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INVITED REVIEW

Changing paradigms in oncology: Toward noncytotoxic treatments for advanced gliomas

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Abstract

Glial-lineage malignancies (gliomas) recurrently mutate and/or delete the master regulators of apoptosis p53 and/or p16/CDKN2A, undermining apoptosis-intending (cytotoxic) treatments. By contrast to disrupted p53/p16, glioma cells are live-wired with the master transcription factor circuits that specify and drive glial lineage fates: these transcription factors activate early-glial and replication programs as expected, but fail in their other usual function of forcing onward glial lineage-maturation—late-glial genes have constitutively “closed” chromatin requiring chromatin-remodeling for activation—glioma-genesis disrupts several epigenetic components needed to perform

Abbreviations: 2-HG, 2-hydroxyglutarate; AKG, alpha-ketoglutarate; ARID1A/B, AT-rich interactive domain-containing protein 1A/B (MTFs); ATF3, activating transcription factor 3 (MTF); ATP, adenosine triphosphate; ATRX, alpha thalassemia x-linked mental retardation (SWI/SNF protein); BAF, ATP-dependent BRG1/BRM associated factor (SWI/SNF complex); CBP, CREB binding protein (HDAC); CDA, cytidine deaminase; CDKN2A, cyclin-dependent kinase inhibitor 2A (gene encoding p21); CHD4, chromodomain helicase DNA binding protein 4 (ISWI protein); CPEC, cyclopentenyl cytosine; CTPS2, CTP synthase 2; DAXX, death domain associated protein 6; DHODH, dihydroorotate dehydrogenase; DLX, distal less X (family of developmental transcription factors); DNA, deoxyribonucleic acid; DNMT1/3A/3B, DNA methyl transferase 1/3A/3B; EANO, European Association of Neuro-Oncology; EGFR, epidermal growth factor receptor; EP300, gene encoding HDAC p300; ESC, embryonic stem cell; EZH2, enhancer of zeste 2 (PRC2 subcomponent); G34R/V, mutation resulting in an exchange of glycine residue 34 on histone 3.3 for arginine or valine; GBM, glioblastoma multiforme; GFAP, glial fibrillary acidic protein; GSX2, GS homeobox 2 (MTF); GTP, guanosine triphosphate; H3F3A, gene encoding histone variant 3.3; H3K27ac, acetylated lysine residue 27 on histone 3; H3K27me2/3, di/tri-methylated lysine residue 27 on histone 3; H3K4me3, tri-methylated lysine residue 4 on histone 3; HDAC, histone deacetylases; HIST1H3B/C, genes encoding histone variant 3.1; IDH 1/2, isocitrate dehydrogenase 1/2; ISWI, imitation switch (gene family of ATP-dependent chromatin remodelers); K27M, mutation resulting in an exchange of lysine residue 27 on histone 3.1 or histone 3.3 for methionine; KDM1A, Jumonji histone lysine demethylase 1A (H3K4me3 demethylase); KDM6A/B, Jumonji histone lysine demethylase 6A/B (H3K27me3 demethylases); KMT, histone lysine methyl transferase; me-CpG, methylated deoxycytosine residues preceding deoxyguanine residues; MRI, magnetic resonance imaging; MTF, master transcription factor; MYC, derived from the name of the avian virus “myelocytomatosis” (MTF); MYCL1, MYC lung carcinoma derived 1; MYCN, MYC neuroblastoma derived; NANOG, of Irish origin “Tir na nÓg”, meaning “land of eternal youth” (MTF); NFIA, nuclear factor 1 A (MTF); NSC, neuronal stem cell; OLIG2, oligodendrocyte transcription factor 2 (MTF); PDGFR, platelet-derived growth factor receptor; POU5F1, POU class homeobox 1 (gene encoding the MTF Oct-4); PRC2, polycomb repressor complex 2; RANO, response assessment in neuro-oncology; RUNX2, RUNX family transcription factor 2 (MTF); SET-domain, Su(var)3-9, Enhancer-of-zest and Trithorax (protein domain of specific KMTs); shRNA, short hairpin ribonucleic acid; SMARC, SWI/SNF related, matrix associated, actin-dependent regulators of chromatin (different SWI/SNF and ISWI family proteins involved in chromatin remodeling); SOX2/10, sex-determining region Y (SRY) box 2/10 (MTFs); SUZ12, suppressor of zeste 12 (PRC2 subcomponent); SWI/SNF, switch/sucrose nonfermentable (family of ATP dependent chromatin remodelers); TET1/2, ten-eleven translocation methylcytosine dioxygenase 1/2; TP53, tumor protein p53 (gene encoding protein p53); UTP, uridine triphosphate; WHO, World Health Organization.

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this work, and simultaneously amplifies repressing epigenetic machinery instead. Pharmacologic inhibition of repressing epigenetic enzymes thus allows activation of late-glial genes and terminates glioma self-replication (self-replication = replication without lineage-maturation), independent of p53/p16/apoptosis. Lineage-specifying master transcription factors therefore contrast with p53/p16 in being enriched in self-replicating glioma cells, reveal a cause-effect relationship between aberrant epigenetic repression of late-lineage programs and malignant self-replication, and point to specific epigenetic targets for noncytotoxic glioma-therapy.

KEYWORDS
cancer epigenetics, epigenetic, glioma, glioma therapy, neurooncology

1 | THE PATHWAY OBJECTIVE OF CONVENTIONAL ONCOTHERAPY: P53/P16-DEPENDENT APOPTOSIS

A time-traveling oncologist from the 1970s would readily recognize the principles and practice of much current oncotherapy (reviewed in Reference 1), since pharmacology-pathway goals have remained steadfast: terminate malignant replications/self-replications by activating the metazoan program of apoptosis (also known as cytotoxicity).[2] Apoptosis is a replication-control program that senses cell stress or injury as stimuli to upregulate the master regulator p53 (*TP53*) and its key co-factor p16 (*CDKN2A*); these in turn activate gene expression modules that force cell cycle exits as an interlude for repair, then if the stress persists, compel orderly cell self-destructions. Some tissue lineages, mainly testicular, lymphoid and myeloid, can transform and advance to disseminated malignancy without *TP53* or *CDKN2A* deletions and/or mutations (reviewed in Reference 3). The apoptosis master switch is therefore available for activation, enabling cures even in advanced cases. Underscoring the centrality of p53/p16 to these treatment successes, p53/p16 loss-of-function or dominant-negative alterations typify relapsed/refractory testicular, lymphoid and myeloid malignancies.[3]

Transformation of most other lineages into advanced malignancy, however, usually does entail attenuation or deep inactivation of the p53/p16-system. Cancers derived from these lineages thus demonstrate (a) anticipatory resistance to cytotoxic therapy (*primary refractory*), or (b) ready selection by cytotoxic therapy for the most apoptosis-attenuated malignant subclones, that resist subsequent treatments (*relapsed-refractory*),[3] a clinical reality demonstrated by glial malignancies (gliomas). The World Health Organization (WHO) and European Association of Neuro-Oncology (EANO) use histological features such as microvascular proliferation and necrosis, and molecular features such as *IDH1*, *IDH2*, *ATRX* mutations and *CDKN2A* deletions, to categorize gliomas into grades with predictable, different natural histories and treatment outcomes[4,5]: *Oligodendroglioma, IDH-mutant, 1p/19q-codeleted, WHO grade 2/3* are oligodendroglial-lineage transformations in which *TP53* or *CDKN2A* inactivating mutations/deletions are infrequent, and these malignancies have better overall survivals than astroglial-lineage malignancies *astrocytoma, IDH-mutant, WHO grade 2/3* that routinely incorporate *TP53* inactivating mutations (>99% of cases).[6-8] Moreover, frequent deletion of *CDKN2A* characterizes the progression of astrocytomas from grade 2/3 to grade 4,[4,9] and *glioblastomas* (*GBM, IDH wild-type, WHO grade 4*) that have poor prognoses despite surgery and intense radiotherapy/chemotherapy, have rates of p53/p16-pathway inactivation exceeding 85%[6,10] (Grade 1—curable by surgery; Grade 2/3—overall survival durations of several years; Grade 4—overall survival durations months to few years[4,5]).

Thus, durable remissions as pay-offs for short- and long-term toxicities of aggressive chemotherapy and radiation are expected only for the few glioma types containing genetically intact p53/p16-systems (reviewed in Reference 3), and it would seem logical to develop new treatments not reliant on p53/p16/apoptosis.

