Differential acetylation of histones H3 and H4 in paternal and maternal germline chromosomes during development of sciarid flies

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Summary

A classic example of chromosome elimination and genomic imprinting is found in sciarid flies (Diptera. *Sciaridae*), where whole chromosomes of exclusively paternal origin are discarded from the genome at different developmental stages. Two types of chromosome elimination event occur in the germline. In embryos of both sexes, the extrusion of a single paternal X chromosome occurs in early germ nuclei and in male meiotic cells the whole paternal complement is discarded. In sciarids, early germ nuclei remain undivided for a long time and exhibit a high degree of chromatin compaction, so that chromosomes are cytologically individualized. We investigated chromatin differences between parental chromosomes in *Sciara ocellaris* and *S. coprophila* by analyzing histone acetylation modifications in early germ nuclei. We examined germ nuclei from early embryonic stages to premeiotic larval stages, male meiotic cell and early somatic nuclei following fertilization. In early germ cells, only half of the regular chromosome complement is highly acetylated for histones H4 and H3. The chromosomes that are highly acetylated are paternally derived. An exception is the paternal X chromosome that is eliminated from germ nuclei. At later stages preceding the initiation of mitotic gonial divisions, all chromosomes of the germline complement show similar high levels of histone H4/H3 acetylation. In male meiosis, maternal chromosomes are highly acetylated for histones H4 and H3, whereas the entire paternal chromosome set undergoing elimination appears under-acetylated. The results suggest that histone acetylation contributes towards specifying the imprinted behavior of germline chromosomes in sciarids.

Key words: Histone acetylation, Chromosome elimination, Imprinting, Sciara

Introduction

An extreme example of genomic imprinting is found in sciarid flies (Diptera. *Sciaridae*), where paternally derived whole chromosomes are selectively eliminated from the genome at different developmental stages (reviewed in Gerbi, 1986; Goday and Esteban, 2001). During the early cleavages that follow the zygote constitution ($A_mA_pX_mX_pX_p$), all somatic cells lose one or two paternal X chromosomes (X_p) depending on the sex of the embryo (females or males respectively). At later embryonic stages, in germ cells of both sexes a single X_p chromosome is discarded. Finally, during the last larval stage while female meiosis is orthodox, in male meiosis I the whole paternal complement is eliminated. As a consequence, only maternal chromosomes are retained in the sperm nucleus, and these chromosomes will be then recognized as paternal after fertilization. An additional particular feature of male meiosis is the non-disjunction of the maternal X chromosome (X_m) that determines the characteristic 3X constitution of the zygote in sciarids. In some sciarid species such as *Sciara coprophila*, there are additional heterochromatic chromosomes, which are restricted to the germline and are known as L chromosomes (for germline-limited). L chromosomes in the zygote may vary in number owing to non-disjunction occurrence during male gonial mitosis. At early embryonic cleavages, all L chromosomes are discarded from somatic cells, and later in development, all but two are also eliminated from embryonic germ cells. In Fig. 1 we schematically summarize the present understanding of germline chromosome features common to sciarid flies that are relevant for this work.

Since the early studies on the chromosome cell cycle of sciarids, there has been an increasing understanding at the cellular level of the tissue-specific mechanisms underlying the different chromosome-elimination events (reviewed in Gerbi, 1986; Goday and Esteban, 2001). A less-studied aspect, as revealed by classic cytology in *S. ocellaris* and *S. coprophila* (Berry, 1939; Berry, 1941; Dubois, 1932; Rieffel and Crouse, 1966), refers to the peculiar behavior and chromatin organization of germ cells during development. Following somatic chromosome elimination in the early embryo, germ cells migrate from the posterior pole plasm to the gonad site, remaining undivided until the beginning of the second larval instar. Despite the interphase appearance of the germ nuclei, chromatin inside is atypically condensed so that the chromosomes are cytologically distinguishable from each other (Berry, 1941; Rieffel and Crouse, 1966). Moreover, ultrastructural studies in *S. ocellaris* showed that the level of chromatin condensation corresponds to prometaphase/metaphase chromosomes rather than interphase chromosomes

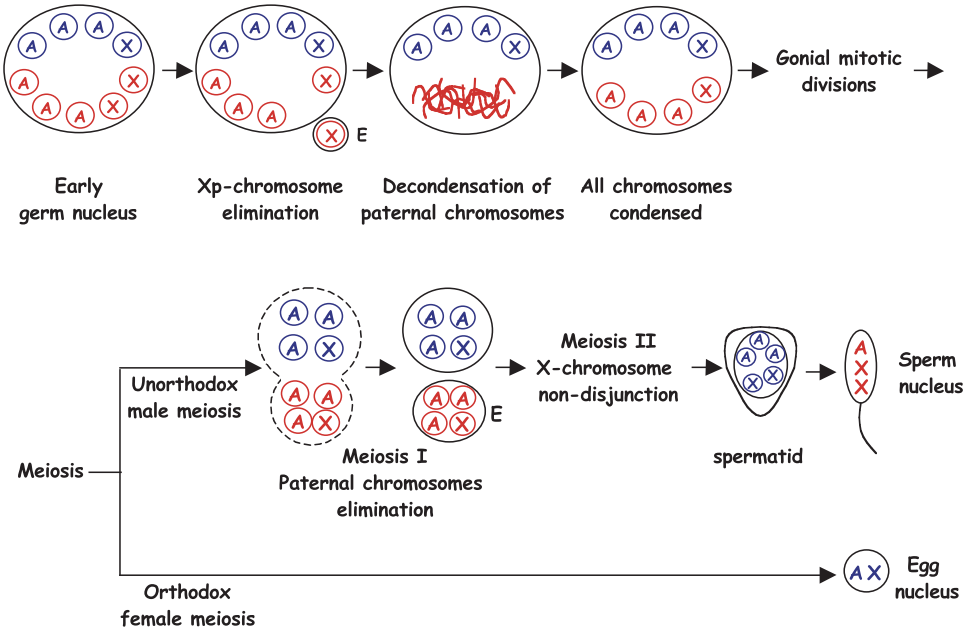

Fig. 1. Scheme summarizing the most relevant chromosomal events of germline nuclei at the resting stage and male meiosis in sciarids. Maternally derived chromosomes are represented in blue and paternally derived chromosomes are represented in red. Early embryonic germ nuclei contain three maternal and paternal autosomes (A) plus one maternal and two paternal X-chromosomes (X). E refers to the occurrence of chromosome elimination. Note that the parental origin of meiotic chromosome products in the sperm nucleus and egg are represented as they will be recognized after fertilization (paternal and maternal, respectively).

(Perondini and Ribeiro, 1997). It is during this long non-dividing period of germ cells known as the 'resting stage' that one paternal X chromosome is extruded from the nuclei through the nuclear membrane by a process that is still not fully understood (Berry, 1939; Berry, 1941; Perondini and Ribeiro, 1997). The elimination of a single X_p chromosome occurs synchronously in all germ cells of both sexes, reducing the germline complement of three sex chromosomes ($A_mA_pX_mX_pX_p$) to two sex chromosomes ($A_mA_pX_mX_p$) (Berry, 1939). Genetic evidence has demonstrated that the eliminated Xp is one of the undysjoined dyad in male meiosis (Crouse, 1965). Whether there is a random selection for which paternal X chromosome is eliminated, and how the germ cell identifies one of the two X_p chromosomes has remained open questions since they were posed by Berry (Berry, 1941).

