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Supplemental information

Dual targeting of EZH1 and EZH2

for the treatment of malignant rhabdoid tumors

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Figure S1

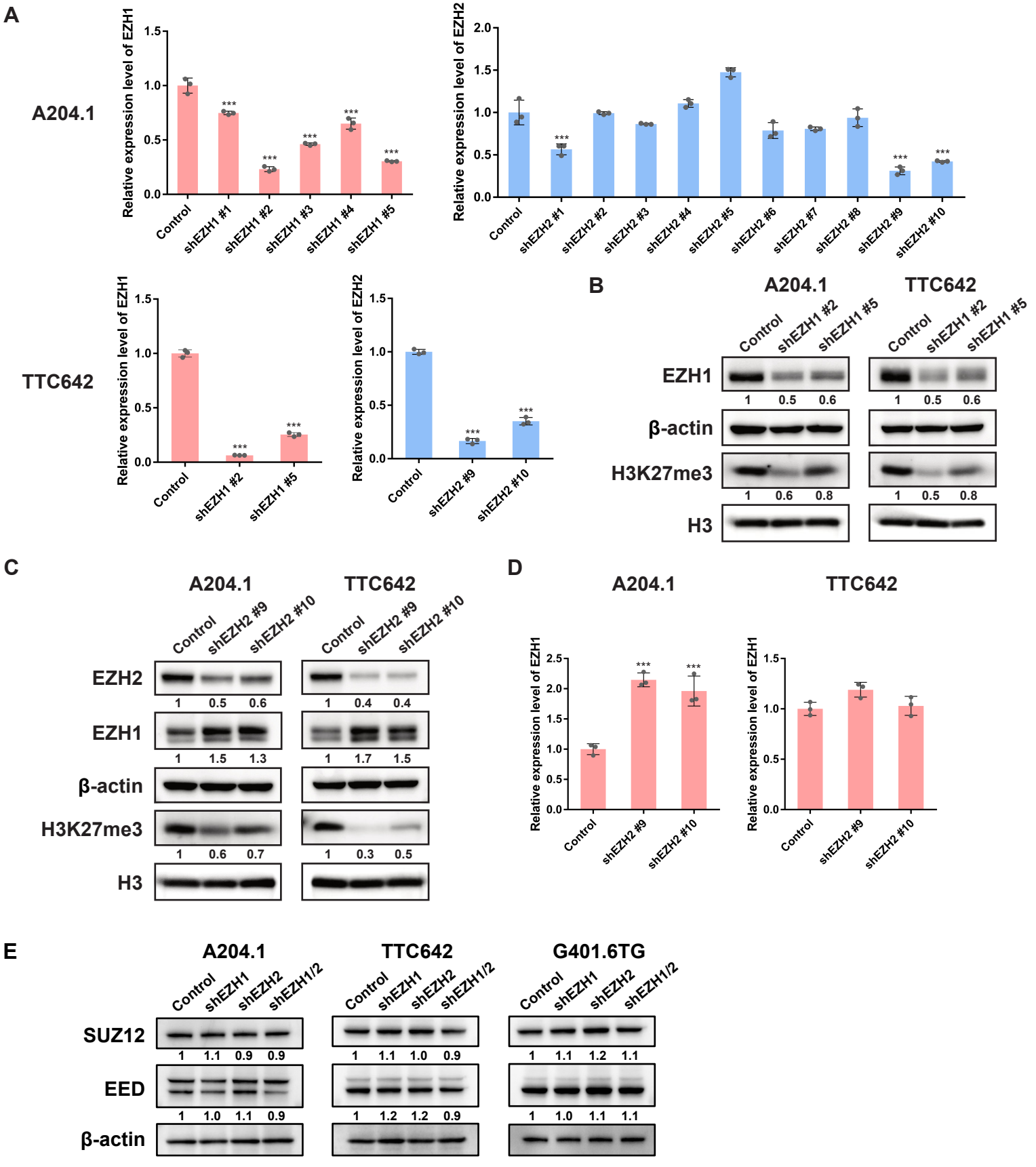
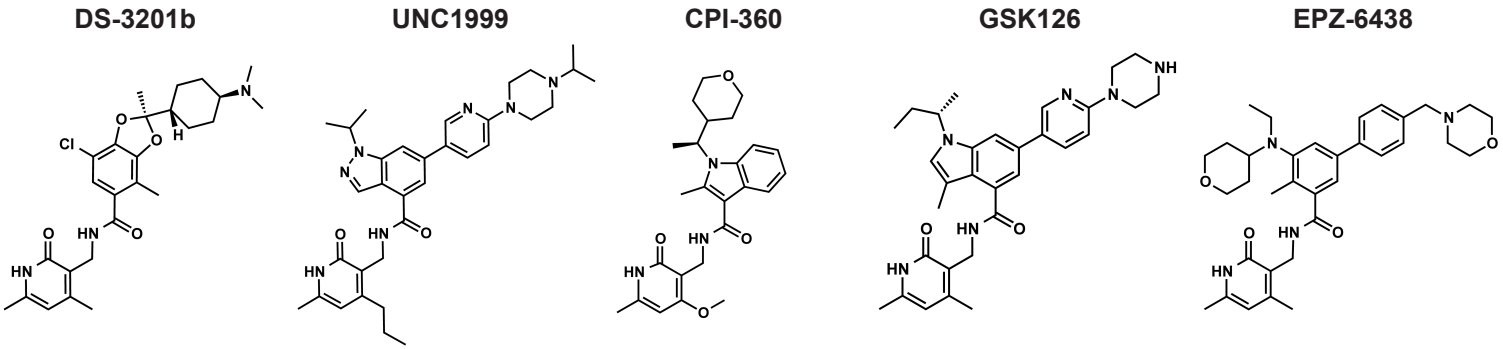


Figure S1. Effect of EZH1 and EZH2 deficiency on H3K27 methylation and EZH1 expression.

(A) Expression of *EZH1* and *EZH2* mRNAs. Cells were infected with retroviral sh*EZH1* or sh*EZH2* vectors. Next, transduced cells were selected by culturing for 3 days with 1 μg/ml of puromycin. To confirm knockdown (KD) efficacy and determine short hairpin RNA (shRNA) sequences, the relative expression level of each mRNA was evaluated by qRT-PCR (n = 3, mean ± SD). The expression level of the Control is indicated as "1". ***p < 0.001 *vs.* Control. **(B and C)** Expression of the EZH1, EZH2, and H3K27me3 proteins in *EZH1* **(B)** or *EZH2* **(C)** knockdown (KD) cells. The expression level of each protein was determined by western blotting analysis. β-actin and H3 were used as internal controls. The numbers below EZH1, EZH2, and H3K27me3 indicate the band density relative to Control (taken as "1"). The sequences of sh*EZH1* #2 and sh*EZH2* #9 were picked up for further experiments in Figure 1. **(D)** Expression of *EZH1* mRNA in *EZH2* KD cells (n = 3, means ± SD). The expression level of the Control is indicated as "1". ***p < 0.001 *vs.* Control. **(E)** Expression of SUZ12 and EED proteins in cells single or double KD of EZH1 and EZH2. The expression level of each protein was determined by Western blotting analysis. β-actin was used as the internal controls. The numbers below SUZ12 and EED indicate each band density relative to Control (taken as "1").

