Inducible protein expression in stably transfected cells paves the way toward in-cell NMR studies in defined physiological states and 3D tissue cultures.

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ABSTRACT: In-cell NMR spectroscopy is the sole technique for characterizing protein structure, dynamics, and interactions in living human cells at atomic resolution. However, its applications have been restricted to asynchronous single-cell suspensions. We developed a strategy based on target protein inducible overexpression in stably transfected cells, allowing the acquisition of high-resolution in-cell NMR spectra in physiologically defined cellular states and 3D tissue models.

INTRODUCTION

In addition to providing unique atomically resolved insights into protein structure and dynamics within the intracellular space of living (human) cells[1,2], in-cell NMR spectroscopy has recently emerged as a source of valuable information for drug screening and development[3–6].

The success of in-cell NMR applications critically relies on the ability to deliberately increase the concentration of the protein(s) of interest within the interior of the cell. This increase, usually accompanied by isotopic labeling, allows for separating NMR signals linked to the target protein from those originating from other cellular components[2]. This elevated protein concentration is achieved by introducing the recombinant protein from outside the cells[7–10] or by in situ transient protein overexpression[11]. However, these methods are intrusive and/or require specific cell culture manipulations that disrupt the normal functioning of cells, making it impossible to maintain precise control over their physiological state during in-cell NMR experiments.

To address this problem, we proposed an alternative approach: inducing the overexpression of the target protein within stably transfected cells. By adopting this established molecular biology concept to meet the sensitivity requirements and workflow of in-cell NMR experiments, we could gain external control over the physiological state of the cells,

enabling NMR experiments on synchronized cells and 3D cell cultures.

RESULTS AND DISCUSSION

To implement this approach, we employed the PiggyBac Cumate Switch Inducible Vector (System Biosciences, US) (Fig. 1A) for several reasons: i) The TTAA-specific transposon piggyBac is rapidly becoming a highly useful vector for genetic engineering of a wide variety of species, comprising insects, yeast, and mammals, including humans[12]; ii) it allows inducible protein overexpression under the control of the cumate operator; the inducibility of protein expression is essential for the cell's physiological state and protein isotopic labeling control; iii) it allows for the insertion of multiple copies of the gene of interest at various genomic locations; this might be vital for achieving the protein overexpression levels required for NMR detection; and iv) it contains a gene coding for green fluorescent protein (GFP) under the control of an internal ribosomal entry site (IRES) following the stop codon of the target protein, which facilitates indirect monitoring of target protein expression via GFP fluorescence while not affecting the target protein in-cell NMR readout (GFP resonances are too broad to be observed in cells, most likely due to interactions with intracellular components, decreasing its rotational diffusion rate[13,14]).