Following the X_p elimination event, an additional remarkable event was observed in the germ nuclei of *S. ocellaris* nuclei when stained with classic dyes (Berry, 1941; Rieffel and Crouse, 1966). Two distinct groups of chromosomes could be distinguished inside the nucleus, as light- or dark-staining, owing to differences in the degree of chromatin condensation. Berry deduced, in *S. ocellaris*, that each distinct chromosome group constitutes an entire genome (Berry, 1941). Similarly, the analysis in *S. coprophila* of reciprocal translocations altering chromosome morphology permitted the identification of two groups of chromosomes (Rieffel and Crouse, 1966). The paternal chromosome set (lacking the X_p chromosome that has been previously eliminated) corresponds to the light-staining set with an uncoiled chromatin state, whereas the maternal set remains dark-stained and highly condensed (Rieffel and Crouse, 1966). These studies also revealed that such differences between the paternal and maternal chromosomes were maintained in germ cells until the late first larval instar of both sexes, before the beginning of gonial mitotic divisions (Berry, 1941; Rieffel and Crouse, 1966). It has been questioned whether this period could be associated with changes to or reversion of the imprinting of the sets of homologs (Rieffel and Crouse, 1966). However, this highly unusual differential chromatin behavior associated to the parental origin of chromosomes in sciarid flies has not been explored further.

In view of these findings, we decided to explore in *Sciara* chromosomes the existence of molecular chromatin modifications in relation to its paternal/maternal origin. A candidate for producing rapid and reversible DNA modifications, susceptible to be cytologically detected, is that of histone acetylation of certain lysine residues at the N-terminal domains of the core histones. Histone acetylation is a dynamic process regulated by two classes of enzymes: the histone acetyltransferases (HATs) and histone deacetylases (HDACs). Changes in acetylation have been correlated with changes in the transcription, replication and packaging of DNA, and these modifications are thought to play an important role in the control of chromatin and chromosomal functions (reviewed in Cheung et al., 2000; Strahl and Allis, 2000; Turner, 2000).

In the present work, we have examined histone acetylation patterns in *S. ocellaris* and *S. coprophila* germline by immunolabeling with site-specific antibodies for histone H4 acetylated at lysines 5, 8, 12, and 16 and with an antibody for the histone H3 di-acetylated form (Lys9 and Lys14). The distribution of acetylated histones has been analyzed in: **(1)** germ cells of embryos of both sexes, prior to and after X_p chromosome (and L chromosomes) elimination; **(2)** germ cells of larvae of both sexes from the first instar stage to premeiotic divisions; **(3)** male meiotic divisions undergoing the elimination of the whole paternal complement; **(4)** early embryos following fertilization.

We show here that in early germ cells of both *Sciara* species, the paternally derived half of the regular chromosome complement is highly acetylated for histone H4 and histone H3, whereas in the maternally derived half of the complement, H4/H3 acetylation is cytologically undetectable. The unique X_p chromosome, which is discarded from germ nuclei, is an

exception, as it is not acetylated. Acetylation of histone H4 is predominantly found at lysines 8 and 12. The observed differences in the histone acetylation pattern between paternal and maternal chromatin are maintained during decondensation of paternal chromosomes inside the resting stage nuclei of young larvae. At later larval stages, in germ cells of both sexes, all chromosomes become highly acetylated and no differences are observed up to meiotic division. During male meiosis, whereas maternal chromosomes maintain a high level of histone H4 and H3 acetylation, the whole paternal complement that is eliminated appears under-acetylated. In light of our data, we discuss histone acetylation status and germline chromosomes elimination events in *Sciara*. We also propose a model that relates histone acetylation, chromosome elimination and intranuclear chromosome arrangements in germline cells.

Materials and Methods

Fly culture and egg collection

S. ocellaris and *S. coprophila* were raised at 20°C. *S. coprophila* strain 6980 was kindly provided by Susan Gerbi (Brown University, Providence, Rhode Island). The duration of the different developmental stages from embryo to pupal stage was determined as described elsewhere (Rieffel and Crouse, 1966). To analyze whole embryos at the early fertilization stage, eggs were collected and fixed every 20 minutes from 0 to 6 hours. To analyze embryonic germ nuclei at the resting stage, prior to and after chromosome elimination, eggs collected from 10-60 hours were fixed every 10 hours and 60-70 hour egg collections were fixed every 2 hours.

Fixation

To analyze embryonic germ nuclei, embryo contents were squashed on microscope slides following the CSS technique (Goday et al., 1999). The buffer solution used to crack the embryos consisted of 6 mM $MgCl_2$, 1% citric acid and 1% Triton X-100. Slides were then fixed in 3.7% formaldehyde for 20 minutes at room temperature. To analyze whole embryos after fertilization they were dechorionated and fixed in methanol following previously described procedures (de Saint-Phalle and Sullivan, 1996). To analyze post-embryonic germ cells, ovaries and testes were removed from larvae dissected in the above buffer solution. They were immediately squashed in a drop of the same buffer, frozen in liquid N_2 to remove the coverslips and fixed in 3.7% formaldehyde for 20 minutes at room temperature.

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 for 10 minutes. They where then incubated with 2% BSA for 1 hour at room temperature.

The primary antibodies were anti-acetyl-histone H4 (Lys5), anti-acetyl-histone H4 (Lys8), anti-acetyl-histone H4 (Lys12), anti-acetyl-histone H4 (Lys16), anti-acetylated histone H3 (Lys9 and Lys14) (Upstate Biotechnology); and the secondary antibody was Cy3-conjugated anti-rabbit antibody (Jackson). All antibodies were diluted in 1% BSA in PBS. Primary antibodies were dilute 1:60 and secondary antibodies 1:600. Incubation of primary antibodies was at 4°C overnight followed by washing in PBS for 3×10 minutes. Incubation of secondary antibody was at room temperature for at least 4 hours or at 4°C overnight. DNA was visualized with 4′6-diamidino-2-phenylindole (DAPI) staining (0.1 μg/ml) and preparations mounted in antifading solution. Observations were made under epifluorescence optics with a Zeiss axiophot microscope equipped with a Photometrics CCD camera.

Results

In *Sciara*, the constitution of the early embryonic germ nuclei is that of the zygote (A_p A_m X_m X_p X_p). In *S. ocellaris* there are nine chromosomes (three pairs of autosomes plus three X-chromosomes), whereas in *S. coprophila* additional L chromosomes are also present (generally three). As already described (Berry, 1941; Rieffel and Crouse, 1966), chromosomes can be distinguished inside the nucleus owing to their atypical high level of chromatin condensation.

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

We first investigated histone acetylation in germ nuclei of *Sciara* embryos at the beginning of the resting stage period, when all chromosomes of the complement exhibit a high level of chromatin condensation. Fig. 2 shows the immunolocalization of acetylated histone H4 at lysine 8 (H4Ac8) in *S. ocellaris* germ nuclei prior to (A-A″), during (B-B″), and after (C-C″) Xp chromosome elimination. In all cases only four chromosomes showed bright overall fluorescence, indicative of high levels of H4 acetylation. 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-acetylated. In germ nuclei undergoing X_p chromosome elimination (Fig. 2B-B″), it was possible to observe that the chromosome expelled from the nuclei was always devoid of H4Ac8 labeling. Moreover, in some of the nuclei where the longest autosome pair of the complement was recognizable by DAPI staining (designated by arrows in Fig. 2C-C″), only one of the two homologous chromosomes exhibited H4Ac8 labeling. We observed a similar behavior in *S. coprophila* germ nuclei prior to (Fig. 2D-D″) and after chromosome elimination (data not shown). Again, only four of the chromosomes showed high levels of H4Ac8. In addition, all of the L chromosomes in these *S. coprophila* germ cells were devoid of staining.

