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Unravelling the evolutionary origins of X chromosome inactivation in mammals: insights from marsupials and monotremes

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Abstract Determining the evolutionary origin of X inactivation mechanisms in mammals requires knowledge of features of X inactivation across all three major mammal lineages; monotremes, marsupials and eutherians. In the past, research into X inactivation in marsupials and monotremes lagged far behind the major advances made in understanding the mechanisms of X inactivation in human and mouse. Fragmentary knowledge of the genic content and sequence of marsupial and monotreme X chromosomes has been alleviated by the recent release of genome sequences for two marsupials and one monotreme. This has lead to a number of important findings, among which is the absence of *XIST* in marsupials and monotremes, and the surprising finding that X-borne genes in platypus are subject to stochastic transcriptional inhibition rather than whole chromosome inactivation. Availability of sequence data, and new techniques for studying expression and chromatin modification, now make rapid advance possible.

Keywords X chromosome inactivation · dosage compensation · marsupial · monotreme

Abbreviations

ATR	ataxia telangiectasia and Rad3 related
BRCA1	breast cancer 1
DMRT1	doublesex and mab-3 related transcription factor 1
G6PD	glucose–6-phosphate dehydrogenase
GLA	galactosidase, alpha
H3K4me2	histone H3 dimethylation on lysine 4
H3K9ac	histone H3 acetylation on lysine 9
H3K9me2	histone H3 dimethylation on lysine 9
H3K27me3	histone H3 trimethylation on lysine 27
H4Kac	histone H4 acetylation
HP1β	heterochromatin protein 1β
HP1γ	heterochromatin protein 1γ
HPRT1	hypoxanthine phosphoribosyltransferase 1
LINE1	long interspersed nuclear element 1
MHM	male hypermethylated
MSCI	meiotic sex chromosome inactivation
MYA	million years ago
PGK1	phosphoglycerate kinase 1
RNA-FISH	ribonuleic acid fluorescent in situ hybridisation
RT-PCR	reverse transcriptase polymerase chain reaction

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SLC1A1	solute carrier family 1 member 1
SLC16A2	solute carrier family 16, member 2
SNP	single nucleotide polymorphism
SNuPE	single nucleotide primer extension
TSIX	XIST antisense RNA
XCI	X chromosome inactivation
XIC	X inactivation centre
XIST	X-inactive specific transcript
Xite	X-inactivation intergenic transcription element
γH2AX	Phosphorylated histone H2AX

Introduction

The X chromosome of all species of placental mammals ('eutherians'; *eu*=true, *therian*=beast) have a suite of about 1100 genes that is virtually identical across species, and whose order is also highly conserved even between human and many other species (rodents are an exception). The Y chromosome is very much smaller, having lost all but a few of these genes (Graves et al. 2006), leaving genes on the X present in two doses in XX females and a single dose in XY males. The chromosome-wide silencing of genes on one X chromosome in eutherians compensates for this difference in gene dosage. This inactivation of one X chromosome occurs randomly in cells of the early embryo, and is stable and somatically heritable.

For over 50 years, X chromosome inactivation and the mechanisms behind it have been extensively studied in humans, mice and common domestic mammals. The X chromosome in eutherians is inactivated by a complex, multistep process, of which many of the major steps are understood. The initiation of the silencing process during embryogenesis is under the control of the X inactivation centre (XIC) (Brockdorff et al. 1991; Brown et al. 1991), a master locus encoding several non-coding RNAs, including the *XIST* (X Inactive Specific Transcript) gene. In each cell of the embryo, interplay of the XICs of each X chromosome is critical for counting the number of X chromosomes, choosing the one to inactivate and initiation of silencing (Bacher et al. 2006; Xu et al. 2006; Augui et al. 2007). The *XIST* transcript then coats the X chromosome destined for inactivation and initiates formation of a repressive compartment from which RNA polymerase II and other transcription factors are excluded (Chaumeil et al. 2006). The future inactive X then undergoes a series of chromatin changes through histone modifications and DNA methylation (reviewed by Heard 2005). The condensed chromatin of the inactive X (visible within the nucleus as a Barr body in humans) undergoes replication later in the cell cycle than the active X.

To begin tracing the evolutionary origins of this complex process, we need to look beyond these familiar eutherian mammals to distantly related members of the class Mammalia. Marsupials (infraclass Metatheria) and monotremes (subclass Prototheria) last shared a common ancestor with eutherian mammals 147 and 166 million years ago (MYA) respectively (Bininda-Emonds et al. 2007). Although eutherians, marsupials and monotremes share the common characteristics of mammals, such as fur and feeding their young milk, each of the three major lineages also differ in many unique attributes. Prominent unique features are their modes of reproduction and, in the case of monotremes, their sex chromosomes, both of which could have implications for their strategies for dosage compensation.

Here we summarize the history of X inactivation studies in marsupials and monotremes, report on the latest findings and discuss how they can answer some of the questions about the evolution of dosage compensation in mammals.

Marsupials

Marsupials give birth to immature young and most of their development occurs postnatally while the young are attached to a teat, often within the confines of a pouch. There are 270 species of marsupials, 200 of which live in Australasia and 70 in the Americas (one in North America and 69 in South America). Australian and American marsupials last shared a common ancestor around 70 MYA (Kirsch et al. 1997), so a comparison between American and Australian marsupials is similar in evolutionary terms to the human-mouse comparison which has been so informative for determining the common characteristics of X inactivation in eutherian mammals.

