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## In-Cell Protein Structures from 2D NMR Experiments

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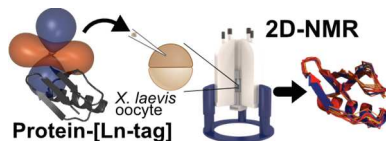
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3 1 ABSTRACT.  
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7 2 In-cell NMR spectroscopy provides atomic resolution insights into the structural properties of  
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10 3 proteins in cells, but it is rarely used to solve entire protein structures *de novo*. Here, we  
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12 4 introduce a paramagnetic lanthanide-tag to simultaneously measure protein pseudocontact shifts  
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14 5 (PCSs) and residual dipolar couplings (RDCs) to be used as input for structure calculation  
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16 6 routines within the Rosetta program. We employ this approach to determine the structure of the  
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18 7 protein G B1 domain (GB1) in intact *Xenopus laevis* oocytes from a single set of 2D in-cell  
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20 8 NMR experiments. Specifically, we derive well-defined GB1 ensembles from low concentration  
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22 9 in-cell NMR samples ( $\sim 50 \mu\text{M}$ ) measured at moderate magnetic field strengths (600 MHz), thus  
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26 10 offering an easily accessible alternative for determining intracellular protein structures.  
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32 12 TOC GRAPHICS  
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45 15 **KEYWORDS** Cellular structural biology, in-cell NMR, protein structure determination, NMR  
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47 16 spectroscopy, pseudocontact shifts, residual dipolar couplings.  
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3 1 Physical methods to delineate structural insights into the three-dimensional properties of  
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5 2 biomolecules such as X-ray crystallography, NMR spectroscopy or electron microscopy,  
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8 3 typically require experimental conditions and sample states that are vastly different from the  
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10 4 crowded intracellular environments in which these molecules natively occur<sup>1</sup>. For these reasons,  
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12 5 considerable effort is put into the development of biophysical methods to directly study  
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14 6 biomolecules inside live cells. While high-resolution X-ray crystallography and single molecule  
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16 7 electron microscopy are inherently excluded from such *in vivo* experiments, due to the  
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18 8 requirement of crystalline or vitrified samples and the use of high-energy X-ray or electron  
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20 9 beams to generate experimental data, solution NMR spectroscopy can provide non-destructive  
