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Long Non-coding RNAs in the X-inactivation Center

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Abstract

The X-inactivation center is a hotbed of functional long non-coding RNAs in eutherian mammals. These RNAs are thought to help orchestrate the epigenetic transcriptional states of the two X-chromosomes in females as well as of the single X-chromosome in males. To balance X-linked gene expression between the sexes, females undergo transcriptional silencing of most genes on one of the two X-chromosomes in a process termed X-chromosome inactivation. While one X-chromosome is inactivated, the other X-chromosome remains active. Moreover, with a few notable exceptions, the originally established epigenetic transcriptional profiles of the two is maintained as such through many rounds of cell division, essentially for the life of the organism. The stable divergent transcriptional fates of the two X-chromosomes, despite residing in a shared nucleoplasm, make X-inactivation a paradigm of epigenetic transcriptional regulation. Originally proposed in 1961 by Mary Lyon, the X-inactivation hypothesis has been validated through much experimentation over the last fifty years. In the last 25 years, the discovery and functional characterization has firmly established X-linked long non-coding RNAs as key players in choreographing X-chromosome inactivation.

Keywords

Xist; Tsix; Polycomb group; Jpx; Tsx; Ftx; RepA; X-inactivation; X-chromosome inactivation; histone modifications; epigenetic regulation

A key set of insights in X-inactivation came through chromosomal translocations and truncations involving the X-chromosome. Through the study of these aberrant X-chromosomes in mice and mouse embryonic stem cells (ESCs), as well as in human disorders, a region on the X-chromosome was pinpointed as being both necessary and sufficient to bring about inactivation. One of the most well-studied translocations is the mouse T16H Searle's translocation, a reciprocal translocation between the X-chromosome and chromosome 16 (Eicher et al., 1972). Cytological assessments suggested that only one of the translocation products, 16^X, but not the other, X¹⁶, can undergo inactivation (Rastan, 1983, Takagi, 1980). Based on these observations, a region required for X-inactivation – the X-inactivation center – was predicted to reside distal to the T16H breakpoint (Rastan, 1983). A second mutation, termed HD3, in mouse ESCs truncated the X-chromosome but did not impede X-inactivation (Rastan and Robertson, 1985). Thus, the X-inactivation center was delimited to the interval between the T16H and HD3 breakpoints. Initial banding studies of these chromosomes followed by comparative genetic analyses of multiple rearranged X-chromosomes in mice, including the T16H translocation, narrowed the X-inactivation center to roughly 8 centimorgan (Brown, 1991, Keer et al., 1990, Rastan and Brown, 1990). These mapping experiments pinpointed the mouse T16H breakpoint to lie just proximal to the *Zfx*

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locus (Keer et al., 1990). Mapping the HD3 breakpoint would have similarly delineated the distal end of the X-inactivation center, but the instability of this particular ESC line seems to have precluded molecular mapping (Brown, 1991).

The human X-inactivation center was also defined by X-chromosomal abnormalities. In humans, the X-inactivation center was mapped distal to the *AR*, *CCG-1*, *RPS4X*, and *PHKA* loci and proximal to *Pgk1* (Brown et al., 1991a, Brown et al., 1991b). A comparison of the X-inactivation center regions of mice and humans demonstrated that they both belonged to a conserved linkage group (Brown, 1991).

Xist

Amongst the first and perhaps the most iconic of all long non-coding RNAs, the *X-inactive specific transcript* or *Xist* maps to the X-inactivation center (Figure 1). Since its discovery in 1991, a large body of work has anointed *Xist* as the epicenter for the epigenetic inactivation of the X-chromosome. *XIST* was first identified based on hybridization of a human cDNA probe to female but not male samples (Brown et al., 1991a). This cDNA clone intriguingly mapped to the human X-inactivation center (Brown et al., 1991a, Brown et al., 1991b). The sex-specific expression and the location of the transcript within the X-inactivation center made *XIST* a compelling candidate regulator of X-inactivation. The mouse homolog, *Xist*, was identified shortly thereafter, and similarly found to show inactive X-specific expression (Borsani et al., 1991, Brockdorff et al., 1991). *Xist* RNA was subsequently found to physically coat the inactive-X chromosome, and studies in mice demonstrated that *Xist* remains associated with the inactive-X during mitosis (Figure 2) (Brown et al., 1992, Clemson et al., 1996, Jonkers et al., 2008). The presence of *Xist* on the mitotic inactive-X supports its role as the transmitter of the epigenetic state of the inactive-X from one cell division cycle to the next. In human cells, however, *Xist* RNA appears to dissociate from the X-chromosome during mitosis (Clemson et al., 1996, Hall and Lawrence, 2003).

