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Dynamic interplay and function of multiple noncoding genes governing X chromosome inactivation

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Abstract

There is increasing evidence for the emergence of long noncoding RNAs (IncRNAs) as important components, especially in the regulation of gene expression. In the event of X chromosome inactivation, robust epigenetic marks are established in a long noncoding Xist RNA-dependent manner, giving rise to a distinct epigenetic landscape on the inactive X chromosome (Xi). The X inactivation center (*Xic* is essential for induction of X chromosome inactivation and harbors two topologically associated domains (TADs) to regulate monoallelic *Xist* expression: one at the noncoding *Xist* gene and its upstream region, and the other at the antisense *Tsix* and its upstream region. The monoallelic expression of *Xist* is tightly regulated by these two functionally distinct TADs as well as their constituting IncRNAs and proteins. In this review, we summarize recent updates in our knowledge of IncRNAs found at the *Xic* and discuss their overall mechanisms of action. We also discuss our current understanding of the molecular mechanism behind Xist RNA-mediated induction of the repressive epigenetic landscape at the Xi.

Introduction

The XX/XY sex-determination system is the most common designation in mammals whereby SRY on the Y-chromosome mainly acts to determine sex and activates a cascade of genetic pathways to develop testis in mice and humans. In this system, females have two X chromosomes in somatic cells, while males have one X and one Y chromosome. Since more than 1,300 genes reside on the X chromosome, there is a potential risk for females to possess a twofold abundance of X-linked genes, which is harmful for cellular viability and embryonic development. To overcome this problem, the mammalian females have developed a unique dosage compensation system called X chromosome inactivation (XCI) to balance the X-linked gene dosage between females and males [1]. As a result of XCI, which is induced at an early stage of embryonic development, one of the two X chromosomes becomes inactive (Xi), meaning the majority of genes on the Xi are

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transcriptionally silenced. About 15% of X-linked genes on the Xi in humans and 3–13% of X-linked genes in mice escape XCI; dubbed escape genes, these may have female-specific roles during development [2–4]. Epigenetic marks for active transcription, such as trimethylation of histone H3 lysine-4 (H3K4me3), are known to be enriched at escape loci on the Xi, indicating the establishment of transcriptionally active compartments within an otherwise transcriptionally inactive environment [5].

Although XCI occurs in early developmental stages, the exact process varies across species [6]. XCI is triggered by overexpression of Xist lncRNA from the Xi, followed by Xist RNA coating and chromatin modifications across the Xi. In mice, XCI can be divided into two distinct forms: imprinted and random XCI [7]. *Xist*, a master regulator of XCI, is transcribed from the paternal X-chromosome (Xp) at low levels as early as the two-cell embryonic stage. Timing of X-linked gene silencing on the Xp is diverse in each X-linked gene and the gene silencing is induced from the 4-cell stage to the post-blastocyst stage, which is referred to as imprinted XCI [8–11]. During the peri-implantation stage, cells in the trophectoderm and primitive endoderm, which give rise to extraembryonic tissue such as the placenta, sustain imprinted XCI; meanwhile, the epiblast lineage cells at the inner cell mass (ICM) erase imprinted XCI and reactivate X-linked gene expression [8,12]. *Tsix*, an antagonist and antisense long noncoding gene to *Xist*, and the germline factor PRDM14 cooperatively play a critical role in X-chromosome reactivation at the ICM in the blastocyst [13]. X-chromosome reactivation is then followed by random XCI, wherein either the Xp or Xm is randomly inactivated in the epiblast lineage around gastrulation [14]. Once random XCI is completed, the Xi is inherited to the next generation of cells. Mouse embryonic stem (ES) cells derived from the ICM of the blastocyst provides us an ideal system to study random XCI. Using an *ex vivo* embryoid body differentiation method, we can recapitulate random XCI during early embryo development [15]. The random nature of XCI in somatic cells results in mosaicism of cells, which contributes to its physiological diversity and is usually beneficial for the survival of females. However, defective XCI causes developmental anomalies and embryonic lethality during embryogenesis and diseases such as cancer [16,17]. Proper XCI regulation is hence indispensable to mammalian females.

In this review, we aim to cover recent findings regarding the operation of XCI, with a special focus on describing the molecular mechanism underlying: (1) induction of monoallelic *Xist* expression through the cooperation of multiple lncRNAs at the X-inactivation center and (2) Xist RNA-induced recruitment of repressive factors along the Xi. An elucidation of the molecular mechanism driving XCI would provide valuable insight towards understanding lncRNA-mediated gene regulation, which is critical for various biological processes.

2. Molecular mechanism to induce monoallelic *Xist* upregulation - Dynamic interplay of multiple long noncoding genes within *Xic*

To date, numerous lncRNAs are known to be expressed all over our genome and an increasing number of reports have indicated that lncRNAs regulate a wide variety of biological processes [18–21]. Xist RNA is one of the extensively studied representations of a regulatory lncRNA, which triggers dynamic alteration of the epigenetic landscape on the

future Xi. The *Xist* gene is located within the X-inactivation center (*Xic*), a genetic locus which has been mapped within XqD and Xq13 on the X chromosome in mice and humans, respectively, through a series of cytological experiments using X-autosome translocation [22–24]. Prior studies have determined *Xist* is exclusively expressed from the Xi and is required for induction of XCI [25–28]. Furthermore, a series of transgene experiments delineated the *Xic* region [29–31]. When a mouse 450-kb multicopy DNA fragment containing *Xist* was introduced into autosomes in male ES cells, the autosomes containing the fragment underwent inactivation similarly to the Xi in female cells. This suggests that the 450-kb fragment can fulfill the functions of the *Xic* [29]. Since a single copy of a YAC transgene containing *Xist* in mice could not lead to random inactivation [32], copy number of transgenes could be a critical factor for transgenic *Xic* function. Interestingly, the same single copy transgene inherited paternally induced autosome inactivation associated with H3K27me3 accumulation and late replication, which were observed in the inactivated Xp in the imprinted XCI [33], suggesting different factors might be required for inducing *Xist* expression in imprinted and random XCI. To date, the minimal *Xic* region required for induction of inactivation has been reduced to ~100 kb including *Xist* [34]. Several lncRNAs within the *Xic* have been identified (*Tsix, Xite, DxPas34, Tsx, Jpx/Enox, Ftx* and *RepA*) which act cooperatively together for the induction of monoallelic *Xist* expression from the future Xi in order to initiate XCI [35,36]. Furthermore, various approaches surveying a neighboring region of the *Xic* have identified several additional factors, such as lncRNA Linx, as potential transcriptional regulators of *Tsix*, in addition to the E3 ubiquitin ligase Rnf12 as an activator of *Xist* [37,38]. These findings indicate that various RNA and protein factors are involved in coordinating the regulation of *Xist* mono-allelic expression from the Xi.

