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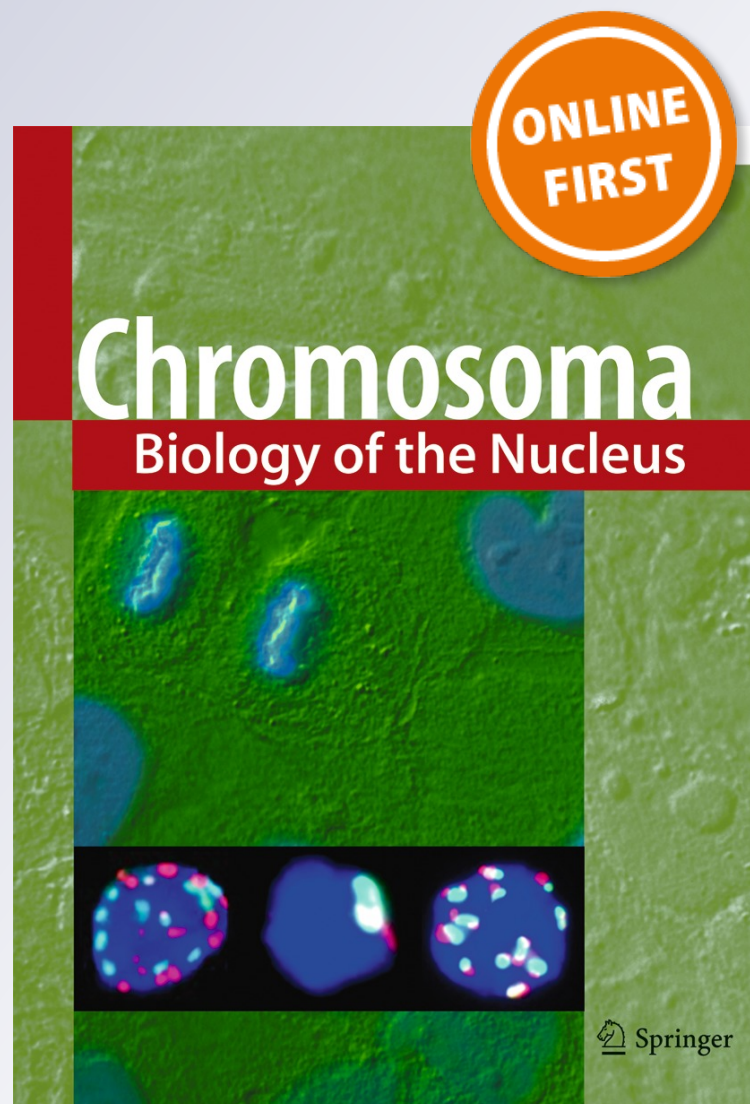
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RESEARCH ARTICLE

Protein markers of synaptic behavior and chromatin remodeling of the neo-*XY* body in phyllostomid bats

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Abstract The *XX*/*XY* system is the rule among mammals. However, many exceptions from this general pattern have been discovered since the last decades. One of these non-conventional sex chromosome mechanisms is the *multiple sex chromosome system*, which is evolutionary fixed among many bat species of the family *Phyllostomidae*, and has arisen by a translocation between one original gonosome (*X* or *Y* chromosome), and an autosome, giving rise to a "neo-*XY* body." The aim of this work is to study the synaptic behavior and the chromatin remodeling of multiple sex chromosomes in different species of phyllostomid bats using electron microscopy and molecular markers. Testicular tissues from adult males of the species *Artibeus lituratus*, *Artibeus planirostris*, *Uroderma bilobatum*, and *Vampyrodes caraccioli* from the eastern Amazonia were analyzed by optical/electron microscopy and immunofluorescence of meiotic proteins involved in synapsis (SYCP3 and SYCE3), sister-chromatid cohesion (SMC3), and chromatin silencing (BRCA1, γ-H2AX, and RNApol 2). The presence of asynaptic axes—labeled by BRCA1 and γ-H2AX—at meiotic prophase in testes that have a normal development of spermatogenesis, suggests that the basic mechanism that arrests spreading of transcriptional silencing (meiotic sex chromosome inactivation (MSCI)) to the autosomal segments may be per se the formation of a functional synaptonemal complex between homologous or non-homologous regions, and thus, this SC barrier might be probably related to the preservation of fertility in these systems.

