Supplementary Information

Effective assessment of lanthanide ion delivery into live cells by paramagnetic NMR spectroscopy

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Materials and methods

The amino acid sequence of fusion protein LanM-GB1

The fusion protein is constructed by connecting lanmodulin (residues 29-133) (LanM)[1] and GB1 with a flexible *GGS* linker (red). The specific amino acid sequence is shown as following. The four lanthanide binding motifs in LanM are shown as underline.

MVDIAAFDPDKDGTIDLKEALAAGSAAFDKLDPDKDGTLDAKELKGRVSEADLKKLDPDNDGTLDKKEYLAAV EAQFKAANPDNDGTIDARELASPAGSALVNLIR***GGS***MQYKLILNGKTLKGETTTEAVDAATAEKVFKQYANDN GVDGEWTYDDATKTFTVTE

Protein expression

The plasmid of fusion protein LanM-GB1 was cloned into the PET28a vector and expressed in *E. coli* BL21-Condon plus using a high-density protocol.[2-3] The target protein LanM-GB1 was purified through a DEAE column and following a Superdex75 gel filtration. Finally, about 15 mg of purified uniformly ^{15}N-labeled LanM-GB1 was obtained per 250 mL medium.

In-cell sample preparation

The plasmid of fusion protein LanM-GB1 was cloned into the PET28a vector and expressed in *E. coli* cells. The cells were first incubated in the 50 mL LB medium at 310 K to OD_{600} of 0.8 - 1.0. Then the cells were collected and transformed into a 25 mL modified M9 medium, which contained glucose as the carbon source, $^{15}NH_4Cl$ as the nitrogen source, and other essential nutrients.[3] After induction by isopropyl-β-D-thiogalactoside (IPTG) at 310 K for 3 h, the cells were collected and washed twice using 10 mL 20 mM pH 7.2 HEPES buffer each time by 3000 rpm centrifugation at 298 K. For NMR titration, the collected cell pellets were resuspended by the NMR buffer to prepare the in-cell NMR sample with a cell concentration of 15 % (m/m, wet weight). For cell sample incubated with Ln^{3+}, the collected cell pellets were resuspended by 3 mL incubation buffer and further incubated for 3 h at 150 rpm shaking at 310 K. Then the cells were collected gently by centrifugation at 3000 rpm and 298 K, and washed twice by 20 mM pH 7.2 HEPES buffer. The resulting cells were resuspended by the NMR buffer to prepare the in-cell NMR sample with a cell concentration of 15 % (m/m).

After NMR measurement, the in-cell NMR sample (500 uL) was centrifuged gently using 3000 rpm for 5 min at 298 K. The supernatant was used to record ^{15}N-HSQC spectra to monitor protein leakage. The cell sediment was resuspended by 1 mL 20 mM HEPES buffer at pH 7.2 and then lysed

by sonication for 10 min in an ice bath. The resulting cell lysate was about 7% (m/m) and centrifuged for 5 min at 12000 rpm before NMR measurement.

M9 medium: 40 mM Na_2HPO_4, 10 mM KH_2PO_4, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, a trace metal mixture, 0.1 % glucose (m/m), 0.8 g/L $^{15}NH_4Cl$, 1.0 mM kanamycin.

NMR buffer: 0.1 % glucose (m/m), 0.8 g/L $^{14}NH_4Cl$, 1.0 mM kanamycin, 1.0 mM chloramphenicol, 10 % D_2O (v/v), 20 mM HEPES pH 7.2.

Incubation buffer: 0.1 % glucose (m/m), 0.8 g/L $^{14}NH_4Cl$, 0.1 mM $CaCl_2$, 1.0 mM $MgSO_4$, a trace metal mixture, 150 mM salt (NaCl: KCl = 6:1), 1.0 mM kanamycin, 1.0 mM chloramphenicol, 20 mM HEPES pH 7.2, and various concentration of Ln^{3+} (0.5 mM) or $LnCit_2^{3-}$ (0.5 mM, 1.0 mM, 2.0 mM).

NMR measurement

All NMR experiments were recorded in 20 mM HEPES buffer at pH 7.2 and 298 K on a Bruker Avance neo 600 MHz NMR spectrometer equipped with a QCI-cryoprobe. For Ln^{3+} titration of LanM-GB1 in vitro, 0.1 mM LanM-GB1 was prepared in 20 mM HEPES buffer at pH 7.2 containing 7 % D_2O (v/v) and titrated using a 10 mM Ln^{3+} nitrate (Ln = Y, Yb) stock solution. ^1H, ^{15}N-HSQC spectra were recorded through a standard pulse sequence using Watergate for water suppression using 180 × 2048 data matrices for in vitro measurements and 140 × 2048 data matrices for in-cell measurements. All the ^{15}N-HSQC experiments of the in-cell sample were recorded for about 42 min with 16 scans to assure the cell viability during measurement.