2.1. Xist lncRNA

Xist plays crucial roles in both imprinted and random XCI [16,28]. *Xist* resides at the central region of the *Xic* locus on the Xi (Fig. 1) and is expressed from promoter P1 and P2 in a differentiation-specific manner [25–27,39,40]. Nascent Xist RNA is processed to −17 kb-length RNA by splicing, and different polyadenylation sites and alternative splicing patterns contribute to the generation of multiple isoforms of Xist RNA [41–43]. Interestingly, some isoforms of Xist RNA is produced in a differentiation- or sex-specific manner [43,44].

Xist lncRNA initiates XCI in *cis* by covering the entire Xi and recruiting multiple chromatin modifying enzymes for repressive epigenetic modification, including polycomb repressive complex 2 (PRC2) for histone H3 trimethylation at lysine 27 (H3K27me3), during embryogenesis [45–47]. Exclusion of RNA polymerase II occurs during the first wave of transcriptional alteration as induced by Xist RNA, which is followed by the loss of active histone markers like histone H3K4 methylation and histone H3 acetylation [48]. Then, repressive epigenetic modifications such as H3K27me3 and H2Aub which are catalyzed by PRC2 and PRC1, respectively, are deposited onto the Xi [48–50]. Consistent with the Xist RNA-dependent recruitment of PRC2 to the Xi, deposition of H3K27me3 and PRC2 is well-correlated with Xist RNA recruitment onto the Xi during X-inactivation: a limited number of binding loci were seen to frequently overlap with CpG islands in gene-rich regions at the initiation of XCI, along with a robust number of binding loci at the intergenic regions and

chromosome-wide spreading as differentiation progressed [5,51]. Thereafter, further repressive epigenetic modifications such as histone macro H2A and DNA methylation are incorporated into the entire Xi in an Xist RNA-dependent manner and are maintained through subsequent generations for stable X-linked gene silencing [52,53].

2.2. Functionally distinct topologically associating domains (TADs) which regulate monoallelic *Xist* expression

Xic can be divided into two topologically associating domains (TADs) or active chromatin hubs (ACHs) which could facilitate the interplay of long noncoding genes within the same TAD to activate or repress *Xist* [37,54]. Interestingly, each TAD is functionally distinct in *Xist* expression. The Tsix TAD is located downstream of *Xist* and contains long noncoding genes and IncRNA-associated elements (*Tsix, Dxpas34, Xite*, Tsx and *Linx*) and primarily plays a role in upregulating *Tsix* expression, which in turn downregulates *Xist* expression. The Xist TAD contains *Xist* itself and its upstream region including long noncoding genes, *Jpx* and *Ftx*, and the protein-coding *Rnf12* gene encoding an ubiquitin ligase, all of which eventually promote *Xist* upregulation at the onset of random XCI [55–57]. These two TADs are separated by a boundary region, which resides around the *Xist/Tsix* region [37,58]. Interestingly, functionally relevant genes and elements for *Xist* regulation reside within the same TAD in close proximity to each other and exhibit similar expression profiles. Thus, genes that facilitate coordinated expression and cooperative action in *Xist* regulation are clustered within the Xist and Tsix TADs. Disruption of the boundary between Xist and Tsix TADs by *Xist-Tsix* deletion induces ectopic contacts between sequences in the Xist and Tsix TADs, altered organization of the Tsix TAD, and long-range transcriptional misregulation of genes within the Xist TAD [37]. Furthermore, the deletion of boundary elements between the Xist and Tsix ACHs also leads to aberrant XCI in female mutant ES cells [58], suggesting an essential role of TAD/ACH in XCI to both separate functionally distinct chromosomal domains as well as to cooperatively induce *Xist* expression.

2.2.1. Tsix TAD antagonizes *Xist* expression though *Tsix* upregulation—As a central player in the Tsix TAD, the expression of *Tsix* is cooperatively promoted by the other Tsix TAD elements. *Tsix* is important as an antagonist of *Xist* expression in *cis* on the future active X chromosome (Xa) and is required in both imprinted and random XCI [59–61]. *Tsix* is transcribed to the antisense orientation of *Xist* from minor and major promoters [60,62]. Based on a study using ex vivo differentiation of mouse ES cells, *Xist* and *Tsix* exhibit an interesting expression pattern in random XCI which reflects *Tsix* function in *Xist* expression [62]. Prior to the onset of random XCI, both *Tsix* and to a lesser extent *Xist* are biallelically expressed from both X chromosomes. At the onset of random XCI, *Tsix* expression becomes monoallelic and designates the future Xa; consequently, *Xist* expression is upregulated on the future Xi and repressed on the future Xa. When the XCI is established, the expression of *Tsix* is repressed on both alleles. While random XCI is tightly linked with cellular differentiation, *Tsix* expression is regulated by pluripotent factors [63–65]. *Tsix* is known to exert its antagonistic function against *Xist* by promoting DNA methylation and histone modification at the promoter of *Xist* [66–69]. Since a *Tsix* deficient X chromosome does not undergo XCI in male embryonic lineages and ES cells, there is a *Tsix*-independent mechanism for the prevention of *Xist* upregulation [59,70]. Double mutation of *Eed*, a

