Supplementary Methods

Lentivirus production and stable cell line generation

The lentivirus was packaged in HEK293T cells using pMD2.G (Addgene #12259) and psPAX2 (Addgene #12260) system. Virus was directly added into cell culture medium. Stable expression cell lines were selected by puromycin (1-2μg/mL). U251 and U87 cells with stably expressed wild-type or mutated-IDH1 were generated using lentivirus transduction. The doxycycline-inducible pLVX-TetOne-Puro vector (Clontech, Mountain View, CA) carrying IDH1 R132C or R132H variants were transduced into U251 MG and U87 MG by lentivirus transduction.

RNA interference

Small interference RNA oligos targeting NRF2 were designed and synthesized by Integrated DNA Technologies (IDT, Coralville, IA). Oligos were transfected into cells using Lipofectamine RNAiMAX transfection reagent according to the manufacturer's protocol. RNAi sequence used in the present study are shown as follow. siNrf2-1.F: AAA GUG AUA GAU CAG AAA CAU CAA UGG; siNrf2-1.R: AUU GAU GUU UCU GAU CUA UCA CUT T; siNrf2-2.F: GAA ACU ACU GAU UCA ACA UAC UGA CAC; iNrf2-2.R: GUC AGU AUG UUG AAU CAG UAG UUT C; siNrf2-3.F: CCU GAU UAG UAG CAA UGA AGA CUG GGC; siNrf2-3.R: CCA GUC UUC AUU GCU ACU AAU CAG G. Negative control RNA were purchased from Qiagen (Qiagen, Hilden, Germany).

ROS level measurement

Cellular ROS level was measured using either ROS-Glo H_2O_2 assay kit (Promega, Madison, WI) or H_2DCFDA (Thermo Fisher) staining. ROS-Glo assay was performed according to manufacturer's

protocol and luminescence was detected using a Polarstar Optima plate reader (BMG LABTECH, Ortenberg, Germany). As for H_2DCFDA staining, cells were incubated with H_2DCFDA dye (Thermo Fisher) for 15 min at 37°C. Cells were then harvested and analyzed by flow cytometry. Mitochondrial ROS was measured using MitoSOX-Red (Thermo Fisher) staining, cells were incubated with MitoSOX-Red for 15min at 37°C and analyzed by either confocal imaging or flow cytometry.

D-2-HG level measurement

Cellular D-2-HG level was measured using colorimetric D-2-HG assay kit from Biovision (Milpitas, CA) according to the manufacturer's instructions. The assay measurement was performed using Polarstar Optima plate reader (BMG LABTECH). D-2-HG level was normalized to protein quantification.

Real-time PCR

Total RNA was extracted form cultured cells using PureLink RNA mini kit (Thermo Fisher), and reverse transcript to DNA using Superscript IV VILO Master Mix (Thermo Fisher). Genes related to oxidative stress were analyzed by RT2 Profiler PCR array (PAHS-065YA-12, Qiagen) or with Power SYBR Green Master Mix. Primers used in the present study include *NQO1* (QT00050281), *HMOX1* (QT00092645), *NFE2L2* (QT00027384) and *ACTB* (QT00095431).

Chromatin Immunoprecipitation (ChIP) assay

ChIP assay was performed using ChIP-IT Express Enzymatic kit (Active Motif, Carlsbad, CA) following the manufacturer's protocol. Control IgG and antibody targeting NRF2 (Active Motif) was

used. Real-time RCP amplification was carried out using ChIP products with primers of *NQO1* promoter ARE region, Forward: 5' TTT GCT GAG TCA CCA GTG C 3', Reverse: 5' ATG CCC TTT TAG CCT TGG CA 3'. *HMOX1* promoter ARE region, Forward: 5' TTT GCT GAG TCA CCA GTG C 3', Reverse: 5' TAA AGC TGC CCT TTC ACC TC 3'. Input DNA of each sample was used as control.

Immunoblotting

Total protein was extracted from cultured cells using RIPA lysis buffer supplemented with protease and phosphatase inhibitor cocktail (Thermo Fisher) and resolved on 4-12% Bis-Tris gel (Invitrogen). Protein was electrotransferred onto PVDF membrane, blocked in Superblock PBST (Thermo Fisher) and probed with indicated primary antibodies at 4°C overnight. Membranes were subsequently incubated with HRP labeled secondary antibodies and visualized using Bio-Rad ChemiDoc Imaging System. The primary antibodies used in the present study include: NRF2 (CST, Danvers, MA, 1:1,000), GCLC (Abcam, Cambridge, UK, 1:1,000), HMOX1 (Proteintech, Rosemont, IL, 1:1,000), PGD (Abcam, 1:1,000), NQO1 (Abcam, 1:1,000), Ubiquitin (Abcam, 1:2,000), PARP1 (CST, 1:1,000), cleaved-PARP (CST, 1:1,000), ɣH2A.X (CST, 1:1,000), EGFP (Thermo Fisher, 1:1,000) HA (Covans, 1:1,000) and β-actin (Sigma).

