Supplement of

Inter-residue through-space scalar $^{19}F–^{19}F$ couplings between CH_2F groups in a protein

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Supporting Information

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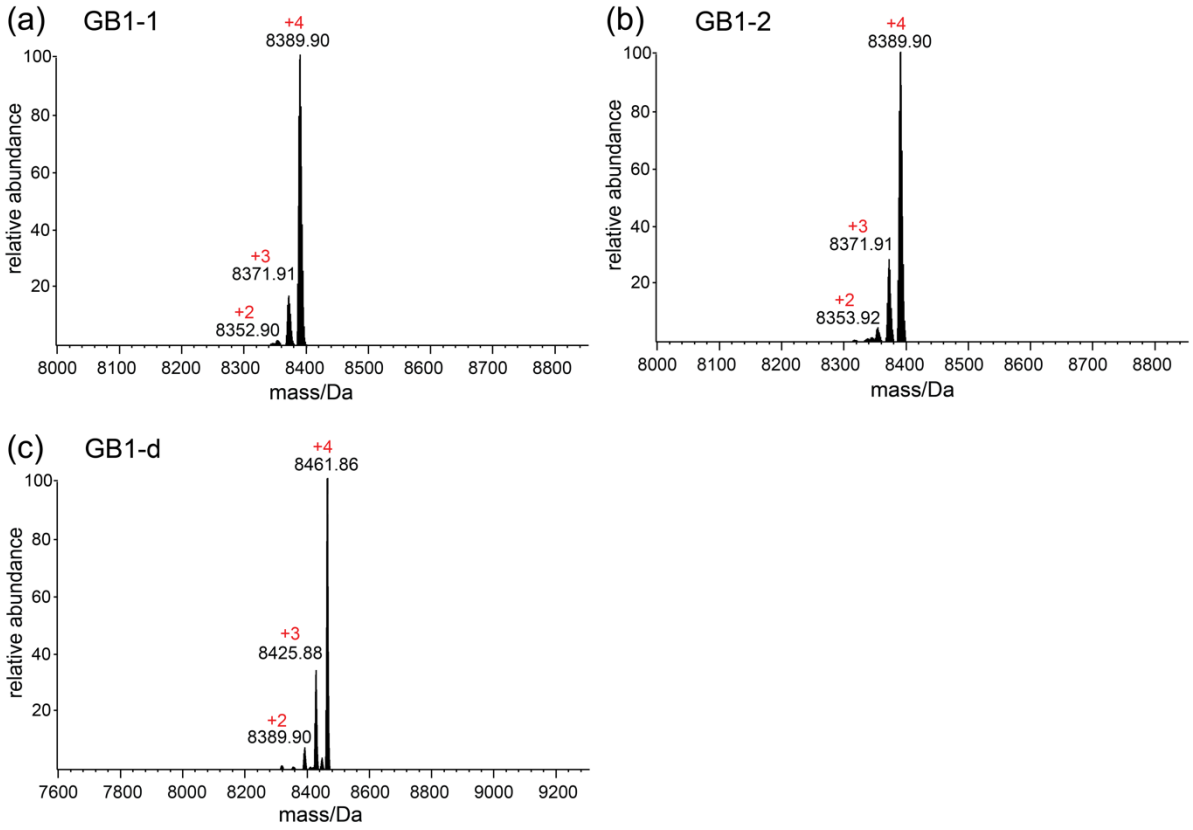

Figure S1. Intact protein mass spectra of GB1 made with (a) Fleu1, (b) FLeu2 and (c) diFLeu. The red numbers indicate the number of FLeu residues in the protein. The expected mass of the full-length protein with four fluorinated leucine residues is 8,390.1 Da for the samples made with FLeu1 and FLeu2, and 8,462.1 Da for the sample made with diFLeu.

