SUPPORTING INFORMATION

Supporting Information

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Visualizing Proteins in Mammalian Cells by ^{19}F NMR Spectroscopy

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Abstract: In-cell NMR spectroscopy is a powerful tool to investigate protein behavior in physiologically relevant environments. Although proven valuable for disordered proteins, we show that in commonly used ^{1}H-^{15}N HSQC spectra of globular proteins, interactions with cellular components often broaden resonances beyond detection. This contrasts ^{19}F spectra in mammalian cells, in which signals are readily observed. Using several proteins, we demonstrate that surface charges and interaction with cellular binding partners modulate linewidths and resonance frequencies. Importantly, we establish that ^{19}F paramagnetic relaxation enhancements using stable, rigid Ln(III) chelate pendants, attached via non-reducible thioether bonds, provide an effective means to obtain accurate distances for assessing protein conformations in the cellular milieu.

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Experimental Procedures

Protein expression and purification

Coding sequences for the T2Q GB1 (pET-11a, 6.2 kDa), T2Q/Q32C GB1 (pET-11a, 6.2 kDa), D22N/D36R/E42K GB1 (pET-41, 6.3 kDa), K63R ubiquitin (pET-26B, 8.6 kDa), V2Q CypA (pET-21, 18.0 kDa) and HIV-1 capsid CTD dimer (Met144-Leu231, pET-21, 19.7 kDa) were inserted into pET vectors and proteins were expressed in Rosetta DE3 cells. For expression, E. coli Rosetta DE3 cells were grown at 18 ºC for 16 hrs in modified M9 medium, containing 4 g/L U-$^{12}C_6$-glucose and 1 g/L $^{15}NH_4Cl$ as carbon and nitrogen sources, respectively. For 5F-Trp labeling, 20mg/L of 5F indole (Sigma-Aldrich, St. Louis, MO, USA) was added at OD_{600} = 0.6, followed by induction after 20 mins with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich, St. Louis, MO, USA) and subsequent growth at 18 °C for 16 h. For 3F-Tyr labeling, 1 g/L glyphosate (Sigma-Aldrich, St. Louis, MO, USA) was added at OD_{600} = 0.4 and 70 mg/L 3F-Tyr (Sigma-Aldrich, St. Louis, MO, USA), 60 mg/L 5F-Trp (Sigma-Aldrich, St. Louis, MO, USA), 60 mg/L Phe (Sigma-Aldrich, St. Louis, MO, USA) were supplemented at the same time. Protein expression was induced at OD_{600} = 0.6-0.8 with 0.5 mM IPTG and cells were grown at 18 °C for 16 h. Cells were harvested by centrifugation at 4000 × g for 10 mins and lysed by sonication.

Cell debris was removed by centrifugation at 18000 × g for 30 mins. All proteins were purified as described previously[1]. The D22N/D36R/E42K GB1 was purified over an ion exchange HP SP column (GE Healthcare, Chicago, IL), followed by gel filtration on a Superdex 75 column (GE Healthcare, Chicago, IL). The commonly used T2Q variant of GB1 was used to prevent Met+/- N-terminal heterogeneity. All proteins employed in this study are devoid of any N- or C-terminal tags.

Mammalian cell culture and electroporation

Proteins were delivered into cells by electroporation, according to the procedure developed by the Selenko group[2]. A2780 cells (Sigma-Aldrich, St. Louis, MO, USA) were seeded onto a T75 flask (Thermo Fisher Scientific Inc.) from cryogenically stored stock cells in RPMI-1640 medium (Gibco), containing 10% Fetal Bovine Serum (Sigma-Aldrich, St. Louis, MO, USA) until they reached 70~80% confluency. These initial cells were harvested and seeded into 4 × T175 flasks and grown to yield about 150-300 million cells for electroporation per experiment. Each flask was washed twice with prewarmed Dulbecco's Phosphate Buffered Saline (DPBS) (Thermo Fisher Scientific Inc.) before trypsinization. Cells were detached using 5 mL of trypsin/EDTA (TrypLE™ Express, Gibco) at 37 ℃ for 5 mins, followed by addition of 30 mL prewarmed RPMI-1640 medium with 10% fetal bovine serum (FBS) and centrifugation at 90 × g for 10 mins. The cell pellet was resuspended in 30 mL prewarmed DPBS and pelleted by centrifugation for electroporation. The protein solutions for electroporation were 1 ml of 2 mM K63R Ub, 2 mM CypA, 2 mM CA-CTD and 5 mM GB1 and GB1 variants in electroporation buffer (100 mM sodium phosphate, 15 mM magnesium chloride, 15 mM HEPES, 5 mM potassium chloride, 2 mM reduced glutathione, 2 mM ATP pH 7.0). After passage through a sterile 0.22 µm filter (Sigma-Aldrich St. Louis, MO, USA) the protein solution was added to the cell pellet, which was resuspended by gently tapping the tube to generate a uniform cell slurry. An Amaxa Nucleofector I (Lonza Inc.) was used with program B28, and 12-16 cuvettes, each with 100 µL (10-20 million cells) of cell suspension, were electroporated twice with a ~30 s interval. After electroporation, 1 mL prewarmed RPMI-1640 medium was added to each cuvette and cells were gently suspended by pipetting up and down three times prior to transfer into 50 ml prewarmed RPMI-1640 medium in four T175 flasks.

