

A cross-species comparison of escape from X inactivation in Eutheria: implications for evolution of X chromosome inactivation

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Abstract Sex chromosome dosage compensation in both eutherian and marsupial mammals is achieved by X chromosome inactivation (XCI)—transcriptional repression that silences one of the two X chromosomes in the somatic cells of females. We recently used RNA fluorescent in situ hybridization (FISH) to show, in individual nuclei, that marsupial X inactivation (in the absence of *XIST*) occurs on a gene-by-gene basis, and that escape from inactivation is stochastic and independent of gene location. In the absence of similar data from fibroblast cell lines of eutherian representatives, a meaningful comparison is lacking. We therefore used RNA-FISH to examine XCI in fibroblast cell lines obtained from three distantly related eutherian model species: African savannah elephant (*Loxodonta africana*), mouse (*Mus musculus*) and human (*Homo sapiens*). We show that, unlike the orthologous marsupial X, inactivation of the X conserved region (XCR) in eutherians generally is complete. Two-colour RNA-FISH on female human, mouse and elephant interphase nuclei showed that XCR loci have monoallelic expression in almost all nuclei. However, we

found that many loci located in the evolutionarily distinct recently added region (XAR) displayed reproducible locus-specific frequencies of nuclei with either one or two active X alleles. We propose that marsupial XCI retains features of an ancient incomplete silencing mechanism that was augmented by the evolution of the *XIST* gene that progressively stabilized the eutherian XCR. In contrast, the recently added region of the eutherian X displays an incomplete inactivation profile similar to that observed on the evolutionarily distinct marsupial X and the independently evolved monotreme X chromosomes.

Introduction

The mammalian X and Y chromosomes are morphologically and genetically differentiated. The X chromosome is large and gene rich, whereas the Y chromosome is highly degenerated and bears few functional genes. This chromosomal sexual dimorphism introduces disequilibrium of X-borne gene dose between females (with two Xs) and males (with just one X). In therian mammals, subclasses Eutheria ('placental' mammals) and Metatheria (marsupials), the imbalance is corrected by X chromosome inactivation (XCI), a paradigmatic epigenetic phenomenon in which two homologous X chromosomes are expressed differently within the same cell. The result is transcriptional silencing of one of the two X chromosomes in female somatic cells (Graves and Gartler 1986).

Most data on XCI have been gathered for humans and mice (which belong to the same superordinal eutherian clade). It was originally proposed that inactivation of one X occurs randomly early in embryogenesis, and is stable and somatically heritable (Lyon 1961). Expression of the non-coding regulatory RNA, *Xist* (X-inactive specific transcript), initiates

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XCI during early embryogenesis, although its expression is regulated differently between eutherian mammals (Okamoto et al. 2011). The accumulation and spread of *Xist* from an X chromosome inactivation centre (XIC) rapidly ensures chromosome-wide transcriptional silencing by triggering recruitment of inactive histone modifications and variants to the inactive X (Xi), and to DNA methylation sites on Xi (reviewed in Heard 2005). The transcriptional silencing is almost complete along the mouse X chromosome (Yang et al. 2010), and RNA-fluorescent in situ hybridization (FISH) for five loci showed monoallelic expression of these genes in the majority of cells (Chaumeil et al. 2006). In contrast, approximately 15% of genes are expressed from the inactive X in humans (Carrel and Willard 2005). Most of these genes are located on the short arm of the X, reflecting the different evolutionary history of the eutherian Xp (see below).

Comparative genomics (Veyrunes et al. 2008) has dated the origin of the therian sex chromosomes after the divergence of Prototheria (monotremes) from the therian ancestor (~166 million years ago), but before the therian radiation (~148 million years ago; dates from Bininda-Emonds et al. 2007). Comparative mapping between marsupials and eutherians reveals that the eutherian X consists of two evolutionary blocks (Graves 1995)—(1) an ancient conserved region (X conserved region (XCR)) that encompasses the entire long arm (Xq) and proximal short arm (Xp) of human X that is homologous to the marsupial X, and (2) a region recently added (XAR) to the sex chromosomes in the eutherian ancestor that is autosomal in marsupials. This latter region constitutes most of human Xp, where most human ‘escapee’ genes are located. Almost complete conservation of the gene content of the eutherian X (excluding the atypical mouse (Amar et al. 1988)), and even of gene order (Rodriguez Delgado et al. 2009; Raudsepp et al. 2004), suggests that there is strong selection against rearrangements on the X, consistent with the hypothesis that these would interfere with XCI (Ohno 1967).