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22 10 atomic-resolution information on individual biomolecules in cells. Specifically, in-cell NMR  
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24 11 spectroscopy<sup>2,3</sup> takes advantage of the isotope-labeling effect to selectively ‘visualize’ isotope-  
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26 12 enriched, NMR-active proteins, RNA or DNA against the backdrop of all other non isotope-  
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28 13 labeled and NMR-inactive intracellular components. This enables direct NMR measurements  
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30 14 under truly physiological *in vivo* conditions. Following this rationale, in-cell NMR has been used  
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32 15 to derive insights into intracellular protein conformations,<sup>4</sup> conformational equilibria,<sup>5</sup> folding  
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34 16 and stability behaviors,<sup>6-8</sup> protein dynamics,<sup>9</sup> protein-protein and quinary protein interactions,<sup>9,10</sup>  
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36 17 physiological redox states,<sup>11</sup> metal-binding properties<sup>12</sup> and post-translational protein  
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38 18 modifications.<sup>9,13,14</sup> By contrast, the use of in-cell NMR to determine entire protein structures in  
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40 19 live cells is generally hampered by the limited lifetimes of in-cell NMR samples, their inherently  
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42 20 low concentrations of intracellular, isotope-enriched biomolecules (i.e. protein, RNA or DNA)  
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44 21 and their concomitantly poor spectral qualities. Especially lengthy 3D and 4D NMR experiments  
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46 22 - commonly used to derive long-range distance restraints for calculating biomolecular structures  
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48 23 - suffer from these drawbacks.<sup>15</sup> Several advances in NMR methods, including faster acquisition  
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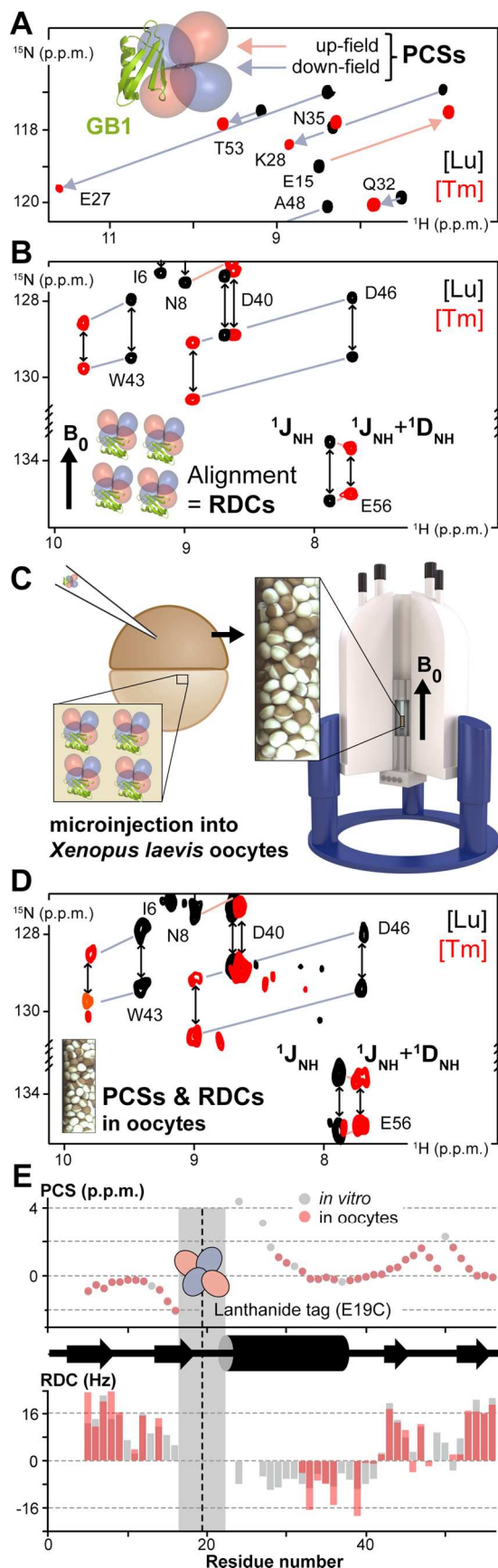
1 routines and non-uniform sampling procedures<sup>16-18</sup> have helped to ameliorate some of these  
2 shortcomings and enabled the first and only intracellular protein structure to be determined by in-  
3 cell NMR spectroscopy in bacteria, although at exceedingly high, non-physiological intracellular  
4 protein concentrations in the millimolar range<sup>4</sup>. As a result, and given the general poor sensitivity  
5 of 3D and 4D NMR experiments even with such enhancing techniques, comprehensive structure  
6 determination efforts of proteins in live cells are deemed impractical and unfeasible. Here, we  
7 present an alternative approach to determine intracellular protein structures in live eukaryotic  
8 cells that solely relies on 2D NMR experiments and paramagnetic protein tagging to  
9 simultaneously induce pseudocontact shifts (PCSs) and residual dipolar couplings (RDCs). In  
10 turn, we demonstrate how these structural parameters suffice to calculate high-precision in-cell  
11 protein structures with the Rosetta program.

