Histone Variant H3.3: A versatile H3 variant in health and in disease

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Histones are the main protein components of eukaryotic chromatin. Histone variants and histone modifications modulate chromatin structure, ensuring the precise operation of cellular processes associated with genomic DNA. H3.3, an ancient and conserved H3 variant, differs from its canonical H3 counterpart by only five amino acids, yet it plays essential and specific roles in gene transcription, DNA repair and in maintaining genome integrity. Here, we review the most recent insights into the functions of histone H3.3, and the involvement of its mutant forms in human diseases.

histone variants, H3.3, histone chaperones, development, tumorigenesis

INTRODUCTION

Chromatin consists of repeating units called nucleosomes, which are composed of an octamer of canonical histone proteins H2A, H2B, H3 and H4, and 147 bp of DNA wrapped around the octamer (Luger et al., 1997). Histones possess tails on both C- and N-termini, both of which are subject to a great number of post-translational modifications (PTMs), including methylation, acetylation, phosphorylation and ubiquitination (Kouzarides, 2007). These PTMs play important roles in the regulation of dynamic processes related to nucleosomes, including nucleosome turnover and chromatin remodeling. As a consequence, highly specific chromatin states can be established to regulate DNA replication and transcription, and maintain genome stability (Kouzarides, 2007). In addition to the mechanisms mentioned above, another regulatory layer of chromatin state exists, namely- the exchange of canonical histones with histone variants. Histone variants possess characteristics absent from canonical histones and contribute to the regulation of the structure and function of nucleosome and chromatin (Henikoff and Ahmad, 2005).

In mammals, various canonical H3 as well as variant forms of H3 have been identified. Canonical H3 are H3.1 and H3.2, which differ at only one amino acid residue, with Cys96 in H3.1 and Ser96 in H3.2 (Szenker et al., 2011). Apart from the canonical H3, multiple variants of H3 have now been identified: the replacement variant H3.3, the centromere-specific variant CENP-A, the testis-specific variant H3t and H3.5, and the primate-specific variants H3.X and H3.Y (Filipescu et al., 2013). H3.3 differs from H3.1 by five amino acids substitutions (S31A, A87S, I89V, G90M, and S96C) (Figure 1A). H3.3 Ser31 is located at N-terminal tail and can be phosphorylated (Hake et al., 2005). The residues Ala87, Ile89 and Gly90 are located in the α2 helix of the histone-fold domain, and are important for H3.3 deposition at specific loci in the genome by different histone chaperones (Filipescu et al., 2013). In this review we will focus on the recent progress in our understanding of the

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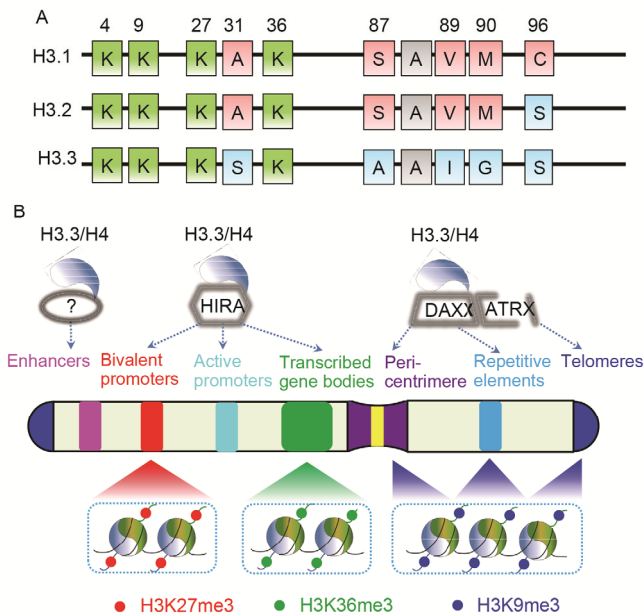*Corresponding author (email: liguohong@sun5.ibp.ac.cn)

Figure 1 Genomic distribution of H3.3. A, Schematic view of the amino acid difference between canonical H3 (H3.1/H3.2) and replacement H3(H3.3), and the lysine residues enriched in the N-terminus of H3. B, H3.3 is deposited at various chromatin environments by either HIRA complex or DAXX/ATRX. HIRA complex is responsible for the incorporation of H3.3 to active promoters, bivalent promoters and transcribed gene bodies. H3.3 is essential for the maintenance of H3K27me3 at bivalent promoters and is enriched with H3K36me3 in transcribed gene bodies. ATRX/DAXX deposits H3.3 to pericentromeric regions, repetitive elements and telomeres. H3.3 specific enrichment of H3K9me3 is required for the repression of ERVs and imprinted DMRs.

biological functions of histone H3.3, as well as its role during development and tumorigenesis.

THE PROPERTIES OF H3.3 COMPARED WITH ITS CANONICAL COUNTERPARTS

The expression patterns of H3.3 coding genes

In the human species, the genes encoding H3.1 and H3.2 lack introns and do not have a polyA signal, though they contain a palindromic termination element, which is involved in the regulation of transcriptional termination (Marzluff et al., 2002). Ten H3.1 genes have been identified (*HIST1H3A* to *HIST1H3J*), all of which are located in the histone cluster 1 on chromosome 6p22-p21.3 (Marzluff et al., 2002). H3.2 is coded by three genes, namely *HIST2H3A, HIST2H3C and HIST2H3D* on chromosome 1 (Marzluff et al., 2002). These H3 genes are all expressed simultaneously at S phase, together with the other canonical histone genes (Frank et al., 2003). These histones are incorporated into the chromatin in a DNA replication-dependent manner (RD). In contrast, there are only two H3.3 genes, namely *H3F3A* and *H3F3B,* both of which contain introns and a polyadenylation signal (Frank et al., 2003). These are located on chromosome 1 and 17, respec-

tively, and have different 5′-UTR (5′ untranslated region) , which suggests they may be subjected to different mechanisms of transcriptional regulation (Frank et al., 2003). H3.3 is expressed and incorporated into chromatin throughout the entire cell cycle, through both DNA replication-dependent (RD) and -independent (RI) mechanisms (Ahmad and Henikoff, 2002). It is worth noting that H3.1 can also be incorporated into chromatin at DNA repair sites through DNA synthesis-dependent mechanisms.

