Supplementary Materials for

Autoregulatory control of mitochondrial glutathione homeostasis

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This PDF file includes:

Other Supplementary Materials for this manuscript include the following:

Material and Methods

Cell lines and reagents

Human cell lines HEK293T and HeLa were purchased from ATCC and FreeStyle™ 293-F Cells were purchased from ThermoFisher. HEK293T and HeLa cells (except for the viability assay) were cultured in DMEM medium (Gibco) containing 4.5 g l^{-1} glucose, 110 mg l^{-1} pyruvate, 4 mM glutamine, 10% FBS, 1% penicillin/streptomycin. FreeStyle™ 293-F Cells were cultured in FreeStyle™ 293 Expression Medium supplemented with 0.5% penicillin/streptomycin and 2% FBS.

Antibody against Ubiquitin (A300-317A-T) was purchased from Bethyl Laboratories; Citrate synthase (14309S), IRP2 (37135S), LC3B (3868S), NRF2 (12721S), RCAS1 (12290S), Calreticulin (12238S), HA-tag, C29F4 (3724S), LAMP1(D2D11) (9091P), TFRC (13208S) and XPD (11963S) antibodies were obtained from Cell Signaling Technology; RFP antibody (600-401-379) was purchased from Rockland; FLAG-M2 antibody (F1804) and FLAG-M2-FITC (F4049) antibody were from Sigma-Aldrich; Total OXPHOS Human WB Antibody Cocktail (ab110411), AFG3L2 (ab154990), GCLM (ab126704), GLRX5 (ab221121), ISCU (ab154060), SLC25A12 (ab200201) and SLC25A28 (ab103758) antibodies were purchased from Abcam; HIF1A (610958) antibody was purchased from BD Bioscience; β-tubulin (GTX101279), GAPDH (GTX627408) and PPAT (GTX102725) antibodies were from GeneTex; SLC25A37 (PA5-26720) antibody was from Invitrogen; CHAC1 (15207-1-AP), CHCHD4 (21090-1-AP), FECH (14466-1-AP), GCLC (12601-1-AP), HSCB (15132-1-AP), MRM1 (16392-1-AP), MRPL11 (15543-1-AP), MRPS12 (15225-1-AP), MRPS23 (18345-1-AP), MRPS35 (16457-1-AP), NDUFS1 (12444-1-AP), NDUFS7 (15728-1-AP), NFU1 (25598-1-AP), NQO1 (11451-1-AP), POLD1 (15646-1-AP), SLC25A11 (12253-1-AP) and SLC25A39 (14963-1-AP) antibodies were from Proteintech; SPG7 (C-5, sc-514393) was from Santa Cruz Biotechnology. ATP5A1 (439800) antibody was from Thermo Fisher Scientific. α-tubulin antibody (AA4.3-s) was from Developmental Studies Hybridoma Bank. Anti-mouse IgG–HRP (7076S) and anti-rabbit IgG–HRP (7074S) were purchased from Cell Signaling Technology. Antibodies donkey anti-rabbit Alexa Fluor 488 (A21206) and goat anti-mouse Alexa Fluor 568 (A10037) were from ThermoFisher.

Other reagents included in the study: Trolox (AC218940010) from Acros Organics , GSH (glycine-$^{13}C_2$, ^{15}N) (CNLM-6245-50) from Cambridge Isotope Laboratories, BSO (14484), Pepstatin A (9000469), Bafilomycin A1 (11038), tetrahydro-L-biopterin (hydrochloride) (BH4, 81880), CHX (14126), E64D (13533) and MitoQ (29317) from Cayman Chemical Company, Methoxy PEG Maleimide, 5000 (A3125) from JenKem Technology, reduced glutathione (210181401) from MP Biomedicals, $^{55}FeCl_3$ (NEZ043001MC) from Perkin Elmer, MG132 (S2619), Roxadustat (S1007), Liproxstatin-1 (S7699) and Erastin (S7242) from Selleckchem, BSO (B2515), Cystine (168149), GSH reduced ethyl ester (G1404), N-ethylmaleimide (E3876), Deferoxamine mesylate (DFO) (D9533), α-Tocopherol (258024), ammonium iron (III) citrate (FAC, F5879) and N-acetyl-L-cysteine (A9165) from Sigma-Aldrich.

Cell proliferation and viability assays

For HEK293T cells, approximately 1,000 cells per well were seeded on day 0 in clear-bottom 96-well plates with 200 µl culture media. On the indicated days, 40 µl CellTiter-Glo reagent (Promega) was added per well. The reaction was allowed to equilibrate for 10 minutes at room temperature and luminescence was read on a SpectraMax M3 plate reader (Molecular Devices). Cell quantity was normalized to the luminescence levels on day 0.

For viability assay of HeLa cells, approximately 15,000 cells per well were seeded in RPMI 1640 media (Gibco) containing 2.0 g l^{-1} glucose, 4 mM glutamine, 10% FBS, 1% penicillin/streptomycin on day 0 in clear-bottom 96-well plates with 200 µl culture media. Drug treatment was initiated one day after cell seeding that lasted for four days. At the end of the drug treatment, 40 µl CellTiter-Glo reagent (Promega) was added per well. The reaction was allowed to equilibrate for 10 minutes at room temperature and luminescence was read on a SpectraMax M3 plate reader (Molecular Devices). Cell viability was normalized to the luminescence levels of the DMSO-treated group.

Structural prediction and alignment of SLC25 family proteins

Structural models of SLC25 family proteins were retrieved from AlphaFold Protein Structure Database (https://alphafold.ebi.ac.uk/) (*13*). The retrieved structural models were aligned with UCSF ChimeraX using the Matchmaker function in which SLC25A39 was used as the reference structure (*33*). Alignment was performed between the specific chain in the reference structure SLC25A39 and best-aligning chain in match structures, with Needleman-Wunsch algorithm, BLOSUM-62 matrix and default penalty scores.

Generation of knockout, knockdown and cDNA-overexpression cell lines

sgRNAs were cloned into lentiCRISPR-v1 vector (Addgene) (for *SLC25A39*) linearized with BsmBI (NEB R0739L) by ligation with T4 DNA ligase (NEB M0202L), or lentiCRISPRv2-Opti (Addgene) (for *AFG3L2, HSCB, ISCU, GLRX5, FECH, CHCHD4, NFU1*) linearized with BsmBI (NEB R0739L) by Gibson assembly using NEBuilder® HiFi DNA Assembly Master Mix (NEB E2621L). For Co-IP or ^{55}Fe-binding assays involving sgRNAs for knocking out *HSCB*, we used a double-sgRNA vector to enhance knock-out efficiency (*34*). Briefly, a pair of primers (listed in the Oligonucleotides and gene fragments sequences section) containing the sgRNA sequences for *HSCB* were used to PCR-amplify a plasmid carrying the following sequence:

Lenti-MultiOpti-Template:
GTTTAAGAGCTATGCTGGAAACAGCATAGCAAGTTTAAATAAGGCTAGTCCGTTATCAACTT
GAAAAAGTGGCACCGAGTCGGTGCTTTTTTAAGCTTGGCGTAACTAGATCTTGAGACAAATG
GCAGTATTCATCCACAATTTTAAAAGAAAAGGGGGGATTGGGGGGTACAGTGCAGGGGAAA
GAGTCCGGCAAGTTTGTGGAATTGGTTTAACATAACAAATTGGCTGTGGTATATAAAATTAT
TCATAATGATAGTAGGAGGCTTGGTAGGTTTAAGAATAGTTTTTGCTGTACTTTCTATAGTGA
ATAGAGTTAGGCAGGGATATTCACCATTATCGTTTCAGACCCACCTCCCAACCCCGAGGGGA
CCCAGAGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACAAGGCTGTTAG
AGAGATAATTAGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAATACGTGACGT
AGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCAT
ATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGA
AACACCG

The amplicon was then ligated into lentiCRISPRv2-Opti plasmid by Golden Gate assembly using BsmBI (NEB R0739L) and T4 DNA ligase (NEB M0202L) in T4 DNA ligase buffer following the manufacturer's protocol. sgRNA-expressing vectors were packaged into lentivirus by co-transfecting with packaging vectors Delta-VPR and pCMV-VSV-G into HEK293T cells using the XTremeGene 9 transfection reagent (Roche).

For the generation of mitochondrial glutathione import-deficient HeLa cells, we cloned 6 sgRNAs (3 sgRNAs against SLC25A39 and 3 sgRNAs against SLC25A40) into the lentiCRISPRv2-Opti vector, using the same strategy of Golden Gate assembly. HeLa cells were transiently transfected with the CRISPR knockout plasmid, selected for 2 days with puromycin and sorted into single-cell clones. Knockout efficiency was confirmed by Sanger sequencing.

For acute knockout of other genes of interest, 250,000 HEK293T cells were seeded per well in 6-well plates, and 24 hours later, 1ml of lentivirus-containing media was added per well in the presence of 4 μg/mL polybrene and spin-infected by centrifugation at 2,200 rpm for 80 minutes on a Beckman Allegra X-14R centrifuge. Twenty-four hours later, cells were trypsinized and re-seeded in a 10 cm dish with 1 μg/ml puromycin in culture media. Puromycin was removed after three days, and the cells were allowed to recover for two days before experiments downstream.

For constructing overexpression cell lines, gBlocks (IDT) or synthetic gene fragments (Twist Bioscience) containing the cDNA of interest were cloned into pMXS-IRES-BLAST/pMXS-IRES-puro (linearized with BamHI and NotI), or pLV-EF1α-IRES-puro (linearized with EcoRI and BamHI) by Gibson assembly (NEB). For complementing mitochondrial GSH-deficient HeLa cells, the ORFs of GFP,

SLC25A39 or SLC25A39(C88/94S) were cloned into pCW57.1-puro vector (Addgene 41393, linearized with NheI and AgeI). Expression vectors based on pMXs vector were used to package retrovirus by co-transfecting with packaging vectors Gag-Pol and pCMV-VSV-G into HEK293T cells using the XTremeGene 9 transfection reagent (Roche). Expression vectors based on pLV and pCW57.1 vectors were used to package lentivirus by co-transfecting with packaging vectors Delta-VPR and pCMV-VSV-G into HEK293T cells. For overexpression of genes of interest, cells were transduced by spin-infection in the presence of virus supernatant and 4 μg/mL polybrene. Cells expressing cDNAs from the pCW57.1 vector were titrated with Doxycycline to control SLC25A39 protein expression levels close to endogenous levels in wild-type HeLa cells.

Immunoblotting

Cells were washed in ice-cold PBS, mechanically detached from culture dishes, and pelleted by centrifugation at 1,000g for 5 minutes. Unless otherwise stated, cells were lysed in membrane lysis buffer (2% SDS, 0.1% CHAPS in TBS) with 1:200 protease inhibitor cocktail (EMD Millipore 535140) and homogenized by sonication. Protein concentration was measured by Pierce™ BCA® Protein Assay Kits (Thermo Scientific, 23225), adjusted to the same levels across samples, and added Laemmli sample buffer to the final concentration of 1X. Protein samples were boiled at 95°C for 5 minutes, resolved on 10%–20% SDS-PAGE gels (Novex, ThermoFisher) and analyzed by standard immunoblotting protocol. Briefly, after transfer and blocking with 5% BSA in TBS + 0.1% Tween-20 (TBS-T), PVDF membranes (Milipore) were incubated with primary antibodies at 4°C overnight. Membranes were washed with TBS-T and incubated with secondary antibody incubation at room temperature for 1h. After thoroughly washed with TBS-T, membranes were wetted with ECL Chemiluminescent Detection Dystem (Perkin Elmer LLC) and exposed on Premium Autoradiography Films (Thomas Scientific) with SRX-101A Film Processor (Konica Minolta).

Immunofluorescence

HEK293T cells were seeded on a coverslip pre-coated with poly-D-lysine in 6-well plates. On the day of the experiment, cells were washed three times with PBS and fixed with 4% formaldehyde in PBS for 15 minutes, washed again and permeabilized with 0.1% Triton-X 100. After blocking by 10% BSA, the cells were stained with the indicated primary antibodies overnight at 4°C. Coverslips were then washed with PBS for 3 times, stained with secondary antibodies and DAPI (100 ng/ml). After 3 more PBS washes, the coverslips were mounted on glass slides in Molecular Probes ProLong Gold Antifade Mountant (Thermo Fisher Scientific, P36934) and sealed with nail polish. Confocal imaging was performed on Nikon A1R MP multiphoton microscope with confocal modality, using Nikon Plan Apo γ 60X/1.40 oil immersion objective.

Quantitative real-time PCR

Total RNA from HEK293T cells was extracted using Ambion TRIzol LS Reagent (Fisher Scientific, 10-296-010) and RNA concentration was measured by absorption at 260 nm. cDNA was synthesized with LunaScript RT SuperMix Kit (New England Biolabs, E3010S) diluted with RNAse-free water for quantitative real-time PCR using Applied Biosystems Power SYBR Green PCR Master Mix (Fisher Scientific, 43-676-59) according to the manufacturer's instruction.

FACS-based CRISPR screen

For the mitochondrial protease CRISPR screen, sgRNA sequences were designed using the CRISPick portal (https://portals.broadinstitute.org/gppx/crispick/public) (*35*) and ordered from Integrated DNA Technologies (IDT). For the whole-mitochondrial (MITO-sgRNA) library, mitochondrial-localized protein candidates were selected from the IMPI or MitoCarta2.0 databases (*16*, *17*) and ssDNA oligonucleotide pool was ordered from Agilent. Pooled oligonucleotide was Gibson-assembled as ssDNA or dsDNA after PCR amplification into linearized LentiCRISPRv2-Opti modified vector (in which the FLAG tag on Cas9 was removed), using NEBuilder® HiFi DNA Assembly Master Mix (NEB E2621L) or Gibson Assembly Master Mix (NEB E2611). Assembly product was used to transform Endura™ ElectroCompetent Cells (Lucigen 60242-2) by electroporation on a BioRad Gene Pulser with the following

settings: 25 µF, 200 Ohms and 1600 Volts. Transformed bacteria were allowed to grow overnight shaking at 32°C. Library plasmid was extracted using QIAGEN Plasmid Midi Kit (Qiagen 12143) or QIAGEN Plasmid Maxi Kit (Qiagen 12162) and packaged into lentivirus.

A single-cell clone of HEK293T cells expressing 3xFLAG-*SLC25A39* cDNA was generated by seeding HEK293T cells expressing 3xFLAG-SLC25A39 in 96-well plate at the average density of <0.5 cells per well. This clone was then expanded and used for the screen. Briefly, cells approximately 1,000X the number of sgRNAs in the library were infected at MOI of 0.5. Infected cells were selected 48 hours post-infection with 2 µg/ml puromycin for three days. Puromycin was then withdrawn, and cells were allowed to recover for two days. The cells were then harvested by trypsinization, washed with and resuspended in ice-cold PBS; ice-cold methanol was then added dropwise with the gentle swirling of the cell suspension to the final concentration of 80%. The cells were then stored in -20 for up to a week until the day of sorting. Before sorting, cells were rehydrated by adding 100% volume of PBS dropwise; the cells were then pelleted and washed twice with ice-cold PBS and resuspended in FACS buffer (0.5% BSA and 2 mM EDTA in PBS). The cells were blocked by rotating at 4°C for 30 minutes in FACS buffer and stained with 1:2000 FLAG-M2-FITC antibody (Sigma, F4049) for 1 hour rotating at 4°C. The cells were then washed twice with FACS buffer and passed through a 70µm cell strainer (BD Falcon 352235) and sorted on a Sony MA900 cell sorter based on FITC signal intensity. For the mitochondrial protease screen, approximately 2% of SLC25A39-hi and 10% of SLC25A39-lo cells were collected. For the whole-mitochondrial protein screen, approximately 2.5% of SLC25A39-hi and 2.5% of SLC25A39-lo cells were collected. Genomic DNA from pre-sorted and sorted cells was extracted by Qiagen Blood & Cell Culture DNA Midi Kit (Qiagen 13343); sgRNA sequences were amplified by PCR and sequenced on the Illumina MiSeq or NextSeq 500 Sequencers. Gene scores were calculated as the median log2 fold change of sgRNA abundance in a sorted fraction over pre-sort control.

SLC25A39 ancestral sequence reconstruction and the analysis of evolutionary origin for the loop region

Phylogenic tree of the *SLC25A39* and *SLC25A40* gene families was curated from Treefam database (*36*). Sequences were aligned with MAFFT-LINSI (*37*), and the AAML module in PAML 4.9 (*38*) was used to reconstruct the most probable ancestral sequences based on the alignments and the Treefam phylogenetic tree. For each of the ancestral lineage, we only considered residues that had more than 50% coverage among the species in this lineage, as the reconstruction can be unreliable at sites with alignment gaps. Human SLC25A39 residues 42-72 were searched against the UniprotKB sequence database using HMMER (*39*) with an E-value cutoff of 1e-5. The search was repeated using the most ancestral SLC25A39 (Anc39) loop region. Additionally, TBLASTN (*40*) was used to search the sequence in human SLC25A39 and the most ancestral SLC25A39 (Anc39) loop region against a non-redundant nucleotide collection database using an E-value cutoff of 0.05. Taxonomy information of the significant homologous sequence was collected for further analysis.

