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H3S10 Phosphorylation Marks Constitutive Heterochromatin During Interphase in Early Mouse Embryos Until the 4-Cell Stage

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Abstract. Phosphorylation of histone H3 at Ser10 (H3S10P) has been linked to a variety of cellular processes, such as chromosome condensation and gene activation/silencing. Remarkably, in mammalian somatic cells, H3S10P initiates in the pericentromeric heterochromatin during the late G2 phase, and phosphorylation spreads throughout the chromosomes arms in prophase, being maintained until the onset of anaphase when it gets dephosphorylated. Considerable studies have been carried out about H3S10P in different organisms; however, there is little information about this histone modification in mammalian embryos. We hypothesized that this epigenetic modification could also be a marker of pericentromeric heterochromatin in preimplantation embryos. We therefore followed the H3S10P distribution pattern in the G1/S and G2 phases through the entire preimplantation development in *in vivo* mouse embryos. We paid special attention to its localization relative to another pericentromeric heterochromatin marker, HP1 β and performed immunoFISH using specific pericentromeric heterochromatin probes. Our results indicate that H3S10P presents a remarkable distribution pattern in preimplantation mouse embryos until the 4-cell stage and is a better marker of pericentromeric heterochromatin than HP1 β . After the 8-cell stage, H3S10P kinetic is more similar to the somatic one, initiating during G2 in chromocenters and disappearing upon telophase. Based on these findings, we believe that H3S10P is a good marker of pericentromeric heterochromatin, especially in the late 1- and 2-cell stages as it labels both parental genomes and that it can be used to further investigate epigenetic regulation and heterochromatin mechanisms in early preimplantation embryos.

Key words: Aurora, Embryo, Heterochromatin, Histone, Phosphorylation

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Nowadays, it is known that the way in which the chromatin is positioned in the nucleus inside the cell can direct all the nuclear and chromatin functions essential for the cell cycle and development [1–3].

It is believed that chromatin organization and nuclear architecture are governed by epigenetic mechanisms that are not random. Therefore, the chromatin and nucleus are controlled by these epigenetic modifications to achieve such a spatial organization and structure leading to the creation of functional nuclear compartments during cell cycle progression and development [4, 5].

Epigenetic modifications have been the focus of intense investigation. This includes DNA methylation and posttranslational histone modifications such as methylation, acetylation, phosphorylation, ubiquitination and ADP ribosylation. It is through these processes that chromatin and nuclear organization can be modulated to change gene expression. Many epigenetic modifications have been studied in different organisms, and it is believed that some of them are conserved in a variety of metazoan, fungi, plants and protozoa [6].

It is known that the same histone modification can have different

functions depending on the samples studied, implying that there are some specific factors orchestrating the event. Histone phosphorylation of H3 at Ser10, for instance, has been linked to a variety of cellular processes, such as chromosome condensation and segregation, regulation of gene expression (activation of transcription or gene silencing), apoptosis and DNA damage repair [7]. This modification is clearly involved in cell cycle progression [8]. In mammalian cells, this modification initiates in the late-replicating/early condensing heterochromatin surrounding the centromeres during the G2 phase and is coincident with the initiation of chromosome condensation of centromeres [9]. At the beginning of mitosis, in prophase, this phosphorylation spreads throughout the euchromatin in the chromosome arms [10]. This distribution pattern is maintained until anaphase, when histone H3 gets dephosphorylated. Because of this distribution pattern and timing, phosphorylation of histone H3 at Ser10 (H3S10P) is often referred to as a marker of mitosis. On the other hand, it is believed that H3S10P can also function as an “open-chromatin factor” in interphase for a subset of genes, allowing many different elements to access the chromatin, keeping it in a more open state enabling transcription [11–13]. Remarkably, H3S10P seems to be highly dependent on posttranslational modification of neighboring amino acid residues. This is the case of H3 (tri) methylation at Lys9 (H3K9me3), which gave rise to the binary switch hypothesis. It is, for example, known that constitutive heterochromatin is enriched with H3K9me3 and that the heterochromatin protein HP1 β is recruited

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to these sites, thus mediating heterochromatin organization and gene silencing [14]. Conversely, it was shown that when cells start mitosis, the same histone H3 is phosphorylated at Ser10, thereby promoting the ejection of HP1 β [15]. This probably facilitates the release of tightly bound factors from the chromatin, inducing its rearrangement to higher-order structures that are required for chromosome condensation [16].

It has been shown that mitosis specific H3S10 phosphorylation occurs via Aurora B kinase [17]. The Aurora kinase family consists of evolutionary conserved serine/threonine kinases that are important for centrosome duplication, mitotic spindle assembly, chromosome condensation, alignment and segregation [18]. The mammalian Aurora family includes three protein kinases referred as Aurora A, B and C [19]. Among these kinases, Aurora B is said to function in both early and late mitotic events, including chromosomes segregation and cytokinesis [20]. Immunofluorescence studies have shown a temporal and spatial relationship between Aurora B, chromosome condensation and phosphorylation of H3S10 [21]. It has also been shown that in opposition to the kinases, mitotic histone H3 phosphorylation is also regulated by at least one phosphatase protein [22]. Work carried out with *Xenopus* egg extract has shown that the phosphatase PP1C can directly dephosphorylate histone H3 at Ser10 and that its inhibition activates Aurora B, increasing the levels of H3S10P [23]. Thus, a balance between kinases and phosphatases activity appears to be in place to regulate histone H3 phosphorylation [24].