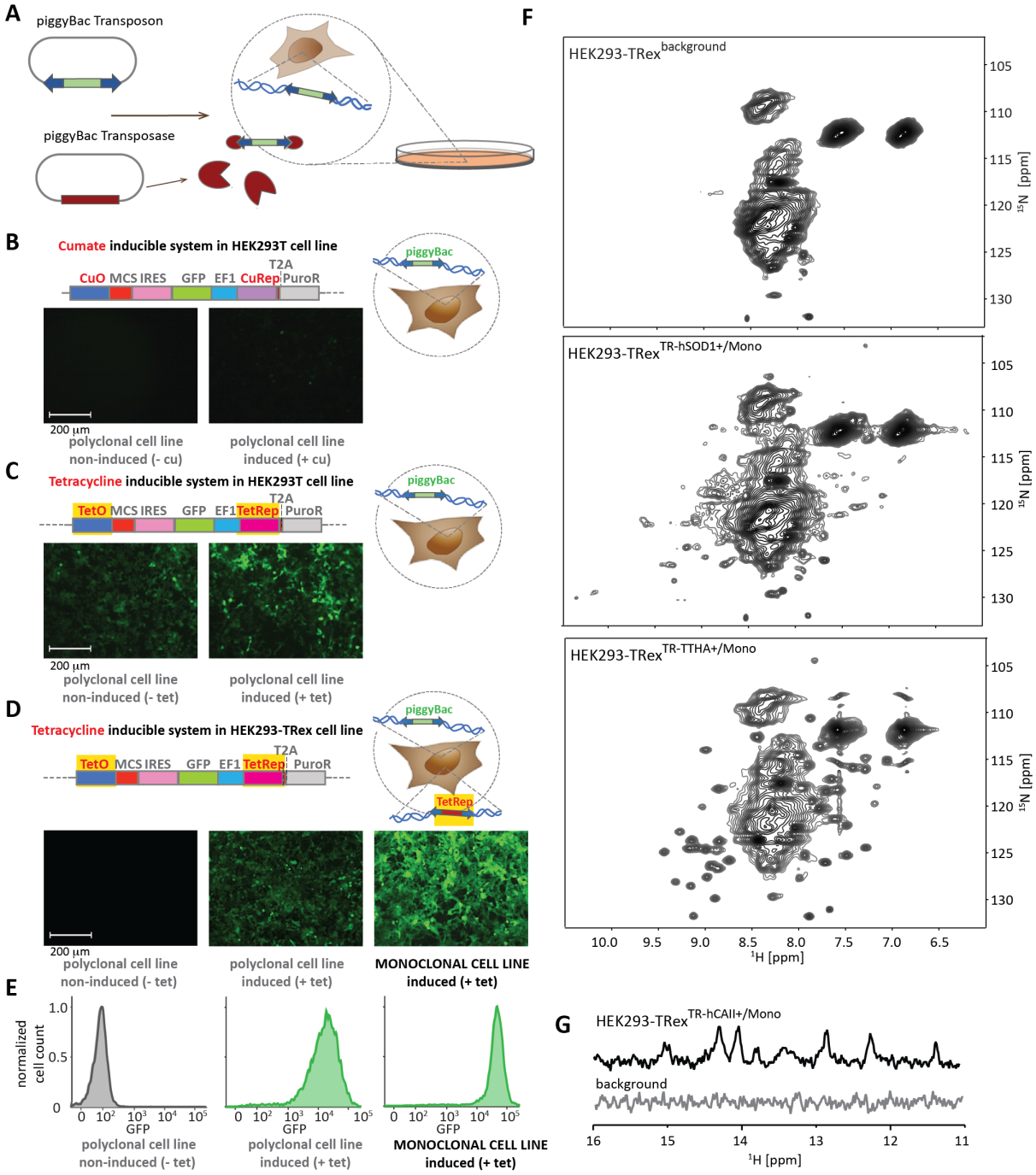

Fig. 1. (A) Schematic representation of stable cell line production using piggyBac transposon. (B), (C) and (D) show confocal images revealing GFP expression levels in hSOD1 stably transfected HEK293T and HEK293-TRex polyclonal cell lines before (LEFT) and 48 hours after adding expression inducer (RIGHT); cumate in (B), tetracycline in (C) and (D). (E) Flow cytometry (FCM) plot: non-induced HEK293-TRex$^{TR\text{-}hSOD1/Poly}$ cells (LEFT), HEK293-TRex$^{TR\text{-}hSOD1+/Poly}$ (MIDDLE), and HEK293-TRex$^{TR\text{-}hSOD1+/Mono}$ (RIGHT) cells 48 hours after expression induction. (F) ^{1}H-^{15}N 2D SOFAST-HMQC in-cell NMR spectra: non-induced HEK293-TRex$^{TR\text{-}hSOD1}$ (TOP) and HEK293-TRex$^{TR\text{-}hSOD1+/Mono}$ (MIDDLE), and HEK293-TRex$^{TR\text{-}TTHA+/Mono}$ (BOTTOM) cells 48 hours after expression induction. (G) The imino regions of ^{1}H 1D in-cell NMR spectra of induced (TOP) and non-induced (BOTTOM) HEK293-TRex$^{TR\text{-}CAII+/Mono}$ cells.

We inserted the human superoxide dismutase 1 (hSOD1) gene into the PiggyBac Cumate Switch Inducible Vector (Supplementary Fig. 1A), generating a stable HEK293T cell line (HEK293T$^{CuR\text{-}hSOD1/Poly}$). Upon cumate exposure, hSOD1 expression in HEK293T$^{CuR\text{-}hSOD1+/Poly}$ cells monitored indirectly via a GFP reporter was low (Fig. 1B, Supplementary Fig. 2A) and insufficient for NMR readout (*data not shown*). To enhance expression, we replaced the cumate operator (CuO) and repressor (CuR) with the tetracycline operator (TetO) and repressor (TetRep; TR) (Supplementary Fig. 1B). A stable cell line generated using this modified vector expressed hSOD1 at increased levels in the presence of tetracycline; however, hSOD1 expression leaked in the absence of tetracycline (Fig. 1C, Supplementary Fig. 2B). To suppress leakage of target protein expression, we integrated the modified vector into a cell line stably expressing the tetracycline repressor Flp-In T-REx 293 (HEK293-TRex); hSOD1 expression in the resulting polyclonal cell line (HEK293-TRex$^{TR\text{-}hSOD1+/Poly}$) was rigorously controlled with tetracycline (Fig. 1D, Supplementary Fig. 2C), with levels resembling those in HEK293T cells (Fig. 1C). Moderate differences in the hSOD1 expression levels, as revealed via the GFP reporter, were observed among cells in the polyclonal culture (Fig 1D, E, and Supplementary Fig. 2C) likely stemming from differences in the number of gene copies and differences in the genomic integration sites. Nevertheless, the differences were much lower than those typically encountered when using transient transfection (Supplementary Fig. 3B).

