

Microenvironmental Perfusion Gradients Shape the CpG Methylome to Drive Glioblastoma Intratumoral Heterogeneity

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Abstract

Glioblastoma (GBM) exhibits intense intratumoral heterogeneity, primarily driven by abnormal vasculature that establishes distinct perfusion and oxygen gradients. Although these perfusion-defined niches possess unique transcriptomic and metabolic signatures, the upstream hierarchical drivers of these variations remain unresolved. Here, we combined Perfusion-based Fluorescent Dye Labeling of Cells (PFDLC) with high-density methylation profiling microarray to evaluate how regional perfusion variations shape niche-specific CpG methylation landscapes in GBM cells. We isolated perivascular, intermediate, and hypoxic cancer cell subpopulations based on their proximity to functional blood vessels. Pan-genomic profiling revealed that the most extensive CpG methylation variations occur at the microenvironmental extremes, closely tracking the tumor's perfusion gradient. These altered sites target transcriptionally critical promoter regions and CpG islands linked to separate, complementary oncogenic cascades. Multi-omic integration of our niche-specific methylome and transcriptome datasets revealed that promoter hypomethylation exercises a dominant role over hypermethylation in driving active transcription. Integrating these methylomic signatures with niche-specific transcriptomes identified a core set of hypoxia-regulated genes — ATP-binding cassette transporter A1 (ABCA1), Glycogen branching enzyme 1 (GBE1), Glycoprotein nonmetastatic melanoma protein B (GPNMB) and C-X-C motif chemokine ligand 14 (CXCL14) — where hypomethylation correlates with transcriptional upregulation. Elevated expression of these candidates clinically associates with highly aggressive tumor phenotypes, therapy resistance, and reduced patient survival. Multi-modal cross-species validations across tumor xenografts, patient tissue immunohistochemistry, single-cell RNA sequencing, and spatial transcriptomics confirmed the robust enrichment of these malignant targets within perfusion-deprived hypoxic microdomains. Collectively, these findings define a perfusion-methylome-transcriptome axis that reproducibly marks the most aggressive, treatment-refractory compartment of GBM, nominating ABCA1, GBE1, and GPNMB as candidate biomarkers for hypoxia-informed patient stratification and epigenetically directed precision therapeutic strategies.

Introduction

Glioblastoma multiforme (GBM), an isocitrate dehydrogenase (IDH) wildtype glioma, is among the most aggressive and lethal primary brain cancers, posing a formidable challenge in modern medicine.^[1] Despite aggressive multimodal therapies—including surgical resection, fractionated radiotherapy, chemotherapeutic regimens centered on temozolomide (TMZ) and bevacizumab, and emerging immunotherapeutic approaches—the median patient survival remains a dismal 12 to 15 months.^[1, 2] This persistent therapeutic resistance highlights the urgent need for a more profound understanding of the complex biological mechanisms driving GBM pathogenesis. A key factor limiting therapeutic efficacy is intratumoral heterogeneity (ITH), which exacerbates tumor malignancy.^[2, 3] While genetic and epigenetic alterations have long been recognized as drivers of ITH, the dynamic tumor microenvironment (TME) has recently emerged as a critical modulator of this heterogeneity.^[4] Blood vessels within the TME provide essential oxygen, nutrients, and growth factors, supporting tumor growth. However, the rapid

proliferation of GBM cells often outpaces normal angiogenesis, leading to aberrant vasculature and differential perfusion - a key determinant of the ITH observed in GBM.^[5-8] This differential perfusion creates distinct microenvironments, including hypoxic, intermediate and perivascular niches, with unique metabolic and transcriptomic profiles.^[9] This study is a direct continuation of our group's previous work, which established that perfusion gradients differentially influence distinct tumor niches - dictating intratumoral metabolic zonation and phenotypic diversification^[9], and programming cancer cell stemness and functional output through vascular-derived paracrine cues independently of genetic drivers^[10], together with our recent synthesis of how perfusion gradients drive spatial heterogeneity across the tumor.^[8] Building on this body of work, we reasoned that the same perfusion gradients dictating metabolic zonation may also imprint stable epigenetic marks that lock cancer cells into niche-specific transcriptional states, providing a mechanistic bridge between the vascular microenvironment and molecular subtyping of GBM.

DNA methylation, particularly, 5-methyl cytosine (5-mC) modifications at CpG dinucleotides, plays a crucial role in shaping the epigenetic landscape of cancer cells and influencing gene expression.^[11-13] Aberrant DNA methylation patterns, including both hypermethylation and hypomethylation, have been implicated in GBM pathogenesis.^[14, 15] For example, promoter hypermethylation of the O-6-Methylguanine-DNA methyltransferase (MGMT) gene is associated with improved response to TMZ,^[15] while hypomethylation of the Melanoma antigen family A1 (MAGEA1) gene can promote tumor progression.^[16] The TME, can further modulate DNA methylation patterns, adding another layer of complexity to GBM heterogeneity. Hypoxia, for example, regulates the hypomethylation of Oz/ten-m homolog1 (ODZ1) promoter, which increases its expression and supports the migration of GBM cancer cells.^[17] Consistent with this, recent studies of the GBM molecular landscape have reiterated that microenvironment-responsive epigenetic reprogramming, alongside genetic drivers, underlies therapy resistance and tumor evolution, reinforcing the need for spatially resolved methylome studies.^[18]

While previous studies have examined genome-wide methylation changes in GBM^[14, 19] and highlighted the impact of TME factors on DNA methylation,^[20] a comprehensive understanding of how differential perfusion shapes the CpG methylation landscape in the GBM niches and contributes to epigenetic heterogeneity remains elusive. To address this gap, we have combined perfusion-based Fluorescent Dye Labeling of Cells (PFDLC) with niche-specific methylome analysis to isolate cancer cells based on distance from the nearest functional blood vessels and analyzed genome-wide CpG methylation signatures. We examined how intratumoral perfusion gradients correlate with DNA CpG methylation patterns in GBM. Our findings reveal that the highest perfusion gradient across the tumor map directly to the most extensive differences in CpG methylation. Consequently, we identified distinct hypermethylated and hypomethylated CpGs across various genetic and CpG island (CGI) features. These epigenetic variations were most pronounced at the microenvironmental extremes - the highly perfused perivascular niche and the poorly perfused hypoxic niche. Furthermore, functional pathway analyses of the differentially methylated genes (DMGs) uncovered unique oncogenic pathway associations with the

hypoxic niche. Integrating the methylome with the PFDLC-derived niche-specific transcriptome, we identified hypermethylated/downregulated and hypomethylated/upregulated genes. Among the hypomethylated/upregulated genes, high expression of ATP-binding Cassette Transporter A1 (ABCA1), Glycogen Branching Enzyme 1 (GBE1), Glycoprotein Nonmetastatic Melanoma Protein B (GPNMB), and C-X-C motif Chemokine Ligand 14 (CXCL14) correlated with poor survival and aggressive malignant phenotypes in the TCGA dataset. Furthermore, they showed the highest upregulations in the core hypoxia histological features in the Ivy GAP dataset. The elevated expressions of these genes in hypoxic niches were validated in tumor xenografts, GBM patients, multiple patient single-cell transcriptomic and spatial transcriptomic datasets and samples. In summary, this study presents a niche-specific genome-wide CpG methylation analysis as a function of perfusion in GBM, identifying four hypomethylated/upregulated genes, ABCA1, GBE1 and GPNMB, with potential roles in tumor aggressiveness and malignancy.

Methods

1. Cell Culture

The U87MG glioblastoma cell line (UGFP), a kind gift from Prof. Eli Keshet (Hebrew University of Jerusalem, Israel), was used in this study. These cells were stably transduced with green fluorescent protein (GFP). Cells were maintained in complete Dulbecco's Modified Eagle Medium (DMEM; Himedia) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Pen-strep; Himedia). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Routine mycoplasma testing was performed, and early passage cells were used for all experiments.

2. Perfusion-based Fluorescent Dye Labelling of Cells (PFDLC) in GBM Xenograft Model

Non-obese diabetic-severe combined immunodeficient (NOD-SCID) mice (male and female; 6 to 8 weeks; approximately 20 grams in body weight) were used for the xenograft tumor model. Perfusion-based Fluorescent Dye Labeling of Cells (PFDLC), combined with flow cytometry-mediated cell sorting, was employed to isolate cancer cells based on their proximity to blood vessels.^[9, 21] NOD/SCID mice were subcutaneously injected on either dorsal flank with 10⁶ UGFP cells. Following inoculation, mice were monitored until they regained normal activity, and tumor volume was measured every other day. 3 to 4 weeks post-inoculation, when tumors reached a sufficient size (8–10 mm in length), live, anesthetized mice were perfused via tail vein injection with Hoechst 33342 dye (5 mg/mL [Thermo Fisher Scientific] in 1X PBS [Lobachemie]). Mice were then euthanized, tumors were surgically dissected, and separate portions of the tumor were used for cellular fractionation through flow-cytometry-based sorting and immunohistochemistry.

