SUPPLEMENTARY INFORMATION

Multiple recurrent genetic events converge on control of histone lysine methylation in medulloblastoma

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Table of Contents:

- 1) Supplementary Figure 1: Gains and losses in the medulloblastoma genome
- 2) Supplementary Figure 2: The medulloblastoma genome (100K SNP array)
- Supplementary Figure 3: Copy number aberrations on chromosome 17 in medulloblastoma
- **4)** Supplementary Figure 4: Loss of heterozygosity (LOH) in the medulloblastoma genome

Excel Spreadsheets, Methods, & Tables:

- Supplementary Note: Clinical and sample information for the medulloblastoma cohort analyzed by SNP array
- 2) Supplementary Methods
- **3)** Supplementary Table 1: Known copy number variants identified in the medulloblastoma genome

- 4) Supplementary Table 2: Regions of genomic gain and loss in the medulloblastoma genome
- 5) Supplementary Table 3: GISTIC analysis of medulloblastoma copy number data
- 6) Supplementary Table 4: Recurrent regions of amplification in medulloblastoma
- 7) Supplementary Table 5: Homozygous deletion of known and candidate tumor suppressor genes in medulloblastoma
- 8) Supplementary Table 6: List of primers used in the study

Supplementary Figure 1a-x: Gains and losses in the medulloblastoma genome

Chromosomal views showing regions of genomic gain (red) and loss (green) along each chromosome, for tumors analyzed on either the 100K or 500K Affymetrix SNP array platforms. For the X chromosome, tumors from males and females are displayed separately. Copy number output is from CNAG.

Supplementary Figure 1a





Supplementary Figure 1b





Supplementary Figure 1c





Supplementary Figure 1d





Supplementary Figure 1e





Supplementary Figure 1f





Supplementary Figure 1g





Supplementary Figure 1h





Supplementary Figure 1i





Supplementary Figure 1j





Supplementary Figure 1k





Supplementary Figure 11





Supplementary Figure 1m





Supplementary Figure 1n





Supplementary Figure 10





Supplementary Figure 1p





Supplementary Figure 1q





Supplementary Figure 1r





Supplementary Figure 1s





Supplementary Figure 1t





Supplementary Figure 1u





Supplementary Figure 1v





Supplementary Figure 1w







— Homozygous Deletion

Supplementary Figure 1x





Supplementary Figure 2: The medulloblastoma genome (100K SNP array)

Global view of regions of gain and loss across the genome in a series of 89 medulloblastomas genotyped on the Affymetrix 100K SNP array platform. Output is from GenePattern SNP Viewer. Regions of gain are shown in red, regions of loss are shown in blue.



Supplementary Figure 3: Copy number aberrations on chromosome 17 in medulloblastoma

Loss of chromosome 17p concurrent with gain of chromosome 17q was observed in ~28% of medulloblastomas profiled on the 100K (A) and 500K (B) SNP array platforms. This is consistent with the known rate of occurrence of isochromosome 17q in medulloblastoma.



Supplementary Figure 4: Loss of heterozygosity (LOH) in the medulloblastoma genome

(A) Genome-wide depiction of regions of inferred LOH (blue) and regions of inferred retention of heterozygosity (yellow) in a series of 123 medulloblastomas (500K SNP array). Output is from dChipSNP. (B) Bar graph exhibiting that some chromosomes always achieve LOH through deletion, other chromosomes have predominantly copy-number neutral LOH, and some demonstrate both mechanisms.





Α



Supplementary Methods

Cell lines and cell culture. We purchased all media and reagents for culturing mammalian cells lines from Wisent unless otherwise stated. Medulloblastoma cell lines ONS76, UW228 and MED8A were grown as a monolayer in DMEM supplemented with 10% FBS. DAOY and D283 cell lines were grown as monolayers in AMEM with 10% FBS. The RES256 cell line was grown adherent in DMEM/F12 media supplemented with 2% FBS. D425, D458 and D556 were grown as suspension cultures in IMEM with 20% FBS, 10 mM HEPES and 0.225% sodium bicarbonate. MHH-MED-1 and D341 were grown in suspension in DMEM and AMEM with 10% FBS, respectively. For generation of DAOY and D283 stable cell lines, we selected stable transfectants in G418 at a concentration of 0.5 mg/ml and 2.0 mg/ml, respectively. For routine passaging of stable cell lines, we maintained cells in 0.2 mg/ml G418. NIH3T3 and Eco-Pheonix cells were grown in DMEM supplemented with 10% FBS. We cultured 293E cells in DMEM supplemented with 10% FBS and 300 µg/ml G418. All media were supplemented with a 1× antibiotic/antimycotic solution. Cerebellar granule cell progenitors (GCPs) were cultured in NB-B27 (Neurobasal medium; Invitrogen) supplemented with B27 (Invitrogen), 2 mM L-glutamine (Invitrogen), 1 mM Na-pyruvate (Invitrogen) and penicillin/streptomycin.

