

H4 replication-dependent diacetylation and Hat1 promote S-phase chromatin assembly in vivo

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ABSTRACT While specific posttranslational modification patterns within the H3 and H4 tail domains are associated with the S-phase, their actual functions in replication-dependent chromatin assembly have not yet been defined. Here we used incorporation of trace amounts of recombinant proteins into naturally synchronous macroplasmidia of *Physarum polycephalum* to examine the function of H3 and H4 tail domains in replication-coupled chromatin assembly. We found that the H3/H4 complex lacking the H4 tail domain was not efficiently recovered in nuclei, whereas depletion of the H3 tail domain did not impede nuclear import but chromatin assembly failed. Furthermore, our results revealed that the proper pattern of acetylation on the H4 tail domain is required for nuclear import and chromatin assembly. This is most likely due to binding of Hat1, as coimmunoprecipitation experiments showed Hat1 associated with predeposition histones in the cytoplasm and with replicating chromatin. These results suggest that the type B histone acetyltransferase assists in shuttling the H3/H4 complex from cytoplasm to the replication forks.

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INTRODUCTION

The basic subunit of eukaryotic chromatin consists of 146 base pairs of DNA wrapping around a histone octamer composed of two copies of each of the four core histones: H2A, H2B, H3, and H4 (Luger *et al.*, 1997). Assembly of chromatin during the S-phase is believed to be mechanistically coupled to concomitant DNA replication (Almouzni and Mechali, 1988; Smith and Stillman, 1989). Nucleosome assembly is a multistep process in which H3/H4 is first deposited onto DNA, followed shortly by the deposition of H2A/H2B dimers (Wolffe, 1998; Annunziato and Hansen, 2000).

Each core histone has an unstructured N-terminal tail that extends out from the nucleosome, which can be posttranslationally modified by specific enzymes involved in chromatin activities (Wolffe and Hayes, 1999). In addition, it has been suggested that histone

acetylation is critical in replication-coupled chromatin assembly (Annunziato and Hansen, 2000). Early work showed that in naturally synchronous *Physarum*, lysine 5 of H4 exhibited the greatest turnover of acetyl groups during S-phase (Pesis and Matthews, 1986). Annunziato and colleagues have shown that newly assembled histones present a conserved pattern of acetylation on H4 at lysines 5 and 12 in a variety of eukaryotes (Sobel *et al.*, 1995; Chang *et al.*, 1997; Benson *et al.*, 2006). Consistent with this idea, biochemically fractionated cellular extracts revealed that H3/H4 containing chromatin assembly factor 1 complex (CAC) exhibited, at least partly, this conserved acetylation pattern (Verreault *et al.*, 1996). Although these results suggested an important function of the histone tail domain of H3 and H4 in replication-coupled chromatin assembly, structural studies of the histone chaperone anti-silencing function 1 (ASF1) failed to be confirmatory and did not reveal any physical interaction with the amino-terminal tail domains of histones (English *et al.*, 2006). Indeed, determination of the structure of the globular domain of ASF1 bound to H3/H4 by x-ray crystallography showed that the chaperone interacts with the C-terminus of H3 and H4 (English *et al.*, 2005; English *et al.*, 2006). Although ASF1 did not exhibit cell cycle stage specificity and is implicated in both replication-dependent and replication-independent chromatin assembly, it has been shown that this factor assists chromatin assembly factor 1 (CAF-1) during assembly of newly synthesized DNA into chromatin in vitro (Tyler *et al.*, 1999; Mello *et al.*, 2002). Furthermore, analyses of chromatin assembly by CAF-1 revealed that the amino-terminal tail domains of H3 and H4 are dispensable for replication-dependent

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Abbreviations used: ASF1, anti-silencing function 1; BrdU, bromodeoxyuridine; CAC, chromatin assembly complex; CAF-1, chromatin assembly factor 1; DAPI, 4',6-diamino-2-phenylindole; GFP, green fluorescent protein; HAP, hydroxyapatite; HAT, histone acetyltransferase; HU, hydroxyurea; IP, immunoprecipitation; MNase, micrococcal nuclease; NLS, nuclear localization signal; ORC, origin recognition complex; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen.

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chromatin assembly in vitro (Shibahara *et al.*, 2000), despite the fact that CAC purified from human cell extracts contained H4 acetylated on lysines 5 and 12 (Verreault *et al.*, 1996). Thus a direct functional link between replication-dependent patterns of acetylation on histones H3 and H4 and replication-coupled chromatin assembly has not been established.

Several molecular genetic approaches have been used to address the issue of the function of histone tail domains and the role of the replication-dependent pattern of acetylation in H3 and H4. Yeast genetic experiments have revealed important roles of the amino-terminal regions in vivo (Morgan *et al.*, 1991; Lenfant *et al.*, 1996; Ling *et al.*, 1996; Blackwell *et al.*, 2007). Deletion of both H3 and H4 amino-terminal tail domains results in lethality (Ling *et al.*, 1996; Blackwell *et al.*, 2007). However, the presence of either the H3 or H4 tail domain prevents cell death, although cells suffer a variety of defects, including a slower growth rate (Ling *et al.*, 1996; Blackwell *et al.*, 2007). Intriguingly, whereas these data suggest that amino-terminal tail domains have redundant functions, substitution of four lysines of H4 subject to reversible acetylation (K5, K8, K12, and K16) for arginine or asparagine is lethal (Megee *et al.*, 1990). However, this phenotype was not observed when simultaneous substitutions at positions 5, 8, and 12 were performed (Thompson *et al.*, 1994). Nevertheless, viability of cells bearing substitutions of single lysine for arginine revealed that individual residues were not essential, although mutants were slower in completing DNA replication (Megee *et al.*, 1990). Further analyses showed that in absence of the H3 tail domain, H4 K5/K12 diacetylation is dispensable for nucleosome deposition in yeast (Ma *et al.*, 1998). Yet simultaneous mutagenesis of the first three sites of acetylation (K5, K8, and K12) led to cell lethality and deficiency in nucleosome assembly in vitro (Ma *et al.*, 1998). To ascertain the essential function of histone tail domains of H3 and H4 in chromatin assembly in vivo, Wolffe and colleagues have microinjected tagged mRNA into *Xenopus* embryos (Freeman *et al.*, 1996). However, the formation of hybrid histone complexes composed of mutant exogenous histone and endogenous histone did not allow the determination of a function of the tail domain. An additional strategy using chimeric proteins composed of peptides fused to green fluorescent protein (GFP) has been used to identify nuclear localization signals (NLS) within the core histone tail domains (Mosammamaparast *et al.*, 2002b; Blackwell *et al.*, 2007). However, the absence of the histone fold domain, which mediates histone complex formation, hindered elucidation of the actual role of the tail domains in structurally relevant histone complexes. Indeed, while the histone tail fusion proteins revealed NLS in the amino-terminal tails of H2A and H2B (Mosammamaparast *et al.*, 2002a), H2A/H2B dimers lacking both tail regions exhibit nuclear localization (Thiriet and Hayes, 2001). Therefore the function of histone tail domains and the replication-dependent pattern of acetylation within living cells remains to be determined.

As originally defined, type B histone acetyltransferases (HATs), in contrast to type A HATs, specifically acetylate free histones and are initially located in cytoplasm (Brownell and Allis, 1996; Parthun *et al.*, 1996), although nuclear localization has been shown in a number of cell types since (Verreault *et al.*, 1998; Lusser *et al.*, 1999; Ai and Parthun, 2004). To date the only member of type B HAT is the Hat1 that specifically acetylates free H4 at lysine residues 5 and 12 (Parthun, 2007). Interestingly, this specificity of Hat1 corresponds to a specific replication-dependent pattern of acetylation. A highly conserved homolog of Hat1 has been isolated from human, chicken, maize, *Xenopus laevis*, and yeast extracts (Eberharter *et al.*, 1996; Parthun *et al.*, 1996; Ahmad *et al.*, 2000). Importantly, despite its conservation throughout eukaryotic evolution, Hat1 is not essential

for viability in yeast and vertebrate cells, similar to the acetylation pattern of newly synthesized histone H4 (Ai and Parthun, 2004; Barman *et al.*, 2006). These observations may be explained, at least in part, by redundant pathways that involved acetylation of H4 by Hat1. In an effort to understand the link between histone acetylation and replication-coupled chromatin assembly, a sensitive genetic assay that monitored subtle changes in chromatin structures was carried out (Kelly *et al.*, 2000; Parthun, 2007). These experiments revealed that Hat1 might play a role in telomeric silencing that involved the catalytic activity of the enzyme and its subcellular localization. Furthermore, Hat1 has been shown to bind in yeast nuclei to Hif1p, a histone chaperone that selectively interacts with H3 and H4 (Ai and Parthun, 2004). In vitro assays identified Hif1p as a chromatin assembly factor when supplemented with a yeast cytosolic extract. These results suggested that Hat1 might be linked to chromatin assembly (Ai and Parthun, 2004; Parthun, 2007). The caveat to the hypothesis is that, although the H4 replication-dependent pattern of acetylation seems to occur throughout the genome, deletion of both Hat1 and Hif1p is not lethal and thus does not prevent replication-coupled chromatin assembly, perhaps due to the redundancy of pathways involved in chromatin replication. Therefore, despite a large panel of studies for understanding the function of the histone tail domains of H3 and H4 in replication-coupled chromatin assembly and the H4 replication-dependent pattern of acetylation, these remain elusive (Ransom *et al.*, 2010).

