Oncogenic Mechanisms of Histone H3 Mutations

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Recurrent missense mutations in histone H3 were recently reported in pediatric gliomas and soft tissue tumors. Strikingly, these mutations only affected a minority of the total cellular H3 proteins and occurred at or near lysine residues at positions 27 and 36 on the amino-terminal tail of H3 that are subject to well-characterized posttranslational modifications. Here we review recent progress in elucidating the mechanisms by which these mutations perturb the chromatin landscape in cells through their effects on chromatin-modifying machinery, particularly through inhibition of specific histone lysine methyltransferases. One common feature of histone mutations is their ability to arrest cells in a primitive state refractory to differentiation induction, highlighting the importance of studying these mutations in their proper developmental context.

Chromatin, the combination of DNA and its interacting proteins, is the physiologically relevant form of eukaryotic genomes. The basic repeating unit of chromatin is the nucleosome, comprised of two copies of the core histone proteins H2A, H2B, H3, and H4 that together form an octamer wrapped by two superhelical turns of DNA (Luger et al. 1997). Historically, nucleosomes were thought to mainly provide structural support for genome packaging. However, research from the past two decades has revealed a remarkable role of nucleosome composition, modification, and positioning in virtually all DNA-based processes, including replication, transcription, and damage repair (Jenuwein and Allis 2001; Ernst and Kellis 2010).

Posttranslational modifications (PTMs) of histones are critically involved in chromatin-mediated gene regulation (Jenuwein and Allis 2001). It is believed that histone PTMs exert their effects through direct physical modulation of nucleosome–DNA contacts and/or recruitment of downstream "reader" protein complexes. To date more than 100 histone PTMs have been identified (Huang et al. 2014), many of which are dynamically controlled by enzymes catalyzing their addition ("writers") or removal ("erasers"). Among them, PTMs of several lysine residues located at the amino-terminal tail of histone H3 have been extensively characterized (Fig. 1A). For example, H3 lysine 27 (H3K27) can be acetylated by p300/CBP and H3K27ac is preferentially located at promoters and/or enhancers of genes that are actively transcribed (Ogryzko et al. 1996). In contrast, methylation of H3K27, catalyzed by the Polycomb repressive complex 2 (PRC2) and removed by KDM6 family demethylases, is a mark associated with gene

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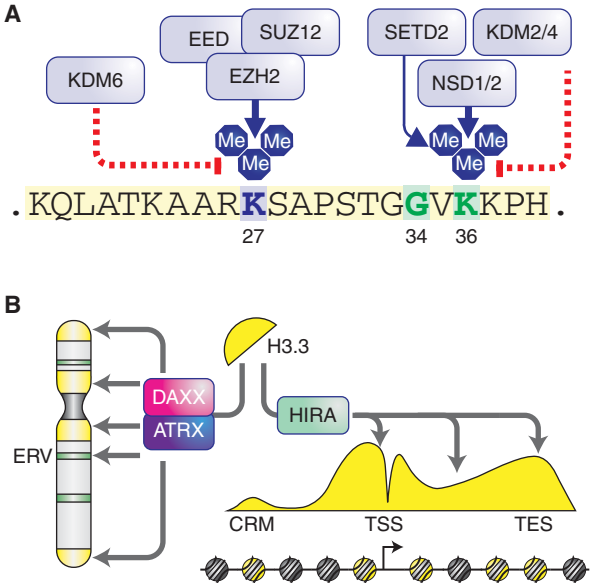

Figure 1. Posttranslational modification of the histone variant H3.3 and its chaperone/deposition machinery. (*A*) The amino-terminal tail of histone H3.3 (shown here) and other histone H3 proteins are subject to methylation of lysine residue 27 by the Polycomb repressive complex 2 (PRC2), containing core subunits EED, SUZ12, and EZH2. Removal of H3K27 methylation is performed by KDM6 family demethylases, including JMJD3 and UTX. Lysine residue 36 is subject to methylation by multiple enzymes, including the NSD family enzymes and SETD2. Removal of H3K36 methylation is performed by the KDM2 and KDM4 family demethylases. (*B*) Histone variant H3.3 is deposited at pericentric heterochromatin, telomeres, and certain endogenous retroviral elements (ERV) by the ATRX/DAXX heterodimeric complex. In contrast, H3.3 is deposited at euchromatin regions such as promoters and gene bodies by the histone chaperone HIRA (see Banaszynski et al. 2010 and Maze et al. 2014 for details and references).

silencing (Margueron and Reinberg 2011). Methylation of H3 lysine 36 (H3K36), depending on the context, can regulate transcriptional elongation, RNA processing, and DNA damage sensing (Kuo et al. 2011; Carvalho et al. 2014; Simon et al. 2014; Wen et al. 2014). In mammals, several enzymes targeting H3K36 have been reported (Wagner and Carpenter 2012). Although SETD2 is the only methyltransferase that can generate trimethylation of H3K36 (H3K36me3), multiple methyltransferases catalyze mono- and dimethylation of H3K36 (H3K36me1/2), including NSD1/2/3 and ASH1L. Conversely, members of the KDM2 and KDM4 families act as H3K36-specific demethylases.

