Histone H3 phosphorylation and non-disjunction of the maternal X chromosome during male meiosis in sciarid flies

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Summary
An extremely unorthodox method of chromosome segregation is found in sciarid flies (Diptera, Sciaridae), where at male meiosis, the whole paternal complement is eliminated and the maternal X chromosome undergoes non-disjunction. At meiosis I, a monopolar spindle directs the segregation of maternal chromosomes to the single pole, whereas paternal chromosomes are discarded. At meiosis II, although maternal autosomes segregate normally, the X chromosome remains undivided. A cis-acting locus within the heterochromatin proximal to the centromere is known to regulate X centromere activity. By immunofluorescence analysis in spermatocytes from Sciara ocellaris and Sciara coprophila, we investigated histone H3 phosphorylation at Ser10, Ser28, Thr3 and Thr11 during male meiosis. We found that chromosome condensation and H3 phosphorylation patterns differ between chromosomes of different parental origin at the time of paternal set elimination. Importantly, at meiosis II, the maternal X chromosome differs from the rest of the chromosomes in that its centromeric region does not become phosphorylated at the four histone H3 sites. We provide here the first evidence linking the under-phosphorylated H3 status of the X chromosome centromeric region with its meiotic non-disjunction in sciarid flies. Our findings strongly support the idea that the deficiency in local H3 phosphorylation inactivates the X centromere at the transition from meiosis I to meiosis II.

Key words: H3 phosphorylation, Sciara, Meiosis, Chromosome non-disjunction, Chromosome elimination

Introduction
A classical example of unorthodox chromosome segregation is found in sciarid flies (Diptera, Sciaridae) where the selective elimination of paternal chromosomes takes place at different times during development (Metz, 1925; Metz, 1926b; Metz, 1933) (reviewed in Gerbi, 1986; Goday and Esteban, 2001). One of the most complex and bizarre chromosome behaviours occurs in sciarid male meiosis, where the whole paternal chromosome complement is discarded so that only maternally derived chromosomes are included in the sperm nucleus. An additional exceptional feature of male meiosis is the occurrence of non-disjunct of the maternal X chromosome (Xm), which determines the characteristic 3X male meiosis is the occurrence of non-disjunction of the maternal X chromosome at meiosis I and non-disjunction of Xm chromosome at male meiosis II have been the subject of several studies (reviewed in Gerbi, 1986; Goday and Esteban, 2001). Since the early findings on the chromosome cell cycle of sciarids, the mechanisms that eliminate paternal chromosomes at meiosis I and non-disjunction of Xm chromosome at male meiosis II have been the subject of several studies (reviewed in Gerbi, 1986; Goday and Esteban, 2001). In Fig. 1 we summarise the most relevant chromosomal events occurring during male meiosis that are common to sciarid flies. As shown (Fig. 1A), at the first meiotic division there is no pairing of homologous chromosomes at prophase and the chromosomes do not align in a metaphase-like array (Metz, 1925; Metz, 1926a; Metz, 1926b; Fuge, 1994). Instead, they proceed directly from prometaphase to an ‘anaphase-like’ stage (Fig. 1B). This atypical behaviour is accompanied by the formation of a monopolar spindle generated from a single polar complex that contains ‘giant centrioles’ surrounded by a large amount of pericentriolar material, from which numerous microtubules radiate (Kubai, 1982; Fuge, 1994; Esteban et al., 1997). During the anaphase-I-like stage, the monopolar spindle directs the segregation of maternal chromosomes to the single pole, whereas paternal chromosomes move in the opposite direction into a cytoplasmic bud to be discarded later (Metz, 1925; Smith-Stocking, 1936). Importantly, cytological and ultrastructural data indicate that the two parental sets of chromosomes occupy distinct nuclear compartments in germ nuclei from the initial stages of development until the occurrence of meiosis (Rieffel and Crouse, 1966; Kubai, 1982; Kubai, 1987; Goday and Esteban, 2001; Goday and Ruiz, 2002). Moreover, the precocious intranuclear segregation of the maternal chromosomes with respect to the paternal ones is considered to be essential in determining which chromosomal set will be lost during male meiosis I (Kubai, 1987; Goday and Esteban, 2001).

The outstanding feature of meiosis II is the different kinetic behaviour of the maternal X chromosome with respect to maternal autosomes (Metz, 1925; Crouse, 1943) (reviewed in Esteban et al., 1997). By metaphase II (Fig. 1C), whereas maternal autosomes align in a typical metaphase plate, the non-disjoining Xm chromosome does not move away from the polar complex formed at meiosis I. Importantly, microtubules radiating from the single pole interact with the Xm centromere and maintain the non-disjunction of this chromosome throughout meiosis (Esteban et al., 1997). Finally, at anaphase II (Fig. 1D,E), the autosomal chromatids segregate to opposite poles in the conventional manner by means
of an asterless bipolar second meiotic spindle (Metz, 1926a; Abbott and Gerbi, 1981; Esteban et al., 1997).

