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► To cite this version:

Franck Court, Elisa Le Boiteux, Anne Fogli, Mélanie Müller-Barthélémy, Catherine Vaurs-Barrière, et al.. Transcriptional alterations in glioma result primarily from DNA methylation independent mechanisms. *Genome Research*, 2019, 29 (10), pp.1605-1621. 10.1101/gr.249219.119 . hal-02304180v1

HAL Id: hal-02304180

<https://hal.science/hal-02304180v1>

Submitted on 3 Oct 2019 (v1), last revised 12 May 2020 (v2)

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Transcriptional alterations in glioma result primarily from DNA-methylation independent mechanisms

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Running title: Bases of transcriptional alterations in cancer

Keywords: Glioma, Epigenetics, transcriptional defects, CpG-island

Abstract

In cancer cells, aberrant DNA methylation is commonly associated with transcriptional alterations, including silencing of tumor suppressor genes. However, multiple epigenetic mechanisms, including polycomb repressive marks, contribute to gene deregulation in cancer. To dissect the relative contribution of DNA methylation-dependent and -independent mechanisms to transcriptional alterations at CpG-island/promoter-associated genes in cancer, we studied 70 samples of adult glioma, a widespread type of brain tumor, classified according to their Isocitrate Dehydrogenase (*IDH1*) mutation status. We found that most transcriptional alterations in tumor samples were DNA methylation-independent. Instead, altered histone H3 tri-methylation at lysine 27 (H3K27me3) was the predominant molecular defect at deregulated genes. Our results also suggest that the presence of a bivalent chromatin signature at CpG island promoters in stem cells predisposes not only to hypermethylation, as widely documented, but more generally to all types of transcriptional alterations in transformed cells. In addition, the gene expression strength in healthy brain cells influences the choice between DNA methylation- and H3K27me3-associated silencing in glioma. Highly expressed genes were more likely to be repressed by H3K27me3 than by DNA methylation. Our findings support a model in which altered H3K27me3 dynamics, more specifically defects in the interplay between polycomb protein complexes and the brain-specific transcriptional machinery, is the main cause of transcriptional alteration in glioma cells. Our study provides the first comprehensive description of epigenetic changes in glioma and their relative contribution to transcriptional changes. It may be useful for the design of drugs targeting cancer-related epigenetic defects.

Introduction

Cancer is a complex disease that results from the disruption of key pathways, including those regulating cell survival and division. Besides genetic lesions, epigenetic alterations also contribute to tumorigenesis mainly by leading to abnormal gene expression (Flavahan *et al.*, 2017).

Together with genome-wide DNA hypomethylation, DNA hypermethylation of CpG islands (CGIs) is a well-defined feature of cancer cells, and is believed to be the main cause of aberrant gene repression (Baylin and Jones 2016). CGIs are key regulatory genomic regions of few hundred base pairs in size characterized by high frequency of CpG dinucleotides. In humans, about 70% of promoters are associated with CGIs that generally remain unmethylated during somatic development, regardless of the gene expression status (Deaton and Bird 2011). Conversely, it has been shown that in tumors, DNA hypermethylation of their CGI/promoter leads to aberrant silencing of some tumor suppressor genes, such as *BRCA1* (Dobrovic and Simpfendorfer 1997), *RBI* (Greger *et al.*, 1994) and *MLH1* (Herman *et al.* 1998). However, the primary role of this defect in the widespread cancer-associated genes silencing, and more broadly in cancer biology, is still being questioned. Indeed, an increasing number of studies showed that in tumors, DNA hypermethylation affects primarily CGI/promoters that control genes already repressed in the matched normal tissue (Gal-Yam *et al.*, 2008; Hinoue *et al.*, 2012; Sproul *et al.*, 2011; Sproul *et al.*, 2012; Court and Arnaud 2017). Moreover, in some tumor types, such as glioma or breast cancer, patients with a CpG island methylator phenotype (CIMP), a signature identified in various human malignancies and defined by the concomitant hypermethylation of multiple CGIs (Suzuki *et al.*, 2014), have a better clinical prognosis compared with patients without CIMP (Noushmehr *et al.* 2010; Fang *et al.* 2011).

Therefore, other DNA methylation-independent epigenetic alterations at CGI/promoters might contribute to the genome-wide pattern of aberrant gene repression observed in cancer cells. During normal development, promoters/CGIs are dynamically marked by the permissive H3K4me3 and/or the repressive H3K27me3 histone marks (Mikkelsen *et al.*, 2007). When in combination, these marks constitute the so-called bivalent chromatin signature that maintains genes (for example, developmental genes in stem cells) repressed, but ‘poised’ for activation, since the bivalent mark can resolve into either H3K4me3 or H3K27me3 (Bernstein *et al.*, 2006). Alterations in the control of these chromatin signatures also could lead to gene silencing (Court and Arnaud, 2017). This hypothesis is supported by the observation that genes encoding methyltransferases and demethylases that regulate H3K27me3 and H3K4me3 deposition, such as *EZH2*, *KMT2 (MLL)* family members and *KDM6A*, are translocated or mutated and/or their expression is altered in many malignancies (Suvà *et al.*, 2013; Dawson 2017). Such defects had been documented in a handful of studies. In detail, analyses in established prostate (Gal Yam *et al.*, 2008; Kondo *et al.*, 2008) and urothelial (Dudziec *et al.*, 2012) cancer cell lines and in colorectal tumor samples (Hahn *et al.*, 2014) highlighted that gene silencing can be mediated just by H3K27me3.

Chromatin-based alterations could also lead to gain of gene expression in tumors. Hahn *et al.*, showed that genes associated with CGI/promoters displaying a bivalent chromatin signature in normal colon can be ectopically expressed in the matched tumor samples following H3K27me3 loss (Hahn *et al.*, 2014). Moreover, Bert *et al.*, (Bert *et al.*, 2013) identified in prostate cancer cell lines some genomic domains characterized by altered chromatin signatures associated with aberrant gene expression.

Therefore, it is clear that different epigenetic alterations at promoters/CGIs could contribute to the abnormal gene expression pattern of cancer cells. Because of the absence of dedicated integrative studies, many questions remain concerning the bases and extent of these alterations as well as their relative contribution to aberrant loss/gain of gene expression in cancer cells.

Glioma, which is derived from glial cells, is one of the most widespread brain tumor types. In 2007, the World Health Organization (WHO) classified gliomas in four grades (I-IV) according to their histology. Malignant anaplastic astrocytoma (a subset of the WHO grade III gliomas) and glioblastoma multiforme (GBM; WHO grade IV) account for about half of all gliomas, and are the most deadly and aggressive forms. The median survival time after diagnosis of patients with GBM does not exceed 18 months despite the aggressive treatments. At the molecular level, aggressive gliomas are characterized by expression of wild type isocitrate dehydrogenase (*IDHwt*) genes (*IDH1* and *IDH2*), while gliomas with better prognosis express mutated *IDH* (*IDHmut*) (Cohen *et al.*, 2013). Consequently, the recently released 2016 WHO classification of diffuse gliomas (Louis *et al.*, 2016), which we used in this study, is primarily based on the *IDH1* mutation status (*IDHmut vs IDHwt*). *IDH1* mutation results in CIMP-positive tumors (Turcan *et al.*, 2012) with a better clinical prognosis compared with CIMP-negative tumors (Noushmehr *et al.*, 2010; Cohen *et al.*, 2013). Other epigenetic regulators also could be involved in glioma development/progression, for instance the polycomb repressors EZH2 and BMI1 (Häyry *et al.*, 2008; Suvà *et al.*, 2009; Bruggeman *et al.*, 2009) and KMT2 family members that are mutated in a subset of GBM (Parsons *et al.*, 2008; Brennan *et al.*, 2013). Here, we used glioma as a model to investigate the molecular bases of transcriptional alterations of CGI/promoter-associated genes in cancer.

Results

CGI methylation poorly contributes to transcriptional alterations in glioma

For this study, we used 70 clinically well-characterized primary glioma samples (the patients' demographic and main molecular and clinical features are provided in Supplemental Table S1). We classified samples according to their *IDH1* status ($n = 55$ *IDHwt*, and $n = 15$ *IDHmut*). This first level of classification, upstream of the 1p/19q co-deletion status according to the 2016 WHO classification (Louis *et al.*, 2016), clearly discriminated two tumor classes relative to aggressiveness, with a significant survival advantage for patients with *IDHmut* tumors (HR=0.32, 95% CI [0.14-0.71], $p=0.005$) (Supplemental Table S1 and Supplemental Fig. S1A).

Genome-wide analysis of DNA methylation at CGIs, using the Infinium HumanMethylation450 (HM450K) BeadChip Arrays, showed that DNA methylation defects were more widespread in *IDHmut* samples, constituting a Glioma CIMP (G-CIMP) subclass (Supplemental Fig. S1B). In agreement with the literature (Noushmehr *et al.*, 2010; Turcan *et al.*, 2012; Louis *et al.*, 2016), in our cohort, aggressive gliomas were characterized by *IDHwt* and absence of G-CIMP, whereas *IDHmut* gliomas showed a G-CIMP profile and had a better prognosis.

To more precisely define the CGI/promoter alterations in our glioma samples, we analyzed the DNA methylation profiles of 14,714 genes with a single CGI-rich promoter that could be assessed using the HM450K array. Most of these genes (76.0%) were protein-coding genes, and the others were antisense transcripts (10.7%), long intergenic non-coding RNAs (lincRNA; 6.5%), and pseudogenes (6.8%) (Fig. 1A). As some CGI/promoters can control more than one gene, these 14,714 genes are associated with 11,795 CGI/promoters. About 90% of these CGI/promoters were unmethylated in non-tumor control brain samples (mean β -value <0.2), and most of them remained unmethylated also in glioma samples. Among these CGI/promoters, 11.6% ($n=1,369$; associated with 1,623 genes) were aberrantly hypermethylated in *IDHwt* samples, and 22.8% ($n=2,692$; 3,198 genes) in *IDHmut* samples, contributing to their CIMP-positive status. Conversely, CGI/promoter hypomethylation, although more limited than hypermethylation, was more common in *IDHwt* ($n=198$ CGI/promoters associated with 235 genes; 1.7%) than in *IDHmut* ($n=14$ CGI/promoters associated with 22 genes; 0.12%) samples (Fig. 1B; Supplemental Fig. S1C-D).

To evaluate the consequences of these DNA methylation changes, we analyzed by RNA-seq eight *IDHwt* and five *IDHmut* glioma samples. Most genes with aberrantly hyper- or hypomethylated CGI/promoters (79.9% in the *IDHwt* and 87.4% in the *IDHmut* group) showed no significant transcriptional change compared with control brain samples ($|\log_2 Fc| > 2$; $p < 0.05$) (Fig. 1C,D). The number of genes with altered expression was similar between glioma subtypes despite their different DNA methylation profiles. Among the genes with aberrantly hyper- or hypomethylated CGI/promoters, 223 and 265 were downregulated (82 in common), and 150 and 140 were upregulated (44 in common) in *IDHwt* and *IDHmut* glioma samples, respectively, compared with controls (Fig. 1C,D). These findings indicate that aberrant CGI/promoter methylation minimally affects gene transcription in glioma. However, the deregulated genes included some putative tumor suppressors, such as *RASL10A* and *HTATIP2* (Schmidt *et al.*, 2012; Dong *et al.*, 2015), and some putative oncogenes, such as *HOXD9* and *CXCL1*, the overexpression of which was, counterintuitively, associated with methylation gain (Supplemental Table S2).

Gene transcription alterations are more widespread in *IDH*wt glioma samples

Detailed analysis of the transcriptional landscape of tumor samples showed that transcriptional alterations were more widespread in *IDH*wt than in *IDH*mut glioma samples (1,670 and 1,024 deregulated genes, respectively; $FDR < 0.05$; $|\log_2 Fc| > 2$) (Fig. 2A), particularly for CGI/promoter-associated genes (Supplemental Fig. S2A).

Copy number variation (CNV) analyses in the same samples showed that, as previously reported, Chromosome 7 gain and Chromosome 10 loss characterized *IDH*wt samples (Louis *et al.*, 2016), while the 1p/19q co-deletion was mainly present in *IDH*mut samples (Fig 2B). By integrating these data with the gene expression profiles, we identified 92 genes in *IDH*wt and 37 genes in *IDH*mut samples, respectively, in which expression alteration correlated with CNV ($p < 0.05$) (Fig. 2B, and Supplemental Fig. S2B-C, Supplemental Table S3). For instance, upregulation of epidermal growth factor receptor (*EGFR*) and the histone methyltransferase *EZH2* (both located on Chromosome 7) correlated with increased copy number in *IDH*wt samples. Conversely, overexpression of *HOXA13*, also located on Chromosome 7, did not correlate with CNV (Fig. 2C; Supplemental Fig. S2C).

Altogether, affected genes without CNV (mostly protein-coding genes) represented about 11% of all CGI/promoter-associated genes in *IDH*wt samples (841 downregulated and 737 upregulated genes), and 6.7% in *IDH*mut samples (556 downregulated and 431 upregulated) (Fig. 2D, Supplemental Table S2).

Most transcriptional alterations are DNA methylation-independent

To understand the bases of such transcriptional alterations we next focused our analyses on *IDH*wt glioma samples. We used paired RNA-seq and HM450K data from 8 *IDH*wt samples to concomitantly determine the DNA methylation and transcriptional changes in the 1,578 affected genes. In these *IDH*wt samples, we could identify four main transcriptional defect types (Fig. 3A): gain or loss of gene expression associated with CGI/promoter hypermethylation (referred to as ‘Meth+/Exp+’ and ‘Meth+/Exp-’ defects, respectively), and gain or loss of gene expression without changes in CGI/promoter methylation status (*i.e.*, the CGI/ promoter remained unmethylated; referred to as ‘No Meth/Exp+’ and ‘No Meth/Exp-’ defects, respectively). More than 93% of aberrantly repressed genes did not display any significant DNA methylation alteration at their CGI/promoter (No Meth/Exp-). About 47% of the affected genes showed gain of expression that was associated with DNA hypermethylation at their CGI/promoter in 6% of them (Meth+/Exp+).

To evaluate the robustness of this classification, we first analyzed the HM450K data of all 55 *IDH*wt glioma samples of our cohort and confirmed that DNA hypermethylation was associated with genes classified in the Meth+/Exp+ and Meth+/Exp- groups, and absence of methylation with genes classified in the No Meth/Exp+ and No Meth/Exp- groups. The only exception was a subset of “methylable” genes that gained methylation in some samples (Fig. 3B). Next, we concomitantly assessed, in 42 *IDH*wt glioma samples, the DNA methylation and expression, by RT-qPCR, of

randomly selected genes from the Meth+/Exp+, No Meth/Exp+, and No meth/Exp- groups. Genes in the Meth+/Exp+ group were methylated and ectopically expressed in all analyzed *IDHwt* glioma samples. The seven genes from the No Meth/Exp- group were aberrantly repressed in all analyzed samples, and their CGI/promoter mostly unmethylated (Fig. 3C). For instance, the candidate tumor suppressor gene *BINI* was unmethylated in all analyzed samples. *PCSK6* and *HOXD1* provided examples of “methylable” genes. They were methylated in a subset of samples, but their expression was repressed in all of them. The six genes from the No Meth/Exp+ group were all overexpressed and most of them, including the tumor progression-associated *VEGFA* and *E2F2* genes, tended to be unmethylated in all analyzed samples. This group also included some “methylable” genes (see Fig. 3B), such as *KDR* (the tyrosine kinase receptor for VEGFA) that was overexpressed in all samples, while its CGI/promoter was methylated only in a subset of gliomas. These “methylable” genes displayed an overall significant gain of DNA methylation in the 55 *IDHwt* samples. Therefore, we reclassified them from their initial No Meth/Exp+ and No Meth/Exp- groups (based on the analysis of 8 *IDHwt* samples) into the Meth+/Exp+ and Meth+/Exp- groups, respectively, for the subsequent analyses.

To assess the reproducibility of these observations, we performed the same analyses in an independent cohort of 134 *IDHwt* samples described in Ceccarelli *et al.*, 2016. In these samples, we confirmed the existence of the four main transcriptional defect groups, with proportions similar to those observed in our cohort. Overall, the number of affected genes was smaller in this validation cohort (970 vs 1,564), possibly due to the different RNA-seq strategies used. Indeed, Ceccarelli *et al* used mainly non-stranded RNA-seq in which sense-antisense overlapping transcripts were discarded from the analyses, whereas we used an oriented RNA-seq approach that considered independently sense and antisense transcripts. Nonetheless, all four defect categories significantly overlapped in the two cohorts (minimal P value $<1 \times 10^{-40}$; Fisher’s exact test), demonstrating the robustness of our observations (Fig. 3D).