component of PRC2, and *Tsix* in male ES cells leads to *Xist* hyperactivation upon differentiation: this does not occur in cells carrying either single mutation, suggesting that PRC2 is involved in the repression of *Xist* [71]. Although *Tsix* was believed to be involved in choosing the Xi upon initiation of random XCI [59,66–68], a recent report proposes that *Tsix* is only involved in the maintenance of random XCI to prevent *Xist* induction from the Xa, not both the "counting" and "choice" steps at the onset of X-inactivation [72]. The observation of bi-allelic Xist RNA clouds in a subset of *Tsix* mutant female ES cells and epiblast cells at the onset of random XCI prompted the authors to propose that non-random XCI in *Tsix* heterozygous mutant female cells is due to secondary cell selection. That is, cells exhibiting bi-allelic Xist RNA clouds choose the wild-type X-chromosome to become the Xi, while the *Tsix*-mutant X becomes the Xa. However, this *Tsix*-mutant Xa eventually reverts to an inactive state due to a failure in the repression of *Xist* induction. To support this model, the authors found that *Tsix* heterozygous mutant epiblast stem cells (EpiSCs), which represent an early phase of random XCI, exhibited a wide-range of skewed X-chromosome choices in each undifferentiated EpiSC clones, which became almost exclusively skewed in the favoring of *Tsix* mutant X-chromosomes as the Xi in a majority of EpiSC clones upon differentiation. Their findings stand in contrast to previous works which found *Xist* expression to be partially upregulated and completely skewed in favor of the *Tsix* mutant X-chromosome in *Tsix* heterozygous mutant undifferentiated ES cells [59,73–76]. Due to a lack of Xist RNA cloud formation in *Tsix* mutant undifferentiated ES cells, it is possible that further *Xist* induction and Xist RNA cloud formation would be dependent on cell differentiation. On the other hand, it appears as if the initial *Xist* induction and Xi/Xa choice can be completed within *Tsix* heterozygous mutant female ES cells without additional support. Further cautious examination would be necessary to clarify whether *Tsix* is required for the choice between Xi and Xa or maintenance of the Xa.

In imprinted XCI, the disruption of maternal *Tsix* results in ectopic *Xist* expression from the maternal X chromosome (Xm) in the extraembryonic tissues and early embryonic lethality in females [60,61], attributed mainly to the inactivation of both Xp and Xm. These data suggest a crucial role for *Tsix* in the maintenance of *Xist* repression in *cis* on the Xm in extraembryonic tissues. Since *Tsix* expression cannot be detected until the blastocyst stage, and *Xist* is monoallelically expressed from the Xp even in the female embryo carrying a maternal *Tsix* mutation, it can be inferred that *Tsix* expression is not required for initial monoallelic *Xist* expression at the early stage of imprinted XCI. Thereafter, *Tsix* is critical for the prevention of *Xist* expression from the Xm as trophectodermal progenitor cells differentiate [77].

Two enhancer-like elements preserve *Tsix* expression and repress *Xist* on the future Xa at the onset of random XCI [73,78,79]. One of the enhancer elements for *Tsix, Xite* (X-inactivation intergenic transcription elements), is located between the major and minor *Tsix* promoters and lies 20–32 kb downstream of *Xist. Xite* mutation results in a significant downregulation of *Tsix* coupled with *Xist* upregulation on the *Xite* mutant X chromosome upon differentiation, leading to a skewed XCI which favors the mutant X chromosome as the Xi. A 1.2-kb core enhancer element embedded within the *Xite* is associated with differentiation-specific bidirectional low-level transcription and DNaseI hypersensitive sites,

both of which are characteristic features of an enhancer. During ES cells differentiation, the expression pattern of *Xite* is similar to that of *Tsix* but at a lower level: *Xite* expression is highest in undifferentiated cells and downregulated upon differentiation. The other enhancer element for *Tsix* is *DXPas34*, which resides <1-kb downstream of the *Tsix* major promoter and comprises a 34 mer tandem repeat and flanking sequence [73,79]. *DXPas34* as well as *Xite* promotes *Tsix* only in undifferentiated ES cells prior to XCI and in differentiating cells during the early stage of XCI; their enhancer activity gradually diminishes upon cellular differentiation. However, once the XCI is established, *DXPas34* is needed for proper repression of *Tsix* [73]. While each *Xite* and *DXPas34* enhancer unit of *Tsix* can act in a separate fashion, their combination leads to a greater stimulation of the Tsix promoter [79].

Other noncoding genes in the Tsix TAD are also implicated in *Tsix* repression [37,80]. *Tsx* (Testes specific X-linked gene) located 40 kb downstream to the 3' end of *Xist* was originally proposed as a coding gene, but further investigation indicated *Tsx* is a non-coding gene [80,81]. *Tsx* is comprised of 7 exons, and is transcribed from the opposite orientation of *Xist. Tsx* expression is found in many tissues with the highest expression level seen in testis. The expression of *Tsx* decreases upon differentiation similar to that of *Tsix* and *Xite*. Since the *Tsx* knockout mutant leads to repression of *Tsix* and abnormal accumulation of *Xist* RNA onto the Xa in both male and female ES cells during differentiation, *Tsx* is proposed to function in *Tsix* activation [80]. *Linx*, which resides within the Tsix TAD, has recently been identified as a long noncoding gene that may be a potential regulator for *Tsix* expression. Although its function in *Tsix* expression remains unknown, there is a positive correlation between *Linx* and *Tsix* expression reminiscent of the correlation between *Tsix* and *Xite* expression, indicating a possible similarity in function [37]. Further investigation will reveal the role of *Linx* in XCI, especially for *Tsix* and *Xist* regulation.

2.2.2. Xist TAD promotes *Xist* upregulation—Several long noncoding genes and one protein-coding gene have been identified as a positive regulator for Xist within the Xist TAD. RepA RNA, a 1.6kb-long IncRNA, is transcribed from the same DNA strand as *Xist* [82]. *RepA's* transcription start site is located within repeat A, which is located ~300 bp downstream of *Xist's* P1 promoter and contains 7.5 tandem repeats of a 28 nucleotide-long sequence which may fold into specific secondary structures [83,84]. RepA RNA is present in both male and female undifferentiated ES cells, but is only maintained in female cells after XCI [82]. RepA RNA knock-down in ES cells showed poor embryonic body differentiation with less *Xist* expression and H3K27me3 levels on the Xi compared with control cells. Furthermore, RepA RNA interacts with Ezh2, a component of the PRC2 complex, *in vivo* and *in vitro*. Thus, RepA RNA is proposed to function in upregulation of *Xist* expression by altering chromatin modification at the *Xist* promoter.