The genome-wide distribution of H3.3.

Histone proteins, which are translated in the cytoplasm, are chaperoned into the nucleus for assembly into chromatin (Li et al., 2013). To date, numerous histone chaperones have been identified. For the histone H3 family, both common chaperones and histone form-specific chaperones are described. Common chaperones include Asf1a, Asf1b and NASP, and specific chaperones include CAF1 (chromatin assembly factor 1), HIRA (histone cell cycle regulator), DAXX (death domain-associated protein) and HJURP (Holliday junction recognition protein) (Hamiche and Shuaib, 2013). Both common and specific chaperons bind H3 in the form of its H3/H4 hetero-dimer. NASP transports H3/H4 into the nucleus and supplies them to Asf1a/b, which in turn delivers H3.1/H4 to CAF1 or H3.3/H4 to HIRA and DAXX (Hamiche and Shuaib, 2013). The crystal structure of the DAXX-H3.3-H4 complex reveals that both Ala87 and Gly90 of H3.3 can mediate the specific interaction between DAXX and H3.3 (Liu et al., 2012). It would be of great interest to also acquire the crystal structure of CAF1 complexed with H3.1/H4 and/or HIRA complexed with H3.3/H4, to identify the mechanisms by which chaperones distinguish the minor sequence difference between canonical H3 and H3.3.

Currently known H3.3 histone chaperones are DAXX complex and HIRA complex (Lewis et al., 2010; Tagami et al., 2004), both of which deposit H3.3 into the genome throughout the entire cell cycle of actively dividing cells and in quiescent cells (Figure 1B). In a pioneering cytological study, using immunostaining and DNA FISH (Fluorescent *in suit* Hybridization), H3.3 was discovered to be deposited at specific, active chromatin regions in *Drosophila* cells (Ahmad and Henikoff, 2002). Subsequently, the replacement of H3.3 was studied on a genome-scale by ChIP-chip (chromatin immunoprecipitation ("ChIP") with DNA microarray ("chip")) method. It was found that the H3.3 signal covered active genes and transposons, and positively correlated with H3K4 methylation intensity and RNA polymerase II (RNAP II) signal. Importantly, the male X chromosome, which has stronger transcriptional activity compared to autosomes due to dosage-compensation (Straub et al., 2005), also displayed higher H3.3 density across the chromosome (Mito et al., 2007). There is, now, growing evidence that the HIRA complex is responsible for the deposition of H3.3 at actively transcribed genes. As a

consequence, new nucleosomes can form, ultimately ensuring genome stability. Moreover, in recent studies in mouse ES cells using chromatin immunoprecipitation combined with high-resolution genome-wide sequencing (ChIP-seq), it was revealed that H3.3 is also enriched at a subset of inactive genes, telomeres and repetitive elements such as endogenous retroviral elements (ERVs) (Elsasser et al., 2015; Goldberg et al., 2010), where ATRX/DAXX is required for H3.3 deposition and the maintenance of a repressive chromatin state (Elsasser et al., 2015; Goldberg et al., 2010; Lewis et al., 2010; Voon et al., 2015). Meanwhile, H3.3 is also distributed in other regions such as *cis*-regulatory elements (Chen et al., 2013; Goldberg et al., 2010; Mito et al., 2007), and plays an important part in the regulation of gene expression (Chen et al., 2013). However, the chaperon that is responsible for deposition of H3.3 at these regions remains to be identified (Filipescu et al., 2013; Goldberg et al., 2010; Szenker et al., 2011). Taken together, these results suggest that H3.3 is deposited across the genome by dedicated chaperone pathways with respect to chromatin contexts (Figure 1B).

The regulation of chromatin dynamics by variant H3.3

Recent biochemical and biophysical studies on the stability of mono-nucleosomes and the dynamics of nucleosomal arrays have convincingly demonstrated that the presence of H3.3 has no effect on mono-nucleosomes' stability (Chen et al., 2013; Thakar et al., 2009). Rather, it was found that H3.3 presence impairs the inter-fiber interactions of nucleosomal arrays (Chen et al., 2013). In comparison, H2A.Z, a H2A variant, enhances both the stability of mono-nucleosomes and the compaction of chromatin fibers. Interestingly, H3.3 incorporation overrules the effect of H2A.Z on chromatin compaction and results in the maintenance of a relatively open chromatin state (Chen et al., 2013). While the precise mechanisms for this phenomenon are unknown, it is clear that the interplay between histone variants is crucial for the regulation of chromatin function *in vivo*. Previously, it was reported that H3.3-containing chromatin regions are less resistant to salt extraction, a condition which suggests that a relatively open chromatin structure is maintained there (Henikoff et al., 2009). In addition, nucleosomes containing H2A.Z/H3.3 double variants are found enriched at active promoters, enhancers and insulator regions in human cells, and the instability of these nucleosomes facilitates the access of transcription factors and co-factors to these regulatory regions (Jin et al., 2009). In addition, H3.3 levels at promoter regions are positively correlated with expression levels of the respective genes (Jin et al., 2009). Although genome-wide analysis has shown that there is a positive correlation between H3.3 signal and transcriptional activity, direct evidence for a causal relationship is still lacking. Some hints about this correlation come from genetic studies in various model systems, including those of flies and mice.