2 | REPLICATION: THE HEART OF EVOLUTION, INCLUDING NEOPLASTIC EVOLUTION

All cancers share an essential foundation of relentless replication, the engine that drives evolution. Synchronized duplication of life's materials and machines is inexplicably complex. Neoplastic evolution is unlikely to reinvent such complexity, refined over millennia, and therefore hijacks it from the normal cellular contexts in which it occurs: (a) tissue stem cells, and/or (b) lineage-committed progenitors.[3] In the adult mammalian brain, neuronal stem cells (NSC) reside in neuroglial-genic niches in the dentate gyrus of the hippocampus and in subventricular zones lining the lateral ventricles.[11-13] NSC replicate rarely, for example, once every several months, and these replications can be naturally decoupled from onward lineage-differentiation for self-replications needed to maintain NSC pools through life-span (linear proliferation kinetics over long time-scales).[11,14,15] Instead of self-replication, daughter cells may commit toward neuronal or glial lineage-fates, to produce lineage-committed progenitors.[12-14]

Lineage-committed progenitors replicate every day for exponential proliferation kinetics over short time scales.[14] Each replication is coupled to the acquisition of neuronal- or glial-lineage programs (lineage-maturation), culminating after 4 or more cell divisions in activation of final neuronal or glial lineage-fate programs that terminate proliferation (terminal-differentiation).[11,14,16]

3 | WHICH OF THESE NORMAL REPLICATION CONTEXTS DO GLIOMAS HIJACK?

Malignant clonal expansion requires a substantial proportion of replications to be self-replications, a biological and mathematical requirement that can be computationally modeled.[17] Since NSC are the only normal brain cells to self-replicate, an intuitive assumption is that glioma self-replications derive from NSC (Figure 1A). To examine this assumption it is useful to recap the role of "master transcription factors" (MTFs) in cell fate determination. MTFs are sequence-specific, DNA-binding proteins that cooperate in specific combinations (MTF circuits) to activate the hundreds to thousands of genes that define specific cell fates and functions. The MTF circuit that creates NSC, SOX2/POU5F1/NANOG, has been reproducibly identified (reviewed in Reference 18), as has the MTF circuit NFIA/ATF3/RUNX2 that commits NSC to glial-lineage fates.[20,21]

What then is the MTF combination highly expressed in glioma cells? Gliomas express the MTF circuit SOX2/POU5F1/NANOG, which converts other somatic cells into NSC (and also into embryonic stem cells, ESC) in the same pattern as that observed in normal whole brain (Figure 1A,B): SOX2 is highly expressed in gliomas, but SOX2 is normally highly expressed through glial-lineage maturation,[20] and accordingly, is also highly expressed in normal brain that consists mainly of terminally-matured cells. The other two MTF in the NSC circuit POU5F1 and NANOG are expressed at barely detectable levels and are not more elevated in aggressive GBMs than less aggressive grade 2/3 oligodendrogliomas or astrocytomas (Figure 1B). Instead, the MTF circuit that forces NSC commitment into the glial-lineage NFIA/ATF3/RUNX2 is expressed at log levels higher than POU5F1 or NANOG, with further upregulation in GBM vs grade 2/3 oligodendrogliomas or astrocytomas (Figure 1B).

4 | WHY DOES NEOPLASTIC EVOLUTION SELECT TO TRANSFORM IN LINEAGE-SPECIFYING MTF CIRCUIT CONTEXTS?

MYC, and its paralogues MYCN and MYCL, are the ancient MTF that regulate hundreds of genes essential for nutrient supply, energy production, provision of cellular building blocks, cell cycle entry and progression (replication).[23] The emergence of multicellularity (metazoa) occasioned MYC subordination to MTF circuits that create diverse lineages and hierarchies[24]: MTF circuits that create tissue stem cells permit only low-grade MYC activity (shown in several tissue contexts, reviewed in Reference 3) presumably because quiescence protects the genomes of these cells vital to a multicellular organism overall life-span. In contrast, MTF circuits that commit cells into lineage activate and cooperate with MYC (or MYCN or MYCL) to propel replications every 1 to 2 days (reviewed in Reference 3), to in this way ensure the transit amplification needed to replenish specialized tissue cells lost to daily wear-and-tear.[3,15,25,26] "Transit" amplification is transitory because lineage-specifying MTF circuits simultaneously activate lineage-differentiation programs that cascade (lineage-maturation) toward activation of final specialized-fate programs which antagonize MYC and terminate replication (terminal-differentiation).[3,16,26] Gliomas thus express: (a) high levels of both glial lineage-specifying MTF and MYC (or MYC paralogues; Figure 1B); (b) high levels of early glial-lineage programs (https://biologic.crick.ac.uk/astrocyte) and MYC-target genes (Figure 1C); (c) strong positive correlation between early-glial and MYC-target gene expression (Figure 1D,E); (d) strong negative correlation between late-glial and MYC-target gene expression (Figure 1D,E); (e) more aggressive glioma subtypes display a left-shift away from late-glial toward early-glial gene expression (Figure 1C,D); and (f) this left-shift independently predicts and stratifies for worse overall survival, even within WHO/EANO glioma subtypes (Figure 2). Similar observations have been made by others,[27-31] and glial-lineage of gliomas is evident also from (a) histo-morphological examination; (b) biomarkers measured by immunohistochemistry or other methods, for example, OLIG2, GFAP, NES, PDGFR and EGFR[32]; (c) functional properties, for example, responses to neurotransmitters like glutamate[33-35]; (d) global and/or single-cell comprehensive gene expression profiles.[27,36,37]

5 | CLARIFYING CANCER "STEM" CELL TERMINOLOGY

Self-replicating cancer cells are often referred to in the literature as "stem" or "stem-like" (cancer "stem" cells), which is true in so far as they self-replicate, but the terminology obscures that by MTF content, dependency, and many other parameters, these cells phenocopy lineage-committed progenitors.[38-45] Tens of glioma cell lines that indefinitely self-replicate in vitro, as well as other human and murine glioma cells shown to initiate GBM in mice, faithfully recapitulate the high lineage-specifying MTF circuit/early-glial/MYC-target gene expression configuration observed in bulk glioma samples.[46-52] Self-replicating malignant cells in other tissues are also characterized by high expression and dependency on lineage-specifying MTF circuits that activate and cooperate with MYC for transit-amplification (reviewed in[3]). Thus, normal self-replication is restricted to tissue stem cells, but malignant self-replication is not (self-replication = replication without lineage-maturation; Figure 1A).

Oncogenic mutations, however, can originate in stem cells as far upstream as germ-line, for example, in familial gliomas, then propagate downstream into lineage-committed progenitors, wherein phenotypic consequences and clonal advantage most prominently emerge[53] (reviewed in Reference 3). Upstream mutations can also skew downstream commitment decisions, for example, *ATRX* mutations skew NSC commitment decisions toward astro-over oligodendroglial or neuronal lineage fates.[54]

