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Review

Epigenomic Reprogramming as a Driver of Malignant Glioma

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SUMMARY

Malignant gliomas are central nervous system tumors and remain among the most treatment-resistant cancers. Exome sequencing has revealed significant heterogeneity and important insights into the molecular pathogenesis of gliomas. Mutations in chromatin modifiers—proteins that shape the epigenomic landscape through remodeling and regulation of post-translational modifications on chromatin—are very frequent and often define specific glioma subtypes. This suggests that epigenomic reprogramming may be a fundamental driver of glioma. Here, we describe the key chromatin regulatory pathways disrupted in gliomas, delineating their physiological function and our current understanding of how their dysregulation may contribute to gliomagenesis.

INTRODUCTION

Chromatin is the physiological state in which genetic material is packaged in the nucleus and consists of genomic DNA and its interacting proteins. The basic repeating subunit of chromatin is composed of two copies of the four core histone proteins H2A, H2B, H3, and H4, which together form an octamer around which DNA is wrapped to form the nucleosome (Luger et al., 1997). Unlike the DNA sequence, chromatin structure is highly dynamic, allowing information encoded within the DNA sequence to be transmitted in different ways in a cell-type-specific fashion. Cellular signals reach the nucleus to affect gene expression through the activity of DNA binding transcription factors—this engagement occurs in the context of chromatin, which can be either permissive or restrictive, thereby providing an additional layer of regulation to tailor gene expression and cellular phenotypes. Phenotypic variations that cannot be explained by alterations in the DNA template are often referred to as being ''epigenomic'' in nature, although mechanistic details into how epigenomic states are inherited during differentiation and development remain poorly understood.

The chromatin landscape can be modulated through several mechanisms including (1) post-translational modifications (PTMs) on histones and DNA methylation, (2) remodeling of histone:DNA and histone:histone contacts within nucleosomes and their genomic positions, and (3) the incorporation of minor histone ''variants'' with distinct function into nucleosomes (Allis and Jenuwein, 2016). Modifications on histones and DNA are established by ''writer'' enzymes (Brownell et al., 1996), removed by ''eraser'' enzymes (Taunton et al., 1996), and exert function in some cases when recognized by ''reader'' proteins (Jones et al., 1998), which mediate specific effects on chromatin (i.e., opening or closing of the chromatin fiber). On DNA, methylation of cytosines at position 5 on the pyrimidine ring (5mC) is the more abundant modification and is often associated with gene repression when present at transcription factor binding sites at gene promoters (Razin and Riggs, 1980). In contrast, an impressive array of PTMs have been described on histone proteins (Huang et al., 2014), perhaps the most well characterized being acetyl or methyl modifications on specific lysine residues of the unstructured amino-terminal ''tail'' of histone H3. PTMs have characteristic genomic locations and can either mediate, or are correlated with, specific transcriptional states. For example, methylation of H3-lysine 27 (H3K27me3) mediates gene repression (Cao et al., 2002; Müller et al., 2002; Pengelly et al., 2013), while acetylation of multiple residues on H3 are associated with gene activation (e.g., H3K9ac and H3K27ac) (Allfrey et al., 1964; Brownell et al., 1996). The second broad class of proteins to shape the chromatin landscape are ''remodelers'' (Hirschhorn et al., 1992), which alter access for chromatin binding factors by changing nucleosome position or via direct exclusion. Some remodelers can also replace the histones within nucleosomes with variant isoforms, which alter chromatin structure locally for specialized functions (Goldberg et al., 2010). For example, canonical H3 can be replaced by the variant H3.3, whose deposition is replication independent and important for transcriptional regulation in post-mitotic cells in the brain (Maze et al., 2015).

The discovery of mutations in chromatin regulators at a high frequency across human cancer was an unexpected finding from pivotal sequencing efforts (The Cancer Genome Atlas Research Network, 2008; Mardis, 2018). This is exemplified in malignant gliomas where, remarkably, genes involved in most of the chromatin regulatory mechanisms described above are either directly mutated or indirectly dysregulated, making glioma an instructive model of epigenomic reprogramming in cancer (The Cancer Genome Atlas Research Network, 2008; Verhaak et al., 2010; Wu et al., 2012; Schwartzentruber et al., 2012; Brennan et al., 2013). Gliomas arise in the central nervous system (CNS) and were historically classified based on their morphological resemblance to specific glial cells

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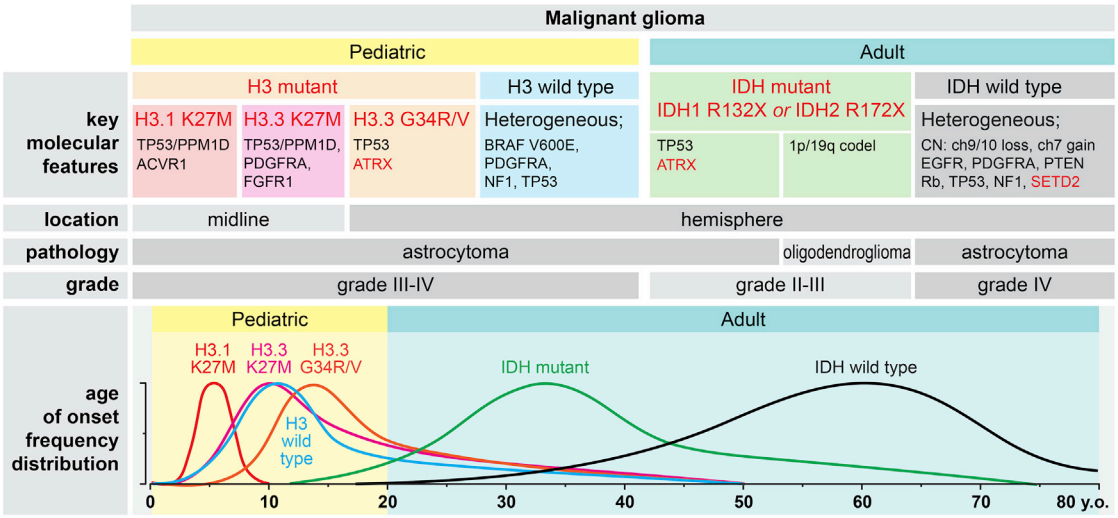

Figure 1. Simplified Molecular Subgrouping of Malignant Gliomas Defined by Common Somatic Mutations

Subtypes broadly defined by mutational status of H3 (in children) and IDH (in adults) with common accompanying molecular alterations for each subtype shown. Alterations in genes that regulate chromatin function are shown in red. Subtypes were initially identified in either adults or children, although they are distributed across age groups at different frequencies, e.g., H3 mutations were defined in children but also occur in young adults. The designation "H3K27M-midline glioma" is replacing "diffuse intrinsic pontine glioma or DIPG" to reflect a variety of midline anatomical locations (e.g., pons, spinal cord) in which these mutations occur. IDH mutant subtypes are further defined by either ATRX loss ("astrocytomas") or 1p/19q co-deletion ("oligodendrogliomas"). IDH wild-type group in adults and H3 wild-type group in children are highly heterogeneous and can be further subdivided based on DNA methylation analysis (reviewed in Sturm et al., 2014). Grade denotes typical grade at presentation, although tumors can present clinically at different points in their anaplastic evolution.