12 Tagging of proteins with different metals of the lanthanide series is known to induce strong  
13 metal-specific distance- and orientation-dependent PCS effects on individual NMR-active atomic  
14 nuclei.<sup>19,20</sup> Such PCSs serve as powerful long-range distance restraints in structure calculation  
15 routines and they can be derived from simple 2D NMR experiments, with different types of  
16 lanthanide-binding protein tags (Fig. 1a).<sup>21-24</sup> Optimizing the rigidity and linker-lengths of  
17 individual tag structures also enables partial alignments of coupled proteins with respect to the  
18 external magnetic field, thus giving rise to measurable RDCs and, thereby, additional  
19 orientational restraints (Fig. 1b).<sup>25-27</sup> In a first step, we designed a modified version of the  
20 classical tetraaza-carboxylic DOTA chelator, known for its excellent metal coordinating  
21 properties, which we termed DOTA-M7Py (Fig. S1). This tag can be covalently coupled to the  
22 sulfhydryl moiety of cysteine residues forming a non-reducible thioether bond.<sup>28-30</sup> With regard  
23 to in-cell PCS and RDC measurements, DOTA-M7Py displays several attractive features. First,

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3 1 it is inherently rigid and adopts exclusively the square anti-prismatic  $\Lambda(\delta\delta\delta\delta)$  stereo-confi-  
4 guration for the *4S,3R*-Lu derivative.<sup>31</sup> Second, it is neutral after binding to lanthanide metals.  
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8 3 Third, its linker portion is short, which reduces tag mobility and generates larger PCS effects,  
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10 4 thus providing higher precision structural information. Fourth, it features both hydrophilic and  
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12 5 hydrophobic properties (Fig. S1), which augment stable positioning on most protein surfaces,  
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14 6 further enhancing PCSs. Fifth, its thioether bond is expected to withstand the reducing  
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16 7 environment of the cytoplasm while maintaining DOTA's outstanding affinity towards  
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18 8 lanthanide metals ( $K_d < 10^{-25}$  M).<sup>27</sup>  
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22 9 We initially prepared diamagnetic DOTA-M7Py[Lu], and paramagnetic DOTA-M7Py[Tm]  
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24 10 and DOTA-M7Py[Tb] complexes, which we coupled to the Streptococcal protein G B1 domain  
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26 11 (GB1) via cysteine residues that we introduced by site-directed mutagenesis at individual GB1  
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28 12 positions, i.e., E19C, K28C and E42C. Using purified GB1 samples we recorded 2D  $^1\text{H}$ - $^{15}\text{N}$   
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30 13 HSQC spectra at 600 MHz, which revealed the expected PCS effects for the paramagnetic  
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32 14 species (up to 6 p.p.m., Fig. 1a; Fig. S2 & S3; Table S1). We also detected strong cross-peak  
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34 15 splitting in 2D  $^1\text{H}$ - $^{15}\text{N}$  IPAP-HSQC spectra due to paramagnetic alignment of the GB1 domain  
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36 16 and resulting RDC effects<sup>32</sup> (amplitudes reaching 25 Hz at 293 K, 600 MHz, Fig. 1b; Fig. S2 &  
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38 17 S4; Table S2). In agreement with the temperature dependency of the tag's mobility, we obtained  
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40 18 30 % higher or lower PCS and RDC values at 277 K and 310 K, respectively (Table S1 & S2).  
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42 19 Moreover, NMR spectra of the different DOTA-M7Py[Tm]-tagged GB1 samples (E19C, K28C  
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44 20 and E42C) revealed both positive and negative PCSs, as well as larger overall RDCs,<sup>19,20</sup> which  
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46 21 is particularly useful for structure calculation routines. Therefore, we resorted to using DOTA-  
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48 22 M7Py[Tm]-GB1 samples in all further experiments.  
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1 Next, we microinjected tagged GB1 carrying either diamagnetic (Lu) or paramagnetic metals  
2 (Tm) into *Xenopus laevis* oocytes for in-cell NMR measurements.<sup>3,33,34</sup> We recorded 2D <sup>1</sup>H-<sup>15</sup>N  
3 HSQC spectra at effective NMR concentrations of ~25 μM (intracellular GB1 concentrations  
4 ~50 μM), which revealed PCSs that were virtually indistinguishable from the respective *in vitro*  
5 samples with an overall RMSD of 0.04 p.p.m., corresponding to 24 and 2.4 Hz in the <sup>1</sup>H and <sup>15</sup>N  
6 dimensions, respectively (Fig. 1e; Fig. S2, S5 & S6). We did not detect sample degradation or  
7 metal leakage for up to 24 hours, thus indicating the excellent stability of metal-loaded DOTA-  
8 M7Py in *Xenopus* oocytes. Similarly, we measured in-cell RDCs that were comparable to those  
9 obtained *in vitro* (Fig. 1d-1e, Fig. S7, Table S1 & S2). Because intracellular viscosity leads to  
10 faster T2 relaxation and, accordingly, enhanced <sup>15</sup>N signal decays, we chose to record in-cell  
11 RDC experiments with the <sup>15</sup>N free-induction decay (FID) set to 36 Hz, as opposed to 17 Hz for  
12 RDC measurements *in vitro* (cf Material and Methods). This resulted in average GB1 <sup>15</sup>N line  
13 widths of ~30 Hz, compared to ~12 Hz *in vitro*, which, concomitantly, increased the RMSD of  
14 RDCs measured *in vitro* versus in cells by 5 Hz, explaining also the larger differences of PCS  
15 and RDC RMSDs.



**Figure 1.** (A) Superposition of  $^1\text{H}$ - $^{15}\text{N}$  2D NMR spectra of purified GB1(E19C) coupled to DOTA-M7Py carrying diamagnetic Lutetium (Lu, black) or paramagnetic Thulium (Tm, red). Pseudocontact shift (PCS)-induced up- and down-field chemical shift changes are indicated (subset view). The inset depicts the GB1(E19C) ribbon structure (green) with paramagnetic iso-surfaces drawn at 2 p.p.m. (blue & red) (B) Superposition of 2D IPAP-HSQC spectra of GB1 with peak splitting due to amide scalar- ( $^1J_{\text{NH}}$ ) and residual dipolar-coupling (RDC, i.e.  $^1D_{\text{NH}}$ ). Paramagnetic GB1 alignment with respect to the external magnetic field ( $B_0$ ) is shown schematically. (C) Overview of GB1 sample preparation in *Xenopus laevis* oocytes and (D) superposition of GB1 NMR spectra displaying in-cell PCS and RDC effects (at 600 MHz). (E) Residue-resolved quantification of *in vitro* & in-cell PCS and RDC data at 293 K and 600 MHz.