To improve the uniformity of protein expression, a crucial factor for reliably interpreting in-cell NMR experiments, we isolated high-expression clones from HEK293-TRex$^{TR\text{-}hSOD1+/Poly}$ cells based on the GFP reporter intensity. The expansion of these clones yielded monoclonal cell lines (HEK293-TRex$^{TR\text{-}hSOD1/Mono}$), which displayed higher and more uniform hSOD1 expression levels than did HEK293-TRex$^{TR\text{-}hSOD1+/Poly}$ (Fig. 1E, and Supplementary Fig. 2C vs. 2D). Most significantly, with HEK293-TRex$^{TR\text{-}hSOD1+/Mono}$, we achieved sufficient protein expression levels for a positive in-cell NMR readout (Fig. 1F, Supplementary Fig. 4). Finally, we tested this procedure with two additional model proteins, TTHA (Fig. 1F, Supplementary Fig. 5) and human carbonic anhydrase II (hCAII) (Fig. 1G, Supplementary Fig. 5), confirming that this expression system is suitable for in-cell NMR measurements.

To achieve NMR readout in cell cycle-synchronized HEK293-TRex$^{TR\text{-}hCAII+/Mono}$ cells, we induced protein overexpression for 48 hours while subjecting the cell culture to 14 and 24 hours of mimosine or RO3306 and nocodazole treatment for G_1/S and G_2/M-phase synchronization[15-17], respectively (Fig. 2A, B). To maintain cell viability and synchronization during prolonged in-cell NMR spectra acquisition, we continuously supplied fresh medium supplemented with mimosine and nocodazole to G_1/S and G_2-phase synchronized cells, respectively, using an NMR bioreactor[18]. Nocodazole treatment of G2-phase synchronized cells was employed to prevent potential completion of mitosis during the measurement, trapping cells at G2/M. The resulting in-cell NMR spectra of CAII in G_1/S- and G_2/M-phase synchronized cells exhibited the expected signals from Zn^{2+}-coordinated His residues in the CAII active site[3] in the region between 11.0 and 15.5 ppm; the spectra showed comparable S/N ratios and resolutions and did not exhibit substantial differences, consistent with the housekeeping role of CAII (Fig. 2A, B). Upon the addition of methazolamide (MZA), a validated CAII inhibitor, both spectral patterns changed and matched the previously reported spectrum of the CAII-MZA-bound state[3] (Fig. 2A, B). These data demonstrate the capacity of in-cell NMR to assess protein structure and interactions with drug-like molecules in different cell cycle phases.

To extend the in-cell NMR concept to 3D cell cultures[19], a crucial step toward high-resolution NMR protein studies in tissues/organs, we separately used HEK293-TRex$^{TR\text{-}TTHA+/Mono}$ and HEK293-TRex$^{TR\text{-}hCAII+/Mono}$ cells to form spheroids. Spheroid formation and protein expression occurred simultaneously over 48 hours, resulting in spheroids < 250 µm in size (Fig. 2C, Supplementary Fig. 3E). Before NMR data acquisition, the spheroids were immobilized in a gel matrix to facilitate bioreactor-assisted measurements and prevent their fusion. The resulting NMR spectra of spheroid-based samples from HEK293-TRex$^{TR\text{-}TTHA+/Mono}$ and HEK293-TRex$^{TR\text{-}hCAII+/Mono}$ cells exhibited the expected patterns. They demonstrated comparable signal-to-noise ratio (S/N ratio) and resolution to those obtained from the corresponding single-cell suspensions (Fig. 2C). Notably, the introduction of MZA to the medium supplied to HEK293-TRex$^{TR\text{-}hCAII+/Mono}$-based spheroids induced a change in the spectral pattern within the 11.0 to 15.5 ppm region, corresponding to the spectrum of the CAII-MZA-bound state (Fig. 2C – red).

