Advances in understanding chromosome silencing by the long non-coding RNA Xist

Takashi Sado1 and Neil Brockdorff2

1Division of Epigenomics, Medical Institute of Bioregulation, Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
2Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK

In female mammals, one of the two X chromosomes becomes genetically silenced to compensate for dosage imbalance of X-linked genes between XX females and XY males. X chromosome inactivation (X-inactivation) is a classical model for epigenetic gene regulation in mammals and has been studied for half a century. In the last two decades, efforts have been focused on the X inactive-specific transcript (Xist) locus, discovered to be the master regulator of X-inactivation. The Xist gene produces a non-coding RNA that functions as the primary switch for X-inactivation, coating the X chromosome from which it is transcribed in cis. Significant progress has been made towards understanding how Xist is regulated at the onset of X-inactivation, but our understanding of the molecular basis of silencing mediated by Xist RNA has progressed more slowly. A picture has, however, begun to emerge, and new tools and resources hold out the promise of further advances to come. Here, we provide an overview of the current state of our knowledge, what is known about Xist RNA and how it may trigger chromosome silencing.

1. Introduction

The present sex chromosomes in mammals are believed to have evolved from a pair of morphologically identical autosomes. Although this must have directly or indirectly benefited the mechanism for sex determination or efficient reproduction, the advent of two morphologically different sex chromosomes, which generated homogametic and heterogametic animals in the same species, caused an imbalance in the dosage of sex-chromosome-linked genes. To circumvent this, female mammals evolved a mechanism by which one of the two X chromosomes in females is genetically silenced during early development. Since Mary Lyon first described the X chromosome inactivation (X-inactivation) hypothesis in 1961 [1], it has intrigued and occupied many researchers. Significant progress has been made, and X chromosome inactivation now stands as a classical model that illustrates the principles of epigenetic gene regulation in mammals.

The discovery of the X inactive-specific transcript (Xist) gene in the early 1990s [2–6] brought a breakthrough in our understanding of the molecular basis for X-inactivation. Xist was initially identified on the basis of its location in the classically defined master control region for X-inactivation, called the X chromosome inactivation centre (Xic/XIC). The gene was found to be expressed only from the inactive X chromosome and to produce a transcript that does not encode protein, but rather plays a role as a functional RNA that associates in cis with the X chromosome from which it originated. These observations led to the idea that Xist RNA recruits proteins involved in heterochromatinization of the X chromosome. Targeted disruption of Xist unequivocally showed that it is essential for X-inactivation to occur in cis. The X chromosome bearing a null mutation of Xist never undergoes inactivation in the mouse [7] and differentiating female embryonic stem (ES) cells [8], which recapitulate the process of X-inactivation [9,10].

Xist has been at the centre of the studies in X-inactivation during the last two decades. Efforts have been largely directed towards the importance of Xist in X-inactivation and how Xist is differentially regulated between two X chromosomes during development. We have learned that Xist regulation

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plays a central role in the initiation of X-inactivation [7,8,11,12], and its antisense gene, known as Tsix, negatively regulates Xist in cis through chromatin modifications at the onset of X-inactivation [13–18] (figure 1). Recent studies further propose that the differential expression of Xist is spatio-temporally regulated between two X chromosomes prior to the onset of X-inactivation by a physical association of the Xic region referred to as ‘pairing’, suggesting a means by which two X chromosomes may communicate with one another prior to selecting a single X chromosome for silencing [19,20]. Other studies have suggested that pluripotency factors, which bind their recognition sequences present in potential regulatory regions of Xist and Tsix, balance their expression to suppress or induce X-inactivation in a developmentally regulated manner [21–23]. More recently, an X-linked factor, Rnf12, has been shown to have a role in ensuring that Xist is activated only on a single X chromosome in female cells [24]. Similar function has been attributed to the non-coding RNA (ncRNA) Jpx, produced by a gene located immediately upstream of the Xist locus and the regions known to be bound by pluripotency factors. TSS, transcription start site.

