Electronic Supporting Information

Title

Impact of cellular health condition on protein folding state in mammalian cells

Authors

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Materials and Methods

Preparation of recombinant protein samples

Synthetic DNA fragments encoding the human ubiquitin gene containing three alanine substitutions (L8A, I44A, V70A, designated as hUB-3A) and the human adenylate kinase 1 (hAK1) gene were subcloned into the pET-23a vector to generate the hUB-3A expression vector and the pET-28a vector for hAK1 expression. These proteins were recombinantly expressed in the *Escherichia coli* BL21 (DE3) strain (Invitrogen), grown in M9 minimal medium containing 0.5 g/L of ^{15}N NH_4Cl (Cambridge Isotope Laboratories) as the sole nitrogen source for uniformly ^{15}N labeled samples. Cells were grown at 37 °C to an OD_{600} of 0.7 - 0.8, and then induced with 1 mM isopropyl-β-thiogalactopyranoside (Nacalai Tesque) for 4 h at 37 °C. hUB-3A was purified as described previously[1]. hAK1 was expressed as the fusion protein with an N-terminal hexa-Histidine (His6) and human Small ubiquitin-like modifier (His6-hSUMO3) purification tag. hAK1 was purified by Ni-NTA affinity chromatography. After cleavage of the tag by the catalytic domain of human SENP2 protease in the N-terminal GST-fused from, the proteins were re-purified by Ni-NTA affinity chromatography to remove the residual His6-hSUMO3 and uncleaved fusion protein. The proteins were further purified by HiLoad™ 26/600 Superdex™ 75 pg (GE Healthcare) size-exclusion column chromatography.

In-cell NMR sample preparation

The ^{15}N-labeled protein delivery into HeLa cells (Summit Pharmaceuticals International Corp.) by electroporation was performed as described previously[2,3], with a few modifications. HeLa cells were grown at 37 °C under a 5 % CO_2 atmosphere in DMEM medium with high-glucose (Thermo Fisher Scientific), containing 10 % (vol/vol) of FBS (Thermo Fisher Scientific), 200 U/mL of penicillin, and 200 mg/mL of streptomycin (Nacalai Tesque), until the cells reached about 80% confluency. For in-cell NMR samples, 17-20 × 10^6 cells were used. Cells were collected by TrypLE™ Express (Thermo Fisher Scientific) treatment and centrifugation. The collected cells were washed twice in the electroporation buffer (EP buffer) as described[3], and resuspended in EP buffer containing 1.0 - 1.3 mM of stable isotope-labeled proteins by adjusting the cell density to 17-20 × 10^6 cells/mL. Aliquots (100 µL) of the cell suspension were dispensed into 2 mm gap electroporation cuvettes and electroporated with a NEPA21 Super Electroporator (Nepa Gene) with a single high power pulse of 110 V, 15 ms to form pores in the cell membrane, and after a 50 ms interval, two lower power pulses of 20 V, 50 ms per pulse to deliver the protein into the cells. The reverse polarity was applied for the two lower pulses. The total energy transfer from the electroporator was 4.0 - 4.5 J. Aliquots (200 µL) of medium containing FBS and antibiotics were transferred into cuvettes, and the mixed solution was added to three Type-I collagen coated 100 mm diameter culture dishes, filled with culture medium containing serum and antibiotics. The cell samples were used for in-cell NMR measurements after recovery for 3 h at 37 °C under a 5 % CO_2 atmosphere. For the in-cell NMR sample without medium flow and gel encapsulation, the cells were harvested, washed and re-suspended in DMEM medium containing FBS, antibiotics and 10 % D_2O. The cell viability after the in-cell NMR experiments was assessed by trypan blue staining.

Preparation of alginate hydrogel encapsulated HeLa cell beads for in-cell NMR

The alginate hydrogel encapsulated HeLa cell beads were prepared by the centrifuge-based method[4]. The surviving cells after protein delivery by electroporation were detached with Accumax Cell Dissociation Solution (Innovative Cell Technologies). The harvested cells were washed twice in HEPES buffer (30 mM HEPES, pH 7.2, 120 mM NaCl) and then mixed with a 2 % (w/v) sodium alginate solution. The mixed suspension was transferred into the handmade centrifuge-based droplet preparation device (Fig. 2A). The bottom of the device was filled with a 150 mM $CaCl_2$ solution. The cell suspension transfer device was centrifuged at 60 × *g* for 5 min at 20 °C, for gelation. The cell beads were washed twice in DMEM medium and transferred into a 100 mm diameter dish containing 10 mL DMEM medium with FBS and antibiotics. After 1 h incubation at 37 °C under a 5 % CO_2 atmosphere, the cell beads were transferred into the custom-made NMR sample tube (Shigemi) for our medium flow system for in-cell NMR.

