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Short Communication

Loss of centromeric histone H2AT120 phosphorylation accompanies somatic chromosomes inactivation in the aberrant spermatocytes of *Acricotopus lucidus* (Diptera, Chironomidae)

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Abstract

In the germ line of the chironomid *Acricotopus lucidus* two cells with quite different chromosome constitutions result from the last unequal gonial mitosis. In the male, the future primary spermatocyte receives all the germ line-limited chromosomes (=Ks) together with somatic chromosomes (=Ss), and later on undergoes meiotic divisions, while the connected aberrant spermatocyte gets only Ss and remains undivided with chromosomes inactivated in a metaphase-like condensed state. This raises the question whether the centromeres of the permanently condensed Ss of the aberrant spermatocyte remain active during meiosis of the connected regular spermatocyte. Active centromeres exhibit an epigenetic phosphorylation mark at threonine 120 of histone H2A. To visualise the centromeric H2A phosphorylation of the Ss in the aberrant spermatocyte, meiotic stages were immunostained with different anti-phospho histone H2AT120 antibodies. Clear H2AT120ph signals appear at the centromeres of the Ss during prophase, persist on the metaphase-like condensed Ss during meiosis I of the connected primary spermatocyte and disappear during transition to meiosis II. The centromeres of the Ss and Ks of the regular spermatocytes display H2AT120ph signals from prophase I to anaphase II. The loss of the H2AT120 phosphorylation detected on the centromeres of the Ss of the aberrant spermatocyte indicating their deactivation supports the idea of a programmed inactivation of the Ss to block the entry of the germ line-derived aberrant spermatocyte, lacking Ks, into meiosis, and thus to prevent the generation of sperms possessing only Ss. This mechanism would ensure the presence of the Ks in the germ line.

Keywords: Histone H2AT120 phosphorylation · Centromere inactivation · Chromosome inactivation · Aberrant spermatocyte · *Acricotopus lucidus*

Introduction

A programmed inactivation of all chromosomes of a germ line-derived cell takes place in males of the chironomid *Acricotopus lucidus*. The cell originates from the last unequal gonial mitosis, in which all germ line-limited chromosomes (Ks) move unseparated to only one pole, while the somatic chromosomes (=S) stay back in the metaphase plane (see Fig. 1b) and then segregate equally. The daughter cell, that receives all Ks and two sets of Ss differentiates into a primary spermatocyte, and the other, that receives only Ss becomes an aberrant spermatocyte. Both cells remain connected by a cytoplasmic canal (Staiber 2008). The doubling of the Ks in the primary spermatocyte by this differential mitosis compensates for the elimination of about half of the Ks in the first division of the primordial germ cell (Bauer and Beermann 1952; White 1973; Redi et al. 2001). With entry of the primary spermatocyte into meiosis the Ss in the aberrant spermatocyte condense and remain condensed in a metaphase-like state during both meiotic divisions (Staiber 2008, 2012). Since the sister chromatids of the condensed Ss do not separate, this raises the question as to whether their centromeres are still active.

On dicentric maize metaphase chromosomes Dong and Han (2012) demonstrated, that only the active centromere is phosphorylated at threonine 133 of histone H2A (H2AT133), which corresponds to human H2AT120 and to yeast H2AS121, while the inactive centromere had lost this epigenetic phosphorylation mark. This histone phosphorylation was detected at active centromeres of mitotic and meiotic chromosomes in maize (Dong and Han 2012), and in mammals, it is also present at active centromeres in mitosis and meiosis (Baarends et al. 2007, Yamagashi et al. 2010; Liu et al. 2013). The phosphorylation of H2AT120 at the centromeres generated by the protein kinase Bub1 is necessary for the centromeric localisation of shugoshins, which protect cohesin at the centromeres from cleavage by separase (Lee et al. 2008; Watanabe, 2012).

