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Epigenetic regulation of glioblastoma

Impact on tumor recurrence and treatment resistance

INÊS NEVES

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Abstract

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Glioblastoma (GB) remains one of the most aggressive and lethal brain cancers, characterized by profound heterogeneity and resistance to standard therapies. The current treatment regimen with surgical resection and chemoradiotherapy is not curative and GB will almost always recur in proximity to the resection cavity. This thesis explores the molecular and phenotypic complexity of GB through a series of investigations that utilize advanced multiomic approaches to explore the interplay between epigenetic regulation, lineage specificity, and tumor microenvironment interactions.

Paper I employs single-nucleus RNA sequencing, ATAC sequencing, and whole exome sequencing to compare the central tumor mass with the invasive edge in GB patients revealing that peritumoral cells exhibit distinct phenotypes marked by increased invasiveness, immune activation, and mesenchymal-like states while showing reduced proliferative capacity. These cells possess fewer genetic alterations but undergo significant epigenetic reprogramming, suggesting that targeting the immune-driven epigenetic changes could be a promising therapeutic strategy to prevent tumor recurrence.

Paper II investigates the influence of *TP53* mutational status on epigenetic regulation in GB. Two epigenetically distinct subgroups—ATAC-C2 and ATAC-C3—were identified, correlating with divergent survival outcomes. ATAC-C2 tumors, linked to disruptive TP53 mutations, exhibit a mesenchymal, immune-activated phenotype and resistance to standard therapy. In contrast, ATAC-C3 tumors, which retain functional p53 activity, demonstrate better therapeutic responsiveness. This underscores the therapeutic potential of targeting mutation-specific p53 reactivation and alternative agents to counteract resistance mechanisms.

Paper III focuses on enhancer signatures and their role in defining GB subgroups with divergent survival rates. By integrating ATAC-seq and CUT&Tag data, we identify distinct enhancer landscapes that drive mesenchymal-like and neural progenitor-like phenotypes in ATAC-C2 and ATAC-C3 subgroups, respectively. The results highlight that enhancer signatures are more predictive of patient prognosis than traditional transcriptome-based subtyping. Furthermore, the findings reveal lineage-specific transcription factor networks that shape each subgroup's aggressiveness and therapeutic response, paving the way for novel epigenetic therapeutic strategies.

Together, these papers contribute to a deeper understanding of GB biology by elucidating the epigenetic mechanisms underlying tumor heterogeneity, invasion, recurrence, and resistance. They highlight the significance of personalized therapeutic approaches tailored to the unique molecular landscapes of GB subgroups, emphasizing the potential of targeting immune-activated states, TP53 vulnerabilities, and enhancer-driven transcriptional programs.

Keywords: Glioblastoma, Peritumoral cells, Chromatin Profiles, Epigenetic regulation

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

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To all those who have faced cancer, their loved ones, and the survivors.

"Don't let anyone rob you of your imagination, your creativity,
or your curiosity. It's your place in the world; it's your life.
Go on and do all you can with it, and make it the life you want to live."

— Mae Jemison

List of Papers

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

I. **Neves, Inês**[*]., Lu, Xi[*]., Jokinen, Veera[*]., Latini, Francesco[*]., Maturi, Nagaprathyusha., Sundström, Anders., Dang, Yonglong., Uppman, Irem., Bergström, Tobias., Ryttlefors, Mats., Chen, Xingqi[#]., Swartling. Fredrik J.[#]., Uhrbom, Lene[#]. (2025). Multiome analysis of local peritumoral glioblastoma cells uncovers phenotypically unique subpopulations. *Manuscript.*

II. **Neves, Inês**., Vikström, Elin., Sundström, Anders., Lu, Xi., Rendo, Verónica[*]., Uhrbom, Lene[*,#]. (2025). Lineage-dependent epigenetic regulation in glioblastoma is influenced by p53 mutational status. *Manuscript.*

III. Xing, Pengwei[*]., **Neves, Inês**[*]., Salehi, Maede., Yadav, Ram Prakash., Xie, Minglu., Jokinen, Veera., Lundin, Emma., Wallin, Johan., Sjöblom, Tobias., Uhrbom, Lene[#]., Chen, Xingqi[#]. (2025). Enhancer signatures define glioblastoma subgroups with divergent patient survival. *Manuscript.*

** shared author*
 # corresponding author

Contents

Abbreviations

AC	Astrocyte
ANXA	Annexin
ATAC	Assay for Transposase-Accessible Chromatin
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CNS	Central nervous system
CSC	Cancer stem cell
ChiP	Chromatin immunoprecipitation
CUT&RUN	Cleavage Under Targets and Release Using Nuclease
CUT&Tag	Cleavage Under Targets and Tagmentation
DCX	Doublecortin
DNMTS	DNA methyltransferases
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FGF	Fibroblast growth factor
GB	Glioblastoma
GEMM	Genetically engineered mouse model
GEMs	Gel Beads-in-Emulsion
GFAP	Glial fibrillary acidic protein
GSC	Glioma stem cell
HAT	Histone acetyltransferases
HDAC	Histone deacetylases
HGCC	Human Glioblastoma Cell Culture
HOPX	Homeodomain-only protein
IDH	Isocitrate Dehydrogenase
IL-10	Interleukin 10
MES	Mesenchymal
MDM2	Murine double minute 2
NF1	Neurofibromatosis type 1
NGS	Next-generation sequencing
NPC	Neural precursor
OLIG1	Oligodendrocyte transcription factor 1
OLIG2	Oligodendrocyte transcription factor 2
OMG	Oligodendrocyte Myelin Glycoprotein

OPC	Oligodendrocyte precursor cell
SB100	S100 calcium-binding protein B
SOC	Standard of care
SOX11	SRY-box transcription factor 11
PDC	Patient-derived cell culture
PDGFRA	Platelet-derived growth factor receptor alpha
PDX	Patient-derived xenograft
PI3K	Phosphoinositide 3-kinase
PTEN	Phosphatase and tensin homolog
PTMs	Post-translational modifications
PRC1	Polycomb Repressive Complex 1
PRC2	Polycomb Repressive Complex 2
RB	Retinoblastoma
RCAS	Replication Competent ALV LTR with a Splice acceptor
RTK	Receptor Tyrosine Kinase
TCGA	Tumor Cancer Genome Atlas
TGF-β	Transforming growth factor-β
TF	Transcription factor
TME	Tumor microenvironment
TMZ	Temozolomide
TP53	Tumor suppressor protein 53
TSSs	Transcription start sites
TTF	Tumor-treating fields
VIM	Vimentin
WHO	World Health Organization

Introduction

Cancer

Cancer remains one of the most significant health challenges of the 21st century, affecting millions worldwide and posing a profound burden on society. It is a multifaceted disease characterized by uncontrolled cell growth, invasion of surrounding tissues, and the potential to spread to distant organs, known as metastasis. Cancer is the leading cause of death worldwide, with an estimated 10 million deaths and an estimated 20 million new cases in 2023[1]. In Sweden, approximately 75,000 people were diagnosed with cancer in 2023. The most common type of cancer in Sweden is skin, followed by prostate, breast, and colorectal cancers[2]. Despite significant improvements in overall cancer survival since the 1970s, cancers such as brain tumors, pancreatic cancer, and lung cancer remain incurable[3].

Hallmarks of cancer

Cancer is a complex disease arising from genetic and epigenetic alterations that disrupt tightly regulated processes governing cell proliferation, differentiation, and death[3].

The hallmarks of cancer encompass fundamental capabilities acquired during the development of a malignant tumor and currently embody eight hallmarks, two enabling characteristics, and four emerging traits common to all cancers[4–6] (**Figure 1**).

The key differences between cancer cells and normal cells lie in their behavior related to growth control, apoptosis, cell division, response to growth signals, invasion, metabolic processes, and interactions with the immune system. These differences collectively facilitate tumor growth, progression, and spread. Normal cells are extremely limited in their ability to grow and proliferate. This happens because normal cells often respond to specific growth signals, undergo programmed cell death, have limited division capacity, and are recognized and monitored by the immune system[7].

