Spatial and temporal homogeneity of driver mutations in diffuse intrinsic pontine glioma

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Diffuse Intrinsic Pontine Gliomas (DIPGs) are deadly paediatric brain tumours where needle biopsies help guide diagnosis and targeted therapies. To address spatial heterogeneity, here we analyse 134 specimens from various neuroanatomical structures of whole autopsy brains from nine DIPG patients. Evolutionary reconstruction indicates histone 3 (H3) K27M—including H3.2K27M—mutations potentially arise first and are invariably associated with specific, high-fidelity obligate partners throughout the tumour and its spread, from diagnosis to end-stage disease, suggesting mutual need for tumorigenesis. These H3K27M ubiquitously-associated mutations involve alterations in TP53 cell-cycle (TP53/PPM1D) or specific growth factor pathways (ACVR1/PIK3RI). Later oncogenic alterations arise in sub-clones and often affect the PI3K pathway. Our findings are consistent with early tumour spread outside the brainstem including the cerebrum. The spatial and temporal homogeneity of main driver mutations in DIPG implies they will be captured by limited biopsies and emphasizes the need to develop therapies specifically targeting obligate oncohistone partnerships.

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Diffuse Intrinsic Pontine Gliomas (DIPGs) represent 10–15% of all paediatric brain tumours and have a dismal median survival of 9–12 months. They seemingly arise as de novo tumours in the pons where surgical options are limited. Recently, we and others have identified recurrent somatic gain-of-function mutations leading to lysine 27 to methionine (p.Lys27Met, K27M) substitution in histone H3 in ~80% of DIPGs. Despite a low mutational rate relative to other cancers, these H3K27M mutants appear to be often associated with other mutations in cell cycle regulatory genes (TP53, PPM1D), chromatin remodeler (ATRX) or growth factors (ACVR1) (refs 3–8).

Spatial and temporal tumour heterogeneity is viewed as a major obstacle to accurate diagnosis and successful targeted therapy in cancer. Many adult cancers including high-grade gliomas (HGG) show intra-tumour heterogeneity9–11, which confounds determining the complete genetic landscape when only a fraction of a tumour is sampled. In DIPG, published studies have solely used biopsied or limited autopsied specimens without assessing the full spatial and temporal tumour heterogeneity. Despite the fact that H3K27M mutations are identified in more DIPG tumour samples7–8,12 the homogeneity of histone and its partner mutation(s) across tumour mass is yet to be determined. It is not currently clear to what extent the tumour may be composed of a mixture of clonal populations carrying distinct mutations. In addition, while DIPGs invariably exhibit tumour spread outside of the pons and brainstem13, there are to date no comprehensive molecular studies of these invading cells. Such infiltrating tumour cells may originate from specific clones present in the primary site, as described for other cancers, including other paediatric tumours14,15. Alternately, malignant cells may seed early during tumour evolution, as recently shown for low-grade gliomas in the pons and brainstem13, there are to date no comprehensive molecular studies of these invading cells. Such infiltrating tumour cells may originate from specific clones present in the primary site, as described for other cancers, including other paediatric tumours14,15. Alternately, malignant cells may seed early during tumour evolution, as recently shown for low-grade gliomas in adults17 and/or may genetically diverge from the primary tumour as seen in metastatic cancer including medulloblastoma16.

Therefore, in order to define DIPG heterogeneity, we sought to: (i) determine whether the currently recommended needle biopsies18 robustly define DIPG tumour biology; (ii) infer the temporal order of specific driver mutations in DIPG; and (iii) fully characterize the molecular signature of the primary, regional and distant subclones, and define their evolutionary relationships. Our results suggest that histone H3K27M mutations arise first and are associated with specific, obligate partners involving alterations in TP53 cell-cycle (TP53/PPM1D) or specific growth factor pathways (ACVR1/PIK3R1), which are present throughout the tumour as well as its spread outside the brainstem.