In the present report, we used a powerful in vivo approach that prevents deleterious effects observed in genetic experiments to examine the function of histone tail domains of H3 and H4 in the S-phase as well as the replication-dependent pattern of acetylation and the role of Hat1 in replication-coupled chromatin assembly. Spontaneous incorporation of trace amounts of tagged histone complexes into naturally synchronous macroparasitidia of *Physarum* has been shown not to perturb cell cycle progression and has allowed the analyses of primary pathways used by the cell for histone transport and deposition (Thiriet and Hayes, 2001, 2005). Our results demonstrate that the tail domains of H3 and H4 have distinct functions in replication-coupled chromatin assembly, as H3/H4 dimers lacking the H4 tail domain did not accumulate in nuclei, while the H3 amino-terminal domain was required only for histone deposition into chromatin. Detailed analyses of the replication-dependent pattern of H4 acetylation revealed that exclusive acetylation of lysine residues 5 and 12 are required for proper assembly of histones into chromatin. Furthermore, immunoprecipitation (IP) experiments provide evidence for the formation of a Hat1-containing histone complex in the cytoplasm and Hat1 coprecipitates with newly replicated chromatin. These results provide significant insights into the primary pathways by which newly synthesized H3 and H4 are supplied to the replication fork during replication-coupled chromatin assembly within living cells.

RESULTS

Exogenous FLAG-tagged H3/H4 complex is incorporated into nuclei and assembled in chromatin in a replication-dependent manner

To verify the incorporation of exogenous histones into *Physarum* and to determine their fate in the cell, we introduced H3/FH4 into half of a macroparasitidium at the beginning of the S-phase and used the other half as control. After 1 h incorporation, plasmodium fragments were fractionated and analyzed by SDS-PAGE and Western blotting (Figure 1A). The Western blot analyses revealed that, although only trace amounts of exogenous proteins are detected in the total fraction (exogenous histones were estimated to ~1:10,000

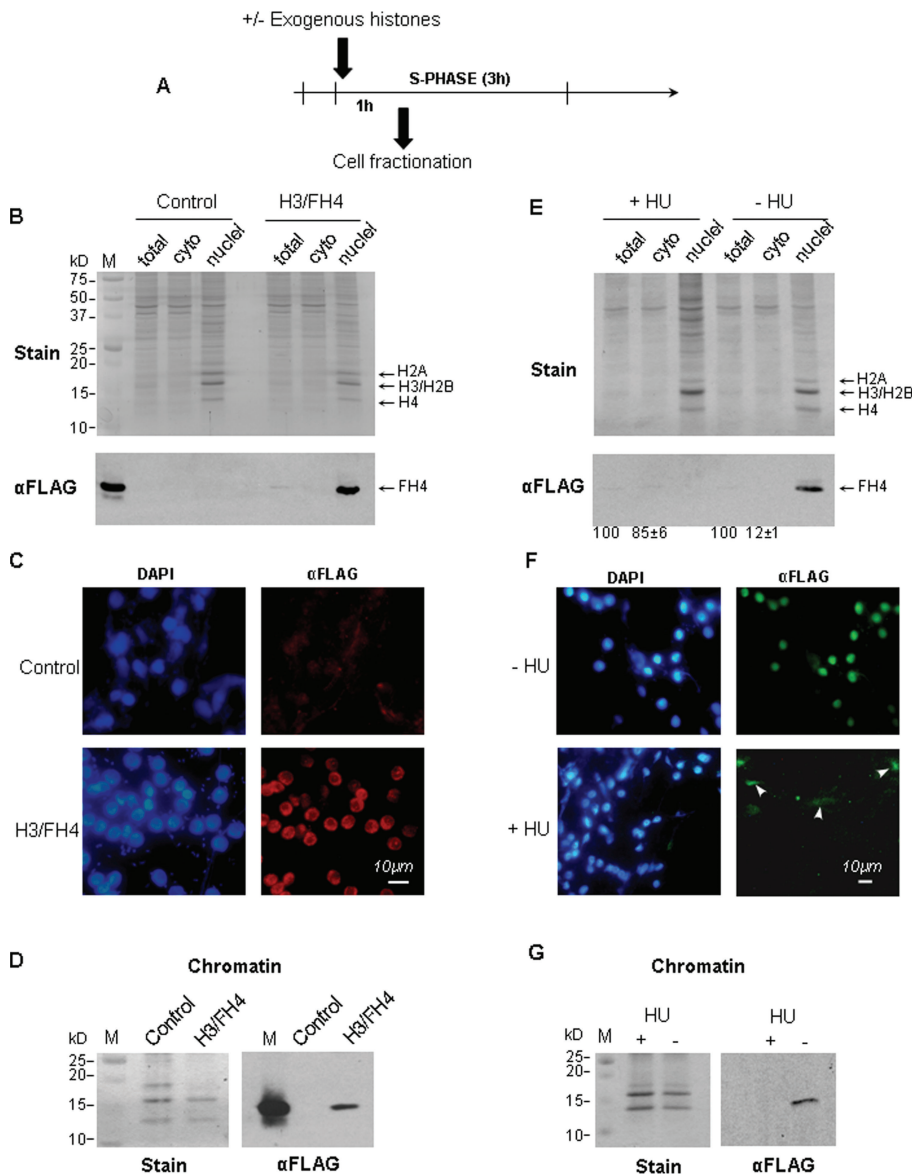


FIGURE 1: Exogenous FLAG-tagged H3/H4 histones incorporated into nuclei and assembled in chromatin in a replication-coupled manner. (A) Experimental scheme: *Macroparasitum* is cut in two halves in early S-Phase (S-phase lasts 3 h in *Physarum*). One half is treated with a solution containing purified histones, and the other half is used as control. The cell fragments are harvested after 1 h treatment and analyzed. (B) Exogenous histones H3/H4 are incorporated into nuclei. Following cell fractionation, samples (total, cytoplasm, and nuclei) without exogenous histones (control) and with exogenous histones (H3/FH4) are resolved in SDS-PAGE and analyzed by Western blotting with anti-FLAG antibody. (C) FLAG-tagged exogenous H3/H4 colocalized with DAPI-stained *Physarum* nuclei. Cell explants were squashed, fixed, and stained with anti-FLAG antibody. Slides were then observed by fluorescence microscopy. (D) Exogenous histone H3/H4 complex is assembled in chromatin. Nuclear fraction from (B) was used for preparing soluble chromatin with hydroxyapatite beads and high-salt wash. Chromatin-bound fractions are then analyzed by SDS-PAGE and Western blotting. (E) Accumulation of exogenous H3/H4 into nuclei depends upon replication activity. *Plasmodium* fragments are treated with exogenous histones in the presence and absence of hydroxyurea (HU). Cell fractions (total, cytoplasm, and nuclei) are analyzed similarly to (B). The percentages below the blot correspond to the quantification of overexposed blots (unpublished data) and represent the percentage of histone in cytoplasmic fractions relative to histone incorporated into the macroplasmidia. (F) HU treatment blocks nuclear import of exogenous histones. Fluorescence microscopy observations of cell explants treated as in (C). Control treated with exogenous H3/H4 in the absence of HU revealed a colocalization of FLAG signal with DAPI-stained nuclei. HU-treated cells concomitantly with exogenous histone incorporation showed the absence of accumulation into nuclei, but cytoplasmic aggregates are observed (arrowheads). (G) Blocking replication prevents chromatin assembly. Nuclear fractions from (E) were analyzed as in (D).

of endogenous histones), they were recovered in nuclei (Figure 1B). Microscopy observations of cell smears immunostained with anti-FLAG antibody confirmed the nuclear localization of the exogenous proteins (Figure 1C). We previously showed that nuclear localization of the exogenous proteins did not necessarily imply their assembly into chromatin (Thiriet and Hayes, 2001). We thus wanted to verify that full-length exogenous histones were assembled in chromatin. We developed an affinity technique to exclusively detect histones assembled into chromatin using hydroxyapatite (HAP) beads. As a control, FLAG-tagged histones added directly to soluble chromatin *in vitro* were not detected in the high-salt wash, while native, properly assembled histones were detected (Supplemental Figure S1). Thus chromatin was prepared from nuclear fractions by micrococcal digestion, and chromatin bound to HAP after high-salt wash was analyzed by SDS-PAGE and Western blotting (Figure 1D). The blots stained with anti-FLAG antibody showed that exogenous FLAG-tagged histones were assembled into chromatin, in a manner identical to endogenous proteins.

