Mol. Cells *28*, 149-154, September 30, 2009
DOI/10.1007/s10059-009-0127-7

Minireview

Molecules and Cells

Histone Modifications During DNA Replication

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Faithful and accurate replication of the DNA molecule is essential for eukaryote organisms. Nonetheless, in the last few years it has become evident that inheritance of the chromatin states associated with different regions of the genome is as important as the faithful inheritance of the DNA sequence itself. Such chromatin states are determined by a multitude of factors that act to modify not only the DNA molecule, but also the histone proteins associated with it. For instance, histones can be posttranslationally modified, and it is well established that these posttranslational marks are involved in several essential nuclear processes such as transcription and DNA repair. However, recent evidence indicates that posttranslational modifications of histones might be relevant during DNA replication. Hence, the aim of this review is to describe the most recent publications related to the role of histone posttranslational modifications during DNA replication.

INTRODUCTION

In the eukaryotic nucleus the DNA is wrapped around nucleosomes, the basic building block of chromatin. Each nucleosome is composed of a core particle, consisting of a 147 bp piece of DNA wrapped around an octamer of histones (two molecules of H2A, H2B, H3 and H4), and linker DNA bound to histone H1 connecting the core particles. Furthermore, nucleosomes interact with each other to form a highly compacted structure referred to as the 30 nm fiber.

Both the nucleosome and the 30 nm fiber represent a significant barrier to nuclear processes such as DNA replication. Therefore, chromatin has to first be "decompacted" in order for the replication machinery to gain access to origins of replication (Falbo and Shen, 2006). Next, during replication fork formation and progression, nucleosomes are disrupted and the DNA is duplicated. The chromatin structure associated with the parental strands is replicated along with the DNA sequence and inherited by the daughter strands. Thus, parental nucleosomes ahead of the replication fork are disrupted and histones are transferred behind the replication fork onto either the leading or the lagging strand to reconstitute the chromatin structure. Finally, newly synthesized histones are incorporated in the new nucleosomes by a replication-dependent de novo nucleosome assembly pathway to fully restore nucleosome density in the daughter strands (Groth et al., 2007).

Newly synthesized histones, as well as assembled histone proteins, are subjected to a wide range of posttranslational modifications such as acetylation, methylation, phosphorylation, etc. Moreover, these modifications are known to be important in the establishment of chromatin structures that are essential for many nuclear processes such as transcription, centromere function, gene silencing, etc (Groth et al., 2005; Taddei et al., 1999). Hence, correct re-establishment of these epigenetic marks during DNA replication is essential to ensure proper inheritance of chromatin structures. However, DNA replication can also provide the cell with an opportunity to modify chromatin structures that influence transcription, for example, during differentiation. In fact, it was described that transcriptional activation of certain developmentally regulated genes depends upon DNA replication (Fisher and Mechali, 2003).

Currently, it is unclear how, and to what extent, these epigenetic marks are reproduced during replication. In fact, some histone posttranslational modifications are inherited from the parental histones while others are incorporated with the newly synthesized ones. Additionally, some of these marks are transient during replication and actively involved in the replication process itself, while others simply mark the DNA to lead future events (Groth et al., 2007). In this review, we focus on the histone posttranslational modifications that are involved in the process of replicating the DNA and summarize the current knowledge of histone posttranslational modifications that are necessary for proper DNA replication, including those involved in avoidance of replication related DNA damage.

Phosphorylation of linker histones

The H1 histone family of linker histones is the most divergent class of histones. H1 histones are known to be involved in chromatin condensation as well as in limiting the access of regulatory factors to nucleosomes (Zlatanova and Doenecke, 1994) during transcription of several genes. Interestingly, mass spectrometry analysis has shown that H1 can also be posttranslationally modified at several residues by phosphorylation, acetylation, methylation, ubiquitination, etc (Deterding et al., 2008; Garcia et al., 2004; Sarg et al., 2006; Wisniewski et al., 2008). In particular, H1 phosphorylation has been intensively studied since its discovery, and accumulated evidence indicates that this modification is important during DNA replication (Balhorn et al., 1972).

