The oncometabolite D-2-hydroxyglutarate promotes DNA hypermethylation at lineage-specific enhancers controlling microglial activation in IDH^{mut} gliomas

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[ǂ]Dr. Laurent Capelle passed away in June 2023. This work is dedicated to his memory.

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Summary

Tumor-associated macrophages and microglia (TAMs) are highly abundant myeloid cells in gliomas, with their phenotype and immune response determined by ontogeny and microenvironment. TAMs display distinctive transcriptional programs according to the IDH mutation status but the underlying regulatory mechanisms remain largely unknown. Herein, we uncover that CD11B+ myeloid cells in human IDH^{mut} gliomas exhibit DNA hypermethylation predominantly at distal enhancers. This hypermethylation was linked to decreased expression of genes involved in inflammatory responses and glycolytic metabolism, and the inactivation of transcription factors that regulate microglial responses to environmental stimuli. Prolonged exposure of human primary microglia to D-2-hydroxyglutarate (D-2HG) inhibited TET-mediated 5mC oxidation, resulting in a reduced accumulation of global 5hmC levels. We confirmed high 5mC/5hmC ratios at lineage-specific enhancers, by analyzing CpGs at single-base resolution. D-2HG-treated microglia show reduced proinflammatory capacity and enhanced oxidative phosphorylation, consistent with the remodeled enhancer landscape. Conversely, depletion of D-2HG following treatment of a glioma patient with an IDH mutant inhibitor was associated with enhanced microglial responses, as assessed by snRNA-seq. Our findings provide a mechanistic rationale for the hyporesponsive state of microglia in IDH^{mut} gliomas and support the concept that oncometabolites may disrupt the function of immune cells residing in the tumor microenvironment.

Keywords: D-2HG, 5mC, 5hmC, Glioma, TAMs, IDH mutation, Tumor Microenvironment

Introduction

Diffuse gliomas are the most frequent malignant primary brain tumors and are refractory to standard therapies[1]. A high proportion of these tumors bear an IDH mutation, which confers a neomorphic ability to convert α-ketoglutarate (α-KG) to (D)-2-hydroxyglutarate (D-2HG)[2]. It is believed that D-2HG drives cellular transformation by inhibiting α-KG-dependent dioxygenases, including the TET family of DNA demethylases, KDMs histone demethylases, and FTO/ALKBH RNA demethylases, ultimately leading to widespread epigenomic dysregulations and blocking of cell differentiation[3]. Much less is known about the tumor cell-extrinsic effects of this oncometabolite, which accumulates in the tumor microenvironment (TME)[4,5]. Emerging evidence indicates that D-2HG released by IDH^{mut} cells suppresses T cells and impairs the metabolism of infiltrating macrophages, dendritic cells, and CD8+ T cells by non-epigenetic mechanisms[6-10]. It remains to be determined whether D-2HG is able to reshape the epigenetic landscape of the TME, particularly in resident microglia, which are abundant in IDH^{mut} gliomas and are exposed to this oncometabolite from tumor initiation.

Tumor-associated macrophages and microglia (TAMs) represent almost 40% of the tumor mass and play critical roles in tumor progression[11]. As such, these cells may represent prognostic biomarkers and therapeutic targets[12,13]. TAMs are plastic cells, with their phenotype and immune response determined by both ontogeny and microenvironment[14,15]. Previous studies in IDH^{wt} glioma models uncovered that resident microglia and monocyte-derived macrophages (MDMs) display distinctive transcriptional and chromatin landscapes[16]. Transcriptional differences have also been observed in the myeloid compartment of gliomas as a function of the IDH mutation status[17,18]. In IDH^{wt} tumors, MDMs and microglial cells exhibit expression changes associated with activation, including high expression of MHC class genes[17]. On the other hand, in IDH^{mut} gliomas, these cells display a hyporeactive phenotype similar to that of homeostatic microglia in non-tumor tissues[17].

The relative proportion of MDMs and microglial cells differs according to the IDH^{mut} status[17-21] and/or tumor grade[22,23]. Glioma progression entails a process of cellular competition, wherein infiltrating macrophages overtake the niche occupied by resident microglia[24,25]. Recent single-cell RNA-seq studies of the myeloid compartment in human gliomas have further unveiled subsets of MDMs with mesenchymal-like, lipid, and hypoxic/glycolytic signatures, as well as subsets of inflammatory and oxidative phosphorylation (OXPHOS)-related microglia[20-22,24,26], some of which have been associated with clinical outcomes. Additionally, it has been reported that myeloid cells express superimposed immunomodulatory activity programs, irrespective of the ontological origin[23]. The epigenetic changes, i.e., DNA methylation, that drive the transcriptional programs and cell states of TAMs in human glioma, and the effects of tumor-derived signals on the TAM methylome, remain largely uncharacterized.

Inhibition of TET-mediated demethylation by D-2HG in tumor cells accounts for DNA hypermethylation characteristic of IDH^{mut} gliomas[27]. Considering the elevated concentrations of this oncometabolite in the TME and its uptake by myeloid cells[9,28], we hypothesized that such a mechanism could be also at play in CD11B+ myeloid cells of these tumors, thus contributing to both epigenetic intratumoral heterogeneity and altered immune functions. Herein, to investigate whether and how TAMs in IDH^{mut} gliomas display functional DNA methylation changes, we first profiled the bulk transcriptome and methylome of CD11B+ myeloid cells isolated from a well-characterized series of human gliomas and non-tumor tissues. Our findings reveal that myeloid cells in IDH^{mut} tumors exhibit DNA hypermethylation at enhancer elements that regulate gene networks involved in microglia activation. We demonstrate the direct effects of D-2HG on TET-mediated DNA demethylation at lineage-specific enhancers in primary cultures of human microglia, thereby providing a mechanistic foundation for the hyporesponsive behavior of these cells in IDH^{mut} gliomas. These results have implications for therapeutics aimed at inhibiting the IDH mutant enzyme or repolarizing TAMs.

Results

Study design

To determine the mechanistic underpinnings of TAM phenotype linked to the IDH status, we analyzed bulk DNA methylome and/or transcriptome of CD11B+ myeloid cells isolated by magnetic sorting from adult-type human diffuse gliomas (n=36) comprising IDH^{wt}, IDH^{mut} oligodendrogliomas and IDH^{mut} astrocytomas. In addition, we analyzed available methylome data of CD11B+ cells from normal brains, isolated using a similar method and profiled with the same technology[29] (Fig. 1a). To adjust for potential confounding variables, samples were matched by age and sex while being fully characterized for their clinical, pathological and genetic features (Supplementary Table 1). The contamination of CD11B+ fractions by tumor cells was assessed by analysis of IDH1 R132H or TERT C228T/C250T promoter mutations through droplet digital PCR (ddPCR) and further verified by RNA-seq-based analysis of IDH variant allelic frequency (Extended Data Fig. 1a and 1b). The estimated purity of the CD11B+ samples included in downstream analyses reached ~95%. This analysis rules out the possibility that TAMs from our study cohort harbored the IDH1 R132H mutation to a significant extent, as previously reported[30].

CD11B+ myeloid cells in human IDH^{mut} gliomas display global DNA hypermethylation

To confirm the DNA methylation status of the cohort in relation to the IDH mutation, we first profiled glioma cell-enriched CD11B- fractions isolated from both IDH^{wt} and IDH^{mut} tumors (n=17, Supplementary Table 1) using MethylationEPIC (EPIC) arrays. Analysis of methylation levels across all probes targeting CpG sites confirmed that IDH^{mut} tumors display global hypermethylation, yet with a high degree of heterogeneity as previously reported[31-33] (Extended Data Fig. 2a and 2b). In total, there were 49,676 differentially methylated CpG sites (absolute $\Delta\beta > 0.2$, FDR < 0.05), of which 92.5% were hypermethylated in IDH^{mut} compared with IDH^{wt} glioma samples (Extended Data Fig. 2c). The distribution per functional genomic region revealed a high proportion of hypermethylated CpGs located in regulatory regions. These regions encompass promoters, defined as constant genomic regions within a distance of -1.5 kb to +0.5 kb respect to transcription start site (TSS), and enhancers,

defined as distal regulatory elements associated with FANTOM robust promoters within a range of up to 500 kb (FDR < 1e-5; Pearson's correlation), as outlined by the FANTOM5 consortium[34]. Our analysis confirms that enhancers are particularly sensitive to hypermethylation in IDH^{mut} glioma cells[35] (Extended Data Fig. 2c and 2d).

We then looked for methylome changes in the fraction of CD11B+ myeloid cells isolated from gliomas (n=26) and from normal brain tissues (n=6) (Supplementary Table 1). Principal component analysis (PCA) revealed a distinct segregation of myeloid cells based on both tissue origin and IDH mutation status in component 1, which accounted for 48% of the variance (Fig. 1b). Despite the substantial heterogeneity of methylome data in CD11B+ samples from IDH^{mut} tumors, we found 37,198 differentially methylated CpG sites (absolute $\Delta\beta > 0.2$, FDR < 0.05) when compared with CD11B+ cells from IDH^{wt} gliomas (Fig. 1c). Strikingly, similar to what was observed in glioma cells, CD11B+ samples from corresponding IDH^{mut} tumors exhibited a bias towards hypermethylation, particularly in regulatory regions (Fig. 1c). It is known that IDH^{wt} glioblastomas are enriched in MDMs, whereas the TME of IDH^{mut} tumors is dominated by tissue-resident microglia[17,18]. However, the asymmetric distribution of the methylation changes we observed indicates that this was not simply an effect of differences in cell composition. Indeed, global hypermethylation was also observed when comparing the DNA methylome of CD11B+ cells isolated from IDH^{mut} tumors with CD11B+ cells isolated from normal brain tissues, both predominantly composed of microglial cells (Fig. 1c). While in this comparison the number of differentially methylated CpG sites and genomic region was higher, there is a notable overlap between regulatory regions that exhibited hypermethylation in both comparisons (Fig. 1d). Of these, the number and methylation level of affected enhancers, along with their overlap between the two comparisons, were greater than those observed for promoters (Fig. 1d). Functional enrichment analyses of genes regulated by hypermethylated regulatory regions intersected in the two comparisons indicated roles in inflammatory responses such as TNF-α, IFN-γ and IL2/IL6 signaling, as well as in complement and hypoxia pathways (Fig. 1e and Supplementary Table 2).

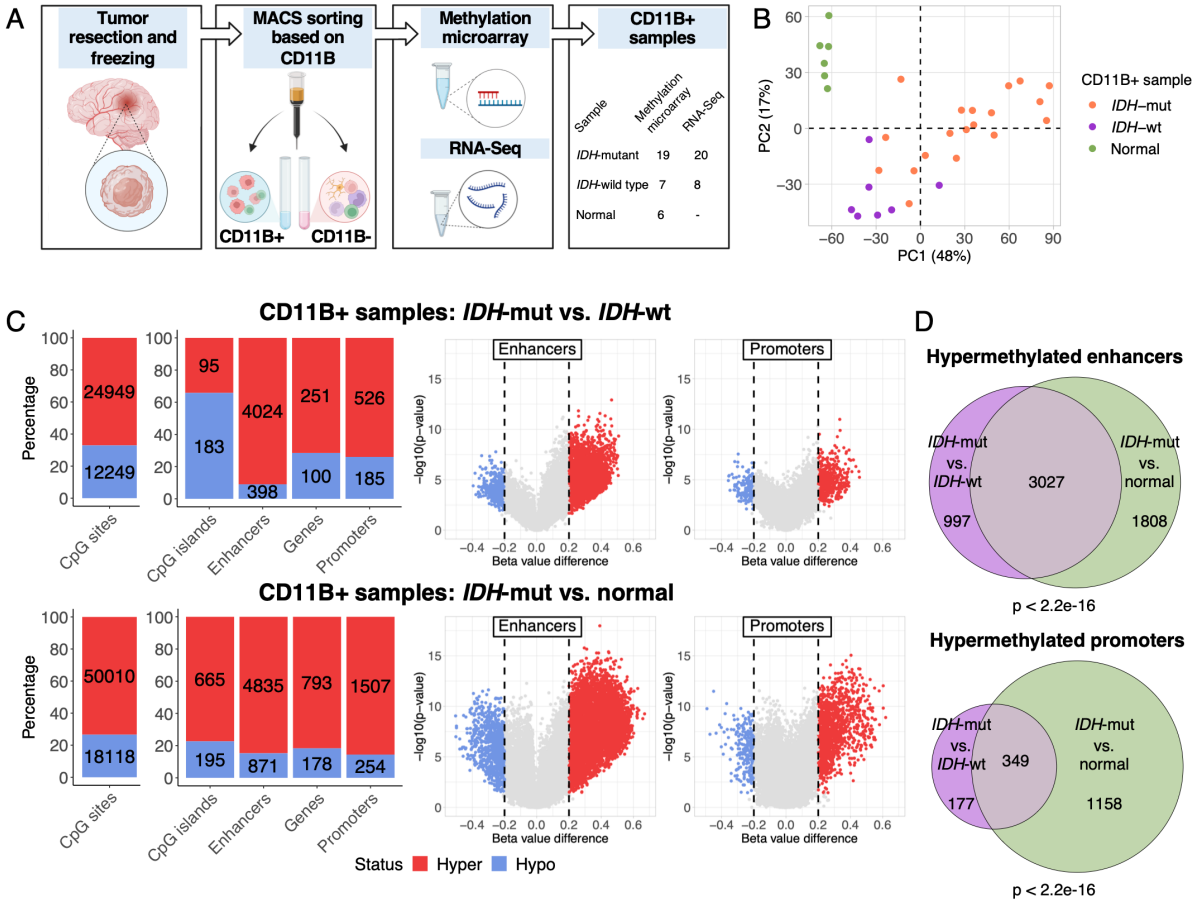

A
Tumor resection and freezing
MACS sorting based on CD11B
CD11B+
CD11B-
Methylation microarray
RNA-Seq
CD11B+ samples
Sample
Methylation microarray
RNA-Seq
IDH-mutant 19 20
IDH-wild type 7 8
Normal 6 -
B
PC2 (17%)
PC1 (48%)
CD11B+ sample
IDH–mut
IDH–wt
Normal
C
CD11B+ samples: IDH-mut vs. IDH-wt
Percentage
24949
12249
95
183
4024
398
251
100
526
185
CpG sites
CpG islands
Enhancers
Genes
Promoters
Enhancers
Promoters
−log10(p−value)
Beta value difference
CD11B+ samples: IDH-mut vs. normal
50010
18118
665
195
4835
871
793
178
1507
254
Status Hyper Hypo
D
Hypermethylated enhancers
IDH-mut vs. IDH-wt
3027
997
IDH-mut vs. normal
1808
p < 2.2e-16
Hypermethylated promoters
IDH-mut vs. IDH-wt
349
177
IDH-mut vs. normal
1158
p < 2.2e-16
E
Hypermethylated enhancers in CD11B+ samples of IDH-mut gliomas
TNFA SIGNALING VIA NFKB
INFLAMMATORY RESPONSE
ALLOGRAFT REJECTION
INTERFERON GAMMA RESPONSE
COMPLEMENT
HEME METABOLISM
IL2 STAT5 SIGNALING
HYPOXIA
APOPTOSIS
IL6 JAK STAT3 SIGNALING
PI3K AKT MTOR SIGNALING
REACTIVE OXYGEN SPECIES PATHWAY
GeneRatio
Count
FDR
F
Motifs enriched in hypermethylated enhancers
Motif p-value % of targets Best match
1e-356 36% SPI1
1e-161 27% AP-1
1e-98 21% CEBP
1e-90 19% RUNX
1e-68 8% IRF
1e-50 13% NFKB
Hypermethylated promoters in CD11B+ samples of IDH-mut gliomas
INFLAMMATORY RESPONSE
IL2 STAT5 SIGNALING
ALLOGRAFT REJECTION
INTERFERON GAMMA RESPONSE
KRAS SIGNALING UP
TNFA SIGNALING VIA NFKB
GeneRatio
Count
FDR
Motifs enriched in hypermethylated promoters
Motif p-value % of targets Best match
1e-35 32% SPI1
1e-16 9% IRF
1e-15 2% RUNX

Figure 1. DNA methylome profiling of CD11B+ cells underscores hypermethylation in the myeloid compartment of IDH^{mut} gliomas. (A) Overview of the *ex vivo* study workflow. Tumor samples (n=36) underwent enzymatic dissociation, followed by CD11B-based magnetic sorting. DNA and/or RNA extraction was performed on both CD11B+ and CD11B- fractions for downstream analyses. Available methylome data of CD11B+ cells from six normal brains were included as controls for comparisons. Clinical, pathological, and genetic annotations of the study cohort are detailed in Supplementary Table 1. **(B)** Principal component analysis performed using methylome data from around 600,000 CpG sites across the genome in CD11B+ samples from the study cohort. **(C)** Stacked bar charts (left) show absolute numbers and relative percentages of CpG sites and of four genomic regions with respect to the methylation status (absolute $\Delta\beta > 0.2$, FDR < 0.05) for the indicated comparisons. Volcano plots (right), colored by methylation status, depict the magnitude and extent of differentially methylated enhancers and promoters for the indicated comparisons. **(D)** Venn diagrams illustrate significant overlaps between hypermethylated enhancers (upper panel) or promoters (bottom panel) in CD11B+ cells from IDH^{mut} gliomas, compared to IDH^{wt} gliomas or normal brain tissues. P-values were computed using over-representation analysis (ORA). **(E)** Bubble plots indicate the hallmark gene sets enriched in the lists of genes exhibiting hypermethylated enhancers (n=3027) and promoters (n=349) in CD11B+ samples from IDH^{mut} gliomas in comparison to CD11B+ samples from IDHwt gliomas and normal brain tissues (Fig. 1D). The size and color code of each dot indicate the number of genes with significant enrichment for the indicated categories (FDR < 0.05). **(F)** Significant sequence motifs (ranked by p-value) enriched in hypermethylated enhancers and promoters in CD11B+ samples from IDH^{mut} gliomas, in comparison to CD11B+ samples from IDH^{wt} gliomas and normal brain tissues (Fig. 1D), were determined using HOMER.

Transcription factors (TFs) bind to specific DNA motifs on promoters and enhancers to control cell-type specific transcriptional programs, and the affinity can be modulated by the methylation status[36-38]. Since a substantial proportion of hypermethylation observed in CD11B+ cells from IDH^{mut} tumors involved regulatory elements in both comparisons (Fig. 1c), we sought to examine enriched TF binding motifs located within hypermethylated regulatory regions. This analysis revealed an overrepresentation of 21 motifs at 349 hypermethylated promoters and 24 motifs at 3027 hypermethylated enhancers (p-value < 10^{-12}) (Fig. 1f). We found SPI1 (PU.1) as the most significantly enriched motif in both hypermethylated regulatory regions (36% of enhancers and 32% of promoters). Interestingly, the other top enriched motifs were bound by key TFs controlling an environment-dependent transcriptional network in microglial cells such as members of C/EBP, AP-1, RUNX, and IRF[39], alongside NF-κβ. Collectively, these results reveal extensive differences in the DNA methylome of TAMs as a function of the IDH mutational status.

Hypermethylation correlates with reduced expression of inflammatory regulons in CD11B+ cells from human IDH^{mut} gliomas

To investigate the transcriptome landscape of TAMs in gliomas, we performed RNA-seq on CD11B+ fractions (n=28, Supplementary Table 1). PCA indicated a good separation between samples derived from IDH^{wt} and IDH^{mut} cases (Extended Data Fig. 3a). Differential expression analysis identified 2714 genes upregulated and 1404 genes downregulated (log2FC > 1 or < -1, respectively; FDR < 0.05) in TAMs from IDH^{mut} compared with those from IDH^{wt} (Supplementary Table 3). We found significant overlaps between the list of downregulated genes in our CD11B+ sample series and those reported by Klemm et al., who further discriminated the expression changes displayed by microglia and MDMs (Extended Data Fig. 3b). Noteworthy, the largest overlap occurred in downregulated genes within microglial cells from IDH^{mut} tumors, with 156 out of the 357 genes falling into this category. Furthermore, significant proportions of genes defining normal microglial signatures also intersected with the list of downregulated genes from our series of CD11B+ fractions in IDH^{mut} gliomas (Extended Data Fig. 3c). Enrichment analysis revealed that these downregulated genes are involved in proinflammatory pathways such as INF-γ/α response and IL6/JAK/STAT3 signaling, cell cycle/proliferation, hypoxia, glycolysis and the acquisition of mesenchymal-like traits (Extended Data Fig. 3d).

