

Spatial and single-cell characterization of human glioblastoma tumor microenvironment reveals malignant cellular communities

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Understanding the complex cellular and spatial organization of glioblastoma (GBM) and its microenvironment is crucial for improving diagnosis and treatment. Here we integrated 121 spatial transcriptomics, single-cell RNA sequencing, single-cell assay for transposase-accessible chromatin using sequencing and patch sequencing profiles from 100 patients to characterize primary GBM tissue. We identified four malignant cellular communities that exhibited consistent patterns of cell-type compositions, gene expression and intercellular interactions across patients. We identified two subpopulations of mesenchymal-like (MES-like) tumor cells: MES-Hyp, colocalized with monocyte-derived brain macrophages in hypoxic regions; and MES-Ast, associated with endothelial cells, pericytes and vascular smooth muscle cells. We also predicted and experimentally verified cell subtypes and ligand–receptor pairs involved in intercellular communications in each cellular community. Furthermore, patch sequencing analysis revealed that synaptic connections with glioma cells were predominantly formed between neurons and oligodendrocyte-progenitor-like tumor cells. Overall, our study provides insights into the spatial organization and intercellular communication in GBM, offering potential therapeutic targets.

Glioblastoma (GBM) is the most prevalent primary malignant glioma in adults, characterized by an extensive cellular heterogeneity within its microenvironment^{1,2}. Four distinct malignant cell subtypes have been identified within GBM—mesenchymal-like (MES-like), astrocyte-like (AC-like), oligodendrocyte-progenitor-like (OPC-like) and neural-progenitor-like (NPC-like)³—and multiple tumor subtypes or states often coexist within a single tumor specimen^{3–5}. Spatial transcriptomic (ST) studies have revealed that human GBM consists of both disorganized and structured regions, featuring a five-layered

organization associated with hypoxia⁶, and exhibits distinct transcriptional programs, including hypoxia/necrosis, angiogenesis and neuronal development⁷. However, a comprehensive characterization of GBM's spatial architecture across a large cohort is still required to elucidate the mechanisms driving tumor progression.

Tumor-associated macrophages (TAMs) represent the most prevalent cell population in the GBM microenvironment^{8–10}. Although reciprocal interactions between tumor cells and TAMs are known to promote GBM progression^{11,12}, the transcriptional

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regulatory mechanisms driving the polarization of TAM subsets toward a malignancy-promoting phenotype remain unclear. In parallel, accumulating evidence indicates that neurons actively support glioma growth through paracrine signaling and direct synaptic connections with glioma cells^{13–15}; however, the specific tumor subtypes preferentially form neuroglial synapses remains unresolved.

In this study, we integrated 32 spatial transcriptome profiles from 27 patients and 118,639 small cytoplasmic RNA sequencing/small nuclear RNA sequencing (scRNA-seq/snRNA-seq) cells from 53 patients. We identified four distinct spatial regions across GBM patients, termed ‘cellular communities (CCs)’, characterized by conserved and recurrent patterns of cell type composition, gene expression and intercellular interactions across multiple patient samples. To validate our findings on cell colocalization, communication and functional relevance, we conducted multiple rounds of *in situ* hybridization (ISH), multiplex immunostaining, perturbation assays and patch sequencing (Patch-seq) experiments. Our study provides a comprehensive road-map of the spatial architecture and intercellular communication in primary GBM tissue, offering valuable insights into the mechanisms driving tumor malignancy.

Results

Spatial organization of GBM

To elucidate the spatial architecture and heterogeneity of GBM, we employed a multiomics approach, generating 38 profiles of scRNA-seq, small cytoplasmic single-cell assay for transposase-accessible chromatin using sequencing (scATAC-seq) and ST from 20 IDH wild-type GBM patients (Extended Data Fig. 1a). Additionally, we integrated 12 publicly available single-cell and spatial transcriptome datasets from 58 GBM patients to expand our cohort (Supplementary Table 1). Experimental validations were performed using multiple rounds of ISH and Patch-seq on samples from 15 GBM patients. In total, our study encompassed 121 data profiles from 100 GBM patients, including 118,639 scRNA-seq/snRNA-seq cells from 53 patients, 20,650 scATAC-seq cells from 20 patients, 78,944 Visium spots from 27 patients, 340,568 ISH cells from 8 patients and 21 Patch-seq cells from 7 patients (Fig. 1a).

We performed microfluidic-based scRNA-seq using the 10x Genomics platform on 17 GBM patients, yielding a total of 30,725 single-cell transcriptomes with an average of 9,831 unique molecular identifiers (UMIs) representing 2,423 genes (Extended Data Fig. 1b). Our dataset was integrated with public scRNA-seq datasets¹⁶ consisting of 51,012 cells from 13 IDH wild-type GBM patients. Given the limitations of scRNA-seq in capturing neurons due to their size and fragility¹⁷, we incorporated 3,860 neurons from 10 IDH wild-type GBM patients using snRNA-seq data from ref. 18. This comprehensive integration resulted in 85,597 high-quality cells and nuclei for further analysis (Extended Data Fig. 1c and Methods).

To achieve robust integration of scRNA-seq profiles, we utilized the Harmony algorithm, focusing on the top 3,000 most recurrent variable genes across all patients (Methods). The integrated scRNA-seq profiles displayed minimal batch effects across datasets (Extended Data Fig. 1d). Through unbiased clustering with Seurat V4¹⁹ and annotation based on canonical lineage marker gene expression and copy number variations (CNVs), we identified 25 major cell types, including 4 tumor cell subtypes, 5 TAM subtypes, 6 T cell subtypes, as well as monocytes, dendritic cells, B cells, plasma cells, NK cells, oligodendrocytes, endothelial cells, pericytes, vascular smooth muscle cells (VSMCs) and neurons (Fig. 1b and Extended Data Fig. 1e). InferCNV⁵ analysis identified 36,247 malignant cells exhibiting large-scale CNVs, including GBM hallmarks of chromosome 7 gain and chromosome 10 loss. These malignant cells were classified into four subtypes: MES-like, AC-like, OPC-like and NPC-like, based on the classification model proposed by Neftel et al.³ (Fig. 1b and Extended Data Fig. 1e–h). This comprehensive single-cell transcriptome map of GBM serves as a foundational reference for subsequent spatial transcriptome analyses.

To explore the spatial distribution of the identified cell types in a two-dimensional context, we performed 10x Visium ST sequencing on 10 slices obtained from 5 IDH wild-type patients (Fig. 1a). Initial preprocessing of histological images using StarDist²⁰ resulted in an average of 8.79 cells and 4,003 genes per spot (Extended Data Fig. 2a). To identify recurrent spatial domains with consistent cell type composition across patients, we integrated our ST data cohort with publicly available ST data from 22 samples: 12 from ref. 7, 7 from ref. 21, 2 from ref. 22 and 1 from the 10x dataset (Extended Data Fig. 2a).

We employed the cell2location algorithm²³ to deconvolute the cell type compositions of each ST spot due to its supervisor performance on this task^{24,25}, using the scRNA-seq profiles and identified cell subtypes as a reference (Methods). Subsequently, we utilized the Splane module of SPACEL²⁶ to jointly analyze all 32 ST slices, identifying 10 recurrent spatial domains based on cell type deconvolution, spatial coordinates and inferred CNV information (Fig. 1c, Extended Data Fig. 2b and Methods). High correlations in gene expression and cellular composition between spatial domains across different slices confirmed the reproducibility of these domains across multiple GBM ST cohorts (Extended Data Fig. 2c).

The distributions of spatial domains across the cohorts are illustrated in Extended Data Fig. 2d,e. Domain 9 and domain 10 (D9, D10) exhibited significantly lower levels of CNVs in chromosome 7 and chromosome 10 compared to D1–D8 (Wilcoxon rank-sum test, $P = 0.0$; Extended Data Fig. 2f–h), indicating that D9 and D10 represent peritumoral domains. These findings were corroborated by haematoxylin and eosin (H&E) staining performed on the same slices. To further characterize the cellular compositions of each spatial domain, we quantified the number of cells belonging to each cell subtype in each ST spot. As expected, peritumoral domains were enriched for normal neurons, astrocytes, oligodendrocytes and normal OPCs (Extended Data Fig. 2i and Methods). The malignant domains, however, could be further grouped into four distinct CCs, each with similar cell compositions and gene expressions, suggesting shared biological functions (Fig. 1d).

Specifically, domains D1 and D2 in CC-1 were enriched for MES-like tumor cells, TAM5-GPNMB and monocytes (Fig. 1d,e). Functional enrichment analysis of spatially variable genes (SVGs) revealed that CC-1 is a central tumor region characterized by a hypoxic cellular state⁶ (Extended Data Fig. 2j and Supplementary Table 2). Domain D3 in CC-2 was enriched for endothelial cells, pericytes and VSMCs, indicating a vascular-rich region consistent with extracellular matrix organization and angiogenesis. Domains D4 and D5 in CC-3 were enriched for NPC-like, OPC-like and AC-like tumor cells and neurons. CC-3 exhibited an upregulation of genes involved in synaptic signaling and cell cycle, suggesting a region associated with neuroglioma synaptic connections and increased tumor cell proliferation and invasion²⁷. Domains D6–D8 in CC-4 were predominantly composed of AC-like tumor cells.

Given the limited spatial resolution of 10x Visium data, the predicted spatial distribution of cells may not fully reflect their actual distribution. To refine the cellular composition of CCs at single-cell resolution, we employed multiple rounds of ISH to detect the spatial distribution of 116 cell marker genes (Supplementary Table 3) on 2 slices from 2 GBM patients (Methods). This approach yielded 136,100 cells at single-cell resolution, with an average of 33.42 transcripts representing 17.63 genes per cell (Extended Data Fig. 3a,b). We annotated the ISH-identified cells by mapping their gene expression to the scRNA-seq profiles using Seurat V4’s label transfer approach (Extended Data Fig. 3c). Additionally, we mapped the ISH cells to the 10x Visium data to identify corresponding spatial domains (Extended Data Fig. 3d and Methods). Within expectation, all four CCs were identified in the ISH data with cell type compositions closely matching those observed in the 10x Visium profiles (Fig. 1f and Extended Data Fig. 3e). By integrating large-scale spatial transcriptome data at varying resolutions, we have delineated the spatial landscape and cellular organization within primary GBM tumors.

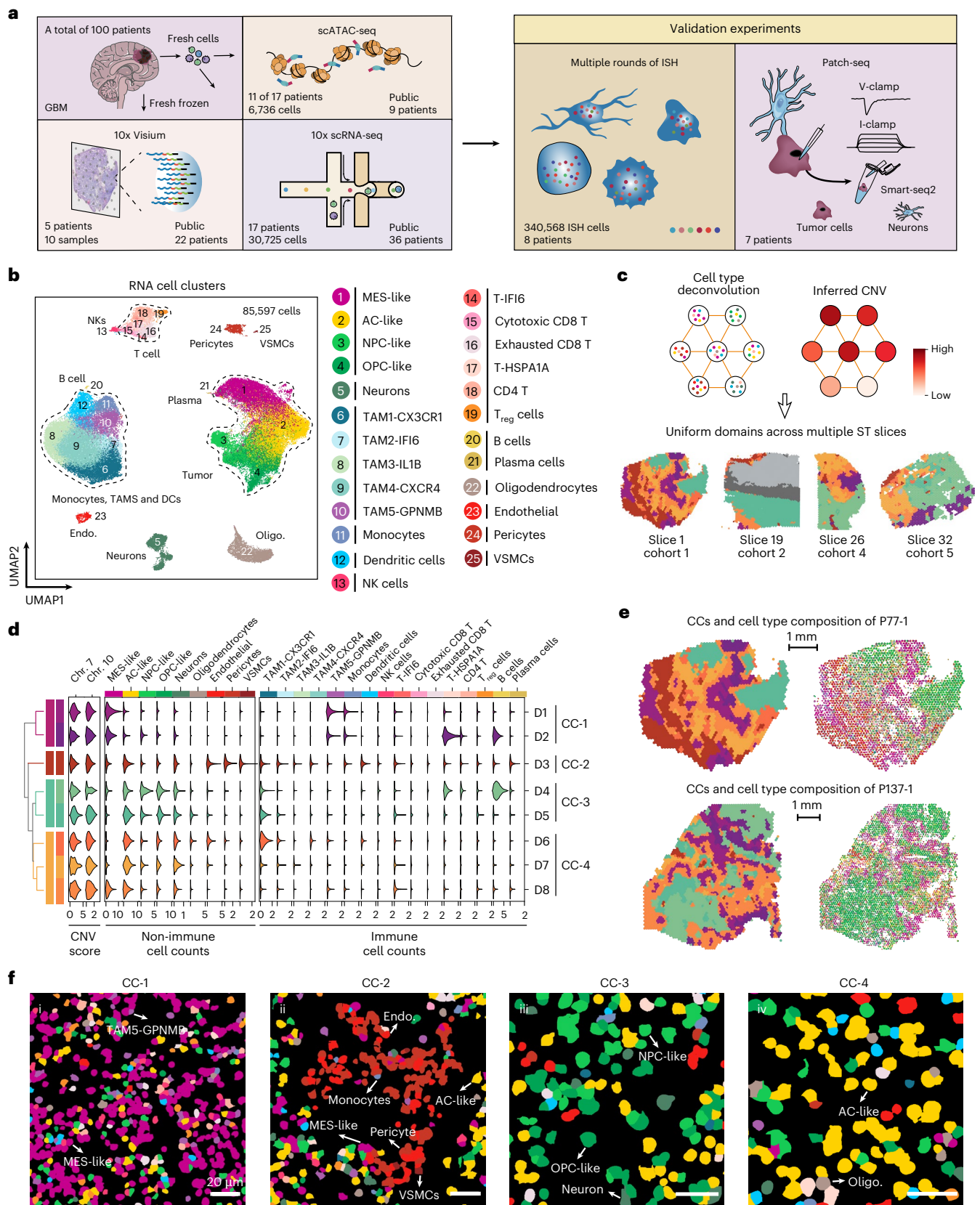


Fig. 1 | Identification and cellular composition of four CCs. a, Study schematic. **b**, Uniform manifold approximation and projection (UMAP) visualization of scRNA-seq data from 30 GBM patients ($n = 81,737$) and snRNA-seq data of neurons from 10 GBM patients ($n = 3,860$). Cells are colored by cell clusters. **c**, Schematic diagram illustrating the strategy employed for defining uniform domains cross 32 ST slices. **d**, Cellular composition of spatial domains within the

tumor core. The hierarchical clustering in the left panel shows the identification of CCs. **e**, Distribution of spatial domains and 25 cell types predicted by cell2location in P77-1 (top) and P137-1 (bottom). Each cell type is color-coded as shown in **b**. **f**, ISH data showing the spatial distribution of 25 cell types in representative fields of view of 4 CCs. DC, dendritic cell; endo., endothelial; I-clamp, current clamp; oligo., oligodendrocyte; V-clamp, voltage clamp.

Two subpopulations of MES-like tumor cells in GBM

Previous studies have indicated the existence of two distinct subtypes of malignant MES-like tumor cells in GBM, based on limited single-cell transcriptome data³ or ST profiles with non-single-cell resolution⁶. To comprehensively characterize the heterogeneity and biological functions of MES-like tumor cells, we integrated our dataset with the GBMap database²⁸, constructing a scRNA-seq profile comprising 92,256 high-quality malignant cells, including 24,112 MES-like, 41,902 AC-like, 12,369 NPC-like and 13,873 OPC-like tumor cells (Extended Data Fig. 4a–c). Of these, 18,139 MES-like tumor cells were collected from 19 patients, each with more than 200 MES-like tumor cells, across 8 datasets for subsequent analysis.

Focusing on these 18,139 MES-like tumor cells, we performed Leiden clustering on the transcriptomic data and identified 54 gene sets, whose expression consistently differentiated two subtypes of MES-like tumor cells across patients, datasets and assay technologies, highlighting the intrinsic heterogeneity within MES-like tumor cells (Fig. 2a and Methods). We selected the top 100 genes as signature markers most effective in distinguishing these two subtypes, and they were indeed distinctly expressed in two clusters of MES-like tumor cells, irrespectively of the interindividual heterogeneity observed across patients (Extended Data Fig. 4d and Supplementary Table 4). One subtype was enriched with hypoxia-related pathways, which we designated MES-Hyp, and the other was enriched with integrin and extracellular matrix organization, designated MES-Ast (Extended Data Fig. 4e).

To further investigate the biological characteristics of MES-Hyp and MES-Ast, we conducted a hypergeometric test comparing the signature genes of these subtypes with published gene sets representing malignant cell subtypes in GBM^{3,6,7}. MES-Hyp signature genes significantly overlapped with the MES-Hyp state identified by Greenwald et al.⁶ and the MES2 state described by Neftel et al.³, whereas MES-Ast signature genes specifically overlap with the MES-Ast state by Greenwald et al. and the MES1 state by Neftel et al. (Fig. 2b and Extended Data Fig. 4f,g). Notably, a significant overlap between MES-Ast and AC-like cells was observed, suggesting a potential evolutionary connection between these subtypes.

Moreover, MES-Hyp cells exhibited a hypoxic state characterized by the upregulation of hypoxia-related genes *NDRG1* and *ERO1A* (Extended Data Fig. 4h). Furthermore, these cells expressed multiple cytokines, including *VEGFA* and *IL6* family members *LIF* and *IL11*. Notably *LIF* has been implicated in promoting the emergence of protumoral CD206⁺ TAMs²⁹, indicating that MES-Hyp cells may drive myeloid cell differentiation. In contrast, MES-Ast cells displayed high expression levels of *GAP43* and *CHI3L1* (Extended Data Fig. 4i), suggesting a role in promoting microtubule formation and facilitating tumor network integration^{30,31}. Additionally, MES-Ast cells showed elevated expression of *COL1A2* and *COL5A2*, indicating an association with extracellular matrix organization and collagen formation (Extended Data Fig. 4i). Collectively, these findings provide robust evidence for the existence of two functionally distinct subtypes of MES-like tumor cells in GBM, derived from a comprehensive analysis of large-scale datasets.

