USCAP

REVIEW ARTICLE

The epigenetic dysfunction underlying malignant glioma pathogenesis

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Comprehensive molecular profiling has dramatically transformed the diagnostic neuropathology of brain tumors. Diffuse gliomas, the most common and deadly brain tumor variants, are now classified by highly recurrent biomarkers instead of histomorphological characteristics. Several of the key molecular alterations driving glioma classification involve epigenetic dysregulation at a fundamental level, implicating fields of biology not previously thought to play major roles glioma pathogenesis. This article will review the major epigenetic alterations underlying malignant gliomas, their likely mechanisms of action, and potential strategies for their therapeutic targeting.

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INTRODUCTION

The advent of high-throughput genomics and molecular profiling technology has dramatically altered both neoplastic and non-neoplastic disease classification, delineating more biologically and clinically uniform entities that often transcend conventional histopathological categorization. This is particularly evident in brain tumor taxonomy, where this fundamental shift in approach has led to both the refinement of existing diagnostic categories and the creation of several novel tumor types. Diffusely infiltrating gliomas, also called diffuse gliomas, remain the most common and deadly primary brain tumors and their taxonomic evolution over the past decade exemplifies the more general trends in diagnostic pathology described above. Neoplastic categories based on the histopathological features of astrocytes and oligodendrocytes have been revised and optimized with the integration of disease-defining molecular markers, stratifying biologically, clinically, and prognostically distinct entities in both adult and pediatric populations. Moreover, above and beyond tumor classification, these developments have revealed novel pathogenic mechanisms, involving epigenetic regulator genes not previously been implicated in oncogenesis. Characterizing this pathobiology is now an active area of research on multiple fronts. This review will describe recent developments in diffuse glioma classification, including those informing the World Health Organization (WHO) 2021 blue book, with an eye towards molecular abnormalities inducing epigenetic dysregulation, their downstream biological consequences, and strategies for therapeutic targeting.

Epigenetic dysregulation defines large subsets of diffuse glioma

Historically, diffuse gliomas have been classified solely on the basis of histological characteristics into high-grade glioblastomas, lower-grade astrocytomas, or oligodendrogliomas, with a portion of lower-grade tumors expressing both glial phenotypes (oligoastrocytomas). Over time, however, it has become clear that morphology alone does not sufficiently predict clinical behavior, with tumor progression varying notably across each histological subtype. More recently, the WHO, informed by large genomic profiling studies like those of the Cancer Genome Atlas, has extensively revised diffuse glioma classification to incorporate highly penetrant molecular abnormalities[1]. Intriguingly, many of these key biomarkers, including mutations in genes encoding isocitrate dehydrogenase enzymes (*IDH1* and *IDH2*), H3 histone monomers (*H3F3A* and *HIST1H3B*), and the histone chaperone α-thalassaemia mental retardation X-linked (*ATRX*), induce disruptions in normal epigenetic functionality, revealing previously unappreciated oncogenic mechanisms.

Mutations in *IDH1/2* represent the most important classifying biomarkers for gliomas, defining lower-grade astrocytomas and oligodendrogliomas in their entirety. IDH mutations, as codified in the WHO 2016 and WHO 2021 blue books (WHO 2016 and 2021), designate predominantly lower-grade (WHO grade 2 and 3) diffuse gliomas as distinct from IDH-wildtype glioblastoma (GBM), the archetypical WHO grade 4 primary brain tumors. IDH-wildtype GBM almost invariably arises de novo in a fully malignant state characterized by the aggressive histopathological features of microvascular proliferation and necrosis; and although exclusively low-grade morphology is occasionally encountered, rapid clinical progression is the rule[1,2]. By contrast IDH-mutant gliomas, while ultimately deadly, typically progress at a more measured pace through repeated cycles of treatment and recurrence, before emerging as high-grade lesions. In adults, IDH-mutant gliomas are further subcategorized by coincident deletion of chromosomal arms 1p and 19q (1p/19q codeletion). Oligodendrogliomas, WHO grades 2 and 3, are now defined by the concurrent IDH mutation and 1p/19q codeletion, and while ultimately deadly, can be associated with extended clinical course, with median survival times exceeding 8 years[1]. By contrast, IDH-mutant

