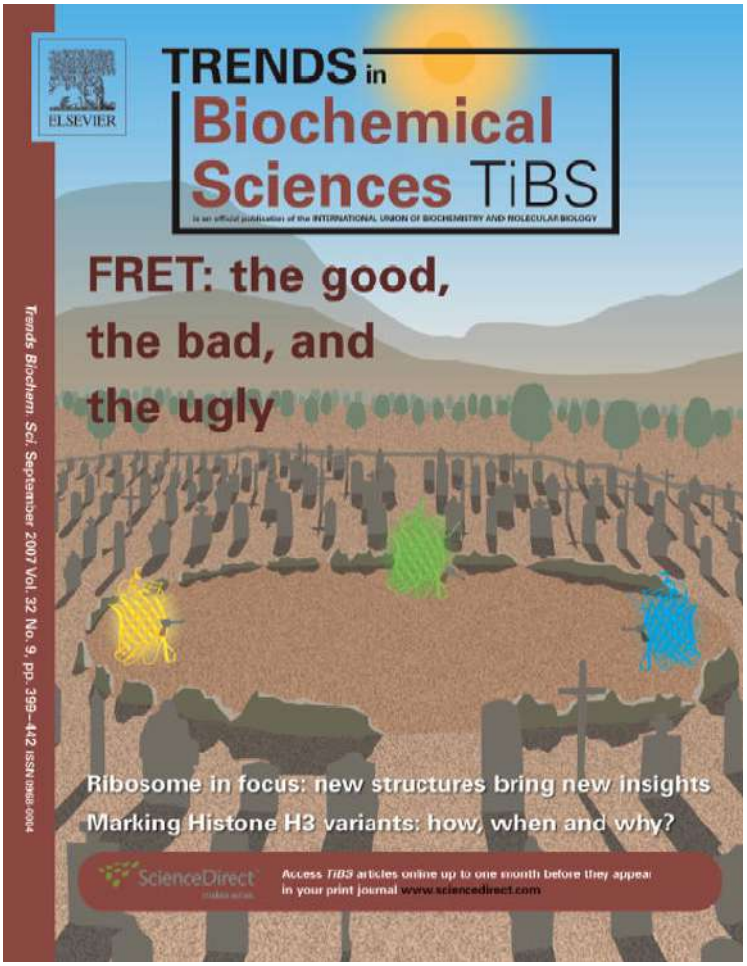

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Marking histone H3 variants: How, when and why?

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DNA in eukaryotic cells is compacted into chromatin, a regular repeated structure in which the nucleosome represents the basic unit. The nucleosome not only serves to compact the genetic material but also provides information that affects nuclear functions including DNA replication, repair and transcription. This information is conveyed through numerous combinations of histone post-translational modifications (PTMs) and histone variants. A recent challenge has been to understand how and when these combinations of PTMs are imposed and to what extent they are determined by the choice of a specific histone variant. Here we focus on histone H3 variants and the PTMs that they carry before and after their assembly into chromatin. We review and discuss recent knowledge about how the choice and initial modifications of a specific variant might affect PTM states and eventually the final epigenetic state of a chromosomal domain.

Replicative histones and replacement histones

In the nucleosome, DNA is wrapped around a histone octamer, comprising a central core made of a tetramer of histones H3–H4 flanked by two dimers of histones H2A–H2B. Duplication of this basic unit during DNA replication necessitates doubling the amount of histones for proper packaging of the replicated material, a task that is facilitated through two sources of histones: those of parental origin, obtained through recycling; and those newly synthesized to guarantee a full complement. For the latter source, a set of histone genes, known as the 'canonical' or 'replicative' histone genes, are expressed in a manner that is tightly coordinated with S phase. In addition to these replicative histone genes, another set of histone genes produces transcripts throughout the cell cycle and during quiescence, the protein products of which are called 'replacement' histone variants. As discussed later, these two types of histone are incorporated into DNA by different chromatin assembly pathways.

Until recently, histone variants were thought simply to function in a redundant manner, whereby the replacement variants are essentially another supply that can substitute for the replicative ones. Several recent examples have revealed, however, that at least in some situations replacement histones have specific features that distinguish them from their replicative counterparts and can have a profound impact on cellular functions (reviewed in Ref. [1]).

The number of histone H3 variants differs among species (Table 1). In mammals, they comprise four main members [2]: H3.1 and H3.2 are the replicative histones expressed during S phase; H3.3 is the replacement histone expressed throughout the cell cycle; and centromeric protein A (CENP-A) is specifically present at centromeres. In addition, another variant found in testis is called H3t [3]. Human H3.1 and H3.2 are 99% identical; and H3.3 is 96% identical to H3.1, differing at five amino acid positions. Human CENP-A is highly divergent and shares only 46% identity with H3.1, whereas H3t differs from H3.1 by just four amino acids (Figure 1a).

In this review, we highlight recent findings that help us to follow better the modifications made to histone H3 variants from their synthesis until their incorporation into specific chromatin domains (Box 1). We focus specifically on the human H3.1 and H3.3 variants to evaluate the extent to which specific chromatin assembly pathways affect their final destination, and how post-translational modifications (PTMs) are imposed before the deposition of these histones onto DNA. We also discuss how H3 variants and their PTMs contribute to marking specific chromosomal domains. In particular, we examine possible roles of H3 variants: first, in establishing epigenetic information early during development; and second, in ensuring inheritance of this information into daughter cells in a stable cell lineage.