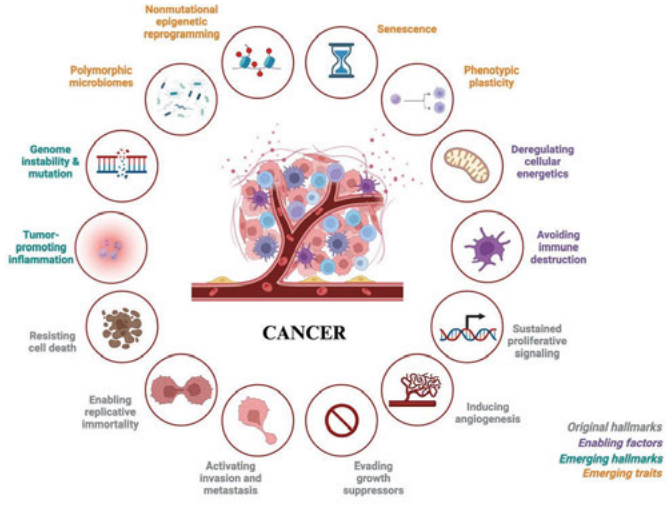

Figure 1: The hallmarks of cancer and their enabling characteristics. Adapted from[6]. Image produced using Biorender.com

Cancer cells, on the other hand, need to obtain an unlimited capacity to proliferate. To do so, they need to achieve sustained proliferative signaling, evade growth suppression, resist cell death, and enable replicative immortality. Cancer cells constantly stimulate their growth signals, leading to uncontrolled cell division[8]. They can activate telomerase and continue to divide beyond normal cell limits[9]. Cancer cells frequently develop mechanisms to avoid growth suppressors, which would halt their growth[4], and often develop resistance to apoptosis, allowing them to survive and accumulate genetic abnormalities[10].

Cancer cells also interact with their surrounding microenvironment, leading to their restructuration. They can develop strategies to evade immune recognition and destruction and promote angiogenesis to provide the tumor with nutrients and oxygen[5], altering metabolic pathways and promoting rapid tumor growth[6]. Lastly, cancer cells can invade and metastasize to colonize different parts of the body. For this, they can use phenotypic plasticity to escape the state of terminal differentiation and enable several disruptions of cellular differentiation[6].

Behind these processes lie characteristics that allow them to develop. These characteristics can include genomic instabilities, mutations, and non-mutational epigenetic reprogramming. As a result, genomic reorganization and gene expression regulation are modulated[6].

Glioblastoma

The classification of brain tumors has undergone significant changes over the past few decades, with five editions of the World Health Organization (WHO) *Classification of Tumors of the Central Nervous System* being released. Traditionally, in earlier editions, brain tumors have been classified based on their histopathological features and immunohistochemical expression of lineage-specific proteins. Tumors have been further classified into astrocytomas, oligodendrogliomas, and ependymomas with grades I-IV[11,12]. Further revisions emphasized the importance of molecular markers and introduced classification that considered both histopathological and molecular markers[13,14]. In the 2016 revision, the WHO classified glioblastoma (GB) by combining histopathological and molecular characteristics. GB was then classified into three subtypes based on the presence or absence of isocitrate dehydrogenase (IDH) mutations: GB IDH-wildtype (90% of the tumors), IDH-mutant (10% of the tumors), and GB IDH-NOS for cases where IDH mutation evaluation could not be performed. In the 2021 edition, the new WHO classification separates diffuse gliomas based on their IDH mutation status. IDH-mutant tumors, which were previously classified as glioblastoma IDH-mutant, are now labeled as astrocytomas of grade 2-4 with alterations in *IDH1, IDH2, ATRX, TP53,* and *CDKN2A/B*. IDH-WT glioblastomas are classified as grade 4 and are characterized by *TERT* promoter, chromosomes 7/10, and *EGFR* alteration[15].

GB is the most common malignant brain tumor, accounting for approximately 15% of all intracranial neoplasms and about 50% of all primary malignant brain tumors[16,17]. The annual incidence is around 3 to 4 cases per 100,000 population[17]. GB can affect individuals of any age but primarily impacts older adults, with the highest incidence occurring in patients aged 55 to 85 years. This rapidly progressing disease presents symptoms that largely depend on the tumor's location, mainly manifesting as neurological deficits such as hemiparesis and aphasia, along with tumor-associated edema that results in increased intracranial pressure[18,19]. GB's notorious ability to invade nearby brain structures makes complete resection impossible[20].

The standard-of-care (SOC) for glioblastoma includes maximal surgical resection, radiation therapy, chemotherapy, and tumor-treating fields (TTF). Typically, standard radiotherapy is given in daily fractions of 2 Gy for 30 days, totaling 60 Gy, along with concomitant temozolomide (TMZ) from the first to the last day of radiotherapy, followed by six cycles of adjuvant temozolomide[21]. Optune® (tumor-treating fields) consists of low-intensity, intermediate-frequency, alternating electrical fields that disrupt cancer cell division[22]. Despite numerous advances in the field, glioblastoma remains incurable, with a median overall survival of 12-18 months, and the 5-year survival rate remains at 7%[23].

The majority of GB research has traditionally focused on bulk tumor analysis, primarily due to the accessibility of core tumor tissues during surgical resection. This emphasis, however, overlooks the critical role of infiltrating tumor cells located at the tumor margins, which are often responsible for recurrence and therapeutic resistance. Studying these peripheral "edge" cells presents significant challenges, as they intermingle with healthy brain tissue, making surgical extraction difficult and limiting the availability of pure samples for analysis. Despite these obstacles, some studies have successfully investigated the properties of these infiltrating cells. For instance, research has shown that glioblastoma cells at the tumor margin exhibit distinct molecular and phenotypic traits compared to core tumor cells, including altered metabolic profiles and increased resistance to therapy. The Ivy Glioblastoma Atlas Project has provided insights into these differences[24], while other studies have identified key regulatory factors, such as PLAGL1 and CD109[25], that drive the transition from edge to core phenotypes and contribute to tumor progression. Additionally, single-cell sequencing efforts have begun to reveal the heterogeneity within infiltrative populations[26], shedding light on their role in therapeutic evasion. Given the critical function of these cells in disease recurrence, further research into their biology is essential for developing more effective, targeted treatments for GB.

Despite these advancements, studies focusing specifically on infiltrative glioblastoma cells remain scarce, and the number of available samples is often limited due to the difficulty of isolating these cells without contamination from surrounding brain tissue. This scarcity underscores the need for further dedicated research efforts to fully understand the biology of infiltrative tumor populations and develop more effective therapeutic strategies.

Recurrence

Despite extensive progress in understanding GB biology, therapeutic advances have been minimal over the past 20 years[27]. The lack of screening, early detection, and cure by surgery contribute to the high mortality associated with this disease. GB is defined by high proliferation and invasion in the normal neighboring brain. At diagnosis, cancer cells have often infiltrated and migrated to distant brain regions, and these disseminated cells are frequently implicated in treatment failure. However, studies investigating GB recurrence following SOC have shown that 80-90% of recurrences occur in proximity to the primary resection cavity[28], with approximately 80% being exclusively local[28,29]. Recurrence is almost inevitable, leading to a poorer prognosis and a median overall survival of 2-9 months[23].

Recent studies have delved into the molecular landscape of recurrent GB, revealing that these tumors often shift toward a mesenchymal phenotype, which is associated with increased aggressiveness and therapeutic resistance[30,31]. To advance the understanding of GB recurrence, comprehensive datasets such as those developed by the *Glioblastoma Longitudinal AnalySiS (GLASS) Consortium* have provided valuable resources for studying recurrence patterns through integrated genomic and transcriptomic analyses[32].

Glioblastoma tumor microenvironment

GB is characterized by a highly heterogeneous tumor microenvironment (TME) composed of malignant cells, stromal elements, immune cells, an aberrant vasculature, and a dynamically remodeled extracellular matrix. This complex ecosystem sustains tumor growth and drives its invasiveness and resistance to therapy[33,34]. For instance, the disorganized, leaky blood vessels in glioblastoma contribute to hypoxic regions that further exacerbate malignant behavior, while enzymes degrading the extracellular matrix facilitate tumor invasion[35,36,37].

Within this multifaceted TME, microglia and macrophages play a particularly pivotal role. These cells—including brain-resident microglia and infiltrating peripheral macrophages—are actively co-opted by glioblastoma to support tumor progression[38]. Under the influence of tumor-derived signals, they often polarize toward an M2-like, immunosuppressive phenotype that is marked by the secretion of anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor-β (TGF- β), which dampen effective anti-tumor immune responses[38,39]. Moreover, these reprogrammed immune cells

release matrix metalloproteinases that break down the extracellular matrix, thereby facilitating tumor cell migration and further invasion into the surrounding brain tissue[33]. This creates a vicious cycle in which tumor cells and the TME mutually reinforce each other's pro-tumorigenic activities[34].