Xist has been shown to be instrumental in the two forms of inactivation found in mice, the pre-eminent experimental model system of X-inactivation: imprinted and random X-inactivation. During imprinted X-inactivation, the paternally-inherited X-chromosome is preferentially inactivated (Takagi and Sasaki, 1975). Imprinted X-inactivation initiates at the 4- to 8- cell stage of zygotic development, and is accompanied by *Xist* induction only from the paternal-X (Okamoto et al., 2004, Kalantry et al., 2009, Namekawa et al., 2010). Quite unusually, *Xist* RNA upregulation results in coating *in cis* by the *Xist* RNA of the paternal-X (Figures 2 & 3) (Okamoto et al., 2004, Kalantry et al., 2009, Patrat et al., 2009, Sheardown et al., 1997, Mak et al., 2004). By the blastocyst stage of development (64–128 cell stage), most genes on the paternal-X have either undergone complete silencing or will do so shortly thereafter. Strikingly, at the peri-implantation stage of development (128–256 cell stage), the paternal-X undergoes reactivation but only in the epiblast lineage (Mak et al., 2004, Sheardown et al., 1997, Williams et al., 2011). These cells, which will give rise to all the tissue-types of the fetus, subsequently undergo random X-inactivation (Rastan, 1982, McMahon et al., 1983). In random X-inactivation, either the maternally-inherited or paternally-inherited X-chromosome is stochastically selected for inactivation. In contrast to the embryonic lineages, the extra-embryonic lineages, which give rise to the placenta and the yolk sac, maintain imprinted inactivation that of the paternal-X (Harper et al., 1982, Takagi and Sasaki, 1975, Takagi et al., 1978, West et al., 1977).

At the onset of both imprinted and random X-inactivation, *Xist* RNA is induced from and coats the X-chromosome that will become inactivated, thus suggesting a causal role in inactivation itself. In agreement, mutational studies have shown that *Xist* is essential for

both imprinted and random X-inactivation in mice. Embryos that inherit a paternally-transmitted Xist mutation die due to compromised extra-embryonic development, consistent with a defect in imprinted X-inactivation (Marahrens et al., 1997, Kalantry et al., 2009). Analysis of the epiblast-derived tissues, which have earlier undergone random X-inactivation, indicates that all fetal cells harboring a heterozygous Xist mutation will preferentially inactivate the wild-type X-chromosome (Marahrens et al., 1998, Kalantry et al., 2009). In differentiating female ESCs, which are derived from the epiblast lineage and are the favored *in vitro* random X-inactivation model system, X-inactivation is also biased in cells heterozygous for a null Xist mutation (Penny et al., 1996). These biases in random X-inactivation suggest that Xist may be required *in cis* to bring about silencing of the chromosome from which it is expressed. However, Xist-heterozygosity biases the *choice* of which X-chromosome becomes inactivated, such that the wild-type X is preferentially selected to become inactivated; the mutant-X therefore never has the option of being inactivated. Thus, strictly speaking, the biased choice step which necessarily precedes random X-inactivation precludes knowing if Xist is required for inactivation itself (see the Tsix section below for a discussion of X-chromosome choice).

The most convincing evidence supporting a role for Xist in triggering silencing is via transgenes that ectopically express Xist (Plath *et al.* 2002; Wutz and Jaenisch 2000; Wutz, Rasmussen, and Jaenisch 2002). In cultured ESCs, Xist transgenes can variably induce silencing of reporter constructs or endogenous genes surrounding the insertion site. Silencing is dependent on the genomic site of integration, the expression level, copy number of the transgene, as well as the inclusion of Xist regulatory regions present in the transgene. For example, a multi-copy 450 kb mouse transgene has been shown to induce Xist RNA expression and coating, as well as silencing of a LacZ reporter within the transgene in male ESCs (Lee et al., 1996). Additionally, fibroblast cells that were derived from adult chimeric mice generated by injecting the transgenic ESCs into wild-type embryos displayed silencing of four endogenous autosomal genes spread across the length of the transgenic chromosome (Lee et al., 1996, Lee and Jaenisch, 1997). The conclusion in these studies was that the entire X-inactivation center function can be recapitulated by the 450 kb transgene sequence. Of note, however, is that haploinsufficiency for large regions of autosomes, which would occur in these cells if the Xist transgene resulted in extensive silencing of endogenous autosomal genes, typically results in early embryonic lethality, as suggested by studies of monosomic embryos and embryos harboring large chromosomal deletions (Baranov, 1983, Magnuson et al., 1985). The extensive contribution of transgenic ESCs to adult chimeric mice, which were estimated to show up to 90% chimerism, suggests that silencing of endogenous genes by this transgene may be weak (Lee et al., 1996, Lee and Jaenisch, 1997).

While multi-copy transgenes can bring about Xist induction and potentially gene silencing, single copies of similarly large transgenes are unable to induce silencing in ESCs. A single-copy 460 kb X-inactivation center transgene including Xist showed negligible Xist induction in a number of adult cell types and was insufficient to silence a linked LacZ reporter cassette in mice, leading to the conclusion that the transgene does not contain sequences within it to induce Xist expression (Heard et al., 1996, Heard et al., 1999). The same animals, however, display imprinted Xist expression in early mouse embryos; Xist is only induced when the transgene is paternally-inherited (Okamoto et al., 2005). Ectopic Xist RNA expression and coating in this study correlates with transcriptional silencing of a gene within the transgene construct; whether endogenous genes near the insertion site are also silenced, though, is not known. The fact that the development of these animals is not defective argues against large-scale inactivation of endogenous loci that reside at or near the transgene integration site. Moreover, given the failure of transgenic Xist expression in cells that undergo random X-inactivation, the ability of the same transgene to express Xist and silence during imprinted X-inactivation is paradoxical. This differential silencing ability may suggest divergent

mechanisms that influence both the expression and function of Xist RNA during imprinted versus random X-inactivation.

