Methylation of histone H3 at Lys4 differs between paternal and maternal chromosomes in *Sciara ocellaris* germline development

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Summary

An outstanding example of programmed chromosome elimination and genomic imprinting is found in sciarid flies (Diptera, *Sciaridae*), where whole chromosomes of paternal origin are selectively discarded from the genome during development. In early germ cells a single paternal X chromosome is eliminated in embryos of both sexes and in male meiotic cells the whole paternal complement is discarded. In sciarids, differential acetylation of histones H3 and H4 occurs between chromosomes of different parental origin, both in early germ nuclei and in male meiotic cells (Goday and Ruiz, 2002). We here investigated histone methylation modifications between chromosomes in germline cells of *Sciara ocellaris*. In early germ nuclei, maternal chromosomes show high levels of di- and trimethylated histone H3 at Lys4, whereas this histone modification is not detected in paternal chromosomes. In male meiosis, only the eliminated paternal chromosomes exhibit high levels of di- and trimethylated histones H3 at Lys4 and dimethylated H4 at Lys20. In early germ nuclei, RNA polymerase II associates to maternally-derived chromosomes but lacks phosphorylation of the C-terminal domain on Ser2. We found that histone H3 methylation at Lys4 does not correlate with transcriptional activity in early *Sciara* germline nuclei. The results support the conclusion that specific covalent chromatin modifications are involved in the imprinted behaviour of germline chromosomes in *Sciara*.

Key words: Histone methylation, Chromosome elimination, Imprinting, *Sciara ocellaris*, Sciarid flies

Introduction

The programmed elimination of paternally derived chromosomes during development in sciarid flies, constitutes a remarkable classic example of genomic imprinting (for reviews, see Gerbi, 1986; Goday and Esteban, 2001). In *Sciara ocellaris*, during the early cleavages that follow the zygote constitution ($A_mA_pX_mX_pX_p$), all somatic cells eliminate one or two paternal X chromosomes (X_p), depending on the sex of the embryo (female or male, respectively). Two other types of chromosome elimination event occur in the germline. The first one takes place in embryos of both sexes and involves the exclusion of a single X_p chromosome from all germ nuclei. A second elimination event occurs at the last larval stage where, although female meiosis is orthodox, in male meiosis I the whole paternal chromosome complement is discarded. As a consequence, only maternal chromosomes are included in the sperm nucleus and these chromosomes are recognized as paternal after fertilization. Another relevant feature of sciarid male meiosis is the non-disjunction of the maternal X chromosome (X_m) that establishes the characteristic 3X constitution of zygote.

The peculiar behaviour and chromatin organization of germ cells during development in sciarid flies has been the subject of classic cytological studies (Dubois, 1932; Berry, 1939; Berry, 1941; Rieffel and Crouse, 1966). In *S. ocellaris*, following the fifth embryonic nuclear division, two nuclei migrate to the posterior end of the egg giving rise to the pole cells (Dubois, 1932; Berry, 1941). After somatic chromosome elimination takes place in the early embryo, germ cells become located at the gonad site and remain undivided until the beginning of the second larval instar. A remarkable feature of this unique interphase condition, designated the 'resting stage', is that the chromatin inside the nucleus is atypically condensed so that individual chromosomes are cytologically distinguishable (Berry, 1941; Rieffel and Crouse, 1966). It is during the resting stage period that one X_p chromosome is synchronously expelled from all germ nuclei through the nuclear membrane by a process still not fully understood (Berry, 1939; Berry, 1941; Perondini and Ribeiro, 1997). The consequence of this germline elimination process is that the zygotic complement of three sex chromosomes ($A_mA_pX_mX_pX_p$) is reduced to two ($A_mA_pX_mX_p$). Genetic evidence has demonstrated that the eliminated X_p chromosome is one of the undisjoined dyad in male meiosis; how germ cells identify one of the two X_p chromosomes was questioned earlier (Berry, 1941; Crouse, 1965).

Following X_p elimination, there is an additional atypical event in sciarid flies germ nuclei: the two groups of homologous chromosomes differ in their degree of chromatin condensation. Paternal chromosomes unravel and are light-stained with classic dyes, whereas maternal chromosomes remain condensed and are dark-stained (Berry, 1941). In *S. ocellaris*, these differences are very evident after hatching and are maintained until the late first larval instar, just before the

beginning of gonial mitosis where the two chromosomal sets are both regularly condensed (Berry, 1941).

More recently, the organization of germline chromosomes of sciarid flies has been further analyzed by looking at molecular chromatin modifications in relation to the parental origin of chromosomes (Goday and Ruiz, 2002). From this analysis, it emerged that during germline development there is a differential acetylation of histones H3 and H4 between paternal and maternal chromosomes. In early germ cells at the resting stage, only half of the regular chromosome complement is highly acetylated for histone H3 at Lyts9 and Lys14 (H3K9ac, H3K14ac) and for histone H4 at Lys8 and Lys12 (H4K8ac, H4K12ac). The chromosomes that are highly acetylated are paternally derived, with the sole exception of the X_p chromosome that undergoes elimination from the germ nuclei. This chromosome, as well as the whole maternal complement, remains under-acetylated for histones H3 and H4. At later larval stages, preceding the initiation of mitotic gonial divisions, all chromosomes of the germline complement exhibit similar levels of histone H3/H4 acetylation. At male meiosis I, whereas maternal chromosomes maintain a high level of histone H3 and H4 acetylation, the whole paternal complement that is eliminated appears under-acetylated (Goday and Ruiz, 2002). In Fig. 1 we schematically summarize the present understanding of germline chromosomal events and the distribution of acetylated histones H3 and H4 during germline development of *S. ocellaris*.

In view of these findings, we decided to explore whether other covalent chromatin modifications, such as histone methylation, might differentially associate to paternal and/or maternal chromosomes in germline cells of sciarids. In the present work we have examined histone methylation patterns in the *S. ocellaris* germline by immunolabelling with specific antibodies against histone H3 methylated at different lysine residues (Lys4, Lys9, Lys36 and Lys79) and for histone H4 methylated at Lys20. We have analyzed: (1) germ cells of embryos of both sexes, prior to and after X_p elimination; (2) germ cells of larvae of both sexes from the first instar stage to the end of the resting stage; (3) male meiotic divisions undergoing the elimination of the whole paternal complement.

We show here that in early germ cells of *S. ocellaris*, the maternally derived half of the regular chromosome complement is highly methylated for histone H3 at Lys4. By contrast, in the paternal group of chromosomes as well as in the X_p chromosome undergoing elimination, H3K4 methylation is cytologically undetectable. Both di- and trimethylated forms of histone H3 at Lys4 (H3K4me2 and H3K4me3, respectively) were found to associate with maternal chromosomes throughout the resting stage. At later larval stages, no differences in the histone H3 methylation pattern are observed up to the time of meiotic division. During male meiosis and in contrast to what was found in early germ nuclei, maternal chromosomes appear H3K4 under-methylated, whereas paternal chromosomes undergoing elimination show high levels of H3K4 methylation.

In different systems from yeast to mammals, a correlation between transcriptionally competent or active sequences and H3K4me2 and H3K4me3 patterns has been established (Santos-Rosa H et al., 2002; Ng et al., 2003). Consequently, we examined whether the abundance of this histone modification in maternal chromosomes at the resting stage might correlate with transcriptional activity. By using specific antibodies, we show that RNA polymerase II (RNA pol II) specifically associates to maternal chromosomes but is not phosphorylated at Ser2, supporting the view that *S. ocellaris* early germ nuclei are transcriptionally silent. Taken as a whole, the present results are consistent with a model that relates histone covalent modifications, chromosome elimination and intranuclear chromosome arrangements in germline cells.