Dot blotting

Genomic DNA was isolated using Qiagen DNeasy Blood and tissue kit. Isolated genomic DNA was denatured in 0.1 M NaOH for 10 min at 95°C and chilled on ice. Then, one microgram DNA was applied to Amersham Hybond-N nylon membrane (GE Healthcare) using a Bio-Dot Microfiltration Apparatus (Bio-Rad, Hercules, CA). The membrane was washed with 2x SCC and UV cross-linked at 1200 J/m^2. The membrane was blocked in superblock (Thermo Scientific) for 30 min at room

temperature. Mouse anti 8-oxoG mAb (Sigma, 1:1000) was added to the membrane and incubated at 4°C overnight. Then, the membrane was incubated with HRP labeled donkey anti-mouse IgG at room temperature for 30 min and visualized using Bio-Rad ChemiDoc Imaging System. The membrane was stained with methylene blue as loading control.

Immunofluorescence

Cells were treated with Brusatol 48 hr, fixed in 4% paraformaldehyde and penetrated with 0.3% Triton X-100. Cells were blocked in superblock and incubated with primary antibody targeting γH2A.X (CST, 1:1000) or 8-oxoG (Sigma, 1:500) overnight at 4°C. Then, cells were washed using PBS and incubated with fluorescent conjugated secondary antibody. Cell nucleus was stained with Hoechst 33342. Samples were visualized by Zeiss LSM710 confocal microscope.

Annexin V/PI apoptosis assay

Cells were treated with 40 nM Brusatol for 72-96 hr. Cell apoptosis level was analyzed by Annexin V/PI apoptosis kit (Thermo Fisher) per manufactory's protocol. Generally, cells were harvested and incubated with a mixture of Annexin V-FITC and PI for 20 min on ice. Cell samples were analyzed by FACS Canto II (BD, Franklin Lakes, NJ) flow cytometer.

Caspase 3/7 activity assay

Caspase 3/7 activity was measured using the Caspase-Glo 3/7 Assay (Promega). Cells were treated with 40nM Brusatol for 24 hr and Caspase 3/7 activity was measured according to the

instruction. Luminescence was measured by Epoch plate reader (BioTek, Winooski, VT) and normalized to protein quantification.

DNA oxidative damage ELISA assay

DNA oxidative damage ELISA assay was performed using Cayman DNA/RNA Oxidative Damage ELISA kit (Cayman Chemical, Ann Arbor, MI), following the manufacturer's protocol. Genomic DNA of samples were extracted, and 100 ng DNA was loaded into each test. Absorbance at 405 nm was measured by Epoch plate reader (BioTek).

DNA fragmentation electrophoresis assay

Genomic DNA was isolated from cells using QIAamp DNA Blood Kit. Equal amount of genomic DNA (300-500 ng) were resolved by electrophoresis on 4-20% TBE gel (Invitrogen). The gel was stained with SYBR Safe and visualized on Bio-Rad ChemiDoc Imaging System.

Cell proliferation

BrdU (Sigma) was directly added to medium at a final concentration of 10 μM and incubated for 2-3 hr under growth condition. Cells were fixed with 4% PFA for 15 min at room temperature. Permeabilization of cells was achieved using 0.3% Triton X-100 in PBS. Cells were treated with 2N HCl for 10 min and neutralized with 0.1M Sodium borate, pH 8.5, for 30 min. Cells were stained with mouse anti-BrdU antibody (BD Biosciences) overnight at 4°C. Cells were labeled with Donkey anti mouse Alexa Fluor 488 IgG and visualized by Zeiss LSM780 confocal microscope.

Sphere formation assay

BTIC lines GSC627, GSC711, TS603 and MGG152 were seeded in low attachment 6-well plate at 10,000 cells per well. Cells were incubated with 40nM or 100nM Brusatol for at least 2 weeks to allow formation of stem cell spheres. Phase contrast images were taken from 10 randomly selected fields. Spheres number and size (diameter) were quantified using ImageJ software.

Limiting dilution assay

Cells were seeded in 24-well plates at concentrations of 500, 250, 120, 60, 30 and 15 cells per well. Cells were treated with DMSO or 40nM Brusatol for 12 days after plating and analyzed by light microscopy. Positive wells were defined as groups of cells larger than 100 μm in diameter. The negative rate for each sample is calculated. *IDH1* wild-type BTIC GSC923 and $IDH1^{R132H}$ mutation BTIC TS603 were used.

Cell viability analysis

The CCK-8 assays (Dojindo, Rockville, MD) or directly cell count was used to determine cell proliferation according to the manufacturer's protocol. CCK-8 absorbance was measured by Epoch plate reader (BioTek) at 450 nm. Cell count was measured by Vi-CELL Cell counter (Beckman Coulter, Brea, CA). All of the experiments were done in triplicate and results are presented as mean ± SEM.