In vivo, the majority of chromatin assembly taking place during S-phase is most likely coupled to DNA replication. To verify whether nuclear transport and assembly of exogenous histones depended upon replication activity, we introduced exogenous FLAG-tagged H3/H4 into macroplasmidia concomitantly with hydroxyurea (HU) treatment. We have previously established that this drug strongly inhibits DNA replication in *Physarum* (Thiriet and Hayes, 2001). To ensure that the incorporation was efficient, we used control cell fragments treated with H3/FH4 (Figure 1E). Immunocytochemical results showed a noticeable difference between cell fragments treated and untreated with HU. While Western blot analyses of total cell extracts showed that incorporation of exogenous histones was similar in both HU-treated and untreated cells, HU treatment of cells resulted in cytoplasmic localization of exogenous histones, in contrast to the nuclear localization of the untreated control as revealed by the quantification of overexposed blots (Figure 1E, bottom, and unpublished data). Examination of *Physarum* smears stained with anti-FLAG antibody confirmed the apparent hindrance in nuclear import of exogenous H3/H4 by HU and the presence of cytoplasmic aggregates (Figure 1F, arrows). As expected from the cellular localization results, analyses revealed the absence of exogenous histones in chromatin from cells treated with HU

(Figure 1G). We conclude that exogenous H3/H4 complex was efficiently incorporated into *Physarum* cells. Furthermore, the results indicate that nuclear import and assembly into chromatin of exogenous H3/H4 are coupled to DNA replication.

Histone tail domains of H3/H4 exhibit distinct functions in replication-coupled chromatin assembly

Because the H3/H4 tail domains were suggested to play a role in both chromatin assembly and nuclear import (Morgan *et al.*, 1991; Ling *et al.*, 1996; Blackwell *et al.*, 2007), we next investigated the requirement for these domains in replication-coupled chromatin assembly *in vivo*. We first generated H3/H4 lacking specific tail domains and examined by standard salt-dialysis reconstitutions that the different purified complexes can efficiently form nucleosomes (Figure 2A). The incorporation of the mutant complexes into *Physarum* after 1 h in S-phase was then analyzed by Western blotting (Figure 2B). To enable comparison of the results from different mutants for nuclear import and chromatin assembly, macroplasmidia were cut in two halves. One fragment was treated with H3/H4 lacking one or both tail domains, and the other fragment was treated with identical amounts of full-length H3/H4 as control. Determination of the subcellular localization by Western blot analyses revealed that the H3 amino tail is not required for the nuclear import of exogenous histones. Actually, we found that H3/H4 complex lacking the H3 amino tail was incorporated into nuclei with a greater efficiency than the full-length H3/H4 control (Figure 2B, H3n/FH4 panel). In contrast, incorporation of complexes lacking the H4 tail domain led to a much reduced accumulation in *Physarum* nuclei (Figure 2B, H3/FH4n and H3n/FH4n panels), perhaps due to increased degradation, reduced efficiency of transport, or both.

To determine whether H3/H4 lacking one or both tail domains actually assembled into chromatin, we prepared soluble chromatin by micrococcal nuclease (MNase) and purified nucleoprotein complexes with HAP followed by analyses of the protein composition by SDS-PAGE and Western blot (Figure 2C). Clearly, exogenous H3/H4 was detected in chromatin from control cells treated with full-length H3/H4 while no exogenous histones were detected in chromatin from cells treated with any tailless H3/H4 complexes. Surprisingly, given the efficiency of import of the H3n/FH4 dimer in nuclei, the absence of this complex in chromatin revealed that the H3 tail domain is required for chromatin assembly.

