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Fifty years of X-inactivation research

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Summary

The third X-inactivation meeting 'Fifty years of X-inactivation research', which celebrated the fiftieth anniversary of Mary Lyon's formulation of the X-inactivation hypothesis, was an EMBO workshop held in Oxford, UK, in July 2011. This conference brought together the usual suspects from the field, as well as younger researchers, to discuss recent advances in X-inactivation research. Here, we review the results presented at the meeting and highlight some of the exciting progress that has been made. We also discuss the future challenges for the field, which aim to further our understanding of the developmental regulation of X inactivation, the randomness (or skewing) of X inactivation, and the diverse strategies used by mammalian species to mediate X inactivation.

Key words: X chromosome, X-inactivation hypothesis, Epigenetics, Xist

Introduction

X-chromosome inactivation (XCI) refers to the silencing of one of the two X chromosomes in females during early development, thus ensuring dosage compensation for X-linked gene products between XX females and XY males. The heteropyknotic structure that corresponds to the inactive X chromosome (Xi) was first noted by Barr in 1949 (Barr and Bertram, 1949), but it was in 1961 that Mary Lyon proposed her seminal hypothesis, based on genetic evidence in mice, for the early silencing of one of the two X chromosomes, at random, during early development, and for the clonal propagation of the inactive state during subsequent cell divisions (Lyon, 1961).

This workshop, which was organised by Neil Brockdorff and Tatyana Nesterova (University of Oxford, UK), was a historical event honoured by the presence of Mary Lyon herself as well as by a rich gathering of experts, both young and old. There was a particularly memorable session, chaired by Sohaila Rastan, in which some of the 'pioneers' of the field, including Bruce Cattanach, Stan Gartler, Arthur Riggs, Nobuo Takagi and Marilyn Monk, recounted the exciting early years where genetics, embryology and molecular biology were applied to lay the foundations of the field and, in many ways, the foundations of modern epigenetics. The workshop attendees also witnessed the official birth of 'Lyon's law': Hunt Willard (Duke University, Durham, USA) reminded us that because XCI has been pretty much an established fact for quite some time since its discovery 50 years ago, it seems about time that it should formally be promoted from a mere hypothesis to a full-blown law! Thus, Lyon's law for XCI in XX mammals can join Ohno's law for X-chromosome synteny (Ohno, 1967). This should not to be confused with Ohno's hypothesis that the active X chromosome must be upregulated, compared with autosomes, to ensure equal dosage between the single X and autosomes (Ohno, 1967), which will be discussed below.

In mammals, XCI actually comes in different flavours. In early mouse embryos, imprinted, paternal XCI is found initially. Imprinted XCI is also found in all tissues of marsupials. However, in the somatic tissues of most eutherian species, including mice, XCI is random. Indeed, eutherian females consist of mosaic cell populations in which either the maternal X or the paternal X is silenced (Fig. 1), an observation that led to the discovery of XCI in the first place (Lyon, 1961). XCI is established early in development and studies in mice and humans have shown that it is initiated by a unique locus: the X-inactivation centre (Xic) (Fig. 2). The Xic produces a long non-coding transcript, X inactive specific transcript (Xist), which associates in cis with the X chromosome from which it is expressed, induces a series of chromatin modifications as well as the spatial reorganisation of the chromosome; this ultimately results in the silencing of nearly all genes on this chromosome, although some genes can escape from XCI. Once established, the inactive state is believed to be generally irreversible in somatic cells and stably maintained through mitotic divisions. Reactivation of the Xi can occur during early development, first in the inner cell mass of the mouse blastocyst and then in the developing germ line, just before meiosis, thus ensuring that the two X chromosomes are active during oogenesis [for recent reviews on XCI, see Augui et al. (Augui et al., 2011) and Wutz (Wutz, 2011)].

In recent years, an increasing number of molecular and biochemical insights have contributed to the molecular dissection of the XCI process. We now have a better understanding of the complex regulatory network controlling *Xist* expression, with the identification of new molecular players (Jonkers et al., 2009; Tian et al., 2010), a link with key pluripotency factors (Navarro et al., 2008; Donohoe et al., 2009; Navarro et al., 2010), and the involvement of nuclear dynamics through chromosome pairing (Xu et al., 2006; Augui et al., 2007). By contrast, the mechanism of binding and propagation of Xist RNA along the Xi remains poorly understood, although recent insights implicate Yin Yang 1 (YY1) as a possible candidate protein for bridging Xist RNA to its own locus (Jeon and Lee, 2011). The roles of nuclear scaffold proteins and of the local chromatin environment also appear crucial (Chow et al., 2010; Hasegawa et al., 2010; Tang et al., 2010). However, many unanswered questions still remain. As developments in the field accelerate with the power of new technologies, it was timely for experts to meet and discuss recent progress and future challenges for the field, while also celebrating its origins.

