Evolution from XIST-Independent to XIST-Controlled X-Chromosome Inactivation: Epigenetic Modifications in Distantly Related Mammals

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Abstract

X chromosome inactivation (XCI) is the transcriptional silencing of one X in female mammals, balancing expression of X genes between females (XX) and males (XY). In placental mammals non-coding XIST RNA triggers silencing of one X (Xi) and recruits a characteristic suite of epigenetic modifications, including the histone mark H3K27me3. In marsupials, where XIST is missing, H3K27me3 association seems to have different degrees of stability, depending on cell-types and species. However, the complete suite of histone marks associated with the Xi and their stability throughout cell cycle remain a mystery, as does the evolution of an ancient mammal XCI system. Our extensive immunofluorescence analysis (using antibodies against specific histone modifications) in nuclei of mammals distantly related to human and mouse, revealed a general absence from the mammalian Xi territory of transcription machinery and histone modifications associated with active chromatin. Specific repressive modifications associated with XCI in human and mouse were also observed in elephant (a distantly related placental mammal), as was accumulation of XIST RNA. However, in two marsupial species the Xi either lacked these modifications (H4K20me1), or they were restricted to specific windows of the cell cycle (H3K27me3, H3K9me2). Surprisingly, the marsupial Xi was stably enriched for modifications associated with constitutive heterochromatin in all eukaryotes (H4K20me3, H3K9me3). We propose that marsupial XCI is comparable to a system that evolved in the common therian (marsupial and placental) ancestor. Silent chromatin of the early inactive X was exapted from neighbouring constitutive heterochromatin and, in early placental evolution, was augmented by the rise of XIST and the stable recruitment of specific histone modifications now classically associated with XCI.
may be involved in the stabilization and somatic heritability of the silent state [20]. Little is known about the recruitment of these modifications, except that Xist RNA seems to recruit the Polycomb complex PRC2 responsible for H3K27me3 [17,18,20], and the protein(s) responsible for H4K20me1 [20].

Novel insight into the mechanism of placental mammal XCI can be gained by comparing human and mouse epigenetic components to those of distantly related mammals. Work on the distantly related afrotherian mammals (placental mammals such as the elephant) revealed an Xi displaying classic features of XCI (i.e. Barr body formation and late replication compared to the active X [25,26]) and bearing the XIST gene [9], suggesting that many features of placental mammal XCI were established before the placental (eutherian) radiation.

Marsupial (metatherian) mammals are even more distantly related to humans and mice, having diverged from placental mammals 148MYA. XCI also occurs in marsupials. There are several molecular and phenotypic differences between marsupial XCI, and human and mouse XCI, which offers important insights into how this process works, and how it evolved. Like the Xi in human and mouse, the marsupial Xi replicates late in S phase [27,28], and sex chromatin (Barr body) has been observed in some species and tissues [29]. However, contrary to human and mouse, marsupial XCI has been described as incomplete, tissue-specific [30], and unstable [31]. Differential histone H4 acetylation was associated with XCI in human and mouse, whereas X inactivation is random in placental mammals. This led to the suggestion that marsupial imprinted XCI could be gained by comparing human and mouse epigenetic features underpinning XCI, as it represents only a fraction of the cell cycle with a specific condensed state of chromosome, and may not represent histone modifications on Xi in the rest of the cell cycle. A recent report showed a H3K27me3 enrichment on opossum Xi at different frequencies in brain and liver tissues of opossum (Monodelphis domestica) [42]. Here we conducted a detailed, extensive and comparative analysis of the pattern of active and repressive histone marks in interphase nuclei of phylogenetically important mammalian species: two marsupial models (tammar wallaby and opossum) and an afrotherian (elephant). We observed a specific pattern of inactive marks on the marsupial Xi, with a variable degree of stability throughout cell cycle, and strikingly different from the pattern seen in all placental mammals studied so far, including the elephant (examined herein). These results shed new light on the evolution of X chromosome inactivation and the role of the XIST gene.

Results

Profile of H3K27me3 on the inactive X chromosome of distantly related mammals

In placental mammals (human/mouse) the inactive X chromosome (Xi) can be easily recognized by detecting XIST RNA accumulation with RNA FISH, or by immuno-staining of the trimethylation of lysine 27 of histone H3 (H3K27me3) [17,18,20].

