CSIRO PUBLISHING

Australian Journal of Zoology, 2013, **61**, 13–23
http://dx.doi.org/10.1071/ZO12113

Review

Marsupial X chromosome inactivation: past, present and future

Janine E. Deakin

Division of Evolution, Ecology and Genetics, Research School of Biology, The Australian National University, Canberra, ACT 0200, Australia. Email: janine.deakin@anu.edu.au

Abstract. Marsupial and eutherian mammals inactivate one X chromosome in female somatic cells in what is thought to be a means of compensating for the unbalanced X chromosome dosage between XX females and XY males. The hypothesis of X chromosome inactivation (XCI) was first published by Mary Lyon just over 50 years ago, with the discovery of XCI in marsupials occurring a decade later. However, we are still piecing together the evolutionary origins of this fascinating epigenetic mechanism. From the very first studies on marsupial X inactivation, it was apparent that, although there were some similarities between marsupial and eutherian XCI, there were also some striking differences. For instance, the paternally derived X was found to be preferentially silenced in marsupials, although the silencing was often incomplete, which was in contrast to the random and more tightly controlled inactivation of the X chromosome in eutherians. Many of these earlier studies used isozymes to study the activity of just a few genes in marsupials. The sequencing of several marsupial genomes and the advent of molecular cytogenetic techniques have facilitated more in-depth studies into marsupial X chromosome inactivation and allowed more detailed comparisons of the features of XCI to be made. Several important findings have come from such comparisons, among which is the absence of the *XIST* gene in marsupials, a non-coding RNA gene with a critical role in eutherian XCI, and the discovery of the marsupial *RSX* gene, which appears to perform a similar role to *XIST*. Here I review the history of marsupial XCI studies, the latest advances that have been made and the impact they have had towards unravelling the evolution of XCI in mammals.

Received 6 November 2012, accepted 17 February 2013, published online 12 March 2013

Introduction

Mammals have an XX/XY sex determination system in which males are the heterogametic sex, with a gene-rich X chromosome and a highly degraded, gene-poor Y chromosome. This difference between the X and Y chromosomes creates an imbalance for X-borne gene dosage between the sexes. In 1961, Mary Lyon proposed that female eutherian mammals compensate for this imbalance by inactivating one X chromosome in a random fashion. The inactivation was thought to take place early in embryogenesis and to be maintained throughout subsequent cell divisions (Lyon 1961). This hypothesis was proposed after observing a speckled phenotype in female mice heterozygous for X-linked genes (Lyon 1961). Population studies on the human X-linked *G6PD* gene led to similar conclusions, with heterozygous women possessing both active and deficient *G6PD* erythrocytes (Beutler *et al*. 1962), indicating that some cells express only the maternal X chromosome whereas others express only the paternal X. This hypothesis was supported by examining G6PD variants in cultures established from single cells from heterozygous women. Only one G6PD variant was observed in each culture (Davidson *et al*. 1963). Further support for an inactive X chromosome (Xi) was offered by observations at the cytogenetic level, which included the presence of a heterochromatic sex chromatin body (Barr Body) observed in female and not male interphase cells (Barr and Carr 1962) and the late replication of one X chromosome during mitosis (Taylor 1960), both of which are hallmarks of the inactive X chromosome in eutherians.

Over the past 50 years, X chromosome inactivation (XCI) has been extensively studied, particularly in mouse and, to a lesser extent, in humans. The steps involved in this complex process are still being elucidated but are known to involve the initiation of silencing from a master locus known as the X inactivation centre (XIC) (Brockdorff *et al*. 1991; Brown *et al*. 1991). The XIC is critical for counting the number of X chromosomes in a cell and choosing the X to inactivate (Bacher *et al*. 2006; Xu *et al*. 2006; Augui *et al*. 2007). It encodes several non-coding RNAs, among which is *XIST* (X Inactive Specific Transcript), a 17-kb-long non-coding RNA responsible for coating the inactive X in *cis* and initiation of chromosome-wide-silencing (Borsani *et al*. 1991). The *XIST*-coated chromosome then undergoes chromatin remodelling via histone modifications and DNA methylation (reviewed in Heard 2005), and is ultimately observed as condensed chromatin (Barr body) and late replicating.

The initial finding of this remarkable dosage compensation mechanism in such divergent species as human and mouse led to questions regarding its evolution and, in particular, whether XCI was peculiar to the eutherian mammals or more broadly subscribed to amongst members of the class Mammalia.

Mammals diverged from a common ancestor between 161 and 217 million years ago (Phillips *et al*. 2009) and are represented by three major lineages: Prototheria (monotremes), Metatheria (marsupials) and Eutheria (placentals). The closest lineage to eutherian mammals is that of the marsupials, which last shared a common ancestor at least 160 million years ago (Luo *et al*. 2011). They are represented by over 300 species, distributed between the Americas and Australasia. Marsupials, with large, easily distinguishable chromosomes, and the ability to generate species and subspecies hybrids, have proven particularly valuable for unravelling the evolutionary origin of XCI.

Imprinted XCI

As in eutherians, an early study on replication timing of the X chromosomes in two marsupial species demonstrated asynchronous replication of the X chromosomes in females, suggesting that marsupials did subscribe to XCI. The assumption was that, since they shared this common feature with eutherians, XCI in marsupials would be achieved in a similar fashion (Marshall Graves 1967). However, a subsequent study established that this was not the case. Sharman (1971) examined replication timing in subspecies and species crosses, where the X chromosome of each subspecies or species could be easily distinguished on the basis of size. The results established that the paternally derived X chromosome was late replicating in each case, a striking difference to the random XCI observed in eutherians.

Examining XCI in marsupials proved difficult without knowing the gene content of the X chromosome in marsupials. A hypothesis proposed by Susumo Ohno (now referred to as Ohno's Law) proved important for facilitating work in this area (Lyon 1992). Ohno posited that genes X-linked in one mammalian species would be located on the X chromosome in all mammalian species because translocations between the X and autosomes would be selected against as they risk disrupting the dosage compensation mechanism (Ohno 1967). Hence, it was assumed that genes known to be X-linked in human or mouse would be located on the X chromosome in marsupials. This provided a starting point for investigating XCI in marsupials.

Two papers published at the same time as the finding of late replication of the paternal X (Sharman 1971) reported two genes known to be X-linked in humans were found to be likewise X-linked in marsupials (Cooper *et al*. 1971; Richardson *et al*. 1971). The late replication of the paternal X was associated with silencing of the paternal *G6PD* allele in red blood cells from euro (*Macropus robustus erubescens*) × wallaroo (*M. r. robustus*) hybrid animals (Richardson *et al*. 1971). Variants of the PGK enzyme in hybrids of eastern (*Macropus giganteus*) and western (*M. fuliginosus*) grey kangaroos and backcrosses further demonstrated silencing of the paternal allele in red blood cells and, importantly, that the silenced allele could be active in the subsequent female offspring (Cooper *et al*. 1971). Furthermore, this mode of expression was not limited to red blood cells but was observed in a range of somatic tissues (Cooper *et al*. 1971). These studies were the first to demonstrate genomic imprinting in mammals, a phenomenon where genes are expressed in a parent-of-origin fashion.

Surprisingly, despite this major difference in the nature of XCI between eutherians and marsupials, studies of paternal XCI have been relatively limited. This pattern of XCI has been directly confirmed for only three genes using isozymes (*G6PD*, *GLA*, *PGK*) (reviewed in Cooper *et al*. 1993; Deakin *et al*. 2009) and indirectly for *HPRT1* using somatic cell hybrids (Marshall Graves and Dawson 1988).

Several different species have been examined for each gene but differences are observed between species, between genes and even for the same gene in different tissues within a species, making it difficult to draw concrete conclusions about the nature of marsupial XCI (Fig. 1). For instance, in the Virginia opossum (*Didelphis virginiana*), *PGK1* was expressed exclusively from the paternally derived X chromosome whereas *G6PD* showed partial expression from the paternal X in most tissues (Samollow *et al*. 1987). In contrast, *G6PD* showed complete silencing of the paternal copy in somatic tissues in macropod species (*M. robustus* and *M. rufogriseus*) (Johnston *et al*. 1978). Differences have also been observed in *G6PD* expression throughout development in *D. virginiana*, with increasing expression of the paternal allele associated with increasing age (Samollow *et al*. 1995).

It was difficult to determine whether the partial expression of the paternal allele observed for some genes was the result of partial expression from the X in all cells or due to a mosaicism, with some cells expressing only the maternal allele while others expressed both. However, experiments on cloned fibroblasts failed to detect any indication of mosaicism (Johnston *et al*. 1978), suggesting that there was a reduced level of expression from the paternal allele in every cell.

