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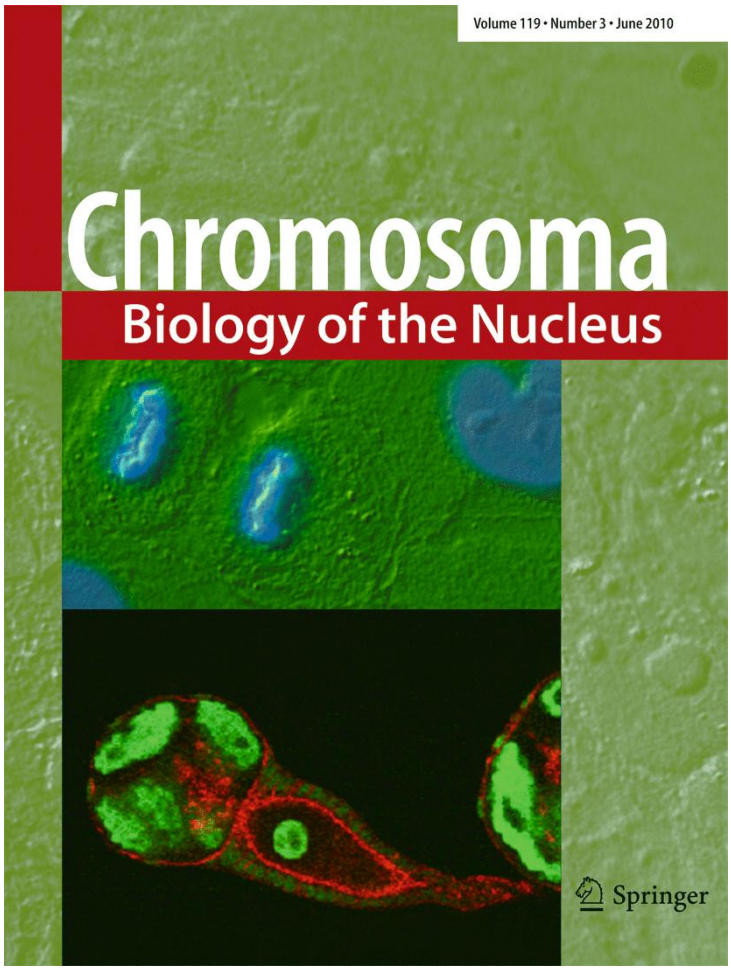

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RESEARCH ARTICLE

Heterochromatin and histone modifications in the germline-restricted chromosome of the zebra finch undergoing elimination during spermatogenesis

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Abstract In the zebra finch (*Taeniopygia guttata*) a germline-restricted chromosome (GRC) is regularly present in males and females. While the GRC is euchromatic in oocytes, in spermatocytes this chromosome is cytologically seen as entirely heterochromatic and presumably inactive. At the end of male meiosis, the GRC is eliminated from the nucleus. By immunofluorescence on microspreads, we investigated HP1 proteins and histone modifications throughout male meiotic prophase, as well as in young spermatid stages after the GRC elimination. We found that in prophase spermatocytes the GRC chromatin differs from that of the regular chromosome complement. The GRC is highly enriched in HP1β and exhibits high levels of di- and tri-methylated histone H3 at lysine 9 and tri- and di-methylated histone H4 at lysine 20. The GRC does not exhibit neither detectable levels of di- and tri-methylated histone H3 at lysine 4 nor acetylated histone H4 at lysine 5 and 8. The results prove the heterochromatic organization of the GRC in male germline and strongly suggest its transcriptional inactive state during male prophase. Following elimination, in young spermatids the GRC lacks HP1β signals but maintains high levels of methylated histone H3 at lysine 9 and methylated histone H4 at lysine 20. The release of HP1 from the GRC with respect to its elimination is discussed.

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Introduction

The programmed elimination of chromosomes during development is a remarkable biological process that is present in taxonomically scattered groups of organisms from lower animals to vertebrates. In most of them, this process occurs during the somatic/germline separation at early embryonic cleavages as well as during gametogenesis (for review see White 1973, Gerbi 1986, Goday and Esteban 2001). The loss of parts of the genome during development can be envisaged as a drastic form of chromosome silencing or inactivation.

An example of chromosome elimination in birds was first described in the zebra finch (*Taeniopygia guttata*, Passeriformes), where the elimination of a germline-restricted chromosome (GRC) occurs during early embryogenesis and in male meiosis (Pigozzi and Solari 1998). In early embryos of both sexes, the GRC is regularly discarded from all somatic cells while it is maintained in the primordial germ cells. The cellular mechanisms leading to the somatic GRC elimination still remain unknown. However, as found in other organisms undergoing chromosome elimination in the embryonic soma (see for review Goday and Esteban 2001), it is possible that the GRC might be lost due to modifications in its segregation properties at early somatic divisions. As a consequence of the somatic elimination process, a single GRC is present in the zebra finch germline cells of both sexes. Despite of it, the GRC behaves differently between males and females because it is

only transmitted through the females since it is invariably discarded in males at the time of the meiotic divisions (Pigozzi and Solari 2005). While in the spermatocytes the GRC is a univalent with the associated chromatin densely packed during meiotic prophase, in the oocytes, the GRC is euchromatic, recombines and forms a bivalent (Pigozzi and Solari 1998 and 2005). As reported, the GRC recombines with a copy of itself and the duplication of this chromosome together with its recombining activity constitute features that assure a stable behavior during female meiosis and a regular maternal inheritance.

In males, the highly condensed state of the GRC chromatin together with its differential staining properties lead to the proposal that this chromosome becomes heterochromatic and transcriptionally silent in the male germline (reviewed in Pigozzi and Solari 2005).

The presence of the GRC in addition to the zebra finch regular chromosomal complement was revealed by classic cytological techniques applied to zebra finch germline cells (Pigozzi and Solari 1998). From these studies, it emerged that from early stages of prophase spermatocytes (leptozygotene) a prominent heteropycnotic chromatin body is present, reaching its highest degree of condensation at pachytene where it morphologically resembles the XY body of mammalian spermatocytes (Pigozzi and Solari 1998). At metaphase I, the GRC appears as a large univalent along with the regular chromosomal complement whereas at methaphase II, the GRC is seen as a round and densely packed chromatin body associated to about one half of metaphases II. Finally, the GRC is visualized as a DAPI bright round body in the perinuclear cytoplasm of the secondary spermatocytes indicating that its elimination occurs during the meiotic divisions (Pigozzi and Solari 1998). Electron microscopy of thin-sectioned seminiferous tubules show that the GRC expelled from the nucleus is present as a compact chromatin body in the cytoplasm of secondary spermatocytes and young spermatids (Pigozzi and Solari 2005). These evidences are indicative that the GRC divides reductionally during the first division and it is excluded from the nucleus during the reformation of the nuclear membrane after the first anaphase. A schematic representation of the GRC behavior during male meiosis is shown in Fig 1.

Further valuable information on the peculiar GRC behavior during male meiosis was achieved by immunodetection of the proteinic meiotic axes present at first meiotic prophase. Unlike in females, where the GRC forms a regular synaptonemal complex, in males the GRC is represented by a single axis that contains the cohesin subunit SMC3 with its associated chromatin densely packed until late pachytene (Pigozzi and Solari 2005).