Incorporation of histones H3.1 and H3.3 into chromatin

The idea that histone H3 variants might show distinct properties was first established with the most divergent form, CENP-A, which has unique properties essential for centromere function [4]. More recently, work in *Drosophila* has shown that H3.3 can potentially make a link between variants in chromatin structure and transcriptional states [5]. Using green fluorescent protein fusion proteins, Ahmad and Henikoff *et al.* [5] found that the replicative H3 is incorporated only during S phase, whereas H3.3 can be incorporated at any phase of the cell cycle and accumulates in actively transcribing ribosomal repeat genes. These findings led to the hypothesis that H3.3 might be a mark of transcriptional activation.

In human cells, nuclear complexes containing H3.1 (the replicative H3) and H3.3 have been isolated that show both

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Table 1. Histone H3 variants in different organisms[a]

Organism	Replication dependent (replicative)	Replication independent (replacement)	Centromere
Saccharomyces cerevisiae	None	Hht1, Hht2 (H3.3-like)	Cse4
Schizosaccharomyces pombe	None	H3 (H3.3-like)	Cnp1 (SpCENP-A)
Neurospora crassa	None	H3 (H3.3-like)	H3v
Caenorhabditis elegans	H3	H3.3	HCP-3
Drosophila melanogaster	H3 (homologous to H3.2)	H3.3	Cid
Xenopus laevis	H3 (homologous to H3.2)	H3.3	CENP-A
Arabidopsis thaliana	H3	H3.2 (H3.3-like)	CenH3
Mammals	H3.1, H3.2	H3.3	CENP-A

[a]Owing to a lack of sufficient information at the time of writing, the H3t variant has been omitted from the table. Adapted, with permission, from Ref. [10].

distinct protein composition and specific chromatin assembly properties. The histone chaperone Chromatin assembly factor-1 (CAF-1) was exclusively found in the H3.1 complex, whereas the histone chaperone Histone regulation A (HIRA) was associated only with H3.3 [6,7]. These two chaperones support DNA-replication-coupled (RC) and DNA-replication-independent (RI) chromatin assembly, respectively ([6]; reviewed in Refs [8–10]). How the specific associations between H3.1 and CAF-1, and between H3.3 and HIRA occur, and how H3.1 and H3.3 are specifically incorporated into the DNA by the RC and RI pathway, respectively, remain a mystery.

Given that the bulk of H3.1 expression is largely coupled to DNA replication and H3.3 is expressed throughout the cell cycle, a simple explanation might be based on this difference in the timing of expression of the variants. If true, variant availability would be the only parameter to determine the specificity of assembly. Although this parameter must be considered, however, it is not sufficient enough to explain the observed specificity. Indeed, even when H3.1 is constitutively expressed throughout the cell cycle, it remains specifically bound to CAF-1 and is incorporated only by the RC pathway [5,6].

Notably, amino acids located in the histone fold domain of *Drosophila* H3 (Ala87, Ile89 and Gly90, which are part of the α2 helix; Figure 1) are central to preventing incorporation through the RI pathway [5]. Thus, these observations suggest that the α2 helix somehow has an essential role in the choice of pathway. Whether this role is due to a lack of association with HIRA, such that RC deposition becomes active by default, needs to be investigated. Furthermore, the amino-terminal tail of the replicative