Immunohistochemistry and TUNEL assay

The tissue sections were deparaffinized and rehydrated through graded ethanol washes. Antigen retrieval was conducted by boiling in Citrate buffer (pH 6.0) for 5 min. The slides were then

incubated with 3% hydrogen peroxide for 10 min and were blocked with 10% normal goat serum at room temperature for 10 min. After removing excess blocking buffer, the slides were incubated with primary antibodies, NRF2 (CST, 1:1000), NQO1 (abcam, 1:500), GCLC (abcam, 1:500), Ki67 (abcam, 1:1000), PCNA (Santa Cruz, 1:1000), at 4°C overnight. The slides were then washed with PBS and incubated with HRP labeled secondary antibody at room temperature for 30 min. Bound antibodies were detected using diaminobenzidine (DAB). The slides were then immersed in running tap water, and 0.1% hematoxylin was used for counterstaining. Finally, slides were analyzed using light microscopy. TUNEL assay was performed using DeadEnd Colorimetric TUNEL System (Promega) following the manufacturer's protocol. The slides were detected using DAB and counterstain using hematoxylin.

Reagents and treatment conditions

Brusatol (Sigma, St. Louis, MO), ML385 (MedChem Express, Monmouth Junction, NJ), AGI-5198 (Selleckchem, Houston, TX), ivosidenib (Selleckchem) and Vorasidenib (Selleckchem) were dissolved in DMSO. ROS scavenger NAC and Catalase was purchased from Sigma. For brusatol and ML385 treatment, cells were treated in cell culture media at 37°C in a humidified air with 5% CO_2, with final concentration of 40 nM brusatol or 5 μM ML385. Generally, for gene expression detection, like real-time PCR, ChIP and Western Blot, cells were treated for 24 hr. For oxidative stress and DNA damage related assay like ROS detection, DNA fragmentation, γH2A.X staining and comet assay, cells were treated for 48 hr. For cytotoxicity related assay like cell viability and cell apoptosis, cells were treated for 72 hr or 96 hr. For ubiquitination assay, Cells were treated for 72 hr after transfection of NRF2-EGFP and ubiquitin-HA plasmids. For *IDH1* mutation inhibitors, cells were treated in culture media with 1 μM AGI-5198, 0.5 μM ivosidenib or 1.5 μM Vorasidenib for at least 3 passages (passage every

two days), then cells were harvested for further analysis. For rescue experiments using NAC or Catalase, cells were treated with drugs with freshly made 2.5 mM NAC or 500U/mL Catalase.

Immunoprecipitation and Ubiquitination assay

Immunoprecipitation was performed as previously described [1]. In brief, NRF2-EGFP and Ubiquitin-HA plasmids were transfected into U251 cells using lipofectamine 3000 (Thermo Fisher). Cells were treated with 10μM MG-132 for 6 hr before harvesting. Two hundred microgram of whole cell lysate was incubated with Dynabeads and monoclonal antibody against EGFP (Thermo Fisher). Bounded protein was washed, eluted and analyzed by western blot for ubiquitination.

CHX pulse chase protein half-life measurement

CHX pulse chase assay was performed as previously described [2]. NRF2-EGFP plasmid was transfected into U251 cells with transduced IDH1 expression. Cells were exposed to CHX (Sigma, St. Louis, MO) to halt protein synthesis. NRF2 protein residue was determined by western blot and densitometry analysis.

DNA fragmentation analysis by comet assay

The comet assay was performed as previously described [3]. Briefly, cells were harvested after treatment and resuspended in molten agarose. After lysed in lysis solution, cells were resolved at 0.6 V/cm for 30 min in alkaline electrophoresis solution. DNA was stained with SYBR Safe (Thermo Fisher) and visualized under Zeiss LSM710 confocal microscope.