Cells were allowed to recover for 4~6 hrs to restore normal morphology as assessed by light microscopy, harvested by trypsinization and centrifugation, as described above, for in-cell NMR experiments. In brief, the medium was aspirated and cells were washed three times with DPBS to remove any detached dead cells. 5 mL trypsin/EDTA was added for trypsinization at 37 ℃ for 5 mins. Flasks were gently tapped to help cell detachment and 30 mL prewarmed RPMI-1640 medium was added to deactivate the trypsin. The cells were resuspended by up and down pipetting and transferred into 50 mL Falcon centrifuge tubes. Pelleted cells were washed with 30 ml pre-warmed L-15 medium (Thermo Fisher Scientific Inc.) to remove any residual RPMI-1640. About 30-100 million electroporated cells (counted using a NucleoCassette) were harvested and resuspended in 0.5-1.0 mL L-15 medium containing 10% FBS (Sigma-Aldrich, St. Louis, MO, USA) and 10% D_2O, were transferred to 3 mm or 5 mm Shigemi NMR tubes (Sigma-Aldrich, St. Louis, MO, USA), depending on the number of cells, and used for in-cell NMR studies.

Preparation of cell lysate

After each in-cell NMR experiment, the cells (30-100 million) were removed from the NMR tube and mixed with NMR buffer (20 mM sodium phosphate, 150 mM sodium chloride, 5 mM DTT, pH 7.0) to a final volume of 500 ml and subjected to 8 freeze (liquid N_2) and thaw (room temperature) cycles. Completion of cell lysis was confirmed by Trypan blue (Thermo Fisher Scientific Inc.) staining. The cell lysate was centrifuged at 13000 × g for 10 mins to remove cell debris, and the supernatant was used for analysis by SDS-PAGE and preparation of NMR samples.

Protein quantification

The volume of the cell lysate was determined by weighing the cell lysate (assuming 1 mg = 1 ml), and a 1D ^{19}F NMR spectrum was collected for the cell lysate and compared to a 100 µM reference sample of the pure protein, using identical acquisition and processing parameters. Integral peak areas were measured for the lysate samples and compared to those in the reference samples. The average volume of a A2780 cell was taken as ~1.1 pL (V_{cell}), as reported by the Selenko group[2]. The intracellular protein concentration was calculated according to the following formula:

$$C_{in\text{-}cell} = \frac{C_{lysate} * V_{lysate}}{N_{cells} * V_{single\text{-}cell}}$$

All intracellular protein concentrations for the proteins studied are summarized in Table S2. The concentrations determined from the lysate samples represent a lower limit since some NMR invisible species may be present.

Assessment of protein leakage and cell viability

After the NMR experiments, the cell suspensions in the NMR tubes were gently mixed, and 2 ml were removed and stained with Trypan blue to check for cell viability. In general, the cell viability was greater than 90% in each experiment. The remaining cells were centrifuged at 150 × g for 5 min in a 1.5 ml Eppendorf tube. The supernatants were transferred back into the empty NMR tubes, and ^{19}F spectra were collected under identical conditions as the in-cell spectra. In all cases, no significant protein leakage signal was detected.

Preparation of the CypA/CsA complex

Cyclosporin A (CsA, Sigma-Aldrich, St. Louis, MO, USA) stock solution (20 mM, dissolved in DMSO-d6) was added into 1 mL prewarmed 10% FBS L-15 medium and sterile filtered. CypA containing cells after the NMR experiments were gently resuspended in

1 mL prewarmed 10% FBS L-15 media containing 100 μM CsA and incubated at 37 ℃ for one hour prior to recording the in-cell spectrum.

Labeling with BrPSPy-DO3A-Ln(III)

Purified Q32C 5F-Trp U-^{15}N GB1 protein was incubated with 5 equivalents of BrPSPy-DO3A-Gd(III) in reaction buffer (20mM Tris, pH 7.6) for 16 h at 4 ℃. ESI mass spectroscopy was performed every 4 hrs to assess the progress of the reaction. The final tagged protein exhibited a mass increase of 668.9 Da, in excellent agreement with the theoretical value (669 Da). Final purification of tagged protein involved passage over a desalting column (Cytiva HiPrep^{TM} 26/10) in NMR buffer to remove any excess of BrPSPy-DO3A-Gd(III). For the preparation of BrPSPy-DO3A-Y(III) tagged Q32C 5F-Trp U-^{15}N GB1, 5-fold Y^{3+} was added to the metal-free BrPSPy-DO3A tag before incubation with the Q32C GB1 protein. Subsequently, the pH of the reaction buffer was adjusted to ~7.5 by slowly adding 1 M Tris buffer; the reaction was allowed to proceed for 16 h at 4 ℃. Labeling with the BrPSPy-DO3A-Ln(III) was carried out as illustrated in scheme S1.

