Supplemental Methods

Cas9 and dCas9-KRAB genome-wide enrichment screening

The human CRISPRi-v2 library (83969, Addgene) and the human Brunello library (73178, Addgene) were used for DAOY$^{dCas9\text{-}KRAB}$ and DAOY^{Cas9} genome-wide enrichment screens, respectively. For amplification, libraries were diluted in ddH_2O to a final concentration of 50ng/µL, and 2µL was added to 25µL of Endura electrocompotent cells (60242, Lucigen) for electroporation in a 1.0mm GenePulser electroporation cuvette (1652083, BioRad) at 10µF, 600Ohms, and 1800V using a Gene Pulser Xcell electroporation system (1652100, BioRad). Following the addition of 975µL of recovery medium (80026, Lucigen) immediately after electroporation, suspensions were transferred to separate tubes containing another 1mL of recovery medium. At least 4 electroporations were performed per amplification and were rescued for 1 hour at 37°C at 220rpm. Electroporation reactions were pooled, and a 1:40000 dilution was plated on a 10 cm^2 LB agar plate supplemented with ampicillin (14417, Cayman Chemical). In parallel, 4mL was plated on Bioassay plates (43111, Thermo Fisher Scientific) containing LB agar supplemented with ampicillin. The next day, colonies were scraped after adding 10mL of LB and pelleted at 15,000g for 20 minutes, and library plasmids were isolated using a HiSpeed Plasmid Maxi Kit (12663, Qiagen) with ~0.45g of bacteria per column.

To produce lentivirus libraries for DAOY^{Cas9} and DAOY$^{dCas9\text{-}KRAB}$ genome-wide enrichment screens, pooled library plasmids were co-transfected with the lentiviral packaging plasmids psPAX2 and pMD2.G (12260 and 12259, Addgene) into 40 15 cm^2 plates and harvested, filtered, and concentrated as described below. Cells were transduced using spinfection at a multiplicity of infection (MOI) of 0.2-0.5. Viral titers and MOI calculations were performed as described previously (41). In brief, viral titers were estimated by seeding $3x10^6$ cells per well into a 12 well plate in growth media supplemented with 10µg/mL polybrene (TR-1003-G, Sigma-Aldrich), and increasing amounts of viral volume were added per well. The plate was centrifuged at 2,000 rpm for 2 hours at room temperature, and the next day, cells were counted and split into duplicate wells with one well receiving 1µg/mL puromycin (P8833, Sigma-Aldrich) for 2-3 days. Cells were counted and the MOI was calculated by dividing the cell count from the puromycin well by the replicate without puromycin, and the virus volume yielding an MOI closest to ~0.3 was selected for genome-wide enrichment screens. To do so, DAOY^{Cas9} and DAOY$^{dCas9\text{-}KRAB}$ cells were transduced with their respective pooled lentiviral libraries so each sgRNA was represented

100x. Infected cells were expanded for 7d under puromycin selection, and $60x10^6$ cells per condition were seeded into a 15 cm^2 plates and treated with either abemaciclib 0.1μM (the concentration achieving ~90% growth inhibition) or DMSO as a vehicle control. Cells were passaged when confluent, but a minimum of 33% of cells were kept from each passage. Fresh media containing either abemaciclib (17740, Cayman Chemical) or an equal volume of vehicle control (DMSO) was replenished every 3-4 days, and cell pellets containing a minimum of $100x10^6$ cells were collected from each condition after 10 days of selective pressure.

Genomic DNA was extracted as described previously (42). In brief, 6mL of NK Lysis Buffer (50mM Tris, 50mM EDTA, 1% SDS, pH 8) supplemented with 30μL of 20mg/mL Proteinase K (PR-V3021, Fisher Scientific) was added to cell pellets and incubated overnight at 55°C. The next day, 30μL of 10mg/mL RNase A (19101, Qiagen) was added to lyse the samples, which were inverted 25 times, and incubated at 37°C for 30 minutes. Samples were cooled on ice, and 2mL of 7.5M ammonium acetate (A1542, Sigma-Aldrich) was added. Samples were vortexed for 20 seconds, and then centrifuged at >4,000g for 10 minutes. The supernatant was decanted into a new 15mL conical tube and 5mL of 100% isopropanol was added, inverted 50 times, and centrifuged at >4000g for 10 minutes. The supernatant was discarded again and 6mL of freshly prepared 70% ethanol was added to each tube, inverted 10 times, and centrifuged at >4000g for 1 minute. The remaining ethanol was aspirated off and the pellet was allowed to air dry until slightly translucent. Genomic DNA pellets were re-suspended in 200μL of EB Buffer (19086, Qiagen) and left at room temperature overnight to fully resuspend.

The sgRNA library that was enriched in each sample was prepared for sequencing using a two-step PCR reaction, where the first step amplified the region containing the sgRNA cassette maintaining representation, and the second step added appropriate sequencing adaptors (Supplemental Table 3). 100 bp paired-end reads were sequenced on an Illumina HiSeq 4000 to at least 11 million unique reads per sample at the Center for Advanced Technology at the University of California San Francisco, resulting in at least 100x coverage per sgRNA.

Sequencing data were demultiplexed and mapped to sgRNA sequences using MaGeCK-VSIPR (43). Genes were ranked using the same algorithm, which considers sgRNA enrichment as well as the number of sgRNAs targeting each gene. Positive enrichment gene candidates were prioritized for further analysis, as opposed to negative enrichment gene candidates, due to insufficient sequencing depth to accurately identify negatively enriched genes in our study, and

more broadly, the propensity for false positives with negative selection (44). Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) analyses were performed using String-DB. The top 100 genes as ranked by sgRNA enrichment from the MaGeCK analysis were input into the STRING portal to discover significant GO or KEGG pathways (Supplemental Figure 2B) (45). For combined analysis of DAOY^{Cas9} and DAOY$^{dCas9\text{-}KRAB}$ genome-wide enrichment screens, the top 100 genes from each screen were aggregated with repeat genes appearing only once (Figure 1C).

