Guided by RNAs: X-Inactivation as a Model for lncRNA Function

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Abstract

The recent revolution in sequencing technology has helped to reveal a large transcriptome of long non-coding RNAs (lncRNAs). A major challenge in the years to come is to determine what biological functions, if any, they serve. Although the purpose of these transcripts is largely unknown at present, existing examples suggest that lncRNAs play roles in a wide variety of biological processes. Exemplary cases are lncRNAs within the X-inactivation center. Indeed, lncRNAs dominate control of random X-chromosome inactivation (XCI). The RNA-based regulatory mechanisms of XCI include recruitment of chromatin modifiers, formation of RNA-based subnuclear compartments, and regulation of transcription by antisense transcription. XCI and lncRNAs now also appear to be very relevant in the development and progression of cancer. This perspective focuses on new insights into lncRNA-dependent regulation of XCI, which we believe serve as paradigms for understanding lncRNA function more generally.

Introduction: LncRNAs and X-Inactivation

Chromosome-based sex determination systems create an imbalance in the dosage of X-linked genes between the two sexes. As a result of the X–Y system of sex determination, female mammals must correct for its double dosage of X-linked gene expression by transcriptionally silencing the majority of genes on one X-chromosome in a process known as X-chromosome inactivation (XCI) [1]. This process is driven by a series of long non-coding RNAs (lncRNAs) (Table 1). Because of the involvement of many lncRNAs and distinct mechanisms of action, XCI is an excellent model system for studying lncRNA function.

One of the first lncRNAs identified was Xist [18–21], a 17-kb transcript expressed from the X-inactivation center (XIC) solely from the inactive X (Xi) [18,19]. *Xist* deletions prevent X-inactivation *in cis* [2,22] and forced Xist expression is sufficient to induce chromosome-wide silencing [14,23,24]. Xist RNA coats the Xi [25], and the spreading of Xist RNA along one X-chromosome *in cis* initiates chromosome-wide silencing. Whereas Xist expression designates the Xi, Tsix expression demarcates the active X (Xa), also *in cis* [11,14,26–28]. *Tsix* is antisense to *Xist* and serves as a potent antagonist of *Xist* expression. It is therefore an excellent example of a natural antisense transcript that represses its sense partner. Non-coding genes have also been implicated in activating *Xist*, including *Jpx*, *RepA*, and *Ftx* [4,15,17]. In this review, we will summarize recent advances in the control of XCI by lncRNAs and conclude with new insight into how one lncRNA (Xist) influences the development of cancer, a disease for which a role of the X-chromosome has long been suspected.

XIST RNA: The Recruiter of Silencing Complexes

Xist RNA is one of the first examples of an RNA that recruits a chromatin-modifying complex to specific sites (Fig. 1). Polycomb repressive complex 2 (PRC2) is attracted to the X-chromosome by Xist RNA through a repeated motif at the 5′ end of the

Table 1. Summary of lncRNAs and proposed interacting protein partners for X-inactivation

RNA	Function	*cis*- or *trans*-acting	Known protein interactors
Xist	Required for initiation of X-inactivation [2]	*cis*, can in some cases act *in trans* at autosomal Xist transgenes [3]	PRC2 [4], YY1 [3], hnRNP-U [5], ASF [6]
Tsix	Represses Xist expression by silencing the Xist promoter [7–10], also required for X-chromosome pairing, counting the number of XICs, and mutually exclusive allelic choice [11–13]	*cis* [14]	Dnmt3a [7,8]
RepA	Independent transcript from Xist 5′ end, helps activate Xist [4]	*cis* [4]	PRC2 [4]
Jpx	Activator of Xist transcription; counting of X-chromosomes [15,16]	*trans* (mild *cis* preference) [15]	CTCF [16]
Ftx	Potential activator of Xist expression [17]	Unknown [17]	Unknown

RNA, known as "Repeat A" [4]. The Repeat A motif directly interacts with EZH2, the catalytic subunit of PRC2, both *in vivo* and *in vitro*. PRC2 in turn decorates the X-chromosome and silences it as it trimethylates histone H3 at lysine 27 (H3K27me3) [29–32]. Along the X, PRC2 first binds ~150 "strong sites", which have canonical features of known PRC2 binding sites, including a CpG-rich content and the presence of bivalent domains [33]. From the strong sites, PRC2 migrates laterally and locally, giving rise to thousands of non-canonical domains that may represent sites of dynamic spreading along the X chromatin [33]. H3K27me3 density also spreads out from the strong sites, and H3K27me3 occupancy is anti-correlated with LINE density [33,34], an intriguing finding given a long-standing hypothesis that LINE elements serve as "booster elements" that help X-inactivation spread across the whole chromosome [35]. When expressed ectopically from autosomal transgenes, Xist RNA also recruits PRC2 and silences genes located *in cis* [3,29,36], demonstrating that Xist RNA is both necessary and sufficient to recruit PRC2 and inactivates genes on a multi-megabase scale.

