REVIEW

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Histone chaperones in nucleosome assembly and human disease

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Nucleosome assembly following DNA replication, DNA repair and gene transcription is critical for the maintenance of genome stability and epigenetic information. Nucleosomes are assembled by replication-coupled or replication-independent pathways with the aid of histone chaperone proteins. How these different nucleosome assembly pathways are regulated remains relatively unclear. Recent studies have provided insight into the mechanisms and the roles of histone chaperones in regulating nucleosome assembly. Alterations or mutations in factors involved in nucleosome assembly have also been implicated in cancer and other human diseases. This review highlights the recent progress and outlines future challenges in the field.

In eukaryotic cells, chromatin encodes epigenetic information and governs genome stability[1,2]. How epigenetically determined chromatin states are propagated to daughter cells during mitosis, in a process termed epigenetic inheritance, is one of the challenging questions in the chromatin and epigenetics field[3,4]. One key process contributing to epigenetic inheritance is assembly of the nucleosome, the basic repeating unit of chromatin. The nucleosome consists of 145–147 base pairs of DNA wrapped around a histone octamer containing one histone $(H3–H4)_2$ tetramer and two histone H2A–H2B dimers[5]. As nucleosomes pose as barriers for DNA-related processes, they must first be disassembled to allow DNA replication, DNA repair and transcription machineries to access the DNA. Following DNA replication during S phase, nucleosomes are assembled, incorporating both parental histones and newly synthesized histones, in a process called replication-coupled nucleosome assembly. Nucleosome assembly during gene transcription and histone exchange occur throughout the cell cycle in a replication-independent manner[1,2].

Early studies suggested that nucleosome assembly occurs in a stepwise manner: the histone $(H3–H4)_2$ tetramer, including both old and new H3–H4, is deposited first, and this is rapidly followed by deposition of two H2A–H2B dimers[6]. Supporting this model, non-nucleosomal intermediates containing $(H3–H4)_2$ tetramers and DNA, called tetrasomes, are formed when histones are incubated with DNA in the presence of histone chaperones *in vitro*[7]. Histone chaperones are key proteins that function at multiple steps of nucleosome formation (**Box 1**; **Table 1**). Canonical histone H3 (which, in higher eukaryotic cells, refers to H3.2 and H3.1, which differ by one amino acid in humans) is deposited onto DNA by the histone chaperone CAF-1 during DNA replication–coupled nucleosome assembly (**Fig. 1a**,**b**). The histone H3 variant H3.3, differing from canonical H3 by four or five amino acids, is deposited, along with histone H4, by the histone chaperones HIRA and Daxx in replication-independent nucleosome assembly[8–10]. In this review, we focus our discussion on how canonical and variant histones are deposited during replication-coupled and replication-independent nucleosome assembly with the help of histone chaperones. Furthermore, we discuss how alterations in nucleosome-assembly factors contribute to human diseases.

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Replication-coupled nucleosome assembly

Early studies indicated that the assembly of replicated DNA into nucleosomes is coupled to DNA replication[11,12], and a recent study measuring the length of lagging-strand DNA (Okazaki fragments) in budding yeast firmly established the idea that DNA replication is coupled to nucleosome assembly. Okazaki fragments were found to be similar in size to the repeat length of nucleosomal DNA. Furthermore, deletion of Cac1, the large subunit of histone chaperone CAF-1, involved in replication-coupled nucleosome assembly, caused increased Okazaki-fragment length[13]. These results suggest that lagging-strand DNA synthesis is inherently coupled to the assembly of replicated DNA into nucleosomes. In addition, uncoupling DNA synthesis to nucleosome assembly could contribute to the genome-instability phenotypes observed in cells lacking nucleosome-assembly factors[14–16]. Thus, it is important to understand how nucleosome assembly is coupled to DNA replication.

During S phase, parental nucleosomes ahead of the DNA replication fork are disassembled to facilitate DNA replication, and parental H3–H4 molecules are segregated as $(H3–H4)_2$ tetramers[17], yet the molecular mechanism whereby parental $(H3–H4)_2$ tetramers are transferred to replicated DNA is unknown. In contrast, significant progress has been made in understanding how newly synthesized H3–H4 is deposited onto DNA. Therefore, we will summarize how H3–H4 complexes are assembled, highlight the functions of post-translational modifications on new H3–H4 in nucleosome assembly factors and discuss the interactions between nucleosome assembly and the DNA replication machinery. Incorporation of H2A–H2B will be discussed in a later section for reasons described therein. We suggest that mechanisms regulating histone synthesis, histone nuclear import and the deposition of new H3–H4 molecules onto replicating DNA all contribute to the inherent coupling of nucleosome assembly to DNA replication (**Box 1**).

BOX 1 Histone chaperones and their functions

R. Laskey coined the term 'histone chaperone' to describe the function of nucleoplasmin in the prevention of histone-DNA aggregation during nucleosome assembly[117]. Histone chaperones are now broadly defined as a group of proteins that bind histones and regulate nucleosome assembly[1]. In general, histone chaperones can be classified as either H3–H4 or H2A–H2B chaperones on the basis of their preferential histone binding. Currently, it is not clear what determines the specificity observed for some histone chaperones for H3–H4 or H2A–H2B, although it is probably a combination of structural features and the aid of histone chaperone–binding factors. Some histone chaperones, like FACT, bind both H3–H4 and H2A–H2B[85,86]. In addition to canonical histones, a unique histone chaperone probably exists for each histone variant[8]. Histone variants are a group of proteins that adopt similar structural folds and share sequence homology with their corresponding canonical histones. The majority are variants of histone H3 or H2A.