Much less is known about the nature and molecular mechanisms of XCI outside of eutherian mammals. The marsupial XCI is paternally imprinted (Cooper et al. 1971; Sharman 1971) and appears to be incomplete, unstable and tissue specific (reviewed in Deakin et al. 2009). Like the eutherian inactive X, the marsupial X also becomes heterochromatic and exhibits marks of transcriptionally silent chromatin; there are, however, some fundamental epigenetic differences between the Xi in the two lineages (Chaumeil et al. 2011; Rens et al. 2010). Most interestingly, marsupial XCI occurs in the absence of an *XIST* homologue (Duret et al. 2006; Hore et al. 2007). Moreover, RNA-FISH recently demonstrated that the penetrance of XCI in marsupials is far from complete (Al Nadaf et al. 2010). Whereas X-borne genes are thought to be transcribed from one allele in human and mouse cells (with the exception of

escapee genes), marsupial cells show reproducible locus-specific frequencies of nuclei that display either mono- or biallelic expression. This is similar to RNA-FISH results for X loci in monotremes (platypus; Deakin et al. 2008a), even though their sex chromosomes share no homology to those of therians (Veyrunes et al. 2008).

Despite the enormous amount of work done on XCI in mouse and human cells, it is not possible to make a direct comparison with the cell-specific data obtained from marsupials (Al Nadaf et al. 2010) and monotremes (Deakin et al. 2008a). Moreover, equivalent data are lacking from eutherians more distantly related to humans and mice. Using two-colour RNA-FISH, we examine XCI at the cellular level in mouse (*Mus musculus*) and human (*Homo sapiens*), and a representative basal eutherian mammal, the African savannah elephant (*Loxodonta africana*). We show that inactivation of genes on the ancient region (XCR) of the eutherian X is complete in almost all nuclei. However, many genes located in the evolutionarily distinct added region (XAR) showed reproducible frequencies of nuclei with either one or two active X alleles, a pattern similar to that of XCR genes in marsupials.

Materials and methods

Cell culture and coverslip preparation

Human cell lines were supplied courtesy of Dr. Jeff Craig, Murdoch Children's Research Institute, Australia. Male and female mouse and elephant fibroblasts cell lines were established from ear clips. All cell lines were cultured (in DMEM/10% foetal bovine serum) to a density of 70–80% on gelatine-coated coverslips at 37°C in 5% CO₂. Coverslips were rinsed with phosphate-buffered saline (PBS) and then permeabilized for 10 min on ice in CSK buffer (100 mM NaCl, 300 mM sucrose, 10 mM PIPES pH 6.8)/0.5% Triton X 100 (Sigma)/2 mM vanadyl ribonucleoside complex (Sigma). Cells were subsequently fixed in 3% paraformaldehyde/1× PBS for 10 min at room temperature, and then washed twice for 5 min each in 70% ethanol, and stored at –20°C. Before hybridization, coverslips were dehydrated in 80%, 95% and 100% ethanol washes (3 min each) and air-dried.

Probes and probe preparation

Probes used in this study are large BAC clones containing the genes of interest. These genes are distributed along the length of the X chromosome of both the XCR and XAR (Online Resource 1 and 4). BACs bearing 12 human and mouse genes of interest (11 of which were in common) were identified using BAC tracks on the Ensembl or UCSC

genome browsers (mouse RPCI-23 and RPCI-24, human RP-11; BAC PAC Resources, CHORI). For elephant, 24 X borne BAC clones (representing 16 X-specific loci; some BACs overlapped; Rodriguez Delgado et al. 2009), isolated from a male-derived BAC library (VMRC-15; BAC PAC Resources, CHORI), were used as probes. However, BACs containing five genes (*AMELX*, *AR*, *PLP1*, *RBMX* and *SOX3*) produced no RNA-FISH signals. Results are only presented for the 11 expressed loci. Of these 11 elephant markers, six were in common with both human and mouse (Table 1). For the other five elephant-specific markers, RNA-FISH was not conducted in human and mouse because they were close to loci already included in this study. One to two micrograms of BAC DNA was prepared (as described in Veyrunes et al. 2008) and labelled by nick translation reaction with SpectrumOrange dUTP or SpectrumGreen dUTP (Enzo life Sciences, New York, NY, USA).