NMR spectroscopy

All ^{19}F spectra were recorded on a 14.1 T Bruker AVANCE spectrometer, equipped with a CP TXO F/C-H-D triple-resonance, z-axis gradient cryoprobe at 283 K. ^{19}F chemical shifts were referenced to trifluoracetic acid. ^{19}F spectra were collected with 4,096 data points and a spectral width of 20 ppm using a recycle delay of 1.5 s, except when indicated otherwise. The carrier frequency was set to -123 ppm and -134 ppm for 5F-Trp and 3F-Tyr labeled proteins, respectively. The number of scans varied from 4,096 to 18,000 depending on the signal-to-noise ratio of the spectra. The spectra were baseline corrected with a polynomial fit and subjected to Lorentzian line shape fitting using MestReNova software to extract the intensities, linewidths and chemical shifts. The total experimental time and the SNR (signal-to-noise ratio) of the in-cell ^{19}F spectra for the proteins in this study were as follows: GB1 (1 h, 11), D22N/D36R/E42K GB1 (1 h, 18), K63R Ub (2 hrs, 17), CA CTD (1 hr, 28), CypA (3.5 hrs, 15) and CypA-CsA (4.5 hrs, 9). The line broadening (lb) factors were: GB1 (50 Hz), D22N/D36R/E42K GB1 (50 Hz), K63R Ub (100 Hz), CTD (100 Hz), CypA (100 Hz) and CypA-CsA (100 Hz). The same lb factors were applied to the in-buffer, in-cell and supernatant ^{19}F spectra for the same protein. ^{1}H-^{15}N HSQC spectra were recorded for GB1 and D22N/D36R/E42K GB1 with an interscan delay of 1.5 s, 256 scans and 128 complex points in ^{15}N dimension. ^{1}H-^{15}N SOFAST-HMQC spectra were recorded for K63R Ub, CTD dimer and CypA with an interscan delay of 200 ms, 256 scans and 128 complex points in ^{15}N dimension, with the exception that 512 scans and 64 complex points in ^{15}N dimension were used for CypA and CypA-CsA in the cell. Data were processed in Topspin (Bruker) or NMRpipe[3] and analyzed in NMRFAM-Sparky[4].

R_1 and R_2 measurement and analysis

^{19}F R_1 and R_2 rates were measured by inversion recovery[5] and CPMG[6], respectively, using a recycle delay of 2s. Data processing and analysis were performed in Topspin (Bruker) and MestReNova. Relaxation rates were obtained by fitting the intensity changes to single exponential functions ($I_t=I_0$*exp(-R*t)). 1D ^{19}F spectra were collected before and after in-cell R_1, R_2 measurements to make sure no intensity changes occurred due to cells settling in the NMR tube over time.

^{19}F transverse relaxation and [^{1}H-^{15}N]-TRACT experiments[7] were used to determine the rotational correlation time for WT GB1 in cells (Figure S7). ^{19}F T_2 is dominated by chemical shift anisotropy (CSA) with negligible dipolar interactions (DD) to surrounding protons[1b]. In addition, cross-correlation effects between CSA and DD are also minimal due to fast proton spin flips. Therefore, the rotational correlation time can be estimated exclusively from the R_2 value using the formula below:

$$R_2=\frac{1}{20}\omega_F^2\delta_\sigma^2\tau_c\left(1+\frac{\eta^2}{3}\right)\left(4+\frac{3}{1+\omega_F^2\tau_c^2}\right)$$

The ^{19}F R_2 provides an upper limit for τ_c since binding-associated chemical exchange may contribute to R_2 in the cell. Therefore, we also recorded ^{1}H-^{15}N-TRACT experiments using a 2 s recycle delay with an increment delay of 2 ms and 10 ms in the cell. Both methods yielded a very similar τ_c value (18.7 ± 1.9 ns by TRACT and 16.4 ± 0.9 ns by ^{19}F R_2), suggesting that chemical exchange induced line-broadening effects can be discounted for GB1 in cells. For proteins for which no in-cell ^{1}H-^{15}N HSQC spectrum was visible, we suggest that ^{19}F R_2 provides a reasonable estimate for τ_c in cells. Furthermore, any contribution from chemical exchange can also be suppressed by using high frequency π pulses or a spin lock field during the R_2 measurement.

^{19}F paramagnetic relaxation enhancement measurements and analysis

A series of 1D ^{19}F spectra were recorded for GB1 with different relaxation delays to determine R_2 values. The ^{19}F spectra for BrPSPy-DO3A-Gd(III) tagged GB1 in buffer were recorded with 0.3 ms, 1.0 ms, 2.0 ms, 3.0 ms, 4.0 ms and 5.0 ms relaxation delays; The ^{19}F spectra for BrPSPy-DO3A-Y(III) tagged GB1 in buffer were recorded with 0.3 ms, 1.0 ms, 1.6 ms, 3.0 ms, 5.0 ms, 8.0 ms, 10.0 ms, 16.0 ms, 20.0 ms, 25.0 ms, 40.0 ms and 50.0 ms relaxation delays; For BrPSPy-DO3A-Gd(III) tagged GB1 in cells, ^{19}F spectra were recorded with 0.3 ms, 1.0 ms, 1.6 ms and 3.0 ms relaxation delays. Only four time points were recorded to ensure cells were healthy throughout the NMR experiment. For BrPSPy-DO3A-Y(III) tagged GB1 in the cell, ^{19}F spectra were recorded with 0.3 ms, 1.0 ms, 1.6 ms, 2.0 ms, 3.0 ms, 4.0 ms, 6.0 ms and 8.0 ms relaxation delays. Transverse relaxation rates were determined for tagged GB1 in solution and in cell by fitting the intensity decays at different time points to a single exponential function, and the ^{19}F-PRE rate (Γ_2) was calculated by taking the difference of two R_2 values ($\Gamma_2=R_{2,\,para}-R_{2,\,dia}$). ^{19}F PRE experiments were performed at two different

concentrations (Figure S8) to exclude the contribution of inter-molecular PRE arising from protein-protein/tag interactions. The distance r between the ^{19}F nucleus and Gd^{3+} was calculated based on the measured Γ_2 value according to the Solomen-Bloembergen equation:

$$\Gamma_2=\frac{1}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \gamma_F{}^2 g^2 \mu_B{}^2 S(S+1) r^{-6}\left\{4\tau_c+\frac{3\tau_c}{1+(\omega_F\tau_c)^2}\right\}$$

