Supplemental Material

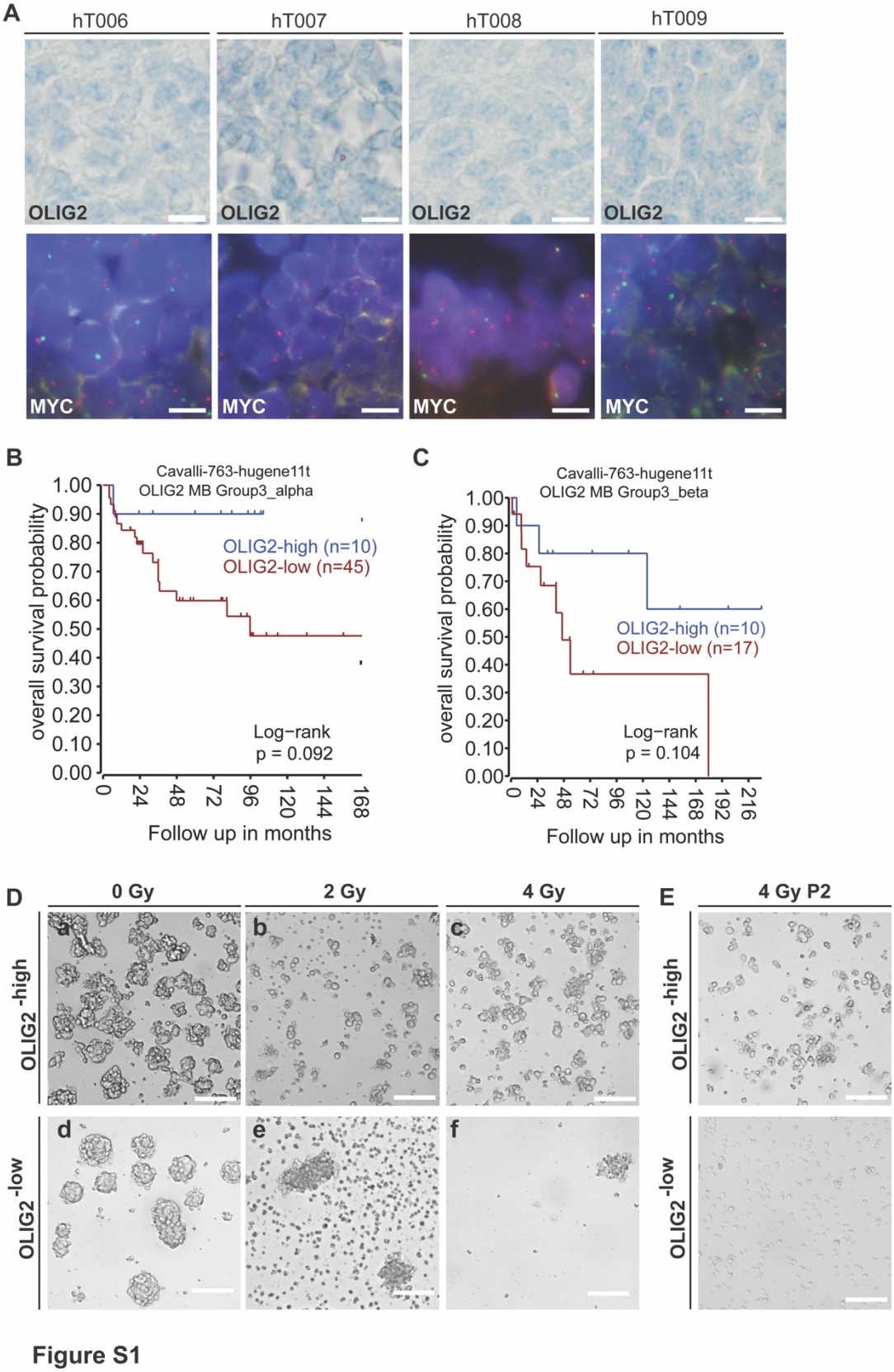

Figure S1

Figure S1. OLIG2^{-high} *MYC*-amplified MB cells are resistant to irradiation compared to OLIG2^{-low} *MYC*-amplified MB cells *in vitro*

(A) OLIG2 or MYC expression in non-MYC amplified tumors resected from patients, as assessed by IHC (OLIG2) or FISH (MYC). No OLIG2 expression was detected in the non-*MYC*-amplified MB tumors examined. IHC, scale bars = 25 μm. FISH, scale bars = 10 μm.