Figure S2

A



B

target	DS-3201b	UNC1999	CPI-360	GSK126	EPZ-6438
EZH1	8.4	45	102.3	680	392
EZH2	2.5	< 10	0.50	9.9	11

C

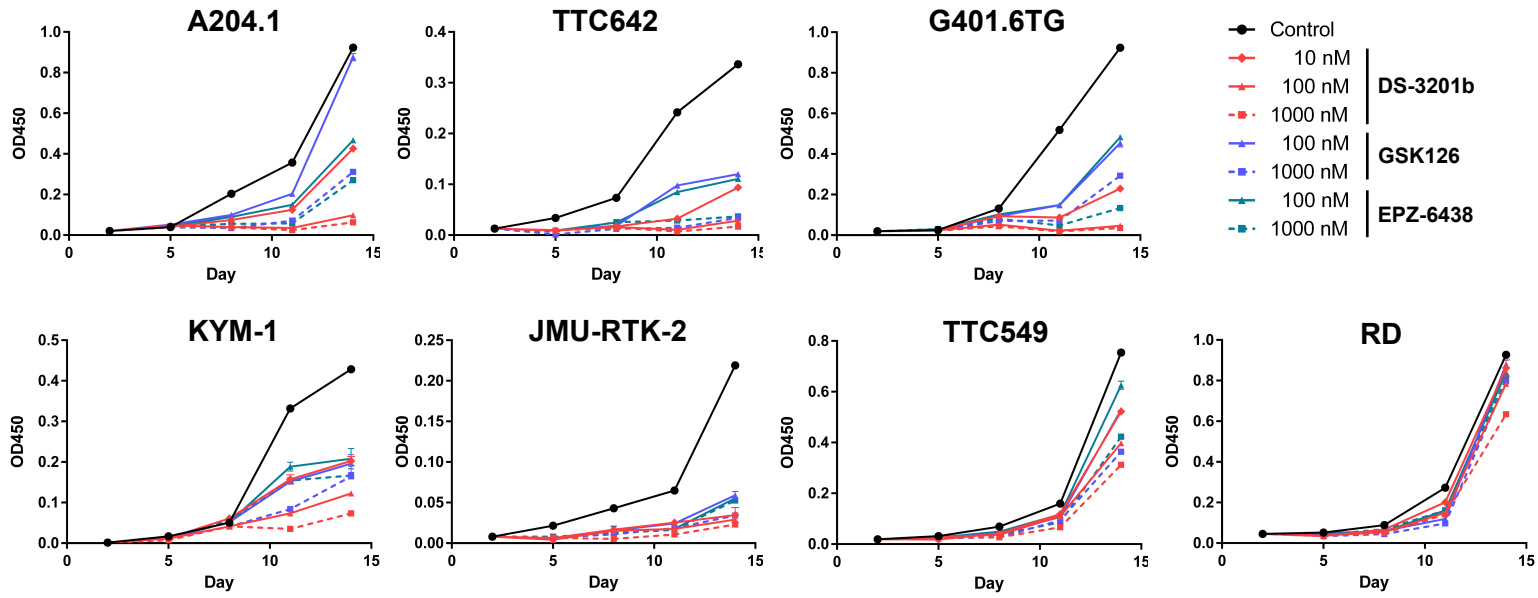


Figure S2. Characteristics of EZH inhibitors.

(A) Chemical structures of EZH inhibitors. **(B)** Officially released *in vitro* IC_{50} values of EZH inhibitors against EZH1 and EZH2. **(C)** The effect of an EZH1/2 dual inhibitor and EZH2-selective inhibitors on the cell growth. 6 MRTs (A204.1, TTC642, G401.6TG, KYM-1, JMU-RTK-2, and TTC549) and *SMARCB1*-wild type RMS (RD) cells were treated with different concentrations of DS-3201b, GSK126, or EPZ-6438 for the indicated durations, and relative cell growth was evaluated by a WST-8 assay (n = 3, means ± SD). Differences were statistically evaluated by two-way ANOVA followed by Tukey's multiple comparisons test. Statistical significance between control and each drug treatment (DS-3201b, GSK126, or EPZ-6438) is $p < 0.001$ in all cell lines. Statistical significance between DS-3201b and each EZH2 inhibitor (GSK126 or EPZ-6438) at the same concentration was observed on day 14 in all cell lines.

