HISTONE VARIANTS MEET THEIR MATCH

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Abstract | A fascinating aspect of how chromatin structure impacts on gene expression and cellular identity is the transmission of information from mother to daughter cells, independently of the primary DNA sequence. This epigenetic information seems to be contained within the covalent modifications of histone polypeptides and the distinctive characteristics of variant histone subspecies. There are specific deposition pathways for some histone variants, which provide invaluable mechanistic insights into processes whereby the major histones are exchanged for their more specialized counterparts.

ORPHAN GENE
A protein-coding region that bears little or no homology to genes in distant species.

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The nucleosomes form the basic repeating units of chromatin in eukaryotes. The composition of the individual nucleosomes is fundamentally similar and consists of an octameric core of four types of histones — H2A, H2B, H3 and H4 — around which 147 bp of DNA is wrapped. Each octamer contains two copies of each histone. Modulation of the fundamental nucleosome units contributes to the dynamic structural characteristics of chromatin, which are heritable and impact on transcription and, therefore, cellular identity. This entails various post-translational modifications of the histone proteins and also the incorporation of variant histone subspecies. These variant or 'replacement' histones were discovered on the basis of the small — and sometimes even large — differences in their amino-acid sequence relative to the major histone species (FIG. 1).

The nucleus is characterized by distinct chromatin domains. The dynamics, maintenance and post-translational modifications in these domains have sparked intensive interest in the field of chromatin biology, and recent discoveries have helped elucidate their structural and functional regulation (BOX 1). Some of these specialized domains in chromatin are enriched for the specific histone variants, which operate with other factors to ensure the proper functioning of these domains. In this review, we highlight the roles of these variant histones, their modes of deposition by specific chaperones and also how these might relate to other known histone exchangers.

Histone deposition and exchange

The expression of the major histones is tightly regulated during the cell cycle, and the histones are deposited onto DNA in a process that is strictly coupled to DNA replication. However, histone variants are expressed from a set of genes known as ORPHAN GENES, which are not subject to this stringent regulation. These genes are expressed throughout the cell cycle and their products are deposited during, as well as after, the completion of S phase. These variants have evolved particular characteristics that impact on the transcriptional capacity of the nucleosomal regions they inhabit, some of which are described briefly in this review (for a comprehensive review, see REF. 1). Chromatin is further compacted by the incorporation of the linker histone H1, which has been reported to have eight isoforms in higher eukaryotes. This topic has been extensively covered in a forthcoming review by Kimmins and Sassone-Corsi[2], and we will therefore not address histone H1 or its variants.

Although previous studies have indicated low levels of histone exchange in the absence of transcription or replication[3], the first direct visual evidence was obtained by using cells that expressed green fluorescent protein (GFP)-tagged histones in conjunction with photobleaching of a small area of the nucleus. The recovery of fluorescence in these 'bleached' areas was scored for the level of histone mobility — in other words, histone exchange. These analyses indicated that histones are not readily replaced. In fact, histones H3 and H4 were

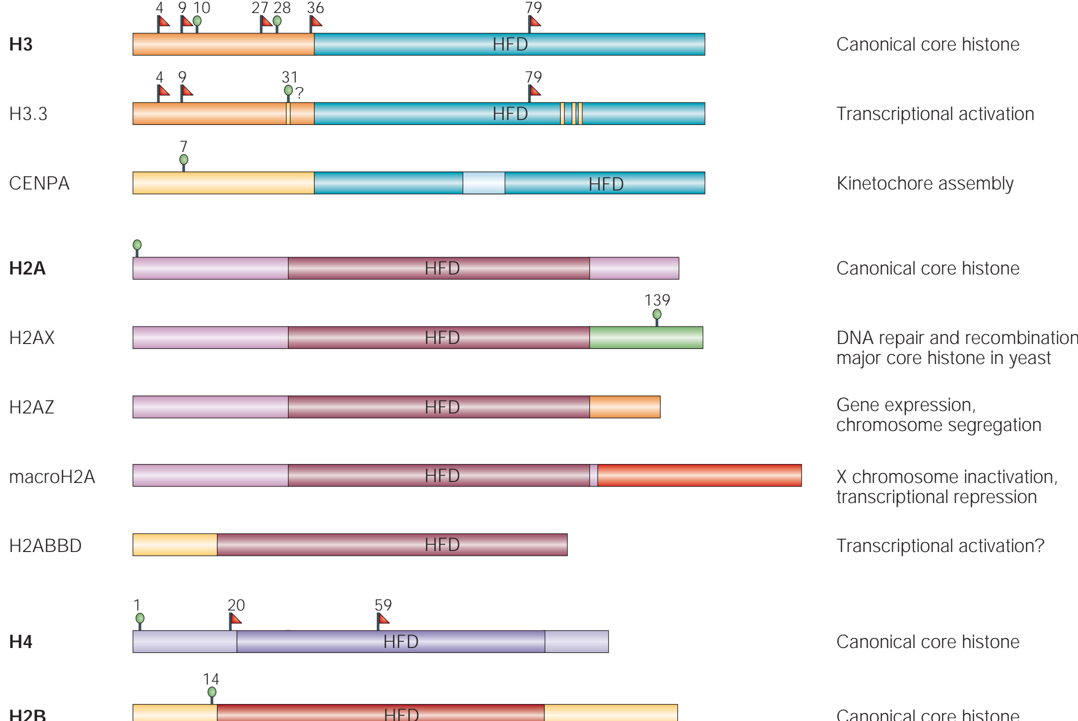

Figure 1 | **Canonical core histones and their variants.** The major core histones contain a conserved histone-fold domain (HFD). In addition, they contain N- and C-terminal tails that harbour sites for various post-translational modifications. For simplicity, only well-established sites for lysine methylation (red flags) and serine phosphorylation (green circles) are shown (other types of modifications, such as ubiquitylation, are not shown). In the histone H3.3 variant, the residues that differ from the major histone H3 (also known as H3.1) are highlighted in yellow. Three of these residues are contained in the globular domain and one resides in the N terminus. This N-terminal residue (Ser31) has been speculated to be a potential site for phosphorylation on H3.3. The centromeric histone CENPA has a unique N terminus, which does not resemble other core histones. Two sites of phosphorylation have been identified in this region, of which Ser7 phosphorylation has been shown to be essential for completion of cytokinesis. The region in the globular domain that is required for targeting CENPA to the centromere is highlighted in light blue. Histone H2A variants differ significantly from the major core H2A in their C terminus. The C terminus of H2AX harbours a conserved serine residue (Ser139), the phosphorylation of which is an early event in response to DNA double-strand breaks. A short region in the C terminus of H2AZ is essential for viability in *Drosophila melanogaster*. MacroH2A has an extended C-terminal macro domain, the function of which is unknown. Finally, the H2ABBD is the smallest of the H2A variants and contains a distinct N terminus, which lacks all of the conserved modification sites that are present in H2A. The C terminus is also truncated and lacks the docking domain that is found in other H2A species. The histones H4 and H2B are also shown, including their known methylation and phosphorylation sites. The proposed functions of the variants are listed.

found to show almost no recovery after photobleaching, whereas histones H2A and H2B showed slightly higher levels of exchange[4]. However, the linker histone H1 showed recovery within a few minutes, which indicated that it has a high rate of diffusion[5].