(B-C) Correlation of OLIG2 expression with patient survival in Group 3α MB (B) or Group 3β MB (C).

(D) OLIG2^{-high} (MB002) or OLIG2^{-low} (RCMB28) *MYC*-amplified MB PDX cells were treated with radiation at the indicated dose and cultured for 7 days in a medium that favors the growth of stem cells. Bright field. Scale bars =100 μm.

(E) Irradiated OLIG2^{-high} (MB002) cells (top) and or OLIG2^{-low} (RCMB28) cells (bottom) after passaging. Bright field. Scale bars =100 μm.

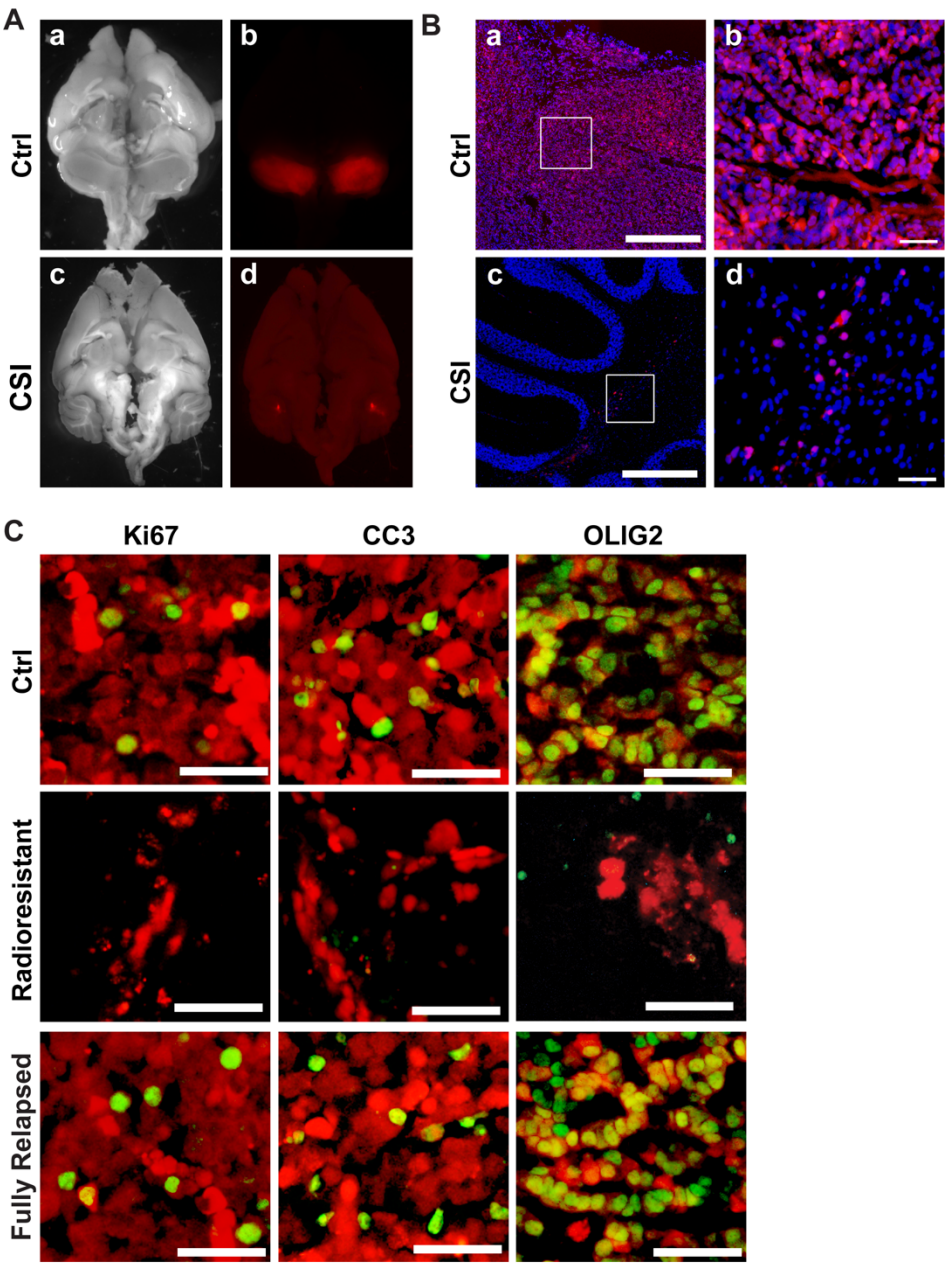

Figure S2

Figure S2. Characterization of radioresistant tumor cells in Med-211FH

(A) Images of brain tissue from tumor-bearing animals without (a, b) or with (c, d) irradiation. Whole mount images in bright field. Tumor cells are indicated in red (mCherry).

(B) Sagittal sections from tumors without (a, b) or with (c, d) irradiation stained with DAPI (blue). Tumor cells are indicated in red (mCherry). Scale bars = 500 μm in a and c. Images for b and d are high magnification closeup of images in the inset boxes of a and c, respectively. Scale bars = 50 μm in b and d.

