HHS Public Access
Author manuscript
Oncogene. Author manuscript; available in PMC 2021 January 04.

EZH2 targeting in medulloblastoma reduces tumor growth through epigenetic reactivation of the BAI1/p53 tumor suppressor pathway

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Abstract

Medulloblastoma is a malignant pediatric tumor for which new therapies are urgently needed. We demonstrate that treatment with EPZ-6438 (Tazemetostat), an enhancer of zeste homologue 2 (EZH2) inhibitor approved for clinical trials, blocks MB cell growth *in vitro* and *in vivo,* and prolongs survival in orthotopic xenograft models. We show that the therapeutic effect is dependent on epigenetic reactivation of adhesion G protein-coupled receptor B1 (*ADGRB1*), a tumor suppressor gene that controls p53 stability by blocking Mdm2. Histone 3 trimethylated on lysine 27 (H3K27me3), a marker of silent chromatin conformation is present at the *ADGRB1* promoter, and inhibition of EZH2, the catalytic component of the Polycomb Repressive complex 2 (PRC2) that methylates H3K27, switches the gene into an active chromatin status and reactivates BAI1 expression. Mechanistically, targeting EZH2 promotes transition from H3K27me3 to H3K27ac at the promoter, recruits the C/EBPβ (CREB-binding protein) and CBP transcription factors and activates *ADGRB1* transcription. Taken together, our results identified key molecular players that regulate *ADGRB1* gene expression in MB, demonstrate that reactivation of BAI1 expression

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underlies EPZ-6438 anti-tumorigenic action, and provide pre-clinical proof-of-principle evidence for targeting EZH2 in patients with MB.

Keywords

adhesion GPCR; ADGRB1; BAI1; TP53; EZH2; C/EBPβ; CBP; medulloblastoma

Introduction

MB is the most malignant brain tumor in children, and is subdivided into four major distinct groups (WNT, SHH, Group 3, Group 4). Genomic studies evidenced a high rate of alterations in epigenetic regulators, with group-specific clustering of aberrations [1]. H3K27me3, a marker of suppressive chromatin written by EZH2, the catalytic component of PRC2, is enriched in Groups 3 and 4, and correlates directly with *EZH2* overexpression. In WNT and SHH groups, *EZH2* expression is increased at the transcriptional level [2], and loss-of-function mutations in the *CREBBP* gene, which encodes the CBP acetyltransferase that writes H3K27ac marks, are found in WNT-MBs, suggesting that alteration in histone H3 trimethylation by several genetic mechanisms is a general feature across MB groups and an opportunity for therapeutic targeting [3].

Targeting EZH2 has anti-tumor effects in experimental cancers, and drugs targeting EZH2 (Tazemetostat, GSK2816126 and CPI-1205) are currently in clinical trials for lymphomas and advanced solid cancers [4]. However, a challenge with epigenetic therapy is that broad genome reprogramming is expected to occur, which will be tissue type and context-dependent, and in addition to induction of tumor suppressors, might also awaken cryptic oncogenes. Therefore, it is important to establish the key genes that drive response to epigenetic therapy so as to select patients who are most likely to benefit, predict routes of therapeutic resistance, and emergence of unwanted side effects. The effectiveness of targeting EZH2 in different MB groups with clinically approved drugs has not been established, and the key target tumor suppressor gene(s) suppressed by H3K27me3 in MB and other cancers remain to be identified.

Adhesion G protein-coupled receptors are the second largest GPCR subfamily in humans. Their long N-termini contain various modular domains involved in cell adhesion, migration, polarity and guidance, all highly relevant processes in tumor cell biology [5]. *ADGRB1* encodes brain-specific angiogenesis inhibitor 1 (BAI1), an orphan adhesion GPCR highly expressed in brain that has potent anti-tumorigenic activities [6–9]. BAI1 expression is downregulated in human MB samples and *Adgrb1* loss destabilizes p53 and promotes MB development in mice, suggesting it is a new tumor suppressor in MB [10]. However, the mechanisms underlying *ADGRB1* transcriptional silencing and potential for epigenetic reactivation towards therapeutic gain remain largely unknown.

Here, we investigated the therapeutic impact of targeting H3K27me3 with EPZ-6438, a clinical EZH2 inhibitor in cell culture and orthotopic MB xenografts in MB. We examined the importance of epigenetic reactivation of the *ADGRB1/p53* tumor suppressor axis in the anti-tumor effects and defined the underlying key transcription factors involved.

