Trends in Cancer

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Review

EZHIP's role in diffuse midline glioma: echoes of oncohistones?

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The enhancer of zeste inhibitory protein (EZHIP) is typically expressed during germ cell development and has been classified as a cancer-testis antigen (CTA) in various cancers. In 2020, 4% of diffuse midline gliomas (DMGs) were shown to aberrantly express EZHIP, mirroring the DMG hallmark histone H3 K27M (H3K27M) oncohistone mutation. Similar to H3K27M, EZHIP is a negative regulator of polycomb repressive complex 2 (PRC2), leading to global epigenomic remodeling. In this opinion, we explore the similarities and disparities between H3K27M- and EZHIP-DMGs with a focus on their shared functional hallmark of PRC2 inhibition, their genetic and epigenomic landscapes, plausible differences in the cell of origin, and therapeutic avenues. Upcoming research on EZHIP will help better understand its role in gliomagenesis and DMG therapy.

Highlights

Enhancer of zeste inhibitory protein (EZHIP) is a cancer-testis antigen normally expressed in germ cells but becomes aberrantly expressed in various types of cancer, including pediatric diffuse midline glioma (DMG).

EZHIP is a histone 3 lysine 27 trimethylation (H3K27me3) oncohistone mimic and a negative regulator of the polycomb repressive complex 2 (PRC2), which plays a significant role in driving tumorigenesis in DMG.

The cell of origin is unknown for EZHIP-DMG, but EZHIP expression in germ cells may coincide with the timing window for the abnormal expression seen during neural development.

There are two main themes for targeting EZHIP-DMG, specifically exploring EZHIP-specific targets and harnessing the K27M mimetic feature of EZHIP to repurpose therapies used in H3K27M-DMG.

The evolving role of EZHIP in cancer

EZHIP (*CXorf67 or CATACOMB*) is a monoexonic gene located on the X chromosome (Xp11.22) and is only expressed in the placenta, testis, and ovarian follicles (https://www.proteinatlas.org/ENSG00000187690-EZHIP). Functionally, EZHIP regulates germ cell differentiation through suppression of **PRC2** (see Glossary) and consequently through the reduction of global levels of the repressive H3K27me3 mark [1].

EZHIP is categorized as a **CTA**, given its role in germ cell development [2]. CTAs become aberrantly expressed in normal somatic cells to promote tumorigenesis [3]. In fact, since 2014, accumulating evidence has linked *EZHIP* expression to various types of cancer. First, translocations involving the *EZHIP* gene that resulted in pathogenic fusion proteins were identified in two cases of endometrial stromal sarcoma expressing the fusion protein MBTD1-CXorf67, which contained the 3′ end of EZHIP and was able to decrease the catalytic activity of PRC2 [4,5]. More recently, an *EWSR1-EZHIP* fusion was found in one hemisphere astroblastoma case [6]. Second, missense mutations concentrated in a hotspot region close to the N terminal in the *EZHIP* gene were found in 9.4% of posterior fossa ependymoma type A (PFA), 5.8% of endometrial carcinomas, 2.7% of melanomas, 1.4% of adenocarcinomas, and less than 1% of adult low-grade gliomas, glioblastomas, and carcinomas [7]. *In silico* analyses suggested that this region may correspond to a protein–protein interaction domain; however, *in vitro* experiments revealed that these mutations can still bind to PRC2 to reduce H3K27me3 levels similar to wild-type (WT) EZHIP [7]. A novel variant of uncertain significance at the serine-rich region of the C terminal of EZHIP (S443F) was the only reported alteration in a non-subclassified pediatric high-grade glioma case with hemispheric location [8]. However, its functional impact was not studied. Therefore, it remains unknown if these missense mutations or variants have any differential role to their WT counterpart. Finally, and most commonly, aberrant expression of *EZHIP* has been reported in 11.4% of non-small cell lung cancer [2], 9.4% of Merkel cell carcinoma [9], 95% of PFA [10,11], and 4% of DMG [12–14]. Mechanistically, aberrant *EZHIP* expression has been explained by DNA promoter hypomethylation in PFAs [7] and DMGs [12].

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Despite the different forms of expression of *EZHIP* in cancer as WT, carrying missense mutations, or as translocations, the downstream molecular consequence converges on the inhibition of the PRC2 complex with subsequent global reduction of its catalytic deposition of histone methylation marks (H3K27me2/3). This seems to be one of the major drivers of EZHIP oncogenesis [7,11,13].

