Supplemental Information

Supplemental Figures

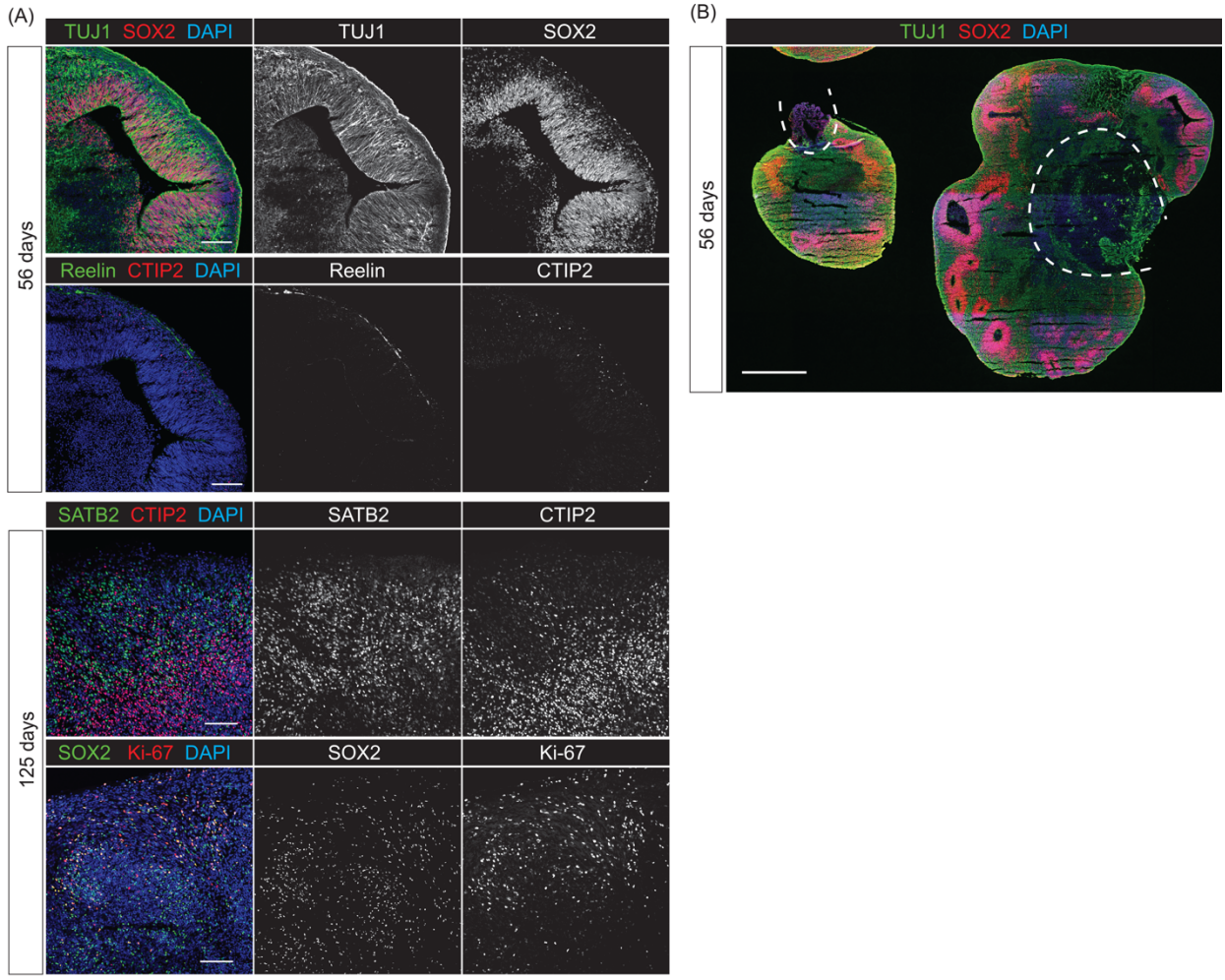

Figure S1. Human cerebral organoids development. Related to Figure 1.
(A) Immunostaining for neurons (TUJ1, green) and progenitors (SOX2, red) shows evidence for cortical plate formation. Staining for Reelin (green) is positive in the dorsal cortical region and also CTIP2 (red), an early-born deep layer neuron marker, at 56 days of culture in human cerebral organoids. At 125 days, the CTIP2 and SATB2 (red), a late-born upper layer marker, expression pattern has a distribution consistent with a cortical layer structure. Scale bar = 100µm (B) Entire organoid view at 56 days. Immunostaining of TUJ1(green) and SOX2(red) shows evidence for cortical plate formation on the surface of organoids, however they also contain some populations of non-neuronal differentiated areas (non-neuronal areas are demarcated by the dashed white line). Scale bar = 1mm