Results

Distribution of methylated histones in germ nuclei prior to and after X_p chromosome elimination

In *Sciara ocellaris*, the constitution of the early embryonic germ nuclei is that of the zygote ($A_pA_mX_mX_pX_p$), there are nine chromosomes, three pairs of autosomes plus three X

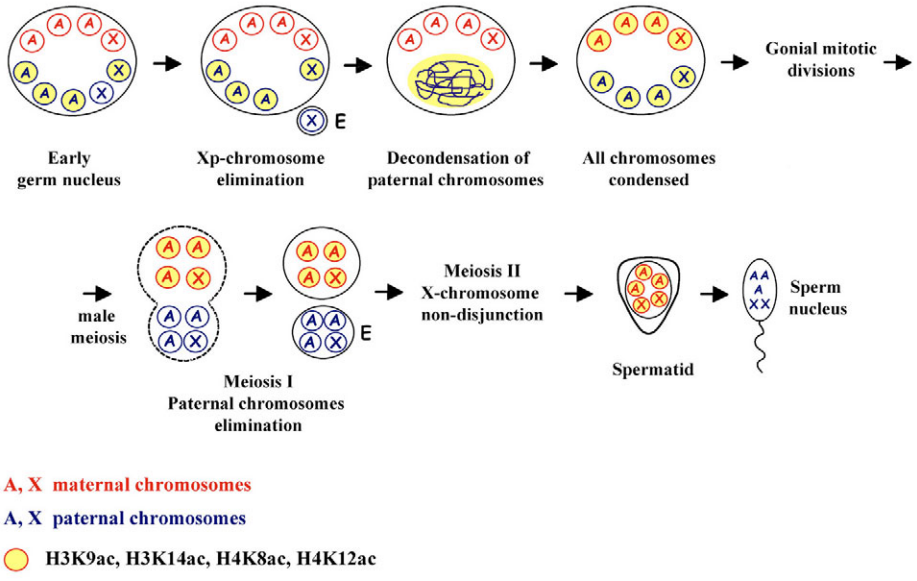

Fig. 1. Scheme summarizing the most relevant chromosomal events and the distribution of acetylated histones H3 (H3K9ac and H3K14ac) and H4 (H4K8ac and H4K12ac) in early germline nuclei at the resting stage and male meiosis in *S. ocellaris*. Maternally derived chromosomes are in red and paternally derived chromosomes in blue. Early germ nuclei contain three maternal and three paternal autosomes (A) plus one maternal and two paternal X chromosomes (X). Chromosomes containing highly acetylated histones H3 and H4 are marked in yellow, whereas chromosomes containing under-acetylated histones H3 and H4 are unmarked. E, chromosomal elimination events. The figure has been modified from Goday and Ruiz (Goday and Ruiz, 2002).

chromosomes. We first investigated histone methylation in germline nuclei of *S. ocellaris* embryos at the beginning of the resting stage period when, as already described (Berry, 1941; Rieffel and Crouse, 1966), all chromosomes of the complement display a high level of chromatin condensation so that they can be distinguished inside the nucleus. We first performed the immunodetection of H3K4me2 in *S. ocellaris* embryonic germline nuclei. As shown in Fig. 2, prior to, during and after (panels A-A'', B-B'', C-C'', respectively) X_p chromosome elimination, four chromosomes showed bright fluorescence, indicative of high levels of H3K4me2. By contrast, the rest of the chromosomes, five in pre-elimination nuclei (Fig. 2A-A'') and four in the post-elimination nuclei (Fig. 2C-C''), remained unstained and therefore under-dimethylated at H3K4. In germ nuclei undergoing X_p chromosome elimination (Fig. 2B-B''), it was observed that the chromosome expelled from the nuclei was always devoid of H3K4me2 labelling.

In view of these results, we then analysed the presence of H3K4me3 in early embryonic germline nuclei in *S. ocellaris*. As shown in Fig. 3, the antibody against H3K4me3 stained again only four chromosomes of the complement (A''-C'') and no labelling of the X_p chromosome undergoing elimination was detected (B-B''). Moreover, in the same cytological preparations it was possible to observe that somatic nuclei at interphase exhibited H3K4me3 staining at discrete nuclear regions, whereas chromosomes at mitosis displayed a high level of H3K4me3 labelling (Fig. 3A,B,A'',B''). Identical results were obtained with somatic cells when the antibody against H3K4me2 was used (data not shown). In order to investigate whether the observed distribution of H3K4me2 and H3K4me3 was coincident with the same four chromosomes of the complement, we simultaneously immunolabelled *S. ocellaris* germ nuclei with both antibodies. From this analysis (data not shown), it emerged that only four chromosomes were highly stained, so that we concluded that the same four chromosomes of the complement undergo high levels of H3K4me2 and H3K4me3, in contrast to the rest of the complement.

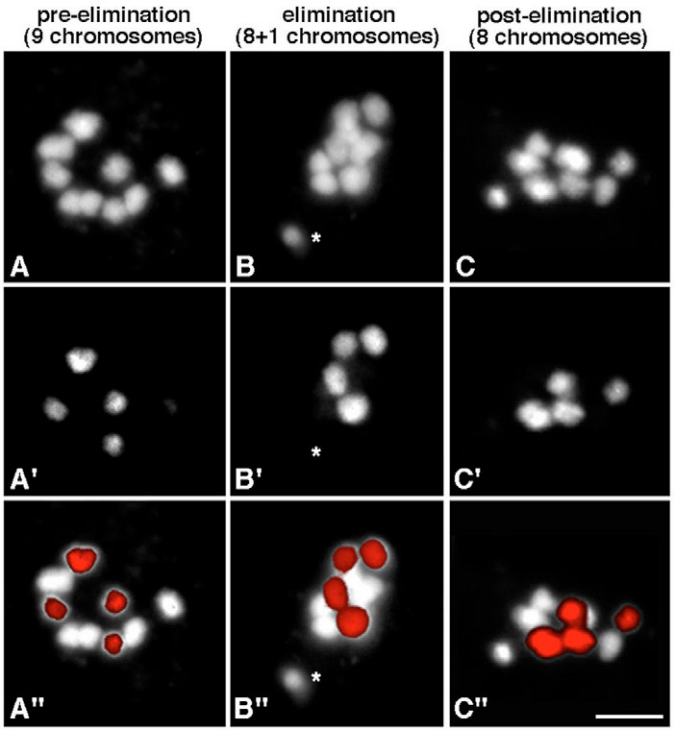

Fig. 2. Distribution of histone H3K4me2 in *S. ocellaris* embryonic germ cell nuclei at the resting stage. (A-C) Chromosome staining with DAPI, (A'-C') indirect immunolabelling with anti-H3K4me2 antibody and (A''-C'') superimposed images with antibody staining in red. (A) *S. ocellaris* germ nuclei before, (B) during and (C) after the occurrence of X_p-chromosome elimination. In all cases only four chromosomes of the complement are strongly labelled (A''-C''). (B-B'') A *S. ocellaris* germ nucleus undergoing elimination; the X_p chromosome is expelled from the nucleus (asterisk in B) and is not stained (asterisks in B' and B''). Bar, 10 μm.

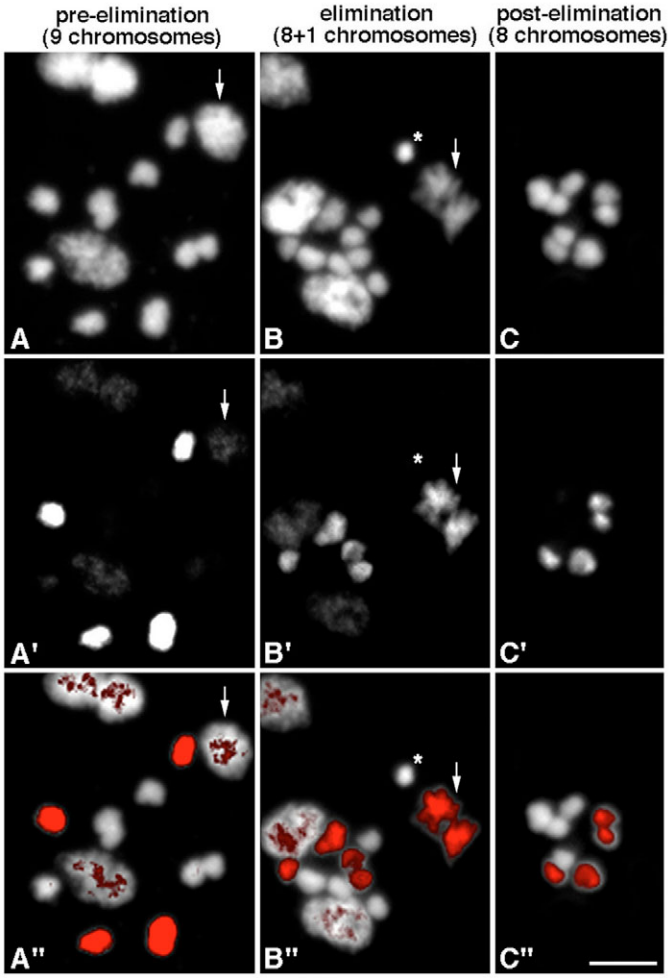

Fig. 3. Distribution of histone H3K4me3 in *S. ocellaris* embryonic germ nuclei at the resting stage. (A-C) Chromosome DAPI staining, (A'-C') indirect immunolabelling with anti-H3K4me3 antibody and (A''-C'') superimposed images where antibody staining is in red. (A-C) *S. ocellaris* germ nuclei (A) prior to, (B) during and (C) after the occurrence of X_p chromosome elimination. In all cases only four chromosomes of the complement are highly labelled (A'-C' and A''-C''). (A-A'') Somatic nuclei exhibit some H3K4me3 labelling at discrete nuclear sites (arrow). (B-B'') A *S. ocellaris* germ nucleus undergoing elimination where it is seen that the X_p chromosome expelled from the nucleus (asterisk in B) is not stained (asterisks in B' and B''). Somatic chromosomes at mitosis are highly H3K4me3 labelled (arrow in B-B''). Bar, 10 μm.