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Introduction

The usual sex chromosome system in mammals is *XX*/*XY* (reviewed in Solari 1994), the males being those who have the sex chromosomes *X* and *Y* and the females being homogametic *XX* (with a few exceptions; Solari 1994). In male meiotic cells, the *X* and *Y* chromosomes synapse partially and form a condensed chromatin body termed the "*XY* body" (reviewed in Solari 1994). Functionally, the *XY* body represents a transcriptionally silenced chromatin domain at male meiotic prophase that remains silenced even at later stages, when spermatid elongation occurs (reviewed in Handel 2004 and in Turner 2007).

Even as the *XX*/*XY* system is the rule among mammals, several exceptions from this general pattern have been discovered since five decades ago (reviewed in Fredga 1970). One instance is the *multiple sex chromosome system* which is widely spread in different eutherian and metatherian mammals (mammals will be dealt here, as they share the specific meiotic sex chromosome inactivation (MSCI) (see below) differently from other vertebrates and invertebrate sex systems). Thus, multiple sex chromosomes are present in marsupials (Sharp 1982), rodents (Ratomponirina et al. 1986), primates (Solari and Rahn 2005), and gazelles (Kingswood et al. 1994).

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Particularly, the presence of a multiple sex chromosome system among many bat species of the family *Phyllostomidae* is well known (Hsu et al. 1968; Beçak et al. 1969; Yonenaga et al. 1969; Baker 1973; Baker et al. 1979; Kasahara and Dutrillaux 1983; Tucker and Bickham 1986; Solari and Pigozzi 1994; Noronha et al. 2001, 2004). This kind of sex chromosome mechanism has arisen by a translocation between one original gonosome, either the X or the Y chromosome, and an autosome, giving rise to a trivalent XY_1Y_2 (Solari and Pigozzi 1994) or to a closed bivalent called "neo-X and neo-Y" (Noronha et al. 2009). As a marker of this heterogeneous origin, Kasahara and Dutrillaux (1983) showed that the autosomal segment of the trivalent in the phyllostomid bat *Artibeus lituratus* has early replication, even when linked to the late-replicating segment of the X chromosome.

From a functional viewpoint, the origin of these multiples can be compared to the occasional occurrence of de novo translocations in mammalian spermatocytes. For instance, in human males, it has been suggested that there is a strong association between the presence of gonosome-autosome rearrangements and the failure of spermatogenesis during meiotic prophase (reviewed in Van Assche et al. 1996; Martin 2008). However, infertility arising from de novo translocations should be differentiated from evolutionary fixed, multiple sex chromosomes that conserve fertility. Recent studies of meiotic proteins in human patients which are carriers of a variety of chromosomal rearrangements showed that the common features shared by all these cases are the abnormal localization of RAD51 and BRCA1 proteins and the presence of transcriptionally silenced γ-H2AX-labeled chromatin domains on asynaptic regions of rearranged chromosomes (Sciurano et al. 2007, 2012a). On the other hand, the presence of asynaptic segments of rearranged chromosomes—which is evolutionary fixed in many species of phyllostomids—does not affect either the normal development of spermatogenesis or their fertility.

The aim of the present work is to study the synaptic behavior and chromatin remodeling of multiple sex chromosomes in four different species of bats from the *Phyllostomidae* family and to suggest possible clues for the appearance and evolutionary permanence of these systems.

Materials and methods

Adult males of the following four different species of bats (family *Phyllostomidae* and subfamily *Sternodermatinae*) were collected from natural populations from State of Pará, Brazil, eastern Amazon region: *A. lituratus* ($n=3$), *A. planirostris* ($n=1$), *Vampyrodes caraccioli* ($n=2$), and *Uroderma bilobatum* ($n=1$). These animals were captured by specialized technicians under adequate care and deposited in the mammalian collection of the Instituto de Ciências

Biológicas, Universidade Federal do Pará, Brazil (permission no. 13248 from Instituto Chico Mendes de Conservação da Biodiversidade—ICMBio).

The testicular tissue was processed for light microscopy and electron microscopy and for immunolocalization of proteins as previously described by Sciurano and Solari (2014). Briefly, two pieces of tissue were processed for semithin (0.5 μm thick) and thin (0.08 μm thick) sections to analyze the seminiferous epithelium in detail with the light microscope and the ultrastructure of the XY body in spermatocyte nuclei by EM, respectively. A third piece of testicular tissue was used to perform spreadings of synaptonemal complex and fluorescence immunostaining techniques.