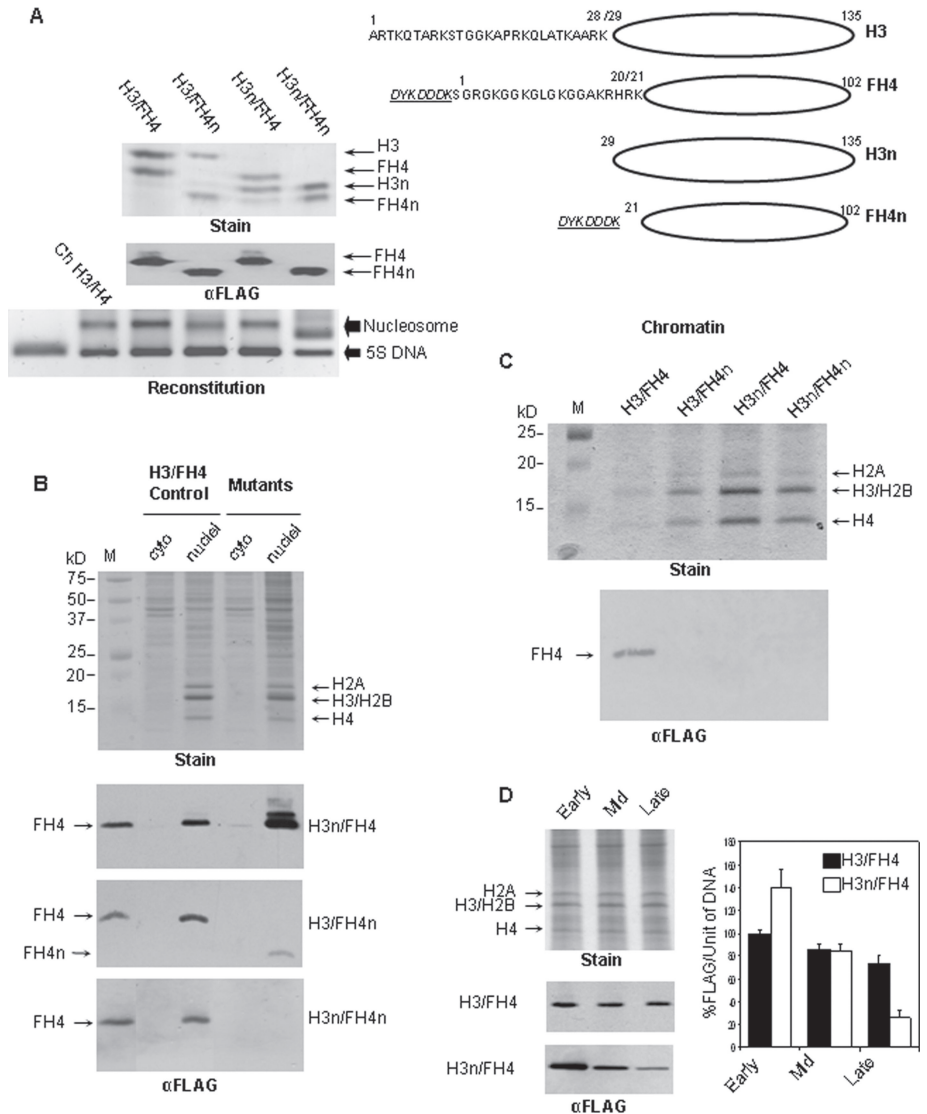


FIGURE 2: Tail domain regions of H3 and H4 exhibit distinct functions in cellular localization and chromatin assembly requirement. (A) Histone mutants of H3 and H4 were used to determine the function of the histone tail domains during S-phase. Schemes represent the histones that are expressed in *Escherichia coli*. The underlined and italicized sequences at the amino termini of FH4 and FH4n correspond to FLAG sequences that are used throughout our experiments. The histone complexes have been assessed by SDS-PAGE and Western blotting, and their ability to form nucleosome *in vitro* has been evaluated relative to purified chicken erythrocyte H3/H4 (Ch H3/H4). (B) Cellular localization of exogenous H3/H4 complexes. Exogenous histone incorporations and cell fractionation are carried out similarly to Figure 1. Each mutant complex is incorporated in half macroplasmidium, and the other half is treated with full-length histones and used as control. Shown on the top is a representative SDS-PAGE of cell fractionations. The bottom panels correspond to blots obtained with the different mutant complexes, H3n/FH4, H3/FH4n, and H3n/FH4n, respectively. (C) Chromatin analyses revealed the requirement of H3 and H4 tail domains in replication-coupled chromatin assembly. Chromatin is prepared from different nuclear fractions and analyzed by SDS-PAGE and Western blotting. Note that the absence of immunodetection with H3n/FH4 indicates that the exogenous complex is not disrupted on incorporation and does not exchange with *Physarum* histones. (D) Depletion of the H3 amino-tail domain affects stability of exogenous complexes in nuclei. H3/FH4 and H3n/FH4 are incorporated into *Physarum* at the beginning of S-phase, and plasmodium fragments are harvested after 1 h (early), 2 h (mid), and 3 h (late), respectively. Nuclei are isolated and analyzed by SDS-PAGE and Western blotting, wherein a constant amount of DNA is loaded. The graph represents the quantitation of three different experiments in which 100% was arbitrarily affected to full-length exogenous histones in early S-phase (mean ± SEM).

Because the H3-tailless-containing complex exhibited a better efficiency of accumulation into nuclei than the full-length control complex, but an inefficient chromatin assembly, we wanted to

examine the stability of the complexes once imported into nuclei (Figure 2D). Incorporation experiments were carried out with exogenous H3n/FH4 into half a macroplasmidium at the beginning of the S-phase, and the other half was treated with an identical amount H3/FH4 and used as control. Unlike previous experiments, cell fragments were harvested after 1 h S-phase (early), 2 h S-phase (mid), and 3 h S-phase (late), respectively. Nuclei were then isolated and analyzed by SDS-PAGE and Western blot. We found that although H3-tailless-containing complex is efficiently incorporated into nuclei, the H3n/H4 complex is almost completely degraded after 3 h S-phase (>80%), whereas the full-length complex remained relatively constant. These results show that a lack of the H3 tail domain inhibits assembly into chromatin and leads to degradation of histone complexes.

Histone H4 acetylation at positions 5 and 12 are required for chromatin assembly

It has been well documented that newly synthesized H3/H4 histones exhibit an evolutionarily conserved pattern of acetylation, especially histone H4, which is acetylated on lysines 5 and 12 (Sobel *et al.*, 1995; Annunziato and Hansen, 2000). Interestingly, we found that lacking the H4 tail domain of H3/H4 impedes the nuclear recovery of the complex. We assumed that the absence of accumulation of the complex within the nucleus might be caused by the loss of replication-dependent acetylable residues within the H3/H4n complex. To verify this hypothesis, we generated histone mutants by substituting K5 and K12 for glutamine (FH4-Q5/12) and for arginine (FH4-R5/12), which are commonly used for mimicking acetylated lysine and unacetylated lysine, respectively (Figure 3A) (Megee *et al.*, 1990; Blackwell *et al.*, 2007; Wang and Hayes, 2008). Histone mutant complexes were then purified and incorporated into *Physarum* at the beginning of S-phase for 1 h (Figure 3B). Importantly, incorporation analyses revealed that mimicking replication-dependent acetylation at positions 5 and 12 of H4 (FH4-Q5/12) improved nuclear import of H3/H4 complex compared with the wild type. In contrast, preventing acetylation of H4 at lysines 5 and 12 (FH4-R5/12) inhibited nuclear import of the histone complex. Furthermore, the failure to recover the exogenous FH4-R5/12 by Western blot following incorporation and by anti-FLAG IP of the cytoplasmic and nuclear soluble fractions suggested that the FH4-R5/12-containing complex was degraded (Supplemental Figure S2). Chromatin assembly was then examined to determine whether acetylation at positions 5 and 12 of H4 affects histone deposition into chromatin (Figure 3C). The results showed that assembly of histone mutants reflects the efficiency of nuclear import of these histone complexes. These results strongly suggested

that acetylation at positions 5 and 12 facilitates nuclear import of histones and chromatin assembly *in vivo*.

Hat1 binds H3/H4 complex in cytoplasm and localizes in nuclei

The results discussed previously indicate that a specific replication pattern of acetylation is involved in nuclear import and the proper chromatin assembly of the H3/H4 complex. Given that in the wide variety of organisms from yeast to mammals as well as in *Physarum* (Lopez-Rodas *et al.*, 1992; Lusser *et al.*, 1997) the only histone acetyltransferase responsible for the acetylation of lysines 5 and 12 of non-nucleosomal H4 is Hat1, we presumed that Hat1 might have a function in the nuclear import of newly synthesized H3/H4 and perhaps is involved in their assembly in chromatin. To further investigate the role of Hat1 in H3/H4 replication-coupled nuclear import and chromatin assembly, we incorporated exogenous H3/FH4 histone complex for a short period in S-phase (10 min) and carried out pull-down

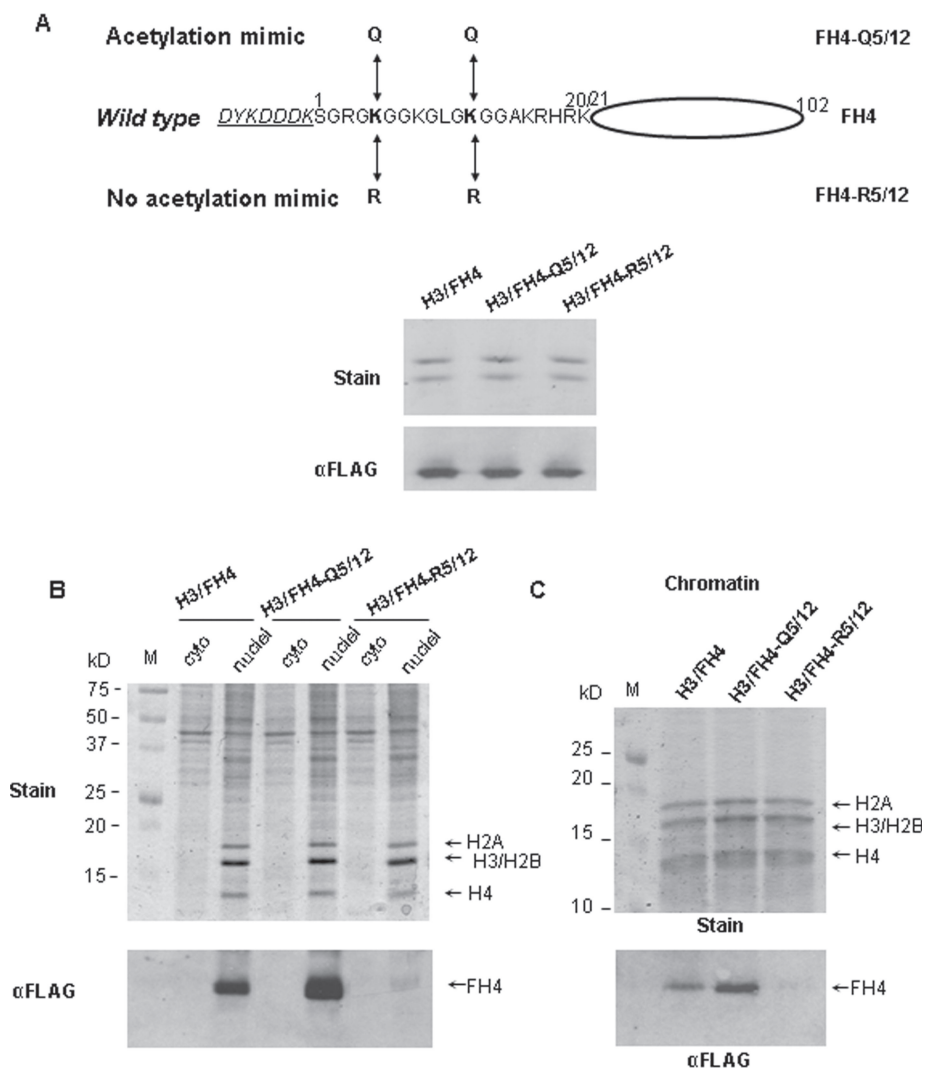


FIGURE 3. Histone H4 acetylation is required for nuclear import and facilitates chromatin assembly. (A) Mutations of lysine residues 5 and 12 of H4. Scheme represents the substitutions of lysines 5 and 12 to glutamine (Q) to mimic acetylation and to arginine (R) to prevent acetylation. (B) Cellular localization of the different histone complexes. Exogenous histones were incorporated for 1 h in early S-phase, similarly to Figure 1, and cells were fractionated before electrophoretic analyses of the fractions. (C) Chromatin assembly analyses of exogenous mutant complexes. Chromatin is prepared from nuclear fractions obtained after exogenous histone complex treatment, H3/FH4, H3/FH4-Q5/12, and H3/FH4-R5/12, respectively.