The presence of the Ks in the germ line is indispensable for a normal gametogenesis, as demonstrated by their experimental elimination from the germ line in the cecidomyiids *Wachtliella persicariae* (Geyer-Duszynska 1966) and *Mayetiola destructor* (Bantock 1970).

This gives rise the idea that the Ss of the germ line-derived aberrant spermatocyte of *A. lucidus* are permanently inactivated in a metaphase-like condensed state, to block the entry of the aberrant spermatocyte into meiosis, and thereby preventing the formation of sperms without Ks (Staiber 2008).

In the present study, the phosphorylation status of H2AT120 at the centromeres of Ss and Ks in regular and aberrant spermatocytes of *A. lucidus* was visualised during meiosis by immunofluorescence using different antibodies against H2AT120ph. Loss of the H2AT120 phosphorylation mark from the centromeres of the Ss of the aberrant spermatocyte, indicating their deactivation, strongly supports the concept of a programmed inactivation of the Ss to inhibit the entry of the aberrant spermatocyte into meiosis.

Materials and methods

Chromosome preparation and immunofluorescence

Chromosome preparations of spermatogonial mitoses and male meiotic stages of young fourth instar larvae, and prepupae of *Acricotopus lucidus* (Diptera, Chironomidae), were made as described in Staiber (2012). Preparations were treated with 0.4% Triton® X-100 in phosphate buffered saline (PBS), pH 6.9, under agitation for 4 h, blocked with 4% non-fat dry milk in PBS, pH 7.4, for at least 4 h, and were then covered with different polyclonal rabbit anti-phospho histone H2AT120 antibodies (abcam, # ab111492, product no longer available, gift of A. Houben; Active Motif , #39392, Lot 30508001; Active Motif, # 61195, Lot 31511001; Millipore, # ABE418, Lot Q2282924) and anti-phospho histone H2AT133 (a custom antibody prepared by LifeTein (USA) as described in Dong and Han (2012), gift of A. Houben) diluted 1:50 to 1:150 in PBS, pH 7.2, with 3% bovine serum albumin (BSA), and incubated in a moist chamber under plastic coverslips at 5°C for about 40 h. After four washes of 10 min each in PBS, pH 7.4, 0.2% Tween® 20, preparations were overlayed with FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, Dianova, Hamburg, Germany) diluted 1:50 in PBS with 3% BSA at 5°C overnight. Following three final washes chromosomes preparations were embedded in Vectashield Mounting Medium with DAPI, 1.5 µg/ml (Vector Laboratories, Burlingame, CA, USA). Images were taken with a Canon 450D and a Canon 600D digital camera on a Zeiss Axioplan epifluorescence microscope using a Plan-Neofluar 100/1.3 objective, and were further processed with Corel Photo-Paint and CorelDraw software (Ottawa, ON, Canada).

Results and discussion

In spermatogonial cells of *A. lucidus* in addition to the regular somatic chromosomes (=Ss; 2n = 6) a varying number of germ line-limited chromosomes (=Ks; n = 6 to 16) occurs (Fig. 1a) (Staiber 1988). About half of the Ks are eliminated in the first division of the single primordial germ cell of a testis by remaining in the equatorial plane (Bauer and Beermann 1952). To compensate for this reduction, in the last spermatogonial mitosis prior to meiosis all Ks migrate as unseparated sister chromatids to one pole, while the Ss stay back in the equatorial plane (Fig. 1b). With the arrival of the Ks at the pole the Ss segregate equally. The daughter cell left with only with Ss, and thus possessing a chromosome complement as somatic cells (Fig. 1c; ganglion cell with tightly paired homologous Ss), develops as an aberrant spermatocyte and remains permanently connected by a cytoplasmic canal with the primary spermatocyte containing all the Ks (Fig. 1d). With entry of the primary spermatocyte into meiosis all Ss of the aberrant spermatocyte condense, but do not become organised in the equatorial plane, and are arrested in a metaphase-like condensed state during both meiotic divisions and early spermiogenesis (Fig. 1d, e). This raises the significant question about the activity of the centromeres of the Ss during this time.

