Supplementary Information

Combining in-cell NMR and X-ray fluorescence microscopy to reveal intracellular maturation states of human superoxide dismutase 1

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Supplementary Methods

Cell growth and transfection

HEK293T cells were cultured and transfected as previously described.[1] For protein overexpression, cells were transfected with the pHLsec[2] vector containing the cDNA of human SOD1 (1–154, GenBank: NP_000445.1) and human CCS (1–274, GenBank: NP_005116.1). Control cells were transfected with empty pHLsec vector. Cells expressing either SOD1 or CCS alone were transfected with a 1:1 mixture of cDNA and empty vector; cells co-expressing SOD1 and CCS were transfected with a 1:1 mixture of the two cDNA.

In-cell NMR experiments

Samples for in-cell NMR spectroscopy were prepared by treating cells transfected with either SOD1 alone or SOD1 and CCS with 10 μM $ZnSO_4$. For copper delivery experiments cells were incubated with 50 μM $CuCl_2$ 48 h after transfection.[1] NMR experiments were acquired at a 950-MHz Bruker Avance III spectrometer equipped with a CP TCI CryoProbe. 1D ^{1}H and 2D ^{1}H-^{15}N SOFAST HMQC[3] spectra were acquired at 305 K. Identical NMR experiments were acquired on the supernatant of each cell sample to exclude protein leakage out of the cells. Cell viability was checked by trypan blue staining after the experiments, and remained above 90%.

Immunofluorescence microscopy

Cells were grown either on glass coverslips or on Si_3N_4 membranes (Silson, 1.5 mm wide, 100 nm thick window) in 6-well plates. Cells were transfected with different levels of SOD1 and CCS, and were treated with different amounts of metals during protein expression. Alternatively, cells were first grown and transfected on the bottom of the 6-well plates and, after protein expression, they were trypsinized and re-seeded on Si_3N_4 membranes for immunofluorescence and XRF analysis. The cells were fixed with 4% paraformaldehyde (in PBS, pH 7.4) and permeabilized with 0.1% Triton X-100 (in PBS, pH 7.4). Cells were rinsed in PBS after each step. Blocking was carried out with 10% normal goat serum (Invitrogen) in PBS. Cells were incubated with rabbit polyclonal anti-SOD1 antibody (Abcam: ab16831, diluted 1:500) and mouse monoclonal (3A1) anti-CCS antibody (Abcam: ab16964, diluted 1:500); Alexa Fluor 488 Goat Anti-Rabbit IgG and Alexa Fluor 568 Goat Anti-Mouse IgG (Life Technologies) fluorescent secondary antibodies were used (both diluted 1:1000). Cells were counterstained with DAPI and washed with PBS. Cells grown on glass coverslips for intracellular protein localization were mounted on microscope slides with an antifade reagent (Prolong Gold, Life Technologies); cells grown on Si_3N_4 membranes were thoroughly washed with milli-Q H_2O and subsequently air-dried. Optical micrographs of the cells were

acquired through a 10x 0.3NA air objective, using a Leica DM6000B microscope and a Leica DC350FX camera). Leica fluorescence filter set A4, L5 and N3 were used for DAPI, Alexa Fluor 488 and Alexa Fluor 568, respectively.

X-ray fluorescence microscopy

XRF microscopy measurements were performed at the TwinMic beamline of Elettra Synchrotron (Trieste, Italy).[4] The TwinMic microscope was set up in Scanning Transmission mode where the sample is raster-scanned across a microprobe delivered by a zone plate diffractive optics. For the present measurements an X-ray beam spot size of 1 μm diameter was chosen at energy of 1.225 keV to get best excitation of Cu and Zn L emission lines and a sufficient lateral resolution, able to highlight the features of interest. The X-ray photons transmitted by the sample are collected by a Fast Readout CCD through a X-ray - visible light converting system, generating absorption and phase contrast images simultaneously.[5,6] At the same time the XRF signal is acquired by 8 Silicon Drift Detectors facing the sample in an annular way.[7,8] The XRF maps were obtained by deconvoluting and batch-fitting the XRF spectra with PyMCA software.[9]

