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Invasion and Cellular Plasticity in Glioblastoma

From Regulators to Functional Models

REBECKA STOCKGARD

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Abstract

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The adult brain tumor glioblastoma is characterized by extensive heterogeneity and invasion, with intertumoral and intratumoral variation. This heterogeneity is partly driven by cellular plasticity, which permits glioblastoma cells to transition between cellular states. The plastic cells invade the brain, commonly via perivascular spaces, and diffuse invasion into the white matter, termed invasion routes. In this thesis, we investigate whether cellular states influence invasion routes using patient-derived models and develop new tools for real-time monitoring of glioblastoma across models.

In Paper I, we connect cellular states to invasion routes by characterizing six patient-derived cell culture and xenograft models using single-cell RNA profiling and spatial proteomics. We connect bulk-forming capacity and perivascular invasion to MES-like and OPC-like cells, driven by *ANXA1*. NPC-like and AC-like cells, we link to diffuse invasion, driven by *HOPX* and *RFX4*. Perturbation of these genes shifts cellular state composition and invasion routes, suggesting that cellular state shapes invasion.

To directly monitor cellular states, paper II introduces the CRISPR-tag, which we use to fluorescently label genes representative of cellular states in patient-derived cells. *In vitro*, we observe differences and oscillations in protein levels. *Ex vivo*, we monitor CRISPR-tagged cells and detect spatially-dependent expression of cellular-state markers. During differentiation treatment, *SOX2* expression remains high outside the tumor core, whereas it is lost in the central tumor regions. *ANXA1*-expressing cells display higher expression closer to a vessel. We performed a whole-genome knock-out screen to identify genetic dependencies that increase or decrease *ANXA1* expression and identified several candidate genes that regulate *ANXA1* expression.

Finally, in Paper III, we characterize eleven patient-derived cell cultures in a zebrafish model and monitor tumor initiation and growth using AI. The freely swimming fish are automatically imaged every four to six hours and display heterogeneity in growth and survival. Further characterization using light-sheet imaging revealed intratumoral variability in bulk-forming ability, tumor spread, and the presence of cells near vessels.

Together, our findings suggest an association between cellular states and invasion routes and introduce new tools to monitor cellular plasticity and tumor growth.

Keywords: Glioblastoma, Cellular states, Plasticity, Invasion, Genome engineering

Rebecka Stockgard, Department of Immunology, Genetics and Pathology, Neurooncology and neurodegeneration, Dag Hammarskjölds väg 20, Uppsala University, SE-751 85 Uppsala, Sweden.

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It is our choices that show what we truly are, far more than our abilities.

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Doroszko M*, **Stockgard R***, Uppman I, Heinold J, Voukelatou F, Bhagavanbhai Mangukiya H, Millner T, Skeppås M, Ballester Bravo M, Elgendy R, Berglund M, Elfineh L, Krona C, Kundu S, Koltowska K, Marino S, Larsson I, Nelander S. (2025) The invasion phenotypes of glioblastoma depend on plastic and reprogrammable cell states. *Nature Communications,* 2025 Jul 19;16:6662

II. **Stockgard R***, Heinold J*, Skeppås M, Nordquist C, Nyberg E, Kruse F, Kleindienst S, Uppman I, Bhagavanbhai Mangukiya H, Nelander S. CRISPR-tag enables real-time mapping and perturbation of cell-state plasticity in glioblastoma. Manuscript.

III. Almstedt E*, Rosén E*, Gloger M, **Stockgard R,** Hekmati N, Koltowska K, Krona C, Nelander S. Real-time evaluation of glioblastoma growth in patient-specific zebrafish xenografts. *Neuro oncology*, 2022 May 4;24(5):726-738.

* Indicates equal contribution.

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Related work by the author

I. Rosén E, Mangukiya HB, Elfineh L, **Stockgard R**, Krona C, Gerlee P, Nelander S. Inference of glioblastoma migration and proliferation rates using single time-point images. *Commun Biol.* 2023 Apr 13;6(1):402.

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Abbreviations

3R	Replacement, reduction, and refinement
AC	Astrocyte
AI	Artificial Intelligence
ANXA1	Annexin A1
AQP1	Aquaporin 1
AQP4	Aquaporin 4
ARF	p14ARF - ARF tumor suppressor
BBB	Blood-brain barrier
bFGF	Basic fibroblast growth factor
BMP4	Bone morphogenetic protein 4
Cas	CRISPR-associated
CD44	CD44 molecule
CDK4	Cyclin-dependent kinase 4
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CHI3L1	Chitinase 3-like 1
Chr	Chromosome
CL	Classical
CNN	Convolutional neural network
COL1A1	Collagen type I alpha 1 chain
CRISPR	Clustered regularly interspaced short palindromic repeats
CSF	Cerebrospinal fluid
DCX	Doublecortin
DLX5-AS1	Distal-less homeobox 5 antisense RNA 1
DLX6-AS1	Distal-less homeobox 6 antisense RNA 1
DSB	Double-strand break
E2F	E2F transcription factors
E-cadherin	Epithelial cadherin
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
FACS	Fluorescence-activated cell sorting
GB	Glioblastoma
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GPC	Glial progenitor cell
GRN	Gene regulatory network
gRNA	guide RNA
GSC	Glioblastoma stem cell
HDAC2	Histone deacetylase 2

HDR	Homology-directed repair
HGCC	Human glioblastoma cell culture
HOPX	Homeodomain-only protein X
IDH	Isocitrate dehydrogenase
IRF2BP2	Interferon regulatory factor 2 binding protein 2
IRF2BPL	Interferon Regulatory Factor 2 Binding Protein Like
KO	Knock-out
MAP4K4	Mitogen-activated protein kinase kinase kinase kinase 4
MDM2	Murine Double Minute 2
MES	Mesenchymal
MET	MET proto-oncogene, receptor tyrosine kinase
METTL17	Methyltransferase-like 17
MGMT	O6-methylguanine-DNA methyltransferase
MMP	Matrix metalloproteinase
N-cadherin	Neural cadherin
NES	Nestin
NEU	Neuronal
NF1	Neurofibromin 1
NF2	Neurofibromin 2
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NHEJ	Non-homologous end joining
NK	Natural killer
NOD-SCID	Non-obese diabetic severe combined immunodeficiency
NPC	Neural progenitor cell
NSG	NOD-SCID Gamma
OLIG1	Oligodendrocyte transcription factor 1
OLIG2	Oligodendrocyte transcription factor 2
OMG	Oligodendrocyte myelin glycoprotein
OPC	Oligodendrocyte progenitor cell
PDC	Patient-derived cell
PDGFRA	Platelet-derived growth factor receptor alpha
PDOX	Patient-derived orthotopic xenograft
PDX	Patient-derived xenograft
PI3K	Phosphoinositide 3-kinase
PN	Proneural
PS	Perineuronal satellitosis
PTEN	Phosphatase and tensin homolog
PV	Perivascular
RAS	Rat sarcoma
RB	Retinoblastoma 1
RFX4	Regulatory factor X4
RNP	Ribonucleoprotein
RTK	Receptor tyrosine kinase
S100B	S100 calcium-binding Protein B
scRNA-seq	single-cell RNA-sequencing
SMAD3	SMAD family member 3
SOX2	Sex-determining Region Y-box transcription factor 2
STMN1	Stathmin 1
STMN2	Stathmin 2

STMN4	Stathmin 4
TERT	Telomerase reverse transcriptase
TF	Transcription factor
TGF-β	Transforming growth factor beta
TMZ	Temozolomide
TNF-α	Tumor necrosis factor alpha
TP53	Tumor protein p53
VGLL4	Vestigial-like family member 4
VIM	Vimentin
WM	White matter
wt	wild-type
zPDX	zebrafish patient-derived xenograft

Introduction

An adult human consists of roughly 3-4 $\times$ 10^{13} cells. Long before we were anything else, we were only one cell - a zygote - formed from an egg and a sperm. This cell contains all the necessary information to form a human being.

The early embryo contains pluripotent stem cells that can become, differentiate into, any cell in our body. These cells follow well-defined pathways, responding to both internal and environmental signals that control gene expression and guide their migration and cellular fate. Although all cells contain the same genome, they can express genes to varying degrees, thereby adopting different phenotypes. As a result, cells acquire new features, migrate to reach their positions, and ultimately, differentiate into specialized cells, such as skin, nerve, or immune cells, and organize into tissues that form organs. There is a balance in our bodies between cell growth, differentiation, migration, and cell death that maintains tissue homeostasis. This allows new cells to replace old or damaged cells or tissue. However, this balance can be disrupted.

Cells acquire mutations or epigenetic changes over time through stochastic events, which may be influenced by environmental exposure. This can lead to a cell that divides uncontrollably, evades cell death, and progresses to a cancer cell. Importantly, cancer does not necessarily invent new mechanisms. It activates properties stemming, for instance, from normal development or wound healing. One such property is *cellular plasticity*, the capacity of cells to change phenotype without changing their genotype. Thereby, enabling it to adapt to changing environments despite no new genetic mutations. This trait can promote migratory behavior and *invasion* into surrounding tissue.

This thesis focuses on how cellular plasticity shapes invasion in the brain cancer glioblastoma (GB) and on how targeting underlying gene regulations, combined with new tools for real-time cell monitoring, can help understand and potentially target plasticity-driven invasion.

Cancer

Solid tumors are growths of cells classified as either benign or malignant. A benign tumor comprises cells closely resembling normal cells, growing slowly over an extended period of time, and does not spread beyond its own boundaries. Benign tumors can grow to a very large size and pose a danger by exerting pressure on surrounding organs. A malignant tumor comprises malignant cancer cells. Cancer is characterized by uncontrolled proliferation and invasion of malignant cells into adjacent normal tissue. However, cancer is not a single disease but a diverse array of diseases that can arise in any cell. For instance, carcinomas arise from epithelial cells, whereas blastomas arise from immature precursor cells [1].

The severity of the cancer increases with greater cell abnormality and the degree of spread, and is categorized into stages and grades. The stage defines the location and dissemination of the cancer in the body, while the grade describes the phenotype of the cancer cells [1-3]. In Sweden, the incidence rate is around 700 per 100,000 persons, where prostate, breast, and skin cancer are the most common diagnoses [4]. If possible, solid tumors are removed by surgery. Furthermore, cancer patients are often treated with chemotherapy and radiation, or with other therapies such as immuno-, hormone-, or anti-angiogenic therapy. These can be used alone or in combination [5, 6]. Early detection is of high importance for improved outcomes [7, 8].

The Hallmarks of Cancer

As a cell changes from a normal to a malignant cell, it acquires features common to cancer. These were collectively described as the Hallmarks of Cancer in 2000, expanded in 2011 and 2022, and include fundamental hallmarks, enabling characteristics, and emerging hallmarks (*Figure 1*) [7-9].

