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New and Xisting Regulatory Mechanisms of X Chromosome Inactivation

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Abstract

During development, female mammals silence one of their two X chromosomes to compensate for the presence of a single X in males. This process, termed X chromosome inactivation (XCI), is a quintessential epigenetic phenomenon and involves a complex interplay between non-coding RNAs and protein factors. Progress in this area of study has consequently resulted in new approaches to study epigenetics and regulatory RNA function. Here we will discuss recent developments in the field that have advanced our understanding of XCI and its regulatory mechanisms.

INTRODUCTION

X chromosome inactivation (XCI) evolved in mammals to balance X-linked gene expression levels between males (XY) and females (XX) [1–4]. During development females undergo two forms of XCI: Imprinted and Random. Imprinted X inactivation is encountered during early embryogenesis, where the paternal X chromosome (Xp) is preferentially silenced. While this state is maintained in extra-embryonic tissues throughout development, all imprinted epigenetic marks on Xp are erased in cells of epiblast lineage, which will form the future embryo-proper, to initiate another round of XCI where either Xp or Xm are silenced randomly (random XCI) [5].

Mouse embryonic stem (mES) cells are derived from the epiblast of early embryos in which both X chromosomes are active. mES cells offer a tractable system to study random XCI because they recapitulate this process upon differentiation *in vitro* and can be genetically manipulated. In the undifferentiated state, each cell contains two active X chromosomes (Xa). Upon differentiation each cell first counts the number of X chromosomes within the cell and then randomly chooses to inactivate one X chromosome. After a choice is made, Xist RNA is upregulated on the future inactive X (Xi) and a gradual chromosome wide silencing process is initiated. Once established, this silent state is transmitted through each round of cell division in a stable and heritable manner.

In the past few years, substantial progress has been made in understanding the regulation of XCI. Novel roles for long non-coding RNAs as well as their interplay with various protein

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factors have been identified [6], revealing detailed mechanisms involved in this process (Fig.1). Links have been uncovered connecting XCI to the pluripotency network. In this review, we will discuss recent advances in the field with an emphasis on regulatory RNAs and protein factors involved in X chromosome silencing.

New insights into lncRNA regulation of XCI

Xist

Xist is a 17kb RNA that is transcribed exclusively from Xi and coats it in *cis* [7–10]. It is comprised of several repetitive units, Repeats A–F. *Repeat A* is located at the 5' end of *Xist* and is the region conferring silencing ability to this RNA [11]. The motif within Xist RNA is now known to recruit Polycomb Repressive Complex 2 (PRC2) to the Xi [12]. PRC2 in turn catalyzes histone H3 Lysine 27 trimethylation (H3K27me3), a mark associated with repressed chromatin. Subsequent events involved in the maintenance of Xi include incorporation of the histone variant MacroH2A and DNA methylation [1]. As Xist is a key molecule that triggers chromosome-wide silencing, much effort has been devoted to understanding how *Xist* is regulated. In addition to transcription factors and *cis*-elements within the *Xist* locus, many regulatory factors are lncRNAs or loci encoding them. Like transcription factors, RNA regulators of *Xist* come in two flavors: activators and repressors (Table 1A). In the next subsections, we discuss recent developments in this arena.

RepA, a Repeat A RNA—Previous transgenic studies showed that the Repeat A region of *Xist* is required for silencing in ES cells, as deleting Repeat A on a transgene precluded Xist from silencing genes *in cis* [11]. When a similar deletion was introduced into mice, the X-chromosome bearing the deletion could no longer be silenced. Interestingly, this deletion prevented *Xist* transcription [13,14] and is consistent with prior work showing that the Repeat A region produces a shorter transcript independently of Xist. Called RepA for "Repeat A RNA", this 1.6 kb RNA directly binds PRC2 through the 28-nt Repeat A motif and is believed to target the H3K27me3 modification to the *Xist* promoter to (paradoxically) facilitate *Xist* transcription [12].

RS14—Previous studies have suggested that the *Xic* is partitioned into two distinct active chromatin hubs: one mediating long range interactions between the *Xist* promoter and the proximal region of the *Jpx* gene (an *Xist* activator, discussed below) and the second encompassing *Xite* and the promoter region of *Tsix*, the repressor of *Xist* [15]. A well-conserved region, termed RS14 that lies within the end of the *Xist* gene, separates these two domains. Computational analyses uncovered binding sites for the chromatin insulator protein CTCF within RS14. Deletion of RS14 (RS14Δ) in mES cells exhibited a female specific defect in XCI. RS14Δ cells on differentiation failed to efficiently upregulate Xist RNA while maintaining a higher than normal level of Tsix. These results suggest that RS14 via its CTCF binding sites may function as an insulator element, in the absence of which *Tsix* transcription persists and spreads into the *Xist* gene and represses the complementary gene to prevent XCI [16].

Negative Regulation of Xist

Tsix—The lncRNA Tsix was the first described negative regulator of Xist [17]. *Tsix* is transcribed in the antisense orientation to *Xist* and represses *Xist* transcription in *cis*. Another non-coding locus, *Xite*, functions as a *cis*-acting activator of *Tsix* [18]. Deletion of *Tsix* results in skewing the choice of which X chromosome to inactivate, since in the absence of *Tsix* on one chromosome, *Xist* is upregulated and commences the process of XCI [19].

Previous studies showed that Tsix inhibits *Xist* expression on the future Xa in several different ways. First, *Tsix* expression blocks loading of the RepA-PRC2 complex onto the 5' end of *Xist*, in an act that correlates with suppression of *Xist* induction [12]. *Tsix* transcription also establishes repressive chromatin at the 5' end of *Xist*, in part by stimulating DNMT3a activity on the *Xist* promoter to lock in its off state [20,21]. Recent studies further added to this by showing that *Tsix* transcription must proceed through the 5' end of *Xist* for repression to occur, as indeed preventing *Tsix* transcription (by introducing polyA cassettes) through the *Xist* promoter obstructed the establishment of a repressive environment at the promoter [22]. These changes are crucial for efficient silencing of *Xist* on the future Xa.

Tsix may also be involved in suppressing *Xist* expression by recruiting the RNAi machinery. The involvement of the RNAi pathway in XCI has long been speculated, given that *Tsix* makes a complementary transcript along the entire length of *Xist*. Ogawa and colleagues showed that Xist and Tsix form a long duplex RNA that is processed into small RNAs of 22–42 nt [23]. Appearance of small RNAs is dependent on Dicer and is correlated with suppression of *Xist* expression. Given the intimate connection between the process of differentiation and XCI, further studies are needed to determine whether Dicer has a direct role during XCI, as $dcr^{\Delta/\Delta}$ ES cells are incapable of differentiation [24,25]. While conflicted about the exact role of Dicer in XCI, several independent studies agree that Xist levels increase significantly in undifferentiated $dcr^{\Delta/\Delta}$ cells. This may be explained by DNA hypomethylation at the *Xist* promoter in male cells, as the levels of the DNA methyltransferase, DNMT3a, are reduced upon Dicer deficiency [25]. However, accumulation of Xist was also observed in female cells where promoter regions of both Xs are normally hypomethylated, implying that the RNAi machinery is required to keep Xist levels low in the pre-XCI state [23,24].