The genetic control of X<sub>y</sub> chromosome segregation at male meiosis was examined in *Sciara coprophila*, where three heterochromatic blocks proximal to the centromere contain rDNA sequences (Gerbi and Crouse, 1976; Crouse, 1977; Crouse et al., 1977; Crouse, 1979). A cis-acting locus, the *controlling element* (*CE*), regulating X-centromere activity was identified in the middle heterochromatic block (Crouse, 1960; Crouse, 1977; Crouse, 1979; Gerbi, 1986). Its translocation to an autosome provokes non-disjunction of the recipient autosome, whereas the X chromosome lacking the *CE* segregates normally (Crouse, 1979). In view of this, it has been hypothesised that the *CE* inhibits normal centromeric function in the X<sub>y</sub> chromosome (Gerbi, 1986). However, how this occurs and which specific DNA sequences are involved remains undetermined.

In the present work, we have re-examined the process of male meiosis in *Sciara ocellaris* and *S. coprophila* in an attempt to further understand the cellular mechanisms leading to both paternal chromosome elimination and X<sub>y</sub> chromosome non-disjunction. With this in mind, we decided to analyse conserved chromatin modifications involved in normal chromosome segregation during mitosis and meiosis. A good candidate was that of histone H3 phosphorylated at four N-terminal residues, Ser10, Ser28, Thr3 and Thr11 (reviewed in Nowak and Corces, 2004; Xu et al., 2009). Cell-cycle-dependent phosphorylation of histone H3 (H3-P) occurs in most eukaryotes and high levels of H3-P constitute a conserved mark of mitotic cell division. As a general rule, condensed metaphase chromosomes attain high levels of phosphorylated histone H3 and upon exit of mitosis or meiosis, a global dephosphorylation of histone H3 occurs. Moreover, an increasing amount of data coming from different systems support the idea that the four H3-P forms are coordinated both in space and time during mitosis and meiosis (Xu et al., 2009).

Phosphorylation of H3S10 (H3S10-P) (Gurley et al., 1978; Wei et al., 1998; Hsu et al., 2000; Giet and Glover, 2001) and of H3S28 (H3S28-P) (Goto et al., 1999), are carried out by the mitotic kinase Aurora B that is a component of the chromosomal passenger complex (CPC) (Giet and Glover, 2001; Ruchaud et al., 2007). Both H3-P modifications are highly conserved among eukaryotes and are crucial for higher-order chromatin compaction during mitosis and meiosis (reviewed in Hsu et al., 2000; Nowak and Corces, 2004). The temporal correlation of H3S10-P with chromosome condensation during cell cycle progression was first demonstrated in *Tetrahymena* using antibodies against H3S10-P (Wei et al., 1998). Accordingly, mutation of the site H3S10 in *Tetrahymena* disrupts proper chromosome condensation and segregation both at mitosis and meiosis (Wei et al., 1999). A number of studies have shown that in mammals, the cell-cycle-dependent phosphorylation of H3S10 and H3S28 begins at the pericentromeric chromosome regions and spreads throughout the chromosomes during the G2–M phase transition (Goto et al., 1999; Hsu et al., 2000). A similar distribution of H3S10-P linked to chromosome condensation has also been found in flies where depletion of Aurora B provoked deficiencies of chromosome condensation at mitosis (Giet and Glover, 2001). Moreover, in *Drosophila* spermatocytes undergoing the first meiotic division, metaphase I chromosomes show prominent H3S10-P signals that decrease substantially at anaphase and telophase (Krishnamoorthy et al., 2006).

Phosphorylation of H3T3 (H3T3-P) by the kinase Haspin (Dai et al., 2005) and of H3T11 (H3T11-P) by Dlk/ZIP (Preuss et al., 2003) were found to temporally associate with mitosis in turkey and mammalian cells (Polioudaki et al., 2004; Dai et al., 2005). The timing of H3T3 phosphorylation and dephosphorylation is similar to that of H3S10-P, although the strongest presence of H3T3-P at the inner centromeric regions of the chromosomes suggested a more direct role of H3T3-P in regulation of kinetochore assembly and functional activity (Dai et al., 2005). In this regard, recent findings in mammalian cultured cells revealed for the first time an essential functional role of H3T3 phosphorylation at the centromere site (Wang et al., 2010). Phosphorylated H3T3 is crucial for the
Fig. 2. Distribution of histone H3S10-P in S. ocellaris spermatocytes in the first meiotic division. (A–C′) Chromosome DAPI staining; m and p refer to the maternal and paternal chromosome complement, respectively; asterisk indicates the position of the single spindle pole (polar complex or ‘pc’ visualised by anti-tubulin staining). (A′–C′) Indirect immunolabelling with the H3S10-P antibody and merged images where antibody staining is in red. (B′) Double-immunolabelling with H3S10-P antibody (red) and anti-tubulin antibody (green). (A, A′) Prophase nuclei undergoing chromosome condensation; H3S10-P labelling is detected in all chromosomes as chromatin condensation increases (nucleus on the right in A′); arrow indicates mitochondria. (B–B′) Anaphase-like stage where the maternal four chromosomes facing the pole (pc in B′) appear less condensed than the paternal set segregating towards the bud; antibody labelling is restricted to the four paternal chromosomes (B′). (C, C′) End of meiosis I; a partial view of a cyst showing four spermatocytes arranged radially with respect to the lumen of the cyst; maternal chromosomes remain at the pole and lack H3S10-P staining; paternal chromosomes, tightly grouped, exhibit H3S10-P labelling. Scale bars: 10 μm.