Bioinformatics

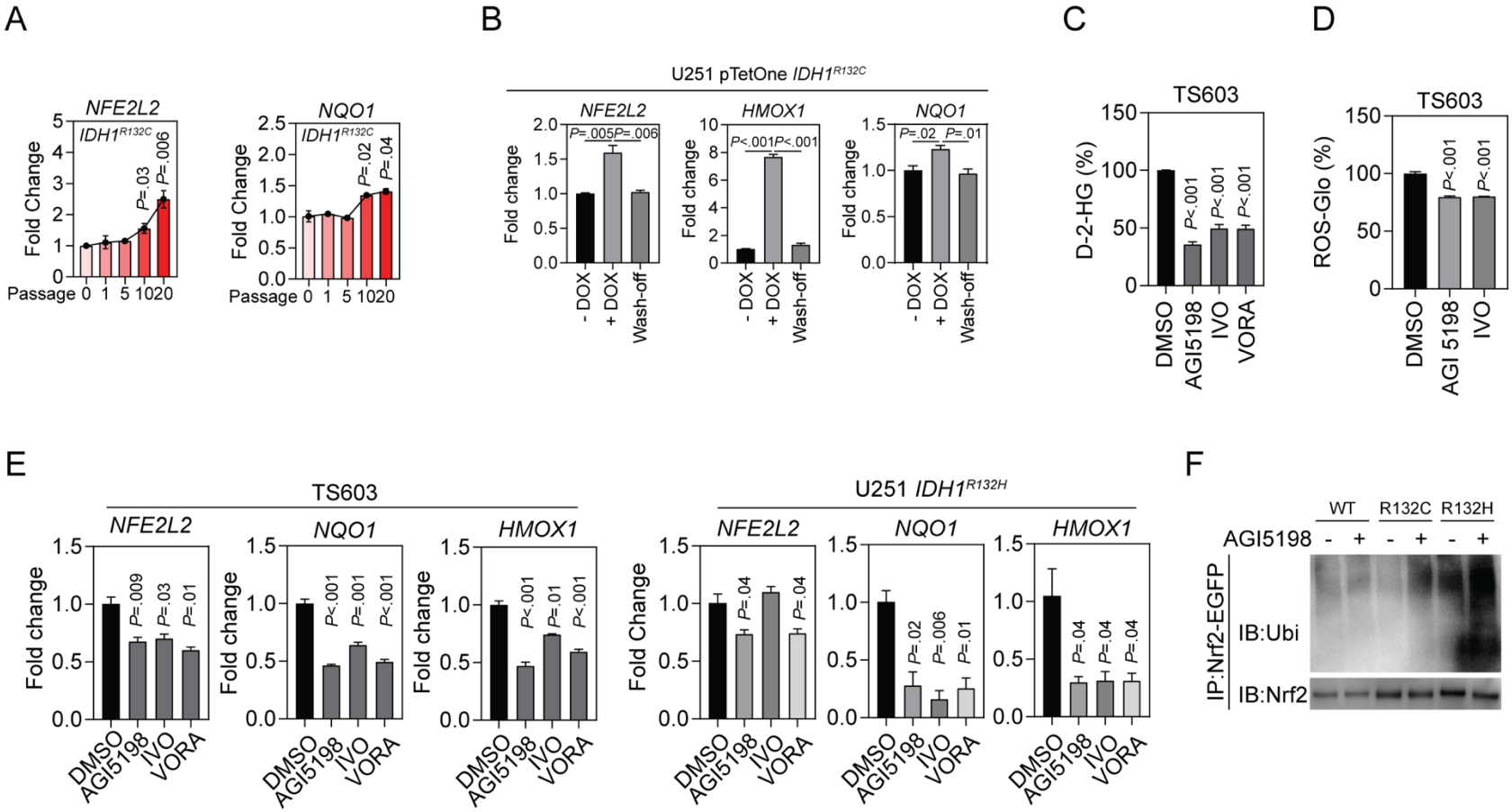
For Cox regression analysis, Lower Grade Glioma (WHO grade II/III) data generated from The Cancer Genome Atlas were downloaded from cBioPortal (http://www.cbioportal.org/) and Firehose (https://gdac.broadinstitute.org/) using R's libraries cgdsr and RTCGA Toolbox in 10/2016. 451 samples with known *IDH1/IDH2* mutation status (366 *IDH*-mutant, and 85 *IDH*-wt) and available RNASeq mRNA expression data were used in the analysis. R's survival package was used to perform Cox proportional hazards ratio analysis on overall survival using the *IDH*-mutation status and expression of antioxidant gene as covariates. Both covariates were found to be statistically significant in the model, with *IDH*-mutant status being associated with better prognosis (hazard ratio 0.22 and 95% confidence interval [0.13, 0.37]), and higher expression of *GCLM* being associated with poorer prognosis (hazard ratio 1.825 and 95% confidence interval [1.2674, 2.63]). The *p*-value associated with the *GCLM* coefficient is 0.001, and the Likelihood ratio test has a *p*-value=0. Survival curves for the *IDH* mutant case were plotted using expression of *GCLM* at 25% and 75%. We tested for the proportional hazards assumption using R's implementation of Grambsch et al.'s test (https://academic.oup.com/biomet/article/81/3/515/257037). The *p*-value of the chi-square test between transformed survival time and scaled Schoenfeld residuals was non-significant, and hence the proportional hazard assumption holds.

For Kaplan-Meier analysis, RNASeq expression data were downloaded with R's TCGABiolinks library and processed using the recommended settings. 94 IDH-WT Low-grade glioma (LGG) and 140 IDH1-WT glioblastoma (GBM) samples were submitted to Kaplan-Meier analysis, stratifying the samples by the median expression of the gene of interest; 1 sample of LGG was excluded due to missing survival information. Analysis was performed using R's survminer library.

References:

1. Yang C, Huntoon K, Ksendzovsky A, et al. Proteostasis modulators prolong missense VHL protein activity and halt tumor progression. Cell Rep 2013;3(1):52-9.
2. Yang C, Sun MG, Matro J, et al. Novel HIF2A mutations disrupt oxygen sensing, leading to polycythemia, paragangliomas, and somatostatinomas. Blood 2013;121(13):2563-6.
3. Lu Y, Liu Y, Yang C. Evaluating In Vitro DNA Damage Using Comet Assay. J Vis Exp 2017(128).