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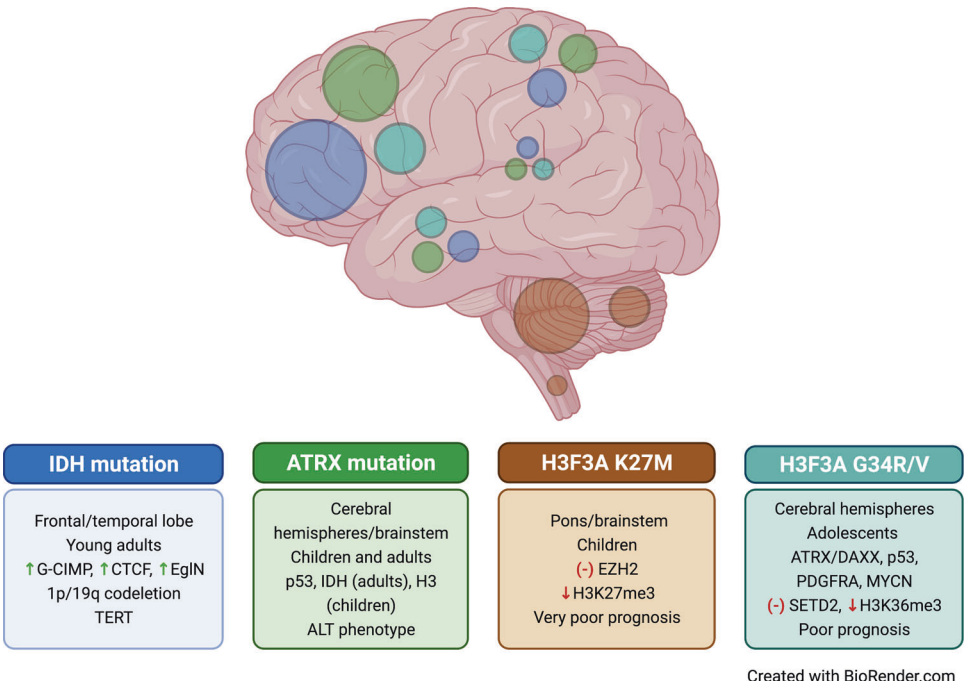

Fig. 1 Hallmark molecular alterations in malignant glioma. IDH mutations are the most common mutation observed in glioma, often presenting in the frontal and temporal lobes. These tumors exhibit the hallmark G-CIMP phenotype, dysregulate the CTCF-binding motif and enzymatic activity of EglN, and often present with 1p/19q chromosomal arm codeletion and *TERT* promotor mutations. IDH mutated, 1p/19q codeleted glioma confer the most favorable survival among malignant glioma. *ATRX* mutations arise in multiple glioma variants, each with distinct clinical behavior, across the adult and pediatric populations. These tumors have concurrent mutations in *TP53*, *IDH1/2* in adults, and H3 in children. Notably, these tumors do not have activating mutations in *TERT* and instead maintain replicative immortality through a homologous-recombination-dependent and telomerase-independent mechanism known as ALT. *H3F3A* K27M-mutant glioma are found in midline regions, particularly the pons and brainstem, and are enriched in the pediatric population. K27M-mutant gliomas are extremely aggressive and confer very poor survival. Unlike K27M-mutant tumors, H3F3A G34R/V-mutant tumors are found strictly in the cerebral hemispheres, primarily in adolescents and young adults, and share marginally improved survival. These mutations are often concomitant with mutations in the *ATRX/DAXX* complex, MYCN, and PDGFRA. Taken together, these hallmark molecular alterations guide glioma classification and are fundamentally driven by potentially targetable epigenetic dysregulation.

astrocytomas, WHO grades 2 and 3, do not harbor 1p/19q codeletion, instead featuring combined loss-of-function mutations in ATRX and TP53 in the vast majority of cases, and exhibit a somewhat more aggressive biological behavior than their oligodendroglial counterparts (Fig. 1)[1,3]. WHO 2021 now also recognizes a grade 4 IDH-mutant astrocytoma, effectively replacing the glioblastoma, IDH-mutant diagnostic category established in WHO 2016. IDH-mutant astrocytomas, WHO grade 4, exhibit the defining molecular features of their lower-grade counterparts along with microvascular proliferation, necrosis, and/or homozygous deletion in CDKN2A/B, the latter having been repeatedly associated with unfavorable prognosis within this glioma subclass[4,5].

Despite similar histopathology, pediatric diffuse gliomas are defined by molecular alterations that only partially overlap with those commonly seen in adults. Of note, mutations in *H3F3A* and *HIST1H3B* are commonly featured as first reported for diffuse intrinsic pontine glioma (DIPG) and hemispheric high-grade glioma[6–8]. H3 mutations come in two major varieties: the first occurring at position 27 as a lysine-to-methionine substitution (K27M) and the second occurring at position 34 as either a glycine-to-arginine (G34R) or a glycine-to-valine (G34V) substitution[6,7,9]. Parenthetically, while the preceding mutational nomenclature is more extensively utilized, WHO 2021 employs a more standard nomenclature that accounts for N-terminal methionine residues in the relevant polypeptides (i.e. K28M and G35R/V). Interestingly, K27M-mutant gliomas arise exclusively in midline structures of the central nervous system (CNS), while G34R/V gliomas are localized to the cerebral hemispheres (Fig. 1)[10]. WHO 2021 has now codified these distinctions into two diagnostic categories, namely diffuse midline glioma, H3 K27M-altered, and hemispheric glioma, H3 G34-altered. Both variants behave aggressively, on the order of WHO grade 4. Intriguingly, both also feature high rates of coincident *ATRX* and *TP53* mutations, drawing parallels with adult glioma counterparts and further emphasizing the profound epigenetic dysfunction likely underlying their pathogenesis (Fig. 2)[6].

As the preceding two paragraphs definitively demonstrate, abnormalities involving epigenetic regulator genes form the foundation of glioma classification for both adults and children. For the remainder of this review, we will explore the physiologic consequences of these molecular alterations on chromatin landscapes, gene expression, and genomic instability, along with strategies for their therapeutic targeting. We will also describe recent efforts to apply epigenomic profiling to clinically relevant brain tumor classification on a larger scale.