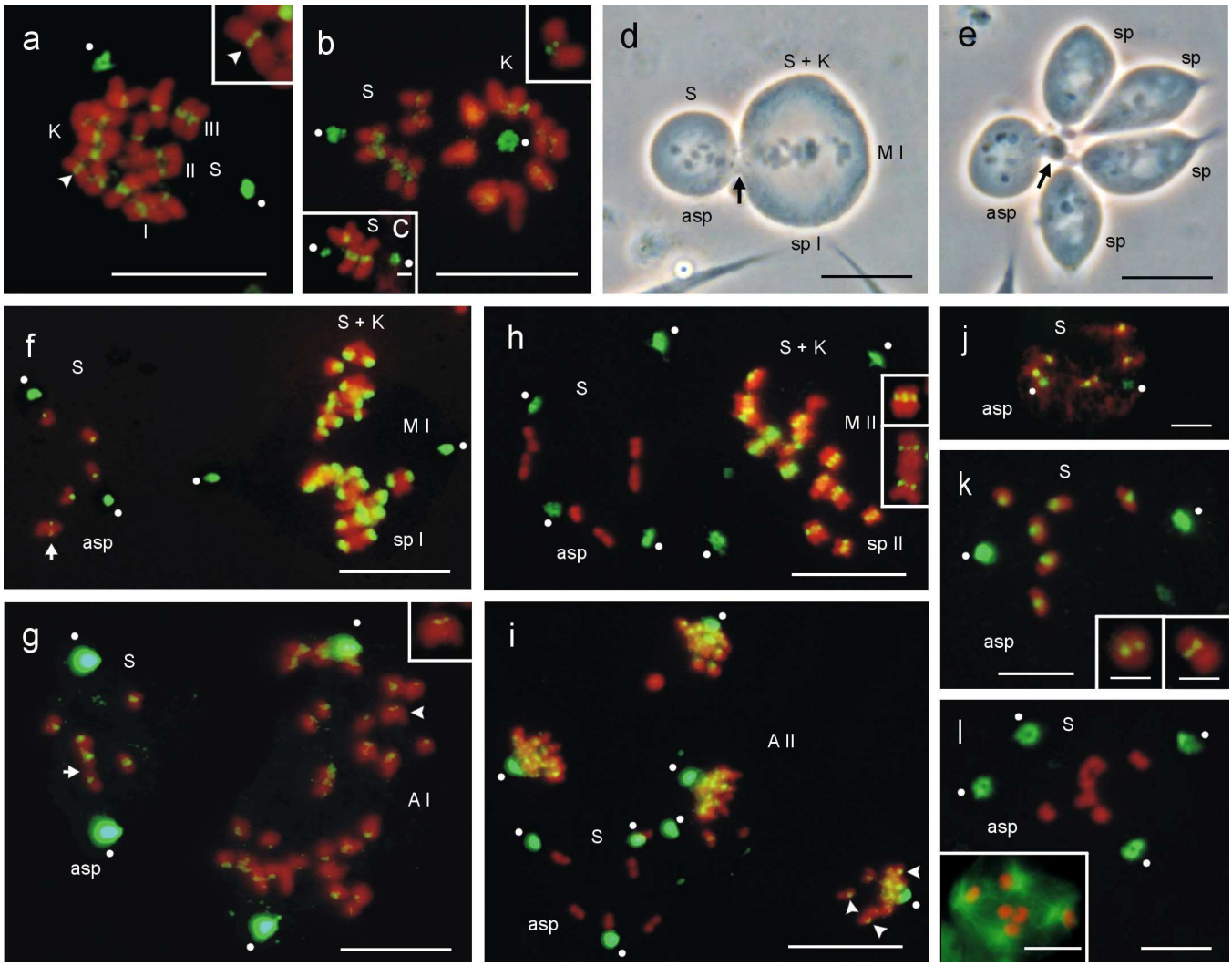

Fig. 1 a Spermatogonial metaphase with 6 Ss and 10 Ks, **b** male differential gonial mitosis with monopolar moving Ks and remaining Ss, and **c** ganglion metaphase with tightly paired homologous Ss immunostained with an anti-H2AT120ph antibody (abcam). Clear fluorescence signals indicate H2A phosphorylation at all centromeres of Ss and Ks. The strong signals at the spindle poles (*dots*) result from a cross-reactivity of this antibody with an unknown pericentrosomal protein. *Inserts* in **a** and **b** display a K in higher magnification of the gonial metaphase (**a,** *arrowhead*) and a monopolar migrating K from an other differential mitosis (**b**) both clearly showing two H2AT120ph signals on the centromeres of the sister chromatids. **d** Living primary spermatocyte (*spI*) with Ss and Ks in metaphase I (*M I*) and the affiliated aberrant spermatocyte (*asp*) with six metaphase-like condensed Ss. Both cells are connected by a permanent cytoplasmic canal (*arrow*). Phase contrast. **e** Syncytial complex of four young spermatids (*sp*) resulting from the two meiotic divisions and of the arrested aberrant spermatocyte with the six condensed Ss. **f-l** Immunostaining with anti-H2AT120ph (**f**, **h-l** abcam**;** lower insert in **h** Active Motif) and anti-H2AT133ph (**g**) . **f**, **g** All centromeres of the Ss and Ks of the primary spermatocytes (*spI*) in metaphase I (*MI*) (**f**) and in mid anaphase I (*A I*) (**g**) are clearly labelled and also the centromeres of the Ss of the connected aberrant spermatocytes (*asp*). *Insert* in **g** shows a poleward moving chromosome with two signals of the sister centromeres (arrowhead in **g**). The cross-reactivity of the antibodies with an unknown pericentrosomal protein indicates the position of the spindle poles (*dots*). **h** In metaphase II cells (*M II*, only one is shown) and **i** in late anaphase II (*A II*) only the centromeres of the Ss and Ks