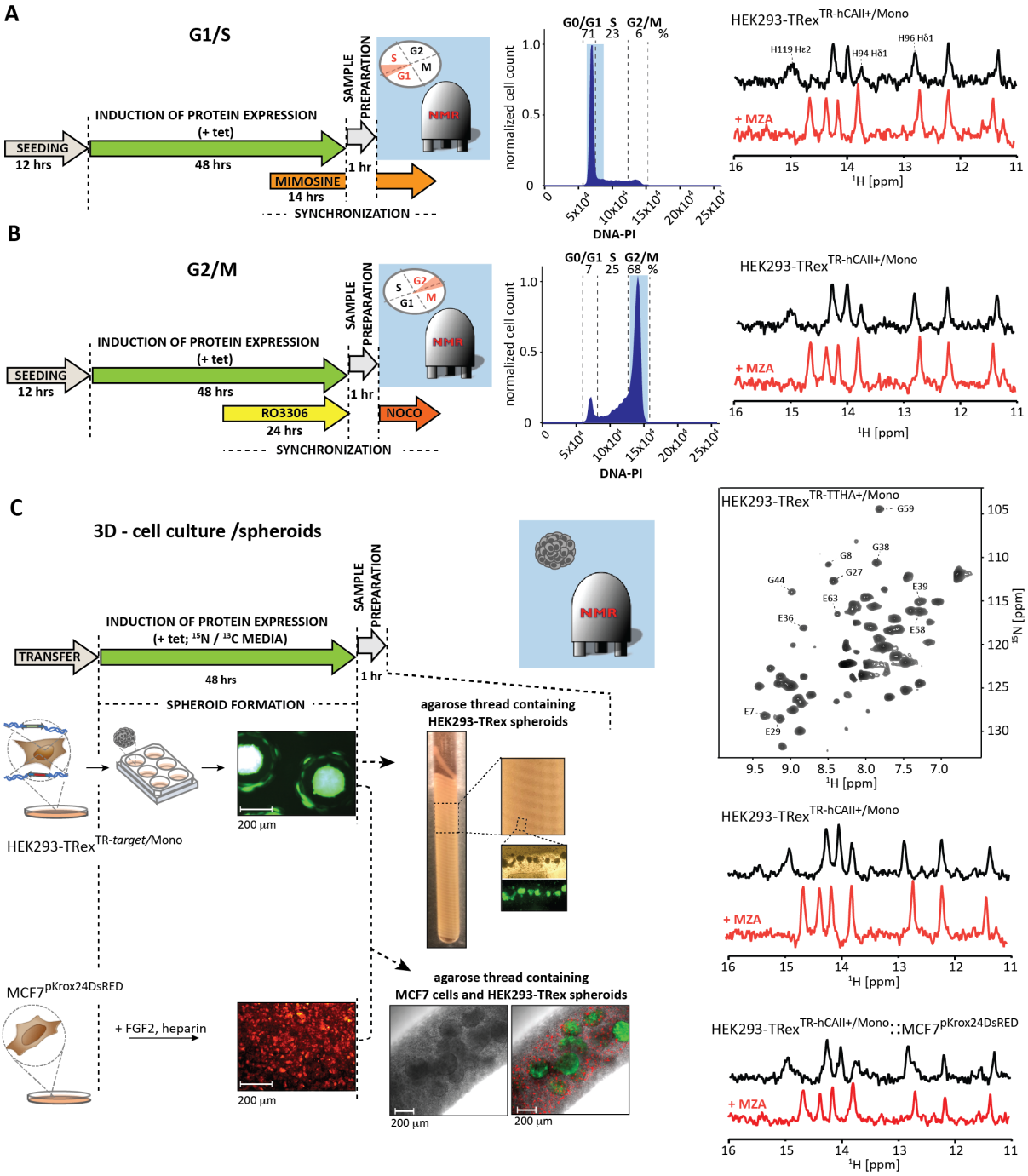

Fig. 2. (A) and (B) - LEFT: Overview of the stable cell line manipulations leading to the acquisition of in-cell NMR spectra at the G_1/S and G_2/M boundary of the cell cycle, MIDDLE: The propidium iodide (PI) DNA content staining of G_1/S (A) and G_2/M (B) synchronized HEK293-TRex$^{TR\text{-}hCAII+/Mono}$ cells before gel immobilization and NMR analysis, RIGHT: ^{1}H 1D in-cell NMR spectra of G_1/S (A) and G_2/M (B) synchronized HEK293-TRex$^{TR\text{-}hCAII+/Mono}$ cells 48 hours after expression induction before (black) and after (red) addition of MZA. Note: Signals arising from the zinc-coordinating histidines (H94, H96, and H119)[3] in the active site of CA2 are indicated. (C) – LEFT: Overview of the stable cell line manipulations leading to the acquisition of in-cell NMR spectra in 3D cell culture (spheroids); MIDDLE: Sample preparation - (microscope) images of HEK293-TRex$^{TR\text{-}TTHA+/Mono}$ spheroids (green), MCF7^{pKrox24DsRED} cells (red; DsRED expression induced by addition of FGF2), and the spheroid-based in-cell NMR samples preparations; RIGHT: background-subtracted ^{1}H-^{15}N 2D SOFAST-HMQC in-cell NMR spectrum of spheroid preparation from induced HEK293-TRex$^{TR\text{-}TTHA+/Mono}$ cells (the assignment of imino protons from selected Gly and Glu residues was transferred from ref. 22), and ^{1}H 1D in-cell NMR spectra of HEK293-TRex$^{TR\text{-}hCAII+/Mono}$ and HEK293-TRex$^{TR\text{-}hCAII+/Mono}$::MCF7^{pKrox24DsRED} spheroids before and after treating them with the CAII inhibitor (MZA).