The popular model for nucleosome deposition onto DNA is that it is coupled to DNA replication and occurs in a stepwise manner, initiated by the deposition of the H3–H4 tetramer followed by the deposition of the H2A–H2B dimers[6,7]. The addition of post-translational modifications could occur before or after deposition. However, for DNA-replication-dependent deposition of the tetramer, the acetylation of lysines 5 and 12 of the H4 tail are thought to be necessary[8,9]. The significance of acetylation at these residues is debatable as they are not required for chromatin assembly in budding yeast, and deletion of H3 and H4 N-terminal tails does not compromise the chromatin-assembly factor-1 (CAF1)-dependent assembly of these histones onto DNA *in vitro*[10,11]. Nonetheless, Kadonaga and co-workers isolated a DNA-replication-dependent histone-deposition complex — known as replication-coupling assembly factor (RCAF) — which included the anti-silencing factor-1 (ASF1; see below) and CAF1, as well as the histones H3 and H4 with appropriate acetylated residues[12].

Whether other histone modifications that are important in defining 'chromatin domains' are established before or subsequent to histone deposition is not clear. However, owing to the disparate expression pattern of enzymes that incorporate stable histone marks during the cell cycle, such as lysine methylation, some of these modifications are likely to be incorporated during mitosis and, therefore, post-replication[13,14]. Other complexes that contain HISTONE LYSINE METHYLTRANSFERASE activity seem to be expressed in normal cells during S phase, and so a

HISTONE LYSINE METHYLTRANSFERASE
An enzyme that catalyses the transfer of methyl groups onto the ε-amino residue of lysines in histones.

Box 1 | **Definition of chromatin domains**

Historically, chromatin domains have been broadly classified into two forms, euchromatin and heterochromatin.

Euchromatin

This is the region of chromatin that is decondensed and is thought to represent loci that are transcriptionally active. Genes in this region replicate early, the chromatin contains hyperacetylated histones and stains poorly in the nucleus. Active genes in this region are enriched for methylation at Lys4 of histone H3 (H3-K4), H3-K36 and H3-K79.

Heterochromatin

This is highly compacted chromatin with regions of silenced DNA. It replicates late, contains hypoacetylated histones and high levels of DNA methylation. Heterochromatin is further classified into pericentric or constitutive heterochromatin and facultative heterochromatin.

Pericentric or constitutive heterochromatin

This is the region that is juxtaposed to centromeres on the chromosome and contains large blocks of ALPHA SATELLITE REPEATS **in humans (known as major satellite repeats in mice). This region contains H3 tri-methyl K9 and mono-methyl K27, and H4 tri-methyl K20** (REFS 84–86)**. As the name suggests, it is irreversibly silenced and remains so throughout the cycles of cell division.**

Facultative heterochromatin

This type of heterochromatin has the ability or 'faculty' to become transcriptionally active again. A classic example of facultative heterochromatin is the inactive X chromosome in mammals, which is characterized by the presence of H3 tri-methyl K27 and di-methyl K9, and H4 mono-methyl K20 (REFS 87–90)**. Silenced euchromatic genes contain methylated H3-K9, H3 tri-methyl K27 and H4 mono-methyl K20** (REF. 85)**.**

methylation mark at a specific histone residue (for example, at Lys27 of H3; H3-K27) is probably incorporated during DNA replication[15]. Whether this modification is incorporated into the histone before deposition onto DNA or immediately after deposition is at present unknown.

The correct incorporation of histones onto DNA requires the assistance of additional factors. This function is fulfilled by HISTONE CHAPERONES. Chaperones are thought to function in coordination with CHROMATIN-REMODELLING FACTORS to mediate the accurate positioning of nucleosomes on a DNA template. Several histone chaperones have been identified and characterized both biochemically and genetically (for details, see REF. 16). Recently, some chaperones were shown to function in the deposition of specific histone variants. Although histones H2B and H4 were once thought to be invariable, recent studies have identified two testis-specific H2B variants in humans, the functions of which have yet to be determined[17,18]. However, no H4 variant has been reported so far.

Next, we discuss the different histone H3 and H2A variants and their modes of deposition, in particular H3.3 and H2AZ, as these have been most well studied.

ALPHA SATELLITE REPEAT
Large highly repetitive stretches of (A+T)-rich DNA sequences in the human genome that are usually untranscribed.

HISTONE CHAPERONE
A protein that escorts histones to DNA for deposition.

CHROMATIN-REMODELLING FACTOR
A protein that alters the dynamic organization of nucleosomes to help in the activation or repression of gene expression.

HISTONE ACETYLTRANSFERASE
An enzyme that catalyses the addition of an acetyl group to specific lysine residues in histones.

H3 variants

Four different isoforms of histone H3 have been reported: H3.1, H3.2 and H3.3, which are similar, and CENPA, the centromeric histone H3, which shows a wide variability in amino-acid composition between species and even within the same species when compared with the other H3 isoforms (FIG. 1; BOX 2). Of these H3 variants, H3.3 and CENPA have been studied the most intensively and have been found to carry out distinct functions.

H3.3. Recent studies showed that the variant histone H3.3 is present at transcriptionally active loci[19]. Deposition of the GFP-tagged variant H3.3 was observed during DNA-replication-coupled (RC) processes as well as in a DNA-replication-independent (RI) manner. During the RC phase, GFP–H3.3 was found throughout the genome; however, during RI assembly, GFP–H3.3 was found predominantly at rDNA arrays, which indicates incorporation at sites of active transcription. On the other hand, cells transfected with the GFP-tagged core histone H3 (H3.1) and exposed to the S-phase inhibitor aphidicolin were inhibited for GFP–H3.1 deposition onto DNA. Moreover, artificial expression of H3.1 outside S phase did not result in its incorporation into chromatin, which showed that deposition of H3.1 is tightly coupled to DNA replication. Importantly, even though H3.1 and H3.3 differ by only four amino acids (FIG. 1), these three residues in the globular domain are crucial for their distinctive deposition during the cell cycle. The H3.1-specific residues apparently impede its assembly outside S phase. Mutation of any of the H3.1-specific residues to the corresponding residue in H3.3 led to both RC and partial RI deposition.

