Supplementary Information

^{19}F-PCS measurements on proteins in live cells

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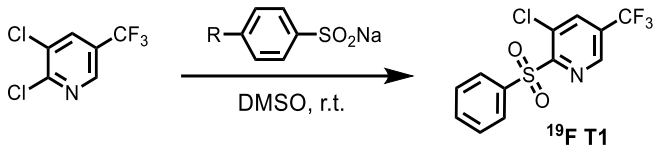
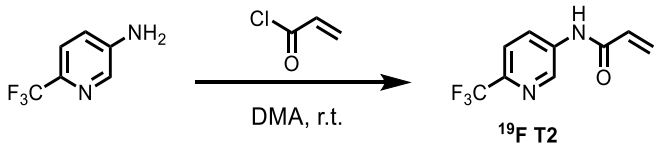
1. Synthesis and characterization of ^{19}F-tags

General information.

Unless otherwise stated, all materials and solvents were purchased commercially and used without further purification. Unless otherwise stated, all experiments were performed under ambient air conditions. All ^{1}H-NMR, ^{13}C-NMR, ^{19}F-NMR spectra were recorded on Bruker NMR spectrometer at a magnetic field with a proton frequency of 800 MHz. Chemical shifts were reported in ppm on the scale of an internal standard (NMR descriptions: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet). Coupling constants, J, are given in Hz.



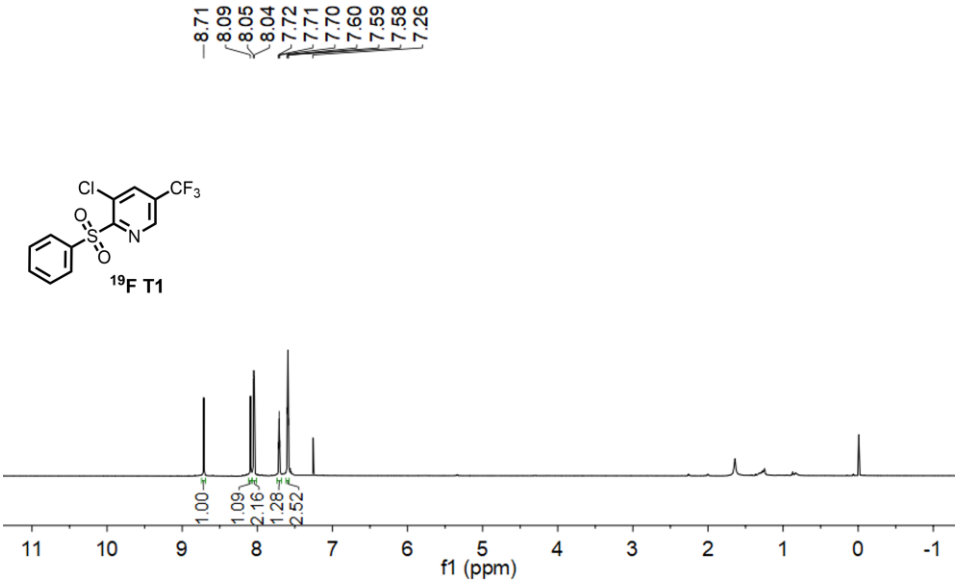
Synthesis of 3-chloro-2-(phenylsulfonyl)-5-(trifluoromethyl)pyridine (^{19}F T1): a mixture of 2,3-dichloro-5-(trifluoromethyl)pyridine(2 mmol, 0.43g), benzenesulfinic acid sodium salt (5 mmol, 0.8 g) and dimethyl sulfoxide (20 mL) were stirred at room temperature for 6 hours. The mixture was diluted with ethyl acetate and filtered through Celite. The filtrate was washed with water, dried over sodium sulfate and concentrated under reduced pressure. Purification of the crude product was achieved by column chromatography (petroleum ether: ethyl acetate 5 : 1) to provide **^{19}F T1** (0.21 g, yield about 62%) as a white solid.^{1}H NMR ($CDCl_3$, 800 MHz): δ 8.71 (s, 1H), 8.09 (s, 1H), 8.05 (d, *J* = 8.2 Hz, 2 H), 7.72-7.70 (m, 1H), 7.60-7.58 (m, 2H). ^{13}C NMR ($CDCl_3$, 201 MHz): δ 157.70, 143.54 (d, J_{C-F} = 3.6 Hz), 138.00 (d, J_{C-F} = 3.32 Hz), 137.98, 137.37, 136.65, 134.61, 130.91, 129.79, 129.17, 127.58. ^{19}F NMR ($CDCl_3$, 753 MHz): -62.50 ppm. LC-MS: m/z, $C_{12}H_7ClF_3NO_2S$, $[M+Na]^+$, calculated 343.98, found 343.92.



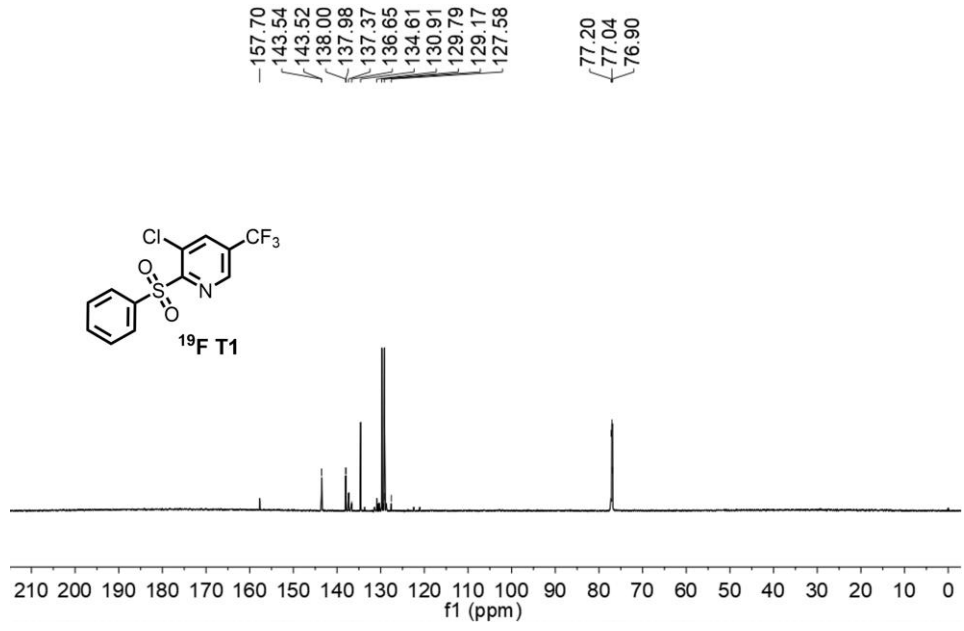
Synthesis of N-(6-(trifluoromethyl)pyridin-3-yl)acrylamide (^{19}F T2): a mixture of 6-(trifluoromethyl)pyridin-3-amine (6.17 mmol, 1 g), acryloyl chloride (6.79 mmol, 0.6 g) and N, N-dimethylethanolammonium (DMA, 20 mL) were stirred at room temperature for 2 hours. Gradually add 70 mL of water to the reaction solution, a white solid precipitated, filtered, dried, to obtain **^{19}F T2** as a white solid (0.87 g, yield about 65%).^{1}H NMR ($CDCl_3$, 800 MHz): δ 8.67 (s, 1H), 8.51 (d, *J* = 8.02 Hz, 1H), 7.84 (s, 1H), 7.69 (d, *J* = 8.46 Hz, 1 H), 6.53 (d, J = 16.42 Hz, 1 H), 6.32-6.68 (m, 1H), 5.91 (d, J = 10.21 Hz, 1 H). ^{13}C NMR ($CDCl_3$, 201 MHz): δ 164.13, 143.47,143.30, 140.78, 137.08, 130.02, 129.86, 127.39, 121.14(d, J_{C-F} = 2.5 Hz). ^{19}F NMR ($CDCl_3$, 753 MHz): -69.85 ppm. LC-MS: m/z, $C_9H_7F_3N_2O$, $[M+Na]^+$, calculated 239.05, found 239.01.

^{1}H NMR, ^{13}C NMR and ^{19}F NMR spectra of ^{19}F-tags

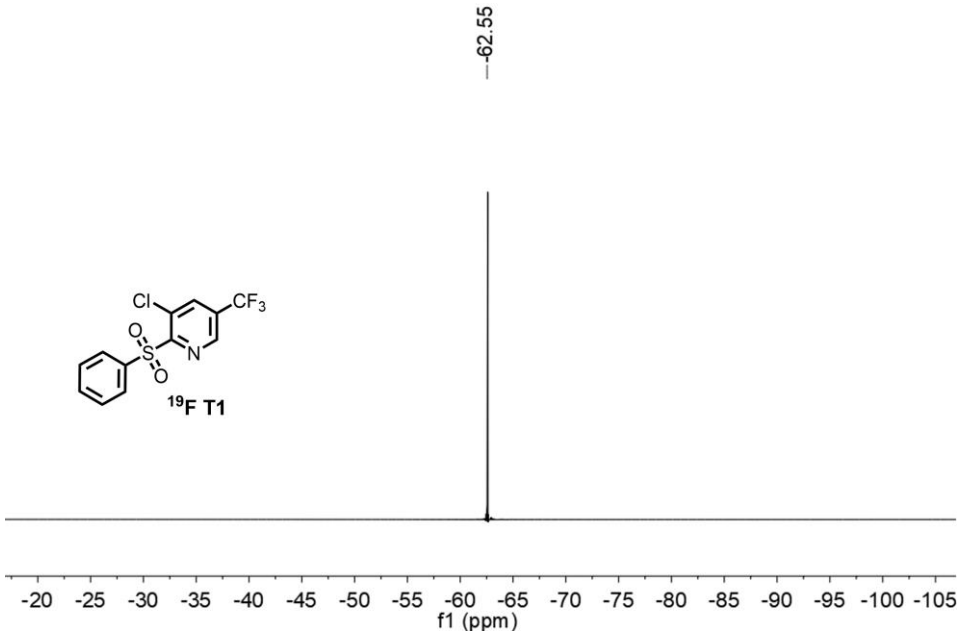
^{1}H NMR (800 MHz, $CDCl_3$) of tag **^{19}F T1**