Calculation of chemical shift perturbation

The chemical shift perturbation $\Delta\delta$ was calculated following the equation

$$\Delta\delta = \sqrt{\Delta\delta_H{}^2 + (\Delta\delta_N/10)^2} \qquad (1)$$

where $\Delta\delta_H$ and $\Delta\delta_N$ are the chemical shift differences of backbone amides in the hydrogen and nitrogen dimension, respectively.

Determination of concentration of Yb^{3+} in *E. coli* cells

The concentration of LanM-GB1 in the lysate was determined by comparing the peak volume of resonance signals of the GB1 domain between in lysate and free LanM-GB1 in vitro. The concentration of LanM-GB1-Yb in the lysate was determined by comparing the peak volume of

resonance signals of the GB1 domain between free LanM-GB1 and paramagnetic LanM-GB1-Yb species in cell lysate. The concentration of LanM-GB1 and LanM-GB1-Yb in *E. coli* cells was achieved using their concentration in cell lysate dividing the population of the lysate (~7 %). The cell pellets after co-incubation with $YbCit_2^{3-}$ were digested using nitric acid and analyzed by ICP-MS (Agilent 7800).

CW band EPR measurement

All the CW band EPR spectra were recorded at 298 K using a Bruker ELEXSYS E580-10/12 EPR spectrometer equipped with a super-high sensitivity CW-EPR resonator. About 20 μL sample was filled into a capillary (inner diameter = 0.5 mm) for EPR measurements. The EPR spectra of LanM-GB1-Gd were recorded for the sample of 1.0 mM LanM-GB1, 4.5 mM Gd^{3+}, and 20 mM HEPES pH 7.2 buffer. The in-cell sample of LanM-GB1-Gd was prepared as the in-cell NMR sample of LanM-GB1-Yb except for using $GdCit_2^{3-}$ (1.0 mM) instead of $YbCit_2^{3-}$ in the buffer. For the blank control, the *E. coli* cell sample was prepared as the LanM-GB1-Gd in-cell sample except for without isopropyl-β-D-thiogalactoside (IPTG) induction. The *E. coli* cell sample without co-incubation with 1.0 mM $GdCit_2^{3-}$ was also prepared for control.

