All roads lead to chromatin: multiple pathways for histone deposition

Qing Li[*], **Rebecca Burgess**[*], and **Zhiguo Zhang**

Department of Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, 200 First Street SW, Rochester, MN 55905

1. Introduction

Chromatin, a complex of DNA and associated proteins, governs diverse processes including gene transcription, DNA replication and DNA repair [1]. The fundamental unit of chromatin is the nucleosome, consisting of 147bp of DNA wound approximately 1.6 turns around a histone octamer of one $(H3-H4)_2$ tetramer and two H2A-H2B dimers. In order to form nucleosomes, $(H3-H4)_2$ tetramers are deposited first, followed by the rapid deposition of H2A-H2B. Therefore, it is believed that the assembly of $(H3-H4)_2$ tetramers into nucleosomes is the rate-limiting step of nucleosome assembly. Moreover, assembly of H3-H4 into nucleosomes following DNA replication, DNA repair and gene transcription is likely to be a key step in the inheritance of epigenetic information and maintenance of genome integrity. Understanding how assembly of H3-H4 into nucleosomes is regulated is extremely important and will be the focus of this review.

In general, nucleosome assembly is classified into replication-coupled (RC) nucleosome assembly and replication-independent (RI) nucleosome assembly (Figure 1A and Table 1). Both RC and RI nucleosome assembly processes occur in both yeast and mammalian cells despite the fact that yeast cells have only one form of histone H3, which is most similar to the mammalian H3 variant H3.3. In mammalian cells, H3.3 is mainly assembled into nucleosomes in a RI manner while the canonical histone H3 (H3.1 and H3.2), whose expression peaks during S phase in mammalian cells, is assembled into nucleosomes in a RC manner. H3.1 and H3.2 differ by only one amino acid and therefore, throughout the review, we will refer to H3.1 as the canonical histone H3 in mammalian cells. While H3 molecules are different between yeast and mammalian cells, most of the histone chaperones, a group of proteins that bind histones and promote nucleosome assembly and/or exchange without being final products, are conserved from yeast to human cells [2]. Histone chaperones are key factors in regulating nucleosome assembly. Further regulation comes from post-translational modifications of the histone proteins. Therefore, we will separate our discussion below into the regulation of RC and RI nucleosome assembly, highlighting the roles of histone chaperones and modifications on newly synthesized H3 and H4 in these two processes.

Corresponding author: Zhang.Zhiguo@mayo.edu, Phone: 507-538-6074, Fax: 507-284-9759.
[*]These two authors contributed equally to this work.

2. Replication coupled (RC) nucleosome assembly

Nucleosomes are barriers for DNA replication and therefore, nucleosomes ahead of the replication fork must be temporarily disassembled or remodeled in order for the DNA replication machinery to gain access to the DNA. Immediately following DNA replication, replicated DNA is assembled into nucleosomes using both parental and newly synthesized histone proteins. Numerous studies indicate that the assembly of replicated DNA into nucleosomes is coupled to the on-going DNA replication [3, 4]. It is hypothesized that coupling nucleosome assembly to DNA replication ensures proper inheritance of chromatin structure, propagation of epigenetic marks on histones to daughter cells and maintenance of genome integrity. Supporting this hypothesis, mutations in genes involved in DNA RC nucleosome assembly result in increased sensitivity to DNA damaging agents and compromised maintenance and inheritance of heterochromatin states in yeast and mammalian cells [5-9].

At the molecular level, two separate pathways are likely involved in RC nucleosome assembly. First, parental histones in front of the replication fork are transferred onto the replicated DNA. While how this process is coupled to ongoing DNA replication remains elusive, recent evidence indicates that $(H3-H4)_2$ tetramers are transferred as a single unit for nucleosome formation. While it has been known for a while that parental histones do not mix with newly synthesized histones to form nucleosomes during S phase of the cell cycle [10-12], several studies proposed that parental $(H3-H4)_2$ tetramers may split into two dimers for nucleosome formation [13, 14]. Recently, using stable isotope labeling of amino acids in cell culture (SILAC) combined with quantitative mass spectrometry (MS), it has been shown that parental $(H3.1-H4)_2$ tetramers do not mix with newly synthesized $(H3.1-H4)_2$ tetramers, whereas parental H2A-H2B and newly-synthesized H2A-H2B can be found within one nucleosome following DNA replication [15]. These results not only clarify a major question in the field, but also reinforce the idea that the assembly of $(H3-H4)_2$ tetramers, both parental and newly synthesized, is likely to be a key step in the inheritance of chromatin states and high order chromatin structure. Since only one daughter cell receives the parental $(H3-H4)_2$ tetramer at any given DNA position, epigenetic marks on H3-H4 in the individual nucleosome cannot be maintained. Instead, epigenetic marks on H3-H4 can only be maintained in a group of nucleosomes in a functional domain.

Second, newly synthesized H3-H4 can be deposited onto replicating DNA to form nucleosomes in a histone chaperone dependent manner. Below, we introduce the histone chaperones involved in RC nucleosome assembly and then discuss how newly synthesized $(H3-H4)_2$ tetramers are deposited onto replicating DNA during S phase of the cell cycle.