with g_F as the fluorine gyromagnetic ratio, S the electron spin quantum number of the Gd^{3+} (S=7/2), μ_0 the vacuum permeability, ω_F the Larmor frequency of the fluorine, and $\tau_c=(\tau_r^{-1}+\tau_s^{-1})^{-1}$, with τ_r the protein rotational correlation time and τ_s the electron relaxation time of Gd^{3+} (~10 ns). It is worth noting that τ_c for GB1 cannot be determined accurately since τ_r extracted from ^{19}F R_2 or TRACT experiments represents a population averaged value due to non-specific interaction in the cell. Since τ_c values for free and bound GB1 could be significantly different in the cell, Γ_2 may be influenced. Assuming that binding does not affect the structure and isotropic tumbling of GB1 in the cell, the same distance (r) can be used to back-calculate the theoretical Γ_2, using a simplified two-state fast exchange model based on Solomen-Bloembergen equation. This yields a distribution of distances between ^{19}F and Gd atom. Our simulation results (Figure S6) show that the distance could vary between 13.8 and 14.6 Å, taking into account the experimental error of Γ_2 and non-specific binding,

$$\tau_{r,observed}=p^*\tau_{r,free}+(1-p)^*\tau_{r,bound}$$

$$\Gamma_{2,observed}=(1-p)^*\Gamma_{2,free}+p^*\Gamma_{2,bound}$$

$$\frac{1}{\tau_{c,}}=\frac{1}{\tau_{r,i}}+\frac{1}{\tau_e};\text{i=free or bound}$$

with $\tau_{r,observed}$ as the population-averaged protein rotational correlation time measured by ^{19}F R_2 (19 ns) and $\tau_{r,free}$, $\tau_{r,bound}$ the rotational correlation times for free and bound GB1 in the cell, respectively. For GB1 in the cell, $\tau_{r,free}$ is estimated to be 12 ns, assuming that the intracellular viscosity is ~2-fold higher than that of aqueous buffer, and in our simulation $\tau_{r,bound}$ was varied between 80 and 1000 ns. $\Gamma_{2,observed}$ is defined as the measured Γ_2 of tagged GB1 in the cell, and $\Gamma_{2,free}$ and $\Gamma_{2,bound}$ are the theoretical Γ_2 values for a distance r in the free and bound GB1, respectively. The population of bound GB1 (p) in the cell is below 10% when $\tau_{r,bound}$ is 80 ns in our simulation, a reasonable value since no chemical shift changes are seen between the resonance of 5-^{19}F-Trp GB1 in buffer and in cells. The predicted average distance of in the Gd-F distance distribution generated by MtsslWizard[8] for the model of BrPSPy-DO3A-Gd(III)-tagged GB1 is 14.6 ± 1.0 Å, in excellent agreement with the average distance in buffer and the upper and lower distance boundaries in the cell, implying that the GB1 structure is not affected by the cellular environment.

Supplementary Figures, Scheme and Tables

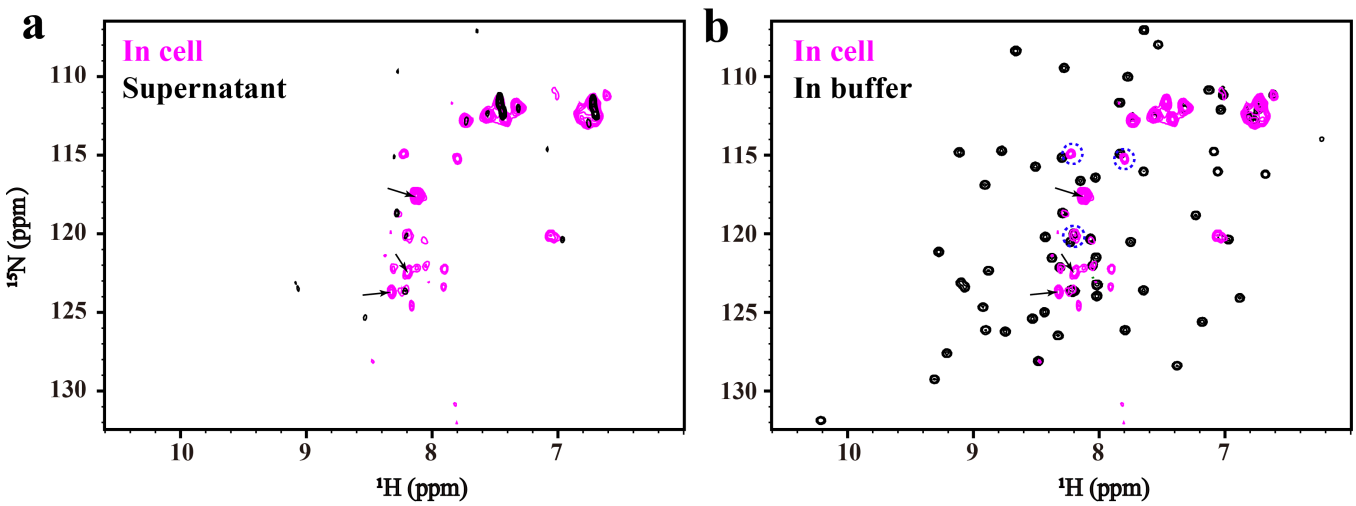


Figure S1. In-cell ^{1}H-^{15}N HSQC spectra of 5F-Trp U-^{15}N D22N/D36R/E42K GB1. (a) Superposition of in-cell (magenta) and supernatant (black) ^{1}H-^{15}N HSQC spectra of 5F-Trp U-^{15}N D22N/D36R/E42K GB1. (b) Superposition of in-cell (magenta) and in-buffer (black) ^{1}H-^{15}N HSQC spectra of 5F-Trp U-^{15}N D22N/D36R/E42K GB1. Amide resonances that originate from cellular background are indicated by the black arrows. A few potential GB1 amide resonances present in the in-cell spectrum are enclosed in blue dashed circles.