Early work on X inactivation in marsupials was carried out on several Australian species (largely kangaroos), and the North American opossum

(*Didelphis virginiana*). In more recent years, research has focussed on just two model marsupial species, the tammar wallaby (*Macropus eugenii*) and the South American opossum *(Monodelphis domestica*). The genomes for both of these species have been sequenced (Human Genome Sequencing Center at Baylor College of Medicine 2009; Mikkelsen et al. 2007), providing a valuable resource for studies on X inactivation. The tammar wallaby, a member of the kangaroo family (Macropodidae), is small and easily bred in captivity. Captive animals are derived from two genetically isolated populations (Kangaroo Island in the south and Garden Island in the west of Australia) which differ in many fixed polymorphisms (Zenger et al. 2002). Hybrids between these two populations are an asset for studies where it is important to determine the parental origin of alleles. Opossums (*M.domestica*) are the marsupial version of the lab mouse or rat, being easily housed and bred in the laboratory, and able to produce many young at once, which is particularly valuable for research into X inactivation during development.

The Marsupial X Chromosome

Marsupials have an XX female, XY male sex chromosome system. The marsupial X shares homology with two-thirds of the eutherian X chromosome and corresponds to an ancient (150MYA) conserved region of the human X chromosome (Fig. 1), whereas a recently added region of the human X is autosomal in marsupials (Graves 1995). Many genes from this added region escape inactivation in humans, presumably reflecting its recent arrival (Carrel and Willard 2005; Johnston et al. 2008). Given the high degree of homology between the marsupial and eutherian X, one might predict that dosage compensation would be similar in these two groups, having arisen from a common mechanism.

There are several clues in the organization of the X that this may not be the case. Despite conservation of gene content, gene order between marsupials and eutherians has not been retained. Several major rearrangements between the opossum and human X were revealed during the assembly of the opossum genome (Mikkelsen et al. 2007). Even more surprisingly, gene order is poorly conserved between marsupials, with many rearrangements detected between the opossum and wallaby X chromosomes (Deakin et al. 2008b). This contrasts with the eutherian X, which has undergone very little rearrangement since their divergence from marsupials. A suppression of rearrangement on the X in eutherians was suggested to be a consequence of selection against disruption of the chromosome-wide dosage compensation system (Ohno 1967). Moreover, LINE1 elements are concentrated on the human and mouse X chromosome, and their concentration correlates to the degree of inactivation, suggesting that they act as 'booster stations' for spread of silencing along the X chromosome (Lyon 1998; Carrel et al. 2006). Sequencing of the opossum X chromosome shows that LINE1 elements have only passively accumulated on the X and are unlikely to play a critical role in the inactivation process in marsupials (Mikkelsen et al. 2007).

These differences in structure and organization of the marsupial and eutherian X chromosomes raise the possibility that marsupials have a significantly different dosage compensation mechanism, having independently evolved at least some steps of the X inactivation mechanism. Does the data on marsupial X-inactivation allow us to recapitulate what steps are in common (and therefore ancient) and what steps are more recent embellishments in one or other lineage? First we will summarize what is known about marsupial X inactivation, and then consider this information with respect to evolution.

Paternal X inactivation

As in eutherians, one X of marsupials was found to be late replicating, leading to the assumption that marsupial X inactivation is similar to inactivation in human and mouse (Graves 1967). However, biochemical and cytogenetic studies in heterozygous and hybrid animals showed that it is qualitatively different.

The somatic tissues of eutherian mammals are mosaics of cells in which the active X is the one inherited from the mother or the father. This observation revealed that the decision of which X to silence is made randomly in cells of the inner cell mass (Lyon 1961). In contrast, early work on isozyme expression showed that marsupial X-inactivation is non-random, with the paternally-derived X being preferentially silenced (Richardson et al. 1971). In hybrid animals with distinguishable maternal and paternal X chromosomes,