3. Flow-Cytometry and Sorting of Cancer-Cell Populations

Tumor portions were mechanically and enzymatically dissociated by finely mincing and incubating in a C-tube with DMEM containing hyaluronidase (10 mg/mL; Sigma-Aldrich), collagenase D (10 mg/mL; Gibco), and DNase I (5 mg/mL; Sigma-Aldrich) on a Gentle-MACS dissociator. Following dissociation, the cell suspension was filtered through a 70 μ m filter, and enzymatic activity was quenched by adding ice-cold DMEM (Himedia). Cells were centrifuged, the supernatant was removed, and the cell pellet was resuspended in FACS buffer (2% FBS and 1 mM EDTA [SRL] in 1X PBS [Lobachemie]). Stroma-free cancer cell populations were isolated by flow cytometry based on Hoechst 33342 dye uptake. Following removal of cell doublets and selection of pure cancer cell populations (high GFP-expressing cells), the cells were further subdivided into three fractions based on proximity to blood vessels: HH (perivascular), HM (intermediate), and HL (hypoxic). These fractions were used for niche-specific methylome and transcriptomic analyses. The flow cytometry data were analyzed by Floreada.io software.

4. Immunohistochemistry

Tumor xenograft and GBM patient tissue portions were fixed in 4% paraformaldehyde (PFA; Himedia) for 3–4 hours at 4°C, followed by overnight saturation in 30% (wt/vol) sucrose (SRL). Tumors were then embedded in O.C.T (Tissue-tek O.C.T.; Sakura Finetek) and sectioned using a cryotome (Medimeas; MCM-ST). Sections were permeabilized with 1X PBST (0.2% Triton X-100 [SRL] in 1X PBS [Lobachemie]) and blocked with 5% bovine serum albumin (BSA; SRL) in 1X PBST for 1.5 hours. Sections were then incubated overnight at 4°C with primary antibodies against GLUT1 (1:100; CST), CD31 (1:100; CST), ABCA1 (1: 100; Thermo Fisher Scientific), GBE1 (1:50; Thermo Fisher Scientific), GPNMB (1:100; Thermo Fisher Scientific) and CXCL14 (1:100; Thermo Fisher Scientific). After washing with 1X PBST, sections were incubated with appropriate secondary antibodies (1:400; CST) for 1.5 hours. Patient sections were co-immunostained with the hypoxia marker GLUT1. Nuclei were counterstained with DAPI (1 μ g/mL; SRL). All sections were mounted with CC mounting medium (Thermo Fisher Scientific) and visualized using an inverted confocal microscope (Leica; TCS SP8).

5. PFDLC-based Niche-Specific Methylome Analysis

Genomic DNA was isolated from PFDLC generated HH, HM, and HL cancer cell fractions using the QIAamp® DNA Mini Kit (Qiagen), followed by bisulphite treatment and fragmentation to generate probes. DNA methylation was assessed using the Illumina 850k EPIC microarray platform. Methylation levels were quantified as β -values, representing the ratio of methylated probe intensity (M) to the sum of methylated and unmethylated probe intensities (M + U), where $0 \leq \beta \leq 1$. The data was analyzed using the ChAMP package in R. After probe filtering (removal of probes with $p > 0.01$; probes with bead count > 3 in at least 5% of samples; non-CpG site binding probes; small nucleotide polymorphism [SNP] binding probes; probes aligning to multiple locations and probes located on X and Y chromosomes), CpG site binding probes were identified and differential methylation studies were done for the 3 fractions. Each

probe corresponded to a single CpG site. To quantify differential methylation, the $\Delta\beta$ value was calculated that represented the difference in β -values for a single CpG between cell fractions: $\Delta\beta$ (HL-HH) = $\beta_{HL} - \beta_{HH}$ for HL versus HH; $\Delta\beta$ (HL-HM) = $\beta_{HL} - \beta_{HM}$ for HL versus HM; and $\Delta\beta$ (HM-HH) = $\beta_{HM} - \beta_{HH}$ for HM versus HH. To distinguish hypermethylation and hypomethylation; $\Delta\beta > 0.1$ defined CpG hypermethylation, while $\Delta\beta < -0.1$ defined CpG hypomethylation. For example, a $\Delta\beta$ CpG value of -0.3 between HL and HH ($\beta_{HL} - \beta_{HH}$), indicated that the CpG was hypomethylated in the HL fraction relative to the HH fraction. The percentage of niche versus niche hyper and hypomethylated CpGs were determined for genetic and CGI features. Differentially methylated genes (DMGs) were identified and KEGG and gene ontology (GO) databases were used for enrichment pathways analyses using R Cluster Profiler for HL versus HH fraction.

6. PFDLC - based Niche - Specific Transcriptome Analysis

RNA from PFDLC generated HH, HM and HL cell fractions were isolated using RNeasy mini kit (Qiagen). Libraries were prepared and sequenced according to the single-cell Cell-Seq2 protocol on the Illumina Hi-Seq platform.^[22] Following quality control, transcript reads were mapped to the human genome, and quantification and normalization were done to produce normalized expression values for the identified genes. Differentially expressed genes (DEGs) were identified as having p value < 0.05.

7. Integrated Methylome and Transcriptome Analysis

From the HL versus HH methylome (5), the hypermethylated ($\Delta\beta > 0.1$) and hypomethylated ($\Delta\beta < -0.1$) CpGs located within all genetic features, excluding only intergenic repeats (IGRs), and promoter exclusive CpGs were taken for the two separate integrated analyses. From niche-specific transcriptome (6), subsets of genes were considered upregulated ($\log_2FC > 1$, HL/HH) and downregulated ($\log_2FC < -1$, HL/HH) for both analyses. Hypomethylated/upregulated and hypermethylated/ downregulated genes were generated by intersecting the methylome and transcriptome data.

8. Single-Cell Transcriptome Analysis

Three publicly available datasets were analysed using Seurat package in R (Table below). Following quality control, an integrated dataset was generated (44099 cells). Cluster resolution to 27 seurat clusters was performed through unsupervised clustering (resolution = 0.5). The previously established Cellular Hypoxia Prediction Framework (CHPF) algorithm datasets were used, along with PFDLC DEGs ($\log_2FC > 2$ for HL/HH), to demarcate hypoxic (16329 cells) and normoxic (27770 cells) cells based on binary scores.^[23] Expression of selected genes were mapped to the hypoxic and normoxic cells in the dataset. Continuity in hypoxic gradient was captured through hypoxia scoring based solely on expression of the PFDLC DEGs. k – means clustering (k = 6) further stratified the continuous scores to generate N1, N2, N3 and H1, H2 and H3 subclusters in increasing order of score values. DEGs were identified for all

the subclusters and ranked to distinguish upregulated genes in each of the hypoxia subcluster as compared to the pooled normoxia subclusters populations. Expression of selected genes was determined in the subclusters. Pathway enrichment analysis using the KEGG database for the six subclusters was done from the DEGs using Cluster Profiler package in R.

GEO dataset ID	Patient count	Total cells	Reference
GSE125587	10	21750	[24]
GSE117891	14	> 6000	[25]
GSE131928	28	24131	[26]

Table

Description of datasets used for single-cell RNA sequencing analysis.

9. Spatial Transcriptome Analysis

Three publicly available IDH – wild type GBM tissues with accession codes GSM5838699 (S1 sample), GSM5838698 (S2 sample) and Parent Visium, CG000239 (S3 sample) were used for analyses using Seurat package in R. Following spot-level quality control (n- feature > 200, n-count > 500, mitochondrial DNA < 25%), normalized samples were resolved into seurat clusters through unsupervised clustering (resolution = 0.5) for cluster identification and total RNA reads (n - count) and expressed genes (n - feature) were mapped. Cell-type labeling within sample spots was done through integrated automated annotation (utilizing DEGs in single-cells per cluster) and manual annotation (cross- referencing DEGs to known markers) to identify eight categories - cancer cells, stromal cells (immune cells and cancer-associated fibroblasts), vascular cells (red blood cells, endothelial cells and pericytes), neuronal cells, astrocytes, supportive cells (schwann cells, ependymal cells, oligodendrocytes), neuroepithelial cells, and neural stem cells. Hypoxic niche demarcations within samples were based on dual hypoxia and vascular proximity scores. The PFDLC transcriptome DEGs ($\log_2FC > 2$, for HL/HH) were utilized to identify spots with high hypoxic scores. Expression values for 27 known angiogenic markers were used to identify spots with high vascular proximity scores. Scores were binarized at median values to generate normoxic/hypoxic and high vascular/low vascular values, and Pearson correlation was calculated between the scores. For comprehensive perfusion- based spatial zonation per sample, each spot was assigned to one of four mutually exclusive zones based on dual hypoxia / vascular scores - hypoxic core zone (above-median hypoxia, below-median vascular), palisading zone (above-median hypoxia, above-median vascular), normoxic distal zone (below-median hypoxia, below-median vascular), and well-perfused zone (below-median hypoxia, above-median vascular). Zone-wise gene expression analysis was done for selected genes by dividing each gene into high, medium, and low expression groups using tertile expression thresholds.

