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Dynamics of histone H3 phosphorylation at threonine 3 during meiotic maturation in mouse oocytes

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ABSTRACT

Various histone residues are post-translationally modified during the cell cycle. Among these, histone H3 phosphorylation at threonine 3 (H3T3ph) is newly characterized and has been considered to be crucial for chromosome dynamics during mitosis. However, little is known about the role of H3T3ph during mouse oocyte maturation. In the present study, we examined H3T3ph expression and localization during oocyte meiosis. Our results showed that H3T3ph was tightly associated with condensed chromosomes during meiotic maturation. H3T3ph along the chromosome arms was dissociated at anaphase/telophase I, but centromeric H3T3ph remained intact. Moreover, the inhibition of H3T3ph with the small molecule inhibitors CHR-6494 and 5-Itu impaired segregation of homologous chromosomes during meiosis. Partial inhibition of H3T3ph revealed that centromeric Aurora B/C kinase is sufficient to complete meiosis I, but Aurora B/C kinase along the chromosome arms is required to ensure accurate homologous chromosome segregation. Therefore, our results demonstrate that H3T3ph is a universal regulator of chromosome dynamics during oocyte meiosis and mitosis.

1. Introduction

Histones are a family of basic proteins that associate with DNA and help condense it into chromatin. In all eukaryotes, histones form octamers containing two copies of each of the four histone subunits, H2A, H2B, H3 and H4, which wrap DNA. This basic building block of chromatin is referred to as a nucleosome. The N-terminal tails of histones protrude from the nucleosome surface and are subjected to multiple post-translational modifications such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and ADP-ribosylation [1]. These modifications are central to modulating chromatin structure and function.

Histone phosphorylation has long been implicated in the regulation of various aspects of the cell cycle, including chromosome condensation and segregation [2]. For example, phosphorylation of serine 10 on H3 (H3S10ph) is crucial for chromosome condensation and cell cycle progression during mitosis and meiosis [3,4]. Histone H3 is also phosphorylated at serine 28 (H3S28ph) during mitosis, and this phosphorylation coincides with chromosome condensation [5]. These two mitotic phosphorylations are catalyzed by Aurora B kinase, a catalytic component of the chromosomal passenger complex (CPC), which plays a key role in controlling chromosome segregation and cytokinesis during mitosis [6]. In meiosis, these modifications are also mediated by Aurora C kinase, a germ cell-specific homologue of Aurora B kinase [7,8]. Phosphorylation of threonine 3 on H3 (H3T3ph) by Haspin kinase was recently reported as another mitotic histone H3 phosphorylation [9]. H3T3ph accumulates specifically at centromeres during prometaphase and plays a key role in CPC localizing to mitotic chromatin [10–15]. Enriched CPC at the centromere locally activates its kinase subunit, Aurora B, which leads to downstream phosphorylation of a variety of substrates, including the kinetochore and the spindle assembly checkpoint (SAC) proteins [13,16–18]. Therefore, mitotic histone H3 phosphorylation has a key role in chromosome dynamics during cell cycle progression.

Although histone H3 phosphorylation during mitosis has been well studied, relatively little is known about histone H3 phosphorylation during oocyte meiosis. Recently, the dynamics of H3S10ph and H3S28ph during mouse oocyte maturation have

Abbreviations: SAC, spindle assembly checkpoint; CPC, chromosome passenger complex; kMT, kinetochore-microtubule; ACA, anti-centromere antibody.

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been investigated [19–21]. However, the data are still limited and controversial because these reports reached different conclusions on the expression and localization of H3S10ph and H3S28ph during meiotic maturation. Compared with H3S10ph and H3S28ph, H3T3ph has been very recently reported in mammalian oocytes [22]. In the present study, we examined the expression, localization, and function of H3T3ph during mouse oocyte meiotic maturation. Using small molecule inhibitors that target Haspin kinase, CHR-6494 and 5-iodotubercidin (5-Itu), we found that H3T3ph is required to recruit CPC into the chromosomes. We also found that CPC localization is not restricted to the centromeres, but widely distributed at the entire chromosomes in mouse oocytes. Partial inhibition of H3T3ph mostly along chromosome arms revealed that centromeric CPC is sufficient to complete meiosis I, but arm CPC is required to ensure accurate homologous chromosome segregation.