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3 1 Finally, we used in-cell PCS and RDC data as input for GPS-Rosetta, a program that integrates  
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5 2 PCSs from multiple paramagnetic centers into unified distance constraints in structure  
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7 3 calculation routines.<sup>23</sup> Following the fragment-based rationale used by Rosetta, we generated  
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9 4 input libraries of 3- and 9-residue fragments of known protein structures, excluding the structure  
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11 5 of GB1 and homologous folds. Using these fragments, we generated 10,000 GB1 structures, out  
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13 6 of which we collected the 100 lowest-energy models and compared their conformations to  
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15 7 experimentally determined GB1 structures, i.e. X-ray crystallography (PDB code: 2QMT<sup>35</sup>) and  
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17 8 solution NMR (PDB code: 1GB1<sup>36</sup>, 2PLP<sup>37</sup>). We found poor convergence of individual models,  
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19 9 with a median backbone C $\alpha$  RMSD of 1.85 Å (Fig. 2a). Next, we added a PCS-based ‘weight-  
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21 10 ing’ function to steer GB1 models towards conformations that recapitulated the measured values.  
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23 11 We used 72, 86 and 96 PCS constraints from the E19C, K28C and E42C GB1 mutants,  
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25 12 respectively, and obtained a substantially improved convergence of GB1 structures. The newly  
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27 13 determined average C $\alpha$  RMSD of the 100 lowest-energy models was 0.98 Å, and 0.64 Å  
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29 14 between the closest model and the crystal structure (Fig. 2b). Lastly, we used the RDC module of  
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31 15 Rosetta to include measured RDCs as additional input in our structure calculation routines,  
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33 16 which yielded a similar improved convergence of GB1 models (average C $\alpha$  RMSD of 1.04 Å for  
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35 17 the 100 lowest-energy structures, 0.64 Å for the closest model and the X-ray structure) (Fig. 2c).

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37 18 Upon closer inspection of the 10 lowest energy structures, we noticed a remarkable difference  
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39 19 between structures obtained with PCS data alone and the ones for which PCS and RDC values  
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41 20 were used. In both ensembles, loop L1 (residues N8 to E15) connecting strands  $\beta$ 1 and  $\beta$ 2 of  
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43 21 GB1 displayed two distinct conformations. One identical to the X-ray structure with an average  
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45 22 C $\alpha$  RMSD of 1.1 Å, and one with a larger C $\alpha$ -deviation and average RMSD of 1.6 Å. In PCS  
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47 23 models, only three out of ten structures adopted the X-ray L1 conformation. In PCS+RDC  
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1 models, five out of ten structures did. Previous solution NMR data indicated that L1 is highly  
 2 flexible with backbone order parameters ( $S^2$ ) in the range of 0.5-0.6 ( $S^2$  of GB1 regions with  
 3 secondary structure  $\sim 0.8$ ).<sup>37-40</sup> These *in vitro* solution conformations of L1 are similar to those  
 4 observed in GB1 crystals with an L1 C $\alpha$  RMSD of 1.35 Å (Fig. 2D). From this we concluded  
 5 that combined PCS and RDC data from single 2D in-cell NMR experiments are sufficient to  
 6 determine well-defined protein structures within PCS Rosetta. Our results further confirmed that  
 7 the overall structural features of GB1 in *Xenopus* oocytes are similar to those observed *in vitro*.<sup>34</sup>  
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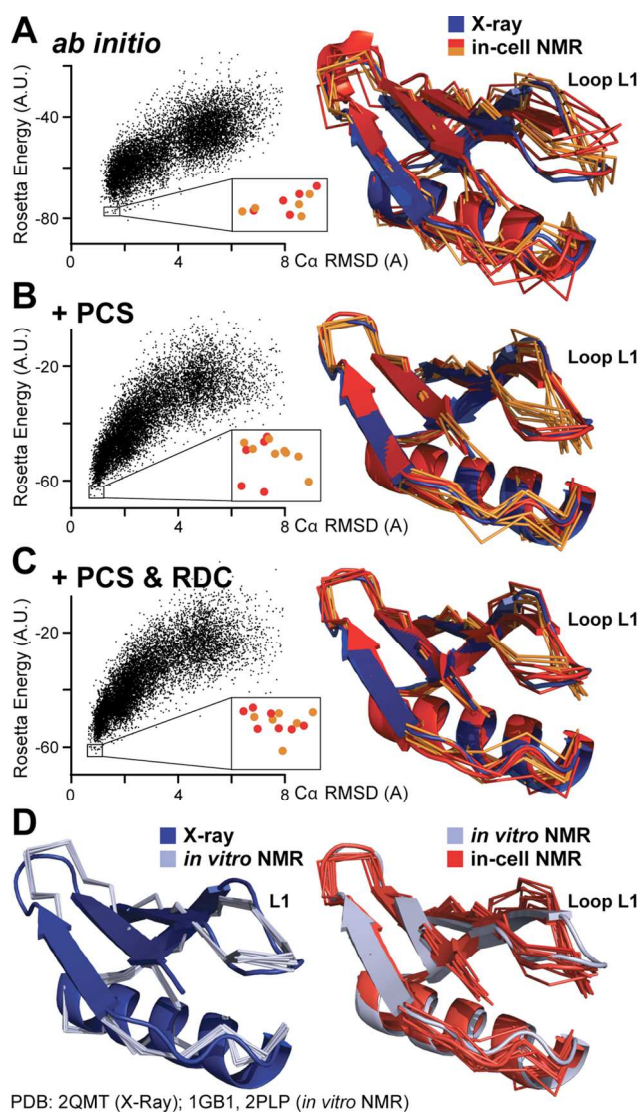