Here, we summarise the key results presented at the workshop, dividing our review into five themes that were discussed throughout the meeting: the *Xist* regulatory network; the search for X-linked loci affecting the randomness of XCI; X chromosome-wide silencing and escape events; the upregulation of genes on the active X (Xa); and, lastly, the regulation of XCI in mammals other than mice.

Teasing apart the *Xist* regulatory network

One of the holy grails in the past 20 years of XCI research has been to understand more about the non-coding RNA Xist, which is one of a growing list of long non-coding RNAs (lncRNAs) found in complex genomes and involved in various developmental and

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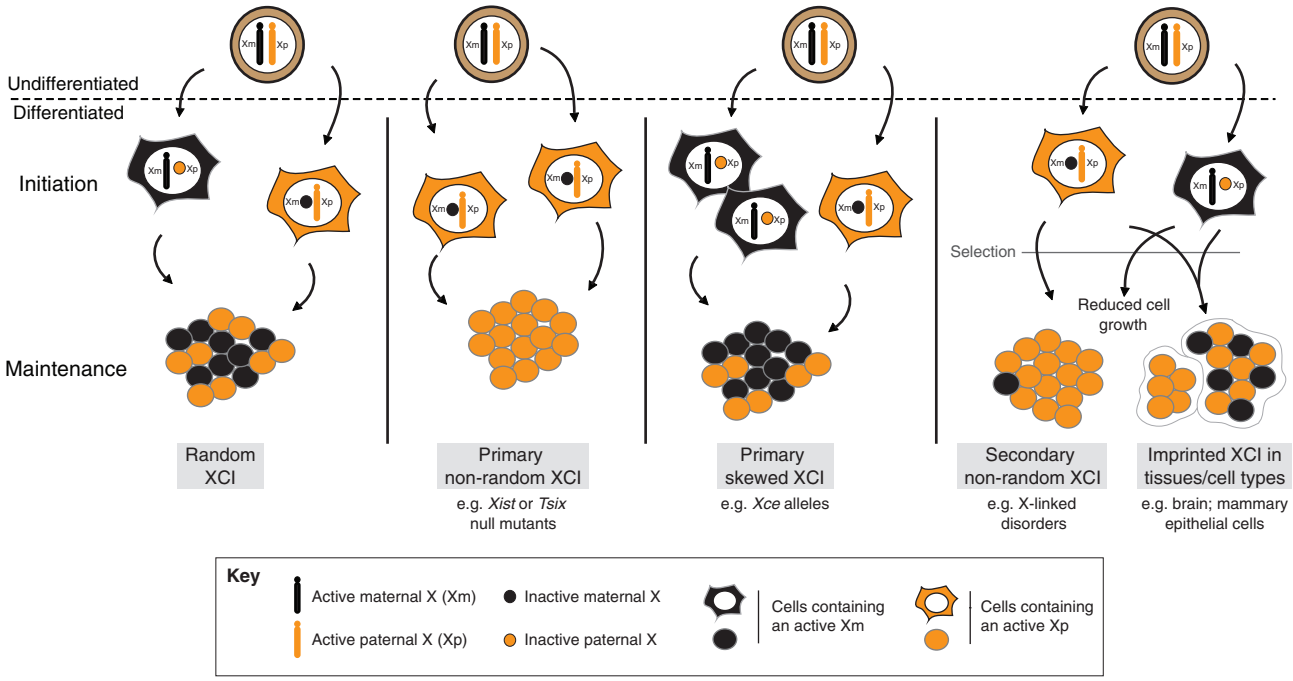

Fig. 1. Random and non-random X inactivation. Both the paternal (Xp; orange) and maternal (Xm; black) X chromosomes are initially active in undifferentiated female cells. The random 'choice' of which X chromosome to inactivate leads to a mosaic population of cells in which either the Xm (black cells) or the Xp (orange cells) is active. Non-random X-chromosome inactivation (XCI) patterns can arise through different situations. Examples of partial (skewed) or complete non-random XCI are shown. The disruption of *Xist* or *Tsix* results in a complete and primary non-random XCI pattern. Heterozygosity of the *Xce* locus leads to skewed XCI patterns, already from the initiation stage, with an unequal number of cells having inactivated one of the two X chromosomes. Secondary non-random XCI can arise from cell selection after a random initiation step. In this situation, it is proposed that cells expressing one X chromosome rather than the other would be privileged in their cell growth and/or survival. This can be due to deleterious X-linked mutations carried by one of the X chromosomes, or following imprinting of one X chromosome in specific cell or tissue types (e.g. as observed in the brain for Xp and in mammary epithelial cells for Xm).

