Supplementary Methods

SB Injections

Mice were injected according to a previously described protocol (1). Briefly, plasmids were mixed in mass ratios of 1:2:2:2:2 (20 μg plasmid in a total of 40 μL plasmid mixture) with in vivo-jetPEI® (Polyplus Transfection, 201-50G) (2.8 μL per 40 μL plasmid mixture) and dextrose (5% total) and maintained at room temperature for at least 20 minutes prior to injection. Anesthesia was performed by placing the pup on ice for 2 minutes and then on a neonatal stereotaxic stage cooled to 2-8°C to maintain anesthesia. The fourth ventricle (3 mm posterior to the λ-suture and 3 mm deep) was targeted (2). Plasmid uptake and tumor development and progression was monitored as previously described (1,3). Animals were monitored daily for signs of morbidity (ataxia, impaired mobility, hunched posture, seizures, or scruffy fur). Symptomatic mice were transcardially perfused using Tyrode's solution and fixed with 4% paraformaldehyde (PFA) (1,3,4).

Immunohistochemistry (IHC) of paraffin embedded brains

Immunhistochemistry staining was performed as previously described (1,3). Briefly, after perfusion, mouse brains were harvested and post-fixed in 4% PFA then paraffin embedded. Tissue was sectioned using a rotary microtome. Heat mediated antigen retrieval was performed using either 1X Rodent Decloaker (Biocare Medical, RD 913) or citrate buffer (10 mM citric acid, 0.05% non-ionic detergent, pH 6) at 125°C for 30 seconds and at 90°C for 10s followed by quenching of endogenous peroxides using 0.3 % H2O2 for 30 minutes, and permeabilization with 0.025% Triton in TBS. Sections were blocked with 10% horse serum, 0.1% BSA in TBS) for 30 minutes and then incubated overnight at 4°C with primary antibody diluted in 0.1% BSA in TBS. For 3,3′-Diaminobenzidine (DAB) staining the VECTASTAIN ABC HRP Kit (Vector Laboratories, PK-

4000) was used. The Alexa Flour 488 Tyramide SuperBoost Kit (Thermo Fisher Scientific, NC1136352) was used for the phospho Smad1/Smad5/Smad8 (Ser463/465) (MilliporeSigma, AB3848-I) antibody. Bright-field images were obtained using Olympus MA BX53 microscope. Flourescent images were obtained using confocal microscopy (Carl Zeiss: MIC-System). A list of all antibodies used is included in Supplementary Table 1.

Primary Neurospheres (NS)

Mouse neurospheres (NS) were generated from tumors that were developed using the SB system by injection of the following plasmid combinations (1) (i) shp53 and NRAS, or (ii) shp53, NRAS, and ACVR1m into the lateral ventricle (1.5 mm AP, 0.7 mm lateral, and 1.5 mm deep from the λ-suture) following previously described protocols (1,3-5). When animals became symptomatic they were anesthetized then transcardially perfused with Tyrode's solution. The brains were harvested and tumors were identified by fluorescence expression. The tumor was then extracted and placed in 300 µL of media in an Eppendorf tube. Then, the tumor was mechanically dissociated using a sterile plastic pestle that fit the walls of the Eppendorf tube. This was followed by incubation with 1 mL of a cell dissociation buffer (Accutase, 423201), then filtered through a 70 µm strainer and maintained in neural stem-cell media [DMEM/F12 with L-Glutamine (Gibco, 11320-033), B-27 supplement (Gibco, 12587- 010), N-2 supplement (Gibco, 17502-048), Penicillin-Streptomycin (Corning, Cellgro, 30-001-CI), and Normocin (InvivoGen, ant-nr-1)] at 37 °C, 5% CO2. hFGF and hEGF (Shenendoah Biotech,100-26, 100-146) were supplemented twice weekly at 1 µL (20 ng/µL each stock, 1000x stock) per 1 mL media. Primary mutant ACVR1 glioma NS were transduced with pLVX-OVA to generate mACVR1-OVA NS and selected with 5 ug/mL puromycin (Sigma, P8833). pLVX-OVA was generated by cloning cytoplasmic ovalbumin from

pCI-neo-cOVA (Addgene plasmid #25097) into pLVX-mCherry-c1 (Takarabio, 632561) by Nde1 and EcoR1 directed cloning.

