Dynamic Distribution of Thr3-Phosphorylated Histone H3 in CHO Cells in Mitosis

(mitosis / Thr3-phosphorylated H3 / immunofluorescence labelling / chromatin condensation)

H. ZHOU, L.-P. SONG, D.-W. LI, R.-M. LIU, J.-T. CHEN, X.-T. HUANG

Department of Biochemistry and Molecular Biology, College of Life Sciences, Nankai University, Tianjin, China

Abstract. The phosphorylation of histone H3 at Ser10, Ser28, Thr11 and Thr3 of the amino terminal has been proved related to mitosis of the mammalian cells. However, the function of the Thr3 phosphorylation of H3 remains unclear. In this study, indirect immunofluorescence labelling and laser confocal microscopy were used to examine the cellular dynamic distribution of Thr3-phosphorylated H3 at mitosis in CHO cells. The results showed that the Thr3 phosphorylation began at early prophase and spread throughout the chromosomes at late prophase. At metaphase, most of the Thr3-phosphorylated H3 was distributed along the entire chromosomal arms and maintained until early anaphase. During late anaphase and telophase, the fluorescent signal of Thr3-phosphorylated H3 disappeared from chromosomes. There was a precise spatial and temporal correlation between H3 phosphorylation of Thr3 and stages of chromatin condensation. The timing of Thr3 phosphorylation and dephosphorylation in mitosis were similar to that reported for Thr11 phosphorylation of H3. The Thr3-phosphorylated H3 localized along the arms of chromosomes during metaphase and early anaphase. It was different from the Ser10-phosphorylated H3, which localized at telomere regions, and Thr11-phosphorylated H3, which localized at centromeres. The results suggest that the Thr3 phosphorylation of histone H3 may play a specific role, which is different from Ser10 phosphorylation and Thr11 phosphorylation in mitosis.

Dynamic changes in the organization of chromatin are emerging as key regulators in nearly every aspect of DNA metabolism in eukaryotes. Histones, the main protein components of chromatin, undergo extensive post-translational modifications, particularly acetylation, methylation, phosphorylation, ubiquitination and ADP-ribosylation. The various modifications of histones serve different functions in the dynamic changes of chromatin during transcription, replication, DNA repair, recombination, and cell division (Fischle et al., 2003). One of the most thoroughly analysed modifications is the phosphorylation of histone H3. The H3 molecule is phosphorylated at four different sites, serines 10/28 (Wei et al., 1998; Gernand et al., 2003; Li et al., 2005) and threonines 3/11 (Preuss et al., 2003; Hara et al., 2004), all located at its N-terminal tail.

In mammals, phosphorylation of histone H3 at serines 10 and 28 seems to be crucial for cell cycle-dependent chromosome condensation and segregation, but has also been linked to activation of transcription (Thomson et al., 1999; Wei et al., 1999; Prigent and Dimitrov, 2003). Phosphorylation of H3 at Ser10 initiates in late G2, in the pericentromeric region, and spreads over the whole chromosome arms thereafter (Hendzel et al., 1997). It assumes its highest level during prometaphase/metaphase when chromosomes are highly condensed, and lasts until anaphase upon which it disappears. The strong correlation of H3 phosphorylation and chromosome condensation suggests a causal relationship. In contrast, the phosphorylation of histone H3 at Thr11 is restricted to the centromeres; it may, together with phosphorylated CENP-A (Zeitlin et al., 2001), serve as a recognition code for kinetochore assembly (Preuss et al., 2003).

In the present study, we used indirect immunofluorescence labelling on CHO (Chinese hamster ovary) cells with the antibody that was specific for phosphorylated histone H3 at Thr3 to examine the dynamic cellular distribution of this protein at mitosis. Our results showed that a precise spatial and temporal correlation existed between H3 phosphorylation of Thr3 and the process of chromatin condensation. The behaviour of Thr3 phosphorylation and dephosphorylation in mitosis was different from that of Ser10 and Thr11 phosphorylation and dephosphorylation. It suggested that the different modifications of the histone H3 implicate different biological function.

Received August 10, 2006. Accepted September 26, 2006.

This work was supported by "Chuangxin" Foundation of Nankai University.