experiments to examine the association of Hat1 with histone complexes (Figure 4A). Cell fractionation analyses revealed that exogenous histones are found in nuclei even after only 10 min of incorporation (Figure 4B). We then examined the localization of Hat1 in cytoplasmic and nuclear fractions by Western blotting (Figure 4C). We found that in both control cells and H3/FH4-treated cells, Hat1 antibodies recognized a peptide of ~50 kDa corresponding to Pp Hat1, which was localized almost exclusively in nuclei during S-phase. We then determined whether Hat1 was associated with free H3/FH4. To carry out these experiments, we prepared soluble extracts from cytoplasmic fractions by removing insoluble organelles and membranes and from nuclear fractions by pelleting chromatin with MgCl₂. Cytoplasmic and nuclear soluble fractions were incubated with anti-FLAG antibody, and immunoprecipitated proteins were analyzed by Western blotting (Figure 4D). We found that soluble H3/FH4 histones were recovered only in the cytoplasmic and not the nuclear fractions, indicating that even after a short period of incorporation of 10 min in S-phase, exogenous histones are rapidly assembled into chromatin following nuclear import. We then examined whether Hat1 coimmunoprecipitated with exogenous histones. Anti-Hat1 antibodies revealed a specific band of ~50 kDa that was not observed in the control fractions and demonstrated the presence of Hat1-containing H3/FH4 complexes in the cytoplasmic soluble fraction, consistent with a recent analysis of cytoplasmic H3/H4 chaperone complex mammalian cell (Jasencakova *et al.*, 2010). Using acetylation-specific antisera, we found that immunoprecipitated histone H4 in the cytosolic fraction was acetylated at position 12, consistent with the presence of Hat1 as it is the only HAT able to perform H4 acetylation in cytoplasm (Brownell and Allis, 1996; Parthun, 2007). However, we failed to detect acetylation of H4 at position 5 or 8 (unpublished data). Interestingly, the subcellular localization difference of exogenous histones and Hat1 between cell fractionation analyses (Figure 4, B and C) and IP experiments (Figure 4D) reflects the direct detection limitation within cytoplasmic fractions as we estimate that our IPs are ~2000-fold more concentrated.

To confirm the role of Hat1 in the nuclear import of H3/H4, we examined the efficiency of the incorporation of exogenous histones that do not allow the formation of the Hat1/histone complex. It has been proposed that Hat1 binding to H4 is facilitated by acidic patches on the surface of the

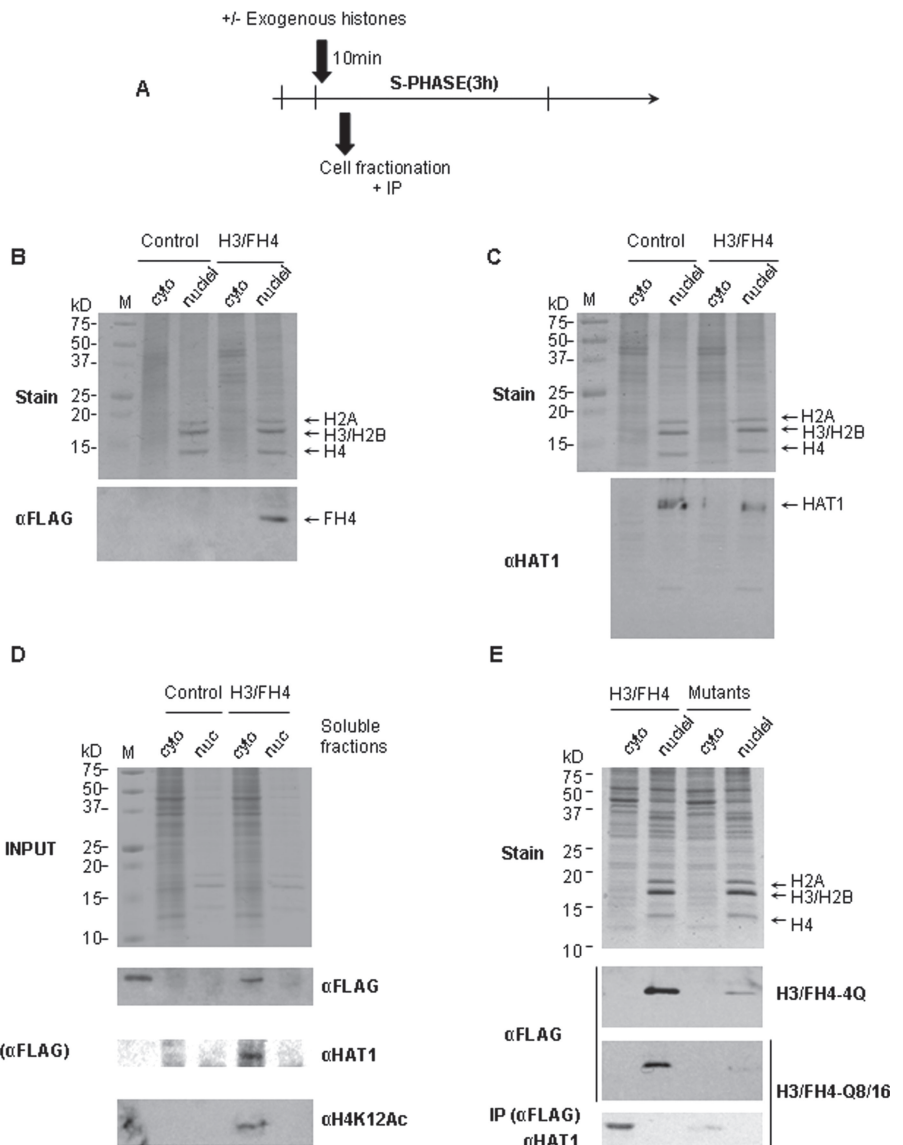


FIGURE 4: Hat1 binding to H3/H4 in cytoplasm is required for the delocalization of the tertiary complex (Hat1-H3/H4) into nuclei. (A) Experimental scheme of the incorporation of exogenous histones. H3/FH4 complex is incorporated in *Physarum* plasmodium in early S-phase for a short period of 10 min. Cells are then harvested and fractionated before carrying out immunoprecipitation (IP) experiments. Plasmodium fragment untreated with exogenous histones was used as control. (B) Cellular localization of exogenous H3/FH4. Following cell fractionations, cytoplasmic (cyto) and nuclear fractions (nuclei), respectively, were analyzed by SDS-PAGE and Western blotting. Note that even after the 10-min treatment with exogenous histones, H3/FH4 complex is recovered into nuclear fraction. (C) Cellular localization of HAT1. Cytoplasmic (cyto) and nuclear (nuclei) fractions from control and H3/FH4-treated cells are analyzed by SDS-PAGE and Western blotting. In control and H3/FH4-treated fragments, anti-HAT1 antibodies recognize a band of ~50 kDa corresponding to the molecular mass of HAT1. (D) IP of FLAG-containing complexes. Soluble fractions from cytoplasmic and nuclear fractions are prepared from control and exogenous histone-treated cells (Input) and used for immunoprecipitating FLAG-containing complexes. Immunoprecipitated proteins are analyzed by Western blotting using specific antibodies, anti-FLAG (α FLAG), anti-HAT1 (α HAT1), and anti-H4K12Acetyl (α H4K12Ac), respectively. The input corresponds to 1:2000 of the IP. Note that analyses with anti-H4K8Acetyl and anti-H4K5Acetyl did not reveal H4 acetylation (unpublished data). (E) Preventing Hat1 binding to H4 reduces the accumulation of H3/H4 in nuclei. The complexes H3/FH4 (control), H3/FH4-4Q (K5, K8, K12, and K16 substituted to Q), and H3/FH4-Q8/16 (K8 and K16 substituted to Q) were incorporated in *Physarum* cells for 1 h in early S-phase. Following cell fractionations, the cytoplasmic (cyto) and the nuclear (nuclei) fractions were resolved in SDS-PAGE and analyzed by Western blotting. IP of FLAG-containing complexes in soluble cytoplasmic and soluble nuclear fractions was carried out as in (D) on cells treated with H3/FH4 and H3/FH4-Q8/16, and the presence of Hat1 was assessed by Western blotting.