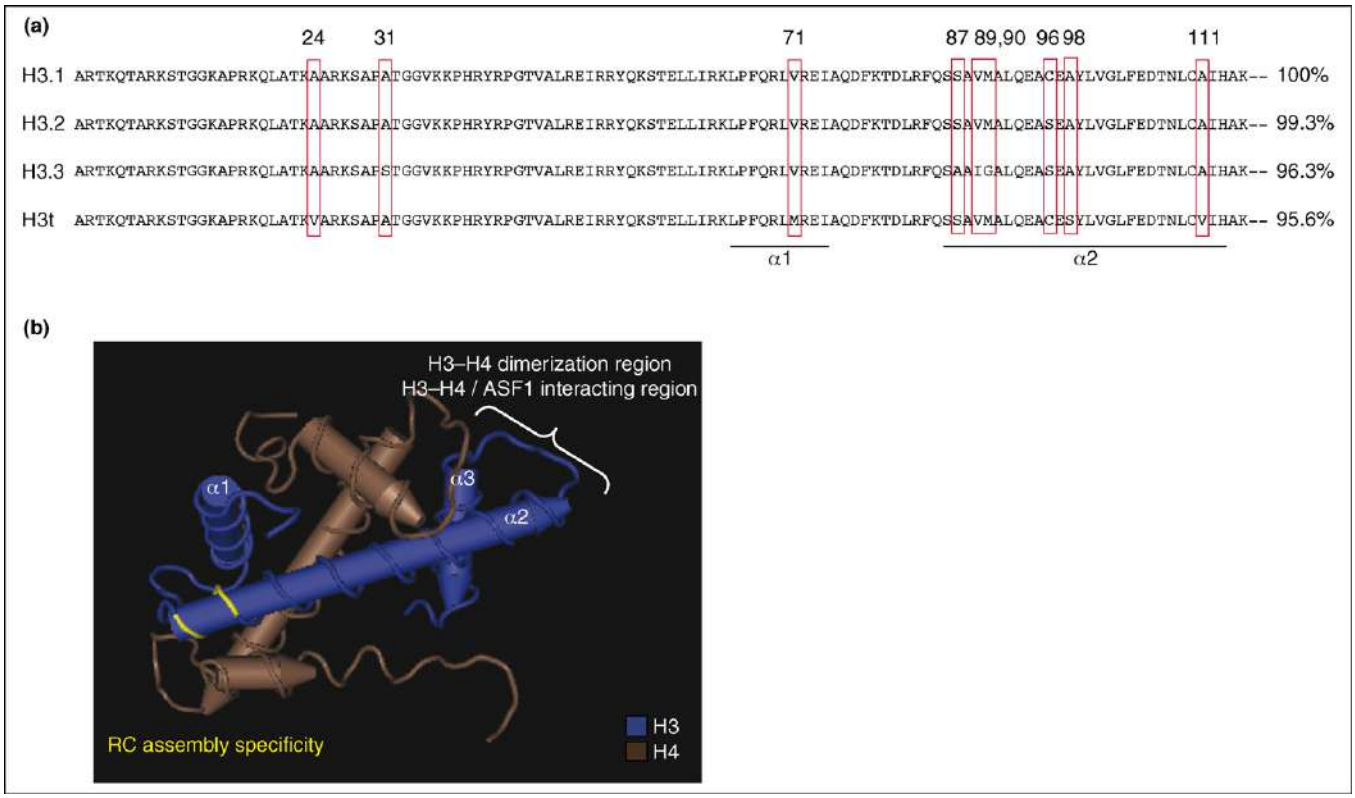

Figure 1. Histone H3 variants and their interaction with H4. **(a)** Sequence alignment of the main mammalian histone H3 variants: H3.1, H3.2, H3.3 and H3t. The amino acids that differ among the sequences relative to H3.1 are highlighted. Amino acids 87, 89 and 90 are key to preventing histone H3.1 incorporation into chromatin through the DNA-replication-independent (RI) pathway. The percentage of homology is shown on the right, taking the H3.1 sequence as 100%. The bars labeled α1 and α2 represent the residues that constitute two of the three α helices forming the histone fold domain. **(b)** Ribbon diagram of the structure of the H3–H4 dimer. H3 is shown in blue and H4 in brown. The dimerization region, which overlaps with the region of ASF-1 interaction (the C terminus of α2 and α3 in histone H3), is highlighted. Amino acids 87, 89 and 90 are indicated in yellow. Coordinates are taken from Ref. [13].

Box 1. The histone code and H3 barcode hypotheses

Histones can be covalently modified by several post-translational modifications (PTMs), including acetylation, methylation, phosphorylation and ubiquitination [52]. In all species, these modifications occur mainly in the amino-terminal tail of the histones, but they can also occur in the globular domain. Interestingly, recent studies have shown that there are some species differences in the modifications of histone H3, which become more complexes in mammals, in particular with respect to methylation marks [53].

The 'histone code' hypothesis proposes that specific PTMs and/or combinations of modifications regulate gene expression by two possible mechanisms: (i) changing the chromatin structure into an 'activated' or 'repressed' transcriptional state, or (ii) acting as a docking site for transcriptional regulators that associate with chromatin [54,55]. Several protein domains have been identified that recognize specific modifications. Among them are chromodomains, bromodomains and plant homeobox domain (PHD) domains, which recognize H3K9me, acetylated histones, and H3K4me, respectively (reviewed in Ref. [56]). An example is H3K9me3, which can recruit HP1 through its chromodomain to heretochromatic regions [29,30].

Extensive characterizations of the PTMs of histone H3 variants have shown that H3.1, H3.2 and H3.3 have distinct PTM patterns (see text). These observations have led to the 'H3 barcode hypothesis' [40], which proposes that each H3 variant can act as a signature to create different chromosomal regions.

histone H3 is necessary for RC but not RI assembly [5] (Figure 1b). This observation is intriguing, given that the tail has been found to be dispensable for CAF-1-dependent deposition of histones *in vitro* [11].

How histone variants interact specifically with particular histone chaperones remains unknown, and it is attractive to envisage that histone PTMs might affect the selectivity of these interactions. Recent structural analyses of the histone chaperone Assembly factor-1 (ASF-1) have shed light on its interaction with H3–H4 [12–15]. The region of ASF-1 that binds to H3–H4 is not compatible with the existence of H3–H4 tetramers and, instead, promotes ASF-1 interaction with H3–H4 dimers (Figure 1b). Interestingly, the small CAF-1 subunit Retinoblastoma-associated protein 48 (RbAp48) and HIRA both contain WD-40 repeat domains, which are predicted to have exposed β sheets. These domains are important in the interaction with H3–H4 dimers. Consistent with the preference of ASF-1 for H3–H4 dimers, CAF-1 and HIRA are both thought to interact with dimers of H3–H4 [6], suggesting that a common interacting feature shared by several histone chaperones might function along the whole assembly line. Distinct features of other domains most probably contribute to the specificity of choice of distinct chromatin assembly pathways.