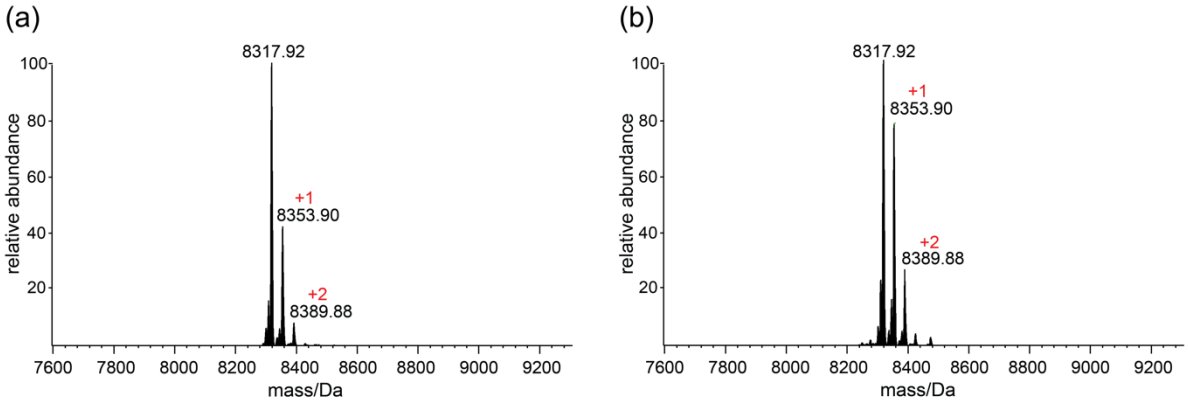

Figure S2. Intact protein mass spectra of GB1 made with mixtures of diFLeu and canonical Leu. (a) Sample prepared with 0.5 mM L-leucine and 4 mM diFLeu in the amino acid mixture of the CFPS reaction. (b) Same as (a) but using 0.25 mM L-leucine and 4 mM diFLeu. The red numbers indicate the number of diFLeu residues in the protein. The expected mass of GB1 containing one or two diFLeu residues is 8,354.1 Da and 8,390.1 Da, respectively.

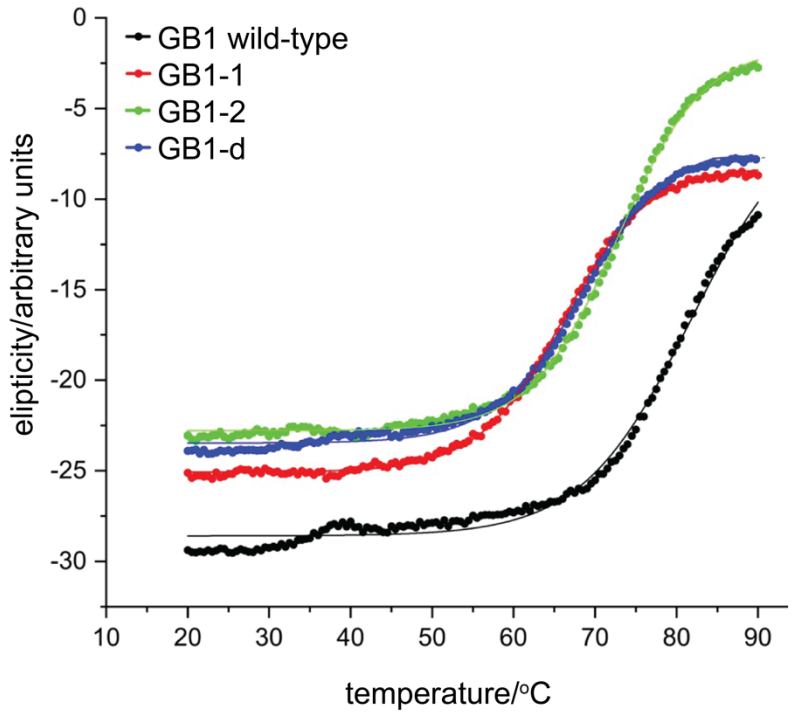

Figure S3. Thermal stability analysis by circular dichroism (CD) spectroscopy. The CD signal of wild-type GB1 (black) and GB1 with FLeu-1 (red), FLeu-2 (green) and diFLeu (blue) was monitored at 216 nm in a 1 mm quartz cuvette using a Chirascan spectrometer (Applied Photophysics). Parameters used: protein concentration 0.3 mg mL^{-1}, pH 7.5, heating rate 1 °C min^{-1}. Blanks with buffer measured in the same cuvette were subtracted from the data. The melting temperatures obtained from the fits are 81 °C for wild-type GB1, 66 °C for GB1 made with FLeu1 (GB1-1), 72 °C for GB1 made with FLeu2 (GB1-2) and 68 °C for GB1 made with diFLeu (GB1-d).