NMR spectroscopy

NMR experiments were performed at 310 K on Bruker Avance III 800 MHz or 900 MHz spectrometers equipped with a cryogenic TCI probehead. The 2D ^{1}H-^{15}N SOFAST-HMQC spectra[5] were acquired for all in-cell NMR experiments. The number of scans was 128 or 256 and the acquisition data size was 512 (^{15}N) × 32 (^{1}H) complex points. In some of the in-cell NMR measurements, data points in the indirect dimension were sampled non-uniformly and processed by compressed sensing[6]. The interscan delay was approximately 0.2 s for each duration of about 30 minutes or 60 minutes. The 2D data sets were combined to improve the signal to noise ratio. Data processing was performed with the programs NMRPipe[7] and mddNMR[8], and the processed data were analyzed with the program Sparky[9].

Confocal laser scanning microscopy

For fluorescent labeling of hUB-3A and hAK1, the proteins were treated with Alexa Fluor® 488 5-SDP Ester (Thermo Fisher Scientific). The unreacted labeling reagent was separated by using a PD MiniTrap G-25 desalting column (GE Healthcare). The ratio of labeled protein to unlabeled protein was approximately 0.1. After protein delivery into HeLa cells by electroporation, the cells were allowed to recover for 2 h at 37 °C under a 5 % CO_2 humidified atmosphere on a Type-I collagen coated 35 mm glass bottomed dish (IWAKI), filled with culture medium containing serum and antibiotics. The intracellular distribution of proteins in HeLa cells was analyzed by a confocal laser scanning microscope (FLUOVIEW FV10i-DOC; OLYMPUS) equipped with a 60 × objective lens. For nuclear staining, cells were treated with Hoechst 33342 (NucBlue® Fixed Cell ReadyProbes® Reagent; Thermo Fisher Scientific).

Figure S1

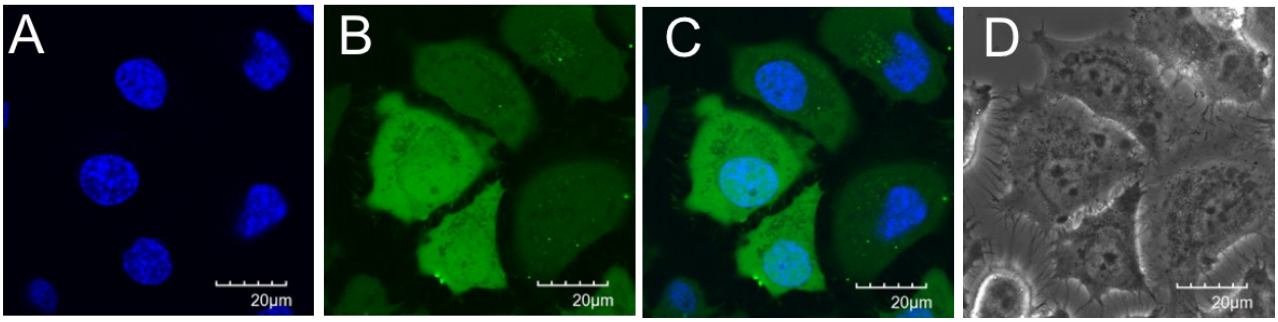

Figure S1 Intracellular distribution of hAK1 delivered into HeLa cells. The fluorescent (A-C) and phase contrast (D) images of living HeLa cells containing the delivered hAK1, at 2h after electroporation. (A) Nuclear staining with Hoechst 33342. (B) Intracellular distribution of Alexa488-labeled hAK1. (C) Superimposition of (A) and (B). Scale bars, 20 μm.

