Trends in Cell Biology

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Review

The many faces of H3.3 in regulating chromatin in embryonic stem cells and beyond

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H3.3 is a highly conserved nonreplicative histone variant. H3.3 is enriched in promoters and enhancers of active genes, but it is also found within suppressed heterochromatin, mostly around telomeres. Accordingly, H3.3 is associated with seemingly contradicting functions: It is involved in development, differentiation, reprogramming, and cell fate, as well as in heterochromatin formation and maintenance, and the silencing of developmental genes. The emerging view is that different cellular contexts and histone modifications can promote opposing functions for H3.3. Here, we aim to provide an update with a focus on H3.3 functions in early mammalian development, considering the context of embryonic stem cell maintenance and differentiation, to finally conclude with emerging roles in cancer development and cell fate transition and maintenance.

Highlights

H3.3 is a highly conserved nonreplicative histone variant.

In mammalian cells, H3.3 is enriched in both active chromatin and suppressed heterochromatin.

H3.3 is associated with development and cell fate maintenance.

H3.3 is also involved in heterochromatin formation and silencing of developmental genes.

Multiple H3.3 mutations cause cancer or neurodegeneration.

H3.3, a multifunctional nonreplicative H3 histone variant

Chromatin in living mammalian cells is composed of histone proteins, DNA, and other structural proteins. An octamer of histones (two copies each of the four core histones H2A, H2B, H3, and H4) wrapped by 147 bp of DNA makes up the core particle of the nucleosome [1]. The four core histones have replicative forms that are synthesized during S-phase and are inserted into the chromatin to fill voids created after the chromosome is replicated and additional nonreplicative variants that are expressed independently of S-phase [2]. Their incorporation into chromatin is dynamic and influences chromatin structure and composition (Box 1).

In mammals, the core histone H3 has two replicative variants, H3.1 and H3.2 [3], and several nonreplicative variants, among them H3.3. H3.3 is expressed steadily throughout the cell cycle (except in mitosis), and its incorporation usually involves active replacement of H3 in the nucleosome [2]. H3.3 differs from the replicative H3.1 by four amino acids. This difference is conserved between metazoan species and even seemed to have reemerged independently a few times in evolution [2]. Interestingly, H3.3 is encoded by two different genes in mammals on separate chromosomes, *H3.3A* and *H3.3B*, which, despite considerably varying in their gene structure, gene length, and untranslated regions, code for the exact same protein in both human and mouse.

Here we review recent discoveries pertaining to the function of H3.3 in **embryonic stem cells** (**ESCs**) (see Glossary), pluripotency, cell fate, and early differentiation. Recent discoveries shed light on its dual, almost contradicting, action in controlling both active and suppressive chromatin in stem cells and early development and highlight its distinctive roles in ESCs, regulating pluripotency and metabolic genes on the one hand and, e.g., endogenous retroviral elements on the other. Crucially, the identification in recent years of H3.3 mutations in aggressive types of cancer (Table 1) opened the door for mechanistic studies of these mutations, revealing not only their role in tumor biology and cancer progression but also, more generally, in cellular

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plasticity and reprogramming. Thus, H3.3's many actions lie at the heart of understanding cell fate, differentiation, and cancer.

Histones are heavily modified by post-translational modifications

Chromatin regulation is modulated not only by the dynamic incorporation of histones but also by their post-translational modifications (PTMs) that can take several chemical forms [4]. Histone modifications are reversible [5] and are carried out by a variety of different enzymes to impose or remove a given modification [4]. Different proteins can interact with different histone modifications [6], making the regulation of the chromatin complex, adaptive, and dynamic.

Among the four histones, histone H3 is most extensively modified, and the modifiable residues of its tail are highly conserved [7]. Although there are exceptions, numerous extensively researched modifications are closely linked to particular chromatin environments. These include repetitive regions, transposon and viral elements, and pericentromeric regions and **telomeres**. Histone modifications and downstream proteins can cause the transition from transcriptionally active to silent chromatin – referred to as **facultative heterochromatin** – and *vice versa*, thus representing a regulatory mechanism with consequences for cell fate and pathology. H3 trimethylated lysine 9 (H3K9me3) marks heterochromatin; H3K27me3, repressed genes; H3 monomethylated lysine 4 (H3K4me1), enhancers; H3K4me3 and H3 acetylated lysine 9 (H3K9ac), active promoters; H3K27ac, active enhancers; and H3K36me3, gene bodies of active genes [7]. Bivalent chromatin is the combination of the two 'opposing' marks H3K27me3 and H3K4me3 in a single promoter, and even the same nucleosome [8], but most likely not on the same histone tail [9]. This marking is abundant (although not exclusive) in ESCs [10,11], most prominently at silent developmental genes [12]. During differentiation, many bivalent genes are resolved and become monovalent [13]. Histone modifications thus have vital roles in regulating cell fate transitions, and, as we will see in the following section, mutations in modifiable residues could have dire consequences for cellular plasticity and maintenance.

