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A unique late-replicating XY to autosome translocation in *Peromyscus melanophrys*

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Abstract We report on the characterization of the *Peromyscus melanophrys* karyotype and sex chromosome system. Classic studies reported the sex chromosome system of this species may be as complex as an $X_1X_1X_2X_2/X_1X_2Y_1Y_2$ and provided conflicting identification of the X chromosome. Using *Peromyscus maniculatus* chromosome paints, we have positively identified the sex chromosomes and clarified the sex determining system that once perplexed *Peromyscus* researchers. The sex chromosomes are characterized by a unique autosomal translocation of DNA shared between both the X and Y chromosomes. The translocated material is late replicating and heterochromatic yet retains the active chromatin conformation. Thus, autosomal regions derived from translocations involving repeat-rich material may retain some epigenetic marks specific to the sex chromosomes despite loss of epigenetic silencing activity.

Keywords translocation · *Peromyscus* · sex chromosome · X inactivation

Abbreviations

FISH	Fluorescence in situ hybridization
WCP	Whole chromosome paint
BrdU	5-bromodeoxyuridine
Xi	Inactive X chromosome
PAR	Pseudoautosomal region

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Introduction

The *Peromyscus* genus is a classical cytogenetic model that has been extensively studied. *Peromyscus* karyotypic diversity has occurred at an elevated rate (Greenbaum and Baker 1978; Hamilton et al. 1992; Robbins and Baker 1981), characterized by heterochromatic re-patterning through additions, deletions, and translocations (Bowers et al. 1988).

Previous karyotypic studies of *Peromyscus melanophrys* have provided contradictory data regarding the evolution of the sex chromosome complement. Hsu and Arrighi (1968) first described the polymorphic *P. melanophrys* karyotype after examining five specimens; they identified the X chromosome as a large biarmed chromosome and concluded that the Y chromosome varies from a small acrocentric to a tiny metacentric chromosome. Within the male specimens, the large chromosomes were consistently left unpaired in the resolved karyotype (Hsu and Arrighi 1968). Zimmerman (1974) also identified a large, unpaired subtelocentric chromosome in males that was not found in females. Both of these analyses were based on solid staining and low resolution, G-banded chromosome preparations; although, the former study also included meiotic analyses and replication timing studies. The identification of the X chromosome is unclear as each study described the X chromosome as either the largest biarmed chromosome in the karyotype or a mid-sized subtelocentric chromosome (Hsu and Arrighi 1968; Zimmerman 1974). To explain the karyotypic disparity between *P. melanophrys* males and females, Zimmerman (1974) suggested that either males have a pericentric inversion that is always heterozygous and never found in females or there was a Y-autosome translocation which resulted in an unique $X_1X_1X_2X_2/X_1X_2Y_1Y_2$ sex chromosome system.

Multiple sex chromosome systems have been previously identified in a variety of species. For example, an $X_1Y_1Y_2$ sex chromosome system has been described in two other rodent species, *Taterillus* (Dobigny et al. 2002, 2004a, b) and *Nannomys* (Veyrunes et al. 2004). A similar sex chromosome system has even been identified in *Rumex*, a plant genus (Cunado et al. 2007). The duck-billed platypus has a very complicated male sex chromosome system: $X_1Y_1X_2Y_2X_3Y_3$ $X_4Y_4X_5Y_5$ (Rens et al. 2004). These multiple sex chromosome complements have all evolved from X-autosome translocations.

Using cross species chromosome painting, we have determined that *P. melanophrys* contains an XY sex chromosome system. However, X and Y chromosome-derived repeated DNA has been translocated to chromosome 1 in this species and has retained its late-replicating, heterochromatic state, although it has lost its signature of unichromosomal, closed chromatin that normally characterizes the inactive X (Xi) of female mammals.

Materials and methods

G-Banding

Slides were incubated in 0.05% trypsin/phosphate buffer solution (PBS) for 20–40 s then rinsed in cold PBS.

Slides were stained in 5% Giemsa in pH6.8 Gurr Buffer for 5 min, rinsed in dH2O, and air-dried.

Fluorescence in situ hybridization

We employed fluorescence in situ hybridization (FISH) of flow sorted whole chromosome paints (WCPs) from *Peromyscus maniculatus* to correctly identify *P. melanophrys* sex chromosomes as per Mlynarski et al. (2008). Following paint confirmation in *P. maniculatus* (Mlynarski et al. 2008), WCPs for chromosomes 1, X, and Y were amplified and labeled as per Mlynarski et al. (2008). Three different combinations of indirectly labeled *P. maniculatus* WCPs were hybridized to *P. melanophrys* and *P. maniculatus* metaphase chromosomes. The probes contained 5 μg of Dig-11-dUTP (Roche) labeled *P. maniculatus* chromosome 1 and 5 μg of Biotin-16-dUTP (Roche) labeled X, 5 μg of Dig-11-dUTP (Roche) labeled chromosome 1 and 5 μg of Biotin-16-dUTP (Roche) labeled Y, and 5 μg of Dig-11-dUTP (Roche) labeled X and 5 μg of Biotin-16-dUTP (Roche) labeled Y. In addition to the 10-μg of labeled DNA, each probe was precipitated with 10 μg of *P. maniculatus* cot-1 DNA, 10 μg of salmon sperm, and 40 μl ice cold ethanol. Hybridization and post-hybridization washes were performed as per Brown et al. (2002). Probes were co-detected using anti-digoxigenin rhodamine and fluorescein isothiocyanate-conjugated avidin (FITC avidin) fluorescently labeled antibodies. Images were captured as per Mlynarski et al. (2008).

