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Chromatin assembly during S phase: contributions from histone deposition, DNA replication and the cell division cycle

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Abstract. During S phase of the eukaryotic cell division cycle, newly replicated DNA is rapidly assembled into chromatin. Newly synthesised histones form complexes with chromatin assembly factors, mediating their deposition onto nascent DNA and their assembly into nucleosomes. Chromatin assembly factor 1, CAF-1, is a specialised assembly factor that targets these histones to replicating DNA by association with the replication fork associated protein, proliferating cell nuclear antigen, PCNA. Nucleosomes are further organised into ordered arrays along the DNA by the activity of ATP-dependent chromatin assembly and spacing factors such as ATP-utilising chromatin assembly and remodelling factor ACF. An additional level of controlling chromatin assembly pathways has become apparent by the observation of functional requirements for cyclin-dependent protein kinases, casein kinase II and protein phosphatases. In this review, we will discuss replication-associated histone deposition and nucleosome assembly pathways, and we will focus in particular on how nucleosome assembly is linked to DNA replication and how it may be regulated by the cell cycle control machinery.

Key words. DNA replication; chromatin assembly; histone; nucleosome; protein kinase.

Introduction

In proliferating cells, the replication of eukaryotic chromosomes occurs during S phase of the cell division cycle. The passage of the replication fork does not only result in an exact duplication of the DNA but also generates a requirement for an assembly of the newly replicated DNA into chromatin. The basic structural unit of chromatin is the nucleosome core particle consisting of 146 bp of DNA wrapped in 1.75 left-handed superhelical turns around a central core histone octamer containing two copies of each histone H2A, H2B, H3 and H4 [1]. The bulk synthesis of new histone proteins for assembly into nucleosomes also occurs during S phase and is coupled to ongoing DNA replication [2]. Arrays of nucleosome core particles are further compacted into higher-order chromatin structures involving binding of linker histones and a plethora of nonhistone chromatin proteins. This assembly not only allows packaging of the genomic DNA to fit into the nucleus but it is also involved in the regulation of essential DNA processes such as gene expression, replication, recombination and repair.

The assembly of nucleosomes during DNA replication is achieved by two different pathways. The first pathway is known as the parental nucleosome transfer, where histones from parental DNA are recycled by direct transfer and deposition onto the replicated daughter DNA branches. This recycling of parental histones only contributes to the assembly of half of the replicated DNA into chromatin. The other half is assembled by the second pathway known as de novo nucleosome assembly, which is mediated by chromatin assembly factors that can act as histone chaperones targeting soluble histones to the sites of assembly at DNA replication forks. These reactions have been discussed in a series of recent reviews [3–6].

Here, we shall not replicate these reviews in detail, but we will focus on publications adding new perspectives to our understanding of how nucleosome assembly is linked to DNA replication and how it may be regulated by the cell cycle control machinery (fig. 1).

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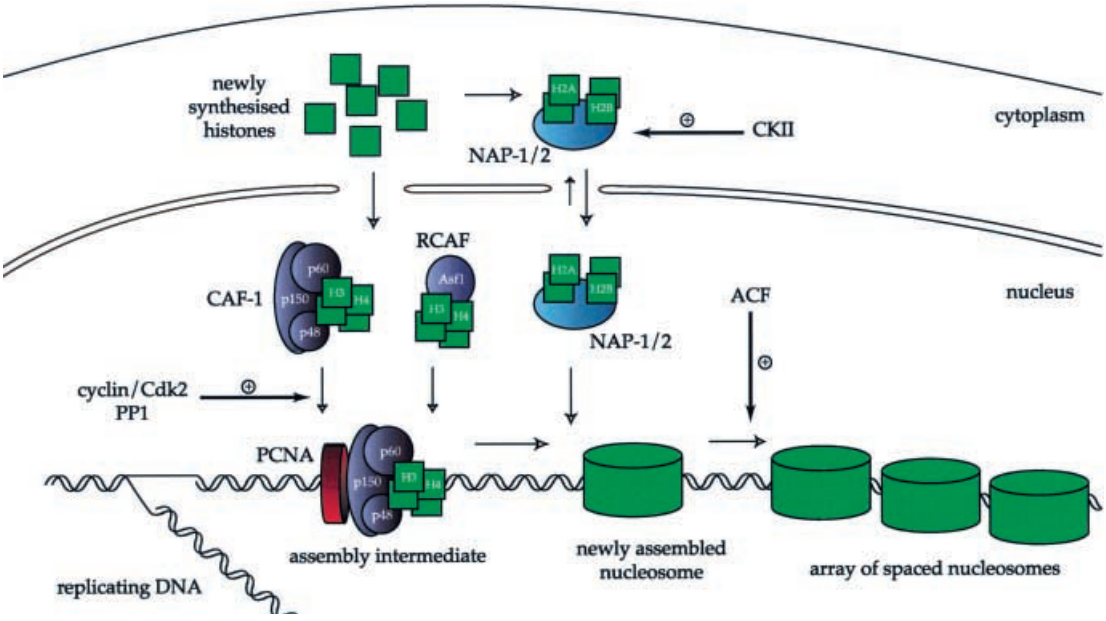