Cell Culture

DAOY cells (HTB-186, ATCC), HEK293T cells (CRL-3216, ATCC), ONS76 cells (gift from T. Milde), and UW228 cells (gift from T. Milde) were cultured in DMEM medium (11960069, Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS) and glutaMAX (35050079, Life Technologies) at 37°C in a humidified atmosphere at 5% CO_2. Abemaciclib was dissolved in DMSO and used at the following concentrations: 0.1μM, 0.33μM, 0.5μM, 1μM (dose-titration, Supplemental Figure 1D, 1E, 5I, 5L), 2μM (conditioned resistance cells, Supplemental Figure 2D), and also at 0.1μM for enrichment screens and clonogenic assays. Conditioned cells were generated by treating DAOY cultures with abemaciclib 0.25μM and increasing the concentration by 0.25μM each week until a final concentration of 2μM was achieved. For ciliation and Hedgehog signaling assays, DAOY, UW228, and ONS76 cultures were transitioned to OptiMEM (51985091, Thermo Fisher Scientific) and treated with recombinant Sonic Hedgehog (SHH) 1μg/mL (1845-SH, R&D Systems) or vehicle control. KIRA6 (Figure 1L) was dissolved in DMSO and used at a concentration of 500μM (19151, Cayman Chemical). GSK2606414 (Supplemental Figure 5A) was dissolved in DMSO and used at a concentration of 250μM (1337531-89-1, Calbiochem). For clonogenic assays, 1000 cells were seeded in a 10 cm^2 plate and grew for 10 days. Cells were fixed in methanol for 30 minutes and stained with 0.01% crystal violet (C6158, Sigma-Aldrich) for 1 hour. Plates were rinsed with water three times, allowed to air dry, digitally scanned, and colony area was quantified by the ImageJ Plugin ColonyArea (46).

DAOY^{Cas9} and DAOY$^{dCas9\text{-}KRAB}$ cell generation

DAOY^{Cas9} or DAOY$^{dCas9\text{-}KRAB}$ cells were generated by producing lentiCas9-Blasticidin (52962, Addgene) or pHR-UCOE-SFFV-dCas9-BFP-KRAB (gift from J. Weissman) lentiviruses

as described above. DAOY cells were transduced with lentiviruses and selected with either 5μg/mL blasticidin (B-800-25, GoldBio), or sorted for the top ~25% BFP expression on a Sony SH800 FACS, respectively. Single cells were sorted into 96-well plates and expanded. Clones were tested by immunoblot for the FLAG epitope fused to Cas9, or the HA epitope fused to dCas9-KRAB. Clones with high expression were selected and used for experiments by transducing $DAOY^{Cas9}$ or $DAOY^{dCas9\text{-}KRAB}$ cells with sgRNA lentiviruses, and selected with puromycin 1μg/mL for at least 3 days before mechanistic or functional interrogation.

Genome-wide enrichment screening validation

Targeted sgRNA protospacer sequences (Supplemental Table 3) with proper overhangs were cloned into either the two-vector systems lentiGuide-Puro (52963, Addgene) or pCRISPRia-v2 (84832, Addgene) following a BsmBI (R0580, New England Biolabs) restriction digest or a BstXI (R0113, New England Biosystems) and BlpI (R0585, New England Biolabs) restriction digest, respectively. For one-vector systems used in UW228 and ONS76 experiments, these same protospacer sequences with proper overhangs were cloned into pLVhU6-sgRNA hUbC-dCas9-KRAB-T2A-Puro (71236, Addgene) following a BsmBI digest. Cloning was confirmed by Sanger sequencing and plasmids were isolated from midipreps (12415, Qiagen). Lentiviruses were produced and DAOY, ONS76, and UW228 cells were transduced as described below.

Lentiviral production and transduction

Plasmids were co-transfected into HEK293T cells with the lentiviral packaging plasmids psPAX2 and pMD2.G. In brief, $3.5x10^6$ HEK 293T cells were seeded in a poly-D-lysine (P6407, Sigma-Aldrich) coated 10 cm^2 plate the day before transfection. For each 10 cm^2 plate, the following DNAs were diluted into 600μL of OptiMEM: 4.5μg construct, 6μg psPAX2, and 1.5μg pMD2.G. Separately, 36μL of TransIT-Lenti transfection reagent (MIR6606, Mirus) was diluted into 600μL of OptiMEM, briefly vortexed, and incubated for 5 minutes. The DNA and Mirus solutions were combined, and allowed to incubate at room temperature for 20 minutes. Meanwhile, the cell culture media on HEK293T cells was replaced with 9mL of pre-warmed DMEM supplemented with 1% FBS and glutaMAX, and the transfection mixture was added dropwise onto cells. Viral particles were harvested both 48 hours and 72 hours following transfection by passing the cell culture medium through a 0.45μM PVDF syringe filter (F5510, Denville Scientific). All

previously stated amounts were doubled when producing virus from a 15 cm^2 plate, when larger quantities of virus were desired. The filtered supernatant was used for transduction directly, or concentrated in 100kD Amicon Ultra Centrifugal Filter Units (UFC910024, EMD Millipore), and diluted in complete growth medium supplemented with 10µg/mL polybrene before addition to $DAOY^{Cas9}$, $DAOY^{dCas9\text{-}KRAB}$, $ONS76^{dCas9\text{-}KRAB}$, or $UW228^{dCas9\text{-}KRAB}$ cells. For long-term storage, aliquots of lentiviral particles were snap-frozen and stored at -80°C.

Histology and light microscopy

Histology and light microscopy were performed as described previously (11). In brief, mouse cerebella were fixed overnight in 4% paraformaldehyde, washed in PBS, embedded in paraffin, and sagittal sections were stained with H&E. Tumor section and fresh brain samples were imaged on an inverted light microscope (Zeiss).