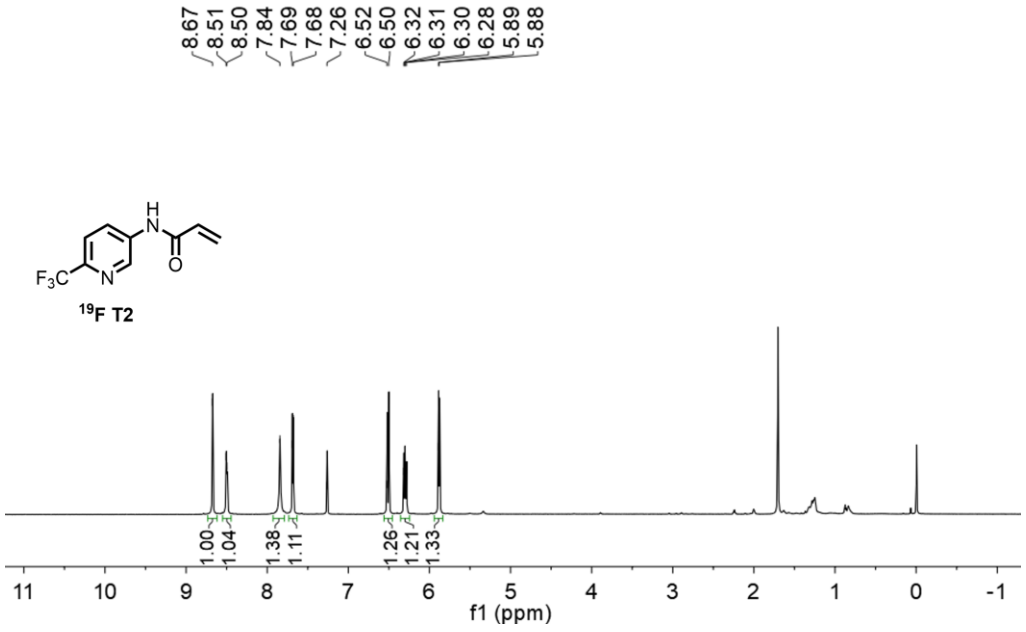
^{13}C NMR (201 MHz, $CDCl_3$) of tag **^{19}F T1**



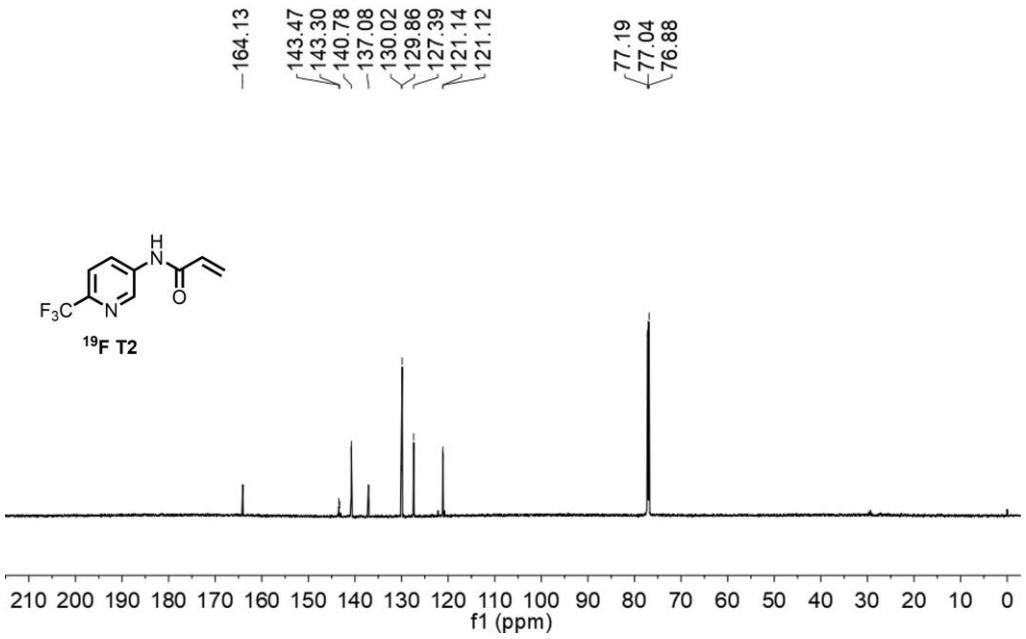
^{19}F NMR (753 MHz, $CDCl_3$) of tag **^{19}F T1**



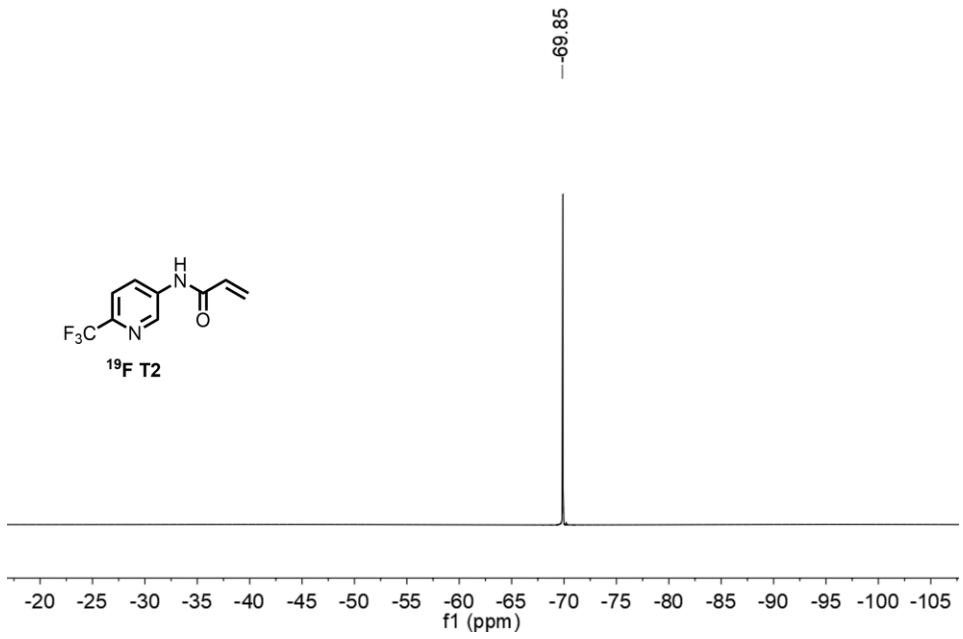
^{1}H NMR (800 MHz, $CDCl_3$) of tag **^{19}F T2**



^{13}C NMR (201 MHz, $CDCl_3$) of tag **^{19}F T2**



^{19}F NMR (753 MHz, $CDCl_3$) of tag **^{19}F T2**



2. Protein expression and purification

The plasmid used in this study was transformed into *Escherichia coli* BL21 (DE3) Codon Plus strain for protein expression. The protein expression and purification process of human ubiquitin (Ub) cysteine mutants D39C, G47C, E64C, S57C, T12C, and G35C were similar to those described previously.[1,2] The protein samples were made by overexpression of the target protein using the high-cell-density method.[3] Briefly, the cells transformed with the plasmid of interest were grown in 600 mL LB medium at 37 °C till OD_{600} of 0.7-0.9 was achieved, the cell culture was gently spun down at 3000 rpm for 5 min, and the cells were washed with MilliQ water and then resuspended in 250 mL of minimal M9 medium with $^{15}NH_4Cl$ as the only nitrogen source. The cells were allowed to recover by incubation at 37 °C for 40 min. Protein expression was then induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 8-10 hours at 37 °C while shaking at 200 rpm. The target protein was purified via a DEAE column (DEAE Sepharose FF, Cytiva), followed by size exclusion chromatography (HiLoad 16/600 Superdex 75, GE Healthcare Biosciences).

3. NMR spectroscopy

All the ^{1}H-NMR, ^{13}C-NMR and ^{19}F-NMR spectra were recorded on the Avance Neo 800 MHz spectrometer equipped with a H(F)-TCI cryoprobe at 298 K. All the ^{15}N-HSQC spectra were recorded on a Bruker Avance 600 MHz NMR spectrometer equipped with a QCI-cryoprobe. All NMR spectra were processed with Topspin 3.2 and analyzed with Topspin 2.1.

4. Reactivity assay for the interaction of tags (Para T1, ^{19}F T1, ^{19}F T2) with protein free thiols

To maintain the reduced state of the protein sulfhydryl groups, 1-2 equivalents of tris (2-carboxyethyl) phosphine hydrochloride (TCEP) were first added to the purified protein solution and incubated at room temperature for 15 minutes. Following this step, the TCEP is removed from the protein solution by a PD-10 desalting column, balanced with a 20 mM MES buffer (pH 6.4).