2.1. Histone chaperones involved in de novo nucleosome assembly of H3-H4

The classic histone chaperone regulating RC nucleosome assembly is chromatin assembly factor 1 (CAF-1), identified in human cells using a SV40 replication-coupled nucleosome assembly assay [3, 16, 17]. CAF-1 is a three subunit (p150, p60 and RbAp48 in humans) protein complex known to bind histones H3-H4 containing modifications indicative of newly synthesized H3-H4 [18, 19]. Thus, CAF-1 is likely involved in promoting de novo nucleosome assembly of newly synthesized H3-H4. Whether CAF-1 has any role in the transfer of parental H3-H4 during S phase of the cell cycle is not known. In human cells, CAF-1 preferentially co-purifies with the canonical histone, H3.1, in comparison to H3.3, indicative of CAF-1's role in the deposition of H3.1-H4 during S phase of the cell cycle [13]. However, how CAF-1 recognizes H3.1-H4 preferentially over H3.3-H4 remains to be determined.

Anti-silencing factor 1 (Asf1) was discovered as a histone chaperone that promotes CAF-1 dependent nucleosome assembly [20, 21]. Asf1 purified from yeast to human cells cannot promote RC nucleosome assembly by itself using an *in vitro* SV40 DNA RC nucleosome assembly assay [22, 23], consistent with the idea that Asf1's role in RC nucleosome assembly is not direct deposition of H3-H4 onto replicating DNA. Several studies indicate that Asf1 is likely involved in regulating the modification and/or nuclear import of newly synthesized H3-H4. For example, in budding yeast, Asf1 is essential for acetylation of histone H3 lysine 56 (H3K56Ac) [24-27], a modification on newly synthesized H3 [28]. Moreover, genetic studies indicate that Asf1's role in nucleosome assembly and other processes in budding yeast are likely due to its ability to regulate H3K56Ac [26]. In human cells, there are two sequence homologs of Asf1, Asf1a and Asf1b. Both Asf1a and Asf1b are required for H3K56Ac in human cells, with Asf1a being the predominant form in regulating H3K56Ac [29, 30]. In addition, it is known that Asf1a and Asf1b associate with H3-H4 prior to the import of newly synthesized H3-H4 into the nucleus, suggesting that Asf1 in human cells may regulate nuclear import of new H3-H4 [31]. In addition to binding newly synthesized H3-H4 and promoting RC nucleosome assembly of newly synthesized H3-H4, Asf1 has other functions. First, yeast cells lacking Asf1 exhibit increased supercoiling of an endogenous 2μ plasmid, suggesting a role for Asf1 in nucleosome disassembly [32]; however, Asf1's role in nucleosome disassembly is unclear. It is possible that Asf1 alone disassembles nucleosomes by removing H3-H4. Alternatively, Asf1 may collaborate with ATP dependent chromatin remodeling complexes to disassemble nucleosomes. Second, in *S. pombe*, Asf1 binds the histone deacetylase Clr6 and is required for deacetylation of histone H3 lysine 9 [33]. In *S. cerevisiae*, however, Asf1 is required for efficient acetylation of H3 lysine 9 [34]. These distinct functions of Asf1 in budding yeast and fission yeast are further highlighted in the fact that Asf1 is essential for cell growth of *S. pombe*, whereas *S. cerevisiae* cells lacking Asf1 are viable. Third, human Asf1 binds histones with marks representing parental histones, suggesting that Asf1 may have a role in parental H3-H4 transfer [35]. How Asf1 is involved in parental histone transfer remains to be determined.

In *S. cerevisiae*, Rtt106 is another H3-H4 histone chaperone found to be important for mediating de novo histone deposition. Rtt106's function as a histone chaperone was originally identified in a yeast genetic screen for enhancers of silencing defects of mutant PCNA cells defective for CAF-1 binding [36]. Genetic and biochemical evidence suggest that Rtt106 functions in parallel with CAF-1 to promote RC nucleosome assembly [37, 38]. However, it remains to be resolved exactly how Rtt106 functions in parallel with CAF-1 in nucleosome assembly. It is possible that CAF-1 and Rtt106 deposit H3-H4 at distinct chromatin regions. Alternatively, CAF-1 and Rtt106 may function distinctly as histone chaperones for either the leading or lagging strand. Further supporting distinct roles for CAF-1 and Rtt106, Rtt106 binds dsDNA with no sequence specificity, and this, along with the histone binding ability, appears to be important for Rtt106's function in transcriptional silencing [39]. Therefore, Rtt106 may have roles outside of RC nucleosome assembly.

As the assembly of newly-synthesized H3-H4 is tightly coupled with DNA replication, it is not surprising that several of the histone chaperones involved in RC nucleosome assembly exhibit interactions with DNA replication proteins, suggesting that the replication fork machinery recruit histone chaperones to the fork for histone deposition. CAF-1 is recruited to the replication fork through a direct interaction between the large subunit of CAF-1, p150, and PCNA [40, 41], the processivity factor for the DNA polymerases, an interaction conserved from yeast to humans [42]. In yeast, Asf1 directly binds RFC, a protein complex that loads PCNA onto DNA [43]. In mammalian cells, both Asf1a and Asf1b are found to complex with the replicative helicase MCM, and this association is required for fork progression [35]. It is still unclear as to how Rtt106 is recruited to the replication fork.