Figure 1. Schematic representation of factors involved in chromatin assembly during DNA replication in S-phase cells. Nucleosomes and histones are shown in green, assembly factors in blue and replication fork-associated proteins in red. Assembly reactions are represented by small open arrows and factors involved in positively regulating these reactions are indicated by large filled arrows. See text for further details.

Histone deposition onto replicating DNA

Chromatin assembly factor CAF-1

The assembly of nucleosomes during DNA replication was first studied in cytosolic extracts from human cells. Double-stranded DNA molecules containing the Simian virus 40 (SV40) replication origin are replicated under the control of the virally encoded initiator protein and DNA helicase, T antigen [7]. Nucleosome assembly on replicating DNA is triggered upon the addition of the nuclear chromatin assembly factor 1 (CAF-1), which consists of three subunits, p150, p60 and p48 [8] (fig. 1). CAF-1 was originally purified from human cell nuclei [8], and homologous protein complexes with a similar subunit composition have since been purified and characterised in many different organisms such as yeast, *Drosophila*, chicken, mouse and plants [9–13, 77].

CAF-1 targets newly synthesised and soluble histones H3 and H4 present in the cytosolic extract onto replicating DNA to form a $(H3/H4)_2$ tetramer [14]. Following deposition of the tetramer by CAF-1 as a nucleosome precursor particle, the full nucleosome core particle is completed in a second step, independently of CAF-1, by the association of two H2A/H2B dimers to the H3/H4 tetramer [14]. This latter association may require the activity of other assembly factors that do not depend on DNA replication (fig. 1, and see below).

All three subunits of human CAF-1 bind histone proteins [15–17]. The large subunits p150 and p60 are found to be associated with newly synthesised and acetylated histones H3 and H4 [15, 16], possibly via an internal cluster of negatively charged acidic amino acid residues present on p150 [15]. Interestingly, all three subunits can bind to recombinant histone $(H3/H4)_2$ tetramers which lack the amino-terminal domains of both H3 and H4, and promote deposition of these histones onto replicated DNA [17]. Therefore, the amino-terminal tails of histones H3 and H4 that contain the sites for posttranslational protein modifications such as acetylation and methylation are not required for CAF-1-dependent nucleosome assembly onto replicated DNA [17]. Incidentally, this observation is paralleled by domain requirements for the transfer of parental histones past the replication fork onto the replicated DNA daughter strands ([3], and references therein). It was shown that removal of the amino-terminal histone tails by mild trypsinisation did not prevent this nucleosome transfer [18], creating an intriguing parallel between these concerted assembly reactions.

The smallest subunit of CAF-1, p48, is identical to the protein RbAp48, a member of the conserved p46/p48 family [16]. Both proteins bind free histones H2A and H4 at the helix 1 in the central globular domain of the histone proteins [19]. This domain is not accessible when histones are assembled into nucleosomes [1], explaining the binding of p46/p48 to free but not to nucleosomal histones.

In cytosolic extracts of human cells, p48 is also present in large amounts that are not complexed with p150 and p60 [16, 20]. Interestingly, members of the p46/p48 family have been found to be noncatalytic subunits of histone acetylases and histone deacetylases as well as chromatin remodelling factors in various organisms [12, 19, 21–

23]. The two smaller subunits of CAF-1, p60 and p48 are both members of the WD repeat protein family. Each subunit contains five to seven WD repeats which are likely to be required for physical interaction with each other or with other related WD repeat proteins [15, 16]. In chicken cells, it was shown that the p48 subunit of CAF-1 binds to histone deacetylases and that the WD repeats of p48 are essential for a direct protein-protein interaction with the histone deacetylase chHDAC-2 [12]. Thus, the small histone-binding subunit p48 of CAF-1 can also participate in other histone metabolising activities, providing a possible link between the assembly of new nucleosomes and the alteration of chromatin structure.