Results

The chromosomal complement in S. ocellaris premeiotic germ nuclei is that of eight chromosomes: three pairs of autosomes (two acrocentric and one metacentric) and one pair of X chromosomes (acrocentric). In S. coprophila, additional germline-limited ‘L’ chromosomes are present in a variable number (1–4). L chromosomes are metacentric and, in contrast to the regular chromosomal component, are mostly heterochromatic.

Distribution of phosphorylated H3S10 in S. ocellaris spermatocytes during meiotic division

We performed the immunodetection of H3S10-P in S. ocellaris in male meiosis I when the elimination of the whole paternally inherited complement takes place. As mentioned, in Sciara males, homologous chromosomes do not pair at prophase nor do they align in a metaphase-like array. Instead, they proceed directly from prophase to an ‘anaphase-like’ stage (Gerbi, 1986; Esteban et al., 1997). Intranuclear H3S10-P labelling was detected in all chromosomes at prophase stage when a significant degree of chromosome condensation was achieved (Fig. 2A’, A′; nucleus on the right). Fig. 2B–B′ shows an example of a DAPI-stained first ‘anaphase-like’ figure, together with the corresponding monopolar first meiotic spindle evidenced by anti-tubulin staining. Immunolocalisation of H3S10-P (Fig. 2B′) revealed that the antibody associates exclusively to paternal chromosomes (p, segregating towards the cytoplasmic bud), whereas the maternal set (m, already near to the polar complex) is devoid of staining. In addition, the chromosomes that displayed H3S10-P signals (paternal set) exhibited a considerably higher degree of condensation with respect to the unstained ones (maternal set), as seen by DAPI staining (Fig. 2B). H3S10-P staining differences between the two parental chromosome groups could be observed until the end of the ‘anaphase-like’ stage (Fig. 2C, C′), where paternal chromosomes, usually tightly grouped and highly fluorescent with DAPI, are eliminated in buds into the lumen of the cyst. From these results, we concluded that in the ‘anaphase-like’ stage of meiosis I, the two chromosomal groups differ in H3S10 phosphorylation and in chromosome condensation levels, with the paternal chromosomes phosphorylated and more highly condensed than the maternal homologues. Before this stage, as shown in prophase nuclei (Fig. 2A, A′), both maternal and paternal chromosomes had transiently achieved a similar degree of condensation and significant levels of H3S10-P. Thus, during meiosis I, paternal chromosomes segregating towards the spermatocyte bud remain condensed and H3S10 phosphorylated, whereas the maternal set associated with the polar complex appears to some extent decondensed and under-phosphorylated at H3S10.

We next examined H3S10-P distribution during the second meiotic division. The transition from meiosis I to meiosis II is clearly marked by H3S10 phosphorylation of maternal
chromosomes together with increasing chromosome condensation (Fig. 3A,A'). At this phase, we frequently observed that one of the maternal acrocentric chromosomes was devoid of H3S10-P signals at one chromosomal end (Fig. 3A,A', arrow in m). In some of the spermatocyte squashes, it was also possible to detect a small un-stained chromosomal region in the bulk of the