Collectively, these insights into the tumor microenvironment—especially the role of microglia and macrophages—underscore the potential of reprogramming these cells to shift the balance against tumor progression. Advancements in targeting the molecular pathways that drive their pro-tumorigenic functions promise enhanced therapeutic efficacy and a paradigm shift in glioblastoma treatment, paving the way for innovative interventions that could significantly improve patient outcomes[34,38,40].

Molecular subtypes of glioblastoma

The genomic landscape of glioblastoma is characterized by a wide range of genetic alterations that contribute to tumorigenesis and progression. The most common genetic alterations coincide with three main pathways – receptor tyrosine kinase (RTK) signaling, p53 signaling, and retinoblastoma (RB) signaling[41] (**Figure 2**). 74% of GB harbor aberrations in all three pathways, indicating that deregulation of these is a fundamental requirement for GB pathogenicity[41]. The most frequent genetic alterations in glioblastoma are the gain of chromosome 7 and the loss of chromosome 10. These are commonly associated with *EGFR* amplification, which leads to its constitutive activation. Aberrant *EGFR* signaling results in increased cell proliferation, survival, and invasion, contributing to tumor growth and aggressiveness[42]. Another critical genetic alteration in glioblastoma involves the tumor suppressor gene *TP53*. Mutations in *TP53* are found in nearly 90% of cases of GB[41] and are associated with resistance to therapy and poor prognosis. Loss of *TP53* function can lead to unchecked cell division, impaired DNA repair, and increased potential for genomic instability, promoting tumor growth and survival[43].

Additionally, alterations in the *PTEN* gene are prevalent in glioblastoma and are often associated with poor outcomes. *PTEN* acts as a negative regulator of the PI3K-AKT-mTOR signaling pathway, which plays a central role in cell survival and growth. Loss of *PTEN* function results in hyperactivation of this pathway, leading to increased cell proliferation and survival[44]. The genetic landscape of GB is further diversified by other genetic alterations, such as alterations in the *RB1*, *CDKN2A/B*, *NF1*, and *IDH1/2* genes[45].

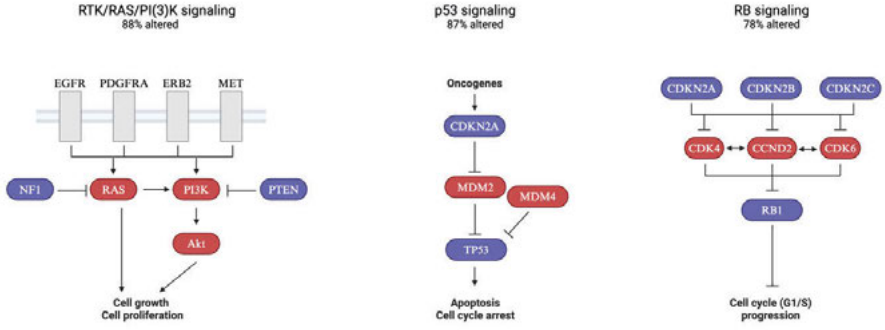

Figure 2: Alterations in the three main signaling pathways in glioblastoma. Red indicates the activation of genetic alterations, while blue indicates the inactivation of genetic alterations. Adapted from[41]. Image produced using Biorender.com

Furthermore, emerging evidence suggests that GB can exhibit intratumoral genetic heterogeneity, with different regions of the tumor harboring distinct genetic alterations[46]. Understanding the genetic diversity within GB is crucial to developing personalized treatment strategies that target specific molecular aberrations.

Glioblastoma can be further classified into molecular subtypes based on gene expression profiles. The number and definition of subtypes have evolved[42,45,47,48], but currently, GB tumors are classified as classical, mesenchymal, or proneural[48]. The classical subtype is characterized by 100% loss of chromosome 10 and amplification of chromosome 7. It is often associated with aberrant expression and amplification of *EGFR*. The classical subtype is often characterized by homozygous deletions of *CDKN2A* and the expression of neuronal stem cell markers[45]. The mesenchymal subtype is defined by *NF1* and *PTEN* deletions[45] and is associated with more aggressive, invasive, inflammatory, and therapy-resistant features[49–51]. The proneural subtype is associated with alterations in the *PDGFRA, IDH1*, and *TP53* genes and is characterized by increased markers of neuronal differentiation[45].

TP53

TP53, often referred to as the "guardian of the genome," plays a crucial role in maintaining cellular integrity by regulating cell cycle, DNA repair, and apoptosis[52]. In GB, *TP53* mutations are common and frequently lead to the loss of function of this tumor suppressor, contributing to tumorigenesis[53]. *TP53* mutations are found in approximately 30-40% of GB cases and are associated with poor prognosis, therapy resistance, and enhanced tumor

invasiveness[54,55]. A key feature of the *TP53* role in tumor suppression is the regulation of cell cycle progression through two primary mechanisms: the activation of p21 and the inhibition of MDM2[52].

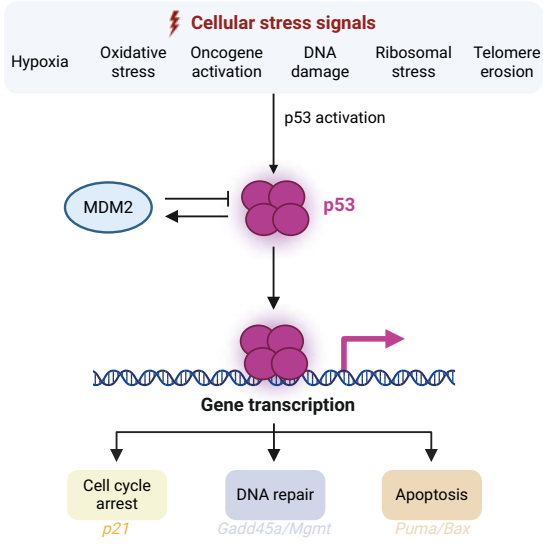

Figure 3: TP53 pathway. Under cellular stress, the p53 protein accumulates and assembles into a tetramer, initiating the transcription of multiple downstream genes involved in key cellular processes such as cell cycle arrest, DNA repair, and apoptosis. Loss of p53 tumor-suppressor function due to mutation, deletion, or inhibition permits the proliferation of damaged cells, potentially leading to tumor formation. Additionally, p53 and MDM2 engage in an autoregulatory feedback loop, where p53 induces MDM2 expression, and MDM2, in turn, inhibits p53 activity by blocking its transcriptional function. Adapted from[58]. Image produced using Biorender.com

Upon activation, *TP53* transactivates the expression of p21 (*CDKN1A*), a cyclin-dependent kinase inhibitor that blocks cyclin-CDK complexes, inducing cell cycle arrest at the G1/S checkpoint[56]. This allows for DNA repair before the cell proceeds with division, preventing the replication of damaged DNA[57]. In parallel, *TP53* regulates *MDM2*, an E3 ubiquitin ligase that normally keeps *TP53* levels in check by promoting its degradation via proteasome[58]. However, in response to DNA damage, *TP53* is stabilized and activated, upregulating p21 and inducing a feedback loop that also impacts *MDM2* expression. This feedback ensures that *TP53* activity is carefully controlled to prevent excessive or inappropriate cell cycle progression[52,59] (**Figure 3**).

In GB, mutations in *TP53* disrupt this pathway, preventing proper cell cycle arrest and leading to uncontrolled cell proliferation[60,61]. Furthermore, elevated *MDM2* expression is observed in some GBs, further exacerbating *TP53* dysfunction and contributing to tumor aggressiveness and resistance to treatment[62,63]. The loss of functional *TP53* and dysregulation of its interaction with p21 and *MDM2* are key factors in the development of GB[41]. As a result, targeting this pathway—such as through small molecules that inhibit *MDM2* or

restore *TP53* function—has become a promising avenue for therapeutic development in glioblastoma[64–68].

Glioblastoma stem cells

Besides genetic and epigenetic heterogeneity, GB displays significant cellular heterogeneity, with various cell populations coexisting within the tumor[69]. For many years, the existence of diverse cell types, including tumor cells, glioblastoma stem cells (GSCs), differentiated tumor cells, and non-tumor cells such as endothelial cells, macrophages, and other immune cells, has been considered a primary contributor to tumor heterogeneity and therapeutic resistance[70].