Finally, to further characterize these defects, we studied the methylation pattern of the 681 genes that were transcriptionally affected in both *IDHwt* and *IDHmut* glioma samples (377 downregulated and 303 upregulated in both groups, and 1 downregulated in *IDHwt* and upregulated in *IDHmut*). Most of these genes displayed the same methylation pattern in *IDHwt* and *IDHmut* samples. However, “methylable” genes and a subset of unmethylated genes in *IDHwt* gliomas (symbolized by a blue column in Supplemental Fig. S3) tended to be methylated in *IDHmut* samples, suggesting that different molecular pathways can lead to the same aberrant gene expression pattern.

Altogether, this integrative analysis identified four classes of transcriptional defects at CGI/promoter genes in *IDHwt* glioma: aberrant loss and gain of gene expression without DNA methylation defect (most genes), and gene expression defects associated with aberrant DNA methylation either in all samples or in some samples (*i.e.*, at methylable genes). In summary, among the 1,578 CGI/promoter-associated genes affected in *IDHwt* glioma samples, most belonged to the No Meth/Exp- group (n=628), followed by the No meth/Exp+ (n=612), Meth+/Exp- (n=208), and Meth+/Exp+ (n=116) (Supplemental Fig. S4). The classification for each gene is provided in Supplemental Table S2.

The four defect classes include mainly genes with a chromatin bivalent signature in stem cells

Gene Ontology analysis (Fig. 4A, Supplemental Fig. S5) showed that the Meth+/Exp- and No Meth/Exp- groups were enriched in genes involved in transmembrane ion transport, specifically in synapsis and neurons. Conversely, the No Meth/Exp+ group was enriched in genes implicated in general processes, such as cell cycle, cell division and chromosome segregation. Finally, the Meth+/Exp+ group was highly enriched in genes encoding homeodomain proteins, including the HOX family, and implicated in embryonic development.

Studies on the molecular bases of CGI hypermethylation have shown that genes with a bivalent chromatin signature in stem cells are more likely to gain aberrant methylation in cancer cells (Ohm *et al.*, 2007; Deneberg *et al.*, 2011; Court and Arnaud 2017). To evaluate whether such an instructive program could apply to some or all genes of the four defect categories, we evaluated the GCI/promoter chromatin signature of genes in these four categories in human embryonic stem (ES) cells, in neural progenitor cells (NPC), and in brain samples. In agreement with previous findings, most genes in the Meth+ groups showed bivalent signatures in ES cells and NPC. This was true regardless of their aberrant expression pattern (*i.e.*, both Meth+/Exp- and Meth+/Exp+). Similarly (but more unexpectedly), about 36% and 48% of genes in the No meth/Exp+ and No meth/Exp- groups, respectively, showed a bivalent chromatin signature in ES cells (compared with 24% of all studied genes) (Fig. 4B, Supplemental Fig. S5).

In agreement with the resolution of the bivalent signature during development/cell differentiation, the chromatin signature tended to change towards an exclusive H3K4me3 signature, but also to a “none” signature (*i.e.*: depleted for both H3K4me3 and H3K27me3) in brain samples (Fig. 4C). In comparison, most of the transcriptionally unaffected genes maintained their H3K4me3-only signature from ES cell to brain samples. Accordingly, the transcriptionally affected genes displayed a dynamic expression pattern from ES cells to NPC and brain. This was true also for the subset of genes in the No meth/Exp+ and No meth/Exp- groups that maintained an exclusive H3K4me3 signature in ES cells, NPC and brain, but showed loss and gain of expression in brain, respectively (Fig. 4C).

Altogether, these findings suggest that genes with a bivalent chromatin signature in ES cells and/or with a dynamic expression pattern during neural differentiation are more prone to be deregulated in *IDHwt* glioma.

CGI/promoter hypermethylation is associated with gain of expression

As bisulfite treatment cannot distinguish between methylation and 5-hydroxymethylation (5-hmC), we evaluated whether the DNA methylation level detected with the HM450K array at genes classified in the Meth+/Exp+ group could be explained by 5hmC deposition. By using publicly available 5hmC data for *IDHwt* tumors (Johnson *et al.*, , 2016), we found that CGI/promoter regions of Meth+/Exp+ genes were devoid of 5hmC (median level = 1.2%), indicating that the signal detected with the HM450K array was due to DNA methylation (Supplemental Fig. S6A). Note that a similar observation is made at the three other defect groups (Supplemental Fig. S7).

To determine how DNA hypermethylation and expression gain could co-exist in this group of genes, we next assessed their CGI/promoter chromatin signature using publicly available ChIP-seq data for *IDHwt* glioma cells. Compared with healthy brain (control), H3K27me3 level was strongly decreased, while H3K4me3 level was increased in about 65% of these genes and totally depleted in the others (Fig. 5A).

Analysis of the HM450K data on the localization of hypermethylated sites relative to the transcription start site (TSS) showed that at H3K4me3-enriched genes, the gain of DNA methylation occurred at the border of the CGI/promoter, while the TSS area remained unmethylated (Fig. 5B). Analysis of individual loci in glioma samples using strand-oriented RNA-seq data suggested that transcription initiated from H3K4me3-marked TSS embedded in methylated CGIs (Fig. 5C, and Supplemental Fig. S6B). This was observed in several genes that promote gliomagenesis, including *TWIST1* (Mikheev *et al.*, 2018), *CTHRC1* (Liu *et al.*, 2017a) and *FOXD3-AS1* (Chen *et al.*, 2016). The H3K4me3 and DNA methylation signals were mutually exclusive (Fig. 5C and Supplemental Fig. S6B), in agreement with their documented antagonism (Weber *et al.*, 2007).

In H3K4me3-depleted genes, DNA methylation was spread along the entire CGI/promoter, including the TSS (Fig. 5B), suggesting that transcription from these genes could arise from an alternative TSS. Analysis of RNA-seq data supported this hypothesis because genes, such as *HOXC11* and *NR2F2*, showed transcription signal from H3K4me3-enriched regions located away from the documented TSS (Fig 5D and Supplemental Fig. S6C). However, for few genes, such as *HEYL* and *C15orf48* (Supplemental Fig. S6D), transcription apparently initiated from a methylated CGI through an unknown mechanism.

Altogether, these approaches support the hypothesis that in Meth+/Exp+ genes, transcription could be allowed by the absence of DNA methylation at the TSS, or the use of alternative TSS.

E2F- and HOX-target genes are frequently overexpressed in glioma

The publicly available ChIP-seq data indicated that genes in the No Meth/Exp+ group were enriched in H3K4me3 and depleted in H3K27me3 in *IDHwt* glioma cells compared with healthy brain (Fig. 6A). We observed this H3K4me3 gain also in the subset of genes that were constitutively marked by the H3K4me3-only signature in ES cells, NPC and brain (framed in Fig. 6A). To understand the basis of their overexpression in glioma, we determined whether specific motifs were enriched at their CGI/promoters. We found that overall, No Meth/Exp+ genes were putative targets of transcription factors associated with cell cycle pathways, including the Krüppel-like factors (KLF)/specificity protein (SP) and E2F families (Fig. 6B-C). Moreover, most of the H3K4me3-only genes showed specific motif enrichment for the E-26 transformation specific (ETS) and nuclear transcription factor-Y (NF-Y) families. The other No Meth/Exp+ genes were putative targets of homeodomain transcription factors, including HOX proteins (Fig. 6B).

Most of these transcription factors were expressed in healthy controls and remained expressed in glioma samples (Fig. 6C). However, a subset was specifically overexpressed in *IDHwt* glioma samples, including *HOX* genes, *E2F2* and 7, *ETS1* and *ETV1* and 4 (Fig. 6C: RNA-seq data). We

confirmed these findings by RT-qPCR analysis of selected genes in 42 *IDHwt* samples (Fig. 6D). Gene encoding aberrantly overexpressed transcription factors mostly belonged to the Meth+/Exp+ (e.g., *HOXA2* and *HOXD8*) and No meth/Exp+ (e.g., *E2F2*) groups (Supplemental Table S2). This observation suggests that the initial overexpression of few key transcription factors could lead to overexpression of most genes belonging to the No Meth/Exp+ group.

The repressive signature of silenced genes is related to their transcriptional status in healthy brain

To understand how Meth+/Exp- and No Meth/Exp- genes were transcriptionally repressed, we compared their chromatin signature in healthy brain and in glioma cells using publicly available ChIP-seq data. This highlighted a marked H3K27me3 enrichment in both groups in glioma samples compared with controls (Fig. 7A). The only exception was the subset of No Meth/Exp- genes that displayed the constitutive H3K4me3-only signature in ES cells, NPC and brain (framed in Fig. 7A) and maintained this signature in glioma cells. H3K27me3 enrichment in glioma cells led to a bivalent chromatin signature in the large subset of genes that were also marked by H3K4me3 (Fig. 7A). At CGI/promoters of Meth+/Exp- genes, H3K4me3 tended to be reduced and H3K27me3 was enriched (Fig. 7A), suggesting that unlike normal cells (Brinkman *et al.*, 2012), both H3K27me3 and DNA methylation can coexist at CGI/promoters in glioma cells.

ChIP analysis of selected genes from the No Meth/Exp- group (*PCSK6*, *MAL*, *SH3GL3* and *NKAIN2*) confirmed the marked H3K27me3 gain associated with reduced or unchanged H3K4me3 and H3K9ac levels, according to the studied locus, in the seven glioma samples tested (Fig. 7B-C, and Supplemental Fig. S8A). Also, bisulfite analysis of the H3K27me3-immunoprecipitated fraction confirmed that both DNA methylation and H3K27me3 co-existed at CGI/promoters in glioma samples, as exemplified by the methylable *PCSK6* gene (Supplemental Fig. S8B).

Several studies have highlighted that the propensity of genes to be hypermethylated in cancer cells is related to their transcriptional status in the normal tissue (Gal-Yam *et al.*, 2008; Hinoue *et al.*, 2012; Sproul *et al.*, 2011; Sproul *et al.*, 2012; Court and Arnaud 2017). Accordingly, we observed that the transcriptional status in brain discriminated between Meth+/Exp- and No Meth/Exp- genes. Specifically, DNA methylation-independent silencing (No Meth/Exp-) mainly affected genes that were highly expressed in normal brain. Conversely, poorly expressed genes tended to be hypermethylated (Fig. 7D). To more systematically assess the bases of the differences between these groups, we performed principal component analysis for all genes in the Meth+/Exp- and No Meth/Exp- groups. The first principal component accounted for about 44.5% of the variance and allowed separating the two groups (centroid values along the axis: +0.984 and -0.326, respectively) (Fig. 7E). The observation that the first principal component was significantly correlated with the permissive H3K4me3 mark ($R=0.79$; $P < 3.4 \times 10^{-126}$) and the expression level ($R=0.71$; $P < 1 \times 10^{-129}$) in healthy brain (Fig. 7F) supported the hypothesis that the expression status in healthy cells contributes to the choice of silencing pathway used in cancer cells. Additional analyses using normalized RNA-seq data

for 21 different tissues showed that genes from the No Meth/Exp- group were strongly expressed specifically in adult brain tissues (Supplemental Fig. S8C and D).

Thus, besides DNA methylation, H3K27me3 enrichment and chromatin bivalency emerge as major causes of aberrant gene repression in aggressive glioma cells. The choice between these alternative silencing pathways is related to the expression level of the affected genes in healthy tissues/cells.

The four classes of expression defects are observed also in *IDH*mut glioma samples

We next extended our analyses to *IDH*mut glioma. Integrative analysis of differential gene expression and methylation identified the four main defect classes also in these samples (Supplemental Fig. S9 A-C; Supplemental Table S2). DNA methylation-independent defects were the most frequent, although less prominent than in *IDH*wt samples. Specifically, the 556 repressed genes were equally distributed between the No Meth/Exp- (n=294) and the Meth+/Exp- (n=262) groups (Supplemental Fig. S4).

These observations were validated in an independent cohort of 415 *IDH*mut samples derived from Ceccarelli *et al.*, 2016, with a highly significant overlap in the composition of each defect group between cohorts (minimal P value $<1 \times 10^{-40}$; Fisher's exact test) (Supplemental Fig. S9D). We also observed that, like in *IDH*wt samples, genes with a bivalent chromatin signature in ES cells and/or with a dynamic expression pattern during neural differentiation were more prone to be deregulated in *IDH*mut glioma (Supplemental Fig. S10). Then, to understand the molecular bases of these alterations, and in the absence of publicly available ChIP-seq data on *IDH*mut samples, we performed ChIP analyses at selected genes from the Meth+/Exp- group (*PCSK6* and *MAL*) and No Meth/Exp- group (*SH3GL3* and *PCDH10*) in five *IDH*mut samples. We found that H3K27me3 enrichment was associated with reduced or unchanged H3K4me3 and H3K9ac, in function of the studied locus (Supplemental Fig. S11A). This confirms that, like in *IDH*wt samples, gain of H3K27me3 and chromatin bivalency are, besides DNA methylation, the main hallmarks of repressed genes in *IDH*mut glioma cells. Finally, by considering the expression level in healthy brain and performing principal component analysis, we observed that the expression status in healthy cells contributed to the choice of silencing pathway used in *IDH*mut cells, with genes repressed independently of DNA methylation (No Meth/Exp- group) being mainly genes that were highly expressed in brain (Supplemental Fig. S11B-D).

Altogether, these approaches show that the main observations made in *IDH*wt glioma also apply to *IDH*mut glioma.

Discussion

Here we used glioma, one the most widespread brain tumor types, as a model to evaluate the relative contribution of DNA methylation-dependent and -independent mechanisms to transcriptional alteration at CGI/promoter-associated genes in cancer cells. Our study showed that H3K27me3 level changes are the predominant molecular defect at both aberrantly repressed and expressed genes. Moreover, our findings support that H3K27me3 dynamics deregulation, particularly when present in a bivalent context, is the main cause of transcriptional alteration in glioma cells.

Some studies have described H3K27me3-based transcriptional repression in cancer cells (Gal-Yam *et al.*, 2008; Kondo *et al.*, 2008; Dudzic *et al.*, 2012; Statham *et al.*, 2012; Hahn *et al.*, 2014). In colorectal tumors, ectopic gene expression has been associated with aberrant loss of H3K27me3 from CGI/promoters with bivalent chromatin signature (Hahn *et al.*, 2014). Moreover, gene expression changes in primary human clear cell renal cell carcinomas can be attributed to chromatin accessibility alterations, independently of DNA methylation (Becket *et al.*, 2016). Our study further extends these observations and provides, for the first time, a comprehensive description of these alterations in glioma samples and their relative contribution to transcriptional alteration in such tumors. Specifically, our integrative analyses identified and quantified four main types of transcriptional defects in glioma (Fig. 8) that recapitulate the DNA methylation- and H3K27me3-based molecular signatures at aberrantly repressed and expressed CGI/promoter-associated genes. We detected these defects in *IDHwt* and also in *IDHmut* glioma samples (Fig. 8B), indicating that they occur regardless of the G-CIMP status and tumor aggressiveness. Additional studies are required to determine whether the relative distribution of these defects can discriminate different *IDHmut* sub-populations (*e.g.*, classified according to the 1p/19q co-deletion status) and can be associated with specific clinical features. Moreover, these observations prompt to investigate whether they might apply to cancer cells in general.

Our study revisited the relationship between aberrant transcription and DNA methylation in cancer cells. First, we confirmed, that gene expression deregulation is very rarely associated with CGI/promoter DNA hypomethylation in glioma samples, in agreement with the general unmethylated status of CGI/promoters in healthy cells. Unexpectedly, we found that DNA hypermethylation is not the main cause of transcriptional repression at CGI/promoter genes, and that it can be associated also with gain of expression. Indeed, about 16% of ectopically expressed genes were associated with a hypermethylated CGI/promoter in *IDHwt* glioma samples. Specifically, in many genes, ectopic expression was associated with CGI/promoters that gained methylation at their borders, while the H3K4me3-marked TSS was methylation-free. At other genes, extensive methylation of their main CGI/promoter was associated with the use of an alternative promoter. It is not known whether there is a causal link between these events. These two distinct signatures have also been described in prostate

cancer cell lines (Bert *et al.*, 2013), suggesting that an association between CGI/promoter DNA hypermethylation and gain of gene expression is common in cancer.

