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Supporting Information for
Cysteine induces mitochondrial reductive stress in glioblastoma through hydrogen peroxide production

Evan K Noch[1,2,*], Laura Palma[2], Isaiah Yim[2], Nayah Bullen[2], Daniel Barnett[3], Alexander Walsh[3], Bhavneet Bhinder[4,5], Elisa Benedetti[4,5], Jan Krumsiek[4,5], Justin Gurvitch[2], Sumaiyah Khwaja[2], Daphne Atlas[6], Olivier Elemento[4,5], Lewis C. Cantley[7]

[1]Department of Neurology, Division of Neuro-oncology, [2]Sandra and Edward Meyer Cancer Center, Weill Department of Medicine, [3]Graduate School of Medical Sciences, [4]Department of Physiology and Biophysics, Institute for Computational Biomedicine, [5]Caryl and Israel Englander Institute for Precision Medicine, Weill Cornell Medicine, New York, NY, USA; [6]Department of Biological Chemistry, Institute of Life Sciences, The Hebrew University of Jerusalem [7]Department of Cell Biology, Harvard Medical School; [*]Lead Contact

Corresponding Author: Evan Noch, MD, PhD
Email: evan.noch@utsouthwestern.edu

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Supporting Information Text

Methods

Chemicals and Reagents

The following chemicals were purchased from Sigma: N-acetylcysteine (A7250), L-cysteine (168149), L-methionine (M5308), 2-deoxyglucose (2-DG) (D6134), D-cysteine (30095), L-cystine (C6727), glutathione reduced ethyl ester (GREE) (G1404), reduced glutathione (GSH) (G6013), oxidized glutathione (GSSG) (G4376), catalase (C1345), pCMB (C5913), and H_2O_2 (H1009). U-13C6-D-glucose (CLM-1396), 13C5-glutamine (CLM-1822) and 13C3-L-cysteine (CLM-4320-H) were purchased from Cambridge Isotope Laboratories. Hydrogen peroxide lucerin was purchased from Swiss Lumix (GL-61204-5). TXM-CB4 was purchased from Pepmic. Recombinant human Trx1 (LFP0001) and TrxR2 (LFP0019) were purchased from Life Technologies. Recombinant human GR was purchased from Life Technologies (8866-GR).

Vectors

Non-target (NT) (SHC016), human SLC7A11 (TRCN0000043126, shSLC7A11-2; TRCN0000288926, shSLC7A11-5), and human Txn2 (TRCN0000377410, shTxn2-3; TRCN0000333453, Txn2-4) shRNA were purchased from Sigma. Human *TXN*, *TXN2*, *GSR*, *TXNRD2*, and *CAT* expression vectors were purchased from Genscript and cloned into the pLenti-Puro vector within BamHI and EcoRI restriction sites. The 3X-Myc-EGFP-OMP25 (Addgene plasmid number 83355) and 3X-HA-EGFP-OMP25 (Addgene plasmid number 83356) vectors were purchased from Addgene and cloned into the pLenti-Puro vector within BamHI and EcoRI restriction sites. The Trx2 C90S/C93S mutant was prepared from *TXN2* using overlap PCR with the following primers: forward GTGGAGTGGACCCAGCAA and reverse CTTGCTGGGTCCACTCCAC.

Cell culture

667 and 603 glioma cells were obtained from Cameron Brennan at Memorial Sloan Kettering Cancer Center. MGG119 and MGG152 cells were obtained from Daniel Cahill at Massachusetts General Hospital. 667 cells were cultured in 1:1 Neurobasal medium and DMEM/F12 medium supplemented with Glutamax, HEPES, sodium pyruvate, minimal essential amino acids, Pen/Strep, B27 supplement minus vitamin A, heparin (2 μg/ml), and EGF and FGF (20 μg/ml) (all from Life Technologies, Waltham, MA, USA). 603 cells were cultured in the same medium as 667 cells except with the addition of 20 ng/ml PDGF-AA and PDGF-BB (Shenandoah). MGG119 and MGG152 cells were cultured in Neurobasal medium containing Primary human fetal astrocytes were purchased from Thermo Fisher (NC9711462) and cultured in DMEM with 15% FBS. A549 (ATCC Cat# A549, RRID:CVCL_0023), MCF7 (ATCC Cat# MCF7, RRID:CVCL_0031), H1975 (ATCC Cat# CRL-5908, RRID:CVCL_1511), HT-29 (ATCC Cat# HT-29, RRID:CVCL_0320), and HPAF-II (ATCC Cat# CRL-1997, RRID:CVCL_0313) cells were purchased from ATCC. A549 and HT-29 cells were cultured in DMEM with 10% FBS and Pen-strep, MCF7 and HPAF-II cells were cultured in MEM with 10% FBS and Pen-strep, and H1975 cells were cultured in RPMI medium with 10% FBS and Pen-strep. Cell cultures were maintained at 37 °C under 5% CO2.