Immunopurification of mitochondria

Rapid isolation of mitochondria from HEK293T cells were performed as described before (*6*, *41*). Briefly, cells expressing 3×HA-mCherry-OMP25 were allowed to grow to a density of 3×10^8 cells per 15 cm dish. Following two quick washes by cold 0.9% NaCl, cells were harvested by mechanical detachment into KPBS (136 mM KCl, 10 mM KH_2PO_4, pH 7.25) and pelleted via centrifugation at 1,000*g* for 1.5 minutes at 4 °C. Cells were resuspended in 1ml KPBS and homogenized with 20 strokes followed by 10 strokes in a PTFE tissue grinder; 10 µl of homogenates were transferred into 40 µl of 1% Triton lysis buffer for a whole-cell protein sample and another 10 µl of homogenates were transferred into 40 µl of 80% methanol, containing heavy labeled amino acid standards, for whole-cell metabolites profiling. The remaining homogenates were centrifuged at 1,000g for 90 seconds, and the supernatant was incubated with 200 µl of KPBS pre-washed anti-HA magnetic beads (Thermo Scientific Pierce 88837) and rotated for 5 minutes at 4 °C. Post-IP beads were washed 3 times with KPBS, and half of the bead volume was lysed with 1% Triton buffer for protein extracts and the remaining half was extracted in 40 µl 80% methanol with heavy labeled amino acid standards (Cambridge Isotope Labs) by rotating for 10 minutes at 4 °C. Extracted protein or metabolites were centrifuged at 20,000*g* to remove remnant beads and contaminants. Protein

lysates were used for immunoblotting or proteomics analysis and methanol-extracted samples were used for measuring whole-cell and mitochondrial metabolite levels by LC-MS.

Glutathione uptake assay with isolated mitochondria

Mitochondrial glutathione uptake assays were performed as described before (*6*). Briefly, cells expressing 3×HA-mCherry-OMP25 were allowed to grow to a density of 3×10^8 cells per 15 cm dish. Media was removed, following two quick washes by cold 0.9% NaCl, and harvested by mechanical detachment into KPBS (136 mM KCl, 10 mM KH_2PO_4, pH 7.25) and pelleted via centrifugation at 1,000*g* for 1.5 minutes at 4 °C. Cells were resuspended in 1 ml KPBS and homogenized with 20 strokes followed by 10 strokes; 10 µl of homogenates were transferred into 40 µl of 1% Triton lysis buffer for a whole-cell protein sample and another 10 µl of homogenates were transferred into 40 µl of 80% methanol, containing heavy labeled amino acid standards, for whole-cell metabolites profiling. The remaining homogenates were centrifuged at 1,000g for 90 seconds, and the supernatant was incubated with 200 µl of KPBS pre-washed anti-HA magnetic beads (Thermo Scientific Pierce 88837) and rotated for 5 minutes at 4 °C. Post-IP beads were washed 2 times and resuspended in 1 ml KPBS; 300ul beads suspension was retained for elution with 1% triton lysis buffer as protein samples, and 300 µl of suspended beads were used for each uptake experiment. Following the removal of KPBS, mitochondria attached to the beads were incubated in 200µl transport buffer (KPBS, 10 mM HEPES, 0.5 mM EGTA) pre-warmed to 37°C containing 5 mM [$^{13}C_2$,^{15}N]-GSH (Cambridge Isotope Laboratory, CNLM-6245-50) for 5 seconds. Approximately 7 mM KOH was added to the transport buffer to adjust pH to 7.3-7.4. The uptake was stopped by adding 1 ml ice-cold KPBS and the beads were washed three more times with cold KPBS. Polar metabolite was extracted in 40 µl 80% methanol with heavy labeled amino acid standards (Cambridge Isotope Labs) by rotating for 10 minutes at 4 °C. Extracted protein or metabolites were centrifuged at 20,000*g* to remove remnant beads and contaminants. [$^{13}C_2$,^{15}N]-GSH take up by the mitochondria was quantified by LC-MS and normalized to mitochondrial NAD^+ levels.

Degradation assay of SLC25A39 in isolated mitochondria

Mitochondria were immunopurified from HEK293T cells expressing 3×HA-mCherry-OMP25 after 24 hours-treatment with BSO (1 mM) + Erastin (5 µM) as described above. Mitochondria attached to the magnetic beads were resuspended in protease assay buffer (*42*) (250 mM sucrose, 5 mM MgAc, 80 mM KAc, 10 mM Succinic acid, 20 mM NaOH, 20 mM HEPES, 2.5 mM ATP, 1 mM DTT and adjust pH with KOH to 7.4) with or without 20 mM GSH. Samples were incubated for the indicated time in a 37°C water bath. Proteins were eluted by boiling the beads in 1X Laemmli SDS loading buffer for immunoblotting.

Co-immunoprecipitation

HEK293T cells expressing FLAG-tagged proteins or empty vector were seeded in 10 cm dishes to reach a confluency of approximately 50%. Two days before immunoprecipitation, cells were transfected with 3 µg pMXs-AFG3L2(E408Q)-HA-IRES-puro plasmid per dish with XtremeGene 9 DNA transfection reagent (Roche XTG9-RO). Culture media was replaced 24 hours post-transfection and pharmacological treatments were added. On the day of immunoprecipitation, cells were briefly washed, harvested in ice-cold PBS, and pelleted by centrifugation at 1,000 g for 4 minutes. The cell pellet was lysed with 1ml 1% Triton lysis buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1 mM EDTA and 1:200 inhibitor cocktail (EMD Millipore 535140)) by rotating for 15 minutes at 4°C. The lysate was cleared by centrifuging at 13,000 g for 10 minutes, and 60 µl of supernatant was retained as input samples and added with Laemmli sample buffer to final concentration of 1X. The rest of the lysate was mixed with 10 µl anti-FLAG-M2 beads (Sigma-Aldrich M8823, pre-washed for 3 times with PBS) and rotated at 4°C for 2.5 hours. The beads were washed six times with high-salt TBS-T (50 mM Tris-Cl pH 7.4, 300 mM NaCl and 0.05% Tween-20). Proteins were eluted by boiling the beads for 5 minutes at 95°C in 1X Laemmli sample buffer diluted in membrane lysis buffer. The input and IP samples were resolved on a 10%–20% SDS-PAGE gel and analyzed by immunoblotting.

Metabolite profiling

Two days before the experiment, approximately 500,000 HEK293T cells were seeded in 6-well plates. Following pharmacological treatments, cells were rapidly washed with 0.9% NaCl twice and polar metabolites were extracted in 80 % methanol containing ^{15}N and ^{13}C fully-labeled amino acid standards (MSK-A2-1.2, Cambridge Isotope Laboratories, Inc). Cell extracts were shaken for 10 minutes at 4°C, and insoluble components were removed by centrifuging at 21,130g for 10 minutes. The supernatant was dried by nitrogen blow down and stored at -80°C until analysis. LC-MS was performed as described below. Metabolite amount was normalized to total protein amount measured by Pierce™ BCA® Protein Assay Kits (Thermo Scientific, 23225).

LC-MS quantification of whole-cell and mitochondrial metabolites

MitoIP extracts (in 80% methanol) were vortexed for 10 seconds, centrifuged for 30 minutes at (20,000 g, 4°C) and 5 µl of the supernatant was injected into a ZIC-pHILIC 150 × 2.1 mm (5 µm particle size) column (EMD Millipore). Dried polar extracts were resuspended in 60 µL of 50% acetonitrile, vortexed for 20 seconds, centrifuged for 30 minutes at (20,000 g, 4°C) and 5 µl of the supernatant was injected onto a ZIC-pHILIC 150 × 2.1mm (5 µm particle size) column (EMD Millipore). LC-MS analysis was conducted on a QExactive benchtop orbitrap mass spectrometer equipped with an Ion Max source and a HESI II Probe coupled to a Dionex Ultimate 3000 UPLC System (Thermo Fisher Scientific). External mass calibration was performed every 3 days using a standard calibration mixture. Chromatographic separation was achieved using the following conditions: Mobile phase A consisted of 20 mM ammonium carbonate with 0.1% (v/v) ammonium hydroxide (adjusted to pH 9.3) and mobile phase B was acetonitrile. The column oven and autosampler tray were held at 40°C and 4°C, respectively. The chromatographic gradient was run at a flow rate of 0.150 mL/minute as follows: 0-22 min linear gradient from 90% to 40% B; 22-24 min: held at 40% B; 24-24.1 min: returned to 90% B; 24.1-30 min: equilibrated at 90% B.

The mass spectrometer was operated in full-scan, polarity switching mode with the spray voltage set to 3.0 kV, and the heated capillary held at 275°C. The sheath gas flow was set to 40 units and the auxiliary gas flow was set to 15 units. The MS1 data acquisition was performed with the following parameters: scan range of 55-825 m/z, 70,000 resolution, 1×10^6 AGC target, and 80 ms injection time. A pool of all the biological samples was prepared and analyzed using a Top2 data-dependent acquisition method, with polarity switching. The data-dependent MS/MS scans were acquired at a resolution of 17,500, 1×10^5 AGC target, 50 ms max injection time, 1.6 Da isolation width, stepwise normalized collision energy (NCE) of 20, 30, 40 units, 8-sec dynamic exclusion, and loop count of 2. Relative metabolite abundances were quantified using Skyline Daily v22 (MacCoss Lab) using a 3 ppm mass tolerance and a 6 sec retention time from known standards.

TMT proteomics analysis for whole-cell proteome

HEK293T cells expressing the indicated cDNAs were washed twice and harvested with cold TBS. After centrifugation at 300g for 4 minutes, the supernatant was removed and cell pellets were lysed with 150µL of lysis buffer (TBS with 1 mM EDTA, 2% Triton X-100, Protease Inhibitor Cocktail (Sigma-Aldrich, 11836170001) and phosSTOP (Roche 04906837001)) on ice for 15 minutes. The samples were cleared by centrifugation at 21,130 g for 5min. The soluble fraction was collected for proteomics analysis and protein quantification by Pierce™ BCA® Protein Assay Kits (Thermo Scientific, 23225). Protein samples were acetone precipitated, reduced and alkylated and then digested with a mixture of trypsin and LysC, TMT labeled, and fractionated via SCX and High-pH fractionation. Samples were analyzed on the Orbitrap Fusion LUMOs LC-MS/MS instrument operated in high/high mode and peptides were separated on an Easy-Sprayer column using a 120-minute gradient increasing from 2 to 30% solvent "B" in 120 min, where solvent "B" = 80% acetonitrile/0.1% formic acid and solvent "A" = 0.1% formic acid (in water). The acquired spectra were searched against a Uniprot Human database. Proteome Discoverer 2.5 and Sequest were used to perform the database search. Peptide matches were filtered based on a 1% False Discovery Rate. Data were Log2x transformed and normalized by subtracting the median value per column. In total, over 7,800 proteins were quantified. For selecting differentially expressed proteins for gene ontology analysis, one-way ANOVA was performed for each identified protein across the samples, and proteins with

a nominal p-value of <0.01 were chosen. These proteins were subsequently used as input for enrichment analysis using ShinyGo 0.76.2, with all the detected proteins used as background reference (*43*). Enriched pathways were selected using an FDR cutoff of 0.05 and ranked by the fold of enrichment for the genes belonging to the pathways.

TMT proteomics analysis for mitochondrial proteome

Mitochondria were immunopurified as previously described. Mitochondrial proteins were denatured in 8 M Urea, and DTT and IAA were used to reduce disulfide bonds and alkylate cysteines. Chloroform/Water/Methanol extraction was used to isolate proteins and isolated proteins were digested with both LysC and Trypsin. Resulting Peptides were labeled using TMTPro 16-plex labeling reagents, and relative protein abundance across samples as well as labeling efficiency was evaluated. TMT-labelled peptides were mixed 1:1 and purified using RP solid phase extraction. TMT peptides were fractionated using high pH RP fractionation and the resulting 8 fractions were analyzed by LC-MS/MS. Spectra were searched against the human proteome concatenated with common contaminants and filtered with 1% FDR. Quantitation was performed using reporter ions from fragment spectra, requiring a spectral purity of 75%. Protein abundance values were log2 transformed; proteins that were observed in at least 3 out of 3 replicates in at least one treatment group were kept and all other proteins were filtered out. Any missing abundance values were replaced by imputation.

DIA proteomics analysis for mitochondrial proteome upon AFG3L2 knockout

Mitochondria were immunopurified as previously described. Mitochondrial proteins were reduced with dithiothreitol (DTT) and alkylated with iodoacetamide (IAA). Proteins were precipitated with ice-cold acetone overnight and pellets were dissolved in 50mM triethyl ammonium bicarbonate (TEAB) with sequencing-grade modified trypsin (Promega). Peptides were separated across a 60-minute linear gradient on a 100 micrometer x 12 millimeters pulled emitter C18 column at 900 nL/minute. Analysis was carried out using a Q-Exactive HF mass spectrometer (Thermo Fisher) and data was recorded in positive DIA mode. Raw data was analyzed using Spectronaut v.17 (Biognosys) in DirectDIA mode. Spectra were queried against the homo sapiens proteome at 1% FDR. Further statistical analysis was carried out in the Perseus computational environment.

Aconitase assay

The aconitase assay protocol is adapted from Terzi et al (*44*). After washing with cold PBS, cells were lysed with lysis buffer (40 mM KCl, 25 mM tris-Cl (pH 7.5), 1% Triton X-100, 0.1 M fresh DTT, 1 M sodium citrate, and 1 M $MnCl_2$) with a protease inhibitor cocktail (Sigma-Aldrich, 5892791001). Lysates are incubated for 10 minutes on ice, collected and centrifuged at 4°C at 21,000g for 10 min. The supernatant was collected, and protein concentration was quantified using a Bradford protein assay (Biorad, 5000006). Protein was loaded into 8% polyacrylamide/TBE gel supplemented with 1 M sodium citrate and run at 175 V for 4 hours in cold running buffer (25 mM Tris base, 192 mM glycine, and 3.6 mM citrate, pH 8). The gel was washed with distilled water and incubated at 37°C for 5 to 45 min in reaction solution (100 mM tris (pH 8.0), 1 mM nicotinamide adenine dinucleotide phosphate (Sigma-Aldrich, 10128031001), 2.5 mM cis-aconitate (Sigma-Aldrich, A3412), 5 mM MgCl2, 1.2 mM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Thermo Fisher Scientific, M6494), 0.3 mM phenazine methosulfate (Sigma-Aldrich, P9625), and isocitrate dehydrogenase (5 U/ml; Sigma-Aldrich, I2002)). The gel was washed with distilled water until the background was removed and the gel was imaged.

^{55}Fe binding assay

The amount of ^{55}Fe bound to SLC25A39 was determined using a modified protocol based on Patel et al (*45*). Approximately 5×10^6 HEK293T cells were seeded in DMEM containing 1 µCi/ml $^{55}FeCl_3$ 48 hours prior to the experiments. Cells were washed one time with ice-cold PBS containing 10 mM EDTA, 2 mM bathophenanthrolinedisulfonic acid and 50 µM sodium ascorbate and three times with PBS to remove $^{55}FeCl_3$ in the media. Approximately 1.5×10^7 cells were pelleted and lysed with 1 ml lysis buffer containing TBS (50 mM Tris-Cl pH 7.4, 150 mM NaCl) with 1% Triton X-100, 1 mM EDTA, 250 µM TCEP and

protease inhibitor cocktail (EMD Millipore 535140). After 15-minute incubation at 4°C, cell lysate was cleared by centrifuging at 13,000g × 10min. FLAG-tagged proteins were immunoprecipitated with 5 µl anti-FLAG M2 magnetic beads (Sigma M8823) per ml of lysate by rotating at 4°C for 2.5 hours. The supernatant was removed, and beads were washed 4 times with wash buffer (50 mM Tris-Cl pH 7.4, 250 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.05% Tween 20, 5 mM ATP). Bound protein was eluted by boiling at 95°C for 5 minutes in 1 ml PBS + 2% SDS + 10 mM EDTA. For measuring ^{55}Fe radioactivity, 400 µl of elute was mixed with 5 ml of Insta-Gel Plus (Perkin Elmer 6013391) in a mini scintillation vial and counted on a Tri-Carb 2910 TR scintillation counter.

Analysis of ^{55}Fe distribution in cells

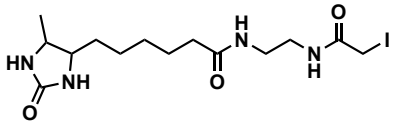
Approximately 1×10^7 HEK293T cells were seeded in DMEM containing 1 µCi/ml $^{55}FeCl_3$ 48 hours prior to the experiments. Drug treatment was initiated 24 hours prior to the assay. The cells were washed one time with ice-cold KPBS containing 10 mM EDTA, 2 mM bathophenanthrolinedisulfonic acid and 50 µM sodium ascorbate and one more time with KPBS to remove $^{55}FeCl_3$ in the media. The cells were resuspended in 1ml KPBS and disrupted by passing five times through a 28.5G needle and 10 µl lysate was taken for measuring whole-cell radioactivity and protein concentration. The remaining homogenates were centrifuged at 1,000g for 90 seconds, and the supernatant was incubated with 200 µl of KPBS pre-washed anti-HA magnetic beads (Thermo Scientific Pierce 88837) and rotated for 5 minutes at 4°C. Post-IP beads were washed 3 times with KPBS and the isolated mitochondria were lysed on beads in 500 µl of 2% SDS + 10mM EDTA in PBS, shaking at 37°C for 5 minutes. For measuring ^{55}Fe radioactivity, 400 µl of elute was mixed with 5 ml of Insta-Gel Plus (Perkin Elmer 6013391) in a mini scintillation vial and counted on a Tri-Carb 2910 TR scintillation counter. Protein concentration was measured with 10 µl elute using the Pierce™ BCA® Protein Assay Kits (Thermo Scientific, 23225).