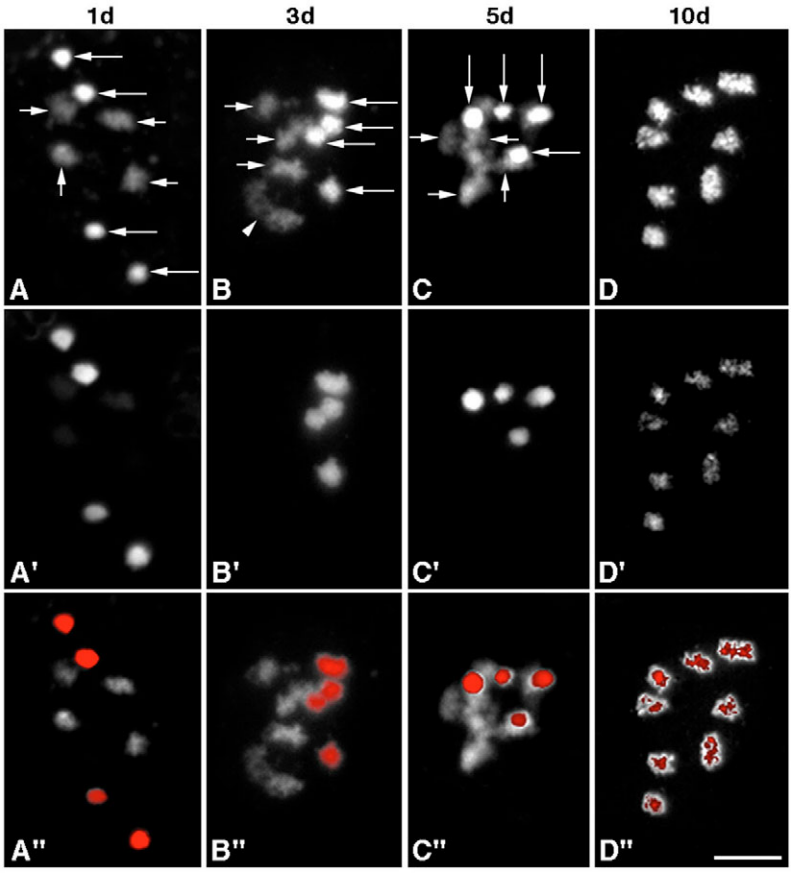

Fig. 4. Distribution of histone H3K4me2 in germ cells of *S. ocellaris* male larvae at 1 day (1d), 3 days (3d), 5 days (5d) and 10 days (10d) from hatching. (A-D) Chromatin DAPI staining, (A'-D') indirect immunolabelling with anti-H3K4me2 antibody and (A''-D'') superimposed images where antibody staining is in red. (A,B) Maternal chromosomes appear condensed (long arrows) whereas paternal chromosomes, less stained with DAPI, initiate decondensation (short arrows and arrowhead); H3K4me2 staining is restricted to the maternal chromosome set. Arrowhead in (B) denotes the paternal chromosome corresponding to the longest autosomes pair. (C) Maternal chromosomes remain condensed (long arrows) whereas paternal ones appear totally decondensed (short arrows); (C',C'') H3K4me2 labelling is restricted to the chromatin corresponding to the maternal chromosome set. (D) All chromosomes appear equally condensed and exhibit similar levels and distribution of H3K4me2 labelling (D',D''). Bar, 10 μm.

We next investigated by immunodetection the presence of H3K9me2, H3K9me3, H3K36me2, H3K79me2 and H4K20me2 in *S. ocellaris* embryonic germline nuclei at the resting stage. In all cases, chromatin was devoid of staining, thus indicating the absence of detectable histone methylation levels (data not shown). We assumed that this constitutes a reliable result since in the same cytological preparations, positive signals were detected in somatic nuclei (data not shown).

Distribution of methylated histone H3 in germ cells of larvae from the first instar stage to premeiotic divisions

To investigate histone H3K4 methylation with respect to the process of paternal chromosome decondensation, germ nuclei of newly hatched larvae of both sexes were stained with DAPI and with the antibodies against H3K4me2 and H3K4me3.

Fig. 4 shows *S. ocellaris* germ nuclei from a 1-day-old (A) and 3-day-old (B) larvae stained with DAPI and each containing four chromosomes highly condensed and brightly fluorescent, plus four chromosomes which have initiated decondensation and appear less fluorescent. At this stage, the group of chromosomes which unravel corresponds, for both sexes, to the paternal set (Berry, 1941; Rieffel and Crouse, 1966). Thus, in Fig. 4A,B it is possible to recognize paternal and maternal chromosomes by DAPI staining (short and long arrows, respectively). The immunolocalization of H3K4me2 in the germ nuclei (Fig. 4A',A'',B',B'') revealed that H3K4me2 labelling corresponds to the maternal chromosomes, whereas paternal chromosomes are devoid of antibody staining. In accordance with previous data (Berry, 1941; Rieffel and Crouse, 1966; Goday and Ruiz, 2002), the maximum degree of *S. ocellaris* paternal chromosomes decondensation is achieved in germ nuclei of larvae that are 3-5 days old (Fig. 4C). At posterior larval stages (from 10 days onwards) the differences in chromatin condensation between the two parental groups of chromosomes are no longer seen in germ nuclei (Fig. 4D). Fig. 4C-C'' shows a germ nucleus from a 5-day-old larva where paternal chromosomes are fully decondensed and unstained, in contrast to maternal chromosomes that remain highly condensed and H3K4me2-labelled. The same result was found in germ nuclei of larvae that were 7-8 days old (data not shown). In Fig. 4D it is possible to observe by DAPI staining, that paternal and maternal chromosomes are similarly condensed and no longer recognizable. Importantly, H3K4me2 labelling of this germ nuclear stage (Fig. 4D',D'') revealed that both paternal and maternal chromosomes showed similar levels of H3K4me2. During the successive gonial mitotic divisions until the occurrence of meiosis (see below) no differential distribution of H3K4me2 in germ nuclei was observed (data not shown). Identical results to those described above for H3K4me2 staining were obtained for the distribution of H3K4me3 in *S. ocellaris* germ nuclei from larvae of both sexes (data not shown).

The above results, together with the previous observations of histone H3 methylation in pre-elimination germ nuclei of early embryos, lead us to conclude that, in *S. ocellaris*, the chromosomes that undergo high levels of histone H3

methylation are those of maternal origin. Paternally derived chromosomes, by contrast, remain H3 undermethylated through most of the resting stage until the time that precedes gonial mitotic divisions. Notably, in *S. ocellaris* early germ nuclei paternal chromosomes were found to be highly acetylated for histones H3/H4, in contrast to maternal ones that appear underacetylated through most of the resting stage (Goday and Ruiz, 2002). From our results, therefore, at early germ nuclei development, maternally and paternally derived chromosomes, in addition to differences in the H3/H4 acetylation patterns, also show relevant differences in histone H3 methylation patterns.