Box 1. Histone variants versus core histones

Core histone proteins package and organize the DNA within all living eukaryotic cells. During cell division, DNA is replicated, and the core, or 'canonical', histones are produced at large quantities to meet the demand of the newly synthesized DNA. As such, they are highly transcribed during S-phase and are therefore referred to as 'replication-dependent' histones. Multiple genes code for the same core protein. These genes are clustered; they are all devoid of introns; and they are the only genes transcribed by RNA polymerase II that are nonpolyadenylated.

Histone variants, or replacement histones, are transcribed throughout the cell cycle, regardless of S-phase and therefore are known as 'nonreplicative' or 'replication-independent.' Unlike the core histones, they are solitary, they contain introns, and their mRNA is polyadenylated. Histone variants provide an additional layer of complexity to chromatin organization and gene regulation by mostly modulating chromatin structure and accessibility. Unlike canonical histones, which are primarily involved in packaging DNA into nucleosomes, histone variants can alter chromatin compaction and influence gene expression. For example, the histone variant H2A.Z is associated with transcriptional activation, promoting accessibility to DNA and facilitating the binding of transcription factors. Another well-studied H2A variant is H2A.X. It is a sensor of DNA damage, becoming phosphorylated upon double-strand breaks and thus initiating a cascade of repair. Histone H3 has two main canonical replication-dependent forms, H3.1 and H3.2, and two main nonreplicative variants, H3.3 and CENP-A. CENP-A is incorporated in centromeric heterochromatin and is important to define the centromere, whereas H3.3 is highly expressed in actively transcribing cells, it is incorporated in active regions of the genome (Figure I), and it is associated with dynamic chromatin remodeling during development and differentiation.

Several histone variants exhibit tissue-specific expression patterns and functions, highlighting their importance in cell type–specific regulation of gene expression. Overall, histone variants provide a regulatory component of chromatin, controlling diverse cellular processes, including transcriptional regulation, chromatin remodeling, and genome stability. They play key roles in maintaining cellular homeostasis, and mutations or altered expression of some could lead to cellular dysfunction, cancer, and neurodegenerative diseases.

Glossary

Constitutive heterochromatin: highly compacted chromatin that is transcriptionally inactive. Includes structural regions of the chromosome, such as centromeres, that lack genes.
DAXX-ATRX: named after its two main subunits, the SWI/SNF-like protein ATRX and DAXX, this chromatin remodeling and histone chaperone complex deposits H3.3/H4 into suppressed chromatin. Mutations in the subunits of this complex are associated with a variety of different cancers.
Embryonic stem cells (ESCs): are derived from the inner cell mass of the blastocyst-stage mammalian embryo. These cells retain their pluripotential and can give rise to all different cell types by *in vitro* differentiation.
Epithelial-to-mesenchymal transition (EMT): the process by which epithelial cells become mesenchymal. Cells undergoing EMT acquire the capability to migrate. This occurs naturally during different stages of development but also in cancer, providing the cells with the capacity to metastasize.
Euchromatin: a form of chromatin that is relatively decondensed and often transcriptionally active during interphase.
Facultative heterochromatin: chromatin regions in which genes are held silenced in a given cell type or a given developmental stage.
Histone cell cycle regulator (HIRA): chromatin remodeler and histone chaperone complex that deposits H3.3/H4 in active chromatin regions. Main subunits of the HIRA complex include the HIRA protein, UBN1/2, and CABIN1.
Polycomb repressive complex 2 (PRC2): a methyltransferase enzymatic complex that methylates H3K27 and regulates expression of many developmental genes. Its main subunits include EZH1/2, possessing the catalytic activity, RBBP4, SUZ12, and EED.
SOX2: a pivotal transcription factor that plays a crucial role in pluripotency and self-renewal, as well as during neural differentiation. It is one of the 'Yamanaka factors,' expression of which in somatic cells induces reprogramming and pluripotency. Many cancers contain *Sox2* aberrations.
Telomeres: a region of repetitive DNA at the ends of chromosomes that protects the chromosomes from premature deterioration, rearrangements, and chromosome fusion.