Pluripotency factors—In pre-XCI cells, *Xist* expression has been proposed to be directly repressed by pluripotency factors such as OCT4, SOX2 and NANOG, which bind to the first intron of *Xist* [26,27]. Pluripotency factors also control Xist levels by activating *Tsix*, which binds OCT4 and REX1 at the 5' end of *Tsix* and binds other crucial factors such as CTCF and c-MYC through juxtaposed motifs [28]. At the same time, OCT4 and SOX2 bind *Xite*, one of *Tsix*'s enhancers [26]. It is therefore proposed that the timing of XCI is controlled by the inherent, developmentally specific action of the pluripotency factors. A latest study shows that, while upregulation of *Xist* by deletions in intron 1 or *Tsix* promoter (ΔCpG) was only modest, a combination of both mutations further enhanced Xist levels [29]. Taken together, the published evidence indicates that *Tsix* transcription and the inhibitory effects of pluripotency factors at intron 1 cooperate to maintain the *Xist* gene in a repressed state in undifferentiated ES cells. Given that these mutations do not result in complete derepression of *Xist*, there are clearly other repressive mechanisms and/or *Xist* activator(s). Evidence for *Xist* activators has indeed recently come to light.

Positive Regulation of Xist

Jpx—One positive regulator of *Xist* is the noncoding *Jpx* locus. Tian and colleagues showed that heterozygous deletion of *Jpx* resulted in massive cell death due to inability to upregulate *Xist* and initiate XCI in female cells [30]. The recapitulation of the *Jpx* knockout by RNAi knockdown of Jpx RNA demonstrates that Jpx acts as an RNA, rather than through associated chromatin or DNA elements. Unlike other lncRNAs of the *Xic*, however, Jpx functions in *trans* to activate *Xist*, as *Xist* upregulation can be restored in $Jpx^{+/-}$ cells by an autosomally expressed *Jpx* transgene. Further analysis suggested that Jpx and Tsix have antagonistic roles, with the two RNAs working in parallel pathways to transactivate *Xist*:

Abrogating Tsix RNA in *Jpx*$^{+/-}$ cells at least partially restores *Xist* upregulation. The mechanism of Jpx-mediated activation remains to be determined.

Rnf12—Another positive regulator to come to light is *Rnf12*, an X-linked gene encoding an E3 ubiquitin ligase [31]. *Rnf12* is located 500 kb upstream of *Xist* and does not reside within a region traditionally considered the *Xic*. However, RNF12 protein has been shown to increase in levels at the onset of XCI [31–33] and, in an ES model, overexpression of *Rnf12* is sufficient to induce ectopic Xist cloud formation in a fraction of male and female nuclei [31,32]. In studies performed in ES models, *Rnf12*$^{+/-}$ mutants show a slight delay in initiation of XCI and *Rnf12*$^{-/-}$ mutants largely fail to initiate XCI supporting the idea that RNF12 is a positive regulator of *Xist* [31,32]. A study in a mouse model, however, has resulted in somewhat different results. Here, it was reported that random XCI and *Xist* expression occur properly in both *Rnf12*$^{+/-}$ and *Rnf12*$^{-/-}$ mouse embryos and ES cells, thereby arguing against *Rnf12* as a necessary activator of *Xist*. Nonetheless, the *Rnf12* deficiency instead caused a problem in imprinted XCI in placental tissues, as evidenced by failure to express Xist RNA from the paternal X-chromosome. Female embryos lacking a normal maternal copy of *Rnf12* die after implantation [34]. Further work is required to understand the exact contribution of *Rnf12* to both random and imprinted XCI.

Pairing: Breaking X-chromosome Symmetry

Prior to Xist upregulation during differentiation, the two X chromosomes in a cell migrate toward each other and pair transiently [35,36]. Previous studies have shown that pairing is essential for the onset of XCI, since X inactivation fails to occur in cells where pairing between the X's is disrupted by the introduction of an autosomal transgene. The minimal region sufficient for pairing was narrowed down to 1–2 kb surrounding the *Tsix and Xite* sequences. Furthermore, the formation of X-X pairs was shown to require active transcription and paired X's have a short half-life of only 30–60 min [37].

The predominant view in the field is that the pairing is a means of counting the number of X chromosomes and for choosing X-chromosomes for inactivation in a mutually exclusive way. Accordingly, factors that affect pairing also affect the number of X chromosomes that are inactivated. Studies have shown that depletion of factors such as OCT4 and CTCF that are implicated in pairing also result in presence of cells with aberrant number of active or inactive X chromosomes (2 Xi's or 2 Xa's) [26,37]. The requirement of CTCF for pairing correlates well with the notion that CTCF is able to mediate long-range chromatin interactions and thereby influence homologous chromosome pairing. Donohoe and colleagues showed that CTCF interacts with the pluripotency factor OCT4 *in vivo*. Depletion of OCT4 resulted in reduced incidence of X-X pairs in the cell [26]. However, since OCT4 and CTCF are known to function as transcription factors, the pairing defect observed could be not only a result of their decreased binding to the *Tsix-Xite* region, but also their indirect effect on *Tsix* transcription. It will be interesting to analyze whether Tsix is involved in the pairing process.

Recently, Masui and colleagues were able to directly observe the process of X-X pairing at *Tsix-Xite* sequences in female ES cells using live cell imaging [38]. They confirmed that X chromosome pairing was indeed a transient event that lasted approximately 45 minutes and occurred prior to Xist RNA upregulation on one allele. They also sought to determine whether *Tsix* down-regulation occurred before or after the X chromosomes had paired. While Tsix levels were reduced after pairing, the levels fluctuated, suggesting that perhaps the transient asymmetry between Tsix levels on the two X chromosomes was a signal that allowed the cell to up-regulate Xist. Another region within the *Xic* named *Xpr* (X pairing

region) was hypothesized to regulate X chromosome pairing, but the hypothesis remains to be tested with a knockout [39,40].

From *Xic* to chromosome-wide silencing

Spreading of Xist RNA

During XCI, Xist RNA to initially surrounds the territory around its own gene and then spreads gradually along the entire Xi in *cis*. Further proof of this process was obtained by a study that employed live cell imaging using a tetracycline inducible Xist RNA fused to MS2 binding sites (Xist-MS2) [41]. The MS2 RNA binding protein recognizes MS2 sites with very high affinity. In this study the transgene containing Xist-MS2 was targeted to an autosomal locus in male ES cells [41]. It was observed that Xist RNA spreading along a chromosome was indeed a gradual process. Using fluorescence recovery after photobleaching (FRAP), the authors showed that Xist binding to chromatin was a dynamic process that required transcription. Surprisingly and in contradiction to previous reports, cell cycle analysis of Xist-MS2 binding to chromatin showed that Xist-MS2 was dislodged from the chromosome during mitosis [42]. It will be worthwhile to analyze whether dissociation from mitotic chromosomes is observed with Xist-MS2 on the X chromosome also.