Considerable studies have been carried out about phosphorylation of histone H3 at Ser10 in different organisms; however, there is little information about this histone modification in mammalian embryos [25–27]. These studies showed that H3S10P behavior in early mouse embryos differs from somatic cells, with genome-wide phosphorylation in interphase. In fact, preimplantation embryos are very interesting to investigate when it comes to chromatin organization simply because of their unique chromatin architecture [28, 29]. To form an embryo, two specialized cells, the gametes, need to undergo genome reprogramming, meaning that their chromatin structure needs to be reshaped in order to create a totipotent zygote. Among the reprogrammed nuclear compartments, there is the pericentromeric heterochromatin that tends to form chromocenters in somatic cells [30]. However, in mammalian preimplantation embryos, pericentromeric heterochromatin is organized in rings around the nucleolar precursor bodies (NPBs) in the pronuclei of 1-cell embryos. It is only at the 2-cell stage that the pericentromeric heterochromatin of different chromosomes clusters together, forming the characteristic heterochromatin domains called chromocenters [31, 32]. Two very well established markers of pericentromeric heterochromatin are the epigenetic modification H3K9me3 and the protein HP1 β , which have been widely used to study chromatin arrangement and nuclear organization in preimplantation embryos [33–38]. However, there is a clear parental asymmetry in early preimplantation embryos until the 4-cell stage, and H3K9me3 (as well as HP1 β) constitutes the major marker for maternal pericentric heterochromatin only. Other epigenetic markers characterize pericentromeric heterochromatin (H4K20me3 or H3K27me3), but none of them have been observed in both parental inherited genomes [36].

Previous studies suggested that perinucleolar heterochromatin in 1-cell embryos could contain H3S10P marker but did not investigate

this hypothesis in details [25–27, 39]. As H3S10P correlates to heterochromatin organization/condensation in different cells and organisms, we decided to further investigate H3S10P in preimplantation mouse embryos with a specific focus on pericentromeric heterochromatin. Therefore, we followed the spatial and temporal pattern of phosphorylation of histone H3 at Ser 10 in *in vivo* mouse embryos from fertilization to the blastocyst stage. We compared its nuclear localization with the HP1 β distribution and performed immunoFISH as well, using specific pericentromeric heterochromatin DNA probes. Our results clearly indicate that H3S10P staining partially colocalizes with HP1 β in 1- and 2-cell embryos, even in interphase, and overlaps with the pericentromeric DNA probes. It labels the heterochromatin rings around NPBs in both parental pronuclei of 1-cell embryos and later on also colocalizes on the chromocenters appearing during the 2-cell stage. Remarkably, we also observed that interphasic H3S10P staining on heterochromatin regions disappears after the 4-cell stage, concomitantly with interphasic Aurora B staining.

Based on these findings, we believe that H3S10P is a good marker of pericentromeric heterochromatin and that it can be used to further investigate epigenetic regulation and heterochromatin mechanisms in early preimplantation embryos.

Materials and Methods

Animal care and handling were carried out according to European regulations on animal welfare.

Embryo production

C57/CBA F1 female mice, 6–8 weeks of age, were superovulated with 5 IU of PMSG (pregnant mare serum gonadotropin) followed by injection with 5 IU of hCG (human chorionic gonadotropin) 48 hours later. For *in vivo* embryo production, females were placed together with males (one by one) after hCG administration. Embryos were collected (at either the 1- or 2-cell stage) in M2 medium and then cultured in M16 at 37 C in a humidified atmosphere containing 5% CO₂ until fixation for immuno-fluorescent staining. Fertilization occurred at about 12 h after hCG injection, which was used as a reference point for embryonic development (hours post hCG, i.e., hphCG).

Embryo immunofluorescent staining

The following antibodies were purchased from the indicated companies: rabbit polyclonal antibody against H3S10P (Abcam #5176, Cambridge, UK), mouse monoclonal antibody against HP1 β (Euromedex #MOD-1A9-AS, Souffelweysheim, France), rabbit polyclonal antibody against Aurora B (Santa Cruz Biotechnology #ARK-2 H-75, Heidelberg, Germany), FITC-conjugated anti-rabbit and TRITC-conjugated anti-mouse secondary antibodies (Jackson ImmunoResearch, Interchim, Montluçon, France).

Embryos at different developmental stages were fixed with 4% PFA (paraformaldehyde) in PBS at 4 C overnight and permeabilized with 0.5% Triton X-100 (15 min, room temperature). The fixed embryos were blocked in PBS containing 2% BSA (1 h at room temperature) and incubated overnight at 4 C with the specific first antibody diluted in 2% PBS-BSA (H3S10P 1:300; HP1 β 1:200; Aurora B 1:100). The embryos were then washed twice in PBS to remove any first antibody excess. After this step, the embryos were incubated with fluorescent

labeled secondary antibody for 1 h at room temperature (1:200). DNA counterstaining was performed with ethidium homodimer 2 (Life Technologies, Saint Aubin, France). Embryos were then postfixed with 2% PFA for 15 min at room temperature, washed and mounted on slides with an antifading agent, Citifluor, under coverslips.

ImmunofISH

FISH pericentromeric DNA probes (major satellites probes) were prepared by PCR on genomic mouse DNA using the primers 5'-CATATTCAGGTCCTTCAGTGTGC-3' and 5'-CACTTTAGGACGTGAAATATGGCG-3' followed by Cy5-labeling through random priming (BioPrime Array CGH Genomic Labeling System, Invitrogen).

For this procedure, H3S10P immunostaining was performed first, and the immunolabeled embryos were then further processed for 3D-FISH. All steps were performed at room temperature unless otherwise specified. The zona pellucida was removed with Tyrode's acid (Sigma Aldrich, Saint Quentin Fallavier, France). The embryos were then rinsed in PBS and gently plated with a minimum amount of PBS on glass slides to allow adherence. They were then postfixed in 4% PFA for 30 min, permeabilized for 30 min in 0.5% Triton X-100 and rinsed once for 5 min in 2x saline-sodium citrate (SSC) (pH 6.3). RNA digestion was performed by incubation with 200 µg/ml RNase (Sigma) in 2xSSC for 30 min at 37°C. After rinsing twice for 5 min in 2xSSC at room temperature, the slide was equilibrated in the hybridization buffer (50% formamide, SSC 2X, Denhardt 1X, 40 mM NaH₂PO₄, 10% dextran sulfate) for 1 to 2 h. Denaturation was performed separately for probes and embryos during 10 min at 85°C in the hybridization buffer. After deposition of the probes onto the slide, embryos were then placed in an humidified chamber at 37°C for 24 h. After rinsing twice in 2xSSC at 42°C, samples were permeabilized in 0.5 Triton X-100 for 10 min, rinsed with PBS for 5 min and DNA counterstained with propidium iodide for 15 min at 37°C (Sigma, 1 µg/ml). The embryos were then postfixed in 2% PFA for 15 min and a last wash was done in PBS for 5 min.