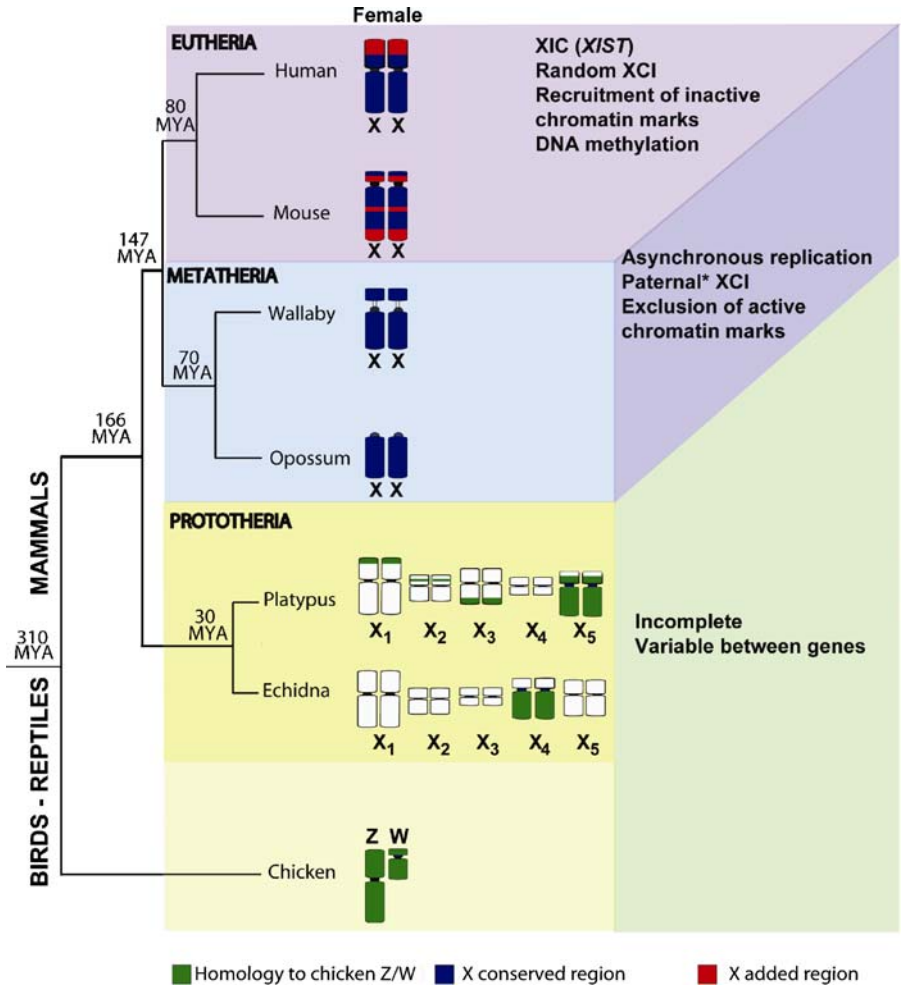

Fig. 1 Dosage compensation features for each of the three major mammalian lineages. The X chromosomes of marsupials and eutherians share homology, whereas the X chromosomes of monotremes share homology with the chicken Z chromosome. Putative lineage-specific characteristics and features common to more than one lineage are indicated. (*Paternal XCI in eutherians is observed in the extraembryonic tissues of rodents and cattle)

the paternal X was found always to be the late replicating one (Sharman 1971). Thus, marsupial X inactivation was the first example of parent-specific gene expression, ("genomic imprinting") to be reported in mammals (Cooper et al. 1971).

Despite its significance with respect to understanding X inactivation and genomic imprinting, paternal-specific silencing of the marsupial X is poorly studied. Paternal X inactivation has been described for a mere four genes spread over nine species in which they were polymorphic (Table 1), and the lack of consistency and small numbers of genes has made it difficult to establish the rules of marsupial X inactivation. Older studies determined the inactivation status of the classic *G6PD, PGK1,* and *GLA* (with indirect evidence about *HPRT1)* using isozyme analysis (reviewed in Cooper et al. 1993). Inactivation was shown to be incomplete and locus-specific. For instance, *G6PD* in *D. virginiana* was incompletely silenced in most tissues whereas *PGK1* showed complete inactivation (Cooper et al. 1993). Confusingly, the patterns of inactivation varied between species, with the paternal *G6PD* allele being completely silenced in somatic tissues from two kangaroo species (*Macropus robustus* and *Macropus rufogriseus*), yet was partially active in tissues from the North American opossum (*Didelphis virginiana*).

More sensitive molecular methods have been since used to detect transcription of two genes (*G6PD* and *PGK1*) on the marsupial X. SNuPE (Single Nucleotide Primer Extension) assay results confirmed isozyme studies showing complete silencing of the paternal copy of *G6PD* in somatic tissues in the wallaroo (*Macropus robustus*) (Watson et al. 2000), and allele-specific RT-PCR was used to show mostly complete inactivation of the paternal *G6PD*

Table 1 Genes and species used to study X inactivation in marsupials

Gene	Species	Method	Inactivation Status	
			Somatic tissues	Cultured fibroblasts
G6PD	*Macropus robustus*	Isozyme	Complete	Incomplete
		SNuPE[1]	Complete	Incomplete
	Macropus rufogriseus	Isozyme	Complete	Incomplete
	Didelphis virginiana	Isozyme	Incomplete	Incomplete
	Monodelphis domestica	Allele-specific RT-PCR[2]	Mostly complete	-
GLA	*Antechinus stuarttii*	Isozyme	Complete	Complete
	Kangaroo hybrids	Isozyme	Complete	Complete
HPRT	*Didelphis virginiana*	Isozyme	-	Incomplete
	Macropus		-	Incomplete
PGK	*Macropus giganteus*	Isozyme	Tissue specific	Incomplete
	Macropus parryi	Isozyme	Tissue specific	Incomplete
	Trichosurus vulpecula	Isozyme	Tissue specific	-
	Didelphis virginiana	Isozyme	Complete	Complete
	Monodelphis domestica	SNuPE[2]	Incomplete	-
SLC16A2	*Macropus eugenii*	RNA-FISH[3]	-	Incomplete

Isozyme data reviewed in Cooper et al. 1993. [1] Watson et al. 2000; [2] Hornecker et al. 2007; [3] Koina et al. 2005.

allele in the South American opossum (*M.domestica)* (Hornecker et al. 2007). However, allele-specific RT-PCR revealed expression of paternal *PGK1* in most tissues. These results were further verified with a SNuPE assay and hot-stop RT-PCR, which provides more quantitative data, demonstrating that the paternal *PGK1* allele was often expressed at the same, or occasionally even greater, level as the maternal copy (Hornecker et al. 2007).

Thus, although the paternal X is usually preferentially inactivated in all marsupials, patterns of gene expression on the marsupial X may differ between loci, between tissues, and between species.