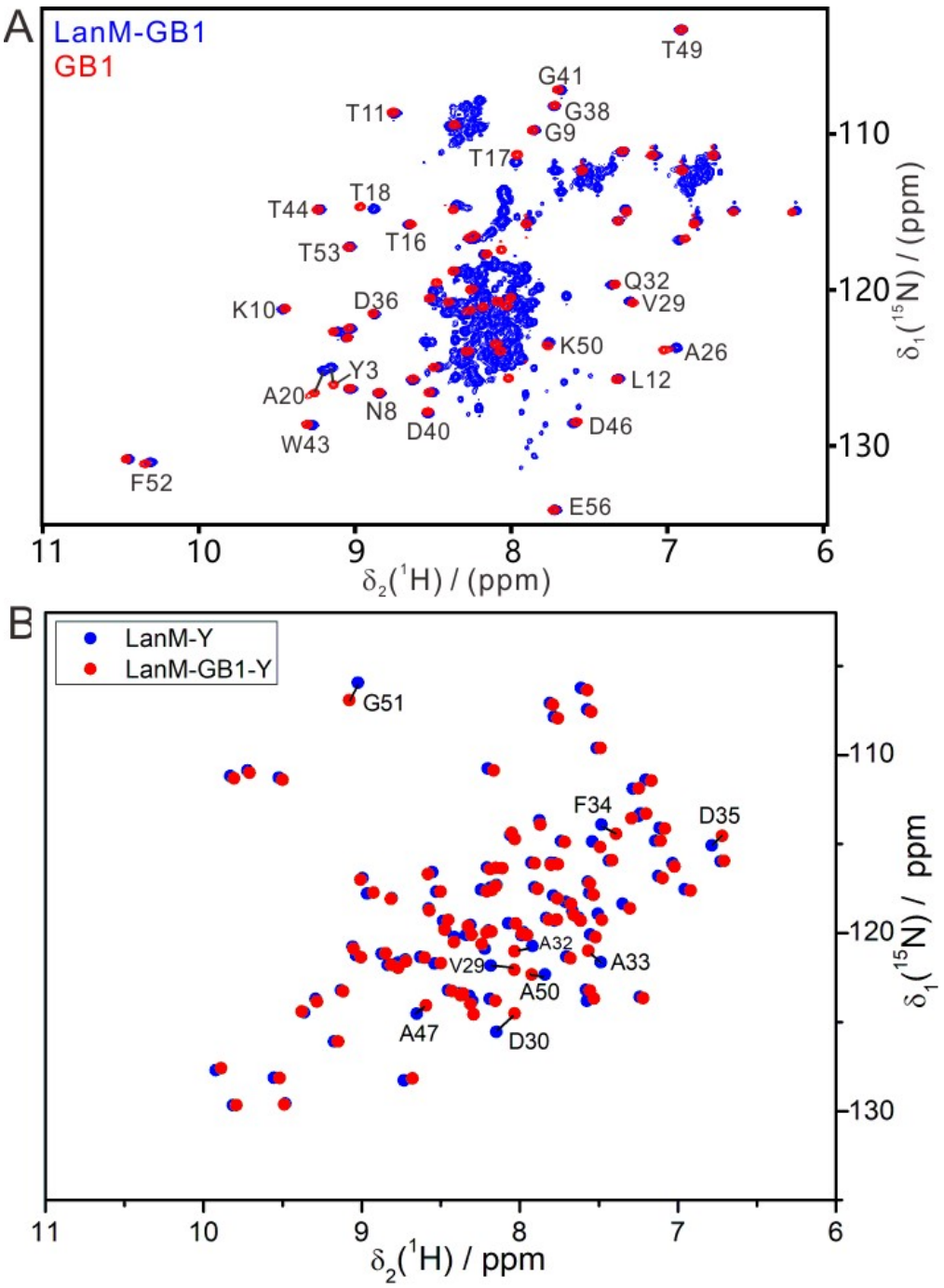

Fig S1. Negligible implications of GB1 to LanM and to interaction of LanM with Ln^{3+} ion in the fusion protein. A) ^{15}N-HSQC spectra overlay of 0.1 mM GB1 (red) and 0.1 mM LanM-GB1 (blue) in 20 mM HEPES buffer at pH 7.2 and 298 K. Assignment of resonance signals of GB1 was labeled. The narrow distribution of signals of LanM in the ^{1}H dimension showed the disordered state of LanM. **B**) The pseudo ^{15}N-HSQC spectra overlay of reported assignments of LanM-Y (blue, BMRB code: 30515)[4] and LanM-Y in the LanM-GB1-Y complex here.

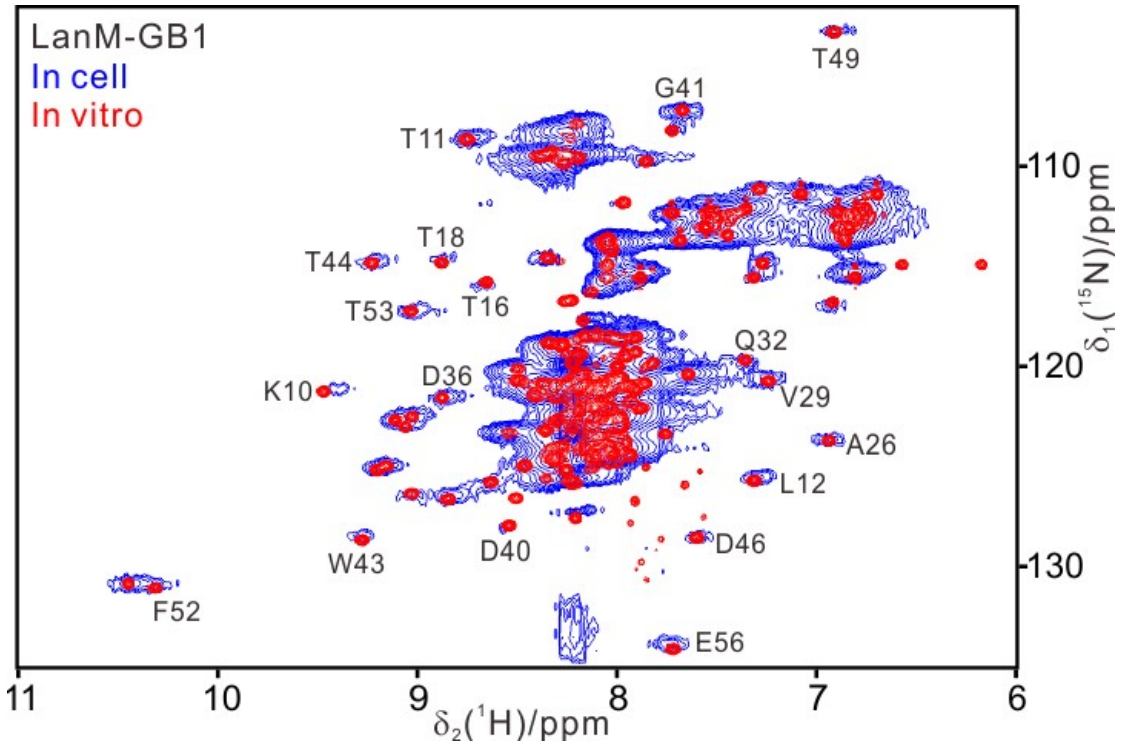

Fig S2. More broadened resonance signals of LanM-GB1 in live *E. coli* cells than that in vitro. ^{15}N-HSQC spectra overlay of LanM-GB1 in ~15 % (wet weight, m/m) *E. coli* cell solution (blue) and 0.1 mM LanM-GB1 in 20 mM HEPES buffer at pH 7.2 (red). Resonance signals of GB1 domain of LanM-GB1 are labeled.