IDH mutation

As indicated above, heterozygous mutations in *IDH1* and, less frequently, *IDH2* essentially define lower-grade astrocytomas and oligodendrogliomas of adults[2], designating a more favorable clinical outcome (median survival of 65 months) relative to those of IDH-wildtype GBM (median survival of 15 months). It is important to note here that IDH-wildtype GBM is pathogenically distinct from its IDH-mutant counterparts, exhibiting diverse mutational landscapes that are not similarly defined by highly recurrent alterations in epigenetic regulatory molecules. This is not to say, however, that epigenomic dysfunction plays no role in the biology of these tumors. Indeed, hypermethylation of O6-methylguanine-DNA-methyltransferase (MGMT) promoter is a predictive marker of sensitivity to alkylating cytotoxic agents in

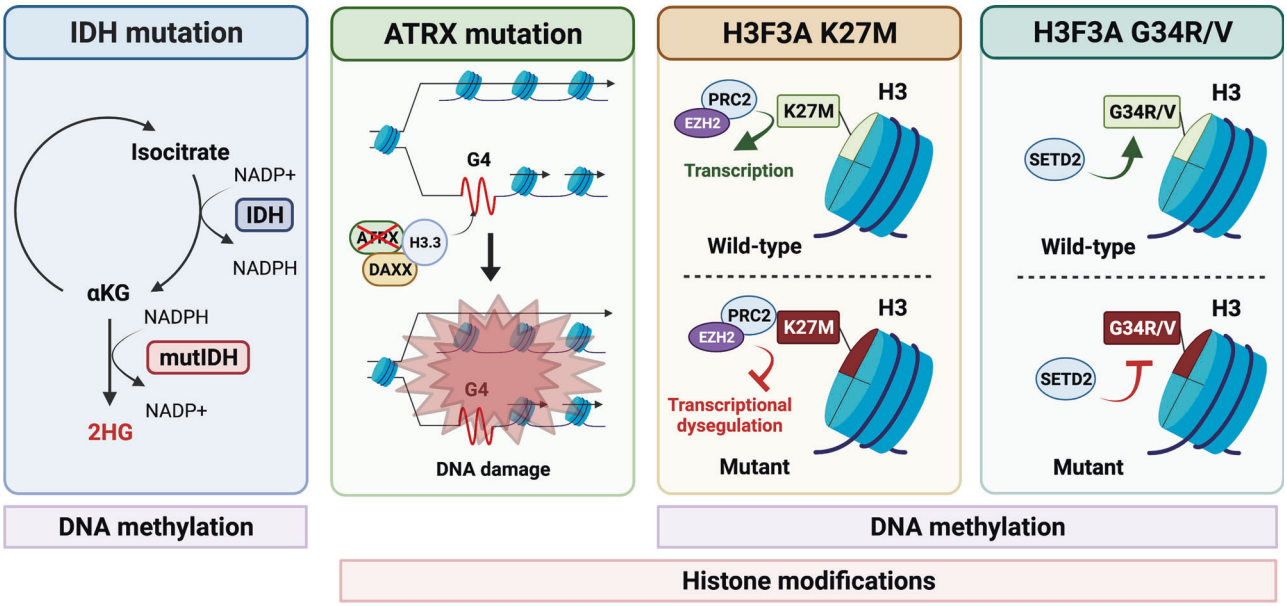

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Fig. 2 Mechanisms of action of molecular and epigenetic alterations in malignant glioma. Normally, IDH1 cooperates with NADP+ to convert isocitrate into α-ketoglutarate in the Krebs cycle. However, in IDH-mutant glioma, mutant-IDH cooperates with NADPH to convert α-KG into oncometabolite 2HG. Loss of function mutations in *ATRX* disrupt the function of the ATRX/DAXX complex to recruit and deposit histone variant H3.3 at sites of replication stress and DNA damage, ultimately leading to genomic instability in ATRX-deficient glioma. K27M mutations bind to and restrict the enzymatic activity of a core subunit of the PRC2, EZH2, sequestering PRC2, inhibiting the deposition of the repressive H3K27me3 mark, and ultimately causing transcriptional dysregulation in diffuse midline glioma. Lastly, G34R/V mutations inhibit the enzymatic activity of SETD2, a critical histone methylase, resulting in differential binding of K36 and disruption of H3K36me3 deposition, ultimately altering epigenetic and transcriptional regulation.

IDH-wildtype disease, specifically temozolomide[11]. *IDH1* and *IDH2* encode two of the three IDH enzymes that play integral roles in essential metabolic processes like the Krebs cycle, lipid metabolism, and oxidative damage regulation[12,13]. Cancer-associated IDH mutations invariably involve active site arginine residues, at codon 132 for IDH1 and codons 140 and 172 for IDH2[2,14], although alternative sites have been reported in rare instances[15,16]. A single mutation in IDH1, which converts arginine 132 to histidine (R132H), represents 90% of IDH mutations in glioma. This serendipitous predomination effectively renders immunohistochemical screening for IDH1 R132H a highly sensitive approach for the assessment of IDH mutational status in relevant tumors[17].