Elephant. We performed immunofluorescence (IF) against H3K27me3 on interphase nuclei of male and female elephant cells. A nuclear territory was enriched in H3K27me3 of histone H3 (H3K27me3) [17,18,20].

Table 1. Profiles of repressive histone modifications associated with constitutive or facultative heterochromatin in placental and marsupial mammals.

| Modification | Constitutive heterochromatin | XIST dependent | XIST independent | Marsupial Xi |
|--------------|-----------------------------|----------------|-----------------|-------------|
| Repressive marks | H3K9me2 | -- | + | -- | 30% |
| | H3K27me3 | -- | + | -- | 30% |
| | H4K20me1 | -- | + | -- | -- |
| | H2AK11ub | -- | + | -- | ? |
| | H3K9me3 | + | -- | + | + |
| | H3K27me1 | + | -- | -- | -- |
| | H4K20me3 | + | -- | + | + |
| Active marks | H3K4me | -- | -- | -- | -- |
| | H3K9ac | -- | -- | -- | -- |
| | H4Kac | -- | -- | -- | -- |

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we showed that this Barr body was decorated by XIST transcripts (Figure 1B). Thus the inactive X chromosome of female basal placental mammals, like other placentals, harbours a stable accumulation of XIST RNA and H3K27me3, demonstrating that this system was established before the divergence of Afrotheria from other placental mammals 105 MYA [43].

**Marsupials.** In order to find a cytological marker for the marsupial Xi, we performed IF for H3K27me3 on interphase nuclei of male and female primary fibroblasts of two marsupial representatives, the tammar wallaby and the opossum. Primary cultures were studied at low passage (<5), with two independent replicates for the tammar wallaby. We used three independent and extensively tested antibodies to confirm our results (one polyclonal [Upstate-Millipore] and two monoclonal [Abcam and [19]); Table 2). In both marsupial species a large nuclear domain was strikingly enriched for H3K27me3 in 30% of nuclei from female, but not male cells (n = 100 for both sexes, for each primary culture and for each antibody) (Figure 1A, centre and right panels). This domain of H3K27me3 enrichment was often (~65%) but not always associated with a DAPI-dense domain, suggesting that this enrichment is not only the result of chromatin compaction (see examples in Figures 1, 2 and 3).

To confirm that this domain was indeed the Xi in the tammar wallaby, we performed IF combined with RNA FISH using a BAC containing UBE1, an X-linked gene that largely escapes X inactivation in wallaby (i.e. is bi-allelically expressed in ~70% of nuclei; [44]). When UBE1 was bi-allelically expressed in cells showing a large H3K27me3 enrichment domain, one of the two signals was located at the border of this domain, confirming that it represented one X chromosome (n = 30) (Figure 1C). When it was mono-allelically expressed the single signal was never associated with the H3K27me3 domain, confirming that the X enriched in H3K27me3 was the Xi.

**Cell cycle specificity of H3K27me3 enrichment on the marsupial Xi.** This H3K27me3 enrichment on the Xi in 30% of marsupial cells (contrasting to almost 100% in placental mammals), as well as the failure to detect it on wallaby mitotic chromosomes [41], led us to hypothesize that H3K27me3

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**Figure 1. Status of H3K27me3 enrichment and XIST-RNA accumulation on the inactive-X in distantly related mammals.**

A. Examples of immunofluorescence for H3K27me3 (in green) on elephant cells (left panel), tammar wallaby cells (centre) and opossum cells (right panel). The top row shows a representative nucleus from female cell, the bottom row from male cell. Arrow heads point the inactive X chromosome enriched in H3K27me3. n = 100.

B. Example of RNA FISH showing accumulation of XIST transcripts (in grey) on the DAPI-dense inactive X chromosome of female elephant cells. Arrow head points the inactive X chromosome containing UBE1 transcripts and enriched in H3K27me3. Asterisk shows UBE1 transcripts from the active X chromosome. n = 30.

C. Immunofluorescence for H3K27me3 (in green) combined with RNA FISH for the UBE1 primary transcripts (in red) in female cells from tammar wallaby. Arrow head points the inactive X chromosome containing UBE1 transcripts and enriched in H3K27me3. Asterisk shows UBE1 transcripts from the active X chromosome. n = 30.

D. Dual immunofluorescence for H3K27me3 (in green) and H3S10ph (in red) on female tammar wallaby cells showing that enrichment in H3K27me3 (arrow heads) on the Xi is restricted to the G1/S phase and the early G2 phase of the cell cycle. n = 90.

