HDAC6 involves in regulating the lncRNA-microRNA-mRNA network to promote the proliferation of glioblastoma cells

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Abstract

Background: Glioblastoma (GBM) is the most aggressive and lethal brain tumor. Although the histone deacetylase (HDAC)/transcription factor axis promotes growth in GBM, whether HDACs including HDAC6 are involved in modulating long non-coding RNAs (lncRNAs) to affect GBM malignancy remains obscure.

Methods: Integrative analysis of microarray and RNA-seq was performed to identify lncRNAs governed by HDAC6. Half-life measurement and RNA-protein pull-down assay combined with isobaric tags for relative and absolute quantitation (iTRAQ)-based proteomic analysis were conducted to identify RNA modulators. The effect of LINC00461 on GBM malignancy was evaluated using animal models and cell proliferation-related assays. Functional analysis of the LINC00461 downstream networks was performed comprehensively using ingenuity pathway analysis and public databases.

Results: We identified a lncRNA, LINC00461, which was substantially increased in stem-like/treatment-resistant GBM cells. LINC00461 was inversely correlated with the survival of mice-bearing GBM and it was stabilized by the interaction between HDAC6 and RNA-binding proteins (RBPs) such as carbon catabolite repression—negative on TATA-less (CCR4-NOT) core exoribonuclease subunit 6 and fused in sarcoma. Targeting LINC00461 using azaindolylsulfonamide, an HDAC6 inhibitor, decreased cell-division-related proteins via the lncRNA-microRNA (miRNA)-mRNA networks and caused cell-cycle arrest, thereby suppressing proliferation in parental and drug-resistant GBM cells and prolonging the survival of mice-bearing GBM.

Conclusions: This study sheds light on the role of LINC00461 in GBM malignancy and provides a novel therapeutic strategy for targeting the HDAC6/RBP/LINC00461 axis and its downstream effectors in patients with GBM.

Keywords: Glioblastoma, Histone deacetylase 6, lncRNA LINC00461, RNA-binding proteins

Background

Glioblastoma (GBM) has a poor prognosis and remains incurable, despite aggressive treatment options including surgery, radiation therapy, and first-line chemotherapy with the drug temozolomide (TMZ). Less than 5% of the patients with GBM survive for 5 years following diagnosis. Approximately 90% of patients suffer from disease relapse within 2 years of treatment, regardless of their treatment.
initial response to prior therapy [1]. Previous studies have revealed that GBM frequently harbors different gene expression patterns, causing patients to exhibit diverse clinical characteristics, survival times, and responses to treatment [2, 3]. Therefore, understanding the molecular mechanisms of GBM and developing novel strategies for treating the disease are urgent.

Although GBM is characterized by chromosome 10q loss, aberrant expression of proteins, such as epidermal growth factor receptor amplification and p16INK4 deletion, and gene mutations, such as TP53, IDH1, and PTEN, the molecular basis of the onset and progression of GBM malignancy is not fully understood [4, 5]. Because protein-coding genes account for < 2% of the whole genome, examining the impact of non-coding genes on the regulation of the glioma phenotype appears essential [6]. Long non-coding RNAs (lncRNAs) are defined as transcripts exceeding 200 base pairs in length; these molecules have a wide variety of biological functions [7]. Accumulating evidence has revealed that many dysregulated lncRNAs contribute to chemo-resistance and poor prognostic features of GBM [8]. As a result, identification of lncRNA as a potential target for treating patients with GBM is urgently needed.

The structure of lncRNA is comparable to that of mRNA, with a 7mGpppG 5' cap and 3'-polyadenosine tail. Similar to mRNA decay, lncRNA stabilization and degradation are regulated by microRNAs, decay-promoting RNA-binding proteins (RBPs), decapping enzymes, and deadenylases [9]. Our previous study has demonstrated that azaindolylsulfonamide (MPT0B291), a histone deacetylase (HDAC) inhibitor selective for HDAC6 (IC50 ≈ 0.0052 μM) [10, 11], induces cellular senescence in stem-like GBM cells and prolongs the survival of mice-bearing temozolomide (TMZ)-resistant xenografts in stem-like GBM cells and prolongs the survival of mice-bearing temozolomide (TMZ)-resistant xenografts through downregulation of Sp1 and its targeted genes associated with drug resistance [10]. Although HDAC6 is known to maintain the acetylation balance of histones and nonhistone substrates, such as α-tubulin, cortactin, HSP90, and a few transcriptional factors [12], whether it participates in the regulation of lncRNAs that affect tumorigenesis remains obscure.

In the current study, we used integrative microarray and RNA-seq analysis to investigate whether HDAC6 modulates lncRNAs. A highly conserved lncRNA, LINC00461, which functions as an essential regulator in glioma formation [13] and regulates the expressions of genes such as DNA topoisomerase II Alpha (TOP2A) [13], B-cell lymphoma 2 (BCL2) [14], and integrin β3 (ITGB3) [15], was identified to be remarkably down-regulated by HDAC6 depletion. Our data highlight LINC00461-associated regulatory networks in GBM malignancies, including treatment resistance and cancer stemness, and provide a novel insight into targeting the HDAC6/RBP/LINC00461 axis as a therapeutic approach for patients with GBM.

**Materials and methods**

**Materials**

MPT0B291 [11], synthesized by Prof. J. P Liou at Taipei Medical University, was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich Corp., St. Louis, MO, USA). Temozolomide (TMZ) and trichostatin A (TSA) were purchased from MedChemExpress (Monmouth Junction, NJ, USA) and Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), respectively.

**Cell culture**

Human GBM cell lines, including U87MG (ATCC HTB-14; American Type Culture Collection, Manassas, VA, USA) and A172 (ATCC CRL-1620), and patient-derived primary GBM (Pt#3 and Pt#5) along with their TMZ-resistant and stem-like cells, were cultured in respective media as described previously [16]. Procedures utilized for establishing TMZ-resistant GBM cells were mentioned previously [16]. To maintain TMZ-resistant cells, 50μM TMZ was added into the culture medium, and their resistance characteristics were confirmed using colony-formation assay. Informed consent obtained from the patients followed the protocols (Nos. 201,006,011 and 201,402,018) approved by the Joint Institutional Review Board (JIRB) of Taipei Medical University (Taipei, Taiwan).

**Transient transfection**

FLAG-tagged CNOT6 (Sino Biological, Inc., Beijing, China) or the empty vector with FLAG tag was transfected into U87MG cells using PolyJet (SignaGen Laboratories, Frederick, MD, USA).