Marsupial inactivation at the cellular level

Partial inactivation of the paternal allele of *G6PD, HPRT1* and *PGK1* observed in fibroblasts of most species and some somatic tissues, could be the result of uniformly low expression from the paternal X in every cell, or to a mix of cells, some with both copies active and others with monoallelic expression (Deakin et al. 2008a). None of these molecular assays discriminate between the two explanations. An alternative method for detecting inactivation at the transcript level is RNA-FISH, a technique that detects the nascent transcript at the site of transcription within individual nuclei. RNA-FISH was used to determine the inactivation status of *SLC16A2* on the tammar wallaby X chromosome. In 76% of nuclei examined, expression from just one allele was observed, and in 17% both were transcribed (a further 6% had no signal and 1% with 3 signals per nucleus) (Koina et al. 2005). It remains to be determined if cells with monoallelic expression are all transcribing only the maternal copy of *SLC16A2,* but these data suggest that partial expression of genes on the inactive X is due to some cells expressing both alleles and others just one.

Obviously information on the activity of many more genes on the marsupial X is required to understand the extent of marsupial X inactivation, and the patterns of variation. Current efforts of our lab are focussed on creating an activity map of the X chromosomes of both the opossum and tammar wallaby. A comparison of these maps between the two species will help to delineate the common features of marsupial X inactivation.

Sex chromosome elimination in marsupials

Several members of the bandicoot family (Peramelidae) take X chromosome inactivation to an extreme, a fascinating but surprisingly under-examined variation

on the paternal X inactivation observed in other marsupials. They eliminate one sex chromosome (one X in females and the Y in males) in somatic tissues at different stages of development (Hayman and Martin 1974). This elimination varies between tissues from loss of one sex chromosome in all cells to loss only in a subset of cells (Hayman and Martin 1965). A polymorphism in the PGK1 protein in Southern brown bandicoots (*Isodon obesulus*) has provided some evidence that it is always the paternal X that is eliminated in female somatic tissues (Johnston et al. 2002). In this species, as in other marsupials, the paternal X is late replicating, as is the Y (Johnston et al. 2002).The mechanism of sex chromosome loss remains unknown, but the preferential elimination of the paternal X and its delayed replication suggest a connection with X inactivation.

Marsupial X inactivation is not controlled by XIST

One of the most striking differences between marsupial and eutherian XCI is the absence of an *XIST* gene from the marsupial X chromosome. *XIST* and other neighbouring non-coding RNAs within the X inactivation centre play critical roles in X inactivation in eutherians, including the counting of the number of X chromosomes, the choice of the X chromosome to be inactivated and the initiation of silencing.

After many years of searching failed to find an *XIST* orthologue in marsupials, careful assembly and sequencing of the regions that flank this gene in humans and mice showed that *XIST* does not exist. In fact, genes flanking the XIC in humans, map to two different regions of the X chromosome in both the opossum and tammar wallaby (Davidow et al. 2007; Hore et al. 2007; Shevchenko et al. 2007; Deakin et al. 2008b), indicating that this region has been significantly rearranged since the divergence of marsupials and eutherians. Comparisons with genes in the orthologous region in birds and frogs suggest that some regions of the non-coding RNAs from within the XIC (including *XIST*) were derived from protein-coding genes, some of which are present in marsupials, but all of which have succumbed to pseudogenisation in eutherians (Duret et al. 2006). Integration of mobile elements during marsupial/eutherian divergence or early in the eutherian lineage may have augmented the function of the proto-*XIST* gene, allowing it to evolve a role in X inactivation (Elisaphenko et al. 2008). Another of these non-coding RNAs is *TSIX*, the non-coding RNA with a role in counting and choice in mice but not humans, also appears to have evolved in early eutherians, with the participation of repeat elements (Cohen et al. 2007).

The absence of *XIST* and disruption of its flanking markers means that X inactivation in marsupials cannot be under the control of the same XIC as it is in eutherians. Is it possible that there is a marsupial specific inactivation centre? Early studies of gene order and expression suggested a polarity in expression (Graves and Dawson 1988). However, the substantial rearrangement of the X between opossum and wallaby suggests that, rather, marsupial X inactivation is achieved on a gene by gene basis (Deakin et al. 2008b). Perhaps the evolution of *XIST* has imposed a chromosome-wide control on the X in eutherians.

Epigenetic marks involved in marsupial XCI

The human inactive X is visible under the microscope as a densely staining sex chromatin or "Barr body". No Barr body was distinguishable in the nucleus of adult possum cells (McKay et al. 1987), although it was obvious in the early embryo of a dasyurid marsupial, *Antechinus stuartii* (Johnston and Robinson 1987).

The heterochromatic state of the eutherian inactive X chromosome is now known to be due to the specific pattern of chromatin marks that distinguish it from its active homologue (reviewed in Heard 2005). Histone modifications appear at the time when inactivation becomes irreversible, making them candidates for being part of the 'lock-in' system of the silent state (Kohlmaier et al. 2004). DNA methylation at 5′CpG dinucleotides which appears later, seems to be involved in the maintenance of the inactive state (Lock et al. 1987; Norris et al. 1991; Sado et al. 2004). Thus, it is of great interest to compare this profile of modifications in marsupials, and to explore their potential role in marsupial X chromosome inactivation.