Immunoblotting

Cells were dislodged with 0.05% trypsin-EDTA (25300120, Thermo Fisher Scientific) and pelleted at 300g for 5 minutes. Pellets were resuspended in Dulbecco's phosphate buffered saline (DPBS) (SH30028.02, GE Healthcare) and re-pelleted at 300g for 5 minutes. Lysates were prepared by re-suspending cell pellets in lysis buffer (1% NP-40, 150mM NaCl, 50mM Tris pH 7.4, protease inhibitor, and phosphatase inhibitor all in DPBS) on ice. Lysates were centrifuged at 22,000g for 15 minutes at 4°C, and the supernatant was transferred to a fresh tube. Protein concentrations were determined using a Bradford assay (5000002, BioRad). Proteins were separated on pre-cast sodium dodecyl sulfate-polyacrylamide 4-20% gels (4561093 or 4561096, BioRad), transferred to nitrocellulose membranes, blocked with 5% milk in Tris-buffered saline (10mM Tris, pH 8.0, and 150mM NaCl) with 0.1% Tween (TBS-T) for 1 hour at room temperature, and incubated with the following primary antibodies for one hour in blocking solution: ATF4 (1:1000, Rabbit, 11815, Cell Signaling Technology), Actin (1:5000, Mouse, 8226, Abcam), eIF2α (1:1000, Rabbit, 9722, Cell Signaling Technology), p-eIF2α (1:1000, Rabbit, 9721, Cell Signaling Technology), RPL23A (1:500, Rabbit, 16386, Proteintech), FLAG (1:1000, Rabbit, 14793, Cell Signaling Technology), GAPDH (1:1000, Mouse, MA515738, Thermo Fisher Scientific), HA (1:1000, Mouse, 2999, Cell Signaling Technology), or HA-tagged HRP conjugate (1:1000, Mouse, 2999, Cell Signaling Technology). Membranes were washed and incubated with

in Peroxidase AffiniPure Goat Anti-Rabbit IgG (1:5000, Goat, 111-035-144, Jackson ImmunoResearch) or Peroxidase AffiniPure Goat Anti-Mouse IgG (1:5000, Goat, 111-035-146, Jackson ImmunoResearch). Membranes were developed using SuperSignal West Pico PLUS or SuperSignal West Femto PLUS Chemiluminescent substrate (34577 or 34096, ThermoFisher Scientific).

Immunofluorescence and Fluorescence Microscopy

Immunofluorescence for DAOY, ONS76, and UW228 cilia was performed on glass coverslips. Cells were fixed in 4% paraformaldehyde for 8 minutes, blocked in 2.5% FBS and 0.1% Triton X-100 (X100, Sigma-Aldrich) in DPBS for 1 hour at room temperature. All antibody incubations were performed for 1 hour in blocking solution at room temperature or overnight at 4°C with anti-acetylated-tubulin (1:1000, Mouse, T7451, Sigma-Aldrich), anti-Smoothened (1:1000, Rabbit, ab72130, Abcam), donkey anti-mouse linked with Alexa Fluor 488 (A21202, Invitrogen), or donkey anti-rabbit linked with Alexa Fluor 568 (A10042, Invitrogen). DNA was stained using Hoescht 33342 (1:1000, H3570, Life Technologies) and coverslips were mounted in ProLong Diamond Antifade Mountant (P36965, Thermo Fisher Scientific).

For staining with Perfringolysin O-647 reagent (PFO) (29), cells were seeded onto glass coverslips and blocked for 15 minutes in ice-cold PBS supplemented with 10mg/mL BSA (A2153, Sigma-Aldrich) on ice. PFO was diluted into blocking buffer (1:45) and incubated with cells for 30 minutes followed by one quick wash in PBS with Hoescht (1:1000). Cells were then fixed in 4% paraformaldehyde for 8 minutes, washed with PBS, and coverslips were mounted in ProLong Diamond Antifade Mountant.

Fluorescence microscopy was performed on a Zeiss LSM 800 confocal laser scanning microscope. Acquisition parameters were controlled with Zeiss Zen 2.3 software. Images were collected at room temperature (25°C) using either a Plan-APOCHROMATIC 63x/1.4 oil-immersion objective or Plan-APOCHROMATIC 10x/0.45 objective. For quantification of PFO signal intensity, 2D maximum projections were saved as 16-bit TIFF images, and regions of interest were drawn around 10 cell clusters. PFO integrated density was divided by the area of measurement to give a signal/area value. Data was normalized to control cells. For quantification of ciliary SMO, 2D maximum projections were saved as 16-bit TIFF images, and SMO signal (SS) was measured by tracing the cilia using acetylated-tubulin as a marker. Background was subtracted

by measuring the equivalent in the regions just above and below (ST and SB) for a final formula of (SS – (ST + SB)/2).

Lipidomic mass spectrometry

Sterol and oxysterol quantitation was carried out by ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) using stable isotope-labelled internal standards (d_7-cholesterol, d_7-7-dehydrocholesterol, $^{13}C_3$-desmosterol, and $^{13}C_3$-lanosterol for sterols and d_7-7-ketocholesterol for oxysterols) as described previously (30, 47). Each sterol or oxysterol level was normalized to the corresponding protein weight of the cognate sample.

Mice

Mice were generated and treated as described previously (11, 30). In brief, *B6.Cg-Tg(Atoh1-cre)1Bfri*/J (011104, *Math1-Cre*), *GT(ROSA)26Sor*$^{tm1(Smo/EYFP)Amc}$/J (005130, *SmoM2*^{c}) and *Ptch1*^{tm1Mps}/J (003081, *Ptch1*^{c}) alleles were obtained from The Jackson Laboratory. The *Cdk6*^{ko} and *Hsd11ß2*^{c} alleles were obtained through collaborations (48, 49). Animals were gavaged with abemaciclib 50µg/g or palbociclib 50µg/g (Pfizer), injected with carbenoxolone 50µg/g (CNX, 3096, Tocris) in the peritoneal cavity, or treated with an equal volume of vehicle control by the same route of administration, daily, for 14 consecutive days from P21 to P35. Tumor weights were normalized to brain weights after euthanasia at P35. Animals for survival studies were monitored until death or protocol-defined neurologic endpoints, such as hydrocephalus or ataxia.