enzyme that interact with lysines 8 and 16 (Makowski *et al.*, 2001; Benson *et al.*, 2007). Therefore we generated H4 mutants in which lysines 8 and 16 were substituted by glutamine to neutralize the positive charges, FH4-4Q presented the neutralization of lysines 8 and 16 and mimicked replication-dependent diacetylation, and FH4-Q8/16 prevented stable binding of Hat1. Histone complexes were incorporated for 1 h in early S-phase, and cells were fractionated and analyzed by SDS-PAGE and Western blotting (Figure 4E). The results showed that in contrast to the control, the histone complexes bearing the mutations on positions 8 and 16 that neutralized lysine charge were not efficiently transported into nuclei. Importantly, the substitution of the four lysines 5, 8, 12, and 16, which prevented Hat1 binding and presented the replication-dependent diacetylation mimic, exhibited an inhibition of the nuclear accumulation of the exogenous protein similar to the complex that prevents only the binding of Hat1, suggesting that acetylation is not sufficient for nuclear import of the H3/H4 complex. To verify that substitution of K8 and K16 to Q affected the Hat1 binding to the H3/H4 histone complex, we carried out coimmunoprecipitation (co-IP) experiments using anti-FLAG antibody (Figure 4E, bottom). Western blot analyses of the immunoprecipitated complexes revealed the presence of Hat1 only in H3/FH4-containing complex, and not in H3/FH4-Q8/16-containing complex.

Altogether, our results demonstrate that exogenous histones bind Hat1 in the cytoplasm and are acetylated at least at position 12 of H4. Furthermore, the determination of the localization of Hat1 in S-phase strongly suggests that histone/Hat1 complex is transferred from cytoplasm to nuclei. Importantly, our data showed that the Hat1-containing histone complex in nuclei is rapidly associated with the chromatin fraction as the IP experiments failed to detect free exogenous histones in nuclear fraction, although we could not conclude from these experiments that Hat1 is associated with chromatin.

Hat1 is in the vicinity of replicating chromatin

To verify whether Hat1 was only involved in nuclear import of H3/H4 or was also critical for supplying newly synthesized histones to assembly factors at replication forks, we developed a novel approach for preparing proteins in the vicinity of the replication forks. Based on the principle that factors involved in replication might be immunoprecipitated with newly synthesized DNA, we carried out short bromodeoxyuridine (BrdU) pulses of *Physarum* cells at the beginning of S-phase. Nuclei were then prepared and fixed with formaldehyde before shearing chromatin by sonication, similarly to chromatin immunoprecipitation experiments (Figure 5A). Soluble chromatin was irradiated with UV to induce DNA breaks and to make more accessible BrdU to specific antibody. Recently, Dejardin and Kingston (2009) have reported a related approach for examining the protein composition in telomeric chromatin using specific sequences

for IP, although we focused not on specific sequences but on newly replicated DNA. To ensure that the IPs were specific for replicating chromatin, we carried out experiments of IP in the presence of an excess of competitor BrdU-labeled bacterial DNA. Clearly, the results revealed that immunoprecipitated peptides were not recovered in the presence of competitor BrdU-containing DNA, demonstrating that visualized peptides in SDS-PAGE were associated with BrdU-labeled chromatin (Figure 5B). Then we wanted to examine whether this approach allowed the recovery of known marks of replication. Western blot analyses of immunoprecipitated replicating chromatin revealed, as expected, the presence of a ~30-kDa band with anti-proliferating cell nuclear antigen (PCNA) antibody (unpublished data). To determine whether Hat1 was associated with BrdU-containing chromatin, we repeated the IP experiments and analyzed by Western blot with anti-Hat1 antibodies (Figure 5C). A band of the expected molecular mass and corresponding to Hat1 was visualized in BrdU chromatin IP, which was not detected in control IP untreated with the thymidine analogue. However, the appearance was fuzzy, compared with Hat1 signal from nuclear fractions, most likely due to the chemical and heat treatments. Similar IP experiments carried out with anti-PCNA antibody showed that Hat1 coimmunoprecipitated with this replication factor (Figure 5C). These results showed that Hat1 can localize in the vicinity of replication sites during S-phase,

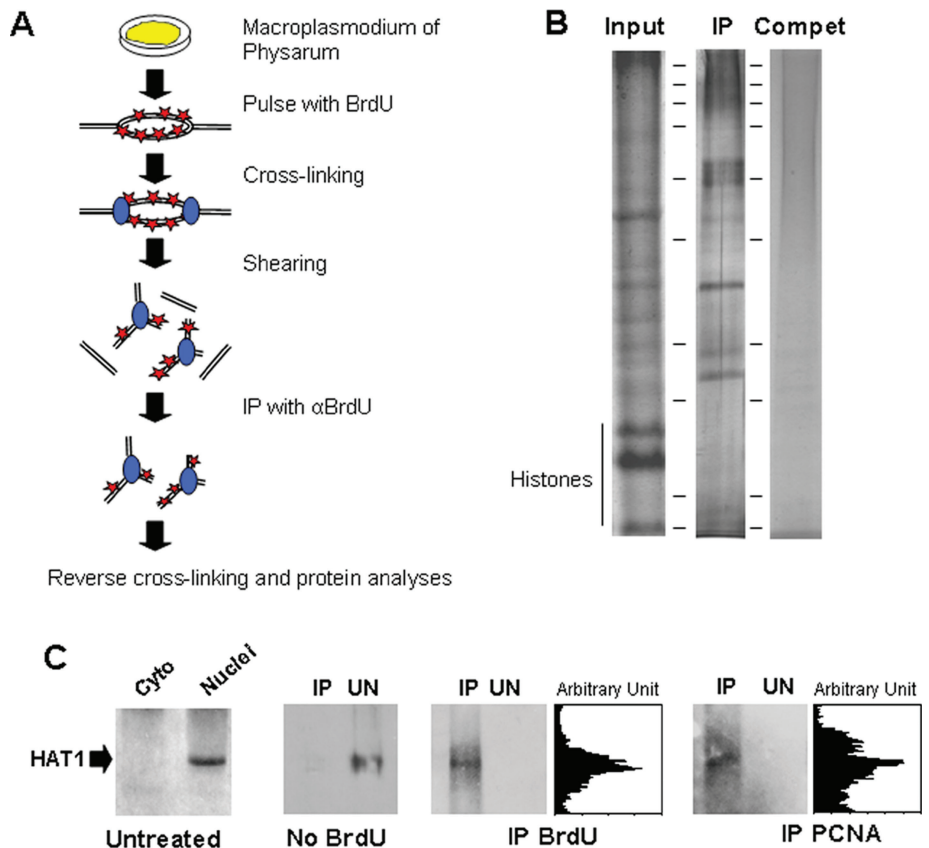


FIGURE 5: Purification and analysis of the replication complex. (A) Experimental scheme of the procedure of purification of replication complex. (B) Silver-stained SDS-PAGE of input (Input), immunoprecipitated material (IP), and immunoprecipitated material in the presence of competitor bacterial DNA (Compet), respectively. The molecular mass markers are 250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kDa, respectively. (C) Analysis of HAT1 in replication complex. Antibodies to HAT1 are used to reveal Western blot of untreated cell fractions (Untreated), bound (IP) and unbound (UN) fractions from chromatin untreated with BrdU (No BrdU), BrdU immunoprecipitated chromatin (IP BrdU), and PCNA immunoprecipitated chromatin in conditions identical to IP BrdU (IP PCNA), respectively. Densitometric profiles of immunostained HAT1 band are shown.

possibly for delivering newly synthesized histones to chromatin assembly factors.

DISCUSSION

We examined the function of H3/H4 tail domains in replication-coupled chromatin assembly using the naturally synchronous model system *Physarum polycephalum*. Previously, we and others have shown that this organism has the unique ability to internalize exogenous protein without noticeable degradation (Bradbury *et al.*, 1973; Thiriet and Hayes, 2001, 2005). We used this characteristic to incorporate trace amounts of exogenous histones and determine their fate in living cells at specific cell cycle stages (Thiriet and Hayes, 2001). Importantly, this approach prevents deleterious cellular effects of histone tail mutations and histone overexpressions as reported with yeast molecular genetic experiments (Lenfant *et al.*, 1996; Ling *et al.*, 1996; Gunjan and Verreault, 2003). Moreover, the internalization of trace amounts of histone complex displays the primary pathways utilized by the cell and does not highlight other secondary, redundant pathways that may contribute to survival.