Further clues regarding the function of H3.3 were derived from the identification of its post-translational modifications. This variant is enriched for the presence of 'marks' that reflect transcriptional competence, such as di- and tri-methylation of Lys4, acetylation at Lys9, Lys18 and Lys23, and methylation at K79. Of note, although H3.3 is present at lower levels in dividing cells, on terminal differentiation, the level of H3.3 increases significantly and contributes to more than half of the total amount of H3 protein in the cell[20]. This confirms again that H3.3 is deposited at all stages of the cell cycle, whereas H3.1 incorporation is restricted to the S phase. So, in differentiated cells, the increased level of H3.3 does not correlate with the amount of transcriptional activity.

Insight into the mechanisms by which histone H3.1 and H3.3 are deposited onto DNA has come from biochemical studies. The purification of H3.1- and H3.3-containing complexes from stable cell lines that contain tagged H3-species revealed that these variants associate with different chromatin-assembly complexes *in vivo*[21]. The H3.1 complex was associated with CAF1, whereas the H3.3-containing complex was associated with the histone chaperone HIRA. Interestingly, both complexes contained the HISTONE ACETYLTRANSFERASE HAT1, which supported previous evidence that histones are transiently acetylated before deposition onto DNA. This also indicated that the complexes represented the pre-deposited forms of histones. Furthermore, both complexes included histone H4 and ASF1, a histone chaperone that was initially shown to promote deposition of histones in a DNA-replication-dependent manner *in vitro*[12]. However, recent *in vivo* studies suggest that deletion of ASF1 in

yeast leads to the formation of more compact chromatin, indicating that ASF1 functions in the disassembly rather than the assembly of chromatin[22]. Whether the H4 histone in the H3.1 complex contained the acetylation pattern that is important for DNA-replication-dependent deposition was not analysed.

CAF1 is one of the most well-studied chaperones and consists of three subunits: p150, p60 and p48. CAF1 interacts with the proliferating cell nuclear antigen (PCNA), a multifunctional protein complex that partakes in various functions such as DNA replication, repair, cell-cycle regulation and chromatin assembly. During the process of DNA synthesis, it functions as a sliding clamp around the replication fork and stimulates the processivity of DNA polymerases δ and ε[23]. PCNA is thought to recruit CAF1 for replication-dependent assembly of nucleosomes[24,25]. CAF1 was now found to specifically interact with H3.1 *in vitro* and to deposit H3.1 onto DNA in a DNA-repair-synthesis-dependent manner. In the case of HIRA, its role as a chaperone was first uncovered in *Xenopus laevis* extracts, which on HIRA depletion were able to support RC but not RI histone deposition. This deficiency was restored when HIRA and H3–H4 tetramers were added[26]. HIRA deposits histone H3.3 independently of DNA synthesis; whether it does so during DNA synthesis as well, is not yet clear.

Importantly, the isolated complexes contained the tagged H3 isoform and histone H4, but were devoid of H2A and H2B. Most importantly, these complexes, which were isolated on the basis of the tag that is present on the H3 isoforms, were also devoid of endogenous H3, despite the fact that endogenous H3 was present in the extracts at levels much greater than those for the tagged H3 isoforms. This latter finding was interpreted as indicating that each complex contained one dimer of H3–H4. Importantly, mononucleosomes that were isolated from these stable cell lines contained endogenous and tagged versions of either H3.1 or the H3.3 variant, but not both isoform types, which suggested that the nucleosomes that formed through these pathways are homogeneous in their H3 composition.

These observations raise at least two important questions. First, under the assay conditions, are the H3–H4 histone polypeptides deposited as tetramers, as previous studies that were carried out *in vivo* have shown, or as dimers? Second, if dimers are deposited, how is the second copy of the H3–H4 dimer (for example, the endogenous one) brought to the tagged H3–H4-dimer–DNA complex? A possibility is that the ASF1 chaperone, which is common to both H3.1- and H3.3-containing complexes, deposits the 'respective' endogenous H3 isoform together with H4. Previous observations support this possibility by demonstrating that ASF1 exists in a RCAF complex, which includes H3 and H4 and that also synergizes with CAF1 (REFS 12,27). These studies show that RCAF or CAF1 alone assemble chromatin inefficiently, but that together, their deposition abilities are stimulated. ASF1 might function in localizing an H3–H4 dimer and subsequently deposit the second H3–H4 dimer. However, as ASF1 was found in both the H3.1 and H3.3 complexes, the important question that remains is how the specificity for the respective H3 isoform (H3.1 or H3.3) is attained?

A more general question that remains is whether there are different pathways for nucleosome deposition during DNA replication and during DNA-repair synthesis. The authors scored for *de novo* nucleosome assembly on naked DNA either in the absence of DNA synthesis (HIRA/H3.3 competent) or in the presence of DNA-repair synthesis (CAF1/H3.1 competent)[21]. Do these complexes have similar roles on DNA templates that are assembled with histones? Another important consideration is that even though the process of DNA synthesis during DNA repair is similar to DNA replication, these processes are mechanistically different. During DNA replication, the DNA strands are separated and the nucleosomes are segregated. This is not the case in DNA-repair synthesis, which encompasses just a 20–30 nucleotide stretch. The nucleosome(s) in this process are probably altered or relocated to facilitate access to the DNA-repair machinery. But even if some nucleosomes are evicted, the integrity of others close to the site of repair need not be jeopardized. When coupled to this nucleosome 'alteration' at the site of DNA damage, the H3.3 and H3.1 complexes might then mediate histone deposition or exchange. With regard to DNA replication, the role of the H3.1 complex might be clarified following its isolation during the S phase of the cell cycle.

