

Xist RNA binds select autosomal genes and depends on Repeat B to regulate their expression

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eLife Assessment

This **valuable** study addresses the potential roles of the master regulator of X chromosome inactivation, the Xist long non-coding RNA, in the regulation of autosomal genes. Using data from mouse cells, the authors propose that Xist can coat specific autosomal promoters, which in turn leads to the attenuation of their transcriptional activity. The evidence from individual genes is interesting, and the model aligns with recently published results from humans. However, despite some improvements during revision, the data and statistical analyses in the current study are not yet strong enough to allow for conclusive inferences, leaving the evidence for mouse cells behaving like human cells **incomplete**. The topic of the work is of broad interest, in particular to colleagues studying gene regulation and noncoding RNAs.

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Abstract

Xist, a pivotal player in X chromosome inactivation (XCI), has long been perceived as a cis-acting long noncoding RNA that binds exclusively to the inactive X chromosome (Xi). However, Xist's ability to diffuse under select circumstances has also been documented, leading us to suspect that Xist RNA may have targets and functions beyond the Xi. Here, using female mouse embryonic stem cells (ES) and mouse embryonic fibroblasts (MEF) as models, we demonstrate that Xist RNA indeed can localize beyond the Xi. However, its binding is limited to ~100 genes in cells undergoing XCI (ES cells) and in post-XCI cells (MEFs). The target genes are diverse in function but are unified by their active chromatin status. Xist binds discretely to promoters of target genes in neighborhoods relatively depleted for Polycomb marks, contrasting with the broad, Polycomb-enriched domains reported for human XIST RNA. We find that Xist binding is associated with down-modulation of autosomal gene expression. However, unlike on the Xi, Xist binding does not lead to full silencing and also does not spread beyond the target gene. Over-expressing Xist in transgenic ES cells similarly leads to autosomal gene suppression, while deleting Xist's Repeat B motif reduces autosomal binding and perturbs autosomal down-regulation. Furthermore, treating female ES cells with the Xist inhibitor, X1, leads to loss of autosomal suppression. Altogether, our findings reveal that Xist targets ~100 genes beyond the Xi, identify Repeat B as a crucial

domain for its in-trans function in mice, and indicate that autosomal targeting can be disrupted by a small molecule inhibitor.

Introduction

The unequal sex chromosome composition between male (XY) and female (XX) placental mammals necessitates dosage compensation of the X chromosome ^{1,2}. X chromosome inactivation (XCI) specifically evolved to ensure equal X-linked gene dosage between the sexes. During early embryogenesis, one of the two X chromosomes in female cells is randomly silenced, leading to a mosaic of cells in which X-linked genes can be expressed from either the maternal or paternal X chromosome ^{3–5}. The long non-coding RNA Xist is an essential regulator of the XCI process ^{6,7}. At the onset of XCI, Xist is expressed exclusively from the future inactive X chromosome (Xi) and then selectively spreads *in cis* along the chromosome to initiate formation of heterochromatin via association with chromatin-modifying complexes and alterations in 3D chromosome structure (reviewed in ^{2,8,9–12}). From early RNA fluorescence in situ hybridization (FISH) experiments, it was shown at a cytological level that Xist binds only to the X-chromosome which transcribes the RNA ^{6,13}. In Xist transgenesis studies, the RNA is also observed to localize exclusively *in cis* to the transgene, even on autosomes ^{14–20}. In more recent years, technical advances in epigenomic mapping of RNA confirmed the early cytological data and provided a map of Xist binding sites *in cis* at kilobase resolution ^{21,22}. Furthermore, genetic analysis of the X-inactivation center revealed a Xi-specific nucleation site for the initial binding of Xist that then enabled the RNA to spread exclusively *in cis* ²³. Altogether, these studies solidified the view that Xist RNA is a *cis*-acting RNA.

However, studies have long documented the potential for Xist to spread beyond the X-chromosome under various non-physiological conditions. When Xist is overexpressed, the RNA can diffuse to bind neighboring chromosomes ^{15,23,24}. Furthermore, when Xist's nucleation site is mutated, Xist will diffuse and bind other chromosomes *in trans* ²³. A recent study has also pointed to broad XIST binding patterns outside of the Xi in human naïve stem cells and suggested autosomal targets ²⁵. These findings prompt interesting questions about the binding dynamics and properties of Xist on autosomes, its potential to spread locally in autosomal domains, and its impact on gene regulation and cellular physiology. To investigate, here we examine the capacity for Xist to spread beyond traditional boundaries. We use mouse embryonic stem cells (mESC) and mouse embryonic fibroblasts (MEF) in order to capture both the establishment and maintenance phases of XCI. Intriguingly, we identify about 100 binding sites on autosomes in cells undergoing XCI as well as post-XCI cells. We demonstrate discrete binding sites, rather than broad binding domains. Transcriptomic analysis reveals a selective downregulation, but not silencing, of associated genes. We also probe a requirement for Xist's Repeat B (RepB) motif and the ability to perturb autosomal effects by treating cells with a small molecule inhibitor of Xist RNA.