**Stable cell line construction**

The expression vector pcDNA3.1(−) carrying LINC00461 inserts (MDBio, Taipei, Taiwan) was transformed into Escherichia coli directly, and the DNA extracted from ampicillin (Sigma-Aldrich Corp.)-resistant bacteria was amplified using PCR. Pt#3 cells transiently transfected with pcDNA3.1-LINC00461 using PolyJet (SignaGen Laboratories). Two days after transfection, cells stably expressing pcDNA3.1-LINC00461 were selected by treating with 0.5 mg/mL genetin (G418; Thermo Fisher Scientific, Waltham, MA, USA).

**RNA interference**

Parental and TMZ-resistant GBM cells (U87MG, A172, and Pt#3) were transfected with 5, 10, or 20nM specific gene siRNA or control non-targeting siRNA
and normalized against the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA and U6 snRNA levels, respectively, for lncRNA or mRNA and miRNA. Oligonucleotides used for TaqMan qPCR assays were purchased from Suu-Flower Co., Ltd. (Taichung City, Taiwan). The primer or probe sequences for each gene are listed in Table S2.

### Animal models

For the subcutaneous tumor model, $1 \times 10^6$ GBM cells (U87MG and Pt#3) were implanted into both dorsal flanks of female nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice (8 weeks old, BioLASCO Co., Ltd., Taipei, Taiwan). Intraperitoneal administration with MPT0B291 (10 mg/kg) or vehicle (DMSO) every other day was initiated when the tumor volume reached a size of 10–20 mm$^3$ after tumor implantation. Tumor volumes were measured using a caliper three times a week and calculated following the modified ellipsoidal formula: tumor volume = $\frac{1}{2}$ (length $\times$ width$^2$) [17]. Tumor weights were recorded at the end of the experiments. For the orthotopic tumor model, $5 \times 10^5$ Pt#3 cells (with or without stable LINC00461 expression) were injected into the right frontal brain area of male Bagg albino (BALB)/c nude mice (9 weeks old, BioLASCO Co., Ltd.) according to detailed procedures reported previously [18]. Intraperitoneal administration with MPT0B291 (10 mg/kg) or vehicle (DMSO) every other day was initiated 5 days after tumor inoculation. Mice weights were recorded three times a week. All protocols for animal experiments were approved by the Institutional Animal Care and Use Committee of the National Health Research Institute (IACUC, NHRI, Tainan, Taiwan). Animal experiments were conducted under the registration number (NHRI-IACUC-106010).

### RNA-in situ hybridization (ISH) and RNA-fluorescence in situ hybridization (FISH)

Digoxigenin (DIG)-labeled probes (Custom LNATM Detection Probes; Table S3) targeting LINC00461 or scramble were purchased from Qiagen (Hilden, Germany). In situ hybridization (ISH) was performed following the manufacturer’s instructions with slight modifications from IsHyb in situ hybridization kit (BioChain Institute Inc., Newark, CA, USA). In brief, 5-μm serial sections of MPT0B291-treated or control xenograft tumors were hybridized with DIG-labeled LINC00461 or scramble probes (2–4 ng/μL) at 55°C for 16 h. For ISH, chromogenic reaction used alkaline phosphatase (AP)-conjugated anti-DIG antibody (1:100) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium provided by manufacturer to detect the expression. For FISH, rhodamine tetramethylrhodamine-isothiocyanate-conjugated anti-DIG (111-025-003, 1:100, Jackson...
ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was used to detect the expression instead. Cell nuclei were counterstained with nuclear fast red (Vector Laboratories, Inc., Burlingame, CA, USA) and 4′,6-diamidino-2-phenylindole (DAPI) (glycerol mounting medium - anti-fade with DAPI; ab188804, Abcam, Inc., Cambridge, UK), respectively, in ISH and FISH staining. Images of FISH staining were captured using ImageXpress Pico Automated Cell Imaging System (Molecular Devices, San Jose, CA, USA).

**Immunohistochemistry**

After antigen retrieval, immunohistochemistry was performed following the manufacturer’s instructions with slight modifications using VECTASTAIN® ABC AP Kits (Vector Laboratories, Inc.). In brief, 5-μm serial sections of MPT0B291-treated or control xenograft tumors were hybridized with an antibody against either Ki-67 (ab16667, 1:100, Abcam, Inc.) or cleaved caspase-3 (#9661, 1:100, Cell Signaling Technology, Inc., Danvers, MA, USA). Average 3,3′-diaminobenzidine staining intensities of each slide normalized by the nuclei number were calculated using the semi-quantitative method [19].

**mRNA stability measurement**

Twenty-four hours after treating with MPT0B291 or vehicle control (DMSO), 10 μg/mL actinomycin D (ActD; Selleckchem, Houston, TX, USA) was added to inhibit transcription in TMZ-resistant Pt#3 and Pt#5 cells. RT-qPCR was used to detect the half-life of LINC00461 at 0, 20, 40, 60, and 120 min after transcriptional inhibition.

**Immunoprecipitation assay**

The cell lysate was prepared using radioimmunoprecipitation assay buffer containing protease inhibitor (Roche, Basel, Switzerland) and then immunoprecipitated with 2 μg anti-HDAC6 (#7612, Cell Signaling Technology, Inc.) or IgG (sc-2025/2027, Santa Cruz Biotechnology, Inc.) at 4°C overnight, followed by incubation with protein A agarose (Merck Millipore, Bedford, MA, USA) at 4°C for 1h. For the immunoprecipitation of FLAG-tagged CNOT6, anti-FLAG M2 affinity gel (Sigma-Aldrich Corp.) was used following the manufacturer’s instructions. The immune complexes were detected by Western blot analyses with anti-CNOT6 (sc-81,231, 1:1000, Santa Cruz Biotechnology, Inc.), anti-HDAC6 (#7612, 1:1000, Cell Signaling Technology, Inc.), and anti-acetyl-lysine (GTX80693, 1:1000, GeneTex Inc., Irvine, CA, USA) antibodies.

**RNA-protein pull-down assay**

Circular plasmid, pcDNA3.1-LINC00461, was converted to a linear template using EcoRV-HF digestion (New England Biolabs Inc., Ipswich, MA, USA) for in vitro LINC00461 synthesis (HiScribe™ T7 Quick High Yield RNA Synthesis Kit; New England Biolabs). The pull-down of RNA-protein complexes was conducted using Pierce™ Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher Scientific) based on the manufacturer’s instructions. Briefly, 50 pmol desthiobiotin-labeled (Pierce™ RNA 3′ End Biotinylation Kit, Thermo Fisher Scientific) androgen receptor (AR) 3′-untranslated regions (UTR) and LINC00461 were captured by 50 μL streptavidin magnetic beads and then interacted with 200 μg lysate extracted from parental and TMZ-resistant Pt#3 cells. Elution of RNA-binding protein complexes was applied for western blotting and isobaric tags for relative and absolute quantitation (iTRAQ)-based proteomic analysis (Biotools Co. Ltd., New Taipei City, Taiwan). 3′-UTR of AR RNA, which contains UC-rich regions for human antigen R binding, is the control system for the pull-down assay.