Besides the concomitant gain of expression and DNA methylation, this group of genes was characterized also by a reduction of H3K27me3 level in glioma cells (compared with controls), suggesting that the interplay between these repressive marks is a driving force in their transcriptional deregulation. In the mouse, widespread DNA methylation depletion triggers redistribution of H3K27me3 (Brinkman *et al.*, 2012; Reddington *et al.*, 2013) that in turn leads to a loss of H3K27me3 and ectopic expression at a subset of polycomb target genes, including *Hox* clusters (Reddington *et al.*, 2013). Reciprocally, analyses in mouse ES cells showed that the area close to CGI/promoters of polycomb target genes is protected from aberrant DNA methylation gain by polycomb proteins (Li *et al.*, 2018). Therefore, the Meth+/Exp+ defect might affect a subset of polycomb-target genes that are particularly sensitive to the H3K27me3 redistribution induced by the genome-wide hypomethylation of cancer cells. Noteworthy, in the aggressive *IDHwt* glioma, this group is enriched in homeodomain genes, and more specifically in *HOX* genes. Deregulation of *HOX* genes contributes to the tumorigenic potential of glioblastoma stem cells, by activating a network of downstream genes (Gallo *et al.*, 2013). Accordingly, we observed that many ectopically expressed genes from the No Meth/Exp+ group are putative HOX transcription factor targets. Altogether, our observations support a domino effect model to account for the gain of expression of CGI/promoter genes in aggressive glioma. In this model, genome-wide hypomethylation leads to ectopic expression (and methylation gain) of Meth+/Exp+ genes (especially *HOX* genes) that then promote gain of expression of target genes from the No Meth/Exp+ group (Fig. 8A). Additional studies are required to test this model and specifically whether No Meth/Exp+ genes are *bona fide* HOX transcription factor targets.

Another key finding of our study is that a bivalent chromatin signature in stem cells may not only predispose genes to hypermethylation, as widely documented (Ohm *et al.*, 2007; Deneberg *et al.*, 2011; Court and Arnaud 2017), but more globally to transcriptional alteration in cancer cells. We observed that genes with CGI/promoters marked by a bivalent chromatin signature in ES cells and NPC were more prone to be deregulated in glioma samples, regardless of the transcriptional defect. This was particularly true for genes with DNA methylation-associated defects, irrespectively of their association with gain or loss of expression (Meth+/Exp+ and Meth+/Exp-), and to a lesser extent, for genes with DNA methylation-independent defects (No meth/Exp+ and No meth/Exp-). This observation suggests that defects in the control of the bivalent chromatin signature, and more specifically of H3K27me3 dynamics, upon differentiation, are one of the main causes of transcriptional deregulation of CGI/genes in cancer cells. Accordingly, we found that aberrant gene repression in glioma samples affected mainly genes with brain-specific expression, and thus more sensitive to bivalency alterations upon neural stem cell differentiation. Besides the functional aberrations of H3K27me3 and H3K4me3 writers and erasers documented in many tumors (Suvà *et al.*, 2013; Dawson 2017), these control defects could also result from transcriptional changes in key tissue-specific transcription factors or co-factors. Studies in mouse ES cells and tissues showed that their transcriptionally inactive status is sufficient to promote the recruitment of the polycomb responsive

complex PRC2 and H3K27me3 deposition at CGI/promoters (Mendenhall *et al.*, 2010; Klose *et al.*, 2013; Riising *et al.*, 2014; Jadhav *et al.*, 2016; Maupetit-Méhouas *et al.* 2016). This suggests that the fate of bivalent chromatin domains upon development/differentiation relies on the interplay between PRC2 and the availability of the *ad hoc* transcriptional machinery. Our observation that the gene transcription strength in healthy brain influences the choice of silencing mechanism at repressed genes in glioma precisely argues for an alteration in this interplay. Specifically, we propose that following the alteration of a subset of brain-specific co-factors, the resulting weakened transcriptional machinery cannot efficiently counteract PRC2 recruitment upon differentiation, leading to aberrant maintenance of chromatin bivalency and to silencing of a subset of genes that are normally specifically expressed in brain. Moreover, gain of function of factors that promote, directly or indirectly, the recruitment of PRC2 at CGI could facilitate this process. This includes for instance the histone demethylase KDM2B (Farcas *et al.*, 2012; Wu *et al.*, 2013; Blackledge *et al.*, 2014) that is critical in various cancers, including glioma (Yan *et al.*, 2018; Staberg *et al.*, 2018). At normally poorly expressed genes, these events associated with the initial weak level of H3K4me3, a mark that prevents recruitment of DNA methyltransferases, would facilitate the subsequent gain of DNA methylation (Fig. 8A).

In addition to genes in which their chromatin signature was altered in glioma, we also identified a subset of genes with an apparent constitutive H3K4me3-only signature in healthy (ES cells to brain) and glioma samples, and that showed either gain of expression (No meth/Exp+) or aberrant repression (No Meth/Exp-) in glioma samples (Fig. 8A). Additional studies are required to establish the molecular bases of these observations. Specifically, it would be interesting to determine whether the regulation of these genes in normal and pathological contexts relies exclusively on the availability of *ad hoc* transcription factor(s), or whether it is also associated with not yet identified repressive histone marks. Moreover, No Meth genes that are ectopically expressed in glioma samples were also expressed in ES cells and NPC (Fig. 4). Similarly to genes with bivalent chromatin signature in ES cells whose aberrant repression in glioma recapitulated their repression in stem cells (Fig. 4), this group of genes could contribute to glioma aggressiveness by maintaining tumor cells in a stem-cell like state.

Our study also provides a framework to explain the counterintuitive observation that patients with CIMP-positive *IDH*mut have a better clinical outcome than patients with CIMP-negative *IDH*wt. Our data indicate that CIMP is observed mainly at genes that are already repressed in healthy brain. Consequently, the number of deregulated genes with CGI/promoters associated with DNA methylation defects is similar between glioma subtypes. Conversely, the higher frequency (about two times) of DNA methylation-independent transcriptional alterations in *IDH*wt than in *IDH*mut samples could contribute to the prognosis difference between glioma subtypes. The CIMP-positive status, by promoting stable gene repression, could also act as a protective mechanism against cancer progression, by limiting the tumor epigenetic plasticity and its ability to adapt to environmental changes, such as metastasis formation or treatment (Sproul and Meehan 2013). Specifically, among the many genes that gain expression in *IDH*wt samples and that are maintained repressed through gain of methylation in *IDH*mut samples (Supplemental Table S4), a dozen are oncogenes with some documented for their

role in glioma biology, such as Spalt-like transcription factor 4 (*SALL4*) (Liu *et al.*, 2017b) and the long non-coding RNA MIR155 host gene (*MIR155HG*) (Wu *et al.*, 2017).

In conclusion, our study on the extent and consequences of epigenetic alterations in glioma indicates that transcriptional deregulations rely mainly on chromatin-based DNA methylation-independent mechanisms. It also shows that the gene expression level in healthy tissue influences the type of silencing pathway used for repression in cancer cells, whereby highly expressed genes are more likely to be repressed by H3K27me3 rather than DNA methylation. Besides providing an original framework to understand the epigenetic basis of carcinogenesis, these observations are also important for the design of drugs to target epigenetic defects in cancer.

Materials and Methods:

Tumor and control samples

Glioma samples ($n=70$) resected between 2007 and 2014 were obtained from Clermont-Ferrand University Hospital Center, France (anonymized samples from the ‘Tumorotheque Auvergne Gliomes’, ethical approval DC-2012-1584). The ethics committees and the respective competent authorities approved this study. The study protocols conform to the World Medical Association Declaration of Helsinki.

Immediately after surgery, samples were snap-frozen and stored in liquid nitrogen. Random sections of each tumor were analyzed under a light microscope after hematoxylin–eosin staining to determine the extent of necrosis and the percentage of tumor cells. All glioma samples included more than 50% of tumor cells. Gliomas were classified according their *IDH1* mutation status: *IDHwt* ($n=55$) and *IDHmut* ($n=15$). *IDH1* genotyping was performed by EpigenDx (Worcester, MA) (EpigenDx pyrosequencing assays ADS1703 and ADS1704) (Fogli *et al.*, 2016).

Eight control brain samples (healthy controls, mean age of 27.3 years, standard deviation ± 2 years) were removed by autopsy 4-16h after accidental death (Brain and Tissue Bank of Maryland). These samples, identified by the Brain and Tissue Bank of Maryland as corpus callosum ($n = 5$) and frontal cortex ($n = 3$), corresponded to white matter enriched in astrocytes and oligodendrocytes from which gliomas originate.

Tumor and control samples were homogenized into powder by cryogenic grinding, equally distributed in at least three vials before use for matched genomic DNA, RNA and chromatin extraction. All samples were stored at -80°C until use. Overall survival (OS) was calculated as the number of days between the surgery date and the patient’s death. Tumor resection was classified as gross total resection, when no enhanced contrast was detected 48h post-surgery, or as partial resection, when enhanced contrast was still detected 48h post-surgery.

The demographic and clinical features are presented in Supplemental Table S1. The related statistical analyses were performed using the Stata software, version 13 (StataCorp, College Station, TX, US) and the R software, version 3.3.1 (R Core Team, 2016). To test the prognostic value of the patients’ characteristics in univariate analyses, OS curves were compared between groups using the log-rank test. Results are expressed as hazard ratios (HRs) and 95% confidence intervals (CIs).

Validation cohorts, obtained from the TCGA research network, were described in Ceccarelli *et al.*, , 2016. We retained *IDHmut* ($n=415$) and *IDHwt* ($n=134$) samples for which matched DNA methylation (HM450K array) and RNA expression (RNA-seq) data were available. Clinical and molecular data on these patients were retrieved from the cBioPortal for Cancer Genomics

(<https://www.cbiportal.org/>) (Gao *et al.*, , 2013; Cerami *et al.*, , 2012); processed RNA-seq and methylation data were obtained from the TCGA web site (<https://portal.gdc.cancer.gov/>) and analyzed as described below. The TCGA ID for each sample is provided in the supplemental table S5.

Genome reference

Arrays (HM450K and Cytoscan HD), as most of the databases used in this study, are based on the hg19 reference. Moreover, compared with GRCh37/hg19, a higher fraction of HM450K probes displays low mapping quality with the GRCh38 assembly (Zhou *et al.*, , 2017). Therefore, we used the hg19 as the genome reference in this study. Realignment to GRCh38 assembly should not significantly affect the conclusions of this work as we determined that among the 11,795 CGI/promoters, all being single CGI/promoters within the associated gene(s), studied here, more than 93 % remained single CGI/promoters (*i.e.*, overlap \pm 1kb with the TSS area) within the associated gene(s) in GRCh38. This proportion is maintained when considering only affected CGI/promoters.

Selection of the genes to be analyzed

The positions of genes and CpG islands (CGI: defined using standard criteria: *GC content* $\geq 50\%$; *length* $> 200bp$; *ratio Obs/Exp CpG* > 0.6) were downloaded from GENCODE annotation release V19, and CpG-island tracks of UCSC hg19 assembly, respectively. For each gene, the promoter region was defined as TSS \pm 1kb. To assess the relationship between CGI/promoter methylation status and gene expression, we first identified the genes associated with only one promoter with CGI features (n=15,350). As our cohort included both men and women, we then excluded genes located on the X or Y Chromosomes. We finally retained 14,714 genes in which the CGI/promoter is covered by at least two probes in the HM450K Illumina array.

DNA methylation analyses

- DNA extraction

DNA was isolated from frozen tissue samples using the QIAamp DNA Mini Kit (Qiagen, Hamburg, GmbH) according to the manufacturer's recommendations.

- Gene-specific bisulfite sequencing

Bisulfite conversion, PCR amplification, cloning, and sequencing were performed as previously described (Arnaud *et al.*, 2003). Details on the primers used are in Supplemental Table S6.

- HM450K analysis

The HM450K array data for controls and gliomas sample (8 normal brain, 55 *IDHwt* and 15 *IDHmut* gliomas) were analyzed as previously described (Maupetit-Méhouas *et al.*, 2018). Specifically, DNA bisulfite conversion and array hybridization were performed by Integragen SA (Evry, France), using the Illumina Infinium HD methylation protocol. β -values were computed using the GenomeStudio control interplate normalization and background subtraction (version 2011.1, manifest files: HumanMethylation450_1 5017482_v.1.2.bpm). For each sample, β -values

with a detection P-value >0.01 were excluded. All probes with a detection P-value >0.01 or lacking signal in more than 5% of our samples were excluded. Finally, 26,507 probes containing common SNPs (dbSNP 147) in their last 5bp or in the CpG sites were discarded. As our patient cohort included both men and women, probes on the X and Y Chromosomes were also excluded from the analysis. After the implementation of these quality filters, a total of 443,691 CpG methylation values were considered suitable for the downstream analysis. Methylation level at the 14,714 genes (11,795 CGIs) was given by the mean β -value of all probes located in their CGI. Differential methylation analyses were performed using the limma R package (Ritchie *et al.*, 2015), as previously described (Court *et al.*, 2014). These analyses concerned the entire groups (8 controls vs 55 *IDHwt*, and 8 controls vs 15 *IDHmut*) or only the samples with matched RNA-seq data (3 controls vs 8 *IDHwt* and 3 controls vs 5 *IDHmut*). The HM450K probes were considered differentially methylated when the FDR was <0.05 and when the β -value difference between groups was >0.1 . To consider only robust methylation variations, CGI/promoters were classified as hyper-methylated or hypo-methylated only if they included at least two probes differentially methylated (gain or a loss of methylation) in their CGI.

To test the robustness of this strategy we performed again the analyses by using Wilcoxon test, instead of limma (Ritchie *et al.*, 2015), and by including the filters described in Zhou *et al.*, (Zhou *et al.*, 2017). For both *IDHwt* and *IDHmut* samples, we identified the same four groups of defects. At the statistical level, the affected probes displayed more than 91% of homology with both tests. In addition, the percentage of affected genes for each defects only marginally changed between our initial conditions and these conditions (Supplemental file S1: Fig. S12).

- **5hmC analysis by data mining**

5hmC data from 30 *IDHwt* glioma samples were retrieved from Jonhson *et al.*, 2016 (GSE73895). β -values, derived from HM450K arrays hybridized with DNA after conversion with oxidative bisulfite (oxBS) and bisulfite only (BS), were computed as described above. The level of 5hmC for each probe and sample was obtained by subtracting the β -values of oxBS samples from their corresponding BS pair.

Expression analysis

- **RNA extraction**

RNA was isolated from frozen tissue samples using the RNeasy Mini Kit (Qiagen, Hamburg, GmbH), according to the manufacturer's recommendations.

- **RT-qPCR expression analyses**

RT-qPCR assays were performed using a microfluidic-based approach, as previously described (Maupetit-Méhouas *et al.*, 2016). First-strand cDNA was pre-amplified for 14 cycles with the pool of primers used for RT-qPCR and the TaqMan PreAmplification Master Mix (Life Technologies, 4488593). Primer sequences are in Supplemental Table S6. RT-qPCR assays were then performed and validated using Fluidigm 96.96 Dynamic Arrays and the Biomark HD system (Fluidigm Corp.),

according to the manufacturer's instructions. The relative expression level was quantified with the 2-dCt. The housekeeping genes *PPIA*, *TBP* and *RPL13A* were used to normalize transcript expression. Each analysis was performed in duplicate.

- **RNA-sequencing**

RNA-seq was performed using total RNA after ribosomal RNA depletion (Ribo-Zero rRNA Removal Kit, Illumina) from 3 brain, 8 *IDHwt* and 5 *IDHmut* glioma samples. RNA processing, rRNA depletion, library preparation and sequencing on an Illumina HiSeq 2500 apparatus were performed by Integragen SA (Evry, France), according to the manufacturer's protocol (mean of 90 million of paired reads per sample). Stranded RNA-seq reads were mapped to the human genome (hg19) using TopHat2 (version 2.1.0) and a transcript annotation file from GENCODE (Release 19) (Kim *et al.*, 2013). The average alignment rate was about 94.5% with a concordant pair alignment rate of 92%. Only properly paired reads were considered for downstream analysis. The reads count per gene was obtained with the HTseq-count script (option: -m intersection-nonempty -s reverse), and the FPKM gene expression level was determined with Cuffquant and Cuffnorm from the Cufflinks suite (version 2.2.1), based on GENCODE V19 transcript annotation (Anders *et al.*, 2015, Trapnell *et al.*, 2010). Strand-specific RNA-seq signals were derived from the RNA-seq alignments using Samtools, genomeCoverageBed and bedGraphToBigWig tools, and visualized using the UCSC Genome Browser (Li *et al.*, 2009; Quinlan and Hall, 2010 ; Kent *et al.*, 2010). Differential expression analyses between controls and glioma samples were based on reads counts using the DESeq2 and edgeR R packages (Love *et al.*, 2014, Robinson *et al.*, 2010). Genes were considered as differentially expressed between groups when $|\log_2\text{-fold change}| > 2$ with an adjusted P-value < 0.05 in both statistical approaches.