Plasmids. For cDNA cloning of mammalian expression constructs, we carried out PCR using Platinum Hi-Fidelity Taq Polymerase (Invitrogen). All additional reagents for PCR were obtained from Invitrogen. Restriction enzymes and DNA ligase (Quick Ligation Kit) were purchased from New England Biolabs (NEB). To generate the L3MBTL3 expression plasmid, we amplified a 627-bp, HA (hemagglutinin) epitope-tagged

fragment, corresponding to the 5' end of mouse *l3mbtl3* (NM 032438; 5'-HA-*L3MBTL3*) by PCR using a cDNA template (Image: 30356672) and the HA-L3MBTL3 sense and antisense primers listed in Supplementary Table 6. The PCR product was cloned into the pCR 2.1-TOPO TA cloning vector (Invitrogen) according to the manufacturer's instructions. pcDNA3.1(+) (Invitrogen) and the 5'-HA L3MBTL3 cDNA were digested with NheI and BamHI, gel-purified and ligated. The resulting 5'-HA-L3MBTL3 cDNA and the *l3mbtl3* library plasmid were digested with *Bam*HI and *NotI*, gel-purified and ligated to create the final pcDNA3.1-HA-L3MBTL3 construct. The pcDNA3.1-GFP plasmid was constructed by digestion of pCAT3Blbp-GFP with NcoI and XbaI, and ligation of the GFP fragment into blunted EcoRI and XbaI sites of pcDNA3.1(+). We generated a C-terminal HA-tagged pcDNA3.1-SAMD3-HA construct by PCR amplification of the SAMD3 cDNA (BC029851) using SAMD3-HA sense and antisense primers. The PCR product was ligated into pcDNA3.1(-) (Invitrogen) at NotI and EcoRI sites. The pcDNA3.1-TMEM200A-HA plasmid was constructed by PCR amplification of the TMEM200A cDNA (BC044246) using TMEM200A-HA sense and antisense primers. The PCR product was ligated into pcDNA3.1(-) at NotI and EcoRI sites. The pBabe-HA-JMJD2C construct (gift from K. Helin, University of Copenhagen) has been previously described. To generate pWZL-HA-JMJD2C-IRES-GFP, the JMJD2C cDNA was subcloned from pBabe-HA-JMJD2C by digestion with NaeI and SalI and directional ligation into a blunted EcoRI site and SalI of pWZL-IRES-GFP (gift of A. M. Kenney, Memorial-Sloan Kettering). All constructs used in this study were fully sequenced (TCAG) in both directions before their use.

Antibodies. For immunoblotting, mouse antibody to HA (anti-HA; F7; Santa Cruz Biotechnology), mouse anti-p27 KIP 1 (DCS-72.F6; Abcam) and mouse anti-β-actin (AC-74; Sigma-Aldrich) were used. Goat anti-mouse and goat anti-rabbit HRPconjugated secondary antibodies were purchased from BioRad. For immunoprecipitation of HA-tagged recombinant proteins, we used rabbit anti-HA (Y-11; Santa Cruz Biotechnology). For chromatin immunoprecipitation experiments, we used ChIP-grade mouse anti-H3K9me2 (ab1220; Abcam) and normal mouse control IgG (sc-2025; Santa Cruz Biotechnology) antibodies; for indirect immunofluorescence, we used mouse anti-HA (HA.11; Covance), mouse anti-GFP (MAB3580; Chemicon) and rabbit anti-H3K9me2 (9753; Cell Signaling Technologies). Goat anti-mouse Alexa-488 and goat anti-rabbit Alexa-594 secondary antibodies were acquired from Molecular Probes. Antibodies used in immunohistochemistry included mouse anti-GLP (EHMT1; B0422; R & D Systems), ChIP-grade mouse anti-H3K9me2 (ab1220; Abcam), mouse anti-Histone H3K9me3 (ab6001; Abcam), ChIP-grade rabbit anti-Histone H3K9me1 (ab9045; Abcam), mouse anti-p27 KIP 1 (DCS-72.F6; Abcam) and rabbit anti-JMJD2C (A300-885A; Bethyl Laboratories).