Figure 2. *In vitro* and in-cell structures of GB1. Scatter plots depict Rosetta energy scores and C $\alpha$  RMSDs of 10,000 GB1 models compared to the GB1 X-ray structure (2QMT). 10 lowest-energy structures are magnified and color-coded according their loop L1 conformations (red/orange). A superposition of their structures with the crystal conformation (blue) is shown on the right. GB1 models with L1 conformations corresponding to the one of the GB1 crystal are shown in red, deviating L1 conformers are colored orange. (A) *Ab initio* GB1 models without using experimental restraints, (B) GB1 models calculated with PCS and (C)

1 PCS+RDC input data. Rosetta energies contain different energy components and are not  
2 comparable. (D) Left: Superposition of high-resolution *in vitro* solution NMR structures of  
3 isolated GB1, i.e. 2PLP<sup>37</sup> (dark blue, ribbon representation) and 1GB1<sup>36</sup> (light blue, ensemble  
4 representation). Right: Superposition of 2PLP (blue) and 10 lowest-energy in-cell GB1 models  
5 (PCS+RDC, red).

6 In summary, we show that in-cell NMR-derived PCS and RDC data suffice to solve a protein's  
7 structure inside cells. Whereas PCS effects decrease with the distance to the coordinated metal,  
8 RDCs are distance-independent and offer valuable structural information for residues distal to  
9 the paramagnetic center. PCS and RDC data can jointly be obtained from single 2D NMR  
10 experiments on in-cell NMR samples of low intracellular protein concentrations, measured at  
11 moderate magnetic field strengths, which makes them easily accessible and highly useful. The  
12 presented approach can further be used for determining glycan and nucleic acid structures,<sup>2,41</sup> as  
13 well as to probe ligand interactions.<sup>42</sup> In addition, the high rigidity of the DOTA-M7Py tag  
14 renders it a useful tool for in-cell EPR studies.<sup>43,44</sup> Given that the intracellular delivery of  
15 paramagnetically tagged proteins into cultured mammalian cells by electroporation is  
16 straightforward,<sup>9</sup> combined PCS and RDC measurements in live cells also hold great promise for  
17 future structure determination efforts in intact mammalian specimens.

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### 19 **Supporting Information.**

20 The Supporting Information is available free of charge on the ACS Publications website. It  
21 contains Material and Methods, Supporting Figures and Tables, in pdf format.

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### 7 **Notes**

8 Any additional relevant notes should be placed here.  
9 The authors declare no competing financial interests.

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### 17 **References**

18 (1) Theillet, F.-X.; Binolfi, A.; Frembgen-Kesner, T.; Hingorani, K.; Sarkar, M.; Kyne, C.;  
19 Li, C.; Crowley, P. B.; Gierasch, L.; Pielak, G. J.; *et al.* Physicochemical Properties of Cells and  
20 Their Effects on Intrinsically Disordered Proteins (IDPs). *Chem. Rev.* **2014**, *114*, 6661–6714.

- 1  
2  
3 1 (2) Hänsel, R.; Luh, L. M.; Corbeski, I.; Trantirek, L.; Dötsch, V. In-Cell NMR and EPR  
4  
5  
6 2 Spectroscopy of Biomacromolecules. *Angew. Chem. Int. Ed.* **2014**, *53*, 10300–10314.  
7  
8  
9 3 (3) Freedberg, D. I.; Selenko, P. Live Cell NMR. *Annu. Rev. Biophys.* **2014**, *43*, 171–192.  
10  
11  
12 4 (4) Sakakibara, D.; Sasaki, A.; Ikeya, T.; Hamatsu, J.; Hanashima, T.; Mishima, M.;  
13  
14 Yoshimasu, M.; Hayashi, N.; Mikawa, T.; Wälchli, M.; et al. Protein Structure Determination in  
15  
16 5 Living Cells by in-Cell NMR Spectroscopy. *Nature* **2009**, *458*, 102–105.  
17  
18  
19  
20 7 (5) Ye, Y.; Liu, X.; Xu, G.; Liu, M.; Li, C. Direct Observation of Ca<sup>2+</sup>-Induced Calmodulin  
21  
22 8 Conformational Transitions in Intact *Xenopus Laevis* Oocytes by <sup>19</sup>F NMR Spectroscopy.  
23  
24 9 *Angew. Chem. Int. Ed.* **2015**, *127*, 5418–5420.  
25  
26  
27  
28 10 (6) Monteith, W. B.; Cohen, R. D.; Smith, A. E.; Guzman-Cisneros, E.; Pielak, G. J. Quinary  
29  
30 11 Structure Modulates Protein Stability in Cells. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 1739–  
31  
32 12 1742.  
33  
34  
35  
36 13 (7) Danielsson, J.; Mu, X.; Lang, L.; Wang, H.; Binolfi, A.; Theillet, F.-X.; Bekei, B.; Logan,  
37  
38 14 D. T.; Selenko, P.; Wennerstrom, H.; et al. Thermodynamics of Protein Destabilization in Live  
39  
40 15 Cells. *Proc. Natl. Acad. Sci. U.S.A.* **2015**, *112*, 12402–12407.  
41  
42  
43  
44 16 (8) Luchinat, E.; Barbieri, L.; Rubino, J. T.; Kozyreva, T.; Cantini, F.; Banci, L. In-Cell  
45  
46 17 NMR Reveals Potential Precursor of Toxic Species From SOD1 fALS Mutants. *Nat. Commun.*  
47  
48 18 **2014**, *5*, 5502.  
49  
50  
51  
52 19 (9) Theillet, F.-X.; Binolfi, A.; Bekei, B.; Martorana, A.; Rose, H. M.; Stuver, M.; Verzini,  
53  
54 20 S.; Lorenz, D.; van Rossum, M.; Goldfarb, D.; et al. Structural Disorder of Monomeric A-  
55  
56 21 Synuclein Persists in Mammalian Cells. *Nature* **2016**, *530*, 1–19.  
57  
58  
59  
60