Results

Epigenomic mapping of Xist binding reveals autosomal targets in mouse cells

To explore the possibility of Xist extending its binding to autosomal sites beyond the X chromosome, we conducted CHART (Capture Hybridization of Associated RNA Targets) ^{21,26} in female ES cells. To capture Xist RNA, we used oligoprobes antisense to Xist and then pulled down interacting chromatin for deep sequencing of the associated DNA. To control for any direct hybridization to DNA that could occur independently of Xist RNA, we conducted CHART in parallel using “sense” probes that should not hybridize to Xist. This helps us distinguish signals genuinely

due to Xist RNA from those that could arise from non-specific probe binding to DNA. We note that some of the sense probes could hybridize to Tsix RNA, but Tsix expression is normally down-regulated by day 4 of ES differentiation²⁷. We also performed CHART using male ES cells, which do not upregulate Xist expression, to define the background level of binding. Notably, male ES cells express Tsix RNA at day 0²⁷ and therefore also assist in excluding non-Xist RNA binding during analysis. Together the male and sense controls allowed us to account for non-specific interactions and background noise. In addition to these controls, we performed 2 biological replicates and analyzed only overlapping signals between the 2 replicates to ensure the reliability and reproducibility of our results. The Pearson and Spearman correlation analysis showed good reproducibility between replicates (Figure S1A). We also normalized to input DNA to account for differences in chromatin preparation and sequencing depth.

Xist RNA is transcriptionally upregulated during female ES cell differentiation when XCI is induced. To profile the dynamics during cell differentiation and XCI, we examined wild-type (WT) female ES cells in the undifferentiated state (day 0) and at three differentiated stages (day 4, 7, and 14). Antisense oligonucleotides targeting Xist efficiently pulled down Xist RNA and associated chromatin targets, in excess of the “background” observed with the sense probe, and male control (Figure S1B). Principal component analysis (PCA) of CHART-seq data from day 4 and Xist coverage on X-linked genes revealed that Xist CHART signals in female cells were distinct from those of sense probe and male controls (Figure S1C). This PCA separation indicates a clear difference in Xist binding in females as compared to controls. As expected, the Xist CHART signal in WT female ES cells displayed strong binding at the Xist locus and genes subject to XCI, such as *Cdkl5* and *Mecp2*, while showing significantly weaker binding at the escapee gene, *Kdm6a*. In contrast, CHART experiments using sense probes and male ES cells, which lack Xist expression, showed minimal binding to these genes (Figure S2A-S2D, S3). Because Xist spreads *in cis* along the Xi to cover much of the chromosome, we first examined total Xist coverage (normalized to input), rather than calling peaks²⁸. Day 0 ES cells showed no enrichment for Xist binding, consistent with Xist being in the uninduced state (Figure 1A). Upon cell differentiation, enriched binding of Xist was consistently observed across all time points (Figure 1A). Greatest enrichment on the X chromosome was observed on day 7 (Figure 1B), in agreement with *de novo* XCI when Xist is needed at highest concentration^{21,29}.

Intriguingly, on day 7, only ~10% of the reads mapped to the X chromosome (Figure S1B), prompting the question of whether Xist might have non-X-linked targets as well. Indeed, although the X chromosome was most enriched for Xist, we noticed that autosomes also showed highly reproducible peaks, beginning at day 4 and increasing across the differentiation time points (Figure 1A). In contrast to broad binding pattern covering the entire X chromosome (Figure S2E), Xist binding patterns on autosomes trended towards sharp peaks (Figure 1C and S2F). To determine if the peaks were statistically significant, we called Xist peaks using MACS2 on the Xist CHART data. We restricted the analysis to autosomal reads to increase the sensitivity of the analysis. We performed peak-calling separately for each CHART biological replicate. Irreproducible Discovery Rate (IDR) analysis indicated a strong correlation between the two replicates (Figure S4A). We used *bedtools intersect* to identify significant peaks that overlapped between the two replicates and only used overlapping peaks in subsequent analyses. Overall, Xist coverages in significant peaks of female cells were substantially greater than in the negative controls (Figure 1E-1F).