Replication banding methods

5-Bromo-2-deoxyuridine (BrdU) was used to distinguish between early and late-replicating chromosome regions within the karyotype. Actively growing *P. melanophrys* fibroblast cells were incubated in 50 μM BrdU for 3 h in the dark. The cells were then

harvested and dropped onto slides as per Mlynarski et al. (2008). The BrdU was visualized following a modified protocol previously described by Diaz-Perez et al. (2006). Slides were denatured in 70°C 70% formamide/2XSSC pH7 for 2 min then immediately dehydrated for 2 min in ice cold 70%, 90%, and 100% ethanol. Following preincubation in blocking buffer (5% bovine serum albumin (BSA)/ 4XT), BrdU was detected using a 1:100 dilution of biotintylated anti-BrdU antibody. A 1:200 dilution of avidin FITC, anti-FITC rabbit, and anti-rabbit AF555 antibodies were layered to amplify the BrdU signal. Images were captured, and coordinates were recorded using the Leica DM6000 and CytoFISH software suite (Leica Microsystems Imaging Solutions Ltd.: www.leica-microsystems.com).

DNase1 in situ nick translation

Male and female *P. melanophrys* slides were treated with DNase1 (4 µl of Roche Dig Nick Translation mix was diluted with 6 µl of dH_2O). Slides were incubated at 37°C for 10 min. Slides were then washed in 10 mM Tris-Cl pH7.4 for 5 min. Slides were rinsed in 4XT then flooded in preincubation buffer (5% BSA/ 4XSSC/0.2% Tween 20) prior to detection with anti-Dig FITC antibody. Images were captured as described above.

C-banding

C-banding was employed to identify blocks of constitutive heterochromatin. As per standard protocols, slides were aged at room temperature overnight, dehydrated, soaked in 0.2 N HCl at room temperature for 20 min, then rinsed in dH_2O. Slides were flooded in saturated $Ba(OH)_2$ for 15 min at room temperature then rinsed in dH_2O and air-dried. Slides were then soaked in 65°C 2XSSC for an hour, serially dehydrated, and air-dried. Slides were stained in 6% Giemsa in pH6.8 Gurr buffer for 35–40 min, rinsed in dH_2O, and air-dried.

Immunocytochemistry/fluorescence in situ hybridization

P. melanophrys female fibroblast cells were grown on slide flasks and processed as previously described (Bulazel et al. 2007; Carone et al. 2009). Immunocytochemistry was preformed as previously described by Carone et al. (2009) with anti-trimethyl H3K27 rabbit-conjugated antibody (Upstate) and detected using anti-rabbit Alexa Fluor 555 (Molecular Probes). Slides were fixed in 4% paraformaldehyde then Carnoy's fixative and aged overnight prior to the FISH with chromosome 1 paint. The probes contained 30 µg biotin labeled chromosome 1, 15 µg of *P. maniculatus* cot-1 DNA, 10 µg of tRNA, and 90 µl ice cold ethanol. Following a two-night hybridization, slides were washed as described by Mlynarski et al. (2008) and co-detected using avidin fluorescein and anti-rabbit Alexa Fluor 555 antibodies.

Results

Sex chromosome identification

Initial G-banding of *P. melanophrys* metaphase chromosomes revealed several chromosomes with size discrepancies. The most notable were that of the heteromorphic X and Y chromosomes in males (Fig. 1). However, in both males and females, another pair was identified that carried a size discrepancy, the p-arms of chromosome 1 (Fig. 1). *P. maniculatus* WCPs were hybridized to *P. maniculatus* male and female metaphase chromosomes to confirm sex chromosome delineation within *Peromyscus*. The X WCP hybridized to the metacentric X chromosomes and the putative pseudoautosomal region (PAR) on the much larger *P. maniculatus* metacentric Y chromosome. The Y WCP hybridized to the Y chromosome and the PAR on the X chromosome. Neither the X nor the Y WCP hybridized to the distal tip of the p-arm on chromosome 1 in *P. maniculatus* (Fig. 2a–c).

