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Characterizing the biology of primary brain tumors and their microenvironment via single-cell profiling methods

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Abstract

Genomic and transcriptional heterogeneity is prevalent among the most common and aggressive primary brain tumors in children and adults. Over the past 20 years, advances in bioengineering, biochemistry and bioinformatics have enabled the development of an array of techniques to study tumor biology at single-cell resolution. The application of these techniques to study primary brain tumors has helped advance our understanding of their intra-tumoral heterogeneity and uncover new insights regarding their co-option of developmental programs and signaling from their microenvironment to promote tumor proliferation and invasion. These insights are currently being harnessed to develop new therapeutic approaches. Here we provide an overview of current single-cell techniques and discuss relevant biology and therapeutic insights uncovered by their application to primary brain tumors in children and adults.

Keywords

gliomas | medulloblastoma | single-cell methods | singlecell RNA-seq | single-cell multi-omics

Over the last 20 years, significant advances have been made in understanding the molecular alterations of primary central nervous system (CNS) tumors in children and adults. Although initial driver events underlie oncogenesis in these tumors, dysregulation of cellular processes driven by these initial genetic changes and the effect of environmental factors, such as cancer treatments, often lead to the accumulation of additional genetic alterations driving an evolutionary drift away from the initial oncogenic clone promoting intra-tumoral heterogeneity. In addition to this genetic heterogeneity, tumor cells also demonstrate phenotypic heterogeneity by implementing distinct transcriptional programs on a background of similar genetic alterations. Therapeutic resistance of many primary brain tumors to targeted therapies provides evidence of this heterogeneity as, for example, targeting of particular genetic alterations in glioblastoma—such as epidermal growth factor receptor, *EGFR*, gene mutations—has not led to a survival benefit.[1] The development and integration of microfluidic, biochemical and bioinformatic technologies applied to the study of cancer biology at the single-cell level have launched a new era in our understanding of primary brain tumors. Through the application of these techniques, we have been able to better characterize the intra-tumoral heterogeneity of primary brain tumors, dissect interactions between cellular sub-populations, and identify developmental programs that are often hijacked by tumors in their quest for proliferation and migration. Importantly, applying these techniques to primary brain tumors holds promise for identifying mechanisms to modulate intra-tumoral heterogeneity or reveal

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common vulnerabilities of cellular subpopulations that could lead to more effective therapies.

Single-Cell Methods to Dissect Brain Tumor Biology

Once isolated from their original tumors, individual cancer cells and tumor infiltrating cells present multiple layers of information that can be analyzed to characterize the heterogeneity of the tumor, including epigenomic and genomic alterations, as well as differential gene and protein expression, Figure 1. Tumor cells can differ in their chromatin structure and patterns of chromatin accessibility; their patterns of DNA methylation and other epigenetic alterations; their genetic alterations (mutations, deletions, amplifications, fusions, rearrangements); their transcriptional profiles; and their intra-cellular and cell surface protein expression. An array of single-cell techniques has been developed to capture specific information at all these different levels, and in some cases, to characterize cells at multiple levels of information (multi-omics).[2]

Profiling regions of chromatin accessibility offers a window into the regulation of gene expression by revealing regions of the genome accessible for transcription as well as binding sequences of regulatory elements, such as promoters, enhancers and insulators. The single-cell assay of transposase accessible chromatin using sequencing (scATAC-seq) relies on a hyperactive derivative of the prokaryotic Tn5 transposase to cleave accessible regions of the genome and insert sequencing adapters, to allow for amplification, barcoding and library construction.[3,4] Single-cell DNA methylation (scDNAme-seq) offers another perspective into the regulation of gene expression. The addition of a methyl group to cytosine (C) residues in humans occurs at cytosine-guanine (CG) dinucleotides, frequently referred to as CpG sites. Regions of the genome with a high frequency of CpG sites are known as CpG islands and are found at or near 40% of the promoters of mammalian cells.[5,6] Methylation of CpG islands leads to a closed chromatin configuration, leading to repression of gene transcription.[5,6] The bisulfite sequencing method—in which DNA is treated with bisulfite before sequencing, leading to the conversion of unmethylated cytosine residues to uracil leaving methylated cytosine residues unaffected—has now been adapted for implementation at the single-cell level through different protocols.[7–9] Additionally, chromatin heterogeneity and DNA-protein interactions can be directly resolved via antibody-mediated approaches such as single-cell chromatin immunoprecipitation followed by sequencing (scChIP-seq) or single-cell CUT&Tag sequencing.[10,11] Genetic variation at the single-cell level can be characterized using single-cell whole-genome sequencing (scWGS) methods, which provide critical information for describing intra-tumoral heterogeneity via copy number alterations (CNAs) and enable phylogenetic analyses to study tumor evolution.[12–14]

Although they help characterize tumor heterogeneity and provide insights into the regulation of gene expression, hence providing insight into determinants of cellular state, scATAC-seq, scDNAme-seq and scWGS do not by themselves directly reveal the functional states associated with tumor cell subpopulations. Single-cell RNA-sequencing (scRNA-seq) has become the most widely adopted technique for the study of intra-tumoral heterogeneity, as it allows for direct characterization of the transcriptome of individual cells, revealing gene expression programs underlying distinct cellular states and facilitating the detection of rare cell subpopulations, as well as allowing for the inference of copy number alterations and mutations depending on sequencing depth and transcript length. scRNA-seq protocols rely on the capture of mRNA transcripts (using an oligo-dT primer that only captures polyadenylated RNA) followed by reverse transcription to obtain complementary DNA, which is subsequently amplified for library construction. Full transcript length scRNA-seq protocols are labor intensive but allow for gene expression quantification, detection of transcript isoforms and inference of CNAs as well as mutations.[15,16] On the other hand, droplet-based platforms targeting the 3′-end or 5′-end of the mRNA transcript readily enable the profiling of thousands of cells (high-throughput) at the expense of coverage per cell.[17] A more recent method, single-nucleus RNA-sequencing (snRNA-seq), enables the transcriptional profiling of frozen tumor samples bypassing the requirement of immediately processing fresh tumor samples as is needed for scRNA-seq, enabling the analysis of previously collected frozen samples for the study of rare tumors or longitudinal analyses of tumor treatment response and evolution.[18]

Intra-tumoral heterogeneity and functional cell states can also be characterized by profiling intra-cellular and cell surface proteins at the single-cell level. Mass cytometry methods such as cytometry by time-of-flight (CyTOF), overcome the limitations of flow cytometry in profiling protein markers, as with the latter, attempts at profiling more than a handful of different fluorophore-tagged antibodies bound to specific proteins lead to overlap of emission spectra and inability to resolve signal from some of the surface molecules. In contrast, CyTOF allows for the simultaneous profiling of over 100 proteins in thousands of cells through the use of antibodies labeled with distinct metal isotopes that are detected and quantified via mass spectrometry.[19,20] Besides being able to profile the biology of individual tumor cells at different layers—from chromatin accessibility to protein expression—there are an increasing number of methods enabling the concurrent profiling of individual cells at different layers, including chromatin accessibility and transcriptome (SHARE-seq[21]), DNA methylation and transcriptome (scM&T-seq, scMT-seq[22,23]), genotype and transcriptome (G&T[24,25]), and transcriptome and cell surface proteins (CITE-seq[26]). These multi-omics techniques facilitate the in-depth interrogation of tumor biologies, such as relating epigenomic regulation and genomic variation with changes in cellular state at the transcriptional and protein expression levels.

Insights from Single-Cell Methods for Primary Brain Tumors

Glioblastoma

Glioblastoma, IDH-wildtype, is a paradigm of intra-tumoral heterogeneity, and its molecular alterations have been

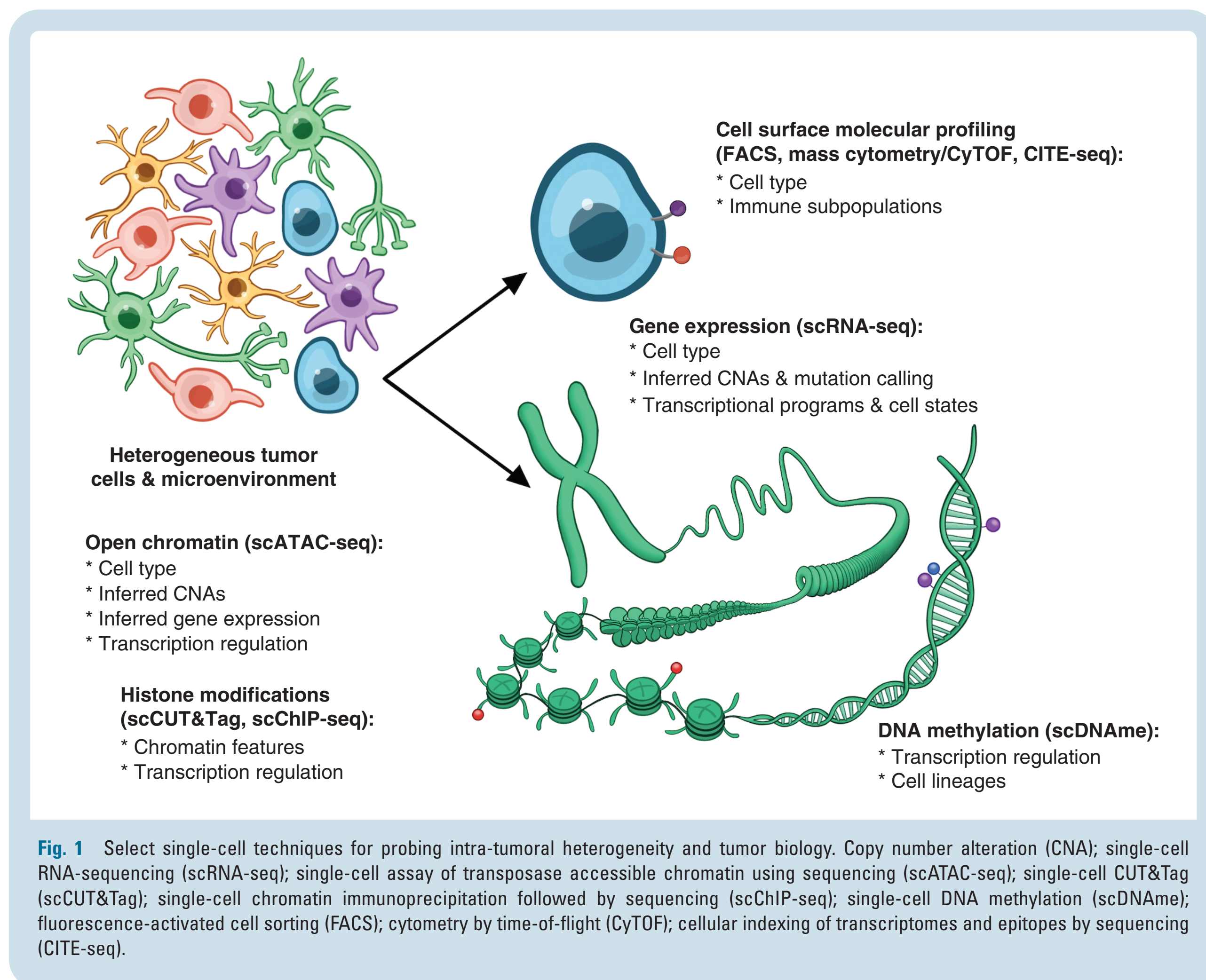

Fig. 1 Select single-cell techniques for probing intra-tumoral heterogeneity and tumor biology. Copy number alteration (CNA); single-cell RNA-sequencing (scRNA-seq); single-cell assay of transposase accessible chromatin using sequencing (scATAC-seq); single-cell CUT&Tag (scCUT&Tag); single-cell chromatin immunoprecipitation followed by sequencing (scChIP-seq); single-cell DNA methylation (scDNAme); fluorescence-activated cell sorting (FACS); cytometry by time-of-flight (CyTOF); cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq).