Furthermore, there appears to be significant coordination among the H3-H4 histone chaperones in order to effectively deposit newly-synthesized H3-H4 at the replication fork. Various studies support a model in which newly synthesized H3-H4 molecules bind Asf1. Asf1 binds H3 through the same H3 interface involved in formation of $(H3-H4)_2$ tetramers, and *in vitro*, Asf1 disrupts $(H3-H4)_2$ tetramers [44]. This raises the following questions, how are $(H3-H4)_2$ tetramers, the first building block of a nucleosome, formed during RC nucleosome assembly? Pull-down experiments have revealed that in mammalian cells, the Asf1-H3-H4 heterotrimer resides in a complex with CAF-1 and HIRA [13]. We have shown that Asf1 is required for an efficient association of H3-H4 with the histone chaperones Rtt106 and CAF-1 in yeast [38]. Therefore, Asf1 may transfer histone H3-H4 dimers to other histone chaperones for deposition onto chromatin [38, 45]. Two possible models help explain on how newly synthesized $(H3-H4)_2$ tetramers are formed (Figure 1B). First, two H3-H4 dimers are deposited sequentially by a histone chaperone onto replicating DNA to form the $(H3-H4)_2$ tetramer for nucleosome formation. Second, a histone chaperone may bind a single $(H3-H4)_2$ tetramer, which in turn, is deposited onto DNA to promote nucleosome formation. Several studies suggest that $(H3-H4)_2$ tetramers can be deposited by histone chaperones via oligomerization of histone chaperone proteins. For instance, Xenopus p150, the large subunit of CAF-1, can form dimers in vitro, and the ability of p150 to dimerize is important for nucleosome assembly [46]. More recently, two members of the NAP (nucleosome assembly protein) family, Nap1 and Vps75, bind histone H3-H4 in a tetramer fashion [47]; however, Nap1 in cells is a H2A-H2B chaperone [48] and Vps75 is a component of Rtt109-Vps75 complex [49]. Therefore, the significance of Nap1 and Vps75 binding H3-H4 remains to be further investigated. Recent studies from our lab indicate that Rtt106 dimerizes and binds a $(H3-H4)_2$ tetramer (Fazly et al., unpublished), further supporting the possibility that $(H3-H4)_2$ tetramers are formed prior to the deposition of H3-H4 onto DNA. Furthermore, Rtt106 directly interacts with CAF-1 [37]. These studies highlight the complex interactions occurring among the histone chaperones for coordinated deposition of histones. Future studies are needed to address how each histone H3-H4 chaperone bind dimeric or tetrameric forms of H3-H4 and how the coordination among the histone chaperones and these different H3-H4 forms enables proper nucleosome assembly (Figure 1B).

2.2. Roles of modifications on newly synthesized H3 and H4 in RC nucleosome assembly

Histone proteins contain a number of post-translational modifications. These modifications aid in regulating a host of cellular processes [50]. It was observed long time ago that newly synthesized histones were acetylated and that these acetylation marks were removed shortly after deposition [51, 52]. Three acetylation marks on newly-synthesized histones are relatively conserved among species: H4 lysine residues 5 and 12 (H4K5,12Ac), H3 N-terminal tail acetylation and H3 lysine 56 (H3K56Ac) [28, 53-55]. In addition to acetylation, monomethylation of histone H3 K9 (H3K9me) is present on H3.1 prior to deposition in mammalian cells [56]. These modifications likely regulate RC nucleosome assembly via one of the following mechanisms. First, histone modifications may regulate the interactions between histones and histone chaperones. Second, histone modifications on newly synthesized histones may be important for regulating histone import as histones are shuttled from the cytoplasm into the nucleus for deposition. Finally, histone modifications on newly synthesized histones may facilitate the inheritance of marks on histones by aiding in the transfer of information from parental histones to the newly synthesized histones (Figure 2 and Table 1). Discussed below are the known and/or predicted functions of specific modifications found on newly synthesized histones.

The most highly conserved mark of newly synthesized histones is diacetylation of histone H4 at lysine residues 5 and 12 (H4K5,12Ac) [53]. This mark is catalyzed by the HAT1

acetyltransferase complex [57-59]. While this modification has been known for a long time, only recently has the function of this modification in RC nucleosome assembly come to light. A detailed study of histone complexes formed from histone synthesis to deposition indicate that H4K5, K12Ac occurs within the cytoplasm before histones associate with Asf1 and before newly-synthesized histones are imported into the nucleus [31]. Furthermore, an analysis of recombinant protein incorporation into the macropasmodia of Physarum revealed that the nonacetylable, H4K5,12R form of H3-H4 inhibits H3-H4 nuclear import while the import of an acetylation mimic, H4K5,12Q, is improved over wild type H3-H4 [60]. In addition, H4K5,12Ac is present on H4 co-purified with histone chaperone CAF-1 [18, 19]. These results suggest H4K5, K12Ac may have multiple roles in nucleosome assembly. First, it may facilitate the nuclear import of H3-H4. Second, it may regulate the function of CAF-1 in nucleosome assembly. In addition, Hat1 also co-purifies with H3.3, raising the possibility that this modification on H4 also impacts the nucleosome assembly of H3.3 [13, 61]. Future studies are needed to determine to what extent H4K5, K12Ac and Hat1 regulate RC nucleosome assembly via nuclear import of H3.1-H4 and/or the regulation of the association between histone chaperones and histones.