The functional role of the GRC in the female germline has yet to be established. On the other hand, since in the male germline the GRC exhibits heterochromatic behavior from early meiotic prophase, it was assumed that this chromosome is dispensable for spermatogenesis. Importantly, the functional inactivation of the GRC in males might prevent meiotic abnormalities caused by a single unpaired element (Pigozzi and Solari 2005). Despite of it, it has not been ruled out that prior its condensation and ultimate elimination, the GRC could also have a functional meaning in the male germline (Itoh et al. 2009). Regarding its molecular organization, a first GRC-specific DNA sequence, of repetitive nature, has been recently identified and found to distribute across a large area of the chromosome (Itoh et al. 2009).

The cellular mechanisms involved in the processes of GRC inactivation and subsequent elimination at late spermatogenesis remain poorly understood. Though, a most relevant feature is that from early meiotic prophase the GRC undergoes major structural chromatin modifications with respect to the rest of the chromosomes, as evidenced by cytological and ultrastructural analyses (Pigozzi and Solari 2005). To date, little is known about the differential chromatin modifications that underlie the high condensation and heterochromatic appearance of this chromosome before it is eliminated from the male germline.

In eukaryote organisms, it is well known that heterochromatin contains conserved proteins belonging to the heterochromatin protein 1 (HP1) family and that such proteins associate to chromatin by interacting specifically with methylated histone H3 at lysine 9 (James and Elgin 1986; reviewed in Grewal and Jia 2007). Studies on the mechanisms of HP1 recruitment to heterochromatin demonstrated that HP1 selectively binds, through the amino chromodomain, nucleosomes containing di- or trimethylated H3K9 (Bannister et al. 2001; Lachner et al. 2001; Fischle et al. 2005). The interactions between methylated histone H3 and HP1 proteins are thought to be crucial for establishing and maintaining heterochromatic domains.

In higher eukaryotes, members of the HP1 family, represented by isoforms α, β, and γ, are major components of constitutive heterocromatin and some forms of facultative, developmentally regulated, heterochromatin (Singh and Georgatos 2002; Li et al. 2002). Although all HP isoforms associate predominantly to pericentromeric heterochromatin, HP1β and HP1γ can also be found at euchromatic domains where they are thought to be involved in gene repression (for review see Grewal and Jia 2007). To our knowledge, the presence of HP1 proteins in avian cells was reported in chicken TD40 cells and in nucleated chicken erythrocytes (Gilbert et al. 2003).

Post-translational modifications of histones constitute an important regulatory mechanism of the chromatin structure. In addition to methylated H3K9 that is considered a hallmark of heterochromatin, other post-translational

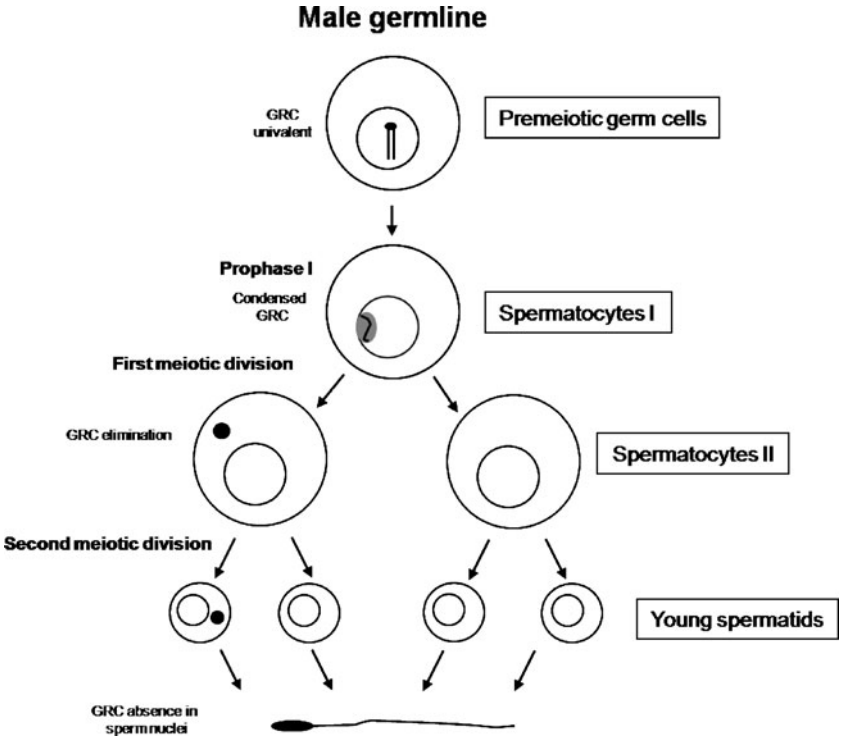

Fig. 1 Schematic representation summarizing the behavior of the germline-restricted chromosome (GRC) in male germ cells

histone modifications such as methylation of histone H4 at lysine 20 have been described as repressive chromatin marks present in highly condensed chromatin and heterochromatic domains (Nishioka et al. 2002; Schotta et al. 2004; Sims et al., 2006). By contrast, methylation of histone H3 at lysine 4 and global acetylation of histone H4 generally correlate with transcriptionally competent chromatin (Santos-Rosa et al. 2002; Ng et al. 2003; Shogren-Knaak et al. 2006).

To better understand the GRC chromatin organization in the zebra finch male germline, we decided to investigate the presence of heterochromatin proteins in meiotic cells. By double immunofluorescence analysis using HP1- and SMC3-antibodies on meiotic spreads, we describe the distribution of an HP1β antigen that differentially associates to the GRC univalent prior to its elimination from the spermatocyte nucleus. In view of this finding, we have explored covalent histone modifications in male meiotic cells and found that the GRC is highly enriched in di- and tri-methylated H3 at lysine 9 (H3K9me2 and H3K9me3, respectively) as well as in di- and tri-methylated histone H4 at lysine 20 (H4K20me2 and H4K20me3, respectively). We also investigated the distribution of di- and tri-methylated histone H3 at lysine 4 (H3K4me2 and H3K4me3, respectively) plus acetylated histone H4 at lysine 5, 8, 12, and 16 (H4K5ac, H4K8ac, H4K12ac, and H4K16ac, respectively) and found that such histone modifications are detectable in the regular chromosomes of the complement, but not in the GRC. From our observations, during male meiotic prophase the GRC is highly enriched in HP1β and in histone modifications essential for HP1 deposition and that constitute known marks of repressive chromatin status. These findings strongly support the heterochromatic conformation of the GRC in zebra finch male germline and in addition, are consistent with the functional inactivate state of this chromosome during gametogenesis. We also report the absence of HP1β labeling in the discarded GRC at the spermatocyte stages and discuss this finding with respect to histone modifications and GRC elimination during meiotic divisions.

Materials and methods

Meiotic spreads preparation and fixation

Meiotic spreads were done from adult male zebra finches, *T. guttata*. Testes were dissected out, placed into Hanks' balanced salt solution, and released from the tunica albuginea. Tubules were treated with a hypotonic extraction buffer for 30 min, and then gently disaggregated in 100 mM sucrose at pH8.5 to give a homogeneous cell suspension. Amounts of about 30 ml of this suspension were dropped onto glass slides covered with a thin layer of 1% PFA, 0.1% Triton X-100. The slides were allowed to dry for about 1 h in a humid chamber and then were washed in 0.4% Photoflo. The spreads were used immediately for FISH and immunostaining or alternatively were kept at −70°C for later use.