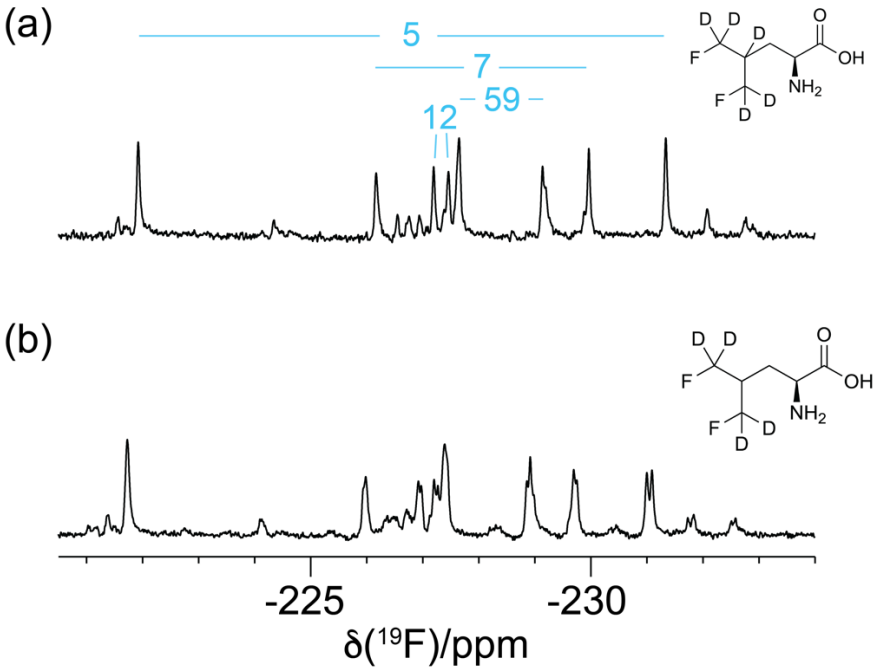

Figure S4. 1D ^{19}F-NMR spectra of GB1 made with diFLeu deuterated at (a) five and (b) four sites as indicated in the chemical structures of the inserts. The spectra were recorded of, respectively, 0.5 and 0.9 mM protein solutions in 20 mM MES buffer pH 6.5, 100 mM NaCl. The spectra were recorded without ^{2}H decoupling, using an acquisition time of 0.1 s and a recovery delay of 1 s. The resonance assignments are shown in cyan. Apart from the deuterium isotope effects on the ^{19}F chemical shifts, the spectra differ by the appearance of $^{3}J_{HF}$ couplings. The spectrum in (b) shows larger splittings at the high-field end of the spectrum while the couplings are unresolved for the low-field resonances, in agreement with the γ-*gauche* effect that predicts a correlation between ^{19}F chemical shifts and $^{3}J_{HF}$ coupling constants.