(e.g., astrocytes or oligodendrocytes) and the degree of anaplasia they exhibit, otherwise known as "grade." Malignant gliomas can broadly be considered those tumors ≥grade III (and ≥grade II in adults) due to their locally invasive behavior, meaning surgery cannot safely remove the entire tumor from the brain. Even with additional treatments such as radiation and/or chemotherapy, they remain inevitably fatal. The histopathological designation of gliomas has been augmented, and in many respects superseded, by molecular characteristics that identify biological subtypes—often defined by chromatin regulators—with specific distributions across age and anatomical location in the brain (Figure 1).

Here, we review the main chromatin pathways that are dysregulated in malignant glioma, using their function in normal physiology as a primer to illustrate how they may promote cancer. We speculate as to why aberrant chromatin drives cancer in specific contexts and highlight some of the fundamental questions that need to be addressed to translate mechanistic insights into therapies for what remains a largely incurable disease.

MISREGULATION OF H3K27 METHYLATION: H3K27M MUTATION AND EZHIP

H3 lysine 27 methylation is a PTM that functions as an important effector mechanism in gene repression mediated by a group of evolutionarily conserved multi-protein complexes collectively called the Polycomb repressive complexes (Schuettengruber et al., 2017). These complexes were discovered in *Drosophila* as critical regulators of cell fate during development, as evidenced by abnormal body patterning induced by mutations in Polycomb proteins (Lewis, 1978). Two Polycomb repressive complexes (PRC1 and PRC2) act cooperatively to repress genes. PRC2 is responsible for establishing H3K27 methylation, predominantly through EZH2 (Enhancer of zeste homolog 2), and to a lesser extent, EZH1 enzymatic ("writer") subunits. H3K27 can be mono-, di-, or trimethylated (H3K27me1, H3K27me2, and H3K27me3, respectively), with H3K27me3 the best characterized PTM. The functional importance of H3K27 as a substrate for methylation was inferred by genetic experiments in which H3 lysine-27 was mutated to arginine, which cannot undergo methylation at that site, thus leading to aberrant cell fate decisions, phenocopying Polycomb mutants (Pengelly et al., 2013). H3K27me3 interacts directly with a second complex, PRC1, through its "reader" component, a chromobox (CBX) protein. CBX proteins also have nucleosome-compacting (Francis et al., 2004; Lau et al., 2017) and phase-separating activity (Plys et al., 2019; Tatavosian et al., 2018), which may be essential for PRC1-mediated repression, potentially by restricting access of transcription factors to genes (Figure 2A). Polycomb repression is both heritable through cell division ("epigenetic memory") (Angel et al., 2011; Berry et al., 2015) and capable of being dynamically reversed when necessary, making it a versatile repressive mechanism, particularly in development.

There has been a surge in interest in the function of H3K27 methylation in glioma following landmark studies identifying mutations in histone H3 at high frequency in pediatric glioma, implicating mutant histones as potential oncogenes in cancer for the first time (Schwartzentruber et al., 2012; Wu et al., 2012). One class of the mutations described occurred recurrently at H3K27, leading to a lysine-to-methionine substitution (H3K27M), the exact site at which PTMs such as H3K27me3 is established. Interestingly, these H3K27M tumors occur in midline brain structures (Aihara et al., 2013; Schwartzentruber et al., 2012; Sturm et al., 2012; Wu et al., 2012), possibly reflecting a propensity for this mutation to transform a cell of origin in a specific anatomical location. The strict occurrence of these

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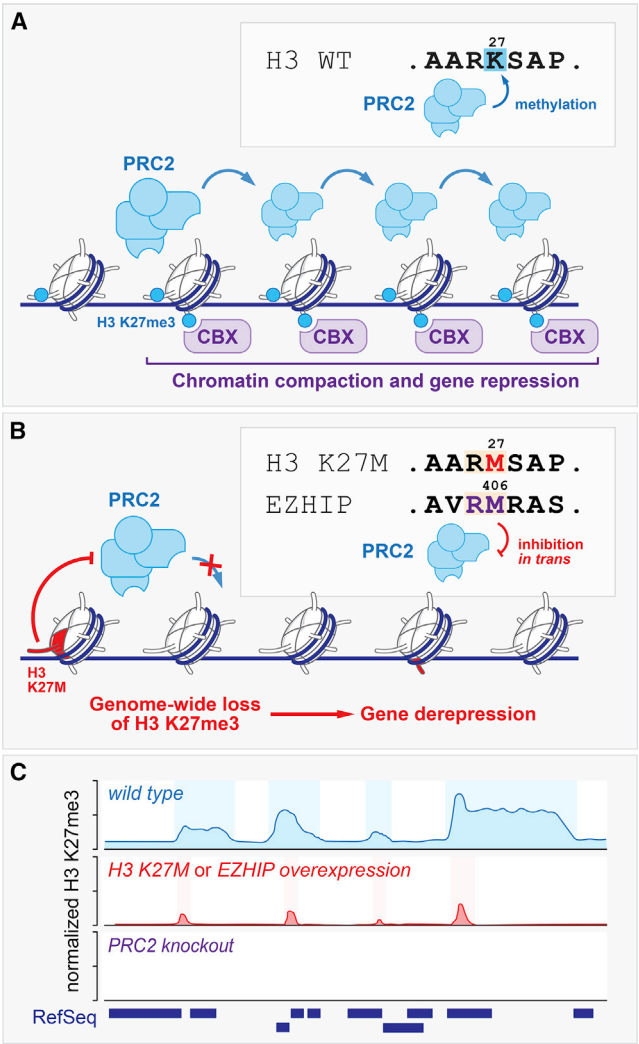

Figure 2. Reprogramming of the H3K27 Methylation Landscape by H3K27M Mutation and EZHIP

(A) PRC2 establishes H3K27me3 through its enzymatic component EZH2. H3K27me3 is recognized by a CBX "reader" protein in the PRC1 complex, which compacts chromatin to repress genes.

(B) PRC2 is unable to methylate the methionine residue in H3K27M, but also has reduced methylation activity in *trans*, i.e., at nucleosomes that contain wild-type *H3*. Amino acid sequence adjacent to K-to-M mutation aligns with EZHIP sequence showing similarity, an example of "oncohistone mimicry."