Spatial distribution and colocalization of two MES-like subpopulations

To further investigate the spatial distribution of the two MES-like cell subpopulations, we first employed Tangram³² to map the scRNA-seq data onto the ST slices, given its demonstrated superior performance for single-cell and spatial data integration²⁴. This analysis revealed that MES-Hyp cells were primarily localized in CC-1, whereas MES-Ast cells were more widely distributed across CC-2, CC-4 and CC-1 (Extended Data Fig. 5a–d and Methods). We then conducted an independent high-resolution ISH analysis using confocal microscopy to examine the spatial distribution and colocalization of MES-Hyp and MES-Ast cells at single-cell resolution. In this analysis, we assessed

36 cell-type-specific marker genes (Supplementary Table 3), detecting an average of 2.66 transcripts per gene in individual cells, which exceeds the 1.80 transcripts per gene observed in fluorescence microscopy ISH data (Extended Data Fig. 5e). The ISH data were annotated by mapping them to the scRNA-seq profiles using Seurat V4's label transfer approach.

Through ISH analysis, we identified MES-Hyp cells (*CD44*⁺, *LIF*⁺, *IL11*⁺ and *COL1A2*⁺) and MES-Ast cells (*CD44*⁺, *COL1A1*⁺, *COL1A2*⁺ and *LIF*⁺) in GBM, exhibiting significantly higher expression of signature genes for each subtype (Extended Data Fig. 5f–h). To further explore the spatial distribution and colocalization of cell subsets within GBM, we divided the ISH slices into windows 1,200 pixels (192 μ m) square and performed clustering based on the cell type composition within each window (Extended Data Fig. 5i,j and Methods). This analysis identified three regions with distinct cellular compositions—region 1, enriched in MES-Hyp and TAM5-GPNMB cells; region 2, enriched in AC-like, OPC-like, NPC-like and MES-Ast tumor cells; and region 3, characterized by enrichment of MES-Ast cells and endothelial and pericyte cells (Fig. 2c–e)—corresponding to CC-1, CC-4 and CC-2 in Fig. 1.

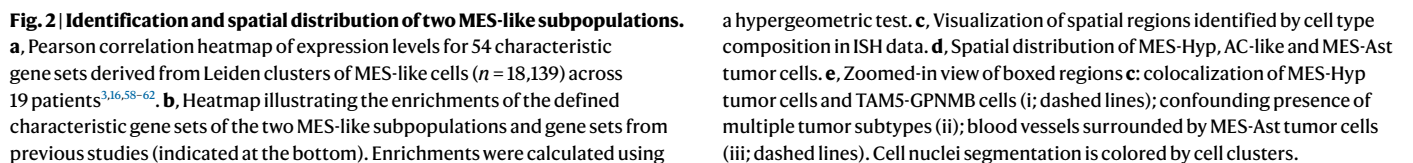
Colocalization probability analysis revealed that MES-Hyp cells significantly colocalized with TAM5-GPNMB cells (Wilcoxon rank-sum test, $P = 4.3 \times 10^{-9}$), whereas MES-Ast cells significantly colocalized with endothelial and pericyte cells (Wilcoxon rank-sum test, $P = 0.00012$) (Extended Data Fig. 5k and Methods). These ISH findings are consistent with the cell type distributions identified through 10x Visium ST analysis. Collectively, these results reveal the spatial distribution of the two MES-like tumor cell subpopulations in GBM at single-cell resolution.

Tumor cell transitions in GBM

GBM tumor cells exhibit a high degree of plasticity, with reversible phenotypic states, including transitions between OPC-like/NPC-like and MES-like cells^{11,33}, as well as between AC-like and MES-like and between OPC-like and NPC-like states³⁴. We performed pseudotime analysis using Palantir³⁵ on our scRNA-seq data and observed continuous transcriptomic shifts among AC-like, MES-Ast and MES-Hyp tumor cells (Fig. 3a and Methods), suggesting that MES-Ast cells may represent an intermediate state between AC-like and MES-Hyp tumor cells. To validate these shifts at the chromatin accessibility level, we performed pseudotime analysis using publicly available scATAC-seq data consisting of 13,914 tumor cells from 9 GBM patients³⁶ (Methods). We observed similar state transitions among AC-like, MES-Ast and MES-Hyp tumor cells (Fig. 3b).

To further explore the spatiotemporal dynamics underlying these transitions, we mapped our scRNA-seq profiles onto ST slices using Tangram³² and subsequently employed scVelo³⁷ to reconstruct spatiotemporal trajectories of tumor cells (Methods). Notably, the inferred trajectories displayed clear directional patterns predominantly pointing toward the CC-1 region, where MES-Hyp cells were markedly enriched (Fig. 3c). These results provide additional evidence supporting the temporal transitions initially observed at both the transcriptomic and chromatin accessibility levels.

To experimentally validate the predicted tumor cell transitions, we generated patient-derived GBM organoids (GBOs) and conducted high-resolution ISH analysis using a 20-gene panel (Supplementary Table 3) to examine the spatial distribution of different tumor subtypes in GBOs. We observed a clear spatial gradient in cell type composition, transitioning from AC-like cells at the periphery through MES-Ast cells toward MES-Hyp cells in the organoid core (Fig. 3d). Over a 4-week culture period, the relative proportions of AC-like and MES-Ast tumor cells progressively declined, whereas the proportion of MES-Hyp tumor cells increased substantially, particularly in the central regions of the GBOs (Fig. 3d–k). Concurrently, the hypoxic area (*ADM*⁺) expanded in correlation with organoid growth (Extended Data Fig. 6a–h), highlighting hypoxia as a potential driving force for tumor cell transition. Notably, among all tumor subtypes, MES-Hyp exhibited the lowest expression level of *MKI67* in both GBOs



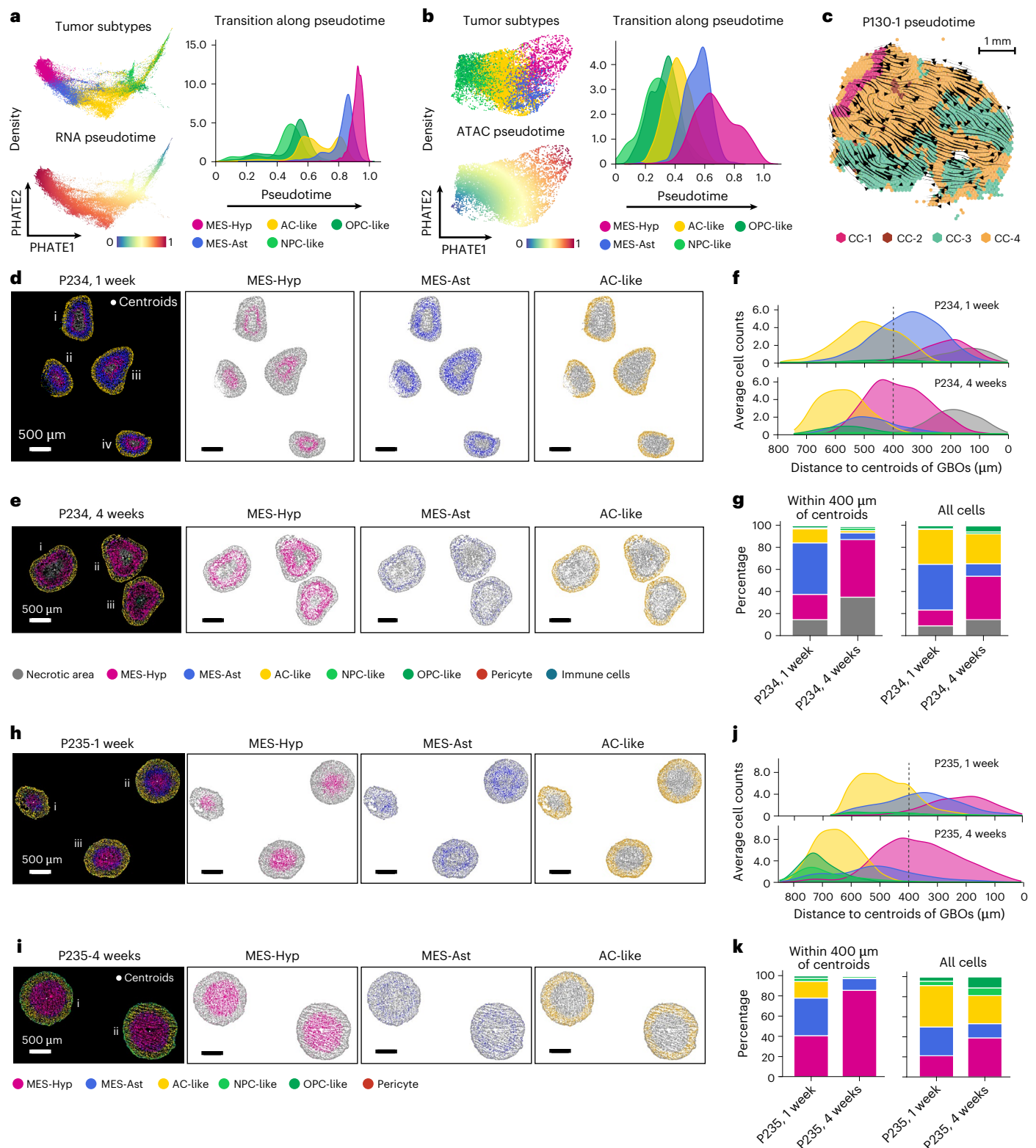


Fig. 3 | Characterization of spatial and temporal transitions of tumor cells.

a, b, Low-dimensional representation of tumor cells based on scRNA-seq (**a**, $n = 19$ patients) and scATAC-seq (**b**, $n = 9$ patients). Top-left: cells colored by tumor cell identity. Bottom-left: colored by pseudotime ordering. Right: density distributions of tumor subtypes along the pseudotime trajectory.
c, Spatiotemporal pseudotime trajectory in representative slice P130-1.
d–g, ISH images of P234-derived GBOs cultured for 1 week (**d**) and 4 weeks (**e**), with corresponding density distributions of tumor subtypes along the

periphery-to-centroid axis (**f**) and stacked bar plot showing proportions of cell types within 400 μm of the centroids (left) and overall composition (right) (**g**). In **d**, area = 0.92 mm² (i), 0.71 mm² (ii), 1.11 mm² (iii), 0.60 mm² (iv). In **e**, area = 1.26 mm² (i), 1.22 mm² (ii), 1.17 mm² (iii). **h–k**, ISH images of P235-derived GBOs cultured for 1 week (**h**) and 4 weeks (**i**), with corresponding density distributions (**j**) and stacked bar plots (**k**) as in **f** and **g**. In **h**, area = 0.66 mm² (i), 1.20 mm² (ii), 1.09 mm² (iii). In **i**, area = 1.74 mm² (i), 1.94 mm² (ii).

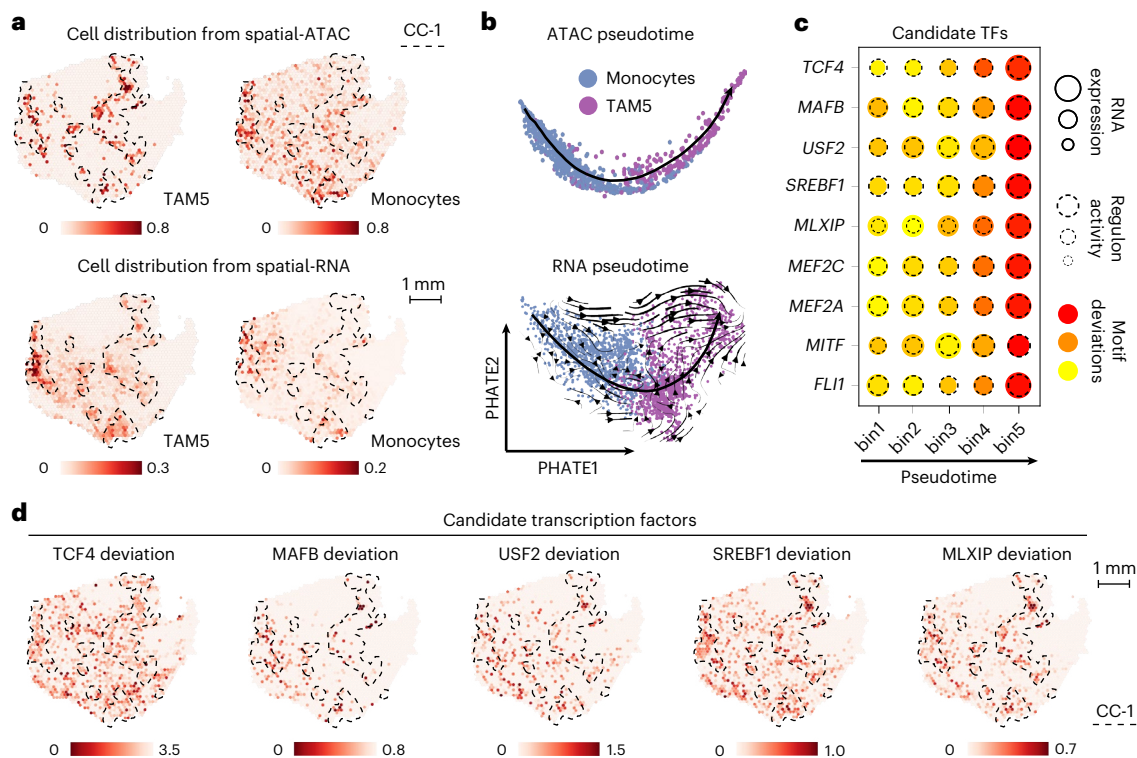


Fig. 4 | Transcriptional regulation driving monocyte polarization. **a**, Top: proportions of TAM5-GPNMB and monocytes subpopulations within scATAC-seq clusters on representative slice P77-1. Bottom: the same subpopulations in scRNA-seq clusters. **b**, Low-dimensional representation and ordering of pseudotime trajectories for monocyte-derived TAM clusters based on scATAC-seq data (top) and scRNA-seq data (bottom). **c**, Dot plot illustrating nine TFs

with significant increases in expression, motif deviations and SCENIC-estimated regulon activity along pseudotime trajectory. Color indicates motif deviations of the TF, dot size of solid circles represents RNA expression of the TF and dot size of dashed circles indicates SCENIC-estimated TF regulon activity. **d**, Visualization of binding site accessibility for five candidate TFs on representative slice P77-1.

and primary tumors (Extended Data Fig. 6i,j), indicating that the expansion of MES-hyp cells is unlikely to result from elevated proliferative activity. These experimental findings support our computational predictions of dynamic transcriptomic shifts among AC-like, MES-Ast and MES-Hyp tumor subpopulations.

Transcriptional regulators drive monocyte polarization

In our dataset, TAMs accounted for 81.5% of immune cells within the GBM microenvironment. Previous studies have reported that TAMs in brain tumors can originate from microglia cells or peripheral blood monocytes^{9,38} and exhibit high heterogeneity and plasticity⁸. We observed that TAM1-TAM4 expressed higher levels of homeostatic microglia marker genes, including *TMEM119* and *CX3CR1* (Extended Data Fig. 1e). In contrast, TAM5-GPNMB exhibited elevated expression of monocyte-derived brain macrophage marker genes *CD163* and *TGFBP*, suggesting a microglial origin for TAM1-TAM4 subtypes and a monocyte origin for TAM5-GPNMB. Furthermore, TAM5-GPNMB uniquely expressed higher levels of *GPNMB* (Extended Data Fig. 1e), and GPNMB-high TAMs in GBM were found to promote malignancy by transferring myelin-derived lipids to adjacent MES-like tumor cells¹², fueling cancer cell proliferation and recurrence. Therefore, we aimed to elucidate the potential regulators driving the transformation of monocyte into TAM5-GPNMB.

We conducted plate-based scATAC-seq experiments on 11 IDH wild-type GBM patients and produced 6,736 high-quality cells with an average of 54,983 unique fragments mapped to the genome per cell (Extended Data Fig. 7a). We then integrated these scATAC-seq data with scRNA-seq and ST data to assess the spatiotemporal profiling of chromatin accessibility and gene expression in GBM (Methods). We first estimated the gene scores for each cell in the scATAC-seq profile using

ArchR³⁹. Then, we anchored these cells onto the scRNA-seq-defined clusters by using Signac⁴⁰ (Extended Data Fig. 7b and Methods). We then utilized Tangram to map these cells onto the 10x Visium ST slices based on gene scores and obtained a spot-level spatial-ATAC-seq profile (Methods).

As positive controls, we observed a significant enrichment of TAM5-GPNMB and monocytes in CC-1 (Wilcoxon rank-sum test, $P = 0.0$ for TAM5-GPNMB; Fig. 4a) and OPC-like and NPC-like tumor cells in CC-3 according to this spatial-ATAC-seq profile (Extended Data Fig. 7c). These findings are consistent with the deconvolution results obtained from spatial-RNA-seq analysis, providing confidence in the prediction of spatial chromatin accessibility.

We employed Palantir³⁵ to perform a pseudotime analysis and observed a clear transformation from monocytes to M2-like TAM5-GPNMB cells at both the RNA transcription and chromatin accessibility levels (Fig. 4b and Methods). Investigating gene regulation along this trajectory, we identified nine transcription factors (TFs) that demonstrated significant increases in RNA expression, motif deviations and regulome activity predicted by SCENIC⁴¹, along the pseudotime trajectory (Fig. 4c).

Among these TFs, SREBF1 is an established regulator of M2 macrophage polarization⁴², and MAFB has been implicated in restricting local monocyte proliferation before macrophage differentiation⁴³. We observed abundant binding sites and increased chromatin accessibility of these TFs around key marker genes of TAM5-GPNMB cells, including *GPNMB*, *CD163* and *ABCA1* (Extended Data Fig. 7d). In addition, the spatial chromatin accessibility of these TF binding sites was highly correlated with the spatial distribution of TAM5-GPNMB cells (Fig. 4d), further indicating that these TFs are likely key transcriptional regulators driving the transformation of monocytes into TAM5-GPNMB in GBM.

Spatial interactions of cells in CCs

Cells in GBM are not static; instead, they interact with cells nearby within the CC. This intercellular communication is crucial in driving protumor functions within GBM microenvironment⁴⁴. We then conducted an extensive analysis to investigate the specific cell–cell interactions occurring within each CC and the corresponding ligand–receptor (LR) pairs involved in these interactions. To achieve this, we utilized the CellChat LR pairs library⁴⁵ and employed COMMOT⁴⁶ (which integrates RNA expression levels and spatial distances of LR pairs) to infer interaction strength in the 10x Visium data. We explored all the conservative LR pairs with significantly elevated RNA expression levels in each of the four CCs across at least 20% of the 32 slices (*t*-test and false discovery rate (FDR) $< 1.0 \times 10^{-20}$) (Methods). This yields 37, 300, 50 and 13 LR pairs specifically enriched in CC-1, CC-2, CC-3 and CC-4, respectively, indicating pervasive cell–cell communication in GBM (Fig. 5a,b and Supplementary Table 5).

