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Lysine-specific histone demethylase 1A (KDM1A/LSD1) inhibition attenuates DNA double-strand break repair and augments the efficacy of temozolomide in glioblastoma

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Abstract

Background. Efficient DNA repair in response to standard chemo and radiation therapies often contributes to glioblastoma (GBM) therapy resistance. Understanding the mechanisms of therapy resistance and identifying the drugs that enhance the therapeutic efficacy of standard therapies may extend the survival of GBM patients. In this study, we investigated the role of KDM1A/LSD1 in DNA double-strand break (DSB) repair and a combination of KDM1A inhibitor and temozolomide (TMZ) in vitro and in vivo using patient-derived glioma stem cells (GSCs).

Methods. Brain bioavailability of the KDM1A inhibitor (NCD38) was established using LS-MS/MS. The effect of a combination of KDM1A knockdown or inhibition with TMZ was studied using cell viability and self-renewal assays. Mechanistic studies were conducted using CUT&Tag-seq, RNA-seq, RT-qPCR, western blot, homologous recombination (HR) and non-homologous end joining (NHEJ) reporter, immunofluorescence, and comet assays. Orthotopic murine models were used to study efficacy in vivo.

Results. TCGA analysis showed KDM1A is highly expressed in TMZ-treated GBM patients. Knockdown or knockout or inhibition of KDM1A enhanced TMZ efficacy in reducing the viability and self-renewal of GSCs. Pharmacokinetic studies established that NCD38 readily crosses the blood-brain barrier. CUT&Tag-seq studies showed that KDM1A is enriched at the promoters of DNA repair genes and RNA-seq studies confirmed that KDM1A inhibition reduced

their expression. Knockdown or inhibition of KDM1A attenuated HR and NHEJ-mediated DNA repair capacity and enhanced TMZ-mediated DNA damage. A combination of KDM1A knockdown or inhibition and TMZ treatment significantly enhanced the survival of tumor-bearing mice.

Conclusions. Our results provide evidence that KDM1A inhibition sensitizes GBM to TMZ via attenuation of DNA DSB repair pathways.

Key Points

- KDM1A inhibition attenuates DNA double-strand break repair pathways and enhances temozolomide (TMZ)-mediated DNA damage in glioma stem cells.
- Combination of KDM1A inhibitor and TMZ treatment improves the survival of tumor-bearing mice compared to monotherapy.

Importance of the Study

Glioma stem cells (GSCs) possess excellent DNA repair capacity and efficiently repair DNA lesions caused by standard therapies which contribute to therapy resistance. Epigenetic modifier KDM1A/LSD1 is highly expressed in glioblastoma (GBM). However, the mechanistic role of KDM1A in temozolomide (TMZ) resistance, identifying a KDM1A-specific inhibitor that effectively crosses the blood-brain barrier (BBB), and whether KDM1A inhibition sensitizes GSCs to TMZ is a major knowledge gap. Our work shows that KDM1A is essential for the DNA double-strand break repair capacity of GSCs and KDM1A inhibition attenuates the homologous recombination and non-homologous end joining repair leading to TMZ sensitization. Furthermore, KDM1A inhibitor NCD38 has excellent BBB permeability and knockdown or pharmacological inhibition of KDM1A potentiates the efficacy of TMZ in vitro and in vivo. Our results suggest that a combination of KDM1A inhibitor with TMZ could serve as a novel therapeutic strategy for GBM.

Glioblastoma (GBM) remains the most common and deadliest malignant primary brain tumor, with current 5-year survival statistics at a dismal 7.2%.[1–3] Standard of care consists of surgical resection of the bulk tumor, followed by external beam radiation therapy alongside adjuvant chemotherapy with temozolomide (TMZ).[4,5] Despite decades of research, effective treatment options for GBM have remained elusive. Glioma stem cells (GSCs), also known as glioblastoma tumor-initiating cells, demonstrated to be critical in GBM tumor development, invasiveness, and recurrence.[6–8] Chemo and radiation therapy resistance is linked with increased DNA damage response (DDR) signaling and tumor cell survival, which is enhanced by deregulated oncogenic and epigenetic pathways.[9,10] GSCs are known to exhibit high DNA repair capacity[7,11] and harbor altered DDR signaling pathways including the preferential activation of DNA damage sensing pathways such as ATR/CHK1, ATM/CHK2, and repair pathways such as PARP1, and enhanced activation of the G2/M checkpoint, that aid GSCs to repair DNA double-strand breaks.[7,12–14] Identifying mechanisms regulating DDR in GSCs may reveal new paradigms to curb GBM growth and recurrence, and ultimately a strategy to improve patient outcomes.

Lysine-specific histone demethylase 1A (KDM1A/LSD1), which demethylates mono- and di-methylated lysine residue 4 and 9 on histone H3,[15] has been identified as a potential target in GBM.[16–18] KDM1A is a key player in the maintenance of pluripotency and high levels of KDM1A are required to maintain the undifferentiated state of human embryonic stem cells.[19,20] Recent studies implicate KDM1A in DDR through its recruitment to sites of DNA double-strand breaks in osteosarcoma cells.[21] It is currently unknown whether KDM1A plays a role in DNA repair signaling in GSCs. Currently, the available KDM1A inhibitors largely exhibit different mechanisms of action and cell-specific inhibitory activities and few of them are in clinical trials for small cell lung cancer and myeloid neoplasms,[22,23] however, the blood-brain barrier (BBB) permeability of these inhibitors is not established. We have identified the novel KDM1A-specific inhibitor NCD38 as a promising drug for GBM therapy.[16,24] We reason that KDM1A inhibition could enhance the efficacy of TMZ in GBM.

In this study, we examined whether KDM1A inhibition enhances TMZ efficacy in GBM and investigated its mechanism of action. We observed that KDM1A knockdown or inhibition sensitized patient-derived GSCs to TMZ treatment. Mechanistic studies identified that KDM1A is enriched at promoters of several DNA double-strand break (DSB) repair genes and KDM1A inhibition suppressed the DNA repair gene expression and DNA DSB repair pathways in GSCs. Pharmacokinetic studies showed that the KDM1A inhibitor NCD38 has excellent BBB permeability and combination therapy of KDM1A knockdown or inhibition with TMZ potently enhanced the mice survival in orthotopic GBM models.

Materials and Methods

Cell Culture and Patient-Derived GSCs

Patient-derived primary GSCs were established from discarded specimens obtained from GBM patients undergoing surgery at UT Health San Antonio under an IRB-approved biorepository. The specimens were collected in accordance with the declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of UT Health San Antonio. Patients provided informed consent for surgery and the use of their tissues for research. All patient-derived GSCs were cultured as neurospheres as described previously.[16] See Supplementary Materials and Methods for details.

KDM1A shRNA and gRNA Transduction

For KDM1A knockdown studies, GSCs were transduced with lentiviral control shRNA or previously validated KDM1A shRNA.[25,26] For KDM1A knockout, Scramble and KDM1A-specific gRNA were transduced into GSCs possessing TET-inducible Cas9. Cas9 stimulation was done by treating GSCs with 50 ng/mL doxycycline. See Supplementary Materials and Methods for details.

Cell Viability Assays

The effect of the combination of KDM1A knockdown/inhibitor with TMZ on the viability of GSCs was determined using CellTiter-Glo 2.0 Cell Viability Assay according to the manufacturer's instructions. See Supplementary Materials and Methods for details.

