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Oncogenic 3D genome conformations identify novel therapeutic targets in ependymoma.

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67 Abstract

68 Ependymoma is a tumor of the brain or spinal cord. The two most common and aggressive molecular
69 groups of ependymoma are the supratentorial *RELA*-fusion associated group and the posterior fossa
70 ependymoma group A. In both groups, tumors occur mainly in young children and frequently recur
71 after treatment¹. Although the molecular mechanisms underlying these diseases have recently been
72 uncovered, they remain difficult to target and innovative therapeutic approaches are urgently needed.
73 Here, we use genome-wide chromosome conformation capture (Hi-C), complemented with CTCF
74 (insulators) and H3K27ac (active enhancers) ChIP-seq as well as gene expression and whole-genome
75 DNA methylation profiling in primary and relapsed ependymoma tumors and cell lines to identify
76 chromosomal rearrangements and regulatory mechanisms underlying aberrant expression of genes that
77 are essential for ependymoma tumorigenesis. In particular, we observe the formation of new
78 topologically associating domains ('neo-TADs') by intra- and inter-chromosomal structural variants,
79 tumor-specific 3D chromatin complexes of regulatory elements, and the replacement of CTCF
80 insulators by DNA hyper-methylation as novel oncogenic mechanisms in ependymoma. Through
81 inhibition experiments we validated that the newly identified genes, including *RCOR2*, *ITGA6*,
82 *LAMC1*, and *ARL4C*, are highly essential for the survival of patient-derived ependymoma models in a
83 disease subtype-specific manner. Thus, this study identifies potential novel therapeutic vulnerabilities
84 in ependymoma and extends our ability to reveal tumor-dependency genes and pathways by oncogenic
85 3D genome conformations even in tumors that lack known genetic alterations.

86

87 Main

88 Tumors of the central-nervous system (CNS) are the most common cancers in children aged 0-14 years
89 and a leading cause of death during childhood²⁻⁴. Intracranial ependymomas are segregated on the
90 basis of anatomical location (supratentorial versus infratentorial or posterior fossa) and further divided
91 by DNA methylation and expression profiling into distinct molecular groups that reflect differences in
92 the age of onset, gender predominance, response to therapy, and genetic aberrations that drive the
93 disease^{1,5,6}. The supratentorial *RELA*-fusion associated group is characterized by recurrent complex
94 chromothripsis events in chromosome 11 that lead to different types of *C11orf95-RELA* fusion genes,
95 which have been shown to drive tumorigenesis in this group of tumors^{7,8}. In contrast, initial DNA
96 sequencing studies showed an absence of recurrent mutations or gene fusions in posterior fossa
97 ependymoma group A (PFA), suggesting that these tumors might be epigenetically driven^{5,7}. Indeed,
98 global loss of histone H3 lysine 27 trimethylation (H3K27me3), a histone modification associated with
99 the negative regulation of gene expression, was identified as a marker for PFA tumors⁹. Recent studies

have revealed that EZH inhibitory protein EZHIP (previously known as *CXorf67*), which is aberrantly expressed in most PFA ependymomas, causes downregulation of H3K27me3 by inhibiting EZH2 in the polycomb repressive complex 2 (PRC2)^{10,11}. The few PFA ependymomas that do not overexpress *EZHIP* appeared to harbor K27M mutations in H3.1 or H3.3, which also inhibit EZH2. Furthermore, gain of chromosome arm 1q, present in ~25% of all PFA tumors, has been associated with a particularly poor survival of PFA patients, but the underlying driver mechanism remains unknown^{10,12}. Since there are no small molecules available directly targeting the C11orf95-RELA fusions or EZHIP, and since it is not yet known whether EZHIP alone drives tumorigenesis in PFA, a better understanding of the tumor driving mechanisms and how they can be targeted is urgently needed. New insights into the regulation of gene expression during normal and diseased human development have recently been gained by analyzing 3D chromatin architectures¹³⁻¹⁵. Therefore, we have combined Hi-C with complementary molecular profiling techniques of ependymoma tumors and cell lines to investigate whether changes in intra- or inter- chromosomal DNA interactions in these tumors may lead to activation of specific oncogenes and may identify novel targets and tumor dependencies (**Figure 1a**).

115 ***The 3D genome organization of ependymoma tumors***

We have performed Hi-C¹⁶ followed by deep sequencing in 14 PFA and RELA ependymoma samples, comprising eleven tumors (fresh frozen or FFPE) and three cell lines (**Figure 1b, Suppl. Figure 1a**). Most samples were also analyzed by chromatin immunoprecipitation targeting the histone modification H3K27ac, which is associated with active chromatin, followed by sequencing (ChIP-seq, n=9), gene expression (RNA-seq, n=11), whole genome sequencing (WGS, n=12) and DNA methylation (n=14, **Suppl. Table 1**). PFA and RELA ependymoma groups can be clearly distinguished using various molecular profiling techniques including DNA methylation¹⁷ (**Suppl. Figure 1b**). Unsupervised clustering of the Hi-C data also clusters ependymoma tumors into the expected groups, demonstrating pronounced group-specific 3D tumor genome conformations (**Figure 1c, Suppl. Figure 1c,d**). By an integrative analysis of the Hi-C data, enhancers (defined by H3K27ac ChIP-seq enrichments), and gene expression, we observed that genes are generally expressed at higher levels when their promoters physically interact with enhancers or other gene promoters (**Figure 1d**). A large percentage (~63-66%) of enhancer-associated genes (EAGs) previously predicted to be regulated by PFA or RELA enhancers¹⁸ can be confirmed to have chromatin interactions between gene promoters and enhancers by the analysis of subgroup-specific Hi-C maps (**Figure 1e**). For example, the *Tenascin C* (*TNC*) promoter physically interacts with distal PFA enhancers (**Figure 1f**), which are positively correlated with *TNC* expression across a cohort of 24 tumors from six different intracranial ependymoma groups (**Figure 1g, Suppl. Figure 1e**). Overall, we found that more than twice as many

134 genes as previously reported¹⁸ are potentially regulated by proximal and distal ependymoma enhancers
135 (**Figure 1h, Suppl. Table 2,3**). For example, the gene encoding Eukaryotic Translation Elongation
136 Factor 1 Alpha 2 (*EEF1A2*) interacts with subgroup specific enhancers (**Figure 1i**) and is specifically
137 upregulated in *RELA* -fusion associated tumors (**Figure 1j**). These and other regulatory dependencies
138 were not recognized in our previous study¹⁸, due to lack of data on chromatin interactions in
139 ependymoma samples and because TAD annotations from a fetal lung fibroblast cell line (IMR90)
140 were used instead (**Suppl. Figure 1f,g**).

141

142 ***Transcriptional activation of RCOR2 by neo-TAD formation in supratentorial RELA ependymoma***