Processing of tumor samples and cell lines. Fresh-frozen medulloblastoma specimens were stored at -80 °C before being processed for extraction of nucleic acid. Tissue samples were pulverized under liquid nitrogen using a mortar and pestle and partitioned for extraction of genomic DNA and total RNA. For genomic DNA isolation, we subjected approximately 25–50 mg of crushed tissue to digestion using SDS/Proteinase K (Roche) for 3 h at 50 °C. Homogenates were extracted three times with buffer-saturated phenol (Invitrogen) before precipitation of DNA with two volumes of anhydrous ethanol

and 10% (v/v) 10 M ammonium acetate. Precipitated DNA was washed three times with 70% ethanol and resuspended in reduced EDTA-TE (10 mM Tris, 0.1 mM EDTA; pH = 8.0). We quantitated samples by Nanodrop and assessed the integrity of isolated DNA by agarose gel electrophoresis before submission for SNP array. For RNA, crushed tumor specimens were resuspended in 1 ml Trizol (Invitrogen), passed through a 20-gauge needle 5–10 times, and incubated at room temperature for 10 min. Following addition of 0.2 ml chloroform (Sigma-Aldrich), samples were mixed briefly and cleared by centrifugation at 12,000g for 15 min at 4 °C. Total RNA was precipitated by addition of 0.5 ml isopropanol, incubation at room temperature for 10 min, and subsequent centrifugation at 12,000g for 10 min at 4 °C. RNA pellets were washed once with 75% ethanol and dissolved in DEPC water (Invitrogen). Nanodrop-quantitated samples were evaluated by Bioanalyzer before any downstream analysis.

Real-time quantitative PCR. Copy number aberrations (CNAs) detected by SNP array analysis were validated by real-time PCR using the ^{$\Delta\Delta$}Ct method. For each experiment, a minimum of two diploid loci (as predicted by SNP array analysis) were amplified and used for normalization, and a normal brain sample was included as a calibration template. Reactions were carried out in triplicate using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) with 25 ng of template per reaction as suggested by the supplier using a Chromo4 Real-Time System (Bio-Rad). We designed all primers using Primer3 and sequences are available upon request. For candidate gene expression profiling, we subjected 2 µg of total RNA to random hexamer-primed reverse transcription using SuperScript III (Invitrogen) according to the manufacturer's instructions. PCR reactions were done as described above using 10 ng of cDNA per reaction with β -actin as a reference gene for data normalization. Normal fetal and adult cerebellum RNA samples (Biochain) served as controls for calibration. Primers were designed with PerlPrimer software and sequences are listed in Supplementary Table 6. To compare levels of EHMT1 expression between 9q diploid and 9q monosomic samples, a two-sample Wilcoxon test was applied. For *SMYD4*, *JMJD2C*, *JMJD2B* and *BMI1*, a Wilcoxon signed rank test was used, and *P* values were calculated using exact methods where ties in ranks were not present.

Cell proliferation assays. Cellular proliferation assays were done using the CellTiter 96 Aqueous One Solution Proliferation Assay (Promega) according to the manufacturer's instructions. Briefly, 1×10^3 cells were seeded in triplicate on 96-well plates and colorimetric readings (absorbance of 490 nm) were taken at 24, 48, 72 and 96 h time points following 2 h incubations with proliferation reagent. To qualitatively assess cell growth in culture, we seeded 5×10^3 cells in triplicate on 10-cm dishes and cultured cells for 7 d before crystal violet staining. Cells were washed twice with ice-cold PBS, followed by fixation with ice-cold methanol for 10 min on ice. Fixed cells were stained at room temperature with crystal violet (Sigma-Aldrich) solution (0.5% crystal violet (w/v), 25% methanol), rinsed 2–3 times with Milli-Q H2O, and allowed to dry at room temperature.

Cell cycle analysis and cell viability assays. Cell cycle analysis was done by propidium iodide (PI) staining of medulloblastoma cell lines and by measuring DNA content using a Becton Dickinson FACScan. Briefly, asynchronous cells were grown on 10-cm dishes and collected by centrifugation at 400*g* for 5 min at 4 °C. Cell pellets were resuspended in staining media (1× Hank's balanced salt solution (HBSS), 10 mM HEPES-NaOH, pH