**Cell proliferation assay**

Parental and TMZ-resistant cells (U87MG and A172) at a density of 10⁴ cells per well were seeded in 24-well tissue culture plates overnight. After downregulation of LINC00461 using siRNA, cell proliferation was determined by adding 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, BIONOVAS Biotechnology Co., Ltd., Toronto, CA) for 30 min at 37°C. After the reaction, the formazan products were dissolved in 0.3 mL DMSO (Sigma-Aldrich Corp.), and the absorbance was spectrophotometrically measured at 550 nm every 24h by an ELIZA reader (Bio-Rad Laboratories, Inc.).

**Cell-cycle analysis**

The cell cycle was evaluated by flow cytometry. Briefly, parental and TMZ-resistant U87MG cells at a density of 7.5 × 10⁴ cells per well were seeded in 6-cm petri dishes. After downregulation of LINC00461 using siRNA, cells were collected and fixed with 70% ethanol at 4°C overnight. Fixed cells were incubated with 0.5 mL PBS containing 0.1% Triton X-100 (Pharmacia & Upjohn Company LLC, North Peapack, NJ, USA) on ice for 15 min and then stained with propidium iodide (Sigma-Aldrich Corp.)/RNase A (BIONOVAS Biotechnology Co.) solution (20 μg/mL propidium iodide and 0.3 mg/mL RNase A in PBS) for 30 min in darkness. Stained cells were measured by flow cytometry (Guava easyCyte, Merck Millipore). Cell-cycle distribution was analyzed by calculating percentages of cells at subG1, G0/G1, S, and G2/M phases.
Western blot analysis
For western blot analysis, anti-Sp1 (#07-645, 1:1000, MilliporeSigma, Burlington, MA, USA), anti-HDAC1 (H3284, 1:1000, MilliporeSigma), anti-HDAC2 (#05-814, 1:1000, MilliporeSigma), anti-HDAC6 (#7612, 1:1000, Cell Signaling Technology, Inc.), anti-CNOT6 (sc-81,231, 1:1000, Santa Cruz Biotechnology, Inc.), anti-fused in sarcoma (FUS) (A5921, 1:1000, ABclonal Inc., Woburn, MA, USA), anti-Acetyl-lysine (GTX80693, 1:1000, GeneTex Inc.), anti-PABP (ab21060, 1:1000, Abcam, Inc.), anti-TOP2A (MAA4197, 1:1000, MilliporeSigma), anti-Ki67 (A11005, 1:1000, ABclonal Inc.), anti-MELK (A3530, 1:1000, ABclonal Inc.), anti-DLGAP5 (A2197, 1:1000, ABclonal Inc.), anti-CD168 (A11666, 1:1000, ABclonal Inc.), anti-MCM10 (A5427, 1:1000, ABclonal Inc.), anti-TPM2 (A2534, 1:1000, ABclonal Inc.), anti-cyclin D1 (EIA6234, 1:1000, EnoGene Biotech Co., Ltd., New York, NY, USA), anti-CDK1 (sc-8395, 1:1000, Santa Cruz Biotechnology, Inc.), and anti-alpha Tubulin (66031-1-lg, 1:10,000, Proteintech Group, Inc., Rosemont, IL, USA) were used as primary antibodies. HRP-linked anti-mouse IgG (sc-2004, 1:5000, Santa Cruz Biotechnology, Inc.) and HRP-linked anti-rabbit IgG (sc-2005, 1:5000, Santa Cruz Biotechnology, Inc.) were used as secondary antibodies. The signals were amplified using an enhanced chemiluminescence reagent (ECL, GE Healthcare, Chicago, IL, USA) and captured using the ChemiDoc Touch Imaging System (Bio-Rad Laboratories, Inc.). The intensity of each band was determined by Image Lab, and the relative target protein levels were normalized against the tubulin level.

Single-cell RNA sequencing (scRNA-seq)
Fresh tumor tissues from four patients with GBM were prepared for scRNA-seq following the protocol established by 10x Genomics® Single Cell (Pleasanton, CA, USA). Data analysis was performed using Cell Ranger (10x Genomics®) and Seurat (29608179). Informed consent obtained from the patients followed a protocol (IRB No. EC1080202) approved by the Research Ethics Committee of the National Health Research Institute (NHRI, Miaoli, Taiwan).

Analysis of databases
Next-generation sequencing (NGS) databases of human GBM transcriptome obtained from The Cancer Genome Atlas (TCGA) (https://portal.gdc.cancer.gov) were collected and processed as described previously [10]. The expression fold change and significance level (t-test) of 50 cell-division-related genes, which appeared in the intersection dataset of LINC00461 depletion and MPT0B291 treatment, between normal and GBM NGS data were calculated. The correlation of gene expression status between TCGA-GBM and LINC00461-silenced datasets was computed using Pearson’s correlation coefficient (PCC). Prognostic implications of cell-division-related genes in high-grade glioma patients were analyzed using PROGgeneV2 (http://www.progtools.net/gene/) [20]. Three public databases, miRDB (http://mirdb.org/), miR-WALK (http://mirwalk.umm.uni-heidelberg.de/), and starBase (http://starbase.sysu.edu.cn/index.php) were used to predict the lncRNA-miRNA-mRNA networks.

Bioinformatic analyses
The heatmap, which displayed the log2 (fold change) values of selected lncRNAs from microarray datasets, was generated using online software Morpheus (https://software.broadinstitute.org/morpheus). Functional analysis of data from microarray and RNA sequencing was performed using ingenuity pathway analysis (IPA, Qiagen). The overlap between molecules in our datasets and a particular function was calculated using the right-tailed Fisher’s exact test. Prediction of activation or inhibition of pathways was examined using a Z-score. The heatmap was generated using a MultiExperiment Viewer by the log2 (fold change) values of selected genes.

