Electronic supplementary information

L-cysteine-mediated modulation of copper trafficking in prostate cancer cells: an *in vitro* and *in vivo* investigation with ^{64}Cu and ^{64}Cu-PET

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Supplementary methods

In vitro cell uptake of ^{64}Cu

Cells were seeded in 24-well plates 16-24 hours before the experimental protocol. Seeding densities (cells/well) were as follows: DU145: 125,000, PC3: 150,000, IGROV1, SKOV-3: 200,000 and MDA-MB-231: 250,000. Immediately before the experiment, cells were washed twice with 400 µL of phosphate-buffered saline (PBS, D5652, Sigma). 200 µL of the incubation medium containing 10 kBq of [^{64}Cu]$CuCl_2$ was applied. Incubation media were: Hank's Buffered Salt Solution (HBSS, H8264 Sigma), DMEM, human serum (obtained from healthy volunteers) or mouse serum (M5905, Sigma) supplemented with amino acids as indicated in the manuscript. Incubation media were mixed with [^{64}Cu]$CuCl_2$ by vortexing 5 minutes prior to incubation. Plates were kept in the humidified incubator at 37 C° under 5% CO_2 for 7-180 minutes. Afterwards, the incubation media were removed and wells were washed twice with 400 µL of ice-cold PBS. Cells were then lysed using 200 µL of 0.1 M NaOH and wells were washed with 400 µL of PBS. Incubation media, all washes and cell lysates were collected in Eppendorf tubes and measured in the Wallac Gamma Counter.

To obtain the concentration of ^{64}Cu in the intracellular and extracellular fractions, radioactive counts associated with the cell pellet and with the supernatant were divided by their corresponding total volumes. Diameters of the cells used for volume calculations (using a sphere formula) were obtained from Nexcelom Bioscience database[1] and are as follows: MDA-MB-231: 15.50 µm, DU145: 15.07 µm, PC3: 18.08 µm, IGROV-1: 16.66 µm, SK-OV-3: 17.20 µM. Diameter of A375 cells (17.50 µm) was estimated based on the published microscopic images[2].

Western blotting of subcellular fractions

Abcam's cell fractionation kit (109719) was used to prepare highly enriched cytoplasmic, mitochondrial and nuclear fractions. All steps were performed as described in the fractionation kit protocol, except that buffer A was customised so that it did not contain EDTA (ethylenediaminetetraacetic acid). Protein concentration in the subcellular fractions was measured by Thermo Scientific™ Pierce™ BCA Protein Assay Kit (23227). Samples for western blotting were diluted in NuPage 4x LDS buffer (NP0008, Invitrogen) to the final protein concentration of 1 µg/µl and processed according to manufacturer's instructions. 50 mM DTT was added for reducing conditions. 8 µl of samples (8 µg of protein) was loaded onto NuPAGE 4 to 12% Bis-Tris gels (NP0322PK2, Invitrogen), along with the protein ladder (1610374, Bio-Rad). Gels were run in NuPAGE MOPS SDS Running Buffer (NP0001, Invitrogen) and transferred onto 0.45 µm PVDF Transfer Membranes (88585, Thermo Fisher). Membranes were blocked for 1 hour at RT in 5% milk in TBST buffer (Tris-buffered

saline with 0.1% Tween-20). All antibodies were diluted in 5% bovine serum albumin in TBST and 5 x 5 minutes washes were done after each blocking and incubation step. Incubation with primary antibodies was done overnight at 4°C. Rabbit primary antibodies were from Cell Signalling: MAPK/ERK kinase (MEK) 1/2 (9122, used 1:1000), apoptosis inducing factor (AIF) (D39D2, used 1:500). Secondary horseradish peroxidase (HRP)-conjugated anti-rabbit mouse antibody was from Santa Cruz Biotechnology (sc-2357, used 1:5000), incubation was done for 1 hour at RT. Proteins were visualised by chemiluminescent detection using Pierce ECL Plus Western Blotting Substrate (Thermo Scientific 32134) and GE Healthcare Amersham Hyperfilm (10752067).