DNA methylation is an important mechanism of gene expression regulation. To evaluate the impact of methylation on the observed transcriptional changes in CD11B+ cells isolated from IDH^{mut} gliomas, we intersected the lists of differentially expressed genes with the lists of differentially methylated promoters or enhancers. This analysis involved 36 CD11B+ samples, with 18 of them having both data available (Supplementary Table 1). Contrary to IDH^{mut} glioma cells, where methylation and expression changes have been shown to be uncoupled[35,40], myeloid cells from these tumors displayed confident overlaps in the expected direction: for instance, reduced gene expression was strongly associated with hypermethylation at promoters and enhancers (p-value=2.5e-14 and p-value=7.7e-18, respectively, over-representation analysis (ORA) (quadrant IV, Fig. 2a), while the occurrence

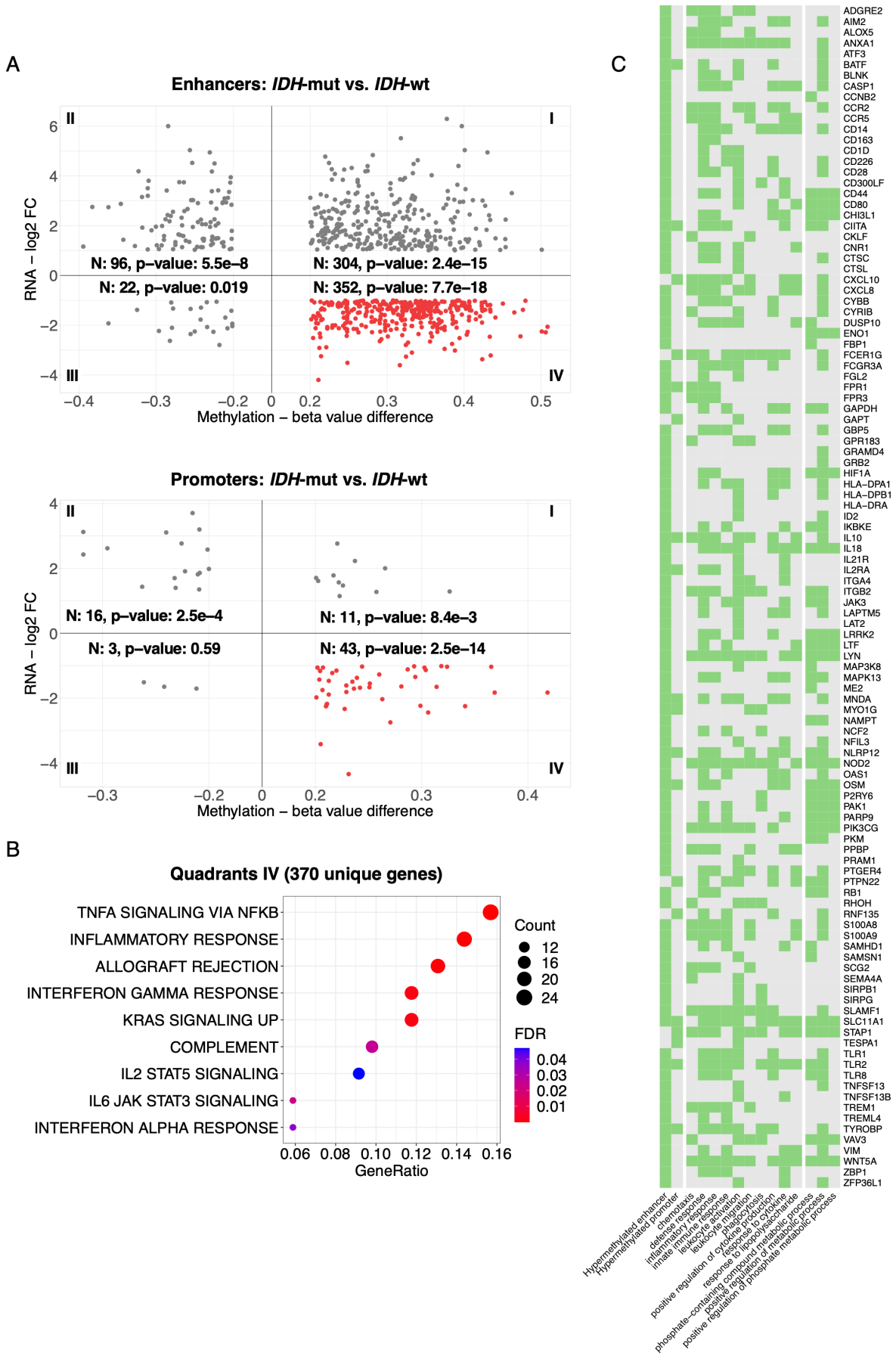

A
Enhancers: IDH-mut vs. IDH-wt
II
I
III
IV
N: 96, p–value: 5.5e–8
N: 304, p–value: 2.4e–15
N: 22, p–value: 0.019
N: 352, p–value: 7.7e–18
RNA – log2 FC
Methylation – beta value difference
Promoters: IDH-mut vs. IDH-wt
N: 16, p–value: 2.5e–4
N: 11, p–value: 8.4e–3
N: 3, p–value: 0.59
N: 43, p–value: 2.5e–14
B
Quadrants IV (370 unique genes)
TNFA SIGNALING VIA NFKB
INFLAMMATORY RESPONSE
ALLOGRAFT REJECTION
INTERFERON GAMMA RESPONSE
KRAS SIGNALING UP
COMPLEMENT
IL2 STAT5 SIGNALING
IL6 JAK STAT3 SIGNALING
INTERFERON ALPHA RESPONSE
GeneRatio
Count
12
16
20
24
FDR
0.04
0.03
0.02
0.01
C
ADGRE2
AIM2
ALOX5
ANXA1
ATF3
BATF
BLNK
CASP1
CCNB2
CCR2
CCR5
CD14
CD163
CD1D
CD226
CD28
CD300LF
CD44
CD80
CHI3L1
CIITA
CKLF
CNR1
CTSC
CTSL
CXCL10
CXCL8
CYBB
CYRIB
DUSP10
ENO1
FBP1
FCER1G
FCGR3A
FGL2
FPR1
FPR3
GAPDH
GAPT
GBP5
GPR183
GRAMD4
GRB2
HIF1A
HLA–DPA1
HLA–DPB1
HLA–DRA
ID2
IKBKE
IL10
IL18
IL21R
IL2RA
ITGA4
ITGB2
JAK3
LAPTM5
LAT2
LRRK2
LTF
LYN
MAP3K8
MAPK13
ME2
MNDA
MYO1G
NAMPT
NCF2
NFIL3
NLRP12
NOD2
OAS1
OSM
P2RY6
PAK1
PARP9
PIK3CG
PKM
PPBP
PRAM1
PTGER4
PTPN22
RB1
RHOH
RNF135
S100A8
S100A9
SAMHD1
SAMSN1
SCG2
SEMA4A
SIRPB1
SIRPG
SLAMF1
SLC11A1
STAP1
TESPA1
TLR1
TLR2
TLR8
TNFSF13
TNFSF13B
TREM1
TREML4
TYROBP
VAV3
VIM
WNT5A
ZBP1
ZFP36L1
Hypermethylated enhancer
Hypermethylated promoter
chemotaxis
defense response
inflammatory response
innate immune response
leukocyte activation
leukocyte migration
phagocytosis
positive regulation of cytokine production
response to cytokine
response to lipopolysaccharide
phosphate–containing compound metabolic process
positive regulation of metabolic process
positive regulation of phosphate metabolic process

Figure 2. Integration of significant changes in the transcriptome and methylome displayed by CD11B+ myeloid cells in IDH^{mut} gliomas. (A) Cartesian diagrams represent genes (dots) exhibiting significant changes in both expression (Y-axis) and methylation (X-axis) at promoters or enhancers in CD11B+ cells from IDH^{mut} vs. IDH^{wt} gliomas. The total number of genes displaying differential methylation and expression changes is indicated for each quadrant. Over-representation of canonical methylation/expression associations (quadrants II and IV) and under-representation of non-canonical associations (quadrants I and III) were evaluated using ORA and URA, respectively. Quadrants IV (highlighted in red) indicate genes of interest exhibiting downregulation and hypermethylation at either promoters or enhancers **(B)** Bubble plot indicates the "hallmark gene sets" enriched in the integrative gene list (n=370) obtained by combining quadrants IV from both promoters and enhancers analyses. The size and color code of each dot indicate the number of genes with significant enrichment in the corresponding categories (FDR < 0.05). **(C)** The plot illustrates a subset of genes (n=110) from the integrative list that are enriched (FDR < 0.05) in Gene Ontology biological processes related to immune response and metabolism. The hypermethylation status at promoter and enhancer regions is also indicated. Positive or negative hits are represented in green or gray, respectively.

of genes showing non-canonical associations such as overexpression coupled with hypermethylation at promoters and enhancers, was lower than expected (quadrants I, Fig. 2a) (p-value=8.4e-3 and 2.4e-15, under-representation analysis (URA). This integrative analysis of methylome and transcriptome changes revealed 370 unique genes with a hypermethylated enhancer and/or promoter linked to downregulated expression in CD11B+ cells from IDH^{mut} tumors (Supplementary Table 4).

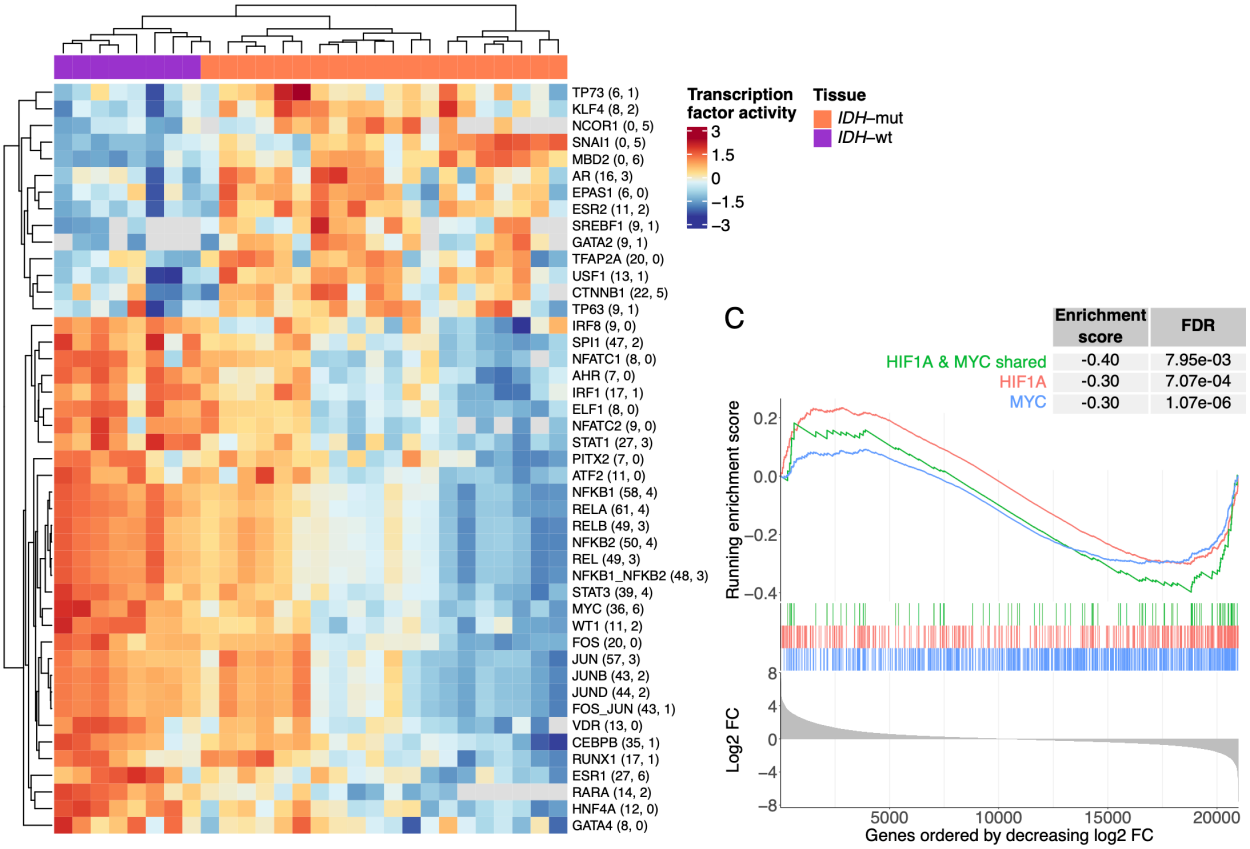
The integrative gene list contained key factors involved in TNF-α signaling via NF-κβ, IL6/JAK/STAT3 signaling, and response to IFN-γ/α (Fig. 2b and Supplementary Table 4). Biological processes such as chemotaxis, leukocyte activation, and positive regulation of metabolism also appeared enriched in this gene list (Fig. 2c). Among the genes whose downregulation was associated to hypermethylation at both regulatory regions, we found CIITA, the most important transactivator of MHC class II, the cytokine oncostatin M (OSM), and myeloid receptors such as formyl peptide receptor 1 (FPR1), and Toll-like receptor 2 (TLR2) (Fig. 2c). Hypermethylation at either promoter or enhancer together with reduced expression was observed for HLA genes, proinflammatory cytokines CXCL10, IL18, and CXCL8, chemokine receptors CCR2/5, FPR3 and TLR1/8, master regulators of glycolysis/hypoxia HIF1A, PKM, ENO1, CD44, and signaling components or enzymes

involved in cytokine production and NAD biosynthesis, such as TYROBP and NAMPT, respectively (Fig. 2c). Therefore, hypermethylation appears to affect various levels of signaling regulation within inflammatory pathways and, possibly, the activation of myeloid cells.

To assess the influence of DNA hypermethylation on TF binding at the enhancers highlighted in quadrants I and IV (Fig. 2a), we estimated TF activity by examining high-confidence TF-gene interactions (n=1,209 human TFs)[41,42] in our RNA-seq data of CD11B+ samples. Analysis of regulons identified 45 TFs having differential activity (FDR <0.05; Wilcoxon's test) according to the IDH mutation status of corresponding tumors, with the majority of TFs showing a decreased activity in IDH^{mut} gliomas (Fig. 3a). Consistent with the binding motifs enriched in the entire repertoire of hypermethylated enhancers (Fig. 1f), this analysis emphasizes that lineage-determining and stimulus-related microglial TFs are repressed from DNA binding by methylated CpGs in CD11B+ cells from IDH^{mut} gliomas. Among these, SPI1 (PU.1), AP-1 complex (ATF2, FOS and JUN), and CEBPB, have previously been shown to be sensitive to methylation[43-45], while NFATC2, RELA and STAT3 have been associated with microglial super-enhancers[39]. Similar results were observed when using the whole set of hypermethylated promoters (Extended Data Fig. 4). In addition, these analyses revealed low activity of MYC and HIF1A (Fig. 3a and Extended Data Fig. 4).

On the other hand, a few TFs appeared activated in these cells, including TP63, TP73, SNAI1, CTNNB1, EPAS1/HIF2A and KLF4. In these cases, methylated CpGs could promote TF binding[46]. Upon closer examination of specific TFs inactivated in CD11B+ cells from IDH^{mut} gliomas, a network inference analysis also suggests that TF-target genes implicated in inflammation and antigen presentation are effectively downregulated as a result of disrupted cooperative effects of core microglial TFs (Fig. 3b). Similarly, targets that are commonly regulated in a positive manner by both MYC and HIF1A, which play roles in the response to hypoxia and glycolysis, were more enriched than the targets regulated by each individual TF in the list of downregulated genes in CD11B+ cells from IDH^{mut} vs. IDH^{wt} tumors (Fig. 3c).

A


TP73 (6, 1)
KLF4 (8, 2)
NCOR1 (0, 5)
SNAI1 (0, 5)
MBD2 (0, 6)
AR (16, 3)
EPAS1 (6, 0)
ESR2 (11, 2)
SREBF1 (9, 1)
GATA2 (9, 1)
TFAP2A (20, 0)
USF1 (13, 1)
CTNNB1 (22, 5)
TP63 (9, 1)
IRF8 (9, 0)
SPI1 (47, 2)
NFATC1 (8, 0)
AHR (7, 0)
IRF1 (17, 1)
ELF1 (8, 0)
NFATC2 (9, 0)
STAT1 (27, 3)
PITX2 (7, 0)
ATF2 (11, 0)
NFKB1 (58, 4)
RELA (61, 4)
RELB (49, 3)
NFKB2 (50, 4)
REL (49, 3)
NFKB1_NFKB2 (48, 3)
STAT3 (39, 4)
MYC (36, 6)
WT1 (11, 2)
FOS (20, 0)
JUN (57, 3)
JUNB (43, 2)
JUND (44, 2)
FOS_JUN (43, 1)
VDR (13, 0)
CEBPB (35, 1)
RUNX1 (17, 1)
ESR1 (27, 6)
RARA (14, 2)
HNF4A (12, 0)
GATA4 (8, 0)
Transcription factor activity
3
1.5
0
−1.5
−3
Tissue
IDH–mut
IDH–wt
C
Enrichment score
FDR
HIF1A & MYC shared -0.40 7.95e-03
HIF1A -0.30 7.07e-04
MYC -0.30 1.07e-06
Running enrichment score
0.2
0.0
−0.2
−0.4
Log2 FC
8
4
0
−4
−8
5000
10000
15000
20000
Genes ordered by decreasing log2 FC

B

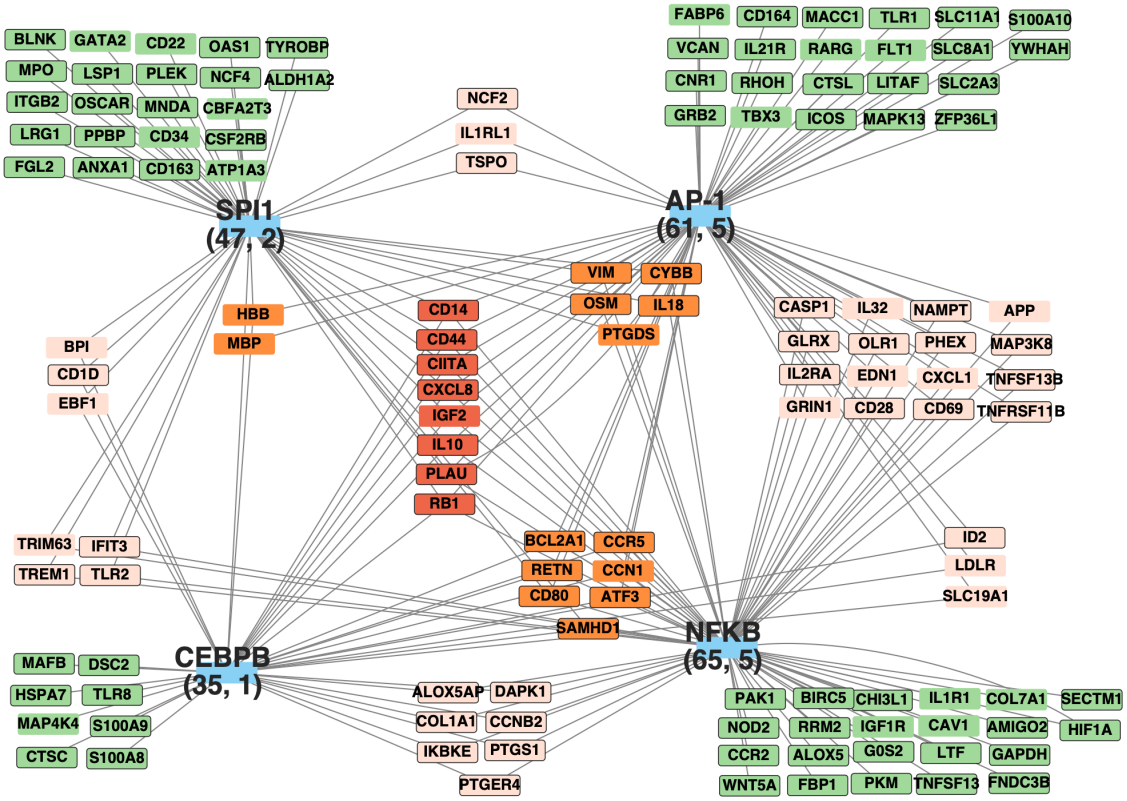

BLNK
GATA2
CD22
OAS1
TYROBP
MPO
LSP1
PLEK
NCF4
ALDH1A2
ITGB2
OSCAR
MNDA
CBFA2T3
LRG1
PPBP
CD34
CSF2RB
FGL2
ANXA1
CD163
ATP1A3
SPI1
(47, 2)
NCF2
IL1RL1
TSPO
FABP6
CD164
MACC1
TLR1
SLC11A1
S100A10
VCAN
IL21R
RARG
FLT1
SLC8A1
YWHAH
CNR1
RHOH
CTSL
LITAF
SLC2A3
GRB2
TBX3
ICOS
MAPK13
ZFP36L1
AP-1
(61, 5)
VIM
CYBB
OSM
IL18
PTGDS
HBB
MBP
CD14
CD44
CIITA
CXCL8
IGF2
IL10
PLAU
RB1
CASP1
IL32
NAMPT
APP
GLRX
OLR1
PHEX
MAP3K8
IL2RA
EDN1
CXCL1
TNFSF13B
GRIN1
CD28
CD69
TNFRSF11B
BPI
CD1D
EBF1
TRIM63
IFIT3
TREM1
TLR2
BCL2A1
CCR5
RETN
CCN1
CD80
ATF3
SAMHD1
ID2
LDLR
SLC19A1
MAFB
DSC2
HSPA7
TLR8
MAP4K4
S100A9
CTSC
S100A8
CEBPB
(35, 1)
ALOX5AP
DAPK1
COL1A1
CCNB2
IKBKE
PTGS1
PTGER4
NFKB
(65, 5)
PAK1
BIRC5
CHI3L1
IL1R1
COL7A1
SECTM1
NOD2
RRM2
IGF1R
CAV1
AMIGO2
HIF1A
CCR2
ALOX5
G0S2
LTF
GAPDH
WNT5A
FBP1
PKM
TNFSF13
FNDC3B

Figure 3. Gene regulatory network analysis reveals lower activity of core microglial TFs linked to hypermethylated enhancers in CD11B+ cells from IDH^{mut} gliomas. (A) The heatmap depicts TFs (n=45) exhibiting differential activity (FDR <0.05; Wilcoxon's test) in CD11B+ cells according to the IDH mutation status of corresponding tumors. The TFs targets used for this analysis, retrieved from the CollecTRI database were searched among the genes displaying upregulated/downregulated expression and hypermethylation at enhancers in myeloid cells from IDH^{mut} gliomas (quadrants I and IV, Fig. 2A). The number of targets regulated positively and negatively by each TF is indicated in brackets. Unsupervised clustering was performed using the z-scores of TF activity, where red and blue colors indicate high and low activity, respectively, and non-significant activity is colored in gray. **(B)** Gene regulatory network of four representative TFs (blue background) having lower activity in CD11B+ cells from IDH^{mut} compared to IDH^{wt} gliomas. TFs targets have a red, orange, pink or green background if they are controlled by four, three, two or one TF, respectively. Targets with or without black box come from quadrant I (hypermethylated enhancers and upregulated expression) or quadrant IV (hypermethylated enhancers and downregulated expression) of Fig. 2A, respectively. **(C)** Gene set enrichment analysis of positive gene targets of HIF1A, MYC (retrieved from CollecTRI database) and those in common between these TFs. Genes are ranked using log2 fold change from differential expression analysis of CD11B+ samples in human gliomas (IDH^{mut}, n=14 vs. IDH^{wt}, n=8).

Of note, shared targets of these TFs involved in glycolysis (CD44, ENO1, GAPDH, HIF1A, ID2), and targets of HIF1A involved in NADH regeneration and mitochondrial function (ENO2, NAMPT, PKM, PFKFB4, SDHB) also appear in the integrative gene list (Fig. 2c). These findings were validated in published RNA-seq data of microglia and MDMs from IDH^{mut} vs. IDH^{wt} gliomas, with the most marked differences being displayed by microglia respect to MDMs (Extended Data Fig. 5). Overall, these results highlight enhancers as main targets of microenvironmental perturbations affecting microglial activation in IDH^{mut} gliomas and raise questions about the underlying mechanisms driving hypermethylation.