2. Materials and methods

2.1. Oocyte collection and culture

All mice were 3- to 5-week-old CD-1 female mice (Koatech). The guidelines of the Institutional Animal Care and Use Committees were followed for all animal procedures.

Oocytes were prepared as described previously [23]. Briefly, female mice were injected with 5 IU of pregnant mare serum gonadotrophin (Calbiochem), and the ovaries were collected 44–48 h later. Oocytes were recovered from the ovaries and placed in M2 medium (Sigma) supplemented with 200 μM IBMX (Sigma) to maintain the GV stage. The oocytes then were cultured in IBMX-free media to resume meiosis. The oocytes were fixed at specific stages for analysis. All oocytes were cultured at 37 °C in a 5% CO_2 atmosphere.

In the inhibitor treatment experiments, the medium was supplemented with the indicated concentrations of CHR-6494 (Calbiochem), 5-Itu (Calbiochem), or ZM449437 (Calbiochem). As a control, oocytes were treated with an equal volume of dimethyl sulfoxide (DMSO).

2.2. Immunofluorescence analysis

Oocytes at specific stages were fixed in 4% paraformaldehyde for 20 min and permeabilized in 0.5% Triton X-100 for 20 min. Oocytes were then incubated with primary antibody followed by Alexa Fluor-conjugated 488 and 594 secondary antibodies (1:500; Jackson ImmunoResearch). DAPI was used for DNA staining. At least 20 oocytes were examined for each group, unless otherwise stated. Antibodies were H3T3ph (1:500; Millipore), H3S10ph (1:500; Millipore), anti-centromere antibody (ACA, 1:50; Antibodies Incorporated), and Survivin (1:800; Cell Signaling).

For aneuploidy analysis, oocytes pretreated for 2 h in 200 μM monastrol (Sigma) were fixed and stained with the indicated antibodies, as described previously [24].

2.3. Image analysis

Images were acquired using a confocal laser-scanning microscope (LSM 710; Zeiss) equipped with a C-Apochromat 63 × /1.2 water immersion objective. Optical sections were obtained at 1 μm intervals and converted into maximum intensity projections. Data analysis was performed using ZEN 2010 LSM software (Zeiss) and ImageJ (National Institutes of Health).

2.4. Statistical analysis

Statistical analysis was performed with GraphPad Prism (GraphPad Software). Data are representative of at least three independent experiments unless otherwise specified. Values were analyzed by Student's t-test, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Expression and localization of H3T3ph during oocyte meiosis

To examine the H3T3ph distribution during oocyte meiosis, we collected mouse oocytes corresponding to the germinal vesicle (GV), GV breakdown (GVBD), pro-metaphase I (Pro-MI), metaphase I (MI), anaphase I/telophase I (AI/TI), and metaphase II (MII) stages. At the GV stage, H3T3ph appeared as several dots associated with chromatin in the nucleus. After GVBD, in Pro-MI and MI, H3T3ph began to concentrate in the chromosomes, with strong enrichment along the interstitial axes of the chromosomes arms. At AI/TI, the H3T3ph signal gradually decreased in the chromosome arms but remained at the centromere. Interestingly, residual amounts of H3T3ph were detectable at the midbody during TI. At MII, H3T3ph was primarily localized in the inner centromere. As the distance between sister kinetochores increased, H3T3ph staining was localized along the area between kinetochores. Close examination by monastrol treatment further confirmed H3T3ph localization pattern between kinetochores at the univalent sister chromatids during MII stage (Fig. 1B).

