REVIEW ARTICLE

X-chromosome inactivation and escape

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Abstract

X-chromosome inactivation, which was discovered by Mary Lyon in 1961 results in random silencing of one X chromosome in female mammals. This review is dedicated to Mary Lyon, who passed away last year. She predicted many of the features of X inactivation, for e.g., the existence of an X inactivation center, the role of L1 elements in spreading of silencing and the existence of genes that escape X inactivation. Starting from her published work here we summarize advances in the field.

[Disteche C. M. and Berletch J. B. 2015 X-chromosome inactivation and escape. *J. Genet.* **94**, 591–599]

Introduction

This review is in honour of Mary Lyon who passed away last year. She has inspired my work and that of the members of my laboratory who have studied the regulation of the mammalian X chromosome. I initially met Mary Lyon in Boston when I was a post-doctoral fellow in Samuel Latt's laboratory at Harvard. Her visit was a highlight of my training. Throughout my career I continued to enjoy meeting her at the International Mammalian Genome Society conferences and the X inactivation meetings.

Lyon's law

> *'It is here suggested that this mosaic phenotype is due to the inactivation of one or other X chromosome early in embryonic development.'* (Lyon 1961)

> *'Thus, the general picture concerning heterozygotes for sex-linked genes in man is one of variable expression, which accords with the predicted result of random inactivation of one or the other X chromosome.'* (Lyon 1962)

Mary Lyon formulated her X-chromosome inactivation (XCI) hypothesis in 1961 based on her observations in female mice heterozygous for a mutation in an X-linked gene that controls coat colour (for e.g. tabby), and based on known facts at the time that X0 mice are viable (Welshons and Russell 1959) and that female cells contain a heteropyknotic X chromosome (Ohno and Hauschka 1960). She interpreted the variegated coat colour as due to clonal growth of cells with random silencing of one X chromosome (Lyon 1961). Similar variegation in coat colour is seen in female mice with the Cattanach X;autosome insertion (figure 1a). Mary Lyon predicted that her XCI hypothesis, now deemed a law, would be applicable to humans (Lyon 1962). A main consequence of XCI is to equalize the dosage of X-linked gene expression (dosage compensation) between male and female mammals (see Gartler, same issue). A second type of dosage compensation balances expression between X-linked genes and autosomal genes by upregulation of genes on the active X chromosome (Disteche 2012; Deng *et al.* 2014). Thus, XCI also prevents overexpression of X-linked genes in female cells with two X chromosomes (Lin *et al.* 2011). Early studies in female mouse preimplantation embryos demonstrated evidence of halving X-linked gene expression, thus pinpointing the timing of random XCI (Epstein *et al.* 1978; Kratzer and Gartler 1978). Silencing of one allele of X-linked genes is then clonally inherited in female somatic cells. Mary Lyon favoured the existence of three major steps for XCI: initiation, spreading and maintenance (Lyon 1988).

X inactivation centre and XCI initiation

> *'One would therefore not expect all points on the X to act independently with regards to inactivation. There might be some center or centres from which the inactivation spreads.'* (Lyon *et al.* 1964)

Based on analyses of X;autosome translocations in which silencing can spread into the attached autosome, both Lyon

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Keywords. X chromosome; X inactivation; escape from X inactivation.

and Russell suggested the possibility of an X-inactivation centre (XIC) required for the onset of XCI (Lyon *et al.* 1964; Russell 1964). Location of the XIC on the mouse and human X chromosomes was further defined by examining patterns of XCI in multiple additional cases of X;autosome translocations or other types of X rearrangements (Brockdorff *et al.* 1991; Leppig *et al.* 1993). The search was on to find the key molecular element(s) within the XIC essential for initiation.