exhibit strong phosphorylation signals, while the centromeres of permanently condensed Ss of the connected aberrant spermatocytes have lost the H2AT120 phosphorylation. The tetrapolar arrangement of the doubled centrosomes in the aberrant spermatocytes is obvious, as seen on the four signals of the unknown pericentrosomal protein. **j** The centromeric H2AT120 phosphorylation begins during prophase condensation of the Ss in the aberrant spermatocyte. **k** The H2AT120ph antibody clearly labels the centromeres of the metaphase-like condensed Ss in an aberrant spermatocyte with bipolar arranged

centrosomes. *Inserts*: Strongly squashed Ss show that the centromeres of both sister chromatids are phosphorylated. **l** The centromeres of the Ss of an aberrant spermatocyte with doubled and tetrapolar arranged centrosomes are devoid of phosphorylation signals. *Insert*: Tetrapolar microtubule asters in an aberrant spermatocyte stained with Tubulin Tracker ™ Green. *K* germ line-limited chromosome, *S* somatic chromosomes, *I, II, III,* somatic chromosomes I-III. *Scale bars* represent 10 µm (**a**,**b**,**d**-**i**), 5 µm (**j**-**k**) and 1 µm (**c**, *inserts* in **k**)

The phosphorylation of centromeric histone H2A at threonine 120 is necessary to recruit the cohesin protector shugoshin to the centromeres, and to localize the chromosomal passenger complex (CPC) in mitosis and meiosis (Watanabe 2012; Wang and Higgins 2013). This phosphorylation site of H2A and some amino acids in the N-terminal direction (-LLPKKTp-) are evolutionarily conserved (Aihara et al. 2004; Dong and Han 2012; Demidov et al. 2014). In dicentric chromosomes of maize only the active centromere is phosphorylated at H2AT133, corresponding to human H2AT120, but not the inactive one (Dong and Han 2012). The amino acid sequence of histone H2A, and thus the position of the phosphorylated threonine, is unknown in *A. lucidus*. In the following, this site in *A. lucidus* is denoted as H2AT120.