To definitively identify the sex chromosomes, *P. maniculatus* WCPs specific to the X, Y, and chromosome 1 were hybridized to male and female *P. melanophrys* metaphase chromosomes. Chromosomes were karyotyped based on the inverted DAPI banding patterns from the FISH images (Fig. 2). The X paint hybridized to two large acrocentric chromosomes with matching G-banding patterns in females (Supplemental Fig. 1). In males, the X paint hybridized to one large acrocentric chromosome with the same G-banding pattern seen in females, and to the PAR on the Y chromosome (Figures 1 and 2d, f). The Y paint

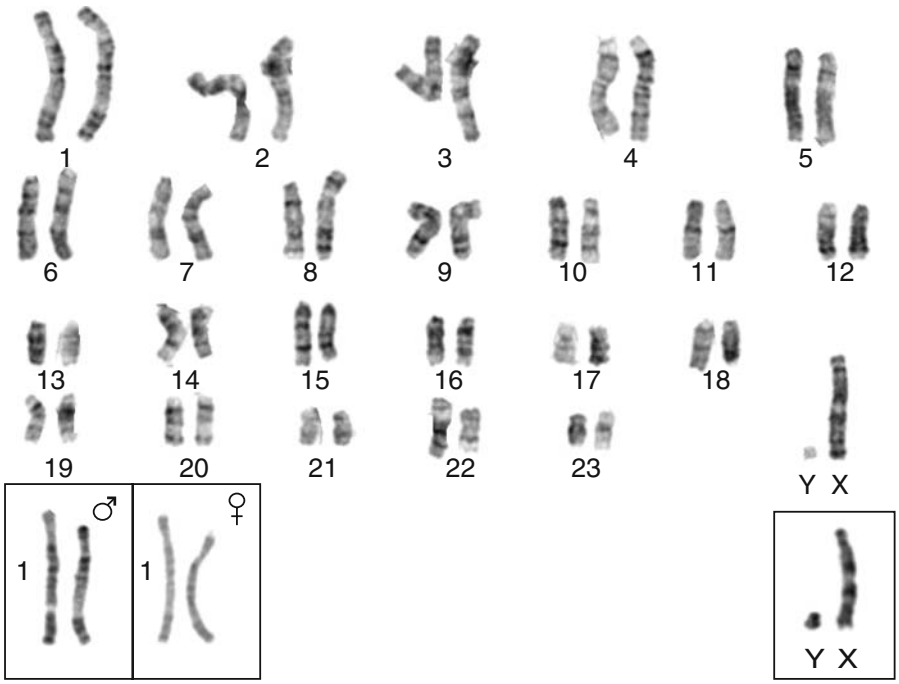

Fig. 1 G-banded male *Peromyscus melanophrys* metaphase chromosomes. The variable sizes of chromosome 1 homologues from male and female specimens are *highlighted in boxes* (*lower left*). The sex chromosomes from a different male are also displayed in a *box* (*lower right*). Scale bars represent 5 μm

hybridized to the minute *P. melanophrys* Y chromosome and to the p-arm of the X chromosome, which likely represents the PAR that is present on both the X and Y chromosome (Fig. 2e). The X and Y paint also hybridized to the distal tip of the p-arm on chromosome 1 in males and females. Increased blocking with 20 μg of Cot-1 DNA abolished the X and Y signal on the p-arm of chromosome 1 (results not shown). The chromosome 1 paint was used to confirm the observed cross-hybridization was present on the chromosome 1 pair in both males and females (Fig. 2d, e). The hybridization of the X, Y, and chromosome 1 paint clarified the sex determining system and identified an X/Y translocation, likely containing repeated material, at the distal tip of chromosome 1.

BrdU replication timing

The replicating timing properties of the X/Y translocation present on chromosome 1 was assayed to determine if this region retained its late-replicating status, which is characteristic of the inactive X in females and the Y chromosome in males. A BrdU treatment time-course was performed to identify the proper conditions to distinguish between early and late-replicating DNA in *P. melanophrys*. After 2.5 and 3 h of exposure to BrdU, the inactive X of females and the Y chromosome of males, as well as other late-replicating regions within the genome, were labeled with BrdU. FISH with the X, Y, and chromosome 1 paints was performed post-BrdU treatment to positively identify these specific chromosomes (Fig. 3). Both chromosome 1 p-arms had incorporated BrdU, but the labeling patterns are not uniform between the homologs. One homolog of each chromosome 1 pair had a distinctive increase in BrdU labeling on the p-arm that extended to the telomere (Fig. 3, Supplemental Fig. 2). The same discrepancy in hybridization pattern between chromosome 1 pairs was identified in both males and females.

In situ nick translation

In situ nick translation was performed using DNaseI followed by fluorochrome incorporation on metaphase chromosomes to identify transcriptionally active and inactive chromosomal regions. The active X chromosome of females was highly sensitive to DNase1 digestion. The inactive X of females and the Y chromosome of males were not sensitive to DNase1. Small, discontinuous regions of DNase1 sensitivity punctuated the autosomes (Supplemental Fig. 3). The distal tip of the chromosome 1 p-arm of