Figure S3

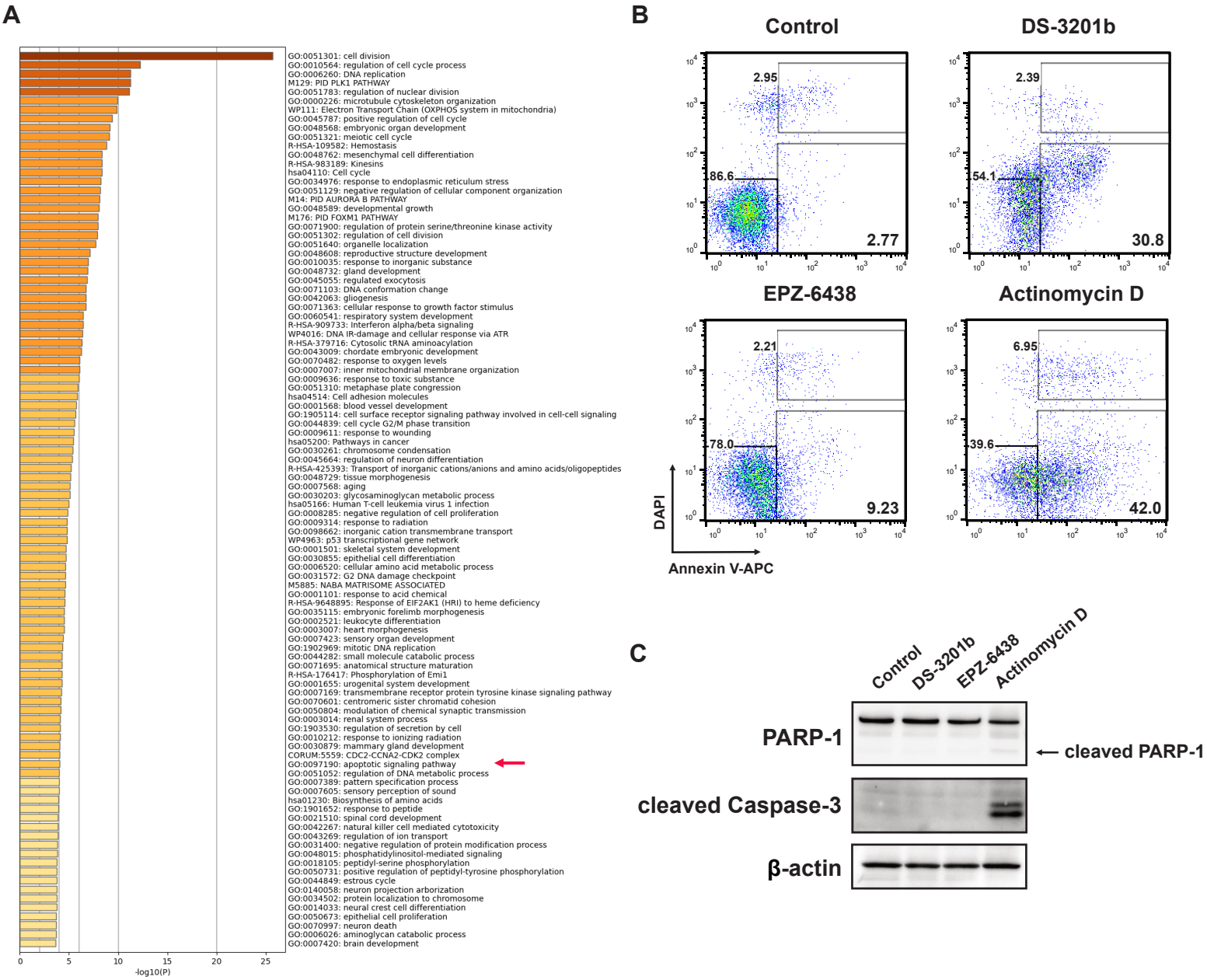


Figure S3. Apoptosis analysis of EZH inhibitor-treated cells.

(A) Enrichment analysis of differentially expressed genes (FDR-adjusted $p < 0.05$; fold change > 2) using Metascape. A204.1 cells were treated with DS-3201b (100 nM) for 14 days, followed by RNA-sequencing (RNA-seq) analysis. Bar graph colored by *p*-value shows the top 100 enriched terms altered by DS-3201b treatment. The term “apoptotic signaling pathway” (GO: 0097190) is indicated by a red arrow.

(B) Representative images of apoptosis analysis. A204.1 cells were treated with DS-3201b (100 nM) or EPZ-6438 (100 nM) for 14 days, followed by apoptosis analysis using Annexin V-APC and DAPI. Actinomycin D (1 mg/ml for 2 days) was used as a positive control. Cells in the early stage of apoptosis are presented in the lower right quadrants (Annexin V-APC-positive and DAPI-negative). The numbers in the dot plots indicate the percentage of cells in the respective quadrants. **(C)** Assessment of PARP-1 and caspase-3 cleavage in DS-3201b-, EPZ-6438-, and actinomycin D-treated cells. A204.1 cells were exposed to DS-3201b (100 nM) or EPZ-6438 (100 nM) for 14 days, or to actinomycin D (1 mg/ml) for 2 days, followed by western blotting.

Figure S4

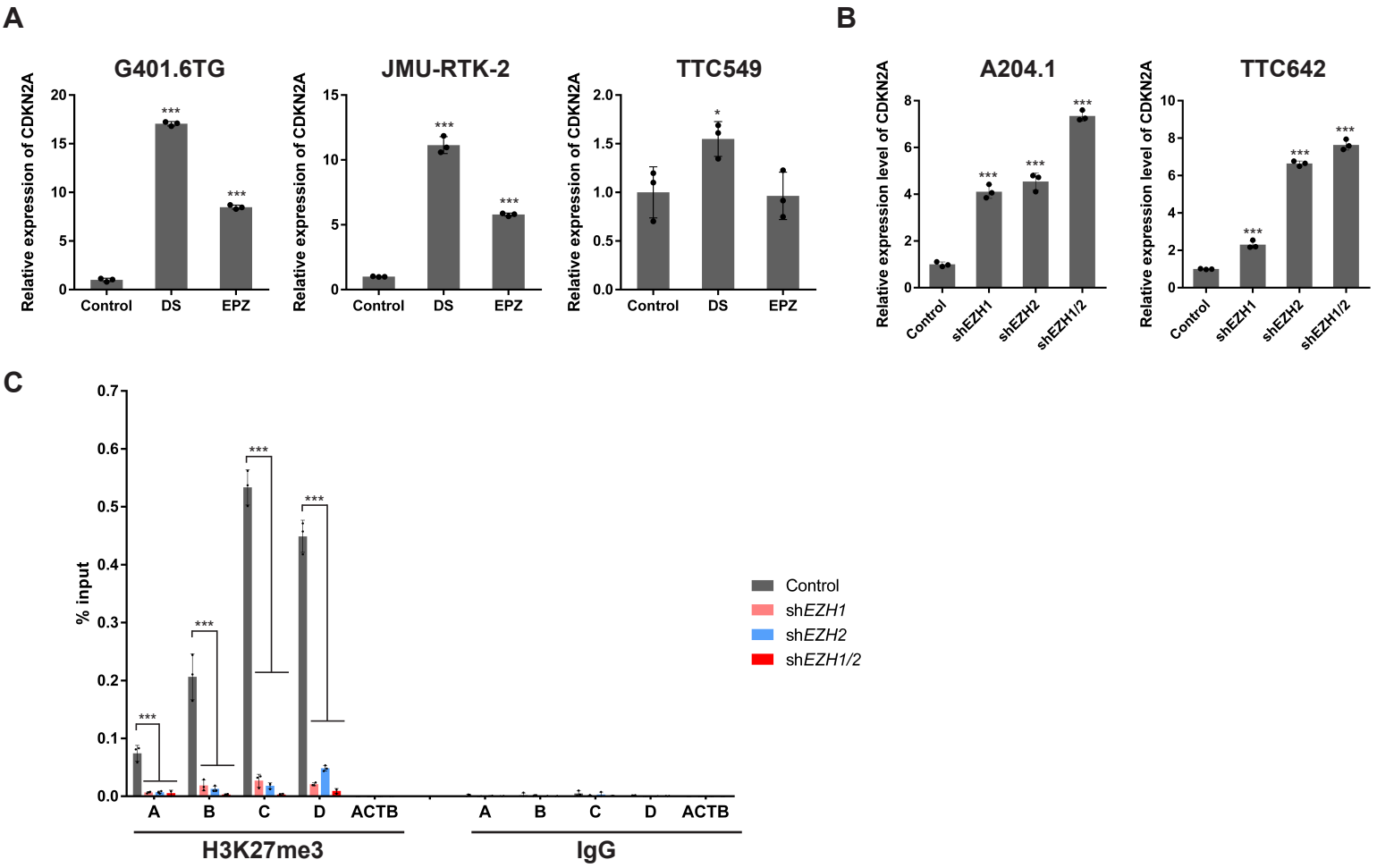


Figure S4. Upregulation of CDKN2A after EZH1/2 inhibition and depletion.