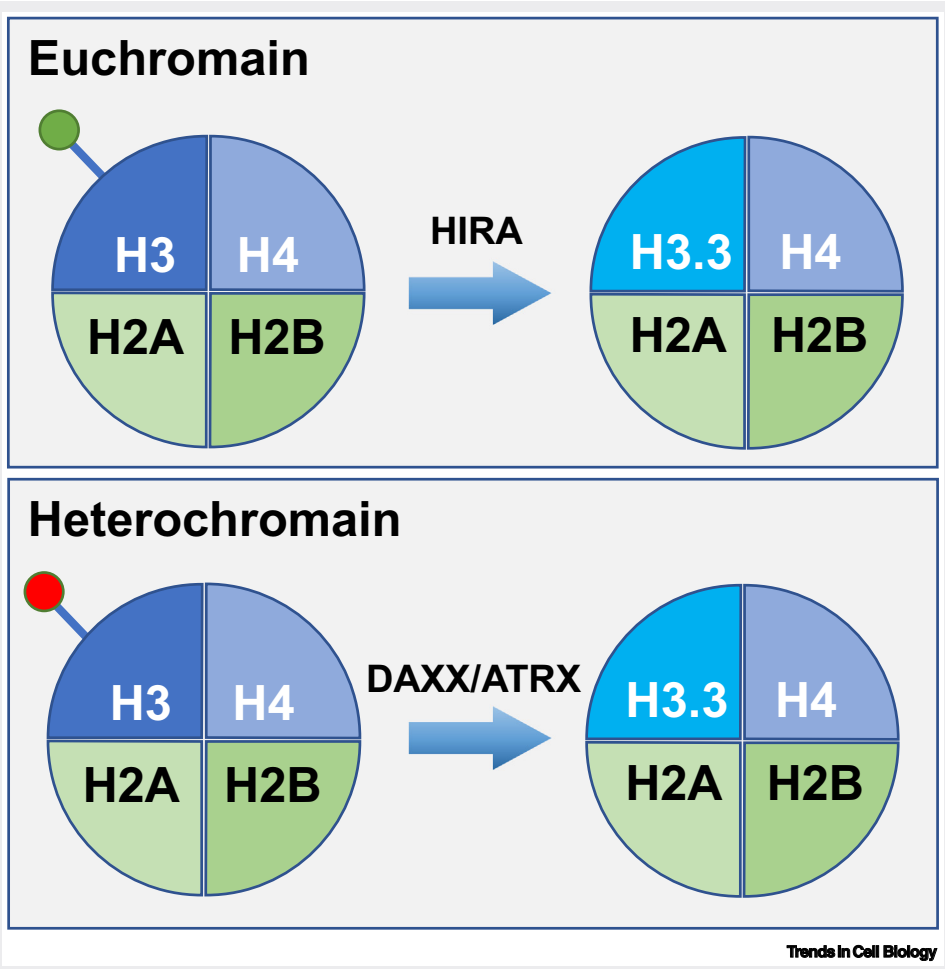

Figure I. H3.3 incorporation pathways. In euchromatin, H3.3 is incorporated via the HIRA pathway (top), whereas in heterochromatin, it is incorporated via the DAXX/ATRX pathway (bottom).

Table 1. H3 mutations in cancer

Mutation	Disease	Refs
K27M	Diffuse midline glioma (DMG), posterior fossa ependyma (PFE), acute myeloid leukemia (AML)	[60,101,102]
K27I	Diffuse midline glioma (DMG), acute myeloid leukemia (AML)	[83,101]
G34V	Glioblastoma multiforme (GBM)	[60,62]
G34R	Glioblastoma multiforme (GBM), osteosarcoma	[60,62]
G34W	Osteosarcoma; giant cell tumor of the bone (GCTB)	[62]
G34L	Osteosarcoma; giant cell tumor of the bone (GCTB)	[62]
K36M	Chondroblastoma; head and neck carcinoma	[62,103]
Overexpression	Lung cancer metastasis	[97]
Multiple missense mutations	Neurodegeneration; cortical atrophy; developmental delay	[55]

H3.3 contributes to heterochromatin formation in the early embryo

As a nonreplicative variant, H3.3 is deposited in distinct regions of the genome by two separate chaperone systems [3,14]. **Histone cell cycle regulator (HIRA)** is responsible, at least in part [15], for the incorporation of H3.3 in active chromatin [3,14], whereas **death domain associated protein (DAXX)–ATP-dependent X-linked helicase (ATRX)** is required for the incorporation of H3.3 in telomeres and heterochromatin [16]. **Constitutive heterochromatin** generally refers to regions of chromatin that remain condensed and transcriptionally silenced [17].

H3.3 is crucial for early development in mammals, and knockout (KO) of the four H3.3-encoding alleles is embryonically lethal in mice [18,19]. At the two-cell stage, H3.3 is involved in establishing heterochromatin and repressing genes [20,21]. In line with this, a point mutation in H3.3 rather than H3.1 leads to developmental arrest as early as the two-cell stage in mouse embryos [20]. Interestingly, H3.3-KO in murine embryos has little influence on gene expression patterns or on ATRX recruitment to heterochromatin, and instead it impairs heterochromatin formation, which in turn triggers mitotic failure, genome instability, and chromosomal aberrations (e.g., lagging chromosomes), resulting in embryonic lethality [19]. Potentially explaining these phenotypes is the idea that H3.3 acts as a placeholder for the centromeric specific H3 variant, CENP-A (centromeric protein A) [22].