Although it is tempting to consider replicative variants as a single class with essentially identical properties and to extend the biochemical findings on the human H3.1 to all of the replicative H3 variants, it remains to be investigated whether H3.2 (called H3 in *Drosophila*) is indeed associated with CAF-1 or with another histone chaperone. Lastly, incorporation of CENP-A into centromeric regions might involve another pathway. Clues pointing to this possibility have been provided by *Drosophila*, in which the deposition of CENP-A is performed by RbAp48, the smallest subunit of CAF-1 [16].

Clearly, much work is needed to determine whether the proposed mechanism is conserved in other species; however, the fact that H3.1 and H3.3 variants are present in different chromatin assembly pathways might have an impact on both the fate of the histones and their metabolism. As a result, it is important to evaluate how the PTMs associated with each of the variants dictate a particular assembly pathway.

Post-translational modifications on histone H3 variants

Initial analyses of PTMs present on distinct H3 variants were carried out on total acid-extracted material, in which histones incorporated into chromatin are largely over-represented relative to non-nucleosomal histones. This approach in alfalfa showed that, at steady state, there is a two-fold enrichment in acetylation of the histone variant H3.3 as compared with the replicative histone H3 [17]. Further analyses in other species, including *Drosophila* [18], plant [17,19] and mammals [20–22], essentially confirmed these findings. When compared to H3.1 (the replicative H3), these analyses showed that H3.3 is enriched in marks generally considered as representative of a 'transcriptionally active' state such as Lys4 (K4) and K36 methylation, and K9, K18 and K23 acetylation (Box 1).

These modifications can be attributed to the pool of histones incorporated into chromatin, which represent most of the total histone population (Table 2) [21]. Interestingly, comparison of the H3.1 and H3.2 PTMs in mammals shows that H3.2 is enriched in modifications associated with transcriptional repression, such as K27 that is dimethylated (K27me2) and trimethylated (K27me3), whereas H3.1 carries both active and repressive marks [20]. These findings suggest that H3.1 and H3.2 have potentially different roles, despite their comparable synthesis time and high amino acid sequence conservation (they differ by only one amino acid).

Currently, a picture emerges in which (i) the presence of H3.2 indicates heterochromatic sites, and (ii) H3.1 preferentially localizes to genes that can be switched to become active or repressed, depending on the metabolic state of the cell and developmental stage. This feature is reminiscent of the 'bivalent domains' found in stem cells and the chromatin signature of pluripotent cell lines [23,24]. In stem cells, bivalent domains show large chromosomal regions in which H3 is enriched in K27 methylation (a repressed mark) and small patches in which H3 is enriched in K4 methylation (an activated mark). Although this study [23] did not distinguish the status of individual H3 variants, finding out how distinct variants and their PTMs are used will be important for our understanding of specific marking in stem cells.

Clearly, histone marking is more complex than was originally anticipated, and recent studies challenge the simple distinction between transcriptionally active and transcriptionally repressive modifications with a more complex partitioning. Examples include the bivalent domains cited above, in which typically active (H3K4 methylated) and repressive (H3K27 methylated) marks are found in close proximity. Another example is the H3K9me3 modification, initially described as a mark of

Table 2. Known methylation and acetylation states on histone H3 variants and their associated H4

Modification type	Histone modified	Histone residue modified	Modification state	Non-nucleosomal H3 variant present[a]		Nucleosomal H3 variant present		
				H3.1	H3.3	H3.1	H3.2	H3.3
Methylation	H4	K20	me1	nd	nd	+	nd	+
			me2	–	–	+	nd	+
	H3	K4	me1	–	–	+	+	+
			me2	–	–	+	nd	++
			me3	–	–	+	nd	++
		Peptide (9–17)	me1	++	+	+	++	+
			me2/ac2	–	+	+++	++	+
			me3/ac	+	++	+	+	+
		K9	me2	–	+	+++	++	+
			me3	–	–	++	++	+
		K27	me1	–	–	+	+	++
			me2	–	–	+	++	+
			me3	–	–	++	+++	+
		K36	me1	–	–	+	+++	+++
			me2	–	–	+	+	+++
		K64	me1	nd	nd	+++	+	+
		K79	me1	–	–	+	+	+++
			me2	–	–	+	+	+++
			me3	–	–	–	nd	–
		K122	me1	nd	nd	+	+	+++
			me2	nd	nd	+	+	+
Acetylation	H4	Peptide (4–17)	monoAc	+	++	++	nd	+
			diAc	+++	+++	+	nd	+
			triAc	+	++	+	nd	++
			tetraAc	–	–	+	nd	++
	H3	Peptide (9–17)	me3/ac	+	++	+		+
		K9	ac	–	–	+	+	+++
		K14	ac	+	++	–	–	+
		K9/K14	diAc	–	+	+	+	++
		K18/K23	monoAc	+	++	+	+	+

[a]nd, not determined; –, modification absent; +, modification present.

transcriptionally silenced regions such as heterochromatin, but also found associated with genes that are actively transcribed [25]. Similarly, H3K36me has been typically associated with transcriptional elongation; however, it has also been associated with the suppression of transcriptional initiation in coding regions through the recruitment of histone deacetylates [26]. These examples illustrate the complexity of the histone marking that we are only beginning to appreciate.