RNA fluorescence in situ hybridization (FISH)

In all instances, 1 µg of labelled test gene DNA was co-precipitated with 1 µg of the control gene DNA, and RNA-

FISH was performed as described in Al Nadaf et al. (2010). Images were captured on a SPOT RT Monochrome charge-coupled device camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) and analysed in IP lab (Scanalytics Inc., Fairfax, VA, USA).

Hybridization efficiencies for the X-linked test genes were determined by the frequency of male nuclei with one signal. Using this efficiency measure, the binomial $p^2+2pq+q^2=1$ was used to determine the expected frequency of female nuclei that have one signal due to inefficient hybridization.

Results

We tested inactivation status of X-borne loci in fibroblasts cultured from three eutherian species (human, mouse and elephant) using RNA-FISH (Fig. 1). The probes used in this study are derived from genes distributed along the length of the X chromosome of both the XCR and XAR (Fig. 2 and Online Resource 1). To control for probe accessibility and cell polyploidy, two-colour RNA-FISH was utilized; two probes, one containing the X-borne gene of interest, and the second containing the autosomal housekeeping gene, *GBA*, were labelled with different colour fluorochromes, in human and mouse. A pseudoautosomal region gene (*XG*) was used for elephant cell lines (Fig. 1 and Online Resource 1). Both alleles of the control gene needed to be detected before the cell was included in the analysis. At least 100 diploid nuclei were scored for each test gene (Online Resource 2). We used these data to create an XCI activity map for each species (Fig. 2).

Expression from X chromosomes

Male interphase cells (XY) were used to test the efficiency and specificity of probe hybridization (a single signal is expected for an X-borne gene; Fig. 1a and Online Resource 2). Loci with no X-gene expression in male nuclei were probably not expressed in fibroblasts and were eliminated from the analysis (Online Resource 2).

In female cells (XX), nuclei expressing a gene from only one of the two X chromosomes (1X-active) were observed as a single signal, whereas cells expressing a gene from both X chromosomes (2X-active) were observed as two signals (Fig. 1b, c). Our RNA-FISH results identified a number of genes in all three species that displayed complete inactivation (one signal in >90% female nuclei, comparable to the frequency in male cells; Table 1 and Online Resource 2). Loci with two signals in more than 10% of cells in the population were considered to escape inactivation to varying extents (13–81% for different loci). Importantly, none of the loci showed two signals in all nuclei (complete escape from inactivation; Table 1 and Online Resource 2).