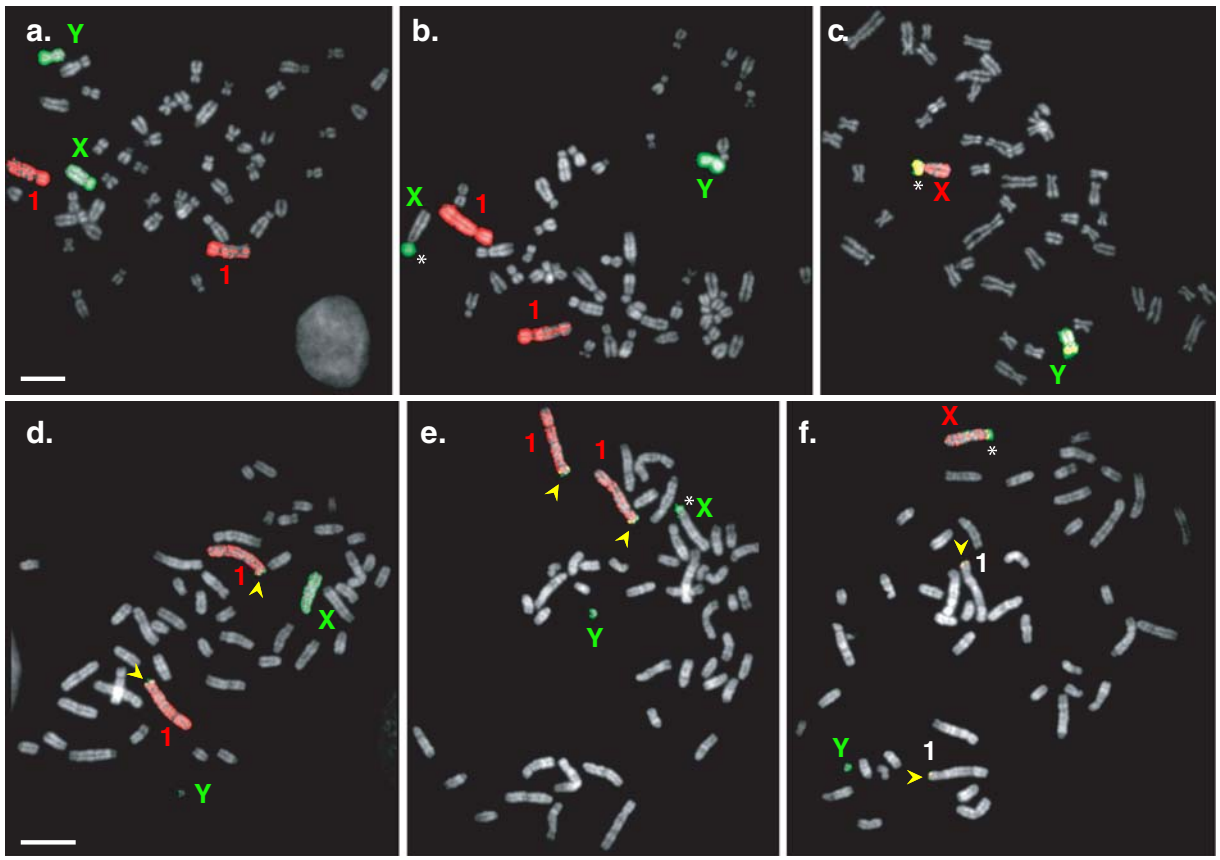

Fig. 2 *Peromyscus maniculatus* chromosome paints specific to chromosome 1, X, and Y hybridized to male metaphase chromosomes. **a**–**c** *P. maniculatus* metaphase spreads. **d**–**f** *Peromyscus melanophrys* metaphase spreads. Scale bars represent 10 μm. **a** *P. maniculatus*-derived Chr1 (*red*) and X (*green*) chromosome paint probes hybridized to male *P. maniculatus* metaphase chromosomes. **b** *P. maniculatus*-derived Chr1 (*red*) and Y (*green*) chromosome paint probes hybridized to male *P. maniculatus* metaphase chromosomes. Pseudoautosomal region (PAR) is indicated with an *asterisk*. **c** *P. maniculatus*-derived X (*red*) and Y (*green*) chromosome paint probes hybridized to male *P. maniculatus* metaphase chromosomes. Sex chromosomes are indicated; PAR is indicated with an *asterisk*. **d** *P. maniculatus*-derived Chr1 (*red*) and X (*green*) chromosome paint probes hybridized to male *P. melanophrys* metaphase chromosomes. The *yellow arrows* indicate the region where the X chromosome paint hybridizes to the distal tip of the p-arm. **e** *P. maniculatus*-derived Chr1 (*red*) and Y (*green*) chromosome paint probes hybridized to male *P. melanophrys* metaphase chromosomes. The *yellow arrows* indicate the region where the X chromosome paint hybridizes to the distal tip of the p-arm. PAR is indicated with an *asterisk*. **f** *P. maniculatus*-derived X (*red*) and Y (*green*) chromosome paint probes hybridized to male *P. melanophrys* metaphase chromosomes. Sex chromosomes are indicated; PAR is indicated with an *asterisk*. The *yellow arrows* indicate the region where the X and Y chromosome paints hybridize to the distal tip of the p-arm

both homologs was DNase1-sensitive in both female and male specimens, as shown in Fig. 4.

