Manuscript submitted to: Volume 2, Issue 2, 110-126.

AIMS Genetics DOI: 10.3934/genet.2015.2.110

Received date 3 November 2014, Accepted date 1 March 2015, Published date 4 March 2015

Review

Imprinted X chromosome inactivation: evolution of mechanisms in distantly related mammals

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Abstract: In females, X chromosome inactivation (XCI) ensures transcriptional silencing of one of the two Xs (either in a random or imprinted fashion) in somatic cells. Comparing this silencing between species has offered insight into different mechanisms of X inactivation, providing clues into the evolution of this epigenetic process in mammals. Long-noncoding RNAs have emerged as a common theme in XCI of therian mammals (eutherian and marsupial). Eutherian X inactivation is regulated by the noncoding RNA product of *XIST*, within a *cis*-acting master control region called the X inactivation center (XIC). Marsupials XCI is *XIST* independent. Instead, XCI is controlled by the long-noncoding RNA *Rsx*, which appears to be a functional analog of the eutherian *XIST* gene, insofar that its transcript coats the inactive X and represses activity of genes *in cis*. In this review we discuss XCI in eutherians, and contrast imprinted X inactivation in mouse and marsupials. We provide particular focus on the evolution of genomic elements that confer the unique epigenetic features that characterize the inactive X chromosome.

Keywords: X chromosome; XCI; imprinting; noncoding RNA; epigenetic regulation; marsupial; *XIST*; RSX; X inactivation center; evolution

Abbreviations

MYA: Million years ago
MSY: Male specific region of the Y
XCR: X conserved region
MSCI: Meiotic sex chromosome inactivation
XCI: X-chromosome inactivation
Xp: Paternally inherited X chromosome
Xi: Inactive X chromosome

XIC: X Inactivation Center
RepA: Repeat A
Rsx: RNA on the silent X

The class Mammalia (mammals) is divided in to two subclasses: Prototheria (monotremes) and Theria (marsupials and eutherians). All therian mammals have male heterogamety, with an XX female: XY male sex chromosome system (Figure 1), or some simple variant. This sex chromosome system arose before the marsupial/eutherian split, ~ 180 million years ago (MYA) [1], from an ordinary pair of autosomes after a mutation in the *Sox3* gene resulted in the birth of the testis-determining gene *Sry* [2].

Genes advantageous to males accumulated on the proto Y near *Sry* either by transposition from autosomal sites or by mutation of existing genes. Recombination with the X was suppressed across this region so that the male advantageous genes were only inherited with the testis-determining gene, giving rise to the male specific region of the Y (MSY). Lack of recombination led to progressive gene loss on, and degradation of, the MSY [3]. Thus, in all therian species the X and Y are morphologically distinct.

1. Mammal sex chromosomes

The marsupial X chromosome is ~ 2/3 the size of the eutherian X, and is homologous to the long arm (Xq) and proximal short arm (Xp) of human X chromosome (called the X conserved region; XCR). In contrast, the short arm of the human X chromosome (distal to Xp11.22) is orthologues to marsupial autosomes, so was added to the eutherian X before the radiation of eutherians (~ 105 MYA), but after their divergence from marsupials [4] (Figure 1).

Monotremes, which diverged from therian mammals ~ 200 MYA, comprise one extant platypus and four extant echidna species, all with a complex of serially translocated sex chromosomes. In the model monotreme, platypus (*Ornithorhychus anatinus*), males have five X and five Y chromosomes, and females have 5 pairs of X chromosomes [5]. These sex chromosomes do not bear the *Sry* gene or share homology with the sex chromosome of therian mammals [6] (Figure 1). Instead, they share extensive homology with the independently evolved bird ZW sex chromosome system [6,7]. Thus, sex chromosomes have evolved multiple times throughout amniote evolution [1] (Figure 1).

2. Mammal sex chromosome dosage compensation

In spite of the lethal effect whole chromosome monosomy has for any autosome [8], such grand sex chromosome imbalances are present in many distantly related species. Ohno [9] suggested that copy number imbalance of the X with the autosomes (1X: 2A) in males resulted in the almost twofold upregulation of the X. This led to overexpression from the two Xs in females, which resulted in down-regulation of one X in that sex [10].