Size-exclusion chromatography of intracellular ^{64}Cu

Following incubation of cells with [^{64}Cu]$CuCl_2$ and PBS washes, 100 µL of Triton-X lysis buffer was used to harvest cells. Triton-X buffer composition was: 150 mM NaCl, 0.1% (v/v) Triton X-100 (Sigma, T8532), 50 mM TRIS-HCl (Trisma base from Sigma, 93350), 10% (v/v) glycerol. Lysates were spun at 16,000 relative centrifugal force (RCF) for 10 minutes and the supernatant was used for further analysis. 50 µL of the cell lysates or size standards (1 mg/mL GSH, mouse serum from Sigma, 10 mM L-cysteine) were loaded onto PD MidiTrap G-25 columns (GE Healthcare), previously equilibrated with 15 mL of the mobile phase (0.9% (w/v) NaCl with 1 mM sodium ascorbate). The void column volume was collected in the first 1 mL of the eluting buffer and named fraction 0; applied samples were then eluted in 30 x 100 µL fractions. Radioactivity of the fractions and columns was measured in the Wallac Gamma Counter.

Supplementary figures

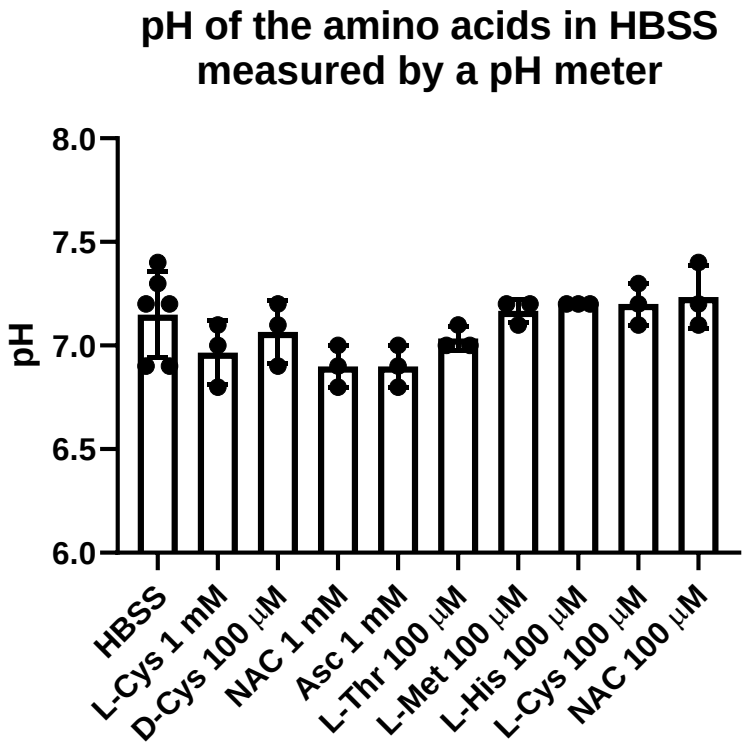

Fig. S1 pH measurement of the amino acid solutions prepared in HBSS at the concentrations used in the *in vitro* ^{64}Cu uptake assays. Measurements were done in triplicates at room temperature using a benchtop pH meter (SevenCompact S220, Mettler Toledo) and show that the pH of all solutions was between 6.9 and 7.2. There were no significant differences between the pH values for HBSS and amino acids, tested using one-way Anova with a *post-hoc* Dunnett's test for multiple comparisons.