While it is clear that Xist RNA spreads PRC2 to targets on the X-chromosome, mechanisms that localize Xist RNA itself are just beginning to emerge. Localization begins with loading of Xist RNA at a "nucleation center" located within exon 1 of the *Xist* locus [3]. The transcription factor, YY1, is required for Xist RNA loading onto the nucleation site. Knocking down YY1 or mutating its three binding sites within

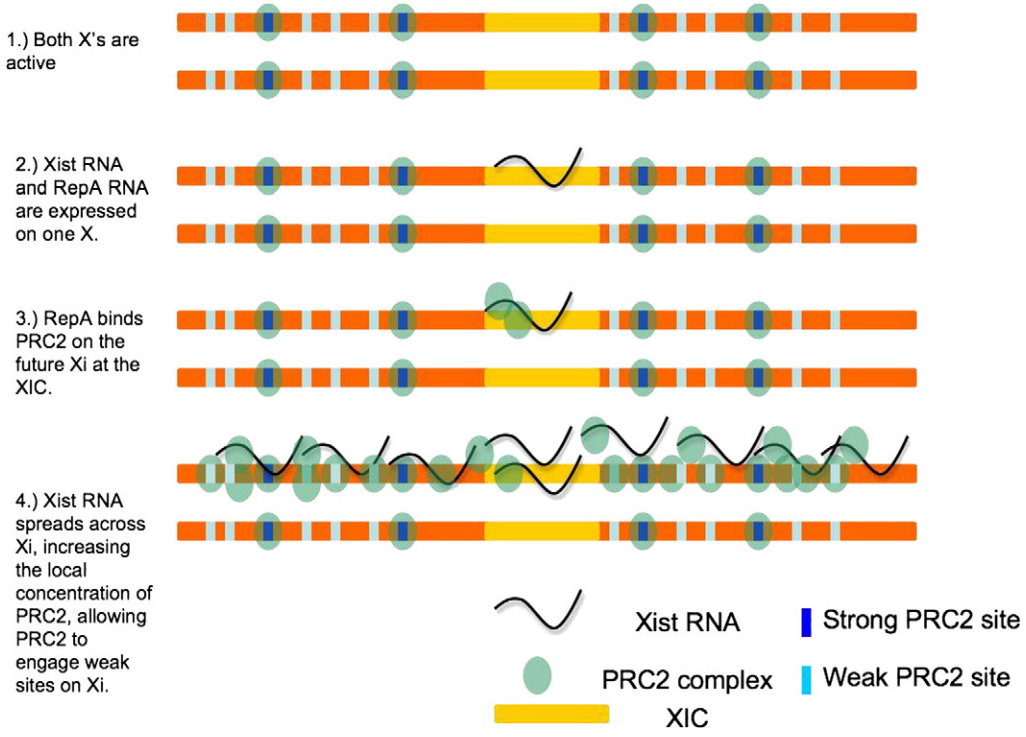

Fig. 1. Model for Xist RNA-mediated recruitment of PRC2.

the nucleation center eliminates Xist loading and furthermore blocks formation of the prominent cloud of Xist RNA seen in RNA fluorescence in situ hybridization experiments. Xist RNA directly binds YY1 *in vivo* and *in vitro*, and YY1 in turn directly contacts three YY1-binding sites near "Repeat F" within *Xist* exon 1 [3]. Thus, YY1 is a "bivalent" protein (capable of binding both RNA and DNA) and acts as "bridge" between Xist DNA and RNA. In this way, YY1 tethers the Xist–PRC2 complex to the nucleation site and positions the complex to spread throughout the rest of the chromosome. It is not currently known how Xist RNA binds to other sites along the Xi. These results suggest that recruitment of PRC2 in mammals may involve not only a lncRNA but also an adaptor protein such as YY1. YY1 is a homolog of the *Drosophila* gene *pleiohomeiotic* (PHO) [37], which has been shown to recruit PRC2 to polycomb response elements in *Drosophila* [38]. Some data suggest that YY1 can interact with several components of the mammalian PRC2 [39] but may not be a core component.