GSCs represent a small subpopulation of cells within the tumor that share characteristics with neural stem cells[71]. These cells can self-renew and differentiate into different cell types found in the tumor[72], and new tumors can be initiated when transplanted into mice[73]. GSCs were considered the driving force behind tumor initiation and resistance[71] , as they can survive therapy and give rise to a new population of tumor cells[72]. In addition to GSCs, GB tumors contain differentiated tumor cells with varying levels of differentiation. These cells exhibit diverse phenotypic traits, including proliferation, migration, and invasion capabilities. Subpopulations of highly migratory tumor cells have been identified and are believed to play a crucial role in tumor invasion and metastasis[74].

The traditional cancer stem cell (CSC) model posits a hierarchical organization in GB, where a distinct subpopulation of CSCs drives tumor heterogeneity and growth. However, recent research has challenged this perspective, emphasizing the role of cellular plasticity in GB. Studies have shown that GB can transition between different states in a non-hierarchical, reversible manner, influenced by cues from the microenvironment. This dynamic interconversion implies that all tumor cells may contribute to tumor propagation and resistance rather than merely a specific CSC subset[75–77].

Glioblastoma cell states and dynamics

With the development of new technologies, such as single-cell RNA sequencing, the complex heterogeneity of glioblastomas became evident at the single-cell level. In 2014, a study by *Patel et al*[26] showed that cells from patient tumors with distinct transcriptional profiles could be clustered into four meta-

modules representing cellular processes such as the cell cycle and hypoxia. Several subsequent single-cell studies have further classified GB cell states[78–81]. The most prominent classification was introduced by *Neftel et al.* in 2019,[82] which includes four meta-modules where tumor cells can reside and mimic different types of neural cells influenced by the tumor microenvironment. These four meta-modules can be further described as neural progenitor-like (NPC-like) states, characterized by the upregulation of markers of neural progenitors (*DCX, SOX11*). The upregulation of markers of oligodendrocytic lineages, such as *OLIG1* and *OMG,* defines the oligodendrocyte progenitor-like (OPC-like) state. Both NPC- and OPC-like states can be defined as more immature or undifferentiated states.

The astrocyte-like (AC-like) state is defined by the expression of markers typical of normal astrocytes and radial glia, including *GFAP, SB100*, and *HOPX.* In contrast, the mesenchymal-like (MES-like) state is characterized by the expression of genes typically found in non-cancerous cells or cells undergoing mesenchymal processes, such as *VIM* and *ANXA*. Unlike NPC- and OPC-like states, AC- and MES-like states are associated with more mature and differentiated states.

This study also highlighted the plasticity of these cells, with multiple transitions observed between the four states. The tumor initiation capacity of the meta-modules cell population was further evaluated through orthotopic xenografts of sorted cells. Interestingly, regardless of the selected cell population to initiate the PDX, the resulting tumor contained all the initial states in a similar distribution. This plasticity could also be recapitulated in human glioblastoma, as primary samples infected with lentiviruses harboring unique barcodes were injected, leading to the identification of human GB cells in the resulting tumors that shared genetic barcodes and were distributed across different cell states.

To further study the underlying heterogeneity of GB, methods such as single-cell ATAC-seq can help identify different populations based on their chromatin accessibility profiles. In fact, by analyzing the landscape of chromatin accessibility, one can infer the activity of different transcript factors in individual cell populations, as well as identify enhancers and promoters that are active in specific populations of GB[83]. This type of information can provide insight into possible regulatory networks and elements that control the development and progression of GB[84]. Integrating single-cell ATAC-seq data with other single-cell omics data can offer a comprehensive view of cellular

heterogeneity and potential regulatory mechanisms in GB. In 2019, *Wang et al.*[85] performed single-cell RNA and single-nuclei ATAC-seq of glioblastoma samples. When analyzing the samples, the authors noted that glioblastoma follows a single axis of variation between the mesenchymal and proneural subtypes, further supported by the identification of transcription factors associated with both proneural and mesenchymal subtypes.

In addition to differences in tumor composition, glioblastomas can exhibit variations in genetic mutations, gene expression patterns, and cellular composition across different regions of the tumor. In 2013, *Sottoriva et al*[86] described a multiregional sampling of tumor fragments, revealing that most patients displayed different subtypes within the same tumor. Another study later discovered evidence of regional mutational heterogeneity in multiple tumors affecting clinically relevant genes such as *TP53* and *RB*[87]. A third study examined the molecular heterogeneity of GB using biopsies obtained through neuronavigation in four distinct areas of the tumor. *Aubry et al*[88] conducted a genome-wide screening across three molecular levels (genome, transcriptome, and methylome) and found that glioblastomas have a pronounced intratumor architecture that reflects the surgical sampling.

Together, GB's heterogeneity is highly complex and consists of several levels. Not only is the tumor composed of different cell types, but the same cells can occupy different states at different times. To further increase the complexity, different regions of the tumor can exhibit different genetic mutations, gene expression patterns, and even methylation profiles.

Models of glioma

Given the poor prognosis associated with glioblastoma, the search for innovative therapies that truly enhance survival rates has become the primary objective. There is a significant need for models to study the disease that can deliver reproducible and reliable results efficiently. However, selecting the optimal model that reflects the complexity of the tumor system is challenging. An effective model should be user-friendly, disease-relevant (i.e., recapitulating genetic alterations, etiology, heterogeneity, etc.), and account for tumor-host interactions and the role of the tumor microenvironment[89]. The most suitable model may vary depending on the study or research question. Overall, when studying glioblastoma, researchers can employ short-term cultures, long-term cultures, organoids, and xenografts. To date, most knowledge about glioblastoma has been derived from bulk tumor data.

Patient-derived cell lines (PDCs)

One of the most important tools in studying GB has been the use of cell cultures. They play a fundamental role in understanding basic glioma biology and conducting controlled experiments. Researchers can easily manipulate these cells to investigate various molecular and cellular processes, such as proliferation, migration, and drug responses. Although they are easy to culture and study, cell lines lack the complexity of the tumor microenvironment in living organisms[90]. Additionally, they can acquire genetic changes over time, potentially leading to deviations from the original tumor characteristics[91].

The first studies using glioma cell line models used cell lines derived from rats[92] and mice[93] tumors of the central and peripheral nervous system. Later, glioblastoma cells were immortalized and cultured[94]. This contributed to a better understanding of the biology of the disease. Until now, the glioblastoma cell lines U87MG, U252, T98G, and LN-229 remain the most widely used[95]. While a fast way to obtain results and reproducibility, cell lines do not provide a reliable model to understand the underlying mechanism of GB as they fail to recapitulate the complexity of the tumor microenvironment. In addition,

successive passaging of the cell lines selects cells with the highest fitness, decreasing the heterogeneity that is characteristic of the original tumor. Selection imposed by cell passaging can result in genetic drift, accumulation of chromosomal alterations, and potential phenotypical alterations[96,97]. Nevertheless, the use of cell lines continues to be widespread in cancer research globally.

Due to the extensive heterogeneity characteristic of glioblastoma, the use of primary cell cultures derived from patients has been accepted as a standard for studying GB *in vitro*. All traditionally used glioma cell lines are grown in media containing serum. Tumor cells grown in a serum-containing media lose their tumor stem cells, resulting in the growth of cells that are vastly different from the original tumor[98]. To bypass this problem, patient-derived cell lines can be maintained in a serum-free medium supplemented with fibroblast growth factor (bFGF) and epidermal growth factor (EGF)[98]. Cells grown under serum-free conditions resemble the primary tumor[98] more closely. However, it is important to point out that the useful life of patient-derived cell lines is limited. After 20-30 passages, cell lines start to exhibit transcriptional and genomic changes in metabolic and signaling pathways (e.g. the Wnt signaling pathway[91]).

In 2015, *Xie et al.*[99] developed a library of patient-derived cell lines that can be maintained under serum-free conditions to contribute to GB-relevant model systems. This library, called the *Human Glioblastoma Cell Culture* (HGCC) resource, is a biobank composed of cell lines that have been studied and are tumorigenic, present common glioblastoma genetic alterations, and represent all the defined molecular subtypes.