Immunostaining and microscopy

After fixation, slides were washed in PBS (3×10 min) and then in PBS containing1% Triton X-100 for 10 min. They were then incubated with 2% BSA for 1 h at room temperature. The primary antibodies were mammalian anti-SMC3 (Chemicon); CREST serum (The binding site, UK) anti-HP1β (Abcam); anti-HP1α (Millipore); anti-H3K9me, anti-H3K9me2, anti-H3K9me3, anti-H4K20me2, anti-H4K20me3 (Abcam); anti-H3K4me2, anti-H3K4me3, anti-H4K5ac, anti- anti-H4K8ac, anti-H4K12ac, anti-H4K16ac (Upstate Biotechnology); anti-PH3(Ser10) (Millipore); anti-γH2AX (Millipore). Secondary antibodies were Texas Red-conjugated anti-human, FITC- and Cy3-conjugated anti-rabbit (Jackson ImmunoResearch), FITC-conjugated anti-mouse (Southern Biochtechnology), and Cy3-conjugated anti-rat (Jackson ImmunoResearch). 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 and 1:600 for Cy3-conjugated antibodies. Incubation of primary antibody was at 4°C overnight followed by washing in PBS 3 × 10 min. Incubation of secondary antibody was at room temperature for at least 1 h 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. Images were processed using Adobe Photoshop program. All the observations after the immunostaining were performed in more than 50 meiotic cells for each antibody.

Results

The zebra finch has a diploid number of 80 chromosomes including seven pairs of macrochromosomes, 32 pairs of microchromosomes and the sex chromosomes ZZ/ZW in males and females, respectively. In addition to the regular chromosomal complement, a single accessory chromosome (GRC) can be recognized in spermatogonial metaphases as the largest chromosome in the set (Pigozzi and Solari 1998). In agreement with these observations, as shown in Fig. 2, the largest axis observed in synaptonemal complex spreads from pachytene spermatocytes corresponds to the GRC univalent.

HP1β localization in male germline cells

To investigate HP1 proteins in zebra finch meiotic spreads, we employed antibodies against mammalian HP1β and HP1α, known to recognize the HP1 isoforms in avian

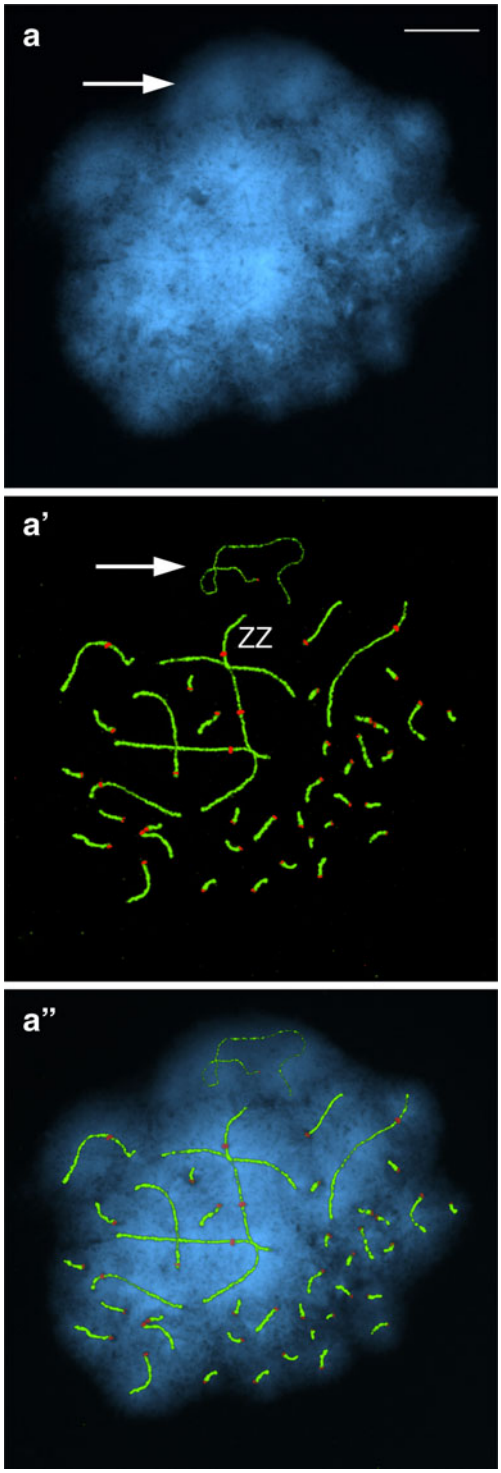

Fig. 2 Double immunofluorescence labeling of a pachytene spermatocyte with SMC3 antibody (*green*) to identify the meiotic axes, a CREST serum (*red*) to identify the centromeres and chromatin DAPI staining (*blue*). The single axis corresponding to the GRC (*arrow*) can be clearly observed along with the complete set of autosomal bivalents and the ZZ bivalent. *Bar* indicates 10 μm

chicken cells (Gilbert et al. 2003). We first performed double immunostaining with HP1β- and SMC3-antibodies in male germ cells (Fig. 3). In all prophase nuclei of spermatocytes from leptotene to diplotene stages, HP1β antibody clearly decorates the chromatin corresponding to the GRC univalent while no labeling was detected in the rest of chromosomes. Fig. 3A, B shows HP1β in a leptotene and a pachytene nuclei. It appears, therefore, that in zebra finch spermatocytes the GRC chromatin is specifically enriched in HP1 antigens recognized by HP1β antibody. This result strongly suggests the presence of an HP1β avian homologue in the GRC and is consistent with previous data supporting the heterochromatic nature of such chromosome in male germ cells (Pigozzi and Solari 1998; Pigozzi and Solari 2005; Itoh et al. 2009). Moreover and importantly, in young spermatids, where it is possible to visualize the discarded GRC as a DAPI bright chromatin body closely associated to the spermatid nucleus, HP1β labeling was no longer detected (Fig. 3C–C").

A similar analysis was performed with an HP1α antibody in germline male cells prior to the GRC elimination and from our results, all chromosomes in the