D-2HG inhibits TET-mediated 5mC oxidation and promotes DNA hypermethylation at lineage-specific enhancers in human microglia

We hypothesized that D-2HG, the oncometabolite produced by the IDH^{mut} glioma cells and released into the TME, could impact the DNA methylome of CD11B+ cells. Ten-eleven translocation (TET) enzymes are α-KG-dependent dioxygenases that mediate the first step of active DNA demethylation by catalyzing the conversion of 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC)[47] (Extended Data Fig. 6a). Inhibition of TET-mediated DNA demethylation by D-2HG results in decreased levels of 5hmC along with increased levels of 5mC after several rounds of cell replications, ultimately establishing the G-CIMP-

high phenotype of IDH^{mut} gliomas[27,48]. To explore whether this mechanism is at play in the myeloid cells of these tumors, we analyzed global levels of 5hmC and 5mC, as well as the cumulative cell replication history, in an independent series of CD11B+ and CD11B- fractions isolated from human gliomas, and from normal tissues used as controls (Extended Data Fig. 6b and 6c). We confirmed a higher 5mC/5hmC ratio in CD11B- cells of IDH^{mut} gliomas compared to non-tumor tissues (Extended Data Fig. 6b). Remarkably, a significant increase in 5mC/5hmC ratio was also observed in corresponding myeloid cells along with a higher cumulative number of cell divisions as indicated by epigenetic clock analysis (Extended Data Fig. 6c). These results suggest that TET inhibition contributes to the global DNA hypermethylation observed in myeloid cells (most likely microglia) from IDH^{mut} gliomas.

To ascertain the effect of D-2HG on TET-mediated DNA demethylation in human microglia, we set up primary cultures obtained from tissue aspirates of patients who underwent surgery for glioma or drug-resistant epilepsy (Fig. 4a). Microglial cells, maintained in a serum-free defined culture medium[49], are viable for up to 15 days. Over 95% of these cells express the markers ionized calcium-binding adapter molecule 1 (IBA1) and transmembrane protein 119 (TMEM119) (Fig. 4b), whereas less than 5% of cells were proliferating (KI67+) (not shown). Furthermore, these cells maintain a stable RNA-seq microglial signature over time (Extended Data Fig. 7a) and are functional, as evidenced by the proinflammatory response upon lipopolysaccharide (LPS) stimulation at 48 hours or 14 days (Extended Data Fig. 7b and 7c). To evaluate the uptake of D-2HG by human microglial cells, we measured intracellular levels after 48 hours of treatment at a concentration estimated to occur in the TME[4,5], which does not affect cell viability. We found that the levels of D-2HG reached by microglial cells were comparable to those observed in *bona fide* IDH^{mut} cells (Extended Data Fig. 8a and 8b, and Fig. 4c) and were sufficient to induce a significant decrease in TET enzymatic activity (Fig. 4d and Extended Data Fig. 8c). We then exposed primary microglia cultures to D-2HG for a prolonged period and observed that global levels of 5hmC in treated and control microglia have opposite trends over time, leading to a significant decrease of 5hmC levels in treated cells at 14 days (Fig. 4e and Extended Data Fig. 8d).

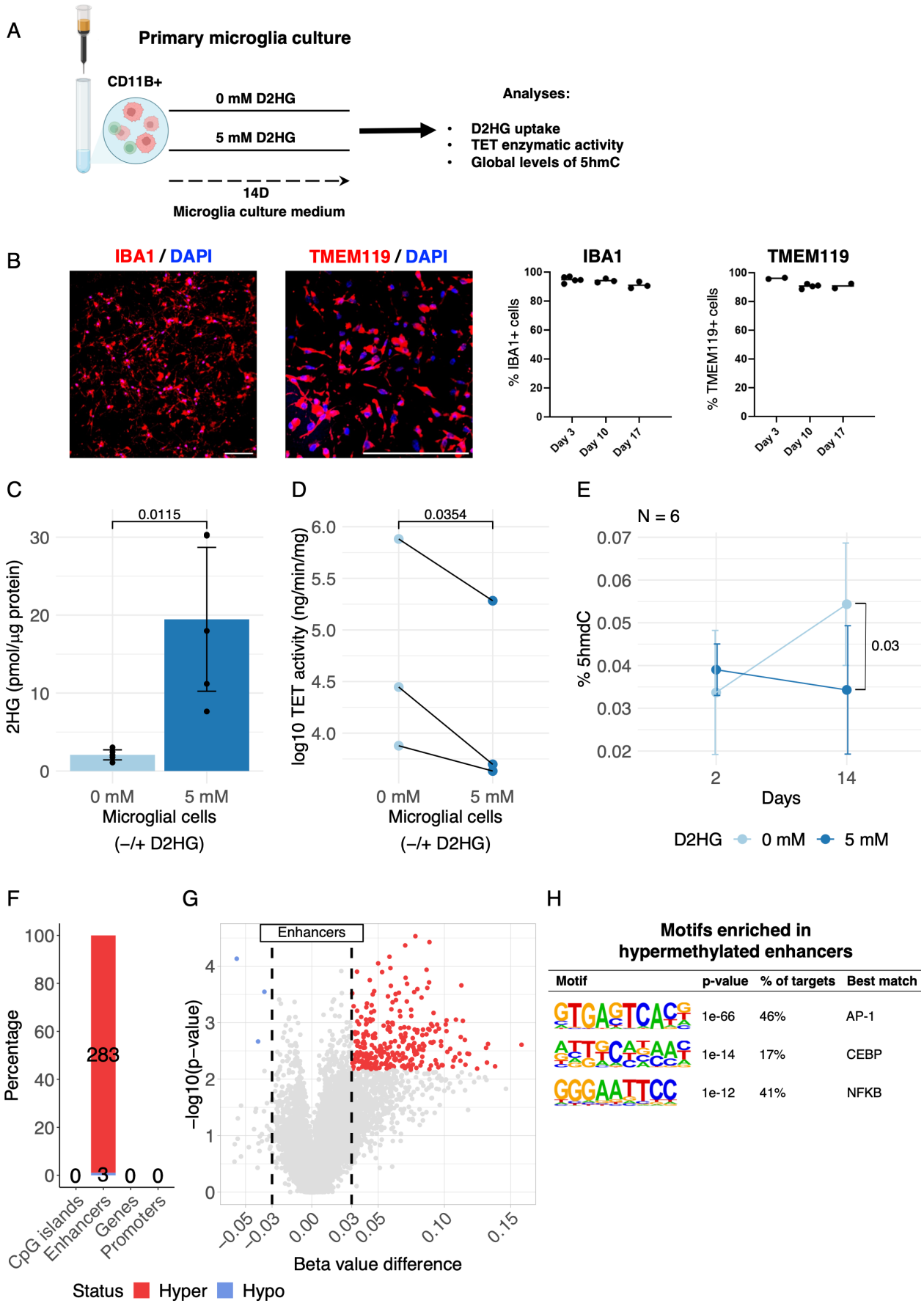

A
Primary microglia culture
CD11B+
0 mM D2HG
5 mM D2HG
14D
Microglia culture medium
Analyses:
D2HG uptake
TET enzymatic activity
Global levels of 5hmC
B
IBA1 / DAPI
TMEM119 / DAPI
IBA1
TMEM119
% IBA1+ cells
% TMEM119+ cells
Day 3
Day 10
Day 17
C
0.0115
2HG (pmol/µg protein)
0 mM
5 mM
Microglial cells
(−/+ D2HG)
D
0.0354
log10 TET activity (ng/min/mg)
0 mM
5 mM
Microglial cells
(−/+ D2HG)
E
N = 6
% 5hmdC
0.03
Days
D2HG
0 mM
5 mM
F
Percentage
283
0
3
0
0
CpG islands
Enhancers
Genes
Promoters
Status
Hyper
Hypo
G
Enhancers
−log10(p−value)
Beta value difference
H
Motifs enriched in hypermethylated enhancers
Motif
p-value
% of targets
Best match
1e-66
46%
AP-1
1e-14
17%
CEBP
1e-12
41%
NFKB

Figure 4. D-2HG uptake by human primary microglial cells inhibits TET function. (A) Overview of the experimental workflow for establishing primary microglia cultures treated with D-2HG, and analyses assessing the inhibition of TET-mediated DNA demethylation. The tissue collection procedures and culture conditions are thoroughly detailed in the Methods section. **(B)** Representative immunofluorescence images show the preserved expression of microglial markers TMEM119 (confocal microscopy) and IBA1 (Apotome) in primary cells at 12 days in culture (scale bars: 100 µm). Estimated percentages of TMEM119+ and IBA1+ cells in primary cultures at different time points are indicated on the right. **(C)** The quantification of intracellular levels of 2HG was determined using LC/MS in primary human microglia obtained from five surgical aspirates with IDH^{wt} (n=2) or IDH^{mut} (n=3) status. Data are represented as the mean with a 95% CI; p-value was calculated using a one-sided paired t-test. **(D)** TET enzymatic activity measured as detection of hydroxymethylated product (ng) after 1-hour incubation of the substrate with protein extracts from microglia untreated and treated with 5 mM D-2HG over 48 hours. Primary cultures were generated from surgical aspirates with IDH^{wt} status (n=3). The p-value was calculated using one-sided paired t-test. **(E)** Assessment of global levels of 5hmC, determined by LC-MS/MS, in primary human microglia obtained from six surgical aspirates with IDH^{wt} (n=5) or IDH^{mut} (n=1) status after 48 hours and 14 days of treatment with 5 mM D-2HG (added every 2-3 days). Data are represented as the mean with a 95% CI; p-value was calculated using a one-sided paired t-test. **(F)** DNA methylation profiling of primary microglia was conducted with EPIC array. Stacked bar chart shows absolute numbers and relative percentages of functional genomic regions respect to the methylation status (absolute $\Delta\beta > 0.03$, FDR < 0.25) in D-2HG treated vs. untreated primary microglia cultured for 14 days. Samples were obtained from eight surgical aspirates with IDH^{wt} (n=5) or IDH^{mut} (n=3) status. **(G)** Volcano plot illustrates the magnitude and extent of differentially methylated enhancers in D-2HG treated vs. untreated primary microglia. **(H)** Motif enrichment analysis of hypermethylated enhancers in D-2HG-treated primary microglia. Significant sequence motifs (ranked by p-value) were determined using HOMER.

No concomitant changes in 5mC levels were observed in D-2HG-treated microglia (data not shown). These results suggest that TET-mediated demethylation in non-dividing human microglia is inhibited by D-2HG. However, contrary to the *ex vivo* situation (Extended Data Fig. 6b and 6c), the low proliferation rate of cultured microglia and the reduced time window (only 2 weeks) precluded our ability to demonstrate D-2HG-induced global hypermethylation. Nonetheless, further analysis of DNA methylome in microglial cells treated with D-2HG for 14 days, using the same EPIC array for the analysis of *ex vivo* CD11B+ cells in gliomas, revealed subtle yet reproducible hypermethylation at 283 enhancers (absolute $\Delta\beta > 0.03$, FDR < 0.25) (Fig. 4f and 4g). Importantly, these enhancers showed significant enrichment for motifs of lineage-determining and stimulus-responsive TFs as in the *ex vivo* study, including AP-1, C/EBP and NF-κβ, but not SPI1 (PU.1) (Fig. 4h).

While EPIC arrays offer sufficient coverage of enhancers, only a single probe captures methylation changes across an enhancer region[50]. Moreover, this profiling method does not discriminate 5hmC from 5mC, which could provide valuable insights into impaired TET function. Considering the high turnover of DNA methylation at enhancers, which relies on TET activity rather than replicative passive dilution of 5mC[51], we wondered whether D-2HG affects TET-mediated demethylation at enhancers genome-wide. To investigate this possibility, we performed a DNA methylome and hydroxymethylome profiling of microglial cells treated with D-2HG for 48 hours or 14 days. For this experiment, we used microglial preparations from normal brain tissues of three individuals with drug-resistant epilepsy to exclude tumor influences other than released D-2HG, and we applied a reduced representation and non-bisulfite sequencing approach[52]. The extensive coverage enables a more confident assessment of 5mCG and 5hmCG levels in enhancers, yet only across 20% of the enhancers analyzed with EPIC arrays, which cover ~58% of FANTOM annotated enhancers[50]. Changes of 5mCG and 5hmCG levels in the entire enhancer regions were most evident at 14 days of treatment respect to 48 hours, with a marked difference for 5hmCG, reflecting its accumulation over time in D-2HG-treated cells (Fig. 5a). This result indicates that D-2HG-related changes in genomic 5hmC content, initially detected by LC-MS/MS, mostly occur in non-CpG contexts, and shows that TET function is not affected in the entire enhancers regions, at least *in vitro*. Moreover, while EPIC arrays revealed some lineage-specific enhancers that underwent hypermethylation following D-2HG treatment (Fig. 4f-4h), only a few of them were covered by the reduced representation sequencing approach.

To ascertain whether inhibition of TET-mediated demethylation by D-2HG affects a subset of lineage-specific enhancers, we pursued the following analytical strategy with our sequencing data (Fig. 5b). We aimed to use as ground truth the enhancers in CD11B+ cells from normal brain tissues, which eventually become hypermethylated in myeloid cells from IDH^{mut} gliomas, likely corresponding to microglia (Fig. 1c). Considering that the highest level of TET activity occurs around TFs binding motifs located in enhancers[51,53], we then focused our analysis on TFs binding motifs within hypermethylated enhancers found in both CD11B+

cells from IDH^{mut} gliomas (Fig. 1f) and D-2HG-treated microglia (Fig. 4h), potentially impacting TF occupancy (Fig. 3a and 3b).

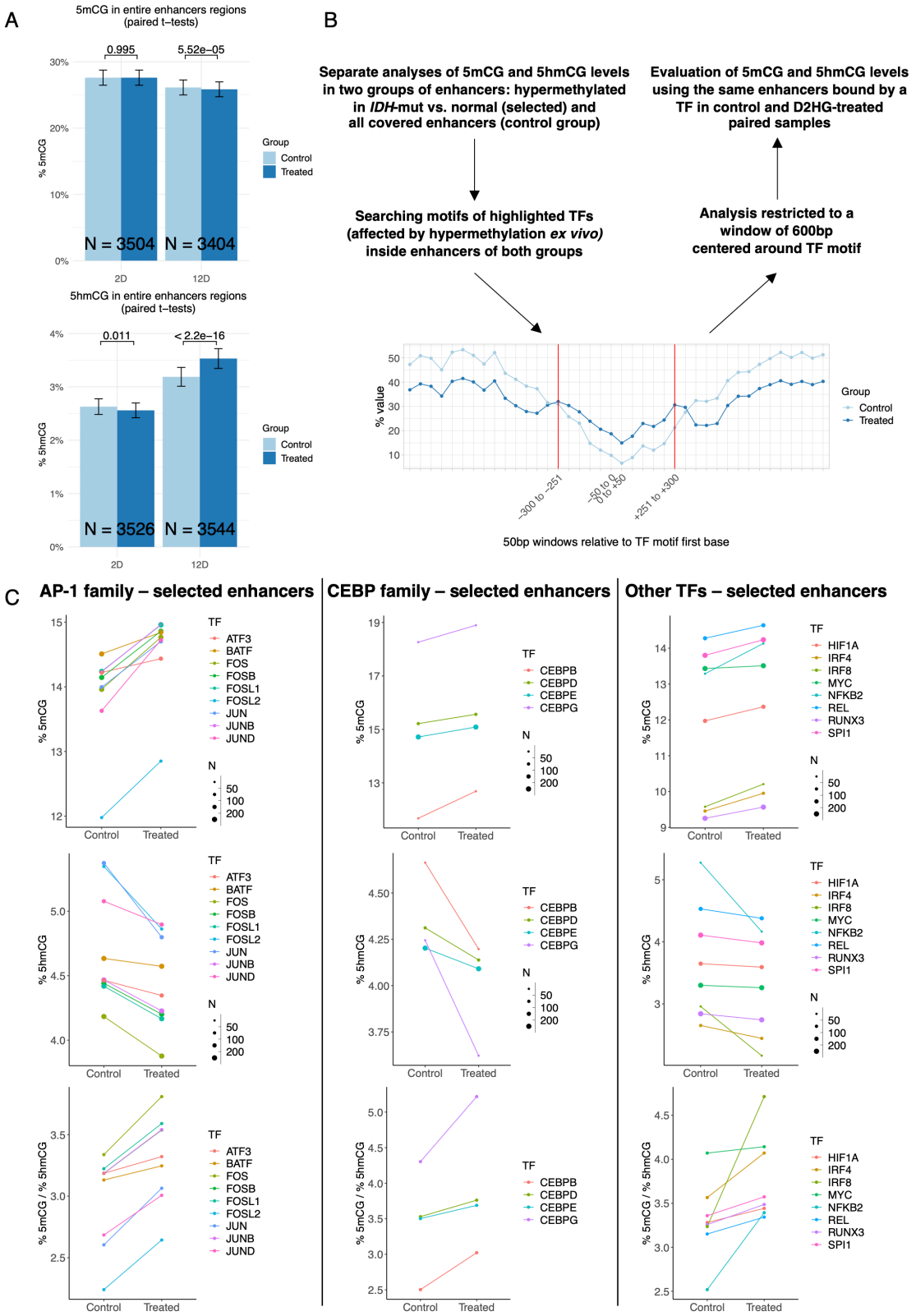


Figure 5. Reduced representation non-bisulfite sequencing reveals high 5mCG/5hmCG ratios at lineage-specific enhancers in D-2HG-treated primary microglia. (A) Bar plots show the percentages of 5mCG and 5hmCG at the whole set of covered enhancers (full-length regions) in primary human microglia treated with D-2HG for 48 hours or 14 days and in paired untreated controls. The number of paired enhancers for each comparison is indicated. Only common enhancers across patients were analyzed. **(B)** Workflow analysis of 5mCG and 5hmCG levels quantification in defined windows around TFs binding motifs, located in lineage-specific enhancers, in D-2HG-treated microglia for 14 days and in paired untreated controls. **C)** Plots showing a separate analysis of 5mCG and 5hmC percentages (upper) around TFs binding motifs for the indicated TFs in D-2HG-treated microglia and paired untreated controls. Only common enhancers across patients were analyzed. The size of each dot indicates the number of enhancers analyzed in a paired manner between conditions. The 5mCG/5hmCG ratios (bottom), which are higher in D-2HG-treated microglia than in paired controls, are also shown. The TFs binding motifs for this analysis are located in enhancers exhibiting hypermethylation in CD11B+ cells from IDH^{mut} gliomas vs. normal brain tissues in the *ex vivo* study (Fig 1C).

Notably, higher 5mCG/5hmCG ratios were detected around binding motifs of members of the AP-1, C/EBP and NF-κβ TFs families in D-2HG-treated primary microglia compared to paired untreated controls with these motifs being located in hypermethylated enhancers of CD11B+ cells from IDH^{mut} gliomas vs. normal brain tissue (Fig. 5c). Similar results were obtained when this analysis was extended to motifs of SPI1 (PU.1), IRF4/8, RUNX3, HIF1A and MYC, which also appeared to be relevant in the *ex vivo* situation (Fig. 5c). In sharp contrast, 5mCG and 5hmCG levels in the proximity of binding motifs located in the whole set of covered enhancers, exhibit opposite trends (Extended Data Fig. 9a). Notably, the genes regulated by the selected enhancers bound by key TFs (Fig 5c), that exhibited hypermethylation/hypohydroxymethylation, were enriched among the downregulated genes, indicating a direct impact of D-2HG-induced hypermethylation on gene expression (Extended Data Fig. 9b and Supplementary Table 5). Taken together, these results strongly support that D-2HG has the potential to impede TET-mediated DNA demethylation selectively at lineage-specific enhancers, leading to a blunted microglial cell response.

D-2HG dampens the LPS-induced inflammatory response and increases basal oxidative metabolism in human microglial cells

Lineage-specific enhancers that were affected by D-2HG treatments in our *in vitro* experiments have been shown to be transcribed upon macrophage stimulation[54]. Thus, we

hypothesized that the methylome primed by D-2HG could hinder the activation of enhancers governing the proinflammatory capacity of human microglia. To address this issue, we evaluated transcriptional changes in cells pre-treated with D-2HG for 14 days and subsequently challenged with LPS for 6 hours (Fig. 6a).

Gene set enrichment analysis (GSEA) indicated that treatment with D-2HG attenuates LPS-induced microglial activation (Fig. 6b). Specifically, these cells exhibit an M2-like phenotype characterized by enhanced OXPHOS and fatty acid metabolism alongside marked suppression of the inflammatory response pathways including TNF-α signaling via NF-κβ (Fig. 6b). Additionally, in RNA-seq data from the *ex vivo* study, we find reduced expression of proinflammatory cytokines in CD11B+ cells from IDH^{mut} compared to IDH^{wt} gliomas (Extended Data Fig. 10a). We then prompted to evaluate the effect of D-2HG on the expression of LPS-induced cytokines using RT-qPCR in an independent series of primary microglia preparations. We confirmed that the expression of TNFA and IL6 was abrogated by D-2HG in LPS-challenged cells compared to paired controls (p-value=0.02 and 0.07, respectively) (Extended Data Fig. 10b). Similarly, assessment of mitochondrial respiration parameters demonstrated that D-2HG-treated microglia exhibit an increase in basal and maximal respiration even after LPS stimulation (Extended Data Fig. 10c). This observation suggests that this oncometabolite impedes the glycolytic shift that accompanies cellular activation and instead favors OXPHOS metabolism. All in all, these results indicate that D-2HG dampens microglia proinflammatory response to LPS, likely through direct effects on TET-mediated demethylation at enhancers governing microglial activation.

Lastly, to determine whether suppressed microglial cell signaling linked to D-2HG-driven enhancer hypermethylation can be restored upon targeted therapy, we analyzed microglial cells from single-nucleus RNA-seq (snRNA-seq) data obtained from one oligodendroglioma patient treated with ivosidenib, an inhibitor of the IDH1 mutant enzyme that reduces D-2HG levels in tumor bulk (Fig 6c and Supplementary Table 1)[55].