To systematically predict which cell subtypes interact via specific LR pairs, we defined a connectivity score as the product of (1) the average gene expressions of the ligand in each cell subtype, (2) the average gene expressions of the corresponding receptor in each cell subtype and (3) the interaction strength of the LR pairs obtained from COMMOT. We selected the top 10% of interactions based on the highest connectivity scores and clustered the corresponding LR pairs according to their associated signaling pathways (Methods). To experimentally validate critical cell–cell interactions predicted by our computational analyses, we also performed PhenoCycler multiplex imaging using a 21-antibody panel (Supplementary Table 6), targeting selected cell type markers and key LR pairs (Methods).

Our analysis provided valuable insights into intercellular communication within each CC. In CC-1, interactions were predominantly observed between MES-Hyp cells and TAM5-GPNMB, monocytes and AC-like tumor cells (Fig. 5c), with the highest spatial proximity observed between MES-Hyp cells and TAM5-GPNMB macrophages (Extended Data Fig. 8a and Supplementary Table 7). Previous studies have shown that VEGF can chemoattract macrophages via NRP1 (ref. 47), suggesting that MES-Hyp cells may recruit TAM5-GPNMB or monocytes to CC-1 through VEGF signaling. Consistent with this, interactions between MES-Hyp cells and TAM5-GPNMB or monocytes were primarily mediated by *VEGFA*, *NRP1/NRP2* and *MIF*, *CD74/CD44* LR pairs (Fig. 5c and Extended Data Fig. 8b). PhenoCycler multiplex imaging analysis further demonstrated a clear spatial colocalization of MES-Hyp tumor cells (NDRG1⁺) with TAM5-GPNMB (GPNMB⁺), alongside elevated VEGFA expression within MES-Hyp tumor cells (Fig. 5d and Extended Data Fig. 9a,b). Additionally, *LIF*, *LIFR* pairs, known to facilitate the transition of tumor cells to a MES-like state¹¹, were predominant in the interactions between MES-Hyp and AC-like cells.

In CC-2, interactions primarily occurred between MES-Ast cells and vascular cells, including endothelial cells, pericytes and VSMCs, through collagen-formation-related LR pairs (Fig. 5e and Extended Data Fig. 8a). MES-Ast cells expressed high levels of collagen genes (*COL1A2*, *COL5A2*, *COL6A2*), whereas vascular cells expressed the corresponding receptors (*ITGA1*, *ITGB1*, *CD93*) (Extended Data Fig. 8c). PhenoCycler assays confirmed spatial proximity between MES-Ast tumor cells expressing high levels of collagen proteins (*COL1A2* and

COL6A2) and adjacent vascular cells marked by elevated *ITGA1* expression (Fig. 5f and Extended Data Fig. 9c,d). Additionally, MES-Ast cells were found to interact with endothelial cells through *TGFB2*, *ENG* and *TGFB3*, *TGFB2* LR pairs. To experimentally validate the function of this interaction, we treated patient-derived GBOs with recombinant TGFβ2 protein and observed significant vascular remodeling, characterized by increased proportions of proliferating (*MKI67*⁺) endothelial cells and pericytes compared to untreated controls (17.5% versus 12.6% in patient P244; 38.4% versus 18.8% in patient P245; Extended Data Fig. 8f,g). VEGFA treatment was included as a positive control. These results confirm the angiogenic role of TGFβ signaling in GBM.

In CC-3, interactions were predominantly found between neurons and OPC-like tumor cells (Fig. 5g), with the highest proximity observed between these two cell subtypes (Extended Data Fig. 8a). Previous studies have shown that neurons interact with tumor cells through neuroligin-3 to promote robust glioma cell proliferation and feedforward expression of *NLGN3* in glioma cells^{13,15}. Consistent with these findings, we observed elevated expression of *NLGN3* in OPC-like tumor cells, and our analysis revealed strong interactions between neurons and OPC-like tumor cells mediated by synaptic cell-adhesion molecules, such as the *NRXN1-3*, *NLGN1-3* and *EFNB3*, *EPHB1-3* LR pairs (Fig. 5g and Extended Data Fig. 8a,d). PhenoCycler multiplex imaging confirmed spatial colocalization of NRXN1-positive neurons and NLGN3-expressing OPC-like tumor cells (Fig. 5h and Extended Data Fig. 9e,f). Spatial permutation analysis further indicated that these interactions are non-random: colocalization scores with true labels were significantly higher than those from randomized label maps (Extended Data Fig. 9g; paired *t*-test, $P = 3.7 \times 10^{-172}$ for CC-1, $P = 7.4 \times 10^{-149}$ for CC-2 and $P = 1.4 \times 10^{-109}$ for CC-3). Interestingly, tumor cells may provide positive feedback to neurons, potentially inhibiting neuronal senescence and promoting neurogenesis^{48,49}. We predicted that OPC-like tumor cells could act on neurons through *GDF11* and members of the activin receptors *ACVR2A* and *ACVR1B*. Supporting this prediction, a subpopulation of OPC-like tumor cells with high GDF11 expressing was detected within neuron (NeuN⁺)-enriched regions (Fig. 5h). In contrast, CC-4 lacked sufficiently strong cellular interactions (Fig. 5i).

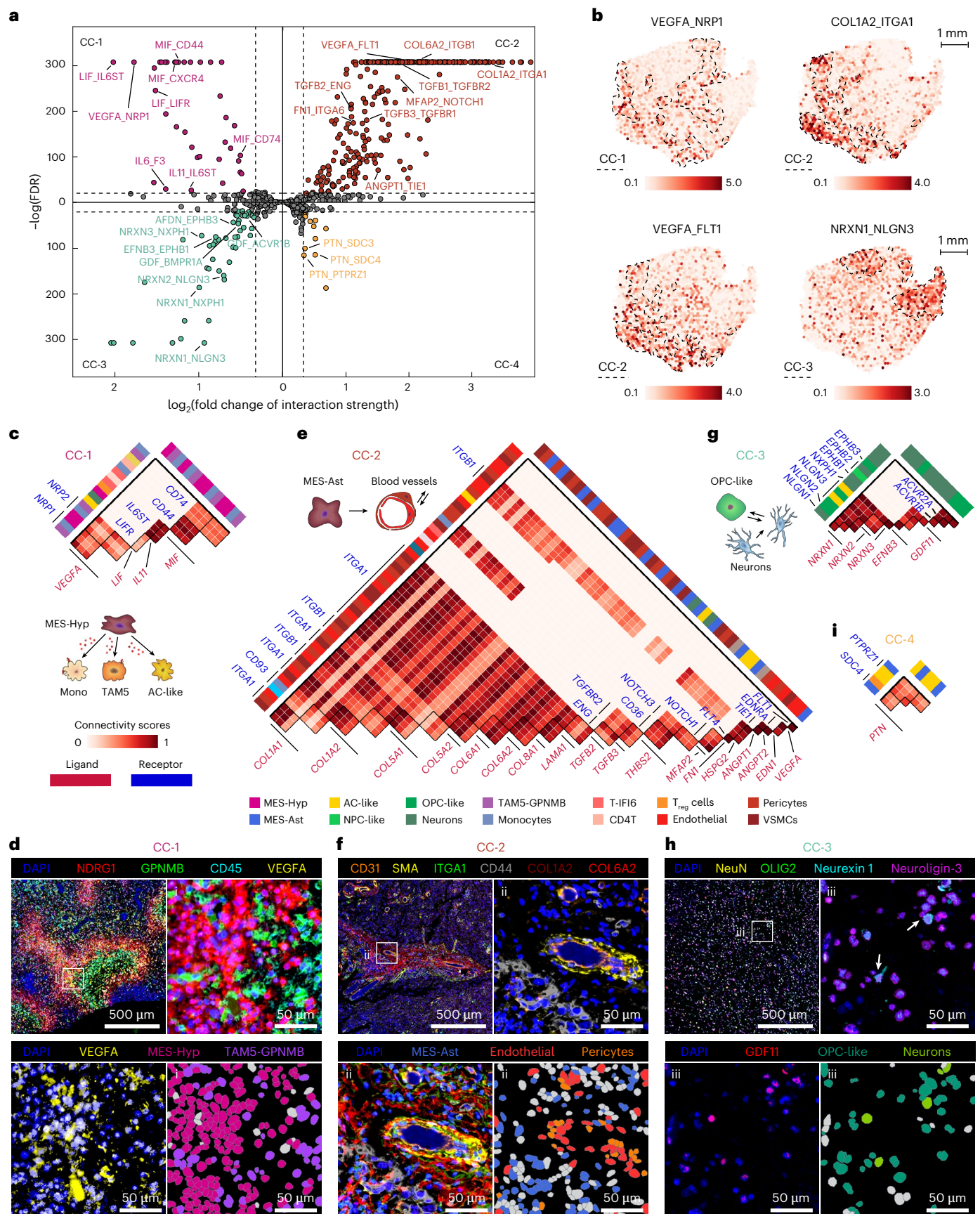
Thus, through ST and PhenoCycler multiplex imaging analysis, we have uncovered key cell subtypes and molecular LR pairs involved in intercellular interactions in primary GBM. Understanding these complex interactions provides valuable insights into potential therapeutic strategies for treating this disease.

OPC-like tumor cells form synaptic connections with neurons in CC-3

Previous studies have indicated that direct synaptic communication between neurons and tumor cells can promote glioma growth and invasion^{14,27,50}. We asked whether such neuroglioma synapses occur preferentially in certain tumor subtypes. Our previous findings revealed a notable enrichment of OPC-like, NPC-like and AC-like tumor cells with neurons in CC-3 (Fig. 1d–f). Additionally, our ISH results demonstrated a frequent clustering of OPC-like and NPC-like cells around neurons (Fig. 6a–c and Extended Data Fig. 10a). Furthermore, genes associated with synaptic vesicles and postsynaptic signaling were expressed at significantly higher levels in CC-3 compared to

Fig. 5 | Conserved intercellular interactions within CCs. **a**, Four-quadrant diagram illustrating CC-specific intercellular communications within CCs. Each quadrant corresponds to a distinct CC. The exact positions of interactions (dots) reflect the FC of interaction strength (horizontal axis) and FDR of interaction strength (vertical axis) of this CC compared to other CCs. **b**, Visualization depicting the strength of representative CC-specific intercellular communications for CC-1, CC-2 and CC-3 on a representative slice, P77-1. **c,e,g,i**, Heatmaps showing connectivity scores of CC-specific interactions between cell types within CC-1 (**c**), CC-2 (**e**), CC-3 (**g**) and CC-4 (**i**). Red gene

names represent ligand genes, and blue gene names represent receptor genes. Labels on both sides of the triangle indicate the interacting cell types. **d,f,h**, Representative PhenoCycler fluorescence images of CC-1 (**d**), CC-2 (**f**) and CC-3 (**h**) in primary tumor sections. Top-left: overview of each CC. Top-right and bottom-left: magnified regions highlighting cell-type markers and intercellular interactions. Bottom-right: cell membrane segmentation colored by cell clusters. Experiments were performed independently on samples from six patients with consistent results; additional representative fields of view are shown in Extended Data Fig. 9a–f.



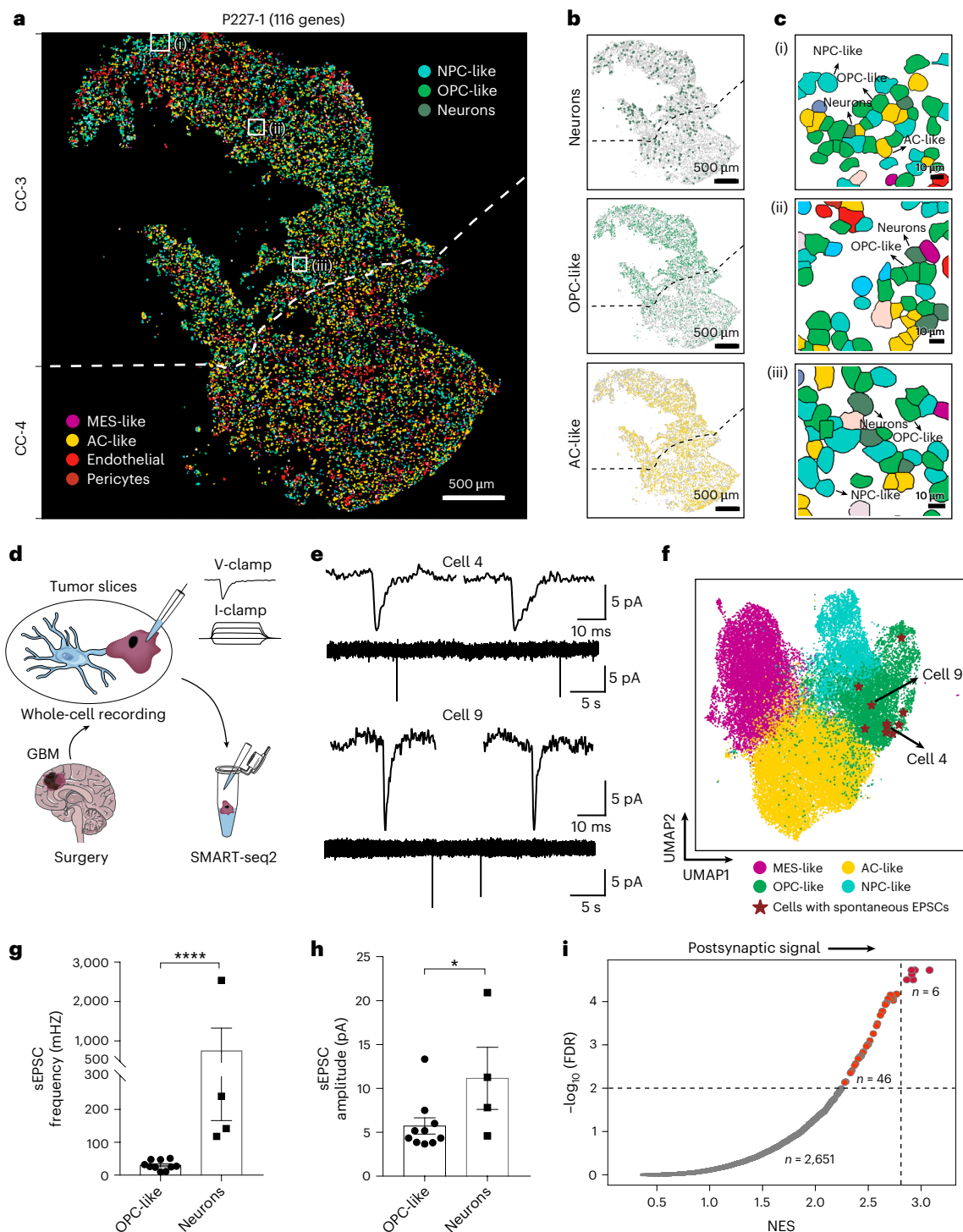


Fig. 6 | Validation of the specific tumor subtype that form synapses with neurons. **a–c**, Visualization of cell type spatial distribution in an entire frozen section, P227-1 (**a**). Spatial distribution of neurons, OPC-like and AC-like tumor cells (**b**). Zoomed-in view of boxed regions (i–iii) in **a** (**c**): NPC-like and OPC-like tumor cells surrounding neurons. Cell nuclei segmentation is colored by cell clusters. **d**, Schematic diagram depicting the Patch-seq experiment process. **e**, Representative traces of sEPSC in cell 4 and cell 9 obtained from fresh tumor slices of GBM patients. **f**, UMAP visualization of Patch-seq data from cells with sEPSCs (star-shaped dots) mapped by Seurat into low-dimensional representation of tumor cells. **g,h**, Frequency (**g**) and amplitude (**h**) of sEPSCs

in OPC-like tumor cells and neurons. Data shown in **g,h** are presented as averages $\pm$ s.e.m. OPC-like tumor cells, $n = 10$ cells; neurons, $n = 4$ cells. Statistical significance was assessed using a two-sided exact permutation test (frequency, $P = 9.9 \times 10^{-4}$; amplitude, $P = 0.042$). **i**, Scatter plot depicting gene set enrichment analysis on all OPC-like cells ($n = 5,660$), ranked by the expression levels of postsynaptic signaling genes. (1) Positive regulation of cell cycle, (2) chemical synaptic transmission, (3) mitotic sister chromatid segregation, (4) mitotic chromosome condensation, (5) chromosome condensation, (6) mitotic nuclear division. Each point represents a gene set, with the x axis indicating the NES and the y axis representing the FDR. Dot size reflects the NES of each gene set.

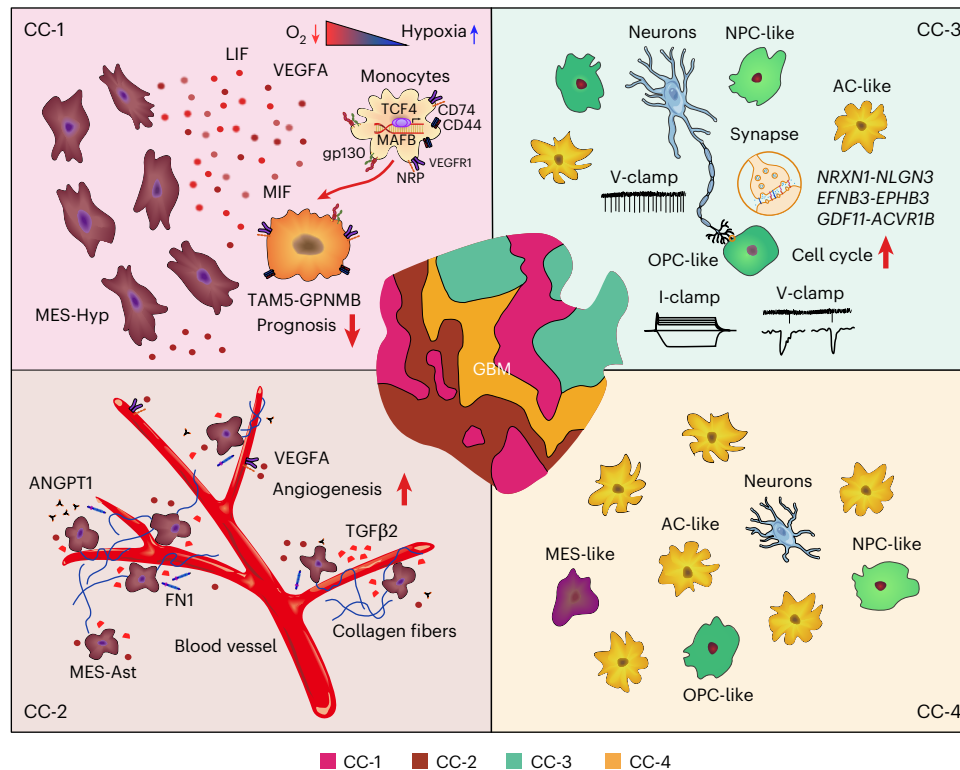


Fig. 7 | Graphical schematic of tumor-promoting interactions across CCs.