Antibodies

The following primary antibodies were used: Cytochrome C (Abcam, ab13575, RRID:AB_300470), Trx1 (Proteintech, 14999-1-AP, RRID:AB_2272597), Trx2 (Cell Signaling Technology, 14907S, RRID:AB_2798645), TrxR1 (Cell Signaling Technology, 15140S, RRID:AB_2798725), TrxR2 (Cell Signaling Technology, 12029, RRID:AB_2797803), GR (Abcam, ab137513, RRID:AB_2732913), Citrate synthase (Cell Signaling Technology, 14309S, RRID:AB_2665545), Lamin A/C (Cell Signaling Technology, 4777S, RRID:AB_10545756) Cathepsin C (Santa Cruz Biotechnology, sc-74590, RRID:AB_2086955), p70 S6K (Cell Signaling Technology, 2708T, RRID:AB_390722), GM130 (BD Biosciences, 610823, RRID:AB_398142), Calnexin (Abcam, ab31290, RRID:AB_868628), beta-actin (Abcam, ab6276, RRID:AB_2223210), OXPHOS antibody cocktail (Abcam, ab110413, RRID:AB_2629281).

Differential Proteomics Analysis
We downloaded the LC-MS/MS protein-quantitation data and associated clinical data of CPTAC's 6 Discovery Studies, each of which characterized one of the following cancer types: clear cell renal cell carcinoma (ccRCC)(1), GBM(2), lung adenocarcinoma (LUAD) (3), lung squamous cell carcinoma (LSCC)(4), and uterine corpus endometrial carcinoma (UCEC) (5). We used base R functions (6) and dplyr (7) to isolate the spectral counts for only those peptides which uniquely mapped to individual proteins. We then generated two unsupervised hierarchically clustered heatmaps for each cancer with clinical data annotations, one for redox enzyme expression and another for the expression of cysteine- and methionine-metabolizing proteins, using the R package ComplexHeatmap (8). Within each cancer type's dataset, we also used base R functions and dplyr to calculate the ratio of each redox enzyme's median expression in cancer tissues to its median expression in normal samples. These ratios were visualized using the ggplot2 and ggforce (9).

Metabolomics analysis
Metabolites were extracted using pre-cooled 80% methanol. Samples were then centrifuged at 4°C for 15 minutes at 14,000 rpm. The supernatants containing polar metabolites were dried down using a SpeedVac. Targeted LC/MS analyses were performed on a Q Exactive Orbitrap mass spectrometer (Thermo Scientific) coupled to a Vanquish UPLC system (Thermo Scientific). The Q Exactive operated in polarity-switching mode. A Sequant ZIC-HILIC column (2.1 mm i.d. × 150 mm, Merck) was used for separation of metabolites. Flow rate was set at 150 µL/min. Buffers consisted of 100% acetonitrile for mobile B, and 0.1% NH_4OH/20 mM CH_3COONH_4 in water for mobile A. Gradient ran from 85% to 30% B in 20 min followed by a wash with 30% B and re-equilibration at 85% B. Data analysis was done using El-MAVEN (v0.12.0). Metabolites were identified based on exact mass within 5 ppm and standard retention times.