Distribution of methylated histone H3K4 in young embryos prior to the germ nuclei resting stage period

In view that differential methylation of histone H3K4 in maternal and paternal chromosomes was found during the germ nuclei resting stage period, we investigated whether such differences could already be observed in pregerminal nuclei at earlier stages of embryo development. In *S. ocellaris*, the pole cells (in number of 30-32) are generated by successive divisions of two pre-germ cells at the posterior end of the embryo (Berry, 1941). To this purpose, we immunostained whole embryos at the early syncitial stage (1-3 hours of development) containing different numbers of germ nuclei, with both anti-H3K4me2 and H3K4me3 antibodies. This analysis revealed that, as shown in Fig. 5 for anti-H3K4me2, germ cells at both interphase (Fig. 5A-B'') and mitosis (Fig. 5C-C'') do not exhibit any labelling and, therefore, do not contain detectable levels of histone H3 methylated at Lys4. Somatic nuclei, by contrast, both at mitosis (Fig. 5A-B'') and at interphase (Fig. 5C-C''), exhibit positive staining. Identical results were obtained when anti-H3K4me3 antibody was used (data not shown). From these results, we concluded that the differences in the methylation levels of H3K4 between chromosomes of different parental origin become visible, as described above, when germ nuclei undergo the resting stage phenomenon.

Distribution of methylated histones in male meiotic cells

We have investigated histone H3 and H4 methylation during the first meiotic division in *S. ocellaris* males, when the elimination of the whole paternal complement takes place. In sciarid flies, the differential segregation of maternal chromosomes from paternal ones is accomplished by a monopolar first meiotic spindle (capturing maternal chromosomes) and by non-spindle microtubules generated in a cytoplasmic bud (capturing paternal chromosomes) (Esteban et al., 1997). Exceptional features of the male first meiosis are that homologous chromosomes do not pair at prophase, they do not align in a metaphase-like array but they proceed directly from prometaphase to an anaphase-like stage (for reviews, see Gerbi, 1986; Esteban et al., 1997). Fig. 6A-C shows examples of DAPI-stained first anaphase-like figures where maternal chromosomes (m) face the single pole and paternal chromosomes (p), closely grouped, segregate towards the opposed bud region of the spermatocyte. The immunolocalization of H3K4me2, H3K4me3 and H4K20me2 (Fig. 6A',A'',B',B'',C',C''), revealed that the antibodies associate exclusively to discrete regions of the paternal group of chromosomes whereas the maternal set is devoid of staining. Since, at this stage, paternal chromosomes are usually highly grouped (Esteban et al., 1997), it was not possible to discern whether specific chromosomal regions are enriched in the observed histones H3/H4 modifications. However, the eliminated paternal chromatin exhibited H3K4me2-, H3K4me3- and H4K20me2-labelling throughout spermiogenesis (Fig. 6D,E). We next investigated whether differences between paternal and maternal chromosomes were also detectable with respect to H3K9me2 and H3K9me3. The results of this immunodetection analysis showed that all chromosomes exhibit a similar level of labelling restricted at specific chromosomal regions apparently corresponding to pericentromeric and centromeric regions (data not shown). Moreover, no differences in the staining pattern between paternal and maternal chromosomes were detected with respect to H3K36me2 and H3K79me2,

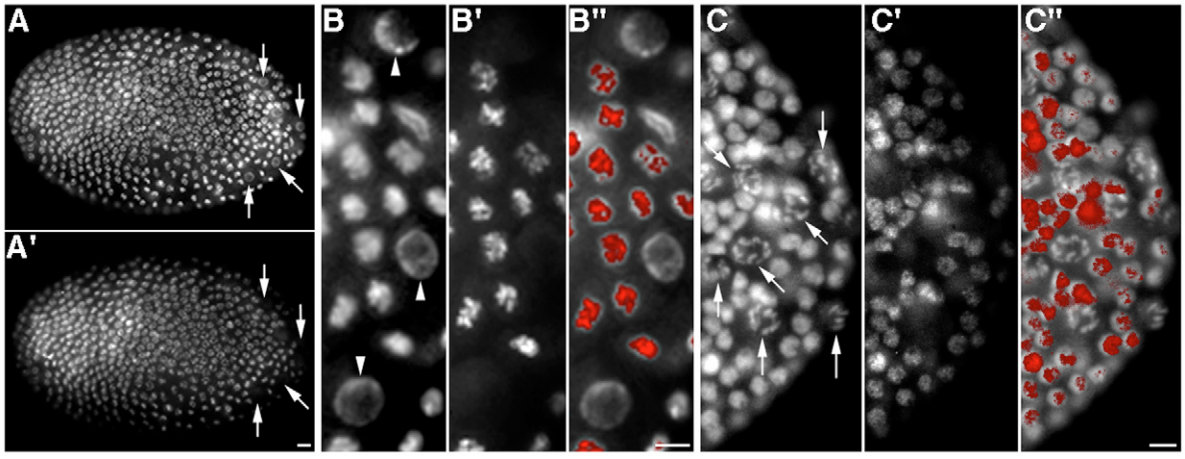

Fig. 5. Distribution of histone H3K4me2 in *S. ocellaris* embryos at the early syncitial stage. (A-C) chromatin DAPI staining, (A'-C') indirect immunolabelling with anti-H3K4me2 antibody and (B'',C'') superimposed images where antibody staining is in red. (A) A young whole embryo showing that the germ nuclei at the polar region of the embryo (arrows in A) are devoid of labelling (arrows in A'). (B-B'') Enlarged images of the same embryo where it is seen that the antibody labelling is restricted to the somatic mitotic chromosomes whereas germ nuclei at interphase (arrowheads in B) remain unstained. (C-C'') Partial view of the polar region of an embryo showing dividing germ cells (arrows in C) lacking antibody staining in contrast with somatic nuclei at interphase that display H3K4me2 staining. Bars, 10 μm.

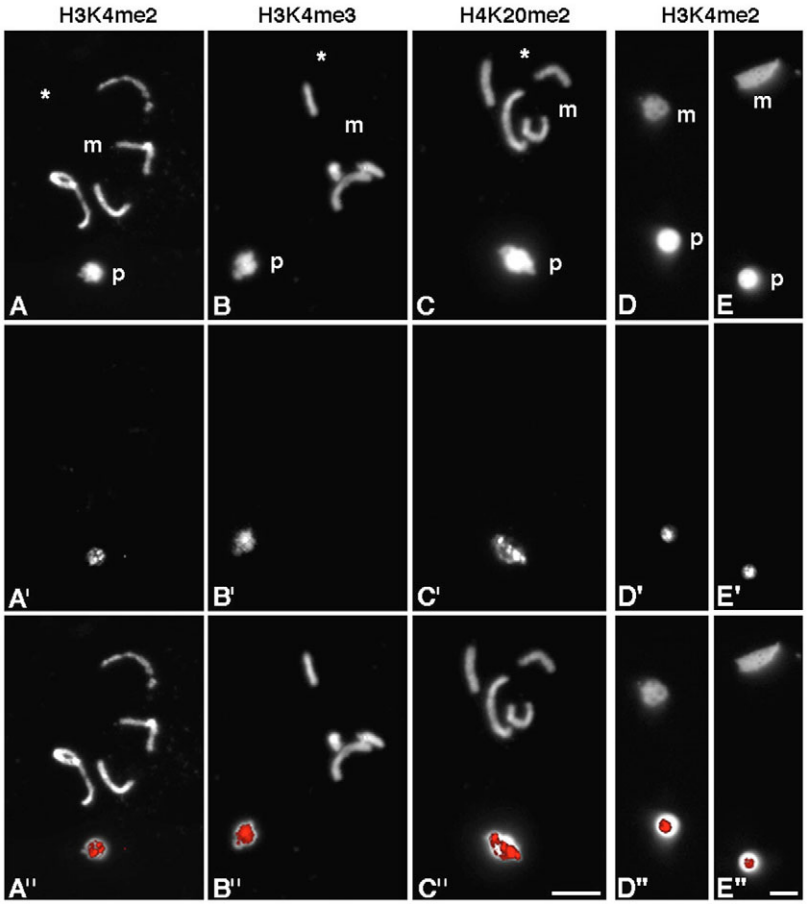

Fig. 6. Distribution of methylated histones in *S. ocellaris* spermatocytes undergoing paternal chromosomes elimination at first meiotic division (A-C) and in spermatids (D,E). (A-C) DAPI-staining of anaphase-like stage figures. m, maternal group of chromosomes; p, paternal chromosomes; * indicates position of the single spindle pole. (D,E) DAPI staining of (D) early and (E) late spermatids. (A'-E') Indirect immunolabelling with antibodies against histones H3 and H4 as indicated, and (A''-E'') superimposed images where the antibody staining is in red. At first meiosis (A''-C''), the four maternal chromosomes that face the pole are devoid of staining whereas the opposed paternal group of chromosomes, closely grouped, show antibody labelling. During spermiogenesis (D,E), antibody labelling is observed in the eliminated paternal chromatin. Bars, 10 μm.

with all chromosomes homogeneously stained (data not shown).