Neurosphere Formation and Limiting Dilution Assays

For neurosphere assays, single-cell suspensions of GSCs were seeded in 96-well plates (5 cells/well) and treated with vehicle (DMSO 0.1% v/v), NCD38 (3 μM), TMZ (25 or 50 μM), or in combination for 7–10 days. Neurospheres were imaged and quantitated using NIH ImageJ. Extreme limiting dilution assay was performed by seeding decreasing numbers (20, 10, 5, and 1 cells/well) of dispersed GSCs in 96 well ultra-low attachment plates and treated with vehicle (DMSO 0.1% v/v), NCD38 (3 μM), TMZ (25 or 50 μM), or in combination. After 10–14 days, the number of wells containing neurospheres per each plating density was recorded and the stem cell frequency between control and treatment groups was calculated using extreme limiting dilution assay software (http://bioinf.wehi.edu.au/software/elda/) and statmod package in R.

Single Cell Alkaline Gel Electrophoresis (Comet Assay)

GSCs were treated with vehicle, NCD38 (4 μM), TMZ (25 μM), or in combination. After 24 h, samples were subjected to comet assay. Briefly, cells were embedded in low melt agarose, lysed with alkaline lysis solution, pulsed for 10 min, and stained with SYBR gold solution. See Supplementary Materials and Methods for details.

qPCR-based Homologous Recombination Assay

Homologous recombination (HR) Assay Kit (Norgen Biotek Cat# 35600, Ontario Canada) was used to determine the HR efficiency in GSCs following KDM1A knockdown or inhibitor treatment according to the manufacturer's instructions. See Supplementary Materials and Methods for details.

DR-GFP Reporter Assay

HR efficiency after KDM1A knockdown or inhibitor treatment was determined using DR-U2OS and DR-HeLa cell lines containing a single integrated copy of the DR-GFP reporter. See Supplementary Materials and Methods for details.

Non-homologous End Joining Reporter Assay

To assess the non-homologous end joining (NHEJ) capacity of GSCs, we utilized the reporter system as previously described using pEGFP-Pem1-Ad2, a gift from Dr. Vera Gorbunova.[27] See Supplementary Materials and Methods for details.

RNA-seq, RT-qPCR, and Chromatin Immunoprecipitation Assay

RNA-seq experiments were performed as described previously.[16] Patient-derived GSCs were treated with vehicle or NCD38 (3 μM) or TMZ (50 μM) or NCD38+TMZ for 24 h. RNA was isolated using RNeasy mini kit and subjected to RNA-seq on the Illumina HiSeq 3000 system using Illumina TruSeq stranded mRNA-seq library preparation kit by UTHSA Genome Sequencing Facility. Chromatin immunoprecipitation assay was performed using Pierce™ Magnetic Chromatin immunoprecipitation Kit (Thermo Fisher Scientific, Waltham, MA, USA) as per the manufacturer's protocol. See Supplementary Materials and Methods for details.

CUT&Tag Sequencing and Data Analysis

CUT&Tag sequencing was performed as described previously.[28] Briefly, GSC 082209 cells were treated with vehicle or NCD38 (5 μM) for 24 h and 250 000 cells per condition were used. Data analysis on CUT&Tag sequencing reads was performed by the nf-core/cutandrun bioinformatic analysis pipeline. See Supplementary Materials and Methods for details.

Western Blotting

GSC samples were pelleted down and lysed in RIPA buffer and transferred to PDVF membranes. See Supplementary Materials and Methods for antibodies and their concentrations.

γH2AX, RAD51, and 53BP1 Foci Formation Assays

DNA DSB were evaluated using γ-H2AX, RAD51, and 53BP1 foci on GSCs seeded on poly-l-ornithine/fibronectin-coated coverslips and treated for 48 h. See Supplementary Materials and Methods for further details.

Pharmacokinetic and Brain Bioavailability Studies

A pharmacokinetic study of NCD38 was conducted in male Sprague Dawley rats following intravenous and oral administration of the compound NCD38. See Supplementary Materials and Methods for details.

In vivo Orthotopic Tumor Model

All animal experiments were conducted according to institutional guidelines after obtaining UT Health San Antonio IACUC approval. NOD.CB17-*Prkdc*^{scid}/NCrCrl mice 8–10 weeks old were purchased from Charles River (Wilmington, MO). Orthotopically implanted GBM tumor-bearing mice were randomized to receive either vehicle, NCD38, TMZ, or in combination via oral gavage. See Supplementary Materials and Methods for details.

Immunohistochemistry

Immunohistochemistry experiments were performed as described.[16] Briefly, tumor sections were incubated overnight with Ki67 (1:200), cleaved caspase3 (1:100), and γH2AX (1:100) primary antibodies. See Supplementary Materials and Methods for details.

Statistical Analysis

Differences between the control and experimental groups were analyzed using a two-tailed student's *t*-test or ANOVA, as applicable, using GraphPad Prism 9 software. See Supplementary Materials and Methods for details.

Results

KDM1A Knockdown Enhances the Efficacy of TMZ in Reducing Viability, Neurosphere Formation, and Self-renewal of GSCs

The importance of KDM1A in tumorigenicity and stemness has been underscored in numerous cancer types.[25,29,30] In gliomas, patients with high KDM1A expression exhibit poor overall survival compared to low KDM1A expression populations (Supplementary Figure 1A–C). Additionally, compared to non-tumor samples, GBM patient tumors express higher levels of KDM1A (Supplementary Figure 1D–F). Importantly, among patients receiving standard TMZ/IR, we observed that non-responsive patients exhibited higher levels of KDM1A compared to their responsive counterparts (Figure 1A). Further analysis showed that KDM1A is highly expressed in recurrent GBM samples compared to primary tumors (Figure 1B). We established a collection of GSCs obtained from GBM patient samples at the UTHSA. These patient-derived GSCs were subtyped based on established TCGA gene expression-based molecular classifications (Supplementary Figure 1G). Using these samples, we examined whether KDM1A is associated with the resistance phenotype. To test this hypothesis, we transduced patient-derived GSCs with control or 2 different KDM1A-specific shRNAs and treated them with increasing doses of TMZ. Results from cell viability assay show KDM1A knockdown, compared to control, sensitizes GSCs to TMZ (Figure 1C–D; Supplementary Figure 2A–C. Additionally, KDM1A knockdown reduces neurosphere formation (Figure 1E–F), neurosphere growth (Figure 1G–H; Supplementary Figure 2D–G), and self-renewal capacity (Figure 1I–J) of TMZ-treated GSCs compared to their respective controls or monotherapy. Importantly, we have validated these findings using KDM1A knockout (KO) GSCs generated using an inducible CRISPR/Cas9 system. Cell viability assay results show that KDM1A-KO significantly sensitized GSCs to TMZ treatment (Supplementary Figure 2H–J). Interestingly, the reintroduction of KDM1A in KDM1A-KO cells significantly reversed this effect. Furthermore, we validated these findings using cell viability and clonogenic studies in known TMZ-sensitive (U251), TMZ-resistant (T98G), and acquired TMZ-resistant (U251 TMZ-R) GBM lines (Supplementary Figure 3A–K). Altogether, these findings suggest KDM1A is associated with a therapy-resistant phenotype and that KDM1A knockdown enhances TMZ efficacy in GSCs.