(C) IHC staining with antibodies (green) against Ki67, CC3 or OLIG2 in the untreated control tumors (top), residual radioresistant tumor cells (middle) or fully relapsed tumors post radiotherapy (bottom). Scale bars = 50 μm. Tumor cells are indicated in red (mCherry) in all panels.

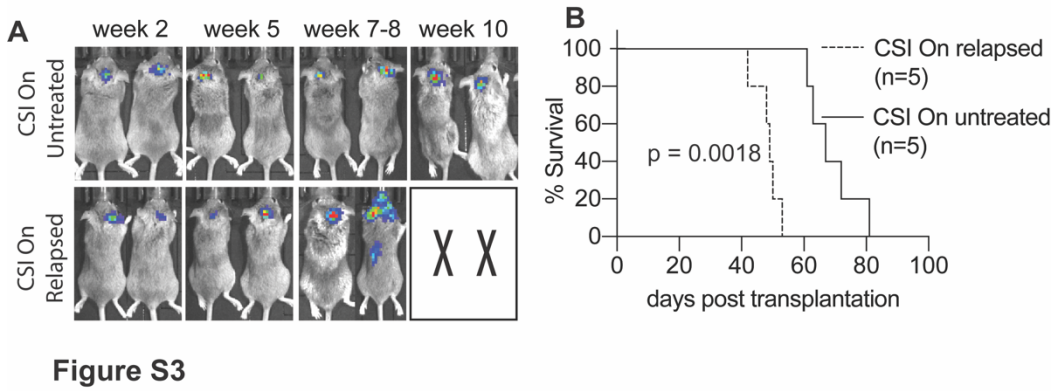

Figure S3

Figure S3. Relapsed tumors are less sensitive to re-irradiation than primary tumors
(A) IVIS images of representative mice bearing primary tumor cells (top panels), or relapsed tumors (bottom panels), both treated with radiation.
(B) Survival analysis by Kaplan Meier (n=5 each group). Logrank test, p=0.0018.

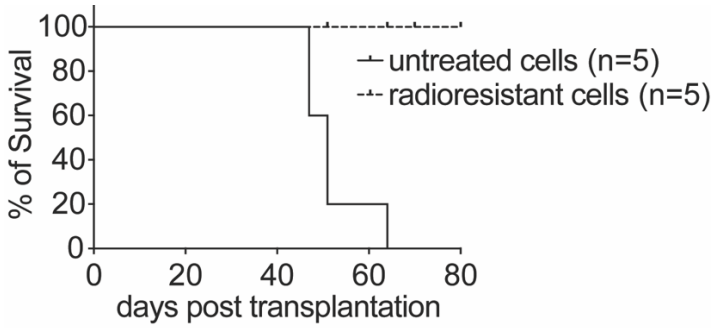

Figure S4

Figure S4. Freshly isolated residual radioresistant tumor cells without culture did not give rise to tumors post-transplantation. Survival curves for animals transplanted with 2,000 freshly FAC-sorted cells from untreated tumors, or from radioresistant tumor cells. Survival analysis by Kaplan Meier (n=5 each group).

