Advances in Experimental Medicine and Biology 1283

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Histone Mutations and Cancer

Springer

Advances in Experimental Medicine and Biology

Volume 1283

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2019 Impact Factor: 2.450 5 Year Impact Factor: 2.324

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ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISBN 978-981-15-8103-8 ISBN 978-981-15-8104-5 (eBook)
https://doi.org/10.1007/978-981-15-8104-5

Acknowledgement

Thanks to the fantastic group of authors who have generously given their time, energy, and support to share their passion and knowledge in this book. Without their help, this book could not have been born. We would like to extend our sincere gratitude and appreciation for all of the support and contributions provided by them.

Contents

Overview of Histone Modification

1

Yanjun Zhang, Zhongxing Sun, Junqi Jia, Tianjiao Du, Nachuan Zhang, Yin Tang, Yuan Fang, and Dong Fang

Abstract

Epigenetics is the epi-information beyond the DNA sequence that can be inherited from parents to offspring. From years of studies, people have found that histone modifications, DNA methylation, and RNA-based mechanism are the main means of epigenetic control. In this chapter, we will focus on the general introductions of epigenetics, which is important in the regulation of chromatin structure and gene expression. With the development and expansion of high-throughput sequencing, various mutations of epigenetic regulators have been identified and proven to be the drivers of tumorigenesis. Epigenetic alterations are used to diagnose individual patients more accurately and specifically. Several drugs, which are targeting epigenetic changes, have been developed to treat patients regarding the awareness of precision medicine. Emerging researches are connecting the epigenetics and cancers together in the molecular mechanism exploration and the development of druggable targets.

Keywords

Histone · Epigenetics · Methylation · Phosphorylation · Acetylation · Histone mutation · Cancer

Epigenetics refers to the study of heritable information other than individual DNA sequence. Since the discovery of the DNA sequence as the genetic code in biology, people have found that other heritable information could also be passed to offspring. Within cells, there are three main systems that record epigenetics: histone modifications, DNA methylation, and RNA-based mechanism.

In eukaryotic cells, DNA is wrapped into chromatin in the nuclei. Nucleosome, the basic unit of chromatin, is comprised of 146–147 base pairs of DNA and a histone octamer with one H2A–H2B tetramer and two H3–H4 dimers [1]. The N- and C-terminal tails of these histone proteins can be post-translationally modified, such as acetylation, phosphorylation, methylation, SUMOylation, and ubiquitination [2]. These post-transcriptional modifications can change the electronic charge and structures of these histone tails, which bind to the DNA, to alter the chromatin status and subsequent gene expression [3]. To date, histone modifications have been found to play important roles in a variety of cellular processes.

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D. Fang, J. Han (eds.), *Histone Mutations and Cancer*, Advances in Experimental Medicine and Biology 1283, https://doi.org/10.1007/978-981-15-8104-5_1

1.1 Histone Methylation

Histone methylation, which usually occurs at the lysine (K) residues of histone H3 and H4 by adding methyl groups, is one of the most important post-transcriptional modifications [4]. This methylation is catalyzed by the histone methyltransferase (HMT), which uses *S*-adenosyl methionine (SAM) as the substrate to transfer methyl groups onto the lysine residues of histones [5]. The lysine residues of histones can be mono-, di-, and trimethylated (me1, me2, and me3, respectively) to act as the active or repressive marks of gene expression (Fig. 1.1). In general, H3K4, H3K36, and H3K79 are considered as active marks which occupy the actively transcribed gene regions in chromatin. H3K9, H3K27, and H4K20 are known as repressive marks that are usually associated with the silenced gene expression and condensed chromatin [6]. It is also worth noting that these histone marks are not exclusive to each other in the chromatin. There are bivalent or trivalent domains with two or three histone marks in the same genomic loci, respectively [7–9]. Until the first demethylase, lysine-specific demethylase 1 (LSD1), was found to erase the methylation at H3K4, histone methylation was once believed to be irreversible [10].

1.1.1 H3K4 Methylation

H3K4 methylations are highly enriched at enhancer regions, promotor regions, and transcription start sites (TSSs). H3K4me1 is enriched at the enhancer regions [11], where it is associated with H3K27ac or H3K27me3 to mark the active or repressive enhancers, respectively [12]. Unlike other H3K4 methylations which are enriched at intergenic regions, H3K4me2 marks the 5′ end of transcribed genes [13]. H3K4me3 shows canonical distributions at the promoters of actively transcribed genes and poised genes associated with differentiation [14, 15]. In oocytes, H3K4me3 is non-canonically arranged as broad domains at the super-enhancers which regulate oncogenes and tumor suppressors. These broad H3K4me3 domains can be inherited in preimplantation embryos and then be erased in the late two-cell embryos when canonical H3K4me3 peaks are established [16]. In yeast, Set1, which forms COMPASS (Complex Proteins Associated with Set1), is the only enzyme responsible for all H3K4 methylations [17, 18]. There are six Set1 homologies (SET1A, SET1B, MLL1, MLL2, MLL3, and MLL4) and other five H3K4 methyltransferases (SMYD1, SMYD2, SMYD3, SET7/9, and PRDM9) reported in human cells [19].

1.1.2 H3K9 Methylation

In eukaryotes, chromatin is organized as the euchromatin and heterochromatin [20]. The euchromatin is remained at the open status which will facilitate the access to transcription factors and active gene expression, while the heterochromatin is tightly packed to maintain gene silencing. H3K9 methylations, especially H3K9me2 and H3K9me3, are generally associated with gene repression and heterochromatin formation [21]. Besides the heterochromatin and silenced genes, H3K9me1 is also detected surrounding the TSSs associated with active genes [22]. In mammalian cells, H3K9me1 and H3K9me2 are present both in the cytoplasm and nucleus, while H3K9me3 is only detected in the nucleus [23]. Moreover, histones are expressed in the cytoplasm and then assembled into the chromatin by the help of histone chaperons. This distribution pattern of H3K9 methylation indicates that there is a stepwise methylation from H3K9me1/me2 to H3K9me3 during the nucleosome assembly process. Indeed, H3K9 is co-translationally mono- and dimethylated by SETDB1 when bound to the ribosomes [24]. Prdm3 and Prdm16, which contain a PR domain, can also direct the methylation of cytoplasmic H3K9me1 [25]. The cytoplasmic H3 with K9me1 is then assembled into chromatin and used as a substrate to reinforce H3K9me3 and heterochromatin. Moreover, G9a and GLP (G9a-Like Protein) can form heterodimers and

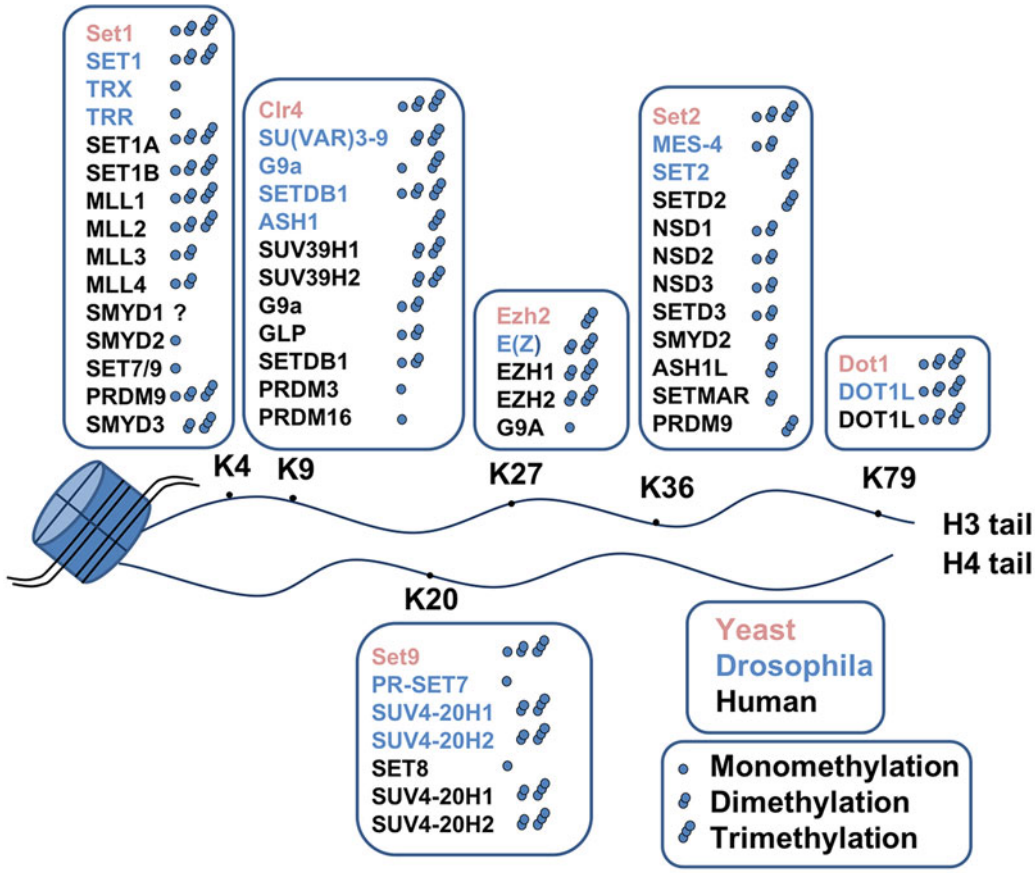

Fig. 1.1 The histone methyltransferases and their lysine targeting sites on histones. The enzymes from yeast are marked red, from *Drosophila melanogaster* are marked blue, from human are marked black

homodimers to catalyze the H3K9me1 and H3K9me2 in euchromatin [26]. H3K9me2 and H3K9me3 are catalyzed by SUV39H1 and SUV39H2 in heterochromatin, including pericentromeric regions [27]. HP1α and HP1β bind to H3K9me3 through the chromodomains and form multimers to link adjacent nucleosomes. SUV39H1/2 can be further recruited to methylated H3K9 by HP1α/β and themselves [21, 28]. These multiple interactions among HP1α/β, SUV39H1/2, and H3K9me3 ensure the spread of H3K9me3 at the heterochromatin regions. Moreover, HP1α/β can function as the scaffolds to further interact with other proteins involved in heterochromatin formation, such as chromatin remodelers, transcriptional repressors, and histone deacetylases [21, 28–30].

1.1.3 H3K27 Methylation

H3K27 methylation is usually considered as a hallmark of gene repression. H3K27me3 forms broad domains at promoters of silenced genes [31–34]. In addition, H3K27me3 is enriched at poised enhancers along with a low level of H3K4me1 in mouse and human embryonic stem cells (ESCs) [12, 35]. Through this enrichment at promoters and enhancers, H3K27me3 plays an important role in the repression of development-associated genes. In addition to the enrichment at poised enhancers, H3K27me2 is correlated with the promoters of both active and repressive genes [36]. Unlike H3K27me2 and H3K27me3, H3K27me1 is distributed at the actively transcribed gene promoters [36]. H3K27 methylation

is catalyzed by PRC2 complex, which contains four core subunits (Ezh2, Suz12, EED, and RbAP46/48), and methylates preferentially nucleosomal histone H3K27 [33]. While G9a is a histone methyltransferase for H3K9me1/me2, it also contributes to H3K27 monomethylation [37]. The mechanism of how PRC2 complex is recruited on chromatin is interesting. In *Drosophila* cells, the PRC2 complex recognizes specific DNA sequences, so-called Polycomb Response Elements (PREs), by the DNA binding protein in the complex [38]. These specific DNA sequences have not been found in human cells. In addition, there's no such DNA binding proteins in the core subunits of PRC2 complex [39, 40]. Several models including protein binding partners and non-coding RNAs have been proposed [41–44]. For example, the non-canonical subunit Jarid2 is found to target PRC2 to the strong H3K27me3 loci in mouse ESCs [45, 46]. Jarid2 belongs to Jumonji (Jmj) family, the majority of which functions as a demethylation enzyme. However, Jarid2, lacking the core demethylase domain, binds to the PRC2 complex and functions as a regulator for the enzymatic activity of PRC2 complex to enhance its enzymatic activity [47–50]. The presence of long non-coding RNAs (lncRNAs) can enhance the interaction between Jarid2 and PRC2 in vitro and stimulates the recruitment of PRC2 to chromatin in vivo [48, 51]. As a partner of PRC2, Jarid2 is highly expressed in mouse ESCs and plays a role in the process of ES cell differentiation [45, 46]. In ESCs, the repressive H3K27me3 mark and active H3K4me3 mark present at the same promoters of development genes. These bivalent promoters can preset the gene expression at a "poised" status to be rapidly activated and repressed, which ensures the quick response of gene transcription during development [7].

1.1.4 H3K36 Methylation

H3K36 methylation is commonly considered as an active mark which is enriched at intergenic and intragenic regions. In actively transcribed gene bodies, H3K36 methylations gradually shift from monomethylation to trimethylation through the promoter to the 3′ end [52]. In yeast, H3K36me3, which is enriched at the actively transcribed genes, recruits the histone deacetylase (HDAC) Rpd3S to the chromatin, resulting in the deacetylation of histones [53]. This histone deacetylation process serves as a "safeguard" to prevent the aberrant transcriptional initiation. In human cells, H3K36 methylation preserves the repressed chromatin status after gene transcription through the interplay of H3K9 methylation and transcriptional elongation, which is independent of the histone acetylation [54]. In addition, H3K36me3 recruits DNA methyltransferase 3A (DNMT3A) to methylate DNA, which is a redundant pathway to inhibit spurious transcription initiation [55]. H3K36me2 is enriched both at the gene bodies and intergenic regions. In the intergenic regions, H3K36me2 can also recruit DNMT3A to regulate the establishment and maintenance of DNA methylation. Moreover, inhibition of H3K36me2 redistributes DNMT3A from the intergenic regions to H3K36me3-enriched gene bodies, resulting in the reduction of DNA methylation at intergenic regions [56]. The functions of H3K36me2 at the gene body regions are still poorly understood. However, several reports have shown the correlations between the increased H3K36me2 and abnormal transcription [57]. Additionally, H3K36 methylation can inhibit the enzymatic activity of PRC2 complex, therefore, to prevent the PRC2-mediated methylation of H3K27 [58]. Other than the gene transcription [59, 60] and DNA methylation [58, 61], the H3K36 methylation has also been reported to regulate RNA splicing [62–64], DNA repair [65], and m^6A RNA modification [66]. In yeast, Set2 is the only enzyme catalyzing H3K36 methylation [67]. There are nine H3K36 methyltransferases found in mammalian cells. While NSD1-3, SMYD2, SETMAR, ASH1L, and SETD3 direct the mono- and dimethylation of H3K36, only SETD2 and testis-specific PRDM9 can catalyze H3K36me3 [68].

1.1.5 H3K79 Methylation

H3K79 methylation is enriched at the coding regions and correlated with the active transcription. Unlike other histone marks presenting at the unstructured histone tails, H3K79 methylation is located at the globular domains of histone H3 [69]. Yeast Dot1 protein and its mammalian homolog DOT1L are responsible for all of these three forms of H3K79 methylation [70, 71]. Intriguingly, H3K79 methylation exhibits a trans-tail histone modification pattern with other histone marks like H2B ubiquitination and H4K16ac. Depletion of H2B ubiquitination in yeast abolishes H3K79me2 and H3K79me3 [72]. In vitro histone methyltransferase assay and structural analysis have shown that H2B ubiquitination is indispensable for the methyltransferase activity of DOT1L [73]. Dot1 requires the N-terminal tail of H4 for its enzymatic activity in vitro. Moreover, elevated H4K16ac induces the upregulation of H3K79 methylation in vivo [74]. Several lines of evidences have shown that H3K79 methylation functions in the disruption of telomeric silencing, DNA damage responses, and transcription elongation [71, 75, 76].

1.1.6 H4K20 Methylation

H4K20 methylation is a repressive hallmark of histone modification. H4K20me1 marks the coding regions of lowly transcribed genes and is accumulated in parental nucleosomes during cell division [77]. H4K20me1/me2 recruits ORC1 (Origin Recognition Complex Subunit 1) and LRWD1 (Leucine-Rich Repeats and WD Repeat Domain Containing 1) at the replication origin to regulate the DNA replication [78]. More importantly, the chromatin remodeler protein L3MBTL1 is directly recruited by H4K20 methylation to induce chromatin condensation [79]. H4K20 is monomethylated by SET8, followed by a stepwise methylation from H4K20me1 to H4K20me2/me3 through SUV4-20H1/H2 [32, 80]. In addition, several lines of evidences have implicated H4K20 methylation in genome stability, DNA damage responses, chromatin compaction, DNA replication, and nucleosome turnover [80–84].

1.2 Histone Acetylation

Histone acetylation is regulated by the balance between histone acetyltransferases (HATs) and histone deacetylases (HDACs). Acetylation can reduce the positive charge of the lysine residues, which will then inhibit the binding between histone tails and negatively charged DNA, leaving the underlying DNA exposed [85]. Therefore, histone acetylation is usually considered as an active histone mark [86, 87]. Moreover, the acetylation and methylation in the same lysine residues can act as antagonists to inhibit each other, leading to the crosstalk between different histone marks [88].

In humans, there are 18 HDACs belonging to four classes: the class I Rpd3-like proteins (HDAC1-3 and HDAC8), the class II Hda1-like proteins (HDAC4-7, HDAC9, and HDAC10), the class III Sir2-like proteins (SIRT1-7), and the class IV protein (HDAC11) [89]. The class I and class II HDACs, which catalyze the metal-dependent hydrolysis of acetylated histones, share a conserved group of amino acid residues at active sites [89, 90]. While the class I, II, and IV HDACs are zinc-dependent, the class III HDACs use NAD^+ to generate nicotinamide and metabolite 2′-*O*-acetyl-ADP-ribose during the process of deacetylation [89, 91]. Lots of HDAC inhibitors have been developed and characterized in tumors. They can cause cell differentiation and/or apoptosis, leading to the inhibition of tumor growth in animals [92, 93].

There are lots of lysine residues which can be acetylated in histones, including H3K4, H3K9, H3K27, H3K36, H3K79, H4K5, H4K12, H4K20, and so on. Hereafter, we will focus on the well-studied H3K27ac. In general, H3K27ac is localized at the promoters and enhancers of actively transcribed genes, where it co-exists with H3K4me3 [12, 35]. With the help of H3K27ac, enhancers which are marked by

H3K4me1 could be divided into three groups: active enhancers that are marked by H3K27ac and H3K4me1; poised enhancers, enriched with H3K27me3 and H3K4me1; primed enhancers, which are solely marked by H3K4me1 [12, 94, 95]. In addition, the H3K27ac can form large broad domains in the intergenic regions, which are so-called super-enhancers, to further promote gene expression [96]. Through the regulation of key regulatory genes, these super-enhancers participate in various cellular processes including stem cell differentiation and tumorigenesis [97].

The histone acetyltransferases p300 and cyclic AMP response element-binding protein (CBP) were firstly found by two independent groups in 1996 [98, 99]. CBP often acts in conjugation with p300 to form a CBP/p300 complex, which can further recruit other histone acetyltransferases like PCAF (p300/CBP-associated factor). Bi-allelic mutations of CBP and p300 have been observed in several cancers including colon cancer, breast cancer, and gastric cancer [100, 101]. Chromosomal translocation of CBP and p300 is also identified in acute myeloid leukemia to induce the aberrant cell growth and tumorigenesis. This oncogenic effect is further supported by a CBP inactive mouse model which shows the defects in hematopoietic differentiation [102]. Bromodomain and extraterminal domain (BET) proteins, including BRD1, BRD2, BRD3, BRD4, and BRDt, can recognize H3K27ac through their bromodomains [103, 104]. The BET proteins act as scaffolds to recruit other transcription factors and RNA polymerase II to regulate gene expression [105]. The overexpression and translocation mutations of BET proteins are identified in the tumorigenesis of cancers [104, 106]. Blocking the binding between BRD4 and chromatin by BET inhibitors, JQ1, results in anti-proliferation and differentiation in BRD4-dependent tumors [107]. H3K27ac and BRD4 co-occupy a small number of exceptionally large super-enhancers that are associated with prominent genes in multiple myeloma cells, including the oncogene MYC [108]. Treatment of multiple myeloma cells by JQ1 results in preferential loss of BRD4 on the super-enhancers and defects of transcriptional extension, thereby preferentially affecting genes associated with super-enhancers including MYC [108]. Although the small molecule BET inhibitors, including JQ1, have immediate therapeutic potentials for a variety of malignancies, they may still cause serious damages to normal cells proliferated in the MYC-dependent manner [109]. In addition, BET inhibitors may have deleterious effects on the underlying gene regulation of human learning and memory [104]. Therefore, after the discovery of preclinical activities of JQ1 in tumors, several other BET inhibitors have been developed and entered into the preclinical trials, with more and more BET inhibitors under development [110].

1.3 Histone Phosphorylation

Phosphorylation, one of the most common post-translational modifications, also occurs at serine and tyrosine residues of histone proteins [111]. Histone phosphorylation participates in a wide range of cellular processes, including gene expression, cell cycle regulation, DNA damage repair, and asymmetric cell division [112]. Two of the important methylation sites, H3K9 and H3K27, share the same subsequent serine residue that can be phosphorylated, forming a "KS" domain. This close position between these two modification sites leads to the hypothesis that H3 phosphorylation may alter, at least in part, the affinity of readers or writers of the contacted lysine residues [113]. This crosstalk between histone phosphorylation and methylation allows the spatiotemporal control of histone marks, leading to the complicated but precise regulation of chromatin structure [114]. In mammalian cells, the H3S10 phosphorylation starts at pericentromeric heterochromatin regions, maximal abundance in the metaphase, followed by a rapid decrease upon transition into anaphase [115]. H3S10ph, mediated by Aurora-B, dissociates HP1 from chromatin and prevents the formation of condensed heterochromatin [116]. Repo-man/PP1 is shown to dephosphorylate H3S10ph at anaphase, so that HP1 can interact with H3K9me3 during replication, maintaining the heterochromatin environment [117]. During

mitosis, H3S10ph drives the release of Swi6 to open up chromatin, resulting in the outer centromere transcription and, therefore, restoration of H3K9me2 and heterochromatin [118]. Moreover, the binding of PRC2 and H3K27me3 is abolished when the neighboring S28 is phosphorylated [119]. Dephosphorylations of H3S10 and H3S28 can also regulate the gene transcription by controlling the chromatin binding of HP1 and PRC2 [120].

H3T3ph, which is phosphorylated by the Haspin kinase, contributes to the accurate positioning of the Chromosomal Passenger Complex (CPC) in inner centromeres. In addition, Haspin kinase acts through H3T3ph to modulate the activation of Aurora-B at centromeres, which helps kinetochore-microtubule attachment and error correction during mitosis [121, 122]. In addition to the regulation of H3K9me2 [123], H3T11 is phosphorylated by the death-associated protein-like kinase (Dlk) to regulate kinetochore assembly during the prophase to early anaphase [124].

Stem cells can maintain the stemless and produce differentiated cells by asymmetric cell division, of which one daughter cell sustains the stem cell status and the other daughter cell goes through cell differentiation [125]. During the germline stem cells (GSCs) asymmetric division in male *Drosophila*, canonical histone H3 is selectively segregated into the two daughter cells, whereas old histones from the parent cell are deposited into the undifferentiated stem cell and the newly synthesized histones are enriched in the differentiating daughter cell [126]. H3T3ph acts as a transient landmark that distinguishes sister chromatids with identical genetic code but different epigenetic information [127]. Loss of the tight control of H3T3ph in GSCs randomizes the segregation of sister chromatids enriched with old or new histone H3, resulting in the GSCs development defects.

1.4 Chromatin Remodeler

Chromatin remodeling is the process of dynamic changes of chromatin structure. The high-order chromatin structures are uneven, whereas some regions are loosened and some regions are condensed. In the loosened regions of chromatin, the DNAs are at an "open" status and could be accessed by other proteins which control the gene expression, including transcription factors and RNA polymerases [128]. It's hard for transcription-associated proteins to retrieve the condensed regions that are at a "close" status. Therefore, the gene expression of these regions will be repressed. Chromatin remodeling alters the "close" and "open" status of chromatin as a "gate" to regulate the gene expression and cellular processes.

Chromatin remodeling is carried out by chromatin remodeling complexes (Table 1.1) [129]. The ATP-dependent chromatin remodeling complexes use the energy of ATP hydrolysis to disrupt the binding between DNA and histones, leading to the changes of nucleosome position [130]. These remodeling complexes can be divided into four families, SWI/SNF, ISWI, CHD, and INO80, depending on the sequence and structure of ATPases in the complexes (Fig. 1.2).

SWI/SNF, which exists in both eukaryotes and prokaryotes, is the earliest ATP-dependent chromatin remodeler found in yeast [131]. The core subunit of SWI/SNF is swi2/snf2 which possesses a DNA-stimulated ATPase activity and destabilizes histone-DNA interactions in reconstituted nucleosomes [132]. Moreover, the ATPases of SWI/SNF family contain a bromodomain which can recognize and bind to the acetylated histones [133].

ISWI, firstly found in *Drosophila*, presents as distinct variants in different species. The ISWI family proteins alter nucleosome positioning to facilitate chromatin condensation [134]. In addition, ISWI activates gene expression by directly facilitating transcription-regulator-mediated transcription [135]. The ATPase of ISWI family has a HAND-SANT-SLIDE domain in its C-terminal region which recognizes and binds to the lysine of histone H3 [136]. In vivo, ISWI is associated with DNA repair [137] and cell fate determination [138].

The ATPase of CHD complex contains a chromodomain [139, 140]. The CHD family is

Table 1.1 The name and number of subunits and the catalytic subunits of some representative remodelers from four families in yeast, *Drosophila melanogaster*, and human

Family	Remodeling complex	Number of subunits	Catalytic subunits	Species
SWI/SNF	SWI/SNF RSC	12 17	Swi2/snf2 Sth1	Yeast
	BAP PBAP	11 12	Brm/Brahma Brm/Brahma	*Drosophila*
	BAF PBAF	10 12	hBrm/BrgI BrgI	Human
ISWI	ISWI ISWIb ISWI2	2 3 2	ISWI1 ISWI1/ISWI2 ISWI2	Yeast
	NURF ACF CHRAC	4 2 4	ISWI ISWI ISWI	*Drosophila*
	ACF CHRAC NoRC RSF WICH NURF	2 4 2 2 2 3	Snf2H Snf2H Snf2H Snf2H Snf2H Snf2L	Human
CHD	CHD1	1	Chd1	Yeast
	CHD1 CHD2 NuRD	1 1 6	Chd2 Chd2 Mi-2	*Drosophila*
	CHD1 CHD2 NuRD	1 1 7	Chd1 Chd2 Chd3/Chd4 (Mi-2α/Mi-2β)	Human
INO80	*y*INO80 SWR1	15 16	Ino80 Swr1	Yeast
	*d*INO80 Tip60	7 16	Ino80 Domino	*Drosophila*
	INO80 SRCAP TRRAP/Tip60	15 9 16	hIno80 SRCAP P400	Human

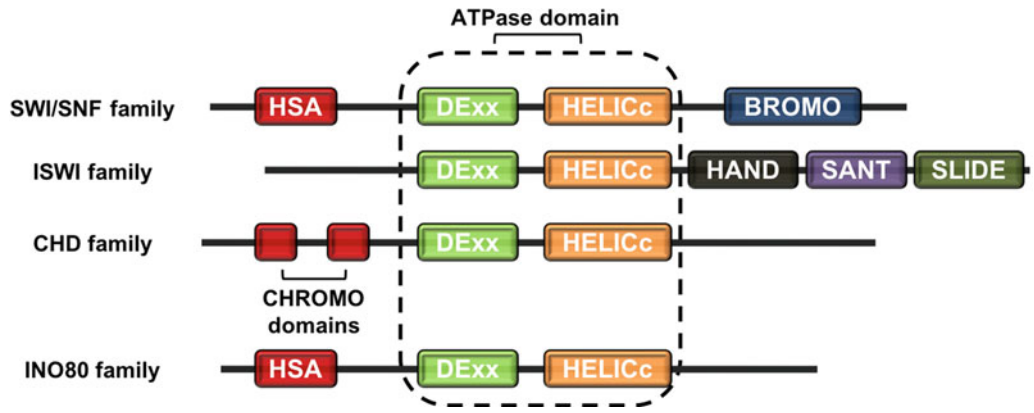

Fig. 1.2 The scheme showing the domains of ATPases in four different groups of ATPase-dependent chromatin remodelers

then divided into three subfamilies depending on the identities of their subunits. Chd1 and Chd2, which belong to the first subfamily, contain a DNA binding domain preferentially binding to AT-rich regions [141]. The second subfamily, including Chd3 and Chd4, has a pair of zinc-finger-like domains in their N-terminal regions instead of C-terminal regions [142]. The last subfamily is distinguished by the additional motifs like SANT domain or BRK domain in the C-terminal region [143].