Data analysis

Optical fluorescence images and XRF elemental maps were analyzed using ImageJ.[10] The concentration values of SOD1 (expressed in molar concentration) and CCS (expressed as relative overexpression level) for each cell were obtained as follows. The total fluorescence intensity for each cell F_i is considered proportional to the total amount of protein: $F_i = \alpha \cdot c_i V_i$, where c_i and V_i are protein concentration and volume, respectively. The total X-ray scattering intensity S_i is proportional to the cell volume: $d \cdot V_i = S_i$, giving $F_i = \alpha/d \cdot c_i S_i$. Therefore, the protein concentration in each cell can be calculated via the formula:

$$c_i = \frac{d}{\alpha} \cdot \frac{F_i}{S_i}$$

The scale factor d/α was calculated by measuring the average fluorescence intensity $\bar{F}_0$ of cells expressing endogenous levels of protein $\bar{c}_0$ (10 μM for SOD1 and 1× for CCS), which were analyzed by optical fluorescence microscopy but not by XRF, and using the averaged X-ray fluorescence intensity $\bar{S}$ of the cells analyzed by XRF:

$$\frac{d}{\alpha} = \bar{c}_0 \cdot \frac{\bar{S}}{\bar{F}_0}$$

Supplementary Figures

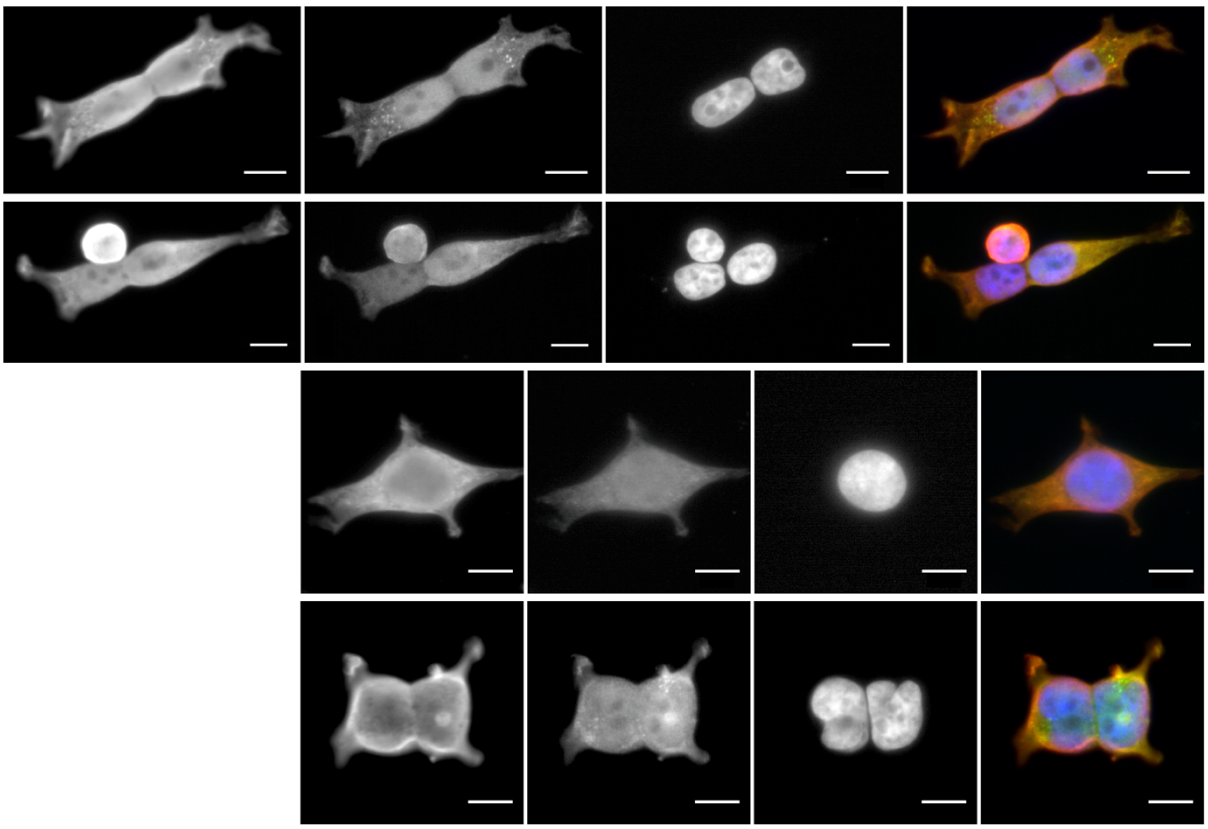

Fig. S1. Intracellular localization of SOD1 and CCS is mainly cytoplasmic. HEK293T cells co-expressing SOD1 and CCS. Cells were fixed, double-stained with anti-SOD1, anti-CCS primary antibodies, fluorescent secondary antibodies and detected by epi-fluorescence microscopy. Nuclei were counterstained with DAPI (blue). From left to right: red channel (CCS), green channel (SOD1), blue channel (DAPI), composite. Both SOD1 and CCS are mainly localized in the cytosol, and to a lesser extent in the nucleus. In some cells a small fraction of SOD1 appears localized in vesicles. Scale bar = 10 μm.