Intriguingly, we found hundreds of significant peaks across autosomes between days 4-14 of differentiation (Figure 1D, S4B, Tables S1-S6). Notably, Xist binding sites on autosomes were disproportionately located in promoter regions (Figure 1A and 1G), hinting at a potential role for Xist in autosomal gene regulation. We conclude that, in addition to the Xi of differentiating ES cells, mouse Xist RNA selectively binds ~100 autosomal targets, preferentially at promoter regions. This promoter-dominant profile contrasts with XIST patterns identified in human cells, which tend to demonstrate broad regions of coverage over genes²⁵. Although the Gene Ontology (GO)

analysis of these Xist autosomal target genes did not yield statistically significant enrichment for specific biological processes or pathways, a closer examination reveals that these genes have diverse functions. They are involved in processes such as cancer development (*Bcl7b*), POLII transcription regulation (*Med16*), amino acid transport (*Slc36a4*), RNA binding (*Rbm14* and *Stau2*), among other processes. Together with previously published work in human cells²⁵, our findings suggest that Xist may have a broad regulatory impact on a variety of autosomal genes, extending its influence beyond X-chromosome inactivation.

Deleting RepB causes a loss of binding to autosomal target genes

Prior work showed that Xist's Repeat B (RepB) element is essential for Polycomb recruitment^{30–33}. RepB is also required for proper spreading and localization of Xist RNA to the Xi: Without RepB, the Xist RNA cloud was observed to adopt a dispersed appearance consistent with diffusion of the RNA away from the Xi³². Given the partial loss of attachment to the Xi, here we asked if the Xist diffusion could lead to enhanced binding to autosomal targets. We conducted CHART experiments in RepB deletion (Δ RepB) female ES cells at various differentiation stages and quantitated the degree of X-linked versus autosomal binding (**Figure 2**). Although Xist retained partial binding to the X-chromosome, there was a significant decrease in localization along X-linked genes along all differentiation days, especially at day 7 (**Figure 2A**), consistent with RepB being essential for attachment of Xist to the Xi³². Intriguingly, however, the loss of Xi binding was not accompanied by any significant increase in Xist coverage at the same autosomal targets, at any differentiation day (**Figure 2A**). In fact, there appeared to be decreased binding to the ~100 autosomal genes (**Figures 2E–2F**, S4C–S4D). However, there was not a change up- or down-wards in the number of autosomal targets (**Figures 2B–2C**, S4B, S4E, Tables S1–S6). A similar number of Xist peaks across autosomes in Δ RepB cells was observed and the autosomal targets remained similar. Moreover, in the absence of RepB, Xist retained a preference to bind promoter regions (**Figure 2D**). Thus, RepB is required not only for Xist to localize to the X-chromosome but also for its localization to the ~100 autosomal genes.

Xist RNA down-regulates autosomal genes in a RepB-dependent manner

The promoter-specific binding pattern of Xist on autosomal targets contrasts sharply with the broad binding pattern of Xist on the Xi (**Figure 1A**) and with broad regions identified for human autosomal genes²⁵. Given the preference for autosomal promoters, we investigated whether Xist modulates expression of the associated autosomal genes. To address this question, we conducted transcriptome sequencing on WT female ES cells across day 0, 4, 7, and 14, and compared the profile to those of Δ RepB female ES cells. The RNA-seq data showed that Xist expression increased during the establishment phase of XCI in differentiating ES cell, reaching its peak at day 7 (**Figure S5A**), consistent with super-resolution data indicating that Xist is present at ~300 copies/cell during de novo XCI relative to the ~100 copies/cell during the maintenance phase²⁹. Xist expression followed a similar dynamic in Δ RepB female cells (**Fig. S5A**). However, deletion of Xist's RepB resulted in increased X-linked genes expression in differentiating female ES cells (**Figures 3D** and S5B–5C), consistent with a previous report³². To analyze the characteristics of genes bound by Xist on autosomes, we categorized all refSeq genes based on their expression levels at each differentiation day. The genes were divided into 5 quintiles (Q1 silent, Q2 low, Q3 moderate, Q4 high, Q5 highest). Consistent with Xist's behavior on the X-chromosome^{21,22}, Xist also favored binding to actively expressed genes on autosomes (**Figure 3A**). To examine gene expression levels as a function of distance from the Xist binding site, we divided neighboring genes into 10- to 100-kb bins and observed that genes at <10 kb showed highest Xist levels (**Figure 3B**, **Figure S6A–S6B**). These findings support Xist's favoring actively expressed genes. We then examined Polycomb marks in relation to the autosomal Xist domains. In agreement with the Xist's predilection for active genes, we observed lower coverage of Polycomb marks associated with Xist RNA^{12,17,30–34}, H3K27me3 and H2AK119ub (associated with