10. TCGA and Ivy GAP Patient Datasets Analysis

The adult GBM TCGA patient (HG-UG133A array gene expression) dataset was used in this study. 425 patients analyzed were divided into high and low expression groups based on median $\log_2$ FC expression values. Selected genes expression according to G - CIMP and subtype status was analysed. Clinical-pathological tables for low and high gene expression groups were made for age, gender, G – CIMP and subtype status. Univariate and multivariate survival analysis was done using high/low gene expression groups, age, gender, GBM subtype and G – CIMP status. Hazard ratios were calculated. For Ivy GAP dataset, 271 patients were chosen. Expression of selected genes as fragment per kilobase of transcript per million of reads (FPKM) was analysed in each histological feature.

11. Data Visualisation and Statistical Analysis

Built in ChAMP, Seurat functions were utilized to systematically pre-process, harmonize and curate the datasets for heatmaps, PCA and uniform manifold approximation and projection (UMAP) dimensionality reduction. TCGA dataset was retrieved from UC Xena and Ivy GAP from the Ivy GAP website. All plots were created using ggplot2, GraphPad Prism, and jamovi. Descriptive statistics were generated for all quantitative data (test type and details mentioned in figure legends).

12. Data Availability

The PFDLC - methylome analysis data is submitted in the Array Express database under accession number E-MTAB-15449 at EMBL-EBI. The PFDLC - transcriptomic analysis data is available in Array Express database under accession number E-MTAB-6882 at EMBL-EBI.

Results

1. Genome-Wide Methylome Analysis Uncovers Epigenetic Divergence Driven by Intratumoral Perfusion Gradients

To determine how microenvironmental variations in blood supply shape the GBM epigenetic landscape, we performed a genome-wide methylome analysis across distinct perfusion-driven tumor niches. To establish a human GBM microenvironment, GFP-expressing U87MG cells were implanted into the dorsal flanks of immunocompromised mice. Using this xenograft model, we employed Perfusion-based Fluorescent Dye Labeling of Cells (PFDLC) via tail-vein injection of Hoechst 33342 to isolate cancer cells based on vascular proximity. Importantly, this approach overcame the cell-yield limitations of previous models, providing sufficient material for high-resolution downstream molecular analyses (**Figure S1a**).^[9, 21]

Flow cytometry was employed to isolate a pure population of cell doublet-free and stromal-free GFP+ cancer cells, which were fractionated into three distinct subpopulations: Hoechst-High (HH; perivascular niche), Hoechst-Medium (HM; intermediate niche), and Hoechst-Low (HL; hypoxic niche) (**Figure S1b**). The spatial architecture of this perfusion gradient was confirmed via immunohistochemistry (IHC), verifying the close proximity of HH cells to CD31 + endothelial cells and the enrichment of the GBM hypoxia marker Glucose Transporter 1 (GLUT1) in distal HL cells (**Figure S1c-d**).^[27, 28]

Following high-resolution microarray profiling and stringent quality-control filtering of over 850,000 unique methylation sites, 726,338 high-confidence CpG sites were retained for pan-genomic analysis (Fig. 1a). Unsupervised Principal Component Analysis (PCA) demonstrated robust and highly reproducible clustering of biological replicates corresponding to their respective niches (Fig. 1b). This distinct segregation underscores the distinct epigenetic signature embedded within each microenvironment. A heatmap of the top 100 differentially methylated CpGs demonstrated a clear gradation across the perfusion spectrum, with progressive methylation shifts occurring from perivascular to hypoxic domains (Fig. 1c). While specific CpG subsets displayed a linear decrease in methylation from the perivascular toward the hypoxic zone, an opposing cluster showed a progressive increase in methylation along the same axis, demonstrating the dynamic epigenetic reprogramming driven by the local TME.

To define the magnitude of these perfusion-induced epigenetic variations, we conducted a niche-versus-niche bivariate analysis. Pairwise scatter plots revealed that the most extensive CpG methylation divergence occurred at the microenvironmental extremes, identifying 98,355 differentially methylated CpGs between the extremes – perivascular (HH) and hypoxic (HL) subpopulations (Fig. 1d–f). Pan-genomic Manhattan plots highlighting the chromosomal distribution of the significant differentially methylated sites revealed non-random autosomal occupancy, with pronounced hotspots localized on chromosome 1 (10.3%) and chromosome 2 (7.87%) (Fig. 1g–i). Together, these results establish intratumoral perfusion gradients as a primary determinant of niche-specific CpG methylation.

2. Perfusion-Mediated Epigenetic Zonation Targets Functional Promoter Regions and Alters Oncogenic Signaling Pathways

To quantify perfusion-induced epigenetic variance and its impact on gene regulation, we classified differentially methylated CpGs into hypermethylated and hypomethylated groups using established criteria (details in Methods, section 5).^[29, 30] The most substantial variations occurred between the two extreme microenvironments (HL versus HH), encompassing 27,874 hypermethylated CpGs (28.3%) and 18,474 hypomethylated CpGs (18.8%) out of 98,355 total differentially methylated sites (Figs. 2a, S2a-b). Circos plots revealed a non-uniform distribution of these modifications across the autosomes, with chromosomes 1 (hypermethylated – 10.31%, hypomethylated – 9.99%) and 2 (hypermethylated – 8.09%, hypomethylated – 8.43%) emerging as primary hotspots for perfusion-dependent methylation clusters (Figs. 2b, S2c-d).

Because aberrant DNA methylation heavily influences gene expression and downstream cellular networks,^[29, 31] we next examined the distribution of hypermethylated and hypomethylated CpGs across various defined genomic loci and CpG island (CGI) features (Figs. 2c-d, S2e-h). Within transcriptionally vital promoter regions (comprising merged TSS1500 and TSS200 features) of the HL versus HH fractions, hypermethylated CpGs (5,401; 60.8%) noticeably outnumbered hypomethylated sites (3,487; 39.2%) (Fig. 2c). An identical pattern emerged within CGIs, where hypermethylation accounted for 63.9% (1,376 CpGs) compared to 36.1% (776 CpGs) for hypomethylation (Fig. 2d). These localized variations highlight a direct role for perfusion-driven epigenetic modifications in gene regulatory mechanisms, establishing a firm biological rationale to focus on the microenvironmental extremes of the perivascular and hypoxic niches.

To define the downstream functional consequences of these niche-specific signatures, we derived the differentially methylated genes (DMGs) for pathway annotations. KEGG pathway analysis revealed that the top 10 enriched terms for hypermethylated genes in the HL fraction involved critical oncogenic signaling cascades, including the Transforming Growth Factor-beta (TGF- β), Ras-associated protein 1 (Rap-1), and Programmed Death-Ligand 1/Programmed Death-1 Receptor (PD-L1/PD-1) immune checkpoint pathways (Fig. 2e). In contrast, hypomethylated genes preferentially were enriched in the Mitogen-activated protein Kinase (MAPK), Wnt, and Hedgehog pathways (Fig. 2f). These findings were corroborated by Gene Ontology (GO) analyses, which showed that primary processes governing GBM progression—such as DNA transcription factor binding, protein kinase activity, and actin cytoskeleton remodeling—were heavily modulated by both arms of the differential methylation spectrum (Fig. 2g-h). Uniquely, hypermethylation primarily targeted DNA damage responses and chromatin remodeling machinery (Fig. 2g), whereas hypomethylation predominantly affected autophosphorylation and active gene transcription (Fig. 2h). Together, these data indicate that hypoxic niches drive distinct hyper- and hypomethylation signatures that engage specific oncogenic programs, worsening ITH.

3. Perfusion-Induced Hypomethylation Activates Malignant Oncogenic Gene Signatures in Hypoxic Niches

Aberrant DNA methylation is a well-established mechanism that disrupts physiological gene expression patterns, driving oncogenesis across diverse malignancies.^[11–13, 32] Given the prominent role of the TME in altering the expression of malignancy-associated genes,^[14, 17, 19, 33] we integrated our niche-specific PFDLC methylome (excluding intergenic repeat [IGR] CpGs) with the transcriptome to link perfusion-dependent CpG methylation to downstream transcriptional output. This identified 73 genes as hypomethylated/upregulated (HoU) and 15 genes as hypermethylated/downregulated (HeD) in the hypoxic (HL) fraction relative to the perivascular (HH) fraction (**Figure S3a**). To refine this observation to the most functionally significant loci, we focused on transcriptionally vital promoter-region CpGs. An identical intersection strategy narrowed the candidate list to 36 HoU genes and 8 HeD genes, validating the pronounced regulatory influence of regional hypomethylation and consequent enhanced expression of downstream genes (Fig. 3a–c).