For immunolocalization of meiotic proteins, primary antibodies were used as follows: a rabbit anti-SMC3 (ab3914, Millipore Corp., USA) at 1:100, a rabbit anti-SYCP3 at 1:100 (ab15093,Abcam Ltd., UK), a rabbit anti-SYCE3 at 1:300 (Prof. M. Alsheimer, University of Würzburg, Germany), a rabbit anti-BRCA1 at 1:10 (C20, Santa Cruz Biotech, CA, USA), and human CREST serum at 1:100 (Laboratorios IFI, Buenos Aires, Argentina) were incubated at 4 °C. The other primary antibodies were incubated at 37 °C: a rabbit anti-γ-H2AX at 1:1000 (ab2893, Abcam Ltd., Cambridge, UK) and a mouse anti-RNA-Pol2 (65223, Progen Biotech, DE) at 1:100. All incubations were performed overnight in a humid chamber. After washing, the corresponding secondary antibodies were incubated for 2 h. Slides were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (0.2 μg/ml) and mounted in glycerol with 1,4-diazobicyclo-(2,2,2)-octane (DABCO) antifade. All the spermatocyte microspreads were examined using a LEICA DM microscope (Leica Microsystems, Wetzlar, Germany) and photographed with a Leica DFC 300 FX digital camera (Cambridge, UK). The separate images were superimposed using the program Adobe Photoshop CS (Adobe Systems Inc., San Jose, CA, USA).

Results

The sex trivalent XY_1Y_2 in *A. lituratus* and *A. planirostris* along the pachytene stage

The analyses of 80 pachytene spermatocyte nuclei by electron microscopy and hundreds of spermatocytes by immunofluorescence show a typical trivalent XY_1Y_2 in both species (Fig. 1). This trivalent is composed by the following three chromosomal elements: the product of the translocation between the X chromosome and the autosome (neo-X), the intact autosome-like chromosome Y_2, and the original Y_1 chromosome (Fig. 1f). These three elements form a neo-XY body with two synapsed regions of very different length and asynaptic axial regions corresponding to the differential regions of the X, Y_1, and Y_2 (Fig. 1a–e). One of the paired regions consists of a
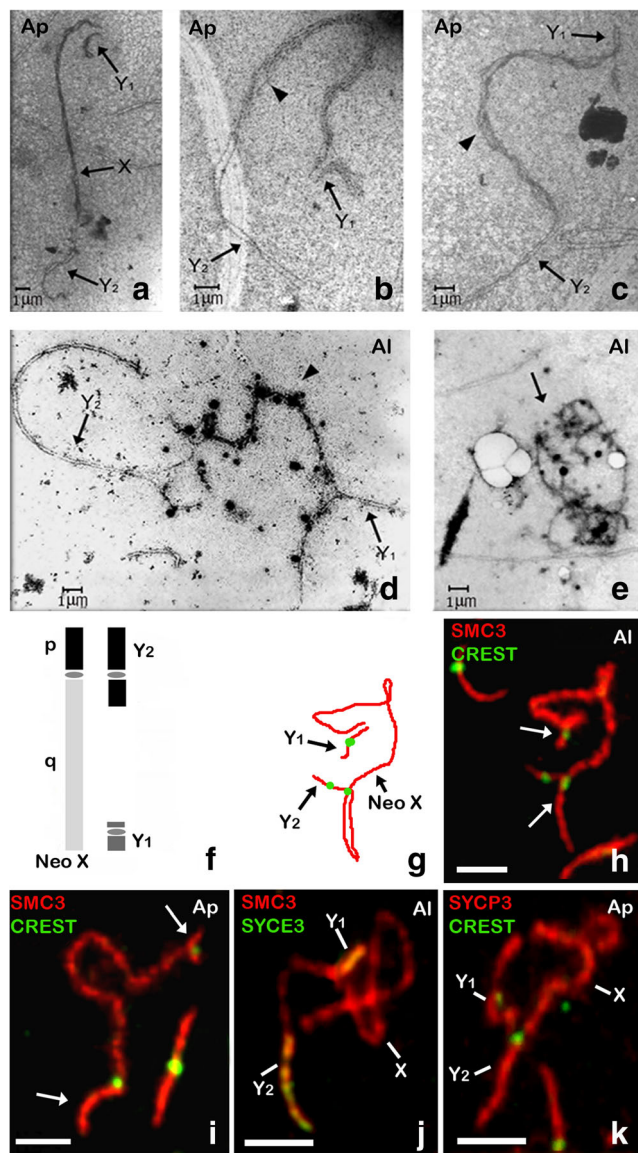
Fig. 1 Pachytene configuration of the sexual trivalent XY_1Y_2 in *A. lituratus* (Al) and *A. planirostris* (Ap) (**a–k**). **a–e** Electron micrographs of synaptonemal complexes showing the behavior of the sexual trivalent from early pachytene (**a**) to late pachytene substages (**e**). In both species, the sex chromosomes form a trivalent with two fully synapsed regions, X-Y_2 and X-Y_1, and asynaptic axial regions corresponding to the differential regions which usually show split axes (**b, c**, *arrowheads*) and little round bodies (**d**, *arrowhead*). The cohesin SMC3 (**h–j**, *red axes*) and the SYCP3 protein (**k**, *red axes*) are present along the single chromosomal axes and the SYCE3 on the synapsed ones (**j**, *yellow-green axes*). Kinetochores are labeled by CREST serum (**h, i, k**, *green foci*). **f** Original X segment, in *light grey*; autosomal segments, *in black*, and original Y segment, *in dark grey*; kinetochores, *in grey elipses*. *Bars* **a–e** 1 μm and **h–j** 5 μm

