OnLine Journal of Biological Sciences 12 (2): 54-61, 2012
ISSN 1608-4217
© 2012 Arnaud and Thiriet, This open access article is distributed under a Creative Commons Attribution (CC-BY) 3.0 license

Histone Tail Modifications of H3 and H4 during the *Physarum polycephalum* Cell Cycle

Marie-Claire Arnaud and Christophe Thiriet
University of Nantes, CNRS, UFIP (FRE 3478),
Epigenetics: Proliferation and Differentiation,
2 rue de la Houssinière, 44322 Nantes Cedex 3, France

Abstract: Problem statement: Histone modifications are required for chromatin activities and are believed to form an epigenetic code. However, the inheritance of the histone post-translational modifications remains unclear. **Approach and Results:** The aim of this study was to evaluate the histone modifications throughout the cell cycle and to determine whether the histone marks are transferred to daughter cells. Using the naturally synchronous model system *Physarum polycephalum* and Western blotting analyses, we showed that the histone modifications are not exclusive of the cell cycle stage. Importantly, we also demonstrated that during mitosis, a substantial fraction of the acetylation and the methylation marks of the histone H3 and H4 is removed. **Conclusion:** The results illustrate that a part of the histone modifications are displaced during mitosis and thus failed to exhibit an inheritance to daughter cells as proposed for the epigenetic marks.

Key words: Cell cycle, nucleus, histone, post-translational modifications, *Physarum polycephalum*

INTRODUCTION

In eukaryotic cell, the genome is associated with proteins to form chromatin. The basic sub-unit of chromatin is the nucleosome wherein two of each H2A, H2B, H3 and H4 form a histone octamer wrapped by ~2 superhelical turns of DNA (Luger *et al*., 1997). The histones are composed of domains, a globular domain involved in histone-histone and histone-DNA contacts and the tail domain which can be subjected to Post-Translational Modifications (PTMs) (Luger and Richmond, 1998; Wolffe, 1999; Wolffe and Hayes, 1999). More than 100 PTMs have been described and they have largely been involved in the chromatin activities. It is generally believed that PTMs function by recruiting factors to chromatin (Strahl and Allis, 2000). Indeed, it has been shown that chromo-domain-containing proteins associate to methylated histones and bromo-domain containing factors bind to acetylated histones (Kouzarides, 2007).

The progression of the cell cycle requires the passage through different stages that are characterized by the chromatin activities. Although in most cell types all stages of the cell cycle are required, the S-phase and mitosis imply drastic changes within the genome. Indeed, in S-phase the genome is replicated and in mitosis the two copies of the genome are transferred into the daughter cells. These two stages of the cell cycle are critical for the epigenetic inheritance as during S-phase the two copies of the genetic material require to display the same epigenetic information and during mitosis the epigenetic information is transferred into the two daughter cells for propagating throughout the generations. It is generally believed that histone PTMs might compose an epigenetic code which should thus be copied in S-phase and transferred to daughter cells during mitosis (Probst *et al*., 2009; Wolffe, 1994).

Although the copy of the histone PMTs following replication is still under debate, it has been shown that during mitosis all PMTs are not all transferred to daughter cells. For instance, deacetylation of histone H3 by HDAC3 was revealed required for mitosis proceeding and HDAC3-depleted cells exhibit acetylation of centromeric H3 at lys4 (Eot-Houllier *et al*., 2008; Li *et al*., 2006). Thus, this demonstrated that histone PTMs can be altered during mitosis and suggested that histone modification associated with mitosis might compose a specific code. Similarly, the histone H3 phosphorylation at Ser10, which is generally believed to be a mitotic mark, exhibit in mammal cell a maximum before mitosis and a decrease during mitosis (Hendzel *et al*., 1997). Nonetheless, one cannot exclude that the variation in the phosphorylation signal resulted in a decrease of the

Corresponding Author: Christophe Thiriet, University of Nantes, CNRS, UFIP (FRE 3478), Epigenetics: Proliferation and Differentiation, 2 rue de la Houssinière, 44322 Nantes Cedex 3, France

accessibility the antibody to the epitope due to the compaction of mitotic chromosome as these experiments were carried out by *in situ* analyses.