Whether the H3–H4 tetramer is displaced during DNA replication or half of the tetramer is displaced, leaving behind the other H3–H4 dimer, is a topic that warrants more investigation. It bears on whether H3–H4 is then deposited or replaced in the form of a tetramer or a dimer during this process. The issue of dimer versus tetramer deposition is an important one in the context of epigenetics. How is the information that is contained within the mononucleosomes, with respect to histone isoform type and histone modifications, which reflect active and inactive chromatin regions, retained when histones are deposited during DNA replication? If, during the process of DNA replication, an H3–H4 dimer remains behind on the template DNA and another must be re-deposited, given the results of Tagami *et al.* — who showed the homogeneity of histone H3.3 or H3.1 isoforms within nucleosomes — the remaining dimer might determine the isoform that is brought in[21]. Modifications that are contained on the remaining dimer might be copied to the new dimer, by as-yet-unknown means. On the other hand, if it is the tetramer that is displaced during DNA replication, how then is the original information safeguarded and restored? The mechanism of nucleosome segregation during DNA replication needs to be revisited, as nucleosome deposition and histone exchange during DNA synthesis (or DNA-repair synthesis), transcription (see below) and DNA replication seem to be more different than was first thought.

CENPA. The centromeric histone CENPA was found to be a histone-H3 variant on the basis of its tendency to co-purify with the other core histones[28]. Sequence

Box 2 | **Histone exchange — why bother?**

Several post-translational modifications have been identified both at the N-terminal tails and the globular domains of histones. These include acetylation, phosphorylation, methylation, polyADP ribosylation and monoubiquitylation[91]. Of these 'marks', methylation has been shown to occur on both lysine and arginine residues. The function of these methyl marks on histones has been extensively investigated recently[92–94]. As several methyl marks have been implicated in the regulation of gene expression, it follows that the addition and removal of these modifications must also be controlled. This stems from the need to return the gene to its original state before the stimulatory or repressive signal. This can be done in two ways. The first is an enzymatic reaction that catalyses the removal of the methyl group. The second is the removal of the entire histone molecule. Unlike acetylation and phosphorylation, both of which have been shown to be dynamic marks that are subject to reversal by deacetylases and phosphatases, respectively, lysine methylation has been found to be relatively stable; so far, no enzymes have been found that 'demethylate' modified lysine residues that are involved in repression (H3-K9, H3-K27 and H4-K20). However, recently, the H3-K4 methyl mark, which is involved in activation, has been shown to be demethylated by the enzyme LSD1 (previously known as p110 or BHC110)[97]. On the other hand, arginine methylation was recently shown to be reversible. This is the consequence of a deimination reaction that converts both unmethylated and mono-methylated but not di-methylated arginines of histone H3 and H4 to citrulline. The enzyme peptidyl arginine deiminase-4 (PADI4 or PAD4) catalyses this reaction, and this in turn antagonizes the activity of the histone arginine methyltransferase CARM1 (REFS 95,96). Whether deimination by PAD4 occurs *in vivo* as a secondary step after the removal of a single methyl moiety from di-methylated arginines remains to be tested.

A histone-exchange reaction would have a dual function *in vivo*. First, it would remove all epigenetic marks on histones and facilitate reprogramming of the gene in question. Second, it would allow for the incorporation of replacement histones that have evolved to carry out diverse functions in cells. The removal of stable epigenetic marks poses a paradox, as such marks are presumably transmitted to the daughter cell. However, it is interesting to note that the lysines that are reported as methylated in H3.1 are completely conserved in variant H3.3, which indicates that H3.3 could be subject to the same modifications. This is also the case for H2A, H2AX and H2AZ. Most of the methylated lysines are conserved, except for one in H2AZ in which there is a shift in position by one amino acid. This means that the integrity of the epigenetic programme need not be perturbed on histone exchange with variant species.

analysis revealed that it shared a similar C-terminal histone-fold domain with H3, but varied extensively in its N-terminal region (FIG. 1). CENPA is localized exclusively to centromeres, but when overexpressed, CENPA spreads along the chromosome arms[29]. Although not much is known about how CENPA is targeted to centromeres, it is known to carry out an essential function(s), as a homozygous knockout of this gene in mice results in lethality[30]. Domain-swap analysis between H3.1 and CENPA has shown that the highly conserved histone-fold domain, but not the variant N terminus, is essential for targeting to the centromeres[29]. This is similar to the findings with H3.3 in that the amino acids within the conserved histone-fold motif have an important role in its localization to chromatin domains.

Interestingly, in fission yeast, the histone chaperones Caf1 and Hira have also been shown to be associated with centromeric chromatin, and deletion of both gives rise to an altered centromeric structure[31]. The localization of Cse4 (the yeast centromeric H3, which is homologous to CENPA) was also affected. In the absence of Caf1 and Hira, although Cse4 was found to localize to centromeric DNA, it was also distributed in non-centromeric regions. This indicates that even though these histone chaperones are not essential for the deposition of Cse4 to centromeric regions, they might be required for imparting specificity to its localization. Whether the same Hira- or Caf1-containing complexes that function in restricting the spreading of centromeric CENPA also function in chromatin assembly during DNA synthesis and repair (see above) remains to be determined, as at least three different complexes that contain Hira and Caf1 were separated during glycerol-gradient sedimentation[21].

Recently, Wieland and colleagues showed that the yeast centromeric histone Cse4 could functionally complement human CENPA. In this study, the phenotype that is induced by depletion of CENPA by RNA interference (RNAi) could be complemented by the ectopic expression of the yeast Cse4 (REF. 32). Structural studies have provided new insights into the mechanism of targeting CENPA to centromeres. CENPA that is complexed with H4 forms a more rigid subnucleosomal structure compared with the H3–H4 tetramer, and this results in reduced solvent accessibility for histone H4 (REF. 33). It is also interesting to note that the globular region of CENPA that varies from H3 (consisting of loop 1 and the $\alpha2$ helix) is conserved across species, and this could account for the complementation of human CENPA by yeast Cse4. In addition, when these regions were replaced with the corresponding H3 regions, targeting to the centromere was abrogated. This is reminiscent of the situation whereby amino-acid substitutions in the H3.1 to the corresponding amino acids in H3.3 in the globular domain confer RI deposition[19].

H2A variants

Four H2A variants have been reported so far — H2AX, H2AZ, macroH2A and H2A-bar-body-deficient (H2ABBD). These variants function in diverse cellular pathways, some of which are discussed below.

H2AZ. H2AZ (for which the yeast homologue is Htz1) is the most studied H2A variant with respect to function.