NCD38 is a Brain Permeable KDM1A Inhibitor and Enhances TMZ Efficacy in GSCs

Recently our group identified novel KDM1A-specific inhibitor NCD38 which selectively inhibits KDM1A more potently than its parent compound 2-PCPA (0.59 ± 0.32 μM vs. 31 ± 12 μM)[24,31] and exhibits antitumor activity in several cancer models.[16,25,30,31] However, its pharmacokinetic and brain bioavailability profiles have not been established. We conducted pharmacokinetic and brain bioavailability studies following intravenous (IV) and peroral administration of NCD38 in male Sprague Dawley rats. Both routes of administration demonstrated favorable pharmacokinetic parameters of NCD38 in the plasma (Figure 2A, Supplementary Table 1), with oral bioavailability of 23% and half-life in each case approaching 2 h (Figure 2A; Supplementary Tables 1 and 2). Importantly, NCD38 was available in both the brain and plasma (Figure 2B, C, Supplementary Tables 3 and 4), with a total brain/plasma ratio exceeding a ratio of 2 at 8-h post-administration in both IV and peroral routes, suggesting NCD38 readily permeates the BBB and exhibits adequate brain bioavailability. Moreover, we performed dose-ranging (1mg/kg, 5mg/kg, or 10mg/kg of NCD38) studies using U251-GSC orthotopic mouse model. Results showed that 10mg/kg was the most effective dose in reducing tumor growth (Figure 2D).

Next, we examined whether KDM1A-specific inhibitor NCD38 enhances TMZ-mediated reduction in cell viability. Similar to KDM1A knockdown, NCD38-treated GSCs were more sensitive to TMZ than monotherapy alone (Figure 2E,

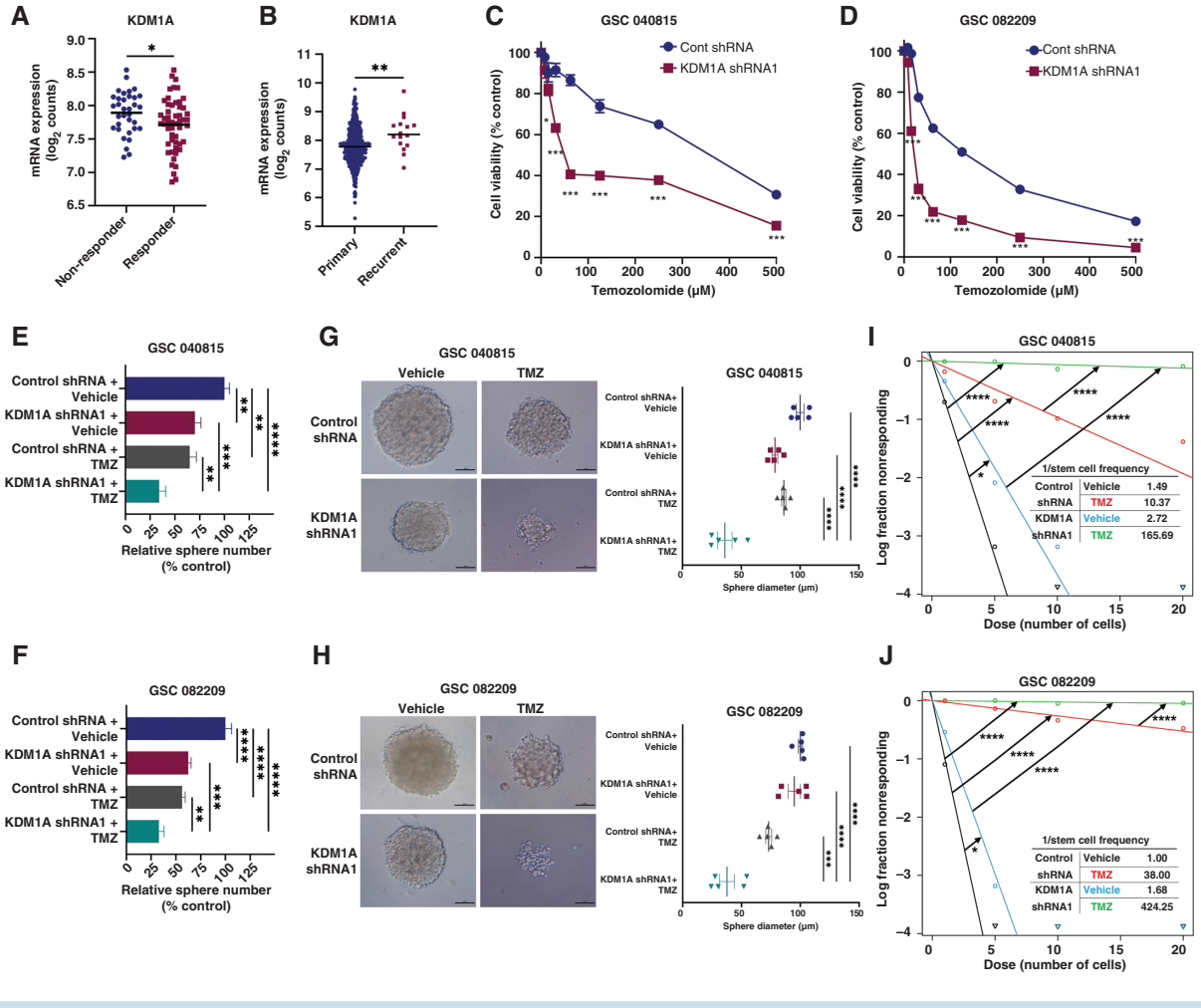

Figure 1. **KDM1A knockdown sensitized Glioma stem cells (GSCs) to TMZ treatment.** **(A)** Box plot of KDM1A was generated using a response based on overall survival at 16 months after radiation and TMZ treatment from ROC plotter datasets. **(B)** KDM1A expression was examined in primary versus recurrent GBM TCGA data sets. **(C–D)** Control and KDM1A knockdown GSCs were treated with either vehicle or TMZ for 5 days and cell viability was determined using CellTiter-Glo assays. **(E–H)** Neurosphere formation of control and KDM1A knockdown GSCs following TMZ treatment was determined. **(I–J)** Self-renewal ability of control and KDM1A knockdown GSCs following TMZ treatment was determined by extreme limiting dilution assays. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$, by t-test or one-way ANOVA.

F; Supplementary Figure 4A). Combination index analysis suggests NCD38 treatment works synergistically with TMZ (Supplementary Figure 4B–D). Importantly, the knockout of KDM1A significantly compromised the activity of NCD38 in GSCs (Supplementary Figure 4E, F). Additionally, combination treatment significantly reduces the self-renewal and neurosphere-forming capacity of GSCs compared to monotherapy (Figure 2G–L). These results suggest NCD38 synergizes with TMZ to reduce GSCs viability and self-renewal.

KDM1A is Enriched at Transcriptional Start Sites of DNA DSB Repair Genes

To understand the mechanistic insights on how KDM1A inhibition sensitizes GSCs to TMZ, we performed genome-wide localization studies of KDM1A in GSCs using CUT&Tag-seq, a cutting-edge technology with greater signal: noise ratio compared to traditional ChIP-sequencing.[28] We detected 75,491 KDM1A peaks in the genome of GSCs ($P < .0001$) (Figure 3A, Supplementary Figure 5A). Of these peaks, the majority (49.11%) resided in promoter regions. Specifically, 36.6% lie within the 1 kilobase pair (kbp) region flanking the transcription start site. Furthermore, KDM1A peaks at intergenic and intronic regions comprised 18.57% and 29.29%, respectively. Importantly, pathway analysis of KDM1A binding genes using gene ontology showed KDM1A binding genes were enriched in DNA repair, cell cycle, and UPR signaling pathways (Figure 3B). Since TMZ resistance phenotype is associated with enhanced DNA repair, we next examined KDM1A-specific enrichment at DNA repair genes. Our results showed KDM1A is highly enriched at the transcriptional start site of numerous DNA repair genes