E. Immunofluorescence for H3K27me3 (in green) combined with a cell proliferation assay to label replicating DNA (in red) showing H3K27me3 enrichment on the Xi in late S phase, at the time of Xi replication (enrichment of H3K27me3 (green) on the replicating Xi (red)). n = 200. DAPI is shown in grey. Scale bar = 1 μm.

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Table 2. Primary antibodies used for the immunofluorescence experiments.

| Antibody                  | Dilution | Species | Supplier                  |
|---------------------------|----------|---------|---------------------------|
| RNA Polymeerase II (CTD4H8) | 1/200    | Mouse   | Upstate-Millipore (05-623) |
| Histone H3K4me2           | 1/200    | Rabbit  | Upstate-Millipore (07-030) |
| Histone H3K9ac            | 1/100    | Rabbit  | Upstate-Millipore (07-352) |
| Histone H3K9me2           | 1/200    | Rabbit  | Upstate-Millipore (07-441) |
| Histone H3K9me3           | 1/300    | Rabbit  | Upstate-Millipore (07-442) |
| Histone H3K14ac           | 1/200    | Rabbit  | Upstate-Millipore (07-353) |
| Histone H3R17me           | 1/200    | Rabbit  | Upstate-Millipore (07-214) |
| Histone H3K27me1          | 1/300    | Rabbit  | Upstate-Millipore (07-448) |
| Histone H3K27me3          | 1/200    | Rabbit  | Upstate-Millipore (07-449) |
| Histone H3K27me3          | 1/200    | Mouse   | Abcam (6147)              |
| Histone H3K27me2/3        | 1/200    | Mouse   | Rougeulle et al., 2004    |
| Histone H4Kac             | 1/200    | Rabbit  | Upstate-Millipore (06-946) |
| Histone H4K5ac            | 1/100    | Rabbit  | Upstate-Millipore (07-327) |
| Histone H4K8ac            | 1/200    | Rabbit  | Upstate-Millipore (07-328) |
| Histone H4K12ac           | 1/300    | Rabbit  | Upstate-Millipore (07-761) |
| Histone H4K20me1          | 1/300    | Rabbit  | Upstate-Millipore (07-440) |
| Histone H4K20me3          | 1/200    | Rabbit  | Upstate-Millipore (07-463) |
| Histone H3S10ph           | 1/500    | Rabbit  | Upstate-Millipore (06-570) |

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accumulation is cell cycle specific. Because primary cells are sensitive to artificial treatment, we used techniques that identified cell cycle stage (rather than cell synchronization) to minimize the risk of interfering with normal accumulation (or loss) of transient modifications.

We first used phosphorylation of serine 10 of histone H3 (H3S10ph) as a marker of cell cycle phases (Table 2). This modification, involved in chromatin condensation, is absent in G1/S and starts to associate with few centromeres in early G2, all centromeres in mid G2, and then spreads to the whole arms of chromosomes in late G2/mitosis [43]. H3K27me3 enrichment was visible in 80% of early G2 phase nuclei, but never in mid to late G2 or mitosis (n = 90) (Figure 1D), supporting its absence on metaphase chromosomes [41]. H3K27me3 enrichment was also detected 15% of G1/S phase.

Therefore, we used a cell proliferation assay to label replicating DNA (Click-it EdU Alexa Fluor 488, Invitrogen) to discriminate between G1 (no nuclear EdU staining), early S phase (overall nuclear EdU staining) and late S phase (EdU staining on centromeres/Xi). The G1/S phase enrichment of H3K27me3 was only detected in S phase, and never in G1 (n = 200). 20% of S phase enrichment was observed in early S phase, and 80% was observed in late S phase (Figure 1E). Thus, in marsupials, H3K27me3 begins to accumulate on Xi in early S phase, but is most prominent on Xi in late S and early G2 phase, around the time of Xi replication [27,28]. Additionally, when H3K27me3 was enriched on Xi, it was located close to the nucleolus in more than 90% of the cases (see examples in Figures 1, 2 and 3).

Exclusion of histone modifications associated with transcription from the afrotherian and marsupial inactive X chromosome

In human and mouse the Xi is depleted for histone modifications associated with active chromatin (euchromatin) [4,13,14,16,46,47,48]. To determine whether this holds true for the afrotherian and marsupial Xi, we performed a series of dual immunofluorescence using H3K27me3 (as a marker for the Xi) in combination with four histone H3 modifications (H3K4me2, H3K9ac, H3K14ac and H3K17me), or histone H4 acetylation (global acetylation as well as single acetylation at K5, K8 and K12) (Figure 2, Table 2). We were also interested in whether or not Xi was lacking RNA Polymeerase II, as was shown in mouse [49] (Figure 2).