The immunofISH experiments were repeated two times with not less than 15 embryos analyzed per time point.

High resolution microscopy

Three dimensionally preserved embryos were observed either with an ApoTome fluorescent microscope or a confocal laser scanning microscope (Zeiss LSM 510, Le Pecq, France). Immunofluorescent stainings were observed using a Carl Zeiss Axio Observer fluorescence microscope equipped with an ApoTome slider (MIMA2 Platform, INRA, Jouy-en-Josas, France). The samples were observed with a 63x Plan-Neofluar oil objective (NA 1.3), and digital optical sections were collected every 1 µm using a Z-series acquisition feature. ImmunofISH experiments were visualized with a Zeiss LSM 510 confocal laser scanning microscope equipped with an oil-immersion objective (Plan Apochromat 63X NA 1.4), and imaging was performed with the 488-, 535- and 633-nm wavelengths of the lasers. Entire embryos were scanned with a distance of 0.37 µm between light optical sections.

Quantitative analyses of H3S10P levels and total DNA contents were estimated using the ImageJ software by quantifying fluorescent signals as follows: 1) the area of each nucleus was outlined manually,

and the mean fluorescence intensity was measured for both H3S10P and EthD-2 images. 2) The mean fluorescence intensities were then divided by the acquisition times of the corresponding signal. 3) These corrected mean fluorescence intensities were multiplied by the nuclear areas to obtain the total fluorescence intensities for both H3S10P and EthD-2. Finally, H3S10P levels were divided by total DNA contents (EthD2 signal) to calculate normalized H3S10P levels.

Results

Distribution pattern of H3S10P from fertilization to implantation

To begin with, we followed the spatial and temporal pattern of H3S10P by immunostaining in *in vivo* fertilized mouse embryos from the 1-cell stage (fertilization) to the blastocyst stage (implantation). Where possible, embryos were fixed at two different time points during the embryonic cell cycle, i.e., in the G1/S-phase (G1 is often very short—except at the 1-cell—and difficult to distinguish from the S phase) or in G2 and then during mitosis [40]. As shown in Fig. 1, we observed an H3S10P signal in the nucleoplasm of interphasic and mitotic blastomeres until the 4-cell stage. During the first embryonic cycle, histone H3 phosphorylation at Ser10 was present in both male and female pronuclei. H3S10P displayed homogeneous staining in the whole nucleoplasm, and intensely marked the periphery of the NPBs (nucleolar precursor bodies) appearing between the S phase (~18 hphCG, n=83) and G2 phase (~28 hphCG, n=56) (Fig. 1A and 1B). During the second cell cycle, the H3S10P signal was also present in the nucleoplasm, and an intense signal was still seen around NPBs of early 2-cell embryos in G1/S phase (~36 hphCG, n=37) (Fig. 1D). Later on, during the G2 phase (~48 hphCG, n=73), pronounced H3S10P staining appeared in foci (Fig. 1E) not associated with any nucleoli, which will then become transcriptionally active [41, 42]. During the first and second mitosis, phosphorylation of histone H3 at Ser10 was detected on the whole chromosomes following a similar pattern to that of somatic cells (Fig. 1C and 1F).

During the 4-cell stage, H3S10P was clearly detected in the interphasic blastomeres uniformly labeling the nucleoplasm and staining more strongly specific regions, i.e., the nucleolar periphery in G1/S phase (~50 hphCG, n=94) and isolated foci in G2 (~58 hphCG, n=86) (Fig. 1G and 1H). However, H3S10P labeling began to differ from the earlier embryonic stages. The diffuse nucleoplasm staining and the perinucleolar ring intensity started to weaken when compared with previous stages, especially during the G1/S phase of the cell cycle.

From the 8-cell to blastocyst stage, H3S10 showed exactly the same phosphorylation/ dephosphorylation kinetics seen in somatic cells. Early interphasic H3S10P staining did not exist anymore, and intense foci only appeared in the G2-phase spreading throughout the euchromatin in the chromosome arms upon mitosis (Fig. 1I to 1O). In 8-cell embryos for instance, when blastomeres were still synchronized, no labeling of H3S10P was seen at all in early interphase (~63 hphCG, n=59) (Fig. 1J), and foci were only noticed at the end of the cell cycle, during the G2 phase (~77 hphCG, n=27) (Fig. 1K). Upon the transition from the 8- to 16-cell stage, labeling was clearly observed in prophase/metaphase/anaphase but not in telophase (Fig. 1L), as already described in mitotic somatic cells. In the more advanced