Measurement of cysteine redox state

Cysteine redox state was measured based on a mass shift assay similar to that described before (*46*). After 24 hours treatment with BSO (1 mM) + erastin (5 µM) or DMSO as control, cells were washed with SHE buffer (250 mM Sucrose, 10 mM HEPES, 1 mM EDTA, pH 7.4) containing 100 mM NEM and lysed in SHE buffer containing 100 mM NEM and 1% SDS. The cell lysate was buffer-exchanged using Micro Bio-Spin 6 chromatography columns (Biorad 732-6221) to remove NEM. The flowthrough was reduced in the presence of 2.5 mM DTT and 1% SDS by incubating at 37°C for 10 minutes. Reduced cysteines were alkylated with 50 mM Methoxy PEG Maleimide, 5000 (JenKem Technology, A3125-1). Proteins were acetone-precipitated and re-solubilized in TBS with 1 % SDS for immunoblotting.

Chemoproteomics profiling of cysteine reactivity

IA-DTB (structure shown below) was made in-house based on published procedures (*47*).



IA-DTB

HEK293T cells overexpressing 3xFLAG-*SLC25A39* cDNA were transduced with LentiCRISPRv2-Opti lentiviral particles targeting *AFG3L2*. Twenty-four hours after infection, cells were selected with 1 µg/ml puromycin for three days and recovered for two days, and seeded in 15-cm plates at the density of 1×10^8 per plate. After one day of growth, the cells were treated for 24 hours with BSO (1 mM) + erastin (5 µM), or BSO (1 mM) + erastin (5 µM) + DFO (20µM) or DMSO as a control. The cells were harvested by mechanical detachment, washed with ice-cold PBS, and pelleted by centrifuging at 1,000g for 5 minutes. The cell pellets were lysed in 500 µl of PBS + 1 mM EDTA + 250 µM IA-DTB probe by sonication with QSonica Q55 sonicator at 20% power for 8 × 2 seconds pulses. The homogenate was incubated at room temperature for 1 hour and added with 500 µl 2X triton lysis buffer (50 mM Tris-Cl pH 7.6, 150 mM NaCl, 1 mM EDTA, 2% Triton-X 100 and 1:100 protease inhibitor cocktail (EMD Millipore 535140)). Cell lysates were rotated at 4°C for 15 minutes and cleared by centrifugation at 13,000g for 10 minutes. The supernatant was added with 1 mM dithiothreitol to quench the unreacted IA-DTB probe and

incubated with 10 µl anti-FLAG-M2 magnetic beads (washed three times with PBS) rotating at 4°C for 2.5 hours. After immunoprecipitation, the beads were washed three times with wash buffer (50 mM Tris-Cl pH 7.4, 250 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.05% Tween 20, 5 mM ATP) and once with PBS. Protein from 10% of the beads were eluted by boiling in 1X Laemmli buffer for quality check with immunoblotting. The rest 90% of the beads were frozen in -80°C for mass spectrometry analysis.

The beads were allowed to warm up to 25 °C (rt) and re-suspended in 80 µl of 2M urea buffer in Tris•HCl (pH 7.5) containing 1 mM DTT, 0.8 µg trypsin (Promega), and 0.8 mM $CaCl_2$. The buffer was prepared by combining 2 M urea (950 µL, Tris•HCl pH 7.5), DTT (25 µl, 40 mM), and trypsin/$CaCl_2$ solution (20 µg trypsin, 60 µl trypsin buffer, 20 µl 100 mM $CaCl_2$). The samples were incubated at rt with shaking for 2 h, and the solution was separated from the beads on a magnet. The beads were washed with 2 M urea buffer in Tris•HCl (pH 7.5) (2 × 60 µl), bringing the final volume of the sample to 200 µl. DTT (10 µl, 180 mM) was added to each sample, following incubation at 65 °C for 20 min. The samples were allowed to cool down to rt, after which iodoacetamide (10 µl, 410 mM) was added and the samples were incubated in a shaker for 30 min at 37 °C. Trypsin/LysC (Promega, 0.5 µg/sample, 20 µg in 40 µL buffer) was added to each sample and the samples were digested overnight at 37 °C. The following day the samples were acidified by adding 20 µL of 20% formic acid and desalted on stage tips according to the modified manufacturer's protocol (ThermoFisher Scientific, #87784). Briefly, the tips were washed with MeOH (100 µl), 50/50 CH_3CN/H_2O (0.1 % FA, 100 µl), and equilibrated with buffer A (5% CH_3CN/H_2O, 0.1 % FA, 3 × 100 µl). The samples were loaded onto the tips, and the eluate was used to wash the original Eppendorf tube and passed through the tips again; the tips were then washed with buffer A, and the peptides were eluted with buffer B (80% CH_3CN/H_2O, 0.1 % FA, 200 µl). The samples were dried using SpeedVac vacuum concentrator, re-suspended in 100 µl EPPS (200 mM, pH 8.0) containing 30% CH_3CN, and labeled with isobaric TMT tags (5 µL/sample, 20 µg/µL, TMT10plex™, ThermoFisher Scientific, #90406) at rt for 1 h. Following the labeling step, the reaction was quenched with 5% hydroxylamine (3 µl/sample), vortexed and incubated at rt for 15 min, before acidification with FA (5 µl/sample). The samples from each TMT10plex were pooled and dried using SpeedVac vacuum concentrator. The samples were then desalted using Sep-Pak C18 cartridges. Briefly, the cartridge was washed with CH_3CN (3 × 1 ml) and equilibrated with buffer A (3 × 1 m). The samples were re-suspended in buffer A with sonication and passed through the cartridge. The cartridge was then washed with the flow-through from the sample (1 × 1 m) and desalted by passing buffer A (3 × 1 m). The peptides were eluted by adding 1 m of buffer B, and the eluent was dried using SpeedVac vacuum concentrator. The final sample was re-suspended in Buffer A (10 µ) and analyzed by mass-spectrometry using the following LC-MS gradient: 5% LC-MS buffer B (CH_3CN, 0.1% FA) in LC-MS buffer A (H_2O, 0.1% FA) from 0-10 min, 5%–20% buffer B from 20-120 min, 20%–45% buffer B from 120-140 min, 45%–95% buffer B from 140-145 min, 95% buffer B from 145-147 min, 5% buffer B from 147-149 min, 95% buffer B from 149-151 min, 5% buffer B from 151-160 min) and standard MS3-based quantification. Briefly, the scan sequence began with an MS1 master scan (Orbitrap analysis, resolution 120,000, 400−1600 m/z, RF lens 40%, normalized AGC Target 250%, automatic maximum injection time, profile mode) with dynamic exclusion enabled (repeat count 1, duration 60 s). The top ten precursors were then selected for MS2/MS3 analysis. MS2 analysis consisted of quadrupole isolation (isolation window 0.7) of precursor ion followed by collision-induced dissociation (CID) in the ion trap (Standard AGC, normalized collision energy 35%, maximum injection time 120 ms). Following the acquisition of each MS2 spectrum, synchronous precursor selection (SPS) enabled the selection of up to 10 MS2 fragment ions for MS3 analysis. MS3 precursors were fragmented by HCD and analyzed using the Orbitrap (collision energy 55%, normalized AGC Target 500%, maximum injection time 118 ms, resolution was 60,000). For MS3 analysis, MS isolation window was set at 0.7. The MS2 and MS3 files were extracted from the raw files using RAW Converter (version 1.1.0.22; available at http://fields.scripps.edu/rawconv/), uploaded to Integrated Proteomics Pipeline (IP2), and searched using the ProLuCID algorithm (publicly available at http://fields.scripps.edu/downloads.php) using a reverse concatenated, non-redundant variant of the Human UniProt database (release-2016_07). Cysteine residues were searched with a static modification for carboxyamidomethylation (+57.02146 Da). *N*-termini and lysine residues were also searched with a static modification corresponding to the TMT tag (+229.1629 Da). Peptides were required to be at least 5 amino acids long and to have at least one tryptic terminus. ProLuCID data was filtered through DTASelect (version 2.0) to achieve a peptide false-positive rate below 1%. The MS3-based peptide

quantification was performed with reporter ion mass tolerance set to 20 ppm with Integrated Proteomics Pipeline (IP2).

Signal intensities for SLC25A39 were extracted from raw IP2 output files and individually filtered to remove low-quality peptides. The following filters were applied: removal of non-unique peptides, removal of half-tryptic peptides and removal of peptides with more than one missed cleavage site. Peptides with identical sequences were aggregated and their signal intensities were summed. The mean of each condition per peptide was calculated and peptides that did not pass the signal intensity filter of >= 5,000 for at least one of the conditions were removed. Signal intensities of filtered peptides identified with both labeled and unlabeled cysteine residues were used to calculate the treated/untreated ratio for each corresponding peptide sequence. Ratios of DMSO control replicates were averaged and final values were reported as % to DMSO control.

Iron-sulfur cluster synthesis

For UV-vis experiments, stock solutions of 50 mM $FeCl_3$ and 50 mM Na_2S were prepared under N_2 with a Schlenk line. From these stocks, $FeCl_3$ and Na_2S were added to 0.1 mM and 0.05 mM, respectively, to an anaerobic solution of 0.25 mM peptide, pH 7.5 to form a $[2Fe\text{-}2S]^{2+}$ cluster. $[4Fe\text{-}4S]^{2+}$ conditions were 0.05 mM $FeCl_3$, 0.1 mM Na_2S, 0.25 mM peptide, pH 7.5. Samples for circular dichroism were prepared with 0.5 mM $FeCl_3$, 0.25 mM Na_2S, 1 mM peptide, pH 7.5. Spectra were decomposed with Fit-FeS (*48*). The preparations of all solutions were carried out under a nitrogen atmosphere using a Schlenk line. The pH of solutions was measured with an Orion Star A211 pH meter with pH and ATC Probes from Thermo Scientific.

UV-Vis absorption and Circular Dichroism spectroscopy

The peptide was synthesized by Lifetein and Bio Basic with 85% purity. For UV-Visible spectroscopy, anaerobic sealed Hellma quartz cuvettes were used, and the solutions were transferred to cuvettes under nitrogen. UV-vis spectra were recorded with a Carry 3500 Multicell Peltier UV-Vis Spectrophotometer (Agilent) with an integration time of 0.02 s, an interval of 1 nm, and bandwidth of 2 nm. Circular Dichroism (CD) spectra were recorded with an Olis Carry-17 spectrophotometer using 5 mm, 1.5 mL Hellma quartz cuvettes with a spectral window of 700-240 nm and a bandwidth of 2 nm. Spectra are the average of five scans.

Peptides with bound Co^{2+} were prepared inside a Genesis 2P glovebox (Vacuum Atmospheres Company) with $O_2 < 1$ ppm. 0.5 mM of Co^{2+} was added to 0.5 mM peptide at pH 7.5 and spectra recorded inside the glovebox with a ThermoFischer Genesys 150 UV-vis spectrophotometer with an integration time of 0.5 s and interval of 1 nm.

Mössbauer spectroscopy

Conditions for the synthesis of $[2Fe\text{-}2S]^{2+}$-peptide were 5 mL of 5 mM peptide, 0.25 mM Na_2S, 0.5 mM $^{57}FeCl_3$, pH 7.5. Subsequently, the sample was precipitated with degassed 50 mL 2-propanol. The solution was centrifuged for 20 min at 8000 rpm with a Centrifuge 5804 R (eppendorf). The solvent was discarded, and the precipitate frozen in liquid nitrogen and lyophilized overnight at -84 °C (Labconco FreeZone Freeze Dryer). Mössbauer spectra were collected at the Department of Mechanical, Aerospace and Biomedical Engineering at the University of Tennessee Space Institute in Tullahoma, TN, USA. The measurements were at 293 K with a 25 mCi ^{57}Co in Rh source held at room temperature. The MS4 spectrometer was operated in constant acceleration mode in transmission geometry. The centroid shift δ is quoted with respect to metallic α–iron at room temperature. The spectrum was least square fitted to extract hyperfine parameters (δ), quadrupole splitting (Δ), full width at half-maximum (FWHM), and intensities (*I*). Units of δ and Δ are mm/s.

Prime editing

Prime editing plasmids based on the PE3 strategy were constructed based on PEA1-puro vector (Addgene 171991 (*49*)). The following pairs of oligos were annealed to form dsDNAs and cloned into PEA1-puro via Golden Gate Assembly. HEK293T cells were transfected with prime editing plasmids and

selected with puromycin for two days; editing efficiency was monitored by PCR amplifying the targeted genomic locus and Sanger sequencing. Additional rounds of editing were repeated if no significant editing is observed. The cells were then sorted into 96-well plates as single clones, and clones with homozygous base substitution as verified by Sanger sequencing were chosen for further analysis.

sgRNA sequence	PrimeEdit-C88-sgF: caccgAGCGGGCACCATTTGGGCAC PrimeEdit-C88-sgR: aaacGTGCCCAAATGGTGCCCGCTc
	PrimeEdit-C94-sgF: caccgCTTGAAACCAGGTGGCACAG PrimeEdit-C94-sgR: aaacCTGTGCCACCTGGTTTCAAGc
PBS-RTT sequence	PrimeEdit-C88S-PBSRTT-F: gtgcTCTGTATCTGTCCCCAAATGGTGC PrimeEdit-C88S-PBSRTT-R: aaaaGCACCATTTGGGGACAGATACAGA
	PrimeEdit-C88C-PBSRTT-F: gtgcTCTGTATCTGTGCCCAAATGGTGC PrimeEdit-C88C-PBSRTT-R: aaaaGCACCATTTGGGCACAGATACAGA
	PrimeEdit-C94S-PBSRTT-F: gtgcTGGTGCTCGCTCTGCCACCTGGTT PrimeEdit-C94S-PBSRTT-R: aaaaAACCAGGTGGCAGAGCGAGCACCA
	PrimeEdit-C94C-PBSRTT-F: gtgcTGGTGCTCGCTGTGCCACCTGGTT PrimeEdit-C94C-PBSRTT-R: aaaaAACCAGGTGGCACAGCGAGCACCA
PE3 nick gRNA sequence	PrimeEdit-PE3nick-sgF: accgCCTCCTCTCTCCAATCCACAgt PrimeEdit-PE3nick-sgR: taaaacTGTGGATTGGAGAGAGGAGG

Mitochondrial stress test

The Agilent Seahorse XF Cell Mito Stress Test was performed on the Seahorse XFe96 Analyzer according to the manufacturer's instructions. Briefly, the cells were seeded at density of 15,000 cells per well, 72 hours prior to the assay. Drug treatment was started 48 hours prior to the assay. One hour before the assay, the cells were washed and conditioned in Agilent RPMI culture media (103576-100) supplemented with 10 mM glucose, 1 mM pyruvate and 200 µM glutamine. Cells were immediately washed again before assay, and oligomycin (final concentration 1.5 µM), FCCP (final concentration 0.25 µM) and Rotenone + Antimycin (final concentration 0.5 µM each) were injected before the 4^{th}, 7^{th} and 10^{th} measurement, respectively. After the assay, the cells were stained with the Molecular Probes NucBlue Live ReadyProbes Reagent (Thermo Fisher Scientific R37605) and the total nucleus number was counted on the Molecular Devices ImageXpress Micro Confocal High-Content Imaging System for normalization.