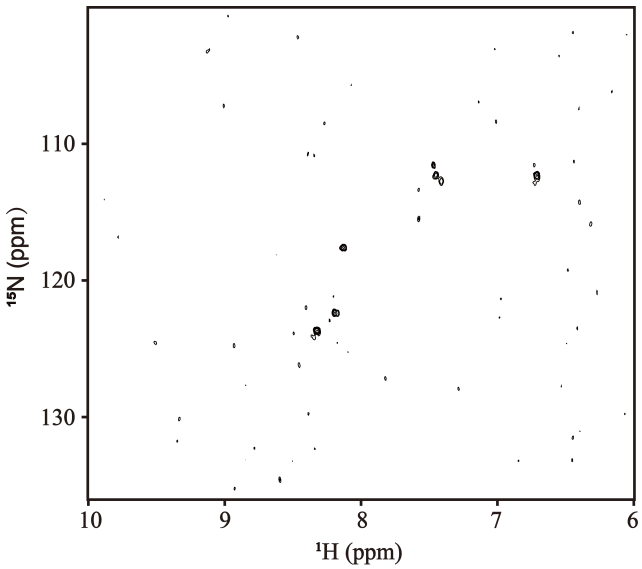


Figure S2. ^{1}H-^{15}N HSQC spectrum of blank cells in L-15 medium containing 10% FBS, 10% D2O.

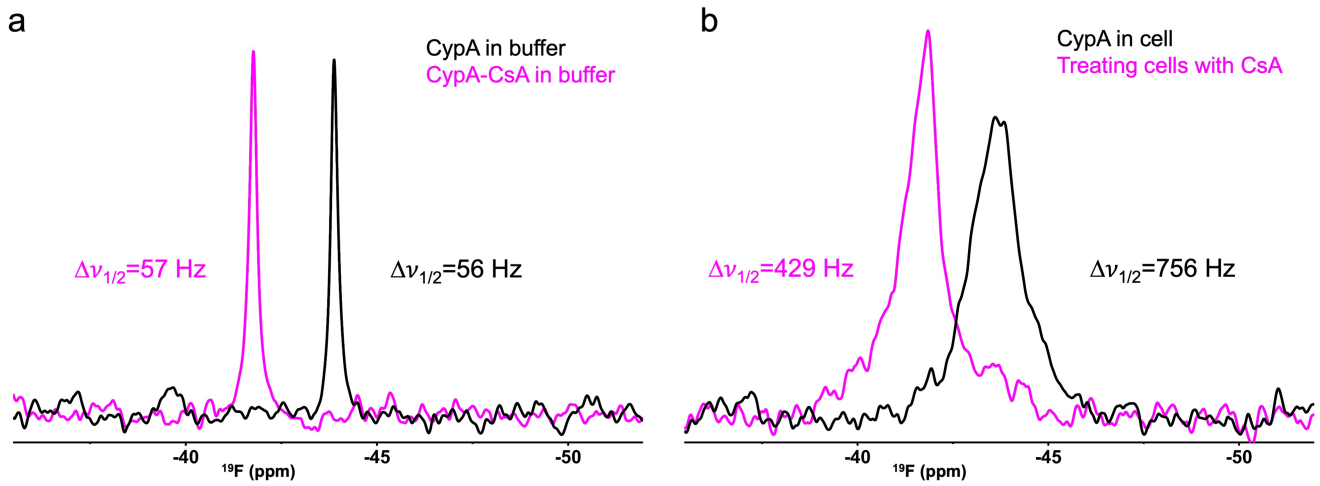


Figure S3. ^{19}F spectra of CypA and CypA-CsA in buffer and in cell. (a) ^{19}F spectra of CypA (black) and CypA-CsA (magenta) recorded using the same protein concentrations (100 μM). Linewidths were extracted by Lorentzian line shape fitting. (b) ^{19}F spectra were recorded for cells treated with 100 μM CsA after in-cell experiments for CypA.