(A) Expression of *CDKN2A* mRNA in DS-3201b- or EPZ-6438-treated cells. Cells were treated with DS-3201b (DS; 100 nM) or EPZ-6438 (EPZ; 100 nM) for 7 days, followed by qRT-PCR (n = 3, means ± SD). The expression level of the Control is indicated as "1". *p < 0.05, **p < 0.01, ***p < 0.001 *vs.* Control. **(B)** Expression of *CDKN2A* mRNA in cells in which *EZH1* or *EZH2*, or both, were knocked down (n = 3; mean ± SD). The expression level of the Control is indicated as "1". ***p < 0.001 *vs.* Control. **(C)** H3K27me3 enrichment at the *CDKN2A* locus in cells in which *EZH1* or *EZH2*, or both, were knocked down. ChIP-qPCR analysis was performed using the primer pairs described in Figure 5E (n = 3; mean ± SD). The statistical significance of the differences between the Control and each KD condition (sh*EZH1*, sh*EZH2*, and sh*EZH1/2*) were evaluated by one-way ANOVA followed by Dunnett's multiple comparisons test. ***p < 0.001 *vs.* Control.

Figure S5

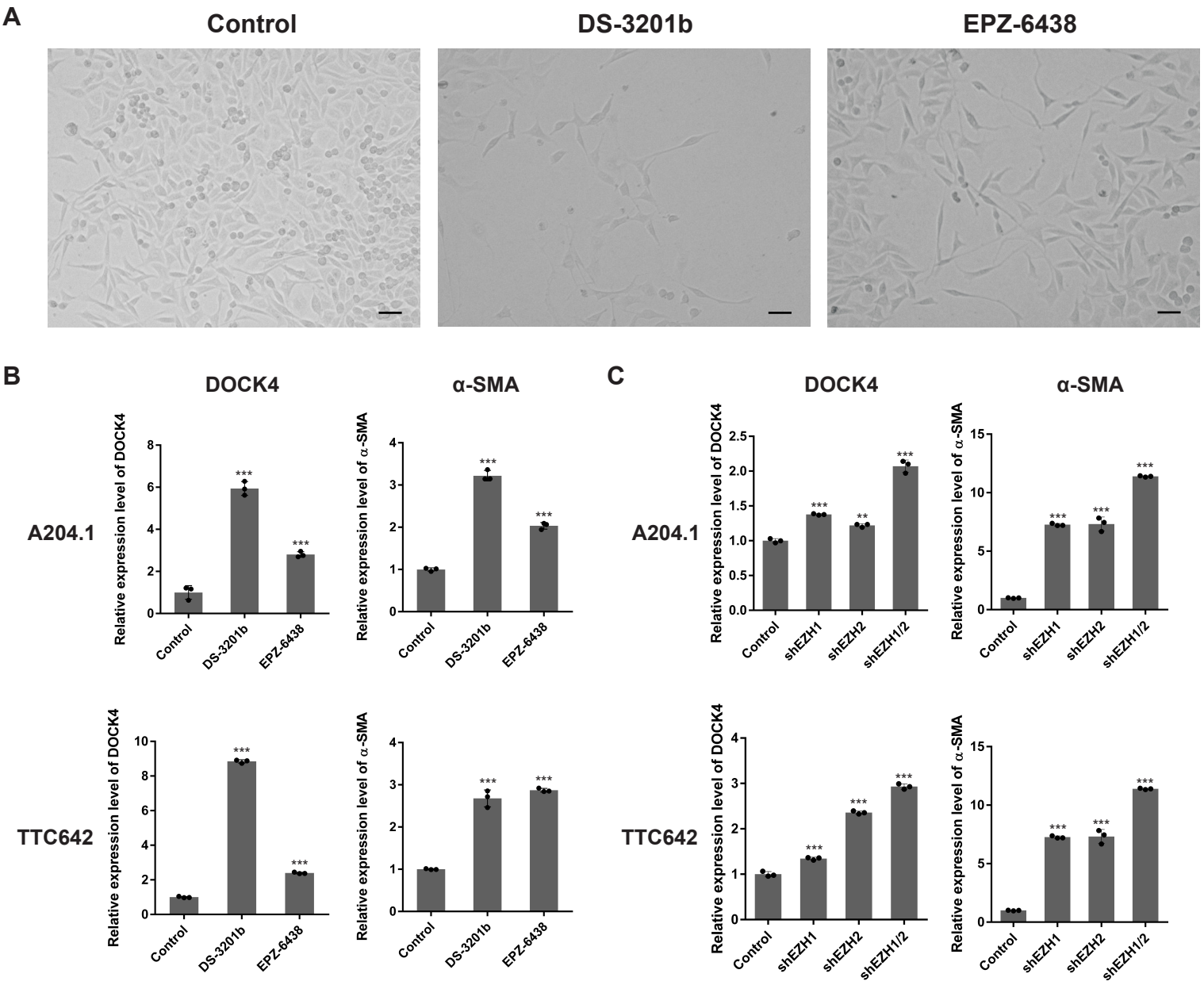


Figure S5. Morphological changes in EZH1/2 dual inhibitor-treated MRT cells.

(A) Representative images showing the morphology of DS-3201b- or EPZ-6438-treated A204.1 cells. Cells were treated with DS-3201b (100 nM) or EPZ-6438 (100 nM) for 7 days, followed by observation under a bright-field microscope. The number of long, thin, spindle-shaped cells increased after treatment with DS-3201b. Scale bars, 50 μm. **(B)** Expression of *DOCK4* and *α-SMA* mRNAs in DS-3201b- or EPZ-6438-treated cells. Cells were treated with DS-3201b (100 nM) or EPZ-6438 (100 nM) for 7 days, followed by qRT-PCR (n = 3, means ± SD). The expression level of the Control is indicated as "1". ***p < 0.001 *vs*. Control. **(C)** Expression of *DOCK4* and *α-SMA* mRNAs in cells in which *EZH1* or *EZH2,* or both, were knocked down (n = 3; mean ± SD). The expression level of the Control is indicated as "1". **p < 0.01, ***p < 0.001 *vs.* Control.