Spatially defined CCs in GBM. In CC-1, MES-Hyp cells express VEGFA, LIF and MIF and are positioned adjacent to monocytes and TAM5-GPNMB within hypoxic regions. In CC-2, MES-Ast cells localize along the vasculature, with expression of

VEGFA, TGFβ2 and FN1 associated with angiogenic niches. In CC-3, neuroglial synapses are detected between neurons and OPC-like tumor cells, with indicated *NRXN1-NLGN3*, *EFNB3-EPHB3*, *GDF11-ACVR1B* axes. CC-4 represents a mixed region enriched for AC-like tumor cells.

other CCs (t -test, $P = 5.0 \times 10^{-82}$ for synaptic vesicles in CC-3 versus CC-4; Extended Data Fig. 10b). These observations strongly suggest that the neuroglia synaptic connections are likely to occur predominantly in CC-3, with potential involvement of OPC-like and NPC-like tumor cells.

To further investigate whether OPC-like and/or NPC-like tumor cells establish synaptic connections with neurons, we conducted a Patch-seq experiment⁵¹ (Fig. 6d). This technique combines whole-cell patch-clamp recording with scRNA-seq to simultaneously capture the electrophysiological and transcriptomic profiles of individual cells. Using smart-seq2, we examined single-cell gene expression in cells without significant action potentials by applying step-current injection on fresh tumor slices from seven IDH wild-type GBM patients (Extended Data Fig. 10c,d). We obtained smart-seq2 data for 10 cells exhibiting spontaneous excitatory postsynaptic currents (sEPSCs) and 11 cells without significant sEPSCs, detecting an average of 2,580 genes per cell (Fig. 6e and Extended Data Fig. 10e).

Remarkably, all 10 cells with sEPSCs (cells 1–10) exhibited upregulation of OPC-like tumor cell marker genes while not expressing MES-like, NPC-like or AC-like tumor cell markers (Extended Data Fig. 10f). To further confirm the identity of these cells, we employed Seurat to anchor these cells to all single-cell transcriptome clusters (Methods). Strikingly, all 10 sEPSCs-positive cells were classified within the OPC-like tumor cell cluster (Fig. 6f). In contrast, the 11 cells without sEPSCs were identified as a mix of OPC-like tumor cells (cells 11–13), NPC-like tumor cells (cell 14), AC-like tumor cells (cell 15), oligodendrocytes (cells 16, 17), TAMs (cell 18–20) and T cells (cell 21). Results from a deep generative model SCANVI^{52,53} matched these cell-type assignments (Extended Data Fig. 10g). Furthermore, we observed that the sEPSCs recorded from OPC-like tumor cells exhibited a significant reduction in both frequency and amplitude compared to those detected in neurons (exact permutation test, $P = 9.9 \times 10^{-4}$ for frequency; $P = 0.042$ for

amplitude) (Fig. 6g,h and Supplementary Table 8), suggesting that the electrophysiological interactions of neuron-to-tumor synapses are weaker than normal neuron–neuron synapses.

A recent study identified a cluster of cells that fire action potentials, termed hybrid cells (HCs), in low-grade glioma by Patch-seq⁵⁴. We then compared the transcriptomes of these spiking HCs with those of our identified sEPSC-positive GBM cells. The CNV analysis confirmed the malignant identity of both cell populations (Extended Data Fig. 10h). Notably, both the HCs and sEPSC-positive GBM cells exhibited upregulation of OPC-like tumor cell marker genes, but not markers characteristic of normal OPC or other tumor cell subtypes (Extended Data Fig. 10f and Methods), further validating our findings.

To examine whether neuron–tumor synaptic communication impacts tumor growth, we performed gene set enrichment analysis on all OPC-like cells ($n = 5,660$) ranked by the expression levels of postsynaptic signaling genes. We identified 52 significantly enriched gene sets ($FDR < 0.01$), with 5 of the top 6 sets being related to cell cycle and cell division, indicating a positive correlation between postsynaptic signaling and tumor cell proliferation (Fig. 6i). Additionally, we observed a positive correlation between postsynaptic signals and the expression of CC-3-specific interacting ligands, such as *NRXN2* and *GDF11*, which are known to promote synapse formation and maintain neuronal lifespan^{49,55} (Extended Data Fig. 10i). Consistent with this, treating primary rat neurons with recombinant GDF11 protein induced significant upregulation of synaptic marker expression (Extended Data Fig. 10j,k), supporting the synaptogenic function of GDF11. These findings suggest the presence of a positive-feedback mechanism wherein OPC-like tumor cells with higher postsynaptic signaling promote interaction with neurons, leading to increased tumor cell proliferation and growth.

In summary, by integrating ST, scRNA-seq, ISH, scATAC-seq and Patch-seq data, we have constructed the spatial landscape of cells in

primary GBM tissue and elucidated the cellular interactions within each CC and their roles in promoting tumor malignancy (Fig. 7). This comprehensive approach provides deeper insights into the complex molecular and cellular dynamics driving GBM progression.

Discussion

Previous studies have identified clinically relevant cell states and ecosystems across various tumor types^{56,57}, emphasizing the importance of systematic, quantitative research on the tumor ecosystem. Here we developed an analytical framework to identify and characterize GBM CCs by integrating single-cell and spatially resolved transcriptomics. The spatial colocalization and interactions of major subpopulations were experimentally confirmed using ISH and PhenoCycler multiplex imaging analysis. Nevertheless, stringent antibody requirements for PhenoCycler limited protein-level validation of additional candidate LR pairs at the protein level, and future adoption of three-dimensional tissue reconstructions will enable more accurate assessment of cellular interactions.

Our analysis identified two subpopulations of MES-like tumor cells. MES-Hyp cells expressed cytokines such as VEGFA and LIF, potentially attracting monocytes to CC-1 and promoting their transformation of monocytes into the M2-like TAM5-GPNMB subpopulation. In contrast, MES-Ast cells may interact with vascular integrins via collagen and drive angiogenesis through ligands such as TGFβ2 and FN1. However, the biological functions and transitions of the tumor cell subpopulations warrant further investigation. Lineage-tracing and gain-of-function/loss-of-function studies under controlled hypoxic conditions could further elucidate the mechanisms underlying transitions from AC-like to MES-Hyp.

We conducted Patch-seq experiments to simultaneously record sEPSCs and profile gene expression in single cells from fresh GBM tumor slices and successfully obtained transcriptomes of 10 cells with sEPSCs, all of which were identified as OPC-like tumor cells, suggesting that the synaptic connections are predominantly formed between neurons and OPC-like tumor cells. Expanding Patch-seq to include a larger number of cells may reveal additional tumor cell subtypes capable of forming synapses with neurons.

Together, this study established a comprehensive framework to characterize the cellular composition, spatial architecture and cell–cell interactions within CCs in GBM; underscore the pivotal role of cellular communication in sustaining tumor cell proliferation, differentiation, invasion and migration in GBM; and highlight potential therapeutic targets for precision medicine. For instance, targeting the crosstalk between OPC-like tumor cells and neurons via GDF11 inhibition offers a potential strategy to improve GBM therapy.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41593-026-02265-5>.

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Methods

Refer to the Supplementary Information for full methodological details.

Specimens and ethics

The collection of GBM specimens was approved by the ethics committee of the First Affiliated Hospital of the University of Science and Technology of China (USTC) (No. 2022-KY-092). Informed consent was provided by individuals donating fresh surgical tissue, and all experiments were conducted in compliance with the approved guidelines. The tissue samples used were destroyed during or after the analysis. A total of 33 patients, who were diagnosed and underwent surgery at The First Affiliated Hospital of USTC, were included in this study. The clinical characteristics of the patients are summarized in Supplementary Table 1.

ST

Fresh GBM tumors were washed in cold phosphate-buffered saline, embedded in optimal cutting temperature compound and stored at -80°C . Tissue blocks were cut into $10\text{-}\mu\text{m}$ sections and processed using the Visium Spatial Gene Expression Kit (10x Genomics) following the manufacturer's instructions. Tissue permeabilization time (6 min) was optimized using the Visium Spatial Tissue Optimization Kit. Sections were H&E-stained and imaged using a TG TissueFAXS PLUS microscope before being prepared for ST. Libraries were sequenced using an Illumina NovaSeq 6000 system with 150-bp paired-end reads.

Tumor dissociation and cell sorting

Fresh tumors were enzymatically dissociated using papain and DNase at 37°C and filtered through $40\text{-}\mu\text{m}$ strainers. Cells were stained with anti-human CD45-APC/Cyanine7 (clone 2D1, BioLegend), $1\text{ }\mu\text{mol l}^{-1}$ calcein AM (Life Technologies) and $0.1\text{ }\mu\text{mol l}^{-1}$ TO-PRO-3 iodide (Life Technologies) for a 30-min incubation at 4°C . Viable single cells (calcein AM⁺/TO-PRO-3⁻) were sorted using a Sony SH800S Cell Sorter.

ScRNA-seq

Sorted viable cells from two to three patients per batch were pooled equally and processed using the Chromium Single Cell 3' (v2 or v3) platform (10x Genomics), loading $\sim 16,000$ cells per channel. Libraries were sequenced on Illumina HiSeq X Ten or NovaSeq 6000 (150-bp paired-end).

ScATAC-seq

A total of 140,000 sorted cells per patient were used for scATAC-seq library construction following established protocols. Libraries were sequenced using the Illumina HiSeq X Ten System (150-bp paired-end), yielding $\sim 100,000$ reads per cell on average.

Multiple rounds of ISH

Padlock probes for rolling circle amplification were designed as previously described, containing a 32-nt target-complementary region spanning the ligation junction, a universal primer sequence and a fluorescent probe-binding sequence. Probe specificity was verified by BLAST, and all probes were synthesized by Sangon Biotech (Supplementary Table 9).

Frozen GBM sections were fixed, permeabilized and hybridized with padlock probes at 37°C . The padlock probe was ligated using Splint R ligase (NEB), and DNA amplification was performed using phi29 DNA polymerase (Thermo Fisher). Iterative imaging was performed through sequential hybridization of fluorescent detection probes with formamide-based stripping between cycles. For the 36-gene panel, images were acquired using a Nikon Eclipse Ti2 microscope ($\times 40$ objective) across four channels (405/488/561/640 nm). Z-stacks with a z-step of $1.5\text{ }\mu\text{m}$ were collected. For the 116-gene panel, imaging was performed on a SOPTOP IRX60 ($\times 40$ objective) across four channels (405/561/640/750 nm). Adjacent images had a 10% overlap for image stitching.

GBOs

Fresh surgically resected GBM tissue was minced into fragments $0.5\text{--}1\text{ mm}$ in diameter in H+GPSA medium and cultured in ultra-low attachment six-well plates containing GBO medium. The plates were maintained at 37°C with orbital shaking (120 rpm) and partial medium exchange (75%) every 48 h. For perturbation assays, GBOs were divided into 3 groups, with 3–5 GBOs in each group, and treated with $20\text{ }\mu\text{g ml}^{-1}$ VEGF-165 (PeproTech) or $10\text{ }\mu\text{g ml}^{-1}$ TGF β 2 (PeproTech) for 3 days. Multiple rounds of ISH targeting 20 cell-type marker genes (Supplementary Table 3) was performed to assess temporal changes in cellular states.

PhenoCycler assay

All antibodies were initially screened and validated through individual staining on formalin-fixed, paraffin-embedded human GBM tissue sections. Antibody dilutions and amounts were used according to the manufacturers' instructions. Standard immunohistochemistry was performed to validate unconjugated antibodies, as listed in Supplementary Table 6. Primary antibodies were subsequently conjugated to nucleotide-barcodes according to Akoya's antibody conjugation manual (Akoya Biosciences).

Formalin-fixed, paraffin-embedded tissue sections ($5\text{ }\mu\text{m}$, substantially thinner than the average diameter of a typical mammalian single cell, to minimize projection artifacts from the z-axis) were incubated with a cocktail of nucleotide-barcoded primary antibodies. Iterative fluorescent reporter hybridization and imaging were performed using the PhenoCycler-Fusion r system (Akoya Biosciences). Blank cycles were incorporated for autofluorescence subtraction. Automated image acquisition, registration, processing and fluidics exchange were executed using the Akoya PhenoCycler-Fusion instrument and Fusion software (v2.3.1, Akoya Biosciences).

Patch-seq

Freshly resected GBM tissue was processed for acute slice electrophysiology. Whole-cell recording was performed on cells adjacent to neurons using glass pipettes ($3.5\text{--}7\text{ M}\Omega$) filled with potassium gluconate-based internal solution (pH 7.2; 305–310 mOsm). Signals were recorded with a MultiClamp 700B amplifier, digitized at 20 kHz and filtered at 1 kHz. sEPSCs were recorded in a voltage clamp at -70 mV . Cells lacking significant action potentials in a current clamp were classified as non-neuronal. After recording, cells exhibiting sEPSCs but without significant action potentials were aspirated and processed for Smart-seq2 library preparation. Libraries were sequenced on an Illumina NovaSeq 6000 (150-bp paired-end).

Rat primary neurons culture and treatment

All experimental procedures involving animals were conducted in accordance with protocols approved by the University of Science and Technology of China Animal Care and Use Committee (Approval No. USTCACUC1902024). Hippocampal neurons from E18 Sprague-Dawley rat embryos were enzymatically digested, mechanically dissociated and cultured on poly-D-lysine (Sigma-Aldrich)-coated plates in neuronal attachment medium. Partial medium replacement (50%) was performed using neurobasal-based medium. Glial proliferation was inhibited by cytosine arabinoside (Sigma). On day 8, neurons were transfected with AAV2/8-pAAV-hSyn-Synaptophysin-mRuby-T2A-H2B-GFP (Taitool Bioscience). On day 14, recombinant GDF11 protein (40 ng ml^{-1} ; PeproTech) was added, and cells were cultured for an additional 4 days.

Immunofluorescence assay

Neurons were fixed, permeabilized and incubated with anti-PSD-95 primary antibody (1:500; Abcam, ab13552) followed by fluorescent secondary antibody. Images were acquired using a Leica THUNDER Imager Live Cell system and quantified with ImageJ.

ScRNA-seq data processing

ScRNA-seq reads were aligned and quantified against GRCh38 using Cell Ranger software (v6.0.2) with the default options. Per batch, doublets were eliminated using scDblFinder (v1.7.7, automatic doublets rate). Cell-cycle genes were removed from dimensionality reduction and clustering. To build a cohort-wide atlas (30 patients), we used Seurat (v4.3.0) to identify highly variable genes (2,000 per patient), selected recurrent features across patients and performed principal component analysis. Batch effects were corrected with Harmony (v1.0.3) on the top 40 principal components using dataset source and patient IDs as covariates. Clustering was performed in Seurat using a shared-nearest-neighbor graph and Louvain clustering (resolution = 1.0).

Malignant cell identification and classification

Malignant versus non-malignant populations were identified by expression-inferred CNVs from 100-gene sliding windows, following prior GBM scRNA-seq frameworks based on hallmark alterations (for example, chr. 7 gain, chr. 10 loss). Non-malignant clusters were annotated by canonical markers (immune, glial and vascular lineages) and lack of large-scale CNVs. Malignant cells were classified into canonical GBM tumor states (MES-like, AC-like, NPC-like, OPC-like) based on established signature genes.

Assigning pooled scRNA-seq cells to individual patients

For pooled scRNA-seq experiments (2–3 donors per run), donor assignment was performed using matched whole-exome sequencing genotypes (Supplementary Table 1). Whole-exome sequencing reads were aligned to GRCh38 using BWA-MEM (v0.7.17), and variants were called with GATK (v4.1.3). Cells were first demultiplexed with Demuxlet and then refined with Souporecell (v2.4) using the scRNA-seq binary alignment map files and the expected number of donors per pool. Souporecell genotype clusters (Supplementary Table 10) were labeled using Demuxlet calls, substantially increasing the fraction of assigned cells.

Integrating external neuronal snRNA-seq

Neuronal snRNA-seq profiles from ref. 18 were integrated into the atlas after excluding low-neuron samples. Dimensionality reduction and batch correction were performed with principal component analysis and Harmony (patient covariate), and neuronal embeddings were merged with the atlas to support joint downstream analyses.

H&E-based cell segmentation

High-resolution H&E images were segmented with StarDist using a pretrained H&E model. Each image was partitioned into 32×32 blocks with an overlap of 128 pixels between adjacent blocks, to which the segmentation model was applied independently and subsequently stitched to produce the final segmentation mask.

ST data analysis

ST (10x Visium) data were processed with Space Ranger (v1.2.1, default) using matched H&E images for alignment. Cell-type composition per Visium spot was estimated with Cell2location (v0.6) using scRNA-seq derived reference signatures (25 cell types). For samples without reliable histology-based cell counts, a fixed cell number per spot was used. Recurrent spatial domains across slices were identified using SPACEL (v1.1.6; Splane module) from (1) spot coordinates, (2) cell2location composition estimates and (3) spot-level CNV scores (chr. 7 and chr. 10) derived from inferCNV (v1.6.0) outputs. Core Splane settings used for cross-slice alignment and spatial smoothing were neighborhood size $k = 1$, adversarial alignment weight $dl = 0.5$ and dropout $g_{nn_dropout} = 0.2$ (others default). This procedure yielded a shared set of recurrent domains across slices. For peritumoral domains, reference signatures were expanded using published normal brain snRNA-seq datasets^{63,64} (astrocytes, OPCs, neurons) to improve deconvolution.

SVGs per CC

For each slice, differentially expressed genes (DEGs) specific to the four CCs were calculated separately using the 'FindMarkers' function in Seurat. DEGs were required to exhibit a $\log_2(\text{fold-change (FC)}) > 0.25$ and $\text{FDR} < 0.01$, with only upregulated DEGs being retained. SVGs were defined as CC-specific DEGs reproducibly detected in at least four slices.

