Supporting Information

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SI Materials and Methods

Antibodies. Before use, antibodies specific for modified histones and HP1α were diluted 1:800 and α5meC was diluted 1:500 in 0.01% (vol/vol) TKCM buffer [120 mM KCl, 20 mM NaCl, 10 mM Tris HCl (pH 7.5), 0.5 mM EDTA, 0.01% (vol/vol) Triton X-100] with 2.5% (vol/vol) goat serum [1% (vol/vol) for 5MeC]. Antibodies against the following histone modifications failed to produce signals: H3K9me2 (Upstate, Abcam), H3K27me3 (Abcam), HP1γ (Chemicon), and macroH2A (Upstate).

Immunofluorescence. ***Histone modification.*** The protocol used for the distribution analysis of histone modifications was adapted from published procedures (1, 2), allowing a more accurate distribution comparison, particularly between the multiple platypus sex chromosomes and autosomes. The platypus has five different X chromosomes, which are recognized by size, centromere position, and DAPI banding; hence, metaphase preparations must be well spread for satisfactory identification.

Chromosome preparation. Cell cultures were incubated with Colcemid (1:100; GIBCO) for 2–4 h, after which cells were collected by mechanical shake-off. Medium was removed after centrifugation, and cells were treated in 75 mM KCl with 0.1% (vol/vol) glycerol for 10–15 min at 37 °C. Three hundred microliters was deposited per slide in a cytospin holder and spun for 5 min at 300 rpm (Cytospin 3, Shandon). One 70% confluent 175-cm^2 flask was sufficient for six to eight cytospin slides. After centrifugation, slides were allowed to dry for 10–20 s; the deposit area was covered with 0.3% (vol/vol) TKCM buffer for 25 min, after which this area was washed three times with 0.01% (vol/vol) TKCM buffer.

Immunofluorescence. Subsequently, the area was covered with 10% goat serum [in 0.01% (vol/vol) TKCM buffer] for 7 min. After shake-off, the area was covered with diluted primary antibody and incubated at room temperature for 30–45 min in a wet box. After three 4-min washes in 0.01% (vol/vol) TKCM buffer, the area was covered with secondary antibody (Alexa-568 goat anti-rabbit antibody, Invitrogen) at a 1:200 ratio in 0.01% (vol/vol) TKCM buffer and incubated in a wet box for 15 min. Slides were fixed with ice-cold 3:1 methanol/acetic acid. Subsequent fixation with 4% (gr/vol) paraformaldehyde for 15 min was found to be redundant, and results were the same with or without paraformaldehyde fixation. Slides were mounted in Vectashield containing DAPI (Vector).

DNA methylation analysis with formamide DNA denaturation. Standard protocols were followed for metaphase preparations (3). Briefly, cell suspensions were dropped on slides and subsequently dehydrated through 70–100% (vol/vol) ethanol series and air-dried. The slides were denatured in 70% (vol/vol) formamide at 65 °C for 1 min and then incubated in ice-cold 70% (vol/vol) ethanol for 5 min, dehydrated through ethanol series, and air-dried. The metaphase area was covered with 10% goat serum [in 0.01% (vol/vol) TKCM buffer] for 7 min. This area was then covered with the primary antibody and incubated for 45–60 min in a wet box at room temperature. Subsequently, the slides were washed three times for 5 min each time with 0.01% (vol/vol) TKCM buffer. The area was covered with the secondary antibody [Alexa-488 goat anti-mouse (Invitrogen), 1:100 ratio in 0.01% (vol/vol) TKCM buffer] and incubated for 30 min, after which the slides were washed as before. The slides were then fixed in 4% (gr/vol) paraformaldehyde for 15 min, washed in PBS (three times for 3 min each wash), and mounted in Vectashield containing DPI. In addition to the above protocol and in light of initial findings, a second DNA methylation protocol was used.

DNA methylation analysis with HCl DNA denaturation. This protocol was similar to the one above, with the exception of the denaturation procedure and antibody preparation. In particular; antibodies were diluted in PBS with 0.05% (vol/vol) Tween (PBT) rather than TKCM buffer. For denaturation, slides were incubated for 20 min in 2 N HCl at 37 °C and incubated in 0.1 M borax (pH 8.4) for 1 min. Finally, slides were incubated for 1 min in PBT, dehydrated through ethanol, and rehydrated in PBT for 3 min. Subsequently, the area with metaphases was covered with 10% (vol/vol) goat serum [in 0.01% (vol/vol) TKCM buffer] for 7 min and handled as above from this step onward.