YY1 (Ying Yang 1)

A long-standing question in the field has been how Xist RNA localizes and spreads in *cis*. What prevents Xist from diffusing throughout the nucleus? Through a series of experiments, a recent study by Jeon and Lee showed that Xist RNA can actually diffuse within the nucleus [43]. For instance, in a female somatic cell line, transgenic Xist expressed autosomally can migrate between the autosome and the Xi, suggesting that Xist has the potential to act in *trans*. What normally prevents Xist RNA molecules from diffusing is tethering by the transcription factor, YY1. Three tandem YY1-binding sites located within *Xist*'s exon 1 were shown to be essential for tethering Xist to chromatin. Because spreading and localization to the rest of the X-chromosome were abrogated when these sites were deleted, it was proposed that this region serves as the Xist nucleation center. YY1 binding is observed preferentially on Xi (not Xa), explaining how diffusible Xist can be secured on Xi alone. Interestingly, YY1 was found to bind Xist via Repeat C, different from the YY1 motif used for DNA binding. This is consistent with previous studies, where Xist RNA localization to Xi was interrupted by blocking Repeat C and its surrounding region [44,45]. Targeting Repeat C with locked nucleic acids (LNAs) resulted in rapid loss of Xist RNA from Xi [45]. While the exact manner of action of Repeat C on Xist RNA localization is not known, it is tempting to speculate that it is through specific binding of YY1.

HnRNP-U (Heterogeneous nuclear ribonucleoprotein U)

HnRNP-U, also known as Scaffold attachment factor A (SAF-A), has been long known to localize on Xi, but its role in XCI has remained elusive until recently [46,47]. Depletion of hnRNP-U results in loss of Xist foci and gene silencing in ES cells [48]. HnRNP-U harbors separate RNA (RGG) and DNA-binding domains (SAF), both of which are required for Xist localization [46–48]. It is noteworthy that like YYI, hnRNP-U binds Xist close to Repeat C, further reinforcing the concept of this region being a functional localization domain. HnRNP-U was originally identified as a nuclear matrix component that binds S/MARs (Scaffold associated region/Matrix attached region), A+T-rich DNA elements involved in structural organization of chromatin [49]. Given that Xist coating is independent of unique X-linked sequences, S/MARs are very attractive *cis*-acting candidates for Xist localization. Study of S/MAR site distribution along the X chromosome could provide valuable clues regarding the silencing by and localization of Xist.

SATB1 (Special A+T-rich binding protein 1)

Another S/MAR-associated protein SATB1 was identified a key determinant of Xist-mediated gene silencing [50]. SATB1 expression in ES cells during differentiation corresponds to the 48-hour time window [51], within which Xist can initiate silencing, and overexpression of SATB1 is sufficient for extending this time span. SATB1 does not colocalize with but resides adjacent to Xist. However, overexpression of Xist can transiently recruit SATB1 to its foci implying their physical interaction. Reciprocally, SATB1-dependent Xist delocalization and redistribution was observed in thymocytes. Therefore, one might speculate that SATB1 organizes chromatin to make it accessible to Xist as well as other silencing factors.

LINE-1

The mammalian genome contains a wealth of repetitive elements such as long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs). It was hypothesized by Mary Lyon that the propagation of the inactive state during XCI might be influenced by the presence of these elements, since they are more abundant on the X chromosome as compared to autosomes [52]. Recently, Chow and colleagues described specific transcription and localization of a subset of LINE elements during XCI and that the formation of a heterochromatic environment was necessary for the transcription of these elements [53]. They showed that when *Xist* was introduced into an autosome, the genes that efficiently underwent inactivation were generally those with a full-length LINE element in the vicinity, further supporting the correlation of LINEs and spreading of inactivation. They suggest that the local density of LINEs may impact on the ability of genes in this region to be drawn into the Xist silent compartment. However, the authors also observed LINE transcription near genes that are prone to escape XCI, implying that LINEs also correlate with gene activity on the Xi. It is hypothesized that transcription of LINEs during XCI serves several purposes, first to draw genes into a repressive compartment established by Xist, second to aid in the successful inactivation of genes present at escape prone regions by making them more susceptible to Xist mediated silencing, and third to potentially aid in escape from XCI. Further work is required to establish a functional link between LINEs and XCI.

Pcl2 (Polycomblike 2)

Although the PRC2 catalyzed histone modification, H3K27me3 has been a longknown marker of Xi and its distribution along the entire chromosome during XCI has been examined at a single gene level [54], the mechanism of PRC2 targeting to specific loci on Xi is still unknown. Recently, Casanova and colleagues characterized PCL2 [55], one of three mammalian homologues of *Drosophila* Polycomblike (PCL) protein that is known to interact with PRC2 and facilitate its activity. Similar to core PRC2 components, PCL2 is highly expressed during early embryogenesis and enriched on Xi at the onset of XCI. Biochemical analyses revealed that PCL2 forms a complex with PRC2. Moreover, knockdown of PCL2 impairs PRC2 recruitment to Xi and Polycomb target loci, implicating its role in PRC2 recruitment. However, it remains unclear how PCL2 facilitates PRC2 recruitment. Given that PRC2 loading on Xi is also Xist-dependent, it is conceivable that Pcl2 acts by stabilizing PRC2 after it is recruited to Xi via Xist-EZH2 interactions [12], rather than initiating recruitment. Further characterization of other polycomblike homologues and their interplay with non-coding RNAs during the process of XCI will aid in understanding their respective roles.

MacroH2A, ASH2L and ATRX

Despite their involvement in XCI, the functional significance of some Xi-coating proteins remains unclear. Histone variant *macroH2A* is one such Xi marker, though its role in gene silencing has long been appreciated. Female macroH2A knockout mice are viable and fertile, and XCI can proceed normally in macroH2A-deficient ES cells, indicating that it is dispensable for Xi silencing [56,57]. Pasque and colleagues recently identified macroH2A as a key factor that makes genes on Xi resistant to reprogramming in the Xenopus oocyte nuclear transfer system [58]. Hence, while not crucial for XCI, MacroH2A may still be responsible for maintaining long-term stability of Xi states.

Besides macroH2A, two more proteins have been reported to mark Xi during late XCI [47,59]. ATRX (α-thalassemia/mental retardation X-linked protein) is known to regulate chromatin structure and function at constitutive heterochromatin domains. Oddly, ASH2L, a component of the trithorax group complex (TrxG) that functions in maintaining gene expression, also showed localization to the Xi, but its role remains elusive. Future studies are anticipated to elucidate exact roles of both proteins in XCI.

Orc2 and HP1a

Another recent study identified 32 protein candidates involved in XCI maintenance through a genome-wide RNAi screen [60]. Among those, origin recognition complex 2 (ORC2) is known to function in pericentric heterochromatin-organization together with HP1 α [61]. Both proteins are found at the pericentric region on Xi and depletion of either resulted in reactivation of a subset of genes on the Xi. However, it is not yet clear whether the role of ORC2-HP1 α is Xi-specific because (1) their localization at pericentric regions is true of all chromosomes; and (2) X-linked genes whose expression was affected by ORC2-HP1 α are located relatively close to the pericentric region. Further experiments with Xa and autosomal controls would be helpful to draw conclusions.