Jpx (also called *Enox* [*expressed neighbor of Xist*]) is located 10-kb upstream of *Xist* and is transcribed in the opposite direction of *Xist* [85,86]. *Jpx* is known as an escape gene in both random and imprinted XCI and *Jpx* expression increases in both male and female ES cells during *ex vivo* differentiation [55,86,87]. The *Jpx* heterozygous knockout results in inefficient *Xist* upregulation and leads to cell death in mutant female ES cells upon differentiation; thus, *Jpx* works as an activator of *Xist* [55]. Interestingly, transgenic

expression of *Jpx* rescues defects in *Jpx* heterozygous mutant cells while anti-Jpx siRNA destroys its ability to promote *Xist* expression; thus, Jpx RNA can promote *Xist* upregulation in *trans*. Since the *Jpx* heterozygous mutation shows mild *Xist* repression on the mutant X chromosome, *Jpx* might function in *cis in vivo*. Moreover, the XCI and differentiation defects observed in *Jpx* heterozygous mutant ES cells can be restored by disrupting *Tsix* function on the same X chromosome, indicating multiple long noncoding genes regulate *Xist* expression in females. Further studies found that the *Jpx* transgene released CTCF from the *Xist* promoter, which led to an upregulation of *Xist* expression, while transgenic CTCF expression reduced *Xist* expression [56]. Based on these findings, it has been proposed that Jpx RNA activates *Xist* by reducing CTCF from the *Xist* promoter at the onset of XCI.

In mice, the *Ftx* (five prime to *Xist*) gene is localized about 150-kb upstream of *Xist*, with the same transcription orientation as *Xist* [85]. The 5' region of *Ftx* and the microRNA cluster which resides in intron 12 of *Ftx* are conserved among species [88]. Similar to *Jpx*, *Ftx* is upregulated during *ex vivo* ES cell differentiation and is also considered as an escape gene [87,88]. In male ES cells, *Ftx* disruption induced gene repression in a wide-range of neighboring regions from *Cnbp2* to *Tsix* [88]. In this *Ftx* mutant male ES cells, the expression of *Xist* was also reduced whereas CpG methylation at the *Xist* promoter was increased. Thus, *Ftx* is proposed as a positive regulator of *Xist*. Since *Ftx* mutation also led to *Jpx* repression, *Ftx* function for *Xist* upregulation might be mediated by *Jpx*. However, *Ftx* might be dispensable for imprinted XCI [89]: *Ftx* mutant homozygous and heterozygous female embryos were both normal and *Ftx* knockout on the paternal X did not affect the silencing of paternal X-linked genes nor expression profiles of imprinted paternal X-linked genes, *Xist* and *Rnf12*. Since this work examined silencing of only a subset of X-linked genes, it is unclear whether the paternal *Ftx* mutation affects the chromosome-wide integrity of imprinted XCI in *Ftx* heterozygous mutant female mice. Like *trans*-action of Jpx RNA in *Xist* activation, *Ftx* on the maternal X might be able to induce *Xist* upregulation in *trans*. Alternatively, it is possible that *Ftx* has different functions in imprinted and random XCI. *Ftx* gene targeting and *ex vivo* differentiation experiments using female ES cells could delineate *Ftx* function in random XCI.

Xpr (X-pairing region) was originally identified as a region required for X-X pairing at the initiation of XCI [90]. Although X-X pairing mediated by *Xpr* is not essential for induction of XCI, *Xpr* cooperates with *Jpx, Ftx* and *Rnf12* to induce *Xist* upregulation upon differentiation [91]. Although *Xpr* overlaps with the protein-coding *Slc16a2* gene, also known as monocarboxylate transporter 8 (Mct8), it is unclear whether the SLC16A2 protein is involved in *Xist* expression. However, since *Slc16a2* is expressed in many tissues and plays a critical role in the development of the central nervous system as a transporter of the thyroid hormone [92,93], it is unlikely to directly control *Xist* expression and XCI. Future studies might lead to the discovery of novel critical elements for *Xist* expression such as an enhancer and an additional regulatory IncRNA within this region.

Protein-coding *Rnf12* is also included within Xist TAD and functions to promote *Xist* upregulation in both imprinted and random XCI [37,38,94]. *Rnf12* encodes an ubiquitin ligase and is the only functional protein-coding gene in *Xist* regulation within Xist and Tsix TADs. While the *Rnf12* transgene induces XCI in male ES cells and produces abnormal XCI

at both X chromosomes in female ES cells, Rnf 12 heterozygous knockout in female ES cells leads to significantly delayed XCI upon differentiation. These results suggest that Rnf12 has a critical role for dose-dependent activation of *Xist* induction [38]. *Rnf12* expression is upregulated upon differentiation to induce *Xist* upregulation at the onset of random XCI and then gradually reduced during the course of differentiation, indicating a differentiation-specific function of Rnf12 at the onset of random XCI. ChIP analysis showed the pluripotent factors Nanog, Sox2 and Oct4 bind together upstream of *Rnf12* and may play a role in the repression of *Rnf12* in undifferentiated ES cells, suggesting a further restriction of *Rnf12* function upon differentiation [95]. Although Rnf12 was originally proposed to activate *Xist* directly in random XCI [57], more recent studies have indicated that *Rnf12* regulates *Tsix* expression by ubiquitination-mediated proteasomal degradation of REX1, a putative transcription factor for *Tsix*, at the initiation of random XCI [65,91]. Although *Rnf12* is essential for Xist upregulation in imprinted XCI, knocking out *Rnf12* leads to only a mild impact on *Tsix* expression; thus, it is likely that *Tsix* repression by *Rnf12* is not involved in activation of *Xist* expression in the embryonic day 3.5–4.5 blastocyst [94]. Two prominent REX1 binding regions reside within *Xist/Tsix*. one exists between *Xist* promoter P1 and P2 while the other exists between *Tsix* promoter and DXPas34. Although it remains unknown how RNF12 directly functions on *Xist* expression, REX1 might act as a repressor at the *Xist* promoter in opposition to its putative function as a transcriptional activator for *Tsix* because REX1 binds upstream of the differentiation-specific *Xist* promoter P2. However, recent *in vivo* studies have provided some evidence to show that *Rnf12* is dispensable in random XCI [96]. Conditional knockout of *Rnf12* in female embryos post imprinted XCI but before random XCI did not result in any defects in the embryos nor reduced fertility. It is possible that a compensation mechanism induces XCI for normal embryonic development since Rnf12 heterozygous knockout female ES cells undergo delayed XCI but can be induced to random XCI upon differentiation [38].