Do H3.1 and H3.3 help to establish specific PTM patterns?

The fact that the H3.1, H3.2, and H3.3 variants have different PTM patterns in their final location in chromatin, and H3.3 is accumulated at active sites of transcription, has led to the proposal that the selective incorporation of specific variants could be a principal determinant for establishing particular PTM patterns [27,28]. How and when these patterns are actually imposed is thus an essential question.

To address these issues, we and our co-workers [21] recently characterized PTM patterns of human H3.1 and H3.3 variants before and after chromatin assembly. Non-nucleosomal histones – that is, histones that have not been assembled into chromatin – do not show PTM patterns that are comparable to their nucleosomal counterparts (Table 2 and Figure 2). A typical hallmark of non-nucleosomal H3/H4 dimers is enrichment in the acetylation of both H4K5 and H4K12; this enrichment is found regardless of the associated H3 variant. Most importantly, in contrast to nucleosomal histones, H3.1 and H3.3 show a general lack of methylation marks, with the exception of K9. This finding argues against a hypothesis in which the selective incorporation of specific variants is the primary determinant for establishing particular PTM patterns. Rather, it implies that PTM patterns are mainly imposed during or after histone deposition and depend on the local environment where the histones have been incorporated.

On the basis of these findings, we can propose a stepwise model to explain the establishment of PTM patterns (Figure 2). In this model, initial marks take place before histones are assembled into chromatin, when they are in a soluble form associated with histone chaperones. Modifications can occur at any time along the line of their transfer from their synthesis point to their final destination. The initial modification step is followed by modifications occurring during or after nucleosome assembly. Of course, cell-cycle modulation and the particular metabolic state of the cell must also be integrated. In addition, given that our analysis was performed with a transformed cell line [21], it will be important to pursue analyses of normal cells and different cell types. It remains crucial, under all circumstances, to understand how pre-existing modifications present on non-nucleosomal histones, if not erased, might affect final states in chromatin domains and how PTM patterns are imposed for specific chromatin regions.

K9 methylation and acetylation patterns in non-nucleosomal H3.1 and H3.3

Mass spectrometric analysis of non-nucleosomal human histones H3.1 and H3.3 has revealed distinct patterns of