A normal cell relies on highly regulated mechanisms controlling cell proliferation, differentiation, movement, and survival to sustain tissue organization and homeostasis [10]. Cancer cells acquire traits that affect normal cellular regulatory mechanisms, such as those controlling proliferation and cell death, by circumventing suppressive growth signals, thereby gaining a

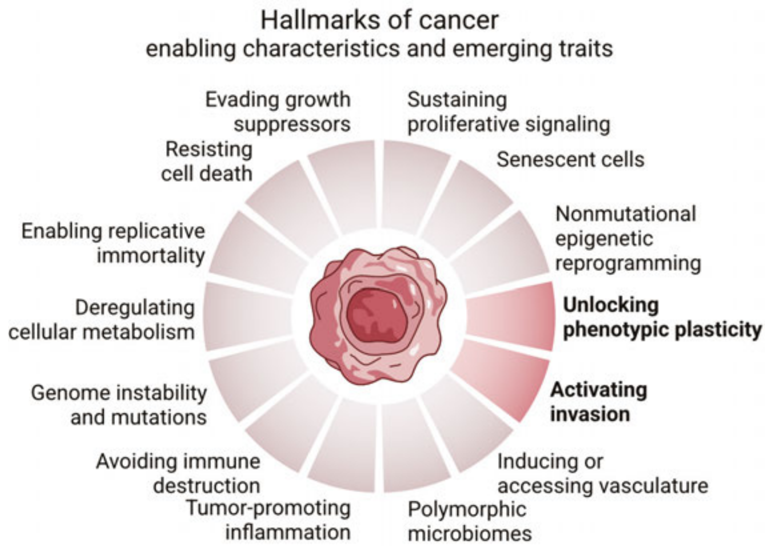

Figure 1. The Hallmarks of Cancer, enabling characteristics and emerging traits. Adapted from [9].

growth and survival advantage. Cancer activates genes that elongate telomeres to tolerate many cell divisions. Genomic instability and mutations enable these hallmarks and accelerate malignancy. Cancer cells induce new blood vessels, angiogenesis, and alter their metabolism to meet elevated nutrient and oxygen demands [11, 12]. Cancer cells evade the immune system, promote tumor-associated inflammation, and remodel their microenvironment to support continued growth [7-9]. Cancer cells can also undergo senescence-like cell-cycle arrest in response to therapy, but later resume cell division, a process associated with treatment resistance [13]. Epigenetic reprogramming is a non-mutational alteration of a cell's gene expression and chromatin structure, conferring dynamic changes and promoting phenotypic changes [8]. Additionally, the microbiome can impact cancer progression. Lastly, an emerging trait and a hallmark of cancer, particularly relevant in Glioblastoma (GB) are phenotypic plasticity and invasion.

Phenotypic plasticity

Phenotypic plasticity refers to a cell's ability to alter its transcriptional or functional state and transition between *cellular states*. This can occur in

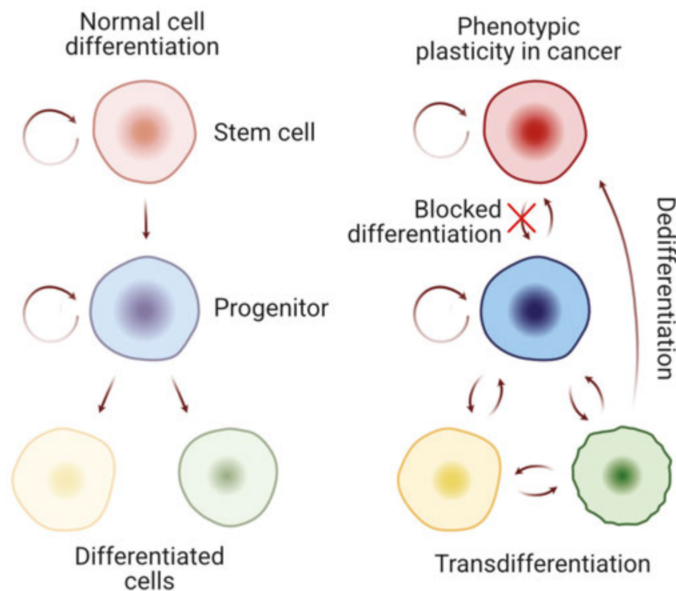

Figure 2. Examples of phenotypic plasticity enabled in cancer. Adapted from [9].

response to intrinsic or extrinsic cues, importantly, without the need for new genetic alterations [14, 15]. While cellular states can be distinguished from one another, they are increasingly recognized to exist along a continuum. The continuum allows for intermediate and transitional states rather than placing cells in discrete categories [15, 16].

In normal development, the differentiation of a stem cell into a specialized cell is highly regulated and progresses in a one-directional manner [17]. In contrast, cancer cells can regain progenitor-like features, or those of alternative lineages, through reversible cellular state transitions [9, 18]. For example, cancer cells may dedifferentiate and acquire stem-like properties [19], remain in a progenitor-like state due to blocked differentiation [20], or switch lineage through transdifferentiation (*Figure 2*) [21]. Phenotypic plasticity has been demonstrated by lineage-tracing studies, in which individual cancer cells can repopulate alternative cellular states over time [22, 23].

Cellular state transitions are one contributing factor to intratumoral heterogeneity, the coexistence of multiple cellular states within a single tumor. This ability of cancer cells is central when they are exposed to an altered microenvironment, such as hypoxia or therapy. It allows cancer cells to alter their phenotype to promote invasion or survival, or to repopulate cellular states lost after treatment [16, 24]. This process is associated with poor clinical outcomes

[25, 26]. Targeting cellular state transitions has therefore been proposed as a therapeutic strategy [27].

Cancer cell migration and invasion

The capacity of malignant cells to migrate from the primary tumor site causes invasion into surrounding tissue and, for many cancers, metastatic progression, leading to organ disruption and therapeutic failure [8].

Invasion of solid tumors involves loss of cell-to-cell adhesion and detachment from the tumor site. In carcinomas, this involves downregulation of E-cadherin, an epithelial adhesion protein [28, 29]. As well as upregulation of N-cadherin [28, 30], an adhesion protein important for migratory neurons and mesenchymal cells [31-33]. These changes facilitate detachment and migration [29, 30, 33, 34].

Further, cancer cells can alter their surroundings to facilitate their invasion [35-39]. The extracellular matrix (ECM) provides both a scaffold and a restriction for invasion. Cancer cells can remodel the cytoskeleton and differentially express adhesion proteins, such as integrins, that bind to the ECM [39-41]. Furthermore, they can express metalloproteinases (MMPs) to degrade the ECM [40, 42] and thereby remove the physical boundary that it constitutes.
Invasion is often described in two forms: amoeboid and mesenchymal *(Figure 3*). The amoeboid mode of invasion is characterized by cells moving as rounded cells, independent of adhesion proteins and MMPs [43]. In contrast, cells that invade in a mesenchymal mode are elongated, use integrins and MMP-dependent ECM interactions [44]. Transitions between these modes have been reported, enabled by perturbations of MMPs or Wnt signaling [45, 46] or by microenvironmental alterations, such as differential ECM composition or hypoxia [47, 48].

Invasion can occur as either a collective or as single cells. Collective invasion can occur in sheets, clusters, or strands, often guided by a leading cell. It allows cells to share forces from the surrounding microenvironment while maintaining cell-to-cell communication, which has been linked to increased migration and metastatic capacity [33, 50-52]. For example, cell-to-cell connections via tumor microtubes confer glioma cells resistance to radiotherapy [53].

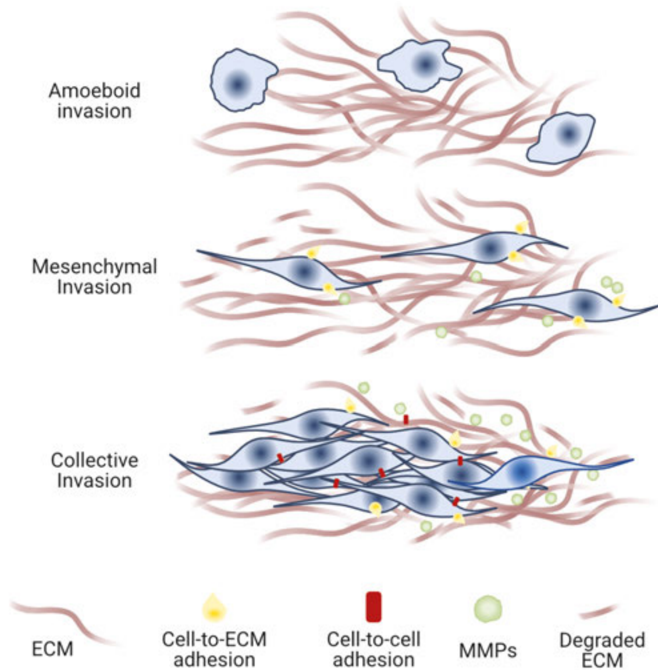

Figure 3. Modes of invasion exploited by cancer cells. First, single-cell amoeboid invasion. Second, single-cell mesenchymal invasion, dependent on cell-to-ECM adhesion and degradation of ECM by MMPs. Lastly, collective mesenchymal invasion depends on both MMP-mediated ECM degradation, cell-to-ECM, and cell-to-cell adhesion. Adapted from [49].

Processes such as epithelial-to-mesenchymal transitions (EMT), where cells transdifferentiate from epithelial-like to mesenchymal-like cells, exemplify both phenotypic plasticity and invasion [24, 26, 54-56]. Epithelial-like cells exhibit strong cell-to-cell adhesion. During EMT, cell-to-cell adhesion loosens, mesenchymal-like properties are upregulated, allowing cancer cells to detach from the collective [30, 34, 48, 57], thereby acquiring invasive potential.

Phenotypic plasticity and invasion are interrelated processes that facilitate cancer progression. Transitions between cellular states can alter invasion modes in response to microenvironmental cues and contribute to therapy resistance.

Glioblastoma

Nearly half of all diagnosed primary malignant brain tumors are GBs. It is highly aggressive, characterized by heterogeneity and extensive invasion of malignant cells into normal brain tissue [58].

Clinical features and therapeutic barriers

GB is an isocitrate dehydrogenase (IDH)-wild type (wt), grade 4 astrocytoma, defined by either microvascular proliferation, necrosis, copy number changes in chromosomes (chr) +7/-10, amplification of *EGFR* located on chr 7, or mutation of the *TERT* promoter, located on chr 5 [59].

Patients with GB may present with neurological symptoms such as headaches, seizures, visual dysfunctions, and changes in behavior [60]. GB is predominantly found in the cerebral hemispheres and is less common in the brainstem, cerebellum, or spinal cord [61]. The median age at diagnosis is around 65 years [58], and the incidence rate is around 3 cases per 100,000 individuals [62, 63]. GB is more common in males, with an incidence rate around 1.6 times higher than in females, and almost twice as common in white individuals as in black individuals. Previous treatment with radiation is a risk factor for developing high-grade gliomas [64, 65]. Methylation of the DNA repair enzyme O6-methylguanine DNA methyltransferase (MGMT) is a prognostic marker in GB. Reduced MGMT expression predicts improved response to alkylating agent temozolomide (TMZ) and extended overall survival [66].