Oligonucleotides and gene fragments sequences

The following oligos were used for cloning sgRNAs into the LentiCRISPRv2-Opti vector:

Opti-sgCTRL	atcttgtggaaaggacgaaacaccgAACCTACGGGCTACGATACGgtttaagagctatgc
Opti-sg*GLRX5*.1	tatatatcttgtggaaaggacgaaacaccgCGTTGTAGGCCGCGTAATCGgtttaagagctatgctggaaacagcatagc
Opti-sg*GLRX5*.2	tatatatcttgtggaaaggacgaaacaccgGACGACCCGGAGCTCCGACAgtttaagagctatgctggaaacagcatagc
Opti-sg*CHCHD4*.1	tatatatcttgtggaaaggacgaaacaccgTGTTCTCCAAACACTAGGGAgtttaagagctatgctggaaacagcatagc
Opti-sg*CHCHD4*.2	tatatatcttgtggaaaggacgaaacaccgCTGGAACTGCCCATGCCTTGgtttaagagctatgctggaaacagcatagc
Opti-sg*FECH*.1	tatatatcttgtggaaaggacgaaacaccgAAGATATGGACTTCCAAGCAgtttaagagctatgctggaaacagcatagc
Opti-sg*FECH*.2	tatatatcttgtggaaaggacgaaacaccgTACAGATACTATAATCAAGTgtttaagagctatgctggaaacagcatagc
Opti-sg*AFG3L2*.1	atcttgtggaaaggacgaaacaccgGAATGAGACTCACTCTAGCAgtttaagagctatgc
Opti-sg*AFG3L2*.2	atcttgtggaaaggacgaaacaccgTTCCACTCACCTGAAAACCCgtttaagagctatgc
Opti-sg*ISCU*.1	atcttgtggaaaggacgaaacaccgTTATGAAAATCCTAGAAACGgtttaagagctatgc
Opti-sg*ISCU*.2	atcttgtggaaaggacgaaacaccgCTACCTTCTTGTGATAGAGTgtttaagagctatgc
Opti-sg*ABCB7*.1	atcttgtggaaaggacgaaacaccgTCACAGTTGCAGTCACACGGgtttaagagctatgc
Opti-sg*ABCB7*.2	atcttgtggaaaggacgaaacaccgAGTCATTAAAAAGTATCACAgtttaagagctatgc
Opti-sg*HSCB*.1	atcttgtggaaaggacgaaacaccgGGCTGAAGTAGTCTCGAGTGgtttaagagctatgc
Opti-sg*HSCB*.2	atcttgtggaaaggacgaaacaccgGGCCGCCGCAGTTCCAACAGgtttaagagctatgc
Opti-sg*NFU1*.1	atcttgtggaaaggacgaaacaccgAGTAATATACCTAGCCAGAGgtttaagagctatgc
Opti-sg*NFU1*.2	atcttgtggaaaggacgaaacaccgAACAGCTTAAAGTTTATACCgtttaagagctatgc
Opti-sg*SPG7*.1	atcttgtggaaaggacgaaacaccgACGGAAAACATACCACAGGAgtttaagagctatgc
Opti-sg*SPG7*.2	atcttgtggaaaggacgaaacaccgCACAGGCCGCCCAAACACCAgtttaagagctatgc

The following oligos were used to construct double or multiple sgRNA-expressing vector using LentiCRISPRv2-opti backbone:

Multi-sg*HSCB*.1-F	ACG CGTCTC ACACCG GGCTGAAGTAGTCTCGAGTG GTTTAAGAGCTATGCTGGAAACA
Multi-sg*HSCB*.2-R	ACG CGTCTC AAAAC CTGTTGGAACTGCGGCGGCC CGGTGTTTCGTCCTTTCCAC
Multi-sgSLC25A39-1F	ACG CGTCTC ACACCG TGACCTCTACGCACCCATGG GTTTTAGAGCTAGAAATAGCAAGTT
Multi-sgSLC25A39-2R	ACG CGTCTC A TGACTGTGCCAG CGGTGTTTCGTCCTTTCCAC
Multi-sgSLC25A39-2F	ACG CGTCTC A GTCATCACCCTG GTTTTAGAGCTAGAAATAGC
Multi-sgSLC25A39-3R	ACG CGTCTC G TCATAGGCAGTG CGGTGTTTCGTCCTTTCCAC
Multi-sgSLC25A39-3F	ACG CGTCTC T ATGACCAACTGA GTTTTAGAGCTAGAAATAGC
Multi-sgSLC25A40-1R	ACG CGTCTC T TATTTATTTTAC CGGTGTTTCGTCCTTTCCAC
Multi-sgSLC25A40-1F	ACG CGTCTC A AATAACTGTGGC GTTTTAGAGCTAGAAATAGC
Multi-sgSLC25A40-2R	ACG CGTCTC T CAAT TAAGTGCT CGGTGTTTCGTCCTTTCCAC
Multi-sgSLC25A40-2F	ACG CGTCTC A ATTG ATCATAGC GTTTTAGAGCTAGAAATAGC
Multi-sgSLC25A40-3R	ACG CGTCTC AAAACGCAACAATTGGTATGCAGGT CGGTGTTTCGTCCTTTCCAC

The following primers were used for RT-qPCR at final concentration of 500 nM:

SLC25A39-qPCR-F	TGCCCTTCTCAGCCCTGTA
SLC25A39-qPCR-R	GGTTCACTCTCACAGCCTCC
ABCB7-qPCR-F	CAGGACAGTTCTTAGATGCTGC
ABCB7-qPCR-R	TTTGGGCCACACATAAGAAAGC
ACTB-qPCR-F	CATGTACGTTGCTATCCAGGC
ACTB-qPCR-R	CTCCTTAATGTCACGCACGAT

Codon-optimized 3xFLAG-SLC25A39 sequence:

ATGGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATTGATTACAAGGATGACG
ATGACAAGGGAGGTTCAGGTGGATCAGCAGACCAGGACCCCGCGGGCATCTCACCTCTCCA
GCAGATGGTCGCATCTGGAACAGGGGCAGTCGTCACAAGTTTGTTCATGACCCCACTTGATG
TAGTGAAAGTCCGGCTTCAATCACAACGCCCTAGCATGGCCAGCGAGCTGATGCCGAGCTCC
AGGCTCTGGTCACTTTCTTATACGAAGCTTCCCTCTTCTCTCCAGTCTACGGGTAAATGTTTG
CTTTATTGTAACGGCGTACTCGAACCTCTGTATTTGTGTCCAAATGGAGCACGCTGCGCCAC
GTGGTTTCAGGACCCAACTCGATTTACCGGCACAATGGACGCATTTGTCAAGATAGTAAGAC
ACGAGGGTACAAGAACGCTTTGGAGCGGCCTCCCTGCTACGTTGGTGATGACGGTTCCCGCA
ACGGCCATATACTTTACAGCCTACGACCAGCTGAAGGCCTTTCTGTGTGGTAGGGCACTTAC
CTCAGACCTTTACGCTCCAATGGTCGCAGGGGCCCTTGCAAGACTTGGTACGGTCACTGTAA
TAAGTCCGCTCGAACTCATGAGGACAAAACTCCAAGCTCAGCACGTGAGCTACCGGGAACT
GGGGGCTTGTGTACGCACAGCGGTCGCGCAAGGCGGCTGGAGGAGTCTGTGGCTGGGTTGG
GGGCCCACGGCCCTCCGGGACGTACCGTTTTCTGCGCTTTATTGGTTTAACTACGAGCTTGTG
AAATCTTGGCTCAATGGATTCCGGCCGAAAGACCAGACCTCCGTTGGAATGTCTTTCGTCGC

CGGGGGCATTTCCGGCACGGTGGCCGCCGTGCTGACCTTGCCATTCGACGTTGTTAAGACCC
AGCGACAGGTCGCTTTGGGGGCAATGGAGGCCGTGCGGGTGAACCCACTCCACGTTGACAG
TACATGGTTGCTGCTCCGCCGCATCCGGGCCGAAAGCGGAACTAAAGGTCTGTTTGCTGGAT
TTCTTCCGCGAATCATTAAGGCTGCGCCATCTTGTGCAATCATGATCTCTACATACGAGTTTG
GAAAATCCTTCTTTCAGAGGCTTAATCAGGACAGACTGCTCGGAGGGTAA

3xFLAG-SLC25A39(delta42-106):

ATGGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATTGATTACAAGGATGACG
ATGACAAGGGAGGTTCAGGTGGATCAGCAGACCAGGACCCCGCGGGCATCTCACCTCTCCA
GCAGATGGTCGCATCTGGAACAGGGGCAGTCGTCACAAGTTTGTTCATGACCCCACTTGATG
TAGTGAAAGTCCGGCTTCAATCACAACGCCCTGGTACGGGTTCTGGTCCAACTCGATTTACC
GGCACAATGGACGCATTTGTCAAGATAGTAAGACACGAGGGTACAAGAACGCTTTGGAGCG
GCCTCCCTGCTACGTTGGTGATGACGGTTCCCGCAACGGCCATATACTTTACAGCCTACGAC
CAGCTGAAGGCCTTTCTGTGTGGTAGGGCACTTACCTCAGACCTTTACGCTCCAATGGTCGC
AGGGGCCCTTGCAAGACTTGGTACGGTCACTGTAATAAGTCCGCTCGAACTCATGAGGACAA
AACTCCAAGCTCAGCACGTGAGCTACCGGGAACTGGGGGCTTGTGTACGCACAGCGGTCGC
GCAAGGCGGCTGGAGGAGTCTGTGGCTGGGTTGGGGGCCCACGGCCCTCCGGGACGTACCG
TTTTCTGCGCTTTATTGGTTTAACTACGAGCTTGTGAAATCTTGGCTCAATGGATTCCGGCCG
AAAGACCAGACCTCCGTTGGAATGTCTTTCGTCGCCGGGGGCATTTCCGGCACGGTGGCCGC
CGTGCTGACCTTGCCATTCGACGTTGTTAAGACCCAGCGACAGGTCGCTTTGGGGGCAATGG
AGGCCGTGCGGGTGAACCCACTCCACGTTGACAGTACATGGTTGCTGCTCCGCCGCATCCGG
GCCGAAAGCGGAACTAAAGGTCTGTTTGCTGGATTTCTTCCGCGAATCATTAAGGCTGCGCC
ATCTTGTGCAATCATGATCTCTACATACGAGTTTGGAAAATCCTTCTTTCAGAGGCTTAATCA
GGACAGACTGCTCGGAGGGTAA

3xFLAG-SLC25A11

ATGGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATTGATTACAAGGATGACG
ATGACAAGGGAGGTTCAGGTGGAAGCGCGGCGACGGCGAGTGCCGGGGCCGGCGGGATAG
ACGGGAAGCCCCGTACCTCCCCTAAGTCCGTCAAGTTCCTGTTTGGGGGCCTGGCCGGGATG
GGAGCTACAGTTTTTGTCCAGCCCCTGGACCTGGTGAAGAACCGGATGCAGTTGAGCGGGG
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AGAAGGCCTGAGGGGCATTTACACTGGGCTGTCGGCTGGCCTGCTGCGTCAGGCCACCTACA
CCACTACCCGCCTTGGCATCTATACCGTGCTGTTTGAGCGCCTGACTGGGGCTGATGGTACTC
CCCCTGGCTTTCTGCTGAAGGCTGTGATTGGCATGACCGCAGGTGCCACTGGTGCCTTTGTG
GGAACACCAGCCGAAGTGGCTCTTATCCGCATGACTGCCGATGGCCGGCTTCCAGCTGACCA
GCGCCGTGGCTACAAAAATGTGTTTAACGCCCTGATTCGAATCACCCGGGAAGAGGGTGTCC
TCACACTGTGGCGGGGCTGCATCCCTACCATGGCTCGGGCCGTCGTCGTCAATGCTGCCCAG
CTCGCCTCCTACTCCCAATCCAAGCAGTTCTTACTGGACTCAGGCTACTTCTCTGACAACATC
TTGTGCCACTTCTGTGCCAGCATGATCAGCGGTCTTGTCACCACTGCTGCCTCCATGCCTGTG
GACATTGCCAAGACCCGAATCCAGAACATGCGGATGATTGATGGGAAGCCGGAATACAAGA
ACGGGCTGGACGTGCTGTTCAAAGTTGTCCGCTACGAGGGCTTCTTCAGCCTGTGGAAGGGC
TTCACGCCGTACTATGCCCGCCTGGGCCCCCACACCGTCCTCACCTTCATCTTCTTGGAGCAG
ATGAACAAGGCCTACAAGCGTCTCTTCCTCAGTGGCTGA

3xFLAG-SLC25A11+SLC25A39(AA42-106)

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3xFLAG-SLC25A11+SLC25A39(AA42-106Ancestral)

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GA

MitoCHAC1: *CHAC1* cDNA was modified by deleting the first 8 amino acids in the ORF and tagging a tandem MTS consisting of the *Lactobacillus brevis COXIV* MTS and human *ACOI* MTS to the N-terminus.

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MitoCHAC1-HA: lower case bases represent HA tag

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SLC25A28

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SLC25A37

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SLC25A37(H60Q/H118Q/H157A/H215A)

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SLC25A37(C124T/C184T/C258I)

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Expression vectors for AFG3L2-E408Q-HA were constructed by Gibson assembly with the following two DNA fragments into pMXs-IRES-puro vectors linearized with NotI and BamHI. Lower-case bases represent flanking sequences for Gibson assembly.

pMXs-AFG3L2(E408Q)-(1-407)-HA:

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pMXs-AFG3L2(E408Q)-(401-812)-HA:

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CGCTTAAgctacgtaaattccgcc

Expression constructs MitoGSHf and CytoGSHf were described before (*6*). For knockdown of *NFS1*, shRNA TRCN0000229755, B6 (Addgene) was used. For knockdown of *ABCB7*, the following shRNA sequences were cloned into pLKO-puro vectors.

sh*ABCB7*.1	CCGGGCACAGAGATATGATGGATTTCTCGAGAAATCCATCATATCTCTGTGCTTTTTG
sh*ABCB7*.2	CCGGGCATTTATCTTGCTGGTCAAACTCGAGTTTGACCAGCAAGATAAATGCTTTTTG

Statistics and reproducibility

GraphPad PRISM 9, Microsoft Excel 16.66.1 and R 4.1.1 were used for statistical analysis. Skyline (v.20.1.1.158) were used for metabolomic analyses and ImageJ FIJI (NIH, Version 1.0) were used for image analysis. Error bars, *P* values and statistical tests are reported in the figure captions. All experiments (except CRISPR screens and proteomics, which were done once) were performed at least twice with similar results.

Both technical and biological replicates were reliably reproduced. Comparison of two mean values was evaluated by two-tailed unpaired *t*-test. Comparison of multiple mean values was evaluated by one-way ANOVA followed by post hoc Tukey test.

Fig. S1

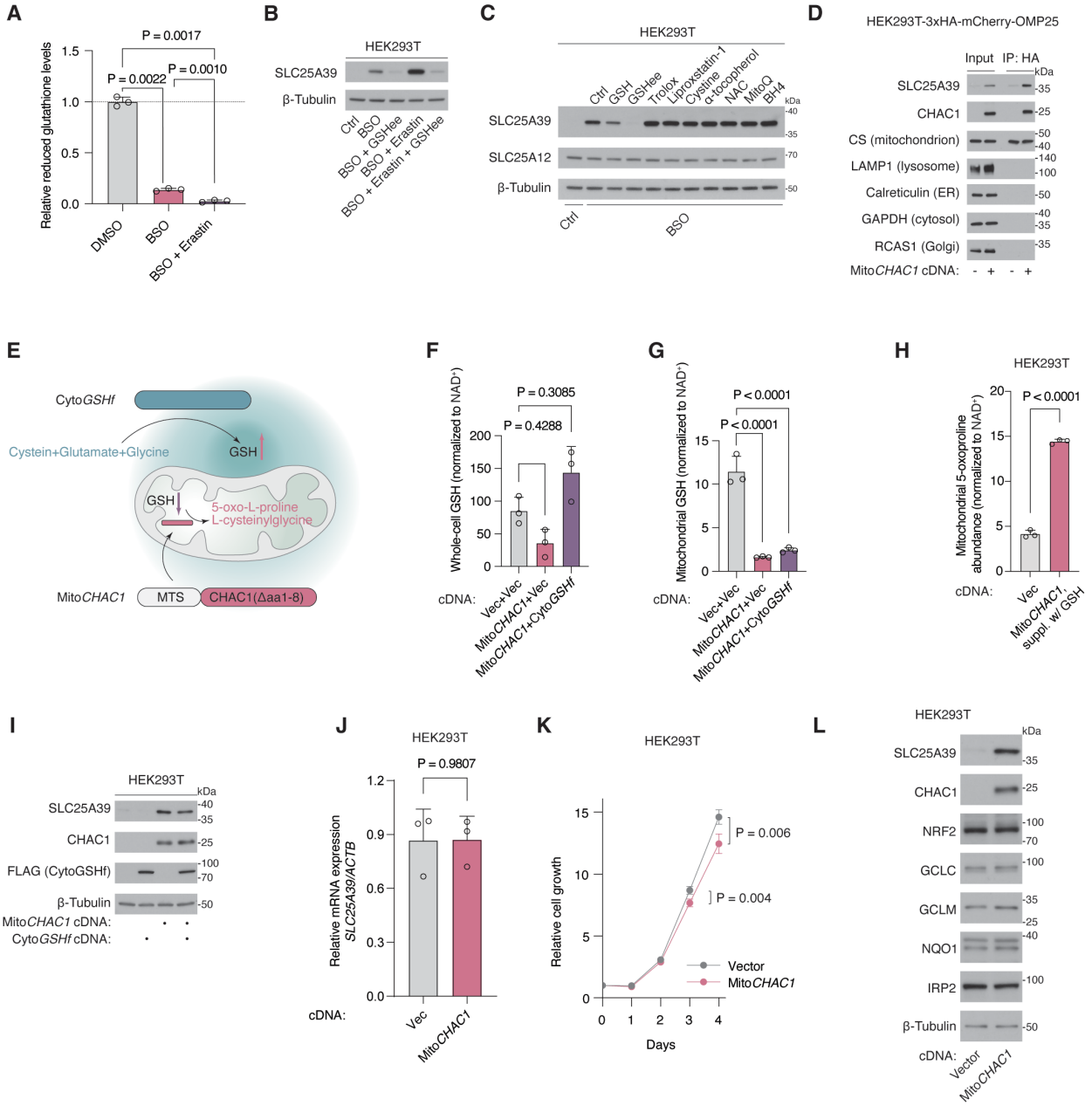


Fig. S1. Depletion of mitochondrial matrix glutathione stabilizes SLC25A39.