Histone chaperones participate in distinct steps of nucleosome assembly. First, after synthesis in the cytosol, histone proteins are imported to the nucleus. Some histone chaperones, such as Nap1, help to shuttle newly synthesized histones from the cytoplasm to the nucleus, in part through regulation of the importin-histone interaction[18,82]. Second, a soluble pool of histones must be maintained to meet challenges during stress conditions. Some histone chaperones, such as NASP, act as histone reservoirs and regulate histone supply[19,58]. Third, histone chaperones and histone-binding proteins, such as RbAp46 and Asf1, directly regulate the enzymatic activity of histone-modifying enzymes, serving to bridge interactions between histones and histone-modifying enzymes[48,118]. Fourth, histone chaperones are directly involved in the deposition of histones onto DNA for nucleosome assembly[8]. **Table 1** summarizes the major histone chaperones discussed in this review and their roles in nucleosome assembly.

New H3–H4 dimers bind various histone chaperones. Newly synthesized H3–H4 molecules appear to form distinct protein complexes shortly following their synthesis in the cytoplasm. Purification of human canonical histone H3.1 from HeLa cytosolic extracts, followed by separation of the protein complexes by chromatography, suggested that new H3.1 associates with the protein chaperone Hsc70 before being assembled into a larger complex containing histone chaperone t-NASP, histone H4 and protein chaperone Hsp90 (ref. 18). H3–H4 then associates with the lysine acetyltransferase Hat1–RbAp46, for acetylation, and histone chaperone Asf1 and importin-4 before nuclear import[18]. More recently, it was observed that depletion of NASP results in reduced amounts of free histones H3–H4 and that NASP protects histones from degradation by chaperone-mediated autophagy, through inhibition of Hsp90 and Hsc70 activity[19]. Thus, new H3.1–H4 forms various complexes with different histone chaperones to regulate free histone abundance and nuclear import, which probably affects the deposition of new H3–H4 onto replicating DNA.

How are new $(H3–H4)_2$ tetramers formed? Once bound to Asf1, new H3–H4 is imported from the cytoplasm to the nucleus. Various studies have shown that one molecule of Asf1 binds an H3–H4 heterodimer to form a heterotrimeric complex[14,20], with Asf1 binding the H3 interface involved in formation of a $(H3–H4)_2$ tetramer[21] (**Fig. 2a**,**b**). Similarly, it has been shown that HJURP (Scm3 in yeast), the chaperone for the centromeric histone H3 variant CENP-A[22–24], binds the CENP-A interface involved in tetramer formation[25,26] (**Fig. 2c**). Thus, Asf1 and HJURP represent a class of H3–H4 chaperones that bind the dimeric form of H3–H4.

One key unresolved question is how $(H3–H4)_2$ tetramers are formed from new H3–H4 dimers complexed with Asf1. Evidence from various studies supports a model in which H3–H4 of the Asf1–H3–H4 complex is transferred to other histone chaperones, such as CAF-1 and Rtt106, for nucleosome assembly. First, in human cells, Asf1 regulates the pool of H3–H4 available to CAF-1 during replication stress[27]. In budding yeast, Asf1 is essential for acetylation of H3 lysine 56 (H3K56ac)[15,28], a mark of newly synthesized H3 (ref. 29). Importantly, Asf1 and H3K56ac are required for the efficient association of H3–H4 with Rtt106 and CAF-1 *in vitro* and *in vivo*[30]. Finally, Asf1 directly interacts with the human p60 (yeast Cac2) subunit of CAF-1 (refs. 31,32). *In vitro*, Asf1 binds H3–H4 with similar affinity as CAF-1 or Rtt106 binding to H3–H4 (refs. 33–35), which raises the question of how H3–H4 can be transferred from Asf1 to other histone chaperones. A recent study indicates that RbAp48, a subunit of CAF-1, binds heterodimeric H3–H4 and that Asf1 can associate with the RbAp48–H3–H4 complex. Interestingly, the affinity of Asf1 for RbAp48–H3–H4 is lower than that for H3–H4 (ref. 36), which suggests that the interaction between Asf1 and H3–H4 is weakened once the Asf1–H3–H4 complex associates with other histone chaperones. Together, these results suggest that the interaction between Asf1 and other histone chaperones may facilitate the transfer of H3–H4 from the Asf1–H3–H4 complex to other histone chaperones.

H3K56ac is located far away from the H3 interface involved in $(H3–H4)_2$ tetramer formation[5], which suggests that Rtt106 and CAF-1 adopt a different mode of interaction with histones compared to that of Asf1 (**Fig. 2b**). Indeed, recent studies indicate that $(H3–H4)_2$ tetramers are probably formed on Rtt106 and CAF-1 before deposition of H3–H4 molecules at the replication fork. Rtt106 contains a dimerization domain at the Rtt106 N terminus and a double pleckstrin homology (PH) domain that is critical for recognition of H3K56ac[35,37–39] (**Fig. 2d**). *In vitro*, both the Rtt106 dimerization domain and the tandem PH domains bind H3–H4, with the Rtt106 dimerization domain binding unacetylated H3–H4 and the tandem PH domains recognizing H3K56ac[35]. In addition, Rtt106 binds a $(H3–H4)_2$ tetramer *in vitro* and *in vivo*[35,37]. Thus, Rtt106 may promote nucleosome assembly by assembling and depositing $(H3–H4)_2$ tetramers. In human cells, CAF-1 dimerizes, and this dimerization is important for CAF-1's ability to assemble nucleosomes[40], which suggests that CAF-1 may deposit either two H3–H4 dimers or an $(H3–H4)_2$ tetramer for nucleosome assembly. Similarly, yeast CAF-1 also probably assembles $(H3–H4)_2$ tetramers before histone deposition[34]. Thus, CAF-1 and Rtt106 are members of a second class of histone chaperones that assemble $(H3–H4)_2$ tetramers before depositing them onto replicating DNA.