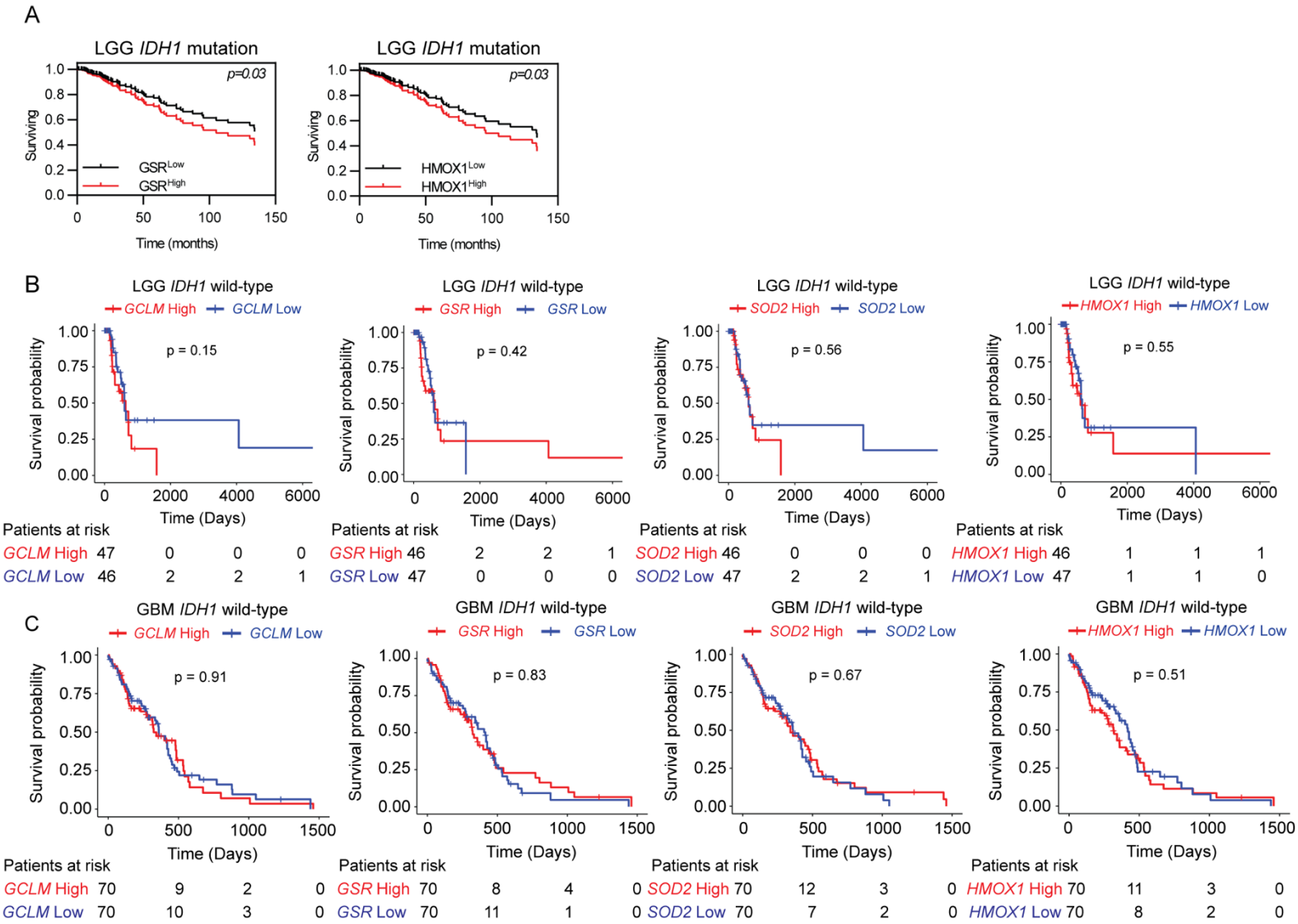
Supplementary Figure 1



Supplementary Figure 1. Analysis of NRF2 pathway in *IDH1*-mutated cells

(A) Gene expression of *NFE2L2* and *NQO1* was measured by real-time PCR after 10-20 passages of DOX-induced mutated-$IDH1^{R132C}$ expression in U251 cells. **(B)** Gene expression level of *NFE2L2, NQO1* and *HMOX1* was measured by real-time PCR after removing mutated-$IDH1^{R132C}$ expression in U251 cells. **(C)** D-2HG was quantified using colorimetric assay kit after inhibition of mutated-*IDH1* function using specific inhibitors for 48 hr in TS603 cells. **(D)** ROS level was measured by ROS-Glo H_2O_2 assay kit in TS603 cells after inhibition of mutated-*IDH1* function for 48 hr. **(E)** mRNA level of *NFE2L2*, *NQO1* and *HMOX1* was measured by Real-time PCR in TS603 and $IDH1^{R132H}$ U251 cells with *IDH1* mutation inhibitors treatment for 3 passages (6 days). **(F)** NRF2 ubiquitination was measured by Western Blot in *IDH1*-mutated U251 cells with *IDH1* mutation inhibitor treatment for 72 hr. P values were calculated using a two-sided Student's *t* test. Error bars represent standard deviation. DOX, doxycycline; D-2HG, D-2-Hydroxyglutarate; DMSO, Dimethyl sulfoxide; IVO, Ivosidenib; VORA, Vorasidenib.