Because H3S10 is also phosphorylated during meiotic maturation, we compared H3T3ph expression and localization with those of H3S10ph. Unlike H3T3ph, which was detectable at the GV stage, no H3S10ph signal was observed in the GV stage. After GVBD, H3S10ph was strongly detected along the entire chromosome and remained until MII, without dissociating from the chromosome during AI/TI (Fig. 1B). The data imply that these two modifications are regulated differently during the meiotic cell cycle.

3.2. Inhibiting H3T3ph with small molecule inhibitors impaired chromosome dynamics during meiosis I

To assess the functional significance of H3T3ph in oocyte maturation, we treated oocytes with CHR-6494, a small molecule inhibitor that inhibits Haspin kinase, thereby preventing H3T3ph. In a preliminary experiment, GV oocytes were cultured with various concentrations of CHR-6494 to optimize the conditions. No oocytes underwent meiosis in the presence of the inhibitor (Fig. 2A). Consistently, CHR-6494 has been shown to cause G2/M arrest in mitotic cells [25]. However, since small molecule inhibitors have various side effects, we confirmed the inhibitory effect of CHR-6494 on meiotic resumption using 5-Itu, another inhibitor that specifically inhibits Haspin kinase [13,16]. Although the effect of 5-Itu was not as dramatic as that of CHR-6494, 5-Itu also inhibited meiotic resumption of mouse oocytes in a dose-dependent manner (Fig. 2A). However, all GV-arrested oocytes resumed meiosis when the inhibitors were washed from the oocytes, excluding the possibility of a cytotoxic effect of the inhibitors (data not shown). To prevent meiotic arrest, we treated oocytes with CHR-6494 after GVBD. After treatment with 1 μM or 10 μM CHR-6494, H3T3ph level decreased by 73.9% and 93.7%, respectively (Fig. 2B, C). A similar result was obtained when oocytes were treated with 5-Itu (Fig. 2D). These results demonstrate that both small inhibitors prevent endogenous H3T3ph in a dose-dependent manner in mouse oocytes.