Previously, we examined the function of H2A/H2B tail domains in replication-coupled chromatin assembly (Thiriet and Hayes, 2001). We found that similarly to yeast, the *Physarum* model exhibited redundant functions of H2A/H2B tail domains during S-phase (Schuster *et al.*, 1986; Thiriet and Hayes, 2001). Clearly, the results presented in the current study revealed that the histone complexes H2A/H2B and H3/H4 are differently regulated. Indeed, while we found that even in the absence of replication activity H2A/H2B dimers are transported into nuclei, H3/H4 in similar conditions reveal an inhibition of nuclear import. The inhibition of exogenous H3/H4 nuclear import concomitant with replication block indicates that histone levels in cell nuclei are not regulated only at the transcriptional level and suggests that, in *Physarum*, replication activity coordinates the import of H3/H4 in nuclei, possibly via a mechanism similar to the yeast-specific factor Rad53 (Gunjan and Verreault, 2003), and perhaps also involves other factors shuttling between cytoplasmic and nuclear compartments during S-phase.

In addition to the tight regulation of the level in nuclei of H3/H4, which contrasted with that observed in H2A/H2B dimer, our results highlight the function of H3 and H4 tail domains in replication-coupled chromatin assembly. Although yeast genetic experiments have examined the effects of tail domain deletions, lethality induced by the deletions unambiguously showed essential functions for the tails, but the actual reason for the phenotype was not clarified (Megee *et al.*, 1990; Megee *et al.*, 1995; Ling *et al.*, 1996; Ma *et al.*, 1998). To improve the knowledge of the requirement of histone tail domains in the cellular process, Wolffe and colleagues microinjected tagged histone mRNA into *Xenopus* embryos (Freeman *et al.*, 1996). However, because histone complexes were composed of exogenous and endogenous histone hybrids, the actual role the histone tail domain in chromatin assembly remained inconclusive. To overcome the redundancy of the histone tail function in histone complex, analyses of the nuclear import of chimeric proteins resulting from the fusion of a histone tail domain with GFP protein were carried out (Mosammamarast *et al.*, 2001; Mosammamarast *et al.*, 2002b; Blackwell *et al.*, 2007). Indeed, by this means, nuclear import of only the histone tail domain was examined, which allowed the determination of sequences that might be involved in nuclear import within the histone tail domain. Nonetheless, the absence of the histone fold domain did not reflect the tail requirement for histone complex in nuclear import, as shown by the identical efficiency of nuclear import of the H2A/H2B dimer in the presence and absence of the histone tail domains (Thiriet and Hayes, 2001).

In contrast, our experiments of the incorporation of trace amounts of structurally relevant mutated exogenous histone complexes did not perturb the cell cycle progression and did not generate hybrid complexes, as demonstrated by the absence of chromatin assembly of H3n/FH4 (Figure 2C) (Thiriet and Hayes, 2001; Thiriet and Hayes, 2005). Therefore this procedure exhibits the function of histone tail domains in the primary pathway that cells utilized for replication-coupled chromatin assembly. Clearly, our results revealed that H3 and H4 tail domains exhibited distinct functions (Figure 2). Indeed, incorporation of complex deleted of the H4 tail domain exhibited an impediment to the recovery within nuclear fractions, suggesting that the presence of H4 tail prevents either the degradation or nuclear import of the complex. In contrast, the absence of H3 tail domain did not affect nuclear import. Nonetheless, we showed that both tails of H3 and H4 are required for chromatin assembly. Our results are consistent with the H4 tail essential requirement in yeast following cell death and growth defect observations (Morgan *et al.*, 1991; Lenfant *et al.*, 1996; Ling *et al.*, 1996; Blackwell *et al.*, 2007).

Histone acetylation has been reported as a conserved feature through evolution in replication-coupled chromatin assembly (Sobel *et al.*, 1995). Although these marks are found in all four core histones in nascent chromatin, H4 presented the most conserved pattern, with acetylations on lysine residues 5 and 12 (Sobel *et al.*, 1995; Annunziato and Hansen, 2000). Furthermore, isolation of H3/H4-containing chromatin assembly complex revealed that H4 is predominantly acetylated on lysines 5 and 12 (Verreault *et al.*, 1996). Our results showed that the replication-associated acetylation of H4 is required for efficient chromatin assembly. Indeed, recapitulating the acetylation–replication pattern was consistent with a function of acetylation in replication-coupled chromatin assembly, while mutations of lysine for preventing acetylation of lysines 5 and 12 exhibited nuclear import deficiency (Figure 3). Interestingly, defects in nuclear accumulation of H4 bearing four alanine substitutions at positions 5, 8, 12, and 16 have been reported in a yeast heterozygous strain expressing both H4-wt and H4-4A. However, the exclusion of the mutant H4-4A was mostly abrogated in the absence of Cac1p (the largest subunit of CAF-1) (Glowczewski *et al.*, 2004). Similarly, our results showed that replication-dependent diacetylation is not sufficient for nuclear import, as binding of Hat1 is critical for the transport of H3/H4 into the nucleus. Indeed, preventing the formation of Hat1–H3/H4 complex by the neutralization of lysine at positions 8 and 16 (Makowski *et al.*, 2001; Benson *et al.*, 2007) interfered with the accumulation of the histones into the nuclear fraction (Figure 4).

In addition to the determination of the relevant function of histone tail domains in replication-chromatin assembly and the requirement of the replication-dependent pattern of acetylation of H4, we also determined a function of Hat1 during replication. Similarly to the analyses of the function of the H3/H4 histone tails, the contribution of Hat1 in replication-coupled chromatin assembly *in vivo* has not been elucidated. Indeed, genetic deletion of Hat1 in yeast and vertebrate cell culture did not display obvious phenotypes (Kelly *et al.*, 2000; Qin and Parthun, 2002), although cells deficient in Hat1 exhibited heightened sensitivity to DNA damaging agents (Benson *et al.*, 2007). Analyses of subtle changes in chromatin structure monitored by a genetic assay of telomeric silencing showed that Hat1 deletion combined with mutations of the histone H3 amino-terminal tail exhibited a significant silencing defect (Kelly *et al.*, 2000; Ai and Parthun, 2004). Interestingly, mutants lacking the yeast CAF-1 subunits displayed similar defects in telomeric silencing (Game and Kaufman, 1999). These results suggested that Hat1 and CAF-1 may operate in a common pathway. Furthermore, yeast Hat1 has been

copurified from nuclear extracts with Hif1 that exhibited chromatin assembly activity *in vitro* when complemented with cytosolic extracts (Ai and Parthun, 2004). However, the function of Hif1 in replication-coupled chromatin assembly *in vivo* remains elusive. Evidence of a function of Hat1 in replication has been provided by tandem affinity purification; thereby Hat1 was shown to associate with the subunits of the origin recognition complex (ORC) (Suter *et al.*, 2007). However, despite the biochemical and genetic evidences of the relevance of the Hat1–ORC complex, the requirement of Hat1 at the replication fork was not established.