(C) Schematic of genome-wide H3K27me3 landscape contrasting wild-type, H3K27M, and PRC2 knockout cells. In H3K27M cells small areas of H3K27me3 are retained at PRC2 nucleation sites, in contrast to PRC2 knockout cells where H3K27me3 is totally ablated.

mutations at lysine 27, in addition to their heterozygous nature—especially given there are a total of 16 genes (and 32 alleles) from which H3 protein can be produced—suggests that the H3K27M mutation has dominant or neomorphic function. Indeed, biochemical data are consistent with this: *in vitro*, H3K27M inhibits the enzyme EZH2 and furthermore, H3K27M glioma tissues or cells in which H3K27M has been experimentally introduced exhibit dramatically reduced H3K27me3 (Bender et al., 2013; Chan et al., 2013; Lewis et al., 2013). The H3K27M mutation in histone H3, along with other recently identified histone mutations in H3 as well as in H2A, H2B, and H4, have been collectively described as "oncohistones" (Nacev et al., 2019). The function of these mutations in tumorigenesis, if any, is not well understood and is under active investigation.

The molecular details of how loss of H3K27 methylation occurs in H3K27M cells are still being unraveled (Figure 2B). The increased affinity of H3K27M for EZH2 *in vitro* (Lewis et al., 2013) and the crystal structure showing tight H3K27M/EZH2 binding (Justin et al., 2016) support a model in which the mutant histone sequesters EZH2 (and PRC2) on chromatin (Lewis et al., 2013). In contrast, studies mapping occupancy of H3K27M and EZH2 on chromatin show localization at predominantly distinct sites, arguing against sequestration (Fang et al., 2018a; Mohammad et al., 2017; Piunti et al., 2017; Stafford et al., 2018). There is evidence of a persistent effect on PRC2 function following disengagement from H3K27M: PRC2 complex purified from H3K27M expressing cells exhibits reduced methyltransferase activity *in vitro* (Stafford et al., 2018). How PRC2 may be "poisoned" following its engagement with H3K27M remains to be seen, although one possibility would be through a conformational allosteric change that diminishes enzyme activity. EZH2 was recently shown to automethylate at specific lysine residues, and this activity is also inhibited by H3K27M (Lee et al., 2019; Wang et al., 2019). It will be important to understand the structural/conformational effect of this automethylation on EZH2 and the entire PRC2 complex.

It is important to note that the inhibition of PRC2 is not total, a minority of loci in the genome retaining H3K27me3 in H3K27M cells (Chan et al., 2013; Mohammad et al., 2017). PRC2-mediated methylation is nucleated on chromatin at particular sites, typically large CpG islands (regions of the genome that contain a large number of CG dinucleotide repeats), then spreads outward establishing broad domains of H3K27me3 (Oksuz et al., 2018). In H3K27M cells, PRC2 is still recruited to its nucleation sites (which are typically distinct from H3K27M incorporation sites in the genome), but broad H3K27me3 domains do not form, suggesting the spreading mechanism is defective (Harutyunyan et al., 2019; Stafford et al., 2018). The generalized loss of H3K27me3 with selective retention at PRC2 nucleation sites is a distinct epigenomic landscape not observed in cancers with loss-of-function mutations in PRC2 components (Figure 2C) (Wojcik et al., 2019). This may explain why other PRC2 mutations are not found in midline gliomas—the retained H3K27me3 sites may silence tumor-suppressor genes, such as *CDKN2A*, which are essential for cell survival (Mohammad et al., 2017). This also explains why inhibiting this residual PRC2 function may be a promising strategy in H3K27M midline gliomas through derepression of tumor-suppressor genes (Mohammad et al., 2017; Piunti et al., 2017).

How might loss of H3K27me3 drive tumorigenesis in neural cells? Given the role of H3K27me3 and the Polycomb system in cell fate regulation, the effect of H3K27M on differentiation has been explored (Funato et al., 2014; Silveira et al., 2019). To faithfully reflect molecular alterations that co-occur in midline gliomas, human neural stem cells were engineered to express H3K27M, a *TP53* short hairpin RNA, and constitutively active platelet-derived growth factor receptor A (PDGFRA) (Funato et al., 2014). This led to impairment of differentiation and acquisition of a more primitive gene expression program, and was sufficient to drive gliomagenesis when the transformed cells were

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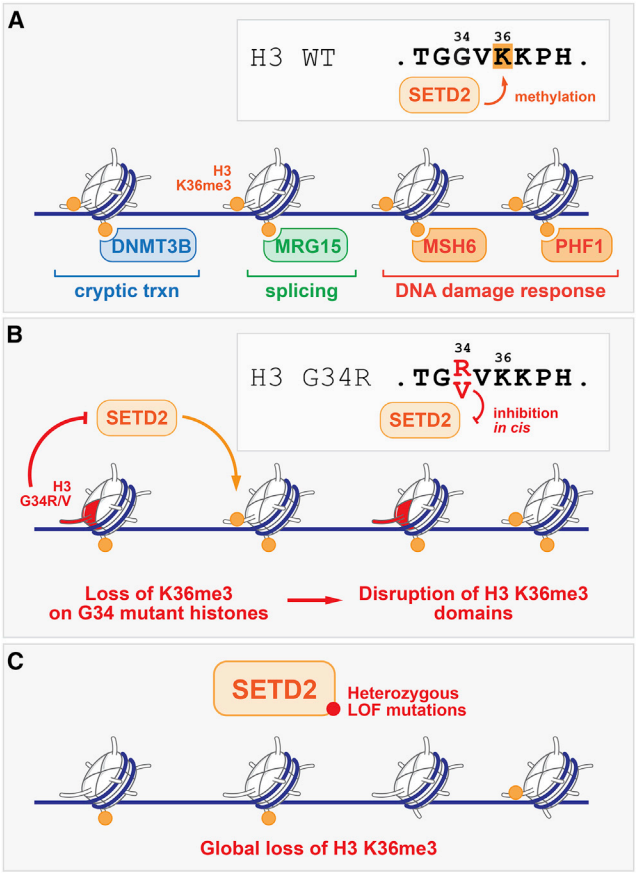

Figure 3. Reprogramming of the H3K36me3 Landscape by H3G34 and SETD2 Mutations

(A) H3K36me3 is coupled with transcription elongation and resides at gene bodies to recruit chromatin effectors to suppress cryptic transcripts (DNMT3B), and regulate splicing (MRG15) and DNA repair (MSH6, PHF1) (see text for details).

(B) H3G34R/V sterically restricts access of SETD2 to H3K36, leading to loss of H3K36me3 in *cis*, i.e., in nucleosomes where the mutant histone is incorporated, while SETD2 remains functional in *trans* on nucleosomes containing wild-type H3.