The anti-phospho H2AT120 antibodies used in this study clearly detected H2AT120ph at the centromeres of all Ss and Ks in spermatogonial metaphases (Fig. 1a), and in differential mitoses (Fig. 1b), and at the centromeres of the Ss in ganglion metaphases (Fig. 1c). In addition, two of the antibodies (anti-H2AT120ph from abcam, and anti-H2AT133ph) showed a cross-reactivity with an unknown pericentrosomal protein, indicating the position of the spindle poles (Fig. 1a-c). No differences in the centromeric H2AT120ph immunostaining were observed between Ss and Ks in gonial metaphases (Fig. 1a). Both sister chromatid centromeres of such metaphase chromosomes, and of monopolar moving Ks in differential mitoses, displayed phosphorylation of H2AT120 (inserts in Figs. 1a and 1b).

In metaphase I of the primary spermatocyte all the centromeres of the S and K bivalents exhibit strong H2AT120 phosphorylation signals (Fig. 1f). H2AT120ph signals, although of less intensity, are also present at the centromeres of the six Ss of the aberrant spermatocyte, which are not aligned in the equatorial plane (Fig. 1f). Sometimes homologous Ss in the aberrant spermatocyte pair, as shown in Figures 1f and 1g (arrows).

At anaphase I, the centromeres of the poleward moving Ss and Ks in the primary spermatocyte, and also of the condensed Ss in the aberrant spermatocyte, show clear H2AT120ph labelling (Fig. 1g). Both sister centromeres of the anaphasic chromosomes are stained by the H2AT120ph antibody (insert in Fig 1g).

At metaphase II, the centromeres of both sister chromatids of the equatorially arranged Ss and Ks of the secondary spermatocytes display strong H2AT120ph signals, while the Ss in the aberrant spermatocyte have lost the centromeric H2AT120 phosphorylation (Fig. 1h; only one secondary spermatocyte is shown). In metaphases II, chromosomes were observed in which both sister chromatids showed H2AT120ph signals only at the outer centromeres (lower insert in Fig. 1h), and also chromosomes in which the sister chromatids displayed additional signals at the inner centromeres (Fig. 1h and upper insert in Fig. 1h). Both distribution patterns of H2AT120ph

signals were also reported for mitotic metaphase chromosomes of HeLa cells (Liu et al. 2013), where 75% of the sister chromatids presented H2AT120ph signals on the kinetochores and 25%, in addition, on the inner centromeres. Liu et al. (2013) also detected that, depending on the microtubule induced tension at the kinetochores, the cohesin protector shugoshin undergoes a redistribution from the inner centromeres to the kinetochores, and that H2AT120ph and cohesin specify these distinct pools of shugoshin (Liu et al. 2013). In mitoses of *Drosophila* S2 cells phosphorylated H2AT119, corresponding to H2AT120ph in humans, is enriched in prometaphase and metaphase in regions between and surrounding the sister chromatid centromeres, and is strongly reduced at the onset of anaphase (Brittle et al. 2007).

With the duplication of the single centrosome in each of the secondary spermatocytes during interkinesis/prophase II, also the two centrosomes in the aberrant spermatocyte having doubled, the resulting four centrosomes have a tetrapolar arrangement, as indicated by the signals of the unknown pericentrosomal protein (Fig. 1h).

At late anaphase II (Fig. 1i) the Ss in the aberrant spermatocyte are still devoid of centromeric H2AT120ph signals, in contrast to the Ss and Ks in the secondary spermatocytes, which exhibit clear centromeric H2AT120 phosphorylation (arrowheads in Fig. 1i).