One of the major modifications specifically important for heterochromatin establishment in mouse embryos is H3.3K27 methylation. A lysine-to-arginine substitution mutation in H3.3, H3.3K27R, was shown to impair the silencing of pericentromeric transcripts, resulting in dysfunctional chromosome segregation and developmental arrest [20]. This was specific to H3.3 because H3.1K27R did not provoke a similar response, suggesting a unique role for H3.3K27 in pericentromeric regions. Similarly, H3.3K4R mutation, which impacts H3.3K4 methylation marks in open chromatin, did not induce a developmental arrest, suggesting that during the early stages of embryonic development, H3.3 is specifically crucial for heterochromatin (Figure 1).

Interestingly, H3.3 incorporation by HIRA also participates in the stabilization of repressive chromatin and the exit from the two-cell stage. This occurs presumably due to H3K9 trimethylation of the incorporated H3.3 and recruitment of the H3K9 methyltransferase Suv39H1 (suppressor of variegation 3-9 homolog 1) by the CABIN1 (calcineurin binding protein 1) subunit of the HIRA complex, enabling the silencing of two-cell stage genes [21]. Because both H3K27me3 and H3K9me3 promote propagation of histone methylation by recruiting and thus spreading the methylating complex [23], modified histones by themselves can promote silencing and contribute to the maintenance of the heterochromatin landscape. These studies portray a vital role for H3.3 in the earliest stages of mammalian development, specifically in the establishment of heterochromatin.

H3.3 maintains heterochromatin in ESCs

As observed in early embryos, H3.3 is partially localized in heterochromatin regions in mouse ESCs, and H3.3 depletion in mouse ESCs results in mitotic defects due to genome instability as a result of disturbed heterochromatin [19]. Both DAXX and ATRX participate in H3.3's incorporation into heterochromatin and in its formation and maintenance [24,25]. Mitotic defects were observed in *Daxx*-KO ESCs, but not in *Hira*-KO ESCs, supporting a possible H3.3-specific role in heterochromatin, but not in **euchromatin**, maintenance in ESCs [26].

Although chromatin proteins are usually less dynamic on heterochromatin than euchromatin [27], presumably due to the more compact environment, H3.3 was shown to maintain dynamic

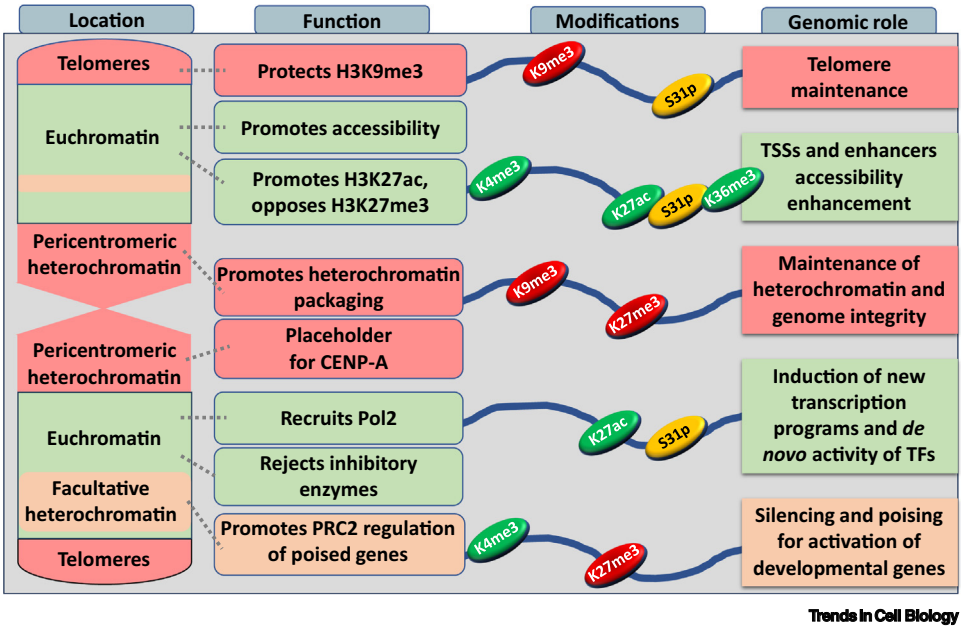

Figure 1. H3.3's different localization, functions, and related modifications. From left to right: a map of H3.3 genomic localization, the functions it plays in these regions, the H3.3 modifications specifying these functions, and the final contribution to the cell. Roles, modifications, and regions related to gene silencing are in shades of red, and those related to activation are in shades of green.

turnover in heterochromatin in mouse ESCs [24]. Interestingly, rapid turnover of H3.3 in endogenous retroviral elements (ERVs) was not accompanied with increased accessibility. Instead, *H3.3*-KO in ESCs induced increased heterochromatin accessibility and perturbed silencing of heterochromatic elements, suggesting that H3.3 acts to protect from spurious transcription in silenced regions, perhaps via DAXX-mediated H3.3 incorporation [24]. This idea is supported by other studies demonstrating that H3.3 is involved in the silencing ERVs and other retroviral elements in ESCs and that its depletion leads to their induction [28].