In *H3f3a* and *H3f3b* double-null adult *Drosophila*, gene expression was found to be changed at a global level. Intriguingly, more genes were up-regulated than down-regulated (Sakai et al., 2009), suggesting a repressive role of H3.3 in the transcriptional regulation. However, these changes were not specific to H3.3 as they were rescued by overexpression of canonical H3. This result suggests that the non-exclusive role that H3.3 plays in transcription is to maintain nucleosome density and genome stability (Sakai et al., 2009). However, gene expression profiling in mouse ES cells either depleted of H3.3 or HIRA, showed minor differences of gene expression levels in a subset of genes when compared to wild type cells. Thus, H3.3 may not be required for maintenance of the transcriptome in all cell types (Banaszynski et al., 2013; Bush et al., 2013).

In a recent study utilizing all-trans-retinoic acid (tRA) to induce activation of target genes, H3.3 positioned at enhancers was found to be essential for the rapid activation of target genes. It was also found that knockdown of H3.3 by siRNA resulted in chromatin compaction and delayed transcriptional response of target genes (Chen et al., 2013). These results suggest that in mouse ES cells, H3.3 may function mainly by maintaining an open chromatin state, which is important for transcription initiation (Li et al., 2010). Furthermore, H3.3 deposits at DNA damage sites and renders chromatin prone for later reactivation of transcription once DNA repair is completed (Adam et al., 2013).

However, recent genome-wide studies found that H3.3 is also deposited at heterochromatin regions such as retroviral elements, telomeres and pericentromeres (Elsasser et al., 2015; Goldberg et al., 2010). For instance, studies of mouse ES cells showed that the DAXX-dependent deposition of H3.3 at telomere regions maintains telomere integrity, a situation which is essential for self-renewal of these cells. Furthermore, to fulfill this function in ensuring telomere integrity, both H3.3K4 and the presence of CBX4 (HP1α) are important (Goldberg et al., 2010; Wong et al., 2010; Wong et al., 2009). More recently, a genome-wide study revealed that ATRX-dependent H3.3 deposition is not limited to telomeres and pericentromeric regions, but also occurs at imprinted loci. The interplay of ATRX and H3.3 at imprinted alleles is essential for the maintenance of H3K9me3 modification, gene repression and proper allelic expression (Voon et al., 2015). In another study, H3.3 was found to be required for the repression of key developmental genes via the recruitment of PRC2 by HIRA; furthermore, suppression of endogenous retrovirus element was dependent on DAXX, H3.3 and KAP1 (Banaszynski et al., 2013; Elsasser et al., 2015). These researches suggest a more complex picture of H3.3 function along the genome.

The dynamics of H3.3 turnover at the genome-wide.

The incorporation into chromatin and eviction from chromatin of H3.3 during cellular activities are highly dynamic

processes. While conventional chromatin immunoprecipitation (ChIP)-based methods measure the steady-state occupancy of H3.3 at specific target regions, they provide no information about the kinetics or turnover of H3.3. However, these characteristics are very important for the diverse function of chromatin proteins (Lickwar et al., 2012). To address this problem, a novel method called CATCH-IT (covalent attachment of tags to capture histones and identify turnover) was used in a recent study to determine the turnover rate of H3.3 along the *Drosophila* genome (Deal et al., 2010). In that report, it was shown that bodies of actively transcribed genes, replication origins and *cis*-regulatory elements showed the highest rate of turnover. This is consistent with the idea that histone turnover can assist the accessibility of the DNA to transcription or replication machinery and *trans*-acting factors. More interestingly, nucleosomes at trithorax group-binding regions turn over faster than those in polycomb-group protein-binding regions; this is consistent with the notion that trithorax group maintains active chromatin state while polycomb-group protein binding results in a repressive chromatin state (Steffen and Ringrose, 2014). In another study, the H3.3-containing nucleosome turnover in a mouse fibroblast genome was captured by inducible expression of histones with epitope at different time points (Kraushaar et al., 2013). This study showed that the turnover rate along the mouse genome varied greatly, falling into three categories: (i) rapid turnover at promoters and enhancers; (ii) intermediate turnover at gene bodies; and (iii) slow turnover at heterochromatic regions. Faster H3.3 turnover at enhancers and promoters is positively correlated with active histone modifications, including H3K4me1, H3K4me3, H3K9ac, H3K27ac and the histone variant H2A.Z, whereas slower turnover is positively correlated with H3K27me3 and H3K36me3 modifications. These results from *Drosophila* and mice studies indicate that there are distinct mechanisms for histone deposition and nucleosome eviction at different functional chromatin regions, mechanisms which are correlated with specific histone marks and chromatin binding proteins.

Although H3.3 displays a highly dynamic behavior at a genome-wide level, it remains unknown whether the $(H3.3-H4)_2$ tetramer is replaced as a unit, or as a $(H3.3-H4)$ heterodimer. Using a double-induction system to control the expression of H3.3, Zhu and colleagues reported that while none of the $(H3.1-H4)_2$ tetramers, and nearly none of $(H3.3-H4)_2$ tetramers (~90%) split, the remaining 10% of $(H3.3-H4)_2$ tetramers did experience splitting during DNA replication, and these splitting events are significantly enriched at the cell type-specific enhancer regions (Huang et al., 2013). Considering the role of enhancers in maintaining cell type-specific transcription programs, the split H3.3 nucleosomes, together with the epigenetic information associated, may serve as epigenetic memory for a specific transcriptional status. It is of note that, during somatic cell nuclear transfer (SCNT), the active transcriptional state of donor nuclei was retained after being transplanted into an enucleated egg, and H3.3 indeed appeared to be necessary for the maintenance of epigenetic memory (Ng and Gurdon, 2008). Although the molecular mechanisms behind memory establishment remain unclear, the splitting phenomenon described above might represent a potential pathway for the inheritance of epigenetic information during cell division.