Under normal physiology, IDH1 cooperates with nicotinamide adenine dinucleotide phosphate (NADP+) to catalyze the production of α-ketoglutarate (α-KG) from isocitrate in the Krebs cycle[18,19]. However, in heterozygous IDH-mutant glioma, the WT-component of the IDH dimer catalyzes the conversion of isocitrate to α-KG while the mutant-component cooperates with NADPH to convert α-KG into R(−)-2-hydroxyglutarate (2HG), an oncometabolite (Fig. 2)[2,14,20,21]. Accumulated 2HG then serves as a competitive inhibitor of the TET family of 5′-methylcytosine hydroxylases, which play a major role in the removal of methyl group moieties from nucleic acid and protein[22–24]. Accordingly, these combined epigenetic disruptions ultimately induce abnormally high levels of methylation involving both DNA and histones in affected cells, a state termed the glioma CpG island hypermethylator phenotype (G-CIMP)[25,26]. Tumors characterized by G-CIMP are tightly associated with underlying IDH mutations, are more commonly found in LGGs in younger patients, and are associated with more favorable outcomes[25].

Recent work supports the notion that induction of G-CIMP by IDH mutation represents a primary oncogenic sequela. Glioma-associated IDH mutations have been identified at significant rates in histologically normal brain samples, underscoring their likely role in tumor initiation[27]. Moreover, while IDH mutations are almost invariably retained in recurrences of IDH-mutant primary gliomas, those few cases for which IDH mutations are lost (via obliterative copy-number alterations) nevertheless maintain crucial elements of the G-CIMP chromatin state[28]. Such work points to dysregulated DNA and histone methylation patterns as oncogenic in and of themselves, although recent data from the Glioma Longitudinal Analysis (GLASS) Consortium suggests that the extent of epigenetic dysfunction in IDH-mutant gliomas wanes over time, calling into question its absolute requirement for tumor maintenance[29].

Interestingly, IDH-mutant gliomas also demonstrate hypermethylation at cohesin and CCCTC-binding factor (CTCF)-binding sites[24]. CTCF functions as a methylation-sensitive insulator that prevents enhancers from binding and activating gene promotors not normally under their direct purview[30–32]. CTCF dysfunction in glioma has previously been associated with a loss of insulation and unregulated gene activation, allowing for aberrant enhancer-gene interactions with the established glioma oncogene PDGFRA[24]. Thus, IDH mutations are thought to promote gliomagenesis in part through the disruption of normal chromatin topology.

EglN prolyl-4-hydroxylases represent still another family of enzymes impacted by 2HG accumulation in IDH-mutated gliomas. In normal cellular physiology, these α-KG-dependent dioxygenases modulate cellular response to hypoxia via regulation of hypoxia inducible factor (HIF) transcription factors[33]. In the presence of oxygen, EglN hydroxylates HIFα, which is then polyubiquitylated by the von Hippel-Lindau (VHL) E3-ubiquitin ligase complex and flagged for proteasomal degradation[34]. By contrast, during hypoxia EglN is inhibited, permitting HIFα to accumulate and activate transcription. Elevated 2HG in IDH-mutant gliomas has been shown to potentiate EglN activity and diminish the extent of HIF activation, promoting oncogenesis in this specific neoplastic context[34].

While numerous studies have implicated IDH mutations and co-occurring molecular alterations in gliomagenesis, only recently were in vivo models developed that adequately validate these findings. Establishing robust models for IDH-mutant glioma has simply proven difficult over time. Initial efforts to express mutant IDH1 knock-in allele by either Nestin-*Cre* or GFAP-*Cre*-mediated approaches resulted most commonly in perinatal lethality, with no gliomas detected in surviving mice[35]. Similarly, employing tamoxifen-inducible Nestin-Cre^{ER} to specifically express IDH^{R132H} in the subventricular zone (SVZ) of adult mice failed to generate tumors, despite increasing levels of 2-HG and DNA methylation, and decreasing levels of α-KG, strongly suggesting that IDH1 mutation alone is insufficient for gliomagenesis[36]. More recently Philip et al. successfully established an IDH-mutant gliomas in mice by using the RCAS/t-va glioma model and postnatally injecting mutant IDH1 to cells expressing Nestin[37,38]. Immortalized astrocytes were transformed in vitro by combining IDH1^{R132H} and PDGFA expression with loss of *CDKN2A*, *ATRX*, and *PTEN* to drive gliomagenesis in vivo[38]. Similarly, Pirozzi et al. used a targeting vector containing IDH1^{R132H} and a Lox-stop-Lox cassette to generate a IDH1-mutant conditional knock-in mouse. In this context, IDH1^{R132H} expression actually downregulated cellular proliferation of NSCs in the SVZ, suggesting that IDH1^{R132H} fundamentally disrupts the microenvironment from which gliomas develop, and hampered the growth of p53-deficient gliomas in vivo, recapitulating the relatively indolent behavior of IDH-mutant gliomas in humans[39]. Taken together, these murine glioma models shed light on the complex biology mediated by mutant IDH expression in the context of gliomagenesis and provide a preclinical foundation for optimizing targeted therapies in the clinic.