ISH data preprocessing and cell type annotation

Multiround ISH images were stitched using ASHLAR software (v1.14.1) with DAPI-based alignment. Nuclei were segmented using DeepCell Mesmer (v0.11.0, nuclear mode), and masks were modestly expanded to approximate cell boundaries (5 pixels for the 36-gene assay and 3 pixels for the 116-gene assay). A Gaussian-based high-pass filter was applied to eliminate background noise. RNA spots were deconvoluted and decoded using the Starfish pipeline (v0.2.2). Both ISH data and 10x Visium data were normalized using the sctransform function in Seurat.

Colocalization analyses in ISH/PhenoCycler

Cell-type colocalization was quantified by defining neighbors within a fixed radius (50 μm) around a reference population (for example, TAM5-GPNMB) and computing the fraction of each cell type found near the reference relative to its total abundance in the slice. Analogous analyses were performed for proximity to vessels and neurons. For PhenoCycler, spatial permutation (random label shuffling; 1,000 iterations) was used to test whether observed proximities exceeded expectations from tissue density.

Identification of two MES-like subpopulations

MES-like malignant cells were analyzed per patient (excluding samples with low MES-like counts). Patient-level clustering was performed in Scanpy (v1.9.1), and cluster marker gene sets were collected. Marker gene sets were filtered for size/strength (DEGs > 50 and $\text{FC} > 2$), resulting in 31 and 23 gene sets representing the characteristics of MES-Ast cells and MES-Hyp cells, respectively. MES-like cells were then divided into two types.

Mapping scRNA-seq cells onto the spatial transcriptome

scRNA-seq cells were mapped to Visium slices using Tangram (v1.0.2). For MES-Ast/MES-Hyp localization, Tangram was trained on an extended gene set derived from recurrent pattern genes. Slices with insufficient representation of key CCs were excluded to reduce mapping artifacts, and cells mapped to only one slice were omitted from cross-slice CC assignment. For broader tumor analyses, Tangram was trained using recurrent highly variable genes across patients.

Pseudotime trajectory analysis of tumor cells using scRNA-seq

Tumor cells from multiple datasets were integrated with Harmony (covariates: patient and assay), embedded with PHATE (v1.0.11) and ordered in pseudotime using Palantir (v1.3.3). A proliferative cell (high *MKI67*) was used as the starting point. Cells from strongly batch-shifted datasets were projected into the shared embedding via k -nearest-neighbors mapping to reduce integration artifacts.

scATAC-seq processing and annotation

Public scATAC-seq data reported in ref. 36 were quality-control filtered (minimum unique fragments; minimum fraction in peaks). In-house scATAC-seq reads were aligned to GRCh38 using APEC (v1.1.0). Signac (v1.9.0) was used to reduce the dimensionality of the scATAC-seq data. ArchR (v1.0.1) was employed to estimate the gene scores, and the identity of each scATAC-seq cell was annotated using Seurat's label-transferring method for downstream analysis of tumor cell state transitions.

Pseudotime trajectory analysis of tumor cells using scATAC-seq

The first 10 components of tumor cells derived from Harmony embedding were utilized as input for PHATE (v1.0.11) to create a transition embedding (k nearest neighbors = 10; decay = 40). Subsequently, Palantir (v1.3.3) was applied to infer the pseudotime of tumor cells, with the cell showing the highest average gene scores for cell-cycle-related genes serving as the starting point for trajectory inference.

Spatiotemporal analysis of tumor cell transitions

Tumor cells were mapped onto ST spots using Tangram with default hyperparameters and were then allocated to the spatial coordinates of Stardist-segmented cells within the Tangram-assigned spots. The resulting spatial coordinates and splicing information were subsequently provided to scVelo (v0.3.2) to infer spatiotemporal trajectories using the default 'stochastic' mode. Velocity vector fields of tumor cells were overlaid with the spatial distribution of CCs.

Mapping scATAC-seq cells to space and TF motif deviation in CCs

Tangram (v1.0.2) was utilized to map scATAC-seq cells to the spatial transcriptome with gene scores estimated by ArchR. The 'clusters' mode was employed in the 'tg.map_cells_to_space' function in Tangram. The predicted results were normalized to the proportion of each cell type in the spots. The average deviation score d_{it} of TF motif t in the cell type i within the scATAC-seq data was initially calculated. Subsequently, the deviation score D_{st} of the TF motif t in spot s was calculated as follows:

$$D_{st} = \sum_{i=1}^{25} d_{it} P_{si} / \sum_{i=1}^{25} P_{si}$$

where P_{si} represents the proportion of cell type i in the spot s .

Monocyte-derived TAM trajectories (scRNA-seq and scATAC-seq)

Monocyte-derived TAMs were embedded with PHATE (v1.0.11). The transition embedding and splicing kinetics of TAMs were provided to scVelo (v0.3.2). Palantir (v1.3.3) was then employed to calculate the pseudotime of monocyte-derived TAMs.

APEC (v1.1.0) peak groups were used for PHATE and Palantir pseudotime inference for scATAC-seq. Monocyte-like cells were designated as the starting point.

CC-specific intercellular communications

The interaction strength of LR pairs within the spots was estimated using COMMOT (v0.0.3), with a spatial distance constraint of 200 μm . The differentially LR pairs for the four CCs were calculated separately using a T -test (FDR < 0.05 and FC > 1). Differential LR pairs in at least 20% of the slices were annotated to signaling pathways based on the CellChat database. LR pairs with FC > 1.25 and FDR < 1.0×10^{-20} were identified as CC-specific LR pairs, and ligand and receptor expression levels were normalized. Connectivity scores were computed as the product of the ligand and receptor gene expression and the relative interaction strength inferred within the CC.

PhenoCycler analysis

PhenoCycler images were segmented using the StarDist plugin (v0.5.0) in QuPath software. Nuclear boundaries were expanded by 5 μm to approximate cellular boundaries. Cell quality control was performed based on established criteria. Cell phenotyping was conducted using workflows adapted from previous studies. Spatial permutation analysis was conducted to quantify whether the observed cellular proximities were biologically meaningful rather than random occurrences arising from the high cell density in GBM tissue.

Patch-seq data preprocessing

In the Patch-seq transcriptome, data were aligned to the GRCh38 reference genome using the STAR aligner (v2.5.3) with default parameters (–outFilterMultimapNmax 10, –outFilterMismatchNmax 10). The gene expression of the cells was quantified using RSEM (v1.3.1) in paired-end mode with STAR specified as the aligner (–star). The transcripts per kilobase million of each cell was used and log2-transformed for downstream analysis. Normal OPC marker genes were defined as the top DEGs between the healthy OPCs from publicly available snRNA-seq datasets and the OPC-like tumor cells identified in this study. Cells with fewer than 1,000 gene detections were excluded from the Patch-seq data. Seurat (v4.3.0) was used to anchor the Patch-seq cells to the reference cells. Patch-seq cells were also annotated using the deep generative model SCANVI of scArches (v0.5.9) for cross validation.

Statements

No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications^{6,7}. The specific statistical test used was indicated for each comparison, and analyses primarily used two-sided Wilcoxon rank-sum tests. For analyses using two-sided t -tests, normality and equality of variances were formally assessed before applying the test. Human tissue and data collection was conducted without randomization. Data collection and analysis were not performed blind to the conditions of the experiments. No samples or data points were excluded from the analyses; criteria for removing low-quality data during preprocessing are clearly described in the Methods.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Processed ST, scRNA-seq, scATAC-seq, multiple rounds of ISH and Patch-seq data generated in this study are available via Zenodo at <https://doi.org/10.5281/zenodo.8085502> (ref. 65). The corresponding raw sequencing data for ST, scRNA-seq and scATAC-seq have been deposited with NGDC GSA for Human under accession code [HRA004942](https://ngdc.cnc.ac.cn/gsa/HRA004942). Publicly available data used in this study include (1) 12 samples of human GBM 10x Visium data from ref. 7 (<https://datadryad.org/stash/dataset/doi:10.5061/dryad.h70rxwdmj>); (2) 1 sample of human GBM 10x Visium data from the 10x dataset (<https://www.10xgenomics.com/datasets>); (3) 7 samples of human GBM 10x Visium data from ref. 21 (<https://doi.org/10.6084/m9.figshare.22434341>); (4) 2 samples of human GBM 10x Visium data from ref. 22 (available in the GEO database under accession code [GSE194329](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE194329)); (5) GBM scRNA-seq data from ref. 16 (https://github.com/mbourgey/scRNA_GBM); (6) GBM snRNA-seq data from ref. 18 (<https://portal.gdc.cancer.gov/projects/CPTAC-3>); (7) scRNA-seq data of MES-like tumor cells from ref. 28 (<https://cellxgene.cziscience.com/collections/999f2a15-3d7e-440b-96ae-2c806799c08c>); (8) 9 GBM scATAC-seq samples from ref. 37 (<https://gdc.cancer.gov/about-data/publications/TCGA-ATAC-Seq-2024>); (9) 5 human brain snRNA-seq samples from ref. 63 (<https://cellxgene.cziscience.com/collections/d17249d2-0e6e-4500-abb8-e6c93fa1ac6f>); (10) 4 human brain snRNA-seq samples from ref. 64 (<https://cellxgene.cziscience.com/collections/283d65eb-dd53-496d-adb7-7570c7caa443>); and (11) Patch-seq data of low-grade glioma from ref. 54 (available in the GEO database under accession code [GSE271961](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE271961)).

Code availability

The analysis performed in this study primarily utilized publicly available software, as described in the Methods section. The custom code for the main analysis can be available via GitHub at <https://github.com/QuKunLab/GBM>.

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Author contributions

K.Q. and J.L. conceived and supervised the project. J.L., C. Chen and C. Cheng processed tumor tissue and prepared the sequencing library of scRNA-seq, scATAC-seq and ST. L.C., C.J., S.F. and X.L. performed the multiple rounds of ISH. S.L., K.L. and C. Chen performed slice electrophysiology and single-cell RNA library preparation. J.L. generated patient-derived GBOs and performed perturbation assays. C. Chen and J.L. performed the data analysis with help from B.L., H.X., K.C., F.Z., R.W., M.F., M.T., Z.L. and S.W. K.Q., J.L., C. Chen, A.-H.T. and C.N. wrote the manuscript with help from all the other authors.

Competing interests

A.-H.T. and K.Q. are cofounders of Bassfish Technology incorporation. The other authors declare no competing interests.

Additional information

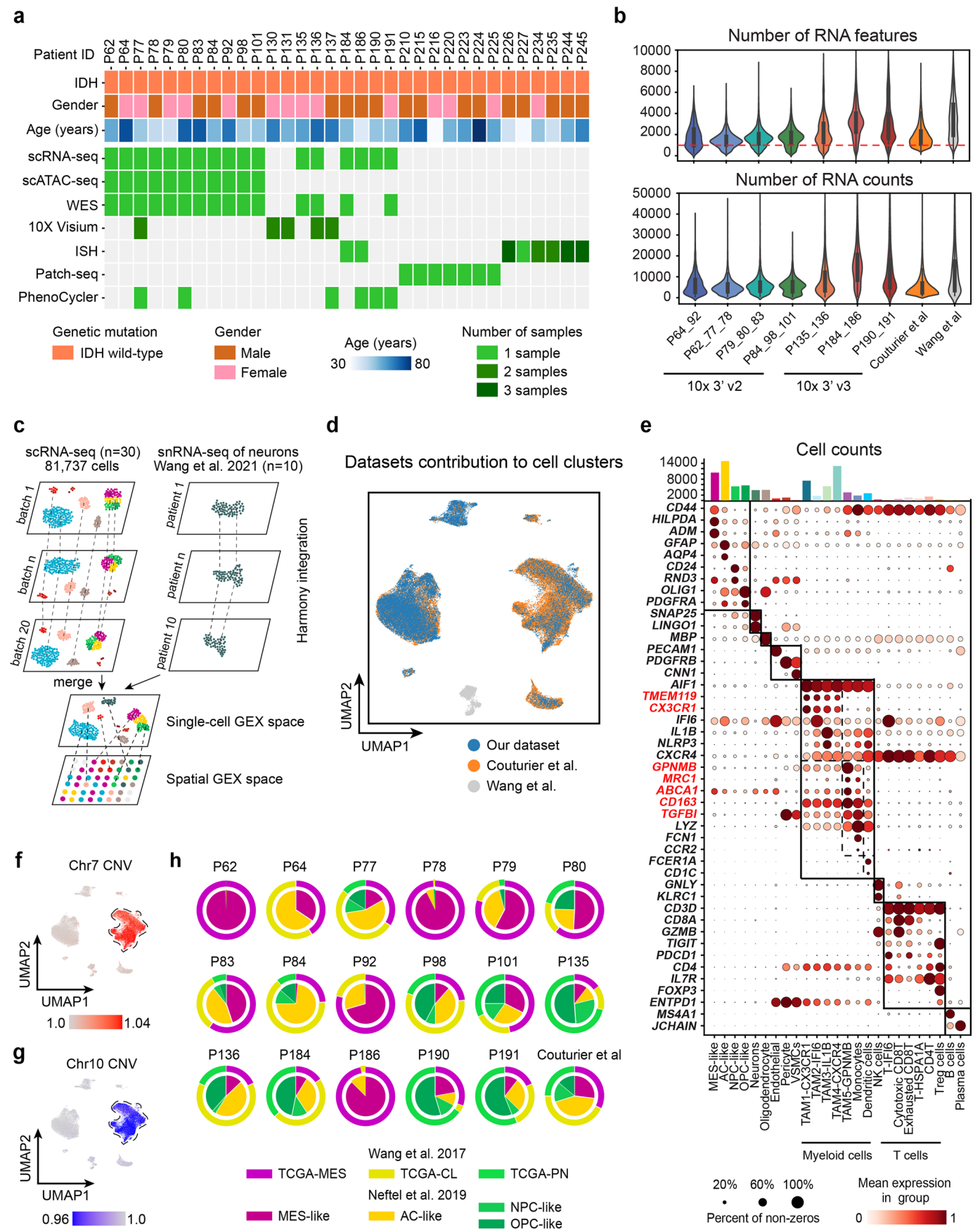
Extended data is available for this paper at <https://doi.org/10.1038/s41593-026-02265-5>.

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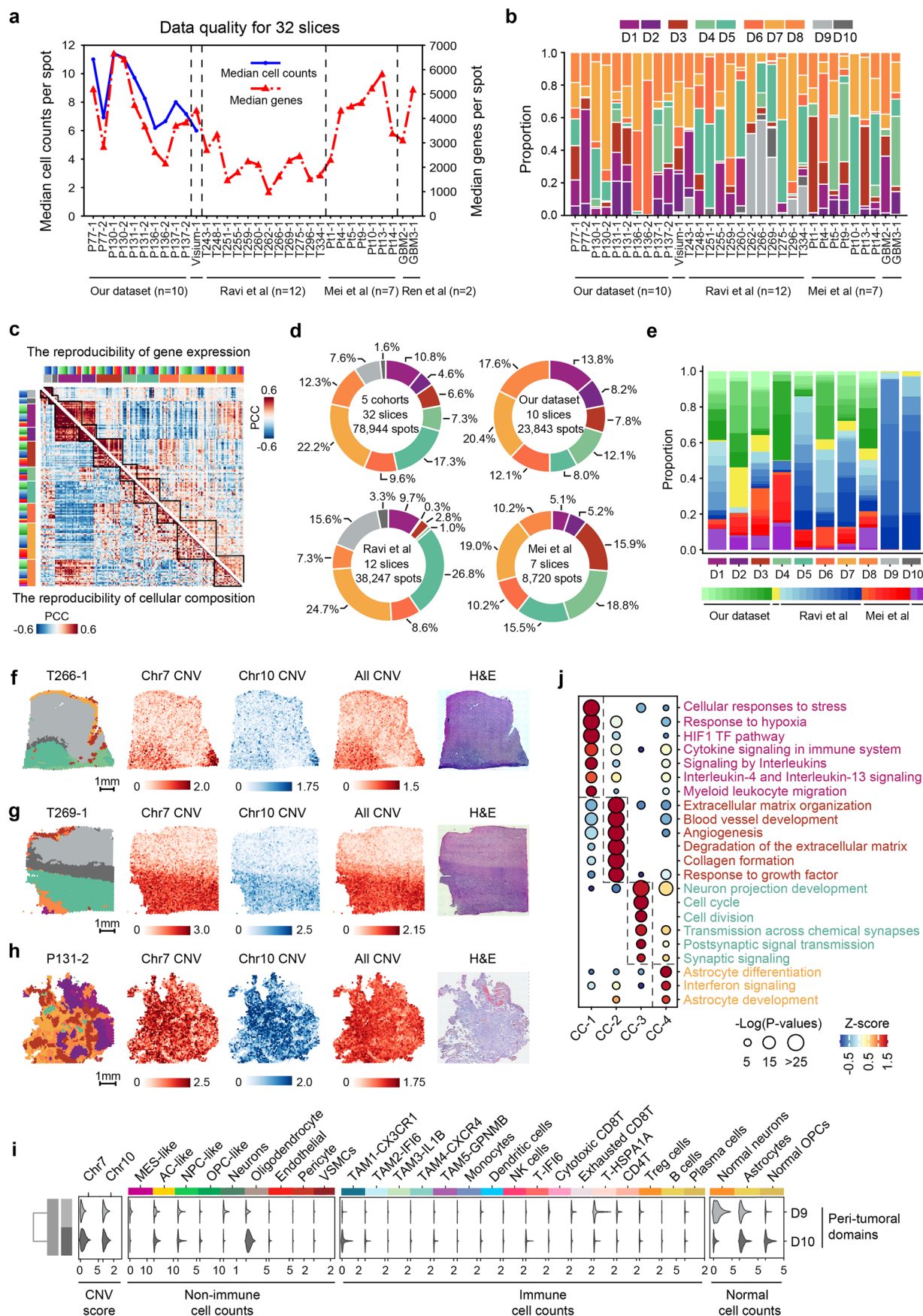
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Extended Data Fig. 1 | See next page for caption.