THE FUNCTIONS OF H3.3 IN DEVELOPMENT

The function of H3.3 in early embryo development

H3.3 takes part in various stages of development, such as gametogenesis, fertilization and differentiation (Bush et al., 2013; Lin et al., 2013; Tang et al., 2015; Wen et al., 2014a; Yuen et al., 2014) (Figure 2A). Knockout animal models for H3.3 or H3.3-specific chaperones have revealed the essential functions of H3.3 during development (Garrick et al., 2006; Michaelson et al., 1999; Roberts et al., 2002; Tang et al., 2015). H3.3 is encoded by two different intron-containing genes in mice, *H3f3a* and *H3f3b*. The two genes have distinct expression patterns and regulatory regions, suggesting they fulfill different cellular functions (Frank et al., 2003). *H3f3a* knockout mice were viable to adulthood, although males were found subfertile; in comparison, *H3f3b* homozygotes-null mice displayed deficiencies in embryonal growth and did not survive birth, with *H3f3b* heterozygote males being sterile (Tang et al., 2015). In addition, a small percentage of homozygous *H3f3b*-HA knock-in mice died within a week after birth (Wen et al., 2014b). Although the cause of such lethality remains unclear, these phenomena indicate that H3.3 is important for early embryonic development. Furthermore, *H3f3b* knockout MEF cells exhibited severe karyotype abnormalities, ectopic CENP-A localization and defective chromosome segregation, thus, suggesting that H3.3 is important for maintaining overall chromosome integrity (Bush et al., 2013).

During *Xenopus* development, H3.3 expression peaks at gastrulation, and the depletion of H3.3 results in gastrulation arrest and abnormal expression of late mesoderm markers (Ng and Gurdon, 2008). Knockdown of H3.3 in fertilized mouse zygotes results in growth arrest at the morula stage (Lin et al., 2013). H3.3-deficient embryos display significantly reduced levels of open chromatin markers (such as H3K36me2 and H4K16Ac) and increased incorporation of linker H1, resulting in over-condensation and mis-segregation of chromosomes (Lin et al., 2013). Together, these findings indicate that H3.3 is involved in establishing a finely balanced equilibrium between open and condensed chromatin states during early mouse development. Knockout of *HIRA* in mice was embryonically lethal, with defects in early gastrulation and disruption in mesodermal development (Roberts et al., 2002); this phenotype was similar to that observed for *H3.3*-deficient embryo in *Xenopus* (Szenker et al., 2012). Moreover, *DAXX* and *ATRX* knockout mice died before E9.5 (Garrick et al.,
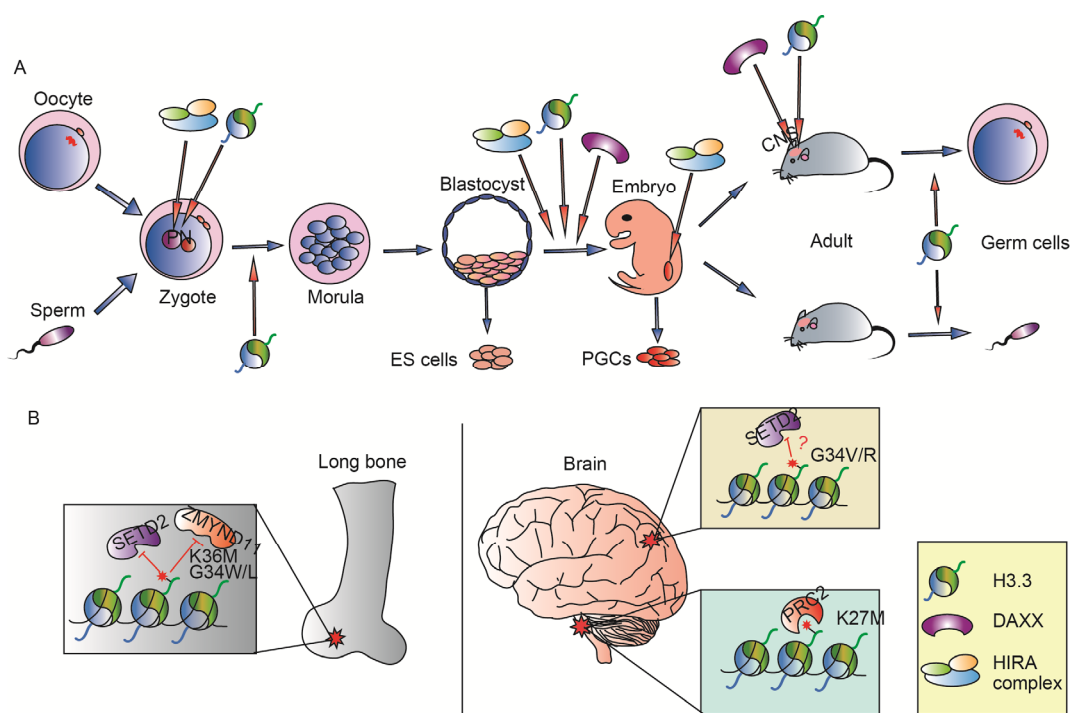
Figure 2 Roles of H3.3 and its chaperones during mammalian development and human cancers. A, H3.3 and its chaperones take part in several development stages during the life cycle of mammal, such as fertilization, early embryo development, PGCs reprogramming and gametogenesis. B, H3.3 mutations in skeletal and brain tissues. K36M and G34W/L mutants are prevalent in a variety of bone tumors and result in the decrease of H3K36me3. The K27M and G34V/R mutants are present in the gliomas. K27M mutation containing nucleosomes bind PRC2 with high affinity and inhibit PRC2 activity, thus leading to a global loss of H3K27me3. The G34V/R mutation containing nucleosomes also inhibit H3K36me3 in the tumor cells.

2006; Michaelson et al., 1999). Thus, these H3.3 chaperones are essential for early embryo development, but whether these phenotypes of chaperone knockout mice are specifically linked to H3.3 requires further investigation.