Results

EZH2 inhibitor EPZ-6438 reduces MB cell growth in a BAI1/p53-dependent fashion

First, we examined whether pharmacological inhibition of EZH2 had anti-tumor effects on MB cells in culture and found that treatment with EPZ-6438 greatly inhibited MB cell growth *in vitro* (Fig. 1a). To determine whether reduced cell growth was related to cell proliferation, we performed cell cycle analysis and found a G0/G1 block (Fig. 1b and Suppl. Fig.1). As p53 is a major regulator of cell cycle progression, we repeated the experiment in cells stably expressing *TP53*-shRNA and observed neutralization of EPZ-6438's inhibitory effect on MB cell growth (Fig. 1b, c). Since BAI1 can stabilize p53 by blocking Mdm2 and is silenced in MB cells [10], we further tested whether the growth inhibitory effect of EPZ-6438 is dependent upon reactivation of BAI1 tumor suppression activity and found *ADGRB1*-shRNAs indeed abrogated the anti-proliferative effect (Fig. 1d). Taken together, these results demonstrate that EPZ-6438 inhibits *in vitro* MB cancer cell growth in a BAI1/ p53-dependent manner.

EZH2 inhibitor EPZ-6438 inhibits MB xenograft formation in the brain and increases animal survival through a BAI1-dependent mechanism

We then explored the feasibility of targeting EZH2 toward therapeutic gain *in vivo*, using three genetically diverse orthotopic human MB xenograft models. MB cells (D556, D425, and ONS-76) were implanted in the cerebellum, and two (D556 and D425) to four (ONS-76) weeks later, mice were treated orally with EPZ-6438 (350 mg/kg). EPZ-6438 has poor brain uptake as it is a substrate for drug efflux pumps [11], so Elacridar (100 mg/kg), a P-glycoprotein inhibitor was administered prior to EPZ-6438 delivery. Kaplan-Meier curves showed that treatment of EPZ-6438 significantly increased median survival in all three models, from 22.5 to 29 days (p=0.04) in D556 xenografts, from 48 to 58.5 days in ONS-76 xenografts (p=0.0008) and from 20.5 to 27 days in D425 xenografts (p=0.006) (Fig. 2a). EPZ-6438 treated tumors were smaller, had reduced expression of proliferation marker Ki67, and had focal areas of apoptosis (Fig. 2b and suppl. Fig. 2) and showed augmented nuclear H3K27ac/ H3K27me3 staining ratio (Fig. 2b). The tumors also displayed enhanced BAI1 staining, so we determined whether EPZ-6438-mediated enhanced survival was BAI1-dependent by repeating the experiment with D556 and ONS-76 cells stably expressing *ADGRB1*-shRNAs. *ADGRB1* knockdown completely abrogated the survival advantage conferred by EPZ-6438 (p=0.62 and 0.31, respectively, log-rank test) in both xenograft models (Fig. 2c), demonstrating that EPZ-6438's anti-tumor effects in MB are BAI1-dependent.

H3K27me3 contributes to *ADGRB1* transcription silencing in MB

To determine whether EZH2-mediated H3K27me3 plays a direct role in *ADGRB1* gene silencing, we first examined whether the H3K27me3 mark is enriched at the *ADGRB1* promoter. Chromatin immunoprecipitation (ChIP) assays showed abundant promoter binding of H3K27me3 in *ADGRB1*-silent MB cell lines (D556 and ONS-76), accompanied by moderate binding of EZH2 (Fig. 3a). Knock-down of endogenous EZH2 protein with two independent siRNAs or overexpression of KDM6A, the H3K27me3 demethylase, both led to a robust reactivation of *ADGRB1* mRNA expression in both cell lines (Fig. 3b, c).

Treatment with pharmacological inhibitors of EZH2 (EPZ-6438 and GSK126) induced a global shift from suppressive to active chromatin in the cells, as evidenced by a transition from trimethylation to acetylation at the H3K27 mark (Fig. 3d, left). ChIP assays confirmed the same changes at the *ADGRB1* promoter (Fig. 3d, middle), and this led to abundant reactivation of *ADGRB1* expression (Fig. 3d, right). Taken together, these results suggest that targeting H3K27me3 is an effective way to reactivate *ADGRB1* gene expression.

C/EBPβ and CBP are involved in *ADGRB1* transcription activation

We next investigated what transcriptional factors are involved in EPZ-6438-mediated *ADGRB1* reactivation. We focused on EPZ-6438 as it is currently in phase II clinical trials. The factors controlling transcription of the *ADGRB1* gene are currently unknown. Analysis of the human *ADGRB1* promoter sequence for potential transcription factor binding sites by the PROMO module [12] predicts several binding sites for C/EBPβ (Fig. 4a). CCAAT/ enhancer binding proteins (C/EBPs) are a family of transcription factors that bind DNA directly through a conserved basic-leucine zipper domain [13]. Moreover, C/EBPβ can bind and recruit histone acetyltransferases p300 and CBP (CREB-binding protein), which serve as transcription coactivators, in part by relaxing chromatin structure through H3K27me3 acetylation [14]. To test whether C/EBPβ, p300 or CBP are involved in epigenetic *ADGRB1* reactivation, we knocked them down individually with two different siRNA pools before treatment with EZH2 inhibitor (Suppl. Fig. 3). Knockdown of C/EBPβ or CBP greatly blocked EPZ-6438-mediated reactivation of *ADGRB1* expression, while p300 knockdown had no effect (Fig. 4b). CBP can bind BRG1 (Brahma-related gene 1), one of two catalytic ATPase subunits of mammalian SWI/SNF remodeling complexes [15]. SWI/SNF complexes interact with transcription factors, co-activators/repressors, and can mobilize nucleosomes at promoters to regulate gene expression [16]. However, in our system, knockdown of BRG1 expression through *SMARCA4* siRNA had no effect on EPZ-6438-induced *ADGRB1* expression (Fig. 4b). Altogether, these results suggest that C/EBPβ and CBP are required for *ADGRB1* reactivation, while p300 and BRG1 are dispensable for this process.