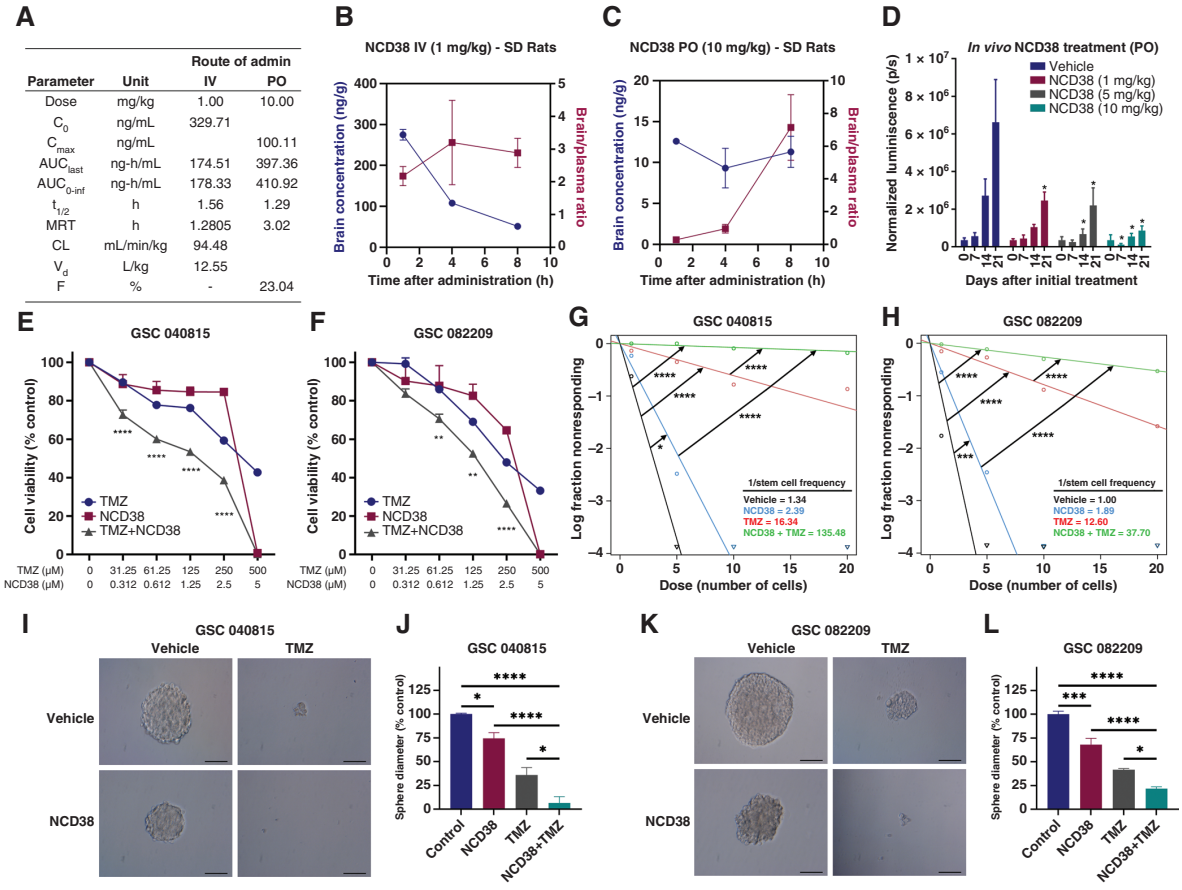

Figure 2. **NCD38 is a brain-permeable KDM1A inhibitor and synergistically enhanced TMZ-mediated reduction in cell viability and self-renewal of Glioma stem cells (GSCs). (A)** Plasma pharmacokinetic parameters of NCD38 following intravenous (IV) and peroral administration in male SD rats. Blood-brain distribution of NCD38 after IV dose of 1mg/kg ($n = 3$) and peroral dose of 10mg/kg ($n = 3$) was determined by LC-MS/MS analysis. Individual plasma concentration vs. time profile of NCD38 after IV **(B)** and peroral **(C)** administration to male SD rats. **(D)** U251-GSCs tumor-bearing mice were treated with vehicle or different doses of NCD38. Tumor growth rate in terms of luciferase intensity was measured using Xenogen IVIS ($n = 6$). **(E–F)** GSCs were treated with NCD38 or TMZ alone and in combination for 5 days and the cell viability was determined by Cell Titer Glo assay. **(G–H)** GSCs were treated with NCD38 or TMZ alone or in combination and their self-renewal ability was determined by extreme limiting dilution assays. **(I–L)**, Effect of NCD38 or TMZ alone or in combination on neurosphere formation was determined. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$, by Student's *t*-test or one-way ANOVA.

(Supplementary Figure 5B). We validated KDM1A binding at select gene promoters using ChIP (Supplementary Figure 5C–D), indicating that KDM1A may regulate their expression via its direct recruitment to the promoter regions.

KDM1A Inhibition or Knockout Reduces the Expression of Genes Involved in DNA Repair

Since GSCs possess high DNA repair capacity, a major contributor to TMZ therapy resistance, we hypothesized that KDM1A inhibition-mediated sensitivity to TMZ may involve attenuation of DNA repair genes. We examined global transcriptional changes by RNA-sequencing using GSCs treated with either vehicle, NCD38 or TMZ monotherapy, or a combination. Initially, we looked at the effect of NCD38 monotherapy and among the top downregulated pathways in NCD38-treated GSCs were related to DNA repair and cell cycle (Figure 3C). A closer examination of differentially expressed genes revealed the downregulation of several important DNA repair genes (Figure 3D–E). Importantly, GSEA analysis indicated that genes altered in NCD38-treated cells showed negative enrichment with the gene sets of HR and NHEJ repair pathways (Figure 3F–G). Further analysis showed that NCD38 treatment increased the enrichment of repressive histone methylation mark H3K9me2 at promoter regions of a subset of DNA repair genes (Supplementary Figure 5E–L).

To further validate the effects of KDM1A on DNA repair pathways in GSCs, we performed RNA-seq using KDM1A-KO GSCs and compared them with NCD38-treated cells. Results show there exists a significant overlap in gene expression between NCD38 versus KDM1A-KO cells (53.5% commonly upregulated; 51% commonly downregulated) (Supplementary Figure 6A–B). Pathway analysis of overlapping downregulated genes showed enrichment