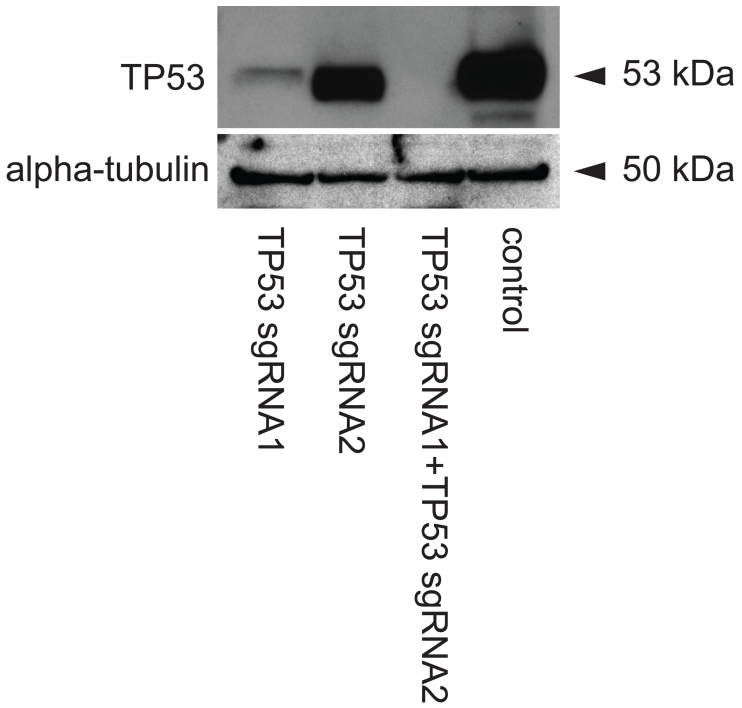

Figure S2. Test of TP53 sgRNA effectiveness. Related to Figure 1A.
Western blot analysis of human TP53 expression in 293T cells transfected CRISPR/Cas9 TP53 sgRNA Plasmid pSpCas9(BB)-2A-Puro (PX459). 293T cells were placed under the selection with Puromycin (2 μg/ml) for 72hrs. We tested two different sgRNAs for the human TP53 locus and both showed significant protein reduction.