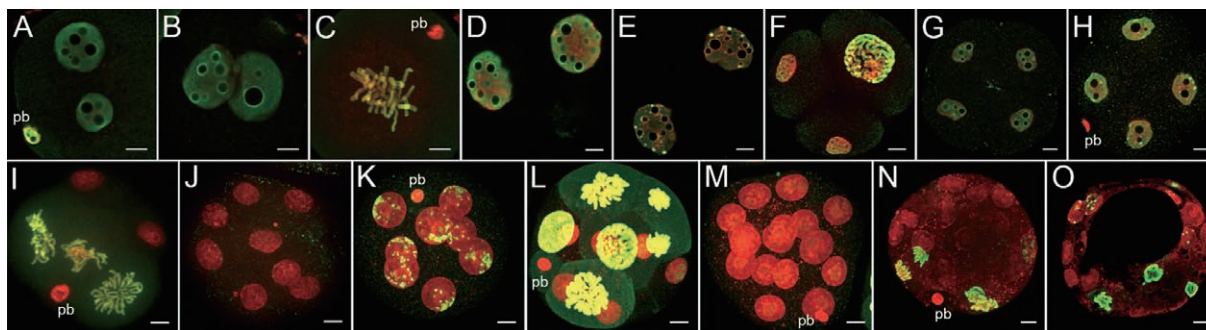


Fig. 1. Distribution of H3S10P during the entire mouse preimplantation development. Immunofluorescent staining of H3S10P (green) and DNA (red) was performed on preimplantation embryos at different time points: at 18 hphCG (A) and 28 hphCG (B) for the 1-cell stage; 30 hphCG for metaphase of the first mitosis (C); 36 hphCG (D) and 48 hphCG (E) for the 2-cell stage; 50 hphCG for the 3-cell (F) and the early 4-cell stages (G); 58 hphCG for the late 4-cell stage (H); 63 hphCG for the transition from the 5-cell (I) to the early 8-cell stage (J); 77 hphCG for the late 8-cell stage (K); 79 hphCG for the 12-cell stage (L); 79 hphCG for the 16-cell stage (M); 89 hphCG for the morula stage (N); and 96 hphCG for the blastocyst stage (O). All the embryos were observed on an inverted ApoTome microscope with 3 different settings; H3S10P acquisition was performed during 100 ms for G1/S stages, 50 ms for G2 stages and 25 ms for mitosis. Each time, z-series were performed. Representative single z-sections are shown here for 1-cell to 4-cell embryos as well as for morulae and blastocysts. Z-series projections are shown for 5-cell to 16-cell embryos. Note that remains of the second polar body are often present (pb). Scale bars: 10 μ m.

stages like morula ($n=51$) and blastocyst ($n=99$), blastomeres were no longer synchronized and various H3S10P staining patterns could be observed simultaneously: no staining, foci or whole-chromosome staining that most probably illustrate Ser10 phosphorylation during cell cycle progression as in somatic cells (Fig. 1N and 1O).

Peculiar pattern of H3S10P in 1-cell and 2-cell stage embryos

Since the nuclear distribution pattern of H3S10P in early preimplantation embryos seemed very peculiar and different from the one observed in somatic cells, we pursued our observations and analyzed more developmental time points at the 1-cell and 2-cell stages (every two-to-three hours from 18 to 48 hphCG: $n=485$ at the 1-cell stage, $n=83$ at mitosis and $n=250$ at the 2-cell stage). Because we used *in vivo* fertilized embryos, some asynchrony can be observed between several mice and describing the 1-cell stage becomes difficult. We therefore used the nomenclature that we previously set up to describe the morphological changes of the pronuclei occurring during the first cell-cycle [43] and classified embryos from PN0 (fertilization) to PN5 (end of the 1-cell stage).

As shown on Fig. 2, an H3S10P signal was present shortly after fertilization in both sets of parental inherited genomes, i.e., the decondensing sperm head and the maternal chromatin going through the last steps of meiosis II (Fig. 2A). H3S10P staining was quite homogeneous, although some dense foci could be observed in the maternal genome. This staining pattern was maintained upon formation of the pronuclei (PN1, Fig. 2B). At PN2, diffuse nucleoplasm H3S10P labeling was still present, but some accumulations appeared at the periphery of the NPBs (Fig. 2C). This perinucleolar staining became more intense in PN3 stage (that corresponds to the beginning of the S phase) and formed complete rings around the NPBs (Fig. 2D). The same signal was observed in PN4 and PN5 embryos, i.e., upon exit of the S phase and during the G2 phase (Fig. 2D, 2E and 2F). During mitosis of the first embryonic cycle, H3S10P staining covered the entire length of all chromosomes arms (Fig. 2G and 2H). The quantification of the H3S10P signal (Fig. 3) underlines

a gradual dephosphorylation of H3S10 during the G1/S phases as previously described [25]. This decrease is then followed by *de novo* phosphorylation at the end of the cell cycle.

After formation of the nuclei in early 2-cell stage (~ 32 hphCG, $n=28$, Fig. 2I), a diffuse H3S10P staining was observed within the nucleoplasm and on the perinucleolar rings. A similar staining was observed in the S-phase, with slightly more intense perinucleolar rings (~ 36 hphCG, $n=37$, Fig. 2J). It is only at the beginning of the G2 phase (~ 42 hphCG, $n=60$, Fig. 2K) that isolated foci of H3S10P, not attached to any NPBs, appeared. Finally, by the end of the 2-cell stage, H3S10P perinucleolar rings had almost completely disappeared; conversely, at that time the number of H3S10P nucleoplasmic foci was much more numerous (~ 48 hphCG, $n=73$, Fig. 2L).

Colocalization with the pericentromeric heterochromatin marker, HP1 β

We observed that the immunodetection of H3S10P in early preimplantation embryos is quite different from somatic cells and that its most intriguing features are the perinucleolar staining detected in the G1 phase at the 1- and 2-cell stages and the transition to intense foci, often not related to any nucleoli, during the G2 phase of the second cell cycle. This correlates with the distribution of pericentromeric heterochromatin around NPBs at the 1-cell stage and the formation of the so-called chromocenters, when the pericentromeric regions of several chromosomes assembled together, as previously described by ourselves and others [31, 32, 35]. Here, we used the well-established pericentromeric marker HP1 β to further characterize the H3S10P distribution in relation to this type of heterochromatin and performed double immunostaining.

In pronuclear stage embryos (20 hphCG, $n=31$), H3S10P and HP1 β uniformly label the nucleoplasm of both pronuclei. Interestingly, colocalization of both markers within the heterochromatin rings around the NPBs is only seen in the maternal pronucleus (Fig. 4A). We were indeed unable to detect the same perinucleolar signal for HP1 β in the paternal PN as previously described [31–33]. The striking