The discovery of EZHIP's abnormal expression in DMGs expanded the spectrum of DMGs with global epigenetic loss of H3K27me3 beyond the well-known **H3K27M** mutation and triggered the reclassification of subtypes from H3K27M-DMGs to H3K27-altered DMGs, which contain both H3K27M and EZHIP-overexpressing subtypes (Figure 1) [15]. In this opinion, we will discuss the recent research of EZHIP as a H3K27M onco-histone mimic in DMG. We will then compare EZHIP- and H3K27M-DMGs by their molecular landscapes and provide our perspective for the cell of origin for EZHIP-DMGs. We will finally propose and discuss two targeting themes by repurposing H3K27M-DMG targets and/or by using EZHIP-specific targets for the currently incurable EZHIP-DMG, and for EZHIP-positive cancers beyond DMG.

EZHIP: an epigenetic regulator via its H3K27M–oncohistone mimic ability

Suppression of PRC2 activity is also associated with the disease-causing H3K27M mutation in DMG [16]. Interestingly, *EZHIP* expression and the presence of H3K27M are mutually exclusive events in both DMGs and PFAs, suggesting comparably detrimental downstream effects [7,13,17]. Furthermore, several studies in PFA have converged to demonstrate that EZHIP is a structural and functional 'mimic' of the H3K27M mutation [5,11,18].

Structurally, EZHIP is a highly disorganized and poorly conserved 503 amino acid (aa) protein, except for a developmentally conserved serine-rich area between 394 and 418 aa, toward the C terminal. In 2019, several seminal publications performed *in vitro* experiments using truncated peptides for EZHIP to identify the regions responsible for enhancer of zeste homolog 2 (EZH2) interaction and for the inhibition of histone methyltransferase activity that drives the reduction of H3K27me3 levels [1,5,11,18]. These studies corroborate the aforementioned highly conserved serine region, with some length variations, which is responsible for both EZH2 interaction and the inhibition of EZH2 activity. These studies also predicted that within this domain, there is a 9- to 15 aa region with a high sequence homology for the hotspot area of H3K27M, which is 23–31 aa long and is called **K27M-like peptide** (**KLP**). Experiments replacing single amino acids at the EZHIP-KLP region demonstrated that methionine 406 (M406), which is the equivalent of M27 in H3, is essential for EZH2 methyltransferase inhibition [5,11,18] and that arginine 405 (R405) is required for the interaction with EZH2 [11].

H3K27M inhibits PRC2 enzymatic activity via two mechanisms: (i) by sequestering PRC2 prior chromatin interaction and/or in the chromatin to abolish H3K27me3 chromatin seeding and/or spreading, respectively [19], and (ii) by H3K27M active chromatin exclusion of PRC2 methyltransferase activity [19–21]. Accumulating evidence suggests that EZHIP shares the PRC2 sequestration model with H3K27M in the chromatin. Coimmunoprecipitation and mass spectrometry assays of exogenous or endogenous EZHIP revealed interactions with components of PRC2 complexes, including EZH2, suppressor of zeste 12 (SUZ12), embryonic ectoderm development (EED), AEBP2, JARID2, PAL1, MTF2, and PHF19 [7,11,18], and chromatin immunoprecipitation followed by sequencing (ChIP-seq) experiments showed co-occurrence between EZHIP and EZH2, suggesting that EZHIP binds to the chromatin via the PRC2 complex [5,22]. The sequestering model was further demonstrated biochemically, showing an allosteric mechanism where EZHIP binds to PRC2 more efficiently when PRC2 binds to the chromatin at nucleosomes containing H3K27me3. This PRC2-EZHIP chromatin complex inhibits the downstream spreading of H3K27me3 and promotes stalling of PRC2 function [22].