regulatory mechanisms. Xist RNA is essential for the initiation of XCI in mice (Penny et al., 1996) and probably in other eutherians. Although its mode of action remains elusive, its unusual mode of regulation in mice is starting to become clearer. Briefly, *Xist* is activated in female but not male cells, and is only expressed from one X chromosome, thus inducing its silencing in a cis-limited fashion. How is this elaborate expression profile put into place? Recent studies have pointed to the X-encoded protein RING finger protein 12 (Rnf12, or Rlim), which acts as a key dosage-sensitive factor that upregulates *Xist* in cells with more than one X chromosome (Fig. 2) (Jonkers et al., 2009). Intriguingly, a new role for Rnf12 as a survival factor specifically for milk-producing alveolar cells in the mammary gland was presented by Ingolf Bach (University of Massachusetts Medical School, Worcester, USA), suggesting that this factor plays highly specialised roles in females.

Xist is only upregulated during early development and it is repressed in undifferentiated ES cells. Several recent studies have identified a link between *Xist* repression and pluripotency factors, although the question of whether they act directly or indirectly on *Xist* is still somewhat open. Past studies have shown that intron 1 of *Xist* is bound by pluripotency factors, such as octamer-binding transcription factor 4 (Oct4, or Pou5f1), Nanog, and sex-determining region Y-box 2 (Sox2) (Navarro et al., 2008). However, a recent knockout by the Gribnau laboratory demonstrated that this region of *Xist* might not be crucial for *Xist* repression (Barakat et al., 2011). Joost Gribnau (Erasmus MC, Rotterdam, The Netherlands) and Pablo Navarro (University of Edinburgh, UK) presented new work showing that *Rnf12* itself is regulated by pluripotency factors (Navarro et al., 2011). Furthermore, Gribnau presented exciting new data showing that this protein acts specifically at the *Xist* promoter to degrade a repressor of *Xist*, the reduced expression protein 1 (Rex1, or Zfp42) stem cell factor, which is itself also regulated by pluripotency factors. Previous work has suggested that Rex1 is also an activator of *Tsix*, the antisense transcription unit to *Xist* (Navarro et al., 2010). Thus, Rex1 appears to act as a repressor of *Xist*, both directly at its promoter and indirectly via *Tsix*, clearly suggesting that pluripotency factors are embedded in the *Xist* regulatory network at several levels.

The Xic has been found to harbour an ever-increasing number of lncRNAs (Fig. 2). Whether this is a peculiarity of the Xic, or simply a result of the intense scrutiny of this part of the genome, remains to be seen. In addition to Xist and its antisense transcript Tsix, the Jpx (Enox) and Ftx lncRNAs were recently characterised (Tian et al., 2010; Chureau et al., 2011). Claire Rougeulle (Université Paris-Diderot, France) presented data showing that Ftx is a conserved lncRNA that is likely to participate in the control of Xist RNA levels in male ES cells, as an *Ftx* promoter deletion leads to decreased *Xist* expression (Chureau et al., 2011). However, its role in XCI remains to be examined. Moreover, Phil Avner (Institut Pasteur, Paris, France) and Elphège Nora from Edith Heard's group (Institut Curie, Paris, France) reported on new lncRNAs that originate from a region upstream of *Tsix* and that might play a role in the choice of which X chromosome to inactivate (see below). Jeannie Lee (Howard Hughes Medical Institute and Harvard Medical School, Boston, USA) described *Tsx*, which encodes a protein expressed in testes (Cunningham et al., 1998), and showed that Tsx may also play a role as a lncRNA that positively regulates *Tsix* (Anguera et al., 2011).