Upregulation of expression from the single X in male is observed in marsupials, where average transcriptional output is near diploid expression levels [11]. However, whether or not the single X is upregulated in male eutherian mammals has remained controversial as a result of inconsistent processing, filtering and analysis methods of transcriptome data [12]. The debate surrounding Ohno's

hypothesis [13,14,15,16,17,18] has spawned a novel view that eutherian dosage compensation evolved to restore balance of X genes, which function in protein complexes or protein networks, with their autosomal partners [12]. In some instances expression of X genes were increased to match the original autosomal level (as proposed by Ohno), and in other instances expression of the autosomal genes was decreased to match the new reduced X level This suggests that hyper-expression evolved on a gene-by-gene basis and affected only a subset of X genes.

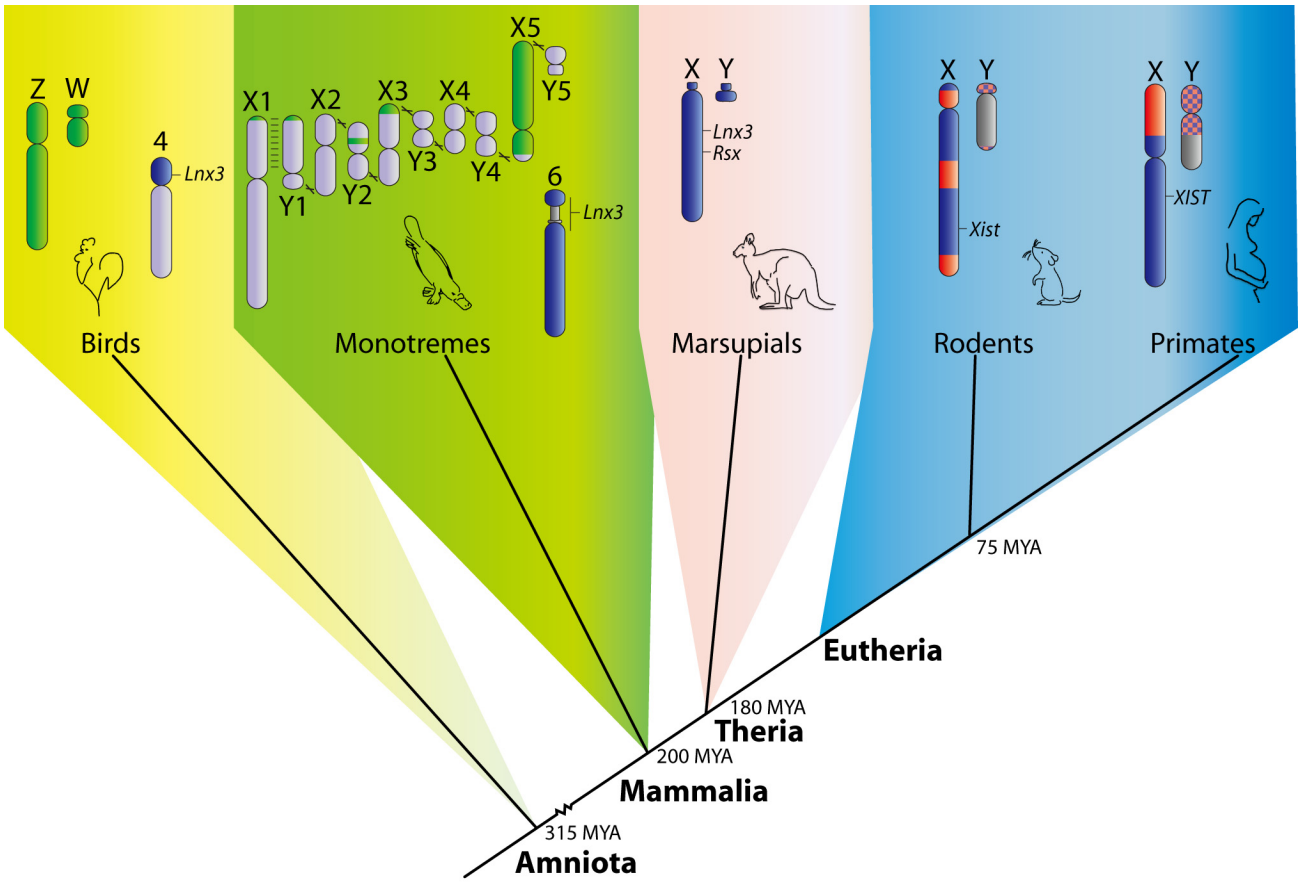

Figure 1. Sex chromosome systems in amniote vertebrates. Eutherian and marsupial mammals both have XY male: XX female sex chromosome systems that share considerable homology (blue). A region that is autosomal in marsupials (red) was added to the X in the eutherian ancestor. Both conserved (blue) and added (red) regions are autosomal in birds and monotremes. In the model monotreme (platypus) males have five X chromosomes and five Y chromosomes, whereas females have five pairs of Xs. Birds have a ZZ male: ZW female sex chromosome system. The bird Z shares homology with platypus X5 (green). The position of *Xist* and its orthologue (*Lnx3*) are shown in each lineage. The marsupial specific *Rsx* is also shown.

In female eutherians and marsupials, down-regulation of X genes to restore parity with the autosomes is achieved by X-chromosome inactivation (XCI); an epigenetic mechanism by which one of the two X chromosomes is silenced in somatic cells. Once silencing has occurred, it is stably

maintained throughout all ensuing cell divisions [19]. Although some features that characterize the inactive X (Xi) chromosome are shared between the two lineages, lineage-specific genetic, and epigenetic differences exist [20,21,22,23,24,25]. These similarities and differences provide insight into the evolution of mammal XCI.

3. XCI in therian mammals

In the male germline of both eutherians and marsupials, sex chromosomes are inactivated during meiosis in a process called meiotic sex chromosome inactivation (MSCI) [26,27,28,29,30]. All X-borne genes tested in opossum round spermatids were reactivated and expressed [28]. In mouse reactivation is only observed for some genes after spermatogenesis, so the paternally inherited X chromosome (X^p) is delivered to the ovum in a partially pre-inactivated state [31,32]. After fertilization, transcription of repetitive elements on the X^p is suppressed [33], but biallelic expression is observed for X-borne genes at the two-cell stage [33,34,35,36].