In contrast, our results provide new insights into the function and the mechanism of Hat1 in replication-coupled chromatin assembly that are consistent with the working model proposed by Parthun (2007) of a function of Hat1 in replication. Indeed, we show that Hat1 associated with H3/H4 in cytoplasm and, as Hat1 is mostly associated with replication forks during S-phase, the results strongly suggest a rapid delocalization in nuclei at replicating chromatin. Most likely, the association between Hat1 and H3/H4 complex is performed via the H4 tail domain in agreement with the reported specificity of the enzyme. Thus our results support the hypothesis that Hat1 is involved in delivering H3/H4 newly synthesized histones to the replication site (see the model proposed in Figure 6). Interestingly, p46 binds to and enhances Hat1 activity and simultaneously binds to helix 1 of H4, positioning therefore the holoenzyme at the amino side of the H3/H4 complex (Verreault *et al.*, 1998). On the other hand, the positioning of chromatin assembly factors, like CAF-1 or ASF-1, has been reported to bind the opposite carboxy side of H3/H4 (English *et al.*, 2006). Among the three subunits of CAF-1, p48 exhibits a high homology with p46 from HAT1 holoenzyme (90% at the amino acid level) (Murzina *et al.*, 2008). Supposedly, p46 and p48 might play important functions in the histone transfer from HAT1 to CAF-1, as the nature of the interaction of H4

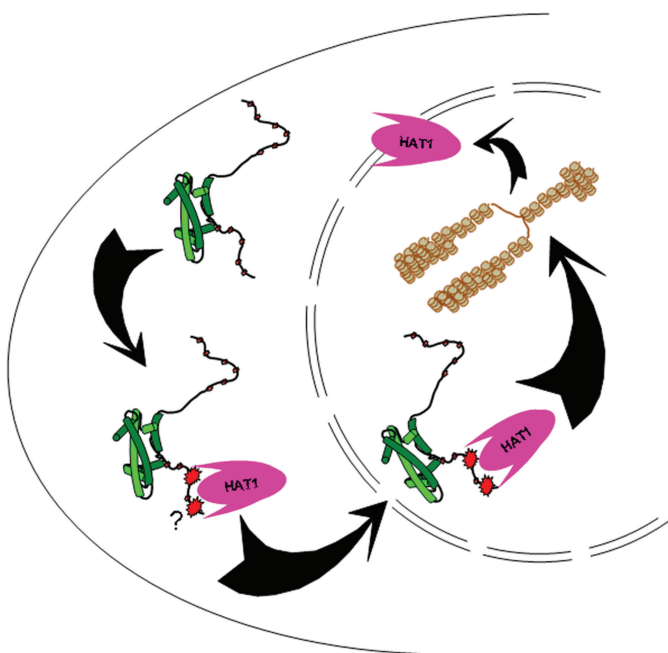


FIGURE 6: Model of the role of HAT1 in the supply of H3/H4 dimer at the replication fork. During S-phase, the newly synthesized H3/H4 dimer associates with HAT1 in the cytoplasm, where H4 is acetylated at least at lysine 12. Concomitantly to DNA replication, the complex composed of HAT1 and H3/H4 is transferred into nuclei and localizes in the vicinity of replicating chromatin for supplying H3/H4 to chromatin assembly factors.

with p48 is different from that of Hat1-p46 (Saade *et al.*, 2009). We are currently investigating the hypothesis that the replication-dependent pattern of acetylation of H4 at position 12 and then 5 by Hat1 might favor the histone transfer from HAT1-containing complex to chromatin assembly factors. Importantly, recent purification of histone-associated nuclear complexes revealed the presence of Hat1 in canonical H3 complex as well as in H3.3 complex (Drane *et al.*, 2010), suggesting that the model that involves Hat1 in nuclear import of H3/H4 complexes might not be restricted to the canonical histones.

MATERIALS AND METHODS

Cultures of *Physarum*

Microplasmidia of *P. polycephalum*, strain TU291, were maintained in liquid culture. Macroplasmidia were prepared as described (Thiriet and Hayes, 1999), and cell cycle progression was determined by observation of the second mitosis following coalescence by phase contrast microscopy (Thiriet and Hayes, 1999).

Preparation of exogenous H3/H4 complexes and incorporation in *Physarum*

Histone genes of full-length and tailless H3 and H4 were generated by PCR and cloned into pET3a vector. FLAG-tagged histone H4 and H4n were obtained by addition of the FLAG coding sequence to the histone gene as described (Thiriet, 2004). Histones bearing lysine substitutions were obtained by PCR using standard mutagenesis procedures. Histones were expressed in bacterial cells and purified in complexes of H3/H4 as previously reported (Supplemental Table S1) (Thiriet, 2004). Incorporation experiments of exogenous histone H3/H4 complexes were carried out as described (Thiriet, 2004; Thiriet and Hayes, 2005). Briefly, macroplasmidia were cut into halves and 200 μ l solution containing 10 mM phosphate buffer at pH 7.4, 5 mM ATP, and \sim 50 μ g/ml histone complexes were spread onto the upper cellular surface at the desired cell cycle stage. Macroplasmidia were then placed at 26°C in the dark for the time indicated in the figure legends before they were harvested for further analyses (Thiriet and Hayes, 1999). For experiments using replication inhibition, 80 mM HU was added to the media 10 min before the histone treatments to ensure that replication was inhibited (Thiriet and Hayes, 2001).

Cell fractionation and preparation of chromatin

Total cell extracts were prepared by Dounce homogenization in 10 ml extraction buffer A (15 mM MgCl₂, 15 mM Tris-HCl, pH 8.0, 5 mM ethylene glycol tetraacetic acid, 0.25 M hexylene glycol, 0.6% surfynol, and 3 mM dithioerythritol) of macroplasmidia. Cytoplasmic extracts were obtained by centrifugation at 700 \times g for 5 min (supernatant fractions). Nuclei were isolated from nuclear pellets by Percoll gradients (buffer A containing 25% Percoll), as described in Thiriet and Hayes (2005), wherein the nuclei form a ring at the bottom of the tube while membrane debris and polysaccharides remain at the top of the gradient. Soluble chromatin was prepared by MNase digestion (Thiriet, 2004) and purified with HAP beads using high-salt washes according to Brand *et al.* (2008). Histones were removed from HAP-bound chromatin with SDS–PAGE loading buffer.

Soluble fractions used in IP of exogenous proteins were obtained by pelleting organelles from cytoplasmic fractions and chromatin precipitation with 10 mM MgCl₂ from nuclear fractions.

Analyses of the incorporation of exogenous proteins

Incorporated proteins were analyzed by SDS–PAGE and Western blotting using anti-FLAG monoclonal antibody M2 as previously

described (Thiriet and Hayes, 2005). Immunocytochemical analyses were performed on macroplasmidium explants as described (Thiriet and Hayes, 2005). Slides were stained with anti-FLAG M2 antibody diluted at 1:1000 and secondary antibodies coupled to either rhodamine or fluorescein diluted at 1:500. After 4',6-diamino-2-phenylindole (DAPI) staining and exhaustive washings with phosphate-buffered saline (PBS), the slides were observed with a Leica fluorescence microscope.

For IP experiments of exogenous histones, soluble cytosolic and nuclear fractions were incubated overnight at 4°C with anti-FLAG beads (Sigma, France). Beads were then pelleted and washed as recommended by the manufacturer before analyses of the immunoprecipitated proteins by Western blotting using anti-FLAG antibody, anti-HAT1 antibodies (Abcam, UK), and anti-H4 K12 acetyl antibodies (Abcam, UK). Note that analyses with anti-H4 K8 Acetyl and anti-H4 K5 Acetyl did not reveal positive signals.

IP of replication forks

In early S-phase, macroplasmidia were incubated with BrdU (50 µg/ml) for 20 min. Nuclei were isolated as described above and cross-linked with 2% formaldehyde for 30 min. Nuclei were then washed with PBS and H₂O and resuspended in lysis buffer (1% Triton X-100, 0.1% SDS). The solution was then sonicated 5 × 30 s duty 50% and output 30% with a Branson Sonifier, and nuclear debris were pelleted by centrifugation. The supernatant was then exposed to UV (1200 J/m²) and treated with 1.5 M HCl for 15 min before neutralization with NaOH. The chromatin solution was diluted two-fold with PBS, 1% Triton X-100, and 0.1% SDS, and 10 µg anti-BrdU (BioLegend, Ozyme, Paris, France) was added overnight at 4°C. Immunocomplexes were recovered using magnetic beads (Ademtech, Pessac, France) according to the manufacturer's instruction and formaldehyde crosslinks reversed by heating 99°C for 30 min before analyses. Competition experiments were carried out similarly, except that BrdU-labeled *Physarum* chromatin was supplemented with ~10 µg BrdU-containing bacterial DNA.

Reconstitution of nucleosome in vitro

In vitro reconstitution of nucleosome was carried out by a salt dialysis method. A *Xenopus* 5S rDNA fragment of 215 base pairs and purified core histones at the correct ratio of H3/H4 and H2A/H2B were mixed in a total volume of 200 µl in 2M NaCl. The different DNA/histones mixtures were dialyzed for ~1 h against several buffers of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and decreasing NaCl concentrations of 1.2, 1.0, 0.8, and 0.6 M. A final dialysis was performed against Tris-EDTA for at least 3 h as previously described (Thiriet and Hayes, 1998). After reconstitution, the complexes were analyzed by nucleoprotein gel electrophoresis using as control the reconstitution of nucleosome with purified chicken erythrocyte histones.

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