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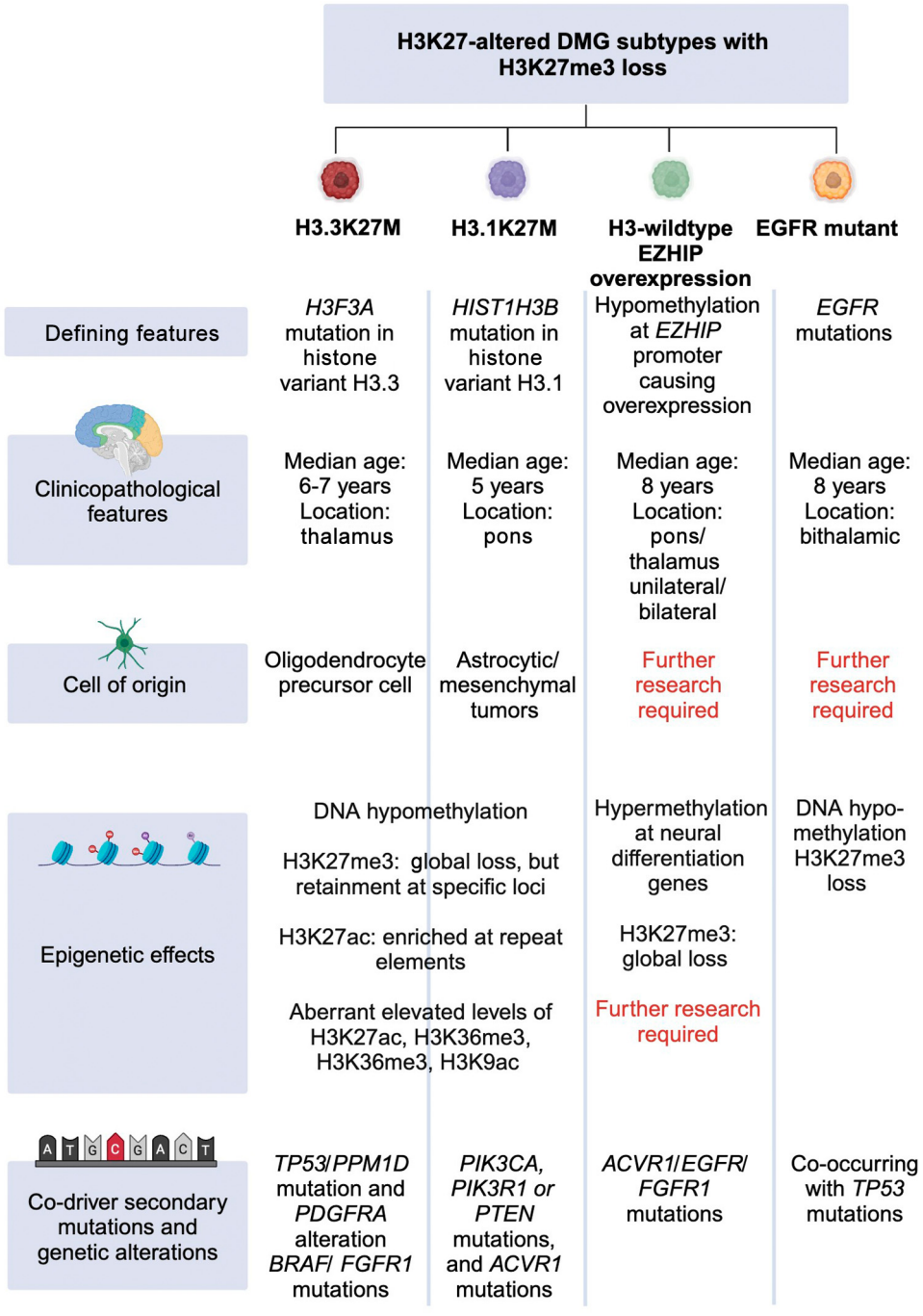

Figure 1. Comparison of H3K27-altered subtypes in diffuse midline glioma (DMG). The gaps in knowledge are highlighted in red letters. Figure created using BioRender.com. Abbreviations: EZHIP, enhancer of zeste inhibitory protein.

Glossary

Cancer-testis antigen (CTA): a group of proteins that are usually located on the X chromosome and normally have germ cell (testis)-restricted expression but become aberrantly expressed in somatic cells, which is seen in several types of cancer.

Histone H3 K27M (H3K27M): a lysine-to-methionine substitution mutation at position 27 of the histone H3 tail, which has been identified as a driver of diffuse midline gliomas.

K27M-like-peptide (KLP): a region toward the C-terminal region of EZHIP with sequence homology to the 23–31 amino acids of histone H3. Specifically, the M406 residue of EZHIP is equivalent to the mutated K27 residue in histone H3.

PRC2: a multiprotein transcriptional repressor that mediates histone (mono-, di-, and tri-)methylation in lysine 27 of histone H3 (H3K27me1/2/3) to ensure gene silencing and epigenetic regulation. Human PRC2 contains four core proteins: the histone-lysine N-methyltransferase enzyme EZH2, EED, SUZ12, and retinoblastoma-associated proteins 46 and 48.

Proteolysis targeting chimera (PROTACs): a molecule that can degrade proteins that may lack an active site and are not directly targetable by small-molecule inhibitors.

To date, most studies have focused on describing the mechanism by which EZHIP interacts and inhibits PRC2 activity and how it compares with H3K27M. However, studies looking into EZHIP's role beyond PRC2 are limited (Box 1). H3K27M nucleosomes do not always co-occupy PRC2 chromatin

Box 1. EZHIP-specific targeting mechanisms

EZHIP inhibits HR-mediated DNA repair

EZHIP suppresses homologous recombination (HR)-mediated DNA repair independently of its role in PRC2 inhibition. EZHIP interacts through its C terminal (residue W425) with PALB2, a member of the HR-mediated DNA repair complex, and this abolishes HR-mediated DNA repair [55]. In fact, the use of a poly(ADP)ribose polymerase inhibitor (talazoparib), which selectively kills cells that have erroneous HR repair, showed an increase in sensitivity in those cells with high *EZHIP* levels [55]. However, talazoparib permeability across the BBB is restricted [56], and thus the use of more BBB-permeable molecules is required for *in vivo* testing of these agents in EZHIP-DMGs.