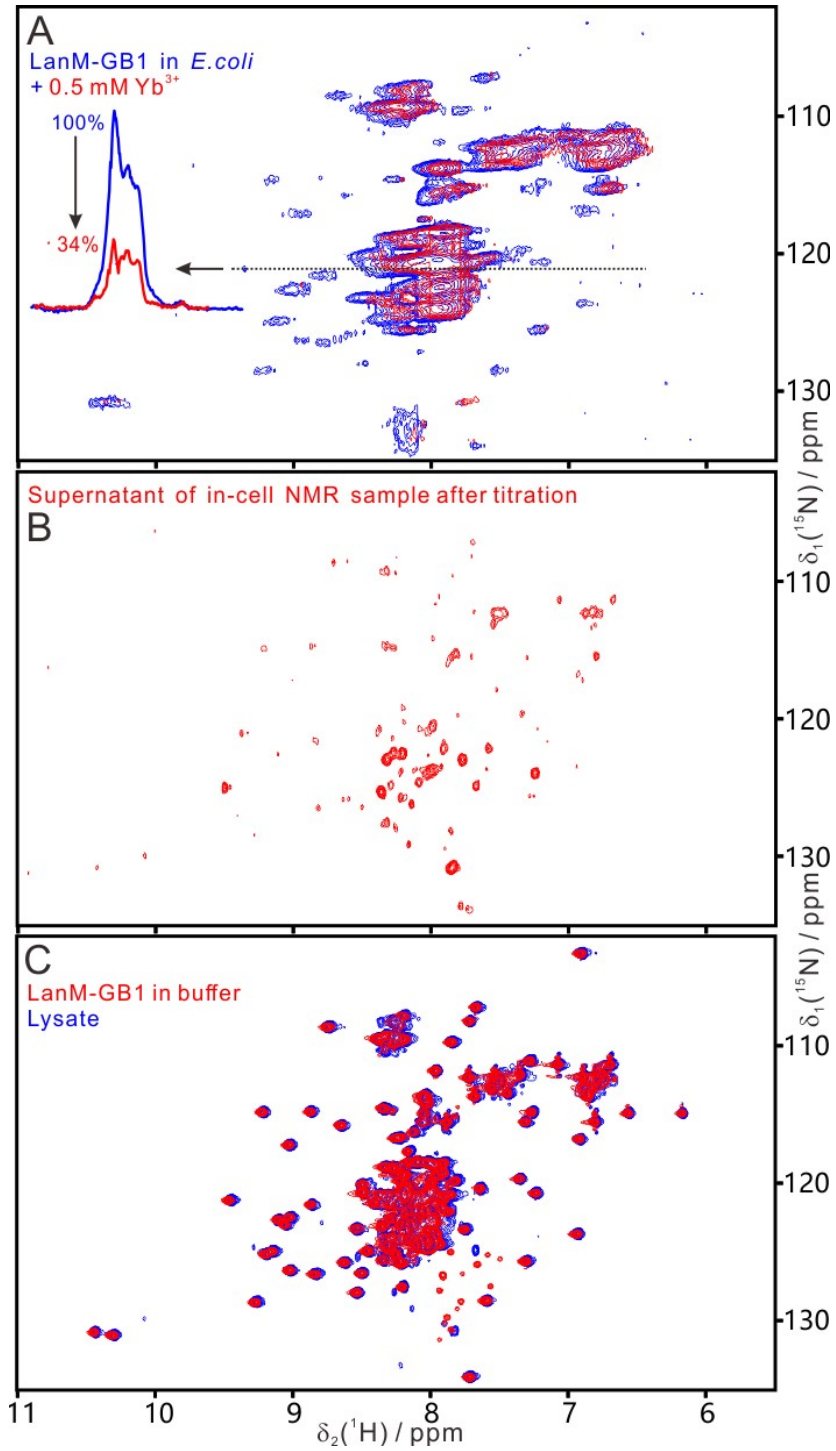

Fig S3. No significant delivery into cells by titrating Yb^{3+} into cell solution at 298 K. A, ^{15}N-HSQC spectra overlay of LanM-GB1 in live *E. coli* cells (blue) and that titrated with 0.5 mM Yb^{3+} (red) in 20 mM HEPES buffer at pH 7.2 and 298 K. The cross-section is shown to characterize the peak intensity decrease. **B**, ^{15}N-HSQC spectra of supernatant separated from the cells sample with addition 0.5 mM Yb^{3+} in (A). **C,** ^{15}N-HSQC spectra overlay of 0.1 mM LanM-GB1 in vitro (red) and lysate of in-cell sample (blue) in 20 mM HEPES buffer at pH 7.2 and 298 K.