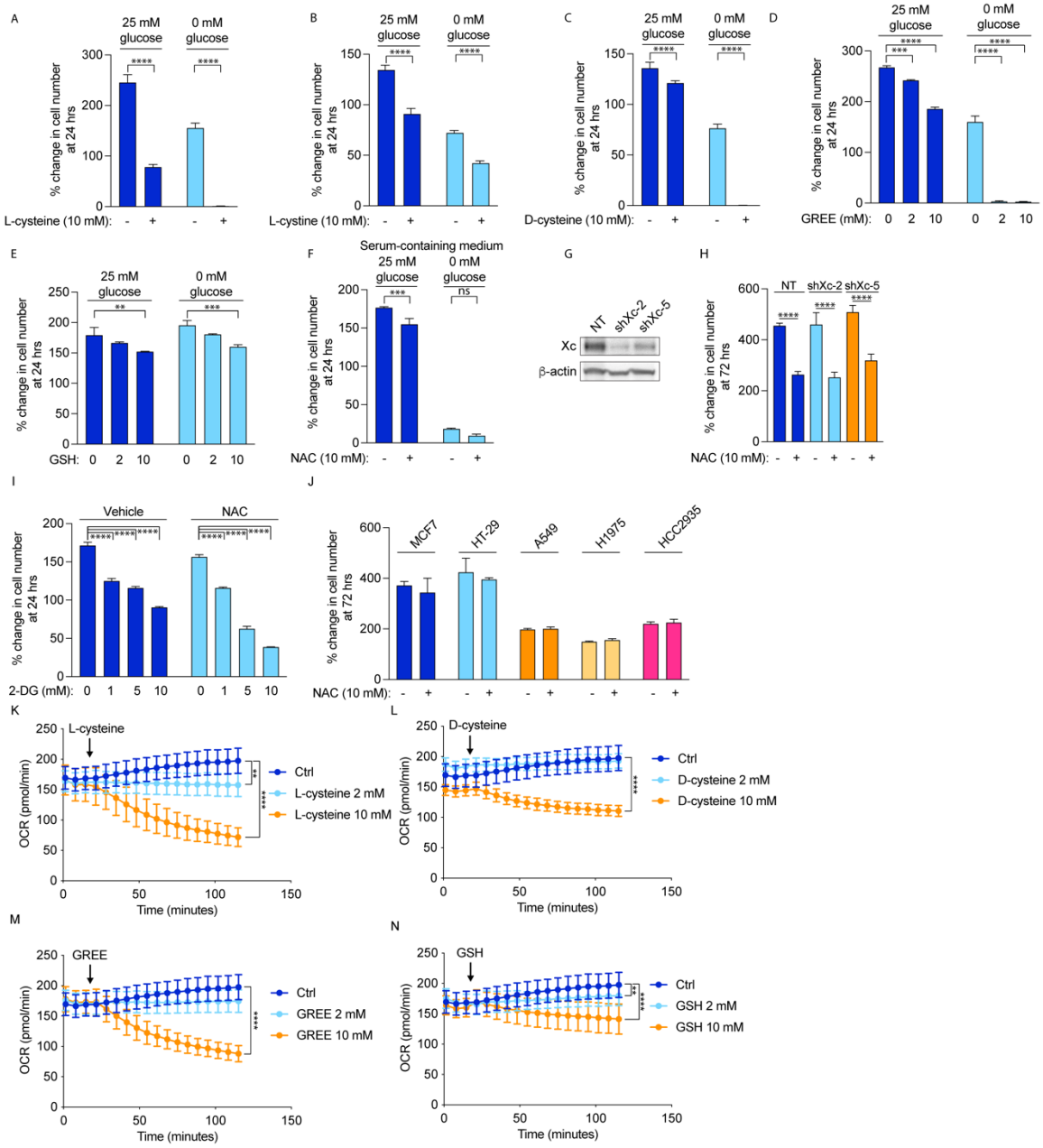

Figure S1 Cysteine-containing compounds suppress growth and reduce oxygen consumption in GBM cells

667 cells were treated with 10 mM L-cysteine (A), 10 mM L-cystine (B), 10 mM D-cysteine (C), 2 or 10 mM glutathione reduced ethyl ester (GREE) (D), or reduced glutathione (GSH) (E) under normal glucose conditions (25 mM) or glucose starvation (0 mM). Growth was measured at 24 hours. (F) 667 cells were grown in DMEM containing 10% FBS and were treated with 10 mM NAC under normal glucose conditions (25 mM) or glucose starvation, and growth was measured at 24 hours. (G) 667 cells were transduced with lentivirus expressing NT shRNA or two SLC7A11 shRNAs, and Xc protein expression was measured by western blot. (H) 667 cells expressing NT or SLC7A11 shRNA were treated with 10 mM NAC, and growth was assessed at 72 hours. (I) 667 cells were treated with 1, 5, or 10 mM 2-deoxyglucose (2-DG) along with vehicle or 10 mM NAC, and growth was measured at 24 hours. (J) MCF7, HT-29, A549, H1975, and HCC2935 cells were treated with 10 mM NAC under normal glucose conditions (25 mM), and growth was measured at 72 hours. 667 cells were treated with 2 or 10 mM L-cysteine (K) 2 or 10 mM D-

cysteine (L), 2 or 10 mM GREE (M), or 2 or 10 mM GSH (N), and oxygen consumption rate was measured using the Seahorse assay. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, ****, $p < 0.0001$.