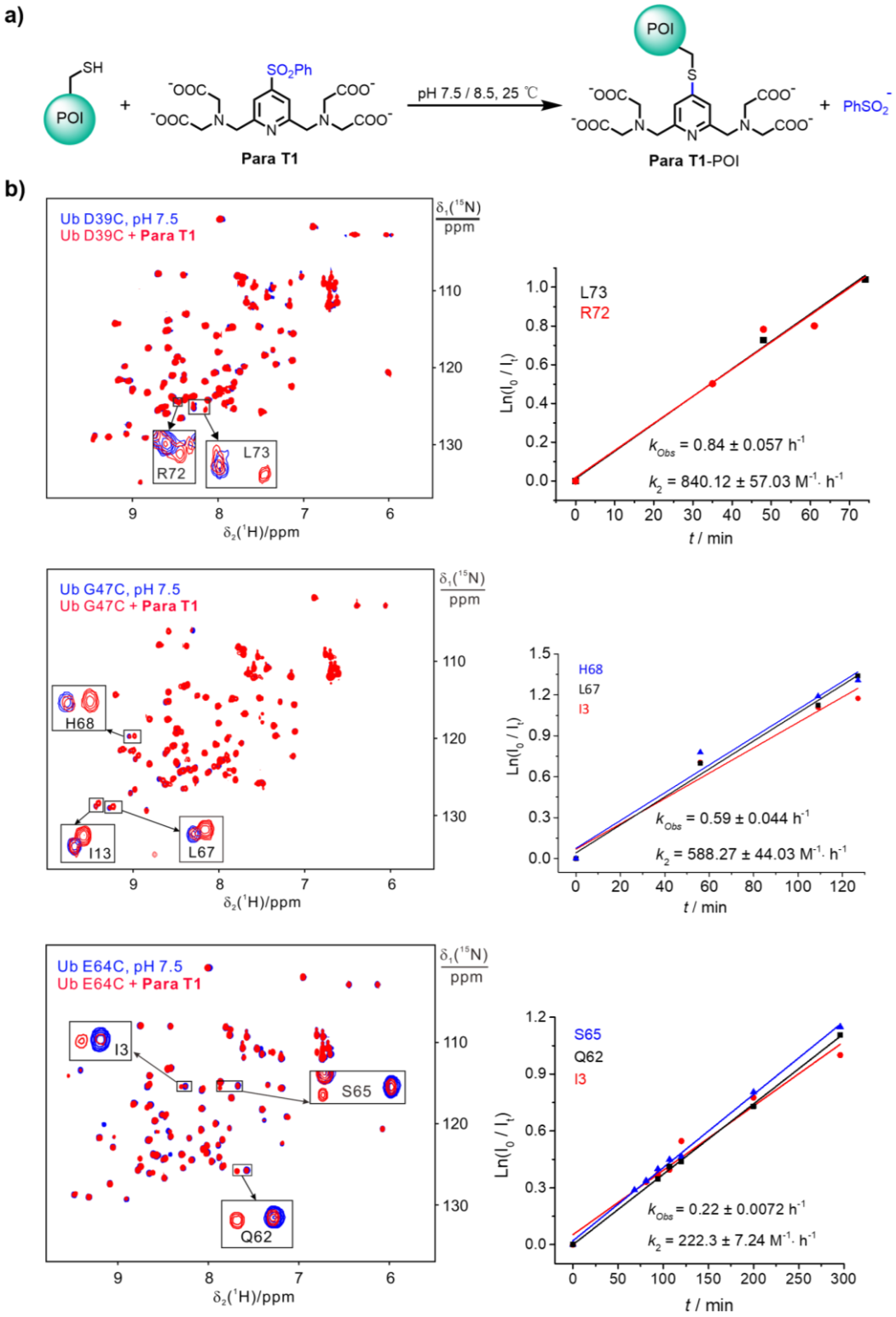
To assess the reactivity of tags (**Para T1, ^{19}F T1, ^{19}F T2**) toward different single cysteine mutations in aqueous solution, the reaction of the tags with target proteins (Ub D39C, G47C, E64C, S57C, T12C, G35C) were evaluated by ^{15}N-HSQC in 20 mM phosphate buffer (including 10% D_2O) at different pH (7.5, 8.5) and 298 K. In general, 0.1 mM target protein was mixed with six or ten equivalent of the tags (**Para T1, ^{19}F T1, ^{19}F T2**), and the reaction was monitored by NMR with incubation time. The crude products mixture were characterized by MALDI-TOF or ESI-QTOF.

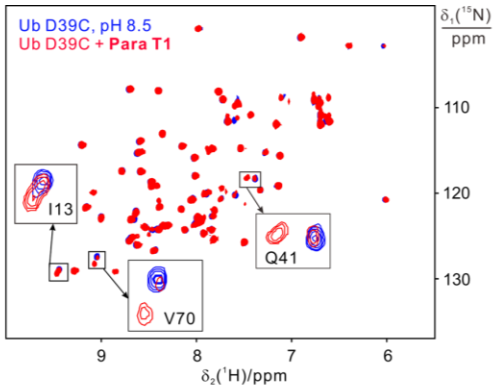
When the initial concentrations of the tags and target proteins vary greatly. In the presence of large excess of tags, the reaction rate is described as in equation (1), and the corresponding second-order reaction rate constant is calculated according to the first-order reaction as shown in Equation (2), and the corresponding second-order reaction rate constant was calculated according to the second-order reaction as follows:

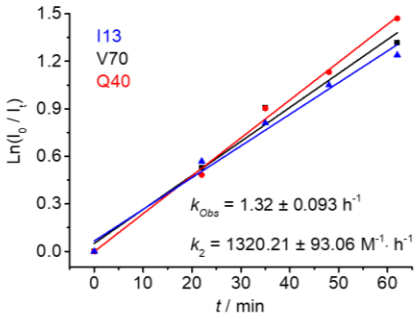
$$\mathrm{Ln}\ \frac{I_0}{I_t} = k_{obs} \cdot t \qquad (1)$$

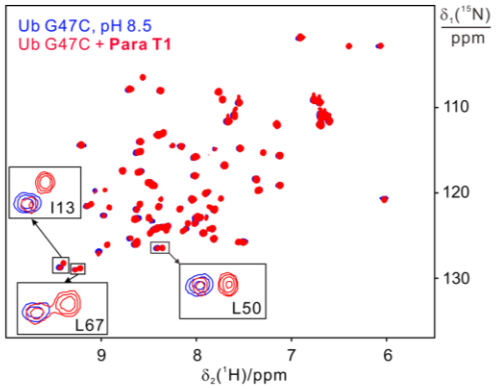
$$k_2 = \frac{k_{obs}}{[\mathrm{Tag}]_0} \qquad (2)$$

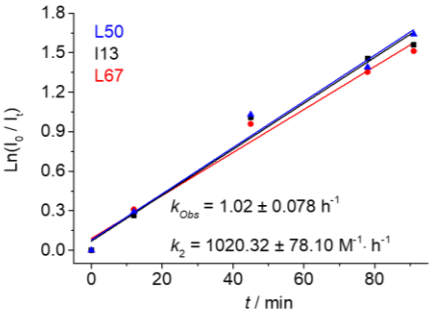
In which k_{obs} is the pseudo-first-order reaction rate constant, k_2 is the second order formation rate constant, t is the reaction time, I_0 is the initial peak intensity of residues in the target protein, I is the peak intensity of residues in the target protein at the time of reaction at t, $[Tag]_0$ is the initial concentration of the tag.

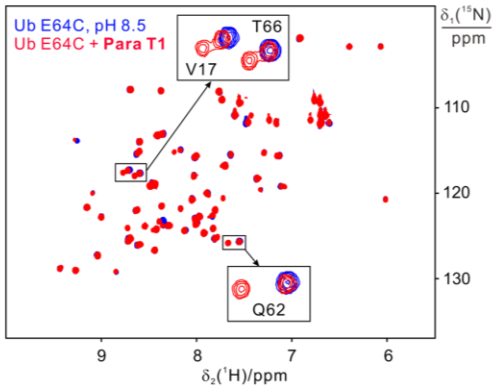


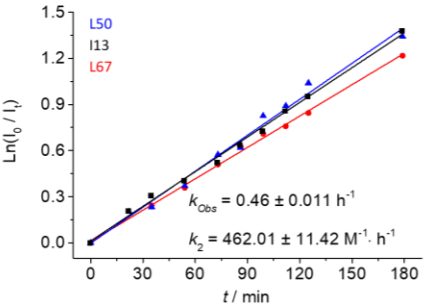

Ub D39C, pH 8.5
Ub D39C + Para T1
δ1(15N) ppm
I13
Q41
V70
δ2(1H)/ppm


I13
V70
Q40
kObs = 1.32 ± 0.093 h-1
k2 = 1320.21 ± 93.06 M-1· h-1
Ln(I0 / It)
t / min

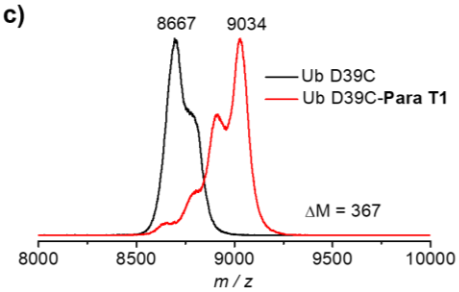

Ub G47C, pH 8.5
Ub G47C + Para T1
δ1(15N) ppm
I13
L50
L67
δ2(1H)/ppm

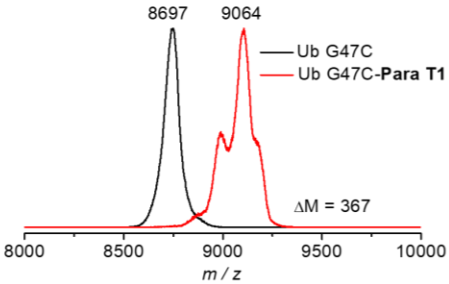

L50
I13
L67
kObs = 1.02 ± 0.078 h-1
k2 = 1020.32 ± 78.10 M-1· h-1
Ln(I0 / It)
t / min