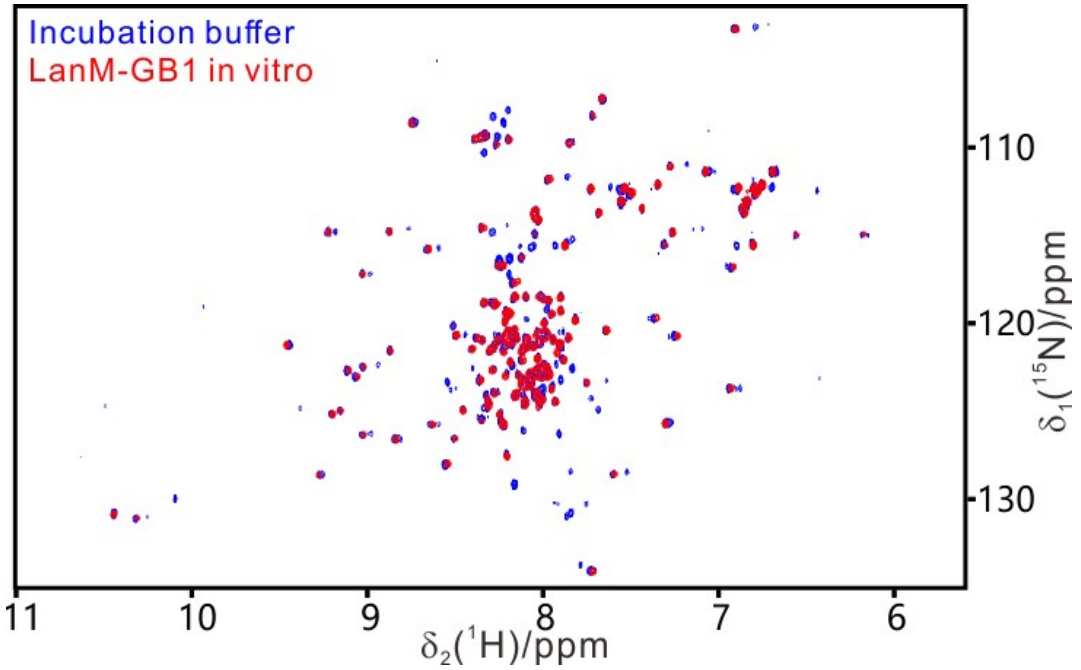

Fig S4. Significant protein leakage during co-incubation *E. coli* cells with 0.5 mM Yb^{3+} by 150 rpm shaking at 310 K. ^{15}N-HSQC spectra overlay of 0.1 mM LanM-GB1 in vitro (red) and incubation buffer of the mixture of *E. coli* cells (IPTG induction 3 h) with 0.5 mM Yb^{3+} (blue) after 3 h shaking at 310 K (detail in experimental section). Significant LanM-GB1-Yb species was also observed in the spectra of incubation buffer.