Molecular and cell biology

For quantitative reverse transcriptase PCR (qRT-PCR), RNA was isolated from cells using the RNeasy Mini Kit using Qiacubes (Qiagen). cDNA was synthesized using the iScript cDNA synthesis kit (1708891BUN, BioRad), and genes of interest were amplified using PowerUp SYBR Green Master Mix (A257342, Thermo Fisher Scientific) on a Life Technologies QuantStudio 6 Flex Real Time PCR System (4485694, ThermoFisher Scientific) (Supplemental Table 3). The ΔΔCt method relative to *GAPDH* expression was used to quantify gene expression.

RNA sequencing

Library preparation on RNA isolated from 6 *Math1-Cre SmoM2^{c} Cdk6$^{ko/ko}$* mouse medulloblastomas (RIN >8) was performed using the TruSeq Stranded mRNA Library Prep Kit (20020594, Illumina). This kit specifically enriches for mRNA using poly-T beads and fragments and ligates sequencing adaptors to the resulting cDNA for next generation sequencing, thereby eliminating ribosomal RNA (rRNA) contaminates. Fifty base-pair single-end reads were sequenced on an Illumina HiSeq 4000 to at least 37 million unique reads per sample at the Center for Advanced Technology at the University of California San Francisco. Quality control of FASTQ files was performed with FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were trimmed with Trimmomatic to remove leading and trailing bases with quality scores below 28, as well as any bases that did not have an average quality score of 28 within a sliding window of 4 bases. Any reads shorter than 37 bases after trimming were removed. Reads were subsequently mapped to the mouse reference genome GRCm38.p6 (https://www.ncbi.nlm.nih.gov/grc/mouse) using HISAT2with default parameters, which successfully mapped ~90% of reads to annotated genes (50). For differential expression analysis, exon level count data were extracted from the mapped HISAT2 output using FeatureCounts (51). Differential expression analysis was done with DESeq2, using the 'apeglm' parameter to calculate log fold changes after setting a false discovery rate of 0.05 (52). Differentially expressed genes were identified as those with log fold changes greater than 1 and an adjusted p-value less than 0.05. For GO and KEGG analyses of differentially expressed genes in 6 *Math1-Cre SmoM2^{c} Cdk6$^{ko/ko}$* versus 3 *Math1-Cre SmoM2^{c}* (11) medulloblastomas, the 126 downregulated transcripts were processed in the STRING portal as described above (45).

Data Accessibility

All raw sequencing data can be accessed at the Gene Expression Omnibus (GEO) under the accession number GSE164311.

Statistics

All experiments were performed with at least 2 biologic replicates, and were performed at least 3 times. Lines represent mean and error bars represent standard error of the mean. Overall survival was estimated using the Kaplan-Meier method and compared by log-rank tests. Two-

tailed Student's unpaired t-test and Tukey's multiple comparisons test were used to compare groups, and in all cases, statistical significance, as denoted by (*-****), was defined as $p \leq 0.05$

Study approval

All animal protocols and experimental protocols were approved by the University of California San Francisco Institutional Animal Care Use Committee (AN174769-02).

Author Contributions

VD and DRR designed the study and analysis. Experiments were performed by VD, JH, AB, AC, ALK, PK, PT, AL and DRR. Data analysis was performed by VD, JH, AB, AC, PT, AL and DRR. The study was supervised by LX, JFR and DRR. The manuscript was prepared by VD and DRR with input from all authors.

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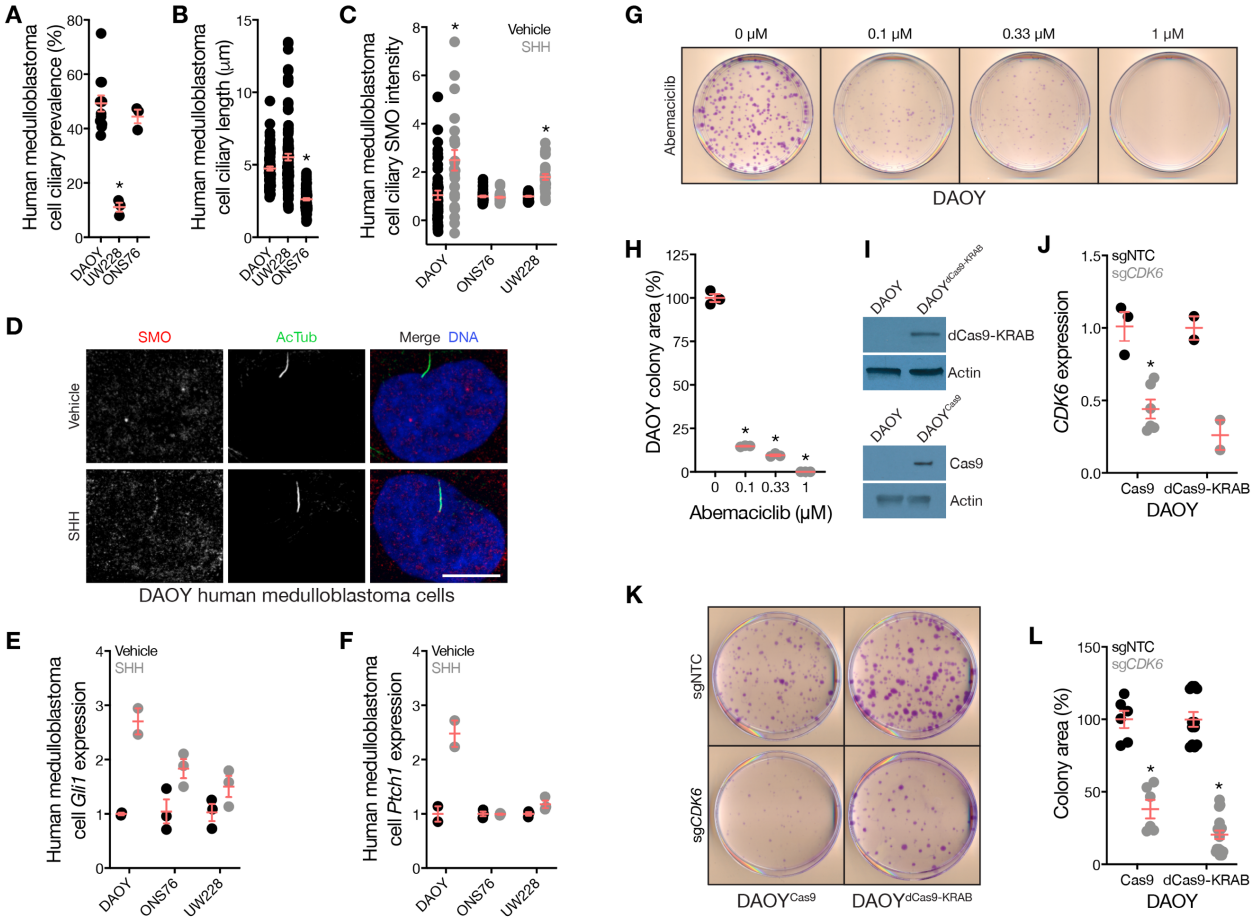
Supplemental Figures