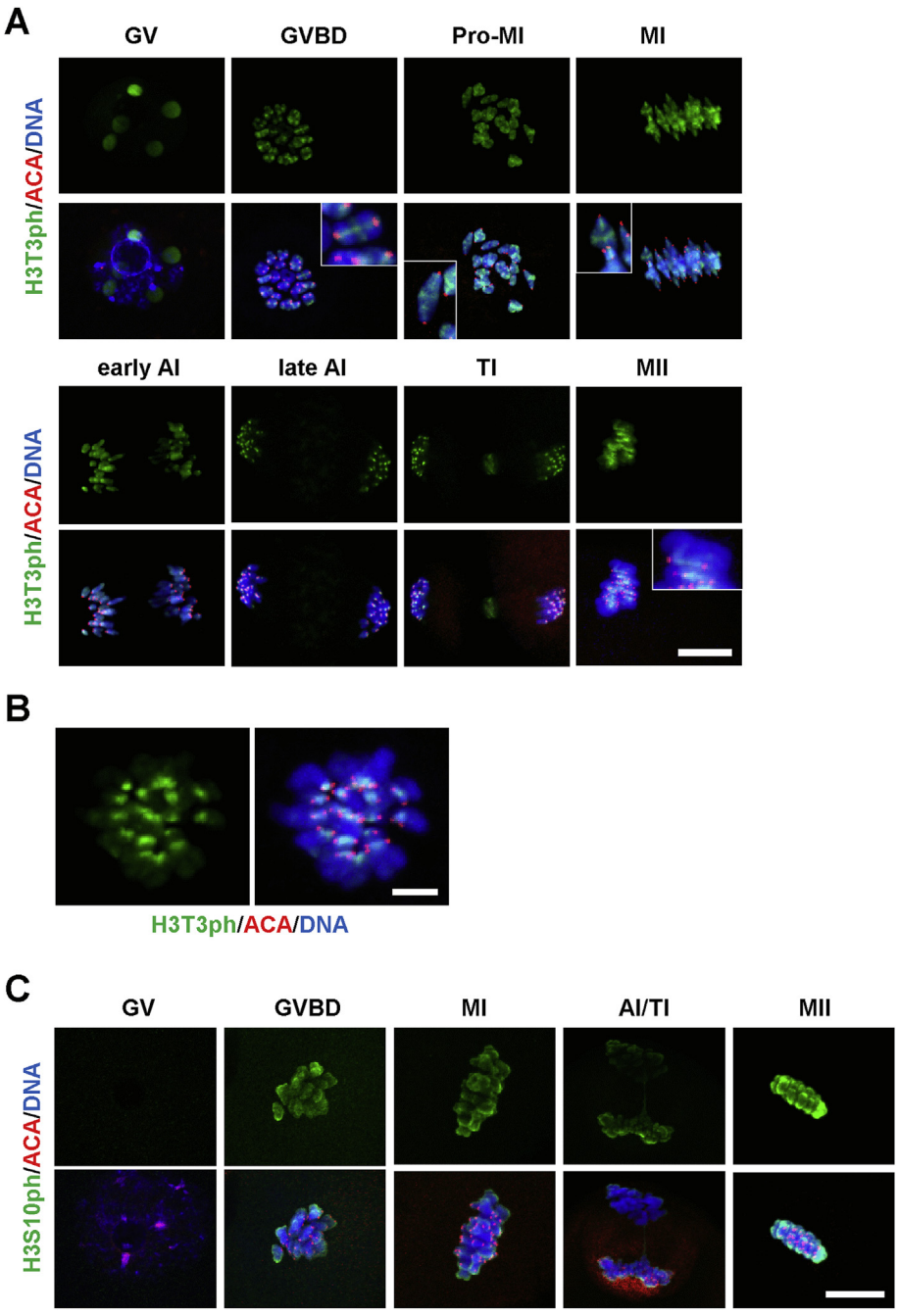

Fig. 1. Expression and subcellular localization of H3T3ph during meiotic maturation. (A, C) Oocytes at various stages were immunostained with antibodies against either H3T3ph (A) or H3S10ph (C). Enlarged images of chromosomes at the GVBD, Pro-MI, MI and MII stages are shown in the inset. Images are obtained from three independent experiments and processed using ZEN 2010 LSM software (Zeiss) to improve the signals. (B) MII oocytes were incubated with monastrol and immunostained with H3T3ph antibody. DNA and inner centromeres were stained with DAPI and anti-centromere antibodies (ACA), respectively. Bar, 20 μm.

Because H3T3ph regulates chromosome segregation during mitosis [10–13,15], we speculated that H3T3ph most likely regulates chromosome dynamics during meiosis. To test this hypothesis, oocytes were cultured in the presence of CHR-6494. In oocytes treated with 0 or 1 μM CHR-6494, the chromosomes were well-aligned at the metaphase plate, while a large proportion of oocytes treated with 10 μM CHR-6494 displayed dispersed and misaligned chromosomes (Fig. 2E, F). We observed similar results in oocytes treated with 5-Itu (Fig. 2D), indicating that H3T3ph is involved in chromosome dynamics during meiosis, as in mitosis.

3.3. Small amounts of CPC are sufficient to complete meiosis I

To determine the effects of H3T3ph inhibition on meiotic maturation, oocytes were cultured in the presence of CHR-6494. At 10 μM CHR-6494, a significant portion of oocytes arrested at MI (Fig. 3A). These oocytes progressed to MII after removing the inhibitor, similar to the rescue from meiotic arrest after removal of the inhibitor (Fig. 3B). This result suggests that the effects of CHR-6494 are reversible and not toxic to the oocytes. Most oocytes normally developed to MII when treated with 1 μM CHR-6494