Fluorescence Microscopy. For each experiment, 20–40 metaphase spreads were scored for each species, including the mouse. Images were captured using Leica QFISH software (Leica Microsystems) and a cooled CCD camera (Photometrics Sensys; Photometrics) mounted on a Leica DMRXA microscope (Leica Microsystems) equipped with 40×, 63×, and 100× objectives. Cy3, FITC, and DAPI signals were captured separately as 16-bit black and white images and merged to a color image. The DAPI image was enhanced with a spatial filter. Relevant chromosomes can be recognized by size, centromere position, and banding pattern (4, 5) (Fig. 2*L*). Line scanning of individual chromosomes was conducted using CW4000 CytoFISH software (Leica Microsystems). All image processing was performed with Leica CW4000 software (Leica Microsystems).

1. Belyaev N, Keohane AM, Turner BM (1996) Differential underacetylation of histones H2A, H3 and H4 on the inactive X chromosome in human female cells. *Hum Genet* 97: 573–578.
2. Bisoni L, Batlle-Morera L, Bird AP, Suzuki M, McQueen HA (2005) Female-specific hyperacetylation of histone H4 in the chicken Z chromosome. *Chromosome Res* 13:205–214.
3. Rens W, O'Brien PC, Yang F, Graves JA, Ferguson-Smith MA (1999) Karyotype relationships between four distantly related marsupials revealed by reciprocal chromosome painting. *Chromosome Res* 7:461–474.
4. Rens W, et al. (2004) Resolution and evolution of the duck-billed platypus karyotype with an X1Y1X2Y2X3Y3X4Y4X5Y5 male sex chromosome constitution. *Proc Natl Acad Sci USA* 101:16257–16261.
5. Rens W, et al. (2001) Karyotype relationships between distantly related marsupials from South America and Australia. *Chromosome Res* 9:301–308.