the focus of intense study. In fact, The Cancer Genome Atlas (TCGA) selected glioblastoma as the first tumor to be profiled via bulk analyses of DNA copy number, gene expression and DNA methylation in 2008.[27] Subsequent TCGA analyses relying on bulk RNA-seq suggested that glioblastomas could be characterized into one of four different subtypes—proneural, neural, classical and mesenchymal—each associated with specific genomic alterations.[28] These results were encouraging as they suggested that glioblastoma could be characterized into one of these subtypes and that each tumor could be treated by targeting specific aberrant pathways. Unfortunately, the promise of effective targeted therapy for glioblastoma has not been realized and single-cell profiling methods provide an explanation. The first study to profile glioblastoma samples using scRNA-seq demonstrated that cells with transcriptional profiles consistent with the proneural, neural, classical and mesenchymal bulk subtypes coexisted within individual tumors, highlighting the intra-tumoral heterogeneity of the tumor.[29]

A follow-up scRNA-seq study profiling glioblastoma patient samples described four distinct cellular states—astrocyte (AC)-like, neural progenitor cell (NPC)-like, oligodendrocyte progenitor cell (OPC)-like and mesenchymal (MES)-like—based specific transcriptional programs, as well as cells with elements of different transcriptional programs, suggesting transitions between cellular states, Figure 2A.[28] The proportions of each cellular state within a tumor are shown to depend on copy number alterations of the *EGFR* (AC-like), *CDK4* (NPC-like) and *PDGFRA* (OPC-like) loci, and on *NF1* mutations as well as influences from the tumor microenvironment for the MES-like state, with pediatric tumors demonstrating a higher proportion of cells in the OPC-like state.[30] Using the transcriptional programs defining each of the cellular states, the authors were able to deconvolve the cellular states of TCGA glioblastoma subtypes, noting that classical and mesenchymal subtypes are predominantly composed of cells in the AC-like and MES-like states, while tumors in the proneural subtypes are enriched by cells in the developmental NPC-like and OPC-like cellular states.[30] Interestingly, and highlighting the limitations of bulk assays for characterizing tumors, the TCGA neural glioblastoma subtype is shown to be mainly composed by non-malignant cells, including neurons and oligodendrocytes.[30] Lastly, through a series of cellular barcoding experiments to track cell lineage in xenografted tumors, this study shows the ability of cellular states to transition into different cellular states, reconstituting the heterogeneity of a tumor once other cellular states have been depleted.[30]

Subsequent single-cell studies have validated this cellular state model of glioblastoma by highlighting developmental hierarchies in cellular states,[31–34] and complemented it, by describing cellular states with specific metabolic vulnerabilities.[32] Single-cell techniques have also been applied to understanding the epigenetic determinants of cellular states in glioblastoma. A recent scDNAme-seq study demonstrated intra-tumoral heterogeneity of DNA methylation in glioblastoma, as well as hypomethylation of regulatory targets of the polycomb repressor complex 2 (PRC2) in the NPC-like and OPC-like (stem-like) cell states when compared to the more differentiated (AC-like, MES-like) cell states, suggesting that hypomethylation of PRC2 targets is an epigenetic mechanism for the regulation of stemness in tumor cells.[35] Another scDNAme-seq study, also confirmed intra-tumoral heterogeneity of DNA methylation in glioblastoma and hypothesizes that randomness in the placement of epigenetic marks might underlie the ability of glioma cells to adapt to environmental stressors.[36] Studies profiling chromatin accessibility in glioblastoma at the single-cell level have also shown heterogeneity of tumor cells, and identified regions of increased chromatin accessibility consistent with stemness in both stem-like and mesenchymal cell states, consistent with the previous findings of plasticity/cell state transitions from transcriptomic data.[34,37]

In addition to revealing the intra-tumoral heterogeneity and plasticity of malignant cells in glioblastoma, single-cell profiling methods have facilitated the characterization of the tumor microenvironment and the discovery of important interactions between malignant and non-malignant cells. Mass cytometry studies have shown that the immune infiltrates of glioblastoma is mainly composed of immunosuppressive macrophages with a smaller compartment of activated microglia,[38,39] a finding that has also been replicated with scRNA-seq studies.[40–42] By concurrently profiling gene expression in macrophages and malignant cells, scRNA-seq has allowed the identification of macrophage-derived oncostatin M (OSM) as an inducer of the MES-like cellular state in glioblastoma, as well as the identification of macrophages with an MES-like transcriptional program.[43] Concurrent scRNA-seq in cytotoxic T lymphocytes and glioblastoma cells, has also led to the recognition of a novel immune-checkpoint inhibition mechanism through the activation of the CD161 receptor in T cells by the CLEC2D ligand found on the surface of glioblastoma and myeloid cells.[44] Single-cell RNA-seq has also been leveraged to evaluate the response of a neoantigen vaccine in glioblastoma by reading the T-cell receptor sequence of infiltrating T cells in patient samples and identifying activated, neoantigen-specific T cells within the tumor.[45] Lastly, scRNA-seq studies have also revealed increased expression of glutamate receptor genes in the stem-like subpopulations of glioblastoma cells, providing evidence of glutamatergic synaptic input as a driver of cell proliferation and tumor progression.[46,47]

IDH-Mutant Gliomas

The discovery of isocitrate dehydrogenase (IDH) mutations in gliomas in 2008 has led to a revolution in our understanding of the biology of a distinct subset of gliomas known to preferentially affect the young and have a more ingravescent disease course compared to glioblastoma.[48] According to 2021 WHO classification of central nervous system tumors, IDH-mutant gliomas comprise astrocytoma, IDH-mutant (Grade 2–4) and oligodendroglioma (Grade 2–3).[49] In addition to the presence of IDH-mutations, these tumors have characteristic molecular alterations, including loss of the *ATRX* chromatin remodeler gene and *TP53* mutations in astrocytoma, and 1p/19q chromosomal codeletion in oligodendroglioma.[49,50] In addition to these canonical molecular alterations, single-cell profiling techniques have advanced our understanding of IDH-mutant gliomas by characterizing their intra-tumoral heterogeneity and revealing unique aspects of their interaction with the tumor microenvironment. An initial characterization of oligodendroglioma by profiling patient samples with scRNA-seq, revealed a developmental hierarchical model of cellular states with an originating stem-like compartment of neural progenitor cell (NPC)-like the transcriptional program, branching into two separate more differentiated, non-proliferating cellular compartments with astrocyte (AC)-like and (OC)-like transcriptional programs, Figure 2B.[51]

Interestingly, profiling of astrocytoma, IDH-mutant, via scRNA-seq revealed an analogous hierarchy of cellular states, with cells in an NPC-like cellular state branching in a development-like fashion towards differentiated AC-like and OC-like cellular states, and without a MES-like cellular state as seen in astrocytoma, IDH-wildtype (glioblastoma).[52] Despite their different histology and specific molecular alterations that characterize oligodendroglioma, and astrocytoma, IDH-mutant, this common developmental hierarchy of cellular states revealed by scRNA-seq highlights the importance of IDH-mutations in driving common oncogenic processes in tumors previously thought of as distinct from each other, and opening the opportunity to develop new treatments for both tumors with therapies targeting IDH-mutations and other downstream vulnerabilities.[51–53]

Investigations of the epigenomic determinants of cellular states in IDH-mutant gliomas have relied on concurrent single-cell profiling of gene expression and DNA methylation.[35,36] DNA methylation is a particularly relevant epigenomic regulation mechanism in IDH-mutant gliomas, as the mutant IDH protein produces the oncometabolite *D*-2-hydroxyglutarate (D-2HG), a competitive inhibitor of the TET family of 5-methylcytosine hydroxylases, preventing their demethylating activity and leading to increased DNA methylation of regulatory elements.[54–58] Single-cell DNA methylation profiling in IDH-mutant gliomas has revealed decoupling of promoter methylation-expression relationships (persistent oncogenic gene expression despite promoter methylation), preferential enhancer hypermethylation, as well as disruption of CTCF-mediated oncogene insulation.[35] More recently, scATAC-seq of 22 IDH-mutant gliomas indicated an additional axis of epigenomic heterogeneity mediated via ATRX loss-of-function, resulting in enhanced immunosuppressive phenotypes, the global increase of accessible chromatin and decrease of CTCF binding, which was further validated by single-cell CUT&Tag for CTCF in engineered mouse glioma cells.[59]

Single-cell profiling has also revealed important insights into the composition of the tumor microenvironment of IDH-mut gliomas. Unlike in glioblastoma, single-cell mass cytometry studies of IDH-mutant gliomas have demonstrated that their immune infiltrate is made up mostly of

activated microglia rather than monocyte-derived macrophages.[38,39] The inhibition of cytotoxic T-cells via activation of the CD161 receptor by the CLEC2D ligand expressed by glioblastoma uncovered by scRNA-seq has also been demonstrated in IDH-mutant gliomas.[44] Single-cell RNA-seq and T-cell receptor sequencing have also been used to probe the specificity of the immune response to a peptide vaccine targeting the canonical IDH1 R132H mutation in IDH-mutant gliomas.[60] Beyond interactions between glioma cells and immune cells in the tumor microenvironment, scRNA-seq has also revealed increased expression of glutamatergic AMPA receptor genes which provide the basis for neuron-glioma synapses in IDH-mutant gliomas that drive tumor proliferation and invasiveness.[46,47]

Single-Cell Profiling of Pediatric Brain Tumors

Pediatric brain tumors have unique pathobiology compared to their adult counterparts.[61] First, pediatric brain tumors are diseases of dysregulated development arising from spatiotemporally restricted precursor cells, often of fetal origin. Second, these precursor cells are transformed by specific genetic or non-genetic events during a defined developmental window, commonly leading to epigenetic dysregulation and cellular arrest in a stem cell-like state permissive to oncogenic transformation. Third, a cellular hierarchy is thereby established, in which not all tumor cells are developmentally arrested to the same extent and some tumor cells appear to be able to differentiate along this hierarchy or take on alternative phenotypes.