Patient-derived xenograft models (PDXs)

Animal models have long been used to study tumor development upon the engraftment of human cells into immunodeficient mice. Different murine models can be used according to their immune response: i) nude mice without T-cell production, ii) non-obese diabetic combined immunodeficiency (NOD-SCID) mice that lack both B-cells and T-cells, and iii) NOD-SCID IL2R-**γ** mice that lack T-cells, B-cells, and NK cell activity[89]. Glioblastoma implantation can be performed on the flank or in the brain, which can provide the CNS environment[95]. PDXs are particularly valuable for pre-clinical drug testing and for understanding the behavior of gliomas in a more complex environment. However, generating and maintaining PDX models is resource-

intensive, as they require specialized animal facilities, and the lack of a functional immune system in the mice may influence tumor behavior.

Genetically engineered mouse models (GEMMs)

As mentioned above, several genetic alterations have been found in GB. Some of these mutations are common to all cancers, while others are glioblastoma-specific. The study of these genetic alterations has led to the development of genetically engineered mouse models. By introducing these mutations, one can study, for example, how they contribute to tumor initiation, progression, and response to treatment[100]. Transgenic mouse models and knockouts can be used to reproduce preclinical characteristics of the disease, such as genetic and epigenetic alterations in tumor suppressor genes or activation of oncogenic pathways[101].

Furthermore, the transduction of genes with viral vectors such as RCAS[39,102] can be used to reproduce the development of the disease. Contrary to patient-derived xenografts, GEMMs allow GB to develop in mice with an intact immune system and do not alter the blood-brain barrier upon injection[100].

Genetically engineered mice offer insight into the genetic underpinnings of glioblastoma and their interaction with the tumor microenvironment. However, the generation of these models involves genetic manipulation, and the resulting tumors may not fully recapitulate the diversity of genetic alterations found in glioblastoma.

Nonmutational epigenetic reprogramming

Epigenetics refers to the non-genetic information that is inherited from parents to offspring. With the advancement of high-throughput sequencing technologies, various mutations in epigenetic regulators have been identified as key drivers of tumorigenesis[103]. These mutations contribute to cancer-related traits independently of genetic variations, both at the onset of cancer and during its progression[104,105]. Among these non-genetic variations are chromatin variants and the presence of specific epigenomic marks. These marks include DNA methylation, histone modifications, histone variants, and nucleosome positioning[106]. The precise coordination of these epigenomic marks is essential for regulating gene expression and maintaining cell identity (**Figure 4**). As a result, the disruption of these marks is a common characteristic of cancer.

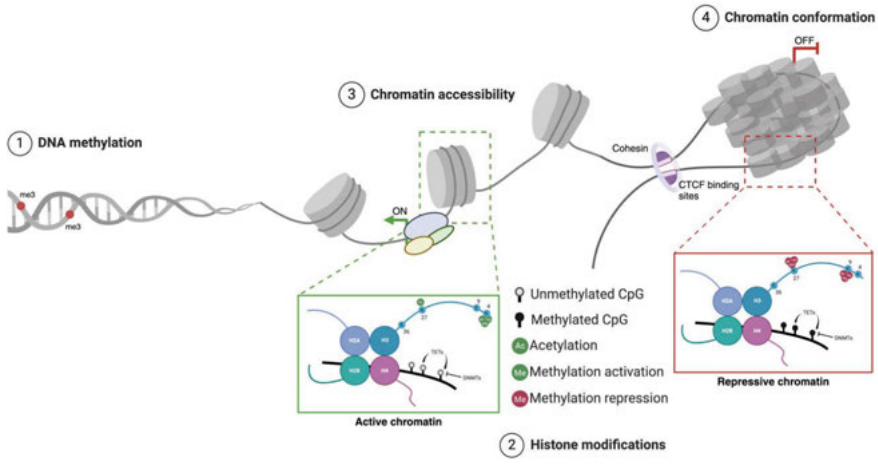

Figure 4: Overview of epigenetic modifications. (1) DNA methylation, where methyl groups are covalently added to the cytosine residues located 5' to a guanine residue in CpG dinucleotides and their derivatives. (2) Histone modifications, which involve covalent post-translational modifications such as trimethylation (me3) or acetylation (ac) of the N-terminal tails of histones. (3) Chromatin accessibility; and (4) Chromatin conformation relaying to the three-dimensional conformation of the genome organization. Adapted from[106,161]. Image produced using Biorender.com

DNA methylation

DNA methylation is a direct chemical modification of DNA in which a methyl group is added to cytosine residues[107], primarily at CpG sites[108,109]. This process is catalyzed by a family of DNA methyltransferases (Dnmts), including Dnmt3a and Dnmt3b, which establish new methylation patterns on previously unmethylated DNA, and Dnmt1, which maintains existing methylation patterns during DNA replication (**Figure 5**)[107]. DNA methylation plays an essential role in gene expression regulation, transposable element silencing, genomic imprinting, and X chromosome inactivation[107].

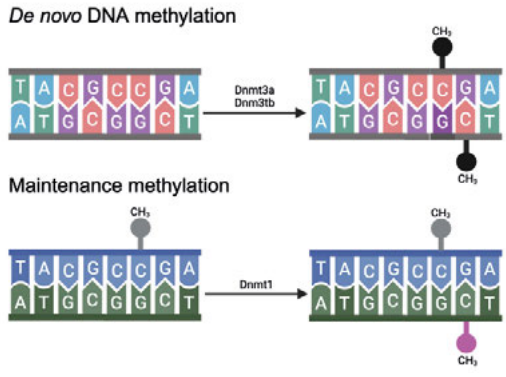

Figure 5: DNA methylation. DNA methyltransferases (Dnmts) catalyze the addition of a methyl group to the fifth carbon of cytosine, forming 5-methylcytosine (5mC). Top: De novo methyltransferases, Dnmt3a and Dnmt3b, introduce new methylation marks (black) onto unmethylated DNA strands. Bottom: Dnmt1 functions as a maintenance methyltransferase, preserving DNA methylation patterns during replication. As DNA replicates, the parental strand (blue) retains its original methylation pattern (gray), while Dnmt1 restores symmetry by adding methyl groups (purple) to the newly synthesized DNA strand (green). Adapted from[107]. Image produced using Biorender.com

CpG islands, which are approximately 1000 base pairs long and enriched in CpG sites, are often found in gene promotors and are typically unmethylated to facilitate transcription[110–113]. When methylated, CpG islands recruit repressive methyl-binding proteins and inhibit gene expression, leading to stable transcriptional silencing[114]. Tissue-specific DNA methylation is more common in CpG island shores – regions adjacent to CpG islands – and plays a significant role in gene regulation[115]. DNA methylation is highly conserved across species[112] and is essential for development and differentiation[116,117].

Aberrant DNA methylation is implicated in various diseases, including cancer[118,116]. In GB, distinct methylation signatures can guide tumor classification. Methylation profiling has critical clinical applications in cancer diagnosis and prognosis[119]. Methylation profiling, combined with next-generation sequencing, enables the stratification of pediatric brain tumors into clinically relevant subgroups[120].

GB can be further categorized into six DNA methylation-based clusters (M1–M6), which correlate with specific genomic features such as copy-number alterations, patient age at diagnosis, and somatic mutations[42]. Additionally, a pan-glioma methylation analysis has identified six distinct methylation subtypes (LGm1–LGm6)[121], highlighting the heterogeneity of GB. Notably, methylation-based clustering aligns only with the *IDH*-mutant subtype, which overlaps with the proneural classification[122].

Histone modifications

Histone modifications play a key role in gene regulation by altering chromatin structure and accessibility. The nucleosome - the fundamental unit of chromatin - consists of DNA wrapped around a histone octamer[123]. Post-translational modifications (PTMs) of histone tails, such as acetylation, phosphorylation, methylation, SUMOylation, and ubiquitination, influence gene expression by modulating chromatin dynamics[124,125].

Histone methylation occurs primarily at lysine (K) residues of histones H3 and H4, with different methylation states (me1, me2, me3) serving as either activating or repressive marks[126–128]. H3K4, H3K36, and H3K79 methylation are generally associated with active transcription, whereas H3K9, H3K27, and H4K20 methylation are linked to gene repression and condensed chromatin states[129].

Bivalent chromatin regions, characterized by the simultaneous presence of activating (H3K4me3) and repressive (H3K27me3) marks, are crucial for gene regulation and cellular differentiation[130,131]. In embryonic stem cells, these bivalent domains maintain genes in a poised state, allowing rapid activation or repression during development[132].