CTA-based cancer immunotherapy

CTAs are known to act as neoantigens and thus show immunogenicity [57]. A recent study in CTAs in lung cancer showed an association between *EZHIP*-expressing cells and $CD138^{+}$ plasma cell infiltration. However, the relevance of these immune cells in immune tolerance/rejection remains unknown [58]. The immune landscape of EZHIP-PFAs remains largely unknown. scRNAseq studies in PFA tumors have mainly focused on tumor heterogeneity, and, while they showed some immune infiltration of T cells and myeloid/microglia cells, further annotation on their immune properties was not carried out [36,37]. Therefore, mining the existing scRNAseq data in PFA tumors may shed light on the immunogenicity of EZHIP-PFA tumors, which will help to guide immunotherapeutic strategies. Immune profiling of EZHIP-DMG has not been done, and as patient samples become available, scRNAseq experiments and spatial transcriptomics will enable this characterization. Given a plausible proimmunogenic role for EZHIP as a CTA, there may be therapeutic potential for EZHIP antigen-based vaccines or EZHIP antigen-specific T cell infusions to induce an immune response [57].

regions. In fact, H3K27M ChIP-seq experiments in DMG cell lines have shown that H3K27M nucleosomes are mainly found at active chromatin regions, including enhancers and promoters [21,23], and these regions co-occur with histone acetylation [24,25], but it remains unknown whether EZHIP has a similar role to H3K27M localizing to active chromatin regions, as a consequence of the lack of EZHIP ChIP-seq studies, particularly in DMGs. Mechanistically, the role of H3K27M nucleosomes in active transcription could be linked to its role in excluding PRC2 from these chromatin regions [19,21]. However, two recent publications suggest that H3K27M also directly correlates with active chromatin independently of PRC2, including through its direct interaction with MLL1 that leads to deregulation of H3K4me3 [26] and the increase of H3.3 turnover at active promoters and enhancers that sustains gene activation and stem-like gene signatures [27]. Therefore, further studies are required to evaluate if EZHIP may share any of these additional molecular roles.

Does EZHIP-DMG resemble the molecular characteristics of H3K27M-DMG beyond the loss of H3K27me3?

H3K27-altered DMGs, in addition to global loss of H3K27me3, have other epigenetic alterations in histone marks, including global gains of H3K27 acetylation and H3K36me2 and in DNA methylation (Figure 1) [19,28]. It is still under debate whether these additional epigenomic changes are direct consequences of H3K27M/EZHIP or if they are indirect consequences of the developmental stalling of the cancer cells resulting from PRC2 inhibition. Further functional studies are needed to address this open question.

Unfortunately, a clearly defined epigenomic landscape for EZHIP-DMGs is currently lacking due to its recent discovery and the low frequency of cases (thus limited availability of tumor tissues), but these studies will be key in better delineating the molecular similarities and differences among EZHIP-DMGs, H3K27M-DMGs, H3 WT DMGs, and EZHIP-PFAs [13]. Currently, the epigenetic data in EZHIP-DMG tumors are mostly limited to DNA methylation or immunohistochemistry of global changes of H3K27me3 [12,13]. DNA methylation profiling has confirmed that EZHIP-DMGs cluster closer to H3K27M-DMGs than EZHIP-PFA, suggesting that these differences may account for a distinctive cell of origin [12,13]. Some differences in DNA methylation patterns have been identified between EZHIP-DMGs and H3K27M-DMGs, including a higher number of hypermethylated CpG sites in homeobox genes involved in the development of EZHIP-DMGs. Additional DNA methylation clustering of EZHIP-DMGs revealed two separate clusters for either *ACVR1* or *EGFR* mutations,

suggesting mutual exclusivity of these mutations for H3 WT cases (Figure 1) [12]. Recently, mutations in *FGFR1* and *BRAF* genes were associated with longer survival in H3K27M-DMG, and *FGFR1* was found in EZHIP-DMG that also had an *ACVR1* mutation [29]. In further analysis of copy number variations, EZHIP-DMGs displayed increased frequency of 1q gains and therefore shared greater similarity with H3.1K27M tumors than H3.3K27M tumors [30]. These discoveries suggest that EZHIP-DMGs are also associated with specific genetic mutations that share similarities with H3K27M-DMG subtypes and that can predict patient prognosis.