During eutherian mammal XCI, the choice of which X is to be inactivated can be either random with regard to the parent of origin, or imprinted, where the paternal X is inactivated in all cells. These two forms of XCI are species specific, but can also occur in different cell types within the same species. During mouse pre-implantation development, exclusive silencing of the X^p leads to establishment of imprinted-XCI [37,38,39] through to the blastocyst stage. Beyond this, imprinted XCI is maintained only in the trophectodermal extra-embryonic cell lineages that give rise to placental tissue, and the primitive endoderm that gives rise to the visceral endoderm and yolk sac [40]. In contrast, in the developing inner cell mass, which gives rise to the embryo proper, the inactive X^p is reactivated, which is then followed by random XCI [40,41,42]. Imprinted XCI was also observed in extra-embryonic cell lineages of rat [43] and cow [44,45]. However, in human, monkey, horse, pig, mule and rabbit random XCI was observed in both embryonic and extra-embryonic cells [46,47].

One of the marked differences between marsupial and eutherian XCI is the choice of X to be inactivated in the embryo proper. XCI in marsupial extra-embryonic, fetal, and adult tissues is imprinted, with the paternally derived X always silenced [24,25,48]. The reason and cause for this difference in choice during X inactivation is not understood.

Although the marsupial inactive X shares some similarities with the eutherian Xi at the cytogenetic level, such as late replication at S phase and heterochromatinization [49,50,51,52,53,54], it differs at the molecular level [22,25]. There are considerable differences in the histone profile of the inactive X between eutherians and marsupials [21] (Table 1), but in general at the onset of XCI, the inactive X loses epigenetic modifications associated with active transcription (e.g. H3K9ac, H4Kac and H3K4me2) and sequentially acquires repressive marks characteristics of silenced chromatin (e.g. H4K20me1 and H3K27me3). In addition, the eutherian Xi exhibits enrichment of histone variants such as macro-H2A, and hypermethylation of promoter sequences stabilizes inactivation once repression has occurred [55] (Table 1). The Xi in marsupial female possum and potoroo metaphase appears hypomethylated [23], in addition promoter DNA methylation appears absent on the Xi for loci tested in opossum [25,56].

Table 1. Comparison of repressive and active epigenetic marks on the inactive X chromosome in eutherian (mouse, human) and marsupials (Opossum).