To examine cell cycle dependent events by biochemical means, generally cell synchronization is required. However, most of these methods utilize drugs that might also affect the epigenetic modifications of histones. To address potential alteration of the epigenetic information by experimental procedures, the model system *Physarum polycephalum* is ideal. Indeed, this organism can grow as giant cell with about five hundred million nuclei perfectly synchronous throughout the cell cycle (Thiriet and Hayes, 1999). Thus no synchronization procedures are required to examine specific cell cycle stages. In addition, as the cell cycle is reproducible, the only determination of a characteristic time point makes possible the determination of any stage of the cell cycle. Interestingly, in *Physarum* pre-mitotic changes of the morphology of nuclei allow the precise determination of the timing of mitosis and consequently the subsequent phases of the cell cycle, the 3h of S phase (*Physarum* lacks the G1-phase) and the 6h of G2-phase. Furthermore, mitosis in *Physarum* is endonuclear without disruption of the nuclear membranes.

In the present report, we have examined modifications of the histone tail domain of H3 and H4 throughout the cell cycle. Our results revealed that most of the analyzed histone PTMs are present throughout the interphase and cannot provide a signature for specific cell cycle stage. Nevertheless, the histone modification specifically associated with mitosis, as the H3 phosphorylation at serine 10, is found in *Physarum*. Importantly, our analyses also demonstrated that part of the histone modifications are removed during mitosis as shown by the decrease in specific antibody reactivity by Western blotting. Specifically, we showed that the loss of H3 Lys9 methylation during mitosis does not result in phosphorylation of Ser10, but rather to an enzymatic demethylation of the lysine residue.

MATERIALS AND METHODS

***Physarum polycephalum* cultures:** Macroplasmodia of the strain TU291 are grown in liquid cultures by using the standard procedures described by (Prescott, 1964; Thiriet and Hayes, 1999). The macroplasmodia result of the coalescence of microplasmodia onto a filter paper placed into a petri dish. The macroplasmodium corresponds to a syncytium with several million naturally and perfectly synchronous nuclei. The evaluation of the cell cycle progression is performed by the determination of the timing of mitosis by phase contrast microscopy of smears of tiny explants of macroplasmodia fixed in ethanol. The duration of the S-phase was determined by pulse labelling of genomic DNA. Cellular fragments from a single macroplasmodium were cultured for 5 min presence of α^{32}P-ATP. The genomic DNA was then prepared and the quantity was determined by optical density measurement at 260 nm. The DNA fractions were then counted by a scintillation counter to determine the amount of radioactive precursor incorporated.

Isolation of the nuclei: At defined cell cycle stages, macroplasmodia were harvested and the cells were homogenized with a potter in homogenization buffer (15mM MgCl2, 15mM Tris-HCl, pH 8.0, 10 mM EGTA, 250 mM hexylene glycol, 0.6% surfynol and 3 mM DTE). The homogenized cell was centrifuged at 700g for 5 min. The pellet containing the nuclei was suspended in the homogenization buffer supplemented with 25% percoll and centrifuged at 25,000g for 35min. The nuclear fraction is recovered at the bottom of the tube and is transferred into a new tube (Thiriet and Hayes, 1999). The nuclei were quantified by the evaluation of the amount of H3 Western blotting. For experiments of phosphatase treatments, the nuclei were incubated for 1h in the presence of phosphatase.

Western blotting analysis: Nuclear fractions were resolved by SDS-PAGE and transferred onto nitrocellulose membrane as described by (Thiriet and Albert, 1995). The blots were blocked for 30 min in 5% milk-containing PBS-T and then incubated for 1 h with primary antibodies. Following exhaustive washings with PBS-T, the membranes were incubated for 1 h with a secondary antibody, revealed by ECL and exposed to films. The quantifications of the blots were performed using image-quant software.