What is the functional consequence for failing to deposit $(H3–H4)_2$ tetramers? Mutations that compromise the ability of Rtt106 to dimerize and bind $(H3–H4)_2$ affect the function of Rtt106 in transcriptional silencing and the response to DNA damage[35,37,38]. A recent study in *Caenorhabditis elegans* revealed that mutations in CAF-1 or in the H3 interface involved in tetramer formation result in the loss of neuronal asymmetry in the *C. elegans* nervous system[41]. These results suggest that Rtt106's and CAF-1's roles in depositing new $(H3–H4)_2$ tetramers during S phase are critical for Rtt106's or CAF-1's role in maintaining genome stability and/or gene expression states. In principle, once a nucleosome is formed by using a new $(H3–H4)_2$ tetramer deposited as a unit by either Rtt106 or CAF-1 onto DNA, this would effectively inhibit formation of mixed nucleosomes containing one parental H3–H4 dimer and one new H3–H4 dimer (**Fig. 2e**). Therefore, deposition of new $(H3–H4)_2$ tetramers by CAF-1 and Rtt106 may serve as a mechanism to ensure that parental

Table 1 Histone chaperones and their functions during nucleosome assembly

Histone chaperone	Histone cargo	Function during nucleosome assembly	Key references
Anti-silencing factor 1 (Asf1)	H3–H4	Histone import; histone transfer to CAF-1 and HIRA; regulation of H3K56ac	14,20,30
Chromatin assembly factor 1 (CAF-1)	H3.1–H4	H3.1–H4 deposition; $(H3–H4)_2$ formation	8,12,34,116
Death domain–associated protein (Daxx)	H3.3–H4	H3.3–H4 deposition at telomeric heterochromatin	9,10
DEK	H3.3–H4	Regulation of H3.3–H4 incorporation and maintenance of heterochromatin	66,67
Histone cell cycle regulation defective homolog A (HIRA)	H3.3–H4	Deposition of H3.3–H4 at genic regions	65
Nuclear autoantigenic sperm protein (NASP)	H3–H4	Histone supply and turnover	19
Regulator of Ty transposition (Rtt106)	H3–H4	Formation and deposition of $(H3–H4)_2$ tetramer	30,35
Holliday junction recognition protein (HJURP)	CENPA–H4	Regulation of incorporation of the H3 variant CENP-A	23,24
Facilitates chromatin transcription (FACT)	H3–H4, H2A–H2B, H2A.X–H2B	Deposition and exchange of H3–H4, H2A–H2B, H2A.X–H2B	85
Nucleosome assembly protein 1 (Nap1)	H3–H4 and H2A–H2B	H2A–H2B nuclear import and deposition	82,84
Chaperone for H2A.Z–H2B (Chz1)	H2A.Z–H2B	H2A.Z–H2B deposition	98
Aprataxin-PNK-like factor (APLF)	Core histones and macroH2A.1–H2B	Regulation of macroH2A.1 incorporation during DNA damage	103

This table lists only histone chaperones that were discussed in this review.

H3.1–H4 does not mix with new H3.1–H4 to form mixed nucleosomes during S phase of the cell cycle[17].

H3 and H4 modifications regulate replication-coupled nucleosome assembly. Histone proteins are marked, by histone-modifying enzymes, with post-translational modifications, such as acetylation, methylation, phosphorylation and ubiquitylation. These marks have distinct functions and regulate a number of cellular processes[42]. New H3–H4 is modified post-translationally, such that it is distinguishable from parental histone H3–H4 (refs. 27,29,43). Recent studies indicate that modifications on new H3–H4 affect replication-coupled nucleosome assembly in various ways, including the regulation of histone protein folding and processing[18,27], histone nuclear import[44] and the interaction between histones and histone chaperones[30,45].

Monomethylation of histone H3 lysine 9 (H3K9me1) is an early mark observed on newly synthesized histone H3 in mammalian cells. Although molecular insight into the function of this modification in nucleosome assembly is still lacking, H3K9me1 may be involved in histone processing following histone synthesis and/or the conversion of new H3K9me1 to trimethylated H3 lysine 9 (H3K9me3), a mark on heterochromatin[18,46]. Supporting the latter idea, mutations in H3K9me1 lysine methyltransferases have been found to affect heterochromatin integrity[47].

Diacetylation of histone H4 at lysines 5 and 12 (H4K5,12ac), catalyzed by Hat1–RbAp46 (refs. 43,48), is detected on newly synthesized histone H4 from yeast and human cells and is likely to be an early modification occurring on new H3–H4 (ref. 18). Histone H4 mutants harboring mutations at H4K5 and H4K12 are imported less efficiently into the nucleus than are wild-type histones[49]. Moreover, Hat1–RbAp46 and H4 K5,12ac regulate the association