In mammals, it is encoded by an essential gene, as homozygous knockout of the gene in mice resulted in embryos that failed to develop beyond gastrulation[34]. The C-terminus of H2AZ contains a short region that is essential for development beyond the larval stages in *Drosophila melanogaster*[35]. In *Tetrahymena thermophila*, H2AZ was found exclusively in the transcriptionally active MACRONUCLEUS and was expressed in the silent MICRONUCLEUS only during CONJUGATION before gene activation[36]. This gave the first indication that H2AZ might be involved in the activation of gene expression. Studies in yeast indicated that Htz1, the yeast orthologue of H2AZ, localized within actively transcribed regions, particularly those that flank heterochromatin that is associated with the Sir silencing complex; specifically, telomeric chromatin, MATING-TYPE LOCUS and rDNA[37]. The Sir silencing complex comprises the Sir2–4 proteins. Sir2 is an NAD^+-dependent histone deacetylase, which preferentially targets H4-K16 *in vivo*, and Sir3 and Sir4 in this complex bind to the H3 and H4 N-terminal tails of deacetylated nucleosomes. The spread of heterochromatin that is mediated by the Sir proteins is thought to be through the deacetylation of H4-K16 by Sir2, followed by the binding of Sir3 and Sir4 to these hypoacetylated regions[38]. As the silenced regions spread in the absence of Htz1 *in vivo*, Hzt1 was postulated to be a participant in the maintenance or establishment of the boundary between heterochromatic and euchromatic regions.

The crystal structure of the H2AZ-containing nucleosome suggests that two H2AZ molecules are preferred over one copy of H2AZ and H2A[39]. Once again, homogeneity of a variant form in the nucleosome is preferred. Previously, H2AZ was shown to localize to pericentric heterochromatin in early mouse development[40]. Depletion of H2AZ by targeted disruption or RNAi resulted in early mouse embryonic lethality[34]. More recently, knockdown of H2AZ levels by RNAi in mammalian cell lines led to chromosomal missegregation and disruption of the normal distribution of the heterochromatin-specific protein HP1α[41]. This indicates that, in higher eukaryotes, H2AZ is also involved in confining HP1α to specific regions and, therefore, in the maintenance of facultative heterochromatin (see BOX 1). Taken together, the reason for chromosome missegregation could be attributed to incomplete chromatin condensation or heterochromatin formation in mitosis. As previous studies have shown a putative role for H2AZ in transcriptional activation or prevention of the spread of repressive chromatin, the pertinent question now arises: how does H2AZ find its way to these euchromatic regions?

Three independent studies revealed the existence of a novel complex that is required for H2AZ deposition in yeast[42–44]. The complex contains, among other polypeptides, Swr1 (a member of the ATP-dependent SWI/SNF family of chromatin-remodelling factors), H2AZ and H2B. The complex was also found to contain Bdf1, a protein containing BROMODOMAINS that interacts with acetylated histone H4. Bdf1 also associates with the yeast TFIID complex and shows similarity to the C-terminal region of human TAF1 (REF. 45).

The H2AZ deposition complex was discovered using divergent approaches and while pursuing diverse goals. Wu and co-workers initiated studies to investigate the role of a lesser-known member of the SWI/SNF family, Swr1. Through purification of a tagged Swr1, they isolated a Swr1 complex that contained H2AZ[42]. Greenblatt and colleagues used a genetic screen to isolate synthetic mutations that function in conjunction with mutants in chromatin-modifying factors and RNA polymerase II transcription elongation, followed by a proteomic approach. This led to the characterization of a complex that contained Swr1 and Bdf1, among other polypeptides. The presence of H2AZ in this complex was discovered when it co-immunoprecipitated with Bdf1 (REF. 43). Rine's group specifically looked for complexes that contain H2AZ and that might facilitate its deposition — they discovered a similar complex, which they called Swr1-Com[44]. The presence of Bdf1 in the Swr1 complex was postulated as a possible candidate to facilitate Swr1/H2AZ localization to active chromatin regions.

The role of the Swr1 complex in H2AZ exchange was directly broached by biochemical studies from the Wu laboratory. The Swr1 complex contains several polypeptides, some of which are also present in the INO80 complex, as well as some polypeptides that are unique to each. Both the Swr1 and INO80 members of the SWI/SNF family differ from the other family members in having a split ATPase domain[46]. When Wu's laboratory discovered that the Swr1 complex contained H2AZ, they used tagged H2AZ to purify two H2AZ-containing complexes that also contained H2B; the Swr1 complex, thereby confirming the integrity of the original association, and a second complex that contained the nucleosome-assembly protein-1 (Nap1) chaperone. The Nap1-containing complex, designated NAP-Z, contains at least five other polypeptides that are yet to be described. In the presence of ATP, the Swr1 complex transferred H2AZ–H2B dimers that were free or those that were associated with Nap1 to immobilized nucleosomes. As free dimers were also exchanged, the chaperone characteristics of Nap1 remain unresolved; Nap1 might function as a source of dimers without escorting them to the chromatin. Nonetheless, these findings clearly established the precedent for a nucleosome-remodelling complex, such as Swr1, to function also in ATP-dependent histone exchange.

All three studies demonstrate the dependency of H2AZ on Swr1 for its deposition onto chromatin *in vivo*. The presence of H2AZ at previously identified regions was significantly reduced in both an H2AZ-deletion mutant strain and, correspondingly, in a Swr1-deletion strain, as evidenced by CHROMATIN IMMUNOPRECIPITATION (ChIP) analyses[42–44]. This substantiates the requirement of Swr1 in H2AZ deposition. Regions that are deficient in H2AZ show significant overlap with reduced gene expression using whole-genome microarrays. However, there were some surprising findings. H2AZ localized to the polyadenylation and 3′ mRNA cleavage sites at higher levels than was seen at the promoters for the *ADH1*, *PMA1* and *GAL1* genes when probed in one study[43]. Also, H2AZ was more prevalent at the *GAL1*

MACRONUCLEUS
The larger of the two nuclei in the unicellular ciliate *Tetrahymena thermophila*. This is the somatic nucleus and is transcriptionally active.

MICRONUCLEUS
The smaller 'germline' nucleus in *Tetrahymena thermophila*, which is transcriptionally silent.

CONJUGATION
A process of sexual reproduction that occurs in some unicellular organisms and that involves the exchange of genetic material between two cells through a so-called sex pilus.

MATING-TYPE LOCUS
The genomic region in yeast that determines the mating type or 'sex' of the haploid yeast cell.

BROMODOMAIN
An evolutionarily conserved domain that has been shown to bind to acetylated residues.