Albeit the resemblance of global reduction of H3K27me3 in H3K27M-DMGs and EZHIP-PFAs, comparisons of the H3K27me3 ChIP-seq profiles between these cancer types have shown differences in H3K27me3 retention at loci-specific regions related to the tumor anatomical location and cell of origin. Loci-specific similarities mainly linked to important regions for the promotion of tumorigenesis [30]. Therefore, it is crucial to specifically identify H3K27me3 sites in EZHIP-DMGs to also understand the extent to which EZHIP modulates H3K27me3 deposition after dysregulation of PRC2. In addition to H3K27me3 deposition, further modeling of 3D topology may reveal insights into heterochromatin architecture. A recent publication showed that EZHIP may regulate the formation of heterochromatin by type B long-range interactions, 'TULIPs', almost exclusively a feature of PFAs [10]. It remains elusive if EZHIP is replicating this formation in DMGs.

The global increase of the active enhancer mark H3K27ac is another distinct feature of H3K27M-DMG. H3K27ac ChIP-seq experiments in cellular models of neural stem cells (NSCs) and embryonic stem cells overexpressing H3K27M demonstrated that, in the chromatin, H3K27ac is locally reduced at pre-existing enhancers and leads to the decreased expression of neurodevelopmental genes [27,31]. Experiments using knockout models for H3K27M in DMG cells further showed that this histone mutation did not result in *de novo* H3K27ac/enhancer formation, but the increase of H3K27ac in the chromatin was mainly found at repetitive elements [28]. Comparisons of H3K27ac levels and location in the chromatin between H3K27M-DMGs and EZHIP-PFAs showed that H3K27ac enhancers were associated with higher expression of astrocytic-like genes in EZHIP-PFA tumors [30] than the oligodendrocyte origin for H3K27M-DMGs [32–34]. Together, these studies point toward an indirect link between H3K27ac enhancers and H3K27M/EZHIP that may be linked with the cell of origin. However, further work defining the enhancer landscape for EZHIP-DMGs is needed to determine if these are DMG-specific or EZHIP-specific enhancers.

In conclusion, in-depth epigenomic and genomic profiling in EZHIP-DMG samples as well as the generation of cellular models for gain or loss of function of EZHIP are needed to determine the driver or passenger roles for EZHIP in regulation of the epigenomic landscape of DMG beyond H3K27me3 and in its association with other genomic alterations.

Defining the cell of origin for EZHIP-DMG

Single-cell RNA sequencing (scRNAseq) has advanced knowledge in identifying the cell of origin and development trajectories for H3K27M-DMG and PFA [33–37]. These studies converged to identify that H3K27M-DMGs had an origin from immature oligodendrocyte precursor cell (OPC)-like state [33–35]. However, each histone mutation, H3.1K27M and H3.3K27M, had a different lineage and positional identity and thus a different cell of origin during distinct waves of OPC specification [33,34]. Multiomic analyses based on single-cell transcriptomics, epigenome, and 3D chromatin structure suggest that H3.1K27M-DMGs originate during the earliest waves of OPC specification at the ventral progenitor stage, expressing *NKX6-1* and being sonic hedgehog dependent, while H3.3K27M-DMGs arise from dorsal progenitors that are $PAX3^{+}$ and bone morphogenetic protein dependent [33]. The cell of origin is also highly dependent on location in the brain. For example, H3.3K27M-DMGs located in the thalamus have a different origin than

the H3.3K27M from the brainstem as they seem to originate after the developmental commitment of posterior parencephalon during dienchephalon development, and this anatomical origin is highly related to the expression patterns and DNA methylation of *HOX* gene clusters [12,33].

Another important cell-intrinsic factor required for the progression to cancer from the cell of origin where the first hit mutation (K27M) occurs is the co-occurrence of specific genetic alterations. For example, *ACVR1* mutations in H3.1K27M-DMGs are required for the aberrant activation of BMP signaling, necessary for gliomagenesis [33], or *PDGFRA* sustained signaling is required for OPC-like cancer cells to proliferate [35]. Further functional experiments controlling the induction of primary and secondary mutations at certain neurodevelopmental stages are needed to expand and validate the findings from multiomic descriptive research in H3K27-altered DMG. Also, single-cell transcriptomic studies in EZHIP-DMGs are lacking, and the cell of origin of this molecular subtype is yet to be determined.

Cell of origin studies in PFA have shown that EZHIP-PFA tumors are found in NSC, gliogenic progenitor-like cell, and ependymal tumor cell states [30,36]. PFAs that do not express *EZHIP* harbor a better prognosis and consist of more differentiated cell populations [37]. Collectively, EZHIP-PFA or H3K27M-DMG originates from an undifferentiated state; however, both the anatomical location and specific undifferentiated progenitor states are different. Recently a rare case classified as H3 K27-altered DMG, a possible EGFR subtype, with ependymal features had a DNA methylation profile similar to the DMG-EGFR mutant subtype (Figure 1) but without EGFR mutations and with EZHIP expression and loss of H3K27me3. In addition, the tumor presented mixed glial and ependymal pathological features [38]. This DNA methylation pattern was shared with another reported case [39], which may suggest that these tumors have a different cell of origin to EZHIP-PFA or H3K27M-DMG.