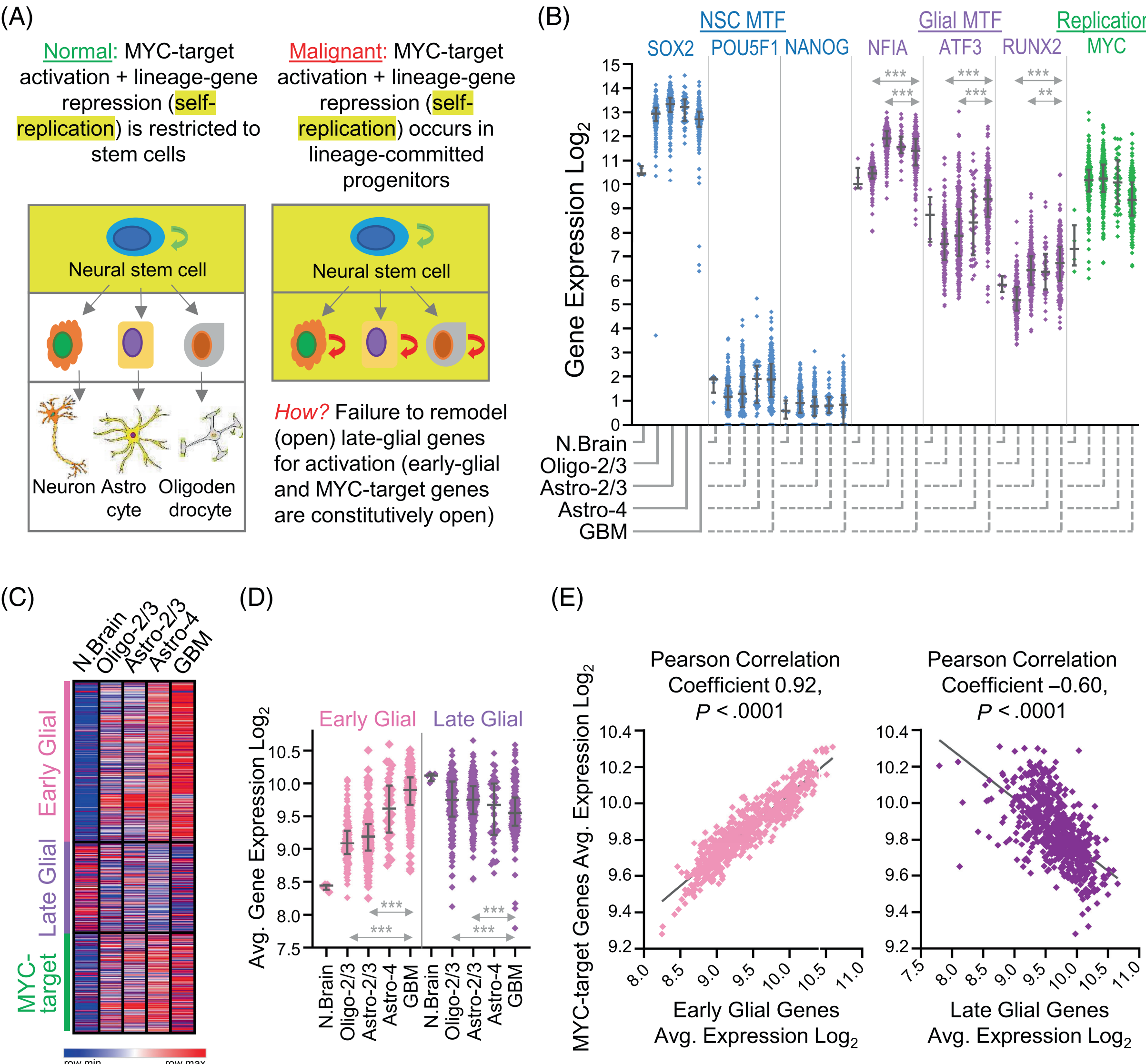


FIGURE 1 (A) Normal self-replication (replication without onward lineage-differentiation) is restricted to tissue stem cells, but malignant self-replication is not. (B) Of the master transcription factor (MTF) circuit SOX2/POU5F1/NANOG[18,19] that produces neural stem cells (NSC), only SOX2 that is stably expressed through glial lineage-maturation[20] is also highly expressed in gliomas, however, the MTF circuit NFIA/ATF3/RUNX2[21] that compels NSC commitment into glioma lineage-precursors is highly expressed. Normal brain (n = 5); Oligo-2/3 = oligodendroglioma, IDH-mutant and 1p/19q-codeleted, WHO grade 2 or 3 (n = 176); Astro-2/3 = astrocytoma, IDH-mutant, WHO grade 2 or 3 (n = 241); Astro-4 = astrocytoma, IDH-mutant, WHO grade 4 (n = 38); GBM = glioblastoma, IDH wild-type, WHO grade 4 (n = 196). TCGA RNA-seq public data, RSEM values (counts normalized by RNA-Seq by Expectation-Maximization). Mann-Whitney two-sided test ***$P < .0001$, **$P < .005$. (C) Consistent with lineage MTF circuit wiring, gliomas significantly upregulate 539 genes that characterize astroglial lineage-commitment/early maturation (early glial)[20] (67 known MYC-target genes excluded), and 337 MYC-target genes identified by chromatin-immunoprecipitation,[22] but there is anomalous suppression of 310 astroglial late lineage-differentiation (late glial) genes.[20] Average expression of each gene in glioma samples of each glioma sub-type (samples as per panel B). (D) More aggressive gliomas demonstrate deeper suppression of late-glial, and more upregulation of early-glial, genes. Average expression per sample of all genes in a category. Lines = median ± IQR, ***$P < .0001$ Mann-Whitney two-sided test (samples as per panel B). (E) Early-glial and MYC-target gene expression positively correlate (67 known MYC-target genes were excluded from early glial genes analyzed); Late-glial and MYC-target gene expression negatively correlate [Color figure can be viewed at wileyonlinelibrary.com]

6 | A CORE FAILURE DRIVING GLIOMA-GENESIS

Gliomas thus coordinately upregulate early-glial and MYC-target genes as expected from their lineage MTF content, the failure is to not then activate late-glial programs that suppress early-glial genes/MYC/MYCN/MYCL. Several experimental conditions have been shown to correct this anomaly, resuming glioma lineage-maturation and hence terminating self-replication/tumor-initiating capacity.[46,47,55,56] To develop such remedies for clinical use, it would be useful to understand how the failure occurs in the first-place.

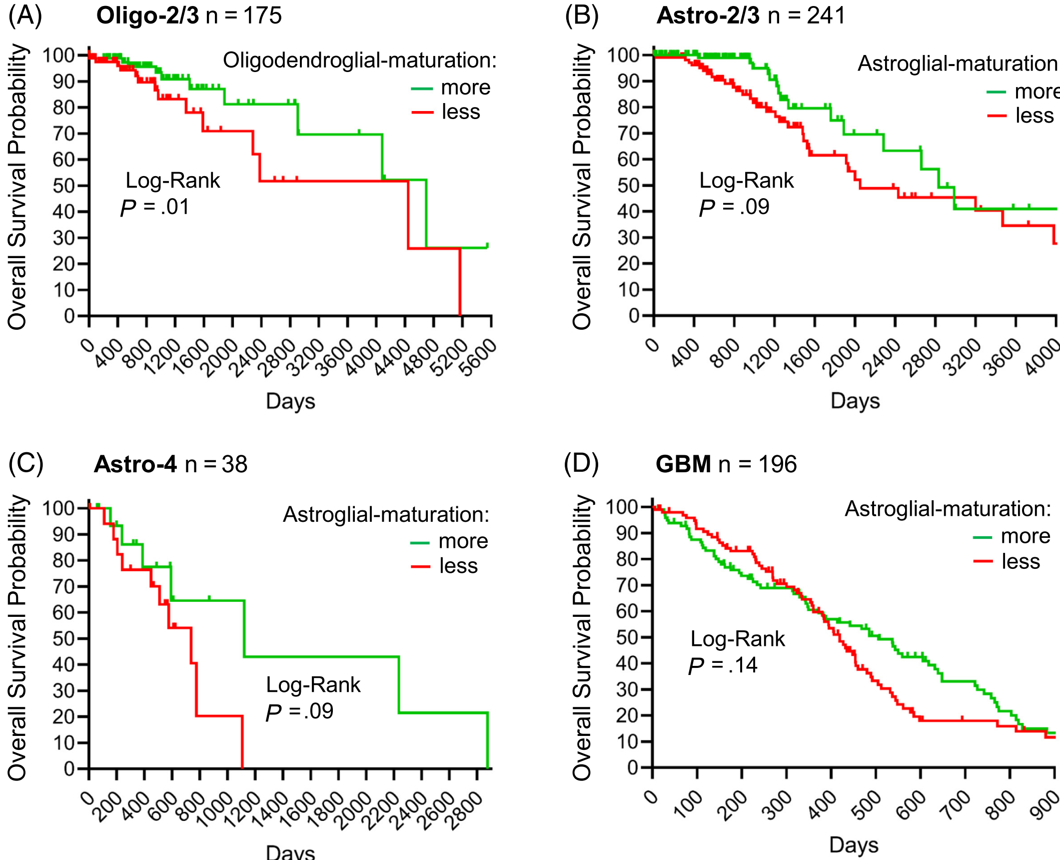


FIGURE 2 Less onward maturation of cells committed into the glial lineage (higher expression of commitment/early-glial genes, lower expression of late-glial genes) independently predicts and stratifies for worse overall survival within well-established EANO/WHO glioma subtypes. Cases within pathologic subgroups were stratified around the median average expression of late oligodendroglial lineage genes (as identified in Reference 27) for Oligo-2/3, or early astroglial lineage genes (as identified in Reference 20) for Astro-2/3, Astro-4 and GBM. Overall survival data TCGA. (A) Oligo-2/3. (B) Astro-2/3. (C) Astro-4. (D) GBM [Color figure can be viewed at wileyonlinelibrary.com]