Statistics
The statistical significance of the difference between the two groups was analyzed using the unpaired Student’s t-test with a two-tailed p-value. For more than two groups, one-way or two-way analysis of variance (ANOVA) was used to examine statistically significant differences depending on the number of variables. The survival curves of different groups were compared using the log-rank test. A p-value of < 0.05 was considered statistically significant. The statistical significance increased in the order: *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Results
HDAC6 inhibition significantly decreases LINC00461 expression
To determine whether HDAC6 affects lncRNA regulation, we analyzed the expression levels of 9445 lncRNAs using microarray. Microarray gene expression data were applied independently on mRNA samples isolated from GBM stem-like cells (serum-free suspension-adapted tumorspheres) [21] and TMZ-resistant cells [18]. A comparison of MPT0B291-treated with DMSO-treated conditions showed that 54 lncRNA probe sets represent reliable microarray signals (signal-to-noise ratio ≥ 1, Fig. 1A). Among these, only two lncRNAs (LINC00461 and LINC01559) were commonly and significantly altered in stem-like (S-U87MG) and TMZ-resistant (R-U87MG) GBM
cells following MPT0B291 treatment (Fig. 1B, Fig. S1). *LINC00461* levels were decreased 0.27-fold and 0.49-fold in S- and R-U87MG cells, respectively, while *LINC01559* levels were upregulated 2.70-fold and 3.64-fold in S- and R-U87MG cells, respectively. To further confirm the effect of HDAC6 on lncRNA regulation, we depleted HDAC6 using siRNA and performed RNA-seq analysis. Four lncRNAs showing altered levels after HDAC6 knockdown were identified (Fig. 1C). Interestingly, a comparison of datasets obtained from MPT0B291 treatment and HDAC6 knockdown revealed that *LINC00461* was the only IncRNA with an obvious reduction after HDAC6 inhibition, suggesting that it is a downstream IncRNA target of HDAC6. Moreover, further confirmation using RT-qPCR analysis showed a significant reduction in *LINC00461* levels by MPT0B291 treatment in both parental and TMZ-resistant U87MG cells (Fig. 1D).
Attenuation of LINC00461 by MPT0B291 remarkably extends the survival time of mice with GBM

Using U87MG xenografts and Pt#3 xenografts in mice, we observed a significant reduction in tumor growth after MPT0B291 treatment (Fig. 2A–C). Moreover, we analyzed the expression of LINC00461 in MPT0B291-treated and control xenografts by RNA ISH and RNA FISH. A decrease of approximately 40% in LINC00461 molecules was visualized within the cytoplasm of MPT0B291-treated xenograft tissues compared to that in control tumors (Fig. 2D–E), confirming the suppressive effect of MPT0B291 on LINC00461 expression in vivo. Furthermore, MPT0B291 treatment extended the survival of mice-bearing intracranial GBM (Fig. 2F). To understand the mechanism by which MPT0B291 treatment prolonged survival through downregulating LINC00461, protein expressions of proliferation (Ki-67) and apoptosis (cleaved caspase-3) marker were measured in xenograft tissues. A significant reduction in Ki-67 expression was observed within the nucleus of MPT0B291-treated xenograft tissues compared to that in control tumors (Fig. S2A). In contrast, the difference in cleaved caspase-3 expression was not significant in the nucleus between MPT0B291-treated and control xenograft tissues (Fig. S2A), suggesting that the extension of survival in mice-bearing GBM is mainly derived from the anti-proliferative effect of MPT0B291. We then evaluated the role of LINC00461 on survival outcomes using the same orthotopic mouse model. Pt#3 cells that stably express LINC00461 were constructed (Fig. 2G) and injected into the right cerebral hemisphere of mice. The LINC00461 high expression group’s median survival was 24 d, while that of the LINC00461 low expression group was 54 d (Fig. 2H). Thus, LINC00461 plays an important role in GBM growth, but HDAC6 inhibition induced by MPT0B291 blocks the oncogenic effect of LINC00461.

HDAC6 and RNA-binding proteins are involved in managing LINC00461 expression

MPT0B291 is known to block the activation of the HDAC6/Sp1 axis [10]. Whether MPT0B291 suppresses LINC00461 expression by a Sp1-dependent mechanism was further investigated. The result showed that LINC00461 expression was substantially decreased by MPT0B291 treatment while it increased with Sp1 depletion (Fig. 3A, bars 1–3). Consistently, RT-qPCR detection after Sp1 knockdown confirmed elevated LINC00461 levels in parental and TMZ-resistant U87MG cells (Fig. 3B), suggesting that MPT0B291-mediated downregulation of LINC00461 is modulated via an Sp1-independent pathway. Furthermore, to explore the mechanism underlying the modulation of LINC00461 expression, we investigated the RNA stability of LINC00461 using dactinomycin to block de novo RNA transcription in cells and found that MPT0B291 treatment caused 2.75-fold and 1.75-fold reduction in the half-life of LINC00461 in TMZ-resistant Pt#3 and Pt#5 cells, respectively (Fig. 3C). Considering that MPT0B291 is a selective inhibitor of HDAC6 and based on HDAC6 knockdown reduction of LINC00461 levels as verified by RNA-seq analysis (Fig. 1C) and RT-qPCR detection (Fig. 3D), HDAC6 was presumed to contribute to the regulation of LINC00461 stability. Additionally, we found that LINC00461 expression was higher in TMZ-resistant GBM cells. Increased expression of LINC00461 may correspond with the malignant behavior of GBM cells.

HDACs are known to regulate post-transcriptional gene expression by altering deadenylase complex activation and affecting polyadenylate tail stability of RNA [22]. Thus, we examined HDAC6 interaction with the catalytic subunits of the deadenylase complex and identified that carbon catabolite repression—negative on TATA-less (CCR4-NOT) core exoribonuclease subunit 6 (CNOT6), a typical protein related to mRNA decay, is associated with HDAC6 in U87MG cells (Fig. 3F). Furthermore, treating cells with HDAC6 inhibitors, MP0B291