C-banding

Our BrdU results indicate that the translocated region on *P. melanophrys* chromosome 1 is late replicating. Given that increasing Cot-1 DNA reduces the hybridization signal, it is likely this late-replicating material is repeated DNA and not strictly euchromatin. Therefore, we performed C-banding on female and male metaphase chromosomes to confirm the presence of heterochromatin in the translocated region. *P. melanophrys* male and female metaphase chromosomes are almost entirely C-band negative. Very small C-band positive blocks of constitutive heterochromatin are present at some centromeres and only a few telomeres (Supplemental Fig. 4). The distal tip of the p-arm on chromosome 1 is also C-band positive in males and females (Fig. 5). Over 50

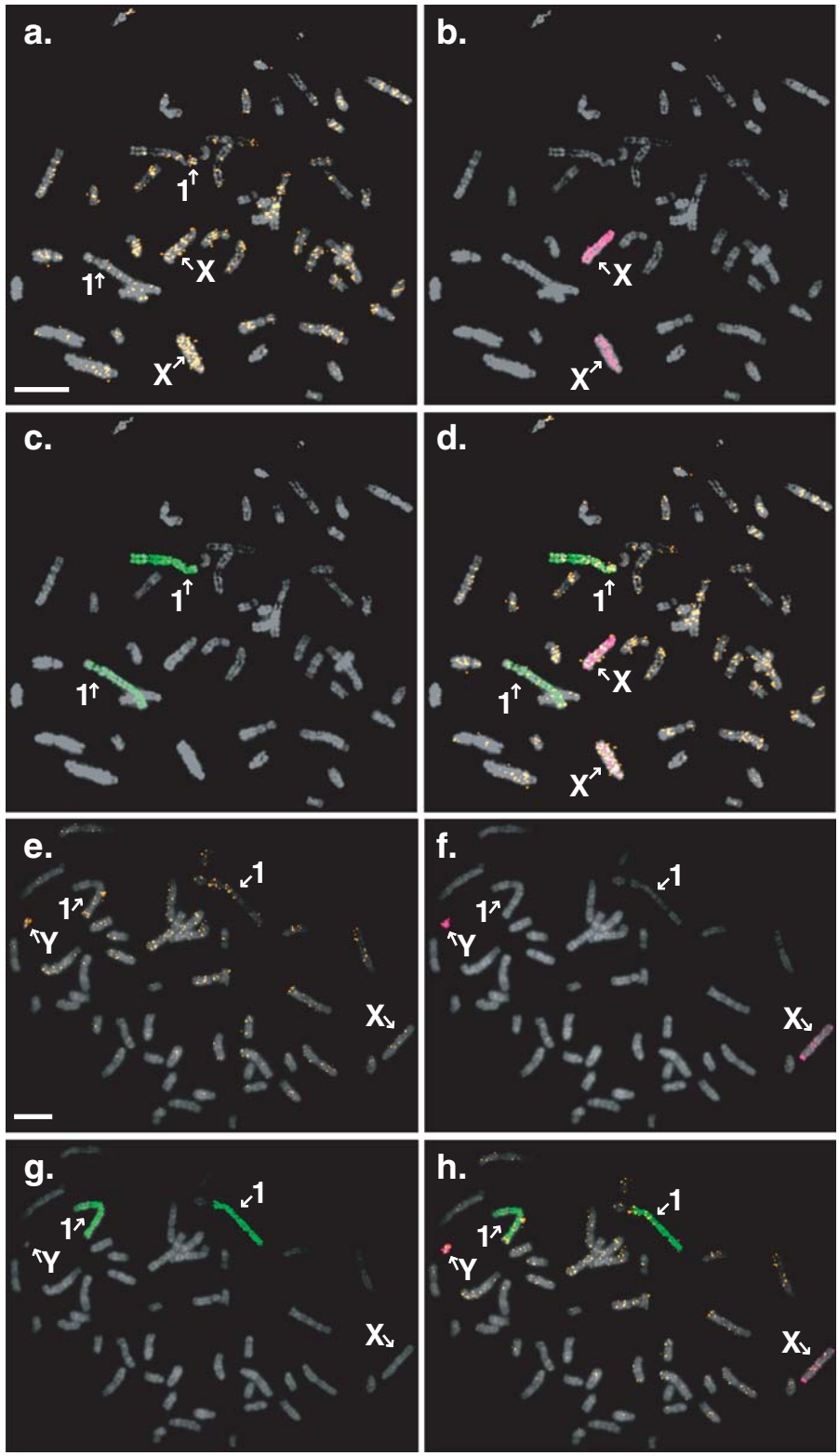

Fig. 3 **a** Female *Peromyscus melanophrys* chromosomes treated with BrdU. BrdU incorporation was detected using fluorescently labeled anti-BrdU antibodies, shown in *orange*. The X chromosome and Chr1 are indicated. Scale bars represent 10 μm. **b** X Chromosome paint (*pink*) hybridized to female *P. melanophrys* BrdU-treated chromosomes. **c** Chromosome 1 paint (*green*) hybridized to female *P. melanophrys* BrdU-treated chromosomes. **d** Merged image of X chromosome paint (*pink*) and chromosome 1 paint (*green*) hybridized to female *P. melanophrys* BrdU-treated chromosomes (*orange*). **e** Male *P. melanophrys* chromosomes treated with BrdU (*orange*). The X, Y chromosomes and chromosome 1 are indicated. **f** Y Chromosome paint (*pink*) hybridized to male *P. melanophrys* BrdU-treated chromosomes. **g** Chromosome 1 paint (*green*) hybridized to male *P. melanophrys* BrdU-treated chromosomes. **h** Merged image of Y chromosome paint (*pink*) and chromosome 1 paint (*green*) hybridized to male *P. melanophrys* BrdU-treated chromosomes (*orange*)