Extended Data Fig. 1 | Clinical information and integrated analysis of single-cell RNA-seq data. **a**, Clinical information related to the samples utilized in all experiments performed in this study. **b**, Violin plots of RNA features and RNA counts per cell in the sc/snRNA-seq dataset ($n = 85,597$ cells from 40 patients). X axis, seven in-house scRNA-seq batches (2–3 patients pooled per batch) and two published datasets (Couturier et al.; Wang et al.). Insets box plots showing median (centre line), 25th–75th percentiles (box) and minima–maxima (whiskers). **c**, Schematic diagram illustrating the strategy employed to integrate the scRNA-seq data ($n = 81,737$) and snRNA-seq data ($n = 3,860$). **d**, UMAP

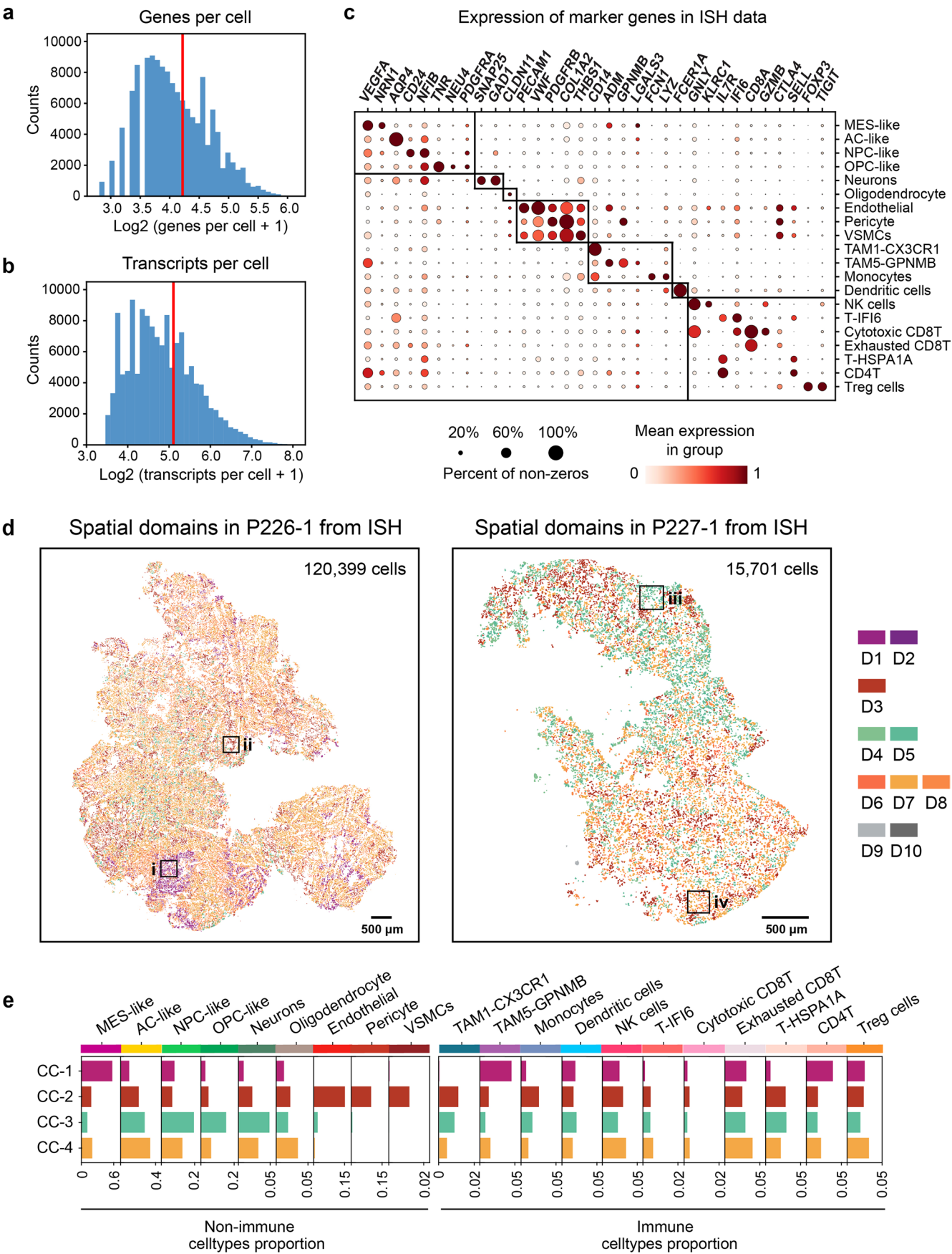
visualization of scRNA-seq data, with cells colored by dataset source. **e**, Dot plot representing the relative gene expression of canonical markers within the scRNA-seq cell clusters. The color represents the scaled average gene expression within cluster, while the dot size indicates percentage of cells within the cluster that express the gene. The upper panel displays the number of cells in each scRNA-seq cell cluster. **f,g**, UMAP visualization of inferred CNV scores for chromosome 7 (**f**) and chromosome 10 (**g**) in the scRNA-seq data. **h**, Donut plots showing the classification results of malignant cells within the scRNA-seq data according to classification models of Wang et al. (outer circle) and Neftel et al. (inner circle).



Extended Data Fig. 2 | See next page for caption.

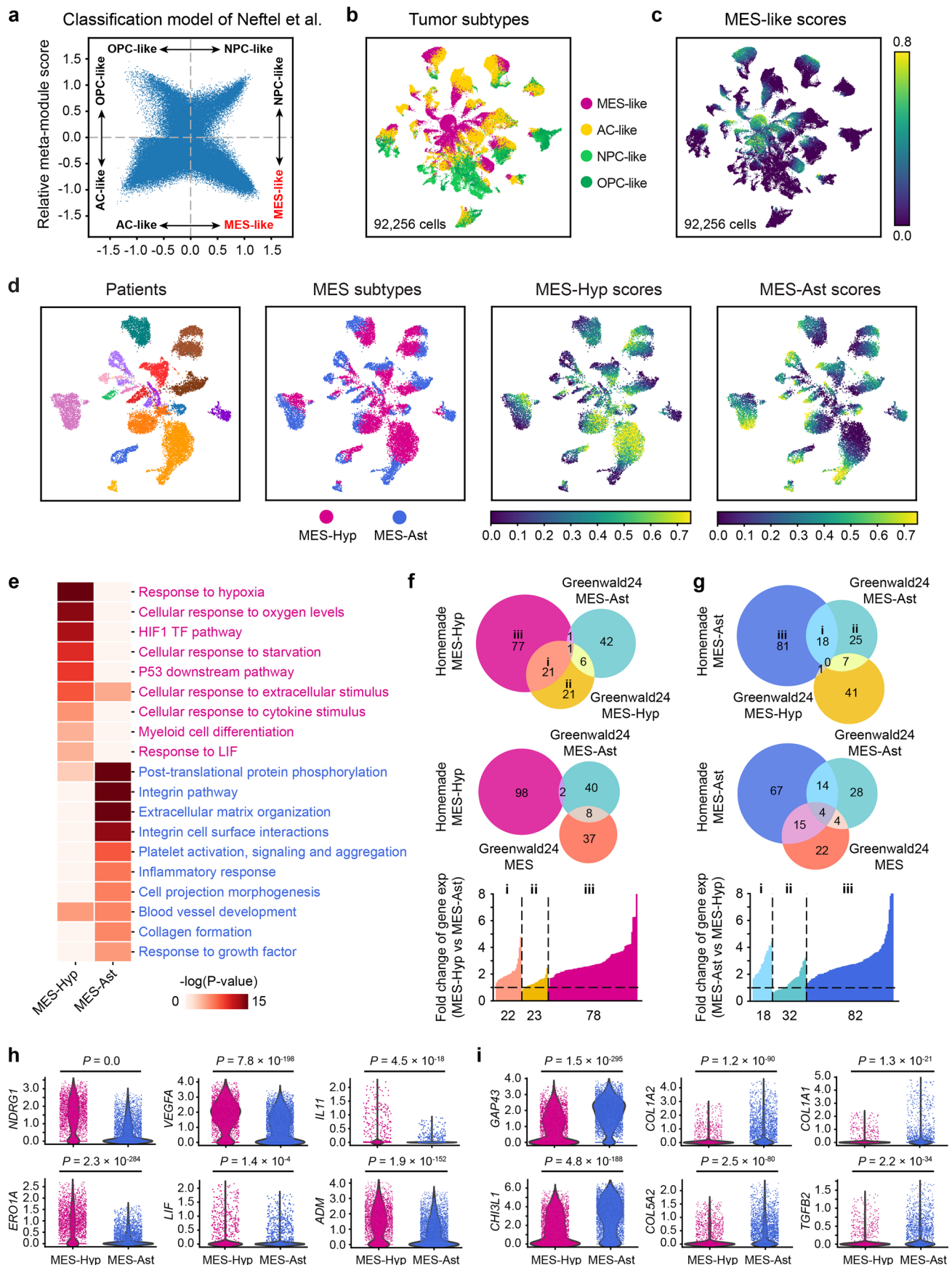
Extended Data Fig. 2 | Distribution and characteristics of spatial domains among ST slices. a, Line graph displaying the median number of genes per spot in our ST dataset ($n = 10$) and publicly available ST datasets ($n = 22$), along with the median cell counts per spot in our ST dataset ($n = 10$) and the 10X dataset ($n = 1$) with high resolution H&E staining images. **b,** Stacked bar plot illustrating the proportion of each spatial domain in our ST dataset and publicly available ST datasets. **c,** Correlations of gene expression (top right panel) and cellular composition (bottom left panel) between spatial domains of different slices. PCC, Pearson correlation coefficient. **d,** Pie chart depicting the relative size of

each domain across the cohort. **e,** Stacked bar plot showing the proportion of samples contributing to each spatial domain. **f–h,** Visualizations depicting the spatial domain distribution, inferred CNV scores of chromosome 7, chromosome 10, all chromosomes, and H&E staining images of T266-1 (**d**), T269-1 (**e**), and P131-2 (**f**). Scale bars, 1 mm. **i,** Cellular composition of spatial domains within the *peri-tumoral* domains. **j,** Functional enrichment analysis for spatially variable genes within each cellular community. Enrichment P values, two-sided hypergeometric test with Benjamini–Hochberg correction.



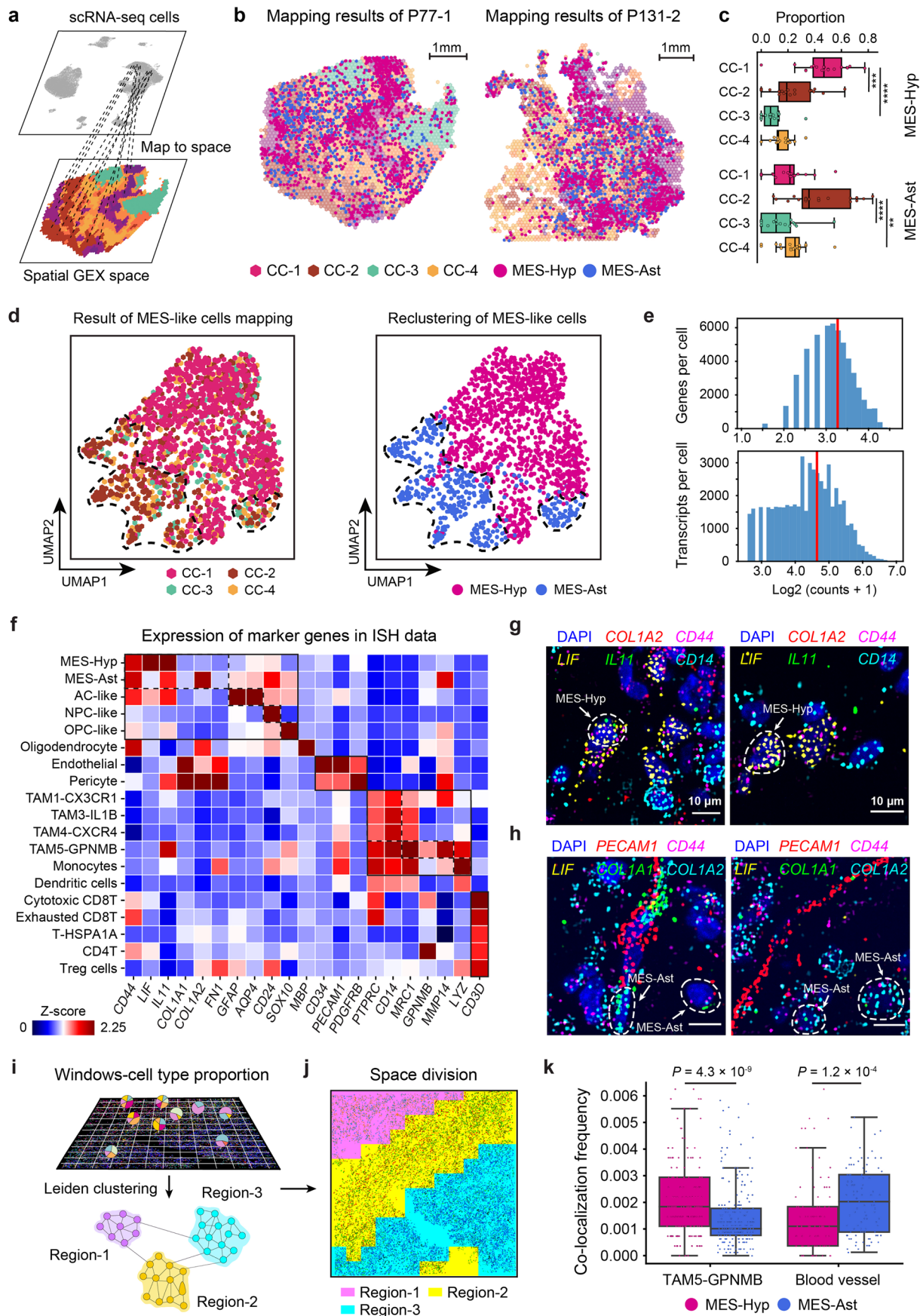
Extended Data Fig. 3 | Identification of cellular composition within cellular communities using ISH data. a,b, Frequency histogram of the distribution of RNA features per cell (**a**), and RNA transcripts per cell (**b**) across 2 patients within the ISH data. The red line indicates the average. **c**, Dot plot representing the relative gene expression of canonical markers within ISH cell clusters. The color represents the scaled average gene expression within clusters, while dot

size indicates the percentage of cells within the cluster that express the gene. **d**, Visualization of spatial domain distribution in entire frozen sections P226-1 (left panel) and P227-1 (right panel). i-iv correspond to zoomed-in views of boxed regions in Fig. 1f. Scale bars, 500 μ m. **e**, Cellular composition of each CC in the ISH data.



Extended Data Fig. 4 | Comparative analysis of characteristic genes of two MES-like subpopulations. **a**, Two-dimensional representation of tumor cellular states. **b,c**, UMAP visualization of tumor cells ($n = 92,256$) from all 8 datasets. Cells are color-coded by tumor subtypes (**b**) and expression levels of the meta-module defining MES-like cells proposed by Neftel et al. (**c**). **d**, UMAP visualization of MES-like tumor cells from all 8 datasets. Cells are color-coded by donors, cell type identity, and expression levels of characteristic gene sets defining the two MES-like subpopulations. **e**, Functional enrichment analysis of the characteristic gene sets for the two MES-like cell types. Enrichment P values,

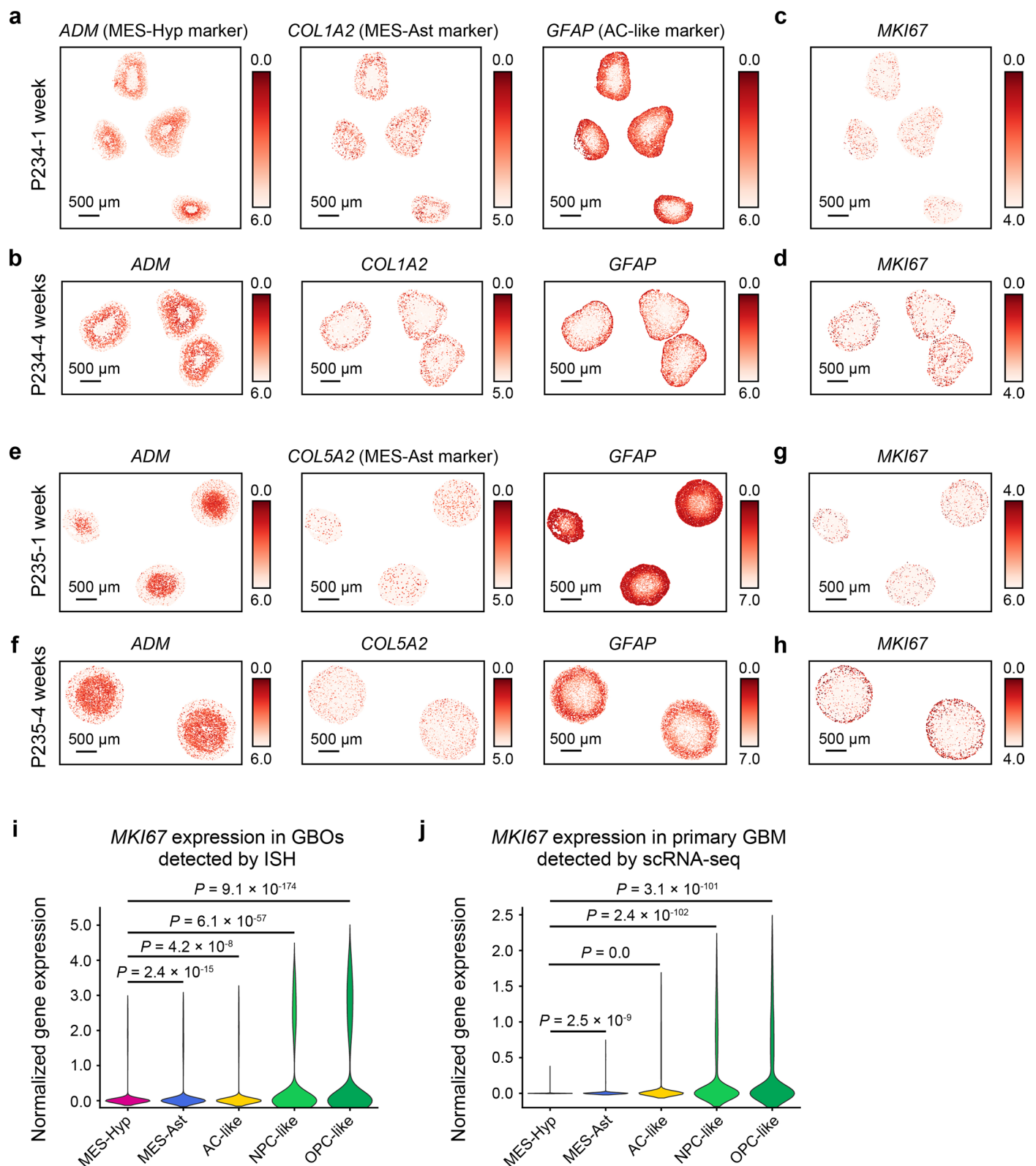
two-sided hypergeometric test with Benjamini–Hochberg correction. **f,g**, Upper and middle panels: Venn diagrams showing the number of overlapping genes between characteristic gene sets of MES-Hyp cells (**f**) and MES-Ast cells (**g**) with published gene sets. Bottom panel: Fold change of gene expression in the two MES-like cell types. **i**, genes overlapping between our defined gene sets and the published gene sets; **ii**, genes unique to the published gene sets; **iii**, genes unique to our defined gene sets. **h,i**, Violin plots comparing the expression of MES-Hyp signature genes (**h**) and MES-Ast signature genes (**i**) between MES-Hyp and MES-Ast cells. Statistical significance, two-sided paired t test; exact P values shown.