Despite advances in diagnostics and treatment therapies for cancer [67], the survival prognosis is very poor for GB patients. The median survival is approximately 15 months, and only 7% survive for 5 years after diagnosis [58, 68]. The current standard of care includes surgical resection of the tumor, radiation, and chemotherapy with TMZ, sometimes with the addition of tumor-treating fields [69, 70]. GB typically recurs after approximately 7 months [69].

Several challenges limit therapeutic success. Firstly, GB is an immune-cold tumor, thus characterized by low T cell infiltration [74] and a microenvironment supported by immunosuppressive microglia [71, 72]. This limits immunotherapies. Secondly, drug delivery is compromised by the blood-brain

barrier (BBB), which constrains the passage of small molecules and immune cells. Additionally, ATP-binding cassette transporter proteins remove substances from the brain, hindering the delivery of an effective drug dose to the tumor site. Although the tumor core may exhibit increased BBB leakiness, allowing some therapies to reach the tumor bulk [73, 74], invasive cells can be found within normal brain tissue in regions with a normal BBB. Thirdly, surgical resection and radiotherapy are spatially controlled to protect the surrounding brain tissue, leaving invasive GB cells behind. These invasive cells represent the main therapeutic challenge, leading to recurrence within 2-4 cm of the primary tumor [75]. Molecular, cellular, and spatial heterogeneity of GB further constitutes therapeutic challenges.

Molecular heterogeneity

One of the most prominent features of GB is its heterogeneity, which is first evident at an intertumoral level. The heterogeneity is structured around recurrent alterations in three pathways associated with tumor suppressors p53 and RB, and receptor tyrosine kinases (RTKs). Alterations in all three pathways are present in about 74% of GB patients [76], leading to aberrant control of the cell cycle, suppression of apoptosis, and promotion of growth (Figure 4).

The RTK/RAS/PI3K pathway is altered in 88% of GB patients and is involved in cell survival and growth, with *PTEN* and *NF1* acting as negative regulators. Amplifications or mutations of the RTKs *EGFR* and *PDGFRA* are common in GB, as are *PTEN* loss or abnormalities, leading to elevated PI3K signaling and increased proliferation [76]. The p53-ARF-MDM2 pathway is altered in 87% of all GB patients. In response to stress or damage, ARF prevents MDM2 from binding to p53, leading to p53 accumulation and activation of its tumor-suppressing functions, such as cell cycle (G2/M) arrest and apoptosis. *TP53* loss or pathway alterations can lead to uncontrolled cell division, inhibition of apoptosis, and genomic instability, in which cells with damaged DNA can continue to proliferate [76]. *RB1* acts as a negative regulator of cell cycle (G1/S) progression. Rb signaling is disrupted in 78% of GB patients [76]. For instance, RB binds to E2F, thereby suppressing transcription of cell-cycle regulatory genes. Phosphorylation or mutations of RB release E2F and promote cell cycle (G1/S) progression [77].

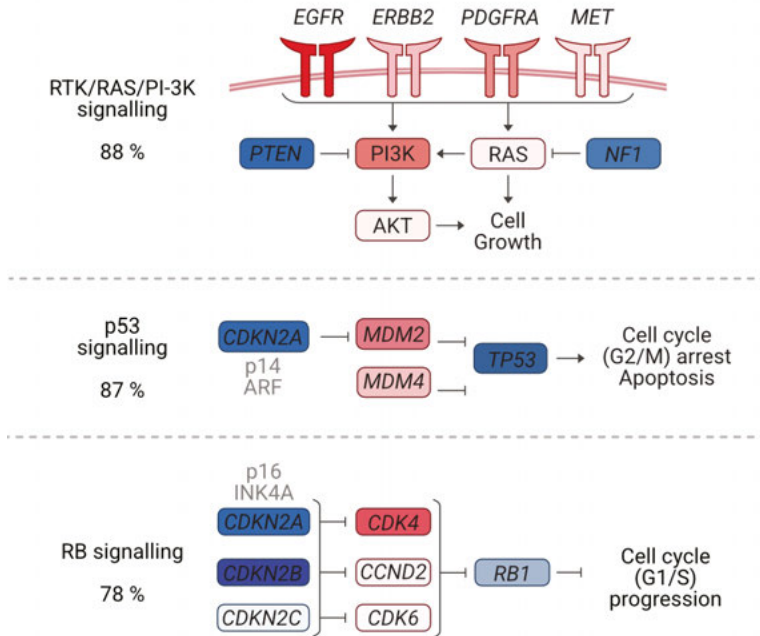

Figure 4. Recurrent genetic alterations in GB. Shades of red denote activating genetic alterations, whereas shades of blue denote inactivating alterations. Adapted from [76].

Trying to impose structure on GB heterogeneity, several efforts have been dedicated to classifying GB. Over the years, the number of defined subtypes and the exact scope of each have changed. The classifications are based on recurrent alterations, common somatic mutations, copy-number aberrations, and RNA expression [78-80].

At the intertumoral level, patients are stratified into three subtypes: Proneural (PN), Classical (CL), and Mesenchymal (MES) [79, 81]. The PN subtype is characterized by high *PDGFRA* and *OLIG2* expression, oligodendrocyte development markers, and p53 mutations. The CL subtype is defined by a gain of chr 7 and a loss of chr 10. Consequently, it commonly includes amplification of *EGFR* located on chr 7. Other genetic aberrations associated with the CL subtype include the absence of *TP53* mutations, *CDKN2A* deletion, elevated *NES* expression, and increased Notch and Sonic Hedgehog signaling [79]. *RB1* mutations are rarely observed together with *CDKN2A* deletion [76]. The MES subtype often harbors *NF1* abnormalities and is associated with mutations of *PTEN*, high expression of *MET*, *CHI3L1*, and *CD44,* as well as elevated NF-κB pathway activity [79, 81].

The subtype classification provides a common language for comparing tumors across patients but offers limited clinical utility [80], whereas age at diagnosis and the extent of surgical resection are more important [68, 82]. The assigned subtype may reflect the dominant subtype of the resected patient material, but may differ depending on the tumor region from which the tissue was resected. Additionally, distinct clonal genetic events occurring in different regions of the tumor can drive region-specific subtypes, contributing to intratumoral heterogeneity [83-85]. For instance, *NF1* and *EGFR* aberrations appear to be exclusive at the intertumoral level [79, 81], but are also evident intratumorally [85]. Furthermore, different RTK alterations, including *EGFR, PDGFRA,* and *MET*, co-occurred within the same tumor but in spatially distinct regions [86]. Multiregional sampling of a GB tumor revealed the coexistence of mutually exclusive events across different cell subpopulations. A sample at the tumor edge harbored *EGFR* amplification, whereas a sample from the same tumor, closer to the tumor core, lacked it, suggesting intratumoral clonal expansion driving invasion [87]. Another study that focused on epigenetic alterations identified distinct methylation patterns across different tumor regions associated with subgroups of expressed RTKs [88].

Intratumoral heterogeneity is not only spatial but also extends temporally. For instance, the mutual exclusivity of genetic events has been found to be reduced upon recurrence [96], along with changes in GB subtype during tumor progression [79, 81, 89]. Consistent with this subtype plasticity, studies by Schmitt et al. and Bhat et al. demonstrated that a MES subtype can be induced from a PN subtype by TNF-α exposure, a shift driven by NF-κB upregulation [90, 91].

Profiling of single-cell RNA-sequencing (scRNA-seq) data from GB patient samples demonstrated that subtype classification constitutes a discrete model of a continuous spectrum and inadequately describes the intratumoral complexity observed at the single-cell level.

Cellular states and plasticity

scRNA-seq studies have demonstrated GB intratumoral heterogeneity, with individual patients harboring heterogeneous cell populations that simultaneously adopt multiple transcriptional programs [81, 92]. These transcriptional programs, often referred to as cellular states, follow established neurodevelopmental lineages while also adopting a reactive and injury-response

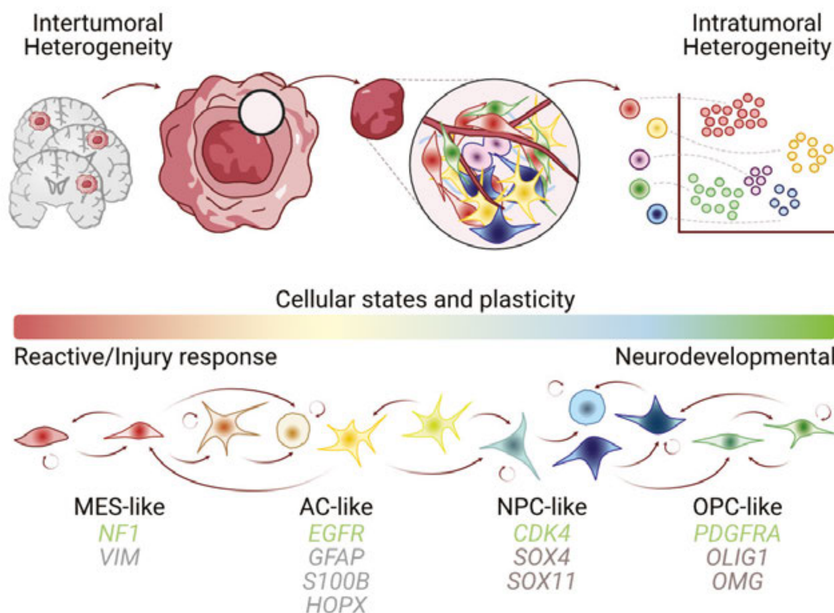

Figure 5. Intertumoral and intratumoral heterogeneity, and cellular state plasticity in GB. Associated alterations in green and representative markers in gray are indicated below each state. Arrows represent transitions between cellular states. Adapted from [93, 94].

transcriptional profile and metabolic states, which further contribute to intratumoral heterogeneity (Figure 5) [95-98].

Neftel et al. published a study in 2019 profiling the single-cell transcriptional landscape of GB, describing four malignant cellular states. These were neural progenitor-like (NPC-like), oligodendrocyte progenitor-like (OPC-like), astrocyte-like (AC-like), and mesenchymal-like (MES-like) cells [94].

NPC-like cells are characterized by *CDK4* amplification, suggesting this as a genetic driver, and can be further identified by increased expression of NPC-specific markers (*SOX4, SOX11,* and *DCX*). NPC-like cells can be further classified into two subgroups, one primed for OPC and one primed for neuronal programs, with elevated expression of *OLIG1* versus *STMN*1, *STMN2*, and *STMN4* and *DLX5-AS1* and *DLX6-AS1* [94].

OPC-like cells are characterized by *PDGFRA* amplification and identified by markers such as *OLIG1* and *OMG*, reminiscent of the oligodendroglia lineage. Notably, NPC-like and OPC-like cells contained the most actively dividing cells and constitute the PN subtype [94].

