Supplementary Materials
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Materials and methods

Gene cloning

The cDNA sequences for SLC3A1 (NM_000341.4) and SLC7A9 (NM_001243036.2) were synthesized and cloned into the pLV3-CMV-MCS-3×Flag-EF1alpha-copGFP-Puro plasmid vector. In addition to the wild-type constructs, single-residue mutant plasmids for SLC7A9 were generated by site-directed mutagenesis. The mutations introduced were A70V, G105R, V170M, A182T, A354T, R333W, P482L, and P482G. For solute carrier family 3 member 1 (SLC3A1), a single-point mutation (M467T) was also introduced. All wild-type and mutant constructs were verified by Sanger sequencing.

Cell Lines and transfection

Human Embryonic Kidney 293 (HEK293) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (volume fraction) fetal bovine serum (FBS, Sigma, USA), 1%（0.1 g/L）penicillin-streptomycin, and incubated at 37 ℃ in a humidified environment with 5% CO_2. Plasmids were transfected into HEK293 cells using Lipofectamine 2000 (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Forty-eight hours post-transfection, cells transiently expressing the exogenous wild-type or mutant transporters were re-seeded into multi-well plates for analysis.

Atomic absorption spectroscopy

HEK293 cells transiently co-expressing SLC3A1 and SLC7A9 were washed three times with cystine-free Hanks' Balanced Salt Solution (HBSS) to deplete endogenous cystine. Cells were then incubated with 200 μmol/L selenocystine in cystine-free HBSS at 37 ℃ for 30 min. Subsequently, cells were washed three times with ice-cold HBSS to remove extracellular selenocystine, followed by two washes with ice-cold phosphate-buffered saline (PBS) to minimize salt interference. Washed cells were harvested and digested using microwave-assisted digestion in concentrated nitric acid (HNO_3) using the following program: 150 ℃ for 10 min, followed by 180 ℃ for 20 min to mineralize organic material and liberate selenium atoms. The acid was evaporated to near-dryness under a fume hood, and digested samples were reconstituted in 2% (volume fraction) nitric acid and analyzed by graphite furnace atomic absorption spectroscopy (GFA-6880, Shimadzu Corporation, Japan). Selenium was quantified at its characteristic absorption wavelength (196 nm) using a selenium-specific hollow cathode lamp (HAS-1, Guobiao (Beijing) Testing & Certification Co., Ltd, China). Quantitation was performed against a matrix-matched standard curve generated from certified selenium reference solutions.

Cystine uptake assay

HEK293 cells were seeded at 2.5×10^4 cells per well in a poly-l-lysine coated black, clear-bottom 96-well plate and cultured overnight at 37 ℃ in a humidified 5% CO_2 atmosphere. The following day, the growth medium was removed. Cells were pre-incubated with pre-warmed HBSS at 37 ℃ for 5 min to ensure cystine deprivation, then washed twice with cystine-free HBSS. For dose-response experiments, cells were incubated with selenocystine at 0, 25, 50, 100, 200, 400, or 600 μmol/L in cystine-free HBSS at 37 ℃ for 30 min. For time-course experiments, cells were incubated with 200 μmol/L selenocystine at 37 ℃ for 0, 10, 20, 30, 45, or 60 min. Control wells received cystine-free HBSS without substrate.

The 30-min end-point used for the standard assay was chosen on the basis of the time-course data, which showed that uptake is linear through approximately 45 min before beginning to saturate. After incubation, cells were washed three times with ice-cold HBSS and lysed with ice-cold methanol. Lysates were then incubated at 37 ℃ for 30 min in 100 mmol/L MES (2-morpholinoethanesulphonic acid) buffer (pH 6.0) containing 10

μmol/L Fluorescein *O*,*O*'-diacrylate (FOdA, Sigma #7262-39-7, USA) and 200 μmol/L tris (2-carboxyethyl) phosphine hydrochloride (TCEP, Sigma #51805-45-9, USA). Fluorescence intensity was measured using a microplate reader (Infinite M200PRO, TECAN, Switzerland) with excitation at 485 nm and emission at 535 nm. Background fluorescence, determined from identically treated blank control wells (without selenocystine), was subtracted from every sample reading. Furthermore, wells transfected with empty pCDNA3.1 vector (without any transporter construct) served as the negative expression control, confirming that non-specific selenocystine incorporation was negligible.