Once histones H3 and H4 are deposited onto replicated DNA, both CAF-1 and the acetylation pattern of histone H4 are rapidly lost in early replicating euchromatin [24]. In contrast, both are maintained for up to 20 min after replication fork passage in heterochromatin [24]. These observations enforce the suggestion that p48 may serve as a molecular link or adapter for the different histone modification complexes such as CAF-1 and HDACs [25]. However, the p46/p48-binding helices 1 of histones H4 and H2A are not accessible when assembled into nucleosomes [19]. A simple subunit swap between the p150/p60 subunits of CAF-1 and the catalytic subunit of a histone deacetylase following nucleosome assembly at the replication fork seems hence unlikely. Therefore, the precise mechanism as to how HDACs are recruited to post-replicative chromatin remains to be elucidated.

In addition to its role in histone deposition, CAF-1 has also been found to interact functionally with a component of heterochromatin [13], namely the heterochromatin protein HP1 (reviewed in [26]). The p150 subunit binds to HP1 via an amino-terminal domain that is dispensable for nucleosome assembly [13]. HP1 and p150 persist on replicated heterochromatin after replication [13, 24]. Mutations in p150 can prevent association with HP1 in heterochromatin outside of S phase in vivo and prevent formation of CAF-1/HP1 complexes on replicating chromatin in vitro [13]. In addition, HP1 can also bind directly to nucleosome core particles and DNA in vitro, possibly via association with the amino terminus of histone H4 [27] or the methylated lysine residue 9 in the amino terminus of histone H3 [A. Bannister et al., personal communication]. These observations suggest that CAF-1 has an additional role in the formation of higher-order heterochromatin structures following DNA replication.

Replication-coupling assembly factor: RCAF

A different chromatin assembly factor was purified from *Drosophila* embryo extracts by a complementation assay using the SV40 DNA replication system supplemented with *Drosophila* CAF-1, where nucleosome assembly is inhibited by addition of excess template DNA [28]. The purified factor was termed replication-coupling assembly factor (RCAF) and consists of a complex containing the *Drosophila* homologue of the yeast antisilencing function 1 protein (ASF1) and the histones H3 and H4 [28] (fig. 1). The histones of RCAF are acetylated at lysines 5 and 12, (for H4) and at lysine 14 (for H3), which mirrors the transient acetylation at these sites during replication-coupled deposition. RCAF acts synergistically with CAF-1 to assemble nucleosomes preferentially on replicating DNA and cannot be replaced by acetylated or bulk histones in the absence of the ASF1 subunit, indicating that the ASF1 subunit is essential for the chromatin assembly activity of RCAF. In yeast, mutations in ASF1 and the large subunit of CAF-1 show cooperative defects in cell growth and transcriptional silencing, indicating separate but coordinated functions for both assembly factors in vivo [28].

Histone deposition and nucleosome assembly in the absence of DNA replication

In yeast cells, a deletion of CAF-1 produced viable cells with orderly assembled bulk chromatin in their nuclei, despite some defects in gene silencing, DNA repair and other functions [11, 29–33]. These effects have been reviewed in detail elsewhere [5, 6]. However, these observations clearly indicate that CAF-1 is not the only factor that can assemble nucleosomes onto replicating DNA. Indeed, many assembly factors are known that assemble nucleosomes onto double-stranded DNA under physiological conditions in the absence of DNA synthesis, which we will discuss below.