7 | THE EPIGENETIC LANDSCAPE AT LATE-GLIAL GENES ENABLES THEIR ONCOGENIC REPRESSION

Here, the term "epigenetics" or chromatin refers to the three-dimensional organization of DNA around histone protein octamers (nucleosomes), that is configured and reconfigured by enzyme-containing multi-protein complexes that methylate or demethylate DNA bases, posttranslationally modify histones, exchange or reposition histones, to in this way facilitate (on) or obstruct (off) transcription of genes by the basal transcription factor machinery.[3,26] Gene repression (off) for example is favored by tri-methylation of lysine 27 on histone 3 (H3K27me3), a histone modification executed by Polycomb Repressor Complex 2 (PRC2) in which EZH2 is the enzyme component, and/or mono-methylation of DNA deoxycytidine residues that precede deoxyguanine residues (me-CpG) by DNA methyltransferases DNMT1, DNMT3A and/or DNMT3B.[57-59] Gene activation (on) on the other hand is favored by H3K27 acetylation (H3K27ac) executed by CBP/p300, and H3K4 trimethylation (H3K4me3) executed by SET-domain containing histone methyltransferases (KMTs).[58,60]

How are me-CpG, H3K27me3 and H3K27ac marks distributed at MYC-target, early- and late-glial genes? Me-CpG is minimal at MYC-target and early-glial but elevated at late-glial genes in the ultimate tissue baseline of ESC (Figure 3A).[62] Relatively high me-CpG at late-glial genes is even higher in grade 2/3 oligodendrogliomas or astrocytomas (*IDH1*- or *IDH2*-mutated gliomas) vs normal brain (Figure 3B).[63-66] Elevated H3K27me3 at late-glial genes in ESC is erased with ontogeny into the normal brain (Figure 3C),[59,62] but this erasure does not occur in analyzed GBM (Figure 3C); H3K27me3 is depleted from late-glial genes in gliomas containing *H3F3A* K27M or G34V mutations (Figure 3C),[67,68] however, acquisition of the H3K27ac "on" mark, that occurs with ontogeny into the normal brain, fails to occur (Figure 3C).[40,69-71] Failure to activate late-glial genes might be explained by different lineage-trajectory, for example, with *H3F3A* G34R/V-mutated gliomas that originate from GSX2/DLX-expressing interneuron-progenitors, however, these tumors also fail to activate late-neuronal programs.[72] Thus, pediatric gliomas, including H3F3A K27M-mutated gliomas, recapitulate key features of adult gliomas: wiring with the glial lineage-commitment MTF circuit (Figure 3D), early-glial and MYC-target gene activation as expected from this (Figure 3E), but the attenuated transition to late-glial

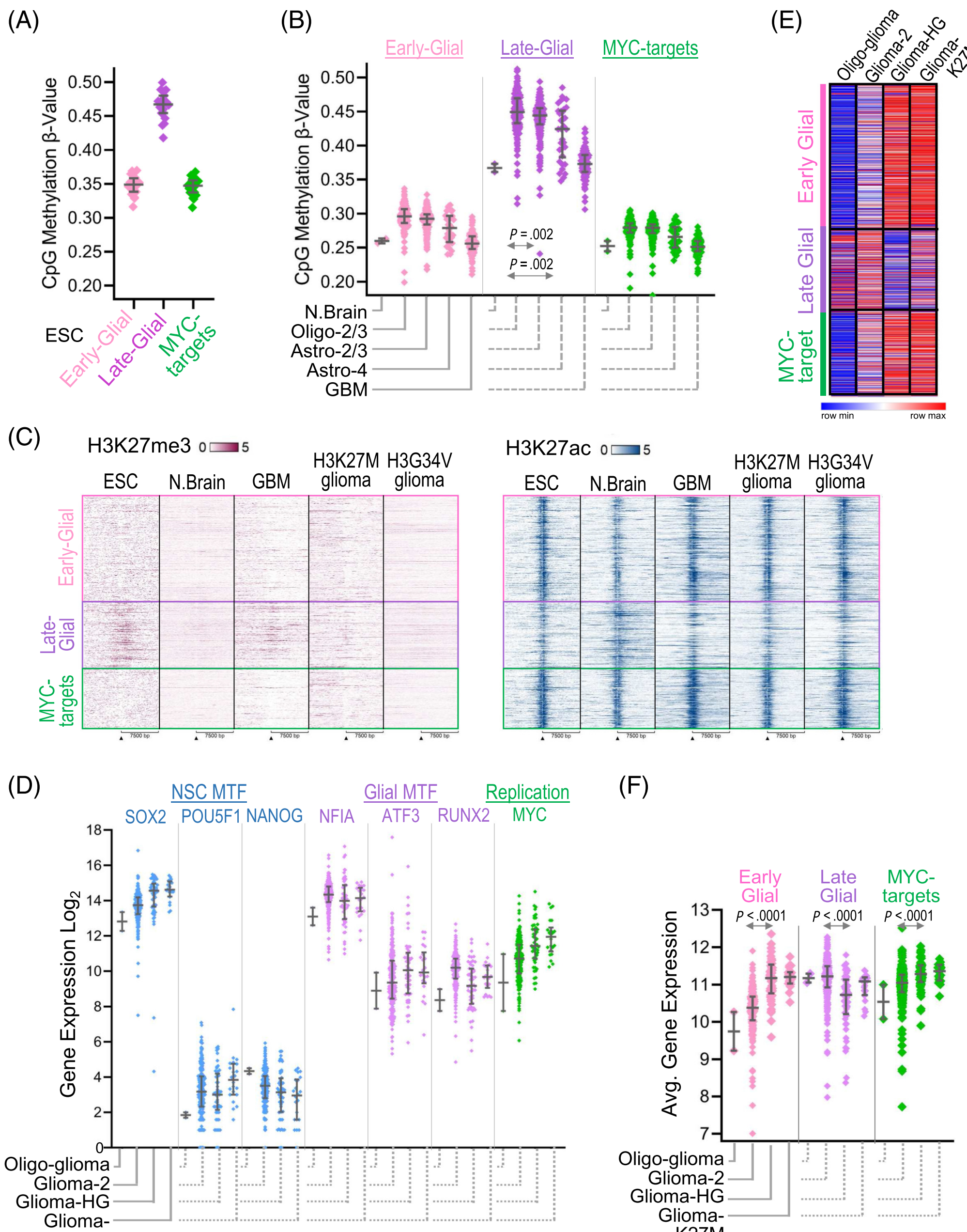


FIGURE 3 Glioma-genesis exploits differences in epigenetic landscape: MYC-target and early-glial genes have a constitutively accessible epigenetic configuration but late-glial genes do not. (A) me-CpG at early-glial, late-glial and MYC-target genes in embryonic stem cells (ESC) (gene groups as per Figure 1) (A) Public data GSE31848116. Median ± interquartile range (IQR). ESC (n = 19). me-CpG measured by Illumina 450K array. (B) me-CpG at early-glial, late-glial and MYC-target genes in normal cerebral cortex vs clinicopathologic types of glioma. me-CpG measured by Illumina 450K array, TCGA public data as per Figure 1. *P*-value Mann-Whitney test 2-sided. (C) H3K27me3 and H3K27ac distributions at early-glial, late-glial and MYC-target genes in ESC, normal brain cortex and gliomas without and with histone 3 gene (H3F3A) mutations. Public ChIP-seq data (FastQ files processed by UseGalaxy suite of tools): ESC H3K27me3—GSM428295 (Encode); Normal cerebral cortex H3K27me3—GSM772833 (Encode); ESC H3K27ac—GSM466732 (Encode); Normal cerebral cortex H3K27ac—GSM1112812 (Encode); GBM (SF9402), H3K27M glioma (SF7761) and H3G34V glioma (KNS42) H3K27me3 and H3K27ac GSE162976117. Plots using EASEQ. (D) Pediatric gliomas recapitulate the glial lineage-specifying MTF (NFIA, ATF3, RUNX2) wiring observed in adult gliomas (Figure 1). Of NSC-specifying MTF, only SOX2 is highly expressed, again as also seen in adult gliomas, and as expected from stable SOX2 expression through normal glial lineage-maturation.[20] Oligo-glioma = oligodendroglioma (n = 2); Glioma-2 = glioma grade 2 (n = 236); Glioma-HG = glioma high-grade (n = 53); Glioma-K27M = glioma containing H3F3A K27M mutation (n = 22). Pediatric Brain Tumor Atlas[61] public data, RSEM values (counts normalized by RNA-Seq by Expectation-Maximization). (E) More aggressive pediatric gliomas display deeper late-glial gene suppression, accompanied by more upregulation of early-glial and MYC-target genes. Heat map collapsed on average expression per gene in all the samples in each subtype (samples as per panel D). (F) Average expression of all early-glial, late-glial and MYC-target genes in each pediatric glioma sample (samples as per panel D). *P* values Mann-Whitney test two-sided [Color figure can be viewed at wileyonlinelibrary.com]