Other forms of dosage compensation in mammals

X chromosome imprinting: Inactivation of Xp

Dosage compensation in the mammal involves a complex cycle of inactivation and reactivation that starts in the parental germline and continues through early embryogenesis. In the pre-implantation mouse embryo, the imprinted form of XCI is observed where the paternal X chromosome (Xp) is exclusively silenced. But how is Xp always favored for inactivation? While most aspects of Xp inactivation is relatively well understood as it shares common gene-silencing mechanisms with random XCI, this fundamental question about imprinted XCI remained unanswered and has fostered many debates in the field. Preferential paternal XCI can result from the pre-inactivated Xp during spermatogenesis (pre-inactivation hypothesis), and/or from the maternal imprint on the *Xic* region precluding the maternal X chromosome (Xm) from silencing (*de novo* model) [5]. Recent studies agree that X-linked genes are initially active on both Xp and Xm at the 2-cell stage and become gradually silenced on Xp during preimplantation development [62–64]. However, repetitive elements such as LINEs and SINEs are already inactive on Xp at the 2-cell stage in an Xist-independent manner, suggesting a contribution for repetitive elements in the paternal germline towards imprinted XCI process [63]. Thus, imprinted XCI is a multistep process that first involves silencing of repetitive elements by the two-cell stage and progressively involves silencing of genic elements in the 8- to 16-cell stages.

Reactivation of Xp during embryogenesis

Imprinted inactivation of Xp is reversed at the blastocyst stage. This reactivation process allows both Xp and Xm get an equal chance to be subjected to random XCI in the future

embryo-proper. X-reactivation can be visualized in epiblast cells by loss of Xist and H3K27me3 on Xp around the time of implantation [65,66]. While the pluripotency factors OCT4 and SOX2 are ubiquitously expressed in both morulae and early blastocysts, NANOG is exclusively expressed in epiblast cells and is thought to be essential for determining the pluripotency of cells [67]. Given roles of pluripotency factors in *Xist* regulation, the key step for X-reactivation would be loss of *Xist* expression. Indeed, a new study has shown that the overexpression of *Tsix* - the negative regulator of *Xist* - is sufficient for reactivating Xp-linked GFP expression in extraembryonic tissues [68], raising the possibility that Tsix is a regulator of X-reactivation *in vivo*, though this idea still needs to be tested formally. Recently, the idea that reactivation depends on suppression of *Xist* has been challenged by Williams and colleagues who observed that some genes are reactivated even before loss of Xist/H3K27Me3 foci on Xp using the fluorescence in situ hybridization method (FISH) [69]. Ectopic extinction of Xist foci by NANOG expression did not affect the reactivation rate of these genes, which implies that Xist downregulation can be uncoupled from genic reactivation. However, both chromosome-wide and RNA-sequencing (at the individual gene level) analyses would be necessary to confirm that X-reactivation could occur without extinguishing *Xist* expression.

Regulation of Xa: dosage compensation between autosomes and the X

XCI evolved as a means to equalize gene dosage between male and female mammals. However, this process results in the imbalance of gene expression between the X chromosome and autosomes. In order to circumvent this, female mammals are hypothesized to upregulate their active X chromosome 2 fold.[70]. Although early studies using microarrays demonstrated that Xa is expressed at a higher level compared to an autosome in mammals [71–73], this hypothesis was recently challenged by Xiong and colleagues. With the availability of the RNA sequencing technology (RNA-seq), which provides more sensitive and quantitative data than microarrays, they analyzed expression patterns of all X-linked genes and found no dosage compensation of Xa [74]. Given that the X is enriched for tissue-specific genes, the population of X-linked genes that are transcriptionally silent is significantly higher than that of autosomal genes [74–76]. Therefore, dosage compensation by upregulation of Xa would be applied to only genes that are actively expressed. Correspondingly, when analyzing RNA-seq data as well as previous microarray data only with actively expressed genes, four independent groups arrived at the same conclusion that the Xa is indeed upregulated in mammals [75–78]. Xa was enriched with active transcription marks such as RNA polymerase II (RNA Pol II) and histone H3 Lysine 4 trimethylation (H3K4me3), compared to autosomes, in both mES and somatic cells [75,76]. Together, these results suggest that dosage compensation between autosomes and sex chromosomes does occur in mammals via Xa upregulation.

CONCLUSIONS

Work in the past two years have significantly advanced our understanding of XCI mechanisms, revealing multiple new regulators of both RNA and protein nature. Collectively, we have seen that *Xist* transcription is strictly regulated by pluripotency factors and ncRNAs that are in turn developmentally regulated. We have also found that the *cis*-specific localization and action of Xist is subject to regulation by a plethora of protein factors. Fittingly, at the 50th anniversary celebration for the discovery of XCI last year (Oxford, UK; July 2011), the breadth and depth of what has been learned were clear to all participants. Much remains unknown, however, and new knowledge raises new questions. Comprehensive yet thorough studies are awaited for exact roles of various ncRNAs and protein factors in XCI regulation, the mechanism of Xist spreading across Xi, as well as details for the action of Xist on gene silencing at the individual gene level. With the recent

evidence of dosage compensation in mammals via Xa upregulation, its molecular mechanisms also need to be addressed. Fortunately, recent developments in technology have enabled identification of DNA sequences bound by non-coding RNA (i.e. the roX RNAs in the fly dosage compensation system) [79,80]. These experimental advances together with high throughput sequencing techniques will surely become valuable tools to understand diverse interplays among Xist, chromatin and other ncRNA or protein regulators during XCI. Having witnessed the identification of different regulatory roles of noncoding RNAs and proteins in gene regulation by using XCI as a model system, we expect that future studies in this field will be instrumental in furthering our understanding of epigenetic gene regulatory networks in the cell.

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REFERENCES AND RECOMMENDED READING

* of special interest

** of outstanding interest

1. Chang SC, Tucker T, Thorogood NP, Brown CJ. Mechanisms of X-chromosome inactivation. Front Biosci. 2006; 11:852–866. [PubMed: 16146776]
2. Lyon MF. Gene action in the X-chromosome of the mouse (Mus musculus L.). Nature. 1961; 190:372–373. [PubMed: 13764598]
3. Payer B, Lee JT. X chromosome dosage compensation: how mammals keep the balance. Annu Rev Genet. 2008; 42:733–772. [PubMed: 18729722]
4. Wutz A. Gene silencing in X-chromosome inactivation: advances in understanding facultative heterochromatin formation. Nat Rev Genet. 2011; 12:542–553. [PubMed: 21765457]
5. Payer B, Lee JT, Namekawa SH. X-inactivation and X-reactivation: epigenetic hallmarks of mammalian reproduction and pluripotent stem cells. Hum Genet. 2011; 130:265–280. [PubMed: 21667284]
6. Lee JT. Gracefully ageing at 50, X-chromosome inactivation becomes a paradigm for RNA and chromatin control. Nat Rev Mol Cell Biol. 2011; 12:815–826. [PubMed: 22108600] Written for the 50th anniversary of XCI this review gives a historical perspective of developments in the field of XCI and also a review of XCI's recent contributions towards understanding RNA and chromatin regulation.
7. Brockdorff N, Ashworth A, Kay GF, Cooper P, Smith S, McCabe VM, Norris DP, Penny GD, Patel D, Rastan S. Conservation of position and exclusive expression of mouse Xist from the inactive X chromosome. Nature. 1991; 351:329–331. [PubMed: 2034279]
8. Brockdorff N, Ashworth A, Kay GF, McCabe VM, Norris DP, Cooper PJ, Swift S, Rastan S. The product of the mouse Xist gene is a 15 kb inactive X-specific transcript containing no conserved ORF and located in the nucleus. Cell. 1992; 71:515–526. [PubMed: 1423610]
9. Hong YK, Ontiveros SD, Chen C, Strauss WM. A new structure for the murine Xist gene and its relationship to chromosome choice/counting during X-chromosome inactivation. Proc Natl Acad Sci U S A. 1999; 96:6829–6834. [PubMed: 10359798]
10. Jonkers I, Monkhorst K, Rentmeester E, Grootegoed JA, Grosveld F, Gribnau J. Xist RNA is confined to the nuclear territory of the silenced X chromosome throughout the cell cycle. Mol Cell Biol. 2008; 28:5583–5594. [PubMed: 18625719]
11. Wutz A, Rasmussen TP, Jaenisch R. Chromosomal silencing and localization are mediated by different domains of Xist RNA. Nat Genet. 2002; 30:167–174. [PubMed: 11780141]