Studies of imprinted XCI at the molecular level on marsupial X-linked genes have been rather limited. In fact, until the sequencing of the human genome, sequence information had been obtained for only three marsupial X-linked genes: *HPRT1* (Kaslow *et al*. 1987; Conaty and Piper 1996); *G6PD* (Kaslow *et al*. 1987; Loebel *et al*. 1995) and *PGK1* (Zehavi-Feferman and Cooper 1992). The identification of an informative polymorphism was then required for this sequence information to be useful for XCI studies. Such polymorphisms were found for *G6PD* in the wallaroo (*M. robustus*) (Watson *et al*. 2000) and for *G6PD* and *PGK1* in the grey short-tailed opossum (*Monodelphis domestica*) (Hornecker *et al*. 2007). The use of molecular techniques, such as SNuPE (Single Nucleotide Primer Extension) assay and allele-specific RT–PCR, permitted allele expression in heterozygous females to be examined at the nucleotide rather than protein level. A SNuPE assay for *G6PD* in the wallaroo correlated with isozyme results for this species, with the paternally derived X allele being completely silenced in somatic tissues (Watson *et al*. 2000). Similarly, allele-specific RT–PCR demonstrated mostly complete silencing of the paternal *G6PD* allele in the opossum (Hornecker *et al*. 2007). In contrast, *PGK1* in the opossum was expressed from the paternal alleles in most tissues, at the same or even greater levels than the maternal allele (Hornecker *et al*. 2007).

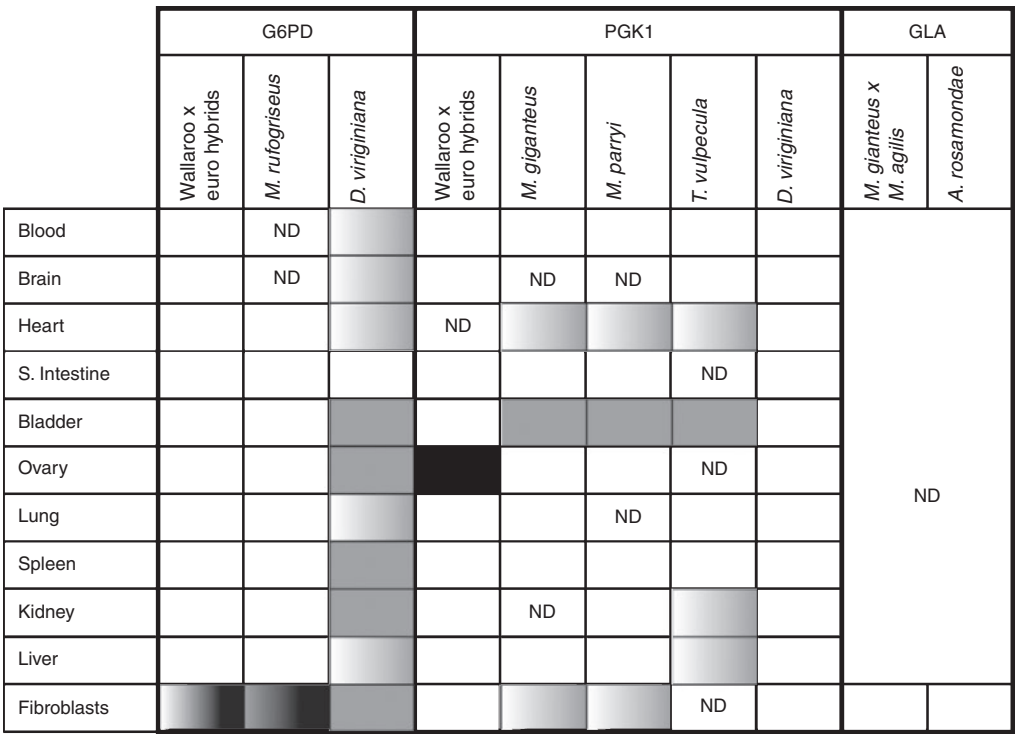

Fig. 1. Expression from the inactive X chromosome detected in isozyme studies. White indicates no expression from the paternal X. Grey indicates partial expression and black complete expression from the inactive X. Differences between individuals of the same species are depicted as gradients. ND, not determined.

From these studies, it was evident that the inactivation status of many more genes would be required to gain a true understanding of the nature of XCI in marsupials.

Gene content of the marsupial X chromosome

For many years, examination of more genes was hampered by the difficulty in isolating X-linked genes in marsupials. Although Ohno's Law had been supported by the finding of several genes X-linked in eutherians being X-linked in marsupials, the isolation of further X-linked genes using the limited information available for eutherian species meant that isolating gene sequences for the marsupial orthologues of human X-linked genes was a slow, painstaking process. It has only been in recent years that the genic content of marsupial X chromosomes has been thoroughly established.

The advent of molecular cytogenetic techniques provided greater insight into the homology between marsupial and eutherian X chromosomes. A region of the X chromosome conserved between therian mammals, corresponding to two-thirds of the human X and known as the X conserved region (XCR), was uncovered by cross-species chromosome painting (Glas *et al*. 1999). This supported Ohno's Law and, interestingly, the X is the only chromosome for which chromosome painting is successful between marsupials and eutherians. The remaining third of the human X chromosome, the X added region (XAR) is autosomal in marsupials and was added to the X chromosome before eutherian radiation 105 million years ago (Marshall Graves *et al*. 1995).

The recent sequencing of three marsupial genomes has elucidated the genic content of this conserved region of the X. Fortunately, the species chosen for sequencing are nicely spread across the marsupial phylogeny, with the grey short-tailed opossum (*M. domestica*) representing the American marsupials, and the tammar wallaby (*Macropus eugenii*) and the Tasmanian devil (*Sarcophilus harrisii*) representing the Australian species. The opossum is a 'laboratory' marsupial, being easily bred in captivity in facilities similar to those used for rodents and producing many young per litter, making it particularly suitable for studies of XCI throughout development (VandeBerg 1983). The tammar wallaby, a member of the kangaroo family, is easily bred in captivity and has the advantage of having two subspecies that display many fixed polymorphisms between their geographically isolated populations (Zenger *et al*. 2002; Wang *et al*. 2011), making it an ideal species for examining paternal XCI. The Tasmanian devil has been sequenced in response to its current struggle with devil facial tumour disease (Miller *et al*. 2011; Murchison *et al*. 2012), which has resulted in it being listed as an endangered species. While its endangered status is not ideal for XCI studies, the sequence information still provides a valuable resource.

The opossum genome has been deeply sequenced, well assembled and anchored to chromosomes (Duke *et al*. 2007; Mikkelsen *et al*. 2007). The opossum X spans ~97 Mb (Renfree *et al*. 2011) and contains 442 annotated protein-coding genes (based on Ensembl 68). The tammar wallaby and devil X chromosomes are larger at 150 Mb (Renfree *et al*. 2011) and 122 Mb (Murchison *et al*. 2012), respectively. The size

differences between the X chromosomes of these species is attributable to additional heterochromatin in the Australian species and does not reflect a difference in overall gene content (Renfree *et al*. 2011). Unfortunately, the two Australian species have not been as deeply sequenced or as well assembled and have required cytogenetic mapping to provide information on gene order (Deakin *et al*. 2008*b*, 2012).

Conservation of gene order on the X in the eutherian lineage is observed from the African elephant (Rodriguez Delgado *et al*. 2009), representing the most basal clade, to humans (rodents being the notable exception). In stark contrast, gene order is scrambled between all three sequenced marsupial species. The conservation of gene order on the eutherian X is supposedly a result of selection against rearrangements that could affect the spread of *XIST* transcripts across the chromosome from the centrally located inactivation centre and thereby disrupt the tightly controlled eutherian XCI mechanism (Mikkelsen *et al*. 2007). In keeping with this hypothesis, extensive searches of the opossum genome sequence failed to identify an orthologue of the *XIST* gene (Duret *et al*. 2006). Analysis of genes flanking *XIST* showed that, although these genes are adjacent in other vertebrates, there is a breakpoint between *XIST* flanking genes in marsupials (Davidow *et al*. 2007; Hore *et al*. 2007; Shevchenko *et al*. 2007; Deakin *et al*. 2008*b*). Thus, *XIST* evolved after the divergence of marsupials and eutherians. The possibility of a marsupial-specific XIC with an *XIST*-like gene could not be ruled out.