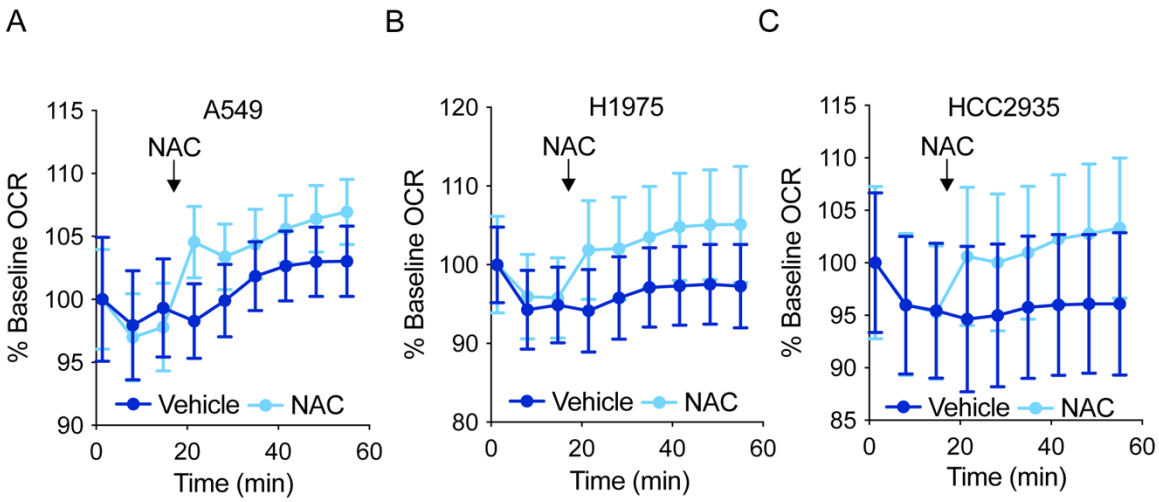

Figure S2 NAC does not reduce oxygen consumption in non-glioma cells
A549 (A), H1975 (B), and HCC2935 (C) cells were treated with 10 mM NAC, and oxygen consumption was measured with the Seahorse assay.