Figure S6

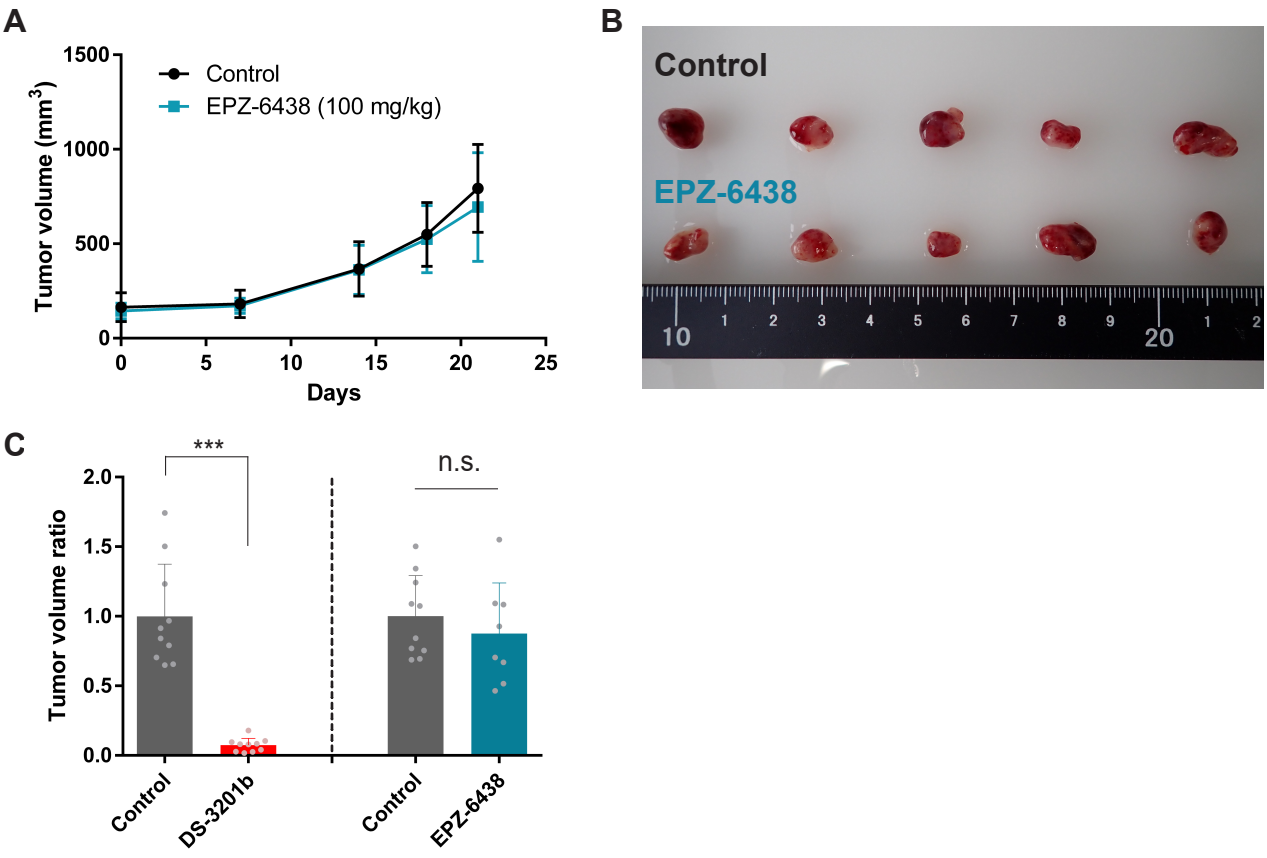


Figure S6. The anti-proliferative effect of an EZH2-selective inhibitor EPZ-6438 in A204.1 xenograft mice.
(A) The tumor volume of A204.1 xenograft mice. A204.1 cells (2×10^6 cells) were suspended in 100 µl of 50% Matrigel prepared in PBS and subcutaneously inoculated into 5-week-old female BALB/c *nu/nu* nude mice. After the mean tumor volume had reached approximately 100 mm^3, the mice were randomized and separated into two groups. The grouping day was set as day 0, and treatment was started at day 1. EPZ-6438 was suspended in 5% DMSO, 40% PEG300, 5% Tween 80, and 50% sterile purified water. The mice were orally administered once daily with 100 mg/kg EPZ-6438 or vehicle control. Differences were statistically evaluated by two-way ANOVA followed by Tukey's multiple comparisons test. The difference between EPZ-6438 and Control was not statistically significant. **(B)** Representative image of tumors from the A204.1 xenograft mice at day 21. **(C)** Tumor volume ratio at day 21. The volume of each Control is indicated as "1". Relative tumor volume of DS-3201b-treated mice is 0.018 and EPZ-6438-treated mice is 0.88. n.s.: not significant, ***$p < 0.001$ *vs.* Control.

Figure S7

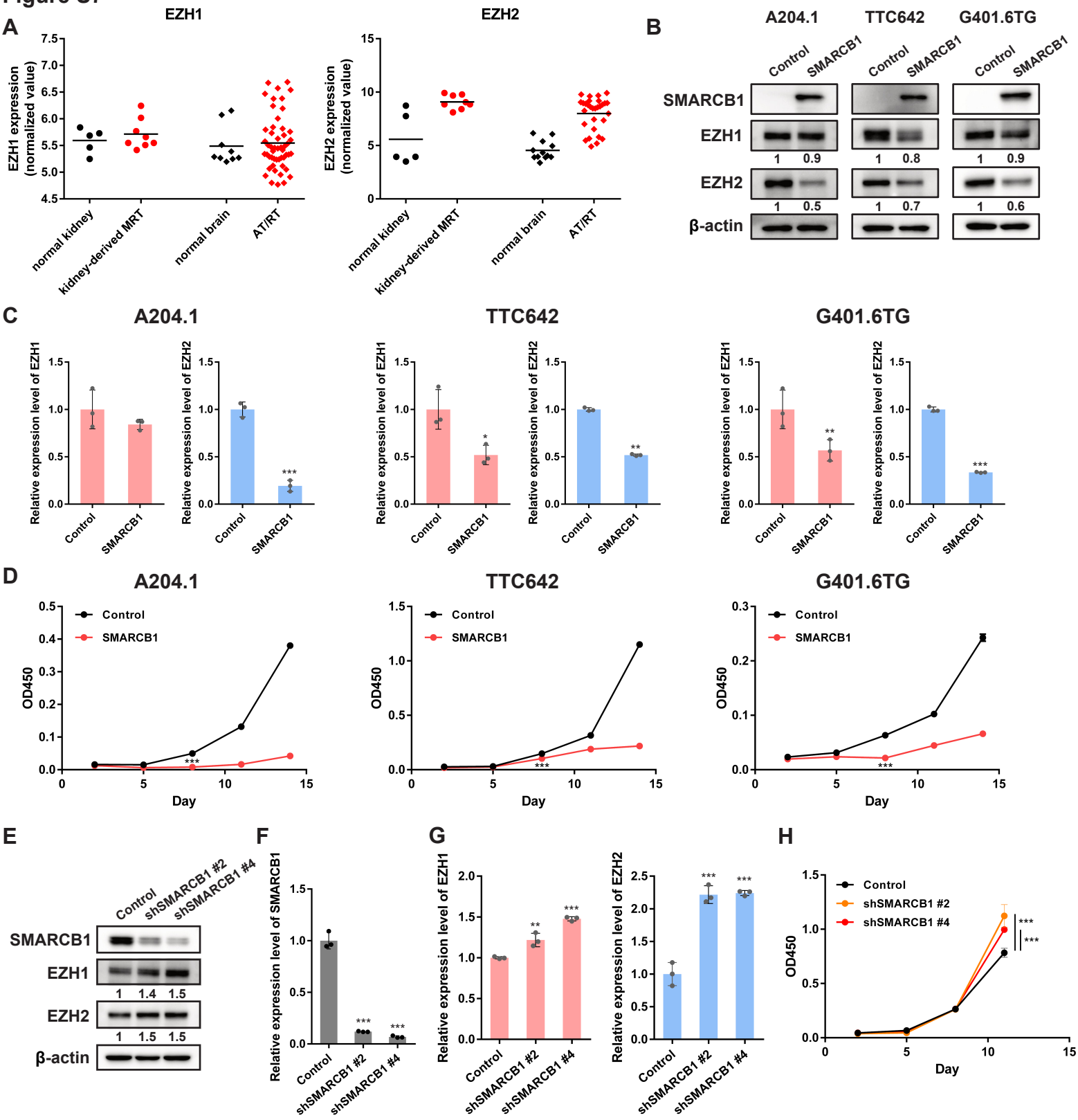


Figure S7. Regulation of EZH1/2 expression by SMARCB1.