		Eutherian Xi			Marsupial Xi
		Mouse		Human	Opossum
		Somatic cells, or embryonic stem cells	Extra-embryonic cells	Somatic cells, or embryonic stem cells	Somatic cells
		Random XCI	Imprinted XCI	Random XCI	Imprinted XCI
Active marks	H3ac	O	O	O	O
	H4ac	O	O	O	O
	H3K4me2	O	O	O	O
Repressive marks	H3K9me2	★	★⌛	★	★1
	H3K9me3	O	★	★	★
	H3K27me3	★	★⌛	★	★2
	H4K20me1	★	★	?	O
	H4K20me3	O	★	★	★
	Macro-H2A	★⌛	★	★	?
	Promoter CpG hyper-methylation	¶⌛	¶⌛	¶	┤

★ = enriched on Xi, O= excluded from Xi, ¶= present, ┤= absent, ? = not determined, 1 = cell cycle specific, 2 = tissue specific; ⌛ = late event

4. Genes that escape inactivation

The final outcome of these modifications is silencing of transcription from most genes on the Xi. However, some genes escape inactivation, and as a result are expressed from both active and inactive X chromosomes [57]. The chromatin state of these genes more closely resembles that of expressed genes on the active X and autosomes, than that of silent Xi loci. The number and identity of genes that escape inactivation is different between species. In human somatic cells 15% of genes on the X escape inactivation [58,59,60], with a higher frequency on the short arm (orthologues to marsupial autosomes) than on the long arm of the human X (homologous to the marsupial X). Furthermore, about 10% of human X-borne genes have variable inactivation status between tissues and/or individuals [60,61]. In mouse somatic cells, almost all X-borne genes are inactivated; only 3% escape [59,62].

In female mouse trophoblast stem cells [63] and extra-embryonic endoderm [64], both of which are subject to imprinted XCI, a larger number of X-borne genes (13% and 15%) are expressed from both X chromosomes. However, different subsets of genes in these extra-embryonic cell lineages are subject to XCI. The inactive X^p in mouse extra-embryonic tissues globally accumulates the same repressive histone marks as the Xi in other somatic cell types [36,64,65]. However, the order in which these modifications appear on the Xi is different. In random XCI enrichment of macro-H2A is a late stage event. In contrast, during imprinted XCI enrichment of H3K27me3 and macro-H2A appear early on the Xi, whereas H3K9me2 accumulation is detected later [42,66]. Similar to Xi in

somatic cells, X-borne promoters are hypermethylated on the inactive X chromosome [67].

In marsupials, a large proportion of X-borne genes escape XCI [20,25], as in the mouse extra-embryonic membranes. Genes on the marsupial Xi exhibit variable levels of incomplete silencing across species, tissue and developmental stage [68,69]. Approximately 15% of genes on the American grey short-tailed opossum X escape inactivation [25]. As such imprinted XCI in marsupials and mouse extra-embryonic tissues is not as complete as random XCI in eutherian somatic tissue. Interestingly, the marsupial Xi lacks the repressive H4K20me1 mark [21], which accumulates on Xi during both imprinted and random XCI in mouse [70] (Table 1). Localization of macro-H2A to the marsupial Xi is unknown.

5. X Inactivation Center (XIC)

The silencing achieved during XCI is triggered by long-noncoding (lnc) RNAs that interact with chromatin regulatory complexes to alter chromosome conformation. Yet despite the central role of RNA-chromatin interactions during XCI, they are not fully understood.