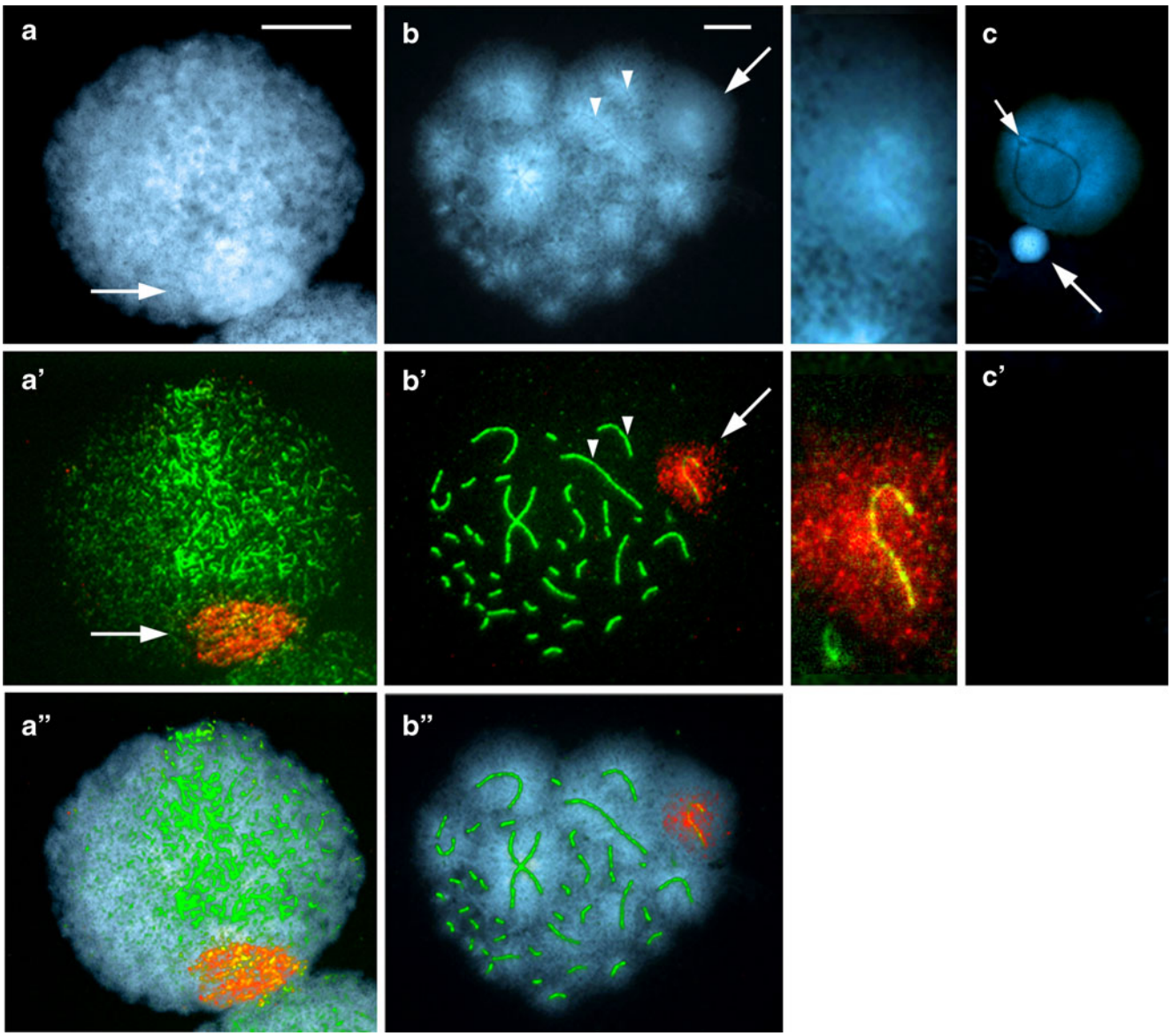

Fig. 3 Double immunofluorescence labeling with anti-HP1β (*red*) and SMC3 (*green*) antibodies in male germ cells counterstained with DAPI (*blue*). Prophase spermatocytes at leptotene (*A–A"*), pachytene (*B–B"*) stages and a young spermatid after GRC elimination (*C*, *C'*). The nuclear region containing the GRC is strongly labeled with the HP1β antibody (*arrows* in *A*, *A'*, *B*, *B'*). At pachytene it is possible to observe that the HP1 staining is restricted to the chromatin associated to the GRC single axis (*inset* in *B*, *B'*).The cohesin axes of pachytene bivalents appear as DAPI negative lines (*arrowheads* in *B*, *B'*). In young spermatids, the eliminated GCR (*arrow* in *C*) is devoid of HP1 staining (*C'*). The *small arrow* in *C* points at the implantation fossa of the developing flagellum. *Bars* indicate 10 μm

spermatocyte nucleus are devoid of labeling (data not shown).

Histones H3 and H4 modifications in male germline cells

Given that the GRC is extensively marked by HP1β antibody, we next investigated methylated histone H3K9, a modification that is prominent in heterochromatin and that is crucial for HP1 deposition (reviewed in Ebert et al. 2006; Grewal and Jia 2007). As shown in Fig. 4A–A", double immunostaining with H3K9me3 and SMC3 antibodies in leptotene and pachytene spermatocytes revealed that the entire GRC chromatin exhibits H3K9me3 labeling. This staining pattern was consistent in every examined spermatocyte, from early until late meiotic prophase stages, including diplotene (not shown). By contrast, the rest of the chromosomes remain unstained and therefore, apparently under-tri-methylated at H3K9. Interestingly, and in contrast with what is observed for HP1β staining, following its elimination the GRC remains highly H3K9me3-labeled, as seen at young spermatid stage (Fig. 4B–B"). Identical results were obtained with the antibody against the di-methylated H3K9 form, whereas no staining was detected using an antibody against the mono-methylated H3K9 form (data not shown). These observations are in good agreement with the presence of HP1β in the GRC from prophase spermatocytes and in addition, strongly support the heterochromatic nature of the GRC in the male germline.