2. Functional domains of Xist RNA

Xist RNA has been generally described as a spliced polyadenylated ncRNA around 17 kb in length. There are two major forms produced through use of alternative termination signals [30]. Xist RNA includes six regions composed of short tandemly arranged repeat sequences, termed A to F, some of which are conserved among species (figure 2). Strauss and co-workers [31] showed that Xist RNA domains or ‘clouds’ seen in the interphase nucleus of female mouse fibroblasts disappear when cells are administered antisense peptide nucleic acids (PNAs) against the C-repeat, but not against the B- and D-repeat. Because steady-state levels of Xist RNA were not affected by PNA treatment, the effect of PNAs appears to be distinct from the transcriptional or posttranscriptional gene silencing caused by siRNA. In addition, the C-repeat PNAs compromise accumulation of Xist RNA on the X chromosome in differentiating female ES cells with an accompanying failure of X-linked gene silencing. A similar effect of locked nucleic acids (LNAs) against the C-repeat was also recently reported [32]. Interestingly, nucleofection of the LNAs against the C-repeat in female embryonic fibroblasts caused a loss of Xist RNA within 1 h, but the effect did not last for long and Xist RNA clouds were reestablished in 8 h. Because there is no change in steady-state levels of Xist RNA upon LNA treatment, as is the case with PNAs, the antisense LNAs, and also probably PNAs, may displace Xist RNA from the X chromosome. It has not, however, been confirmed by northern blotting whether the RNA stripped off from the X chromosome remains intact. A more recent study using ES cells found that YY1—a multifunctional protein that has been implicated in both activation and repression, probably depending on the context of the complex—bound to the C-repeat of Xist RNA and tethered it to YY1 recognition sites present in exon 1 of the Xist gene (figure 2), creating nucleation sites for Xist RNA [33]. Taken together, these results suggest that the C-repeat is a domain involved in the association of Xist RNA with the X chromosome. To identify regions in the Xist RNA sequence required for chromosome silencing and coating, Wutz et al. [26] developed a way to measure the competency of mutant Xist RNA harbouring various deletions to cause chromosome silencing by doxycycline-induced expression from a single copy transgene on a single X chromosome in male ES cells. The most striking finding in this work was that chromosome silencing is severely compromised by deletion of the A-repeat located in the 5’ region of the RNA (figure 2, ∆SA), suggesting that the A-repeat is a domain critical for Xist RNA-mediated silencing. The A-repeat consists of 7.5–8.5 copies of 26-mer tandem repeats separated by U-rich spacers. Although the mechanism of A-repeat function is currently unknown, possible conformational
structures of the A-repeat have been suggested to form a module interacting with proteins required for chromosome silencing [34,35]. An Xist transgene containing a mutation that disrupts the predicted conformation of the A-repeat in vitro has, in fact, been shown to abrogate the silencing in differentiating ES cells [34]. Another study found that a splicing factor, ASF/SF2, binds to the A-repeat in ES cells and that deletion of the repeat compromises proper splicing of Xist RNA and precludes its accumulation, resulting in non-random inactivation of the wild-type X chromosome [36].

The importance of the A-repeat in the endogenous locus has been examined in the mouse. A mutant allele, Xist\textsuperscript{D\textsubscript{SA}}, was produced by gene targeting [37]. Males and females hemizygous and heterozygous, respectively, for Xist\textsuperscript{D\textsubscript{SA}}, which is maternal in origin, were produced but intercrosses between those males hemizygous for Xist\textsuperscript{D\textsubscript{SA}} and wild-type females revealed that the majority of female offspring, all of which should have inherited the Xist\textsuperscript{D\textsubscript{SA}} allele, were lost soon after implantation. Female-specific loss upon paternal transmission of Xist\textsuperscript{D\textsubscript{SA}} was indicative of the failure of imprinted X-inactivation in the extraembryonic tissues, which gives rise to the future placenta and some extraembryonic membranes, suggesting that Xist\textsuperscript{D\textsubscript{SA}} was dysfunctional, similar to null mutation of Xist [36]. Although it was anticipated that this would be attributable to defective silencing by Xist\textsuperscript{D\textsubscript{SA}} RNA, RNA-fluorescence in situ hybridization (FISH) revealed that expression of Xist RNA from the mutated paternal X was severely compromised and no Xist RNA cloud was observed, not only in the trophoblast of developmentally stunted postimplantation embryos, but also in the preimplantation stage embryos. Although this result was apparently consistent with the study in ES cells described earlier, quantitative reverse transcription polymerase chain reaction (qRT-PCR) failed to detect expression of Xist per se from the mutated X, suggesting that the genomic sequence encompassing the A-repeat plays an essential role as a cis-element required for proper upregulation of Xist at the onset of X-inactivation. The effect was not observed by Wutz et al. [26] because in those experiments, expression of mutant Xist RNA was driven by a heterologous inducible promoter.