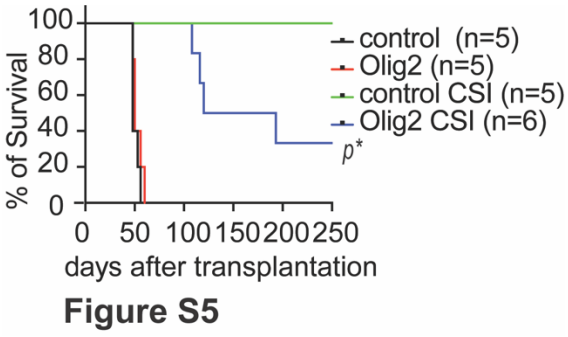

Figure S5. OLIG2 overexpression confers radioresistance in OLIG2^{-low} *MYC*-amplified MB. Survival of animals transplanted with control tumor cells or unpurified OLIG2^{-GFP}-lentivirus-infected tumor cells with or without CSI. Logrank test p^*=0.0005, OLIG2 CSI group *vs.* control CSI group.

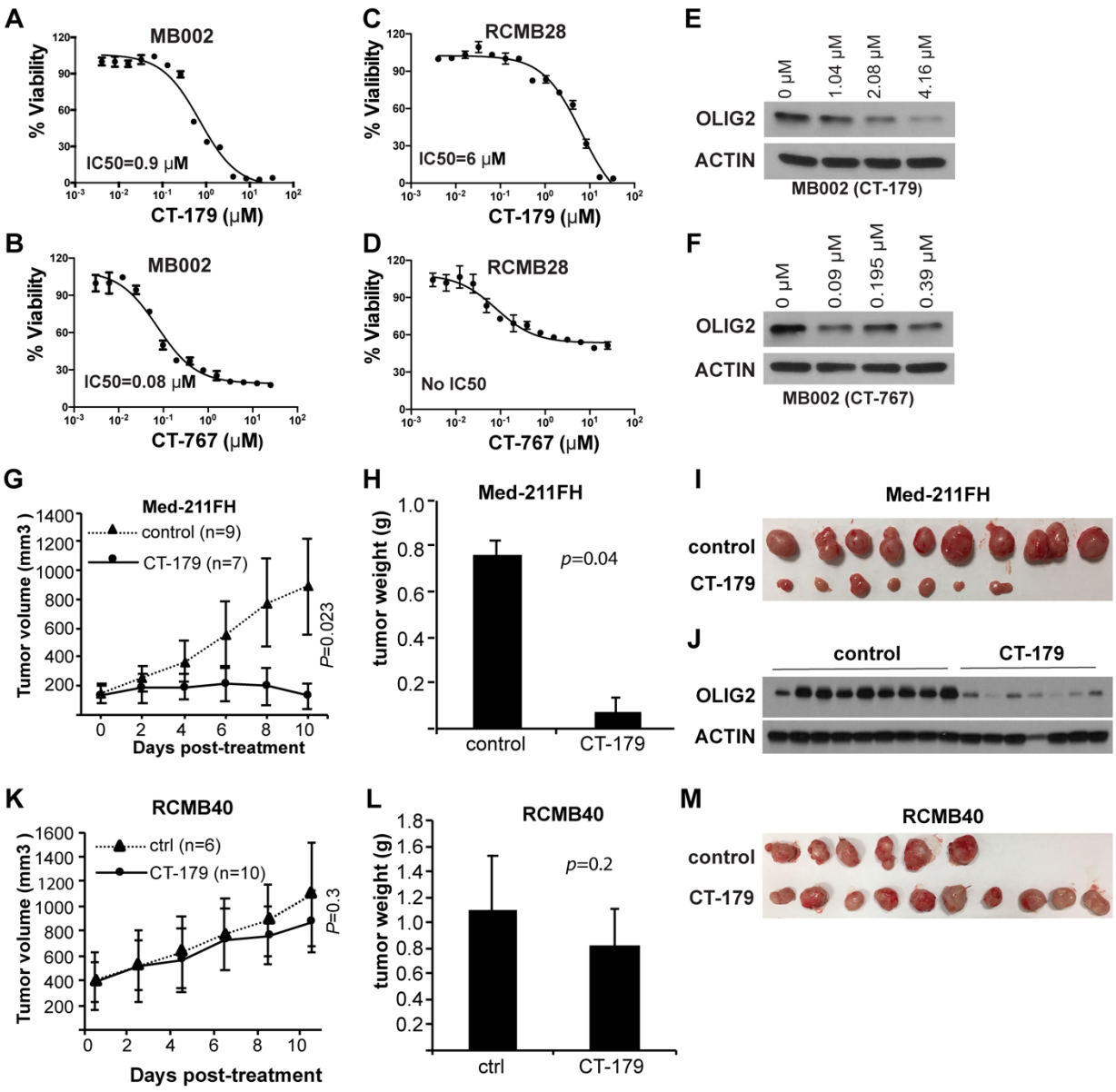

Figure S6

Figure S6. OLIG2 antagonists inhibit the growth of OLIG2^{-high} but not OLIG2^{-low} *MYC*-amplified MB tumors *in vitro* and *in vivo*

(A-D) OLIG2-^{high} (MB002) or OLIG2^{-low} (RCMB28) tumor cells were treated with CT-179 (A, C) or CT-767 (B, D) at indicated concentrations for 72 hours, and cell viability was determined by Cell-Titer-Glo.
(E, F) Western blot analysis of OLIG2 expression in MB002 cells treated with CT-179 or CT-767 for 48 hours.
(G-J) CT-179 inhibits the growth and reduces OLIG2 expression of OLIG2^{-high} MB (Med-211FH) in the flank mouse model. P values determined by Student T test.
(K-M) OLIG2^{-low} MB (RCMB40) does not respond to OLIG2 antagonist in the flank mouse model. P values determined by Student T test.