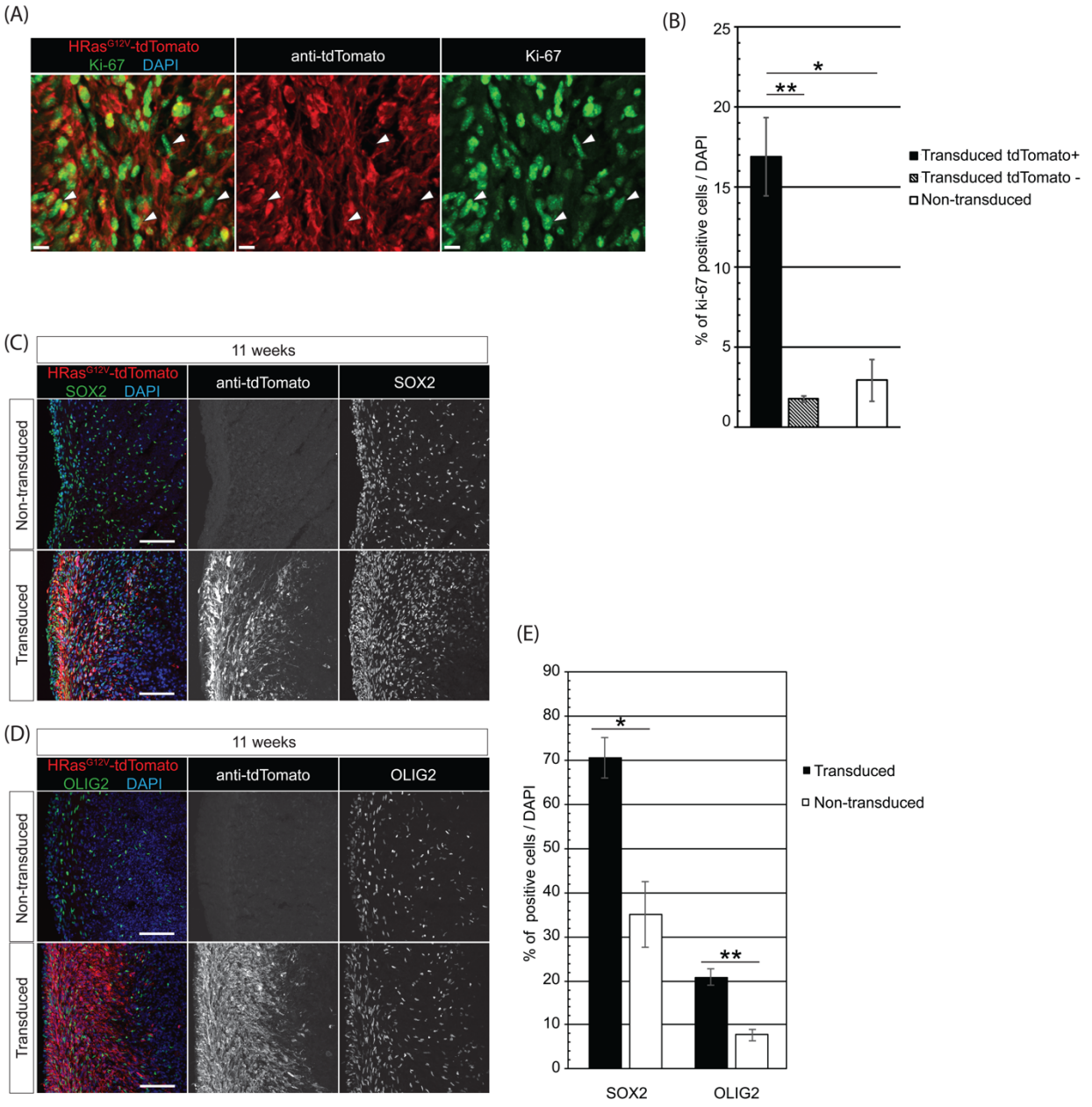

Figure S3. Brain tumor stem cell marker expression of transformed human cerebral organoids. Related to Figure 1C.

(A) anti-Ki-67 immunostaining image of transduced organoids at higher magnification (scale bar = 15µm) shows that most of the Ki-67 positive proliferative cells are HRas^{G12V}-tdTomato positive however, there is a small population which is Ki-67 positive and tdTomato negative (arrowheads). (B) Histogram shows the ratio of Ki-67 positive cells, from the transduced cells which are tdTomato positive (16.9%) and also those from the non-transduced organoid cells (1.7%), which are tdTomato negative. In non-transduced organoids 3.0% of cells are proliferative and Ki-67 positive (p**= 0.007, p* = 0.014). The error bars represent ±SEM. Statistical significance was evaluated using Student's t test. (C-D) HRas^{G12V} transduced cells show a higher proportion expression of stem cell transcription factors SOX2(C)and OLIG2(D). (E)The histogram shows the percentages of SOX2-positive cells and OLIG2-positive cells respectively. These cells were 70.5% positive for SOX2 in RAS/TP53 transformed cells, compared to 35.0% in controls (p* = 0.029). Similarly, OLIG2 was expressed in 20.8% of transduced cells and only in 7.8% of control cells (p** = 0.0089). The error bars represent ±SEM. Statistical significance was evaluated using Student's t test.