While large transgenes that harbor the Xist locus as well as other elements of the X-inactivation center are not always sufficient to induce silencing, single-copy inducible Xist transgenes often are. For example, inducible Xist cDNA transgenes targeted to the *Hprt* locus on the X-chromosome or on autosomes are able to trigger silencing of endogenous genes (Wutz and Jaenisch, 2000, Wutz et al., 2002, Jiang et al., 2013). This silencing function may, however, be due to the artificially high levels of Xist expression from these inducible transgenes. Some evidence also suggests that ectopic Xist induction is able to silence genes in some cell types *in vivo*, not just in cultured cells. In transgenic mice harboring an inducible Xist transgene, Xist induction is able to lead to ectopic X-inactivation in immature hematopoietic precursor cells, but not hematopoietic stem cells or mature cells (Savarese et al., 2006). Similar to studies of Xist transgenes in ESCs, this work suggests that there is a window of opportunity during development when Xist RNA is able to silence; this silencing function is closely linked to the differentiation state of cells as well as, importantly, to the level of Xist expression (Savarese et al., 2006, Wutz and Jaenisch, 2000).

Xist is thought to function by recruiting proteins to the prospective inactive-X to modify its chromatin structure and alter gene expression. Xist RNA expression is followed by the formation of a repressive chromatin state that excludes transcriptional machinery from the inactive-X, potentially by recruiting chromatin-modifying proteins (Chaumeil et al., 2006). These proteins are thought to help establish the heterochromatic and transcriptionally inert chromatin state characteristic of the inactive X-chromosome. Xist RNA is known to recruit Polycomb group proteins, a process in which the RepA non-coding RNA that is encoded within Xist may play a role (see below) (Silva et al., 2003, Plath et al., 2003, Kohlmaier et al., 2004, Zhao et al., 2008). The Polycomb group proteins form two complexes, Polycomb repressive complex 1 (PRC1) and Polycomb repressive complex 2 (PRC2). These complexes catalyze repressive histone modifications that are enriched on the inactive-X, such as ubiquitination of lysine 119 in histone H2A (H2A-K119ub) and trimethylation of histone H3 lysine 27 (H3-K27me3; see Figures 2 & 3) (Gieni and Hendzel, 2009, Margueron and Reinberg, 2010, Sauvageau and Sauvageau, 2010, Surface et al., 2010). While Polycomb group proteins are perhaps the best known of the Xist recruits, a number of other proteins are also localized to the inactive-X, potentially via Xist RNA. Ash2l, a member of the Trithorax group of chromatin modifying proteins, is recruited to the inactive-X following the onset of X-inactivation (Pullirsch et al., 2010). Paradoxically, the trithorax group proteins catalyze H3K4 trimethylation, a chromatin modification typically associated with active transcription (Steward et al., 2006). The recruitment of Ash2l coincides with the recruitment of SAF-A, a nuclear scaffolding factor (Pullirsch et al., 2010). The histone variant macroH2A, a variant associated with transcriptional repression, is enriched on the inactive-X as well (Costanzi and Pehrson, 1998, Perche et al., 2000, Rasmussen et al., 2000).

While important advances have been made in mechanisms underlying Xist function in X-inactivation, numerous crucial gaps remain. First, the temporal and lineage-specific function of Xist in X-linked gene silencing remains unclear; the Xist RNA appears to be required during precise developmental windows in both imprinted and random X-inactivation. Evidence shows that Xist is dispensable during the early initiation phase of imprinted X-inactivation for many X-linked genes assayed (Kalantry et al., 2009). Conversely, Xist is also not required to maintain random X-inactivation in differentiated cells, despite the persistence of Xist RNA coating in somatic cells (Brown and Willard, 1994, Csankovszki et al., 1999, Wutz and Jaenisch, 2000). The data therefore suggest that Xist plays a tightly

regulated, temporally-specific role in controlling X-inactivation. Additionally, in both imprinted and random X-inactivation, gene expression in the absence of Xist varies from gene to gene. Some genes are dependent more on Xist for silencing, while others are less so (Csankovszki et al., 1999, Kalantry et al., 2009).

In addition to questions regarding the context-dependent requirement for Xist RNA in transcriptional silencing, how precisely Xist RNA acts as a catalyst for inactivation – i.e., through which of its recruited proteins – remains largely unknown. Both PRC1 and PRC2 that are recruited to the inactive-X by Xist are dispensable for random X-inactivation (Leeb and Wutz, 2007, Kalantry and Magnuson, 2006, Schoeftner et al., 2006). Mutations in SAF-A, another recruit of Xist, disrupt both Xist localization and X-linked gene silencing in ES cells, though not absolutely (Hasegawa et al., 2010). Both SAF-A and Ash2l, which are recruited to the inactive-X after the onset of X-inactivation, are able to be recruited to the X-chromosome by mutant Xist transcripts that are unable to induce X-linked gene silencing (Pullirsch et al., 2010). Furthermore, a null mutation in macroH2A1 does not result in defective X-inactivation (Changolkar et al., 2007). A paralog of MacroH2A1, macroH2A2, can potentially substitute for macroH2A1. In studies in which both macroH2A genes are knocked-down, X-inactivation is again normal (Tanasijevic and Rasmussen, 2011). These data suggest that additional *trans*-acting factors contribute to X-linked gene silencing. These may include additional proteins recruited by Xist RNA as well as via Xist-independent mechanisms.