H2AT120 phosphorylation signals appear at the centromeres of the Ss during prophase of the aberrant spermatocyte (Fig. 1j) and persist from metaphase I until late anaphase I in the connected primary spermatocyte (Fig. 1k). The inserts in Figure 1k show strongly squashed Ss from aberrant spermatocytes, clearly demonstrating that the centromeres of both sister chromatids of the metaphase-like condensed Ss are phosphorylated. In the interval between telophase I and metaphase II the Ss of the aberrant spermatocyte completely lose the centromeric phosphorylation at H2AT120 and hence centromere activity (Fig. 1l). Both centrosomes have reduplicated and form four tetrapolar arranged microtubule asters as visualised by Tubulin Tracker ^{TM} staining in the insert of Fig. 1l.

The schematic depiction in Fig. 2 summarises the centromeric histone H2AT120 phosphorylation of Ss and Ks in metaphases of gonial mitosis, differential mitosis, meiosis I and meiosis II, and of the Ss in aberrant spermatocytes.

Demidov et al. (2014) demonstrated that an antibody against a phosphopeptide derived from human H2A around the phosphorylation site of T120 (abcam) can be used to identify and visualise the centromeres in a wide variety of mono- and holocentric plant species. In some of these species threonine is replaced by serine and also the position in the H2A sequences can vary. In the histone H2A amino acid sequences of the midges (Nematocera) *Anopheles gambiae* (UniProt No. Q7PD33), *Chironomus thummi thummi* (UniProt No. P21896), *Rhynchosciara americana* (UniProt No.P84056), and of the fly (Brachycera) *Drosophila melanogaster* (UniProt No.P84051), the corresponding phosphorylation site to human H2AT120 is H2AT119. So most probably in *A. lucidus* phosphorylated H2AT119 is detected by the H2AT120ph antibodies used in this study. In *Drosophila*, the phosphorylation of H2AT119 is generated by the nucleosome histone kinase-1 (NHK-1) (Aihara et al. 2004). It is not known which phosphatases dephos-

phorylate H2AT120ph at the centromeres in humans and H2AT119ph at the centromeres in *Drosophila*.