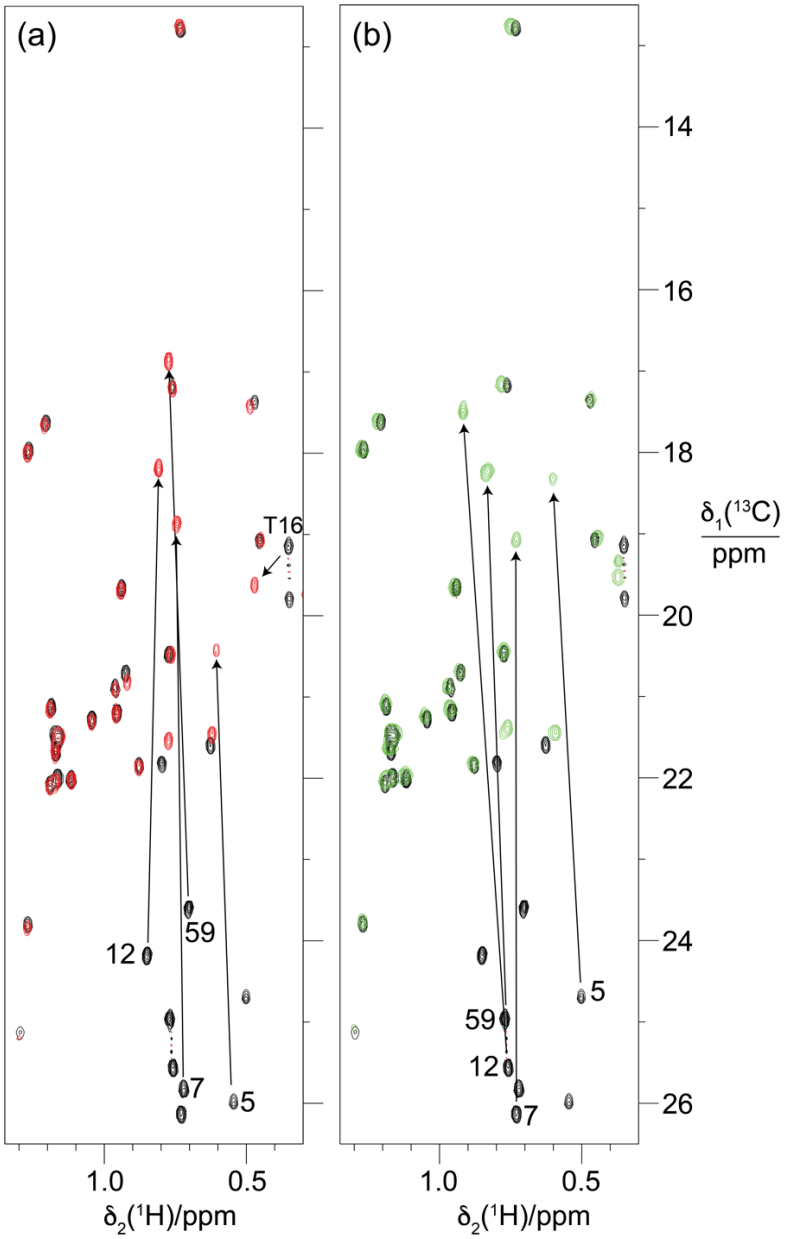

Figure S5. Selected spectral region from the ^{13}C-HSQC spectra of GB1-1 and GB1-2 superimposed on the corresponding spectrum of wild-type GB1 shown in black. The cross-peaks of leucyl residues in wild-type GB1 are labelled with the residue number. Arrows point to the corresponding cross-peaks in GB1-1 (red) and GB1-2 (green). The stereospecific assignments in the wild-type protein were taken from Goehlert et al. (2004). The stereospecific assignment of the CH_3 groups of residue 59 is based solely on the assumption that the change in ^{13}C chemical shift is similar in GB1-1 and GB1-2. (a) ^{13}C-HSQC spectrum of GB1-1, where the change in chemical shifts is caused by FLeu1 residues. The change in chemical shift for the methyl group of Thr16 can be explained by slightly altered ring currents, as it is near residue 5 and makes NOEs with the aromatic ring protons of Phe30 and Tyr33. (b) ^{13}C-HSQC spectrum of GB1-2, where the change in chemical shifts is due to FLeu2 residues.