long synaptonemal complex (SC) between the Y_2 chromosome and the homologous, translocated product in the short arm of the X chromosome, and the other shows a short SC between the original X and Y_1 segments (Fig. 1c, d, g–k). During pachytene stage, a stepwise association of the three components of the trivalent is observed (Fig. 1a–e); at early

substages of pachynema, the Y_2 synapses completely or almost completely with a corresponding segment of the X, while the small Y_1 chromosome shows a slight delay of synapsis with the X chromosome (Fig. 1a, b), until the end of pachynema, when both chromosomes Y_1 and Y_2 become completely synapsed with the X (Fig. 1c–e). At very late substages of pachynema, the asynaptic axial segments of the trivalent split into several strands and show protein excrescences, as it has been described previously in other mammals (Fig. 1d, e).

The fluorescent immunolocalization of structural proteins of the synaptonemal complex agrees with the electron microscopical observations in the sex chromosomes, as well as in the autosomes (Fig. S1b–f in the Supplementary data). In the sexual trivalent, the protein of structural maintenance of cohesion, SMC3, and the major protein component of the lateral elements of the SC, SYCP3, are located along the single-chromosome axes corresponding to X, Y_1, and Y_2 chromosomes (Fig. 1h–k), while the SYCE3 protein, a marker of the central element of the SC, is present on the synapsed regions, X-Y_1 and X-Y_2 (Fig. 1j).

The sexual bivalent neo-X/neo-Y in *U. bilobatum* and *V. caraccioli* along the pachytene stage

In these two species, the sex chromosome system is the result of a translocation of a pair of autosomes to the sex chromosomes, where one autosomal region corresponds to the short arm of the X and the autosomal homolog forms the long arm of the Y (Fig. 2a). In *U. bilobatum*, as well as in *V. caraccioli*, a characteristic behavior of the sex chromosomes is observed as the pachytene stage advances. At early substages of pachynema, the sex chromosomes form a bivalent neo-X/neo-Y with only one long SC (Fig. 4a–h), which is labeled by SYCE3 (Fig. 2d), and two BRCA1-positive unpaired terminal segments (Fig. 4b, f), as usually seen in other mammals. However, as pachynema progresses, these two ends become closer until late pachytene stages, where both ends come together and synapse, forming a short SC (Figs. 2b, c, e, f and 4i–l). At this latter substage, the sexual bivalent neo-X/neo-Y is seen as a close ring with the following two SCs: the longer one, corresponding to the synapsed regions of the original autosome, and the shorter one, corresponding to the original pairing segments of the X and Y chromosomes. These two synaptic regions are labeled by SYCE3 (Fig. 2e, f), and the remaining asynaptic X segment is labeled by BRCA1 (Fig. 4j).

Chromatin remodeling and transcriptional silencing in the multiple sex chromosomes of the analyzed phyllostomid species

The analyses of 40 thin sections of primary spermatocytes in the four analyzed species show a typical XY body at late pachynema (with the special feature of having two SCs at this
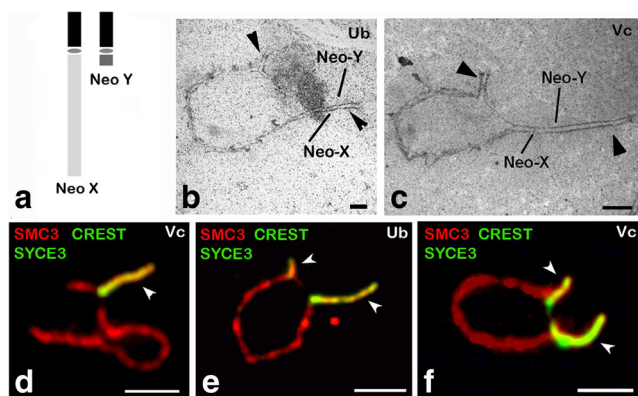
Fig. 2 Pachytene configuration of the sexual bivalent neo-*X*/neo-*Y* in *Uroderma bilobatum* (Ub) and *Vampyrodes caraccioli* (Vc) (**a–f**). In both species, the sex chromosomes form a neo-*XY* bivalent with two synaptic regions (**b**, **c** and **e**, **f**, *arrowheads*) resulting in a closed configuration at late pachytene stage. At the beginning of pachytene stage, the marker of the central element of SC, SYCE3 (**d**, *yellow-green axes*), is only decorating the long SC. As pachynema progresses, the SYCE3 protein appears on both synapsed regions of the closed bivalent. The SMC3 protein is located along the single axes (**d–f**, *red axes*). **a** Original *X* segment, in *light grey*; autosomal segments, *in black*, and original *Y* segment, *in dark grey*; kinetochores, *in grey elipses*. *Bars* **b**, **c** 1 μm and **d–f** 5 μm