Inactivation status at the cellular level

Armed with information regarding the gene content of the marsupial X chromosome, it was possible to more thoroughly examine XCI in marsupials. Given the somewhat leaky nature of inactivation in marsupials, it was important to determine whether the partial expression from the paternal X observed for some genes was indeed due to a low level of expression from the paternal X in all cells as suggested from the isozyme studies on cloned fibroblasts (Johnston *et al*. 1978). This was achieved by using RNA-fluorescence *in situ* hybridisation (FISH), a molecular cytogenetic technique capable of detecting nascent transcripts within interphase nuclei.

RNA-FISH performed on fibroblasts for 32 tammar wallaby genes spread across the X chromosome revealed that no gene was inactivated in every cell at the transcript level, with all genes showing a proportion of cells (5–68%) with biallelic expression (Al Nadaf *et al*. 2010). The four genes previously tested by biochemical studies on marsupials (*G6PD*, *HPRT1*, *GLA* and *PGK1*) were biallelically expressed in 7.5% (*GLA*) to 23.5% (*HPRT1*) of nuclei (Fig. 2). Similar results were found for 12 genes in opossum fibroblasts and four genes in the Tasmanian devil (Al Nadaf 2011). The disadvantage of this technique is that it is impossible to distinguish the maternal and paternal copies of the X. However, we assume that in cells with monoallelic expression that it is the maternal allele being detected. Therefore, the partial expression of the paternal X observed in isozyme studies is most likely the result of expression from the paternal X in a proportion of cells.

Since one X chromosome is late replicating, it could be surmised that the level of escape from inactivation could be

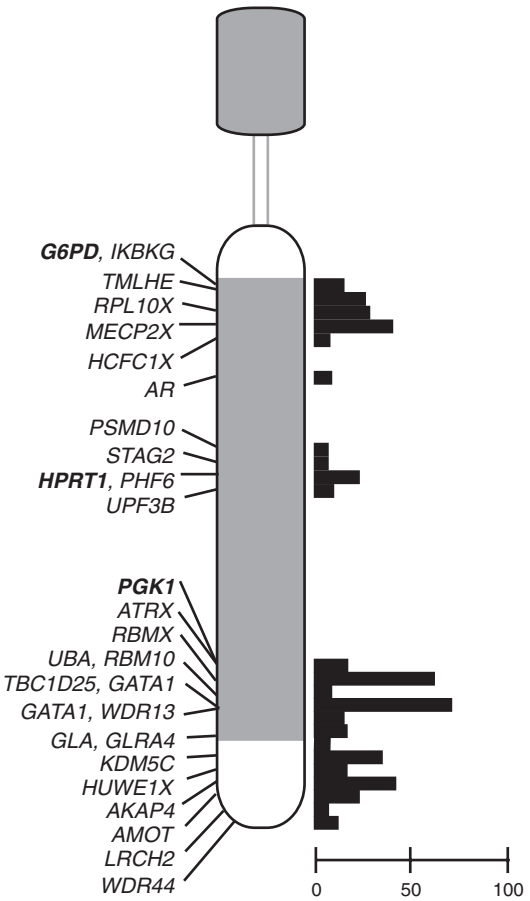

Fig. 2. Gene map of the tammar wallaby X chromosome and the percentage of nuclei for each gene with biallelic expression (black bars).

dependent on the stage of the cell cycle. However, no difference in the proportion of nuclei with biallelic expression in S or G1 phase was observed, indicating that expression from the inactive X is independent of the stage of the cell cycle (Al Nadaf 2011).

This RNA-FISH data could also be used to test the two alternative hypotheses proposed to explain the differences observed between genes and tissues in the extent of inactivation in marsupials. One hypothesis suggested that the silencing of loci on the X chromosome is regulated in a piecemeal manner, rather than across the entire chromosome (VandeBerg *et al*. 1983). The alternative proposed that inactivation was a chromosome-wide phenomenon, which spread from an inactivation centre, resulting in a correlation between the extent of inactivation and position on the X chromosome in relation to the inactivation centre. Inactivation of genes closest to the inactivation centre would be more complete than of those further away (Marshall Graves and Dawson 1988). The activity map of the tammar wallaby X chromosome clearly shows a lack of correlation between gene location and the extent of inactivation (Fig. 2), thereby refuting the latter hypothesis and supporting the former (Al Nadaf *et al*. 2010).

Simultaneous analysis of the expression of neighbouring loci in nuclei with monoallelic expression for both genes showed coordinated expression from the same X chromosome,

suggesting that a chromosome-wide mechanism exists to ensure expression of genes from the same active X (Al Nadaf *et al*. 2010). Al Nadaf *et al*. (2010) also tested the possibility of small domains of coordinate control of gene expression on the inactive X by examining nuclei in which at least one gene of the two X genes located near each other on the tammar wallaby X chromosome were expressed biallelically. Discordant expression was observed for genes with a similar percentage of nuclei displaying biallelic expression, indicative of a lack of local control of expression from the inactive X (Fig. 2). For example, genes *PSMD10* and *STAG2*, both have a 6.7% frequency of biallelic expression, yet displayed discordant expression from the inactive X in 99% of cells. This suggests that expression from the inactive X is independently controlled for each gene and explains the differences observed in the early isozyme studies.

It is important to note that the RNA-FISH experiments mentioned above were performed on fibroblasts, which from isozyme studies were found often to exhibit a higher level of expression of the paternal allele than somatic tissues. RNA-FISH for seven opossum (*M. domestica*) genes from different regions of the X chromosome showed a much tighter level of inactivation in brain and/or liver, with monoallelic expression in 96–100% of nuclei (Mahadevaiah *et al*. 2009). Unfortunately, none of these genes were examined in fibroblasts, making it difficult to draw conclusions as to whether overall somatic tissues display tighter regulation or if the selected subset of genes is more generally subject to virtually complete inactivation.

Discovery of a marsupial-specific *XIST*-like gene

The extensive rearrangement of the X chromosome between species was taken as an indication that a marsupial equivalent of *XIST* was unlikely to be present in marsupials (Deakin *et al*. 2008*b*). Astonishingly, an *XIST*-like gene called *RSX* (RNA on the silent X) has recently been identified in maruspials (Grant *et al*. 2012). The gene was discovered accidently when using a Bacterial Artificial Chromosome (BAC) clone encompassing the *HPRT1* gene in RNA-FISH experiments on female opossum (*M. domestica*) brain tissue. Rather than the typical discreet dot-like signal, a cloud of fluorescence resembling the *Xist* signal detected in mouse ES cells was observed.

The sequence responsible for the formation of this cloud-like signal was narrowed down to a 47-kb region downstream of *HPRT1*, which represents a precursor RNA. The mature *RSX* is a 27-kb non-coding RNA, which shares no sequence homology with *XIST* but does appear to possess some *XIST*-like features, such as a high GC content, enrichment of tandem repeats at the 5′ end of the gene and conserved motifs that may be involved in the formation of stem-loop structures. *RSX* orthologues were subsequently identified in two Australian marsupials, the tammar wallaby and the brushtail possum (*Trichosurus vulpecula*) (Grant *et al*. 2012).

RSX is expressed exclusively in females and, like *XIST*, coats the inactive X in *cis*. Notably, *RSX* transcripts were not detected in the female germline where both X chromosomes are active, associating *RSX* expression with inactivation. Moreover, a *RSX* transgene in mouse ES cells coated the transgenic chromosome and resulted in silencing in more than half the ES cells examined. Thus, *RSX* represents an excellent candidate marsupial-specific X-inactivation centre (Grant *et al*. 2012).

Maintaining inactivation

In eutherians, the inactivated status of the X chromosome is maintained by a series of epigenetic modifications, including accumulation of histone marks associated with repression of transcription (e.g. H3K9me2, H3K27me3, H4K20me1) and loss of marks associated with active chromatin (e.g. H3K4me2, H3K9ac, H4Kac) and DNA methylation (Heard 2005). Given the less stable and incomplete nature of marsupial XCI, determining the extent to which these epigenetic marks are involved has been of considerable interest.

The underacetylation of histone H4 on one X chromosome in tammar wallabies was the first shared molecular feature of XCI identified between marsupials and eutherians (Wakefield *et al*. 1997). Similarly, other histone modifications associated with active chromatin (e.g. H3K4me2, H3K9ac) are depleted on the inactive X in marsupials as they are in eutherians species (Koina *et al*. 2009; Rens *et al*. 2010; Zakharova *et al*. 2011).