INO80 is enriched at the replication fork and DNA damage sites, including holiday junctions and H2A variants (H2A.X and H2A.Z) [144, 145]. SWR1 complex, one of the INO80 complexes, catalyzes the replacement of the canonical H2A-H2B with the H2AZ-H2B variants in vivo [146]. Ino80 and Swr1 complexes, two remodelers of INO80 family in yeast, function antagonistically during DNA double strand breaks, promoting or blocking cell cycle check point adaption through regulating the incorporation of histone H2A variants, respectively [147].

It has been reported that chromatin remodeling complex functions like DNA translocase to induce the sliding of the nucleosomes on DNA strand. Remodelers bind to the histones or DNA sequence, and then the ATPases in the complex will be anchored to disrupt the interactions between histone octamer and DNA, resulting in DNA "bulge" or DNA "loop" for the sliding of histone octamers [148]. For example, ISWI2 can generate a small DNA bulge with about 10 bps between two sites, of which one is at the subunit SHL2 and the other is at the entry/exit of extranucleosomal DNA [149]. In addition, the canonical histones can be replaced by histone variants which are differently post-translational modified from canonical histones, leading to an alternation of the chromatin structure. For example, the Swr1 can stimulate the exchange between H2A.Z and canonical H2A in yeast [150]. Ino80 has an opposite function of Swr1 by removing the H2A.Z out of nucleosome in yeast [151]. Moreover, human CHD1 is involved in the deposition of histone H3 variant H3.3 at actively transcribed genes [152].

Chromatin remodelers function in a lot of cellular processes including transcriptional regulation, chromatin assembly, and DNA damage repair [153]. Mutations of chromatin remodelers are found to be the drivers in a variety of diseases, especially cancers [154, 155]. Many BAF proteins can act as cancer inhibitors, of which the loss or decreased expressions are reported to induce the dysregulation of gene expression as well as tumorigenesis [156]. SWI/SNF, which is believed to be a central tumor-suppressive complex, is one of the hot spot mutations found in pancreatic cancers [157]. In addition, SNF5 is a cancer-related remodeler of which the mutations cause chromosomal instability to induce the tumorigenesis [158].

1.5 Non-coding RNA

In addition to the direct control by proteins, histone modifications are regulated by non-coding RNAs. Small non-coding PIWI-interacting RNA (piRNA), mainly found in the mammalian germ cells and stem cells, is identified after siRNA and miRNA, with a length mostly around 29–30 nucleotides [159, 160]. One well-known function of piRNA is that it recognizes and silences the transposon stable elements in germ cells. The piRNA binds to PIWI protein to form a complex that targets the transcriptional site of a transposon by recognizing its primary transcript and then recruits histone H3K9 methyltransferase to induce H3K9me3 at this locus, leading to the chromatin condensation and gene repression [161]. In addition, long non-coding RNA (lncRNA), which is longer than 200 base pairs, mediates the histone modifications to regulate chromatin status [162, 163]. For example, in patients with capillary dilated ataxia syndrome, the lncRNA JADE promotes transcriptional activation of Jade1, a key component of histone acetylation complex HBO1, and subsequently induces histone H4 acetylation [164]. The lncRNA ANRIL, transcribed from the antisense INK4 locus, recruits PRC1 and PRC2 complexes which induce histone methylation and chromatin condensation, to reduce cellular senescence by

cis-inhibiting the expression of INK4 [165, 166]. In lung cancer, the lncRNA HOTAIR, which acts as an oncogene, recruits PRC2 complex to promote the H3K27me3 at tumor suppressor HOXD, resulting in the repression of HOXD [167]. The 3′ end of HOTAIR binds to the LSDl/CoREST/REST complex to remove active chromatin histone marks and enhance the expressions of targeted genes [168]. Moreover, PRC2 complex, which lacks the DNA binding domain, is recruited to the chromatin through the binding between HOTAIR and PRC2 subunit Jarid2 [51]. The lncRNA HOTTIP is the antisense RNA of the distal transcription of HOXA gene. HOTTIP recruits the KMT2A/MLL complex to promote the H3K4me3 in the HOXA gene cluster, which will subsequently elevate the expression of heteronomous genes by looping DNA to other sites far away from the HOXA gene cluster [169].

Since epigenetics plays important roles in the DNA replication, DNA damage response, transcriptional regulation, and cell cycle progression, it is not surprising that mutations of epigenetic regulators are found to induce the tumorigenesis in tumors.

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The Histone H3 Family and Its Deposition Pathways

2

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Abstract

Within the cell nucleus, the organization of the eukaryotic DNA into chromatin uses histones as components of its building block, the nucleosome. This chromatin organization contributes to the regulation of all DNA template-based reactions impacting genome function, stability, and plasticity. Histones and their variants endow chromatin with unique properties and show a distinct distribution into the genome that is regulated by dedicated deposition machineries. The histone variants have important roles during early development, cell differentiation, and chromosome segregation. Recent progress has also shed light on how mutations and transcriptional deregulation of these variants participate in tumorigenesis. In this chapter we introduce the organization of the genome in chromatin with a focus on the basic unit, the nucleosome, which contains histones as the major protein component. Then we review our current knowledge on the histone H3 family and its variants—in particular H3.3 and CenH3^{CENP-A}—focusing on their deposition pathways and their dedicated histone chaperones that are key players in histone dynamics.

Keywords

Chromatin · Nucleosome · Histone · Histone variant · Histone chaperone · Histone deposition

Abbreviations

ChIP-seq	Chromatin immunoprecipitation sequencing
DSC	DNA synthesis coupled
DSI	DNA synthesis independent
ES	Embryonic stem
KO	Knockout
NCP	Nucleosome core particle
PTM	Posttranslational modification

2.1 Introduction

2.1.1 Chromatin

The term chromatin (from the Greek *chrôma*, "color") emerged in the 1880s when Flemming found a structure in the cell nucleus that strongly absorbed basophilic dyes [1]. Chromatin is a complex nucleoprotein structure comprising mainly DNA (deoxyribonucleic acid) and basic

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D. Fang, J. Han (eds.), *Histone Mutations and Cancer*, Advances in Experimental Medicine and Biology 1283, https://doi.org/10.1007/978-981-15-8104-5_2

proteins (histones). DNA is the heritable genetic material (genome) which consists of about three billion base pairs distributed into 46 chromosomes per cell in humans. This material representing about 2 m of DNA is confined in each cell in a nuclear compartment of few micrometers in diameter. Thus, chromatin organization ensures to compact DNA from the basic unit, the nucleosome, up to a higher level of architecture. Chambon and Kornberg discovered in the 1970s the nucleosome as a repeating unit for the organization of chromatin [2, 3]. This nucleosome comprises about 147 bp (base pairs) of DNA wrapped around a core histone octamer flanked by 20–90 bp of a linker DNA associated with the linker histone H1. The complex 147 bp DNA-core histone octamer (without the linker DNA and H1) constitutes the nucleosome core particle (NCP). The core histone octamer consists of two copies of each core histone H3, H4, H2A, and H2B organized into an $(H3–H4)_2$ tetramer flanked by two (H2A-H2B) dimers. The resolution of the crystal structure of the nucleosome core particle at 2.8 Å in 1997 revealed how histones interact with each other and with DNA and how their amino-terminal tails protrude out of the particle [4]. The nucleosome array forms a 10 nm (nanometer) diameter fiber that resembles "beads on a string" [5]. In the cell, this fiber undergoes different levels of compaction to form the higher-order chromatin structure. In 1928, Heitz observed in the cell nucleus two different types of regions with a light microscope, discrete highly condensed regions, and dispersed lightly packed regions that constitute two types of chromatin, heterochromatin and euchromatin, respectively [6]. Constitutive heterochromatin mainly consists of repetitive DNA sequences that do not contain genes such as telomeres, centromeres, and pericentromeres, whereas euchromatin is mainly comprised of the coding part of the genome harboring genes. Recent progress with chromatin capture technologies has further revealed levels of chromatin organization with interacting chromatin loops and topologically associating domains (TADs) which serve as functional platforms for physical interactions between regulatory elements [7, 8].

Beyond DNA compaction, chromatin organization influences all nuclear functions. Indeed, chromatin is the substrate for the different processes operating on DNA such as replication, transcription, and repair. Thus a proper control of the dynamics of this organization ensures accurate genome function [9–11]. This control is exerted at all levels from the DNA and histones within the nucleosome particle up to the higher-order chromatin architecture in the cell nucleus [12].

2.1.2 Histones

Histones are small basic proteins that are among the most conserved in eukaryotes [13]. The core histones from the H2A, H2B, H3, and H4 families range in size from 11 kDa to 15 kDa, while the linker histones from the H1 family are around 21 kDa. A structurally conserved motif called histone-fold domain is present in all core histones. It consists of three α-helices (α1, α2, and α3) connected by short loops L1 and L2 that mediate heterodimeric interactions between the core histones [14]. The unstructured N-terminal extremity that extends at the surface of the nucleosome is the main region of the histone which is subjected to posttranslational modifications (PTMs) with important consequences on chromatin functions [15]. Numerous PTMs include acetylation, phosphorylation, methylation, ubiquitylation, crotonylation, and the latest described serotonylation [16]. As schematic examples, tri-methylation of the lysine 9 of histone H3 (H3K9me3) mainly associates with silenced regions in the genome, while tri-methylation of the lysine 4 of histone H3 (H3K4me3) generally correlates with transcriptionally active regions. These histone PTMs do generally occur in various combinations that gave rise to the hypothesis of a histone code where these modifications would work sequentially and/or together [17, 18]. They can either modulate the physical properties of the nucleosome and/or regulate the binding of protein partners that recognize specific modifications or RNA and possibly alter higher-

order structure. The histone PTMs are most often reversible providing a system to react to external stimuli for a short-term response, as part of a signaling module. They can also be maintained over cellular divisions and thereby function as a memory module of "epigenetic" nature. Furthermore, as we will discuss, in addition to these PTMs, the choice of distinct histone variants to form a nucleosome particle offers not only various provision of histones but also another way to alter the nature of the NCP with impact on chromatin function.

2.1.3 Replicative Histones and Histone Variants

In most eukaryotes, two types of histones exist for each histone family, the replicative and the non-replicative histones, the latter commonly referred to as non-allelic histone variants [19]. The replicative histones exhibit a high peak of expression during S phase when the doubling of genomic content requires a massive provision of histones. The genes of replicative histones show a peculiar organization in clusters that comprise multiple copies of all core histones and the H1 histone linker [20, 21]. This unique genomic organization and regulation at multiple levels contribute to optimizing the coregulation which is essential for the need of a high peak of expression of replicative histones during S phase. These histone clusters lack introns, have relatively short UTRs, and produce transcripts that do not undergo polyadenylation and harbor a conserved 3′ stem-loop structure which is required for the regulation of mRNA stability [22]. The existence of non-allelic variants for H2A, H2B, and H3 in mammals was first uncovered in 1977 when resolving histones on polyacrylamide gel electrophoresis in presence of non-ionic detergents (triton acid urea gels) [23]. In contrast with replicative histones, the expression of histone variants does not increase during S phase, and each variant harbors a unique temporal expression. They are encoded by multi-exon genes located outside of histone clusters. Transcripts lack 3′ stem-loop and undergo conventional processing through splicing and polyadenylation like most other RNA polymerase II (Pol II) transcripts. The protein sequence of a histone variant is either extremely similar or divergent from its replicative counterparts. Importantly, not only the expression but also the deposition pathways for the replicative histones and the histone variants are distinct. The replicative histones are incorporated into chromatin in a DNA synthesis-coupled (DSC) manner. This occurs mainly at replication forks during S phase when vast amounts of newly synthesized histones are required to ensure chromatin restoration on the duplicated genome. In contrast, the histone variants are incorporated into chromatin in a DNA synthesis-independent (DSI) manner. Their mode of deposition onto DNA and their location in the genome are in general specific for each histone variant [24].

The deregulated expression of histone variants and histone chaperones in various cancers shed light on the importance to connect chromatin and genome stability [25, 26]. The discovery of H3 mutations in pediatric glioblastomas attracted attention on histone variants in cancer development [27, 28]. In particular, mutations of histone H3 at K27M and G34V/R known as "oncohistones" were put forward as major drivers in these glioblastomas [29–32]. Since then, a considerable number of histone mutations—higher than previously recognized—has been identified in human tumors [33]. An increased interest has thus arisen in exploring the histones of the H3 family in greater details along with their modes of incorporation into chromatin by their dedicated chaperones. Progress in this field is therefore of critical importance for understanding normal development and disease like cancer [25].

2.2 The Histone H3 Family

To date in humans, eight members constitute the histone H3 family, two replicative histones (H3.1 and H3.2) and six histone variants. Among these H3 variants, limited information exists for the testis-specific H3.4 (H3.1t) [34], the hominid-specific H3.5 [35], and the two primate-specific

H3.X and H3.Y [36]. Thus, we will focus on the H3.1 and H3.2 replicative histones and H3.3 and CenH3^{CENP-A} histone variants which have been explored in more details.

2.2.1 Replicative H3.1 and H3.2

The two replicative histones H3, H3.1 and H3.2, are encoded by several genes in clusters (Fig. 2.1). They differ by only one residue at position 96 (Fig. 2.2a). Most studies have focused on H3.1 as the representative of H3 replicative histone in mammals, yet H3.2 is the most common replicative histone in eukaryotes. H3.1 is present only in mammals in addition to H3.2. Despite their high similarity, in human, some functional specificity was suggested based on differences in both expression patterns and associated PTMs [37]. As expected for replicative histones, both H3.1 and H3.2 are incorporated into chromatin at replication forks during S phase in a DSC manner in order to duplicate chromatin of the replicated DNA [38–40]. Moreover, deposition of H3.1 outside of S phase occurs at sites of DNA synthesis as observed at UV damaged sites [41]. Indeed, the DNA repair process leads to histones eviction to allow access to the repair machinery and repaired coupled incorporation of H3.1 participates in the chromatin restoration [42]. This is accompanied by a recycling of pre-existing histones [43].

2.2.2 H3.3 Variant

The histone variant H3.3 is encoded by two single genes in mammals (Fig. 2.1). It is closely related to H3.1 and H3.2 with only five and four amino acid residue differences, respectively (Fig. 2.2a). One difference concerns the residue 31 in the N-terminal tail of the histones with a serine in H3.3 instead of an alanine in both H3.1 and H3.2. This serine 31 in H3.3 is phosphorylated during mitosis [44] and at transcribing regions in mouse-activated macrophages [45]; however the exact role of this modification remains elusive. The three other different residues AIG, located in the α2 helix of the histone fold (at positions 87, 89, and 90), are important for specific histone chaperone recognition and the choice of a deposition pathway. An evolutionary analysis suggested that H3.3 is the ancestral form of the replicative H3.1/2 and in budding yeast the unique non-centromeric histone H3 is closely related to H3.3 [19]. However, recent work to "humanize" histones in budding yeast showed that adaptation to H3.1 proved easier in yeast compared to H3.3 in the context of a fully humanized nucleosome [46]. In mammals, two paralogous genes, H3.3A and H3.3B, encode the same H3.3 protein but have different codons (could impact the folding) and distinct untranslated regions (could impact transcription regulation) (Fig. 2.1). This suggests that a distinct transcriptional and posttranscriptional regulation of these two genes could provide different patterns of expression among tissues and during development [47–49]. The H3.3 variant present throughout the cell cycle was first described for its high level of incorporation at active rDNA arrays independently of replication in *Drosophila* [38]. H3.3 is deposited onto DNA in a DSI manner during interphase (G1, S, and G2 phases). H3.3 is the histone H3 predominantly present in chromatin of cells that are not dividing like quiescent or post-mitotic cells, due to its capacity to be incorporated in a pathway independent of DNA synthesis [50–52]. Moreover, upon fertilization and concomitantly with protamine removal, a major reprogramming involves H3.3 incorporation in paternal chromatin before the first round of DNA replication in both *Drosophila* and mouse [53–55]. The genome-wide distribution of H3.3 variant as observed by ChIP-seq analysis in mammalian cells shows a distinct pattern. Enriched in euchromatin at active genes, H3.3 presence is most often correlated with active transcription [56]. More precisely, H3.3 accumulates throughout the body of active genes but also at promoter regions at both active and inactive genes and at genic and intergenic regulatory regions in ES cells. In addition, a significant contribution of this variant is also revealed through enrichment in heterochromatin at both

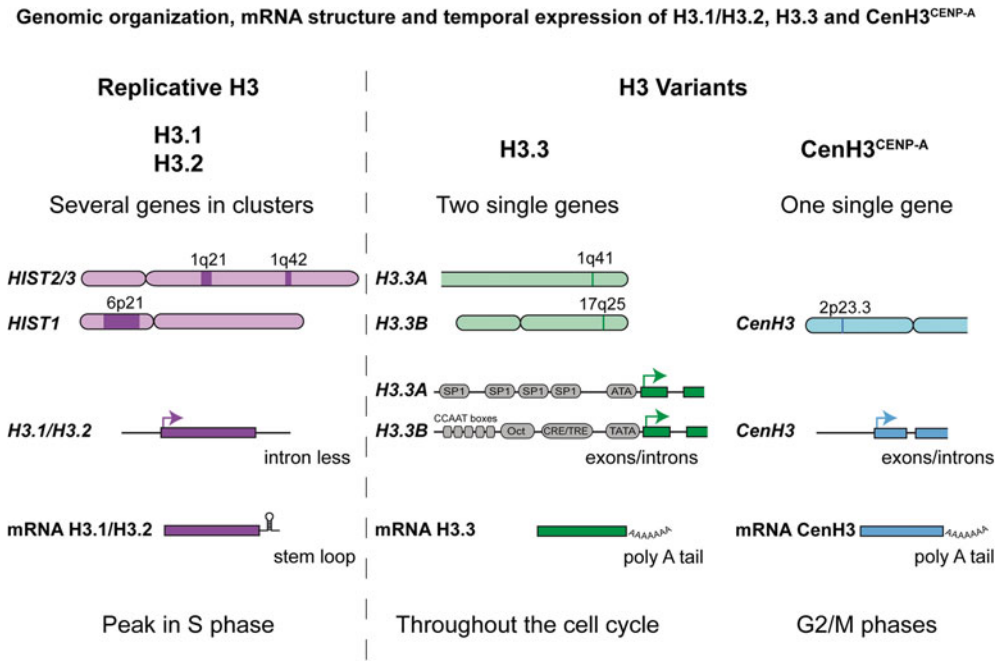

Fig. 2.1 Differences between replicative histones (H3.1 and H3.2) and histone variants (H3.3 and CenH3^{CENP-A}) in human. Replicative histone genes are organized in clusters and lack introns, whereas histone variants are encoded by single genes (two for H3.3 and one for CenH3^{CENP-A}) and have introns. While H3.3A and H3.3B genes encode the same protein, their architecture is different. In particular, their promoter regions contain distinct putative binding sites for transcriptional regulators. Transcripts of replicative histones do not undergo polyadenylation and harbor a 3′ stem-loop structure, while transcripts of histone variants are polyadenylated and processed through splicing like most RNA pol II mRNAs. Replicative histone genes are highly transcribed during S phase which is not the case of histone variant genes that can be transcribed with various timings depending of the variant (throughout the cell cycle for H3.3 and during G2/M phases for CenH3^{CENP-A})

telomere and pericentric heterochromatin in ES cells [56, 57]. Remarkably, the crystal structure of human nucleosome core particles containing H3.1, H3.2, or H3.3 revealed identical structures suggesting a common function in the organization at the level of individual particles (Fig. 2.2b) [58]. However, H3.3 containing nucleosomes in vivo appear more sensitive to salt-dependent disruption [59], arguing that the presence of this variant, the associated PTMs, or combination with other variants (such as H2AZ) may change the properties of the nucleosome towards a more open/active chromatin [60]. Importantly, morpholino experiments revealed a critical role for H3.3 during early development in *X. laevis* [61]. In mice, a double KO of the two genes (H3.3A and H3.3B) results in impaired development and embryonic lethality, no double-KO embryo surviving after stage E8.5 [48]. These defects are proposed to result from heterochromatin structures dysfunction at telomeres and centromeres leading to mitotic defects [48]. How this is entailed remains to be deciphered since it is unclear to which extent these phenotypes arise from the provision of the variant, the deposition mode, or the final organization involving the variant or a combination.

2.2.3 CenH3^{CENP-A} Variant

In 1985, Earnshaw and Rothfield identified CenH3^{CENP-A} as one of the proteins detected by autoantibodies from patients with CREST (calcinosis, Reynaud syndrome, esophageal dysmotility, sclerodactyly, telangiectasia)

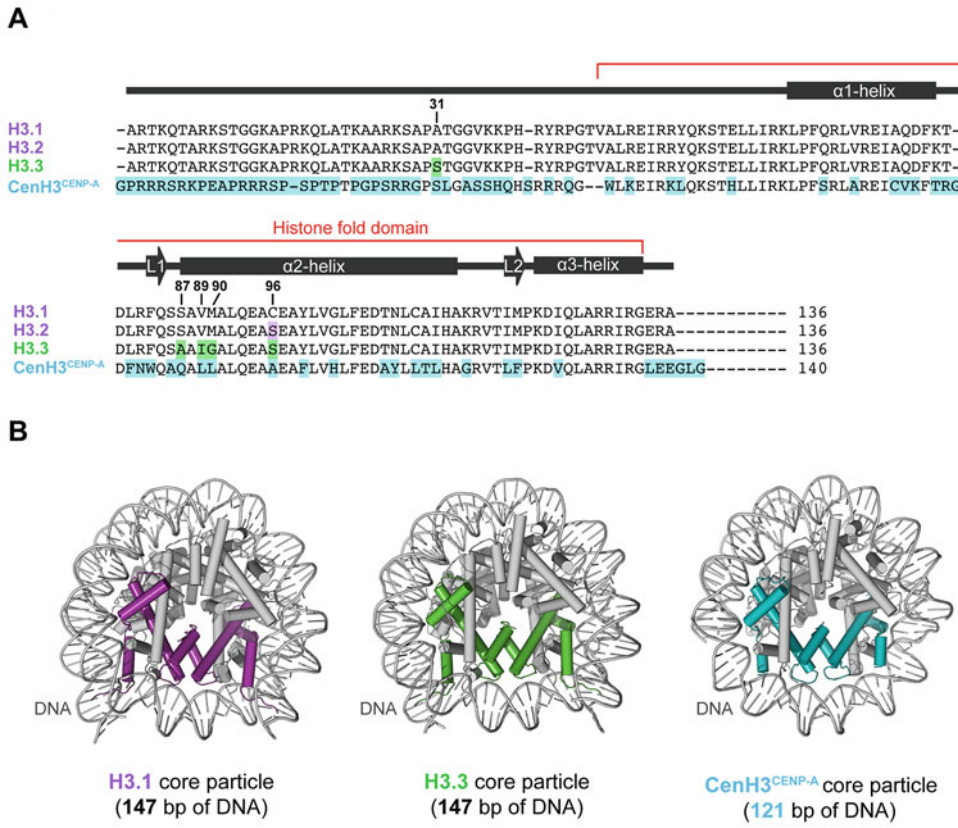

Fig. 2.2 (**a**) Alignment of human amino acid sequences corresponding to the replicative histones H3.1 and H3.2 and the histone variants H3.3 and CenH3^{CENP-A}. Sequences are compared to H3.1, and the residue differences are highlighted. H3.1 and H3.2 differ by only one residue at position 96. H3.3 differs from H3.1 by five residues (at positions 31, 87, 89, 90, and 96) and from H3.2 by four residues (at positions 31, 87, 89, and 90), while the amino acid sequence of CenH3^{CENP-A} exhibits less than 50% identity with H3.1. The histone-fold domain containing three α-helices and two loops is shown. (**b**) Crystal structures of H3.1, H3.3, and CenH3^{CENP-A} nucleosome core particles (NCP). The NCP contains a histone octamer that consists of a tetramer with two H3-H4 dimers ($(H3-H4)_2$) flanked by two H2A-H2B dimers. Histone octamer from both H3.1 and H3.3 NCPs is wrapped by 147 bp of DNA [58], whereas 121 bp of DNA wrapped the histone octamer from CenH3^{CENP-A} NCP [66]

[62]. Then, in 1991 Palmer et al. demonstrated that CenH3^{CENP-A} is a distinctive histone, with some sequence similarity to H3 [63]. This distant histone H3 variant was also called "deviant" given the fact that many segments are not related to H3 [64]. The histone variant CenH3^{CENP-A} is encoded by a single gene (Fig. 2.1) and exhibits less than 50% of amino acid sequence identity with its replicative counterparts H3.1/2 and is highly divergent in various species (Fig. 2.2a). This low level of conservation is in line with a rapid evolution of centromere organization and its components [65]. While H3.1 and H3.3 nucleosomal structures are almost identical, the more compact CenH3^{CENP-A} nucleosome only wraps 121 bp of DNA, and this may impact further the higher-order chromatin organization in these regions (Fig. 2.2b) [66–68]. In human, expression of new CenH3^{CENP-A} occurs in G2/M phases. Its incorporation into chromatin is restricted to late mitosis (telophase)/early G1 by a DSI pathway, leading to centromeric CenH3^{CENP-A} dilution during replication [69]. The deposition of CenH3^{CENP-A} specifically

at centromere (in the centric region) plays a crucial role in chromosome segregation by enabling kinetochore formation in mitosis [70, 71]. This critical need is illustrated in KO mice which are not able to develop beyond the stage E8.5 [72]. The embryos accumulate mitotic problems, further arguing for a major function of this variant in chromosome segregation.

2.3 Histone H3-H4 Chaperones

2.3.1 Histone Chaperone Definition

In the NCP, the basic charge of the histones is neutralized by the phosphate backbone of the DNA. Before incorporation into chromatin or after eviction, free histone in solution could potentially, due to their charge, engage in promiscuous interactions with any acidic partner and even could form aggregates in the cell. This is prevented by dedicated proteins, named histone chaperones which escort non-nucleosomal histones in the cell throughout all their cellular life [73]. Some of them directly buffer the positive charge. Nucleoplasmin, the most prominent protein in *X. laevis* oocyte, thanks to its properties to promote chromatin assembly, was the first protein named "histone chaperone" in 1977 by Laskey [74]. The current definition of a histone chaperone is "a protein that associates with histones and is involved in their transfer but is not necessarily part of the final product" [75]. This definition fully illustrates the general property of a histone chaperone and underlines the fact that in vivo, histones are never left alone from their synthesis to their delivery into or eviction from chromatin. All processes involving histone transfer or modification will thus involve at least one histone chaperone. They have a wide range of functions including histone transport, buffering, storage, histone modification, recycling and deposition onto DNA, as well as nucleosome remodeling. In vitro, all histone chaperones share the fundamental ability to promote a progressive transfer of purified histones onto naked DNA at physiological ionic strength to reconstitute nucleosomes from purified components [76]. Interestingly, no single feature in terms of sequence allows to demarcate a protein as a histone chaperone and some proteins turned out to function as histone chaperones after having been first characterized for other functions. Among histone chaperones, we can consider a first category according to the affinity for either H2A-H2B or H3-H4. Then, within these categories, a further distinction depends on the selectivity for replicative histones and/or for one or several particular histone variants. Here, we focus on H3-H4 chaperones with an emphasis on those involved in histone deposition using newly synthesized histones (new/de novo deposition) or old histones (recycling) (Table 2.1) (for reviews on histone chaperones [24, 77, 78]).