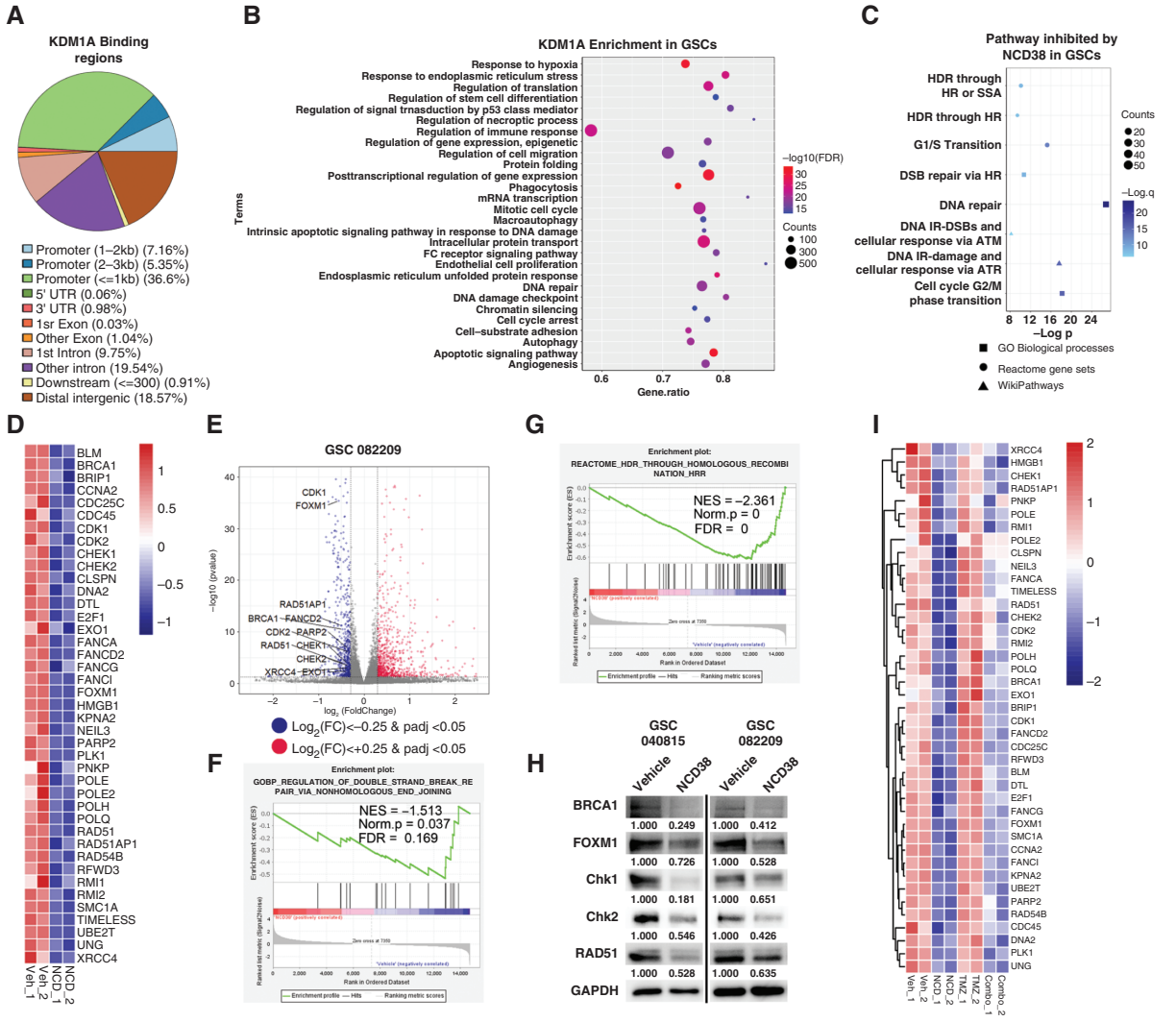

Figure 3. CUT&Tag and RNA-seq analysis identified KDM1A inhibition suppress DNA repair genes expression. (A) Chipseeker annotation of KDM1A peaks to identify their chromosomal locations. **(B)** Functional enrichment of KDM1A binding regions in gene ontology-BP pathways through GREAT online platform. **(C)** GSC082209 were treated with either vehicle or NCD38 (5 μM) for 24 h and subjected to RNA sequencing. Top gene ontology terms of differentially expressed genes were shown. **(D)** Heat map showing downregulation of DNA damage response genes in NCD38 treated group. **(E)** Volcano plots comparing the gene expression levels for the NCD38 versus vehicle-treated GSCs. **(F–G)** GSEA testing correlation of NCD38 regulated genes with signatures of homologous recombination and non-homologous end joining gene sets. **(H)** Western blotting confirms the downregulation of DNA repair genes following NCD38 treatment. **(I)** Heat map showing downregulation of genes in the combination group.

in DNA repair pathways (Supplementary Figure 6C) with significant overlap with DNA repair genes (71 genes), including BRCA1, EXO1, and FOXM1 (Supplementary Figure 6D). Importantly, heatmap analysis showed that a subset of DNA DSB repair pathway genes are downregulated in the KDM1A-KO group (Supplementary Figure 6E). We validated changes in select genes using western blotting and RT-qPCR in KDM1A-KO, KDM1A-KD, as well as NCD38 treated GSCs (Figure 3H; Supplementary Figure 7A–D). Altogether, these results show both KDM1A-KO, -KD, and KDM1A inhibition results in the downregulation of DNA DSB repair genes in GSCs. We also observed that more genes are altered in the KDM1A-KO group compared to the NCD38 group, and this may, in part, reflect KDM1A's ability to regulate gene expression in both demethylase-dependent and independent means, whereas NCD38 only inhibits KDM1A demethylase dependent gene expression.

To further understand the mechanistic basis of the combined effect of NCD38 and TMZ in GSCs, differentially expressed genes were subdivided into 4 major clusters by unsupervised clustering (Supplementary Figure 7E–F). Cluster A3 comprised 140 genes induced by TMZ but repressed by NCD38. Gene ontology analysis showed that these genes were mainly enriched in the cellular response to DNA damage stimulus, DNA recombination, DNA repair, and HR (Figure 3I). Cluster A4 included 420 genes synergistically

induced by NCD38 and TMZ combination treatment implicated in cell death and autophagy. Furthermore, cluster A2 included 646 genes synergistically repressed in combination treatment, with several involved in cell cycle, epithelial to mesenchymal transition, E2F, mTORC1, and MYC signaling. Collectively, these results suggest KDM1A inhibition contributes to attenuation of DNA repair genes.

KDM1A Inhibition Suppresses HR and NHEJ in GSCs

Since RNA-seq and CUT&Tag-seq results suggest that KDM1A plays a role in DNA DSB repair, we examined the effect of KDM1A inhibition on the DNA repair capacity of GSCs. Previous studies have shown that HR and NHEJ repair abilities are responsible for the therapy resistance of GBM. We determined the effect of KDM1A knockdown or inhibition on HR and NHEJ repair assays. First, we utilized a qPCR-based HR assay (Figure 4A) in GSCs and found that KDM1A knockout or inhibition significantly reduces the HR capacity in GSCs (Figure 4B–D). Next, we measured HR efficiency using a well-known HR-specific DR-GFP reporter system in U2OS and HeLa cell lines (Figure 4E). KDM1A knockdown or NCD38 treatment also reduces HR efficiency significantly in these models (Figure 4F–J). Furthermore, we explored whether NHEJ capacity was affected by KDM1A knockdown or inhibition (Figure 4K). As predicted by sequencing results, knockdown or inhibition of KDM1A reduces the NHEJ capacity of GSCs (Figure 4L–O). Altogether, these results highly suggest that KDM1A promotes both HR and NHEJ repair activity of GSCs.

KDM1A Inhibition Enhances TMZ Mediated DNA Damage in GSCs

Since KDM1A inhibition compromised the DNA repair capacity of GSCs, we next examined whether such attenuation of HR and NHEJ repair capacity will lead to persistent DNA damage signaling and unrepaired double-strand breaks. First, we measured γH2AX foci formation