stage). This "neo-*XY* body" is attached to the nuclear envelope and has the following two different regions as regards to its structure: a peripheral region, formed by highly packed chromatin corresponding to the differential axes of the sex chromosomes, and an inner region, having more dispersed chromatin fibers of autosomal-like chromatin, which corresponds to the two synapsed regions (Fig. 3a). This different degree of chromatin remodeling is consistent with the presence of the variant histone γ-H2AX and the exclusion of RNA polymerase type 2 on the asynaptic chromatin of the neo-*XY* body (Figs. 3b, d and 4 (third and fourth columns)). These observations indicate that the neo-*XY* bodies have a peripheral, transcriptionally silenced region in the analyzed species.

In *U. bilobatum* and in *V. caraccioli*, the chromatin of the neo-*XY* body shows a particular behavior. At the beginning of

pachynema, γ-H2AX is present in both asynaptic segments, including the chromosomal ends that synapse later in more advanced stages of pachynema (Fig. 4c, g). Moreover, as pachynema progresses and both ends synapse, BRCA1 and γ-H2AX proteins disappear from the ends (Fig. 4j, k).

Discussion

The formation of a neo-*XY* body

The present work shows that the sex chromosomes in males of the analyzed species of phyllostomid bats form a specific *XY* body, which, because of its particular configuration, can be named *neo-XY body*.

In *A. lituratus*, as well as in *A. planirostris*, the sexual trivalent XY_1Y_2 is formed by two SCs of very different size—the longer X-Y_2 and the shorter X-Y_1—and asynaptic axial regions corresponding to the differential regions of the X, Y_1, and Y_2. The latter regions show thickenings of the chromosomal axes and excrescences. These observations are consistent with those previously described by electron micrographs of synaptonemal complexes and chromosomal axes in *A. lituratus* (Solari and Pigozzi 1994).

On the other side, the sex chromosomes of *U. bilobatum* and *V. caraccioli* show an exceptional bivalent with two synaptic regions interspersed with a long asynaptic region, resulting in a closed configuration at late pachytene stage. This sex composite structure is the product of a translocation of a pair of autosomes to the sex chromosomes, where one autosomal region corresponds to the short arm of the X and its homolog corresponds to the long arm of the Y (see "Results" section). The difference between both types of pachytene configurations is that in the former (that of *Artibeus* sp.), the original pseudoautosomal region is part of the Y_1 chromosome and is separated from the translocated chromosome Y_2, while in the latter (*U. bilobatum* and *V. caraccioli*), both regions
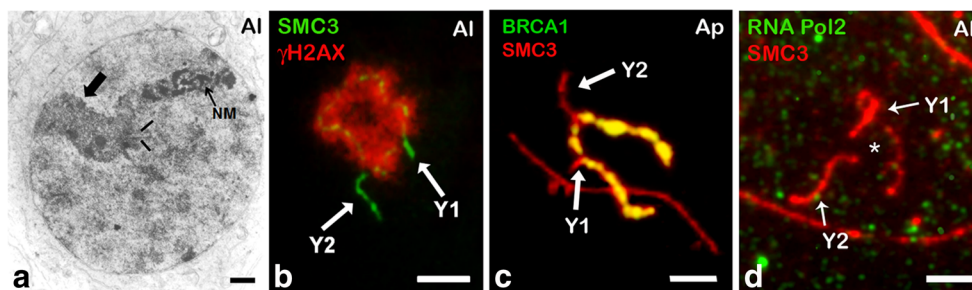