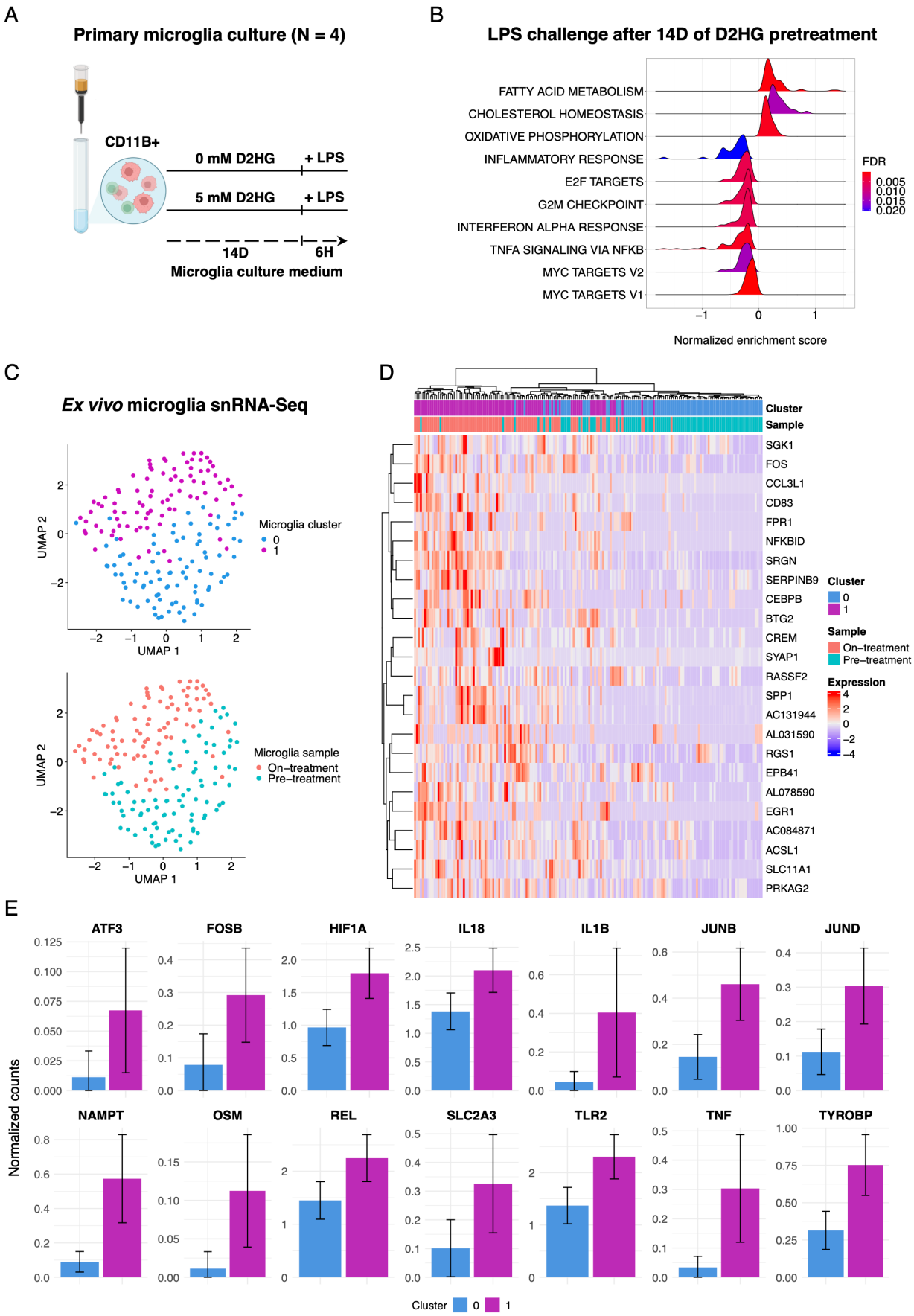

A
Primary microglia culture (N = 4)
CD11B+
0 mM D2HG
+ LPS
5 mM D2HG
+ LPS
14D
6H
Microglia culture medium
B
LPS challenge after 14D of D2HG pretreatment
FATTY ACID METABOLISM
CHOLESTEROL HOMEOSTASIS
OXIDATIVE PHOSPHORYLATION
INFLAMMATORY RESPONSE
E2F TARGETS
G2M CHECKPOINT
INTERFERON ALPHA RESPONSE
TNFA SIGNALING VIA NFKB
MYC TARGETS V2
MYC TARGETS V1
FDR
0.005
0.010
0.015
0.020
Normalized enrichment score
C
Ex vivo microglia snRNA-Seq
UMAP 2
UMAP 1
Microglia cluster
0
1
Microglia sample
On−treatment
Pre−treatment
D
Cluster
Sample
SGK1
FOS
CCL3L1
CD83
FPR1
NFKBID
SRGN
SERPINB9
CEBPB
BTG2
CREM
SYAP1
RASSF2
SPP1
AC131944
AL031590
RGS1
EPB41
AL078590
EGR1
AC084871
ACSL1
SLC11A1
PRKAG2
Expression
E
ATF3
FOSB
HIF1A
IL18
IL1B
JUNB
JUND
NAMPT
OSM
REL
SLC2A3
TLR2
TNF
TYROBP
Normalized counts
Cluster 0 1

Figure 6. D-2HG impairs microglial activation. (A) Experimental workflow for the LPS challenge experiment in primary microglia pre-treated with D-2HG for 14 days. RNA-seq data was generated from human primary microglia obtained from four surgical aspirates with IDH^{wt} (n=3) or IDH^{mut} (n=1) status. Patient-paired approach was adopted for RNA-seq data analysis. **(B)** GSEA ridge plot illustrates "hallmark gene sets" in D-2HG-treated microglia for 14 days, 6 hours after LPS stimulation. Gene sets with positive or negative normalized enrichment score are associated with up or down regulated genes, respectively (FDR < 0.05). **(C)** Uniform Manifold Approximation and Projection (UMAP) plots depict microglial cells analyzed with snRNA-seq from one oligodendroglioma patient pre- and on-treatment with the mutant IDH inhibitor ivosidenib. **(D)** The heatmap illustrates the significantly upregulated genes (log2FC > 1, Bonferroni-adjusted p-value < 0.05) in microglia upon ivosidenib treatment. **(E)** Normalized counts of selected genes in microglia pre and on-treatment. Data are represented as the mean with a 95% CI.

After removing low quality cells, 178 microglial cells remained, as determined by the high expression of markers TAL1 and SLC2A5, and the low/absence of expression of MDMs markers CD163 and TGFBI. These cells encompassed two well-defined clusters corresponding almost entirely to on- (n=87) and pre-treatment (n=91) microglia, on which the analysis was focused (Fig 6c). We noticed a transcriptional shift towards gene upregulation in cluster 1 (on-treatment) compared to cluster 2 (pre-treatment) (1683 genes with log2FC >1, 501 genes with log2FC < -1, respectively). Furthermore, we found 24 upregulated (including the master regulators TFs FOS and CEBPB) (Fig 6d) and 3 downregulated genes (Bonferroni-adjusted p-value < 0.05, log2FC > 1 or < -1, respectively) with a strong enrichment of the upregulated genes in the TNF-α signaling via NF-κβ gene set (FDR=1.5e-4; ORA). Additionally, microglial cells in cluster 1 displayed overexpression of other TFs governing activation, including AP-1 family members (FOSB, ATF3, JUNB, and JUND), REL, and HIF1A, as well as related target genes highlighted in the *ex vivo* and *in vitro* analyses, such as OSM, IL18, IL1B, FPR1, TLR2, NAMPT, TNF, TYROBP, and SLC2A3 (GLUT-3) (Fig 6d and 6e). This result suggested that decreased levels of D-2HG in the TME may reverse the hypermethylation phenotype observed in microglia from IDH^{mut} gliomas, thereby enabling their activation program. Supporting this hypothesis, a significant proportion of genes upregulated following ivosidenib treatment are regulated by enhancers that were found to be hypermethylated in CD11B+ cells from IDH^{mut} gliomas (p-value = 0.0012, ORA).

Discussion

The development of new therapeutic options to cure glioma requires a deep understanding of the epigenetic mechanisms governing transcriptional programs, cell states, and proinflammatory capacities in TAMs. Differences in the transcriptome and chromatin landscapes have been associated with the IDH^{mut} status[17,18,21], but the extent and the role of DNA methylation in TAMs have remained uncharacterized. Herein, through genome-wide profiling of the DNA methylome, we uncovered global and differential distribution of 5mC and 5hmC changes in TAMs according to the IDH^{mut} status of gliomas. Our findings revealed how this differential methylation affects microglial enhancers involved in the regulation of proinflammatory and metabolic pathways.

We found that CD11B+ cells residing in an IDH^{mut} environment display a striking bias towards DNA hypermethylation. TAMs comprise MDMs and microglia in proportions and states of activation that differ according to glioma IDH^{mut} status, subtype, and grade[17-23]. Previous studies reported that most of the TAMs in IDH^{mut} tumors are microglia, whereas IDH^{wt} gliomas are marked by an influx of MDMs[17-20]. In this regard, we do not rule out the possibility that differentially methylated changes observed in CD11B+ cells partly reflect a decreased recruitment of MDMs in IDH^{mut} compared to IDH^{wt} tumors. However, the hypermethylation we observed holds true even when comparing CD11B+ cells from IDH^{mut} glioma to non-tumor brain tissues, both of which are highly enriched in microglia. In addition, a recent study indicated that immunomodulatory activity programs are expressed across myeloid cell types, and demonstrated convergent phenotypes of resident microglia and infiltrating MDMs, with the latter acquiring a microglia-like phenotype in tumors[23]. Moreover, mouse studies indicate that MDMs can adopt a DNA methylation profile very similar to that of microglia after colonizing the central nervous system niche[56]. Therefore, our bulk profiling of CD11B+ cells strongly suggests that microglia undergo a hypermethylated state in IDH^{mut} tumors. This assumption is further substantiated by our analyses of methylation across gene regulatory regions, revealing functional implications related to proinflammatory activation and metabolism in microglia.

We highlighted that the occurrence and extent of differentially methylated changes were more prominent in distal enhancers. DNA methylation at CpG sites within or flanking motifs may prevent TF binding at regulatory regions by inducing steric hindrance, altering DNA shape and rigidity, or influencing nucleosome positioning, thereby affecting the formation of active chromatin[36,38,43,57-59]. A recent study showed that only a minority of enhancers in a given cell type are sensitive to DNA methylation[60]. In line with this, our integrative analysis of methylome and transcriptome changes demonstrates that, among the hypermethylated enhancers displayed by CD11B+ cells from IDH^{mut} vs. IDH^{wt} comparison, 23% may account for the downregulated expression of target genes. We also observed that hypermethylated enhancers were enriched for motifs of lineage-determining TFs, including SPI1 (PU.1) and members of the C/EBP and RUNX families, as well as motifs for stimulus-induced TFs, such as AP-1, IRF, and NF-κB. These TFs are known to regulate the functional properties of myeloid cells. Our interrogation of gene regulatory networks further supports the hypothesis that hypermethylated enhancers are responsible for the reduced activity of core TFs that govern the identity and activation program in human microglial cells[39]. Specifically, hypermethylation seems to disrupt cooperative regulatory functions involving lineage-determining TFs SPI1 (PU.1) and C/EBP, and stimulus-responsive TFs AP-1 and NF-κβ, which in turn affect the expression of key genes involved in inflammatory responses. Hypermethylated enhancers also appear to influence the expression of activating receptors, as well as of genes involved in antigen presentation, an observation previously reported in bulk methylome analysis of IDH^{mut} gliomas[61]. These findings indicate that microglia in these tumors undergo a profound alteration of promoter-enhancer interactions that characterize these cells[62].

In addition to microglial TFs, our data revealed the inactivation of HIF1A and MYC, which possibly reinforces the silencing of shared targets essential for the glycolytic shift of stimulated macrophages[63-66]. Along with a marked downregulation of several target genes, there was low expression of HIF1A, accompanied by hypermethylation at regulatory regions

of HIF1A and some of its targets. In particular, reduced expression of HIF1A targets such as LDHA, ENO1, HK2, PKM, VIM, ITGB2, and PGK1 has also been reported in bulk IDH^{mut} tumors and/or cells[67], which may be attributable to hypermethylation driving the silencing of these genes in addition to the effect of D-2HG on HIF-1α protein destabilization[68]. Collectively, our findings provide evidence that DNA hypermethylation may contribute to a reduced proinflammatory microglial response by targeting enhancers that orchestrate synergistic responses to environmental stimuli driving myeloid cell activation[69-71]. This assumption is supported by our experiments, which demonstrate how oncometabolite signaling modulates the methylome and subsequently affects the activation of human microglia.

Our analysis of single-nucleus RNA sequencing data of two paired oligodendroglioma samples revealed that treatment with an inhibitor of the IDH1 mutant enzyme could unleash the signaling that suppresses microglia activation. This observation supports the notion that D-2HG production plays a major role in the suppressive signaling that affects microglia function and highlights the prognostic and therapeutic value of microglial activation in IDH^{mut} glioma. By establishing primary cultures of human microglia, we have shown that D-2HG is taken up by these cells, inhibits TET-mediated DNA demethylation, and hampers the accumulation of 5hmC genomic content after a 14 days treatment. Although D-2HG-driven global hypermethylation is challenging to reproduce in our microglial cultures, our analysis of methylome and hydroxymethylome at nucleotide resolution clearly demonstrates higher 5mCG/5hmCG ratios at lineage-specific enhancers that were also hypermethylated in *ex vivo* CD11B+ cells from IDH^{mut} gliomas. This result is consistent with the previous demonstration that the highest TET activity is localized to enhancer elements[51,53]. Our observation that the 5hmC turnover is limited in cultures of non-proliferating microglial cells points to a passive demethylation mechanism that could unfold under successive rounds of replication. Nevertheless, a comprehensive characterization of other TET-mediated oxidized derivatives, such as 5fC and 5caC, and an assessment of the activity and the role of thymidine DNA glycosylase[47], will be required to determine the relative contribution of active vs. passive demethylation mechanisms in human microglia.

Regardless of the demethylation mechanism, we speculate that inhibition of TET2 in slowly proliferating microglial cells of IDH^{mut} gliomas, which are exposed to D-2HG over several years, may lead to full-blown hypermethylation, as opposed to the limited changes observed in our non-proliferating microglial cultures exposed to this oncometabolite for no more than two weeks. It is also possible that additional mechanisms contribute to the hypermethylation highlighted in the *ex vivo* context. Nevertheless, our *in vitro* system was instrumental in demonstrating that D-2HG readily affects TET-mediated demethylation at specific enhancers, leading to suppressed expression of targeted genes. A high turnover of DNA methylation at enhancers is crucial for cellular plasticity, differentiation, and responsiveness to environmental changes, for instance, during macrophage activation[72,73]. Thus, the significant overlap of enhancer elements impacted by D-2HG *in vitro* with those exhibiting hypermethylation in myeloid cells from IDH^{mut} gliomas strongly suggests that the methylome of microglial cells primed by D-2HG negatively affects the transcriptional response to inflammatory stimuli.

Consistent with this hypothesis, we observed that D-2HG-treated microglia, when challenged with LPS, display attenuation of the inflammatory response while favoring an OXPHOS metabolism that marks anti-inflammatory M2-like macrophage phenotypes[74,75]. While the hypermethylation we observed may affect the glycolytic shift—a metabolic response documented in iPSC-derived human microglia treated with LPS[76]— our data indicate that hypermethylation could also impact genes involved in cytokine production. Considering the time-dependent effects of D-2HG on the microglia methylome, we propose that an epigenetic drift—involving the selection of hypermethylated enhancers over time—underlies the silencing of master regulators, which impedes not only the proinflammatory response but also the glycolytic reprogramming, both of which are essential for microglial cell activation. This does not exclude the possibility of contributions from chromatin remodeling and non-epigenetic mechanisms to the LPS hyporesponsiveness of D-2HG-treated microglia, including signaling pathways targeted by D-2HG in other cell types[6,9,10,77].

In particular, it has been reported that acute octyl-D-2HG treatment can dampen LPS-triggered cytokine expression in the immortalized mouse BV2 microglial cell line through a mechanism involving AMP-activated protein kinase (AMPK)-mTOR-mediated reduction of NF-κB activity[77]. Whether this mechanism also applies to human primary microglia remains undetermined.

In conclusion, we provide evidence that D-2HG reshapes the enhancer landscape of resident microglia in IDH^{mut} gliomas, thus impairing the regulatory function of core TFs. This priming mechanism precludes the capacity of these cells to mount the inflammatory response and possibly the shift towards a glycolytic metabolism. We speculate that D-2HG effects on myeloid cells promote a cold immune TME that favors tumor progression. Given that the reduced concentration of D-2HG in bulk tumors, induced by inhibitors of the IDH mutant enzyme, is associated with inflammatory and metabolic changes[78], it is important to evaluate the extent of these effects in myeloid cells and to correlate them with the clinical response to this therapeutic option.

Limitations of the study

We acknowledge certain limitations in our study that should be taken into consideration. Our comparative analyses of CD11B+ myeloid fractions from gliomas and non-tumor tissues remained at the bulk level. Moreover, the methylome profiling is limited to CpG residues in the EPIC array and to global levels assessed by LC-MS/MS, whereas no exhaustive analysis of 5hmC at nucleotide resolution was conducted *ex vivo*. While our results indicate that hypermethylation affects the dominant population of resident microglia cells in IDH^{mut} gliomas, employing single-cell multimodal approaches will be necessary to better associate enhancer landscapes with their regulatory roles in specific myeloid cell subsets/states. Lastly, we did not analyze the potential effects of D-2HG on the local chromatin environment, which could also influence enhancer function.

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Author contributions

A.L: Conceptualization, optimization of primary microglial cultures, designed and performed *in vitro* experiments, data collection, analysis and interpretation of results, draft editing. P.P: Conceptualization, data curation, computational and statistical analyses, interpretation of results, data visualization, draft editing. B.M: Provided surgical resection specimens, optimization of primary microglial cultures, draft editing. Q.R., S.J: Performed *ex vivo* experiments and data visualization. Y.H., S.S: Conducted validation experiments, data analysis and interpretation, data visualization, draft editing. P.M: Provided surgical resection specimens. K.L., A.A: Bioinformatics assistance, draft editing. M.V., A.I., C.B., E.H., N.P., K.M., S.T., M.L.S., M.T., F.B: Provided material, data curation. A.D., I.F: Performed LC/MS analysis of 2HG. G.A.K., P.J.K., L.J.M: Performed LC-MS/MS and data analysis of global 5mC and 5hmC levels. E.D., E.E-H: Performed seahorse experiments and data analysis. L.A.S: analysis of

epigenetic clocks and draft editing. A.I: Data analysis and interpretation, draft editing. M.C: Computational analyses, draft editing. M.M: Conceptualization, experimental design, data analysis and interpretation, draft editing. M.S: Conceptualization, experimental design, data analysis and interpretation, draft editing, funding acquisition, supervision. L.J.C-V: Conceptualization, experimental design, data analysis and interpretation, data visualization, writing–original draft, funding acquisition, project administration and supervision. All authors listed revised the manuscript critically for important intellectual content and approved it for publication.

Conflict statement

L.A.S. is a scientific advisor and co-founder of Cellintec L.L.C., which had no role in this research. M.L.S. is equity holder, scientific co-founder, and advisory board member of Immunitas Therapeutics. The other authors declare no competing or financial interests.

STAR Methods

Tumor samples and CD11B sorting

Human brain tissues were obtained with informed consent approved by the institutional ethical committee board in accordance with the Declaration of Helsinki. Samples for analyses were selected from the Pitié-Salpêtrière tumor bank Onconeurotek based on clinical information and validation by expert neuropathologists (K.M., S.T., F.B) of both histological features, and molecular diagnosis as previously described[79]. Cryopreservation was as follows: tumors taken from the neurosurgery room were immediately transported on ice in HBSS (Gibco), cut into 2-5 mm diameter pieces, and submerged in cryotubes containing 1 mL of DMEM/F-12 (Gibco), 20% FBS, and 10% DMSO (Sigma-Aldrich). The cryotubes were placed at -80°C in alcohol-free freezing containers (Corning® CoolCell®). The cryopreserved samples were then stored in liquid nitrogen until they were used. On the day of analysis, tumor pieces were thawed and rinsed in DMEM/F-12. Next, the tissue was enzymatically and mechanically digested for 5-10 minutes at 37°C in an HBSS-papain-based lysis buffer

(Worthington) containing DNAse (0.01%, Worthington) and L-Cysteine (124 µg/mL, Sigma). Enzymatic digestion was stopped with ovomucoid (700 µg/mL, Worthington). The homogenates were sequentially filtered using 100 µm, 70 µm, and 30 µm SmartStrainers (Miltenyi) to remove residual clumps. HBSS was added, and samples were then centrifuged at 300 g for 10 minutes at 4°C. Cell pellets were resuspended in an appropriate volume of cold MACS debris removal solution following the manufacturer's instructions (Miltenyi). The suspension was gently mixed by pipetting 10-12 times, and ice-cold DPBS without calcium and magnesium was added very slowly above the cell suspension to create a transparent gradient. The suspension was centrifuged at 4 °C and 3000 g for 10 min. After centrifugation, the top liquid and solid interphases were discarded, and cell suspensions were resuspended with cold MACS Buffer (0,5% BSA and 2 mM EDTA in PBS). Tubes were gently inverted 3-5 times and then centrifuged at 1000 g for 10 min at 4°C. After centrifugation, the supernatant was completely removed, and red blood cells lysis was performed with ACK buffer (Gibco) for 5 min at room temperature. Next, cells were centrifuged at 4°C and pellets were washed with cold DPBS. After centrifugation, cells were resuspended in MACS buffer and counted. Next, cell pellets were labeled with an appropriate volume of CD11B MicroBeads (Miltenyi) in MACS buffer and incubated for 15 min at 4°C. Subsequently, cells were resuspended in cold MACS buffer and centrifuged at 300 g for 10 min at 4°C. The supernatant was aspirated, and cells were resuspended in cold MACS buffer. LS MACS Separation Columns were placed in the magnetic field of a suitable MACS Separator, and the cell suspension was applied onto the columns. CD11B+ and CD11B- fractions were separately collected on ice. The dry pellets were stored at -80°C until nucleic acid extraction.