Distribution of RNA pol II in germ nuclei at the resting stage

To investigate transcriptional activity in maternal chromosomes we first examined the presence of RNA pol II in the germ nuclei at the resting stage by means of an antibody recognizing both the phosphorylated and non-phosphorylated forms of the large subunit (Progen, ARNA-3). As shown in Fig. 7, prior to (A-A'') , and after (C-C''') X_p chromosome elimination, four chromosomes showed several fluorescent spots, indicative of the presence of RNA pol II. By contrast, the rest of the chromosomes, five in pre-elimination nuclei (Fig. 7A-A'') and four in the post-elimination nuclei (Fig. 7C-C'''), remained unstained and therefore, in our hands, devoid of detectable levels of RNA pol II. To investigate which group of chromosomes exhibited RNA polymerase detection we examined germ nuclei from larvae undergoing paternal chromosomes unravelling. The results indicated that the RNA polymerase labelling is restricted to the condensed maternal set of chromosomes (Fig. 7B-B''). This, was further confirmed in double-immunostained germ nuclei with anti-RNA pol II and H3K4me2 antibodies where the same chromosomes were positive for both antibodies (Fig. 7 C'-C''').

In view of these results (as shown in Fig. 8), we further investigated the transcriptional activity of maternal chromosomes with an antibody against pSer2 CTD RNA pol II recognizing phosphorylation of the C-terminal domain (CTD) of RNA polymerase on Ser2, a modification only present in transcriptionally engaged chromatin (Patturajan et al., 1998). In embryos at 72 hours of development, when somatic nuclei have acquired general competence for transcription, only somatic nuclei are labelled with the anti-pSer2 CTD RNA pol II antibody and no staining was detected in germ nuclei at the resting stage (Fig. 8A-A''). Similar results were obtained for germ nuclei at later developmental stages until the end of the resting stage period (data not shown). These results lead us to conclude that the germ nuclei lack a specific RNA pol II phosphorylation epitope present in transcriptionally active somatic cells. Therefore, we think that the presence of both methylated H3K4 and RNA pol II in maternal chromosomes of *S. ocellaris* germ nuclei at the resting stage cannot be correlated to ongoing RNA pol II transcription. However, at later developmental stages from the beginning of gonial mitotic divisions until the occurrence of meiosis (Fig. 8B-B''), RNA pol II CTD phosphorylation on Ser2 was detected on both maternal and paternal chromatin.

Discussion

In a previous report, we provided the first evidence that specific covalent chromatin modifications, such as acetylation of histones H3 and H4, can be related to the parental origin of chromosomes in germline cells of sciarid flies (Goday and

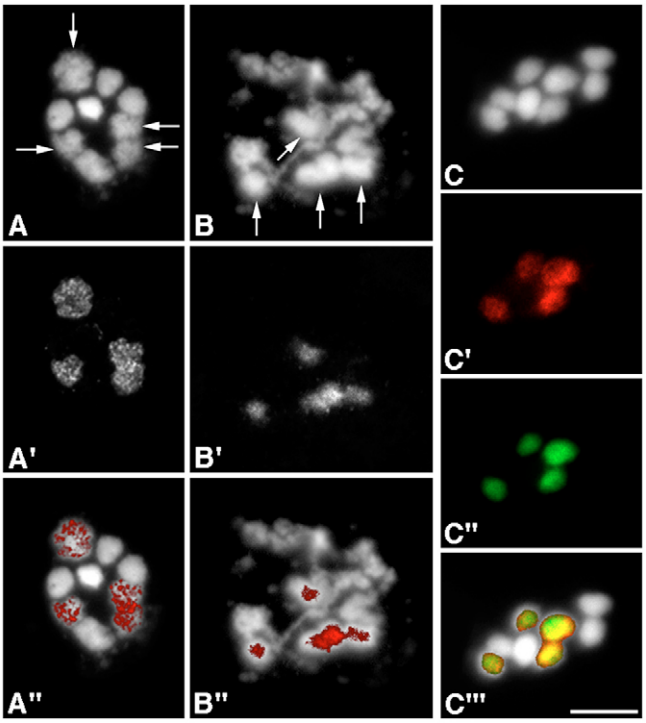

Fig. 7. Distribution of RNA pol II in *S. ocellaris* embryonic germ nuclei at the resting stage (A,C) and male larva germ nucleus at 5 days from hatching (B). (A-C) Chromosome DAPI staining. (A'-C') Indirect immunolabelling with antibody against RNA pol II (red in C') and double staining with antibody against H3K4me2 (green in C''). (A'',B'') superimposed images where RNA pol II staining is in red and (C''') merged image where RNA pol II is in red and H3K4me2 in green. (A-A'') Prior to the occurrence of X_p chromosome elimination (nine chromosomes), RNA pol II is observed in four chromosomes of the complement. (B-B'') RNA pol II staining is restricted to the condensed maternal chromosomes (arrows in B) whereas decondensed paternal chromosomes are devoid of staining. (C-C''') A germ cell nucleus after the occurrence of X_p chromosome elimination (8 chromosomes), showing that labelling of RNA pol II and H3K4me2 is present in the same chromosomes of the complement. Bar, 10 μm.

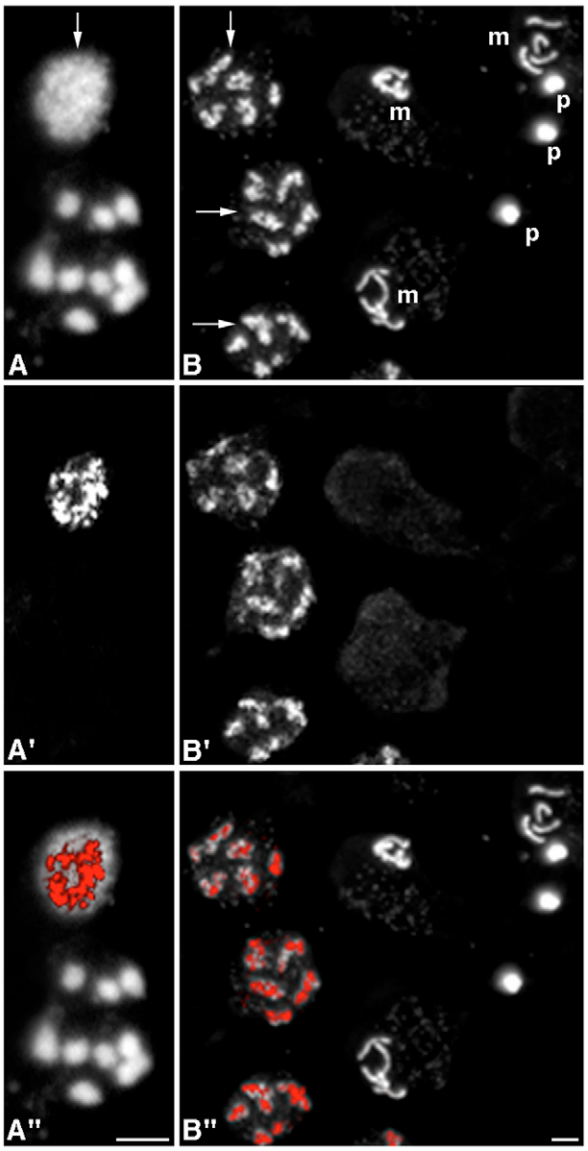

Fig. 8. Distribution of RNA pol II CTD phosphorylated on Ser2 in *S. ocellaris* somatic and germline nuclei of an embryo at 72 hours of development (A) and in male premeiotic nuclei and spermatocytes at first meiotic division undergoing paternal chromosomes elimination. m, maternal group of chromosomes; p, paternal chromosomes (B). (A,B) Chromatin DAPI staining, (A',B') indirect immunolabelling with anti-pSer2 CTD RNA pol II antibody and (A'',B'') superimposed images where antibody staining is in red. (A) A somatic nucleus (arrow) staining positive whereas a germ nucleus at the resting stage is devoid of staining. (B) Partial view of a testis cyst showing that premeiotic germ nuclei (arrows) are clearly stained whereas no staining is detected during meiosis I on both maternal and paternal chromosomes. Bars, 10 μm.