A similar analysis was then performed in embryonic germline nuclei of *S. ocellaris* and S. *coprophila* using antibodies against acetylated histone H4 at lysine 12 (H4Ac12), lysine 5 (H4Ac5) and lysine 16 (H4Ac16). An identical distribution pattern to that shown above for H4Ac8 was observed for the H4Ac12 antibody, whereas no labeling of chromosomes was detected with H4Ac5 and H4Ac16 antibodies (data not shown). Moreover, antibodies against acetylated histone H3 (H3Ac) stained again only four chromosomes of the complement both in *S. ocellaris* (Fig. 3A-A″) and *S. coprophila* (data not shown). In order to investigate whether the observed distribution of H4Ac8, H4Ac12 and H3Ac antibodies was coincident on the same four chromosomes of the complement, we immunolabeled *S. ocellaris* germ nuclei by using different combinations of these antibodies (Fig. 3). From this analysis, it emerged that in all cases only four chromosomes were stained (Fig. 3B,C,D-B″,C″,D″). Thus, we concluded that the same four chromosomes of the complement undergo high levels of histone H4 acetylation at lysines 8 and 12 and of histone H3 acetylation (Lys9 and Lys14). Identical results were obtained for *S. coprophila* germ nuclei (data not shown).

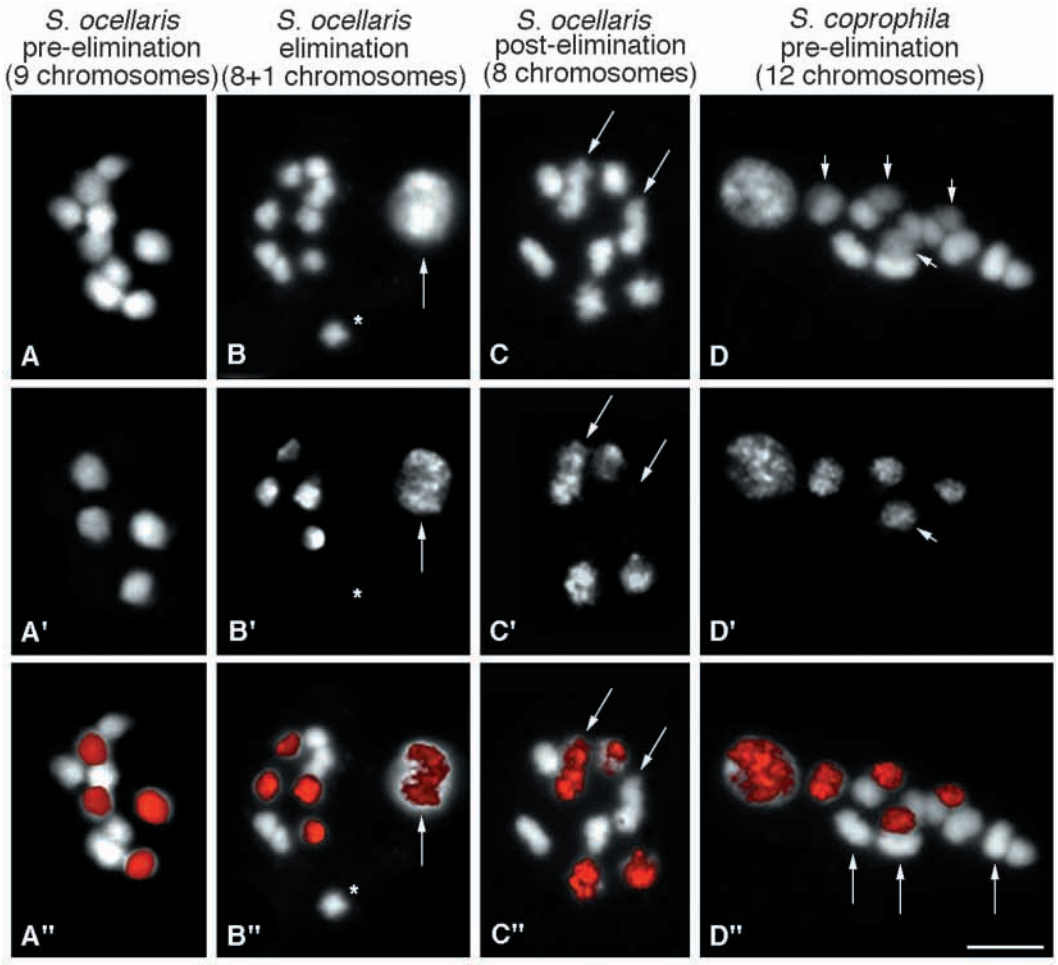

Fig. 2. Distribution of acetylated histone H4 at lysine 8 in *S. ocellaris* and *S. coprophila* embryonic germ nuclei at the resting stage. (A-D) Chromosome DAPI staining, (A′-D′) Indirect immunolabeling with H4Ac8 antibody and (A″-D″) superimposed images. where the antibody staining is in red. (A) *S. ocellaris* germ nuclei prior to, (B) during and (C) after the occurrence of X_p chromosome elimination. (D) A *S. coprophila* nucleus before the elimination of X_p and L chromosomes. In all cases only four chromosomes of the complement are highly H4Ac8-labelled (A′-D′ and A″-D″). (B-B″) a *S. ocellaris* 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″); a labeled somatic nucleus is also shown for comparison (arrows). (C′-C″) A post-elimination *S. ocellaris* nucleus where it is possible to observe that only one of the chromosomes corresponding to the longest autosomes pair is labeled (arrows). (D-D″) The four *S. coprophila* chromosomes that are H4Ac8-labelled (short arrows in D′) correspond to the ones that are slightly less stained with DAPI (short arrows in D); the H4Ac8-unstained eight chromosomes contain three L chromosomes (possibly the ones indicated by long arrows in D″). Bar, 10 μm.

Distribution of acetylated histones in germ cells of larvae from the first instar stage to premeiotic divisions

To investigate histone acetylation 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 H4Ac8, H4Ac12, H4Ac5 and H4Ac16. Fig. 4A shows a *S. ocellaris* germ nucleus from a 1-day-old larvae stained with DAPI and containing four chromosomes highly condensed and brightly fluorescent, plus four chromosomes which have initiated decondensation and appear less fluorescent. As previously demonstrated (Berry, 1941; Rieffel and Crouse, 1966), at this stage, the group of chromosomes that unravel corresponds, for both sexes, to the paternal set. Thus, in Fig. 4A it is possible to recognize paternal and maternal chromosomes by DAPI staining (short and long arrows, respectively). The immunolocalization of acetylated histone H4 at lysine 8 in this germ nucleus (Fig. 4A′,A″) revealed that H4Ac8 labeling corresponds to the paternal chromosomes whereas maternal chromosomes are devoid of staining. By means of DAPI staining, we then followed paternal chromosome decondensation and found that in *S. ocellaris* the maximum degree is achieved in germ nuclei of larvae that are three to five days old. Fig. 4B-B″ shows a germ nucleus from a 5-day-old larva where paternal chromosomes are fully decondensed and are H4Ac8-labelled; maternal chromosomes, in contrast, remain highly condensed and unstained. The same result was found in germ nuclei of larvae that were 7-8 days old (data not shown). The differences in chromatin condensation between the two parental groups of chromosomes were no longer seen in germ nuclei at posterior larval stages (from 10 days on), in accordance with classic data (Berry, 1941; Rieffel and Crouse, 1966). In Fig. 4C it is possible to observe that paternal and maternal chromosomes are similarly condensed and no longer recognizable. Importantly, H4Ac8 labelling of this germ nucleus (Fig. 4C′,C″) revealed that both paternal and maternal chromosomes showed overall staining, indicative of high levels of H4 acetylation. During the successive gonial mitotic divisions until the occurrence of meiosis (see below) no differential distribution of H4 acetylation at lysine 8 in germ nuclei was observed (data not shown). Similar results were obtained in germ cells from *S. ocellaris* larvae of both sexes.