Corresponding author: Xi-Tai Huang, Department of Biochemistry and Molecular Biology, College of Life Sciences, Nankai University, Tianjin, China. Tel.: 86-22-23508874; Fax: 86-22-23508874; e-mail: xthuang@public.tpt.tj.cn

Abbreviations: CHO – Chinese hamster ovary; DAPI – 4'-6-diamidino-2-phenylindole; FITC – fluorescein isothiocyanate; TRITC – tetramethyl rhodamine isothiocyanate.

Material and Methods

Cell culture

CHO cells were employed in this study. Cells were grown in RPMI medium 1640 (Gibco, Gaithersburg, MD) supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained in a humid chamber at 37°C with 5% CO_2.

Acid extraction of histones

Cells were synchronized by adding nocodazole (Sigma, St. Louis, MO) to the media to a final concentration of 0.6 μg/ml. After 18 hours, mitotic cells were collected by mechanical shaking and the other cells by digesting with 0.25% pancreatin as interphase cells as described (Houben et al., 1999). Cells were washed with ice-cold PBS (pH 7.4) two times and suspended in 5 volumes of lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 1.5 mM PMSF) for 30 min on ice. When centrifuged at 1,000 *g* for 10 min at 4 ºC, the histones in the pellets were extracted by 0.4 N H_2SO_4. Then the samples were centrifuged at 12,000 *g* for 10 min at 4 ºC after incubating on ice for 30 min. The soluble proteins were TCA precipitated on ice for 10 min, spun at 12,000 *g*, washed with acetone/0.1% HCl, washed twice more with acetone, and resuspended in loading buffer.

SDS-PAGE and Western blotting

The samples of acid soluble proteins were analysed by 15% SDS-PAGE. The proteins in another parallel gel were transferred onto nitrocellulose membranes according to standard protocols, and then the membranes were blocked in 3% non-fat dry milk in TBS for 1 h. The anti-phospho-H3 Thr3 antibody (Upstate Biotechnology, Lake Placid, NY) was used at a dilution of 1 : 2,000. After washing, bound antibodies were detected with a horseradish peroxidase-conjugated secondary antibody and visualized by diaminobenzidine (DAB, Golden Bridge Biotechnology, Beijing, China).

Immunofluorescence

A succession labelling method was performed for co-detection of Thr3-phosphorylated histone H3 and α-tubulin. For immunofluorescence, CHO cells were cultured on coverslips for 2 days. The cells were fixed in 4% paraformaldehyde at room temperature for 10 min. After washing with PBS, cells were treated with 0.25% TritonX-100 for 5 min at room temperature. Then specimens were treated with 3% BSA in PBS for 45 min at room temperature and subsequently incubated with the anti-phospho-histone H3 Thr3 antibody (Upstate Biotechnology) diluted at 1 : 100 and anti-α-tubulin antibody (Zymed Laboritories, South San Francisco, CA) diluted at 1 : 200) in a humid chamber at 4 ºC overnight. As secondary antibodies, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG were diluted in 1 : 100 with blocking solution for 45 min at room temperature. After final washing, the cells on the coverslips were counterstained with 4'-6-diamidino-2-phenylindole (DAPI) and mounted.

Confocal laser microscopy

Confocal scanning microscopy was performed using a TCS-NT Leica microscope (Lasertechnik, Heidelberg, Germany). An argon-krypton ion laser was adjusted at an excitation wavelength of 345 nm, 488 nm and 568 nm. Fluorescent images were captured in sequential mode. Serial optical sections were taken. Selected three sections were then processed to produce single composite, colour-merged overlay images.

Results

SDS-PAGE and Western blot analysis of Thr3-phosphorylated H3

SDS-PAGE and Western blot analysis were carried out to compare the histone-specific phosphorylation at interphase and metaphase. As shown in Fig. 1, the acid soluble protein samples extracted from synchronized interphase and metaphase cells of CHO were separated into a group of bands by electrophoresis. The core histones H2A, H2B, H3, H4 bands line up on the range of molecular weight 14.4 kD – 20.1 kD (Fig. 1A). Western blotting analysis shows that there was no band found in column I, and only one band in column M that is Thr3-phosphorylated H3 detected by the antibody against Thr3-phosphorylated H3 (Fig. 1B). The results suggest that the antibody is highly selective for Thr3-phosphorylated H3, and the H3 phosphorylation is a special event in mitosis of the cells.