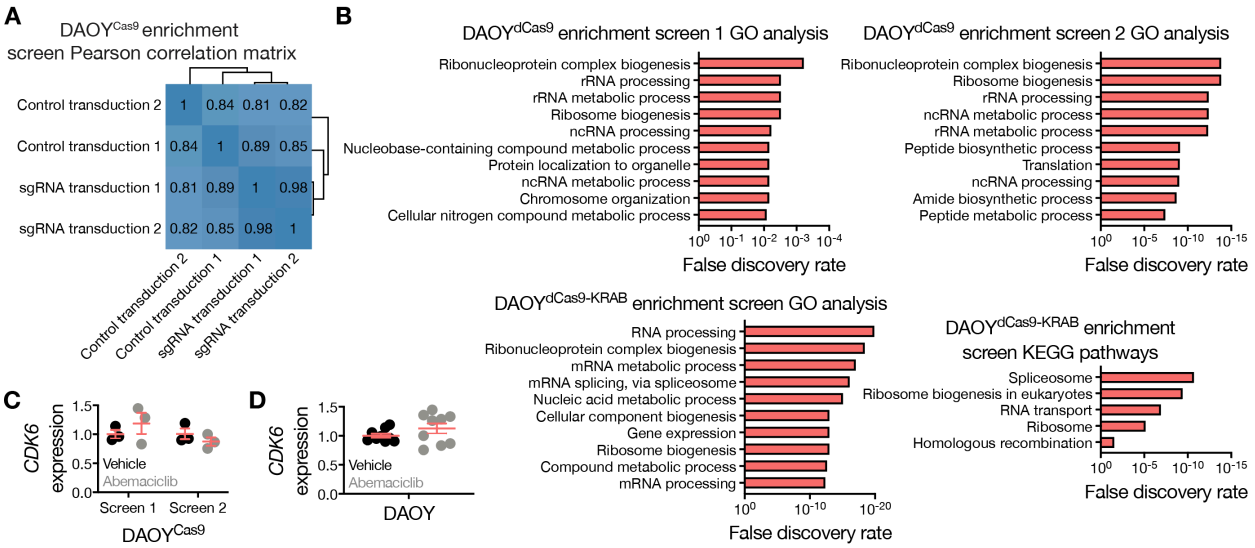
Supplemental Figure 1. Characterization and generation of human Hedgehog-associated medulloblastoma cells for genome-wide enrichment screens.



Supplemental Figure 1. Characterization and generation of human Hedgehog-associated medulloblastoma cells for genome-wide enrichment screens. (A, B) Quantitative immunofluorescence microscopy for the ciliary marker AcTub to quantify ciliary prevalence and length in human HH-associated medulloblastoma cells. *p<0.0001, ANOVA. (C) Quantitative immunofluorescence microscopy for ciliary SMO in human HH-associated medulloblastoma cells with and without Hedgehog pathway stimulation with SHH. *p<0.0001. (D) Immunofluorescence microscopy for SMO (red), AcTub (green) and DNA (blue) in DAOY cells with and without Hedgehog pathway stimulation with SHH. Scale bar, 10μm. (E, F) qRT-PCR assessment of the Hedgehog transcriptional program in human HH-associated medulloblastoma cell lines with and without Hedgehog pathway stimulation with SHH. (G) DAOY clonogenic growth after 10 days of treatment with abemaciclib. (H) DAOY clonogenic assay quantification. *p<0.0001. (I) Immunoblot assessment of stable Cas9 (FLAG tag) or dCas9-KRAB (HA tag) expression in

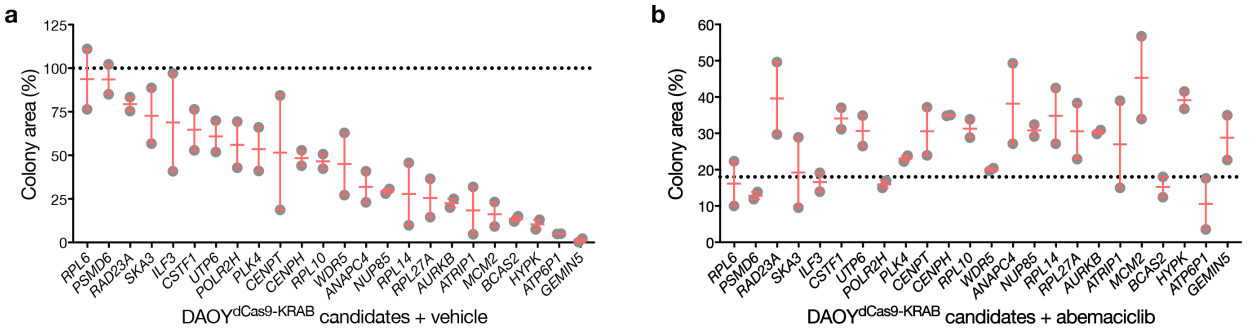
DAOY cells. (J) qRT-PCR assessment of *CDK6* suppression in $DAOY^{Cas9}$ and $DAOY^{dCas9\text{-}KRAB}$ cells. *p=0.0008. (K) $DAOY^{Cas9}$ and $DAOY^{dCas9\text{-}KRAB}$ clonogenic growth after 10 days. (L) $DAOY^{Cas9}$ and $DAOY^{dCas9\text{-}KRAB}$ clonogenic assay quantification. *p<0.0001. Lines represent mean and error bars represent standard error of the mean. The number of samples for each experiment is shown in each panel.