The same analysis was carried out in *S. coprophila* germ nuclei. Fig. 5A shows a germ nucleus from 3-day-old larvae where paternal chromosomes (short arrows) are already totally decondensed, in contrast to maternal (long arrows) and to L

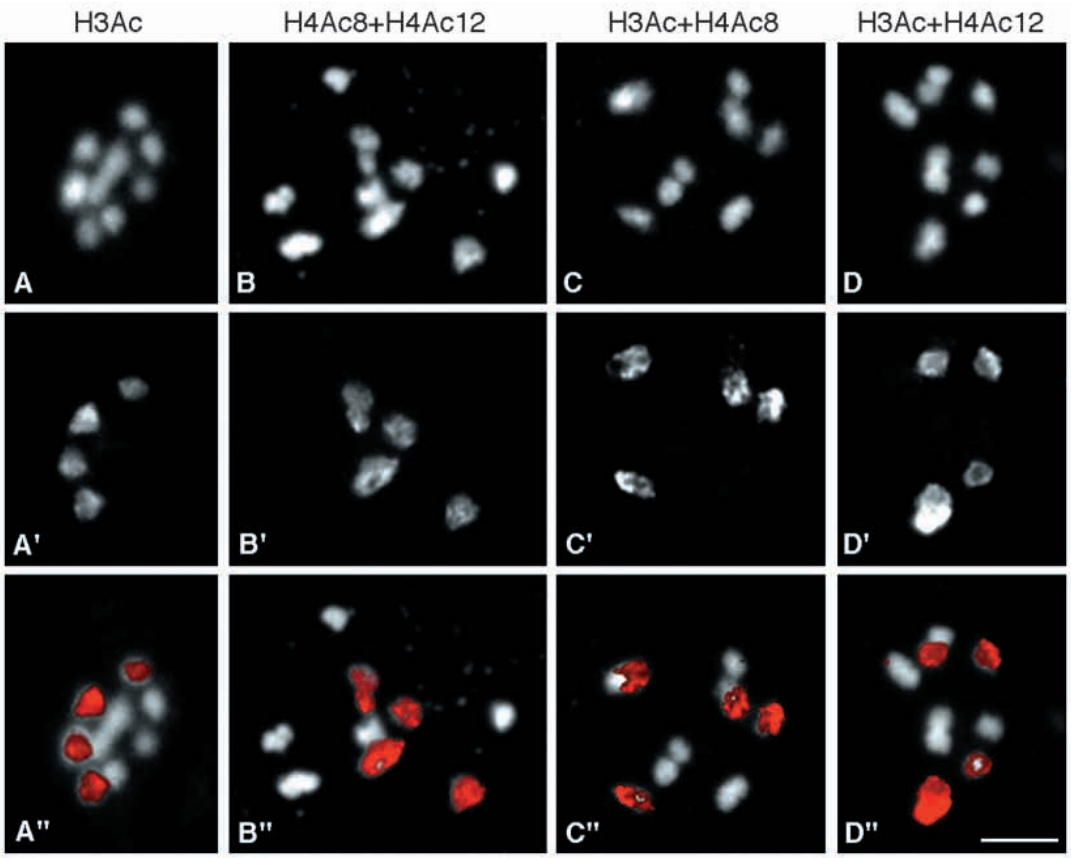

Fig. 3. Colocalization of acetylated histone H3 and acetylated histone H4 at lysines 8 and 12 in *S. ocellaris* embryonic germ nuclei. (A-D) DAPI staining, (A′-D′) indirect immunolabeling with different antibody combinations that are specified in the top of the figure and (A″-D″) superimposed images where antibody staining is in red. In all cases the antibodies labeling is restricted to the same four chromosomes of the complement. Bar, 10 μm.

(arrowheads) chromosomes, which remain highly condensed and DAPI stained. Clustering of maternal homologs and of L chromosomes is clearly observed during this period, in accordance with previous observations (Rieffel and Crouse, 1966). The immunolocalization of H4Ac8 in this germ nucleus (Fig. 5A′,A″) revealed that the H4Ac8 labeling corresponds to the paternal chromatin while the four maternal chromosomes and the two Ls are devoid of staining. Similar to observations in *S. ocellaris*, all chromosomes of *S. coprophila* germ nuclei

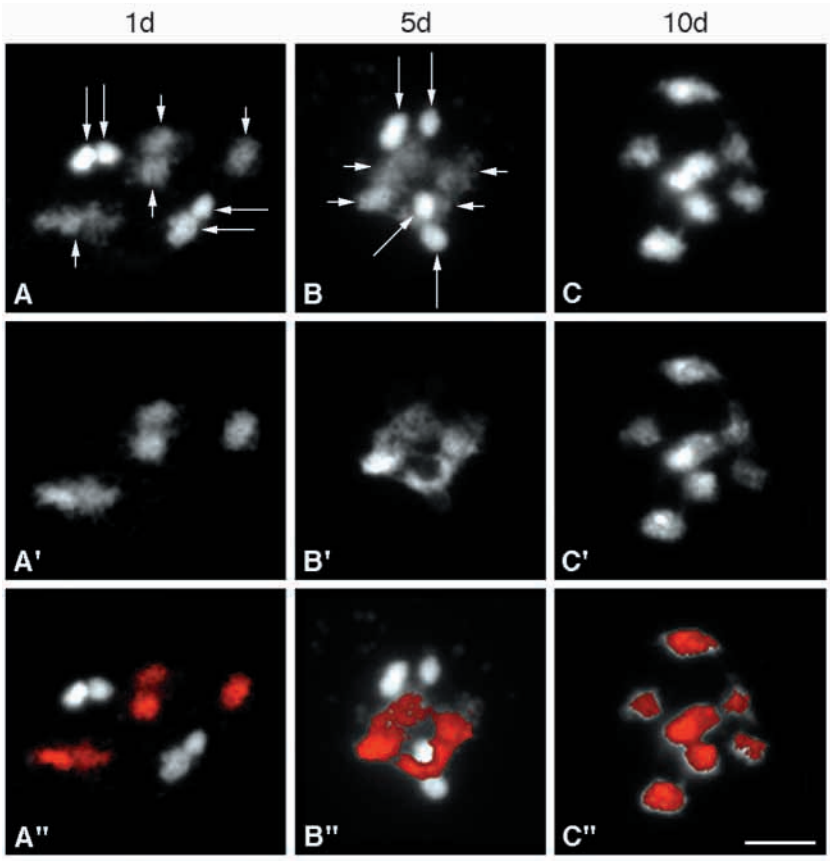

Fig. 4. Distribution of acetylated histone H4 at lysine 8 in germ cells of *S. ocellaris* male larvae at one day (1d), five days (5d) and 10 days (10d) from hatching. (A-C) DAPI staining, (A′-C′) indirect immunolabeling with H4Ac8 antibody and (A″-C″) superimposed images where antibody staining is in red. (A) Maternal chromosomes appear condensed (long arrows) whereas paternal chromosomes, less stained with DAPI, initiate decondensation (short arrows); (A′,A″) H4Ac8-staining is restricted to the paternal chromosome set. (B) Maternal chromosomes remain condensed (long arrows) whereas paternal ones appear totally decondensed (short arrows); (B′,B″) H4Ac8-labeling is restricted to the chromatin corresponding to the paternal chromosome set. (C) All chromosomes appear equally condensed and exhibit H4Ac8-staining (C′,C″). Bar, 10 μm.

were seen condensed at posterior stages. In this nucleus, as shown in Fig. 5B-B″, paternal and maternal chromosomes exhibit overall H4Ac8 labeling whereas L chromosomes are completely devoid of staining.

We next examined *S. ocellaris* and *S. coprophila* germ nuclei of both sexes with H4Ac12, H4Ac5 and H4Ac16 antibodies. Identical results to those described above for H4Ac8 antibody were obtained for acetylation of histone H4 at lysine 12, whereas, again, no nuclear staining was detected with H4Ac5 or H4Ac16 antibodies (data not shown).

The above results, together with the previous observations of histone acetylation in pre-elimination germ nuclei of early embryos, lead us to conclude that in *Sciara* the chromosomes that undergo high levels of histone acetylation are those of paternal origin. From our results, only the paternally derived Xp chromosome, which is eliminated, is not susceptible to become highly acetylated. Maternally derived chromosomes, in contrast, remain under-acetylated through most of the resting stage until just before gonial mitotic divisions.