(**A**) Relative GSH abundance in HEK293T cells treated with DMSO, BSO (1 mM), or BSO (1 mM) + erastin (5 μM) for 24 hours, measured by LC-MS. Data normalized to total cellular protein amount and compared to the average value of DMSO-treated cells. (**B**) Immunoblots showing the levels of SLC25A39 in HEK293T cells treated with DMSO, BSO (1 mM), or BSO (1 mM) + Erastin (5 μM) for 24 hours. β-tubulin was used as a loading control. (**C**) Immunoblots of the indicated proteins in HEK293T cells after treated with BSO (1 mM) for 48 hours and then treated with GSH (10 mM), GSH ethyl ester (GSHee, 10 mM), Trolox (50 μM), Liproxstatin-1 (1 μM), cystine (200 μM), N-acetylcysteine (NAC, 1 mM), α-tocopherol (5 μM), MitoQ (30 nM) or BH4 (4 μM). β-tubulin and SLC25A12 are used as loading controls. (**D**) Immunoblots of the indicated proteins in whole-cell lysate (Input) and immunopurified mitochondria (IP: HA) from HEK293T cells expressing empty vector or Mito*CHAC1* cDNA. (**E**) Schematic of another strategy for simultaneous manipulation of cytosolic and mitochondrial glutathione pool. Mitochondria GSH was depleted by expressing MitoCHAC1, while cytosolic GSH pool was maintained by simultaneous

expression of a bacterial GSH synthase (cyto*GSHf*). (**F**) Whole-cell GSH abundance in HEK293T cells expressing empty vectors, MitoCHAC1, or MitoCHAC1 plus CytoGSHf, quantified by LC-MS. Data were normalized to NAD^+ abundance. (**G**) Mitochondrial GSH abundance in HEK293T cells expressing empty vectors, MitoCHAC1, or MitoCHAC1 plus CytoGSHf, quantified by LC-MS. Data were normalized to NAD^+ abundance. (**H**) Relative abundance of 5-oxoproline in immunopurified mitochondria from HEK293T cells expressing empty vectors or MitoCHAC1, measured through LC-MS. Data were normalized to NAD^+ levels. (**I**) Immunoblots of the indicated proteins in HEK293T cells expressing the cDNAs for empty vectors, Mito*CHAC1*, cyto*GSHf* or both. (**J**) Relative mRNA levels of *SLC25A39* normalized to *ACTB* in HEK293T cells expressing empty vector or MitoCHAC1. (**K**) Relative cell numbers of HEK293T cells expressing empty vector or MitoCHAC1, measured by Cell Titer-Glo viability assay over 4 days of growth. (**L**) Immunoblots of the indicated proteins in HEK293T cells expressing empty vectors or MitoCHAC1. GCLC, GCLM and NQO1 were chosen as representative NRF2 targets. β-tubulin is used as a loading control. (**A**, **F**-**H**, **J**-**K**), Data are mean ± s.d., representing three biologically independent samples. P-values were calculated from one-way ANOVA (**A**, **F**, **G**), Welch's *t*-test (**H**, **J**) or multiple unpaired Welch's *t*-test with Holm-Šídák method (**K**).

Fig. S2

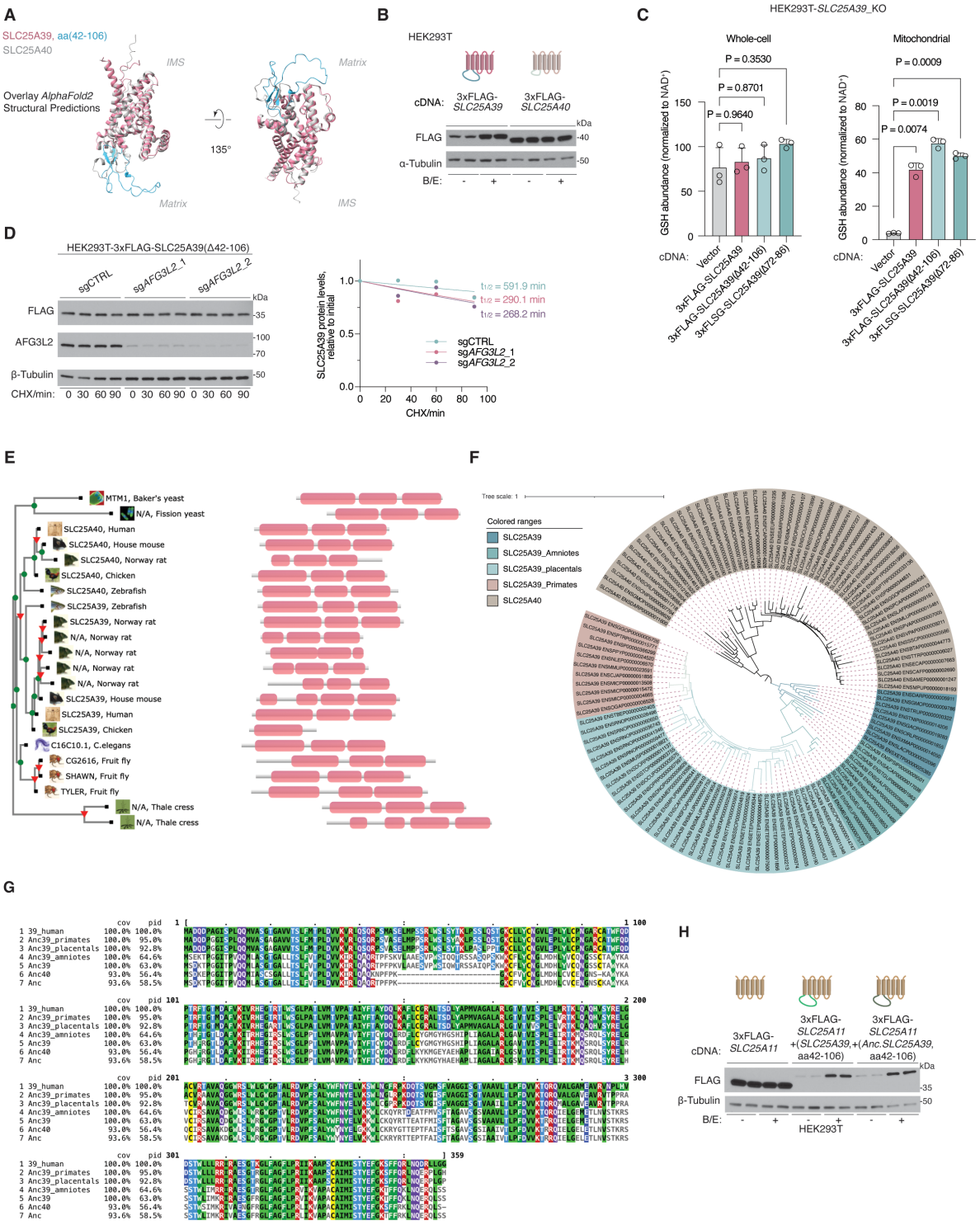
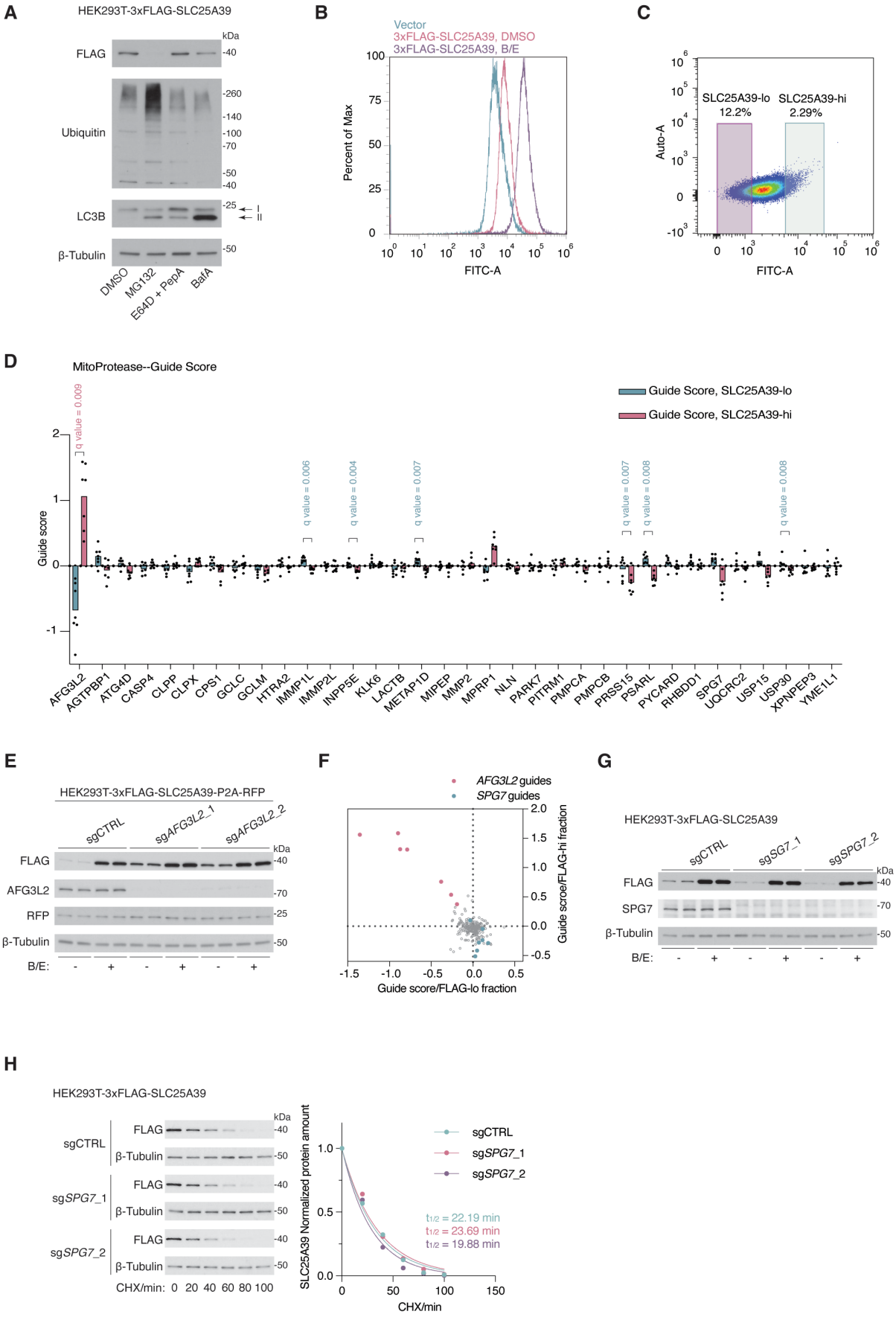


Fig. S2. A conserved matrix-side loop on SLC25A39 mediates its response to mitochondrial GSH.

(**A**) Overlay of AlphaFold2-predicted structural models of SLC25A39 and SLC25A40. SLC25A39 is highlighted in pink, with its amino acids 42-106 highlighted in green. (**B**) Immunoblots of FLAG-tagged proteins and α-tubulin in HEK293T cells expressing 3xFLAG-SLC25A39 or 3xFLAG-SLC25A40, after 24 hours treatment with BSO (1 mM) + erastin (5 μM) or DMSO as control. α-tubulin was used as a loading control. (**C**) Relative abundance of GSH in HEK293T-*SLC25A39*_KO cells expressing empty vector, SLC25A39, SLC25A39 without the matrix-facing loop (SLC25A39-Δ42-106) or SLC25A39 without

amino acids 72-86. Data were measured with LC-MS and normalized to NAD^+ abundance. P-values were calculated from one-way ANOVA. (**D**) Left, immunoblots of the indicated proteins in HEK293T cells expressing 3xFLAG-SLC25A39(Δ42-106) and sgRNAs targeting *AFG3L2* (for reasons mentioned later) or control, upon treatment with cycloheximide (CHX, 50 μg/ml) for the indicated times. β-tubulin was used as a loading control. Right, quantification of FLAG band signal intensity from the immunoblots. Half-life was calculated by the non-linear fitting of FLAG band signal intensity versus time to one phase decay exponential model. (**E**) Phylogenetic tree of *SLC25A39*, retrieved from the TreeFam database. (**F**) Phylogenetic tree of SLC25A39 homolog sequences across vertebrates used for the reconstruction of the ancestral sequence of SLC25A39. (**G**) Multiple sequence alignment of human SLC25A39 amino acid sequence with the inferred sequence of ancestral SLC25A39 and SLC25A40 in the indicated taxa. The last sequence (Anc) represents the common ancestral sequence of SLC25A39 and SLC25A40. (**H**) Immunoblots of FLAG-tagged proteins and α-tubulin in HEK293T cells expressing 3xFLAG-SLC25A39 or a chimeric protein in which the matrix-facing loop (aa42-106) of SLC25A39 or its ancestral sequence is spliced into 3xFLAG-SLC25A11. Cells were treated for 24 hours with BSO (1 mM) + erastin (5 μM) or DMSO as control. β-tubulin was used as a loading control.

Fig. S3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II
β-Tubulin
-50
DMSO
MG132
E64D + PepA
BafA
B
Vector
3xFLAG-SLC25A39, DMSO
3xFLAG-SLC25A39, B/E
Percent of Max
FITC-A
C
SLC25A39-lo 12.2%
SLC25A39-hi 2.29%
Auto-A
FITC-A
D
MitoProtease--Guide Score
Guide Score, SLC25A39-lo
Guide Score, SLC25A39-hi
Guide score
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sgCTRL
sgAFG3L2_1
sgAFG3L2_2
kDa
FLAG
-40
AFG3L2
-70
RFP
-25
β-Tubulin
-50
B/E:
F
AFG3L2 guides
SPG7 guides
Guide scroe/FLAG-hi fraction
Guide score/FLAG-lo fraction
G
HEK293T-3xFLAG-SLC25A39
sgCTRL
sgSG7_1
sgSPG7_2
kDa
FLAG
-40
SPG7
-70
β-Tubulin
-50
B/E:
H
HEK293T-3xFLAG-SLC25A39
kDa
sgCTRL
FLAG
-40
β-Tubulin
-50
sgSPG7_1
FLAG
-40
β-Tubulin
-50
sgSPG7_2
FLAG
-40
β-Tubulin
-50
CHX/min: 0 20 40 60 80 100
SLC25A39 Normalized protein amount
sgCTRL
sgSPG7_1
sgSPG7_2
t1/2 = 22.19 min
t1/2 = 23.69 min
t1/2 = 19.88 min
CHX/min

Fig. S3. Focused CRISPR screen identifies AFG3L2 as the protease for SLC25A39 degradation. (**A**) Immunoblots of the indicated proteins in HEK293T cells expressing 3xFLAG-*SLC25A39* cDNA. Cells underwent 6 hours of treatment with proteasome inhibitor MG132 (50 μM), lysosomal protease inhibitors E64D (25 μM) + Pepstatin A (50 μM), lysosome acidification inhibitor Bafilomycin A (0.5 μM) or DMSO as control. (**B**) Histogram showing FITC (3xFLAG-SLC25A39) signal intensity in flow cytometry analysis of HEK293T cells expressing empty vector or 3xFLAG-*SLC25A39* cDNA. Cells were treated for 24 hours with DMSO or BSO (1 mM) + erastin (5 μM), fixed, and stained with FITC-conjugated anti-FLAG-M2 antibodies. (**C**) Scatter plot showing the gating strategy for sorting SLC25A39-lo and SLC25A39-hi population of HEK293T cells expressing 3xFLAG-*SLC25A39* cDNA. Cells were fixed and stained with FITC-conjugated anti-FLAG-M2 antibodies. (**D**) Bar plot representing the enrichment of sgRNAs (Guide score) in SLC25A39-lo or SLC25A39-hi cell population. Q-values derived from multiple paired *t*-test followed by false discovery rate (FDR) calculation with two-stage step-up (Benjamini, Krieger and Yekutieli) methods. The red annotation indicates that sgRNAs targeting the indicated gene are significantly enriched in the SLC25A39-hi fraction. Green annotation indicates that sgRNAs targeting the indicated gene are significantly enriched in SLC25A39-lo fraction (opposite to the expected behavior of the protease candidate for SLC25A39, so not counted as screen hits). (**E**) Immunoblots of the indicated proteins in HEK293T cells expressing the 3xFLAG-SLC25A39-P2A-RFP construct and sgRNAs targeting control or *AFG3L2*, after 24-hour treatment with BSO (1 mM) + erastin (5 μM) or DMSO as a control. (**F**) Scatter plot showing the enrichment of sgRNAs targeting mitochondrial proteases in the SLC25A39-lo cell fraction (x-axis) and SLC25A39-hi cell fraction (y-axis). Red dots represent sgRNAs targeting *AFG3L2*. Green dots represent sgRNAs targeting *SPG7*. (**G**) Immunoblots of the indicated proteins in HEK293T cells expressing 3xFLAG-SLC25A39 and sgRNAs targeting control or *SPG7*, after 24-hour treatment with BSO (1 mM) + erastin (5 μM) or DMSO as a control. (**H**) Left, immunoblots of the indicated proteins in HEK293T cells expressing 3xFLAG-SLC25A39 and sgRNAs targeting *SPG7* or control, upon treatment with cycloheximide (CHX, 50 μg/ml) for indicated times. β-tubulin was used as a loading control. Right, quantification of FLAG bands signal intensity from the immunoblots. The half-life was calculated by the non-linear fitting of FLAG band signal intensity versus time to one phase decay exponential model.