(C) Loss-of-function mutations of SETD2 lead to global reduction in H3K36me3.

injected *in vivo* (Funato et al., 2014). Although there is broad reduction of H3K27me3, a restricted number of genes are inappropriately activated following loss of H3K27me3, with an enrichment of "bivalent" genes (bearing both H3K27me3 and H3K4me3), which ordinarily are activated only in a highly orchestrated fashion during differentiation (Funato et al., 2014; Larson et al., 2019). Depletion of H3K27M in midline glioma cells derived from patients also promotes expression of neural differentiation genes (Harutyunyan et al., 2019; Silveira et al., 2019). Thus, disruption of normal neural differentiation is the prevailing hypothesis of how H3K27M contributes to oncogenesis. It is notable that many of the differentiation phenotypes described above required additional molecular perturbations alongside H3K27M, and it remains poorly understood whether and how H3K27M alone may confer an oncogenic advantage.

Interestingly, a striking loss of H3K27me3 is also a characteristic feature of posterior fossa ependymomas (PFAs) (Pajtler et al., 2015), a rare glioma variant that develops in the posterior part of the brain and histologically resembles ependymocytes, the cells lining the fluid-filled brain ventricles (Wu et al., 2016). However, only 4% of PFAs express H3K27M, and recent studies have implicated mutation or overexpression of a different gene, *CXorf67*, as a driver of this altered H3K27 methylation landscape in PFAs (Pajtler et al., 2018). Notably, the protein sequence of CXorf67 resembles the region surrounding lysine-to-methionine (K-to-M) sequence within the H3 tail in H3K27M mutants (Figure 2B) and, remarkably, expression of protein derived from this gene, or a synthetic peptide incorporating the K-to-M sequence, inhibits EZH2 via a similar mechanism (Hübner et al., 2019; Jain et al., 2019; Ragazzini et al., 2019; Piunti et al., 2019). Hence, this gene has now been renamed EZH2 inhibitory protein (*EZHIP*) as an example of "oncohistone mimicry." These data lend further support to the idea that hindbrain-derived cells may have enhanced sensitivity to transformation by proteins that antagonize EZH2 function during development.

H3K27me3 domains can block the placement of other PTMs, and the loss of H3K27me3 in H3K27M tumors is also accompanied by downstream epigenomic changes. For example, several studies have shown that expression of H3K27M leads to an increase in H3K27 acetylation on wild-type H3 (Lewis et al., 2013; Piunti et al., 2017). This PTM is frequently present at active regulatory elements (enhancers and promoters), and it has been proposed that H3K27M may induce enhancer reorganization (Piunti et al., 2017) although analysis in isogenic models suggests that the enhancer landscape is determined largely by the cell of origin rather than the presence of the H3K27M mutation (Krug et al., 2019). Increased H3K27ac was also observed in intergenic regions, leading to higher basal endogenous retroviral element (ERV) expression (Krug et al., 2019). These acetylated ERVs may prime H3K27M gliomas for epigenetic therapies aiming to augment antitumor immunity by increasing ERV expression to activate the innate interferon response (Krug et al., 2019). H3K36me2 has also been shown to be increased in the presence of H3K27M (Stafford et al., 2018), forming new domains that invade regions where H3K27me3 is lost. Further study is required to elucidate whether these epigenomic changes are causal or consequential to oncogenesis induced by H3K27M.

MISREGULATION OF H3K36 METHYLATION: H3G34R/V AND SETD2 MUTATIONS

Misregulation of histone lysine 36 methylation (H3K36) has been implicated as an important event in gliomagenesis by two key observations. Firstly, there is a subtype of malignant gliomas in children and young adults characterized by recurrent mutations in H3 at a residue in close proximity to H3K36, leading to substitution of glycine to either arginine or valine (H3G34R/V) (Schwartzentruber et al., 2012; Wu et al., 2012) (Figure 1). Biochemically, substituting G34 with a bulky side chain such as arginine or valine causes steric hindrance and restricts access to H3K36 by the "writer" enzyme SETD2 (SET domain containing 2, histone lysine methyltransferase), leading to loss of H3K36 methylation (Yang et al., 2016) (Figure 3B). Secondly, loss-of-function mutations in SETD2 have been identified at high frequency in malignant gliomas (Brennan et al., 2013; Fontebasso et al.,

2013). Although they are not subtype defining, they represent the second most frequent mutation in a chromatin modifier in adults (Brennan et al., 2013; Zehir et al., 2017). H3G34 mutations differ from SETD2 mutations in that the loss of H3K36me3 occurs in *cis* (i.e., only at nucleosomes where this mutant histone is present) as opposed to more globally in SETD2 mutant cells (Figure 3C).

Insights into how reduced H3K36me3 levels promote glioma may be deduced from understanding its function in normal cells (Figure 3A). Physiologically, H3K36me3 occurs on chromatin at the gene bodies of actively expressed loci, and its establishment is coupled with transcription elongation (Krogan et al., 2003; Li et al., 2002). Functional studies suggest that H3K36me3 plays an important role in regulating the "quality" of transcription by repressing generation of aberrant or unwanted transcripts (McDaniel and Strahl, 2017). For example, loss of H3K36 methylation through mutation of SETD2 (or its homologs) (Kaplan et al., 2003; Neil et al., 2009; Xu et al., 2009) leads to the emergence of cryptic transcripts—RNA species whose production is initiated within the gene body or at other sites outside the canonical promoter region at the 5′ end of a gene. H3K36me3 may prevent cryptic transcript production by promoting DNA methylation in the gene body through direct recruitment of the methyltransferase, DNMT3B (Neri et al., 2017). If cryptic transcripts occur in the antisense direction, they may suppress production of the physiological transcript (and thus the encoded protein) derived from the 5′ promoter in the "sense" direction (Huber et al., 2016; Kim et al., 2016). It has been shown that some cryptic transcripts are translated (Cheung et al., 2008); whether cryptic proteins provide a selective advantage (or neomorphic function) for cancer cells remains to be determined.

H3K36me3 also plays an important role in recruiting RNA splicing factors, thereby regulating exon choice (Luco et al., 2010), and SETD2 mutant cells show genome-wide disruption of splicing as evidenced by the presence of alternative and intron-retaining transcripts (Simon et al., 2014). Spliceosome disruption is an established driver of cancer (Dvinge et al., 2016), broadly by either generating oncogenic splice variants or by abrogating expression of tumor-suppressive isoforms (Simon et al., 2014). Thus, splicing defects secondary to loss of H3K36me3 have plausible potential to promote gliomagenesis, although the specific mis-splicing events and/or protein isoforms which are oncogenic in this context remain to be characterized.

In addition to its described role in regulating transcription, H3K36me3 plays an important role in DNA damage repair, which has a well-established role in tumor suppression (Jeggo et al., 2016). For example, deletion of SETD2 is sufficient to disrupt mismatch repair (MMR) and increase mutation frequency, as MSH6 (MutS homolog 6), a component of the MutSα mismatch recognition complex, interacts with the H3K36me3 mark through its PWWP "reader" domain (Li et al., 2013). This phenotype has also been demonstrated in cells bearing H3G34R mutations (Fang et al., 2018b). Consistent with this observation, unbiased analyses of >1,000 cancer genomes demonstrated that mutation frequency is reduced in H3K36me3-enriched regions of the genome (Clendening et al., 2010). H3K36me3 also plays a role in the early stages of the DNA damage response by recruiting the protein PHF1 to sites of DNA double-strand breaks through its H3K36me3 interacting Tudor domain (Musselman et al., 2012), where it may repair damage through non-homologous end-joining (Musselman et al., 2012).