CHROMATIN IMMUNOPRECIPITATION
(ChIP). A technique by which direct or indirect protein–DNA interactions in chromatin can be studied using antibodies against specific chromosomal proteins.

promoter when repressed. H2AZ was found not only throughout the region near the telomere, as expected, but also overlapped with silenced telomeric regions of chromosome V[43]. So, H2AZ probably does not function alone in establishing the heterochromatic–euchromatic boundary in this region.

So, what does this mean in the context of yeast and higher eukaryotes? The genome in yeast is less complex than in humans, and the finding that H2AZ in yeast localized at large distances from the telomeres strongly indicates a role other than that of impeding the spread of heterochromatin. The genome in higher eukaryotes is more complex as it contains blocks of 'junk' DNA. So, the equivalent Swr1 complex might function in the exchange of H2A for H2AZ to allow a nucleosome environment that would favour the action of chromatin-remodelling factors to facilitate transcription. It has been shown that the incorporation of H2AZ into chromatin stabilizes the octamer within the nucleosome, but impedes oligomerization (and therefore condensation) of chromatin fibres[47,48]. Nakatani and co-workers (personal communication) have isolated a similar Swr1 complex from human cells that contains substoichiometric amounts of FACT ('facilitates chromatin transcription'), a complex that disassembles or reassembles chromatin during RNA polymerase II transit[49,50]. This, in conjunction with the findings from H2AZ knockdown by RNAi, portrays a more global role for the Swr1 homologue in human cells.

H2AX. H2AX is a histone variant in higher eukaryotes, which, although absent in nematodes, is the 'normal' histone H2A in budding yeast[51]. As pointed out in an excellent review by Malik and Henikoff[1], the copy number of the gene seems to correlate directly with the extent of homologous recombination in the organism. For example, yeast, which has high levels of homologous recombination, only has H2AX and not H2A, and nematodes that seem to have little homologous recombination lack H2AX altogether. Similarly, in humans and flies, the copy number of the gene that encodes H2AX is low, which correlates with the low levels of homologous recombination. On the other hand, *T. thermophila* has high levels of homologous recombination and a large number of genes that encode H2AX (approximately similar to the number of genes encoding H2A)[1].

The yeast H2A and the higher eukaryote H2AX histones contain an extension at the C-terminus, which includes the conserved amino-acid sequence SQ(E/D)ϕ (where ϕ denotes a hydrophobic residue). Ser139 in this unique C-terminal region is phosphorylated in response to DNA double-strand breaks (DSBs) and seems to be an early step in the response to DNA damage[52]. Whereas the main kinase that phosphorylates Ser139 is thought to be ATM (ataxia-telangiectasia mutated), the DNA-dependent protein kinase (DNA-PK) has a redundant function in this event[53]. In *D. melanogaster*, the function of H2AX has been taken over by a chimeric molecule that contains the H2AZ globular domain coupled to the C-terminal H2AX tail[54]. The means by which H2AX is targeted to DSBs is unknown at present, but it is thought that H2AX is randomly deposited and that it is phosphorylated around DSBs (see below). The deletion of H2AX in mice, although not lethal, caused a reduction in the number of irradiation-induced foci (IRIF) and resulted in genomic instability and male infertility[55]. It has recently been shown that although DNA-repair factors are recruited to sites of DNA damage in H2AX-deficient cells, their retention is transient and they fail to form IRIF. This was also the case when H2AX-deficient cells were stably transformed with an H2AX form carrying a mutation at its phosphorylation site[56]. So, phosphorylation of H2AX seems to be essential for the formation of efficient repair foci in cells. A function for H2AX that is independent of phosphorylation was observed in male meiosis. H2AX is required for the condensation of the mouse X and Y chromosome pair and for their maintenance in a silenced state during meiosis[57].

Apoptotic DNA damage was found to promote the phosphorylation of another histone, H2B, at Ser14. This phosphorylation has a broad distribution pattern in nuclei[58]. In a recent study, H2B Ser14 was found to be phosphorylated in the absence of H2AX, but its localization at DSBs was compromised in the absence of H2AX Ser139 phosphorylation[59]. As the N terminus of H2B is required for chromosome condensation[60], one possibility to explain this observation is the existence of an interplay between the modifications in the H2AX C-terminus and the H2B N-terminus. Compaction would have two consequences. First, chromosomes would be unable to separate until the DNA-repair process was complete, and second, the concentration of repair factors around the lesion would increase to promote efficient repair. The latter would be possible if H2AX is distributed at regular intervals in the genome to monitor the integrity of chromosomes, thereby functioning as the 'histone guardian of the genome'[61]. Whether phosphorylation of H2AX and H2B can occur together on the same nucleosome remains to be tested.

MacroH2A. MacroH2A is a vertebrate-specific variant, which has two distinct domains — the N-terminus, which is similar to H2A, and a large C-terminus, which has no similarity to other histones[62]. MacroH2A is enriched on the inactive X (Xi) chromosome in mammalian female cells[63]. Although this variant is a hallmark of X INACTIVATION, its presence is not essential for maintenance of the inactivated state. Its deposition occurs after localization of the inactive-X-specific transcript, *Xist,* on the Xi[64]. In the absence of the *Xist* transcript, macroH2A cannot localize to the Xi. This suggests that an RNA molecule might be involved in promoting histone exchange. MacroH2A in undifferentiated embryonic stem (ES) cells (that is, before X inactivation) is concentrated at the centrosomes of the nucleus, where it is tethered by microtubules[65]. At the onset of differentiation, macroH2A shows reorganization with enrichment on the inactive X chromosome. MacroH2A has a general role in silencing, as evidenced by findings that the C-terminal 'macro' domain inhibits the binding of transcription factors and that the N-terminal H2A domain

X INACTIVATION
The process whereby one of the two copies of the X chromosome in female mammals is silenced to compensate for the presence of a single copy in males.