To further test whether C/EBPβ and CBP can directly activate *ADGRB1* transcription, we used expression vectors for C/EBPβ or CBP and co-transfected them with a luciferase reporter vector under the control of the *ADGRB1* promoter (−400 to +100 of TSS) (Fig. 4a) in HEK293 cells. Either C/EBPβ or CBP could induce a 5~7 fold activation of luciferase activity, confirming that these two factors can directly activate *ADGRB1* transcription (Fig. 4c). Consistently, ChIP experiments showed EPZ-6438 treatment promoted abundant binding of both transcription factors to the endogenous *ADGRB1* promoter (Fig. 4d). Taken together, these results indicate that epigenetic reactivation of *ADGRB1* expression by EZH2 inhibition involves recruitment of C/EBPβ and CBP to the *ADGRB1* promoter in MB cells.

Discussion

Targeting aberrant epigenetic programming in medulloblastoma holds promise for new therapy. However, a challenge is to determine the extent of therapy-induced reprogramming and the key genes that drive response, as this will determine which tumors will respond and how resistance might emerge. We showed that treatment with EPZ-6438 (Tazemetostat), an

EZH2 inhibitor approved for clinical trials extends survival in both SHH and Group 3 MB xenograft models, and this was associated with both a reduction in tumor cell proliferation and increase in apoptosis. Remarkably, while this epigenetic therapy reprograms multiple genes across the MB genome, its effects are entirely dependent upon reactivation of the BAI1-p53 tumor suppressor signaling axis.

Evaluation of EZH2 targeting drugs in cancer is ongoing [17, 18], but the critical effectors of epigenetic reprogramming remain unknown. Finding them is important to predict which patients will respond and which pathways will lead to resistance and tumor recurrence. Our work shows that BAI1 is the main effector of EZH2 targeting in MB, and that the therapeutic effect of EPZ-6438 is lost in its absence. EPZ-6438 therapeutic effect is also dependent upon p53, suggesting that BAI1's protective effect on p53 underlies the tumor suppressive effects [10]. Thus, our findings have direct clinical applicability; we can predict that MB having overexpression of Mdm2 or mutated p53 will not respond to EZH2 inhibition and that inactivation of the p53 pathway will lead to therapeutic resistance. Consistently, recent results showed that deletion of EZH2 before or after tumor formation accelerated tumor growth in a MYC-driven Group-3 MB model with *TP53* deletion [19]. This shows that tumors mutated for p53 are resistant to EZH2-mediated anti-tumor effects, and that this may even unveil cryptic oncogenes.

Targeting EZH2 shifts the *ADGRB1* promoter from a transcription suppressive to active state, as evidenced by depletion of H3K27me3 and enrichment of H3K27ac. Mutations in the *CREBBP* gene, which encodes CBP, are frequent in the WNT group, and lead to truncated CBP proteins [3] that lack the histone acetyltransferase (HAT) domain responsible for writing the H3K27ac mark [20], indicating loss-of-function. Our results that knockdown of CBP blocked EPZ-6438 induced *ADGRB1* reactivation, along with the data that overexpression of CBP activates *ADGRB1*-promoter mediated transcription, demonstrate *ADGRB1* is under CBP regulation. Its close homolog p300 could not rescue CBP loss, providing another example of differential target specificity for the p300/CBP coactivators. This suggests that in MBs where EZH2 is not overexpressed, silencing of *ADGRB1* transcription may also be achieved through *CREBBP* mutation and provides a plausible explanation as to why *CREBBP*, but not *EP300* is mutated in MB.

In sum, our preclinical results demonstrate that epigenetic targeting of EZH2 is therapeutic in MB with wild-type p53, providing proof-of-principle for the testing of agents like EPZ-6438 in patients with MB. These studies will have to consider simultaneous blocking of drug efflux pumps, addition of combination therapy to increase efficacy and will need to include strategies to counter emergence of resistance through inactivation of the p53 pathway.