In the next step, HEK293-TRex$^{TR\text{-}hCAII+/Mono}$ spheroids were embedded in a gel matrix containing MCF7 cells (Fig. 2D), which exhibit an epithelial-like morphology[20]. For visualization, we used MCF7 cells stably transfected with the pKrox24 reporter (MCF7^{pKrox24DsRED}), which expresses destabilized red fluorescent protein (DsRED), upon treatment with fibroblast growth factor 2 (FGF2). In this way, we created the simplest model mimicking a complex organ, where 3D spheroids were surrounded by cells of different types, referred to as HEK293-TRex$^{TR\text{-}hCAII+/Mono}$::MCF7^{pKrox24DsRED} spheroids. Taken together, the comparison of the imino region of the 1D ^{1}H in-cell NMR spectra of the HEK293-TRex$^{TR\text{-}hCAII+/Mono}$ and HEK293-TRex$^{TR\text{-}hCAII+/Mono}$:: MCF7^{pKrox24DsRED} spheroids before and after treatment with the CAII inhibitor (MZA) (Fig. 2C) demonstrated the potential for conducting in-cell NMR-based drug screening in 3D tissue culture systems; the use of spheroids, particularly hybrid preparations that consist of different cell types, allows more physiological in vitro modeling of cell proliferation, differentiation, metabolism, cytoskeletal dynamics, and cell-matrix and cell-to-cell interactions than traditional 2D single-cell cultures[21].

As demonstrated here, our approach employing inducible stable cell lines offers the critical advantage of accessing structural information by in-cell NMR in various physiologically defined cellular states. This approach is vital for establishing quantitative and meaningful relationships between structural and biological/functional data. This capability, combined with the development of novel protein labeling strategies, NMR data acquisition schemes, and optimized hardware for more sensitive and selective NMR detection, could revolutionize structural biology and pharmacology.

METHODS

Vector construction

The inducible PiggyBac Cumate Switch vector (pB-CuRep), along with the Super PiggyBac Transposase expressing vector (pB-Transp), were purchased from System Biosciences. The pB-CuRep vector contains the cumate switch combined with the EF1-CymR repressor-T2A-Puro cassette. The expression of the cDNA of interest, inserted into the multi-cloning site (MCS) downstream of the cumate operator (CuO), can be switched on by adding cumate to the cells. Several cloning steps reconstructed the pB CuRep vector from a cumate inducible vector to a vector inducible by tetracycline (pB-TetRep). The CuO was replaced by the tetracycline operator (TetO), and a fragment of the cumate repressor (CuRep; CuR) was replaced by the tetracycline repressor (TetRep; TR). cDNA of human SOD1 (hSOD1) bearing the sequence coding flag-tag (DYKDDDDK) on the 3' end was inserted into MCS of pB-CuRep. cDNAs of human SOD1 (hSOD1), CAII (hCAII), and bacterial TTHA1718 (TTHA) genes, bearing in all cases the sequence coding flag-tag on the 3' end, were individually inserted into MCS of pB-TetRep. **Note**: Introducing the C-terminal flag-tag to wild-type proteins aimed to facilitate the monitoring of hSOD1, TTHA, and hCAII expression levels using flag-tag specific antibodies (Sigma Aldrich, F1804) relative to the expression levels of the GFP reporter (cf. Supplementary Figures 4 and 5).

Cell lines and cell cultivation

polyclonal and monoclonal cell lines was analyzed by Coomassie-stained SDS-PAGE and microscopy (see below).

The HEK293T cell line (ATCC, CRL-3216) and the Flp-In T-REx 293 cell line (ThermoFisher) were employed to establish stable, inducible cell lines for in-cell NMR studies. HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Merck) containing 10% fetal bovine serum (FBS) (Merck) and 1% penicillin/streptomycin (Merck). In cell lines with integrated pB-CuRep or pB-TetRep cassettes, 3 mg/ml of puromycin (Merck) was added to the media. Cumate (System Biosciences) at a concentration of 300 mg/ml or tetracycline (Merck) at 3 mg/ml induced the expression of inserted cDNA. HEK293-TRex cells were cultured in DMEM with 10% fetal bovine serum tetracycline-free (FBS-tet) (Biosera), 1% ZellShield (Minerva Biolabs), and 5 mg/ml blasticidin. For cell lines with integrated pB-TetRep cassettes, 3 mg/ml puromycin was added to the media. Tetracycline at a concentration of 3 mg/ml induced the expression of inserted cDNA. In cell lines expressing metalloproteins like hSOD1 and hCAII, 10 μM $ZnSO_4$ (Sigma Aldrich) was added to the media upon induction of gene expression. For uniform ^{15}N labeling, ^{15}N labeled BioExpress6000 medium (CIL) was used instead of regular DMEM at the time of tetracycline induction (48 hours before in-cell NMR measurement).

The HEK293-TRex and MCF7^{pKrox24DsRED} cell lines were used to prepare an in-cell NMR sample of a 3D spheroid culture system consisting of two different cell lines. The pKrox24(MapErk)DsRED reporter[23] (Addgene #200114) was integrated into the genome of MCF7 cells (ATCC) using piggyBac transposase, as described before.[24] The MCF7^{pKrox24DsRED} cells respond to treatment with recombinant FGF2 (RnD Systems) by inducing the expression of destabilized red fluorescence protein (DsRED). The MCF7^{pKrox24DsRED} cells were cultivated in DMEM containing 20% FBS and 1% penicillin/streptomycin. FGF2 (20ng/ml; RnD Systems #233-FB-025) and heparin (1 μg/ml; Merck Millipore #2106-10VL) were added to the media 24 hours before the in-cell NMR experiment.