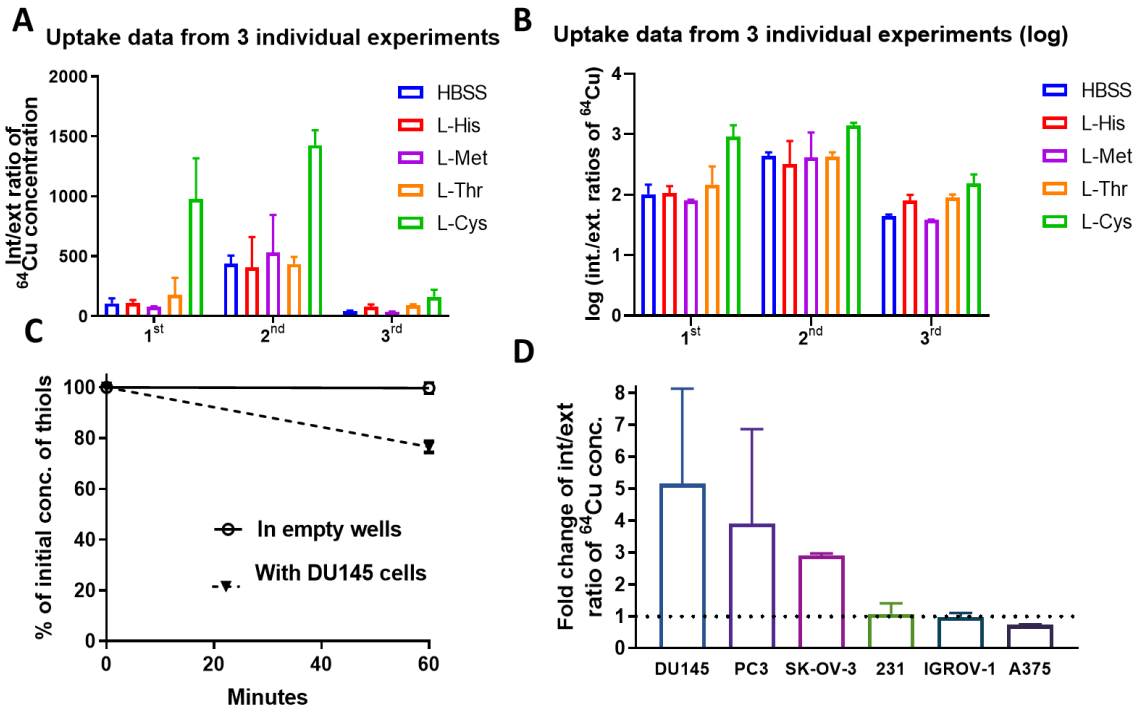

Fig. S2 The effect of added amino acids on ^{64}Cu accumulation in cancer cells. (A) ^{64}Cu accumulation (90 min) in DU145 cells in HBSS (control) with added L-histidine, L-methionine, L-cysteine and L-threonine (100 µM). Graph A represents the intracellular/extracellular ratios of ^{64}Cu concentrations from 3 independent experiments (denoted as 1st, 2nd, 3rd) to demonstrate the variability in the basal uptake in HBSS between experimental days. In order to account for this variability, data was converted to their logarithms (B) and values obtained within one experimental day were matched in further statistical analysis (one-way Anova with *post-hoc* Dunnett's test). Presented bars are means ± SD of technical replicates within each experiment. (C) Oxidation of L-cysteine in the HBSS buffer with [^{64}Cu]$CuCl_2$ (50 kBq/mL). HBSS was mixed with [^{64}Cu]$CuCl_2$ and immediately added to the 24-well plate (either empty or with DU145 cells). Samples of HBSS were removed from the wells upon addition (t=0) and after 60 min incubation at 37°. The concentration of thiol groups was derived using Ellman's reagent and normalised. Graph shows results from one exemplar experiment. (D) ^{64}Cu accumulation in several cancer cell lines in HBSS with added L-cysteine (100 µM). Du145 (90 min, *n*=3) and PC3 (90 min, *n*=3) - prostate cancer, SK-OV-3 (60 min, *n*=2) and IGROV-1 (60 min, *n*=2) - ovarian cancer, MDA-MB-231 (60 min, *n*=2) - breast cancer, A375 (60 min, *n*=2) - melanoma. Graphs represent the intracellular/extracellular ratios of ^{64}Cu concentrations normalised to HBSS control (mean ± SD).