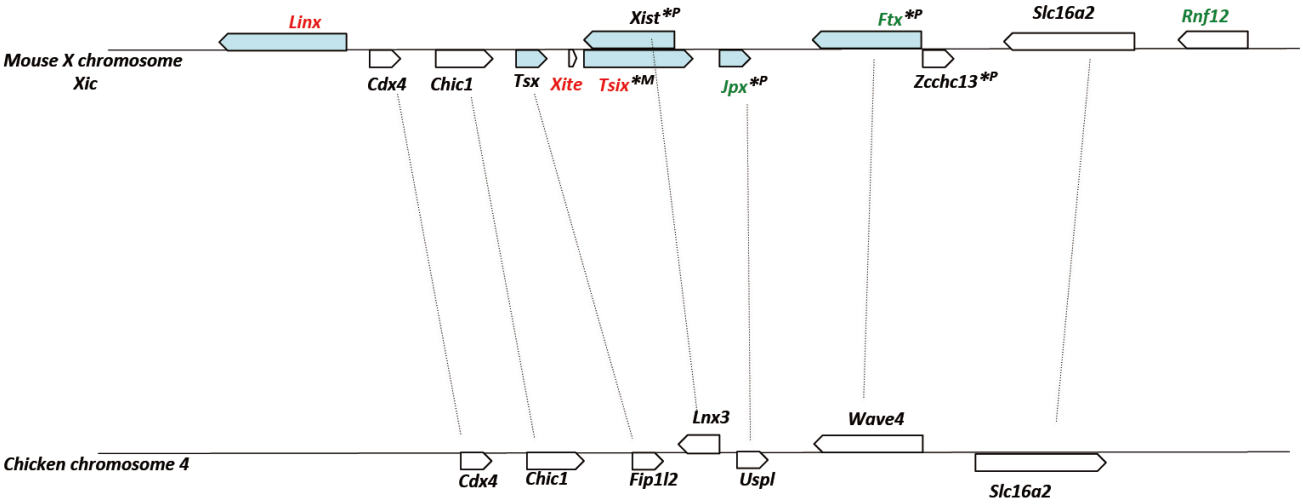

Figure 2. Comparative maps of the X inactivation center (XIC) in mouse and orthologous region in chicken. The mouse XIC spans 8cM (10–20 Mb) and bears several non-coding RNAs as well as protein-coding genes. Only elements around *Xist* are shown. Arrows identify direction of each transcription unit. Protein coding genes are indicated in white and genes producing lncRNAs are blue. Genes with imprinted expression in mouse extra-embryonic tissues are marked by an asterisk, and the expressed allele is indicated (i.e. p = paternally expressed, m = maternally expressed). Putative positive regulators of *Xist* (*Jpx, Ftx, Rnf12*) are labeled in green, and putative negative regulators (*Linx, Xite, Tsix*) are labeled in red. Lines identify homologous genes in chicken.

A region on the eutherian X chromosome called the X Inactivation Centre (XIC) is of key importance in coordinating XCI. The XIC contains several pseudogenes (e.g. *Fxyd6*) and protein-coding genes (e.g. *CDX4*, *CHIC1*, *SLC16A2*) [71], along with the key non-coding RNA genes (e.g. *XIST, TSIX*, *FTX*, *JPX* and others) (Figure 2). The XIC lncRNAs are poorly conserved between eutherian species, with the master regulator (*XIST*) the most conserved element between

sequenced eutherian genomes [72,73,74]. However, there is no ortholog of *XIST* in the marsupial or monotreme genomes, in which the XIC locus has been disrupted [72,75]. Interestingly, in chicken the locus remains intact with protein coding genes that share homology to *XIST* and the mouse *Tsx* gene [73].

6. Evolution of the master regulator—*XIST*

Several exons of the chicken protein-coding gene *Lnx3* share homology with the *XIST* gene [73,76,77] (Figure 3). These homologies reveal that the *XIST* promoter evolved from exons 1 and 2 of the *Lnx3* gene, which is among the most conserved regions of the *XIST* gene between different eutherian species [73,76,77]. The remaining *XIST* exons (that share no homology with *Lnx3*) are likely to have originated via transposition of various mobile elements, presumably endogenous retroviruses, fragments of which were amplified to generate several simple tandem repeats [76,78]. The lack of *XIST* in marsupials, along with it being in all eutherian genomes, means that *XIST* evolved as a key player in XCI in the eutherian ancestor.