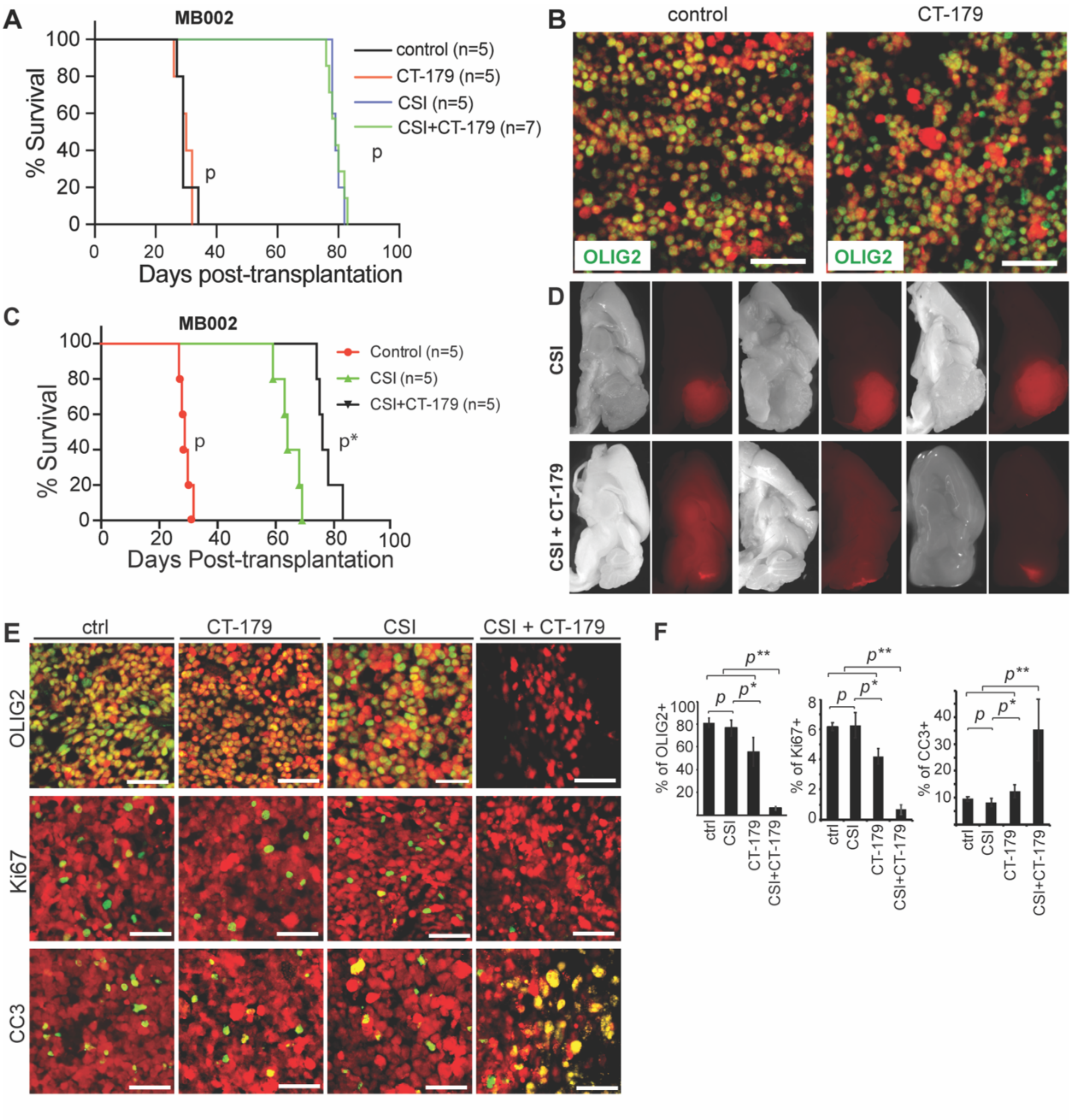
A
MB002
% Survival
Days post-transplantation
control (n=5)
CT-179 (n=5)
CSI (n=5)
CSI+CT-179 (n=7)
p
B
control
CT-179
OLIG2
OLIG2
C
MB002
% Survival
Days Post-transplantation
Control (n=5)
CSI (n=5)
CSI+CT-179 (n=5)
p
p*
D
CSI
CSI + CT-179
E
ctrl
CT-179
CSI
CSI + CT-179
OLIG2
Ki67
CC3
F
% of OLIG2+
% of Ki67+
% of CC3+
ctrl
CSI
CT-179
CSI+CT-179

Figure S7

Figure S7. CT-179 delivered via IT injection after irradiation suppresses the growth of OLIG2 expressing *MYC*-amplified MB cells *in vivo*

(A-B) Mice with intracranial MB002 tumors were treated with vehicle, CT-179, CSI, or CSI + CT-179. CT-179 was delivered by oral gavage. (A) Mice survival curves. Analysis by Kaplan Meier, Logrank test p>0.05, control vs CT-179, CSI vs CSI + CT-179. CT-179 delivered by oral gavage did not inhibit the growth of intracranial OLIG2^{-high} (MB002) tumors. (B) OLIG2 protein level was not reduced in the tumors when CT-179 was delivered by oral gavage, as assessed by IHC staining. Olig2 (green), tumor cells (red, mCherry). Scale bars = 50μm.

(C-D) Intrathecal injection of CT-179 once a week for 3 weeks after irradiation prolongs animal survival compared to control or CSI alone. (C) Mice survival curves analyzed by Kaplan Meier, Logrank test p=0.0001, CSI vs control. p*=0.0018, CSI vs CSI + CT-179. (D) Images of brains from animals treated with CSI (top panels) or CSI + CT-179 (bottom panels). The tumor sizes were much smaller in CSI + CT-179 group compared to CSI alone. Whole mount images in bright field. Tumor cells indicated in red (mCherry).