To prioritize high-confidence candidates, we applied a stringent selection filter that required at least two differentially methylated CpGs within a single promoter. This refined stratification yielded 9 HoU genes - ABCA1, CXCL14, GBE1, GPNMB, Semaphorin 3B (SEMA3B), SPARC-like protein 1 (SPARCL1), Hypoxia-inducible factor 3 alpha (HIF 3α), Cytochrome P450 2E1 (CYP 2E1) and Solute carrier family 27 member 3 (SLC27A3) (Figs. 3c, S3b-f). To determine their clinical and histological relevance, we cross-referenced these candidates with external patient datasets. Notably, 4 of these hypomethylated genes - ABCA1, GBE1, GPNMB, and CXCL14, demonstrated significant enrichment within the Ivy GAP dataset, showing their highest expression patterns localized specifically to palisading necrosis (PAN) and perinecrotic zones (PNZ), which are histological hallmarks of the severe, core hypoxic microenvironment (Fig. 3d–g).^[34] Structural mapping in these genes confirmed that they harbored multiple hypomethylated CpGs across both promoter regions and gene bodies, a configuration highly linked to transcriptional active states (**Figure S3b–f**).^[33, 35]

We next evaluated the molecular and clinical impact of this 4-gene signature using the TCGA repository. The expression of ABCA1, GBE1, GPNMB, and CXCL14 peaked in tumors belonging to the highly aggressive mesenchymal glioblastoma subtype (**Figure S3g–j, k–o**),^[26] and within the highly therapy-resistant non-glioma - CpG island methylator phenotype (N-G-CIMP) cohort (**Figure S3 g–j, p–s**).^[36] Crucially, both univariate and multivariate survival analyses revealed that elevated expression of this 4-gene cohort is significantly associated with accelerated disease progression and poorer patient survival (Fig. 3h–k). Together, these analyses show that hypoxic niches drive targeted CpG hypomethylation of ABCA1, GBE1, GPNMB, and CXCL14, linking this signature to therapy resistance and poor clinical outcomes.

4. In Vivo and Clinical Validation Confirms Enriched Expression of Hypomethylated Target Genes Within the Hypoxic Niches

To validate our multi-omic findings and evaluate whether the identified transcriptionally active, hypomethylated candidates translate into distinct protein expression profiles within the TME, we utilised both in vivo tumor xenografts and clinical patient tissues. We first performed IHC on GBM mouse xenografts generated using our PFDLC pipeline. A marked increase in the protein levels of ABCA1, GBE1, and GPNMB within the poorly perfused, hypoxic (HL) niches compared to the well-perfused perivascular (HH) regions was observed. In contrast, CXCL14 protein expression showed negligible spatial variation across the same perfusion-defined zones, suggesting potential post-transcriptional or niche-specific buffering in vivo (Fig. 4a–d).

To extend these preclinical observations to human disease, we examined GBM tissue specimens obtained from surgical resections. Patient tumor sections were co-immunostained with GLUT1, an established clinical GBM tissue hypoxia marker.^[27] Prominent upregulation of ABCA1 and GBE1 specifically localized to GLUT1^{high} hypoxic zones, in stark contrast to their lower baseline expression in adjacent GLUT1^{low} normoxic areas. Furthermore, moderate spatial upregulation of GPNMB and CXCL14 was observed within these hypoxic domains (Fig. 4e–h). These cross-species validations corroborate

our multi-omic analysis, confirming that ABCA1, GBE1, and GPNMB are consistently upregulated under hypoxia, consistent with hypomethylation-driven transcriptional activation.

5. Single-Cell Transcriptomics Resolve the Expression of ABCA1, GBE1 and GPNMB in the Hypoxic Cells Along the Perfusion Axis

Within the GBM TME, differential oxygenation and nutrient availability create a perfusion-driven continuum of cellular states rather than discrete compartments, a landscape optimally resolved through single-cell transcriptomics.^[24–26, 37] To validate our multi-omic findings at the human single-cell resolution, we analyzed 3 independent GBM single-cell RNA sequencing datasets (**Table** in 8 - materials and methods). Following dataset integration (Fig. 5a; and **Figure S4a-c** - individual datasets), 27 distinct cell clusters were identified, highlighting baseline transcriptional heterogeneity characteristic of GBM tumors (**Figure S4d**). To classify individual cells by their perfusion-driven physiological state, we applied a binary prediction framework. This was achieved by crossing our top PFDLC-derived hypoxic gene signatures ($\log_2FC > 2$ for HL/HH) with the Cellular Hypoxia Prediction Framework (CHPF) algorithm.^[23] This approach successfully segregated the dataset into explicit hypoxic and normoxic cell populations (Fig. 5b), a classification further validated by the restricted, high expression of canonical hypoxia markers GLUT1 and VEGFA (**Figure S4e**).^[27] Crucially, the hypomethylated target genes demonstrated significantly enhanced expression within the designated hypoxic single-cell population, corroborating our previous *in situ* IHC findings (Fig. 5c).

Because binary labels can mask subtle intermediate phenotypes, we went beyond CHPF boundaries to map the continuous hypoxia gradient. Cells were stratified into six distinct subclusters- labeled N1 through N3 for normoxic cells and H1 through H3 for hypoxic cells- ranked by a hypoxia score derived from the top PFDLC-induced hypoxia-elevated genes ($\log_2FC > 2$ for HL/HH) (Fig. 5d–f). Within this ranking, the H3 subcluster represented the highest hypoxic burden and the N1 subcluster represented the lowest baseline, with score variations reflected across the 27 cell clusters (**Figure S4f**). Evaluating our target genes across this sub-clustered gradient revealed that ABCA1 and GBE1 undergo robust upregulation, whereas GPNMB showed moderate, step-wise upregulation that tracks to the increasing hypoxia scores, in the more severe H2 and H3 subpopulations. Conversely, CXCL14 expression was highly restricted, peaking moderately only within the extreme H3 subpopulation (Fig. 5g–j).

To define the functional and metabolic wiring of these stratified cellular states, we performed pathway enrichment analyses across the subclusters. While HIF-1 signaling was globally preserved across all hypoxic cohorts, the most severely hypoxic H2 and H3 subclusters uniquely prioritized HIF-1 signaling, glycolysis/gluconeogenesis, and ferroptosis pathways (Figs. 5k–m, S4 g–j). In contrast, intermediate normoxic cells (the N2 subcluster) were driven by immune-regulatory programs, including T-helper (Th) cell differentiation, phagosome formation, and antigen processing and presentation (**Figure S4i**). Consistent with these microenvironmental pathways, differential expression profiling confirmed that ABCA1, GBE1, and GPNMB reside within the top 100 differentially expressed genes (DEGs) defining the severe H2 and H3 subclusters relative to normoxic subclusters, while all target genes were absent from

the mild H1 subcluster (Figs. 5n-o, S4k). These single-cell analyses confirm that ABCA1, GBE1, and GPNMB are elevated in the most severely hypoxic cellular states, directly linking their expression to canonical hypoxic pathways.

6. Spatial Transcriptomics Links ABCA1, GBE1 and GPNMB Enrichment to Palisading and Hypoxic Core Tumor Topographies

A growing body of evidence highlight the role of spatial architecture in defining gene expression programs within the GBM TME, with hypoxia serving as a key organizer for cancer and stromal cells into distinct higher-order states.^[38–40] To investigate the exact spatial distribution of our epigenetically regulated target genes relative to these localized tissue hypoxia gradients, we performed high-resolution spatial transcriptomic mapping across three independent human IDH-wildtype GBM tissue sections (details in 9- materials and methods). Spot-level clustering initially revealed pronounced spatial transcriptional heterogeneity among the specimens, with sample S3 resolving into the highest complexity at 8 distinct transcriptomic clusters (**Figure S5a-d**). This structural divergence was further reflected in the variable baseline distribution of total unique transcripts (n count) and expressed genes (n features) captured across the tissues (**Figure S5e-g, h-j**). To link these abstract clusters to precise biological identities, cell-type annotation was performed to map the distribution of malignant and non-malignant populations within the tissue spots. This mapping revealed distinct niche compositions - sample S1 was uniquely enriched with supportive and neural stem cells, sample S2 presented prominent astrocyte signatures, and sample S3 was characterized by neuroepithelial cell populations, underscoring the baseline cellular complexity of the GBM spatial architecture (Fig. 6a-d).

Importantly, the structural alignment of cancer and vascular cells confirmed the presence of a functional perfusion gradient across all 3 samples. To map these local microenvironmental dynamics, we calculated dual spatial indices across the tissue sections - a hypoxia score (derived from the top PFDLC-derived hypoxic genes, $\log_2\text{FC} > 2$ for HL/HH) and a vascular proximity score (derived from angiogenic gene expression matrices). This analysis revealed inverse, spatially opposed layouts of high-scoring spots across the tissue landscapes, confirming the existence of a perfusion axis (Fig. 6e–g: **left**). Spots with elevated hypoxia scores showed concurrent high expression of canonical hypoxia markers GLUT1 and VEGFA (**Figure S5k–m**).^[27] Crucially, ABCA1, GBE1, and GPNMB demonstrated highly localized, overlapping profiles of elevated expression within these high-hypoxia spots, whereas CXCL14 exhibited a continuous, unstratified expression layout spanning the entire tissue (Fig. 6e–g: **right**).