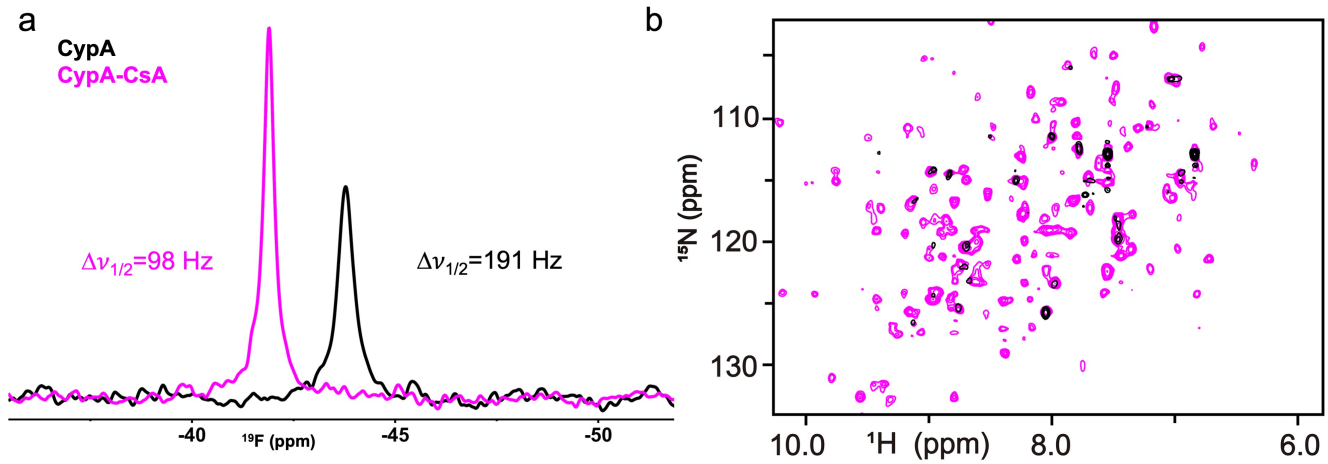


Figure S4. (a) ^{19}F and (b) SOFAST ^{1}H-^{15}N HMQC spectra of CypA (black) and CypA-CsA (magenta) in cell lysate for the same protein concentration (100 µM).

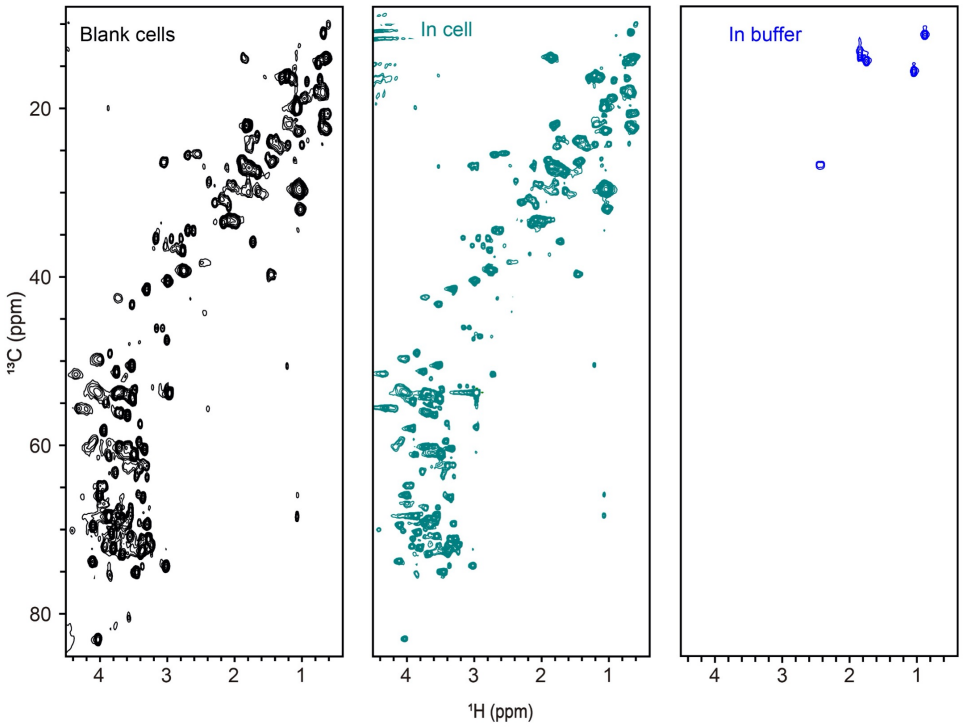


Figure S5. Methyl ^{1}H-^{13}C HMQC spectra of blank cells (left), methyl-^{13}C methionine selectively labelled CypA in cells (middle) and in buffer (right).

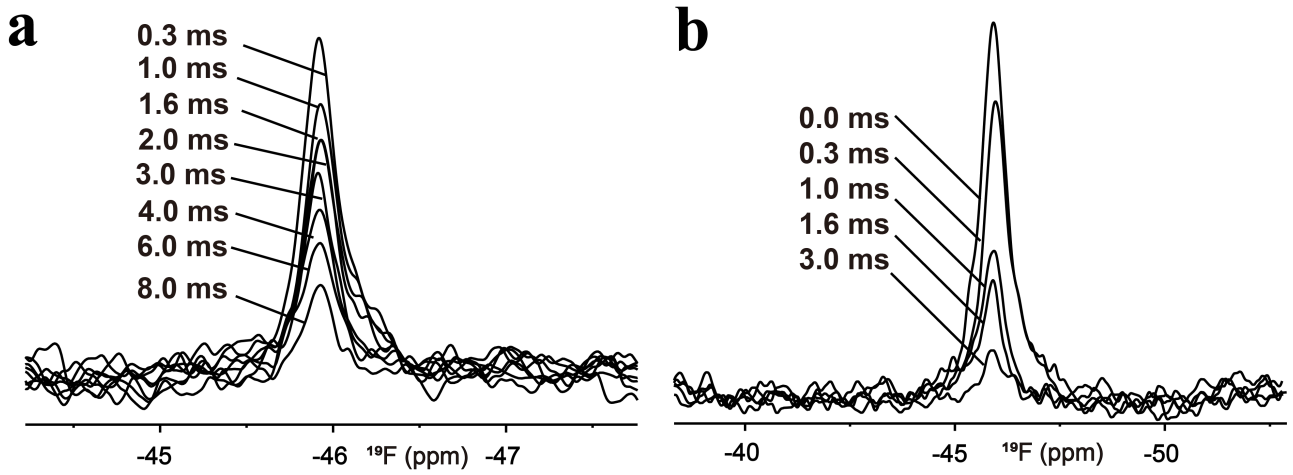


Figure S6. 1D in-cell ^{19}F spectra of BrPSPy-DO3A-Y(III) (a) and BrPSPy-DO3A-Gd(III) (b) tagged 5F-Trp U-^{15}N Q32C GB1 for different relaxation delays.