Single-cell profiling methods have helped elucidate central questions arising from this developmental hypothesis at unprecedented granularity across a variety of pediatric brain tumors. In parallel, increasingly detailed single-cell atlases of the normal developmental and adult brains across different species facilitate annotation and comparison of pediatric cancer cell states to their cellular correspondents during normal neuro-development.[62] Below, we discuss the findings from single-cell genomics investigations across different pediatric brain tumor types pertaining to the outlined developmental model.

Pediatric-Type Diffuse High-Grade Gliomas

Childhood high-grade gliomas (HGG) arise in a spatiotemporal pattern of incidence that segregates according to their driver oncogenic events. Most HGGs can be stratified by point mutations in histone H3: lysine27-to-methionine (K27M) substitutions in the non-canonical variant H3.3 or less commonly in H3.1, and glycine34-to-arginine/valine (G34R/V) substitutions in H3.3.[63–65] Broadly, these oncohistone mutations lead to epigenetic dysregulation, re-programming or arresting a spatiotemporally restricted "cell of mutation" in a pro-tumorigenic cell state.[66–68] The remaining group of H3 wildtype pediatric HGGs is more heterogeneous and subject to ongoing molecular analyses and stratifications. Mounting evidence suggests that many of these may be likewise driven by genetic or non-genetic alterations resulting in epigenetic dysregulation similar to the mechanisms observed in H3-mutant tumors.[49,69,70]

Diffuse Hemispheric Glioma, H3 G34-Mutant

Diffuse hemispheric glioma (DHG), H3 G34-mutant, almost exclusively presents in the cerebral hemispheres of adolescents and young adults. Their unique neurodevelopmental cellular background has been recently indicated to be anchored in a neuron-like instead of glial-like identity by a series of genomic and modeling studies.[71–74] Comparing DHG, H3 G34-mutant tumor bulk transcriptomes to human and mouse brain single-cell atlases by GSEA revealed that these were most similar to the prenatal cortical interneuronal lineage originating from progenitors of embryonal ganglionic eminence structures in the ventral telencephalon.[71] Isogenic modeling and epigenomic studies further indicated that this interneuron-like signature likely reflects cell-intrinsic properties of an initial "cell of mutation" itself rather than re-programming effects induced by the H3 G34R/V mutation, which serves to reinforce this pre-existing interneuronal-like state.[71,72] ScRNA-seq of 16 H3-G34R/V HGGs identified tumor cells expressing neuron-like and AC-like signatures, while the oligodendroglial lineage was absent,[71] Figure 2C. Notably, most tumor cells were found to be highly dysplastic co-expressing both neuron-like and AC-like signatures with incomplete segregation and demonstrated strong inter-tumoral heterogeneity. One axis of heterogeneity was linked to *PDGFRA* gene mutation status with *PDGFRA*-mutant tumors exhibiting a shift towards the AC-like state.[71] The identification of a prominent interneuron-like compartment within diffuse midline glioma, H3 K27M-altered sets the stage for additional questions as to the heterogeneity and phenotype of this cell, such as, if these cells represent the full developmental spectrum of interneuron lineage maturation, potentially reflecting a cellular hierarchy amenable to the concept of differentiation therapy, and whether they exert distinct interactions with their tumor microenvironment given their neuron-like instead of glia-like identity.

Diffuse Midline Glioma, H3 K27M-Altered

Diffuse midline gliomas (DMG) and H3 K27M-altered arise in midline brain regions primarily during the first decade of life. The majority of DMG, H3 K27M-altered, are lethal and thus defined as grade 4 by the presence of the oncohistone mutation irrespective of histology.[49,75] In vitro and in vivo studies have supported the hypothesis that the H3 K27M mutation occurs in neural stem cells of the developing pons during an early window of development,[76–81] engendering an active chromatin landscape reflective of early oligodendroglial lineage.[82–84] ScRNA-seq of primarily pontine pediatric DMG, H3 K27M-altered, indeed identified a substantial proportion of stem-like OPC-like tumor cells besides more differentiated AC-like and OC-like malignant cell populations.[85] This was supported by projecting bulk DMG, H3 K27M-altered transcriptomes to scRNA-seq data of embryonal brainstem using single-sample gene-set enrichment analysis (ssGSEA).[86] Filbin et al. further demonstrated that OPC-like cells constituted the predominant cycling cell population, and in vivo orthotopic xenotransplantation of undifferentiated OPC-like

cells initiated glioma formation, whereas injection of more differentiated AC-like cells was not able to induce tumors. Together, this supports a developmental hierarchy, in which OPC-like cells are at the apex and largely self-renewing, with some tumor cells being able to differentiate to less aggressive AC-like or OC-like lineages, Figure 2D. In contrast to more conventional cancer stem cell models, the OPC-like stem-like cell population in DMG, H3 K27M-altered is not small but rather predominant, which is indicative of a differentiation block. Notably, unlike in other gliomas so far profiled by single-cell transcriptomics, DMG, H3-K27M-altered is the only entity in which neuron progenitor-like or neuron-like cell populations are absent, which further supports the postulated H3 K27M oncohistone mechanism of skewing and arresting cells in the glial, mainly oligodendroglial lineage. These findings are currently being validated in larger patient cohorts, which may further provide power to assess the effect of different midline locations, age groups, and the mutant histone variant (H3.3 versus H3.1) on DMG, H3 K27M-altered cellular states and hierarchy.

The age of onset of DMG, H3 K27M-altered coincides with midline brain structures undergoing developmental waves of myelination coupled with OPC proliferation that has been shown to be neuronal activity dependent.[87] Indeed, it has been demonstrated that H3-K27M DMGs exploit their neuronal tumor microenvironment for tumor growth and progression, via both neuronal activity-regulated mitogen signaling as well as bona fide neuron-glioma synapse formation.[46,88,89] Analogous to normal neuron to OPC synapses fueling OPC proliferation, analysis of DMG, H3 K27M-altered scRNA-seq data indeed demonstrated that glutamatergic synapse genes are enriched in OPC-like cancer cells.[46] The immune cell compartment represents another tumor microenvironment component that has been analyzed at the single-cell level in DMG, H3 K27M-altered. Only a small proportion of immune cells were detected in scRNA-seq analysis of unenriched tumors, consistent with the fact that pediatric gliomas are relatively immune "cold" tumors. Within these, a predominance of immunosuppressive myeloid cells and only very few T-cells were detected.[85,90] These myeloid cells were found to be less inflammatory compared to adult glioma-associated myeloid cells at transcriptome and secretome levels.[90] In a recent first-in-human, phase 1 clinical trial of chimeric antigen receptor (CAR) T cells directed against the disialoganglioside (GD2) in patients with DMG, H3 K27M-altered,[91,92] scRNA-seq following Anti-GD2 CAR T cell treatments demonstrated heterogeneous populations of lymphocytes and myeloid cells correlating with the route of CAR T cell administration, clinical timepoint and response. Specifically, single-cell profiling revealed an immune-activating gene signature in myeloid cells at peak inflammatory timepoints, whereas later timepoints were associated with an immune-suppressive and phagocytosis signature, indicating complex and heterogeneous modes of interaction between tumor microenvironmental compartments in modulating therapy response.

Pilocytic astrocytoma

Pilocytic astrocytoma (PA), the most common primary CNS neoplasm in children, arises in infratentorial regions such as the cerebellum, in the optic pathway of patients with neurofibromatosis type 1 (NF1), and are less frequently seen in the cerebral hemispheres. 70% of PAs are driven by a single-driver somatic gene alteration in form of the *KIAA1549-BRAF* fusion oncogene, resulting in constitutive activation of the MAPK pathway. In a first single-cell transcriptome study of PAs, a strong infiltrate of mainly tumor-associated myeloid cells accounting for ~ 30% of all cells across tumors was discerned at the single-cell level, concordant with bulk transcriptomics data.[93] Within tumor cells, a minor subpopulation of OPC-resembling cells depicted high levels of MAPK-signaling positively correlating with *BRAF* expression, while the majority of tumor cells showed low MAPK-signaling expression and harbored an AC-like signature. Cells with high MAPK-signaling were enriched for mutually exclusive states of active cell proliferation or senescence, which is in line with the concept of oncogene-induced senescence specifically described in PA and potentially accounting for relatively favorable outcomes in this tumor entity. Based on these scRNA-seq characterized tumor cell states, a cellular hierarchy was suggested that places MAPK-signaling high OPC-like cells at the top, parts of which are cycling, become senescent, or differentiate towards more glia-like populations low in MAPK-signaling. When comparing these tumor cell signatures to those in pediatric higher-grade gliomas, PA MAPK-signaling enriched populations bore little resemblance to HGG stem-like cells and were markedly distinguishable by lower expression of neuronal progenitor and NSC marker genes as opposed to higher-grade stem-like populations, thereby suggesting MAPK-high cells may be more committed to the glial lineage as a potential cellular correlates of their favorable clinical outcome.

Medulloblastoma

Medulloblastoma (MB) is a malignant embryonal tumor of the cerebellum and comprises four molecular subgroups (WNT, Sonic Hedgehog/SHH, Group 3, Group 4).[94–96] These four subgroups are characterized by distinct genotypes, DNA methylomes, transcriptomes, age of onset, and prognoses, which again conjures the central developmental hypothesis that each subgroup arises from neuro-developmentally restricted cells of origin in a circumscribed spatiotemporal pattern.