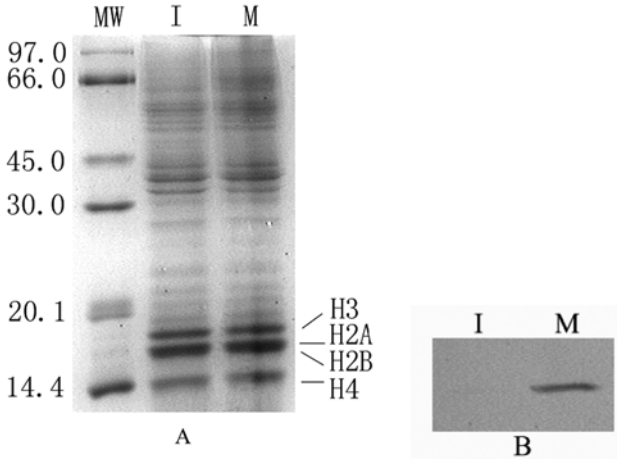

Fig. 1. Histones H3 were analysed by SDS-PAGE with Coomassie brilliant blue (A) and Western blotting (B) MW: protein marker weight; I: interphase acid-soluble nuclear protein; M: nocodazole blocks mitotic acid-soluble nuclear protein.

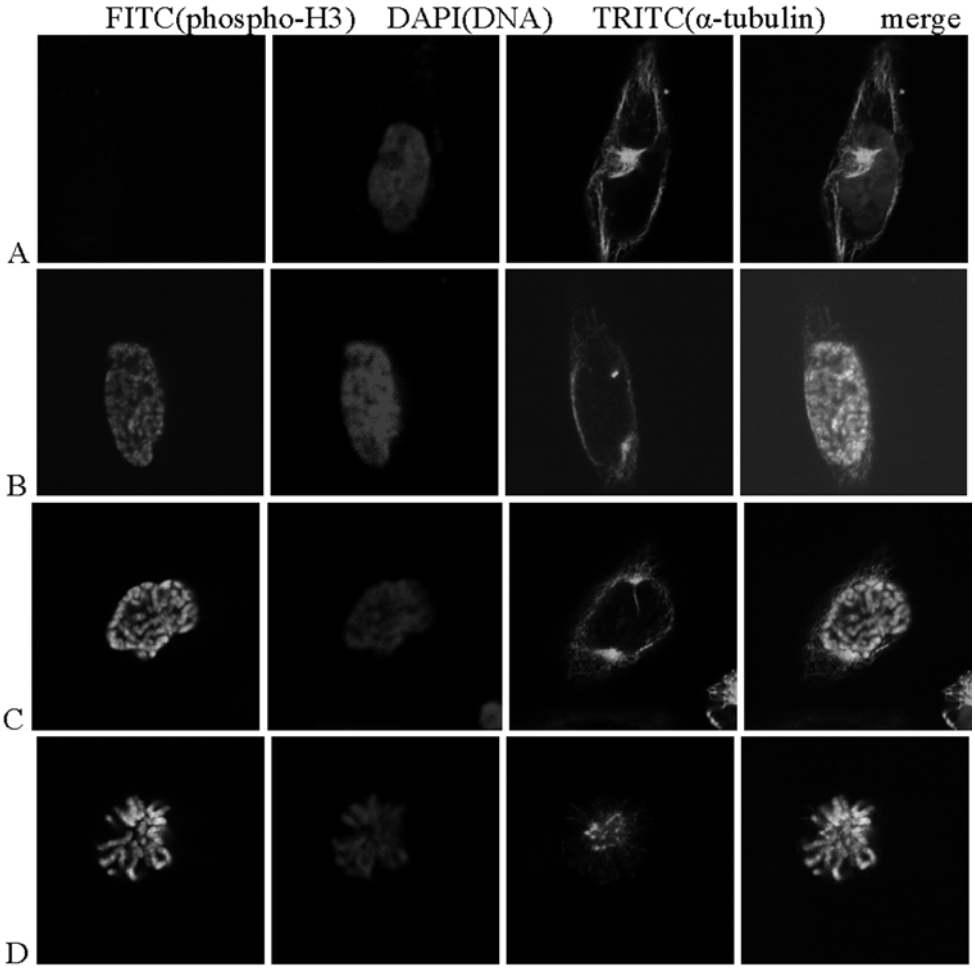

Fig. 2a. Dynamic distribution of Thr3-phosphorylated histone H3 during mitosis in CHO cells. A interphase; B early prophase; C prophase; D early metaphase.