Nucleic acids extractions

RNA and DNA of CD11B+ and CD11B- samples were co-eluted using the AllPrep DNA/RNA Micro Kit (Qiagen, 80284) or AllPrep DNA/RNA Mini Kit (Qiagen, 80204) following the manufacturer’s instructions. In some cases, nucleic acids were purified separately using the Maxwell RSC simplyRNA Cells Kit (Promega, AS 1390) and the Maxwell RSC Blood DNA Kit

(Promega, AS 1400), following the manufacturer's instructions. The yield and quality of total RNA was assessed using the Tapestation 2200 (Agilent) instrument.

Digital droplet PCR

IDH1 R132H, TERT C228T, and TERT C250T mutations were analyzed in CD11B+ and CD11B- fractions from human gliomas using ddPCR. Droplet generation and partition was performed using the Bio-Rad system QX200. Primer sets, probes, and ddPCR™ Supermix for Probes (No dUTP) were used for quantification of absolute copy number in 3 ng of input DNA following the manufacturer's instructions. All primers and probes were obtained from Bio-Rad. Sequence information is available at www.bio-rad.com using the ID numbers: IDH1 p.R132H Hsa, Human (Ref: 10031246 UniqueAssayID: dHsaCP2000055), IDH1 WT for p.R132H Hsa, Human (Ref: 10031249 UniqueAssayID: dHsaCP2000056), TERT C228T_113 Hsa, Human (Ref: 12009308 UniqueAssayID: dHsaEXD72405942), TERT C250T_113 Hsa, Human (Ref: 12003908 UniqueAssayID: dHsaEXD46675715). The data were then analyzed using Poisson statistics to determine the target DNA template concentration in the original sample. Positive controls consisted of tumor DNA from the CD11B- fractions and negative controls contained water instead of DNA.

DNA methylation profiling

Genomic DNA was quantified by Quant-iT dsDNA Broad range Assay (Thermo Fisher Scientific, Waltham, MA, USA) in a Tecan SPARK microplate Reader (TECAN, Switzerland). Total DNA (500 ng) from each sample was sodium bisulfite converted using Zymo EZ-96 DNA Methylation kit and following the manufacturer's recommendations for Infinium assay (Zymo Research, catalog number: D5004). After conversion, DNA concentration of each sample was adjusted to 50 ng/µL with M-elution Buffer or concentrated using speed vacuum. In total 300 ng of converted DNA for each sample was used as a template on the Infinium MethylationEPIC BeadChip following the manufacturer's recommendations. Briefly, bisulfite-converted DNA is whole genome amplified, fragmented and hybridized to the array. After hybridization, unhybridized and no specifically hybridized DNA is washed away, and the

captured product is extended with fluorescent labels coupled to nucleotides. Finally, the arrays are scanned with a high-resolution Illumina scanner (iScan), which acquires light images emitted from fluorophores. The intensities are measured and the methylation signals are extracted and recorded as raw data (IDAT). These assays were performed at the P3S platform (Sorbonne University). DNA methylation data were analyzed using the RnBeads package. In short, after the removal of bad quality samples, data were aligned to the hg19 assembly using the beta Mixture Quantile dilation (BMIQ) method for normalization and adjusted for sex as a covariate. Sex chromosomes were included and sites having a standard deviation below 0.01 across all samples were filtered out. Together with default annotations, we included the enhancers coordinates (entire enhancers regions) as defined in the FANTOM5 enhancer-robust promoter associations. Briefly, using Pearson's correlation, enhancers having and FDR < 1e-5 in an enhancer-promoter pair within 500 kb over cell types and organs were retained. The enhancer-promoter associations are computed between enhancers and all FANTOM robust promoters. Epigenetic clock analysis of CD11B+ cells from the *ex vivo study* was conducted using "epiTOC2" (Epigenetic Timer Of Cancer), which calculates the mitotic age of the cells using the updated total number of divisions per stem cell[80]. This new method approximates age as a weighted estimate based on 163 CpG sites interrogated with EPIC arrays.

RNA sequencing

Library preparations were performed with Kapa mRNA Hyper prep (Roche) following the manufacturer's instructions and sequenced (paired end) with the Illumina Novaseq 6000 Sequencing system with 200 cycles cartridge, to obtain 2*60 million reads 100bases/RNA. Sequencing was performed at the iGenseq platform of the ICM – Paris Brain Institute. For the *in vitro* experiments, we combined Illumina Stranded Total RNA Prep and Ribo-Zero Plus. The quality of raw data was evaluated with FastQC. Sequences were trimmed or removed with Fastp software to retain only good quality paired reads. Star v2.5.3a was used to align reads on the GRCh38 reference genome using default parameters except for the maximum number of multiple alignments allowed for a read which was set to 1. Gene level transcript

quantification was done with rsem 1.2.28. Differential expression analysis was conducted using the quasi-likelihood F-test from the edgeR package. Normalized data were computed using the cpm function. Multiple hypothesis testing was corrected using the False Discovery Rate Benjamini-Hochberg method. Samples from all the *in vitro* experiments were analyzed in a paired manner per individual.

Sequence motif enrichment analysis

Motif enrichment was performed using Hypergeometric Optimization of Motif EnRichment (HOMER) v4.11[81], enabling masking repeats, setting the parameter size to "given" and searching for motifs with a length within the range of 7-14 bp. We used HOCOMOCO v12 CORE as the reference dataset (matrix with threshold level corresponding to a p-value of 0.001)[82].

Transcription factor activity

We used CollecTRI database[41] to infer TF activity, which contains regulons comprising signed TF-target gene interactions. To estimate TF activity at hypermethylated enhancers, we took into account genes with associated overexpression or downregulation (falling in quadrants I and IV, respectively, Fig. 2a). These genes were then interrogated among the targets of each TF of the database. We retained the TFs having at least 5 total targets regulated in a positive or negative manner. Then, we computed the TF activity using the Normalized Enrichment Score (NES) of the Mann-Whitney-Wilcoxon genes set test (MWW-GST)[42]. We applied MWW-GST to each sample normalized data and retained TFs having an FDR < 0.05 for the NES in at least 18 samples and an FDR < 0.05 in the comparison of the activity between groups (Wilcoxon's test). For promoters, we took into account all genes associated with hypermethylation in the IDH^{mut} vs. IDH^{wt} comparison without testing for differential activity between TFs. Heatmaps depicting TF activity at hypermethylated enhancers and promoters were generated using a z-score of the activity.

Primary cultures of human microglia

Primary microglial cultures were prepared using surgical aspirates obtained from patients with either brain tumors or drug-resistant epilepsy, following established surgical protocols. For gliomas, tissue collection involved the removal of both the adjacent "entry cortex" (the tissue surrounding the surgical access point) and the tumor core, whereas in cases of epilepsy surgery, only the normal tissue at the entry point was collected. Upon receipt (averaging 2 hours), tissues were promptly processed following the protocol specified for human tumor samples. After enzymatic digestion and MACS purification, CD11B+ cells were counted and directly seeded in non-coated 12-well plates at a density of 750-1000 K cells/well in DMEM/F12, GlutaMAX medium (Gibco Ref# 10565018) supplemented with N-acetyl-cysteine and insulin [5 µg/mL], apo-Transferrin [100 µg/mL], sodium selenite [100 ng/mL], human TGFβ2 [2 ng/mL], CSF-1 [20 ng/mL], ovine wool cholesterol [1.5 µg/mL], ascorbic acid [75 µM] and penicillin/streptomycin[49]. All reagents were purchased from Sigma and growth factors from Preprotech. Non-permeable D-2HG acid disodium salt (Sigma) was used at a final concentration of 5 mM for all the experiments, with treatments starting at 48 hours culture. Half of the culture medium was renewed every 2-3 days. For the 6 hours LPS challenge experiments, 1% FBS was added to all conditions.

IDH^{mut} and IDH^{wt} cell lines

Human glioma cell lines IDH^{wt} were established by the GlioTEx team (Glioblastoma and Experimental Therapeutics) in the Paris Brain Institute (ICM) laboratory. Cultured cells were maintained at 37 °C, 5% CO2 under neurosphere growth conditions using DMEM/F12 medium (Gibco) supplemented with 1% penicillin/streptomycin, B27 diluted 1:50 (Gibco), EGF (20 ng/mL), and FGF (20 ng/mL) (Preprotech). Human glioma cell lines IDH^{mut} BT88, BT54 were kindly provided by Artee Luchman and Sam Weiss (University of Calgary); BT237, BT138 were kindly provided by Keith Ligon (Dana-Farber Cancer Institute). Cells were grown using DMEM/F12 medium (Gibco) supplemented with B27 (1/100; Gibco), 20ng/ml EGF and FGF (Peprotech). IDH^{wt} and IDH^{mut} cell lines were used as negative and positive controls, respectively, in assays applied to quantify intracellular D-2HG levels. The Idh1 R132H

conditional knock-in (cKI) mouse line (Bardella et al., 2016) crossed with *Rosa26*$^{LSL\text{-}YFP}$ (Srinivas et al., 2001) was used to generate postnatal (P0-P1) neural progenitor cells. Cells were grown in DMEM/F-12 with GlutaMAX™ Supplement (61965-026, ThermoFisher), Penicillin-Streptomycin 1X (15140122, Life Technologies), B27 supplement 1X (17502048, ThermoFisher), N2 supplement 1X (17502048, ThermoFisher), 0.6% w/v D-glucose (G8769, Sigma), 5mM HEPES (15630056, Life Technologies) and 20µg/ mL insulin (I5500, Sigma). Growth factors (20 ng/ mL EGF GMP100-15, Peprotech, and 10 ng/ mL bFGF GMP100-18B, Peprotech) were added to the cultures and replaced every 2-3 days. Cells were maintained in a humidified incubator at 37 °C with 5% CO2. Cultures were infected with a Cre expressing adenovirus (Ad:cre, VB180308-1398jmt, VectorBuilder) after 1 passage, enabling recombination at the Idh1 and Rosa26LSL-YFP locus leading to expression of Idh1 R132H and YFP. Efficient DNA recombination was assessed through YFP visualization in NSC. Cells were cultured for 3-6 passages and assayed for D-2HG levels using a fluorimetric test, TET enzymatic activity, and 5mC/5hmC genomic content using LC-MS/MS.

Immunofluorescence staining

Sterile coverslips were placed in 12 multiple wells and the surface was covered with poly-D-lysine and laminin (Sigma). The plate was incubated at room temperature (RT) for 2 hours and the solution was aspirated; then, it was rinsed with sterile water and allowed to dry. Cells were seeded at a density of 750K cells/well and maintained as described for 3, 10 and 17 days. Cells were washed with PBS (Gibco) and fixed with 4% paraformaldehyde (Electron Microscopy Sciences) in PBS for 10 min at RT. Next, cells were washed with PBS and permeabilized with 0,1% Triton X-100 in 1x PBS for 5 min at RT. Cells were quickly washed with PBS and blocked for non-specific binding sites with Human Fc Block (#564219, BD Pharmingen) for 10 min at RT, and subsequently with 5% donkey in PBS for 30-60 min at RT. Incubation with primary antibodies was overnight at 4°C using anti-Iba1 (#19-19741, Wako; diluted 1/800), Tmem119 (#A16075D, BioLegend; diluted 1/100), or Ki-67 (#AB16667-1001, Abcam; diluted 1/250) in a humidified and light protected chamber. Coverslips were washed with PBS and incubated with secondary antibodies (anti-rabbit #A21206 or anti-mouse

#A21203; diluted 1/1000) in blocking solution for 30 min at RT and protected from light. Subsequently, the coverslips were washed with PBS and mounted with Fluoroshield-DAPI solution (Sigma) and read under an Apotome inverted microscope (ZEISS Apotome 3) and a confocal microscope (A1R HD25 Nikon Inverted). Negative controls (background fluorescence) were obtained omitting primary antibodies. The percentages of TMEM119+ and IBA1+ cells were determined in primary cultures grown in uncoated wells by counting 3 to 5 fields, covering 0.22% of the well surface, at different time points over a two-week period. The fields were randomly selected in the area where the cell density, as assessed by DAPI, was adequate.

Intracellular quantifications of D-2HG

Cultured cells were pelleted by centrifugation to remove the media, followed by two washes with ice-cold DPBS to ensure complete removal of all D-2HG-containing media. Microglial cells were collected by trypsinization (0.25% for 5 min), whereas glioma cells and neurospheres were collected using accutase. Corresponding culture medium supplemented with 10% FBS was added for inactivation. After centrifugation (300 g at 4°C for 5 min) the resulting pellet was washed with cold DPBS. Cell pellets were then preserved at -80°C until used. The concentration of D-2HG in cell lysates was determined using the D-2-Hydroxyglutarate (D-2HG) Assay Kit (Ref MAK320, Sigma) and with LC/MS for validation. For the fluorometric assay, cellular extracts were prepared by adding 75 µl of CelLytic MT Cell Lysis Reagent (Sigma) with Protease Inhibitor Cocktail (Sigma). An aliquot was kept for protein quantification with the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). The collected supernatants were deproteinized by perchloric acid precipitation and neutralized with KOH. A standard curve was prepared with serial dilutions of D-2HG. The equivalent of 5000 pmol was added to a cell extract and used as spike-in internal control. The plate is incubated at 37°C for 30–60 minutes. This assay is based on the oxidation of D-2HG to α-ketoglutarate (αKG) by the enzyme (D)-2-hydroxyglutarate dehydrogenase (HGDH), coupled with the reduction of NAD+ to NADH[83]. The amount of NADH formed is then quantitated by the diaphorase-mediated reduction of resazurin to the fluorescent dye resorufin (λex =

540 nm/λem = 590 nm) using Spectramax i3x microplate reader. The concentration of D-2HG for each sample is estimated as pmol/µg of protein. For the quantification of 2HG using LC-MS, dry cell pellets were lysed in water and the solution was divided in half for 2-HG measurement and protein quantification. NaCl was added and samples were acidified using HCl. A liquid-liquid extraction of organic acids with ethylacetate was performed. Three extractions were performed, organic phases were pooled, dried under nitrogen stream at 30 °C. Then samples were derivated by a standard silylaltion protocol N,O-Bis [trimethylsilyl] trifluoroacetamide and 1% TMCS (Trimethylchlorosilane) under anhydrous conditions using pyridine. Chromatographic separation was performed with a TR-5MS (30m x 0.25 mm x 0.25 mm) column from Thermo Scientific (Waltham, Massachusets, USA). Spectral data acquisitions were performed using XCalibur software (Thermo Electron Corporation, Austin, TX, USA). Sample were placed 30 min at 80°C, and then injected into the GC system. Quantifications were performed using internal standard 2-hydroxyglutaric acid-D3 from Cambridge isotope laboratories (Tewksbury, Massachusetts, USA).

LC-MS/MS assessment of genomic 5mC and 5hmC content

Genomic DNA (gDNA) was isolated as indicated and concentrated by ethanol precipitation. Samples were then digested at 37°C for 12 h in 25 µL reactions containing 1-4 µg of gDNA, 7.5 U of DNA Degradase Plus (Zymo Research), reaction buffer and isotope labeled internal standards [700 pmol 2'-deoxycytidine-15N3 (Cambridge Isotope Laboratories, Inc.); 1.75 pmol 5-methyl-2′-deoxy cytidine-d3 (Toronto Research Chemicals); 1 pmol 5-hydroxymethyl-2′-deoxycytidine-d3 (Toronto Research Chemicals)] for quantification. Prior to LC/MS analysis, samples were diluted 1:1 in mobile phase component A (see below). The LC-MS system consisted of a Shimadzu Nexera UPLC system in-line with a SCIEX 6500 QTrap mass spectrometer equipped with a TurboV Ion spray source and operated in positive ion mode. The analytes were chromatographed on a gradient elution system where mobile phase A was water with 10 mM ammonium formate and 0.05% trifluoroacetic acid (TFA) (Sigma Aldrich); and mobile phase B was 7:1 methanol:acetonitrile (ThermoFisher Scientific), with 10 mM ammonium formate and 0.05% TFA. The column was a Phenomenex Curosil-

PFP column (10 x 0.2 cm 1.7 um) (Phenomenex Inc) held at 42°C. The flow rate was 300 µL/min and the gradient started at % B = 1.0% (held for 30 s) and was then increased to 19% over 5 min. All analytes were detected using selected reaction monitoring (SRM). Analyst software (version 1.6.2) was used to acquire and process the data. Supplementary Table 6 gives the Q1 and Q3 m/z values, collision energy (CE) and declustering potential (DP) for all analytes and their internal standards. Analytes were quantified by stable isotope dilution against their stably labeled internal standard. Data represents a percentage for each analyte relative to the total cytosine pool (dC+5mC+5hmC) in the same sample.

Reduced Representation Enzymatic Methyl sequencing (RREM-seq)

Library preparation was performed using 100 ng of genomic DNA is spiked with 0.95 ng of unmethylated Lambda DNA and 0.05 ng of CpG methylated pUC19. The mixture is digested with 400 units of MspI (New England Bioloabs) in NEB ultra II buffer (New England Biolabs) in a final volume of 57 µL for 2 hours at 37°C. After enzymatic DNA digestion, fragments are repaired and A-tailed using 15 units of Klenow 3'-5' exo- (New England Biolabs). After End-repair and A-tailing, Illumina universal methylated adapters are ligated to DNA fragments using the NEB ultra II ligation module, following the provider's recommendations. After ligation step, DNA fragments are purified and size-selected with a two steps SPRI beads purification in order to select fragments from 40bp to 400bp. Clean-up ligated DNA is eluted in 10 mM Tris pH 8. Purification product is then converted using the Enzymatic methyl-seq module (New England Biolabs) following the provider's recommendations. Briefly, 5mC are converted to 5hmC with TET2, then 5hmC are specifically protected from APOBEC deamination with a T4-BGT glycosylation. After 5hmC/5mC glycosylation, libraries are denatured during 10min at 85°C in 20% Formamide. Then unmodified cytosines are de-aminated during APOBEC reaction. The converted product is amplified by PCR using indexing primers and polymerase KAPA HiFi Hotstart U+ Ready Mix 2X in a final volume of 50 µL for 13 cycles. PCR products are finally purified with a 1.2X SPRI ratio, then controlled by capillary electrophoresis on Fragment Analyzer. After quantification by qPCR libraries are sequenced on an Illumina Novaseq 6000 as paired-end 100b reads.

Reduced Representation hydroxyMethyl sequencing (RREhM-seq)

We used the RREhM-seq protocol published by Sun, Zhiyi et al[52]. with slight modifications. Library preparation was performed using an input of 200 ng with a spike of 1.9 ng of unmethylated Lambda DNA and 0.1 ng of CpG methylated pUC19. Following MspI digestion and reparation with Klenow 3'-5' exo-, pyrrolo-dC Illumina adapters are used instead of 5mC adapters during the ligation step. For the TET2 step during the conversion, TET2 enzyme and Fe2+ are replaced by H_2O to avoid 5mC protection. The product is deaminated, amplified, qualified and sequenced in the same conditions as RREM-seq libraries. RREM-seq and RREhM-seq were performed by Integragen SA (Evry, France).

RREM-seq & RREhM-seq data analysis

FastQ files were aligned using BSseeker2 (https://github.com/BSSeeker/BSseeker2) on hg19 genome with bowtie2. The hg19 index was built with the rrbs option on and selected fragments size between 40bp and 400 bp. The adapters sequence and CCGG motif were removed during the alignment steps. After alignment, methylation calling is performed in order to obtain methylation level and covering for each cytosine positions from the library. Options to remove overlap and bad quality sequences were enabled. For RREM-seq libraries, methylation signal corresponds to 5mC and 5hmC, whereas the methylation signal only concerns 5hmC for RREhM-seq libraries[84]. Levels of total 5mC (methylation and hydroxymethylation) or 5hmC were defined as percentage of the number of reads having the corresponding modification on a specific cytosine divided by the number of reads covering that cytosine (modified or not modified). Methylation-only level was obtained subtracting 5hmC level from the total 5mC level. CpG sites were analyzed with methylKit package[85] using a minimum coverage of 10 reads per base. For the defined window analysis, TFs motifs were retrieved from HOCOMOCO v12 CORE and searched inside entire enhancers regions using Biostrings package[86] with a maximum mismatch of 1 base. The region of interest was defined as a window of 600 bp centered around the first base of the motif. For each patient, only common enhancers windows between treated and control samples were analyzed. Microglia samples used for analyses of 5mC and 5hmC at 2 days (2D) and 14 days

(14D) were obtained from the aspirates of normal tissue from three patients with epilepsy who underwent surgery. One technical outlier from the 5hmC analysis at 14D was excluded from the study. 5mC-only and 5hmC levels are reported as the mean across patients for each time point/condition, using only the common enhancer regions or defined windows shared among patients.

TET enzymatic assay

Nuclei isolation was performed using the nuclear extraction kit (cat. Ab113474, Abcam, Cambridge, MA, USA). Cells were collected by trypsinization (0.25% for 5 min) and counted. Then, cells were resuspended in an appropriate volume of pre-extraction buffer containing dithiothreitol and protease inhibitor cocktail, and incubated on ice for 2 minutes. After centrifugation at 12,000 rpm for 3 minutes, the cytoplasmic extract (supernatant) was carefully removed, leaving behind the nuclear pellet. Two volumes of extraction buffer containing dithiothreitol and protease inhibitor cocktail were added to the nuclear pellet. The extract was incubated on ice for 15 minutes with vortexing for 5 seconds every 3 minutes. After incubation, the suspension was centrifuged for 10 minutes at 14,000 rpm at 4°C, and the resulting supernatant was transferred to a new microcentrifuge vial. Protein quantification from the nuclear extract was performed using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Technical triplicates for this assay were performed using 12 µg of protein from nuclear extracts of microglial cells (untreated and treated with D-2HG), as well as from external controls (neurospheres obtained from the cKI Idh R132H mouse model). To determine TET enzymatic activity, we employed the TET Hydroxylase Activity kit from Abcam (ab156913). In this fluorimetric assay, a methylated substrate is stably coated onto microplate wells. Active TET enzymes bind to the substrate and convert the methylated substrate into hydroxymethylated products. The hydroxymethylated products generated by TET activity can be recognized with a specific antibody. The ratio or amount of hydroxymethylated products, which is proportional to the enzyme activity, was measured fluorometrically using a fluorescent microplate reader with

excitation at 530 nm and emission at 590 nm. The TET enzyme activity is proportional to the relative fluorescent units measured.