The main key element turned out to be a gene that encodes a long noncoding RNA (lncRNA) called X inactive-specific transcript (*Xist*). *XIST* was initially discovered in humans by Carolyn Brown and Hunt Willard who singled out this lncRNA as a critical factor for XCI based on its location within the XIC and its unique expression pattern that is completely female-specific in adult somatic cells (Brown *et al.* 1991; Brown *et al.* 1992). The homologous *Xist* gene was then identified in mice where expression was detected at a critical stage of embryo development (Brockdorff *et al.* 1992; Kay *et al.* 1993). *XIST/Xist* RNA coats the inactive X chromosome in *cis* and thus becomes detectable as a cloud within the nucleus of somatic cells using RNA-FISH (Clemson *et al.* 1996) (figure 1b). Subsequent studies showed that insertion of the XIC including *Xist* on autosomes induces silencing at great distances, and that deletions/mutations of *Xist* perturbs XCI (Penny *et al.* 1996; Lee and Jaenisch 1997). The role of *Xist* and of all elements of the XIC which include several other lncRNAs and controlling elements is still under study (Payer and Lee 2008; Gendrel and Heard 2014). In mice, where there are two waves of silencing in early development imprinted XCI is initiated on the paternal X chromosome at day four after fertilization (Okamoto *et al.* 2005). Imprinted paternal XCI persists in extraembryonic tissues (Takagi and Sasaki 1975; West *et al.* 1977), while it is followed by X reactivation in the inner cell mass and random XCI at the blastocyst stage (Mak *et al.* 2004). XCI depends on levels of *Xist* expression, which are controlled by its antisense *Tsix* and a series of lncRNAs located at the XIC (Galupa and Heard 2015). The XIC also contains the protein-coding gene *Rnf12*, whose product activates *Xist* expression based on its dosage (Gontan *et al.* 2012). Pluripotency factors such as OCT4 and NANOG control *Xist* and *Tsix* expression, preventing XCI in pluripotent cells such as embryonic stem (ES) cells (Navarro *et al.* 2008). Induced differentiation of cultured ES cells triggers the onset of random XCI, which has greatly facilitated experimentation.

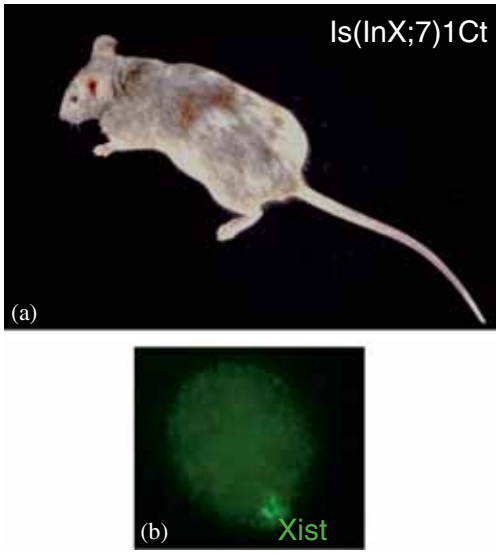

Figure 1. (a) Female mouse with the Cattanach insertion [Is(In7;X)1Ct] shows variegation of her coat colour due to spreading of XCI in the inserted portion of chromosome 7, which silences coat colour markers. (b) Nucleus from a female mouse fibroblast after *Xist* RNA-FISH (green). The inactive X is coated with *Xist* RNA.

Surprisingly, in human and rabbit, XCI onset is delayed and the paternal or maternal X chromosome is randomly silenced in some cells at early stages of development (Okamoto *et al.* 2011), thus skipping the imprinted XCI observed in mouse. The organization and function of the XIC also differ between human and mouse. Whereas the antisense *Tsix* RNA and/or its transcription are critical for regulation of *Xist* in mouse, this is not the case in human (Chang and Brown 2010). Important questions remain, for e.g., about mechanisms that ensures that only one active X per diploid set of autosomes persists. *Tsix* may protect the active X from silencing (Gayen *et al.* 2015). The choice of which X chromosome becomes silenced is also under study; one important element is the X controlling element (*Xce*) locus (Cattanach 1975) whose molecular identity remains elusive (Morey and Avner 2010; Thorvaldsen *et al.* 2012). One possibility is that structural oscillations in topological domains at the XIC may influence choice in individual cells (Giorgetti *et al.* 2014). It has also been proposed that initiation of XCI may be stochastic, with subsequent selection of cells with the appropriate number of X chromosomes expressed, i.e. a single X chromosome per diploid cell (Monkhorst *et al.* 2008).