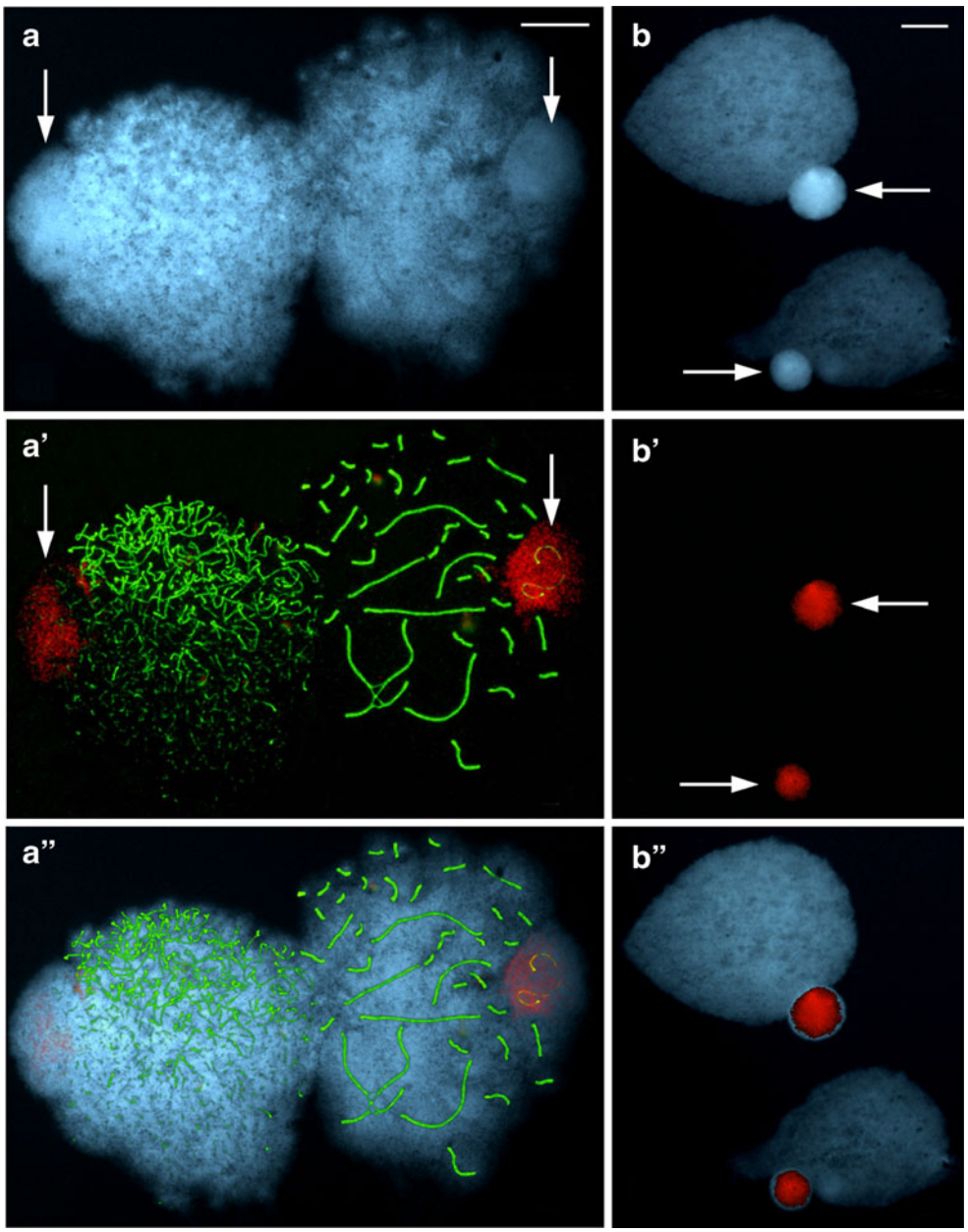

Fig. 4 Double immunofluorescence labeling with H3K9me3 (*red*) and SMC3 (*green*) antibodies in male germ cells counterstained with DAPI (*blue*). *A–A"* Spermatocytes at leptotene (*left* nucleus) and pachytene (*right* nucleus) stage showing the GRC chromatin labeled with H3K9me3 antibody (*arrows*). Notice the differential condensation of GRC chromatin with respect to the rest of the chromosomes (*arrows* in *A*). *B–B"* The eliminated GRC (*arrow*) is strongly labeled with anti-H3K9me3 antibody. The nuclei close to the GRC correspond to young spermatids (*B*). *Bar* indicates 10 μm

Since methylated histone H4K20 is another covalent histone modification that has been related to condensed and silent chromatin (Nishioka et al. 2002; Schotta et al. 2004; Sims et al. 2006) we then analyzed the presence of tri- and di-methylated H4K20 in the GRC. High levels of tri-methylated H4K20 were clearly detected on the GRC chromatin throughout all prophase stages, as shown within a pachytene spermatocyte nucleus (Fig. 5A–A") and also after its nuclear exclusion (Fig. 5B–B"). Identical results were obtained for di-methylated H4K20 (data not shown). All the above results lead us to conclude that, prior to and after its elimination from the spermatocytes, the GRC contains known marks of repressive chromatin status such as methylated H3K9 and methylated H4K20.

In many different systems, a correlation between transcriptionally competent sequences and di- and tri-methylated

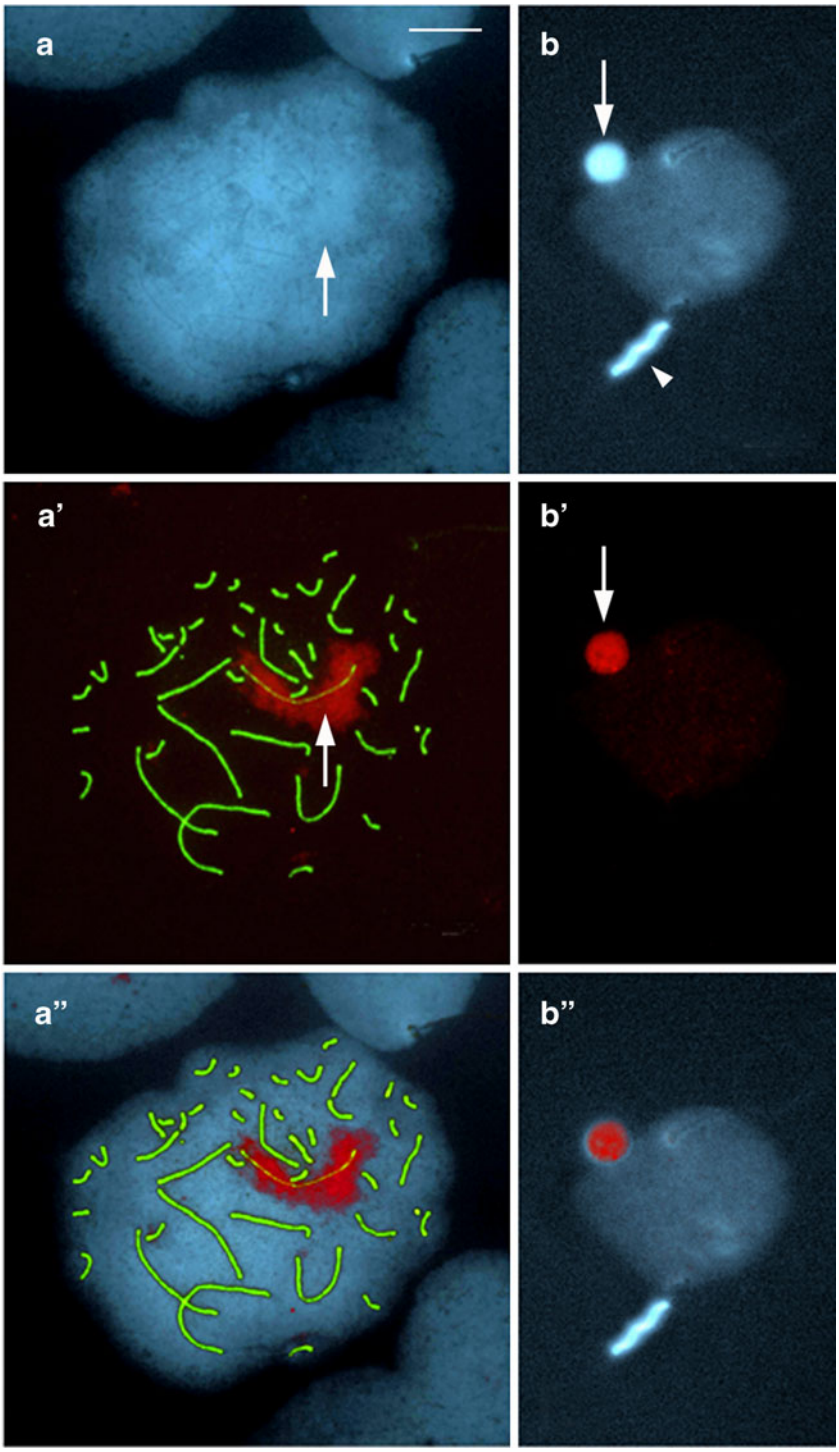

Fig. 5 Double immunofluorescence labeling with H4K20me3 (*red*) and SMC3 (*green*) antibodies in male germ cells counterstained with DAPI (*blue*). *A–A'* Pachytene spermatocyte showing the GRC chromatin labeled with H4K20me3 antibody (*arrow*). *B–B"* The eliminated GRC (*arrow*) is strongly labeled, in contrast to the corresponding young spermatid and a sperm head (*arrowhead*) that are devoid of H4K20me3 signals. *Bar* indicates 10 μm