- 1  
2  
3 1 (10) Majumder, S.; Xue, J.; DeMott, C. M.; Reverdatto, S.; Burz, D. S.; Shekhtman, A.  
4  
5 2 Probing Protein Quinary Interactions by in-Cell Nuclear Magnetic Resonance Spectroscopy.  
6  
7  
8 3 *Biochemistry* **2015**, *54*, 2727–2738.  
9  
10  
11 4 (11) Barbieri, L.; Bertini, I.; Luchinat, E.; Secci, E.; Zhao, Y.; Banci, L.; Aricescu, A. R.  
12  
13 5 Atomic-Resolution Monitoring of Protein Maturation in Live Human Cells by NMR. *Nat. Chem.*  
14  
15 6 *Biol.* **2013**, *9*, 297–299.  
16  
17  
18 7 (12) Banci, L.; Barbieri, L.; Bertini, I.; Cantini, F.; Luchinat, E. In-Cell NMR in *E. Coli* to  
19  
20 8 Monitor Maturation Steps of hSOD1. *PLoS ONE* **2011**, *6*, 1–8.  
21  
22  
23 9 (13) Selenko, P.; Frueh, D. P.; Elsaesser, S. J.; Haas, W.; Gygi, S. P.; Wagner, G. In Situ  
24  
25 10 Observation of Protein Phosphorylation by High-Resolution NMR Spectroscopy. *Nat. Struct.*  
26  
27 11 *Mol. Biol.* **2008**, *15*, 321–329.  
28  
29  
30  
31  
32 12 (14) Binolfi, A.; Limatola, A.; Verzini, S.; Kosten, J.; Theillet, F.-X.; May Rose, H.; Bekei,  
33  
34 13 B.; Stuiver, M.; van Rossum, M.; Selenko, P. Intracellular Repair of Oxidation-Damaged A-  
35  
36 14 Synuclein Fails to Target C-Terminal Modification Sites. *Nat. Commun.* **2016**, *7*, 10251.  
37  
38  
39  
40 15 (15) Rosato, A.; Vranken, W.; Fogh, R. H.; Ragan, T. J.; Tejero, R.; Pederson, K.; Lee, H.-W.;  
41  
42 16 Prestegard, J. H.; Yee, A.; Bin Wu; *et al.* The Second Round of Critical Assessment of  
43  
44 17 Automated Structure Determination of Proteins by NMR: CASD-NMR-2013. *J. Biomol. NMR*  
45  
46 18 **2015**, *62*, 413–424.  
47  
48  
49  
50  
51 19 (16) Waudby, C. A.; Christodoulou, J. An Analysis of NMR Sensitivity Enhancements  
52  
53 20 Obtained Using Non-Uniform Weighted Sampling, and the Application to Protein NMR. *J.*  
54  
55 21 *Magn. Reson.* **2012**, *219*, 46–52.  
56  
57  
58  
59  
60

- 1  
2  
3 1 (17) Hyberts, S. G.; Robson, S. A.; Wagner, G. Exploring Signal-to-Noise Ratio and  
4  
5 2 Sensitivity in Non-Uniformly Sampled Multi-Dimensional NMR Spectra. *J. Biomol. NMR* **2012**,  
6  
7 3 55, 167–178.  
8  
9  
10  
11 4 (18) Palmer, M. R.; Suiter, C. L.; Henry, G. E.; Rovnyak, J.; Hoch, J. C.; Polenova, T.;  
12  
13 5 Rovnyak, D. Sensitivity of Nonuniform Sampling NMR. *J. Phys. Chem. B* **2015**, *119*, 6502–  
14  
15 6 6515.  
16  
17  
18  
19 7 (19) Otting, G. Protein NMR Using Paramagnetic Ions. *Annu. Rev. Biophys.* **2010**, *39*, 387–  
20  
21 8 405.  
22  
23  
24  
25 9 (20) Koehler, J.; Meiler, J. Expanding the Utility of NMR Restraints with Paramagnetic  
26  
27 10 Compounds: Background and Practical Aspects. *Prog. Nucl. Magn. Reson. Spectrosc.* **2011**, *59*,  
28  
29 11 360–389.  
30  
31  
32  
33 12 (21) Schmitz, C.; Vernon, R.; Otting, G.; Baker, D.; Huber, T. Protein Structure  
34  
35 13 Determination From Pseudocontact Shifts Using ROSETTA. *J. Mol. Biol.* **2012**, *416*, 668–677.  
36  
37  
38  
39 14 (22) Rinaldelli, M.; Ravera, E.; Calderone, V.; Parigi, G.; Murshudov, G. N.; Luchinat, C.  
40  
41 15 Simultaneous Use of Solution NMR and X-Ray Data in REFMAC5 for Joint  
42  
43 16 Refinement/Detection of Structural Differences. *Acta Crystallogr. D Biol. Crystallogr.* **2014**, *70*,  
44  
45 17 958–967.  
46  
47  
48  
49 18 (23) Yagi, H.; Pilla, K. B.; Maleckis, A.; Graham, B.; Huber, T.; Otting, G. Three-  
50  
51 19 Dimensional Protein Fold Determination From Backbone Amide Pseudocontact Shifts Generated  
52  
53 20 by Lanthanide Tags at Multiple Sites. *Structure* **2013**, *21*, 883–890.  
54  
55  
56  
57  
58  
59  
60