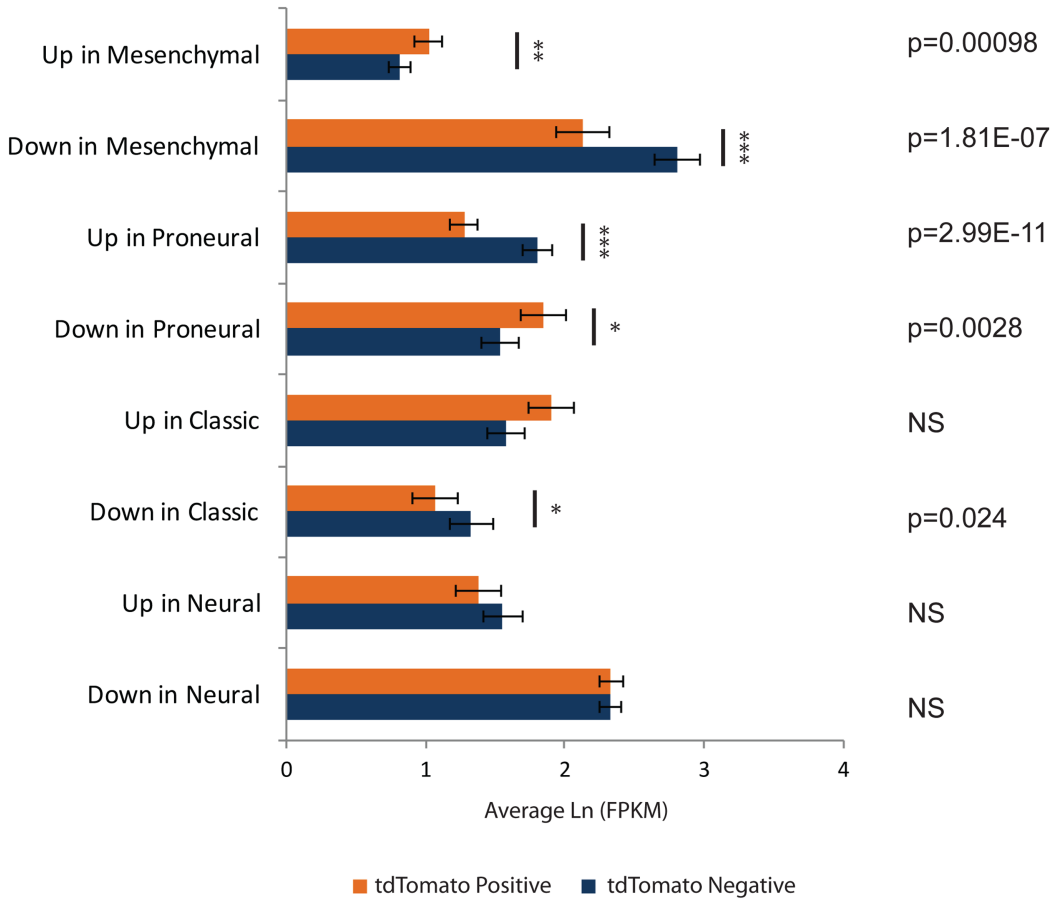

Figure S4. Average normalized gene expression of Verhaak glioma subtype gene sets. Related to Figure 2F. The log-normalized FPKM expression values from data were z-normalized across tdTomato positive POS (orange) and negative NON (navy) samples (Fig 2E) then a two-tailed Welch's t-test was used to determine if the average log-normalized FPKM values of the Verhaak upregulated and depleted genes were significantly different between POS and NON organoids.

Supplemental Experimental Procedures

Plasmid constructs

sgRNA targeted TP53 exon 4 were designed using CRISPRdirect (Naito et al., 2015)(http://crispr.dbcls.jp) For CRISPR-Cas9 TP53 sgRNA plasmid construction, single-stranded DNA oligos were designed to target the human TP53 locus exon 4 (oligo 1: 5-CACCGCCATTGTTCAATATCGTCCG-3′; oligo 2 : 5′- AAACCGGACGATATTG AACAATGGC-3') and cloned into pSpCas9(BB)-2A-GFP (PX458) or into pSpCas9(BB)-2A-Puro (PX459) as previously described (Ran et al., 2013). These plasmids were gifts from Feng Zhang (Addgene plasmid # 48138 and #48139). For Donor plasmid, the $HRas^{G12V}$ IRES tdTomato fragment was cloned into pcDNA3.1(+) between the AflII site and NotI site and then left and right homology arms added. Both homology arms were of 200 bp length with perfect homology with the genomic locus centered on Exon 4 of TP53 and flanked by the sgRNA targeted sequence. The arms with the flanking sgRNA targets were synthesized by gBlock (Integrated DNA Technologies) and then cloned into the ApeI site of pcDNA3.1 construct.

Western blotting

293T cells were maintained in 10% FBS DMEM. CRISPR-CAS9 TP53 sgRNA plasmid was transfected by the calcium phosphate transfection method into 293T cells then selected for 72 hrs with Puromycin (2 µg/ml). After another 48 hrs incubation, cells were scraped and suspended in RIPA buffer (cell signaling technologies) then sonicated at 4°C with three 30 second pulses (1 min pause between pulses). Lysate was centrifuged (14,000g, 10min) then the protein concentration of the supernatant was measured by Bio-Rad Protein Assay (Bio-Rad). 10µg protein extract per lane sample were electrophoresed through NuPAGE™ 3-8 % Tris-Acetate Protein Gels (Thermo Fisher Cat. # EA0375BOX) then gels were blotted on a PVDF membrane (Immobilon-P, Millipore), following the manufacturer's protocol. The membrane was blocked with 5% non-fat dry milk in PBS. For primary antibody rabbit anti-TP53 (Santa Cruz, sc-6243) and mouse anti-alpha tubulin (abcam, ab7291) was used, and for secondary horseradish peroxidase-conjugated anti-Rabbit IgG or anti-Mouse IgG antibody (cell signaling technology #7074, #7076) and ECL chemiluminescence reagent (Amersham) was used for detection.