Wutz et al. [26] also reported that deletion of the C-repeat compromises neither the association of the mutant Xist RNA with the X chromosome nor the silencing function of the RNA. This is at variance with the effect of the antisense PNAs and LNAs against the C-repeat. It seems that the antisense PNAs and LNAs have a more severe impact than the deletion of the C-repeat on the association of Xist RNA with the X chromosome. It will be of interest to see what happens to Xist RNA coating and X-inactivation in the mouse embryo if the C-repeat in the endogenous Xist gene is deleted, and also if the antisense RNA against the C-repeat is expressed from an ectopic site. However, it should be commented that the Wutz et al. [26] study indicated that association of Xist RNA with the X chromosome is not mediated by a single domain, but by functionally redundant sequences dispersed along the transcript length.

![Figure 2. Structure of mutant Xist RNAs defective in silencing.](image-url)
Four alleles that express mutant forms of Xist RNA from the endogenous locus have been described, and these have furthered our understanding of functional domains in Xist RNA; Caparros et al. [38] deleted exon 4 of Xist because its primary sequence is highly conserved and is potentially able to form a stable RNA stem-loop structure. However, the mutant allele, XistEx4del, was transmitted from the father to female pups at the expected ratio, indicating that the XistEx4del allele is competent to establish imprinted inactivation of the paternal X chromosome in the extraembryonic tissues. In the female pups thus obtained, which are heterozygous for XistEx4del, either the wild-type or the mutated X was inactivated in an essentially random fashion, and in fact no allelic bias was observed in expression at the X-linked loci examined. The results, therefore, demonstrate that exon 4 is dispensable for the function of Xist RNA and the mutant RNA produced by the XistEx4del allele is competent to establish both imprinted and random X-inactivation in the extraembryonic and embryonic lineages, respectively. Steady-state levels of the mutant RNA were, however, diminished, and Xist RNA clouds detected by RNA-FISH were less intense than those formed by the wild-type Xist RNA. Because stability of the mutant RNA did not show significant difference from that of the wild-type RNA, it is likely that the deletion of exon 4 affects transcription rate or processing of Xist RNA. That lower expression of XistEx4del when compared with the wild-type does not seem to affect silencing is consistent with an early study that showed considerable variation in steady-state levels of Xist RNA in different mouse strains. Notably, levels in the wild-derived Mus spretus strain were significantly lower than in laboratory strains [3].

The second mutant allele that produces an aberrant Xist transcript is the XistInv allele. Here, a chimeric transcript is produced, consisting of an IRES-EGFP sequence with the proximal 2.3-kb sequence of Xist exon1 under the control of the endogenous Xist promoter [16]. This allele behaves like the null mutant reported by Marahrens et al. [7]. XistInv, when paternally transmitted, results in female-specific embryo lethality occurring soon after implantation, indicating a failure of imprinted paternal X-inactivation in the extraembryonic lineages. By contrast, there is no phenotypic defect upon maternal transmission of the mutation and both hemizygous males and heterozygous females for XistInv are recovered at the expected ratio.

Several studies have demonstrated that Tsix plays a role in establishing the repressive chromatin modifications at the Xist promoter and represses Xist on the X that stays active at the onset of X-inactivation [16–18]. When the allele XistInv was introduced onto an X chromosome deficient for Tsix [16], the XistInv allele on the Tsix-deficient X (X0) expressed the chimeric RNA at a significant level in both male and female mouse foetuses, despite the fact that X0 is active. Interestingly, although these foetuses express the chimeric RNA containing the IRES-EGFP sequence, they do not exhibit green fluorescent protein (GFP) fluorescence. The chimeric RNA may fail to be exported to the cytoplasm for translation, possibly due to the intrinsic property of Xist RNA to be retained in the nucleus. In fact, preliminary results suggest that the chimeric transcript expressed from a single X chromosome in mutant male ES cells is found predominantly in the nucleus but not in the cytoplasm (T. Sado 2005, unpublished result). It is tempting to speculate that any RNA transcribed from the Xist locus or RNA containing an Xist sequence is somehow diverted from the mRNA export pathway. Although this chimeric transcript probably stays in the nucleus and harbours the A-repeat, it is defective in silencing. This is most probably due to the fact that the RNA, which contains only the proximal 2.3-kb sequence of Xist RNA and lacks the rest of it, cannot accumulate on the X chromosome. This is consistent with the finding by Wutz et al. [26], who showed that the same 2.3-kb fragment of Xist RNA (figure 1, ΔEC) does not correctly localize on the X chromosome.