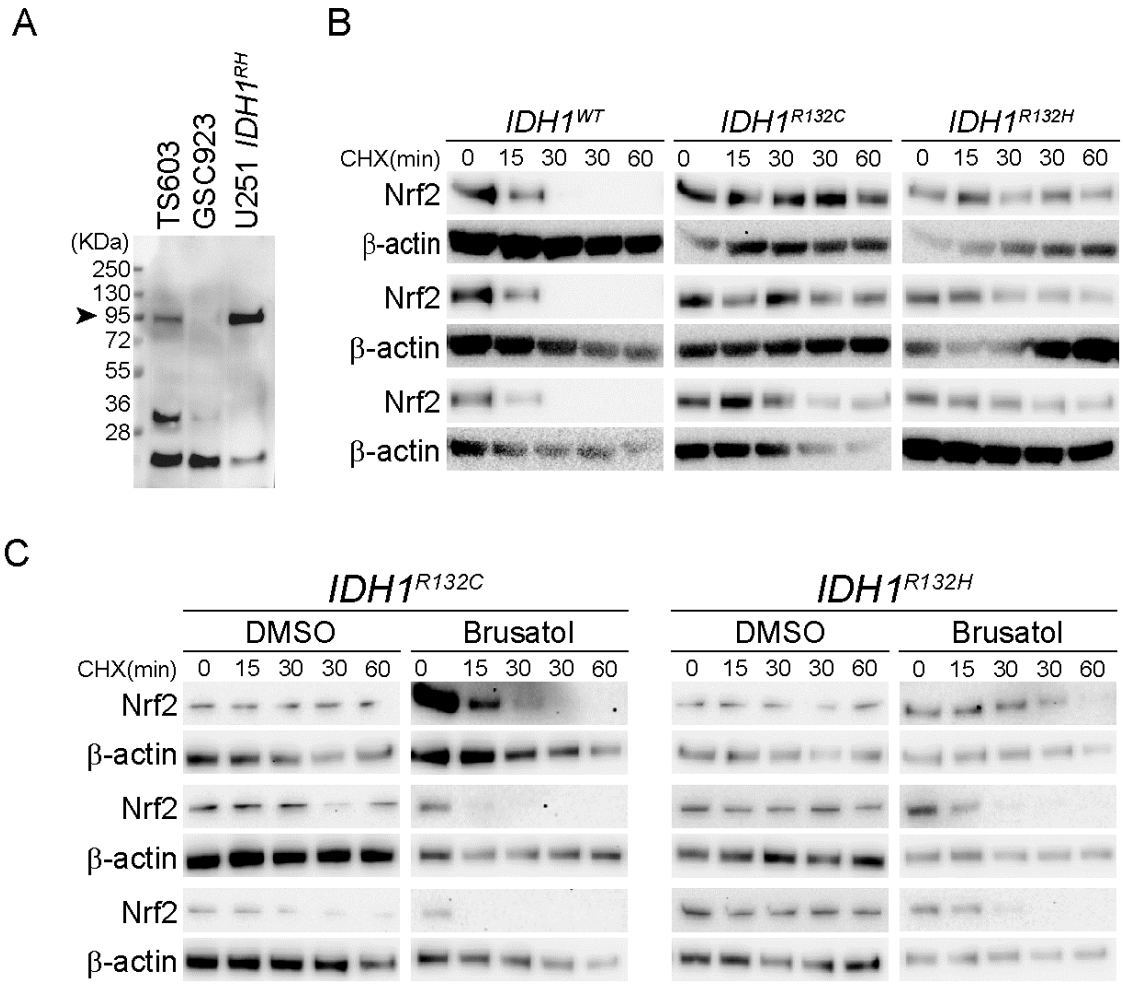
Supplementary Figure 2



Supplementary Figure 2. Correlation of antioxidant pathway and glioma disease outcome

(A) Cox regression analysis of *GSR* and *HMOX1* expression level for *IDH1*-mutated LGG is shown (n=366). **(B)** Kaplan-Meier analysis for *IDH1* wild-type LGG is shown (n=93). Expression level of *GCLM*, *GSR*, *SOD2* and *HMOX1* were analyzed. **(C)** Kaplan-Meier analysis for *IDH1* wild-type GBM is shown (n=140). Expression level of *GCLM*, *GSR*, *SOD2* and *HMOX1* were analyzed. A table of the numbers of patients at risk in *GCLM*, *GSR*, *SOD2* and *HMOX1* low and high expression groups are shown below the graph. The statistical test used was two sides. LGG, Low-grade glioma; GBM, glioblastoma.