We found that all these histone modifications and RNA Pol II were excluded from the Xi in elephant as well as marsupial female cells (100<n<200; Figure 2). In marsupial cells, these euchromatic markers were analysed relative to H3K27me3, i.e., in 30% of interphase cells. Unfortunately technical limitations prohibited the analysis of these active marks in 100% of interphase nuclei as H3K27me3 was the only suitable marker of the Xi available to us: Signals from the X-chromosome paint were faint and unreliable; immunofluorescence with the active marks followed by DNA FISH with X-linked genes resulted in punctate DNA FISH signals that were difficult to correlate with a hole in IF staining; finally, in the next section we describe two histone marks enriched on the marsupial Xi throughout most of the cell cycle, but unfortunately we could not perform combined IF as all the antibodies were polyclonal (raised in rabbit).

However, we confirm here the exclusion of these active marks from the Xi for at least 30% of interphase, which together with our previous study showing that they were also excluded from mitotic chromosomes [41], suggests that exclusion of active marks is a stable feature of the marsupial Xi throughout interphase and metaphase. Moreover, we were able to combine the IF of a stable repressive mark on Xi with RNA Polymeerase II (as this antibody is monoclonal; see last section of result), which confirmed that RNA Polymeerase II was excluded from the Xi throughout the cell cycle. Thus, the stable formation of a transcriptionally inert nuclear compartment devoid of active marks and transcription machinery is a feature of XCI shared across all therian mammals.

Profile of repressive histone marks on inactive X chromosome in distantly related mammals

In human and mouse, the Xi bears a characteristic signature of repressive histone modifications (XCI-marks: H3K9me2, H3K27me3, H4K20me1, H2AK119ub, H2AK119ub, H2AK119ub) (Table 1). This pattern of Xi-associated facultative heterochromatin differs strikingly from constitutive pericentromeric heterochromatin (PCH-marks: H3K9me3, H3K27me1 and H4K20me3) (Table 1) [50,51,52,53]. However, a detailed analysis of human mitotic chromosomes [41,46,47,48]. To determine whether this holds true for the afrotherian and marsupial Xi, we conducted dual immunofluorescences with RNA Pol II (as this antibody is monoclonal; see last section of result), which confirmed that RNA Polymeerase II was excluded from the Xi throughout the cell cycle. Thus, the stable formation of a transcriptionally inert nuclear compartment devoid of active marks and transcription machinery is a feature of XCI shared across all therian mammals.

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elephant cells. As in human and mouse, H3K27me1 and H4K20me3 were only associated with PCH.

**Marsupials.** The pattern of repressive histone marks on the marsupial Xi showed some similarities to other therians, but also displayed some striking differences (n = 100 for each antibody).

As in placental mammals, the Xi positive for H3K27me3 showed an enrichment of H3K9me2 and a depletion of H3K27me1. However, we unexpectedly observed that the marsupial Xi was depleted for H4K20me1; moreover, H4K20me3 and H3K9me3 (PCH-marks in placental mammals) were not only associated with PCH in our marsupial representatives, but were also strongly enriched on the Xi (Figure 3A, bottom panels; Table 1). Because in humans XCI and PCH-marks form non-overlapping regions on the Xi [54], we performed intensity line scans along the enrichment domains. These scans showed that the domains overlapped at least partially in the nucleus, suggesting that in contrast to the human Xi H3K27me3 and H3K9me3/H4K20me3 may be associated with the same sequences on the marsupial Xi (Figure 3B).