Tsix

The anti-sense transcript to Xist, Tsix, was identified following the observation that the region 3' to Xist influences X-chromosome counting, a process during which the cell senses the number of X-chromosomes present and determines how many, if any, to inactivate (Clerc and Avner 1998). In the seminal study by Clerc and Avner, XX female cells inactivate a single X-chromosome, as expected. However, XO female cells that have lost the wild-type X chromosome and which also harbor a 65 kb deletion 3' of Xist on their intact X-chromosome induce Xist RNA and initiate silencing of their single X-chromosome. The expectation is that cells with a single X-chromosome should not activate Xist expression or undergo X-inactivation. Thus, in the absence of the Xist 3' region, the cells failed to correctly identify the number of X-chromosomes present (Clerc and Avner, 1998). The 65 kb deleted segment, therefore, normally controls X-chromosome counting by suppressing Xist.

Shortly after the study by Clerc and Avner, assessment of the Xist 3' region using RNA fluorescence *in situ* hybridization detected an RNA anti-sense to Xist in both male and female ESCs (Lee et al., 1999a). The transcript, termed Tsix (Xist spelled backwards) is expressed in females from both X-chromosomes prior to X-inactivation (Figure 1); however, upon differentiation of female ESCs that triggers X-inactivation, Tsix is downregulated from the Xist-expressing inactive-X and is expressed only from the active X-chromosome. Following the onset of X-inactivation, Xist and Tsix thus show mutually exclusive expression from the inactive and active X-chromosomes, respectively. Unlike Xist, however, Tsix RNA is expressed at relatively low levels and does not coat the active X-chromosome.

Tsix transcription has been proposed to repress Xist at multiple key developmental time points. First, due to the early expression of Tsix, the Tsix RNA has been nominated as the instrument of the oocyte-derived imprint that inhibits Xist expression from the maternally-inherited X chromosome during the onset of imprinted X-inactivation (Lee, 2000, Sado et al., 2001). Continued expression of Tsix is then posited to keep the maternal-X from

undergoing X-inactivation in the extra-embryonic tissues of the developing embryo that maintain imprinted X-inactivation. This function of Tsix is clearly illustrated by the death of embryos harboring maternally-inherited Tsix mutations due to failed development of the extra-embryonic tissues (Lee, 2000, Sado et al., 2001).

Tsix also plays a prominent role in random X-inactivation. As part of its role as a repressor of Xist, Tsix has been proposed to function in the counting and choice processes of random X-inactivation. Random X-inactivation is thought to be a linear three-step process. In the first step, counting, the cell senses how many X-chromosomes it has (Lyon, 1962, Grumbach et al., 1963). If and only if there are two or more X-chromosomes does the choice step ensue. During the choice step, the cell selects which X chromosome will remain active, and which will be inactivated. Following the choice step, X-inactivation initiates (Rastan, 1983).

Evidence for a counting step in X-inactivation is supported by observations of cells harboring abnormal complements of sex chromosomes. While normal XY male cells do not undergo X-inactivation, XXY nuclei inactivate one of their two X-chromosomes. Furthermore, in females, diploid cells with more than two X-chromosomes will inactivate all but one X, while XO cells do not undergo X-inactivation. This suggests that inactivation occurs, in part, as a function of the number of X-chromosomes in the cell. The autosomal complement also plays a critical role in X-chromosome counting. While diploid XX cells always have a single active and single inactive X-chromosome, tetraploid cells maintain two active- and two inactive-Xs (Webb et al., 1992, Monkhorst et al., 2008). Tetraploid cells can therefore tolerate two active Xs. This suggests that both X-linked and autosomal factors contribute to X-chromosome counting, and mediate the decision as to whether to undergo X-inactivation or not.

Tsix was initially implicated as a counting factor based on a series of deletions adjacent to, and upstream of, the Tsix locus. These mutations can lead to aberrant Xist induction in differentiating XO female and XY male ESCs, a phenotype that is considered indicative of a counting defect (Clerc and Avner, 1998, Cohen et al., 2007, Vigneau et al., 2006). The DXPas34 repetitive sequence, located adjacent to Tsix exon 3, has been identified as a regulator of counting based on these genetic studies. DXPas34 functions to enhance Tsix expression, thereby influencing X-chromosome counting (Navarro et al., 2010, Cohen et al., 2007).

Tsix is also suggested to control the choice of which X-chromosome will be inactivated. In Tsix-heterozygous female embryos and ESCs, the Tsix-mutant X-chromosome is observed to always be the inactive-X (Lee and Lu, 1999, Sado et al., 2001). There are two models that could explain this bias. The first and most popular model is a primary non-random X-chromosome choice model, where the Tsix-mutant X is always chosen for inactivation due to ectopic Xist induction from the mutant-X at the onset of inactivation (Sado et al., 2001, Lee, 2000). A second possibility that could give rise to the observed bias is that random X-inactivation occurs normally, with both the wild-type and the mutant X-chromosome equally likely to undergo inactivation. Subsequently, Xist is ectopically expressed from the Tsix mutant X-chromosome if the wild-type X is initially chosen for inactivation. These cells would then rapidly be selected away due to two inactive Xs. Since inactivation of the wild-type X-chromosome is not observed at significant rates in differentiating Tsix-mutant ESCs and embryos, the model of primary non-random choice is favored. Incidentally, a secondary cell-selection effect has been invoked to explain X-inactivation patterns in Xist-heterozygous ESCs (Penny et al., 1996).