Ruiz, 2002). Here, we show that histone H3 methylated at Lys4 differs between both parental genomes during germline development in *S. ocellaris*.

We found that in germ cells of *S. ocellaris* at their resting stage only half of the chromosome complement contains high levels of H3K4me2 and H3K4me3. Moreover, from our results other histone modifications, such as H3K9me2, H3K9me3, H3K36me2, H3K79me2 and H4K20me2, are apparently absent in the germ cells chromatin during this particular developmental stage. Importantly, the group of chromosomes in which histone H3 is highly methylated throughout the resting stage period, corresponds to maternally derived chromosomes. Most interestingly, these chromosomes were previously found to be under-acetylated for histone H3 at Lys9 and Lys14, and for histone H4 at Lys8 and Lys12 (Goday and Ruiz, 2002). Conversely, paternally derived chromosomes, that are highly acetylated for histones H3/H4 (Goday and Ruiz, 2002), do not exhibit significant levels of histone H3K4 methylation throughout the resting stage. Therefore, high levels of methylated histone H3 at Lys4 occur in the chromosome group that lacks significant levels of acetylated histones H3/H4. The present results, together with previous data referring to histone acetylation patterns in the germline of *S. ocellaris*, are summarized in Fig. 9 and Table 1.

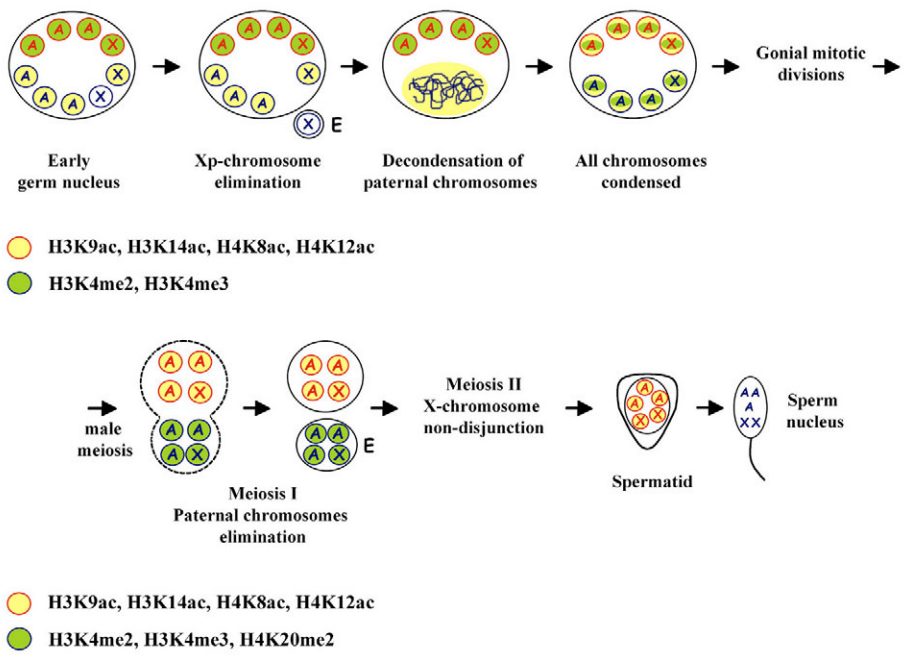

Fig. 9. Scheme summarizing the distribution of acetylated and methylated histones in early germline nuclei at the resting stage and male meiosis in *S. ocellaris*. Maternally derived chromosomes are red and paternally derived chromosomes blue. E, chromosomal elimination events.

Germline chromosome(s) elimination and histone methylation

Differences in histone H3K4 methylation between chromosomes are already observed in embryonic germ nuclei ($A_mA_pX_mX_pX_p$) at the beginning of the resting stage, before a single paternal X chromosome (X_p) is regularly expelled from them. An identical observation was previously reported for the detection of acetylation of histones H3 and H4 in paternal chromosomes, with the sole exception of the X_p chromosome, which remains under-acetylated and is later eliminated (Goday and Ruiz, 2002). In sciarid flies, once the germ nuclei enter the resting stage, the two genomes exhibit similarly high levels of chromatin compaction inside the nuclei. Thus, most probably, the specific, covalent chromatin modifications that distinguish each genome in the *Sciara* germline are established simultaneously, in correlation with the onset of the resting stage. This conclusion is further supported by our observations at earlier embryonic developmental stages, where germline chromatin lacks the H3K4 methylation signals, whereas somatic nuclei exhibit high levels of this histone modification.

From our analysis of the end of the resting stage preceding the initiation of gonial mitotic divisions, differences in histone H3 methylation between chromosomes are no longer detectable and all chromosomes show a similar level and pattern of antibodies staining. An identical observation was also reported for the detection of acetylation of histones H3 and H4 (Goday and Ruiz, 2002) (see Fig. 9). Both findings, strongly support that, in *S. ocellaris* germline development the differential chromatin modifications occurring between the two parental genomes are specifically linked to the resting stage period.

The extrusion of a single X_p chromosome from all embryonic germ nuclei through the nuclear membrane involves a close interaction between X_p chromatin at a specific site of the inner nuclear membrane (Berry, 1941; Rieffel and Crouse, 1966; Perondini and Ribeiro, 1997). This specialized process is still poorly understood and, as suggested, X_p chromosome-associated proteins forming a putative complex with the two core histones H3/H4, may play a role in the chromatin interactions with inner membrane components (Polioudaki et al., 2001; Goday and Ruiz, 2002). Moreover it has been suggested that the under-acetylated histone chromatin condition of one copy of the X_p chromosomes might be specifically required for its elimination (Goday and Ruiz, 2002). In light of the present data, the three copies of the X chromosomes contained in embryonic germ nuclei ($X_mX_pX_p$) differ in their global state of chromatin acetylation and/or methylation, with the eliminated X_p being the sole chromosome that is both H3/H4 under-acetylated and H3K4 under-methylated. Such differences, we think, may be involved in the specification of which X chromosome is eliminated from the germ nucleus during the resting stage. This, in addition, is consistent with the suggestion that such chromosome is

Table 1. Histone modifications associated to the parental origin of chromosomes in early germ nuclei and male meiotic cells of *S. ocellaris*

	Maternally-derived chromosomes A,X	Paternally-derived chromosomes A,X	Paternally-derived chromosomes X(E)
Early germ nuclei (X_p elimination)	H3K4me2	H3K9ac	–
	H3K4me3	H3K14ac	–
		H4K8ac	–
		H4K12ac	–
Male meiosis I (paternal set elimination)	H3K9ac	H3K4me2	
	H3K14ac	H3K4me3	
	H4K8ac	H4K20me2	
	H4K12ac		

(E) refers to the eliminated X_p chromosome.

preferentially confined to a restrictive intranuclear location (Goday and Ruiz, 2002).

Another interesting result involves the histone methylation state of chromosomes in male cells entering the first meiotic division when the process of paternal chromosomes elimination occurs (see Fig. 8). At this developmental stage, and in contrast to premeiotic divisions, only maternal chromosomes exhibit a high level of acetylated histones H3/H4, whereas paternal chromosomes remain under-acetylated for H3/H4 (Goday and Ruiz, 2002). From the present results, only paternal chromosomes undergoing elimination display significant levels of methylated histones H3K4 from the beginning of meiosis I and throughout spermiogenesis. Therefore, at male meiosis, histone H3 methylation status and parental origin of the chromosomes is reversed when compared with that of early germ nuclei. This result is consistent with the reversal of the acetylation status of histones H3 and H4 at male meiosis (see Fig. 9). Interestingly, paternal chromosomes, in addition to methylated H3K4, also exhibit significant enrichment in H4K20 methylation during male meiosis. Since H4K20 methylation is known to be cell-cycle-dependent and to associate to densely packed chromatin (Fang et al., 2002; Rice et al., 2002), this result suggests a differential chromosome condensation between both parental sets during anaphase movements at male meiosis I.