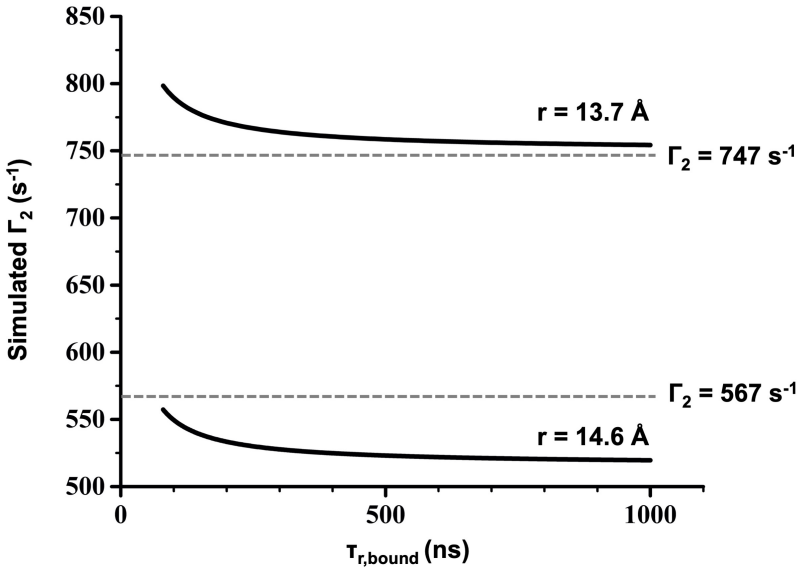


Figure S7. Effects of non-specific interactions on ^{19}F Γ_2 for GB1 in cells. Simulated Γ_2 with $\tau_{r,bound}$ values ranging from 80 to 1000 ns using distances of 13.7 and 14.6 Å, respectively, corresponding to the upper (747 s^{-1}) and lower limit (567 s^{-1}) of experimental Γ_2 values measured in the cell. Details of simulation procedure are described in the Methods part "^{19}F paramagnetic relaxation enhancement measurements and analysis".

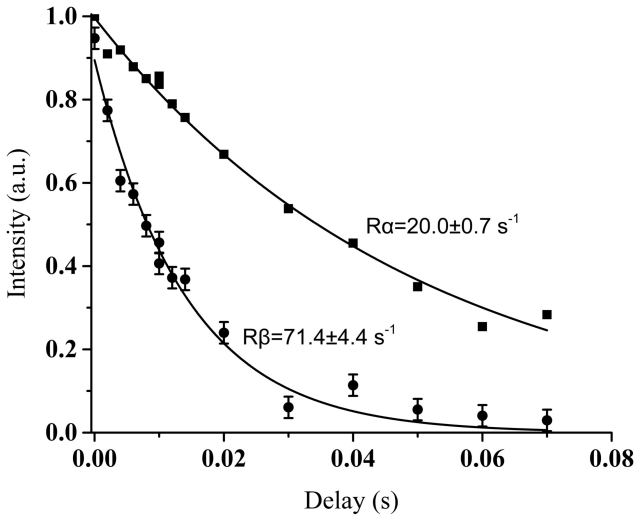


Figure S8. Measurement of τ_c for 5F-Trp U-^{15}N WT GB1 in cells using a 1D ^{1}H-^{15}N TRACT experiment. Intensities of the amide signal envelope (integration over 6.5-10.0 ppm) for different relaxation delays are fitted to an exponential function for extraction α-spin (squares) and β-spin state transverse relaxation rates (circles). The protein rotational correlation time was determined as reported previously[7] and intensity errors were estimated from repeats for a relaxation delay of 10 ms.