- **Gene expression data mining**

Gene expression levels in several human tissues were obtained from the Roadmap Epigenomics project (https://egg2.wustl.edu/roadmap/web_portal/processed_data.html#RNAseq_uni_proc). The transcription levels in different brain regions were retrieved from the GEO database (accession number GSE33587).

Chromatin analyses

- **Chromatin immuno-precipitation from glioma samples**

Anti-H3K9ac (Millipore 06-942), -H3K4me3 (Diagenode 03-050) and -H3K27me3 (Millipore 07-449) antibodies were used to assess the respective marks at selected genes by ChIP of native chromatin isolated from glioma samples and controls, as described in (Maupetit-Méhouas *et al.*, 2016). Input and antibody-bound fractions were quantified by real-time PCR amplification with the SYBR Green mixture (Roche) using a LightCycler 480II (Roche) instrument. Background precipitation levels were determined by performing mock precipitations with a non-specific IgG antiserum (Sigma-Aldrich C-2288), and were only a fraction of the precipitation levels obtained with the specific antibodies. The bound/input ratios were calculated and normalized to the precipitation

level at the *TBP* promoter for the anti-H3K9ac and -H3K4me3 ChIPs and at the *SP6* promoter for the anti-H3K27me3 ChIP. The primers used are described in Supplemental Table S6.

- **ChIP-seq data mining associated with chromatin analyses**

ChIP-seq data from NPC and brain samples were from the NIH Roadmap Epigenomics project (<http://www.roadmapepigenomics.org/>). In detail:

- For H9-derived Neural Progenitor Cells (NPC): Input (GSM772805); H3K4me3 (GSM772736), and H3K27me3 (GSM772801)
- In brain: Input (GSM772991), H3K4me3 (GSM772996), H3K27me3 (GSM772993) and H3K9me3 (GSM670005)

ChIP-seq data for the H3K4me3 and H3K27me3 profiles in glioma-derived cells were obtained from the GEO database (accession numbers: GSM1121888 and GSM1121885, respectively).

To describe the histone modification enrichment in each defect group, the ChIP-seq read densities around TSS (± 2 kb) was represented by a heatmap where each line represents one single promoter. The mean signal around TSS (± 2 kb) for each defect group was compared with the mean signal for all genes included in this study ($n=14,714$) to correct for the bias due to the use of different datasets.

- **Gene classification according to their chromatin signature**

The gene classification according to their chromatin signature (bivalent, H3K4me3-only, H3K27me3-only and none) in human ES cells is from (Court and Arnaud, 2017). For NPC and brain samples, this classification was performed as previously described in (Court and Arnaud, 2017). Briefly, data for input, H3K4me3 and H3K27me3 ChIPs were aligned to the hg19 genome assembly. Peaks were then called with MACS 1.4.2 using the input as control for peak detection (Zhang *et al.*, 2008). Chromatin signatures were classified using an in-house R scripts. Specifically, a bivalent region was defined by the overlapping of H3K4me3 and H3K27me3 peaks for at least 1kb. H3K4me3-only and H3K27me3-only regions were identified as 1 kb peaks for H3K4me3 or H3K27me3 that did not overlap. The rest of the genome was considered as having a “none” chromatin signature. To attribute a chromatin signature to CGI/promoter regions, only the signature spreading across the CGI region was retained.

Copy-number variation (CNV) analyses

CNV analyses were performed using the Genome-Wide Human CytoScan HD Array (Affymetrix, CA, USA) and the samples analyzed by RNA-seq (3 controls, 8 *IDH*wt and 5 *IDH*mut glioma samples). DNA and array data were processed by the Genomic Platform/Curie Institute (France) according to the manufacturer's protocol. Arrays were scanned using an Affymetrix GeneChip Scanner 3000 7G. Scanned data files were analyzed with Affymetrix Chromosome Analysis Suite v3.1 (ChAS) (Affymetrix Inc., USA) using the CytoScanHD_Array.na33.annot.db annotation file on the hg19 genome. To find CNVs, single samples were analyzed using the reference model file “CytoScanHD_Array.na33.r2.REF_MODEL”. To prevent the detection of false-positive CNVs, only alterations that involved at least 50 consecutive probes and spanning more than 100 kb were considered in the analysis. To evaluate genetic alterations at gene level, the mean \log_2 ratio of the CNV fragment overlapping with a CGI/promoter region was assigned to each gene. For genes with a CGI/promoter region not covered by a CNV fragment, the mean \log_2 ratio was considered as null.

Positive and negative mean \log_2 ratios were used to categorize duplicated and deleted regions, respectively. To identify genes the expression alteration of which correlates ($p < 0.05$) with CNV changes, the mean \log_2 ratio of the CNV values and the normalized pseudo RNA-seq counts for each transcriptionally affected gene in the same sample were compared using the Pearson's correlation.

Principal component analysis (PCA)

The PCA was done with the FactoMineR package using molecular features (histones modifications, DNA methylation and expression) associated with genes from Meth+/Exp- and No Meth/Exp- groups in brain (Le *et al.*, 2008). For each gene, the histone modification levels at the CGI/promoter region (TSS ± 1 kb) were based on the average ChIP-seq signal for H3K4me3, H3K27me3 and H3K9me3 obtained in brain samples. The DNA methylation levels were determined by the \log_2 value of the mean β -value, obtained from 8 brain control samples, for all the probes located in the CGI of that gene. For the transcriptional level, the average FPKM value from 3 brain control samples was \log_2 transformed. As the different variables used were defined by different measure units, they were standardized all before the PCA analysis (i.e., centered and scaled).

Functional annotations

InterPro protein functional classification analysis was performed using the functional annotation tools in DAVID 6.8 (<https://david.ncifcrf.gov/>) (Huang *et al.*, 2009). Gene Ontology analyses were done with the GeneRanker tools of the Genomatix suite (<http://www.genomatix.de/>). To identify putative regulatory features linked to the transcriptional defect groups, the CGI positions in genes were analyzed with i-cis Target (<https://gbiomed.kuleuven.be/apps/lcb/i-cisTarget/>) (Herrmann *et al.*, 2012). The comparative analysis tools were then used to identify specific binding sites. As transcription factors could have multiple position weight matrices (PWM), all Normalized Enrichment Scores (NES) given by i-cis Target for a factor were displayed in a boxplot. The tumor-suppressor gene and oncogene lists were obtained from www.ongene.bioinfo-minzhao.org, www.cta.lncc.br and www.uniprot.org with the keywords tumor suppressor (KW-0043) and proto-oncogene (KW-0656).

Data Access

Data generated in this study have been submitted to the NCBI Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) under the accession numbers:

- GSE123678 for the the HM450K DNA methylation data
- GSE123682 for the Cytoscan HD data
- GSE123892 for the oriented RNA-seq data

Custom R scripts used to perform this study are available as Supplemental Code.

Acknowledgments

We thank the CHU Clermont-Ferrand's Neurosurgery department (Prof. JJ. Lemaire) for its help in completing this study, the Platform "Gentyane" (<http://gentyane.clermont.inra.fr/>) for its technical help with the Fluidigm 96.96 Dynamic Arrays, and Zachary-LoL-Arnaud for its precious input. We also thank Dr David Monk and all members of PA's team for critical reading of the manuscript.

Funding

This study was funded by the Plan Cancer-INSERM (CS14085) (to LKT, PV and PA), the Cancéropole CLARA (Oncostarter «Gliohoxas») (to PA), the Fonds de dotation Patrick Brou de Laurière (to PA), the Ligue Contre le Cancer comité de l'Ardèche et du Puy de Dôme (to FC & PA). FC and ELB had a fellowship from Université Clermont Auvergne and La Ligue Nationale Contre le Cancer, respectively. This research is supported by the French government IDEX-ISITE initiative 16-IDEX-0001 (CAP 20-25).

Authors' contributions

FC and PA initiated and supervised the study. FC, AF, CVB, EC, LKT, PV and PA designed the study. TK collected glioma samples from the operating room. JLK, JB, EC and PV characterized the glioma samples and updated the clinical follow-up of the patients. JB and EC collected the patients' data. BP conducted the patients' data statistical analyses. FC, ELB, AF, MMB and CVB performed the experiments. FC performed the bioinformatics analyses. FC, ELB, AF, MMB, CVB, EC and PA analyzed the data. FC produced the figures with CVB and PA input. PA wrote the paper with FC input. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Figure Legends:

Figure 1: Aberrant methylation at CGI/promoters is not the main contributor to transcriptional alteration in glioma

A) Classification of the 14,714 genes analyzed in this study. **B)** DNA methylation level (mean β -values) of the 11,795 CGI/promoters (row) analyzed in *IDHmut* and *IDHwt* glioma and control (normal brain tissue) samples (columns). Left columns show their hyper- or hypo-methylation status in *IDHmut* and *IDHwt* glioma samples compared with controls. **C-D)** Differential expression status of genes associated with hyper- (**C**) or hypomethylated (**D**) CGI/promoters in *IDHwt* (left panel) and *IDHmut* (right panel) glioma samples compared with controls.

Figure 2: Extent of transcriptional alterations in *IDHwt* and *IDHmut* glioma samples.

A) Volcano plot analysis of differential gene expression in *IDHwt* (left) or *IDHmut* (right) glioma samples. Blue and red dots represent genes that were significantly down- or up-regulated, respectively, compared with healthy controls (n=14,714 genes analyzed). **B)** Circular karyotype showing the duplication (in red) and deletion (in blue) frequencies at the 14,714 analyzed genes in *IDHwt* (outer circles) and *IDHmut* (inner circles) samples. Genes showing a significant correlation between CNV and expression are symbolized by an orange (up-regulated) or green (down-regulated) line. **C)** Correlation analysis between CNV and expression levels for the *EGFR* and *HOXA13* genes in *IDHwt* (yellow dots, left panels) and *IDHmut* (blue dot, right panels) glioma samples. Black dots indicate value in healthy controls. *EGFR* overexpression correlated with increased copy number in *IDHwt* glioma samples. **D)** Classification of the genes with expression alterations that did not correlate with CNV.

Figure 3: Four expression defect classes

A) Integrative analysis of differential gene expression and methylation in 8 *IDHwt* glioma samples identified four main defect classes: Gain of expression with gain of methylation (Meth+/Exp+), gain of expression with CGI/promoter remaining unmethylated (No Meth/Exp+), loss of expression with gain of methylation (Meth+/Exp-), and loss of expression with the CGI/promoter remaining unmethylated (No Meth/Exp-). **B)** Differential DNA methylation analysis in all *IDHwt* glioma samples (n=55) vs controls (n=8) (delta of the mean β -value). Glioma samples were grouped in the four classes of expression defects defined in **A**). The methylated and methylable status of genes is indicated in the left column. **C)** Integrative analysis of differential expression and methylation at selected Meth+/Exp+ (upper panel), No Meth/Exp+ (middle panel), and No Meth/Exp- (lower panel) genes in 42 *IDHwt* glioma samples compared with controls (n=8). **D)** Integrative analysis of differential gene expression and methylation in an independent cohort of 135 *IDHwt* glioma samples (validation cohort) also identified the four main defect classes. Odds ratio and significance of the overlap (Fisher's exact test) between the data of the validation cohort and our cohort, for each defect category, are shown on the right panel.

Figure 4: Genes with bivalent chromatin signature in ES cells are more prone to be deregulated in *IDHwt* glioma.

A) Gene Ontology terms (biological processes) enriched in genes from the four defect categories. For each category, the four highest terms are shown. **B)** Distribution of genes of each defect category according to their chromatin signature in human ES cells (none: gray; bivalent: black; H3K4me3-only:

blue; H3K27me3-only: red). As reference, the distribution of the 14,714 genes analyzed in this study according to their chromatin signatures in human ES cells is shown in the left panel. **C)** Expression level and chromatin signatures of genes of the four defect categories in human ES cells, neural progenitor cells (NPC) and healthy brain. For comparison, the same analysis is provided on the right panel for genes without expression defect (unaffected) in *IDHwt* glioma samples.

Figure 5: Expression from genes with methylated CGI/promoter

A) Data-mining derived ChIP-seq read density data for H3K27me3 (purple) and H3K4me3 (blue) in genes with defect 1 in a ± 2 kb window centered on their TSS, in healthy brain (left panel) and *IDHwt*-derived cell lines (right panel). The mean ChIP-seq signal values are shown on the lower panels for “Meth+/Exp+” genes (red line) and for the 14,714 analyzed genes (black line) that were used as normalized reference. **B)** Heatmap showing CpG sites density and their mean methylation level in a ± 2 kb window centered on the TSS of genes with defect 1 and enriched (upper panel) or depleted (lower panel) for H3K4me3 in *IDHwt* glioma samples, compared with healthy controls. The ChIP-seq read density obtained in *IDHwt*-derived cell lines is shown on the right panels. **C)** Genome browser view at the *TWIST1* and *FOXD3* loci to show H3K4me3 enrichment, differential DNA methylation, and the oriented RNA-seq signal. These two loci are representative of genes that initiate from an H3K4me3-marked TSS embedded in a methylated CGI/promoter in *IDHwt* samples. **D)** *HOXC11* is representative of genes in which expression initiates from an alternative TSS in *IDHwt* glioma samples.

Figure 6: Transcription factor binding motifs in the promoters of genes overexpressed in glioma samples

A) Data-mining derived ChIP-seq read density data for H3K27me3 (purple) and H3K4me3 (blue) at “No Meth/Exp+” genes in a ± 2 kb window centered on their TSS, in healthy brain (left panels) and *IDHwt*-derived cell lines (right panels). The mean ChIP-seq signal values are shown on the lower panel for all class 2 defect genes (orange line) and for those that are (dotted green line) or not (dotted pink line) marked by H3K4me3-only in ES cells, NPC and brain. The black line, used as normalized reference, shows the value for all analyzed genes. **B)** Transcription factor motif enrichment in the CGI/promoter of “No Meth/Exp+” genes, calculated using *i-cis* Target and represented as a Normalized Enrichment Score (NES). Enrichment is shown for genes that are (green squares) or not (red squares) marked by H3K4me3-only in ES cells, NPC and brain. When a transcription factor possesses several binding motifs, data are presented as a box plot. **C)** Expression status, assessed by RNA-seq, of the transcription factors identified in **B)**. The middle column shows their expression status in healthy control (n=5) (white, not expressed; gray, expressed: fpkm>1) and the right column their expression in *IDHwt* glioma samples (n=8). The left column shows the motif enrichment in all “No Meth/Exp+” genes (black), and those with H3K4me3-only (green) and without (red) H3K4me3-only (red) in ES cells, NPC and brain. **D)** Expression *vs* controls of selected overexpressed transcription factor identified in **C)** assessed by RT-qPCR in 42 *IDHwt* glioma samples. Details for each sample are provided in the lower panel (p-value by Mann-Whitney *U* test).

