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New twists in X-chromosome inactivation

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Abstract

Dosage compensation, the mechanism by which organisms equalize the relative gene expression of dimorphic sex chromosomes, requires action of a diverse range of epigenetic mechanisms. The mammalian form, 'named X Chromosome Inactivation' (XCI), involves silencing of one X chromosome in the female cell and regulation by genes that make noncoding RNA (ncRNA). With large-scale genomic and transcriptome studies pointing to a crucial role for noncoding elements in organizing the epigenome, XCI emerges as a major paradigm and a focus of active research worldwide. With more surprising twists, recent advances point to the significance of RNA-directed chromatin change, chromosomal *trans*-interactions, nuclear organization, and evolutionary change. These findings have impacted our understanding of general gene regulation and are discussed herein.

INTRODUCTION

In mammals, X chromosome inactivation (XCI) achieves transcriptional silencing of one X chromosome in the XX female cell, thereby equalizing gene dosage to the XY male [1]. This epigenetic phenomenon involves non-coding RNAs (ncRNAs), antisense transcription, histone modifications, and DNA methylation in order to distinguish two genetically identical X chromosomes as active and silent entities within the same nucleus. Because of the numerous interesting epigenetic mechanisms involved, XCI remains an active field of research spanning over half a century. Two forms of XCI have been described: In extra-embryonic mouse tissues and in all tissues of marsupial mammals, XCI is imprinted and the paternal X chromosome is preferentially inactivated (reviewed in [2,3]. In the embryo proper of eutherian mammals, the paternal imprint is supposedly erased and XCI recurs in a random manner. Once silenced, epigenetic mechanisms ensure the maintenance of silencing in future cell divisions. Random XCI is achieved in three genetically separable events. First, the X-chromosome-to-autosome ratio is counted to ensure that one X chromosome is inactivated per female diploid nucleus. Next, one X chromosome is "chosen" in a mutually exclusive fashion to be the future inactive X (Xi). Lastly, silencing is initiated by coating of the future Xi by the noncoding Xist RNA, recruitment of silencing factors, and condensation of the X-chromatin.

In mammals, a region on the X chromosome named the 'X-inactivation center' (*Xic*) regulates the different steps of XCI. The *Xic* is comprised of several genetic elements that make long ncRNAs, including *Xist*, *Tsix*, *Xite*, *DXPas34*, and *Jpx/Enox* [4]. *Xist* encodes a 17kb long, nuclear RNA expressed exclusively from the Xi [5]. *Xist* is essential for the silencing step of XCI, as a chromosome deleted for *Xist* cannot be silenced [6,7]. *Xist* RNA physically associates

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with the X-chromatin and nuclear matrix around the X and coats the Xi [8,9]. In one of the first examples of antisense regulation in mammals, *Xist* is negatively regulated by its antisense gene partner, *Tsix*. On the future Xi, loss of *Tsix* permits upregulation of *Xist* and silencing of the *cis* chromosome. In parallel on the future active X (Xa), persistence of *Tsix* expression prevents upregulation of *Xist* and thereby prevents silencing on that chromosome. *Tsix* is in turn regulated by *Xite*, a gene located ~10 kb upstream of *Tsix* and bears a *Tsix*-specific enhancer.

Here we highlight advances in the field of XCI over the past two years. Greatest advances have been made in the areas of antisense regulation, chromosome-wide changes to chromatin, nuclear organization, and evolutionary progression. Due to space limitations, we regret that other important works cannot be discussed. For a summary of earlier works, we refer the reader to a number of existing reviews [4,10–14].

TSIX-MEDIATED REPRESSION OF XIST

Large-scale genomic analyses in multiple mammalian species have confirmed that disparity between their large genome sizes and their relatively small number of coding genes [15]. Surprising findings by the Fantom 3 project established that 62% of the mouse genome is transcribed [16]. Of 181,000 unique transcripts, half are ncRNAs. In addition, 70% of transcripts have a component that is antisense to another transcript. Such studies suggest that noncoding elements – including antisense transcripts – may play a major role in regulating the epigenome and speak to the importance of learning more.

Perhaps one of the best studied cases of mammalian antisense regulation is *Tsix*, a ~40-kb gene complementary to *Xist*. Due to the sense-antisense nature of *Xist* and *Tsix*, models describing potential mechanisms of *Tsix*-mediated repression of *Xist* invoke either transcriptional or post-transcriptional mechanisms [17]. Genetic evidence points to a mechanism involving either antisense transcription through *Xist* or overlapping sense-antisense RNA [18,19]. Earlier evidence suggested that *Xist* RNA stabilization leads to initiation of XCI on the future Xi [20,21]. However, several recent papers argue against a role for *Xist* stabilization and instead support a model of transcriptional regulation. Specifically, it was shown that *Tsix* transcription through *Xist* modulates *Xist* chromatin structure and determines whether *Xist* will be activated or repressed [22–24].