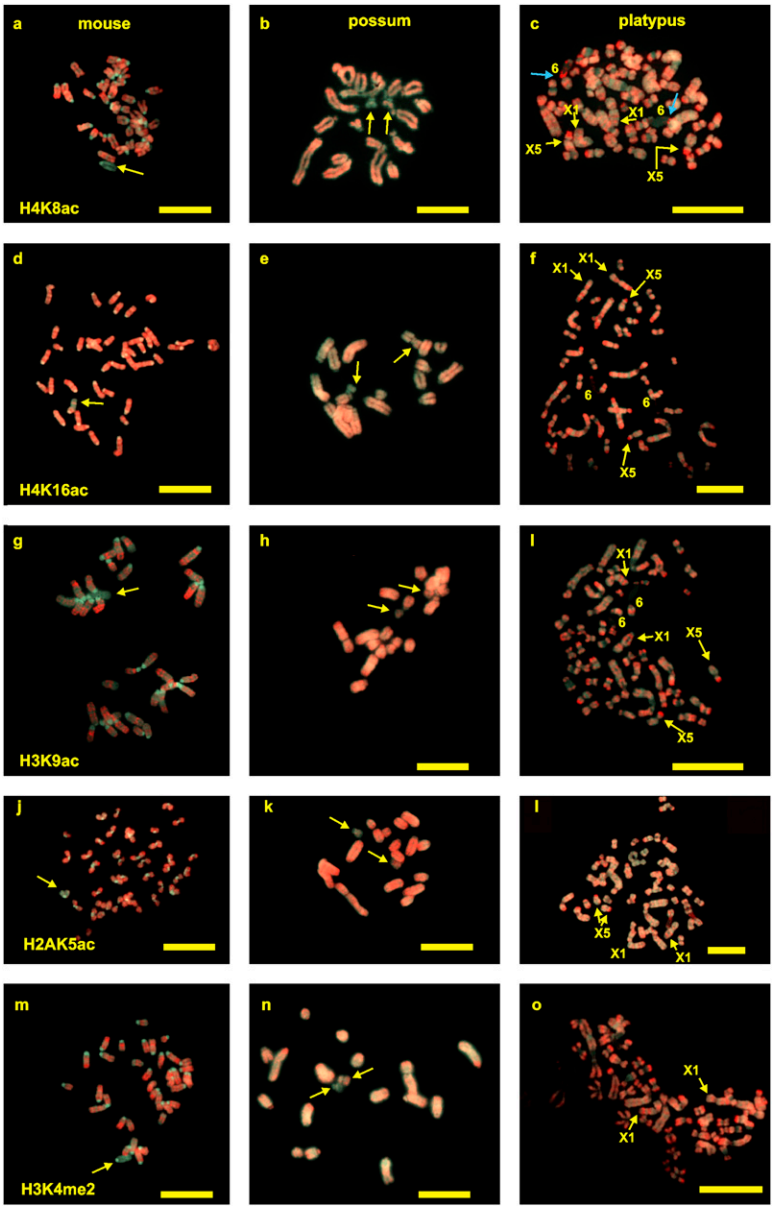

Fig. S1. (*A–O*) Activating modifications in the female mouse, possum, and platypus. The columns represent each species, and the rows present each epigenetic modification. In all images, chromosomes are shown in light green and antibody staining is shown in red. The arrows in the mouse column point to the inactivated X. The arrows in the possum column point to both X homologs. In the platypus column, relevant chromosomes are indicated. Blue arrows in *c* point to an H3K8ac-enriched region on platypus 6. (Scale bar, 10 μm.)

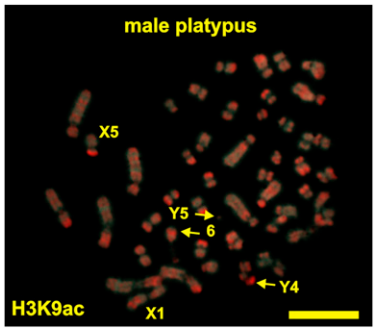

Fig. S2. Distribution of H3K9ac on male platypus chromosomes. The chromosomes are shown in light green, and antibody staining is shown in red. The relevant chromosomes are indicated. (Scale bar, 10 μm.)

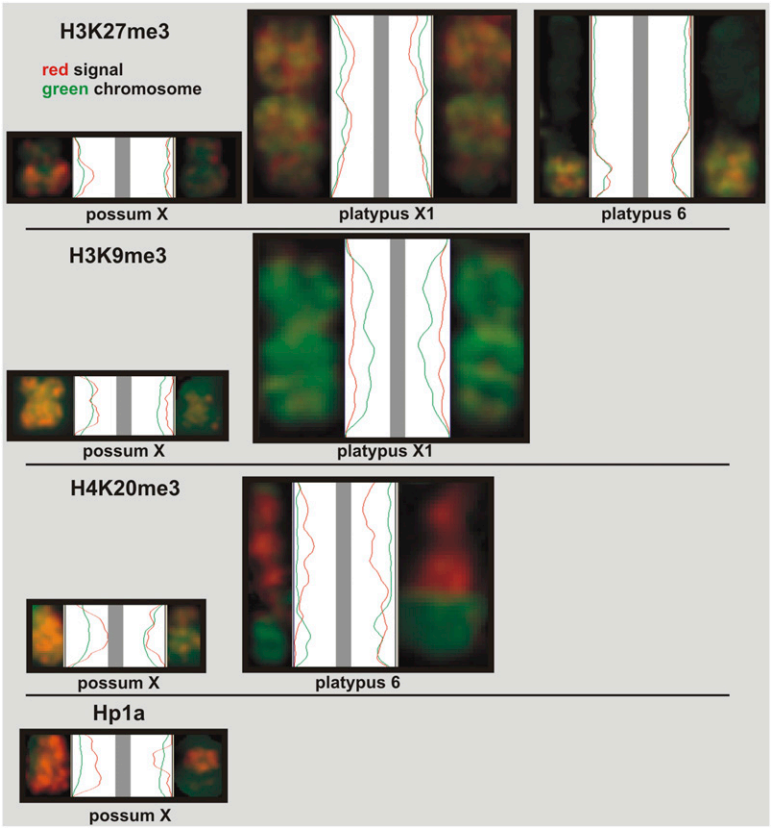

Fig. S3. Line scans of distributions of canonical repressive histone modifications. The green curves correspond to the DAPI staining along the length of the chromosomes. The red curves show the distribution of repressive epigenetic marks. As indicated, each row corresponds to a specific modified histone, the first column represents possum X's, and the other two columns show platypus X_1 and chromosome 6.

Table S1. Antibodies used to detect epigenetic modifications

Antibody	Catalog no.	Source
H4K8ac	06-760	Upstate
H4K16ac	AHP417	Serotec
H3K9ac	Ab12178	Abcam
H3K4me2	07-030	Upstate
H2AK5ac	07-290	Upstate
H3K27me3 and H3K27me3	07-449	Upstate*
H3K9me3	07-523	Upstate
H4K20me3	Ab9053-25	Abcam
Hp1α	MAB3584	Millipore
5MeCyt	MMS-900P-A	Eurogentec Seraing Belgium

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