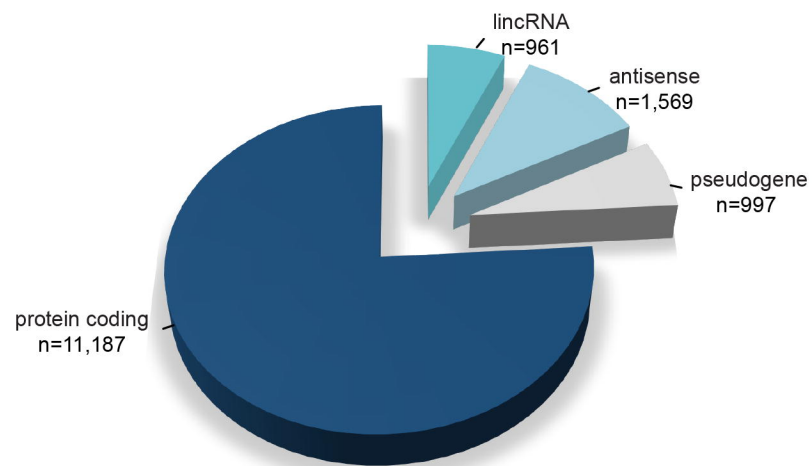
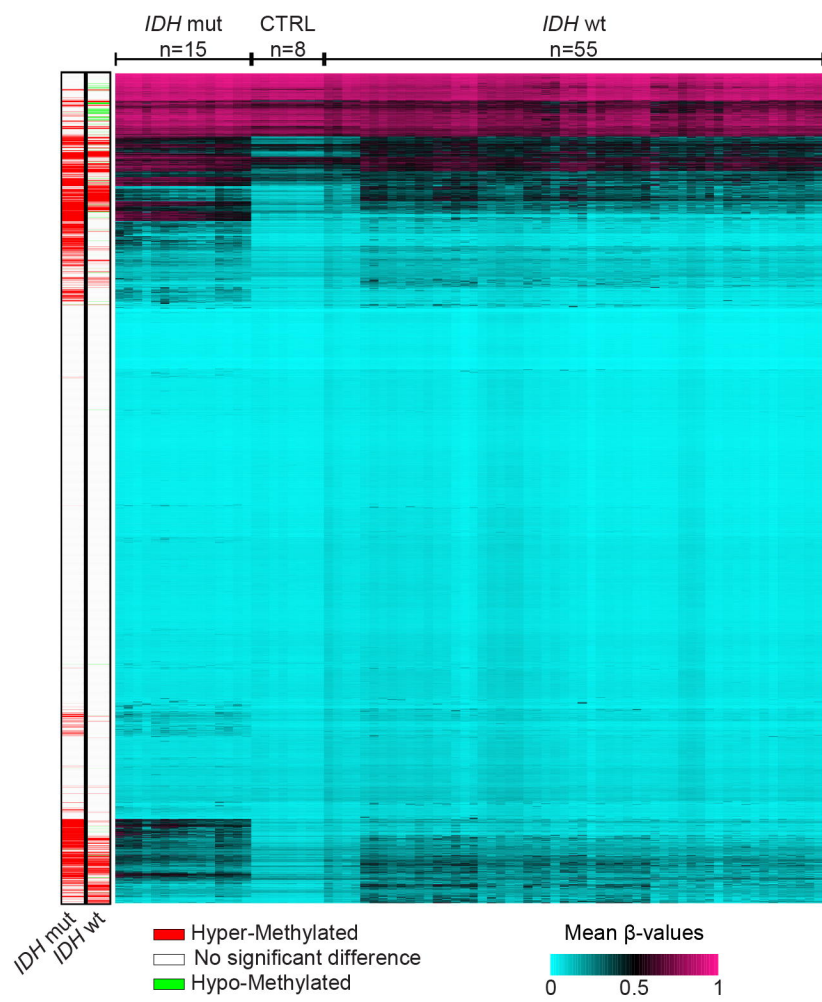
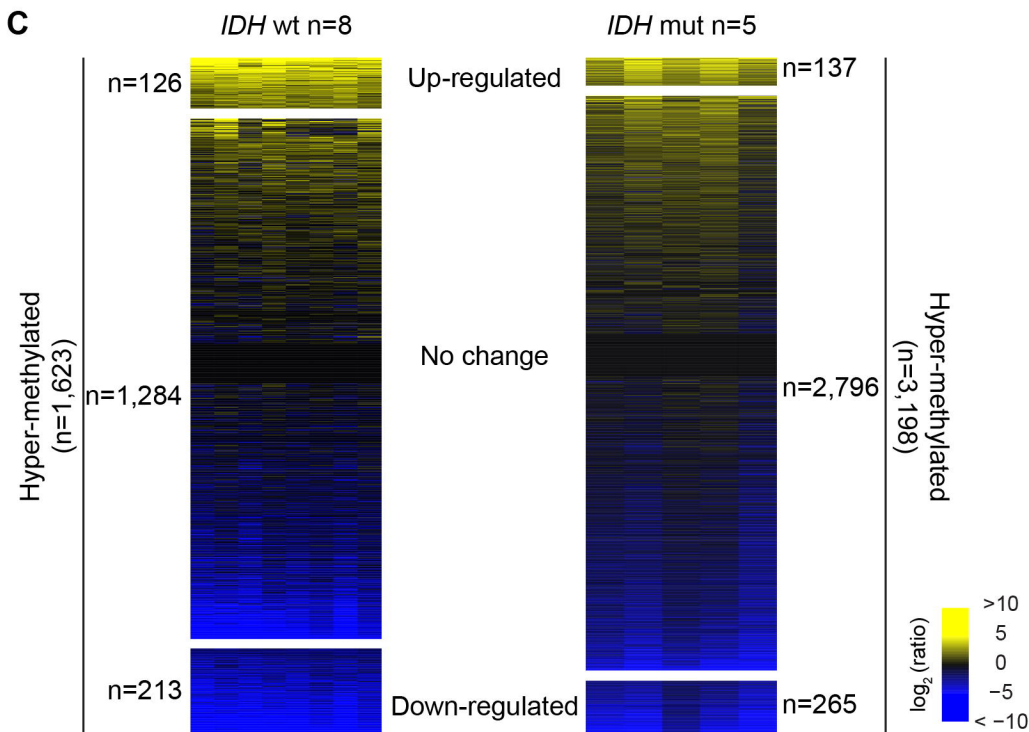
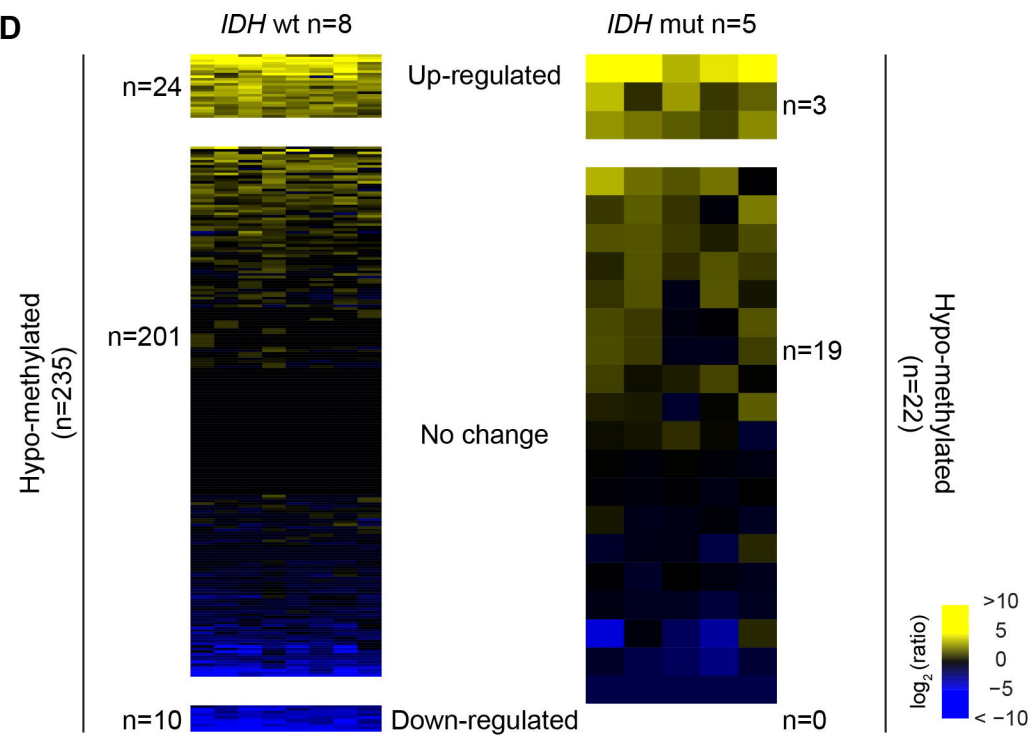
Figure 7: Gene repression is associated with H3K27me3 gain

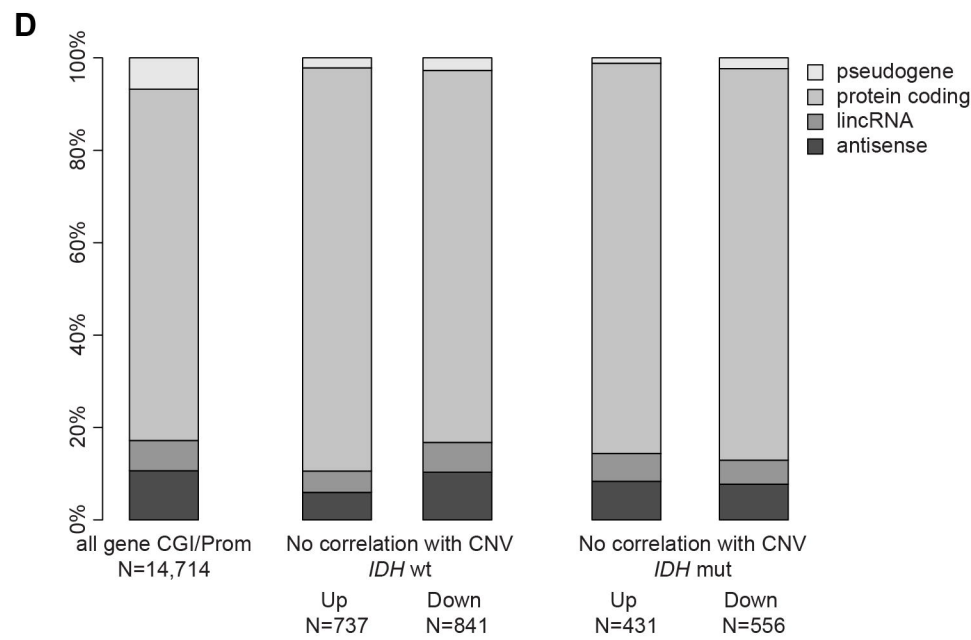
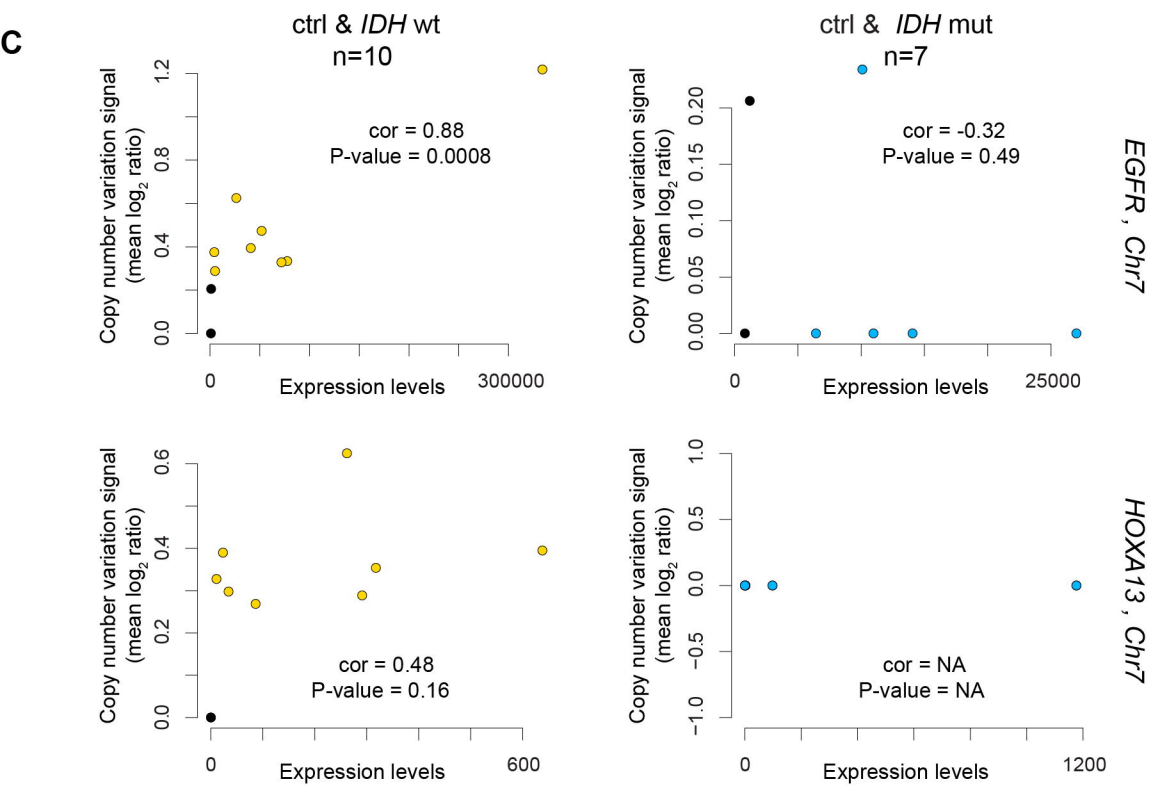
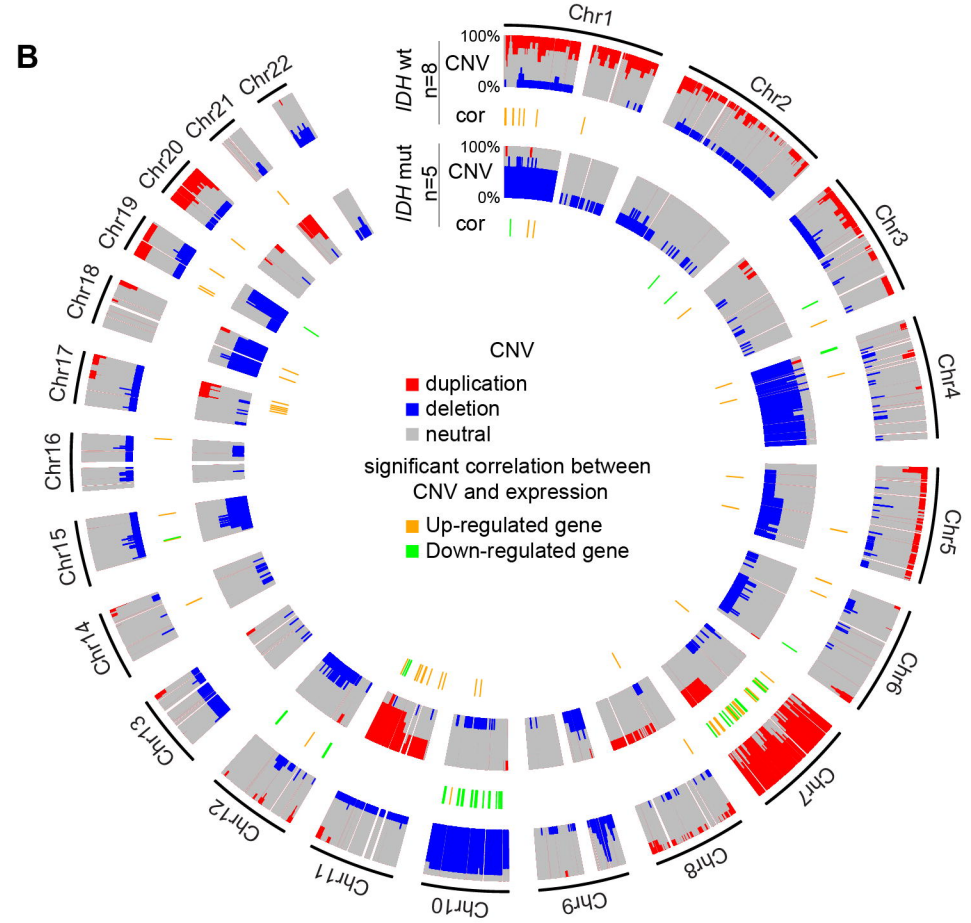
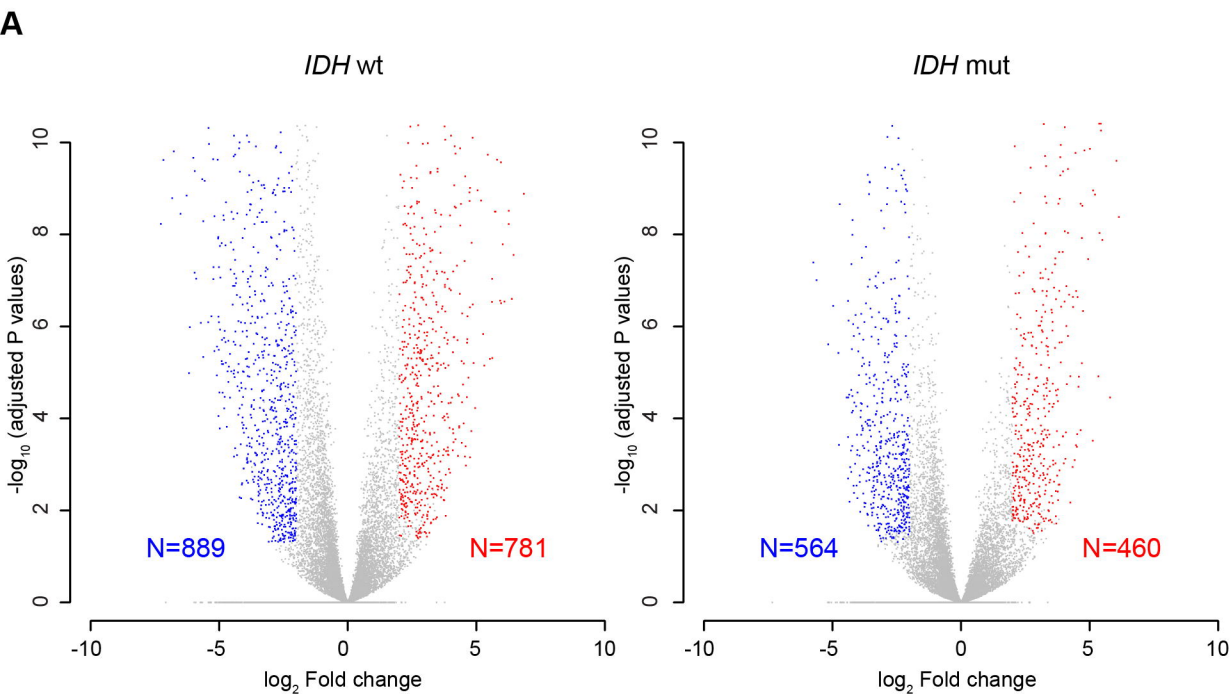
A) Data-mining derived ChIP-seq read density data for H3K27me3 (purple) and H3K4me3 (blue) at “Meth+/Exp-” and “No Meth/Exp-” genes in a ± 2 kb window centered on their TSS, in healthy brain (left panels) and *IDHwt*-derived cell lines (right panels). The mean ChIP-seq signal values are shown in the lower panels for “Meth+/Exp-” (purple line) and “No Meth/Exp-” (blue line) genes. Genes in the “No Meth/Exp-” group were further subdivided in genes marked (dotted light blue line) and not marked (dotted dark blue line) by H3K4me3-only in ES cells, NPC and brain. The black line used as normalized reference shows the value for all analyzed genes. **B)** ChIP analysis of H3K9ac, H3K4me3 and H3K27me3 at selected genes in *IDHwt* (n=7) and control (n=5) samples. The precipitation level was normalized to that obtained at the *TBP* promoter (for H3K4me3 and H3K9ac) and at the *SP6* promoter (for H3K27me3) (p-values calculated with the GENCODE *U* test). **C)** Detail for each