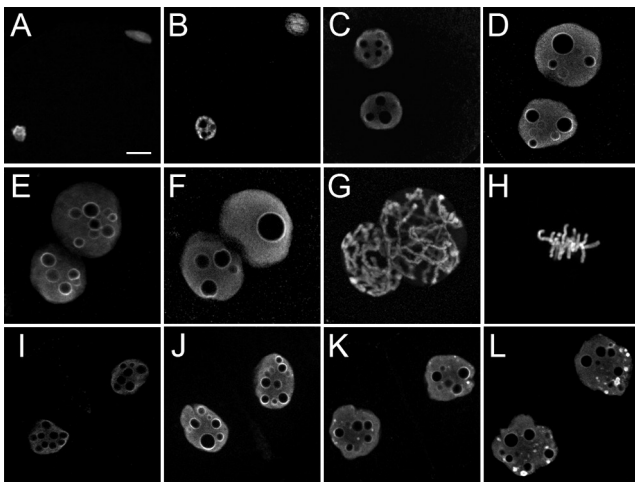


Fig. 2. Distribution of H3S10P during the first and second cell cycle in mouse embryos. Immunofluorescent staining of H3S10P was performed on 1- and 2-cell stage embryos. At the 1-cell stage (from 16 hphCG until 29 hphCG), we classified the embryos into PN0 (A, just after fertilization), PN1 (B, formation of the pronuclei), PN2 (C, pronuclei increase in size but remain at the nuclear periphery), PN3 (D, large pronuclei now in the center of the cytoplasm), PN4 (E, large pronuclei close to each other) and PN5 (F, pronuclei are then apposed). For all these PN stages, we chose to turn the images in order to have the paternal pronuclei in the upper part. Two representative embryos in prophase and anaphase of the first mitosis (29/30 hphCG) are also shown (G and H, respectively). In the last row are representative images of 2-cell embryos at 32 hphCG (I), 36 hphCG (J), 42 hphCG (K) and 48 hphCG (L). All these images are single light-optical sections taken on the ApoTome microscope, except for the two embryos in mitosis, for which Z-series projections are shown. All the images were taken with one of the 3 different H3S10P acquisition settings: 100 ms for G1/S stages, 50 ms for G2 stages and 25 ms for mitosis. Scale bar: 10 μ m.

observation during this stage is that some heterochromatin left in the periphery of the nucleus is stained for HP1 β but not for H3S10P (Fig. 4A, arrowhead). As for early 2-cell embryos (36 hphCG, $n=18$), HP1 β was partially detected around the NPBs (Fig. 4B) with the exception of rare single foci accumulating at the nuclear periphery (Fig. 4B, arrowheads). However, H3S10P was only concentrated around the NPBs forming rings and was excluded from the nucleoplasmic isolated foci (Fig. 4B, arrowheads). In late 2-cell embryos (48 hphCG, $n=21$), HP1 β is localized within the newly formed chromocenters as well as H3S10P; only a few HP1 β foci did not contain H3S10P at the same time (Fig. 4C). Conversely, H3S10P staining, not colocalized with HP1 β , could be observed on some nucleoli and also within small nucleoplasmic aggregates (Fig. 4C, arrowheads). This suggests that H3S10P indeed labels pericentromeric heterochromatin in G1/S phase of early embryos and that supplementary H3S10P-labeled domains appear in late G2, underlying the entrance into mitosis.

In later stages such as blastocysts, H3S10P and HP1 β colocalize in the chromocenters of some blastomeres only, most probably those going through the G2 phase ($n=21$, Fig. 4D). In early interphasic blastomeres, only HP1 β could be observed within pericentromeric heterochromatin, as no H3S10P was ever detected. Conversely, HP1 β staining was diffuse in the nucleoplasm of mitotic blastomeres,

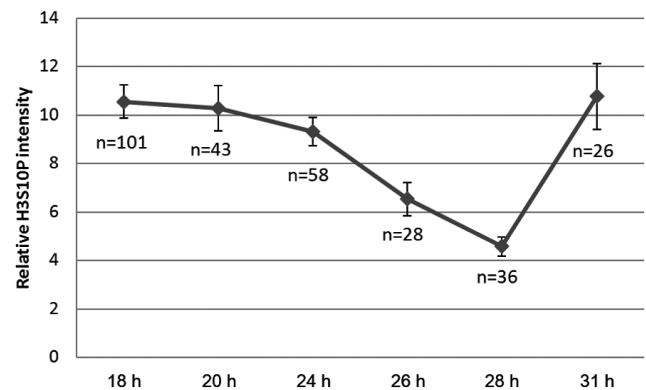


Fig. 3. Quantification of H3S10 phosphorylation levels in 1-cell stage embryos at different time points during the first cell cycle (in hphCG). Relative H3S10P fluorescent signal was estimated using the Image-J software, taking into account the DNA signal (see Materials and Methods). Error bars show standard deviations. The number of pronuclei analyzed is indicated next to each value.

whereas H3S10P was concentrated on chromosomes.

H3S10P colocalization with pericentromeric DNA repeats using immunoFISH

ImmunoFISH is a refined technique that makes it possible to specifically check colocalization of proteins and DNA sequences. We therefore used it to check colocalization of H3S10P with the DNA repeats that constitute pericentromeric heterochromatin using probes for major satellites. This technique was performed under conditions that preserved the 3D nuclear structures of the embryos [35, 44].

In 1-cell embryos, H3S10P staining clearly colocalized with the pericentromeric repeats forming rings around the NPBs of both pronuclei, except for some pericentromeric heterochromatin foci seen in the periphery of the pronucleus ($n=21$, Fig. 5A). As for the 2-cell stage, in earlier examples, the perinucleolar rings labeled with the major satellite probes also stained for H3S10P, showing faithful colocalization ($n=31$, Fig. 5B). At that stage, the very few chromocenters already present were labeled with the major satellite probes and not H3S10P (Fig. 5B, arrowhead). In contrast, for late 2-cell embryos ($n=72$), colocalization of H3S10P with major satellite sequences was obvious on the chromocenters. Scarcely, few nucleoli show some pericentromeric heterochromatin signal. However, it always colocalized with H3S10P (Fig. 5C). Some aggregates of H3S10P that do not correspond to pericentromeric heterochromatin were seen in the nucleoplasm in the late G2-phase, as already suggested by the double immunostaining of H3S10P and HP1 β (Fig. 5C).

Altogether, these observations show that H3S10P labels the pericentromeric heterochromatin of both parental origins at the 1- and 2-cell stages, except for some isolated foci at the nuclear periphery.