Because H3K27me3 is enriched on the marsupial Xi only in late S/early G2 phase, we analyzed whether H4K20me3 and H3K9me3 were also cell cycle dependent or more stably enriched. We performed immunofluorescence followed by DNA FISH (to locate the Xs) with three X-linked genes that are known to escape X inactivation in 40–70% of nuclei (UBE1, ATRX and MECP2) [44]. We consistently observed two clusters of DNA FISH signals (corresponding to the two Xs). In more than 90% of nuclei the H4K20me3 or H3K9me3 domain was invariably situated next to one of the two DNA FISH signals (n = 50) (Figure 3C). Therefore, because these cell lines were not synchronized, H3K9me3 and H4K20me3 must be enriched on Xi throughout at least 90% of the cell cycle. Thus, these two inactive marks, specific to pericentromeric heterochromatin in placental mammals, are stably associated with the Xi in marsupials. We confirmed that RNA Pol II was excluded from the Xi by performing dual IF (with H4K20me3 and RNA Pol II) before DNA FISH (data not shown). Finally, this experiment also demonstrated that UBE1, ATRX and MECP2 were always located at the periphery of the repressive domain, as do genes escaping XCI in mice [49]. Thus, in marsupials, H3K27me3 and H3K9me2 are enriched on Xi in late S phase/early G2, whereas H4K20me3 and H3K9me3 are stably enriched on the marsupial Xi throughout the cell cycle.

**Discussion**

In this study we examined the histone modification profiles of the inactive X chromosome in interphase cells of elephant (a representative of Afrotheria, a clade distantly related to human and mouse), and two marsupial representatives (wallaby and opossum) that represent the therian mammals most distantly related to placental mammals.

**Depletion of active chromatin marks and transcription machinery from the inactive X chromosome is common to all therian mammals**

Here we demonstrated that the Xi of marsupials (and also the elephant) is stably depleted throughout cell cycle of histone marks associated with transcription, which is consistent with their absence from the marsupial Xi during mitosis [32,33,41]. We also showed that RNA polymerase II is excluded from the Xi. Thus, the inactive X chromosome in all therian mammals forms a transcriptionally inert nuclear compartment devoid of active histone marks and transcription machinery, and that this must be an ancestral epigenetic characteristic of the therian X inactivation system.

**Loss of active histone marks, and exclusion of RNA polymerase II, has been ascribed directly or indirectly to the accumulation of XIST transcripts on the placental Xi. Formation of this repressive compartment must be an XIST independent characteristic of XCI in marsupial cells, a feature likely to have been present in the common ancestor to therian mammals. However, this does not necessarily mean that the loss of active histone marks from the Xi is XIST-independent in placental cells, even though it has been demonstrated that this loss of active marks was independent of the gene-silencing function of Xist RNA in mice ES cells [49]. One hypothesis is that there could be another non-coding RNA triggering the formation of the transcriptionally inert compartment in marsupial cells [42], the function of which was supplanted by the rise of XIST in a placental ancestor.

**XIST RNA accumulation and recruitment of inactive histone marks to the inactive X chromosome is conserved across all placental mammals**

The elephant X chromosome was recently demonstrated to have the same gene content and order as the human X chromosome, with only a difference in centromere positioning [58]. This remarkably conserved gene order along the whole placental mammal X chromosome over such long evolutionary time (~105 million years) contrasts with the marsupial X chromosome, which has suffered multiple rearrangements between opossum and wallaby over approximately 63 million years [59].

Genome sequencing revealed that the elephant genome contains XIST [9], dating the origin of this gene after the divergence of marsupials and placentals 145 MYA, but before the placental radiation 105MYA. Here we showed that the Xi is coated by XIST RNA and displays the same specific combination of repressive histone marks as in mouse and human (Table 1). This identical chromatin profile suggests that XIST-dependent XCI was fully established before the placental mammal radiation ~105MY and has been maintained in all lineages (studied thus far) since then. Strong selection against rearrangements that disrupt this conserved cis-acting XCI machinery is consistent with the observed hyper-conservation of the placental mammal X chromosome [58].

**Association of the inactive X chromosome with the two XCI-specific repressive marks is cell cycle specific in tammar wallaby and opossum fibroblasts**

As in placental mammals, the marsupial Xi is enriched for the histone modifications H3K27me3 and H3K9me2. However, although there was a strong enrichment of H3K27me3 it was only observed in 30% of female nuclei, in contrast to enrichment in almost 100% of nuclei in placental mammals. H3K9me2 was enriched less strongly than in placental cells, and only in conjunction with H3K27me3. We showed that enrichment was transient and occurred almost exclusively in late S phase and early G2, around the time when the inactive X is replicating [27,28]. These results are consistent with our previous failure to detect these repressive marks on the inactive X during metaphase [41].
Evolution of X Inactivation Epigenetics