programs, worse in higher grade disease (Figure 3E, F). The constitutive difference in the epigenetic landscape at replication/early-lineage vs late-lineage genes, and exploitation of this epigenetic gradient by neoplastic evolution to decouple replication from lineage-maturation, have been shown for other tissue lineages also.[40,73,74]

8 | MECHANISMS UNDERLYING CHROMATIN-REMODELING FAILURE AT LATE-GLIAL GENES

The following genetic alterations are highly recurrent or pathognomonic of gliomas, and are implicated in chromatin-remodeling failure at late-glial genes.

Amplifications of EZH2 or SUZ12, key components of the PRC2 complex that writes H3K27me3. Almost all GBMs recurrently amplify *EZH2* via whole chromosome 7 gains, and ∼10% also amplify *SUZ12*, driving higher expression (Figure 4A, B).[46,76-79] Supporting functional consequences, immunohistochemical quantification found >95% H3K27me3-positive cells in 41/72 (57%) GBM samples analyzed, and >50% H3K27me3-positive cells in most of the remainder.[80]

Deletions of the H3K27me3 eraser KDM6B; KDM6A decrement in males. KDM6B is a H3K27me2 and H3K27me3-specific demethylase,[60,81,82] and its gene locus at chromosome 17p13.1 is deleted in ∼10% of GBMs with correspondingly suppressed expression (Figure 4A, B; 17p13.1 is also the *TP53* locality). *KDM6A* at Xp11.3 produces another PRC2-counteracting demethylase—substantially lower KDM6A levels in males vs females may contribute to male bias in glioma incidence (Figure 4C).[81]

Missense mutations H3F3A K27M or HIST1H3B/C K27M. These heterozygous histone 3 (H3) gene mutations occur in ∼30% of pediatric high-grade gliomas and ∼100% of diffuse midline gliomas. The resulting amino-acid substitution precludes writing H3K27me3 (off) or H3K27ac (on) marks (dominant-negative effect)[83](Figure 3C), and late-glial genes are as repressed in these gliomas as gliomas without the mutations (Figure 3D-F). That is, H3 K27M functional impact is aberrant repression of late-glial genes, with depletion of H3K27me3, but also H3K27ac.[84]

Recurrent deletions of EP300 (p300) that writes H3K27ac. The histone acetyltransferase p300, encoded by *EP300* at 22q13.2, is deleted in ∼10% of grade 2/3 astrocytomas, increasing to >40% of grade 4 astrocytomas or GBMs, with correspondingly suppressed expression (Figure 4A,B). H3K27ac deposition at late-glial genes is decreased in gliomas compared to normal brain (Figure 3C).

Inactivating mutations/deletions of alpha-thalassemia X-linked mental retardation (ATRX) that mediates H3 exchange. Inactivating mutations in *ATRX* at Xq21.1 characterize astrocytomas and *H3F3A* G34R/V-mutated pediatric gliomas.[4,7,63] ATRX is linked to the histone chaperone DAXX and to histone H3.3 exchange (reviewed in Reference 85)—histone turnover may regulate H3K27me3 amounts, since, in immunohistochemical analyses of a series of astrocytoma samples (n = 41), all had >50%, and most had >95%, H3K27me3-positive cells, while oligodendrogliomas that do not have ATRX inactivating mutations had <5% H3K27me3-positive cells.[80] ATRX has also been implicated in telomere length regulation and DNA repair[54,86,87] and cytogenetic instability resulting from ATRX loss might explain concordance of *ATRX*- with *TP53*-mutations.[30,88-90]

Missense IDH1 or IDH2 mutations that compromise me-CpG erasure by TETs. *IDH1/2* mutations are pathognomonic of oligodendrogliomas and astrocytomas (Figure 4B). Cytoplasmic wildtype IDH1 and mitochondrial IDH2 produce alpha-ketoglutarate (AKG), a mandatory cofactor for Jumonji histone demethylases (KDMs) and Ten-Eleven Translocation (TET) family DNA methylcytosine dioxygenases that erase me-CpG.[91,92] Mutant-IDHs produce an oncometabolite 2-hydroxyglutarate (2HG) that competes with AKG, inhibits TET family enzymes, increases me-CpG, and represses late-glial genes (Figure 3B): mutant-IDH1 or 2HG introduction into differentiating glial cells stalled lineage-maturation,[65,93] and *IDH*- and *ATRX*-mutation in neural precursors increased neuro-glial precursor proliferation and immortalized astrocytes—the increased proliferation was controlled by apoptosis, thus, subsequent *TP53*-mutations caused glioma-genesis.[54,94-96]

TET1 deletions. *TET1* deletions via chromosome 10 losses, or minimal deletion of the *TET1* locus at 10q21.3, typify GBMs, and also occur in astrocytomas, increasing from <10% in grade 2/3 astrocytomas to >40% in grade 4 (Figure 4A,B).[77,78,97-99] Interestingly, me-CpG at late-glial genes is much higher in IDH-mutant gliomas, even though these have intact *TET1*, than in GBMs with *TET1* haploinsufficiency (Figure 3B)—possibly, reduction in TET1 protein amounts disrupts multiprotein coactivator complexes to hence impede gene activation in ways beyond disrupted erasure of me-CpG.[100,101]

Amplifications of DNA methyltransferase 1 (DNMT1) that writes me-CpG. *DNMT1* at 19p13.2 is amplified in ∼40% of oligodendrogliomas and in ∼40% of GBMs,[30,78] driving higher DNMT1 expression (Figure 4A,B). DNMT1 writes/maintains me-CpG onto the newly synthesized DNA strand during S-phase (maintenance methyltransferase) and is also a corepressor recruited into lineage MTF protein hubs.[59,62,73,74]

Deletions of SWI/SNF-family ATP-dependent chromatin remodelers that reposition nucleosomes to allow basal transcription factor access to genes. *ARID1A* (chromosome 1p36.11), *ARID1B* (6q25.3), *SMARCA2* (9p24.3) and *SMARCB1* (22q11) are components of the BAF coactivator complex that repositions nucleosomes for gene activation. Oligodendrogliomas are characterized by 1p deletions and hence *ARID1A* haploinsufficiency (Figure 4A,B). Deletions of *ARID1B* and *SMARCA2* are found in 20-30% of grade 2/3 astrocytomas, increasing to 30% to 70% in grade 4 astrocytomas, and also in 20% to 40% in GBMs, driving lower expression (Figure 4A,B).

Deletions/translocations of genes for other activating machinery. Genes for other key components in the machinery needed to activate genes, for example, cohesins, splicing factors, mediator family members and histone methyltransferases containing SET-domains (KMTs), are frequently deleted and sometimes translocated in gliomas, as for cancers in general.[6,7,10,73]

Alterations to lineage MTF. Genes for lineage MTF themselves, for example, *SOX10*, can be mutated, translocated or deleted, to thereby disrupt mutual cooperation in MTF circuits that mediates exchange of corepressors for coactivators - corepressor/coactivator imbalance in lineage MTF hubs represses instead of activates late-lineage genes[40,74,102,103] (reviewed in Reference 3).