The eutherian inactive X chromosome loses histone modifications associated with transcriptional activity (H3K4me2, H3K9ac, H4Kac) and accumulates marks associated with inactivity (H3K9me2 and H3K27me3) (Keohane et al. 1996; Heard et al. 2001;

Chaumeil et al. 2002; Okamoto et al. 2004). These three active marks are also lost from the marsupial X (Wakefield et al. 1997; Koina et al. 2009). However, in metaphase spreads, no enrichment of repressive marks H3K9me2 or H3K27me3 could be detected on one of the two wallaby X chromosomes (Koina et al. 2009). Indeed, some of our preliminary results suggest that H3K27me3 is not stably enriched on the inactive X in interphase (Chaumeil et al. in preparation). As recruitment of H3K27me3, and probably also of H3K9me2, depends on *XIST* RNA localisation in eutherians (Kohlmaier et al. 2004), their absence on the marsupial inactive X chromosome is consistent with the absence of the *XIST* gene from the marsupial genome. These modifications are suggested to help stabilise the inactive state of the eutherian X (Silva et al. 2003), so their absence from the marsupial X, at least at metaphase, might render XCI less stable in marsupials, as suggested by the observations of Kaslow and Migeon (1987).

In eutherians, X chromosome inactivation is accompanied by methylation of 5′ CpGs (Mohandas et al. 1981; Graves 1982) although this appears to stabilize, rather than initiate, inactivation (Keohane et al. 1998). In marsupials there seems not to be methylation differences between the active and inactive X chromosomes, although this conclusion has been based on the study of just two genes, *G6PD* and *PGK1*. Kaslow and Migeon (1987) failed to find methylation differences in the *G6PD* 5′ CpGs of the American opossum (*Didelphis virginiana*), using methylation sensitive restriction enzymes. More recently, Hornecker et al (2007) used bisulfite sequencing to demonstrate that 5′ CpG islands of *G6PD* and *PGK1* were not differentially methylated in *M. domestica*. The methylation status of the wallaroo *G6PD* 5′ CpG island gene was also determined using bisulfite sequencing, but no differential methylation was detected (Loebel and Johnston 1996).

However, one study is inconsistent with the conclusion that there are no DNA methylation differences between the active and inactive X chromosomes in marsupials. A chromosome-wide examination using methylation sensitive restriction enzymes on metaphase chromosomes followed by *in situ* nick translation showed considerable methylation of the paternal X (Loebel and Johnston 1993). Similarly, it was thought that imprinting of *IGF2* in marsupials was independent of methylation (Weidman et al. 2004), but this has recently been shown to be untrue, with methylation differences demonstrated in a previously unidentified 5′ untranslated exon of the opossum *IGF2* gene (Lawton et al. 2008). Obviously we must probe the methylation status of more genes before we can rule out a role for methylation in marsupial X inactivation.

Thus, these studies demonstrated that loss of active histone marks is a common feature of marsupial and eutherian X chromosome inactivation. On the contrary, it seems that the marsupial inactive X fails to recruit the same inactive marks as the eutherian inactive X, at least in a stable manner. This could be due to the lack of the *XIST* gene in marsupials that is involved in recruiting repressive marks for stabilisation of the silencing state, and could explain, at least partially, why X chromosome inactivation is less stable in marsupials than in eutherians.

Meiotic sex chromosome inactivation (MSCI)

In eutherian mammals, the X (and Y) chromosome is inactivated during spermatogenesis, presumably to account for gene dosage imbalances between X- and Y- bearing sperm (Monesi 1965; Lifschytz and Lindsley 1972). MSCI has recently been discovered in opossum by analysing expression of nine X-borne housekeeping and three germcell-specific genes in meiotic pachytene spermatocytes. Seven of these genes showed a decrease in transcription level during spermatogenesis, suggesting that MSCI does occur in marsupials (Hornecker et al. 2007). Namekawa et al (2007) showed that marsupial MSCI shares many common features with MSCI in eutherians, including the binding of heterochromatin-associated proteins (HP1β and HP1γ) and histone variant γH2AX. However, HP1β and HP1γ binding occurs earlier in the opossum than it does in the mouse, being detectable by early pachytene. MSCI in mouse is not controlled by *Xist* (McCarrey et al. 2002; Turner et al. 2002), but two proteins, BRCA1 and kinase ATR, appear to contribute to chromatin condensation (Turner et al. 2004). Expression of *BRCA1* and *ATR* during opossum spermatogenesis is consistent with a role in MSCI (Hornecker et al. 2007). Following meiosis, silencing of the X chromosome persists into spermiogenesis and is correlated with HP1β and HP1γ and enrichment and H3-K9 trimethylation (Hornecker et al. 2007; Namekawa et al. 2007).

It was proposed (Cooper 1971) that in marsupials, the paternal X, as well as the Y, is inactivated during male meiosis and simply stays inactive throughout fertilization and development. H3K9me3 and/or HP1 in MSCI may represent imprint marks that keep the paternal X inactive or allow subsequent inactivation of paternal X in the embryo. For example, H3K9me3 is also found enriched on the inactive X in interphase in somatic female cells (Chaumeil et al, in preparation).