In addition to acetylation of new H4, newly synthesized histone H3 is also acetylated. While acetylation of H3 lysine residues appears to be conserved among species, the acetylation patterns observed are different [53]. In *S. cerevisiae*, the predominant acetylation on newly synthesized histones is H3K56Ac, a residue located at the DNA entry/exit site of the nucleosome [28]. In the budding yeast, H3K56Ac is catalyzed by the Rtt109-Vps75 complex that utilizes Asf1-H3-H4 complex as the substrate [25-27, 49, 62-64]. While Vps75 is not required for H3K56Ac *in vivo* [49], Vps75 is required for the nuclear import of Rtt109 as well as the stability of Rtt109 [65, 66]. Following deposition, H3K56Ac is deacetylated by Hst3 and Hst4, members of the NAD dependent family of histone deacetylase (HDAC) enzymes, during late S or G2/M phase of the cell cycle [67, 68]. The primary role of H3K56Ac is to regulate RC nucleosome assembly. Supporting this idea, H3K56Ac peaks during S phase [28]. Using chromatinimmunoprecipitation (ChIP) assay, we have shown that H3K56Ac is deposited only onto replicating DNA, but not non-replicating DNA, during early S phase of the cell cycle [38]. This assay recapitulates early observations that nucleosome assembly is coupled to DNA replication [4]. Therefore, this assay is likely to be useful in determining to what extent a gene is involved in RC nucleosome assembly in budding yeast. Finally, H3K56Ac increases the binding affinity of H3-H4 with two histone chaperones, CAF-1 and Rtt106, suggesting that H3K56Ac regulates RC nucleosome assembly by increasing the binding affinity of H3-H4 with specific histone chaperones [38].

Cells lacking Rtt109 or those expressing a non acetylation state H3K56R mutant are much more sensitive towards DNA damaging agents than cells lacking both Rtt106 and CAF-1 [38]. This result suggests that H3K56Ac may also promote nucleosome assembly mediated by other histone chaperones in addition to CAF-1 and Rtt106. Alternatively, it implies that H3K56Ac has other functions in addition to its role in RC nucleosome assembly. Indeed, H3K56Ac is also important for nucleosome assembly following DNA repair, which is important for the recovery from checkpoint activation [69]. Furthermore, H3K56Ac has been shown to be involved in gene transcription [70, 71] as well as the regulation of RI nucleosome assembly (see discussion below)

In mammalian cells, H3K56Ac abundance is relatively low compared to yeast cells [30, 67, 72], leading one to inquire to what extent this modification plays a regulatory role in RC nucleosome assembly. Compared to H3K56Ac in budding yeast, the regulation of H3K56Ac in mammalian cells appears to be more complicated. For instance, while this modification is dependent on Asf1a and Asf1b in mammalian cells [29, 30], there are at least two acetyltransferases, p300/CBP and Gcn5, reported to acetylate mammalian H3K56 [30, 73],

whereas Gcn5 in budding yeast is not required for H3K56Ac [74]. In addition, several H3K56Ac deacetylases including Sirt1, Sirt6, Hdac1 and Hdac2 have been reported [29, 30, 75, 76]. Finally, there are conflicting reports on whether this modification is regulated during S phase of the cell cycle and how H3K56Ac levels change in response to DNA damage agents [29, 30, 73]. These conflicting reports likely result from the following possibilities. First, since the levels of H3K56Ac are low in mammalian cells, much more specific antibodies are needed to detect this modification by Western blot in mammalian cells. Therefore, the antibodies used by different groups from different sources may contribute to such conflicting results. Second, compared to yeast cells that have only one form of H3, human cells contain H3.1 and H3.3, which cannot be differentiated by Western blot. Therefore, different enzymes catalyzing H3K56Ac may target H3.1 and H3.3 differently. Future studies are needed to resolve these issues.

In addition to H3K56Ac within the H3 core domain, acetylation of lysine residues at the N-terminus of H3 also regulates RC nucleosome assembly [77]. Acetylation patterns on the N terminal tails of new H3 vary among species. For instance, in yeast, H3K9 and K27 are acetylated, in *Drosophila*, H3K9 and K14, and in humans, H3K9 and H3K18 [53, 55, 72]. Both yeast Elp3 and Gcn5, two acetyltransferases with well-established roles in gene transcription, function with Rtt109 to acetylate the H3 N-terminal tail of newly-synthesized histones and promote nucleosome assembly in a manner parallel to H3K56Ac [74, 78]. Supporting this idea, Gcn5, although it does not directly regulate levels of H3K56Ac in yeast, is important for proper deposition of H3K56Ac at the replication fork. Furthermore, while H3K56Ac is important for both CAF-1 and Rtt106 to bind H3-H4, Gcn5 and H3 N-terminal tail acetylation appear to only regulate the interaction between CAF-1 and histones H3-H4 [74].

In addition to a role in nucleosome assembly, Gcn5 and acetylation of the H3 N-terminus are likely to have a role in the initiation of DNA replication [79, 80]. The levels of acetylation at multiple lysine residues on H3 and H4 in nucleosomes surrounding a replication origin increase during S phase. Interestingly, in the case of H3, multiple sites tend to be acetylated together. Mutational analysis suggests that acetylation at these sites functions redundantly in origin firing as the quantitative number of acetylation marks is more important in regulating origin firing than acetylation at a particular site [79]. Because nucleosome positioning at origins affects origin firing [81], it would be challenging to separate the roles for Gcn5 and acetylation of lysine residues at the H3 N-terminus in origin firing from their roles in nucleosome assembly.