Intriguing links between IDH mutation, 2HG, and abnormal cellular responses to DNA damage have also been established. For instance, recent work demonstrated that elevated levels of 2HG in IDH-mutant glioma suppressed homology-dependent repair (HDR), rendering cancer cells more susceptible to poly (ADP-ribose) polymerase (PARP) inhibition[40]. This phenotype was reversible upon treatment with IDH inhibitors and was observed in several clinically relevant models, ranging from patient-derived glioma stem cells (GSCs) in vitro to xenograft models in vivo. Additionally, Sulkowski et al. showed that oncometabolites suppress HDR at loci surrounding DNA breaks by inhibiting KDM4B, a lysine methylase, resulting in uncontrolled hypermethylation of histone 3 lysine 9 (H3K9)[41]. This mechanism obstructs an essential H3K9 trimethylation signal for normal HDR function and leads to poor deposition of key HDR factors Tip60 and ATM, impairing end-resection and recruitment of downstream effectors[41]. Taken together, these studies established a previously unknown link between oncometabolite accumulation, DNA damage repair suppression, and genomic instability, while providing an intriguing avenue for targeted therapeutic development.

Selectively targeting IDH mutations and their downstream pathobiology remains an area of active research. Standard-of-care treatment for IDH-mutant diffuse gliomas currently includes maximal surgical resection, followed by radiation and chemotherapy as feasible. Regrettably, these interventions only lead to transient responses, with inevitable disease recurrence and progression the rule. The neomorphic enzymatic activity induced by IDH mutation would seem to lend itself quite well to therapeutic targeting, and multiple pharmacologic IDH inhibitors are currently being investigated as potential treatments for LGG[42–50]. The dual IDH1/2 inhibitor vorasidenib (AG-881) was optimized for enhanced blood-brain barrier penetrance and is now in a first-in-human phase I trial for patients with advanced IDH1/2 solid tumors, with a primary focus on glioma[51]. In preliminary reports, vorasidenib was well tolerated and associated with positive safety profiles at doses <100 mg once daily in patients with recurrent disease; median progression-free survival (PFS) times were 36.8 and 3.6 months in patients with non-enhancing and enhancing glioma, respectively[51]. Due to heterogeneity in prior treatment histories within this study cohort, direct comparisons of associated PFS results to known patient outcomes is difficult. However, in vorasidenib-treated patients treated with non-enhancing disease, median PFS compared favorably to reported outcomes in patients receiving temozolomide and radiation[52,53]. While standard of care in low-risk LGG often employs a watch-and-wait approach following surgical resection, there is an increased risk of accruing additional genetic alterations at recurrence along with development of long-term toxicities to additional radiation or chemotherapy. As such, vorasidenib provides a potential targeted approach for IDH-mutated gliomas as a single-agent that could delay usage of more aggressive and toxic therapies, thereby improving patient quality of life. Accordingly, a randomized, phase III clinical trial (INDIGO) has recently been initiated that will assess the impact of vorasdenib in grade 2 non-enhancing IDH-mutant glioma following surgery alone[51].

As we have seen, the metabolic and epigenetic consequences of IDH mutation are extensive, pointing to additional molecular strategies for therapeutic development above and beyond the mutant enzymatic activity itself, options multiple groups are currently exploring. For instance, Tateishi et al. systematically profiled metabolites following mutant IDH1 inhibition of primary IDH1-mutated cancer cell lines to identify metabolic vulnerabilities potentially amenable to synthetic lethality relationships. This approach led to the discovery that IDH-mutant gliomas are sensitive to NAD+ depletion induced by concomitant nicotinamide phosphoribosyltransferase inhibition, with cytotoxic autophagy ultimately mediated by the intracellular energy sensor AMPK[54]. Turcan et al. investigated the epigenetic dependencies of IDH-mutant gliomas, focusing particular attention on the importance of the G-CIMP chromatin state to tumor maintenance. Specifically, they hypothesized that IDH1 mutations block differentiation, leading to aberrant malignant growth of tumor-initiating cells, and that treating these tumors with the FDA-approved demethylating agent decitabine could release this differentiation block by erasing relevant repressive methylation marks on DNA and histones[55]. Decitabine depletes and degrades the DNA-methyltransferase DNMT1 and has high blood−brain barrier penetrance, making it a favorable pharmacological compound for the treatment of glioma. Remarkably, low doses of decitabine induced differentiation in patient-derived IDH1-mutant glioma stem cells and induced expression of genes associated with glial differentiation[55]. This study highlights the promising potential of implementing DNA demethylating agents and/or modifying the global epigenome in the treatment of IDH-mutant glioma.

ATRX mutation

In recent years, inactivating mutations in SWI/SNF chromatin remodeler ATRX were identified as oncogenic drivers in a variety of cancers, including adult and pediatric diffuse gliomas[1,3,6,56,57]. In glioma, ATRX deficiency almost invariably arises in conjunction with *TP53* mutations and with either *IDH1/2* mutations (adults) or H3 mutations (children) (Fig. 1). Across the broad spectrum of diffuse gliomas, mutations in *ATRX* are also mutually exclusive with promoter mutations in *TERT*, which encodes the catalytic component of telomerase[1,3]. ATRX is a multidomain chromatin-binding protein and helicase whose germline loss of function results in ATR-X syndrome, a congenital condition associated with intellectual disability and α-thalassaemia[58–60]. Early studies established the critical role ATRX plays as a mediator of cell survival during neuronal differentiation, while our group more recently demonstrated that ATRX deficiency drives disease-defining phenotypes by specifically altering genome-wide chromatin accessibility[57,61]. This work highlights the far-reaching impact of mutations in epigenetic modifiers and chromatin remodelers in both normal nervous system development as well as neoplasia.