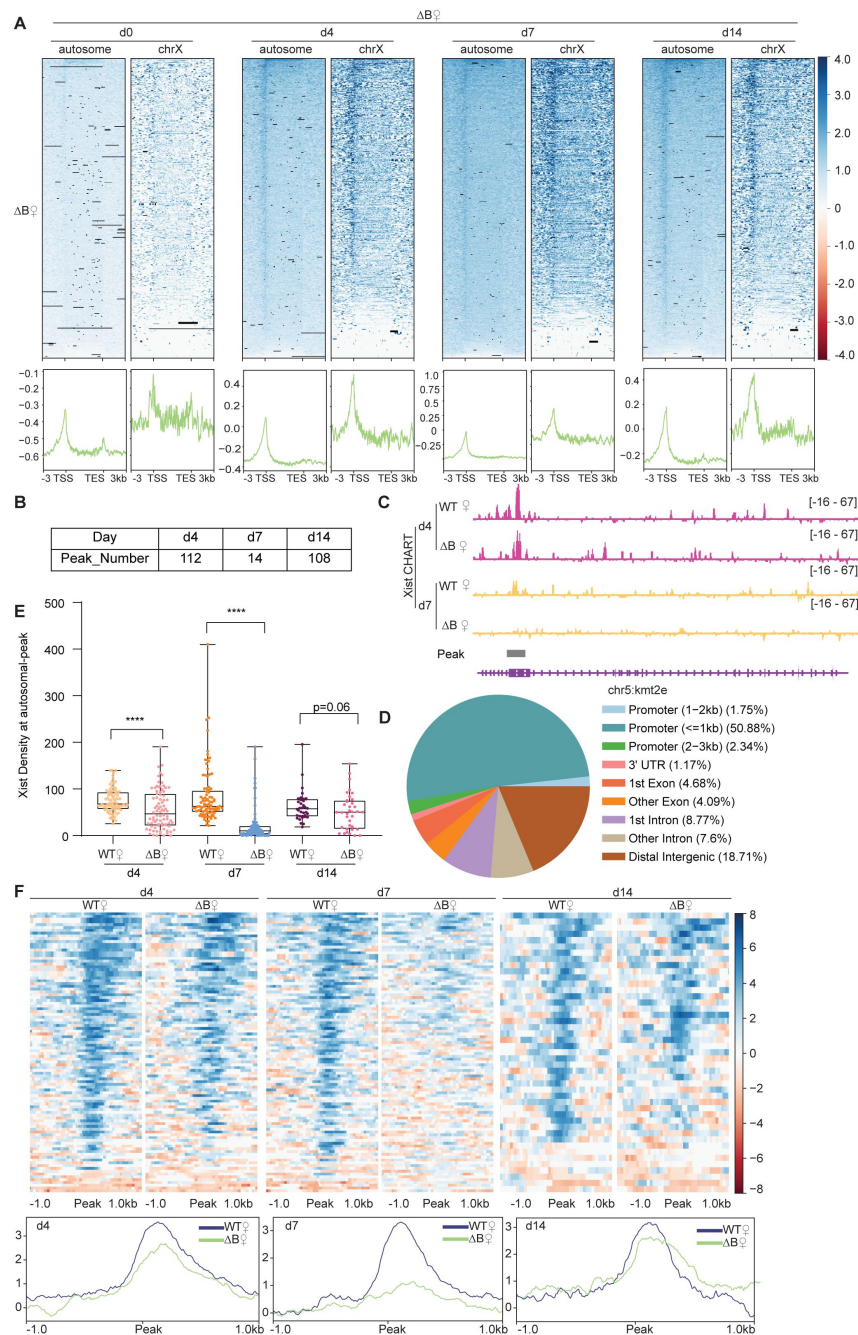


Figure 2.

Xist's Repeat B is required for proper binding of Xist to autosomal genes.

A. Top: Heatmaps of Xist coverage on autosomes and chromosome X genes in WT female ES cells at day 0, day 4, day 7, and day 14. Bottom: Average profiles shown as metagene maps of the data in the top panel.

B. Number of Xist peaks determined by MACS2 peak calling on autosomes in ΔRepB female ES cells at day 4, day 7, and day 14.

C. Representative site-specific binding of Xist on autosome locus (*Kmt2e*) in WT and ΔRepB female ES cells at day 4 and day 7.

D. Feature annotation of Xist binding loci on autosomes by ChIPseeker in ΔRepB female ES cells at day 4.

E. Xist CHART signal coverage on Xist-autosomal peak region in WT and ΔRepB female ES cells at day 4, day 7, and day 14. P-values are determined using the Wilcoxon rank sum test.