Fig. S4

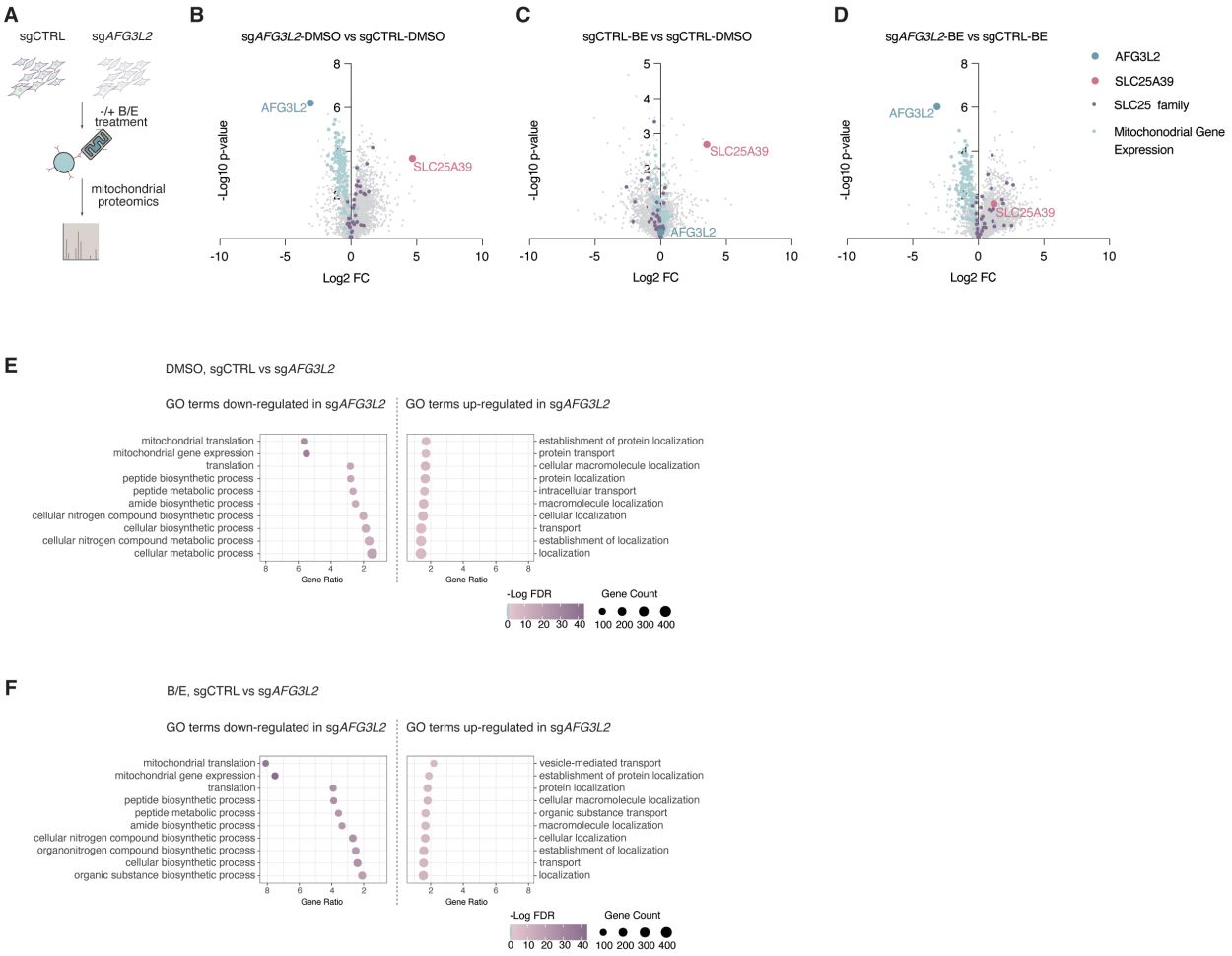

Fig. S4. AFG3L2 mediates the GSH-dependent proteolysis of SLC25A39.
(**A**) Schematic for profiling mitochondrial proteome upon the knockout of *AFG3L2* or control, after 24-hour treatment with BSO (1 mM) + erastin (5 μM) or DMSO as a control. (**B**) Volcano plots representing the fold-change and statistical significance of proteins from immunopurified mitochondria in HEK293 cells expressing the indicated sgRNAs, after 24 hours treatment with DMSO as a control or BSO (1 mM) + erastin (5 μM). The red dot represents *SLC25A39*; the purple dots represent other members of the SLC25 family, the dark green dot represents AFG3L2 and the light green dots represent proteins involved in mitochondrial gene expression. (**C**, **D**) Gene ontology enrichment analyses of differentially expressed proteins from mitochondrial proteomics experiment on HEK293T cells expressing *AFG3L2* or non-targeting control sgRNA, after 24 hours treatment with DMSO as a control or BSO (1 mM) + erastin (5 μM). Genes encoding proteins with nominal p-value <0.05 were chosen as the input gene set.

Fig. S5

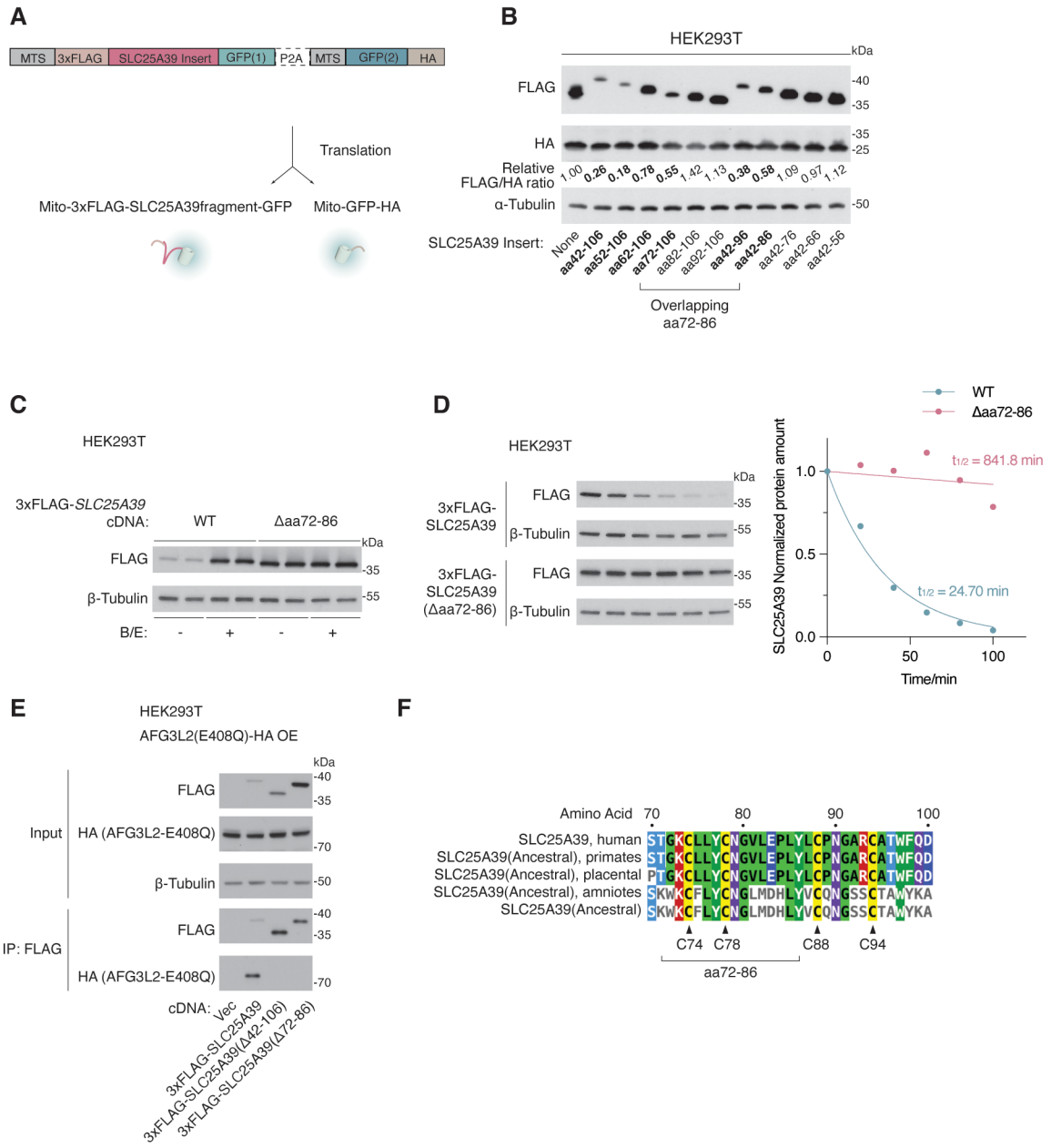

Fig. S5. A short fragment on the matrix-facing loop of SLC25A39 is essential for its proteolysis.

(**A**) Schematic for the construct for analyzing the stability of mitochondria-targeted protein fragments. Serially truncated SLC25A39 matrix-facing loop fragments are fused to a mitochondria-targeted, 3xFLAG-tagged GFP. A co-translated, HA-tagged, mitochondria-targeted GFP is used as an internal control. (**B**) Immunoblots of the FLAG-tagged, HA-tagged proteins and α-tubulin from HEK293T cells expressing the aforementioned construct. The indicated fragments from SLC25A39 were fused to the FLAG-tagged, mitochondria-targeted GFP. FLAG/HA ratio was quantified. α-tubulin was used as a loading control. (**C**) Immunoblots of FLAG-tagged proteins and β-tubulin in HEK293T cells expressing 3xFLAG-SLC25A39 or 3xFLAG-SLC25A39(Δ72-86). Cells were treated for 24 hours with BSO (1 mM) + erastin (5 μM) or DMSO as control. β-tubulin was used as a loading control. (**D**) Left, immunoblot of indicated proteins in HEK293T cells expressing 3xFLAG-SLC25A39 or 3xFLAG-SLC25A39(Δ72-86), after being treated with cycloheximide (CHX, 50 ug/ml) for the indicated times. β-tubulin was used as a loading control. Right,

quantification of FLAG band signal intensity from the immunoblot. Half-life was calculated by the non-linear fitting of FLAG band signal intensity versus time to one phase decay exponential model. (**E**) Immunoblots of the indicated proteins from whole-cell lysates or FLAG-immunoprecipitation from HEK293T cells stably expressing cDNA for empty vector, 3xFLAG-SLC25A39 or 3xFLAG-SLC25A39(Δ72-86), and transiently transfected with *AFG3L2*(E408Q)-HA cDNA. (**F**) Multiple sequence alignment between SLC25A39 and inferred ancestral sequence of SLC25A39 reconstructed from amino acid sequences of SLC25A39 homologs in the indicated taxa. Four conserved cysteines in the matrix-facing loop and amino acids 72-86 were highlighted.

Fig. S6

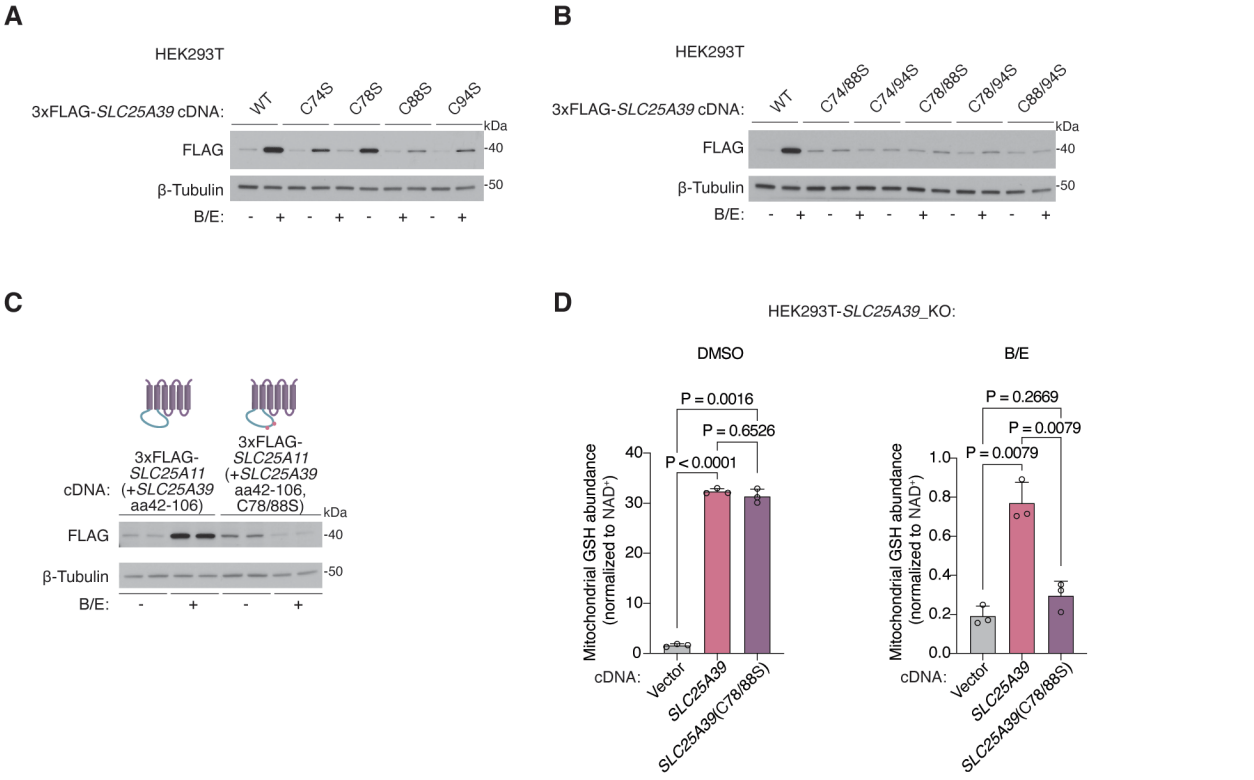

Fig. S6. Conserved cysteine residues on the matrix-facing loop of SLC25A39 are essential for sensing mitochondrial glutathione.

(**A**, **B**) Immunoblots for the indicated proteins in HEK293T cells expressing wild-type or mutant 3xFLAG-*SLC25A39* cDNA. β-tubulin was used as loading control. (**C**) Immunoblots for the indicated proteins in HEK293T cells expressing chimeric proteins where SLC25A11 was spliced with the matrix-facing loop of SLC25A39, or the same loop carrying C78S/C88S mutations. (**D**) Abundance of GSH in immunopurified mitochondria from HEK293T-*SLC25A39*_KO cells expressing cDNAs for empty vector, *SLC25A39*, or *SLC25A39*(C78/88S). Cells were treated for 24 hours with DMSO as control (left) or BSO (1 mM) + erastin (5 μM). GSH abundance was quantified by LC-MS and normalized to mitochondrial NAD^+. Data represent three biologically independent samples. P-values were calculated from one-way ANOVA.

Fig. S7

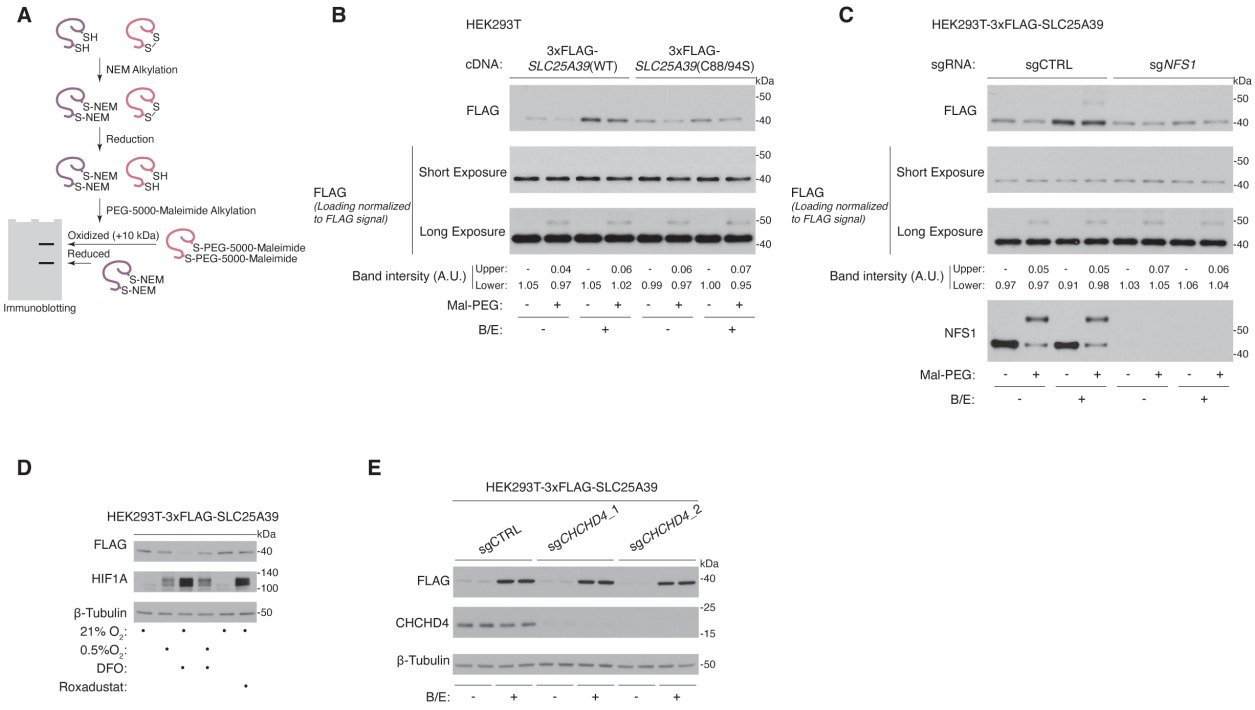

Fig. S7. Disulfide bond formation and relay and hypoxic response do not account for the GSH-sensitive regulation of SLC25A39.