RESULTS

Natural synchrony of *Physarum* nuclei throughout the cell cycle: The determination of the histone PMTs at precise cell cycle stages is of general importance as it is believed that the histone marks are associated with chromatin activities. However, to precisely evaluate the histone modifications during the cell cycle, it is required to carry out the analyses with an organism that does not need artificial synchronization as the procedure might affect the enzymatic factors involved in the epigenetic modifications. Interestingly, the slime mold *Physarum polycephalum* offers the advantage to grow as giant cells with several hundred million nuclei perfectly and naturally synchronous throughout the cell cycle.

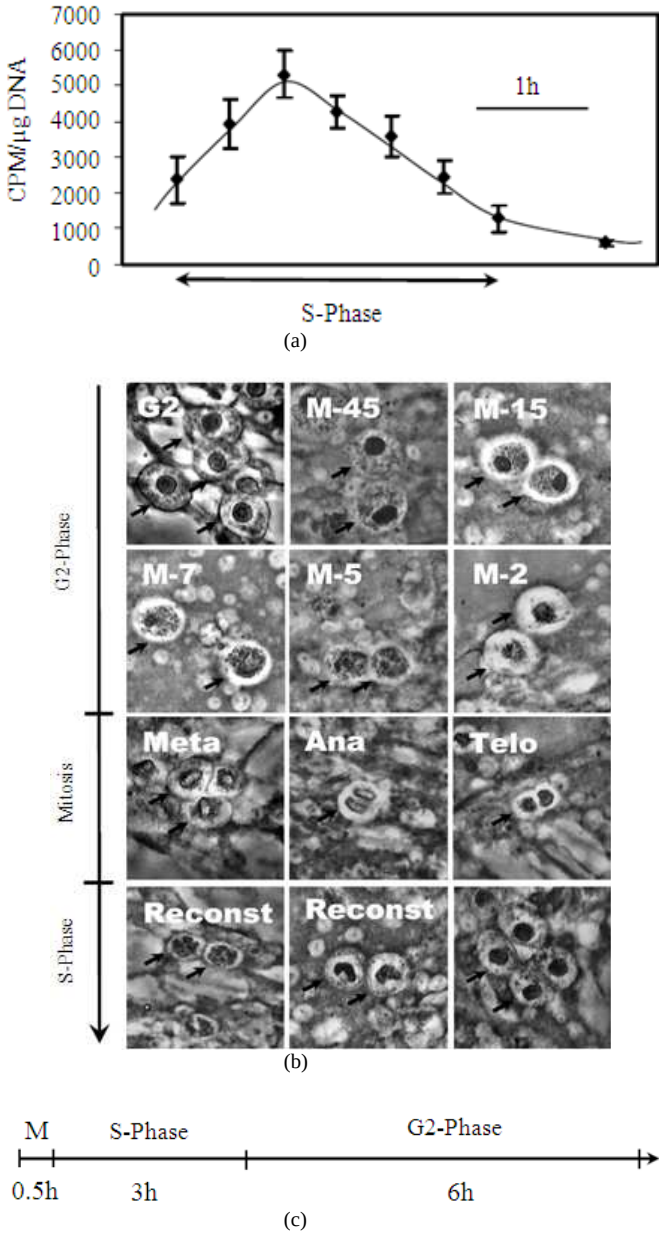

Fig. 1: Natural synchrony of *Physarum polycephalum* macroplasmodium: (a): Determination of the S-phase: the determination of the duration of the S-phase has been performed by the analysis of the period of genomic DNA replication. Fragments from a single macroplasmodium were pulsed with radioactive ATP and the incorporation of the radioisotopes within genomic DNA was determined by measurements of radioactivity in a scintillation counter. (b): Nuclear morphology during the cell cycle: Explants of *Physarum* cells were observed by phase contrast microscopy. At the end the G2-phase the nuclei undergo characteristic changes allowing the precise determination of the timing of the mitosis. The changes correspond to a displacement of the nucleolus to the periphery of the nucleus (M-45; M-15), the disappearance of the nucleolus and the chromatin condensation (M-7; M-5; M-2), the mitosis (Meta; Ana; Telo) and the reconstitution of the nucleolus (Reconst), respectively. (c) The stages of the cell cycle of *Physarum* are precisely timed: The timing of the different cell cycle stages is depicted in the scheme