Fig. 3 Silencing and differential chromatin remodeling in the XY_1Y_2 body of *A. lituratus* (Al) and *A. planirostris* (Ap) (**a–d**). Electron micrographs of thin sections of primary spermatocytes of *A. lituratus* (**a**) show the ultrastructural differences between the condensed chromatin of the differential *X* axis (**a**, *black arrow*), labeled by the variant histone γ-H2AX (**b**, *red area*), and the chromatin that is associated with a segment of SC (**a**, *lines*) of the sexual trivalent without γ-H2AX (**b**). The DNA damage repair protein, BRCA1 (**c**, *yellow-green axes*), is present on the differential axes of the XY_1Y_2 body. The presence of RNApol2 (**d**, *green foci*) indicates areas of transcriptional activity. *Asterisk* depletion of RNApol2; *NM* nucleolar material. *Bars* **a** 1 μm and **b–d** 5 μm

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Fig. 4 Behavior of the neo-*XY* body along the pachynema in *Vampyrodes caraccioli* (Vc) and *Uroderma bilobatum* (Ub) (**a-l**). Parallelism between the ultrastructure of the neo-*XY* pair by EM (*first column*) and the localization of meiotic proteins SMC3 (*second to fourth column*, *red or green axes*), BRCA1 (*second column*, *yellow-green axes*), γ-H2AX (*third column*, *red areas*), and RNApol2 (*fourth column*, *green foci*) by fluorescence immunostaining. From early to late pachynema, the BRCA1 protein is restricted to the asynaptic segments in the sexual bivalent. The histone variant γ-H2AX is located on the chromatin of the differential areas in the neo-*XY* body, and RNApol2 reveals areas of transcription. SMC3 delineates single-chromosome axes. *Asterisks* depletion of RNApol2. *Bars* **a**, **e**, **i** 1 μm and **b**–**d**, **f**–**h**, **j**–**l** 5 μm
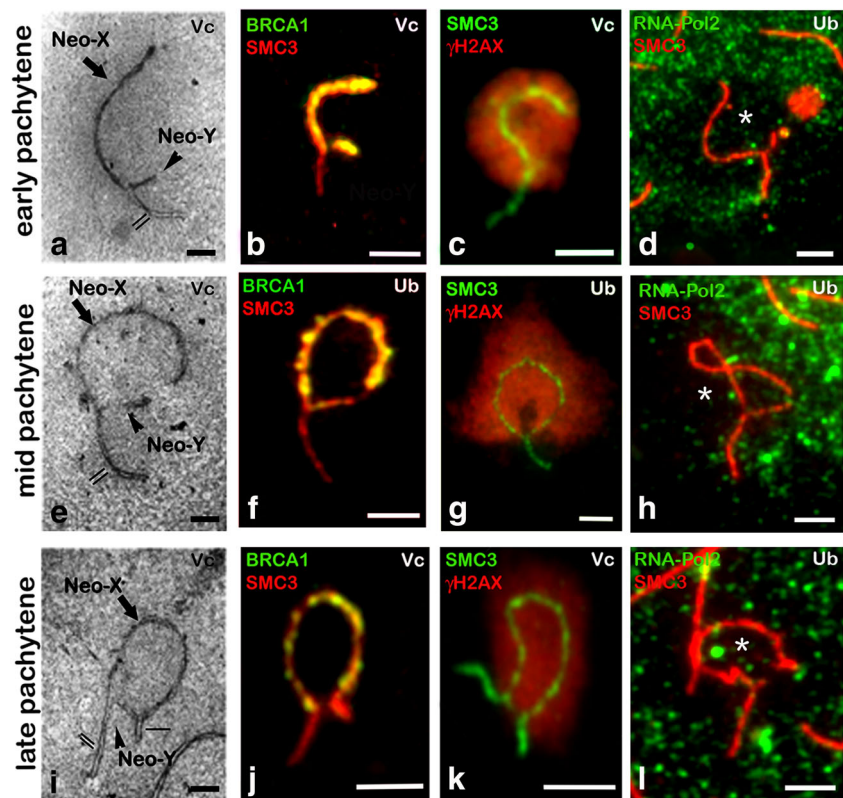
belong to the same chromosomal axis, the neo-*Y*, giving rise to a closed bivalent.

Our observations of different meiotic proteins on the spermatocytes of phyllostomid bats by immunofluorescence techniques are similar to those previously described in other mammalian taxa (Turner et al. 2004, 2005; Franco et al. 2007; Sciurano et al. 2012a, b, 2013; Villagómez and Pinton 2008; Pinton et al. 2009). The common features shared by all these species are the presence of the cohesin SMC3 and the SYCP3 protein on the single-chromosome axes and the SYCE3 and BRCA1 proteins on the synapsed and asynapsed regions, respectively.