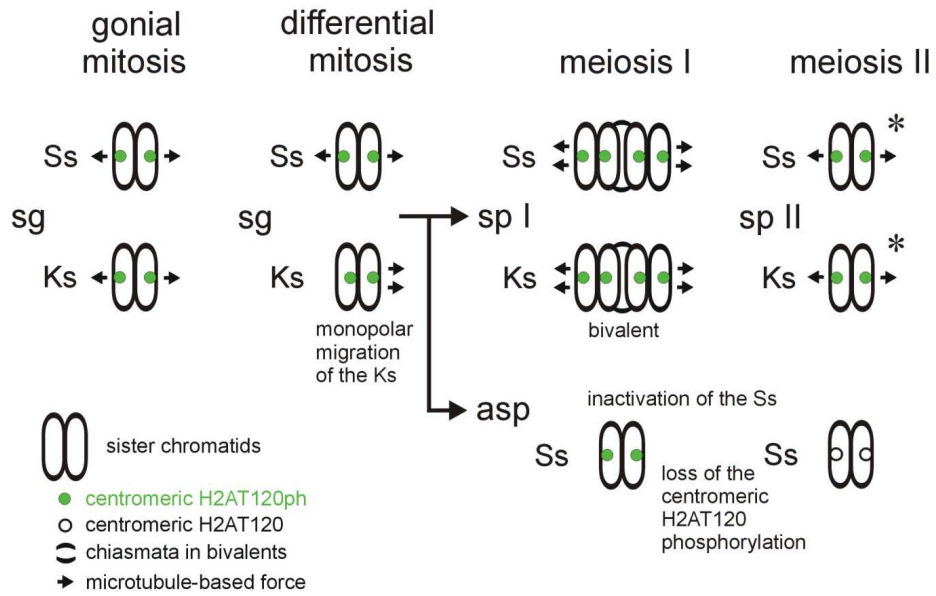

Fig. 2 Diagram summarising the centromeric histone H2AT120 phosphorylation of Ss and Ks in metaphases of gonial mitosis, differential mitosis, meiosis I and meiosis II., and of the Ss in aberrant spermatocytes. *Asterisk*: in metaphases II, also Ss and Ks showing an additional H2AT120ph signal at the inner centromeres were detected (see Fig. 1h and text). *Ss* soma chromosomes, *Ks* germ line-limited chromosomes, *asp* aberrant spermatocyte, *sg* spermatogonium, *sp I* primary spermatocyte, *sp II* secondary spermatocyte

The only study in which the different H2A phosphorylation state of an inactive and an active centromere was analysed is the above-mentioned case of a dicentric maize chromosome (Dong and Han 2012). The group of Han also demonstrated (Fu et al. 2013), that a newly formed active centromere on a chromosome fragment in maize is strongly phosphorylated at H2AT133 in mitotic metaphases (Fu et al. 2013).

It is not known why the Ss of the aberrant spermatocyte do not arrange themselves in the metaphase plane. Perhaps the microtubules cannot attach in a stable way at the kinetochores in prometaphase, or the centromeres of the sister chromatids cannot achieve biorientation because the chromosomal passenger complex (CPC), or components of it, were not localised to the centromeres or are not active (Tsukahara et al. 2010; Carmena et al. 2012).

The Ss of the aberrant spermatocyte do not undergo heterochromatinisation during their inactivation (Staiber 2012). Trimethylated H3K9, as a marker of heterochromatin, could only be detected in the heterochromatin of the centromeric regions of the condensed Ss in the aberrant spermatocyte as also of the Ss in spermatogonial metaphases (Staiber 2012). Such a hetero-chromatinisation of a whole chromosome, with a corresponding trimethylation of H3K9, was established during the meiotic inactivation of the single germ line restricted chromosome (GRC) in the spermatocytes of the zebra finch (Goday and Pigozzi 2010; Schoenmakers et al. 2010). In contrast to the Ss in *A. lucidus*, the inactivation affects only the GRC, not the other chromosomes, and takes place within the early meiotic prophase nucleus.

The dephosphorylation of H2AT120ph at the centromeres of the condensed Ss in the aberrant spermatocyte, detected in the present study, clearly indicates that their deactivation corroborates the idea of a programmed inactivation of the Ss. Since the presence of the Ks in the germ line is essential for a normal development of the gametes, it is strongly supposed that the inactivation of the Ss and their centromeres is required to inhibit the entry of the germ line-derived but K-free aberrant spermatocyte into meiosis, and thereby to prevent the formation of sperms possessing only Ss.

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Conflict of interest The author declares that he has no conflict of interest.

References

Aihara H, Nakagawa T, Yasui K, Ohta T, Hirose S, Dhomae N, Takio K, Kaneko M, Takeshima Y, Muramatsu M, Ito T (2004) Nucleosomal histone kinase-1 phosphorylates H2A Thr 119 during mitosis in the early *Drosophila* embryo. Genes Dev 18:877-888

Bantock CR (1970) Experiments on the chromosome elimination in the gall midge, *Mayetiola destructor*. J Embryol Exp Morph 24:257-286

Baarends WM, Wassenaar E, Hoogerbrugge JW, Schoenmakers S, Sun ZW, Grootegoed JA (2007) Increased phosphorylation and dimethylation of XY body histones in the Hr6b-knockout mouse is associated with derepression of the X chromosome. J Cell Sci 120:1841-1851

Bauer H, Beermann, W (1952) Der Chromosomencyclus der Orthocladiinen (Nematocera, Diptera). Z Naturforsch 7b:557–563