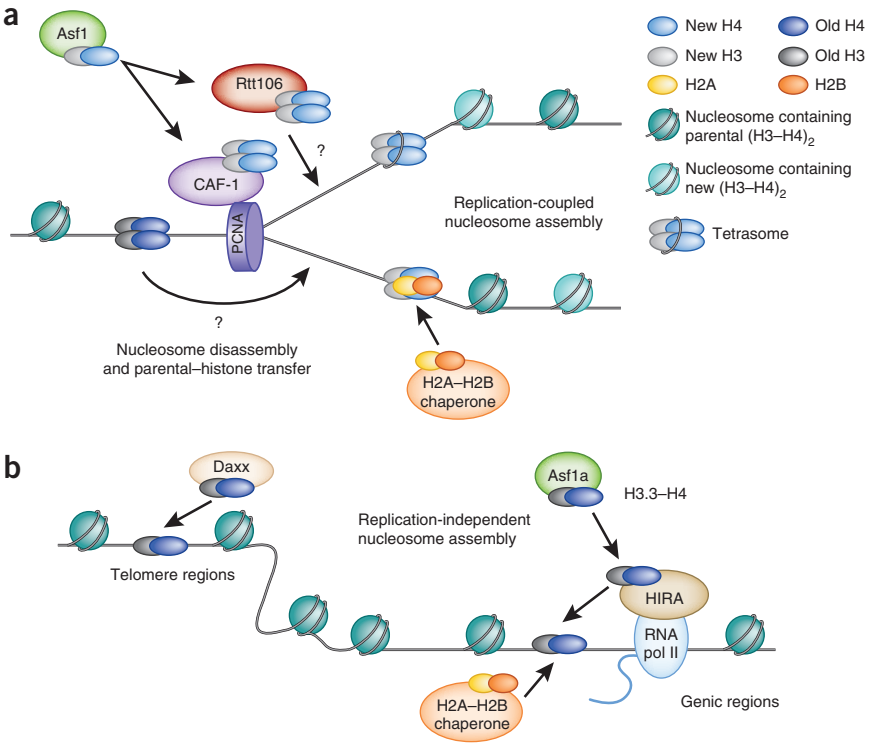

Figure 1 Histone chaperones are key regulators of replication-coupled and replication-independent nucleosome assembly. (**a**) Histone chaperones coordinate to regulate DNA replication–coupled nucleosome assembly. Once newly synthesized histone H3–H4 is imported into the nucleus, the new H3–H4 of the Asf1–H3–H4 complex is transferred to CAF-1 and Rtt106 for $(H3–H4)_2$ formation and deposition onto newly synthesized DNA. Deposition onto replicated DNA depends, in part, on the interaction between CAF-1 and PCNA. Parental histones are also a source of histones for nucleosome assembly following DNA replication. However, the molecular mechanism is relatively unclear. (**b**) HIRA and Daxx mediate replication-independent nucleosome assembly of H3.3–H4. In human cells, H3.3–H4 of the Asf1a–H3.3–H4 complex is transferred to HIRA for deposition of H3.3–H4 at genic regions, possibly through interactions with RNA polymerase II and double-stranded DNA. Daxx facilitates deposition of H3.3–H4 at telomere regions, although mechanisms by which Daxx-mediated histone deposition is regulated are currently unclear.

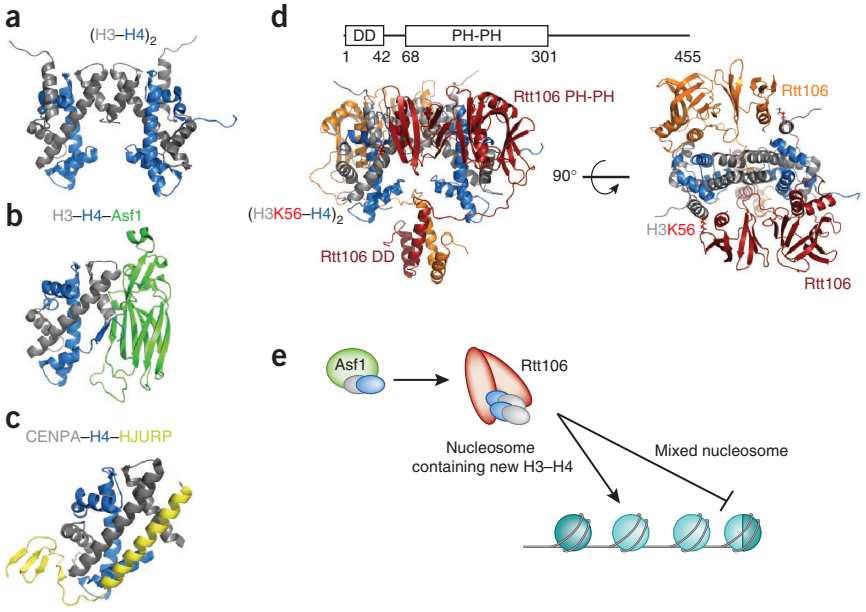

Figure 2 H3–H4 histone chaperones bind a H3–H4 dimer or a $(H3–H4)_2$ tetramer. (**a**) Structure of a $(H3–H4)_2$ tetramer from the budding-yeast nucleosome core particle (PDB 1ID3). (**b**) Asf1 interacts with the H3 interface involved in H3–H4 tetramerization and forms the Asf1–H3–H4 heterotrimeric complex (PDB 2HUE). (**c**) HJURP binds a CENPA–H4 dimer, in part through interactions with the CENPA interface involved in formation of CENPA–H4 tetramers (PDB 3R45). (**d**) A model for Rtt106 binding to a $(H3–H4)_2$ tetramer. Top, Rtt106 contains two domains involved in histone binding: a dimerization domain (DD; PDB 2LHO) and tandem PH domains (PH-PH; PDB 3FSS). Bottom, model of the Rtt106 homodimer binding an $(H3–H4)_2$ tetramer. Model is constructed by using domain structures of Rtt106 (residues 1–301) and atomic coordinates of $(H3–H4)_2$ from the yeast core nucleosome particle. (**e**) Deposition of one new $(H3–H4)_2$ tetramer by Rtt106 effectively inhibits formation of mixed nucleosomes containing one parental and one new H3–H4 dimer.

of importin-4 with H3.1–H4 (ref. 50). These results indicate that H4K5,12ac regulates nucleosome assembly, in part, through the regulation of H3.1–H4 nuclear import.