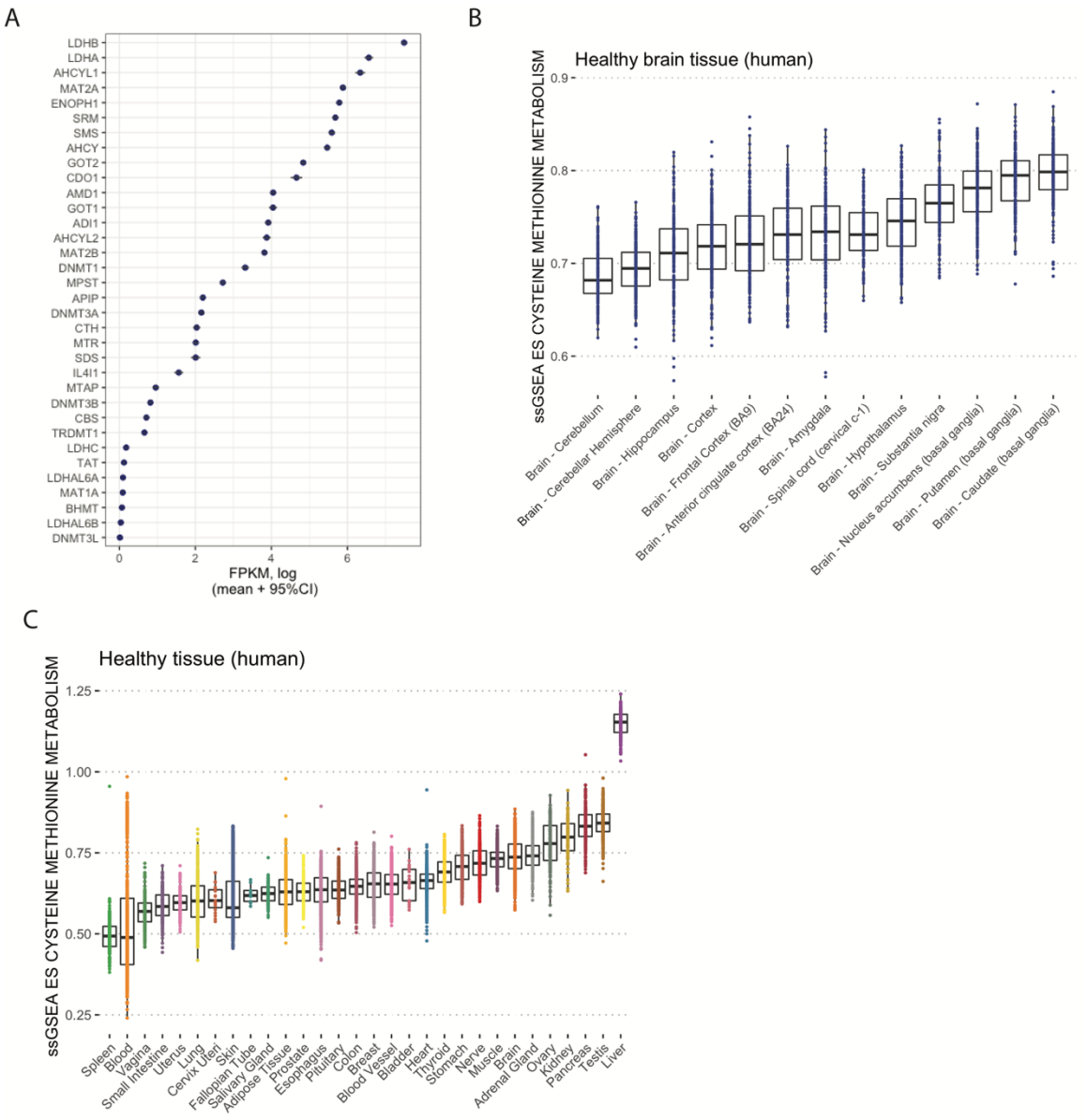

Figure S3 Distribution of cysteine and methionine gene signature in healthy tissues and normal brain

Distribution of cysteine and methionine KEGG genes across TCGA GBM samples (A), normal brain regions (B), and normal human tissues (C).