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Fig. 2 Suppression of LINC00461 by MPT0B291 inhibits tumor growth and improves survival of mice-bearing intracranial GBM. A Growth curves show average tumor volumes from 1 × 10^6 Pt#3 cells and 1 × 10^6 U87MG cells injected into the dorsal flanks of NOD/SCID mice. MPT0B291 was administered after the tumor volume reached a size of 10–20 mm^3 every other day (n ≥ 5 per group). Two-way ANOVA. B Average final tumor weights of control and MPT0B291-treated xenografts (n ≥ 5 per group). Unpaired Student’s t-test. C Images of control and MPT0B291-treated xenografts at the end of the experiment (n ≥ 5 per group). D ISH and FISH detected the expression of LINC00461. Serial sections of control and MPT0B291-treated xenografts were hybridized with either a LINC00461 probe or a scramble probe in ISH and FISH, and cell nuclei were stained with nuclear fast red and DAPI, respectively, in ISH (left 4 panels) and FISH (right 4 panels) staining. Scale bars indicate 10 μm (inset of ISH), 13.07 μm (inset of FISH), and 26.22 μm (FISH). E Relative percentages of LINC00461 expression in the cells of control and MPT0B291-treated xenografts were measured by fluorescent intensity. F The Kaplan–Meier plot represents the survival time of BALB/c nude mice-bearing orthotopically implanted 5 × 10^6 Pt#3 cells. MPT0B291 (10 mg/kg) was administered 5 d after cell implantation every other day (n = 6 per group). Log-rank test. G The expression levels of LINC00461 in Pt#3 cells stably expressing either LINC00461 or empty vector control (pcDNA3.1). Unpaired Student’s t-test. H The Kaplan–Meier plot represents the survival time of BALB/c nude mice-bearing orthotopically implanted 5 × 10^6 Pt#3 cells stably expressing either LINC00461 or empty vector control (pcDNA3.1) (n = 8–9 per group). Log-rank test. The results are shown as mean ± SEM for triplicate samples in each group.
Fig. 2 (See legend on previous page.)
and trichostatin A, led to a decrease in the interaction of HDAC6 and CNOT6 in cells (Fig. S3A), suggesting that deacetylase activity is essential for HDAC6 to bind CNOT6, causing changes in the deacetylase activation. Apart from RNA-degrading enzymes that contribute to RNA degradation, RNA-protective proteins that contribute to the stability of LINC00461 were also investigated. We purified LINC00461 (Fig. 3G) and performed RNA-protein pull-down assay (Fig. S3B) combined with iTRAQ-based proteomic analysis, by which several LINC00461-binding proteins were identified (Fig. 3H). Further investigation of the proteomics data using IPA, two RNA-stabilizing proteins, FUS and vimentin, were discovered as HDAC6-targeted factors that functioned in regulating RNA stability. Interestingly, a previous study has indicated that FUS is associated with several RBPs in the vicinity of the mRNA 3’ end and it controls polyadenylate tail maintenance [23]. To investigate the role of HDAC6 on these RNA-regulating proteins, HDAC6 inhibition was induced by MPT0B291 treatment or HDAC6 knockdown and this caused a significant decrease in FUS expression in both parental and TMZ-resistant GBM cells and a significant increase in CNOT6 expression (Fig. 3I–J and Fig. S3C). Furthermore, the effect of FUS knockdown on the RNA stability of LINC00461 showed a 3-fold reduction in the half-life of LINC00461 in TMZ-resistant Pt#3 cells (Fig. 3K).

**LINC00461 depletion suppresses GBM cell proliferation through cell-cycle arrest**

We examined the effects of LINC00461 on GBM cell proliferation. LINC00461 knockdown in parental and TMZ-resistant GBM cells reduced LINC00461 expression approximately by 40–50% (Fig. 4A). Interestingly, LINC00461 depletion suppressed cell proliferation significantly in both parental and TMZ-resistant GBM cells (Fig. 4B). To further determine the anti-proliferative effect of LINC00461 silencing on GBM cells, we analyzed cell-cycle distribution by flow cytometry. LINC00461 knockdown induced a significant cell-cycle shift from G2/M to G1 phases in parental GBM cells and similarly induced a slight perturbation of cell-cycle progression into G2/M phase in TMZ-resistant cells (Fig. 4C). Recently, topoisomerase II alpha (TOP2A), a key enzyme in DNA replication to maintain cell proliferation, was found to be upregulated by LINC00461 through sponging of miR-411-5p during gliomagenesis [13]. Thus, we examined TOP2A expression, including levels of RNA (Fig. 4D) and protein (Fig. 4E), in our experimental model. The results showed that LINC00461 knockdown significantly attenuated TOP2A expression at the transcriprional level in both parental and TMZ-resistant U87MG cells. Additionally, MPT0B291 treatment showed a similar trend with reduced mRNA and protein expression of TOP2A in these GBM cells (Fig. 4F–G).

**LINC00461 is involved in HDAC6-mediated cell division and survival regulation**

To characterize the role of LINC00461 in GBM cells, we performed a genome-wide analysis of gene expression in LINC00461-silenced U87MG cells, and identified that 115 genes were upregulated and 219 genes were downregulated (Fig. 5A). Interestingly, the 219 down-regulated genes included TOP2A and CCNA2, both containing a gene expressing cyclin A2 to maintain the G2/M transition phase of the cell cycle. A comprehensive analysis conducted by comparing the LINC00461-silenced dataset with the MPT0B291-treated dataset revealed 105 intersection genes (Fig. 5B). The expression of 88 genes showed the same trend in both treatments. Functional analysis by IPA revealed highly significant functional gene classes associated with ‘cell division (50 of

(See figure on next page.)

**Fig. 3** Interaction of HDAC6 and RBPs in regulating LINC00461 stability. A Expression levels of LINC00461 from transcriptome data, including two microarray data as shown in Fig. 1A and one microarray analysis using Sp1 knockdown cells. B Parental and TMZ-resistant U87MG cells were treated with either si-Sp1 to silence Sp1 expression or si-NC as a control. Sp1 protein levels were detected by western blotting. LINC00461 expression was detected by qRT-PCR. Unpaired Student’s t-test. C The amount of LINC00461 was measured in TMZ-resistant Pt#3 and Pt#5 cells in either the presence (green lines) or the absence (gray lines) of MPT0B291 following actinomycin D (ActD) treatment at 0, 20, 40, 60, and 120 min. Two-way ANOVA. The red dashed line indicates the time at which 50% of the lncRNA remained. D A172 cells were treated with either si-HDAC6 to silence HDAC6 expression or si-NC as a control. HDAC6 protein levels were determined by western blotting. LINC00461 expression was detected by qRT-PCR. Unpaired Student’s t-test. E Expression levels of LINC00461 in parental and TMZ-resistant GBM cells (Pt#3 and Pt#5). Unpaired Student’s t-test. F Ingenuity pathway analysis (IPA) in U87MG cells was performed using the antibody against HDAC6 or IgG as a negative control. The IP product was examined by western blotting with CONT6 and HDAC6 antibodies. G Desthiobiotin-labeled LINC00461 was captured by streptavidin magnetic beads. PCR (upper panel) and qRT-PCR (lower panel) detected the amount of LINC00461 binding to beads. H Proteins associated with LINC00461 in TMZ-resistant Pt#3 cells were identified by iTRAQ-based proteomic analysis. Interaction between LINC00461-binding proteins and HDAC6 was further evaluated by IPA analysis. I Effect of MPT0B291 on the protein expression of RBPs (CNOT6 and FUS) at 48 h in parental and TMZ-resistant Pt#3 cells. J Effect of HDAC6 knockdown by siRNA on the protein expression of RBPs (CNOT6 and FUS) in parental and TMZ-resistant Pt#3 cells. The results are shown as mean ± SEM for triplicate samples in each group. K The amount of LINC00461 was measured in TMZ-resistant Pt#3 cells treated with either si-FUS to silence FUS expression (green lines) or si-NC as a control (gray lines) following actinomycin D (ActD) treatment at 0, 20, 40, 60, 120, and 240 min. FUS protein levels were detected by western blotting. Two-way ANOVA. The red dashed line indicates the time at which 50% of lncRNA remained.
Fig. 3 (See legend on previous page.)
88 genes)” and “survival (49 of 88 genes)” (Fig. 5C). Furthermore, we examined the activity of several pathways using IPA, and found that either LINC00461 depletion or MPT0B291 treatment significantly inhibited cell proliferation/survival and induced cell death/senescence in GBM (Fig. 5D). These data indicated that LINC00461 is involved in HDAC6-regulated GBM malignancy. To further confirm the clinical relevance of the 50 cell-division-related genes appearing in the intersection dataset (88 genes), we analyzed the TCGA-GBM next-generation sequencing (NGS) datasets of 124 patients. A strong negative correlation was observed (PCC: −0.452) (Fig. 5E).