Canonically, ATRX interacts with death-associated protein 6 (DAXX) to recruit and deposit histone variant H3.3 at sites of repeat GC-rich sequences, including pericentromeric and telomeric heterochromatin as well as more gene-rich regions (Fig. 2)[57,62,63]. The N-terminal ADD domain of ATRX interacts with two binding pockets on the N-terminal tail of H3.3, unmodified lysine 4 (H3K4me0) or di-/tri-methylated lysine 9 (H3K9me3). These contacts facilitate ATRX recruitment to heterochromatin for deposition of H3.3. Ratnakumar et al. demonstrated that ATRX acts as a negative regulator of the repressive histone variant macroH2A1, independent of its association with DAXX and H3.3. MacroH2A accumulated at telomeres, leading to the suppression of α-globin levels in ATRX-deficient erythroleukemic cells, a process that presumably drives the α-thalassemia phenotype in ATR-X syndrome[64].

While most cancers predominantly rely on telomerase to maintain telomere length, ~10–15%, including ATRX-deficient gliomas, lack telomerase activity and instead rely on a telomerase-independent, homologous recombination (HR)-dependent mechanism known as alternative lengthening of telomeres (ALT)[65–67]. While loss of function mutations in *ATRX* are strongly correlated with ALT and are mutually exclusive of *TERT* promotor mutations—which activate *TERT* expression and telomerase activity—*ATRX* loss alone is insufficient to induce ALT. Instead, recent data indicates that *ATRX* loss cooperates with IDH mutation to drive ALT and abnormal telomere maintenance[68]. ATRX-deficient, mutant IDH1^{R132H} models resolve telomere dysfunction by employing HR and ALT and evade cell death. Interestingly, the re-expression of telomere capping complex protein *RAP1* and the non-homologous end-joining repair factor *XRCC1* suppressed ALT in the ATRX-deficient context, suggesting that DNA damage repair at telomeres is fundamentally altered[68].

Loss of ATRX gives rise to abnormal DNA secondary structures known as G-quadruplexes (G4s) at GC-rich sites throughout the genome. ATRX binds at these genomic loci and is thought to maintain normal DNA conformation through DAXX-dependent incorporation of H3.3 monomers[69]. G4s induce stalled replication forks in ATRX-deficient tumors, impairing DNA replication. Our group recently demonstrated that the accumulation of G4s in ATRX-deficient glioma models enhances replication stress and DNA damage on a global scale, promoting transcriptional dysregulation and genomic instability (Fig. 2)[70].

The exact interaction between ATRX and G4s is still under investigation and as the molecular mechanisms that drive ATRX deficiency in glioma remain poorly understood, translating these recent findings into targeted therapies represents an under-explored area with great potential. For ATRX-deficient gliomas, pharmacologic stabilization of G4 structures may lead to enhanced, selective cytotoxicity by enhancing replication stress and DNA damage in tumor cells vulnerable to this biology. Our group and several others have demonstrated that tumors deficient in replication fork stabilization and DNA damage repair exhibit selective lethality in the context of G4-stabilizing compounds[70–72]. These findings support the notion that ATRX loss, compounded by enhanced accumulation of G4s following treatment with G4 stabilizers, yields cytotoxic levels of DNA damage in a synthetic lethality paradigm. Excitingly, the G4 stabilizer CX-5461, an RNA polymerase I inhibitor first described in BRCA-mutated cancers, is currently in early phase I clinical trials for the treatment of solid tumors and advanced hematologic malignancies[71,73–75]. While the efficacy of this compound in ATRX-deficient glioma remains to be definitively established, preliminary findings from our group in the preclinical setting have been promising.

Histone 3

Unlike adult diffuse gliomas, chromatin dysregulation in pediatric gliomas is a direct result of mutations in histone H3. The four core histone monomers include H2A, H2B, H3, and H4, which package DNA into nucleosome octamers containing two copies of each and are then further compacted into chromatin. These histones are critical for transcription and genomic stability and their regulatory signals to the transcriptional apparatus rely on a series of post-translational modifications[6,76]. H3 subtypes include H3.1 and H3.2, which are regulated by cell cycle and only deposited during S-phase and DNA repair, along with H3.3, which is deposited by the ATRX/DAXX complex in a replication-independent fashion at GC-rich heterochromatic regions of the genome[6,8,63]. Histone H3 mutations are highly conserved somatic alterations and are drivers of tumorigenesis in pediatric diffuse gliomas. As indicated above, the two most common such mutations involve the H3 variant genes *H3F3A* and *HIST1H3B*, yielding either K27M or G34R/V substitutions[6,9,10].