Nucleosome assembly proteins: NAP-1 and NAP-2

In human cells, a 53-kDa protein termed nucleosome assembly factor 1 (NAP-1) has been shown to assemble nucleosomes in the absence of ongoing DNA replication [34] (fig. 1). Homologues of NAP-1 were subsequently found in different organisms such as yeast, *Drosophila*, soybean and mouse [35–39], and a related gene product, hNRP, was also found in humans [40]. NAP-1 binds to all core histones, but shows a preference for H2A and H2B [36, 37, 41, 42]. For histone binding, NAP-1 requires the central domain of the protein, which is also necessary for nucleosome assembly activity, as well as the negatively charged carboxy-terminal domain [35]. Under physiological conditions, NAP-1 mediates nucleosome assembly from core histones on double-stranded DNA, but no regularly spaced arrays of nucleosomes are formed. However, in *Drosophila,* NAP-1 facilitated assembly of regularly spaced nucleosomes onto DNA together with partially purified CAF-1 [36], suggesting a cooperative activity of these two assembly proteins. Comparison of the protein sequences of NAP-1 from different sources

reveals some evolutionarily conserved domains, such as stretches of acidic residues implicated for histone binding [35, 38], as well as nuclear localisation and nuclear export signals [36, 43].

Another related nucleosome assembly factor termed NAP-2 has been isolated from and characterised in human cells [42, 44, 45]. Recombinant NAP-2 binds to all core histones as well as to the linker histone H1 and mediates histone deposition and nucleosome assembly on double-stranded DNA [42]. NAP-2 contains a potential nuclear localisation signal and two clusters of acidic residues. Deletion analysis of NAP-2 showed that both acidic amino- and carboxy-terminal domains, as well as a central domain common to the known Nap-1/2 proteins, are all required for nucleosome assembly activity [42].

An ATP-utilising chromatin assembly and remodelling factor: ACF

In bulk chromatin, nucleosomes are organised in ordered arrays along the DNA. The periodic assembly of nucleosomes and hence an exact positioning on DNA is an ATP-dependent process. One factor has been purified from *Drosophila* which catalyses this event and was subsequently termed ATP-utilising chromatin assembly and remodelling factor, ACF [46] (fig. 1). One subunit of ACF is the ISWI protein, which is also a component of the two chromatin-remodelling factors NURF and CHRAC [47–50]. Deletion analysis of ISWI in *Drosophila* revealed essential functions of ISWI for cell viability and gene expression during development as well as for maintenance of higher-order chromatin structure on the male X chromosome [51]. The other subunit of ACF, Acf1, is unique to ACF, and both subunits together function synergistically in the assembly of chromatin [52]. In addition, ACF acts synergistically with either assembly factor NAP-1 or CAF-1 to deposit histones into extended periodic nucleosome arrays in an ATP-dependent manner [46, 52]. A homologous protein complex was purified from human cell nuclei and termed hACF [53]. It also facilitates ATP-dependent chromatin assembly in the presence of NAP-1. Human ACF consists of two subunits encoded by the Baz1A and the hSNF2h genes, respectively [53]. hSNF2 is related to ISWI, and it is also present in the chromatin-remodelling factor RSF, which is required for transcription initiation on chromatin templates [54]. A factor closely related to, and perhaps identical with, hACF was independently purified and designated as Williams syndrome transcription factor-related chromatin-remodelling factor (WCRF) [55]. The complementary DNAs (cDNAs) coding for the small subunit of WCRF is hSNF2h [55] and for the large subunit appears to be identical to Baz1A [53]. However, a sequence homology search indicated that there is another protein closely related to the large subunit of WCRF in humans, suggesting the presence of a family of related factors [55].

Recruitment of CAF-1 to replicating DNA and sites of DNA repair

The CAF-1 and PCNA connection

To date, CAF-1 is the only chromatin assembly factor that specifically targets replicating or newly replicated DNA for an assembly of nucleosomes and that is capable of discriminating against bulk DNA that is not replicating. This biochemical specificity is mirrored by the intracellular localisation of CAF-1. In S-phase nuclei, the two large subunits of CAF-1 colocalise with the discrete sites of DNA replication, both during euchromatin replication in early S phase and heterochromatin replication in late S phase [56]. This observation supports a physiological involvement of CAF-1 during DNA replication in vivo and has since been reproduced and further confirmed by colocalisation with the replication fork-associated protein, proliferating cell nuclear antigen, (PCNA) in mammalian cell nuclei [13, 20, 24].