Histone H3K4 methylation and transcriptional activity in early *S. ocellaris* germline nuclei

Histone methylation regulates chromatin structure, transcription and the epigenetic state of a cell. Histone methylation, a modification that is dynamically regulated by histone methyltransferases (HMTases), induces specific and distinctive signals depending on the lysine residue that is modified (reviewed in Lachner et al., 2003; Whetstine et al., 2006). Methylation of Lys4, Lys36 and Lys79 on histone H3 has been broadly linked with active transcription from yeast to mammals (Gerber and Shilatifard, 2003; Schneider et al., 2004). In particular, H3K4me2 associates with transcriptionally competent chromatin and H3K4me3 with actively transcribed sequences (Santos-Rosa et al., 2002; Ng et al., 2003). Moreover, genetic and biochemical analyses in *Saccharomyces cerevisiae* have identified a H3K4 methyltransferase that associates with a transcriptionally active RNA pol II (Krogan et al., 2003; Ng et al., 2003).

Although H3K4 methylation has thus far been a histone modification associated with actively transcribed sequences, there are a certain number of examples where this histone modification apparently does not mark active chromatin. For instance, in cultured *Drosophila* and mouse cells, even though H3K4me3 prevalently associates with euchromatin, this histone modification was also detected in the pericentromeric heterochromatin (Spada et al., 2005). Moreover, the abundance of methylated H3K4 does not necessarily seem to be proportional to local levels of transcriptional activity in either euchromatic or heterochromatic domains. Furthermore, in the urochordate *Oikopleura dioica*, a chromatin compartment enriched in heterochromatic histone modifications and DNA methylation was also found to contain high levels of histone H3K4me3 in both diploid and endocycling cells (Spada et al., 2005). Interestingly, H3K4me2 has also been detected on inactive globin genes (Schneider et al., 2004).

During early development, in primordial germline cells of many animals, RNA-pol-II-dependent transcription is globally silenced to prevent somatic differentiation and, thus, maintain the germline fate (for a review, see Martinho et al., 2004). As discussed above, germline chromatin of *S. ocellaris* nuclei preceding the resting stage does not contain detectable levels of methylated H3K4, in contrast to somatic cells. This observation, is consistent with data coming from *Drosophila* where, in early embryos, the germ cell precursors (pole cells) lack H3K4 methylation, whereas, following zygotic transcription, somatic chromatin contains high levels of such histone H3 modification (Schaner et al., 2003). In *Drosophila,* the accumulation of methylated H3K4 in germ cells at later stages of embryonic development (gastrulation stage) links temporally with transcriptional activation (Schaner et al., 2003). Taking these data into consideration, it seems reasonable to assume that the absence of H3K4 methylation in *S. ocellaris* germ nuclei prior to the resting stage, correlates to a quiescent chromatin state during early development.

However, from the beginning of the resting stage and throughout it, both H3K4me2 and H3K4me3 forms were found to associate exclusively with maternal chromosomes. Interestingly, other histone H3 modifications known to be involved in transcription, such as H3K36me2 and H3K79me2, were not detectable during the resting stage. These findings led us to question whether maternal chromatin could be transcriptionally active during this characteristic interphase period of *Sciara* germline development. To address this question, we first investigated the presence of RNA pol II by using an antibody against both the non-phosphorylated and phosphorylated forms of the large subunit. From this analysis, it emerged that, in germline nuclei at the resting stage, only maternal chromosomes contain RNA pol II. However, a second antibody specifically recognizing phosphorylation of the C-terminal domain (CTD) of RNA pol II on Ser2 residue failed to give any staining. Thus, in *S. ocellaris*, RNA pol II associated with maternal chromosomes lacks CTD phosphorylation on Ser2, a modification which is crucial for the elongation and processing phases of transcription (reviewed in Orphanides and Reinberg, 2002). Noteworthy, in *Caenorhabditis elegans* and *Drosophila*, transcriptionally repressed early germ cells are specifically distinguished by undetectable levels of RNA pol II CTD phosphorylation on Ser2 (Seydoux and Dunn, 1997; Batchelder et al., 1999). Moreover, in *Drosophila*, silencing of germline transcription depends upon a non-coding RNA, encoded by the polar granule component (PGC) gene, which is localized to germ plasm (Martinho et al., 2004). In *S. ocellaris*, the lack of RNA pol II phosphorylated on Ser2 favours the idea that germ nuclei are also transcriptionally repressed during the resting stage, in spite of the abundance of H3K4me2 and H3K4me3 detected in maternal chromatin. This assumption is further supported by observations in germ cells at later developmental stages preceding meiosis, where RNA pol II CTD phosphorylation on Ser2 and histone H3K4 methylation were detected at the same time on both parental genomes.

Intranuclear germline chromosome distribution and covalent histone modifications

Cytological and ultrastructural data support the idea that in sciarid flies the two sets of chromosomes occupy distinct

nuclear compartments in germ nuclei from the initial stages of development until the occurrence of meiosis, where paternal and maternal sets assume a non-random arrangement in the prophase nucleus (Rieffel and Crouse, 1966; Kubai, 1982; Kubai, 1987; Goday and Ruiz, 2002). In this respect, it has been suggested that the establishment of intranuclear specification of the chromosomal set domains in early germline nuclei might constitute a significant requirement for the success of non-random meiotic segregation that leads to chromosome elimination (Kubai, 1987; Goday and Esteban, 2001).

We provide here the first evidence than histone methylation can be related to the parental origin of chromosomes in germline cells of *S. ocellaris*. As already known for the distribution of histone acetylation, we found differences in histone methylation between parental chromosome sets both in early germ nuclei as well as during the first male meiotic division (Table 1). Assuming that in these two cell types the chromosome sets occupy discrete nuclear compartments, an attractive hypothesis is that specific histone covalent modifications and a particular intranuclear arrangement of chromosomes are linked. Our present findings on the distribution of histone H3K4 methylation in germline cells of *S. ocellaris*, together with previous data on histone acetylation distribution, are consistent with a model that integrates covalent histone modifications, intranuclear parental chromosome distribution and chromosome elimination in *Sciara* germ cells (Fig. 9). Moreover, they also give further support to the proposal that precise covalent chromatin modifications are involved in specifying the imprinted behaviour of germline chromosomes in *Sciara.* Furthermore, our findings in sciarid flies envisage a new role for H3K4 methylation besides its involvement in chromatin transcription.

Materials and Methods

Fly culture and egg collection

S. ocellaris was raised at 20°C and the duration of the different developmental stages from embryo to pupal stage was determined as described elsewhere (Rieffel and Crouse, 1966). To analyze embryonic germ nuclei at the resting stage, prior to and after chromosome elimination, fertilized eggs collected over 10-60 hours were fixed every 10 hours and eggs collected over 60-70 hours were fixed every 2 hours. To analyze embryonic germ cell nuclei before the resting stage period, eggs were collected and fixed every 20 minutes from 1 hour to 6 hours after deposition.

Fixation

To analyze embryonic germ cell nuclei at the resting stage, embryos were squashed on microscope slides according to the CSS technique (Goday et al., 1999). The buffer that was used to crack the embryos consisted of 6 mM $MgCl_2$, 1% citric acid and 1% Triton X-100 or, alternatively, 1% paraformaldehyde and 0.1% Triton X-100. Slides were then frozen in liquid N_2 to remove the coverslips, postfixed in 3.7% formaldehyde for 20 minutes at room temperature and rinsed in PBS. To analyze post-embryonic germ cells, ovaries and testes were removed from larvae and immediately squashed in a drop of the above buffer, frozen in liquid N_2 to remove the coverslips, fixed in 3.7% formaldehyde for 20 minutes at room temperature and immersed in PBS. Alternatively, ovaries and testes were first fixed in 1% paraformaldehyde and 0.1% Triton X-100 for 3 minutes at room temperature, squashed in a drop of 50% acetic acid and 1% paraformaldehyde and rinsed in PBS after removal of the coverslips. To analyze whole embryos at earlier stages they were dechorionated and fixed in methanol following described procedures (de Saint-Phalle and Sullivan, 1996).