Organoid culture

Cerebral organoids were generated from human Embryonic Stem (ES) cell lines H9 using the protocol previously described.(Lancaster and Knoblich, 2014) Briefly, H9 cells were maintained in mTeSR1 medium and cultured on Matrigel (8.8 $\mu g/cm^2$) (BD Biosciences) coated plates and then transferred into low-attachment 96 well plates in a low-bFGF KnockOut Serum based hESC medium with ROCK inhibitor (9000 cells per well) to generate the embryoid bodies (EB). EBs are then subjected to neural induction in a minimal medium (DMEM-F12 with 1% (vol/vol) N2 supplement, 1% (vol/vol) GlutaMAX supplement and 1% (vol/vol) MEM-NEAA with heparin (final concentration of 1 µg/ml) to develop neuroectodermal tissues and then transferred to droplets of Matrigel, which promotes outgrowth of neuroepithelial buds. The droplets are then transferred into Cerebral organoid differentiation medium (1:1 of DMEM/F12 and Neurobasal (Thermo Fisher, cat. #21103049), 0.5% N2 supplement (Thermo Fisher, cat. #17502048), 2% B-27 without vitamin A (Thermo Fisher, cat. #12587010), 0.25 % insulin solution (Sigma, cat. #I9278-5 ML), 50 µM 2-mercaptoethanol, 1% Glutamax, 0.5% MEM- NEAA, and 1% Antibiotic-Antimycotic (Thermo Fisher, cat. #15240062), then after 4 days of neuroepithelial bud expansion medium changed to Cerebral organoid differentiation medium with vitamin A (2 % B-27 + vitamin A (Thermo Fisher, cat. #17504044)) and start shaking on an orbital shaker at 80 rpm in an incubator at 5% CO_2 37°C for the duration of organoid maintenance.

Organoid electroporation

CRISPR-CAS9 TP53 sgRNA construct and donor CMV $HRas^{G12V}$ tdTomato constructs were co-transfected into 4months old cerebral organoids. Co-electroporation performed using a 3 mm diameter tweezer electrode and electro-square-porator (ECM 830) from BTX Harvard Apparatus. A total of 1 µl of 1.5 µg/ul each plasmid in PBS/ with 0.01% FastGreen was injected into 2-4 locations within the organoid surface and electroporation was performed in PBS at 5 pulses of 45 V were given with a 50 ms pulse duration over a 950 msec interval. The electroporated organoids were quickly placed back into the Cerebral organoid differentiation medium then incubated on an orbital shaker in an incubator with 5% CO_2 at 37°C and kept shaking at 80 rpm. Complete medium Change was performed every 72 hrs. The organoids were visualized and photographed using a Zeiss Axioivert 200 microscope with a 5X objective lens,

Axiocam HRc camera and ZEN 2012 (blue edition) software. The pictures were stitched with Adobe Bridge CS5.1 and Adobe Photoshop CS5.1.

Histological procedures and immunohistochemistry

Organoids were fixed in 4% paraformaldehyde in PBS on ice 2 hrs and washed in PBS then equilibrated in 30% (weight/vol) sucrose at 4°C overnight. Then mounted in the optimal cutting temperature medium (OCT) and cryosectioned at 14 µm thickness and adhered to Superfrost Plus Microscope glass slides (Fisher Cat# 12-550-15). For the brain sections, animals were anesthetized and perfused with PBS followed by 4% paraformaldehyde/PBS. The brains were dissected out and post-fixed in 4% paraformaldehyde/PBS at 4°C overnight then washed in PBS and equilibrated in 30% (vol/vol) sucrose at 4°C overnight. The brains were mounted in OCT then cryosectioned coronally (40 µm) as floating sections in 0.02% NaN_3 PBS. Immunostaining was performed using standard procedures for tissue cryosections. The sections were blocked in blocking buffer, 5% Normal Goat Serum/ 3% BSA/ PBS/ 0.01% tween-20 then incubated in primary antibody solution in blocking buffer at 4°C overnight. After washing in PBS-T (0.1% Tween-20) sections were incubated with secondary antibody solution 2hr and washed with PBS-T then mounted with ProLong™ Gold Antifade Mountant (Thermo Fisher). Primary antibodies include: rabbit anti-Ki-67 (1:300, VECTOR VP-RM04), rat anti-SOX2 (1: 200, eBioscience #14-9811), rabbit anti-Olig2 (1:200, abcam ab109186), mouse anti-GFAP (1:100, Cell Signaling #3670), rabbit anti-SATB2 (1:200, abcam ab34735), rabbit anti-NeuN (1:200, Millipore #ABN78A4), mouse anti-Tuj1 (1:500, BioLegend #801202), rat anti-CD31 (1:50, BD Pharmingen #553370), mouse anti-Reelin (1:200, MBL D223-3). The imaging was performed with a Zeiss LSM 710 confocal microscopy system with the Zeiss Zen Black software package.