A direct molecular link between CAF-1 dependent nucleosome assembly and DNA replication was found by the molecular interaction of CAF-1 with PCNA [57, 58] (fig. 1). PCNA forms a ring-like clamp of three identical subunits around DNA [59]. The first function described for PCNA was that it enhances the processivity of DNA synthesis by serving as a sliding clamp for the replicative DNA polymerases α and δ (reviewed in [60–62]). Recent studies, however, revealed additional interaction partners of this protein, which are involved in regulating the cell cycle, Okazaki fragment joining or DNA mismatch repair (reviewed in [63]). The loading and unloading of PCNA onto and off DNA requires a further protein, replication factor C (RF-C) (reviewed in [63]). RF-C binds to single-strand/double-strand junctions on DNA which are present as intermediate structures at replication forks and at sites of DNA excision repair on damaged DNA. In this aspect, RF-C acts as a clamp loader, forming a complex with PCNA and requires ATP to temporally open the PCNA ring in order to pass DNA strands. Following hydrolysis of ATP by the intrinsic ATP'ase activity of RF-C, PCNA is released from RF-C forming a stable PCNA clamp around the DNA. PCNA can persist on the replicated DNA following engagements with different interacting partners during DNA replication, and it is eventually unloaded by RF-C again.

Human CAF-1 associates with PCNA present on replicated DNA, and can mediate nucleosome assembly on these postreplicative DNA structures (fig. 1), implicating direct coupling of nucleosome assembly to replicating DNA [57] and to DNA undergoing nucleotide excision repair ([58]; reviewed in [5]). This association is mediated by the p150 subunit, most likely by interaction via at least two internal PCNA binding domains within the protein [57, 58]. In conclusion, this association of PCNA with CAF-1 is suggestive of how CAF-1 may be targeted to DNA at the

replication forks and to sites of excision repair synthesis, allowing a subsequent histone deposition and nucleosome assembly.

Linking nucleosome assembly to the cell cycle

Intracellular localisation and phosphorylation of NAP-1/2 by casein kinase II

Localisation studies in *Drosophila* embryos showed that NAP-1 is present in the nucleus during S phase, but is predominantly cytoplasmic during G2 phase [36]. In human cells, NAP-1 is predominantly cytosolic throughout the cell cycle [20]. These observations suggest that NAP-1 may act as a shuttle that delivers core histones from the sites of synthesis in the cytoplasm into the nucleus, cooperating with CAF-1 in the formation of new nucleosomes [36]. Similarly, human NAP-2 is cytoplasmic in G0/G1 and G2 phases and relocates to the nucleus during S phase, suggesting an equivalent shuttle mechanism [42].

Both NAP-1 and NAP-2 are phosphoproteins and can bind to and be phosphorylated by casein kinase 2 (CKII) [43, 45]. CKII activity is required for cell cycle progression from G1 to S phase and further into mitosis [64].

NAP-2 undergoes cell-cycle-dependent changes in the phosphorylation state [45]. In G0/G1 as well as in G2/M phase the protein is phosphorylated, but it is dephosphorylated in S phase. Addition of core histones stimulates the phosphorylation of NAP-2 by CKII [45]. Phosphorylated NAP-2 is complexed with histones and remains in the cytoplasm, whereas dephosphorylated NAP-2 is translocated into the nucleus in S phase. However, NAP-2 is found to be complexed with histones during the entire cell cycle, supporting the idea that phosphorylation is involved in nuclear transport rather than required for histone binding [45] (fig. 1). Furthermore, both NAP-1 and NAP-2 contain consensus sites for phosphorylation by CKII flanking the nuclear localisation signals, suggesting that the nuclear transport of these histone chaperones might be regulated by CKII mediated phosphorylation [43, 45].

A different cell-cycle-associated function for NAP-1 in mitosis was suggested in yeast by interactions of NAP-1 with the B-type cyclin Clb2 and a functional requirement for NAP-1 in Clb2-dependent induction of mitotic events [65, 66]. Genetic and biochemical approaches in yeast have identified a protein kinase called Gin4 that binds to NAP-1 and that is required for the ability of Clb2 and Nap-1 to promote normal progression through mitosis [67]. However, a link between the nucleosome assembly activity and this mitotic function of NAP-1 remains to be established.

An independent role for CKII in the assembly of heterochromatin was suggested by the observation that in *Drosophila* HP1 is phosphorylated by CKII, promoting efficient binding to heterochromatin, and substitution mutations in the CKII phosphorylation target sites of HP1 dramatically reduce the heterochromatin binding activity of HP1 [68].