Two independent studies have resolved the subgroup-specific cellular architectures and putative hierarchies by scRNA-seq. The study by Vladoiu et al.[97] profiled tumors of the SHH, Group 3 and Group 4 subgroups, demonstrating subgroup-specific resemblance to distinct neuronal lineages of the developing cerebellum along varying degrees of differentiation. Hovestadt et al.[98] sequenced tumors encompassing all four sub-groups and identified subgroup-specific tumor cells spanning an axis from undifferentiated to more mature neuron-like states, respectively, with undifferentiated populations often found to be in a cycling state and enriched for translational marker genes as well as subgroup-characteristic signaling markers. For SHH-MB, both studies showed by comparative cross-species analyses that tumor progenitor-like cells

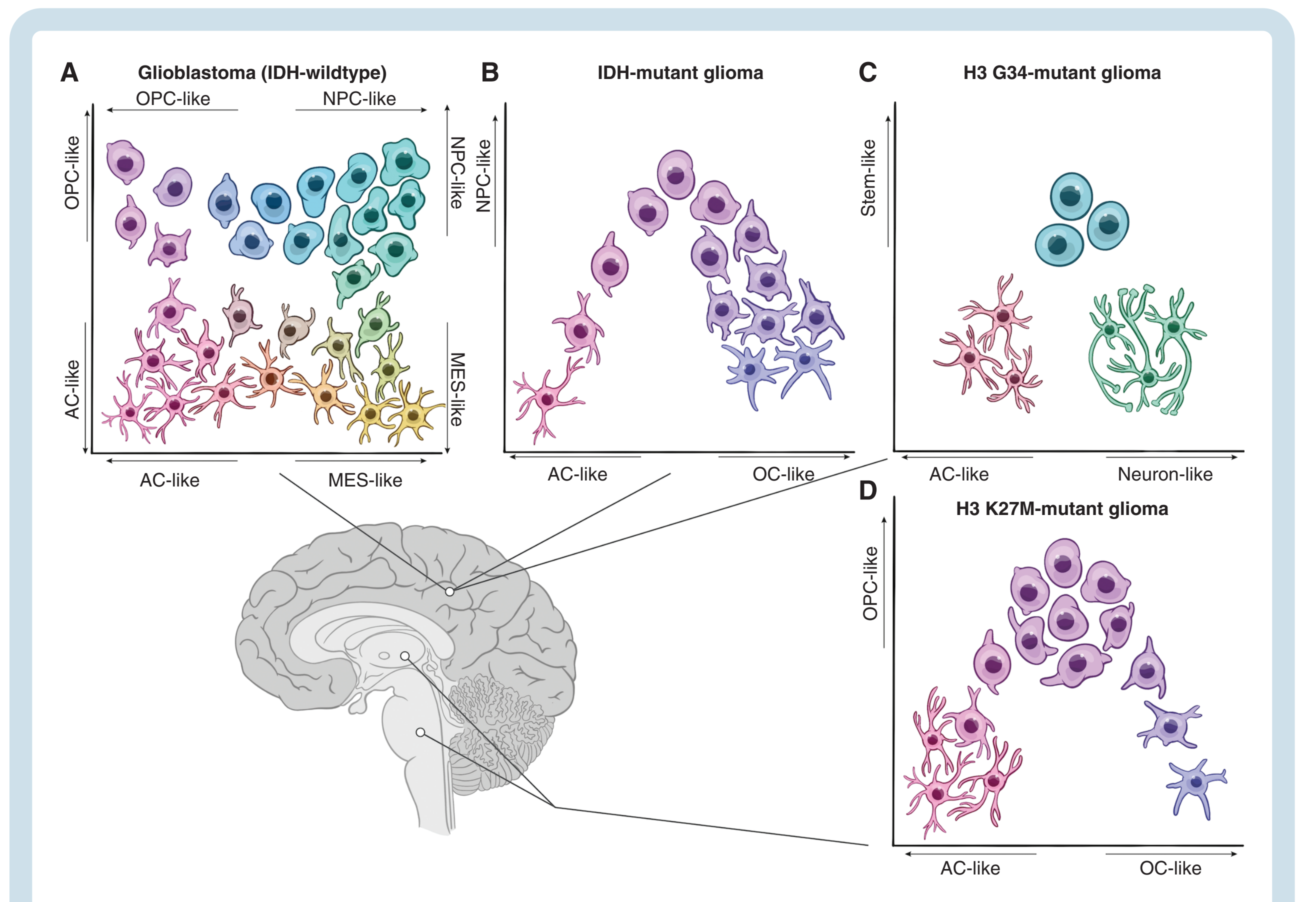

Fig. 2 Intra-tumoral heterogeneity and cellular hierarchies in gliomas. (A) Cellular state model of intra-tumoral heterogeneity in glioblastoma, IDH-wildtype. Four cellular states—astrocyte (AC)-like, neural progenitor cell (NPC)-like, oligodendrocyte progenitor cell (OPC)-like and mesenchymal (MES)-like—coexist within the tumor, with cells able to transition between states. (B) Developmental hierarchical model of cellular states in IDH-mutant gliomas. An originating stem-like cell compartment with a neural progenitor cell (NPC)-like transcriptional program branches into two separate more differentiated, non-proliferating cellular compartments with astrocyte (AC)-like and oligodendrocyte (OC)-like transcriptional programs. (C) Developmental cellular model in H3 G34-mutant glioma. A stem-like cell is directed towards the neuronal lineage mirroring interneuronal development from the embryonic ganglionic eminence. Tumor cells can also take on astrocyte (AC)-like or hybrid AC-like/neuron-like profiles, while tumor cells of the oligodendroglial lineage are lacking. (D) Developmental hierarchical model of cellular states in H3 K27M-mutant glioma with self-renewing oligodendrocyte progenitor cell (OPC)-like cells at the apex and differentiating towards astrocyte (AC)-like and oligodendrocyte (OC)-like cells.

resembled granule neuron progenitor (GNP) cells, that in normal development are stimulated by SHH signaling and give rise to glutamatergic granule cells, the most common neurons in the mature cerebellum, as had been previously indicated by modeling studies.[99–101] Hovestadt and colleagues also identified more differentiated intermediate and mature granule neuron-like populations, and notably, showed that adult SHH-MB harbor higher proportions of undifferentiated GNP-like cells than pediatric SHH-MB, pointing towards distinct biological processes specific to age groups.[98] ScRNA-seq of SHH-driven mouse MB models further indicated differentiation of GNP-like tumor cells upon inhibition of SHH signaling.[102] In WNT-MB, undifferentiated populations that were enriched for WNT signaling marker genes were detected alongside more differentiated neuron-like tumor cells. While previous experimental evidence had suggested lower rhombic lip (LRL) progenitors of the dorsal brainstem as putative cells of origin,[100] this was not evident by scRNA-seq analysis, potentially due to incompleteness of reference atlases or high aberrance of primary human tumor cells. Non-SHH, non-WNT Group 3 and Group 4 -MB were analyzed together in the study by Hovestadt et al.[98] as subsets of these tumors are known to exhibit overlapping signatures. This combined analysis revealed a common continuum of undifferentiated progenitor-like to more mature neuron-like populations. It was demonstrated that Group 3 and Group 4 subgroups were segregated by proportions of either undifferentiated or more mature tumor cells. While Group 3-MB contained considerably lower (up to 10% or completely absent in the case of *MYC*-amplified Group 3-MB) proportions of differentiated neuron-like cells, Group 4-MB almost exclusively consisted of more differentiated neuron-like cells that resembled unipolar brush cells (UBCs) and glutamatergic cerebellar nuclei (GluCN).[98] Together, this hints at an impaired differentiation capacity in Group 3-MB, potentially

correlating with the worse outcome of this subgroup. Interestingly, a subset of DNA methylation classified Group3/4 intermediate tumors did not show the above described strict dichotomy but was characterized by a mixture of undifferentiated and mature neuron-like tumor cell populations, accounting for the more complex molecular subtyping of Group 3 and Group 4-MB.[98] Of note, Vladoiu and colleagues described a resemblance between Group 3-MB bulk transcriptomes and a very early murine multipotent Nestin-positive cerebellar progenitor cell, which had been previously suggested as the Group 3-MB cell of origin.[103] Analyzing Group 3-MB single-cell transcriptomes, the authors detected divergent tumor cells resembling multiple developmental lineages including GCPs, UBCs, Purkinje cells and GABAergic interneurons, which were interpreted as hints toward a putative multipotent cell of origin.[97] This has not yet been supported by other MB scRNA-seq studies.[98,104] Deeper analyses including projections to now available human cerebellar reference atlases and functional validations are required to further resolve the origin of Group 3-MB. Remarkably, cross-subgroup comparisons of tumor cell populations revealed higher similarities between more mature neuron-like populations than between more divergent progenitor-like states.[98] This is in line with observations of higher similarity between more differentiated cell states across glioma types[53] and ependymoma subtypes[105] (see below), and may point towards stronger dysregulation of progenitor-like cells, or increased divergence of spatiotemporally-specific cells of origin manifesting themselves in the state of progenitor-like cells, or both. A recent scRNA-seq study further interrogated the immune cell landscape of all MB subgroups, identifying heterogeneous tumor-associated myeloid cell populations differentially associated with specific MB subgroups.[104]