Presence of Aurora B staining is coincident with an interphasic H3S10P signal

In somatic cells, the kinase responsible for the mitotic phosphorylation of histone H3 at Ser10 is the Aurora B kinase. This kinase phosphorylates the histone H3 at Ser10 starting from the late S/G2 phase and maintains this phosphorylation until anaphase. We

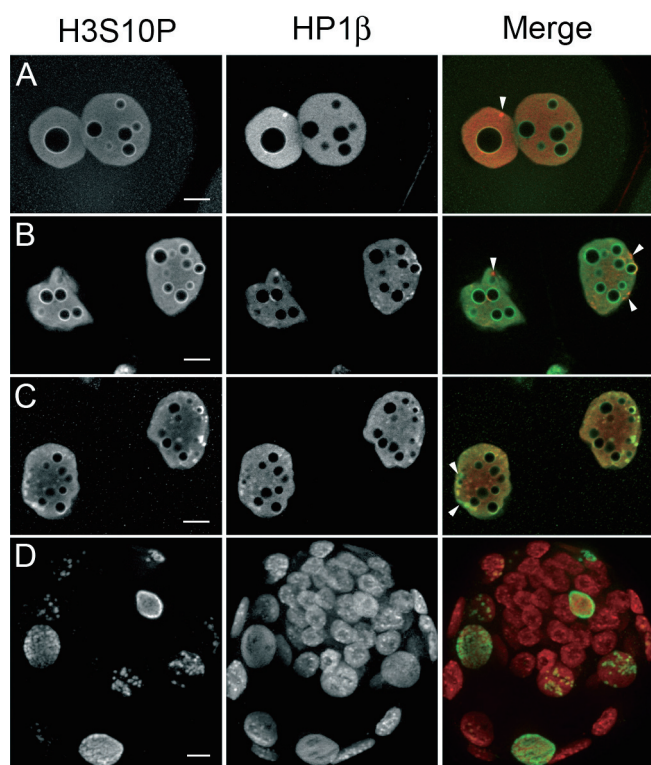


Fig. 4. Distribution of H3S10P in relation to pericentromeric heterochromatin protein HP1 β . The distribution of H3S10P (green) and HP1 β (red) was analyzed in the 1-cell (A, 20 hphCG), early (B, 36 hphCG) and late 2-cell (C, 48 hphCG) and blastocyst stages (D) under the ApoTome microscope. Representative light-optical sections are shown here. Obvious co-localization of H3S10P and HP1 β in the nucleoplasm is observed in 1-cell and early 2-cell stage embryos except for some HP1 β foci, which are often located at the nuclear periphery (arrowheads in A and B). Colocalization of both markers was also observed in late 2-cell embryos, especially within the newly formed chromocenters. However, some H3S10P staining not colocalized with HP1 β could also be observed (arrowheads in C). In blastocysts (D), H3S10P and HP1 β colocalize in some blastomeres only (within the chromocenters). Scale bars: 10 μ m.

therefore decided to perform immunodetection of Aurora B kinase in preimplantation mouse embryos to check whether the presence of an interphasic H3S10P signal was related to the presence of this kinase.

Interestingly, Aurora B staining was detected during the G1/ S phases of 1-, 2- and 4-cell embryos, concomitantly to histone H3 phosphorylation (n=32, n=20 and n=15 respectively; Fig. 6A, 6B and 6C). On the other hand, in 8-cell embryos, Aurora B staining was no longer observed during S phase (n=34 Fig. 6D), which coincides with the lack of an H3S10P signal at this stage. An Aurora B signal was only detected in 8-cell embryos going through the late G2-phase (n=15 Fig. 6E), which is when an H3S10P signal was also present. It therefore seems that Aurora B might well be involved with phosphorylation of histone H3 at Ser10 during interphase in early embryos, as well as with the mitotic phosphorylation of histone H3 at Ser10 during mitosis in late embryos.

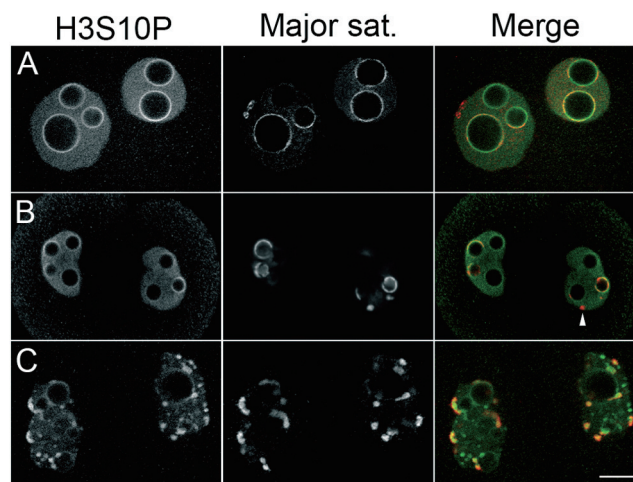


Fig. 5. Colocalization of H3S10P and pericentromeric heterochromatin. Three dimensional immunofISH with H3S10P antibody (green) and major satellite probes for pericentromeric DNA repeats (red) was performed on 1-cell (A, 24hphCG), early (B, 36hphCG) and late 2-cell embryos (C, 48hphCG). Z-series were taken for each embryo on the LSM510 confocal microscope. Single z-sections are shown on this figure. A: Note the colocalization of H3S10P and pericentromeric repeats around the NPBs and presence of some isolated pericentromeric foci at the nuclear periphery. B: Colocalization between H3S10P and pericentromeric repeats is observed in early 2-cell embryos, except for isolated chromocenters (arrowhead). C: In late 2-cell embryos, H3S10P overlaps with the pericentromeric probes in the newly formed chromocenters, but supplementary H3S10P foci appeared. Scale bar: 10 μ m.