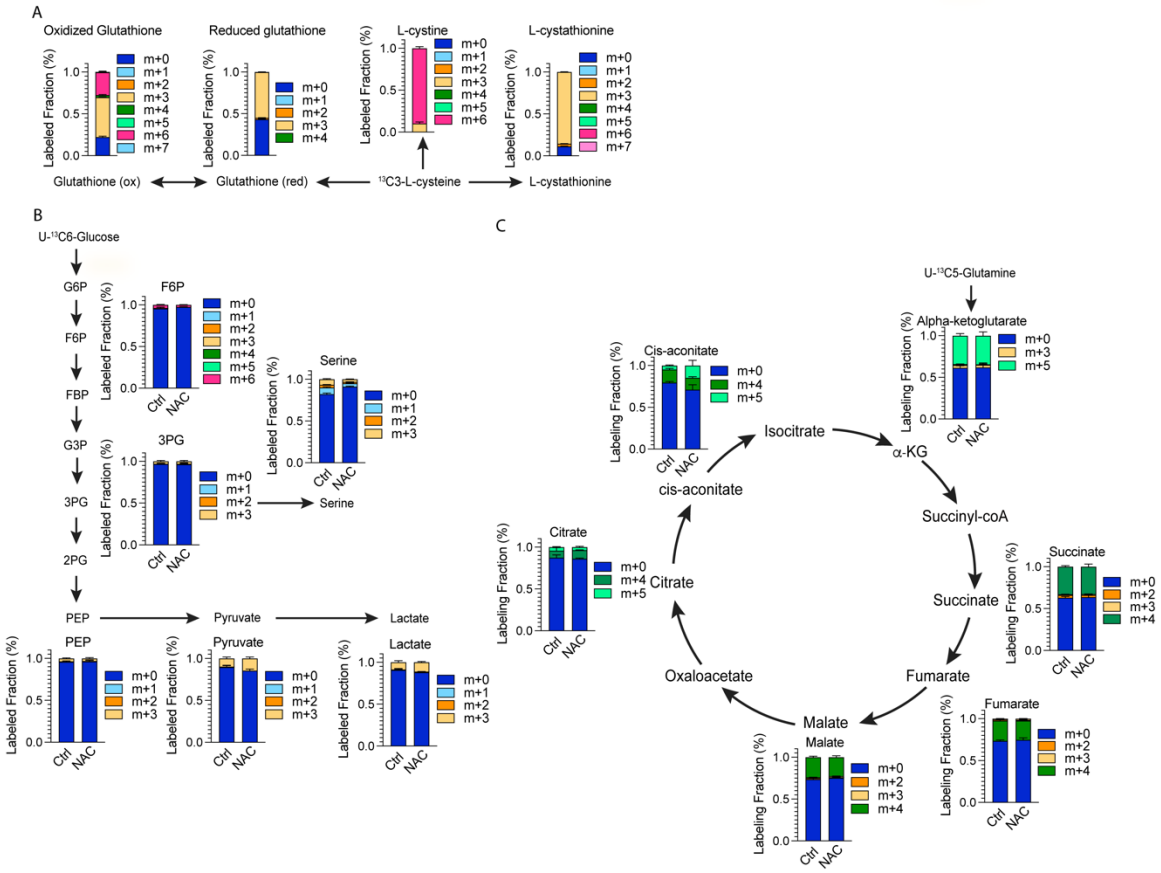

Figure S4 ^{13}C3-L-Cysteine and U-^{13}C6-glucose metabolic flux in GBM cells
(A) 667 cells were treated with 10 mM ^{13}C3-L-cysteine for 5 minutes, and labeled metabolites were measured by LC/MS. (B) GBM cells were treated with 10 mM ^{13}C6-glucose and 10 mM NAC for 5 minutes, and labeled metabolites were measured by LC/MS. (C) GBM cells were treated with 10 mM ^{13}C5-glutamine and 10 mM NAC for 5 minutes, and labeled metabolites were measured by LC/MS.

