI cryoprobe, using t_{1max} = 65 ms, t_{2max} = 140 ms. (b) Correlation between back-calculated and experimental PCSs of backbone amide protons. (c) PCS isosurfaces (blue: +1 ppm, red: -1 ppm) plotted on the PCS-Rosetta structure of ERp29 A163H/Q167H (PDB ID: 2M66[11]).

Table S5. Experimental PCSs and RDCs for the double mutants E24H/A28H and A28H/D32H of ubiquitin.[a]

Res. no.	Res. type	PCS/ppm		RDC/Hz	
		E24H/A28H	A28H/D32H	E24H/A28H	A28H/D32H
2	Gln	-	-0.044	-	-2.05
3	Ile	-0.028	0.014	0.73	0.99
4	Phe	-0.082	0.065	2.03	1.67
5	Val	-0.15	0.137	3.56	1.71
6	Lys	-0.209	0.096	3.46	2.02
7	Thr	-0.164	0.089	-0.16	0.49
8	Leu	-0.246	0053	-2.02	0.65
9	Thr	-0.152	0.047	-3.76	0.57
10	Gly	-0.125	0.048	1.74	2.24
11	Lys	-0.125	0.068	1.23	-0.73
12	Thr	-	0.089	-	1.83
13	Ile	-0.141	0.142	3.49	1.19
14	Thr	-	0.154	2.76	1.82
15	Leu	-0.02	0.086	0.65	1.28
16	Glu	-	-	-	-
17	Val	0.031	-0.123	-1.86	-0.35
18	Glu	0.038	-0.191	0.48	-2.47
20	Ser	0.039	-0.062	2.76	-1.20
21	Asp	0.05	-0.024	-0.84	0.40
22	Thr	0.215	0.162	-1.56	1.60
33	Lys	0.213	-	3.23	-
36	Ile	-0.362	0.198	-2.11	-3.08
40	Gln	-	-0.183	-	-0.20
41	Gln	-	-0.035	-	1.87
43	Leu	-0.636	0.133	-	1.89
44	Ile	-	0.102	-	1.60
45	Phe	-0.145	0.093	2.76	0.97
46	Ala	-0.098	0.043	2.36	0.65
47	Gly	-0.098	0.024	3.00	1.13
48	Lys	-0.079	0.049	1.67	-2.03

49	Gln	-0.084	0.047	2.76	0.12
50	Leu	-0.227	0.099	1.64	1.56
51	Glu	-	0.078	-	-4.30
52	Asp	-	0.151	-	-3.57
53	Gly	0.766	-	-	-
54	Arg	0.401	0.136	-1.40	-0.65
55	Thr	0.172	0.082	2.50	1.34
56	Leu	0.012	0.075	0.40	1.38
57	Ser	0.016	0.015	-2.00	1.55
58	Asp	0.055	0.034	-0.02	0.08
59	Tyr	0.015	0.046	1.60	1.22
60	Asn	0.008	0.012	-3.70	1.70
61	Ile	-0.021	0.017	-2.59	1.50
62	Gln	-0.031	0.017	-3.11	1.73
63	Lys	-	-0.017	-	-0.50
64	Glu	-0.018	-0.008	-1.54	1.22
65	Ser	-0.055	0.012	-3.95	1.21
66	Thr	-0.076	0.053	0.70	1.38
67	Leu	-0.166	0.088	2.73	1.74
68	His	-0.236	0.08	2.80	1.99
69	Leu	-0.24	0.095	0.30	2.07
70	Val	-0.478	0.071	-3.32	1.21
71	Leu	-0.301	0.018	-	0.56
72	Arg	-	-0.037	-	0.56
73	Leu	-0.307	-0.027	-	-1.46
74	Arg	-0.211	-0.085	-1.38	0.16
75	Gly	-0.126	-0.068	-3.00	-0.32
76	Gly	-0.091	-	-1.30	-

[a] Data measured at 25 °C on an 800 MHz NMR spectrometer.

Table S6. Experimental PCSs of GB1 E15H/T17H.[a]

Res. No	Res.Type	PCS/ppm
3	Tyr	0.673
8	Asn	-0.215
9	Gly	-0.031
20	Ala	0.226
21	Val	0.086
22	Asp	0.080
23	Ala	-0.039
26	Ala	-0.016
27	Glu	-0.108
28	Lys	-0.085
29	Val	-0.107
30	Phe	-0.210
31	Lys	-0.195
32	Gln	-0.186
34	Ala	-0.318
35	Asn	-0.162
38	Gly	-0.115
43	Trp	-0.105
44	Thr	-0.196
46	Asp	-0.080
48	Ala	-0.040
49	Thr	-0.017
52	Phe	-0.439
53	Thr	-0.254
54	Val	-0.276
55	Thr	-0.087

[a] Data measured at 25 ^{o}C on an 800 MHz NMR spectrometer.

Table S7. Experimental PCSs of ERp29-C A163H/Q167H.[a]

Res. No	Res.Type	PCS/ppm
172	Ser	0.243
173	Ser	0.183
174	Arg	0.085
175	Glu	0.073
176	Ala	0.112
178	Gln	0.121
185	Gln	-0.055
186	Asp	-0.118
187	Gly	-0.095
190	Gly	-0.083
191	Val	0.026
192	Lys	0.088
193	Glu	0.043
194	Thr	0.015
196	Lys	0.067
197	Lys	0.057
198	Trp	0.090
199	Ala	0.135
200	Ser	0.109
203	Leu	0.251
207	Gly	0.147
210	Leu	0.112
212	Gln	0.081
213	Gly	0.076
214	Glu	0.068
215	Asp	0.056
216	Phe	0.057
218	Ala	0.056
219	Ser	0.041
220	Glu	0.060
224	Ile	0.035
225	Ser	0.005
227	Leu	0.007

234	Glu	0.011
238	Glu	0.004
240	Leu	0.020
241	Gln	0.030
243	Ser	0.041
252	Lys	-0.092

[a] Data measured at 25 °C on an 800 MHz NMR spectrometer.

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