Ub E64C, pH 8.5
Ub E64C + Para T1
δ1(15N) ppm
T66
V17
Q62
δ2(1H)/ppm


L50
I13
L67
kObs = 0.46 ± 0.011 h-1
k2 = 462.01 ± 11.42 M-1· h-1
Ln(I0 / It)
t / min

c)


8667
9034
Ub D39C
Ub D39C-Para T1
ΔM = 367
m / z


8697
9064
Ub G47C
Ub G47C-Para T1
ΔM = 367
m / z

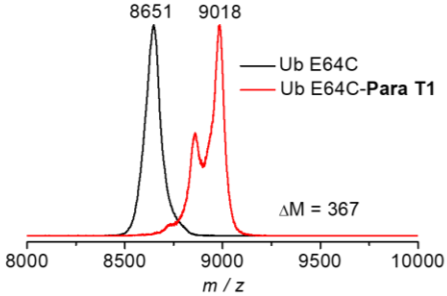
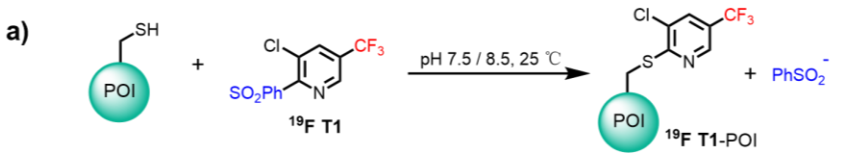
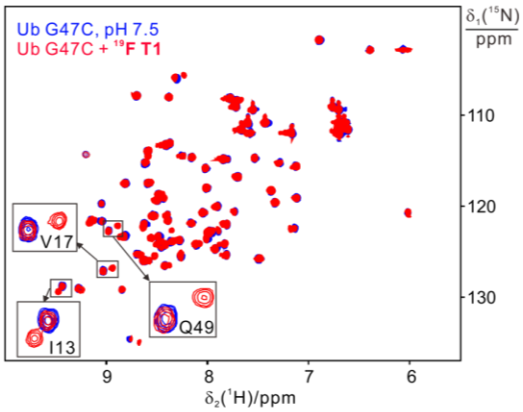
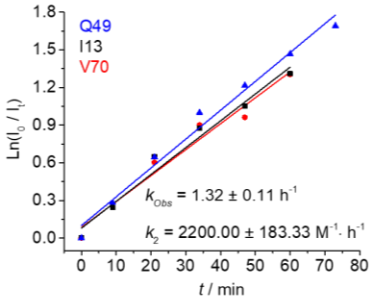

8651
9018
Ub E64C
Ub E64C-Para T1
ΔM = 367
m / z

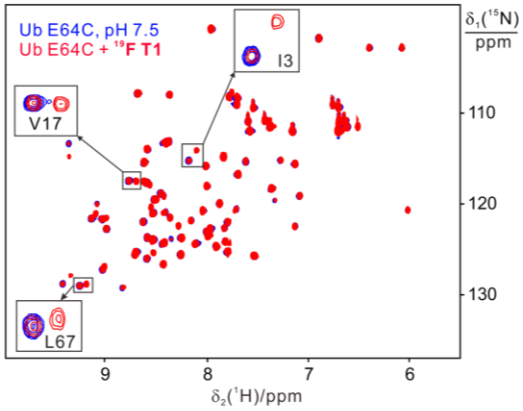
Figure S1. Reactivity assay of **Para T1** with POI (Ub D39C, G47C, E64C). a) The reaction scheme of POI with **Para T1**; b) Superimposition of ^{15}N-HSQC spectra recorded for 0.1 mM ^{15}N POI (blue) after incubation with 1 mM (10 equivalent) **Para T1** for different time (red) in 20 mM phosphate buffer at pH 7.5 and 8.5, respectively. The observed reaction rate constant k_{Obs} and the calculated second order reaction rate constant k_2 at different pH were determined; c) MALDI-TOF mass spectra of ^{15}N labeled POI (black) and after labeling with **Para T1** (red). The observed mass difference between free protein and labeled protein with **Para T1** is consistent with theoretical mass difference (367 Dalton).

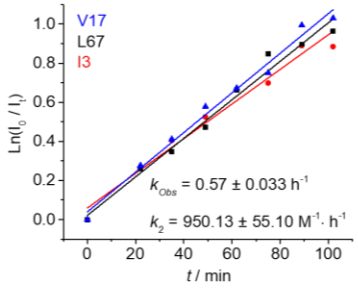

a)
SH
POI
+
Cl
CF3
SO2Ph
N
19F T1
pH 7.5 / 8.5, 25 ℃
Cl
CF3
S
N
+ PhSO2-
POI
19F T1-POI

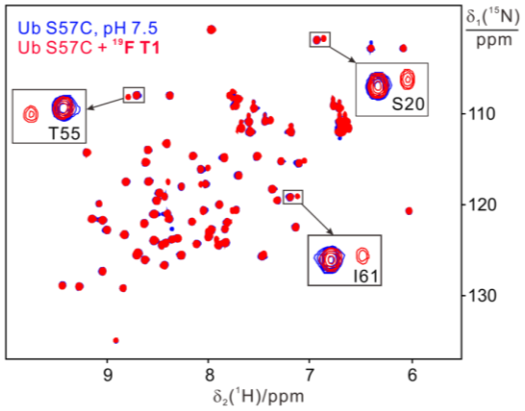
b)

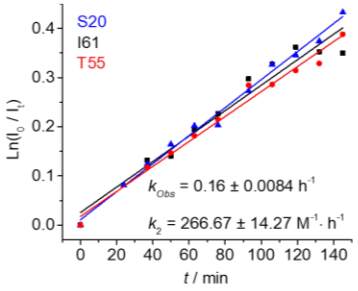

Ub G47C, pH 7.5
Ub G47C + 19F T1
δ1(15N) ppm
110
120
130
V17
I13
Q49
9
8
7
6
δ2(1H)/ppm

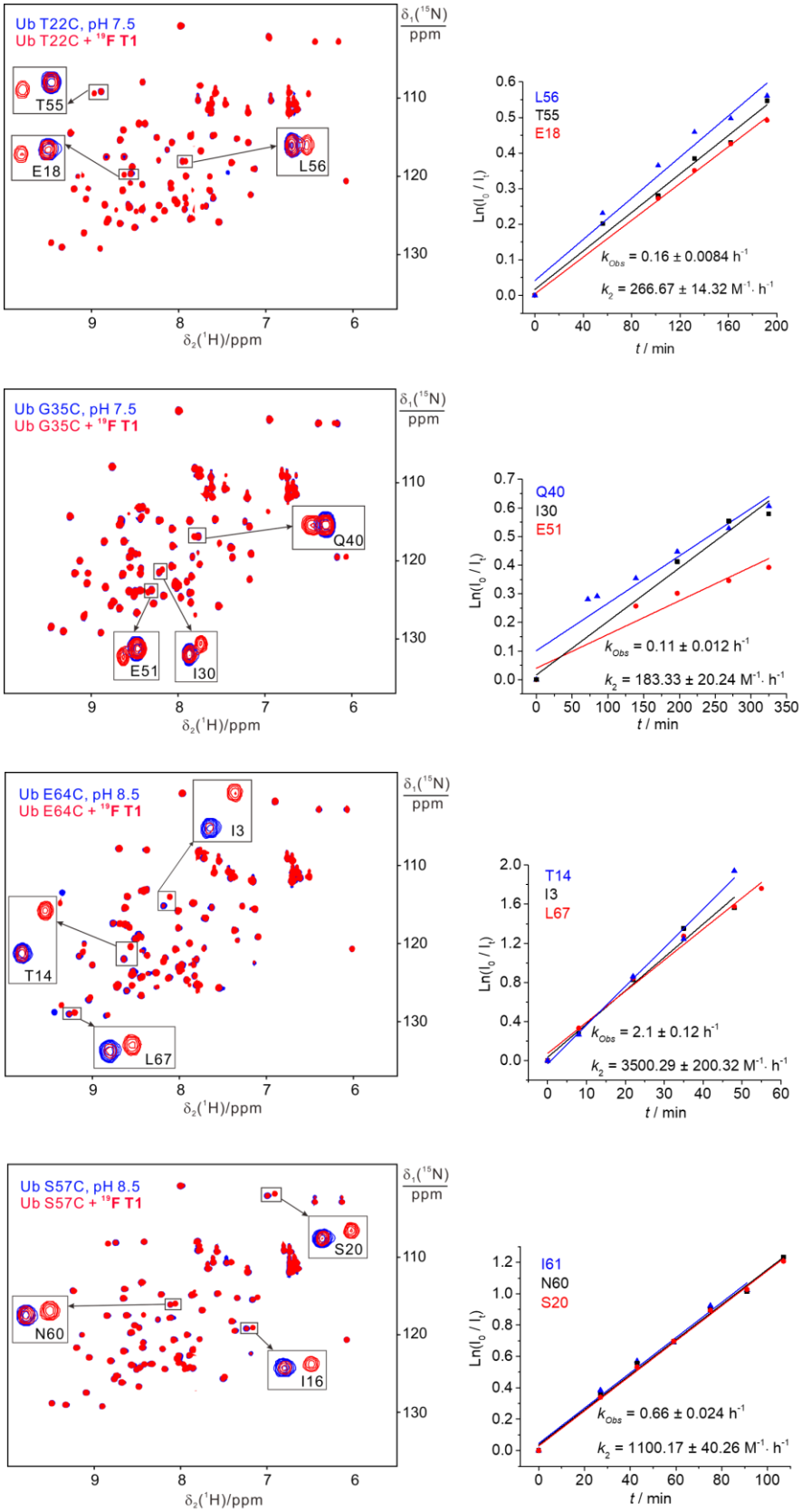

Q49
I13
V70
Ln(I0 / It)
kObs = 1.32 ± 0.11 h-1
k2 = 2200.00 ± 183.33 M-1· h-1
t / min