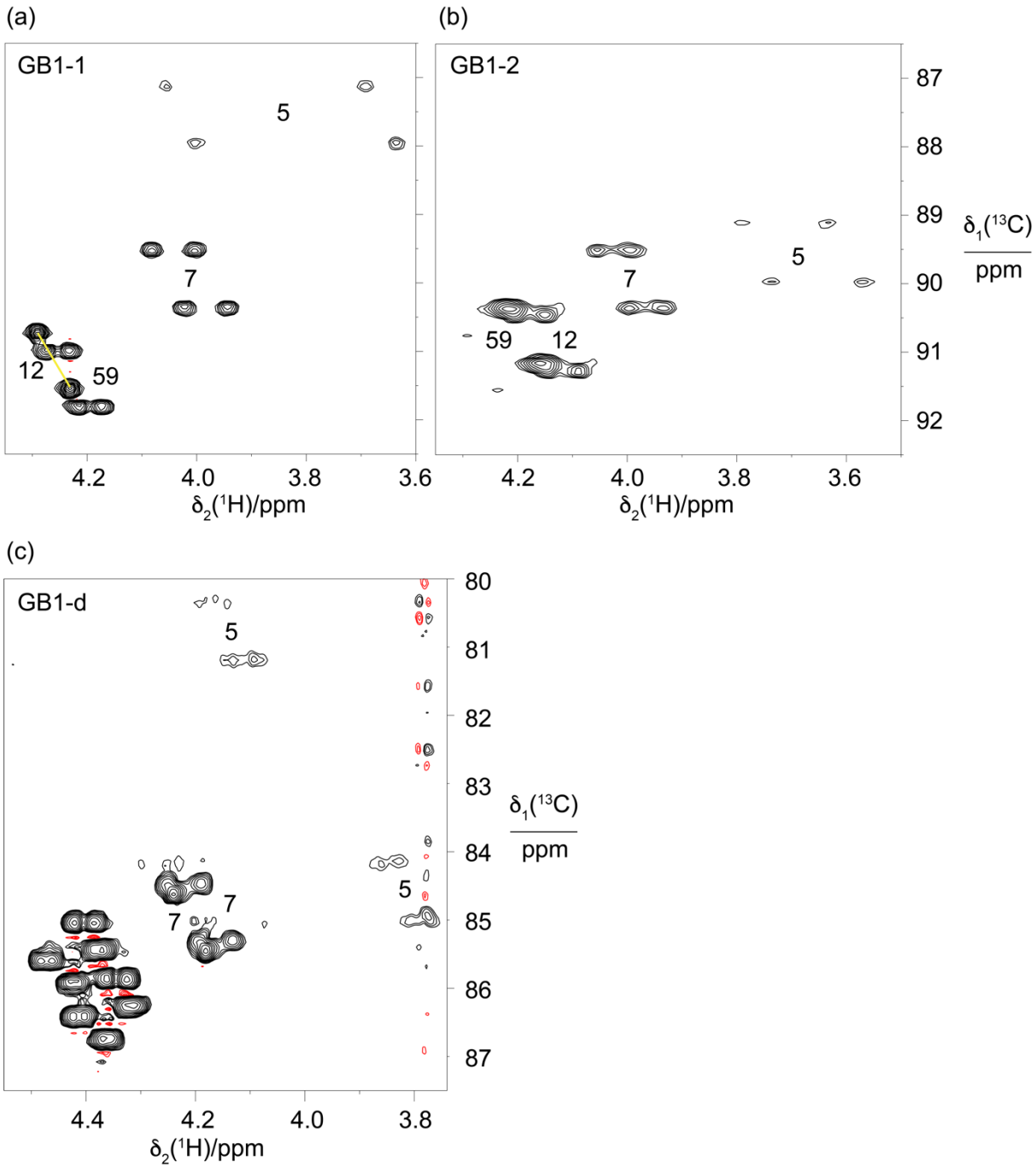

Figure S6. Selected spectral regions from the ^{13}C-HSQC spectra of GB1-1, GB1-2 and GB1-d showing the cross-peaks of the C$^{\delta}$H$_2$F groups. The spectra were recorded on a Bruker 800 MHz NMR spectrometer equipped with a cryoprobe, using t_{1max} and t_{2max} values of about 24 and 100 ms, respectively, and no ^{19}F decoupling. Cross-peaks are split in the vertical and horizontal dimensions by the $^{1}J_{FC}$ coupling constant (about 163 Hz) and the $^{1}J_{HF}$ coupling constant (about 47 Hz), respectively. The cross-peaks are labelled with the residue number. (a) ^{13}C-^{1}H cross-peaks in GB1-1. A yellow line highlights the multiplet splitting due to the one-bond couplings with ^{19}F in residue 12. The resonances were assigned with the help of cross-peaks with the CH$_3$ groups in a TOCSY-relayed ^{13}C-HSQC spectrum. (b) ^{13}C-^{1}H cross-peaks