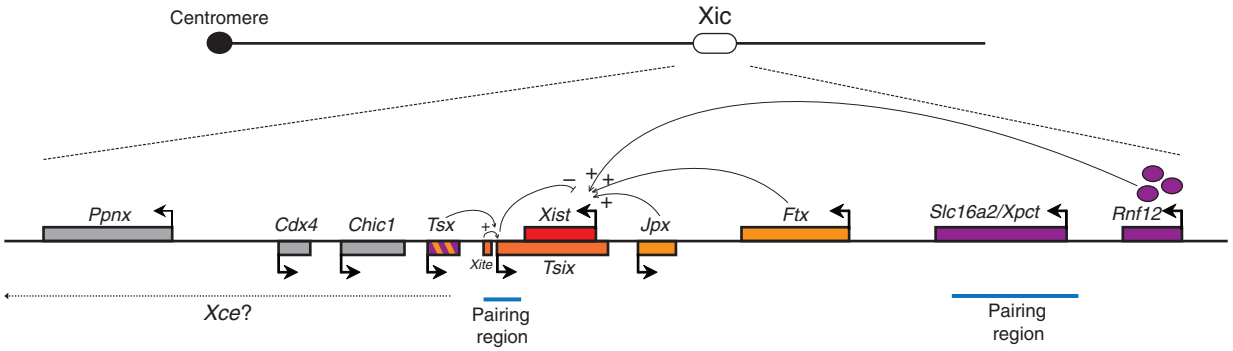

Fig. 2. The X-inactivation centre (Xic) in mouse. The Xic on the X chromosome has been defined as the minimum region necessary and sufficient to trigger XCI (Augui et al., 2011). The region around the *Xist* (red) gene harbours a number of non-coding RNA genes (orange), as well as protein-coding genes (purple or grey), and two homologous 'pairing' regions (blue). All genes marked in colour have been shown to have either a positive (+) or a negative (–) effect on *Xist*/*Tsix* regulation. *Tsx* (striped orange/purple) encodes a protein that is expressed in testes and might also play a role as a ncRNA. *Rnf12* encodes an activator (purple ovals) of *Xist* expression. The potential location of the *Xce* locus, according to genetic mapping, is indicated. *Ppnx* (*4930519F16Rik*); *Cdx4*, caudal type homeobox 4; *Chic1*, cysteine-rich hydrophobic domain 1; *Tsx*, testis-specific X-linked gene; *Xite*, X-inactivation intergenic transcription element; *Tsix*, X (inactive)-specific transcript, antisense; *Xist*, X-inactive-specific transcript; *Jpx* (*Enox*), expressed neighbour of Xist; *Ftx* (*B230206F22Rik*), five prime to Xist; *Xpct* (*Slc16a2*), X-linked PEST-containing transporter; *Rnf12* (*Rlim*), RING finger protein 12.

One interesting feature of Xist discussed at this meeting concerned the highly conserved A-repeat region located in the proximal part of the transcript. Previous work from Anton Wutz had shown that this region is necessary for Xist RNA to establish gene silencing during XCI (Wutz et al., 2002), and the Sado laboratory had reported that this region acts as a positive regulatory element for *Xist* expression (Hoki et al., 2009). This region has also been reported to produce a short independent transcript called *RepA* (Zhao et al., 2008). Barbara Panning (University of California, San Francisco, USA) presented data showing that deletion of the A-repeat region also blocks the accumulation of spliced Xist RNA, resulting in primary non-random XCI (Royce-Tolland et al., 2010). Furthermore, Takashi Sado (Kyushu University, Fukuoka, Japan) presented new data indicating differences in the capacity of a constitutively expressed *Xist* gene at its endogenous locus, with or without the A-repeat region, to induce gene silencing. However, the A-repeat-deleted form of Xist, although defective in silencing, was still competent to recruit histone H3 lysine 27 trimethylation (H3K27me3) in the mouse embryo, consistent with previous data (Wutz et al., 2002). Interestingly, accumulation of both the wild-type and deleted forms of Xist RNA could be found in heterozygous mutant female embryos, indicating that the lack of gene silencing capacity of the A-repeat mutant allele results in prolonged biallelic expression of Xist RNA. This contrasts with the normal situation in females with two wild-type *Xist* alleles, in which only one Xist-coated chromosome is usually seen, pointing to the existence of a feedback mechanism between the two X chromosomes if they both initiate XCI, as previously suggested (Monkhorst et al., 2009; Okamoto et al., 2011).

The window of competence of the Xist RNA silencing function was also discussed, and exciting new data revealed that Xist can definitely trigger XCI in adult cells and outside of the early embryonic developmental time window. Anton Wutz (University of Cambridge, UK) presented recent work showing that the ectopic induction of Xist RNA from the X chromosome at E16.5 induces hair loss, and that this developmental window for Xist function during hair morphogenesis might be dependent on specific factors expressed in precursor cells. This is consistent with previous data from the Wutz laboratory, which showed that Xist can initiate silencing in haematopoietic pro-B cells and also in lymphomas expressing special AT-rich sequence-binding protein 1 and 2 (Satb1/2) (Savarese et al., 2006; Agrelo et al., 2009). Thus, Satb1/2 and other factors, currently under investigation, might define a range of cellular contexts that are permissive for the initiation of XCI.