2.3.2 Dedicated H3-H4 Chaperones

2.3.2.1 H3.1/2-H4 Chaperone

The chromatin assembly factor 1 (CAF-1) is the unique histone chaperone complex that interacts selectively with the replicative variants H3.1/2 (Fig. 2.3). The CAF-1 complex was identified in 1989 on the basis of its ability to promote specifically nucleosome assembly in vitro onto newly synthesized DNA during replication with cytosolic extracts derived from human cells [79]. It consists of three distinct subunits p150/CHAF1A, p60/CHAF1B, and p48/RbAp48/RBBP4 also referred to as "large," "mid," and "small" subunits. They are functionally conserved in *S. cerevisiae* as CAC1, CAC2, and CAC3, respectively. The "large" subunit, p150, provides a scaffold for the other CAF-1 subunits and mediates the recruitment of the complex and interaction with other nuclear factors. In particular, its N-terminal portion contains two important regions: a stretch that is enriched in K/E/R amino acids (KER) predicted to bind DNA and a proliferating cell nuclear antigen (PCNA) interacting peptide (PIP-box) motif [80, 81]. An oscillation between monomeric and homo-oligomeric forms of p150 participates in the regulation of the functional activity of CAF-1 [82, 83]. In addition to histone binding, p150 interacts with heterochromatin protein 1 (HP1),

Table 2.1 Histone H3-H4 chaperones

Chaperone	Human subunit(s)	*S. cerevisiae* subunit(s)	Histone preference(s) in human	Function(s)
CAF-1 complex	p150 (CHAF-1), p60 (CHAF-2), p48 (RbAp48)	cac1, cac2, cac3	H3.1/2-H4	New deposition DSC
HIRA complex	HIRA, CABIN1, UBN1 or UBN2	Hir1p and Hir2p, Hir3p, Hpc2p	H3.3-H4	New deposition DSI
DAXX-ATRX	DAXX-ATRX	NA	H3.3-H4	New deposition DSI
HJURP	HJURP	Scm3	$_{CenH3}$CENP-A	New deposition DSI, recycling?
ASF1	ASF1a or ASF1b	Asf1	H3.1/2-H4 H3.3-H4 CenH3^{CENP-A}?	Transit, buffer, handover, recycling
MCM2	MCM2	Mcm2	H3.1/2-H4 H3.3-H4 $_{CenH3}$CENP-A	Recycling
Polε	POLE3, POLE4	Dpb3, Dpb4	H3-H4	Recycling

NA not available, *DSC* DNA synthesis coupled, *DSI* DNA synthesis independent, *CAF-1* chromatin assembly factor 1, *HIRA* histone regulator A, *Hir* histone regulation, *Hpc2p* histone periodic control 2, *DAXX-ATRX* death domain-associated protein 6-α-thalassemia/mental retardation syndrome X-linked, *HJURP* Holliday junction recognition protein, *Scm3* suppressor of chromosome mis-segregation 3, *ASF1* anti-silencing function 1, *MCM2* mini chromosome maintenance 2, *Polε* DNA polymerase ε

an interaction of importance for the replication of pericentric heterochromatin [84, 85]. The "mid" subunit, p60, with a WD40 propeller fold involved in the binding of H3-H4 dimer, is responsible for histone loading. The "small" subunit, p48, provides less well-characterized accessory interactions and is part of several other chromatin-regulating complexes such as the corepressor mSin3A [86]. It can possibly serve as an interface or link between various complexes. The H3.1 complex purified from human cell extracts retrieved all three CAF-1 subunits required for the deposition of H3.1 onto DNA coupled with DNA synthesis [87] as found later with the H3.2 complex purification [40]. Thus, the deposition of both H3 replicative

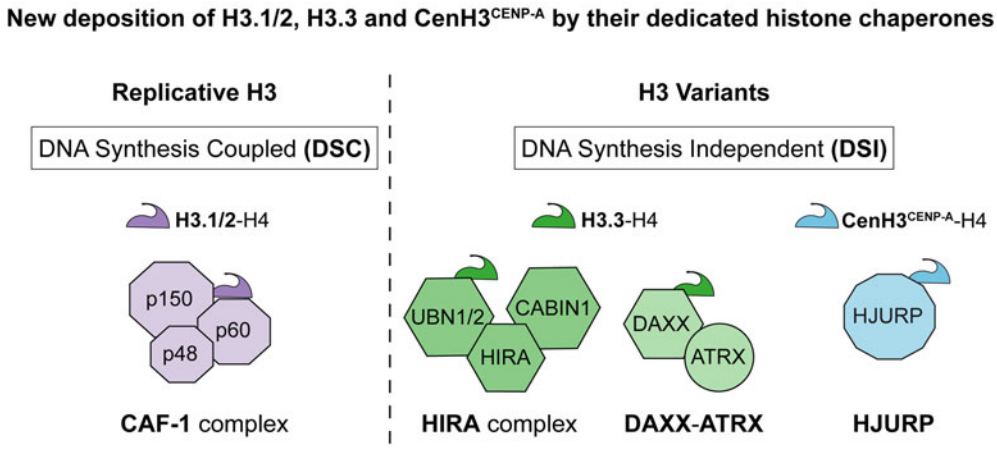

Fig. 2.3 Replicative histones (H3.1 and H3.2) and histone variants (H3.3 and CenH3^{CENP-A}) are de novo deposited onto DNA by their dedicated chaperones using two different nucleosome assembly pathways, DNA synthesis coupled (DSC) and DNA synthesis independent (DSI), respectively. H3.1/2-H4 and CenH3^{CENP-A}-H4 dimers bind to one histone chaperone, CAF-1 and HJURP, respectively, while H3.3-H4 can associate with two distinct histone chaperones, the HIRA complex and DAXX-ATRX. Of note, the homo-oligomerization status of each component is not indicated and only one molecule is represented

histones relies on CAF-1. The current model for histone deposition promoted by CAF-1 involves that the complex binds an H3-H4 dimer and that a transient association of two CAF-1-H3-H4 allows two histone chaperone complexes to concertedly deposit one $(H3\text{-}H4)_2$ tetramer onto DNA [88]. Loss of p150 CAF-1 in homozygous mutants leads to very early developmental arrest at the 16-cell stage in mice (between stages E2.0 and E3.0). These embryos show severe alterations in the organization of cell nuclei and their constitutive heterochromatin [89]. In ES cells, downregulation of CAF-1 can favor the emergence of cells showing properties of totipotent cells [90]. In somatic cells, induction to pluripotency IPS cells is facilitated when CAF-1 is reduced [91]. In T cells, CAF-1 cooperates with DNA methyltransferases and histone-modifying enzymes to maintain silent states of the Cd4 gene [92]. This is in line with the general view according to which CAF-1 can contribute to the maintenance of somatic cell identity by stabilizing chromatin patterns [11].

2.3.2.2 H3.3-H4 Chaperones

H3.3 variants present two selective H3.3 histone chaperones: the histone regulator A (HIRA) complex and death domain-associated protein 6-α-thalassaemia/mental retardation syndrome X-linked (DAXX-ATRX) (for review [93, 94]) (Fig. 2.3).

The HIRA gene was identified in 1995 within a region of chromosome 22q11.2 deleted in most patients with a developmental disorder, the DiGeorge syndrome [95]. The HIRA acronym comes from its amino acid sequence homology to the two *S. cerevisiae* proteins histone regulation 1 and 2 (Hir1p and Hir2p). Initially described as a chaperone involved in a DSI nucleosome assembly pathway using the *X. laevis* egg extract model system [96], the identification of HIRA in the purified H3.3 complex revealed its dedicated function in the deposition of H3.3 [87]. In addition to HIRA, two other proteins, ubinuclein 1 (UBN1) and calcineurin-binding protein 1 (CABIN1), co-purified with H3.3 and turned out later to be part of the HIRA histone chaperone complex [97, 98]. Both UBN1 and CABIN1 interact with HIRA which plays therefore a central platform role in the complex. UBN1, first identified as a nuclear protein interacting with cellular and viral transcription factors [99], is the subunit that directly interacts with the H3.3-H4 dimer [100]. X-ray crystallographic analysis revealed that the Hpc2-related domain (HRD) in UBN1 binds H3.3 in the proximity of the three residues AIG at positions 87-89-90 (in the α2 helix of the histone-fold domain) that are different between H3.1/2 and H3.3 [101]. The Gly90 in H3.3 mediates the specificity for binding to H3.3-H4 over H3.1-H4. CABIN1 was first described as a corepressor of the MEF2 family of transcription factors [102]. To date, its exact function within the HIRA complex remains unclear. Like HIRA, UBN1, and CABIN1 have *S. cerevisiae* counterparts, histone periodic control 2 (Hpc2p), and histone regulation 3 (Hir3p), respectively. They form with Hir1p and Hir2p, the Hir complex which is involved in the incorporation of H3 independently of DNA synthesis in yeast [103]. The HIRA subunit forms a homotrimer that interacts with two CABIN1 subunits [104]. This trimeric structure is required for the functional activity of the HIRA complex in depositing H3.3. Ubinuclein 2 (UBN2), which is a paralog of UBN1 that interacts with HIRA [97], forms with HIRA another complex which appears distinct from the one comprising UBN1 [105]. These two complexes could cooperatively deposit H3.3 onto cis-regulatory regions in mouse embryonic stem cells (mESCs). While yeast exhibits a single Hpc2p, understanding why other eukaryotes evolved with the emergence of two UBN paralogs will be interesting to explore. In a manner that compares with H3.3 KO, HIRA is required for proper development in vertebrates, possibly reflecting their tight functional connection. HIRA KO mice die by stage E10.0 or E11.0 as a consequence of abnormal gastrulation [106].

DAXX was originally described as a Fas death receptor-binding protein that induced apoptosis via JNK pathway activation [107]. ATRX was identified through the discovery of mutations in the corresponding gene in a form of X-linked mental retardation (ATRX syndrome) in young males [108, 109]. It is a member of the SNF2

family of chromatin remodeling factors [110]. Chromatin remodelers consist of a group of protein complexes containing an ATPase subunit that regulate a number of DNA transactions by sliding, removing, and reconstructing nucleosomes [111]. The discovery of DAXX and ATRX in complex with H3.3 suggested a role for these two proteins in the deposition of this variant [56, 57]. Although DAXX and ATRX along with HIRA associate with H3.3, they form distinct H3.3 complexes [112]. Interestingly, in contrast to HIRA, DAXX and ATRX have no known counterparts in budding yeast suggesting a more recent function for this complex possibly in metazoans. In the DAXX-ATRX-H3.3 complex, DAXX is the component that interacts directly with H3.3, while ATRX allows the targeting to heterochromatin [113]. The crystal structure of the histone-binding domain of DAXX bound to the H3.3-H4 dimer revealed the principal determinants of human H3.3 specificity with Ala87 and Gly90 in H3.3. DAXX prefers Gly90 in H3.3 over the hydrophobic Met90 in H3.1 [114, 115]. As mentioned above, the UBN1 subunit in the HIRA complex has nearly identical points of contact in the proximity of H3.3 G90 although the mechanism for H3.3 G90 recognition is likely distinct [101]. Of note, in human cells overexpressing CenH3^{CENP-A}, the strict selectivity of DAXX-ATRX for H3.3 is altered, and DAXX binds the centromeric CenH3^{CENP-A} leading to its mis-localization. This occurs at sites of active histone turnover and involves an unusual heterotypic tetramer containing CenH3 $^{CENP-A}$ -H4 with H3.3-H4 [67, 116–119]. In addition, DAXX can function independently of ATRX to repress endogenous retroviruses, in a process that does not involve H3.3 incorporation into chromatin [120]. Both losses of DAXX and ATRX are embryonic lethal in mice at stage E9.5 [121, 122]. ATRX KO cells exhibit loss of the H3K9me3 heterochromatin modification, loss of repression, and aberrant allelic expression arguing for a role of ATRX in the maintenance of silencing memory at imprinted loci [123].

2.3.2.3 CenH3^{CENP-A}-H4 Chaperone

The histone chaperone dedicated to the centromeric histone H3 variant CenH3^{CENP-A} is the Holliday junction recognition protein (HJURP) (for review [70]) (Fig. 2.3). Described in 2007 as a protein that binds Holliday junction, HJURP was initially involved in the homologous recombination (HR) pathway in the double-strand break (DSB) repair mechanism [124]. As for CAF-1, HIRA, and DAXX-ATRX, the biochemical purification of the protein complex associated with CenH3^{CENP-A} in human cells enabled to identify HJURP as a CenH3^{CENP-A} histone chaperone [125, 126]. The yeast suppressor of chromosome mis-segregation 3 (Scm3) stands as the HJURP counterpart in *S. Cerevisiae*. Despite their conserved function in CenH3 deposition, HJURP/Scm3 homologues exhibit high degrees of sequence divergence among species likely as a consequence of the rapid coevolution of the chaperone and the variant. The selectivity of HJURP for CenH3^{CENP-A} is mediated by the interaction of its CENP-A binding domain (CBD) in the N-terminal part of the protein with the CENP-A targeting domain (CATD) in CenH3^{CENP-A} (composed of the α2 helix and the loop L1) [127]. Structural analysis showed that the CBD of HJURP binds a CenH3^{CENP-A}-H4 dimer [128]. The homodimerization of HJURP, through its HJURP C-terminal domain 2 (HCTD2), is required for CenH3^{CENP-A} deposition, leading to the hypothesis that HJURP dimerization allows to bring two CenH3^{CENP-A} –H4 dimers to form the (CenH3^{CENP-A} –H4)$_2$ tetramer at centromeric DNA [129]. Of note, in addition to its role as chaperone of CenH3^{CENP-A}, HJURP also interacts and recruits CENP-C, another kinetochore component, at centromere [130].

2.3.3 Other H3-H4 Chaperones

Other H3-H4 chaperones, less selective, bind several H3 histones (both replicative and variants) and participate in the nucleosome assembly line. Upstream or downstream the new deposition process, they can be involved in handling soluble

new histones or in recycling nucleosomal histones or both.

The anti-silencing function 1 (ASF1) histone chaperone was initially identified in *S. cerevisiae* in a screen for silencing defects upon overexpression [131]. ASF1 was the first histone chaperone crystallized in complex with H3-H4 [132, 133]. Its domain interacting with histones contains an Ig-like fold that binds the α2-α3 helices of histone H3. Together with CAF-1, ASF1 facilitates chromatin assembly linked to DNA synthesis in vitro [134, 135]. However, ASF1 is not directly involved in the deposition mechanism but likely acts by transferring H3-H4 dimers to the downstream histone chaperones that are depositing the new histones H3-H4. In mammals, two paralogous proteins exist, ASF1a and ASF1b, with distinct cellular roles [136]. ASF1a and ASF1b co-purified with H3.1 and H3.3 complexes arguing for their role in both DSC and DSI assembly lines [87]. Although ASF1a and ASF1b do not exhibit preferences for H3.1/2 or H3.3 per se and can associate with both H3.1/2-H4 and H3.3-H4 dimers, ASF1a harbors a preference for the HIRA complex, whereas ASF1b interacts preferentially with CAF-1 [137]. ASF1a and ASF1b bind a motif named B domain which is present in both HIRA and CAF-1p60 subunits, but how the interaction preferences are achieved is not fully understood [138]. Importantly, ASF1 a and b interact with the B domain of CAF-1p60 or HIRA through a conserved hydrophobic groove at a site opposite to that of their interaction with H3–H4. A ternary complex (CAF-1–ASF1–H3.1– H4) or (HIRA-ASF1-H3.3-H4) could thus represent an intermediate that enables histones to be handed over from one chaperone to the next. ASF1a and b bind H3-H4 at the tetramerization interface and therefore sterically prevent their tetramerization [132, 133, 139]. Furthermore, these chaperones are able to disrupt an $(H3\text{-}H4)_2$ tetramer into two H3-H4 dimers but alone cannot disengage it from DNA. Notably, in addition to participating upstream in the new deposition, ASF1a and b are also involved in old/pre-existing/parental histones recycling during replication in association with mini chromosome maintenance 2 (MCM2) [140, 141].

MCM2 is a subunit of the Cdc45-MCM(2-7)-GINS (CMG) replicative helicase that unwinds DNA and separates the two strands of the double helix prior to the action of DNA polymerases [142]. Although its binding capacity to histones was discovered almost 20 years ago [143], its central role in handling both old and newly synthesized histones during replication was highlighted more recently [140]. Together with other subunits of the helicase (MCM3–7), MCM2 coimmunoprecipitates with H3-H4, enriched in parental histones, from nuclear extracts in S phase human cells. In contrast, only MCM2 co-immunoprecipitates with newly synthesized histones H3-H4 and with ASF1a and b in human cytosolic cell extracts. This suggests that independently from its role at the replication fork, MCM2 could also play a specific role as a histone chaperone. Biochemical studies revealed that the N-terminal tail of MCM2, containing the histone-binding domain (HBD), directly binds histone H3 in vitro [144]. Then, the structural analysis showed that MCM2 HBD can bind both an $(H3\text{-}H4)_2$ tetramer and a dimer of H3-H4 engaged in an interaction with the other chaperone ASF1 [145, 146]. The interaction of MCM2 with histones involving a tetramer-to-dimer transition would be important for the proper dynamics of histones during the passage of the replication fork. Moreover, the finding that MCM2 can bind all H3 (H3.1, H3.2, H3.3, and CENP-A) [145] suggests that this mechanism to handle histones could apply throughout the entire genome [147].

POLE3 and POLE4 are accessory subunits of the mammalian Polε, the polymerase that is active on the leading strand at the replication fork [148]. A recent study discovered that the human POLE3-POLE4 complex binds to histones H3-H4 (either H3.1 or H3.3) as dimer or tetramer [149]. POLE3-POLE4 binds H3-H4 in the context of chromatin during replication excluding the possibility that it chaperones soluble histones. Moreover, POLE3-POLE4 associates with histones carrying modifications characteristic of both newly synthesized and parental histones suggesting that POLE3-POLE4 may handle both new and old histones in the proximity of the

leading strand. Another recent study in *S. cerevisiae* showed that the yeast counterparts Dpb3 and Dpb4 drive the recycling of parental histones onto the leading strand, indicating that their function in histone dynamics at replication fork is likely conserved [150]. We will discuss later how the dynamics of recycling and deposition of histones, respectively, on the leading and lagging strand has combined all these features.

2.4 Deposition of H3-H4 Histones onto DNA

During most of the DNA processes such as replication, repair, or transcription, the nucleosome organization is disassembled then reassembled. The disassembly is generally required to permit access of the actors of the different machineries to DNA, and the reassembly is needed to maintain the chromatin organization to ensure genome integrity. During the reassembly, deposition of both new and old/pre-existing/parental histones occurs. Recycling of old histones contributes to preserve positional information and allows variants and PTMs transmission while new histones deposition could give rise to epigenome fluctuations. The mechanisms of new histone deposition involving histone chaperones have been explored over the last 25 years, while those involved in the deposition/recycling of old histones were under investigation more recently. Of note, the studies on histone dynamics in vivo benefited in particular from the SNAP-tag technology which allows to visualize selectively either newly synthesized or old histones in the cell [69, 151].

2.4.1 Deposition of New H3-H4 Histones

2.4.1.1 New H3.1/2-H4 Deposition by CAF-1

The histone chaperone CAF-1 deposits new replicative histones at the sites of DNA synthesis both during replication when DNA is duplicated and during repair when DNA damage is repaired [79, 152] (Fig. 2.4). Thus, CAF-1 deposits new H3.1/2 onto DNA in a DSC manner both coupled to replication during S phase and independently of replication at sites of DNA repair throughout interphase [41, 104]. CAF-1 is recruited to sites of DNA synthesis mainly by the interaction of its large subunit p150 with the DNA sliding clamp, PCNA [80, 81]. The phosphorylation of CAF-1 p150 by Cdc7/Dbf4 during S phase promotes this interaction by regulating the homo-oligomerization status of p150 [82]. The p60 subunit is also a substrate for phosphorylation by cdk in vitro, which may represent another mechanism by which CAF-1 deposition activity is regulated [153, 154]. The SNAP-technology enabled to follow the deposition of newly synthesized H3.1 in the cell. H3.1 new deposition colocalized with replication sites during S phase and CAF-1 depletion abrogated this new H3.1 deposition [39]. To date, no other histone chaperone proved able to deposit H3.1 in the absence of CAF-1. However, H3.3 deposition promoted by HIRA does occur at replication sites when CAF-1 is depleted [39]. These findings suggest that, when the assembly coupled to DNA synthesis is defective, the gaps left free could be filled up via a compensatory mechanism involving the DSI nucleosome assembly pathway in order to maintain chromatin integrity.

2.4.1.2 New H3.3-H4 Deposition by HIRA

The HIRA complex is involved in the DSI deposition of the new H3.3 histone variant (Fig. 2.4), and this new deposition occurs throughout interphase as visualized in the cell by using the SNAP-tag technology [39]. H3.3 is enriched in the body of transcribed genes, at promoter regions at both active and inactive genes, and also at genic and intergenic regulatory regions [56]. Thus, HIRA-dependent enrichment of H3.3 in the coding regions of genes appears mainly associated with active transcription. The deposition of H3.3 at transcribed genes was underscored by the co-immunoprecipitation of the HIRA complex with both the initiating and elongating forms of the RNA pol II harboring specific phosphorylation at serine 5 and serine 2 into its carboxy-

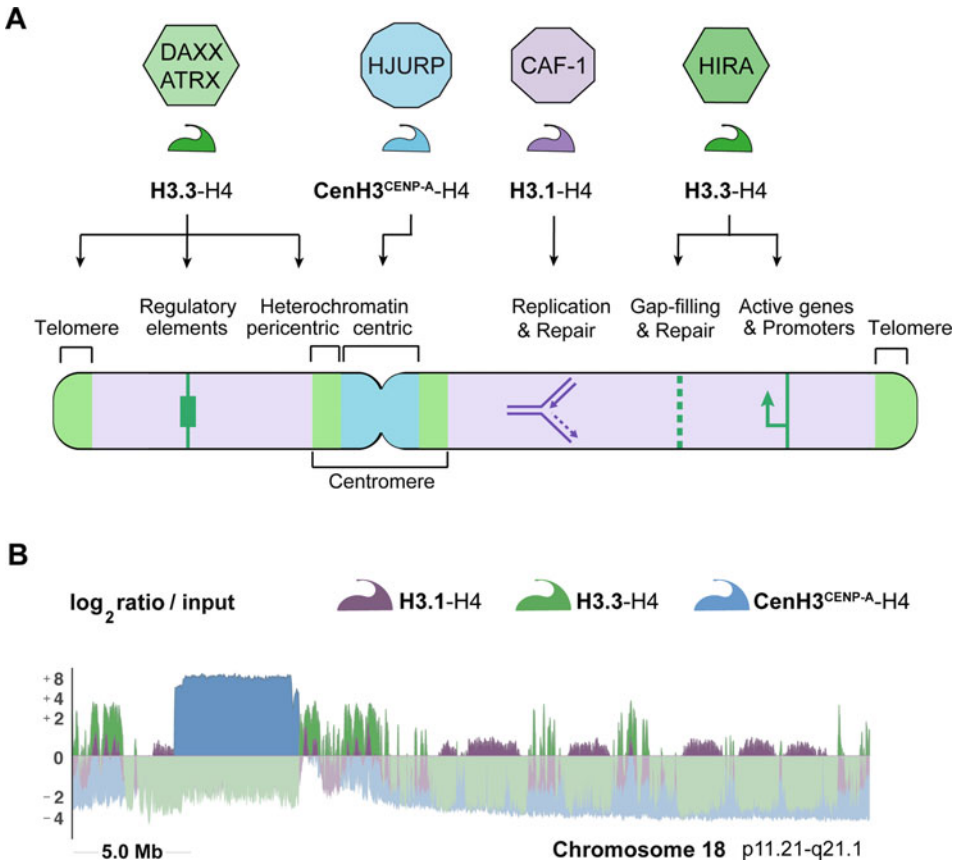

Fig. 2.4 (**a**) Enrichment of H3.1, H3.3, and CenH3^{CENP-A} mediated by their dedicated histone chaperones at specific genomic sites and/or during particular DNA processes in cycling cells. CAF-1 deposits replicative H3.1/2-H4 genome wide mainly during replication but also during DNA repair. The HIRA complex deposits H3.3-H4 at active genes, promoters, sites of DNA repair, and potentially at any transient nucleosome-free region by a gap-filling mechanism, while DAXX-ATRX is involved in the enrichment of H3.3-H4 mainly at heterochromatin (telomere and pericentromere) but also at regulatory elements. HJURP mediates the incorporation of CenH3^{CENP-A}-H4 at centromere (in centric heterochromatin). (**b**) Genomic distribution of H3.1, H3.3, and CenH3^{CENP-A} [20] from published ChIP-Seq data in HeLa cells [67, 141]. The plot shows the enrichment relative to input for all variants at a representative region spanning the centromere and the proximal short and long arms of chromosome 18 (p11.21-q21.1). Enriched regions are highlighted in darker colors, illustrating the partitioning of the genome into chromatin domains associated with specific histone H3

terminal domain (CTD), respectively [39]. The interactions between the HIRA complex and several actors of the transcriptional process further support the link between HIRA-dependent H3.3 deposition and transcription [155–157]. Furthermore, posttranslational modifications of the HIRA subunit can modulate the H3.3 deposition activity of the complex [158, 159].

The HIRA complex can also promote deposition of H3.3 independently of transcription in several circumstances, at UV damage sites where H3.3 deposition occurs upon detection of the DNA damage prior to repair [160], at fertilization in paternal chromatin before the first round of DNA replication [53, 54] and onto viral DNA upon virus infection. In this latter case, depending on the system, H3.3 accumulation onto viral DNA correlated with active or repress viral transcription and with virus latency [161–163].

The HIRA complex shows unique DNA binding properties as compared to other H3-H4 histone chaperones, and its depletion increases DNA

sensitivity to nucleases [39]. The HIRA complex from cell extracts binds to both double-stranded and single-stranded DNA suggesting that it could recognize particular DNA structures [104]. The ability of the HIRA complex to bind naked DNA provides a mechanism of new H3.3 deposition that may operate to avoid nucleosome-free DNA regions which could be deleterious for maintenance of chromatin organization and genome integrity. This leads to the proposal of a nucleosome gap-filling mechanism for the HIRA-dependent H3.3 deposition and a crucial role to maintain chromatin integrity [39, 164].

2.4.1.3 New H3.3-H4 Deposition by DAXX-ATRX

The histone chaperone DAXX-ATRX is responsible for the enrichment of H3.3 in heterochromatin at pericentric regions and telomeres [56, 57]. Although not formally demonstrated, DAXX-ATRX is assumed to be key for the deposition of new histone variant H3.3 at these heterochromatic regions in a DSI manner (Fig. 2.4). Whether the deposition of H3.3 by DAXX-ATRX at these specific locations occurs during a particular time window during the cell cycle and whether it does link to the deposition of H2AZ variant will be interesting to explore [165]. While DAXX directly interacts with H3.3 [114, 115], ATRX recognizes H3K9me3 through its ATRX-Dnmt3-Dnmt3L (ADD) domain and could therefore target DAXX to these locations [113]. DAXX-ATRX also mediates H3.3 deposition at G-quadruplexes (G4) and at endogenous retroviruses through a possible direct binding of ATRX to these structures and transposable elements [166, 167]. These data have led to propose a role for ATRX in suppressing recombination at telomeric repeats by resolving G4 structures through the deposition of H3.3 [168]. At transposable elements, the incorporation of H3.3 is proposed to silence repetitive elements through the regulation of H3K9me3 [167, 169].

DAXX-ATRX also mediates H3.3 enrichment outside of heterochromatin and repetitive elements. DAXX-ATRX together with the HIRA complex was proposed to induce virus latency by enabling H3.3 accumulation onto viral DNA [161]. In the nervous system, the serine residue 669 of DAXX is phosphorylated by the homeodomain-interacting protein kinase 1 (HIPK1), and upon neuronal activation, the calcium-dependent phosphatase calcineurin (CaN) dephosphorylates S669 [170]. This dephosphorylation, by enhancing DAXX activity, increased H3.3 enrichment at promoters and enhancers of immediate early genes leading to their active transcription. However, a major role for the H3.3-mediated activity of DAXX-ATRX is likely linked to its impact on heterochromatin function as shown above.