Ependymoma

Ependymomas (EPNs) are classified based on their anatomic location, histology and molecular alterations.[49,106,107] Recent single-cell transcriptomic studies have further elucidated the cellular lineage compositions and developmental architectures across different EPN subtypes. Gojo et al.[105] profiled a total of 34 EPNs across posterior fossa (14 PF-A, 3 PF-B, 1PF-subependymoma/SE), supratentorial (5 ST-RELA, 1 ST-YAP) and spinal (2 SP-MPE) locations as well as pediatric and adult age groups using full-length and 3'scRNA-seq. In an independent study, Gillen et al.[108] sequenced a total of 26 childhood EPNs from posterior fossa (19 PF-A, 1 PF-B) and supratentorial (5 ST-RELA, 1 ST-YAP1, 2 ST-midline) locations. In PF tumors, Gojo et al. identified 3 immature and 2 more mature tumor cell transcriptional metaprograms. Proliferating cells were restricted to the immature metaprograms that comprised a Neural-Stem-Cell-like (PF-NSC-like), a Neuronal-Precursor-like (PF-NPC-like), and a Glial-Progenitor-like (PF-Glial-Progenitor-like) signature.[105] Conversely, more mature cells were not cycling and expressed either PF-Astroependymal-like or PF-Ependymal-like state, enriched for ciliogenesis markers. Additionally, the authors identified a PF-Immune-Reactive and PF-Metabolic program, enriched for glycolytic and hypoxia response pathways.[105] Inferring cell state trajectories by RNA velocity analysis supported the stem-like PF-NSC-like cell to be at the root of a trifurcate path towards (i) PF-Astroependymal and PF-Ependymal-like, (ii) PF-Glial-Progenitor-like, and (iii) PF-NPC-like tumor cell fates,[105] consistent with a previously described multipotent fetal NSC/radial glia cell of origin giving rise to a broad spectrum of neural lineages.[109,110] PF-A tumors are the most prevalent EPN subtype, occurring predominantly in infants and young children and, together with ST-RELA EPN, harbor the worst prognosis of all EPN subtypes, while PF-B and PF-SE tumors are associated with older patients and better prognoses. In line with this distinction, Gojo and colleagues observed that PF-A EPN harbored higher proportions of immature stem-like cell states accounting for their aggressiveness, whereas PF-B and PF-SE exclusively contained more differentiated tumor cells. Intriguingly, when analyzing these signatures in an extended cohort of 131 PF-EPN patient bulk transcriptomes, the authors showed that the stem-like PF-NPC-like signature was significantly linked to a worse clinical outcome, in contrast to the differentiated PF-Ependymal-like signature that marked significantly higher OS and PFS.[105] This prognostic stratification held true even within PF-A EPN patients only. Moreover, a comparison of matched primary-recurrence PF-A EPN pairs indicated that cycling and undifferentiated cell populations increase upon recurrence, further underscoring the clinical implications of these undifferentiated tumor cell programs in progression and therapy resistance.[105] In the independent study by Gillen et al.[108], undifferentiated tumor populations enriched for ventricular radial glia (vRG) and ventricular progenitor-specific genes as well as more differentiated ependymal-like populations were described, which were likewise associated with worse or more favorable clinical outcomes, respectively. Single-cell transcriptomes of four additional PF-A EPNs were analyzed in the study by Vladoiu and colleagues, in which comparison to a murine cerebellar single-cell atlas suggested similarities of undifferentiated EPN cells to gliogenic progenitors and roof plate-like stem cells.[97] In this study, differentiated cells were completely absent in PF-A tumors, which is in contrast to the other two studies by Gojo et al. and Gillen et al. that identified varying frequencies of more differentiated tumor cells in some PF-A EPNs,[105,108] suggestive of retained differentiation capacity in at least some PF-A tumor cells. Future comparisons to more comprehensive and highly resolved human brain single-cell atlases will enable us to further pinpoint an exact PF-A human progenitor cell identity. Of note, Gillen and colleagues further identified a mesenchymal-like tumor state (termed MECs) that, like the PF-Metabolic signature described by Gojo et al., was enriched for hypoxia-associated genes. Immunohistochemistry (IHC) analyses of cell state-specific marker genes indicated a co-localization of MES-like cells and stem-like tumor cells to necrotic and perivascular zones, hinting at a hypoxic cancer stem cell niche.[108] Notably, a hypoxic microenvironment has been illustrated to drive metabolic changes in PF-EPN stem-like cells resulting in epigenetic deregulation and oncogenesis.[111] This intriguing intersection of a specific cell state with microenvironmental influence was further underscored by recapitulating analogous hypoxia-associated transcriptional

signatures and metabolite composition in a transient tissue niche of embryonic murine development, restricted to a specific time window and region considered as the best murine match to the putative PF-A cell context of origin.[111]

Supratentorial EPNs also display variation regarding age and prognosis. Moreover, they segregate by driver gene-fusion events that are detected in more than 70% of ST-EPN[49] and characterized by a fusion between zinc finger translocation associated (*ZFTA*; formerly *C11orf95*) and a variety of fusion partners, some of them recently identified and validated for their respective tumorigenic capacities.[112–114] Analyzing *ZFTA-RELA*, *ZFTA-YAP1*, and fusion negative midline EPN, Gojo et al. identified two undifferentiated (ST-Radial-Glia-like and ST-Neuronal-Precursor-like), a differentiated ST-Ependymal-like, a metabolic as well as metaprograms specific to each ST-EPN subtype.[105] Again, a favorable prognosis was correlated with the more differentiated ST-Ependymal-like signature, both across all ST EPNs as well as within ST-RELA tumors.[85] In ST-RELA EPN, broad expression of *ZFTA-RELA* fusion gene targets was observed across all cell populations. In the study by Gillen et al., ST-RELA and ST-YAP EPNs were shown to constitute three main clusters, enriched for nervous system development, Wnt signaling, and oxidative stress response pathways, respectively.[108]

Gojo et al. also profiled spinal myxopapillary EPNs (SP-MPE) which are associated with a more favorable prognosis. Surprisingly and indicative of more aggressive tumor cell populations in this subtype, a SP-Progenitor-like population was detected in one tumor, alongside an SP-Ependymal-like, SP-Immune-Reactive and a tumor-specific (SP-variable) population,[105] consistent with the increase in grade (2) according to the new WHO2021 classification.

Last, Gojo and colleagues compared single tumor cell transcriptional programs between EPN subtypes and those identified in different glioma subtypes. While a set of EPN metaprograms were highly correlated with glioblastoma signatures, highlighting common glioneuronal phenotypes, three metaprograms emerged as specific to EPN: the Ependymal-like, ST-Radial-Glia-like and PF-NSC-like populations, indicating putatively distinct cells of origin.[105] Interestingly, in between EPN subtypes, progenitor-like cell states proved to be more different, whereas more differentiated cell states exhibited higher resemblance to each other, which reinforces the underlying notion that spatiotemporally distinct progenitors give rise to different EPN subtypes, but partially retain converging differentiation capacities. This, again, is in line with phenomena of divergent progenitor cell states and converging mature cell states observed in other brain tumor types.

Challenges and Future Directions

Single-cell profiling methods have revolutionized our understanding of brain tumors, unveiling the complex and dynamic heterogeneity of their tumor and microenvironmental cellular components (Table 1). Importantly, these findings reinforce the emerging concept that brain tumor cells are not only isolated dysregulated entities but constitute dynamic organ-like systems that partly mimic the normal brain and employ manifold modes to directly interact with and integrate into their native surroundings,[46,47,115,116] further compounding the necessity to approach these complex ecosystems from a systems biology perspective.

Towards this end, efforts that apply technological advances to primary patient tissues are needed on multiple fronts to tackle the key challenges arising. First, while we now possess high-resolution snapshots of the cellular composition across various brain tumors, we note that we have so far only glimpsed at the tip of the iceberg as samples often remain limited to small biopsies and/or single time points that may only capture a specific region within a tumor. Increased multiregional and longitudinal sampling of primary patient specimens wherever feasible, facilitated by advances in single-nucleus RNA-sequencing of frozen tissues and more recently single-cell sequencing of fixed RNA, is therefore expected to revise and extend our current models of tumor cell states and heterogeneity in the future. Crucially, this will further shed light on the somatic and functional evolution of tumor and microenvironmental cells, and how these respond to therapy and may be driving resistance.[117] Second, we so far lack sufficient understanding of how individual cellular components organize and interact within the tissue complex. Continuously evolving spatial techniques have been recently employed in glioblastoma[43,118] and will continue to become increasingly utilized to characterize tumor cell populations in their native brain environments. This will add to the mounting insights into interactions and niches formed between malignant cells and the diverse microenvironmental components at respective tumor core and infiltrative regions that together contribute to tumor growth and evolution.[117] Spatial approaches can be categorized into next-generation sequencing-based methods (e.g., high-definition spatial transcriptomics[119]; SLIDE-seq[120]), that allow for untargeted whole-transcriptome capture but not yet single-cell resolution, as well as targeted imaging-based approaches (e.g., in situ sequencing/HybISS[121]; seqFISH+[122]; MERFISH[123]; STARmap[124]) that rely on extensive marker gene panels to identify cell populations of interest at true single-cell resolution. Furthermore, spatial techniques extend to the proteomic level (e.g., multiplexed ion beam imaging/MIBI[125]; co-detection by indexing/CODEX[126]; tissue cyclic immunofluorescence/t-cyCIF[127]), allowing for multiplexed targeted (co-)detection and integration with transcriptomics methods to further enhance our capability to study complex brain tumor tissues (see also review by Rao et al.[128]). Finally, single-cell multi-omics approaches will be steered to systematically investigate and integrate additional molecular and functional layers of information into our understanding of individual cell populations, some of which appear to recurrently occur across different brain tumor types. These layers of information extend to insights into the cell populations' clonal dynamics, epigenetic determinants, proteomes, metabolism, and electrophysiology, many of which and in different combinations can already be concomitantly assessed from the same cell.[129–132] Notably, these multi-omics approaches will be of complementary nature and may overcome some of the shortcomings of measuring individual modalities, such as that RNA levels do not necessarily reflect protein levels, which simultaneous proteomic readouts can

Table 1 Select studies characterizing the biology of primary brain tumors via single-cell methods