The result indicated that abnormalities in the expression of the 50 cell-division-related genes are inverted following the HDAC6/LINC00461 inhibition in GBM. Moreover, survival analysis using clinical outcome data showed that several cell-division-related genes (CENPF, DLGAP5, GTSE1, HMMR, KIF14, MCM10, MELK, RACGAPI, TMPO, and TOP2A) have poor prognostic implications in high-grade gliomas (Fig. 5F and Fig. S4A), suggesting that the reducing expressions of these genes through HDAC6/LINC00461 inhibition may improve outcomes in GBM patients. Additionally, the importance of the HDAC6/LINC00461-mediated regulation in clinical GBM was analyzed using single-cell transcriptome sequencing datasets. Surprisingly, higher expressions of LINC00461 and its associated downstream cell-division-related genes mentioned in Fig. 5E were enriched in a specific cluster (Cluster 5) of GBM cells (Fig. 5G–H). Consistently, functional analysis of the conserved markers in Cluster 5 by IPA indicated highly similar functional gene classes as that of the LINC00461-silenced and MPT0B291-treated datasets mentioned in Fig. 5C (Fig. S5A, Table S4).

LINC00461 depletion downregulates cell-cycle-related protein MELK by blocking the interaction between LINC00461 and miR-485-3p

We further investigated downstream targets of LINC00461, especially in cell-cycle-related proteins. Either MPT0B291 treatment or LINC00461 depletion significantly downregulated the expression of several cell-division-regulated factors, including Ki67, maternal embryonic leucine zipper kinase (MELK), DLG-associated protein 5 (DLGAP5), hyaluronan mediated motility receptor (HMMR), minichromosomal maintenance protein 10 (MCM10), and thymopoietin (TMPO) (Fig. 6A–B). Given that LINC00461 is dominantly located in the cytoplasm [13], we explored its function as a miRNA sponge to repress the inhibitory effect of miRNAs on target mRNA. Prediction of the lncRNA-miRNA-mRNA networks (Fig. S6A) using three public databases (miRDB [24], miRWalk [25], and starBase [26]) revealed 13 intersecting miRNAs in the datasets (Fig. S6B). Of these, miR-485-3p, a tumor suppressor in GBM [27] was presumed to inhibit MCM10 and MELK. Thus, we examined whether LINC00461 indeed played a role in sponging miR-485-3p. MPT0B291 treatment-mediated LINC00461 inhibition led to significantly elevated miR-485-3p expression (Fig. 6C) but it significantly reduced MELK expression in parental and TMZ-resistant GBM cells (Fig. 6D). However, the simultaneous overexpression of LINC00461 in cells decreased the induction of miR-485-3p levels (Fig. 6C) and reduction of MELK expression by MPT0B291 (Fig. 6D), suggesting that LINC00461 maintains MELK expression by sponging miR-485-3p.

Discussion

Although lncRNAs were considered non-functional and transcriptional noise initially, cumulative evidence over the past decade has unraveled that lncRNAs perform diverse biological functions [28]. Strategies for targeting lncRNAs in cancer treatment have gained widespread interest because they have vital roles in gene regulation and thus modulate hallmark processes of cancer [29]. Our previous studies demonstrated that HDAC inhibition using inhibitors represses stem-like properties in GBM that provide a potential therapeutic strategy to overcome drug resistance [10, 30]. Nevertheless, those two studies focused on HDAC-regulated protein-coding genes associated with drug resistance, but the role of HDACs on the impact of dysregulated lncRNAs remains
Fig. 4 (See legend on previous page.)
unclear and hence needs further investigation. Here, we investigated the involvement of HDAC6 in LINC00461 regulation by performing microarrays and RNA-seq analysis in stem-like and TMZ-resistant GBM cells with HDAC6 inhibition using MPT0B291 and siRNA.

According to a transition in investigative approaches from lncRNA annotation and molecular characterization to tumorigenic mouse models needed for deciphering the physiological roles of cancer-associated lncRNAs [29], we examined the impact of LINC00461 on GBM growth and survival in vivo. Evidence gathered shows that MPT0B291 treatment significantly reduced subcutaneous tumor growth along with a decreased level of LINC00461 and a negative correlation between LINC00461 expression and survival of mice with intracerebral GBM, suggests a pivotal role for LINC00461 as an oncogenic factor in GBM malignancy. Indeed, a previous study has demonstrated that the expression of C130071C03Rik, the mouse ortholog of LINC00461, is increased not only in precancersous conditions but also during glioma progression [13]. Some characteristics suggest that LINC00461 is a potentially promising target for treating patients with GBM. First, previous literature on LINC00461 demonstrated its oncogenic functions across diverse cancer types [13, 14, 31, 32]. Second, LINC00461 displays a brain-specific expression pattern, which implies reduced unintended toxic effects associated with their targeting. Third, unlike most intergenic lncRNAs that show poor sequence conservation, LINC00461 has a high degree of sequence homology across vertebrates [13], suggesting its essential role among such species [33].

The mechanisms underlying the regulation of mRNA stability have been extensively reviewed [34], whereas little is known about the modulation of lncRNA stability. Considering the inhibitory potency of MPT0B291 on HDAC1 and 2 (IC$_{50}$ ≈ 0.31 and 1.16 μM, respectively) [10, 11], the impact of HDAC1 and HDAC2 on LINC00461 expression was also examined in GBM cells using siRNA-mediated depletion. Interestingly, the silencing of neither HDAC1 nor HDAC2 was involved in modulating LINC00461 expression (Fig. S7A), suggesting that a decrease in LINC00461 expression by MPT0B291 is mainly due to the inhibition of HDAC6 rather than that of HDAC1 or HDAC2. Although several aspects discriminate lncRNAs from protein-coding mRNA, they also share certain similarities [35]. Numerous lncRNAs are subjected to transcriptional editing, for example, splicing, polyadenylation, and 5′ capping, just like mRNAs [35]. Post-transcriptional regulation of lncRNAs is mediated by RBPs in mainly two aspects: stability and localization. Our results identified two HDAC6-associated proteins (CNOT6 and FUS) that might explain a shorter half-life of LINC00461 in response to MPT0B291 treatment (Fig. S7B). Because the enzyme activity of CNOT6 is required for deadenylation by the CCR4-NOT nuclease complex [36], and acetylation enhances CNOT6 activity inducing RNA degradation [22], we predict that high levels of HDAC6 in GBM cells inhibit CNOT6 acetylation, leading to LINC00461 stabilization, while MPT0B291 treatment induces CNOT6 acetylation, promoting LINC00461 degradation (Fig. S7B). FUS-containing 3′-end processing machinery facilitates mRNA stability [23], and lysine status regulated by cysolic adenosine monophosphate–response element binding protein/p300 and histone deacetylase families contribute to FUS function [37]. Therefore, in GBM cells, high levels of HDAC6 may increase the RNA-binding affinity of FUS, resulting in LINC00461 stabilization, whereas MPT0B291 treatment induces FUS acetylation, promoting LINC00461 degradation (Fig. S7B). Although our current results prove that HDAC6 inhibition increases CNOT6 and decreases FUS protein levels, further studies are needed to confirm whether HDAC6 directly regulates the lysine status of these two proteins.