Another interesting story on a related topic came from the presentation of Tatsuya Ohhata from the Wutz group, who addressed the lineage-specific function of *Tsix* in repressing *Xist* and subsequent Xi reactivation. He showed that *Tsix* can repress *Xist* and that this subsequently induces the expression of an X-linked *GFP* transgene in all extra-embryonic lineages of early postimplantation embryos, as well as in the parietal endoderm during development, but not in late-stage trophoblast giant cells or spongiotrophoblast cells (Ohhata et al., 2011). This indicates that the maintenance of imprinted XCI in some extra-embryonic lineages is highly dependent on Xist RNA. This is in contrast to the situation in somatic tissues, where other epigenetic marks seem to take over (Csankovszki et al., 2001).

Choosing an X: *Xce* alleles and more

One of the major unresolved questions in the XCI field concerns how just one of the two X chromosomes is chosen to become inactivated in females. In mice, this situation is initially dealt with by a maternal imprint that robustly silences *Xist* on the maternal X during early development (Tada et al., 2000). This imprint is lost at the blastocyst stage, however, by the time random XCI occurs in the epiblast. How, then, is the random form of XCI ensured? In mice, several factors affect the choice of X chromosome to be inactivated at the onset of XCI. These include *Tsix*, as well as the X-controlling element (*Xce*) locus. *Xce* was originally genetically defined in the 1970s by Bruce Cattanach (Cattanach and Williams, 1972) as a locus, the different alleles of which affect the choice of X chromosome to be inactivated. In females heterozygous for different *Xce* alleles, the X chromosome carrying the 'weaker' allele is more likely to be inactivated than the one carrying a 'stronger' allele, thereby leading to skewed XCI (Fig. 1). Early work mapped *Xce* between the *Tabby* and *Mottled* X-linked markers. Refined genetic mapping using microsatellite-based approaches showed that *Xce* lies in a region 3′ to *Xist* and is independent of *Xist* (Simmler et al., 1993).

Phil Avner presented an analysis of congenic and recombinant inbred lines that has allowed his group to further refine the *Xce* candidate region to an interval of a few hundred kilobases 5′ to *Tsix* (Fig. 2). ChIP-seq data pointed to potential new regulatory elements in the candidate region and, in particular, to a new transcript, the expression pattern of which fits well with *Xce* allelism. Using a completely different approach, based on the identification of long-range interactions using chromosome conformation capture carbon copy (5C) technology to better define the regulatory landscape of *Xist* and *Tsix*, Elphège Nora highlighted that the region responsible for producing this transcript physically interacts with *Xite* and the *Tsix* promoter.

Christina Sheedy (Duke University, Durham, USA) from Hunt Willard's group also presented genetic and quantitative trait loci (QTL) mapping data to map regions associated with skewed XCI patterns. They defined a candidate region that spans 1.8 Mb and includes *Xist*/*Tsix*. Within this region, they identified several large segmental duplications of over 5 kb that vary in number between mouse strains with different *Xce* alleles, in particular in a region lying 350 kb 3′ to *Xist*. By comparing the sequence in different mouse strains, they identified numerous single-nucleotide polymorphisms (SNPs) that correlate with *Xce* allelism in a segment of their candidate region that also overlapped with the *Xce* interval defined by the Avner group. The fact that numerous SNPs within this large region correlate with *Xce* allelism suggests that *Xce* might represent a complex locus or series of elements that influence the balance of *Xist/Tsix* expression. This is also consistent with the 5C analysis presented by Nora, which revealed that *Xist* and *Tsix* lie within large regulatory domains, interacting with numerous potential regulatory elements. Clearly, the extensive regulatory landscape of the Xic and the identity of the *Xce* locus (or loci) will provide food for thought for many years to come.

XCI patterns can be skewed, either due to primary non-random XCI or to secondary selection whereby cells expressing one of the two X chromosomes are selected against (Fig. 1). Primary non-random inactivation can be due to mutations or polymorphisms affecting the initiation step, such as in the *Xist* gene or the *Xce* locus (Rastan, 1982; Marahrens et al., 1998; Lee and Lu, 1999); secondary selection can be linked to deleterious mutations, unfavoured SNPs, or even imprinted genes present on one X chromosome (McMahon and Monk, 1983; Minks et al., 2008) (Fig. 1). This can, of course, be tissue dependent, as different genes may impose such selection and skewing. Recent evidence points to an increasing number of adult or late embryonic tissues in which a skewed pattern of XCI is found due to imprinting. A series of recent studies showed that preferential paternal XCI can occur in the brain (Gregg et al., 2010; Wang et al., 2010). Ingolf Bach presented new work from his group showing preferential maternal XCI. Stemming from analyses of *Rnf12* knockout mice, and based on the observation that a paternally, but not maternally, inherited *Rnf12* knockout allele inhibits alveolar morphogenesis in the female mammary gland, Bach discovered an imprinted maternal XCI pattern in mammary epithelial cells of virgin and lactating females. However, the nature of the imprinted X-linked gene(s) that drive the likely tissue-specific secondary selection steps are not known. Importantly, this represents one of the first examples of preferential silencing of the maternal X. In most other reports of imprinted XCI, including the recent studies showing that this occurs in specific parts of the mouse brain (Gregg et al., 2010; Wang et al., 2010), it is usually the paternal X that is preferentially silenced. Identifying the genes and mechanisms that are the basis of these different parent-of-origin effects on XCI represents an exciting challenge for the future.