2.4.1.4 New CenH3^{CENP-A}-H4 Deposition by HJURP

The histone chaperone HJURP deposits the new histone variant CenH3^{CENP-A} at centromere in a DSI manner during late mitosis (telophase)/early G1 in mammals (Fig. 2.4) [69, 125, 126]. HJURP localizes to centromeres at the time of CenH3^{CENP-A} deposition, and CDK kinases control its timely recruitment to centromeres in late mitosis by changing its phosphorylation status [171, 172]. HJURP interacts with DNA through a specialized domain, which is essential to deposit CenH3^{CENP-A} at centromeres, highlighting that HJURP is not merely escorting CenH3^{CENP-A} but plays an active part in CenH3^{CENP-A} deposition [171]. Of note, although HJURP is the histone chaperone involved in the final step of CenH3^{CENP-A} deposition, numbers of other factors are required for the proper incorporation of this histone variant into centromere (review [70]). CenH3^{CENP-A} posttranslational modifications are important for its deposition. Ser68 phosphorylation of CenH3^{CENP-A} prevents an interaction with HJURP in the pre-deposition complex, helping to prevent premature loading [173]. The crystal structure at the interface of CenH3^{CENP-A} and HJURP shows that Ser68 lies in the histone variant binding domain of HJURP. Moreover, Lys124 ubiquitylation of CenH3^{CENP-A} plays a role in CenH3^{CENP-A} deposition by controlling the stability of the CenH3^{CENPA}-HJURP complex through a regulatory mechanism involving the cullin 4 (CUL4) ubiquitin ligase

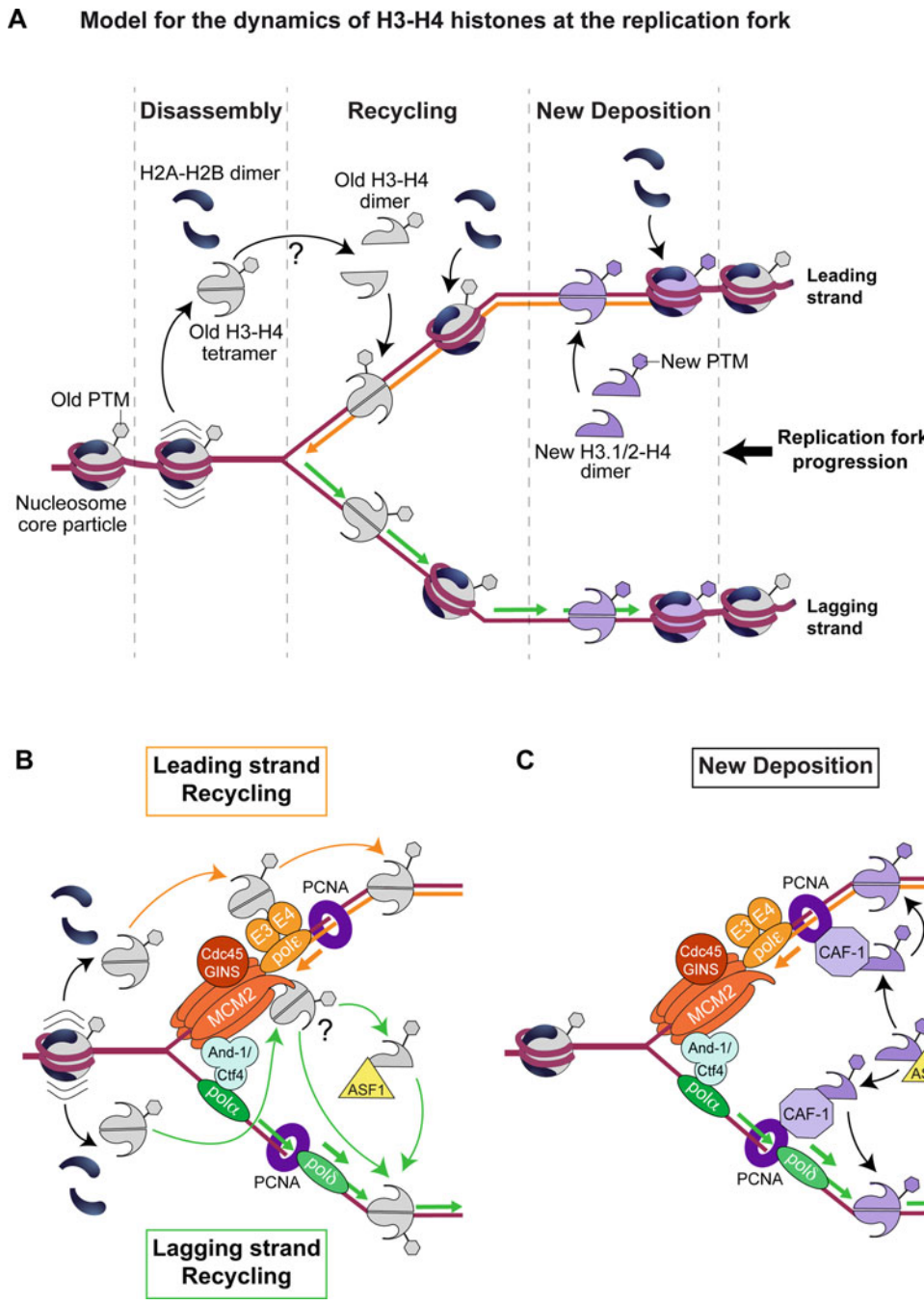

Fig. 2.5 Current model of histone dynamics at the replication fork. (**a**) For each old/parental nucleosome disrupted by the replication fork passage, a H3-H4 tetramer is available (disassembly). The old H3-H4 histones are recycled on newly synthesized DNA either directly as a tetramer or potentially as two dimers (recycling). New H3-H4 dimers are deposited onto newly synthesized DNA to ensure a full complement of nucleosomes on the nascent DNA (new deposition). Recycling of old/parental histones and deposition of new histones are thought to occur randomly on both the leading (in orange) and the lagging strand (in green). (**b**) The mechanisms and the histone chaperones involved in the recycling of old histones on the leading and the lagging strands are distinct. At the replication fork, the CMG (Cdc45-MCM(2-7)-GINS) helicase on the leading strand unwinds the DNA. The homotrimer And-1/Ctf4 links the helicase on the leading strand to the polα on the lagging strand through its interaction with both GINS and polα. While the two accessory subunits of polε (POLE3 and POLE4) mediate the

[174]. The new deposition of CenH3^{CENP-A} only occurs in late mitosis/early G1 phases, thus CenH3^{CENP-A} is diluted during S phase and distributed evenly to both daughter chromosomes in mammals [69]. In addition to deposit new CenH3^{CENP-A}, HJURP may be also required for its retention during S phase. By interacting with the replicative helicase complex, HJURP is proposed to retain and recycle CenH3^{CENP-A} following DNA replication [175]. During S phase, new deposition of both H3.1 (in a DSC manner) and H3.3 (through a possible post-replicative gap-filling mechanism) is observed, filling the gaps generated by diluting CenH3^{CENP-A}. Interestingly, the detected loss of H3.3 only later in G1 phase argues that H3.3 variant could serve as a CenH3^{CENP-A} placeholder [176].

2.4.2 Recycling of Old H3-H4 Histones

Chromatin integrity is critical for cell function and identity but is challenged by DNA processes that involve nucleosome disassembly. How chromatin architecture and the information that it conveys are preserved? For example, during replication, the chromatin structure is affected by the transient disruption of histone-DNA interaction from old/pre-existing/parental nucleosomes located ahead of replication forks (disassembly). Chromatin assembly onto daughter strands relies on two distinct processes: first the transfer of old histones (recycling) and second the deposition of new histones (new deposition). The latter process, as mentioned before, is regulated by the CAF-1 complex that deposits new H3.1/2-H4 histones onto both daughter strands. Experiments in the 1980s with bulk chromatin demonstrated the retention of parental histones on daughter strands [177, 178]. The recycling of old histones with their PTMs and the subsequent modifications of new histones to mirror the parental ones would participate in the maintenance of chromatin identity. The transmission of parental PTMs during replication appears to occur in human cells [179]. This is critical for features to be inherited from one cell to the next.

ASF1 has been the first H3-H4 chaperone implicated in the recycling of old histones during replication [140]. ASF1 was proposed to handle old histones at replication fork via an ASF1-(H3-H4)-MCM2-7 intermediate. An important role for MCM2 emerged based on structural analysis showing that MCM2, in contrast to ASF1, can bind an $(H3\text{-}H4)_2$ tetramer [145, 146]. This mode of binding implies that, once evicted from DNA possibly by the force of the helicase and the activity of remodeling factors, nucleosomal H3-H4 could be directly transferred to MCM2 as a tetramer. After this step old tetrameric $(H3\text{-}H4)_2$ could simply be directly loaded onto the newly synthesized DNA. Alternatively, old H3-H4 could be deposited as dimers after splitting by ASF1 [147]. Although the reassociation of the two parental dimer partners might be favored most of the time, mixing H3.3-H4 dimers, but not H3.1-H4 dimers, was reported with a potential important role in the inheritance of epigenetic traits [180, 181].

During replication, the two daughter chromatids differ in how they are replicated. The leading strand synthesis occurs in the direction of the fork progression while the lagging strand proceeds in interspersed segments in an opposite direction. Recycling old histones on leading and lagging strands exploit distinct mechanisms involving histones chaperones. In

Fig. 2.5 (continued) recycling of old H3-H4 histones on the leading strand, the helicase subunit, MCM2, operates on the lagging strand. It is still not fully understood whether H3-H4 is directly recycled as tetramers or whether they split as dimers before deposition or whether both events happen. Moreover, if H3-H4 split, whether ASF1 handles the H3-H4 dimers before deposition remains unclear. (**c**) To fulfill the requirement for nucleosome assembly, the deposition of newly synthesized H3.1/2-H4 dimers occurs on both strands by the histone chaperone complex CAF-1 through its interaction with the sliding clamp PCNA. The histone chaperone ASF1 would hand over H3.1/2-H4 dimers to CAF-1 before deposition

yeast, while MCM2 operates on the lagging strand, Dpb3-Dpb4 subunits of the polymerase ε act on the leading strand [150, 182]. The function of MCM2 in recycling old histones onto the lagging strand is conserved in human [183]. POLE3-POLE4, the human counterpart of Dpb3-Dpb4, recently described as a H3-H4 chaperone whose depletion affects chromatin at replication fork, could similarly participate in the recycling of old histones onto the leading strand in human [149]. Whether the evicted $(H3\text{-}H4)_2$ tetramer splits in to dimers before recycling/deposition onto the leading strand remains to be explored, in particular in light of possible connection with Asf1. A scheme of the current model for histone dynamics at replication fork, involving old histone recycling and new histone deposition, is shown in Fig. 2.5.

Of note in yeast, cohesion establishment factor 4 (Ctf4), a replisome factor that links the CMG helicase on the leading strand to the DNA polymerase α on the lagging strand (through a direct interaction with both GINS and polα) [184], participates in the recycling of old histones on this latter strand [182]. Ctf4 and its human counterpart, acidic nucleoplasmic DNA-binding protein-1 (And-1), form homotrimers that exhibit homology with the homotrimeric form of the HIRA subunit of the histone chaperone complex HIRA [104]. This intriguing homology could suggest for Ctf4/And-1 and HIRA a similar way of mediating protein and DNA interactions at particular bubble DNA structures as encountered at replication fork for Ctf4/And-1 and perhaps at transcription sites for HIRA.

The existence of distinct mechanisms for recycling old histones on the two daughter strands raises a new interesting hypothesis [185]. Indeed, while ensuring an equal partitioning of old histones for most cells, it may as well offer an opportunity for unequal partitioning. For example, in *Drosophila* male germline and adult midgut, replicative H3 and $CenH3^{CENP\text{-}A}$ are asymmetrically distributed, respectively. The daughter stem cell retains the parental/old histones, while the post-mitotic differentiating daughter cell genome is assembled with new histones [186, 187]. The existence of distinct mechanisms to recycle old histones might be a way to regulate the asymmetric distribution of old histones onto the two daughter cells in the wake of the replication. This could be crucial to initiate a differentiation program by losing parental marks.

2.5 Concluding Remarks and Perspectives

Chromatin can protect DNA from various deleterious threats while remaining flexible to enable the regulation of gene expression and programmed changes in cell identity to occur during normal development. The histone H3 family and its various chaperones are crucial for allowing dynamic accessibility to particular genomic loci. Over recent decades, much progress has been made in the study of histones and their modes of incorporation into chromatin. This is particularly true for histones H3-H4 with the identification of histone chaperones that are often specialized in the deposition of one peculiar H3 variant at a particular time and at specific locations onto the genome. We are unveiling complete histone H3 deposition pathways from their site of synthesis to their sites of delivery, and we are also now currently elucidating how old histones are recycled at replication fork. Open questions remain regarding this network of histones and histone chaperones. For example, histone H3 chaperones are most often protein complexes whose stoichiometry, posttranslational modifications, and functional regulations are still poorly characterized, and understanding how they may link to cell cycle control and cell fate will be extremely exciting. While the deposition process of H3-H4 is now rather well understood, the mechanisms and histone chaperones involved in the deposition of H2A-H2B dimers still needs to be deepened. Processes of old histone recycling started recently to be deciphered at replication fork, but old histone recycling also occurs at DNA repair and transcription sites [43, 188]. Most fascinating is to understand how the marking with particular variants actually experience cell division and can be restored after

the passage of the replication fork. Elucidating therefore the mechanisms that operate during repair and transcription would also be crucial. Furthermore, exploring how this network of histones and histone chaperones is potentially rewired when one of several of the actors are mutated or deregulated in particular during cancer will bring undoubtedly important new findings in the field.

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Histone H3K27M Mutation in Brain Tumors

3

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Abstract

Histones form chromatin and play a key role in the regulation of gene expression. As an epigenetic information form, histone modifications such as methylation, phosphorylation, acetylation, and ubiquitination are closely related to the regulation of genes. In the last two decades, cancer scientists discovered that some histone modifications, including acetylation and methylation, are perturbed in cancer diseases. Recurrent histone mutations, which hinder histone methylation and are implicated in oncogenesis, are recently identified in several cancer disease and called oncohistones. Well-known oncohistones, with mutations on both H3.1 and H3.3, include H3K36M in chondroblastoma, H3K27M in glioma, and H3G34 mutations that exist in bone cancers and gliomas. Oncohistone expression can lead to epigenome/transcriptome reprogramming and eventually to oncogenesis. The H3K27M, H3G34V/R, and H3K36M histone mutations can lead to the substitution of amino acid(s) at or near a lysine residue, which is a methylation target. H3K27M characteristically exists in diffuse intrinsic pontine glioma (pediatric DIPG), and its expression can cause a global decrease of the methylation of histone at the lysine residue. Uncovering the molecular mechanisms of H3K27M-driven tumorigenesis has recently led to the identification of some potential therapeutic targets in diffuse intrinsic pontine glioma. In this chapter, we will review and summarize recent studies on the H3K27M-driven tumorigenic mechanisms and properties and the role of H3.1K27M and H3.3K27M oncohistones in brain tumors.

Keywords

Oncohistones · H3.1K27M · H3.3K27M · Brain tumors · DIPG · Epigenome

Abbreviations

BET	Bromodomain and extra-terminal domain
CGIs	CpG islands
CDKN2A	Cyclin-dependent kinase Inhibitor 2A
DIPG	Diffuse intrinsic pontine glioma
H3K27ac	Histone H3 lysine 27 acetylation
H3K27me3	Histone H3 lysine 27 trimethylation
HMT	Histone methyl-transferase
MAP 2	Microtubule-associated protein 2

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D. Fang, J. Han (eds.), *Histone Mutations and Cancer*, Advances in Experimental Medicine and Biology 1283, https://doi.org/10.1007/978-981-15-8104-5_3

MICA	MHC class I polypeptide-related sequence A
NHEJ	Nonhomologous end joining
PDGFRA	Platelet-derived growth factor receptor-α
PRC2	Polycomb-repressive complex 2
PFA	Posterior fossa type A
Stat3	Signal transducer and activator of transcription 3
TUBB3	Tubulin beta 3

3.1 Introduction

As alkaline proteins, histones exist in the nuclei of eukaryotic cells. They mainly function to control the appropriate DNA package/order the DNA into the nucleosomes units. In addition, histones form chromatin and play a key role in the regulation of gene expression. In the nucleosomes, histone proteins can be modified dynamically by an epigenetic writer(s). Methylation, phosphorylation, and acetylation as well as ubiquitination are key modification processes of histone proteins. Indeed, the posttranslational modification processes of histone proteins are one form of epigenetic information and play significant roles in regulating the gene expression [1–3].

The formation of cancer diseases is widely known to be related to the misregulation or mutation of specific gene expression in cancer cells. However, in the last two decades, cancer scientists have discovered that modification processes of histone proteins, including acetylation and methylation, are perturbed in cancer diseases. Chromatin-remodeling protein mutations often lead to these perturbations in cancer cells. Therefore, much attention has been recently paid for investigating the role of aberrant modifications of histone proteins in cancer development and pathology.

Recently, recurrent histone mutations, which are also implicated in oncogenesis, are identified in several cancer disease and called oncohistones. Well-known oncohistones, with mutations on both H3.1 and H3.3, include H3K36M in chondroblastoma and H3K27M in glioma, as well as H3G34 mutations that exist in bone cancers and gliomas. Both H3.1 and H3.3 have conserved major lysine residues, including K9, K4, K36, and K27, despite their slight differences in the amino acid sequences. Notably, while H3.3 is a variant histone existing in both the gene bodies and promoters of genes that are actively transcribed, H3.1 can be found throughout the genome [4]. In addition, certain histone chaperone complexes, including DAXX–ATRX and HIRA, mediate the incorporation of H3.3 histone onto the chromatin that is cell cycle independent [4, 5]. Notably, all well-documented oncohistones have mutation on or near these residues that are conserved and act as sites for the modifications of histone. Oncohistone expression can lead to transcriptome reprogramming and eventually to oncogenesis [3]. Other types of mutations, such as those on glycine 34 of histone H3, are also found in brain tumors, like H3K27M [6]. These mutations are characterized with the substitution of glycine with valine/arginine (H3G34V/R) and, together with H3K27M, exist in different brain regions [6].

3.2 Role of Oncohistones H3.1K27M and H3.3K27M in Brain Tumors

The oncohistones H3.1K27M and H3.3K27M play important role in brain tumors. One of the most studied brain tumors is the diffuse intrinsic pontine glioma (DIPG). DIPG is an aggressive, glial cell-derived brain tumor that is common in children [7]. DIPG children have a limited median rate of survival (1 year only). Histone H3 somatic mutations, which result in a substitution of lysine with methionine specifically on position 27 (H3K27M), have been identified in 60% of DIPG patients and up to 30% of patients with glioblastoma [6, 8, 9]. Several studies have showed that these somatic mutations occur mostly on the genes that encode histones H3.1 and H3.3, which are *HIST1H3B* and *H3F3A* genes, respectively [10, 11].

In DIPG, H3.1K27M and H3.3K27M tumors represent two different subgroups with a

characteristic clinical manifestation(s). For example, while H3.3K27M tumors exist along the brain midline, tumors with H3.1K27M mutations are only restricted to the pons [10–12]. In addition, H3.1K27M and H3.3K27M tumors have characteristic associating mutations of DNA such as H3.1K27M-associated ACVR1 mutations [13], and H3.3K27M-associated amplified mutations in CCND2, PDGFRA, TP53, and MYC [12, 14]. Moreover, H3.1K27M/H3.3K27M subgroups have characteristic expression profiles. Thus, H3.3K27M tumors have a characteristic oligodendroglial phenotype, while H3.1K27M tumors have a remarkable mesenchymal phenotype. The clinical outcomes also vary between H3.1K27M and H3.3K27M tumors. Thus, patients with H3.1K27M have a longer median survival of 15 months, while patients with H3.3K27M have 11 months' median survival only [10]. In addition, H3.3K27M tumor types display more metastatic relapses and show more radiotherapy resistance [10]. In contrast, expressing both H3.1K27M and H3.3K27M can result in similar epigenomic changes. Notably, a recent immunohistochemical study has detected histone H3 K27M mutation, H3K27me3 (but not H3K27Ac), and posttranslational modifications in pediatric DIPG and suggested H3K27M and H3K27me3 staining for pediatric DIPG diagnosis [15].

3.3 Role of H3K27me3 Landscape Changes in Reprogramed Epigenome and Gene Expression in Brain Tumors

Both trimethylation at histone H3 lysine 27 (H3K27me3) and H3K27ac are mutually exclusive and important in gene regulation. For instance, as a repressive histone mark, H3K27me3 is important for several processes such as the inactivation of X-chromosome, silencing of HOX genes, and genome imprinting [16]. The deposition of H3K27me3 relies on the histone methyl-transferase (HMT), named as EZH2, which represents a subunit of the polycomb-repressive complex 2 (PRC2). H3K27 acetylation (H3K27ac) antagonizes the activity of PRC2 and plays an important role in gene regulation due to its association with both enhancer regions and active gene transcription [17]. Notably, H3K27M expression can influence both the acetylation and methylation of lysine 27 (of Histone H3 protein; [3]).

Despite the general low incidence of H3K27M mutation, which takes place on 1 out of 16 genes that encode histone H3, it can clearly change the reported epigenome(s) of H3K27M-DIPG cells.

In addition, in diffuse intrinsic pontine glioma (DIPG) patient cells, recent data have shown that H3K27M represents 3–17% only of total H3 [18]. However, H3K27M expression in these cells is enough to reprogram the landscape of H3K27me3 [18]. Notably, a remarkable decrease of trimethylated H3K27 has been reported in different types of H3.3K27M brain tumors, including glioblastomas and gliomas [18–21], with an associated gain of H3K27ac in some reports [18, 22, 23]. However, other studies have reported an enhancement of H3K27me3 in a large number of gene loci [20, 24]. Interestingly, the promoter-associated regions loss H3K27me3, while the intergenic regions are associated with gaining the mark [19].

Several studies have demonstrated that H3K27M expression can lead to H3K27me3 loss, which is cell-type independent. In addition, the decrease of H3K27me3 that is observed in DIPG cells can be recapitulated by expressing H3.1K27M/H3.3K27M in 293T, human astrocyte, and murine embryonic fibroblast cells [20, 21]. Remarkably, incubating H3-WT nucleosome with H3K27M peptide can significantly decrease methylation that is mediated by PRC2 (by almost sixfold), suggesting that H3K27M can clearly impede the activity of PRC2 in trans [18]. To exert these activities, H3K27M interacts with the SET-domain (of EZH2), which has a characteristic active site lined with highly conserved residues of tyrosine and plays a key role in EZH2 methyl-transferase activity. Indeed, a substitution of one of these tyrosine residues (Y641N) that exist in the SET-domain can lead to

sensitivity loss to the inhibition of H3K27M. This suggests a hypothesis that the aromatic tyrosine-hydrophobic methionine interactions are important for H3K27M inhibitory effect [3]. A recent structural study by Justin and colleagues that focuses mainly on the crystal structure of K27M peptide-bound EZH2 has confirmed this important hypothesis [25]. Indeed, there are many configuration and charge property similarities between both SET-bound lysine and methionine side chain, and these similarities enable methionine to occupy the "lysine channel" that is lined with the aromatic residues [25]. Interestingly, a 16-fold tighter binding was observed between PRC2 and H3K27M peptide(s) when compared to the wild-type H3 peptide (s) [25]. Moreover, a more recent study has demonstrated that both DIPGs and posterior fossa type A (PFA) ependymomas are partially driven by the activities of peptidyl PRC2 inhibitors (the EZHIP and K27M oncohistone), which lead to promoting tumorigenesis by dysregulating gene silencing [26].

3.4 Involvement of H3K27ac Gain in the Epigenetic Landscape Changes in Brain Tumors

Currently, there are two suggested models that explain H3K27me3 loss in H3K27M tumors/expressing cells: the sequestration model and the H3K27M/H3K27ac exclusion model. Figure 3.1 shows a sequestration model that is supported by several studies and describes both the recruitments and local retaining of EZH2 and the stop of PRC2 complex spreading by H3K27M, and these activities can result in H3K27me3 loss globally [3, 18, 25]. This sequestration model has been opposed recently by a study on H3K27M genome-wide distributions in diffuse intrinsic pontine gliomas [23]. This study has shown that the two subunits of the PRC2 complex, EZH2 and SUZ12, are clearly eliminated from the H3K27M-containing nucleosomes [23]. Notably, H3K27M can be detected at the actively transcribed region(s) and coincides with both RNA polymerase II and H3K27ac in diffuse intrinsic pontine gliomas [23]. This H3K27M- H3K27ac colocalization agrees with previous studies by Lewis and co-workers in pediatric glioblastoma, in which an increased H3K27ac level was detected in the H3K27M-containing oligo-nucleosome array [18]. Interestingly, the study by Lewis and co-workers has suggested another model, H3K27M- H3K27ac exclusion model (Fig. 3.2), in which the H3K27me3 loss is not due to the recruitments of PRC2 by H3K27M but because of the H3K27M/H3K27ac nucleosome-dependent exclusion of PRC2 from the chromatin [18].

3.5 Influence of H3K27M and H3K27me3 Alterations on the Gene Expression in Brain Tumors

Several studies have demonstrated the importance of changes in H3K27me3 occupancy in long-term gene repression in different biological processes. Thus, the expression of H3K27M in cells can lead to a concomitant change in the expression of several differentially expressed genes that are key players of the embryonic morphogenesis, activity of transcription factors, cancer pathways, and differentiation on neurons [19, 20]. These differentially expressed genes include MHC class I polypeptide-related sequence A (MICA) and platelet-derived growth factor receptor-α (PDGFRA) as well as cyclin-dependent kinase inhibitor 2A (CDKN2A) [24, 27, 28]. While MICA downregulation has been suggested in glioma as an important mechanism for the immune evasion, the upregulation of PDGFRA is involved in gliomagenesis [27, 28]. Moreover, the repression of CDKN2A has recently been reported to be involved in H3K27M-driven tumorigenesis [24]. Interestingly, a new model was suggested by which H3K27me2/3 can facilitate both the genome stability maintenance and the nonhomologous end joining (NHEJ) capability in more recent researches [29].

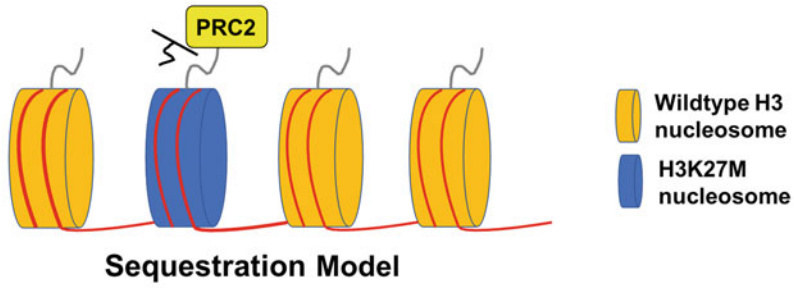

Fig. 3.1 The sequestration model explaining H3K27me3 loss in H3K27M tumors/expressing cells. This model suggests that the PRC2 interactions with H3K27M nucleosome lead to the inhibition of PRC2 complex, as described in detail in the text

3.6 Different H3K27M-Driven Tumorigenic Mechanisms and Their Impacts on the Epigenome

The repressive histone mark, H3K27me3, is globally lost in cells expressing H3K27M. However, some H3K27me3 still existed and/or enriched at some loci in the cells expressing H3K27M [20, 24]. This was supported by a recent study on pediatric gliomas that used Pdgfβ overexpressing murine model and found in wild-type H3 mouse that H3K27me3-enriched CpG islands (CGIs) can gain and retain H3K27me3 in the H3K27M counterparts [24]. These data have led to the suggestion that the H3K27me3 histone mark-retaining loci are strong and important targets for polycomb in gliomas [24], since it is well-known that polycomb complexes target CGIs [30]. Consequently, these data have led to a conclusion that H3K27M incorporations at its strong and important polycomb targets are not apparently sufficient for inhibiting the overall activity of PRC2, and, therefore, the levels of H3K27me3 remain unchanged in this study on pediatric gliomas [24] (Fig. 3.3a, b). Importantly, in H3K27M-DIPG, H3K27me3-gaining genes play important roles in the maintenance of the DIPG cell identity. Yet, the exact mechanisms by which some loci can gain H3K27me3 are still unclear (Fig. 3.3c).

The remarkable gene ontology analyses of H3K27me3-enriched loci in the recent study by Mohammad and colleagues have demonstrated a clear and remarkable enrichment of polycomb targets that play important regulatory roles of key biological/developmental processes, including pattern specification, and transcription regulation as well as embryonic development [24]. Notably, wild-type H3 mice, genes that are associated with H3K27me3 in neural stem cells,

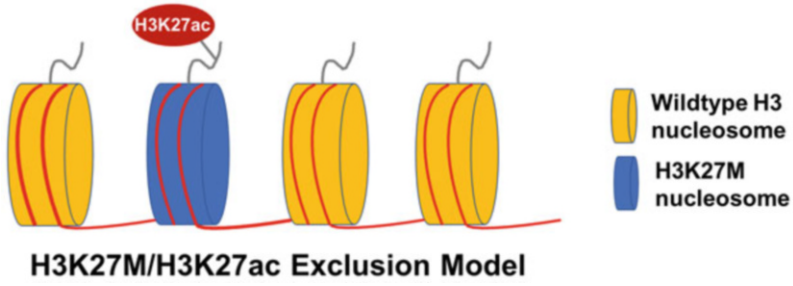

Fig. 3.2 The H3K27M/H3K27ac exclusion model explaining H3K27me3 loss in H3K27M tumors/expressing cells. The H3K27M/H3K27ac exclusion model is based on Piunti et al. [23] studies that demonstrated that H3K27M rather than PRC2 subunits can colocalize with H3K27ac. PRC2 is, therefore, excluded in this model from H3K27M/H3K27ac heterotypic nucleosomes

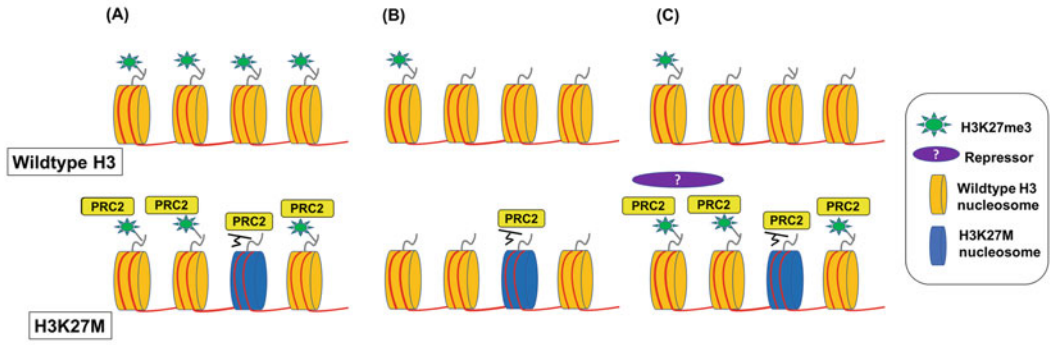

Fig. 3.3 Three models of the landscape of H3K27me3 at different loci in H3K27M-expressing cells. (**a**) At the H3K27me3-enriched strong polycomb target(s), the trimethylation mark persists even when H3K27M nucleosome is present. (**b**) At weak polycomb targets with a scarce H3K27me3, the loss of H3K27me3 is due to H3K27M incorporations. (**c**) The expression of H3K27M can lead to H3K27me3 gain at some loci, in which PRC2 complex is recruited by still- unidentified repressor, as suggested by Mohammad et al. [24]. Adapted from Wan et al. [3]

can completely overlap with that of H3K27M neural stem cells [24]. This has led to a conclusion that some de novo polycomb targets exist in the H3K27M/ PDGFβ model, suggesting that H3K27M-derived DIPG oncogenesis is due to H3K27M supports of a specific transcriptional pattern that is characteristic for the cell-of-origin of DIPG [24]. Nevertheless, it was reported in H3K27M mice that 20 genes that gain H3K27me3, including cyclin-dependent kinase inhibitor 2A (CDKN2A) that is importantly involved in the development of H3K27M-driven DIPG, did not apparently show an enrichment of H3K27me3 in gliomas [24]. This suggests that the oncogenic transformation process involves the selected silencing of these 20 genes [24].