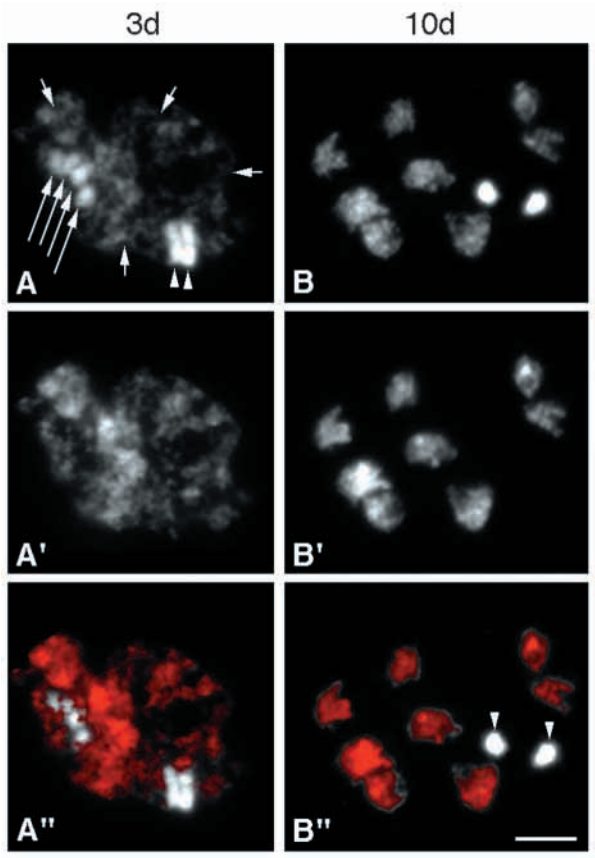

Fig. 5. Distribution of acetylated histone H4 at lysine 8 in germ cells of *S. coprophila* male larvae at 3 days (3d) and 10 days (10d) from hatching. (A,B) DAPI staining, (A′,B′) indirect immunolabeling with H4Ac8 antibody and (A″,B″) superimposed images where antibody staining is in red. (A) Maternal chromosomes (long arrows) and L chromosomes (arrowheads) appear condensed and clustered whereas paternal chromosomes are fully decondensed (short arrows); (A′,A″) Paternal chromatin exhibits H4Ac8-staining whereas maternal and L chromosomes are devoid of staining. (B-B″) All chromosomes are condensed and show H4Ac8-staining except the L chromosomes (arrowheads in B and B″). Bar, 10 μm.

Moreover, when L chromosomes are present, as in *S. coprophila*, these accessory chromosomes do not display detectable histone acetylation in early germ cells or throughout germline development (see below).

Distribution of acetylated histones in male meiotic cells

We have investigated histone H4 and H3 acetylation during the first meiotic division in both *S. ocellaris* and *S. coprophila* males, when the elimination of the whole paternal complement takes place. In sciarids, 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). The relevant features of male first meiosis chromosomes are that homolog chromosomes do not pair at prophase, they do not align in a metaphase-like array and they proceed directly from prometaphase to an anaphase-like stage (rewieved in Goday and Esteban, 2001). Fig. 6A-D shows examples of DAPI-stained first anaphase-like figures of *S. ocellaris* (A-C) and *S. coprophila* (D) spermatocytes where maternal chromosomes (m) face the single spindle pole and paternal chromosomes (p) migrate towards the opposed bud region of the spermatocyte. The immunolocalization of acetylated histone H4 (Lys12 and Lys8) and histone H3 in *S. ocellaris* spermatocytes (Fig. 6A′,A″, B′,B″ and C′,C″) revealed that the labelling corresponds exclusively to the maternal group of chromosomes whereas the paternal set is devoid of staining. Identical results were obtained for *S. coprophila* as shown, as an example, for H4Ac8 antibody in Fig. 6D-D″. On the other hand, L chromosomes (arrows in 6D), which segregate along with maternal chromosomes, lack antibody staining (Fig. 6D″). Moreover, and similar to observations described above for early germ nuclei, no staining was detected with H4Ac5 and H4Ac16 antibodies in spermatocytes of both species (data not shown). As for the second meiotic division, weak and irregular staining of maternal chromosomes was observed, possibly owing to a higher degree of chromosome condensation when compared with that of meiosis I (data not shown).

We then examined histone acetylation in *S. ocellaris* cells during spermiogenesis. Round and elongated spermatids clearly exhibited nuclear staining with H4Ac8, H4Ac12 and H3Ac antibodies in both species. Fig. 7A-A″ shows an example of H4Ac12 nuclear chromatin staining in elongated spermatids of *S. ocellaris*. At late spermatid stages, when changes in histones are known to occur, the antibody labeling is restricted to a few nuclear sites as shown in Fig. 7B-B″ for the H4Ac12 antibody. At later stages in the development of mature spermatozoa (Fig. 7C) when a high compaction of chromatin is achieved, no staining was observed with any of the antibodies. The same results were obtained for *S. coprophila* cells during spermiogenesis (data not shown).

Distribution of acetylated histones in embryos in early fertilization stages

Acetylation of histones H4 and H3 was examined in *S. ocellaris* embryos upon the entrance of the sperm nucleus and during the first somatic division (Fig. 7D-D″). This analysis

revealed that the chromatin of the decondensing sperm nucleus (short arrow in Fig. 7D) exhibits only H4Lys12 labeling (Fig. 7D′,D″) whereas no staining was detected for H4Ac8, H4Ac16, H4Ac5 and H3Ac antibodies (data not shown). On the other hand, chromatin of the maternal meiotic products (long arrows in Fig. 7D) showed only positive staining for histone H3 acetylation (data not shown). Following pronuclear fusion and the organization of chromatin into somatic chromosomes, acetylation of histone H4 at lysine 12 and 8 and of histone H3 could already be observed in all the chromosomes of the complement at first metaphase (Fig. 8A,B,C-A″,B″,C″). Identical results were observed in *S. coprophila* embryos. Note that in *S. coprophila*, as shown during first anaphase stained with H4Lys8 antibody (Fig. 8D-D″), all chromosomes are labeled. L chromosomes, therefore, appear acetylated during the early somatic nuclei that precede their elimination.