Two studies found that, in post-XCI cells and differentiating male ES cells, truncations of *Tsix* lead to an open chromatin structure within *Xist*, allowing for upregulation of *Xist* expression [22,23]. These changes include increased H3-K4 dimethylation, increased RNA Pol II binding, decreased histone H3-K27 methylation, and decreased DNA methylation at the *Xist* promoter, suggesting that *Xist* is upregulated when *Tsix* is downregulated with consequent conversion of the *Xist/Tsix* locus to a more euchromatic configuration. However, a third study directly investigated *Xist* RNA levels and transcription in differentiating female ES cells and came to slightly different conclusions [24]. This study found that *Xist* RNA is indeed regulated by promoter upregulation and not by RNA stabilization, but it also proposed that a transient heterochromatic state in *Xist* preempts *Xist* upregulation. Histone H3-K27 trimethylation, a known heterochromatic mark, was found specifically at the *Xist* promoter of the future Xi, as revealed by allele-specific analysis of a *Tsix*^{+/-} female mutant. On the future Xa, persistent *Tsix* transcription through the *Xist* promoter creates a euchromatic state that correlates with *Xist* repression. Upon silencing of *Tsix* on the future Xi, the *Xist* promoter adopts a more heterochromatic signature, which paradoxically correlates with *Xist* activation. Regardless of the differences among the three studies (which are likely due to differences in experimental systems), all three clearly point to the importance of *Tsix*-directed chromatin change as a key

regulator of *Xist* transcription. All agree that chromatin change occurs co-transcriptionally with *Tsix* expression.

Additional evidence supports the fundamental role of *Tsix* transcription through *Xist*. By eliminating all splicing products of mature *Tsix*, a recent work demonstrated that processed *Tsix* transcripts are dispensable for proper *Xist* repression [25]. Indeed, processed *Tsix* transcripts account for only a fraction of total *Tsix* RNA [26]. Thus, *Tsix* seems more likely to act as a full-length unspliced RNA. *Tsix* RNA has also been implicated in the recruitment of Dnmt3a, a *de novo* DNA methyltransferase, to the *Xist* promoter for stable silencing of the allele on the future Xa [24]. Consistent with this idea, Dnmt3a-deficient embryos have dysregulated *Xist* [27]. *Tsix*-dependent recruitment of Dnmt3a is likely involved in maintaining *Xist* silencing, but does not appear to be involved in the initiation of its silencing. Initiation seems instead to be controlled by changes in histone modifications [22–24].

X-CHROMOSOME CROSSTALK

Because the two X chromosomes in the female must adopt mutually exclusive fates of Xa and Xi, a mechanism of crosstalk must be utilized (in principle) to ensure that no cell inappropriately inactivates both or neither X [28]. Recent reports have shed light onto this mechanism and suggest involvement of physical interactions *in trans* [29,30]. Strikingly, the *Xic* region of the two X chromosomes appear to touch or ‘pair’ just prior to the onset of XCI. The pairing model proposes that the two X chromosomes are epigenetically equivalent before the onset of XCI. Pairing then enables the asymmetric localization of factors upon separation of the Xs. Transgene analysis implicates the noncoding genes, *Tsix* and *Xite*, as mediators of pairing. Deletions of *Tsix* and *Xite* results in either loss or severe delay of pairing with consequences for counting and the pattern of XCI in female cells [31].

More recent work has provided a glimpse of mechanism. Apparently, relatively small and diverse 1–2kb DNA elements which lie within *Tsix* and *Xite* are sufficient to establish ectopic pairing when they are placed onto autosomes [32]. Even sequences of very low complexity, such as the 34mer repeat of *DXPas34*, contain sufficient information to instruct pairing. Interestingly, common to these sequences are binding sites for Ctcf, the zinc finger transcription factor and chromatin insulator. By transient Ctcf RNAi knockdown assays, CTCF was found to be essential for X chromosome pairing. Furthermore, pairing requires new transcription, as inhibition of pol-II transcription by actinomycin D and α -amanitin blocks the formation of new pairs without apparently affecting the stability of already established pairs. The requirement for new transcription suggests that RNA might also be involved, especially given that the essential genes, *Tsix* and *Xite*, make ncRNA.