in GB1-2. (c) ^{13}C-^{1}H cross-peaks in GB1-d. Assignments were obtained by comparison with the short-delay ^{1}H,^{19}F correlation experiment of Figure 9c. Comparison of the chemical shifts of the cross-peaks observed in GB1-d with those in GB1-1 and GB1-2 suggests assignments also for the cross-peaks of residues 12 and 59 not labelled in this figure.

Table S1. DNA and corresponding amino acid sequence of the GB1 construct used in the current work.

Protein	DNA sequence	Amino acid sequence[a]
GB1	ATGGCTTCTATGACCGGTATGACCTACAAACTGATC CTGAACGGTAAAACCCTGAAAGGTGAAACCACCACC GAAGCGGTTGACGCGGCGACCGCGGAAAAAGTTTTC AAACAGTACGCGAACGACAACGGTGTTGACGGTGAA TGGACCTACGACGACGCGACCAAAACCTTCACCGTT ACCGAAGAAAACCTGTATTTTCAGGGCCATCATCAT CACCATCAC	MASMTGMTYKLILNGKTLKG ETTTEAVDAATAEKVFKQYA NDNGVDGEWTYDDATKTFTV TEENLYFQGHHHHHH

[a] The present work uses the sequence numbering of wild-type GB1 starting with the second methionine residue. The N-terminal MASMTG tag was numbered -6 to -1.

Table S2. Yields of purified GB1 per mL inner CFPS reaction mixture obtained with fluorinated leucine analogues.

Protein	Yield/mg
GB1 with FLeu1	2.1
GB1 with FLeu2	2.3
GB1 with diFLeu	2.7
GB1 with diFLeu (0.5 mM L-leucine and 4 mM diFLeu)	1.3
GB1 with diFLeu (0.25 mM L-leucine and 4 mM diFLeu)	1.2

Table S3. Measurements of $^{3}J_{HF}$ couplings by short-delay $^{1}H,^{19}F$ correlation experiments[a]

Residue	^{19}F chemical shift difference (ppm)[b]	Cross-peak intensity ratio[c]	$^{3}J_{HF}$ (Hz)[d]
GB1-1			
12	1.1	11	18
59	0	10	20
7	-0.8	13	17
5	-2.8	4	34
GB1-2			
5	5.2	18	14
12	3.1	15	16
7	1.1	13	17
59	0	9	21
GB1-d			
5	6.5	27	15
7	2.2	12	20
12	1.2	10	21
12	0.9	10	22
59	0.8	9	22
59	-0.8	8	23
7	-1.5	10	22
5	-3.0	3	40
GB1-dd			
5	6.8	> 20	<15
7	3.9	14	19
12	1.8	10	22
12	1.4	11	21
59	0.9	9	23
59	-0.9	8	24
5	-3.6	3	40
7	-4.4	7	26

[a] Based on the spectra shown in Figure 9.

[b] Difference between the measured chemical shift and the chemical shift of the solvent-exposed residue 59 (average chemical shift of residue 59 in the case of diFLeu samples).

[c] Ratio of the integral measured for the H^{γ}–^{19}F cross-peak versus the combined cross-peak intensities of the $C^{\delta}H_2$–^{19}F cross-peaks.

[d] Calculated as described in the main text.

References

Goehlert, V. A., Krupinska, E., Regan, L., and Stone, M. J.: Analysis of side chain mobility among protein G B1 domain mutants with widely varying stabilities. Prot. Sci., 13, 3322–3330, https://doi.org/10.1110/ps.04926604, 2004.