Fig. 3. Distribution of histone H3S10-P in *S. ocellaris* spermatocytes undergoing the second meiotic division. (A–H) Chromatin DAPI staining; m and p refer to the maternal and paternal chromosome complement, respectively; asterisk indicates the position of the single spindle pole. (A'–G') Indirect immunolabelling with H3S10-P antibody and merged images where antibody staining is in red. (B'–D',F',G') Double-immunolabelling with H3S10-P antibody (red) and anti-tubulin antibody (green). (A,A') Meiosis I–II transition; maternal and paternal chromosomes exhibit H3S10-P staining except at one end of one of the acrocentric maternal chromosomes plus at a protruding piece of paternal chromosomes (arrows in m and p). (B–B') Metaphase II; Xm chromosome is H3S10-P labelled except at the chromosome end containing the centromere, as seen by its association to polar microtubules (B'); maternal autosomes at the metaphase plate (two acrocentric and one metacentric, Am in B) are entirely decorated by the antibody (B'); arrows in (B') indicate the asterless second meiotic spindle and arrowhead denote non-spindle bud microtubules. In the lower part of (B,B') arrows indicate a small region of paternal chromatin lacking antibody staining. (C,C') Early anaphase II where maternal autosomes are decorated by the antibody. (D–D') Anaphase II progression showing that the separated maternal autosomes are entirely labelled (D,D'); in D', arrows indicate the second meiotic spindle midzone and arrowhead denotes non-spindle bud microtubules. (E,E') Two nearby spermatocytes showing the maternal autosomal chromatids moving closer to the X chromosome; antibody staining is progressively reduced starting from the centromeric regions (arrows in E'); Xn chromosome also shows a reduction of H3S10-P signals in both chromatids (arrowheads in E'); long arrows in E,E' denote a non-stained region protruding from the paternal chromatin bulk. (F–F') Two nearby spermatocytes in late anaphase where the maternal chromatids have reached the X chromosome at the polar complex. (F') H3S10-P staining is restricted to the tips of the Am chromatids furthest from the direction of movement towards the bud and to paternal chromosomes; insets in F' show enlarged Xn chromosomes where sister chromatids appear are totally separated; arrows in F' indicate the two halves of the second meiotic spindle. (G–G') Spermatocyte at the end of meiosis II; (G) position of the future sperm-nucleus maternal components (Xn+Am) and of the discarded maternal X-null chromatid set (Am) plus paternal chromosomes (p). (G') Antibody labelling is restricted to paternal chromosomes. (H) Spermiogenesis; both spermatid nuclei (arrows) and eliminated chromatin (arrowheads) in the buds are devoid of H3S10-P staining. Scale bar: 10 μm.
eliminated paternal chromosomes (Fig. 3A, A', arrow in p). The analysis of metaphase II (Fig. 3B–B'), where the non-disjoining Xm chromosome remains attached to the polar complex whereas the maternal autosomes (Am) align in a metaphase plate, permitted us to identify the Xm chromosome as that lacking H3S10-P signals at one chromosome end. Moreover, the Xm chromosome end devoid of the H3S10-P label was the one containing the centromere, as seen by its typical spatial location and association with the polar complex microtubules visualised by anti-tubulin staining (Fig. 3B', pc). During anaphase II (Fig. 3C–F'), H3S10-P labelling of maternal autosomes persists until sister chromatids are completely detached (Fig. 3D–D'). At late anaphase II (Fig. 3E–F'), the intensity of H3S10-P staining on the maternal autosomes declined progressively starting from centromeric regions of the chromosomes to the more distal ones. Interestingly, the undivided Xm chromosome that remained attached to the polar complex, also showed a progressive reduction of H3S10-P signal towards the tip of the sister chromatids (Fig. 3E, E'). At the end of anaphase II, when one maternal group of chromatids reaches the undivisioned Xm chromosome to constitute the future sperm nucleus, no H3S10-P signals were detectable in the chromosomes. At this stage, the Xm chromosome sister chromatids can be discerned because appear separated along their length (insets in Fig. 3F'). Thus, at the conclusion of the second meiotic division, H3S10-P was only present in the bulk of paternal chromosomes eliminated at the end of meiosis I (Fig. 3G, G'), p; however, during spermatid differentiation, no staining was detected with antibody against H3S10-P (Fig. 3H).

**Distribution of phosphorylated H3S28 in *S. ocellaris* spermatocytes during meiotic division**

The immunolocalisation of H3S28-P in *S. ocellaris* meiotic divisions (Fig. 4) revealed a very similar pattern to that of histone H3S10-P. Intranuclear staining of prophase chromosomes was detected as chromosomes condense before they enter the ‘anaphase-like’ stage of first meiosis (Fig. 4A, A'). Similarly to H3S10-P staining, at meiosis I, only paternal chromosomes were recognised by the H3S28-P antibody. However, H3S28-P staining appeared less uniform and in a more speckled pattern than that of H3S10-P in the discarded paternal chromosomes. At the meiosis I–II transition (Fig. 4C, C'), when maternal chromosomes located at the polar complex re-condense, they become significantly H3S28 phosphorylated, except at one chromosome tip. As found for H3S10-P staining, during metaphase II it was evident that this chromosome corresponds to the Xm chromosome (Fig. 4D, D'). Similarly, during anaphase II progression (Fig. 4E–F'), a reduction of H3S28-P antibody staining was observed in the separated maternal chromatids, as well as along the Xm chromosome.

**Distribution of phosphorylated H3T3 in *S. ocellaris* spermatocytes during meiotic division**

In contrast to staining with antibodies against H3S10-P and H3S28-P, *S. ocellaris* condensing chromosomes at prophase were devoid of H3T3-P marks (Fig. 5A). H3T3-P signals were first detected at the ‘anaphase-like’ stage of meiosis I (Fig. 5B, B') where, similarly to H3S10-P and H3S28-P staining, H3T3-P labelling was restricted to the highly condensed paternal set of chromosomes. Likewise,
H3T3-P antibody labelling was completely absent in the less condensed maternal set located at the polar complex of meiosis I. Moreover, at metaphase II, although the maternal chromosomes were extensively decorated with H3T3-P antibody (Fig. 5C,C’), the non-disjoining Xm chromosome exhibited, again, lack of antibody staining at the centromeric end. Unlike the HS10-P and H3S28-P staining, at the onset of anaphase, H3T3-P signals were dramatically reduced in all chromosomes, including the Xm chromosome (Fig. 5C,C’). From these results, we conclude that in addition to staining the paternal chromosomes in the late anaphase-like stage of meiosis I, phosphorylated histone H3T3 specifically associates to the metaphase stage of meiosis II and that the Xm chromosome at metaphase is H3T3 underphosphorylated at the centromeric end.