Cell transfection and stable cell line generation

pB-CuRep and pB-Transp were co-transfected into HEK293T cells with FuGene HD Transfection Reagent (Promega). pB-TetRep and pB-Transp were co-transfected in the same manner into HEK293T and HEK293-TRex. The puromycin was added to the media 24 hours after transfection to select efficiently transposed cells. Several polyclonal cell lines (HEK293T$^{CuR\text{-}hSOD1/Poly}$, HEK293T$^{TR\text{-}hSOD1/Poly}$, HEK293-TRex$^{TR\text{-}hSOD1/Poly}$, HEK293-TRex$^{TR\text{-}TTHA/Poly}$, HEK293-TRex$^{TR\text{-}hCAII/Poly}$) were obtained after the selection. The addition of cumate or tetracycline induced the expression of the inserted gene. The intensity of the GFP signal (GFP reporter is co-expressed after tetracycline induction via an IRES element) was analyzed by FCM and fluorescent microscopy 48 hours after the induction. The polyclonal cell lines (HEK293-TRex$^{TR\text{-}hSOD1/Poly}$, HEK293-TRex$^{TR\text{-}TTHA/Poly}$, HEK293-TRex$^{TR\text{-}hCAII/Poly}$) generated monoclonal lines. Monoclonal cell lines (HEK293-TRex$^{TR\text{-}hSOD1/Mono}$, HEK293-TRex$^{TR\text{-}TTHA/Mono}$, HEK293-TRex$^{TR\text{-}hCAII/Mono}$) were obtained by separation of 5 % of the cells with the highest GFP signal on FACSAria Fusion cell sorter (see below). The intensity of GFP fluorescence of individual clones was analyzed 48 hours after tetracycline induction by BD FACSVerse flow cytometer, and the quantity of expressed proteins in the

Polyethylenimine (PEI) transfection was employed to transfect HEK293T cells with EGFP plasmid following a protocol by Banci et al.[25]

in-cell NMR sample preparation from stable cell lines

To express the protein of interest for NMR spectra acquisition, monoclonal cell lines (HEK293-TRex$^{TR\text{-}hSOD1/Mono}$, HEK293-TRex$^{TR\text{-}TTHA/Mono}$, HEK293-TRex$^{TR\text{-}hCAII/Mono}$) were treated with 3 µg/ml tetracycline 48 hours before collecting and loading into the bioreactor.

Cell synchronization in G1/S phase

HEK293-TRex$^{TR\text{-}TTHA/Mono}$ or HEK293-TRex$^{TR\text{-}hCAII/Mono}$ cells with ~ 40 % confluency were treated with tetracycline (3 µg/ml of media) 48 hours before the in-cell NMR experiment. About 33 hours later, the mimosine (Sigma Aldrich) was added to the media, and cells were incubated in 300 µM mimosine for 14 hours. After this, cells were harvested and loaded into the bioreactor (see below). Approximately 6 tissue culture flasks 75 cm^2 (TPP Techno Plastic Products AG) were needed to obtain ~400 µl of the G1/S cellular slurry. The bioreactor medium was supplemented with 300 µM mimosine to keep cells synchronized for 9 hours. A small fraction of the cells was stored for further analysis of cell viability as a synchronization control.

Cell synchronization in the G2/M phase

The initial steps of the G2/M synchronization were the same as in the G1/S synchronization. 24 hours after tetracycline induction, the RO3306 (Sigma Aldrich) was added to the media and HEK293-TRex$^{TR\text{-}TTHA/Mono}$ or HEK293-TRex$^{TR\text{-}hCAII/Mono}$ cells were incubated in 9 µM RO3306 for 24 hours. After this, cells were harvested and loaded into the bioreactor. Approximately 6 tissue culture flasks 75 cm^2 (TPP Techno Plastic Products AG) were needed to obtain ~400 µl of the cellular slurry. The bioreactor medium was supplemented with nocodazole (Sigma Aldrich) (300 ng/ml) to protect cells from escaping mitosis for 9 hours. A small fraction of the cells was stored for further analysis of cell viability and synchronization control.

Spheroid preparation

Spheroids for in-cell NMR were prepared using Elplasia plates (Corning®). Cells HEK293-TRex$^{TR\text{-}TTHA/Mono}$ and HEK293-TRex$^{TR\text{-}hCAII/Mono}$ cells were collected, washed with DPBS filtered on Cell Strainer 40 mm (VWR-Avantor), and counted in the Burker chamber. The cells were collected by centrifugation and resuspended in (isotopically ^{15}N-labeled) media supplemented with tetracycline and other compounds (see above). About 3.5 x 10^7 cells were loaded on one Elplasia 6-well plate, pre-wetted with media-containing tetracycline. Spheroids were collected after 48 hours of incubation, and ~ 350 µl of the spheroid pellet was used for the in-cell NMR sample.