To capture the subtle gradations of the tumor tissue milieu, we utilized the median dual-score thresholds to partition the tissue spots into four mutually exclusive spatial territories: well-perfused, normoxic distal, palisading, and hypoxic core zones (Fig. 6h–j: **left**). More than 50% of the total tissue spots mapped directly to the two hypoxic zones, and negative Pearson's correlation coefficients further substantiated the robust depiction of the perfusion axis for spatial hypoxia model across the samples (Fig. 6k, h–j: **right**). As expected, the validation markers GLUT1 and VEGFA^[27] exhibited maximum high expression

spots in the palisading and hypoxic core zones (**Figure S5n-o**). Characterization of our targets revealed that ABCA1, GBE1, and GPNMB mirrored this spatial trend, showing highest expressions within the hypoxic zones (Fig. 6l–n). Conversely, CXCL14 displayed an inconsistent layout across the tissues, with peak expressions localized to the palisading and normoxic distal zones in samples S1 and S2, but transitioning to the hypoxic core and normoxic distal zones in sample S3 (Fig. 6o). Taken together, these spatially resolved transcriptomic profiles demonstrate that ABCA1, GBE1, and GPNMB are localized within hypoxic tissue zones, confirming their identities as robust, spatially restricted genes of the GBM hypoxic niche.

Discussion

The high degree of ITH characteristic of GBM poses a fundamental barrier to successful therapeutic intervention, enabling rapid adaptation, treatment resistance, and recurrence.^[8, 9] This is consistent with, and directly extends, our previous demonstration that vascular-derived signals dictate cancer cell stemness and functional heterogeneity independently of the underlying genotype^[10], together identifying the tumor vasculature as a unifying, genotype-independent driver of GBM phenotypic diversity. While the past evidence focused predominantly on genetic drivers of ITH,^[41, 42] contemporary models increasingly highlight the dynamic interplay between cancer cells and the physical and metabolic constraints exerted by the TME.^[9, 26, 40] Among the microenvironmental forces driving this heterogeneity, hypoxia stands out as a pervasive feature and a primary determinant of tumor progression and malignancy.^[43] Early studies in GBM have revealed the critical role of differential DNA methylation as an upstream epigenetic driver regulating genes linked to patient survival.^[14] Furthermore, recent findings have highlighted spatial and temporal ITH in DNA methylation profiles across GBM primary and recurrent tumors, associating these patterns with distinct cancer cell expression subtypes and immune cell populations.^[20] While the role of hypoxia in inducing global DNA hypermethylation and hypomethylation in cancers is well established,^[44, 45] a comprehensive, genome-wide understanding of how differential perfusion and its resulting oxygen gradients influence the CpG methylation landscape to alter malignant gene expression has remained largely underexplored.

Our study directly addresses this gap by combining PFDLC^[21] with niche-specific methylome profiling to map CpG methylation patterns in GBM along a precise perfusion gradient. A central finding is that the cancer cell methylome is not static but varies continuously with vascular proximity, with the most significant differential methylation occurring between the perivascular and hypoxic extremes. Interestingly, CpG hypermethylation exceeded hypomethylation in promoter regions and CpG islands within the hypoxic niche. The enrichment of these modifications at transcriptionally vital loci suggests a coordinated regulatory program rather than stochastic epigenetic drift. Functional pathway annotation revealed that these altered loci target separate, complementary oncogenic cascades. While hypermethylation selectively localized to pathways driving tumor invasiveness and immune evasion such as TGF- β , Rap-1 and PD-L1/PD-1 signaling; hypomethylation targeted pathways tied to cell proliferation and survival including Wnt, MAPK, and Hedgehog signaling. Furthermore, the hypermethylated genes

regulate DNA damage response and chromatin remodeling, highlighting how distinct epigenetic switches facilitate survival under hypoxia-induced metabolic stress. Together, local perfusion variations program specific pathway activation through epigenetic modes. Notably, multi-omic integration of our niche-specific methylome and transcriptome datasets revealed that hypomethylation exercises a far more pronounced association with transcriptional activation than hypermethylation does with transcriptional repression. This aligns with emerging evidence suggesting that the magnitude of transcriptional deregulation is not strictly dictated by the total number of differentially methylated CpGs; rather, the regulatory impact is primarily determined by genomic context - such as precise location and sequence variation and functional relevance - particularly through interactions with key transcription factors.^[46–48]

A major strength of our study is the identification of a core 4-gene signature- ABCA1, GBE1, GPNMB and CXCL14- epigenetically activated via hypomethylation within the hypoxic niche and linked with therapy-resistant N-G-CIMP status^[36], aggressive mesenchymal subtype^[26] and poor patient survival outcomes. Recent studies have confirmed independent roles for GBE1 and CXCL14 in hypoxia-mediated tumor malignancy with GBE1 controlling the glycolytic transition in lung adenocarcinomas and gliomas to boost survival^[49, 50] and CXCL14 promoting stemness and invasion in hypoxic glioma cells.^[51] Conversely, while ABCA1 is known to be upregulated under hypoxia in macrophages,^[52] its documented role in gliomas is primarily hypoxia independent relating to cholesterol homeostasis, drug resistance and pro-tumorigenic immune responses.^[53, 54] Similarly, GPNMB has established metastatic roles in breast carcinoma and melanoma,^[55, 56] and promotes GBM tumor progression through glioma stem cell-tumor macrophage cross-talk,^[57] but its transcriptional control under hypoxia remained poorly understood. Here, we have uncovered a previously unrecognised link between the induction of these malignant genes' expression and perfusion-dependent hypomethylation of promoters and gene bodies in the hypoxic niches of GBM. The resolution provided by single-cell and spatial transcriptomics was essential to confirming that these epigenetic states exist as a continuum rather than binary identities. By resolving the "perfusion axis" at the single-cell level, we demonstrated that ABCA1, GBE1, and GPNMB are not merely hypoxia-responsive, but their expression scales with the severity of the perfusion deficit. The spatial zonation analysis further solidified this, showing that these genes specifically demarcate the palisading and hypoxic core (necrotic) zones. The observed inconsistency in CXCL14 suggests that its regulation in the GBM microenvironment is not governed by methylation alone; other independent regulatory pathways or the intersection of multiple signaling networks that govern CXCL14 readouts can't be ruled out.

These findings have significant implications for how we understand and treat GBM. If the malignant phenotype of the hypoxic niche is maintained through epigenetic plasticity along with fixed genetic mutations, it suggests a therapeutic window for epigenetic modifiers. The emergence of locus-specific epigenome editing technologies^[58–60] holds the potential to therapeutically reverse the aberrant methylation signatures, attenuating tumor progression and paving the way towards precision medicine in the future. By identifying the specific CpG sites, genes and pathways that respond to perfusion

gradients, this study moves beyond a descriptive understanding of ITH. We demonstrate that the vascular perfusion- CpG methylation axis is a primary driver of the GBM landscape, bridging the microenvironment and the oncogenic transcriptome, contributing to the resilience of the GBM hypoxic niche. Furthermore, the principles and methodology established here offer a powerful framework for exploring niche-specific multiomics in other solid tumors where intratumoral perfusion gradients play a dominant role in disease progression.

From a precision oncology standpoint, this work has direct translational implications. The hypoxic core and its pseudopalisading margins are increasingly recognised as the most treatment-refractory compartment of GBM ^[61], and our niche-resolved methylome-transcriptome atlas nominates ABCA1, GBE1 and GPNMB, as candidate biomarkers capable of flagging this aggressive, hypomethylation-driven cellular state ahead of overt clinical progression. Because these signatures were independently reproducible across xenograft model, primary patient tissue, and multiple public single-cell and spatial transcriptomic cohorts, they offer a tractable starting point for developing hypoxia-informed molecular stratification tools and for nominating epigenetically druggable vulnerabilities for future precision therapeutic strategies in GBM.

Declarations

Animal Study Ethics

Animal experiments were conducted in accordance with protocols approved by the Institutional Animal Ethics Committee (IAEC/THSTI/254) of BRIC-Translational Health Science and Technology Institute, Faridabad, India, and the guidelines of the Committee for Control and Supervision of Experiments on Animals (CCSEA), Government of India.

Patient Study Ethics

Patient tumor samples were obtained from the Neurosurgery Department at the All India Institute of Medical Sciences (AIIMS), Delhi. Surgical resection of these tumors was performed solely to improve patient survival outcomes. This study was conducted in accordance with ethical guidelines and was approved by the Institutional Ethics Committee of the Indian Institute of Technology Delhi (Ethics approval No. 2021/P028).

Data Disclosure

The authors of the manuscript hereby approve sharing of the methylome and transcriptome data and the codes used for all the relevant analyses upon requirement.

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Author contributions

SK conceptualized the study, provided critical revisions and wrote the manuscript along with RK and SaM. SS performed the experiments, drafted the manuscript, and contributed to study conceptualization and revision. KK conducted the bioinformatic analysis and assisted in manuscript writing and revision. HS conducted the bioinformatic analysis. SaS performed the animal experiments and assisted in manuscript writing. SaK and NP supported the experimental work and participated in manuscript revision. MP, a clinician, performed the surgery, provided GBM patient samples, and contributed to study design. SM offered critical revisions and assisted in study conceptualization.