**Distribution of phosphorylated H3T11 in S. ocellaris spermatocytes during meiotic divisions**

The immunolocalisation of H3T11-P revealed that at prophase all chromosomes were decorated with H3T11-P antibody (arrow in Fig. 6A). At the anaphase-like stage of meiosis I, the antibody exclusively labelled maternal chromosomes and not the paternal set, as seen in all the spermatocytes of a cyst in Fig. 6A’. Thus, at meiosis I, the H3T11-P staining pattern was the reverse of that revealed by H3S10-P, H3S28-P and H3T3-P antibodies. This result was confirmed by double-immunofluorescence with H3T11-P and H3S10-P antibodies, where, as expected, both parental sets were stained (not shown). At the meiosis I–II transition (Fig. 6B,B’), maternal chromosomes remained labelled with H3T11-P, but the Xm chromosome was now unlabelled at the centromeric end, as confirmed next in observations of metaphase II (Fig. 6C,C’). Thus, these results strongly suggest that the Xm chromosome at the onset of meiosis II undergoes dephosphorylation of H3T11 at the centromeric end. Thus, the H3T11-P staining pattern at metaphase II (Fig. 6C’) is identical to that described above for the other histone H3 modifications. At anaphase II (Fig. 6D,D’), however, H3T11-P staining was detected along the chromosomes until the end of anaphase unlike the earlier loss of staining described above for the other antibodies.

**Histone H3 phosphorylation in S. coprophila male meiotic divisions**

We next investigated the distribution of four histone H3-P forms in S. coprophila spermatocytes undergoing meiotic division (Fig. 7). In addition to the ordinary chromosomal complement, S. coprophila poses germline L chromosomes that are paternally and maternally inherited (reviewed in Goday and Esteban, 2001; Greciano and Goday, 2006). In males, all L chromosomes segregate together with the maternal set at the first meiotic division. At the second meiotic division, L chromosomes undergo normal disjunction along with the maternal autosomes.

In meiotic prophase nuclei (Fig. 7A–D), immunostaining with H3S10-P, H3S28-P, H3T3-P and H3T11-P antibodies revealed that the ordinary chromosome complement of S. coprophila exhibits identical staining properties to those found in S. ocellaris. That is, all chromosomes appear phosphorylated for H3S10, H3S28 and H3T11, but not for H3T3. L chromosomes, instead, differ with respect to the ordinary chromosomes in that no labelling was detected with H3S10-P and H3S28-P antibodies (Fig. 7A,B). Because L chromosomes are highly heterochromatic, they display different cell cycle condensation timing. Consequently, the lack of H3S10-P and H3S28-P signals on the L chromosomes possibly reflects a lower condensation level of these chromosomes with respect to the rest, at this particular meiotic stage. However, in all our observations and as shown in Fig. 7D, ordinary chromosomes plus L chromosomes are entirely decorated with H3T11-P antibody.

During S. coprophila meiosis I and meiosis II, the four histone H3-P forms exhibited identical chromosomal distribution to that described for S. ocellaris. At meiosis I (Fig. 7E–H), maternal chromosomes plus L chromosomes were labelled only with H3T11-P antibody, whereas the discarding, more condensed, paternal set exhibited H3S10-P, H3S28-P and H3T11-P signals and lacked H3T11-P labelling. At metaphase II (Fig. 7I–L), the maternal autosomes plus the L chromosomes were decorated with the four antibody staining patterns, whereas the Xm chromosome, which remained attached to the first single pole was devoid of antibody staining at the centromeric end. At anaphase II, the four antibody staining patterns were identical to those found in S. coprophila (not shown). From these results we conclude that in both Sciara species, the centromeric end of the un-disjoined Xm chromosome is specifically under-phosphorylated on histone H3.

Another intriguing observation referred to the staining behaviour of paternal chromosome, which, following meiosis I, remained congregated in the cytoplasmic bud of the spermatocyte. As mentioned above, paternal chromosomes in S. ocellaris usually move as a tight group into the bud, whereas in S. coprophila,
individual paternal chromosomes can be often identified. In the present analysis, on several occasions we first detected in S. ocellaris spermatocytes, a small region protruding from the mass of paternal chromatin that was clearly devoid of H3S10-P and H3S28-P staining (arrows in Fig. 3A, B). Moreover, in S. coprophila, we were able to discern that one of the acrocentric paternal chromosomes clearly lacked H3S10-P and H3S28-P labelling at one chromosome end (Fig. 7J, K). In view of this finding, we decided to investigate whether this particular paternal chromosome could correspond to the X chromosome. For this purpose, we performed in situ hybridisation with a rDNA probe containing the centromere. Moreover, as expected, in other Sciara dividing tissues, histone H3-P patterns are identical between the X chromosomes and the rest of the chromosomes (an example of mitotic neuroblasts immunostained with H3S10-P antibody is shown in Fig. 9).

**Distribution and timing of phosphorylated H3 forms during Sciara male meiosis**

Based on all our results, in Table 1 we summarise the differential distribution of histone H3-P forms in Sciara ordinary chromosomes in relation to the main meiotic stages. At prophase, chromosome condensation correlated with an increase in the levels of H3 phosphorylation at Ser10, Ser28 and Thr11. At the anaphase-like stage, although the condensed paternal chromosomes remained highly phosphorylated, maternal chromosomes decondensed to some extent and became dephosphorylated at histone H3 Ser10 and Ser28 but remain phosphorylated at Thr11. As maternal chromosomes initiate recondensation (meiosis I–II transition) to enter metaphase II, significant phosphorylation at Ser10 and Ser28 occurred again, and in addition, for the first time at H3T3. Thus, the four H3-P modifications spread over the metaphase chromosomes are also found in the X chromosome, but not at the centromeric end. Interestingly, phosphorylation at H3T3 is the only H3 form that is restricted to the metaphase stage.