RT-qPCR

Total RNA from cultured microglia was purified using the Maxwell RSC simplyRNA Cells Kit (Promega). Next, 300 ng of RNA was reverse transcribed to complementary DNA using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Assays were run in triplicate on a Light Cycler 480 instrument (Roche) using the LightCycler 480 SYBR Green Master 2X (Roche). Primer sequences were as follow: TNFA, forward: 5'-GAGCCAGCTCCCTCTATTTA-3', reverse: 5'-GGGAACAGCCTATTGTTCAG-3'; IL-6, forward: 5'-CCTTCCAAAGATGGCTGAAA-3', reverse: 5'-TGGCTTGTTCCTCACTACT-3'; PPIA, forward: 5'-ATGCTGGACCCAACACAAAT-3', reverse: 5'-TCTTTCACTTTGCCAAACACC-3'; ACTB, forward: 5'-CCAACCGCGAGAAGATGA-3', reverse: 5'- CCAGAGGCGTACAGGGATAG-3'. Linearity and efficiency, as well as specific amplifications were verified for all primer sets. Relative quantifications of cytokine expression were normalized to the mean of the two control genes (PPIA and ACTB) and expressed as fold change[87].

Mitochondrial respiration measurement

Real-time measurement of oxygen consumption rate (OCR) was performed using XFe extracellular flux analyzer (Seahorse Bioscience, Billerica, MA) following the manufacturer's instructions. Primary isolated microglia (as detailed in materials and methods) were plated at a density of 1,000,000 cells/well in XFe24 cell culture microplates pre-coated with poly-L-lysine (Sigma). Cells were maintained in 200 µl DMEM/F-12 medium supplemented as previously indicated and treated with 5 mM D-2HG for a period of 12 days. Half of the culture medium was renewed every 2-3 days. The day of the experiment, cells were stimulated with LPS (1µg/mL) for 6 hours. FBS 1% was also added to all experimental conditions. Next, the medium was replaced by Seahorse XF base medium (Agilent, 102353-100) supplemented with 17.5 mM D-Glucose (Dextrose), 0.5 mM sodium pyruvate and 2.5 mM L-Glutamine and incubated for 1 h in CO2 free incubator at 37 °C. The utility plate was hydrated with XF calibrant solution (pH 7.4) (Agilent, 100840-000; 1 mL/well) and incubated overnight (37°C,

CO2-free). The day after, the utility plate with the cartridge containing injector ports and sensors was run on the Seahorse for calibration. The assay medium (Seahorse XF DMEM assay medium, pH 7.4) was prepared immediately before assay. OCR was measured under basal conditions and after sequential injection of the following inhibitors: Oligomycin (1 µM) to stop ATP-synthase, FCCP (1 µM) to dissipate proton gradient, and Rotenone/Antimycin A (0.5 µM each to block electron transport chain (ETC). All the following measurements were carried out with 2 min mix, 2 min delay, and 3 min measure. Three baseline OCR measurements were recorded, followed by assessment of mitochondrial metabolism by injection of oligomycin, FCCP, and a combination of rotenone and antimycin A. The following parameters were deduced: basal respiration (OCR values, used to provide ATP under baseline conditions), ATP-linked respiration (following oligomycin injection, a reduction in OCR values represents the part of basal respiration used to produce ATP), and maximal respiration (the maximal OCR values following FCCP injection). Basal respiration was calculated from the subtraction between the last rate measurement before oligomycin injection and the non-mitochondrial respiration rate (NMRT) determined as the minimum rate measurement after rotenone/antimycin A injection. Maximal respiration was calculated by subtracting the maximum measurement after FCCP injection and the NMRT. ATP-linked respiration was calculated by subtracting the last rate measurement before oligomycin injection and the minimum rate measurement after oligomycin injection. OCR data were normalized by the number of viable cells estimated at the end of the experiment. To do this, the cells were fixed with paraformaldehyde 4%, followed by washes with PBS and DAPI [10 µg/mL] staining. Nuclei were automatically counted in a CellInsight NXT platform.

Single-nucleus RNA sequencing analysis

We downloaded raw data of oligodendroglioma (patient BWH445) from Spitzer et al. study on IDHi[55] GSE260928 and used Seurat (version 5.0.3)[88] to perform data analysis. First, we performed data quality control excluding cells having less than 1500 or more than 3400 genes expressed and less than 2200 or more than 12000 unique molecular identifiers. These stringent thresholds allowed us to have high-quality nuclei also avoiding doublets.

Subsequently, we normalized the entire raw gene count matrix by regularized negative binomial regression using the sctransform function version 2 and clustering cells using 30 principal components. We selected the microglia cluster based on the expression of TAL1, P2RY12, SLC2A5 and the absence of the infiltrating myeloid marker CD163. Selected microglial cells were then analyzed using the raw expression matrix and normalized with the sctransform function version 2 and clustered using 20 principal components. Differential expression analysis was performed using the function FindMarkers.

Statistical analysis and plots generation

Sample sizes and statistical tests are provided in the figure legends. Analyses were performed with R. Normal distribution of data was verified with Shapiro-Wilk's normality test (p-value > 0.05). Plots and analyses were generated using ggplot2[89], the extension ggsignif[90], ComplexHeatmap[91], Cytoscape[92], clusterProfiler[93] and eulerr[94]. Hallmark gene set collection (v2023.1.Hs) for GSEA analyses[95] was downloaded from the Molecular Signatures Database (MSigDB)[96].

Data and code availability

Data and code are available upon request.

Supplementary Tables

Supplementary Table 1: Clinical, pathological and genetic features of the study cohort.

Supplementary Table 2: Genes regulated by hypermethylated enhancers and promoters in CD11B+ cells from IDH^{mut} gliomas.

Supplementary Table 3: Differentially expressed genes in CD11B+ cells from IDH^{mut} vs. IDH^{wt} gliomas.

Supplementary Table 4: Genes regulated by hypermethylated enhancers and/or promoters linked to downregulated expression in CD11B+ cells from IDH^{mut} vs. IDH^{wt} gliomas.

Supplementary Table 5: Genes regulated by the selected enhancers bound by TFs of Fig 5c exhibiting hypermethylation/hypohydroxymethylation in D-2HG-treated microglia.

Supplementary Table 6: Analyte and internal standard Q1 and Q3 *m/z* values, collision energy (CE), and declustering potential (DP) for LC-MS/MS quantifications.

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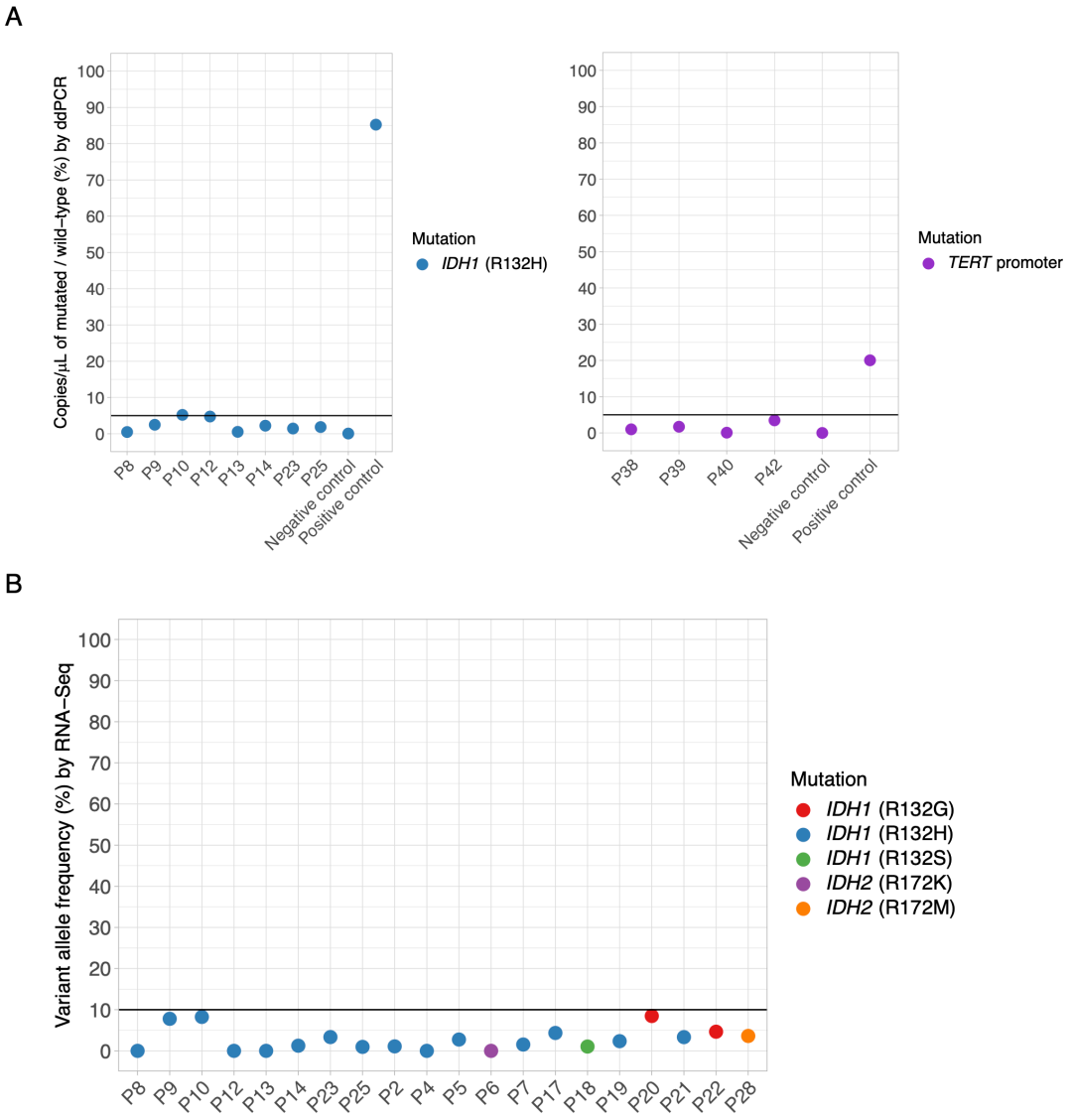
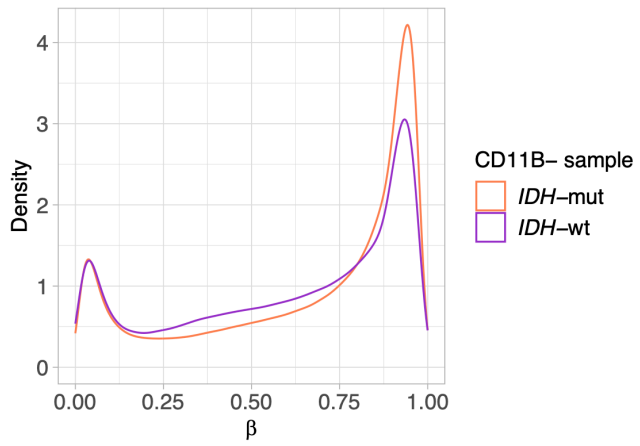
Extended data



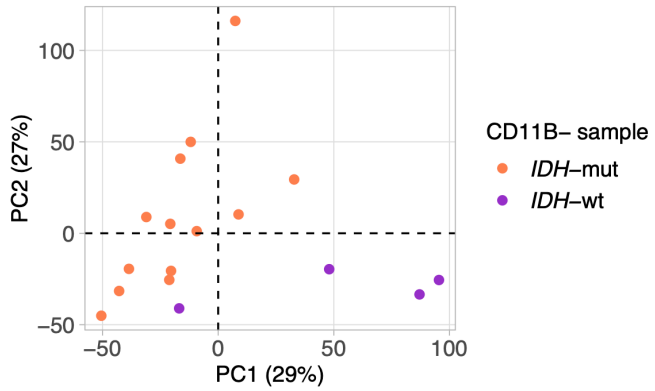
Extended Data Fig. 1. Purity of CD11B+ myeloid fractions isolated from human gliomas. (A) Digital droplet PCR (ddPCR) performed with specific assays to evaluate IDH1 R132H, TERT promoter mutations C228T/C250T, and corresponding wildtype alleles in available DNAs from CD11B+ samples (n=12) isolated from human gliomas with known

mutation status. Less than 5% copies/µL of the indicated mutations relative to their corresponding wildtype alleles were detected in the same reaction for each analyzed sample. Positive and negative controls from bulk tumors for each assay are also shown. The detection of IDHmut and TERT mutations in positive controls differs, possibly reflecting regional clonal heterogeneity. **(B)** Expression frequency of IDH mutated alleles was assessed in RNA-seq data from CD11B+ samples (n=20) isolated from human gliomas with known mutation status using bam-readcount[97]. Compared to ddPCR, this analysis slightly overestimated the contamination in samples P9 and P10.

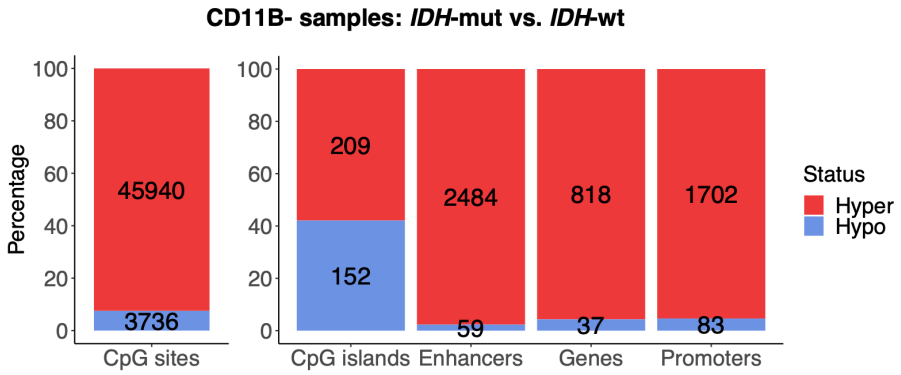
A


Density
β
CD11B– sample
IDH–mut
IDH–wt

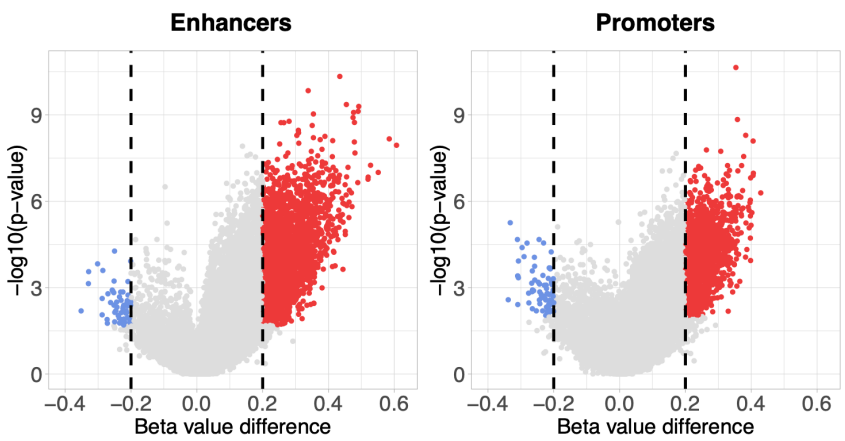
B


PC2 (27%)
PC1 (29%)
CD11B– sample
IDH–mut
IDH–wt

C

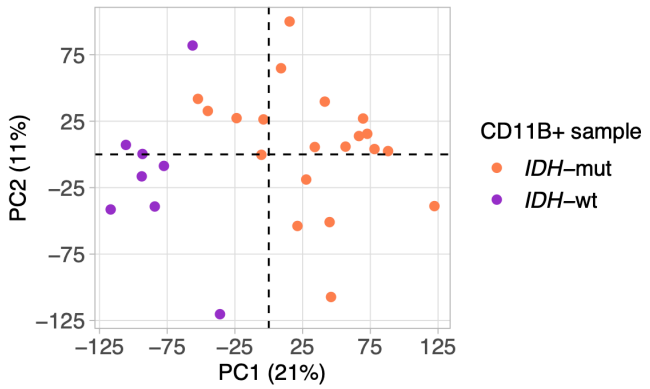

CD11B- samples: IDH-mut vs. IDH-wt
Percentage
45940
3736
209
152
2484
59
818
37
1702
83
CpG sites
CpG islands
Enhancers
Genes
Promoters
Status
Hyper
Hypo

D

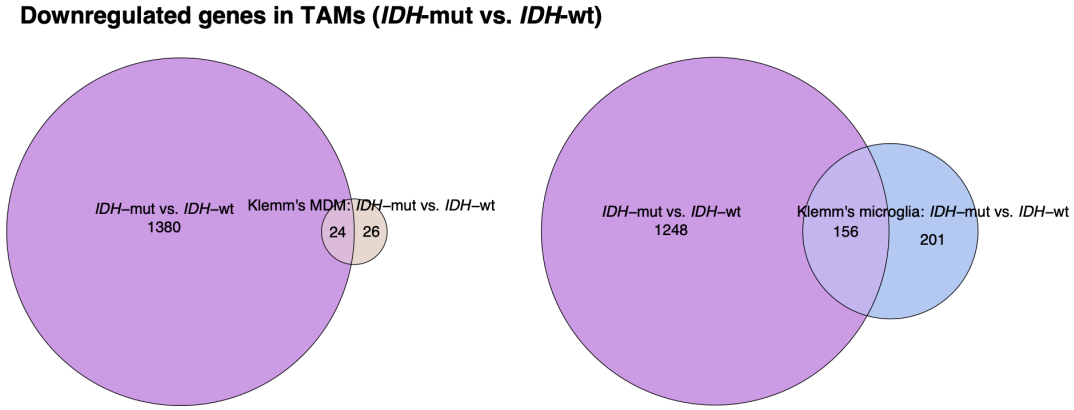

Enhancers
Promoters
−log10(p−value)
Beta value difference

Extended Data Fig. 2. CD11B- fractions enriched of glioma cells display global DNA hypermethylation in IDH^{mut} gliomas. (A) Density plot of DNA methylation levels (β-values) in glioma cells-enriched CD11B- fractions isolated from IDH^{mut} (n=13) and IDH^{wt} (n=4) tumors. **(B)** Principal component analysis performed using methylome data from around 600,000 CpG sites across the genome in CD11B- samples from the study cohort. **(C)** Stacked bar charts show absolute numbers and relative percentages of CpG sites and of four functional genomic regions respect to the methylation status in the CD11B- samples from IDH^{mut} vs. IDH^{wt} tumors. **(D)** Volcano plots depict the magnitude and extent of differentially methylated changes (colored red and blue) located at enhancers and promoters in CD11B- samples from IDH^{mut} vs. IDH^{wt} tumors.

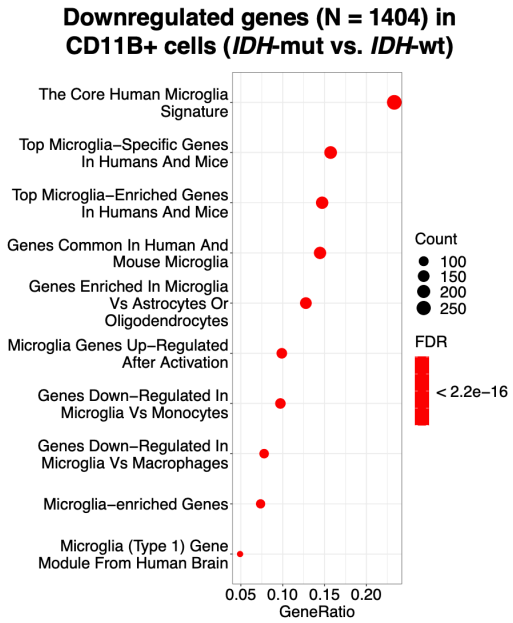
A


75
25
−25
−75
−125
PC2 (11%)
−125
−75
−25
25
75
125
PC1 (21%)
CD11B+ sample
IDH–mut
IDH–wt

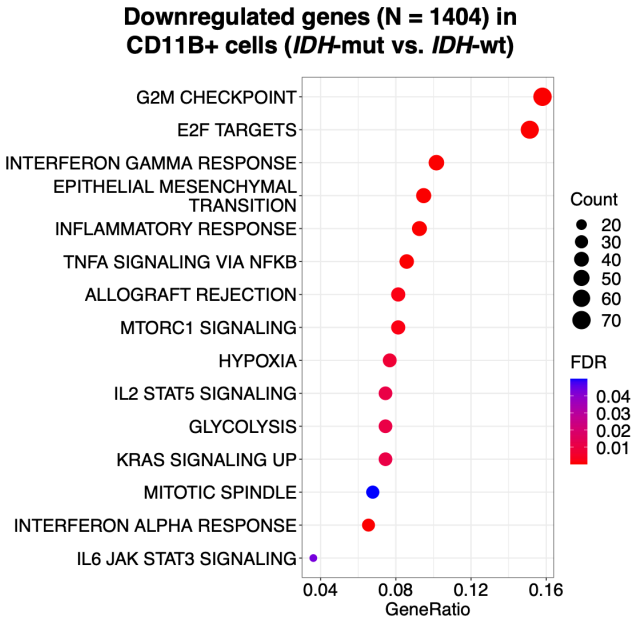
B


Downregulated genes in TAMs (IDH-mut vs. IDH-wt)
IDH–mut vs. IDH–wt
1380
Klemm's MDM: IDH–mut vs. IDH–wt
24
26
IDH–mut vs. IDH–wt
1248
Klemm's microglia: IDH–mut vs. IDH–wt
156
201