Western blot

NS were harvested and re-suspended in RIPA buffer (MilliporeSigma, R0278) with 1X of Halt™ Protease and Phosphatase Inhibitor Cocktail, EDTA-free (100X) (Thermo Scientific, 78441). The Pierce™ BCA Protein Assay Kit (Thermo Scientific, 23227) was used to measure protein concentration. 20 µg of protein extract were run on a 4-12% SDS-PAGE PAGE gel (Thermo Fisher Scientific, NuPAGE®, NP0322BOX) and transferred to nitrocellulose membranes (Bio-Rad, 1620112). The membrane was probed with 1:500 of anti-phospho Smad1/Smad5/Smad8 (Ser463/465) (MilliporeSigma, AB3848-I); 1:500 of anti-Smad1 antibody (Abcam, ab63356); 1:500 of anti-Id1 (Biocheck, BCH-1/37-2); and 1:10,000 of β-actin antibody (MilliporeSigma, A1978). The secondary antibodies used were: 1:4000 of goat anti-rabbit (Dako, Agilent Technologies, P0448) and 1:20,000 of rabbit anti-mouse (Dako, Agilent Technologies, P0260). SuperSignal West Femto (Thermo Fisher Scientific, 34095) was used to for detection.

In vitro experiments with ACVR1 inhibitor

LDN-214117 is a specific ACVR1 inhibitor (6). To determine an effective concentration to inhibit ACVR1 signaling in our mouse NS we plated wild type or mutant ACVR1 cells in a T-25 flask containing growth media supplemented with varying concentrations of LDN-214117 (0.03 µM, 0.1 µM, 0.3 µM, 1 µM); Selleck Chemicals, S7147) or equivalent DMSO control. NS were incubated with inhibitor for 90 minutes and then cells were collected to assess phosphorylation of Smad1/5 and Id2 by western blot.

Immunocytochemistry

SU-DIPG-VI and SU-DIPG-XX1 were obtained from Dr. Michelle Monje at Stanford University (Stanford, Ca) in accordance with an institutionally approved protocol at each institution. Cells were cultured in DMEM with 10% FBS. 1 X 10^5 cells per well were plated on glass slides coated with 2% gelatin. Cells were fixed with 4% PFA and permeabilized with PBS 1X plus 0.3% Tween. ICC was performed with 1:100 ID1 (Biocheck, BCH-1/195-14), pERK1/2 (Cell Signaling, 4370), pMEK1/2 (Santa Cruz, sc-7995-R), and 1:1000 goat anti-rabbit antibody, Alexa Flour 488 (Invitrogen, A -11034).

RNA-seq analysis

RNA-seq analysis was performed in collaboration with the Bioinformatics Core at the University of Michigan. Read files from the University of Michigan Sequencing Core's storage were downloaded and concatenated into a single fastq file for each sample. The quality of the raw reads data for each sample was assessed using FastQC [1] (version v0.11.3) to identify features of the data that may indicate quality problems (e.g. low quality scores, over-represented sequences, inappropriate GC content). The Tuxedo Suite software package was used for alignment, differential expression analysis, and post-analysis diagnostics. Briefly, we aligned reads to the reference genome including both mRNAs and lncRNAs (UCSC mm10) using TopHat (version 2.0.13) and Bowtie2 (version 2.2.1.). The default parameter settings for alignment were used, with the exception of: "--b2-very-sensitive" telling the software to spend extra time searching for valid alignments. We used FastQC for a second round of quality control (post-alignment), to ensure that only highquality data would be input to expression quantitation and differential expression analysis. We performed two different analysis techniques to specify differential expression: Tuxedo and DESeq2, using UCSC mm10.fa as the reference genome sequence. The volcano plot was produced using an R base script and encompasses all genes identified by our RNA-seq