(**A**) Schematic of the workflow for detecting oxidized cysteines via PEG5000-maleimide alkylation. Reduced cysteine residues were blocked by NEM alkylation, and oxidized cysteines were then reduced by DTT treatment and labeled with PEG5000-maleimide (with a molecular weight of ~5 kilodaltons). (**B**) Immunoblots showing FLAG-tagged SLC25A39 or SLC25A39(C78/88S) protein expressed in HEK293T cells after the PEG5000-maleimide alkylation assay, with both short exposure and long exposure. The band intensity of FLAG protein was quantified, and β-tubulin was used as a loading control. (**C**) Immunoblots showing FLAG-tagged SLC25A39 protein expressed in HEK293T cells along with *NFS1* or non-targeting sgRNA after the PEG5000-maleimide alkylation assay, with both short exposure and long exposure. The band intensity of FLAG protein was quantified, and β-tubulin was used as a loading control. (**D**) Immunoblots for the indicated proteins in 3xFLAG-SLC25A39-expressing HEK293T cells, after 8 hours of treatment with DFO (50 μM), Roxadustat (30 μM) or hypoxia of 0.5% O_2. (**E**) Immunoblots for the indicated proteins in HEK293T cells expressing 3xFLAG-SLC25A39 along with Cas9 and a control sgRNA, or sgRNAs targeting *CHCHD4* (MIA40). Cells were treated for 24 hours with BSO (1 mM) + erastin (5 μM) or DMSO as a control. β-tubulin was used as a loading control.

Fig. S8

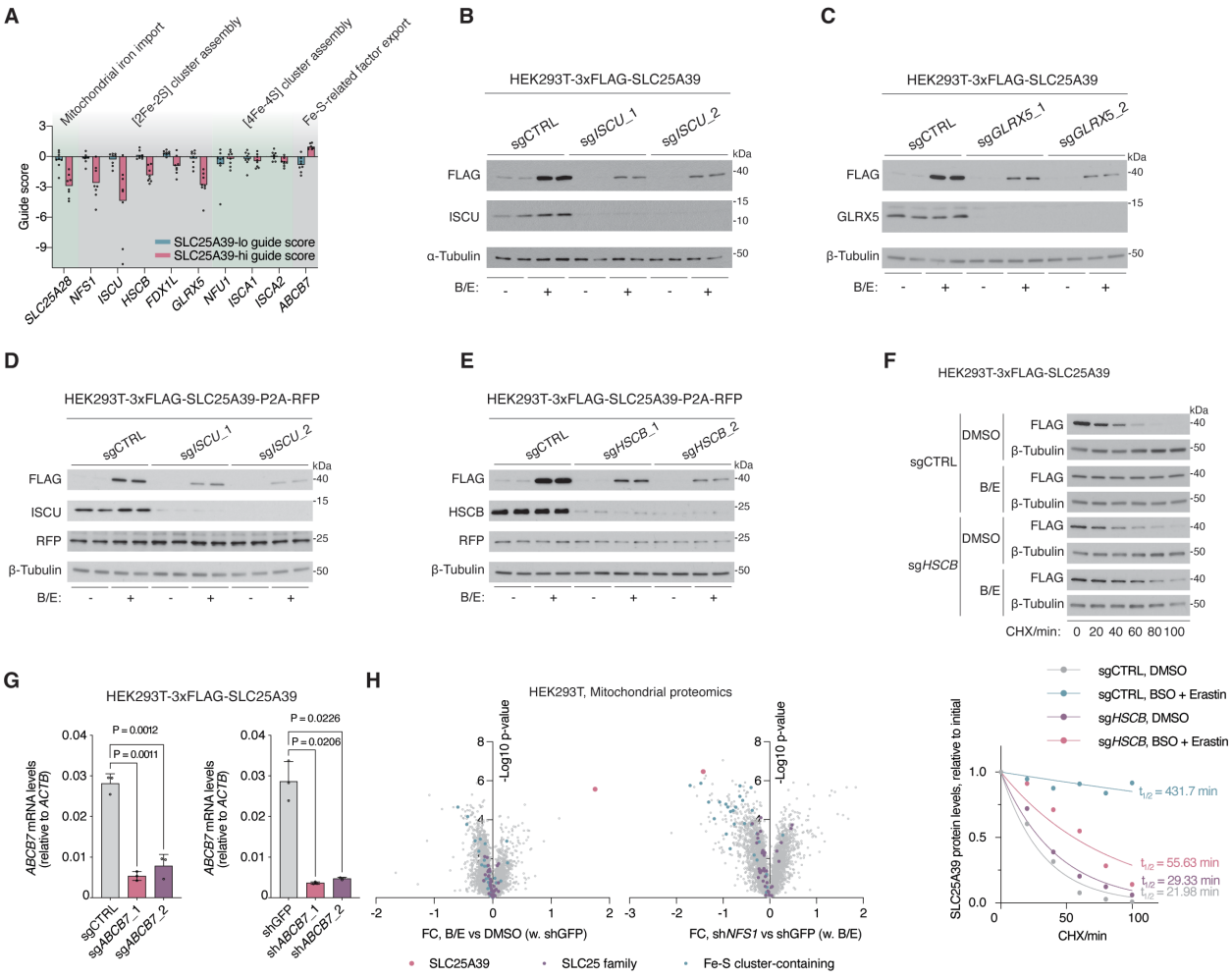

Fig. S8. A putative iron-sulfur cluster mediates SLC25A39 stabilization upon the depletion of GSH. (**A**) Enrichment of sgRNAs (guide scores) in the SLC25A39-hi and SLC25A39-lo fractions for the indicated genes in the MITO-sgRNA CRISPR screen. (**B**) Immunoblots for the indicated proteins in HEK293T cells expressing 3xFLAG-*SLC25A39* cDNA. Cells were transduced with lentivirus expressing sgRNAs targeting *ISCU* or a control sgRNA. α-tubulin was used as a loading control. (**C**) Immunoblots for the indicated proteins in HEK293T cells expressing 3xFLAG-*SLC25A39* cDNA. Cells were transduced with lentivirus expressing sgRNAs targeting *GLRX5* or a control sgRNA. β-tubulin was used as a loading control. (**D**) Immunoblots for the indicated proteins in HEK293T cells expressing 3xFLAG-SLC25A39-P2A-RFP construct. Cells were transduced with lentivirus expressing sgRNAs targeting *ISCU* or a control sgRNA. β-tubulin was used as a loading control. (**E**) Immunoblots for the indicated proteins in HEK293T cells expressing 3xFLAG-SLC25A39-P2A-RFP construct. Cells were transduced with lentivirus expressing sgRNAs targeting *HSCB* or a control sgRNA. β-tubulin was used as a loading control. (**F**) Top, immunoblots for the indicated proteins from HEK293T cells expressing 3xFLAG-*SLC25A39* cDNA, along with sgRNAs targeting *HSCB* or a control sgRNA. Cells were treated with for 24 hours with BSO (1 mM) + erastin (5 μM) or DMSO as a control, and then with cycloheximide (50 μg/ml) for the indicated times before harvested. Bottom, quantification of FLAG band signal intensity from the immunoblots. Half-life was calculated by the non-linear fitting of FLAG band signal intensity versus time to one phase decay exponential model. (**G**) *ABCB7* mRNA levels, quantified by RT-qPCR, of HEK293T cells expressing 3xFLAG-*SLC25A39* cDNA, along with Cas9 and sgRNAs targeting *ABCB7* versus control sgRNA (left), or shRNAs targeting *ABCB7* versus GFP as a control (right). Data were normalized to mRNA levels of *ACTB* by $\Delta\Delta C_T$ method. P-values were calculated from one-way ANOVA. (**H**) Volcano plots representing

the fold-change and statistical significance of proteins from immunopurified mitochondria in HEK293 cells expressing the indicated shRNAs, after 24 hours treatment with DMSO as a control or BSO (1 mM) + erastin (5 μM). The red dot represents *SLC25A39*; purple dots represent other members of the SLC25 family and green dots represent know iron-sulfur cluster-containing protein.

5

Fig.S9

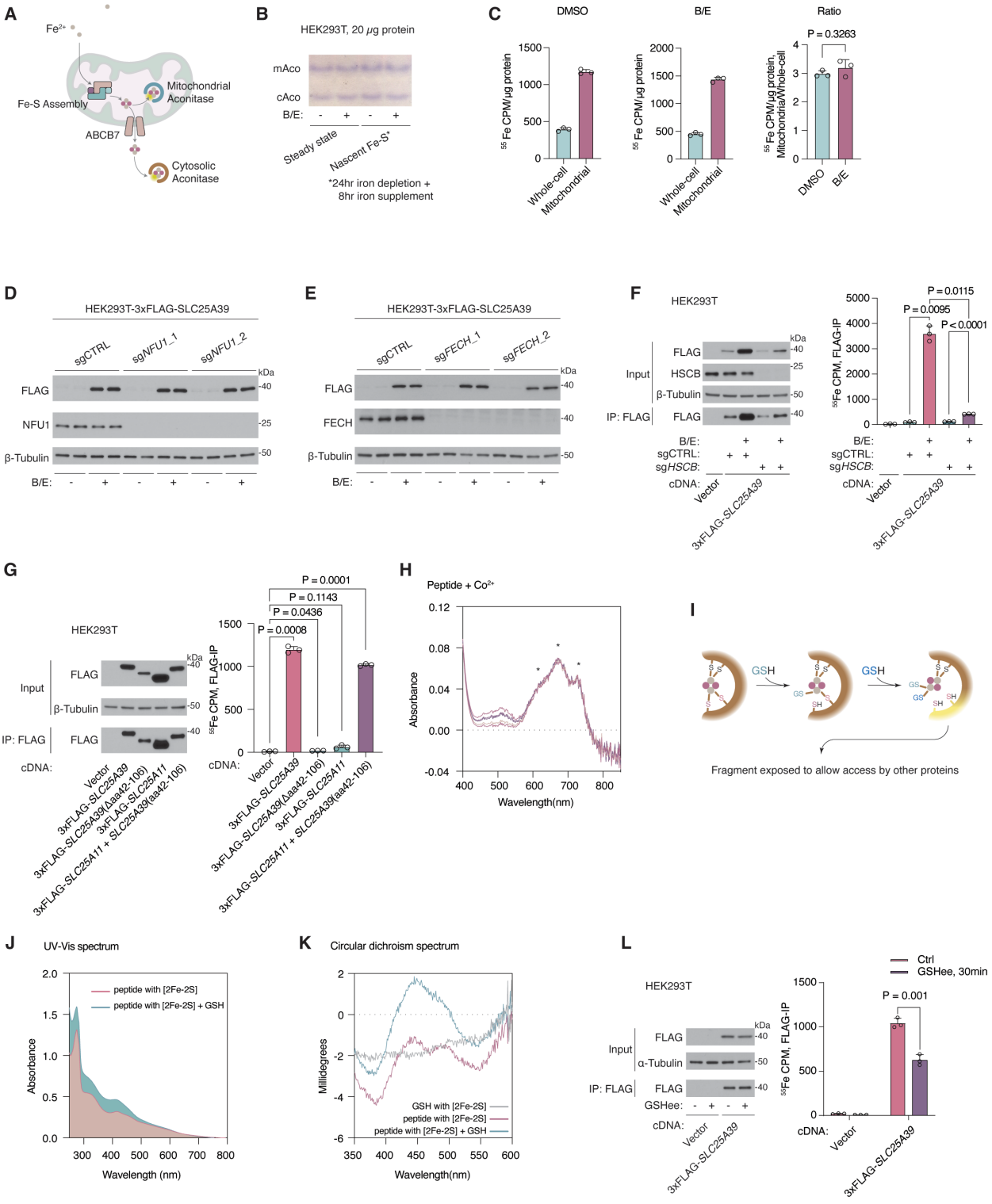

Fig. S9. SLC25A39 associates with a GSH-sensitive [2Fe-2S] cluster.

(**A**) Schematic showing the incorporation of iron-sulfur clusters from the cytosolic or mitochondrial pool into aconitase. (**B**) In-gel aconitase assay showing the activity of aconitase in HEK293T cells treated for 24 hours with BSO (1 mM) + erastin (5 μM) or DMSO as a control. For analyzing nascent iron-sulfur cluster levels, cells were pretreated with 20μM iron chelator deferoxamine (DFO) for 12 hours to deplete the cellular iron pool in the presence of BSO (1 mM) + erastin (5 μM) or DMSO as a control, and iron

(100μg/ml ferric ammonium citrate, FAC) was added for 12 hours to replenish cellular iron. (**C**) ^{55}Fe levels measured by liquid scintillation assay for whole-cell or immunopurified mitochondria from HEK293T cells treated for 24 hours with BSO (1 mM) + erastin (5 μM) or DMSO as a control. Cells were labeled with $^{55}FeCl_3$ for 48 hours prior to the assay. (**D** - **E**), Immunoblots for the indicated proteins in HEK293T cells expressing 3xFLAG-SLC25A39 along with Cas9 and a control sgRNA, or sgRNAs targeting *NFU1* or *FECH*. Cells were treated for 24 hours with BSO (1 mM) + erastin (5 μM) or DMSO as a control. β-tubulin was used as a loading control. (**F**) Left, immunoblots of the indicated proteins from whole-cell lysate or FLAG-immunoprecipitation from HEK293T cells stably expressing cDNAs for empty vector or 3xFLAG-*SLC25A39*, infected with lentivirus expressing control sgRNA or sgRNA targeting *HSCB*, and labeled with radioactive $^{55}FeCl_3$. Cells were treated for 24 hours with BSO (1 mM) + Erastin (5 μM) or DMSO as control. Right, the amount of ^{55}Fe bound to FLAG-immunoprecipitant quantified by liquid scintillation assay, from the identical cells as those in the immunoblot. (**G**) Left, immunoblots of the indicated proteins from whole-cell lysate or FLAG-immunoprecipitation from HEK293T cells stably expressing cDNAs for empty vector, 3xFLAG-*SLC25A39*, 3xFLAG-*SLC25A39* without the matrix-facing loop (Δ42-106), 3xFLAG-*SLC25A11* or a chimeric protein where aa42-106 of SLC25A39 is splice into SLC25A11. Cells were labeled with radioactive $^{55}FeCl_3$ and treated for 24 hours with BSO (1 mM) + erastin (5 μM). Right, the amount of ^{55}Fe bound to FLAG-immunoprecipitant quantified by liquid scintillation assay, from the identical cells as those in the immunoblots. (**H**) The UV-visible spectrum of a synthetic peptide corresponding to aa73-95 of SLC25A39, in complex with Co^{2+}. (**I**) Schematic showing the ligand exchange reaction between GSH and iron-sulfur cluster-containing holoprotein. (**J**) The UV-visible spectrum of a synthetic peptide corresponding to aa73-95 of SLC25A39, in complex with reconstituted [2Fe-2S] cluster, prior to and after the addition of 12.5 mM GSH. (**K**) Circular dichroism spectrum of a synthetic peptide corresponding to aa73-95 of SLC25A39, in complex with reconstituted [2Fe-2S] cluster, prior to and after the addition of 12.5 mM GSH. (**L**) Left, immunoblots of the indicated proteins from whole-cell lysate or FLAG-immunoprecipitation of HEK293T cells stably expressing cDNAs for empty vector or 3xFLAG-*SLC25A39*, labeled with radioactive $^{55}FeCl_3$ and treated for 24 hours with BSO (1 mM) + erastin (5 μM). Cells were incubated with 20 mM GSH ethyl ester (GSHee) or control for 30 minutes immediately before immunoprecipitation. Right, the amount of ^{55}Fe bound to FLAG-immunoprecipitant quantified by liquid scintillation assay, from the identical cells as those in the immunoblots. (**C**, **F**, **G**, **L**), Data are mean ± s.d., representing three biologically independent samples. P-values were calculated from Welch's *t*-test (**C**, **L**) or one-way ANOVA (**F**, **G**).