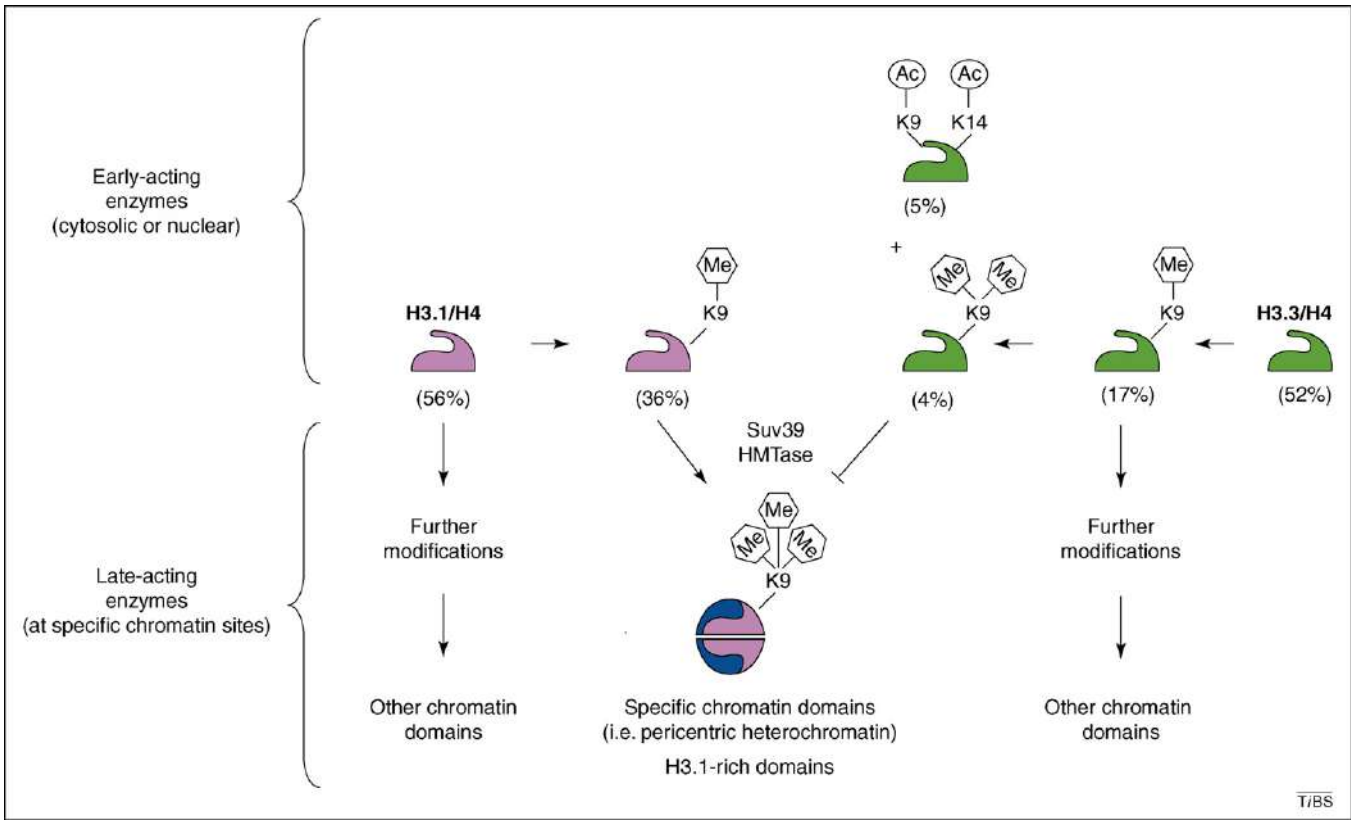

Figure 2. Stepwise model of the establishment and maintenance of PTM patterns. In this model, the H3.1–H4 (pink) and H3.3–H4 (green) dimers represent the different H3–H4 populations, classified on the basis of their PTMs acetylation (Ac) and methylation (Me), before assembly into chromatin. The percentage corresponds to the proportion of the particular modification present relative to the total histone H3 variant. Dimers of H2A–H2B (blue) are shown within the nucleosome structure. For simplicity, histone chaperones are not included. After synthesis, histones H3.1 and H3.3, together with histone H4 and associated chaperones, translocate into the nucleus, where they associate with specific assembly factors: namely, CAF-1 for H3.1–H4, and HIRA for H3.3–H4. Early-acting modifying enzymes function on non-nucleosomal histones to modify H3.1 and H3.3, and also establish the non-nucleosomal PTM pattern for H4 (K5ac, K12ac; not shown), H3.1 (K9me0 and K9me1), and H3.3 (K9me0, K9me1, K9me2, and K9K14ac). The use of different chromatin assembly pathways – replication-coupled and replication-independent – leads to the incorporation of each pre-modified variant. Although pre-modified H3.1 is permissive for further modification either during or after chromatin assembly, a fraction of pre-modified H3.3 is more restrictive. Indeed, the H3.3K9me2 and H3.3K9acK14ac fraction does not permit further modification by the Suv39 enzymes to produce K9me3, as found in H3.1 homogeneous chromatin [21]. Thus, in principle, pre-modified histones represent substrates at the time of deposition or after nucleosomal incorporation (late-acting enzymes). Local dynamics involving late-acting enzymes (histone acetylases and deacetylases, and histone methylases and demethylases) participate in the final equilibrium to obtain specific PTMs.

K9 acetylation and methylation [21]. On the one hand, H3.1 showed a twofold enrichment in H3K9me1 as compared with H3.3, whereas H3K9me2 and acetylated H3K9 (H3K9ac) were below detection limits. On the other hand, a significant fraction of H3.3 was dimethylated at K9 and acetylated at both K9 and K14 [21]. Remarkably, the forms of H3.1 without modification or with K9me1 were both permissive for K9 trimethylation carried out by the H3K9 histone methyltransferase Suppressor of variegation 39 (Suv39). This pattern of H3K9 methylation is known to be important in recruiting Heterochromatin protein 1 (HP1) to pericentric heterochromatin [29,30]. In H3.3, by contrast, the fraction that showed enrichment in K9 dimethylation, or K9 and K14 acetylation, prevented the action of Suv39 [21]. This result emphasizes how important it is to consider pre-existing modifications to evaluate how a given histone-modifying enzyme might or might not work depending on the histone substrate. Clearly, for each enzyme that acts on non-nucleosomal histones, we will have to consider how such potentiation will contribute to the establishment of PTM patterns.

Obviously, nucleosome assembly events associated with DNA replication will influence the transmission and maintenance of the epigenetic state of a specific chromosomal locus (Box 2). How this can affect the maintenance or switching of patterns in loci subjected to nucleosome disruption and reassembly outside S phase needs to be evaluated and is particularly important if there is a significant amount of histone exchange and, potentially, incorporation of new histones. Indeed, genome-wide analysis in *Saccharomyces cerevisiae* has shown that there is a rapid turnover of histones, which is particularly evident at promoters [31]. In this respect, we will have to determine the proportion of histone recycling relative to incorporation of new histones. The importance of histone H3.3 incorporation associated with transcription, as documented in several systems [5,28,32–36], and H3.3 turnover at regulatory regions [37], are examples of such situations of nucleosome disruption and reassembly outside S phase. Most intriguingly, this situation will also have to be considered for H3.1, given that this variant can also become incorporated outside S phase when specific DNA damage gets repaired [38].