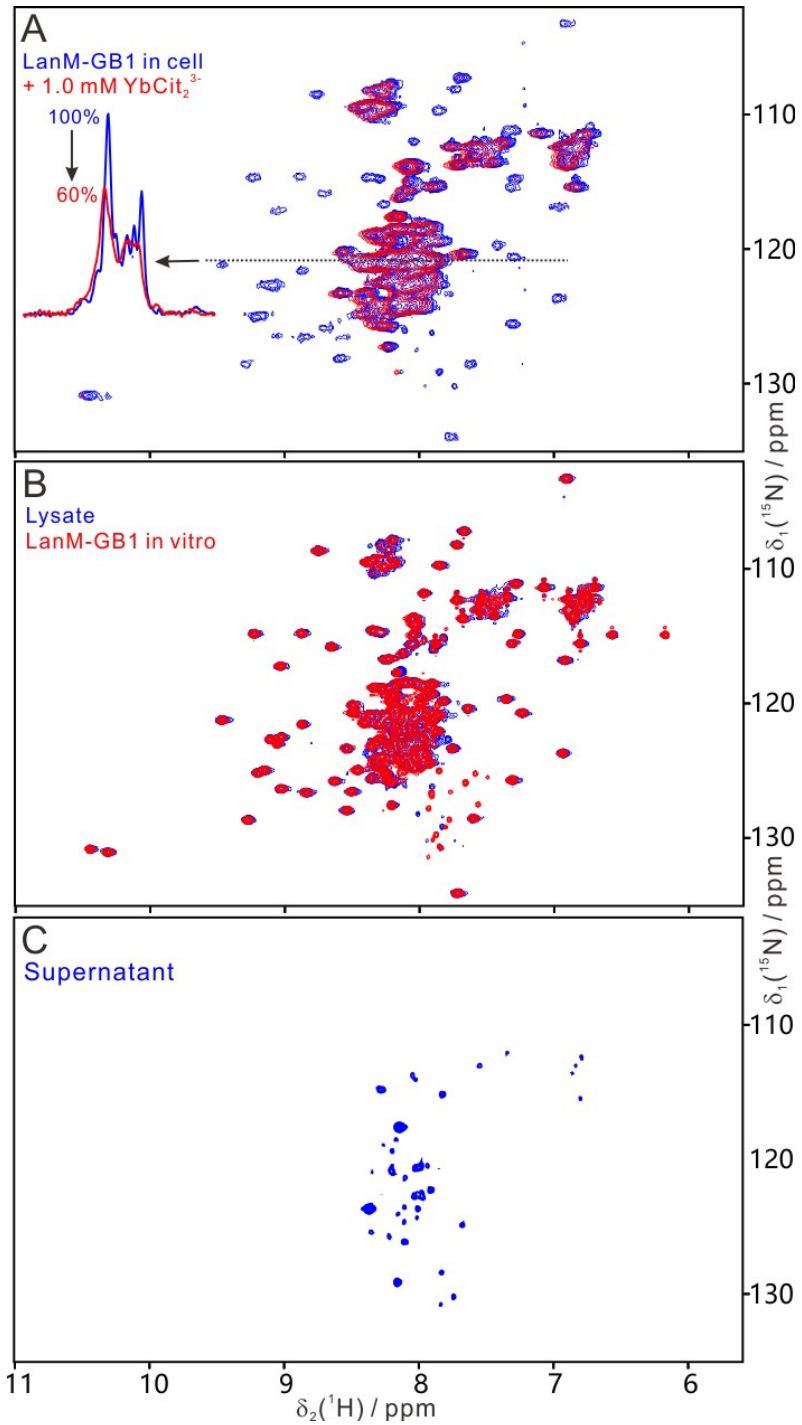

Fig S5. No significant delivery into cells and bound to LanM-GB1 by titrating $YbCit_2^{3-}$ in cell solution at 298 K. A, ^{15}N-HSQC spectra overlay of LanM-GB1 in live *E. coli* cells (blue) and that titrated with 1.0 mM $YbCit_2^{3-}$ (red) in 20 mM HEPES buffer at pH 7.2 and 298 K. The cross-section is shown to characterize the peak intensity decrease. **B**, ^{15}N-HSQC spectra overlay of 0.1 mM LanM-GB1 in vitro (red) and lysate of in-cell sample (blue) in 20 mM HEPES buffer at pH 7.2 and 298 K. **C**, ^{15}N-HSQC spectra of supernatant of in-cell sample after titration of 1.0 mM $YbCit_2^{3-}$. No significant signals of LanM-GB1 were observed.