While the loss of H3K36me3 and resultant failure to recruit H3K36me3 "reader" proteins may be important for oncogenesis, other epigenomic changes that are gained may be worthy of further attention. For example, reduction in H3K36me3 can facilitate H3K27me3 placement, as EZH2 "senses" methylation status at H3K36 and is active when the residue is unmethylated (Jani et al., 2019). It will thus be interesting to define the function of any increases in H3K27me3 in H3G34R/V-induced phenotypes. Additionally, a recent study profiling the chromatin state in embryonic stem cells genome edited to express H3G34R demonstrated relative increases in both H3K36me3 and H3K9me3 in *trans* (Voon et al., 2018). This was attributed to an ability of H3G34R to inhibit the function of lysine demethylase 4B (KDM4B), a dual H3K9/H3K36 demethylase, in a dominant fashion (Voon et al., 2018). These findings are intriguing because this demethylase is a target of the neometabolite, 2-hydroxyglutarate (2-HG) produced by mutant isocitrate dehydrogenase (IDH) (see below). Interestingly, H3G34 and IDH mutant gliomas have several shared features, such as their age distribution, location in the brain, and the co-occurrence of ATRX loss and p53 mutations (Figure 1) (Sturm et al., 2012). One hypothesis is that overlapping effects of IDH and H3G34 mutants on the chromatin landscape promote gliomagenesis and influence the co-mutations that are acquired in the process.

It remains unexplained why H3G34 mutations only occur in the gene encoding the H3 variant, H3.3 (*H3F3A* gene), in contrast to the H3K27M mutation, which can occur in genes encoding either variant H3.3 or canonical H3.1/3.2 (*HIST1H3B* and *HIST2H3C* genes) (Figure 1). H3.3 only differs from canonical H3 by a few amino acids, yet this results in its recognition and deposition into chromatin by distinct chaperones (see below), with a characteristic distribution (Elsässer et al., 2012; Goldberg et al., 2010; Lewis et al., 2010) and in a replication-independent fashion (Ahmad and Henikoff, 2002). How these and other functional differences (Armache et al., 2020; Martire et al., 2019) from canonical H3 interact with mutation at H3G34 are important areas for future investigation.

DISRUPTION OF CHROMATIN THROUGH ONCOMETABOLITES: IDH MUTATIONS

Mutations in IDH have been identified as the earliest somatic event (Johnson et al., 2014; Parsons et al., 2008; Suzuki et al., 2015; Watanabe et al., 2009) in a subtype of gliomas that (1) most frequently present with lower histological grade (often grade II) (The Cancer Genome Atlas Research Network, 2015), (2) arise in the frontal lobes of the brain (Lai et al., 2011), and (3) have a peak age on presentation of between 20 and 35 years (Watanabe et al., 2009) (Figure 1). IDHs are important metabolic enzymes that convert the tricarboxylic acid metabolite isocitrate into α-ketoglutarate (α-KG) (Pavlova and Thompson, 2016). This can occur in the cytosol (IDH1) and mitochondria (IDH2), generating NADPH for metabolic biosynthesis and redox homeostasis (Pavlova and Thompson, 2016). IDH1 is most frequently mutated in gliomas, leading to a heterozygous arginine 132 to histidine mutation (R132H) (Parsons et al., 2008; Verhaak et al., 2010). Less commonly, arginine 172 in IDH2 is heterozygously mutated

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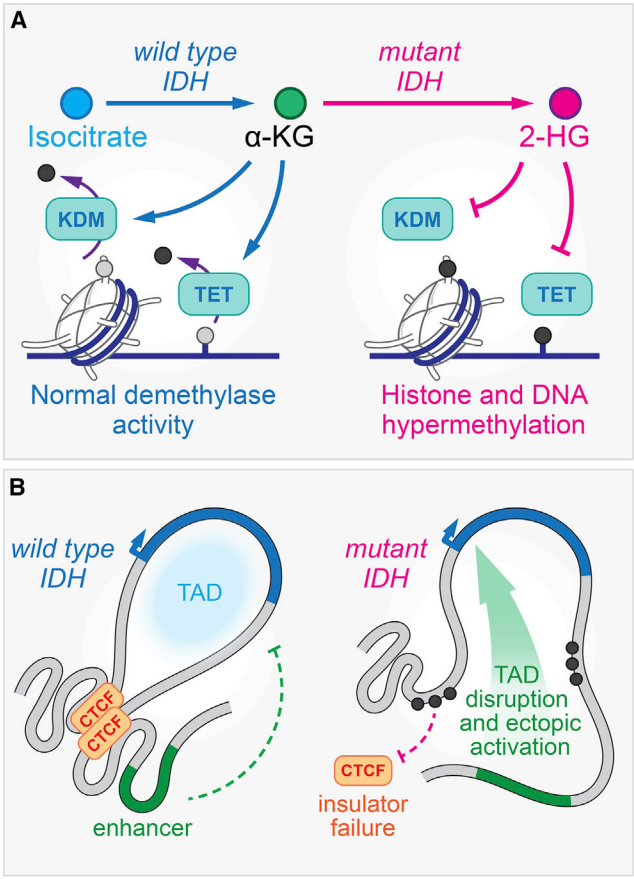

Figure 4. Pleiotropic Effects of IDH Mutation on the Epigenome and Other α-Ketoglutarate-Dependent Pathways

(A) Gain-of-function mutations in IDH lead the enzyme to favor α-ketoglutarate as a substrate, leading to production of the oncometabolite 2-HG. 2-HG inhibits many α-ketoglutarate-dependent enzymes including histone (KDM) and DNA demethylases (TET), leading to aberrant accumulation of methylation on chromatin. Some of these changes such as H3K9 methylation prevent appropriate gene activation required for normal differentiation.

(B) Mutant IDH-induced DNA methylation at CTCF binding sites disrupts chromatin architecture, allowing oncogenes such as *PDGFRA* to hijack enhancers in different TADs.

to histidine in gliomas. These gain-of-function mutations confer neomorphic function upon IDH, which then favors α-KG as a substrate, converting it into 2-HG (Dang et al., 2009; Ward et al., 2010) (Figure 4A). 2-HG may act as a competitive inhibitor of over 60 different enzymes that use α-KG as a cofactor (Rose et al., 2011). These α-KG-dependent enzymes span a variety of functions including metabolism, chromatin regulation, and DNA repair (Rose et al., 2011); thus, mutant IDH has broad potential to directly perturb normal cell physiology.