Although recent studies have shown the oncogenic roles of LINC00461 in tumors [13, 14, 31, 32],...
Fig. 5 (See legend on previous page.)
the molecular mechanisms underlying \textit{LINC00461} regulation remain unclear. To understand the function of \textit{LINC00461} comprehensively, we compared the \textit{LINC00461}-silenced dataset with the clinical TCGA-GBM dataset. Pathway enrichment analysis of intersected genes in these two datasets revealed cell growth, which is consistent with the findings that \textit{LINC00461} promoted GBM proliferation through the regulation of TOP2A expression. TOP2A is a nuclear enzyme that controls and alters the topologic states of DNA during transcription and mitosis, facilitating gene expression and mitotic progression, respectively, in tumor cells [38]. Here, we found that the HDAC6/\textit{LINC00461} axis inhibition downregulated TOP2A expression in parental and TMZ-resistant cells, thereby explaining the perturbation of cell-cycle progression into G2/M phase. In addition, a previous study has demonstrated that \textit{LINC00461} knockdown significantly downregulated expressions of a few cell-division-related genes, including cyclin D1, CDK4, cyclin A2, and cyclin E in U87MG [39]. Although genome-wide analysis with critical criteria for selecting potential \textit{LINC00461} downstream targets from altered genes in the \textit{LINC00461}-silencing dataset only identified cyclin A [39] and TOP2A [13] as presented previously, we measured the expressions of the other cell-division-related genes mentioned above in our GBM cell model. Significant decreases in the expression levels of these cell-division-related genes (cyclin D1, CDK4, cyclin A2, and cyclin E1) following \textit{LINC00461} knockdown were observed in parental and TMZ-resistant U87MG (Fig. S8A). Furthermore, \textit{LINC00461} knockdown significantly downregulated protein expressions of cyclin D1 and CDK1 (Fig. S8B), which is consistent with the findings of a previous report [39]. Except for the \textit{LINC00461}-regulated cell-cycle-related genes, which have already been proved, numerous cell-division-related genes that are modulated by \textit{LINC00461} were revealed in this study for the first time (Figs. 5E and 6). In summary, \textit{LINC00461} displays a widespread regulation of cell-division-related proteins that are indispensable for GBM growth.

Apart from exploring the function of \textit{LINC00461} using the public clinical dataset, we also performed transcriptome profiling of clinical samples at the single-cell level. Recent advances in the development of NGS technologies have overcome conventional profiling methods that assess bulk populations and provide the opportunity to gain insights into the characterization of individual cells [40]. Considering the heterogeneity of tumor biology, we explored whether there is a cluster of cells overexpressing \textit{LINC00461}. Surprisingly, our findings revealed that the cells in Cluster 5 showed highly elevated expressions of \textit{LINC00461} and cell-division-related genes simultaneously and also displayed biomarkers enriched in pathways associated with cell division (Fig. S5A, Table S1). Therefore, the cell characteristics in Cluster 5 are required further study to clarify the clinical malignancy of GBM and the development of treatment strategies.

Like proteins, appropriate subcellular localization patterns of IncRNAs allow for the primary determination of their molecular functions [41]. Our observations in xenograft tumors through both RNA-ISH and RNA-FISH staining demonstrated that \textit{LINC00461} localizes to the cytoplasm rather than to the nucleus. Cytoplasmic IncRNAs modulate mRNA fate by acting as miRNA sponges that compete for miRNA binding [42]. To dissect the regulatory mechanism between \textit{LINC00461} and cell-division-related proteins comprehensively, we used three reliable public datasets to predict IncRNA-miRNA-mRNA networks. Downregulation of MCM10 and MELK proteins by \textit{LINC00461} depletion combined with the induction of miR-485-3p by \textit{LINC00461} inhibition using MPT0B291 suggests that this strategy is effective for identifying the downstream regulatory networks of \textit{LINC00461}.

Conclusions

Recent research has unraveled the indispensable roles of IncRNAs in regulating GBM malignancy, including cancer stem-like features [8]. Nevertheless, the mechanism by which cancer-associated IncRNAs are dysregulated remains unexplored. Here, our results provide an insight into IncRNA regulation by HDAC proteins and lead to a comprehensive understanding of \textit{LINC00461}-associated networks in GBM malignancy, by expanding the utility of the HDAC6/RBPs/\textit{LINC00461} axis as a potential therapeutic approach for treating patients with
**Fig. 6** (See legend on previous page.)

**LINCO0461 stabilization**

**GBM cell**

- HDAC6 deacetylation
- LINCO0461 stabilization

- FUS-

- AAAA (A) tail

- RISC-LINCO0461

- sponge

- miR-485-3p (tumor suppressor)

- Increase expressions of cell division-related proteins

- Promote cell proliferation

**LINCO0461 destabilization**

**GBM cell**

- MPT08291

- CCR4-NOT

- HDAC6 deacetylation

- LINCO0461 destabilization

- RISC-miR-485-3p

- MELK (cell division-related gene)

- Decrease expressions of cell division-related proteins

- Inhibit cell proliferation
GBM (Fig. 6E). However, this research has thrown up many questions that need further investigation. Future research should focus on the identification of the lysine residue of RBPs that is deacetylated by HDAC6, isolating the LINC00461-miRNA-mRNA axis that is the most important compared to the others, and understanding the mechanism of targeting LINC00461-overexpressed cells effectively in clinical practice.