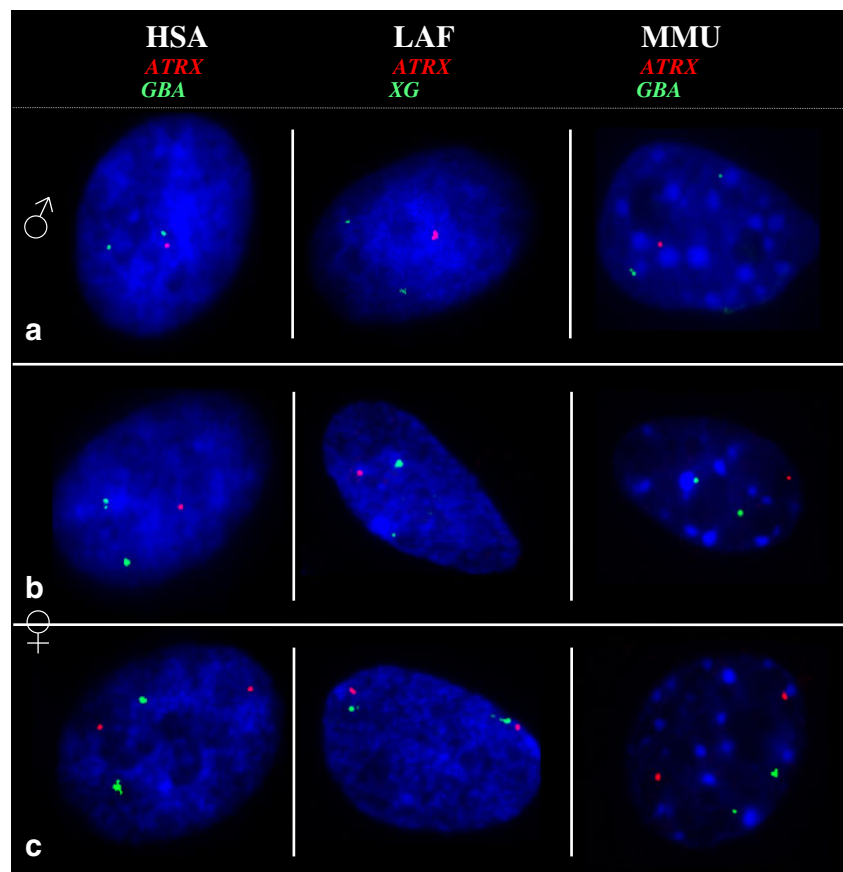
Table 1 Quantitative analysis of female fibroblast RNA-FISH data

Gene name	Percent nuclei with 1 signal		
	HSA	LAF	MMU
<i>STS</i>	59	–	–
<i>EIF1AX</i>	74	25	96
<i>ZFX</i>	–	19	–
<i>GK</i>	–	58	–
<i>USP9X</i>	69	24	91
<i>AMELX</i>	NE	NE	92
<i>POLA1</i>	NE	–	68
<i>UBA1</i>	93	76	97
<i>KDM5C</i>	65	55	94
<i>HUWE1</i>	96	97	84
<i>ATRX</i>	98	90	62
<i>HPRT</i>	–	–	94
<i>PHF6</i>	99	–	90
<i>RBMX</i>	93	NE	89
<i>XIAP</i>	–	92	–
<i>FMRI</i>	–	82	–
<i>G6PD</i>	–	92	–
<i>MECP2</i>	90	–	91

Frequency of nuclei with a single signal for X-borne loci investigated in this study. At least 100 female nuclei were scored (with two signals for the control autosomal gene) for each locus. All data are collected from one cell line in each species. XCR genes are rendered in bold italic

NE no expression in fibroblasts, ‘–’ no data, HSA *Homo sapiens*, LAF *Loxodonta africana*, MMU *Mus musculus*

Fig. 1 RNA-FISH of an X-borne gene (*red*) and an autosomal control (*green*) in male and female fibroblasts of three eutherian species. Transcription from the two autosomal alleles (*green*) is detected in all nuclei. **a** Male fibroblast nuclei show transcription from the single X-borne allele, whereas female fibroblast nuclei show transcription from either one (**b**) or two (**c**) X-borne *ATRX* alleles. Nuclei are counterstained with DAPI (*blue*). HSA *Homo sapiens*, LAF *Loxodonta africana*, MMU *Mus musculus*



Activity maps of eutherian inactive X chromosomes

We constructed an activity map (Fig. 2) for the inactive X of each species in order to determine whether escape from inactivation differed between the evolutionary blocks of the X chromosome, and whether there was any polarity to the frequency of escape (Fig. 2). Genes escaping X inactivation showed an obvious clustering within the XAR of human and elephant (but not mouse), with 29% to 84% nuclei being 2X-active. Conversely, most genes located in the XCR region in all species (except *ATRX*, *FMRI*, *KDM5C* and *UBAI* in at least one of the three species) appear to be subject to X inactivation, with 2X-active expression in less than 10% of nuclei. Three of the four XCR escapee genes have Y homologues in humans, mice or marsupials (*FMRI* only escapes in elephant, for which there is no Y data). *ATRX* (with a Y homologue in marsupials) was expressed in mouse fibroblast nuclei from both X chromosomes in 36% of cells. *KDM5C*, with a Y homologue in most therian species and which escapes inactivation in human and elephant, but not mouse, was expressed from both X chromosomes at frequencies of 33%, 43% and 3%, respectively. *UBAI*, with a Y homologue in mouse and marsupial but not human, was expressed from both X

chromosomes in 21% of elephant, but only in 5% and 1% of human and mouse fibroblast nuclei.