More recently, it was appreciated that changes to the cellular epigenetic state could also result from expression and incorporation of histone variants (Banaszynski et al. 2010; Maze et al. 2014), further increasing the complexity of chromatin regulation. With the exception of H4, all histones are expressed in variant forms differing in primary amino acid sequence, leading to minor or major structural dissimilarities. Importantly, such variations were shown to have functional relevance. For example, in metazoans, variants of H3 include H3.1, H3.2, H3.3, and CENPA (Hake and Allis 2006). CENPA is centromere-specific and structurally dissimilar to the other H3 variants, whereas H3.3 differs from the "canonical" H3.1 and H3.2 by only a few amino acids. However, several lines of evidence suggest that H3.3 plays a distinct role in chromatin biology from canonical H3 (Fig. 1B). First, although canonical H3 is expressed and incorporated into chromatin in a DNA replication-dependent manner,

the expression, assembly, and deposition of H3.3-containing nucleosomes are cell-cycle-independent (Tagami et al. 2004). Second, in proliferating cells, H3.3 is enriched at selected genomic regions, including promoters and gene bodies of highly transcribed or transcriptionally "poised" genes in euchromatin (McKittrick et al. 2004), pericentric heterochromatin and telomeres (Goldberg et al. 2010), and certain classes of endogenous retroviral elements (ERVs) (Elsässer et al. 2015). Third, compared with canonical nucleosomes, distinct sets of factors facilitate the assembly and deposition of H3.3-containing nucleosomes. Deposition of H3.3 to euchromatic regions is mainly mediated by the HIRA complex (Tagami et al. 2004), whereas ATRX/DAXX were identified as the complex that specifically incorporates H3.3 to heterochromatic regions (Lewis et al. 2010; Elsässer et al. 2012, 2015). Depletion of H3.3 in embryonic stem cells leads to aberrant PRC2 binding at developmentally regulated genes, derepression of ERVs, and abnormal cell differentiation (Banaszynski et al. 2013; Elsässer et al. 2015). In mammals, two genes (*H3F3A* and *H3F3B*) encode H3.3 and mice deficient for either H3.3 gene show postnatal death, growth retardation, and infertility (Couldrey et al. 1999; Bush et al. 2013). Therefore, although the specific mechanisms remain to be fully elucidated, it appears that H3.3 is required to establish the proper chromatin states at specific genomic regions to maintain cell identity during development.

As the precise regulation of chromatin is essential for many cellular events, including proliferation and differentiation, not surprisingly chromatin misregulation has been linked to various human diseases, notably cancer. A major finding from recent tumor genome sequencing studies was the discovery that chromatin regulators, including writers, erasers, and readers of histone and DNA modifications and nucleosome remodelers, are frequently altered in malignancies (Shen and Laird 2013). Although aberrant chromatin states are increasingly recognized as an emerging hallmark of cancer, few researchers in the cancer epigenetics field anticipated the reports of recurrent mutations in histone H3 themselves. As detailed in the following sections, these mutations are highly clustered missense mutations of residues at or near well-studied PTM sites. Furthermore, the mutations are always monoallelic and affect only one of the 16 genes encoding H3 in humans. These interesting features have attracted considerable attention from the oncology and chromatin biology communities and recently the mechanisms underlying these so-called "oncohistones" have begun to be unraveled. In this review, we will summarize and discuss efforts to identify and understand histone H3 mutations.

HISTONE MUTATIONS IN CANCER

Recurrent mutations in histone H3 were first reported in pediatric high-grade gliomas (pHGGs) (Fig. 2). Simultaneous reports described H3K27M mutations in the majority of diffuse intrinsic pontine gliomas (DIPGs), a type of pHGG associated with dismal prognosis owing to its location in the brainstem, as well as

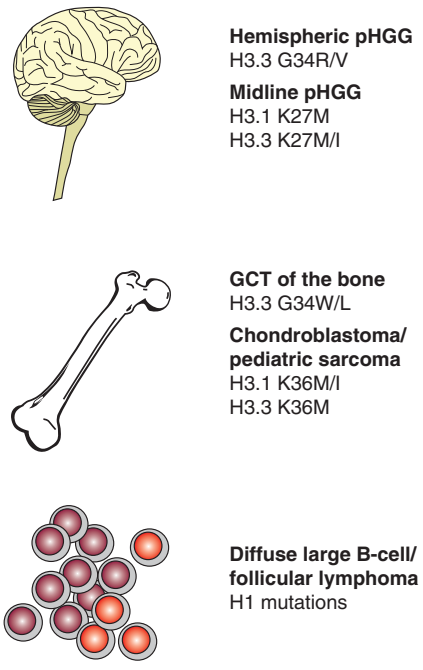

Figure 2. Recurrent histone mutations in human cancer (see text and Fontebasso et al. 2014a; Kallappagoudar et al. 2015). pHGG, Pediatric high-grade glioma; GCT, giant cell tumor.