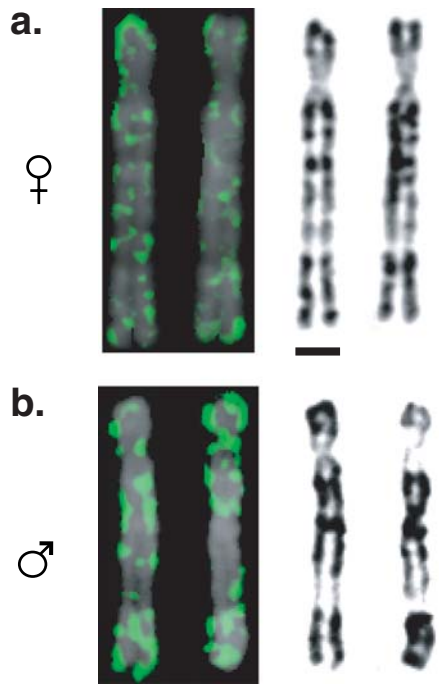

Fig. 4 DNase1 in situ nick translation and corresponding inverted DAPI of *Peromyscus melanophrys* chromosome 1. The *green* probe indicates regions with an accessible chromatin conformation. Scale bars represent 2.5 μm. **a** Female *P. melanophrys* chromosome 1. **b** Male *P. melanophrys* chromosome 1

male and 100 female metaphase chromosome spreads were analyzed; every cell contained a positive C-band on the distal tip of the p-arms of chromosome 1.

Immunocytochemistry/fluorescence in situ hybridization

To determine if the translocated region on chromosome 1 retains the same epigenetic signature as the inactive X chromosome, metaphase chromosomes were simultaneously stained for trimethyl H3K27 and chromosome 1 paint (Fig. 6). Trimethylation at H3K27 is a hallmark of facultative heterochromatin on the Xi (Popova et al. 2006). Over 140 female interphase cells were analyzed; in 79.8% of cells, the fluorescent was distinctly separate; in 19.5% of cells, the trimethyl H3K27 and chromosome 1 signals were adjacent but not overlapping. Thus, while the X-derived chromosome 1 material is late replicating, it has lost the modified histone mark found in Xi.

Discussion

Identification of *P. melanophrys* sex chromosomes

FISH with *P. maniculatus* WCPs specific to chromosome 1, X and Y chromosome was used to definitively identify the sex chromosomes in *P. melanophrys* (Figs. 1 and 2). Previous studies had incorrectly identified the X chromosome as a large biarmed chromosome based on banding similarities to other *Peromyscus* species (Fig. 7; Hsu and Arrighi 1968; Zimmerman 1974). Here, we show that the X chromosome is a large subtelocentric chromosome with a small heterochromatic p-arm containing the PAR; however, the X can sometimes appear as a large acrocentric chromosome, depending on the extent of heterochromatic condensation in each mitotic cell. In contrast to *P. maniculatus*, the *P. melanophrys* X chromosome has a much shorter p-arm, which may have contributed to the original misidentification based on G-band patterns alone. Our WCP data indicate the chromosome Zimmerman (1974) identified as the *P. melanophrys* X chromosome is chromosome 2 (Fig. 1). The *P. melanophrys* Y chromosome is substantially smaller than the Y chromosome in any other *Peromyscus* species. Although the rapid

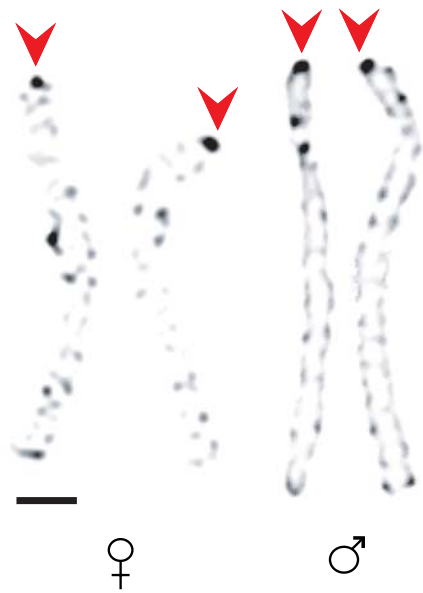

Fig. 5 Images of C-banded *Peromyscus melanophrys* chromosome 1 pair. The C-band positive tuft on the distal tip of both p-arms is present in males and females, as indicated by the *arrows*. Scale bars represent 2.5 μm