2.2.3. Boundary element separates functionally distinct TAD—The boundary between Xist and Tsix TADs resides in the *Xist/Tsix* region. Deletion of *Xist-Xite*, including this putative boundary region led to increased contact between Xist and Tsix TADs, which is not observed in wildtype, and an altered organization of Tsix TAD, suggesting the boundary region is essential for partitioning and compartmentalization of two distinct TADs [37]. Interestingly, within this putative boundary region, the *RS14* element has been previously identified at the 3'-end of *Xist* [58]. *RS14* exhibits high cross-species conservation among eutherian mammals, implying the critical function of this element. *RS14* is associated with the DNaseI hypersensitive site and contains multiple binding sites of CTCF, a critical factor for genome architecture and gene expression. One of the CTCF binding sites within *RS14* has been shown to bind with CTCF *in vivo* and *in vitro* and functions as a chromatin insulator and boundary factor in reporter assay. The deletion of *RS14* failed to induce *Xist* upregulation and instead retained a high expression of *Tsix* from the mutant X in a female-specific manner; hence, *RS14* is presumed to be a boundary element to separate Xist and Tsix ACHs for proper *Xist* and *Tsix* expression. Further studies are required to reveal whether *RS14* is a primary functional boundary element which is necessary for the division of two functionally distinct Xist and Tsix TADs. A recent report using CLIP-seq (UV crosslinking and immunoprecipitation followed by deep sequencing) indicated that CTCF

binds thousands of RNAs including Xist, Tsix and Xite RNAs in mouse ES cells [97]. Knockdown of Tsix and Xite RNAs resulted in reduced occupancy of CTCF at known CTCF binding sites within the *Tsix* and *Xite* region, indicating the potential role of RNA in targeting CTCF. It has been shown that CTCF has multiple roles in XCI: transcriptional regulation of *Xist* [56], X-chromosome pairing through binding to the *Tsix* and *Xite* loci at the onset of random XCI [98–100], and service as a putative boundary factor between the Xist and Tsix TADs/ACHs [58]. Because CTCF binds to multiple sites across the *Xic*, it may also be a contributor to the organization of Xist and Tsix TADs for the coordination of precise *Xist* transcriptional regulation.

3. Mechanism of Xist RNA-mediated gene silencing on the Xi

As mentioned above, monoallelic upregulation of *Xist* expression triggers random XCI. It is not entirely clear how Xist RNA spreads and covers across the future Xi and recruits chromatin modifying enzymes to the Xi. The epigenetic landscape at the Xi undergoes a number of repressive epigenetic modifications such as H3K27me3 and H2AK119ub, brought about by the chromatin modifying enzymes PRC2 and PRC1, respectively. Within the *Xist* gene, six *xist*-specific repetitive elements (repeats A-F) that are conserved among eutherian mammals, have been identified (Fig. 2) [39,101–103]. These unique repeat elements make Xist RNA a hub for a variety of protein factors, which induce multiple layers of repressive modification on the Xi. Repeat A was identified as a crucial element for gene silencing using an inducible *Xist* transgene system [83], and has been the most intensively studied. In addition to induction of gene silencing, the repeat A region of Xist RNA is considered to be a multi-functional element which serves many important roles including: acting as a hub for several chromatin modifying enzymes [82,104], spreading Xist RNA across the Xi [105,106], repositioning active gene regions into the Xist silenced compartment [48], and splicing Xist RNA [107]. All repeat elements reside within exon 1, which is known to be the largest exon, except for repeat E which resides in the second largest exon, exon 6 in humans and exon 7 in mice. These conserved repeat domains interact with various proteins and confer a wide variety of function to Xist RNA, as described below.

3.1. Role of Xist repeat elements in Xist RNA targeting onto the Xi

Although the detailed molecular mechanism underlying c/s-limited action of Xist RNA remains unknown, a recent report proposes that the transcription factor YY1 (Yin Yang 1) anchors Xist RNA within the *Xist* gene locus to function as a nucleation center for chromosome-wide Xist RNA spreading and X-linked gene silencing in *cis* [108]. *In vivo* and *in vitro* data indicates that YY1 binds to Xist RNA through Xist RNA repeat C as well as DNA through YY1 binding sites that are proximal to *Xist* repeat F. Further evidence supports repeat C in Xist RNA playing an important role for Xist RNA localization on the Xi. When the function of Xist RNA repeat C is interfered with by peptide nucleic acids (PNAs) or locked nucleic acids (LNAs), which are complementary to the repeat C domain of Xist RNA, then Xist RNA is displaced from the Xi and a loss of PRC2 complex localization occurs [109,110]. YY1 and its binding sites proximal to repeat F are also shown to be essential for *Xist* upregulation upon differentiation, although previous studies have indicated that YY1 does not affect Xist expression but its localization [108,111]. In addition to YY1,

hnRNP U (also called Saf-A/SP120) is known to be a protein factor required for chromosome-wide Xist RNA localization [112]. Since hnRNP U has DNA- and RNA-binding domains at its N- and C-terminal, respectively, and both domains are essential for Xist RNA localization on the Xi, it has been proposed that hnRNP U anchors Xist RNA at the scaffold/matrix attachment region (S/MAR) on the Xi. Since hnRNP U localization on the Xi depends on Xist RNA, hnRNP U does not seem to simply serve as a bridge between MAR/SAR on the Xi. Cooperative action such as the formation of a ribonucleoprotein complex between hnRNP U and Xist RNA might be required for Xist RNA recruitment to the Xi. The interplay between YY1 and hnRNP U might facilitate the efficient localization and spreading of Xist RNA and X-linked gene silencing.