Abbreviations
ActD: Actinomycin D; AP: Alkaline phosphatase; AR: Androgen receptor; CNOT6: CCR4-NOT core-exonuborneuclease subunit 6; CI: Confidence interval; DIG: Digoxigenin, DLGAPS: DLG associated protein 5; ECL: Enhanced chemiluminescence; FISH: Fluorescence in situ hybridization; FUS: Fused in sarcoma; GBM: Glioblastoma; GEO: Gene expression omnibus; HDAC: Histone deacetylase; HMVR: Hyaluronan mediated motility receptor; IPA: Ingenuity pathway analysis; ISH: In situ hybridization; ITGB3: Integrin β3; IncRNAs: Long non-coding RNAs; iTRAQ: Isobaric tags for relative and absolute quantitation; M241D: Minichromosomal maintenance protein 10; MELK: Maternal embryonic leucine zipper kinase; MPT0B291: Azadiradiolusfonamide compound 12; MTI: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NGS: Next-generation sequencing; Pearson’s correlation coefficient; RBPs: RNA-binding proteins; scRNA-seq: Single-cell RNA sequencing; SNR: Signal-to-noise ratio; TCGA: The cancer genome atlas; TMPO: Thyromperin; TMZ: Temocozolomide; TOPO2A: Topoisomerase II alpha; TRITC: Tetramethylrhodamine-isothiocyanate; TSA: Trichostatin A.

Supplementary Information
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Additional file 1: Figure S1. MPT0B291 commonly alters two IncRNAs (LINC00461 and LINC01359) in stem-like and TMZ-resistant U87MG cells. Volcano plots show overlapping IncRNAs between two microarray data (MS1040791 and MS1060987). Relative expression level (log2) to control (DSMO) of each IncRNA is shown in the x-axis, y-axis indicates SNR (signal-to-noise ratio) value (log2) of each IncRNA. Genes with significant upregulation (red) and downregulation (green) in both microarray datasets are highlighted. Figure S2. MPT0B291 decreases Ki-67 expression in vivo. (A) IHC detected the protein expressions of proliferation (Ki-67) and apoptosis (cleaved caspase-3) marker. Serial sections of control and MPT0B291-treated xenografts were hybridized with either an antibody against Ki-67 (upper two panels) or cleaved caspases-3 (lower two panels), and cell nuclei were stained with hematoxylin. Scale bars indicate 20 μm and 200 μm. Mean intensity of Ki-67 and cleaved caspases-3 staining in the cells of control and MPT0B291-treated xenografts were semi-quantitative determined. Unpaired Student’s t-test. Figure S3. Interaction between HDAC6 and RNA-binding proteins (CNOT6 and FUS). (A) Flag-tagged CNOT6-expressed U87MG cells were treated with 10 μM MPT0B291, 10 μM Trichostatin A (TSA), or DMSO for 2 h. Flag-CNOT6 protein was immunoprecipitated and the precipitated samples were then analyzed by immunoblotting analysis with antibodies of HDAC6 or Flag-M2. (B) Enrichment of H4R from the lysate by deoxthiobiotin-labeled androgen receptor (AR) 3′-UTR validates the efficiency of RNA-protein pull-down assay (left panel). Protein lysates from TMZ-resistant Pt#3 cells were also used for the RNA-protein pull-down assay with deoxthiobiotin-labeled LINC00461, and both poly(A)-binding protein (PABP) and H4R were slightly detected in the pull-down sample, but deoxthiobiotin-labeled LINC00461 did not bind HDAC6 directly. (L = lysate; FT = flow-through in first wash; FF = flow-through in second wash; E = eluate). (C) Quantification of CNOT6 and FUS protein expression from the parental and TMZ-resistant Pt#3 cells either treated with MPT0B291 or si-HDAC6. Unpaired Student’s t-test. The results are shown as mean ± SEM for triplicate samples in each group. Figure S4. Cell division-related genes show poor prognostic implications in high-grade glioma patients. (A) Kaplan–Meier curves compare the survival outcomes in high-grade glioma patients with high (red) and low (green) expressions of cell division-related genes. All survival curves were obtained from PROGeneV2. Log-rank test. Figure S5. Functional analysis of the conserved markers in cluster 5 from scRNA-seq data reveals gene classes associated with cell division and survival. Core analysis using IPA revealed the top ten molecular and cellular functions of highly conserved marker genes in cluster 5 of patient-derived GBM cells. Log(#value) indicates the significance of enrichment for highly expressed marker genes from our dataset. The threshold for significance was set at a p-value < 0.05. Figure S6. Three datasets identify potential interaction networks between 13 miRNAs and cell division-related genes. (A) Pipeline for the identification of potential regulatory LINC00461-miRNA-miRNA networks. (B) The miRNA-miRNA networks were identified using three public databases (miDB, miRWalk, and starBase). Figure S7. The regulation of LINC00461 stability. Protein expression levels of HDAC1 and HDAC2 in parental and TMZ-resistant U87MG cells treated with either si-NC or si-HDAC1/2. Effect of either HDAC1 or HDAC2 depletion on LINC00461 expression in parental and TMZ-resistant U87MG cells. n.s., not significant, unpaired Student’s t-test. (B) A schematic diagram illustrates the proposed regulatory mechanism underlying HDAC6 controls the LINC00461 stability via regulating both the RNA-binding activity of FUS (fused in sarcoma)/PABP and the activity of deadenylases of human Ccr4-Not complex. Figure S8. LINC00461 knockdown downregulates the expressions of cell division-related molecules. (A) Expressions of the cell division-related miRNAs (cyclin D1, CDK4, cyclin A2, cyclin E1, MELK, and MCM10) in parental and TMZ-resistant R-U87MG cells that were treated with either si-NC or si-LINC00461. Unpaired Student’s t-test. (B) Effect of LINC00461 depletion on the protein expression levels of cell division-related molecules (cyclin D1 and CDK1) in parental and TMZ-resistant U87MG cells. Table S1. siRNA targeted-sequences for each gene. Table S2. The primer or probe sequences for each gene used in SYBR green and TaqMan qPCR assays. Table S3. Custom LNA™ detection probes sequences. Table S4. List of biomarkers with fold-change more significant than 1.5 in cluster 5.

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Authors’ contributions
Investigation and validation: A.C.W, W.Y.B, J.P.L, R.Y.S, and T.J.H. Clinical speciﬁc collection: K.Y.C, J.S.L, and P.Y.C. Data curation: A.C.W, K.Y.C, S.M.C, D.Y.H, C.Y.W, P.Y.C, and J.Y.C. Supervision: U.K, W.C.C, P.Y.C, and J.Y.C. Funding acquisition: P.Y.C. and J.Y.C. Writing draft: A.C.W and J.Y.C. All authors have revised and approved the final version of the manuscript.

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Availability of data and materials
All data supporting the findings of this study are available within the article and supplementary data. The RNA-seq data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE182220 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE182220).

Declarations
Ethics approval and consent to participate
Experiments were conducted under a protocol approved by the Joint Institutional Review Board of the Taipei Medical University, Taiwan with the registration number (Nos. 2010006011 and 201402018) and by the Institutional Animal Care and Use Committee of the National Health Research Institute, Taiwan with the registration number (NHRI-IACUC-106010).

Consent for publication
Not applicable.
Competing interests
The authors declare that there are no potential conflicts of interest.

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