AC-like cells exhibit higher expression of astrocytic markers, such as *GFAP* and *S100B*, as well as the radial-glial cell marker *HOPX*. Previous findings overlap with the CL subtype and higher *EGFR* expression [94]. However,

more recent findings did not find an association between *EGFR* amplification and AC-like cells. Instead, it was associated with another emerging cellular state, a glial progenitor cell-like (GPC-like) state [99].

MES-like cells are characterized by *NF1* mutations and increased expression of mesenchymal-related genes, such as *VIM*. Unlike the previously mentioned cellular states, MES-like cells do not recapitulate neurodevelopmental processes. Instead, this cellular state reflects a reactive, stress-induced state associated with interactions in the tumor microenvironment (TME). MES-like cells can be further divided into subgroups: one characterized by stress-associated genes, another by hypoxia-associated genes and chr7 amplification, and a third by distinct MES-like cells [94, 99]. MES-like cell state is associated with and can be promoted by macrophages [84, 100, 101]. Both AC-like and MES-like cells contained a low percentage of actively dividing cells [94].

Apart from the central cellular states mentioned, advances in technology have revealed additional states, expanding the spectrum of GB cellular states. For instance, neuronal-like cells (NEU-like) resembling differentiated neurons and synaptic activity, harboring *TP53* mutations. GPC-like cells, characterized by high expression of, for instance, *EGFR* and TFs related to stemness. Both NEU-like and GPC-like cells contained proliferating cells. Additionally, a subset of AC-like cells expressed cilia genes and was therefore assigned to a separate Cilia-like cell state [99].

Analysis of scRNA-seq data further demonstrates that cells are not confined to a single state but transit between states, occupying a continuum of cellular states. First, individual GBs harbor at minimum two, and more often all four main cellular states. Second, a subset of cells could be classified into multiple groups simultaneously, including intermediates between AC-like/MES-like, NPC-like/OPC-like, and AC-like/OPC-like, with NEU-like cells at the top. This suggests an underlying hierarchy that allows cells to transition between cellular states [94, 99]. Third, lineage tracing of patient-derived cells (PDC) *in vivo* revealed that cells can transit between different cellular states [94]. Functional studies in which cellular states were sorted based on cell-surface markers into distinct subpopulations revealed that a single cell or subpopulation could repopulate other cellular states, both with and without an altered cell-state equilibrium [94, 102].

Studies characterizing recurrent GB have identified both neuronal and mesenchymal phenotypes. On the other hand, there might also be no shift from the primary tumor [89, 96, 103-105]. A mesenchymal shift may be associated with a worse prognosis [85, 89, 105] and with drug and radiotherapy resistance

[78, 89, 90, 106, 107]. These studies highlight the heterogeneous response of GBs to treatment and the difficulty in predicting tumor evolution.

Together, these studies of cellular-state plasticity indicate that GB is a dynamic tumor composed of cells that exist in or transition between cellular states, thereby shaping tumor composition over time. Perturbations to the system, such as surgery or treatment, may alter the proportions of cellular states. The commonly recurring cellular states described earlier likely reflect key aspects of GB tumor biology, and characterizing these cellular states and their transitions is important for understanding tumor biology and evolution. Each tumor likely has different therapeutic susceptibilities, as response to treatment may depend on both present cellular states and transitions between them. Pointing to the importance of a personalized treatment strategy.

Glioblastoma stem cells

The cancer stem cell theory states that many cancers follow a developmental hierarchy, with self-renewing cancer stem cells at the top that can differentiate into progenitor-like and differentiated cancer cells [18, 108]. This suggests that eliminating cancer is achievable by targeting cancer stem cells. On the other hand, the previously mentioned concept of phenotypic plasticity in cancer suggests that cancer cells can transition between stem-like and more differentiated states. This indicates that cancer stem cells are not a static phenotype but rather a part of a dynamic state system [99, 102, 109] that can shift in response to treatment [109, 110].

In GB, a subset of cells has properties resembling neural stem cells and is associated with tumor initiation and self-renewal [111], often termed GB stem cells (GSC). These stem-like cells are commonly identified by increased expression of stemness-associated markers, such as *SOX2* and *NES* [112-114]. Functional studies support an important role of *SOX2* in maintaining stemness in GB. For instance, silencing *SOX2* reduces proliferation in GB cell lines and tumor-initiation capacity [115, 116], whereas forced expression of *SOX2* returns cells to a stem cell-like cellular state and limits astrocytic lineage commitment [117].

Invasion Routes

As previously mentioned, GB is a highly infiltrative disease that invades the brain parenchyma along defined anatomical pathways. Historically, patients diagnosed with GB commonly presented symptoms related to the tumor exerting pressure on the brain, even though invasion of brain parenchyma has always been a fundamental feature of GB [118, 119]. Nowadays, patients often have the bulk of the tumor surgically resected, and therefore, symptoms related to invasion of the brain parenchyma are more apparent [118]. Recurrence often occurs near the primary tumor [105, 120], consistent with invasive cells remaining following surgical resection.

Cancer cells, including GB cells, can alter their surrounding environment as they invade. For instance, GB cells can disrupt white matter tracts, occupy neuronal niches, mimic neuronal activity, remove astrocyte endfeet, and thereby compromise the BBB. GB invasion occurs mainly as mesenchymal invasion [121-124] compared to ameboid invasion [47, 125, 126]. GB can also shift between these modes, for instance, in response to MMP inhibition or impaired ECM degradation. Invasion can occur as either collective [46, 124] or single-cell invasion [46, 127-129].

In 1938, Scherer pathology assessed and studied the invasion patterns of 100 GBs and concluded that GBs invade perivascular spaces and white matter tracts, exhibit perineuronal satellitosis, and invade the subpial space (*Figure 6*) [119]. Not all GBs use all routes, reflecting intertumoral variability [42]. Understanding the invasion routes is important. GB cells invade along multiple structures and cell types and may interact differently with different normal cells in the brain parenchyma. Thus, invasion is not uniform, which implies that targeting invasive cells should account for multiple modes of invasion and be tailored to patient-specific patterns. Most importantly, while preserving the health of surrounding tissue and normal cells [130].

White matter (WM) tract consists of myelinated axons produced by oligodendrocytes, which, in bundles, form long nerve fibers that connect the two hemispheres via the corpus callosum and gray matter [119, 120]. To diffusively invade WM, GB cells have to pass through a dense environment in the brain parenchyma, [131] as small as 20-80 nm *in vivo* [132, 133]. To do this, GB cells can reduce their volume by 33% through water channels (*AQP1* or *AQP4*) and ion channels, and squeeze into the narrow spaces [120]. GB cells can invade along the WM [119] and spread to the other hemisphere via the corpus callosum [120]. GB cells can disrupt WM during invasion, leading to demyelination and axonal injury, and promoting tumor growth [98, 136-138].

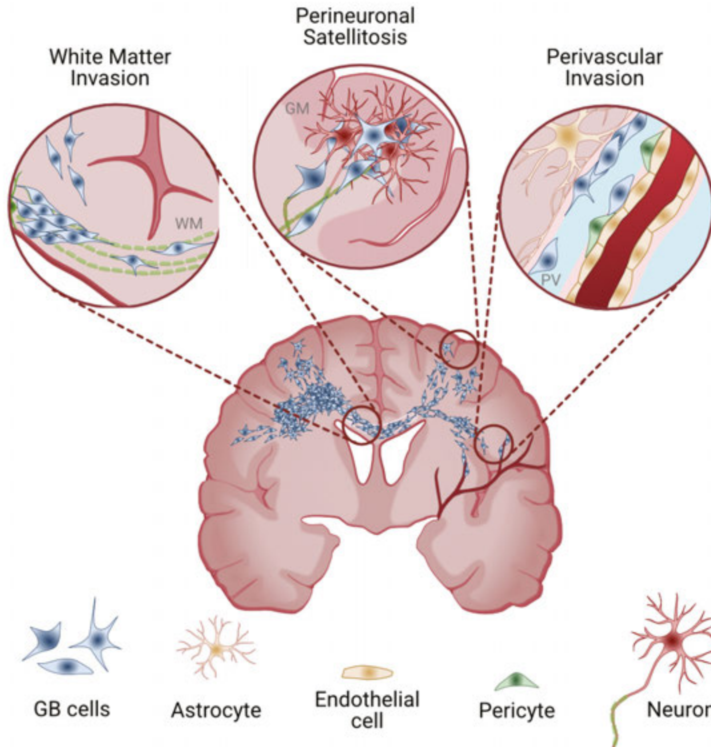

Figure 6. Three common invasion routes in GB are: first, Diffuse invasion of white matter. Next, perineuronal satellitosis. Lastly, Perivascular Invasion. WM: white matter tracts, GM: Gray matter, PV: Perivascular Space. Adapted from [134, 135].

Gray matter is the folded layer of the brain surrounding the WM and containing neuronal bodies. *Perineuronal satellitosis* (PS) is an invasion route characterized by GB cells that encapsulate neurons and dendrites. Additionally, GB cells can eliminate neurons and replace them [119].

The perivascular (PV) space is a cerebrospinal fluid (CSF)-filled compartment surrounding blood vessels in the brain that connects to the subarachnoid space. PV and WM invasion are the two dominant invasion routes in GB [119]. In the PV route, astrocytes and their end feet, together with pericytes, cover the endothelial-lined blood vessels, playing a central role in regulating the BBB [74, 139, 140]. GB cells invading the PV space encounter an unimpeded invasion route, using the surrounding ECM as a scaffold [120, 141]. During PV invasion, GB cells both may and may not displace astrocytic end feet and disrupt the BBB, this appears to be model-dependent [74, 128, 142]. In addition, GB cells can acquire a pericyte-like or endothelial-like phenotype

and integrate into a vascular niche through transdifferentiation [128, 143-146]. The PV space has been proposed as a niche for cancer stem cells [147] and proliferating GB cells have been found near a branching vessel [127].

These invasion modes and routes recapitulate migration observed during development and following injury [98, 119, 120]. Genetic perturbation of *VIM* in GB cells reduced expression of other intermediate filaments, like *GFAP* and MMPs, and reduced migratory potential *in vivo* [148]. Another study perturbed *MAP4K4*, which reduced the expression of *VIM*, increased E-cadherin, and promoted Mesenchymal-to-Epithelial transition. Additionally, knockdown of *COL1A1* in glioma cells reduced invasion *in vivo* [124]. These studies indicate that invasion in GB can be modulated by perturbations in structural proteins, a mesenchymal-like cellular state, ECM composition, and cell-to-ECM interactions. However, studies often discuss only one mode of invasion or treat invasion as a single, uniform process [46, 74, 98, 122, 124, 129, 136, 138, 147, 148]. This raises the question of how cellular states and transitions influence multiple modes of invasion over time.

Spatial organization of cellular states

Recent advances in spatial transcriptomics and proteomics enable the integration of transcriptional cellular states with their anatomical localization [84, 104, 149]. These suggest that GB cellular states exhibit microenvironmental bias within a tumor, including differences in cellular state composition between the tumor core and infiltrative regions.