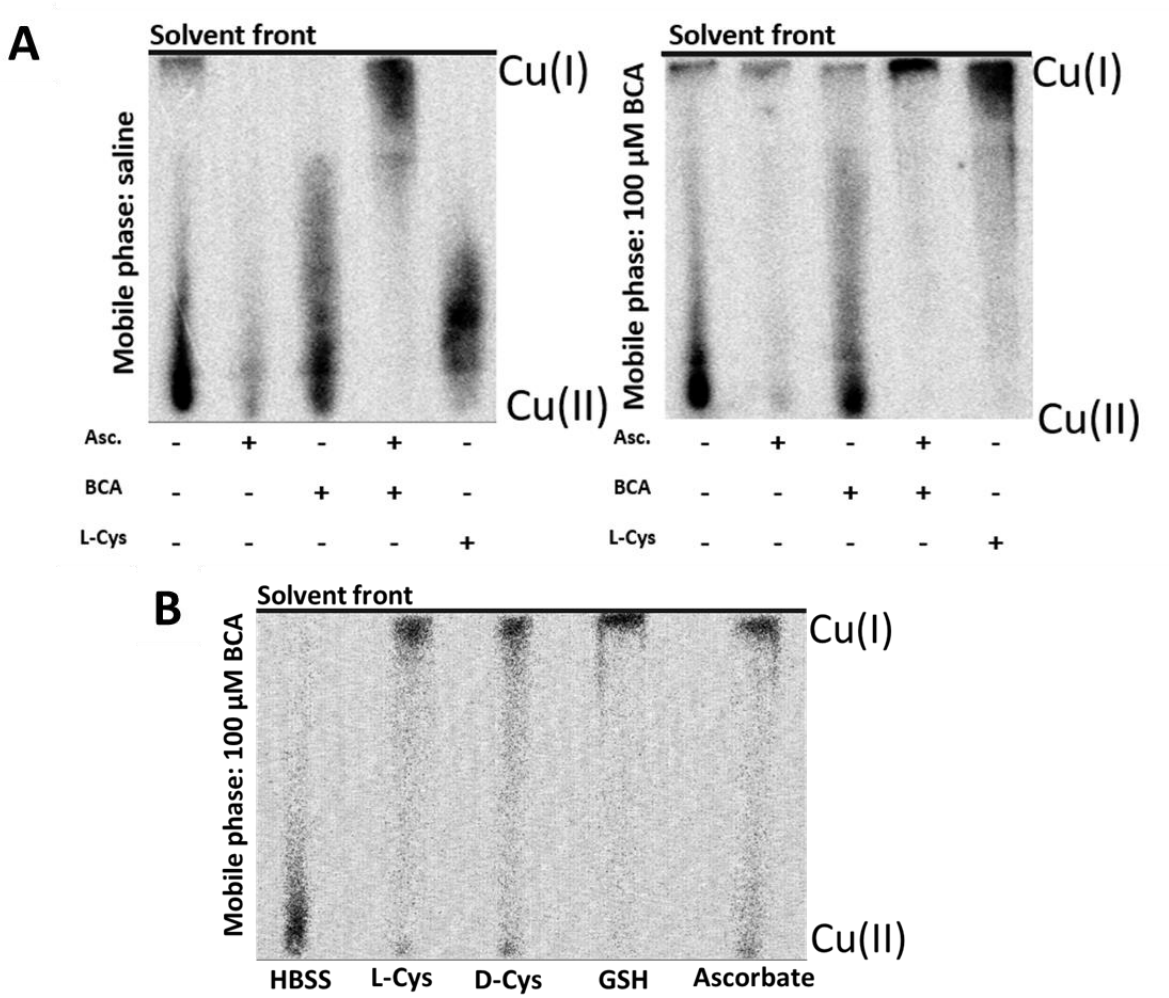

Fig. S3 A-B: Assessing ^{64}Cu(II) to ^{64}Cu(I) reduction by the iTLC SG method. (A) Method validation. [^{64}Cu]$CuCl_2$ was added to 50 µL saline in the presence of reducing agents (1 mM ascorbate or 100 µM L-Cys) and a Cu(I)-specific chelator bicinchoninic acid (BCA, 1 mM). 2 µl of these mixtures were analysed by iTLC SG using either saline (left panel) or 100 µM BCA as mobile phase (right panel). Left panel shows that the shift from a retention factor 0 (^{64}Cu(II)) to 1 (^{64}Cu(I)) only occurs if both a reducing agent and BCA are mixed with ^{64}Cu sample, but does not occur with either of them on their own. Right panel shows that if BCA is present in the mobile phase (100 µM), addition of a reducing agent (ascorbate or L-cysteine) changes the retention factor of ^{64}Cu from 0 to 1. (B) Method from panel A with 100 µM BCA as mobile phase was used to assess ^{64}Cu reduction in HBSS. [^{64}Cu]$CuCl_2$ was added to HBSS containing: L-cysteine or D-cysteine (100 µM), GSH (10 µM) and ascorbate (1 mM), and incubated at RT for 1 h before analysis by iTLC.