Although histone lysine acetylation is predominantly a mark of newly synthesized histones, mammalian H3.1 is monomethylated at H3 lysine 9 (H3K9me) by SETDB1 on more than one third of the H3.1 pool [56]. SETDB1 forms a complex with both CAF-1 and HP1 during S-phase and preferentially methylates free histones over nucleosomal histones, suggesting it methylates histones prior to deposition. It is proposed that H3K9me may serve as a precursor to set up H3K9me3, catalyzed by Suv39, at pericentric heterochromatin sites and thus play a role in the propagation of this histone mark. Consistent with this idea, knockdown of SETDB1 results in lower levels of H3K9me3 [82]. Furthermore, kinetic studies on the levels of methylation of newly synthesized histones suggest that monomethylation of many residues most likely occurs concurrent with or shortly after deposition to enable further higher order methylation at particular sites [83]. A recent report suggests that H3K9me1 is one of the first modifications observed on H3.1 following synthesis, and the levels of H3K9me1 are sharply reduced in conjunction with the appearance of H4K5,12Ac, suggestive of a role of this modification in earlier histone processing [31]. Despite these advances in understanding how this mark occurs on newly

synthesized histones, the exact role of this abundant modification on newly-synthesized H3 remains to be determined.

In summary, modifications on newly synthesized H3 and H4 likely play multiple roles in the regulation of RC nucleosome assembly, whether it be through the regulation of histone nuclear import, histone chaperone and histone interactions, and/or other functions.

3. Replication independent (RI) nucleosome assembly

While canonical histone H3 is strictly incorporated into chromatin during S phase of the cell cycle, the majority of histone H3 variant H3.3 is assembled into nucleosomes in a replication independent (RI) manner [15, 84]. This RI process likely involves the exchange of parental H3 including both canonical H3 and H3 variant H3.3, with new H3.3 to compensate for nucleosome loss and/or replacement of damaged histones in non-dividing cells such as neurons. In addition, RI nucleosome assembly is also important during gene transcription and other cellular processes.

Most of the early studies on RI histone exchange focused on histone H2A-H2B exchange. H2A-H2B exchange occurs namely at actively transcribed regions and the 5' end of the genes, raising the possibility that histone exchange may be an important form of transcriptional regulation [85, 86]. While the frequency of H3-H4 incorporation is much lower than H2A-H2B, H3-H4 exchange does occur. The observation that H3-H4 incorporation independent of DNA replication occurs arose from an elegant study using a chromatin fractionation assay in immature erythrocytes [87]. In addition, histone H3-H4 exchange can be detected in the presence of the transcription inhibitor actinomycin D [88], suggesting that mechanisms other than gene transcription are likely involved in RI histone exchange. Supporting this idea, genome wide mapping of H3.3 shows that H3.3 is enriched at both active and inactive gene promoters [89, 90]. Increased histone exchange is also observed at boundary elements that block the spread of chromatin states in yeast and *Drosophila* [91, 92]. Together, these studies suggest that histone exchange may have an important regulatory role in chromatin dynamics.

Similar to the problem arising during DNA replication, nucleosomes serve as barriers for gene transcription, and therefore, changes in chromatin structure are often a prerequisite for the initiation of gene transcription and transcriptional elongation. Genome wide nucleosome positioning mapping has revealed that two well-positioned nucleosomes flank the transcription start site (TSS) with a nucleosome-depleted region (NFR) in between (reviewed in [93]). In mammalian cells, the NFR is likely to be marked by an unstable nucleosome containing H3.3 and H2AZ [90]. The dynamics of nucleosomes at the promoter likely provide a mechanism for regulating gene expression.

During transcriptional elongation, nucleosomes are temporally disassembled in front of the RNA Polymerase (Pol) II complex to facilitate passage of the transcriptional machinery. Following gene transcription, nucleosomes must be reassembled by recycling the parental histone $(H3-H4)_2$ and/or incorporation of newly synthesized histones (replacement/ exchange). Two different mechanisms are proposed to efficiently restore the chromatin structure following passage of RNA Pol II [94]. First, partial loss or exchange of core histones was observed at highly active transcribed gene regions [95-100]. Second, H2A-H2B exchange, but not H3-H4 exchange, was detected at moderately transcribed gene regions [101, 102]. As mentioned above, genome wide mapping of H3.3 occupancy reveals that H3.3 is enriched at gene bodies of both active and inactive genes in *Drosophila* and mammalian cells [84, 90], suggesting that nucleosome assembly and the exchange of H3.3 occurs in both a transcription dependent and independent manner. Consistent with this idea,

despite having only one form of H3, histone H3-H4 replacement/exchange also occurs at both active and inactive gene promoter regions in budding yeast [103]. Collectively, these studies suggest that histone deposition and/replacement during gene transcription may regulate gene expression and/or maintain gene expression state.