143 The formation of new topologically associating domains ('neo-TADs') through structural variation
144 was recently shown to have a critical role in gene dysregulation and oncogenesis^{19,20}. To dissect the
145 effect of structural variants (SVs) in supratentorial *RELA* tumors on the potential formation of neo-
146 TADs, we used newly developed computational tools for the detection of SVs based on Hi-C data^{19,21}
147 (**Suppl. Table 4**). We first took a closer look at the *C11orf95* and *RELA* gene loci, because it was
148 previously shown that the oncogenic *C11orf95-RELA* gene fusions are a result of chromothriptic
149 events on chromosome 11. As expected, the Hi-C data reproducibly detected structural variants at the
150 *C11orf95* and *RELA* gene loci in the supratentorial *RELA* but not in PFA tumors (**Figure 2a, Suppl.**
151 **Figure 2a**). Furthermore, the Hi-C data captured extraordinarily complex rearrangements within
152 chromosome 11 in some *RELA* ependymoma samples (**Figure 2b**) and revealed that SVs are not
153 restricted to chromosome 11 but also include inter-chromosomal rearrangements (**Figure 2c**). In
154 particular, we observed intra- and inter-chromosomal structural variants in all *RELA* tumors, which
155 lead to the formation of neo-TADs placing the *REST Corepressor 2* (*RCOR2*) gene in a new regulatory
156 environment (**Figure 2d, Suppl. Figure 2b**). *RCOR2* is located ~150kb away of *C11orf95* and has a
157 strong enhancer element upstream of its transcription start site that forms new DNA interactions with
158 the *C11orf95* gene and other nearby enhancer elements by bridging the *C11orf95-RELA* breakpoint
159 (**Figure 2e, Suppl. Figure 2c**). By evaluating global Affymetrix gene expression array data¹ across
160 ependymoma groups, we found that *RCOR2* expression is significantly upregulated in supratentorial
161 *RELA* relative to other ependymoma groups (p-value=1.71e-91, **Figure 2f**) and is highly correlated
162 with *C11orf95* transcription (R=0.66, p-value=6.93⁻¹¹, **Suppl. Figure 2d**). Interestingly, we also
163 identified *RCOR2* as a hit in *RELA* cells in an independent shRNA screen (data not shown). To
164 validate the relevance of *RCOR2* for tumor growth and maintenance, we performed shRNA-mediated
165 knock-down of *RCOR2* expression in patient derived *RELA* and PFA ependymoma cell lines. We
166 observed that *RCOR2* knockdown results in strongly reduced cell survival of supratentorial *RELA* and
167 to a lesser extent of PFA cell lines (**Figure 2g-h**). The on-target effect of shRNAs against *RCOR2* was

confirmed by western blot analysis of RCOR2 protein in RELA endependymoma cells (**Suppl. Figure 2e**). RCOR2 can form a protein complex with the histone de-methylase LSD1, also known as KDM1A, and other transcriptional co-repressors, including HDAC1/2²². LSD1, HDAC1 and HDAC2 are all highly expressed across endependymoma subgroups and HDAC1/2 show pronounced RELA endependymoma-specific expression (**Suppl. Figures 2f-h**). Since there is no available compound against RCOR2, we reasoned that inhibition of other components of the RCOR2/LSD1/HDAC complex may still confer a therapeutic vulnerability for RELA endependymoma tumors. shRNA-mediated inhibition of *LSD1* expression indeed leads to a significant depletion of RELA but not PFA endependymoma cells compared to scrambled shRNA (**Figure 2i, Suppl. Figures 2i,j**). Surprisingly, however, targeting the enzymatic activity of LSD1 with the LSD1 inhibitor ORY-1001²³ had no effect on cell survival using clinically accessible concentrations (**Figure 2j**), suggesting that in this protein complex the protein rather than the enzymatic activity of LSD1 is important. In contrast, targeting the HDAC activity in the complex with Entinostat, an HDAC1-3 inhibitor, strongly inhibited the survival of RELA endependymoma cells, while having less effect on PFA cells (**Figure 2k**). Combining ORY-1001 with Entinostat showed no synergy (data not shown) and also the results for Corin, a dual inhibitor of both LSD1 and HDACs, were not better than those for Entinostat alone (**Figure 2l**). Inhibition of other HDACs with, e.g., HDAC8 and HDAC6/10 inhibitors PCI-34051 and Tubastatin, respectively, had no effect on cell survival (**Suppl. Figure 2k-l**). Altogether, our data show that the CoREST protein complex containing RCOR2, LSD1 and HDAC1/2 has a crucial role in the growth and maintenance of supratentorial RELA endependymoma tumors that can be inhibited by disrupting the complex or by targeting the activity of HDACs.

189

190 ***PFA Ependymomas are dependent on integrin $\alpha 6$***

191 In all PFA tumors the Hi-C data revealed a 3D chromatin cluster that spatially links numerous
192 regulatory sequences and genes located more than 4 million base pairs apart on chromosome 2 (**Figure**
193 **3a-c, Suppl. Figure 3a**). To determine if this chromatin cluster is specific to PFA tumors, we analyzed
194 Hi-C data obtained from RELA endependymoma samples as well as normal human tissues and cell types
195 analyzed by the ENCODE and PsychENCODE consortia^{24,25}. There was no sign of similar DNA
196 interactions in the RELA and non-tumor samples, suggesting that this chromatin cluster is specific to
197 PFA endependymomas (**Figure 3a, Suppl. Figure 3a**). By analyzing the expression of genes in this
198 chromatin cluster in endependymoma and normal human brain samples, we observed that *integrin $\alpha 6$*
199 (*ITGA6*) expression, encoding the receptor of the extracellular matrix protein laminin, is significantly
200 upregulated in PFA compared to RELA endependymoma and also normal human brain tissues (p-value:

1.21e-114, **Suppl. Figure 3b**). As also reported previously¹, gene-ontology analysis shows that ITGA6-associated gene sets, such as *extracellular matrix organization* and *positive regulation of cell migration*, are among the most highly enriched biological processes when comparing overall gene expression profiles of PFA to other ependymoma groups (**Suppl. Table 5**). Recent genome-wide CRISPR-Cas9 inhibition screens have revealed that ITGA6 is highly and specifically essential in PFA ependymoma cell lines compared to glioblastoma (GBM) cell lines and fetal neural stem cells (fNSCs)^{26,27} (**Suppl. Figure 3c**). In addition, we find that *integrin β 4* (*ITGB4*), but not *β 1* (*ITGB1*), is significantly up-regulated in PFA compared to RELA ependymoma and normal brain samples (p-values: 1.48e-119 and 1.42e-90, **Suppl. Figure 3d**), suggesting that the integrin α 6 β 4 heterodimer is the functional form relevant for PFA tumors. Based on these results, we hypothesized that PFA ependymomas are locked in an oncogenic genomic topological configuration that activates *ITGA6* transcription and drives the acquisition and maintenance of stemness. To test this hypothesis, we performed CRISPR-Cas9 mediated *ITGA6* knock-out in patient-derived PFA ependymoma cells by cloning three different sgRNA sequences targeting *ITGA6* into a lentiviral vector expressing Cas9 in conjunction with GFP and subsequently transduced PFA ependymoma cells with the virus. As a result, we observe that transduced PFA ependymoma cells (**Figure 3d**), but neither transduced RELA ependymoma (**Suppl. Figure 3e**) nor glioblastoma cells (**Suppl. Figure 3f**), showed a gradual decrease in cell proliferation over time, validating *ITGA6* as an essential tumor-dependency gene specific for PFA ependymomas.

220

221 ***Transcriptional activation of LAMC1 as a potential resistance mechanism in recurrent PFA*** 222 ***tumors***

Conventional copy-number variation (CNV) analyses previously showed 1q copy-number gains in a subset of very aggressive and recurrent PFA ependymomas (**Suppl. Figure 3g**)¹⁰. By investigating CNVs in PFA tumors, including primary and relapse tumors of the same patient, we observed frequent increases in genomic instability in relapse tumors, while the 1q gain is maintained or emerges during tumor progression (**Suppl. Figure 3h**). To elucidate the molecular mechanisms associated with 1q gain, we systematically searched for SVs in all PFA ependymoma samples using the Hi-C data (**Suppl. Table 6**). As expected, primary PFA EPNs have frequent DNA interactions within chromosomes ('cis') and no DNA interactions indicative of recurrent structural variants (**Suppl. Figure 3i**). However, we observed several complex inter-chromosomal DNA ('trans') interactions indicative of structural variants in PFA ependymoma relapse tumors (**Figure 3e, Suppl. Figure 3j**). When comparing SVs among the analyzed PFA ependymoma relapse samples, we observed a recurring event that leads to an inversion of a ~66 Mb region of chromosome arm 1q into chromosome 8 (chr1-chr8