The spatial organization of GB has intertumoral and intratumoral variation [99, 104, 105]. GB tumor regions have been described as layered or disorganized [104], in which individual GB tumors can harbor both areas. The disorganized areas lack spatial structure, with cellular states intermingling in a non-predictive pattern. In contrast, layered GB regions are shaped by hypoxic areas. These are centered around a necrotic region enriched for MES-like hypoxic cells and depleted of microglia. Cells in the tumor core exhibited epigenetic remodeling and an increased mutational burden. Beyond the tumor core, MES-like cells are increasingly intermixed with vascular and immune cells. Toward the edge of the layered region, OPC-like, NPC-like, and AC-like cells become more prevalent and are increasingly intermixed with non-malignant brain cells (*Figure 7*) [99, 104, 105].

The brain vasculature has been found to serve as a shared microenvironment for macrophages, proliferating malignant cells, OPC-like cells, and, to some extent, MES-like cells [104]. This aligns with previous studies

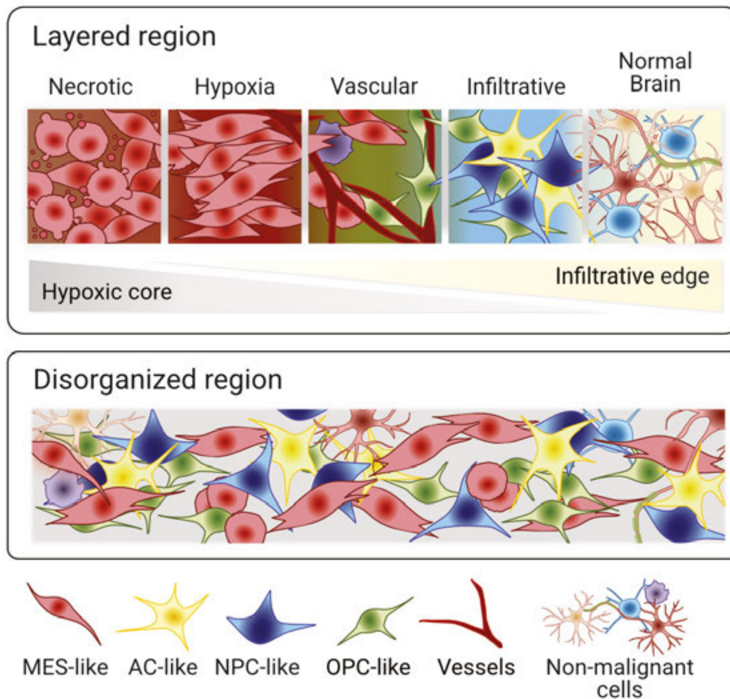

Figure 6. Representative spatial organization of GB cellular states

demonstrating that *OLIG2*+ OPC-like cells migrate along blood vessels, regulated by Wnt signaling, in a *PDGFRA*-driven mouse glioma model [46]. A process resembling OPC migration during development, where *OLIG2*+ and *PDGFRA*+ cells migrate along blood vessels via WNT signaling [150]. Furthermore, MES-like cells were often found in clusters and close to macrophages [104]. GB cells invading the WM have been found to acquire an OPC-like phenotype in response to WM injury, a process regulated by *SOX10*, a TF important in oligodendrocytic lineage differentiation [98].

In the infiltrative area, AC-like, OPC-like, and NPC-like cells frequently co-occur. NPC-like cells have been found close to neurons and oligodendrocytes [104]. Other studies have also found OPC-like and NPC-like cells in the invasive regions, exhibiting increased synaptic activity [129, 151, 152]. Longitudinal live-imaging combined with scRNA-seq demonstrated that OPC-like and NPC-like cells later transitioned into AC-like and MES-like cells, forming a denser population [129]. However, these invasive regions also exhibit a high abundance of AC-like cells that lack synaptic interactions [129, 152].

These observations illustrate intratumoral GB cellular heterogeneity and that GB heterogeneity extends beyond cellular states to encompass spatial and temporal variation. Where specific microenvironments, such as proximity

to vasculature and PV space, WM, and neurons, are occupied by distinct cellular states, yet are not entirely spatially exclusive. The extent of heterogeneity poses a challenge, as targeting one specific cell population or cellular state may not prevent invasion, since cellular plasticity allows transitions and repopulations of other cellular states also associated with invasion.

Investigating Plasticity and Invasion

Experimental models for studying glioblastoma

Despite extensive research, there are limited therapeutic options available for GB patients. Developing new therapeutic options requires experimental models that recapitulate relevant aspects of GB and are generalizable to future patients.

In vitro

The first cell cultures of brain cancer cells from patients were established in the 1950s [153]. During the 1960s, GB cell lines were established and are still widely used. For instance, U87MG (U after Uppsala and MG after Malignant Glioma) [154]. Immortalized cell lines have been of great importance for GB research but do not capture the heterogeneity of GB patients due to clonal selection and expansion. This makes them increasingly homogeneous and deviates from the patient's source material. Additionally, these cell lines are cultured in serum conditions that, over time, will diminish the GSC population [155] and, for instance, reduce *SOX2* expression [116, 156].

PDCs cultured in serum-free conditions supplemented with growth factors EGF and bFGF better recapitulate the original patient material [150]. Still, they are also subject to clonal expansion with time, which affects the population at the genomic and transcriptomic levels [157]. Additionally, long-term culturing of PDC with growth factors, such as EGF, can select for genetic selection, leading to loss of genetic amplifications, like *EGFR* [158]. Biobank efforts of PDCs, such as the Human Glioblastoma Cell Culture (HGCC) resource, provide over 100 patient-derived glioma cell lines [159, 160].

In vivo

While cells cultured *in vitro* are a cost-effective system for studying initial treatment response and growth, they lack environmental context. *In vivo* studies allow the study of interest in a host animal, such as mice or fish. In a xenotransplant model, cells are transplanted into a host animal, allowing their study in a microenvironment with vasculature and stromal cells. The host animal often has a compromised or absent immune system, which prevents rejection of the implanted cells. Cells can be implanted either as a flank or orthotopically. Patient-derived orthotopic xenografts (PDOX), often mentioned

as patient-derived xenografts (PDX), have recapitulated GB features, including intratumoral heterogeneity, microvascular proliferation, and diffuse invasion [161]. There are examples of PDX models for GB in both mice (*Mus musculus*) [94, 161, 162] and zebrafish (*Danio rerio*) [163, 164]. In mice, cells are often transplanted into strains such as nude mice lacking T cells [165], NOD-SCID mice lacking both T cells and B cells, or NOD-SCID gamma (NSG) mice deficient in both T cells, B cells, and natural killer (NK) cells [166].

Zebrafish have been used for decades in developmental and tumor biology, owing to their many advantages over other animal models. First, zebrafish have a high reproductive capacity, allowing hundreds of embryos to be obtained per week [167]. Second, because the zebrafish life cycle is short and rapid, they can serve as models within hours post-fertilization (hpf). For instance, the nervous and circulatory systems develop within 24-72 hpf [168]; however, the immune system does not, a feature necessary for establishing a zebrafish PDX (zPDX) model [169]. Lastly, zebrafish have orthologs of around 70% of human genes that cause disease, as do other genes important for GB pathology, such as those critical for neuronal activity [170].

Genetically modified zebrafish have expanded the repertoire of phenotypes. For example, Casper (roy -/- and nacre -/-) [171], which exhibits a transparent phenotype. This enables imaging of fluorescently labeled cancer cells *in vivo.* The genetic modification (*fli1*:*EGFP*) was used to functionally study interaction with a fluorescent vasculature [172]. Since zebrafish can absorb drugs from the water, they can be utilized for *in vivo* drug testing. Together, these characteristics make zebrafish a useful model for preclinical research assessing new cancer treatments [173-177]. However, a difference between zebrafish and mammalian cells is the temperature at which they are kept: zebrafish are kept at 28°C, whereas temperatures above 35°C impact embryo survival. A zPDX model requires a temperature that balances the two biological systems; therefore, studies often keep their zPDX model around 33°C [160, 161, 164, 176, 178].

As with all model systems, xenograft studies have limitations. For example, due to the lack of a functional immune system and a native human microenvironment, translational assessment of treatment response is limited. Furthermore, PDX models require a trained researcher to inject cells. Lastly, since a single PDX model contains cells from only one patient, it can capture intratumor heterogeneity but cannot capture intertumoral heterogeneity.

Other models

Genetically engineered mouse [179-181] and zebrafish [182] models enable the study of genetic alterations associated with GB and their effects on

treatment response in the context of the innate immune system and BBB. However, their limitations lie in their inability to capture the full spectrum of GB heterogeneity. *Ex vivo* PDX models of GB have been established, for instance, using mouse brain slices. This can be accomplished in different ways, most commonly by transplanting PDC into mouse brain slices [183] or by sectioning an established PDX model [74, 124]. Notably, some protocols maintain *ex vivo* brain slice cultures in serum-containing media [124]. Emerging 3D cell cultures, such as organoid models, can partially depict GB. Derived from either genetically engineered induced pluripotent stem cells [184] or co-culture of PDC with cortical organoids [185, 186]. The co-culture recapitulates a broader range of GB cellular states within 2 weeks, more closely resembling patient samples than *in vitro* cultures [185]. Additionally, it can enable modelling of the patient's immune response if combined with matched peripheral blood mononuclear cells [187]. A co-culture of GB cells with a cortical organoid is relatively short, similar to a zPDX model (days to weeks), excluding the time required to produce organoids. In comparison, a mouse PDX model can extend over several months. However, organoid models miss important aspects found in *in vivo* models, for instance, vasculature and thus a BBB.

Replacement, Reduction, and Refinement

There are pros and cons to every model. When using animal models in research, it is always important to consider the 3Rs: *Replacement, Reduction, and Refinement*. For instance, replacing animals with mathematical models, or advanced cell culture models such as organoids, and limiting the number of animals used, while prioritizing animal welfare.

Single-cell transcriptomic profiling

scRNA-seq provides a snapshot of each cell's transcriptomic state within a sample. Several scRNA-seq protocols exist, each with minor distinctions, but all include isolating single cells. This is often accomplished in microfluidic droplets [188-190]. Each mRNA transcript is labeled inside a droplet with a unique molecular identifier and a barcode that identifies the original transcript and its originating cell. During data processing and analysis, transcript expression can therefore be normalized, quantified, and assigned to the originating cell [188]. A typical analytic pipeline can involve bioinformatic tools to perform quality control and filter for low-quality cells, normalize gene expression for comparison between cells, perform dimensionality reduction and clustering based on transcriptional similarity, and project the results onto a 2-dimensional space for visualization.

Inferring regulatory networks

Cellular states are inferred from the expression of coordinated transcriptional gene programs, which are controlled by gene regulatory networks (GRNs). This consists of interconnected regulators, such as genes, transcription factors (TF), enhancers, and promoters, that together govern gene expression and cellular state. Differences in chromatin organization influence TFs' binding sites at DNA and thereby shape state-specific transcriptional programs. In GB, cellular states are defined by the collective expression of a set of defined genes that together reflect state identity. These coordinated expression patterns imply underlying regulatory factors that drive specific transcriptional cellular states.