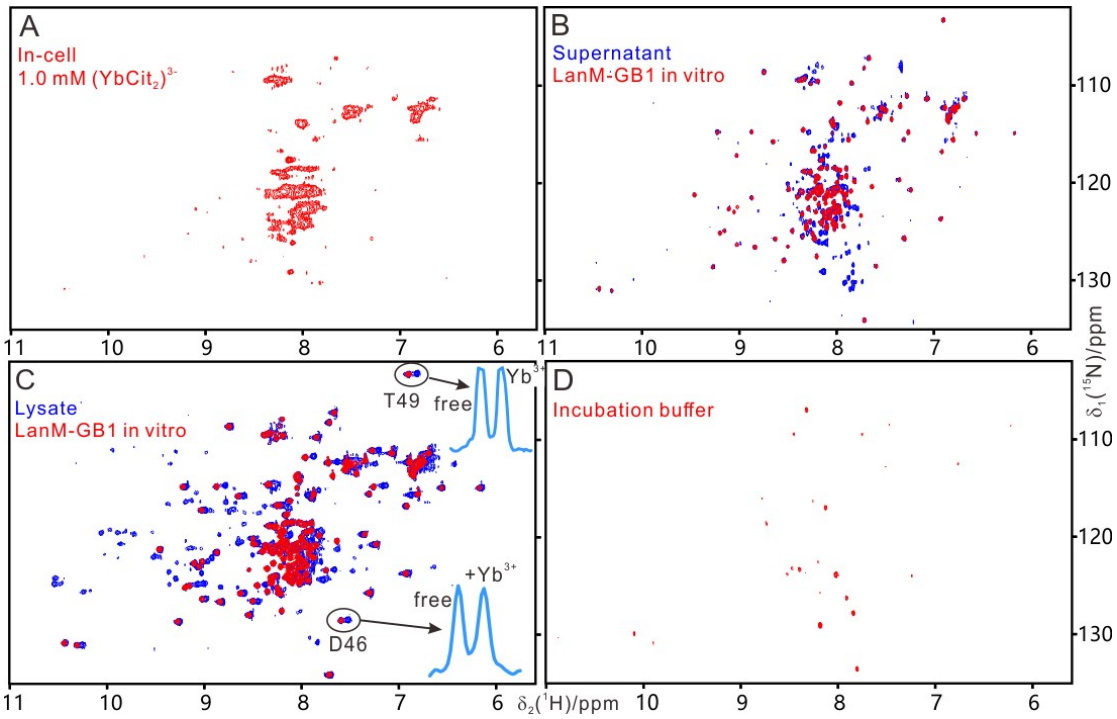

Fig S6. Striking Yb^{3+} delivery into cells by shaking incubation *E. coli* cells with 1.0 mM $YbCit_2^{3-}$ at 310 K. A, ^{15}N-HSQC spectra of the cell solution after shaking culture with 1.0 mM $YbCit_2^{3-}$ for 3 h in 20 mM HEPES buffer at pH 7.2 and 310 K. Only some intense signals of LanM domain were observed. **B**, ^{15}N-HSQC spectra overlay of LanM-GB1 in vitro (red) and supernatant (blue) of in-cell sample showed negligible protein leakage. The intense signals are not from the fusion protein LanM-GB1. **C,** ^{15}N-HSQC spectra overlay of 0.1 mM LanM-GB1 in vitro (red) and lysate of in-cell sample (blue) in 20 mM HEPES at pH 7.2. **D,** ^{15}N-HSQC spectra of the incubation buffer of in cell sample showed no significant protein leakage.

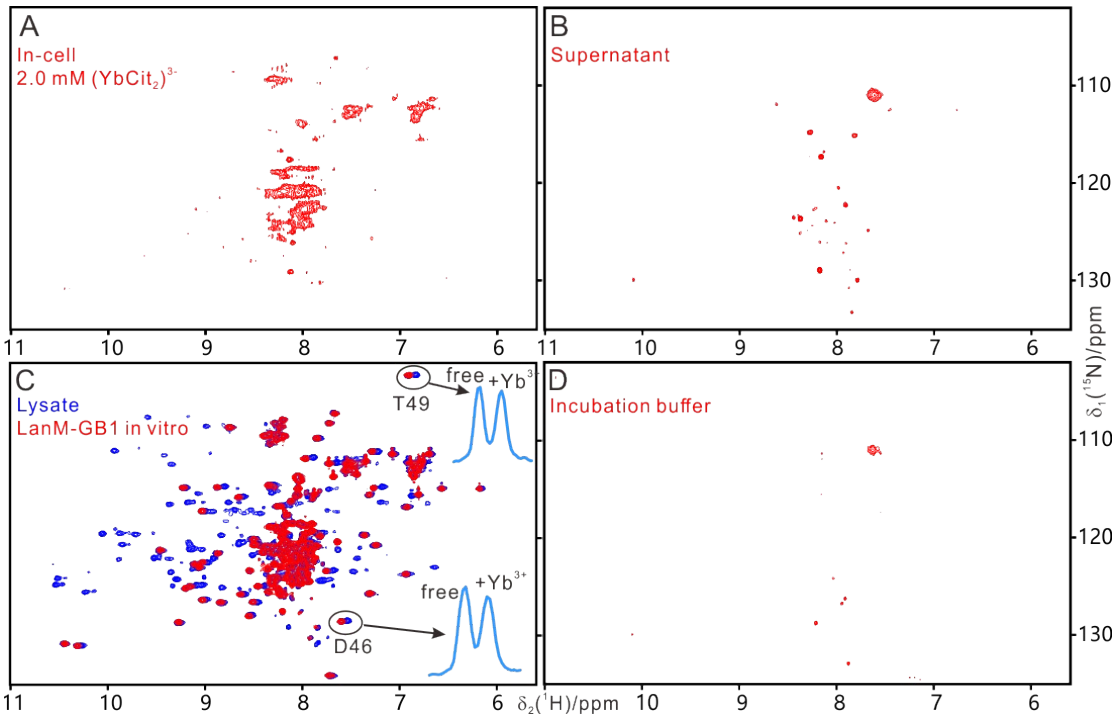

Fig S7. Yb^{3+} delivery into cells by shaking incubation *E. coli* cells with 2.0 mM $YbCit_2^{3-}$ at 310 K. A, ^{15}N-HSQC spectra of the cell solution after shaking culture with 2.0 mM $YbCit_2^{3-}$ for 3 h in 20 mM HEPES buffer at pH 7.2 and 310 K. Only some intense signals of LanM domain were observed. **B**, ^{15}N-HSQC spectra of supernatant of the in-cell sample showed no significant protein leakage. **C,** ^{15}N-HSQC spectra overlay of 0.1 mM LanM-GB1 in vitro (red) and lysate of in-cell sample (blue) in 20 mM HEPES at pH 7.2. **D,** ^{15}N-HSQC spectra of the incubation buffer of in-cell sample showed no significant protein leakage.