Indeed, phosphorylation of H1 is cell cycle regulated. H1 phosphorylation peaks during G2 and mitosis, and decreases

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Received August 31, 2009; accepted September 2, 2009; published online September 17, 2009

Keywords: acetylation, chromatin remodeling, DNA replication, histone, methylation, phosphorylation, post translational modification, ubiquitination

at the end of mitosis during telophase (Bradbury et al., 1974). Thus, during S phase, histone H1 exists in both an unphosphorylated and a low-phosphorylated form. Moreover, the fact that phosphorylated H1 co-localizes with sites of DNA replication *in vivo*, suggests that H1 phosphorylation is a precondition for DNA replication (Alexandrow and Hamlin, 2005; Gurley et al., 1975; Halmer and Gruss, 1996; Yasuda et al., 1981).

In addition, several reports have associated H1 phosphorylation with replication. For instance, in Xenopus extracts, supplementation of histones with H1 from mouse somatic cells causes a decrease in the rate and extent of DNA replication, suggesting that H1 phosphorylation has a role during development that is mediated by DNA replication (Lu et al., 1997). Similarly, a recent report by Thiriet and Hayes suggests that H1 phosphorylation regulates the global timing of replication origins in physarum polycephalum (Thiriet and Hayes, 2008). They observed that genomic DNA is more rapidly replicated in H1 knockout cells than in wildtype, and they found, measured by BrdU or H^3-thymiding incorporation, that the DNA of knockout cells is completely replicated in about 1.5 h, instead of the 3 h that takes to complete replication in wildtype cells. Interestingly, this repressive effect seems to be partially due to an acceleration of the native timing of the replication fork. Indeed, immunoprecipitation analysis at a specific replication origin during S phase showed that H1 is not present at the replicon during origin firing, suggesting that H1 is transiently dissociated from chromatin during replication. Furthermore, this dissociation and the accelerated DNA replication are possibly dependent on H1 phosphorylation, since treatment of cells with okadaic acid (a phosphatase inhibitor) mimics the loss of H1 in knockout cells.

In brief, these experiments strongly suggest that linker histones are necessary to initiate replication, possibly through their role in chromatin decondensation. Interestingly, in mammals, depletion of H1 causes a global defect in higher order chromatin stucture (Fan et al., 2005). Thus, it is possible that spatial and temporal regulation of H1 phosphorylation leads to histone release and, as a consequence, generation of a more replication competent state. However, further research would be necessary to confirm this model, as well as to elucidate the detailed underlying mechanism.

Core histone tail modifications

Acetylation

Histones H3 and H4 synthesized *de novo* during S phase are acetylated at multiple lysine residues within the N terminal tail. In fact, both histones present multiple acetylation sites, described originally in tetrahymena, and subsequently shown to be present in most organisms (Benson et al., 2006; Ma et al., 1998). H4 is acetylated at lysines 5, 8, 12 and 16, while acetylation of H3 occurs at lysines 9, 14, 18 and 23. Interestingly, point mutants that substitute these multiple lysine acetylation sites at the N terminal tail of these histones present reduced cell viability due to defects in chromatin structure during passage through the S phase (Benson et al., 2006; Ma et al., 1998; Sobel et al., 1995). Furthermore, while the acetylation pattern of H3 varies among different species and among different histone variants in humans, H4 diacetylation of K5/K12 is highly conserved (Sobel et al., 1995), indicating that this specific pattern plays an important role in histone deposition during replication. However, the functional significance of this mark during DNA replication remains unknown. Furthermore, very little is known about the role of H4 K8 and K16 during replication.