References	Tumors profiled	Single-cell method	Key findings
Tirosh et al. [51]	Oligodendroglioma	scRNA-seq	Developmental hierarchical model of cellular states (NPC-like branching to AC-like, OC-like)
Venteicher et al. [52]	Astrocytoma, IDH-mutant	scRNA-seq	Developmental hierarchical model of cellular states (NPC-like branching to AC-like, OC-like)
Babikir et al.[59]	Oligodendroglioma, IDH-mutant and astrocytoma, IDH-mutant	ScATAC-seq & scRNA-seq in human tumors, scCUT&Tag for CTCF in engineered mouse glioma cells	ATRX loss linked to increase of open chromatin, decrease of CTCF binding, enhanced infiltration of immunosuppressive macrophages
Neftel et al. [30]	Glioblastoma	scRNA-seq	Four cellular state model with NPC-like, OPC-like, AC-like and MES-like cellular states; plasticity between cellular states
Garofano et al.[32]	Glioblastoma	scRNA-seq	Pathway classification of glioblastoma and discovery of a mitochondrial cellular state
Wang et al.[32]	Glioblastoma; IDH-mutant gliomas	scRNA-seq/ snRNA-seq/ scATAC-seq	Heterogeneity of glioblastoma stem cells (GSCs) with mesenchymal and neural progenitor transcriptional signatures
Friebel et al.[39]	Glioblastoma, IDH-mutant gliomas	scMass_ Cytometry	Characterization of the immune microenvironment of gliomas
Mathewson et al.[44]	Glioblastoma, IDH-wildtype; IDH-mut gliomas	scRNA-seq	Identification of expression of the NK cell immune inhibitor receptor CD161 in cytotoxic T cells, and recognition that the ligand of this receptor—CLEC2D—is expressed in tumor and immunosupressive myeloid cells
Chaligne et al.[35]	Glioblastoma, IDH-wildtype; IDH-mut gliomas	scDNAme-seq/ scRNA-seq	Persistent expression of oncogenic genes despite DNA methylation, as well as disruption of CTCF-mediated insulation, in IDH-mutant gliomas; hypomethylation of regulatory targets of the polycomb repressor complex 2 (PRC2) in glioblastoma stem-like cells
Johnson et al.[36]	Glioblastoma, IDH-wildtype; IDH-mut gliomas	scDNAme-seq/ scRNA-seq	Variability in DNA methylation profiles; randomness in the placement of epigenetic marks might underlie the ability of glioma cells to adapt to environmental stressors
Filbin et al.[85]	Diffuse midline glioma, H3 K27M-altered	scRNA-seq	Developmental hierarchical model of cellular states (OPC-like branching to AC-like, OC-like); tumor-initiating capacity of OPC-like cells in vivo compared to AC-like cells
Chen et al.[71]	Diffuse hemispheric glioma, H3 G34-mutant	ssGSEA of bulk transcriptomes, scRNA-seq	Projection of bulk transcriptomes to prenatal ventral telencephalon/ ganglionic eminence interneuronal lineage; hybrid neuron-like/ AC-like states, absence of oligodendroglial lineage
Reitman et al.[93]	Pilocytic astrocytoma	scRNA-seq	Developmental hierarchical model of cellular states (MAPK-signaling high cycling or senescent OPC-like cells, partly differentiating glia-like MAPK-signaling low cells)
Jessa et al.[86]	WNT medulloblastoma, ETMR, AT/RT, diffuse midline glioma, H3 K27M altered	scRNA-seq	Projection of different pediatric brain tumors onto scRNA-seq atlases of embryonal human and mouse brain
Hovestadt et al.[98]	Medulloblastoma, 4 groups: SHH, WNT, Group 3, Group 4	scRNA-seq	Subgroup-specific developmental hierarchical models from undifferentiated to mature neuron-like states
Vladoiu et al.[97]	Medulloblastoma, 3 groups: SHH, Group 3, Group 4	scRNA-seq	Subgroup-specific resemblance of tumor cells to neuronal lineages of thet developing cerebellum
Riemondy et al.[104]	Medulloblastoma, 4 groups: SHH, WNT, Group 3, Group 4	scRNA-seq	Neuronally differentiated subpopulation expressing photoreceptors; myeloid cell landscape associated with MB subgroups
Gojo et al.[105]	Ependymoma: posterior fossa, supratentorial, spinal	scRNA-seq	Trifurcate developmental hierarchical model of Neural-Stem-Cell-like branching to Ependymal-like, Glial-Progenitor-like, NPC-like; correlation of undifferentiated gene signatures with worse prognosis
Gillen et al.[108]	Ependymoma: posterior fossa, supratentorial	scRNA-seq	Developmental model of undifferentiated and differentiated populations associated with prognosis; mesenchymal tumor cell state enriched ofr hypoxia-associated genes

help account for. Furthermore, in addition to studying single genes and proteins, functional validations will require integrating emerging systems biology approaches such as massively parallel perturbations and readouts of genes and/or proteins including their combinatorial interactions (e.g., Perturb-seq/CRISP-seq/CROP-seq[133–136]) in representative in vitro and in vivo tumor models to measure up to the fast pace and high dimensionality of single-cell genomics data. Tackling these challenges will pave the way toward a holistic understanding of the functional systems constituted by different brain tumor types, and towards translating these insights into diagnostic, prognostic and therapeutic decision-making to advance the care of brain tumor patients.

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References

1. Touat M, Idbaih A, Sanson M, Ligon KL. Glioblastoma targeted therapy: updated approaches from recent biological insights. *Ann Oncol.* 2017; 28(7):1457–1472.
2. Gonzalez Castro LN, Tirosh I, Suva ML. Decoding cancer biology one cell at a time. *Cancer Discov.* 2021; 11(4):960–970.
3. Buenrostro JD, Wu B, Chang HY, Greenleaf WJ. ATAC-seq: a method for assaying chromatin accessibility genome-wide. *Curr Protoc Mol Biol.* 2015; 109:21.29.1–21.29.9.
4. Buenrostro JD, Wu B, Litzenburger UM, et al. Single-cell chromatin accessibility reveals principles of regulatory variation. *Nature.* 2015; 523(7561):486–490.
5. Fatemi M, Pao MM, Jeong S, et al. Footprinting of mammalian promoters: use of a CpG DNA methyltransferase revealing nucleosome positions at a single molecule level. *Nucleic Acids Res.* 2005; 33(20):e176.
6. Miranda TB, Jones PA. DNA methylation: the nuts and bolts of repression. *J Cell Physiol.* 2007; 213(2):384–390.
7. Guo H, Zhu P, Wu X, et al. Single-cell methylome landscapes of mouse embryonic stem cells and early embryos analyzed using reduced representation bisulfite sequencing. *Genome Res.* 2013; 23(12):2126–2135.
8. Guo H, Zhu P, Guo F, et al. Profiling DNA methylome landscapes of mammalian cells with single-cell reduced-representation bisulfite sequencing. *Nat Protoc.* 2015; 10(5):645–659.
9. Gaiti F, Chaligne R, Gu H, et al. Epigenetic evolution and lineage histories of chronic lymphocytic leukaemia. *Nature.* 2019; 569(7757):576–580.
10. Grosselin K, Durand A, Marsolier J, et al. High-throughput single-cell ChIP-seq identifies heterogeneity of chromatin states in breast cancer. *Nat Genet.* 2019; 51(6):1060–1066.
11. Wu SJ, Furlan SN, Mihalas AB, et al. Single-cell CUT&Tag analysis of chromatin modifications in differentiation and tumor progression. *Nat Biotechnol.* 2021; 39(7):819–824.
12. Navin N, Kendall J, Troge J, et al. Tumour evolution inferred by single-cell sequencing. *Nature.* 2011; 472(7341):90–94.
13. Zong C, Lu S, Chapman AR, Xie XS. Genome-wide detection of single-nucleotide and copy-number variations of a single human cell. *Science.* 2012; 338(6114):1622–1626.
14. Navin NE. The first five years of single-cell cancer genomics and beyond. *Genome Res.* 2015; 25(10):1499–1507.
15. Picelli S, Faridani OR, Bjorklund AK, et al. Full-length RNA-seq from single cells using Smart-seq2. *Nat Protoc.* 2014; 9(1):171–181.
16. Hagemann-Jensen M, Ziegenhain C, Chen P, et al. Single-cell RNA counting at allele and isoform resolution using Smart-seq3. *Nat Biotechnol.* 2020; 38(6):708–714.
17. Baran-Gale J, Chandra T, Kirschner K. Experimental design for single-cell RNA sequencing. *Brief Funct Genom.* 2018; 17(4):233–239.
18. Slyper M, Porter CBM, Ashenberg O, et al. A single-cell and single-nucleus RNA-Seq toolbox for fresh and frozen human tumors. *Nat Med.* 2020; 26(5):792–802.
19. Di Palma S, Bodenmiller B. Unraveling cell populations in tumors by single-cell mass cytometry. *Curr Opin Biotechnol.* 2015; 31:122–129.
20. Spitzer MH, Nolan GP. Mass cytometry: single cells, many features. *Cell.* 2016; 165(4):780–791.
21. Ma S, Zhang B, LaFave LM, et al. Chromatin potential identified by shared single-cell profiling of RNA and chromatin. *Cell.* 2020; 183(4):1103–1116. e1120.
22. Angermueller C, Clark SJ, Lee HJ, et al. Parallel single-cell sequencing links transcriptional and epigenetic heterogeneity. *Nat Methods.* 2016; 13(3):229–232.
23. Hu Y, Huang K, An Q, et al. Simultaneous profiling of transcriptome and DNA methylome from a single cell. *Genome Biol.* 2016; 17:88.
24. Macaulay IC, Haerty W, Kumar P, et al. G&T-seq: parallel sequencing of single-cell genomes and transcriptomes. *Nat Methods.* 2015; 12(6):519–522.
25. Macaulay IC, Teng MJ, Haerty W, et al. Separation and parallel sequencing of the genomes and transcriptomes of single cells using G&T-seq. *Nat Protoc.* 2016; 11(11):2081–2103.
26. Stoeckius M, Hafemeister C, Stephenson W, et al. Simultaneous epitope and transcriptome measurement in single cells. *Nat Methods.* 2017; 14(9):865–868.
27. Cancer Genome Atlas Research N. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature.* 2008; 455(7216):1061–1068.
28. Verhaak RG, Hoadley KA, Purdom E, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell.* 2010; 17(1):98–110.
29. Patel AP, Tirosh I, Trombetta JJ, et al. Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *Science.* 2014; 344(6190):1396–1401.
30. Neftel C, Laffy J, Filbin MG, et al. An integrative model of cellular states, plasticity, and genetics for glioblastoma. *Cell.* 2019; 178(4):835–849. e821.
31. Couturier CP, Ayyadhury S, Le PU, et al. Single-cell RNA-seq reveals that glioblastoma recapitulates a normal neurodevelopmental hierarchy. *Nat Commun.* 2020; 11(1):3406.
32. Garofano L, Migliozzi S, Oh YT, et al. Pathway-based classification of glioblastoma uncovers a mitochondrial subtype with therapeutic vulnerabilities. *Nat Cancer.* 2021; 2(2):141–156.
33. Richards LM, Whitley OKN, MacLeod G, et al. Gradient of developmental and injury response transcriptional states defines functional vulnerabilities underpinning glioblastoma heterogeneity. *Nat Cancer.* 2021; 2(2):157–173.