Two other mutations that give rise to modified Xist RNA are hypomorphic alleles, XistInv and XistPVS, recently reported by Senner et al. [39] and Hoki et al. [40], respectively. XistPVS was generated from XistEx4del by a targeted inversion disrupting conserved sequences beginning 6 kb into exon 1 and extending to intron 5. XistPVS was originally generated for the study of Tsix-mediated antisense regulation of Xist by Ohbata et al. [41]. It contains an exogenously introduced 1 kb intron at 0.9 kb from the major transcription start site. These two mutant alleles are quite distinct from the null mutant alleles previously generated by gene targeting in that they produce RNA that coats the X chromosome in cis and is competent to initiate the inactivation process. Also, these hypomorphic alleles, upon paternal transmission, do not cause severe developmental defects in the extraembryonic lineages until some time after implantation.

Female embryos heterozygous for XistPVS of paternal origin are indistinguishable from wild-type male littermates when recovered at E6.5. Growth retardation, however, becomes discernible as early as E7.5, and the further developmental delay is evident by E9.5. Remarkably, these female embryos show no sign of developing a placenta and, as a result, are resorbed by E10.5. Similarly, those female embryos that inherit XistPVS from the father are apparently normal during the early postimplantation stages, and the majority are still essentially indistinguishable from the wild-type male littermates even at E9.5. A subset of the heterozygous females start to show developmental anomalies from E10.5 onwards and many are lost by E13.5. Some, however, continue to develop and can even survive to term. Histological analysis demonstrated that the trophoblast giant cells are the major tissue affected in +/+XistPVS females. Thus, lethality caused by paternal transmission of the XistPVS and XistPVS mutations is apparently ascribed to aberrant inactivation of the paternal X chromosome in the extraembryonic lineages. However, because the effects of mutation become apparent earlier in +/+XistPVS females than +/+XistPVS, it is likely that effects of the respective mutations are more severe in the former than in the latter.

The XistPVS allele produces two distinct transcripts of 12 and 20 kb in length. In both, only the proximal 6-kb sequences are shared with wild-type Xist RNA (figure 2). Expression levels of the respective transcripts, when compared by northern blotting, are lower than those of wild-type Xist in mouse embryonic fibroblasts (MEFs). XistPVS RNA clouds, although detectable by RNA-FISH in the extraembryonic and embryonic tissues, are significantly diminished when compared with the wild-type Xist RNA clouds. This, however, is also the case for XistEx4del RNA, which silences the X chromosome quite efficiently. Analysis of Xist RNA on metaphase chromosomes is, however, needed to determine whether or not binding occurs strictly in cis with the X chromosome, and here there was a clear difference with XistEx4del RNA binding similar to wild-type Xist RNA and XistPVS RNA being barely detectable. This observation suggests that XistPVS RNA is in some way defective in localizing on the X chromosome.
in cis, and this is probably the underlying basis of aberrant X-inactivation. Consistent with this interpretation, H2A mono-
ubiquitylated at lysine 119 (H2AK119u1), deposition of which on the inactive X is dependent on Xist RNA, was strongly
reduced in cells expressing XistIVS RNA.

XistIVS RNA may be viewed to be a chimeric RNA com-
posed of a proximal 6-kb fragment of Xist connected to an
unrelated 6-kb or 14-kb fragment, although the additional
linked sequences actually correspond to the antisense
strand of Xist genomic sequence. Assuming that the exogen-
ous sequences do not impact on the function of the chimeric
RNA, it can be concluded that the proximal 6-kb fragment of
Xist RNA is responsible for the partial silencing function of
XistIVS. This would be consistent with a previous observation
that truncated Xist RNA, which corresponds to a sequence
similar to but not exactly the same as the distal 6-kb fragment
of XistRNA, was less efficient in not only silencing but
also chromosomal association when compared with the full
length Xist RNA [26] (figure 2, A5aC). It will be interesting
in the future to determine if chromatin markers other than
H2AK119u1 are affected when the chromosome is coated
with XistIVS RNA and also to examine their distribution
relative to a normal inactive X chromosome.