Physiologically, *EZHIP* expression peaks during gestation at week 7 after primordial germ cell (PGC) migration and continues in later germ cells (Figure 2A) [1], which coincides with the early stages of embryonic glial development (Figure 2B). Given that the earliest appearance of OPCs is weeks 8–9 [40], whether abnormal *EZHIP* expression also occurs during this window in somatic glial cells as a feature of a CTA is an open question that requires further investigation (Figure 2B).

Potential therapy for EZHIP-DMG: repurposing H3K27M-DMG targets

Effective therapies for DMG are not currently available, and the current standard of care relies on palliative radiotherapy. Thus, there is an urgent need to develop effective treatments for this disease [41]. Ideally, therapies targeting the molecular drivers of the disease should be prioritized. In the case of EZHIP-DMG therapy, two approaches can be considered (Figure 3, Key figure). First, an EZHIP-specific approach using **proteolysis targeting chimeras (PROTACs)** [42] to degrade EZHIP, given its nonessential role in brain function, or exploring targets in EZHIP-specific pathways (Box 1). Second, given the molecular similarities between H3K27M-DMG, EZHIP-DMGs, and EZHIP-PFAs, another approach would include repurposing preclinically validated drugs used for H3K27M-DMG and potentially in EZHIP-PFAs, as discussed below.

Inhibition of the PRC2 complex

EZH2 inhibitors have been trialed for H3K27M-DMG, with prolonged survival observed in mice through the re-expression of PRC2-suppressed genes involved in cell differentiation [43]. This molecular process, as well as the preclinical efficacy using EZH2 and EED inhibitors have also been seen in EZHIP-PFAs [11,44], suggesting that this approach could work for EZHIP-DMG.