Bioreactor setup

The cell / spheroid pellet was mixed with 2% SeaPrep® agarose (Lonza) in DMEM at 37 °C in a v:v ratio of 1:1 (HEK293-Trex cells: agarose) or 2:3:5 v:v:v (spheroids: MCF7-pKrox24^{DsRED} cells: agarose) in the case of preparation of an in-cell NMR sample of a 3D spheroid culture system consisting of two different cell lines. A PEEK capillary with a 0.75 mm inner diameter (or 1 mm inner diameter when preparing a sample from spheroids or spheroids/cell mixture) was filled with the mixture of agarose and cells/spheroid using a 1 ml syringe connected via a Luer adapter. The sample was incubated on ice for 10 minutes to solidify. A thread of the gel was then pushed with a syringe from the capillary into a 5 mm screw-cap NMR tube filled with bioreactor media: DMEM without $NaHCO_3$ (Sigma–Aldrich), 10% D_2O (Eurisotop), 70 mM HEPES (Sigma–Aldrich), ZellShield, supplemented with 10 µM $ZnSO_4$ when studying zinc containing metalloproteins. The NMR cuvette with the sample was connected to a tubing system, ensuring media flow. The flow of the bioreactor medium was driven by an HPLC pump (ECP2010, ECOM, Czech Republic) from a reservoir incubated in a water bath at 37 °C through a vacuum degassing system (DG 4014, ECOM, Czech Republic) to the bottom of the NMR cuvette via a glass capillary connected to the inlet tubing. The fresh medium flowed through the sample, displacing the nutrient-depleted medium drained via the outlet tubing connected to an orifice in the cuvette lid. The flow rate was set to 50 µl/min.

In-cell NMR spectroscopy

In-cell 1D ^1H NMR spectra and 2D ^1H – ^{15}N NMR spectra were recorded on a Bruker Avance NEO 950 MHz NMR (Bruker Corporation, Billerica, MA, USA) equipped with a 5 mm triple-resonance inverse cryoprobe. A JR-echo (1-1 echo) pulse sequence[26] with zero excitation set to the resonance of water and the excitation maximum set to 13 ppm was used to acquire 1D ^1H in-cell NMR spectra. Spectra were processed by the exponential apodization function and baseline corrected. A 2D ^1H – ^{15}N SOFAST-HMQC pulse sequence was used to acquire the in-cell NMR spectra from the proteins isotopically labeled with ^{15}N. The obtained spectra were processed by an exponential apodization function with the line-broadening parameter (LB) set to 10. The NMR spectra were processed and analyzed using Bruker Topspin 4.0 (Bruker Corporation, Billerica, MA, USA) and MNova v14.2.1 software (Mestrelab Research, Spain). All NMR spectra were measured at 37 °C.

Flow cytometry and sorting

FCM analysis of viability and GFP fluorescence: Roughly 10^5 cells were resuspended in 200 µl of DPBS buffer (Sigma-Aldrich). To distinguish the apoptotic, dead cells or cells with compromised membrane integrity from the living cells, the sample was stained with 1 µl (1 mg/ml) of propidium iodide (PI) (Exbio). The total amount of 10^4 cells was analyzed by a BD FACSVerse flow cytometer using BD FACSuite software V1.0.6 (BD Biosciences, USA). To detect the GFP fluorescence in the cells after tetracycline induction, the excitation and emission wavelengths were 488 nm and 527/32 nm, respectively. PI was excited at 488 nm to evaluate the cell viability, and the emission was detected at 700/54 nm.

Analyzing of DNA content in fixed cells stained with PI: The amount of 8x10^6 cells were fixed with ice-cold 70% (v/v) ethanol, incubated over-night at -20 ˚C, washed twice with ice-cold DPBS, and incubated 45 minutes at 37 ˚C in DPBS containing freshly added PI (50 µg/ml) and RNase A (200 µg/ml; Qiagen, 19101). Directly before FACS

analysis, 5mM EDTA was added into the suspension and cells were filtered by Non-sterile CellTrics™ filters with 5µm pores (Sysmex). For FACS analysis, the excitation wavelength was set at 488 nm for PI to visualize the DNA content. The PI emission was detected at 700/54. FACS sorting – generation of monoclonal cell lines: The polyclonal cell lines (HEK293-TRex$^{TR\text{-}hSOD1}$, HEK293-TRex$^{TR\text{-}TTHA}$, HEK293-TRex$^{TR\text{-}hCAII}$) collected 48 hours after tetracycline addition were resuspended in FACS buffer (PBS, 2% FBS, 5mM EDTA) and sorted by FACSAria Fusion cell sorter using BD FACSDiva software V8.0.1 (BD Biosciences, USA). To obtain monoclonal cell lines (HEK293-TRex$^{TR\text{-}hSOD1/Mono}$, HEK293-TRex$^{TR\text{-}TTHA/Mono}$, HEK293-TRex$^{TR\text{-}hCAII/Mono}$), approximately 5 % of the cells with the highest GFP signal were sorted into the 96-well plate by one cell per 1 well. Supplementary Figures 4 and 5 exemplify the employed gating strategy.