In contrast to the XistIVS allele, the XistIVS allele produces
essentially the same RNA as wild-type Xist except for an inser-
tion of an unrelated 16-base sequence at 0.9 kb from the 5′ end
associated with introduction of an exogenous intron.
Expression levels and stability of the RNA arising from the Xis-
tIVS allele is comparable to the wild-type Xist RNA in
trophoblast stem (TS) cells. XistIVS RNA coats the X chromo-
some in cis with an intensity similar to wild-type Xist RNA
and apparently can recruit the machinery responsible for his-
tone H3 trimethylated at lysine 27 (H3K27me3) in TS cells.
In addition, these TS cells contain an asynchronously replicating
X chromosome, a cytogenetic correlate of the inactive
X chromosome. Presumably this corresponds to the chromo-
some bearing the expressed XistIVS allele. Collectively these
observations indicate that the mutated X chromosome carrying
XistIVS has undergone proper inactivation in TS cells. A subset
of cells, however, lose XistIVS RNA and the inactive X associ-
ated histone modification H3K27me3 when TS cells are
induced to differentiate. Furthermore, microarray analysis
revealed that expression levels of X-linked genes become
widely misregulated upon differentiation. Although the
global misregulation became evident only after induction of
differentiation, a small fraction of X-linked genes were not
properly repressed even in the undifferentiated state. These
results suggest that XistIVS can initiate X-inactivation but is
deficient in maintaining the inactivated state. Given that the
H3K27me3 domain excludes active chromatin modifications
such as H3K4me2 and H4Ac, the silent domain has apparently
formed in response to the accumulation of XistIVS RNA in the
nucleus of undifferentiated mutant TS cells.

A loss of XistIVS clouds seen in a subset of differentiated
TS cells could be due to either dissociation of the RNA
from the X chromosome or transcriptional repression of the
XistIVS allele. Since qRT-PCR showed that relative expression
levels of Xist were reduced after differentiation of TS cells car-
rying the paternal XistIVS but unchanged in wild-type, it is
unlikely that XistIVS RNA simply dissociates from the
chromosome. Assuming that the observed effect is due to
transcriptional inhibition of XistIVS, the presence of an
exogenously introduced intron in exon 1 could be causal. In
this scenario, the XistIVS phenotype may not reflect defective
silencing by the mutant RNA but rather failure to maintain
Xist RNA expression in a subset of cells. It may be relevant
that the extra intron in the XistIVS allele is inserted at 0.3 kb
upstream of the three potential YY1 binding sites, which
have been suggested to function as an enhancer [42]. More
recently, these YY1 sites were found to be the sites for touch-
down of Xist RNA bound by YY1 [33]. However, it is also
possible that the XistIVS deficiencies are attributable to loss
of function of the RNA product. The insertion of the 16-base
sequence may directly or indirectly compromise an interac-
tion between XistIVS RNA and factors that are critical
for the stable association of Xist RNA on the X chromosome.
Alternatively, the insertion may partially compromise the
function of the A-repeat that contributes both to silencing
and localization on the X chromosome. Finally, it is possible
that the 16-base insertion disrupts a previously unidentified
silencing element in Xist RNA.

3. Role of Xist RNA in X chromosome silencing

The inactive X chromosome coated with Xist RNA is often
found to reside at the periphery of the interphase nucleus
to form the Barr body. An early study showed that Xist
RNA remains with the nuclear scaffold or matrix fraction
after removal of chromatin by DNaseI digestion and salt
extraction [43]. This implied that the association of Xist
RNA with chromatin of the X chromosome was not mediated
by hybridization of DNA and RNA, but probably by an inter-
action with a protein(s). The finding that scaffold attachment
factor-A (SAF-A), also termed hnRNP U, one of the major
components of the nuclear scaffold, is enriched on the inactive
X chromosome in human and mouse female cells
[44,45] suggested a potential role for this protein in targeting
of the inactive X to the nuclear periphery via association
with Xist RNA. In fact, SAF-A contains two distinct domains,
a SAF box and RGG domain, that have been shown to mediate
SAF-A binding to DNA and RNA, respectively [44,46].
A more recent study showed that knockdown of SAF-A in
mouse tissue culture cells breaks up the Xist cloud, resulting
in a more dispersed nuclear signal, suggesting that SAF-A is
responsible for the retention of Xist RNA on the inactive
X chromosome [47]. Interestingly, of the long and short
forms of Xist RNA (figure 2) detected on a northern blot,
only the former is selectively diminished after a depletion
of SAF-A, suggesting that while the long form is unstable
and readily degraded after dissociation from the inactive X,
the short form is relatively stable and thereby detectable as
dispersed signals in the nucleus. Given that Xist RNA is
polyadenylated only in the short form but not in the long
form [30], stability of the dissociated Xist RNA appears to
depend on the presence or absence of a poly(A) tail. It should
be commented that the functional difference between the long
and short form is unknown. While it appears that the associ-
ation of Xist RNA with the inactive X chromosome depends
on the presence of SAF-A, another study showed that the loss
of Xist RNA resulted in loss of SAF-A from the inactive X
and, therefore, localization of SAF-A is apparently dependent
on the presence of Xist RNA [45]. Thus, although a potential
role of SAF-A in compartmentalization of the inactive X
chromosome is attractive, the picture is currently complicated
and further studies are required.
Several lines of evidence suggest that Xist RNA recruits epigenetic modifiers to the inactive X chromosome, among which Polycomb group (PcG) proteins have been most intensively studied. The importance of PcG proteins in X-inactivation is suggested by the fact that the inactive X chromosome is enriched with H3K27me3 and H2AK119ub1, which are posttranslational modifications mediated by Polycomb repressive complex (PRC) 2 and 1, respectively [48–51]. The recruitment of PRC2 complex in X-inactivation has been attributed to direct biochemical interaction of the catalytic subunit of the complex, Ezh2, with the A-repeats of Xist RNA [52]. Mapping of the candidate RNA interaction domain of Ezh2 suggested that RNA binding requires site specific phosphorylation of Ezh2 [53]. Further to this, others have described an interaction between a different subunit of PRC2, Suz12, and Xist A-repeats [54]. To date these interactions have been demonstrated largely using in vitro assays, and given that none of the PRC2 subunits have a classical RNA binding domain, it will be important in the future to rule out non-specific interactions of Xist A-repeat with charged patches on the surface of either Ezh2 or Suz12 proteins. A key experiment will be to identify amino acids required for interaction with A-repeat RNA in vitro and then test mutant proteins for their ability to associate with the inactive X chromosome in XX cells in vivo.