Enrichment of the repressive mark H3K27me3 was observed in opossum brain (98% of nuclei) and liver (60% of nuclei) (Mahadevaiah *et al*. 2009) and, to a lesser extent, on brushtail possum metaphase chromosomes (50% of metaphases) and in opossum and tammar wallaby fibroblast interphase nuclei (30% of nuclei) (Table 1) (Chaumeil *et al*. 2011). Although the accumulation of H3K27me3 is a shared feature of the inactive X between eutherians and marsupials, there is one notable difference: H3K27me3 accumulation is present throughout the cell cycle in eutherians but is transient in marsupials, starting to accumulate on the inactive X in early S phase with its more prominent accumulation coinciding with Xi replication in late S phase and early G2 phase (Chaumeil *et al*. 2011). The different frequencies at which H3K27me3 accumulation is detected suggests that its enrichment is tissue-specific and may partially explain the differences in level of inactivation of X-borne genes between somatic tissues and fibroblasts. Interestingly, H3K27me3 accumulation is associated with *RSX* expression in opossum ovary cells and monoallelic X-linked gene expression (Grant *et al*. 2012).

Hypermethylation of 5′ CpG islands has been associated with the stabilisation of XCI in eutherians (Hellman and Chess 2007). Investigations of methylation status in marsupials have failed to show any differential methylation of 5′ CpG islands, either by the use of methylation-sensitive restriction enzymes (Kaslow and Migeon 1987) or by bisulfite sequencing (Loebel and Johnston 1996; Hornecker *et al*. 2007). It is important to note that this is based on the examination of just two genes, *G6PD* and *PGK1*. The lack of DNA 5′ CpG methylation may help to explain the reduced stability of XCI observed in marsupials.

Conversely, chromosome-wide techniques have shown global differences in the level of methylation between active and inactive X chromosomes. Loebel and Johnston (1993) showed hypomethylation of the maternal X chromosome by using methylation-sensitive restriction enzymes and *in situ* nick translation on metaphase chromosomes, with similar results observed using immunofluorescence on brushtail possum and potoroo (*Potorous tridactylus*) metaphase chromosomes (Rens

Table 1. Profiles for repressive and active histone marks on the inactive X (Xi)
+, enrichment; –, depletion; +/–, slight enrichment; –/+, slight depletion; %, percentage of nuclei showing enrichment when <90%. Meu, *M. eugenii*; Mdo, *Monodelphis domestica*; Tvu, *Trichosurus vulepcula*

	Eutherian Xi	Interphase nuclei (fibroblasts)		Metaphase			Brain	Liver
		Meu	Mdo	Meu	Mdo	Tvu	Mdo	Mdo
Repressive								
H3K9me2	+	30%	30%	–				
H3K27me3	+	30%	30%	–	+/–	50%	+	60%
H4K20me1	+	–	–	–				
H3K9me3	–	+	+		–/+	+		
H3K27me1	–	–						
H4K20me3	–	–	–			+		
HP1α	–					+		
Active								
H2AK5ac	–					–		
H3K4me2	–	–	–	–	–	–		
H3K9ac	–	–	–	–	–	–		
H4K16ac	–					~50%		
H4K8ac	–					~50%		
H4Kac	–	–	–	–				

et al. 2010). In concordance with this hypomethylation of the inactive X, methylation differences have been observed in the intragenic region of the *HPRT1* and *G6PD* genes in the Virginian opossum (Kaslow and Migeon 1987), and *HPRT1* in the wallaroo. Chong and Piper (1996) correlated a lack of methylation at a site within intron 3 of the inactive copy of *HPRT1* with DNA–protein interactions, suggesting that these proteins may be involved in gene silencing and methylation may inhibit the binding of these proteins within the active copy. The recent development of techniques capable of detecting differential methylation on a genome-wide scale will permit a more thorough investigation of the role of DNA methylation in marsupial XCI both at 5′ and intragenic sites.

Thus, there are some common epigenetic marks of XCI shared between marsupials and eutherians, namely the depletion of active histone marks from the inactive X (e.g. H3K4me2, H3K9ac, H4Kac) and at least some level of enrichment of repressive marks H3K27me3 and H3K9me2. These histone modifications may be a feature of an ancestral XCI mechanism (Zakharova *et al*. 2011). The additional marks present in eutherian XCI may contribute to the more stable inactivation observed in these species.

Does XCI in marsupials result in dosage compensation between the sexes?

Since its discovery, XCI has been assumed to be a mechanism that evolved as a consequence of the evolution of differentiated sex chromosomes, which left an imbalance in X-borne gene expression between the sexes. A recent flurry of investigations into dosage compensation have shown that several animals with differentiated sex chromosomes have varying degrees of compensation and often only a small proportion show complete dosage compensation between males and females (Itoh *et al*. 2007; Deakin *et al*. 2008*a*; Mank 2009; Itoh *et al*. 2010; London *et al*. 2010; Vicoso and Bachtrog 2011; Wolf and Bryk 2011). Does XCI in marsupials achieve dosage compensation between the sexes?

G6PD activity in erythrocytes of several macropod species has been shown to have equal levels of activity in males and females, and thus is effectively dosage compensated (Raphael and Cooper 1978). However, cultured fibroblasts were quite different, with females having up to twice the G6PD activity of males (Raphael and Cooper 1978). The female to male ratio of *G6PD* expression measured by quantitative RT–PCR in tammar wallaby fibroblasts was close to 1.0, indicative of complete dosage compensation (Al Nadaf *et al*. 2010), yet female to male ratios for an additional 11 X-linked genes varied between 1.0 and 3.0. This wide range in female to male ratios was partly attributed to considerable amounts of variation between individuals.

More recently, the transcriptional levels of genes across the entire X chromosome have been compared using RNA-sequencing data (RNA-seq) obtained from sequencing five tissues (brain, cerebellum, heart, kidney and liver) of a female and male opossum. The only tissue to show a slight yet significant deviation from equal expression in females and males was heart (Julien *et al*. 2012). Thus, it seems that for opossum somatic tissues there is efficient dosage compensation between the sexes. In some respects, this is surprising given previous work on *PGK1* gene expression, which demonstrated little if any inactivation of the paternal allele in tissues (Hornecker *et al*. 2007). However, this result would concur with the essentially complete inactivation of one X chromosome observed in opossum brain and liver cells (Mahadevaiah *et al*. 2009). These types of global studies should be performed on more marsupial species and larger numbers of individuals.

The initial studies on measuring enzyme activity in marsupials, in some ways, used the ultimate test to determine whether X chromosome inactivation achieves dosage compensation. Measuring gene expression only provides part of the answer as there could be mechanisms in place at the

post-translational stage to achieve compensation. A lack of compensation at the transcript level and complete compensation at the protein level has been reported for the HSD17B4 enzyme in the zebrafinch brain (London *et al*. 2010). This will most likely be an avenue that XCI studies will take in the future, using some of the sophisticated proteomics techniques now available.

Hypotheses for the evolution of XCI

Determining the evolutionary origin of XCI has been subject to debate for decades, with several alternative hypotheses proposed. After the discovery of imprinted XCI in marsupials, Cooper (1971) hypothesised that the ancestral form of XCI was likely to be paternally imprinted, resembling XCI in marsupials. Support for this hypothesis came upon the discovery of an imprinted form of XCI in the extraembryonic membrane of rodents (Takagi and Sasaki 1975; Wake *et al*. 1976; West *et al*. 1977). This imprinted form of eutherian XCI resembles that of marsupials, being less stable, incomplete and independent of DNA methylation (Huynh and Lee 2005). However, imprinted XCI in mice was found to be dependent on *Xist* to some extent, questioning the common origin of imprinted XCI in therian mammals (Marahrens *et al*. 1997; Okamoto *et al*. 2005). In addition, the paternal X in mouse extraembryonic tissues does not share the same histone-modification profile as the inactive X in marsupials, but is more like that of XCI of eutherian somatic tissues (Chaumeil *et al*. 2011). Recent comparative studies amongst eutherians suggest that the imprinted XCI arose secondarily in the mouse lineage (Escamilla-Del-Arenal *et al*. 2011). Therefore, imprinted XCI must have evolved independently in marsupials and eutherian extraembryonic tissues.

It had been suggested, as an extension of this hypothesis, that imprinted XCI was a carryover from meiotic sex chromosome inactivation, with the paternal X chromosome arriving in the embryo in a preinactivated state (reviewed in VandeBerg 1983; see also Hornecker *et al*. 2007; Namekawa *et al*. 2007). Both the X and Y chromosomes in marsupials are silenced at the pachytene stage during male meiosis and are stably maintained during spermiogenesis (Turner 2007). It was, therefore, proposed that the zygote received an already inactive X from the sperm. However, reactivation of X-linked genes was observed in opossum spermatids and, to some extent, in mouse (Mahadevaiah *et al*. 2009), contesting this hypothesis.