F. Top: Heatmaps of Xist coverage on Xist-autosomal peak region (top 100 peaks) in WT and ΔRepB female ES cells at day 4, day 7, and day 14. Bottom: Average profiles shown as metagene maps of the data in the top panel.

PRC2 and PRC1 respectively), as shown by lower coverages within the 10-kb versus 50-kb neighborhoods (**Figure 3C** [↗](#), **Figure S6C**). These findings also contrasted with observations in human cells, where PRC2/H3K27me3-enriched regions were favored ²⁵[↗](#). Our findings indicate that WT Xist transcripts favor binding to a select set of actively transcribed autosomal genes and these genes are not marked by Polycomb in mouse cells.

Given the association with active autosomal genes, we next asked whether Xist's binding modulates their expression. We performed RNA-seq analysis in two biological replicates in the ES differentiation time series. First, we profiled gene expression in female ES cells relative to male ES cells, reasoning that the lack of Xist RNA in male ES cells might lead to a difference in female versus male expression of autosomal target genes. Indeed, among the ~100 target genes, there was significantly greater expression in male cells (**Figure 3E** [↗](#)-**3G** [↗](#)), suggesting that Xist might also negatively regulate the select genes on autosomes. To test this idea, we took advantage of the Δ RepB mutation, as RepB is required for proper silencing of Xi genes ³²[↗](#). Transcriptomic analysis demonstrated that perturbing RepB resulted in loss of repression of autosomal targets. A detailed examination focusing on the top 100 peaks of Xist-bound genes on autosomes unveiled a down-modulation especially evident on days 4 and 7 of ES cell differentiation (**Figure 3G** [↗](#) and **S7A-S7D**). The Xist-bound autosomal genes on day 14 of ES cell differentiation like *Ces1l* or non-targets of Xist on autosomes such as *Kmt2c* did not demonstrate significant changes in gene expression (**Figure S7E** and **S7F**). We note, however, that unlike on the Xi, autosomal binding of Xist did not lead to full silencing of the target gene. Rather, the effect was a partial repression. In Δ RepB cells, reduced autosomal binding resulted in a blunting of this repression (**Figure 3F** [↗](#)-**3G** [↗](#) and **S7A-S7D**). On the other hand, at day 0 when Xist had yet to be upregulated, Δ RepB female and male ES cells showed no significant expression changes at Xist-autosomal targets (**Figure 3H** [↗](#)). To confirm that the observed changes in autosomal genes are due to Xist binding, we performed a similar analysis on 100 randomly selected autosomal genes that did not have Xist binding ("non-targets"). The non-targets did not show preference for specific autosomal gene region (**Figure S8A-C**) and showed no significant difference in Polycomb enrichment (**Figure S8D**) or gene expression (**Figure S8E**). These results demonstrate that autosomal binding of Xist RNA directly leads to a suppression of gene expression in a RepB-dependent manner.

Effects of Xist overexpression and X1 drug treatment on autosomal genes

To seek further evidence for autosomal effects of Xist binding, we conducted two orthogonal lines of experimentation. First, we asked the reciprocal question and determined whether ectopically overexpressing Xist leads to increased autosomal repression. We examined two previously published transgenic *Xist* cell lines, Tg_1 and Tg_2 ³⁵[↗](#), to test whether — *in the hands of other investigators* — cell lines expressing Xist also demonstrated autosomal targeting (**Figure 4A** [↗](#)). These two transgenic (Tg) females cell lines respond to doxycycline to induce Xist expression on autosomes (specifically chromosome 12, with two different transgene insertion sites). Transcriptomic analysis of these two Tg mouse ES cells upon neuronal differentiation showed a significant suppression of X-linked genes (e.g., *Med14*) in comparison to doxycycline-treated wildtype cells (**Figure 4B** [↗](#) and **S9A**). Autosomal Xist targets, as exemplified by *Bcl7b* and *Rbm14*, were also significantly suppressed beyond what was observed in WT ES cells (**Figure 4C** [↗](#) and **S9B**). In contrast, non-targets of Xist such as *Stau1* did not demonstrate significant changes in gene expression (**Figure 4E** [↗](#) and **4F** [↗](#)). Looking across all autosomal target genes, we observed a significant decrease in mean expression in the Xist overexpressing cell lines (**Figure 4D** [↗](#)), further implicating Xist binding in direct regulation of autosomal gene targets. The fact that the autosomal changes were also observed in datasets generated by other investigators strengthen our conclusions.