Recent attempts to delineate the spreading mechanism of Xist RNA and X-linked gene silencing across the Xi have revealed an unique orderly fashion of Xist RNA and PRC2 spreading upon differentiation [5,51,106]. Using ChlP-seq (chromatin immunoprecipitation with deep sequencing) of Ezh2, it has been revealed that PRC2 binds to a limited number of sites (about 1,500) frequently associated with H3K4me3/H3K27me3 bivalent domains and CpG islands, which are followed by additional binding sites (about 4,000) at intergenic regions and spreading across the Xi as differentiation progresses [5]. Furthermore, recent technical advancements have contributed to uncovering the detailed molecular mechanism of Xist RNA spreading. Using biotinylated antisense oligonucleotides to Xist RNA, CHART-seq (capture hybridization analysis of RNA targets with deep sequencing) and RAP (RNA antisense purification), a genome-wide chromatin region associated with Xist RNA has been isolated and analyzed [51,106]. Binding of PRC2 onto the Xi is correlated with Xist RNA recruitment and H3K27me3 deposition. Interestingly, before the discovery of *Xist*, the well-ordered spreading of XCI had been proposed as the ‘way-station model,’ wherein X-linked gene silencing happens at a number of way-stations across the X-chromosome and then spreads outward along adjacent regions [113]. Similar to this model, the quick and efficient propagation of Xist RNA across the Xi is driven by utilizing spatially proximal regions to the *Xic* and spreading towards distal regions which serve as way-stations to further gene silencing at the onset of XCI [51,106,114]. Consistent with the previous observation of inefficient Xist RNA spreading in the repeat A mutant *Xist* [105], RAP analysis using an inducible *Xist* transgene lacking repeat A showed a reduction in Xist RNA occupancy levels across the Xi, implicating the role of repeat A in the spreading of Xist RNA across the Xi [106]. Interestingly, the repeat A-mutant Xist RNA accumulation was observed on the edges of the active gene-dense. A previous study also showed that transcriptionally active gene regions on the Xi are looped out of the silencing compartment created by Xist RNA lacking repeat A [48]. These results indicate the role of repeat A in spreading Xist RNA to the active gene region for induction of gene silencing. Because repeat A is known as a hub of various chromatin modifying enzymes [82,104], chromatin modifications which are induced in an Xist RNA-dependent manner might be recognized by other Xist RNA-binding factors, thus cooperatively facilitating Xist RNA spreading across the Xi.

3.2. Xist RNA-mediated PRC2 recruitment to the Xi

Previous studies have shown that PRC2 is recruited to the Xi and deposits H3K27me3, one of the hallmarks of facultative heterochromatin, in an Xist RNA-dependent manner [46,47,105]. However, the molecular mechanism behind PRC2 recruitment to the Xi (target chromatin sites) by Xist RNA remains disputed. PRC2 binds to a variety of RNA, which raises the question of how it discriminates between bound and unbound RNA species without a distinct consensus binding sequence [115–119]. Recently, *in vivo* UV cross-linking and immunoprecipitation (CLIP) approaches revealed that PRC2 binds to the 5' region of nascent RNA from a number of genes [117]. Interestingly, the majority of Ezh2-bound nascent RNAs (ezRNAs) originate from transcriptionally active genes with moderate PRC2 binding but without H3K27me3. In the model proposed, PRC2 interacts with a majority of transcription start sites and surveys the transcription state by interacting with ezRNAs. Interaction with ezRNA inhibits the methyltransferase activity of PRC2. Once transcription is repressed, extinction of ezRNA promotes H3K27me3 deposition by PRC2, followed by stable maintenance of gene silencing. The function of ezRNA seems to be restricted at the promoter where ezRNA is transcribed. Since Xist RNA plays a role in repressing chromosome-wide X-linked genes, the molecular mechanism behind Xist RNA's recruitment of PRC2 to its target loci might be distinct from the mechanism driving ezRNAs. The PRC2 core complex consists of four subunits: SUZ12, EED, RBBP4/7, and catalytic subunit EZH2. Among the four core components of PRC2, EZH2 and SUZ12 exhibit RNA binding activity against Xist RNA *in vivo* and *in vitro* [82,84,115,116,120]. Repeat A in the 5' of Xist RNA is crucial for induction of X-linked gene silencing, and repeat A RNA (RepA RNA) containing two long stem-loop structures has been identified as a PRC2 binding motif [82,83]. Since induced mutant *Xist* expression lacking repeat A can still recruit PRC2 to the Xi, albeit only as a small Xist RNA cloud and weak H3K27me3 signal [105], repeat A is not the sole Xist RNA domain to recruit PRC2 to the Xi. Recent studies using *in vitro* RNA electrophoretic mobility shift assays (EMSAs) indicated that PRC2 exhibits promiscuous binding but has a higher binding affinity to RepA RNA [118,121,122]. The catalytic subunit of PRC2, EZH2, binds to RNA in a somewhat non-specific manner, but its combination with the other PRC2 components EED and SUZ12 enhances the specificity of EZH2 binding to target RNAs, including RepA RNA [121]. Interestingly, RNA binding to PRC2 inhibits the methyltransferase activity of PRC2, suggesting that RNA is involved in not only PRC2 recruitment to its targets but also regulation of histone methyltransferase activity [121,123].

Jarid2, a co-factor of the PRC2, is involved in recruiting PRC2 onto the Xi [121,124]. *Jarid2* is known to possess both RNA and nucleosome binding activity, regulate PRC2 recruitment to chromatin, and activate the enzymatic activity of PRC2 [123,125–129]. Since *Jarid2* depletion by shRNA knockdown resulted in impaired recruitment of PRC2 and H3K27me3 modification to the Xi, *Jarid2* plays an important role in PRC2 recruitment to the Xi in an Xist RNA-dependent manner [124]. The region corresponding to repeat F through B in Xist RNA is considered as minimum essential region for JARID2 and PRC2 accumulation to the Xi, although repeat A is also proposed as a JARID2 binding site [121,124]. As mentioned above, while Xist RNA is essential for PRC2 targeting to the Xi, PRC2-RNA interaction represses histone methyltransferase activity of PRC2 [121,123].