In contrast, all genes within the XAR showed incomplete inactivation to a various extent in human and elephant, but not in mouse. The frequency of nuclei showing inactivation ranged from 48% to 71% (52–29% escape) for human, and 25–60% (75–40% escape) for elephant (Table 1 and Online Resource 2). These ‘escapee’ genes have a reproducible (experimental and biological replicates) locus-specific frequency of nuclei with either one or two active alleles (Online Resource 3). In mouse, however, most XAR genes are fully inactivated (Fig. 2). For instance, *EIFAX* and *USP9X*, which escape inactivation in human and elephant, are subject to XCI in mouse.

Discussion

Dosage compensation by means of XCI occurs in all therian mammals, and many of the molecular mechanisms involved in XCI are conserved between human and mouse. Considerable differences exist, however, in the *initiation* of XCI during early embryogenesis among eutherian mammals (Okamoto et al. 2011), with a different percentage of

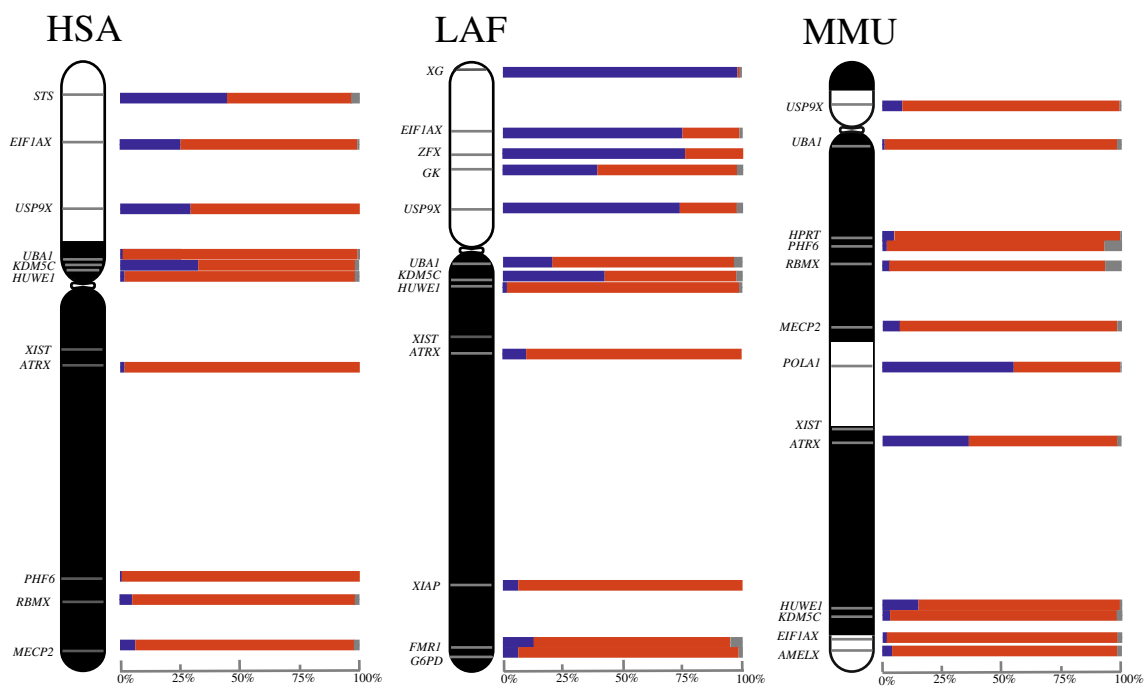


Fig. 2 Activity maps of the human (HSA), elephant (LAF) and mouse (MMU) X chromosomes. *Bars* represent percentage of nuclei in which the X-borne test loci are transcribing from two (blue), one

(red) or zero (grey) alleles. X conserved region is indicate in black. X added region is indicated in white

genes escaping from XCI in mouse (3%) and human (15%) (Carrel et al. 1999; Carrel and Willard 2005; Sudbrak et al. 2001; Yang et al. 2010).