While K27M-mutant diffuse midline gliomas arise most often in the brainstem, in particular the pons, they can be observed throughout the CNS midline from the base of the spinal cord to the thalamus and basal ganglia (Fig. 1). Their aggressive behavior is well-established (median survival 11 months)[77]. We should also note that K27M mutations are not exclusive to diffuse midline glioma, occasionally arising across a diverse set of tumors that include pilocytic astrocytomas, gangliogliomas, and posterior fossa ependymomas[78,79]. While a full discussion of this heterogeneity is beyond the scope of the current article, it speaks to a the more generalizable oncogenic effects induced by K27M mutation and its epigenomic sequelae (see below). K27M mutations are readily detectable by molecular assays, and highly effective immunohistochemical approaches have further streamlined their assessment[80]. Such capabilities are analogous to those regularly employed to identify IDH^{R132H}-mutant gliomas (see above).

The molecular mechanisms driving K27M-mutant diffuse midline glioma have been subjected to extensive study. The mutation itself appears to bind and restrict the enzymatic activity of the methyltransferase EZH2, a core subunit of the Polycomb Repressive Complex2 (PRC2)[81–84]. This inhibition leads to decreased deposition of the normally repressive H3K27me3 chromatin mark, by way of PRC2 sequestration (Fig. 2)[81,83,84]. While some residual EZH2 activity remains in this context, the K27M mutation appears to function by limiting the spread of H3K27me3 from unmethylated DNA at CpG islands, a process that presumably drives gliomagenesis by fundamentally altering transcriptional programs that mediate cellular development and differentiation[85]. Inhibition of H3K27M restores H3K27me3 spread and abolishes tumorigenesis in vivo. Interestingly, as detailed above, gain-of-function mutations in IDH and elevated 2-HG are associated with G-CIMP, which also disrupts the normal methylation patterns of H3K27 residues[23,25,86].

Unlike K27M-mutant tumors, those harboring G34R/V mutations present almost exclusively in the cerebral hemispheres of the brain and confer somewhat longer overall survival (median 18.0 months) (Fig. 1)[77]. These mutations are commonly observed in combination with *ATRX/DAXX* and *TP53* mutations and impact modification of the nearby K36 residue on the H3 tail[6,87–89]. H3G34 mutations inhibit the catalytic activity of the key histone methylase SETD2, leading to differential binding of K36 and disruption of H3K36me3 deposition (Fig. 2)[90,91]. This process fundamentally alters epigenetic and transcriptional landscapes that include markers of stem-cell maintenance, neuronal differentiation, and cellular proliferation – most notably expression of *MYCN*, an oncogenic driver of GBM[89]. Targeting G34R/V-mutant glioma via inhibitors of kinases that stabilize *MYCN* may be a viable treatment option[89].

Fang et al. recently demonstrated that G34R/V mutations inhibit the ability of the H3K36me3 mark to interact with the mismatch repair (MMR) protein MutSα/MSH6 and K36-specific methyltransferases, impairing the catalytic activity of SETD2 and effectively

inducing MMR deficiency and a hypermutator phenotype[91]. Moreover, they found that G34R/V mutations in vitro were associated with enhanced mutational frequency and a concurrent decrease in chromatin-bound MSH6, due to the impaired affinity of MSH6 binding the H3-mutant tail and the diminution of H3K36me3 in G34R/V cells[91].

Chen et al. found that ~50% of G34R/V gliomas present with activating mutations in receptor tyrosine kinase platelet-derived growth factor receptor alpha (PDGFRA), with enriched frequency at recurrence[92]. Transcriptomics of G34R/V tumors using gene set enrichment analysis revealed that these tumors likely arise from cortical GSX2/DLX-expressing interneuron progenitors, rather than from excitatory neuronal or oligodendroglial lineages, that promote PDGFRA overexpression in the context of a dual neuronal and astroglial phenotype[92]. GSX2/DLX-mediated cell fate is believed to actively repress oligodendroglial programs and stall interneuron progenitors in differentiation. As such, targeting the PDGFRA signaling pathway may provide an actionable approach to inhibit gliomagenesis in these aggressive tumors.

Intriguingly, Bressan et al. sought to evaluate the contrasting etiology between K27M and G34R/V tumors by successfully engineering human fetal neural stem cells from various regions of the brain[93]. They observed differential sensitivity to each mutation, based on the region of cellular derivation, that recapitulated oncogenic responses from pediatric high-grade diffuse gliomas. G34R mutations promote strong proliferation in cells derived from the forebrain and induce a cytostatic response in the hindbrain, while K27M mutations demonstrate oncogenic effects in the hindbrain alone[93]. While G34R was not found to induce genome-wide transcriptional or epigenetic changes, its presence impaired recruitment of the transcriptional repressor ZMYND11, likely locking forebrain cells in their pre-existing progenitor state and ultimately promoting tumorigenesis[93].