Supplemental Figure 2. Genome-wide enrichment screens reveal inhibition of ribosome biogenesis underlies resistance to CDK4/6 inhbition in Hedgehog-associated medulloblastoma cells.



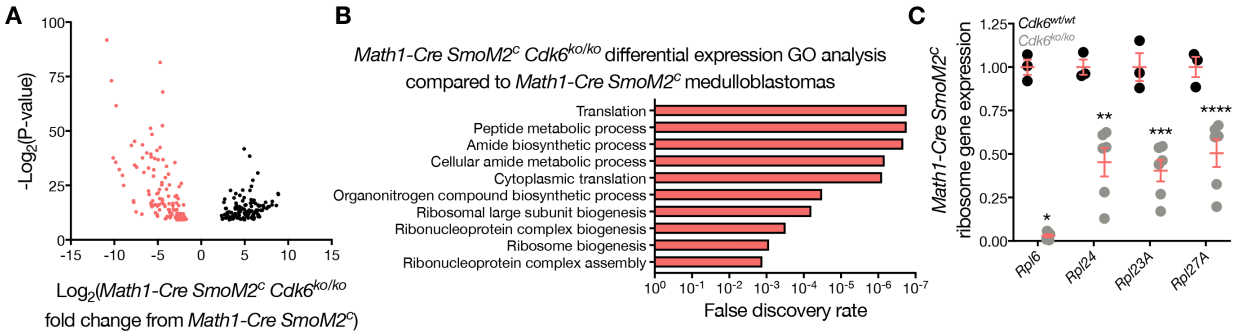
Supplemental Figure 2. Genome-wide enrichment screens reveal inhibition of ribosome biogenesis underlies resistance to CDK4/6 inhibition in Hedgehog-associated medulloblastoma cells. (A) Pearson correlation matrix between 2 independent DAOY^{Cas9} enrichment screens. (B) Suppressed pathways in 2 DAOY^{Cas9} and 1 DAOY$^{dCas9\text{-}KRAB}$ genome-wide CDK4/6 inhibition resistance screens. (C) qRT-PCR assessment of 2 DAOY^{Cas9} genome-wide screens demonstrates no enrichment of *CDK6* expression with abemaciclib treatment. (D) qRT-PCR assessment of parental DAOY cells demonstrates conditioned resistance to abemaciclib does not induce *CDK6*. Lines represent mean and error bars represent standard error of the mean. The number of samples for each experiment is shown in each panel.

Supplemental Figure 3. Validation of genes driving resistence to CDK4/6 inhibition in Hedgehog-associated medulloblastoma cells.



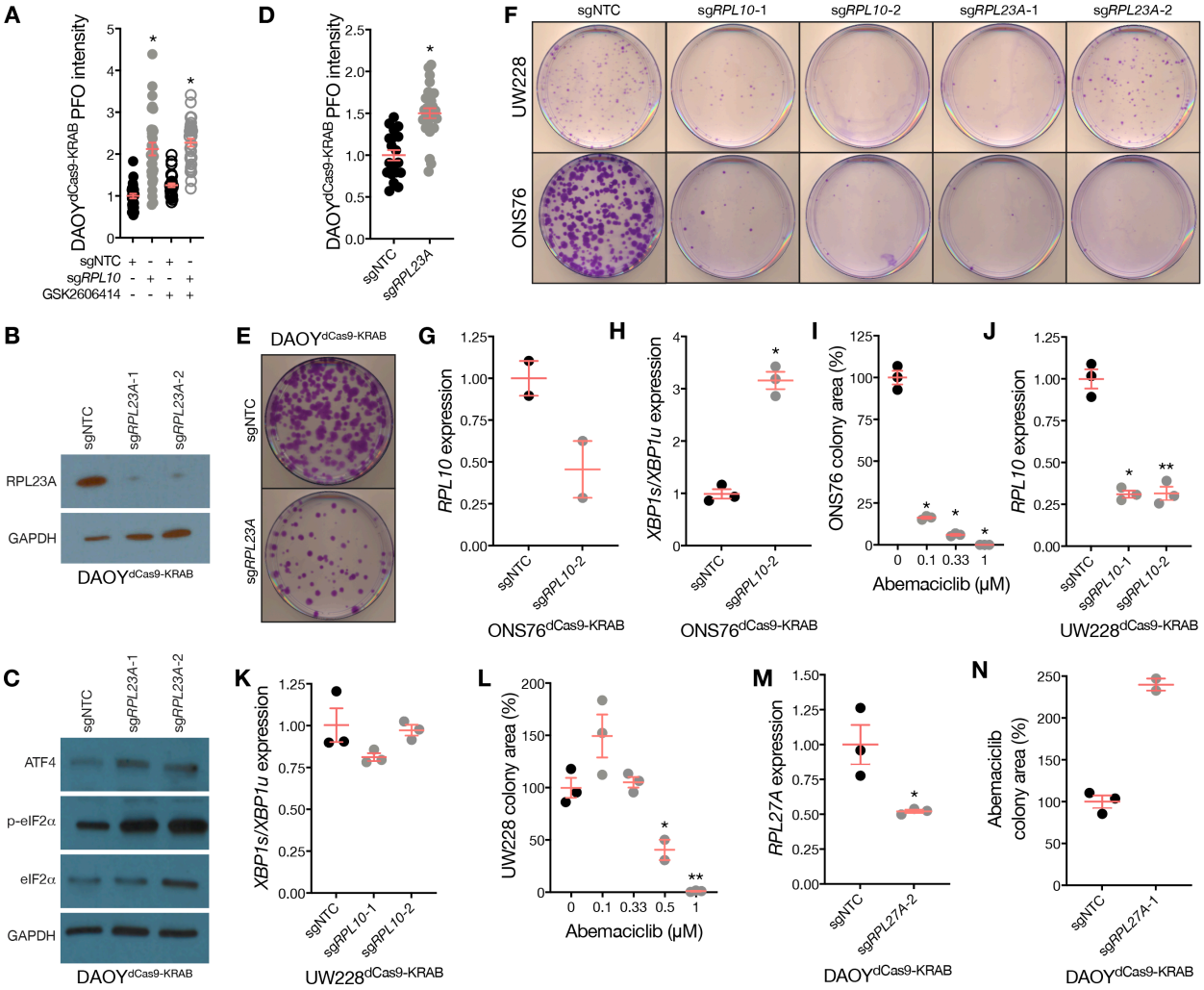
Supplemental Figure 3. Validation of genes driving resistance to CDK4/6 inhibition in Hedgehog-associated medulloblastoma cells. DAOY$^{dCas9\text{-}KRAB}$ clonogenic assay quantification after transduction of the indicated sgRNAs and 10 days of treatment with vehicle control (A) or abemaciclib (B). Dashed lines represent clonogenic growth of DAOY$^{dCas9\text{-}KRAB}$ cells transduced with sgNTCs and treated with vehicle control (A) or abemaciclib (B). All data are normalized to DAOY$^{dCas9\text{-}KRAB}$ cells transduced with sgNTCs and treated with vehicle control. Lines represent mean and error bars represent standard error of the mean. The number of samples for each experiment is shown in each panel.