According to a previous paper (Noronha et al. 2010) in three bat species analyzed with multicolor fluorescence in situ hybridization (FISH), the neo-*XX*/*XY* systems present in two of these species have an autosome translocated to the sex chromosomes that was identified as the pair 15 of *Phyllostomus hastatus*. This observation strongly suggests that the neo-*X*/neo-*Y* system presently studied has a similar origin.

The presence of -H2AX on asynaptic chromatin of multiple sex chromosomes

In previous observations on four bat species, morphological evidence on differences in the degree of chromatin compaction between the original pieces of the *X* and *Y* elements and

the autosomal, translocated regions have been described both at EM (Solari and Pigozzi 1994) and at light microscopic level (Noronha et al. 2009, 2010). The present observations in micrographs of primary spermatocytes show a typical sex chromatin condensation restricted to the asynaptic segments and an autosomal-like chromatin surrounding the synapsed regions of the multiple sex chromosomes. These results were also seen in paraffin and semithin sections of the testicular tissue.

It is widely accepted that the histone variant γ-H2AX is an excellent biomarker of the chromatin remodeling in the *XY* body of different taxa (Fernandez-Capetillo et al. 2003; Franco et al. 2007; Sciurano et al. 2012b, 2013; Sciurano and Solari 2014). In most mammals, the *X* and *Y* chromosomes are highly heterologous and have only a short SC, the pseudoautosomal region, leaving asynaptic segments that are transcriptionally silenced at pachytene stage. This latter phenomenon has been largely demonstrated firstly by incorporation of tritiated uridine (Monesi 1965) and then by detection of nascent transcripts via RNA polymerase 2 or Cot1 RNA FISH and microarray analysis (Turner et al. 2005; Homolka et al. 2012). It has been also suggested that the presence of this asynaptic chromatin on the *XY* body is associated with the presence of several proteins, the mediator of DNA damage checkpoint 1 (MDC1) (Ichijima et al. 2012) and the DNA damage repair protein, BRCA1, and the kinase ATR which triggers the phosphorylation of the variant histone H2AX at

the serine residue 139 (Turner et al. 2004). The phosphorylation of H2AX leads to the silencing of genes throughout the non-synaptic region; this chromosome-wide spreading is a phenomenon called MSCI (reviewed in Turner 2007), which is maintained in post-meiotic cells (Namekawa et al. 2006; Turner et al. 2006).

Our observations show for the first time the presence of γ-H2AX on asynaptic chromatin of the multiple sex chromosomes of fertile males in mammals. In the four analyzed species, the phosphorylated H2AX is limited to the asynaptic chromatin regions of the sex trivalent in the species of the genus *Artibeus*, as well as those of the closed sex bivalent in *U. bilobatum* and *V. caraccioli*. Moreover, a decrease of RNA polymerase type 2 is associated with the presence of γ-H2AX on asynaptic chromatin, revealing that these regions are transcriptionally silenced. Significantly, at the beginning of pachynema, the γ-H2AX is located at the yet unpaired, original pseudoautosomal region (PAR) of the closed bivalent in *U. bilobatum* and *V. caraccioli*. However, as pachytene progresses and both ends of the bivalent synapse and form a short SC, the γ-H2AX mark (and BRCA1) disappears, without any detectable consequences for fertility.

Lack of spreading of the silenced sex chromatin towards the autosomal chromatin

A spreading effect of chromatin inactivation has been assumed to explain the apparent inhibition of gene transcription in the autosomal regions translocated to an *X* chromosome in mammals bearing an *X*-autosome translocation (Russell 1963; Jaafar et al. 1989). The spreading effect from the *X* region towards the autosomal region has been shown both by electron microscopy (Solari 1971) and by radioautography (Jaafar et al. 1989) in the *XY* bodies of spermatocytes of mouse carriers of an *X*-autosome translocation.

In order to explain the conservation of fertility in species of phyllostomid bats which have evolutionary fixed gonosome-autosome translocations, two mechanisms for isolating the translocated, autosomal pieces from the spreading of inactivation have been proposed: (1) the presence of heterochromatin blockage (in *A. lituratus*, Kasahara and Dutrillaux 1983 and *in A. cinereus* and *U. magnirostrum*, Noronha et al. 2010) and (2) the presence of a nucleolar organizing region (NOR) (in *Carollia perspicilatta*, Noronha et al. 2004). However, the C-banding patterns exclude the first option for the presently studied species (Kasahara and Dutrillaux 1983; Solari and Pigozzi 1994; Moreira da Silva et al. 2005; Lemos-Pinto et al. 2012; Gomes 2014). Furthermore, the Ag-NOR staining showed that the nucleolar organizing regions are located in the distal region of the short arm of autosomal chromosomes in all of the mentioned species (Lemos-Pinto et al. 2012; Moreira da Silva et al. 2005; Gomes 2014). Thus, according to the C-banding and NOR analyses, neither the heterochromatin nor

the nucleolar organizing region seems to be the steric hindrance that blocks the spreading of inactivation from the sex chromosomes to the autosomal genes in the translocation product of the analyzed species.