Expressing an *Xist* transgene carrying mutations in the YY1 binding sites led to the very surprising discovery that Xist RNA can act *in trans* [3]. In such situations, transgenic Xist RNA could not bind the nucleation site *in cis* (on the transgene) but was observed to diffuse to the nucleation site of the Xi and then spread along the Xi. Furthermore, when the transgene is not mutated, Xist RNA produced from the Xi could migrate to the transgene nucleation site and spread along the autosome. This observation led to questions about how the Xa does not engage in binding of Xist. Further analysis indicated that YY1 binds only the nucleation site on Xi (not on Xa) [3]. Thus, the *cis*-limited action of Xist RNA occurs in the normal developmental context likely because of developmental programming that blocks YY1 nucleation sites at all but the Xi. Xist is a *cis*-acting RNA only to the extent that sites *in trans* are prevented from binding.

It is now clear that RNA-mediated recruitment of PRC2 is not unique to the inactive X-chromosome, as a myriad of transcripts associate with PRC2 [40–44]. The observation that PRC2 interacts with thousands of transcripts raises interesting questions regarding whether the function of RNA in PRC2 biology may extend beyond the role of targeting PRC2, given that the PRC2 "transcriptome" contains RNAs that are not strictly *cis*-limited. Some lncRNAs may not be directly involved in PRC2 recruitment, but instead modulate PRC2's methyltransferase activity or its interactions with accessory proteins. Many other chromatin modifiers have been shown to interact with RNA, such as Dnmt3a, G9a, PRC1, MLL-WDR5, and LSD1-CoREST (reviewed in Ref. [45]). Further understanding the interaction of the Xist–PRC2 interaction may shed light on the functions of interactions between chromatin modifiers and lncRNAs. It would also be of interest to learn whether YY1 functions as adaptor for PRC2–lncRNA complexes elsewhere in the genome.

TSIX: The Antisense Regulator

Tsix is another long, non-coding transcript that plays a key role in X-inactivation. A considerable amount of genetic analysis has been carried out on *Tsix* towards understanding the antisense mechanism of action. *Tsix* controls *Xist* expression *in cis* by modulating the chromatin structure and DNA methylation status of the *Xist* promoter [7,8]. Anti-sense transcription extending through the *Xist* promoter is required to silence *Xist* in *cis* [9,10,46]. It is possible that Tsix acts as a functional RNA and recruits repressors such as the *de novo* methyltransferase, Dnmt3a [7,8], or titrates activators away from the *Xist* promoter. The act of antisense transcription through the *Xist* promoter could also induce a chromatin state that is refractory for sense transcription; alternatively, it could disrupt RNA polymerase function in the sense direction. Definitive experiments to test these hypotheses must separate transcription of the antisense RNA from the action of the antisense transcript.

Tsix's mechanism of action may extend to other antisense transcripts. There are at least several hundred sense–antisense pairs within mammalian genomes [47–50], many of which are arranged in a structurally similar manner as the *Xist*–*Tsix* pair [49]. In these cases, the antisense transcript might similarly regulate expression of the sense transcript with which it overlaps [51–53]. In several well-studied examples within imprinted loci, allele-specific expression patterns have been proposed to be controlled by expression of an antisense transcript. For example, *Air* silences the paternal *Igf2r* cluster [54–56] and *Kcnq1ot1* silences the paternal *Kcnq1* cluster [57,58]. Both antisense transcripts are implicated in binding of repressive chromatin factors, such as G9a and PRC2 [55,58]. There are likely many other examples of sense–antisense transcription modules that operate like the *Xist*–*Tsix* pair. Thus, uncovering the molecular mechanisms that underpin *Tsix*-mediated regulation of *Xist* may have broad applicability for understanding the role of antisense transcription.