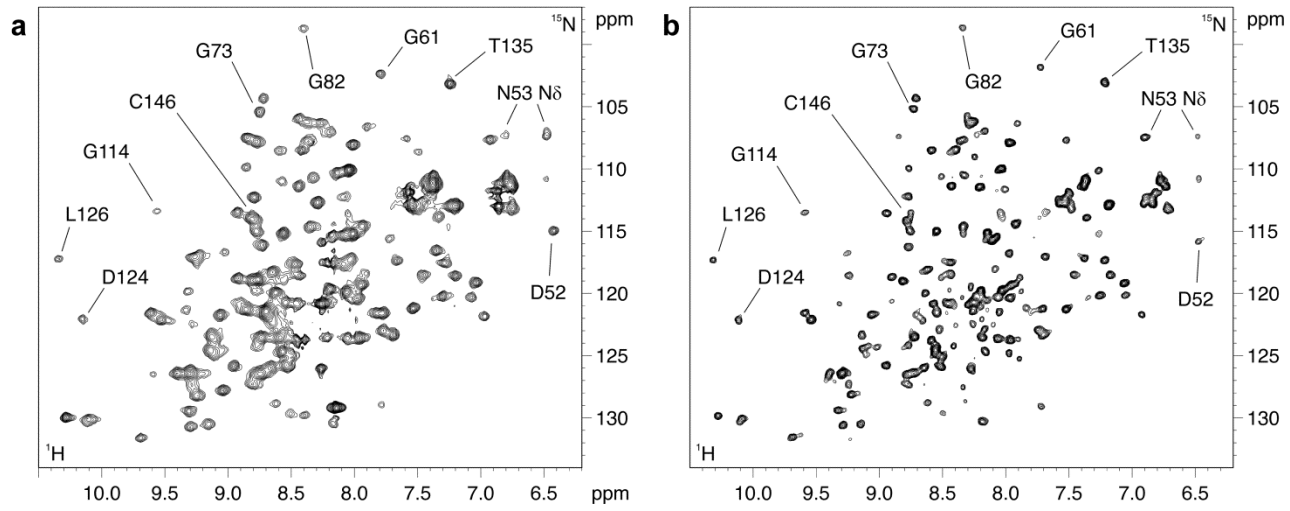

Fig. S2. In cells treated with zinc, SOD1 is present as E,Zn-SOD1^{SH}. (a) ^{1}H-^{15}N correlation NMR spectrum acquired on human cells expressing uniformly ^{15}N-labeled SOD1 in zinc-supplemented medium. (b) ^{1}H-^{15}N correlation NMR spectrum acquired on the purified protein, in the E,Zn-SOD1^{SH} form, which is homodimeric, with one zinc ion bound per monomer and all the cysteines reduced (adapted from (11)). Minimal chemical shifts differences between the two spectra indicate that the intracellular protein conformation is the same as *in vitro*. The assignment of crosspeaks which are indicative of the protein quaternary structure (G114), of its metallation state (G61, G73, G82, D124, L126, T135) and of the cysteine redox state (C146, D52, N53 Nδ) is reported on both spectra.