JARID2 promotes PRC2 histone methyltransferase activity by attenuating PRC2-RNA interaction and promoting PRC2 binding to chromatin [121,123,128], suggesting that JARID2 is a crucial player for PRC2 recruitment to the Xi and for the regulation of PRC2 enzymatic activation.

ATRX, known as a member of the SWI/SNF chromatin remodeler family, also binds to the repeat A motif and promotes localization of the PRC2 to the Xi [130]. Since ATRX knockdown in female mouse embryonic fibroblast (MEF) and *Xist*-inducible female ES cells resulted in severely impaired Ezh2 localization and H3K27me3 accumulation on the Xi, the role of ATRX in Xist RNA-dependent PRC2 recruitment to the Xi is proposed. *In vitro* experiments proved that ATRX has a high affinity for both RepA RNA and double strand DNA. Since inducible-Xist expression lacking repeat A abolished the localization of ATRX to the *Xist* locus, RepA RNA is essential for ATRX targeting to the Xi. Furthermore, in the presence of ATP, ATRX enhances the binding of PRC2 to RepA RNA; meanwhile, ATRX itself binds less well with RNA and DNA in the presence of ATP. On the basis of these findings, it is proposed that ATRX binds to RepA RNA and remodels its configuration, which in turn promotes PRC2 binding to RepA RNA. ATRX ChlP-seq data showed that the distribution of ATRX overlaps with that of EZH2, H3K27me3 and Xist RNA, suggesting the link between ATRX and Xist RNA-dependent PRC2 recruitment.

Collectively, PRC2 recruitment and spreading in XCI is a complex process regulated by multiple co-factors. Induction of *Xist* triggers downstream processes, including the concerted post-translational histone modifications and dynamic alteration of chromatin structure. It is interesting and should be delineated how these epigenetic modifications communicate and are coordinated to establish the highly coordinated heterochromatic landscape of the Xi.

3.3. SPEN/SHARP as a Novel Xist Repeat A binding Protein

Most recently three studies utilizing ChlRP-MS (Chromatin isolation of RNA purification-mass spectrometry), RAP-MS (RNA antisense purification-mass spectrometry) and iDRiP (identification of direct RNA interacting proteins), which are potential ways of harnessing the protein interactome associated with any RNA of interest, were able to comprehensively identify various potential Xist RNA binding proteins [104,131,132]. Two of them, hnRNP U and SPEN (mouse homolog of *Drosophila* Split ends)/SHARP (SMAT and HDAC associated repressor protein), were common in all three of the independent works. While hnRNP U was already known to bind directly with Xist RNA and have an essential role in Xist RNA localization on the Xi [112], SPEN/SHARP was novel and its critical function in XCI was unknown. SPEN/SHARP is a transcriptional repressor that recruits histone deacetylase (HDAC) complexes and contains RNA-recognition motifs [133,134]. Interestingly, SPEN/SHARP could not bind to Xist RNA lacking repeat A, suggesting specific binding of SPEN/SHARP to repeat A, a region which is essential for Xist RNA-mediated gene silencing [83,104]. Furthermore, knockdown of SPEN/SHARP did not affect Xist RNA localization but instead resulted in a failure to exclude RNA polymerase II from the Xi territory, poor recruitment of the PRC2 complex to the Xi, and compromised X-linked gene silencing. SPEN/SHARP is known to interact with various repressor complexes

such as the SMRT nuclear compressor complex that interacts with HDAC3 histone deacetylase and the MBD3-NuRD (Nucleosome Remodeling and Deacetylation) complex [133,135,136]. Similar to SPEN/SHARP, HDAC3 knockdown also led to defective X-linked gene silencing with normal Xist RNA localization, suggesting that Xist RNA can induce X-linked gene silencing through HDAC activity recruited to the Xi by interaction between SPEN/SHARP and repeat A of Xist RNA. Because deacetylation of H3K27ac can permit H3K27me3 modification by PRC2 and gene silencing [137], SPEN/SHARP repressor complex-mediated deacetylation of H3K27ac might be essential to trigger H3K27me3 deposition onto the Xi at the onset of XCI.

Interestingly, iDRiP only identified PRC1 and PRC2 components while ChlRP-MS identified components of PRC1 but not PRC2 [82,121,122]. ChlRP-MS, RAP-MS and iDRiP identified 30, 10 and ~250 proteins, respectively, as potential Xist RNA binding proteins. Only three proteins were commonly isolated among these three approaches, suggesting that various experimental conditions used in different methods could largely affect the isolation of RNA-protein complexes. It is curious how Xist RNA binding proteins identified in the recent works interact with and cooperatively play with proteins such as PRC2, which is recruited to the Xi in a Xist RNA-dependent manner to establish facultative heterochromatin on the Xi.

Conclusions and perspectives

Over the past decade, our knowledge about lncRNA biology has increased intensely, owing to advances and developments in analytical tools and techniques. Further advancements in both the techniques to investigate and in the instruments to analyze lncRNAs would propel forward our understanding of their function in various biological processes including XCI. Indeed, most recently a study utilizing ChlRP-MS, RAP-MS and iDRiP were able to comprehensively identify novel Xist-RNA binding proteins [104,131,132]. XCI is executed through a complex molecular mechanism in which multiple lncRNAs, protein factors and chromosomal elements are involved. Discovery of novel lncRNAs and their associated proteins, as well as characterizing lncRNAs at the *Xic*, could provide additional insight towards the molecular mechanism of lncRNA function in XCI. Further investigations in this direction could enhance our understanding and delineate the detailed molecular mechanism underlying the establishment of such highly ordered and robust repressive Xi.

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Highlights

X-chromosome inactivation is an excellent model of long noncoding RNA (lncRNA)-mediated gene regulation. This review summarizes recent updates in our knowledge of lncRNAs and protein factors found at the X-inactivation center (*Xic*) and discusses their overall mechanisms of action in inducing monoallelic *Xist* expression. We also discuss our current understanding of the molecular mechanism behind Xist RNA-mediated induction of the repressive epigenetic landscape at the inactive X-chromosome.