12. Zhao J, Sun BK, Erwin JA, Song JJ, Lee JT. Polycomb proteins targeted by a short repeat RNA to the mouse X chromosome. Science. 2008; 322:750–756. [PubMed: 18974356]

13. Hoki Y, Kimura N, Kanbayashi M, Amakawa Y, Ohhata T, Sasaki H, Sado T. A proximal conserved repeat in the Xist gene is essential as a genomic element for X-inactivation in mouse. Development. 2009; 136:139–146. [PubMed: 19036803]

14. Royce-Tolland ME, Andersen AA, Koyfman HR, Talbot DJ, Wutz A, Tonks ID, Kay GF, Panning B. The A-repeat links ASF/SF2-dependent Xist RNA processing with random choice during X inactivation. Nat Struct Mol Biol. 2010; 17:948–954. [PubMed: 20657585]

15. Tsai CL, Rowntree RK, Cohen DE, Lee JT. Higher order chromatin structure at the X-inactivation center via looping DNA. Dev Biol. 2008; 319:416–425. [PubMed: 18501343]

16. Spencer RJ, del Rosario BC, Pinter SF, Lessing D, Sadreyev RI, Lee JT. A boundary element between Tsix and Xist binds the chromatin insulator Ctcf and contributes to initiation of X-chromosome inactivation. Genetics. 2011; 189:441–454. [PubMed: 21840866]

17. Lee JT, Davidow LS, Warshawsky D. Tsix, a gene antisense to Xist at the X-inactivation centre. Nat Genet. 1999; 21:400–404. [PubMed: 10192391]

18. Ogawa Y, Lee JT. Xite, X-inactivation intergenic transcription elements that regulate the probability of choice. Mol Cell. 2003; 11:731–743. [PubMed: 12667455]

19. Lee JT, Lu N. Targeted mutagenesis of Tsix leads to nonrandom X inactivation. Cell. 1999; 99:47–57. [PubMed: 10520993]

20. Sado T, Hoki Y, Sasaki H. Tsix silences Xist through modification of chromatin structure. Dev Cell. 2005; 9:159–165. [PubMed: 15992549]

21. Sun BK, Deaton AM, Lee JT. A transient heterochromatic state in Xist preempts X inactivation choice without RNA stabilization. Mol Cell. 2006; 21:617–628. [PubMed: 16507360]

22. Ohhata T, Hoki Y, Sasaki H, Sado T. Crucial role of antisense transcription across the Xist promoter in Tsix-mediated Xist chromatin modification. Development. 2008; 135:227–235. [PubMed: 18057104]

23. Ogawa Y, Sun BK, Lee JT. Intersection of the RNA interference and X-inactivation pathways. Science. 2008; 320:1336–1341. [PubMed: 18535243]

24. Kanellopoulou C, Muljo SA, Dimitrov SD, Chen X, Colin C, Plath K, Livingston DM. X chromosome inactivation in the absence of Dicer. Proc Natl Acad Sci U S A. 2009; 106:1122–1127. [PubMed: 19164542]

25. Nesterova TB, Popova BC, Cobb BS, Norton S, Senner CE, Tang YA, Spruce T, Rodriguez TA, Sado T, Merkenschlager M, et al. Dicer regulates Xist promoter methylation in ES cells indirectly through transcriptional control of Dnmt3a. Epigenetics Chromatin. 2008; 1:2. [PubMed: 19014663]

26. Donohoe ME, Silva SS, Pinter SF, Xu N, Lee JT. The pluripotency factor Oct4 interacts with Ctcf and also controls X-chromosome pairing and counting. Nature. 2009; 460:128–132. [PubMed: 19536159]

27. Navarro P, Chambers I, Karwacki-Neisius V, Chureau C, Morey C, Rougeulle C, Avner P. Molecular coupling of Xist regulation and pluripotency. Science. 2008; 321:1693–1695. [PubMed: 18802003]

28. Navarro P, Oldfield A, Legoupi J, Festuccia N, Dubois A, Attia M, Schoorlemmer J, Rougeulle C, Chambers I, Avner P. Molecular coupling of Tsix regulation and pluripotency. Nature. 2010; 468:457–460. [PubMed: 21085182] This paper reports that the antisense repressor of *Xist, Tsix* is regulated by pluripotency factors. ChIP analyses revealed the requirement of the Dxpas34 repeat for the binding of the pluripotency factors, REX1, KLF4 and c-MYC. Transcription elongation of *Tsix* was shown to depend on REX1.

29. Nesterova TB, Senner CE, Schneider J, Alcayna-Stevens T, Tattermusch A, Hemberger M, Brockdorff N. Pluripotency factor binding and Tsix expression act synergistically to repress Xist in undifferentiated embryonic stem cells. Epigenetics Chromatin. 2011; 4:17. [PubMed: 21982142]

30. Tian D, Sun S, Lee JT. The long noncoding RNA, Jpx, is a molecular switch for X chromosome inactivation. Cell. 2010; 143:390–403. [PubMed: 21029862] This paper identifies the X-linked Jpx RNA as a positive regulator of *Xist* expression. Unlike other noncoding loci of the *Xic, Jpx* is

trans-acting. Even a single allele deletion of *Jpx* is sufficient to prevent *Xist* upregulation and cause female cell lethality.