(E) IHC staining with antibodies (green) against OLIG2, Ki67, or CC3 in the tumors from animals treated with CSI alone or CSI plus increased dose of CT-179 twice weekly. Red is mCherry, indicating tumor cells. Scale bars = 50 μm.

(F) Quantification of percentage of Olig2^{+}, ki67^{+} or CC3^{+} tumor cells in MB002 tumor bearing animals treated with vehicle, CT-179, CSI, or CSI plus increased dose CT-179 (n=5 for each group). Student T test p>0.05, p*<0.05-0.01, p**<0.01.

Table S1. Comparison of gene expression profiles of Spontaneously relapsed tumors to the neurosphere-derived transplantation tumors and the untreated primary tumors in the Excel file.

Experimental Procedures

RNA-Seq Data Analysis

The single-end reads from the MB002 PDX line primary tumor, spontaneous recurrent tumor and neurosphere-derived tumors were aligned to human genome hg38 using STAR v2.4.1 tool(1). Gene expression counts were computed using featureCounts function of the Subread package v1.4.6 with the RefSeq h38 anntoation based. Alignment and counts data quality control was performed using Qualimap(2). Differentially expressed genes between primary tumors, spontaneous recurrent tumor and neurosphere-derived tumors were detected using DESeq2(3) with minimum adjusted p-value limit of 0.0001 and a log2 fold change >1 or <-1. Heatmaps and clustering were performed with the R packages “gplots” and “WGCNA” respectively. Gene Enrichment Analysis was performed using the GSEA(4) v4.1.0 software (http://software.broadinstitute.org/gsea/index.jsp) and the following Molecular Signatures Database (MSigDB): hallmark gene sets (H), the curated gene sets (C2) and the ontology gene sets (C5) . Venn diagrams were generated using the differentially expressed genes

from DESEq2 for the following comparisons: Spontaneous versus Primary tumors, Neurosphere-derived tumors versus Primary tumors and Neurosphere-derived tumors versus Spontaneous tumors.

Survival Analysis of Patients

Overall survival functions were estimated using the Kaplan-Meier method and p-values were calculated using the log-rank test. The statistical analysis was performed in the R statistical environment using the R packages survival (v2.41–3) and survminer (v0.4.0). Gene expression and overall survival data from 31 Group 3_gamma medulloblastoma tumors were downloaded from a previously published dataset (5).

Lentivirus Plasmids

Lentivirus plasmids used include pWPI-mCherry-Luciferase (a gift from Jun Wang at Sanford Burnham Prebys Medical Discovery Institute) and pWPI-*OLIG2*-IRES-GFP. To create pWPI-*OLIG2*-IRES-GFP, OLIG2 was PCR-amplified from pMXs-OLIG2 that was a gift from Kevin Eggan (Addgene plasmid # 32933)(6). The primers used for Olig2 fragment amplification are: Forward primer, CGACATTTAAATTTAATGGACTCGGACGCCAGC; reverse primer, ATACCGTCGAGATTATCACTTGGCGTCGGAGGTG. The vector of pWPI-IRES-GFP (a gift from Didier Trono, Addgene plasmid # 12254) was linearized by restriction enzyme PacI, and Olig2 fragment was subcloned into the vector using In-Fusion® HD Cloning Kit (Clontech) following the user manual.

Cell Culture and Neurosphere Assay

To culture PDX MB cells, we used neural stem cell culture composed of a 1:1 mixture of Neurobasal Media-Vitamin A (Gibco) and Ham's F-12-based medium (Gibco), supplemented with Non-Essential Amino Acids (Gibco, 1:100), B27 (Gibco, 1:50), 2 mM glutamine, 5 mM HEPES (Gibco), 20 ng/mL of bFGF (Peprotech), 20 ng/mL of EGF (Peprotech), and 20 ng/mL of heparin (Sigma Aldrich). To determine whether the radioresistant tumor cells can persist and self-renew in longer-term culture,

FAC-sorted mCherry^{+} radioresistant tumor cells from cerebellum and bulk tumor cells were cultured for 7 days at a density of 2,000 cells/mL in stem cell medium to promote neurosphere formation. Then every 7 days the neurospheres were dissociated into single-cell suspensions and replated to form new neurospheres. Total cell number was counted with a hemocytometer. Cells were passaged 6 times before experiments were terminated.