Indeed, the presence of acetylation marks in citosolic histones led originally to the hypothesis that tail acetylation is necessary for nuclear import of histones. However, the replacement of the four acetylatable lysines for arginines in H4 does not affect nuclear import (Glowczewski et al., 2004). Nevertheless, multiple papers indicate that H4 tail acetylation is dispensable for replication. First, the K5/K12 diacetyl mark is transient, since it is present in newly synthesized histones, but is removed 20 to 60 minutes after completion of replication (Taddei et al., 1999), indicating that this mark might be important during chromatin maturation (Annunziato and Seale, 1983). Second, Hat1-RbAp46, the enzyme that catalyzes K5/K12 acetylation at H4, was shown to be indispensable for replication-couple nucleosome assembly, and a recent report suggests that Hat1 catalytic activity is involved in the formation of H3-H4 pre-deposition complexes in the citosol (Barman et al., 2008). Moreover, lack of this enzyme renders the cells sensitive to drugs that induce DNA damage as a consequence of replication (Barman et al., 2006), suggesting that K5/K12 diacetylation could also be important for recovery of stalled replication forks. However, the specific function of the diacetyl mark remains an enigma.

As previously mentioned, H4 tails are also acetylated at K8 and K16. Although these epigenetic marks are poorly understood, a recent report using *in vitro* assays with a plasmid containing an ARS sequence indirectly suggests that K16 acetylation might be necessary to recruit the MCM complex to sites of replication initiation (Chiani et al., 2006). Although this conclusion may appear premature, the fact that K16 acetylation was shown to inhibit formation of the higher order 30 nm structure (Shogren-Knaak and Peterson, 2006; Shogren-Knaak et al., 2006), supports the hypothesis that K16 acetylation might be present in newly synthesized histones to help keep an open chromatin state that leads to MCM recruitment at ARS during replication initiation.

Remarkably, the crystal structure of the Hat1 K5/K12 acetyl transferase may have highlighted some clues about the role of K16 and K8 acetylation in replication. Indeed, a model derived from the enzyme crystallographic structure predicts that the tail region between K8 and K16 is engaged in electrostatic interactions that stabilize the Hat1- tail binding (Dutnall et al., 1998). The model also predicts that abrogation of the positive charge at K8 and K16 by acetylation reduces the ability of the Hat1 to interact with histone tails. Furthermore, this model is supported by *in vitro* experiments with tail peptides showing that acetylation of K5/K12 by the histone acetyltranferase is impaired when the peptides are acetylated at K8 and K16 (Makowski et al., 2001). Clearly, very little is known about the role of histone tail acetylation during DNA replication and chromatin maturation. Thus, extensive research needs to be done to elucidate the role of these acetylation marks and the steps during replication where these marks are relevant.

H4K20

Another mark found to be specifically present during replication is the methylation of histone H4 at Lys 20. Histone H4 can be mono, di or tri-methylated at Lys20. In mammalian cells, K20 mono-methylation depends on the PR-set7 (Set8) enzyme, while di and tri methylation are dependent on other methyl transferases (Fang et al., 2002). Interestingly, each methyl mark is involved in very different and specific activities. To illustrate, tri-methylation is a selective mark of pericentric heterochromatin, while mono and di-methylation are broadly distributed along the chromatin (Karachentsev et al., 2005; Schotta et al., 2004). Although the biological function of H4K20 is poorly understood, the recent observation that Set8 expression peaks during S phase led two groups to investigate whether Set8 and H4K20 might have a specific function during DNA replication

(Rice et al., 2002).

Indeed, two recent papers by Jorgensen et al and Huen et al. provide strong evidence that Set8 plays a key role in replication fork progression in normal, as well as in transformed human cells. They showed that Set8 mRNA depletion slows down replication fork progression (assessed by H3 thymidine incorporation) and induces accumulation of DSB that are replication dependent. More importantly, Set8 immuno-precipitates with PCNA through a short PIP interacting box sequence, suggesting H4K20 mono-methylation during S phase depends on the Set8-PCNA interaction (Huen et al., 2008; Jorgensen et al., 2007). Despite the fact that the authors did not observe any abnormality in other replication related proteins, the possibility of an indirect-transcription related effect could not be completely discarded by those studies. However, these experiments strongly suggest that mono-methylation of H4K20 by Set8 is coupled to S phase by a physical interaction between Set8 and PCNA.