in thalamic gliomas (Schwartzentruber et al. 2012; Wu et al. 2012). H3K27M mutations were not detected in other pediatric brain tumors including medulloblastomas and ependymomas (Wu et al. 2012) and seemed to be specific for midline pHGGs as they were subsequently found in pHGGs involving the cerebellum and the spinal cord (Sturm et al. 2012). Overall, pHGGs were characterized by additional mutations affecting growth factor signaling (e.g., RAS and PI3K) and the RB1 and TP53 pathways for cell-cycle regulation (Buczkowicz et al. 2014; Fontebasso et al. 2014b; Taylor et al. 2014; Wu et al. 2014). Evolutionary reconstruction of individual DIPG tumors revealed that H3K27M mutations arose early and were accompanied by an obligate partner mutation in a member of either of these pathways (e.g., *PIK3R1* or *TP53*), suggesting that both are needed for tumorigenesis (Nikbakht et al. 2016). H3K27M mutations were also reported in rare cases of pediatric low-grade gliomas (Jones et al. 2013; Zhang et al. 2013) as well as in thalamic gliomas in adults under the age of 50 (Aihara et al. 2014). Notably, DIPG patients carrying the H3K27M mutation had worse overall survival compared with patients lacking the mutation (Khuong-Quang et al. 2012; Feng et al. 2015). Because of its prevalence and association with patient outcomes, the detection of H3K27M mutations by immunohistochemistry is increasingly being considered for diagnostic purposes in pediatric gliomas. Antibodies directed against H3K27M have been developed and were shown to be 100% sensitive and specific for the mutation (Bechet et al. 2014; Venneti et al. 2014).

The majority of pHGG-associated H3K27M mutations affect the variant histone H3.3, whereas others are found in canonical histone H3.1/2. Intriguingly, H3.3K27M mutations differ from H3.1/2K27M mutations in several clinical features. H3.1K27M-mutant gliomas were restricted to the brainstem, unlike H3.3K27M-mutant gliomas, which were found in the brainstem in addition to other midline locations (Fontebasso et al. 2014b; Castel et al. 2015). DIPG patients with H3.1K27M were, on average, 2 years younger than those with H3.3K27M (Castel et al. 2015) and had a distinctive set of co-occurring mutations. Missense mutations in *ACVR1*, which encodes the bone morphogenetic protein (BMP) type I receptor ALK2, were significantly associated only with H3.1K27M but not H3.3K27M (Buczkowicz et al. 2014; Fontebasso et al. 2014b; Taylor et al. 2014; Wu et al. 2014). Many of the *ACVR1* mutations were identical to those found in the autosomal dominant syndrome fibrodysplasia ossificans progressiva in which gain-of-function mutations in *ACVR1* lead to heterotopic ossification (Shore et al. 2006; Chaikuad et al. 2012). Phosphorylation of SMAD1/5/8, downstream events of ALK2 activation, were increased in *ACVR1*-mutant DIPG tumors (Buczkowicz et al. 2014; Fontebasso et al. 2014b), and expression of mutant *ACVR1* transgenes was sufficient to increase phosphorylation of SMAD1/5/8 in astrocytes (Wu et al. 2014) and DIPG cell lines (Taylor et al. 2014). Therefore, ALK2 inhibitors currently in development to treat fibrodysplasia ossificans progressiva could be therapeutically effective against a subset of pHGGs. In contrast, amplification of *PDGFRA* was significantly associated only with H3.3K27M (Buczkowicz et al. 2014; Castel et al. 2015). Other H3.3K27M-mutant gliomas, particularly in the thalamus, were associated with mutations in both *FGFR1* and *NF1* (Jones et al. 2013). H3.1K27M and H3.3K27M DIPGs also have distinct gene expression profiles, although it remains unclear to what extent this is attributable to differences in the H3 variants as opposed to the unique co-occurring mutations in each subgroup or the possibility that each subgroup arises from a distinct cell of origin (Castel et al. 2015).

Unlike midline pHGGs, many pHGGs located in the cerebral hemispheres carried H3.3G34R or H3.3G34V mutations (Fig. 2) (Schwartzentruber et al. 2012; Wu et al. 2012). H3.3G34R/V-mutant tumors were typically diagnosed during adolescence, rather than in early childhood like most H3K27M-mutant tumors. They had distinct gene expression and DNA methylation signatures from H3K27M-mutant tumors and H3 mutational status could be predicted based on the expression of FOXG1

exclusively in H3.3G34-mutant gliomas and OLIG2 exclusively in H3K27M-mutant gliomas (Sturm et al. 2012). Mutations in ATRX, which heterodimerizes with the histone chaperone DAXX to specifically incorporate H3.3 at pericentric heterochromatin and telomeres, were significantly associated with H3.3G34-mutant pHGGs although they were also observed at a lower frequency in wild-type (WT) and H3K27M-mutant pHGGs. Similar to other cancer types, ATRX-mutant pHGGs show alternative lengthening of telomeres (Schwartzentruber et al. 2012). Notably, mutations in the H3K36 methyltransferase SETD2 were also seen in high-grade gliomas involving the cerebral hemispheres in adolescents and were mutually exclusive with H3 mutations (Fontebasso et al. 2013).