The cyclin-dependent kinase inhibitor 2A (CDKN2A) plays important roles in both cell stress and oncogenic activation by encoding p16 tumor suppressor protein, which can terminate the cell cycle during both the cell stress and oncogenic activation [31]. Notably, the homozygous CDKN2A deletion is rare in pediatric high-grade glioma, despite the findings by Brennan and co-workers that it can be detected in >55% of adult high-grade glioma [32]. These findings have led to the conclusion that the gene repression mediated by PRC2 could be considered as an alternative pathway for silencing the expression of CDKN2A [33]. Indeed, a recent study using H3.3K27M murine model by Cordero et al. [34] has shown that CDKN2A repression could lead to the acceleration of gliomagenesis [34]. In addition, the DIPG growth relies on CDKN2A repression since inducing p16 expression in DIPG cell lines can lead to the growth arrest of these cells [24].

3.7 Other H3K27M-Driven Tumorigenic Mechanisms in the Brain

There are other H3K27M-driven tumorigenic mechanisms, in addition to the silencing of CDKN2A. It is well reported that H3K27M is localized to the transcriptionally active regions, together with RNA polymerase II and H3K27ac. It also coincides with that of the members of the family of bromodomain and extra-terminal domain (BET)-containing proteins such as BRD4 and BRD2.

Both the BET domain-containing proteins, BRD2 and BRD4, are also important H3K27M-driven oncogenic mechanisms, and this was confirmed by inhibiting both BRD2 and BRD4 using JQ1 in H3K27M-DIPG cells. This approach helps with dissecting the functional roles of these BET domain-containing proteins, which also play important roles in the transcription elongation,

in H3K27M-DIPG cells. Because of their importance in the transcription elongation, Piunti and co-workers found that inhibiting both BRD2 and BRD4 using JQ1 leads to antitumor effects without CDKN2A derepression and can effectively suppress the activity of well-known transcribed genes that are also direct targets since they are occupied by H3K27M and BRD2/4. These data suggest other oncogenic pathway and H3K27M-driven tumorigenic mechanism in the brain that are independent of p16 [3, 23]. In addition, the study by Piunti et al. [23] has shown evidences that H3K27M can occupy the active enhancer region, which is characteristically marked by H3K27ac/H3K4me1. This recent data suggests another oncogenic-driven mechanism, in which H3K27M can contribute to the super enhancers' formation [23]. In addition, since almost 25% of DIPGs harbor active ACVR1 mutations, which are associated with H3.1K27M mutations, a remarkable recent study has shown that both H3.1K27M and ACVR1 R206H can promote the initiation of tumors and increase gliomagenesis, through activating the signal transducer and activator of transcription 3 (Stat3) [35].

Furthermore, other co-occurring mutations were shown recently to contribute to H3K27M-driven tumorigenesis. For example, in H3.3K27M-expressing mice used to study high-grade gliomas, ATRX knockdown can lead to the formation of focal tumor, while the overexpression of PDGFRA results in the reduction of tumor latency [36].

3.8 H3K27M-DIPG Therapeutic Approaches and Strategies

Currently, the precise and detailed H3K27M-driven tumorigenic mechanisms in the brain are still not clear**.** However, recent studies on H3K27M-DIPG have led to the identification of some important druggable targets, including small molecules that are effective against H3K27M-DIPG in vivo and in culture, using different approaches such as the reverse of the H3K27ac gain, the rescue of H3K27me3, and targeting the activities of residual EZH2.

3.8.1 The Reverse of H3K27ac Gain Approach

Several studies have provided evidences that the trimethylation loss at K27 can consequently lead to the acetylation that is gained at K27 also [10, 11]. This suggests a possible therapeutic strategy/approach that aims to reverse these epigenetic alternations. For instance, Grasso and colleagues have provided evidences that the HDAC inhibitor, panobinostat, is an effective therapy in H3K27M-DIPGs [37].Thus, treatment of K27M-DIPG cell lines with panobinostat has led to increased cell apoptosis, reduced cell viability, downregulated MYC target genes, and enhanced H3K27ac as well as the rescue of H3K27me3 levels [37]. This important study has also shown that panobinostat can decrease H3K27M-DIPG cell viability by acting synergistically with GSKJ4, which is an inhibitor of the demethylases of H3K27me2/3, which are responsible for catalyzing the H3K27me2/3 demethylation [37]. In addition, recent studies on the bromodomain and extra-terminal domain (BET) inhibitor, JQ1, have demonstrated its anti-tumorigenic activity in DIPG cell lines, probably by decreasing the levels of H3K27ac [23]. Other effects of JQ1 on H3K27M-DIPG cells include the promotion of both certain neuronal-like morphological change(s) and anti-proliferation activities by stimulating the differentiation markers of mature neurons, the tubulin beta 3 (TUBB3) and microtubule-associated protein 2 (MAP2), and the cell cycle arrest marker p21, respectively, in these cells [3, 23].

3.8.2 The Rescue of H3K27me3 Approach

Several recent studies show evidences that the rescue of H3K27me3 is an important approach

to develop an effective K27M-DIPGs therapy since H3K27M expression leads to decreasing

H3K27me3 [3]. Studies using the demethylase inhibitor GSKJ4, which has remarkable anti-tumorigenic activities in vivo/ in culture [38], represent a good example for this rescue H3K27me3 approach. For example, the treatment of human H3K27M-DIPG cell lines with GSKJ4 increased cell apoptosis but decreased both cell viability and clonogenic activities [3, 39]. Mechanistically, the GSKJ4 anti-tumorigenic activities in H3K27M-DIPG cell lines are probably due to the inhibition of the well-known H3K27me3 demethylase, JMJD3 [38, 40]. Further analyses of the GSKJ4-treated cell transcriptome are needed to uncover GSKJ4 effects in H3K27M-DIPG cells.

3.8.3 Targeting the Activities of Residual EZH2 Approach

Since residual EZH2 activities play an important role in DIPG cell growth, both targeting and inhibiting EZH2 activities are considered as important approaches for the treatment of diffuse intrinsic pontine glioma (DIPG) [23, 24]. This was supported by a recent study showing decreased proliferation, probably by derepressing p16, and reduced colony-forming abilities of H3K27M-DIPG cell lines after treatment with the highly selective EZH2 inhibitor, EPZ6438 [24]. The EPZ6438 anti-proliferative effects on H3K27M-DIPG cell lines may be also p16-independent effects [23]. Interestingly, treatments of DIPG cells with EPZ6438 cause an increase of p16, while the treatment with JQ1 upregulates the other key regulator of the cell cycle, p21. Further in vivo and in vitro research studies are needed to explore whether treatments with EPZ6438 and JQ1 together can synergistically cause the suppression of DIPG cell growth.

3.9 Conclusion, Perspective, and Future Direction

Since 2012, several studies on the recurrent mutation of H3K27M in gliomas have highlighted the importance of histone mutation-driven tumorigenesis [6, 8, 9]. This was well exemplified in the role of H3.3K27M oncohistone in the diffuse intrinsic pontine glioma (DIPG) that is well-investigated. Indeed, better understanding of H3.3K27M-driven oncogenic mechanisms has recently helped with the identification of some potential DIPG therapeutic targets. For example, a recent study on ACVR1 and H3.1K27M mutations has identified LDN212854 compound as a potential treatment for DIPGs [35]. In addition, the identification and characterization of pediatric glioma-exclusive H3K27M have led to the conclusion that both types of gliomas (pediatric and adult) are different on the molecular levels and, therefore, require different therapeutic strategies and approaches.

Despite accumulated data on the role of oncohistones in brain tumors, more studies are still needed. For example, more research is still needed to uncover the details of the occurrence of oncohistones in specific tumor types that is currently mostly unknown. H3K27M, for example, exists on both H3.3 and H3.1. H3.1 is characteristic in its ubiquitous expression and unformal distribution in the genome. Nevertheless, current research studies have only identified H3.1K27M in the diffuse intrinsic pontine glioma (DIPG). Identification of factors that play a role in the H3.1K27M tumor-type specificity is needed to better understand the H3K27M-driven tumors in the brain.

It is well-known that cancer development is associated with perturbations of histone methylations and changes of histone methyltransferases. Morin and colleagues have, for instance, identified EZH2-inactivating mutations in two types of lymphomas (diffuse large B cell lymphoma and follicular lymphoma; [41]). In contrast, studies by Kanu and co-workers have not detected H3K36M in renal cancer cells that contain inactivating mutations of SETD2 [42], while Lehnertz et al. [43] have recently reported a low frequency (1/415) of H3K27M in follicular lymphomas. Remarkably, inactivating SETD2, together with EZH2, was found to cause methylation abnormalities at histone H3 lysine residues. More studies are, therefore, still needed to clarify and identify the

reasons of infrequency of H3K36M and H3K27M in these cancer types.

Furthermore, the posttranslational modifications of histone do not only include the methylation of lysine on histone H3 but extend to include the acetylation of lysine and serine/threonine phosphorylation as well as ubiquitination. Notably studies have related cancers with many newly discovered writers and reader/eraser mutations of these histone tail modifications [44]. Importantly, further studies are needed to identify the functional roles of more oncohistones in brain tumors.

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Histone Mutations and Bone Cancers

4

Earnest L. Taylor and Jennifer J. Westendorf

Abstract

Primary bone tumors are rare cancers that cause significant morbidity and mortality. The recent identification of recurrent mutations in histone genes *H3F3A* and *H3F3B* within specific bone cancers, namely, chondroblastomas and giant cell tumors of bone (GCTB), has provided insights into the cellular and molecular origins of these neoplasms and enhanced understanding of how histone variants control chromatin function. Somatic mutations in *H3F3A* and *H3F3B* produce oncohistones, H3.3G34W and H3.3K36M, in more than nine of ten GCTB and chondroblastomas, respectively. Incorporation of the mutant histones into nucleosomes inhibits histone methyltransferases NSD2 and SETD2 to alter the chromatin landscape and change gene expression patterns that control cell proliferation, survival, and differentiation, as well as DNA repair and chromosome stability. The discovery of these histone mutations has facilitated more accurate diagnoses of these diseases and stratification of malignant tumors from benign tumors so that appropriate care can be delivered. The broad-scale epigenomic and transcriptomic changes that arise from incorporation of mutant histones into chromatin provide opportunities to develop new and disease-specific therapies. In this chapter, we review how mutant histones inhibit SETD2 and NSD2 function in bone tumors and discuss how this information could lead to better treatments for these cancers.

Keywords

Bone · Chromatin · Oncohistones · H3K36M · H3G34 · Chondroblastoma · Giant cell tumor · Osteosarcoma

Abbreviations

CAF-1	Chromatin assembly factor 1
DRD2	Dopamine receptor D2
GCTB	Giant cell tumor of bone
H3	Histone 3
HIRA	Histone regulator A
HMTase	Histone methyltransferase
K	Lysine
KDM	Lysine demethylase
KMT	Lysine methylation transferase
Me	Methyl group
MMSET	Multiple myeloma SET domain
NSD	Nuclear receptor SET domain-containing
PRC2	Polycomb-repressive complex 2
PRMT	Protein arginine methyltransferases

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D. Fang, J. Han (eds.), *Histone Mutations and Cancer*, Advances in Experimental Medicine and Biology 1283, https://doi.org/10.1007/978-981-15-8104-5_4

PTM	Posttranslational modification
RRM2	Ribonucleotide reductase regulatory subunit M2
SETD	Su(var)3-9, Enhancer of Zeste, Trithorax domain-containing
SRI	Set2 Rpb1 interacting domain
WHSC1	Wolf-Hirschhorn syndrome gene (aka, NSD2)

4.1 Introduction

Primary bone tumors are rare cancers that produce significant morbidity and mortality in children and adults. Bone neoplasms cause debilitating pain, impede skeletal growth, and weaken bones. Benign bone tumors are treated surgically if they are painful or prominent, but many require no treatment. Malignant bone tumors will be treated surgically if possible and with antineoplastic regimens, such as adjunct chemotherapy and radiation. In some cases, amputation or major resection is required, which results in lifelong disability and medical care. Children treated with radiation and chemotherapy for bone cancers are at increased risk for other cancers and require continuous monitoring.

Bone cancers are categorized by the cell type from which they originate, by the type of tissue they produce (e.g., bone (*osteo-*) or cartilage (*chondro-*)), by appearance on radiographs or pathology sections, and increasingly by the presence of reoccuring genetic mutations. For example, recurrent chromosomal translocations characterize Ewing's sarcoma and Ewing-related family of tumors [1]. Recently, somatic mutations in histone genes were found in more than 90% of chondroblastomas and giant cell tumors of the bone (GCTB) [2, 3]. This discovery launched experimentation to understand how mutated histones (oncohistones) contribute to tumorigenesis and enhanced basic understanding of chromatin organization. This chapter will review fundamentals of bone formation and homeostasis and then focus on how genetic mutations within histone genes cause changes to the epigenome and drive tumorigenesis in bone tissues. Ways in which this information can lead to new therapeutic strategies for debilitating bone tumors will be discussed.

4.2 Bone Development, Homeostasis, and Bone Tumors

Bones are highly dynamic organs not just during growth phases but throughout life. Bones provide structural support to skeletal muscles, protect our internal organs, store calcium and other minerals, and produce endocrine factors that affect overall health and function of other tissues, including the skeletal muscle, the gut, and the brain. Bones are formed by two processes: intramembranous ossification and endochondral ossification [4]. During intramembranous ossification, mesenchymal progenitor cells condense and differentiate into osteoblasts that produce an extracellular matrix rich in collagen 1 and mineralization promoting factors that stimulate hydroxyapatite incorporation into the matrix. This process forms the clavicle (collar bone) and many flat bones in the skull. Most bones form by endochondral ossification when mesenchymal progenitors differentiate into chondrocytes, which produce matrices rich in collagen 2, proteoglycans, and mineralization inhibitors. Vascular and neuronal invasion of these cartilaginous structures promotes recruitment of hematopoietic cells, including monocytes that differentiate into osteoclasts and carve out the marrow cavity. Mesenchymal progenitor cells that differentiate into osteoblasts are also recruited and gradually replace the chondrocytic matrix with the mineralized matrix.

Long bone growth continues until the cartilaginous epiphyseal growth plate closes. Chondrocytes in growth plates are organized in columnar structures and horizontal zones (Fig. 4.1). The most distal zone of growth plates on each end of a long bone contains proliferative cells that maintain the chondrocyte pool in developing bones. After proliferation ceases, chondrocytes undergo hypertrophy, and this enlargement in cell size drives longitudinal bone lengthening. Most hypertrophic chondrocytes

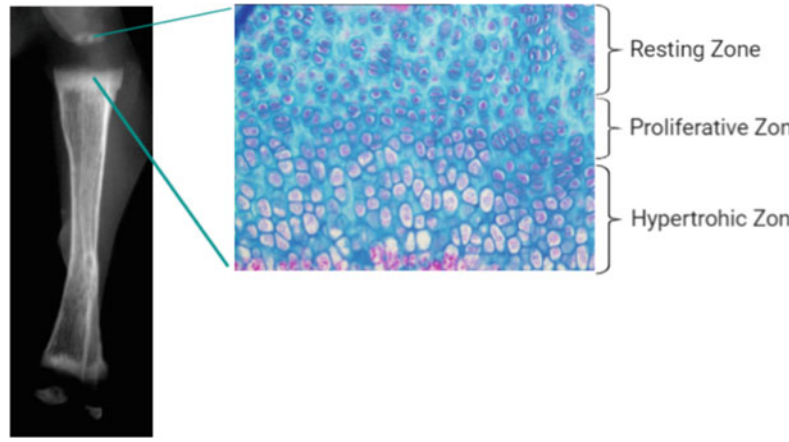

Fig 4.1 Endochondral ossification. The growth plate is a cartilaginous structure at the ends of long bones where endochondral ossification occurs. It is subdivided into three distinct zones: the resting, proliferative, and hypertrophic zones

will die by apoptosis, but some survive and take residence in the bone marrow where they are poised to contribute to fracture healing [5].

After bone growth ceases, bones remain highly dynamic organs throughout life to keep bones strong and healthy. Every day, osteoblasts (bone forming cells) and osteoclasts (bone resorbing cells) are actively remodeling and refreshing the skeleton to ensure that the skeleton remains strong and that the body has a sufficient supply of essential minerals and growth factors. The tight communication between osteoblasts and osteoclasts is skewed during the menopause and with aging such that bone resorption exceeds bone formation and the skeleton weakens. This communication is also altered by primary and metastatic bone tumors that find bone to be a nurturing environment. Bone tumors have a lytic, blastic, or mixed bone phenotype and affect bone strength. Blastic tumors will produce a collagen 1-rich matrix, but the collagen is secreted in woven patterns that are mechanically weaker than the normal lamellar pattern. In contrast, osteolytic tumors activate osteoclasts that resorb bone matrix, lowering bone density and releasing growth factors to sustain their growth in a vicious cycle [6]. Primary bone tumors are relatively rare but frequently occur in children and young adults who are actively growing. Primary bone tumors that become metastatic typically colonize the lungs or other bones. Increased understanding of the molecular mechanisms that drive carcinogenesis, colonization, and metastasis of bone neoplasms will lead to better treatments that optimally treat the bone niche as well as the tumor.

4.3 Histones and Epigenetics

Histones are cellular proteins responsible for the storage, organization, and accessibility of DNA in the nucleus. Proteins from four histone families (H2A, H2B, H3, and H4) form an octamer consisting of two H3-H4 dimers and two H2A-H2B dimers upon which 166 nucleotides of DNA circle twice to form a nucleosome (Fig. 4.2). Proteins from a fifth histone family (H1) stabilize each nucleosome and link it to adjacent nucleosomes. Canonical histones are synthesized during S phase and incorporated into nucleosomes by chromatin assembly factor (CAF)-1-dependent mechanisms [7]. Histone variants may substitute for the core canonical histones in nucleosomes as genomic DNA is used during the cell cycle. Replication-independent histone variants are produced throughout the cell cycle and are inserted into nucleosomes by distinct molecular mechanisms involving histone regulator A (HIRA) [8]. Two canonical forms of H3 (H3.1 and H3.2) and the histone variant H3.3 have been linked to cancers [9]. Histone variant H3.3 varies from H3.1 and H3.2 by just five amino acids in the core domain

Fig. 4.2 Nucleosome structures. The nucleosome is the basic unit of organization and consists of eight histone proteins consisting of two H2A, H2B, H3, and H4 with two full loops of DNA. Together with a short amount of linker DNA that extends toward the next nucleosome and another histone protein type (linker histone H1), each histone octamer organizes approximately 200 bp of DNA. N-termini of histones extend from the nucleosome core. One tail from a H3 molecule is shown here. The amino acids changed by genetic mutations in bone tumors are indicated

and is present in open or active chromatin (i.e., euchromatin).

In addition to being scaffolds for genomic DNA, histones play an important communication role in cell nuclei as posttranslational modifications (PTM) generate codes that control gene transcription or other molecular events and allow cells to respond to their environment [10]. Canonical and variant histones within the nucleosome octamer are arranged such that their amino-termini can extend into the environment, away from the protein core and DNA (Fig. 4.2). This allows enzymes and other proteins to bind and posttranslationally modify select amino acids (e.g., lysines, arginines, and serines) in histones in response to environmental cues. Hundreds of PTMs exist [11], but methylation, acetylation, and phosphorylation are the best understood. In euchromatin (active or open chromatin), PTMs are added to histones by enzymes collectively referred to as "writers," removed by enzymes called "erasers," and functionally interpreted by proteins called "readers" [12, 13]. Writers and erasers typically have no DNA binding activity and are recruited to specific genomic regions by sequence-specific transcription factors. Readers are attracted by the presence or absence of the PTM and build a platform for the recruitment of other complexes that regulate gene expression and chromatin structure. The regulation of histone modifications allows for timely gene expression while maintaining nuclear structure.

4.3.1 Lysine Methylation of Histones

Methylation of histones H3 and H4 is achieved by histone methyltransferases (HMTase) and requires S-adenosyl methionine as the methyl donor [14]. Histone methylation can involve the transfer of one to three methyl groups on a single lysine or arginine residue (Fig. 4.3), resulting in mono-, di-, or tri-methylated states (me^1, me^2, and me^3, respectively). Methylation of specific lysines and arginines within histones controls transcriptional activation, repression, or elongation. For example, methylation of H3K4, H3K36, and H3K79 is typically associated with open chromatin and gene activation, while methylation of H3K9, H3K27, and H4K20 is associated with transcriptional repression of genes [15]. Beyond regulating gene expression, histone methylation may regulate other functions, such DNA repair and stability.

HMTases are recruited to specific regions of the genome by combinations of transcription factors, histone PTMs, DNA methylation, as well as noncoding RNAs [16–18]. HMTases are subdivided based on structure and function into three groups: (1) SET (Su(var)3-9, Enhancer of Zeste, Trithorax) domain-containing lysine methyltransferases, (2) non-SET-domain-containing lysine methyltransferases, and (3) protein arginine methyltransferases (PRMT). SET domain-containing HMTases demonstrate substrate specificity for lysines in histone tails, while HMTases lacking SET domains (e.g., DOT1) methylate lysines in the histone core sequence. The SET domain and flanking peptide sequences form flexible β-strand and β-sheet structures that can recognize many lysine substrates, including partially methylated lysines. The activity and/or expression of several HMTases are altered in many human cancers [19, 20]. In bone tumors, the functional activities of SET domain-containing HMTases, NSD2 and

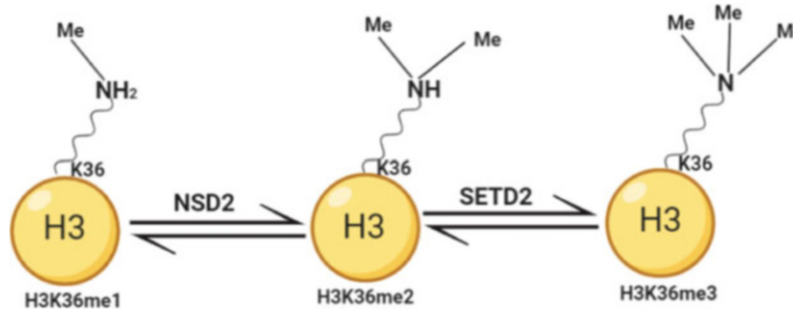

Fig. 4.3 The enzymatic activities of histone methyltransferases, NSD2 and SETD2, are reduced by oncohistones. NSD2 adds a second methyl group to H3K36me1 to create H3K36me2. SETD2 adds the third methyl group to H3K36me2 to generate H3K36me3

SETD2, are suppressed, and global histone H3K36 di- and tri-methylation are consequently reduced [21, 22].

4.3.2 Nuclear Receptor SET Domain-Containing (NSD) Methyltransferase 2

NSD2 is a SET domain-containing HMTase that is generally associated with open and active chromatin. It preferentially methylates the 36th amino acid (lysine) of H3 when it is incorporated in nucleosomes creating H3K36me2 and H3K36me3 (Fig. 4.3) but can also generate H3K4me3, H3K27me3, and H4K20me3 [23]. *NSD2* is one of several genes on the short arm of chromosome 4 that is deleted in Wolf-Hirschhorn syndrome (*WHSC1*), a condition affecting many tissues, including the skeleton, and that is characterized by significant growth and cognitive delays. Also known as *MMSET*, *NSD2* is also involved in a chromosomal translocation, t(4;14), present in 15% of patients with multiple myeloma, which is a hematopoietic malignancy of plasma cells that arises in the bone marrow. This chromosomal rearrangement places *NSD2* downstream of an immunoglobulin promoter, which drives its transcription and overexpression in B-lineage cells [24]. Myeloma cells harboring this translocation also have increased global levels of H3K36me2 [23, 25]. Overexpression of catalytically active NSD causes aberrant enrichment of H3K36me2 at normally silent oncogenes (e.g., MET, PAK1, RRAS2, TGFA), triggering increased expression of these tumor-promoting pathways and transformation of primary B cells [26]. In musculoskeletal tumors that will be discussed below, NSD activity is inhibited by mutant histones. Thus, proper regulation of NSD2 activity and H3K36 methylation is necessary for maintaining cellular homeostasis and preventing cancer.

4.3.3 Su(Var)3-9, Enhancer of Zeste, Trithorax Domain-Containing (SETD) Methyltransferase 2

SETD2 is an HMTase that preferentially methylates H3K36me2 to create H3K36me3, open chromatin regions, and promote DNA repair and chromosomal stability. SETD2 is the primary H3K36 tri-methyltransferase responsible for the bulk of H3K36me3 in most cell types [27] (Fig. 4.3). SETD2 is highly conserved from *Drosophila* to humans and contains three functional regions: (1) triplicate AWS-SET-PostSET domains, (2) WW domain, and (3) Set2 Rpb1 interacting (SRI) domain. The AWS-SET-PostSET domains mediate histone H3K36-specific activities by transferring methyl groups from S-adenosyl-L-methionine to the amino group of lysine residues in histones [27, 28]. The WW domain contains two tryptophan residues that are 20 amino acids apart and mediates interactions of SETD2 with other proteins containing PPxYpSPpTP sequences, including the Huntington disease protein [29, 30]. Abnormal expression of the WW domain in SETD2 has been linked to various

cancer types and Alzheimer's disease [31–33]. The SRI domain facilitates interactions with hyperphosphorylated RNA polymerase II and couples histone H3K36me3 with transcriptional activation and elongation [34]. Deletion of the SRI domain of SETD2 eliminates the interaction between RNA polymerase II and reduces H3K36me3 and transcription elongation [35].

Loss of function from mutations in SETD2 and mutations altering its prime substrate H3K36 has been linked in numerous solid tumors and chemotherapy resistance. Various cancers (clear cell renal cell carcinomas, T-cell lymphoma, breast cancer, and leukemia) harbor inactivating mutations in SETD2 [36]. SETD2 was deemed as a tumor suppressor after a missense mutation that inactivated the protein that was identified in clear cell renal cell carcinoma patients [37]. SETD2 levels were also significantly reduced in breast cancers compared to adjacent noncancerous tissue [38].

4.4 Histone Mutations in Bone Tumors

In a number of adult and pediatric cancers, somatic mutations in the *H2F2A* and *H3F3B* genes that encode H3.3 variants disrupt homeostatic control of histone PTMs, particularly histone methylation, by producing oncohistones that competitively inhibit the activity of HMTases, NSD2 and SETD2. The oncohistones cause chaos in the intricate processes controlling gene expression and consequently lead to activation of oncogenic pathways and/or inhibition of tumor suppressors. The amino acid changes in histone H3 proteins are highly specific to certain cancers. Recurrent H3K27M and H3G34V/R mutations have been discovered pediatric high-grade gliomas [39], and several years later, H3K36M and H3G34W/L are found in over 90% of chondroblastomas and giant cell tumors of bone, respectively [2, 3]. The effects of these mutations in bone cells are discussed below.

4.4.1 Chondroblastomas

Chondroblastomas are aggressive rare bone tumors that present with pain in long bones. They are thought to arise from immature chondrocytes within secondary ossification centers in the epiphyses of long bones (Fig. 4.4). Chondroblastomas do not produce normal cartilage. Rather the matrix surrounding individual chondroblasts becomes calcified, producing a chicken-wire pattern on x-ray. Chondroblastomas are typically removed surgically by curettage. The rate of chondroblastoma recurrence ranges from 5% to 40%. The overall survival prognosis for a patient diagnosed with a chondroblastoma in early stages is 80–90%.