(A)

	DNMT1	TET1	EZH2	SUZ12	KDM6B	EP300	ARID1A	ARID1B	SMARCA2	SMARCB1
Pearson's r	0.31	0.64	0.48	0.22	0.20	0.46	0.45	0.43	0.49	0.55
P-value	<.0001									

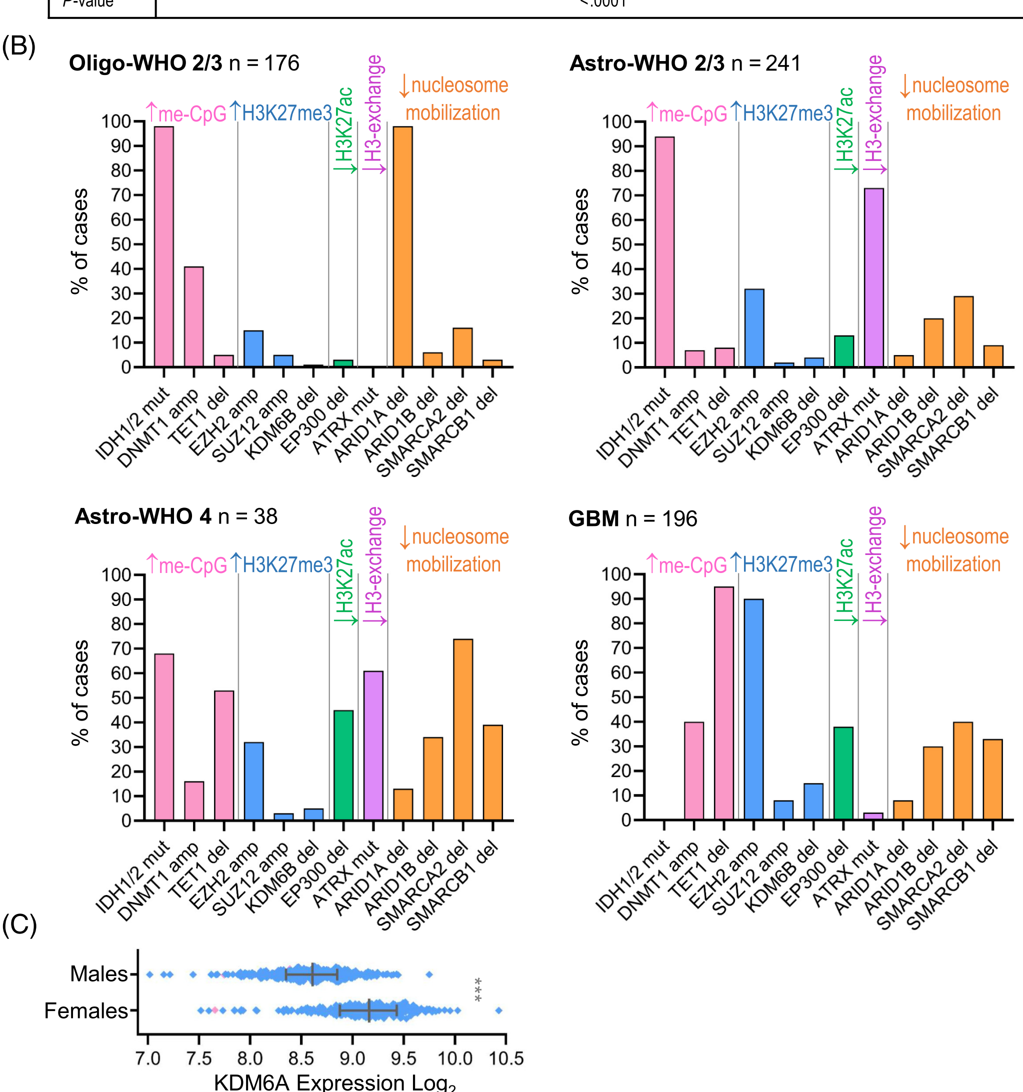
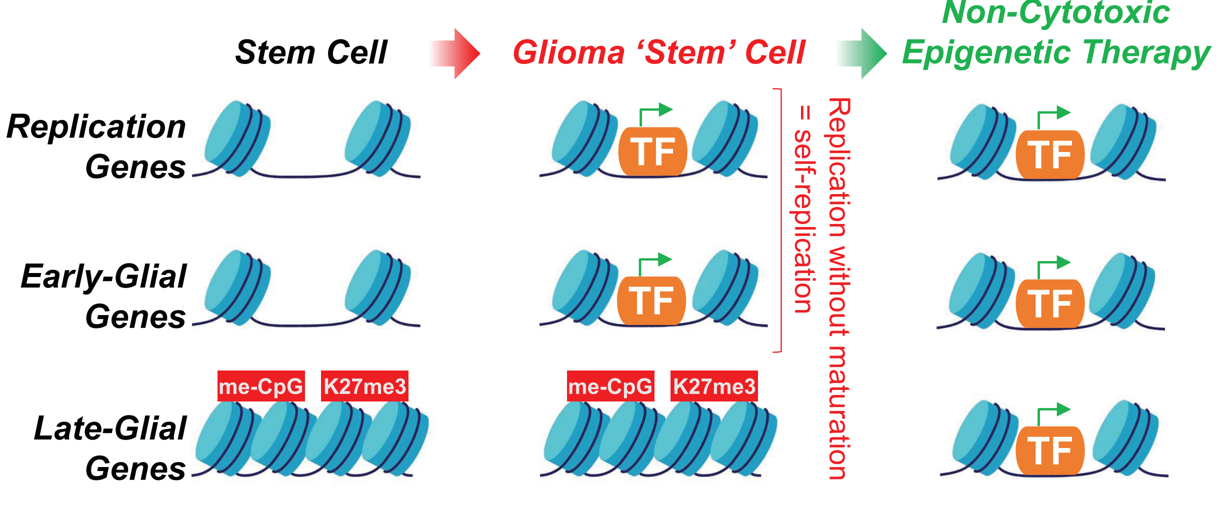


FIGURE 4 Gliomas contain recurrent genetic alterations expected to preserve or increase H3K27me3 and me-CpG, and simultaneously decrease H3K27ac and other chromatin remodeling needed to activate late-glial genes. TCGA public data (n = 651). (A) Significant correlation between gene copy number and expression of the chromatin remodelers. The GISTIC2 method[75] produced segmented copy number variant data mapped to genes to produce gene-level estimates. Gene-level transcript estimates by RNA-Sequencing were analyzed as $\log_2(x + 1)$ transformed RSEM normalized counts. Pearson correlation coefficients, *P*-value two-sided. (B) Glioma-genesis alters several classes of chromatin remodelers, at frequencies that increase with the aggression of disease. Percentage of cases in each WHO/EANO glioma sub-group with the indicated gene copy number or mutation changes. Gene-level copy number estimates generated by the GISTIC2 method were thresholded to estimated values −2, −1, 0, 1, 2 representing homozygous or single-copy deletion (del), diploid normal copy or low-level or high-level copy number amplification (amp). (C) The H3K27 demethylase KDM6A is significantly less expressed in gliomas in males vs females. ***$P < .0001$, Mann-Whitney test, two-sided [Color figure can be viewed at wileyonlinelibrary.com]

Glioma-genesis thus impedes, in several orthogonal ways, the epigenetic work that replicating glial-precursors must exercise to transition to terminal glial-fates. Each ectopic replication caused by this friction against lineage-maturation is an opportunity to select another mutation or copy number alteration to further hinder epigenetic work needed to mature, thereby escalating grade, replications and pace of disease in a merciless clinical reality[17] (reviewed in Reference 104).