NUCLEAR ORGANIZATION AND COMPARTMENTS

In 1949, Barr and Bertram described a unique nuclear structure present only in XX female cat neurons [33]. Although they did not associate this structure with an Xi (that realization would only come later with the identification of XCI by Mary Lyon [1]), they noted that the structure frequently resided near the nucleolus. Nearly 60 years later, the significance of this association became clearer with the discovery that the Xi visits a perinucleolar compartment during S phase when it undergoes DNA replication [34]. This compartment is enriched for Snf2h, a component of the ISWI ATPase chromatin remodeling complex. Perinucleolar localization was furthermore shown to depend on *Xist*. Deleting *Xist* on the Xi results in loss of nucleolar association and reactivation of some previously silenced genes. Perinucleolar localization to a compartment rich in silencing factors provides a means of replicating not only the DNA but also the associated epigenetic factors for the maintenance of heterochromatin. This temporal and spatial separation of chromatin replication may explain how euchromatin and heterochromatin can be maintained in distinct states despite residing in the same nucleus.

The role of nuclear organization in regulating XCI has been further demonstrated by two other recent studies [35,36]. By detailed cytologic analysis, the studies showed that the core of the 'Barr body' consists of silenced nongenic sequences involving centromeric and other repetitive DNA elements – the so-called 'Cot1' fraction of the genome. Genic elements that are silenced by XCI move into the core region, while genes that escape apparently lie outside of or at the edge of the Xi core. Surprisingly, while genic silencing requires an intact *Xist* allele -- notably the Repeat A element [37], nongenic silencing can occur even in its absence. Without Repeat A, nongenic regions are silenced, but genic regions are not. It was shown that Repeat A is required to relocate genic sequences into the repressive nuclear compartment. Thus, XCI can be separated into genic and nongenic silencing. The significance of nongenic silencing is currently not known.

CHROMATIN MODIFYING PROTEINS FOR XCI

What *Xist* RNA recruits to the X chromosome has been an active field of research since *Xist* was first discovered 17 years ago. No interacting factors have been published to date. In the search for silencing proteins, BRCA1 has emerged as a major point of debate. BRCA1 is known to function in DNA damage pathways as a ubiquitin ligase and play a role in meiotic sex chromosome inactivation [38]. BRCA1 mutations predispose carriers to hereditary breast and ovarian cancer. Interestingly, many breast cancers associated with BRCA1 deficiency no longer display an obvious Barr body, suggesting that the second X has either been lost or reactivated. Following up on this longstanding observation, a report from several years ago proposed that BRCA1 protein is responsible for localizing *Xist* RNA to the Xi and implied the high frequency of Barr body loss in breast cancers may be attributed to loss of BRCA1 [39]. The study reported co-localization of *Xist* RNA and BRCA1 on the Xi, absence of *Xist* RNA on the Xi in BRCA1-deficient tumor cell lines, and reactivation of the Xi when BRCA1 is knocked down by RNAi in normal cells. In tumor cells, *Xist* RNA can apparently be relocated to the Xi when BRCA1 is restored. These findings provided a first candidate transfactor involved in *Xist* localization and suggested a link between XCI and cancer.

However, more recent reports have failed to reproduce the original connection between *Xist* RNA and BRCA1 in the same cell lines described previously [40,41]. The authors found that *Xist* RNA and BRCA1 do not colocalize and that BRCA1 RNAi does not disrupt *Xist* coating of the Xi. Similarly, X-linked gene expression and XCI were not significantly disrupted in somatic cells and mammary tumors in BRCA1-deficient mice. In response, the original authors have in fact provided additional evidence to support the original claims [42]. For example, conditionally deleting BRCA1 in mice was shown to decrease *Xist* RNA staining of the Xi. These disparate sets of data are clearly hard to reconcile with each other and will require additional work to resolve the differences.

Other groups have focused on the polycomb group proteins as regulators of XCI. *Xist* RNA upregulation is quickly followed by a number of chromatin changes, including the recruitment of Polycomb Repressive Complexes 1 (PRC1) and 2 (PRC2) to the Xi (reviewed in [11,14]). At other loci, these complexes are known to catalyze histone H2A-K119 ubiquitination and histone H3-K27 methylation and to be essential for the gene repression. For this reason, several labs have focused on characterizing PRC1 and PRC2 components genetically. The results suggest that polycomb function in XCI is complex and may be partially redundant. Surprisingly, when individually knocked out, *Ring1b* (a component of PRC1) was reported to not be required for random XCI [43,44], although *Ring1b* deletion clearly resulted in a lack of H2A-K119 ubiquitination and derepression of lineage-specific genes in ES cells.