X chromosome-wide silencing and escape

The important topic of how chromosome-wide silencing is achieved and how it affects most, but not all, X-linked genes was also covered. Anne-Valerie Gendrel (Institut Curie, Paris, France) presented a study carried out in the Brockdorff group examining the DNA methylation dynamics of X-linked genes during XCI using differentiating mouse ES cells. She found that the majority of CpG islands on the Xi acquire methylation slowly throughout ontogeny, which is in line with previous studies showing that DNA methylation is a late step in the XCI process, and that this is dependent on the chromosomal protein structural maintenance of chromosomes flexible hinge domain containing 1 (Smchd1) (Blewitt et al., 2008). However, a subset of ~10% of CpG islands shows rapid methylation kinetics, indicating that these are methylated, at least in part, independently of Smchd1. What is particular to these early-methylating islands remains to be determined. Moreover, the kinetics of methylation and inactivation do not seem to be linked, indicating that methylation is involved in the long-term maintenance of silencing, and highlighting that the trigger for the initiation of gene silencing is still unknown.

Although XCI entails silencing of the majority of genes on the Xi, a subset of genes can, in fact, escape XCI. A number of talks explored this topic and, in particular, attempted to analyse the properties of genes that are silenced versus those that escape inactivation. Recent studies have shown that the number, distribution and identity of 'escapees' differ strikingly in mouse and human. At least 15% of X-linked genes (i.e. several hundred genes) have been reported to escape in humans, whereas only a few cases (13) are known in mouse (Carrel and Willard, 2005; Yang et al., 2010). Carolyn Brown (University of British Columbia, Vancouver, Canada) was the first speaker in the workshop to give an update on XCI escapees. Her group has compared DNA methylation differences between male and female human tissues to predict XCI status (Cotton et al., 2011) and found an 85% correlation with expression data, indicating hemi-methylation of silenced genes and hypomethylation at promoters of escapees (Carrel and Willard, 2005). They are now extending this analysis to unbalanced X/autosome translocations in order to define cis-acting elements that are important for the spreading of XCI, an ongoing question in the field since Gartler and Riggs hypothesized that 'way station' elements present on the X chromosome play a role in the efficient propagation of silencing (Gartler and Riggs, 1983). Previous analyses have shown that relatively few autosomal, as compared with X-linked, genes are silenced in X/autosome translocations (White et al., 1998; Sharp et al., 2002); preliminary analysis from the Brown laboratory suggests that this might correlate with higher concentrations of LINE-1 elements near genes that are subject to inactivation. The implication of LINEs in XCI efficiency is consistent with another hypothesis proposed by Mary Lyon – that LINEs might represent way stations for XCI (Lyon, 1998) – which was recently supported by studies in mouse ES cells (Chow et al., 2010; Tang et al., 2010). The LINE-enrichment of the X chromosome in humans and mice (Boyle et al., 1990; Bailey et al., 2000) (but not marsupials, see below) supports the idea that such elements might indeed have been selectively retained in the course of evolution to facilitate XCI, at least in eutherians.

Laura Carrel (Penn State College of Medicine, Hershey, USA) presented a comparative analysis of a conserved escape gene domain among several species. Surprisingly, this study revealed that mouse, the favoured model for XCI studies, is actually the exception and not the rule, given that the methylation profiles in dog, rabbit, cow and primates closely mirror those observed in human. Their attention is now focused on defining the regulatory sequences, and the role of

non-coding RNAs in particular, that could account for these differences. This talk highlighted one of the differences between mammalian species in dosage compensation of the X chromosome, a recurrent theme during this conference.

Hunt Willard shared with the audience his view of some of the most fundamental issues and open questions in the field, in particular the need for population- and family-based studies in order to estimate the variability in XCI patterns between populations. He reported new work from his group concerning the allele-specific distribution of RNA polymerase II (Pol II) across the Xi and the Xa in a human ENCODE cell line showing skewed XCI (Kucera et al., 2011). They found that Pol II binding is significantly reduced over the Xi, with the exception of the pseudo-autosomal region and escape genes, which led them to define several gene classes; in particular, monoallelically expressed genes with or without Pol II at their promoters, suggesting that some silenced genes might be poised for expression in specific tissues or in other individuals. This leads to the idea that lineage-specific escape, or transient expression of genes from the otherwise silent X, might occur at specific developmental times or in specific tissues.