Supplemental Figure 4. RNA sequencing of Hedgehog-associated medulloblastomas demonstrates inhibition of ribosome biogenesis underlies tumor growth after genetic deletion of *Cdk6*.



Supplemental Figure 4. RNA sequencing of Hedgehog-associated medulloblastomas demonstrates inhibition of ribosome biogenesis underlies tumor growth after genetic deletion of *Cdk6*. (A) Volcano plot of differentially expressed genes (absolute fold change >2, FDR<0.05, adjusted p<0.05). (B) Suppressed pathways in *Math1-Cre SmoM2^{c} Cdk6$^{ko/ko}$* medulloblastomas compared to *Math1-Cre SmoM2^{c} Cdk6$^{wt/wt}$* tumors. (C) RNA sequencing of ribosome gene expression in HH-associated medulloblastomas. Lines represent mean and error bars represent standard error of the mean. *p<0.0001, **p=0.002, ***p=0.0003, ****p=0.0004. The number of samples for each experiment is shown in each panel.

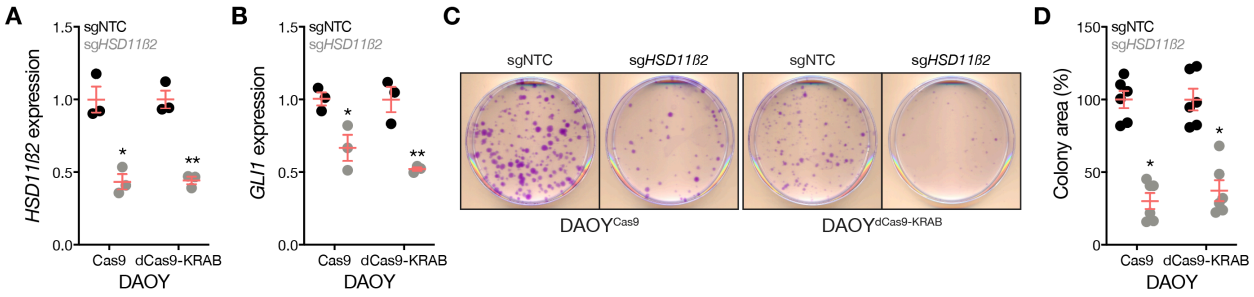
Supplemental Figure 5. Ribosome biogenesis, the unfolded protein response, and CDK4/6 inhibition in Hedgehog-associated medulloblastoma cells.



Supplemental Figure 5. Ribosome biogenesis, the unfolded protein response, and CDK4/6 inhibition in Hedgehog-associated medulloblastoma cells. (A) PFO cholesterol staining and microscopy quantification in DAOY$^{dCas9\text{-}KRAB}$ cells after treatment with GSK2606414, a UPR inhibitor that blocks PERK upstream of non-lipogenic signaling. *p<0.0001. (B) Immunoblot assessment of *RPL23A* suppression in DAOY$^{dCas9\text{-}KRAB}$ cells. (C) Immunoblot assessment of UPR activation through phosphorylation of eIF2α and induction of ATF4 in DAOY$^{dCas9\text{-}KRAB}$ cells. (D) PFO cholesterol staining and microscopy quantification in DAOY$^{dCas9\text{-}KRAB}$ cells. p<0.0001. (E) DAOY$^{dCas9\text{-}KRAB}$ clonogenic growth shows *RPL23A* suppression impairs cell viability. (F) UW228 and ONS76 clonogenic growth shows *RPL10* or *RPL23A* suppression impairs cell viability. (G) qRT-PCR assessment of *RPL10* suppression in ONS76 $^{dCas9\text{-}KRAB}$ cells. (H) qRT-PCR assessment of the ratio of spliced to unspliced *XBP1* as a measure of UPR activation in ONS76 $^{dCas9\text{-}KRAB}$ cells.