Ub E64C, pH 7.5
Ub E64C + 19F T1
δ1(15N) ppm
110
120
130
I3
V17
L67
9
8
7
6
δ2(1H)/ppm


V17
L67
I3
Ln(I0 / It)
kObs = 0.57 ± 0.033 h-1
k2 = 950.13 ± 55.10 M-1· h-1
t / min


Ub S57C, pH 7.5
Ub S57C + 19F T1
δ1(15N) ppm
110
120
130
S20
T55
I61
9
8
7
6
δ2(1H)/ppm


S20
I61
T55
Ln(I0 / It)
kObs = 0.16 ± 0.0084 h-1
k2 = 266.67 ± 14.27 M-1· h-1
t / min


Ub T22C, pH 7.5
Ub T22C + 19F T1
T55
E18
L56
δ1(15N) ppm
δ2(1H)/ppm
L56
T55
E18
Ln(I0 / It)
kObs = 0.16 ± 0.0084 h-1
k2 = 266.67 ± 14.32 M-1· h-1
t / min
Ub G35C, pH 7.5
Ub G35C + 19F T1
Q40
E51
I30
δ1(15N) ppm
δ2(1H)/ppm
Q40
I30
E51
Ln(I0 / It)
kObs = 0.11 ± 0.012 h-1
k2 = 183.33 ± 20.24 M-1· h-1
t / min
Ub E64C, pH 8.5
Ub E64C + 19F T1
I3
T14
L67
δ1(15N) ppm
δ2(1H)/ppm
T14
I3
L67
Ln(I0 / It)
kObs = 2.1 ± 0.12 h-1
k2 = 3500.29 ± 200.32 M-1· h-1
t / min
Ub S57C, pH 8.5
Ub S57C + 19F T1
S20
N60
I16
δ1(15N) ppm
δ2(1H)/ppm
I61
N60
S20
Ln(I0 / It)
kObs = 0.66 ± 0.024 h-1
k2 = 1100.17 ± 40.26 M-1· h-1
t / min

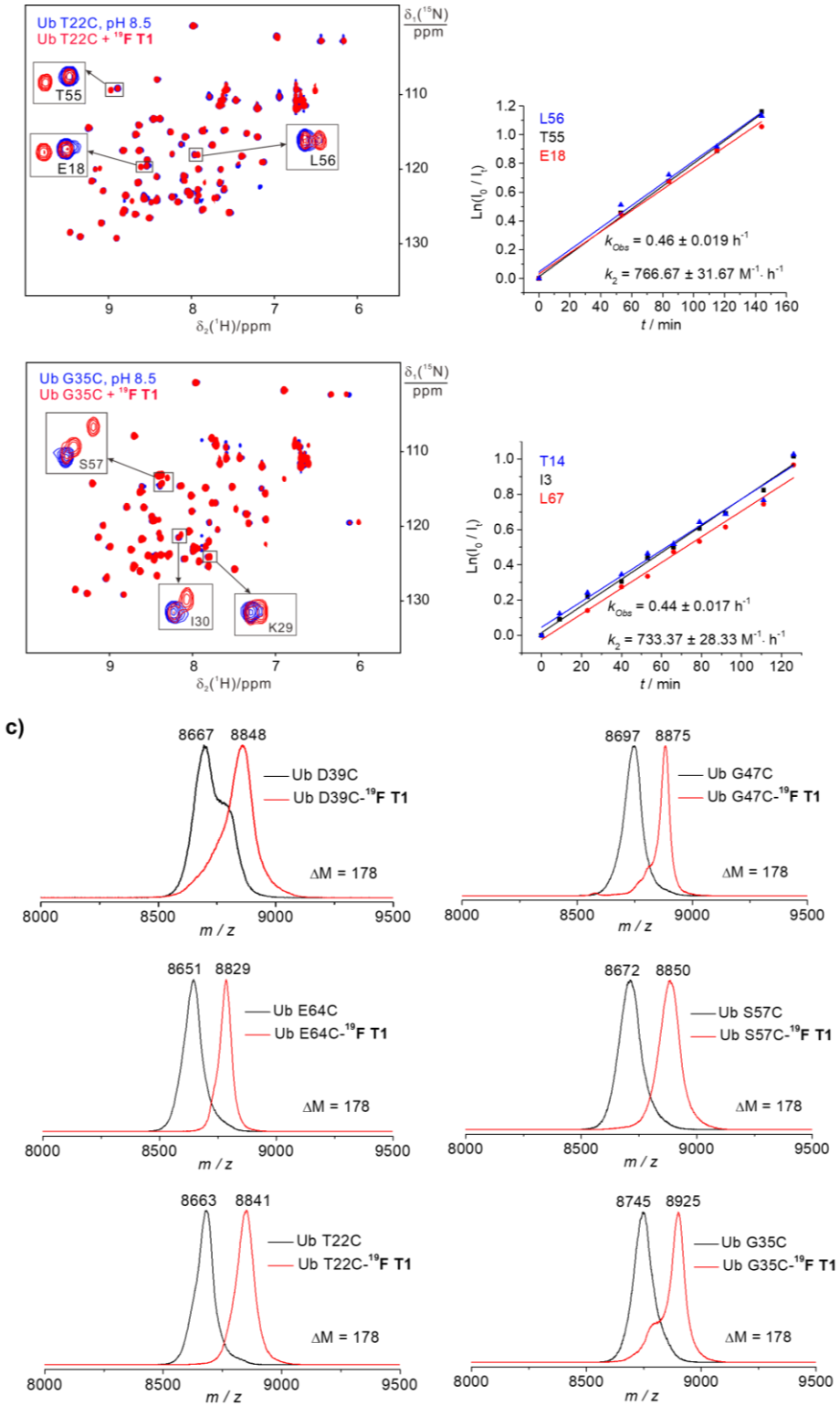
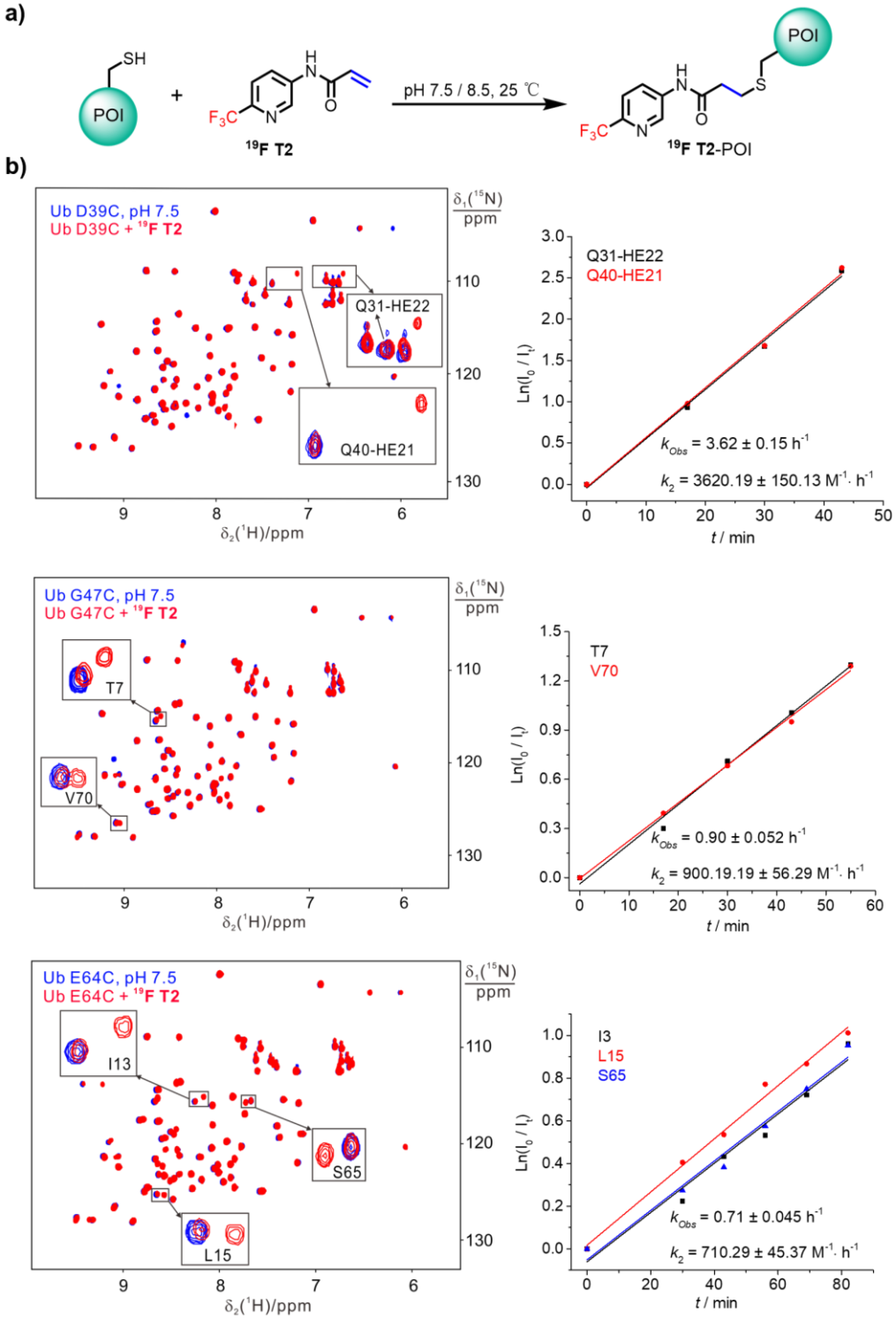
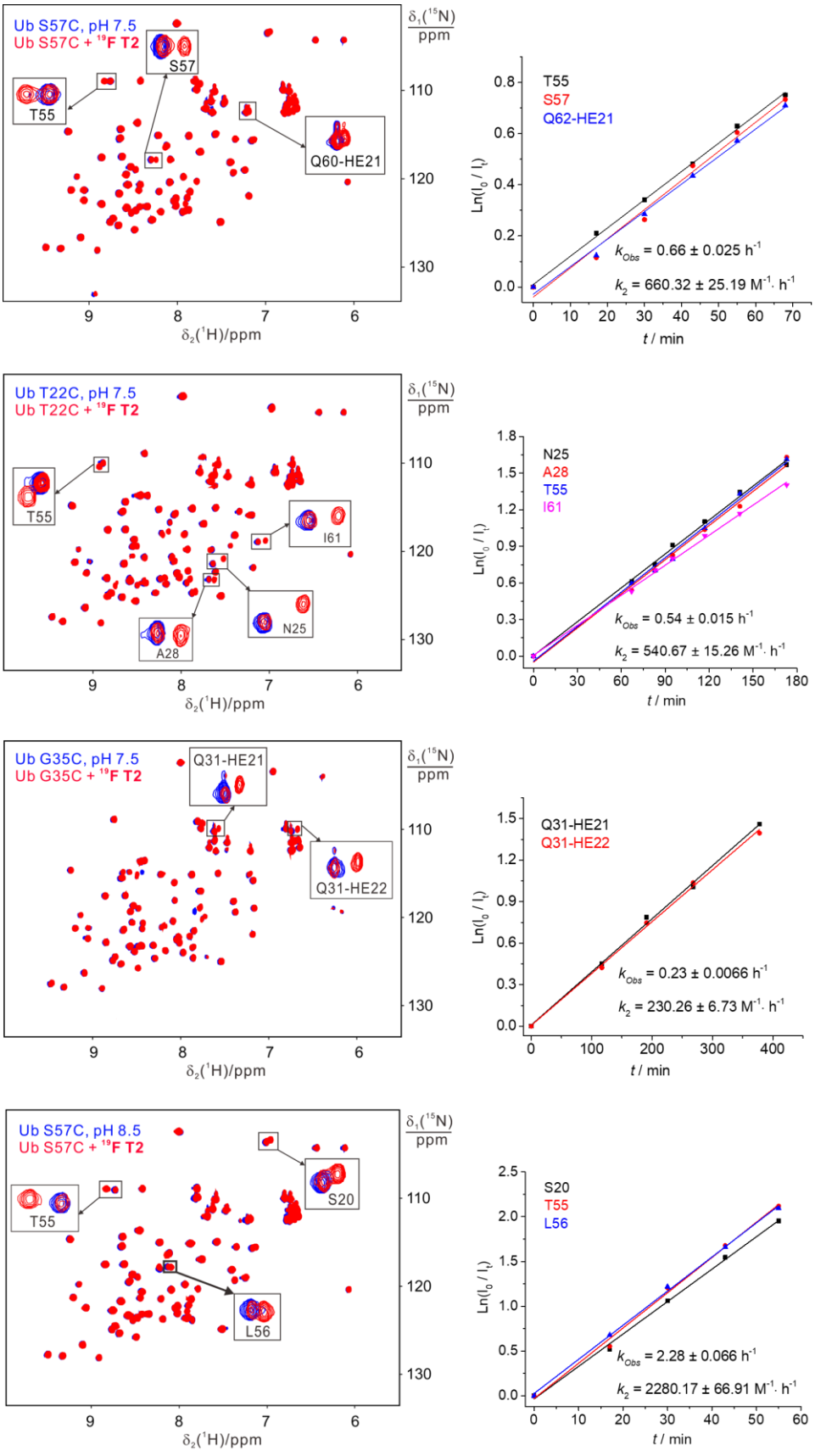