7.2, 2% calf serum (vol/vol), 10 mM NaN₃) and fixed by adding ice-cold 80% ethanol, vortexing briefly, and incubating for 30 min to overnight at 4 °C. Fixed cells were collected by centrifugation at 400g for 5 min at 4 °C and pellets resuspended and incubated in 2 mg/ml RNAase A solution (Qiagen) for 5 min at room temperature. Staining was done by adding propidium iodide solution (HBSS, 0.1 mg/ml propidium iodide, 0.6% NP-40 (vol/vol)) and incubating for 30 min at room temperature protected from light. Cells were collected by a final spin, resuspended in staining media, and filtered into polystyrene tubes fitted with a cell-strainer cap. We collected data using CELLFIT software. To assess cell viability in DAOY stable cell lines, we used the Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen) according to the manufacturer's instructions. Briefly, 48 h before analysis, medulloblastoma cells were seeded at a density of 5×10^5 cells in 10 cm dishes. Asynchronous cells were trypsinized, washed twice with ice-cold PBS, and resuspended in 1× binding buffer at a concentration of 1×10^6 cells/ml. A fraction (~1 × 10⁵) of cell solution was then mixed with Annexin V-FITC and propidium iodide, vortexed gently, and incubated for 15 min at room temperature in the dark. Stained cells were then diluted in 1× binding buffer and analyzed by flow cytometry using a FACScan.

Retrovirus production. To generate ecotropic retroviruses, Eco-Phoenix cells were seeded at a density of 5.5×10^6 cells in 10-cm dishes 24 h before transfection with 8 µg of Eco-Phi helper plasmid and 8 µg of viral construct using Fugene6 (Roche) as directed by the supplier. Twenty-four hours post-transfection, virus producer cells were washed once with PBS and then grown in serum-free DMEM at 32 °C. Viral supernatants were collected successively for 2 d, pooled, filtered through a 0.45-µm filter, flash-frozen, and

stored at -80 °C. Amphotropic viruses were produced in 293E cells (provided by A. M. Kenney) by transfecting cells with 10 µg each of *vsv-g*, *gag-pol*, and viral construct as described above. Supernatants were collected and stored as above.

Cerebellar granule cell progenitor (GCP) isolation and transduction. GCPs were isolated from P5-P7 mouse cerebella by gradient centrifugation through Percoll. For immunofluorescence (IF) microscopy experiments, 7.5×10^5 freshly isolated cells were plated on poly-ornithine (Sigma-Aldrich) coated coverslips (Fisher Scientific) in 24-well dishes and treated with recombinant Sonic Hedgehog protein (provided by A. M. Kenney, Memorial-Sloan Kettering) at a final concentration of 3–5 µg/ml. Three hours post-isolation, conditioned NB-B27 media was removed and stored as cells were incubated with viral supernatant supplemented with 8 µg/ml polybrene (Sigma-Aldrich) for 3 h at 32 °C. Following viral transduction, conditioned media was replaced and cells were cultured for an additional 48–96 h at 37 °C. Transduction of NIH3T3 and medulloblastoma cells was done in a similar manner in their normal growth media.

Immunofluorescence and immunohistochemistry. For immunofluorescence experiments, cells grown on coverslips in 24-well plates were fixed with 4% (w/vol) paraformaldehyde (PFA; Sigma-Aldrich) in PBS for 15 min at room temperature. Cells were washed twice with PBS, permeabilized with 1% Triton X-100 (Sigma-Aldrich) in PBS for 5 min, and washed three times with PBS, before blocking for 1 h in 5% (vol/vol) normal goat serum (NGS; Sigma-Aldrich) in PBS-T (PBS, 0.1% Triton X-100). Blocked cells were washed once with PBS, and incubated in primary antibody diluted in 2.5% NGS/PBS-T for 1 h at room temperature. Stained cells were washed three times and incubated with fluorophore-conjugated secondary antibodies in PBS-T for 1 h at room temperature, before three final washes with PBS and mounting of coverslips on glass slides with VECTASHIELD containing DAPI (Vector Laboratories). We used a Quorum spinning disk confocal microscope for microscopy and acquired images with Volocity 4.4. IHC for EHMT1 and H3K9me2 on primary tumors was done using two independent medulloblastoma tissue microarays (John Hopkin's University and St. Jude Children's Research Hospital). Staining for EHMT1, H3K9me2, H3K9me1, H3K9me3, p27, and JMJD2C in the developing mouse cerebellum was done at the Department of Pathology at Toronto General Hospital.