X inactivation in an ancestral therian mammal

Is MSCI ancestral? Is imprinted X inactivation ancestral? After the demonstration of paternal X inactivation in marsupials, paternal X inactivation was observed in the extra-embryonic tissues of rodents (Takagi and Sasaki 1975; Wake et al. 1976; West et al. 1977). Paternal X inactivation was not found in humans (Zeng and Yankowitz 2003) but was recently demonstrated in cattle (Dindot et al. 2004), suggesting that it was ancestral in eutherians and lost in humans. It has been proposed that imprinted X inactivation is ancestral since it occurs in both marsupials and eutherians (Cooper et al. 1971, 1993). Supporting this hypothesis is the observation that imprinted inactivation in mouse extra-embryonic tissues, like marsupial X inactivation, is less stable and incomplete, and does not involve DNA methylation (Huynh and Lee 2005). However this imprinted X inactivation in mice relies on *Xist*, which cannot be the case in marsupials (Marahrens et al. 1997; Okamoto et al. 2005). In mice, imprinted inactivation of the paternal X occurs very early in development, and is stabilized in the extraembryonic tissues. However, the paternal X is reactivated in blastocysts prior to undergoing random X inactivation (Huynh and Lee 2003; Mak et al. 2004; Okamoto et al. 2005).

An extension of this hypothesis is that imprinted X inactivation is a carryover from MSCI, with the suggestion that the paternally derived X arrives in the embryo in a "pre-inactivated" state. This state persists in marsupials and the extraembryonic tissues of eutherians but has been lost in the somatic cells of eutherians with the more recent evolution of randomness (Huynh and Lee 2005). Demonstration that the paternal X arrives inactive in the embryo would be very important, for it would establish an evolutionary link between MSCI and X inactivation. However, in mice the paternal X arrives active in the zygote and initiation of its inactivation at the 4 cell stage is independent of its inactivation during meiosis (Okamoto et al. 2004, 2005; Patrat et al. 2009). A more likely explanation is that an imprint is deposited on the paternal X chromosome during MSCI that allows the subsequent inactivation of this chromosome during early development of marsupials and eutherians. Continued investigations of marsupial MSCI and XCI are required to test this hypothesis.

Future directions for marsupial dosage compensation studies

Many basic questions regarding X inactivation in marsupials remain unanswered. Does X inactivation in marsupials result in full or partial (or any?) dosage compensation between males and females? Is there dosage compensation between the X and autosomes as there is in eutherians? Is inactivation via transcriptional repression as it is in eutherians (Graves and Gartler 1986)? Is there any evidence of differential methylation? Is inactivation controlled across the X by a marsupial *XIST*-like gene, or does inactivation occur on a gene-by-gene basis? Armed with knowledge of the genic content and sequence of the marsupial X chromosome, we are able to work towards answering these questions.

Monotremes

Monotremes are a basal branch of mammals, a subclass (Prototheria) that diverged from the eutherian-marsupial lineage about 166 million years ago. They have a curious mix of reptilian and mammalian features, exemplified by feeding their young milk, like all other mammals, yet laying eggs similar to those of snakes. Monotremes are represented by four species of echidna and just one extant species of platypus that is limited to Australia. These animals are difficult to breed reliably in captivity (Temple-Smith and Grant 2001), making it troublesome to obtain tissue samples for dosage compensation studies, and virtually impossible to acquire monotreme embryos. However, advances are being made in monotreme husbandry which may improve our chances of obtaining such samples in the future.

Despite the lack of a laboratory model monotreme, we have recently gained insight into dosage compensation for the platypus. This has followed the clarification, greatly assisted by the platypus genome project (Warren et al. 2008), of the genic content of the platypus sex chromosomes.

Unique sex chromosome system

The unique features of monotremes include bizarre sex chromosomes. Like other mammals, they have male heterogamety, but their sex chromosome system is rather complex. The platypus (*Ornithorhynchus anatinus*) has ten sex chromosomes; females have five pairs of X chromosomes ($X_1X_2X_3X_4X_5$) and males have five different X ($X_1X_2X_3X_4X_5$) and five different Y chromosomes ($Y_1Y_2Y_3Y_4$ Y_5) (Grützner et al. 2004; Rens et al. 2004). In echidnas (*Tachyglossus aculeatus*), females have five pairs of X chromosomes and males have five X chromosomes ($X_1X_2X_3X_4X_5$) and four Y chromosomes ($Y_1Y_2Y_3Y_4$) (Rens et al. 2007). In both of these species, the X and Ys form a multivalent translocation chain during male meiosis, in which X and Y chromosomes pair within terminal pseudoautosomal regions to form a chain of alternating X and Y chromosomes, which segregate into X-bearing (female-determining) and Y-bearing (male-determining) sperm (Grützner et al. 2004).

The gene content of the X chromosomes was expected to be related to that of the therian X. However, contrary to earlier reports, monotreme sex chromosomes share no homology with the those of therians (Veyrunes et al. 2008). Genes from the therian X chromosome, including those flanking *XIST* in eutherians, lie on chromosome 6 (Waters et al. 2005; Hore et al. 2007; Veyrunes et al. 2008). Intriguingly, monotreme X chromosomes share homology with the Z chromosome of birds (Fig. 1), and include the bird candidate sex determining gene *DMRT1* (Rens et al. 2007; Veyrunes et al. 2008). The large platypus X_5 contains the largest region homologous to the chicken Z (Veyrunes et al. 2008), with smaller areas of homology spread over X_1, X_2 and X_3. Platypus X chromosomes also share some homology with chicken chromosomes 2, 3, 12, 13, 16 and 17.