Although an ancestral imprinted form of XCI has now been almost entirely disregarded, testing of this hypothesis has had a major impact on the field and our understanding of XCI in marsupials as well as eutherians. A shared origin of marsupial and eutherian XCI has been questioned and alternative hypotheses proposed.

The evolution of XCI has been intimately linked to the degradation of the Y chromosome. Comparisons of the genic content of marsupial and eutherian Y chromosomes indicates independent degradation of the Y chromosome in the two lineages (Murtagh *et al*. 2012) and, as a consequence, independent genesis of XCI in marsupials and eutherians (Al Nadaf *et al*. 2010). *SRY*, the testis-determining factor in therian mammals, is the gene charged with leading the suppression of recombination between the proto-X and Y chromosomes. Therefore, XCI is likely to have arisen in the region surrounding *SOX3*, the X-borne gametologue of *SRY* (Gribnau and Grootegoed 2012). Indeed, high doses of the SOX3 protein are capable of triggering testis differentiation (Sutton *et al*. 2011), suggesting that *SOX3* is a particularly dosage-sensitive gene. Several genes central to the XCI mechanism are located near *SOX3* on the proto-X, supporting the idea that this is the site at which XCI originated, at least in eutherians.

In marsupials, *SOX3* expression is not detected in the developing testes (Pask *et al*. 2000) and therefore may not be a dosage-sensitive gene requiring compensation in marsupials. If this is the case, then marsupial XCI may have arisen in a different region on the X chromosome. In keeping with this idea, *RSX*, the putative marsupial equivalent of *XIST*, is located adjacent to *HPRT1* and *PHF6X* (the gametologue of *PHF6Y*) (Grant *et al*. 2012) in a different region of the proto-X chromosome to *SOX3*. This would support an independent origin of XCI in the two marsupial lineages.

Another possibility, although not mutually exclusive, is that XCI arose from an ancient mechanism used to regulate transcription that has been exapted into independently evolved mechanisms for dosage compensation. In marsupials, XCI, in at least some cell types, is incomplete with stochastic expression from the inactive X (Al Nadaf *et al*. 2010). Even in eutherians with their more complete level of inactivation, genes in the eutherian XAR are more prone to a stochastic form of expression (Al Nadaf *et al*. 2012). This mode of expression may be indicative of an ancestral mechanism, as this recent addition to the X may not have been fully recruited into the more complete silencing mechanism apparent for the ancient XCR. Intriguingly, genes on monotreme X chromosomes, which share no homology to those on the conserved region of the therian X, also exhibit a stochastic form of expression in females (Deakin *et al*. 2008*a*). Recruitment of additional layers of control, such as histone modifications, may have then been selected from a common 'epigenetic toolbox' (Al Nadaf *et al*. 2012). This ancient stochastic form of transcriptional regulation may be a more general mechanism from which genomic imprinting (Ohlsson *et al*. 2001) or even the now more widely observed random monoallelic expression of some genes (Gimelbrant *et al*. 2007) evolved, with layers of epigenetic complexity added depending on selective pressures.

The study of marsupial XCI has come a long way since its first discovery. As highlighted here, many questions still remain unanswered. However, annotated genome sequence assemblies for three marsupial species are making it possible to capitalise on the latest technological advances in epigenomics. Research is now able to examine the entire X chromosome rather than the handful of genes upon which it relied for so many years, ensuring rapid progress towards unravelling the evolution of this remarkable epigenetic mechanism.

Acknowledgements

It is evident from this review that Des Cooper played an instrumental role in the early studies of X chromosome inactivation in marsupials, being one of the first to discover paternal XCI in marsupials and to hypothesise on the evolution of this fascinating epigenetic mechanism. He inspired and encouraged researchers working in this field. His support of the tammar wallaby genome project and leadership role within the Australian Research

Council (ARC) Centre of Excellence in Kangaroo Genomics resulted in the availability of genomic resources that have greatly advanced this area of research and will have a continued impact on the field in the future.

I thank Dr John VandeBerg for helpful comments on the manuscript. An ARC Discovery grant awarded to Paul Waters, Janine Deakin and Jennifer Graves has supported JED's research on marsupial X chromosome inactivation. JED is supported by an ARC Future Fellowship.

References

Al Nadaf, S. (2011). Evolution of X chromosome inactivation in therian mammals. Ph.D. Thesis, The Australian National University, Canberra.

Al Nadaf, S., Waters, P. D., Koina, E., Deakin, J. E., Jordan, K. S., and Graves, J. A. (2010). Activity map of the tammar X chromosome shows that marsupial X inactivation is incomplete and escape is stochastic. *Genome Biology* **11**, R122. doi:10.1186/gb-2010-11-12-r122

Al Nadaf, S., Deakin, J. E., Gilbert, C., Robinson, T. J., Graves, J. A. M., and Waters, P. D. (2012). A cross-species comparison of escape from X inactivation in Eutheria: implications for evolution of X chromosome inactivation. *Chromosoma* **121**, 71–78. doi:10.1007/s00412-011-0343-8

Augui, S., Filion, G. J., Huart, S., Nora, E., Guggiari, M., Maresca, M., Stewart, A. F., and Heard, E. (2007). Sensing X chromosome pairs before X inactivation via a novel X-pairing region of the XIC. *Science* **318**, 1632–1636. doi:10.1126/science.1149420

Bacher, C. P., Guggiari, M., Brors, B., Augui, S., Clerc, P., Avner, P., Eils, R., and Heard, E. (2006). Transient colocalization of X-inactivation centres accompanies the initiation of X inactivation. *Nature Cell Biology* **8**, 293–299. doi:10.1038/ncb1365

Barr, M. L., and Carr, D. H. (1962). Correlations between sex chromatin and sex chromosomes. *Acta Cytologica* **6**, 34–45.

Beutler, E., Yeh, M., and Fairbanks, V. F. (1962). The normal human female as a mosaic of X-chromosome activity: studies using the gene for G-6-PD-deficiency as a marker. *Proceedings of the National Academy of Sciences of the United States of America* **48**, 9–16. doi:10.1073/pnas.48.1.9

Borsani, G., Tonlorenzi, R., Simmler, M. C., Dandolo, L., Arnaud, D., Capra, V., Grompe, M., Pizzuti, A., Muzny, D., Lawrence, C., Willard, H. F., Avner, P., and Ballabio, A. (1991). Characterization of a murine gene expressed from the inactive X chromosome. *Nature* **351**, 325–329. doi:10.1038/351325a0

Brockdorff, N., Ashworth, A., Kay, G. F., Cooper, P., Smith, S., McCabe, V. M., Norris, D. P., Penny, G. D., Patel, D., and Rastan, S. (1991). Conservation of position and exclusive expression of mouse Xist from the inactive X chromosome. *Nature* **351**, 329–331. doi:10.1038/351329a0

Brown, C. J., Lafreniere, R. G., Powers, V. E., Sebastio, G., Ballabio, A., Pettigrew, A. L., Ledbetter, D. H., Levy, E., Craig, I. W., and Willard, H. F. (1991). Localization of the X inactivation centre on the human X chromosome in Xq13. *Nature* **349**, 82–84. doi:10.1038/349082a0

Chaumeil, J., Waters, P. D., Koina, E., Gilbert, C., Robinson, T. J., and Graves, J. A. (2011). Evolution from XIST-independent to XIST-controlled X-chromosome inactivation: epigenetic modifications in distantly related mammals. *PLoS ONE* **6**, e19040. doi:10.1371/journal.pone.0019040

Chong, S., and Piper, A. A. (1996). Methylation sensitive protein binding to an intragenic active X-specific methylated region in the *M. robustus* Hprt gene. *Somatic Cell and Molecular Genetics* **22**, 461–476. doi:10.1007/BF02369438

Conaty, J., and Piper, A. A. (1996). Full-length cDNA sequence of the X-linked HPRT gene of an Australian marsupial, the wallaroo (*Macropus robustus*). *Mammalian Genome* **7**, 74–76. doi:10.1007/s003359900019

Cooper, D. W. (1971). Directed genetic change model for X chromosome inactivation in eutherian mammals. *Nature* **230**, 292–294. doi:10.1038/230292a0

Cooper, D. W., VandeBerg, J. L., Sharman, G. B., and Poole, W. E. (1971). Phosphoglycerate kinase polymorphism in kangaroos provides further evidence for paternal X inactivation. *Nature: New Biology* **230**, 155–157.