(A) Scatter plot of EZH1 and EZH2 expression in primary MRT compared with normal tissues. Gene expression data were obtained from the Gene Expression Omnibus database repository (GEO) and normalized before analysis. Kidney, $p = 0.0425$; Central nervous system, $p = 0.0006$. **(B)** Expression of SMARCB1, EZH1, and EZH2 proteins in SMARCB1-overexpressing cells. Cells were infected with a retroviral SMARCB1 expression vector, and transduced cells were selected by culture with 5 μg/ml of blasticidin for 10 days. Cells were then subjected to western blotting. The numbers below EZH1 and EZH2 indicate each band density relative to Control (taken as "1"). **(C)** Expression of *SMARCB1* mRNA in SMARCB1-overexpressing cells. To confirm overexpression efficacy, the relative expression level of mRNA was evaluated by qRT-PCR ($n = 3$; mean ± SD). The expression level of the Control is indicated as "1". *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs.* Control. **(D)** Effect of SMARCB1 overexpression on growth of MRT cells. Relative cell growth was measured by a WST-8 assay for the indicated durations ($n = 3$; mean ± SD). Differences were evaluated statistically by two-way ANOVA followed by Tukey's multiple comparisons test. A statistically significant difference between Control cells and SMARCB1-overexpressiong cells was observed on Day 8 (for all cell lines). SMARCB1 *vs.* Control is ***$p < 0.0001$ for all cell lines. **(E–G)** Expression of SMARCB1, EZH1, and EZH2 proteins **(E)** and mRNAs **(F and G)** in *SMARCB1* KD cells. *SMARCB1* wild-type rhabdomyosarcoma RD cells were infected with retroviral sh*SMARCB1* vectors, and transduced cells were selected by culture with 1 μg/ml of puromycin for 3 days. KD efficacy was confirmed by western blotting and qRT-PCR. **(E)** The numbers below EZH1 and EZH2 indicate each band density relative to Control (taken as "1"). **(F and G)** $n = 3$; mean ± SD. The expression level of the Control is indicated as "1". **$p < 0.01$, ***$p < 0.001$ *vs.* Control. **(H)** Effect of *SMACB1* KD on MRT cell growth. Relative cell growth was measured by a WST-8 assay for the indicated durations ($n = 3$; mean ± SD). Differences were evaluated statistically by two-way ANOVA followed by Tukey's multiple comparisons test. A statistically significant difference between the Control and each KD condition (sh*SMARCB1 #2* and sh*SMARCB1 #4*) was observed on Day 11. sh*SMARCB1 #2 vs.* Control is ***$p < 0.0001$, and sh*SMARCB1 #4 vs.* Control is ***$p < 0.0001$.

Figure S8

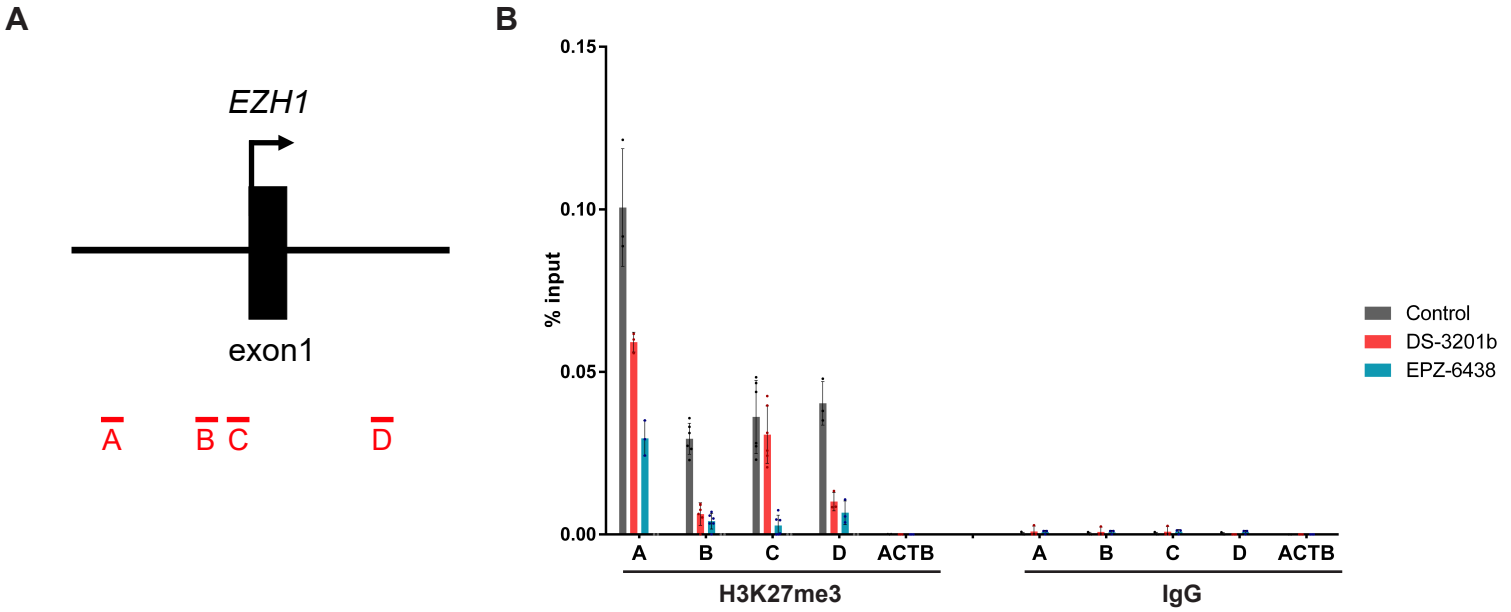


Figure S8. Regulation of EZH1 expression by EZH2.

(A) Schematic of the *EZH1* locus and the locations of the primer pairs used for ChIP-qPCR analysis. **(B)** H3K27me3 enrichment at the *EZH1* locus. A204.1 cells were treated with DS-3201b (100 nM) or EPZ-6438 (100 nM) for 7 days, followed by ChIP-qPCR analysis. Normal rabbit IgG was used as a negative control for the immunoprecipitation. The primer pair specific for ACTB was used as a negative control for the qPCR (n = 6; mean ± SD).