**Discussion**

An intriguing finding of this work is that at the onset of the second meiotic division, the maternal X chromosome undergoes normal H3 phosphorylation except at the centromeric end, which is under-phosphorylated for the four H3-P forms. We provide here the first evidence linking the under-phosphorylated H3 status of the X...
chromosome centromeric region with meiotic non-disjunction of this chromosome in sciarid flies.

Histone H3 phosphorylation, chromosome condensation and paternal chromosome elimination at meiosis I

Many studies indicate that histone H3 phosphorylation is required for chromatin condensation before chromosome segregation at both mitosis and meiosis (Gurley et al., 1978; Van Hooser et al., 1998; Goto et al., 1999; Wei et al., 1999; Giet and Glover, 2001). Accordingly, we found significant levels of H3S10-P, H3S28-P and H3T11-P in Sciara spermatocytes undergoing chromosome condensation at prophase. Moreover, our results also confirmed that the timing of H3S10 and H3S28 phosphorylation (and dephosphorylation) is very similar, as previously reported in other dividing cell types (Nowak and Corces, 2004; Xu et al., 2009). By contrast, H3T11-P does not always overlap with phosphorylation of H3S10 and H3S28, given that the H3T11-P modification was also found in less condensed chromosomes that were under-phosphorylated at H3S10 and H3S28 (see Table 1 and L chromosomes in Fig. 7). However, H3T3-P specifically associates with chromosomes displaying the highest degree of compaction during the meiotic process (metaphase II).

A detailed description of the cellular mechanisms involved in the highly atypical separation of the two parental sets during male meiosis I was given in previous studies (Gerbi, 1986; Goday and Esteban, 2001). An important feature is that, before the anaphase movements of meiosis I, each parental set of chromosomes in the prophase nuclei occupies distinct compartments, with the maternal set always closest to the polar complex of the monopolar spindle (Kubai, 1982; Kubai, 1987). In view of this observation, it was concluded that the first meiotic spindle maintains a pre-existing segregation of the two chromosome sets while the distance between them increases (Kubai, 1982). The proximity of maternal chromatin to a single giant pole generating numerous microtubules ensures that, following breakdown of the nuclear membrane, the maternal chromosomes are retained at the polar complex during meiosis I (Gerbi, 1986; Goday and Esteban, 2001). When covalent histone modifications were analysed during germline development in S. ocellaris, differences in the acetylation or methylation of histones H3 and H4 were found between maternal and parental chromosomes (Goday and Ruiz, 2002; Greciano and Goday, 2006). Concerning male meiosis I, whereas maternal chromosomes at the polar complex are acetylated at histones H3 and H4, the eliminating paternal chromosomes are, instead, under-acetylated and methylated at histones H3 and H4 (Goday and Ruiz, 2002; Greciano and Goday, 2006). Here, we show that phosphorylation of histone H3 differs between the two separating parental sets of chromosomes at male meiosis I, and that such differences in H3 phosphorylation coincide with the extent of chromosome condensation. Thus, the unravelling of maternal chromosomes (at the polar complex) correlates temporarily with dephosphorylation at H3S10 and H3S28, whereas the condensed paternal set (segregating to the bud) remains phosphorylated at H3S10 and H3S28. This finding is in good agreement with previous data, where Sciara paternal chromosomes at meiosis I were found to exhibit significant enrichment in H4K20 methylation (Greciano and Goday, 2006), a histone modification associated with densely packed chromatin (Rice et al., 2002). The present data on histone H3 phosphorylation give further support to our previous model that relates intranuclear chromosome arrangements, histone covalent modifications and chromosome elimination in Sciara germline nuclei (Greciano and Goday, 2006).

Histone H3 phosphorylation and Xm chromosome non-disjunction at meiosis II

From our analysis, the Sciara male meiosis I–II transition is marked by the presence of the four H3-P forms, coincident with a further compaction of maternal chromosomes before metaphase II entry.
H3 phosphorylation in *Sciara* male meiosis

(see Table 1). Therefore, H3 phosphorylation of maternal chromosomes congregated at the polar complex site precedes the migration of the autosomes to align in a metaphase II plate. A particularly good example is seen in Fig. 4C' where *S. ocellaris* maternal chromosomes stained with the anti-H3S28-P antibody line up with the centromeres orientated towards the single pole in the primary spermatocyte. Taking into consideration all our observations, we conclude that it is at this particular 'pre-metaphase stage' when local deficiencies in H3 phosphorylation are established at the centromeric end of the *Xm* chromosome. In view of our results, it seems very reasonable to question whether such H3 phosphorylation modifications are responsible for the persistent chromatid disjunction depends on the deficiency in H3 phosphorylation at the centromeric region. We believe that this is so, and we discuss this further below in light of recent data that demonstrate the crucial role of histone H3 phosphorylation in centromere function during mammalian cell division (Kelly et al., 2010; Wang et al., 2010).