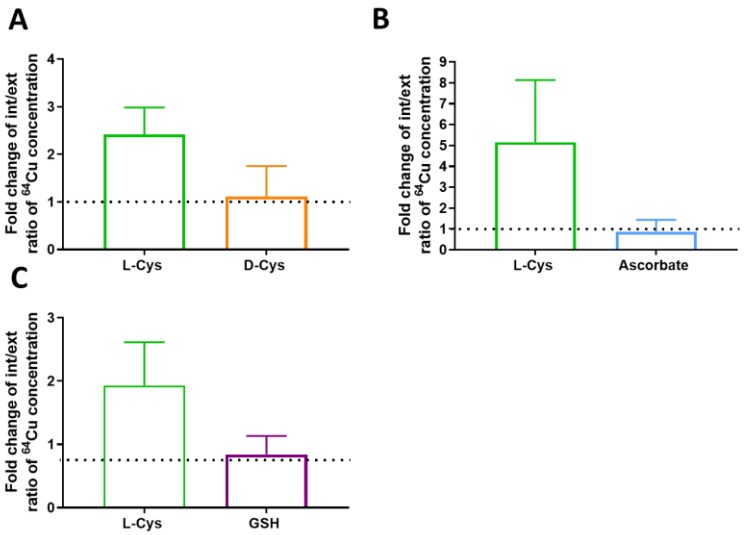

Fig. S4 The effect of reducing agents on ^{64}Cu accumulation in DU145 cells, tested in three separate experiments (A, B and C), which for clarity were presented together in Fig. 3. A: L-Cys and D-cysteine (D-Cys) - 100 µM, 90 min. B: L-Cys - 100 µM, ascorbate – 1 mM, 90 min. C: L-Cys - 10 µM, glutathione (GSH) - 10 µM, 60 min, *n*=3. ^{64}Cu(II) to ^{64}Cu(I) reduction by all reductants was demonstrated by ITLC SG method, shown in Fig S3B. All experiments were done in HBSS and normalised to HBSS control.

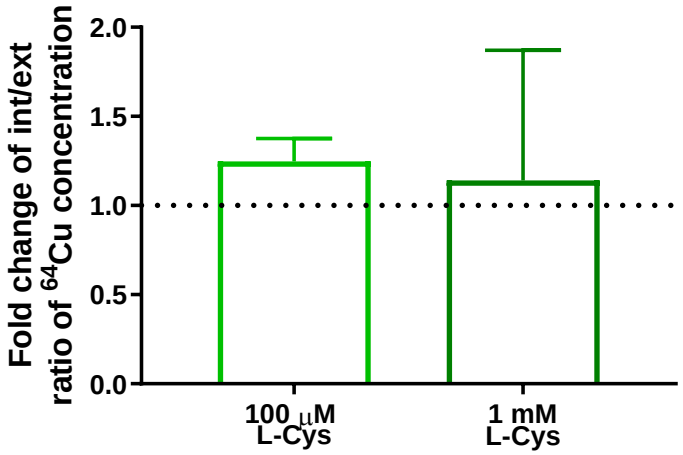

Fig. S5 The effect of L-Cys on ^{64}Cu accumulation in DMEM. Graphs represent the intracellular/extracellular ratios of ^{64}Cu concentrations normalised to HBSS control (mean ± SD, *n*=3). DU145 cells were incubated with ^{64}Cu in DMEM with/without added L-Cys for 60 min.