RNA's Relationship to Large-Scale Chromosome Interactions

In addition to its role as an inhibitor of *Xist* expression on the Xa, *Tsix* plays a role in inter-chromosomal contacts hypothesized to be crucial for X-chromosome choice and for properly demarcating only one Xa. Before *Xist* is upregulated, the two *Xic*'s

of the female cell transiently come into close contact with each other in the nucleus (Fig. 2) [59–62]. This transient "pairing" of the two *Xic*'s may allow a redistribution of transcriptional activators between the two alleles [61,63–65], resulting in asymmetric binding of activators to one X-chromosome and thereby enabling expression of Tsix RNA only on one chromosome. Tsix and its enhancer, *Xite*, are necessary for pairing [61] and are also each sufficient to induce pairing when integrated onto an autosome [62]. Interestingly, inhibition of transcription with actinomycin D prevents the formation of new pairing complexes but has little effect on the half-life of paired complexes already formed, suggesting that RNA may be required to attract two X-chromosome to each other but not to keep them paired [62]. Whether transcripts emanating from *Tsix* and *Xite* are required is currently not known, though the two loci are clearly necessary and sufficient to induce pairing.

It seems likely that RNAs may generally participate in long-range chromosome interactions. There are several pieces of evidence implicating RNA as a structural component that determine higher-order structures. RNA has long been known to known to co-fractionate with chromatin in eukaryotic nuclear extracts [66–69]. The nuclear matrix consists of a network of ribonucleoprotein particles (reviewed in Ref. [70]), and it has been suggested that Xist RNA is a component of the nuclear matrix, interacting with factors such as hnRNP-U/SAF-A and ASF [5,6,71,72]. Recent experiments more directly implicate a relationship between non-coding RNA and chromatin architecture. A newly discovered class of RNAs that appear to activate nearby genes in *cis* called "activating RNAs" (a-RNAs) [73] are required for three-dimensional contacts between enhancers and promoters of nearby genes regulated by a-RNAs [74]. These RNAs are hypothesized to be predominately *cis*-acting non-coding transcripts that