Numerous questions remain regarding the precise role of Tsix in X-inactivation. First, the role of Tsix in counting is highly contested. Mutations that abrogate Tsix RNA expression sometimes, but not always, lead to aberrant Xist induction (Lee, 2000, Luikenhuis et al., 2001, Morey et al., 2001, Sado et al., 2001, Ohhata et al., 2006, Vigneau et al., 2006). Since Xist is not always induced in cells lacking Tsix, Tsix RNA itself may not be directly involved in counting. The DXPas34 enhancer of Tsix has also been implicated in X-chromosome counting, and was initially presumed to act through Tsix RNA (Navarro et al., 2008, Cohen et al., 2007). While deletion of DXPas34 results in ectopic Xist induction that is consistent with a counting defect, an overdose of the DXPas34 genomic segment unexpectedly leads to failure of Xist induction (Lee, 2005). This genomic segment is proposed to function in counting by sequestering proteins that would normally activate Xist, i.e., by repressing Tsix.

Questions also remain about the mechanisms underlying Tsix-mediated regulation of Xist. DNA methylation and chromatin modifications of the Xist promoter region have been proposed as mechanisms through which Tsix may influence Xist expression. Tsix transcription across the Xist promoter indeed leads to DNA methylation and accumulation of repressive histone modifications at Xist promoter (Navarro et al., 2006, Sado et al., 2005). DNA methylation changes induced by Tsix, however, may not be a primary mechanism for regulation of Xist, as loss of both Dnmt3a and Dnmt3b, the *de novo* methyltransferases shown to associate with Tsix, does not lead to defects in X-inactivation (Sado et al., 2004).

Understanding the regulation of Tsix expression itself is also a work in progress. Induction of Tsix is dependent on the recruitment of Rex1, a pluripotency factor, to the Tsix locus (Navarro et al., 2010). Interestingly, *Rex1*^{-/-} female and male mice are born at the same rate, and show no defects in survival, suggesting that while Rex1 may contribute to Tsix regulation, it is not required for the establishment or maintenance of X-inactivation (Masui et al., 2008). Tsix regulation is also mediated by Xite, a non-coding RNA lying upstream of Tsix that promotes Tsix expression (Ogawa and Lee, 2003). The DXPas34 repetitive element additionally serves a dual role as both an enhancer and repressor of Tsix (Cohen et al., 2007). While these regulators of Tsix have been identified, the temporal requirement of these elements in regulating Tsix, Xist, and X-inactivation *in vivo* at the onset of both imprinted and random X-inactivation needs more scrutiny.

The Tsix RNA is also thought to be involved in the reactivation of the inactive paternal X-chromosome prior to random X-inactivation. The reactivation of the paternal-X is characterized by loss of Xist RNA coating in epiblast precursor cells, a process posited to be mediated by Tsix, though again no direct genetic evidence supports this assertion (Sheardown et al., 1997, Mak et al., 2004, Navarro et al., 2009, Nesterova et al., 2011). In contrast, reactivation is not disrupted in the epiblast lineage of embryos harboring paternally-inherited Tsix mutations, suggesting that Tsix may in fact be dispensable during reactivation of the inactive-X (Kalantry and Magnuson, 2006). Moreover, surprisingly, X-linked gene reactivation appears to occur prior to the loss of Xist coating during reactivation (Williams et al., 2011). If Tsix is involved in Xist repression and X-reactivation, how Tsix is induced from the inactive paternal-X is also unclear. Careful analysis of the expression and function of Tsix in these early embryonic stages in future studies may help elucidate the precise role of the Tsix long non-coding RNA in these processes.

Jpx/Enox

Jpx, also known as Enox (*Expressed neighbor of Xist*), is a non-coding RNA whose transcription starts around 10kb upstream of Xist and in the antisense orientation to Xist (Figure 1) (Johnston et al., 2002). Further studies of Jpx were inspired by the observation that a transgene containing an 80kb region of the X-inactivation center, including Xist, Tsix,

and the Tsix regulator Xite, was not capable of inducing Xist, suggesting that additional factors surrounding the *Xist* locus are required to recapitulate X-inactivation center function (Lee et al., 1999b). *Jpx* has been proposed to serve as an *Xist* activator and is required for inactivation to occur (Tian et al., 2010, Sun et al., 2013).

Jpx is expressed both in male and female ESCs, and becomes upregulated over the course of differentiation. This upregulation mimics *Xist* induction during differentiation of female ESCs and was used to suggest the involvement of *Jpx* RNA in *Xist* regulation. *Jpx* was subsequently shown to escape inactivation, consistent with its increased expression in females (Tian et al., 2010). However, *Jpx* is upregulated in both differentiating male and female ESCs, which may suggest an X inactivation-independent role.