To identify candidate regulators, computational approaches have been applied to reconstruct GRNs from scRNA-seq data. Such methods often identify patterns of co-expressing genes across individual cells. One commonly used framework is SCENIC, which identifies genes whose expression correlates with a TF across cells and evaluates motif enrichment in the cis-regulatory regions of target genes [191]. An alternative framework, scregclust, identifies TFs and kinases as candidate regulators of coordinated gene expression programs. It clusters co-expressed genes into sets and iteratively refines them, while nominating a group of regulators whose expression predicts each set across single cells [192].

Integration of chromatin accessibility data provides complementary information on accessible promoter and enhancer regions [193, 194]. When combined with scRNA-seq, nominated regulators can be assessed for both their expression and accessibility to the target of interest. Although these methods can nominate candidate regulators, they remain correlative and do

not establish causality. Functional validation is therefore required to determine the causal relationships between predicted regulators and gene expression programs. For instance, genetic perturbation allows for direct measurement of a regulatory process.

Capturing temporal dynamics

Because cells are lysed during sample preparation for scRNA-seq, any temporal information about gene expression is lost. To address these limitations, computational methods emerged to infer temporal resolution from endpoint transcriptomic data. For instance, Monocle infers one-directional trajectories and orders cells along a pseudo-time path based on gene expression changes [195]. Another method is RNA velocity, which predicts the direction of the upcoming transcriptional state using information from unspliced and spliced mRNAs [196]. These methods provide predictive insights into potential cellular state transitions. However, they infer cellular transitions rather than directly observe real-time plasticity.

Therefore, combining modelling approaches with experimental tools provides a complementary means of obtaining temporal information. Early publications demonstrating cellular plasticity in GB relied on fluorescence-activated cell sorting (FACS)-based screening of protein surface markers as a proxy for cellular states. This was followed by re-sorting, demonstrating that purified cellular-state populations later repopulate other cellular states [94, 102]. This assumes that cellular states can be depicted solely from protein surface markers. Other approaches combine lentiviral delivery of DNA barcodes with scRNA-seq to couple clonal *in vitro* lineages to the transcriptome, enabling the reconstruction of prior directionality between cellular states [109].

Image-based experiments provide an alternative approach to capturing temporal information. A single endpoint image provides a scalable alternative for quantifying proliferation at the bulk level and *in vitro* spatial organization in response to treatment [197]. Such approaches do not capture the dynamics underlying these patterns. Live monitoring enables temporal continuity and observation of morphological changes over time, preferably allowing the observation of state transitions within a relevant microenvironment. Live monitoring has been performed across several experimental GB models, ranging from *in vitro* [197], to *ex vivo* mouse brain slices [74, 124, 183], and to *in vivo* in mice and fish [124, 127, 129, 198, 199]. For visualization, cells are commonly transduced with a lentiviral construct that encodes a fluorescent marker, resulting in stable fluorescent expression throughout the cell. This enables monitoring of invasion, treatment response, and morphological changes.

In addition to capturing cell density and morphology over time, transcriptional states can be monitored using artificial promoters, designed to reflect defined transcriptional activity. In these systems, endogenous TFs drive the expression of fluorescent reporters. For example, GB subtype-specific promoters have been developed to monitor transcriptional activity. This provides a relative measure of TF activity in the cells [91]. The reported activity depends on the promoter's design accuracy and the reporter's half-life. Alternative strategies rely on a fluorescent reporter to tag the protein of interest. This is typically achieved by inducing DNA breaks and exploiting the cell's own repair machinery, non-homologous end-joining (NHEJ) or homology-directed repair (HDR) [200, 201], in the presence of a donor template. This can enable insertion of a reporter sequence at the endogenous locus. The measured intensity reflects protein levels with native regulatory processes controlling protein output. Tagging an endogenous protein with a fluorescent reporter may influence function, folding, or localization, depending on the design and insertion site of the construct. Knock-in efficiency can vary across targets and cell lines[201], potentially affecting the ability to model heterogeneous cell populations [201]. Additionally, quantification of protein levels does not inform on post-translational modifications that regulate protein activity, necessitating complementary approaches [202].

Time-lapse image acquisition provides temporal resolution as a complement to transcriptomic or proteomic profiling of endpoint measurements. However, to obtain quantitative, biologically relevant information from visual data requires additional processing, image analysis, to turn images into features that can be compared across time and conditions. Important aspects of this process include, first, preprocessing; second, segmentation; third, extraction of quantitative features; and also, temporal analysis and tracking. First, preprocessing is used to minimize noise and correct for image artefacts. Second, segmentation enables separating individual objects, such as tumors or cells, from one another and from the background, for quantification. In simple systems, intensity-based thresholding may be sufficient, complemented by Gaussian filters, erosion and dilation, and the watershed algorithm to remove noise, remove small objects, fill holes, and separate very near cells [203]. For example, enabling the quantification of a fluorescent nucleus that does not overlap with other nuclei. In heterogeneous, dense regions or complex *in vivo* systems, convolutional neural networks (CNNs) can be used for tasks such as segmentation. These models are usually trained on annotated images that act as the ground truth for the specific task. Third, quantitative features can be extracted within segmented regions, including proliferation, morphological changes, fluorescence intensity, and spatial organization relative to the

surrounding microenvironment [203, 204]. Lastly, in live-monitoring experiments, a further step is to track cells and link the extracted features of segmented objects over time. Manual tracking of cells across consecutive frames is labor-intensive and not scalable for large datasets. Automated tracking algorithms link cells across consecutive frames and can be used to trace lineages. State-space models, such as Kalman Filtering, provide a framework for estimating cell movement over time, assuming constant velocity and Gaussian noise. Other frameworks include more flexible Particle filters and deep-learning-based frameworks, which can accommodate more complex movement or irregularities [205, 206].

CRISPR-based approaches in functional studies

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is a gene-editing technology enabling targeted modification of DNA in living cells and organisms [207]. A guide RNA (gRNA) and a CRISPR-associated (Cas) protein are combined to form the CRISPR/Cas system protein as a ribonucleoprotein (RNP). The gRNA guides the RNP complex to a specific genomic locus, where Cas, with endonuclease activity, can induce a DNA break [207].

Different Cas versions induce different DNA breaks. Cas9 induces blunt-end double-stranded breaks (DSBs), and Cas12 induces staggered DSBs [208]. Engineered variants of Cas9, such as Cas9 D10A, preferentially induce single-stranded nicks rather than DSB. Using two guide RNAs complexed with Cas9 D10A, two nicks can be induced near the DNA site of interest on opposite DNA strands. This generates staggered overhanging DNA ends, a strategy that reduces off-target effects, as single-stranded nicks are more efficiently repaired than DSBs [207].

The induced DNA break is repaired by the cell's inherent repair mechanisms, primarily HDR or NHEJ. HDR is a repair mechanism with higher fidelity, normally in S/G2/M, since it relies on a template to repair the induced break. Co-delivery of a donor template containing the sequence of interest, flanked by homology arms that mirror the target site, enables HDR-mediated insertion [207, 209]. NHEJ is an error-prone repair mechanism active throughout the cell cycle that introduces mutations, such as insertions or deletions, during the repair of DNA breaks [209]. These mutations can cause frameshifts or premature stop codons, leading to truncated or non-functional proteins. This is commonly used to generate gene knock-outs (KOs) for loss-of-function studies.

A whole-genome KO screen is an unbiased approach to identify genes that regulate a given phenotype [210, 211]. These screens are commonly used to discover genes that sensitize cells to drugs or to identify other genetic

dependencies, such as upstream regulators of a phenotype. Typically, cells are transduced with a pooled gRNA library, followed by selection based on a phenotype, such as FACS, drug treatment, or time. Analysis of both enriched and depleted gRNAs enables identification of genes essential for the phenotype of interest.

Perturb-seq is a high-throughput system connecting CRISPR/Cas editing with scRNA-seq to study gene expression changes at the single-cell level. By comparing the transcriptomes of, for example, perturbed cells with those of control cells, information about how gene perturbation influences cell dynamics can be uncovered [212, 213].

Present investigations

Underlying these three investigations were central questions that motivated this work. Previous studies had established that GB exhibit extensive cellular heterogeneity and invasion into the brain, and that these vary across patients, within each tumor, and during tumor progression. However, at the time this thesis was initiated, it remained uncertain how plasticity and invasion were interrelated and what governed cellular states and their transitions. Therefore, this thesis aims to identify regulators of these processes and understand how cellular plasticity shapes the choice of invasion route. Addressing these questions required experimental approaches that move beyond static measurements. Such tools should enable monitoring of tumor growth and invasion at both the single-cell and tumor levels, *in vitro* and in more relevant microenvironments.

To begin, we first investigated whether distinct cellular states are associated with specific invasion routes and if there are distinct regulators of this. Next, we sought to develop a tool for live monitoring of cellular states and to investigate whether we could use it to investigate regulators governing cellular states. Lastly, we investigated whether tumor growth and survival during treatment could be monitored automatically in a high-throughput manner *in vivo.*

Paper I: The invasion phenotypes of glioblastoma depend on plastic and reprogrammable cell states

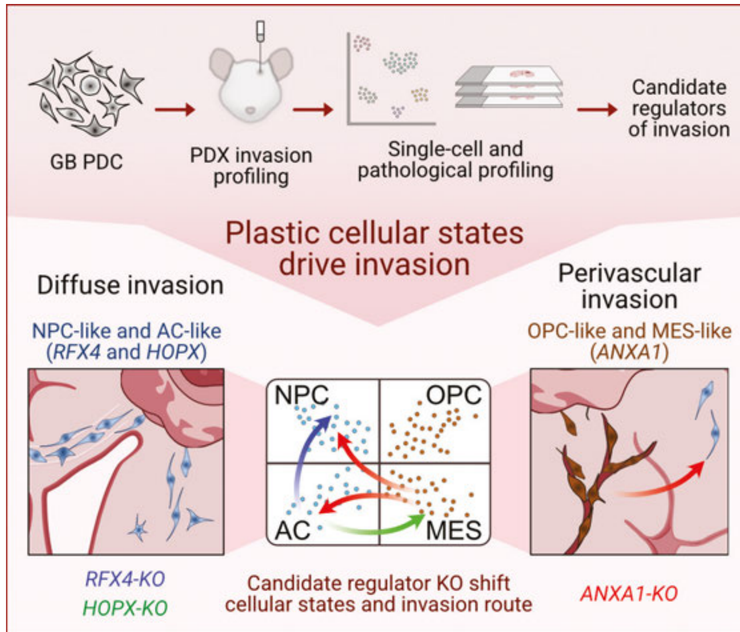

Figure 8. Graphical abstract for Paper I

Paper I investigated whether cellular states occupy distinct invasion routes and whether this is governed by specific regulators. Previous investigations had established the routes of invasion in GB, including PV invasion and diffuse invasion into the brain parenchyma [46, 119, 120, 127]. Invasion had been linked to multiple cellular states in different studies [98, 124, 127, 129, 138, 143, 150]. It remained uncertain whether distinct cellular states exhibit preferences for invasion routes, and specifically, how this is regulated.