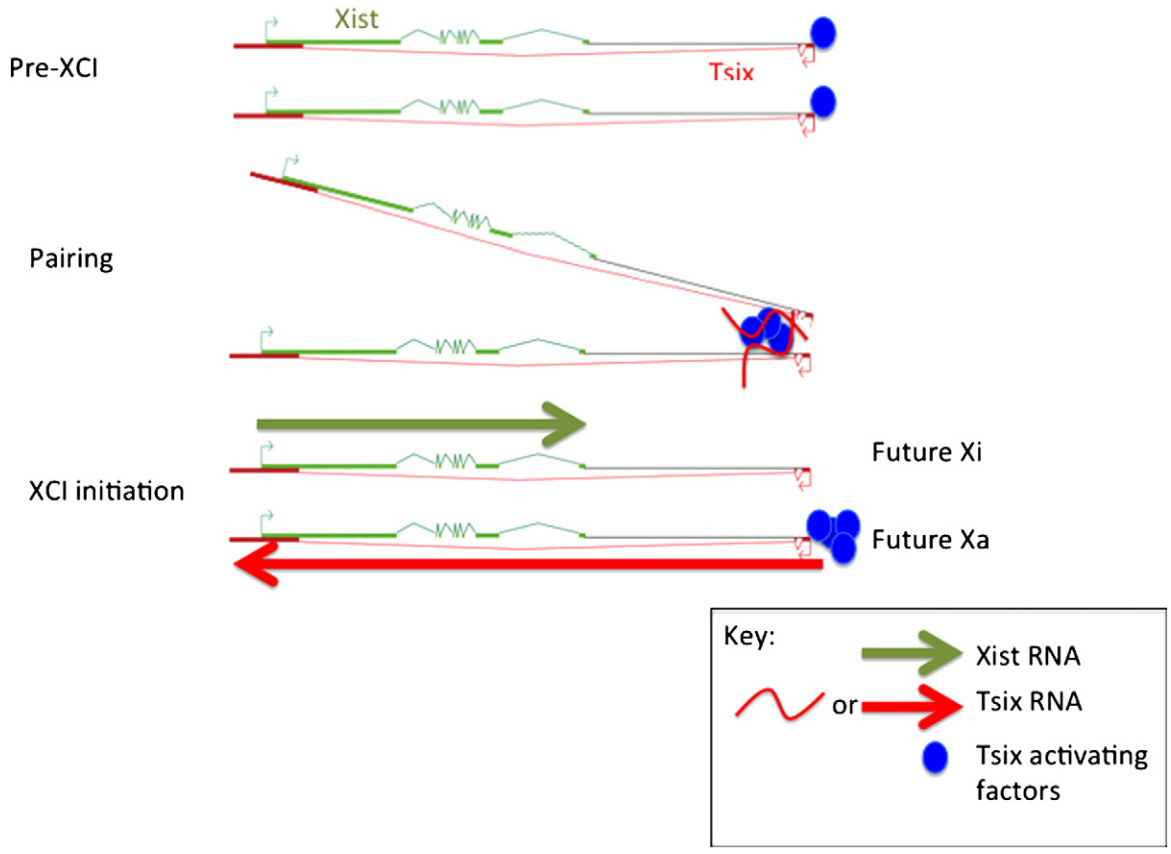

Fig. 2. Model for XIC pairing before XCI onset. The two X-chromosomes are epigenetically identical and euchromatic in the pre-XCI stage. The two Xs are brought together by *Tsix* and *Xite* (pairing) during cell differentiation to enable cross-talk and mutually exclusive choice of Xa and Xi. Because it is thermodynamically favorable to do so, hypothetical transcription factors, potentially OCT4 and CTCF (blue circles), that were previously randomly distributed between the two *Tsix*/*Xite* alleles stochastically shift to one X, which would then become future Xa. This shift results in monoallelic *Tsix* expression and differential chromatin modifications within the Xist region, which lead to repression of Xist on Xa and upregulation of Xist on Xi.

function like enhancers in enhancer assays. However, unlike classical enhancers, a-RNA activity can be blocked by small interfering RNAs targeting the non-coding transcripts. Chromatin conformation assays revealed looping interactions between several a-RNAs and their target gene's promoters. These looping interactions were disrupted by knockdown of the a-RNA. Another lncRNA that appears to function through looping interactions is HOTTIP, a putative activator that operates through a change in chromatin conformation in the *HoxA* cluster [75]. Additionally, two chromatin conformation studies [76,77] suggested that Xi assumes a more "randomized" organization relative to Xa and autosomes, thereby indirectly implicating Xist RNA in generating the less ordered configuration. Indeed, an Xi-specific deletion of Xist partially restored long-range contacts on Xi, suggesting that Xist acts to disrupt long-range chromatin contacts [76]. These may be only a handful of examples of an entire class of lncRNAs involved in large-scale chromatin interactions.

Positive LncRNA Regulators of XCI

Some lncRNAs of the *Xic* appear to be transcriptional activators. They include RepA, Jpx, and Ftx, all proposed to be activators of *Xist* (Table 1). The molecular mechanisms that cause these RNAs to induce Xist are currently unknown, though recent studies have suggested several intriguing possibilities.

RepA RNA is transcribed from an independent transcription unit within exon 1 of *Xist* and consists of a repeated motif (Repeat A) that directly binds and targets PRC2 to the *Xist* promoter [4]. RepA is believed to induce Xist expression by increasing H3K27 trimethylation of the *Xist* promoter via its recruitment of PRC2 [4]. As PRC2 is a repressive complex, this may seem to be a counterintuitive way to activate *Xist* transcription. However, *Xist* may prefer a heterochromatic environment. Indeed, *Xist* remains active in the repressive context of the Xi; it may actually be induced by repressive signals. This intriguing possibility has to be tested more directly to determine whether PRC2 recruitment to the *Xist* promoter actually induces *Xist* expression. Consistent with the idea, a mouse deletion of RepA results in loss of *Xist* induction [78].

Jpx's activating influence is supported by genetic analysis. When female cells are deleted for *Jpx*—even on just one allele—the cells can no longer induce *Xist* expression [15]. *Jpx* does not apparently function as an enhancer, as standard enhancer assays failed to reveal any activating influence on the *Xist* promoter. Furthermore, its ability to activate *Xist in trans* (when *Jpx* is placed in an autosomal context) also argues against an enhancer mechanism [15]. Thus, Jpx's action is distinct from enhancer-associated RNAs [73,79,80]. Because a post-transcriptional knockdown of Jpx phenocopies a *Jpx* knockout, the transcript itself (not just the *Jpx* gene or transcription) must be the activating force. The latest work indicates that Jpx RNA is part of the X-chromosome counting mechanism and activates *Xist* by titrating away a repressive autosomal factor, CTCF, that normally binds and blocks the *Xist* promoter [16].