analysis. In order to identify significantly differentially expressed genes, we set a log2 fold change cut-off of 0.585 and –log10(FDR) greater than 1.3, where circles represent individual genes and colors as indicated (red-upregulated, green-downregulated) shown in Figure 3A. The data was analyzed using Advaita Bio's iPathwayGuide (https://www.advaitabio.com/ipathwayguide). This software analysis tool implements the 'Impact Analysis' approach that takes into consideration the direction and type of all signals on a pathway, the position, role and type of every gene, etc., as described in (7-10). Gene Ontology (GO) enrichment analysis was performed using iPathwayGuide (http://www.advaitabio.com/ipathwayguide), displaying up- and down-regulated genes associated with the same GO and was shown in Figure 3C. GO Biological Processes, selected for relevance to phenotype, were plotted in a horizontal bar graph using graphpad shown in Figure 3B. All 100 differentially expressed genes between wt-ACVR1 and mutant-ACVR1 NS were converted to excel with their corresponding gene names in uppercase in first column and log2-fold change values in second column to create the rank file. This rank file is used as an input for gene set enrichment analysis (GSEA) pre-ranking on program downloaded from Broad Institute (http://software.broadinstitute.org/gsea/index.jsp). In addition, Broad Institute provides a gene set containing all GOs (c5.all.v7.0.symbols.gmt), which is also required to perform the pre-ranking using 2000 permutations and gene set size range of 0 to 200. The enrichment map is generated using the Cytoscape platform and requires the rank and gmt files along with the positive/negative GSEA report files. Each node represents an individual GO, the size is reflective of the number of genes within that set, and the red color signifies positively regulated pathways. We set a pvalue cut off of 0.001 and FDR of 0.5. The stringency of our FDR was lowered due to encompass the underlying biological pathways differentially regulated by ACVR1 are shown in yellow in Figure 3D. The enrichment score plots for Regulation of BMP signaling Pathway and

Regulation of Cell Differentiation are shown in Figure 3E and 3F respectively, which are images provided in the GSEA Pre-ranking report.

Intratumoral injection of adenoviral vectors and radiation treatment

We used first generation Ad.hCMV.hsFLT3L (Ad-Flt3L) + Ad.hCMV.TK (Ad-TK)(11,12). Five days post tumor implantation (dpi) mice were assigned to four treatment groups: (i) control group: empty Ad(Ad0)/saline); Ad0 was delivered intratumorally (i.t) and saline intraperitoneally (i.p.), (ii) gene therapy: Ad-TK(1×10^8 plaque-forming units (pfu)/Ad-Flt3L(2×10^8 pfu of Ad-TK) /GCV; Ad-TK/Ad-Flt3Ldelivered i.t., ganciclovir (GCV) (Biotang; RG001-1g) was administered i.p. at 25 mg/kg/daily for 10d, starting 1d post-gene therapy, (iii) Standard of care (IR): Ad0/saline+IR; an overall dose of 20 Gy IR was administered (2 Gy/d for 10d), and (iv) Gene therapy + standard of care: Ad-TK/Ad-Flt3L/GCV+IR. Intratumoral injections of adenoviral vectors was delivered in μL volume in three locations to depths of 4.4, 4.5, and 4.6 mm, at the coordinates detailed above. Sample size was n = 5 for each treatment group. For the functional analysis of T cells in the tumor microenvironment (TME), 8 days post tumor implantation mice were assigned to either the control group or the gene therapy group detailed above. Sample size was n = 3; where per sample it was necessary to pool tumor tissue from three mice for the control and five mice for the gene therapy group due to the size of the tumor.

Flow cytometry

For flow cytometry experiments were performed using protocols described before (11-13). Flow data were acquired on a FACSAria flow cytometer (BD Biosciences) and analyzed using Flow Jo version 10 (Treestar). Prior to staining cells with antibodies, live/dead staining was carried out using fixable viability dye (eBioscience). Then, cells were resuspended in PBS containing 2% fetal bovine serum (FBS) (flow buffer) and non-specific antibody binding was blocked with

CD16/CD32 (Biolegend, 101335). All stains were carried out for 30 minutes at 4°C with 3X flow buffer washes between live/dead staining, blocking, surface staining, cell fixation, intracellular staining and data acquisition. The fixation/ permeabilization staining kit (BD Biosciences, 554714) was used for all intracellular stains. Antibody information is included in supplementary table 1. For T cell functional analysis within the TME, the cells from the tumor mass were stained with anti-mouse CD45, CD3, CD8, and SIINFEKL-H2Kb-tetramer-PE. For IFNγ stains, single cell suspensions generated from the tumor mass were stimulated with 25 ug/mL of mACVR1-OVA lysate for 24 hours in 10% FCS-containing media followed by 6 h incubation with Brefeldin and monensin. Cells were stained with CD3 and CD8 antibodies, followed by intracellular staining for IFNγ. To assess tumor cell stemness tumor cell suspensions generated from wt-ACVR1 and mACVR1 implanted tumors were stained for CD133, CD44, and Aldh1.