Results
Genomic landscape of primary and extended tumours. We analysed 134 punch cores from nine DIPG whole brain specimens obtained at autopsy as previously described19. Selected punch cores represented multiple spatial regions of the primary tumour and adjacent areas within the brainstem (average of six per autopsy encompassing the pons, midbrain and medulla), cerebellum and various cerebral neuroanatomical regions (average of 10 per autopsy) (Supplementary Tables 1–3; Supplementary Fig. 1). Sixty-seven samples representative of tumour and normal brain areas based on histological assessment were subjected to whole exome sequencing (WES) (Fig. 1; Supplementary Tables 1–4 and Supplementary Data 1–9, Supplementary Fig. 2). Oncogenic mutations associated with HGG5,3–8 were identified in 41/67 (61%) of punch cores and a vast majority (35/37; 95%) of punch cores taken from the brainstem (Supplementary Tables 1 and 2). Surprisingly, samples with oncogenic mutations also included distal supratentorial areas (3/20), a few of which were deemed radiologically and histologically normal (Fig. 1, Supplementary Tables 1–3, Supplementary Figs 2A–E and 4). In eight DIPGs, we observed K27M mutations in canonical H3.1 (2/8) or non-canonical H3.3 (6/8). In one patient, we identified K27M in HIST2H3C, which encodes the canonical histone 3.2 (H3.2K27M) (Fig. 1) a novel mutation recently described in one paediatric DIPG sample20. H3K27M mutations were ubiquitous in all 41 samples with oncogenic content, and were always associated with at least one additional partner driver mutation: TP53, PPM1D, ACVR1 or PIK3R1 (Fig. 1).

Clonal evolution of diffuse intrinsic pontine glioma. To decipher clonal evolution of DIPG, we employed a strategy resembling previously successful multi-region tumour sequencing studies14. Such approaches aim to reconstruct evolutionary relationship among multiple samples from the same patient based on the presence or absence of newly emerging somatic mutations14,17, and the relative mutation allele frequencies21,22. We used our WES data (70 × mean coverage) to select mutations—based on their presence in our and other available DIPG datasets3–8—for further screening using deep amplicon sequencing (~4,000 × mean coverage) in the initial sample set and in other geographically proximal and distal samples (N = 134). Mutation allele frequencies were corrected for aneuploidy and presence within copy number variant (CNV) regions whenever possible, and subsequently used to reconstruct evolutionary trajectories (Fig. 2; Supplementary Figs 5, 6 and 7; Supplementary Table 5). Remarkably, evolutionary analysis showed obligate association of specific combinations of at least four principal driver genes in our sample set: H3K27M/TP53 and/or PPM1D, H3K27M/ACVR1 and H3K27M/PIK3R1 (Fig. 2; Supplementary Fig. 7). The association between the histone mutation and obligate partner(s) was invariably maintained throughout all samples from an autopsy brain. This is in striking contrast to adult cancers, including HGG, which have large numbers of mutations and potentially long evolutionary histories resulting in complex clonal structures8,10,17. This also contrasts isocitrinate dehydrogenase (IDH)-mediated tumorigenesis, where IDH mutations are associated with genetic alterations that change over the course of the disease either through partnership with a different mutated gene or through acquisition of additional/different mutations in the same gene during tumour evolution17.

Temporal tumour homogeneity of main driver mutations. The tight association of principal driver mutation pairs implies early and nearly concurrent appearance during tumour evolution. Accordingly, in several cases the order of the partner mutations could not be resolved (Fig. 2; Supplementary Fig. 7). However, variations in the relative allele frequencies across spatial samples in DIPG1, DIPG5, DIPG7 and DIPG9 enabled estimation of the most likely mutation order, suggesting H3K27M as the earliest tumorigenic event in DIPG (Fig. 2, Supplementary Fig. 7). A clear example of such clonal evolution is illustrated by DIPG5 (Fig. 2a) harboring three principal driver mutations (H3.3K27M/TP53/PPM1D). PPM1D regulates TP53 cell-cycle checkpoint and mutations in both genes have been described as mutually exclusive in gliomas8,23. DIPG5 is the sole reported exception to this rule, providing valuable evolutionary insights. We identified two major clones carrying distinct combinations of driver mutations, H3.3K27M-TP53 and H3.3K27M-PPM1D, indicating the H3.3K27M mutation as the initial event, followed by TP53 mutation in one clone, and PPM1D mutation in another. This suggests that the early presence of oncohistone mutations promotes subsequent alterations of the TP53 pathway, and that this partner association—which is required for full transforming
potential in DIPG—is subsequently maintained throughout the tumour.

Further analysis of combined data from our cohort and published genomic DIPG datasets (N = 130) showed the presence of H3K27M in 116/130 (89%) of patients (29 H3.1K27M, 1 H3.2K27M, 86 H3.3K27M, Supplementary Table 6) and this finding, along with the analysis of published diagnosis biopsy datasets, indicates that the H3K27M obligate partnership is maintained throughout the disease course.