Recurrent mutations in histone H3 were subsequently reported in specific types of bone and cartilage tumors (Fig. 2). H3.3K36M mutations were found in nearly all chondroblastomas, a benign tumor of the active growth plate of bones, and rarely in malignant conventional and clear-cell chondrosarcomas (Behjati et al. 2013). Antibodies directed against H3K36M were 100% specific and sensitive for the mutation and may prove useful for diagnosis (Amary et al. 2016; Lu et al. 2016). Interestingly, although H3.3K27M mutations occurred exclusively in *H3F3A*, H3.3K36M mutations predominately were found in *H3F3B* despite similar expression levels and identical amino acid sequence of both isoforms (Behjati et al. 2013). In addition, nearly all giant cell tumors of the bone had H3.3G34 mutations mostly to tryptophan (and in one case, to leucine), whereas osteosarcomas infrequently had the H3.3G34R mutation originally identified in pHGGs (Behjati et al. 2013; Joseph et al. 2014; Sarungbam et al. 2016). Two patients with postzygotic H3.3G34W mutations presented with paragangliomas and recurrent giant cell tumors of the bone, suggesting that somatic mosaic mutation of H3.3G34 may be the basis for a new nonhereditary cancer syndrome (Toledo et al. 2015). No H3 mutations were detected in other types of bone and cartilage tumors including chondromyxoid fibromas, chordomas, and chondromas (Behjati et al. 2013).

Recurrent mutations in the linker histone H1 have also been reported in diffuse large B-cell lymphomas (Lohr et al. 2012) and follicular lymphomas (Okosun et al. 2014). Many of these mutations are believed to be loss-of-function (Okosun et al. 2014), but their contribution to oncogenesis remains unexplored and therefore will not be discussed in this review.

MECHANISM OF H3K27M-MEDIATED ONCOGENESIS

Initial characterization of the histone PTMs in H3K27M-mutant DIPG patient tumors and cell lines revealed a global reduction of H3K27me3 compared with H3 WT DIPG tumors (Chan et al. 2013; Lewis et al. 2013; Venneti et al. 2013), despite similar expression of EZH2 (Venneti et al. 2013). K27M-mutant H3 contributed to only approximately 3%–17% of the total H3 proteins in DIPG samples (Lewis et al. 2013), which is consistent with the fact that only one of the 32 H3-encoding alleles harbors the mutation and suggests that the global reduction in H3K27me3 was caused by a dominant effect of the H3K27M mutation. Indeed, expression of an H3K27M transgene in 293T cells was sufficient to reduce H3K27me2 and H3K27me3 globally despite the mutant histone accounting for only 1% of total H3 protein (Lewis et al. 2013). Similar effects were seen regardless of whether the transgene was H3.1 or H3.3 (Chan et al. 2013) and were consistent across a range of different cell types including astrocyte, fibroblast, and glioma cell lines (Bender et al. 2013; Chan et al. 2013). This effect was restricted to the mutation of the lysine residue at position 27 to methionine (and to a lesser extent isoleucine), but not to other amino acids (Lewis et al. 2013). The dominant nature of the mutation was further shown by the loss of H3K27me3 on endogenous WT H3 proteins (Bender et al. 2013; Lewis et al. 2013).

In-depth biochemical and molecular work has revealed that the H3K27M mutation achieves global reductions in H3K27 methylation through inhibition of the PRC2 complex in *trans* (Fig. 3A,B). Addition of H3K27M peptides (Lewis et al. 2013) or mononucleosomes