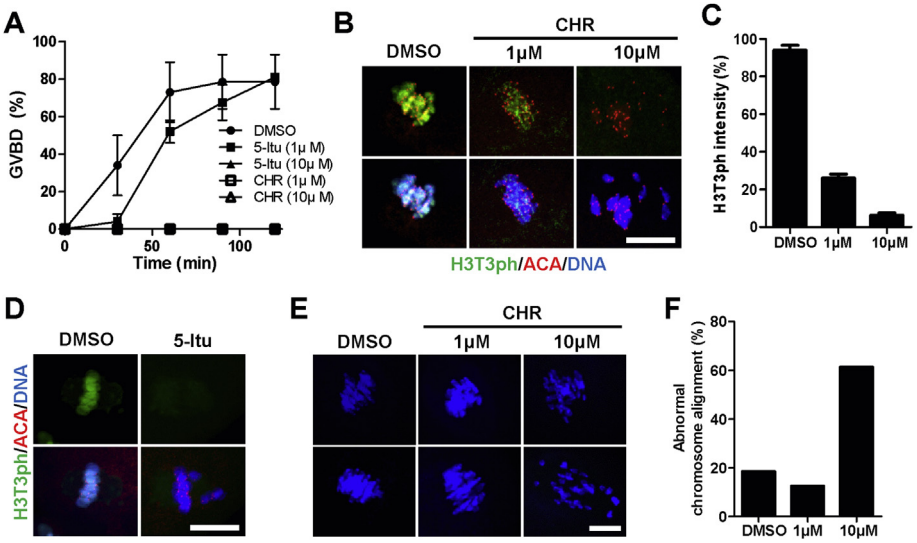

Fig. 2. Inhibition of H3T3ph with small molecule inhibitors. (A) GV oocytes were cultured with indicated concentrations of either CHR-6494 or 5-Itu for 2 h, and GVBD was scored. (B–F) To overcome meiotic arrest, oocytes were treated with inhibitors at 2 h after GVBD and cultured until MI stage. (B) MI oocytes treated with CHR-6494 were fixed and immunostained with H3T3ph antibody. DNA and inner centromeres were stained with DAPI and anti-centromere antibody (ACA), respectively. Bar, 20 μm. (C) H3T3ph levels shown in (B) were quantified. (D) Oocytes were treated with 5-Itu and stained with H3T3ph antibody. DNA and inner centromeres were stained with DAPI and anti-centromere antibody (ACA), respectively. Bar, 20 μm. (E) Representative images of chromosomes in oocytes treated with CHR-6494. Bar, 20 μm. (F) Quantitation of the data in (E).

(Fig. 3A), despite significantly reduced H3T3ph level (Fig. 2C, D). Given that CPC is responsible for chromosome segregation during meiosis I and that H3T3ph recruits CPC to chromosomes during mitosis [6–9,11,12,14,15,18], we determined whether small amounts of H3T3ph are sufficient to recruit CPC to chromosomes during meiosis I. To test this possibility, we detected endogenous Survivin, a CPC component, using immunostaining [6]. Survivin level decreased significantly after CHR-6494 treatment, similar to H3T3ph level (Fig. 3C). However, a significant proportion of Survivin remained at the centromeric region, while Survivin localized at the chromosome arms decreased dramatically. These results suggest that a small amount of CPC, mostly localized at the centromeres, is sufficient to complete meiosis I. To investigate this possibility, we determined the catalytic activity of CPC by