### Molecular characterization of tumour spread

Molecular studies of punch cores representing various neuroanatomical locations showed tumour extension outside the brainstem (Supplementary Tables 1–3; Supplementary Fig. 3). Remarkably, tumour spread was detected in proximal (for example, the cerebellum, 4/8 patients with sampled material) and distal (thalamus 4/4, hippocampus/ventricle/frontal or occipital lobes 3/7) brain locations, and in rare cases in areas with no clinical, radiological or histopathological evidence of tumour (Supplementary Figs 2, 3, 4). Interestingly, re-analysis of the published datasets showed two cases where the matched ‘normal’ frontal lobe samples had H3K27M/partner mutation frequencies similar to the primary tumour. Furthermore, clustering analysis of our DNA methylation data showed distant tumour spread to be more similar to H3K27M mutant tumours than to normal brain (Supplementary Fig. 8). Thus, molecular designation of histologically ‘normal’ brain DIPG specimen warrants more in-depth molecular scrutiny. Evolutionary studies of tumour dissemination were consistent with a mixture of early and late tumour spread. Some sites harbored most of the secondary mutations observed in the primary tumours suggestive of late spread, while others lacked the secondary mutations found in the primary tumour site suggestive of early spread (Fig. 3a, Supplementary Fig. 9).

In addition to the main driver mutations discussed above, several patients carry recurrent mutations that are clearly subclonal (present in some but not all tumour areas in a patient).
and occur at later stages of tumour evolution (Fig. 1). These include mutations in the chromatin remodeler ATRX (DIPG5 and DIPG9), as well as the activating PIK3CA H1047R mutation24, which affects the catalytic domain of PIK3CA (DIPG2, DIPG3 and DIPG6) (Fig. 1). In DIPG3 gross visual inspection of the pons indicated two morphologically distinct areas (Pons 1 and 2, Fig. 3b). Histological examination identified area Pons 1 as low-grade infiltrative astrocytoma (WHO II) and Pons 2 as high-grade astrocytoma (WHO IV, glioblastoma, Fig. 3b). Both areas carried HIST2H3C/ACVR1 mutations inferred to occur early in tumour evolution (Fig. 3b). However, only Pons 2 (WHO IV) carried PIK3CA H1047R. RNAseq analyses showed enrichment of overexpressed genes involved in vasculature development and angiogenesis pathways in Pons 2, in keeping with prior studies linking PI3K activation to angiogenesis25 (Supplementary Data 10). Alterations affecting the PI3K pathway are prevalent both in our sample set (4/9) and in published DIPG datasets (31/116, 26%). Our data suggests that PIK3CA mutations are not essential to tumour initiation in DIPG but may confer localized survival, growth and angiogenic advantage in the areas they are subsequently acquired.

**Discussion**

Genomic analysis of autopsy DIPG brains provides a fascinating opportunity to reconstruct the evolution of this deadly tumour, and has important implications on potential therapeutic interventions. Despite the sample number limitation based on disease incidence, this study provides insight into the spatial and temporal evolution of the tumour genome in DIPG helping derive a general model for this disease.
Our analysis suggests that H3K27M mutations are the initial oncogenic event in DIPGs. However, these mutations are not solely sufficient for tumour formation, as they require, nearly universally, obligate partners for effective tumorigenesis. There is some disagreement in the cancer field as to the definition of ‘driver’ versus ‘passenger’ mutations. The designation passenger has been used for any variant that is not absolutely essential to the tumorigenesis. However, for the purpose of this study—and more generally—we postulate three categories of mutations: (1) main driver mutations are essential to initiate and continue tumorigenesis; (2) accessory driver mutations can further promote and accelerate tumour growth but are not absolutely essential; (3) passenger mutations are neutral and do not affect the tumour. H3K27M and their obligate partners appear thus to be the main driver mutations, since they always co-occur together throughout each tumour.