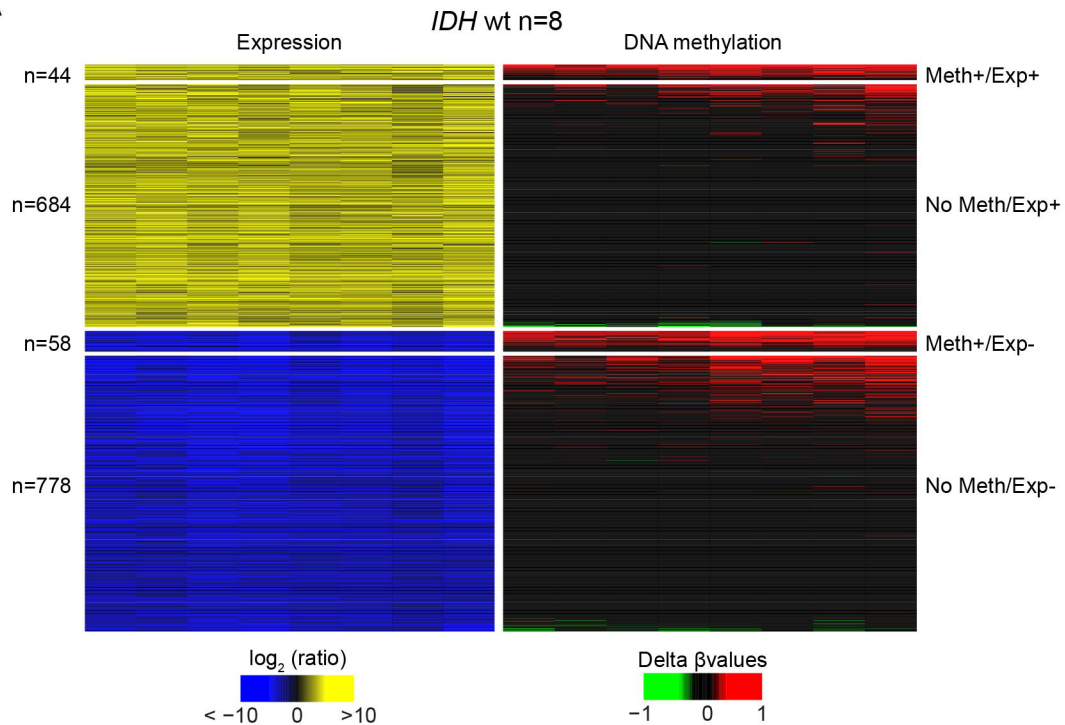
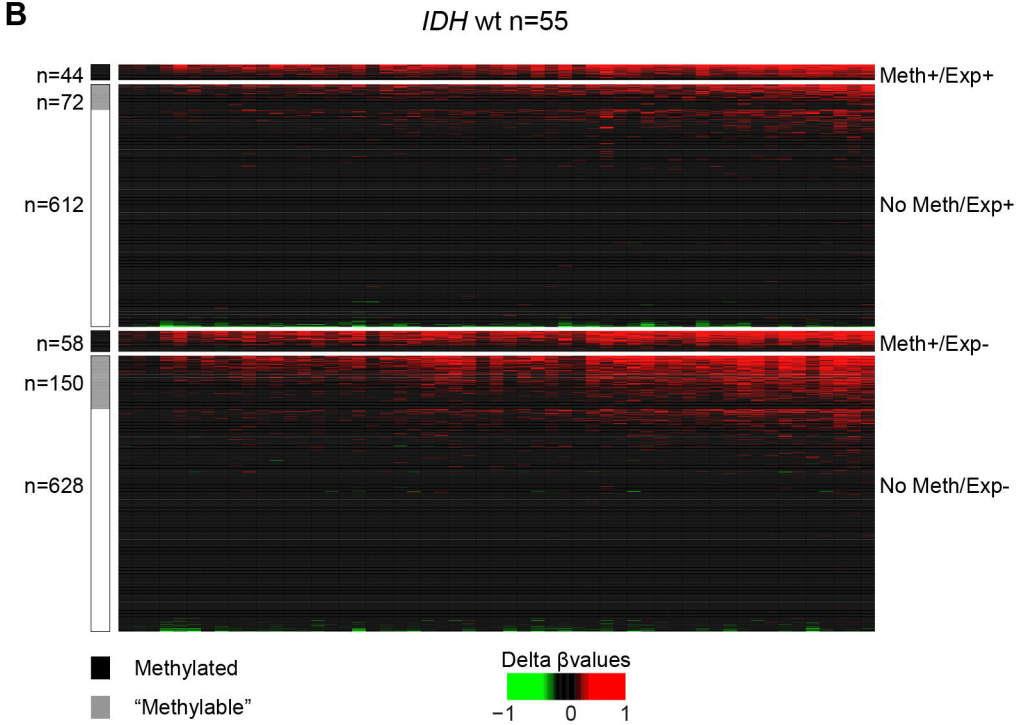
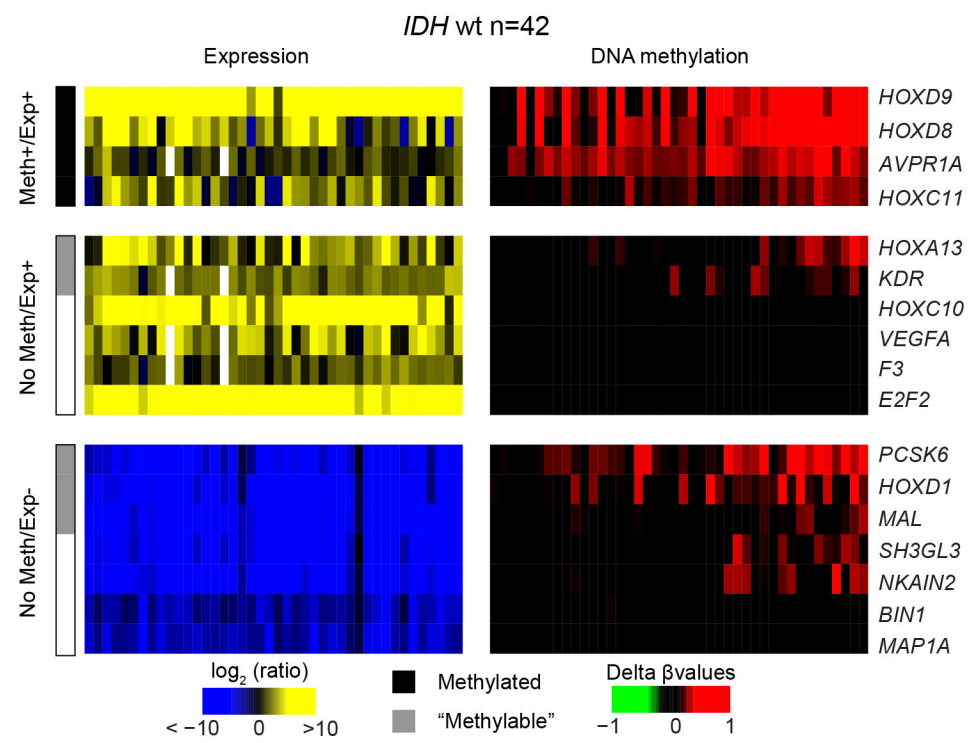
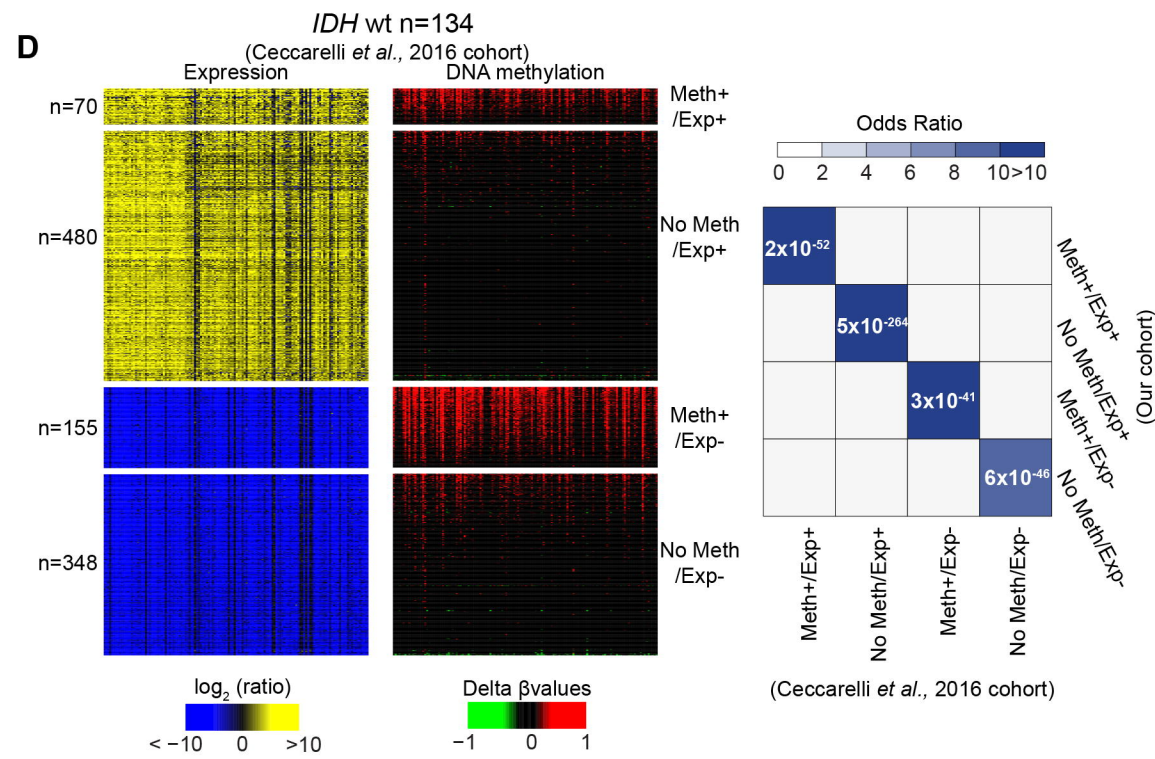
samples of the ChIP analysis at the *PCSK6* locus. Heat maps of the expression and methylation values are in the upper panel. **D**) Expression level of “Meth+/Exp-” (purple column) and “No Meth/Exp-” (blue column) genes and of all analyzed genes (white column) in healthy controls. **E-F**) Principal component analysis. Two-dimensional scatter plot of the values of each “Meth+/Exp-” (purple dots) and “No Meth/Exp-” gene (blue dots) along the 1st (Dim 1) and 2nd (Dim 2) principal component (**E**). For each class defect, the centroids are shown by colored squares. **F**) H3K4me3 and expression levels in healthy brain are the variables that most contributed to and were significantly correlated with the first principal component.

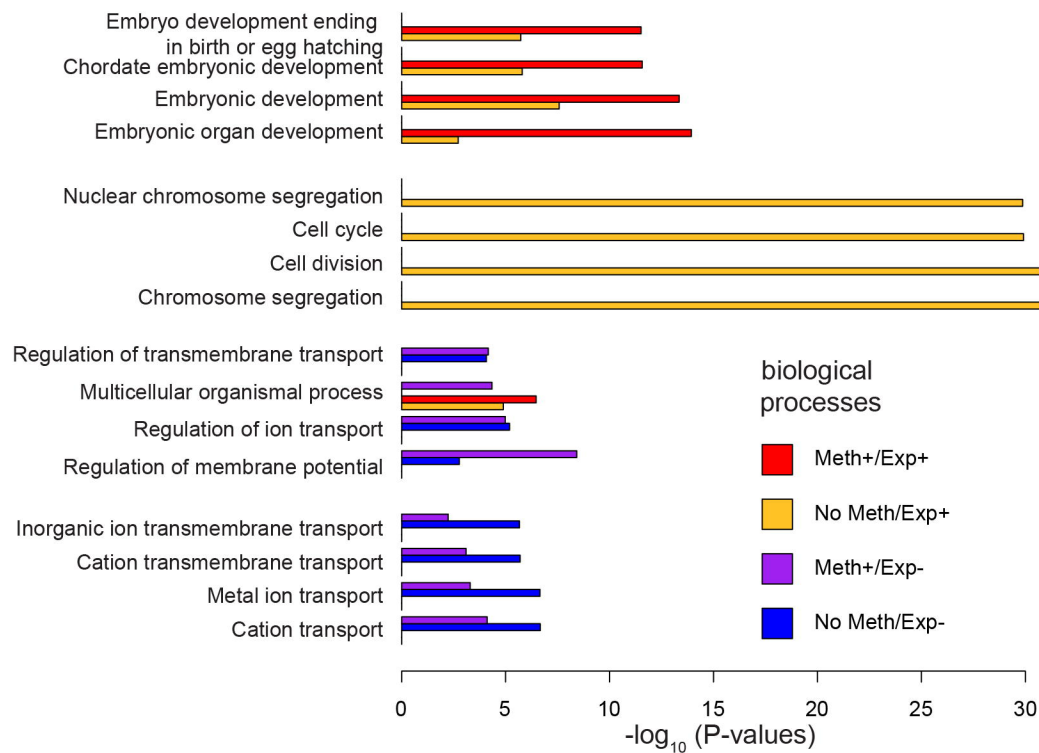
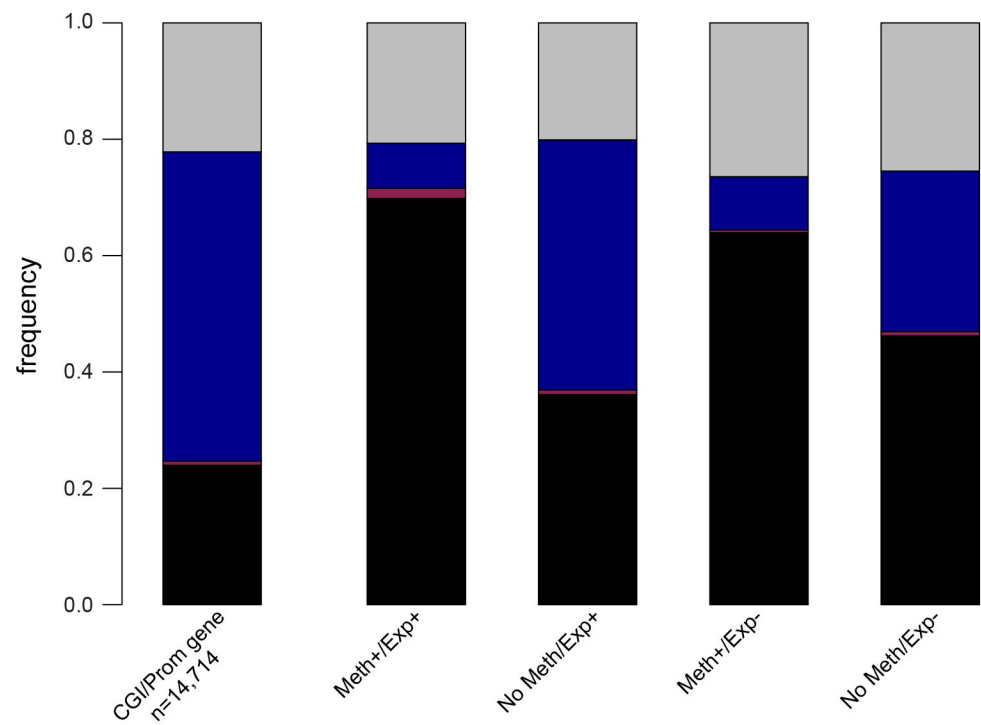
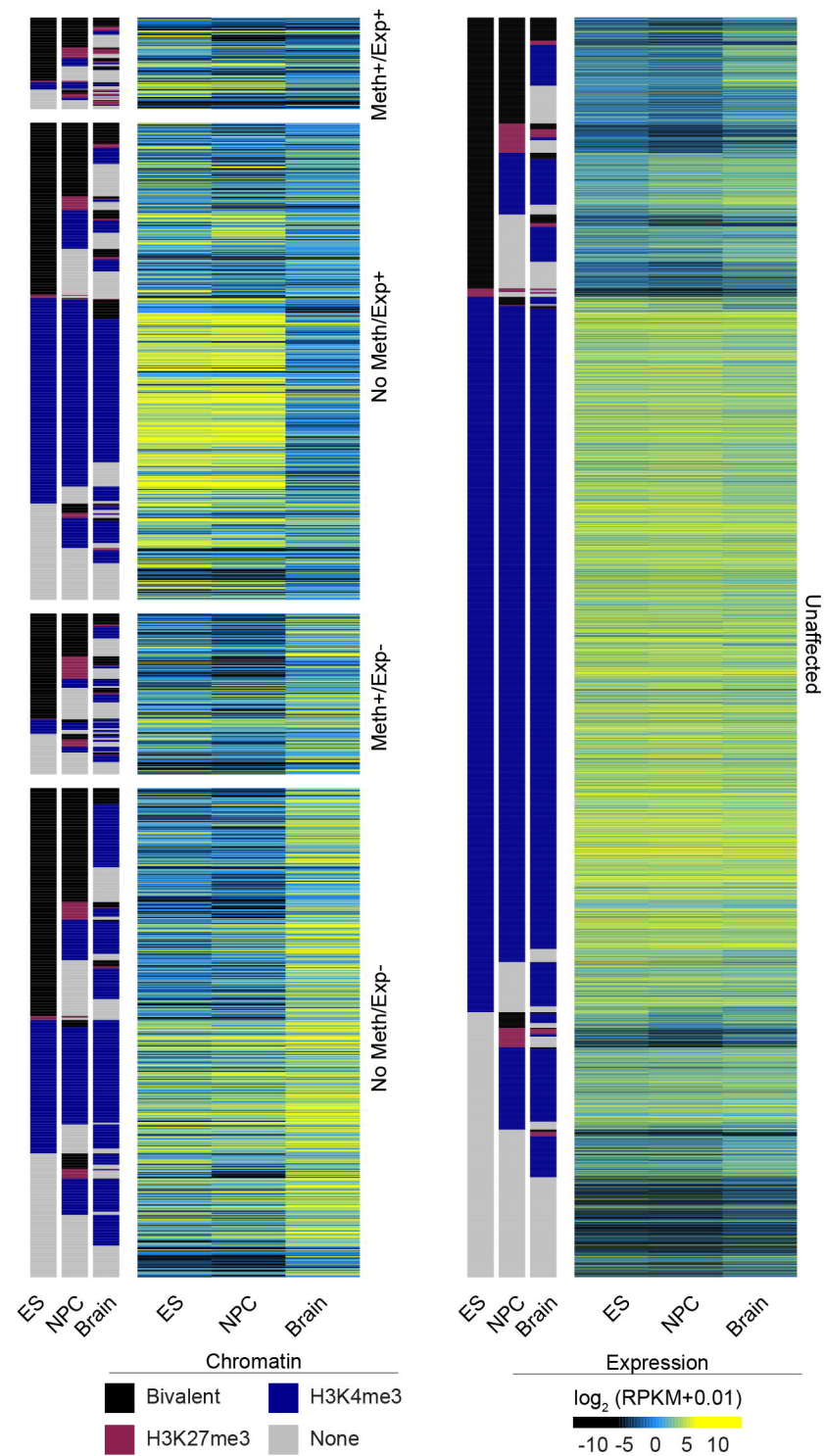
Figure 8: Working model.