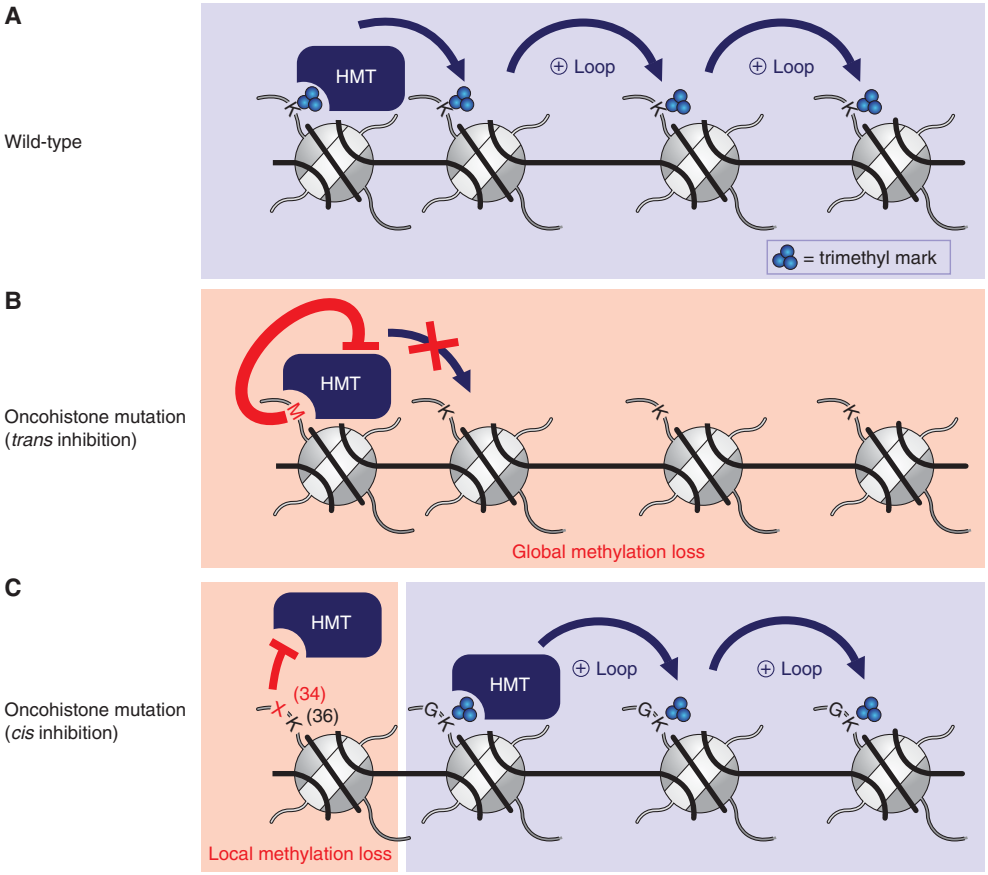

Figure 3. Inhibition of histone lysine methyltransferases (HMTs) by histone H3 oncohistone mutations. (*A*) Unimpeded activity of HMTs in the absence of a mutation (wild-type). (*B*) H3K9M, H3K27M, and H3K36M mutations dominantly inhibit their respective HMTs in *trans*. Consequently, nonmutant nucleosomes are hypomethylated (global methylation loss). (*C*) H3G34 mutations inhibit H3K36 HMTs in *cis* such that only nucleosomes containing the mutation are unable to be methylated (localized methylation loss).

(Brown et al. 2014), or heterotypic nucleosome arrays containing three WT nucleosomes and one spatially defined H3K27M mutant nucleosome (Brown et al. 2015), inhibited recombinant PRC2 activity in a dose-dependent and competitive manner. The IC_{50} for H3K27M inhibition of PRC2 calculated in these in vitro assays is less than the estimated nuclear concentration of a 3%–5% fraction of the total H3 pool (Lewis and Allis 2013), suggesting that such a mechanism could be responsible for the global reduction of H3K27me3 in cells. Although nucleosomes isolated from H3K27M-expressing cells were able to inhibit the activity of recombinant PRC2, their effect on the activity of H3K27 demethylases JMJD3 and UTX was relatively minimal (Bender et al. 2013).

Insights into the interaction between H3K27M and PRC2 have begun to elucidate the structural basis of the inhibition. H3K27M peptides with photo-cross-linkers inserted at or near the mutated residue both pulled down EZH2 from the recombinant PRC2 (Lewis et al. 2013). EZH2 is also enriched on mononucleosomes containing H3K27M isolated from cells (Bender et al. 2013; Chan et al. 2013). Screening of H3K27-mutant peptides found that mutation of K27 to amino acids that have a long, hydrophobic side chain with minimal branching such as methionine, isoleucine, and

the unnatural amino acid norleucine were among the most potent inhibitors of PRC2 activity (Lewis et al. 2013; Brown et al. 2014). The inhibitory properties of isoleucine led to the prediction of H3K27I mutation in pHGGs (Lewis and Allis 2013), which was indeed recently identified in a DIPG patient tumor (Castel et al. 2015). These three side chains are thought to be compatible with binding to the active site of the SET domain of EZH2, which normally uses an aromatic cage to bind the unbranched, hydrophobic side chain of lysine. Mutation of one of these aromatic residues in EZH2 rendered the protein less sensitive to inhibition by H3K27M (Lewis et al. 2013). Recently, the crystal structure of *Chaetomium thermophilum* PRC2 bound to a H3K27M peptide in the presence of *S*-adenosyl-L-homocysteine (SAH) was solved and suggests that a residue adjacent to the missense mutation, H3 arginine 26 (H3R26), occupies the active site and prevents substrate binding (Jiao and Liu 2015). Interestingly, in the human PRC2:H3K27M complex the methionine 27 side chain was positioned in the lysine 27 access channel (Justin et al. 2016). Further structural and biochemical studies of the PRC2-H3K27M interaction will be important to uncover potential ways to reverse the inhibition of PRC2 (Brown et al. 2014).