These K27MH3.3 partner mutations are mostly genetic alterations affecting the TP53 pathway, mainly TP53 and less frequently PPM1D genes. H3K27M mutants also favor association with ACVR1 gain of function mutations and in rare cases (1 in our dataset and 2 in other reported cases) PIK3R1 mutations. Interestingly, even if more frequent, PIK3CA mutations, including PIK3CA H1047R, clearly fall into the accessory driver category, since they were invariably sub-clonal in our dataset and in some other reported cases. Similarly, a PTEN mutation was also found in some but not all tumour areas in DIPG2. Our analysis also indicated a clear clonal subclassification of the two regions, with 18 SNVs and 1 CNA found only in DIPG2, and 11 SNVs and 3 CNAs unique to DIPG2. Intriguingly, DIPG2 carries the activating PIK3CA H1047R mutation, which occurs early in the evolution of this sub-clone judging by its high allelic frequency. PIK3CA H1047R is associated with multi-potency and PI3K activation with angiogenesis and growth and this mutation likely contributes to tumour aggressiveness and high-grade features of Pons 2 compared with Pons 1 in DIPG3. Scale bar = 500 μm.

Figure 3 | Tumour spread in DIPG. (a) Tumour spread in DIPG2 in the thalamus, cerebellum and brainstem. Tumour extension in the thalamus harbors secondary mutation PIK3CA, MAX and PTEN, which indicates late spread from both Pons 1 and Pons 2. Extension towards the cerebellum is relatively early in the tumour evolution as it lacks secondary mutations found in the primary tumour and other brainstem spread. (b) Evolution of tumour in patient DIPG3. Autopsy revealed two morphologically and histologically distinct regions of the tumour, indicated as DIPG3 Pons 1 (low-grade) and DIPG3 Pons 2 (high-grade). Exome sequencing identified 11 SNVs and several large scale CNAs common to both regions. Shared alterations included H3.2 K27M and ACVR1 G328V mutations that are likely the main driver mutations in this patient. The analysis also indicated a clear clonal subclassification of the two regions, with 18 SNVs and 1 CNA found only in DIPG3 Pons 1, and 11 SNVs and 3 CNAs unique to DIPG3 Pons 2. Intriguingly, DIPG3 Pons 2 carries the activating PIK3CA H1047R mutation, which occurs early in the evolution of this sub-clone judging by its high allelic frequency. PIK3CA H1047R is associated with multi-potency and PI3K activation with angiogenesis and growth and this mutation likely contributes to tumour aggressiveness and high-grade features of Pons 2 compared with Pons 1 in DIPG3. Scale bar = 500 μm.
cells. Indeed, H3K27M mutants show a dose-dependent effect in inducing cellular proliferation26,27. Canonical H3 variants are the most abundant histones in cells and need cell-cycle division (S-phase) for synthesis, whereas non-canonical H3.3 variants represent ~5% of all H3 in cells and are present throughout the cell cycle. Accordingly, H3K27M mutations in cell cycle-dependent canonical histones may co-occur with alterations affecting ACVR1, a growth factor that induces cell division, resulting in the synthesis of wildtype and mutant H3.1 proteins. H3K27M mutations in the non-canonical H3.3 histone are mainly associated with mutations affecting the TP53 pathway, as these possibly offer evasion from cell death and senescence and provide the needed opportunity for the mutant H3.3K27M to exert its effect over a longer period, effectively reshaping the epigenome and drive tumour formation.

The previously unsuspected homogeneity for main driver mutations across the course of the disease uncovered in this study indicate that efforts to cure DIPG should be directed at the oncohistone partnership, as other genetic alterations are generally sub-clonal. Our findings further indicate that needle biopsies are recommended to orient care and are representative of the main drivers in DIPG even if the regional heterogeneity of other secondary targetable alterations, such as PIK3CA mutations, may not be fully captured. Based on early tumour spread, efforts to cure DIPG should aim for early systemic tumour control as opposed to regimens focused on the pons.

Methods

Patient samples. All patient samples were collected with informed consent in accordance with the respective Ethics Review Boards of the Children’s National Health System, Washington, DC, USA and the University of Queensland Child Health Research Center, Brisbane, Australia. DIPG post-mortem specimen procurement was performed as previously described28. Briefly, the brainstem and cerebellum were removed en bloc from the whole brain, and dissected into ~9 transverse sections. The cerebral cortex was dissected into ~11 coronal sections. The brainstem, cerebellum and cerebral cortex sections were alternately frozen or fixed in formalin. A total of 158 samples were studied by immunohistochemistry and molecular analyses, representing various neuroanatomical locations such as frontal, parietal, temporal, occipital lobes, thalamus, lateral ventricles, hippocampus, midbrain, pons, medulla and cerebellum (Supplementary Tables 1–3; Supplementary Fig. 1). For molecular studies and cerebral cortex sections were alternatively frozen or fixed in formalin. A total of 6