XCI spreading

> *'It is suggested that interspersed repetitive elements of the LINE type, in which the X chromosome is particularly rich, act as booster elements to promote the spread of XIST mRNA.'*
> (Lyon 1998)

Gartler and Riggs originally proposed that there would be way-stations that help spreading of silencing along the inactive X chromosome (Gartler and Riggs 1983). However, how exactly *Xist* RNA spreads along the X chromosome is still controversial (Engreitz *et al.* 2013; Simon *et al.* 2013). One possibility is that *Xist* RNA binds to preferred sites in a saltatory way and spreads from a limited number of recruitment sites (Pinter *et al.* 2012). High resolution microscopy of single cells show fewer *Xist* RNA molecules over the inactive X chromosome than expected from previous studies on bulk cells, suggesting a hit-and-run model (Sunwoo *et al.* 2015). Importantly, new studies have identified proteins recruited by

Xist RNA, which directly or indirectly facilitate gene silencing (Chu *et al.* 2015; McHugh *et al.* 2015; Minajigi *et al.* 2015). For example, SHARP (also called SPEN) interacts with *Xist* RNA and recruit SMRT that activates the histone deacetylase HDAC3 for silencing (McHugh *et al.* 2015). Among the most enriched proteins recruited by *Xist* RNA is the nuclear matrix protein HnrnpK, which is involved in recruitment of the PRC1 and PRC2 complexes for deposition of the repressive marks H2AK119ub and H3K27me3 (Hasegawa *et al.* 2010; Chu *et al.* 2015; McHugh *et al.* 2015; Minajigi *et al.* 2015). Histone modifications including deacetylation of histones, methylation of H3K27 and ubiquitination of H3K119 are early events in establishing silencing (Jeppesen and Turner 1993; Boggs *et al.* 2002; Plath *et al.* 2002; Heard and Disteche 2006; Marks *et al.* 2009). The A-repeat within the *Xist* gene is essential for gene silencing (Wutz *et al.* 2002) and is a key element for the binding interactors (Chu *et al.* 2015). Thus, important new elements are being discovered that connect *Xist* RNA to the deposition of specific epigenetic modifications put in place to implement stable and heritable gene silencing (see maintenance) (Gendrel and Heard 2014).

> *'... and that there will be a similar effect when autosomal genes are translocated to the X-chromosome'* (Lyon 1961)

Silencing via *XIST/Xist* RNA spreading in *cis* can also recruit silencing factors along autosomal segments attached to the inactive X following a translocation or insertion (figure 1a). Silencing is less efficient along autosomal regions (Sharp *et al.* 2002), suggesting that specific elements enriched on the X help spreading and maintenance. Mary Lyon proposed that such elements might be LINE1 repeats, which are particularly abundant on the X chromosome (Lyon 1998). Indeed, the core of the condensed inactive X chromosome is enriched in L1 elements (Chow *et al.* 2010; Deng *et al.* 2015). Accordingly, inefficient discontinuous spreading is observed along autosomal segments with few L1 elements (Tang *et al.* 2010). However, cell selection plays an important role in the observed patterns of inactivation in X;autosome translocations, which should be interpreted with caution (Disteche *et al.* 1979). An interesting application of the power of *XIST* in inducing *cis* autosomal silencing is the correction of trisomy 21 by insertion of a highly expressed *XIST* transgene on one human chromosome 21 in trisomic cells (Jiang *et al.* 2013). This restores normal gene expression and cellular phenotypes, thus offering hope for helping individuals with Down's syndrome or other autosomal trisomies, at least in cells accessible to treatment such as bone marrow.

XCI maintenance

> *'Thus, it is at present considered that methylation is part of the mechanism for stabilizing inactivation, after spreading has occurred'* (Lyon 1992)

Early studies identified a key molecular feature that locks silencing, i.e. DNA methylation at CpG islands of X-linked genes (Riggs 1975; Gartler and Riggs 1983). Particularly telling were experiments in which a methylated DNA plasmid containing the X-linked gene *HPRT* was shown to remain silent after transfection into HPRT-deficient cells, but became competent after removal of DNA methylation by 5-azacytidine (Liskay and Evans 1980; Venolia *et al.* 1982; Venolia and Gartler 1983). Maintenance of XCI is also ensured by the histone modifications that are progressively added throughout early development (see spreading). Later events include replacement of histone H2A by macrohistone H2A (Costanzi and Pehrson 1998), and DNA methylation of CpG islands implemented by the methylases Dnmt3a/b and maintained by Dnmt1 (Norris *et al.* 1991). Different genes become silent at different times in concordance with epigenetic changes (Gendrel *et al.* 2012). Maintenance of XCI requires synergy of *Xist* RNA, histone modifications and DNA methylation (Csankovszki *et al.* 2001). However, loss of any one of the element does not necessarily affect silencing. For example, EED, a component of the PRC2 complex that mediate H3K27me3 is dispensable for initiation and maintenance of XCI in embryos (Kalantry *et al.* 2006).