in sample EPD210FH, **Figure 3e**), or into chromosome 3 (chr1-chr3 in sample RD-19-157, **Suppl. Figure 3j**). In both cases, the breakpoints on chromosome 1q are located near the gene locus of *laminin subunit γ 1* (*LAMC1*). Examination of the Hi-C data shows that both SVs led to the formation of neo-TADs, which place *LAMC1* into new regulatory environments (**Figure 3f,g**). Inspecting RNA-seq expression data revealed that *LAMC1* is expressed almost three times higher in the two 1q+ PFA ependymoma relapse tumors than in primary PFA ependymoma tumors (**Figure 3h**). The same pattern of increased *LAMC1* expression in PFA relapse cases was found in a larger Affymetrix ependymoma data cohort¹ (**Figure 3i**). By a direct comparison of three relapse tumors that developed within 18 years after the primary diagnosis of a PFA ependymoma patient, we observed upregulation of *LAMC1* specifically in the third and fatal relapse tumor with marked chr1q gain (**Suppl. Figure 3h,k**). These results suggest that transcriptional activation of *LAMC1* by the formation of SV-induced neo-TADs is a resistance mechanism in recurrent 1q+ PFA EPN tumors that potentially promotes proliferation and stemness by further enhancing an already excessive integrin signaling. Based on these results, we hypothesized that *LAMC1* expression is essential for the proliferation and growth of PFA ependymoma tumors. To test this hypothesis, we performed genetic (CRISPR-Cas9) inhibition experiments against *LAMC1* and observed strongly reduced cell growth in the same PFA cell line EPD210FH that harbors the chr1-chr8 translocation (**Figure 3j, Suppl. Fig. 3l**) but not in RELA (**Suppl. Figure 3m**) ependymoma models.

Hypermethylation disrupts CTCF binding in PFA ependymoma

It has recently been shown that DNA methylation-mediated insulator dysfunction can lead to altered chromosomal topology thereby activating oncogenic programs (**Figure 4a**)^{28,29}. Given the global loss of repressive H3K27me3^{10,11} and a previously reported DNA methylation phenotype in PFA ependymoma⁵, we hypothesized that similar molecular mechanisms may drive oncogene activation in this tumor type. Therefore, we analyzed 7 PFA (n=4) and RELA (n=3) tumors using Whole Genome Bisulfite Sequencing (WGBS) and CTCF ChIP-seq (**Suppl. Table 1**). As expected, genome wide CpG methylation is high in PFA and RELA ependymomas with low levels of methylation at functional regulatory elements, such as promoters, enhancers and insulators (**Suppl. Figure 4a**). By comparative analysis of DNA methylation at CTCF binding sites, we found that DNA hypermethylation replaces 2,387 CTCF binding sites in PFA tumors, but conversely is associated with the loss of only 178 CTCF binding sites in supratentorial RELA tumors (**Figure 4b-d**). The loss of CTCF binding through DNA hypermethylation is a predominant event in PFA ependymoma (**Figure 4c**) and can be associated with the formation of new enhancer-gene DNA loops and transcriptional activation of the target genes (**Suppl. Table 7**). To investigate whether such potential DNA methylation-mediated insulator

dysfunctions can be linked to the transcriptional activation of genes essential for PFA ependymoma, we compared our results with those of a genetic inhibition screen in PFA cell lines²⁶. Among others, we observed localized hypermethylation in PFA tumors associated with the loss of a CTCF binding site and the formation of DNA interactions between non-coding regulatory enhancer elements and the *ADP Ribosylation Factor Like GTPase 4C (ARL4C)* gene (**Figure 4e-f**). *ARL4C* transcription is significantly (**p-value:** 4.25e-55) upregulated in PFA tumors compared to other ependymoma groups (**Suppl. Figure 4b**) and is highly correlated with the activity of the enhancer elements that physically interact with the *ARL4C* gene locus in PFA tumors (**Figure 4g, Suppl. Figure 4c**), but not in RELA tumors. It has been shown that *ARL4C* promotes migration, invasion and proliferation in colorectal and lung cancer³⁰ and recent genome-wide CRISPR-Cas9 inhibition screens revealed that *ARL4C* is essential for the proliferation of PFA ependymoma compared to glioblastoma cell lines (**Suppl. Figure 4d**)^{26,27}. By genetic (CRISPR-Cas9) inhibition experiments we validated that *ARL4C* is highly and specifically essential for the growth of PFA ependymoma (**Figure 4h**) compared to RELA ependymoma and glioblastoma models (**Suppl. Figure 4e-f**). These results not only provide additional evidence for the relevance of *ARL4C* in PFA ependymoma tumors, but also shed light on the various molecular mechanisms that potentially lead to oncogenic activation of gene expression through genome-wide epigenetic alterations.

Discussion

By investigating 3D ependymoma genomes using Hi-C, we have identified multiple oncogenic chromatin conformations and novel tumor-dependency genes, pathways and potential therapeutic targets in RELA and PFA ependymoma. We show that structural variants in supratentorial tumors not only lead to *C11orf95-RELA* fusion genes, but also result in the formation of new regulatory environments that are recurrently associated with the aberrant overexpression of *RCOR2*. *RCOR2* is the scaffold protein in the CoREST complex that further contains LSD1 and HDAC1 and HDAC2. The complex is associated with gene silencing and is known to play a role in cancer development³¹. Here, we have shown that both *RCOR2* and *LSD1* expression is essential in RELA ependymoma, but not or to a lesser extent in PFA ependymoma, and that the cells are sensitive to HDAC1/2 inhibitors in line with our previous observations³². However, inhibition of the enzymatic activity of LSD1 had no effect. These results suggest that the activities of HDAC1/2 may be critical in regulating CoREST repressor functions in RELA ependymoma. Recent work in small cell lung cancer (and Merkel cell carcinoma) also implicated that disrupting the CoREST complex, but not the inhibition of LSD1's enzymatic activities is required for blocking cancer cell proliferation³³. Further studies identifying the

303 components of the CoREST complex and identifying drugs that can disrupt the complex will be
304 instrumental in developing an effective CoREST-targeted therapy for RELA endymoma.
305 Furthermore, we have shown that PFA endymomas are not only characterized by diminished histone
306 methylation and increased acetylation at histone 3 lysine 27 (H3K27), as recently reported²⁶, but also
307 exhibit a tumor-specific 3D chromatin organization. Through targeting of *ITGA6*, a gene involved in
308 a PFA-specific chromatin cluster, we demonstrate the importance of integrin signaling for maintained
309 tumor growth, specifically in PFA tumors. ITGA6 has been described as a marker for cancer stem cells
310 (CSCs) in several cancer types³⁴⁻³⁸, where disruption of ITGA6 function suppresses the CSC phenotype
311 and the maintenance of stem cells³⁴. Our results provide evidence for an epigenetic dysregulation event
312 that promotes integrin signaling and the acquisition of stemness in PFA endymoma. The significance
313 of integrin signaling for PFA tumor progression is further promoted by the recurrent transcriptional
314 activation of *LAMC1* in PFA relapse tumors which frequently harbor gains of chromosome 1q.
315 Although relapse tumors often show increased genomic instability, our Hi-C data showed for the first
316 time an unexpected complexity of intra- and inter- chromosomal rearrangements underlying some
317 chromosome-arm-wide copy number variations. Our results suggest that transcriptional activation of
318 *LAMC1* by the formation of SV-induced neo-TADs is a potential resistance mechanism in recurrent
319 1q+ PFA EPN tumors that promotes proliferation and stemness by further enhancing already excessive
320 integrin signaling. For other tumor types, LAMC1 has already been shown to be involved in tumor
321 cell invasion and metastasis³⁹. Thus, strategies that target integrin signaling, including ITGA6 and
322 LAMC1, may reveal new vulnerabilities and overcome resistance to therapy in the treatment of PFA
323 EPN relapse patients. Insulator dysfunction and oncogene activation by hypermethylation of CTCF
324 binding sites has recently been described in IDH mutant gliomas and in SDH-deficient gastrointestinal
325 stromal tumors (GISTs)^{28,29}. Here, we show that PFA endymoma is another tumor type with a global
326 epigenetic phenotype in which there is hypermethylation of CTCF binding sites and associated
327 changes in genome topology. By genetic inhibition of *ARL4C* in a PFA endymoma model, we
328 provide evidence that insulator dysfunction is a potential oncogenic mechanism in PFA endymoma
329 tumors and that tumor-dependency genes can be identified by 3D tumor genome profiling. Altogether,
330 our study has identified several new group specific tumor dependencies in endymoma, opening up
331 avenues for potential novel therapeutic interventions that are highly needed in this disease, especially
332 for RELA and PFA endymoma. Our results will also be important for other (pediatric) cancers,
333 especially those that relapse, where the drivers might be known, but where therapeutic options are
334 scarce. Hi-C studies in these tumors may also reveal unknown tumor dependencies and new
335 therapeutic targets.