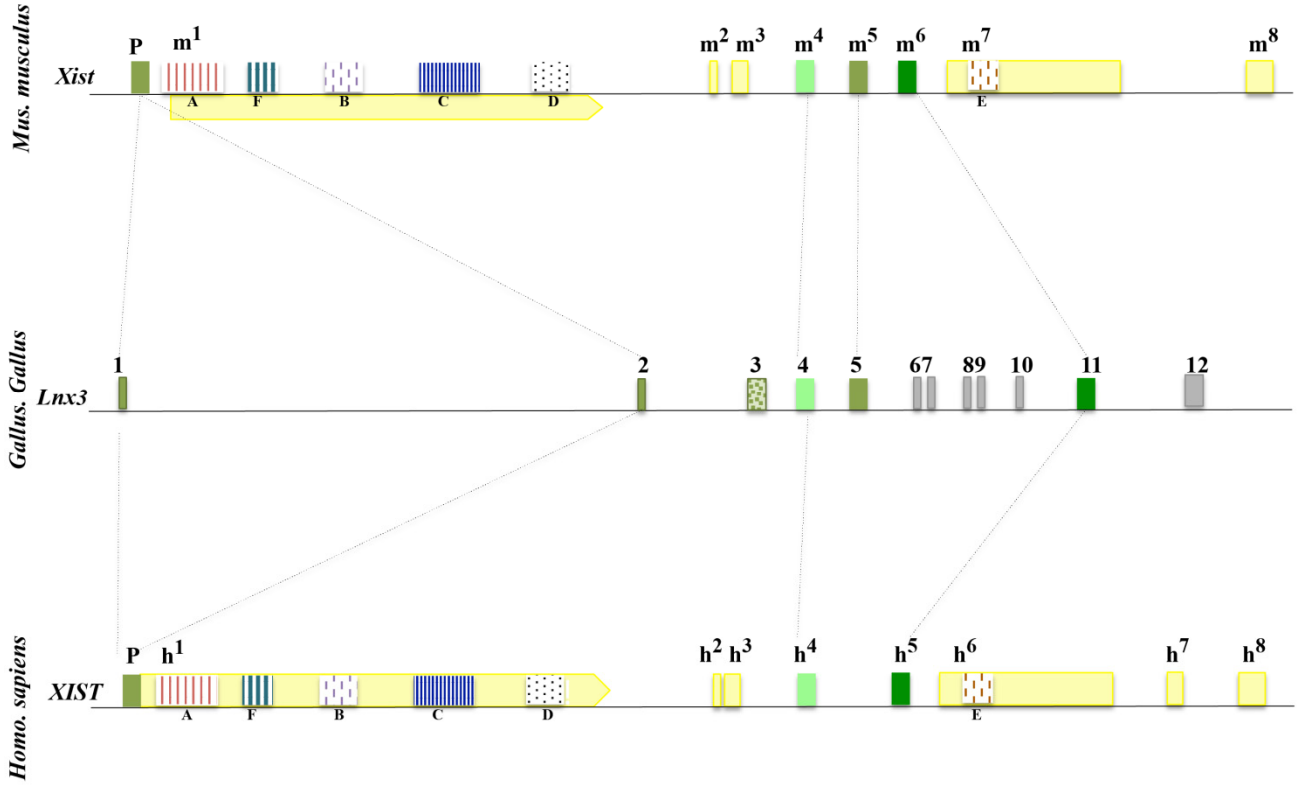

Figure 3. Homology between the eutherian *Xist*, and the protein coding *Lnx3* gene in chicken. Human and mouse *Xist* both have eight exons. Functional domains of *Xist* include the tandem repeats (labeled A to F). The *Xist* promoter (P) originates from exons 1 and 2 of *Lnx3*. Colors shared between chicken *Lnx3* and human/mouse *Xist*, identify homologies and, therefore, origin of *Xist* exons. Yellow *Xist* exons originated from mobile elements. Human exons are numbed h^1–h^8, and mouse exons m^1–m^8. Homology with exon 3 of *Lnx3* is detectable in the human and mouse genome, but gives rise to pseudo-exons (not shown).

The ancestral *XIST* gene presumably consisted of ten exons [74,76]. Two large exons (1 and 8) together constitute about 90% of *XIST* and contain tandem repeats (denoted A to H) [74] (Figure 3). Tandem repeat A is conserved in all eutherian species [75,76,78,79,80,81,82,83,84], whereas presence, absence, or amplification of the other repeats is species specific [76,83]. Six of the ancestral exons are conserved across Eutheria [73,76,77,78], with the remaining four (2, 6, 9, 10) either functional- or pseudo-exons depending on species [74,85]. Thus, human *XIST* encodes a 19kb transcript [86], whereas the mouse *Xist* transcript is 17kb [87]. Despite variable intron-exon structures between species, *XIST* exons are GC dinucleotide rich compared to the introns. The proportion of GC richness is constant between species (39.8–42.2%) and similar to the whole XIC locus.