In Paper I *(Figure 8*), we identified candidate regulators of invasion-route-associated cellular state across six PDX models by combining scRNA-seq and spatial proteomics. We identified that MES-like and OPC-like cells in our PDX models form bulk tumors and invade along a PV invasion route, a process we linked to *ANXA1*. NPC-like or AC-like cells more often invade diffusively into white matter tracts, with *HOPX* and *RFX4* identified as candidate regulators. Furthermore, CRISPR/Cas9 was used to perturb regulators of PV and diffuse invasion. KO of *ANXA1* in bulk-forming and PV-invading cells shifted the preferred invasion route toward a diffuse phenotype,

accompanied by a shift from MES-like cellular state to AC-like and NPC-like cellular states. Mice xenografted with *HOPX* KO, *RFX4* KO, and *ANXA1* KO cells had significantly prolonged survival in mice, compared to controls.

While these findings establish a link between regulators governing cellular states and invasion routes, understanding how cellular plasticity dynamically impacts invasion requires tools that enable real-time monitoring of cellular states and their spatial context.

Paper II: CRISPR-tag enables real-time mapping and perturbation of cell-state plasticity in glioblastoma

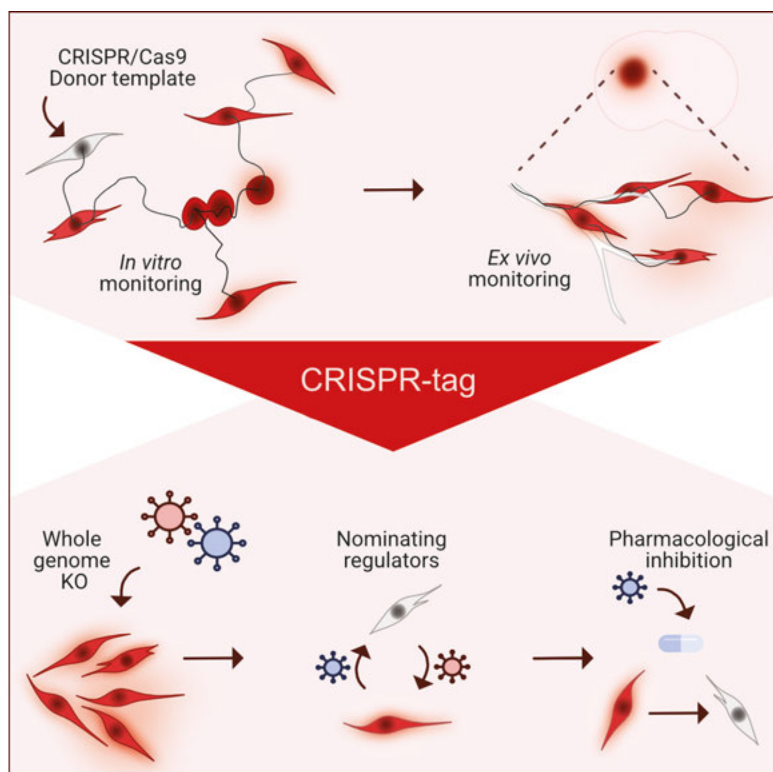

Figure 9. Graphical Abstract for Paper II

We sought to develop a tool to monitor cellular state dynamics in real time. In Paper II, we investigate whether cellular states can be monitored using endogenous fluorescent proteins across different microenvironmental contexts and whether these reporters can be leveraged to identify regulators (*Figure 9*).

GB cells exhibit a wide range of cellular states that resemble different types of brain cells at varying stages of differentiation [79, 92, 94, 96, 97, 109, 144]. These cellular states can change within individual cells over time, due to cues from the microenvironment or treatment [98, 100, 105, 129, 151]. Finding strategies that target cellular-state plasticity to control invasion will be of high importance.

Here, we developed CRISPR-tag, a CRISPR/Cas9 technique for labeling gene-specific cellular states with fluorescent proteins in PDCs, in principle enabling *in vitro* and *ex vivo* monitoring of up to 20 genes. As a proof-of-

concept, we used CRISPR-tag to fluorescently tag *SOX2*. Tracking individual cells over time using the Kalman filter enabled real-time monitoring of *SOX2* expression, a stemness-associated marker in GB, during BMP4-induced differentiation. Displaying a heterogeneous response to treatment, both between PDCs and within a PDC.

In an *ex vivo* brain environment, *SOX2* monitoring during BMP4-induced differentiation revealed a differential spatial response: GB cells within the core gradually decreased *SOX2* expression, whereas invasive cells maintained *SOX2* expression. *Ex vivo* monitoring of *ANXA1* demonstrated increased expression in GB cells located near vessels.

We performed a whole-genome KO screen targeting *ANXA1* to identify causal regulators of MES-like-associated PV invasion. KO of *HDAC2* and *METTL17* were enriched in cells expressing low levels of ANXA1. In contrast, KO of *NF2, VGLL4, IRF2BPL*, and *IRF2BP2* were enriched in cells expressing high levels of ANXA1. Pharmacological inhibition with the HDAC inhibitor BRD4884 in *ANXA1*-tagged PDC decreased *ANXA1* expression in a dose-dependent manner.

Together, we establish an approach to monitor stemness and a MES-like cellular state *in vitro* and *ex vivo* using CRISPR-tag. In combination with whole-genome perturbation screening, this allowed us to identify candidate regulators of the MES-like cellular state. Further studies of how cellular plasticity influences tumor growth and invasion require a scalable model that allows live monitoring and testing of candidate regulators and their pharmacological modulators. This motivated the development of a scalable model to perform larger screening efforts.

Paper III: Real-time evaluation of glioblastoma growth in patient-specific zebrafish xenografts

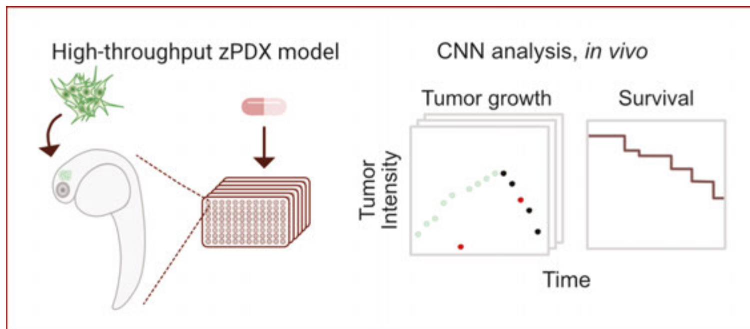

Figure 10. Graphical abstract of Paper III.

In Paper III, we developed a high-throughput zebrafish PDX (zPDX) model of GB, using both live imaging and AI monitoring. This approach offers a scalable alternative to mouse xenografts, enabling the study of tumor initiation, GB growth, and invasion (*Figure 10*).

11 patient-derived HGCC cell lines were modified to express GFP and transplanted into 24-hour-old zebrafish embryos. The freely swimming zebrafish was automatically monitored in a 96-well plate format every four to six hours, up to five days after fertilization. A CNN was used for image analysis. Using our pipeline, we enabled monitoring of tumor growth in individual fish and estimated survival after treatment. We further characterized the invasive growth of each zPDX using light-sheet imaging. Our 11 GB zPDX models demonstrated patient-specific variation in growth and in response to a proteasome inhibitor.

Discussion and Future Perspectives

Emerging evidence connects cellular plasticity in GB to invasion [90, 94, 103, 120, 129], further suggesting that the transition between cellular states impacts tumor progression and response to therapy [107, 109, 192, 214]. If cellular states in GB are temporary, reversible, and microenvironment-dependent, new therapeutic strategies ought to target plasticity within a more biologically relevant microenvironment. It will require approaches that move beyond static measurements, enabling direct observation of how cells adapt and alter invasion mode in response to therapies.

This thesis centers on how cellular plasticity relates to invasion phenotypes in GB and develops new tools to measure and model tumor growth and plasticity-driven invasion. It provides complementary approaches for studying GB across scales, where the individual papers either address the regulation of cellular states and invasion (Paper I and Paper II), a new tool for monitoring cellular states (Paper II), or a system designed to quantify tumor growth and invasion within a more physiologically relevant microenvironment (Paper II and Paper III).

Paper I

In Paper I, we address whether cellular states are interconnected via distinct invasion routes and whether underlying regulatory programs determine this.

We provide evidence supporting a causal relationship between ANXA1 and the ability of U3013MG MES-like cells to form a tumor bulk and invade PV. In contrast, cells lacking *ANXA1*, AC-like, or NPC-like cells invaded diffusely into the brain parenchyma. Perturbation of *HOPX* and *RFX4* shifted the cellular state from an AC-like state to a more NPC-like and MES-like state, still invading diffusely.

Together, these findings suggest that the MES-like cellular state may be an acquired identity and more amenable to perturbation, which is consistent with other studies [84, 100, 101]. In contrast, NPC-like and AC-like cells appear to reflect a more committed identity, corroborated by previous findings [91, 106], linked to diffuse invasion.

Introducing *ANXA1* into the diffusely invasive cell line U3180MG resulted in a bulkier phenotype in a zPDX model. This observation raised the

possibility that enforced ANXA1 expression, shifting GB cellular states to form a bulk-forming phenotype, could make them more surgically accessible than diffusely invading cells. However, any such shift would have to be weighed against the risk of increased PV invasion and its influence on recurrence, as well as other biological effects.

An interesting possibility is that the microenvironment biases state transition probabilities rather than strictly dictate a cell's fate. On the other hand, a genetic mutation, perturbation, or treatment might constrain plasticity, making cells more or less prone to transition to a cellular state. Invasion route shifts due to perturbation of the system might be a consequence of altered transition dynamics rather than a static identity [109, 192].

Expanding the scope of Paper I, we aim to perform a pooled KO-screen targeting cellular states and invasion in GB, enabling a large-scale assessment of how genetic perturbations influence these traits *in vivo* using a Perturb-seq approach.

Paper II

Paper II addresses a concern that targeting cellular plasticity cannot be properly assessed in a static system. By introducing temporal and quantitative measures of cellular states, we provide an experimental approach to assess the targetability of cellular plasticity.

In Paper II, we observe oscillatory patterns of SOX2 expression in our PDCs, raising the question of whether dynamic expression actively contributes to cellular state transitions. Therefore, we further aim to analyze oscillatory patterns across multiple CRISPR-tags. It would be particularly interesting to identify whether the interplay among multiple reporters within a single cell's oscillatory pattern influences cellular state transitions.

The identified candidates may regulate indirectly by modulating a broader cellular state program rather than acting on the *ANXA1* locus or the protein itself. The perturbation of these genes may increase the likelihood that cells transition to or from the *ANXA1*-driven MES-like cellular state, rather than directly suppressing *ANXA1* expression. For instance, loss of *HDAC2* can alter chromatin accessibility [215], potentially influencing the stability of the *ANXA1*-driven MES-like cellular state by altering the chromatin landscape. Possibly, by modulating the regulation of genes involved in EMT and TGF-β signaling pathways [29, 215, 216]. Analysis of chromatin accessibility or lineage tracing under perturbation or inhibition could determine whether these perturbations affect the balance of cellular state transitions rather than regulating *ANXA1* expression. Additionally, repeating experiments across several patient cell lines is relevant to increase the generalizability of findings.