H3K4 methylation is primarily enriched at enhancers, promoters, and transcription start sites (TSSs)[133]. H3K4me1 is predominantly found at enhancers, where its association with H3K27ac or H3K27me3 marks active or repressive enhancers, respectively[134,135]. H3K4me3 is typically enriched at promoter regions of actively transcribed and poised genes[136].

H3K27 methylation is a hallmark of gene repression. H3K27me3 is enriched at poised enhancers and silenced gene promoters, playing a key role in repressing developmental genes[137,138]. H3K27me2 marks both active and repressed gene promoters, while H3K27me1 is found at actively transcribed

gene promoters[139]. The Polycomb Repressive Complex 2 (PRC2) complex catalyzes H3K27 methylation, preferentially targeting nucleosomal histone H3K27[140].

Histone acetylation, regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), reduces the positive charge of lysine residues, weakening histone-DNA interactions and promoting transcriptional activation[141]. H3K27ac is a well-studied active histone mark, localized at promoters and enhancers of actively transcribed genes, often co-existing with H3K4me3[142,143]. Enhancers can be categorized into three groups based on H3K27 and H3K4 status: active enhancers (H3K27ac and H3K4me1), poised enhancers (H3K27me3 and H3K4me1), and primed enhancers (H3K4me1 only)[144].

Crosstalk between epigenetic mechanisms

Epigenetic mechanisms, including DNA methylation, histone modifications, and non-coding RNAs, interact to regulate gene expression and maintain cellular identity[106]. These modifications influence chromatin structure and transcriptional activity without altering the underlying DNA sequence.

There is significant crosstalk between DNA methylation and histone modifications. DNA methylation is often linked to histone PTMs, with Dnmts recognizing specific histone marks[145]. Repressive histone modifications, such as those catalyzed by Polycomb group proteins (PRC1 and PRC2), can precede DNA methylation, suggesting a coordinated mechanism for gene silencing[146,147]. Additionally, proteins that recognize methylated DNA can recruit histone deacetylases, reinforcing transcriptional repression[148].

H3K4 methylation is associated with active gene promoters and can prevent DNA methylation[149], while H3K27 methylation is linked to gene silencing and often correlates with DNA methylation at CpG islands[150] (**Figure 6**). Some histone-modifying enzymes, such as KDM2B, protect CpG islands from *de novo* methylation, ensuring proper gene regulation[151,152]. These interactions between histone modifications and DNA methylation contribute to stable but dynamic gene expression patterns during development and in response to environmental signals.

High-throughput sequencing

High-throughput sequencing, also known as next-generation sequencing (NGS), has revolutionized numerous fields in biology by providing rapid and cost-effective methods to analyze genetic material. In genomics, it enables whole-genome sequencing, variant discovery, and comparative genomics, which have deepened our understanding of genetic diversity and disease susceptibility[153–155]. In transcriptomics, RNA-seq offers detailed analyses of gene expression and alternative splicing, shedding light on complex cellular processes[156]. Epigenomic studies have similarly benefited from NGS with methods such as ChIP-seq for mapping DNA-protein interactions[157], ATAC-seq for assessing chromatin accessibility[158], and DNA methylation profiling for characterizing epigenetic modifications across the genome[159], while single-cell analysis techniques have allowed to explore cellular heterogeneity at an unprecedented resolution[160].

Epigenomic technologies

Epigenetics has transformed our understanding of gene regulation by offering insights into the complex interactions between chromatin structure and gene expression. Unlike genetic modifications, epigenetic mechanisms regulate gene activity through chemical changes to DNA, histones, and chromatin accessibility. These modifications play a critical role in cellular differentiation, development, and disease pathology.

To further investigate these regulatory landscapes, various high-throughput epigenomic technologies have been developed[161]. These techniques enable researchers to map chromatin accessibility, histone modifications, and DNA-protein interactions across the entire genome, offering a comprehensive view of gene regulatory mechanisms.

Among these methods, the Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq)[83] and Cleavage Under Targets and Tagmentation (CUT&Tag)[162,163] have emerged as powerful and effective tools for studying chromatin dynamics.

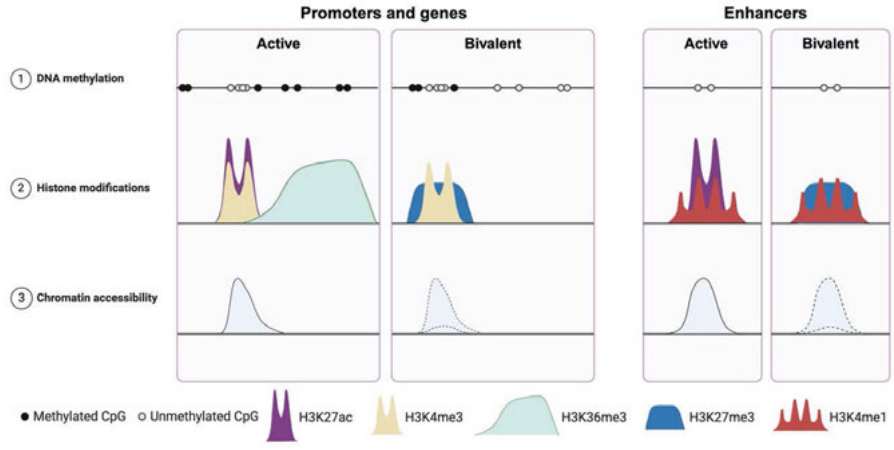

Figure 6: Epigenomic modifications measured using epigenomic technologies. Promoters of actively expressed genes exhibit low DNA methylation, elevated H3K27ac, and high histone H3 trimethylation at lysine 4 (H3K4me3) and are found in accessible chromatin. Actively transcribed gene bodies are enriched in H3K36me3 and display intermediate DNA methylation levels. Poised or bivalent promoters contain both active (H3K4me3) and repressive (H3K27me3) histone marks and may or may not be located in accessible chromatin. Active enhancers are distinguished by low DNA methylation, high levels of histone H3 monomethylation at lysine 4 (H3K4me1) and H3K27ac, and increased chromatin accessibility. Similar to bivalent promoters, bivalent enhancers possess both active (H3K4me1) and repressive (H3K27me3) histone marks. Adapted from[161]. Image produced using Biorender.com

ATAC-seq

ATAC-seq is a powerful molecular tool for studying the accessibility of chromatin across the genome[83]. Chromatin accessibility refers to whether a specific region of DNA is open and accessible to transcription factors, RNA polymerase, and other cellular machinery to bind and regulate gene expression[164]. This can lead to insights into which genes are likely to be actively regulated[165] and how chromatin accessibility plays a role in various biological processes, such as cancer[166]. ATAC-seq leverages a hyperactive Tn5 transposase enzyme to fragment and tag the entire genome. The fragmented DNA is PCR-amplified and sequenced[83] . Regions with higher chromatin accessibility undergo more tagmentation. After sequencing, data analysis involves mapping the reads to the genome to pinpoint open chromatin regions – evaluated based on the number of reads counts after mapping to the genome - and investigating motif enrichment[167].

Several reports in recent years have overlooked chromatin accessibility and the epigenetic landscape in GB. In 2021, *Guilhamon et al.*[168] examined

chromatin accessibility at the single-cell level in four glioblastoma tumors. The authors identified three states defined by unique transcription factors present in varying proportions within the tumors. Another study by *Lu et al.*[169] compared nine mouse cell lines, each with different origins, to sixty patient-derived glioblastoma cell lines. The authors not only found that the assay could distinguish the mouse cell lines based on their origin, but they also identified three clusters among the human samples based on their chromatin accessibility profiles, each exhibiting different phenotypic properties and patient survival outcomes.

CUT&Tag

Chromatin immunoprecipitation with sequencing (ChIP-seq) faces challenges such as low signals, high background, and the necessity for a high number of cells[170]. Alternatives like CUT&RUN offer improved specificity and lower background levels but still require extensive library preparation and are not ideal for single-cell applications[171]. To address these limitations, CUT&Tag uses a hyperactive Tn5 transposase fused with Protein A (ProteinA-Tn5), enabling targeted tagmentation of chromatin components directly in live cells, producing sequence-ready libraries quickly and efficiently, even from low cell numbers or single cells. Briefly, the cells are fixed and incubated with a primary antibody specific for the target protein or histone modification of interest, followed by incubation with a secondary antibody. The cells are treated with transposomes (ProteinA-Tn5) and NGS adapters. After, any unbound transposomes are washed away. As Tn5 needs Mg2+ to work, this is added to start the process, cutting the chromatin near where proteins are bound and adding the NGS adapter DNA sequences at the same time. This allows for chromatin cutting and library preparation to happen in one step[162,163,172].