Materials and methods

Cell culture

Human MB cell lines ONS-76 (SHH Group; *TP53* WT), ONS-76-shTP53, D556-Med (Group 3; *TP53* WT), D556-shTP53, D425 (Group 3; *TP53* WT), were authenticated by STR profiling, tested free of mycoplasma and cultured as described [10]. Experiments were

repeated 3 times and representative data are shown. For cell viability experiments, MB cells (25×10^3 cells cultured on 6-well plates in triplicate) were treated with 10 μM EZH2 inhibitor EPZ-6438 (Chemietek, Indianapolis, IN) for 5 days, live cells were counted by trypan blue exclusion using a hemocytometer. For cell cycle analysis, cells were fixed in 70% ethanol/PBS at −20 °C overnight. Propidium iodide (PI) was used to stain DNA. Cell cycle profile data were acquired with a FACS Canto flow cytometer (Becton Dickinson) and analyzed with FlowJo 9 (Tree Star, Inc.). For knockdown experiments, cells were transiently transfected by two different gene-specific siRNA pools with TransIT-X2 transfection reagent (Mirus, Madison, WI). EZH2-siRNAs were from Cell Signaling Technology (6509) and OriGene (SR320099). Control-siRNAs were from Santa Cruz (sc-37007) and OriGene (SR30004). EP300-siRNAs were from Santa Cruz (sc-29431) and OriGene (SR320041). CEBPB-siRNAs were from Santa Cruz (sc-29229) and OriGene (SR300760). SMARCA4-siRNAs were from Santa Cruz (sc-29827) and OriGene (SR321835). CBP-siRNAs were from Santa Cruz (sc-29244) and OriGene (SR300976). For stable ADGRB1-knockdown, ONS-76 cells were infected with ADGRB1-specific shRNA-expressing lentiviral particles (sc-45208-V, Santa Cruz) in the presence of polybrene (5 μg/ml) (Santa Cruz, Dallas, TX). The lentiviruses contain three to five expression constructs each encoding target-specific 19–25 nt (plus hairpin) shRNAs. Infected cells were selected with puromycin (1 μg/ml) (Santa Cruz) for 4 weeks.

Chromatin immunoprecipitation (ChIP) assays

ChIP was performed as described [9] using 100 μg of genomic DNA/sample. Antibodies (2 μg) used were: histone H3K27me3 (ab6002, Abcam), histone H3K27ac (#8173, Cell Signaling), EZH2 (ab191250, Abcam), C/EBP β (sc-7962, Santa Cruz) and CBP (#7389, Cell Signaling). *ADGRB1* promoter primers used: 5'-GCT CAC TCT GAC CCT CTG CTC TTTC-3' (forward) and 5'-CGT CCC AGC AGC AGC AGC AG-3' (reverse).

Western blotting

Western blotting was performed as described [9] on 30 μg of protein/sample with antibodies against EZH2 (E7031, Sigma-Aldrich; 1:2000 dilution), actin (sc-1616, Santa Cruz; 1:5000), HA-tag (sc-805, Santa Cruz; 1:2000), histone H3K27me3 (ab6002, Abcam; 1:1000), histone H3K27ac (ab4729, Abcam; 1:1000), total histone H3 (#4499, Cell Signaling; 1:1000), p53 (ab28, Abcam; 1:2000), C/EBP β (sc-7962, Santa Cruz; 1:2000), p300 (#54062, Cell Signaling; 1:500), CBP (#7389, Cell Signaling; 1:500), BRG1 (#49360, Cell Signaling; 1:500), cleaved caspase 3 (#9661, Cell Signaling; 1:1000), and cleaved PARP (#5625, Cell Signaling; 1:1000).

Reverse Transcriptase PCR (RT-PCR)

Total RNA was isolated with Trizol reagent (Thermo Fisher Scientific) and first-strand cDNA prepared using AMV RNA PCR kit (TaKaRa) from 1 μg total RNA. PCR amplification for *ADGRB1* mRNA detection was carried out as described [9].

Immunohistochemistry (IHC)

IHC was carried out as described [9]. Briefly, deparaffinized slides were subjected to antigen retrieval by boiling (20 min, 100°C) in 0.01 M Tris HCL (pH 10) and incubated overnight at 4°C with anti-Ki67 (ab15580, Abcam; 1:1,000), Cleaved caspase 3 (1:1000), histone H3K27me3 (1:1000), histone H3K27ac (1:1000), p53 (1:1000), and BAI1 (NB110–81586, Novus, 1:1000) antibodies. Immunostaining was detected with the avidin-biotin complex method, using diaminobenzidine (Abcam) as the chromogen.

Orthotopic MB xenograft models

All protocols for mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Emory University. Eight-week-old Female outbred athymic nude mice (Hsd:Athymic Nude-Foxn1^{nu}; Harlan/Envigo) marked by tattoos [21] were injected with tumor cells (5×10^5 in 5 μl) into cerebellum as described [10]. Two weeks later, surviving mice were randomly divided into two groups (8–13 per group) to minimize variance. The treatment group received oral delivery of Elacridar and EPZ-6438 (350 mg/kg) 5 days/week as described [17]. Elacridar was orally administered at a dose of 100 mg/kg 4 hours before EPZ-6438 treatment. The control group mice received vehicle. No blinding was performed.