By expanding the scope of Paper II, we aim to systematically analyze cellular state reporters and transitions *ex vivo* using a multi-CRISPR-tag reporter system, live monitoring, and mathematical modeling. This approach would allow us to determine how microenvironmental and system perturbations reshape the cellular state landscape. Such an approach can identify strategies to influence plasticity-driven invasion.

Paper III

We established a scalable GB model for studying tumor initiation, quantifying tumor growth, and evaluating treatment response *in vivo*.

Leveraging the method developed in Paper III, we further aim to perform a pharmacological screening of CRISPR-tagged cells (Paper II) to find druggable state-steering cellular state dependencies. Identified hits will be evaluated in the high-throughput zPDX model developed in Paper III. Further assessment of candidate drugs will be performed using 2-photon microscopy to evaluate phenotypic tumor growth, invasion, and interactions with vasculature or neurons *in vivo*. Thus, it will provide as a tool to monitor therapeutic interventions targeting cellular state transitions *in vivo.*

Relevance

The work presented in this thesis is foundational and at an early stage of research. However, there are some potential translational implications with a long-term perspective. Our findings suggest that invasion depends on plasticity. The identification of regulators that link cellular states and invasion highlights intervention points. However, perturbing individual regulators did not eliminate invasion; instead, it altered the preferred invasion route. Displaying that these cells are highly adaptable, and that invasion is a hardwired entity. Therefore, targeting plastic invasion might require perturbing multiple key intervention points, either simultaneously or sequentially, to constrain plastic cells into a more therapeutically vulnerable state. In this context, identifying regulators of multiple cellular states (Continued work of Paper II) may represent strategies that could be combined with other therapeutic approaches.

The experimental methods developed in this thesis may facilitate future discovery efforts. CRISPR-tagged cells enable real-time monitoring of cellular state dynamics and can be used to screen compounds, including drug repurposing, that influence plasticity and invasion. When combined with a fast, scalable zebrafish model, it provides a tool for automated *in vivo* screening of cellular plasticity using PDCs.

In the long term, the mentioned approaches may inform strategies targeting plasticity-driven invasion in GB.

Limitations

In vitro PDCs offer a scalable and controlled environment but are oversimplified, as they lack the environmental context of the human brain. Complementary approaches have therefore been considered. PDX models capture many aspects of GB growth and invasion, as shown in Paper I. All our PDX models are established in immunocompromised mice or fish and therefore cannot assess interactions with the human immune system. Additionally, all PDC and PDX models used in this thesis are derived from HGCC (Sweden) [159, 160] and were established from tumor cores, thereby excluding cells at the invasive edge. Additionally, including other cohorts would further extend the understanding of our findings. Large-scale studies include patient samples from multiple cohorts, capturing broader variability in genetics, treatments, and backgrounds [99, 105], but require accounting for more variables. Consistent with this, our findings in Paper I were corroborated in an independent cohort.

The four-cellular-state model proposed by Neftel et al. [94] is useful for interpreting intratumoral heterogeneity, but it simplifies the tumor diversity. GB cells exist along a continuum between the four main cellular states, and can be microenvironment-dependent [100, 129, 151]. Cellular states are inferred from static scRNA-seq data, where large transcriptional programs do not always reflect protein abundance, post-translational modifications that modulate activity, or the cell's functional state. With time, the cellular state space of GB has expanded [99] and depends on the investigation referred to [96, 97]. Future novel methods will likely expand our current understanding of GB. On this note, fluorescent reporters enable monitoring of protein abundance in a cell, but a single reporter does not capture the full spectrum of cellular states. Therefore, future studies should include simultaneous monitoring of several cellular state reporters. In line with this, we are continuously expanding our repertoire of CRISPR-tagged genes and PDCs.

Conclusion

GB invasion is not only a consequence of underlying genetic mutational profiles but of the cellular state plasticity that endlessly changes during tumor progression and treatment. Many strategies used to study and target invasion overlook the spatial and temporal resolution that shape it.

By integrating regulatory perturbation, endogenous state monitoring, and scalable *in vivo* modelling, this work provides evidence that invasion is linked to cellular plasticity and represents a potentially targetable process. Understanding how cellular states are stabilized or redirected may provide information on how to constrain plasticity-driven invasion.

Populärvetenskaplig sammanfattning

Vår kropp består av celler. Celler innehåller DNA, vilket kan liknas vid en instruktionsbok. Instruktionerna är uppdelade i kapitel, där varje kapitel förklarar olika funktioner i kroppen. Inom varje kapitel finns det specifika instruktioner (gener), och olika celltyper läser olika kapitel. En hjärncell läser till exempel endast kapitlet om hjärnan och använder således bara gener som är till för hjärnceller, och en hudcell använder endast gener som är till för hudceller. När en cell vill använda en gen, skapar den RNA: Det kan liknas vid att skriva ned ett recept på en lapp från DNA-boken, och receptet används sedan för att tillverka en produkt, kallad proteiner.

Över tid uppstår förändringar i DNAt, kallade mutationer. I många fall kan cellen reparera dem, men ibland ger dessa förändringar nya egenskaper. En cell kan till exempel bli väldigt bra på att föröka sig (dela sig), eller på att undvika död. Vårt immunförsvar upptäcker de flesta fall av sådana förändrade celler, men ibland kan de gömma sig och börja föröka sig och till och med sprida sig i kroppen. Dessa celler ger upphov till folksjukdomen cancer.

Glioblastom är en elakartad hjärncancer hos vuxna, och prognosen för överlevnad är mycket dålig; de flesta överlever 15 månader efter diagnos. I dagsläget behandlas glioblastom med kirurgi, cellgifter och strålning. Men glioblastom återkommer nästan alltid, eftersom cancercellerna redan har spridit sig i hjärnan och således inte kan opereras bort. Dessa cancerceller är inte bara en enda typ av cell, utan de kan likna flera olika celltyper, exempelvis: nervceller, oligodendrocyter, och astrocyter, eller mesenkymala celler, även om de fortfarande är cancerceller. Glioblastomceller kan också byta identitet, tillstånd (*cell state*) utan nya mutationer. Denna egenskap kallas för *plasticitet*. Till exempel kan cancercellerna öppna andra kapitel i DNA-boken som de egentligen inte borde läsa, så som kapitel om sårläkning eller kapitel som endast används under utvecklingen av ett embryo. Detta kan hjälpa cancercellen att sprida sig, eller att undvika behandling med cellgifter och strålning. Glioblastomceller är extra bra på detta och sprider sig gärna i hjärnan genom att klättra på blodkärl eller gräva små tunnlar. Forskning tyder på att olika cellidentiteter har olika preferenser eller strategier för olika vägar, och att cellerna också kan byta väg under resans gång. Detta gör just glioblastom till en mycket besvärlig sjukdom att behandla.

I Delarbete I har vi kopplat de olika vägar som cancercellerna tar i hjärnan till olika celltillstånd. Vi kallar detta för en viss typ av *invasion*. Vissa celltyper sprider sig oftare längs med blodkärl medan andra sprider sig mer diffust i hjärnan. Vi försökte förstå vilka gener som driver en viss typ av invasion, genom att analysera RNA (recepten) och proteiner (produkten). Med hjälp av CRISPR, ett verktyg för att klippa och klistra i DNA, tog vi bort gener som vi tror styr olika typer av invasion. När vi avlägsnade genen *ANXA1* ändrades dessa cellers tillstånd och invasionen längs med blodkärl försvann. Dessvärre spred sig då dessa celler istället diffust i mushjärnor. Vi lyckades koppla generna *RFX4* och *HOPX* till diffus invasion, men vid borttagning av dessa gener ändrade vi inte invasionstypen utan bara cellernas tillstånd.

I Delarbete II färglägger vi olika celltillstånd för att i mikroskop kunna följa hur cellerna byter tillstånd i realtid. Vi märker olika produkter (proteiner) med fluorescerande färger och kan följa variationer i hur mycket produkt de tillverkar över tid. En väldigt grön cell har väldigt mycket protein, medan en mindre grön cell har mindre protein. Vi följer dessa celler i plastskålar och i mushjärnvävnad. Vi transplanterar in de färglagda cellerna i en mushjärna för att sedan skiva mushjärnan i tunna skivor. Skivorna kan hållas vid liv några dagar, och genom att fotografera hjärnvävnaden med de färglagda cellerna kan vi följa cellerna i realtid och studera dem i en hjärnmiljö. Vi såg då att celler i olika delar av tumören reagerar olika på behandling. I ett annat försök tar vi bort alla gener som finns i vårt DNA, en efter en, för att se hur genen *ANXA1* påverkas. Till exempel, tar vi bort genen *HDAC2* minskar mängden av proteinet ANXA1 (som byggts av receptet i genen *ANXA1*) i cellen, medan borttagning av genen *NF2* ökar mängden ANXA1.

I Delarbete III utvecklar vi en fiskmodell av glioblastom. Att använda möss för experiment är både långsamt och kostsamt, så vi undersökte istället om fisk kan vara ett skalbart alternativ. Vi transplanterar in redan färglagda cancerceller från glioblastompatienter i hjärnan på 1 mm långa, genomskinliga fiskembryon, vilket gör att vi kan se cellerna genom fiskens huvud. Fiskarna placeras i en fyrkantig plastskål med 96 olika brunnar, en för varje fisk. Sedan följs upp till 6 plastskålar i ett mikroskop som tar bilder på fiskarna var fjärde timme tills fiskarna är fem dagar gamla. Detta genererar en stor mängd bilder, så vi utvecklade ett AI-verktyg för att hjälpa till att analysera bilderna. På så sätt kan vi mäta hur cellerna växer och svarar på olika typer av behandling. I ett försök ser vi att celler från olika patienter växer olika, vissa bildar en kompakt tumör, medan andra sprider sig diffust, och vissa patientceller hittas nära blodkärl i fiskens hjärna.

Denna avhandling undersöker kopplingen mellan glioblastomcellers förmåga att byta tillstånd och hur de invaderar hjärnan. Vi identifierar vad som

styr detta och utvecklar verktyg för att kunna följa plasticitet i realtid. Om invasion drivs av att cellerna byter tillstånd, kan vi kanske lära oss att styra om eller låsa denna förändring, och därigenom lyckas begränsa cancerns spridning.

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Within these pages lies a fragment of my soul, shaped by frustrating experiments, incomprehensible qPCR results, and countless attempts that yielded nothing but non-fluorescent cells. Stubbornness has its limitations, but in the end, sheer determination finally enabled us to tag a gene with a fluorescent protein. With that came a turning point: hope returned along with a renewed sense of purpose for the remainder of my PhD. My only wish now is that no one asks me what I will do after this.

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