Use of generative AI and AI-assisted technologies in the writing process

During the preparation of this study, the authors used Grammarly and Gemini as English-language editing tools to improve the language and readability. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the publication's content.

Conflicts of Interest

The authors declare no conflicts of interest.

Clinical trial number: not applicable

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Figures

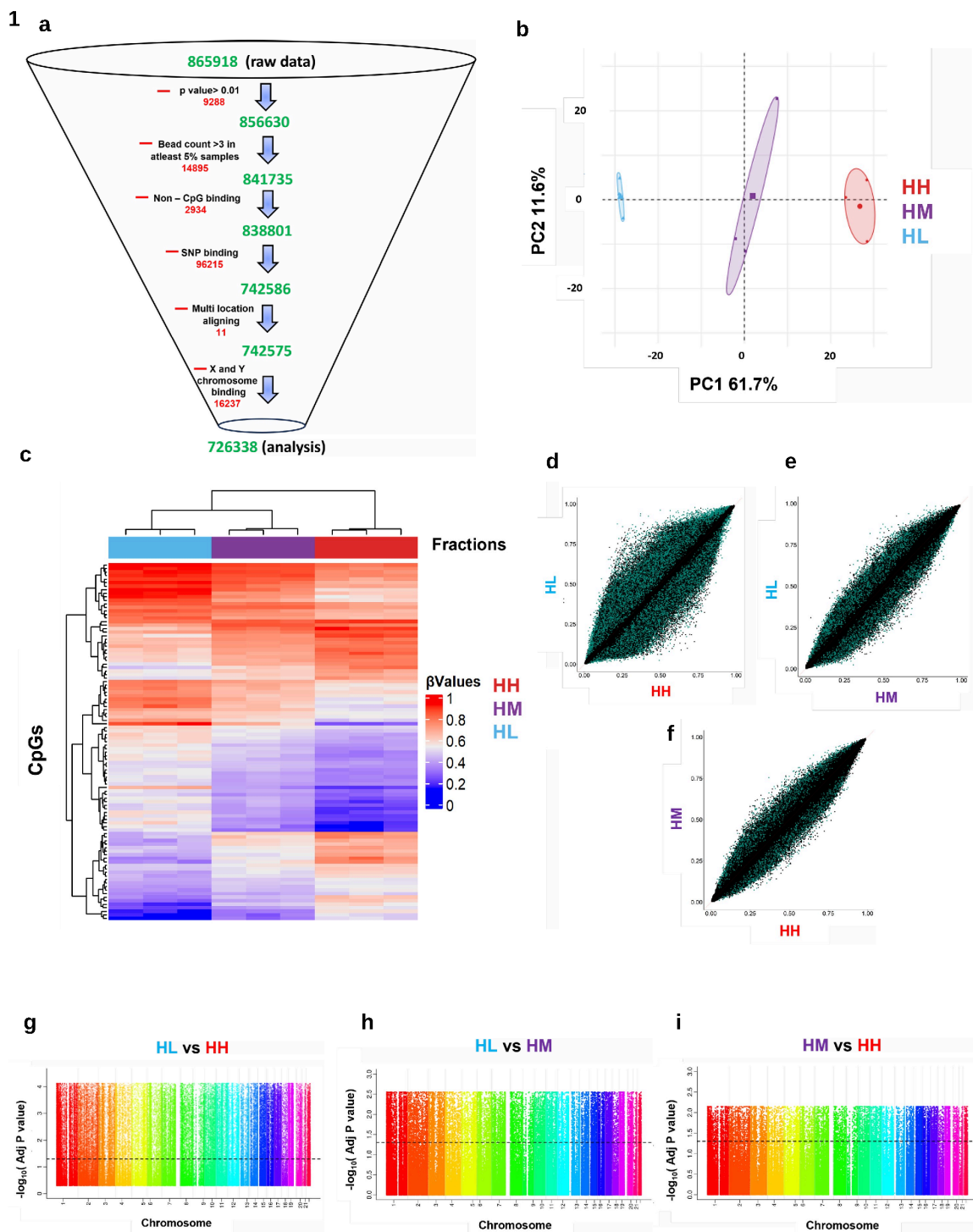


Figure 1

Intratumoral perfusion gradients align with genome-wide CpG methylation signatures. (a) Schematic of the selection strategy for niche-specific CpG methylome analysis. Probes correspond to single CpG sites. (b) Principal Component Analysis (PCA) of differentially methylated CpGs across perivascular (HH), intermediate (HM), and hypoxic (HL) niches. (c–f) Heatmap of the top 100 differentially methylated CpGs (c) and bivariate niche-versus-niche comparisons (d–f), demonstrating maximum divergence

between HL and HH fractions. (g-i)Manhattan plots illustrating the chromosomal distribution of significant differentially methylated CpGs identified in bivariate analyses. Data are derived from sorted tumor cell fractions isolated from n = 3 independent NOD/SCID mouse xenografts.

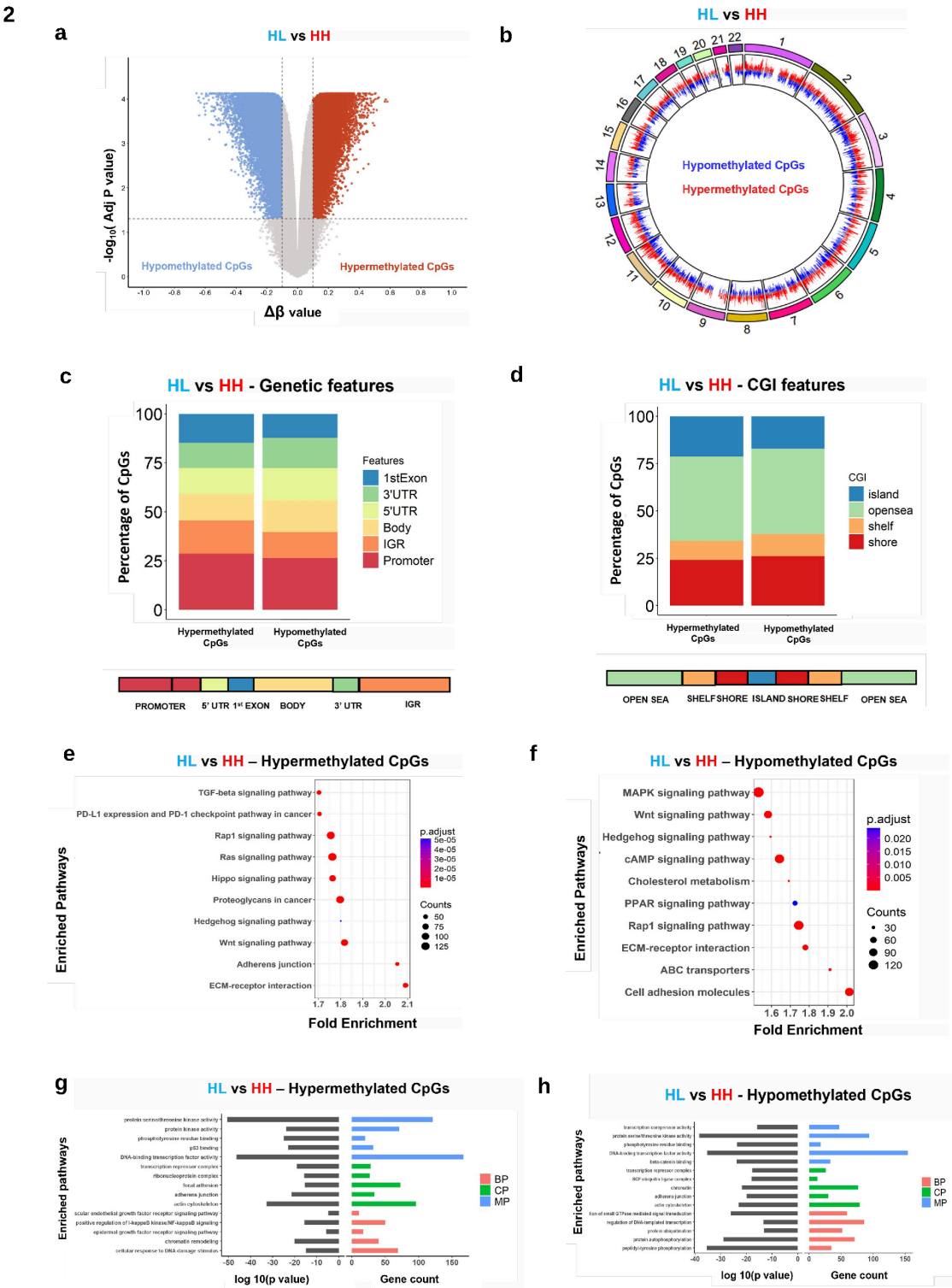


Figure 2

Perfusion-induced hypoxia dictates CpG methylation patterns across genomic regulatory elements and oncogenic pathways. **(a)** Volcano plot showing significant hypermethylation ($\Delta\beta > 0.1$) and hypomethylation ($\Delta\beta < -0.1$) in the HL versus HH fraction. **(b)** Circos plot depicting chromosomal occupancy of hypermethylated and hypomethylated CpGs. **(c, d)** Distribution of differentially methylated CpGs across genetic features **(c)** and CpG island (CGI) regions **(d)**. TSS: transcription start site (integrated TSS200 and TSS1500 defining the promoter); UTR: untranslated region; IGR: intergenic repeats. **(e, f)** KEGG pathway enrichment for genes associated with hypermethylated **(e)** and hypomethylated **(f)** CpGs. **(g, h)** Gene Ontology (GO) analysis of molecular processes (MP), biological processes (BP), and cellular processes (CP) impacted by hypermethylation **(g)** and hypomethylation **(h)**.