Immunoblotting. Medulloblastoma cells cultured on 10-cm dishes were washed twice with ice-cold PBS and lysed on ice with RIPA (150mM NaCl, 1% (vol/vol) NP-40, 0.5% (w/vol) DOC, 0.1% (w/vol) SDS, 50mM Tris-Cl, pH 8.0) for 10 min. Cell lysates were cleared by centrifugation at 13,000 rpm for 10 min at 4 °C. For preparation of nuclear extracts, cells were washed with ice-cold PBS and collected by centrifugation at 1,000 rpm for 5 min at 4 °C. The cytoplasmic fraction was removed by incubating cells in buffer A (10mM KCl, 1.5mM MgCl₂, 0.5mM DTT, 10mM HEPES-KOH, pH 7.9) with protease inhibitors (Complete Mini; Roche) on ice for 10 min. Nuclei were pelleted and proteins extracted in RIPA buffer with inhibitors for 30 min at 4 °C with rotation. Nuclear extracts were then prepared by removing chromatin pellets after centrifugation at 13,000 rpm for 10 min at 4 °C. For immunoprecipitation of epitope-tagged proteins, nuclear lysates were incubated with primary antibodies overnight at 4 °C. Immune complexes were captured by incubation with Protein A Sepharose beads (Sigma-Aldrich) for 1 h at 4 °C and washed 3–4 times in wash buffer (PBS, 0.1% (vol/vol) NP-40) before SDS-PAGE and immunoblotting. Briefly, proteins were separated by SDS-PAGE,

transferred to PVDF, and blocked in 5% (w/vol) skim milk in TBS-T (TBS, 0.1% (w/vol) Tween 20) for 1 h at room temperature. Blots were incubated with primary antibodies overnight at 4 °C, washed three times in TBS-T, and probed for 1 h at room temperature with the appropriate HRP-conjugated secondary antibodies diluted in TBS-T. Blots were then subjected to enhanced chemiluminescence (Western Lightning Chemiluminescent Reagent Plus; PerkinElmer) and exposed to X-ray film (Bioflex; InterScience).

Cytoband	Minimal common region (Mb)	No. amplified	No. focal gain	Total focal	Focal gain (%)	No. large gain	Large gain (%)	Notable gene(s)
1q24.2	165.799- 165.821	2	1	3	1.4	40	18.9	ATP1B1
2p24.3	16.007-16.156	4	6	10	4.7	29	13.7	MYCN
2q14.2	119.359- 120.174	2	0	2	0.9	20	9.4	MARCO, DBI
2q14.2	121.186- 121.717	2	0	2	0.9	20	9.4	GLI2
4q12	54.826-55.233	3	0	3	1.4	22	10.4	PDGFRA
7p21.1- p15.3	20.657-21.017	2	0	2	0.9	56	26.4	ESTs
7q11.21	62.941-63.211	3	2	5	2.4	61	28.8	ESTs
8p11.21	41.593-42.547	2	1	3	1.4	15	7.1	MYST3
8q24.21	128.809- 128.809 ^a	15	4	19	9.0	14	6.6	MYC ^a
13q33.3-q34	109.098- 109.910	2	0	2	0.9	27	12.7	IRS2
14q22.3	56.392-56.451 ^b	2	19	21	9.9	23	10.8	OTX2 ^b
18q21.31	53.363-53.511	2	1	3	1.4	29	13.7	ATP8B1
^a Minimal common region maps to a single SNP based on array coverage. SNP is adjacent to <i>MYC</i> locus. ^b Minimal common region is adjacent to <i>OTX2</i> locus based on array coverage								

Supplementary Table 4: Recurrent regions of amplification in medulloblastoma

Cytoband	Minimal common region (Mb)	No. samples	Copy number range (HMM)	Known/candidate TSG	
1p32.3	51.106-51.150	1	0.0	CDKN2C	
2q22.1	141.617-141.930	4	0.0	LRP1B	
2q34	212.891-212.941	1	0.0	ERBB4	
4q28.2	129.960-130.245	1	0.0	PHF17	
4q28.3	134.019-134.427	1	0.0	PCDH10	
8p22	16.026-16.066	1	0.0	MSR1	
9p21.3	21.913-21.972	4	0.0	CDKN2A	
9p21.3-p21.2	23.513-26.573	1	0.0	TUSC1	
9q31.2-q31.3	106.805-108.856	1	0.0	KLF4	
9q34.13	132.681-132.806	1	0.0	TSC1	
10q23.31	89.509-89.677	1	0.0	PTEN	
11p11.2	46.794-48.121	1	0.0	PTPRJ	
11q22.3-q23.3	107.935-115.354	1	0.0	DDX10, PPP2R1B, IGSF4	
16q23.1	77.287-77.372	1	0.0	WWOX	
17p12	11.572-12.009	1	0.0	MAP2K4	
20p13-p12.3	2.389-5.921	1	0.0	RASSF2	
Xp11.3	43.579-44.135	1	0.0	NDP	
Xp11.3- p11.23	45.600-48.918	1	0.0	GATA1	

Supplementary Table 5: Homozygous deletion of known and candidate tumor suppressor genes in medulloblastoma