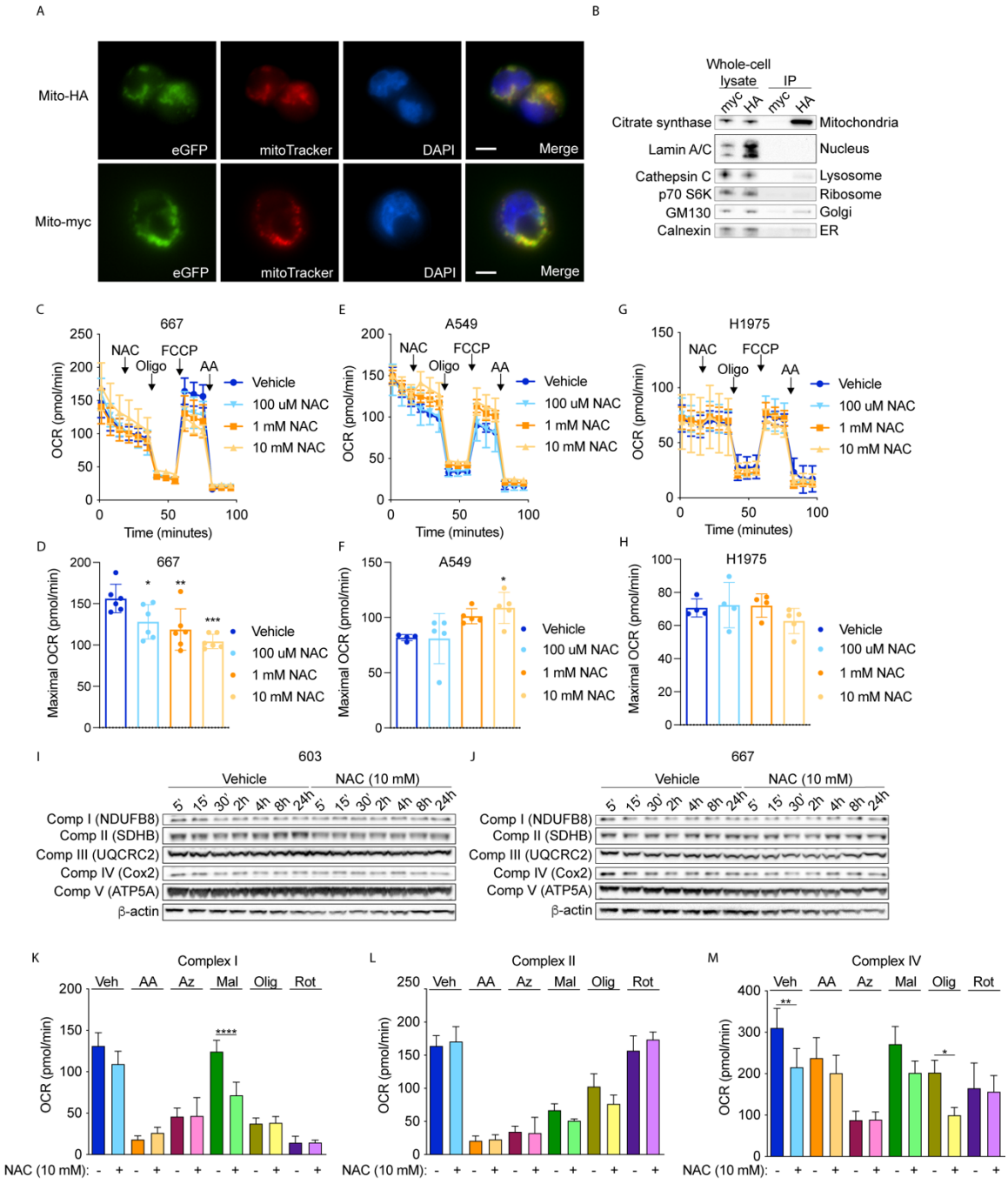

Figure S5 Immunoprecipitation of 667 mitochondria using an HA-tag based method
(A) 667 cells expressing HA-OMP25-EGFP or Myc-OMP25-EGFP were incubated with 100 nM Mitotracker Deep Red and 1 mg/mL Hoechst stain, followed by confocal microscopy. Scale bars are 10 mm. (B) 667 cells expressing HA-OMP25-EGFP or Myc-OMP25-EGFP were immunoprecipitated using HA magnetic beads, and lysates were run on SDS-PAGE along with whole-cell lysates prepared before immunoprecipitation. The indicated cell compartment-specific antibodies were used. (C-H) OCR and maximal OCR were measured in mitochondria isolated from 667 (C, D), A549 (E, F), and H1975 cells (G, H). 603 (I) or 667 (J) cells were treated with 10 mM NAC for the indicated times, and mitochondrial complex expression was examined by western blot using the OXPHOS antibody cocktail. Isolated mitochondria were immunoprecipitated from 667 cells, and the oxygen consumption rate from Complex I (K),

Complex II (L), and Complex IV (M) activity was measured in an electron flow assay after incubation with 10 mM pyruvate, 2 mM malate, and 4 mM ADP. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$.

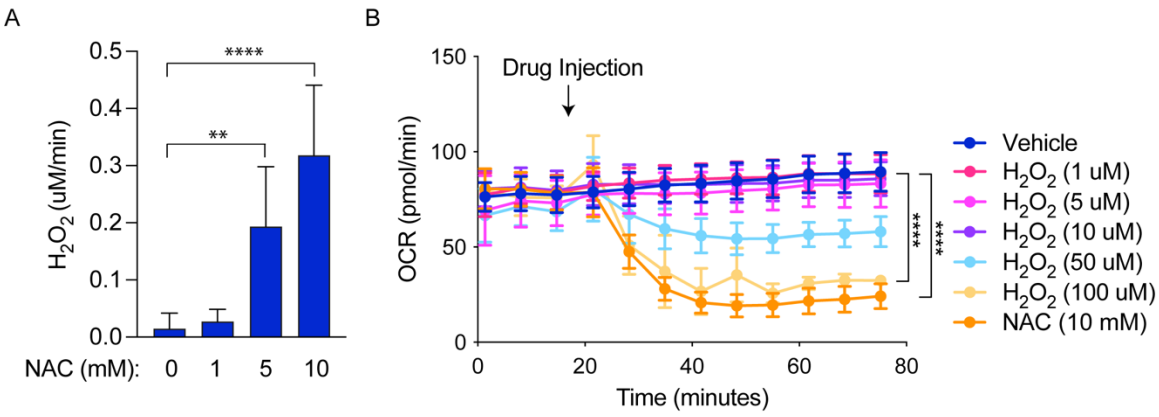

Figure S6 GBM cells induce greater OCR reduction than 100 mM H_2O_2
(A) 1, 5, or 10 mM NAC was added to mitochondrial assay buffer, and H_2O_2 production was measured with Amplex Red reagent. (B) 10 mM NAC or 1, 5, 10, 50, or 100 mM H_2O_2 was added to 667 cells, and oxygen consumption was measured with the Seahorse assay. **, $p < 0.01$, ****, $p < 0.0001$.