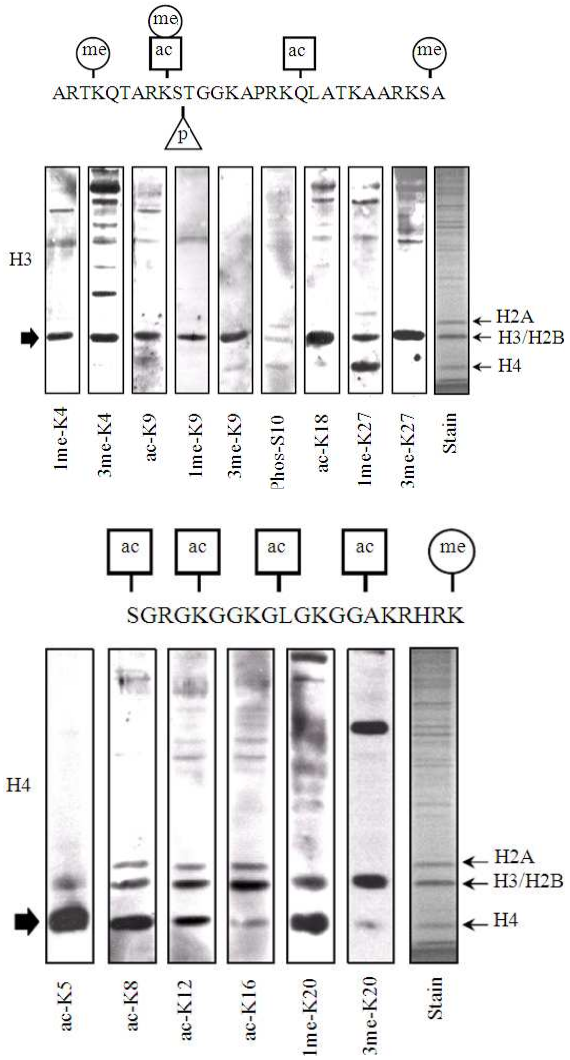

Fig. 2: Reactivity of the nuclear proteins to antibodies directed against histone peptides harboring post-translational modification. The histone tail domains of H3 and H4 and the post-translational modifications examined in the present study are depicted. The analyses of the reactivity of the antibodies directed against the histone marks have been carried out by Western blotting of total nuclei. The expected immunostained band is noted on the left of each panel with an arrow. A typical blot stained with ponceau is shown on the right of the H3 and H4 panels

Therefore, this organism is an ideal model system for examining processes at specific cell cycle stages. The Synchrony of the nuclei of single macroplasmodium can be verified by incorporation of radioactive DNA precursor pulsed for 5 min every 30 min during S-phase followed by the quantification of the radioactivity within genomic DNA (Fig. 1a). Clearly, the stage corresponding to the incorporation of the radioactive precursor is limited to 3 h and represents the stages wherein the genomic DNA is replicated. Interestingly, these experiments revealed that the rate of replication is not constant throughout the S-phase as demonstrated by the biphasic curve. If these analyses exhibited the synchrony of the genome replication, obviously the determination of the S-phase is not a well-suited approach to determine the cell cycle stage. Indeed, it would be more appropriate to determine the cell cycle stage based on the morphology of organelles that are altered during the cell cycle. In this regard, the nucleus is an excellent candidate as demonstrated by the observation by phase contrast microscopy of smears of *Physarum* cell explants wherein changes of the nuclei are detected prior to mitosis. These changes within the nucleus morphology precisely characterized the timing prior to mitosis (from 45 min to 2 min prior to mitosis) and relate to the disappearance of the nucleolus and chromatin condensation associated with mitosis (Fig. 1b). Therefore, as previously reported the cell cycle at the plasmodial stage in *Physarum* is composed of three distinct phases, which are the mitosis that lasts 20-30 min followed by 3h of S-phase (*Physarum* lacks the G1-phase at the plasmodial life cell stage) and 6h of G2-phase, respectively (Fig. 1c).