Based on the strong degree of conservation among SET domain–containing proteins, the widely adaptable inhibition of histone methyltransferases by H3 "K-to-M" mutations was appreciated even before reports surfaced of H3K36M mutations in patients (Behjati et al. 2013; Lewis et al. 2013). Analogous to H3K27M, the introduction of exogenous H3K9M and H3K36M into 293T cells was able to reduce global levels of H3K9me3 and H3K36me3, respectively (Lewis et al. 2013). Recombinant H3K9-specific methyltransferases, SUV39H1 and G9a, were also each shown to be inhibited by the addition of H3K9M peptides in *trans* and in an *S*-adenosyl methionine (SAM)-dependent manner (Lewis et al. 2013; Jayaram et al. 2016; Justin et al. 2016). In contrast, introduction of H3K4M had minimal effect on global H3K4me3 levels in cells, a finding Lewis and colleagues suggest may be a result of inhibition of the H3K4 demethylases LSD1 and LSD2 by H3K4M counterbalancing any potential inhibition of H3K4 methyltransferases by H3K4M (Karytinos et al. 2009).

Although much interest has been directed toward understanding the inhibition of methyltransferases by H3 "K-to-M" mutations, these analyses may overlook additional cellular targets of these mutations. Indeed, histone demethylases have alternatively been implicated as being responsible for the alteration of H3K9 methylation in cells expressing H3K9M. Unbiased mass-spectrometry analysis of immunoprecipitated mononucleosomes containing H3K9M revealed an enrichment for the K9 demethylase KDM3B but a depletion for HP1, a reader of H3K9me3, in native conditions (Herz et al. 2014). In this model, H3K9M serves to recruit KDM3B to genomic loci where it demethylates adjacent WT H3K9, thereby releasing HP1 and decompacting the surrounding chromatin (Herz et al. 2014). It will be important to further validate this model by directly testing the ability of H3K9M to potentiate the activity of KDM3B in *cis* on nucleosomal templates as well as to assess whether deletion of KDM3B can blunt local and/or global changes in H3K9 methylation induced by the H3K9M mutation. Nonetheless, these findings highlight that multiple non–mutually exclusive mechanisms may shape how H3 "K-to-M" mutations alter the chromatin landscape in cells.

Despite global depletion of H3K27 methylation in H3K27M-expressing cells, genome-wide profiling of the chromatin landscape with high-throughput sequencing revealed a striking gain of H3K27me3 at selected genomic loci. The existence of these regions was first observed when comparing H3K27M-mutant DIPG cell lines with human neural stem cells (NSCs) (Chan et al. 2013) and also when comparing H3K27M and H3 WT DIPG tumor samples (Bender et al. 2013). Although H3K27me3 was reduced in mutant DIPG cell lines at every type of genetic element examined (including promoters, 5′ UTRs, coding exons, introns, 3′ UTRs, and intergenic regions), ~60% of the H3K27me3 peaks that remained were not present in NSCs (Chan et al. 2013). A similar reduc-

tion in overall H3K27me3 peaks was observed in H3K27M-mutant DIPG tumors compared with H3 WT tumors, with gain of H3K27me3 at limited loci that were significantly enriched for intergenic regions (Bender et al. 2013). Integrated RNA-seq and ChIP-seq analyses in both cell lines and tumors revealed that expression of genes that gained H3K27me3 in H3K27M-mutant samples was down-regulated (e.g., the long isoform of CDK6, p16Ink4a, MICA). Conversely, genes that lost H3K27me3 showed enhanced expression (Bender et al. 2013; Chan et al. 2013). In addition, H3K27M-mutant DIPG tumors showed global DNA hypomethylation, which was associated with the loss of H3K27me3 at promoter regions (Bender et al. 2013). A better understanding of the mechanism through which H3K27me3 is established and maintained at certain regions will likely require assessing how EZH2 localization and H3K27 methylation change genome-wide following the introduction and removal of H3K27M in the same cell type.

New models to study DIPG biology have been developed in light of the discovery of H3K27M mutations and the recognition of their ability to perturb the chromatin landscape in cells. Human embryonic stem cell–derived NSCs have been used to elucidate the oncogenic nature of H3K27M based on their role as the presumed cells-of-origin for DIPG. Introduction of H3K27M selectively increased cell proliferation in NSCs, but not in embryonic stem cells (ESCs), ESC-derived astrocytes, or human fibroblasts (Funato et al. 2014). In the setting of TP53 knockdown and the presence of a constitutively active form of PDGFRa, addition of the H3K27M mutation enabled NSCs to acquire several neoplastic properties (Funato et al. 2014). These included the ability to suppress apoptosis on growth factor withdrawal, sustained proliferation following irradiation, increased invasiveness, impaired differentiation into astrocytes and oligodendrocytes, and formation of tumors when transplanted in vivo (Funato et al. 2014). None of these attributes were observed with H3K27M expression alone, or when the combination of PDGFRa activation and p53 knockdown was not paired with H3K27M, consistent with the observations that these mutations tend to co-occur in patient tumors. Leveraging this model, a small molecule screen was performed, which identified the menin inhibitor MI-2 to selectively decrease the proliferation of transformed H3K27M-expressing NSCs and an H3K27M DIPG cell line, but not H3 WT NSCs (Funato et al. 2014). Treatment with MI-2 also led to the removal of the differentiation block and slowed in vivo growth of orthotopic tumor xenografts, indicating that menin inhibition might be a potential therapeutic option for DIPG patients (Funato et al. 2014).