The function of H3.3 in gametogenesis

Drosophila with null mutations in both H3.3 genes survive to adulthood; however, both females and males are sterile, with chromosome condensation and segregation of germlines defective (Sakai et al., 2009). In mice, *H3f3a*-null males are subfertile, and *H3f3b* heterozygotes-null males are sterile (Tang et al., 2015). These phenotypes in both mice and *Drosophila* suggest that H3.3 is critical for fertility. H3.3 is expressed throughout the mouse seminiferous tubule, and depletion of *H3f3b* resulted in male testes atrophy, abnormal sperm morphology, and was also shown to disrupt the expression patterns of spermatogenesis-related genes (Yuen et al., 2014). In mammalian males, the X and Y chromosomes are transcriptionally silenced during the meiosis of spermatogenesis, a process called meiotic sex-chromosome inactivation (MSCI). H3.3 was specifically incorporated into the XY chromosome during the MSCI process (van der Heijden et al., 2007). During spermatogenesis, the male germ cells undergo an extensive chromatin remodeling process: histones are largely exchanged for protamine and protamine-like proteins, resulting in the formation of highly condensed chromatin. However, not all

nucleosomes were replaced, with a small number of H3.3-containing nucleosomes being retained. These nucleosomes are enriched in all those genomic regions that are important for development (Yuen et al., 2014). In mouse sperm, H3.3 is enriched at high-CpG rich promoters that are not methylated (Erkek et al., 2013). H3.3 was also found to be essential during foliculogenesis and primary oocytes development (Tang et al., 2015). Furthermore, a mutation in *yemanuclein (yem)*, the homolog of UBN1 subunit of HIRA complex in *Drosophila*, was found to affect chromosome segregation in the first oocyte meiotic division (Meyer et al., 2010). Taken together, these findings reveal that H3.3 affects the meiosis of germ cells and plays an important role in the gametogenesis. Furthermore, HIRA protein was enriched in the nucleus in mouse primordial germ cells (PGCs) undergoing reprogramming (Hajkova et al., 2008). As an H3.3-specific antibody remains unavailable due to the minor sequence difference to canonical H3, it is still not known whether H3.3 is also enriched in PGCs nuclei. Thus, the *H3f3b*-HA knock-in mouse will be a long-awaited model system that should provide exciting insights into the function of H3.3 in PGCs.

The function of H3.3 in fertilization

During the late haploid phase of spermatogenesis, the majority of the histone in sperm chromatin is replaced by protamine, resulting in sperm head condensation. Following

fertilization, this process is reversed, and new maternal H3.3 is preferentially incorporated into the paternal pronucleus (PN), replacing protamine and altering chromatin organization (Akiyama et al., 2011). It has been shown for both *Drosophila* and mice that the deposition of H3.3 on paternal pronucleus is dependent on the presence of the HIRA complex (Inoue and Zhang, 2014; Loppin et al., 2005). Similarly, in *HIRA* mutant mouse zygotes, nucleosomes cannot assemble at the paternal pronucleus, and as a consequence , both parental genomes show deficiencies in DNA replication as well as rRNA transcription (Inoue and Zhang, 2014; Lin et al., 2014). These results show that HIRA-mediated H3.3 deposition is important for reprogramming of the parental genome during fertilization. In addition, H3.3 is specifically enriched in the paternal pericentromeric chromatin, and is essential for the establishment of pericentromeric heterochromatin in the mouse embryo. Mutation of H3.3 K27, but not of H3.1 K27, results in de-repression of pericentromeric transcripts, HP1β mislocalization, dysfunctional chromosome segregation and developmental arrest (Santenard et al., 2010). In paternal pronucleus (PN), overexpression of a H3.3 K4M mutant before fertilization causes a significant reduction in global H3K4me3, decreasing the level of minor zygotic gene activation (ZGA) in PN, and as a consequence, reducing the survival rate of the embryo (Aoshima et al., 2015). Together, these studies suggest that H3.3 affects embryo development after fertilization through specific post-translation modification of its N-terminal tail.

The function of H3.3 in the epigenetic reprogramming during somatic cell nuclear transfer

During SCNT (somatic cell nuclear transfer), the donor nucleus proteins are exchanged by the oocyte proteins, resulting in extensive remodeling of the donor chromatin and the reactivation of pluripotency genes such as *Sox2*, *Oct4*, *Nanog* (Gurdon and Wilmut, 2011). The cytoplasm of mature mouse oocytes contains abundant amounts of both *H3f3a* and *H3f3b* mRNAs; however, these maternal transcripts are largely degraded at the first embryonic cleavage (20 h after oocyte activation), whereas zygotic H3.3 mRNA increases after the two-cell stage (Wen et al., 2014a).

H3.3 takes part in both the transition of epigenetic memory and the transcriptional reprogramming of donor nuclei. On one hand, H3.3 is incorporated into transcriptionally active regions of the donor cell, thus maintaining gene expression patterns after nuclear transfer. For example, the transferred embryos can remember the expression of *MyoD* with the incorporation of H3.3 at the *MyoD* promoter in *Xenopus*, and the H3.3 K4 mutant eliminates this memory (Ng and Gurdon, 2008). On the other hand, H3.3 is required for the reactivation of key pluripotency genes of the donor nucleus. It has been shown that exogenous H3.3 deposits at not only the regulatory regions of *Oct4*, but also other genomic regions, including the major satellite repeats

and rDNA (Jullien et al., 2012). In addition, knockdown of maternal H3.3 results in compromised reprogramming and failure to reactivate key pluripotency genes, such as *Oct4* in mouse embryos (Wen et al., 2014a). These results indicated that H3.3 is required for epigenetic reprogramming and embryonic development of SCNT embryos.