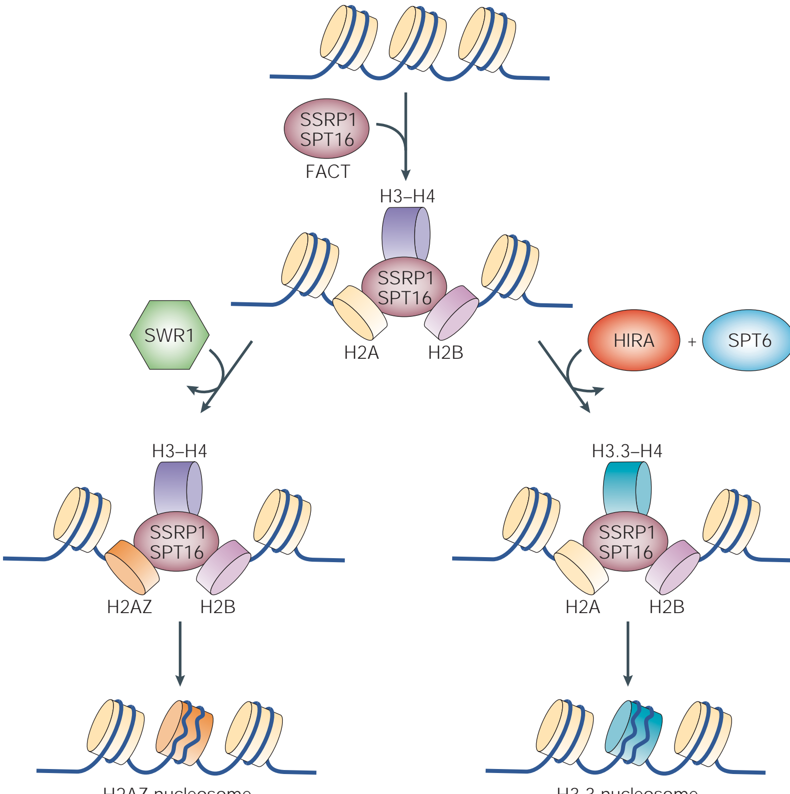

Figure 2 | **Synergism between SWR1, HIRA and FACT.** The major histones can be replaced by their variants to allow for a more transcriptionally competent chromatin state. Here, we show a model for the synergy between the H2AZ and H3.3 exchange complexes with FACT ('facilitates chromatin transcription'), which disassembles and reassembles chromatin during transcription. In one situation, displacement of an H2A–H2B dimer by the SPT16 subunit of FACT could allow exchange of the displaced H2A with H2AZ by SWR1 (a member of the ATP-dependent SWI/SNF family of chromatin-remodelling factors), which leads to an altered nucleosome that is homogeneous in its composition of H2AZ (see main text). In a second situation, the SSRP1 subunit of FACT could coordinate with the elongation factor SPT6 and the histone chaperone HIRA to replace H3 with H3.3. Both of these events would result in the formation of chromatin that is more amenable to transcription, either on the basis of the intrinsic structure of the variant nucleosome or by the presence of post-translational modifications on the variant histones.

interferes with the activity of nucleosome-remodelling factors[66]. The C-terminal of macroH2A contains a LEUCINE-ZIPPER MOTIF that has been implicated in protein dimerization. Such dimerization in macroH2A-containing nucleosomes might facilitate inter-nucleosome interactions, thereby promoting the compaction of large chromatin domains.

H2ABBD. H2ABBD is the most recently isolated H2A variant and little is known about it, with the following exceptions. It is excluded from the Xi chromosome in mammalian cells and colocalizes with H4 that is acetylated at Lys12, which is indicative of a euchromatic function[67]. Stability and structural studies on nucleosomes that have been reconstituted with this variant led to the conclusion that nucleosomes are more 'open' or less stable than conventional H2A-containing nucleosomes[68,69]. Interestingly, photobleaching studies[68] showed that the mobility of H2ABBD in the nucleus was found to be faster than that of H2A.

LEUCINE-ZIPPER MOTIF
A leucine-rich protein domain that mediates interactions with other proteins with a similar domain.

Chaperones and exchange factors

Two histone-variant-specific exchange activities have been identified so far — the HIRA and the Swr1 complexes, which catalyse the replacement of H3.3 and H2AZ, respectively. Apart from these, several ATP-dependent chromatin-remodelling factors have been shown to catalyse the displacement of H2A–H2B dimers[70]. Of the remodelling factors tested, SWI/SNF, among others, efficiently catalyses this displacement. The loss of an H2A–H2B dimer agrees with genetic studies, which showed that the depletion of H2A–H2B *in vivo* alleviates the requirement of SWI/SNF at a subset of promoters[71]. Whether this ability of SWI/SNF to displace dimers is an early step in an exchange reaction remains to be determined.

An important factor in facilitating transcription through chromatin is FACT, which removes one copy of the H2A–H2B dimer in a transcription-coupled manner[72,73]. FACT consists of two subunits, SSRP1 and SPT16 (REF. 50). Through SPT16, FACT binds to nucleosomes, but not H3–H4 tetramers, with the SPT16 subunit making contacts with the H2A–H2B dimer[49]. Interestingly, SSRP1 binds to H3–H4 tetramers but not as part of intact nucleosomes. One interpretation of these observations is that SSRP1 helps to stabilize the H3–H4 tetramers, and assists in promoting the reassembly of nucleosomes after the transit of RNA polymerase II. FACT dislocates one dimer, leaving behind a hexasome, and this makes chromatin more accessible to the transit of RNA polymerase II. So, with one H2A–H2B dimer displaced from the nucleosome, FACT can then function synergistically with SWR1, or another dimer-exchange factor, to allow the incorporation of an H2AZ–H2B dimer. A homogeneous population of H2AZ-containing nucleosomes could arise if the removal of one dimer destabilizes the nucleosome and promotes the removal of a second H2A–H2B dimer. As the crystal structure of H2AZ argues against the presence of a mixed population of H2AZ and H2A in the same nucleosomes (as discussed above), whether FACT binds preferentially to the H2A–H2B dimer relative to H2AZ–H2B remains to be investigated. In such a case, a hypothetical functional interaction between FACT and SWR1 could result in the complete displacement of H2A from the nucleosomes with replacement by H2AZ (FIG. 2).