Extended Data Fig. 5 | See next page for caption.

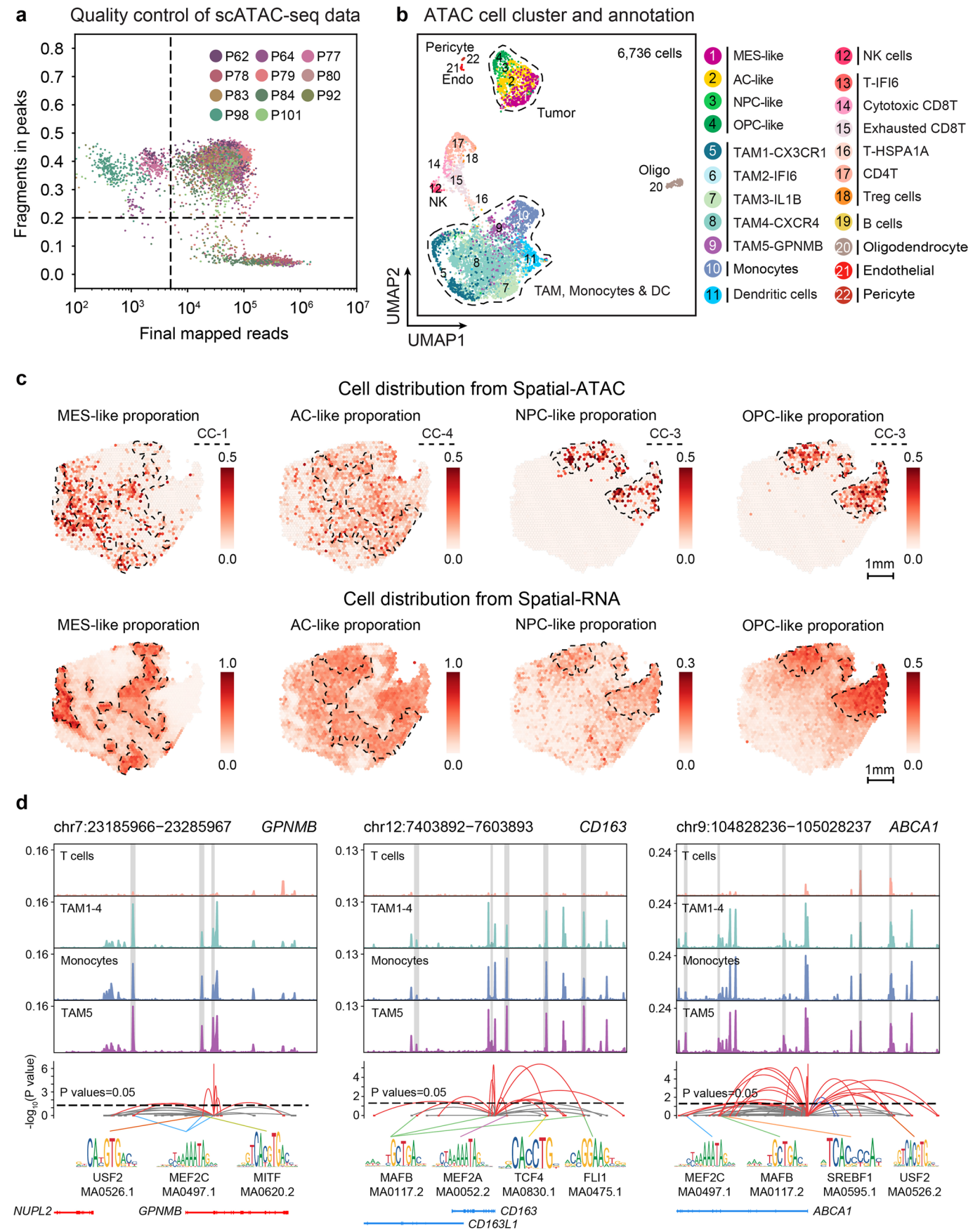
Extended Data Fig. 5 | Co-localization relationships between cell types identified through high-resolution ISH data. **a**, Schematic diagram illustrating the strategy for mapping scRNA-seq data onto the ST slices. **b**, Visualization of the locations of scRNA-seq cells mapped onto representative slices P77-1 and P131-2 using Tangram. **c**, Proportions of two MES-like subtypes across four CCs per patient. Each dot represents one patient ($n = 21$); patients with <5 mapped MES-like cells excluded. Centre line, median; box, 25th–75th percentiles; whiskers, $1.5 \times \text{IQR}$. Two-sided Wilcoxon rank-sum tests: MES-Hyp, CC-1 vs CC-2 ($P = 1.2 \times 10^{-4}$), CC-1 vs CC-3 ($P = 1.6 \times 10^{-5}$); MES-Ast, CC-2 vs CC-3 ($P = 3.4 \times 10^{-5}$), CC-2 vs CC-4 ($P = 2.2 \times 10^{-3}$). **d**, UMAP of scRNA-seq data from MES-like tumor cells. Left, cells colored by mapped CC; right, cells colored by MES-like subtype identity. **e**, Frequency histograms of RNA features per cell (top) and RNA transcripts per cell (bottom) in ISH data acquired by high-resolution confocal microscopy. Red line, mean. **f**, Heatmap showing relative expression of canonical

marker genes within ISH cell clusters. **g**, Representative images of multiple rounds of ISH imaging for DAPI, *COL1A2*, *CD44*, *LIF*, *IL11*, and *CD14* in a frozen section. Arrows indicate MES-Hyp cells. Scale bars, $10 \mu\text{m}$. **h**, Representative images of multiple rounds of ISH imaging for DAPI, *PECAM1*, *CD44*, *LIF*, *COL1A1*, and *COL1A2* in a frozen section. Arrows indicate MES-Ast cells. Scale bars, $10 \mu\text{m}$. **i, j**, Schematic diagram (**i**) illustrating the strategy utilized for calculating spatial regions (**j**) in a frozen section from a GBM patient based on the similarity of cell type composition. **k**, Co-localization frequency of MES-Hyp and MES-Ast cells with TAM5-GPNMB ($n = 213$) and blood-vessel cells ($n = 95$; endothelial cells, pericytes and VSMCs) in high-resolution ISH. TAM5-GPNMB and vascular cells restricted to the top 15% marker-high cells. Centre line, median; box, 25th–75th percentiles; whiskers, $1.5 \times \text{IQR}$. Two-sided Wilcoxon rank-sum test; exact P values shown.



Extended Data Fig. 6 | Temporal transitions of patient-derived glioblastoma organoids. a,b, ISH images of P234-derived GBOs cultured for 1 week (**a**) and 4 weeks (**b**), showing normalized expression of ADM (MES-Hyp marker and hypoxia-responsive gene), COL1A2 (MES-Ast marker), and GFAP (AC-like marker). **c,d,** ISH images of P234-derived GBOs at 1 week (**c**) and 4 weeks (**d**), showing normalized expression of the proliferation marker MKI67. **e-h,** ISH images

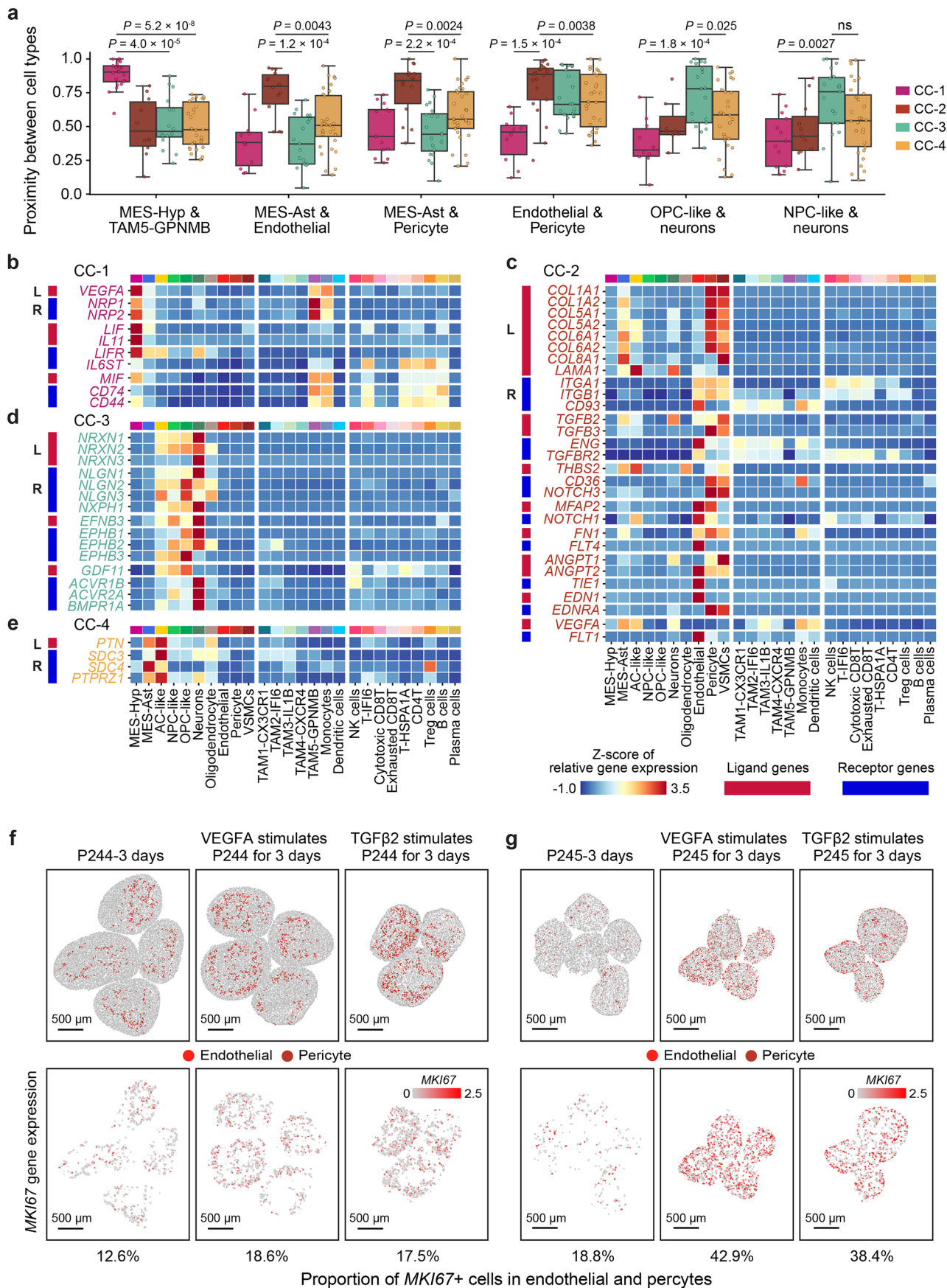
of P235-derived GBOs cultured for 1 and 4 weeks, revealing similar temporal expression patterns as in **a-d**. **i,j,** Violin plots showing normalized expression of the proliferation marker MKI67 across tumor subtypes in patient-derived GBM organoids (**i**) and in primary GBM detected by scRNA-seq (**j**). Statistical significance, two-sided paired *t* test; exact *P* values shown.



Extended Data Fig. 7 | Spatial chromatin accessibility profiling of GBM.

a, Scatter plot showing final mapped reads per cell and fragments in peaks per cell across 11 patients from scATAC-seq data. **b**, UMAP visualization of scATAC-seq data ($n = 6,736$). Cells are colored by clusters obtained by mapping the gene scores from scATAC-seq data to scRNA-seq reference data. **c**, Upper panels depict the proportions of MES-like, AC-like, NPC-like, and OPC-like subpopulations in scATAC-seq clusters on representative slice P77-1, while lower panels display

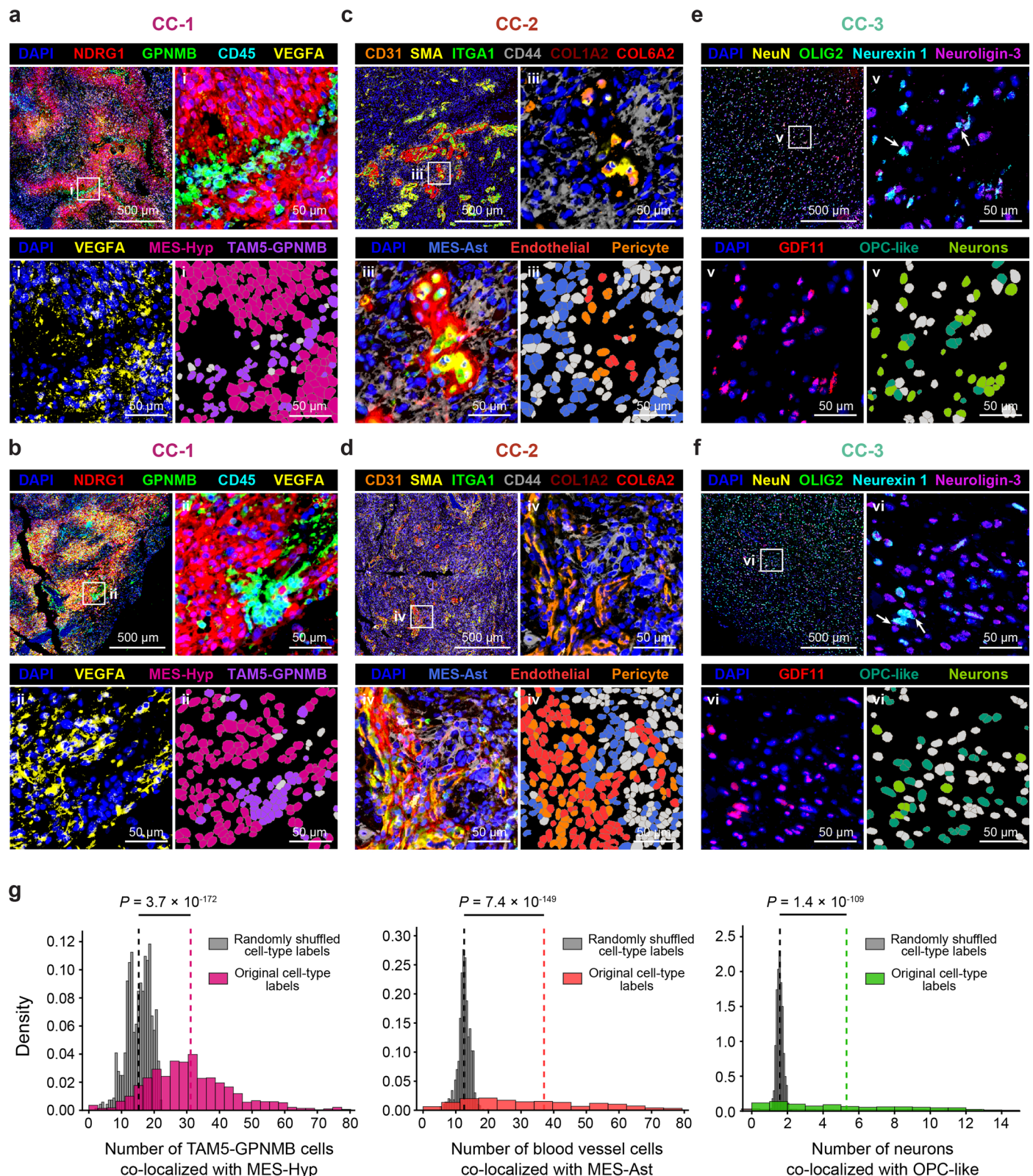
these subpopulations in scRNA-seq clusters. Scale bars, 1 mm. **d**, Aggregated scATAC-seq tracks illustrating chromatin accessibility peaks for representative genes. Loops denote the p value of the correlation between peak accessibility and RNA expression, with loop height reflecting significance. Grey bars highlight scATAC-seq peaks with increased chromatin accessibility in the TAM5-GPNMB subpopulation. Lower panels display motifs located within the peaks indicated by the connecting lines.



Extended Data Fig. 8 | See next page for caption.