Supplemental Methods

Antibodies

For Western blotting, antibodies against the following proteins were purchased from Cell Signaling Technology (Danvers, MA, USA): EZH1 (#42088), EZH2 (#5246), cleaved Caspase-3 (#9661), PARP-1 (#9542), and SMARCB1 (#91735). Anti-H3K27me3 antibody (07-449) was obtained from Millipore (Billerica, MA, USA). Anti-H3 antibody (ab1791) was purchased from Abcam (Cambridge, MA). The quantity loaded was verified using anti-β-actin antibody (#3700; Cell Signaling Technology).

The following antibodies were used for immunohistochemistry: anti-H3K27me3 (#9733; Cell Signaling Technology), and anti-CDKN2A/p16INK4a (ab108349; abcam).

Vectors

Lentiviral short hairpin RNA (shRNA) vectors (EZH1 #2: TRCN0000002439, EZH1 #5: TRCN0000355735, EZH2 #9: TRCN0000286227, EZH2 #10: TRCN0000293738, SMARCB1 #2: TRCN0000310111, SMARCB1 #4: TRCN0000308015) were obtained from Merck. To generate double knockdown vectors, synthesized oligonucleotides (shEZH1 #2 and shEZH2 #9) were cloned into retroviral vectors pSINsi-DK II (3664; Takara, Otsu, Japan) with promoter cassette. The sequence 5'- CGCTAAATACTGGCAGGCGTT-3' targeting LacZ was used as a negative control. Human *EZH1* cDNA open reading frame (ORF) cloned into a tetracycline-inducible gene expression lentiviral vector (TRE3G-ORF-P2A-eGFP-PGK-Tet3G-bsd) was provided from Transomic Technologies

(Huntsville, AL, USA). Human *SMARCB1* cDNA ORF cloned into lentiviral expression vector (pLOC) was purchased from Horizon Discovery (Cambridge, UK). The virus was produced in 293LT cells transfected using GeneJuice transfection reagent (70967; Merck) with viral constructs along with VSV-G and gag-pol. For lentivirus production, Rev was also co-transfected. To prepare high-titer viral solutions, viral supernatants were collected for two days followed by ultracentrifugation-based concentration at 50,000 × g for 2 h. The titers of viral solutions were confirmed by Lenti-X qRT-PCR Titration Kit (631235; Takara) and Retrovirus Titer Set (6166; Takara), according to the manufacturer's protocols. The lentiviral-mediated GFP expression was analyzed using a JSAN cell sorter (Bay bioscience, Kobe, Japan) and FlowJo software (TreeStar, Ashland, OR, USA). The knockdown and overexpression efficacy were confirmed by immunoblotting and qRT-PCR.

Cell growth assay

For evaluation of the cell proliferation, A204.1, TTC549, and RD cells (1×10^3 cells), G401.6TG, and JMU-RTK-2 cells (5×10^2 cells), and TTC642, and KYM-1 cells (2.5×10^2 cells) were plated in 48 well flat bottom plate at day 1. The basal cell numbers were measured at day 2 by the absorption spectrum of WST-8 formazan dye (OD 450) using a Cell Counting Kit-8 (CK04; Dojindo, Kumamoto, Japan) following the manufacturer's protocol. The relative cell growth was evaluated every 3 days. For assessment of the anti-proliferative effect of inhibitors, the cells were treated with the inhibitors on day 2. The half-maximal inhibitory concentration of the compounds (IC_{50}) was estimated by using

GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA).

Histone protein extraction

Harvested cells were suspended in hypotonic lysis buffer comprising 10 mM Tris-HCl (pH 8.0), 1 mM KCl, 1.5 mM $MgCl_2$, and 1mM DTT containing 1% protease inhibitors (11836170001; Roche, Basel, Switzerland) and 1% phosphatase inhibitors (P5726 and P0044; Sigma-Aldrich, St. Louis, MO, USA). The cells were lysed by 0.1% Triton-X with mechanical shearing and obtained nuclear fractions. The nuclei were incubated with 0.4 N H_2SO_4 overnight at 4°C and the supernatants containing histones were collected, followed by TCA-precipitation.

Reverse-transcription (RT)-PCR

The following probes and primers were used: *ACTB* (Hs03023943_g1); *EZH1* (Hs00940463_m1); *EZH2* (Hs01016789_m1); *CDKN2A* (Hs00923894_m1); *CDKN2C* (Hs00176227_m1); *CDKN1A* (Hs00355782_m1); *SMARCB1*-Fwd, 5'-GGCATCAGAAGACCTACGCCTT-3'; *SMARCB1*-Rev, 5'-CTCCATCTCAGCGTCTGTCAGA-3'; *CD133*-Fwd, 5'-CAGAGTACAACGCCAAACCA-3'; *CD133*-Rev, 5'-AAATCACGATGAGGGTCAGC-3'; *DOCK4*-Fwd, 5'-GCATGTGGATGATTCCCTGCAG-3'; *DOCK4*-Rev, 5'-GGAGGTGATGTAACACGACAGG-3'; *α-SMA*-Fwd, 5'-GTGGCTATTCCTTCGTTACT-3'; *α-SMA*-Rev, 5'-GGCAACTCGTAACTCTTCTC-3'. The level of ACTB mRNA was quantified for internal control.

Gene expression analysis

RNA-sequencing (RNA-seq) analysis was consigned to Kyusyu Pro Search LLP (Fukuoka, Japan). Total RNA samples were extracted using TRIzol (15596026; ThremoFisher) according to the manufacturer's instructions. 500 ng total RNA was ribosomal RNA-depleted using NEBNext rRNA Depletion Kit (E6310; New England Biolabs, Ipswich, MA, USA) and converted to Illumina sequencing library using NEBNext Ultra Directional RNA Library Prep Kit (E7420; New England Biolabs). The library was validated with Bioanalyzer (Agilent Technologies) to determine size distribution and concentration. Paired-end sequencing (2 × 36 bases) was performed with NextSeq 500 sequencer (Illumina, San Diego, CA, USA). Sequence reads were mapped to the human genome (hg19) and quantified for annotated genes by CLC Genomics Workbench (v10.1.1; Qiagen, Venlo, Netherlands). To estimate the expression pattern of transcripts among DS-3201b treatment, EPZ-6438 treatment and control sets, the read counts were normalized by quantile method for total count in individual samples using the CLC Genomics Workbench. Filtering characteristics of FDR adjusted $P < 0.05$ and fold-change > 2 were used to identify the differentially expressed genes. For drawing heatmap and gene enrichment analysis, Log2-converted normalized values were used. Gene enrichment analysis was conducted with Metascape (http://metascape.org.).

Cell cycle analysis

Cell cycle analysis was performed by quantitation of the cellular DNA content with

propidium iodide (PI) staining. DS-3201b- or EPZ-6438-treated cells were harvested at day 8 and 14, and fixed with 70% cold ethanol overnight at 4 °C. The cells were then incubated with RNase A (R4642; Sigma-Aldrich) for 15 min at 37 °C followed by 30 min of staining with PI (421301; Biolegend, San Diego, MA, USA). The stained cells were analyzed by flow cytometry using a JSAN cell sorter. The collected data were analyzed by FlowJo software.