Which are the key enzymes inhibited by 2-HG to drive tumorigenesis? Studies indicate α-KG-dependent demethylases (of histones and DNA) may be critical targets of 2-HG in driving tumor development by reshaping the epigenomic landscape (Lu et al., 2012; Schvartzman et al., 2019; Turcan et al., 2012) (Figure 4A). For example, 2-HG inhibits demethylation at several histone sites—H3K9, H3K27, H3K36, H3K79—leading to the accumulation of methylation on chromatin (Lu et al., 2012). The composite effects of these changes are poorly understood, although H3K9 methylation is one of the earliest changes induced by mutant IDH and may be mechanistically important (Lu et al., 2012). In normal cells, H3K9 methylation is enriched at heterochromatin where it recruits HP1 "reader" proteins (Bannister et al., 2001; James and Elgin, 1986; Lachner et al., 2001; Nakayama et al., 2001), which compact chromatin and act as scaffolds for other chromatin modifiers (H3K9 methylases and histone deacetylases) to establish a transcription-refractory state. During normal differentiation, H3K9 methylation is dynamically reorganized (Hawkins et al., 2010; Nicetto et al., 2019) such that lineage-specific genes are activated where loss of H3K9 methylation occurs, while stem cell genes and genes pertaining to other lineages gain H3K9 methylation (Hawkins et al., 2010; Nicetto et al., 2019). In the context of mutant IDH, this process becomes disorganized and there is a block in cellular differentiation as lineage-specific transcription factor access is restricted by aberrant retention of H3K9 methylation (Lu et al., 2012; Modrek et al., 2017; Schvartzman et al., 2019). Interestingly, 2-HG-driven increase in H3K9 methylation was recently demonstrated to impair recruitment of DNA damage repair factors to sites of double-stranded DNA breaks, resulting in potential vulnerability to therapies targeting DNA damage pathways (Sulkowski et al., 2017, 2020)

Through inhibition of the TET (ten-eleven translocation) family of DNA demethylases, 2-HG is sufficient to induce accumulation of DNA methylation at CpG islands, leading to a characteristic epigenomic phenotype called G-CIMP (CpG island methylator phenotype) (Turcan et al., 2012). DNA methylation at promoter CpG islands has long been associated with gene repression (Comb and Goodman, 1990; Prendergast and Ziff, 1991); the mechanism underlying this is incompletely understood but is in part characterized by interfering with transcription factor binding (Stadler et al., 2011). By increasing promoter DNA methylation, mutant IDH can broadly silence multiple putative tumor-suppressor genes. Notably, in acute myeloid leukemia, IDH and TET mutations occur in a mutually exclusive manner (Figueroa et al., 2010)—one interpretation of this is that these two distinct mutations are alternative molecular routes to produce a similar downstream effect, i.e., dysregulation of DNA methylation. TET mutations are observed in glioma, although they are extremely rare and not mutually exclusive with IDH1 mutations (Zehir et al., 2017); hence there are likely distinct, lineage-specific differences in the impact of these mutations on DNA methylation and oncogenesis.

Recent work has highlighted the presence of CpG island DNA methylation at non-promoter sites in IDH mutant cells (Bledea et al., 2019; Flavahan et al., 2016; Modrek et al., 2017), which may have distinct effects on chromatin structure and gene expression (Figure 4B). The transcription factor CTCF (CCCTC binding factor) binds to "insulator sites," regulating the structure of the genome into domains called TADs (topologically associated domains), which insulate genes in one domain from activation by enhancers in different domains (Dixon et al., 2012; Nakahashi et al., 2013). DNA methylation can in certain circumstances prevent CTCF binding, and indeed loss of these insulation sites in an IDH mutant glioma model increased expression of the *PDGFRA* oncogene, potentially by abrogating the TAD boundary and permitting the *PDGFRA* gene access to an active enhancer in a distinct domain (Flavahan et al., 2016). Another study

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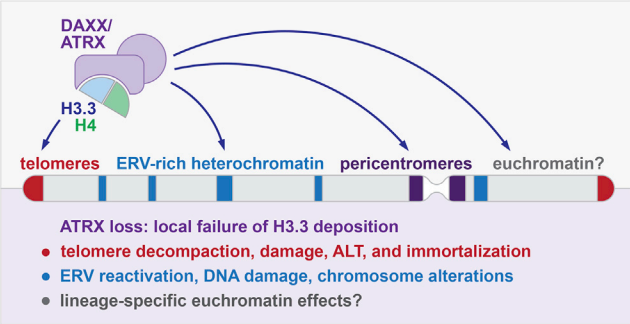

Figure 5. Consequences of ATRX Loss in Different Chromatin Regions

ATRX acts in concert with DAXX as a chaperone to deposit the histone variant H3.3 into heterochromatic regions bearing H3K9me3 at telomeres and pericentromeres, endogenous retroviral elements, and some euchromatic sites. Loss of ATRX and H3.3 deposition at telomeres leads to ALT and supports cell immortalization. Consequences of ATRX loss in euchromatin are poorly defined but may affect gene expression.

highlighted extensive methylation of putative enhancer CpG islands in IDH mutant tumors (Bledea et al., 2019), the transcriptional impact of which will require further characterization (Bledea et al., 2019). In summary, the data showing that mutant IDH disrupts DNA methylation genome-wide is robust, although further work is needed to decipher the transcriptional impact of these changes at different genomic locations (promoter versus gene body versus enhancer), and determine which changes are oncogenic or simply incidental.

CHROMATIN REMODELER DYSFUNCTION: ATRX MUTATION

Loss-of-function mutations in ATRX (Alpha-thalassemia/mental retardation syndrome, X-linked) frequently co-occur with IDH mutations and define a specific IDH mutant subgroup, which has an astrocytic appearance histopathologically (IDH mutant astrocytoma, Figure 1) (Jiao et al., 2012; Liu et al., 2012). In addition, similar ATRX mutations occur in pediatric gliomas where they are uniformly present in the H3G34-mutant gliomas (Figure 1), frequently present in the rare entity called anaplastic astrocytoma with piloid features (Reinhardt et al., 2018) and occasionally in H3K27M mutant gliomas (Khuong-Quang et al., 2012; Schwartzentruber et al., 2012; Wu et al., 2012). Genetic mouse modeling has shown that ATRX loss accelerates glioma formation (Koschmann et al., 2016), supporting a role for this event as a driver; however, ATRX has a complex function physiologically, and how its loss promotes tumorigenesis is poorly understood.