Other approaches taken to therapeutically target H3K27M-mutant DIPG focused on reversing the reduction of H3K27me3 through manipulating various forms of chromatin-modifying machinery. For example, a small molecule inhibitor of the H3K27 demethylases JMJD3 and UTX known as GSKJ4 was able to increase global levels of H3K27me2/3 in pediatric glioma cell lines expressing H3K27M (Hashizume et al. 2014). GSKJ4 more potently inhibited the growth of these lines both in vitro and in vivo compared with other glioma cell lines that were H3 WT (Hashizume et al. 2014). The efficacy of GSKJ4 may be largely through its effect on JMJD3, as knockdown of JMJD3 but not UTX slowed the growth of H3K27M-expressing cell lines (Hashizume et al. 2014). An alternative method of restoring H3K27me3 levels in cells involves "detoxifying" the H3K27M nucleosomes by weakening their interaction with PRC2 (Brown et al. 2014). PRC2 interacts with the entire H3 N-terminal tail, and its inhibition by peptides containing K27Nle was substantially weakened when residues next to position 27 were deleted or altered (Brown et al. 2014). PTMs, including K4 methylation, polyacetylation, and S28 phosphorylation, all reduced inhibition of PRC2 activity by H3K27Nle peptides in vitro (Brown et al. 2014). Furthermore, the expression of an H3K27M, S28E (mimicking a phosphoserine) double mutant transgene partially rescued the global reduction of H3K27me2/3 in cells (Brown et al. 2014). This strategy has now been taken one step closer to the clinic as H3K27M DIPG cell lines were found to be selectively sen-

sitive to histone deacetylase (HDAC) inhibitors (Grasso et al. 2015). Knockdown of either HDAC1 or HDAC2 showed a similar effect. The clinically approved multi-HDAC inhibitor panobinostat led to a dose-dependent increase in both global H3 acetylation and H3K27me3, likely reflecting diminished inhibition of PRC2 and thereby contributing to a normalization of the H3K27M-induced aberrant gene expression signature (Grasso et al. 2015). Several clinical trials are ongoing to test the safety and efficacy of panobinostat and other HDAC inhibitors in patients with DIPG.

MECHANISM OF H3K36M-MEDIATED ONCOGENESIS

The discovery of H3.3K36M mutations in the vast majority of chondroblastomas and in rare cases of chondrosarcomas (Behjati et al. 2013) prompted the initial description of global reduction in H3K36me3 in 293T cells (Lewis et al. 2013) to be revisited in a more developmentally relevant context. The introduction of an H3K36M transgene into murine mesenchymal progenitor cells blocked their ability to differentiate into chondrocytes and promoted tumor formation when the cells were subcutaneously transplanted into mice (Lu et al. 2016). The tumors did not appear by histology as chondroblastomas or chondrosarcomas but rather as undifferentiated sarcomas and indeed H3K36M was found to also block the differentiation of these cells into adipocytes and osteocytes. Screening a small panel of 10 pediatric undifferentiated soft tissue sarcomas identified one tumor with an H3.1K36M mutation and another with an H3.1K36I mutation. H3K36I similarly blocked the differentiation of murine mesenchymal progenitor cells and its presence in patient tumors mirrors the existence of H3K27I mutations in pediatric gliomas (Castel et al. 2015). Consistent with these findings, CRISPR-Cas9-mediated knock-in of the K36M mutation to the endogenous *H3F3B* allele in immortalized human chondrocytes bestowed similar oncogenic properties, including impaired differentiation and suppressed alkaloid-induced apoptosis (Fang et al. 2016).

Expression of either H3K36M or H3K36I resulted in global reductions in H3K36me2/3 in murine mesenchymal progenitor cells, an effect that appeared to be independent of whether the mutation occurred in H3.1 or H3.3 (Lu et al. 2016). Interestingly, reductions in H3K36me2/3 levels were positively correlated to enrichment of H3.3K36M, suggesting that in addition to its global effects, H3K36M may also exert a localized inhibitory influence (Fang et al. 2016). Nucleosomes containing H3K36M inhibited purified SETD2 and NSD2 activity in vitro, and knockdown of H3K36 methyltransferases recapitulated the impact of H3K36M on the epigenome, transcriptome, and cellular differentiation (Fang et al. 2016; Lu et al. 2016). These results lend support for a critical role of methyltransferase inhibition downstream of the H3K36M mutation.