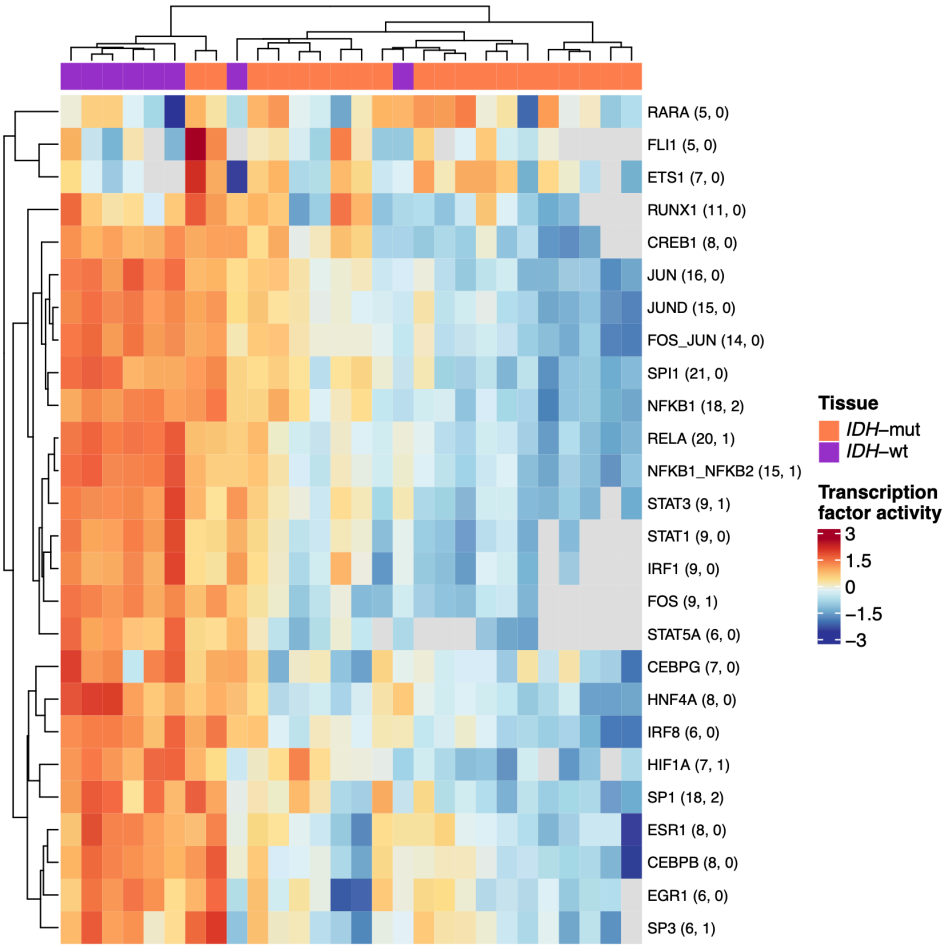
C


Downregulated genes (N = 1404) in CD11B+ cells (IDH-mut vs. IDH-wt)
The Core Human Microglia Signature
Top Microglia–Specific Genes In Humans And Mice
Top Microglia–Enriched Genes In Humans And Mice
Genes Common In Human And Mouse Microglia
Genes Enriched In Microglia Vs Astrocytes Or Oligodendrocytes
Microglia Genes Up–Regulated After Activation
Genes Down–Regulated In Microglia Vs Monocytes
Genes Down–Regulated In Microglia Vs Macrophages
Microglia–enriched Genes
Microglia (Type 1) Gene Module From Human Brain
0.05
0.10
0.15
0.20
GeneRatio
Count
100
150
200
250
FDR
< 2.2e−16

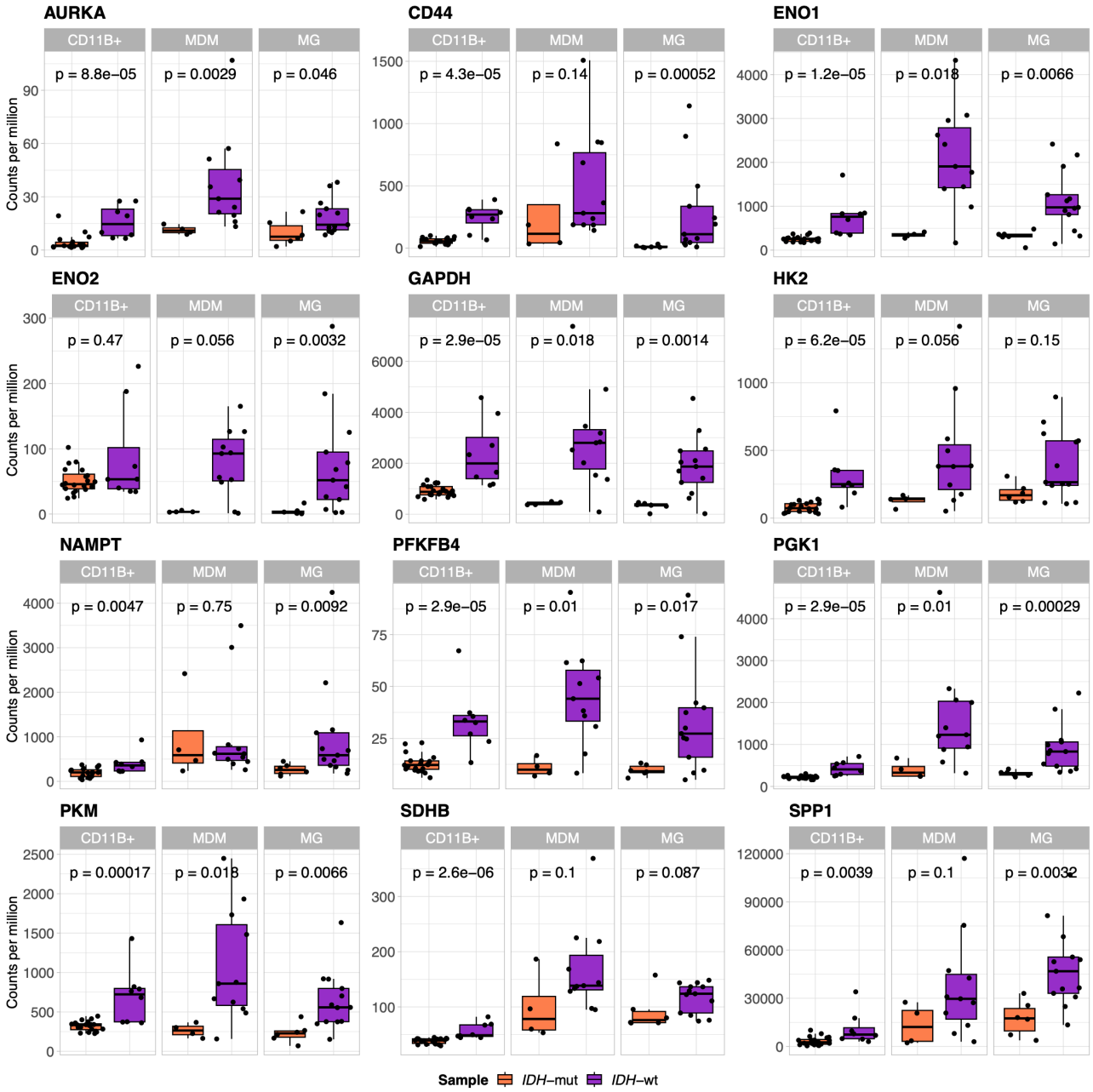
D


Downregulated genes (N = 1404) in CD11B+ cells (IDH-mut vs. IDH-wt)
G2M CHECKPOINT
E2F TARGETS
INTERFERON GAMMA RESPONSE
EPITHELIAL MESENCHYMAL TRANSITION
INFLAMMATORY RESPONSE
TNFA SIGNALING VIA NFKB
ALLOGRAFT REJECTION
MTORC1 SIGNALING
HYPOXIA
IL2 STAT5 SIGNALING
GLYCOLYSIS
KRAS SIGNALING UP
MITOTIC SPINDLE
INTERFERON ALPHA RESPONSE
IL6 JAK STAT3 SIGNALING
0.04
0.08
0.12
0.16
GeneRatio
Count
20
30
40
50
60
70
FDR
0.04
0.03
0.02
0.01

Extended Data Fig. 3. Genes with reduced expression in CD11B+ cells from IDH^{mut} human gliomas belong to microglial signatures. (A) Principal component analysis performed using RNA-seq data from *ex vivo* CD11B+ myeloid cells isolated from IDH^{mut} (n=20) and IDH^{wt} (n=8) tumors. **(B)** Venn diagrams illustrate significant overlaps between downregulated genes displayed by CD11B+ cells fraction from IDH^{mut} gliomas (this study), and downregulated genes displayed by MDMs (left) or microglial cells (right) in Klemm et al. study. P-values were computed using overrepresentation analysis (ORA). **(C)** Enrichment analysis of the downregulated genes in CD11B+ cells fraction from IDH^{mut} gliomas in the present study using HDinHD microglia signatures dataset[98]. **(D)** Enrichment analysis of the downregulated genes in CD11B+ cells fraction from IDH^{mut} gliomas (this study) using hallmark signatures dataset.

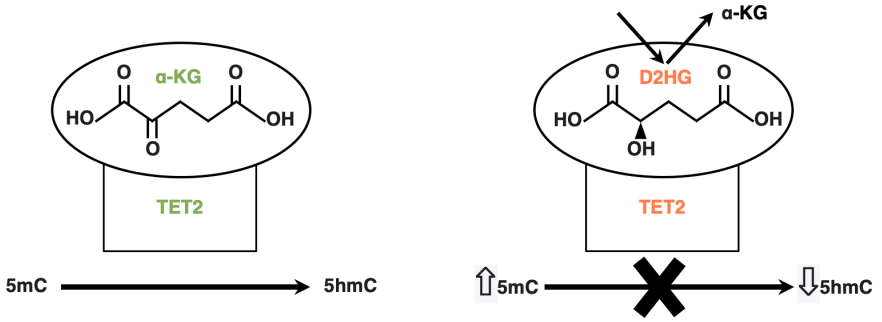


Extended Data Fig. 4. Transcription factors activity controlling genes with hypermethylated promoter in CD11B+ cells from human gliomas. The heatmap depicts TFs (n=26) exhibiting differential activity (FDR <0.05; Wilcoxon's test) in CD11B+ cells according with the IDH mutation status of corresponding tumors. The TF targets used for this analysis, retrieved from the CollecTRI database, were searched among the genes displaying hypermethylation at promoters in CD11B+ cells fraction from IDH^{mut} vs. IDH^{wt} comparison (Fig. 1D). The number of targets regulated positively and negatively by each TF is shown in brackets. Unsupervised clustering was performed using the z-scores of TF activity, where red and blue colors indicate high and low activity, respectively. Non-significant activity is shown in gray.

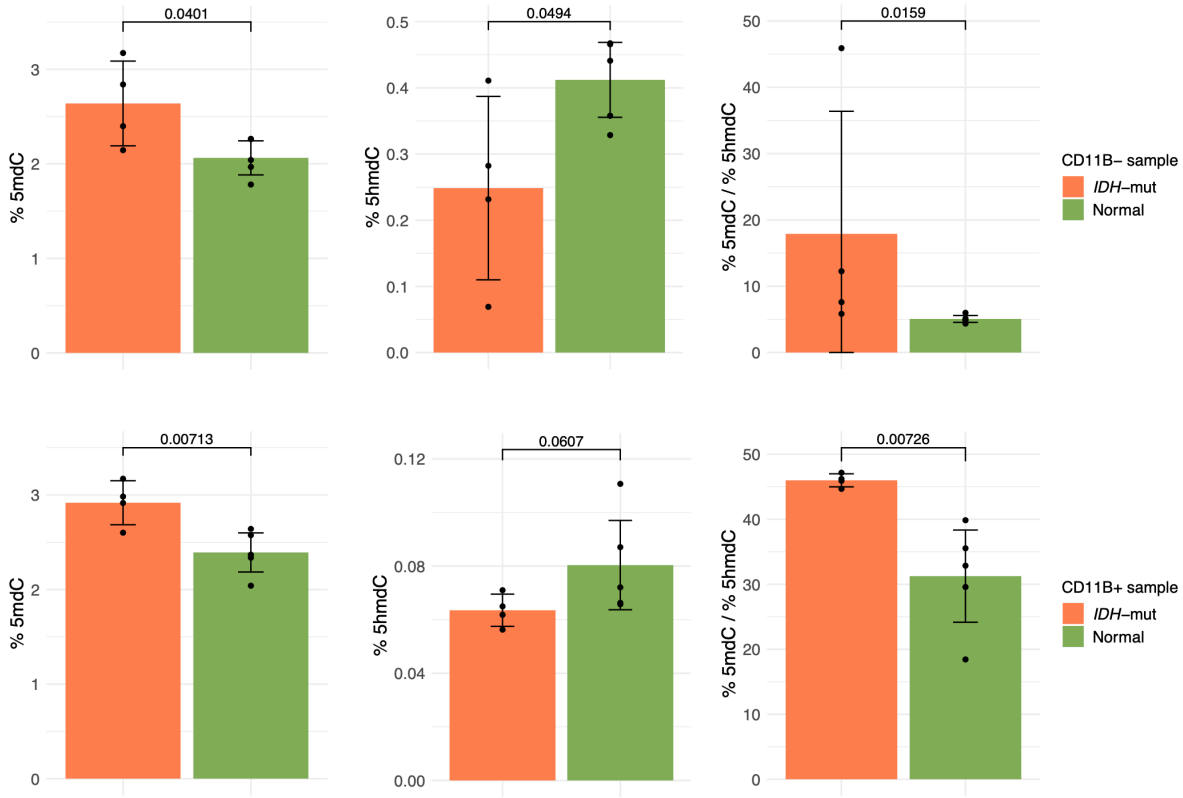


Extended Data Fig. 5. Positive targets of HIF1A/MYC display low expression in microglia and MDMs from IDH^{mut} gliomas. Box plots showing normalized RNA-seq expression data for representative targets of HIF1A/MYC in CD11B+ myeloid cells from the present study cohort (IDH^{mut}, n=20 vs. IDH^{wt}, n=8), as well as in myeloid cells from the Klemm et al. study comprising MDMs (IDH^{mut}, n=4 vs. IDH^{wt}, n=11) and microglia (IDH^{mut}, n=6 vs. IDH^{wt}, n=13) used as validation set. P-values were computed using Wilcoxon's test.

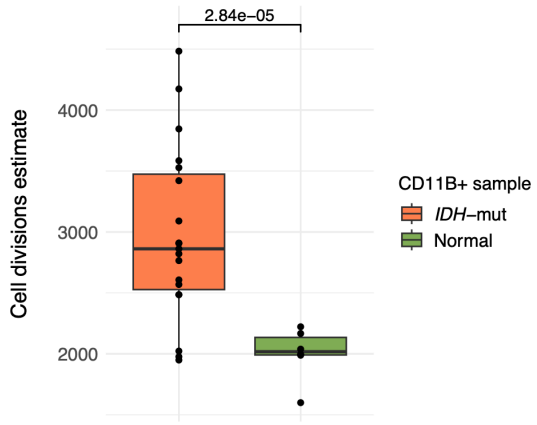
A


α-KG
HO
OH
TET2
5mC
5hmC
α-KG
D2HG
HO
OH
OH
TET2
5mC
5hmC

B

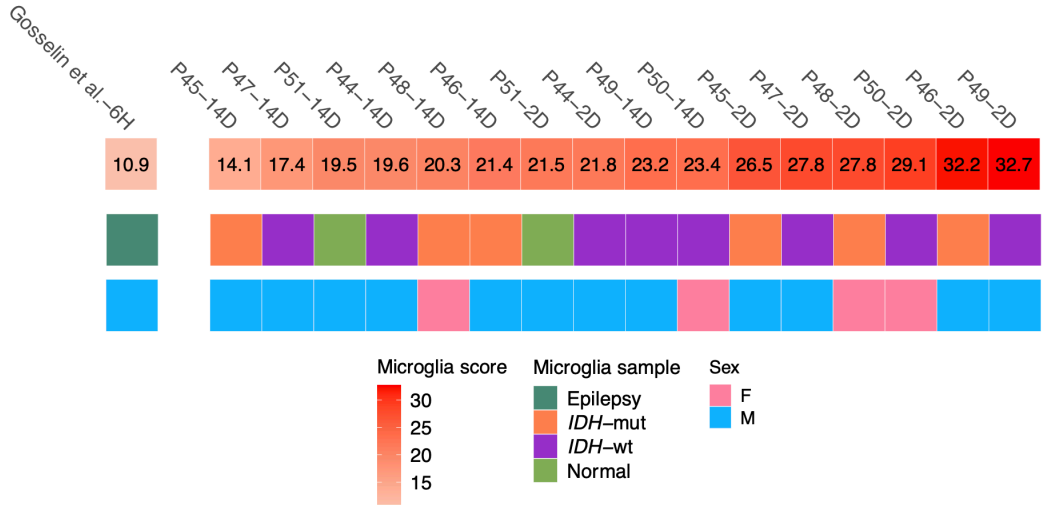

0.0401
% 5mdC
0.0494
% 5hmdC
0.0159
% 5mdC / % 5hmdC
CD11B– sample
IDH–mut
Normal
0.00713
% 5mdC
0.0607
% 5hmdC
0.00726
% 5mdC / % 5hmdC
CD11B+ sample
IDH–mut
Normal

C

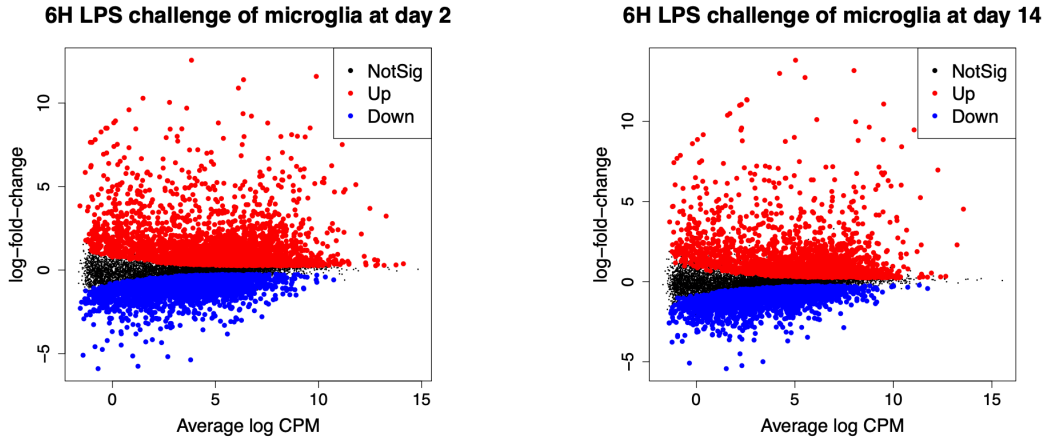

2.84e−05
Cell divisions estimate
CD11B+ sample
IDH–mut
Normal

Extended Data Fig. 6. LC-MS/MS analysis of 5mC and 5hmC genomic content and cell divisions estimate in CD11B+ myeloid cells from IDH^{mut} gliomas and normal brain tissues. **(A)** Schematic representation of α-KG-dependent enzymatic oxidation of 5mC by TET2 and effect of competitive inhibition of TET2 by D-2HG over time in bulk IDH^{mut} gliomas. **(B)** Global levels of 5mC and 5hmC (left) and 5mC/5hmC ratios (right) were measured in CD11B- (upper) and CD11B+ (bottom) fractions from IDH^{mut} gliomas (n=4) and from normal tissues collected during epilepsy surgery (n=5), which served as controls for comparisons. Data are represented as the mean with a 95% CI; p-values were calculated using one-sided Welch's test except for comparison of ratios in CD11B- (one-sided Wilcoxon's test). **(C)** Epigenetic mitotic clock analysis was conducted by applying the epiTOC2[80] model to methylome data (EPIC arrays) of CD11B+ fractions from IDH^{mut} gliomas (n=19) and from normal brain tissues (n=6). The model (adjusted by age) estimates the cumulative number of stem cell divisions in the myeloid compartment. The p-value was calculated using Welch's test.