H3K4 has been well established (Santos-Rosa et al. 2002; Ng et al. 2003). To further investigate chromatin differences between the GRC and the rest of the chromosomes in male meiotic cells, we analyzed the distribution of H3K4me2 and H3K4me3 modifications before and after the process of GRC elimination (Fig. 6). Both in pachytene spermatocytes (Fig. 6A–A") and in young spermatids (Fig. 6B–B"), the H3K4me2 antibody associates to intranuclear discrete foci that evidently do not correspond to the GRC chromatin. Moreover, during prophase I it is possible to observe that the H3K4me2 signals mostly distribute to intranuclear regions that exhibit less condensed DNA, as seen with DAPI staining (arrowheads in Fig. 6A). Identical results were obtained with respect to the distribution of tri-methylated H3K4 (data not shown). These results could be indicative of a lack of transcriptional competency of the GRC at the analyzed meiotic stages, i.e., from leptotene to diplotene. In view of it, and considering that global histone H4 hyper-acetylation is generally absent in heterochromatic domains, we also explored the presence in the GRC of acetylated H4 at lysines 5, 8, 12, and 16. As shown in Fig. 7 both H4K5ac and H4K8ac antibodies recognize multiple discrete sites

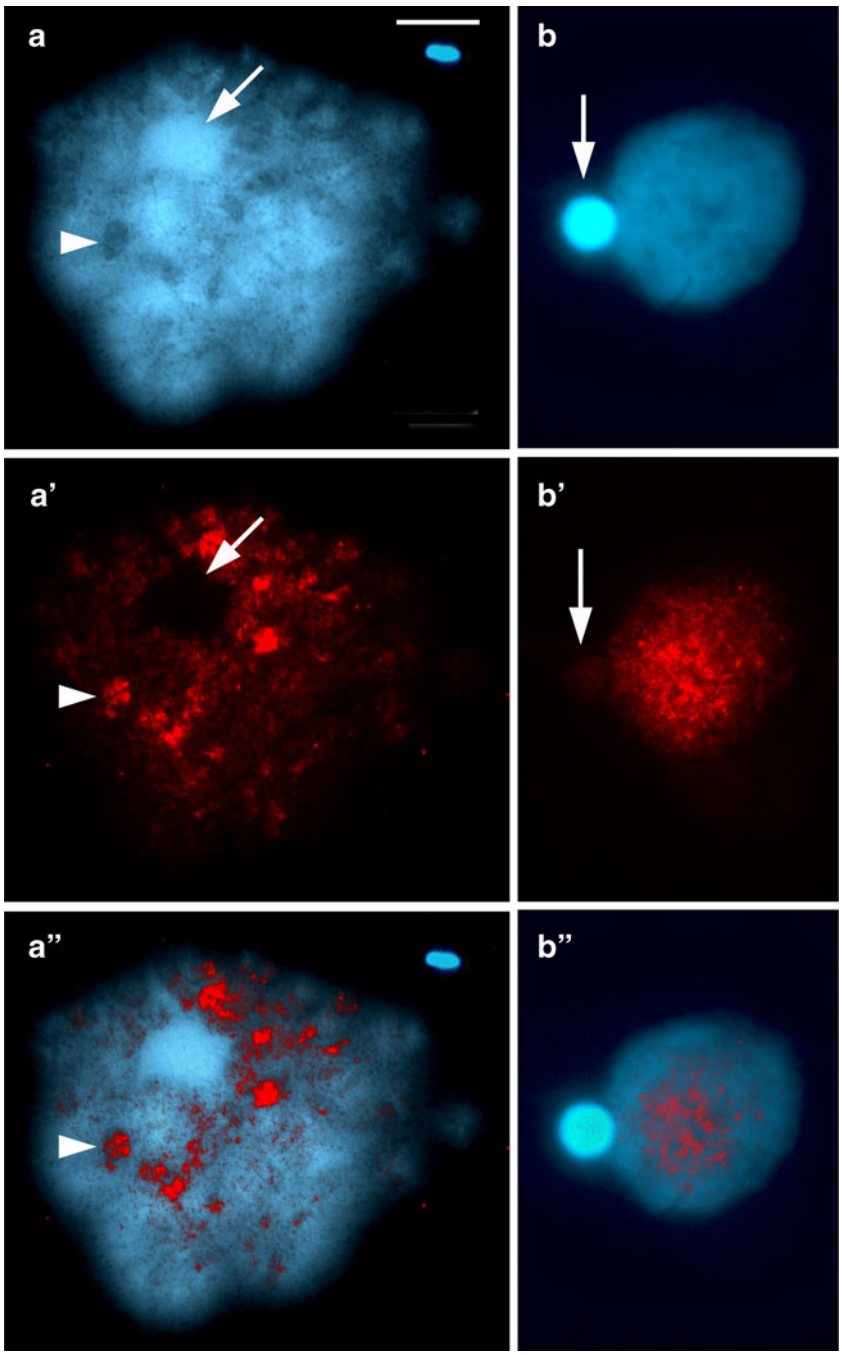

Fig. 6 Indirect immunolabeling with H3K4me2 antibody (*red*) in male germ cells counter-stained with DAPI (*blue*). *A–A"* A pachytene spermatocyte showing that the GRC chromatin is devoid of H3K4me2 staining (*arrows*). Bright H3K4me2 signals are seen at discrete foci that correspond to intranuclear regions apparently containing less condensed DNA, as seen with the DAPI staining (*arrowheads*). *B–B"* A young spermatid showing discrete H3K4me2 labeling within the nucleus, whereas the eliminated GRC (*arrow*) is devoid of staining. *Bar* indicates 10 μm