Profiling the perturbations in the chromatin landscape induced by expression of H3K36M revealed one mechanism by which differentiation blockade is achieved. H3K36me2 was found to be significantly reduced at intergenic domains, which was associated with an increase in H3K27me3 at the same regions. This is consistent with the known inhibition of PRC2 methyltransferase activity by nucleosomes carrying H3K36me2/3 in vitro and the mutual exclusivity of H3K27me3 and H3K36me2/3 genome-wide (Schmitges et al. 2011; Yuan et al. 2011). The increase in intergenic H3K27me3 served to compete with gene-associated H3K27me3 for the recruitment of H3K27me3 "readers" and led to a dilution of the H3K27me3-binding canonical PRC1 complex away from its target genes where it normally represses gene expression. As a consequence, genes that were normally silenced by PRC1 became derepressed on expression of H3K36M. These genes were highly enriched for mesenchymal tissue development regulators and consistently, knockdown of PRC1 components significantly blocked mesenchymal progenitor cell differentiation (Lu et al. 2016).

As H3K36 methylation has also been implicated in the regulation of transcriptional elongation (Wen et al. 2014), RNA processing (Simon et al. 2014), and the DNA damage

response (Carvalho et al. 2014), the H3K36M mutation may rely on additional oncogenic mechanisms besides dilution of PRC1. Indeed, defective homologous recombination was noted in chondrocytes expressing H3K36M (Fang et al. 2016). Further study is needed to address whether dysregulation of these processes contributes to H3K36M-mediated oncogenesis.

MECHANISM OF H3G34R/V-MEDIATED ONCOGENESIS

Less is known about H3G34 mutations in cancer, but they appear to work through a different mechanism than H3 "K-to-M" mutations. Expression of H3G34R or H3G34V in 293T cells both had no effect on global H3K27me2/3 or H3K36me3 levels (Lewis et al. 2013). Instead, H3K36me2/3 was exclusively reduced on exogenous H3G34-mutant nucleosomes but not on endogenous WT nucleosomes (Lewis et al. 2013). Consistent with this finding, recombinant SETD2 was less efficient at methylating H3K36 using H3G34-mutant nucleosomes in vitro (Lewis et al. 2013). Thus, H3G34 mutations appear to exert their effect in *cis*, in contrast to H3K27M and H3K36M mutations, which are able to inhibit SET-domain methyltransferases in *trans* (Fig. 3C). Of note, whereas H3K27M and H3K36M mutations have been reported in H3.1 and H3.3, H3G34 mutations have only been identified in H3.3, further suggesting that they may result in local chromatin changes in genomic regions enriched for H3.3. How H3G34 mutations contribute to oncogenesis remains largely unexplored, although an initial report points to MYCN as being the most highly differentially expressed gene in a H3G34-mutant glioma cell line (Bjerke et al. 2013). Knockdown of MYCN decreased viability of H3G34-mutant cells and expression of H3G34-mutant transgenes in astrocyte and glial cells was sufficient to upregulate MYCN (Bjerke et al. 2013). Further work is needed to assess how H3G34 mutations act to regulate MYCN expression as well as other genes associated with early brain development (Bjerke et al. 2013).

CONCLUDING REMARKS

The application of next-generation sequencing to an increasing number and array of human cancers is revealing mutations where no one expected—perhaps most surprisingly in histones, the fundamental building blocks of chromatin. Strikingly, these mutations occur at or near residues on the amino-terminal tail of histone H3 that have well-characterized writers, readers, and erasers. As more types of cancer are sequenced and as appreciation for the dominant nature of these "oncohistone" mutations grows, it will be interesting to determine if new histone mutations, "K-to-M" or otherwise, are identified. One common feature of histone mutations is their ability to arrest cells in a primitive state refractory to differentiation induction. This is in line with the exquisite tissue and lineage specificity of these mutations and highlights the importance of modeling and studying oncohistones in the appropriate developmental context.

Despite rapid progress in elucidating the molecular mechanism of H3K27M, H3K36M, and H3G34R/V mutations, many questions remain. Although the effects of H3K27M and H3K36M mutations seemed to be independent of the H3 isoforms in cell culture models, patient tumors carrying H3.1/2 "K-to-M" mutations show clear pathological and clinical distinctions from H3.3 "K-to-M" mutant tumors. Further research is warranted to resolve this discrepancy. In addition, more work is needed to understand the persistence of H3K27me3 enrichment at limited genomic loci in H3K27M-mutant gliomas and whether or how it may contribute to oncogenesis. This will likely require a renewed focus on changes to the chromatin landscape beyond genic regions to include intergenic loci. Why specific oncogenes and tumor suppressors co-occur with different oncohistones, such as H3K27M with specific oncogenic signaling pathways or H3G34R/V with ATRX mutations, is also unknown. At another level, the remarkable anatomical and age specificity of oncohistone-associated cancers begs for studies aimed at determining underlying cells-of-origin and developmental timing

issues. If our current knowledge of histone mutations is any indication, answering these questions and making further progress in developing new therapies for patients will require integrating our understanding of chromatin structure and biochemistry with developmental pathways and the hallmarks of cancer.

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