REVIEW

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Histone chaperones in nucleosome assembly and human disease

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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-27.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**).

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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 pro-

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 hetero31.0121omatinH,11	

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

DeeH3.3–H4 H3.H4

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 bind-

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.

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 hmnana9 02(m)16(n5n)19(t)688.1(i)3(nd9 02(m)16(n5cT(t)688.1)-14(3(s)-5(e)3( ))-23()-12(oa)46T)-16(i2( )9(t)-2(l16(n)18(min)4(

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