It was initially thought that recruitment of PRC1 complex to the inactive X is solely attributable to binding of the chromodomain of the core PRC1 subunit, CBX2/4/7/8, to H3K27me3 mediated by PRC2 [50,51]. However, it has been shown that PRC1 complexes and associated H2AK119ub1 are enriched on the inactive X in the absence of PRC2 and H3K27me3 [55]. In a recent study, H3K27me3 independent recruitment of PRC1 has been shown to be attributable to distinct PRC1 like complexes, RYBP-PRC1, in which the protein RYBP is included [56]. Both CBX family proteins and another core PRC1 subunit, MPH1/2/3, are excluded from RYBP-PRC1. The existence of an H3K27me3 independent pathway for PRC1 recruitment to the inactive X raises further challenges in terms of understanding interactions between Xist RNA and PcG proteins.

Interestingly, the X chromosome coated with Xist RNA lacking the A-repeat, which is defective in silencing, manifests the enrichment of H3K27me3 and H2AK119ub1 in differentiating ES cells, albeit at a reduced level [52,55]. This implies that PcG recruitment is not synonymous with gene silencing on the inactive X [57]. This may indicate that elements in Xist RNA other than the A-repeats have the capacity to recruit PcG complexes. Alternatively, PcG recruitment may be secondary to other changes on the inactive X that are conferred both by wild-type and by A-repeat deficient Xist RNA. The domain corresponding to the Xist cloud has also been shown to exclude active epigenetic modifications such as acetylation of core histones and also RNA polymerase II (RNAPII), suggesting that a transcriptionally silent domain or compartment is formed by the accumulation of Xist RNA (figure 3). Interestingly, RNAPII exclusion is also observed in response to expression of Xist RNA lacking the A-repeat [58], suggesting that a transcriptionally silent domain is still formed. Consistent with this, a more recent study demonstrated that expression of Xist RNA lacking the A-repeat is associated with a reduction in the active chromatin modifications H4 acetylation and H3K4 methylation, as detected at the cytogenetic level [45].

DNA-FISH using X-linked sequences showed that while repetitive sequences on the X chromosome are included in the domain of Xist RNA lacking the A-repeat, genic regions are often found outside or at the periphery of the domain [58]. These findings raise the idea that one role of Xist RNA is to create a transcriptionally silent domain that represses repetitive sequences by recruiting PRCs and by excluding

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**Figure 3.** Silencing-defective Xist RNA lacking the A-repeat can form a silent chromatin domain. Repressed X-linked gene loci are included in the silent chromatin domain formed by wild-type Xist RNA (left). Silencing-defective Xist RNA lacking the A-repeat can form a silent compartment, characterized by enrichments of macroH2A, SAF-A and Ash2L as well as typical repressive epigenetic modifications, but fails to incorporate the X-linked gene loci into the silent compartment (right).
RNAPII in a manner independent of A-repeat function, and a second role is to incorporate genic regions on the X chromosome from outside or at the periphery of the Xist RNA domain into the interior in a manner dependent on the A-repeat. Therefore, silencing depends on whether or not genes are translocated into the Xist RNA domain (figure 3). It will be important to elucidate how the silent domain is formed by the accumulation of Xist RNA in order to establish how Xist RNA induces silencing.