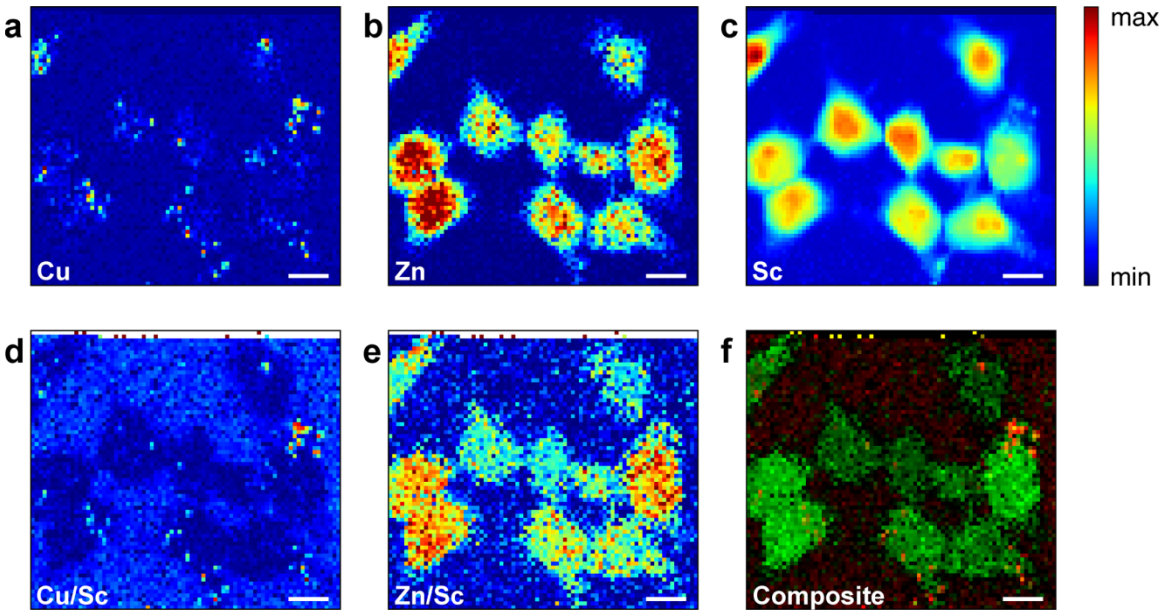

Fig. S3. XRF microscopy image analysis. (a-c) colour-coded maps obtained by deconvolution analysis from the X-ray fluorescence spectral data showing the spatial distribution of copper (a) and zinc (b), and the X-ray scattering intensity (c). To take into account differences in sample thickness, the copper and zinc maps were divided by the scattering map, and the obtained maps (d, e) are proportional to the element concentration averaged over the sample thickness in each pixel. (f) shows the resulting composite image showing both copper (red) and zinc (green) concentration maps. Scale bar = 10 μm.

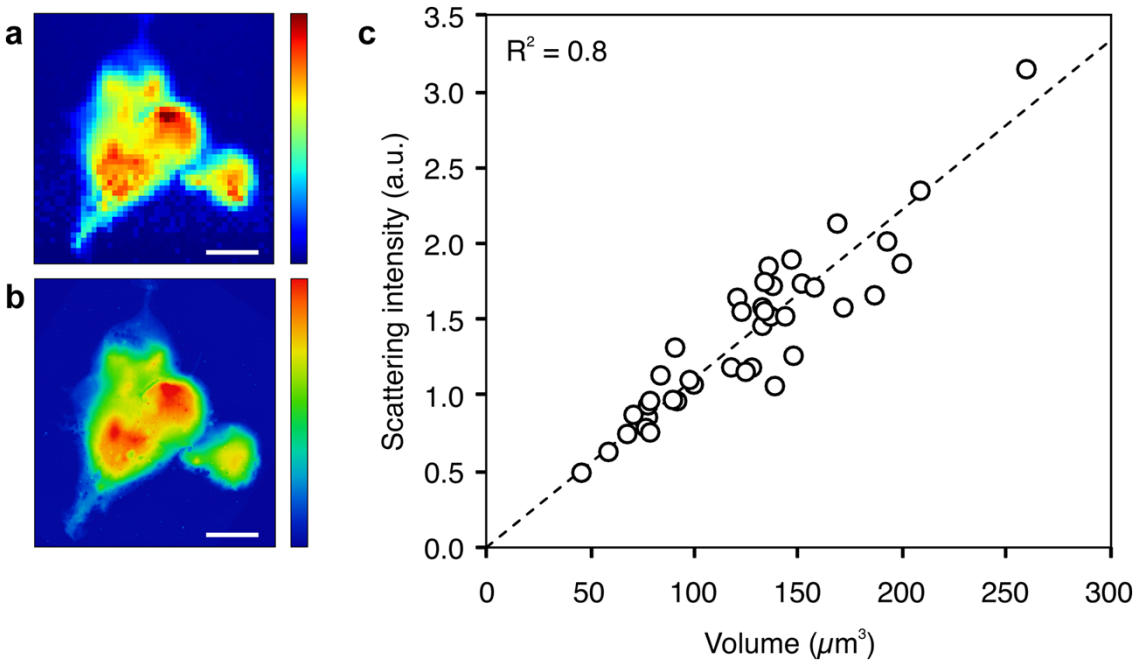

Fig. S4. The X-ray scattering intensity is proportional to the sample thickness. (a) colour-coded X-ray scattering intensity map obtained by deconvolution analysis from the X-ray fluorescence spectral data; (b) colour-coded surface scan of the same sample area obtained by AFM. Scale bar = 10 µm. (c) Total X-ray scattering intensity of each cell plotted against the cell volume measured from the AFM scans. Each dot corresponds to a single cell. A linear relationship between the two values is observed ($R^2 = 0.8$).

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