Acetylation of histone H4 lysine 91 (H4K91ac) is found on H4 associated with the Hat1–RbAp46 complex[51]. Cells harboring an H4K91 mutant are sensitive to DNA-damaging agents and exhibit defects in transcriptional silencing[51], two phenotypes observed in cells lacking genes known to be involved in nucleosome assembly[1]. Although it is not clear which enzyme is responsible for this modification in budding yeast, HAT4, a recently identified lysine acetyltransferase located at the Golgi apparatus, acetylates H4K79 and H4K91 in human cells. Depletion of HAT4 in mammalian cells results in increased DNA-damage sensitivity and defects in proliferation[52]. Thus, HAT4 may influence nucleosome assembly through the regulation of H4K91ac.

In budding yeast, H3K56ac and acetylation of several lysine residues at the H3 N terminus, including H3K9 and H3K27, are found on newly synthesized H3 (ref. 53). Acetylation of these lysine residues probably regulates nucleosome assembly, in part, through the regulation of the interactions between histones and histone chaperones, as mentioned above[30,53]. To what extent H3K56ac and H3 N-terminal tail acetylation function in replication-coupled nucleosome assembly in mammalian cells remains to be determined, although both marks are detected on H3 (refs. 27,54). Together, these studies indicate that histone modifications function at distinct steps of replication-coupled nucleosome assembly, including regulation of histone folding, nuclear import and the interactions between new H3–H4 and histone chaperones.

Interactions with the DNA replication machinery. How nucleosome assembly is coupled to DNA replication at the molecular level remains poorly understood. One contributing mechanism could be the direct interaction between histone chaperones and protein factors involved in DNA replication. For instance, early studies demonstrated that CAF-1 interacts with proliferating cell nuclear antigen (PCNA), the processivity factor for DNA polymerases, and that this interaction is required for CAF-1's function in nucleosome assembly and transcriptional silencing in budding yeast[55–57]. In human cells, chromatin-bound Asf1 is found in a complex with the replicative helicase MCM, and the Asf1-MCM interaction depends on the ability of Asf1 to bind H3–H4 (ref. 58). In budding yeast, Asf1 interacts with replication factor C, a complex involved in the loading of PCNA onto DNA[59]. Asf1 is also localized to the replication fork in *Drosophila melanogaster* cells, although the protein-protein interactions regulating this localization are unclear[60]. Finally, the histone chaperone FACT (described below) interacts with MCM and DNA polymerase[61,62]. As more physical interactions between nucleosome-assembly factors and DNA replication machinery are discovered, the challenge is to determine the extent to which these interactions contribute to the intimate coupling of nucleosome assembly to ongoing DNA replication (**Fig. 1a**).

Replication-independent assembly and histone exchange

Just as nucleosomes pose as barriers for the DNA replication machinery, they also pose as barriers for the transcriptional machinery. Nucleosomes are therefore remodeled and then reassembled, following gene transcription, by DNA replication–independent nucleosome assembly. In addition, histone exchange occurs, which is believed to mark regulatory elements such as transcription start sites[63]. Replication-independent nucleosome assembly is probably the predominant mode of histone replacement in nondividing cells such as neurons and senescent cells[64]. Therefore, factors involved in replication-independent nucleosome assembly, including histone chaperones and histone modifications, are important for gene transcription and the maintenance of epigenetic memory[64]. Below, we highlight recent advances in understanding replication-independent nucleosome assembly in higher eukaryotic cells.

Histone chaperones facilitate replication-independent assembly. As mentioned above, in mammalian cells, the assembly of histone variant H3.3, along with H4, into nucleosomes by the replication-independent pathway is mediated by multiple histone chaperones, including HIRA, Daxx and DEK. HIRA is the canonical histone chaperone involved in the replication-independent nucleosome assembly of histone H3.3–H4 (ref. 65) (**Fig. 1b**). Cells lacking HIRA exhibit reduced H3.3 occupancy at the gene bodies of both active and repressed genes, with no apparent effect on the localization of H3.3 at telomeres and other regulatory elements, which suggests that HIRA is required for the assembly and exchange of H3.3 at genic regions[10] and that other H3.3 chaperones are involved in the deposition and exchange of H3.3 at telomeres and regulatory elements. Indeed, several groups

have shown that Daxx, which forms a complex with the chromatin-remodeling factor ATRX, is a H3.3 histone chaperone[9,10]. Although it remains to be determined whether Daxx regulates H3.3 occupancy at telomeric heterochromatin, it is known that cells lacking ATRX exhibit defects in H3.3 occupancy at telomeres and pericentric DNA regions[10], which suggests that Daxx–ATRX is involved in H3.3 deposition at telomeric regions. In addition to HIRA and Daxx, the human homolog of *D. melanogaster* DEK is probably another H3.3 histone chaperone with a role in maintaining heterochromatin integrity, in part, through interactions with HP1α (refs. 66,67). Together, these studies indicate that H3.3 is deposited at different chromatin regions by distinct histone chaperones.

What factors aid in the recruitment of H3.3–histone chaperone complexes to different chromatin loci? HIRA binds double-stranded DNA and RNA polymerase, which provides a possible mechanism whereby HIRA-mediated nucleosome assembly of H3.3 is linked to gene transcription[68]. The Daxx binding partner ATRX binds repetitive DNA sequences[69], and the ADD domain of ATRX recognizes hallmark chromatin signatures of heterochromatin, such as H3K9me3, MeCP2 and HP1α (ref. 70). Thus, it is possible that ATRX recruits Daxx to telomeric heterochromatin for H3.3 deposition. Together, these studies suggest that HIRA and Daxx are recruited to distinct chromatin loci through different mechanisms, to regulate H3.3 occupancy at destined chromatin loci.