Figure 3. Patterns of repressive marks on the inactive-X of distantly related mammals. A. Dual immunofluorescence with H3K27me3 (in green) combined with H3K9me2, H3K9me3, H4K20me1 or H4K20me3 (in red) in mouse (top left), elephant (top right), tammar wallaby (bottom left) and opossum (bottom right) female cells. n = 100. B. Line scans of H3K27me3, H4K20me3 (left) and H3K9me3 (right) intensities in the Xi territory. C. Sequential immunofluorescence for H4K20me3 (in green; left) or H3K9me3 (in green; right) followed with a DNA FISH for 3 X-linked genes UBE1, ATRX and MECP2 (in red) in tammar wallaby female cells. The arrow head indicates the inactive X chromosome enriched in H3K27me3, asterisks indicates the genes from the active X chromosome. n = 50. DAPI is shown in grey. Scale bar = 1 µm.

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It was recently shown that H3K27me3 is enriched in 60% and 98% of interphase cells from opossum liver and brain respectively [42] which, together with our data showing enrichment in 50% of opossum and wallaby fibroblasts, suggests that the stability of H3K27me3 accumulation is tissue-specific and/or specie-specific in marsupials. A very recent study found H3K27me3 enriched on 50% of the mitotic Xi from Australian common brush-tail possum fibroblast cells [35]. This discrepancy with our current study (and previous study; [41]) may be explained by specie-specific accumulation of H3K27me3 on Xi (wallaby vs. brushtail possum).

Nevertheless, all these studies demonstrate quite variable stability of H3K27me3 on the marsupial Xi in time (cell cycle stages) and space (cell types, species). This is not unexpected given that this mark is XIST dependent in placental mammals [17,18,20]. Even though it remains possible that H3K27me3 (and H3K9me2) is recruited by an unknown non-coding RNA in marsupials, these studies suggest that one important effect of the newly evolved XIST gene was to stabilize H3K27me3 recruitment to Xi.

It is interesting to note that most inactive X chromosomes enriched with H3K27me3 are associated with the nucleolus. It has been shown that in mouse cells, the Xi is targeted to the perinucleolar compartment in mid-to-late S phase, and that this could be involved in maintaining its heterochromatic state by facilitating recruitment of repressive marks, especially H3K27me3 [60]. Location of the marsupial Xi next to the nucleolus may also help define its epigenetic state; however, this mechanism is triggered by the XIST RNA in mice, so in the absence of XIST it is unclear how this mechanism operates in marsupials.

Marsupial inactive X chromosome displays a profile of repressive marks similar to that of constitutive heterochromatin

Surprisingly, the marsupial inactive X is strongly and stably enriched in H3K9me3 and H4K20me3 (>90% of nuclei), marks that are generally associated with pericentric heterochromatin. This supports the recent findings that H3K9me3 and H4K20me3 is present on the mitotic brushtail possum Xi [35]. Thus, these marks are enriched on Xi throughout the entire cell cycle and, therefore, not transient. Moreover, our data suggests that the XCI-marks (H3K27me3) and PCH-marks (H3K9me3/H4K20me3) are organised differently to the mutually exclusive arrangement observed in human cells [54]; rather, they seem to occupy the same sub-nuclear compartment formed by the Xist. Conversely, the marsupial Xi showed no enrichment of H4K20me1, a modification that is enriched on the placental mammal Xi and which is consistent with studies suggesting that accumulation of this mark on Xi is XIST dependent [20]. Thus, the marsupial Xi displays a signature of repressive epigenetic marks very different to that of Xi-facultative heterochromatin associated with XIST RNA in placental mammals, and more similar to pericentric heterochromatin (Table 1).

Evolution of transcriptionally silent chromatin on the inactive X chromosome

In this study we compare the epigenetic profiles of imprinted (marsupials) XCI to random (placental mammals) XCI in adult somatic cells. Female mouse cells also undergo a transient stage of imprinted paternal X inactivation during early development (the X0 is then reactivated in blastocysts for subsequent random inactivation but remains inactive in extra-embryonic tissues [4,37,38]). However, in contrast to the marsupial Xi, the inactive murine Xp does not harbour strong H4K20me3/H3K9me3 enrichment; instead its epigenetic signature is very similar to that observed in random placental mammal XCI. The constitutive heterochromatin marks associated with marsupial inactive X chromatin might, therefore, represent an ancestral epigenetic system of transcriptional silencing on Xi that was exapted from neighbouring constitutive heterochromatin.