To elucidate the role of *Jpx*, Tian *et al.*, deleted *Jpx* in male and female ESCs (Tian et al., 2010). In males, *Jpx* loss did not display a marked effect on expression of X-linked genes. Female ESCs heterozygous for *Jpx*, however, showed a severe phenotype upon differentiation. The cells have growth defects, high levels of cell death, as well as a significant decrease in nuclei with *Xist* RNA coating (Tian et al., 2010). The conclusion drawn from these data is that female cells expressing only half their normal levels of *Jpx* (equal to that in males) are deficient in *Xist* induction. Of note, though, the mutant female cells do display some low-level *Xist* expression, suggesting *Jpx*-independent activation of *Xist* in female cells.

In over-expression studies, a *Jpx* transgene rescued defective *Xist* induction and cell death in heterozygous *Jpx* mutant female cells (Tian et al., 2010). Both the growth phenotype as well as levels of *Xist* expression were brought back to normal with exogenous *Jpx*. These data therefore suggest that *Jpx* can, unusually, act in *trans* to activate *Xist*.

In wild-type ESCs, the same transgene leads to very low levels of ectopic *Xist* induction in both males and females (Sun et al., 2013). Tested with two different promoters, increased *Jpx* expression concurred with higher *Xist* expression, suggesting that *Jpx* activation of *Xist* works in a dose-dependent manner. It should be noted, though, that the *Jpx* transgene-mediated ectopic induction of *Xist* in male ESCs observed by Sun *et al.*, was not recapitulated in an independent study (Jonkers et al., 2009).

If *Jpx* activates *Xist*, then it should antagonize *Tsix* function, which normally represses *Xist*. In agreement, cells heterozygous for both a *Jpx* and a *Tsix* mutation on the same X-chromosome do not appear to suffer the same degree of cellular lethality that *Jpx* heterozygosity alone causes. Moreover, *Xist* expression in these cells is restored (Tian et al., 2010). Thus, if the *Xist* repressor *Tsix* is absent, *Jpx* is not needed to activate *Xist*.

Further experiments in which *Tsix* and *Jpx* levels are modulated support the opposing activities of the two long non-coding RNAs in *Xist* regulation. Male *Tsix*-mutant ESCs displayed low levels of ectopic *Xist* expression. The addition of a genomic *Jpx* transgene increased the level of *Xist* expression in *Tsix*-mutant cells (Sun et al., 2013). The level of *Xist* induction by *Jpx* in a *Tsix*-mutant male background is also greater than in wild-type male cells (Sun et al., 2013). Nevertheless, the increase in *Xist* coating is relatively small, though *Jpx* levels are doubled and equal to that of females. This finding therefore reinforces the idea that activators in addition to *Jpx* RNA function to upregulate *Xist* during X-inactivation. It appears that the effect *Jpx* has on *Xist* expression is most obvious in a *Tsix*-mutant background. The *Xist* inhibitory effects of *Tsix* seem to overpower potential *Xist* activating function of *Jpx*.

Jpx is proposed to activate *Xist* through the zinc finger protein CTCF (Sun et al., 2013). CTCF has binding sites upstream of the *Xist* promoter, and CTCF binding is thought to

normally inhibit Xist expression. In male ES cells, CTCF binding remains constant upon differentiation. However, in female cells CTCF binding is reduced on the inactive-X early during differentiation, which corresponds to the period when inactivation is commencing. Increasing levels of CTCF reduced Xist expression, but a Jpx transgene restored normal levels of Xist RNA. Sun *et al.*, therefore conclude that Jpx RNA and the Xist promoter DNA may compete for binding of CTCF, and Jpx is required to remove CTCF in order for Xist expression to occur. Consistently, CTCF binds Jpx RNA in a dose-dependent manner (Sun *et al.*, 2013). A conclusive role for Jpx in Xist activation, though, awaits genetic loss- and gain-of-function studies in mice.

Tsx

The Tsx non-coding RNA is located approximately 40kb from the 3' end of Xist, and transcribed in the antisense orientation to Xist (Figure 1) (Simmler *et al.*, 1996). Tsx is expressed at high levels in the testes and to a much lesser extent in the adult male and female brain (Anguera *et al.*, 2011).

Once thought to be protein coding, Tsx (Testes-specific X-linked) was postulated to encode a 144 amino acid protein of almost 16 kDa (Simmler *et al.*, 1996). However, immunostaining with anti-Tsx antiserum later showed premeiotic, testes specific staining that is inconsistent with Tsx mRNA expression (Cunningham *et al.*, 1998). Based on this fact, a recent study has looked more closely at the coding potential of the Tsx locus (Anguera *et al.*, 2011). Anguera *et al.*, tested whether putative Tsx open reading frames (ORFs) can express proteins. No protein was detected from multiple constructs containing Tsx ORFs, leading to the conclusion that Tsx may actually be non-coding. An alternative interpretation is that the ORFs may in fact be encoding a protein but a protein is not expressed due to other, for example, technical, reasons.

In order to study the affects of Tsx *in vivo*, Anguera *et al.*, also generated Tsx-mutant mice (Anguera *et al.*, 2011). Homozygous deletion of Tsx led to a small decrease in fertility of females that resulted in a sex-ratio distortion favoring, paradoxically, female offspring. Moreover, hemizygous males did not display decreased fertility. However, during pachytene-stage of spermatogenesis, when Tsx expression is normally at its highest, mutant male testes did show unusually high levels of apoptosis.