Recognition of *Physarum* histone modifications with specific antibodies: The natural synchrony of *Physarum* cells at the plasmodial stage offers the opportunity to examine the histone modifications throughout the cell cycle using Western blotting analyses. However, as the commercial antibodies recognized conserved modifications of histones, it was required to verify that the *Physarum* histones present a high homology. The *Physarum* histone H4, the cloning the gene revealed that the protein is highly conserved as only two amino-acid residues are different from the human protein (Wilhelm *et al*., 1984). For the histone H3, we have identified two genes that encoded for two H3 proteins that exhibited slight differences in the globular domain, but the tail domain is identical to the human histone. Based on the high conservation of the histone H3 and H4, we examined the reactivity of a variety of commercial antibodies directed against modifications within the histone tail domain of H3 and H4 (Fig. 2). These experiments were carried out using nuclei isolated from asynchronous cell population and the nuclear proteins were resolved on SDS-PAGE prior to perform Western blotting analyses.

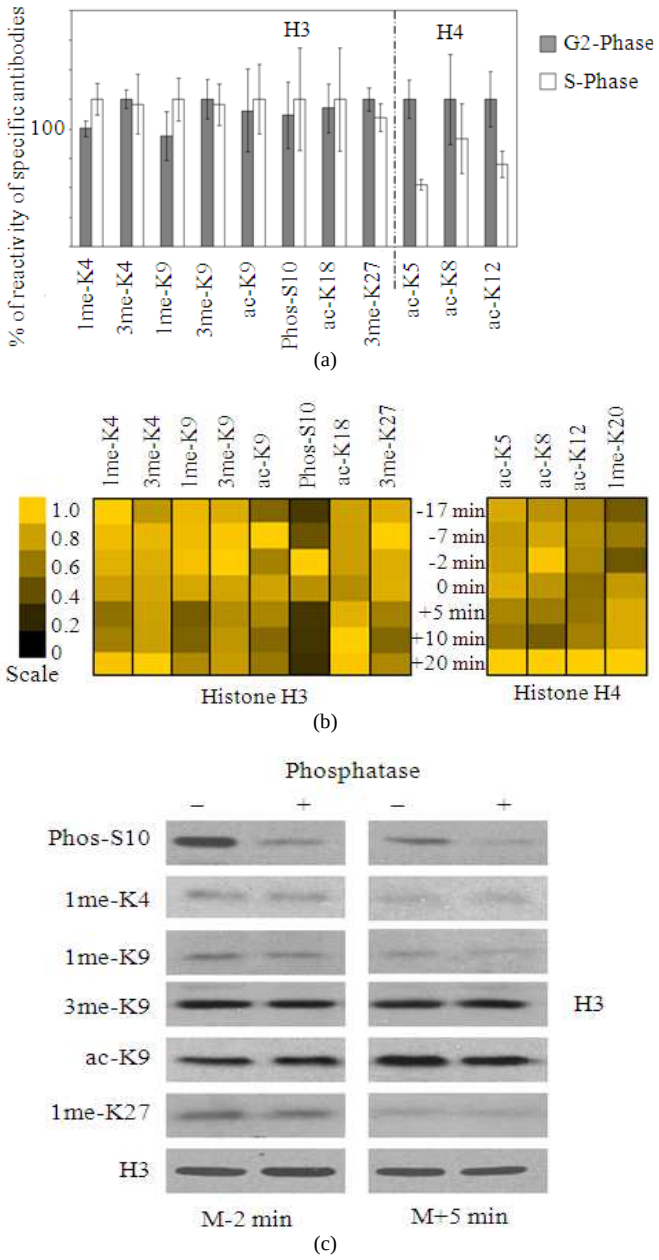

Fig. 3: Variation of the histone modifications throughout the cell cycle: (a): Evaluation of the reactivity of the histone modification specific antibodies. The analyses were carried out during G2-phase and S-phase. The evaluation of the reactivity was determined by densitometry using Image-Quant software and the 100% reactivity for each antibody was arbitrarily affected to the stage presenting the maximal reactivity. The error bars represent the variations within a defined cell cycle stage. (b): Variation of the histone modifications during mitosis. The histone modifications were evaluated as in A. The diagram corresponds to densitometric changes of the reactivity of the different antibodies. The 1.0 value has been arbitrarily affected to the maximum reactivity between Metaphase-17 min and Metaphase+20 min. (c): Methylation detection is not altered by Serine phosphorylation: The nuclei isolated 2 min prior to metaphase and 5 min after metaphase were untreated (-) and treated (+) with phosphatase. The reactivity of the different antibodies to H3 was then estimated by Western blotting