Given that data on escapee genes outside of the rodent and primate lineages are scarce, we studied the expression of loci distributed on the X chromosomes in three eutherian species (human, mouse and elephant). We conducted RNA-FISH experiments on fibroblasts of both sexes, similar to those performed on representative marsupial (the tammar wallaby) and monotreme (the platypus) species (Al Nadaf et al. 2010; Deakin et al. 2008a), to address whether escape from inactivation is due to expression from the inactive X in a proportion of cells or by expression of alleles on Xi in all cells.

Our data show that most genes on the ancient part of the X are completely inactivated (1X-active), but loci on the recently added region showed stochastic escape (2X-active) in a proportion of cells.

Monoallelic expression on XCR and biallelic expression on XAR

Genes subject to XCI were mostly located on the conserved region of the X chromosome (XCR) in each of the three species (Table 1), consistent with previous findings in humans and mice (Carrel and Willard 2005; Chaumeil et al. 2006; Yang et al. 2010). This confirms that X inactivation

is complete for most loci on this ancient region of the eutherian X, and is different from the pattern of expression observed for orthologous loci on the marsupial X (Al Nadaf et al. 2010). The three XCR genes that were the exception were *ATRX*, *KDM5C* and *UBA1*. They showed considerable expression from both loci (>20%) in one or more of the eutherian species tested. These loci all have Y homologues in humans, mice or marsupials: *KDM5C* has an active Y homologue in eutherians (Agulnik et al. 1994) and also a copy on the marsupial Y (Waters et al. 2001). There is a *UBA1* Y homologue in mouse and marsupials (Mitchell et al. 1998, 1992), and *ATRX* has a Y homologue in marsupials (Pask et al. 2000). However, having a Y copy does not necessitate escape. For instance, XCR genes, which also have a Y copy in either eutherians or marsupials (such as *PHF6*, *HUWE1*, *MECP2* and *RBMX*), do not escape with high frequencies. The XCR findings contrasted sharply with those for genes on the XAR, which are autosomal in marsupials, and were 2X-active in many nuclei—a pattern indicative of escape from inactivation (Fig. 2).

Our human and mouse RNA-FISH results are in broad agreement with expression data (microarray and RNA-seq) from populations of cells and from cell hybrids (Carrel et al. 1999; Carrel and Willard 2005; Sudbrak et al. 2001; Yang et al. 2010), although there were some inconsistencies. In mouse, *ATRX* displayed biallelic expression in 40%

of nuclei; this contrasts with previous findings that *ATRX* is subject to XCI in mouse (Yang et al. 2010). This could be due to the tissue-specific escape of *ATRX* from XCI in mice (Garrick et al. 2006; Patrat et al. 2009). Moreover, we detected only 7% biallelic expression of *UBA1* in human fibroblast nuclei which was previously reported to escape inactivation in human (Carrel and Willard 2005), but not mouse (Yang et al. 2010). Finally, in contrast to an earlier study (Yang et al. 2010), we did not detect biallelic expression of *Kdm5c* in mouse. This is unlikely to be due to a low efficiency of hybridization (measure as 98% in controls) and is probably due to the tissue and strain specificity of *Kdm5c* escape from XCI in mice (Carrel et al. 1996; Sheardown et al. 1996).

Expression from the inactive X in different eutherian species

It is noteworthy that escape from inactivation appears to be much more common in cells derived from human and elephant than from cells of the mouse (Fig. 2). This is consistent with previous observations (Yang et al. 2010) where few genes escape inactivation on the mouse X. This difference in frequencies of escape from inactivation might explain the less severe phenotype in XO mice (Ashworth et al. 1991) than XO humans (with Turner's syndrome).