ATRX protein contains two highly conserved domains, which are sites of increased mutation frequency in glioma: the ADD (ATRX-DNMT3-DNMT3L) domain and the ATP-dependent helicase domain. The ADD region of ATRX is a dual "reader" domain that recognizes the combination of H3K9me3 and unmethylated H3K4 (Eustermann et al., 2011; Iwase et al., 2011) found in certain areas of constitutive heterochromatin to which ATRX is recruited. H3K4 methylation is largely correlated with active chromatin (promoter and enhancer elements) and may block ATRX recruitment through the ADD domain. This illustrates an example of an elaborate "code," whereby PTM status at different positions on the H3 amino tail are "sensed" to regulate effector/chromatin interactions (Strahl and Allis, 2000). What function does ATRX serve in heterochromatic regions (Figure 5)? Several studies show that ATRX forms a chaperone complex with another protein called DAXX (Death-domain associated protein) to deposit the histone variant H3.3 to telomeres and pericentromeric chromatin (Drané et al., 2010; Goldberg et al., 2010; Lewis et al., 2010). How deposition of H3.3 regulates heterochromatin function is still being elucidated: studies suggest it is essential for maintaining genomic integrity and normal replication in heterochromatin, and its loss can induce chromosome instability (Clynes et al., 2014; De La Fuente et al., 2015; Huh et al., 2012).

Loss of ATRX function at telomeric heterochromatin promotes a phenomenon called alternative lengthening of telomeres (ALT) (Figure 5). This is a recombination-mediated replication mechanism whereby telomeres become lengthened via use of telomeric DNA as a copy template for replication (Cesare and Reddel, 2010). Induction of ALT has been proposed as the major evolutionary advantage conferred to cancer cells by ATRX loss (Clynes et al., 2015; Heaphy et al., 2011), as it enables maintenance of telomere length, which is important for cellular immortality by preventing senescence associated with telomere shortening (Cesare and Reddel, 2010). Repetitive DNA at telomeres contains a high content of guanine, which is prone to form secondary DNA structures called G4 quadruplexes, which can impede both transcription and DNA replication (Varshney et al., 2020). ATRX co-localizes with regions predicted to adopt these structures and is thought to either suppress or reverse their formation through deposition of H3.3—in the absence of ATRX, homologous recombination of telomeres is promoted. One suggestion is that repeat DNA at heterochromatin can destabilize nucleosomes, and H3.3 deposition can help maintain nucleosome density optimal for heterochromatin formation (Lewis et al., 2010).

Although there is a compelling correlation between loss of ATRX function and ALT, it is notable that a proportion of cells in which ATRX is not expressed do not develop ALT (Clynes et al., 2014; Fishbein et al., 2015; Brosnan-Cashman et al., 2018; Danussi et al., 2018). This suggests that a specific cellular context and/or additional changes are needed to induce ALT. Moreover, it is likely that ATRX loss confers selective advantages to cancer cells through ALT-independent mechanisms. A recent study showed that deletion of ATRX in neural stem cells induced transcriptional changes associated with astrocytic differentiation and increased migratory capacity (Danussi et al., 2018), both characteristic features of the IDH mutant astrocytomas in which ATRX loss invariably co-occurs (Danussi et al., 2018). Notably, ATRX was preferentially enriched at gene promoters and enhancers and at gene bodies in neural stem cells, raising the possibility of a direct role in active chromatin regulation (Danussi et al., 2018). Further work will be necessary to explore this hypothesis and the molecular function of ATRX, DAXX, and H3.3 in these regions.

OVER-REPRESENTATION OF CHROMATIN MUTATIONS IN PEDIATRIC GLIOMA

It is noteworthy that chromatin regulators are most frequently mutated in the glioma subtypes that occur in children and young adults (Figure 1). This reflects a pattern observed across cancer

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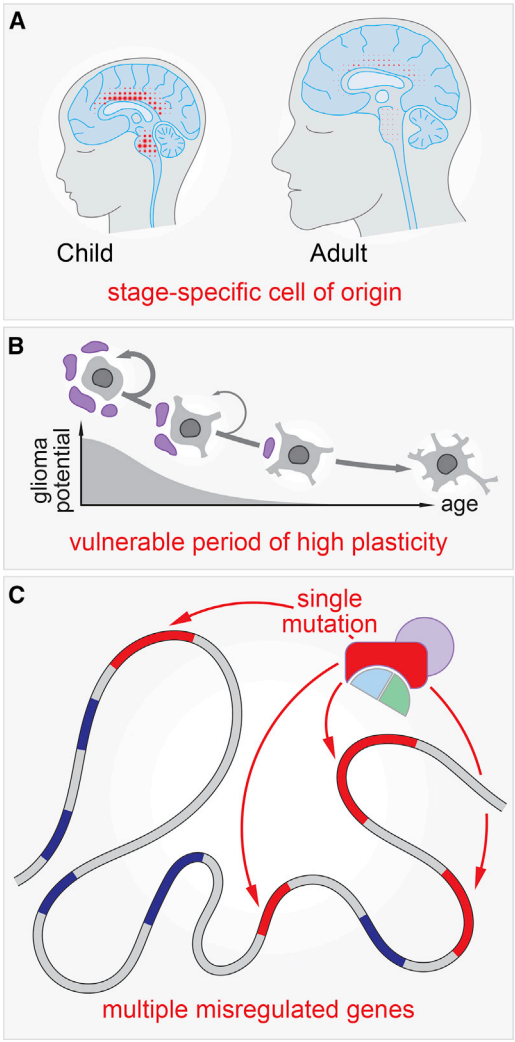

Figure 6. Putative Hallmarks of Chromatin Factor Mutations that Drive Gliomagenesis in Young Patients

(A) Context-dependent effects of chromatin may be oncogenic in a developmentally restricted cell of origin.

(B) Developmentally active period may be more sensitive to perturbation in chromatin factors given their function in establishing and maintaining cell identity.

(C) "Single hit" may disrupt multiple cancer hallmarks, as chromatin factors reside at hundreds of different genes regulating distinct pathways.

types (Schwartzentruber et al., 2012; Sturm et al., 2012; Verhaak et al., 2010; Wu et al., 2012). The biological basis of this is unclear, although a number of non-mutually exclusive hypotheses can emerge (Figure 6A). Chromatin regulators have highly cell-type-specific effects, likely due to (1) cell-type-specific patterns of genomic occupancy for chromatin factors and (2) cell-type-specific activity of genes regulated by chromatin. Hence, the high frequency of chromatin regulator mutations in younger individuals may reflect a propensity to transform a particular cell of origin, which may be only accessible or abundant during an age-restricted window or in a microenvironment unique during a specific developmental stage. Indeed these mechanisms may contribute to the distinct age distributions of H3 and IDH mutations, even among young individuals (Figure 1).