Surprisingly, we found that several antibodies exhibited cross-react with histone and non-histone proteins. Although the undesired antibody reactivity can generate artifacts *in situ*, the analyses in Western blotting prevent misinterpretation of the immunochemical signals. Nonetheless we decided to exclude of our analyses the antibodies directed against H4 acetyl K16 and H4 trimethyl K20, as we found very high non-specific signals. Although the poor reactivity was also observed for the conserved mitotic mark of H3 (H3 phospho serine 10) (Hendzel *et al*., 1997), we thought that the weakness of the signal might be associated with the low frequency of the mitotic cells in the asynchronous population.

A variation of the histone modifications throughout the cell cycle: The Western blotting analyses during the cell cycle did not exhibit drastic changes of reactivity for most antibodies (Fig. 3a). However, consistent with the known modification associated with replication-coupled chromatin assembly, we found that during S-phase H4 acetylation at K5 and K12 was significantly higher than in G2-phase (Ejlassi-Lassallette *et al*., 2011; Sobel *et al*., 1995). In contrast, for the other modifications of the histones H3 and H4, the reactivity of the specific antibodies did not exhibit significant alterations throughout the interphase. Thus we concluded that none of the histone modifications were not exclusive of the specific cell cycle stage, although during the interphase the reactivity of the antibodies to H3 phospho S10 was very weak (Fig. 2).

Then we wanted to verify whether the histone modifications are readily epigenetic marks and inheritable from mother cell to daughter cells. The mitosis in *Physarum* is without cytokinesis and take place without disruption of the nuclear envelope. Therefore we isolated nuclei at a precise timing prior to metaphase and at defined stages of the mitosis. As previously, the nuclei were resolved on SDS-PAGE and histone modifications were analyzed by Western blotting (Fig. 3b). Surprisingly, the quantification of the Western blots revealed that during the 20 min of mitosis the reactivity of the antibodies was altered. Consistently with *in situ* analyses in mammal cells, the most striking change of the specific antibody reactivity was detected for the H3 phospho S10, which exhibited a higher reactivity 2 min prior to metaphase and a decrease of the reactivity in anaphase. Although changes of the histone acetylation have been reported during mitosis, the variation of the H3 methylation was unexpected. Nonetheless, it is important to note that except the methylation of K4 of H3, the other methylations of H3 are adjacent to a serine phosphorylation. Therefore, we could not exclude that the alteration of the methylation of the H3 was due to the serine phosphorylation, which would affect the epitope recognition by the methyl specific antibodies. To rule out this hypothesis, we isolated nuclei from cells harvested 2 min prior to metaphase and in anaphase and treated half of the nuclei with phosphatase. The nuclei were then resolved on SDS-PAGE and analyzed by Western blotting (Fig. 3c). Clearly, the treatment of the nuclei with phosphatase revealed a decrease of the signal detected with the anti-H3 phospho S10 antibodies at both premitotic and mitotic stages. However, the analyses of the reactivity of the antibodies to modifications of adjacent lysine residues were not affected by the removal of the phospho group from the serine. Thus we concluded that during mitosis the lysine 9 and 27 of H3 were partially demethylated. Furthermore, as the antibodies to methylated H3 that were used in these experiments were not capable to bind the a peptide exhibiting K9 methyl and S10 phospho (data from the manufacturer Active Motif) strongly suggested that the phosphorylation and the methylation of H3 were on distinct histone proteins.