3.1 Histone chaperones involved in replication-independent nucleosome assembly

Like RC nucleosome assembly, RI nucleosome assembly/exchange is mediated by histone chaperones. Multiple histone chaperones including Asf1, Hir1, Rtt106 and Spt6 have been shown to be involved in RI nucleosome assembly in budding yeast. The Hir1 complex (consisting of Hir1, Hir2, Hir3, and Hpc2 in budding yeast) is the major histone chaperone involved in RI nucleosome replacement [104, 105]. In higher eukaryotes, HIRA, the sequence homolog of Hir1, is well known to promote the assembly of histone H3.3 into chromatin independently of DNA replication [13, 106, 107]. Similar to RC nucleosome assembly mediated by CAF-1, Asf1 is proposed to deliver H3-H4 to HIRA for subsequent nucleosome assembly.

In human cells, Asf1a and Asf1b appear to have distinct functions in regard to the regulation of RI nucleosome assembly. For instance, HIRA preferentially binds Asf1a over Asf1b [108]. Moreover, depletion of Asf1b, but not Asf1a, results in cell proliferation defects [109]. Interestingly, both Asf1a and HIRA promote the formation of senescence associated heterochromatin foci [110]. These results raise the possibility that Asf1a is the primary histone chaperone that transfers histones to HIRA for nucleosome assembly.

While it is still not clear why cells need so many histone chaperones for RI nucleosome assembly, based on studies of HIRA and Daxx (Death domain containing protein) in mammalian cells, it is possible that these histone chaperones promote RI nucleosome assembly at distinct chromatin regions to regulate gene expression at different chromatin domains. Daxx has been identified as another H3.3 histone chaperone in mammalian cells [61, 89, 111]. In contrast to HIRA which is required for deposition of H3.3 at genic regions, Daxx is required for the localization of H3.3 at telomeric repeats [89]. Interestingly, Daxx contains a Rtt106-like domain at the C terminus, and this domain contributes to the H3.3 binding affinity [61]. Therefore, it is possible that while Daxx does not share significant homology with Rtt106, Daxx may be the functional homolog of Rtt106 in human cells. In addition to Daxx and HIRA, other H3.3 chaperones will likely be identified because it has been shown that the H3.3 occupancy at regulatory elements is independent of either Daxx or HIRA [89].

Unlike RC nucleosome assembly where the coordination between DNA replication and nucleosome assembly is carried out by the recruitment of histone chaperone complexes via direct interactions with the replication machinery, it is still unclear as to how the histone chaperones involved in RI nucleosome assembly are recruited to chromatin. Accumulating evidence suggests that histone chaperones likely interact with transcriptional machinery and chromatin remodeling complexes to promote RI nucleosome assembly and regulate gene expression. For instance, Spt6 interacts with RNA Pol II physically and promotes nucleosome disassembly in order to facilitate the passage of RNA Pol II followed by nucleosome reassembly [112]. Similarly, the histone chaperone complex FACT, which has multiple roles, was first indentified through co-purification with RNA Pol II and is important for both transcription initiation and elongation (Reviewed in [113]). Second, *Drosophila* Asf1 interacts with the Brahma chromatin remodeling complex [114]. Third, HIRA interacts with the chromatin remodeling complex Chd1 [115]. Lastly, Daxx interacts with the SWI/SNF type chromatin-remodeling factor ATRX (α-thalassemia/mental retardation syndrome protein), and the deposition of H3.3 onto telomere and pericentric DNA repeat regions depends on ATRX [61, 89]. Supporting this idea, it has been shown that

ATRX is localized at repetitive DNA sequences [116]. It is worth noting that some histone chaperones, such as DEK, can serve as transcription coactivators [117]. These studies suggest that multiple factors may aid in recruiting histone chaperone complexes for RI nucleosome assembly and such events may serve as another form of regulation. Further studies should be carried out to determine how these different modes of RI nucleosome assembly pathway impact gene transcription.

3.2 Role of histone modifications in RI nucleosome assembly

Unlike modifications on H3 and H4 that function in RC nucleosome assembly, the roles of modifications on new H3 and H4 in RI nucleosome assembly has not been well explored. However, several studies suggest that posttranslational modifications on H3 and H4 also likely regulate RI nucleosome assembly as well. For instance, genome wide measurements of H3K56Ac turnover in yeast cells reveal that H3K56Ac marks the most dynamic nucleosomes [103]. This result suggests that in addition to its role in RC nucleosome assembly, H3K56Ac likely also impacts RI nucleosome assembly. Supporting this idea, Rtt109 and Asf1, two regulators of H3K56Ac, promote histone replacement/exchange during gene transcription. Interestingly, Vps75, a histone chaperone facilitating the enzymatic activity of Rtt109, inhibits histone replacement, suggesting a possible feedback mechanism for nucleosome replacement [103]. In mammalian cells, quantitative mass spectrometry analysis of histone modifications uncovered that histone turnover rates depend upon both site-specific histone modifications and the amino acid sequence of histone variants [118]. In general, histones containing active marks have a faster turnover rate than histones containing repressive marks, consistent with the idea that gene transcription influences histone turnover and/or assembly. Interestingly, the turnover rate of the H3K56Ac peptide is faster than many other histone modifications [118]. Despite these interesting observations, detailed studies on the role of modifications on H3 and H4 in RI nucleosome assembly are needed to understand to what extent modifications on H3 and H4 function in RI nucleosome assembly/exchange. Furthermore, since specific chromatin domains have distinct nucleosomal histone modifications [119, 120], it is also possible that these histone modifications also regulate histone turnover rates and drive the histone deposition pathways. Recently, our laboratory has found that phosphorylation of histone H4 S47 (H4S47ph), catalyzed by the Pak2 kinase, promotes HIRA mediated nucleosome assembly of H3.3-H4 and inhibits CAF-1 mediated nucleosome assembly of H3.1-H4. This suggests that modifications on histone H4 can help specify nucleosome assembly of H3.1 and H3.3 [121].