Another related factor may be the initial mechanism of transformation mediated by mutated chromatin factors, which may be distinct from mutations in non-chromatin genes. Mutations in chromatin regulators often co-occur alongside other classes of mutation in more traditional oncogenic pathways such as growth factor signaling, genomic stability, and cell cycle control (Schwartzentruber et al., 2012; Sturm et al., 2012; Verhaak et al., 2010; Wu et al., 2012). One interpretation of these data is that mutations of chromatin regulators occupy a distinct and specific "niche" in oncogenesis—but what might this niche be? Perturbation of normal differentiation appears to be a common feature and a potential candidate as a more general phenotype induced by chromatin mutations in glioma and other cancers (Funato et al., 2014; Lu et al., 2012). Evidence from single-cell transcriptomics has nominated specific glial precursors in the developing brain as the cells from which different pediatric gliomas arise (Filbin et al., 2018; Jessa et al., 2019). We propose that dysregulation of the chromatin landscape may be particularly impactful during early development, when a larger number of cells are undergoing both self-renewal and fate transitions, which require highly coordinated chromatin remodeling. Moreover, if it is correct to consider epigenetic regulation broadly as a "lock-down" mechanism after traditional transcription factor-mediated gene regulatory programs are initiated, this window of "plasticity" may be more prone to error earlier in development when epigenetic machinery is being established *de novo* (Figure 6B).

Finally, cancers such as glioma in younger individuals in general bear a lower total number of mutations than gliomas in the elderly (Gröbner et al., 2018). We postulate that some chromatin regulators, through their ability to regulate hundreds of genomic loci, have the potential to simultaneously dysregulate multiple cancer hallmarks with a single hit, meaning that fewer additional mutations are required to enter a fully transformed state (Figure 6C).

PROSPECTS FOR THERAPEUTIC TRANSLATION

Not a single targeted therapy has shown efficacy in malignant glioma in a randomized control trial, highlighting the difficulty in identifying tractable therapeutic targets and developing brain-penetrant small molecules to engage those targets in the CNS (Fine, 2015). The recognition that aberrant chromatin is central to glioma development has generated much interest in mechanism-based therapies targeting chromatin regulators. The most direct strategy is to directly inhibit gain-of-function proteins, e.g., mutant IDH and H3. Mutant IDH is particularly amenable to small-molecule inhibition given its defined enzymatic domain, which can be engaged with a small molecule. However, while mutant IDH may be an early driving event, more established tumors may not rely on the effects of the mutation to drive tumor growth once additional molecular events have been acquired. This is evidenced by variable responses to IDH inhibition in preclinical glioma models (Johannessen et al., 2016; Rohle et al., 2013; Tateishi et al., 2015) and other studies which suggest that deletion of mutant IDH may be selected for as tumors evolve in patients or *in vitro* cell culture (Luchman et al., 2013; Mazor et al., 2017). In the context of leukemia, however, IDH inhibition has shown success in human clinical trials (DiNardo et al.,

2018), and the ultimate test of IDH as a therapeutic target in glioma will be determined through similar human clinical studies that are now under way.

For other gain-of-function mutants such H3K27M and H3G34R, which are not enzymes, more creative therapeutic approaches may be necessary such as inhibition of protein-protein interactions or targeted degradation (Bondeson and Crews, 2017). For H3K27M, such approaches are supported by data suggesting that the mutant histone is required by tumors to maintain the oncogenic state (Harutyunyan et al., 2019; Silveira et al., 2019).

Targeting chromatin through non-oncogene dependencies is another promising anticancer strategy that may have utility in glioma. Histone deacetylase inhibitors were the first class of approved "epigenetic" therapy (Marks, 2010), followed by DNA methylation inhibitors (Kaminskas et al., 2005) and, most recently, EZH2 inhibitors in sarcoma (Rothbart and Baylin, 2020; Stacchiotti et al., 2019). EZH2 inhibition in this context built on basic research delineating a synthetic lethal relationship between the SWI/SNF complex (mutated in ~30% of cancers) and PRC2 (Wilson and Roberts, 2011). The loss of SWI/SNF complex on chromatin permits recruitment of PRC2 in *cis* and placement of H3K27me3 (Kadoch et al., 2017; Wilson et al., 2010). This compensatory change is essential for cell survival, and blocking PRC2 activity exhibits antitumor activity in this context (Knutson et al., 2013). This is an example of synthetic lethality in chromatin factors that oppose each other to maintain transcriptional control, and other similar therapeutic opportunities will likely arise as our understanding of chromatin crosstalk in glioma deepens, given the dynamic and highly interconnected manner in which chromatin factors regulate the epigenome. Already it has been shown that the inhibitory effect of H3K27M on EZH2 *in vitro* can be partially reversed by the presence of histone acetylation on nearby lysines (Brown et al., 2014). Building on this finding, histone deacetylase inhibitors, which increase steady-state histone acetylation, are in clinical trials for H3K27M glioma, based on encouraging activity in preclinical studies (Grasso et al., 2015).

Finally, a growing number of studies show that chromatin regulation is an important mechanism of immune evasion in cancer (Pan et al., 2018), with clinical trials combining chromatin-targeting agents with checkpoint blockade now under way (Daver et al., 2019; Gandhi et al., 2018). Such mechanisms are relatively unexplored in malignant glioma and will be important areas of future investigation.

CONCLUSION

Epigenomic reprogramming is now recognized as a key feature in the molecular etiology of malignant glioma. Important initial progress has been made to define the proximal biochemical effects of mutated chromatin regulators in glioma, although key questions remain. A fundamental challenge in studying chromatin regulators lies in their inherent ability to simultaneously affect hundreds of different genomic loci and, hence, biological pathways, even in some cases acting on non-chromatin substrates. Deciphering which events are essential for tumorigenesis and which are passenger events will require use of systems-based technologies that can induce simultaneous perturbations in a controlled fashion, and an ability to causally link these changes to oncogenic phenotypes. Such techniques are emerging and have the potential to reveal important insights when employed in the context of genetically engineered glioma models that have been developed (Funato et al., 2014; Larson et al., 2019; Mohammad et al., 2017; Pathania et al., 2017). These models will also permit detailed interrogation of the functional interplay between changes in the epigenomic landscape caused by chromatin mutations, cell of origin, and aberrations in signaling pathways, all of which co-occur in a characteristic manner in different glioma subtypes (e.g., ACVR1 with H3.1K27M, and PDGFRA/FGFR1 mutations with H3.3K27M, Figure 1). Finally, a wealth of evidence has shown that the chromatin landscape is modulated by non-genetic stimuli including aging, inflammation, and metabolic state within the local microenvironment, all of which can signal to chromatin. The role of these factors in glioma initiation, maintenance, and progression are relatively understudied and will be an important area for exploration in the future. Exciting progress has been made in unraveling fundamental mechanisms that utilize a plethora of epigenetic regulators drawing upon biochemical and cell-based studies. Extending these studies in faithful animal models of glioma with insights gleaned from human genetics will undoubtedly lead to much-needed advances in the treatment of these cancers.

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AUTHOR CONTRIBUTIONS

Conceptualization, R.E.P. and C.D.A.; Writing – Original Draft, R.E.P.; Writing – Review & Editing, R.E.P., C.D.A., and A.A.S.; Visualization, A.A.S. and R.E.P.

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