X chromosome 3D structure

> '*The cytology evidence was provided by Ohno and Hauschka (1960), who showed that in cells of various tissues of female mice one chromosome was heteropycnotic.'* (Lyon 1962)

The inactive X chromosome forms the Barr body (Barr and Bertram 1949) visible as a condensed heteropycnotic structure in interphase nuclei of female cells (Ohno and Hauschka 1960). The modalities of condensation of the inactive X are only beginning to be deciphered using genome-wide analyses of chromatin structure by chromatin conformation studies including Hi-C. Both in human and mouse, the inactive X chromosome forms a bipartite structure of two superdomains separated by a boundary (Rao *et al.* 2014; Deng *et al.* 2015; Minajigi *et al.* 2015). The superdomains differ between human and mouse but the boundary between domains is partially conserved and contains the macrosatellite locus *Dxz4* (Deng *et al.* 2015), which binds CTCF specifically on the inactive X (Chadwick 2008; Horakova *et al.* 2012a, b). CTCF is a zinc finger protein widely known to help organize chromatin in topologically associated domains (TADs) (Dixon *et al.* 2012). In mouse, the boundary between superdomains on the inactive X appears to represent a nucleolus-associated domain (NAD) (Deng *et al.* 2015).

> *'Knowledge of the fine structure of the embryo at this stage may provide some clue whether or not attachment of the X chromosome to a site is a likey mechanism'* (Lyon 1971)

Mary Lyon suggested that the inactive X may occupy a preferred site in the nucleus (Lyon 1971). Such preferred locations for the inactive X are proximity to either the nuclear membrane (Barr and Bertram 1949) or the nucleolus (Zhang *et al.* 2007). These preferred locations are in agreement with findings in other systems, suggesting that the lamina and/or the nucleolus represent 'Velcro' elements for heterochromatin (Padeken and Heun 2014). Interestingly, *XIST* interactors include proteins that help anchor chromosomes to the nuclear membrane such as the lamin B receptor (LBR) (Chu *et al.* 2015; McHugh *et al.* 2015; Minajigi *et al.* 2015). Our own data suggest that proximity of the inactive X to the nucleolus may be facilitated by specific elements such as the lncRNA genes, *Firre* and *Dxz4*, which bind CTCF specifically on the inactive X. Knockdown of *Firre* causes loss of the repressive mark H3K27me3 on the X chromosome, suggesting a role in maintenance of heterochromatin potentially related to positioning (Yang *et al*. 2015).

X reactivation

> *'These observations provide the first evidence with a true X-linked gene (Oct) for an age-related decrease in the stability of the X-inactivation mechanism.'* (Wareham *et al.* 1987)

X chromosome regulation in females represents a cycle of inactivation and reactivation (Gartler and Riggs 1983; Gartler *et al.* 1992). In precursor female germ cells both X chromosomes become active by a process of reactivation that progresses along the X, genes closest to *Xist* being reactivated last (Sugimoto and Abe 2007). This reactivation ensures that each haploid female germ cell contains an active X chromosome. Interestingly, haploid cells derived from female germ cells have a high X:autosome expression ratio due to upregulation of the active X (Leeb and Wutz 2011). Immediately after fertilization, there is reactivation of the paternal X which is largely silenced in sperm (Okamoto *et al.* 2004). A second wave of reactivation occurs in the inner cell mass at blastocyst stage prior to the onset of random XCI (Mak *et al.* 2004). In cells where reactivation occurs *XIST* becomes silent and repressive histone marks are lost (Ohhata and Wutz 2012).