The function of H3.3 in cell differentiation

Pluripotent embryonic stem cells possess the capacity for self-renewal and the potential to differentiate into multiple lineages. The differentiation potential of ES cells is established by marking lineage-specific genes prior to their expression. In ES cells, the promoters of lineage-specific genes are simultaneously marked by both activation-associated H3K4me3 and repression-associated H3K27me3 modifications. These regions are referred to as "bivalent domains", and these bivalent genes are poised for activation during differentiation. A recent study reported that H3.3 is enriched at these bivalent domains in mouse ES cells (Goldberg et al., 2010). When the ES cells differentiated to neuronal precursor cells (NPCs), the genome-wide distribution patterns of H3.3 were changed. For the bivalent genes activated in NPCs, H3.3 is maintained at the TSS and also incorporated into the gene body. In contrast, for the remaining repressed bivalent genes in NPCs, the H3.3 deposition is decreased around the TSS sites (Goldberg et al., 2010). Furthermore, in H3.3 knockdown ES cells, the expression patterns of bivalent genes and the developmental potential ability was changed during the differentiation process. Therefore, H3.3 appears to be essential for ES cell differentiation, but the underlying molecular mechanisms remain to be elucidated.

Further studies indicate that H3.3 plays an important role in the maintenance of specific chromatin states achieved through an interaction with Polycomb repressive complex 2 (PRC2). PRC2 regulates gene expression through histone H3 lysine 27 trimethylation (H3K27me3). In mouse ESCs, H3.3 incorporation at bivalent regions facilitates the recruitment of PRC2 to ensure the establishment of H3K27-me3 marks. Upon H3.3 depletion, the levels of H3K27me3 were found to be reduced at these promoters, but the levels of H3K4me3 remained unchanged (Banaszynski et al., 2013). In *Drosophila*, H3.3 was also found to be enriched at PRC2 binding regions (Mito et al., 2007). These findings strongly suggest that H3.3 facilitates a type of specific chromatin state for PRC2 binding at bivalent domains.

Using a murine C2C12 differentiation system, it is reported that H3.3 is also deposited at the bivalent domains of skeletal muscle gene (SKM) prior to the differentiation by chromodomain helicase DNA-binding domain 2 (Chd2) (Harada et al., 2012). In addition, H3.3 also localizes at the transcriptional regulatory regions of *MyoD* gene mediated by HIRA (Yang et al., 2011). Knockdown of Chd2 or H3.3 decreases the incorporation of H3.3 into the SKM gene loci,

and reduces the levels of H3K27me3 and H3K4me3 at SKM bivalent domains, resulting in myogenic differentiation impairment. Therefore, H3.3 may be essential not only for gene transcription, but may also be important for establishing bivalent domains required for reliable and fast switching on or off the expression of genes during differentiation.

H3.3 AND DISEASE

H3.3 participates in the regulation of chromatin structure and is involved in the maintenance of specific chromatin states, which are important for transcriptional regulation during development. Recently, the function of H3.3 has been highlighted by recurrent somatic mutations identified in gliomas and a subset of skeletal neoplasms. These mutations include K27M, G34R/W/V/L, and K36M in both *H3F3A* and *H3F3B* genes (Figure 2B) (Behjati et al., 2013; Schwartzentruber et al., 2012; Sturm et al., 2012; Wu et al., 2012) .

Pediatric high-grade gliomas (pHGGs), including glioblastomas (GBM) and diffuse intrinsic pontine gliomas (DIPGs) are highly aggressive brain tumors with leading mortality and morbidity in children. In recent efforts to understand their molecular pathogenesis, two recurrent somatic *H3F3A* mutations resulting in amino acid substitutions (K27M and G34R/V) were found almost exclusively in pediatric GBM and DIPGs by whole genome exon sequencing (Schwartzentruber et al., 2012; Sturm et al., 2012; Wu et al., 2012). In the DIPG group, K27M mutation occurred in the entire H3 family, with a high preference of *H3F3A* (60%) over *HIST1H3B* (18%), and the mutation of these two H3 isoforms was found to be mutually exclusive. While no G34R/V mutation was identified in the DIPG samples, 14% of the GBM samples harbor G34R or K27M mutations in *H3F3A* and *HIST1H3B*. In another population study, H3.3 mutation was also found to be highly specific for GBM samples, and the prevalence was significantly higher in the pediatric group than the young adult group (Schwartzentruber et al., 2012). K27M and G34R/V mutations of *H3F3A* were found in these samples with an almost equal mutation rate; however, they displayed specific temporal and spatial distributions. The K27M was more prevalent in younger children and G34R/V in a relative older group. The spatial distribution difference within the central nervous system was that K27M located in tumors along the middle line location, including spinal cord, thalamus, pons, and brainstem; while G34R/V located in the cerebral hemisphere (Figure 2B). While there are two genes—*H3F3A* and *H3F3B* coding the same H3.3 protein in human genome, these mutations have not been detected in *H3F3B* gene so far, indicating that the origin of these somatic mutations may be related to the unique mRNA-untranslated region or regulatory element of the *H3F3A* gene (Albig et al., 1995; Witt et al., 1997). Furthermore, in all tumor samples tested, only one allele of *H3F3A* gene was found mutated for both

K27M and G34R/V substitutions, and they occurred in a mutually exclusive fashion. Together, these data indicate that K27M mutation and G34R/V may originate via different pathways, related to specific clone precursors, genesis niche and develop stage (Yuen and Knoepfler, 2013).

The mutation spectrum of H3.3 was found extended to K36M and G34W/L in a variety of bone and cartilage tumors. This spectrum also showed tumor type-specific H3.3 mutations (Behjati et al., 2013). While the K36M substitution was found predominantly in *H3F3B* gene in chondroblastoma, G34W/L mutation exclusively existed in *H3F3A* gene. In rare cases, *H3F3A* p.K36M and *H3F3B* p.K36M mutations occurred in conventional chondrosarcoma and clear-cell chondrosarcoma, respectively. In osteosarcoma, a G34R substitution was found in both *H3F3A* and *H3F3B* genes.