336

Figures

Figure 1: 3D tumor genome profiling identifies PFA and RELA specific chromatin conformations and ependymoma enhancer associated genes.

(a) Overview of the major results obtained by the application of genome-wide chromosome conformation capture (Hi-C) in ependymoma brain tumors.

(b) Characteristics of ependymoma samples analyzed by Hi-C. One group of PFA ependymoma samples has no apparent copy-number variants, while the other group of PFA samples exhibits chromosome 1q gains associated with an unfavorable outcome.

(c) Unsupervised hierarchical clustering of PFA and RELA ependymoma tumors based on DNA interactions (Hi-C) stratifies the samples into the expected molecular groups.

(d) Integrative analysis of enhancers (H3K27ac ChIP-seq), chromosome conformation (Hi-C) and gene expression (RNA-seq) shows that genes are more strongly expressed when their promoters physically interact with other promoters or with enhancers. Shown are tumors (3x PF-A, 3x RELA) for which sample-matched H3K27ac ChIP-seq, RNA-seq, and HiC data are available. The center line, box limits and whiskers indicate the median, upper/lower quartiles and 1.5× interquartile range respectively. P-values from the bootstrap t-test are included.

(e) Re-evaluation of genes previously predicted to be regulated by PFA ependymoma enhancers confirms that the promoter regions of approximately 66% of these genes (n=1,028) physically interact with PFA ependymoma enhancers. Similar results (63%, n=1,229) are obtained for RELA ependymomas.

(f) The *TNC* promoter physically interacts with two distal enhancers (E1 and E2), whereby the interaction with the more proximal enhancer E1 is much more pronounced in PFA than in RELA tumors.

(g) *TNC* expression is positively correlated with the activity of the PFA-specific enhancer E1 (*chr9:118146925–118163777*), which is located 390kb upstream of the *TNC* transcription start site. Here, a cohort of 24 tumors from six different intracranial ependymoma groups was examined.

(h) The integrative analysis of Hi-C, enhancer and gene expression data reveals that more than twice as many genes as previously reported¹⁸ are regulated by proximal and distal ependymoma enhancers.

(i) The Hi-C data identifies a cluster of DNA interactions between a RELA-specific superenhancer (SE) and the *EEF1A2* gene. This regulatory dependency was not recognized previously because Hi-C data obtained from IMR90 cells, which is commonly used as a reference, does not show any DNA interactions and topologically associated domains at the *EEF1A2* gene locus.

370 **(j)** *EEF1A2* expression is positively correlated with the RELA-specific SE
371 (*chr20:62060923–62127745*) highlighted in panel i. This regulatory dependency is further supported
372 by the many RELA-specific DNA interactions observed in the Hi-C data.

373

374 **Figure 2: Transcriptional activation of *RCOR2* by neo-TADs in *RELA* ependymoma**

375 **(a)** The Hi-C data reliably detect the structural variants that lead to the *C11orf95-RELA* fusion gene
376 in supratentorial RELA-fusion associated tumors. Green boxes highlight SVs predicted by the
377 computational methods applied.

378 **(b)** Chromothriptic rearrangements of chromosome 11 in a patient-derived RELA cell line (RELA
379 BT165) visualized using Hi-C data.

380 **(c)** Structural variants in RELA tumors are not limited to chromosome 11 but also involve other
381 chromosomes. Shown is an inter-chromosomal structural variant that includes chr11 and chr22 in
382 tumor sample 7EP41.

383 **(d)** Reconstruction of the *C11orf95-RELA* breakpoint in a supratentorial tumor (4EP35) using Hi-C
384 data reveals the formation of a neo-TAD that involves DNA interactions between *RCOR2* and the
385 *C11orf95-RELA* fusion gene. RNA-seq and H3K27ac ChIP-seq data of this sample are included as
386 additional tracks.

387 **(e)** Genome browser visualization of the *C11orf95* and *RCOR2* genomic region shows RELA
388 ependymoma-specific enhancers and DNA interactions.

389 **(f)** Boxplot of *RCOR2* gene expression across ependymoma groups using Affymetrix gene
390 expression data (n=393). The center line, box limits, whiskers and points indicate the median,
391 upper/lower quartiles, 1.5× interquartile range and outliers, respectively. *RCOR2* is significantly
392 upregulated in RELA tumors (anova p-val.: 1.71e-91).

393 **(g-i)** shRNA time-course knockdown experiments in RELA (EP1NS) and PFA (EPD210FH)
394 ependymoma cell lines using a scrambled control and two shRNA constructs each targeting either
395 *RCOR2* in EP1NS **(g)**, *RCOR2* in EPD210FH **(h)** or *LSD1* in EP1NS **(i)**. All constructs are GFP
396 tagged and GFP positive cells are sorted by FACS. Results are normalized to day 0 and error bars
397 represent SEM from two independent experiments.

398 **(j-l)** Dose response curves of single-compound treatment with ORY-1001 **(j)**, Entinostat **(k)** or Corin
399 **(l)** of RELA (EP1-NS) and PFA (EPD210FH) ependymoma spheroids over a 72-hour time-course
400 using Celltiter-Glo cell viability assays. For each sample the results are presented as percentage of
401 the Luminescence signal from control condition (i.e. water for ORY-1001 and DMSO for Entinostat
402 and Corin as a vehicle). Data are presented as SEM from three independent experiments per tumor
403 type.

404

405 **Figure 3: PFA Ependymomas are dependent on integrin signaling**

406 **(a)** Hi-C DNA interaction matrices wherein a ~5 million base pair segment of chromosome 2 is
407 aligned along the diagonals shown for PFA (9EP1, left) and RELA (4EP53, middle) tumors and
408 normal cerebellum astrocytes (CAs, right). Off-diagonal signals indicate DNA interactions between
409 different genomic sites. The chromatin complex (highlighted by dashed circles) spatially links
410 several genes, including *ITGA6*, and various regulatory elements located more than 4 million base
411 pairs apart and is recurrently observed in all PFA ependymoma tumors, but in none of the other
412 samples analyzed.

413 **(b)** Hi-C DNA interactions of a PFA tumor (sample BT214) wherein the same ~5 million base pair
414 segment of chromosome 2 shown in panel (a) is aligned horizontally. Circles and dashed lines
415 highlight long-range DNA interactions.

416 **(c)** Genome browser view of the PFA-specific chromatin cluster shown in panels (a) and (b). The
417 included data tracks show long-range DNA interactions in PFA tumors ('Hi-C loops') as well as
418 gene expression and H3K27ac in RELA and PFA tumors.

419 **(d)** Genetic (CRISPR-Cass9) time-course knockout of *ITGA6* in PFA ependymoma cells
420 (EPD210FH) using an unspecific control and three individual sgRNA constructs. All constructs are
421 GFP tagged and GFP positive cells are sorted by FACS. Results are normalized to day 0 and error
422 bars represent SEM from two independent experiments.

423 **(e)** The conventional copy number profile of a PFA relapse sample (EPD210FH) shows high
424 genomic instability including gain of chromosome arm 1q (presented vertically on the left). The
425 genome-wide DNA interaction (Hi-C) map obtained from the same sample identifies complex inter-
426 chromosomal structural variants including an inversion that involves chr1q and chr8. These trans-
427 SVs reveal the complexity of genomic rearrangements underlying some copy-number gains and
428 losses observed by common copy number variation analyses.