Discussion

In this study, we used indirect immunofluorescence, immunofISH and high resolution microscopy to investigate the spatial distribution of an epigenetic modification, phosphorylation of histone H3 at serine 10, in preimplantation mouse embryos. In somatic cells, histone H3 phosphorylation initiates during G2 in pericentric foci and is lost during anaphase. However, this epigenetic modification clearly presents unusual kinetics in mammalian embryos as it is present from early interphase through mitosis over several embryonic cycles. In this study, we performed for the first time a precise observation during the whole preimplantation period, trying to distinguish G1/S versus G2 phases of each embryonic cycle. Our results show that histone H3 can be phosphorylated at serine 10 from early interphase through mitosis over several embryonic cycles. In the interphase of these early developmental stages, H3S10P stains not only the entire nucleoplasm but also the pericentromeric heterochromatin, i.e., the perinucleolar regions or the chromocenters. By looking more precisely at pericentromeric heterochromatin, we clearly observed a shift during the 2- and 4-cell stages, when this heterochromatin moves away from the nucleoli and clusters together, assembling the chromocenters [31, 32]. Remarkably, at the 4-cell stage, the interphasic staining of H3S10P was weaker when compared with earlier embryonic stages and disappeared at the 8-cell stage. This probably reflects the shift from the H3S10P embryonic phosphorylation pattern to the somatic one. Indeed, in 8-cell embryos, H3S10P was solely noticed in the chromocenters at the end of G2 phase and as expected on the

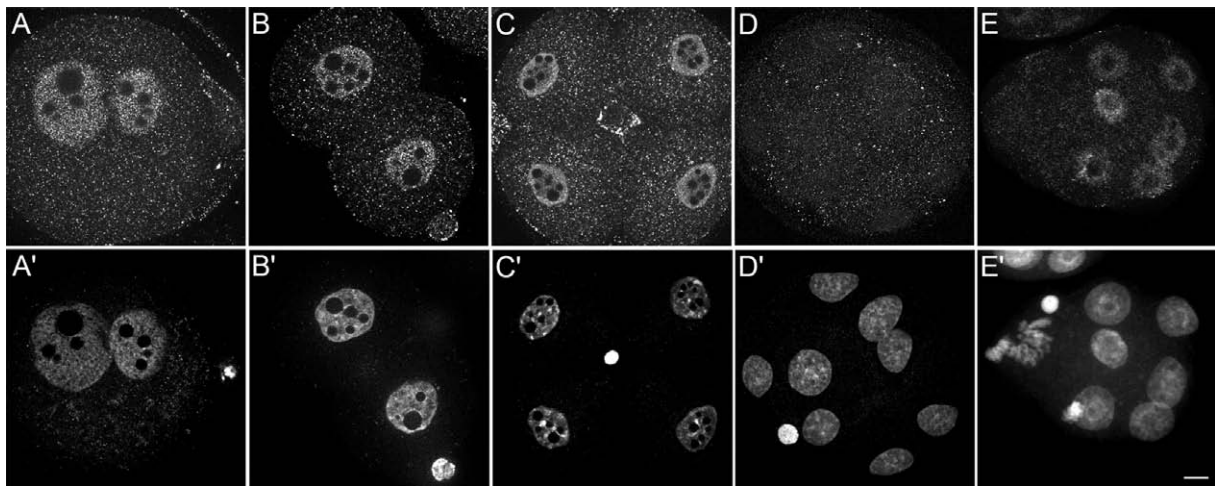


Fig. 6. Detection of Aurora B kinase in preimplantation mouse embryos. Aurora B immunofluorescent staining (A–E) with DNA counterstaining (A'–E') was performed during the S phase in 1-cell (A, 24 hphCG), 2-cell (B, 36 hphCG) and 4-cell embryos (C, 50 hphCG) and samples were observed on an inverted ApoTome microscope. A diffuse staining was observed in all these embryos (single light-optical sections from z-series). On the other hand, 8-cell embryos in the S phase did not show any signal (D, 63 hphCG, projection of z-series); it appeared only in late 8-cell embryos (E, 74/78 hphCG, projection of z-series). Scale bar: 10 μ m.

chromosomes of mitotic blastomeres. The same spatial distribution was observed in morula and blastocyst stages with H3S10P labeling the chromocenters in G2 and the chromosomes during mitosis.

These results are in agreement with previous global observations made by Huang and colleagues, who compared *in vivo* versus *in vitro* fertilized preimplantation mouse embryos [27]. Unfortunately Huang and colleagues did not specify the fixation time points they used, except for the 1-cell stage, which was observed 12 h after insemination, i.e., during the S phase. Similarly, in early stage embryos, Wang and colleagues published that H3S10P is uniformly distributed during interphase of 2-cell embryos without mentioning the timing of fixation [39]. They also observed that H3S10P labeling covered the entire length of the chromosomes arms during mitosis and pointed out that the intense signals they observed with H3S10P are likely to be pericentromeric heterochromatin. Although these results do not mention clear embryo staging, they are all in agreement with our own observations.

Conversely, a report was recently published about H3S10P phosphorylation/dephosphorylation dynamics in 1- and 2-cell mouse embryos. The authors of that report state that H3S10P is present in pronuclei and blastomere nuclei in the early G1 phase but not during the early and middle S phase. They also argue that *de novo* phosphorylation of H3S10 in these embryos begins in the pericentromeric heterochromatin when these domains are starting replication [25]. Also we observed the same dephosphorylation/*de novo* phosphorylation dynamics; we never observed complete disappearance of the signal. The intriguing point here is that all research groups used the same commercially available antibody against H3S10P [25, 27, 39, our study]. It seems more likely that the discrepancies seen could be due to the culture medium used by Teperek-Tkacz and colleagues after embryo collection: the M2 medium from Sigma [25]. This medium is usually recommended for embryo harvest and handling outside a CO₂ incubator but not for culture. In fact, CZB medium [27] or M16 (our study) are more appropriate for embryo culture.

It has been shown that the mitotic phosphorylation of histone H3 at Ser10 is mediated by one of the kinases of the Aurora family, named Aurora B [45]. In mammalian cells, colocalization of Aurora B and H3S10 phosphorylation was detected from the late G2 phase until metaphase [46]. The few studies done so far in oocytes pointed out a role for Aurora B as the kinase behind the phosphorylation of histone H3 at Ser 10. It is believed that this kinase is responsible for the chromatin remodeling and spindle formation during meiosis I [24]. In our study, we observed for the first time Aurora B staining during the G1/ S phases of 1-, 2- and 4-cell embryos, concomitantly to the presence of interphasic histone H3 phosphorylation. Similarly, after the 8-cell stage, Aurora B staining follows H3S10P kinetics and can be detected only upon mitosis. It therefore seems that Aurora B and H3S10P are closely related in preimplantation embryos. This is confirmed by Aurora kinase inhibition with a specific drug, Zm447439, which impairs phosphorylation of histone H3 at Ser10 and causes aberrant chromosome condensation during mitosis in 1-cell stage embryos [25].