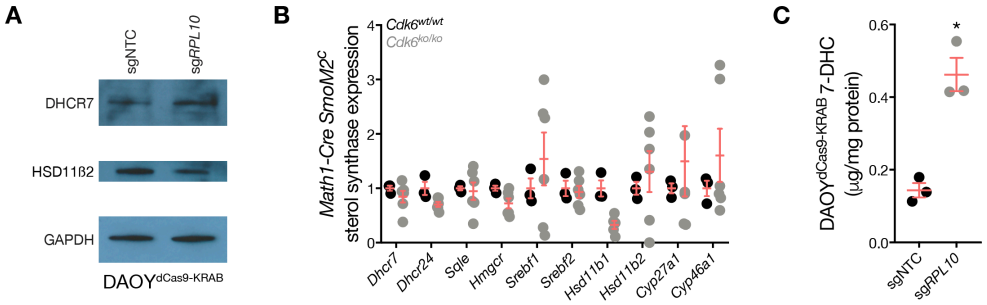
*p=0.0002. (I) ONS76 clonogenic assay quantification after 10 days of treatment with abemaciclib. *p<0.0001. (J) qRT-PCR assessment of *RPL10* suppression in UW228 $^{dCas9\text{-}KRAB}$ cells. *p=0.0001, **p=0.0003. (K) qRT-PCR assessment of the ratio of spliced to unspliced *XBP1* as a measure of UPR activation in UW228$^{dCas9\text{-}KRAB}$ cells. (L) UW228 clonogenic assay quantification after 10 days of treatment with abemaciclib. *p=0.01, **p=0.0002. (M) qRT-PCR assessment of *RPL27A* suppression in DAOY$^{dCas9\text{-}KRAB}$ cells. *p=0.01. (N) DAOY$^{dCas9\text{-}KRAB}$ clonogenic growth after 10 days of abemaciclib treatment demonstrates *RPL27A* suppression confers resistance to CDK4/6 inhibition. Lines represent mean and error bars represent standard error of the mean. The number of samples for each experiment is shown in each panel.

Supplemental Figure 6. Genetic inhibition of *HSD11ß2* blocks the Hedgehog transcriptional program and proliferation in Hedgehog-associated medulloblastoma cells.



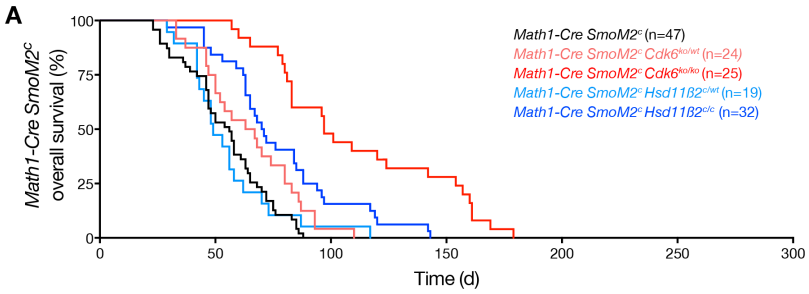
Supplemental Figure 6. Genetic inhibition of *HSD11ß2* blocks the Hedgehog transcriptional program and proliferation in Hedgehog-associated medulloblastoma cells. (A) qRT-PCR assessment of *RPL10* suppression in DAOY^{Cas9} and DAOY$^{dCas9\text{-}KRAB}$ cells. *p=0.002, **p=0.0005. (B) qRT-PCR assessment of *GLI1* in DAOY^{Cas9} and DAOY$^{dCas9\text{-}KRAB}$ cells. *p=0.04, **p=0.01. (C) DAOY^{Cas9} and DAOY$^{dCas9\text{-}KRAB}$ clonogenic growth after 10 days. (D) DAOY^{Cas9} and DAOY$^{dCas9\text{-}KRAB}$ clonogenic assay quantification. *p<0.0001. Lines represent mean and error bars represent standard error of the mean. The number of samples for each experiment is shown in each panel.

Supplemental Figure 7. Inhibition of ribosome biogenesis increases Smoothened-activating lipids, but does not increase lipid synthase expression in Hedgehog-associated medulloblastomas.



Supplemental Figure 7. Inhibition of ribosome biogenesis increases Smoothened-activating lipids, but does increase lipid synthase expression in Hedgehog-associated medulloblastomas. (A) Immunoblot assessment of SMO-activating sterol synthases expression in DAOY$^{dCas9\text{-}KRAB}$ cells. (B) RNA sequencing of sterol synthase expression in HH-associated medulloblastomas. (C) Lipidomic mass spectrometry for the SMO-activating precursor lipids 7-dehydroxycholesterol (7-DHC) in DAOY$^{dCas9\text{-}KRAB}$ cells. *p=0.0031. Lines represent mean and error bars represent standard error of the mean. The number of samples for each experiment is shown in each panel.

Supplemental Figure 8. Genetic deletion of *Cdk6* or *Hsd11ß2* attenuates the growth of Hedgehog-associated medulloblastoma.



Supplemental Figure 8. Genetic deletion of *Cdk6* or *Hsd11ß2* attenuates the growth of Hedgehog-associated medulloblastoma. (A) Kaplan-Meier curves of mouse survival from medulloblastoma from this study (black) compared to littermates from others (11, 30).

Supplemental Tables

Supplemental Table 1. Genome-wide enrichment screen candidates driving resistance to CDK4/6 inhibition in human DAOY medulloblastoma cells. Candidates were scored based on positive enrichment (sgRNAs that increased in representation with abemaciclib treatment compared to control treatment, suggestive of gene suppression) and negative enrichment (sgRNAs that decreased in representation with abemaciclib treatment compared to control treatment, suggestive of gene activation). Num, number of sgRNAs targeting an individual gene within the library. Goodsgrna, number of sgRNAs influencing the positive or negative rank of a candidate.

Supplemental Table 2. Differentially expressed genes in *Math1-Cre SmoM2^{c} Cdk6$^{ko/ko}$* medulloblastomas compared to *Math1-Cre SmoM2^{c}* medulloblastomas.

Supplemental Table 3. Protospacer and primer sequences.