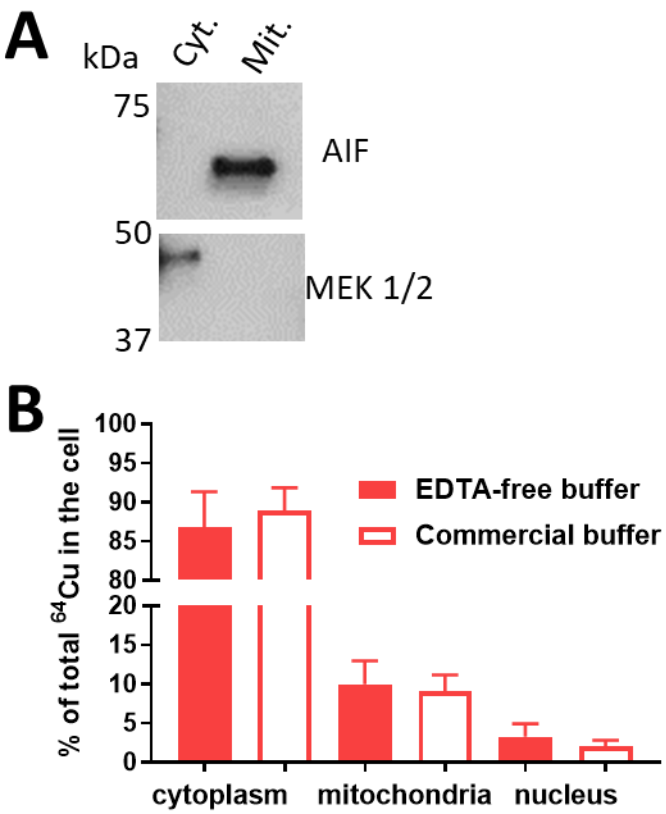

Fig. S6 Intracellular localization of ^{64}Cu taken up by DU145 cells. DU145 cells were incubated with ^{64}Cu for 90 min in HBSS with 1% FBS. Cells were harvested and separated to cytoplasmic, mitochondrial and nuclear fractions using Abcam fractionation kit with original buffer A ('commercial buffer') or EDTA-free buffer A. The rest of the kit components were used as per manufacturer's instructions. (A) Samples fractionated with EDTA-free buffer were analysed by western blotting. Briefly, gels were loaded with 8 µg of protein/lane and immunoblotted with primary antibodies against MAPK/ERK kinase (MEK) 1/2 (present in cytoplasm) and apoptosis inducing factor (AIF, present in mitochondria), followed by secondary HRP-conjugated antibodies and chemiluminescent detection. (B) Effect of EDTA in the fractionation buffer on ^{64}Cu distribution across fractions (n=4).