We have demonstrated that during anaphase II histone H3 dephosphorylation at Ser10 and Ser28 begins at the centromeric regions of the chromosomes and extends along the chromosome arms. This is consistent with observations of the distribution of H3S10-P in anaphase chromosomes from *Drosophila* syncytial mitosis, where dephosphorylation events originate at the centromere region (Su et al., 1998). In this respect, similar observations were obtained in early *Sciara* embryonic somatic divisions (not shown), as well as in neuroblast cells (Fig. 9), suggesting that a gradual H3S10 and H3S28 dephosphorylation pattern might be a general occurrence. Consistently, the un-disjoined *Xm* chromosome lacking H3-P at the centromeric end, also exhibits non-uniform dephosphorylation of H3S10 and H3S28 that, in this chromosome, initiates at the proximal region of chromosome arms. So it seems that, at least in the case of the *Xm* chromosome, phosphorylation or dephosphorylation of histone H3 does not necessarily begin at the centromere site to extend then along the chromosome arms. However, from our observations in *Sciara* spermatocytes at anaphase II, a gradual loss of H3T3 and H3T11 phosphorylation along the chromosome arms could not be discerned. It appears that histone H3T3 phosphorylation is dramatically reduced at the onset of anaphase in all chromosomes, whereas phosphorylated H3T11 persists until very late anaphase (see Table 1). Taken as a whole, the results support differential phosphorylation and dephosphorylation of H3S10 and H3S28 that, in this chromosome, commences at the centromere region and extends along the chromosome arms.

### Table 1. Summary of histone H3 phosphorylation distribution during the first and second male meiotic divisions in *Sciara*

| Meiosis I | Meiosis II |
|-----------|-----------|
| Prophase | Anaphase-like | Meiosis I–II transition | Metaphase | Early anaphase | Late anaphase |
| m | p | m | p | m | m | p |
| A<sub>m</sub>X<sub>m</sub> | A<sub>p</sub>X<sub>p</sub> | A<sub>m</sub>X<sub>m</sub> | A<sub>m</sub> | X<sub>p</sub> | A<sub>m</sub> | X<sub>m</sub> | A<sub>m</sub> | X<sub>m</sub> | A<sub>m</sub> | X<sub>m</sub> |
| H3S10-P | + | + | + | + | + | + | + | + | + | + |
| H3S28-P | + | + | + | + | + | + | + | + | + | + |
| H3T3-P | + | + | + | + | + | + | + | + | + | + |
| H3T11-P | + | + | + | + | + | + | + | + | + | + |

m, maternal chromosomes; p, paternal chromosomes; A<sub>m</sub>, maternal autosomes; X<sub>m</sub>, maternal X chromosome; A<sub>p</sub>, paternal autosomes; X<sub>p</sub>, paternal X chromosome; +/+ denotes antibody staining for a large part of the chromosome except at the centromeric end. Paternal chromosomes are only shown in meiosis I when they are eliminated. L chromosomes (not included in table) behave identically to maternal chromosomes from the initiation of the first meiotic segregation process.
dephosphorylation timing between histone H3-P forms along the meiotic process in Sciara. Importantly, all the H3-P forms first arise at the meiosis I–II transition, and remain until the onset of anaphase II.

**Histone H3 phosphorylation and X_m centromere inactivation**

The kinase Aurora B phosphorylates H3S10 and H3S28 and both H3-P modifications are implicated in chromatin compaction, which in turn, is required for chromosome congression and proper chromosome segregation during mitosis and meiosis (Gurley et al., 1978; Wei et al., 1998; Wei et al., 1999; Goto et al., 1999; Hsu et al., 2000; Giet and Glover, 2001) (reviewed by Nowak and Corces, 2004). Aurora B is a component of the CPC (chromosomal passenger complex) that also contains INCENP, Survivin and Borealin/Dasra (Giet and Glover, 2001; Ruchaud et al., 2007). At the beginning of the M phase, the CPC associates with condensing chromatin, accumulates at the inner centromere at metaphase and relocates onto the central spindle at anaphase (Giet and Glover, 2001; Carmena and Earnshaw, 2003). Importantly, the CPC controls chromatin-dependent spindle assembly and processes at the centromere (Ruchaud et al., 2007). Depletion of Aurora B kinase in Drosophila S2 cells greatly reduces the levels of H3S10 phosphorylation and produces important alterations in the condensation of mitotic chromosome (Giet and Glover, 2001). One of the roles of Aurora B, moreover, is to ensure chromosome bi-orientation at metaphase and to correct mono-oriented attachments to the spindle (Shannon and Salmon, 2002). Taking this information into consideration and given that at metaphase II, the mono-oriented X_m chromosome is clearly under-phosphorylated for H3S10 and H3S28 at the centromere region, it seems reasonable to assume that this region in the X_m chromosome is deficient for Aurora B activity and that it is most probably less condensed than a regular centromeric region.