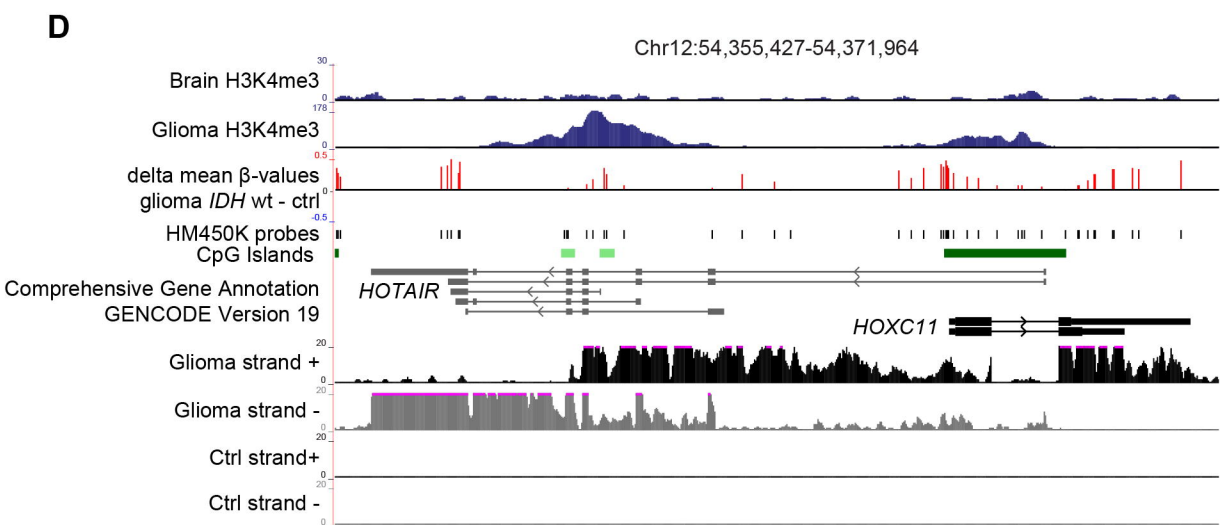
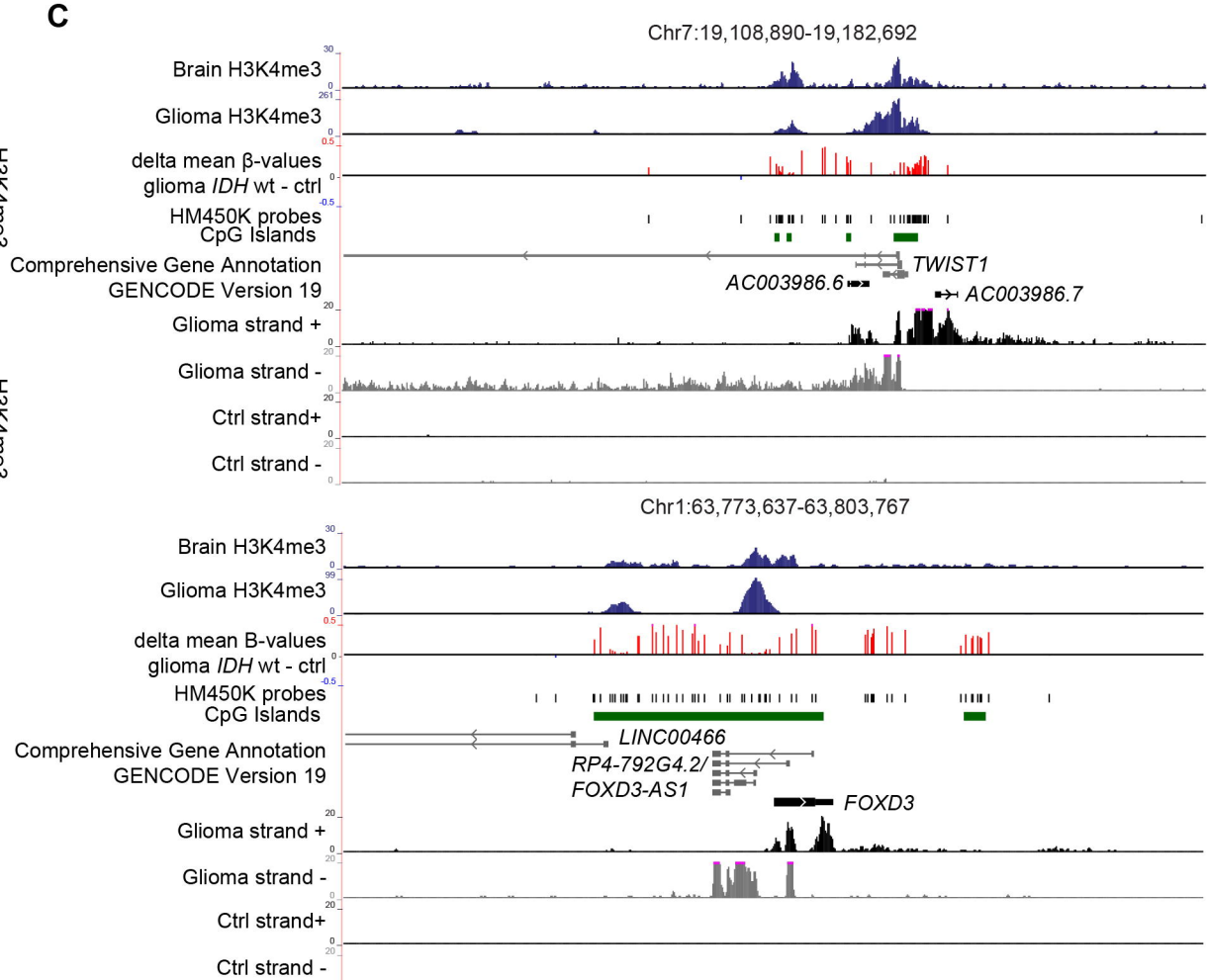
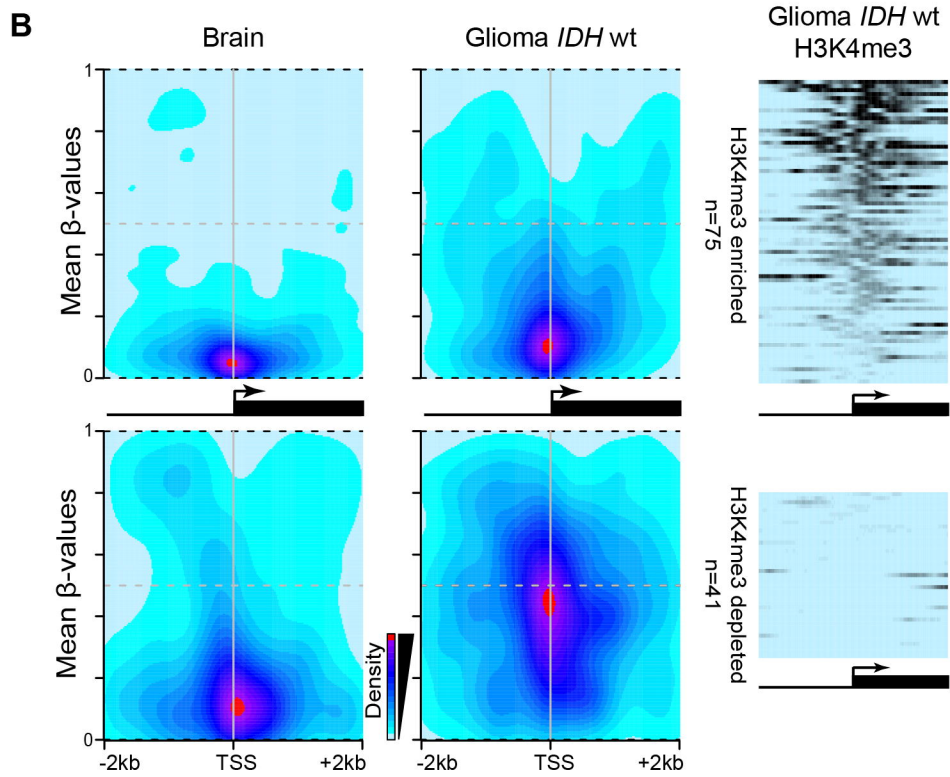
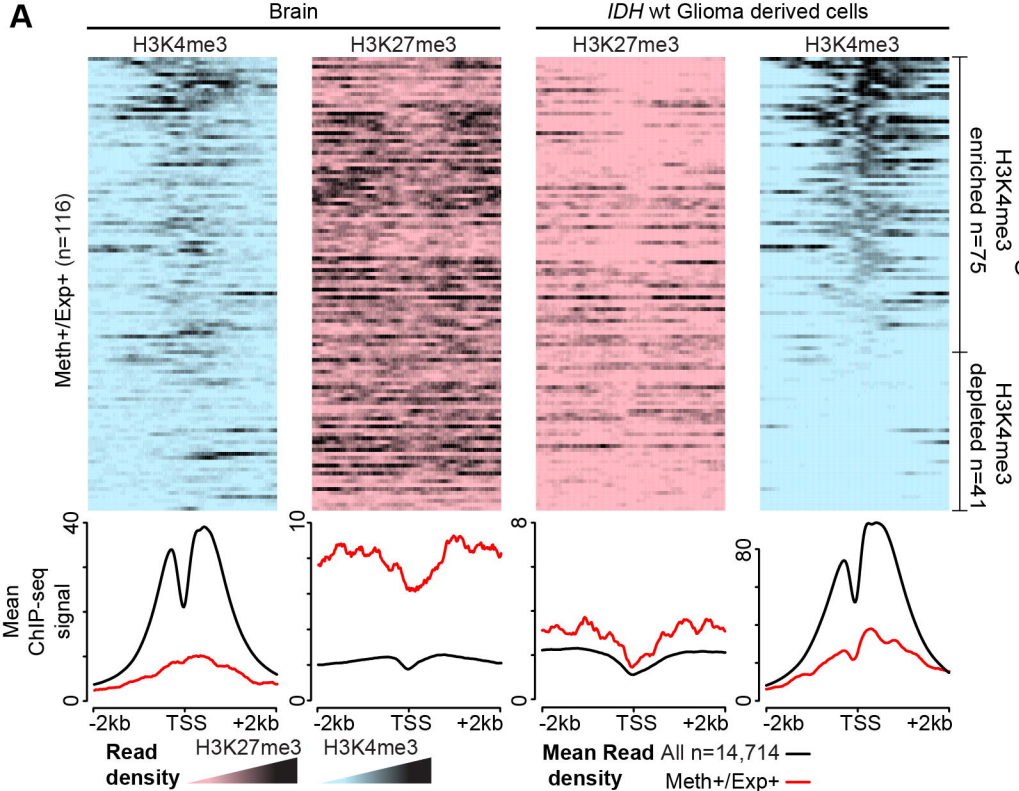
A) In glioma, alterations in the control of the H3K27me3 signature could be one of the main contributors to the four types of transcriptional defects observed at CGI/promoter-controlled genes (upper panels). In this model, genome-wide hypomethylation induces H3K27me3 redistribution that could lead to ectopic expression of genes that are normally repressed by polycomb proteins, including some genes encoding transcription factors. These overexpressed transcription factors could then promote the aberrant expression of their target genes (dotted arrow). Similarly, alterations in the interplay between the polycomb complex and the transcriptional machinery could affect H3K27me3 fate during ES and/or neural stem cell differentiation. Specifically, this alteration could lead to the aberrant maintenance of bivalency and silencing at a subset of genes that are normally specifically expressed in brain. At genes that are normally poorly expressed in healthy brain, this process is associated with gain of DNA methylation in glioma. Beside defects in the H3K27me3 signature, we also identified a subset of genes that are apparently constitutively associated with H3K4me3-only, regardless of their expression status in brain and glioma (lower panel). The mechanisms underlying their transcriptional deregulation remain to be determined. **B**) Percentage of unaffected and affected CGI/promoter-controlled genes for each of the four defects described in the article, in *IDH*wt and *IDH*mut glioma samples from our cohort.