Although a deeper comprehension of the epigenetic mechanisms that could act as a natural barrier in the multiple sex chromosomes of fertile species is needed (reviews in Zamudio et al. 2008 and Khalil and Wahlestedt 2008), it is suggested that the formation of a functional synaptonemal complex between homologous or non-homologous regions may be per se the basic mechanism that arrests spreading of transcriptional silencing to the autosomal segments, and thus, this SC barrier might be probably related to the preservation of fertility in these systems (as also seen in other species when heterosynapsis occurs; Pinton et al. 2009). In fact, most of translocated segments of sexual trivalents in *A. lituratus* and *A. planirostris*, as well as those of the sexual closed bivalent in *U. bilobatum* and *V. caraccioli*, are fully synapsed at late pachynema, and γ-H2AX is absent on these SC segments.

The avoidance of the spreading effect is probably a *conditio sine qua non* for the persistence of chromosomal rearrangements in fertile species with multiple sex chromosomes systems; otherwise, a severe autosomal imbalance would exist in male genomes. However, the molecular nature of this mechanism remains unknown.

Asynapsis and male fertility in phyllostomid bats

One of the most interesting features of multiple sex chromosome systems is the presence of asynaptic segments at meiotic prophase in testes that show a normal development of spermatogenesis. This fact contradicts the claim that silent asynaptic segments of rearranged chromosomes may lead to spermatogenic impairment, as known in mice and humans (Burgoyne et al. 2009; Sciurano et al. 2012a).

In *A. lituratus* (Pigozzi and Solari 1994), as well as in *C. perspicillata* (Noronha et al. 2009), no deleterious signs have been detected in primary spermatocytes despite the presence of multiple sex chromosome systems. Indeed, the sex trivalents in spermatocyte nuclei are divided by alternate segregation giving rise to gametes with a different haploid number—one gamete with the two putative *Y* chromosomes (Y_1 and Y_2) moving to one pole and the other with an *X* to the opposite pole—thus ensuring the production of balanced gametes. These observations would support the ideas that the spermatogenic disturbances in infertile male carriers of gonosome-autosome translocations (including humans) are due to a gene imbalance in spermatocyte nuclei at pachytene, a crucial state for the progress of meiotic and post-meiotic processes.

It is reasonable to think that there should be a selective advantage in the rearrangement of sex chromosomes in phyllostomid bats. One of the possible advantages could be that the short recombination region (*X*-Y_1 in *A. lituratus* and

A. planirostris or the even shorter pairing segment in the closed bivalent of *U. bilobatum* and *V. caraccioli*) could be replaced by a new longer recombination region (X-Y_2) or the longer one in the closed bivalent. Our observations show two peculiarities in the analyzed systems: (1) the delay of synapsis between the Y_1 and the X chromosomes compared to X-Y_2 synapsis in *Artibeus* and (2) at the beginning of pachynema, the presence of BRCA1 and γ-H2AX on the distal end of the neo-Y chromosome in *U. bilobatum* and *V. caraccioli*, strongly suggesting that this region is transcriptionally silenced at that time. Thus, the translocation of a new chromatin mass from an autosome to the sex chromosomes may contribute an additional chromatin stretch to maintain the needed occurrence of recombination and the regular segregation of the sex chromosomes. Moreover, this added autosomal chromatin could be evolutionary of pseudoautosomal nature, as it was suggested in some species of marsupials (reviewed in Graves et al. 1998). However, further studies should be performed to determine if the neo-XY bodies maintain two actual recombinant regions: (1) the original recombination region and (2) the new one in the autosomal pairing region. Such an analysis should include the presence of recombination markers (like the MLH1 protein) in both regions and the demonstration—by sequence comparisons—the presence or not of PAR genes in both synapsed regions. Preliminary results show the presence of one recombination nodule (RN) which corresponds to one chiasma in the autosomal region of the sexual trivalent in *A. lituratus* (Fig. S1a in the Supplementary data and Fig. 2 in Pigozzi and Solari (1994)). However, the presence of a RN/chiasma in the shorter pairing region could not be determined in the present observations.

The present work shows that multiple sex chromosomes are interesting biological systems, because they are natural models that successfully bypass the risk of meiotic arrest and infertility and allow the analysis of biological phenomena involved in the fixation of a new chromosomal rearrangement in fertile mammals.

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