X reactivation can also occur in somatic cells in relation to ageing as Mary Lyon first described (Wareham *et al.* 1987). Aberrant reactivation is also observed in congenital or acquired diseases (see below). For example, abnormal X-linked gene expression is seen in ICF syndrome, which is due to a mutation in the methylase Dnmt3b (Hansen *et al.* 2000). Persistence of *XIST/Xist* in somatic cells is not necessarily required for stable silencing (Brown and Willard 1994). However, an induced *Xist* deletion caused X reactivation and cancer in mice after a long period of time (Yildirim *et al.* 2013). Reactivation can also be induced in iPS cells following dedifferentiation of somatic cells (Lessing and Lee 2013). While this can easily be induced in mouse by adding pluripotent factors this is not always the case in human cells where variable patterns are observed (Lessing and Lee 2013). The presence of two active X chromosomes is rarely observed in undifferentiated human ES cell lines unless they are in a 'naive' state (Ware *et al.* 2014). Interestingly, X reactivation in human pluripotent stem cells is accompanied by coating with the lncRNA *XACT* prior to loss of *XIST* RNA (Vallot *et al.* 2015).

Escape from XCI

> *'... it is still possible that inactivation of one X does not take place in man, or that it differs in some way from the process in the mouse... '*
>
> *'The other possible explanation is that the X chromosome of man has a short pairing segment, that is not normally inactivated, and that it is duplication or deficiency of this region which gives rise to the abnormal phenotypes observed.'* (Lyon 1962)

Mary Lyon hypothesized that some genes, probably located in the pseudoautosomal region (PAR) of pairing between the X and Y chromosomes, would escape XCI (Lyon 1962). She puzzled about differences in phenotypes between X0 mice that can reproduce and 45,X women who have abnormal phenotypes and are infertile. Subsequent studies have shown that the mouse and human PARs contain very different sets of genes (Disteche *et al.* 1992). In addition, genes outside the PAR can also escape XCI with significant differences in the list of escape genes between mouse and human (Berletch *et al.* 2010). In human, about 15% of X-linked genes escape XCI compared to 3–7% in mouse (Carrel *et al.* 1999; Yang *et al.* 2010). Some of the escape genes that reside outside of the PAR have retained a Y-linked paralogue (Lahn and Page 1997). In fact, a subset of these X/Y genes are conserved on the sex chromosomes in multiple mammalian species, possibly because they encode for critical proteins and are highly dosage-sensitive (Bellott *et al.* 2014; Cortez *et al.* 2014).

Escape from XCI can vary between tissues and individuals. We recently completed a study of XCI and escape in multiple mouse tissues using RNA-seq to test allele-specific expression in F_1 mice with skewed XCI based on frequent SNPs (figure 2) (Berletch *et al.* 2015). While, a subset of escape genes were common between tissues, others were tissue-specific. Interestingly, many genes found to escape XCI in adult mouse tissues differ from those reported in trophoblastic cells derived from placenta in which XCI is paternally imprinted, suggesting significant differences between imprinted and random XCI (Calabrese *et al.* 2012; Corbel *et al.* 2013; Finn *et al.* 2014). In addition, levels of expression from the inactive X can vary for a given gene, suggesting tissue-specific dosage effects of escape. Thus, escape from XCI may be a source of tissue-specific sex differences. In human, SNP analyses have also shown tissue and individual

Figure 2. Differences and commonalities in the distribution of genes (pink) that escape X inactivation on the mouse X chromosome (centromere to the left) between tissues *in vivo*: ovary (top), spleen (middle), and brain (bottom). Pink bars indicate the position of escape genes on schematics of the mouse X chromosome (centromere at left) (see Berletch *et al.* 2015).

variability (Cotton *et al.* 2013). Differential methylation levels at X-linked CpG islands and gene bodies has also helped identify escape genes in many human tissue types (Lister *et al.* 2013; Cotton *et al.* 2015; Schultz *et al.* 2015). Indeed, escape genes are often depleted in repressive marks associated with XCI and enriched in marks associated with active gene transcription (Berletch *et al.* 2011). In addition, escape genes are located at the periphery of the silent domain of the inactive X chromosome where they apparently interact with each other (Splinter *et al.* 2011; Deng *et al.* 2015).