- 1  
2  
3 1 (24) Brewer, K. D.; Bacaj, T.; Cavalli, A.; Camilloni, C.; Swarbrick, J. D.; Liu, J.; Zhou, A.;  
4  
5 2 Zhou, P.; Barlow, N.; Xu, J.; *et al.* Dynamic Binding Mode of a Synaptotagmin-1-SNARE  
6  
7 3 Complex in Solution. *Nat. Struct. Mol. Biol.* **2015**, *22*, 555–564.  
8  
9  
10  
11 4 (25) Salmon, L.; Blackledge, M. Investigating Protein Conformational Energy Landscapes  
12  
13 5 and Atomic Resolution Dynamics From NMR Dipolar Couplings: a Review. *Rep Prog Phys*  
14  
15 6 **2015**, *78*, 126601.  
16  
17  
18  
19 7 (26) Su, X.-C.; McAndrew, K.; Huber, T.; Otting, G. Lanthanide-Binding Peptides for NMR  
20  
21 8 Measurements of Residual Dipolar Couplings and Paramagnetic Effects From Multiple Angles.  
22  
23 9 *J. Am. Chem. Soc.* **2008**, *130*, 1681–1687.  
24  
25  
26  
27 10 (27) Häussinger, D.; Huang, J.-R.; Grzesiek, S. DOTA-M8: an Extremely Rigid, High-  
28  
29 11 Affinity Lanthanide Chelating Tag for PCS NMR Spectroscopy. *J. Am. Chem. Soc.* **2009**, *131*,  
30  
31 12 14761–14767.  
32  
33  
34  
35 13 (28) Liu, W.-M.; Skinner, S. P.; Timmer, M.; Blok, A.; Hass, M. A. S.; Filippov, D. V.;  
36  
37 14 Overhand, M.; Ubbink, M. A Two-Armed Lanthanoid-Chelating Paramagnetic NMR Probe  
38  
39 15 Linked to Proteins via Thioether Linkages. *Chem. Eur. J.* **2014**, *20*, 6256–6258.  
40  
41  
42  
43 16 (29) Toda, N.; Asano, S.; Barbas, C. F. Rapid, Stable, Chemoselective Labeling of Thiols with  
44  
45 17 Julia-Kocięński-Like Reagents: a Serum-Stable Alternative to Maleimide-Based Protein  
46  
47 18 Conjugation. *Angew. Chem. Int. Ed.* **2013**, *52*, 12592–12596.  
48  
49  
50  
51 19 (30) Yang, Y.; Wang, J.-T.; Pei, Y.-Y.; Su, X.-C. Site-Specific Tagging Proteins via a Rigid,  
52  
53 20 Stable and Short Thiolether Tether for Paramagnetic Spectroscopic Analysis. *Chem. Commun.*  
54  
55 21 **2015**, *51*, 2824–2827.  
56  
57  
58  
59  
60