Structure prediction of SLC3A1 and SLC7A9

The wild-type structures of SLC7A9 and the SLC3A1-SLC7A9 complex were predicted using the AlphaFold Server (https://alphafoldserver.com) developed by DeepMind, which runs AlphaFold3 as described by Abramson et al (Abramson et al., 2024). Structures were generated in November 2024. For mutant structure prediction, single-residue amino-acid substitutions were introduced by editing the input protein sequence prior to submission (for example, the A354T sequence was generated by replacing alanine with threonine at position 354 of SLC7A9). Each mutant sequence was submitted as an independent prediction run, and no post-prediction side-chain or backbone editing was performed. Per-residue predicted local distance difference test (pLDDT) scores (Table S2) provided by AlphaFold3 were used to evaluate the reliability of the predicted models: residues with pLDDT>70 were considered reliably modeled, and residues with pLDDT<50 were treated as intrinsically disordered and excluded from structural interpretation. The predicted wild-type and mutant structures were integrated and analyzed for conformational changes using UCSF ChimeraX (version 1.7.1, University of California, USA).

Molecular docking of cystine with SLC7A9

The file containing the structure of cystine was downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/). The cystine molecule and the AlphaFold3-predicted SLC7A9 structure were prepared for docking using AutoDock Tools (Scripps Research Institute, USA), including the removal of water molecules and the addition of hydrogen atoms. Molecular docking was performed using AutoDock Vina (version 1.1.2) using the following parameters: the docking boxes were centered on three key regions of SLC7A9—the putative entry site (near Gly41), exit site (near Ser54), and interior. A total of 1000 docking runs per region were performed for a comprehensive exploration of potential binding modes. The pose with the lowest binding energy (most negative kcal/mol) was selected as the predicted binding mode. All docking results were saved in the Protein Data Bank (PDB) format for subsequent analysis.

Statistics

Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA). All experiments were performed in at least three independent biological replicates (separate transfections on different days), each with three technical replicates per condition. Data were presented as mean±standard deviation (SD). Time-dependent responses were analyzed using linear regression, and dose-dependent relationships were fitted by nonlinear regression (Michaelis-Menten or Agonist vs. response). The reported values, *K*m (Michaelis constant, a measure of substrate affinity) and *V*max (maximum reaction velocity), were defined as the best-fit estimates with their standard errors. One-way ANOVA was used for analyzing differences between groups. *P* values<0.05 were considered as statistically significant.

Reference

Abramson J, Adler J, Dunger J, et al., 2024. Accurate structure prediction of biomolecular interactions with AlphaFold 3. *Nature*, 630(8016):493-500.
http://doi.org/10.1038/s41586-024-07487-w

Table S1 Residual selenocystine transport activity of SLC7A9 and SLC3A1 variants relative to wild-type

Variant	Gene	ClinVar classification	Residual activity (% WT±SD)	Functional category
A70V	SLC7A9	Likely pathogenic	79.17±6.14	Mild
A182T	SLC7A9	Conflicting classifications	77.40±1.96	Mild
G105R	SLC7A9	Pathogenic/Likely pathogenic	53.43±8.70	Moderate
R333W	SLC7A9	Pathogenic/Likely pathogenic	41.28±6.65	Moderate
V170M	SLC7A9	Pathogenic	<5	Severe
A354T	SLC7A9	Pathogenic	<5	Severe
P482L	SLC7A9	Pathogenic	<5	Severe
M467T	SLC3A1	Pathogenic/Likely pathogenic	<5	Severe

WT: wild-type; SD: standard deviation; SLC3A1: solute carrier family 3 member 1; SLC7A9: solute carrier family 7 member 9. Values are mean±SD of three independent biological replicates. Classification thresholds: mild>60%, moderate 20–60%, severe<20% of wild-type activity.

Table S2 Mean pLDDT scores of AlphaFold3-predicted structures

Group	pLDDT (Mean±SD)
SLC3A1 WT	87.05±0.23
WT	84.91±0.19
P482L	85.99±0.38
P482G	85.59±0.80
A354T	86.33±0.08
SLC3A1-SLC7A9 WT tetramer	81.16±0.15
SLC3A1-SLC7A9 P482G tetramer	82.61±0.07
SLC3A1-SLC7A9 P482L tetramer	82.81±0.14
SLC3A1-SLC7A9 A354T tetramer	83.08±0.10

pLDDT: predicted local distance difference test; WT: wild-type; SD: standard deviation; SLC3A1: solute carrier family 3 member 1; SLC7A9: solute carrier family 7 member 9.