9 | RESUMING LINEAGE-MATURATION, INSTEAD OF ACTIVATING APOPTOSIS, TO TERMINATE MALIGNANT SELF-REPLICATIONS

Demonstrating cause-effect, inhibiting corepressors terminates glioma self-replication via lineage-maturation, shown by several groups (Figure 5): small molecule inhibitors of EZH2 (MC4040,



1. Chromatin is constitutively 'open' at ***Replication*** and ***Early-Glial*** genes, but 'closed' at ***Late-Glial*** genes

2. Glioma-genesis selects to disrupt machinery that opens chromatin. **Glial-lineage TF** thus activate replication and early-glial, but not late-glial genes (**malignant self-replication**)

3. Drugs to inhibit chromatin-closing enzymes allow glial-lineage TF to activate **terminal glial-fates**

TF = transcription factor me-CpG = methylated CpG K27me3 = histone 3 lysine 27 trimethylation

FIGURE 5 Summary. Glioma-genesis selects to impede chromatin-remodeling needed to activate late-glial lineage genes, thus converting the exponential replications of glial-lineage committed progenitors into self-replications (glioma "stem" cells). Inhibiting repressing epigenetic enzymes enables glial-lineage transcription factors, already highly expressed in glioma stem cells, to activate late-glial genes and hence terminate malignant self-replications, without a need for an intact apoptosis program [Color figure can be viewed at wileyonlinelibrary.com]

MC4041, tazemetostat) decreased glioma cell proliferation without apoptosis-induction, but by resumed onward lineage-differentiation (upregulation of p27 and E-cadherin).[105] Tazemetostat also decreased glioma self-replication in vitro and in vivo in other studies, again not by apoptosis, although terminal-differentiation was not specifically analyzed.[106] EZH2 downregulation with short hairpin RNA or with a small molecule (DZNep) impaired glioma self-replication in vitro and tumor-initiation in vivo.[46] Consistent with a nonapoptosis pathway, there was no significant effect on glioma cell viability, even as sphere morphology (a measure of self-replication) and proliferation were reduced.[46] In *H3F3A* K27M-mutated gliomas, EZH2 inhibition decreased proliferation in vitro and increased survival in mice.[107] Another study looked specifically for a cytotoxic effect of EZH2 inhibition in the glioma cells to explain the cytoreduction, and did not find any.[108]

ATP-dependent chromatin remodelers of the ISWI and CHD families, for example, CHD4, SMARCA5, oppose SWI/SNF ATP-dependent chromatin remodelers. That is, they execute a linchpin epigenetic repression event of re-positioning nucleosomes to obstruct access to genes by basal transcription factor machinery. CHD4 depletion using shRNA promoted astrocyte differentiation in vitro,[109] implying ISWI/CHD-family members are candidate targets for therapy.

Mitochondrial outputs other than AKG, for example, cytidine triphosphate (CTP), also facilitate lineage-differentiation: small molecules that inhibit de novo pyrimidine synthesis and decrease CTP, including dihydroorotate dehydrogenase (DHODH)-inhibitors (several available) and the cytidine triphosphate synthase 2 (CTPS2)-inhibitor cyclopentenyl cytosine (CPEC), release cancer cells including glioma cells to terminal-differentiation.[110-112] Implicating CTP specifically, CTP-restoration with exogenous cytidine prevented terminal-differentiation induction by the DHODH-inhibitor leflunomide.[113] These results imply that CTP operates as a cofactor in a corepressor complex, and interestingly, key DNA packaging proteins in prokaryotes are CTP-dependent and related to eukaryotic condensins.[114]

The deoxycytidine analog decitabine, a clinical pro-drug approved to treat myeloid malignancies, inhibits and depletes DNMT1 from dividing cells. Self-replication of *IDH1*-mutated glioma cells was terminated by decitabine treatment in vitro, without activation of apoptosis, but with activation of neuronal/glial lineage-differentiation genes, and with morphology changes of terminal-differentiation.[115] The cytidine analog 5-azacytidine also inhibits/depletes DNMT1: long-term administration of 5-azacytidine to mice with *IDH1*-mutated anaplastic astrocytoma significantly decreased tumor growth; histological examination indicated terminal-differentiation was the pathway of tumor

TABLE 1 Clinical trials of noncytotoxic epigenetic drugs (or pro-drugs) to treat gliomas

Trial Identifier	Drug	Phase	Status	Prior XRT/ Chemo[a]	Glioma subtype/contrast-enhancement on MRI	Results
NCT02073994	Ivosidenib (IDH1-inhibitor)	I	Not recruiting	Yes	Glioma, IDH-mutant (n = 66); noncontrast (n = 35) and contrast-enhancing (n = 31)	ORR 2.9% and growth rate reduction[b] by 14% in 24 evaluable patients with noncontrast-enhancing gliomas
NCT02481154	Vorasidenib (IDH1/2-inhibitor)	I	Not recruiting	Yes	Glioma, IDH-mutant (n = 52); noncontrast (n = 22) and contrast-enhancing (n = 30)	ORR 18% in noncontrast-enhancing gliomas
NCT04164901	Vorasidenib (IDH1/2-inhibitor)	III	Recruiting	No	Glioma, IDH-mutant (WHO II); noncontrast-enhancing	Pending
NCT03343197	Ivosidenib (n = 12); Vorasidenib (n = 13)	I	Not recruiting	Yes	Glioma, IDH-mutant (n = 25); noncontrast enhancing	2-HG levels in resected glioma tissue substantially lowered by both ivosidenib and vorasidenib
NCT03684811	Olutasidenib (IDH1-inhibitor)	Ib/II	Not recruiting	Yes	Glioma, IDH-mutant (n = 24); contrast-enhancement not described in interim results	ORR 4%
NCT04458272	DS-1001b (IDH1-inhibitor)	II	Not recruiting	Yes	Glioma, IDH-mutant (n = 38); noncontrast (n = 9) and contrast-enhancing (n = 29)	ORR 22% in noncontrast-enhancing gliomas; ORR 14% in contrast-enhancing gliomas
NCT02746081	BAY1436032 (IDH1-inhibitor)	I	Not recruiting	Yes	Glioma, IDH mutant (n = 49); noncontrast (n = 2) and contrast-enhancing (n = 33); GBM (n = 14)	ORR 11% in gliomas (RANO criteria for contrast-enhancing gliomas)
NCT03666559	5-Azacytidine (DNMT1-inhibitor)	II	Recruiting	Yes	Glioma, IDH-mutant; contrast-enhancement not described	Pending
NCT03922555	Decitabine (DNMT1-inhibitor) + cedazuridine (CDA-inhibitor)	I	Recruiting	Yes	Glioma, IDH-mutant; noncontrast enhancing	Pending
NCT00238303	Vorinostat (HDAC-inhibitor)	II	Completed	Yes	GBM (n = 52)	ORR 4% (Mac Donald criteria); Modest increase in PFS (median 11.2 months; range 6.8-28) and OS (median 5.7 months; range 0.7-28)[c]
NCT00641706	Vorinostat + bortezomib (proteasome inhibitor)	II	Completed	Yes	GBM (n = 37)	ORR 3% (modified Mac Donald criteria); No improvement in PFS and OS[c]
NCT01738646	Vorinostat + bevacizumab (VEGF-antibody)	II	Completed	Yes	GBM (n = 40)	No improvement in PFS and OS[c]
NCT01266031	Vorinostat + bevacizumab	II	Completed	Yes	GBM (n = 49)	No improvement in PFS and OS[c]
NCT00762255	Vorinostat + bevacizumab + irinotecan	I	Completed	Yes	GBM (n = 19)	Significantly increased OS in patients receiving higher doses of vorinostat compared to patients receiving lower doses (10.1 vs 5.7 months respectively)[c]
NCT00939991	Vorinostat + bevacizumab + temozolomide	I/II	Completed	Yes	GBM (n = 39)	ORR 43.6% (RANO criteria for contrast-enhancing gliomas); No improvement in PFS and OS[c]
NCT00268385	Vorinostat + radiation therapy + temozolomide followed by vorinostat + temozolomide	I/II	Not recruiting	No	GBM (n = 107)	No improvement in PFS and OS[c]
NCT01189266	Vorinostat + radiation therapy followed by vorinostat	I/II	Completed	No	Pediatric diffuse intrinsic pontine glioma (n = 76)	No improvement in PFS and OS[c]

TABLE 1 (Continued)

Trial Identifier	Drug	Phase	Status	Prior XRT/ Chemo[a]	Glioma subtype/contrast-enhancement on MRI	Results
NCT00859222	Panobinostat (HDAC-inhibitor) + bevacizumab	I/II	Completed	Yes	GBM (n = 24) and anaplastic astrocytoma (WHO III; n = 15)	ORR 29.2% for GBM cohort and 26.7% for astrocytoma (WHO III) cohort (RANO criteria for contrast-enhancing gliomas); No improvement in PFS and OS[c]
NCT00302159	Valproic acid (HDAC-inhibitor) + radiation therapy + temozolomide	II	Completed	No	GBM (n = 30)	ORR 0% (RANO criteria for contrast-enhancing gliomas); Improved OS (70% after 12 months; CI 76-98) and PFS (70% after 6 months; CI 57-87)[c]
NCT00879437	Valproic acid + radiation therapy followed by valproic acid and bevacizumab	II	Completed	No	Pediatric diffuse intrinsic pontine glioma (DIPG; n = 18) and high grade glioma (HGG; n = 14)	ORR 55% for DIPG cohort and 42% for HGG cohort (RANO criteria for contrast-enhancing gliomas); No improvement in PFS and OS[c]

Note: Overall objective response rate (ORR) per Radiology Assessment in Neuro-Oncology (RANO) criteria for gliomas with and without contrast enhancement on MRI unless indicated otherwise.