DISCUSSION

The histone modifications have been correlated with chromatin activities and are believed to form an epigenetic code (Strahl and Allis, 2000). With the regard, the mark associated with the histones should be inheritable when the genome is copied during S-phase and during the cell division. To verify the inheritance of the histone modifications, the slime mold *Physarum polycephalum* is an ideal model. Indeed, with the natural and perfect synchrony of million nuclei within a single cell, it is possible to isolate nuclei at specific cell cycle stages. Most analyses of the histone modifications at defined cell cycle stage have been carried out by *in situ* studies using specific antibodies or radioactive precursors. However, it is well-established that chromatin structure is not homogeneous within the nucleus and also depends upon the cell cycle stage. For instance, heterochromatin and euchromatin exhibit different compaction state as well as interphasic and mitotic chromosomes. Therefore, it is likely that the accessibility of histone modifications by the antibodies can be affected by the compaction state of chromatin. In contrast, the use of radioactive precursor is not affected by the compaction of chromatin. However, the pulse of radioactive precursors allows the detection new modifications rather than total modifications and the

determination of the specific location of the modification requires nontrivial additional analyses.

Although the use of histone modification antibodies is useful and have been largely developed over the past years, their specificity can be affected by the recognition of non-histone proteins. Hence, experiments of *in situ* immunostaining as well as those of chromatin immunoprecipitation require the verification within the biological systems of the specificity announced by manufacturers. Indeed, the histone modification antibodies are usually obtained by immunizing animals with modified peptides and although the procedure raises antibodies specific to the antigen does not mean that the antibodies will recognize a single molecule within the nucleus in further experiments. Our analyses by Western blotting of the histone modifications throughout the cell cycle prevented the artifact of considering the total nuclear reactivity of the antibodies as the protein of interest was first resolved on SDS-PAGE. Interestingly, we found that most histone marks are found throughout the cell cycle. These results showed that the within the bulk chromatin, the quantity of histone marks is not affected by the cell cycle stage, but does not provide information about the turnover of the marks within a considered nucleosome. Nonetheless, the conserved marks of acetylation of K5 and K12 of H4 associated with the nuclear import and the histone deposition of newly synthesized H3/H4 complex are significantly greater during the phase of the genome replication (Ejlassi-Lassallette *et al*., 2011; Sobel *et al*., 1995).

In contrast to the interphase, the analyses of the histone marks through mitosis revealed significant alterations. Consistent with previous works, we found that Serine 10 phosphorylation of H3 is associated with mitosis. The fine analyses of the precise timing of the phosphorylation revealed in mammal cells and in *Physarum* that the mitotic modification is restricted to specific timing prior to anaphase (Hendzel *et al*., 1997). Histone acetylation has been correlated with transcription activity. The loss of transcription during suggested that histones might be deacetylated during the cell division. In agreement with this idea, it has been shown in mammal cells that the enzymatic activity responsible for the removal of the Histone Acetylation (HDAC) is present during mitosis (Eot-Houllier *et al*., 2008; Li *et al*., 2006). In *Physarum*, we found that the acetylation of the histone H3 and H4 significantly decreased of ~50% but did not completely disappear. It is unclear whether the remaining acetylation marks are associated with chromatin structure inaccessible to the HDAC or specific chromosomal regions. Unexpectedly, we also found that the methylation marks of H3 at lysine 9 and 27 drastically decreased in mitosis. We thus verified that the decrease in lysine methylation did not result in the impediment of the epitope by the adjacent serine phosphorylation. Clearly, we showed that the removal of the Phospho group of the serine residues did not affect the reactivity of the anti-methyl lysine of H3 antibodies. Thus, these results strongly suggested that the demethylation of the histone is associated with mitosis in *Physarum* and the serine phosphorylation and the lysine methylation are not adjacent within the H3 protein. Similarly to the acetylation marks, the methylation removal is not total during mitosis and it would be interesting to define whether the remaining modifications are associated with specific chromatin structures or genome regions.

CONCLUSION

The histone modifications are believed to form an epigenetic code that is inheritable. Our results revealed that the inheritability of the histone modifications to daughter cells is not a general feature. Indeed, at least part of the acetylation and methylation marks of H3 and H4 are removed during mitosis. The removal of the acetylation marks by HDAC3 has been reported in mammal cells and is required for normal mitotic progression. For the demethylase activity the function in mitosis remains obscure. Thus, it will be interesting to identify the enzyme responsible for this activity and the effect on the mitotic progression.