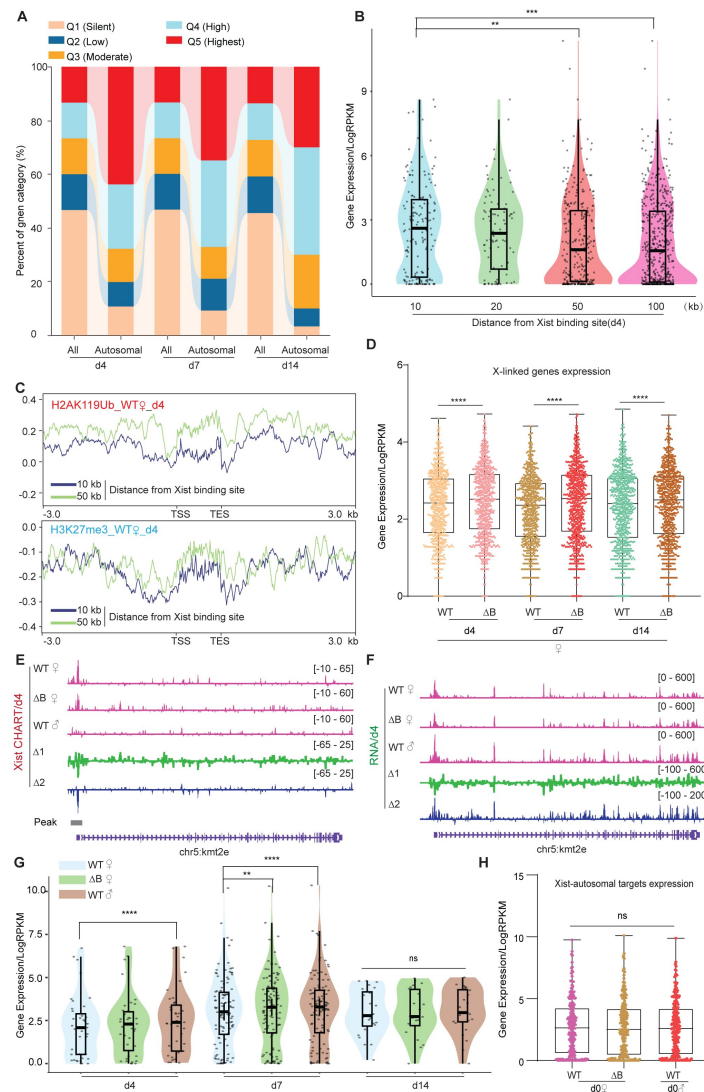


Figure 3.

Autosomal target genes are actively transcribed.

A. Xist preferentially targets actively expressed genes. The proportion of overall genes (label as “All”) and Xist targets on autosomes expression levels (label as “Autosomal”) in different expression categories. Genes are classified into distinct categories (Q1-Q5) based on RPKM values, representing various levels of expression. Category Q1 includes non-expressed genes (RPKM=0), while categories Q2, Q3, Q4, and Q5 represent 25%, 50%, 75%, and 100% expression, respectively.

B. Genes bound by Xist exhibit elevated expression levels compared to surrounding regions. The analysis of gene expression within the 10, 20, 50, and 100-kilobase binding regions of Xist is conducted in WT female ES cells at day 4. P-values are determined using the Wilcoxon rank sum test.

C. The H2AK119ub and H3K27me3 (Chip-Seq) average profile of genes within the 10 and 50-kilobase binding regions of Xist in WT female cells at day 4.

D. Evaluation of gene expression levels for XCI genes in WT and Δ RepB female ES cells at day4, day7, and day 14. P-values are determined using the Wilcoxon rank sum test.

E-F. Representative ChART-seq (F) and RNA-Seq (G) patterns of an autosomal gene bound by Xist (*Kmt2e*) at day 4. Change in coverage ($\Delta 1$ and $\Delta 2$) is shown below ($\Delta 1$ for Δ RepB $\varnothing$ - WT $\varnothing$, and $\Delta 2$ for WT σ - WT $\varnothing$).

G. Assessing gene expression levels of Xist targets on autosomes (10 kb within the peak region) in WT, Δ RepB female ES cells, and male ES cells at different time points. P-values are determined using the Wilcoxon rank sum test.

H. Gene expression levels for Xist targets on autosomes (identified in day4 and day7) in undifferentiated WT, Δ RepB, female and male ES cells (day 0) show no obvious changes. 2 biological replicates were used. P-values are determined using the Wilcoxon rank sum test.