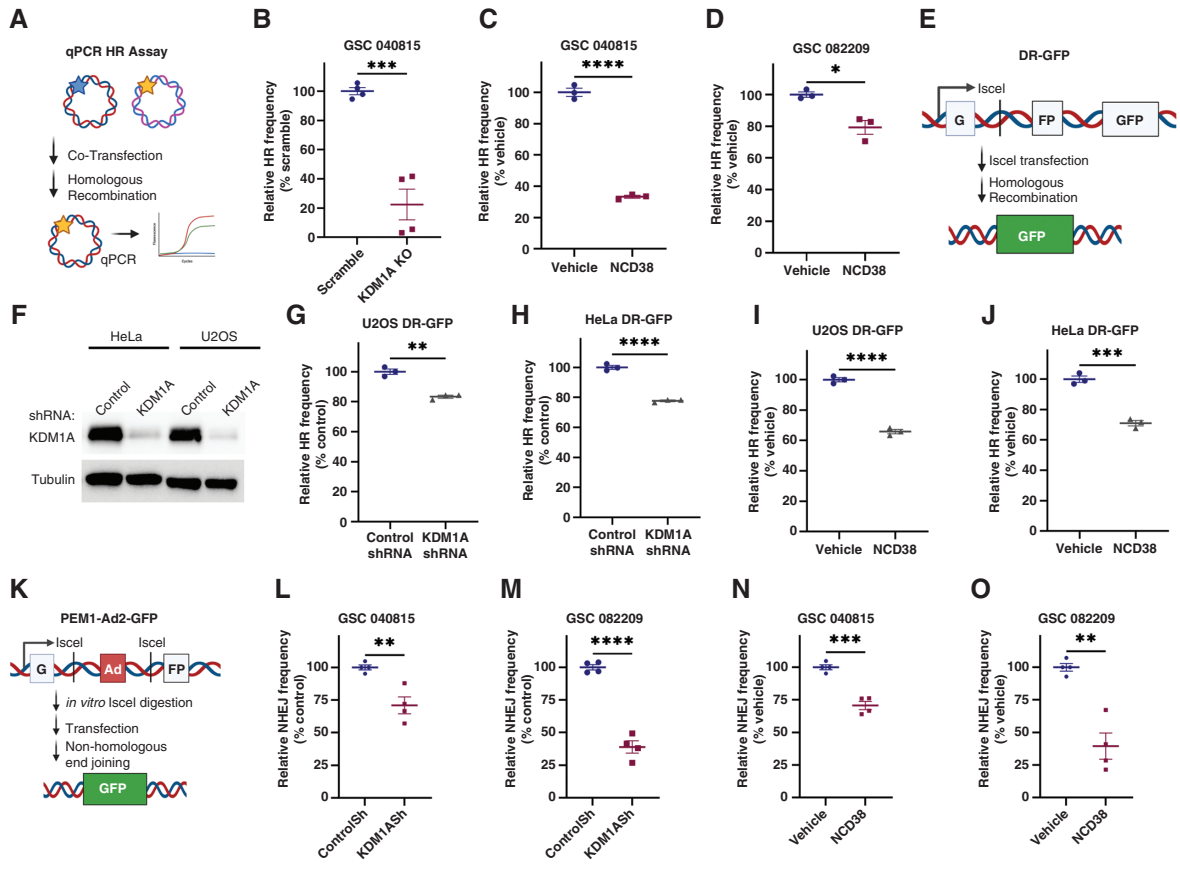

Figure 4. KDM1A inhibition reduces both homologous recombination (HR) and non-homologous end-joining (NHEJ) repair of Glioma stem cells (GSCs). (A) schematic of qPCR-based HR reporter assay. **(B–D)** Effect of KDM1A-KO or NCD38 on the HR repair activity of GSCs was measured. **(E)** Schematic of DR-GFP reporter assay. **(F)** KDM1A knockdown in HeLa and U2OS cells was confirmed using western blotting. **(G–J)** Effect of KDM1A knockdown or NCD38 treatment (72 h) on the HR activity of U2OS and HeLa cells that stably express DR-GFP reporter plasmids was determined using flow cytometry. **(K)** Schematic of NHEJ reporter assay. **(L–M)** Effect of KDM1A knockdown on NHEJ repair activity of GSCs in terms of GFP+ cells were measured using flow cytometry. **(N–O)** NHEJ repair activity of GSCs was determined after 72 h treatment of NCD38. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$, by Student's t-test.

in KDM1A knockdown, knockout, and NCD38-treated GSCs in combination with TMZ. Knockdown of KDM1A increased the γH2AX levels at basal conditions (Figure 5A, Supplementary Figure 8A). Further treatment with TMZ substantially increased γH2AX levels in KDM1A KD cells compared to control shRNA cells and this was corroborated in NCD38 and TMZ combination treatment (Figure 5A, B; Supplementary Figure 8A). Furthermore, knockdown or pharmacologic inhibition of KDM1A significantly increased the γH2AX foci formation in GSCs (Figure 5C–F; Supplementary Figure 8B–H). Next, we assessed the RAD51 foci formation, a marker of HR repair

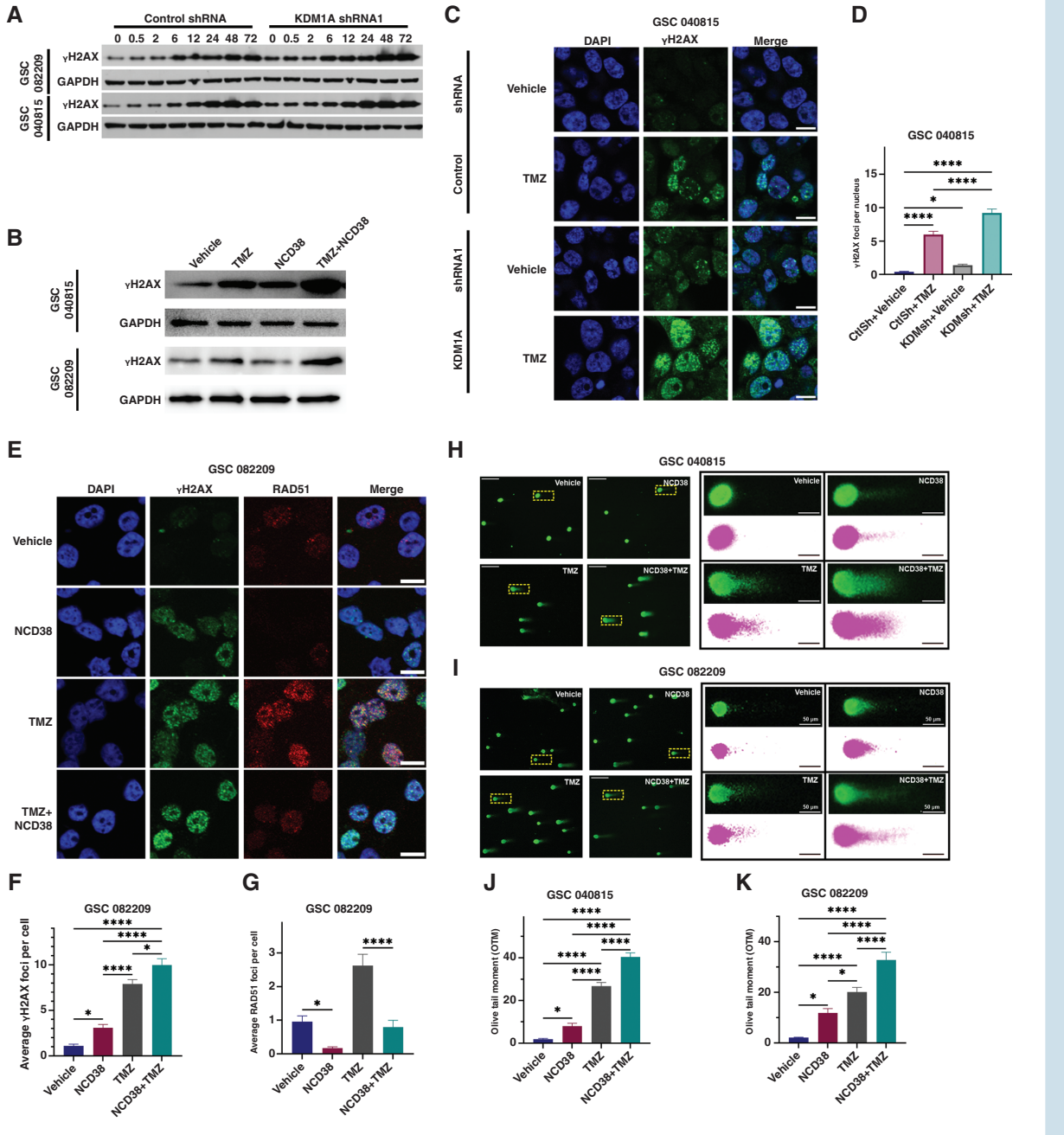

Figure 5. KDM1A inhibition enhances TMZ-mediated DNA damage in Glioma stem cells (GSCs). (**A–B**) Control or KDM1A knockdown or NCD38-treated GSCs were treated with TMZ and the level of γH2AX protein was determined using western blotting. (**C–D**) Control and KDM1A knockdown GSCs were treated with TMZ for 48 h and the γH2AX foci formation was measured using immunofluorescence. (**E–G**) GSCs were treated with either NCD38 or TMZ alone or in combination for 48 h and the γH2AX and RAD51 foci formation was determined using immunofluorescence. (**H–K**) GSCs treated with vehicle or NCD38 for 24 h were subjected to alkaline comet assay. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$, by one-way ANOVA.