Multiple groups have attempted to generate mouse models of K27M and G34R/V-mutant gliomas, often combining genetically engineering with retroviral or retrotransposon-mediated somatic gene transfer[82,94,95]. While several models carrying the K27M mutation have been established, few demonstrate oncogenic dependency on the H3 mutation itself. Of note, Pathania et al. were able to induce neoplastic transformation in utero by combining $H3.3^{K27M}$ and Trp53 loss[96]. The resulting tumors recapitulated hallmark features of K27M-mutated glioma, presenting as proliferative and diffuse clonal lesions with depletion of H3K27me3 and Olig2 positivity. ATRX knockdown in this model resulted in more proliferative lesions and induction of PDGFRA lead to enhanced tumor invasion. Moreover, $H3.3^{K27M}$-tumor cells generated by this model were capable of serial engraftment in recipient mice, further enabling usage in preclinical contexts. Interestingly, induction of $H3.3^{G34R}$ in combination with Trp53 loss in this setting did not result in tumor formation. The limited capacity of G34R/V mutation to induce transformation in the murine context was further demonstrated by more recent work combining the alteration with *PDGFRA* mutation[92].

A variety of potential treatment approaches for H3-altered pediatric gliomas have been explored, particularly for K27M-mutant tumors. Mount et al. demonstrated sensitivity to chimeric antigen receptor (CAR)-expressing T cells in K27M-mutant glioma cells that present with enhanced expression of disialoganglioside GD2[97]. Similarly, GD2-targeted CAR T cells lead to nearly complete tumor regression in patient-derived K27M-DMG orthotopic xenograft mouse models. While similar CAR T cell approaches have been well tolerated in clinical trials for neuroblastoma[98–100], lethal hydrocephaly due to peritumoral neuroinflammation was observed a select group of K27M-DMG xenografted mice in this study. Accordingly, diligent monitoring will be required for effective translation into human patient populations. Recent CRISPR screening revealed that knockout of KDM1A, which encodes for lysine-specific demethylase 1 (LSD1), leads to sensitivity to histone deacetylase (HDAC) inhibitors in K27M-mutant tumors[101]. Furthermore, Anastas et al. demonstrated that the HDAC and LSD1 inhibitor Corin dramatically inhibits tumor growth both in vitro and in vivo by inducing cell cycle arrest and cell death[101]. Taken together, this work sheds light on encouraging therapeutic strategies to redefine the current approach to treating K27M tumors.

The findings detailed above advance our current understanding of histone 3 mutated glioma subtypes, while also highlighting cellular etiology and pathways that lead to gliomagenesis. The hallmark molecular alterations of K27M and G34R/V mutations provide potential prognostic markers that can be leveraged into therapeutic targets, redefining our approaches to treating these aggressive and terminal tumors in pediatric and young adult populations.

Epigenetic signatures and brain tumor diagnostics

As we have seen above, epigenetic dysregulation profoundly influences the pathogenesis of diffuse gliomas, both in adults and children. More broadly, however, epigenomic profiles play defining roles across the brain tumor landscape, regardless of whether they serve as fundamental neoplastic drivers themselves. This reality has become starkly apparent in multiple studies over the past 10 years employing global methylation profiling to effectively delineate robust molecular subclasses in an array of brain tumor groups, notably gliomas, ependymomas, medulloblastomas, and meningiomas[102–106]. Recently, Capper et al. expanded on this approach, establishing a comprehensive system of classifying all CNS tumors, across all ages, using DNA methylation profiling. They generated a random forest algorithm to generate 10,000 binary decision trees that then identified eight methylation class families[102]. They then determined the clinical utility of these data by analyzing 1,104 diagnostic CNS tumors and comparing these cases against standard histopathological analyses. DNA methylation profiling of these cases revealed an 88% match to established DNA methylation classes, most notably assigning 171 cases that previously were unclassified based on histopathology alone[102]. This classification system is now available on a free web platform and includes additional data on DNA copy-number and MGMT promoter methylation status (a key discriminatory feature when diagnosing GBMs), thus allowing for comparative and novel biological insights.

With regard to diffuse gliomas, DNA methylation profiling largely recapitulates the diagnostic categories established by the highly recurrent biomarkers discussed above (e.g. *IDH1/2* mutation, 1p/19q codeletion, etc.)[107]. However, its effective usage enables the proper diagnosis of diffuse gliomas incorrectly classified as other CNS tumor entities, as exemplified in recent work on tumors histopathologically designated as supratentorial primitive neuroectodermal tumor (PNET)[108]. Global methylation profiling has also facilitated the identification of novel neoplastic entities, by identifying tumor clusters with shared epigenetic signatures. Further histopathological and molecular analyses subsequently identify other salient, classifying characteristics. This approach has recently delineated pilocytic astrocytoma with anaplastic features[109], polymorphous low-grade neuroepithelial tumor of the young[110], isomorphic diffuse glioma[111], and neuroepithelial tumor with PLAGL1 fusion[112]. Taken together, these studies demonstrate the diagnostic efficacy of leveraging novel epigenetic findings to guide classification of brain tumors and signal the increasing importance of epigenetic profiling in the field moving forward.

Concluding remarks

The extensive molecular annotation of cancer has yielded many striking insights, including a greater awareness of global epigenomic landscapes and the roles they play in driving cell identity, phenotype, and neoplastic behavior. That markers of

epigenetic dysfunction now guide glioma classification and broader brain tumor diagnostics is only the beginning. The larger and more pressing task that remains is to effectively characterize the molecular mechanisms induced by epigenetic dysfunction in these key oncogenic contexts and translate this knowledge into more effective treatment strategies to improve the lives of affected patients.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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