Cooper, D. W., Johnston, P. G., and Graves, J. A. M. (1993). X-inactivation in marsupials and monotremes. *Seminars in Developmental Biology* **4**, 117–128. doi:10.1006/sedb.1993.1014

Davidow, L. S., Breen, M., Duke, S. E., Samollow, P. B., McCarrey, J. R., and Lee, J. T. (2007). The search for a marsupial XIC reveals a break with vertebrate synteny. *Chromosome Research* **15**, 137–146. doi:10.1007/s10577-007-1121-6

Davidson, R. G., Nitowsky, H. M., and Childs, B. (1963). Demonstration of two populations of cells in the human female heterozygous for glucose-6-phosphate dehydrogenase variants. *Proceedings of the National Academy of Sciences of the United States of America* **50**, 481–485. doi:10.1073/pnas.50.3.481

Deakin, J. E., Hore, T. A., Koina, E., and Marshall Graves, J. A. (2008*a*). The status of dosage compensation in the multiple X chromosomes of the platypus. *PLOS Genetics* **4**, e1000140. doi:10.1371/journal.pgen.1000140

Deakin, J. E., Koina, E., Waters, P. D., Doherty, R., Patel, V. S., Delbridge, M. L., Dobson, B., Fong, J., Hu, Y., van den Hurk, C., Pask, A. J., Shaw, G., Smith, C., Thompson, K., Wakefield, M. J., Yu, H., Renfree, M. B., and Graves, J. A. (2008*b*). Physical map of two tammar wallaby chromosomes: a strategy for mapping in non-model mammals. *Chromosome Research* **16**, 1159–1175. doi:10.1007/s10577-008-1266-y

Deakin, J. E., Chaumeil, J., Hore, T. A., and Marshall Graves, J. A. (2009). Unravelling the evolutionary origins of X chromosome inactivation in mammals: insights from marsupials and monotremes. *Chromosome Research* **17**, 671–685. doi:10.1007/s10577-009-9058-6

Deakin, J. E., Bender, H. S., Pearse, A. M., Rens, W., O'Brien, P. C., Ferguson-Smith, M. A., Cheng, Y., Morris, K., Taylor, R., Stuart, A., Belov, K., Amemiya, C. T., Murchison, E. P., Papenfuss, A. T., and Marshall Graves, J. A. (2012). Genomic restructuring in the tasmanian devil facial tumour: chromosome painting and gene mapping provide clues to evolution of a transmissible tumour. *PLOS Genetics* **8**, e1002483. doi:10.1371/journal.pgen.1002483

Duke, S. E., Samollow, P. B., Mauceli, E., Lindblad-Toh, K., and Breen, M. (2007). Integrated cytogenetic BAC map of the genome of the gray, short-tailed opossum, *Monodelphis domestica*. *Chromosome Research* **15**, 361–370.

Duret, L., Chureau, C., Samain, S., Weissenbach, J., and Avner, P. (2006). The Xist RNA gene evolved in eutherians by pseudogenization of a protein-coding gene. *Science* **312**, 1653–1655. doi:10.1126/science.1126316

Escamilla-Del-Arenal, M., da Rocha, S. T., and Heard, E. (2011). Evolutionary diversity and developmental regulation of X-chromosome inactivation. *Human Genetics* **130**, 307–327. doi:10.1007/s00439-011-1029-2

Gimelbrant, A., Hutchinson, J. N., Thompson, B. R., and Chess, A. (2007). Widespread monoallelic expression on human autosomes. *Science* **318**, 1136–1140. doi:10.1126/science.1148910

Glas, R., Marshall Graves, J. A., Toder, R., Ferguson-Smith, M., and O'Brien, P. C. (1999). Cross-species chromosome painting between human and marsupial directly demonstrates the ancient region of the mammalian X. *Mammalian Genome* **10**, 1115–1116. doi:10.1007/s003359901174

Grant, J., Mahadevaiah, S. K., Khil, P., Sangrithi, M. N., Royo, H., Duckworth, J., McCarrey, J. R., VandeBerg, J. L., Renfree, M. B., Taylor, W., Elgar, G., Camerini-Otero, R. D., Gilchrist, M. J., and Turner, J. M. (2012). Rsx is a metatherian RNA with Xist-like properties in X-chromosome inactivation. *Nature* **487**, 254–258. doi:10.1038/nature11171

Gribnau, J., and Grootegoed, J. A. (2012). Origin and evolution of X chromosome inactivation. *Current Opinion in Cell Biology* **24**, 397–404. doi:10.1016/j.ceb.2012.02.004

Heard, E. (2005). Delving into the diversity of facultative heterochromatin: the epigenetics of the inactive X chromosome. *Current Opinion in Genetics & Development* **15**, 482–489. doi:10.1016/j.gde.2005.08.009

Hellman, A., and Chess, A. (2007). Gene body-specific methylation on the active X chromosome. *Science* **315**, 1141–1143. doi:10.1126/science.1136352

Hore, T. A., Koina, E., Wakefield, M. J., and Marshall Graves, J. A. (2007). The region homologous to the X-chromosome inactivation centre has been disrupted in marsupial and monotreme mammals. *Chromosome Research* **15**, 147–161. doi:10.1007/s10577-007-1119-0

Hornecker, J. L., Samollow, P. B., Robinson, E. S., VandeBerg, J. L., and McCarrey, J. R. (2007). Meiotic sex chromosome inactivation in the marsupial *Monodelphis domestica*. *Genesis (New York, N.Y.)* **45**, 696–708. doi:10.1002/dvg.20345

Huynh, K. D., and Lee, J. T. (2005). X-chromosome inactivation: a hypothesis linking ontogeny and phylogeny. *Nature Reviews. Genetics* **6**, 410–418. doi:10.1038/nrg1604

Itoh, Y., Melamed, E., Yang, X., Kampf, K., Wang, S., Yehya, N., Van Nas, A., Replogle, K., Band, M. R., Clayton, D. F., Schadt, E. E., Lusis, A. J., and Arnold, A. P. (2007). Dosage compensation is less effective in birds than in mammals. *Journal of Biology* **6**, 2. doi:10.1186/jbiol53

Itoh, Y., Replogle, K., Kim, Y. H., Wade, J., Clayton, D. F., and Arnold, A. P. (2010). Sex bias and dosage compensation in the zebra finch versus chicken genomes: general and specialized patterns among birds. *Genome Research* **20**, 512–518. doi:10.1101/gr.102343.109

Johnston, P. G., Sharman, G. B., James, E. A., and Cooper, D. W. (1978). Studies on metatherian sex-chromosomes. 7. Glucose-6-phosphate-dehydrogenase expression in tissues and cultured fibroblasts of kangaroos. *Australian Journal of Biological Sciences* **31**, 415–424.

Julien, P., Brawand, D., Soumillon, M., Necsulea, A., Liechti, A., Schutz, F., Daish, T., Grutzner, F., and Kaessmann, H. (2012). Mechanisms and evolutionary patterns of mammalian and avian dosage compensation. *PLoS Biology* **10**, e1001328. doi:10.1371/journal.pbio.1001328

Kaslow, D. C., and Migeon, B. R. (1987). DNA methylation stabilizes X chromosome inactivation in eutherians but not in marsupials: evidence for multistep maintenance of mammalian X dosage compensation. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 6210–6214. doi:10.1073/pnas.84.17.6210

Kaslow, D. C., Migeon, B. R., Persico, M. G., Zollo, M., VandeBerg, J. L., and Samollow, P. B. (1987). Molecular studies of marsupial X chromosomes reveal limited sequence homology of mammalian X-linked genes. *Genomics* **1**, 19–28. doi:10.1016/0888-7543(87)90100-5

Koina, E., Chaumeil, J., Greaves, I. K., Tremethick, D. J., and Graves, J. A. (2009). Specific patterns of histone marks accompany X chromosome inactivation in a marsupial. *Chromosome Research* **17**, 115–126. doi:10.1007/s10577-009-9020-7

Loebel, D. A., and Johnston, P. G. (1993). Analysis of DNase 1 sensitivity and methylation of active and inactive X chromosomes of kangaroos (*Macropus robustus*) by *in situ* nick translation. *Chromosoma* **102**, 81–87. doi:10.1007/BF00356024

Loebel, D. A., and Johnston, P. G. (1996). Methylation analysis of a marsupial X-linked CpG island by bisulfite genomic sequencing. *Genome Research* **6**, 114–123. doi:10.1101/gr.6.2.114

Loebel, D. A., Longhurst, T. J., and Johnston, P. G. (1995). Full-length cDNA sequence of X-linked G6PD of an Australian marsupial, the wallaroo. *Mammalian Genome* **6**, 198–201. doi:10.1007/BF00293013

London, S. E., Itoh, Y., Lance, V. A., Wise, P. M., Ekanayake, P. S., Oyama, R. K., Arnold, A. P., and Schlinger, B. A. (2010). Neural expression and post-transcriptional dosage compensation of the steroid metabolic enzyme 17beta-HSD type 4. *BMC Neuroscience* **11**, 47.