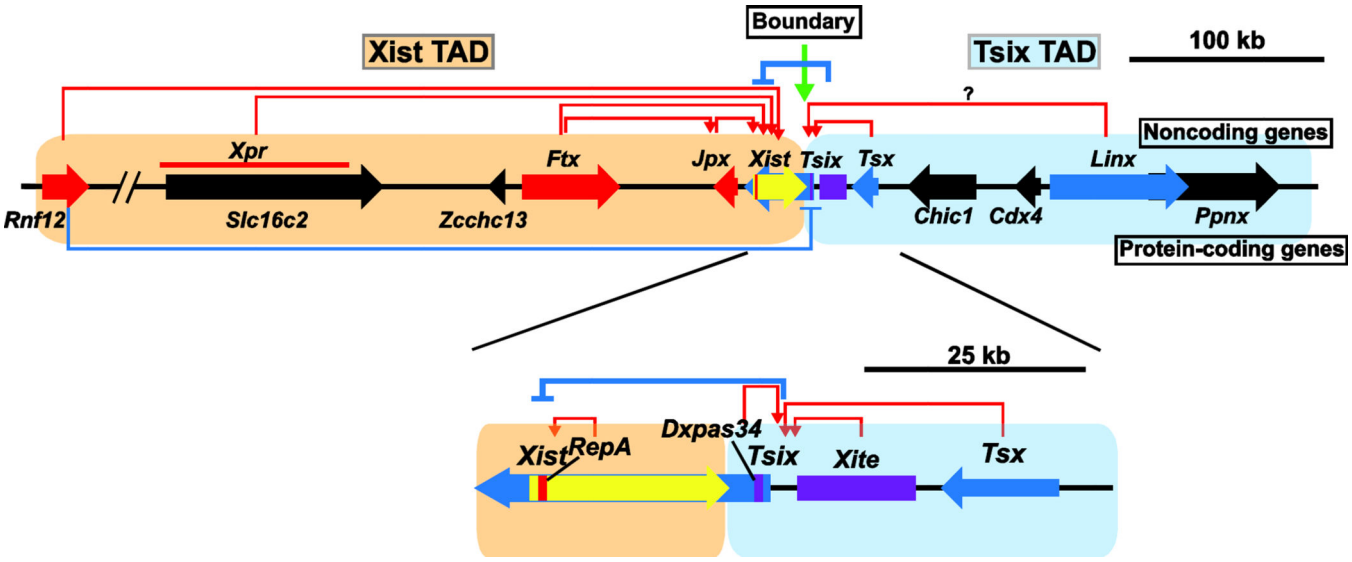

Fig. 1.

Functionally distinct topologically associating domains (TADs) at the *Xic*. The schematic of Xist and Tsix TADs, long noncoding genes, and a protein-coding gene at the *Xic* locus is shown; each of these plays a role in XCI. Yellow bold arrow indicates *Xist*. Red and Blue bold arrows indicate genes that activate and repress *Xist*, respectively. Black arrows indicate non-XCI-related genes to date. Purple boxes indicate enhancer-like region for *Tsix*. Names of long noncoding and protein-coding genes are shown above and below these bold arrows, respectively. Red and blue arrows indicate gene's function as activator and repressor for *Xist* expression, respectively. The green arrow indicates the *Xist/Tsix* TAD boundary.

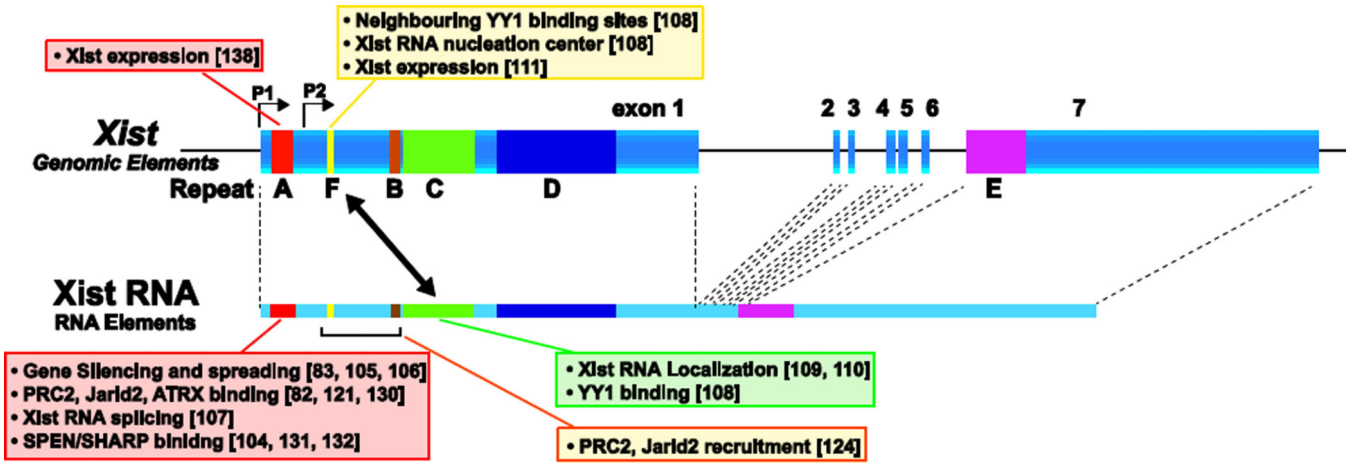

Fig. 2.

Xist repeat elements confer a variety of function to *Xist*. Summary of function of *Xist* repeat elements within Xist RNA and the *Xist* gene. While a majority of essential repeat elements on the *Xist* gene and Xist RNA are located across exonl, many have not yet been explored across the *Xist* gene and *Xist* RNA. Colored bold line indicates *Xist* exon structure and *Xist* repeat elements (A-F). Xist RNA is shown below the Xist gene map. Function of each repeat in genomic elements within *Xist* and Xist RNA are shown above and below the map, respectively, with reference numbers in brackets. Two-headed arrow indicates interaction between a genomic element within Xist and Xist RNA through YY1.