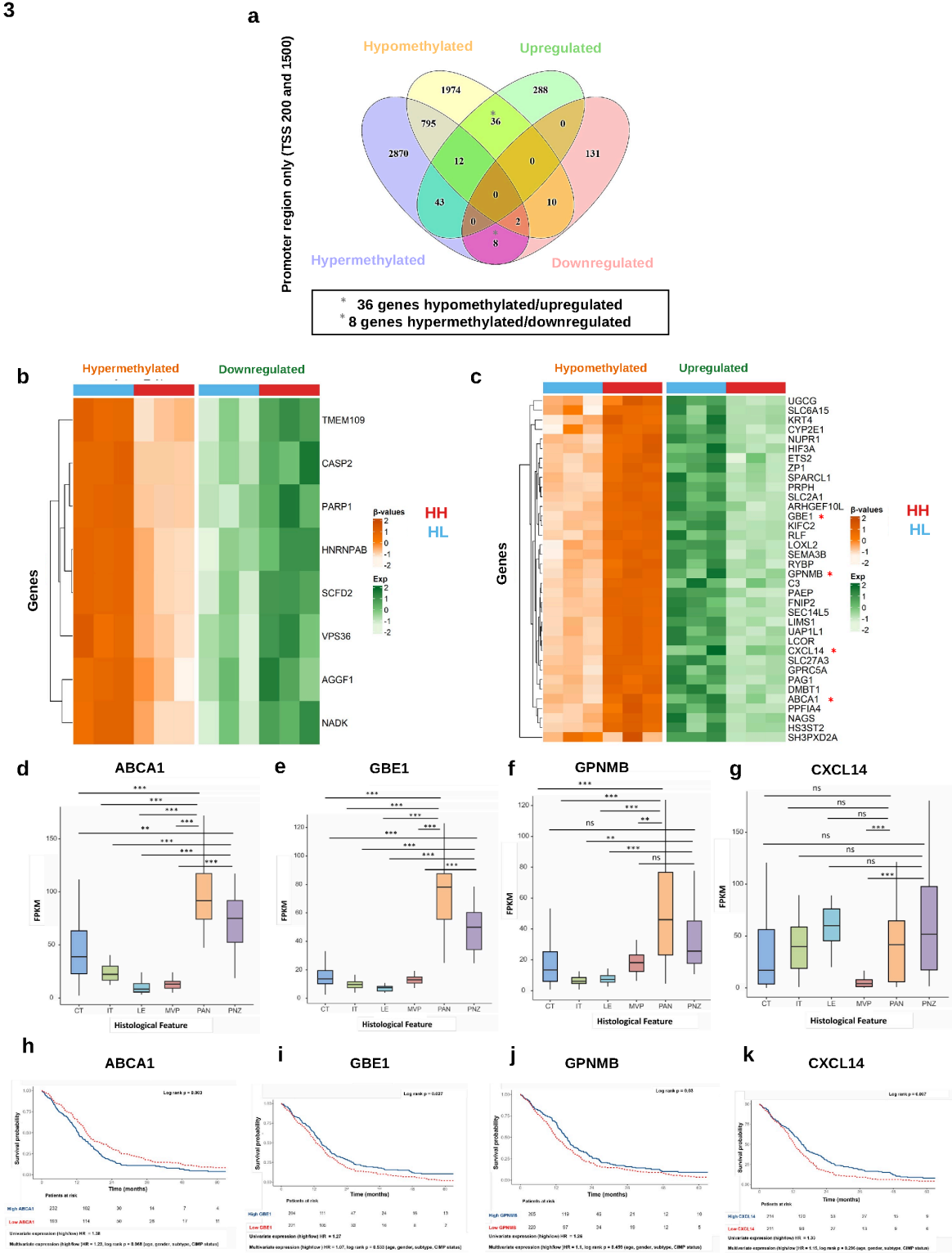


Figure 3

Hypoxia-driven CpG hypomethylation of ABCA1, GBE1, GPNMB, and CXCL14 correlates with malignancy and poor clinical outcomes. (a) Venn diagram integrating methylome and transcriptome data, identifying 36 hypomethylated/upregulated and 8 hypermethylated/downregulated genes in the promoter region. (b, c) Heatmaps showing relative β values and FPKM expression for hypermethylated/downregulated (b) and hypomethylated/upregulated (c) candidates. (d–g) Expression

4

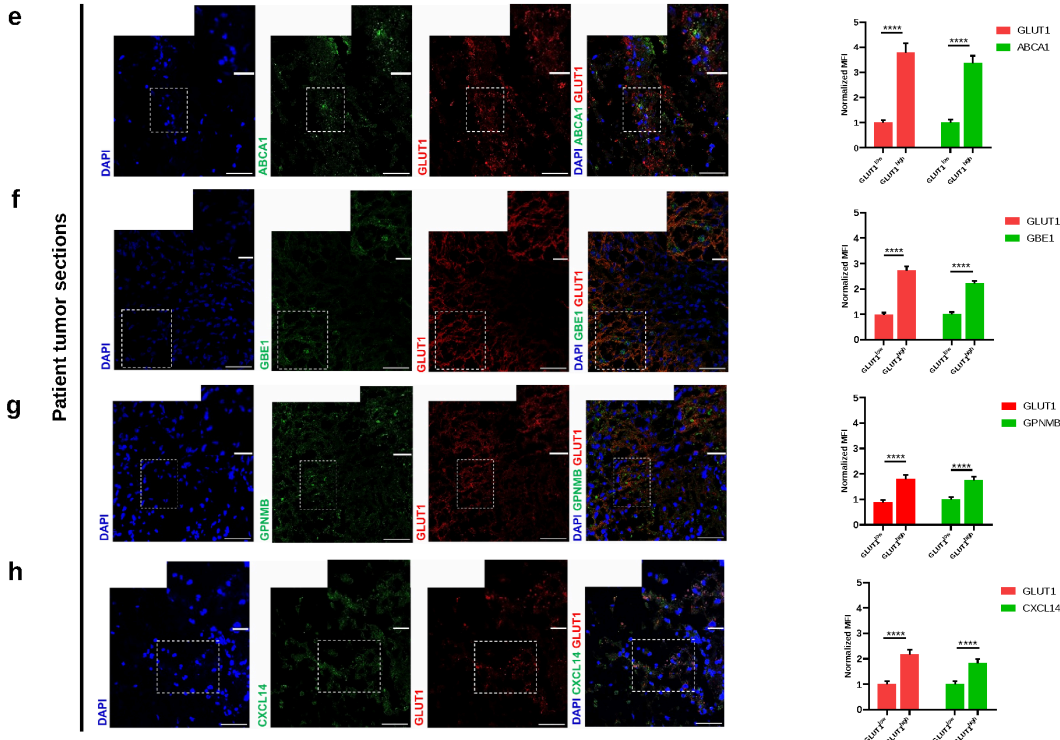


Figure 4

Spatial validation of target gene expression in hypoxic niches of GBM xenografts and patient tissues. **(a–d)** Immunohistochemical (IHC) analysis of ABCA1, GBE1, GPNMB, and CXCL14 in tumor xenografts, comparing perivascular (HH) and hypoxic (HL) niches. **(e–h)** Protein expression of target genes in patient sections, stratified by GLUT1^{high} (hypoxic) and GLUT1^{low} (normoxic) regions. Quantifications represent Mean Fluorescent Intensity (MFI) from 12–16 ROIs per group (HH and HL; GLUT1^{high} and GLUT1^{low}; n = 3 per model). Statistical significance determined by Wilcoxon rank-sum test. Scale bars: 10 μ m (2 μ m insets). Data are mean $\pm$ SEM. ****p<0.0001, ***p<0.001, ns: non-significant.