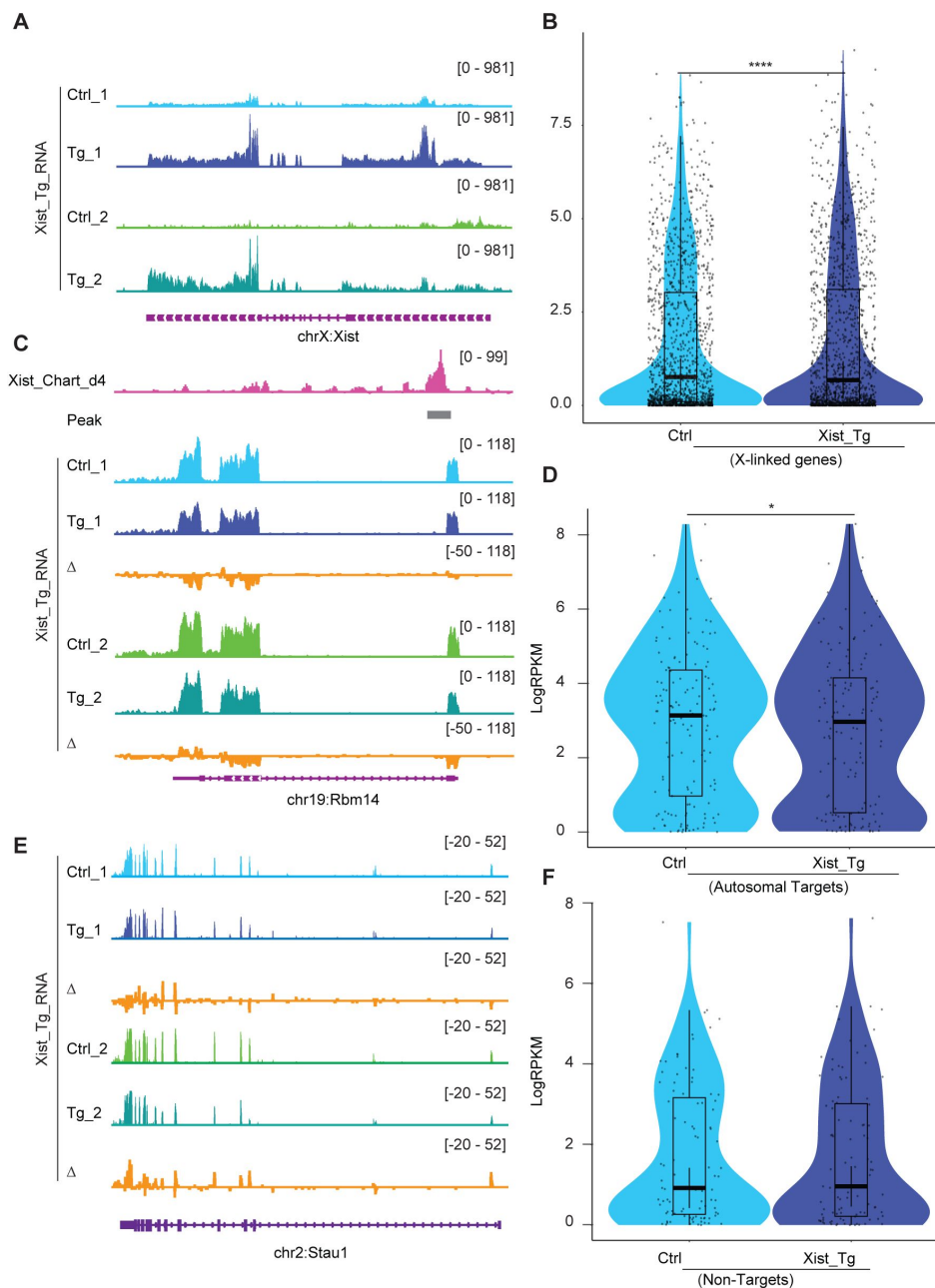


Figure 4.

Xist binding suppresses, but does not silence, autosomal gene expression.

A. RNA-seq track shows the Xist expression level in ectopic doxycycline responsive Xist overexpressed ES cells (129/Sv-Cast/Ei) of neuronal differentiation (Tg-1 and Tg-2 have different insertion sites), the control (Ctrl) was doxycycline-treated wildtype cell.

B. Analysis of gene expression levels for XCI genes in Xist Tg ES cells (129/Sv-Cast/Ei) at of neuronal differentiation. P-values are determined using the Wilcoxon rank sum test.

C. RNA-seq track illustrating expression alongside representative autosome genes with Xist binding (*Rbm14*) in Xist Tg ES cells (129/Sv-Cast/Ei) of neuronal differentiation. Change in coverage (Δ) is shown below (Tg - Ctrl).

D. Analysis of gene expression levels for Xist autosomal targets in Xist Tg ES cells (129/Sv-Cast/Ei) of neuronal differentiation. P-values are determined using the Wilcoxon rank sum test.

E. RNA-seq track illustrating expression level of autosome genes without Xist binding (*Stau1*) in Xist Tg ES cells (129/Sv-Cast/Ei) of neuronal differentiation. Change in coverage (Δ) is shown below (Tg - Ctrl).