34. Wang L, Babikir H, Muller S, et al. The phenotypes of proliferating glioblastoma cells reside on a single axis of variation. *Cancer Discov.* 2019; 9(12):1708–1719.
35. Chaligne R, Gaiti F, Silverbush D, et al. Epigenetic encoding, heritability and plasticity of glioma transcriptional cell states. *Nat Genet.* 2021; 53(10):1469–1479.
36. Johnson KC, Anderson KJ, Courtois ET, et al. Single-cell multimodal glioma analyses identify epigenetic regulators of cellular plasticity and environmental stress response. *Nat Genet.* 2021; 53(10):1456–1468.
37. Guilhamon P, Chesnelong C, Kushida MM, et al. Single-cell chromatin accessibility profiling of glioblastoma identifies an invasive cancer stem cell population associated with lower survival. *Elife.* 2021; 10:e64090.
38. Klemm F, Maas RR, Bowman RL, et al. Interrogation of the microenvironmental landscape in brain tumors reveals disease-specific alterations of immune cells. *Cell.* 2020; 181(7):1643–1660. e1617.
39. Friebel E, Kapolou K, Unger S, et al. Single-cell mapping of human brain cancer reveals tumor-specific instruction of tissue-invading leukocytes. *Cell.* 2020; 181(7):1626–1642. e1620.
40. Darmanis S, Sloan SA, Croote D, et al. Single-cell RNA-seq analysis of infiltrating neoplastic cells at the migrating front of human glioblastoma. *Cell Rep.* 2017; 21(5):1399–1410.
41. Pombo Antunes AR, Scheyltjens I, Lodi F, et al. Single-cell profiling of myeloid cells in glioblastoma across species and disease stage reveals macrophage competition and specialization. *Nat Neurosci.* 2021; 24(4):595–610.
42. Muller S, Kohanbash G, Liu SJ, et al. Single-cell profiling of human gliomas reveals macrophage ontogeny as a basis for regional differences in macrophage activation in the tumor microenvironment. *Genome Biol.* 2017; 18(1):234.
43. Hara T, Chanoch-Myers R, Mathewson ND, et al. Interactions between cancer cells and immune cells drive transitions to mesenchymal-like states in glioblastoma. *Cancer Cell.* 2021; 39(6):779–792.e11 e711.
44. Mathewson ND, Ashenberg O, Tirosh I, et al. Inhibitory CD161 receptor identified in glioma-infiltrating T cells by single-cell analysis. *Cell.* 2021; 184(5):1281–1298. e1226.
45. Keskin DB, Anandappa AJ, Sun J, et al. Neoantigen vaccine generates intratumoral T cell responses in phase Ib glioblastoma trial. *Nature.* 2019; 565(7738):234–239.
46. Venkatesh HS, Morishita W, Geraghty AC, et al. Electrical and synaptic integration of glioma into neural circuits. *Nature.* 2019; 573(7775):539–545.
47. Venkataramani V, Tanev DI, Strahle C, et al. Glutamatergic synaptic input to glioma cells drives brain tumour progression. *Nature.* 2019; 573(7775):532–538.
48. Yan H, Parsons DW, Jin G, et al. IDH1 and IDH2 mutations in gliomas. *N Engl J Med.* 2009; 360(8):765–773.
49. Louis DN, Perry A, Wesseling P, et al. The 2021 WHO classification of tumors of the central nervous system: a summary. *Neuro Oncol.* 2021; 23(8):1231–1251.
50. Gritsch S, Batchelor TT, Gonzalez Castro LN. Diagnostic, therapeutic, and prognostic implications of the 2021 World Health Organization classification of tumors of the central nervous system. *Cancer.* 2022; 128(1):47–58.
51. Tirosh I, Venteicher AS, Hebert C, et al. Single-cell RNA-seq supports a developmental hierarchy in human oligodendroglioma. *Nature.* 2016; 539(7628):309–313.
52. Venteicher AS, Tirosh I, Hebert C, et al. Decoupling genetics, lineages, and microenvironment in IDH-mutant gliomas by single-cell RNA-seq. *Science.* 2017; 355(6332).
53. Suva ML, Tirosh I. The glioma stem cell model in the era of single-cell genomics. *Cancer Cell.* 2020; 37(5):630–636.
54. Xu W, Yang H, Liu Y, et al. Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. *Cancer Cell.* 2011; 19(1):17–30.
55. Turcan S, Rohle D, Goenka A, et al. IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype. *Nature.* 2012; 483(7390):479–483.
56. Hon GC, Song CX, Du T, et al. 5mC oxidation by Tet2 modulates enhancer activity and timing of transcriptome reprogramming during differentiation. *Mol Cell.* 2014; 56(2):286–297.
57. Lu F, Liu Y, Jiang L, Yamaguchi S, Zhang Y. Role of Tet proteins in enhancer activity and telomere elongation. *Genes Dev.* 2014; 28(19):2103–2119.
58. Ginno PA, Gaidatzis D, Feldmann A, et al. A genome-scale map of DNA methylation turnover identifies site-specific dependencies of DNMT and TET activity. *Nat Commun.* 2020; 11(1):2680.
59. Babikir H, Wang L, Shamardani K, et al. ATRX regulates glial identity and the tumor microenvironment in IDH-mutant glioma. *Genome Biol.* 2021; 22(1):311.
60. Platten M, Bunse L, Wick A, et al. A vaccine targeting mutant IDH1 in newly diagnosed glioma. *Nature.* 2021; 592(7854):463–468.
61. Filbin M, Monje M. Developmental origins and emerging therapeutic opportunities for childhood cancer. *Nat Med.* 2019; 25(3):367–376.
62. Cardona-Alberich A, Tourbez M, Pearce SF, Sibley CR. Elucidating the cellular dynamics of the brain with single-cell RNA sequencing. *RNA Biol.* 2021; 18(7):1063–1084.
63. Schwartzentruber J, Korshunov A, Liu XY, et al. Driver mutations in histone H3.3 and chromatin remodelling genes in paediatric glioblastoma. *Nature.* 2012; 482(7384):226–231.
64. Wu G, Broniscer A, McEachron TA, et al. Somatic histone H3 alterations in pediatric diffuse intrinsic pontine gliomas and non-brainstem glioblastomas. *Nat Genet.* 2012; 44(3):251–253.
65. Sturm D, Witt H, Hovestadt V, et al. Hotspot mutations in H3F3A and IDH1 define distinct epigenetic and biological subgroups of glioblastoma. *Cancer Cell.* 2012; 22(4):425–437.
66. Lewis PW, Muller MM, Koletsky MS, et al. Inhibition of PRC2 activity by a gain-of-function H3 mutation found in pediatric glioblastoma. *Science.* 2013; 340(6134):857–861.
67. Bender S, Tang Y, Lindroth AM, et al. Reduced H3K27me3 and DNA hypomethylation are major drivers of gene expression in K27M mutant pediatric high-grade gliomas. *Cancer Cell.* 2013; 24(5):660–672.
68. Piunti A, Hashizume R, Morgan MA, et al. Therapeutic targeting of polycomb and BET bromodomain proteins in diffuse intrinsic pontine gliomas. *Nat Med.* 2017; 23(4):493–500.
69. Jain SU, Rashoff AQ, Krabbenhoft SD, et al. H3 K27M and EZHIP impede H3K27-methylation spreading by inhibiting allosterically stimulated PRC2. *Mol Cell.* 2020; 80(4):726–735 e727.
70. Hubner JM, Muller T, Papageorgiou DN, et al. EZHIP/CXorf67 mimics K27M mutated oncohistones and functions as an intrinsic inhibitor of PRC2 function in aggressive posterior fossa ependymoma. *Neuro Oncol.* 2019; 21(7):878–889.
71. Chen CCL, Deshmukh S, Jessa S, et al. Histone H3.3G34-mutant interneuron progenitors Co-opt PDGFRA for gliomagenesis. *Cell.* 2020; 183(6):1617–1633 e1622.
72. Bressan RB, Southgate B, Ferguson KM, et al. Regional identity of human neural stem cells determines oncogenic responses to histone H3.3 mutants. *Cell Stem Cell.* 2021; 28(5):877–893 e879.
73. Funato K, Smith RC, Saito Y, Tabar V. Dissecting the impact of regional identity and the oncogenic role of human-specific NOTCH2NL in an hESC model of H3.3G34R-mutant glioma. *Cell Stem Cell.* 2021; 28(5):894–905. e7 e897.
74. Bjerke L, Mackay A, Nandhabalan M, et al. Histone H3.3. mutations drive pediatric glioblastoma through upregulation of MYCN. *Cancer Discov.* 2013; 3(5):512–519.