Several histone-binding proteins have been shown to function together with the elongating form of RNA polymerase II during active transcription. One such molecule is Spt6. The role of Spt6 as an elongation factor is evident by its ability to increase the rate of transcription by RNA polymerase II on naked DNA template[74]. It was also shown that Spt6 interacts weakly with both RNA polymerase II and the elongation factor DSIF (DRB-sensitivity-inducing factor), which is comprised of two subunits, Spt4 and Spt5 (REFS 75,76). Spt6 colocalizes with the phosphorylated, elongating form of RNA polymerase II on *D. melanogaster* polytene chromosomes[77,78] as well as with FACT. The function of Spt6 as a chaperone comes from yeast studies, which

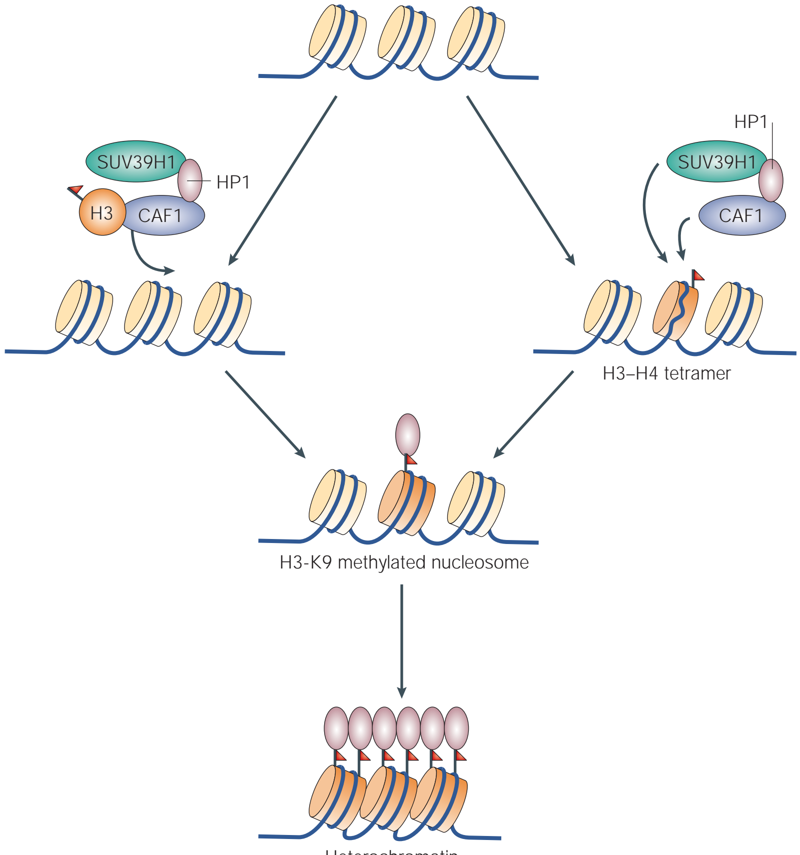

Figure 3 | **Mechanism of repression by histone-exchange complexes and histone methyltransferases.** The chromatin-assembly factor-1 (CAF1) has been shown to interact with the heterochromatin-binding protein HP1, which, in turn, has been associated with the H3-K9 (histone H3 methylated at Lys9) methyltransferase SUV39H1. The methylation of histone H3 that is associated with CAF1 could occur before (left) or after (right) the tetramers have been deposited onto DNA, but before the incorporation of H2A–H2B dimers. This would be followed by the transfer of HP1 from the CAF1 complex to the methylated residue. The stimulation of tri-methylation of H4-K20 by the SUV420H1 and SUV4202 enzymes, which depend on the activity of SUV39H1 and the presence of HP1, would then occur. The establishment of both these marks would lead to the formation and propagation of pericentric heterochromatin.

showed that Spt6 interacts genetically with the histone H3 globular domain. Further biochemical characterization showed that Spt6 binds preferentially to the yeast histones H3 and H4 (REF. 79), and *in vitro* experiments showed that Spt6 transfers H3–H4 onto DNA, albeit rather inefficiently. As Spt6 interacts with Spt5 *in vivo*, the presence of the DSIF elongation factor might be required for increasing the deposition potential of Spt6. In addition, the Spt16 subunit of FACT genetically interacts with Spt6 (REF. 80). This communication could result in a mechanism whereby destabilization of the nucleosome by FACT could facilitate the exchange of an H3–H4 tetramer by Spt6. The exchange function of Spt6 in coordination with a variant-specific exchanger such as HIRA could facilitate the replacement of H3.1 by H3.3 during transcription (FIG. 2).

Exchange of methylated histones by CAF1

The deposition of H3.3 and H2AZ seems to contribute to the establishment of euchromatin, whereas the only histone variant that is implicated in the formation of repressive chromatin is macroH2A, but this is a unique case (as discussed above). This leads us to question the mechanism whereby methylated histones are deposited onto chromatin in the formation of more generalized repressive domains. Of particular interest is the methylation of H3 at Lys9 (H3-K9), as it is by far the most studied histone modification with respect to function and has been clearly shown to repress transcription through the recruitment of HP1. Interestingly, most enzymes that modify H3 at Lys9 seem to be efficient in catalysing this reaction on octamers but not nucleosomes. With the exception of ESET (ERG-associated protein with a SET domain), other H3-K9 methyltransferases such as SUV39H1 and G9A modify Lys9 on core histone substrates, and their activity is inhibited when histones are presented in the form of nucleosomes *in vitro*. This being the case, how do methylated H3-K9 histones find their way to chromatin? CAF1 as well as SUV39H1 bind to HP1 *in vivo*, thereby establishing their association indirectly[81,82]. The H3–H4 dimers or tetramers complexed with CAF1 and HP1 could be methylated by SUV39H1 and deposited onto chromatin. HP1 could then be transferred to the methylated H3-K9 residue, leading to the formation and propagation of repressive chromatin (FIG. 3). Recent studies have confirmed the presence of a replication-specific pool of HP1α that localizes to the boundaries of pericentric heterochromatin in a CAF1-dependent manner. Although the CAF1–HP1α complex is distinct from the H3.1 complex (see above) owing to the absence of the histone H3 and H4 polypeptides, it can assemble newly synthesized cytosolic histones into chromatin as efficiently as the H3.1 complex[83]. Although SUV39H1 cannot methylate nucleosomes *in vitro*, the possibility that tetramers that are assembled on DNA are a favourable substrate cannot be excluded. In this aspect, the dimer versus tetramer deposition of H3–H4 becomes an important issue. If methylated dimers are added to DNA, are the second dimers that are added also methylated? If not, are nucleosomes that are 'hemi-methylated' sufficient to mediate repression? Future efforts to address the scope of modifications within the same nucleosome are needed.

Concluding remarks

Two histone-variant-specific exchange complexes have been described recently — HIRA and the Swr1 complex, which deposit histones H3.3 and H2AZ onto chromatin, respectively. Recent and exciting advances in studies of histone variants, their function and specific exchange complexes have enriched our understanding of the regulation of gene expression. Further studies are required to establish a link between complexes that disrupt nucleosome structure and those that swap histones on remodelled chromatin. In addition, the discovery of new chaperones that are involved in the assembly of the other variants into chromatin will give us a fuller appreciation of how diverse, and yet prescribed, this process is.

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Competing interests statement
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