Figure S2. Reactivity assay of **^{19}F T1** with POI (Ub D39C, G47C, E64C, S57C, T12C, G35C). a) The reaction scheme of POI with **^{19}F T1**; b) Superimposition of ^{15}N-HSQC spectra recorded for 0.1 mM ^{15}N POI (blue) after incubation with 0.6 mM (6 equivalent) **^{19}F T1** for different time (red) in 20 mM phosphate buffer at pH 7.5 and 8.5, respectively. The observed reaction rate constant k_{Obs} and the calculated second order reaction rate constant k_2 at different pH were determined; c) MALDI-TOF mass spectra of ^{15}N labeled POI (black) and after labeling with **^{19}F T1** (red). The observed mass difference between free protein and labeled protein with **^{19}F T1** is consistent with theoretical mass difference (178 Dalton).


a)
SH
POI
+
F3C
N
H
N
O
19F T2
pH 7.5 / 8.5, 25 ℃
POI
S
19F T2-POI
b)
Ub D39C, pH 7.5
Ub D39C + 19F T2
Q31-HE22
Q40-HE21
δ1(15N) ppm
δ2(1H)/ppm
Q31-HE22
Q40-HE21
Ln(I0 / It)
kObs = 3.62 ± 0.15 h-1
k2 = 3620.19 ± 150.13 M-1· h-1
t / min
Ub G47C, pH 7.5
Ub G47C + 19F T2
T7
V70
δ1(15N) ppm
δ2(1H)/ppm
T7
V70
Ln(I0 / It)
kObs = 0.90 ± 0.052 h-1
k2 = 900.19.19 ± 56.29 M-1· h-1
t / min
Ub E64C, pH 7.5
Ub E64C + 19F T2
I13
S65
L15
δ1(15N) ppm
δ2(1H)/ppm
I3
L15
S65
Ln(I0 / It)
kObs = 0.71 ± 0.045 h-1
k2 = 710.29 ± 45.37 M-1· h-1
t / min


Ub S57C, pH 7.5
Ub S57C + 19F T2
S57
T55
Q60-HE21
δ1(15N) ppm
δ2(1H)/ppm
T55
S57
Q62-HE21
Ln(I0 / It)
kObs = 0.66 ± 0.025 h-1
k2 = 660.32 ± 25.19 M-1· h-1
t / min
Ub T22C, pH 7.5
Ub T22C + 19F T2
T55
I61
N25
A28
N25
A28
T55
I61
kObs = 0.54 ± 0.015 h-1
k2 = 540.67 ± 15.26 M-1· h-1
Ub G35C, pH 7.5
Ub G35C + 19F T2
Q31-HE21
Q31-HE22
Q31-HE21
Q31-HE22
kObs = 0.23 ± 0.0066 h-1
k2 = 230.26 ± 6.73 M-1· h-1
Ub S57C, pH 8.5
Ub S57C + 19F T2
T55
S20
L56
S20
T55
L56
kObs = 2.28 ± 0.066 h-1
k2 = 2280.17 ± 66.91 M-1· h-1