Cell counts and statistical analysis

The organoids were stained with anti-tdTomato antibody for enhancement of tdTomato signals. Then co-stained with the neural progenitor markers SOX2, OLIG2, and proliferating cell marker Ki-67 and DAPI nuclear counterstaining. Then imaged using a Zeiss LSM 710 with a 20X objective lens Z sections 10µm thick. Then nuclear (DAPI+, SOX2+, OLIG2+, Ki-67+) were each counted with Imaris (BITPLANE ver. 8) for three fields of 400 µm × 400 µm x 10 µm thickness from different cortical areas. Each SOX2+, OLIG2+, Ki-67+ nuclear counts were normalized by the number of DAPI positive nuclei. Data are presented as mean ± SEM and were analyzed using paired two-tailed Student's t test to determine significance. Statistical significance p values < 0.05 were considered significant.

Cell sorting and RNA sequencing

The transduced organoids were dissociated in 500 µl of Accutase containing 0.025 U/µL Benzonase nuclease (Sigma) for 30 min then passed through a mesh filter to create a single-cell suspension. Cells were collected by centrifugation at 300 × g for 5 min and resuspended in cerebral organoid differentiation medium (1 to $3x10^6$ cells per ml). FACS sorting was done with a Becton-Dickinson Influx™ cytometer eliminated with gate exclusion of dead cells performed by DAPI staining and sorted into TriZol LS directly. Data was analyzed with the FlowJo software. RNA was purified by Direct-zol™ RNA MicroPrep (zymo research) and cDNA libraries were prepared using the TruSeq RNA Sample Prep kit (Illumina). RNA sequencing was performed using a HiSeq 2500 Sequencing System (Illumina).

RNA-seq Analysis

Sequenced reads were quality-tested using FASTQC v0.11.5 and aligned to the hg19 human genome using the STAR aligner version 2.5.1b. Mapping was carried out using default parameters, filtering non-canonical introns and allowing up to 10 mismatches per read and only keeping uniquely mapped reads. The genome index was constructed using the gene annotation supplied with the hg19 Illumina iGenomes collection and sjdbOverhang value of 100. Raw or FPKM (fragments per kilobase per million mapped reads) gene expression was quantified across all gene exons with HOMER using the top-expressed isoform as proxy for gene expression.

Glioblastoma signature analysis

Subtype-specific gene signature centroids were obtained and k-means clustering was used (Cluster 3 v 1.5.2) to identify sets of genes upregulated or depleted in neural, proneural, mesenchymal, or classical GBM subtypes. The log-normalized FPKM expression values from data were z-normalized across tdTomato positive (POS) and negative (NON) samples, centered, and visualized with TreeView v1.1.6r4. The log-normalized FPKM values for each of the 8 gene signature sets were tested for differences in mean between POS and NON samples (Welches 2-tailed t-test) to show altered expression of signature genes between NON and POS samples. All subtype signature genes were then pooled and their log-normalized expression values were k-means clustered (Cluster 3) to find if these genes were either up in POS organoids, down in POS organoids, or part of an ambiguous set. Then for each of the 8 sets of marker genes, the proportion belonging to the up in POS cluster compared to the proportion that are in the down in POS cluster were plotted, and significance was evaluated with a binomial test.

Cell culture and genotyping

The transduced organoids were dissociated in 500 µl of Accutase containing containing 0.025 U/µL Benzonase nuclease (sigma) for 30 min cells were centrifuged at 300 × g for 5min and re-suspended in 2 ml of Cerebral organoid differentiation medium then plated on a Matrigel coated 6 well plate. The genomic DNA was prepared from cultured cells using DNeasy Blood & Tissue Kits (Qiagen) for genotyping. For Sanger sequencing, the TP53 locus was amplified by PCR with specific primers (forward primer 5'- AGACCTGTGGGAAGCGAAAAT-3' and reverse primer 5'-TTCTGGGAGCTTCATCTGGAC-3' or 5'-ATGCGGCATCAGAGCAGATT-3') and cloned into pGEM-T easy and then analyzed by Sanger sequencing using the T7 primer. For Southern blot analysis, 2µg of genomic DNA was digested with Hind III at 37°C overnight and separated by 0.8% agarose gel electrophoresis, and then blotted onto Hybond-N+ nylon membranes (Amersham Biosciences). DIG-labeled probes were produced by PCR DIG Probe Synthesis kit (Roche) and hybridization using DIG Easy Hyb (Roche) was performed following the manufacturer's protocol. For a probe template, 1412bp of the tdTomato coding sequence was amplified by PCR using specific primers (forward primer 5'- GGGCGAGGAGGTCATCAAAGA-3' and reverse primer 5'-ACAGCTCGTCCATGCCGTA-3') and also 1416bp of the TP53 locus was amplified by PCR using specific primers (forward primer 5'-AGACCTGTGGGAAGCGAAAAT-3' and the reverse primer 5'- AGCTGCTCACCATCGCTATC-3')