Mice normally exhibit a significant increase in Tsx expression during meiosis I of spermatogenesis (Anguera *et al.*, 2011). This is the stage of male meiosis during which meiotic sex chromosome inactivation (MSCI) occurs. In MSCI, the X and Y chromosomes are made transcriptionally inert, due to the lack of synapsis along most of the X and Y chromosomes (Turner *et al.*, 2005).

Despite the stringent silencing of X-linked genes during MSCI, Tsx is one of the few X-chromosomal loci that escape MSCI (Namekawa *et al.*, 2006). It could be postulated from this observation that Tsx may play a role in MSCI; however, the apoptotic spermatocytes of Tsx-mutant males do not show a defect in MSCI (Anguera *et al.*, 2011). The cause of apoptosis in these mutant cells is unidentified, although it doesn't seem to be MSCI related, and the observed cell death does not cause male fertility defects.

The close proximity of Tsx to the Xist locus suggested involvement of Tsx in X-inactivation, and loss-of-function studies in ESCs support a role for Tsx in Xist regulation (Anguera *et al.*, 2011). Both female and male ESCs express Tsx in undifferentiated cells, but female expression is significantly higher than in males, consistent with two Tsx alleles in females versus one in males. Upon ESC differentiation, Tsx is downregulated in females and this coincides with Xist upregulation and initiation of X-inactivation. Both female and male

Tsx-mutant ESCs show an increase in Xist coating on the active-X in small numbers of nuclei (Anguera et al., 2011). This finding suggests that Tsx may play a role in repressing Xist expression. Tsix, which represses Xist expression, is also greatly reduced in Tsx-mutant cells. The downregulation of Tsx, along with the position of Tsx just upstream of Tsix, suggests that Tsx may serve to activate Tsix RNA expression. Thus, Tsx mutations may only indirectly lead to Xist upregulation, by causing a decrease in Tsix expression. X-inactivation has not yet been assessed in Tsx mutant mice; however, the female fertility defect seen in Tsx-homozygous mutants leads to a relative increase in female offspring, making it unlikely that infertility is caused by an X-chromosome inactivation defect.

Ftx

The *Ftx* transcript, produced in the sense orientation to *Xist*, is posited to activate *Xist*. *Ftx* is localized about 150 kb upstream of *Xist*, is roughly 63 kb in length, and is composed of 15 exons (Figure 1) (Chureau et al., 2011). The *Ftx* genomic region generates various isoforms through a combination of different promoters, alternative splicing, and transcriptional termination. Recent experimental data also indicate the presence of two micro RNA clusters, miR-374 and miR471, embedded within intron 12 of *Ftx* (Miska et al., 2004, Suh et al., 2004). *Ftx* is expressed ubiquitously in adult tissues and the transcript is restricted to the nucleus (Chureau et al., 2011).

Ftx RNA is upregulated during the onset of X-inactivation in differentiating female mouse ESCs. In both randomly and imprinted inactivated lineages, *Ftx* partially escapes X-inactivation, but is expressed at lower levels from the inactive X-chromosome compared to the active-X (Chureau et al., 2011, Kunath et al., 2005, Mak et al., 2002).

Through a genetic deletion, Chureau *et al.*, functionally characterized *Ftx* in male ESCs (Chureau et al., 2011). *Ftx* deletion led to an interesting pattern of transcription for the genes in the vicinity of *Ftx*: genes transcribed in the same direction as *Ftx*, but not ones transcribed in the opposite orientation, were affected. The *Ftx* deletion led to a significant reduction in expression of surrounding genes, with a greater effect seen for genes closer to *Ftx*, suggesting a preferential role for *Ftx* in regulating genes that lie near it and which are transcribed in the same 5' to 3' orientation. Of note, absence of *Ftx* led to a significant decrease in *Xist* RNA levels and a change in the DNA methylation profile at the 5' end of *Xist* (Chureau et al., 2011). It should be pointed out that *Xist* expression is normally quite low in male ESCs and is not upregulated upon differentiation. Therefore, a decrease in Xist levels upon *Ftx* ablation is challenging to interpret. A deletion of *Ftx* in female ESCs would be more informative, since Xist expression is normally induced upon differentiation in female ESCs.

In the *Ftx*-mutant ESCs, DNA methylation is increased at a CpG island in exon 1 of *Xist*; this increase in DNA methylation coincided with a reduction in histone H3 lysine 4 dimethylation (H3-K4me2), a mark of transcriptional activation, at the *Xist* promoter. These findings suggested that *Ftx* plays a part in configuring the chromatin architecture in and around the *Xist* locus. The *Ftx* genomic region also harbors histone H3 lysine 9 (H3-K9me2) and H3-K27me3 methylation marks that are associated with transcriptional silencing (Heard et al., 2001, Rougeulle et al., 2004). Both of these marks, however, were found to be largely unaltered in the absence of *Ftx* in ESCs. *Ftx* therefore appears to regulate transcription by modulating the surrounding chromatin environment, including *Xist*.