Interestingly, since H4K20 is necessary for mitosis and chromatin condensation, it is possible that Set8 is required during replication simply to maintain the H4K20 mono-methylation status necessary for subsequent condensation and mitosis (Huen et al., 2008). Nevertheless, the fact that replication fork progression is impaired in the *set8* mutant suggests that H4K20 has a functional role at the replication fork. In addition, Rad51 depletion impairs replication fork movement only after Set8 depletion, suggesting that Set8 acts downstream of Rad51 to resolve recombination mediated DNA intermediates (Jorgensen et al., 2007). However, it is also possible that absence of Rad51 channels the damage avoidance pathways to the Rad6-PCNA ubiquitination mediated damage tolerance pathway (Ulrich, 2007). If so, it would be interesting to investigate whether H4K20 mono-methylation is necessary for PCNA ubiquitination and subsequent recruitment of Rad18 and Rad6 to replication forks to avoid replication related DSB generation.

H3K9 methylation

Methylation of H3 at Lys 9 has traditionally been described as a mark that determines heterochromatic regions, but recent evidence indicates that this modification might have a protagonist role in DNA replication as well. In fact, a paper published by Loyola et al. supports the general view that while most methylation marks (K4, K27, K36) are imposed after histone deposition, H3K9me1 and H3K9me2 are the only marks present in histone dimers previous to deposition during S phase, suggesting that both methylation marks may have a role in DNA replication (Loyola et al., 2006). Furthermore, H3.1, a replication dependent histone, is mono-methylated at Lys9, while H3.3, a replication independent histone, is di-methylated or acetylated at Lys9 (Loyola et al., 2006). Since H3.1 and H3.3 are assembled for deposition by two different pathways (Ahmad and Henikoff, 2002; Tagami et al., 2004), it is possible that these pathways, in fact, determine the pre-depositon posttranslational modification (PMT) pattern. Moreover, it is also plausible that after histone deposition the presence of these marks influence the final PMT pattern in nucleosomes by recruitment of other histone modifying enzymes, such us Suv39 (Loyola et al., 2006). In fact, it was shown that Suv39 preferentially acts on histone tails that are specifically mono-methylated at Lys9 (Loyola et al., 2006).

Additionally, a recent report indicates a possible functional link between H3K9/36 methylation and DNA replication (Kim et al., 2008). The authors showed that mutants that do not methylate these residues, and mutants of the enzymes responsible for their respective methylation, *clr4* and *set2*, are sensitive to hydroxyurea (HU), an agent that blocks replication fork progression and induces replication checkpoint activation. They propose a model where these methylation marks and respective binding proteins (such us swi6/HPB1) interact with proteins involved in replication fork stabilization and checkpoint activation. Even though it is possible that these marks have a direct role at replication forks during checkpoint activation, extensive research is necessary to rule out the possibility of an indirect effect as well as to elucidate the underlying mechanism. Finally, it is worth mentioning that a remarkable characteristic of methylation marks is their low turnover, which supports the idea that this mark could have a preponderant role in epigenetic memory instead of in the replication process itself (Volkel and Angrand, 2007).

Histone core domain acetylation: H3 K56 acetylation

In addition to N-terminal acetylation, newly synthesized histones are acetylated at residues located in the globular domain of histones prior to their incorporation into the chromatin during S phase (Hyland et al., 2005; Masumoto et al., 2005; Ozdemir et al., 2005; Xu et al., 2005). Indeed, the H3 lysine 56 residue was found to be acetylated in *Saccharomyces cerevisiae* by mass spectrometry (Masumoto et al., 2005; Xu et al., 2005). Moreover, this residue is strategically localized at the entry-exit points of the nucleosome core, blocking a direct electrostatic interaction between H3 and nucleosomal DNA (Davey et al., 2002). This observation, and the fact that H3K56 acetylation increases during S phase, but disappears during G2, strongly supports the idea that this posttranslational modification might be important during a replication related activity (Masumoto et al., 2005).