Assessment of damage associated molecule release

To assess for release of damage associated molecules after treatment with either radiation (3G) or Ad-TK (500 MOI)/GCV (25μM), 10,000 mACVR1 NS were plated on 6-well plates. The next day they were treated with Ad-TK (500 MOI) and/or radiation (3G). Following a 48 hour incubation they were treated with GCV (25 μM). The following day they were stained with calreticulin or high mobility group box 1 (HMGB1) antibodies following flow cytometry protocols described above. To assess ATP release, we used the ATP determination kit following manufacturer's instructions (Invitrogen, A22066) to measure ATP levels in media after 48 hours of treatment. Media was used to generate the ATP standard curve.

T cell Proliferation analysis

Splenocytes from 21 dpi mACVR1-OVA brainstem glioma-bearing mice treated with saline or gene therapy, as detailed above, were labeled with Carboxyfluorescein succinimidyl ester (CFSE)

per manufacturer's instructions and cultured with 100 nM SIINFEKL peptide (Anaspec, 60193-1; dissolved in H20 and stored in -80 °C) for 4 days. As a positive control we stimulated splenocytes isolated from untreated non-tumor bearing mice with Concanavalin A. As a negative control we used unstimulated splenocytes. Cells were then stained with CD3 and CD8, and T cell proliferation was assessed based on CFSE dye dilution.

ELISA

IFNγ expression in supernatants from the T cell proliferation assay was determined using anti-IFNγ ELISA kit (R&D) following the manufactures instructions. Briefly, to a 96-well plate 50 µl of assay diluent was added followed by 50 µl of sample, or standard; plate was incubated at 25 °C for 2 hours. Then, wells were washed five times with wash buffer and incubated for 2 h at 25 °C with 100 µl of conjugate solution. Wells were washed a further five times with wash buffer and incubated for 30 min at 25 °C with 100 µl of substrate solution. The reaction was stopped by adding 100 µl of stop solution to each well and the absorbance was acquired at 450 nm (background was subtracted by setting the wavelength at 540 nm). This assay was performed by the Rogel Cancer Center Immunology Core at the University of Michigan.

Cytotoxic T cell assay

Splenocytes from saline or gene therapy treated mice were incubated with mACVR1-OVA NS or B16F10 melanoma cells for 24 hours at the indicated ratios (1:1, 10:1, 20:1). Lysis of tumor cells was assessed by using Annexin-V. mACVR1 NS undergoing apoptosis were identified as Annexin-V^+/CD45-/$Katushka^+$ (14,15).

Complete blood count (CBC) and serum chemistry

Collected blood was transferred to EDTA tubes for CBC (RAM Scientific, 077058) or in serum separation tubes (Sarstedt, 41.1378.005). CBC and serum chemistry was performed by the In-Vivo Animal Core (IVAC) at the University of Michigan.

Neuropathological analysis

To assess the safety of vector delivery into the brainstem, non-tumor bearing animals were treated with Ad-TK/Ad-Flt3L gene therapy or saline. GCV was administered intraperitoneally one day later for 7 days. One day after the last dose of GCV was administered animals were euthanized and brains were harvested and processed for histology as detailed above. Hematoxylin and eosin (H&E) staining was performed to assess gross histopathological features (16). Immunohistochemistry staining for glial fibrillary acidic protein (GFAP) to mark astrocytes, myelin basic protein (MBP) to mark myelin sheaths and oligodendrocytes, and Iba1 to mark microglial cells.

Hematoxylin and eosin (H&E)

Liver tissue sections (5 μm thick) from tumor bearing animals treated with saline, radiation, gene therapy, and gene therapy and radiation were stained with H&E (16).

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