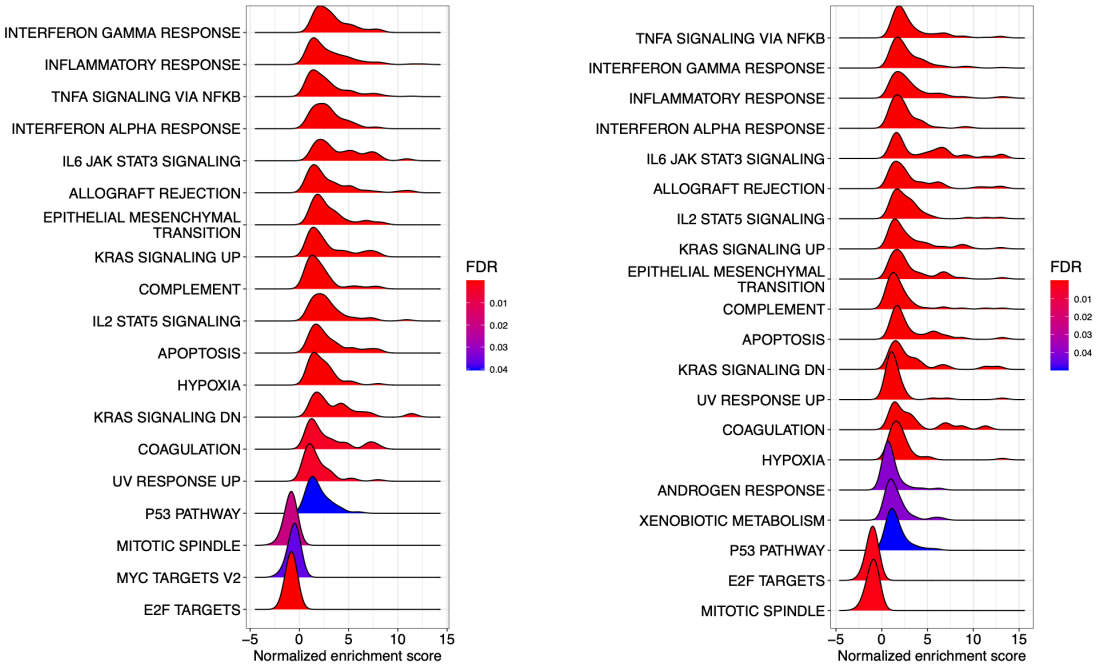
A


Gosselin et al.–6H
P45–14D
P47–14D
P51–14D
P44–14D
P48–14D
P46–14D
P51–2D
P44–2D
P49–14D
P50–14D
P45–2D
P47–2D
P48–2D
P50–2D
P46–2D
P49–2D
10.9
14.1
17.4
19.5
19.6
20.3
21.4
21.5
21.8
23.2
23.4
26.5
27.8
27.8
29.1
32.2
32.7
Microglia score
30
25
20
15
Microglia sample
Epilepsy
IDH–mut
IDH–wt
Normal
Sex
F
M

B

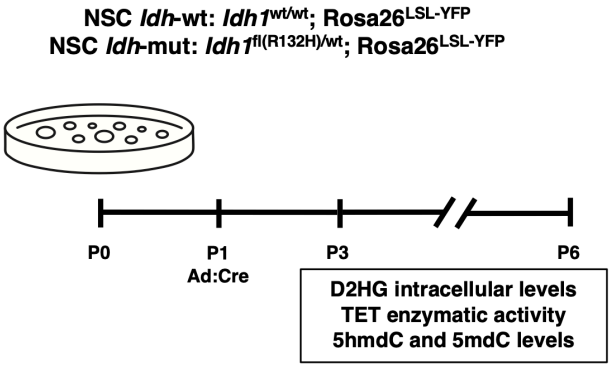

6H LPS challenge of microglia at day 2
6H LPS challenge of microglia at day 14
NotSig
Up
Down
log–fold–change
Average log CPM

C

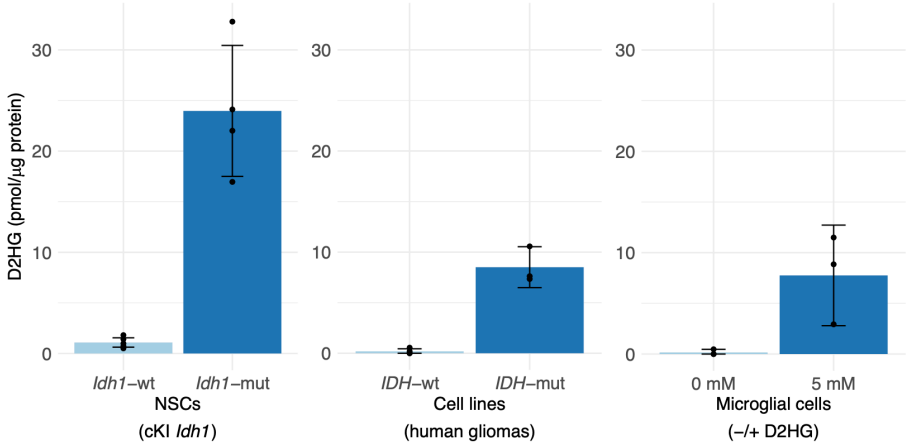

INTERFERON GAMMA RESPONSE
INFLAMMATORY RESPONSE
TNFA SIGNALING VIA NFKB
INTERFERON ALPHA RESPONSE
IL6 JAK STAT3 SIGNALING
ALLOGRAFT REJECTION
EPITHELIAL MESENCHYMAL TRANSITION
KRAS SIGNALING UP
COMPLEMENT
IL2 STAT5 SIGNALING
APOPTOSIS
HYPOXIA
KRAS SIGNALING DN
COAGULATION
UV RESPONSE UP
P53 PATHWAY
MITOTIC SPINDLE
MYC TARGETS V2
E2F TARGETS
FDR
Normalized enrichment score
TNFA SIGNALING VIA NFKB
INTERFERON GAMMA RESPONSE
INFLAMMATORY RESPONSE
INTERFERON ALPHA RESPONSE
IL6 JAK STAT3 SIGNALING
ALLOGRAFT REJECTION
IL2 STAT5 SIGNALING
KRAS SIGNALING UP
EPITHELIAL MESENCHYMAL TRANSITION
COMPLEMENT
APOPTOSIS
KRAS SIGNALING DN
UV RESPONSE UP
COAGULATION
HYPOXIA
ANDROGEN RESPONSE
XENOBIOTIC METABOLISM
P53 PATHWAY
E2F TARGETS
MITOTIC SPINDLE
FDR
Normalized enrichment score

Extended Data Fig. 7. Assessment of the functionality of primary microglial cultures. **(A)** Analysis of a human microglia signature at 2 days (2D) and 14 days (14D) using RNA-seq data from eight independent primary cultures. A microglial score was determined as follows: we took the microglia signature genes and the raw gene expression matrix from Gosselin et al[39]. Subsequently, after normalizing the expression data, we calculated the median of the differences of each gene from the microglia signature in our samples with respect to the five negative samples used as controls in Gosselin et al. study. Microglia score was defined as the average of these five medians. The plot (upper), showing such microglia score per sample, demonstrates that the signature is nearly twice as high, even at 14 days of culture, when using defined culture conditions (as in our study), in comparison to the study by Gosselin et al. study (culture shock at 6 hours), irrespective of tissue origin and sex (as indicated at the bottom). **(B)** The plots illustrate expression changes using four primary microglial cultures analyzed with RNA-seq in a paired manner, 6 hours after LPS stimulation at 2 days (left) and 14 days (right). Red and blue colors indicate upregulation and downregulation, respectively, with FDR <0.05, in LPS-stimulated microglia vs. control. **(C)** Ridge plots illustrate hallmark gene sets GSEA analyses in LPS-stimulated microglia at 2 days (left) and 14 days (right) of culture. Activated (>0) and inactivated (<0) gene sets with FDR < 0.05 are shown.

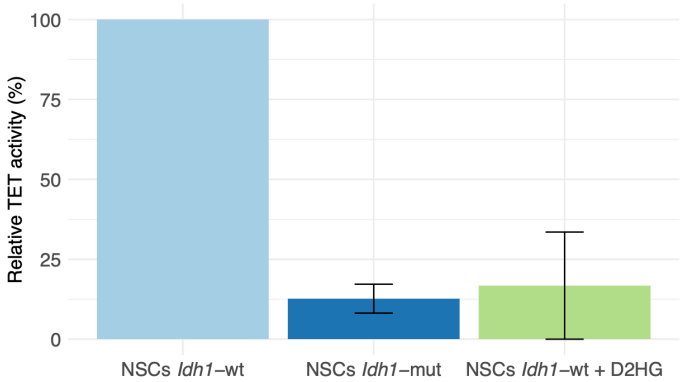
A


NSC *Idh*-wt: *Idh1*$^{wt/wt}$; Rosa26$^{LSL-YFP}$
NSC *Idh*-mut: *Idh1*$^{fl(R132H)/wt}$; Rosa26$^{LSL-YFP}$
P0
P1
Ad:Cre
P3
P6
D2HG intracellular levels
TET enzymatic activity
5hmdC and 5mdC levels

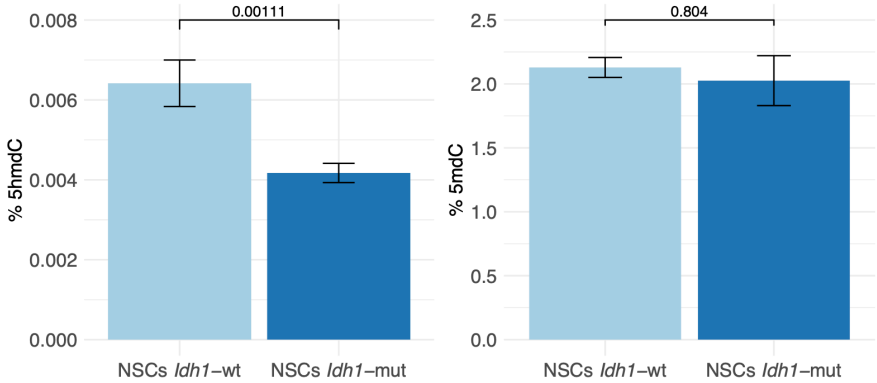
B


D2HG (pmol/µg protein)
30
20
10
0
Idh1–wt
Idh1–mut
NSCs
(cKI *Idh1*)
IDH–wt
IDH–mut
Cell lines
(human gliomas)
0 mM
5 mM
Microglial cells
(–/+ D2HG)

C

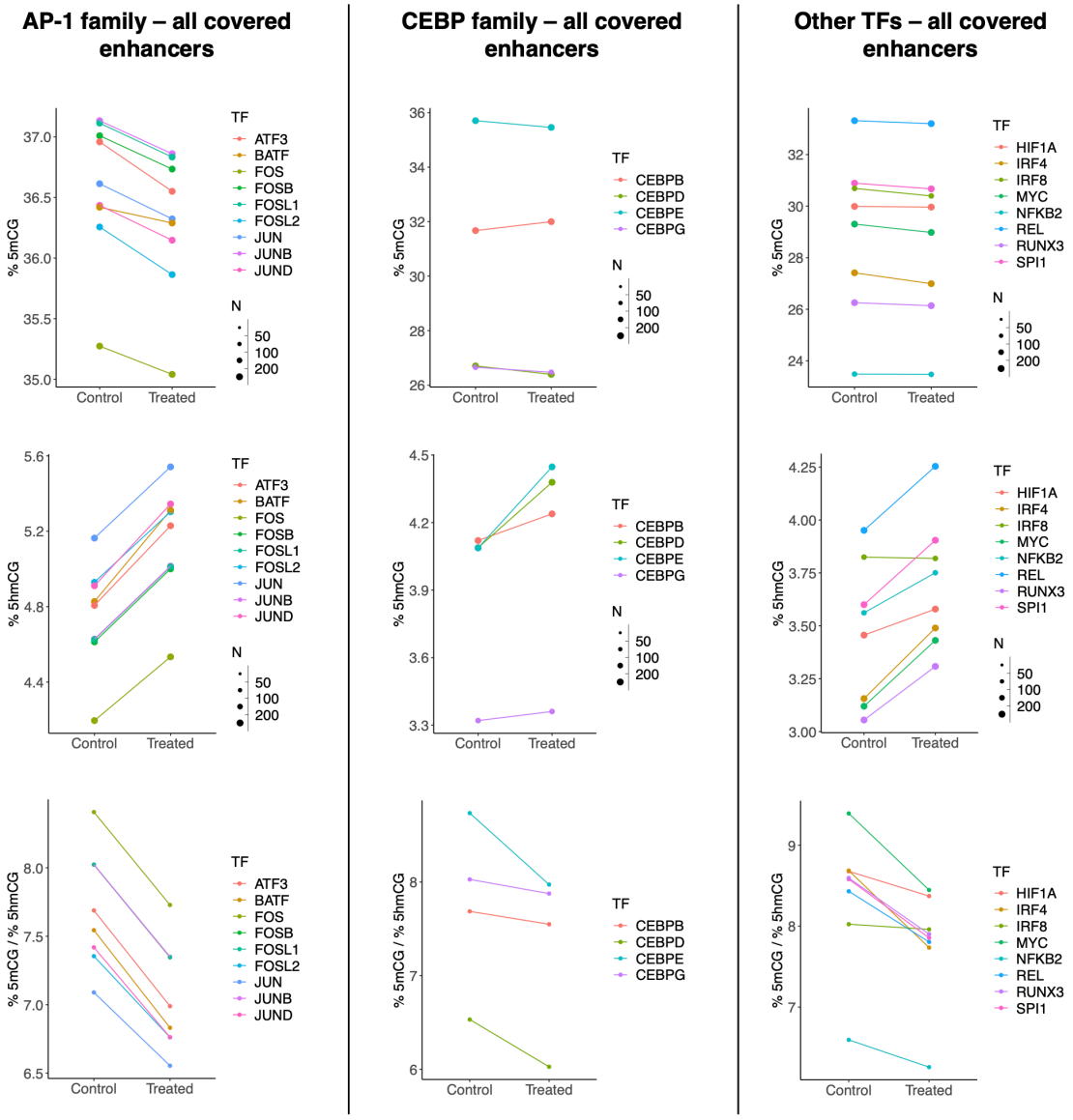

Relative TET activity (%)
100
75
50
25
0
NSCs *Idh1*–wt
NSCs *Idh1*–mut
NSCs *Idh1*–wt + D2HG

D

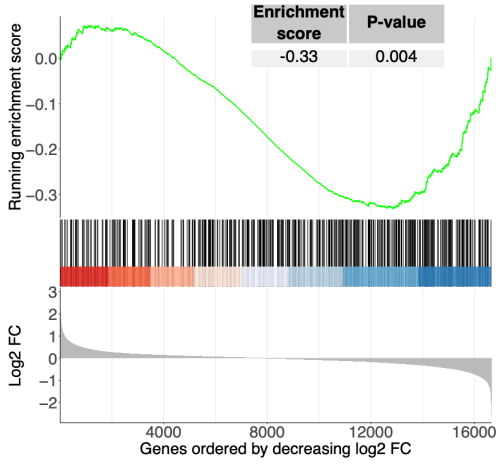

% 5hmdC
0.008
0.006
0.004
0.002
0.000
0.00111
NSCs *Idh1*–wt
NSCs *Idh1*–mut
% 5mdC
2.5
2.0
1.5
1.0
0.5
0.0
0.804
NSCs *Idh1*–wt
NSCs *Idh1*–mut

Extended Data Fig. 8. Assessment of D-2HG levels and TET function in external controls. (A) Workflow procedure for inducing the *in vitro* expression of Idh1 R132H in neural progenitor cells (NSCs) obtained from a conditional knock-in IDH^{mut} mouse model (cKI Idh1), used as external isogenic controls in three assays. Cells collected at day 14 for analyses underwent 3-6 passages. **(B)** Intracellular levels of D-2HG were determined using a fluorimetric assay in primary microglia treated with D-2HG [5 mM] for 48 hours. Microglia were obtained from samples with IDH^{wt} status (n=3). External controls for comparison (cKI model Idhwt n=5; Idhmut n=4, and patient-derived glioma cells IDH^{wt} n=4; IDH^{mut} n=3) are also shown. The levels of D-2HG in each sample were normalized to the corresponding micrograms of protein to enable comparisons among cell types. Data are represented as the mean with a 95% CI. **(C)** TET enzymatic activity assessed in nuclei extracts from NSCs after 14 days in culture. The plot shows a dramatic reduction of TET activity in IDH^{mut} cells with respect to the IDH^{wt} control. Data are represented as the mean with a 95% CI from technical replicates (n=2). A negative internal control, consisting of D-2HG with a final concentration of 400 µM, added to the IDH^{wt} extract during the assay, is also indicated (green color). **(D)** Quantification of 5hmC and 5mC global levels determined by LC-MS/MS in IDH^{mut} and IDH^{wt} NSCs controls after 14 days in culture. Data are represented as the mean with a 95% CI from technical replicates (n=4). P-values were calculated using one-sided Welch's test.

A

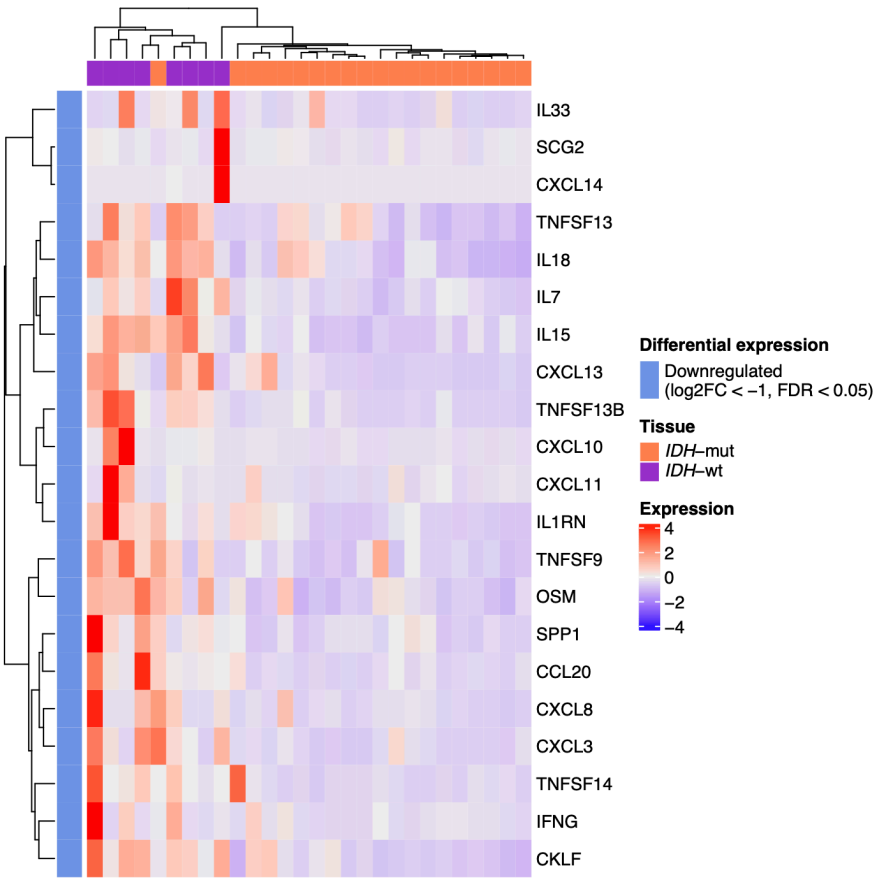

AP-1 family – all covered enhancers
CEBP family – all covered enhancers
Other TFs – all covered enhancers
% 5mCG
% 5hmCG
% 5mCG / % 5hmCG
Control
Treated
TF
ATF3
BATF
FOS
FOSB
FOSL1
FOSL2
JUN
JUNB
JUND
CEBPB
CEBPD
CEBPE
CEBPG
HIF1A
IRF4
IRF8
MYC
NFKB2
REL
RUNX3
SPI1
N
50
100
200

B

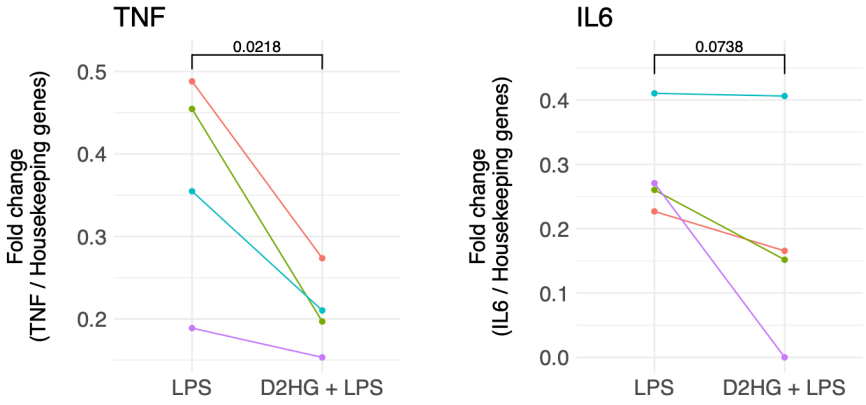

Enrichment score
P-value
-0.33
0.004
Running enrichment score
Log2 FC
Genes ordered by decreasing log2 FC

Extended Data Fig. 9. Single-base resolution analysis of 5mCG and 5hmCG in the proximity of TFs motifs in all covered enhancers of D-2HG-treated primary microglia. **(A)** Plots showing separate analysis of 5mCG and 5hmCG percentages (upper) in the proximity of binding motifs, residing in all covered enhancers, for the indicated TFs in D-2HG-treated microglia and paired untreated controls. The size of each dot indicates the number of motifs analyzed in a paired manner between conditions. The 5mCG/5hmCG ratios at analyzed regions are also shown. The whole set of covered enhancers (used as controls) comprises the selected enhancers of Fig. 5c. **(B)** GSEA analysis using ranked genes by decreasing log2FC of D-2HG-treated vs. control samples of RNA-seq. The plot shows a significant bias towards decreased expression of genes regulated by D-2HG-driven hypermethylated/hypohydroxymethylated enhancers bound by TFs depicted in Fig 5c. Both methylome/hydroxymethylome and RNA-seq analyses were conducted using the same samples and in a paired manner.

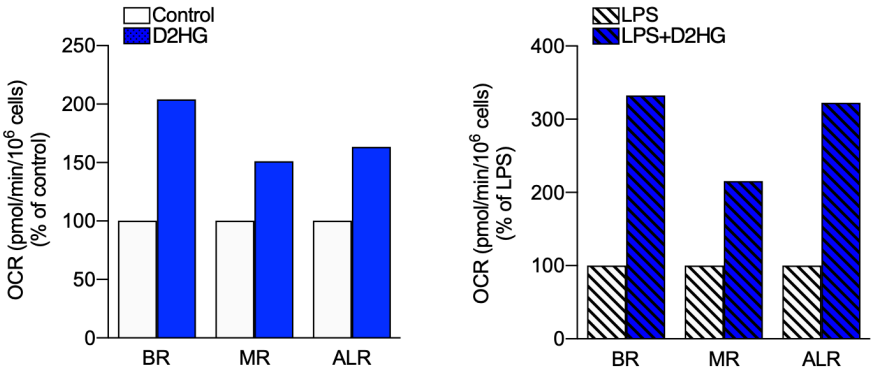
A


IL33
SCG2
CXCL14
TNFSF13
IL18
IL7
IL15
CXCL13
TNFSF13B
CXCL10
CXCL11
IL1RN
TNFSF9
OSM
SPP1
CCL20
CXCL8
CXCL3
TNFSF14
IFNG
CKLF
Differential expression
Downregulated
(log2FC < −1, FDR < 0.05)
Tissue
IDH–mut
IDH–wt
Expression
4
2
0
−2
−4

B


TNF
0.0218
Fold change
(TNF / Housekeeping genes)
0.5
0.4
0.3
0.2
LPS
D2HG + LPS
IL6
0.0738
Fold change
(IL6 / Housekeeping genes)
0.4
0.3
0.2
0.1
0.0
LPS
D2HG + LPS

C


Control
D2HG
OCR (pmol/min/10^6 cells)
(% of control)
250
200
150
100
50
0
BR
MR
ALR
LPS
LPS+D2HG
OCR (pmol/min/10^6 cells)
(% of LPS)
400
300
200
100
0
BR
MR
ALR

Extended Data Fig. 10. Validation experiments on primary microglia treated with D-2HG for 14 days and challenged with LPS. (A) Heatmap depicting the expression of proinflammatory cytokines in CD11B+ cells from IDH^{mut} (n=20) and IDH^{wt} (n=8) human gliomas using RNA-seq data. **(B)** The plots indicate the expression of TNFA and IL6 (relative to housekeeping controls) in paired D-2HG-treated and control samples as determined by RT-qPCR. Primary microglia were obtained from surgical aspirates with IDH^{mut} status (n=4). P-values were calculated using one-sided paired t-tests. **(C)** Assessment of oxygen consumption rate (OCR) in paired D-2HG-treated and control samples as determined by XFe extracellular flux analyzer. Basal respiration (BR), maximal respiration (MR) and ATP-linked respiration (ALR) parameters are indicated for the comparisons control vs. D-2HG, and LPS vs. LPS+D-2HG. Primary microglia were obtained from surgical aspirate of one IDH^{wt} meningioma. Cells were maintained, treated, and collected under the same conditions as previously shown (Fig. 6a).