Fig. S10

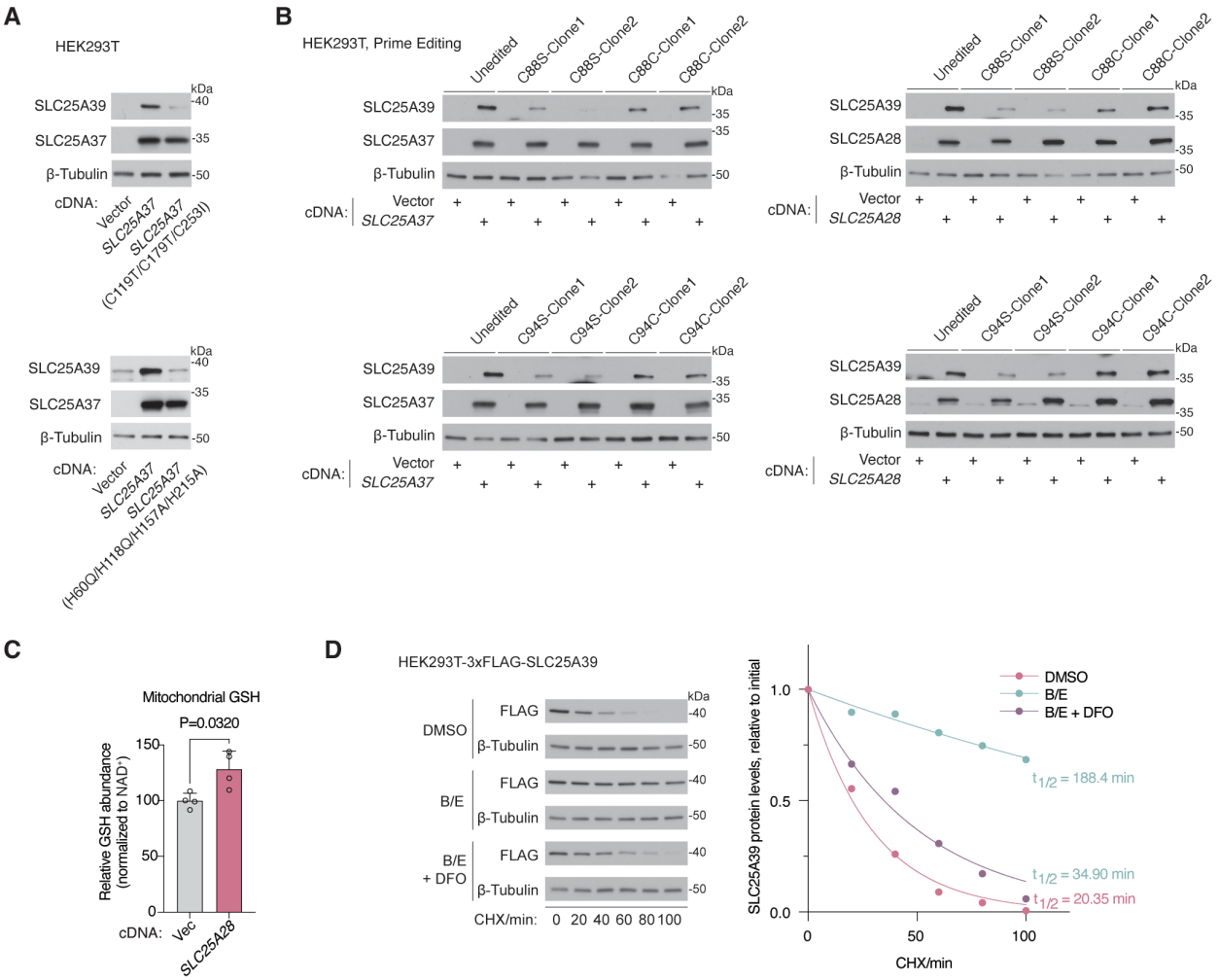

Fig. S10. Mitochondrial iron overload stabilizes SLC25A39.

(**A**) Immunoblots of the indicated protein from HEK293T cells expressing SLC25A37, or transport-deficient SLC25A37(C119T/C179T/C253I) or SLC25A37(H60Q/H118Q/H157A/H215A) mutants, or an empty vector as a control. (**B**) Immunoblots of the indicated protein from parental HEK293T cells or HEK293T cells subjected to prime editing to introduce cysteine-to-serine mutations at C88 or C94. Prime editing that introduces synonymous mutations was used as controls. Cells were transduced with lentiviral vectors expressing SLC25A37, SLC25A28 or an empty vector as a control. (**C**) Relative abundance of GSH in mitochondria isolated from HEK293T cells overexpressing cDNAs of Mitoferrin 2 (*SLC25A28*) or empty vector. Data are mean ± s.d., representing four biologically independent samples. P-value was calculated from Welch's *t*-test. (**D**) Left, immunoblots of the indicated proteins in HEK293T cells expressing 3xFLAG-SLC25A39 treated with cycloheximide (CHX, 50 μg/ml) for the indicated times. Cells were pre-treated with BSO (1 mM) + erastin (5 μM) and DFO (20 μM) or DMSO as a control for 24 hours. β-tubulin was used as a loading control. Right, quantification of FLAG bands signal intensity from the immunoblots above. Half-life was calculated by non-linear fitting of FLAG bands signal intensity versus time to one phase decay exponential model.

Fig. S11

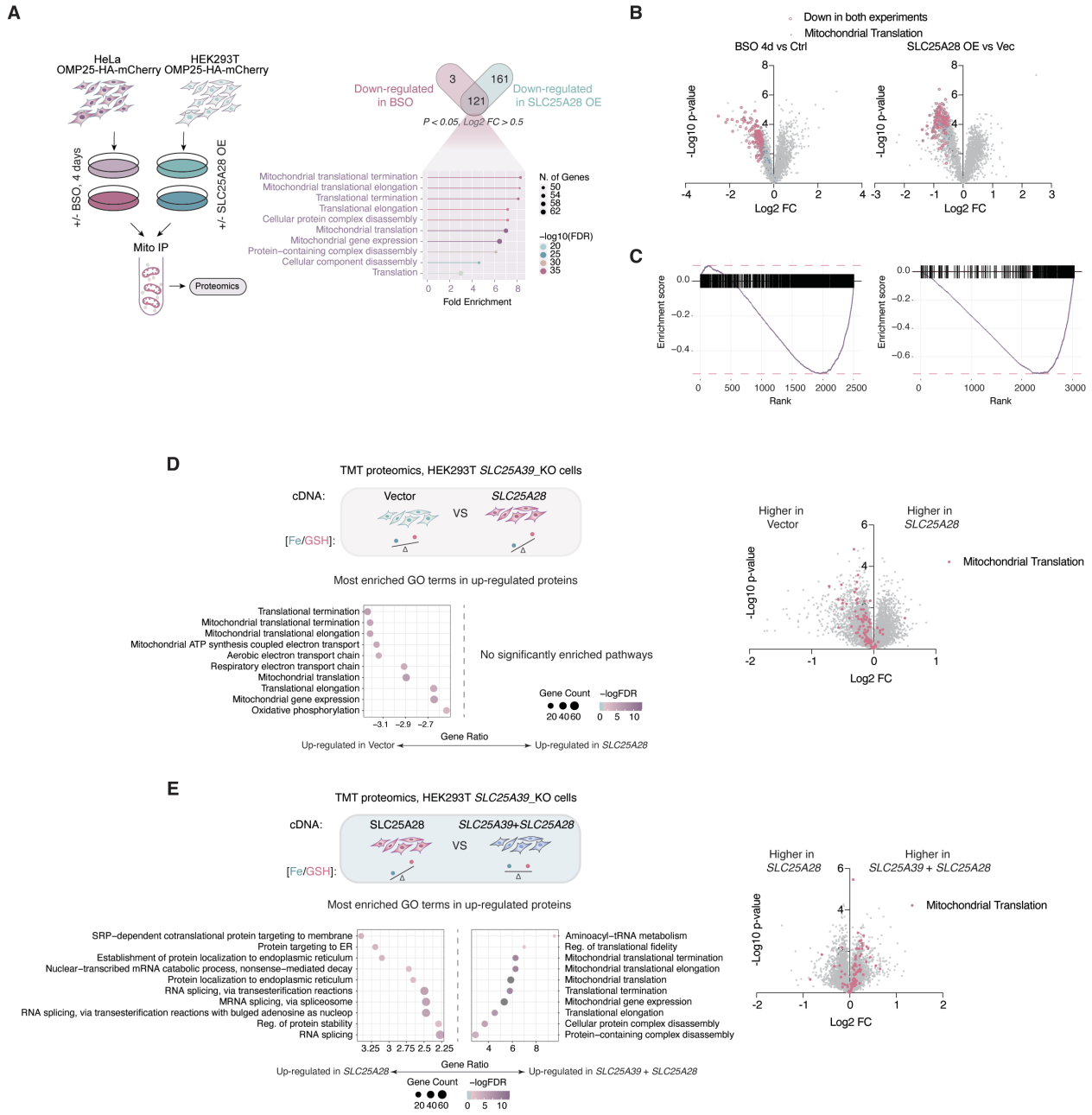

Fig. S11. Aberrant Fe/GSH balance leads to changes in mitochondrial proteome.

(**A**) Left, schematic for the workflow for quantitative proteomics with immunopurified mitochondrial from HeLa or HEK293T cells with disrupted Fe/GSH balance. Hela cells were pretreated with 1 mM BSO or control for 4 days. HEK293T cells were transduced with lentiviral vectors carrying an empty vector or *SLC25A28* cDNA. Right, Venn diagram showing the overlap between the down-regulated proteins across the indicated comparisons, and the gene ontology enrichment analysis for the commonly down-regulated proteins. (**B**) Volcano plot showing the log2(fold change) and -log10 (p-value) of mitochondrial protein levels from HeLa or HEK293T cells with disrupted Fe/GSH balance. Purple dots denote proteins that are downregulated in both experiments. Green dots denote proteins involved in mitochondrial translation. (**C**) Gene set enrichment analysis plots cross-comparing the downregulated proteins in HeLa or HEK293T cells with disrupted Fe/GSH balance. Left, enrichment analysis for downregulated proteins in HEK293T cells overexpressing SLC25A28 against the mitochondrial proteome changes in Hela cells treated with BSO

versus control. Right, enrichment analysis for downregulated proteins in Hela cells treated with BSO against the mitochondrial proteome changes in HEK293T cells overexpressing SLC25A28 versus empty vector. (**D**) Left, gene ontology enrichment analysis of differentially expressed proteins from TMT-proteomics experiment on HEK293T-*SLC25A39*_KO cells overexpressing empty vector or *SLC25A28* cDNA. Genes encoding proteins with nominal p-value <0.05 were chosen as the input gene set. Right, Volcano plot showing the log2(fold change) and -log10(p-value) of protein levels from HEK293T cells overexpressing empty vector or *SLC25A28* cDNA. Proteins involved in mitochondrial translation are shown in purple. (**E**) Left, gene ontology enrichment analysis of differentially expressed proteins from TMT-proteomics experiment on HEK293T-*SLC25A39*_KO cells overexpressing *SLC25A28* cDNA or *SLC25A39* + *SLC25A28* cDNA. Genes encoding proteins with nominal p-value <0.05 were chosen as the input gene set. Right, Volcano plot showing the log2(fold change) and -log10(p-value) of protein levels from HEK293T cells overexpressing *SLC25A28* cDNA or *SLC25A39* + *SLC25A28* cDNA. Proteins involved in mitochondrial translation are shown in purple.

Fig. S12

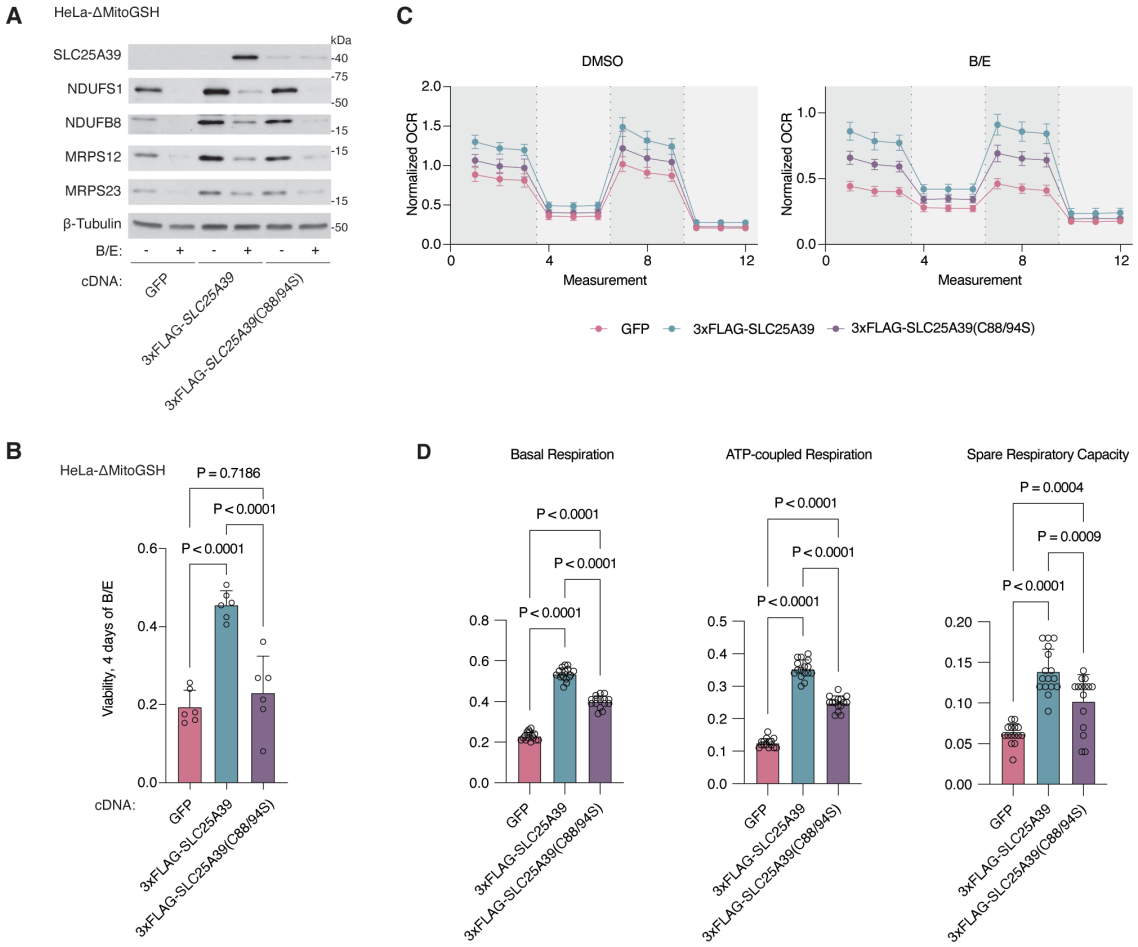

Fig. S12. Feedback regulation of SLC25A39 protects mitochondrial function under GSH limitation. (**A**) Immunoblots of the indicated proteins from HeLa cells defective in mitochondrial glutathione uptake, complemented with either an empty vector, wildtype SLC25A39 or regulation-defective SLC25A39(C88/94S) mutant. Cells were treated with for 72 hours with BSO (1 mM) + erastin (5 μM) or DMSO as a control. (**B**) Viability of HeLa cells defective in mitochondrial glutathione uptake, complemented with either an empty vector, wildtype SLC25A39 or regulation-defective SLC25A39(C88/94S) mutant, after 4 days treatment with BSO (1 mM) + erastin (5 μM) or DMSO as a control. Data are mean ± s.d., representing six biologically independent samples. P-values were calculated from one-way ANOVA. (**C**) Normalized oxygen consumption rate and (**D**) quantification of basal, ATP production-coupled respiration and spare respiratory capacity of HeLa cells defective in mitochondrial glutathione uptake, complemented with either an empty vector, wildtype SLC25A39 or regulation-defective SLC25A39(C88/94S) mutant, after 48 hours treatment with BSO (1 mM) + erastin (5 μM) or DMSO as a control. Data are mean ± s.d., representing 15 or 16 biologically independent samples. P-values were calculated from one-way ANOVA.

Data S1. HMMER search result for SLC25A39 loop region homologs

Amino acids 42-72 of human SLC25A39 protein and the homologous region of the reconstructed ancestral sequence were used as queries to search against UniprotKB sequence database.

Data S2. Gene scores and guide scores for all CRISPR screens

Related to Fig. 2E, 3B, S3D, S8A.

Data S3. Proteomics results for isolated mitochondria with Ctrl vs AFG3L2 knockout under DMSO vs B/E treatment

Related to Fig. S4.

Data S4. TMT proteomics results for isolated mitochondria with Ctrl vs *NFS1* knockdown and DMSO vs B/E treatment

Related to Fig. 3E and S8H.

Data S5. TMT proteomics results for SLC25A39 cysteine reactivity, with IA-DTB labeling

Related to Fig. 4F.

Data S6. Mitochondrial proteomics results for cells with aberrant Fe/GSH ratio

Related to Fig. S11A-S11C.

Data S7. Whole-cell TMT proteomics results for HEK293T-*SLC25A39*_KO cells overexpressing Vector, SLC25A28, or SLC25A28 + SLC25A39

Related to Fig. 5E, S11D and S11E.