429 **(f)** Re-construction of the structural variant that involves chr1q and chr8 in the PFA relapse sample
430 EPD210FH using Hi-C data. This structural variant results in the formation of a neo-TAD that places
431 the *LAMC1* gene locus in a new regulatory environment.

432 **(g)** Re-construction of the structural variant that involves chr1q and chr3 in the PFA relapse tumor
433 RD-19-157 using Hi-C data obtained from FFPE material. This structural variant also results in the
434 formation of a neo-TAD that places the *LAMC1* gene locus in a new regulatory environment.

435 **(h)** Boxplot of RNA-seq expression analysis revealed that *LAMC1* is expressed almost three times
436 higher in the two 1q+ PFA ependymoma relapse tumors than in primary PFA tumors, suggesting that
437 the transcriptional activation of *LAMC1* by the formation of SV-induced neo-TADs is a common

438 resistance mechanism in recurrent 1q+ PFA EPN tumors. The center line, box limits, whiskers and
439 circles indicate the median, upper/lower quartiles, 1.5× interquartile range and samples, respectively.

440 (i) Boxplot of Affymetrix gene expression data shows that *LAMC1* tends to be upregulated in a
441 larger cohort of relapse PFA in comparison to primary PFA ependymoma tumors. The center line,
442 box limits, whiskers and points indicate the median, upper/lower quartiles, 1.5× interquartile range
443 and outliers, respectively.

444 (j) Genetic (CRISPR-Cas9) time-course knockout of *LAMC1* in PFA ependymoma cells
445 (EPD210FH) using an unspecific control and three individual sgRNA constructs. All constructs are
446 GFP tagged and GFP positive cells are sorted by FACS. Results are normalized to day 0 and error
447 bars represent SEM from two independent experiments.

448

449 **Figure 4. Hypermethylation replaces CTCF binding sites in PFA ependymoma.**

450 (a) Proposed mechanism of epigenetic oncogene activation in PFA ependymoma tumors. Top: The
451 oncogene is separated from an enhancer by a CTCF insulator, which forms a topological barrier.
452 Below: The CTCF insulator is replaced by DNA methylation so that the enhancer can contact the
453 oncogene and enhances its expression.

454 (b) The volcano plot shows significant differential CTCF binding sites between PFA and RELA
455 ependymoma tumors (min *p-value*: 0.1). CTCF binding sites significantly hypermethylated in PFA
456 relative to RELA tumors are marked in orange (min *q-value*: 0.05).

457 (c) Comparison of CTCF binding strength (CTCF ChIP-seq, x-axis, min *p-value* 0.1, min fold
458 change: 0.5) and DNA methylation (WGBS, y-axis, min *q-value*: 0.05, min difference: 0.1) at CTCF
459 binding sites that show significant differences between PFA and RELA ependymoma tumors.

460 (d) Heatmap of WGBS-derived DNA methylation at the 300 most significant CTCF binding sites
461 lost in three PFA (left) and three RELA (right) ependymoma tumors. The heatmaps and the
462 composite panels on top show that CTCF binding sites are commonly replaced by DNA methylation
463 in PFA tumors.

464 (e) Genome browser visualization of PFA ependymoma-specific DNA loops that associate two PFA
465 enhancers (E1 and E2) with the *ARL4C* gene locus located ~3,520 kbp away from the *ARL4C*
466 transcription start site.

467 (f) WGBS-derived DNA methylation and CTCF ChIP-seq data from PFA and RELA ependymoma
468 tumors show that a CTCF binding site separating the *ARL4C* gene from the E1 and E2 PFA
469 enhancers is replaced by DNA methylation in PFA tumors.

470 **(g)** *ARL4C* gene expression is positively correlated with the activity of enhancer E2
471 (*chr2:237763494–237764993*) across a cohort of 24 ependymoma tumors from six different
472 intracranial ependymoma groups.
473 **(h)** Genetic (CRISPR-Cas9) time-course knockout of *ARL4C* in PFA ependymoma cells
474 (EPD210FH) using an unspecific control and three individual sgRNA constructs. All constructs are
475 GFP tagged and GFP positive cells are sorted by FACS. Results are normalized to day 0 and error
476 bars represent SEM from two independent experiments.

477

478

479 **Supplementary Figure 1**

480 **(a)** The number of DNA contacts obtained in the individual ependymoma samples as a quality
481 measure for the Hi-C data.
482 **(b)** The tSNE dimensionality reduction visualization and unsupervised clustering of DNA
483 methylation data from a cohort of ependymoma samples (n=1,182) separates PFA and RELA
484 ependymomas into separate groups. The samples analyzed in this study by Hi-C are highlighted in
485 red and orange. Blocks marked with an asterisk show a magnification of selected samples, for better
486 visualization.
487 **(c)** Proportions of topologically associated domains (TADs) shared between ependymoma tumors.
488 The comparison is performed among PFA (n=3) and RELA (n=3) ependymoma tumors that were
489 also included in our previous enhancer-mapping study¹⁸. Hi-C data from two normal cerebellum
490 astrocytes provided in the ENCODE database⁴⁰ were included as controls. The mean proportion of
491 common TADs is 0.569 and the greatest similarity is found in TADs from samples of the same group
492 (PFA, RELA and cerebellar astrocytes), while the common TAD proportions in the individual
493 sample groups are smaller.
494 **(d)** Numbers of common TADs between EPN PFA (n=3) and RELA (n=3) samples from enhancer
495 landscape study. The highest number of same TADs is among all samples.
496 **(e)** *TNC* expression is positively correlated across a cohort of 24 tumors from six different
497 intracranial ependymoma groups with the activity of the PFA ependymoma-specific enhancer E2
498 (*chr9:118275447–118288653*) located upstream of the *TNC* transcription start site.
499 **(f-g)** Hi-C data visualization of a genomic region that includes the *EEF1A2* gene. The association
500 between a RELA ependymoma super enhancer (SE) and the up regulation of *EEF1A2* transcription
501 was not detected previously due to the lack of annotated TADs at this gene locus in the Hi-C data
502 from IMR90 samples. TADs derived from IMR90 (blue), RELA (RELA BT, red), and PFA
503 (EPD210FH, orange) ependymoma samples are visualized in boxes.

504

505 **Supplementary Figure 2**

506 **(a)** Visualization of the Hi-C data at the extended *C11orf95* and *RELA* gene locus in PFA
507 ependymoma tumors shows the absence of structural variants.

508 **(b)** Reconstruction of the *C11orf95-RELA* breakpoint in *RELA* ependymoma tumors (7EP41 and
509 11EP22) using Hi-C data reveals the formation of a neo-TAD that involves DNA interactions
510 between *RCOR2* and the *C11orf95-RELA* fusion gene. RNA-seq, CTCF and H3K27ac ChIP-seq data
511 of these samples are included as additional tracks.

512 **(c)** Chr11 genome browser visualization showing DNA loops that span the *C11orf95* and *RELA* gene
513 loci.

514 **(d)** Correlation between the expression of *RELA* and *RCOR2* ($R=0.663$, $p\text{-val}=6.93\text{e-}11$) in *RELA*
515 ependymoma tumors ($n=76$) profiled by Affymetrix gene expression arrays.

516 **(e)** Knock-down of *RCOR2* expression in *RELA* ependymoma cells. Western blots show protein
517 levels 4 days post infection with indicated shRNAs. B-actin is used as a loading control.

518 **(f-h)** Boxplots of *LSD1*, *HDAC1* and *HDAC2* gene expression across ependymoma subgroups using
519 Affymetrix gene expression data ($n=393$, anova p-values: $9.77\text{e-}15$, $1.02\text{e-}27$, $1.20\text{e-}14$). The center
520 line, box limits, whiskers and points indicate the median, upper/lower quartiles, $1.5\times$ interquartile
521 range and outliers, respectively.

522 **(i)** Knock-down of *LSD1* expression in ependymoma cells. Western blots show protein levels 4 days
523 post infection with indicated shRNAs. B-actin is used as a loading control.