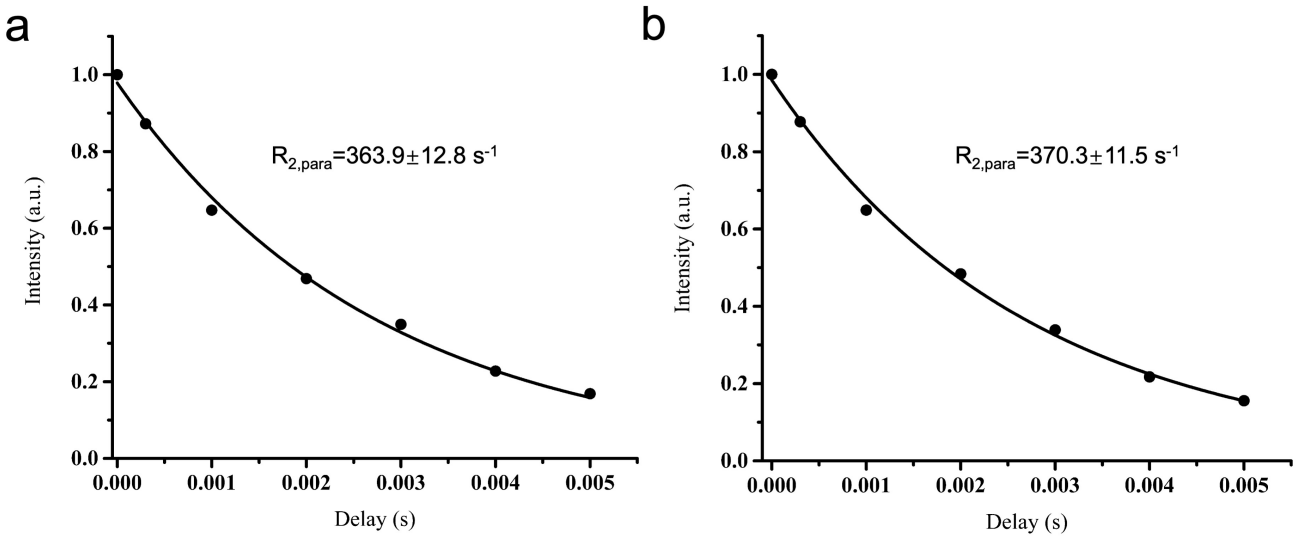
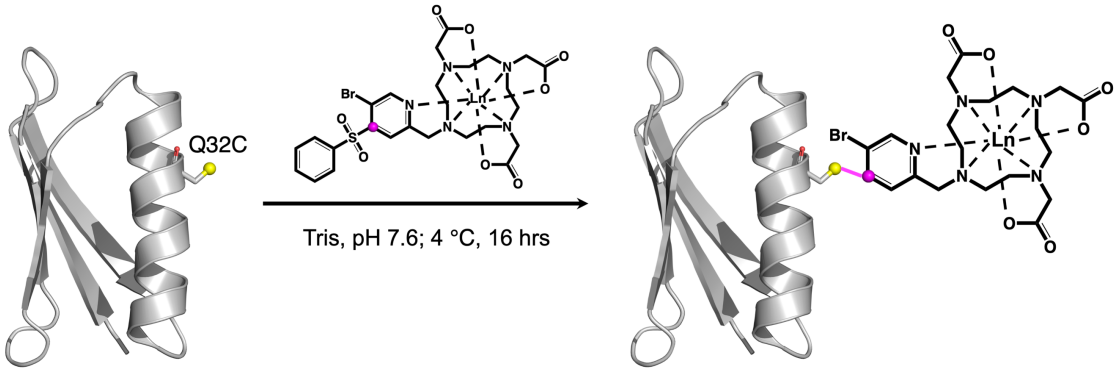


Figure S9. In-buffer R_2 measurements of BrPSPy-DO3A-Gd(III) tagged 5F-Trp U-^{15}N Q32C GB1 at concentrations of 66 μM (a) and 200 μM (b), respectively, for identical acquisition conditions. Exponential fits of the intensities for different relaxation delays yielded very similar R_2 values as indicated. The experimental errors are estimated from the signal-to-noise ratio (SNR) of the ^{19}F spectra and are smaller than each individual point, since the in-buffer spectra have a very high SNR.



Scheme S1. Ligation scheme of GB1 Q32C with BrPSPy-DO3A-Gd/Y(III).

SUPPORTING INFORMATION

Table S1. ^{19}F chemical shifts and linewidths

Protein delivered	^{19}F chemical shifts (ppm)		^{19}F peak linewidth[a] (Hz)		
	In buffer	In cell	In buffer	In cell	In lysate
5F-Trp U-^{15}N GB1	-45.8	-45.8	23	70	23
5F-Trp U-^{15}N D22N/D36R/E42K GB1	-45.9	-45.9	22	161	28
3F-Tyr U-^{15}N K63R Ub	-56.8	-56.7	75	376	104
5F-Trp U-^{15}N CA CTD	-47.7	-47.6	97	367	139
5F-Trp U-^{15}N CypA	-43.8	-43.6	56	756	191
5F-Trp U-^{15}N CypA-CsA	-41.9	-41.8	57	370	98
5F-Trp U-^{15}N GB1_BrPSPy-DO3A-Gd(III)	-45.9	-45.9	142	303	158
5F-Trp U-^{15}N GB1_BrPSPy-DO3A-Y(III)	-45.9	-45.9	30	87	40

[a] Linewidth determined by Lorentzian line shape fitting.

SUPPORTING INFORMATION

Table S2. sample details

Protein delivered	# of cells (million)	protein conc. in lysate (μM)	volume of lysate (μL)	intracellular protein conc. (μM)
5F-Trp U-^{15}N GB1	87	58	557	338
5F-Trp U-^{15}N D22N/D36R/E42K GB1	92	42	459	191
3F-Tyr U-^{15}N K63R Ub	34	50	242	324
5F-Trp U-^{15}N CA CTD	92	86	525	446
5F-Trp U-^{15}N CypA	35	49	330	420
5F-Trp U-^{15}N CypA-CsA	51	21	280	105
5F-Trp U-^{15}N GB1_BrPSPy-DO3A-Gd(III)	57	47	450	337
5F-Trp U-^{15}N GB1_BrPSPy-DO3A-Y(III)	120	17	500	64

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Author Contributions

A.M.G. conceived and directed this study. W.Z., A.G., F.B., and M.L. prepared proteins. W.Z. prepared A2780 cells and delivered proteins. X-C.S. synthesized the BrPSPy-DO3A-Gd(III) tag. W.Z., A.G., and F.B. conducted in-cell NMR experiments. W.Z. processed and analyzed NMR data. W.Z. prepared the initial draft of the manuscript. All authors reviewed, commented on, and approved the manuscript. Correspondence and requests for materials should be addressed to A.M.G. (amg100@pitt.edu).