whose inhibition is known to sensitize glioma cells to alkylating agents.[32] As expected, TMZ treatment promoted RAD51 foci formation. However, when combined with NCD38 treatment, we observed a marked attenuation of TMZ-induced RAD51 foci formation in GSCs, consistent with our sequencing results and supporting the effect of NCD38 to compromise HR (Figure 5E–G, Supplementary Figure 8D, F), which we also observed in TMZ resistant T98G cells (Supplementary Figure 8G–I). We assessed the 53BP1 foci formation, a marker of DNA DSB repair and NHEJ following NCD38 treatment in GSCs and found TMZ treatment increased 53BP1 foci formation. However, when combined with NCD38 treatment, we noted a marked attenuation of TMZ-induced 53BP1 foci formation, consistent with our reporter assays supporting the effect of NCD38 to reduce NHEJ repair in GSCs (Supplementary Figure 8B, C). Furthermore, we measured the effect of combination therapy on DNA damage by alkaline comet assay and found that combination treatment significantly increased the olive tail moment of treated GSCs when compared to monotherapy (Figure 5H–K). Thus, these results provide strong evidence that KDM1A inhibition reduced the ability of GSCs to repair TMZ-mediated DNA damage.

KDM1A Knockdown or Inhibition Enhances Efficacy of TMZ in Orthotopic Xenograft Models

To determine whether KDM1A is a potential target to enhance the efficacy of TMZ, we injected patient-derived GSCs transduced with either control shRNA or KDM1A shRNA into the right cerebrum of NOD-SCID mice (Figure 6A). We found TMZ treatment significantly decreased tumor progression and enhanced survival of KDM1A knockdown tumor-bearing mice when compared to controls (Figure 6B–C). Next, to determine whether the combination of KDM1A inhibitor NCD38 with TMZ enhances overall survival, we treated patient-derived GSC 040815, GSC 082209, and U251 GSCs tumor-bearing mice with NCD38, TMZ, or in combination. As shown, NCD38 and TMZ combination treatment significantly reduced tumor progression (Figure 6D, Supplementary Figure 9A) and enhanced overall survival in all tumor models (Figure 6E, F; Supplementary Figure 9B) when compared to monotherapy. Immunohistochemical analysis of tumor sections showed a marked reduction of the proliferation marker Ki67 after combination treatment (Figure 6G–H) and a significant increase in apoptosis marker cleaved caspase3 and DNA DSB marker γH2AX (Figure 6G, I–J). These findings are in agreement with the data obtained using KDM1A knockdown tumor samples (Supplementary Figure 8C–F). These results suggest KDM1A knockdown or inhibitor treatment significantly enhanced TMZ efficacy and improved the overall survival of GSC tumor-bearing mice.

To further understand the clinical association of KDM1A in DSB repair in human GBM samples, we analyzed the publicly available TCGA patient database. There is a positive correlation between KDM1A and several DNA repair genes that we identified as attenuated in response to NCD38 treatment, including EXO1, RAD51, and BRCA1 (Figure 6K–M). Interestingly, we also observed a similar trend not only in GBM but also in several other cancer types (Figure 6N). Altogether this data provided preclinical evidence that KDM1A inhibition potentiates the efficacy of TMZ in enhancing survival in murine GBM models.

Discussion

GSCs possess exceptional DNA repair capacity and efficiently repair the DNA lesions caused by standard chemo and radiation therapy which often contributes to therapy resistance. Understanding the mechanisms of therapy resistance and identifying the drugs that enhance the therapeutic efficacy of the current standard of care may help to extend the survival of GBM patients. Overexpression of KDM1A has been documented in several human malignancies including GBM. However, the major knowledge gap is due to the lack of suitable KDM1A-specific inhibitor that effectively penetrates the brain parenchyma and the mechanism by which KDM1A inhibition sensitizes GSCs to existing therapies. Our findings filled this knowledge gap by demonstrating that (1) KDM1A inhibitor NCD38 has excellent BBB permeability, (2) KDM1A knockdown or inhibition potentiates TMZ efficacy in reducing cell viability and self-renewal capacity of GSCs, (3) KDM1A epigenetically regulates expression of DNA DSB repair genes, (4) KDM1A inhibition attenuates HR and NHEJ repair capacity of GSCs and exacerbates TMZ-induced DNA damage, and (5) combination of KDM1A knockdown or inhibition with TMZ treatment enhances survival of tumor-bearing mice.

KDM1A plays a vital role in multiple oncogenic processes including cancer stemness and chemoresistance.[33,34] Chemotherapy treatment causes interconversion of non-GSCs and GSCs, leading to enhanced chemoresistance.[35] GSCs possess enhanced DNA repair capacity compared to other cells within the tumor[36] and activation of DDR contributes to TMZ resistance.[37] We observed high KDM1A expression in therapy-resistant nonresponding populations compared to responders and in recurrent GBM compared to primary GBM. Analyses of NCD38-regulated genes showed downregulation of DNA repair pathways with the reduction in HR and NHEJ capacity being the prime mechanisms otherwise sensitizing GSCs to TMZ. Accordingly, our results demonstrated that knockdown or pharmacological inhibition of KDM1A sensitizes GSCs to TMZ treatment suggesting the role of KDM1A in TMZ resistance.

Accumulating evidence suggests both HR and NHEJ play a vital role in TMZ resistance in GBM. Knockdown of HR genes RAD51 or BRCA2 sensitizes GBM to TMZ.[32] It has been shown that higher levels of RAD51, BRCA2, Chk2 and Ku70 were overexpressed in GBM cells and RAD51 knockdown enhances TMZ[37] as well as radiotherapy responses in GBM.[38] Inhibition of NHEJ factor DNA-PK sensitized GBM cells to radiation, as well as to TMZ albeit to a lesser extent.[32] Furthermore, in pediatric GBM cell lines, NHEJ factors LIG4 and XRCC4 were identified as TMZ-sensitizers,[39] and depletion of LIG4 sensitized A172 GBM cells to TMZ.[40] Our findings provide evidence that KDM1A plays a vital role in both HR and NHEJ and inhibition of KDM1A impairs the HR and NHEJ repair pathways which contribute