CUT&Tag has proven to be a transformative tool in cancer research, particularly in understanding epigenetic regulation and tumor heterogeneity[172,173]. Studies have demonstrated its utility in mapping oncogenic fusion proteins in leukemia, uncovering both common and subtype-specific chromatin regulatory mechanisms[172]. Similarly, research on histone mutations in gliomas has leveraged CUT&Tag to reveal how epigenomic alterations drive tumor progression and therapy resistance[173]. These findings highlight CUT&Tag's ability to profile chromatin modifications with high sensitivity, making it an essential tool for studying drug resistance, tumor evolution, and potential epigenetic vulnerabilities.

Single-nuclei multiome sequencing

The Chromium Multiome ATAC + Gene Expression platform enables the simultaneous profiling of chromatin accessibility and gene expression within the same single cell. This integrated approach allows to directly link gene expression patterns with regulatory elements, facilitating the discovery of novel gene regulatory interactions.

The workflow begins with the isolation of nuclei from fresh or frozen tissue samples. These nuclei undergo a transposition reaction using the Tn5 transposase, which preferentially inserts sequencing adapters into regions of open chromatin. Subsequently, the transposed nuclei are encapsulated into Gel Beads-in-Emulsion (GEMs) within the Chromium instrument. Each gel bead carries unique barcoded oligonucleotides that tag both transposed DNA and captured mRNA transcripts from the same nucleus. After reverse transcription and amplification, separate libraries are constructed for ATAC and gene expression profiling. These libraries are then sequenced, and the resulting data is analyzed to map chromatin accessibility and gene expression profiles back to individual cells. This comprehensive single-cell multiomic analysis provides insights into the regulatory mechanisms governing gene expression[174].

This technology has been applied in various studies, such as investigating intra-tumoral heterogeneity in canine osteosarcoma[175], developing regression models to identify functional regulatory elements[176], and optimizing nuclei isolation methods for multiomic single-cell assays[177].

Present investigations

The current investigations in this thesis explore the complex molecular and phenotypic characteristics of GB, focusing on the interplay between epigenetic regulation, lineage specificity, and tumor microenvironment interactions. Through comprehensive multiomic analyses, these studies provide novel insights into the heterogeneity and aggressiveness of GB, emphasizing the need for targeted therapeutic approaches.

Paper I - Multiome analysis of local peritumoral glioblastoma cells uncovers phenotypically unique subpopulations

GB remains one of the most aggressive and lethal brain cancers, with a median survival of less than 15 months despite advances in surgery, radiotherapy, and chemotherapy. A major clinical challenge is the tumor's highly invasive nature and its propensity to recur near the resection cavity[78,178]. This recurrence pattern suggests that the peritumoral region—specifically the infiltrative margin beyond the contrast-enhancing tumor core—harbors cells that are resistant to conventional treatments. Yet, these cells have been understudied compared to those in the tumor bulk, leaving a significant gap in our understanding of GB relapse mechanisms.

To address this, we collected paired tissue samples from both the central tumor mass and the invasive edge (beyond the 5-ALA fluorescent border) in eleven GB patients. This careful sampling preserved tissue integrity and minimized contamination between regions, allowing for a robust multi-omic analysis employing single-nucleus RNA sequencing (snRNA-seq), single-nucleus ATAC sequencing (snATAC-seq), and whole exome sequencing (WES) to compare the two locations.

Functionally, the invasive edge cells were found to be markedly different from bulk tumor cells: while they exhibit significantly higher invasive properties, they are less capable of self-renewal and tumor propagation. Genomic analysis

revealed that these edge cells harbor fewer genetic alterations, indicating they are evolutionarily earlier and suggesting that their progression is primarily driven by epigenetic, rather than mutational, changes. Phylogenetic reconstruction showed that most subclones in the core tumor are also present at the edge, pointing to continuous cellular exchange between these regions.

At the molecular level, snRNA-seq highlighted a shift from the less differentiated, stem-like states of the core cells to more differentiated, injury-like states in the edge cells. This shift coincides with an upregulation of genes associated with invasion, immune activation, and mesenchymal transformation. In parallel, snATAC-seq analysis uncovered distinct regulatory circuits: while core cells are enriched for transcription factors linked to stemness (such as OLIG1, OLIG2, and SOX2), edge cells display motifs related to inflammation, immune response, and invasion (including TP53, NFKB, and interferon regulatory factors). These differences underscore the unique, immune-activated, and mesenchymal-like phenotype of the invasive edge cells.

Collectively, these findings suggest that the peritumoral GB cells, although less proliferative than bulk tumor cells, possess distinct molecular vulnerabilities. Targeting their immune-driven epigenetic reprogramming and the associated regulatory networks may offer a promising therapeutic strategy to prevent tumor recurrence and improve patient outcomes.

Paper II - Lineage-dependent epigenetic regulation in glioblastoma is influenced by p53 mutational status

The tumor suppressor protein p53, encoded by the *TP53* gene, plays a crucial role in maintaining genomic stability, regulating cell cycle arrest, apoptosis, and DNA repair. In GB, mutations in *TP53* are common and often lead to a loss of tumor suppressor activity or a dominant-negative effect, facilitating tumor progression and resistance to apoptosis. The type of *TP53* mutation can significantly impact the tumor phenotype and its sensitivity to therapy, with *TP53* mutations strongly associating with the mesenchymal subtype of GB, which is more invasive, immune-activated, and resistant to treatment. Epigenetic regulation is a key factor in GB's heterogeneity and therapy resistance. Chromatin accessibility, histone modifications, and enhancer activity collectively define the transcriptional programs that characterize distinct GB subtypes and cellular states. Notably, chromatin accessibility profiles have been shown to predict patient survival better than gene expression patterns, underscoring the importance of epigenetic regulation in GB progression. However,

the interplay between *TP53* mutational status and epigenetic regulation in GB remains poorly understood. This study identifies two epigenetically distinct GB subgroups, ATAC-C2 and ATAC-C3, which exhibit significant survival differences. ATAC-C2 tumors are linked to shorter survival and higher resistance to therapy, while ATAC-C3 tumors have better survival and therapeutic responses. This division is independent of MGMT promoter methylation, a known biomarker for GB therapy prediction, suggesting that other factors—particularly epigenetic—are at play. Key to these differences is the *TP53* mutational status. ATAC-C2 tumors primarily carry disruptive *TP53* mutations that impair p53 signaling, contributing to their aggressive phenotype and therapy resistance. Conversely, ATAC-C3 tumors retain wild-type p53 activity or mutations that preserve p53 function, leading to better clinical outcomes. Further examination reveals that the distinct epigenetic profiles of these subgroups are closely linked to *TP53* mutations. Chromatin accessibility analysis shows that ATAC-C2 tumors exhibit increased accessibility at regions associated with immune-inflammatory and stress response pathways, reflecting a mesenchymal, immune-activated, and invasive phenotype. ATAC-C3, in contrast, shows elevated accessibility at regions associated with cell cycle regulation and p53 signaling, consistent with a less aggressive phenotype. This study also highlights differential therapeutic responses between the two subgroups. ATAC-C2 cells exhibit significant resistance to SOC regimen, while ATAC-C3 cells are more sensitive. Potential therapies targeting this resistance include PRIMA-1, which restores mutant p53 functionality. Finally, we employed Connectivity Map (CMAP) to identify promising treatments for the resistant ATAC-C2 subgroup. Two drugs, MG-132 (a proteasome inhibitor) and SIB-1893 (a mGluR-5 receptor antagonist) show efficacy in reducing ATAC-C2 cell viability, independent of p53 status. These findings open new therapeutic avenues for GB, emphasizing the importance of tailoring treatments to the unique mutational and epigenetic profiles of GB subtypes.