Statistical analysis

Results were analyzed using either two-tailed Student's t-test, 2-way analysis of variance (ANOVA) or Log-rank test in Graphpad Prism 5.0 software to assess statistical significance. Sample size was determined based on our prior experience with similar experiments. P values <0.05 were considered to be statistically significant. All histogram data represent mean ± SEM (standard error of the mean).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Abbreviations

ADGRB1	Adhesion G-protein coupled receptor B1
BAI1	Brain-specific angiogenesis inhibitor 1
EZH2	Enhancer of zeste homolog 2
PRC2	Polycomb repressive complex 2
MBD2	Methyl-CpG binding domain protein 2
KDM6A	Lysine-specific demethylase 6A

CBP	CREB-binding protein
MB	medulloblastoma
ChIP	chromatin immunoprecipitation
H3K27me3	histone 3 trimethylated on lysine 27

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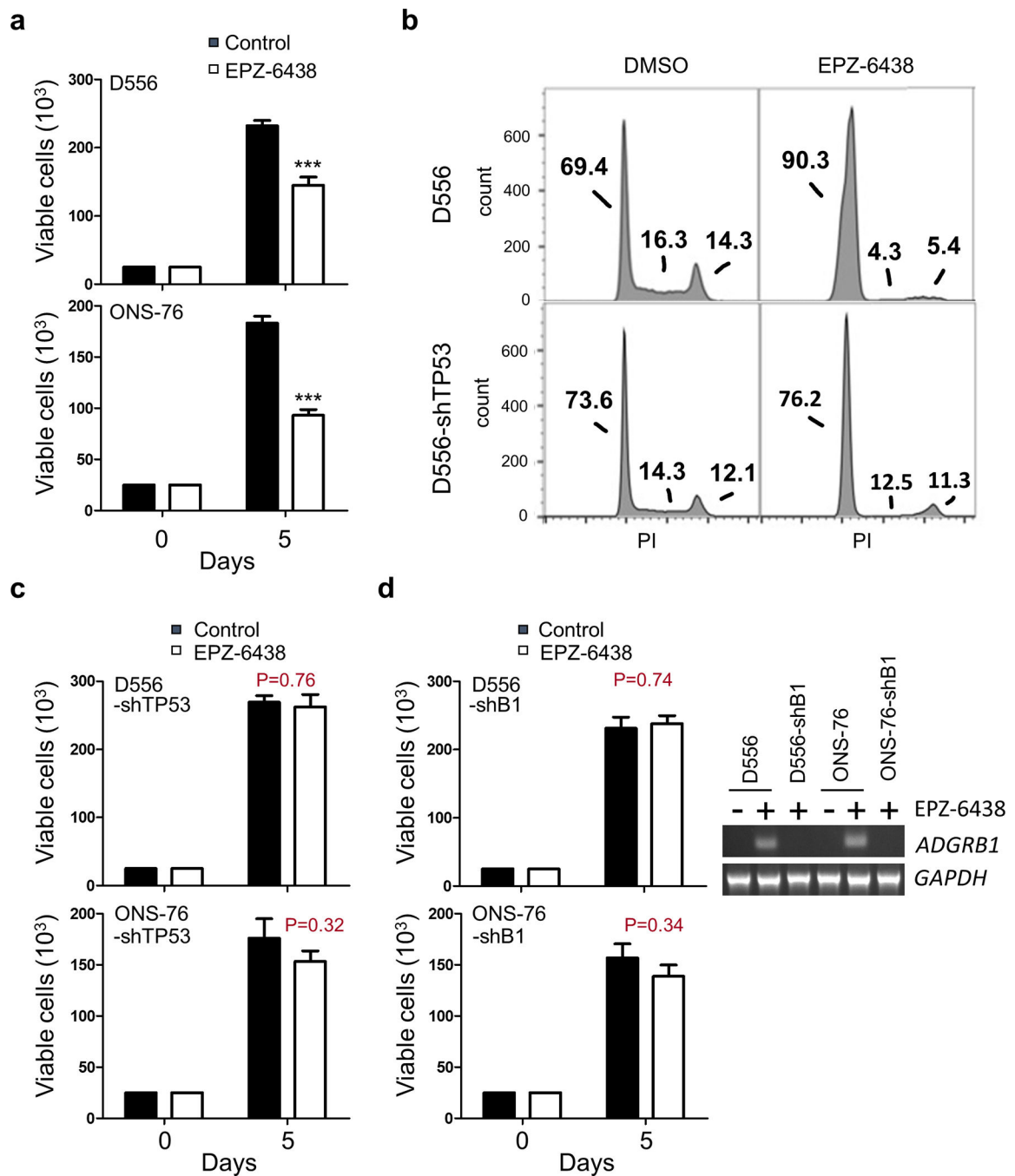21. Van Meir EG. Identification of nude mice in tumorigenicity assays. Int J Cancer 1997; 71: 310. [PubMed: 9139859]

Figure 1.