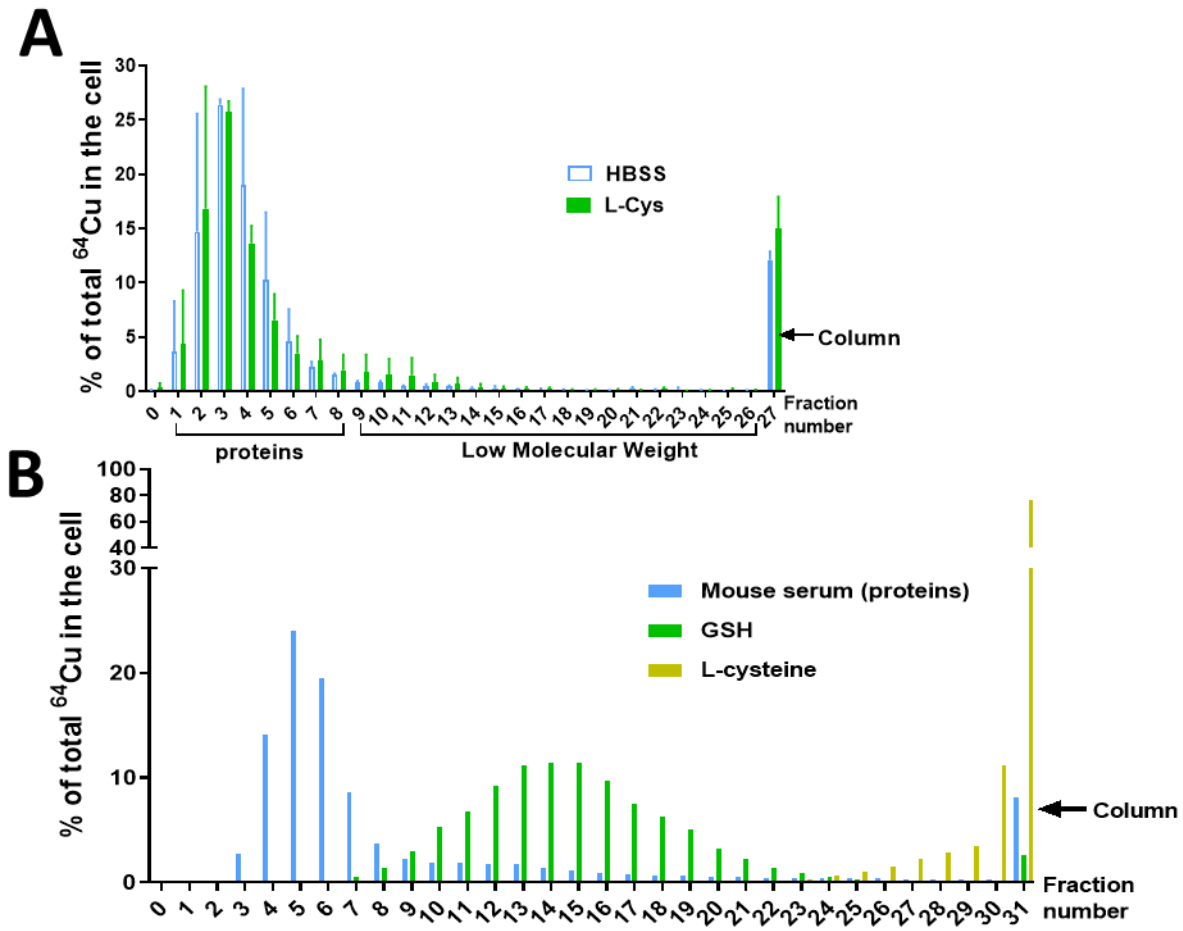

Fig. S7 Intracellular speciation of ^{64}Cu taken up by DU145 cells. DU145 cells were incubated with ^{64}Cu for 90 min in HBSS with 1% FBS, with/without 100 µM L-Cys. Cells were then harvested and lysed using 0.1% Triton-X buffer. Cell lysates were fractionated (100 µL fractions) by size-exclusion chromatography using Sephadex column and saline with 1 mM sodium ascorbate as mobile phase, *n*=2. Radioactivity in the fractions was measured by gamma counting. Graph shows that regardless of the addition of L-cysteine, majority of the intracellular ^{64}Cu associates with proteins. (B) Size standards for the size-exclusion chromatography profile. Mouse serum, GSH or L-cysteine were incubated with ^{64}Cu for 15 minutes and processed as above. Samples 1-8 were assigned as protein fractions since they predominantly contained radioactivity associated with mouse serum, without a significant signal from the radiolabelled GSH (low molecular weight standard).

1. C. L. Kuksin D, Analyzing NCI-60 Cancer Cell Lines, https://www.nexcelom.com/training-and-support/white-papers/accurately-measure-cell-size-of-nci-60-cancer-cell-lines/ (accessed April 2020).

2. M. A. Gonda, S. A. Aaronson, N. Ellmore, V. H. Zeve and K. Nagashima, Ultrastructural studies of surface features of human normal and tumor cells in tissue culture by scanning and transmission electron microscopy, *J Natl Cancer Inst*, 1976, **56**, 245-263.