Extended Data Fig. 8 | Spatial proximity and Ligand-receptor expression of interacting cell types. a, Box plot showing spatial proximity between interacting cell types across four CCs. Each dot represents one tissue slice ($n = 32$). Proximity was calculated per slice and CC only when the CC contained ≥ 50 spots enriched for each interacting cell type. Centre line, median; box, 25th–75th percentiles; whiskers, $1.5 \times \text{IQR}$. Two-sided Wilcoxon rank-sum test; exact P values shown. **b–e**, Heatmaps showing the relative expression levels of ligands and receptors

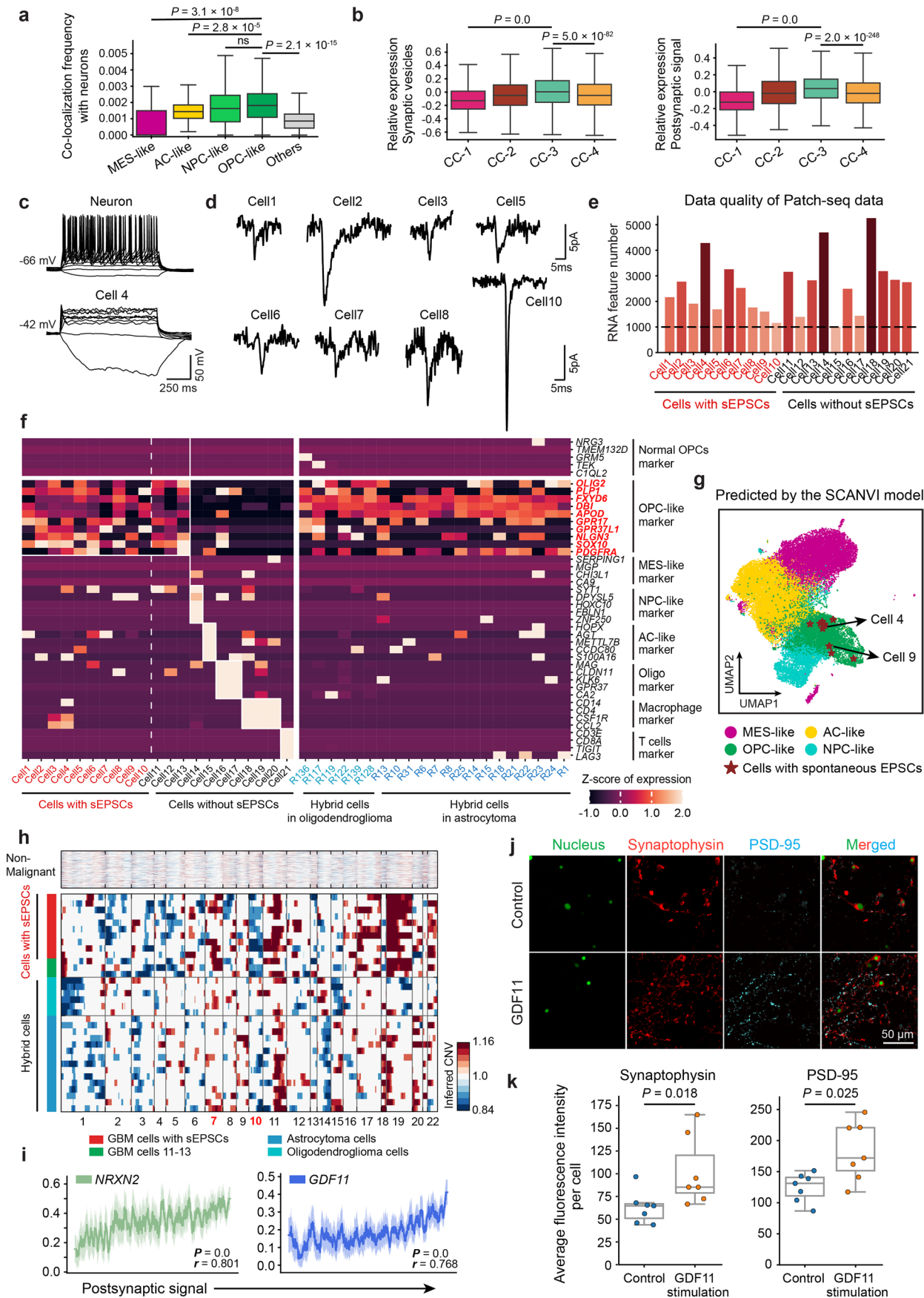
(Methods) involved in representative cellular community-specific intercellular communications with high connectivity scores for CC-1 (**b**), CC-2 (**c**), CC-3 (**d**), and CC-4 (**e**) based on scRNA-seq data integrated with publicly available datasets of MES-like tumor cells. **f, g**, ISH images illustrating the spatial distribution of endothelial and pericytes and *MKI67* expression within these populations in control, VEGF-A treated, or TGF- β 2 treated GBOs derived from patients P244 (**f**) and P245 (**g**).



Extended Data Fig. 9 | Spatial proteomic validation of conserved intercellular interactions within cellular communities on primary tumor sections.

a–f, Representative PhenoCycler fluorescence images of CC-1 (**a,b**), CC-2 (**c,d**), and CC-3 (**e,f**). Top left, overviews of each cellular community. Scale bars, 500 μ m. Top right and bottom left, magnified regions highlighting cell-type markers and intercellular interactions. Bottom right, cell membrane

segmentation colored by cell clusters. Scale bars, 50 μ m. **g**, Frequency histograms showing TAM5-GPNMB cells within 100 μ m of MES-Hyp cells (left), blood vessel cells within 100 μ m of MES-Ast cells (middle), and neurons within 100 μ m of OPC-like cells (right), under original cell-type labels and averaged results from 1,000 iterations of random label permutation. Statistical significance, two-sided paired *t* test; exact *P* values shown.



Extended Data Fig. 10 | See next page for caption.

Extended Data Fig. 10 | Gene expression profiles and electrophysiological recordings in GBM. **a**, Box plot showing spatial co-localization between neurons and MES-like, AC-like, NPC-like and OPC-like tumor cells in ISH data. Each dot represents one neuron ($n = 152$); boxes summarize neuron-wise co-localization. Centre line, median; box, 25th–75th percentiles; whiskers, minima and maxima within $1.5 \times \text{IQR}$. Two-sided Wilcoxon rank-sum test; exact P values shown. **b**, Box plots of synaptic vesicle and postsynaptic signaling gene expression across CCs. Boxes summarize spots per CC. Centre line, median; box, 25th–75th percentiles; whiskers, $1.5 \times \text{IQR}$. Two-sided t test; exact P values shown. **c**, Action potentials in neurons (top) and cell 4 (bottom). **d**, Representative spontaneous excitatory postsynaptic currents (sEPSCs) recorded from 8 cells in fresh GBM slices. **e**, Number of RNA features in cells with (cells 1–10) and without (cells 11–21) sEPSCs. Dashed line, 1,000-feature cutoff. **f**, Heat map of canonical marker genes in GBM cells with and without sEPSCs and hybrid cells in low-grade

glioma. OPC-like markers highlighted in red. **g**, UMAP of Patch-seq cells with sEPSCs (stars) mapped to tumor cells using SCANVI. **h**, Inferred CNVs (100-gene sliding windows). Rows, individual cells; non-malignant cells lacking CNVs (top) followed by malignant cells (with CNVs) ordered as indicated. **i**, Correlation between NRXN2, GDF11 and postsynaptic signaling gene expression in OPC-like tumor cells (scRNA-seq; $n = 5,660$). Two-sided Pearson correlation; shaded bands, 95% confidence intervals (Student's t distribution, based on SEM). **j**, Representative immunofluorescence images of primary rat neurons under control and GDF11 treatment. Synaptophysin-mRuby-expressing neurons with H2B-GFP-labeled nuclei were stained for PSD-95. Scale bars, 50 μm . **k**, Quantification of synaptophysin and PSD-95 fluorescence intensity per cell in control and GDF11-treated neurons ($n = 7$). Centre line, median; box, 25th–75th percentiles; whiskers, $1.5 \times \text{IQR}$. Two-sided Wilcoxon rank-sum test; exact P values shown.

Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed
☐	☒ The exact sample size (<i>n</i>) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ The statistical test(s) used AND whether they are one- or two-sided <i>Only common tests should be described solely by name; describe more complex techniques in the Methods section.</i>
☐	☒ A description of all covariates tested
☐	☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒ For null hypothesis testing, the test statistic (e.g. <i>F</i> , <i>t</i> , <i>r</i>) with confidence intervals, effect sizes, degrees of freedom and <i>P</i> value noted <i>Give P values as exact values whenever suitable.</i>
☐	☒ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☐	☒ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐	☒ Estimates of effect sizes (e.g. Cohen's <i>d</i> , Pearson's <i>r</i>), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection	No software was used for data collection.
Data analysis	Software for performing PhenoCycler assay: Akoya PhenoCycler-Fusion software (v2.3.1). Analysis of electrophysiological recordings: Clampfit 10.3 software. Image processing: ImageJ (v2.1.0), Stardist (v0.8.3). Preprocessing of sequencing data: CellRanger(v6.0.2), SpaceRanger(v1.2.1), APEC (v1.1.0), STAR (v2.5.3), and RSEM (v1.3.1). scRNA-seq data processing: scDblFinder (v1.7.7), Harmony (v1.0.3), Seurat (v4.3.0), Demuxlet (v1.0), souporcell (v2.4), Scanpy (v1.9.3), inferCNV (v1.6.0), Palantir (v1.3.3), and PHATE (v1.0.11). Deep learning model predict cell types: scArches (v0.5.9). Genomic data processing: BWA-MEM (c0.7.17), GATK (v4.1.3). Spatial transcriptome related analysis: Cell2location (v0.6), SPACEL (v1.1.6), COMMOT (v0.0.3), Tangram (v1.0.2), and scVelo (v0.3.2). ISH data processing: ASHLAR (v1.14.1), DeepCell (v0.11.0) and Starfish (v0.2.2). scATAC processing: Signac (v1.9.0) and ArchR (v1.0.1). PhenoCycler analysis: QuPath (v0.5.1). The analysis performed in this study primarily utilized publicly available software, as described in the Methods section. The custom code for the main analysis can be accessed at https://github.com/QuKunLab/GBM .

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

Processed spatial transcriptomics, scRNA-seq, scATAC-seq, multiple rounds of ISH, PhenoCycler, and patch-seq data generated in this study are available at the Zenodo data archive (<https://doi.org/10.5281/zenodo.17117252>).

The corresponding raw sequencing data for spatial transcriptomics, scRNA-seq, and scATAC-seq have been deposited with NGDC GSA for Human under the accession code HRA004942.

The sources of publicly available data used in this study are as follows:

- (1) 12 samples of human GBM 10X Visium data from Ravi et al. (2022), <https://datadryad.org/stash/dataset/doi:10.5061/dryad.h70rxwdmj>;
- (2) 1 sample of human GBM 10X Visium data from the 10X dataset, <https://www.10xgenomics.com/datasets>;
- (3) 7 samples of human GBM 10X Visium data from Mei et al. (2023), <https://doi.org/10.6084/m9.figshare.22434341>;
- (4) 2 samples of human GBM 10X Visium data from Ren et al. (2023), available in the GEO database under the accession GSE194329;
- (5) GBM scRNA-seq data from Couturier et al. (2020), https://github.com/mbourgey/scRNA_GBM;
- (6) GBM snRNA-seq data from Wang et al. (2021), <https://portal.gdc.cancer.gov/projects/CPTAC-3>;
- (7) scRNA-seq data of MES-like tumor cells from Ruiz-Moreno et al. (2022), <https://cellxgene.cziscience.com/collections/999f2a15-3d7e-440b-96ae-2c806799c08c>;
- (8) 9 GBM scATAC-seq samples from Sundaram et al. (2024), <https://gdc.cancer.gov/about-data/publications/TCGA-ATAC-Seq-2024>;
- (9) 5 human brain snRNA-seq samples from Jorstad et al. (2023), <https://cellxgene.cziscience.com/collections/d17249d2-0e6e-4500-abb8-e6c93fa1ac6f>;
- (10) 4 human brain snRNA-seq samples from Siletti et al. (2023), <https://cellxgene.cziscience.com/collections/283d65eb-dd53-496d-adb7-7570c7caa443>;
- (11) Patch-seq data of low-grade glioma (LGG) from Curry et al. (2024), available in the GEO database under the accession GSE271961.

Research involving human participants, their data, or biological material

Policy information about studies with [human participants or human data](#). See also policy information about [sex, gender \(identity/presentation\), and sexual orientation](#) and [race, ethnicity and racism](#).

Reporting on sex and gender

Of the 33 patients whose data were newly generated for this study, 14 were female and 19 were male. Detailed information regarding the patients can be found in Supplementary Table 1.

Reporting on race, ethnicity, or other socially relevant groupings

All the 33 patients whose data were newly generated for this study were of Han Chinese ethnicity.

Population characteristics

Clinical information of all patients used for scRNA-seq, scATAC-seq, WES, 10X Visium, ISH, Patch-seq, PhenoCycler, and Patient-derived GBOs experiments can be found in Supplementary Table 1.

Recruitment

All individuals donating surgical tissue provided informed consent prior to surgery, and all experiments were conducted in accordance with approved guidelines. Patients were consecutively recruited from individuals undergoing surgical resection for primary glioblastoma at The First Affiliated Hospital of USTC. No self-selection was involved. No additional selection criteria were applied beyond clinical diagnosis and tissue availability. Major recruitment bias is unlikely.

Ethics oversight

The collection of GBM specimens was approved by the ethics committee of the First Affiliated Hospital of the University of Science and Technology of China (USTC) (No. 2022-KY-092).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

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☒ Life sciences

☐ Behavioural & social sciences

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For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

Sample details for all experiments are provided in Supplementary Table 1. Regarding spatial transcriptomics, we obtained 32 spatial transcriptome profiles from 27 patients, comprising 78,944 spots. For scRNA/snRNA-seq experiments, we collected 81,737 single-cell and 3,860 single-nucleus transcriptomics profiles from 30 and 10 patients, respectively. In scATAC-seq experiments, 6,736 single-cell chromatin accessibility profiles were generated from 11 patients. To investigate GBM tumor cell transformation, an additional 13,914 scATAC-seq profiles were collected from 9 individuals. For ISH experiments, we captured 340,568 ISH cells. In the spatial proteome assay, profiles from a total of 6 patients were obtained. Finally, in patch-seq experiments, we simultaneously recorded electrophysiological information and single-cell transcriptomes from 21 cells across 7 patients. No statistical methods were used to pre-determine sample sizes. All available GBM samples

that met the inclusion criteria were included during data collection. Sample sizes were sufficient to support reproducible and statistically robust analyses.

Data exclusions	Please refer to the Methods section for detailed information. In the case of scRNA-seq/snRNA-seq data, we excluded low-quality cells based on three criteria: (1) the number of detected genes, (2) mitochondrial transcript content and (3) doublet scores. For scATAC-seq data, low-quality cells were excluded based on two criteria: (1) unique fragments mapped to the genome, (2) cells with a fraction of detected fragments in peaks. For ISH data, cells with less than 5 detected genes were considered low-quality and removed. Similarly, in patch-seq data, cells with fewer than 1,000 detected genes were deemed low-quality and excluded.
Replication	Reproducibility was assessed using independent biological samples and orthogonal validation assays. For instance, we identified four distinct cellular communities with consistent and well-defined cell type co-occurrence patterns across 32 GBM slices, and a meta-module analysis of MES-like cells was performed using data from 19 patients across 8 datasets. The predicted intercellular communications were validated in multiple PhenoCycler sections. Functional assays were repeated in independent patient-derived organoids with similar outcomes. No finding failed replication.
Randomization	For the sequencing data analysis in this study, all available GBM samples were included during the data collection phase; therefore, randomization was not applicable to the sequencing analyses. For experiments involving group allocation, such as organoid perturbation assays, organoids were randomly assigned to three groups. Within each group, three to five organoids were randomly allocated.
Blinding	Blinding of group allocation was not feasible in this study. Analyses were conducted by comparing distinct groups across different cellular communities.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

For 21 antibodies used in PhenoCycler assays.
 Antibody, Company, Catalog number, Clone number:
 Anti-human CD44, Akoya Biosciences, 4450041, 156-3C11;
 Anti-human CD31, Akoya Biosciences, 4450017, EP3095;
 Anti-human SMA, Akoya Biosciences, 4450049, 1A4;
 Anti-GFAP, Abcam, ab218309, EPR1034Y;
 Anti-Iba1, Abcam, ab283342, EPR16589;
 Anti-human CD163, Akoya Biosciences, 4250079, EPR19518;
 Anti-human Collagen IV, Akoya Biosciences, 4550122, N/A;
 Anti-Olig2, Abcam, ab220796, EPR2673;
 Anti-NeuN, Invitrogen, 702022, 14H6L24;
 Anti-human CD45, Akoya Biosciences, 4450042, AKYP0074;
 Anti-Doublecortin, Abcam, ab222921, EPR19997;
 Anti-Lysozyme, Abcam, ab185129, EPR2994(2);
 Anti-GPNMB, R&D, AF2550, Polyclonal;
 Anti-Integrin alpha 1 antibody, Abcam, ab243032, CL7207;
 Anti-COL1A2 antibody, Abcam, ab308456, EPR27415-30;
 Anti-VEGFA antibody, Abcam, ab185238, EP1176Y;
 Anti-NDRG1 antibody, Abcam, ab226082, EPR5593;
 Anti-Neuroigin 3, R&D, MAB6088, 566209;
 Anti-Neurexin-1, Invitrogen, 703650, 22H29L23;
 Anti-human GDF-11, R&D, MAB19581, 743833;
 Anti-Collagen VI alpha 2, Abcam, ab250255, EPR7889(N).
 For flow cytometry:
 APC/Cyanine7 anti-human CD45, BioLegend, 368516, 2D1.
 All antibodies were used at the concentrations recommended by the manufactures.

Validation

Antibodies used in this study are commercially available. The vendors conducted verification experiments for the antibodies. All

Validation

primary antibodies were independently validated in-house by immunostaining on tissue sections prior to use in this study.

Animals and other research organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

Laboratory animals

Embryonic Day 18 (E18) Sprague-Dawley rat embryos.

Wild animals

No wild animals were used in this study.

Reporting on sex

This experiment involved hippocampal neurons from embryonic day 18 (E18) Sprague-Dawley rat embryos; sex information was not collected.

Field-collected samples

No samples were collected from the field for this study.

Ethics oversight

All experimental procedures involving animals were conducted in accordance with protocols approved by the University of Science and Technology of China Animal Care and Use Committee (Approval No. USTCACUC1902024).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Plants

Seed stocks

N/A

Novel plant genotypes

N/A

Authentication

N/A

Flow Cytometry

Plots

Confirm that:

- ☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- ☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- ☒ All plots are contour plots with outliers or pseudocolor plots.
- ☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

The cells were co-stained with anti-human CD45-APC/Cyanine7 (clone 2D1, BioLegend), 1 $\mu\text{mol/L}$ calcein AM (Life Technologies), and 0.1 $\mu\text{mol/L}$ TO-PRO-3 iodide (Life Technologies) for a 30-minute incubation at 4 °C. Subsequently, the cells were washed and resuspended in 1 ml of DMEM/F12 medium (Gibco).

Instrument

SH800S Cell Sorter (Sony Biotechnology)

Software

SONY Sorter software

Cell population abundance

The post-sort fractions achieved a purity exceeding 98%

Gating strategy

Standard forward scatter height versus area criteria were applied to discriminate doublets, allowing only singleton cells to be gated. Viable single cells were identified as positive for calcein AM and negative for TO-PRO-3.

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.