Approximately 95% of chondroblastomas contain a heterozygous mutation in *H3F3B* that replaces lysine 36 with methionine (K36M) in the histone variant H3.3 [3]. Mutant H3K36M molecules are integrated into the genome at sites of active transcription and produce global reductions in H3K36 di- and tri-methylation (HeK36me2 and H3K36me3) in human chondrocytes [21, 22]. Recurrent H3K36M mutations reprogram the transcriptome in chondroblastomas by binding with high affinity to HMTases, NSD2/MMSET and SETD2, and inhibiting their ability to methylate H3K36 (Fig. 4.3). H3K36M did not affect other HMTases, ASH1L and NSD1. Crystal structures of SETD2 bound to H3K36M or H3K36I peptides show that the mutant residues are positioned into the catalytic sites of SETD2 where they block enzymatic activity [40]. Elucidating the specific role of the H3K36me2 interaction in stabilizing NSD2 will be important in further understanding the function of NSD2 and the H3K36me2 modification under normal physiologic conditions and also in musculoskeletal cancers.

In addition to changing H3K36 methylation patterns, increases in H3K27me3 patterns were observed in cells expressing the mutant H3K36M, suggesting that the loss of H3K36 methylation provides a nucleosomal substrate for PRC2 [22]. The overall consequence is altered

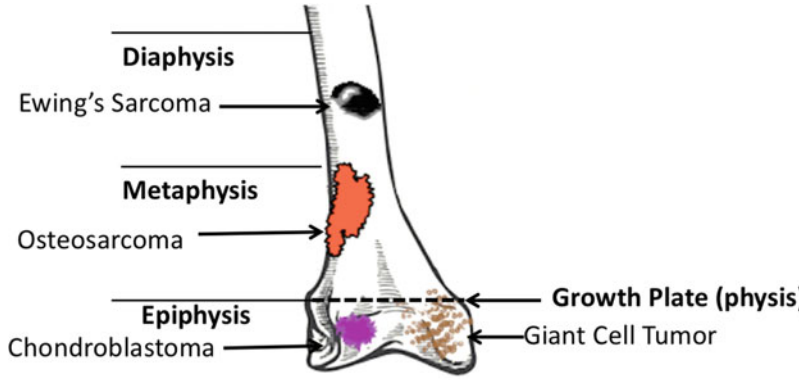

Fig. 4.4 Primary bone tumors present in specific regions of bone. Chondroblastomas and giant cell tumors are typically benign and occur in the epiphyses. Osteosarcomas are malignant tumors that produce immature bone in the metaphyseal region

expression of genes that support chondrocytic proliferation, colony formation, cell survival, and DNA repair, along with suppression of genes (e.g., BMP2, RUNX2) controlling chondrocyte differentiation [21, 22]. It is difficult to precisely replicate this human disease in mice because of its natural anatomical location in developing skeletons, but subcutaneous injection of mesenchymal progenitor cells expressing H3K36M into mice generated undifferentiated sarcomas in mice. These data as a whole demonstrate that H3K36M expression is a driver of neoplasia in mesenchymal cells of the skeleton.

4.4.2 Giant Cell Tumors of Bone (GCTB)

Giant cell tumors of bone are rare but locally aggressive cancers that cause pain and swelling and can destroy surrounding bones and joints. Though typically benign, some GCTBs produce lung metastases, and high-grade sarcomas can form near the benign GCTB [41]. GCTBs usually occur in the epiphyses of the long bones within the appendicular skeleton (Fig. 4.4) and are diagnosed by X-ray or other imaging techniques. These rare cancers (one per one million people) typically form near the knee of young adults (aged 20–40 years) but are also found in the hips, shoulders, wrists, and lower back. Giant cell tumors on average have a 16% mortality rate [42], and treatment options include surgical resection or curettage followed by bone grafting. Radiation and other treatments that can damage the affected joint are reserved for cases where surgery is not possible.

GCTBs are heterogeneous and consist of three cell types. Mesenchymal cells of the osteoblast lineage are the neoplastic component of the tumor and express mutant H3.3 proteins, as well as osteoblast products, osteocalcin, and alkaline phosphatase. The other two cell types (mononuclear histiocytic cells and multinucleated giant cells) originate from hematopoietic progenitors, express CD68 but not mutated histones, and serve to support the mutant osteoblastic cells. The giant cells that give the disease its pathological identity form as a result of fusion between several individual mononuclear cells into a single, larger cells. These large cells resemble osteoclasts and cause bone resorption and destruction.

Somatic *H3F3A* mutations have been linked to over 92 percent of GCTBs [3] and are a molecular marker that separates GCTBs from other tumor types, including more malignant and metastatic bone tumors (e.g., osteosarcomas). The majority of mutations in *H3F3A* alter G34 in H3.3 to W, creating H3G34W, but other substitutions (G34V, G34R, and G34L) have also been discovered [43]. These mutations are created by single-base-pair changes in *H3F3A* that convert the W codon (GGG) to Arg/R ("AGG" and "CGG"), Trp/W ("TGG"), Val/V ("GTG"), Glu/E ("GAG"), or Ala/A ("GCG") [21]. G34 itself is not posttranslationally modified, but it is a crucial

residue for enzymatic processes that affect other nearby residues in histone tails, including H3K36 [44]. Reductions in H3K36me2 levels were noted in cells expressing G34W by chromatin immunoprecipitation assays, even though NSD2 was bound to the mutant chromatin [45]. Thus, G34 mutations in GCTBs likely inhibit lysine HMTases specific to H3K36 (NSD1, NSD2) to reduce global H3K36 methylation levels. RNA processing was also blocked by the G34W substitution in H3.3 [46]. Thus, the normal functions of H3.3 fail when there is an accumulation of H3.3G34W substitutions, which leads to hyperproliferative activity and cancers in the epiphyses of long bones.

4.4.3 Other Bone Tumors

Since the discovery of mutant and oncogenic histones (oncohistones) in gliomas, chondroblastomas, and GCTBs, many other tumor genomes have been searched for histone gene variants. This effort identified additional skeletal tumors harboring such histone mutations but at very low relative frequencies. H3.1 K36M/I mutations were found in a case of pediatric undifferentiated mesenchymal sarcomas [22], and mutations in *H3F3A* and *H3F3B* that produce mutant H3.3G34R or H3.3G34W substitutions were found in less than one percent of osteosarcomas [43].

Osteosarcomas are malignant tumors of osteoblast origin that produce immature woven bone, which is mechanically weaker than normal lamellar bone. It is the most common type of cancer that arises in the skeleton and is usually found at the metaphyseal region of long bones. Most people diagnosed with osteosarcoma are under the age of 25 and are male. Patients with high-grade osteosarcoma in one location have a survival rate of about 68% [47]. Treatment usually includes a combination of surgery and chemotherapy. The presence of H3F3AG34W/R mutations in osteosarcomas is associated with epigenetic deregulation of oncogenic pathways such as PTEN [48]. The close relationship between *H3F3A* G34W/R mutant osteosarcomas and *H3F3A* G34W/L mutant GCBTs is consistent with a similar cellular origin from the osteoblastic lineage. More studies are necessary to determine how these H3F3AG34W/R mutations contribute to osteosarcoma pathogenesis and patient survival.

4.5 Therapeutic Opportunities for Bone Tumors Harboring Oncohistones

The discoveries that most chondroblastomas and GCTBs harbor histone mutations that block HMTase activity, alter histone methylation patterns, and promote cell proliferation and survival have not only allowed for stratification of tumor types but also stimulated discussion on alternative therapeutic options specifically for tumors expressing oncohistones. At least three strategies to eliminate these cancers exist: (1) eliminate the mutant *H3F3A* or *H3F3B* allele through selective gene editing or RNA editing; (2) prevent biochemical interactions between oncohistones and HMTases or HIRA proteins; and (3) modify the activity of genes whose expression are differentially expressed as a result of alterations in the histone methylome. The first two strategies will require further structural information on the histone genes and protein complexes involved, as well as identification of active and specific agents (either biomolecules or chemicals) and effective drug delivery mechanisms. The third strategy involves indirect targeting of the oncogenic driver but could be more rapid if existing drugs can be repurposed. Two promising examples of this strategy are described next.

Ribonucleotide reductase subunit M2 (RRM2) is essential for regulating cellular dNTP levels [49]. RRM2 expression declines in SETD2-deficient cells. The WEE1 kinase inhibitor, AZD1775, also reduces RRM2 levels and synergizes with SETD2-deficiency to further deplete cells of dNTP, leading to S phase arrest and cancer cell death. Thus, WEE1 inhibition can selectively kill SETD2- and H3K36me3-deficient cancers by starving them of

deoxyribonucleotides. It remains to be determined whether or not WEE1 inhibitors are efficacious on musculoskeletal tumors with H3K36me3 deficiency.

Understanding how oncohistones change gene expression profiles has led to new a therapeutic strategy for high-grade gliomas that harbor H3K27M mutations. In these tumors there is a global reduction in H3K27me2 and H3K27me3 levels but focal increases in H3K27me3 in genes associated with cancer progression [50]. Many gliomas with the H3K27M mutations overexpress the dopamine receptor D2 (DRD2). A DRD2/3 antagonist, ONC201, that blocks oncogenic AKT/ERK signaling pathways and anti-apoptotic in these tumors is currently in clinical trials for high-grade midline brain tumors [51]. If successful, this drug that can penetrate the blood-brain barrier will provide a much-needed option for inoperable brain tumors that have a poor prognosis. Moreover, this approach could be used to identify new therapies for bone tumors.

4.6 Conclusion and Perspectives

Advances in genome-wide sequencing technologies have facilitated the discovery of somatic histone mutations (oncohistones) in bone and brain cancers. These genetic mutations have further improved our understanding of epigenetics and mechanistic links between tumor epigenomes and cancer progression. Until recently, epigenomic and genetic alterations have been considered separate mechanisms contributing in tumorigenesis, but genomic sequencing has shown mutations in histone genes can drastically change the epigenome, leading to global chromatin chaos and activation of carcinogenic pathways within cells. Much remains to be learned about how H3K36 and H3G34 mutations drive the formation of chondroblastomas and GCTBs, as well as osteosarcomas and other musculoskeletal tumors. Furthering this knowledge could produce new therapies that eliminate these painful cancers and prevent fatal metastases.

Disclosures The authors have no conflicts of interest.

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Histone H3G34 Mutation in Brain and Bone Tumors

5

Lei Qiu and Junhong Han

Abstract

H3G34 mutations occur in both pediatric non-brainstem high-grade gliomas (G34R/V) and giant cell tumors of bone (G34W/L). Glioblastoma patients with G34R/V mutation have a generally adverse prognosis, whereas giant cell tumors of bone are rarely metastatic benign tumors. G34 mutations possibly disrupt the epigenome by altering H3K36 modifications, which may involve attenuating the function of SETD2 at methyltransferase. H3K36 methylation change may further lead to genomic instability, dysregulated gene expression pattern, and more mutations. In this chapter, we summarize the pathological features of each mutation type in its respective cancer, as well as the potential mechanism of their disruption on the epigenome and genomic instability. Understanding each mutation type would provide a thorough background for a thorough understanding of the cancers and would bring new insights for future investigations and the development of new precise therapies.

Keywords

Glioblastoma · Giant cell tumors of bone · Epigenome · Genomic instability · Oncohistones

5.1 Oncohistone H3G34R/V Mutation in Brain Tumors

H3G34 mutations mostly arise in brain and bone tumors. Depending on the tumor type, the amino acid substitutions are different. For example, *H3F3A* G34R/V mutations often occur in pediatric non-brainstem high-grade gliomas (HGGs), whereas *H3F3A* G34W/L mutations are usually observed in patients with giant cell tumors of bone (GCTB) [1–3].

H3G34R/V mutant tumors are typically located in the cerebral cortex in young adults with a median age of 18 years old, older than for H3K27M mutant tumors [4–7]. Almost all G34R/V mutations happened in *H3F3A*, and they almost always overlap with mutant TP53/ATRX [8]. However, one group in Japan demonstrated that G34R-mutant tumors might also occur in the basal ganglia and deep-seated region, away from the thalamus [9]. Meanwhile, they suggested that G34R/V mutations played a role in the pathogenesis of astroblastoma [9].

H3F3A G34-mutant HGGs arise as primary malignancies like most pediatric HGGs. There is no evidence for lower-grade precursors

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D. Fang, J. Han (eds.), *Histone Mutations and Cancer*, Advances in Experimental Medicine and Biology 1283, https://doi.org/10.1007/978-981-15-8104-5_5

[10]. H3G34R mutant cases are characterized by poorly differentiated/PNET (primitive neuroectodermal tumors) -like tumor cells with very high pleomorphism and high cell density [11]. MRI features of H3G34 mutant diffuse gliomas may be very heterogeneous, where some cases may not even fulfill the imaging criteria of HGGs [12].

Glioblastoma (GBM) patients with G34R/V mutation have a generally adverse prognosis, with reported median progression-free survival (PFS) of 8–9 months and overall survival (OS) of 12–22 months [13, 14], still better than the PFS and OS for other GBM molecular subtypes (not including IDH1-mutant tumors) [15–17]. This may be explained by the frequent methylation in *MGMT* promoter, which relates to boosted temozolomide responsiveness [14].

It is not clear why G34V/R mutation preferably occurred on *H3F3A* over *H3F3B* and why R is a much more frequent mutant than V, since the G34 codon is the same in both genes. Meanwhile, single point mutations can cause both R and V substitutions [18].

5.2 Oncohistone H3G34W/L Mutation in Bone Tumors

H3F3A mutations were also identified in more than 90% of GCTB, leading to G34W/L substitutions [2, 3]. GCTB are young adult benign tumors that are locally aggressive and may cause extensive bone destruction [19]. In most of the cases, H3G34W/L mutations were only found in stromal cells but not in osteoclasts or their precursors [2].

More recent studies have identified G34W mutations in a cancer syndrome (including pheochromocytomas, paragangliomas, and GCTB) [20] where G34W mutation is thought to occur post-zygotically during development rather than as a somatic mutation, since G34W mutation was detected in distinct tumor types within the same individual, whereas their germline *H3F3A* was wild type [18]. A study based on GCTB-derived primary cell lines as well as isogenic knock-in *H3F3A* G34W and WT cell lines has indicated that the H3.3 G34W mutation promotes cell proliferation [21].

H3.3 G34W mutation occurs much more frequently may be because it requires only one base substitution alone, whereas H3.1 G34W or H3.1/H3.3 G34L mutations require substitution of at least two nucleotides [22].

5.3 Impact of H3G34 Mutation on the Epigenome

The functional mechanisms of H3G34 mutations in DNA replication-coupled nucleosome assembly and/or gene transcription are less straightforward to interpret than the mechanisms proposed for H3K27M/K36M mutations. Although H3G34 cannot be posttranslationally modified, it sits only two residues from K36, a residue that undergoes methylation during transcriptional elongation, and four residues from P38, a residue that adopts conformational change to control K36 methylation [23]. Therefore, it is possible that H3G34 mutations may influence K36 accessibility to histone-modifying complexes, thus modifying H3K36 methylation or acetylation. Generally, H3G34 mutations locally block H3K36 methylation on the same and nearby nucleosomes but do not dominantly inhibit bulk H3K36me3 [1, 24–26].

It is possible that introducing a bulky residue (W) or a charged (R) in place of G34 may lead to changes in the accessibility or activity of K36 modifying enzymes or H3K36me3 readers [22]. Indeed, in vitro assay had revealed that G34 mutants were unable for H3K36 methylation mediated by SETD2 [27], suggesting that mechanisms of H3G34 and H3K36M mutations may be similar, where they regulate target gene expression by attenuating SETD2 function in transcriptional elongation (Fig. 5.1). Ectopic expression of Flag-H3.3G34W/L mutant also resulted in increased H3K27me3, reduced H3K27ac, and reduced H3K9me3 levels compared to WT H3.3 [26]. H3.3G34L cells showed a more remarkable H3K27me3 increase than that in H3.3G34W cells, whereas H3K27ac has a little bit change [26]. Consistent with H3K27me3 and

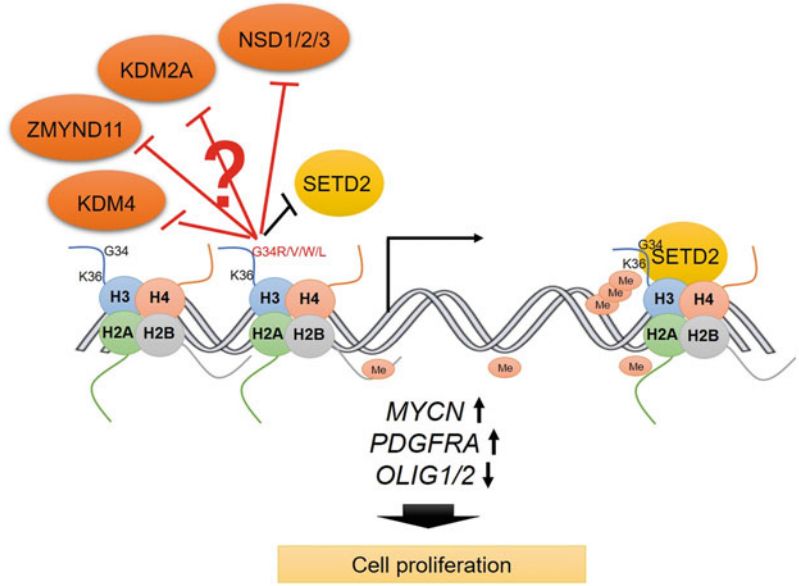

Fig. 5.1 A working model of the mechanism how G34 mutations might disrupt H3K36 methylation and gene expression

H3K36me3 changes, the binding of PRC1 (CBX8 and RING2) and PRC2 complex components (EZH2, SUZ12, and EED) is increased, while the binding of H3K36me3 reader (ZMYND11) to H3G34 mutants is reduced [26]. Interestingly, H3G34 mutations also inhibit histone binding to the H3.3 chaperone HIRA [26], whose complex member UBN1 is known to bind to the H3($A_{87}AIG_{90}$) segment that is far from the H3G34 region [28]. Subsequent studies also detected alterations in H3K27me3 and H3K9me3 in a subset of G34R mutant tumors [9, 26, 29]. G34 mutations generally do not affect methylation on H3K4 and H3K79 [26].

Fang et al. also showed that H3G34 mutations significantly reduced in vivo H3K36me2/me3 on the tail of mutant H3 and impacted in vitro function of SETD2, NSD1, and NSD2 [30]. In G34R mutant fission yeast model, Yadav et al. found that both H3K36me3 and H3K36ac levels decreased, whereas H3K36me2 amassed on the mutant tail [31]. It is difficult to separate out the effect of losing only K36me3 [18], since Set2 itself mediates all three levels of H3K36 methylation in fission yeast [32]. Mammalian studies showed consistent H3K36me3 reduction, suggesting that G34R disturbs SETD2-mediated K36 trimethylation. However, the accumulated H3K36me2 suggests that Set2 can still methylate the G34R-mutant H3 tail in fission yeast, consistent with results from ChIP studies showing that Set2 still interacts with G34R-mutant chromatin [31].

A sequencing study comparing pediatric HGGs to non-cancer samples identified *SETD2* mutations in 15% of pediatric HGGs [33], suggesting that disruption of H3K36 methylation is essential for HGG carcinogenesis. *SETD2* mutant HGGs localize to the cerebral hemispheres along with H3.3G34R/V mutant tumors and frequently contain *IDH1* mutations, suggesting that H3.3G34R/V and *SETD2/IDH1* mutations may function together to interrupt the modification of K36me3 in tumors [33].

Structural analyses revealed that the H3G34 residue was completely buried within a very narrow channel flanked by F1668 and Y1671 of SETD2, which could not hold a larger amino acid at position 34 [18, 30, 34, 35]. Although SETD2 and other SET domain methyltransferases share considerable sequence homology at this region [34, 35], F1668 and Y1671 seem to be essential for the size restriction of the G34 channel in SETD2 [18]. The reason why H3.3G34R/V mutant histones retained mono-methylation and some di-methylation of K36 may be explained by

the shorter side chains (Leu) at both positions in NSD1, 2, and 3, providing a larger channel in these methyltransferases [18, 25]. Surprisingly, while NSD1, 2, and 3 bind more strongly to the G34 mutant H3.3 than the wildtype nucleosomes, they are defective in K36 methylation on the G34 mutant histones [26]. Similar to SETD2, the H3G33-G34 motif occupies a narrow channel in KDM2A, a demethylase that has activity on K36me2/me1 but not on K36me3, G34 substitutions are thus predicted to attenuate KDM2A binding to H3 [36].

Since histones can be methylated on multiple arginine residues [37], Lowe et al. also tested if G34R mutant might provide a new methylation site on the histone tail [18]. They demonstrated in fission yeast that only 1% of the G34R peptide was methylated under enzyme excess conditions in vitro and the methylation on arginine residue of H3G34R mutant was not found in vivo [18]. Whether H3.3G34R is methylated in pediatric cortical HGGs is still obscure.

A recent study revealed that G34 mutants might also have dominant effects on H3 biology and sought to shed light on their mechanism [18]. The study conducted by Voon et al. found that H3G34R mutation in mouse embryonic stem cells triggered H3K36me3 increase at some genomic regions, though the overall H3K36me3 levels were unchanged [27]. They hypothesized that the K36me3 elevation was induced by KDM4 suppression and demonstrated in support that the transcriptional and K36me3 profiles of G34R-mutant cells were both similar to those of KDM4-deficient cells [27]. They also confirmed a correlation between the KDM4-binding sites and K36me3 accumulated regions in G34R cells [27]. Since KDM4 is a H3K9me3 demethylase, they noticed that H3K9me3 levels increased at KDM4-binding sites in G34R cells [27]. These results suggested a correlation between the loci altered by G34R mutation and the KDM4-binding sites.

One possible mechanism whereby this suppression in demethylase activity was that G34R mutation disrupted KDM4 interaction with H3.3 [18]. In consistence to this hypothesis, in vitro demethylation assays revealed that G34R mutant suppressed the KDM4A, B, and C demethylase activity toward H3K36me3 [27]. Structural studies indicated that the interaction between H3.3G34R mutant and KDM4 was weak, since KDM4 active site could not hold an amino acid bigger than glycine [38, 39]. This suggests that the reduction of KDM4 activity at its target genes may result in H3K36me3 enrichment and trans-effects on the chromatin landscape [18]. However, KDM4 immunopurification assay with cells expressing exogenous histones showed that KDM4s bound to G34R more tightly compared to wild-type H3.3 [27], indicating that KDM4 accumulation at G34R deposition sites might decrease H3K36me3 at these sites [18]. Therefore, KDM4 enrichment might be working in combination with the reduction of SETD2 activity at H3.3G34R loci, to guarantee a robust K36me3 loss [18].

ZMYND11, a tumor suppressor and a H3K36me3 reader, is another interesting protein that may be influenced by the changed local H3K36 methylation state [40, 41]. Although the function of ZMYND11 in pediatric HGGs or GCTB has not been demonstrated yet, it is found that H3G34V/R mutations disrupt the interaction between ZMYND11 and the H3.3K36me3 peptide [41]. ZMYND11 delocalization may contribute to the tumorigenesis of H3K36M and H3G34 mutant tumors by locally decreasing H3K36 methylation [22].

It is important to elucidate the detailed mechanisms of how H3G34 mutants affect the activities of SETD2 and other H3K36 methyltransferases, and how they may contribute to tumorigenesis. The reason why G34 instead of other K36 neighboring residues is targeted remains unknown. It would also be great curious to investigate whether ZMYND11 or SETD2 mutation arises in these tumor types and if their knockdown in the suitable cell types may recapitulate features of the diseases.

5.4 H3G34 Mutation and Genomic Instability

H3K36 methylation is important for genomic stability, since H3K36 modification may influence the choice of DNA damage repair pathway

between non-homologous end-joining (NHEJ) and homologous recombination (HR) [42]. H3K36 methylation status may also determine the timing of origin activity during DNA replication [43] and manipulate the mismatch repair pathway by recruiting hMutSα, a mismatch recognition complex, onto chromatin [44]. H3G34R/V mutant cells demonstrated slightly elevated mutation frequency, consistent with a reduced amount of chromatin-bound MSH6 due to decreased MSH6 affinity for binding the mutant H3 tail and K36me3 reduction in H3G34R/V cells [30]. Microsatellite instability is very high in pediatric HGGs [45]. A recent study has revealed that histone chaperon HIRA restores transcription after DNA damage repair by depositing the H3.3 histones into ultraviolet C (UVC) radiation-damaged regions [46]. Chicken bursal lymphoma DT40 cells harboring G34R/V mutant H3.3 or lacking histone H3.3 are sensitive to UV [47]. Interestingly, G34 and K36 mutations occur exclusively in H3.3 which plays a critical role in chromatin repair [48]. Pediatric HGG is distinguished by frequent somatic coding mutations, suggesting possible DNA damage repair deficiency [49]. Therefore, it may be beneficial to further investigate the influence of H3G34 mutations in cancer DNA damage pathways [22].

The pediatric HGG bearing H3G34V mutation has a great change of the genome-wide H3K36me3 pattern, leading to an altered transcriptional signature. ChIP-seq data identified 156 genes that were differentially enriched in RNA polymerase II and H3K36me3 in the KNS42 cell line (derived from a H3G34V mutant pediatric HGG patient) when compared to a *H3F3A* wild-type pediatric GBM cell line [1]. This gene set was enriched for regulators of forebrain and cortical development (*DLX6* and *FOXA1*) that were upregulated from embryonic and early fetal time points and downregulated by mid-late fetal development [50]. H3G34W expression provokes transcriptional dysregulation and altered splicing patterns, causing frequent exon inclusion, which may lead to freak transcript stability, open reading frame extension, and alternative start site usage to promote cell proliferation [21]. Aberrant H3K36me3 and RNA polymerase II enrichment may also result in the transcription of oncogenes or micro-RNAs with oncogenic functions as well as prevent the expression of tumor-suppressor, thus boosting the growth of the respective tumors [51]. Gene expression analyses also uncovered gene expression patterns that were different in samples with the H3K27M mutation versus samples with the H3G34R/V mutation, suggesting that each mutation favors a specific gene expression signature [51].

One of these dysregulated genes is the *MYCN* oncogene (Fig. 5.1). It has been shown that the forced overexpression of stabilized MYCN protein in neural stem cells of the developing mouse forebrain had an ability to produce GBMs [52]. Studies have also demonstrated the mechanism by which the initiating tumorigenic insult is delivered at the correct time and place [53] during neurogenesis. Therefore, targeting MYCN protein stabilization through inhibiting the responsible kinases in *H3F3A* G34-mutant pediatric GBM provides a potential novel approach to treat this subgroup of patients [1]. The potential link between H3.3 and MYCN is remarkable given the association of H3.3 with actively transcribed genes and the function of MYCN in the maintenance of global euchromatin in neural stem cells [54] and neuroblastoma [55].

All H3.3G34 mutant tumors are found to also bear the inactive mutations in the ATRX (alpha thalassemia/mental retardation syndrome X-linked protein)/DAXX (death domain-associated protein) chaperone complex, which facilitates H3.3 deposition into telomeric and pericentromeric regions. Demethylated DNA were found at chromosome ends in H3G34R/V mutant groups, indicating a connection between H3G34R/V mutations and ALT [1]. Therefore, H3G34R/V mutations may not simply alter H3K36me3 levels and activate potential cancer driver genes, but mutations in ATRX/DAXX or H3.3 may interrupt their proper interaction, resulting in abnormal H3.3 deposition near telomeric regions and causing ALT [56]. It is also found that partial H3.3 functional loss in the mouse led to genomic instability [57] and H3.3 is linked to functional complexes involved

in DNA double-strand break repair (DDR), HR [17, 58], and somatic hypermutation [59].

G34 mutant tumors tend to also have high frequency on *TP53* (88%) and *ATRX* (95%) alterations, as well as *PDGFRA* amplification (approximately 27%), 2q loss (67%), and 4q loss (70%) [6, 60]. Cytogenetic analysis comparing GBM subtypes revealed a highly frequent and specific 3q and 4q loss among H3G34 mutant tumors [14].

PDGFRA amplification was more frequently found in GBM cases than in cases with primitive neuroectodermal tumors (PNET) morphology (36% vs. 5%, respectively), whereas an opposite trend was found in *CCND2* amplification (5% vs. 27%) [14]. It is reasonable to speculate that the variability of these high copy number aberrations may contribute to the morphological heterogeneity in H3G34 mutant tumors [14]. But then, these aberrations are not general molecular events since they were identified in only less than 1/3 of the studied tumors [14].

H3.3G34 mutations are associated with global DNA hypomethylation, particularly prominent in sub-telomeric regions [61]. Promoter methylation differences led to differential expression of genes in a tumor origin-dependent manner, suggesting that both DNA methylation and gene expression may be a consequence of tumor origin altered by histone mutations [1, 61–63].