Distribution of Thr3-phosphorylated H3 in CHO cells during mitosis

To confirm the dynamic distribution pattern of phosphorylated H3 in CHO cells during mitosis, the cells were subjected to immunofluorescence examination. The results showed that no labelling with anti-phosphohistone H3 (Thr3) antibodies was observed in the interphase cells (Fig. 2a, A), and Thr3 phosphorylation was detected in cells with chromosome condensation concurrently at early prophase cells (Fig. 2a, B). Upon progression of mitosis, Thr3 phosphorylation of histone H3 was enhanced at whole arms of the condensed chromosomes collected in the central region of the metaphase plate (Fig. 2a, b, D-F). The fluorescence signal on the chromosomes was maintained until early anaphase (Fig. 2b, G), but the intensity of staining declined substantially during anaphase and was neither present on decondensing chromosomes in telophase (Fig. 2b, G) nor in the midbody of cytokinesis (Fig. 2b, J). Overall, the timing of Thr3 phosphorylation and dephosphorylation was similar to that reported for Ser10-phosphorylated H3 at prophase and metaphase. In contrast, there were differences in the location of the two modifications. The signal of Thr3 phosphorylation spread following the arms of the chromosomes, but was absent from centromeres and did not occur at kinetochore or midbody. The results suggest that Thr3 phosphorylation of histone H3 is coupled to the chromatin condensation. The evidence suggests that the Thr3 phosphorylation plays a different role from Ser10 and Thr11 phosphorylation of histone H3 in mitosis of the CHO cells.

Discussion

During cell division of mammalian cells histone H3 is phosphorylated not only at Ser10 (Houben et al., 1999; Kaszas and Cande, 2000; Manzanero et al., 2000) and Ser28 (Gernand et al., 2003), but also at Thr11 (Preuss et al., 2003) and Thr3 (Hara et al., 2004). Remarkably, although the cell cycle-dependent process of histone H3 phosphorylation at Ser10/28 and at Thr11/3 seems to be highly conserved during evolution of animals, the distribution of H3 carrying these individual modifications is quite different (Houben et al., 2005). In mammalian chromosomes, phoshorylated