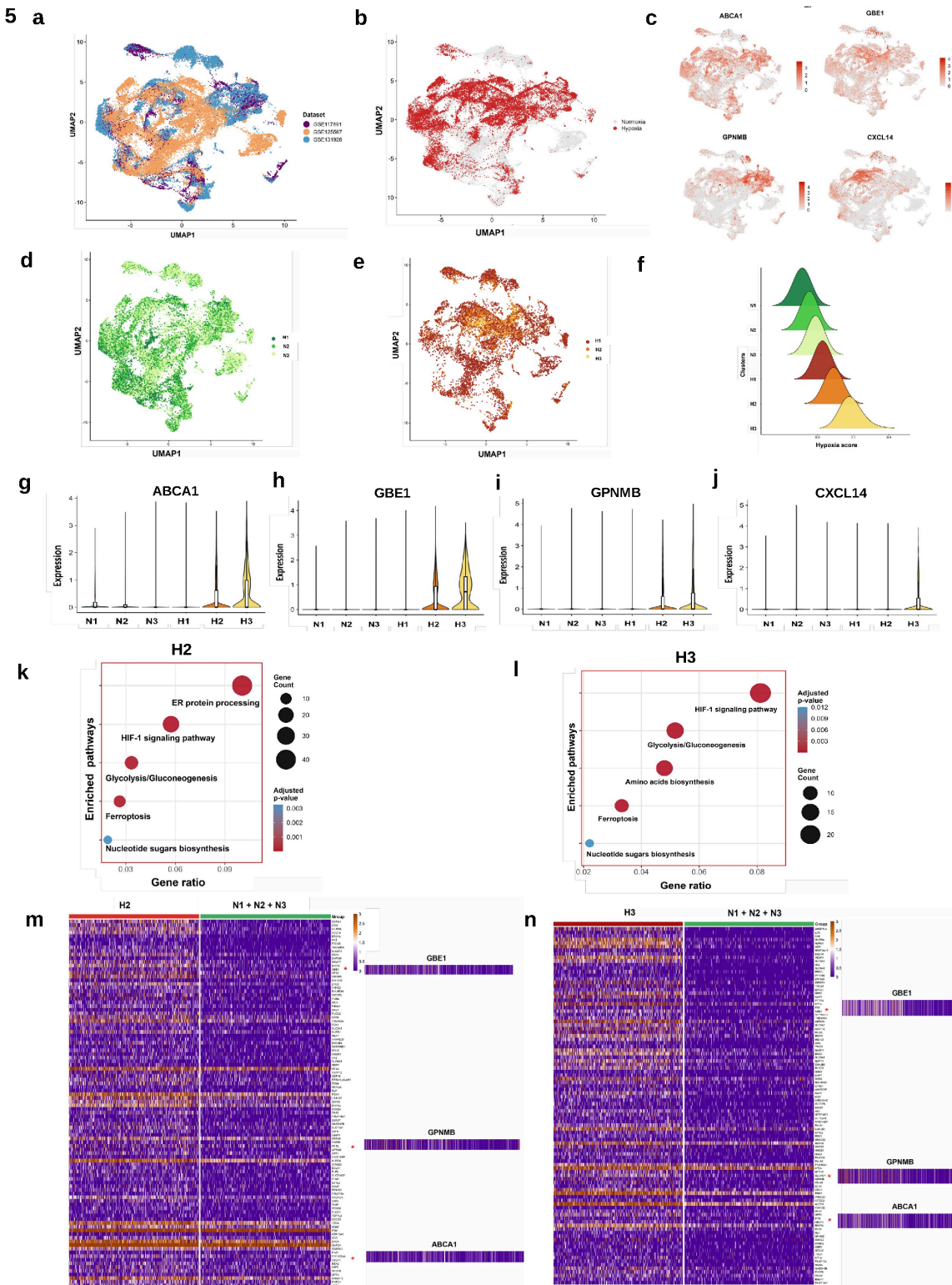


Figure 5

Single-cell analysis resolves target gene enrichment and oncogenic signaling along the hypoxia gradient. (a) UMAP integration of three independent GBM scRNA-seq datasets (totaling >50,000 cells). **(b)** UMAP projection of hypoxia (16329 cells) and normoxia (27770 cells) cellular states based on CHPF and PFDLC signatures. **(c)** Feature plots showing expression of ABCA1, GBE1, GPNMB, and CXCL14. **(d–f)** Subclustering of cells into normoxic (N1–N3) **(d)** and hypoxic (H1–H3) **(e)** states based on PFDLC-

derived hypoxia scores (f). (g-j) Violin plots illustrating target gene expression across subclusters. (k, m) KEGG enrichment for the most hypoxic subclusters (H2, H3). (n, o) Heatmap of the top 100 DEGs in H2 and H3 subclusters versus normoxic subclusters, highlighting ABCA1, GBE1, and GPNMB expression.

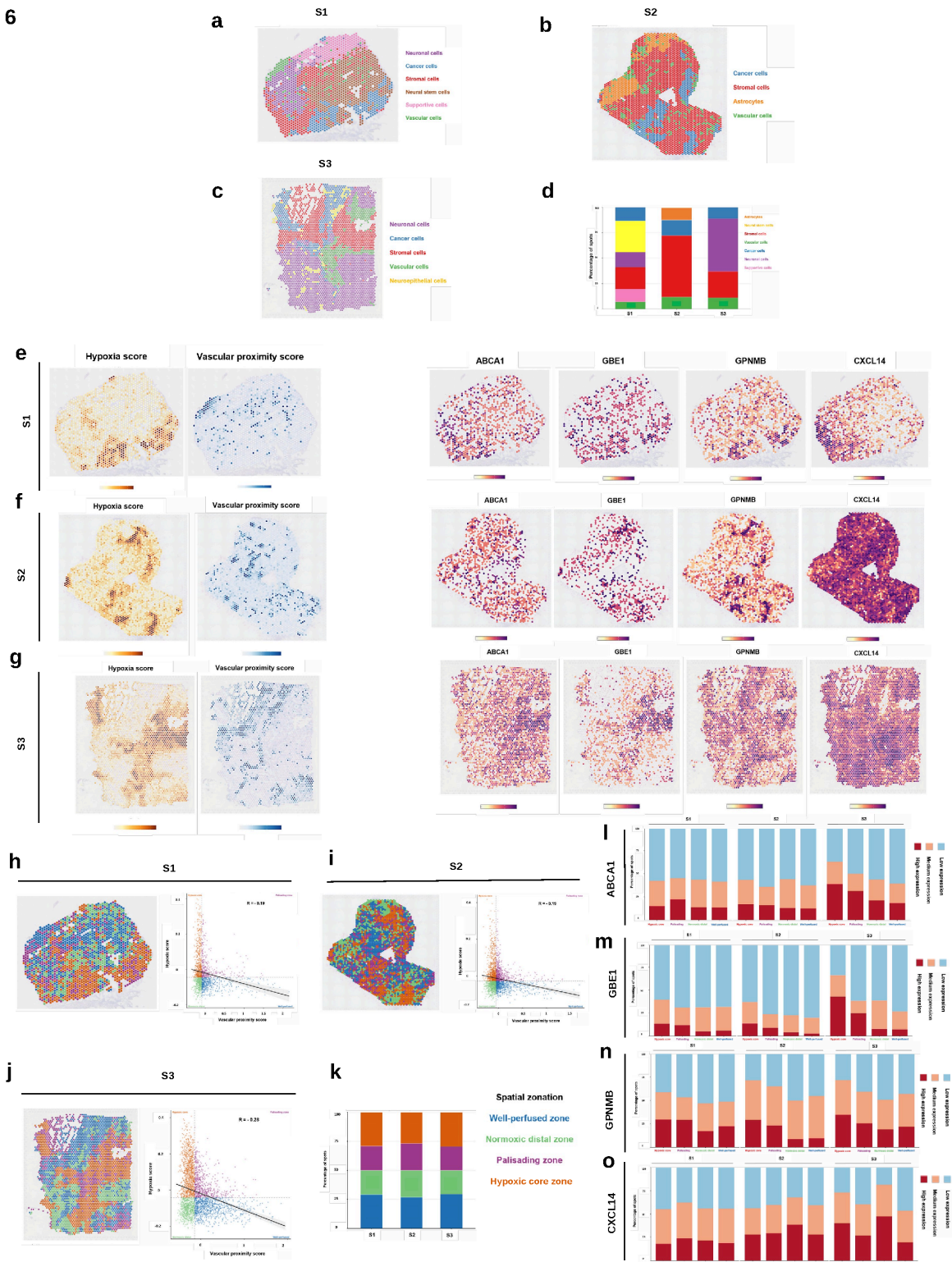


Figure 6

Spatial transcriptomics define the topographical enrichment of ABCA1, GBE1, and GPNMB in the GBM hypoxic zones. (a–d) Cell type mapping and spot percentages across three IDH-wildtype patient sections (S1–S3). **(e–g)** Spatial mapping of hypoxia scores (PFDLC-derived) versus vascular proximity (angiogenic signature) alongside target gene expression. **(h–k)** Dual-score spatial zonation into well-perfused, normoxic distal, pseudo palisading, and hypoxic core zones **(left)**. Pearson’s correlation **(right)** confirms the spatially inverse associations between hypoxia and vascularization. Zone-wise spot percentages for the sections **(k)**. **(l–o)** Tertile-based expression showing the frequency of high, medium and low expression spots for target genes across defined spatial zones.

Supplementary Files

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