Telomeres, the regions at the ends of the chromosomes, are made up of repetitive DNA sequences, which, through the action of telomerase and its complex, protect the DNA from degradation. In the mammalian genome, telomeres are marked by H3K9me3. H3.3 is essential for the formation of proper telomere chromatin because H3.3 knockdown (KD) using RNA interference (RNAi) induces telomere dysfunction in mouse ESCs [29]. Interestingly, phosphorylated H3.3S31 (H3.3S31ph) marks both active chromatin and telomeres in ESCs [29], and H3.3S31ph in heterochromatin was shown to protect H3K9me3 by inhibiting the lysine demethylase KDM4B, thus preventing H3K9me3 demethylation [25]. These observations suggest a dual role for H3.3 in maintaining both active and suppressive chromatin in ESCs.

But the role of H3.3 in heterochromatin regulation is not limited to ESCs. Depletion of H3.3 in mouse embryonic fibroblasts (MEFs) disrupted telomere maintenance and structure [19], and H3.3 was further shown to maintain H3K9me3 and to suppress transcription from repetitive elements in neurons [30] and hematopoietic cells [31]. Regardless, the relative abundance of H3.3 in heterochromatin [24], as well as specifically of H3.3S31ph in telomeres, decreases upon ESC differentiation [29], hinting at some prominent selective role for heterochromatic/**telomeric** H3.3 in undifferentiated ESCs (Figure 1).

H3.3 contribution to euchromatin in ESCs

H3.3 in undifferentiated ESCs

As mentioned already, H3.3 is incorporated into active chromatin regions by HIRA [3,14]. This HIRA-mediated incorporation of H3.3 associates with an increase in the abundance of active chromatin marks in mouse ESCs [32,33]. Accordingly, *H3.3*-KO induces a global reduction in H3K27ac and a global enrichment in H3K27me3 [32]. Interestingly, HIRA also regulates the histone H2A variant H2A.Z incorporation into promoters of active genes [34], and it was suggested that the incorporation of H3.3-H2A.Z into nucleosomes destabilizes them [35], rendering the chromatin more active. In mouse ESCs, H3.3 shows very limited dynamics on chromatin globally using photobleaching assays [36], but genome-wide sequencing approaches revealed high turnover in regulatory regions [37]. Specifically, H3.3 localization and high turnover in mouse ESCs was mapped to the transcription start site of active genes and superenhancers [38,39]. H3.3 incorporation and turnover were associated with DNA accessibility and transcription [38–40] and with active histone marks [37]. Along these lines, H3.3 was also shown to facilitate RNA pol2 activity and transcription factor (TF) binding at mouse ESC promoters [33]. However, whether H3.3 directly regulates chromatin accessibility is somewhat debatable. One study did not observe changes in chromatin accessibility, especially around enhancers, in mouse ESCs depleted for H3.3 [32], whereas another recent study, mentioned already, reported decreased accessibility around promoters and regulatory elements in H3.3-KO ESCs [33]. Either way, these changes in chromatin accessibility and histone marks did not induce any major changes in gene expression [32,33].

The subtle effects of *H3.3*-KO on gene expression patterns in undifferentiated mouse ESCs raises the question whether H3.3 incorporation is a by-product of transcription or whether it plays an active regulatory role in transcription [41]. In recent years, accumulating evidence in various different cell types, including ESCs, supports the latter. In mouse neurons, for example, H3.3 incorporation and turnover were shown to regulate gene expression upon neuronal activation, inducing synaptic plasticity and connectivity [42,43]. In macrophages, H3.3 was shown to be involved in the activation of immune responses [44] and in interferon (IFN)-mediated transcription [45,46].

In mouse ESCs, the most concrete evidence for a direct role for H3.3 in transcriptional regulation was shown in a study that explored the effects of H3.3K4A mutations [47]. The authors knocked out one of the two H3.3 genes and mutated the other one into the H3.3K4A version. Although phenotypes and gene expression changes were mostly noticeable during ESC differentiation and in differentiated cells, the mutant H3.3K4A undifferentiated ESCs also showed transcriptional aberrations. These aberrations, the authors found, were primarily linked to nucleosome deposition and were partially rescued by ectopic expression of WT-H3.3, but not by H3.1, linking these observed changes specifically to H3.3 incorporation. Altogether, these observations suggest that, also in ESCs, H3.3 regulates transcription [47] and that the relatively minor changes in gene expression observed in H3.3-KO ESCs suggest that ESCs have the ability to compensate for the loss of H3.3.