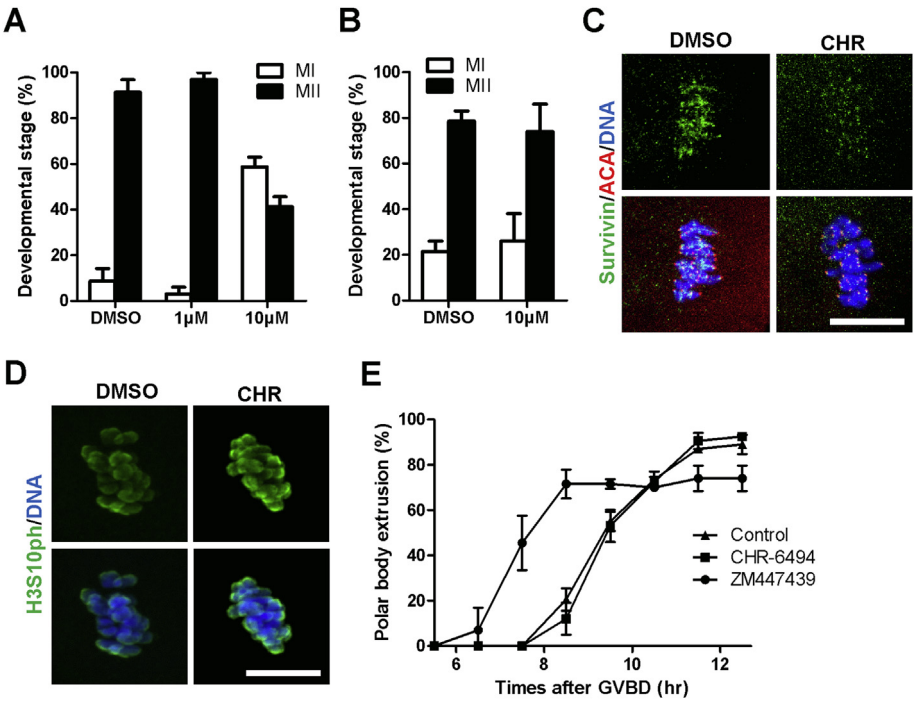

Fig. 3. A small amount of CPC localized at the centromeres is sufficient to complete meiosis I. Oocytes were treated with the inhibitors at 2 h after GVBD and cultured for 13 h. (A) Developmental stages of oocytes treated with CHR-6494 were determined. (B) The MI-arrested oocytes in A were further cultured for 5 h without CHR-6494, and developmental stages were determined. (C, D) Oocytes treated with 1 μM CHR-6494 were fixed at MI and immunostained with either Survivin (C) or H3S10ph (D) antibody. DNA and inner centromeres were stained with DAPI and anti-centromere antibody (ACA), respectively. Bar, 20 μm. (E) Oocytes were treated with either 1 μM CHR-6494 or 10 μM ZM447439 at 2 h after GVBD. The polar body extrusion was scored after 13 h.

measuring H3S10ph level. In CHR-6404-treated oocytes, the H3S10ph level was comparable to that in control oocytes, suggesting that Aurora B/C kinase activity did not change, although a large proportion of CPC was dissociated from the chromosome arms but not from the centromeres (Fig. 3D). Moreover, these oocytes extruded the polar body with normal kinetics, whereas oocytes treated with Aurora inhibitor ZM447439 prematurely extruded the polar body (Fig. 3E). Inhibition of Aurora B/C activity during meiosis has previously been shown to accelerate the completion of meiosis I by inactivating SAC [7,26]. Therefore, our results suggest that a small amount of CPC, mostly localized at the centromeres, provides sufficient Aurora B/C kinase activity and allows completion of meiosis I by segregating homologous chromosomes.

3.4. H3T3ph is required for faithful segregation of homologous chromosomes during meiosis

Although oocytes with small amounts of H3T3ph progressed to MII, these oocytes might have suffered defects during segregation of homologous chromosomes during meiosis I. To investigate this possibility, MII oocytes treated with CHR-6494 were collected and immunostained. Control oocytes treated with DMSO had normal well-aligned chromatids with high H3T3ph level. However, oocytes treated with CHR-6494 had no H3T3ph, and sister chromatids were highly disordered (Fig. 4A, B). Similar phenotypes were observed when oocytes were treated with 5-Itu (Fig. 4C). Moreover, the incidence of aneuploidy was abnormally high in oocytes treated with CHR-6494 (Fig. 4D). Given that only centromeric CPC was present when H3T3ph was partially inhibited (Fig. 3C), this result suggests that the CPC localized along the chromosome arms is required for accurate segregation of homologous chromosomes during meiosis.