It has been shown using a conditional allele of Xist that the inactive X chromosome in female MEFs can maintain the inactive state even after Xist RNA has been removed [59]. This was consistent with an earlier finding that a human inactive X chromosome does not require the XIC for maintenance of X-inactivation once the inactive state is established in human cultured cells [60]. That the loss of XistIVS RNA in differentiating TS cells appears to facilitate reactivation of the hitherto inactive X seems to run counter to the idea that Xist is not required to maintain X-inactivation in differentiated cells. A recent study, however, demonstrates that maintenance of X-inactivation is Xist-RNA-dependent in some differentiated cell types [61]. Ohhata et al. [61] show that induced expression of Tsix by doxycycline could counteract Xist and shut it off in the visceral endoderm, leading to reactivation of the X-linked GFP transgene that had been repressed on the inactive X chromosome. This effect was not seen in the trophoblastic lineages. These observations imply that, contrary to the prevailing view, there is a lineage-specific difference in Xist RNA-dependency for the maintenance of X-inactivation, and that some of the extraembryonic tissues may require Xist RNA to maintain the silent state. In fact, it has been demonstrated that Xist RNA, DNA methylation and histone hypoacetylation synergistically contribute to the maintenance of the inactive X chromosome in female MEFs [62]. Given that DNA methylation seems to be less important for the maintenance of X-inactivation in the extraembryonic lineages than in the embryonic lineage [63], it may be reasonable to assume that the extraembryonic lineages depend more on Xist RNA than the embryonic lineage.

4. Rsx, an Xist-like molecule in marsupials

Mammalian species in which Xist homologues have been identified have provided a useful resource for defining conserved and hence potentially important sequences and features of Xist RNA. In marsupial mammals, X-inactivation resembles that of eutherian mammals in many, albeit not all, aspects (table 1). With this in mind, it was assumed for a considerable time that marsupials have an Xist homologue. This however has been shown not to be the case [78]. Specifically, the Xist gene appears to have evolved from the protein-coding gene Lnx3, which in marsupials retains normal protein coding capacity and is expressed in both sexes. It was further shown that the evolution of Xist RNA involved acquisition of sequences from common repeat elements present in ancestral lineages [79]. It follows that marsupials must have evolved their X-inactivation mechanism independently. A recent study has described a marsupial cis-acting ncRNA, termed Rsx (RNA on the silent X), that appears to be equivalent to Xist RNA in eutherians [64]. Rsx shares a number of key properties with Xist. The locus transcribes a large RNA that is spliced and polyadenylated, lacks obvious protein coding potential and is only expressed in females. Rsx expression leads to formation of RNA clouds in the interphase nucleus resembling those produced by Xist RNA. Similar to Xist, Rsx sequence includes multiple copies of simple repeat sequences that may form

<table>
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<td>nt</td>
<td></td>
</tr>
<tr>
<td>RNAPII depletion</td>
<td>yes</td>
<td>yes</td>
<td>[72]</td>
</tr>
<tr>
<td>H3K4me2 depletion</td>
<td>yes</td>
<td>yes</td>
<td>[72,73]</td>
</tr>
<tr>
<td>H3K9me2</td>
<td>yes</td>
<td>yes</td>
<td>[72]</td>
</tr>
<tr>
<td>H3K9me3</td>
<td>no$^c$</td>
<td>yes</td>
<td>[72–74]</td>
</tr>
<tr>
<td>H4K20me1</td>
<td>yes</td>
<td>no</td>
<td>[72]</td>
</tr>
<tr>
<td>H4K20me3</td>
<td>no</td>
<td>yes</td>
<td>[72,73]</td>
</tr>
<tr>
<td>MacroH2A</td>
<td>yes</td>
<td>nt</td>
<td></td>
</tr>
<tr>
<td>CpG island methylation</td>
<td>yes</td>
<td>no</td>
<td>[75,76]</td>
</tr>
</tbody>
</table>