Targeting EZH2 blocks cell culture growth in an ADGRB1-p53 dependent manner. **a,** EPZ-6438 treatment (10 μM, 5 days) inhibits both D556 and ONS-76 cell proliferation. ***, p<0.0001, 2-way ANOVA, n=3. **b,** EPZ-6438 induces G1 phase arrest only in D556 cells while not in D556-shTP53 cells. **c,** treatment of EPZ-6438 in D556 and ONS-76 cells with stable p53 knockdown had no effect on cell growth. p=0.76 and 0.32, respectively, 2-way ANOVA, n=3. **d,** stable knockdown of BAI1 in both cell lines blocks EPZ-6438's beneficial

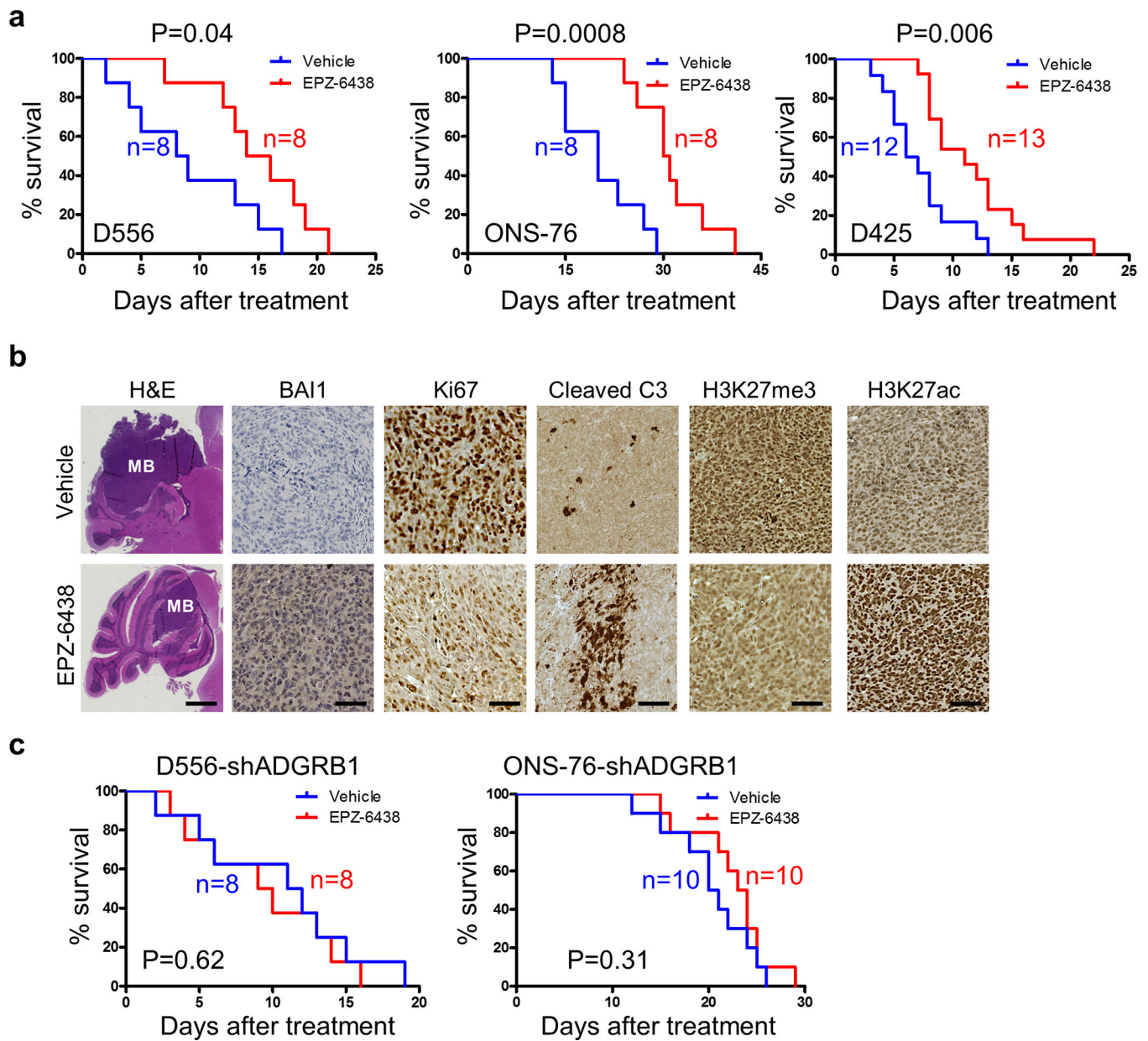effect. p=0.74 and 0.34, respectively, 2-way ANOVA, n=3. (−), DMSO (0.1%) vehicle control.
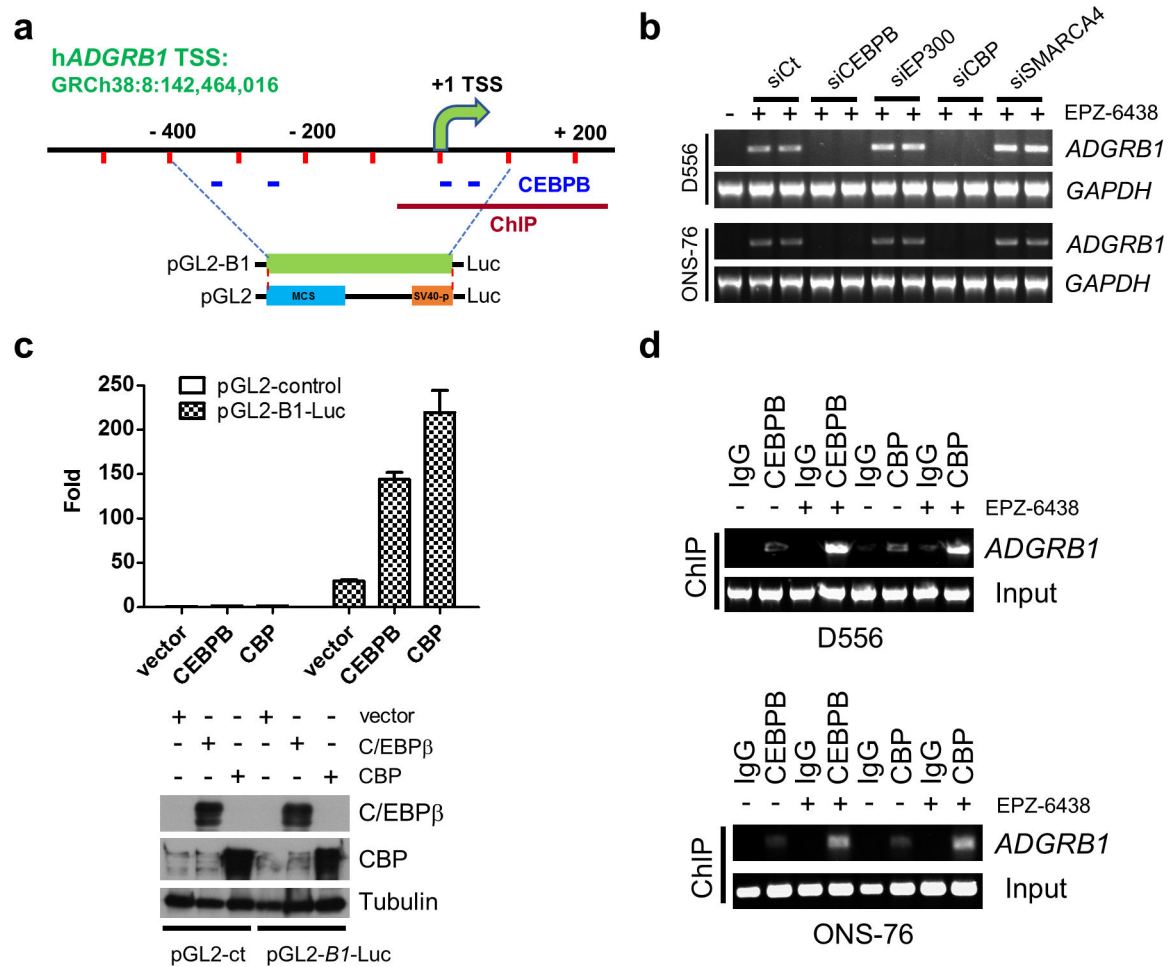
Figure 2.
Targeting EZH2 blocks tumor growth in orthotopic MB models. **a,** Kaplan-Meier survival curves show effect of EPZ-6438 (350 mg/kg i.p.; 5 days/week) on the survival of mice harboring either D556, ONS-76 or D425 intracranial xenografts. P=0.04, 0.0008 and 0.006, respectively, log-rank test. **b**, effect of EPZ-6438 on tumor size (H&E), growth (Ki67), apoptosis (cleaved caspase 3), expression of BAI1, H3K27me3 and H3K27ac in tumors from D556-xenografts by IHC. Scale bar 1 mm (in H&E images) and 50 μm (in IHC images). **c**, Kaplan-Meier survival curves shows effect of EPZ-6438 on the survival of mice harboring intracranial xenografts of D556 and ONS-76 cells with lentiviral shRNA-mediated abrogation of BAI1 (p=0.62 and 0.56, respectively, log-rank test).
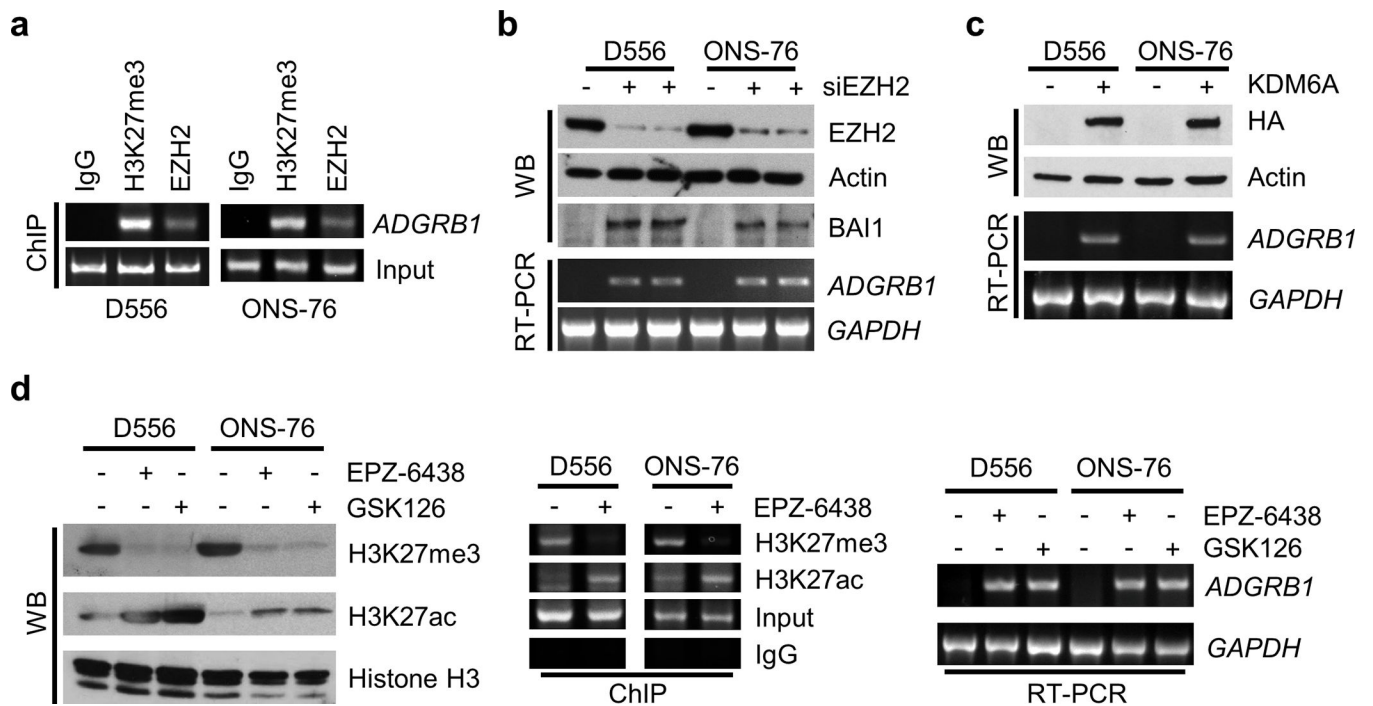
Figure 3.
Targeting EZH2 reactivates *ADGRB1* expression. **a,** ChIP shows enrichment of H3K27me3 and EZH2 at the *ADGRB1* promoter in D556 and ONS-76 cells. **b,** knockdown of EZH2 reactivates *ADGRB1* expression. D556 and ONS-76 cells were transfected with 2 different EZH2-siRNA pools and expression of EZH2 and BAI1 was determined by western blotting and *ADGRB1* expression by RT-PCR. (−), negative control siRNAs. **c,** D556 and ONS-76 cells were transfected with HA-tagged KDM6A-expression vectors, and KDM6A expression was determined by western blotting for HA and *ADGRB1* expression was determined by RT-PCR. **d**, Left, EZH2 inhibitors induce a global decrease of H3K27me3 expression, with increase of H3K27ac marker. Middle, ChIP assay shows treatment of the EZH2 inhibitor EPZ-6438 in D556 and ONS-76 cells depletes H3K27me3 from ADGRB1 promoter. Right, EZH2 inhibitors reactivate *ADGRB1* expression in MB cells by RT-PCR analysis. (−), DMSO (0.1%) control. All blots show representative images from three independent experiments with similar results.

Figure 4.
C/EBPβ and CBP mediate *ADGRB1* activation. **a,** schematic diagram shows *ADGRB1* promoter and putative C/EBPβ binding sites. **b**, EPZ-6438 induced *ADGRB1* reactivation can be blocked by knockdown of either C/EBPβ or CBP. Panel shows *ADGRB1* expression by RT-PCR. **c**, overexpression of C/EBPβ or CBP in HEK 293 cells activates *ADGRB1* promoter driven luciferase activity. **d**, EPZ-6438 promotes binding of C/EBPβ and CBP to the *ADGRB1* promoter in D556 and ONS-76 cells by ChIP analysis. All blots show representative images from three independent experiments with similar results.