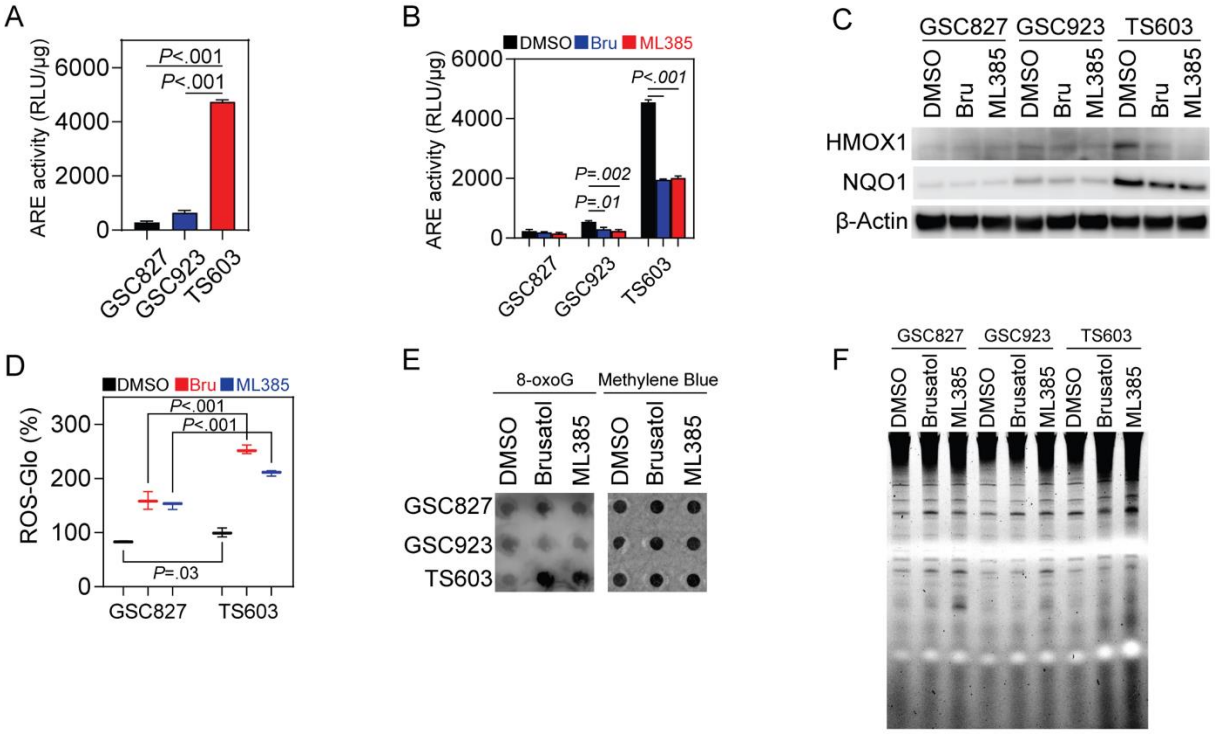
Supplementary Figure 3



Supplementary Figure 3. NRF2 half-life assay in U251 cells with *IDH1* mutation

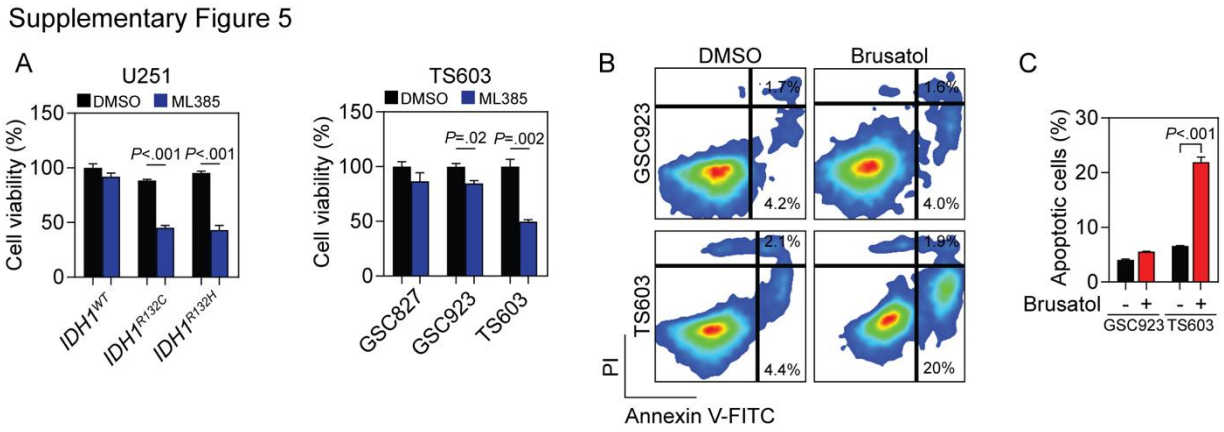
(A) Western Blot with protein ladder shows the molecular weight of NRF2 (100kDa). **(B)** NRF2 protein half lives were measured by CHX pulse chase assay using Western Blot in *IDH1*-mutated U251 cells. This experiment was repeated 3 times. **(C)** NRF2 half lives were measured by CHX pulse chase assay using Western Blot in *IDH1*-mutated U251 cells after brusatol treatment. This experiment was repeated 3 times. CHX, cycloheximide.