Immunostaining and microscopy

After fixation, slides and embryo suspensions were processed identically. Essentially, they were washed in PBS (3×10 minutes) and then in PBS containing 1% Triton X-100 for 10 minutes. They were then incubated with 2% BSA for 1 hour at room temperature. Primary antibodies were anti-H3K4me2, anti-H3K4me3 (Abcam and Upstate Biotechnology); anti-H3K9me2 and anti-H3K9me3 (Abcam); anti-H3K36me2 (Upstate Biotechnology); anti-H3K79me2; anti-H4K20me2; anti-RNA pol II (Progen); anti-pSer2 CTD RNA pol II (Covalence, clone H5). Secondary antibodies were FITC- and Cy3-conjugated anti-rabbit (Jackson), and FITC-conjugated anti-mouse (Southern Biotechnology). All antibodies were diluted in 1% BSA in PBS. Primary antibodies were diluted 1:50 to 1:100 and secondary antibodies were diluted 1:50 for FITC-conjugated antibody and 1:800 for Cy3-conjugated antibody. Incubation of primary antibodies was at 4°C overnight followed by washing in PBS 3×10 minutes. Incubation of secondary antibody was at room temperature for at least 1 hour or at 4°C overnight. DNA was visualized with 4′6-diamino-2-phenylindole (DAPI) staining (0.1 μg/ml) and preparations mounted in anti-fading solution. Observations were made under epifluorescence optics with a Zeiss axiophot microscope equipped with a Leitz CCD camera.

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References

Batchelder, C., Dunn, M. A., Choy, B., Suh, Y., Cassie, C., Shim, E. Y., Shin, T. H., Mello, C., Seydoux, G. and Blackwell, T. K. (1999). Transcriptional repression by the Caenorhabditis elegans germ-line protein PIE-1. *Genes Dev.* **13**, 202-212.

Berry, R. O. (1939). Observations on chromosome elimination in the germ cells of *Sciara ocellaris*. *Proc. Natl. Acad. Sci. USA* **25**, 125-127.

Berry, R. O. (1941). Chromosome behavior in the germ cells and development of the gonads in *Sciara ocellaris*. *J. Morphol.* **68**, 547-583.

Crouse, H. V. (1965). Experimental alterations in the chromosome constitution of *Sciara*. *Chromosoma* **16**, 391-410.

de Saint-Phalle, B. and Sullivan, W. (1996). Incomplete sister chromatid separation is the mechanism of programmed chromosome elimination during early *Sciara coprophila* embryogenesis. *Development* **122**, 3775-3784.

Dubois, A. M. (1932). Elimination of chromosomes during cleavage in the eggs of *Sciara* (Diptera). *Proc. Natl. Acad. Sci. USA* **18**, 352-356.

Esteban, M. R., Campos, M. C. C., Perondini, A. L. P. and Goday, C. (1997). Role of microtubules and microtubule organizing centers on meiotic chromosome elimination in *Sciara ocellaris*. *J. Cell Sci.* **110**, 721-730.

Fang, J., Feng, Q., Ketel, C. S., Wang, H., Cao, R., Xia, L., Erdjument-Bromage, H., Tempst, P., Simon, J. A. and Zhang, Y. (2002). Purification and functional characterization of SET8, a nucleosomal histone H4-lysine 20-specific methyltransferase. *Curr. Biol.* **12**, 1086-1099.

Gerber, M. and Shilatifard, A. (2003). Transcriptional elongation by RNA polymerase II and histone methylation. *J. Biol. Chem.* **278**, 26303-26306.

Gerbi, S. A. (1986). Unusual chromosomes movements in sciarid flies. In *Germ-Line-Soma Differentiation. Results and Problems of Cell Diferentiation*. Vol. 13 (ed. W. Hennig), pp. 71-104. New York: Springer.

Goday, C. and Esteban, M. R. (2001). Chromosome elimination in sciarid flies. *BioEssays* **23**, 242-250.

Goday, C. and Ruiz, M. F. (2002). Differential acetylation of histones H3 and H4 in paternal and maternal germline chromosomes during development of sciarid flies. *J. Cell Sci.* **115**, 4765-4775.

Goday, C., Panzera, Y. and Esteban, M. R. (1999). A simple cytological technique to analyze nuclear divisions during preblastodermic development in *Drosophila*. *Chromosome Res.* **72**, 445-448.

Krogan, N. J., Dover, J., Wood, A., Schneider, J., Heidt, J., Boateng, M. A., Dean, K., Ryan, O. W., Golshani, A., Johnston, M. et al. (2003). The Paf1 complex is required for histone H3 methylation by COMPASS and Dot1p: linking transcriptional elongation to histone methylation. *Mol. Cell* **11**, 721-729.

Kubai, D. F. (1982). Meiosis in *Sciara coprophila*: structure of the spindle and chromosome behavior during the first meiotic division. *J. Cell Biol.* **93**, 655-669.

Kubai, D. F. (1987). Nonrandom chromosome arrangements in germ line nuclei of *Sciara coprophila* males: the basis for nonrandom chromosome segregation on the meiosis I spindle. *J. Cell Biol.* **105**, 2433-2446.

Lachner, M., O'Sullivan, R. J. and Jenuwein, T. (2003). An epigenetic road map for histone lysine methylation. *J. Cell Sci.* **116**, 2117-2124.

Martinho, R., Kunwar, P., Casanova, J. and Lehmann, R. (2004). A noncoding RNA is required for the repression of RNApolII-dependent transcription in primordial germ cells. *Curr. Biol.* **14**, 159-165.

Ng, H. H., Robert, F., Young, R. A. and Struhl, K. (2003). Targeted recruitment of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity. *Mol. Cell* **11**, 709-719.

Orphanides, G. and Reinberg, D. (2002). A unified theory of gene expression. *Cell* **108**, 439-451.

Patturajan, M., Schulte, R. J., Sefton, B. M., Berezney, R., Vincent, M., Bensaude, O., Warren, S. L. and Corden, J. L. (1998). Growth-related changes in phosphrylation of yeast RNA polymerase II. *J. Biol. Chem.* **273**, 4689-4694.

Perondini, A. L. P. and Ribeiro, A. F. (1997). Chromosome elimination in germ cells of *Sciara* embryos: involvement of the nuclear envelope. *Invertebr. Reprod. Dev.* **32**, 131-141.

Polioudaki, H., Kourmouli, N., Drosou, V., Bakou, A., Panayiotis, A. T., Singh, P. B., Giannakouros, T. and Georgatos, S. D. (2001). Histones H3/H4 form a tight complex

with the inner membrane protein LBR and heterochromatin protein 1. *EMBO Rep.* **21**, 920-925.

Rice, J. C., Nishioka, K., Sarma, K., Steward, R., Reinberg, D. and Allis, C. D. (2002). Mitotic-specific methylation of histone H4 Lys 20 follows increased PR-Set7 expression and its localization to mitotic chromosomes. *Genes Dev.* **16**, 2225-2230.

Rieffel, S. M. and Crouse, H. V. (1966). The elimination and differentiation of chromosomes in the germ line of *Sciara*. *Chromosoma* **19**, 231-276.

Santos-Rosa, H., Schneider, R., Bannister, A. J., Sherriff, J., Bernstein, B. E., Emre, N. C., Schreiber, S. L., Mellor, J. and Kouzarides, T. (2002). Active genes are tri-methylated at K4 of histone H3. *Nature* **419**, 407-411.

Schaner, C. E., Deshpande, G., Schedl, P. D. and Kelly, W. G. (2003). A conserved chromatin architecture marks and maintains the restricted germ cell lineage in worms and flies. *Dev. Cell* **5**, 747-757.

Schneider, R., Bannister, A., Myers, F., Thorne, A. W., Crane-Robinson, C. and Kouzarides, T. (2004). Histone H3 lysine 4 methylation patterns in higher eukaryotic genes. *Nat. Cell Biol.* **6**, 73-77.

Seydoux, G. and Dunn, M. A. (1997). Transcriptionally repressed germ cells lack a subpopulation of phosphorylated RNA polymerase II in early embryos of Caenorhabditis elegans and Drosophila melanogaster. *Development* **124**, 2191-2201.

Spada, F., Vincent, M. and Thompson, E. M. (2005). Plasticity of histone modifications across the invertebrate to vertebrate transition: histone H3 lysine 4 trimethylation in heterochromatin. *Chromosome Res.* **13**, 57-72.

Whetstine, J. R., Nottke, A., Lan, F., Huarte, M., Smolikov, S., Chen, Z., Spooner, E., Li, E., Zhang, G., Colaiacovo, M. et al. (2006). Reversal of histone lysine trimethylation by the JMJD2 family of histone demethylases. *Cell* **125**, 467-481.