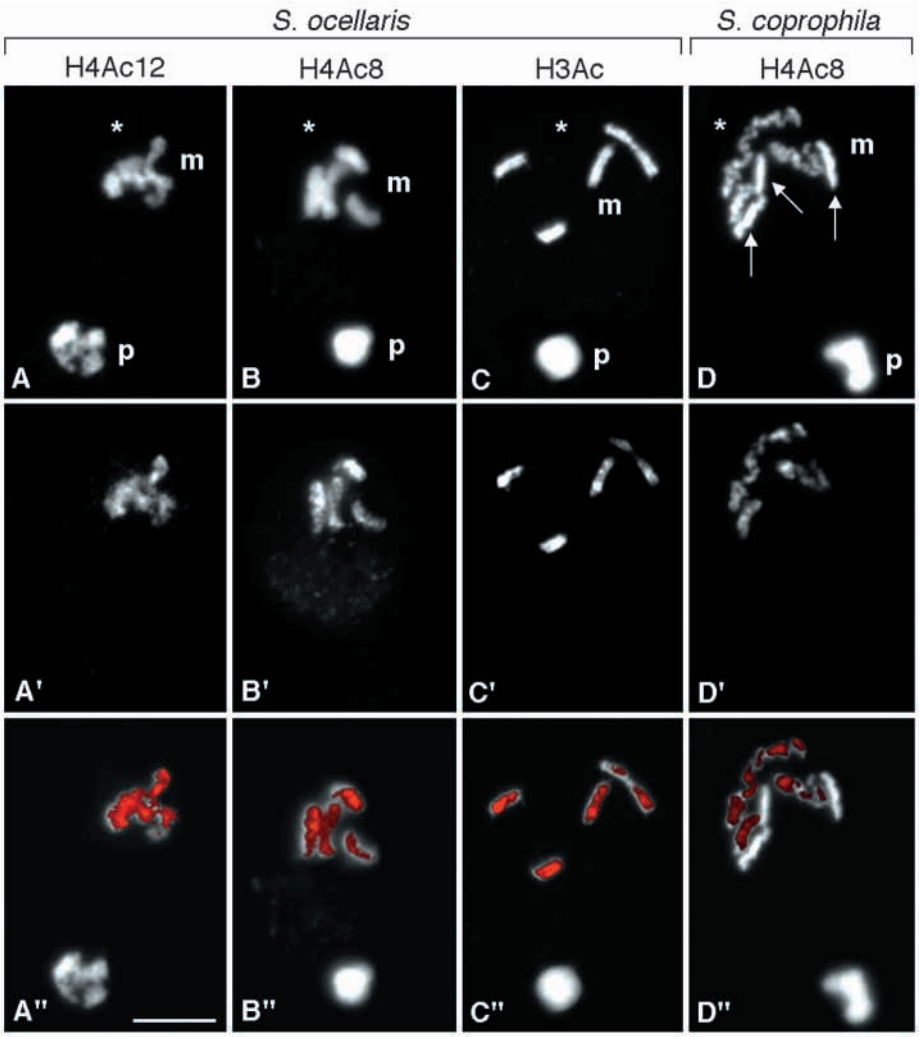

Fig. 6. Distribution of acetylated histones in *S. ocellaris* (A-C) and *S. coprophila* (D) spermatocytes undergoing paternal chromosomes elimination at first meiotic division. (A-D) DAPI staining of anaphase-like stage figures where m refers to the maternal group of chromosomes, p refers to paternal chromosomes and * marks the position of the single spindle pole. (A′-D′) Indirect immunolabeling with the antibody specified in the top of the figure and (A″-D″′) superimposed images where antibody staining is in red. In all cases (A″-D″), the antibody labeling is restricted to the four maternal chromosomes that face the pole whereas the opposed paternal group of chromosomes is devoid of staining. Arrows in D indicate *S. coprophila* L chromosomes that migrate together with the maternal group of chromosomes and do not exhibit antibody labeling (D′-D″). Bar, 10 μm.

Discussion

By indirect immunofluorescence with antisera to acetylated histones H4 and H3, we show here a distinctive and reproducible chromatin-labeling pattern in germline nuclei throughout development of sciarid flies. Fig. 9 summarizes the acetylation patterns of chromosomes in germline cells. In early germ cells of *S. ocellaris* and *S. coprophila*, only half of the regular chromosome complement undergoes high levels of histone H4/H3 acetylation. The differential histone acetylation labeling of germline chromosomes demonstrated here is consistent with the early data on the existence of chromatin staining differences between both parental genomes in germ cells (Berry, 1941; Rieffel and Crouse, 1966). Further, the present results show that in early germ cells at resting stage, the group of chromosomes in which histones H4 and H3 are highly acetylated corresponds to paternally derived chromosomes.

Germline chromosome(s) elimination and acetylation of histones

Differences in histone acetylation 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 X_p chromosome is invariably expelled from them. At this developmental stage, the two genomes exhibit similar high levels of chromatin compaction inside the nucleus. Most interestingly, the X_p chromosome, which will be discarded from the germ nuclei, does not appear to have significant levels of H4/H3 acetylation, in contrast to the rest of the paternal chromosomes. Therefore, the two copies of the paternally inherited X chromosomes differ in their chromatin acetylation state. Such differences, we think, are most probably involved in the specification of which paternal X chromosome is eliminated, or retained, from the germ nucleus.

The mechanisms involved in the extrusion of X_p chromosome through the nuclear membrane are not fully understood. An interesting piece of information comes from ultrastructural observations on *S. ocellaris* germ nuclei prior to, and during, Xp elimination (Perondini and Ribeiro, 1997).

The condensed chromatin of each individual chromosome inside the germ nucleus localizes within a short distance from the inner nuclear membrane with few points of contact. At the time of X_p elimination, this particular chromosome appears slightly more condensed than the others and closely attached to the inner nuclear membrane. The formation and posterior constriction of a nuclear bulge containing the X_p chromosome constitutes part of the elimination mechanism (Perondini and Ribeiro, 1997). Since this is a regular and highly specialized process that involves a close association between X_p chromatin and the inner nuclear membrane, it is conceivable that it occurs at a specific anchorage nuclear region. That X_p chromosome is preferentially extruded to the cytoplasm from a limited nuclear area has already been noted by classic observations in *S. ocellaris* germ cells (Berry, 1941). We suggest that the X_p chromosome to be eliminated is preferentially confined to a restrictive intranuclear location, permitting the interactions of components of this condensed chromosome with inner nuclear membrane proteins such as LBR (lamin B receptor), the major constituent of the inner nuclear membrane. Such interactions, could, in turn, require the under-acetylated chromatin state of this particular X_p chromosome. Consistent with this idea, recent experiments have shown that the association of the mammalian chromosomal heterochromatic protein 1 (HP1) with LBR through the core histones is regulated by H3/H4 acetylation (Kourmouli et al., 2000; Polioudaki et al., 2001). From these analyses a new mechanism for anchoring domains of under-acetylated chromatin to the inner nuclear membrane has been proposed (Polioudaki et al., 2001). In view of the present results, we think that the identification of X_p chromosome-associated proteins forming a putative complex with the two core histones H3/H4 and LBR could provide interesting information to better understand the regulation of X_p elimination from *Sciara* germ nuclei.

In *S. coprophila*, acetylation of histones H4 and H3 was not observed in L chromosomes, regardless of their number, throughout germline development. Since the accessory L chromosomes are cytologically heterochromatic, this result is in accordance with several studies showing that histones H3 and H4 are largely hypoacetylated in heterochromatic chromosomal regions in organisms as diverse as yeast, flies and mammals (for a review, see Jeppesen, 1993; Grunstein, 1998; Wu and Grunstein, 2000). The number of L chromosomes in *S. coprophila* zygote is most commonly two, but it may vary owing to the occurrence of nondysjunction during male gonial mitoses (Crouse et al., 1971; Rieffel and Crouse, 1966). The discarding of all but two L chromosomes from embryonic germ cells is then carried out to prevent their increasing accumulation with each generation (Metz, 1938; Rieffel and Crouse, 1966). The same timing and nuclear extrusion mechanism as that seen for X_p chromosome, is apparently involved in L chromosome elimination, which may either precede or follow X_p elimination (Rieffel and Crouse, 1966). Therefore, it is conceivable that, similar to observations with the X_p chromosome, an under-acetylated histone chromatin condition is required for the elimination of L chromosomes. Results presented here show that in the germline all L chromosomes are under-acetylated for histones H4 and H3. Hence the L chromosomes that are eliminated from the embryonic germ cells could be randomly chosen.