4. Discussion

Meiosis involves two consecutive divisions in each round of DNA replication. During this process, homologous chromosomes and sister chromatids segregate from each other during meiosis I and II, respectively. Successful chromosome segregation requires the time-sensitive and spatial coordination of chromosomal events. This process is mainly regulated by a multi-protein complex called chromosomal passenger complex (CPC), which comprises Aurora B/C kinase and its regulatory subunit, INCENP; Survivin; and Borealin [6]. H3T3ph has been recently identified and shown to recruit CPC to the chromosomes during mitosis.

Unlike somatic cells where CPC is located near centromeres throughout prophase and until metaphase [6], CPC is localized along the chromosome arms and centromeres in mouse oocytes [7]. Given that a primary role of H3T3ph is recruitment of CPC to chromosomes and subsequent CPC activation [10,12], H3T3ph, which is mainly localized at centromeres during mitosis, might be distributed along the chromosome arms and centromeres during meiosis. We observed H3T3ph in the interstitial axes of chromosome arms and centromeres in mouse oocytes (Fig. 1A). This localization is similar to that of Rec8, a cohesin subunit in oocytes. Whereas most mitotic cohesin is released from chromosome arms in prophase, meiotic Rec8 remains intact along the interstitial axes of homologous chromosomes extending over the centromeres and arm regions until MI. Meiotic Rec8 disappears from the chromosome arms at the onset of anaphase I but persists at centromeres until MII [27]. Despite this similar localization pattern during meiosis, it has been recently demonstrated that Rec8 is not changed when H3T3ph is perturbed [22]. Instead, H3T3ph is associated with condensin. However, unlike Rec8 Sgo2 localized at the chromosome arms was reduced when H3T3ph was disrupted [22]. Moreover, condensin has been shown to be localized to sister chromatid axes rather than along the interchromatid regions [28]. Therefore, it seems likely that H3T3ph might be involved in chromosome cohesion as well as condensation during meiosis. In this regard, it is possible to speculate that H3T3ph regulates chromosome cohesion independent of Rec8. Indeed, it has been recently reported a novel cohesin complex containing Rad21-like proteins instead of Rec8 [29]. Further studies will be required for clarifying relationship