31. Jonkers I, Barakat TS, Achame EM, Monkhorst K, Kenter A, Rentmeester E, Grosveld F, Grootegoed JA, Gribnau J. RNF12 is an X-Encoded dose-dependent activator of X chromosome inactivation. Cell. 2009; 139:999–1011. [PubMed: 19945382]
32. Barakat TS, Gunhanlar N, Pardo CG, Achame EM, Ghazvini M, Boers R, Kenter A, Rentmeester E, Grootegoed JA, Gribnau J. RNF12 activates Xist and is essential for X chromosome inactivation. PLoS Genet. 2011; 7:e1002001. [PubMed: 21298085] Ref 31 and 32 together describe the E3 ubiquitin ligase, RNF12, as a positive regulator of *Xist* expression. Overexpression of RNF12 was shown to induce ectopic Xist clouds and deleting *Rnf12* results in delayed *Xist* upregulation.
33. Navarro P, Moffat M, Mullin NP, Chambers I. The X-inactivation trans-activator Rnf12 is negatively regulated by pluripotency factors in embryonic stem cells. Hum Genet. 2011; 130:255–264. [PubMed: 21544581]
34. Shin J, Bossenz M, Chung Y, Ma H, Byron M, Taniguchi-Ishigaki N, Zhu X, Jiao B, Hall LL, Green MR, et al. Maternal Rnf12/RLIM is required for imprinted X-chromosome inactivation in mice. Nature. 2010; 467:977–981. [PubMed: 20962847] In this paper authors generaged *Rnf12* knockout mice and show that RNF12 is essential for imprinted XCI. However, ES cells derived from $Rnf12^{-/-}$ knockout embryos are capable of random XCI, in contrast to results from studies in ES cells (ref 31–32). Combined, these studies imply the existence of other XCI activation pathway(s) through which cells can circumvent the absence of RNF12 in ES cells.
35. Bacher CP, Guggiari M, Brors B, Augui S, Clerc P, Avner P, Eils R, Heard E. Transient colocalization of X-inactivation centres accompanies the initiation of X inactivation. Nat Cell Biol. 2006; 8:293–299. [PubMed: 16434960]
36. Xu N, Tsai CL, Lee JT. Transient homologous chromosome pairing marks the onset of X inactivation. Science. 2006; 311:1149–1152. [PubMed: 16424298]
37. Xu N, Donohoe ME, Silva SS, Lee JT. Evidence that homologous X-chromosome pairing requires transcription and Ctcf protein. Nat Genet. 2007; 39:1390–1396. [PubMed: 17952071]
38. Masui O, Bonnet I, Le Baccon P, Brito I, Pollex T, Murphy N, Hupe P, Barillot E, Belmont AS, Heard E. Live-cell chromosome dynamics and outcome of X chromosome pairing events during ES cell differentiation. Cell. 2011; 145:447–458. [PubMed: 21529716] This article describes the first use of live cell imaging to observe X chromosome pairing in mouse embryonic stem cells. Direct visualization of pairing confirmed that it is a transient event lasting ~45 min. Tsix levels are observed to become asymmetrical following pairing.
39. Augui S, Filion GJ, Huart S, Nora E, Guggiari M, Maresca M, Stewart AF, Heard E. Sensing X chromosome pairs before X inactivation via a novel X-pairing region of the Xic. Science. 2007; 318:1632–1636. [PubMed: 18063799]
40. Sun S, Fukue Y, Nolen L, Sadreyev RI, Lee JT. Characterization of Xpr (Xpct) reveals instability but no effects on X-chromosome pairing or Xist expression. Transcription. 2010; 1:46–56. [PubMed: 21327163]
41. Ng K, Daigle N, Bancaud A, Ohhata T, Humphreys P, Walker R, Ellenberg J, Wutz A. A system for imaging the regulatory noncoding Xist RNA in living mouse embryonic stem cells. Mol Biol Cell. 22:2634–2645. [PubMed: 21613549]
42. Duthie SM, Nesterova TB, Formstone EJ, Keohane AM, Turner BM, Zakian SM, Brockdorff N. Xist RNA exhibits a banded localization on the inactive X chromosome and is excluded from autosomal material in cis. Hum Mol Genet. 1999; 8:195–204. [PubMed: 9931327]
43. Jeon Y, Lee JT. YY1 tethers Xist RNA to the inactive X nucleation center. Cell. 2011; 146:119–133. [PubMed: 21729784] This paper identified YY1 as a factor that dictates the *cis* specific localization of Xist. It demonstrated that YY1 binds both Xist RNA and DNA thereby bridging Xist and Xi. While Xist can migrate in trans, it is prevented from doing so because of YY1 specifically bound to nucleation sites on the Xi.
44. Beletskii A, Hong YK, Pehrson J, Egholm M, Strauss WM. PNA interference mapping demonstrates functional domains in the noncoding RNA Xist. Proc Natl Acad Sci U S A. 2001; 98:9215–9220. [PubMed: 11481485]

45. Sarma K, Levasseur P, Aristarkhov A, Lee JT. Locked nucleic acids (LNAs) reveal sequence requirements and kinetics of Xist RNA localization to the X chromosome. Proc Natl Acad Sci U S A. 2010; 107:22196–22201. [PubMed: 21135235]
46. Helbig R, Fackelmayer FO. Scaffold attachment factor A (SAF-A) is concentrated in inactive X chromosome territories through its RGG domain. Chromosoma. 2003; 112:173–182. [PubMed: 14608463]
47. Pullirsch D, Hartel R, Kishimoto H, Leeb M, Steiner G, Wutz A. The Trithorax group protein Ash2l and Saf-A are recruited to the inactive X chromosome at the onset of stable X inactivation. Development. 2010; 137:935–943. [PubMed: 20150277]
48. Hasegawa Y, Brockdorff N, Kawano S, Tsutui K, Nakagawa S. The matrix protein hnRNP U is required for chromosomal localization of Xist RNA. Dev Cell. 2010; 19:469–476. [PubMed: 20833368] This paper showed that hnRNP U is required for Xist localization. Knockdown hnRNP U resulted in loss of Xist foci and this further led to loss of gene silencing in ES cells. Interaction between hnRNP U and Xist was confirmed *in vivo*.
49. Romig H, Fackelmayer FO, Renz A, Ramsperger U, Richter A. Characterization of SAF-A, a novel nuclear DNA binding protein from HeLa cells with high affinity for nuclear matrix/scaffold attachment DNA elements. Embo J. 1992; 11:3431–3440. [PubMed: 1324173]
50. Agrelo R, Souabni A, Novatchkova M, Haslinger C, Leeb M, Komnenovic V, Kishimoto H, Gresh L, Kohwi-Shigematsu T, Kenner L, et al. SATB1 defines the developmental context for gene silencing by Xist in lymphoma and embryonic cells. Dev Cell. 2009; 16:507–516. [PubMed: 19386260]
51. Wutz A, Jaenisch R. A shift from reversible to irreversible X inactivation is triggered during ES cell differentiation. Mol Cell. 2000; 5:695–705. [PubMed: 10882105]
52. Lyon MF. The Lyon and the LINE hypothesis. Semin Cell Dev Biol. 2003; 14:313–318. [PubMed: 15015738]
53. Chow JC, Ciaudo C, Fazzari MJ, Mise N, Servant N, Glass JL, Attreed M, Avner P, Wutz A, Barillot E, et al. LINE-1 activity in facultative heterochromatin formation during X chromosome inactivation. Cell. 2010; 141:956–969. [PubMed: 20550932]
54. Marks H, Chow JC, Denissov S, Francoijs KJ, Brockdorff N, Heard E, Stunnenberg HG. High-resolution analysis of epigenetic changes associated with X inactivation. Genome Res. 2009; 19:1361–1373. [PubMed: 19581487]
55. Casanova M, Preissner T, Cerase A, Poot R, Yamada D, Li X, Appanah R, Bezstarosti K, Demmers J, Koseki H, et al. Polycomblike 2 facilitates the recruitment of PRC2 Polycomb group complexes to the inactive X chromosome and to target loci in embryonic stem cells. Development. 2011; 138:1471–1482. [PubMed: 21367819]
56. Changolkar LN, Costanzi C, Leu NA, Chen D, McLaughlin KJ, Pehrson JR. Developmental changes in histone macroH2A1-mediated gene regulation. Mol Cell Biol. 2007; 27:2758–2764. [PubMed: 17242180]
57. Tanasijevic B, Rasmussen TP. X chromosome inactivation and differentiation occur readily in ES cells doubly-deficient for macroH2A1 and macroH2A2. PLoS One. 2011; 6:e21512. [PubMed: 21738686]
58. Pasque V, Gillich A, Garrett N, Gurdon JB. Histone variant macroH2A confers resistance to nuclear reprogramming. EMBO J. 2011; 30:2373–2387. [PubMed: 21552206]
59. Baumann C, De La Fuente R. ATRX marks the inactive X chromosome (Xi) in somatic cells and during imprinted X chromosome inactivation in trophoblast stem cells. Chromosoma. 2009; 118:209–222. [PubMed: 19005673]
60. Chan KM, Zhang H, Malureanu L, van Deursen J, Zhang Z. Diverse factors are involved in maintaining X chromosome inactivation. Proc Natl Acad Sci U S A. 2011; 108:16699–16704. [PubMed: 21940502]
61. Prasanth SG, Prasanth KV, Siddiqui K, Spector DL, Stillman B. Human Orc2 localizes to centrosomes, centromeres and heterochromatin during chromosome inheritance. EMBO J. 2004; 23:2651–2663. [PubMed: 15215892]