Whole-exome sequencing. Genomic DNA was extracted from multiple post-mortem samples patient using the standard extraction methods as described by Qiagen. Nextera Rapid Capture Exome kit was used to prepare the paired-end libraries according to the manufacturer’s instructions using on average 36 ng of total starting genomic DNA. Sequencing was performed on Illumina HiSeq 2000 using rapid-run mode with 100 bp paired-end reads. Next, adaptor sequences were removed; reads were trimmed for quality using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx-toolkit). An in-house program was employed to detect reads mapping to the genome that were the presence of exclusively paired-reads to be used in further steps of the analysis. We next aligned the reads using Burrows-Wheeler Aligner (BWA) 0.7.7 to hg19 as reference genome. Indel realignment was performed using the Genome Analysis Toolkit (GATK)39 (http://www.broadinstitute.org/gsa/wiki). We next marked the duplicate reads using Picard (http://picard.sourceforge.net/) and excluded them from further analyses as previously described8. The coverage of consensus coding sequence (CCDS) bases was assessed using GATK. The average coverage over all the samples was 70 ×. The majority of samples had >90% of CCDS bases covered by at least 10 reads and >83% of CCDS bases covered by at least 20 reads.

We called SNVs and indels using SAMTools (http://samtools.sourceforge.net/) mpileup with the extended base quality alignment quality adjustment (−E)0.31. Next we filtered them for quality so that at least 10% of reads supporting each variant call. We used both ANNOVAR32 and in-house tools to annotate the variants and to identify whether these variants affect protein-coding sequence and if they had previously been observed in datasets including the 1,000 Genomes Project data set (November 2011), the National Heart, Lung and Blood Institute (NHLBI) Grand Opportunity (GO) exomes or in ~3,000 exomes previously sequenced at our center. Results of whole exome sequencing are summarized in Supplementary Table 3 and presented for individual patients as follow: DIPG1 in Supplementary Data 1; DIPG2 in Supplementary Data 2; DIPG3 in Supplementary Data 3; DIPG4 in Supplementary Data 4; DIPG5 in Supplementary Data 5; DIPG6 in Supplementary Data 6; DIPG7 in Supplementary Data 7; DIPG8 in Supplementary Data 8; DIPG9 in Supplementary Data 9.

Exploratory targeted high-depth DNA sequencing of hotspot mutations. Genomic DNA from DIPG samples were used for high-depth sequencing using Illumina MiSeq platform. The MiSeq panel covers exons in 16 Histone H3 isoforms (H3F3A and H3F3B), 3 Histone H4 isoforms (HIST1H4A, HIST1H4B and HIST1H4C) and 16 Histone H2A isoforms (HIST1H2A, HIST1H2B, HIST1H2C and HIST1H2D). Genomic DNA samples were sequenced using the MiSeq sequencing platform (Illumina) with an average coverage of >20,000 × of the analogous K27M base change across the three H3 variants.

To estimate allele frequency of mutations identified using whole exome sequencing genomic DNA from the same samples was also used for high-depth sequencing using the MiSeq sequencing platform with an average coverage of >4,000 ×. Reads were mapped to the reference genome (human hg19) using the BWA genome alignment90. Alignment files were fed to an in-house program to calculate different exons’ allele frequency at the desired positions.

RNA sequencing. We used QIAGEN RNeasy lipid tissue mini kit to extract RNA from tumour DIPG (Pons 1 and Pons 2) according to manufacturer’s instructions. Libraries were prepared using RNeasy depletion method followed by preparation from Epicentre (manufacturer) to achieve greater coverage of mRNA. Paired-end sequencing was performed on the Illumina HiSeq 2000 platform.
DNA methylation analysis. Methylation profiling data was analysed as previously described. The raw data were subject to quality control and preprocessing utilizing the R package minfi, and normalized for technical variation between the Infinium I and II probes using the SWAN method. We removed probes on sex chromosomes (chrX, Y), those containing SNPs (dbSNP: http://www.ncbi.nlm.nih.gov/SNP/) as well as non-specific probes that bind to multiple genomic locations. Unsupervised hierarchical clustering was performed using average linkage and Pearson rank correlation distance on the top 5,000 most variable probes selected based on standard deviation of beta values (β-values).