Is new H3.3–H4 deposited as a dimer or tetramer? It is known that during S phase, a small fraction of parental $(H3.3–H4)_2$ tetramers split into two dimers of H3.3–H4 and form mixed nucleosomes containing both new and old H3.3–H4; this is in contrast to parental H3.1–H4 molecules, which rarely split[17]. In budding yeast, mixed nucleosomes are primarily localized to highly transcribed regions or regulatory elements[71]. Therefore, in contrast to new H3.1–H4 molecules that are likely to be deposited in a tetrameric form, new H3.3–H4 may be deposited in both dimeric and tetrameric forms. Two recent independent studies have shown that the histone-binding domain (HBD) of Daxx forms a complex with the H3.3–H4 heterodimer[72,73]. Remarkably, two H3.3-specific residues, Gly90 and Ala87 of H3.3, are principal determinants for Daxx's preferential recognition of H3.3 over H3.1. Ala87 is recognized by a shallow hydrophobic pocket of Daxx, whereas Gly90 binds to a polar environment that discriminates against Met90 of H3.1 (ref. 72). The structure of the Daxx HBD–H3.3–H4 complex also reveals that Daxx HBD–H3.3–H4 competes with DNA for histone binding. In fact, unlike full-length Daxx, the Daxx HBD–H3.3–H4 complexes cannot form tetrasomes[73], which suggests that the observed structure of Daxx HBD–H3.3–H4 complexes must undergo major conformational changes during the assembly of H3.3–H4 into nucleosomes. Future studies are needed to determine whether HIRA uses a similar mechanism to recognize H3.3–H4 and to elucidate how HIRA and Daxx promote formation of H3.3–H4–containing nucleosomes.

Histone modifications in replication-independent assembly. Acetylation marks on newly synthesized histones are important, not only for the regulation of replication-coupled nucleosome assembly but also for replication-independent nucleosome assembly. For example, in addition to its role in replication-coupled nucleosome assembly, H3K56ac promotes histone exchange and turnover in budding yeast[74,75]. Rtt109 and Gcn5, two enzymes catalyzing acetylation of new H3 (refs. 30,53), have been shown to acetylate histone H3 lysine 4 (H3K4ac), a mark correlated with transcriptional activation[76]. Thus, acetylation events on new H3 affect both replication-coupled and replication-independent nucleosome assembly. Because some of these modifications regulate histone–histone chaperone interactions in replication-coupled nucleosome assembly, it is possible that similar mechanisms are used to regulate replication-independent nucleosome assembly.

In addition to acetylation, other modifications probably affect the deposition of H3.3–H4. For example, phosphorylation of histone H4 serine 47 (H4S47ph), catalyzed by the p21-activated kinase 2 (Pak2), is present on histone H4 that co-purifies with Asf1a and Asf1b in mammalian cells. H4S47ph promotes nucleosome assembly of H3.3–H4 and inhibits nucleosome assembly of H3.1–H4 by increasing the binding affinity of H3.3–H4 with HIRA and reducing the association of H3.1–H4 with CAF-1. Thus, in addition to specific residues on H3.1 and H3.3, H4S47ph provides another mechanism by which replication-coupled and replication-independent nucleosome assembly are differentially regulated[45]. Pak2 is an autoinhibitory kinase[77], and it remains unclear how Pak2 is activated to phosphorylate H4S47 to regulate the H3.3 deposition.

Finally, in addition to modifications on new H3, certain modifications on nucleosomal histones, such as methylation of H3 lysine 4 (H3K4me), correlate with active gene transcription and high histone turnover[78]. Although it is possible that these modifications regulate the interactions between histone chaperones and histones, it is more likely that these modifications regulate histone turnover indirectly through their impact on gene transcription.

Deposition and exchange of H2A–H2B dimers

Following deposition of an $(H3–H4)_2$ tetramer on the DNA, two H2A–H2B dimers, including both parental and new H2A–H2B, are incorporated to complete nucleosome formation. Once assembled into nucleosomes, H3–H4 are relatively stable, whereas nucleosomal H2A–H2B molecules undergo rapid exchange with free H2A–H2B[17,79], even during S phase, which suggests that the deposition and exchange of H2A–H2B during DNA replication and gene transcription may not be differentially regulated. Thus, below, we discuss H2A–H2B deposition without differentiating between DNA replication and gene transcription.

Histone chaperones for H2A–H2B deposition and exchange. Similar to that of H3–H4, H2A–H2B deposition and exchange require histone chaperones. The classic H2A–H2B chaperone is Nap1, which shares sequence homology with a large class of histone chaperones, including Nap1-like proteins (NAPL) in human cells and Vps75 in yeast[80]. Although *in vitro* studies indicate that Nap1 binds $(H3–H4)_2$ tetramers and H2A–H2B dimers with similar affinity[81], Nap1 preferentially binds H2A–H2B *in vivo*[82], which suggests that there exists a regulatory mechanism for the specific interactions between Nap1 and H2A–H2B in cells.

Nap1 functions at various steps of H2A–H2B deposition and exchange. First, Nap1, a nucleocytoplasmic shuttling protein, facilitates histone import by mediating the interactions between importin Kap114 and H2A–H2B[82]. Second, using an *in vitro* nucleosome-assembly reconstitution assay, Nap1 was found to deposit both H3–H4 and H2A–H2B for nucleosome formation[7] and to function with the ATP-dependent chromatin and remodeling factor (ACF) complex to assemble regularly spaced nucleosomes[83]. Third, Nap1 promotes nucleosome formation by disassembling nonproductive histone-DNA interactions[84]. Thus, Nap1 regulates nucleosome assembly by facilitating H2A–H2B import, disrupting nonproductive histone-DNA interactions and directly depositing histones onto DNA.