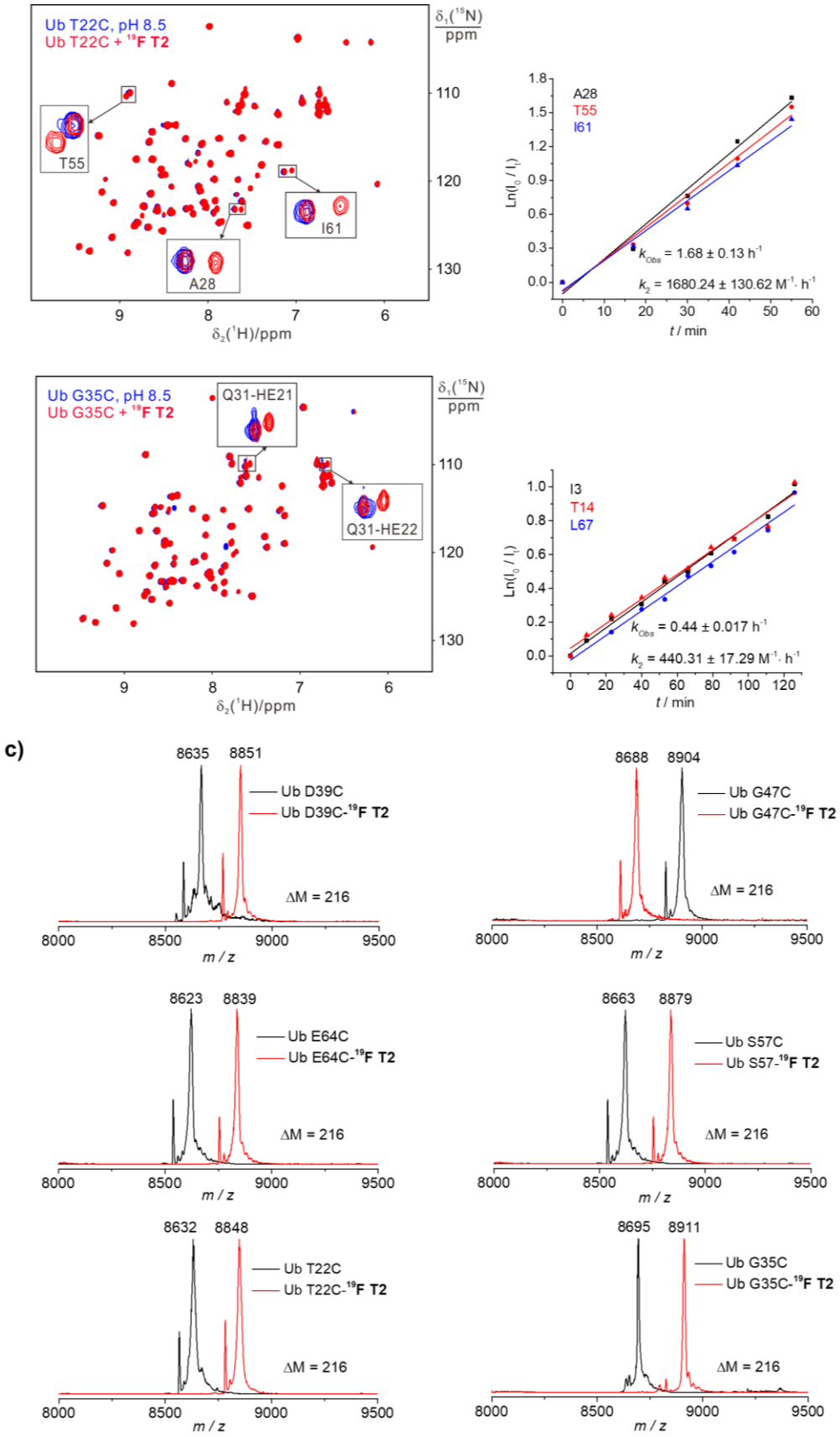


Figure S3. Reactivity assay of **^{19}F T2** with POI (Ub D39C, G47C, E64C, S57C, T12C, G35C). a) The reaction scheme of POI with **^{19}F T2**; b) Superimposition of ^{15}N-HSQC spectra recorded for 0.1 mM ^{15}N POI (blue) after incubation with

1 mM (10 equivalent) **^{19}F T2** for different time (red) in 20 mM phosphate buffer at pH 7.5 and 8.5, respectively. The observed reaction rate constant k_{Obs} and the calculated second order reaction rate constant k_2 at different pH were determined; c) ESI-QTOF mass spectrometry of ^{15}N labeled POI (black) and after labeling with **^{19}F T2** (red). The observed mass difference between free protein and labeled protein with **^{19}F T2** is consistent with theoretical mass difference (216 Dalton).

5. Site-specific dual labelling of proteins with paramagnetic and ^{19}F tags

1.0 mL 0.4 mM freshly prepared ubiquitin variants was mixed with 3.0 equivalents of **para T1** in 20 mM phosphate buffer, and the pH of the above reactant mixture was adjusted to 8.0. The solution was incubated at 30℃ for about 3-5 h. Complete loading of the protein with **para T1** was then confirmed by MALDI-TOF mass spectrometry, and the excess **para T1** was removed by ultrafiltration. The Single-labeled products of ubiquitin with **para T1**, were separately mixed with 3 equivalents **^{19}F T1** or **^{19}F T2** in 20 mM phosphate buffer. The pH was adjusted to 8.5, incubated at 30℃ for no more than 2 hours, and characterized by MALDI-TOF mass spectrometry. Due to the inevitable loss of protein during ultrafiltration, the overall yield is about 75-90%.

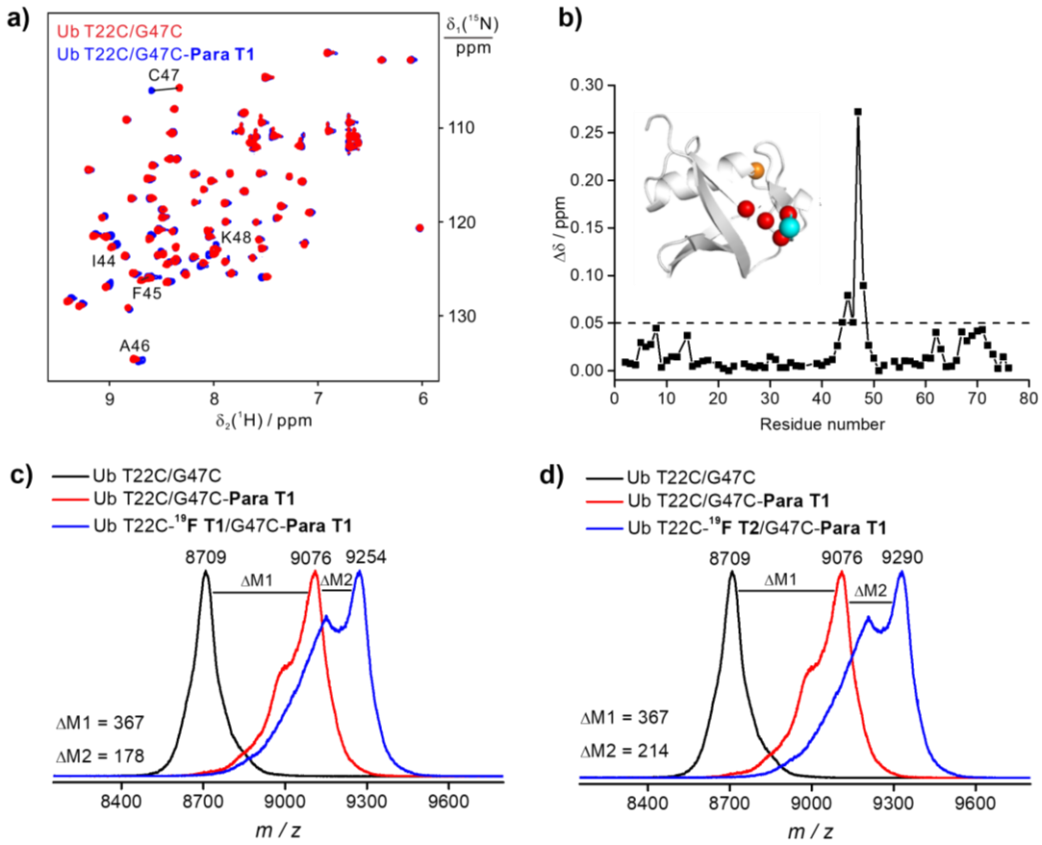


Figure S4. a) Overlay of ^{15}N-HSQC spectra of 0.1 mM ubiquitin T22C/G47C (red) and T22C/G47C-**para T1** conjugate (blue); b) Chemical-shift differences between Ub T22C/G47C and Ub T22C/G47C-**para T1** conjugate, calculated as $\delta = ((\Delta\delta H)^2 + (\Delta\delta N/10)^2)^{1/2}$. The N atoms of the main chain of amino acids whose chemical-shift difference is greater than 0.05 ppm are shown as red spheres and marked on the structure of ubiquitin (PDB code: 1UBI). Among them, the α-C of C47 is represented by a cyan sphere, and the α-C of C22 is represented by an orange sphere. (C)

MALDI-TOF data of Ub T22C/G47C and its sequential reaction with **para T1** and **^{19}F T1**; (D) MALDI-TOF data of Ub T22C/G47C and its sequential reaction with **para T1** and **^{19}F T2**.