However, mitotic histone H3 phosphorylation is the result of a balance of competing kinase and phosphatase activities [23], and Aurora B kinase may indeed be negatively regulated through interactions with phosphatases like PP1 or PP2A [16, 17]. When we attempted to perform immunostaining to detect PP1 in preimplantation embryos, we detected a diffuse spotted signal in anaphase/telophase both during the first and second cell cleavages. Quantification of the signal intensity however suggested that PP1 concentration is much lower in these embryos than in later stages (data not shown).

Interestingly, it was recently shown that another member of the Aurora kinase family, Aurora C, might also be involved in meiotic progression and phosphorylation of histone H3 at Ser10 in mouse oocytes [47]. Aurora C could therefore also be a good candidate for H3S10P regulation during interphase of the first four cell cycles in preimplantation mouse embryos. Further study is clearly required to elucidate all the mechanisms behind this peculiar kinetic of H3S10

phosphorylation.

Several authors have already suggested that H3S10P interphasic staining observed in early embryos likely corresponds to pericentromeric heterochromatin domains [25–27, 39]. Here, we show for the first time the colocalization of H3S10P and pericentromeric heterochromatin in 1- and 2-cell embryos by double immunostainings and immunoFISH. Indeed, immunoFISH with major satellite DNA probes clearly shows that H3S10P labels the major satellite sequences that form these pericentromeric heterochromatin rings around the NPBs in 1- and 2-cell embryos. When we compared H3S10P marker with HP1 β , we clearly saw a better colocalization of H3S10P and pericentromeric probes in the heterochromatin rings around the NPBs, especially as it labels the paternal PN when HP1 β does not. Remarkably, some HP1 β /major satellite foci isolated at the nuclear periphery in 1-cell embryos did not contain any H3S10P labeling. These foci had already been previously described both by immunostaining and immunoFISH [31, 35]. At this stage, it has been suggested that chromosomes form “cartwheels” around the NPBs [31]. It would be interesting to see whether the absence of H3S10P from some pericentromeric domains would explain why some chromosomes are not associated with NPBs.

When looking at early 2-cell embryos, it appears that HP1 β is partially detected in the pericentromeric heterochromatin rings around the NPBs, although in lower amounts than H3S10P. At the end of the second cell cycle, when the heterochromatin undergoes impressive rearrangement, H3S10P and the major probes overlap in the newly formed chromocenters that also contain HP1 β as previously shown [35]. It seems possible that this heterogeneity of pericentromeric labeling by the two markers during the second cell cycle in fact corresponds to differences between the two parental genomes. It has also been previously suggested that in 2-cell embryos, the HP1 β -positive chromocenters contain maternal inherited pericentromeric heterochromatin already labeled in the 1-cell stage whereas HP1 β -negative chromocenters correspond to the paternal part [32]. Altogether this suggests that H3S10P is faithfully following all the pericentromeric heterochromatin movements in early embryos better than HP1 β .

It is also worth noticing that H3S10P is part of the switch involving H3K9me3 and HP1 β . It is known that when histone H3 is phosphorylated at serine 10, HP1 β is ejected from the complex in cells in culture [48]. However, this is not the case in early stage embryos, since our immunostaining results showed that H3S10P colocalizes with HP1 β even in G1/S phase. This is especially true for the maternal inherited genome, as the paternal pronucleus does not stain for HP1 β in the enriched intense heterochromatin areas [31, 32] or only stains very weakly [34]. The work done by Mateescu and collaborators states that phosphorylation of histone H3 at serine 10 is not enough by itself to eject HP1 β from the complex formed with H3K9me3 and that H3K14 acetylation is also required [49]. Consequently, further studies need to be carried out to understand the mechanism behind HP1 β association/ejection.

Some other epigenetic marks also have an asymmetric distribution between the two parental genomes, e.g., H3K27me3 or H3K9me3 [36]. Altogether, it appears that the paternal pronucleus is positive for H3K27me3 and H3S10P but negative for H3K9me3 and HP1 β . On the contrary, the maternal pronucleus is positive for H3K9me3, H3S10P

and HP1 β . It is tempting to speculate that in the paternal pronucleus, the lack of H3K9me3 and HP1 β and presence of H3S10P, could explain why the paternal genome is more accessible to transcription factors being replicated and transcribed earlier than the maternal one. Conversely, the presence of H3K9me3, HP1 β and H3S10P in the maternal pronucleus would account for the more repressive state of the maternal heterochromatin and could explain its very late replication timing [50, 51].

Despite of all these findings about phosphorylation of histone H3 at Ser10, there is still need for some elucidation about this epigenetic modification during embryonic development. The presence of H3S10P from fertilization until the 4-cell stage suggests that H3S10P is involved with gene expression, whereas in mitosis, it most likely recruits factors that act on chromatin condensation. In interphase of the later stages, when H3S10P is only present in the heterochromatin clumps at the end of the G2 phase, this epigenetic modification must function as a signal indicating to the cell that replication is complete and that mitosis can proceed (as in somatic cells). It is tempting to speculate that the purpose of H3S10P during interphase of 1- and 2-cell embryos is to function as a factor holding the open state of the pericentromeric heterochromatin in order to allow transcription to happen. It has indeed been recently shown that pericentromeric transcripts are involved in the recruitment of HP1 β and formation of chromocenters [52, 53]. Interestingly, these transcripts are present in 1-cell/2-cell embryos, whereas a sharp downregulation is observed at 4-cell/8-cell embryos. This kinetic correlates with the peculiar H3S10P dynamic within pericentromeric heterochromatin. It would therefore be very interesting to use H3S10P as a marker to unveil the fundamental mechanisms behind pericentromeric heterochromatin movements in other species with later formation of chromocenters, e.g., bovine [37].

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