Immunohistochemistry Staining and Antibodies

Antibodies used for immunostaining include Ki67 (Abcam, ab16667, 1:100), Nestin (Millipore, MAB353, 1:100), OLIG2 (Millipore, AB9610 and MABN50, 1:800), Synaptophysin (Abcam, ab8049, 1:500), Tuj1 (Abcam, AB18207, 1:100), GFAP (Agilent, Z0334, 1:1000), and Cleaved caspase-3 3 (Cell Signaling Technologies, 9661S, 1:1000). Secondary antibodies included anti-mouse and anti-rabbit antibodies conjugated to Alexa Fluor 488, 594 or 647 (all from Invitrogen, and used at 1:200).

For immunohistochemical staining of frozen sections, animals were perfused with PBS followed by 4% paraformaldehyde (PFA). Whole brain and spinal cord were removed, fixed with 4% PFA overnight, cryoprotected in 30% sucrose for 24 hrs, then embedded in Tissue Tek-OCT (Sakura Finetek). Frozen blocks were cut at 12 μm into sagittal series of sections. Sections were permeabilized with 0.1% Triton X-100 in PBS for 30 minutes and blocked for one hour with 10% normal donkey serum in PBS containing 0.1% Triton X-100, followed by overnight incubation with primary antibodies at 4°C. Sections were then stained with secondary antibody for 2 hrs at room temperature, counterstained with DAPI (Invitrogen), and mounted with Fluoromount-G (Southern Biotech). Stained slides were visualized under the microscope.

Western-blotting and Antibodies

To evaluate gene expression at the protein level, tumor cell pellets were lysed with Pierce RIPA buffer (Thermo Fisher Scientific) supplemented with proteinase and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Protein lysates were separated on an 8-16% Mini-Protean TGX gel (Bio-Rad) by

electrophoresis, then transferred to a Immobilon-P membrane (EMD Millipore). Membranes were blocked in 5% nonfat dry milk (Bio-Rad) for one hour at room temperature, and incubated with primary antibodies to OLIG2 (1:1000, Millipore, MABN50), MYC (1:1000, abcam, ab32072) or beta-actin (1:3000, Abcam, ab8226) at 4°C overnight. Membranes were then stained with HRP-conjugated secondary antibodies (1:5,000, GE Life Sciences) for one hour at room temperature. Bands were visualized using Amersham ECL (GE Healthcare).

Fluorescence In Situ Hybridization

FISH analyses were performed on 2 μm thin sections from formalin-fixed, paraffin-embedded tumor tissue. A Zyto*Light* ® SPEC MYC/CEN 8 Dual Color Probe (Zytovision, Bremerhaven, Germany) was used to detect *MYC* amplifications according to the manufacturer's recommendations. We use the following definition for *MYC* amplification: >5% of nuclei within the sample (of 200 counted) have a strong speckled staining pattern (indicative of the formation of double-minutes or homogenously-stained regions), and the test probe show a copy number ≥ 4 times of the reference copy number signal.

Cell Survival Assay *in Vitro*

To assess the effect of OLIG2 inhibitor on cell growth *in vitro*, tumor cells were freshly isolated from tumor-bearing mice and were plated into 384-well Greiner plates. After treatment with the indicated concentrations of CT-179 and CT-767 for 72 hrs in stem cell medium, cell viability was then assessed using the CellTiter-Glo Luminescent Cell Viability assay (Promega) on a Fluostar spectrophotometer (BMG Laboratories).

***In Vivo* Bioluminescent Imaging**

Tumor-bearing animals were subjected weekly to bioluminescence imaging using IVIS spectrum. Briefly, mice were given intraperitoneal injections of 150 μg/g Xenolight D-Luciferin potassium salt (Perkin Elmer) and anesthetized with 2.5% isoflurane. 10 minutes post-injection, the animals were

imaged using the IVIS® Lumina III (IVIS-200) imaging system.

Supplemental References

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