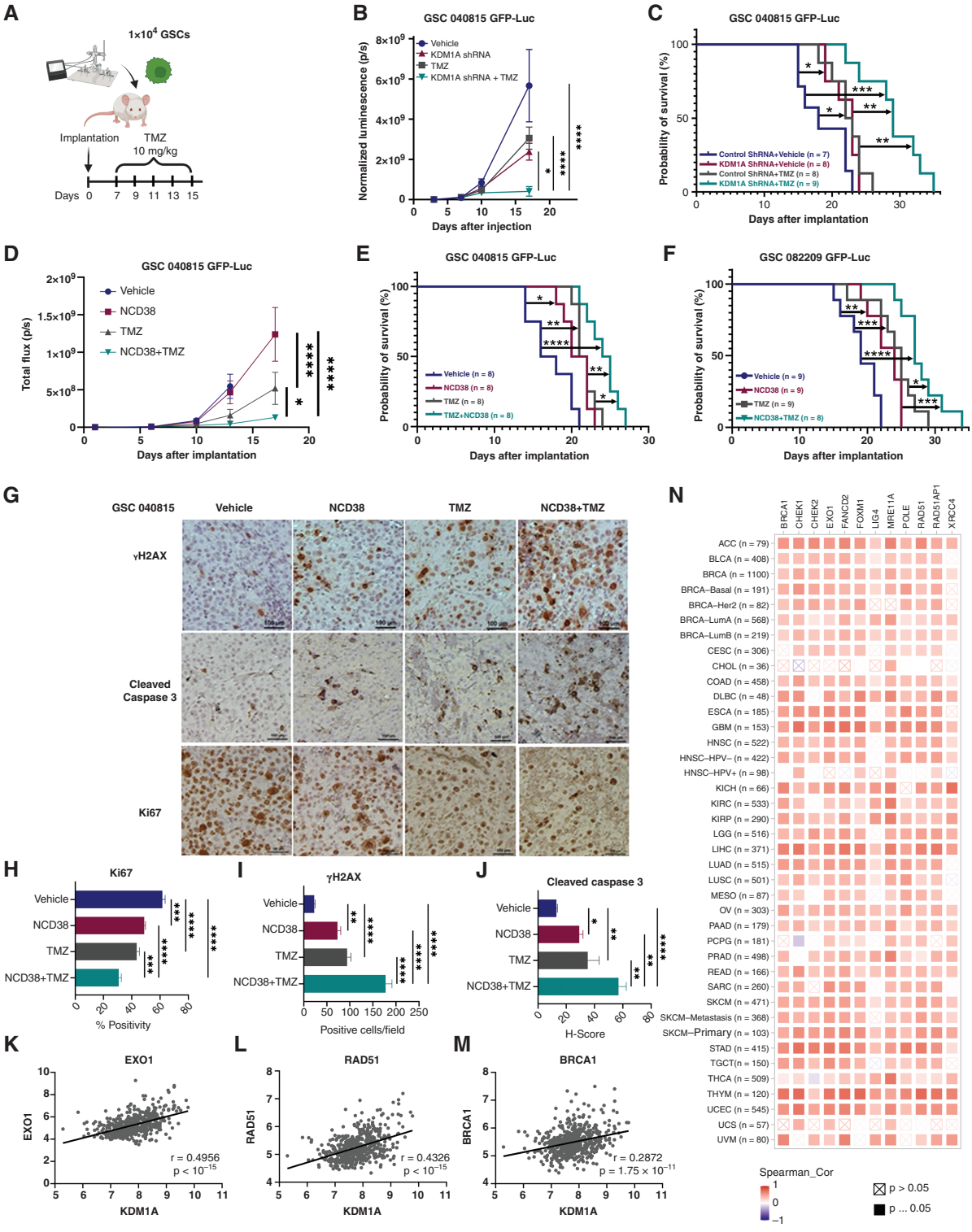

Figure 6. **KDM1A knockdown or inhibition in combination with TMZ enhances the survival of tumor-bearing mice.** **(A)** GSCs that stably express control or KDM1A shRNA were labeled with GFP-Luc and implanted intracranially into NOD-SCID mice. **(B)** Mice were treated with vehicle or TMZ and tumor growth in terms of luciferase activity was monitored using Xenogen IVIS. **(C)** Survival plotted using Kaplan–Meier curves. **(D–F)** GSC040815 and GSC082209 implanted mice were treated with vehicle, NCD38, TMZ, and NCD38+TMZ. Survival was plotted using Kaplan–Meier curves. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$ by log-rank test. **(G–J)** Tumor tissues collected from GSC040815 groups were subjected to IHC staining for Ki67, γH2AX, and Cleaved Caspase3. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$ by one-way ANOVA. **(K–M)** Scatter plots from TIMER2.0 database illustrate expression level correlations between KDM1A and EXO1, RAD51, and BRCA1 in GBM TCGA data sets. Pearson correlation r and *P*-value computed for each dataset. **(N)** Heatmap from TIMER2.0 database showing the expression level correlations between KDM1A and DNA repair genes in pan-cancers.

to the sensitizing effect of TMZ in GSCs. Previous studies have shown KDM1A also regulates MYC, ATF4, and HIF-1 pathways in GBM cells.[16,41–43] Since DNA damage/repair is the target mechanism for first-line therapeutic regimens, including TMZ, radiation, and PARP inhibitors, exploiting this phenomenon in GBM cells provides a distinct advantage over other targets and is readily translatable for GBM therapy.

Emerging studies implicate KDM1A in the regulation of DDR in a cell/tissue-specific manner. KDM1A is shown to be recruited to the DNA damage sites in RNF168 dependent manner, and its depletion sensitizes tumor cells to γ-irradiation[21,44] and localized generation of hydrogen peroxide produced by KDM1A affects the function of proximally located DNA repair proteins in U2OS cells.[45] Furthermore, KDM1A interacts with and destabilizes the tumor suppressor FBXW7 abrogating its functions in growth suppression, NHEJ repair, and radioprotection in lung cancer cells.[46] Our study results are in agreement with previous observations and demonstrated the enrichment of KDM1A at HR and NHEJ repair pathway genes. Furthermore, KDM1A inhibition impairs the HR and NHEJ repair capacity of GSCs possibly via the transcriptional attenuation of a subset of DNA repair genes. A previous study showed that KDM1A knockdown resulted in a modest increase in HR in U2OS cells,[21] contrary to our current findings. We believe that this discrepancy is due to differing treatment periods as well as prior knockdown of KDM1A before I-SceI transfection. In our study, we provided strong evidence that both KDM1A knockdown or inhibition decreases HR in GSCs and in other cancer cell lines such as HeLa and U2OS.

KDM1A inhibitor NCD38 was developed based on a novel concept of the direct delivery of phenylcyclopropyl amine to the KDM1A active site and exhibited antiproliferative activity in leukemia models.[24,30,31] In this study, we established the pharmacokinetic profile of NCD38 and demonstrated that NCD38 in combination with TMZ enhances the survival of GBM tumor-bearing mice while maintaining good BBB permeability. A recent study showed that KDM1A/LSD1 inhibitor DDP_38003 is able to penetrate the brain parenchyma which is evident from the studies of cellular thermal shift assay conducted on brain homogenates and exhibit antitumor activity in GBM.[41] However future studies are needed to establish the safety, efficacy, and off-target effects of KDM1A inhibitor that are needed for further clinical development. Our studies may provide a strong rationale to initiate phase I clinical trials for the combination therapy of NCD38 and TMZ in GBM patients.

In summary, our findings establish the potential of KDM1A inhibition to enhance the efficacy of TMZ in GSCs in vitro and in vivo via attenuation of DNA DSB repair pathways and the combination of TMZ and KDM1A inhibitor NCD38 may represent a novel therapeutic strategy for treating GBM.

Supplementary Material

Supplementary material is available online at *Neuro-Oncology* (http://neuro-oncology.oxfordjournals.org/).

Keywords

DNA repair | glioma stem cells | glioblastoma | KDM1A/LSD1 | temozolomide

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Conflict of Interest Statement
None.

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