- 1  
2  
3  
4 1 (31) Opina, A. C. L.; Strickland, M.; Lee, Y.-S.; Tjandra, N.; Andrew Byrd, R.; Swenson, R.  
5  
6 2 E.; Vasalatiy, O. Analysis of the Isomer Ratios of Polymethylated-DOTA Complexes and the  
7  
8 3 Implications on Protein Structural Studies. *Dalton Trans.* **2016**, *4*, 4673–4687.  
9  
10  
11 4 (32) Ottiger, M.; Delaglio, F.; Bax, A. Measurement of J and Dipolar Couplings From  
12  
13 5 Simplified Two-Dimensional NMR Spectra. *J. Magn. Reson.* **1998**, *131*, 373–378.  
14  
15  
16  
17 6 (33) Hänsel, R.; Luh, L. M.; Corbeski, I.; Trantirek, L.; Dötsch, V. In-Cell NMR and EPR  
18  
19 7 Spectroscopy of Biomacromolecules. *Angew. Chem. Int. Ed.* **2014**, *53*, 10300–10314.  
20  
21  
22  
23 8 (34) Selenko, P.; Serber, Z.; Gadea, B.; Ruderman, J.; Wagner, G. Quantitative NMR Analysis  
24  
25 9 of the Protein G B1 Domain in *Xenopus Laevis* Egg Extracts and Intact Oocytes. *Proc. Natl.*  
26  
27 10 *Acad. Sci. USA* **2006**, *103*, 11904–11909.  
28  
29  
30  
31 11 (35) Schmidt, H. L. F.; Sperling, L. J.; Gao, Y. G.; Wylie, B. J.; Boettcher, J. M.; Wilson, S.  
32  
33 12 R.; Rienstra, C. M. Crystal Polymorphism of Protein GB1 Examined by Solid-State NMR  
34  
35 13 Spectroscopy and X-Ray Diffraction. *J. Phys. Chem. B* **2007**, *111*, 14362–14369.  
36  
37  
38  
39 14 (36) Gronenborn, A. M.; Filpula, D. R.; Essig, N. Z.; Achari, A.; Whitlow, M.; Wingfield, P.  
40  
41 15 T.; Clore, G. M. A Novel, Highly Stable Fold of the Immunoglobulin Binding Domain of  
42  
43 16 Streptococcal Protein G. *Nature* **1991**, *253*, 657–661.  
44  
45  
46  
47 17 (37) Bouvignies, G.; Meier, S.; Grzesiek, S.; Blackledge, M. Ultrahigh-Resolution Backbone  
48  
49 18 Structure of Perdeuterated Protein GB1 Using Residual Dipolar Couplings From Two Alignment  
50  
51 19 Media. *Angew. Chem. Int. Ed.* **2006**, *45*, 8166–8169.  
52  
53  
54  
55  
56  
57  
58  
59  
60

- 1  
2  
3 1 (38) Shapiro, Y. E.; Meirovitch, E. Slowly Relaxing Local Structure (SRLS) Analysis of  $^{15}\text{N}$ -  
4 H Relaxation From the Prototypical Small Proteins GB1 and GB3. *J. Phys. Chem. B* **2012**, *116*,  
5 4056–4068.  
6  
7  
8  
9  
10  
11 4 (39) Lamley, J. M.; Lougher, M. J.; Sass, H. J.; Rogowski, M.; Grzesiek, S.; Lewandowski, J.  
12 R. Unraveling the Complexity of Protein Backbone Dynamics with Combined  $(^{13}\text{C})$  and  $(^{15}\text{N})$   
13 Solid-State NMR Relaxation Measurements. *Phys. Chem. Chem. Phys.* **2015**, *17*, 21997–22008.  
14  
15  
16  
17  
18  
19 7 (40) Vögeli, B.; Kazemi, S.; Güntert, P.; Riek, R. Spatial Elucidation of Motion in Proteins by  
20 Ensemble-Based Structure Calculation Using Exact NOEs. *Nat. Struct. Mol. Biol.* **2012**, *19*,  
21 1053–1057.  
22  
23  
24  
25  
26  
27 10 (41) Canales, Á.; Mallagaray, Á.; Berbís, M. Á.; Navarro-Vázquez, A.; Domínguez, G.;  
28 Cañada, F. J.; André, S.; Gabius, H.-J.; Pérez-Castells, J.; Jiménez-Barbero, J. Lanthanide-  
29 Chelating Carbohydrate Conjugates Are Useful Tools to Characterize Carbohydrate  
30 Conformation in Solution and Sensitive Sensors to Detect Carbohydrate–Protein Interactions. *J.*  
31 *Am. Chem. Soc.* **2014**, *136*, 8011–8017.  
32  
33  
34  
35  
36  
37  
38  
39  
40 15 (42) Guan, J.-Y.; Keizers, P. H. J.; Liu, W.-M.; Lohr, F.; Skinner, S. P.; Heeneman, E. A.;  
41 Schwalbe, H.; Ubbink, M.; Siegal, G. Small-Molecule Binding Sites on Proteins Established by  
42 Paramagnetic NMR Spectroscopy. *J. Am. Chem. Soc.* **2013**, *135*, 5859–5868.  
43  
44  
45  
46  
47  
48 18 (43) Martorana, A.; Bellapadrona, G.; Feintuch, A.; Di Gregorio, E.; Aime, S.; Goldfarb, D.  
49 Probing Protein Conformation in Cells by EPR Distance Measurements Using  $\text{Gd}^{3+}$  Spin  
50 Labeling. *J. Am. Chem. Soc.* **2014**, *136*, 13458–13465.  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

- 1  
2  
3 (44) Bleicken, S.; Jeschke, G.; Stegmueller, C.; Salvador-Gallego, R.; García-Sáez, A. J.;  
4  
5  
6 2 Bordignon, E. Structural Model of Active Bax at the Membrane. *Molecular Cell* **2014**, *56*, 496–  
7  
8 3 505.  
9  
10  
11 4  
12  
13  
14  
15  
16  
17  
18  
19  
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21  
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