Despite the fact that DNA hypomethylation is typically related to increased gene expression, G34 mutations are responsible for downregulating the expression of differentiation genes [61]. Among these genes are a couple of members from the Olig transcription factor family [64]. For example, *OLIG1/2* hypermethylation leads to low OLIG1/2 expression levels in these tumors (Fig. 5.1). This pattern is very similar to that of embryonic stem cells, where epigenetic suppression of OLIG1/2 has been proposed as a barrier of neural lineage commitment [65]. Olig-2 is involved in early central nervous system development and linked to oligodendroglial differentiation. Moreover, it is commonly expressed in glioma cells and is considered as a glial differentiation marker [66]. In Schafer's study, using immunohistochemistry, they analyzed the nuclear Olig-2/FoxG1 expression in a large series of gliomas and found that K27M-mutant tumors carried a FoxG1low/Olig-2high and G34-mutant tumors showed a FoxG1high /Olig-2low profile, which was in accordance with previous data reporting in eight K27M-mutant tumors a Olig-2 +/FoxG1-immunoprofile and in six G34-mutant tumors an Olig-2-/FoxG1+ profile [61, 67].

Nevertheless, Olig-2 expression has recently been found in a low metastatic CNS-PNET subgroup (defined as "oligo-neural" group) as well [68]. Furthermore, the H3G34 mutant mRNA signature seems to demonstrate transcriptional patterns of early CNS developmental stages, suggesting a specific cellular origin and tumor initiation time [14]. Therefore, it may be indicated that the driving H3G34 mutation impacts poorly differentiated neuroepithelial embryonic cells or progenitors, which would develop either glial or neuronal differentiation patterns, respectively, hence explaining the morphological heterogeneity of H3G34 mutant HGG [14]. It is also possible that this particular expression pattern is an outcome from the reprogramming of the H3G34 mutation itself [14].

Chromatin is critical and fundamental for the control of DNA replication, DNA damage repair, gene transcription, and other aspects of genomic stability, including maintenance of telomere integrity and high-fidelity chromosome segregation during cell division [18]. H3.3G34 mutations disrupt H3K36 methylation, RNA polymerase II enrichment, and DNA methylation, leading to aberrant gene expression signature, DNA damage repair, and possibly disrupted chromatin stability and ALT. Further studies would be beneficial since detailed mechanisms of how H3.3G34 mutations dysregulate these biological processes are still unclear.

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Epigenetic-Targeted Treatments for H3K27M-Mutant Midline Gliomas

6

Victor M. Lu and David J. Daniels

Abstract

Diffuse intrinsic pontine glioma (DIPG) is a lethal midline brainstem tumor that most commonly occurs in children and is genetically defined by substitution of methionine for lysine at site 27 of histone 3 (H3K27M) in the majority of cases. This mutation has since been shown to exert an influence on the post-translational epigenetic landscape of this disease, with the loss of trimethylation at lysine 27 (H3K27me3) the most common alteration. Based on these findings, a number of drugs targeting these epigenetic changes have been proposed, specifically that alter histone trimethylation, acetylation, or phosphorylation. Various mechanisms have been explored, including inhibition of H327 demethylase and methyltransferase to target trimethylation, inhibition of histone deacetylase (HDAC) and bromodomain and extraterminal (BET) to target acetylation, and inhibition of phosphatase-related enzymes to target phosphorylation. This chapter reviews the current rationales and progress made to date in epigenetically targeting DIPG via these mechanisms.

Keywords

Diffuse intrinsic pontine glioma · DIPG · H3 K27M · DMG · Trimethylation · Phosphorylation · Acetylation · Demethylase · HDAC · BET

6.1 Introduction

Diffuse intrinsic pontine glioma (DIPG), now characterized by the World Health Organization (WHO) as diffuse midline glioma of the brainstem with H3K27M mutation, is a devastating disease that predominately affects young children [1]. Although rare, DIPG is one of the leading causes of cancer-related morbidity and mortality in children, and despite best practice of radiation therapy, the tumors inevitably progress with average survival of less than 12 months [2–4]. Therefore, there is a great need to investigate possible other therapeutic modalities that may have a greater impact on prognosis.

The establishment of stereotactic surgery in recent years as a safe approach in well-selected patients has allowed us to explore the molecular nature of DIPG, which historically was viewed as unnecessary by some due to the lethality of this diagnosis. Molecular analyses of the biological tissue has uncovered the vast majority of these tumors possess a histone H3 gene mutation that most commonly occurs at either H3.1

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D. Fang, J. Han (eds.), *Histone Mutations and Cancer*, Advances in Experimental Medicine and Biology 1283, https://doi.org/10.1007/978-981-15-8104-5_6

(HIST1H3B/C) or H3.3 (H3F3A) and results in a H3K27M mutation (histone 3 lysine substitution for methionine at site 27 (H3K27M)) [5, 6]. This specific mutation has since been established to prognosticate a worse prognosis in pediatric brain tumors overall [7]. In the largest pretreatment biopsy study to date, mutations in these genes were detected by sequencing in 57 of 62 (92%) subjects diagnosed with presumed DIPG based on radio-clinical parameters [6]. These findings reaffirmed the previous clinical reports of the H3K27M mutation being prototypical of DIPG and confirmed its pathognomonic significance [5, 8]. It was this accumulating evidence that eventually gave way to WHO reclassification of DIPG tumors in 2016 [1]. Not only have these relatively recent advancements in our genetic understanding of DIPG uncovered the growing relevance of the H3K27M mutation in these tumors, they have also uncovered a number of novel epigenetic modifications to residues of the H3 histone tail that likely contribute to the tumorigenic burden of this disease [9–11].

6.2 The Histone and Epigenetic Landscape

Understanding the biology of the H3K27M mutation, and the subsequent posttranslational modifications (PTMs), is required to develop effective targeted therapies for DIPG. In brief, histones are the primary protein component of chromatin in eukaryotic cells, synthesized during DNA synthesis. DNA is packaged around a histone core made of eight histone bodies, and this octamer is able to help regulate DNA transcription activity based on selective and reversible PTMs found on the tails of the histone bodies [12, 13]. These PTMs effectively act as molecular switches for the genes and their transcription, which may be altered in cancers based on the presence of aberrant PTMs [14]. At the H3 N-terminal tails, PTMs include methylation at lysine and arginine sites, acetylation at lysine sites, and phosphorylation at serine sites [14–17]. There is a paradigm within the histone literature to refer to enzymes that facilitate/catalyze these PTMs as "writers," e.g., methyltransferases and histone acetyltransferases (HATs), and to enzymes that act to remove PTMs as "erasers," e.g., demethylases and histone deacetylases (HDACs) [18]. Finally, there is another set of enzymes termed "readers," which recognize and translate these PTMs into cellular programs [19]. The rationale in targeting these epigenetic enzymes, primarily that of writers and erasers, is to alter the epigenetic landscape of DIPG tumors in such a way their associated tumorigenic tendencies can be silenced or even reversed.

Biologically, the H3K27M mutation in DIPG tumors results in significant epigenetic changes in methylation and acetylation at multiple H3 sites [20, 21]. There is effectively a global loss of H3K27 trimethylation (H3K27me3) when the H3K27M mutation is present, which was the earliest detected, and most defined, epigenetic change in DIPG and observed in 100% of the H3K27M cases (Fig. 6.1) [6, 9, 15, 22]. This epigenetic change, along with other PTMs such as acetylation at H3K27 (H3K27ac) and alterations in phosphorylation at adjacent H3S28 (pH3S28), is theorized to accommodate and drive the tumorigenic burden of DIPG [15–17, 23]. Baker and colleagues [21] recently demonstrated that targeted H3K27M depletion restores H3K27me3, increases differentiation, and extends latency of diffuse intrinsic pontine glioma growth in vivo, highlighting the significance of this mutation and its consequent epigenetic alterations. Given the lack of alternative mutations characteristic for this type of tumor, targeting the altered epigenetic landscape presents a worthy therapeutic approach to ameliorate the dismal prognosis of DIPG (Table 6.1), with one avenue being the reversal of associated epigenetic changes such as H3K27me3 restoration.

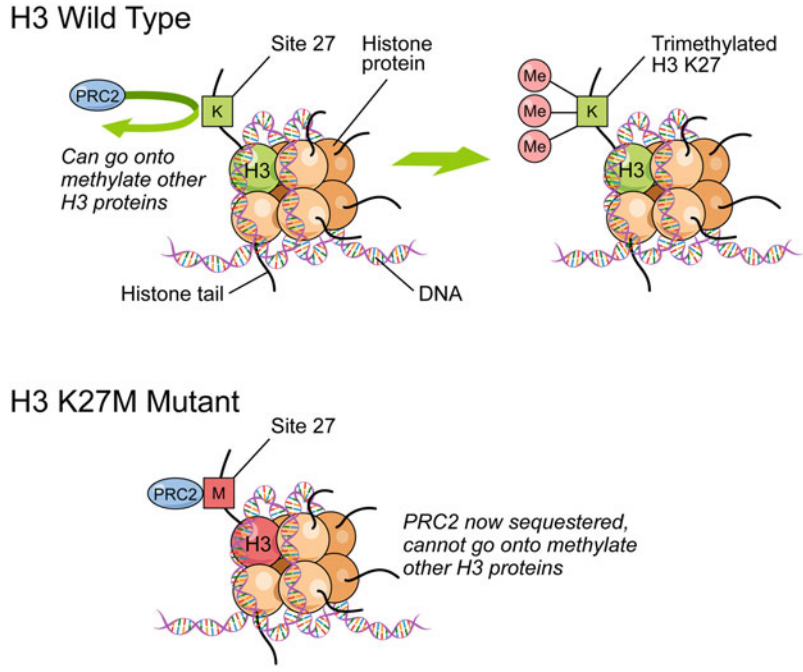

Fig. 6.1 Epigenetic consequence of the H3 K27M mutation. In the H3 wild type, the PRC2 complex is able to (tri-) methylate the lysine (K) at site 27 of the histone tail of histone 3 (H3 K27). When the H3 K27 is substituted for a methionine (M), i.e., the H3 K27M mutation, the PRC2 complex is sequestered, meaning that it is no longer available to methylate other H3 proteins at the K27 site, resulting in global loss in H3 K27 trimethylation

6.3 H3K27M and Lysine Methylation

6.3.1 Molecular Biology of Lysine Methylation

The specific residue at which lysine methylation occurs dictates the biological consequence of the PTM, as across the H3 body there are distinct transcriptional sites. Methylation at H3K4 and K36, for instance, are typically associated with transcriptionally active chromatin, whereas methylation at H3K9 and H3K27 are more associated with transcriptionally repressed chromatin and gene expression [20]. With respect to DIPG, H3K27 is primarily methylated by histone-lysine *N*-methyltransferase enhancer of zeste homolog 2 (EZH2), a component of the polycomb repressive complex 2 (PRC2), and is demethylated in its trimethylated form by the KDM6 subfamily K27 demethylases jumonji domain-containing 3 (JMJD3) and ubiquitously transcribed tetratricopeptide repeat, X chromosome (UTX) [24–27]. When trimethylated (H3K27me3), this H3 PTM is recognized by the PRC1 complex which represses transcription by multiple mechanisms, including ubiquitination of H2A at K119 and compaction of chromatin [18]. The loss of H3K27me3 due to the inhibition of PRC2 activity by the H3K27M mutation in DIPG cells therefore alleviates transcription repression of potential oncogenes to increase tumorigenic burden [9]. Therefore, reversal or restoration of the H3K27me3 loss, by either augmenting repressed PRC2 methyltransferase activity or inhibiting JMJD3/UTX demethylase activity, is a potentially targeted therapeutic approach for H3K27M tumors.

6.3.2 Targeting Methylation

6.3.2.1 Demethylase Inhibition

To date, the attempts to target the loss of H3K27me3 in DIPG have focused primarily on inhibiting demethylase activity. David James and colleagues [28] reported the first, and to date only, evidence of how directly targeting lysine methylation in DIPG can be of therapeutic benefit by

Table 6.1 Details of relevant publications targeting the H3K27 epigenome of the H3K27M-mutant midline gliomas

Study	Location	Epigenomic target	Intervention	Mechanism	Diagnosis (es)	Primary conclusion(s)
Hashizume et al. 2014 [28]	Chicago, USA	Lysine methylation	GSKJ4	Demethylase (JMJD4) inhibitor	DIPG	Favorable antitumor effects in vitro and in vivo
Mohammad et al. 2017 [31]	Copenhagen, Denmark	Lysine methylation	GSK343 and EPZ6438	Methyltransferase (EZH2) inhibitor	DIPG	Favorable antitumor effects in vitro and in vivo
Wiese et al. 2016 [35]	Goettingen, Germany	Lysine methylation	EPZ6438	Methyltransferase (EZH2) inhibitor	DIPG and H3K27M-wild-type glioma	Similar antitumor effects in vitro between DIPG and H3K27M-wild-type glioma
Grasso et al. 2015 [44]	Portland/ Stanford, USA	Lysine acetylation	Panobinostat and vorinostat	HDAC inhibitor	DIPG	Favorable antitumor effects in vitro with H3K27me3 restoration
Hennika et al. 2017 [49]	Durham, USA	Lysine acetylation	Panobinostat	HDAC inhibitor	DIPG and H3K27M-wild-type glioma	Similar antitumor effects in vivo between DIPG and H3K27M-wild-type glioma
Piunti et al. 2017 [23]	Chicago, USA	Lysine acetylation	JQ1	BET inhibitor	DIPG	Favorable antitumor effects in vitro and in vivo
Zhang et al. 2017 [56]	Guangzhou, China	Lysine acetylation and methylation	JQ1 and EPZ6438	BET inhibitor and methyltransferase (EZH2) inhibitor	DIPG	Favorable antitumor effects in vitro and in vivo

their work testing GSKJ4 in vitro and in vivo. GSKJ4 was developed by GlaxoSmithKline as the first small-molecule catalytic site inhibitor that was selective for the H3K27me3-specific JMJ subfamily and was first tested in T-cell acute lymphoblastic leukemia with encouraging results [27, 29]. Of great interest was in addition to reduced cell proliferation in DIPG, James and colleagues [28] demonstrated that the GSKJ4 downregulated genes experience restoration of H3K27me3 upon treatment, signaling its translational therapeutic potential.

James and colleagues [28] found that when exposed to GSKJ4, multiple H3K27M-mutant DIPG cell lines demonstrated evidence of decreased cell viability, increased S-phase arrest, increased apoptosis, and decreased clonal growth when compared to wild-type gliomas, H3G34V-mutant gliomas, and isogenic human astrocyte cell lines. Specificity for JMJD3 in DIPG was confirmed by siRNA depletion studies, with similar results found only when JMJD3 was targeted, but not UTX. In orthotopic H3K27M xenograft models, GSKJ4 resulted in not only decreased cell proliferation and increased apoptotic activity within the tumor but also prolonged survival compared to H3K27-wild-type models. Most relevant, postmortem examination of these tumors demonstrated significant H3K27me3 restoration confirming on target drug effects with GSKJ4 treatment. To date however GSKJ4 is not FDA-approved, and thus despite these promising results, there has been no clinical trial established to investigate how these effects could translate in DIPG patients, or any other pediatric cancer for that matter.

6.3.2.2 Methyltransferase Inhibition

An extension of targeting lysine methylation in DIPG has been inhibiting the EZH2 methyltransferase component of the PRC2 complex. Although this may initially present as counterintuitive to the paradigm of H3K27me3 restoration, it derives from the observation that there are still low levels of H3K27me3 in H3K27M mutant tumors which EZH2 is responsible for [15]. The significance of this is that the retained trimethylated regions are posited to contribute to the transcriptional program specific for K27M-mutant DIPG by means of increasing EXH2 affinity at oncogene promoter regions [30, 31]. It has been proposed that this locus-specific retention of H3K27me3 acts together with the more universal decline of H3K27me3 to promote tumorigenesis by a concerted silencing of tumor suppressor genes, such as with EZH2 and silencing of p16 [9, 23, 32]. The precise mechanism of this phenomenon remains to be fully elucidated.

Mohammad et al. [31] reported that small-molecule inhibition of EZH2 by GSK343 and EPZ6438 [33, 34] resulted in favorable antitumor effects in vitro and in vivo using patient-derived H3K27M-mutant DIPG cell lines, with the expected loss of H3K27me3 at sites that had originally retained methylation despite having the H3K27M mutation. However, the significance of this aspect in targeting methylation modifications in DIPG remains unclear, for these implications are not universal. Wiese et al. [35] reported that in their in vitro studies, using one of the same EZH2 inhibitors EPZ6438, this did not result in any significant cytotoxic difference in H3K27M-mutant DIPG and H3K27-wild-type pediatric glioma cell lines. These differences may be explained in part by varying EZH2 expression levels in DIPG.

A degree of caution is recommended when considering the targeting of EZH2 until the role of the retained H3K27me3 in DIPG is better distinguished from that of the lost H3K27me3. This is because although overexpression of EZH2 may restore H3K27me3 at lost H3 sites in DIPG, it also has been correlated with tumor cell proliferation and invasive growth in multiple adult cancers [36], including brain tumors [37]. Admittedly, even though evidence in pediatric brain tumors is more scarce, anecdotal reports of similar trends in medulloblastoma by Taylor and colleagues [38] would indicate alternative pathways that increase tumorigenesis independent of H3K27me3 restoration following EZH2 inhibition is not an impossible scenario in the setting of DIPG.

6.4 H3K27M and Acetylation

6.4.1 Molecular Biology of Lysine Acetylation

Acetylation of H3 lysine sites reduces the charged attractions between DNA and the histone core within the nucleosome, resulting in greater exposure of DNA in an open chromatin structure which becomes more susceptible to active transcription [39]. Acetylation is achieved by "writer" HATs, and this occurs in natural antagonism to "eraser" HDACs, which remove this PTM. Typically, HATs associate themselves with active genes of transcription, and HDACs associate themselves with inactive genes. The relevance of H3 acetylation is that it is able to recruit specific bromodomain and extraterminal (BET) proteins, which contain bromodomain-containing 4 (BRD4), that recognize acetylated H3 lysines including H3K27ac [40]. Subsequently, transcription initiator cofactors are recruited to respective promoters and ultimately lead to active transcription after phosphorylation by RNA Pol II [41]. Although not clear, it has been suggested that aberrant acetylation at H3K27 in the presence of H3K27M contributes to DIPG tumorigenesis by dysregulating the PRC2 balance between the oncogenes involved in maintaining an undifferentiated state versus oncogenes involved in the differentiation process [23, 42, 43]. There is evidence to suggest that increase in H3K27ac results in partial restoration of the H3K27me3 loss in DIPG by means of alleviating H3K27M-induced PRC2 inhibition, leading to investigations if H3K27ac can be augmented

Table 6.2 Details of relevant clinical trials targeting the H3K27 epigenome of the H3K27M-mutant midline gliomas

Clinical trial identifier	Epigenomic target	Intervention	Phase	Organizing site	Diagnosis	Start date	Finish date	Target size	Current status[a]
NCT02717455	Lysine acetylation (HDAC)	Panobinostat	I	St. Jude Children's Research Hospital, USA	DIPG	June 2016	October 2019	40	Recruiting
NCT03566199	Lysine acetylation (HDAC)	Panobinostat by CED	I/II	University of California, San Francisco, USA	DIPG	May 2018	September 2020	24	Recruiting
NCT03632317	Lysine acetylation (HDAC)	Panobinostat with everolimus	II	University of Michigan, USA	DIPG	June 2019	September 2025	32	Recruiting
NCT02420613	Lysine acetylation (HDAC)	Vorinostat with temsirolimus	I	M.D. Anderson Cancer Center, USA	DIPG	October 2015	October 2020	18	Recruiting
NCT01189266	Lysine acetylation (HDAC)	Vorinostat	I/II	National Cancer Institute, USA	Pediatric high-grade glioma	August 2010	November 2020	80	Active, not recruiting
NCT02296476	Lysine acetylation (BET)	MK-8628 (OTX015)	II	Private, USA	Glioblastoma	October 2014	October 2015	12	Terminated due to lack of activity

[a]As of June 2019

therapeutically to rescue the H3K27M-induced H3K27me3 loss phenotype and result in antitumor effect for DIPG [44, 45].

6.4.2 Targeting Acetylation

6.4.2.1 HDAC Inhibition

The use of HDAC inhibitors has emerged in the cancer field as a potent potential therapy in many cancer types [46, 47]. Correspondingly, there has been much hope that such promising responses could be translated for DIPG patients, in particular given its association with H3K27me3 restoration.

Panobinostat (LBH589) is the most investigated HDAC inhibitor in the DIPG world, with it being a Food and Drug Administration (FDA)-approved nonselective small molecule trialed originally in hematological malignancies with encouraging clinical outcomes [48]. Grasso et al. [44] screened 83 compounds across 14 patient-derived DIPG cell lines and observed sensitivity of multiple cell lines to HDAC inhibitors, with panobinostat the most effective in terms of reducing cell viability. These results showed for the first time since the characterization of the H3K27M mutation that this class of epigenetic therapy may also be of benefit in DIPG, and the epigenetic rescue of H3K27me3 by increasing H3K27ac was validated in their in vitro studies; however the mechanism by which this happens is currently not known [45]. Subsequent in vivo modelling by Grasso et al. [44] showed that in orthotopic H3K27M-wild-type DIPG xenografts, systemic panobinostat led to both tumor volume reduction and prolonged survival compared to vehicle-treated controls. Unfortunately, they did not report the outcomes of in vivo H3K27M-mutant xenografts. However, they did demonstrate synergy between panobinostat and demethylase inhibitor GSKJ4 in H3K27M-mutant DIPG, highlighting the potential for combinatorial therapy and epigenetic interplay to lead to greater therapeutic impact in DIPG.

Next, Becher and colleagues [49] sought to extend the findings of Grasso et al. [44] by utilizing a genetically engineered mouse model (GEMM) of DIPG driven by H3.3-K27M expression with known global loss of H3K27me3, which was thought to be another biological model in which therapeutic hypotheses could be validated. Although they did observe increased H3K27ac in response to the therapy, they also observed multiple results that would temper the implications of the prior study. First, panobinostat showed efficacy against all patient-derived and murine DIPG and brainstem glioma cell lines, irrespective of H3 mutation status. Second, in vivo, extended consecutive daily treatment of both genetic and orthotopic xenograft models with 10 or 20 mg/kg systemic panobinostat consistently led to significant toxicity. Third, when reduced to well-tolerated doses of panobinostat, there was no overall survival difference compared to vehicle-treated GEMM models. Taken collectively, the work by Becher and colleagues [49] suggested that although there may be promise in HDAC inhibition to treat DIPG, this may not be specific to the epigenetic landscape of DIPG and, furthermore, compounded by the universal importance of histone acetylation in normal physiology, that off-target effects following systematic administration cannot be discounted.

Nevertheless, due to the initial encouraging results of panobinostat found by Grasso et al. [44], as well in clinical trial of other cancers [50, 51], a number of clinical trials (clinicaltrials.gov) have been established to investigate whether or not the theorized epigenetic targeting benefits of HDAC inhibition in DIPG can be translated into the clinic (Table 6.2). In brief, NCT027171455 (previously identified as NCT02899715) is a Phase I trial and was the first trial of panobinostat in DIPG spurred by the results of Grasso et al. [44], investigating safety and feasibility of systemic delivery of panobinostat by means of dose escalation; NCT03566199 is a Phase I/II trial evaluating panobinostat in nanoparticle formulation delivered to the brainstem by means of convection-enhanced delivery for DIPG patients with history of radiation therapy; and NCT03632317 is a Phase II trial investigating systematic delivery of 30 mg/m^2 panobinostat in combination with

3 mg/m^2 kinase inhibitor everolimus in newly diagnosed DIPG. All three trials are US-based and are currently active and recruiting at the time of writing.

It is worth noting finally that another FDA-approved HDAC inhibitor vorinostat (suberanilohydroxamic acid) had also been identified as a therapy of interest in DIPG from a 51-drug-screen as 1-of-8 nonmolecularly charged, lipophilic, and relatively small-sized drugs likely to passively diffuse through the blood brain barrier (BBB) [52]. Although Grasso et al. [44] showed vorinostat possessed antitumor effects in DIPG models, these effects were not as pronounced as that of panobinostat, with concerns emerging for how effectively the drug did penetrate the BBB. Nevertheless, there are two active US-based clinical trials examining the therapeutic benefit of vorinostat in DIPG – NCT02420613 is a Phase I trial investigating vorinostat in combination with kinase inhibitor temsirolimus in newly diagnosed and progressive DIPG, and NCT01189266, a Phase I/II study investigating vorinostat in combination with local radiation therapy in newly diagnosed DIPG only.

6.4.2.2 BET Inhibition

The BET bromodomain of BRD4 recognizes H3K27ac and binds to the exposed chromatin structures caused by the acetylation to activate transcription [41, 53]. It has been hypothesized that competitive binding of BET inhibitors to the bromodomain pocket can displace the BRD4 from active chromatin of H3K27ac in DIPG cells, therefore resulting in the inactivation of pertinent oncogene transcription [41, 54].

Piunti et al. [23] investigated in their works the application of BET inhibitor JQ1 [55] to H3K27M-mutant DIPG, which similar to other tested epigenetic therapies was first evaluated in the setting of hematological malignancies with encouraging in vitro antitumor effects and downregulation of specific oncogenes. Their in vitro work in patient-derived DIPG cell lines demonstrated significant dose response in cell viability, which was accompanied by the expected decrease in H3K27ac. Corresponding to transcription downregulation, RNA sequencing revealed that after JQ1 treatment, the genes transcriptionally modulated by JQ1 showed H3K27M, active transcription marks, and BRD2/4 occupancy around their promoters indicating they are direct targets affected by JQ1 treatment. In terms of in vivo results, they observed favorable tumor shrinkage and prolonged survival in orthotopic DIPG xenograft models following administration of JQ1, with the corresponding epigenetic change of H3K27ac reduction, compared to vehicle-treated controls. These findings were successfully recapitulated using another BET inhibitor (I-BET151). Finally, compared to the demethylase inhibitor GSKJ4, the authors observed significantly longer overall survival when using JQ1.

To date, there is no clinical trial established to investigate BET inhibition in DIPG, although we note such trials do exist for malignant adult glioblastoma (NCT02296476). One consideration to bear in mind moving forward is the potential of combinatorial epigenetic therapy in DIPG. With respect to BET inhibition, Zhang et al. [56] demonstrated combinational therapy with JQ1 and EZH2 inhibitor EPZ6438 resulted in synergistic in vitro antitumor effects in patient-derived H3K27M-mutant DIPG cell lines, as well as prolonged overall survival in orthotopic DIPG xenograft models when combined versus individual therapy. Therefore, future clinical trial designs may benefit from incorporating epigenetic therapy from more than one target PTM.

6.5 H3K27M and Phosphorylation

The final epigenetic histone PTM in H3K27M tumors to consider is that of phosphorylation. Although H3K27 residues do not typically undergo phosphorylation, the adjacent serine H3S28 can be phosphorylated in the presence of H3K27me3 [57]. It is thought that pH3S28 causes the displacement of the PRC2 complex from the H3K27me3 peptide, resulting in H3K27me3 loss and increased transcriptional activity of otherwise repressed oncogenes. It should be noted that the activation of these polycomb group target genes

likely depends on a multiple number of factors in addition to the pH3S28, including recruitment of specific transcription factors and other H3 epigenetic changes [58, 59]. Therefore, the target potential of phosphatase/kinase and dephosphatase enzymes remains an area that has yet to be explored in DIPG relevant to the H3K27 site. However, a recent study by Schramm et al. [60] identified serine/threonine protein phosphatase 2A (PP2A) as a top depleted hit in patient-derived DIPG cell lines using next-generation sequencing and validated its lethal potential by genetic knockdown of the PP2A structural subunit PPP2R1A. Furthermore, therapeutic phosphatase inhibition by LB-100 treatment resulted in more favorable antitumor and apoptotic effects in H3K27M-mutant versus wild-type patient-derived cell lines. The epigenetic relevance of this finding is that PP2A is known to interact with the serine residues across histone H3 such as H3S28 [61]. Therefore, pH3S28 may prove another worthy epigenetic target to consider for H3K27M-mutant DIPG in the future.

Looking forward, it is known that phosphorylation occurs at other H3 serine sites which themselves may have possible significance in the case of managing these H3K27M tumors. A Phase I trial of a selective aurora kinase inhibitor AT9283 in a series of solid pediatric brain tumors, including DIPG, showed decrease in phosphorylation at H3S10 in 17 of 18 patients treated at high dose, indicating a rescue role possibly [62]. Furthermore, phosphorylation at H3S31 has been linked with both protection of euchromatin from the spreading of pericentric heterochromatin and a role in marking some H3.3 for replacement with canonical histone H3, which could prove relevant in the context of mutated H3.3K27M [63].