Although the echidna has a similar multiple XY complex, the platypus and echidna chains are not entirely homologous. Chromosome painting has shown that platypus X_4 corresponds to echidna chromosome 27, whereas echidna X_5 shares homology with platypus chromosome 12 (Rens et al. 2007), suggesting that four elements of the chain were in place by the time platypus and echidnas diverged, but different members were added independently in the platypus and echidna lineages. The gene content of the echidna X chromosomes is not completely known, but the region containing *DMRT1* and other genes on platypus X_5 have been localised to echidna X_4 (Rens et al. 2007).

This unexpected homology between the platypus XY and the bird ZW chromosomes suggests that early mammals had a bird-like sex chromosome system that was usurped by the evolution of the *SRY* gene from *SOX3* on an autosome that became redefined as an XY pair (Graves 2008).

Variable dosage compensation in the platypus

The need for dosage compensation in monotremes would appear to be extreme, since the multiple X chromosomes of the complex account for approximately 15% of the haploid genome. They are mostly unpaired by the Y chromosomes, which are smaller (5%) and largely heterochromatic. Thus at least 12% of the genome is present in twice the dose in females as in males.

The different origin of the monotreme and therian sex chromosomes might suggest an independent origin of dosage compensation in this lineage. Are platypus X chromosomes inactivated as in mouse and man? Or is there a partial system of dosage compensation as has been described for birds, or a unique monotreme dosage compensation system? The homology between monotreme and bird sex chromosomes suggests that dosage compensation in these two lineages may be expected to share some common features, except that gene dosage is in the opposite direction; in birds, it is 2:1 in favour of males, and in platypus 2:1 in favour of females, so how a common mechanism for dosage compensation could function is not obvious.

Early studies of replication timing of the large X_1 in platypus and echidna fibroblasts found no asynchronous replication of the unpaired region of this chromosome (Murtagh 1977; Wrigley and Graves 1988). Although echidna lymphocytes showed asynchronous replication of the short arm of X_1 (Wrigley and Graves 1988), this region pairs with Y_1 and would not require dosage compensation (Grützner et

al. 2003). These inconsistent results did not make a compelling case for X inactivation.

Now that genes have been assigned to platypus X chromosomes, and considerable sequence is available, more sophisticated molecular studies can be performed. These show, surprisingly, that platypus dosage compensation is partial and differs between genes (Deakin et al. 2008a). Female to male expression ratios were determined for 19 genes located on platypus X chromosomes. Nine of these genes lie in pseudoautosomal regions. Expression ratios ranged from 0.7 – 1.2, indicating that their Y homologues are active. Two pseudoautosomal genes had ratios around 2.0, suggesting that either their Y copies are not expressed or that the sequence of the Y homologues has diverged from that of the X copy. Of the 10 genes in X-specific regions, half had a female to male ratio close to one (complete compensation), two had an intermediate ratio of around 1.4 (partial compensation), and three showed no evidence of compensation, having a ratio close to two (Deakin et al. 2008a) (Table 2). Considerable variation between individuals was observed.

This range of levels of compensation between genes resembles the variable and incomplete dosage compensation observed in birds (Ellegren et al. 2007; Itoh et al. 2007). Microarray comparisons of male: female expression ratios in chicken and zebrafinch produced a distribution of genes, with some fully compensated, some uncompensated, but most somewhere between (Itoh et al. 2007). The distribution of compensated and uncompensated genes along the Z chromosome was not random: a region in which most genes are not compensated ('peak') has been found on the long arm of the chicken Z, and one region contains genes that are generally compensated ('valley'). Although Mank and Ellegren (2009) argue that these two regions are an artefact of the analysis approach, it is intriguing that this 'valley' of compensation lies near the *MHM* (male hypermethylated) locus on chicken Zp (Melamed and Arnold 2007), which transcribes a non-coding RNA only in females which accumulates near the *DMRT1* locus (Teranishi et al. 2001). With data for only ten X-specific genes in platypus, it is difficult to determine if a similar trend exists in this species. Intriguingly, two genes on X_5 (*DMRT2* and *SLC1A1*) found in the valley of compensation on the chicken Z appear not to be compensated in platypus, suggesting that XX females are more tolerant of higher expression of these genes than ZZ males.

As for marsupials, partial expression in monotremes could be due to a uniformly lower expression of one allele than the other in all cells, or to a mixture of cells in which one or both alleles is active. Examination of the probability of expression via RNA-FISH strongly favoured the latter explanation. Genes showed different frequencies of two X-active nuclei, ranging from 20% to 53%, with an average of 45% of nuclei with biallelic expression. This suggests

Table 2 Female:male expression ratios and frequency of monoallelic expression for platypus X-specific genes (Deakin et al. 2008a)

Chromosome	Gene	Female:Male Ratio	Percent of nuclei with monoallelic expression
Compensated			
X_1	Ox_plat_124086	1.10	46
X_5	*ZNF474, LOX**	1.01, 1.06	53
X_3	*APC*	1.17	48
X_5	*SHB*	1.23	53
Partially Compensated			
X_5	*FBXO10*	1.37	50
X_5	*EN14997*	1.40	61
Not compensated			
X_5	*SEMA6A*	1.82	74
X_5	*DMRT2*	2.04	47
X_5	*SLC1A1*	2.78	45

*These genes were contained within a single BAC used as a probe for RNA-FISH

some form of transcriptional silencing (Deakin et al. 2008a). Single Nucleotide Polymorphisms (SNPs) found in three of these X-specific genes were biallelically expressed, with equal expression of each allele, indicating that the monallelic expression detected is stochastic, and not imprinted as it is in marsupials (Deakin et al. 2008a). This stochastic expression may be co-ordinately regulated in platypus as two genes lying 500kb apart always showed inactivation of the same X. Studying genes that are further apart will show if coordination is at a local level or is chromosome-wide.