Brittle AL, Nanba Y, Ito T, Ohkura H (2007) Concerted action of Aurora B, Polo and NHK-1 kinases in centromere-specific histone 2A phosphorylation. Exp Cell Res 313:2780-2785

Carmena M, Wheelock M, Funabiki H, Earnshaw WC (2012) The chromosomal passenger complex (CPC): from easy rider to the godfather of mitosis. Nat Rev Mol Cell Biol 13:789-803

Demidov D, Schubert V, Kumke K, Weiss O, Karimi-Ashtiyani R, Butlar J , Heckmann S, Wanner G, Dong Q, Han F, Houben A (2014) Anti-phosphorylated histone H2AThr120 - a universal marker for centromeric chromatin of mono- and holocentric plant species. Cytogenet Genome Res 134:150-156

Dong Q, Han F (2012) Phosphorylation of histone H2A is associated with centromere function and maintenance in meiosis. Plant J 71:800-809

Fu S, Lv Z, Gao Z, Wu H, Pang J, Zhang B, Dang Q, Guo X, Wang XJ, Birchler JA, Han F (2013) De novo centromere formation on a chromosome fragment in maize. Proc Natl Acad Sci USA 110:6033-6036

Geyer-Duszynska I (1966) Genetic factors in oögenesis and spermatogenesis in Cecidomyiidae. In: Darlington CD, Lewis KR (eds) Chromosomes today., vol. 1. Oliver and Boyd, Edinburgh, pp 174–178

Goday C, Pigozzi MI (2010) Heterochromatin and histone modifications in the germline-restricted chromosome of the zebra finch undergoing elimination during spermatogenesis. Chromosoma 119:325-336

Lee J, Kitajima TS, Tanno Y, Yoshida K, Morita T, Miyano T,Miyake M, Watanabe Y (2008) Unified mode of centromeric protection by shugoshin in mammalian oocytes and somatic cells. Nat Cell Biol 10:42-52

Liu H, Jia L, Yu H (2013) Phospho-H2A and cohesin specify distinct tension-regulated Sgo1 pools at kinetochores and inner centromeres. Curr Biol 23:1927-1933

Redi AA, Garagna S, Zacharias H, Zuccotti M, Capanna E (2001) The other chromatin. Chromosoma 110:136–147

Schoenmakers S, Wassenaar E, Laven JSE, Grootegoed JA, Baarends WM (2010) Meiotic silencing and fragmentation of the male germline restricted chromosome in zebra finch, Chromosoma 119:311-324

Staiber W (1988) G-banding of germ line limited chromosomes in *Acricotopus lucidus* (Diptera, Chironomidae). Chromosoma 97:231-234

Staiber W (2008) Centrosome hyperamplification with the formation of multiple asters and programmed chromosome inactivation in aberrant spermatocytes during male meiosis in *Acricotopus*. Cell Tissue Res 334:81-91

Staiber W (2012) Germ line-limited and somatic chromosomes of *Acricotopus lucidus* differ in distribution and timing of alterations of histone modifications in male gonial mitosis and meiosis. Chromosome Res 20:717-734

Tsukahara T, Tanno Y, Watanabe Y (2010) Phosphorylation of the CPC by Cdk1 promotes chromosome bi-orientation. Nature 467:719-23

Wang F, Higgins JGM (2013) Histone modifications and mitosis: countermarks, landmarks, and bookmarks. Trends Cell Biol 23:175-184

Watanabe Y (2012) Geometry and force behind kinetochore orientation: lessons from meiosis. Nat Rev Mol Cell Biol 13:370-382

White MJD (1973) Animal cytology and evolution, 3rd edn. Cambridge University Press, Cambridge, pp 500–546

Yamagashi Y, Honda T, Tanno Y, Watanabe Y (2010) Two histone marks establish the inner centromere and chromosome bi-orientation. Science 330:239-243