62. Kalantry S, Purushothaman S, Bowen RB, Starmer J, Magnuson T. Evidence of Xist RNA-independent initiation of mouse imprinted X-chromosome inactivation. Nature. 2009; 460:647–651. [PubMed: 19571810]

63. Namekawa SH, Payer B, Huynh KD, Jaenisch R, Lee JT. Two-step imprinted X inactivation: repeat versus genic silencing in the mouse. Mol Cell Biol. 2010; 30:3187–3205. [PubMed: 20404085] This paper showed that imprinted XCI in the early embryo occurs in two sequential steps, the first involving repetitive elements silencing on Xp evident by the 2-cell stage. This is then followed by genic silencing in the morula-blastocyst stages. The study resolves the controversy between earlier studies on the timing of imprinted XCI in the early embryo.

64. Patrat C, Okamoto I, Diabangouaya P, Vialon V, Le Baccon P, Chow J, Heard E. Dynamic changes in paternal X-chromosome activity during imprinted X-chromosome inactivation in mice. Proc Natl Acad Sci U S A. 2009; 106:5198–5203. [PubMed: 19273861]

65. Mak W, Nesterova TB, de Napoles M, Appanah R, Yamanaka S, Otte AP, Brockdorff N. Reactivation of the paternal X chromosome in early mouse embryos. Science. 2004; 303:666–669. [PubMed: 14752160]

66. Okamoto I, Otte AP, Allis CD, Reinberg D, Heard E. Epigenetic dynamics of imprinted X inactivation during early mouse development. Science. 2004; 303:644–649. [PubMed: 14671313]

67. Silva J, Nichols J, Theunissen TW, Guo G, van Oosten AL, Barrandon O, Wray J, Yamanaka S, Chambers I, Smith A. Nanog is the gateway to the pluripotent ground state. Cell. 2009; 138:722–737. [PubMed: 19703398]

68. Ohhata T, Senner CE, Hemberger M, Wutz A. Lineage-specific function of the noncoding Tsix RNA for Xist repression and Xi reactivation in mice. Genes Dev. 2011; 25:1702–1715. [PubMed: 21852535]

69. Williams LH, Kalantry S, Starmer J, Magnuson T. Transcription precedes loss of Xist coating and depletion of H3K27me3 during X-chromosome reprogramming in the mouse inner cell mass. Development. 2011; 138:2049–2057. [PubMed: 21471155] In this paper authors reported that some X-linked genes were reactivated even before loss of Xist foci as well as H2K27Me3 from Xp suggesting a potential mechanism of Xp reactivation that occurs independently of Xist.

70. Ohno, S. Sex Chromosomes and Sex-Linked Genes. Berlin: Springer-Verlag; 1967.

71. Gupta V, Parisi M, Sturgill D, Nuttall R, Doctolero M, Dudko OK, Malley JD, Eastman PS, Oliver B. Global analysis of X-chromosome dosage compensation. J Biol. 2006; 5:3. [PubMed: 16507155]

72. Lin H, Gupta V, Vermilyea MD, Falciani F, Lee JT, O'Neill LP, Turner BM. Dosage compensation in the mouse balances up-regulation and silencing of X-linked genes. PLoS Biol. 2007; 5:e326. [PubMed: 18076287]

73. Nguyen DK, Disteche CM. Dosage compensation of the active X chromosome in mammals. Nat Genet. 2006; 38:47–53. [PubMed: 16341221]

74. Xiong Y, Chen X, Chen Z, Wang X, Shi S, Zhang J, He X. RNA sequencing shows no dosage compensation of the active X-chromosome. Nat Genet. 2010; 42:1043–1047. [PubMed: 21102464]

75. Deng X, Hiatt JB, Nguyen DK, Ercan S, Sturgill D, Hillier LW, Schlesinger F, Davis CA, Reinke VJ, Gingeras TR, et al. Evidence for compensatory upregulation of expressed X-linked genes in mammals, Caenorhabditis elegans and Drosophila melanogaster. Nat Genet. 2011; 43:1179–1185. [PubMed: 22019781]

76. Yildirim E, Sadreyev RI, Pinter SF, Lee JT. X-chromosome hyperactivation in mammals via nonlinear relationships between chromatin states and transcription. Nat Struct Mol Biol. 2011; 19:56–61. [PubMed: 22139016]

77. Kharchenko PV, Xi R, Park PJ. Evidence for dosage compensation between the X chromosome and autosomes in mammals. Nat Genet. 2011; 43:1167–1169. author reply 1171-1162. [PubMed: 22120048]

78. Lin H, Halsall JA, Antczak P, O'Neill LP, Falciani F, Turner BM. Relative overexpression of X-linked genes in mouse embryonic stem cells is consistent with Ohno's hypothesis. Nat Genet. 2011; 43:1169–1170. author reply 1171-1162. [PubMed: 22120049] Ref 74–78: Nguyen *et al.* present microarray analysis of somatic human and mouse tissues and show that the general

transcription level of Xa is twice that of autosomes. X linked gene upregulation was not observed in germ cells since these cells do not show a disparity in X-autosome expression. This is the first evidence of dosage compensation in mammals. Lin *et al.* confirm X upregulation in mouse embryonic stem cells. Microarray analysis revealed that both male and female ES cells upregulate their X chromosome to achieve balance in X to autosome expression. Xiong *et al.* analyze RNA sequencing data and include all genes on the X chromosome for their analysis regardless of expression. They conclude based on their findings that dosage compensation does not exist in mammals. Deng *et al.*, Kharchenko *et al.*, Lin *et al.* and Yildirim *et al.* (Ref. 75–78) analyze existing RNA sequencing data and only include genes that are expressed from the X chromosome in their analysis, and demonstrate that Xa upregulation does occur for X-autosome dosage compensation in mammals. Additionally, Deng *et al.* and Yildirim *et al.* show that the Xa is enriched for active chromatin marks.