Apoptosis analysis

Apoptotic cells were detected by flow cytometry. DS-3201b- or EPZ-6438-treated cells were harvested at day 14 and stained with Annexin V-APC (640920; Biolegend) and DAPI (D212; Dojindo) in Annexin V binding buffer (422201; Biolegend) at room temperature for 10 min. The stained cells were analyzed using a JSAN cell sorter and FlowJo software.

Chromatin immunoprecipitation (ChIP) assay

Cells (1-5 × 10^7) were cross-linked with 1% formaldehyde at room temperature for 7 min, quenched by adding glycine to 125 mM, and incubated for 10 min on ice. The cells were then incubated with lysis buffer 1 (50 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, and 0.25% Triton X-100) for 10 min on ice, followed by incubation with lysis buffer 2 (10 mM Tris-HCl pH 8.0, 200 mM NaCl, and 1 mM EDTA) for 10 min on ice. The lysate was resuspended in FA lysis buffer (140 mM NaCl, 1 mM EDTA, 0.1% DOC, 1% TritonX-100, 0.1% SDS, and 50 mM Tris-HCl, pH 7.5) and sonicated using an S220 Focused-ultrasonicator (Covaris, Woburn, MA, USA) to shear chromatin into fragments

with an average length of < 1 kb. After centrifugation, the supernatants were diluted 10-fold with dilution buffer (0.01% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, and 150 mM NaCl). The samples were immunoprecipitated with 2 μg of the indicated antibodies overnight at 4°C. Immunocomplexes were pulled down with Dynabeads Protein G (Invitrogen), washed with wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, and 150 mM NaCl), and eluted with elution buffer (1% SDS and 0.1 M NaHCO3) for 15 min at 65°C. The cross-links were reversed by incubation with 5 M NaCl overnight at 65°C and continuous incubation with 0.5 M EDTA, 1M Tris-HCl (pH 6.5), and 20 mg/ml proteinase K for 2 h at 55°C. DNA was purified by phenol/chloroform extraction, followed by isopropanol precipitation with Ethachinmate (318-01793; Nippon Gene, Tokyo Japan). The presence of the target gene sequences in both the input DNA and immunoprecipitated DNA was detected by quantitative PCR using gene-specific primers and SYBRGreen (Roche). The following antibodies were used for immunoprecipitation: anti-H3K27me3 (07-449; Millipore), and rabbit IgG (#2729; Cell Signaling Technology). Sequences of the primers used for RT-PCR as follows: *ARF* exon 1β (primer A in Figure 4I)-Fwd, 5'-GTGGGTCCCAGTCTGCAGTTA-3'; *ARF* exon 1β-Rev, 5'-CCTTTGGCACCAGAGGTGAG-3'; 500 bp upstream of *p16-CDKN2A* exon 1α (primer B in Figure 4I)-Fwd, 5'-ACCCCGATTCAATTTGGCAG-3'; 500 bp upstream of *p16-CDKN2A* exon 1α-Rev, 5'- AAAAAGAAATCCGCCCCCG-3'; *p16-CDKN2A* exon 1α (primer C in Figure 4I)-Fwd, 5'-AGAGGGTCTGCAGCGG-3'; *p16-CDKN2A* exon 1α-Rev, 5'-TCGAAGCGCTACCTGATTCC-3'; 350 bp downstream of *p16-CDKN2A* exon 1α

(primer D in Figure 4I)-Fwd, 5'- GCCAAGGAAGAGGAATGAGGAG-3'; 350 bp downstream of *p16-CDKN2A* exon 1α-Rev, 5'-CCTTCAGATCTTCTCAGCATTCG-3'; 2 kb upstream of *EZH1* exon 1 (primer A in Figure S2A)-Fwd, 5'- TCCCTTGCCCCTATCCATTT-3'; 2 kb upstream of *EZH1* exon 1-Rev, 5'- TATCTTGAAGGCAGCGGGAA-3'; 250 bp upstream of *EZH1* exon 1 (primer B in Figure S2A)-Fwd, 5'- TTCAGCGTTTGAAGCCAACC-3'; 250 bp upstream of *EZH1* exon 1-Rev, 5'- AGACCGGTGTCTGGAGAACT-3'; *EZH1* exon 1 (primer C in Figure S2A)-Fwd, 5'- CGCACTACTGTCATTTGCCG-3'; *EZH1* exon 1-Rev, 5'-AGTGGCTCTGCGAAGGTTTC-3'; 2kb downstream of *EZH1* exon 1 (primer D in Figure S2A)-Fwd, 5'-AGCATAAACCTTGACAATGATAGC-3'; 2kb downstream of *EZH1* exon 1-Rev, 5'- GGGAGAGAGAAGTACTTAAAAACGC-3'; *ACTB*-Fwd, 5'-TGTGGACATCTCTTGGGCAC-3'; *ACTB*-Rev, 5'-CAGGAGCGTACAGAACCCAG-.

GEO gene expression data

Gene expression data from kidney derived MRT and normal kidney have been deposited in the Gene Expression Omnibus (GEO) database (GSE11151, and GSE11482). These data sets include: 11 MRTs of the kidney, and 5 normal kidney samples. Gene expression data for 51 AT/RT and 9 pediatric normal brain samples were also downloaded from GEO (GSE35493, and GSE64019). All downloaded data were normalized together using gcRMA (as implemented in Bioconductor).

Immunohistochemistry (IHC)

The tumor tissues from A204.1 xenografted mice were fixed with 4% paraformaldehyde phosphate buffer solution (161-20141; Fujifilm Wako, Osaka, Japan), embedded in paraffin, sectioned, and mounted on microscope glass slides (CRE-01; Matsunami Glass, Osaka, Japan). The slides were deparaffinized in xylene and rehydrated by passage through graded ethanol. For antigen retrieval, the sections were incubated in HistoVT One (#06380-76; Nacalai Tesque) for 20-40 min at 90℃ and cooled down to room temperature. After 3 times wash with diluted water, the sections were blocked with 3% hydrogen peroxide for 10 min, followed by Blocking One Histo (#06349-64; Nacalai Tesque) for 10min. After that, primary antibodies were diluted in Signal Stain Antibody Diluent (#8112; Cell Signaling Technology), added to the sections, and incubated overnight at 4℃. The sections were washed 3 times with TBS-T and incubated with Signal Stain Boost IHC Detection Reagent (HRP, Rabbit, #8114; Cell Signaling Technology) as secondary antibody for 30 min, and then washed 3 times with TBS-T. The signals were visualized with Signal Stain DAB substrate Kit (#8059; Cell Signaling Technology). The slides were counterstained by using hematoxylin (1.09249; Sigma-Aldrich). Histopathological images were acquired by using a BZ-9000 microscope (Keyence, Osaka, Japan). The histopathological sections were reviewed by the American College of Veterinary Pathology (ACVP) or Japanese College of Veterinary Pathology (JCVP)-board-certified veterinary pathologists.