Is stochastic monoallelic expression, such as that described for the platypus, a basic mechanism from which X inactivation arose? Ohlsson et al. (2001) proposed that X inactivation and genomic imprinting arose from stochastic expression that later became more strictly controlled. A recent study has shown that stochastic monoallelic expression is widespread on human autosomes, affecting perhaps as many as 1000 genes in the human genome (Gimelbrant et al. 2007). Most genes with stochastic expression also showed some level of biallelic expression, similar to that seen in the platypus. Further support for this hypothesis comes from experiments on mouse tetraploid cells, in which each X chromosome was found to have an independent probability of initiating X chromosome inactivation (Monkhorst et al. 2008). Interestingly, the "probability-promoting factor" is encoded outside of the *Xist-Tsix-Xite* region (Monkhorst et al. 2008) and the inactivation of a single X is locked in by a feedback mechanism, controlled by the X inactivation centre, that suppresses the inactivation of the active X (Mlynarczyk-Evans et al. 2006). Incomplete platypus dosage compensation may result from the absence of such a feedback mechanism in a species with no *XIST*.

Future directions for monotreme dosage compensation studies

It will be important to extend these studies to many more platypus genes, potentially matching the microarray studies in birds. It would also be very advantageous to extend these studies to the echidna, which has many advantages as a monotreme model, especially if improved husbandry makes captive breeding more successful, and eggs available. To date, no molecular studies have been conducted on echidna dosage compensation. Echidna sex chromosomes share only four of the five XY elements of the chain so comparison of dosage compensation between platypus and echidna could provide information on whether differences in gene content direct dosage compensation.

Examining X inactivation in monotremes early in development could help answer many questions about the evolution of X inactivation. Do monotremes have imprinted X inactivation early in development that is then lost, as in eutherians? Is dosage compensation more tightly controlled during development?

At this stage, it is unknown the extent (if any) to which chromatin modifications play a role in platypus dosage compensation. Hence, the logical next step is to examine these modifications in the platypus. Of particular interest would be those found in both marsupials and eutherians, such as the marks of the active X.

Concluding Remarks

An understanding of dosage compensation in marsupials and monotremes will help us to unravel the evolutionary origins of dosage compensation in mammals. Our discovery that the X chromosomes in platypus have a bird-like partial dosage compensation system as well as homology to the bird Z chromosome, changes our picture of how and when X chromosome inactivation evolved in mammals (Fig. 1).

Our discovery that the therian XY chromosome pair is represented by an autosome in platypus, as well as in birds, dates the origin of the therian XY system to 145-166MYA, comparatively recently. The ancestral mammal almost certainly had ZW or XY chromosomes with homology to the bird ZW chromosome pair (Graves 2008). Our discovery that dosage compensation in the platypus is only partial and locus specific, similar to birds, strongly suggests that monotremes have retained an ancestral compensation system.

The occurrence of X chromosome inactivation in marsupials as well as eutherians suggests that the marsupial and eutherian X inactivation systems share a common evolutionary origin. Observations that MSCI and paternal X inactivation occurs in marsupials and the extraembryonic tissues of eutherians, and is less stable, incomplete and lacking differential DNA methylation, suggests that the ancestral system involved paternal-specific

inactivation, and ultimately evolved from MSCI (Huynh and Lee 2005).

However, the major differences in inactivation phenotype, and in the molecular mechanism of X inactivation in marsupials and eutherians, suggests an ongoing sophistication of X inactivation in eutherians. In particular the finding that imprinted XCI in mice depends on *Xist* (Marahrens et al. 1997; Okamoto et al. 2005), but is *XIST*-independent in marsupials (Duret et al. 2006; Davidow et al. 2007; Hore et al. 2007; Shevchenko et al. 2007) would require that an ancestral paternal, incomplete X inactivation mechanism was brought under chromosome-wide control by acquisition of the *XIST* locus early in eutherian evolution. Differences in the molecular mechanism of inactivation suggest that a more complete and stable system evolved with the addition of repressive histone marks and DNA methylation on the inactive X, which may be linked to the action of the non-coding *XIST* RNA.

Despite the differences in dosage compensation mechanisms between monotremes and therians, both systems may use similar molecular changes from an ancient molecular toolbox. As suggested by Ohlsson et al. (2001), X inactivation may have originally arisen from stochastic monoallelic expression that is now known for many genes on all chromosomes (Gimelbrant et al. 2007), and this hypothesis gains support from observations of the stochastic nature of the initiation of X inactivation in mice (Monkhorst et al. 2008). Our discovery of the stochastic expression of platypus X genes suggests that monotreme dosage compensation also represents a specialization of stochastic monoallelic expression.

This hypothesis proposes that alteration of chromatin structure (involving histone modification, DNA methylation, change in replication time) changes the probability of transcription of genes. Further characterisation of X inactivation in marsupials and monotremes will help to identify common elements of epigenetic control of gene action in mammals, and to determine how they were built into the complex system by which the eutherian X is inactivated.

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