$^a$Instability observed in tissue culture cells but not in tissues.
$^b$DAPI dense structure observed in various studies.
$^c$Has been observed in some species [77].
stem-loop structures resembling those predicted to be formed by XiA-A-repeats. Strikingly, expression of Rsx in a mouse ES cell line leads to formation of an RNA cloud and, moreover, following differentiation of the ES cells, genes localized on the same chromosome, in cis with the Rsx transgene, are silenced. This is similar to human XIST transgenes in mouse ES cells that also silence only following in vitro differentiation [80], although differentiation is not required for silencing by mouse Xist RNA in mouse ES cells [81]. Despite the obvious similarity of Rsx and Xist, it is clear that Rsx arose entirely independently in the marsupial lineage, a remarkable example of convergent evolution. That chromatin modifications of the marsupial inactive X closely resemble those seen in eutherians (table 1), together with the fact that Rsx can function in mouse ES cells, suggests that the mechanism by which these ncRNAs silence the chromosome could be related. Marsupial X-inactivation is different in some respects, notably it is always the paternal X chromosome could be related. Marsupial X-inactivation is different in some respects, notably it is always the paternal X that is inactivated, silencing is relatively unstable in marsupial tissue culture cells, and, possibly linked to this, there is apparently no DNA methylation at CpG islands of genes on the marsupial inactive X chromosome [66,75]. It remains to be determined whether these reflect fundamental differences in mechanism or only in detail. If the silencing mechanism is indeed shared, then the discovery of Rsx offers an unprecedented opportunity to further elucidate the mechanism of Xist-mediated silencing in eutherians.

5. Concluding remarks
One of the most important unanswered questions in the X-inactivation field is, how does Xist RNA mediate chromosome silencing? One can predict that Xist RNA recruits specific proteins required for gene silencing and heterochromatinization and, in fact, a number of such proteins have been shown to localize on the inactive X chromosome (table 2). For some of these proteins, X chromosome localization has been found to depend on the presence of Xist RNA, although it should be noted that this does not discriminate between direct and indirect recruitment. Other proteins localizing to the inactive X have not yet been tested for Xist RNA dependency and it will be important in the future to establish this. However, it is likely that key factors required for silencing have yet to be identified. This is suggested both from genetic analysis of the known X-inactivation factors, and also because with the exception of SAF-A/hnRNP U, none of the these factors has a known bona fide RNA binding domain, a feature that would be predicted to occur in critical factors that interact directly with Xist RNA.

A further point relating to this is that although Xist RNA lacking the A-repeat is defective in silencing, it can bring about recruitment of PRCC1 and PRCC2 to the inactive X chromosome, and in addition can establish chromosome wide loss of H4 acetylation and H3K4 methylation. A-repeat deficient RNA is also able to recruit macroH2A, SAF-A and Ash2L [45]. Either these proteins interact with regions of Xist RNA other than the A-repeats or their recruitment is secondary to the establishment of a transcriptionally silent domain that occurs using A-repeat deficient transgenes [58]. As discussed earlier, it has been suggested that the silent domain corresponds to common repeat sequences on the X chromosome and it follows that these could also be the site of recruitment of other factors. Thus, the function of the A-repeats would be to allow the silencing factors to spread from common repeat elements into genes.

We thank colleagues in the field for discussions and apologise to those whose results we were unable to cover owing to space constraints. We thank James Turner for providing advanced access to his results. Takashi Sado is funded by the Grant-in-Aid for Scientific Research on Innovative Areas from the Ministry of Education, Science, Sports, and Culture of Japan. Neil Brockdorff is funded by the Wellcome Trust (grant no. 081383).

Table 2. Proteins known to localize on the inactive X chromosome.

<table>
<thead>
<tr>
<th>proteins or complexes</th>
<th>dependency on Xist RNA</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRCC1 (Mel-18/Bm11, Cbx2, Phc, Ring1A/B, RYBP, etc.)</td>
<td>yes</td>
<td>[51,56,62,83]</td>
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<tr>
<td>PRCC2 (Ezh2, Eed, Suz12, etc.)</td>
<td>yes</td>
<td>[48,49,84]</td>
</tr>
<tr>
<td>macroH2A</td>
<td>yes</td>
<td>[45,85]</td>
</tr>
<tr>
<td>PARP1</td>
<td>nt</td>
<td></td>
</tr>
<tr>
<td>SAF-A/hnRNP U</td>
<td>yes</td>
<td>[44,45,47]</td>
</tr>
<tr>
<td>Atrx</td>
<td>nt</td>
<td>[86]</td>
</tr>
<tr>
<td>SmcHD1</td>
<td>nt</td>
<td>[87]</td>
</tr>
<tr>
<td>Ash2L</td>
<td>yes</td>
<td>[45]</td>
</tr>
</tbody>
</table>

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