Phosphorylation of H3T3 is dependent on Haspin, a conserved kinase that functions in mitosis and has homologues in all the main phyla, including fungi, animals and plants (Dai et al., 2005; Higgins, 2010). Haspin, localises predominantly to chromosomes, and phosphorylates histone H3T3 during mitosis at the chromosome arms and at the inner centromeres between the regions delineated by the centromere-specific histone CENP-A (Polioudaki et al., 2004; Dai et al., 2005; Dai et al., 2006; Higgins, 2010). Interestingly, Haspin RNAI in mammalian cells causes partial metaphase figures with numerous misaligned chromosomes, where many of them become trapped near the spindle poles (Dai et al., 2005). Such chromosomes, as demonstrated using anti-centromere antibodies, are constituted by mono-oriented sister chromatid pairs (Dai et al., 2005). It is noteworthy that the Sciara X_m chromosome at metaphase II displays an identical cytological phenotype to that described for misaligned chromosomes in Haspin-depleted cells. Considering that the centromeric region of X_m chromosome lacks H3T3-P, it is possible to assume that, similarly to Aurora B, the centromere of the X_m chromosome is deficient in Haspin kinase activity. This could explain the misalignment of X_m chromosome at metaphase II and its permanent attachment to the spindle pole until the end of anaphase II.

Recently, the essential functional role of Haspin during mitotic division has been further investigated in mammalian cells (Kelly et al., 2010; Wang et al., 2010). From such studies, it emerged that Haspin is required for the accumulation of CPC at the centromeres, and that the CPC subunit Survivin binds directly to phosphorylated H3T3 (Wang et al., 2010). Most important, H3T3-P is recognised by an evolutionarily conserved binding pocket in the BIR domain of Survivin (Kelly et al., 2010). As concluded, H3T3-P positions the CPC to the centromeres to regulate Aurora-B-selected targets and the interaction between H3T3-P and CPC is mediated by Survivin (Kelly et al., 2010; Wang et al., 2010). Taking into consideration these data and our results, it is highly predictable that the mere lack of H3T3-P in the centromeric region of the X_m chromosome (at the meiosis I–II transition) prevents the CPC recruitment and thus leads to a non-functional centromere.

**The CE and histone H3 phosphorylation in the X chromosome**

Early work in sciarid flies identified a cis-acting locus, the CE, which regulates X-centromere activity during S. coprophila male meiosis and is contained in the heterochromatin proximal to the X centromere (Crouse, 1960; Crouse, 1977; Crouse, 1979; Gerbi, 1986). Although the molecular nature of the CE is still unknown, it has been considered that it is capable of modifying normal centromere function of the X chromosome during male meiosis II (Gerbi, 1986). The present results support our view that the CE modifies the X_m centromeric function, causing X_m chromosome non-disjunction, by inhibiting global H3 phosphorylation at the centromeric chromatin. Therefore, it seems that the CE governs the inability of the chromatin at this particular X chromosome region to become H3 phosphorylated, unlike the rest of the chromosome. Consistently, we found that H3 phosphorylation was also inhibited at the centromeric region of the paternal X chromosome, which together with the paternal autosomes, is eliminated at meiosis I in both Sciara species. In this regard, the deficiency in H3 phosphorylation at the X_m centromeric region does not affect the X_p chromosome segregation modality at meiosis I, in either S. ocellaris or S. coprophila. This interesting observation gives further support to the conclusion that the presence of organised kinetochores in paternal chromosomes seems not to be necessary for their regular elimination at meiosis I (Goday and Esteban, 2001).

An understanding of the organisation of the heterochromatic sequences confining the CE and the mechanisms established to inhibit chromatin H3 phosphorylation in cis are important questions that remain to be answered.

**Materials and Methods**

**Fly culture**

S. ocellaris and S. coprophila were raised at 20°C as described elsewhere (Rieffel and Crouse, 1966).

**Fixation**

Sciara prepupae were dissected in 15 mM Tris-HCl pH 7.4, 60 mM KCl, 15 mM NaCl, 0.5 mM espermidine, 0.1% Triton X-100 solution to remove the testes. For each experiment with a different H3-P antibody at least ten testes were processed. The testes were immediately fixed with 1% paraformaldehyde, 0.1% Triton X-100 in PBS for 3 minutes. They were then incubated in 50% acetic acid, 1% parafomaldehyde for 1 minute and squashed. Slides were frozen in liquid N2 to remove coveslips, postfixed in 3.7% formaldehyde for 10 minutes and extensively washed in PBS. For mitotic neuroblasts analysis brains were removed from third instar larvae and fixed as above.

**Immunostaining and microscopy**

All slides were washed in PBS (3 × 10 minutes), incubated in PBS containing 1% Triton X-100 (10 minutes) and in PBS with 3% BSA and 0.1% Tween 1% at room temperature. The primary antibodies were rabbit polyclonal anti-H3S10-P, anti-H3S28-P, anti-H3T3-P, anti-H3T11-P (Upstate Biotechnology) diluted 1:40 to 1:100; mouse monoclonal anti-beta-tubulin (Amersham) diluted 1:200. Secondary antibodies were FITC- and Cy3-conjugated anti-rabbit (Southern Biotechnology) and RhD-conjugated anti-mouse Ig (Dakopatts). Secondary antibodies were diluted 1:50 for FITC-conjugated antibody and RhD-conjugated anti-mouse and 1:800 for
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