Epigenetic modifications on X chromosomes in marsupial and monotreme mammals and implications for evolution of dosage compensation

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X chromosome dosage compensation in female eutherian mammals is regulated by the noncoding *Xist* RNA and is associated with the differential acquisition of active and repressive histone modifications, resulting in repression of most genes on one of the two X chromosome homologs. Marsupial mammals exhibit dosage compensation; however, they lack *Xist*, and the mechanisms conferring epigenetic control of X chromosome dosage compensation remain elusive. Oviparous mammals, the monotremes, have multiple X chromosomes, and it is not clear whether they undergo dosage compensation and whether there is epigenetic dimorphism between homologous pairs in female monotremes. Here, using antibodies against DNA methylation, eight different histone modifications, and HP1, we conduct immunofluorescence on somatic cells of the female Australian marsupial possum *Trichosurus vulpecula*, the female platypus *Ornithorhynchus anatinus*, and control mouse cells. The two marsupial X's were different for all epigenetic features tested. In particular, unlike in the mouse, both repressive modifications, H3K9me3 and H4K20Me3, are enriched on one of the X chromosomes, and this is associated with the presence of HP1 and hypomethylation of DNA. Using sequential labeling, we determine that this DNA hypomethylated X correlates with histone marks of inactivity. These results suggest that female marsupials use a repressive histone-mediated inactivation mechanism and that this may represent an ancestral dosage compensation process that differs from eutherians that require *Xist* transcription and DNA methylation. In comparison to the marsupial, the monotreme exhibited no epigenetic differences between homologous X chromosomes, suggesting the absence of a dosage compensation process comparable to that in therians.

DNA methylation | histone modifications | X chromosome inactivation

In mammals, X inactivation has evolved to solve the difference in X chromosome gene dosage between homogametic female mammals and heterogametic male mammals. Inactivation of one of the two female X chromosomes provides an equal dose in eutherian mammals and marsupials. In the mouse, X-chromosome inactivation (XCI) is imprinted in early development and is then reprogrammed at the blastocyst stage to become random, where, in response to expression of *Xist* RNA from the future inactive X (Xi), repressive histone modifications, histone variants, and DNA methylation are acquired (1, 2). This results in transcriptional repression of most X-linked genes. In particular, the eutherian Xi is late in replicating and is distinguished from the active X by a specific set of covalent histone modifications. The Xi is hypoacetylated on the NH₂-terminal lysines of nearly all histones (3, 4) and lacks H3K4me2 and H3K4me3 but carries H3K9me2, H3K9me3, and H3K27me3 (1). In addition, Xi chromatin is enriched in the histone variants macroH2A1 and macroH2A2 (5). In human and bovine models, H3K27me3 and H3K9me3 are spatially distributed in nonoverlapping regions (6, 7). These stable histone modifications appear shortly after the accumulation of *Xist* RNA along the X with H3K9 methylation as the first modification occurring at the onset of XCI (8, 9). The *Xist* gene, together with the noncoding transcripts that contribute to its regulation, *Tsix* and

Xite, is located within an X-inactivation center (Xic) that also regulates X chromosome counting and choice (10).

In marsupials, less is known about XCI; however, aspects are comparable but not identical to those of eutherian species. In female marsupials, dosage compensation also involves chromosome-wide X inactivation, but marsupials do not have an ortholog of the eutherian *Xist* gene (11, 12) and XCI is not random but imprinted, with the paternally inherited X being inactivated. The eutherian *Xist* inactivation system therefore evolved within a time interval of 28 million years: after marsupial divergence about 148 Mya and before the major radiation of placental mammals 120 Mya (13). The marsupial therefore provides a useful model for studying the evolution of dosage compensation and epigenetic silencing mechanisms in mammals. Interestingly, it has recently been suggested that pre-implantation eutherian imprinted X inactivation is less dependent on *Xist* than the later random inactivation process (14). In the tammar wallaby, *Macropus eugenii*, analyses indicating H4 hypoacetylation of one of the two X chromosomes suggested mechanistic similarities with eutherians; however, a more recent study by the same group suggested absence of repressive histone modifications and proposes that the Xi chromosome in marsupials fails to recruit repressive modifications as seen in eutherians (15, 16).

Monotremes have a peculiar sex chromosome constitution. Monotremes have an X-Y system that appears unrelated to that of the marsupial and eutherian (17, 18). They diverged 166 Mya at the base of the mammalian branch (19). Strikingly, the male platypus has five different X chromosomes and five different Y chromosomes (20), and the male echidna has five different X chromosomes and four different Y chromosomes (17). Hence, the female platypus and echidna have the five X chromosomes in pairs and no Y chromosomes. In monotremes, the sex chromosomes form a chain at male meiosis linked by their pairing regions (17, 20, 21). These pairing regions do not cover the sex chromosomes entirely but are separated by a differential region. In particular, platypus X₁ and X₅ contain large differential regions. Monotreme sex chromosomes share considerable homology with chicken chromosomes Z, 2, 12, 13, and 17 and with certain human autosomes (17). Genes orthologous to the human Xq are found on platypus chromosome 6 (22) and echidna chromosome 16 (17); hence, the platypus does not require dosage compensation. Nonetheless, the five X-differential regions that account for about 12% of the platypus genome contain genes whose products are predicted to be more abundant in the female platypus than in the male platypus unless some form of dosage compensation takes place. Such genes remain to be characterized, and, to our knowledge, no assessment of epigenetic

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centric chromatin. In mouse metaphases and, in contrast to all interphase nuclei where it was present, HP1 α could only be detected in less than 30% of the spreads (Fig. 2J). Fig. 2L shows that platypus chromosome 6 X₁ and X₅ can be recognized by its size, centromere position, and banding pattern.