Copy number variation analysis. To study copy number variations in our samples we developed an in-house program to calculate the deviation of B allele frequency from 50% as well as normalized coverage from whole exome sequencing data (adapted from methods used in FishingCNV33 and ExomeAI34). Different CNV events (duplication, deletion and copy neutral LOH) were called based upon the B allele imbalance and the status of the normalized coverage as follow: deviation from 50% B allele frequency and an increase in normalized coverage was considered as amplification, deviation from 50% B allele frequency and decrease in normalized coverage as deletion and deviation from 50% B allele frequency and no change in the normalized coverage was considered as potential copy neutral loss of heterozygosity (CNLOH) and copy neutral LOH (CN-LOH, respectively). The results of our CNV detection are presented in Supplementary Fig. 5 and Supplementary Table 14.

OncoScan verification of the CNV events. OncoScan FFPE Assay Kit, provided by Affymetrix, is a platform based on Molecular Inversion Probe technology, used to assess copy number and loss of heterozygosity using small amounts of DNA from FFPE samples. We performed this assay on several samples in order to verify our WES based CNV detection. Genomic DNA was quantified using PicoGreen protocol (Quant-IT PicoGreen dsDNA Products, Invitrogen, P-75898) and read on SpectraMAX GeminiXS Spectrophotometer. The OncoScan FFPE Assay Kit was used according to the manufacturer’s instructions (Affymetrix). A GeneAmp PCR system 9700 Thermal Cycler (Life Technologies) was used from the anneal stage to the denaturation stage. QC gels of the PCR and HaeIII digest products were run. CNV events were called using the normalized data using Nexus Express for OncoScan 3.1 (Affymetrix). The OncoScan plots are represented in Supplementary Fig. 6.

Constructing evolutionary trees. We used PhyloWGS35 to reconstruct the tumour phylogeny, which uses a Bayesian approach to infer cellular frequencies from mutation allele frequencies. It applies the hierarchical process to cluster mutations with similar cellular frequencies and the tree-structured stick-breaking process to model the clonal evolutionary tree. For multi-region samples from the same patient, we normalized the read counts used for phylogenetic tree construction by copy number counts from CNV analysis. Read counts were corrected for the CNV events (Supplementary Fig. 5; Supplementary Table 3) for each sample as follow: in case of duplication Ref = Ref, Alt = Alt/2; in case of deletion Ref = Ref + Alt, Alt = Alt; in case of a gene on chromosome X Ref = Ref + 2 + Alt, Alt = Alt; in case of Copy Neutral LOH Ref = Ref + Alt/2 and Alt = Alt/2. The reconstructed trees were redrawn in Graphviz by using in-house scripts adapted from AncesTree36 to show the trajectories of mutations with contribution ≥0.05.

Differential expression and gene set enrichment analysis. We aligned RNASeq data from DIPG-Pons 1 and DIPG-Pons 2 (RNA extraction and sequencing protocol described before using STAR 2.3.0 (ref. 37), and analysed differentially expressed genes using DeSEQ (ref. 36). The top 200 over expressed and top 200 under expressed genes in DIPG-Pons 2 (the sub-clone with PIK3CA activating mutation) were analysed for gene set enrichment using both AmiGo 2 tool37 (http://amigo.geneontology.org/amigo) provided by Gene Ontology and DAVID38 (https://david.ncifcrf.gov/). We used PANTHER Overrepresentation Test (release 20,150,430) for analysis type by Amigo 2 (annotation version and release date: GO Ontology database released 2015-08-06). The top pathways found by AmiGo 2 to be enriched with enrichment folds higher than 5 and (Bonferroni<0.05) were retained. We used functional annotation clustering and set the fold change in DAVID and filtered the results with enrichment folds higher than 5 (Bonferroni corrected P value<0.05). We used the set of genes with at least 50 RNASeq reads in both DIPG Pons1 and 2 combined as background gene set in this analysis (Supplementary Data 10).

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Author contributions
E.P., L.M., D.F. and D.B. performed experiments. E.P., L.M., H.N., R.L., T.G., M.K., E.I.H., M.O., A.S., C.-Y.H., J.K., N.J., J.N. and J.M. analysed the data and produced figures and tables. K.L.L., B.E., W.J.I., A.S.M., C.S., K.E.W., R.J.P. provided tissue samples. N.J., J.N. and J.M. provided project leadership and designed the study.

Additional information
Accession codes: The whole-exome sequencing data for all tumours (along with RNA sequencing data for DIPG3), have been deposited in the European Genome Archive (EGA) database under the accession code EGAS00001001654. The DNA methylation profiles have been deposited in the Gene Expression Omnibus (GEO) database under the accession code GSE77353.

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