Analysis of PRC2 knockouts revealed equally perplexing results [43,45,46]. Because H3K27 tri-methylation marks the Xi, XCI was examined in embryos lacking one of the subunits of

PRC2, Eed. Eed is believed to be a canonical component of PRC2, though Eed itself is not the H3-K27 methylase (Ezh2 is the catalytic subunit). Unexpectedly, when using an X-linked GFP reporter line, one report indicated that Eed is not required for establishment of random XCI [45]. Similar results were obtained by a second report using other gene expression assays [43]. Furthermore, it was reported using the X-linked GFP reporter that, while Eed is not required for the establishment of imprinted XCI in extraembryonic tissues of the mouse [47], the protein is needed to maintain silencing of the paternal X [46].

These surprising findings implied that Eed protein – though not necessarily PRC2 -- is not required for random XCI but is required for maintenance of imprinted XCI. They also suggest that Ring1b – and again not necessarily PRC1 as a whole – may be dispensable for random XCI. One possibility is that PRC2 and PRC1 perform redundant functions during XCI and a phenotype may not be evident unless both complexes are knocked out [43]. For the Eed knockouts, the somewhat contradictory results may in part be explained by use of different assays and an X-linked GFP transgene which may not represent the activity status of the rest of the Xi. Also, maternal pools of Eed may still present in the perimplantation embryo to establish imprinted and perhaps also random XCI. More surprises will undoubtedly surface in this system.

XCI EVOLUTION

While XCI is largely studied in mouse and human systems, the significance of XCI in the marsupial has not been overlooked. Marsupials undergo imprinted X inactivation, likely reveal an ancestral form of XCI, and should therefore help elucidate the evolutionary history of dosage compensation in mammals. Recent work comparing the genomes of vertebrate species suggests that the *Xic* region shows broken synteny in marsupials [48–50] and that the *Xist/Tsix* genes evolved in eutherians from a protein coding gene [51]. *Xist/Tsix* is unique to eutherians and may have been fundamental in the evolution of random XCI. Because of the lack of *Xist* in marsupials, it has been postulated that marsupial and eutherian XCI evolved independently of each other [51,52]. However, it is also known that the eutherian form of imprinted XCI in the extraembryonic tissues depends on *Xist/Tsix*. Thus, it seems as plausible that random and imprinted XCI would be mechanistically related and that random XCI represents an evolutionary progression from imprinted XCI [2,3,12]. It is possible that an *Xist*-independent form of XCI evolved first in the early mammal (200–300 million years ago), that this form of imprinted XCI persisted in the early eutherian, and that it eventually gave way to random XCI with the appearance of the *Xic*.

Many have speculated about the role of meiotic sex chromosome inactivation (MSCI) in both the mechanism and evolution of imprinted XCI [2,3,12] [53,54]. During the pachytene stage of meiosis I, both marsupials and eutherians both the X and Y chromosomes [55,56]. Very recent work showed unexpectedly that the sex chromosomes only partially reactivate after meiosis is completed [56–59]. During the postmeiotic stages, ~85% of X-linked genes remain transcriptionally suppressed. Termed ‘postmeiotic sex chromatin’ (PMSC), the X and Y heterochromatin is enriched in HP1 α , HP1 β , and histone H3-K9 methylation in the marsupial and mouse [55–57]. MSCI is thought to descend from MSUD (meiotic silencing by unpaired DNA)[60,61] and apparently does not require *Xist* [62,63]. These findings are consistent with the hypothesis that inheritance of a pre-inactivated spermatogenic X chromosome may provide the *Xist*-independent mechanism for imprinted XCI in marsupials and that aspects of this form of silencing may be retained in eutherian mammals, accounting in particular for imprinted paternal XCI in the early embryo [2,3,12,53,56][64]. Further work is required to establish both the potential evolutionary and mechanistic links.

FUTURE QUESTIONS IN XCI

While substantial progress has been made in our understanding of XCI in recent years, there is clearly still much that remain unanswered. Some hints of what will come derive from some of the most recent developments. For example, DNA methylation may play a role in both silencing of Xi and maintaining activation on Xa. Silenced chromatin regions are usually hypermethylated, while active regions are undermethylated [65]. However, allele-specific DNA methylation analysis of human Xi revealed surprisingly that the Xa has twice the methylation as the Xi, with hypermethylation occurring on Xa gene bodies and hypomethylation occurring at CpG islands [66]. Another study uncovered a pattern of distinct X-chromosome states even in pre-XCI cells in a manner that predicts which X will become Xi, implying that choice may occur earlier than prevailing models postulate [67]. Furthermore, it has also been reported that a region of ~300 kb upstream of *Xist* is involved in pairing and that it initiates pairing even in the pre-XCI state, 1–4 days earlier than previously proposed [68]. Finally, several reports now sport the idea that dosage compensation in the mammal may involve both X-silencing on Xi and X-upregulation on Xa, suggesting yet another layer of regulation [69,70]. The next few years will for sure uncover more intrigue.

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