Figure S2

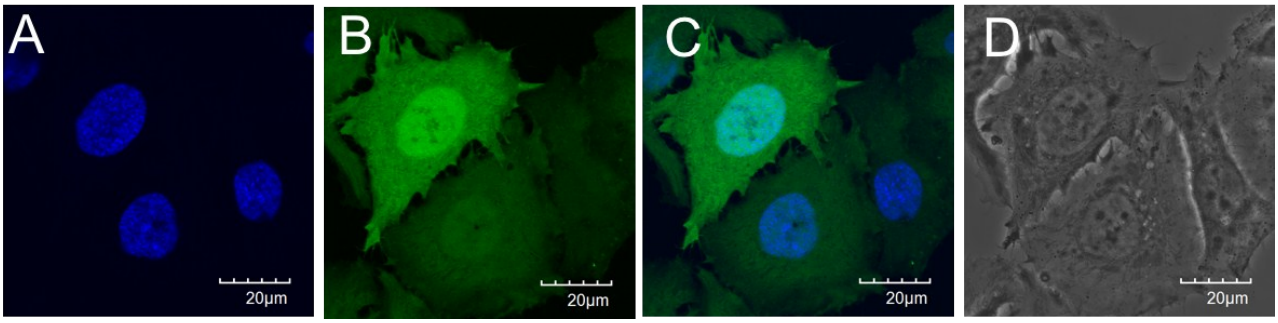

Figure S2 Intracellular distribution of hUB-3A delivered into HeLa cells. The fluorescent (A-C) and phase contrast (D) images of living HeLa cells containing the delivered hUb-3A at 4h after electroporation. (A) Nuclear staining with Hoechst 33342. (B) Intracellular distribution of Alexa488-labeled hUb-3A. (C) Superimposition of (A) and (B). Scale bars, 20 μm.

Figure S3

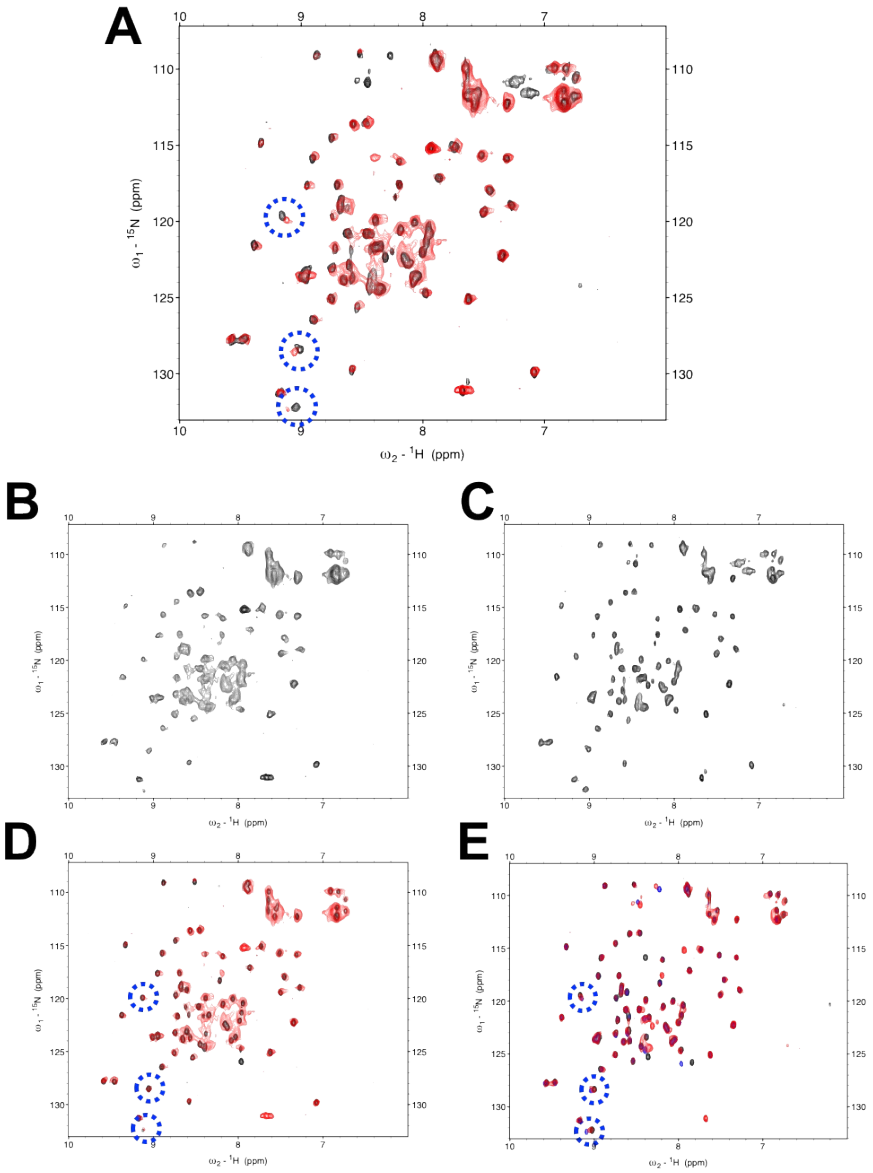

Figure S3 ^{1}H-^{15}N SOFAST-HMQC spectra of hUB-3A in HeLa cells. (A) Superimposition of in-cell NMR spectra of ^{15}N labeled hUB-3A in the gel encapsulated HeLa cell beads with the bioreactor (red) and in the cell suspension (black). (B), (C) In-cell NMR spectra of ^{15}N-labeled UB-3A with the bioreactor (B), and in the cell suspension (C). (D) Superimposition of the in-cell NMR spectrum of ^{15}N-labeled hUB-3A with the bioreactor (red) and the reference spectrum of the diluted protein solution at pH 6.8 (black). (E) Superimposition of the in-cell NMR spectrum of ^{15}N-labeled hUB-3A in the cell suspension (red) with the reference spectrum of the diluted protein solution at pH 5.8 (black) and 6.2 (blue). Cross-peaks highlighted with dotted circles represent those used as pH probes.