H3K56 is acetylated by the Rtt109 acetyltransferase that works together with the histone chaperone Vps75 to introduce the acetylated mark in newly synthesized H3-H4 dimers presented by the Asf1 complex (Driscoll et al., 2007; English et al., 2006; Han et al., 2007a). Rtt109 has no apparent similarity in sequence with any known HAT, suggesting it might recognize its substrate using a unique mechanism. In fact, the recently published crystal structure of Rtt109 suggests a model in which histone acetylation by Rtt109 is performed in a two step mechanism where the acetyl group is first attached to the side chain of the HAT and then transferred to the substrate (Lin and Yuan, 2008). Moreover, and coincidently with the fact that Rtt109 acetylates H3K9 in the presence of Vps75, the crystal structure of the recognition motif suggests that this acetyltransferase could have different substrates depending on the binding chaperone (Lin and Yuan, 2008).

Several lines of investigation indicate that acetylation of K56 is important to preserve genomic integrity. First of all, cells that lack Rtt109 acetyltransferase or cells that express an H3 point mutant that cannot be acetylated (*K56R*) present an increased frequency of chromosome breaks (Allis et al., 2007; Driscoll et al., 2007; Han et al., 2007a). Next, mutations that abolish acetylation of K56 cause hypersensitivity to genotoxic agents that interfere with DNA replication, such us HU and camptothecin (CPT) (Masumoto et al., 2005; Recht et al., 2006). Finally, the *rtt109* mutant exhibits synthetic lethal-slow grow phenotypes with proteins involved in DNA replication and DSB repair (Collins et al., 2007; Han et al., 2007a). Briefly, this evidence indicates that acetylation of H3K56 has an obvious, but not-yet-elucidated role in preserving genome integrity.

Paradoxically, failure to deacetylate H3K56 has also severe consequences in the integrity of chromosomes, despite the fact that deacetylation of H3K56 occurs in G2/M. H3K56 deacetylation is dependent on the NAD-dependent deacetylases Hst3

and Hst4 (Celic et al., 2006; Maas et al., 2006; Masumoto et al., 2005), two proteins related to the Sir2 histone deacetylase (Celic et al., 2006), and cells lacking the Hst3 and Hst4 deacetylases, in which H3K56 is constitutively acetylated, are sensitive to genotoxic agents that block replication fork progression and exhibit marks of replication linked DNA damage (Celic et al., 2006; 2008; Maas et al., 2006). For instance, it was described that the deacetylases Hst3 and Hst4, as well as deacetylation of K56, are necessary to maintain the silenced state of heterochromatin (Yang et al., 2008). Thus, it seems that K56 acetylation has to be carefully regulated during the cell cycle to preserve the integrity of the genome.

Despite the robust evidence indicating H3K56 is important to preserve genome integrity during DNA replication, the molecular mechanism involved has remained an enigma. Unlike histone tails, acetylation of K56 does not affect the nuclear import of H3 (Li et al., 2008). Furthermore, acetylation of H3K56 and acetylation of lysine residues in the N terminal tail of H3 and H4 have been shown to function non-redundantly in nucleosome assembly (Li et al., 2008). These observations led Li et al. to hypothesize that acetylation of H3 at Lys 56 acts through a genetic mechanism that is different from the acetylation of the N terminal region of H3 and H4 (Li et al., 2008).