CAF-1 and reversible phosphorylation by cyclin/Cdk complexes and PP1

CAF-1 is concentrated at sites of DNA replication in S phase cell nuclei, but outside of S-phase no significant staining was observed by immunofluorescence in the nuclei of undamaged cells [56]. However, the presence of CAF-1 protein is clearly not restricted to S-phase cells, and it is detectable in the nucleus of proliferating cells throughout interphase by immunocytochemistry [69] or in nuclear extracts of G1- and G2-phase cells and even in mitotic cells [20]. The p60 subunit of CAF-1 undergoes characteristic changes in its phosphorylation state at phase transitions during the cell division cycle [20]. In interphase cells, p60 is predominately phosphorylated, but a significant proportion of p60 becomes dephosphorylated upon entry into S phase [20, 70]. In mitosis, p60 becomes hyperphosphorylated, coincident with the release of CAF-1 from chromatin and the loss of nucleosome assembly activity [20]. Furthermore, a phosphorylated form of p60 is specifically recruited to chromatin in ultraviolet (UV) irradiated G2 phase cells undergoing DNA repair [70]. These observations suggest an involvement of reversible phosphorylation events on p60 when CAF-1 is recruited for nucleosome assembly during both DNA replication and repair. Differences in the relative levels of p60 phosphorylation as observed in these two situations [20, 70] may be reconciled by different template structures, multienzyme complexes providing interacting surfaces for CAF-1 or general kinase and phosphatase levels at sites of replication and repair in S- and G2-phase nuclei, respectively.

A recent analysis provided functional evidence for a requirement of reversible protein phosphorylation for nucleosome assembly by CAF-1 during DNA replication in extracts from human cells [71]. Inhibition of cyclin/CDK2 and of the reverse activity of protein phosphatase 1 (PP1) both inhibited nucleosome assembly by CAF-1 during DNA replication significantly, and supplementation with purified cyclin/Cdk2 or PP1 negated the inhibition and restored CAF-1 activity [71]. Inhibiting both activities together was additive, suggesting that these activities are required for different aspects of CAF-1 dependent nucleosome assembly (fig. 1).

The phosphorylation level of p60 changed in response to the presence of Cdk2 and PP1 inhibitors in the assembly reaction [71], suggesting that CAF-1 is a target for reversible phosphorylation during nucleosome assembly. Indeed, the p60 subunit contains several consensus sites for

CDK phosphorylation in its carboxy terminus, and it is a direct substrate for the S-phase-specific protein kinase complexes cyclin A/Cdk2 and E/CDK2 as well as the mitotic cyclin B1/CDK1 in vitro [71]. Cyclins A, E and CDK2 are nuclear proteins during S phase [72, 73]. Both cyclin A and CDK2 have also been shown to colocalise with replication foci [74], supporting a physiological role of these cyclin/Cdk complexes for phosphorylation of CAF-1 during chromatin replication in vivo. In mitosis, hyperphosphorylation of p60 coincides with dissociation of CAF-1 from metaphase chromosomes and causes inactivation of its nucleosome assembly activity [20, 71], suggesting a functional role for cyclin B1/Cdk1 in the regulation of CAF-1 during mitosis.

The catalytic subunit of protein phosphatase 1 is present in the cytosol during G1 phase but becomes nuclear during S and G2 phase [75]. Specifically, one isoform of PP1, PP1δ, is associated with chromatin [76]. This intranuclear localisation correlates with the temporal and spatial occurrence of a dephosphorylated form of p60 in vivo [20], supporting physiological roles for PP1 in dephosphorylating CAF-1 during nucleosome assembly on replicating DNA in S phase.

The reversible and opposing phosphorylation reactions mediated by cyclin/Cdk and PP1, respectively, may modulate a reversible binding and release of CAF-1 to histones, to newly synthesised DNA, to PCNA or a combination of these possibilities during nucleosome assembly. Future experiments will be required to test these models. In conclusion, these observations provide a functional link between the cell cycle machinery and the nucleosome assembly activity by CAF-1 during DNA replication. Therefore, the same protein kinases/phosphatases regulating DNA replication and S-phase progression are also involved in regulating nucleosome assembly on replicating DNA.

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Note added in proof. After submission of this manuscript, subunits of CAF-1 in the plant *Arabidopsis thaliana* were identified as *Fasciata* gene products. Mutants showed defects in postembryonic root and shoot development, possibly by disrupting specific gene expression patterns in the apical meristems [77].

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