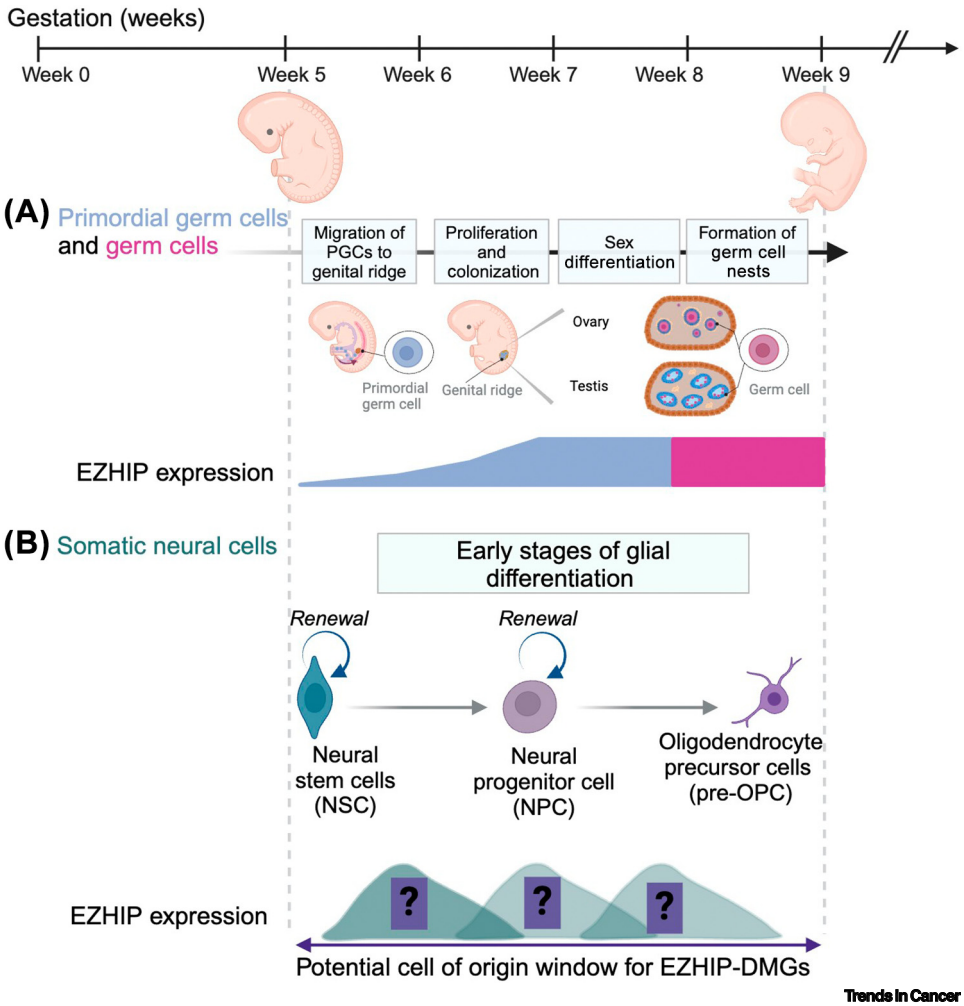

Figure 2. Open questions for the cell of origin for enhancer of zeste inhibitory protein diffuse midline glioma (EZHIP-DMG), which may mirror the germ cell development timing window. (A) Gestation weeks 5–9 are considered where EZHIP expression is normally seen to peak in primordial germ cells (PGCs; purple) and germ cells (pink). (B) During this window, we hypothesize that EZHIP becomes abnormally expressed in somatic neural cells/or oligodendrocyte precursor cells (OPCs) cells to promote gliomagenesis, which is a feature of cancer-testis antigens (CTA) such as EZHIP. Question marks show hypothesized abnormal expression of EZHIP in glioma development. Figure created using BioRender.com.

Modulators of histone acetylation

The inhibition of histone deacetylases (HDACs) has shown promising results in DMG *in vitro* and *in vivo* [45]. However, the HDAC inhibitor (HDACi) panobinostat has poor clinical efficacy, severe toxicity, and poor blood–brain barrier (BBB) permeability [46]. One plausible molecular rationale for increasing HDACi efficacy is through the creation of a pervasive acetylation landscape that induces the expression of endogenous retroviruses persuading an immune response [28] or through the activation of enhancers that regulate cell differentiation [27,31]. Despite a global gain of H3K27ac, the accumulation at repetitive elements has not been explored in *EZHIP*-expressing tumors. So, the efficacy of HDACi is yet to be proven in these cancers. H3K27ac marks astrocytic-like enhancers in EZHIP-PFAs [30], suggesting that HDACi may have an antitumor effect by suppressing stem cell properties and promoting cell differentiation.

Key figure

Overview of areas of research for enhancer of zeste inhibitory protein diffuse midline glioma (EZHIP-DMG)