As mentioned previously, developmental genes poised for activation are bivalently marked with both H3K4me3 and H3K27me3. H3.3 was shown to counteract the recruitment of the H3K27 methyltransferase complex, **Polycomb repressive complex 2 (PRC2)**, thereby limiting H3K27me3 deposition [48]. Around developmental genes, however, it acts to maintain H3K27, but not H3K4, methylation [49]. This demonstrates that in ESCs, H3.3 specifically facilitates PRC2 regulation of bivalent promoters, thus playing a role in regulating lowly expressed but highly regulated developmental genes. This is similar to the situation in mouse hematopoietic stem cells (HSCs), where

H3.3 was shown to maintain bivalent chromatin [50], but in contrast to the situation in MEFs, where H3.3 is incorporated selectively into active genes [37]. In human ESCs (hESCs) as well, HIRA-mediated H3.3 incorporation around promoters was shown to regulate pluripotency by controlling the expression of both metabolic and pluripotency-related genes [51]. Taken together, H3.3 seems to play an active and important role in modulating chromatin structure and nucleosome turnover in ESCs and serves distinct and crucial functions in various different cell types (Figure 1).

H3.3 in ESC differentiation

H3.3 maintains pluripotency and self-renewal capacity in ESCs by regulating the expression of pluripotency genes and developmental genes [52]. However, H3.3 is also required for the initiation of ESC differentiation. *H3.3*-KO mouse ESCs fail to initiate differentiation and maintain their stem cell state. When ESCs are coaxed to differentiate, H3.3 incorporation in active chromatin enables *de novo* transcription during differentiation and TF binding to lineage-specific TF motifs [33]. H3.3 incorporation regulates pluripotency-related genes (e.g., **Sox2**) and enables the transition from a pluripotent toward a differentiated state [47]. Upon ESC differentiation, H3.3 is evicted from pluripotency genes [16] and from ESC-specific enhancers [39,53] and instead incorporates around lineage-specific elements such as neurogenic genes upon their activation by RA-induced differentiation. H3.3 was further shown to play an active role in differentiating neural stem cells (NSCs) in mouse, because H3.3 suppression led to a reduction in proliferating PAX6-positive NSCs and promoted premature terminal neuronal differentiation [54]. Moreover, H3.3 gradually accumulates in mature neurons, becoming the predominant form in the postmitotic brain [42]. It is therefore not entirely surprising that some 40 different missense mutations spread across the entire coding region of the two H3.3 encoding genes (*H3.3A*; *H3.3B*) were shown to be associated with neurological phenotypes, including seizures, cortical atrophy, neurodevelopmental delays, and early- or late-onset neurodegeneration [55] (Figure 2).

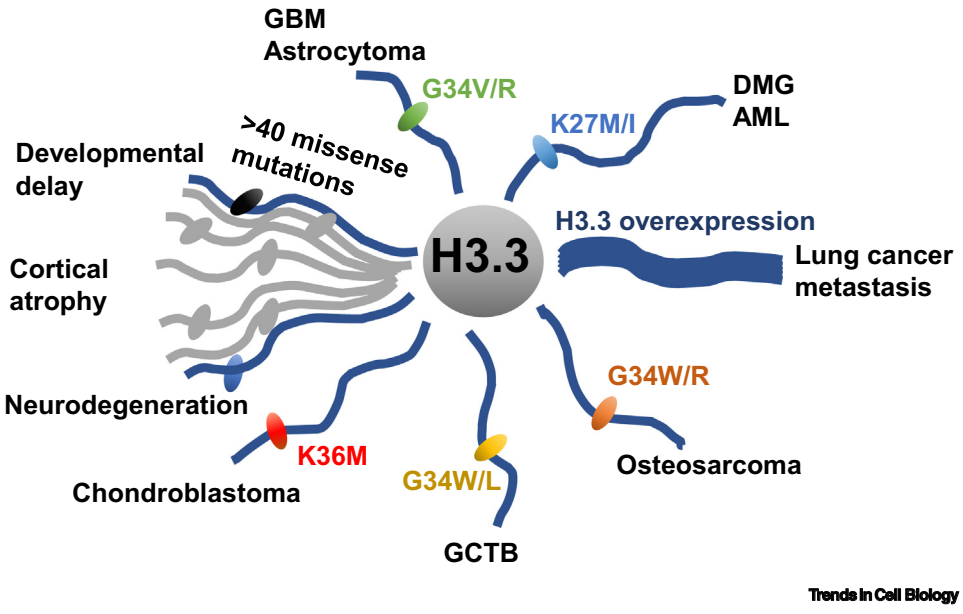

Figure 2. H3.3 mutations and human disease. Shown are the different H3.3 mutations and the diseases they are associated with. Although most mutations lead to specific types of cancer, the phenotypes in the neurological patients include seizures, brain atrophy, neurodevelopmental abnormalities, and neurodegeneration but were never associated with cancer, suggesting other underlying mechanisms. Abbreviations: AML, acute myeloid leukemia; DMG, diffuse midline glioma; GBM, glioblastoma; GCTB, giant cell tumor of the bone.