Jpx could act in other ways as well. *Jpx* is *trans*-acting, but has a mild *cis* preference [15]. Consistent with a *cis* preference, chromosome conformation analysis suggests that the *Jpx* locus makes contacts with the *Xist* promoter following the onset of differentiation and XCI [81]. It is possible that Jpx RNA mediates the formation of these chromatin contacts, as has been shown for several other a-RNAs [74]. Another possibility is that the chromatin contacts form independently of Jpx RNA, and juxtaposing nascent Jpx transcripts to the *Xist* promoter allows Jpx to recruit activators or titrate repressors from the *Xist* promoter. Studying these a-RNAs may provide several important model systems for evaluating the functions of mechanisms of recently discovered a-RNAs [73] and enhancer RNAs [79,80].

XIST and Cancer

Much work has centered on the role of *Xist* in initiating X-inactivation in the early developing embryo. However, it is now clear that *Xist* has important roles in later development and in adults as well, long after the establishment of XCI. In mouse embryonic fibroblasts [82], a conditional deletion of *Xist* from the Xi leads to partial X-reactivation. Since XCI silences several hundred genes, some of which are oncogenes, improper *Xist* expression could potentially be a mechanism underlying tumorigenesis [83,84]. Early experiments suggested a tantalizing connection between X-linked gene dosage and cancer. Loss of XCI and downregulation of XIST expression are commonly observed in basal-like cancer, BRCA1-null triple negative breast cancer [85–91], and ovarian cancer lines [92,93]. Loss of XIST is most commonly caused by X isodisomy, where Xi is lost and Xa is amplified. Reactivation of Xi may be an alternative mechanism leading to loss of XIST and overexpression of the X [87,89,92,93]. These observations suggest a correlation between X-chromosome dysfunction and cancer. The link between XCI and cancer was recently determined to be causal, with the finding that deleting *Xist* in the blood compartment leads to a highly aggressive myeloproliferative neoplasm and myelodysplastic syndrome (mixed MPN-MDS) with 100% penetrance and lethality in mice [94]. This result clearly demonstrates that loss of Xist RNA and overexpression of the X in adult tissues can lead to cancer.

As Xist RNA promotes the initiation or progression of cancer, it may be reasonable to propose reactivation of *Xist* as a therapeutic strategy in cancer. It is known that ectopic Xist expression in adult mice can lead to ectopic gene silencing *in cis* and cell death in the immature precursors of the immune system [95]. Therapeutic strategies may include small molecules. Indeed, there is precedence when one looks to Angelman syndrome, a congenital disorder caused by maternal deletion or mutation of the imprinted *Ube3a* allele. *Ube3a* is known to be controlled by a long antisense transcript from the *Snrpn* locus [96–99]. Topoisomerase inhibitors cause loss of imprinting of the silent paternal *Ube3a* allele by downregulating the antisense transcript in neurons *in vitro* and in mice, which may provide a strategy for rescuing the genetic defect that causes Angelman syndrome [100]. In tumors, *Xist* expression might also be reactivated by small molecules, offering a novel therapeutic approach that would target epigenetically functional lncRNAs.

Conclusions

LncRNAs have received a great deal of attention over the past few years as regulators of gene expression [34,45,101]. Thousands of new transcripts have been identified in the mammalian genome, with estimates upwards of 200,000 [21]. A key challenge is to determine which transcripts are functional and how these transcripts regulate physiological processes. RNAs of the *Xic* may provide a framework towards understanding how those in the rest of the genome operate. Within the *Xic*, both *cis*-acting (e.g., Tsix) and *trans*-acting (e.g., Jpx) lncRNAs can be found. These X-linked lncRNAs can also be either activating (Jpx) or repressive (Xist). They interact with transcription factors (e.g., YY1, CTCF) and chromatin-modifying complexes (e.g., PRC2, Dnmt3a). The *Xic* also provides a model by which to study sense–antisense pairs and the role of RNA in large-scale chromatin architecture. It has furthermore become clear that lncRNAs of the *Xic* are crucial not only during early embryogenesis when dosage compensation takes place but also throughout adult life. Basic homeostatic functions of the lncRNAs have been revealed when deleting one member (Xist) results in a lethal disease (cancer). X-inactivation and other allelic phenomena have often proven to be fertile grounds for uncovering unexpected biology [96]. The very first lncRNAs were discovered by applying classical genetic techniques to find genes that control X-inactivation and imprinting. In the future, emerging genomic and nanoscale technologies will likely provide many new surprising insights into how the non-coding genome contributes to normal physiology and disease.

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Abbreviations used:
lncRNA, long non-coding RNA; XCI, X-chromosome inactivation; XIC, X-inactivation center; Xi, inactive XXa, active X; PRC2, polycomb repressive complex 2; a-RNA, activating RNA.

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