524 **(j)** shRNA time-course knockdown experiments in a PFA ependymoma cell line (EPD210FH) using
525 a scrambled control and two shRNA constructs targeting *LSD1*. All constructs are GFP tagged and
526 GFP positive cells are sorted by FACS. Results are normalized to day 0 and error bars represent
527 SEM from two independent experiments.

528 **(k-l)** Dose response curves of the HDAC8 inhibitor PCI-34051 **(k)** and the HDAC 6/10 inhibitor
529 Tubastatin **(l)** treatment of *RELA* (EP1NS, red) and PFA (EPD210FH, orange) ependymoma
530 spheroids over a 72-hour time-course using Celltiter-Glo cell viability assays. For each sample, the
531 results are presented as percentage of the Luminescence signal from control condition (DMSO as a
532 vehicle). Data are presented as SEM from three independent experiments per tumor type.

533

534 **Supplementary Figure 3**

535 **(a)** DNA interaction matrices derived from HiC data wherein a ~ 5 million base pair segment of
536 chromosome 2 (172,407,031-177,907,030 bp) is aligned along the diagonals. Off-diagonal signals
537 indicate DNA interactions between different genomic sites. The chromatin complex at the *ITGA6*

locus recurrently forms in all PFA EPN tumors analyzed (shown for samples 9EP1, 9EP9, and 7EP18 in the top row). This chromatin complex is not present in RELA endependymoma tumors (middle row). Moreover, normal human cell types analyzed by the ENCODE and PsychENCODE consortia, such as cerebellar astrocytes (CA), neural progenitor cells (NPCs) and embryonic fibroblasts (IMR90, bottom row), do not show signs of similar DNA interactions, suggesting that this chromatin complex is characteristic for PFA endependymoma tumors.

(b) Differential gene expression analysis of Affymetrix array data identified *ITGA6* as significantly (FDR<0.01, two-sided Wilcoxon test) upregulated in PFA compared to RELA endependymoma tumors and normal brain samples (n=200 PFA, n=76 RELA, and n=225 normal human brain samples). In the boxplot the center line, box limits, whiskers and points indicate the median, upper/lower quartiles, 1.5× interquartile range and outliers, respectively.

(c) *ITGA6* has been observed as an essential gene specifically in PFA endependymoma cell lines compared to glioblastoma stem cells (GSCs) and fetal neural stem cells (fNSCs) in a published CRISPR-Cas9 knock-out screen²⁶. In the boxplot the center line, box limits and whiskers indicate the median, upper/lower quartiles and 1.5× interquartile range, respectively.

(d) Expression of two alternative *ITGA6* heterodimer partner proteins *ITGB1* and *ITGB4*. While *ITGB1* and *ITGB4* are highly expressed in PFA and RELA endependymoma tumors compared to normal brain samples, only *ITGB4* is significantly upregulated (FDR<0.01, two-sided Wilcoxon test) in PFA compared to RELA endependymoma tumors, suggesting that the integrin $\alpha6\beta4$ heterodimer is the functional form relevant for PFA endependymoma tumors (n=200 PFA, n=76 RELA, and n=225 normal human brain samples). In the boxplots the center line, box limits, whiskers and points indicate the median, upper/lower quartiles, 1.5× interquartile range and outliers, respectively.

(e) Genetic (CRISPR-Cas9) time-course knockout of *ITGA6* in RELA endependymoma cells (EP1-NS) using an unspecific control and three individual sgRNA constructs. All constructs are GFP tagged and GFP positive cells are sorted by FACS. Results are normalized to day 0 and error bars represent SEM from two independent experiments.

(f) Genetic (CRISPR-Cas9) time-course knockout of *ITGA6* in glioblastoma (GBM2) cells using an unspecific control and three individual sgRNA constructs. All constructs are GFP tagged and GFP positive cells are sorted by FACS. Results are normalized to day 0 and error bars represent SEM from two independent experiments.

(g) Example of a copy number profile of a PFA endependymoma tumor (16EP7) harboring a 1q gain. The CNV profile was obtained from DNA methylation array data.

(h) Copy number profiles of a primary and three relapse PFA endependymoma tumors from the same patient (RD-19-157). The CNV profiles were obtained from DNA methylation array data.

572 **(i)** Visualization of Hi-C data obtained from a primary PFA ependymoma tumor (16EP7) harboring a
573 1q gain shows frequent DNA interactions within chromosomes but no DNA interactions indicative
574 of inter-chromosomal structural variants.

575 **(j)** The conventional copy number profile of a PFA relapse sample (RD-19-157) shows high
576 genomic instability including gain of chromosome arm 1q (presented vertically on the left). The
577 genome-wide DNA interaction (Hi-C) map obtained from the same sample identifies complex inter-
578 chromosomal structural variants including an inversion that involves chr1q and chr3.

579 **(k)** Expression of *LAMC1* in the three relapse tumors of patient RD-19-157 shows up-regulation of
580 *LAMC1* in the third relapse tumor that harbors a 1q gain.

581 **(l)** Western blots show efficacy of sgRNAs targeting *LAMC1* and control sgRNA in EPD210FH
582 cells. Mixture of different clones of infected EPD210FH cells are used to obtain protein extracts at
583 day 5 post infection with indicated sgRNAs. B-actin is used as a loading control.

584 **(m)** Genetic (CRISPR-Cas9) time-course knockout of *LAMC1* in RELA ependymoma (EP1-NS)
585 cells using an unspecific control and three individual sgRNA constructs. All constructs are GFP
586 tagged and GFP positive cells are sorted by FACS. Results are normalized to day 0 and error bars
587 represent SEM from two independent experiments.

588

589 **Supplementary Figure 4**

590 **(a)** Mean methylation of PFA and RELA ependymoma tumors at promoters, enhancers and CTCF
591 binding sites. The center line, box limits, whiskers and points indicate the median, upper/lower
592 quartiles, 1.5× interquartile range and outliers, respectively.

593 **(b)** Boxplot showing *ARL4C* gene expression across ependymoma subgroups (Affymetrix gene
594 expression data for n=393 ependymoma tumors). The center line, box limits, whiskers and points
595 indicate the median, upper/lower quartiles, 1.5× interquartile range and outliers, respectively. *ARL4C*
596 is significantly upregulated in PFA compared to the other ependymoma types (anova p-val.: 4.25e-
597 55).

598 **(c)** Positive correlation of *ARL4C* gene expression and the activity of enhancer E1
599 (*chr2:237545700–237546199*) across a cohort of 24 ependymoma tumors from six different
600 intracranial ependymoma groups.

601 **(d)** *ARL4C* has been observed as an essential gene in PFA ependymoma cell lines in a published
602 CRISPR-Cas9 knock-out screen²⁶. In the boxplot the center line, box limits and whiskers indicate
603 the median, upper/lower quartiles and 1.5× interquartile range, respectively.

604 **(e)** Genetic (CRISPR-Cas9) time-course knockout of *ARL4C* in RELA ependymoma (EP1-NS) cells
605 using an unspecific control and three individual sgRNA constructs. All constructs are GFP tagged

606 and GFP positive cells are sorted by FACS. Results are normalized to day 0 and error bars represent
607 SEM from two independent experiments.

608 **(f)** Genetic (CRISPR-Cas9) time-course knockout of *ARL4C* in glioblastoma (GBM2) cells using an
609 unspecific control and three individual sgRNA constructs. All constructs are GFP tagged and GFP
610 positive cells are sorted by FACS. Results are normalized to day 0 and error bars represent SEM
611 from two independent experiments.

612

613 **Supplementary Tables**

614

615 **Supplementary Table 1:** Cohort of ependymoma tumor samples and available data types.

616

617 **Supplementary Table 2:** Enhancer associated genes supported by DNA loops in PFA ependymoma.

618

619 **Supplementary Table 3:** Enhancer associated genes supported by DNA loops in RELA
620 ependymoma.