Specific H3.3 modifications are crucial for the ability of ESCs to further differentiate. For example, the H3.3K4 mutation discussed in the preceding text impaired neuronal differentiation of mouse ESCs, leading to a reduced number of differentiated cells and to an increased percentage of SOX2-positive neuronal progenitors [47]. Similarly, mutations in H3.3K27 also impair mouse ESC differentiation, leading to the retention of pluripotency-related gene expression programs supporting stemness [56]. Aberrant differentiation and increased proliferation were also reported in human ESCs expressing H3K27M mutations [57]. Further supporting a role for H3.3 in controlling stemness, expansion of blood multipotent progenitors was observed in mouse models harboring H3.3K9M, and severe differentiation defects and early lethality were recorded in mice expressing H3.3K36M [58]. Overall, H3.3 is crucial for differentiation, operating not only in undifferentiated ESCs themselves but also, perhaps more important, at the onset of the differentiation process, shifting its targets from pluripotency- to differentiation-related elements (Figure 1).

H3.3 misregulation of cell fate and differentiation contributes to cancer formation

Cancer cells must proliferate and adjust to new environments, imposing pressure on the epigenetic landscape to support their needs. It is therefore not surprising that mutations in genes encoding proteins that shape the epigenetic landscape – chromatin remodelers and histone chaperones, including DAXX and ATRX – are frequently found in many cancers [2]. In addition, over the past decade, researchers identified multiple somatic mutations in histone-coding genes in many different types of cancer. This was unexpected because each histone protein is encoded by multiple different genes, and therefore the resulting mutant histone constitutes only a small fraction of the total histone. However, these mutated histones act in a dominant-negative fashion, and therefore a single somatic mutation is sufficient to drive cancer. This led to the coining of the term ‘oncohistones’ [59].

In 2012, the exomes of several patients with glioblastoma were sequenced, and a significant portion was found to carry mutations in H3.3. Among these, H3.3K27M was found to be the most prevalent, mostly in gliomas [60,61]. H3.3G34V/R mutations were also identified in glioma cases [60,62]. Since then, driver mutations in H3.3 were identified in a variety of different cancers, including chondroblastoma, giant cell tumors of the bone, giant cell tumors of soft tissue, and more [63–67] (Figure 2). H3.3’s function and dysfunction in cancer provide another clue to the role that H3.3 and its modifications play in the maintenance of cell identity and reprogramming.

In one prominent example, the cancer driver mutation H3.3K36M was shown to reduce global H3K36me2/3 levels and induce cancer in the human bone marrow [68] in cells arising from mesenchymal stem cells (MSCs). H3.3K36M impairs the differentiation of mesenchymal progenitor cells in both mouse and human, resulting in undifferentiated sarcomas [69]. H3K36M was shown to act via two different pathways: first, by controlling DNA methylation, leading to the recommissioning of pluripotency enhancers instead of somatic enhancers, and second, by opposing PRC2-mediated silencing and regulating mesenchymal gene expression [70]. PRC2 propagation is limited by H3K36me2 boundaries [71], and H3K36 methylation regulates mesenchymal cell identity by counteracting the propagation of H3K27 methylation silencing toward lineage-specific genes, inhibiting reprogramming [70,72,73]. Reduction in H3K36me3 is followed by enhanced PRC2-dependent silencing of lineage-specific genes, inducing cellular reprogramming. A similar mechanism was suggested for cancers induced by H3.3G34 mutations [74], as well as H3.3G34V/R interactions with histone methyltransferases KDM4B or ZMYND11, as well as with DNA methyltransferases DNMT3A and DNMT3B [75].

Unlike H3.3K36M, which was mostly identified in bone-related tumors, H3.3K27M-related tumors likely arise from oligodendrocyte progenitor cells (OPCs) [76], which originate from

neuroepithelial cells [77]. H3.3K27M influences different cell types in different ways. In a mouse model of diffuse midline glioma (DMG) involving *p53*-KO and the overexpression of PDGF-A (or PDGF-B), H3.3K27M was shown to have distinct or even opposite effects in different cell types [78]. In OLIG2-positive cells, it promotes **epithelial-to-mesenchymal transition (EMT)** but suppresses it in NESTIN-positive cells. The origin OPCs of H3.3K27M tumors were shown to be positive for OLIG2 [79], suggesting that DMG is supported by EMT. Mechanistically, H3.3K27M mutations inhibit PRC2 activity in a dominant fashion, inducing a global reduction in H3K27me2/3 [80]. The idea that reduced H3K27me3 could promote EMT lends support from the observation that depleting the PRC2 component SUZ12 induces expression of mesenchyme genes, such as *Gata4* [81]. This is in contrast to H3.3K36M, which promotes mesenchymal-to-epithelial transition (MET) [70]. This distinctive influence of the two H3.3 mutations can suggest an interesting relationship between the abundance and balance of H3.3K27 methylation and H3.3K36 methylation and epithelial or mesenchymal identity.