In *Saccharomyces cerevisiae* CAF1, ASF1 and Rtt106 are the main chaperones implicated in the assembly of H3-H4 new dimers into DNA. CAF-1 and Rtt106 have a partially redundant function in chromatin assembly during replication while CAF1 and ASF1 were shown to be functional linked to H3K56 acetylation (Han et al., 2007b; Huang et al., 2005). In a recent paper, Li et al. used *in vitro* and *in vivo* approaches to show that H3K56 acetylation promotes S phase chromatin assembly mediated by CAF1 and Rtt106 (Li et al., 2008). First, they showed that H3 association with the histone chaperones CAF-1 and Rtt106 requires Lys 56 acetylation. Second, using *in vitro* DNA synthesis dependent and independent nucleosome assembly assays, they demonstrated that H3K56 acetylation is necessary for CAF1 and Rtt106 mediated H3-H4 assembly into newly replicated chromatin. Next, they performed chromatin immunoprecipitation to extrapolate these *in vitro* results to S phase chromatin assembly *in vivo* and showed that the H3K56 mark is incorporated into replicating DNA in a process that depends, partially, on CAF1 and Rtt106 (Li et al., 2008). From these observations, they proposed a model in which ASF1 binds to newly synthesized H3-H4 dimers and presents those dimers for H3K56 acetylation by the Rtt109-Vps75 complex. Then, the acetylated dimers are transferred to CAF1 and Rtt106 for deposition into newly synthesized chromatin and nucleosome formation (Li et al., 2008).

Li et al.'s paper clearly implicates H3K56 in the assembly of nucleosomes during normal replication, and suggests that improper assembly can be the cause of genomic instability in the mutants defective in the H3K56 acetylation pathway. In support of this idea, a recent paper from Chen et al. presents some evidence about the relevance of H3K56 acetylation in chromatin reassembly at sites of DSB after completion of repair (Chen et al., 2008). However, this research is based on the generation of a single DSB using the HO Mat system and it is well established that the mechanism leading to DSB avoidance during replication is significantly different from the mechanisms that resolve DSB after an HO cut (Duro et al., 2008). Thus, it would be interesting to explore whether the DNA damage avoidance pathway at the replication fork is dependent on the acetylation of H3K56 and responsible for the genomic instability of the H3K56 mutants, particularly since Miller et al. have recently proposed that mutations in DNA replication factors like PCNA alter H3K56 acetylation (Miller et al., 2008). Since PCNA is the main player during DNA damage avoidance in replication, and activation of this pathway is mediated by PCNA ubiquitination, it would be exciting to explore whether acetylation of H3K56 is directly involved in the DNA damage avoidance during replication. Also, it would be important to investigate whether this defect in the DNA damage avoidance pathway could be the main cause of the sensitivity to genotoxic agents in the Rtt109 and H3K56 point mutants.

It can be derived from the literature that most of the research done so far on the acetylation of H3K56 has been performed in yeast. In fact, most of the knowledge described above was derived from the study of *Saccharomyces cerevisiae* and *Saccharomyces pombe*. Hence, some important questions remain unanswered. Specifically, the implications of K56 acetylation in human cells is still an interesting and unexplored field. Indeed, acetylation and methylation of H3K56 have recently been found in HeLa cells (Garcia et al., 2007; Masumoto et al., 2005), even though the Rtt109 homolog in humans has yet not been identified. Moreover, the presence of CAF1, Asf1, Rtt109 and Rtt106 in the yeast *S Pombe,* added to the presence in humans of homologs of proteins that belong to the H3K56 pathway, such as Asf1, Vps75 and sirtulins, strongly suggests that H3K56 acetylation might have similar roles in yeast and human cells. As a fact, it was shown that human Asf1 regulates the flow of histones during replication (Groth et al., 2005), clearly indicating that extensive work should be done to elucidate the role of H3K56 acetylation in humans.

CONCLUSION

The last few years have witnessed the discovery of a multitude of new histone posttranslational modifications, followed by intensive research defining the role of these modifications in different nuclear functions. While it is clearly established that some of these posttranslational modifications are necessary for DNA replication, there is still a vast field of knowledge to be explored. Particularly, the underlying mechanisms that lead to genomic instability due to improper histone modifications during replication remains poorly defined. Furthermore, several posttranslational histone modifications have been described to be present during S phase, but it remains to be elucidated whether they have a crucial role in replication. Finally, a detailed dissection of the mechanisms involved, as well as the discovery of new histone marks, would be important in the development of new knowledge to understand complicated processes like cancer that clearly depend on the correct duplication of the DNA, as well as the chromatin marks associated with it.

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