621

622 **Supplementary Table 4:** Structural variants in RELA ependymoma samples as identified by
623 (a) hicBreakFinder. No additional filter was applied. (b) HiC SV/trans with adjusted filtering.

624

625 **Supplementary Table 5:** DAVID Gene Ontology analysis results for differentially expressed genes
626 specific for PFA compared to other ependymoma subgroups.

627

628 **Supplementary Table 6:** Structural variants in PFA ependymoma samples as identified by
629 (a) hicBreakFinder. No additional filter was applied. (b) HiC SV/trans with adjusted filtering.

630

631 **Supplementary Table 7:** Enhancer-associated genes supported by DNA loops that are potentially
632 formed due to the replacement of CTCF binding sites by DNA methylation in PFA ependymoma
633 tumors.

634

635 **Supplementary Table 8:** Overview of shRNA and sgRNA oligo sequences applied in experimental
636 validation.

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638 **Supplementary Table 9:** Drugs selected for experimental validation

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Supplementary Table 10: Overview of antibodies applied in experimental validation.

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Contributions

K.O., A.C., M.K. and L.C. prepared the manuscript and figures. K.O., O.C., A.C., M.P. J.T.R., E.F.J. and A.S. performed data analysis and visualization. A.C., D.E.P., M.M., and J.M.H. performed experimental validations, N.G.C., M.L., D.M., S.N., and M.S. processed and analyzed tumor material, R.B., S.C., K.K., R.A.H. and D.R. generated Hi-C libraries from frozen and FFPE tumor material and cell lines, K.W.P., T.M., S.M., A.S., H.C., J.C., R.W.R., K.A.M., S.A.K., M.D.T., F.B., J.R., J.P.M., S.M.P., F.A. and J.R.D. contributed to the study design and data interpretation. L.C. designed the study and L.C. and M.K. co-supervised the project.

667 **Methods**

668

669 **Chromosome conformation capture**

670 Hi-C on frozen tumor tissue sample was carried out using protocols previously described for tissue Hi-
671 C experiments⁴¹. In brief, frozen tissues are pulverized using a mortar and pestle kept cold on a bed of
672 dry ice into a fine powder. The tissue powder was then transferred to a 15mL conical tube containing
673 5mLs of DPBS and fixed with 2% formaldehyde for 10 minutes. The fixation was quenched by
674 addition of 0.2M Glycine. The fixed tissue was pelleted by centrifugation, washed 1x with DPBS, and
675 then flash frozen until ready for further processing.

676 For Hi-C experiments, the fixed frozen tissue pellets were first resuspended in 3mLs of lysis buffer
677 (10mM Tris-HCl pH 8.0, 5mM CaCl₂, 3mM MgAc, 2mM EDTA, 0.2mM EGTA, 1mM DTT, 0.1mM
678 PMSF, 1X Complete Protease Inhibitors). The sample was transferred to an M-tube and dissociated
679 using a GentleMACS Tissue dissociator (Miltenyi) using the “Protein M-tube” setting. The sample
680 was removed from the M-tube into a 50mL conical. The M-tube was washed with 3mLs of lysis buffer
681 with 0.4% Triton X-100 added, and this wash was combined with the original 3mLs of sample for a
682 total volume of 6mLs with final concentration of 0.2% Triton X-100. The sample was then passed
683 through a 40μm cell strainer. The strainer was washed with an additional 2mLs of lysis buffer with
684 0.2% Triton X-100. The sample was then centrifuged and washed with 1mL of lysis buffer with 0.2%
685 Triton X-100. After centrifugation, the sample was resuspended in 0.5% SDS and processed with
686 previously described *in situ* Hi-C method⁴² using the MboI enzyme. Libraries were prepared using the
687 Illumina TruSeq LT sequencing adaptors. Initial QC sequencing was first performed on a MiSeq to
688 assess library quality, and if sufficient, was subject to production scale sequencing on the HiSeq X or
689 NovaSeq platform, respectively.

690

691 **Chromosome conformation capture from FFPE material**

692 Hi-C experiments on FFPE material were carried out by Arima Genomics, Inc (San Diego, CA).
693 Dewaxed and re-hydrated FFPE tissue was used as input to a modified version of the Arima-HiC Kit
694 protocol. After the Arima-HiC protocol, Illumina-compatible sequencing libraries were prepared by
695 shearing the proximally ligated DNA and then size-selecting DNA fragments using SPRI beads. The
696 size-selected fragments containing ligation junctions were enriched using Enrichment Beads (provided
697 in the Arima-HiC Kit), and converted into Illumina-compatible sequencing libraries using the Swift
698 Accel-NGS 2S Plus kit (P/N: 21024) reagents. After adapter ligation, DNA was PCR amplified and

699 purified using SPRI beads. The purified DNA underwent standard QC (qPCR and Bioanalyzer) and
700 sequenced on the NovaSeq following manufacturer's protocols.

701

702 **Hi-C data processing**

703 The sequencing reads alignment to hg19 human genome reference and chromatin contacts calling was
704 performed using HiCPro 2.9.0 toolkit⁴³ allowing the bin sizes 5,10,50,100,250 and 500 Kbp. Main
705 visualization and normalized full contacts extraction was performed with JuiceBox v0.7.5 toolkit⁴⁴.
706 Per sample loop calling was applied from FitHiC v2.0.6 method⁴⁵ on bin sizes 5 Kbp with maximum
707 distance between bins 50 Mbp. TAD calling was performed based on 50 Kbp bins resolution using
708 TopDom tool⁴⁶.

709

710 **Unsupervised clustering of Hi-C data**

711 Hi-C data processing produced interaction matrices in .juicebox format for 3 RELA and 8 PFA
712 ependymoma tumors. For each tumor, unsupervised clustering features were computed using the
713 *Eigenvector* utility from the Juicer Tools analysis toolkit.⁴⁷ Briefly, the Eigenvector utility computes
714 A/B compartments as the first principal component of the Pearson correlation matrix of each intra-
715 chromosomal contact matrix.⁴⁸ A/B compartments were computed using Knight-Ruiz normalization
716 at 1Mb resolution. Unsupervised hierarchical clustering was performed on these features using Pearson
717 correlation distance and average linkage, using the *heatmap.2* function from *gplots* R package.

718

719 **Genes and enhancers connections via loops**

720 The ChIP-seq derived enhancer signals along with genomic locations of group-specific enhancers and
721 normalized RNA-seq gene expression profiles from ependymoma tumors cohort (n=25) were obtained
722 from published materials of the corresponding study¹⁸. Genome was fragmented into 5 Kbp bins and
723 output from FitHiC loop calling tool was used to find contacts between genes and enhancers. For this
724 purpose the genes were assigned to bins based on the location of transcription start site (TSS, 2500
725 Kbp upstream and downstream of the gene start loci), while enhancers based on the overlap. Loop
726 boundary correspondence was assigned to gene and/or group-specific enhancer lying either within the
727 bin or in the closest upstream/downstream bin. Enhancer associated gene was considered to be
728 supported by loop if the TSS of it was lying in one loop anchor while enhancer in the other. Correlation
729 analysis was performed based on the usage of updated InTAD package⁴⁹ v1.9.2.

730

731 **Gene expression analysis**

The global ependymoma tumor gene expression data integration was performed based on the usage of corresponding R2 platform materials with focus on Affymetrix dataset from combined ependymoma tumors cohort with integration of normal brain tissues (n=618). Major of these ependymoma tumor Affymetrix materials were obtained from the corresponding main study¹ (GEO: GSE64415) with additional external inclusions (GEO: GSE50161, GSE50385, GSE21687, GSE3526). The gene ontology analysis was performed using DAVID tool⁵⁰ based on the usage of differentially expressed genes between PFA and other ependymoma groups achieved with R2 platform from the EPN global Affymetrix dataset. The RNA-sequencing materials from target EPN cohort samples were analyzed as previously described¹⁸.