Although many H3.3-related driver mutations act in a dominant-negative fashion to globally shift the epigenetic landscape of the cell, recent evidence suggests that it may not be the entire story. H3.3K36M and H3.1K36M both inhibit H3K36 methylation, but H3.1K36M does not lead to cancer [82]. Other than H3.3K27M, H3.3K27I is the only additional K27 mutation that inhibits PRC2 [80], and, interestingly, it seems to induce a similar disease [83], suggesting that PRC2 inhibition plays a direct role in the development of this cancer. However, loss of function of PRC2 by other mechanisms induces different types of cancers. In addition, PRC2 inhibition occurs in all cell types carrying H3.3K27M mutations, but changes in proliferation rates were observed only in a cell type–specific context [84]. Also, K27M in both H3.1 and H3.3 inhibit PRC2 in a dominant fashion. Yet, these two proteins induce nonidentical diseases [85] associated with different ages of onset, survival durations, and responses to therapy [83]. So, although the distribution of H3K27me3 differs between cells carrying H3.1K27M and H3.3K27M [86,87], potentially explaining some of the phenotypic differences, these diverging disease outcomes suggest the involvement of a pathway that is not entirely dependent on global PRC2 inhibition.

As noted in the preceding text, PRC2 regulates the expression of developmental genes in ESCs, preventing their spontaneous differentiation. In cells carrying H3.3K27M, some genes get activated, but they are not, by and large, PRC2 targets [88,89]. Interestingly, although PRC2 activity is significantly diminished globally in H3.3K27M-mutant cells, residual PRC2 activity is retained or even enhanced around strong PRC2 targets [87,90–93]. This activity is necessary for the proliferation of cancer cells because it represses their differentiation into neurons [88,94].

H3.3 mutations that drive glioma are usually associated with characteristic additional mutations [95]. H3.3K27M mutations, for example, are often accompanied with ATRX mutations [96] but never with HIRA mutations, suggesting that H3.3 incorporation into active chromatin is beneficial for the cancer cells. Indeed, incorporation of H3.3K27 mutated histones in active genes impaired differentiation also when PRC2 was intact, suggesting a PRC2-independent role for the local incorporation of H3.3 [56]. Incorporation of WT-H3.3 was also shown to support the gain of pluripotency in certain stages of reprogramming [73], suggesting that H3.3K27M histones can promote pluripotency using more than one pathway.

Overexpression of WT-H3.3 was also associated with cancer. H3.3 overexpression is associated with lung cancer severity and perturbation of cell identity and is involved in EMT [97]. Also, HIRA-dependent H3.3 incorporation was shown to participate in EMT and metastasis formation in carcinoma and skeletal muscle cancer [98,99]. However, WT-H3.3's role in cancer is context dependent because H3.3 was involved in termination of cancer of murine erythroleukemia cells

[100]. Taken together, keeping H3.3 intact is key for maintaining a healthy cell, and multiple pathways can lead to cancer and other types of pathologies, not unlike its role in the maintenance of the stem cell state.

Concluding remarks and future perspectives

As we have seen, H3.3 participates in the regulation of gene expression in various ways. On the one hand, it can enhance open chromatin landscape and promote transcription, and, on the other hand, it can act to enhance PRC2 inhibition of poised genes and inhibit their expression. In addition, in constitutive heterochromatin, it suppresses the expression of repetitive elements. Future studies should reveal how H3.3 achieves these different feats and decipher its specific role in each cellular context. Despite H3.3's contribution to chromatin accessibility and gene regulation, ESCs seem to cope well in its absence, although they fail to properly differentiate. Although some evidence suggests that in undifferentiated ESCs H3.3 depletion leads to premature expression of trophoblast-related genes, the general lack of phenotype calls for further investigation of potential compensatory mechanisms. Alternatively, phenotypes might be revealed once H3.3-depleted ESCs are challenged.

H3.3 is also necessary for stabilization of a recently acquired cell identity, and increased H3.3 incorporation and unbalanced H3.3 modifications landscape can perturb cell identity and lead to cancer. H3.3 mutations are major cancer drivers, and even noncancerous H3.3 mutations induce upregulation of genes involved in mitotic processes and increased cell proliferation [55]. But although we have learned a great deal about the different types of H3.3 mutations involved in cancer, the precise mechanisms that contribute to, e.g., the development of specific types of tumors or the appearance of these tumors in particular developmental stages remain largely enigmatic (see Outstanding questions). Studying these mutations in different cellular contexts can shed light on the specific functions of the different H3.3 modifications that are altered and the role they play in health and disease, with the hope of ultimately translating these findings into therapy.

Outstanding questions

How does H3.3 achieve its opposing functions in euchromatin and heterochromatin?

Given its crucial role, how do embryonic stem cells cope without H3.3 as undifferentiated cells?

What are the PRC2-independent actions of H3.3 mutations in cancer?

How do H3.3 mutations lead to neurodegeneration?

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Declaration of interests

The authors declare no competing interests.

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