Paper III - Enhancer signatures define glioblastoma subgroups with divergent patient survival

Enhancers are distal regulatory elements that govern the transcriptional programs driving GB heterogeneity. Marked by H3K4me1 and H3K27ac, these chromatin regions serve as critical hubs for lineage-specific transcription factor (TF) binding and loop formation with gene promoters. By integrating ATAC-seq and histone modification (CUT&Tag) data, this study identifies two epigenetically distinct GB subgroups—ATAC-C2 and ATAC-C3—that

display markedly different patient survival outcomes. ATAC-C2 is associated with a more aggressive, mesenchymal-like phenotype characterized by extensive active enhancers, immune-inflammatory gene programs, and elevated resistance to SOC. In contrast, ATAC-C3 exhibits higher levels of H3K27me3-mediated repression, reduced enhancer activity, and a more differentiated neural lineage-like phenotype that correlates with longer survival and enhanced therapeutic responsiveness.

These subgroup-specific enhancer landscapes are more predictive of patient prognosis than traditional transcriptome-based subtyping, underscoring the central role of epigenetic regulation in GB. ATAC-C2 tumors show widespread enhancer-promoter looping that drives the expression of pro-inflammatory and epithelial-mesenchymal transition (EMT) genes, enriched for binding sites of TFs such as NF-κB, STAT3, and TWIST1. Conversely, ATAC-C3 tumors display relatively constrained chromatin interactions, favoring neural progenitor TF networks (e.g., POU3F1, SOX15, FOXC2) and reflecting a more lineage-committed state. Cross-species comparisons between human and mouse GCCs further reveal conserved oligodendrocyte progenitor cell (OPC)-like enhancer signatures in ATAC-C2, pointing to a less differentiated origin with high plasticity. Meanwhile, the more differentiated ATAC-C3 tumors exhibit partially species-specific neural progenitor enhancer networks.

Collectively, these investigations contribute to a deeper understanding of glioblastoma biology by revealing the intricate epigenetic regulation, lineage-specific transcriptional programs, and tumor microenvironment interactions that drive glioblastoma heterogeneity, invasion, and therapy resistance. They provide a comprehensive resource for elucidating the molecular mechanisms underlying glioblastoma progression and highlight novel therapeutic targets. These findings establish a solid foundation for future research aimed at personalized medicine approaches, emphasizing the need for innovative therapeutic strategies tailored to the unique molecular and epigenomic landscapes of glioblastoma subgroups.

Future perspectives

GB is shaped by a complex interplay of epigenetic regulation, genetic alterations, and tumor microenvironment interactions. The distinction between invasive edge cells and core cells, the influence of *TP53* mutational status, and the role of enhancer architectures point to the need for a multifaceted, personalized approach.

Paper I underscored the importance of the peritumoral region—the infiltrative margin that extends beyond the contrast-enhancing tumor core. By comparing matched bulk and edge samples, we showed that peritumoral edge cells are highly invasive yet less proliferative than their counterparts in the tumor core. Notably, edge cells harbor fewer genetic alterations and instead rely on epigenetic reprogramming to drive their invasive behavior. This suggests that targeting the immune-driven and mesenchymal-like states of these cells could prevent local recurrence, which predominantly arises in the peritumoral niche.

Paper II delved into the impact of *TP53* mutational status on GB progression and therapy response. Here, two epigenetically defined GB subgroups designated ATAC-C2 and ATAC-C3, were shown to exhibit significant differences in patient survival and treatment outcomes. ATAC-C2 tumors predominantly harbor disruptive *TP53* mutations, display a more mesenchymal phenotype, and are notably resistant to SOC. In contrast, ATAC-C3 tumors typically retain functional p53 activity and respond better to conventional therapies. This work further highlighted the potential for mutation-specific p53 reactivation strategies, such as the small molecule PRIMA-1, as well as other agents like MG-132 and SIB-1893 that can effectively target the aggressive, p53-impaired ATAC-C2 subgroup.

Paper III expanded on these subgroup distinctions by focusing on enhancer activity and lineage-specific transcriptional programs. Using a multi-omics approach (ATAC-seq, CUT&Tag, and chromatin conformation analyses), we identified how enhancer landscapes more accurately predict patient outcomes than transcriptome-based subtyping. This again delineated two major epigenetic states (ATAC-C2 and ATAC-C3), revealing that ATAC-C2 is driven by

extensive enhancer-promoter looping that activates immune-inflammatory and epithelial-mesenchymal transition (EMT) gene networks. In contrast, ATAC-C3's more restrictive chromatin architecture supports a lineage-committed, neural progenitor-like state correlated with improved survival. Notably, cross-species comparisons in human and mouse glioblastoma stem cells reinforced the idea that these enhancer-driven phenotypes reflect fundamental developmental origins.

Collectively, these findings highlight several promising strategies for improving GB treatment:
Targeting the invasive edge: The peritumoral niche harbors cells that are highly invasive yet less proliferative, relying on epigenetic rather than genomic alterations. Future therapies could focus on modulating the immune-rich and mesenchymal-like microenvironment in this region. Combination treatments that inhibit key epigenetic regulators or disrupt inflammatory signaling pathways may reduce local recurrence by preventing edge-cell infiltration.

Exploiting p53 vulnerabilities: In tumors with disruptive *TP53* mutations (ATAC-C2), reactivating p53 via agents like PRIMA-1 can potentially restore apoptosis pathways. However, because p53 mutations vary among patients, a personalized approach may be necessary—one that screens for specific mutations that can be successfully targeted. Additionally, alternative agents such as proteasome inhibitors (MG-132) or mGluR-5 antagonists (SIB-1893) could offer a broader treatment option in these aggressive, p53-impaired tumors.

Enhancer-driven therapeutics: Given the strong correlation between enhancer signatures and patient survival, therapeutic strategies that disrupt critical enhancer-promoter interactions can hold great promise. Small molecules or biologics targeting lineage-specific transcription factors might selectively weaken the aggressive, enhancer-driven programs in ATAC-C2 tumors. On the other hand, reinforcing the more lineage-committed state of ATAC-C3 tumors could help maintain their responsiveness to SOC therapies, potentially extending survival.

Personalized epigenetic profiling: Routine clinical implementation of chromatin accessibility and enhancer mapping could stratify patients into these epigenetic subgroups, allowing oncologists to customize treatments with better precision. By integrating epigenetic profiling with conventional biomarkers like MGMT promoter methylation, clinicians may identify optimal

therapeutic combinations—for instance, pairing SOC chemotherapy with agents that block specific enhancer circuits or restore p53 function.

Future investigations will likely focus on developing targeted drugs that simultaneously address the immune-activated and mesenchymal phenotypes of aggressive GB subgroups and invasive, relapse-causing edge cells; reestablish tumor-suppressive pathways in p53-deficient contexts; and rewire enhancer-driven transcriptional networks. By capitalizing on these, the field can move closer to more effective therapies that can overcome the profound challenges posed by GB transcriptional and epigenetic heterogeneity and therapy resistance.

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For the longest time, I didn't think I'd ever be writing these words. Finishing my PhD felt like reaching Mount Doom—distant, impossible, and full of orcs. Honestly, it was about as likely as Michael running a successful branch without somehow burning down the building. It took countless sleepless nights, enough self-doubt to make Gollum's inner monologue look optimistic, and more tears than when Michael left Scranton. I look back at these five years, and I don't even recognize myself. And that's not a bad thing. Five years can change a person, just like five seasons can turn Ryan from the temp to a wannabe business mogul to… well, a guy with bleached hair hiding from his responsibilities. My brain is now fully developed (or at least science says so), and I'm in my late 20s. But if I'm being real, deep down, I'm still 18—the age when you think you're Aragorn but are actually more like Pippin. My PhD journey has been a rollercoaster, kind of like the Mines of Moria—exciting, terrifying, and full of surprises. I've had some of my best and worst moments during this ride. And in a twisted kind of way, I'm grateful for all of it. Even the dark times, because, as Samwise Gamgee said, "*It's like in the great stories… Folk in those stories had lots of chances of turning back, only they didn't. They kept going because they were holding onto something.*" If you're reading my thesis and feeling overwhelmed, remember this: ***You hold all the power***. You can close this document anytime, just like you can close the DVD menu of *The Return of the King* when you realize there are seven different endings. But if you decide to keep going, remember: "*Even darkness must pass. A new day will come. And when the sun shines, it will shine out the clearer.*" You can do this because you're incredible. You're like the Sam to someone's Frodo, the Jim to someone's Pam, the Dwight to someone's Michael. And when this journey ends, you'll look back and realize it was totally worth it. Just remember: there's some good in this world, and it's worth fighting for. Or in this case, worth reading for.

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