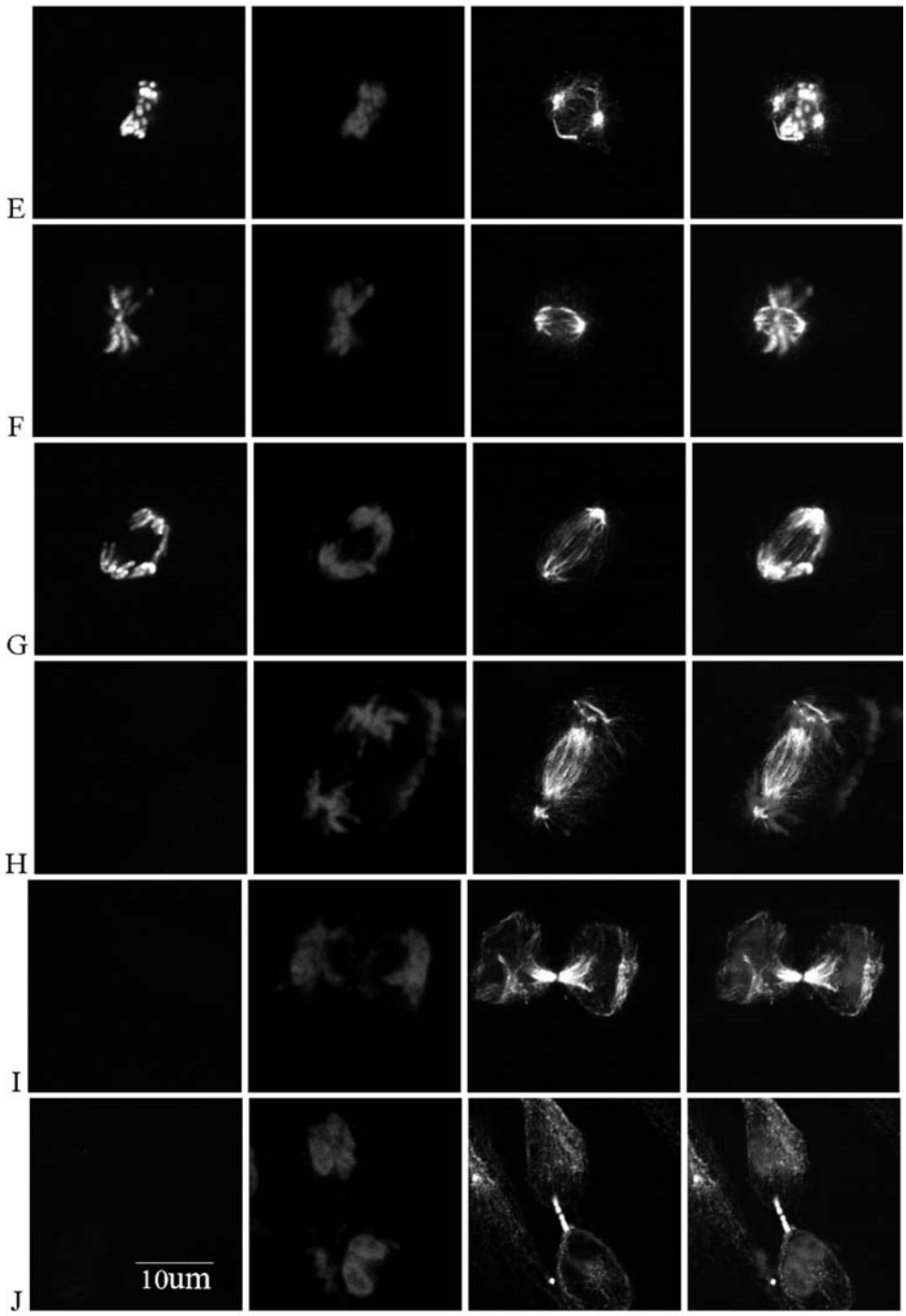

Fig. 2b. Dynamic distribution of Thr3-phosphorylated histone H3 during mitosis in CHO cells. E, F metaphase; G anaphase; H telophase; I late telophase; J cytokinesis.

Ser10 and Ser28 is distributed along the chromosomal arms at prophase (Hendzel et al., 1997; Houben et al., 1999; Crosio et al., 2002) and phoshorylated Thr11 is confined to centromeric chromatin (Preuss et al., 2003). Our results showed that the timing of Thr3 phosphorylation and dephosphorylation was similar to that reported for Ser10-phosphorylated H3 (Hendzel et al., 1997; Li et al., 2005), but there were differences in the locations of the two modified histones. Thr3-phosphorylated H3 was distributed at the arms of the condensed chromosomes at metaphase, while Ser10-phosphorylated H3 localized at the periphery of the metaphase plate (Houben et al., 1999; Li et al., 2005), i.e. the Ser10-phosphorylated H3 localized at ends of the chromosomes collected in the central spindle at metaphase. During late anaphase and telophase no labelling with anti-phospho-H3 Thr3 antibodies was observed, while Ser10-phosphorylated H3 takes part in the formation of

midbody and plays a crucial role in cytokinesis (Li et al., 2005). The distribution of Thr3-phosphorylated histone H3 was different from that reported for Thr11-phosphorylated H3, which is confined to centromeric chromatin (Preuss et al., 2003). The distribution of Thr3-phosphorylated histone H3 did not occur at centromeres.

The evidence showed that the phosphorylation of histone H3 at all sites, Ser10, Ser28, Thr11 and Thr3 begins at pre-prophase and reaches the maxium at late prophase or metaphase (Hans and Dimitrov, 2001), coinciding with the process of the chromosome condensation during mitosis. This situation was also detected by biochemical analysis (Bradbury et al., 1973). It means that all phosphorylation events are related to the chromosome condensation. The specific localization of the phosphorylated histones implicates different functions in mitosis. It is true that the "histone code" hypothesis obeys the phosphorylation of histone H3 during mitosis in mammalian cells. Obviously, the details of the relationship between the functions and different sites of phosphorylation should be thoroughly investigated.

References

Bradbury, E. M., Inglis, R. J., Matthews, H. R., Sarner, N. (1973) Phosphorylation of very lysine-rich histone in Physarum polycephalum – correlation with chromosome condensation. *Eur. J. Biochem.* **33**, 131-139.