Luo, Z. X., Yuan, C. X., Meng, Q. J., and Ji, Q. (2011). A Jurassic eutherian mammal and divergence of marsupials and placentals. *Nature* **476**, 442–445. doi:10.1038/nature10291

Lyon, M. F. (1961). Gene action in the X-chromosome of the mouse (*Mus musculus* L.). *Nature* **190**, 372–373. doi:10.1038/190372a0

Lyon, M. F. (1992). Some milestones in the history of X-chromosome inactivation. *Annual Review of Genetics* **26**, 17–29. doi:10.1146/annurev.ge.26.120192.000313

Mahadevaiah, S. K., Royo, H., VandeBerg, J. L., McCarrey, J. R., Mackay, S., and Turner, J. M. (2009). Key features of the X inactivation process are conserved between marsupials and eutherians. *Current Biology* **19**, 1478–1484. doi:10.1016/j.cub.2009.07.041

Mank, J. E. (2009). The W, X, Y and Z of sex-chromosome dosage compensation. *Trends in Genetics* **25**, 226–233. doi:10.1016/j.tig.2009.03.005

Marahrens, Y., Panning, B., Dausman, J., Strauss, W., and Jaenisch, R. (1997). Xist-deficient mice are defective in dosage compensation but not spermatogenesis. *Genes & Development* **11**, 156–166. doi:10.1101/gad.11.2.156

Marshall Graves, J. A. (1967). DNA synthesis in chromosomes of cultured leucocytes from two marsupial species. *Experimental Cell Research* **46**, 37–57. doi:10.1016/0014-4827(67)90407-7

Marshall Graves, J. A., and Dawson, G. W. (1988). The relationship between position and expression of genes on the kangaroo X chromosome suggests a tissue-specific spread of inactivation from a single control site. *Genetical Research* **51**, 103–109. doi:10.1017/S0016672300024113

Marshall Graves, J. A., Ferguson-Smith, M. A., McLaren, A, Mittwoch, U, Renfree, M. B., and Burgoyne, P (1995). The evolution of mammalian sex chromosomes and the origin of sex determining genes. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **350**, 305–312. doi:10.1098/rstb.1995.0166

Mikkelsen, T. S., Wakefield, M. J., Aken, B., Amemiya, C. T., Chang, J. L., Duke, S., Garber, M., Gentles, A. J., Goodstadt, L., Heger, A., Jurka, J., Kamal, M., Mauceli, E., Searle, S. M. J., Sharpe, T., Baker, M. L., Batzer, M. A., Benos, P. V., Belov, K., Clamp, M., Cook, A., Cuff, J., Das, R., Davidow, L., Deakin, J. E., Fazzari, M. J., Glass, J. L., Grabherr, M., Greally, J. M., Gu, W. J., Hore, T. A., Huttley, G. A., Kleber, M., Jirtle, R. L., Koina, E., Lee, J. T., Mahony, S., Marra, M. A., Miller, R. D., Nicholls, R. D., Oda, M., Papenfuss, A. T., Parra, Z. E., Pollock, D. D., Ray, D. A., Schein, J. E., Speed, T. P., Thompson, K., VandeBerg, J. L., Wade, C. M., Walker, J. A., Waters, P. D., Webber, C., Weidman, J. R., Xie, X. H., Zody, M. C., Graves, J. A. M., Ponting, C. P., Breen, M., Samollow, P. B., Lander, E. S., Lindblad-Toh, K., and Platf, B. I. G. S. (2007). Genome of the marsupial *Monodelphis domestica* reveals innovation in non-coding sequences. *Nature* **447**, 167–177. doi:10.1038/nature05805

Miller, W., Hayes, V. M., Ratan, A., Petersen, D. C., Wittekindt, N. E., Miller, J., Walenz, B., Knight, J., Qi, J., Zhao, F., Wang, Q., Bedoya-Reina, O. C., Katiyar, N., Tomsho, L. P., Kasson, L. M., Hardie, R. A., Woodbridge, P., Tindall, E. A., Bertelsen, M. F., Dixon, D., Pyecroft, S., Helgen, K. M., Lesk, A. M., Pringle, T. H., Patterson, N., Zhang, Y., Kreiss, A., Woods, G. M., Jones, M. E., and Schuster, S. C. (2011). Genetic diversity and population structure of the endangered marsupial *Sarcophilus harrisii* (Tasmanian devil). *Proceedings of the National Academy of Sciences of the United States of America* **108**, 12348–12353. doi:10.1073/pnas.1102838108

Murchison, E. P., Schulz-Trieglaff, O. B., Ning, Z., Alexandrov, L. B., Bauer, M. J., Fu, B., Hims, M., Ding, Z., Ivakhno, S., Stewart, C., Ng, B. L., Wong, W., Aken, B., White, S., Alsop, A., Becq, J., Bignell, G. R., Cheetham, R. K., Cheng, W., Connor, T. R., Cox, A. J., Feng, Z. P., Gu, Y., Grocock, R. J., Harris, S. R., Khrebtukova, I., Kingsbury, Z., Kowarsky, M., Kreiss, A., Luo, S., Marshall, J., McBride, D. J., Murray, L., Pearse,

A. M., Raine, K., Rasolonjatovo, I., Shaw, R., Tedder, P., Tregidgo, C., Vilella, A. J., Wedge, D. C., Woods, G. M., Gormley, N., Humphray, S., Schroth, G., Smith, G., Hall, K., Searle, S. M., Carter, N. P., Papenfuss, A. T., Futreal, P. A., Campbell, P. J., Yang, F., Bentley, D. R., Evers, D. J., and Stratton, M. R. (2012). Genome sequencing and analysis of the tasmanian devil and its transmissible cancer. *Cell* **148**, 780–791. doi:10.1016/j.cell.2011.11.065

Murtagh, V. J., O'Meally, D., Sankovic, N., Delbridge, M. L., Kuroki, Y., Boore, J. L., Toyoda, A., Jordan, K. S., Pask, A. J., Renfree, M. B., Fujiyama, A., Graves, J. A., and Waters, P. D. (2012). Evolutionary history of novel genes on the tammar wallaby Y chromosome: implications for sex chromosome evolution. *Genome Research* **22**, 498–507. doi:10.1101/gr.120790.111

Namekawa, S. H., VandeBerg, J. L., McCarrey, J. R., and Lee, J. T. (2007). Sex chromosome silencing in the marsupial male germ line. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 9730–9735. doi:10.1073/pnas.0700323104

Ohlsson, R., Paldi, A., and Graves, J. A. (2001). Did genomic imprinting and X chromosome inactivation arise from stochastic expression? *Trends in Genetics* **17**, 136–141. doi:10.1016/S0168-9525(00)02211-3

Ohno, S. (1967). 'Sex Chromosomes and Sex Linked Genes.' (Springer-Verlag: Berlin.)

Okamoto, I., Arnaud, D., Le Baccon, P., Otte, A. P., Disteche, C. M., Avner, P., and Heard, E. (2005). Evidence for *de novo* imprinted X-chromosome inactivation independent of meiotic inactivation in mice. *Nature* **438**, 369–373. doi:10.1038/nature04155

Pask, A. J., Harry, J. L., Renfree, M. B., and Marshall Graves, J. A. (2000). Absence of SOX3 in the developing marsupial gonad is not consistent with a conserved role in mammalian sex determination. *Genesis (New York, N.Y.)* **27**, 145–152. doi:10.1002/1526-968X(200008)27:4<145::AID-GENE30>3.0.CO;2-7

Phillips, M. J., Bennett, T. H., and Lee, M. S. (2009). Molecules, morphology, and ecology indicate a recent, amphibious ancestry for echidnas. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 17089–17094. doi:10.1073/pnas.0904649106

Raphael, K. A., and Cooper, D. W. (1978). Studies on Metatherian sex-chromosomes. 8. Evidence for an absence of dosage compensation at glucose-6-phosphate-dehydrogenase locus in cultured-cells of *Macropus rufogriseus. Australian Journal of Biological Sciences* **31**, 425–431.