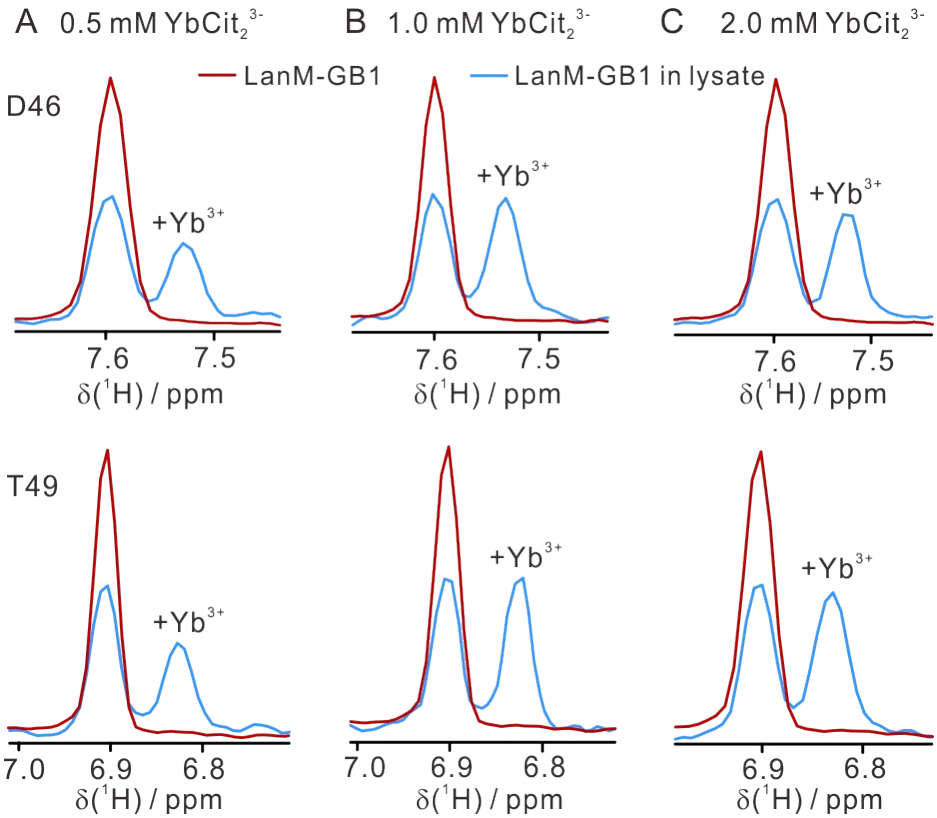

Fig S8. Concentration determination of LanM-GB1 and LanM-GB1-Yb in lysates of in cell samples. Cross-sections of signals of residues D46 and T49 of GB1 in ^{15}N-HSQC spectra were shown for 0.1 mM LanM-GB1 *in vitro* (red) and lysates of in cell samples (blue) incubated with 0.5 mM $YbCit_2^{3-}$ (**A**), 1.0 mM $YbCit_2^{3-}$ (**B**), 2.0 mM $YbCit_2^{3-}$ (**C**), respectively. The concentration of LanM-GB1 and LanM-GB1 in lysates of in cell samples were determined by the peak volume ratio of signals in the ^{15}N-HSQC spectra relative to that *in vitro*.

References

1. J. A. Cotruvo Jr., E. R. Featherston, J. A. Mattocks, J. V. Ho, T. N. Laremore, Lanmodulin: A Highly Selective Lanthanide-Binding Protein from a Lanthanide-Utilizing Bacterium. *J. Am. Chem. Soc.* **2018**, *140*, 15056-15061.
2. J. Marley, M. Lu, C. Bracken, A method for efficient isotopic labeling of recombinant proteins. *J. Biomol. NMR* **2001**, *20*, 71–75.
3. C. Cao, J. L. Chen, Y. Yang, F. Huang, G. Otting, X. C. Su, Selective (15)N-labeling of the side-chain amide groups of asparagine and glutamine for applications in paramagnetic NMR

spectroscopy. *J. Biomol. NMR* **2014**, *59*, 251-261.

4. E. C. Cook, E. R. Featherston, S. A. Showalter, J. A. Cotruvo Jr. Structural Basis for Rare Earth Element Recognition by Methylobacterium extorquens Lanmodulin. *Biochemistry* **2019**, *58*, 120-125.