In vitro assays [21] have shown that all of the modifications carried by the non-nucleosomal H3.1 are permissive for heterochromatin formation, whereas H3.3

Box 2. Epigenetics

The information encoded in the DNA (genetic information) of each organism is transmitted from one cell generation to the next one through faithful replication and chromosome segregation. However, transmission of 'DNA-independent' information to the next generation is also important. This type of information is termed 'epigenetic'. A classical example of a mark that is considered as epigenetic is DNA methylation, which occurs on the cytosines of CpG sequences. High levels of CpG methylation correlate with heterochromatic regions, whereas unmethylated CpG is found in genomic regions where histones are hyperacetylated [57]. Therefore, histone PTMs, particularly histone acetylation and H3K9me, have been considered as crucial in determining specific epigenetic states and are tightly linked to DNA methylation.

has a subpopulation that is totally refractory to modification by the Suv39 enzyme. These observations raise the key question of how an appropriate population of modified histones (e.g. H3.1K9me1) can be targeted to a particular chromatin domain (e.g. heterochromatin domains). Of course, when the available set of patterns is permissive for further modifications, a proper local concentration of appropriated enzymes will be the principal parameter ensuring (or not) a self-sustained pattern.

Further insights into these issues will require us to understand better how the deposition machineries connect to specific loci and should be investigated for each of the specific histone chaperone complexes. In this context, it is interesting to note that CAF-1, which interacts with H3.1, can form a distinct subcomplex with HP1 that is important in the formation of heterochromatin [39]. It will be important to understand how CAF-1 changes partners (from histones to HP1) and to determine the importance of the respective contribution of these interactions to cell survival in mammals.

We should emphasize that we have touched only the tip of the iceberg and that other modifications need to be analyzed in the non-nucleosomal histones, particularly the methylation states of histone H4 (e.g. H4K20) and modifications on histones H2A–H2B and related variants such as MacroH2A, H2AZ and H2AX.

Do H3 variants establish different chromosomal domains?

DNA in the cell is organized into different chromosomal domains, which in many cases reflect their transcriptional activity. We still do not know in detail how these regions are formed and transmitted to the daughter cells, although histone PTMs are thought to play an important part. Hake and Allis [40] recently proposed that histone variants, in particular H3 variants, might also have a role in establishing these domains. They proposed that H3 variants could function as a 'histone H3 barcode' to index the genome (Box 1). In this model, H3.3 would localize and determine transcriptionally active regions, whereas H3.1 would mark constitutive heterochromatin, and H3.2 would mark silent regions in facultative heterochromatin. Thus, there would be a hierarchy in the marking system, with the choice of the variant giving rise to a determined fate. A prediction from this hypothesis is thus that certain modifications will be determined strictly by the choice of the variant.

Our recent analysis of short nucleosomal arrays isolated with tagged human H3.1 or H3.3 has led us to question this hypothesis [21]. We found that, within the vicinity of a H3.3 nucleosome, the adjacent H3.1-containing nucleosomes have modifications associated with transcriptional activation, in a manner that compares to the PTM pattern of H3.3 [21]. Thus, 'transcriptionally active PTMs' can also be enriched on H3.1. Therefore, histone PTMs are not intrinsic to a specific variant – at least in the case of H3.1, which seems rather permissive and largely influenced by the surrounding chromatin. This finding implies that H3.1 cannot be considered to be a determinant of silenced regions and that it can also index activated regions. This observation is also supported by the finding that H3.1 can have both activating and repressive modifications [20]. Of course, it would be interesting to examine whether H3.2, which shows only repressive marks [20], can be found in the vicinity of H3.3 and, if so, whether it can acquire a marking similar to H3.3. Such an observation would be important in that H3.3, either directly or indirectly, would appear to be dominant over the other variants in imposing local marking.

We therefore propose that a particular PTM pattern in a given chromosomal domain, as discussed earlier (Figure 2), will result from a combination involving the choice of a specific chromatin assembly pathway (the variant and histone-modifying enzyme acting before histone deposition) with the local neighborhood (local action of histone-modifying enzymes on chromatin).

How are variants and PTMs transmitted to the next generation?

Given that the interplay between histone variants and their PTMs are thought to have a crucial role in setting up chromosomal domains, how this information is transmitted to daughter cells is an essential matter for the stability of a cell lineage. This transmission is eminently important during DNA replication, when parental histones are displaced and redistributed on daughter strands and *de novo* incorporation provides the complement needed to ensure complete chromatin assembly of the replicated DNA.

On the basis of the stability of purified histone H3–H4, until recently it was thought that they were always tetrameric when free of DNA; however, recent evidence supports the view that, in fact, H3–H4 can exist as dimeric entities when they associate with histone chaperones [6,13,22]. Thus, newly synthesized histones are probably delivered in this form at the replication fork [9]. An unresolved issue, thus, is the fate of parental histones: is it possible that the parental tetramers split? An attractive hypothesis is that the H3–H4 tetramer is split between the two DNA copies in the form of H3–H4 dimers, which are stabilized by their association with histone chaperones. This mechanism could be a way of keeping the same information in the two new cells (reviewed in Ref. [9]). Although global analysis of H3–H4 inheritance carried out in the 1980s and 1990s argues against this hypothesis [41], it would be interesting to re-examine it.

As we discussed earlier, several theories regarding the inheritance of a specific chromatin marking have been

developed to explain how, once a cellular identity is acquired, it can be faithfully transmitted to the next generation. How early during embryogenesis a particular marking contributes to the initial specification of cell fate is another challenging issue, as is understanding how this information can be erased or reprogrammed.

Do histone variants contribute to cell fate during embryogenesis?