We propose a model for the evolution of the X-chromosome inactivation process whereby silencing of the paternal X chromosome in the therian mammal ancestor was achieved by the accumulation of constitutive heterochromatin marks, as well as H3K27me3 and H3K9me2 (Figure 4). The evolution of XIST in the placental mammal ancestor enabled these two marks (together with H4K20me1) to be stably recruited to establish new XIST-dependent X inactivation machinery. Thus, with strong epigenetic signatures of constitutive heterochromatin, and weaker signatures of Xi-specific facultative heterochromatin, the marsupial inactive X chromosome could represent an intermediate stage in the process of recruitment of constitutive heterochromatin epigenetic marks into early therian dosage compensation, to the more complex and stable XIST-dependent heterochromatin observed in placental mammals.

Our findings give new insight into the evolution of this complex epigenetic regulation of the X chromosome, and suggest new avenues of investigation to deepen our understanding of this crucial process. It will be particularly important to identify the enzymes responsible for the repressive marks on the marsupial Xi, how they are recruited to the Xi chromatin (another non-coding RNA?), and how the cell-cycle specific pattern of the recruitment of H3K27me3 operates (is it linked to replication and which enzyme(s) are involved?)

Materials and Methods

Ethics statement

The study was approved, and all samples were collected and held under The Australian National University Animal Experimentation Ethics Committee proposal numbers R.CG.11.06 and R.CG.14.08.

Cell culture

Male and female primary fibroblast cell cultures from mouse (Mus musculus), tammar wallaby (Macropus eugeniæ), grey short-tailed opossum (Monodelphis domestica) and African savanna elephant (Loxodonta africana) were established from ear clips. Cells were cultured at 35°C or 37°C in 5% CO2 and 45% DME/45%Amniomax C100/10% fetal calf serum (Gibco, Invitrogen).

Immunofluorescence and RNA FISH

Immunofluorescence on interphase cells was performed as previously described [61,62]. Cells were grown on coverslips
coated with 0.5% gelatin, then fixed in 3% paraformaldehyde/1× PBS for 10 minutes at room temperature (RT), permeabilized in 0.5% triton/1× PBS/(2 mM Vanadyl Ribonucleoside Complex was added when subsequent RNA FISH was to be performed) for 5 minutes on ice and blocked in 1% BSA/1× PBS (0.4 U/μl of RNAguard [Amersham/Pharmacia] was added when subsequent RNA FISH was to be performed). Cells were incubated with primary and secondary antibodies in blocking solution sequentially for 1 hour at room temperature in dark and humid chamber (see Table 2). After three washes in 1× PBS, coverslips were mounted onto slides with Vectashield® containing DAPI (Vector laboratories).

The replication assay was performed following the manufacturer’s instructions (Click-iT EdU Alexa Fluor 488, Invitrogen). Cells were incubated for 10 minutes in the presence of 10 μM EdU just prior to detection.

For subsequent RNA FISH, preparations were post-fixed (after the last wash in 1× PBS) in 3% paraformaldehyde/1× PBS for 10 minutes at RT, then rinsed in 2× SSC before overnight hybridization with the probe at 37°C in a dark humidity chamber. A BAC containing UBE1, an X-linked gene, was used as a probe (Mc_KBa-51D22; Arizona Genomics Institute, Tucson AZ, USA). It was labelled in a nick translation reaction with SpectrumOrange dUTP (Enzo Diagnostics, NY, USA) following manufacturer’s instructions. 300 ng of labelled BAC probe was precipitated with 10 μg glycogen and 1 μg of M. eugenii sheared genomic DNA and resuspended in 15 μl of hybridization buffer (50% formamide, 20% dextran sulfate, 2× SSC, 1 mg/ml BSA, 10 mM Vanadyl Ribonucleoside Complex). The probe was then denatured for 10 min at 70°C and pre-annealed for 30 min at 37°C before overnight hybridization. Slides were washed three times in 50% formamide/2× SSC (adjusted to pH 7.2) and three times in 2× SSC for 5 min each at 37°C before being mounted with Vectashield® containing DAPI.