The histone chaperone FACT consists of two conserved subunits, Spt16 and SSRP1 (ref. 85). *In vitro*, FACT preferentially binds H2A–H2B over H3–H4, and the association of FACT with H2A–H2B is

mediated mainly through Spt16, whereas SSRP1 preferentially binds H3–H4 (ref. 86). In budding yeast, the N terminus of Spt16 has been shown to bind H3–H4 *in vitro*[87], and Pob3, the SSRP1 homolog, contains tandem PH domains[88], a motif also found in the H3–H4 chaperone Rtt106 (refs. 35,38,39). Thus, FACT may function as a chaperone for both H3–H4 and H2A–H2B.

Several roles have been proposed for FACT in nucleosome dynamics during gene transcription and DNA replication. On the basis of an early *in vitro* study, FACT was proposed to mediate removal of H2A–H2B from nucleosomes to facilitate RNA polymerase II transcription through a nucleosome template[85]. Recently, it has been shown that yeast FACT binds nucleosomes with high affinity[89]. In the presence of FACT, nucleosomal DNA is more susceptible to digestion by restriction endonucleases at sites placed at different locations in a nucleosome, which suggests that FACT can alter DNA–H2A–H2B and DNA–H3–H4 interactions without the removal of H2A–H2B[89]. Inactivation of Spt16 in budding-yeast cells results in the loss of nucleosomal H3 and H2A and increased association of exogenously expressed H3 at several loci tested[90]. On the basis of these results, it was proposed that FACT is involved in H3–H4 nucleosome assembly following gene transcription. Recently, a global loss of nucleosome occupancy has been observed in cells expressing an H2BK123 mutant[91]. Ubiquitylation of the equivalent H2B K120 residue facilitates human FACT function in gene transcription *in vitro*[92]. Therefore, it is possible that the observed loss of H3 and H2A in FACT mutants is linked to ubiquitylation of H2B. Future studies are needed to determine how inactivation of FACT affects loss of chromosomal H3.

Histone chaperones for H2A variants. Similar to H3 variants, different histone chaperones and associated proteins may mediate deposition and exchange of H2A variants. In general, compared to chaperones for H3–H4 and H2A–H2B, chaperones for H2A variants are less well defined. Here, owing to space constraints, we briefly discuss histone chaperones that regulate deposition of three H2A variants, H2A.X, H2A.Z and macroH2A.

H2A.X shares considerable homology with canonical H2A. The C terminus of H2A.X is phosphorylated at Ser139 in response to DNA damage, and this phosphorylation is important for the recruitment of downstream factors involved in DNA-damage signaling and DNA repair[93]. Because of their high degree of sequence similarity, it is thought that canonical H2A–H2B chaperones may be also involved in the deposition and exchange of H2A.X. Indeed, FACT has been shown to mediate exchange of H2A.X–H2B for canonical H2A–H2B in a manner regulated by H2A.X phosphorylation[94].

H2A.Z is an H2A variant enriched at +1 and −1 nucleosomes near the nucleosome-free region (NFR) surrounding the transcriptional start site in budding yeast[95]. In mammalian cells, NFRs may be marked by labile nucleosomes containing H3.3 and H2A.Z[96]. Swr1 (Swi2/Snf2-related ATPase), a member of the SWI/SNF family of ATPase remodeling complexes, is essential for the incorporation of H2A.Z–H2B into chromatin[97]. The histone chaperones Nap1 and Chz1 associate with H2A.Z–H2B dimers and are hypothesized to transfer dimers to Swr1 for exchange of canonical H2A–H2B, with Nap1 mediating H2AZ–H2B nuclear import and Chz1 presenting the dimer to Swr1 (refs. 98,99). Together, these studies demonstrate that incorporation of H2A.Z–H2B into nucleosomes is mediated by the action of both histone chaperones and chromatin-remodeling complexes.

MacroH2A, an H2A variant containing a large C-terminal tail, has two paralogs, macroH2A.1 and macroH2A.2. MacroH2A is enriched at heterochromatin, including the inactivated X chromosome in female mammals[100] and senescence-associated heterochromatin foci[101], consistent with the idea that macroH2A is mainly involved in gene repression. Although histone chaperones specific for macroH2A are currently not known, it has been shown that aprataxin-PNK-like factor (APLF), a protein involved in the DNA-damage response[102], binds to core histones and macroH2A *in vitro* and *in vivo*. Depletion of APLF results in defects in the recruitment of macroH2A.1 to DNA damage sites[103], which suggests that APLF is a macroH2A.1 chaperone involved in the DNA-damage response.

Alterations in nucleosome-assembly factors in human disease

Numerous studies in eukaryotic cells from yeast to humans indicate that mutations in factors involved in nucleosome assembly result in defects in genome stability and gene expression[1,2]. Therefore, misregulation of histone chaperones or histone-modifying enzymes regulating nucleosome assembly may promote development of human disease. Because histone-modifying enzymes probably have other cellular functions beside their roles in nucleosome assembly, we discuss recent reports highlighting a role for histone chaperones in disease pathogenesis.

The human *HIRA* gene is located in a small region on chromosome 22 that is frequently deleted in DiGeorge syndrome (DGS), a congenital developmental disorder characterized by heart defects and poor immune-system function[104]. Although there are at least six genes located in this critical region, a reduction of HIRA in chick cardiac neural-crest cells results in increased incidence of persistent truncus arteriosus[105], a phenotypic change observed in DGS. In mice, a higher abundance of HIRA transcripts are detected in the cranial neural folds, frontonasal mass and circumpharyngeal neural crest and limb buds, structures that are often altered in DGS patients[106]. These results suggest that HIRA haploinsufficiency contributes to the development of DGS. Future studies are needed to determine how reduced HIRA contributes to the observed developmental defects of DGS patients.