In mammals, the male and female chromatin undergoes dramatic reorganization after fertilization, enabling the zygote to gain the totipotent state that is required for the formation of a whole new organism. These chromatin changes are not only restricted to DNA methylation and histone PTMs. Indeed, recent work has shed light on the role of histone chaperones, particularly CAF-1, in these early developmental states. Studies on embryos with mutations in p150, the largest CAF-1 subunit, have shown that CAF-1 is involved in establishing proper pericentric heterochromatin structure [42].

Similar to histone chaperones, the behavior of histone variants during these reprogramming events has been recently analyzed. Studies in *Drosophila* and mouse have shown that, when the paternal DNA decondenses, maternal H3.3 gets incorporated [43,44]. Therefore, the paternal pronucleus becomes enriched in H3.3, whereas the maternal pronucleus does not show enrichment of any particular histone H3 variant. Given that this deposition happens before DNA replication and transcriptional activation occurs, incorporation of H3.3 cannot be coupled to either genome activation or DNA synthesis. Similarly, massive remodeling of chromatin occurs in the mammalian male sex chromosome during meiosis, leading to the exclusive incorporation of histone H3.3 [45]. This process, however, results in inactivation of the sex chromosome.

Interestingly, *Drosophila* HIRA, provided as a maternal pool, has been implicated in deposition of H3.3 onto the paternal chromatin [43]. A specific point mutation in HIRA causes defects in the decondensation stage [46]; thus, HIRA seems to be essential for histone deposition in the paternal pronucleus. In mouse, paternal pronuclei are also enriched in the histone chaperone HIRA and a similar mechanism might be at work [47]. Intriguingly, in contrast to the *Drosophila* HIRA point mutation, HIRA knockout mice die at the gastrula stage [48]. Therefore, it is possible that mammals can bypass the requirement for HIRA in sperm decondensation, but have a strict requirement for it at later stages. Whether this difference is species-related, or reflects the different means of altering HIRA function – namely, deletion versus point mutation – remains to be elucidated.

The asymmetry of variant incorporation in the maternal and paternal pronucleus in both *Drosophila* and mouse offers an attractive means to distinguish the origin of the genetic material and thus could affect parental imprinting. Intriguingly, the asymmetry in the H3.3 variant that occurs during fertilization is not observed in *Caenorhabditis elegans*, where the maternal pools of HIRA and H3.3 variant are distributed equally among the male and female pronuclei [49]. Given the recent proteomic identification of highly basic proteins resembling invertebrate protamines in *C. elegans* [50], it would be interesting to consider whether this difference relates to the use of distinct sperm-specific proteins.

Interestingly, the asymmetry between paternal and maternal chromosomes is also shown at chromosomal domains. Indeed, recent work has shown that, in pericentric heterochromatin, the maternal and paternal nuclei have different epigenetic marks: H3K9me3, H4K20me3 and HP1β are present in the maternal nucleus, whereas H3K9me1 and HP1β in the paternal one [51]. This observation is intriguing, because H3K9me3 has been proposed to be a platform to anchor HP1β [29,30], suggesting that other mechanisms are involved in the anchoring of HP1β to these domains in the paternal nucleus. It is possible, however, that the H3K9me1 is pre-marking pericentric domains for further modifications at later stages during development. It will certainly be interesting to identify which enzymes are responsible for these modifications.

Concluding remarks

We have discussed the possible roles of histone variants and their PTMs in determining different epigenetic states on chromosomal regions. In addition, we have examined recent evidence addressing how and when these patterns are formed in cycling cells, and have highlighted future trends in the field (Box 3). A current challenge is to understand how these patterns are defined initially during early embryogenesis, when the cellular fate is acquired, and what is essential to maintain a particular state. Given that most of the modifications involved in such a marking system are reversible, understanding their control will help us to comprehend not only key developmental steps, but also how and when marks can potentially be erased or reprogrammed.

Box 3. Future trends in the H3 variant field

- The histone chaperones CAF-1 and HIRA specifically recognize and interact with H3.1 and H3.3, respectively. This specificity is intriguing, given that the two variants differ in only five amino acids. Structural analysis will help us to understand how this specificity is achieved. In addition, it should help to elucidate how H3.1 and H3.3 are incorporated into chromatin specifically by the respective RC and RI pathways.
- Histone chaperones essential for the H3 variants H3.1 and H3.3, and possibly for CENP-A, have been identified. But what about H3.2 and H3t: are there other chaperones for these variants?
- Given that most PTM analyses to define specific patterns have been performed in cell lines, it will be important to examine normal cells and compare different cell types. Such studies will shed light on how PTMs might affect cell specification. In addition, it will be important to investigate changes in PTMs during the cell cycle (e.g. H3S10p is a mark of mitosis).
- We have evidence that the methylation and acetylation pattern of non-nucleosomal histones H3.1 and H3.3 can affect the final PTM status in chromatin: in other words, nucleosomal modifications. But what about the modification status of the other histones before and after assembly: can pre-modification in general affect secondary modifications?
- New approaches have been designed to examine combinatorial patterns of PTMs [58,59]. These techniques will facilitate investigation into whether modifications on the same polypeptide are restricted and, if so, how.

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