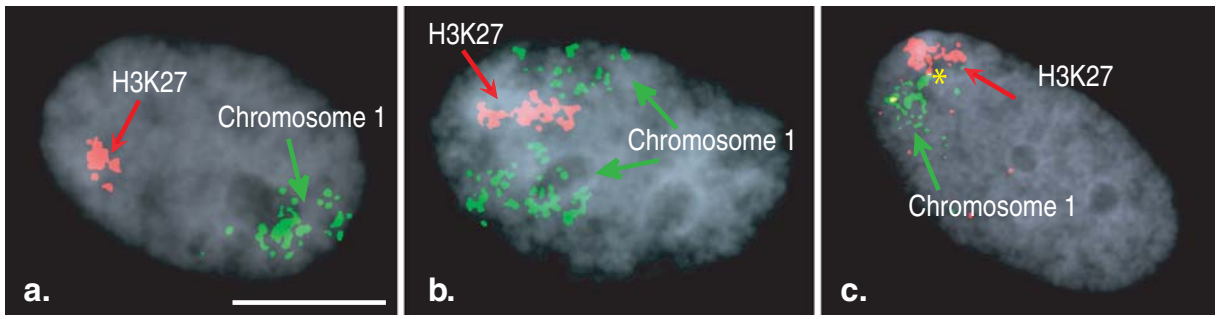

Fig. 6 Immunocytochemistry with an antibody specific to trimethyl H3K27 (*red*) followed by fluorescence in situ hybridization with *Peromyscus maniculatus*-derived Chr1 paint (*green*) on female *Peromyscus melanophrys* interphase cells. Scale bars represent 10 μm. **a** The trimethyl H3K27 signal and Chr1 signal do not co-localize. Distinct and separate hybridization patterns were found in 79.8% of cells inspected. **b** In 19.5% of cells, the trimethyl H3K27 signal and Chr1 signal are adjacent but do not overlap. **c** Only a single cell (shown here) had a possible overlap of trimethyl H3K27 signal and Chr1 signal

reduction of the *P. melanophrys* Y chromosome is unique amongst *Peromyscus* species, it is not unique among mammals. The X WCP co-hybridizes to a small region of the *P. melanophrys* Y chromosome; presumably, this hybridization is due to the homology between the X and Y and thus likely represents the PAR. Aside from the homology between the X and Y PAR, the Y chromosome does not recombine with any other chromosomes, resulting in little selective pressure to maintain Y chromosome content aside from that required to remain sex chromosome limited (such as a sex determining locus; Toder et al. 2000).

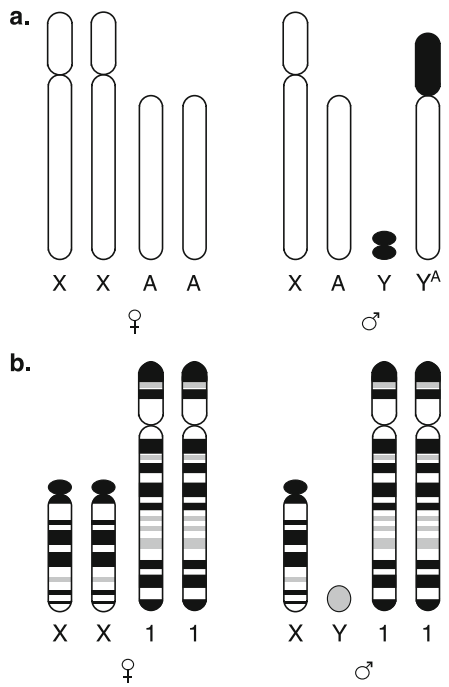

Fig. 7 **a** Ideogram of *Peromyscus melanophrys* multiple sex chromosomes, adapted from Zimmerman (1974). **b** Revised ideogram of *P. melanophrys* sex chromosomes as identified by fluorescence in situ hybridization, G-bands according to Greenbaum et al. (1994)

Previous *P. melanophrys* studies describe the karyotype as highly polymorphic (Hsu and Arrighi 1968; Zimmerman 1974). Both studies identified an unmatched pair of large chromosomes in the male karyotype (Hsu and Arrighi 1968; Zimmerman 1974); using FISH, we were able to determine that the unmatched chromosome pair in males is due to the misidentification of the X chromosome. Our data shows another heteromorphic chromosome pair in the *P. melanophrys* karyotype: there is a notable size discrepancy in the p-arm of chromosome 1 in both male and female specimens. This is not likely a population polymorphism given the number of individuals from an outbred colony examined in this study and the observed frequency of 100% for this heteromorphism. Despite the size discrepancy, the G-banding pattern is consistent for this chromosome in both males and females.