**Immunofluorescence combined with DNA FISH**

We performed sequential immunofluorescence/DNA FISH experiments adapted from previous protocols [62]. Cells were

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**Figure 4. A model for the evolution of mammalian X-chromosome inactivation.** Arrows above the phylogeny show epigenetic features underlying X chromosome inactivation (solid arrows indicate stable components of XCI within the clades, whereas dashed lines indicate unstable repressive modification). We propose that in the ancestral therian mammal neighbouring centromeric constitutive heterochromatin histone modifications were exapted to reduce transcription from the paternal X chromosome in females. H3K27me3 and H3K9me2 were also recruited to the Xi in a cell-cycle and/or cell-lineage dependent manner. After the divergence of placentals from marsupial mammals, the XIST gene evolved and H3K27me3 and H3K9me2 (along with H4K20me1) were stably recruited into the XCI machinery. In placentals mammals the original silencing machinery was restricted, and organised in mutually exclusive domains with the Xi-specific facultative heterochromatin. In marsupials, much of the original silencing machinery that was recruited from constitutive heterochromatin remains in place.

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BAC probes were co-precipitated (300 ng each) and prepared for SpectrumOrange dUTP as described for RNA FISH. The three for DNA FISH. BAC DNA was labelled by nick translation with ATRX 143H14; Victorian Institute of Animal Science, Attwood, VIC, Australia, ATRX (Me_VIA-43E9) and UBE1 were used as probes for DNA FISH. BAC DNA was labelled by nick translation with SpectrumOrange dUTP as described for RNA FISH. The three BAC probes were co-precipitated (300 ng each) and prepared for overnight hybridization as described above.

**XIST RNA FISH**

Coverslips with male or female cells were fixed and permeabilized as described above. These were dehydrated in an ethanol series before hybridization with the XIST probe. The following primers were designed to amplify XIST exonic sequence from elephant genom DNA. 

**LAF XIST 1f** - AGTGGTGATGACCATTCCCTTTTG 
**LAF XIST 1r** - TCTTGGCAATAGGTGTTGACCAGC 
**LAF XIST 2f** - GCCGTAACTTATGAGCCAGCTAC 
**LAF XIST 2r** - TCTGGCTTTTTGTTCCTCCTAGTC 
**LAF XIST 3f** - GCCAATTAGGTGACGCTGCC 
**LAF XIST 3r** - TGCCATTGTTTTTGCTTCTACG 
**LAF XIST 4f** - GAACAGTAAAAGGGCTAAGGGTTTG 
**LAF XIST 4r** - GCTCAAGTGTCTTCCTGACTAAGC 

One primer pair (pair 1) amplified part of exon 4, and the remainder amplified different regions of exon 6, PCR conditions were: 94°C, 2'; followed by 30 cycles of 94°C, 30s/56°C, 30s/ 72°C, 30s. The four amplicons were sequenced to confirm the identity, 50 ng of each product was labelled with SpectrumOrange dUTP in a PCR under the same cycling conditions described above. Labelled products were co-precipitated, the pellet resuspended in 10 μl of deionized formamide and then denatured at 75°C for 7 minutes. After cooling on ice for 2 minutes, 10 μl 2× hybridization buffer (4×SSC, 40% dextran sulphate, 2 mg/ml BSA, 10 mM Vanadyl Ribonucleoside Complex) was added. 10 μl of the probe was hybridised to a coverslip with female nuclei, and 10 μl to a coverslip with male nuclei, overnight at 37°C in a darkened and humidity chamber. After hybridization, coverslips were washed three times for 5 minutes each in 50% formamide/2×SSC at 42°C, and three times for 5 minutes each in 2×SSC at 42°C. Coverslips were rinsed with distilled water and mounted with Vectashield® containing DAPI.

**Microscopy and analyses**

Two-dimensional microscopy was performed using a Zeiss Axialan epifluorescence microscope. Images were captured on a SPOT RT Monochrome CCD (charge-coupled device) camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) and analyzed using IPLab imaging software (Scanalytics Inc., Fairfax, VA, USA).

Sequenctial three-dimension microscopy was performed using a Delta Vision microscope system (Applied precision, Issaqah, WA, USA). 3D stacks were acquired with z planes separated by 0.2 μm and deconvolved using the Softworx software algorithm (conservative ratio method, 7 iterations). For sequential immuno-DNA FISH experiments, nuclei were first imaged and their coordinates were recorded with the Softworx software after immunofluorescence to assist recaptured after DNA FISH.

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**Author Contributions**

Conceived and designed the experiments: JC PDW JAMG. Performed the experiments: JC PDW EK. Analyzed the data: JC PDW. Contributed reagents/materials/analysis tools: JC PDW CG TJR JAMG. Wrote the paper: JC PDW CG TJR JAMG.

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