79. Chu C, Qu K, Zhong FL, Artandi SE, Chang HY. Genomic Maps of Long Noncoding RNA Occupancy Reveal Principles of RNA-Chromatin Interactions. Mol Cell. 2011; 44:667–678. [PubMed: 21963238]

80. Simon MD, Wang CI, Kharchenko PV, West JA, Chapman BA, Alekseyenko AA, Borowsky ML, Kuroda MI, Kingston RE. The genomic binding sites of a noncoding RNA. Proc Natl Acad Sci U S A. 2011; 108:20497–20502. [PubMed: 22143764]

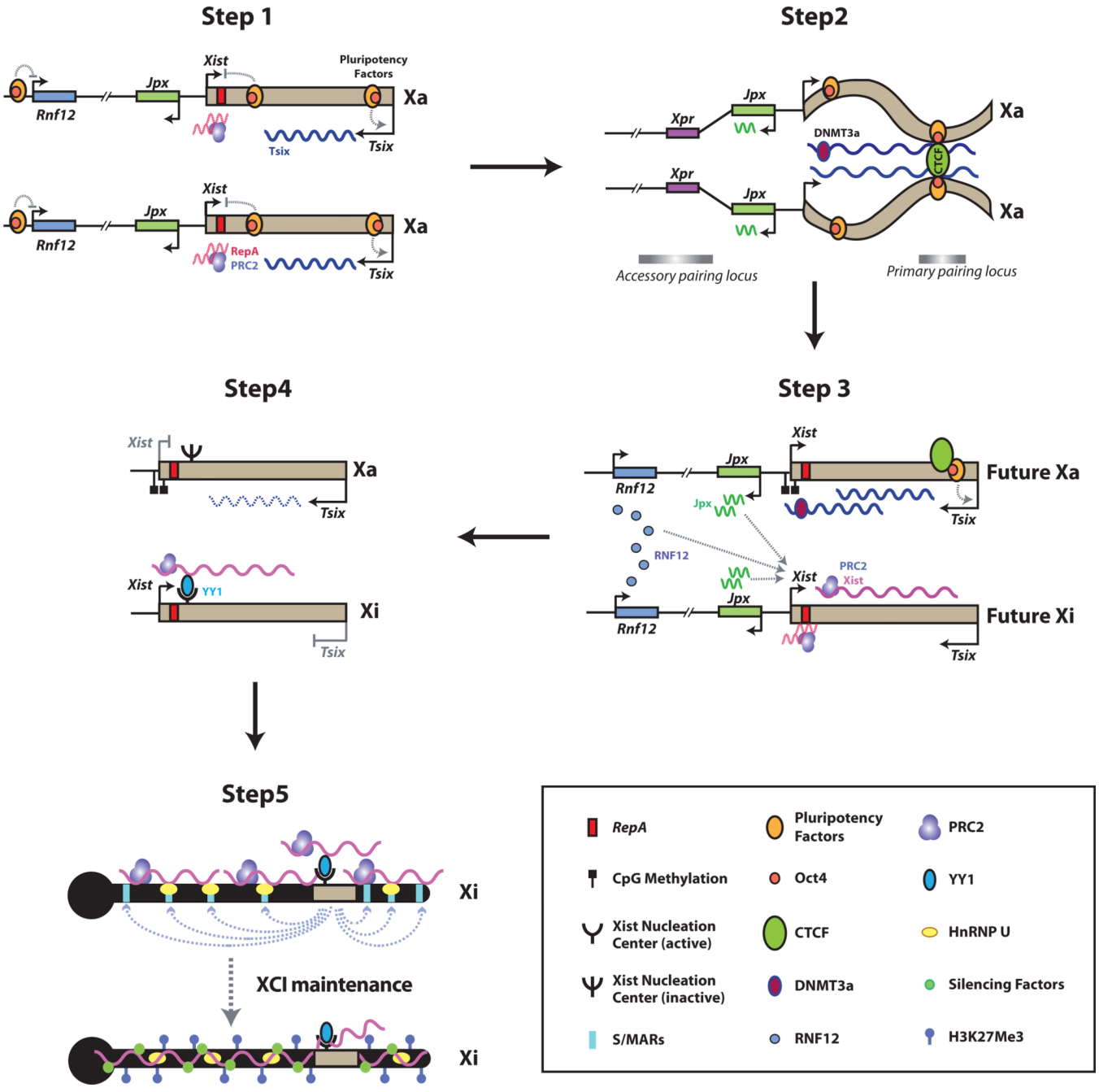

Figure 1. Random X chromosome inactivation in mouse embryonic stem cells upon differentiation

Step 1 – In undifferentiated ES cells, OCT4 and other pluripotency factors repress expression of *Xist* and the *Xist* activator *Rnf12*. Pluripotency factors also directly activate *Tsix* expression. *Tsix* is expressed from both X chromosomes and prevents activation of *Xist* in *cis*.

Step 2 – At the onset of differentiation, X chromosomes pair via interaction between OCT4 and CTCF resulting in uneven distribution of transcription/pluripotency factors on the two Xs. This leads to simultaneous activation of Xist from the future Xi at the same time

repressing *Tsix* from the same allele. Tsix recruits the DNA methyltransferase DNMT3a which methylates *Xist* promoter thereby silencing it on the future Xa.
Step 3 – RepA RNA is expressed from the future Xi and recruits the PRC2 complex. *Jpx* and *Rnf12* are upregulated from both the Xi and Xa. A combination of all three events leads to activation of *Xist* expression. Xist in turn recruits PRC2 to the Xi.
Step 4 –YY1 tethers Xist to the Xi and mediates spreading in *cis* along the entire Xi.
Step 5 – Xist tethered onto Xi spreads via hnRNP U and S/MAR sites. Eventually, Xist coats the entire Xi and recruits various silencing factors to maintain Xi in a repressed state.

Table 1
Factors affecting Xist Expression and Localization

A - Factors known to affect transcription of Xist RNA. Boxes shaded in pink represent negative regulators of Xist expression and boxes in green indicate positive regulators of Xist.
B - Factors known to affect localization of Xist RNA.

A

Factors	Regulators of Xist Expression		References
Tsix	Antisense *cis* acting repressor of *Xist* transcription		[17]
Xite	Enhancer of *Tsix* transcription		[18]
Pluripotency Factors	Via *Xist* Repression	OCT4, SOX2, NANOG	[24,25]
	Via *Tsix* Activation	OCT4, SOX2, REX1, c-MYC	[24,26]
	Via *Rnf12* Repression	NANOG, OCT4, SOX2	[31]
Jpx	IncRNA activator of *Xist*. Can function in *trans*		[28]
RNF12	*Trans* acting activator of *Xist* expression		[29,30,32]
RepA	RNA transcribed within *Xist* Exon 1 Harbors Repeat A and activates *Xist* transcription		[3]

B

Factors	Regulators of Xist Localization	References
YY1	Tethers Xist to the *Xic* and controls spread of Xist in *cis*	[42]
HnRNP U/ SAF-A	Interacts with Xist and required for localization to the Xi	[46]
SATB1	Xist deposition/ Required for Xist mediated silencing	[48]
Repeat C	Required for Xist localization (YY1 binding region)	[42,43]