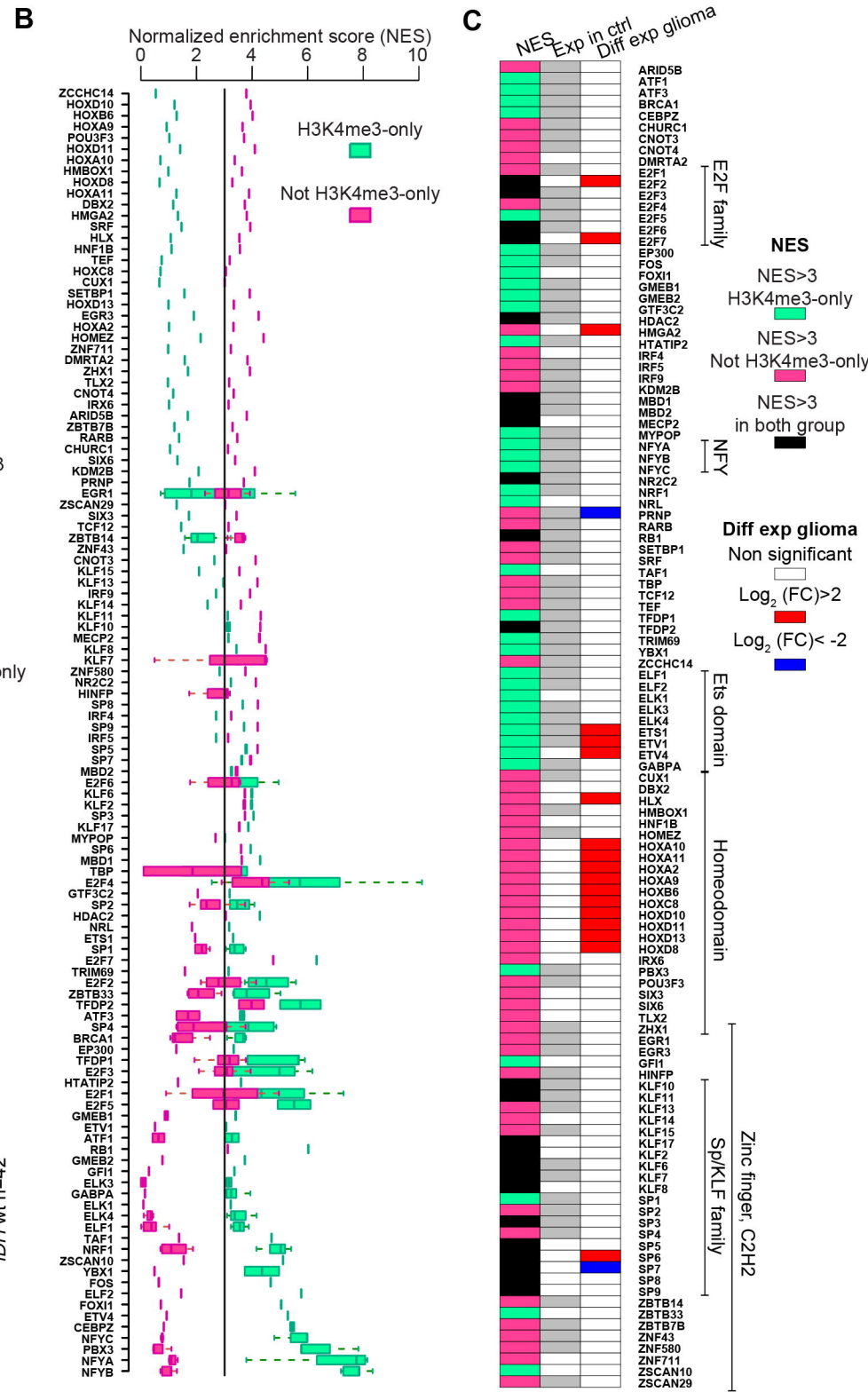
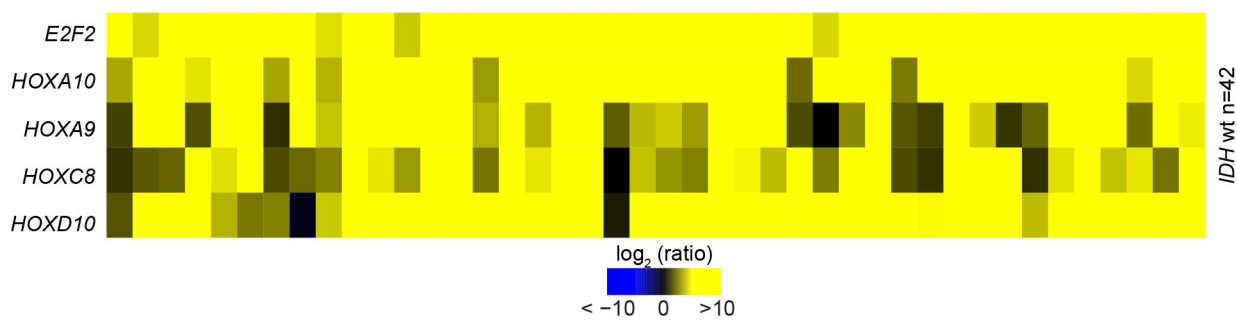
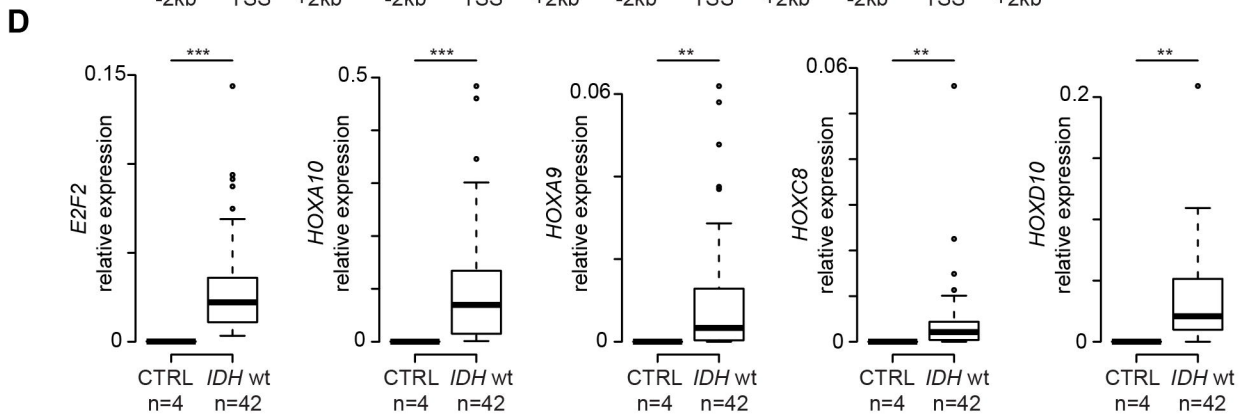
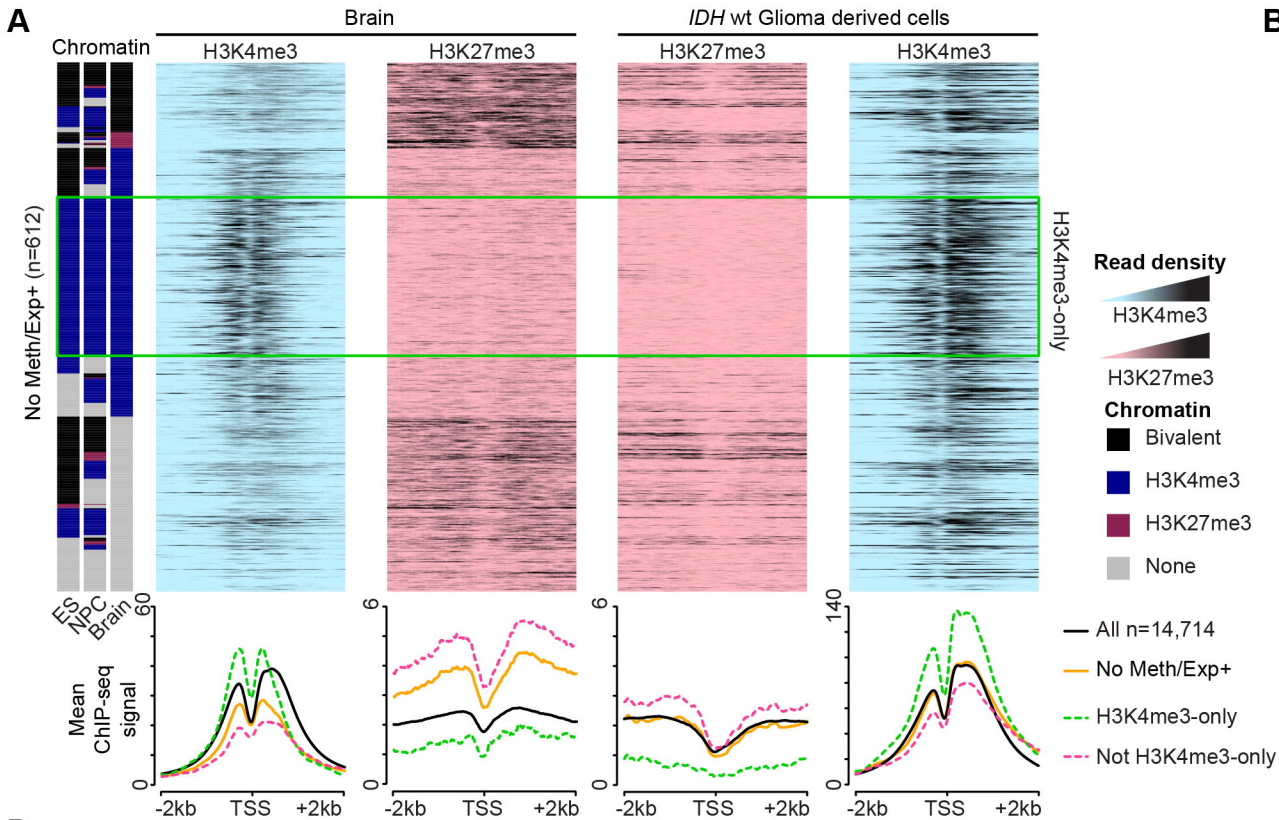
A**B****C****D**

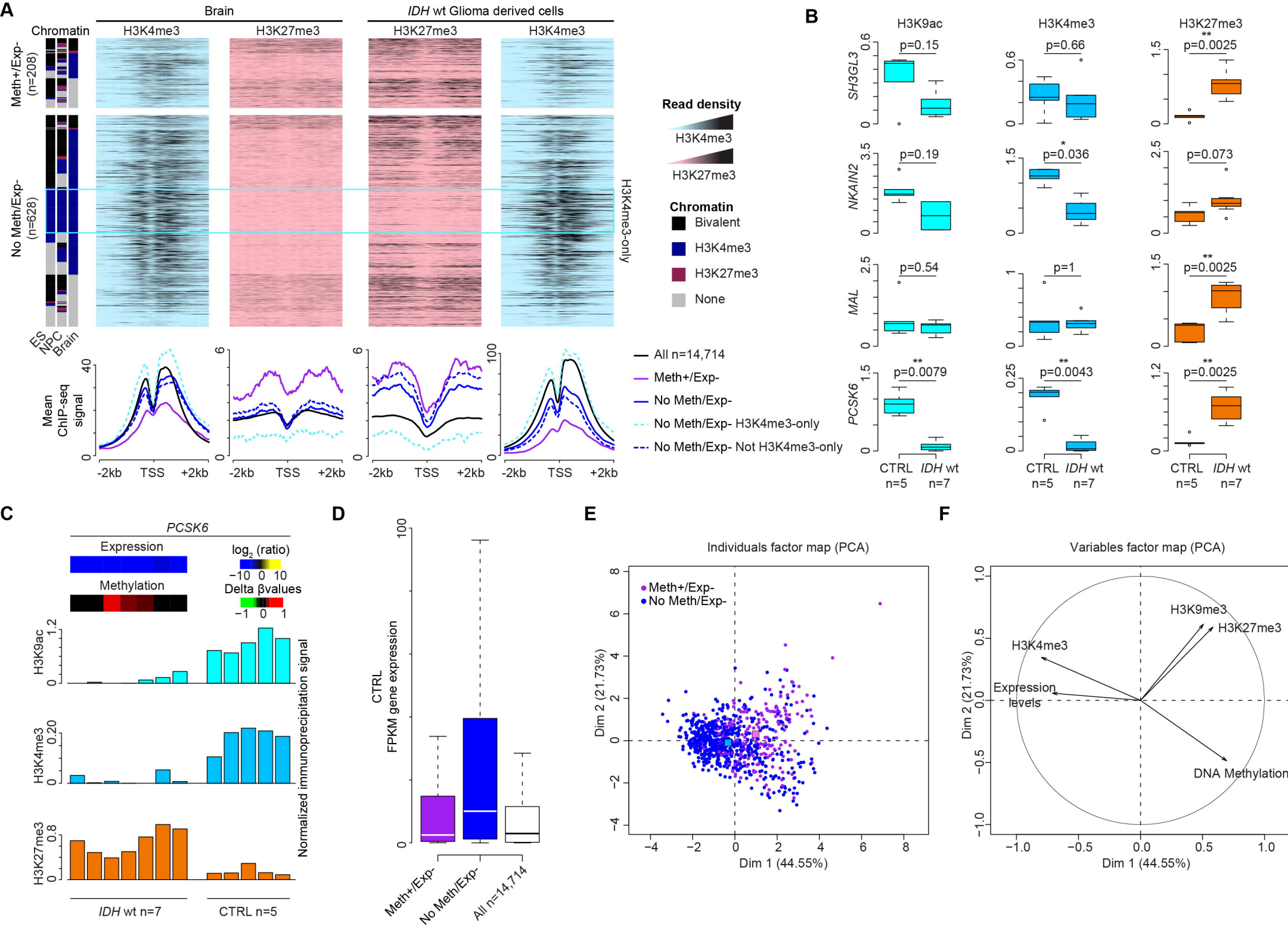


A**B****C****D**

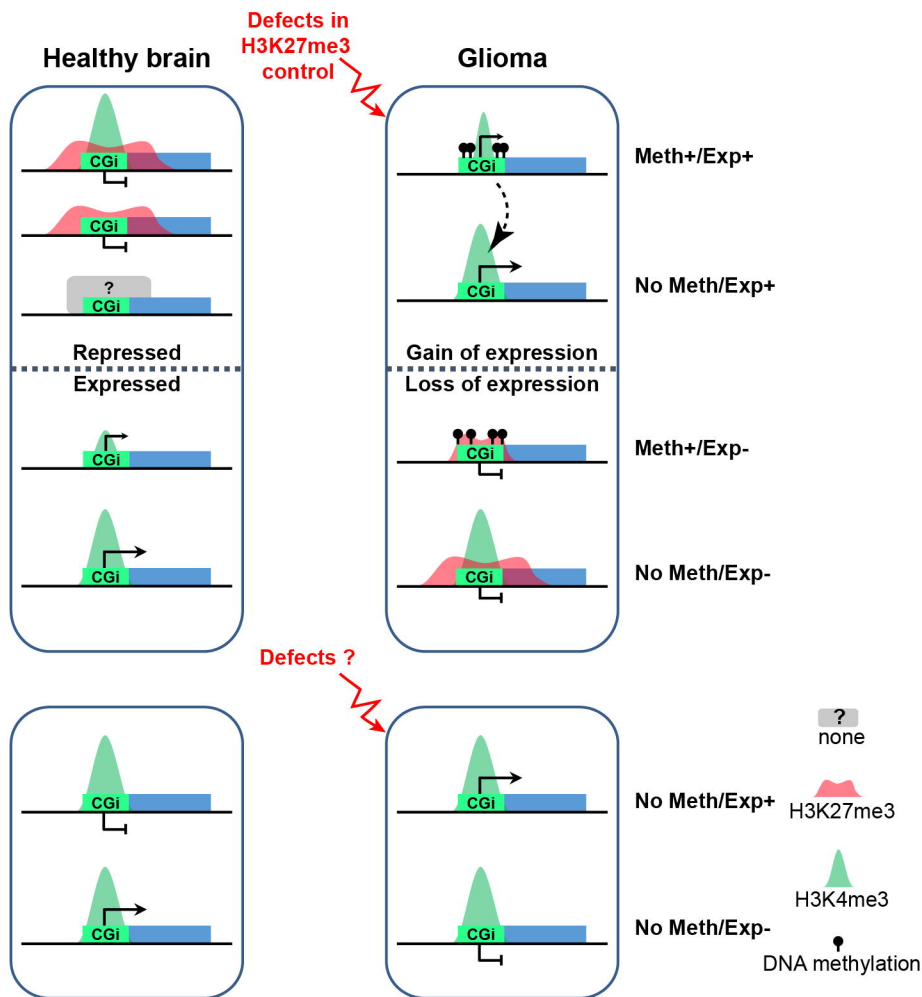
A**B****C**







A



B

	<i>IDH</i> wt % of genes	<i>IDH</i> mut % of genes	
Unaffected	89.5	93.5	
Meth+/Exp+ (DNA methylation-associated)	0.8	0.9	Ectopic expression
No Meth/Exp+	4.1	2	
Meth+/Exp- (DNA methylation-associated)	1.4	1.7	Aberrant repression
No Meth/Exp-	4.2	2	