4. Summary and future directions

Over the last several years, we have witnessed explosive interest in addressing the regulation of nucleosome assembly pathways by histone chaperones and histone modifications and the functional implications of RC and RI nucleosome assembly in the inheritance of chromatin structure and regulation of gene expression. However, there is still a plethora of unanswered questions waiting to be addressed.

Emerging evidence indicates that nucleosomes are much more dynamic than previously recognized. In addition, deposition of new H3-H4 and transfer of parental H3-H4 during DNA replication and DNA repair proceed with rapid speed. Therefore, technical limitations have made it extremely difficult to spatially and temporally monitor the dynamics of histone deposition in live cells. Hopefully, advances in single cell imaging will enable us to design approaches to analyze how parental histones H3-H4 are transferred onto replicated DNA during S phase of the cell cycle and how multiple histone deposition pathways function coordinately to promote nucleosome assembly [122].

Second, in addition to regulating gene transcription, H3.3 likely has roles in heterochromatin formation [123]. Studies are needed to address how H3.3 performs these functions and to what extent these different functions are linked to histone chaperones that deposit H3.3 to distinct chromatin domains.

Third, yeast models have aided immensely in understanding regulatory mechanisms for nucleosome assembly, such as H3K56Ac and the regulation of histone chaperone interactions with H3-H4. It remains not well explored on how RC and RI nucleosome assembly pathways are regulated in higher eukaryotes. Given that nucleosome assembly in mammalian cells is much more complex, it is likely that histone modifications play a significant role in regulating RC and RI nucleosome assembly in mammalian cells. Indeed, we have recently discovered that phosphorylation of histone H4 promotes HIRA mediated nucleosome assembly and inhibits CAF-1 mediated nucleosome assembly [121]. Moreover, covalent modifications on histone chaperones are also detected and these modifications likely impact the function of histone chaperones [124].

Lastly, several histone chaperones have been shown to interact with ATP dependent nucleosome remodeling complexes for nucleosome assembly and/or gene regulation. We suspect that future studies will reveal additional interactions among histone chaperones and chromatin remodeling complexes. The challenge will be to determine how histone chaperones coordinate with chromatin remodeling complexes during different cellular processes.

Acknowledgments

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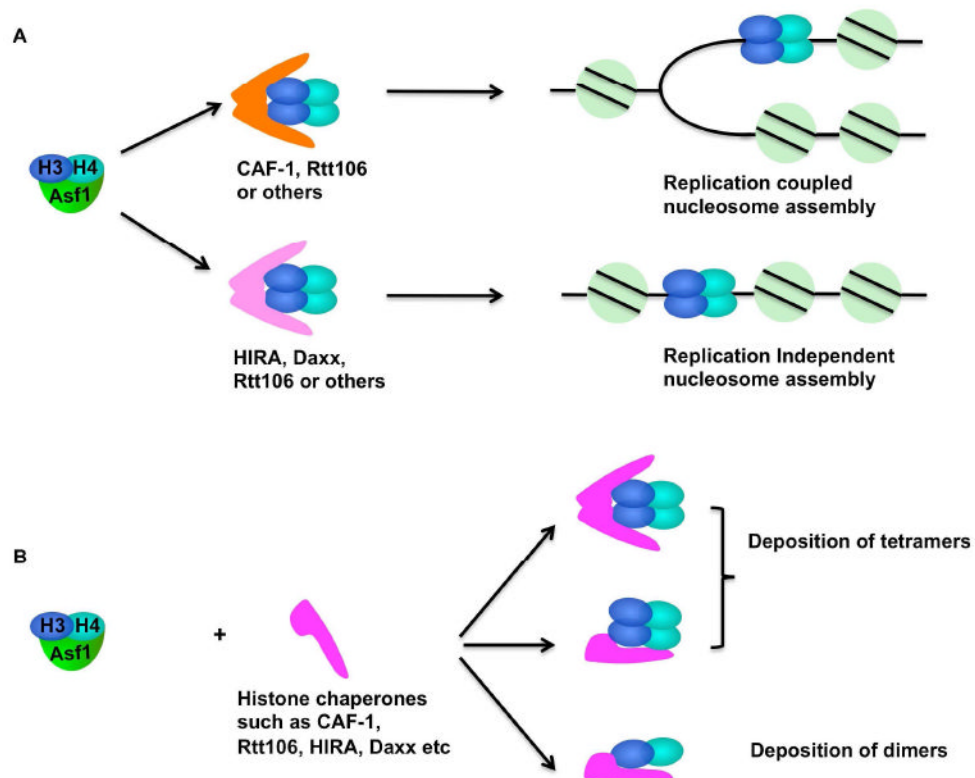
Research highlights

We review literatures on replication-coupled and replication-independent nucleosome assembly.