From our analysis of the end of the resting stage preceding the initiation of gonial mitotic divisions, differences in histone acetylation between chromosomes are no longer detectable. However, in male cells entering first meiotic division, only maternal chromosomes exhibit a high acetylation of histones H4 and H3; so that the situation is reversed when compared to that of early germ nuclei. Therefore, like the X_p chromosome discarded from early

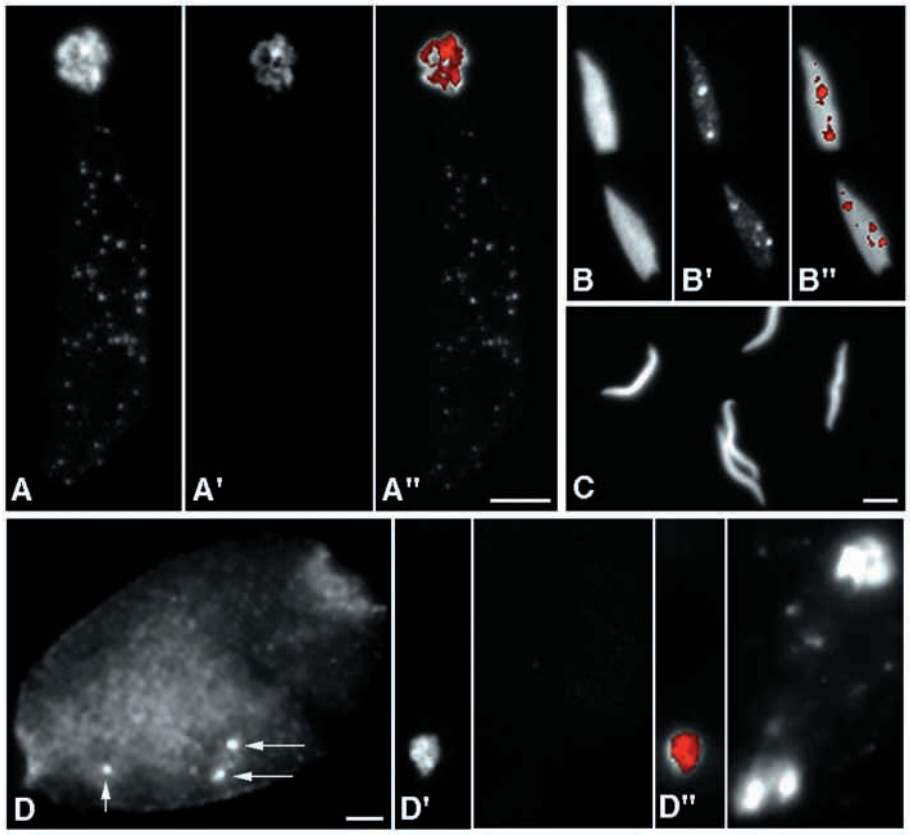

Fig. 7. Distribution of histone H4 acetylation at lysine 12 during *S. ocellaris* spermiogenesis (A-C) and in an embryo at early fertilization stage (D). DAPI staining of elongated spermatids (A), late spermatids (B), mature spermatozoa (C) and the whole embryo (D). (A′,B′,D′) Indirect immunolabeling with H4Ac12 antibody and (A″,B″,D″) superimposed images where antibody staining is in red. In A′,A″ H4Ac12 distributes on the nuclear chromatin whereas in B′,B″ the labeling is restricted to a few nuclear sites. In C no labeling is observed. In D the short arrow points to the sperm nucleus and long arrows to the haploid maternal meiotic products. (D′,D″) Enlarged images of the sperm nucleus and maternal chromosomes where it is seen that H4Ac12-labelling localizes to the decondensing sperm nucleus chromatin and not to the maternal chromatin. Bar, 10 μm.

germ nuclei, paternal chromosomes are under-acetylated when they are eliminated from the male meiotic nucleus. From what is known, the cellular mechanisms established in meiotic cells to assure the discarding of all paternal chromosomes differ from those responsible for X_p chromosome elimination from germ nuclei (reviewed in Goday and Esteban, 2001). Nevertheless, an attractive hypothesis is that a common requirement for both elimination events in germline cells is that the chromosome exhibits an under-acetylation condition of histones H4 and H3.

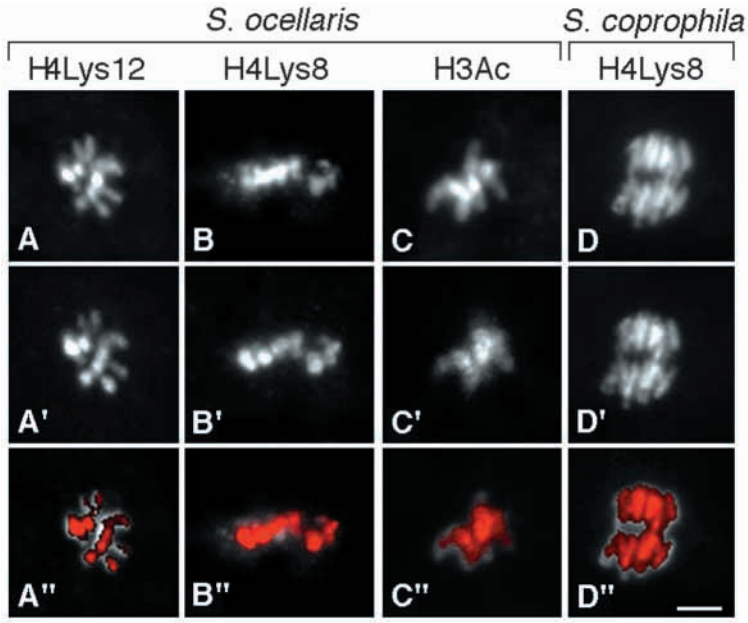

Fig. 8. Distribution of acetylated histones in *Sciara* embryos at first somatic division. DAPI staining of *S. ocellaris* first metaphase chromosomes (A-C) and of *S. coprophila* first anaphase chromosomes (D). (A′-D′) Indirect immunolabeling with the antibodies specified in the top of the figure. (A″-D″) Superimposed images where antibody staining is in red. Bar, 5 μm.

Intranuclear germline chromosome distribution and histone acetylation

Increasing experimental data currently support the existence of chromosome territories in interphase nuclei of different cell types and their relation to the spatial organization and functional nuclear processes (reviewed in Sadoni et al., 1999; Cremer and Cremer, 2001). Interestingly, intranuclear clustering of maternal and L chromosomes was frequently observed in the present work (see Fig. 5 for an example). Aggregation of chromosomes of the same parental origin and of L chromosomes was already reported for *S. coprophila* germ nuclei (Rieffel and Crouse, 1966). A plausible interpretation of these cytological data is that, in the early germ nucleus, there is a distinct localization of the maternal/paternal chromosomes and L chromosomes. In this regard, previous ultrastructural studies in *S. coprophila* indicated that in male meiotic cells, paternal and maternal chromosomal sets assume a non-random arrangement in the prophase nucleus (Kubai, 1982; Kubai, 1987). This chromosome compartmentalization was thought to account for the differential chromosome segregation that leads to paternal chromosome elimination (Kubai, 1987). This is consistent with the absence of homolog chromosome pairing and metaphase alignment in sciarid male meiosis, so that chromosomes proceed directly from prometaphase to an anaphase-like stage (reviewed in Gerbi, 1986; Goday and Esteban, 2001). Moreover and importantly, it has been suggested that the intranuclear specification of the chromosomal set domains might be already established in premeiotic germ cells and may constitute a significant requirement for the success of non-random meiotic segregation (Kubai, 1987; Goday and Esteban, 2001). The observed intranuclear chromosome aggregation in early germ nuclei at resting stage reported here gives further support to this view.

Elimination of chromosomes from male germ cells occurs during the meiotic divisions in the gall midge Cecidomyiidae (White, 1973; revised in Kloc and Zagrodzinska, 2001), which is similar to results in sciarids. Interestingly, ultrastructural studies in *Monarthropaplus buxi* have shown that non-eliminated chromosomes and eliminated chromosomes are separated in the spermatogonium nucleus by a complex system of intranuclear lamellae (Jazdowska-Zagrodzinska and Matuszewski, 1978). The spatial compartmentalization of the two sets of chromosomes was also seen in the oocytes that lack chromosome elimination. It will be interesting to determine if such structures are also seen in *Sciara*.