75. Louis DN, Perry A, Reifenberger G, et al. The 2016 World Health Organization classification of tumors of the central nervous system: a summary. *Acta Neuropathol.* 2016; 131(6):803–820.
76. Monje M, Mitra SS, Freret ME, et al. Hedgehog-responsive candidate cell of origin for diffuse intrinsic pontine glioma. *Proc Natl Acad Sci USA.* 2011; 108(11):4453–4458.
77. Funato K, Major T, Lewis PW, Allis CD, Tabar V. Use of human embryonic stem cells to model pediatric gliomas with H3.3K27M histone mutation. *Science.* 2014; 346(6216):1529–1533.
78. Cordero FJ, Huang Z, Grenier C, et al. Histone H3.3K27M represses p16 to accelerate gliomagenesis in a murine model of DIPG. *Mol Cancer Res.* 2017; 15(9):1243–1254.
79. Pathania M, De Jay N, Maestro N, et al. H3.3(K27M) cooperates with Trp53 loss and PDGFRA gain in mouse embryonic neural progenitor cells to induce invasive high-grade gliomas. *Cancer Cell.* 2017; 32(5):684–700 e689.
80. Larson JD, Kasper LH, Paugh BS, et al. Histone H3.3 K27M accelerates spontaneous brainstem glioma and drives restricted changes in bivalent gene expression. *Cancer Cell.* 2019; 35(1):140–155 e147.
81. Haag D, Mack N, Benites Goncalves da Silva P, et al. H3.3-K27M drives neural stem cell-specific gliomagenesis in a human iPSC-derived model. *Cancer Cell.* 2021; 39(3):407–422 e413.
82. Nagaraja S, Quezada MA, Gillespie SM, et al. Histone variant and cell context determine H3K27M reprogramming of the enhancer landscape and oncogenic state. *Mol Cell.* 2019; 76(6):965–980 e912.
83. Nagaraja S, Vitanza NA, Woo PJ, et al. Transcriptional dependencies in diffuse intrinsic pontine glioma. *Cancer Cell.* 2017; 31(5):635–652 e636.
84. Krug B, De Jay N, Harutyunyan AS, et al. Pervasive H3K27 acetylation leads to ERV expression and a therapeutic vulnerability in H3K27M gliomas. *Cancer Cell.* 2019; 36(3):338–339.
85. Filbin MG, Tirosh I, Hovestadt V, et al. Developmental and oncogenic programs in H3K27M gliomas dissected by single-cell RNA-seq. *Science.* 2018; 360(6386):331–335.
86. Jessa S, Blanchet-Cohen A, Krug B, et al. Stalled developmental programs at the root of pediatric brain tumors. *Nat Genet.* 2019; 51(12):1702–1713.
87. Gibson EM, Purger D, Mount CW, et al. Neuronal activity promotes oligodendrogenesis and adaptive myelination in the mammalian brain. *Science.* 2014; 344(6183):1252304.
88. Venkatesh HS, Johung TB, Caretti V, et al. Neuronal activity promotes glioma growth through neuroligin-3 secretion. *Cell.* 2015; 161(4):803–816.
89. Venkatesh HS, Tam LT, Woo PJ, et al. Targeting neuronal activity-regulated neuroligin-3 dependency in high-grade glioma. *Nature.* 2017; 549(7673):533–537.
90. Lin GL, Nagaraja S, Filbin MG, et al. Non-inflammatory tumor microenvironment of diffuse intrinsic pontine glioma. *Acta Neuropathol Commun.* 2018; 6(1):51.
91. Majzner RG, Ramakrishna S, Yeom KW, et al. GD2-CAR T cell therapy for H3K27M-mutated diffuse midline gliomas. *Nature.* 2022; 603(7903):934–941.
92. Mount CW, Majzner RG, Sundaresh S, et al. Potent antitumor efficacy of anti-GD2 CAR T cells in H3-K27M(+) diffuse midline gliomas. *Nat Med.* 2018; 24(5):572–579.
93. Reitman ZJ, Paolella BR, Bergthold G, et al. Mitogenic and progenitor gene programmes in single pilocytic astrocytoma cells. *Nat Commun.* 2019; 10(1):3731.
94. Taylor MD, Northcott PA, Korshunov A, et al. Molecular subgroups of medulloblastoma: the current consensus. *Acta Neuropathol.* 2012; 123(4):465–472.
95. Northcott PA, Robinson GW, Kratz CP, et al. Medulloblastoma. *Nat Rev Dis Primers.* 2019; 5(1):11.
96. Hovestadt V, Ayrault O, Swartling FJ, et al. Medulloblastomics revisited: biological and clinical insights from thousands of patients. *Nat Rev Cancer.* 2020; 20(1):42–56.
97. Vladoiu MC, El-Hamamy I, Donovan LK, et al. Childhood cerebellar tumours mirror conserved fetal transcriptional programs. *Nature.* 2019; 572(7767):67–73.
98. Hovestadt V, Smith KS, Bihannic L, et al. Resolving medulloblastoma cellular architecture by single-cell genomics. *Nature.* 2019; 572(7767):74–79.
99. Schuller U, Heine VM, Mao J, et al. Acquisition of granule neuron precursor identity is a critical determinant of progenitor cell competence to form Shh-induced medulloblastoma. *Cancer Cell.* 2008; 14(2):123–134.
100. Gibson P, Tong Y, Robinson G, et al. Subtypes of medulloblastoma have distinct developmental origins. *Nature.* 2010; 468(7327):1095–1099.
101. Oliver TG, Read TA, Kessler JD, et al. Loss of patched and disruption of granule cell development in a pre-neoplastic stage of medulloblastoma. *Development.* 2005; 132(10):2425–2439.
102. Ocasio J, Babcock B, Malawsky D, et al. scRNA-seq in medulloblastoma shows cellular heterogeneity and lineage expansion support resistance to SHH inhibitor therapy. *Nat Commun.* 2019; 10(1):5829.
103. Pei Y, Moore CE, Wang J, et al. An animal model of MYC-driven medulloblastoma. *Cancer Cell.* 2012; 21(2):155–167.
104. Riemondy KA, Venkataraman S, Willard N, et al. Neoplastic and immune single-cell transcriptomics define subgroup-specific intra-tumoral heterogeneity of childhood medulloblastoma. *Neuro Oncol.* 2022; 24(2):273–286.
105. Gojo J, Englinger B, Jiang L, et al. Single-cell RNA-Seq reveals cellular hierarchies and impaired developmental trajectories in pediatric ependymoma. *Cancer Cell.* 2020; 38(1):44–59.e9 e49.
106. Pajtler KW, Witt H, Sill M, et al. Molecular classification of ependymal tumors across all cns compartments, histopathological grades, and age groups. *Cancer Cell.* 2015; 27(5):728–743.
107. Pajtler KW, Mack SC, Ramaswamy V, et al. The current consensus on the clinical management of intracranial ependymoma and its distinct molecular variants. *Acta Neuropathol.* 2017; 133(1):5–12.
108. Gillen AE, Riemondy KA, Amani V, et al. Single-cell RNA sequencing of childhood ependymoma reveals neoplastic cell subpopulations that impact molecular classification and etiology. *Cell Rep.* 2020; 32(6):108023.
109. Johnson RA, Wright KD, Poppleton H, et al. Cross-species genomics matches driver mutations and cell compartments to model ependymoma. *Nature.* 2010; 466(7306):632–636.
110. Taylor MD, Poppleton H, Fuller C, et al. Radial glia cells are candidate stem cells of ependymoma. *Cancer Cell.* 2005; 8(4):323–335.
111. Michealraj KA, Kumar SA, Kim LJY, et al. Metabolic regulation of the epigenome drives lethal infantile ependymoma. *Cell.* 2020; 181(6):1329–1345 e1324.
112. Arabzade A, Zhao Y, Varadharajan S, et al. ZFTA-RELA dictates oncogenic transcriptional programs to drive aggressive supratentorial ependymoma. *Cancer Discov.* 2021; 11(9):2200–2215.
113. Kupp R, Ruff L, Terranova S, et al. ZFTA Translocations constitute ependymoma chromatin remodeling and transcription factors. *Cancer Discov.* 2021; 11(9):2216–2229.
114. Zheng T, Ghasemi DR, Okonechnikov K, et al. Cross-species genomics reveals oncogenic dependencies in ZFTA/C11orf95 fusion-positive supratentorial ependymomas. *Cancer Discov.* 2021; 11(9):2230–2247.
115. Venkataramani V, Schneider M, Giordano FA, et al. Disconnecting multicellular networks in brain tumours. *Nat Rev Cancer.* 2022; 22(8):481–491.
116. Osswald M, Jung E, Sahm F, et al. Brain tumour cells interconnect to a functional and resistant network. *Nature.* 2015; 528(7580):93–98.

117. Varn FS, Johnson KC, Martinek J, et al. Glioma progression is shaped by genetic evolution and microenvironment interactions. *Cell.* 2022; 185(12):2184–2199 e2116.
118. Ravi VM, Will P, Kueckelhaus J, et al. Spatially resolved multi-omics deciphers bidirectional tumor-host interdependence in glioblastoma. *Cancer Cell.* 2022; 40(6): 639–655. e613.
119. Vickovic S, Eraslan G, Salmen F, et al. High-definition spatial transcriptomics for in situ tissue profiling. *Nat Methods.* 2019; 16(10):987–990.
120. Zhao T, Chiang ZD, Morriss JW, et al. Spatial genomics enables multi-modal study of clonal heterogeneity in tissues. *Nature.* 2022; 601(7891):85–91.
121. Gyllborg D, Langseth CM, Qian X, et al. Hybridization-based in situ sequencing (HybISS) for spatially resolved transcriptomics in human and mouse brain tissue. *Nucleic Acids Res.* 2020; 48(19):e112.
122. Eng CL, Lawson M, Zhu Q, et al. Transcriptome-scale super-resolved imaging in tissues by RNA seqFISH. *Nature.* 2019; 568(7751):235–239.
123. Xia C, Fan J, Emanuel G, Hao J, Zhuang X. Spatial transcriptome profiling by MERFISH reveals subcellular RNA compartmentalization and cell cycle-dependent gene expression. *Proc Natl Acad Sci USA.* 2019; 116(39):19490–19499.
124. Wang X, Allen WE, Wright MA, et al. Three-dimensional intact-tissue sequencing of single-cell transcriptional states. *Science.* 2018; 361(6400).
125. Angelo M, Bendall SC, Finck R, et al. Multiplexed ion beam imaging of human breast tumors. *Nat Med.* 2014; 20(4):436–442.
126. Goltsev Y, Samusik N, Kennedy-Darling J, et al. Deep profiling of mouse splenic architecture with CODEX multiplexed imaging. *Cell.* 2018; 174(4):968–981 e915.
127. Lin JR, Izar B, Wang S, et al. Highly multiplexed immunofluorescence imaging of human tissues and tumors using t-CyCIF and conventional optical microscopes. *Elife.* 2018; 7:e31657.
128. Rao A, Barkley D, Franca GS, Yanai I. Exploring tissue architecture using spatial transcriptomics. *Nature.* 2021; 596(7871):211–220.
129. Mimitou EP, Lareau CA, Chen KY, et al. Scalable, multimodal profiling of chromatin accessibility, gene expression and protein levels in single cells. *Nat Biotechnol.* 2021; 39(10):1246–1258.
130. Lareau CA, Ludwig LS, Muus C, et al. Massively parallel single-cell mitochondrial DNA genotyping and chromatin profiling. *Nat Biotechnol.* 2021; 39(4):451–461.
131. Ludwig LS, Lareau CA, Ulirsch JC, et al. Lineage tracing in humans enabled by mitochondrial mutations and single-cell genomics. *Cell.* 2019; 176(6):1325–1339 e1322.
132. Cadwell CR, Palasantza A, Jiang X, et al. Electrophysiological, transcriptomic and morphologic profiling of single neurons using Patch-seq. *Nat Biotechnol.* 2016; 34(2):199–203.
133. Dixit A, Parnas O, Li B, et al. Perturb-seq: dissecting molecular circuits with scalable single-cell RNA profiling of pooled genetic screens. *Cell.* 2016; 167(7):1853–1866 e1817.
134. Adamson B, Norman TM, Jost M, et al. A multiplexed single-cell CRISPR screening platform enables systematic dissection of the unfolded protein response. *Cell.* 2016; 167(7):1867–1882 e1821.
135. Jaitin DA, Weiner A, Yofe I, et al. Dissecting immune circuits by linking CRISPR-pooled screens with single-cell RNA-seq. *Cell.* 2016; 167(7):1883–1896 e1815.
136. Datlinger P, Rendeiro AF, Schmidl C, et al. Pooled CRISPR screening with single-cell transcriptome readout. *Nat Methods.* 2017; 14(3):297–301.