ACKNOWLEDGEMENT

This study was supported by grant to C.T. from “la Ligue contre le Cancer” (Région Grand Ouest).

REFERENCES

Ejlassi-Lassallette, A., E. Mocquard, M.C. Arnaud and C. Thiriet, 2011. H4 replication-dependent diacetylation and Hat1 promote S-phase chromatin assembly *in vivo*. Mol. Biol. Cell, 22: 245-55. DOI: 10.1091/mbc.E10-07-0633

Eot-Houllier, G., G. Fulcrand, Y. Watanabe, L. Magnaghi-Jaulin and C. Jaulin, 2008. Histone deacetylase 3 is required for centromeric H3K4 deacetylation and sister chromatid cohesion. Genes Dev., 22: 2639-2644. DOI: 10.1101/gad.484108

Hendzel, M.J., Y. Wei, M.A. Mancini, A.V. Hooser and T. Ranalli *et al*., 1997. Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. Chromosoma, 106: 348-360. DOI: 10.1007/s004120050256

Kouzarides, T., 2007. Chromatin modifications and their function. Cell, 128: 693-705. DOI: 10.1016/j.cell.2007.02.005

Li, Y., G.D. Kao, B.A. Garcia, J. Shabanowitz and D.F. Hunt *et al*., 2006. A novel histone deacetylase pathway regulates mitosis by modulating Aurora B kinase activity. Genes Dev., 20: 2566-2579. DOI: 10.1101/gad.1455006

Luger, K. and T.J. Richmond, 1998. The histone tails of the nucleosome. Curr. Opin Genet. Dev., 8: 140-146. PMID: 9610403

Luger, K., A.W. Mader, R.K. Richmond, D.F. Sargent and T.J. Richmond, 1997. Crystal structure of the nucleosome core particle at 2.8 a resolution. Nature, 389: 251-260. PMID: 9305837

Prescott, D.M., 1964. Methods in Cell Physiology. 1st Edn., Academic Press, New York, ISBN-10: 012564101X, pp: 465.

Probst, A.V., E. Dunleavy and G. Almouzni, 2009. Epigenetic inheritance during the cell cycle. Nat. Rev. Mol. Cell Biol., 10: 192-206. DOI: 10.1038/nrm2640

Sobel, R.E., R.G. Cook, C.A. Perry, A.T. Annunziato and C.D. Allis, 1995. Conservation of deposition-related acetylation sites in newly synthesized histones H3 and H4. Proc. Natl. Acad. Sci. USA., 92: 1237-1241. PMID: 7862667

Strahl, B.D. and C.D. Allis, 2000. The language of covalent histone modifications. Nature, 403: 41-45. DOI: 10.1038/47412

Thiriet, C. and J.J. Hayes, 1999. Histone proteins *in vivo*: Cell-cycle-dependent physiological effects of exogenous linker histones incorporated into *Physarum polycephalum*. Methods, 17: 140-150. DOI: 10.1006/meth.1998.0725

Thiriet, C. and P. Albert, 1995. Rapid and effective western blotting of histones from acid-urea-Triton and sodium dodecyl sulfate polyacrylamide gels: Two different approaches depending on the subsequent qualitative or quantitative analysis. Electrophoresis, 16: 357-361. PMID: 7607170

Wilhelm, M.L., B. Toublan, R. Jalouzot and F.X. Wilhelm, 1984. Histone H4 gene is transcribed in S phase but also late in G(2) phase in Physarum polycephalum. EMBO J., 3: 2659-2662. PMID: 16453570

Wolffe, A.P. and J.J. Hayes, 1999. Chromatin disruption and modification. Nucl. Acids Res., 27: 711-720. DOI: 10.1093/nar/27.3.711

Wolffe, A.P., 1994. Inheritance of chromatin states. Dev. Genet., 15: 463-470. PMID: 7834905

Wolffe, A.P., 1999. Chromatin: Structure and Function. 3rd Edn., Academic Press, San Diego, ISBN-10: 0080926606, pp: 447.