Structural variants (SV) discovery from Hi-C data

SV discovery from Hi-C data was performed using two independent toolkits. The first toolkit, hicBreakFinder (https://github.com/dixonlab/hic_breakfinder), was adjusted for the usage on hg19 human genome reference with taking into account additional filtering lists of false positives obtained from external cohorts¹⁹. Shortly, the tool scans for abrupt shifts in chromosomal connections in order to find possible outliers representing inter/intra-chromosomal events based on the selected threshold ($t=0.6$) and reports them in resolutions 1Mb, 100Kb and 10Kb. Final combined result contains the highest resolution for detected SV. The second toolkit, Hi-C structural variant discovery or HiCsv, consists of two parts and was adjusted for the usage of hg38 genome as the most up-to-date reference genome. First part of this toolkit, HiCtrans²¹, focuses on inter-chromosomal translocations: it scans the inter-chromosomal contact matrices over multiple Hi-C resolutions for each possible pair of chromosomes from a given sample and predicts candidate SVs based on the changepoint analysis using binary segmentation. The intra-chromosomal translocations are also detected in this toolkit based on the dual pattern of off-diagonal enrichment and diagonal depletion of chromatin interactions in a Hi-C map across genomic regions. HiCsv detects enrichment of interactions through FitHiC2 algorithm⁴⁵ and uses an insulation score-based estimation (similar to TAD finding⁵¹) to identify depletion in interaction frequency. Finally, it applies a density-based clustering of enriched Hi-C interactions with high insulation scores to discover structural variants.

CTCF ChIP-sequencing

ChIP-sequencing procedure was prepared and performed as previously described¹⁸. Shortly, ChIP flash-frozen for ependymoma tumours was performed using 5 µg CTCF antibody per ChIP Active Motif. Enriched DNA was quantified and barcoded. Following library amplification, DNA fragments were sequenced using Illumina HiSeq 2000 100-bp paired-end sequencing.

766

767 **CTCF ChIP-seq data analysis**

768 Reads alignment was performed to hg19 reference with BWA v0.5.10⁵². Duplicate alignments were
769 removed using Picard (<http://broadinstitute.github.io/picard>). Peak calling was performed using Macs
770 v1.4⁵³. Differential RELA peaks between EPN PFA and RELA were detected using DiffBind R
771 package⁵⁴ with min adjusted p-value limit 0.05.

772

773 **Whole genome bisulfite sequencing (WGBS)**

774 WGBS procedure was prepared and performed as previously described⁵⁵. Shortly, 5 µg of genomic
775 DNA were sheared using a Covaris device. After adaptor ligation, DNA fragments were isolated and
776 bisulphite converted using the EZ DNA Methylation kit (Zymo Research). PCR amplification of the
777 fragments was performed followed by library aliquots pooling. Sequencing was performed Illumina
778 HiSeq 2000 machine.

779

780 **WGBS data analysis**

781 Initial reads processing was performed using methylTools v0.9.4 as previously described⁵⁵.
782 Differentially methylated regions were detected using methylKit v0.2.6 tool⁵⁶ with min adjusted p-
783 value limit 0.05. Combined visualization of the methylation profiles within CTCF target regions was
784 performed using the EnrichedHeatmap R package. Search of target loops was performed based on the
785 presence of overlapping the DMRs with differential CTCF peaks within.

786

787 **Cell culture**

788 HEK293T cells (CRL-1273, American Type Culture Collection) were cultured as previously described
789 (citation). EPD210FH cells were grown in NeuroCult NS-A Basal Medium (STEMCELL
790 Technologies) supplemented with NeuroCult Proliferation Supplement (STEMCELL Technologies),
791 2mM L-glutamine 1% Penicillin/Streptomycin, 75ng/ml bovine serum albumin (BSA) and 20ng/ml of
792 EGF (Peprotech) and FGF-basic (Peprotech). EP1NS cells were grown in Neurobasalmedium A (Life
793 Technologies) supplemented with 1µg/ml of Heparin (Sigma), 2mM L-Glutamine and 20ng/ml of EGF
794 and FGF-basic. Cells were cultured as neurospheres in tissue culture flasks. When they were cultured
795 as an adherent culture, flask was additionally coated with Laminin (L2020, Sigma) for EPD210FH
796 cells and with Geltrex (A1569601, Life Technologies) for EP1NS cells. Pediatric patient-derived SU-
797 pcGBM2 cells were cultivated as neurospheres as previously described. (Katrin Schramm paper,
798 2019). All cells were routinely tested free of mycoplasma contamination and authenticated by Single

799 Nucleotide Polymorphism profiling (Multiplexion GmbH). All cell models were grown at 37°C with
800 5% CO₂.

801

802 **Lentiviral Transduction**

803 shRNA plasmids and sgRNA plasmids were constructed as previously described (STK3 paper). All
804 oligos are ordered from Sigma. Target sequences of all oligos are listed in Supplementary Table S8.
805 Transduction was performed in the presence of protamine sulfate (final concentration 5 ug/mL, Sigma-
806 Aldrich) Transduced cells were further cultured and GFP signal was analyzed by BD FACS Canto for
807 GFP expression. Reduction of the percentage of GFP-positive cells indicates that the infected cells
808 expressing a particular shRNA/ sgRNA have a growth disadvantage in comparison to the non-infected
809 cells.

810

811 **Drug treatments**

812 All drugs were prepared according to protocols provided by company (Supplementary Table S9) Cells
813 were seeded into 96-well cell culture treated plates at a density of 5000 cells in 100 µl respected
814 medium per well. On the next day morning, cells were treated with increasing concentrations (200 nM,
815 400 nM, 800 nM, 1.6 µM, 3.2 µM, 6.4 µM and 12.8 µM) of each drug or equivalent dilutions of
816 solvent. Cell viability was assessed after 72 hours using the CellTiter-Glo[®] luminescent cell viability
817 assay (Promega) and an automated plate reader Mythras. All samples were assayed in triplicates and
818 normalized to the average values of the corresponding mock control on the same plate and analyzed
819 using Prism 8 (GraphPad).

820

821 **Western blot analysis**

822 For knockdown and/or knockout studies, cells were infected as described above and cultured for either
823 4 days (knockdown) or 5 days (knockout). Before harvesting, cells were washed with phosphate
824 buffered saline (PBS) and collected as pellets. Then, pellets were lysed in RIPA buffer (Sigma-
825 Aldrich) supplemented with protease and phosphatase inhibitors for 30 minutes on ice. After
826 centrifugation at 13,000 rpm for 10 minutes at 4 °C, the supernatants were collected and protein
827 concentrations were determined using a Bicinchoninic acid (BCA) assay (Sigma-Aldrich) with
828 Pierce[™] bovine serum albumin standards (Thermo Fisher). Lysates were mixed with NuPAGE[®]
829 LSD Sample buffer (Life Technologies) supplemented with 10 % 2-mercaptoethanol and denatured
830 for 5 minutes at 95 °C. Afterwards they were subjected to sodium dodecyl sulfate-polyacrylamide gel
831 electrophoresis according to standard procedures using 4-12 % Bis-Tris gels and afterwards transferred
832 to polyvinylidene difluoride membranes. Membranes were incubated with respective primary

antibodies at 4 °C overnight (Supplementary Table S10). Horseradish peroxidase-conjugated anti-rabbit (1:5,000, Santa Cruz, sc-2054) secondary antibody were applied for 1 hour at room temperature and chemiluminescent detection was carried out using Amersham™ ECL™ or ECL™ Prime Western Blotting detection reagents (GE Healthcare). The same membranes were stripped with stripping buffer (Sigma) according to protocol and incubated with conjugated beta-actin antibody as a loading control.

839

Data availability

The novel sequencing data raw materials (HiC, CTCF, WGBS) will be included into the European Genome-phenome archive (<https://www.ebi.ac.uk/ega/home>) under the accession number: GAS00001002696; this source already contains other data types (RNA-seq, H3K27ac) for the corresponding target tumor samples.

845

Code availability

Scripts for processing the raw data and generating figures can be obtained upon request.

848

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Figures

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