7. The role of *Xist*

In mouse, *Xist* is transcribed only in females by RNA polymerase II, solely from the Xi. Analysis of the CpG dinucleotide methylation patterns in the promoter region has shown that the active *Xist* allele (on the inactive X) is completely unmethylated [88]. In contrast, the silent maternal *Xist* allele is fully methylated [88]. Although *Xist* RNA is spliced and polyadenylated, it is absent from polysomes [80,89] and remains in the nucleus where it coats and forms a "*Xist* cloud" on the X to be inactivated [80,90], the spreading of *Xist* RNA along one X chromosome *in cis* initiates the chromosome-wide silencing.

LINE1 retrotransposons are enriched on the X (mouse X ~ 28.5%, autosomes ~ 14.6%) [91], so were proposed to be anchor points for *Xist* to ensure efficient spreading of the machinery responsible for silencing [92]. A significant decrease in LINE1 density at regions containing genes that escape inactivation [93] supports this hypothesis, although a less direct role for LINEs in the spreading process seems more probable [94]. Accordingly, LINEs were proposed to moderate spatial organization of the transcriptionally silent nuclear territory of the inactive X chromosome, into which X-borne genes are recruited as they are silenced [95,96].

Xist expression is followed by the formation of a repressive chromatin state that excludes transcriptional machinery from the inactive X [95]. Repeat A (RepA), at the 5' end of *Xist*, recruits the polycomb repressive complexes PRC1 and PRC2 to the Xi. Polycomb repressive complexes decorate the Xi and catalyze the characteristic repressive histone modifications of Xi. A number of other proteins are also localized to the Xi, potentially trafficked via *Xist* RNA, including nuclear scaffolding factors such as SAF-A [97] and the histone variant macro-H2A [98,99,100,101].

8. Activators and repressors of *XIST*

During the morula/blastocyst stage in mice, a few days after initiation of imprinted XCI, *Tsix* is expressed exclusively from the maternally derived X chromosome to inhibit expression from the maternally derived *Xist* [102,103,104]. However, during random XCI *Tsix* demarcates whichever X remains active (X^a) [105,106,107] and its expression prevents *in cis* transcription of *Xist* and, ultimately, inactivation of that X [104]. *Tsix* in rodents spans more than a 40 kb region that encompasses the entire transcription unit of *Xist* [108]. In primates, cow and dog there are many species-specific repeat-element insertions, and large deletions, that disrupt the overall structure of *TSIX* [109]. In human, *TSIX* appear to be an expressed pseudogene unable to repress *XIST*, and

overlaps only with the last two exons of *XIST* [109,110]. In contrast to mouse *Tsix,* the human *TSIX* is expressed with *XIST* from the Xi in the fetal cells, throughout gestation, but cease transcription shortly after birth [110]. Thus, *Tsix* function seems limited to rodents.

Ftx and *Jpx* potentially upregulate *Xist*, and are conserved in mouse, human, and cow [71,111], evolving from the protein coding genes *Wave4* and *Uspl*, respectively [76]. Both genes escape imprinted XCI and are expressed predominantly from the paternal allele at the pre-implantation stage [112]. Deletion of the *Ftx* promoter leads to decreased *Xist* expression in male embryonic stem (ES) cells [111], indicating that it is a positive regulator of *Xist*. However a recent study shows that *Ftx* disruption did not affect embryo survival, or expression of *Xist* and other X-borne genes during pre-implantation, thus is dispensable for imprinted inactivation [113]. Whether *Ftx* is involved in random XCI in post-implantation embryos is yet to be determined.

Jpx is located just downstream of the *Ftx* locus, and approximately 10 kb upstream of *Xist* [71,114,115]. *Jpx* escapes XCI and can upregulate *Xist* expression on the Xi [71,115,116] by evicting CTCF from the *Xist* promoter [117]. Deletion of a single *Jpx* allele in XX female ES cells results in failed accumulation of *Xist* on either X, and inactivation is prevented [116].

Although the function of *XIST* may be well conserved in eutherians, other elements of the XIC (even those with sequence conservation) may not have conserved function (Table 2). However, poor sequence conservation of noncoding RNAs in the XIC does not necessarily indicate a lack of function [118,119], as maintaining secondary structure (and therefore function) of lncRNA molecules may only require short stretches of sequence preservation. This poor conservation might indicate their adaptation to function in specific genomic environments, suggesting that regulation of XCI is at least partially species-specific. For instance, the recently evolved lncRNA gene *XACT* is only present in human and chimpanzee, but not in macaques or more distantly related species [120]. *XACT* is expressed from and coats the active X in female human embryonic stem cells and early differentiating cells, and may contribute to protecting the Xa from inactivation [120].