6. Comparison of PCS obtained by ^{15}N-HSQC and ^{19}F NMR spectra.

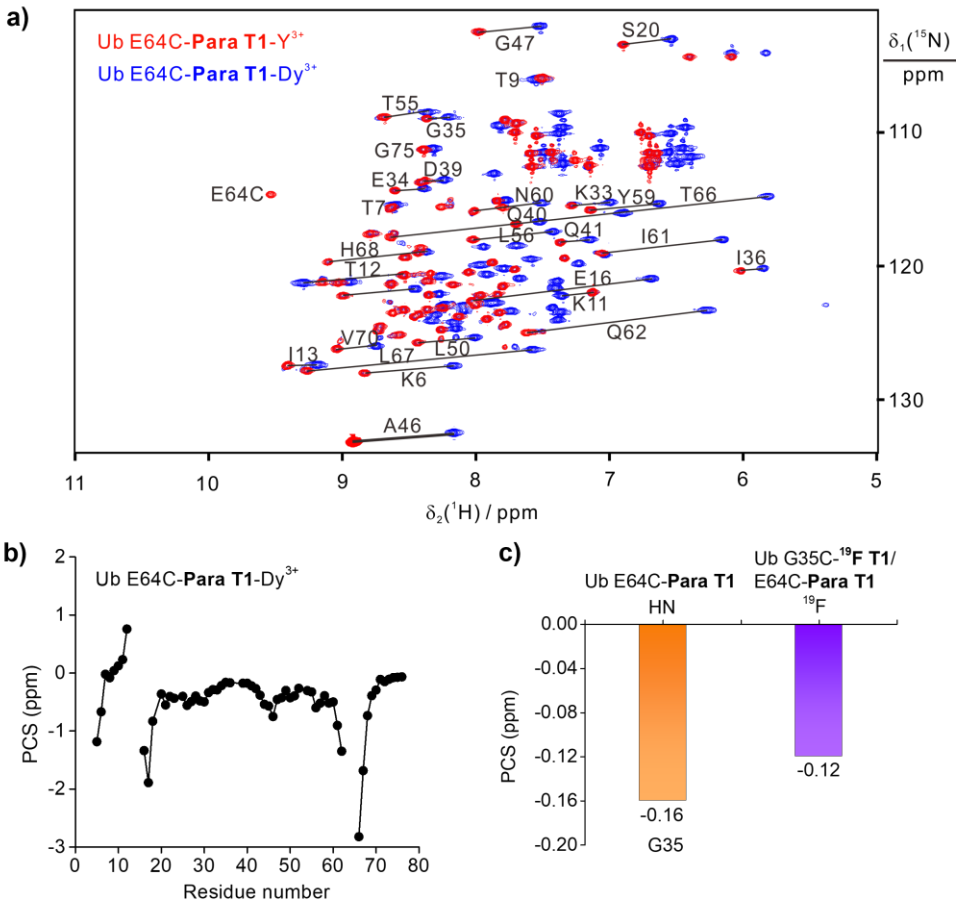


Figure S5. Comparison of PCS obtained by ^{15}N-HSQC and ^{19}F NMR spectra. a) Superimposition of ^{15}N-HSQC spectra of 0.1 mM Ub E64C-**Para-T1**-Y^{3+} (Red) and 0.1 mM Ub E64C-**Para-T1**-Dy^{3+} (Blue). The spectra were recorded at 298 K and pH 6.4 with a ^{1}H NMR frequency of 600 MHz in 20 mM MES buffer; b) PCSs of the backbone amide proton of G35 in Ub E64C-**Para T1**-Dy^{3+} complex (0.1 mM) in 20 mM MES buffer at pH 6.5; c) Comparison of PCS of the backbone amide proton of G35 in Ub E64C-**Para T1**-Dy^{3+} and ^{19}F-PCS of Ub G35C-**^{19}F T1**/E64C-**Para T1**-Dy^{3+} were determined by ^{15}N-HSQC and ^{19}F NMR, respectively.

7. Preparation of *E. coli* cell lysates.

The *E. coli*. BL21 (DE3) Condon Plus cells were grown in 300 mL of LB medium at 37 °C until an optical density (OD) at 600 nm increased to 1.0. Then, the cells were spun down at 6000 rpm for 8 min at 4 °C. The cell pellet was weighted about 1.0 g and the cells were resuspended in 4 mL of lysis buffer (20 mM MES buffer, pH 6.4) and lysed by sonication on ice. Insoluble materials were removed by centrifugation at 12,000 rpm for 10 min, the supernatant was used as crowding medium for NMR analysis.

8. In-Cell NMR experiments.

The HEK293T cells were seeded on 10 cm dishes and cultivated in Dulbecco's Modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin solution) at 37°C under 5% CO_2 humidified atmosphere. Cells were divided at 70-80 % confluence and passaged no more than six times before NMR experiments. For in-cell NMR samples, 28-32 × 10^6 cells were used as starting material for electroporation per experiment.

According to the previously published method[4], the target protein was delivered into HEK293T cells by electroporation. Ub G35C-**^{19}F T1**/E64C-**Para T1** (Y^{3+}/Dy^{3+}) was added to freshly prepared and sterile filtered electroporation buffer (EPB: 100 mM Na_2HPO_4, 5 mM KCl, 15 mM $MgCl_2$, 15 mM HEPES, 2 mM ATP (Thermo Fisher), 2 mM reduced glutathione (Sigma) at pH 7.0) to final concentrations of 2 mM. Cells were pelleted again and mixed with Ub G35C-**^{19}F T1**/E64C-**Para T1** (Y^{3+}/Dy^{3+}) in EPB at 70-80 × 10^6 cells per mL. 4 cuvettes, each containing 100 µL aliquots (8 × 10^6 cells) were then transferred to 2 mm cuvettes and electroporated on an Amaxa Nucleofector I (Lonza). For control experiments, cells were subjected to mock electroporation with EPB alone. Immediately after electroporation, 1 mL of pre-warmed DMEM was added to each cuvette and the cells were gently suspended by pipetting up and down three times before being transferred to 5 ml of prewarmed DMEM medium in two 10 cm dishes.

Cells were allowed to recover for 4 hours to restore normal morphology as assessed by light microscopy, dishes were washed three times with pre-warmed PBS and harvested the cells. Cells were washed twice with pre-warmed PBS before further analysis. About 12-14 × 10^6 electroporated cells were harvested and resuspended in 80-100 µL NMR buffer (DMEM supplemented with 90 mM glucose, 5 mM HEPES, and 10% D_2O), and the cell sample was transferred into a 3 mm NMR tube, followed by ^{19}F NMR measurements.

After the in-cell NMR experiment, the cells were removed from the NMR tube and the cell supernatant was collected by gentle centrifugation (1400 rpm for 3 minutes) for ^{19}F NMR measurement. The cell pellets were mixed with NMR buffer (DMEM supplemented with 90 mM glucose, 5 mM HEPES, and 10% D_2O) to a final volume of 180 µL and subjected to 15 cycles of freezing (liquid N_2) and thawing (room temperature). The cell lysate was centrifuged at 12000 × g for 10 mins to remove cell debris, and the supernatant was used as cell lysate for ^{19}F NMR measurement.

9. Trypan Blue Test.

Cell viability measurements before and after 2 h NMR measurements were performed by Trypan blue exclusion test. In-cell samples were prepared as described above, about 12-14 × 10^6 electroporated cells were harvested and resuspended in 80-100 µL NMR buffer (DMEM supplemented with 90 mM glucose, 5 mM HEPES, and 10% D_2O), and the cell sample was transferred into a 3 mm NMR tube, followed by ^{19}F NMR measurements. Before and after 2 h NMR measurements, 5 µL of the cell suspension was diluted 3-6 times with PBS, and an equal volume of 0.4% Trypan blue solution was added and incubated at room temperature for 3 min. Then, the cell viability was analyzed under a microscope.

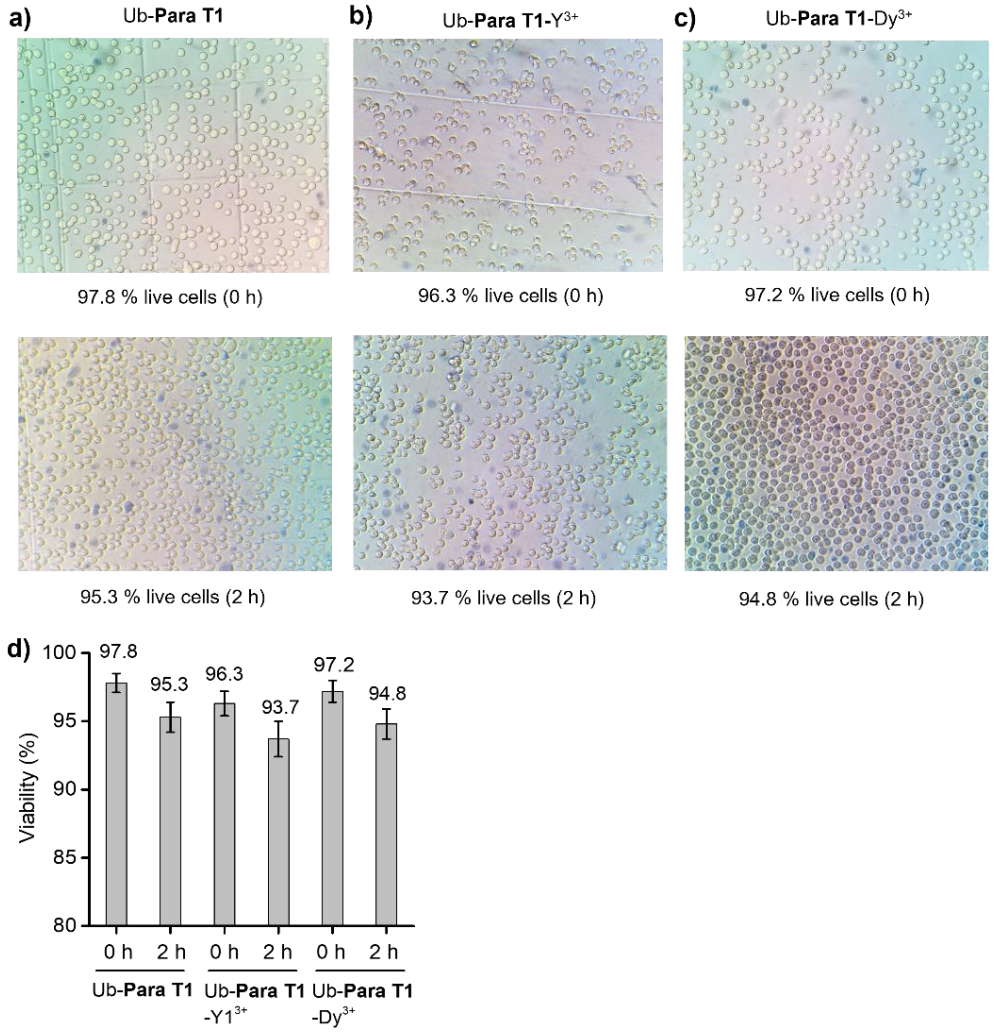


Figure S6. Cell viability before (0 h) and after NMR experiments (2 h) was assessed by trypan blue staining. a) Ub G35C-^{19}**F T1**/E64C-**Para T1**; b) Ub G35C-^{19}**F T1**/E64C-**Para T1**-Y^{3+}; c) Ub G35C-^{19}**F T1**/E64C-**Para T1**-Dy^{3+}; d) Bar graph presenting results from a viability assay using trypan blue staining.

10. References

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