Renfree, M.B., Papenfuss, A.T., Deakin, J.E., Lindsay, J., Heider, T., Belov, K., Rens, W., Waters, P.D., Pharo, E.A., Shaw, G., Wong, E.S., Lefevre, C.M., Nicholas, K.R., Kuroki, Y., Wakefield, M.J., Zenger, K.R., Wang, C., Ferguson-Smith, M., Nicholas, F.W., Hickford, D., Yu, H., Short, K. R., Siddle, H.V., Frankenberg, S.R., Chew, K.Y., Menzies, B.R., Stringer, J.M., Suzuki, S., Hore, T.A., Delbridge, M.L., Patel, H., Mohammadi, A., Schneider, N.Y., Hu, Y., O'Hara, W., Al Nadaf, S., Wu, C., Feng, Z.P., Cocks, B.G., Wang, J., Flicek, P., Searle, S.M., Fairley, S., Beal, K., Herrero, J., Carone, D.M., Suzuki, Y., Sugano, S., Toyoda, A., Sakaki, Y., Kondo, S., Nishida, Y., Tatsumoto, S., Mandiou, I., Hsu, A., McColl, K. A., Lansdell, B., Weinstock, G., Kuczek, E., McGrath, A., Wilson, P., Men, A., Hazar-Rethinam, M., Hall, A., Davis, J., Wood, D., Williams, S., Sundaravadanam, Y., Muzny, D.M., Jhangiani, S.N., Lewis, L.R., Morgan, M.B., Okwuonu, G.O., Ruiz, S.J., Santibanez, J., Nazareth, L., Cree, A., Fowler, G., Kovar, C.L., Dinh, H.H., Joshi, V., Jing, C., Lara, F., Thornton, R., Chen, L., Deng, J., Liu, Y., Shen, J.Y., Song, X.Z., Edson, J., Troon, C., Thomas, D., Stephens, A., Yapa, L., Levchenko, T., Gibbs, R. A., Cooper, D.W., Speed, T.P., Fujiyama, A., Graves, J.A.M., O'Neill, R. J., Pask, A.J., Forrest, S.M., and Worley, K.C. (2011). Genome sequence of an Australian kangaroo, *Macropus eugenii*, provides insight into the evolution of mammalian reproduction and development. *Genome Biology* **12**, 414. doi:10.1186/gb-2011-12-12-414

Rens, W., Wallduck, M. S., Lovell, F. L., Ferguson-Smith, M. A., and Ferguson-Smith, A. C. (2010). Epigenetic modifications on X chromosomes in marsupial and monotreme mammals and implications for evolution of dosage compensation. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 17657–17662. doi:10.1073/pnas.0910322107

Richardson, B. J., Czuppon, A. B., and Sharman, G. B. (1971). Inheritance of glucose-6-phosphate dehydrogenase variation in kangaroos. *Nature: New Biology* **230**, 154–155.

Rodriguez Delgado, C. L., Waters, P. D., Gilbert, C., Robinson, T. J., and Graves, J. A. (2009). Physical mapping of the elephant X chromosome: conservation of gene order over 105 million years. *Chromosome Research* **17**, 917–926. doi:10.1007/s10577-009-9079-1

Samollow, P. B., Ford, A. L., and VandeBerg, J. L. (1987). X-linked gene-expression in the Virginia opossum – differences between the paternally derived Gpd and Pgk-a loci. *Genetics* **115**, 185–195.

Samollow, P. B., Robinson, E. S., Ford, A. L., and VandeBerg, J. L. (1995). Developmental progression of Gpd expression from the inactive X-chromosome of the Virginia opossum. *Developmental Genetics* **16**, 367–374. doi:10.1002/dvg.1020160410

Sharman, G. B. (1971). Late DNA replication in the paternally derived X chromosome of female kangaroos. *Nature* **230**, 231–232. doi:10.1038/230231a0

Shevchenko, A. I., Zakharova, I. S., Elisaphenko, E. A., Kolesnikov, N. N., Whitehead, S., Bird, C., Ross, M., Weidman, J. R., Jirtle, R. L., Karamysheva, T. V., Rubtsov, N. B., VandeBerg, J. L., Mazurok, N. A., Nesterova, T. B., Brockdorff, N., and Zakian, S. M. (2007). Genes flanking Xist in mouse and human are separated on the X chromosome in American marsupials. *Chromosome Research* **15**, 127–136. doi:10.1007/s10577-006-1115-9

Sutton, E., Hughes, J., White, S., Sekido, R., Tan, J., Arboleda, V., Rogers, N., Knower, K., Rowley, L., Eyre, H., Rizzoti, K., McAninch, D., Goncalves, J., Slee, J., Turbitt, E., Bruno, D., Bengtsson, H., Harley, V., Vilain, E., Sinclair, A., Lovell-Badge, R., and Thomas, P. (2011). Identification of SOX3 as an XX male sex reversal gene in mice and humans. *The Journal of Clinical Investigation* **121**, 328–341. doi:10.1172/JCI42580

Takagi, N., and Sasaki, M. (1975). Preferential inactivation of the paternally derived X chromosome in the extraembryonic membranes of the mouse. *Nature* **256**, 640–642. doi:10.1038/256640a0

Taylor, J. H. (1960). Asynchronous duplication of chromosomes in cultured cells of Chinese hamster. *The Journal of Biophysical and Biochemical Cytology* **7**, 455–463. doi:10.1083/jcb.7.3.455

Turner, J. M. (2007). Meiotic sex chromosome inactivation. *Development* **134**, 1823–1831. doi:10.1242/dev.000018

VandeBerg, J. L. (1983). Developmental aspects of X chromosome inactivation in eutherian and metatherian mammals. *The Journal of Experimental Zoology* **228**, 271–286. doi:10.1002/jez.1402280211

VandeBerg, J. L., Johnston, P. G., Cooper, D. W., and Robinson, E. S. (1983). X-chromosome inactivation and evolution in marsupials and other mammals. *Isozymes: Current Topics in Biological Medical Research* **9**, 201–218.

Vicoso, B., and Bachtrog, D. (2011). Lack of global dosage compensation in *Schistosoma mansoni*, a female-heterogametic parasite. *Genome Biology and Evolution* **3**, 230–235. doi:10.1093/gbe/evr010

Wake, N., Takagi, N., and Sasaki, M. (1976). Non-random inactivation of X chromosome in the rat yolk sac. *Nature* **262**, 580–581. doi:10.1038/262580a0

Wakefield, M. J., Keohane, A. M., Turner, B. M., and Graves, J. A. (1997). Histone underacetylation is an ancient component of mammalian X chromosome inactivation. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 9665–9668. doi:10.1073/pnas.94.18.9665

Wang, C. W., Webley, L., Wei, K. J., Wakefield, M. J., Patel, H. R., Deakin, J. E., Alsop, A., Graves, J. A. M., Cooper, D. W., Nicholas, F. W., and Zenger, K. R. (2011). A second-generation anchored genetic linkage map of the tammar wallaby (*Macropus eugenii*). *BMC Genetics* **12**, 72.

Watson, D., Jacombs, A. S., Loebel, D. A., Robinson, E. S., and Johnston, P. G. (2000). Single nucleotide primer extension (SNuPE) analysis of the G6PD gene in somatic cells and oocytes of a kangaroo (*Macropus robustus*). *Genetical Research* **75**, 269–274. doi:10.1017/S0016672300004523

West, J. D., Frels, W. I., Chapman, V. M., and Papaioannou, V. E. (1977). Preferential expression of the maternally derived X chromosome in the mouse yolk sac. *Cell* **12**, 873–882. doi:10.1016/0092-8674(77)90151-9

Wolf, J. B., and Bryk, J. (2011). General lack of global dosage compensation in ZZ/ZW systems? Broadening the perspective with RNA-seq. *BMC Genomics* **12**, 91. doi:10.1186/1471-2164-12-91

Xu, N., Tsai, C. L., and Lee, J. T. (2006). Transient homologous chromosome pairing marks the onset of X inactivation. *Science* **311**, 1149–1152. doi:10.1126/science.1122984

Zakharova, I. S., Shevchenko, A. I., Shilov, A. G., Nesterova, T. B., VandeBerg, J. L., and Zakian, S. M. (2011). Histone H3 trimethylation at lysine 9 marks the inactive metaphase X chromosome in the marsupial *Monodelphis domestica*. *Chromosoma* **120**, 177–183. doi:10.1007/s00412-010-0300-y

Zehavi-Feferman, R., and Cooper, D. W. (1992). PCR derived cDNA clones for X-linked phosphoglycerate kinase-1 in a marsupial, the tammar wallaby (*Macropus eugenii*). *Biochemical and Biophysical Research Communications* **187**, 26–31. doi:10.1016/S0006-291X(05)81453-6

Zenger, K. R., McKenzie, L. M., and Cooper, D. W. (2002). The first comprehensive genetic linkage map of a marsupial: the tammar wallaby (*Macropus eugenii*). *Genetics* **162**, 321–330.

Handling Editor: Katherine Belov