Lentiviral Production and Cell Infection

pCSC-SP-PW-tdTomato was co-transfected with packaging plasmids (pMDL, Rev and VSVg) into 293T cells by the calcium phosphate transfection method. Cell culture supernatants were collected 48 hrs after and 72 hrs after the transfection and concentrated by ultracentrifugation at 50,000 x g for 2 hrs at RT. The viral pellets were re-suspended in HBSS and applied on top of a 20% sucrose/HBSS cushion then re-concentrated by 50,000 x g centrifugations for 90 min at RT. The viral pellets were re-suspended in HBSS, then aliquots were stored at -80°C. SK429, SK2176 were plated on 24 well plates then allowed to adhere overnight. 2µl of the virus solution (1 x 10^6 viral particle/µl) was added to each well and the cells were incubated for 24 hrs then media was changed to fresh virus free medium. The cells were incubated for an additional 5days and then sorted for tdTomato positive and negative cell fractions which were then expanded and cryopreserved.

Mouse orthotopic xenografts

The transduced organoid derived cell lines or human patient derived tumor cell lines were dissociated with TrypLE. Single cell suspensions in Cerebral organoid differentiation medium were stereotaxically injected (3×10^5 cells per animal) into the hippocampus (AP -2.3 mm, ML 1.5 mm, DV 2.0 mm) of NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ mice as previously described(Marumoto et al., 2009).

Sphere co-culture with intact organoids

The transduced organoid derived cell lines were cultured on non-coated plastic dishes with Cerebral organoid differentiation medium to induce sphere formation. Human patient derived glioblastoma cell lines were transferred into low-attachment 96 well plates in a in Cerebral organoid differentiation medium at a density of 18,000 cells per well. 72 hrs after the tumorsphere formation, these were transferred into 6well plates and mixed with intact human cerebral organoids (10 sphere with 5 organoids per well) and incubated on an orbital shaker in an incubator kept at

5% CO_2 37°C and kept shaking at 80rpm. For maintenance, a complete change of medium was performed every 72 hrs and the organoids were visualized and photographed using Zeiss Axiovert 200 microscope with a 5X objective lens, Axiocam HRc camera and ZEN 2012 (blue edition) software. The pictures were stitched with Adobe Bridge CS5.1 and Adobe Photoshop CS5.1.

Supplemental References

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Resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat anti-tdTomato	MyBioSource	Cat# MBS448092
Rabbit anti-Ki-67	VECTOR	Cat# VP-RM04
Rat anti-SOX2	eBioscience	Cat# 14-9811
Rabbit anti-Olig2	abcam	Cat# ab109186
Chicken anti-GFAP	Millipore	Cat# AB5541
Rabbit anti-SATB2	abcam	Cat# ab34735
Rat anti-Ctip2	abcam	Cat# ab18465
Rabbit Anti-NeuN (rabbit) Antibody, Alexa Fluor® 488 Conjugate	Millipore	Cat# ABN78A4
Mouse anti-Tuj1	BioLegend	anti-Tubulin β 3 (TUBB3) Previously Covance Cat# MMS-435P#801202
Rat anti-CD31	BD Pharmingen	Clone MEC 13.3 Cat# 553370
Mouse anti-Reelin	MBL International Corporation	(CR-50) mAb Cat# D223-3
Rabbit anti-TP53	Santa Cruz	Cat# sc-6243
Mouse anti-alpha tubulin	abcam	Cat# ab7291
Horseradish peroxidase-conjugated anti-Rabbit IgG	Cell Signaling Technology	Cat# 7074
Horseradish peroxidase-conjugated anti-Mouse IgG	Cell Signaling Technology	Cat# 7076
Bacterial and Virus Strains		