Other somatic mutations have been found simultaneously with H3.3 mutations in pHGGs, including mutations in *TP53* (Khuong-Quang et al., 2012; Schwartzentruber et al., 2012), *ATRX* (Fontebasso et al., 2014; the St. Jude Children's Research Hospital-Washington University Pediatric Cancer Genome, 2014), *DAXX* (Khuong-Quang et al., 2012; Schwartzentruber et al., 2012), *NF1* (Schwar- tzentruber et al., 2012), *PDGFRA* (Paugh et al., 2013), *ACVR1* (Buczkowicz et al., 2014; Fontebasso et al., 2014; Taylor et al., 2014; the St. Jude Children's Research Hospital-Washington University Pediatric Cancer Genome, 2014) and *SETD2* (Wu et al., 2014). While *TP53* mutation was found in half of all sequenced GBM samples, it showed no preferential co-occurrence with K27M or G34R/V, similar to the *NF1* mutation and *PDGFRA* mutation. Although *ATRX/DAXX* mutations overlapped with H3.3 K27M mutations, this correlation was not found to be statistically significant. Surprisingly, all samples with the G34R/V mutation and *ATRX/DAXX* mutation also co-existed with a *TP53* mutation (Schwartzentruber et al., 2012). Although somatic mutations of *ATRX/DAXX* have been reported in a large proportion of pancreatic neuroendocrine tumors (PanNETs), no *TP53* or *H3F3A* mutation was found to co-exist. Furthermore, these mutations were associated with better prognosis clinically. Mutations of *ACVR1* were found in pediatric midline high-grade astrocytomas (mHGAs) containing a K27M mutation. Such a situation results in ligand-independent activation of the kinase, which further results in enhanced BMP (bone morphogenetic protein) signaling. Interestingly, these mutations of *ACVR1* were linked to the presence of a *HIST1H3B* mutation (H3.1 K27M) and were associated with a younger age of onset of disease. Inactivating mutations of the histone H3 K36 trimethyltransferase-*SETD2* were found in a subgroup of pediatric HGG bearing H3.1K27M substitution. These mutations may interact with H3.3 mutation and provide selective advantages for glioma genesis.

H3.3K27M and tumorigenesis

The N-terminal tail of H3 is positioned outside of the nucleosome, and contains several positively charged residues, including lysine 4, lysine 9, lysine 27, and lysine 36 as indicated in Figure 1A. These residues can be both methylated or acetylated, and play important roles in the regulation of chromatin structure and transcriptional activity during normal development and tumorigenesis. H3K27 can be methylated specifically by PRC2, producing either H3K27me2 or H3K27me3. H3K27me3 can recruit Polycomb repressive complex (PRC1), which catalyzes ubiquitination on H2A K119. Interestingly, the modification H2AK119ub can, in turn, recruit PRC2, thus, forming a positive feedback to maintain a repressive chromatin state. This repressive memory is involved in the body planning during the development of *Drosophila* and X chromosome inactivation in mammals.

Therefore, the question remains of what the consequence is of the K27M mutation in the pHGGS? In the DIPG and pHGGs patient samples bearing H3.3 K27M mutation or H3.1K27M mutation, global levels of both H3K27me2 and H3K27me3 were dramatically reduced, while H3K27ac levels increased mildly, albeit only one allele was mutated and expressed among all the H3 isoforms. EZH2 expression was high in GBMs, but absent in other tumors. However, no significant differences in EZH2 expression were observed between *H3F3A* K27M mutant and wild type GBMs (Bender et al., 2013; Chan et al., 2013; Lewis et al., 2013). Moreover, this phenomenon can be recaptured by introducing an H3.3K27M or H3.1K27M mutation in cultured cell lines with no effect on the protein levels of all the PRC2 core subunits. In both tumor samples and transformed cell lines, K27M mutation do not have any effect on other histone modifications, such as H3K27me1, H3K4me3, H3K9-me3, H3K9Ac, H3K36me3 (Chan et al., 2013; Lewis et al., 2013). However, in DIPG samples containing H3.3G34R/V or H3.1G34R/V mutations, the global level of H3K27me2 and H3K27me3 did not change. These results argued that the global change of H3K27me2/3 was specific to K27M mutation. These results also suggest that the K27M mutation, either on H3.3 or H3.1, represses H3K27 methylation activity of PRC2 toward wild type H3 histones in-*trans*. It is to note that H3K27M peptide directly binds EZH2 with high affinity. Similarly, using immunoprecipitation, EZH2 and EED were found enriched on H3.3K27M mutant nucleosomes, not, however, on the wild-typecounterpart. Using an *in vitro* histone methyltransferase (HMT) assay, it was found that both nucleosomes containing H3K27M and H3K27M peptide greatly inhibit PRC2 activity (Lewis et al., 2013). Taken together, these data suggest that K27M containing nucleosomes bind the catalytic subunit of PRC2 as a mutated substrate with high affinity to inhibit the methyltransferase activity of PRC2. This results in a global loss of H3K27me2 and H3K27me3 modification in pHGGs.