Supplementary Figure 4



Supplementary Figure 4. Oxidative DNA damage in BTIC lines with NRF2 blockade

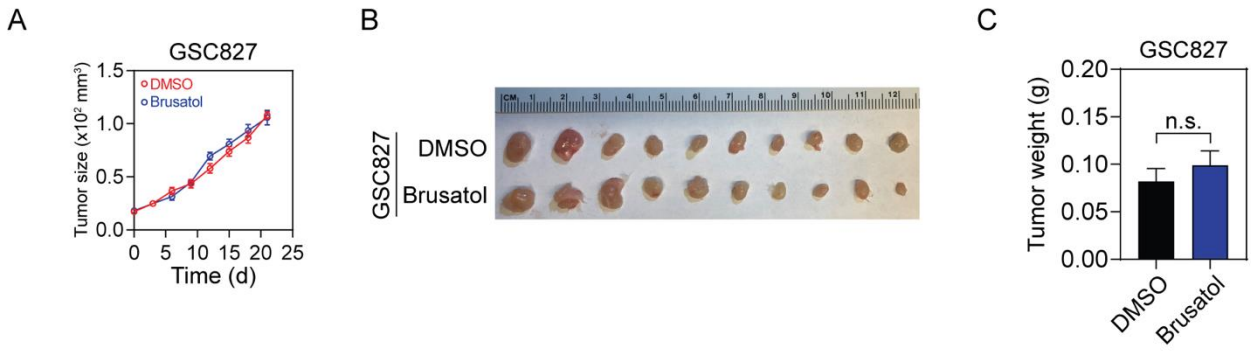
(A) ARE activity was measured by luciferase reporter assay in *IDH1*-mutated BTIC TS603, and *IDH1* wild-type BTIC GSC827 and GSC923. **(B)** ARE activity was measured by luciferase reporter assay in TS603 cells with NRF2 inhibitors brusatol and ML385 treatment. **(C)** The expression of NRF2 downstream target NQO1 and HMOX1 was measured by Western Blot in BTIC cell lines with brusatol and ML385 treatment. **(D)** ROS level was measured by measured by ROS-Glo H_2O_2 assay kit in TS603 and GSC827 cells with brusatol and ML385 treatment. **(E)** Dot blot measures DNA oxidation in BTIC cell lines with brusatol and ML385 treatment, Methylene Blue was used as loading control. **(F)** DNA fragmentation was measured by ellectrophoresis in BTIC cell lines with brusatol and ML385 treatment. P values were calculated using a two-sided Student's *t* test. Error bars represent standard deviation. DMSO, Dimethyl sulfoxide; Bru, Brusatol; BTIC, Brain tumor initiating cell.



Supplementary Figure 5. The vulnerability of *IDH1*-mutated BTIC to NRF2 inhibition.

(A) Cell viability was measured by cell counting in *IDH1*-mutated U251 cells (left panel) and BTICs (right panel) treated with NRF2 inhibitor ML385 for 72 hr. **(B)** Cell apoptosis was measured by Annexin V/PI flow cytometry analysis in *IDH1*-mutated BTIC TS603 with brusatol treatment. **(C)** Quantification of apoptotic cells percentage in Supplementary Figure 5B is shown. P values were calculated using a two-sided Student's *t* test. Error bars represent standard deviation. DMSO, Dimethyl sulfoxide; PI, propidium iodide.

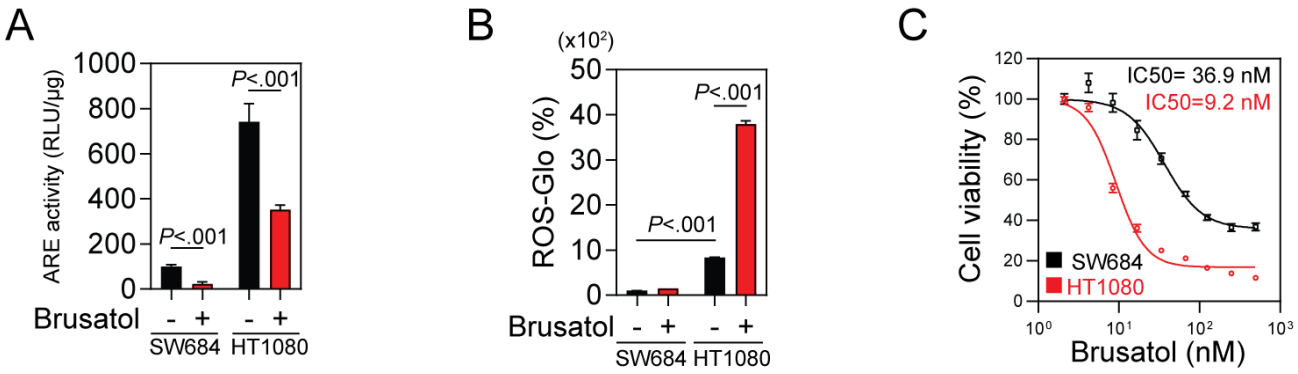
Supplementary Figure 6



Supplementary Figure 6. *IDH1* wild-type BTICs treated with brusatol in vivo.

(A) Tumor growth curve is shown for GSC827 xenograft after brusatol treatment. n=10 for DMSO group, n=10 for Brusatol group. **(B)** Image of tumors is shown after excision at the end point of the experiment. **(C)** Quantification of tumor weight is shown. P values were calculated using a two-sided Student's *t* test. Error bars represent standard deviation. n.s. represents no significant.

Supplementary Figure 7



Supplementary Figure 7. The vulnerability of *IDH1*-mutated fibrosarcoma to NRF2 inhibition.

(A) ARE transcription activity was measured by luciferase reporter assay in *IDH1*-mutated fibrosarcoma cell HT1080 and *IDH1* wild type cell SW684 after brusatol treatment. **(B)** ROS level was measured by ROS-Glo H_2O_2 assay kit in HT1080 cells with brusatol treatment. **(C)** Dose response curve of cell viability was measured by CCK8 assay in SW684 and HT1080 cells with brusatol treatment. Data was fit to nonlinear regression. P values were calculated using a two-sided Student's *t* test. Error bars represent standard deviation. ROS, reactive oxygen species.