Along this line, Jeanne Lawrence (University of Massachusetts Medical School, Worcester, USA) reported that the human Barr body is composed of a repeat-rich heterochromatic core, and that not only escapees, but also the silent genes studied, are located in the periphery of the Barr body (Clemson et al., 2006). An interesting question is whether these silent genes correspond to those that are more prone to reactivation in certain cell types or tissues? This organisation, together with other published and unpublished findings, support a model for XCI involving broad silencing of intergenic repeats (Hall and Lawrence, 2010). This role for repeats is similar to previous findings in the mouse (Chaumeil et al., 2006; Chow et al., 2010), although in this species it was shown that escapees tend to be present outside the Xist RNA domain of the Xi, whereas silent genes tend to be present within its silent, repetitive core (Chaumeil et al., 2006).

Revisiting the active X chromosome and Ohno's hypothesis

In 1967, Susumu Ohno proposed that X-linked genes should be expressed at twice the level of autosomal genes per active allele to regain gene balance (Ohno, 1967). This form of dosage compensation exists in *Drosophila*, which exhibits upregulation of the single male X chromosome (Straub and Becker, 2011). This process in *Drosophila* is regulated by male-specific lethal (MSL), an RNA-protein-containing complex that is involved in acetylation of histone H4 at lysine 16 via the histone acetyltransferase (HAT) Males absent on the first (MOF) (Akhtar and Becker, 2000).

Asifa Akhtar (Max-Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany) presented recent progress from her laboratory on the mechanisms of dosage compensation in *Drosophila*. She reported that the MOF HAT activity is intrinsically regulated to perform X chromosomal and autosomal regulation using distinct domains. Furthermore, in an effort to understand how hypertranscription of the male X chromosome is achieved, her group is analyzing differences in Pol II recruitment on autosomal and X-linked genes in males and females. Their work suggests that enhanced Pol II recruitment at X-linked gene promoters can contribute to hypertranscription of the male X chromosome.

Although X chromosome upregulation clearly occurs in flies, is this also the case in mammals? A number of papers have presented evidence using microarray expression data analysis to suggest that genes on the Xa in males and females are upregulated twofold on average (Gupta et al., 2006; Nguyen and Disteche, 2006; Lin et al., 2007), thus supporting Ohno's hypothesis. This observation was recently challenged by the analysis of publicly available RNA-seq data from human and mouse samples (Xiong et al., 2010), which apparently revealed no dosage compensation of the Xa chromosome. Christine Disteche (University of Washington, Seattle, USA) described her group's recent efforts to further examine these RNA-seq data sets and reported that the X chromosome carries an unusually high number of silent and low-expressing genes compared with autosomes, probably because there is a significant proportion of highly tissue-specific genes on the X chromosome, in particular genes that are expressed in the germ line and in the brain. When such genes are included in the analysis, the median expression ratios calculated between the X chromosome and autosomes become biased toward low values, thereby 'artificially' refuting Ohno's hypothesis (Deng et al., 2011). Bryan Turner (University of Birmingham, UK) also presented studies of undifferentiated female ES cells that support the Xa upregulation theory (Lin et al., 2007). The Disteche group has begun to investigate the mechanism behind gene upregulation of the mammalian X chromosome. They showed that X-linked genes are enriched at their 5′ end with Pol II that is phosphorylated at serine 5, raising interesting parallels with the situation in *Drosophila*. These data provide us with the first glimpse of the mechanism that controls Xa upregulation in mammals. Thus, Ohno's hypothesis might soon also be promoted to Ohno's (second) law!

Regulation of XCI in marsupials and other species

There has been an increasing realisation since the discovery of XCI, and thanks to the study of XCI in non-eutherian mammals such as marsupials and monotremes, that the strategies for X-chromosome dosage compensation can, in fact, vary extensively from one species to another. A number of presentations at the meeting illustrated this and some also showed that even among eutherians there can be major differences.