Differences in histone acetylation between parental chromosome sets were found here both in early germ nuclei as well as during the first male meiotic division. If *Sciara* chromosome sets (and L chromosomes) occupy distinct nuclear compartments in these two cell types, it is tempting to speculate that histone acetylation levels and a specific intranuclear arrangement of chromosomes might be linked. This hypothesis, as shown in Fig. 9, favors a model that integrates histone H4/H3 acetylation, intranuclear parental chromosome distribution and chromosome elimination in *Sciara* germ cells.

On the other hand, the results revealed a long-term maintenance of distinct global histone acetylation states between both parental genomes during early development of *Sciara* germ cells. Recent studies on the regulation of histone acetylation of chromatin during cell cycle support a role for the spatial organization of HATs and HDACs enzymes within the cell nucleus in cultured cells (Kruhlak et al., 2001). In the interphase nucleus, these enzymes locate in both chromatin and non-chromatin domains (Hendzel et al., 1998). Acetylation/deacetylation of chromatin is correlated to changes in HATs/HDAC localization and association with the chromatin. These studies support that HAT and HDAC activity is spatially regulated through the assembly into discrete subnuclear compartments (Kruhlak et al., 2001). Considering these findings, it also seems reasonable to speculate that in *Sciara* early germ nuclei, a spatial proximity, or compartmentalization, of chromosomes from the same parental origin would favor the specific recruitment and catalytic activities of HATs/HDACs.

Modifications by histone acetylation correlate to changes in the transcription, replication and packaging of DNA. Acetylation of histone H4 has been investigated in the mealybug *Planococcus citri* (Homoptera, Coccidea)

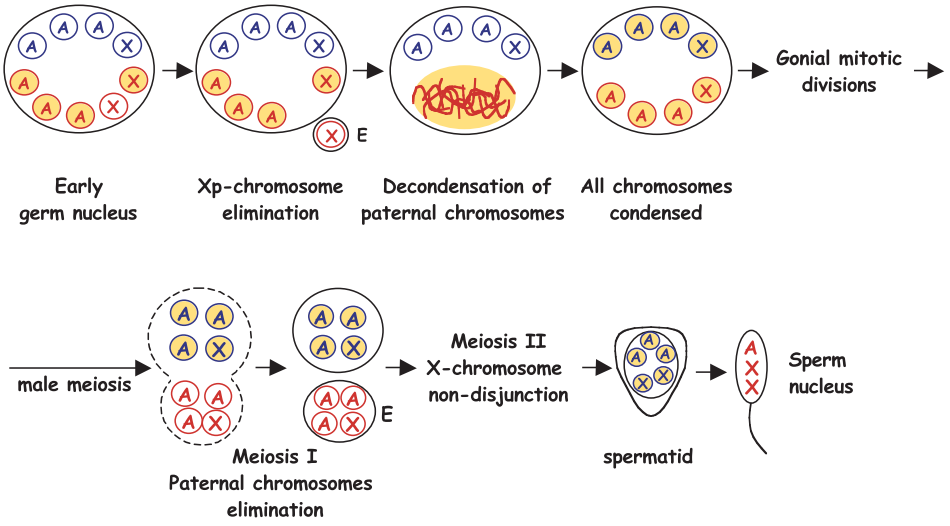

Fig. 9. Scheme summarizing the distribution of acetylated histones H4 (Lys8 and Lys12) and H3 (Lys9 and Lys14) in early germline nuclei and male meiosis of *S. ocellaris*. Maternally derived chromosomes are represented in blue and paternally derived chromosomes are represented in red. Chromosomes containing highly acetylated histones H4 and H3 are marked with yellow color, whereas chromosomes containing under-acetylated histones H4 and H3 are unmarked. E refers to chromosomal elimination events.

(Berlowitz and Pallotta, 1972; Ferraro et al., 2001), where the whole paternal chromosome set becomes heterochromatic and genetically inactive in male embryos (Brown and Wiegmann, 1969). In *P. citri* male somatic cells, differences in the levels of global histone H4 acetylation were found between both parental genomes: the paternal heterochromatic genome was hypoacetylated relative to the maternal euchromatic genome (Ferraro et al., 2001). Such differences were correlated with the differential transcriptional activity of both genomes (Ferraro et al., 2001). In *Sciara* nuclei at resting stage, the degree of chromatin compaction of chromosomes is only slightly lower than that of metaphase chromosomes (Perondini and Ribeiro, 1997). Such a high level of chromatin condensation apparently excludes transcriptional activity during this atypical nuclear stage. In this respect, by means of anti-RNA polymerase II antibodies we detected no labeling in germ nuclei at resting stage, in contrast to regular *Sciara* interphase nuclei (data not shown). The same negative result was obtained in germ nuclei undergoing decondensation of the highly acetylated paternal chromosomes (data not shown). From these data, therefore, we cannot correlate high levels of histone H4/H3 acetylation to chromatin transcriptional activity in germ nuclei at their resting stage.

Site-specific histone acetylation in *Sciara* chromatin

Histone acetylation sites show a high degree of evolutionary conservation, but important differences are known to exist in the control of histone H4 acetylation in different species (for a review, see Munks et al., 1991; Strahl and Allis, 2000). From our results, in *Sciara* germ cells chromatin, from the four possible acetylable lysine residues of histone H4 (Lys16, Lys12, Lys8 and Lys5), sites 8 and 12 are predominantly acetylated, in contrast to sites 5 and 16. A similar preferential site-specific acetylation of H4 was also found in somatic cells of early embryos (see below). However, we do not exclude the possibility that a much lower extent of H4 acetylation at lysines 5 and 16 might be undetectable by the present immunolabeling procedures. Whether there is a low level of acetylation of lysines 5 and 16, our findings strongly support the suggestion that lysine sites 8 and 12 of histone H4, are fundamental in determining the differential acetylation patterns of histone H4 in *Sciara* germline chromosomes. The same is true for lysines 9 and 14 of histone H3. We note that other possible acetylation sites of histone H3 (Lys18 and Lys23) have not been investigated in the present work.

Another interesting result to note refers to the acetylation of histones in the early developmental events that follow fertilization in *Sciara*. At late stages of sperm development, none of the antibodies for acetylated histones stain mature sperm, as already described in other systems including *Drosophila* (Rastelli and Kuroda, 1998). It is thought that this could be due to the histones being replaced by non-histone proteins. Following the sperm nucleus entrance in *Sciara* embryos, a high level of acetylated histone H4 is detectable in paternal chromatin but not in maternal chromatin. At this particular developmental stage, sperm-specific proteins are known to be replaced by histones. From our results, the acetylation of H4 in the decondensing sperm nucleus is, apparently, exclusively due to H4 lysine 12 acetylation.

Histone acetylation in somatic cells of early embryos

We examined *Sciara* embryos to explore whether a distinct distribution of acetylated histones might also occur in early somatic nuclei at the onset of embryonic development. In both *Sciara* species, following the organization of chromatin into first somatic chromosomes, highly acetylated histones H4 and H3 (at Lys12, 8 and Lys9, 14; respectively) are already detectable at first mitotic division (Fig. 8). In this respect, in mouse embryos, before zygotic genome activation, chromosomes at first mitosis show hyperacetylated H4 banding patterns (Adenot et al., 1997). Moreover, in *Sciara* a homogeneous distribution pattern of acetylated H4 and H3 was seen in the interphase nuclei of successive preblastoderm divisions (data not shown). These findings strongly suggest that in early somatic nuclei, no global differences in histone H4 and H3 are established between parental genomes. They also favor the idea that the distribution patterns of acetylated histones H4 and H3 in the chromosomes of germ nuclei are germline specific and are established during early germline differentiation.

In conclusion, we provide here the earliest evidence than covalent chromatin modifications, such as histone acetylation, can be related to the parental origin of chromosomes in germline cells of *Sciara*. In light of our data, we propose that histone acetylation contributes towards specifying the imprinted behavior of germline chromosomes in sciarids. In view of the present findings, we are currently interested in exploring other chromatin modifications, such as histone methylation, that may give further insights in the complex chromosomal system of sciarids.

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