Translocations and X inactivation

Previous studies have shown that transcriptionally active regions have a specific chromatin conformation that permits access of transcriptional machinery to the DNA; this active chromatin state is sensitive to DNase1 digestion (Gazit et al. 1982; Kerem et al. 1984, 1983; Pfeifer and Riggs 1991). Using DNase1 for in situ nick translation on metaphase chromosomes, we were able to distinguish between the active

and inactive X chromosomes in *P. melanophrys* females and visualize active autosomal regions. The X/Y chromosome-derived, translocated region on the chromosome 1 pair was sensitive to DNase1 digestion, which indicates that this region has lost its epigenetic signature of X inactivation and is transcriptionally active. Our results agree with previous X-autosome translocation studies that concluded the X inactivation center (Xic) is required *in cis* for the propagation and spread of the X inactivation signal (Duthie et al. 1999; Lee and Jaenisch 1997; Popova et al. 2006; Sharp et al. 2001, 2002). For example, Xist does not extend into the autosomal chromomome 4^x in the Mus T37H translocation (Popova et al. 2006). Several X-autosome translocation studies demonstrated that in the absence of a physical association with the Xic, X inactivation signal was restricted to the X chromosome and did not spread to the adjacent autosomal material (Ashley 2002; Duthie et al. 1999; Shao and Takagi 1991).

Replication timing and heterochromatin

Transcriptionally active regions typically replicate early in S-phase, whereas inactive regions replicate late in S-phase (Ashley 2002; Diaz-Perez et al. 2006; Gilbert 2002; Goren and Cedar 2003; Keohane et al. 1999). The discrepancy between the replication timing of the active and inactive X chromosomes in females exemplifies the correlation between replication timing and gene expression (Goren and Cedar 2003). However, there is not a direct relationship between replication timing and gene expression (Gilbert 2002). Sharp et al. (2001) demonstrated long-range inactivation of autosomal regions can occur without a delay in replication timing. Conversely, they claim that autosomal genes located in late-replicating regions are always inactive (Sharp et al. 2002). Our DNase1 results indicate that the translocated region in *P. melanophrys* is transcriptionally active, but we also wanted to determine if the translocated region replicated early or late in S-phase (i.e., did it retain its Xi signature). To accomplish this, we used BrdU to analyze the replication timing properties of this region. The inactive X of females and the Y chromosome of males were heavily labeled with BrdU after a 3-h incubation period, as expected. The distal tip of the chromosome 1 p-arm also incorporated BrdU, although differentially between the homologs, which indicates that the translocated region retained its late-replicating state. Interestingly, the delay in replication timing was isolated to the distal tip of the p-arm and did not spread beyond the boundary of the translocated region. Thus, one homolog retains the replication timing signature of Xi.

The different types of heterochromatin are all characterized by a delay in replication timing and nucleosomal patterning with $H3K9me_3$ and hypoacetylated histones (Dillon 2004). Constitutive heterochromatin is present in the centric and pericentric regions throughout the cell cycle (Dillon 2004; Peters et al. 2002). Facultative heterochromatin is developmentally regulated and only found on the inactive X chromosome in females (Chow and Brown 2003; Dillon 2004; Heard 2005). Methylation of H3K27 is the distinguishing feature between constitutive and facultative heterochromatin; constitutive H3K27 is mono-methylated (Dillon 2004), whereas H3K27 is tri-methylated in facultative heterochromatin (Dillon 2004; Plath et al. 2003; Silva et al. 2003).

As expected, the pericentric regions *P. melanophrys* chromosomes were C-band positive (results not shown). The translocated region on the distal tip of *P. melanophrys* chromosome 1 was also C-band positive, which confirms the presence of heterochromatin (Fig. 5). Interestingly, the C-band staining was most pronounced in the translocated region on *P. melanophrys* chromosome 1. Although C-banding techniques cannot distinguish between constitutive heterochromatin and intercalary heterochromatic blocks (IHBs), a type of heterochromatin found at the gonosome–autosome junction in X-autosome translocations (Deuve et al. 2006; Dobigny et al. 2004a; Macaisne et al. 2006), it is unlikely that the C-band positive translocated region on *P. melanophrys* chromosome 1 is an IHB. According to previous studies, IHBs are only present when the autosomal segment is structurally linked to the X chromosome (Dobigny et al. 2004a; Macaisne et al. 2006). In *P. melanophrys*, the gonosome material is translocated to chromosome 1, so there is not a physical association between the autosome and X chromosome. In addition, antibodies specific to trimethylated H3K27 did not co-localize with the chromosome paint, indicating a lack of facultative heterochromatin on chromosome 1 (Dillon 2004; Plath et al. 2003; Silva et al. 2003).

While our work shows that repeated DNAs translocated from the X chromosome to an autosome retain some of the epigenetic signatures of Xi (late replication timing of one homolog), other epigenetic features of Xi have been lost (differential DNase I hypersensitivity and trimethyl-H3K27 modification). Given our WCP data, it is likely the majority, if not all, of the translocated material is derived from repeats on the X chromosomes. The expectation is that the p-arms of both chromosome 1 homologs would share replication timing status among these results. In contrast, there appears to be a chromosome-specific disparity in timing. Whether this is specific to parental origin of the chromosome 1 homolog (maternal vs paternal) is unknown. Likewise, it is unknown whether other marks of Xi, specifically those associated with repeated DNAs, are found to differentially mark one chromosome 1 homolog, such as non-coding RNAs or an enrichment for LINEs. Understanding the mechanism of repeat translocation, the process of losing uniparental epigenetic modifications, and the potential effects of retaining differential replication timing signatures on sister homologs will provide important clues into the evolution of Xi and potentially the derivation of genomic imprinting at autosomal loci.

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