Abbreviations: OS, overall survival; PFS, progression-free survival.

[a]Yes = Patients received one or more rounds of radiotherapy/chemotherapy prior to study inclusion.

[b]Growth rate reduction assessed by longitudinal MRI measurements of three-dimensional tumor volume before and after treatment.

[c]Compared to intervention without HDAC-inhibitor or historic control. No glioma clinical trial data is available at this time for EZH2-, KDM1A- or DHODH-inhibition.

cytoreduction.[116] 5-azacytidine also suppressed IDH-wildtype GBM growth in vitro and in xenografts.[117] 5-azacytidine and decitabine, unfortunately, have pharmacology limitations for treating gliomas (or other solid tumors) in humans, one being that both are rapidly inactivated in solid tissues by the catabolic enzyme cytidine deaminase (CDA).[118,119] To address this limitation, a combination of decitabine with the CDA-inhibitor cedazuridine is in glioma clinical trials (Table 1). Another limitation is that both are pro-drugs that require activation by uridine cytidine kinase 2 and deoxycytidine kinase respectively, pyrimidine metabolism enzymes that are intrinsically much more highly expressed in hematopoietic cells - neutropenia can thus clinically pre-empt achievement of DNMT1-targeting in solid tumor tissue, and methods to overcome this limitation are being explored.[118,120,121]

IDH1-mutated glioma cells were released to terminal differentiation by small molecule inhibitors of mutant-IDH1 in preclinical studies.[122-125] Clinical trial results have been reported for the mutant-IDH1-inhibitors ivosidenib (Agios), olutasidenib (Forma Therapeutics), DS-1001b (Daichi Sankyo), BAY1436032 (Bayer), and the dual mutant-IDH1/IDH2-inhibitor vorasidenib, to treat relapsed/refractory gliomas[126-130]: objective response rates by Response Assessment in Neuro-Oncology (RANO) criteria ranged from ~3% to 29% (Table 1). These low to modest response rates compare unfavorably to high response rates and regulatory approval of mutant-IDH-inhibitors to treat IDH-mutated myeloid malignancies. By way of possible explanation, IDH-mutant gliomas contain numerous mutations and copy number alterations impacting several classes of epigenetic enzymes (Figure 4), compared to few such alterations in IDH-mutant myeloid malignancies. Clinical treatment narrowly specific for mutated-IDH may therefore have less impact on relieving aberrant repression of late-lineage genes in glioma vs myeloid cancer cells.[88-90,131-134] In this regard, even in IDH-mutant myeloid malignancies, clinical practice often combines mutant-IDH-inhibitors with the DNMT1-targeting agents 5-azacytidine or decitabine.

One caveat with magnetic resonance imaging (MRI) assessment of glioma-response is that advancing glioma can be difficult to distinguish from radiation-induced changes in the normal brain.[132,135] This measurement problem is not expected in a trial evaluating vorasidenib as a first-line treatment of IDH-mutant gliomas, results of which are pending[136] (Table 1).

Histone deacetylases (HDAC) and lysine demethylase 1A (KDM1A) are implicated in the repression of lineage-differentiation programs in cancer cells broadly including glioma cells, and accordingly, HDAC- and KDM1A-inhibitors induce terminal-differentiation in vitro and in preclinical in vivo studies.[137-141] Several HDAC inhibitors are approved to treat peripheral T-cell lymphomas, but none are approved to treat cancers of other lineages: vorinostat, panobinostat and valproic acid combined with standard treatments have been evaluated in glioma clinical trials, but without clear evidence of added benefit (Table 1).[142-151] Limited success in translating the preclinical observations into clinical therapy could reflect that HDACs and KDM1A have nonhistone substrates such that even on-target drug effects produce clinical toxicities that restrict exposures needed to achieve intended epigenetic pharmacodynamic effects in solid tumor tissue.

Next steps? HDACs, KDM1A, DNMT1, CHD4, EZH2, DHODH (or CTPS2) and mutant-IDH1/2 are thus validated preclinically as targets for inhibition to compel p53/p16-independent glioma cell cycling exits. However, no major successes have occurred with limited attempts at clinical translation to date (Table 1). Reasons for this, and thus potential solutions, can be determined: some targets, for example, HDACs, KDM1A, have wide cell-physiology roles such that even specific, on-target actions of small molecule inhibitors cause toxicities, including cytotoxicity, that limits feasible clinical exposures needed to achieve tumor pharmacodynamic effect. DNMT1 is a target that can in principle be safely engaged, shown by safety and effectiveness of noncytotoxic DNMT1-targeting regimens of decitabine or 5-azacytidine in patients with myeloid malignancies, including fragile elderly patients with the p53-inactivated disease. However, for drug-metabolism reasons, these pro-drugs have very limited distribution and activation in glioma and other solid tumor tissue—potential solutions for this have been proposed but need clinical evaluation. CHD4 does not yet have a small molecule inhibitor for clinical evaluation, although at least one is in pre-clinical development. Safe clinical inhibitors for EZH2 and DHODH are available, but results from glioma clinical trials are not available—the preclinical data supports the pursuit of clinical trials. Arguably, clinical trials with inhibitors of mutant-IDH are the only ones in which intended molecular pharmacodynamic effects were sufficiently achieved in glioma-tissue, but even so, responses were minimal to modest—glioma-genesis selects to alter epigenetic enzymes from several classes, and oncotherapy should counter like-wise; combining noncytotoxic drugs, all aiming to renew lineage-maturation, is routine treatment practice for some myeloid malignancies—p53/p16-attenuation and pathobiology of malignant self-replication recommends this approach to gliomas too.[152]

10 | CONCLUSION

Genetic attenuation of the p53/p16-apoptosis pathway in glioma cells contributes to poor outcomes with apoptosis-intending (cytotoxic) treatments. Normal p53-intact cells are meanwhile destroyed, causing significant toxicity. Contrasting with attenuated p53/p16, self-replicating glioma cells highly express glial lineage-specifying MTF circuits that cooperate with MYC to activate exponential proliferation, but fail in their other usual function of also driving maturation along lineage-axes: late-glial genes have constitutively "closed" chromatin requiring chromatin-remodeling for activation, and neoplastic evolution selects to disrupt the epigenetic machinery that performs this work. Pharmacologic inhibition of repressing epigenetic enzymes recouples to lineage-maturation and hence terminates malignant self-replication, independent of p53/p16/apoptosis, justifying clinical development oriented to epigenetic molecular pharmacodynamic effects without cytotoxicity.

AUTHOR CONTRIBUTIONS

Conceptualization and design: Nikolaus von Knebel Doeberitz and Yogen Saunthararajah. Funding: Daniel Paech and Yogen Saunthararajah. Collection and assembly of data: Nikolaus von Knebel Doeberitz and Yogen Saunthararajah. Visualization of data: Nikolaus von Knebel Doeberitz and Yogen Saunthararajah. Validation of data: All authors. Manuscript writing: Nikolaus von Knebel Doeberitz and Yogen Saunthararajah. Review and editing of the manuscript: All authors. Final approval of the manuscript: All authors. Accountable for all aspects of the manuscript: All authors. The work reported in the paper has been performed by the authors unless clearly specified in the text.

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CONFLICT OF INTEREST

Ownership: Yogen Saunthararajah—EpiDestiny. Income: None. Research support: none. Intellectual property: Yogen Saunthararajah—patents around tetrahydrouridine, decitabine and 5-azacytidine (US 9259469 B2; US 9265785 B2; US 9895391 B2) and cancer differentiation therapy (US 9926316 B2). Stefan Pusch—eligible to royalties as co-inventor of BAY1436032. The corresponding patents are under the administrative supervision of the DKFZ technology transfer office. All other authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data analyzed are in public databases—The Cancer Genome Atlas (TCGA), Encode, GEO Database, Cancer Cell Line Encyclopedia (CCLE), European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI)—as specifically indicated in each figure legend. The data that support the findings of our study are available from the corresponding author upon reasonable request.

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