Crosio, C., Fimia, G. M., Loury, R., Kimura, M., Okano, Y., Zhou, H., Sen, S., Allis, C. D., Sassone-Corsi, P. (2002) Mitotic phosphorylation of histone H3: spatio-temporal regulation by mammalian Aurora kinases. *Mol. Cell Biol.* **22**, 874-885.

Fischle, W., Wang, Y., Allis, C. D. (2003) Histone and chromatin cross-talk. *Curr. Opin. Cell Biol.* **15**,172-183.

Gernand, D., Demidov, D., Houben, A. (2003) The temporal and spatial pattern of histone H3 phosphorylation at serine 28 and serine 10 is similar in plants but differs between mono- and polycentric chromosomes. *Cytogenet. Genome Res.* **101**, 172-176.

Hans, F., Dimitrov, S. (2001) Histone H3 phosphorylation and cell division. *Oncogene* **20**, 3021-3027.

Hara, P., Yolanda, M., Niki, K. (2004) Mitotic phosphorylation of histone H3 at threonine 3. *FEBS Lett.* **560**, 39-44.

Hendzel, M. J., Wei, Y., Mancini, M. A., Van Hooser, A., Ranalli, T., Brinkley, B. R., Bazett-Jones, D. P., Allis, C. D. (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.

Houben, A., Wako, T., Furushima-Shimogawara, R., Presting, G., Künzel, G., Schubert, I., Fukui, K. (1999) The cell cycle dependent phosphorylation of histone H3 is correlated with the condensation of plant mitotic chromosomes. *Plant J.* **18**, 675-679.

Houben, A., Demidov, D., Rutten, T., Scheidtmann, K. H. (2005) Novel phosphorylation of histone H3 at threonine 11 that temporally correlates with condensation of mitotic and meiotic chromosomes in plant cells. *Cytogenet. Genome Res.* **109**, 148-155.

Kaszas, E., Cande, W. Z. (2000) Phosphorylation of histone H3 is correlated with changes in the maintenance of sister chromatid cohesion during meiosis in maize, rather than the condensation of the chromatin. *J. Cell Sci.* **113**, 3217-3226.

Li, D. W., Yang, Q., Chen, J. T., Zhou, H., Liu, R. M., Huang, X. T. (2005) Dynamic distribution of Ser-10 phosphorylated histone H3 in cytoplasm of MCF-7 and CHO cells during mitosis. *Cell Res.* **15**, 120-126.

Manzanero, S., Arana, P., Puertas, M.J., Houben, A. (2000) The chromosomal distribution of phosphorylated histone H3 differs between plants and animals at meiosis. *Chromosoma* **109**, 308-317.

Preuss, U., Landsberg, G., Scheidtmann, K. H. (2003) Novel mitosis-specific phosphorylation of histone H3 at Thr11 mediated by Dlk/ZIP kinase. *Nucleic Acids Res.* **31**, 878-885.

Prigent, C., Dimitrov, S. (2003) Phosphorylation of serine 10 in histone H3, what for? *J. Cell Sci.* **116**, 3677-3685.

Thomson, S., Clayton, A. L., Hazzalin, C. A., Rose, S., Barratt, M. J., Mahadevan, L. C. (1999) The nucleosomal response associated with immediate-early gene induction is mediated via alternative MAP kinase cascades: MSK1 as a potential histone H3/HMG-14 kinase. *EMBO J.* **18**, 4779-4793.

Wei, Y., Mizzen, C. A., Cook, R. G., Gorovsky, M. A., Allis, C. D. (1998) Phosphorylation of histone 3 serine 10 is correlated with chromosome condensation during mitosis and meiosis in Tetrahymena. *Proc. Natl. Acad. Sci. USA* **95**, 7480-7484.

Wei, Y., Yu, L., Bowen, J., Gorovsky, M. A. and Allis, C. D. (1999) Phosphorylation of histone H3 is required for proper chromosome condensation and segregation. *Cell* **97**, 99-109.

Zeitlin, S. G., Barber, C. M., Allis, C. D., Sullivan, K. F., Sullivan, K. (2001) Differential regulation of CENP-A and histone H3 phosphorylation in G2/M. *J. Cell Sci.* **114**, 653-661.