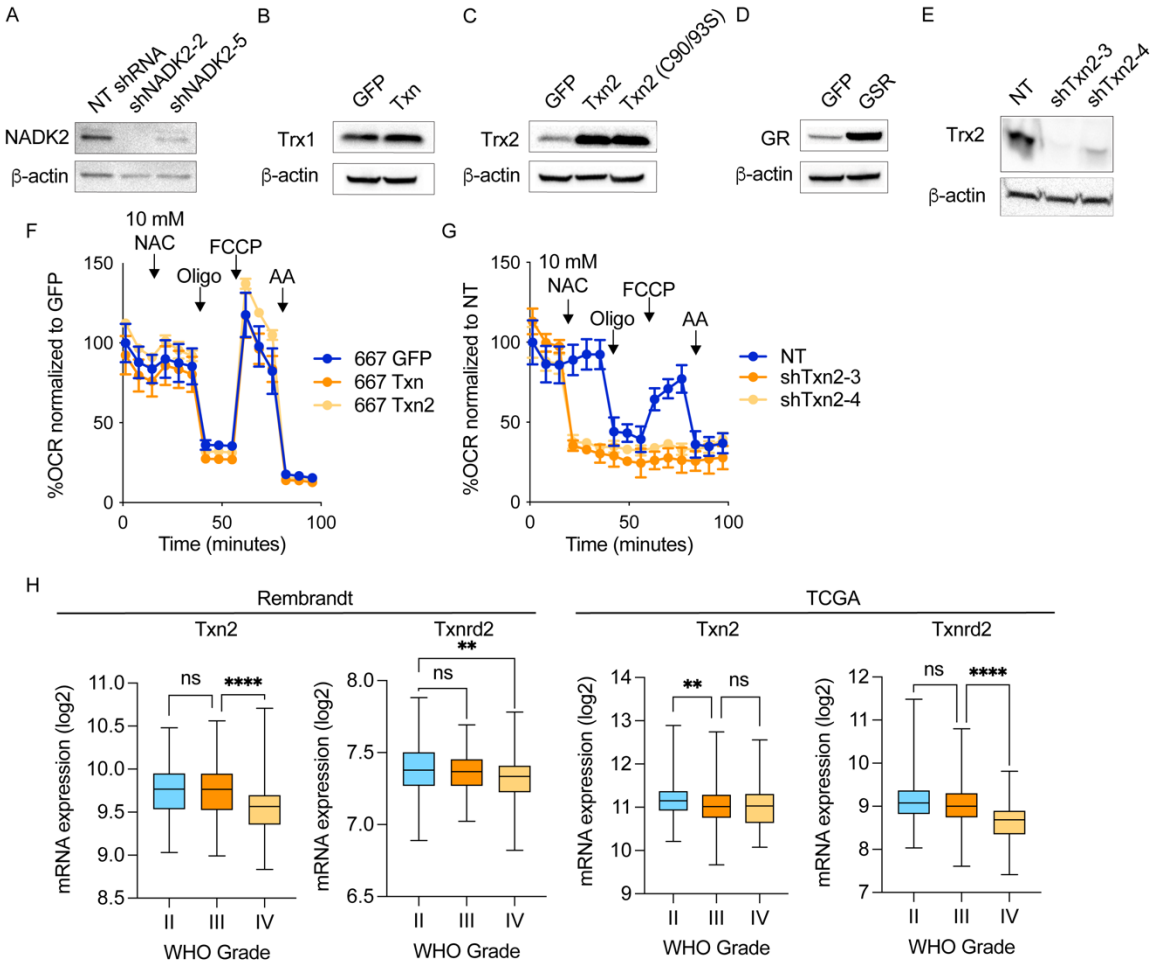

Figure S7 Genetic overexpression of redox enzymes in 667 cells
667 cells were transduced with lentivirus expressing non-targeting shRNA (NT) or NADK2 shRNA (A). 667 cells were transduced with lentivirus expressing GFP or TXN1 (B), TXN2 or TXN2 (C90/93S) (C), or GSR (D). (E) A549 cells were transduced with lentivirus expressing NT shRNA or shRNAs targeting Txn2 (shTxn2-3 and shTxn2-4). Whole-cell lysates were subjected to immunoblot with the indicated antibodies. (F) Mitochondria were isolated from 667 cells expressing GFP, Trx1, and Trx2, and OCR was measured after treatment with 10 mM NAC. (G) Mitochondria were isolated from A549 cells expressing non-targeting shRNA (NT) and shRNAs targeting Txn2 (shTxn2-3 and shTxn2-4), and OCR was measured after treatment with 10 mM NAC. (H) Txn2 and Txnrd2 expression levels across WHO Grade II-IV gliomas were assayed in the Rembrandt and TCGA databases. **, $p < 0.01$; ***, $p < 0.001$.

SI References

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