Escape genes that lack a functionally equivalent Y paralogue are a potential source of sex-specific differences in gene expression and thus, candidates for sex-specific phenotypes (Berletch *et al.* 2011). One example is the histone demethylase KDM6A encoded by a gene that escapes XCI in multiple species. *Kdm6a* is more highly expressed in female cells and regulates a set of reproduction-related homeobox genes (*Rhox6* and *Rhox9*) in a female-specific manner (Berletch *et al.* 2013). Whether other escape genes contribute to sex-specific differences is still under study.

XCI, X aneuploidy and disease

> *'Facts that remain unexplained are that an X0 female and an XXY male show any abnormality, and that an X0 female in man differs in phenotype from that in mouse'* (Lyon 1962)

Escape gene dosage is dependent on the number of X chromosomes present, thus escape genes are candidates for phenotypes associated with X aneuploidy. Indeed, aberrant copy number of escape genes (PAR or non-PAR) is thought to be associated with abnormal phenotypes in Turner and Klinefelter syndromes (Zinn *et al.* 2007; Tartaglia *et al.* 2010a, b). For example, loss of one copy of the *SHOX* gene located in the PAR explains the short stature in Tuner syndrome, whereas three copies of this gene in XXY individuals explain the tall stature in Klinefelter syndrome (Blaschke and Rappold 2006). Specific escape genes have been implicated in mental impairment; for example, *KDM5C* and *IQSEC2* deletions or mutations cause X-linked intellectual disability both in males and females, consistent with dosage sensitivity (Santos-Reboucas *et al.* 2011; Simensen *et al.* 2013; Fieremans *et al.* 2015). Further, cognitive deficiencies have been reported in individuals carrying microduplications of *KDM5C* and *IQSEC2* associated with abnormally high expression (Moey *et al.* 2015). Similarly, mutations and deletions in *KDM6A* have been discovered in patients with Kabuki syndrome characterized by intellectual disability, growth retardation, skeletal abnormalities, and visceral malformations (Lederer *et al.* 2012; Miyake *et al.* 2012). Female carrier of mutations also show abnormalities, consistent with dosage anomalies (Lindgren *et al.* 2013). Some of these patients show symptoms overlapping with Turner syndrome, termed Turner–Kabuki syndrome, suggesting a potential link to other genes that escape XCI.

X-linked mutations cause diseases with widely different consequences in males and females. Males are often affected because they have only one X chromosome, so that recessive mutations cause abnormal phenotypes. Females can compensate by having patches of cells that express the normal allele, or by strong selection (skewing) for cells that express the normal allele (Deng *et al.* 2014). Skewing of XCI can be very extensive or only affect the tissue in which proper expression is critical (Migeon 2014). Random distribution of patches of cells with one X active can be extensive as shown by a recent study of female mice with a different X-linked fluorescent reporter on each allele, in which *in situ* visualization of XCI distribution revealed surprisingly extensive skewing of XCI (Wu *et al.* 2014). For example, one mouse had half of her brain with silencing of the maternal X and the other with silencing of the paternal X.

Mutations in escape genes and abnormal dosage have been linked to noncongenital disease as well. For example, *KDM6A* mutations have been observed in renal carcinoma as well as other cancer types (van Haaften *et al.* 2009; Dalgliesh *et al.* 2010). Interestingly, KDM6A seems to function as a gender-specific tumour suppressor in T-cell acute lymphoblastic leukaemia, where only males with the disease had inactivating mutations in the demethylase (Van der Meulen *et al.* 2015). Additionally, aberrant hypomethylation of the X chromosome along with loss of part or one entire X can occur in breast cancer cells (Sun *et al.* 2015). Extensive reactivation of the X chromosome has been documented in breast cancer (Chaligne *et al.* 2015).

Summary

In summary, the X chromosome inactivation law proposed by Mary Lyon has helped us to understand not only basic principles of gene silencing, heterochromatin structure and

nuclear organization, but has also led to discoveries of new master switches such as the lncRNA *Xist* and to a better understanding of X-linked diseases and of sex-specific differences.

Acknowledgements

This work was supported by grants GM046883 and GM113943 (CMD) from the National Institutes of Health. JB is also supported by grant MH105768 from the National Institutes of Health. We thank X. Deng for critical reading of the manuscript.

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Received 21 August 2015; accepted 27 August 2015
Published online: 6 November 2015