Recent deep-sequencing studies have revealed mutations in the Daxx–ATRX–H3.3 pathway in several cancers. Mutations in genes encoding ATRX and Daxx are observed in pancreatic neuroendocrine tumors (PanNETs)[107]. PanNETs containing ATRX-Daxx mutations have abnormal telomeres, which supports a role for Daxx in H3.3 deposition at telomeres[108]. In a separate study, mutations in components of the Daxx–ATRX–H3.3 pathway were identified in 44% of pediatric glioblastoma tumor samples. Given this high incidence, it has been suggested that these mutations are 'driver' mutations that promote pathogenesis of this particular form of cancer[109]. Patients with mutations in components of the Daxx–ATRX–H3.3 pathway exhibit defects in telomeres as well as altered transcription profiles. Frequent H3.3 mutations occurred at H3.3 lysine 27 and H3 glycine 34 (ref. 109), two residues implicated in gene regulation[42]. Therefore, mutations in ATRX, Daxx and histone H3.3 may promote tumorigenesis by altering gene expression and/or telomere integrity.

DEK is overexpressed in a number of cancers and promotes epithelial transformation[110]. In addition, the fusion protein formed between histone chaperone hDEK and CAN owing to chromosomal translocation was detected in a small subset of acute myeloid leukemia[111]. The DEK-CAN fusion results in reduced interactions of DEK with histones and recruitment of HP1 to heterochromatin[66,67], which suggests that the DEK-CAN fusion protein promotes leukemia progression through misregulation of transcriptional repression. Together, these studies are consistent with the idea that histone–chaperone complexes are important for the regulation of telomeres, gene transcription and heterochromatin, the alterations of which ultimately contribute to disease pathogenesis.

Mutations in codanin-1 are associated with congenital dyserythropoietic anemia type I (CDAI), a rare disorder. Examination of erythrocytes from CDA1 patients revealed defects in heterochromatin structure and HP1 localization[112]. Recently, codanin-1 was found to co-purify with Asf1a and Asf1b (refs. 45,113). Codanin-1 binds Asf1 through the same Asf1 surface as do HIRA and CAF-1, which implies competition with HIRA and CAF-1 for Asf1 binding[113]. Codanin-1 residues mutated in CDAI patients are far removed from the Asf1 binding site, yet codanin-1 mutant proteins harboring these mutations exhibited defects in Asf1 binding[113]. These results suggest that CDAI may be caused by alterations in nucleosome assembly and highlight the importance of proper regulation of distinct steps of nucleosome assembly.

Finally, alterations in histone chaperone expression have been documented as potential prognostic markers for different cancers. Asf1b, one of the two isoforms of Asf1 in mammalian cells, is required for cell proliferation, and higher Asf1b is associated with increased metastasis and shorter survival of breast cancer patients[114]. High CAF-1 p60 correlates with adverse outcomes in renal, endometrial and cervical cancer[115]. Because Asf1b and CAF-1 are involved in cell proliferation, increased protein abundance of these factors in cancer cells could be due to the enhanced proliferation status of cancer cells. Alternatively, increased amounts of these chaperones may alter nucleosome assembly, resulting in genome instability and the promotion of tumorigenesis. Further investigation is needed to determine the extent to which the altered abundance of histone chaperones observed in human cancer is the consequence or the cause of tumorigenesis.

Concluding remarks

Great strides have been made in understanding how replication-coupled and replication-independent nucleosome assembly pathways are regulated by histone chaperones and histone modifications. In addition, connections between defects in nucleosome assembly and human disease have been increasingly documented. The studies highlighted in this review underscore the importance of nucleosome-assembly factors in maintaining genome integrity and gene expression state and provide additional impetus for future studies aimed at determining how nucleosome assembly pathways are regulated.

Although our understanding of the molecular mechanisms underlying the nucleosome assembly of new H3–H4 has significantly improved over the past few years, many questions remain to be addressed. For instance, how are new H3–H4 molecules transferred from Asf1 to other histone chaperones, including CAF-1, HIRA and Rtt106? Is this transfer a passive process that depends solely on dissociation of histone H3–H4 from Asf1 or an active process that depends on interactions between Asf1 and CAF-1 or between Asf1 and Rtt106? What factors and/or post-translational modifications regulate histone transfer from Asf1 to other histone chaperones? With the development of techniques to monitor the deposition of new H3, using immunofluorescence[68], and to measure the binding affinity of histone chaperones and histones[84], significant progress will probably be made in addressing these questions in the near future.

With the application of deep-sequencing technology, progress is also expected in understanding how parental $(H3–H4)_2$ tetramers are transferred to replicated DNA. Does this transfer require the transient interaction between DNA and H3–H4 only or require a histone chaperone that temporarily holds parental $(H3–H4)_2$ tetramers close to the site of DNA replication? Finally, how is the transfer of parental H3–H4 coupled to ongoing DNA replication and the deposition of new H3–H4?

We also expect progress in the understanding of how deposition of H2A and H2A variants is regulated. It is likely that, similar to H3 and H3 variants, each H2A variant may have a unique chaperone and that post-translational modification of each variant regulates its deposition and exchange.

In the coming years, we expect additional documentation to link alterations in histone chaperones and other regulators of nucleosome assembly to human disease. One of the challenges is determining how alterations in factors involved in nucleosome assembly lead to human disease and what we can infer and learn about the physiological functions of these factors. For instance, to what extent do mutations in nucleosome-assembly factors drive tumorigenesis and disease? As mutations in factors in nucleosome assembly probably affect gene transcription, genome stability and telomere maintenance, is the disease phenotype a manifestation of defects in all these functions? By addressing these questions, we expect to gain additional insight into nucleosome assembly pathways as well as to aid the development of new therapeutic agents targeting alterations in nucleosome-assembly factors.

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The authors declare no competing financial interests.

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