SDS PAGE

SDS-PAGE was performed on Mini Protean Vertical Electrophoresis (BioRad). Cells or spheroids from monoclonal cell lines were collected 48 hours after tetracycline addition and lysed in RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0,5% sodium deoxycholate 0,1% SDS) supplemented with cOmplete™ EDTA-free Protease Inhibitor Cocktail (Roche). Total protein concentration was determined by Bradford assay (BioRad). Equal amounts of proteins were run on 16% Tris-Tricine SDS-PAGE in Tris-Tricine running buffer. Afterward, the gels were stained with Coomassie blue solution.

Microscopy

The microscopy images of the living cells monitoring GFP signal intensity after tetracycline/cumate treatment were obtained using a CELEROMICS optical system connected to an inverted fluorescence microscope NIB-100F (Novel Optics). Light transmission images were obtained with objectives PLAN PH 4x/0.1 and PLAN PH 10x/0.25. The objective PLAN PH 4x/0.1 and PLAN F 10x/0.3 were used for fluorescence mode. GFP signal was analyzed with an excitation wavelength of 460-490 nm and detected at emission wavelengths over 520 nm. The excitation wavelength for DsRed was set to 480-550 nm, and the emission was detected at over 590 nm. The images were collected and analyzed in CELEROMICS software. For the immunofluorescence analysis, the monoclonal cells were plated on 0.02% gelatine-coated coverslips in 24 well plates and treated with tetracycline. 48 hours after tetracycline addition, coverslips were washed once with DPBS (Sigma Aldrich) and fixed in a 4% paraformaldehyde (ChemCruz) and 0.02% sucrose (AppliChem) DPBS solution for 10 min at room temperature. The cells were washed twice with DPBS and incubated overnight at -20 °C with ice-cold methanol. Samples were washed with DPBS and blocked for 30 minutes with 3% BSA and 0.05% Tween20 in DPBS (PBS-BSA-T) solution for 30 minutes and incubated over-night at 4 °C with mouse monoclonal anti-flag antibody (Sigma Aldrich, F1804, dilution 1:500) in PBS-BSA-T solution and washed three times with DPBS - 0.05% Tween20 (PBS-T) solution. Subsequently, the samples were incubated with goat anti-mouse Alexa 594 conjugated antibody (Jackson Immune Research, 115-585-146, dilution 1:500) PBS-BSA-T solution. Cells were washed three times with PBS-T solution, and coverslips were dried and mounted with VECTASHIELD® PLUS Antifade Mounting Medium with DAPI (H-2000) (Vector Laboratories). The microscopy images were obtained using a Zeiss LSM 800 confocal microscope with an Apochromat 63x / 1.40 OIL objective. The GFP signal was analyzed with an excitation wavelength of 488 nm, for the GFP signal was detected at emission wavelengths of 502-558 nm. The excitation wavelength for DAPI was set to 405 nm, and the emission was detected at 400- 488 nm. The excitation wavelength for Alexa 594 was set to 594 nm, and the emission was detected at 604-700 nm. The images were collected and analyzed in ZEN Blue 2.6 and 3.1 software. The microscopy images of the samples mimicking the tissue (a small fraction of agarose threat containing mixture HEK293-TRex$^{TR\text{-}hCAII}$ spheroids and MCF7^{pKrox24DsRED} cells) were obtained using a Zeiss LSM 700 Axio scanning confocal microscope with a N-Achroplan 10x/0.3 Wdip objective. GFP signal was analyzed with an excitation wavelength of 488 nm and detected at 300-550 nm emission wavelengths. The excitation wavelength for DsRED was set to 555 nm, and the emission was detected at 560-800 nm. The images were collected and analyzed in ZEN Blue 2.6 and 3.1 software.

ASSOCIATED CONTENT

Supporting Information

Supplementary Figures 1 - 5 are available in the file Rynes_et_al_SI_20032024 (PDF)

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S.F.-T., L.T., J.R., L.Banci, P.K., L.M., and E.L. conceived and designed the study; E.I., M.K., E.L., B.F., L.Barbieri., K.K., and S.F.T. performed cloning; J.R., E.I., M.K., and S.F.T. conducted in-cell NMR measurements; M.K., E.I., J.R. T.J., K.K., B.F., L.M., and G.R.-L. contributed to developing protocol for acquiring in-cell NMR data in distinct cell cycle phases and 3D cell cultures; J.R., E.I., M.K., T.J., K.K. and S.F.-T. performed data processing; E.L., L.Banci, S.F-T., and L.T. supervised research activities; J.R., E.I., M.K., T.L., and S.F.-T. took care of data visualization; L.T. and S.F.-T. wrote the first original draft; all authors contributed to the manuscript review & editing; L.T., E.L., L.Banci, P.K., B.F., G.R., and S.F.T. secured the funding. [#]J.R., E.I. and M.K. contributed equally to this paper.

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