PRC2 and H3K27me3 have been reported to regulate transcriptional activity of genes, and were important for the maintenance of stem cell identity and normal development across diverse organisms. Disruptions of the PRC2-H3K27me3 pathway have been found in many tumors, and contribute to tumorigenesis dependent on differing cellular contexts (Hock, 2012). Using ChIP-seq, it was found that more than three quarters of the H3K27me3 peaks were lost across genic and intergenic regions without chromatin context specificity (Bender et al., 2013; Chan et al., 2013). However, the effect of K27M mutation on the alternation of H3K27me3 is more complicated in specific genomic regions. In contrast with the dramatic change of H3K27me3, both EZH2 and H3K4me3 showed almost no change across the genome (Chan et al., 2013). The neural transcription factor OLIG2 has been reported to repress the p53-signaling pathway and promote tumorigenesis of malignant glioma (Mehta et al., 2011). Interestingly, its up-regulation in DIPG correlated with a decrease in H3K27me3 and increase in H3K4me3 in its promoter region. Other cancer-associated genes such as *p16INK4A* and *CDK6* were repressed with increased H3K27me3 in the promoter regions in DIPG. Moreover, in pHGGs samples, DNA methylation also showed genome-wide changes, with more hypo-methylated regions than hyper-methylated regions detected. This reality contributed to the dysregulation of the cancer transcriptome (Bender et al., 2013). However, in DIPGs harboring no K27M mutation, H3K27me3 level was found to be normal (Lewis et al., 2013), suggesting that other oncogenesis pathways exist in pHGGs.

H3.3K36M and tumorigenesis

H3K36me3, the product of methyltransferase SETD2, is found throughout actively transcribed regions, and is positively correlated with transcriptional activity. H3K36me3 and SETD2 have been demonstrated to regulate both transcriptional elongation and alternative splicing events (Wagner and Carpenter, 2012). Several proteins have been reported to read H3K36me3 modifications by virtue of possessing a PWWP domain, including DNMT3a and Brpf1 (Dhayalan et al., 2010; Vezzoli et al., 2010; Wu et al., 2011). A recent report described for the first time, a reader for H3.3-specific K36me3 called BS69/ZMYND11 (Wen et al., 2014c). BS69 has been identified as a tumor suppressor, as it inhibits transcriptional activation regulated by E1A and c-Myb (Hateboer et al., 1995; Ladendorff et al., 2001). H3K36me3 of H3.3 recruits BS96 to the elongation region of specific genes to regulate RNA alternative splicing via interaction with RNA splicing regulators (Guo et al., 2014). In another study, BS69/ZMYND11 was reported to regulate transcription elongation via its recognition of H3K36me3 on H3.3. Thus it repressed transcriptional programs essential for tumor proliferation (Wen et al., 2014c).

The K36M mutation has been found in *H3F3B* at high prevalence in Chondroblastoma, and was reported to abolish H3K36me3 modification when introduced in 293T cells. In

DIPG samples containing H3.3G34R/V or H3.1G34R/V mutations, global levels of histone modifications remain unchanged, including H3K36me3. However, using immunoprecipitation, a marked loss of H3K36me3 on G34R/V mutations containing-nucleosomes can be observed. This result indicates that H3K36me3 is regulated indirectly through Gly34, possibly because it is involved in the recruitment of methyltransferase. Surprisingly, in pediatric GBM tumors, H3K36me3 and Pol II were found aberrantly enriched within genic regions of several key transcription factors, including *MYCN*, *Sox2* and *DLX6*, which may be involved in the elevated transcription of these genes, thus resulting in the reprogramming of the transcriptional states of the tumor towards an earlier developmental stage (Bjerke et al., 2013). Taken together, H3.3 mutations can promote pHGGs tumorigenesis via a hijacking H3K36me3 pathway either directly (K36M mutation) or indirectly (G34R/V/W/L mutations), thus functioning within a specific tissue environment. As discussed above, the epigenetic information of H3K36me3 can be decoded by various readers; thus, these readers will act in a misguided manner upon pathological changes in H3K36me3 patterns.

Although there is no evidence that Lys27 and Gly34 residue can regulate the genome-wide deposition of H3.3, it still needs to be determined whether these mutations directly impact the interaction between H3.3 histones and their dedicated chaperone complexes, and whether these mutations cause a mis-deposition of H3.3 along the cancer genome.

CONCLUSIONS

H3.3 is an important histone variant that is deposited into the specific loci of chromatin by dedicated chaperones to regulate the various cellular activities. H3.3 can be incorporated into both the euchromatin and heterochromatin by different chaperones. The HIRA complex mediates the distribution of H3.3 at transcription start sites and gene bodies of transcribed genes. DAXX/ATRX mediates the incorporation of H3.3 into heterochromatin sites, such as telomeres, pericentromeres and repetitive elements. This diverse distribution suggests that H3.3 not only takes part in transcriptional regulation, but also in the establishment and maintenance of the heterochromatin state. Therefore, H3.3 has different functions at different chromatin environments. In addition, H3.3 is deposited at specific intergenic regions independent of HIRA and DAXX, with an unknown deposition pathway (Figure 1B). H3.3 is retained in the sperm chromatin during spermatogenesis, which raises the question as to whether there is a testis-specific H3.3 chaperone. As ChIP-seq technology advances, it will be possible to obtain finer and more comprehensive maps of H3.3 across the genome at detailed spatial and temporal resolution, both during development and in specific tissues.

Recent studies showed convincingly that H3.3 is im-

portant for development, whereas mutated H3.3 forms are implicated in tumorigenesis. Whether H3.3 regulates these processes directly by influencing the chromatin structure or indirectly through other mechanisms, however, remains unknown. The somatic mutations of H3.3 were recently reported in a number of malign tumors, such as glioblastomas and skeletal neoplasms. The relationship between these mutants and tumorigenesis is under intensive research. Apart from the PRC2-H3K27me3 and H3K36me3 pathways studied recently, there are other potential mechanisms. ATRX/DAXX mediates the H3.3 deposition in telomeres in ES cells to maintain the integrity of chromatin. The co-existence of *ATRX* and *DAXX* mutations with H3.3 mutant hinted that the ATRX/DAXX/H3.3 axis disruption may cause the instability of the telomere and facilitate tumorigenesis. If this is true, the ATRX/DAXX/H3.3 axis may provide a new therapeutic target for gliomas and other tumors.

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