Experimental and biological replicates indicate that all but one gene in this study escape X inactivation at a reproducible frequency. The exception to this was the variable expression of *USP9X* in human, which showed almost complete inactivation in one cell line (91%), and some escape from inactivation in two others (69% and 70%). Variable XCI status in approximately 10% of X-linked genes in females has also been identified (Carrel and Willard 2005). Our study was performed on fibroblast cell lines, and since the extent of escape of individual loci often varies between different marsupial tissues (Cooper 1971), replicating it using cells from other tissue sources is a priority.

Gene content (and even gene order) on the eutherian X chromosome is conserved, representing the largest conserved block in the eutherian genome. This probably reflects selection against rearrangements that could disrupt the regulation of the XCI system. Mouse is a notable exception to this in that the ancient and added regions of the X have been rearranged—perhaps facilitating the spread of tight repressive control into the XAR in this species. The marsupial X, however, has been significantly rearranged between the opossum and wallaby (Deakin et al. 2008b), which may reflect an incomplete dosage compensation system (Al Nadaf et al. 2010; Chaumeil et al. 2011).

Stochastic expression of genes on the X

Our observation of the frequencies of human, elephant and mouse nuclei with 1X-active and 2X-active expression patterns implies that escape from inactivation is a stochastic (probabilistic) process in eutherians (Fig. 2). Different genes show reproducible frequency (between experimental replicates and among individuals) of 2X-active and 1X-active nuclei in a population of female fibroblast cells. Thus, escape from inactivation of a locus on the eutherian XAR is controlled by the probability of its expression, rather than its reduced expression from the inactive X in all nuclei.

A strikingly similar conclusion was drawn from data describing the inactivation of loci on the marsupial X (genes on the marsupial X chromosome were expressed from one or both alleles at a frequency characteristic of each locus; Al Nadaf et al. 2010). However, genes that escape inactivation on the marsupial X lie on the ancient conserved region of the eutherian X which, with the exception of few genes, are subject to inactivation (see above). Even more remarkable, the pattern of inactivation on the eutherian XAR and the marsupial XCR is similar to inactivation on the monotreme X chromosomes (Deakin et al. 2008a)—a chromosome that shares no homology with marsupial or eutherian sex chromosomes. Thus, we have observed almost identical inactivation patterns on the independently evolved (evolutionarily distinct) regions of mammalian sex chromosomes.

Evolution of X chromosome inactivation

We propose, therefore, that the stochastic inactivation that characterizes XCR genes in marsupials, and XAR genes in eutherians, represents the original mechanism of X inactivation in the common ancestor of marsupials and eutherians (i.e. ~148 million years ago). The major difference in inactivation phenotype, and in the molecular mechanism of XCI in marsupials and eutherians (Rens et al. 2010; Chaumeil et al. 2011), suggests an ongoing sophistication of the XCI machinery on the eutherian XCR that coincided with the rise of *XIST* and the XIC.

This ancient mechanism was subsequently maintained in the marsupial and early eutherian lineages, but at some point before the eutherian radiation (~105 million years ago), XCI became more tightly regulated with the evolution of the *XIST* gene. This tighter regulation spread until it was virtually complete on the ancient eutherian X. Following the addition of XAR ~148–105 million years ago, the genes in this region came under stochastic regulation, similar to that observed on the marsupial X. In the mouse where XCR and XAR are intermixed, recruitment of the added region into tighter *XIST*-controlled regulation is virtually complete.

In human and elephant (and presumably all structurally similar eutherian X chromosomes), however, recruitment of XAR into tight X inactivation remains incomplete.

Random monoallelic expression has been described in eutherians (Singh et al. 2003) for many autosomal loci and could be an ancient mechanism of transcriptional control (Ohlsson et al. 2001). This monoallelic expression is similar to the stochastic inactivation we observed for genes on the evolutionary distinct regions of the therian sex chromosomes (the eutherian XAR and marsupial XCR) and the independently evolved monotreme sex chromosomes. Therefore, incomplete stochastic inactivation is likely common to the initial recruitment of genes into mammalian dosage compensation systems which, in turn, may have been exapted independently (in monotreme and therian mammals) from autosomal monoallelic expression.

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