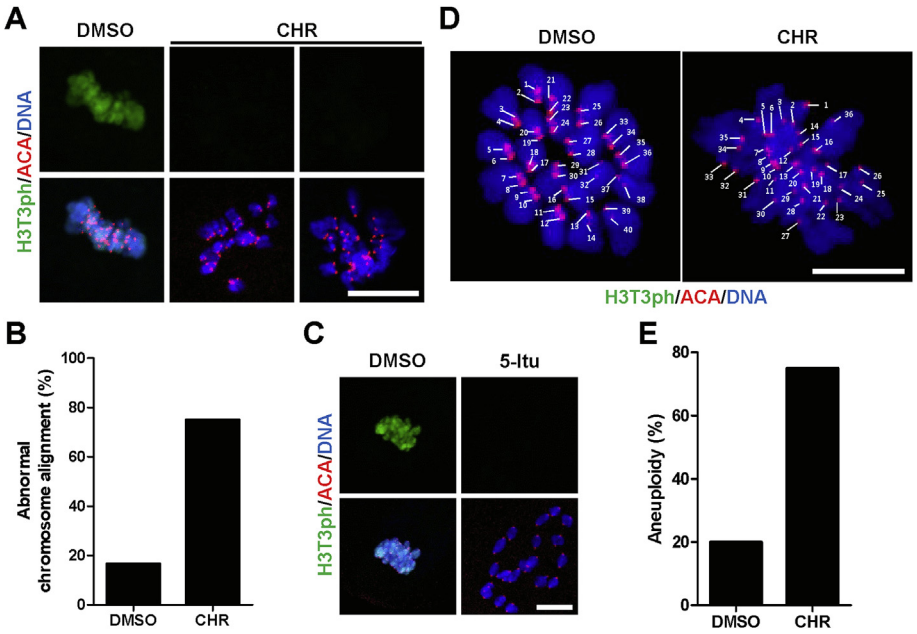

Fig. 4. Partial inhibition of H3T3ph impaired homologous chromosome segregation. (A–C) MII oocytes treated with either 1 μM CHR-6494 (A) or 1 μM 5-Itu (C) were immunostained with H3T3ph. DNA and inner centromeres were stained with DAPI and anti-centromere antibody (ACA), respectively. Bar, 20 μm. (B) Quantitation of chromosome misalignment in (A). (D) Chromosome spread in oocytes treated with CHR-6494. Note that 40 sister chromatids are observed in DMSO group, whereas 35 sister chromatids are detected in CHR-6494 group. Bar, 10 μm. (E) Quantitation of the data in (D).

between H3T3ph and chromosome cohesion during homologous chromosomes segregation.

Erroneous kinetochore-microtubule (kMT) attachments are corrected by CPC in the centromeres during mitosis [6,30,31]. However, during meiosis, CPC localizes along the chromosome arms and centromeres, and the role of this additional CPC localization at the chromosome arms remains elusive [7]. Here, we found that the partial inhibition of H3T3ph delocalized Survivin from the chromosome arms but not from the centromere (Fig. 3C). In this condition, oocytes had comparable levels of H3S10ph, suggesting that Aurora B/C kinase activity did not change (Fig. 3D). Because we treated the oocytes with inhibitor after GVBD, Aurora B/C kinase activity present on the chromosome arms before GVBD might have contributed to H3S10ph, which remained until MI. In agreement with our observation, H3S10ph was not reduced when H3T3ph was inhibited in mitotic cells [13]. Given that vaccinia-related kinase 1 (Vrk1) has been reported to phosphorylate H3S10 [32], it is also possible that H3S10ph might be phosphorylated by other kinases in oocytes. Nevertheless, the CPC localization was obviously restricted to the centromeres, and these oocytes entered meiosis II with normal kinetics. Given that the timing of polar body extrusion is primarily regulated by SAC activity [33–35], centromeric CPC might be sufficient to regulate SAC activity during oocyte meiosis, as it is in mitosis [36]. However, this possibility is unlikely because these oocytes had a high incidence of aneuploidy and chromosome misalignment at MII. Therefore, we assume that Aurora B/C kinase activity, which is mostly localized to the centromeres, allows a partial recruitment of SAC to the kinetochore. This partial recruitment was sufficient for the meiotic cell cycle to progress with normal kinetics but not to allow erroneous kMT attachments to correct during meiosis. In this condition, Aurora B/C kinase activity localized to the chromosome arms seemed to ensure faithful chromosome segregation during meiosis. Centromeric Aurora B/C kinase activity might also be sufficient to establish but not maintain kMT attachments. Thus, the attachments are prone to disconnect during chromosome segregation at anaphase I, leading to aneuploidy at MII. Therefore, localization of Aurora B/C kinase primarily along the chromosome arms might be essential for accurate chromosome segregation during meiosis.

Here, we report that H3T3ph was dynamically regulated during meiosis and that the inhibition of this modification with small molecule inhibitors induced aberrant chromosome segregation. Furthermore, the partial inhibition of H3T3ph revealed that Aurora B/C kinase activity localized either along the chromosome arms or at the centromeres is essential for accurate chromosome segregation during meiosis. Therefore, our results indicate that H3T3ph is a universal regulator of chromosome dynamics during meiosis as well as mitosis. Further studies of the relationships among H3T3ph, CPC, and other associated proteins will provide further insight into the mechanisms that regulate homologous chromosome segregation during meiosis.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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