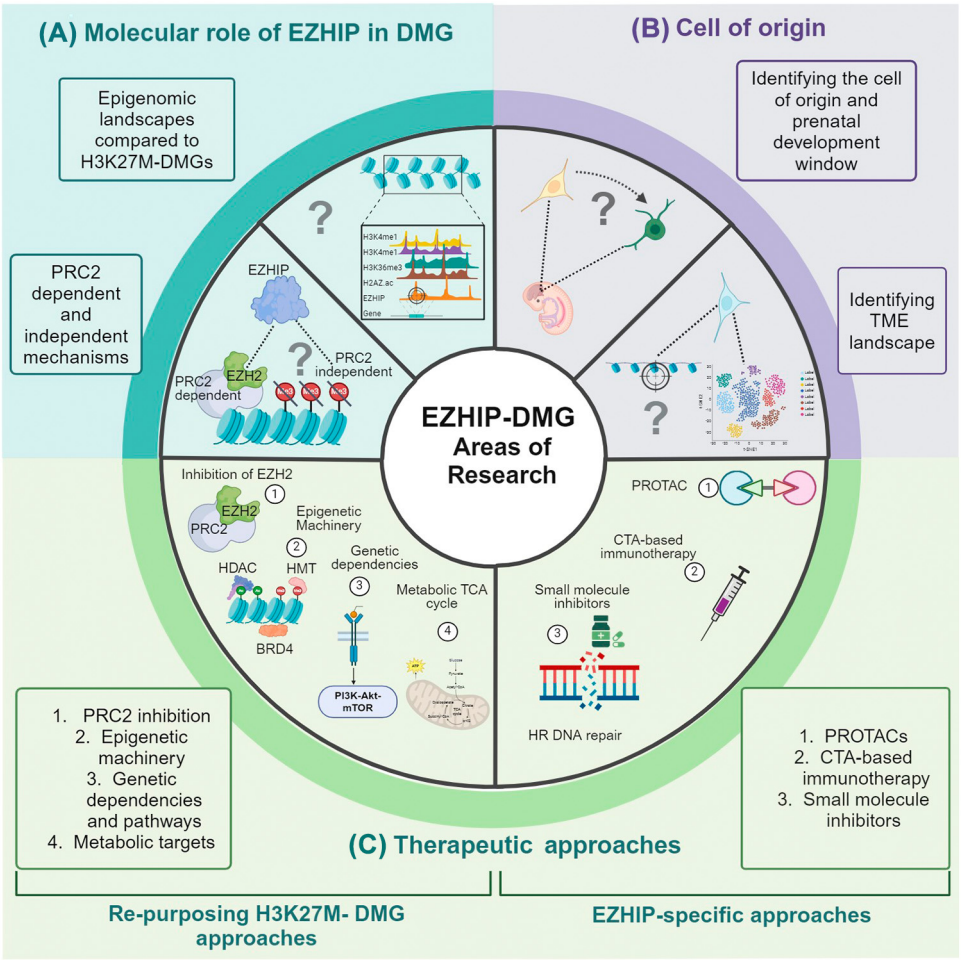

Figure 3. (A) The molecular roles of EZHIP in DMG need further work, including establishing the epigenetic landscape in EZHIP-DMG compared with H3K27M-DMGs and understanding the polycomb repressive complex 2 (PRC2)-dependent and -independent modes of action. (B) Further research is required for identifying the cell of origin and key events during this vulnerable developmental window and the impact of the tumor microenvironment (TME) in shaping these tumors. (C) Therapeutic options may involve a combination of repurposing H3K27M-DMG drugs and exploring EZHIP-specific targets. Figure created using BioRender.com. Abbreviations: CTA, cancer-testis antigen; HDAC, histone deacetylase; HMT, histone methyltransferase; HR, homologous recombination; PROTACs, proteolysis targeting chimeras; TCA, tricarboxylic acid.

Additionally, inhibitors for bromodomain and extra-terminal motif (BET) have been studied in H3K27M-DMG to inhibit the recruitment of bromodomain proteins to heterotypic H3K27M H3K27ac nucleosomes and thus suppress tumorigenic histone acetylation [5]. However, it remains elusive if EZHIP can integrate into chromatin to form EZHIP–K27ac complexes.

Targeting genetic dependencies and/or their signaling pathways

The acquisition of secondary driver mutations is a requirement for tumorigenesis in H3K27M-DMG [47], and EZHIP-DMGs have shown prevalent mutations with a plausible driver nature (Figure 1) [12,29,30]. EZHIP-DMG displays targetable mutations such as *ACVR1* with anaplastic lymphoma kinase (ALK) inhibitors [48], *EGFR* mutations with tyrosine kinase inhibitors [47], and loss of function of p53, which is reversible by APR-246 [49]. Further expanding the mutation spectrum of EZHIP-DMG is warranted to discover additional targetable dependencies [47].

Disruption of cancer metabolic pathways

Therapy targeting integrated metabolic pathways and consequently the epigenome has been suggested for EZHIP-PFAs [44,50]. These tumors rely on glucose [44], and experiments inducing the expression of *EZHIP* in NSCs resulted in increased glycolysis and mitochondrial tricarboxylic acid (TCA) cycle metabolism [50]. The use of the antidiabetic drug metformin resulted in improved preclinical efficacy in PFA models and mechanistically showed a reduction in *EZHIP* expression, and inhibition of the TCA cycle showed increased levels of H3K27me3 [50]. Similarly, H3K27M-DMGs have vulnerabilities in methionine metabolism and have enhanced glycolysis [51]. Consequently, the use of the mitochondrial-targeting drug ONC201 has provided promising therapeutic effects in H3K27M-DMGs through the modulation of metabolic and epigenetic pathways [52,53]. Metformin has also been tested in H3K27M-DMGs in conjunction with paxalisib as a combinatorial treatment to restore glucose homeostasis and overcome resistance [54]. Together this suggests that EZHIP-DMGs are likely to be tumors with high glycolysis and thus may benefit from treatments based on metabolic modulation like ONC201 and metformin.

Concluding remarks

This opinion has discussed the evolving research in the field of EZHIP-DMG by drawing comparisons with the H3K27M-DMG subtype and elucidating research gaps (Figure 3). The slow progress in unravelling the oncogenic molecular processes and therapeutic opportunities of EZHIP-DMG is attributed to its recent discovery, the rarity of its occurrence, and the absence of adequate preclinical models. However, we envision that the upcoming years will yield more EZHIP-DMG models to help elucidate epigenetic and genetic dependencies (see Outstanding questions and Figure 3A). These advancements will not only clarify the role of EZHIP in oncogenesis but also help to define the cell of origin of EZHIP-DMG (Figure 3B), which are both crucial to unveil potential targets in EZHIP-DMG and support the development of EZHIP-specific inhibitors for EZHIP-positive cancers beyond DMG (Figure 3C).

Outstanding questions

To what extent does EZHIP mimic the H3K27M mutation in DMG?

What are the similarities and differences between epigenetic and genetic landscapes of EZHIP-DMG compared with H3K27M-DMG?

What is the timing window during prenatal development that cells become vulnerable to EZHIP for cellular transformation?

What is the role of the N-terminal region of EZHIP, and how does this correlate with hotspot mutations seen in this region?

Are there additional independent effects of PRC2 and/or EZHIP-specific effects?

Can targeting metabolic and epigenetic factors affect the proliferation of EZHIP-DMG?

As CTAs are immunogenic, what are the other immune-targetable features of EZHIP's expression in DMG?

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Declaration of interests

M.D.D. is a parent to a child lost to DIPG and the founder and a director of the not-for-profit charity RUN DIPG, Ltd.

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