We highlight the functions of histone chaperones in nucleosome assembly.

We discuss the roles of histone modifications in nucleosome assembly.

We speculate the future direction of the nucleosome assembly field.

Figure 1.
Nucleosome assembly of new H3-H4. (A) There are two major nucleosome assembly pathways: replication coupled (RC) nucleosome assembly and replication independent (RI) nucleosome assembly. Histone chaperone Asf1 binds a H3-H4 dimer, which will be transferred to histone chaperones that are involved in RC or RI nucleosome assembly. (B) Models of formation of histone $(H3-H4)_2$ tetramers, the first building block of a nucleosome. Two H3-H4 dimers from Asf1 can be transferred to a histone chaperone to form one $(H3-H4)_2$ tetramer on a monomeric or dimeric form of the histone chaperon. Alternatively, a H3-H4 heterodimer will be transferred from Asf1 to another histone chaperone, which will deposit two H3-H4 dimers sequentially onto DNA for formation of a $(H3-H4)_2$ tetramer.
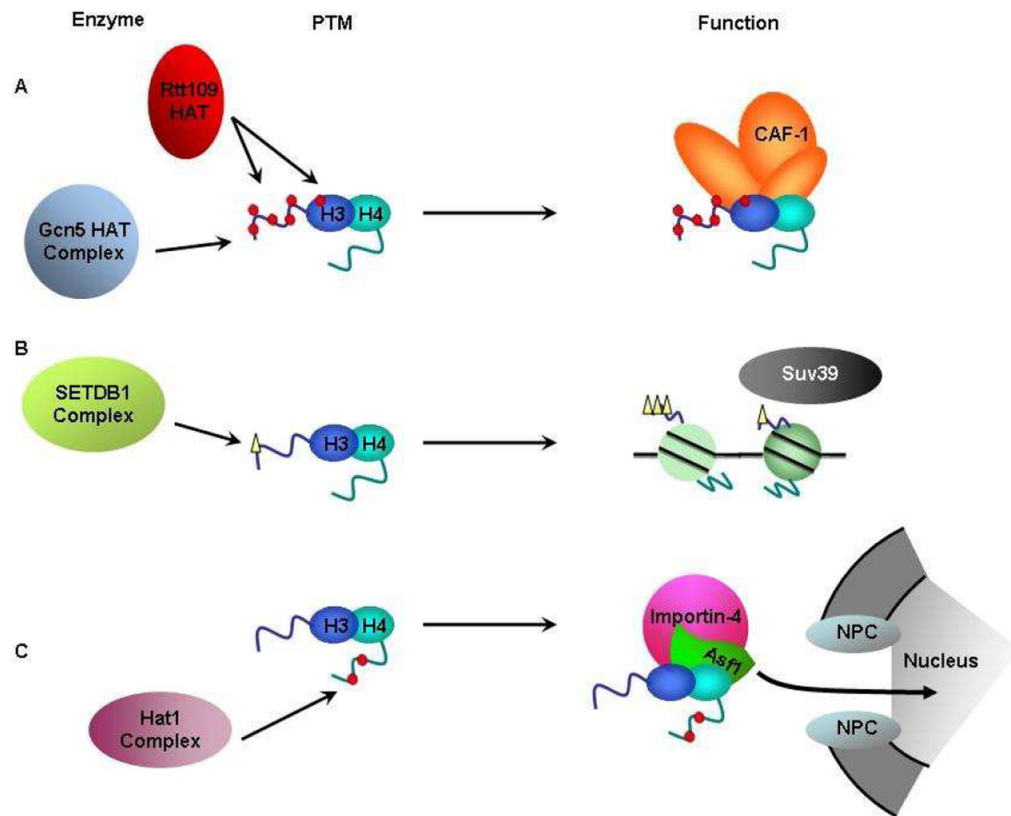
Figure 2.
Roles for post-translational modifications on newly-synthesized histones in nucleosome assembly. Described are the enzymes, post-translational modification (PTM) and function associated with known modifications observed on newly-synthesized H3-H4. (A) Acetylation of the H3 N-terminal tail by Gcn5 and Rtt109 and H3K56 (within the H3 core domain) by Rtt109 regulates the interaction between histone chaperones (like CAF-1) and H3-H4. (B) H3K9me, catalyzed by SetDB1 (in complex with CAF-1 and HP1alpha), serves as a substrate for Suv39, and thereby contributing to the epigenetic inheritance of the H3K9me3 mark. (C) Hat1 catalyzes H4K5,12Ac, which may help regulate histone import from the cytoplasm into the nucleus.

Table 1

A summary of factors involved in RC and RI nucleosome assembly.

Nucleosome Assembly Pathway	Replication-Coupled (RC)	Replication-Independent (RI)
Biological Process	DNA replication	Transcription/others
Function	Epigenetic inheritance	Histone turnover Gene expression
Histone Chaperone	Asf1 CAF-1 Rtt106 (Yeast)	Asf1 HIRA(HIR1) Daxx Rtt106 (Yeast)
Histone H3	H3.1	H3.3
Histone H3 Modifications[1]	H3K56Ac H3 N-terminal tail Ac	H3K56Ac
Histone H4 Modifications	H4K5,12Ac	H4S47ph

[1]We mentioned solely those modifications that have been shown to have a role in regulating the indicated nucleosome assembly pathways.