In marsupials, the inactivated X was recognized by all four repressive marks. In contrast to the mouse control cells, H3K9me₃, H4K20me₃, and HP1 α were clearly enriched on one of the X chromosomes in all observed metaphases (Fig. 2E, H, and K). H3K27me₃ was found in 50% of observed metaphases on the possum Xi (Fig. 2B). This is consistent with recent work showing H3K27me₃ on the *M. domestica* Xi (28). All four repressive marks were enriched at pericentric and telomeric regions of the possum chromosomes. Because H3K9me₃ was epigenetically dimorphic on the possum X chromosomes, it was not surprising to detect an enrichment of HP1 α on the possum Xi, because H3K9me₃ binds HP1 α (29). As in humans (30), HP1 α may recognize and facilitate heterochromatin maintenance and gene silencing in the possum Xi.

In the platypus, repressive histone modifications were evident on both chromosome homologs for all sex chromosomes and autosomes. Unfortunately, for the experiment with platypus H4K20me₃, we could not unambiguously distinguish either X₁ or

X₅, although all chromosomes were equally stained. However, both H3K9me₃ and H4K20me₃ were strongly enriched at blocks of constitutive heterochromatin, especially at the satellite region of the platypus NOR-bearing chromosome 6 (Fig. 2F and I), and differential distributions were not observed. The strong enrichment at constitutive heterochromatic blocks is in contrast to H3K27me₃, whose pattern corresponded more to R-banding and which was depleted on the satellite region of platypus chromosome 6 (Fig. 2C). No metaphases with HP1 α protein were detected. However, interphase nuclei showed clusters of this protein, suggesting that HP1 α does colocalize to heterochromatic DNA in a cell cycle-dependent manner.

Fig. S3 shows line scans of the distribution of repressive histone modifications. The inactivated possum X homolog is brighter for all repressive marks, as shown by the higher amplitude of the red curve. Similar to the active histone modifications (see above) the platypus X₁ homologs do not show a differential pattern for the repressive epigenetic marks.

DNA Methylation. Immunostaining with a 5-methylcytosine (meC) antibody was used to visualize the overall DNA methylation state of mouse, possum, potoroo, opossum, and platypus metaphase

Fig. 2. (A–K) Repressive 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 arrow in the mouse column points to the inactivated X. The arrows in the possum column point to both X homologs. In the platypus column, relevant chromosomes are indicated. HP1 α did not stain platypus metaphases. (L) DAPI-stained platypus chromosome 6 and X₁ and X₅ chromosomes. Each chromosome can be recognized by its specific size, centromere position, and banding pattern. (Scale bar, 10 μ m.)

platypus dosage compensation at six genes by quantitative RT-PCR, RNA FISH, and SNP analysis. Findings were consistent with some form of compensation at the transcriptional level for some but not all genes assessed. These results support our conclusion that there is no chromosome-wide X inactivation in monotremes.

Materials and Methods

Cell Culture. The platypus [*O. anatinus*, diploid chromosome number 52 ($2n = 52$)] and brush-tailed possum (*T. vulpecula*, $2n = 20$) fibroblast cultures [established for our previous studies (44)] were grown at 32 °C in standard medium; for platypus cell cultures, the medium was supplemented with an equal volume of Amniomax (GIBCO, Invitrogen) to promote cell growth. Female fibroblast metaphase preparations from the potoroo (*P. tridactylus*, $2n = 12$ female and $2n = 13$ male), whose normal sex chromosome constitution involves an X-autosome translocation, and from a male opossum (*M. domestica*, $2n = 18$) were generated as described previously (45, 46). Mouse ($2n = 40$) primary adult (CBAx57BL6) lung and embryo (129Sv) fibroblasts were generated and grown at 37 °C in standard medium. All cells were harvested at around 60–70% confluency. After hypotonic treatment (*SI Materials and Methods*), mouse cells were added to the harvested marsupial/monotreme cells, pelleted, and spun onto slides so that cells from the control and test species underwent identical processing. Marsupial and monotreme

metaphase chromosomes were identified by cytogenetic analysis, as described previously (20, 45). Mouse sex chromosomes were identified by X-chromosome paints when appropriate (47).

Antibodies and Immunofluorescence. Antibodies used to detect epigenetic modifications, HP1, and DNA methylation are listed in Table S1, along with methodology for their use on chromosome preparations using protocols adapted from published procedures (3, 48) (*SI Materials and Methods*). Chromosome preparations were conducted as described previously (45) and are further outlined in *SI Materials and Methods*.

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) (*SI Materials and Methods*).

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