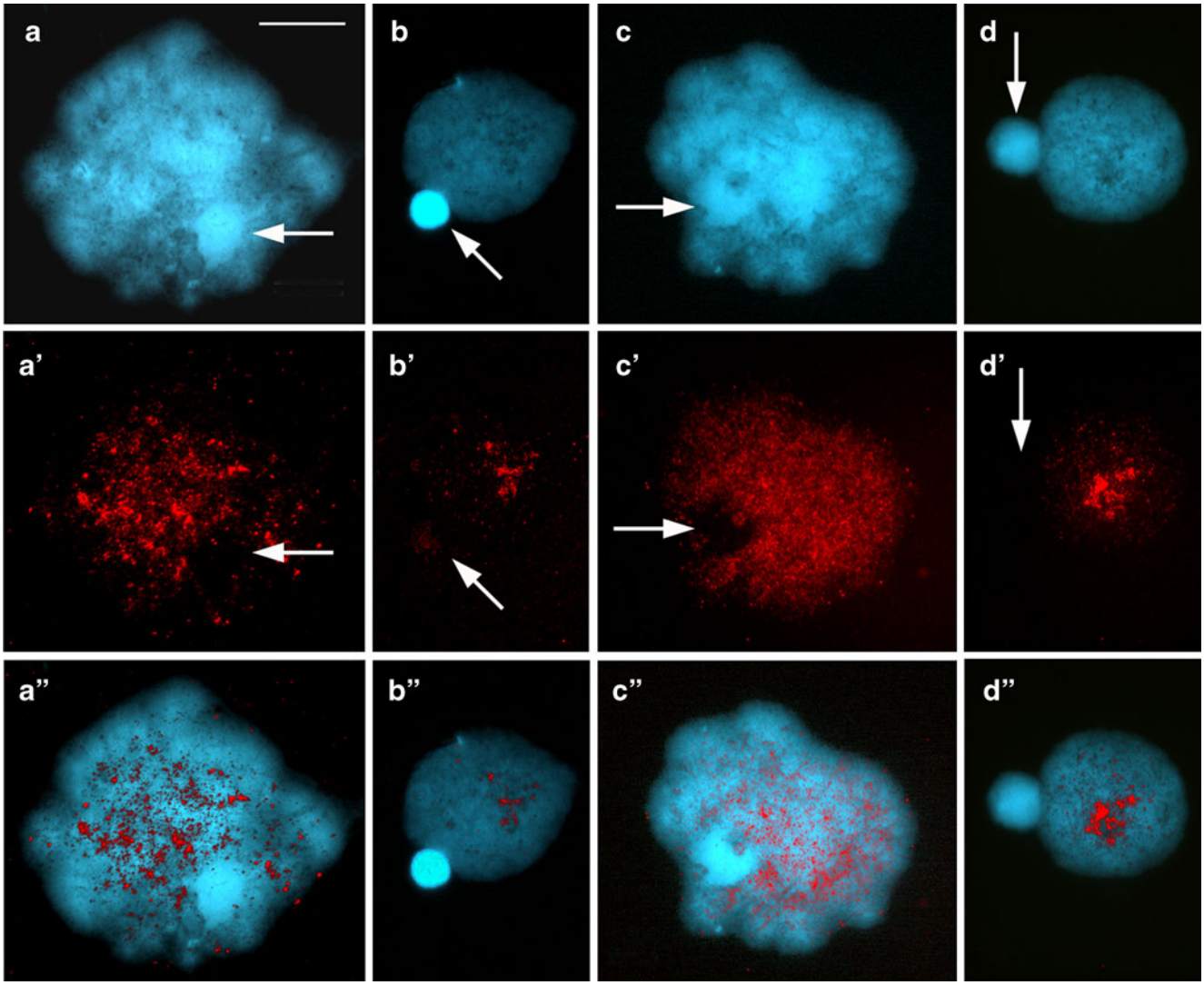

Fig. 7 Indirect immunolabeling with H4K5ac and H4K8ac antibodies (*red*) in male germ cells counterstained with DAPI (*blue*). In pachytene spermatocytes, both H4K5ac (*A'*, *A''*) and H4K8ac (*C'*, *C''*) signals are seen at discrete multiple foci within the nuclei except at the GRC chromatin that lacks H4K5ac and H4K8ac staining (*arrows* in *A*, *A'*, *C*, *C'*). In young spermatids, H4K5ac (*B'*, *B''*) and H4K8ac (*D'*, *D''*) antibodies reveal intranuclear punctuate staining while no staining is observed in the eliminated GRC (*arrow*). *Bar* indicates 10 μm

within the nuclei in pachytene spermatocytes and young spermatids. Importantly, for both antibodies, the GRC chromatin is devoid of labeling, prior and after its elimination. On the other hand, no significant levels of H4K12ac and H4K16ac were detected in this meiotic cell types. All the above results lead us to conclude that, at the analysed stages, the GRC lacks histone modifications, such as methylated H3K4 and acetylated H4, generally associated to transcriptionally active chromatin.

Histone H3S10 phosphorylation

Finally, we have also analyzed the pattern of histone H3 phosphorylated at serine 10 (PH3Ser10) that constitute a known marker of condensed chromosomes undergoing mitotic and meiotic divisions (reviewed in Ito 2007). As shown in Fig. 8B", B"', the PH3Ser10 antibody recognizes the GRC once eliminated but not during meiotic prophase stages (Fig. 8A–A"').

Discussion

From our immunodetection analysis we show that in zebra finch male germline the GRC chromatin differs from that of the regular chromosomal component, as evidenced by the specific association of HP1β antibody to the GRC univalent in prophase nuclei. Besides HP1β, the GRC is

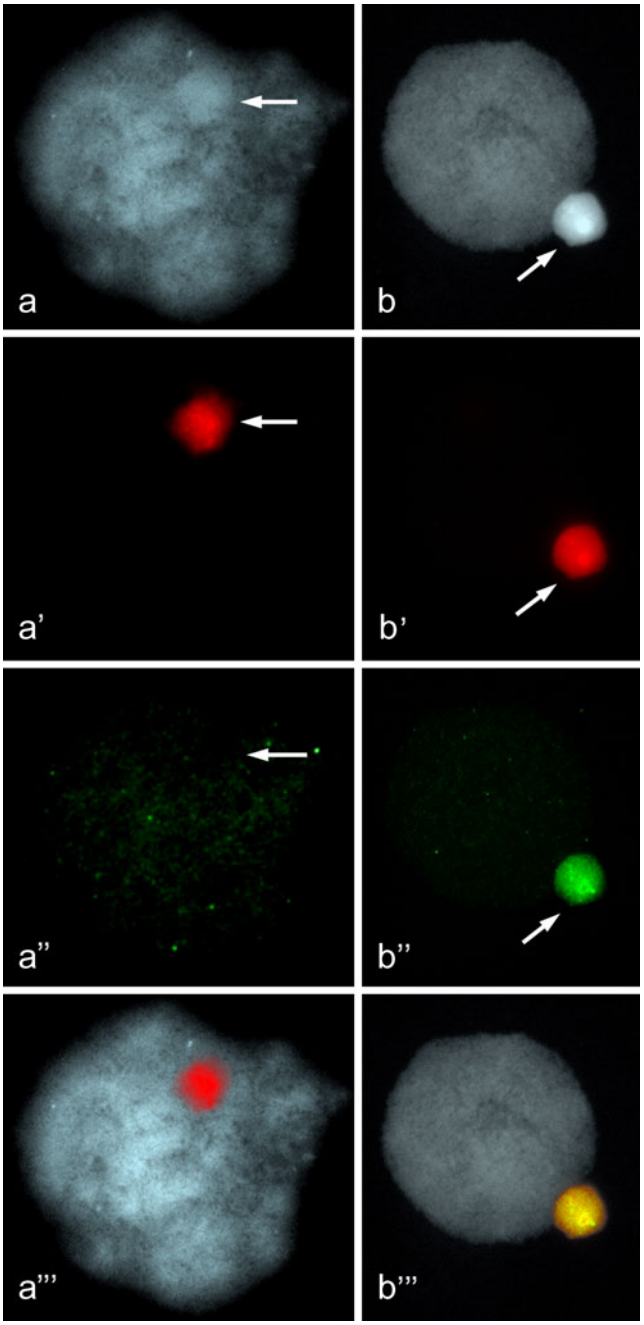

Fig. 8 Double immunofluorescence labeling with H4K20me2 (*red*) and PH3S10 (*green*) antibodies in male germ cells counterstained with DAPI (*blue*). *A-A'''* Pachytene spermatocyte showing that the GRC chromatin (*arrows*) exhibits H4K20me2 labeling (*A'*) and is devoid of PH3S10 staining (*A''*). *B–B'''* Young spermatid with the corresponding eliminated GRC showing that both H4K20me2 and PH3S10 antibodies label the GRC. *Bars* indicate 10 μm