Figure S4

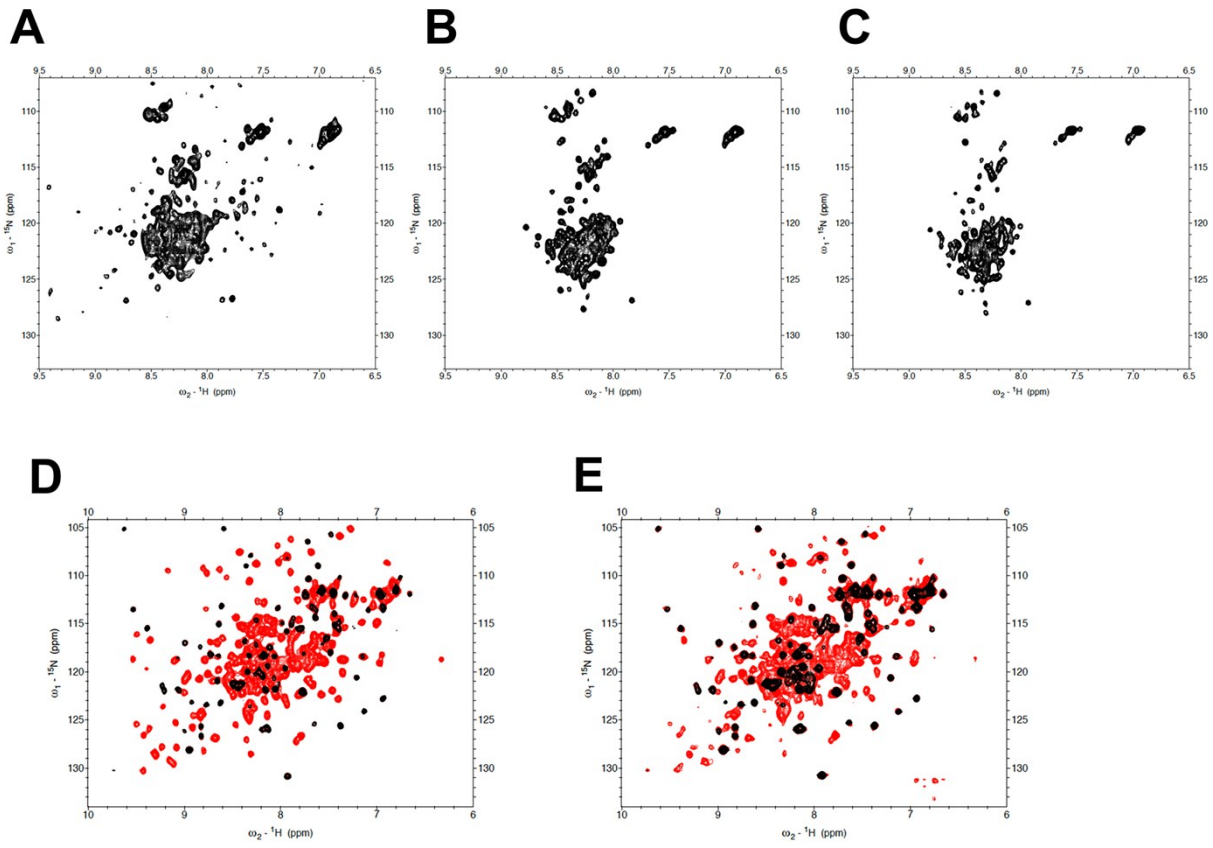

Figure S4 ^{1}H-^{15}N SOFAST-HMQC spectra of hAK1. (A), (B), (C) *in vitro* NMR spectra of purified ^{15}N labeled hAK1 in 5 mM potassium phosphate buffer (pH 6.2) containing 25 mM HEPES, 120 mM KCl, with (A) 100 g/L or (B) 200 g/L or (C) 400 g/L of urea at 37 °C. (D), (E) Superimposition of *in vitro* NMR spectra of purified ^{15}N labeled hAK1 in 5 mM potassium phosphate buffer (pH 6.2) containing 25 mM HEPES, 120 mM KCl, non-labeled lysozyme from egg white of (D) 100 g/L or (E) 200 g/L at 37 °C (red) and samples without ^{15}N labeled hAK1 (black).

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