pCSC-SP-PW-tdTomato (lentivirus)	Salk Institute Verma lab Tiscornia et al., (2006)	
Chemicals, Peptides, and Recombinant Proteins		
mTeSR1 medium	Salk Institute Stem Cell core	N/A
DMEM-F12	Thermo Fisher	Cat# 11330032
KnockOut SR	Thermo Fisher	Cat# 10828028
GlutaMAX	Thermo Fisher	Cat# 35050061
MEM non-essential amino acids	Thermo Fisher	Cat# 11140076
2-Mercaptoethanol	Millipore	Cat# 805740
bFGF	Peprotech	Cat# 100-18B
ROCK inhibitor Y27632	Reagents Direct	Y27632
N2 supplement	Thermo Fisher	Cat# 17502048
B27 – vitamin A supplement	Thermo Fisher	Cat# 12587010
B27 + vitamin A supplement	Thermo Fisher	Cat# 17504044
Antibiotic-Antimycotic	Thermo Fisher	Cat #15240062
Normocin	InvivoGen	Cat# ant-nr-1
Neurobasal medium	Thermo Fisher	Cat# 21103049
Insulin solution	Sigma	Cat# I9278-5ML
Growth factor–reduced Matrigel	BD Biosciences	Cat# 356230
TrypLE	Thermo Fisher	Cat# 12604039
Accutase	Innovative Cell Technologies	Cat# AT104
Paraformaldehyde	Electron Microscopy Sciences	Cat# 15714-S
TRIzol LS Reagent	Thermo Fisher	Cat# 10296-010
ProLong™ Gold Antifade Mountant	Thermo Fisher	Cat# P36930
Benzonase® Nuclease	Sigma	
Critical Commercial Assays		
The NuPAGE® Precast Gel System	Thermo Fisher	https://www.thermofisher.com/us/en/home/brands/product-brand/nupage.html
Amersham ECL Prime Western Blotting Detection Reagent	GE Healthcare Bio-Sciences	Cat# RPN2232
TruSeq RNA Sample Prep kit	Illumina	
Direct-zol™ RNA MicroPrep	Zymo	Cat# R2060
DNeasy Blood & Tissue Kits	QIAGEN	Cat# 69504
Bio-Rad Protein Assay	Bio-Rad	Cat# 5000001
Deposited Data		
hg19 human genome	The Genome Sequencing Consortium	Initial sequencing and analysis of the human genome. Nature. 2001 Feb 15;409(6822):860-921
Verhaak subtypes of clinical samples of glioblastoma	Verhaak et al., 2011	http://tcga-data.nci.nih.gov./
Experimental Models: Cell Lines		
Human ES cell lines (H9)	Salk Institute Stem Cell core	N/A
293T cell	ATCC	293T (ATCC® CRL-3216™)
SK429 human patient derived glioblastoma cell lines	S. Kesari (University California, San Diego	SK429

SK2176 human patient derived glioblastoma cell lines	S. Kesari (University California, San Diego	SK2176
Experimental Models: Organisms/Strains		
Mouse: NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ	The Jackson Laboratory	Stock No: 005557 | NSG
Oligonucleotides		
sgRNA target the human TP53 locus exon 4 (oligo 1: 5-CACCGCCATTGTTCAATATCGTCCG-3′; oligo 2 : 5′-AAACCGGACGATATTGAACAATGGC-3’)	This paper	N/A
TP53 locus PCR for Sanger sequencing primer (forward primer 5’- AGACCTGTGGGAAGCGAAAAT-3’ and reverse primer 5’-TTCTGGGAGCTTCATCTGGAC-3’ or 5’-ATGCGGCATCAGAGCAGATT-3’)	This paper	N/A
TP53 locus PCR for probe template primer (forward primer 5’- AGACCTGTGGGAAGCGAAAAT-3’ and and the reverse primer 5’- AGCTGCTCACCATCGCTATC-3’)	This paper	N/A
tdTomato PCR for probe template primer (forward primer 5’- GGGCGAGGAGGTCATCAAAGA-3’ and reverse primer 5’-ACAGCTCGTCCATGCCGTA-3’)	This paper	N/A
Recombinant DNA		
Plasmid: pSpCas9(BB)-2A-GFP (PX458)	addgene	Plasmid #48138
Plasmid: pSpCas9(BB)-2A-Puro (PX459)	addgene	Plasmid #48139
Software and Algorithms		
CRISPRdirect	Naito Y. et. al., (2015)	http://crispr.dbcls.jp/
FASTQC v0.11.5	Andrews S., (2010)	FastQC: a quality control tool for high throughput sequence data. Available online at:http://www.bioinformatics.babraham.ac.uk/projects/fastqc
STAR aligner version 2.5.1b	Dobin A. et al., (2013)	
hg19 Illumina iGenomes collectio	Illumine (2015)	http://support.illumina.com/sequencing/sequencing_software/igenome.html
HOMER	Heinz S. et al., (2010)	
DeSeq2 package version 1.12.4	Love, M. I., et al., (2014)	
Cluster 3 v 1.5.2	de Hoon, M. J., et al., (2004)	
TreeView v1.1.6r4	Saldanha, A. J. (2004)	
Imaris Ver. 8	Bitplane	
Zeiss Zen Black	Zeiss	
ZEN 2012 (blue edition)	Zeiss	
FlowJo	FlowJo, llc	
Other		
U-bottom ultralow attachment plates, 96 well	Corning	Cat# CLS7007
Ultralow attachment plates, 24 well	Corning	Cat# CLS3473
Orbital shaker	Cole-Parmer	Cat# UX-51820-40