also highly enriched in both H3K9me2 and H3K9me3 modifications during the meiotic prophase. Both results are in good agreement with data coming from other organisms where such post-translational histone modifications are essential for the recruitment of HP1 proteins in heterochromatic domains (reviewed in Grewal and Jia 2007). From our observations, moreover, the condensed state of the GRC at male prophase is also distinguished by hypermethylation of H4K20, a histone modification present in condensed and heterochromatic chromosomal domains (Nishioka et al. 2002; Schotta et al. 2004; Sims et al. 2006). Another significant observation is that H3K4 methylation and H4 acetylation, histone modifications correlated to transcriptionally active chromatin (Santos-Rosa et al., 2002; Ng et al. 2003; reviewed in MacDonald and Howe, 2009) were not detected in the GRC during male prophase. Therefore, the absence of such chromatin modifications is consistent with the presence in the GRC of both methylated H3K9 and H4K20 that constitute repressive marks linked to heterochromatin formation.

In earlier zebra finch studies, the functional inactivation of the GRC in male meiosis was inferred after cytological observations showing the high chromatin compactness and heteropycnotic staining properties of this chromosome (Pigozzi and Solari 1998). The present results, using an immunocytochemical approach in male meiotic cells, lead us to prove the heterochromatic organization of the GRC and to support its transcriptional inactive state prior to its elimination.

The morphological conformation of the GRC at male pachytene stage in zebra finch resembles that of the transcriptionally inactive XY body of mammalian spermatocytes (Pigozzi and Solari 1998). Interestingly, in human late pachytene spermatocytes the whole XY body is decorated by HP1β and HP1γ isoforms and it has been suggested the involvement of such proteins in either increasing the XY body chromatin condensation or in stabilizing its heterochromatic state (Metzler-Guillemain et al. 2003). Moreover, HP1β isoform has been also found to concentrate, among other proteins, in the murine XY body in late pachytene and diplotene spermatocytes (Motzkus et al. 1999; Turner et al. 2001) where H3K9me3 has been observed (Cowell et al. 2002) but not H4K20me3 (Kourmouli et al. 2004). However, in contrast with observations in the XY body, the repressive marks that coat the GRC are evident from early meiotic prophase. As shown here, the chromatin condensation and the appearance of repressive marks on the GRC are already evident at the leptotene stage, unlike XY body formation and meiotic sex chromosome inactivation (MSCI) that occurs immediately as spermatocytes enter pachytene (Solari 1974; reviewed in Turner 2007). In mammals, MSCI is triggered by the phosphorylated form of the histone variant H2AX (γH2AX). It has been shown that γH2AX is within the XY chromatin domain just prior to MSCI, indicating that the chromatin condensation and transcriptional repression are triggered (Mahadevaiah et al. 2001; Turner et al. 2004). In zebra finch spermatocytes, γH2AX labeling can be observed within all the chromatin from leptotene, indicating

the presence of double strand breaks (DSBs) that initiate meiotic crossing over in many organisms (Keeney 2001). During leptotene, the chromatin associated to the GRC shows low levels of γH2AX (Suppl. Fig. 1A–A") and from late zygotene, signals are no longer detectable on the GRC, even though they are still visible mainly on unsynapsed regions of the regular chromosome set (Suppl. Fig. 1B–B"'). In spite of the different dynamics of H2AX phosphorylation during meiotic prophase between the GRC and the mammalian XY body, it cannot be ruled out that H2AX phosphorylation on the GRC at leptotene may have a role in the process of chromatin condensation achieved by additional epigenetic modifications and acquisition of heterochomatin proteins. On the other hand, after its elimination, GRC is heavily labeled with γH2AX (Suppl. Fig. 1C–C"). In mammals, ATM kinase is required for the chromatin-wide phosphorylation of H2AX occurring in leptotene spermatocytes in response to DSBs, while ATR kinase is more likely involved in H2AX phosphorylation in the sex body (Turner et al. 2004; Bellani et al., 2005). Whether, the early phosphorylation of H2AX in zebra finch spermatocytes and its late appearance on the eliminated GRC in the zebra finch also respond to different kinases remain to be established.

Another interesting observation is that following GRC elimination, in second spermatocytes where the GRC is seen as a DAPI bright and round compact body, HP1β labeling is no longer detected. By contrast, hypermethylation of H3K9 and H4K20 signals are very evident in the expelled GRC body. These observations exclude that the compactness of GRC body makes it inaccessible to the antibodies and instead, favor that the loss of HP1β staining correlates with the GRC nuclear exclusion at the time of meiotic divisions. In this respect, it is thinkable that potential modifications in the HP1β epitope could occur or alternatively, the removal of HP1β from the GRC. This latter possibility seems most plausible in view that the release of HP1 proteins from chromatin without major changes in the levels of histone H3K9 methylation has already been demonstrated in different systems. For instance, in mouse fibroblasts endogenous HP1 is lost from DAPI-stained pericentric heterochromatin without the occurrence of alterations in the levels of methylated H3K9 (Mateos-Langerak et al. 2007). Moreover, during terminal differentiation in nucleated chicken erythrocytes, HP1 proteins are totally removed from and replaced by specific condensing factors whereas methylated H3K9 levels experiment only a slight reduction (Gilbert et al. 2003). Furthermore, in mammalian cell cycle studies it has been shown that during mitosis, while the levels of trimethylated H3K9 remain unchanged, the three HP1 isoforms are released from heterochromatin (Minc et al. 1999; Fischle et al. 2005). The dissociation of HP1 is specifically caused by the phosphorylation of histone H3 at serine 10 (H3S10) in nucleosomes during G2/M-phase transition and it is reversed at the end of mitosis, when histone H3 dephosphorylates and HP1 re-associates with chromatin (Fischle et al. 2005; Hirota et al. 2005; Terada 2006).

One of the main conserved functions of histone H3S10 phosphorylation is to regulate chromatin condensation during mitosis and meiosis (reviewed in Ito 2007). The meiotic G2/MI transition involves disassembly of the SC structure, phosphorylation of histone H3, and remodeling of chromatin leading to the condensation of bivalent chromosomes (reviewed in Sun and Handel 2008). As shown here, following the first meiotic division, the discarded condensed GRC exhibits high levels of histone H3S10 phosphorylation in addition to hypermethylated H3K9 and H4K20. Taking into consideration the reported data together with our results, it is conceivable that HP1β could be released from the GRC at the onset of the first division at the time that chromosomes undergo global H3S10 phosphorylation. Further analyses using different methodological approaches are indeed needed to next investigate HP1 proteins and histone modifications during male zebra finch meiotic divisions. Despite of it, an attractive hypothesis is that the loss of HP1β, together with the maintenance of methylated H3K9/H4K20 and phosphorylated H3S10 could represent specific chromatin modifications involved in the GRC elimination process in zebra finch males.

In view of the present findings, we are currently interested in investigating chromatin modifications during GRC elimination from the soma at early embryogenesis in zebra finch of both sexes.

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