6.6 Future Considerations

Major validation of these findings to date is required to justify epigenetic targeting as a viable and feasible approach in DIPG treatment. Concerns about macroscopic and molecular specificity, as well as capacity to overcome the BBB, are the primary translational barriers that impede the interpretation of how effective these epigenetic-targeted therapies can be in practice based on the current literature. Furthermore, whether or not combinatorial therapy, targeting multiple epigenetic changes associated with increased tumorigenic burden in DIPG is an avenue worthy of exploration.

Finally, as we continue to work towards a more robust molecular understanding to DIPG, surgical intervention to gain biological sample for analysis is not without its risks. Therefore, preference for a less invasive modality that can provide biological information about the tumor remains optimal. There is emerging evidence to suggest that a liquid biopsy, targeting biofluids, such as cerebrospinal fluid (CSF) and blood plasma, could be a feasible alternative for brain tumors in general [64, 65]. The promise of liquid biopsy of circulating nucleosomes focusing on H3K27, and even other H3 sites of known phosphorylation, could afford us an insight into the changes in the epigenetic landscape of DIPG and be used to follow response to various targeted treatments [66]. There have been encouraging results to date about the feasibility of this concept in other cancers, and the epigenetic landscape of DIPG would suggest this tumor type too may be amenable to noninvasive monitoring [67, 68].

6.7 Conclusions

The complete epigenetic landscape of H3K27M-mutant DIPG is still being elucidated. Nonetheless, targeting distinct epigenetic changes such as H3K27me3, H3K27ac, and pH3S28 has shown therapeutic potential in vitro and in vivo. However, as there likely remains a complex interplay between epigenetic parameters in the tumorigenic burden of DIPG, it is difficult to ascertain whether or not targeting one specific change in isolation will be sufficient to translate into clinically meaningful benefits. We wait with much anticipation for the finalization of multiple epigenetic (H3K27ac)-based clinical trials in DIPG to begin to address these translational unknowns.

Greater understanding of these molecular interactions will better inform us of how to best

target the collective, characteristic epigenome of DIPG. Furthermore, barriers such as tumor specificity and access to the tumor site will need to be overcome in order to fully evaluate how effective epigenetic-targeted therapies can be in DIPG patients, with the predicted emergence of liquid biopsy, a tool that will assist in bridging this bench-to-bedside divide.

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Histone Lysine-to-Methionine Mutation as Anticancer Drug Target

7

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Abstract

Histone modification stands for a vital genetic information form, which shows tight correlation with the modulation of normal physiological activities by genes. Abnormal regulation of histone methylation due to histone modification enzyme changes and histone mutations plays an important role in the development of cancer. Histone mutations, especially H3K27M and H3K36M, have been identified in various cancers such as pediatric DIPG (diffuse intrinsic pontine glioma) and chondroblastoma respectively. "K to M" mutation results overall downregulation of methylation on these lysine residues. Also, "K to M" mutant histones can inhibit the enzymatic activity of the responsible HMT (histone methyltransferase); for instance, SETD2 indicates H3K36 methylation, and Ezh2 represents H3K27 methylation. In-depth analysis of the mechanism of tumor formation triggered by the K to M mutation results in possible targeted therapies. This chapter is going to briefly introduce the mechanism of histone lysine-to-methionine mutation and review the recently identified targeted therapeutic strategies.

Keywords

Histone modification · Histone lysine-to-methionine mutation · Cancers · Diffuse intrinsic pontine glioma · Chondroblastoma · H3K27M · H3K36M

Abbreviations

DIPG	Diffuse intrinsic pontine glioma
EZH2	Enhancer of zeste homolog 2
H2A	Histone H2A
H2B	Histone H2B
KDM1A	Lysine (K)-specific demethylase 1A
KDM1B	Lysine (K)-specific demethylase 1B

7.1 Introduction

Histones represent the huge family constituted by basic proteins with multicopy and a few introns, which are highly conserved among species [1]. Histones and non-histones serve as scaffolds combine with genomic DNA assemble into nucleosomes, which hiding genetic information in the nucleus [2]. The mass of histones accounts for over 1/2 of all chromosomes. Generally, histones can be classified into four types, including H2A-B and H3-4. Histone monomer forms

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D. Fang, J. Han (eds.), *Histone Mutations and Cancer*, Advances in Experimental Medicine and Biology 1283, https://doi.org/10.1007/978-981-15-8104-5_7

the octamer nuclear structure which is entangled by DNA, forming the basic unit of chromosomes-nucleosomes [3]. Except for H4, at least 55 known histone variants have been found in all canonical histones. Among these variants, most of the differences occurred at the N-terminus of the amino acid sequence, and only H2A is changed at C-terminal [4].

A majority of histones can be produced at S-phase of cell division, which can be assembled with DNA quickly after the formation of the replication fork [5]. In the process of forming new nucleosomes, histone posttranslational modification (PTM) causes changes of epigenetic state of cells [6]. However, the assembly of nucleosomes can be also performed in DNA replication-independent manner [7]. This change in chromatin composition is usually dynamic, which exerts an important part during the remodeling epigenetic inheritance. In recent years, the sequencing technologies have been greatly developed, which are conductive to the identification of recurrent mutations in the histones within many cancer types [8–10]. Among all mutations, methionine substituting lysine in H3K27M and H3K36M are most common [11]. Such mutations in histones may be relevant to cancer genesis, so they are referred to as the "oncohistones"; however, these precise mechanisms of tumor genesis remain largely unclear so far.

7.2 Posttranscriptional Modifications of Histones

In histone tails, amino acid residues can be modified by adding or removing chemical groups through specific enzymes. Such covalent modifications can change chromatin molecular weight, structures, as well as functions [6, 12]. Many specific modified enzymes are found to be involved in catalyzing specific chemical group removal or addition (writers) in the histone. Histone acetyltransferases can add acetyl groups to certain histone lysine residues (such as H3K9 and H3K27), and they can be recovered via the histone deacetylases [13, 14]. Specific methyltransferases add methyls to lysine (mono-, di-, and trimethyl), glutamate, or arginine residue [15]. Generally, repression markers related to gene transcription include the methylation of H3K27 [16]. Markers related to gene activation include H3K9 acetylation, together with methylation of certain arginine residues or lysine residues (including H3K4, H3K36, H3K79) [17]. The methyl can be eliminated via the histone lysine demethylases, and the latter can be classified as two different categories: amino oxidases (including KDM1B and KDM1A targeting H3K9me and H3K4me, respectively) along with hydroxylases (including demethylases that contain the Jumonji domain like KDM6B and KDM6A), and they can scavenge dimethyl and trimethyl in lysine 27 [18, 19]. Additionally, additional modifications have also been detected, including histone serine, tyrosine and threonine residue phosphorylation, citrullination (on methylarginine), as well as ubiquitination [20]. Histone PTMs have been well-recognized readers of its specific domains including chromodomain and bromodomain, along with Tudor domain, and they contribute to recognizing the residues of acetylated lysine and methylated arginine together with methylated lysine, separately [21]. The above proteins usually show interactions with the greater complexes binding to chromatin, affecting many basic biological events, like response to DNA damage, recombination and replication of DNA, remodeling of chromatin, and transcription of genes [22].

Deregulation of epigenetic events involves various disorders in human beings, such as malignancies, dysplasia, and psychiatric and neurological diseases[23].

7.3 Histone H3 Mutations in Cancer

So far, each of the oncohistone identified at present harbors mutations in the conserved residues which are the histone modification targets. Typically, oncohistone expression hinders histone mark deposition, thereby reprogramming the

tumorigenesis-related transcriptome. In 2011, the genome-wide sequencing studies on the diffuse intrinsic pontine glioma (DIPG) as well as additional high-grade gliomas in children found that histone H3 genes harbored recurrent somatic mutations [24, 25]. Previously, it was mainly to study mutations of epigenetic modifiers within various cancers, which represented the initial results about the recurrent mutations for the real histone genes, and aroused wide attention from the fields of oncology and epigenetics. Later, histological and genomic studies proved the potential of histone mutation as the driver mutation in defining different glioma subtypes at clinical, molecular, and anatomical levels [26]. The existing studies regarding the identification and dissection of carcinogenicity of histone H3 lysine-to-methionine mutation are shown in the Table 7.1.

In recent years, histone-encoding genes harboring recurrent driver mutations are depicted within the brain tumors in children, bone giant cell tumors, and chondroblastomas. These mutations usually show heterozygosity and are constituted by substitutions of single amino acids within histone variant tails. H3K27M mutations on the histone H3.2-encoding HIST2H3C and the histone H3.1-encoding HIST1H3C are less than 1%; typically, methionine-to-lysine substitutions of H3.3K36 and H3.3K27 are frequently seen within chondroblastomas and DIPGs, separately. As suggested by existing studies, the histone mutations will change epigenetic profiles within cancer cells while hindering differentiation, yet the precise relevant mechanism of carcinogenesis remains unclear yet. Such mutations are usually exclusively expressed within certain cell lineages; for instance, they are markedly anatomy- and development-specific within brain tumors. Typically, histone mutation has proposed a new treatment strategy incorporating the epigenetic agents.

7.4 Histone Lysine 27 to Methionine Mutation

The histone H3 recurrent somatic mutations will lead to the lysine27-to-methionine substitution (H3K27M), and they have been recognized within about 30% glioblastoma children along with 60% DIPG patients [25, 32, 34]. Such mutations usually take place on H3F3A or HIST1H3B that encode histones H3.3 and H3.1, separately; besides, they are also detected within HIST2H3C or HIST1H3C but at the low frequencies [28, 35]. The lysine27 site in histone H3 has been well-recognized to be methylated or acetylated, which play important role in gene regulation [36, 37]. H3K27me3 is the inactivation histone mark related to the silencing of HOX gene, inactivation of X-chromosome, and imprinting of genome, and they are regulated via proteins in the polycomb group (PcG) [38, 39]. EZH2, the polycomb-repressive complex 2 (PRC2) component, accounts for the specific histone methyltransferase (HMT) related to the deposition of H3K27me3n [40]. By contrast, H3K27 acetylation has been suggested to be related to the enhancer region and the transcription of active genes. H3K27me3 and H3K27ac show mutual exclusiveness to each other, while the latter antagonizes the PRC2 function [37, 41]. H3K27M expression has a certain effect on the acetylation or methylation of H3 on H3K27.

K27M of the H3.3 histone is found along the brain midline, which includes the spinal cord, pons, and thalamus [32]. DIPG mostly occurs in young patients, while thalamic glioma that harbors the H3.3K27M mutation may also be detected among the senile cases [31]. H3.3K27M mutation is accompanied with p53 loss-of-function mutation, together with that in CCND2, MYC, and platelet-derived growth factor receptor-α (PDGFRA) [24, 31, 32, 34, 35, 42–44]. The H3K27M mutation confers a worse prognosis and more frequently metastatic relapses in pediatric gliomas and was deemed for the advanced classification based on the latest World Health Organization classification [45]. However, K27M mutation identified in the canonical histone H3.1 in pontine glioma is associated with mutations of ALK2 [35, 46, 47]. Compared to K27M of H3.3, H3.1K27M cases are associated with an extended median survival (15 months), as well as the enhanced

Table 7.1 Recently identified carcinogenicity of histone H3 lysine-to-methionine mutation

Genes	Histones	Mutations	Cancer types	Percent (%)	References
HIST1H3B/C	H3.1	K27M	DIPG	15–25	(n = 8/48) [27] (n = 23/91) [28] (n = 9/50) [25]
HIST1H3C/E/G/I	H3.1	K36M	HNSCC	4	(n = 22/518) [29] (n = 4/270) [30]
HIST1H3C	H3.2	K27M	Non-BS-PG	1	(n = 1/91) [28]
H3F3A	H3.3	K27M	DIPG Non-BS-PG Thalamic glioma	18–60	(n = 10/18 in thalamic glioma) [31] (n = 55/91) [28] (n = 9/49) [32] (n = 30/50 in DIPG, n = 7/36 in non-BS-PG) [25]
		K36M	Chondroblastoma Conventional Chondrosarcoma	1	(n=5/77 in chondroblastoma, n=1/75 in conventional chondrosarcoma)
H3F3B		K36M	Clear cell chondrosarcoma HNSCC Chondroblastoma	95	(n = 2/518 in HNSCC) [29] (n = 1/15 in clear cell chondrosarcoma; n = 73/77 in chondroblastoma)[33]

Abbreviations: *DIPG* diffuse intrinsic pontine glioma, *non-BS-PG* non-brain stem pediatric glioblastoma, *HNSCC* head and neck squamous cell carcinoma

radiosensitivity. The distinct expression patterns between H3.3K27M and H3.1K27M groups were presented through transcriptomic analyses. Typically, cancer harboring H3.3K27M shows the oligodendroglial phenotype, and tumor harboring H3.1K27M exhibits the mesenchymal phenotype, where the expression of hypoxia- and angiogenesis-related genes increases [28].

7.5 Mechanistic Insights into the Role of H3K27M

Fifteen histone H3-coding genes are identified; however, K27M mutation just takes place on the allele in a histone gene of every cancer; besides, each histone gene mutation shows mutual exclusiveness [48]. Incubating H3K27M peptide using the H3 nucleosome has been reported to downregulate the methylation regulated via PRC2 by six times, which suggests the effect of H3K27M on impeding PRC2 effect [49]. H3.1K27M/H3.3K27M expression within mouse embryonic fibroblasts, human astrocytes, and 293T cells suggests that H3K27me3 is reduced, which is similar with in DIPG cells [50]. In addition, in the presence of H3K27M expression, the H3K27me3 loss has been suggested as the phenomenon independent of cell type. H3K27 is the recognized site of methylation marks, mediated by EZH2 or EZH1 sometimes, and H3K27M mutation can inhibit PRC2 methyltransferase effect. Besides, as suggested by substituting histone H3.3 on lysine 27 to any of the potential amino acid, just substitutions of K27I and K27M (lysine-to-Isoleucine) lead to H3K27me3 loss together with PRC2 inhibition [49]. Similarly, it was found that H3.3K27I mutation within the DIPG samples was related to K27-trimethyl mark global loss [28]. As suggested by the existing studies, nucleosomes that contain H3.3K27M can bind to EZH2 when there is cofactor *S*-adenosyl methionine (SAM), which is affiliative compared with the wild-type histone H3.3, which supports that the H3.3K27M model is likely to isolate the PRC2 complex, thereby avoiding H3K27me3 inhibitory mark spreading [49]. For human PRC2 complex that harbors the K27M substitution that contains one histone peptide, its crystal structure verifies the occupation of catalytic pocket in SET methyltransferase domain by the methionine residue for substitution [51]. However, another study supplies evidence against the sequestration

model, which was found that SUZ12 (the other subunit of the PRC2 complex) and EZH2 have been ruled out of the nucleosome that contains H3K27M. By contrast, H3K27M, along with RNA polymerase II and H3K27ac, is located in the region with active transcription activity. H3K27ac colocalization conforms to one prior work reporting that the oligo-nucleosome array that contained H3K27M showed upregulated H3K27ac expression[52]. These works prove that H3K27me3 loss may be due to PRC2 that is ruled out of chromatin via the H3K27M/H3K27ac nucleosome, rather than PRC2 with higher affinity to H3K27M.

Gene ontology analysis of gene expression in H3K27M mutation cells revealed that the signaling pathways change involved in oncogenesis, transcription factor activity, embryonic morphogenesis, and neuronal differentiation [53, 54]. Genes such as PDGFRA [55], MYC [56], and CDKN2A [57] changes are related to H3K27M-driven tumorigenesis. Furthermore, H3K27M mutations have complicated while interesting influences; K27 trimethyl mark focal gains are still seen in certain genome loci, including tumor suppressor such as p16 (CDKN2A), regardless of the H3K27me3 global loss [53, 54]. According to whole-genome analysis, the H3.3K27M mutation exerts its impact partly depending on H3K27me3 repressive mark by its initial degree. For instance, genomic loci showing weak mark via H3K27me3 are likely to experience H3K27me3 loss in the case of H3K27M mutation, but mutation has little influence on the genomic loci showing increased H3K27me3 expression, which may be because that only little histone H3.3 is incorporated to the above genomic loci. But H3K27me3 marks are required for certain loci such as INK4A/ARF (encoding p16) in the presence of H3K27M mutation, which appear to be unaffected by the initial H3K27me3 degree [8]. P16 is a tumor suppressor gene suppressing cell cycle in the case of oncogene activation or cell stress. Over 55% high-grade glioma adults showed p16 homozygous deletion, which induces p16 expression. In addition, the DIPG cells show growth retardation, which demonstrates that DIPG growth depends on p16 suppression [58]. Noteworthily, inactivation of P16 does not represent the only oncogenesis pathogenesis triggered by H3K27M. It is reported that BET domain containing proteins inhibitor JQ1 displayed good antitumor activity of H3K27M-DIPG cells without repression of P16; as a result, the oncogenesis mechanism may be independent from P16 [52].

7.6 Histone Lysine 36 to Methionine Mutation

The mutation of H3.3K36M was detected among 73/75 (95%) chondroblastoma cases, while 68 patients with K36M had mutation on H3F3B, one of those two genes coding histone H3.3 variant. However, when seven more bone cancers are screened, it is discovered that H3.3K36M shows a low incidence, which demonstrates that K36M mutation is tumor type specific [33]. H3K36M mutation has also been reported within other tumor types, like colorectal cancer or head and neck squamous cell carcinoma (HNSCC) [29, 30].

Chondroblastoma with H3K36M mutation results in global reduction of H3K36 methylation; ChIP-seq analysis suggested that H3K36me2 loss in intergenic regions and H3K36me3 loss occurred in gene bodies in H3K36M mutation cells or chondroblastoma samples [59, 60]. Mechanistically, H3K36M-containing nucleosome is markedly enriched histone methyltransferase (HMTs), sequestrating and inhibiting of the activity of HMT and then resulting the decreased of H3K36 methylation. This H3 methylation perturbation or HMT suppression is also supported by the evidence that using H3K36M peptides can repress the activities of HMTs in vitro [59, 60]. The H3K36M suppression on HMT can be confirmed through analyzing SETD2-combined K36M peptide's crystal structure. K36M peptide is bound to SETD2 in a way just like how K27M is bound to EZH2, and the suppression is modulated through the interaction of tyrosine residues within SET domain with methionine [61, 62]. miRNA knockout demonstrated that the HMTs of NSD2 as well as SETD2 may be

related to H3K36me2 and H3K36me3, separately. Cells depleted of H3K36-directed methyltransferases NSD1/NSD2 and SETD2 in combination had decreased H3K36me2/3 ratio, and H3K27me3 expression increased in comparison with the level within H3.3K36M cells, which indicates that H3K27me3 gain within H3.3K36M cells just takes place in the presence of H3K36me2 as well as H3K36me3 global loss simultaneously. Nucleosomes that contain H3K36me2/3 represent the weak PRC2 substrates in vitro [63, 64] and the K36M-regulated H3K36 methylation loss has provided the novel PRC2 nucleosomal substrates, leading to the upregulated H3K27me3 expression.

ChIP-seq of mesenchymal progenitor cells (MPC) with H3K36M suggested that the increased H3K27me3 expression in the intergenic regions may result in the reduced ratio of genes correlated with the intergenic H3K27me3. Typically, compared with gene-wide mean and upregulated expression, genes highly enriched with H3K27me3 have H3K27me3 loss. It is speculated that H3K27me3 "readers" recruitment will be changed within H3.3K36M cells because of the intergenic H3K27me3 gain together with the relative H3K27me3 abundance loss in specific genes. Cbx2 and Ring1b are the integral parts in a typical PRC1 complex binding to H3K27me3 to suppress the expression of genes, and their localization is evidently downregulated within the specific bound genes of H3.3K36M cells or following H3K36 methyltransferase knockout. The H2AK119ub1 abundance is higher in the intergenic loci with H3K27me3 gain within H3.3K36M cells, which suggests that the PRC1 activity spreads to such regions. As a result, the PRC1 complex is diluted from the suppressed genic loci, resulting in ectopic expression of genes and facilitating the H3.3K36M-induced differentiation obstruction. A number of such genes, including Sox6 and Wnt6, participate mesenchymal stem cell (MSC) self-renewal as well as lineage specification. Besides, according to transcriptomic analysis on the human chondroblastomas, the polycomb-bound genes were derepressed, which were related to the mutations of H3.3K36M [60]. Additionally, human chondrocytes harboring H3.3K36M display some tumor cell hallmarks, such as enhanced colony formation capacity, differentiation defect, and apoptosis resistance [59]. It is demonstrated that H3K36M drives oncogenesis partially via changing cancer-related gene expression.

7.7 Therapeutic Targeting of Histone Lysine-to-Methionine Mutation

Mutation of histone lysine-to-methionine (mainly included H3K27M and H3K36M) causes abnormal histone methylation in cells, which drives oncogenesis or impairs the differentiation of progenitor cell. A lot of works explain that the mechanism of oncogenesis originates from the alteration of H3 methylation landscape upon its lysine-to-methionine mutation. The precise mechanism of histone lysine-to-methionine mutation-driven oncogenesis in many cancers remains to be elucidated, including identification of the novel histone gene mutations. Nevertheless, based on the accumulation of previous studies, several druggable targets for the mutation of histone lysine-to-methionine have been discovered.

7.8 The Targeting Strategies for H3.3K27M

Because H3K27M mutation can be used to be the suppressor for PRC2 complex, resulting in a reduction of H3K27me3, it may be a possible treatment for cancers that harbor the K27M mutations to compensate for the reduced H3K27me3 expression through suppressing JMJD3 and UTX (the histone demethylases specific for H3K27). GSK-J4 serves as the UTX/JMJD3 selective inhibitor [65], which has anticancer effect in vitro and in the H3K27-DIPG xenograft models which accompanied an increase of H3K27me3 levels [66]. The detailed mechanism of GSK-J4 is still controversial for its specificity to demethylases of other families [67]. H3K27ac and H3K27me3 are mutually

exclusive, and H3K27M causes concomitant gain of acetylation, which hampers K27M mutation-mediated PRC2 inhibition [37, 41, 49]. Thus, increasing of H3K27ac is an alternative potential therapeutic approach for K27M mutation tumors. Similar to the above assumption, pan-HDAC inhibitor, LBH589, also exhibits certain effectiveness in vitro as well as within the DIPG orthotopic xenograft models. Furthermore, combination of GSK-J4 with LBH589 synergistically reduces the viability of H3K27M-DIPG cells [68]. But a different study showed that LBH589 exhibited significant toxicity and ineffective at tolerable doses in mice bearing H3.3-K27M-mutant tumors [69]. An alternative strategy to address the increased H3K27ac expression related to the H3K27M level is to target BRD2 and BRD4, which are the readers that contain bromodomain for recognizing acetylated histones. Nucleosomes that contain H3K27M show acetylated H3K27 enrichment, which also generate the BRD2/4 recruitment sites. JQ1, the inhibitor for BET, can be administered to abrogate such recruitment in the meantime of suppressing proliferation through enhancing cancer cell differentiation while extending their survival in mice with xenograft of H3K27M DIPG [52]. In spite of H3K27me3 global loss, K27 trimethyl mark focal gains are detected in some certain loci such as p16, and this may result from certain residual PRC2 activity [52]. It is also put forward to target EZH2 in the treatment of H3K27M-DIPG. The administration of EPZ6438 (the EZH2 inhibitor) or EZH2 genetic ablation can suppress cancer cell survival to extend patient survival in the DIPG mouse model [8]. Suppressing PRC2 effect through SUZ12 or EED knockdown reduces the proliferation or survival of H3K27M mutant cells, further approving that the preserved PRC2 effect exerts a certain part [52]. Therefore, it is worthy of being expected that combination JQ1 with EPZ6438 treatment DIPG in future based on their independent mechanism. Receptor tyrosine kinases such as PDGFRA were activated in H3.3K27 mutant tumors, although there had not been enough data to suggest that these pathways are the critical drivers for cancer genesis and development. However, certain RTK inhibitors exert vital parts in the combination treatments. The use of human embryonic stem cell system to mimic DIPG, inhibitors of MI-463, and MI-503 targeting MEN1 protein were identified as a potential therapeutic drug for H3K27M-DIPG [70]. This drug targeting MEN1 is initially developed for disrupting MEN1-MLL interaction, and this is essential for leukemogenic activity, and pharmacological suppression on MEN1-MLL interaction may be an efficient in vivo therapy to treat MLL leukemias [71].

7.9 The Targeting Strategies for H3.3K36M

Mutation on H3.3K36M results in H3K36me3 mark global loss as well as increased H3K27me3 expression at the intergenic regions that have been occupied by H3K36me2/3 at first. As suggested by a possible explanation, H3K27me3 redistribution helps to dilute PRC1 complex from those suppressed genic loci while resulting in ectopic expression of transcriptional genes to interrupt differentiation [60]. However, no drug that specifically targets H3.3K36M is developed so far. According to the concept of synthetic lethality, the WEE1 suppression is suggested to be the fatal combination within cancer cells with H3K36me3 deficiency [72]. The potential mechanism is regulated via RRM2, the ribonucleotide reductase subunit. RRM2 is under regulation via two pathways. When H3K36me3 is depleted to disrupt the first pathway, the RRM2 level together with dNTP release is reduced. When WEE1 is suppressed to disrupt the second pathway, the CDK activity is abnormal at the S-phase, which thus cause abnormal origin firing and premature RRM2 degradation, along with decreased dNTP pool degree. When these two pathways are disrupted at the same time, RRM2 expression is extremely downregulated, the dNTP pool is depleted, DNA replication is inhibited, finally leading to cell death. AZD1775, the selective small molecular inhibitor for WEE1, is currently being studied for its effect on different cancer types, and it is generally used combined

with cytotoxic chemotherapy or radiotherapy [73]. More efforts should be made to examine whether cancers with H3K36M mutant stand for the extremely sensitive anticancer target of WEE1 inhibitors.

7.10 Conclusion and Perspective

Since histone mutation is discovered to be a key driver of gliomas in children, the role of "oncohistone" in tumorigenesis attracted lots of attention. Mutation of H3K27M and H3K36M is the most common oncogenic events that can be used to be the drivers for sarcomas and gliomas in children. H3K27M can be detected in over 60% DIPGs or in 30% glioblastomas in children, and it leads to the globally reduced H3K27me3. Nonetheless, mutation on H3K36M hinders mesenchymal progenitor cell differentiation and produces the non-differentiated sarcomas, finally increasing H3K27me3 expression and leading to H3K36 global loss (me2 and me). Although great efforts have been made in this field in the past decade, the detailed mechanism and many key questions remain still poorly understood.

H3K27M mutation almost found on both H3.1 and H3.3 and had low frequency on H3.2. H3.1 is ubiquitous expression and evenly distributed in genome. However, H3.1K27M can only be found within DIPG, and the reasons for specificity of H3.1K27M remain unknown. The abnormalities of enzymes of methylation modifications of histones are found in many cancers. For example, EZH2 overexpression can serve as the marker for metastatic or advanced solid cancers, such as breast cancer (BC) and prostate cancer. In addition, EZH2 Y641 mutation has been depicted within lymphoma, which boosts the activity, while mutation inactivation is observed within myeloid cancers with dismal prognosis [74]. The SETD2 methylation site of H3K36, function loss, and the acquired H3K36 mutation can boost the branched evolution of renal cancer, in the meantime rendering resistance to cisplatin for non-small cell lung cancer (NSCLC), respectively [75, 76]. However, there was no H3K27M or H3K36M mutation that has been reported in these cancers. The major enzymes related to the modifications of histone H3K27 or H3K36 residues include "readers," "writers," and "erasers"; however, it is not completely clear whether these enzymes have more abnormal functions related to tumorigenesis. More studies are warranted to reveal target pathway dependence or biological results for histone mutations, together with those precise mechanisms for the specificity to development and lineage. Importantly, it should be noted that histone modification not only restricted methylation but also included phosphorylation of Ser/Thr/Tyr, lysine acetylation, ubiquitination, citrullination, etc.

"Oncohistones" have been identified to be the popular recurrent driver mutations, which arouses increasing attention in epigenetic treatments for histone mutant cancers. Recently, the development of epigenetic drugs has exploded and dozens of drugs targeting epigenetic modifiers are ongoing preclinical trials, such as EZH2 inhibitor EPZ6438, JMJD3 inhibitor GSK-J4, and WEE1 inhibitor AZD1775. Applying epigenetic agents in combination with additional therapies like immunotherapy has been studied [77–79]. Currently, selectivity is one of the biggest challenges of developing drugs targeting epigenetic modifiers. Most currently developed drugs do not show selectivity to certain enzymes; instead, they target molecules that have certain common functions and structure. But epigenetic agents are most advantageous in their good tolerance and low severe adverse reaction rate, even though there are certain concerns on the safety of certain medicines [80]. Additionally, more and more reports indicate that the poor response rates after treatment within short time and developed resistance in the end are common problems, and this can be attributed to the transcriptional plasticity driven by epigenetics responding to environmental stress [81]. Regardless of the above challenges, the understandings to certain cancers in children have attained breakthroughs, and more progresses are expected to be made in the future.

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