Table 2. Sequence conservation of different genes involved in XCI between eutherian (mouse and human) and marsupials (Opossum).

		Eutherian		Marsupial
		Mouse	Human	Opossum
lncRNAs	*Xist*	√	√	×
	Ftx	√	√	×
	Jpx	√	√	×
	Tsix	√	*	×
	XACT	×	√	×
	Rsx	×	×	√

√! = presence, ×! = absence, * = pseudogene.

9. A marsupial *XIST* like gene: *Rsx*

The marsupial *Lnx3* (the precursor of *XIST*) gene has a native open reading frame that is expressed in both males and females. The eutherian XIC locus is disrupted in marsupials, and *Lnx3* presumably functions as a PDZ domain containing ring finger protein rather than an untranslated nuclear RNA similar to *XIST*. Consequently, the X inactivation process in marsupials involves neither *XIST* nor the XIC.

The marsupial X chromosome has multiple large-scale internal rearrangements with respect to both the human X, and between Australian and American representative species [121]. This is contrary to the generally conserved gene order on the eutherian X [122,123], presumably due to purifying selection against rearrangements that perturb interactions between *XIST* and regions of the X intended for inactivation [93]. Extensive rearrangement of the marsupial X chromosome was taken as support for the lack of a *XIST* equivalent [121]. However, *Rsx* (RNA on the silent X) was identified in opossum (and two Australian marsupials), and appears to fulfill some of the functions of *XIST* [22]. As such, the epigenetic mechanisms that silence the inactive X in the somatic cells of marsupials and eutherians share a remarkable degree of convergence.

The mature *Rsx* in opossum is a 27-kb non-coding RNA with several *XIST*-like characteristics, such as a high GC content and enrichment of conserved 5' tandem repeats that may be involved in the formation of stem-loop structures [22]. These are potentially important functional domains necessary for directing protein complexes responsible for chromatin modification that repress transcription. However, further studies are needed to determine the candidate functional domains of *Rsx*.

Rsx is located adjacent to *Hprt* on the marsupial X in a different genomic context to—and shares no sequence homology with—*XIST*. Yet, like *XIST*, *Rsx* is expressed exclusively in female somatic cells [22] and extra embryonic membranes [25], but not in germ cells where both X chromosomes are active [22]. *Rsx* is expressed *in cis* from Xi, around which it forms a "*Rsx*-cloud" that results in repressed gene activity. Moreover, after introduction of *Rsx* into mouse ES cells, *Rsx* RNA coated the transgenic chromosome and resulted in its inactivation in more than half the cells examined [22].

Monoallelic expression from the paternally derived allele of *Rsx*, in both fetal brain and extra embryonic membranes, was shown to be due to different epigenetic characteristics of the active and inactive alleles. *Rsx,* similar to *Xist*, is differentially methylated at its promoter. There is ~ 100% methylation of the maternally derived allele, and virtually no methylation of the paternally derived allele [25]. Furthermore, H3K27me3 repressive mark was absent from the *Rsx* gene body, demonstrating that similar epigenetic mechanisms regulate the independently evolved *Rsx* and *XIST* genes [25].

10. Conclusion

Although independently evolved, there appear to be remarkable functional similarities shared by *Xist* and *Rsx*. However, it remains unknown if *Rsx* can perform all functions attributed to *Xist*, or if it traffics the epigenetic machinery as *Xist* is proposed to do. Since overlapping, but different, suites of repressive chromatin modifications are used to silence the X in eutherians and marsupials, many of these epigenetic tools were likely utilized in the therian ancestor to achieve X chromosome

inactivation. However, it is yet to be determined if *Rsx* was an XCI switch in the therian mammal ancestor that was retained in marsupials, and then replaced by *Xist* in the eutherian ancestor; or if they evolved simultaneously in the two lineages. Perhaps the epigenetic differences observed between eutherian and marsupial XCI merely reflect that these two lncRNAs direct protein complexes that are responsible for different chromatin modification. Finally, the potential existence of a marsupial X inactivation center close to *Rsx*, which bears lncRNAs that may regulate *Rsx* expression, remains a fascinating possibility.

Acknowledgments

P.D.W. was supported by an ARC fellowship.

Conflict of Interest

The authors declare no conflicts of interest in this paper.

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