As Jenny Graves (Australian National University, Canberra, Australia) reminded us, marsupials exhibit dosage compensation by chromosome-wide silencing of the X. However, XCI is not random but is paternally imprinted (Cooper et al., 1971; Sharman, 1971). Furthermore, marsupials have no orthologue of the *Xist* gene (Duret et al., 2006; Elisaphenko et al., 2008). However, it turns out that these are not the only differences with eutherians. The Graves group, which has been studying the mechanisms of dosage compensation in non-placental mammals for decades, has shown that many X-linked genes partially escape paternal XCI in marsupials and that this is locus- and tissue-specific (Deakin et al., 2009). Moreover, the opossum X chromosome does not show any LINE-1 enrichment (Mikkelsen et al., 2007), unlike the situation in humans and mice (Boyle et al., 1990; Bailey et al., 2000), and female-specific methylation does not seem to be correlated with the Xi. Their studies also showed that this partial dosage compensation of one or both alleles in monotremes and birds is stochastic and may be regulated at the level of the probability of transcription (Deakin et al., 2009). XCI might therefore have evolved from an incomplete form in ancestral mammals to a much more widespread and stable process, thanks to the evolution of *Xist* and possibly the retention of LINEs in eutherians, as well as the exploitation of epigenetic modifications such as DNA methylation.

The question of how XCI is actually initiated in marsupials, which lack an *Xist* gene, was addressed by James Turner (MRC National Institute for Medical Research, London, UK). He presented recent work on the characterisation of a novel, large and potentially non-coding RNA that is expressed from the Xi in opossum. In the course of a study using various X-linked BACs to look at gene expression

and XCI in their new model organism, *Monodelphis domestica*, they observed an Xist-like cloud that colocalises with the Xi-specific H3K27me3 mark. They identified a transcription unit that produces a female-specific RNA of 27 kb, which is even longer than the Xist RNA. Interestingly, and similar to the Xist A-repeat region, this novel lncRNA is also biased for repeat sequences at its 5′-proximal end. This exciting finding identifies a new non-coding RNA that is likely to be involved in dosage compensation. Whether XCI is strictly dependent on this transcript in opossum and whether it is also present in other non-placental mammals remain to be determined.

Edith Heard presented data on XCI strategies during early development in eutherians. She showed that in rabbits and humans, both alleles of *Xist* can be activated transiently, before one of them is apparently downregulated (Okamoto et al., 2011). This contrasts with the situation in mice, in which a single Xist RNA-coated chromosome is almost exclusively seen during early development. She also presented preliminary data from peri-implantation mouse embryos showing that biallelic *Xist* activation can also occur, but very transiently and to a lesser extent than in rabbits and humans. This suggests that, in the mouse, *Xist* monoallelic regulation might be much more tightly controlled. One explanation for this could be that the antisense transcript Tsix fine-tunes the monoallelic expression of *Xist*, possibly via transient homologous pairing events, which can create transient asymmetry in *Tsix* expression and hence in *Xist* expression, as recently shown in differentiating mouse ES cells (Masui et al., 2011). How biallelic *Xist* upregulation is resolved in other mammals, such as humans and rabbits, in which *Tsix* may not be present or functional, remains unknown.

Taken together, these studies of species differences in dosage compensation are revealing a much higher level of evolutionary plasticity for XCI than was previously thought. Marsupial XCI is incomplete and variable, but this is nevertheless sufficient for dosage compensation in these mammals. In human XCI, and presumably in other non-rodent species, a higher number of genes escape silencing, indicating that these species are probably more tolerant to some level of expression from the Xi, or that the nature of the escapees provides specific advantages in particular tissues or situations. An important issue to address in this context is the influence of the local genome environment and whether this could account for the differences in escape between mouse and human. Interestingly, the conclusion that emerged from this meeting was that rodents seem to be the outliers to some extent, as they show much more stringent monoallelic regulation of XCI initiation and also much more robust XCI, i.e. less escape. Indeed, the question of whether rodents might actually be more highly evolved, at least in terms of their control of XCI (or whether they are some kind of alien, as Jenny Graves joked!), was raised by several speakers.

Conclusion

In summary, this meeting illustrated how much the XCI field has moved on since its initial discovery 50 years ago, and highlighted the complexity of dosage compensation strategies in mammals. This includes, on the one hand, the many different ways of achieving XCI, and, on the other, the multiple examples of escape from inactivation, as well as the existence of more globally conserved strategies for X upregulation in order to deal with the dosage differences between the X chromosome and autosomes. The workshop provided a number of exciting surprises, including the fact that XCI is clearly not a single, highly conserved phenomenon, and that a plethora of strategies are used in different mammals to achieve the same endpoint: dosage compensation. A further surprise was the breaking of the dogma that XCI is purely an early developmental process, thanks to studies demonstrating that *Xist* can play an initiating role outside of its early time window, both later in development and in certain adult cell types. Finally, the randomness of XCI is also being actively revisited, with new studies on XCI initiation and the *Xce* locus, as well as mounting evidence that non-random, parent-of-origin XCI can be found in certain somatic tissues. The results and discussions presented at this fiftieth anniversary meeting certainly seem to be paving the way for future decades of exciting XCI research!

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Competing interests statement

The authors declare no competing financial interests.

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