While much is known about *Ftx* expression and function, several crucial gaps remain. For example, if *Ftx* in fact normally activates *Xist* expression in females, then its over-expression should be expected to induce *Xist* in males. It is also unclear to what extent *Ftx*

functions in a direct versus an indirect manner. Genomic deletions themselves are acute chromatin modifying events; it is difficult to rule out that the histone modification changes observed upon *Ftx* deletion may in fact be due the removal of a segment of the *Ftx* genomic locus, rather than via loss of the *Ftx* RNA per se. The structural alterations in chromatin may then cause transcriptional changes nearby. More subtle mutations that abrogate expression but leave the locus relatively unchanged may address this conundrum. Finally, it will be important to validate any implied function via cell culture studies through loss- and gain-of-function experiments in animals.

RepA

An obvious extension to the discovery of ncRNAs in the X-inactivation center is that proteins must be recruited by these ncRNAs to bring about epigenetic gene regulation. As we have described above, *Xist* has been long postulated to interact with chromatin modifiers to exert its function; however, the physical interaction of proteins with *Xist* has only recently been described. In 2008, direct interactions between *Xist* RNA and Polycomb group proteins EZH2, SUZ12, and EED, members of the PRC2 Polycomb complex were documented (Zhao et al., 2008). These RNA-protein complexes are not static, rather they seem to follow a time-dependent spread along *Xist* RNA over the course of X-inactivation during ESC differentiation. PRC2 proteins initially bound the 5' end of *Xist* RNA and then encompassed the more 3' regions of the *Xist* RNA (Zhao et al., 2008). PRC2 also displayed a time dependent enrichment on *Xist* genomic DNA. The 5' region of *Xist* that Zhao *et al.*, examined contained a novel promoter activity. This segment harbors a repeat sequence, termed 'A' repeat, which encoded a distinct transcriptional unit, termed *RepA*, in the same transcriptional orientation as *Xist* (Figure 1). *RepA* RNA spans bp 300–1948 in exon 1 of *Xist* and is expressed prior to *Xist* upregulation. *RepA* interacts with PRC2 proteins prior to PRC2 binding to *Xist*. The deposition of PRC2-catalyzed H3-K27me3 at the 5' end of *Xist* paradoxically led to *Xist* upregulation. In agreement with a role for the Polycomb group in inducing *Xist* expression, short hairpin RNA (shRNA)-mediated knockdown of EZH2 or EED led to a reduction in *Xist* levels and decreased H3-K27me3 enrichment in differentiating female ESCs (Zhao et al., 2008). *Xist* RNA then itself is posited to bind PRC2 and thereby promote the spread of H3-K27me3 across the X-chromosome, leading to chromosome-wide inactivation. Of note, however, absence of PRC2 function in the epiblast lineage, which is the source of ESCs, in developing female embryos does not diminish *Xist* expression.

To functionally investigate the *RepA* element, Zhao *et al.*, set out to disrupt *RepA* via shRNA-mediated knock-down. Depletion of the *RepA* led to reduced *Xist* levels and attenuated enrichment of H3-K27me3 on the inactive-X. A caveat in these experiments is that an shRNA targeting *RepA* is expected to also impact *Xist* RNA, since *RepA* is wholly contained within *Xist*. Thus, it is difficult to rule out that a shRNA against *RepA* is not also knocking-down *Xist*. A central role for *RepA* RNA in the recruitment of PRC2 to *Xist* is also questioned by the observation that PRC2 can be recruited to *Xist* in the absence of *RepA* (Plath et al., 2003). Thus, it is possible that other sequences within *Xist* can recruit PRC2, in tandem with or independent of *RepA*.

Conclusion

The discovery and characterization of non-coding RNAs within the X-inactivation center has engendered much enthusiasm. Starting with the discovery of *Xist* in 1991, these non-coding RNAs have transformed our understanding of both long non-coding RNA function as well as of X-inactivation. Yet, much remains unknown on both fronts. Among the key open questions is how precisely do these non-coding RNAs recruit proteins to DNA? A corollary is how can the secondary structure of these non-coding RNAs be accurately recapitulated *in*

vitro, so as to design appropriate mechanistic studies? How do the recruited proteins in turn modify chromatin? Can the non-coding RNA identified to date explain the epigenetic profile of the entire chromosome, or are there other unidentified non-coding RNAs that are instrumental in epigenetic regulation of the two X-chromosomes? Clearly, the excitement will remain unabated for the foreseeable future.

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Abbreviation list

ESCs	Embryonic stem cells
Xist	X-inactive specific transcript
PRC1	Polycomb repressive complex 1
PRC2	Polycomb repressive complex 2
H2A-K119ub	Histone H2A lysine 119 monoubiquitination
H3-K27me3	Histone H3 lysine 27 trimethylation
H3-K9me2	Histone H3 lysine 9 dimethylation
MSCI	Meiotic sex chromosome inactivation
ORF	Open reading frame
shRNA	short hairpin RNA

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Figure 1.
Key long non-coding RNAs in the X-inactivation center.

Figure 2. Enrichment of Xist RNA, Polycomb group protein EED, and H3-K27me3 on the inactive X-chromosome during mitosis. DAPI stains the chromosomes blue.

Figure 3. Mouse blastocyst embryo stained to detect Xist RNA coating (in green), Tsix RNA (green pinpoint), and histone H3 lysine 27 tri-methylation (H3-K27me3; in purple). DAPI stains the nuclei blue.