F. Analysis of gene expression levels for Xist non-targets on autosomes in Xist Tg ES cells (129/Sv-Cast/Ei) of neuronal differentiation. P-values are determined using the Wilcoxon rank sum test.

In the second line of investigation, we deployed X1, a small molecule inhibitor of Xist that was previously shown to block initiation of XCI in female ES cells through Xist's Repeat A (RepA) motif ³⁶. We reasoned that if the mechanism of autosomal gene suppression were similar to XCI, treating cells with X1 could also blunt autosomal gene suppression. Indeed, just as X-linked genes such as *Mecp2* failed to undergo silencing after 5 days of differentiation, transcriptomic analysis showed that autosomal gene targets also failed to be suppressed in the presence of X1 (**Figure 5A-C**). On average, there was a significant upregulation of autosomal targets on day 5 of differentiation and X1 treatment (**Figure 5E**). Again, non-targets of Xist such as *Cbx2* did not demonstrate significant changes in gene expression (**Figure 5D** and **5F**). Analysis of ChIP-seq data for H3K27me3 indicated a reduction of the PRC2 mark within the Xist target gene and flanking regions (**Figure 5G** and **5H**), consistent with X1 blocking RepA's recruitment of PRC2 ³⁶. In contrast, Xist non-target genes show no significant changes in PRC2 signal (**Figure 5I**). These additional perturbation studies reinforce the notion that autosomal binding of Xist RNA directly suppresses autosomal targets. Thus, it is possible to disrupt autosomal Xist binding by administering a small molecule inhibitor of Xist.

Xist RNA also binds to autosomal genes in post-XCI cells

Lastly, because the establishment and maintenance phases of XCI show differential sensitivity to loss of Xist ³⁷⁻⁴⁰, we asked if autosomal repression exhibits a similar sensitivity. To address this, we turned to mouse embryonic fibroblasts (MEF), where XCI is well established. We first conducted CHART assays to determine whether Xist binds to the Xi and autosomes in a similar way in MEFs (**Figure S10A-10B**). Because the autosomal signals displayed peak-like characteristics, we used MACS software to call significant peaks on autosomes to determine if there was an increase in promoter-specific binding of the autosomal genes. We observed that, while Xist continued to favor targeting promoter regions in both WT and Δ RepB MEFs (**Figure 6A-C**), Xist peaks did not increase in size in Δ RepB MEFs; rather, the peaks decreased in size (**Figure 6A-C**) — a result similar to that in Δ RepB ES cells (**Figure 2**). Furthermore, there was a considerable decrease in the number of significant peaks in Δ RepB MEFs when compared to WT MEFs (**Figure 6A**). In WT MEFs, Xist clearly favored Xi binding, but Xist signals could also be visualized on autosomes (Table S7-S10, **Figure 6E**), in agreement with the results in ES cells (**Figure 1**). In Δ RepB MEFs, there was a partial loss of Xi localization, aligning with previous findings ³² (**Figure 6D-F**). Consistent with these findings, RNA-seq analysis of Δ RepB MEFs did not reveal any significant change in expression of Xi genes (e.g., *Mecp2*) and autosomal target genes (e.g., *Rbm14*) (**Figure 7A-D**).

We then asked if the retention of some Xist binding on the Xi could explain the lack of transcriptomic difference in Δ RepB MEFs. To investigate, we utilized a deletion of Xist's Repeat E (Δ RepE), which was previously demonstrated to severely abrogate localization of Xist to the Xi ^{41,42}. We reasoned that the severe loss of Xist binding might unmask a transcriptomic difference. As expected, we observed that Xist signals were somewhat more reduced on the Xi in Δ RepE MEFs compared to Δ RepB cells (**Figure 6E-F**). Despite this reduction, peak coverages in autosomal target genes did not increase in Δ RepE MEFs (**Figure 6E-F**). However, there was an overall decrease in the number of significant autosomal peaks in Δ RepE MEFs relative to WT cells (**Figure 6A**). Regardless, we observed no significant transcriptomic differences in Δ RepE MEFs relative to WT MEFs (**Figure 7A-E**). Additionally, further examination of RNA sequencing data from male and female MEF cells in two published studies ^{43,44} corroborated that the expression levels of these autosomal Xist targets did not exhibit significant changes (**Figure 7F** and **7G**). Altogether, the analysis in MEFs demonstrates that Xist continues to bind autosomal genes in post-XCI somatic cells. However, autosomal binding of Xist in post-XCI cells does not overtly impact expression of the associated autosomal genes. Nonetheless, we cannot exclude more subtle changes that do not meet the significance cut-off.

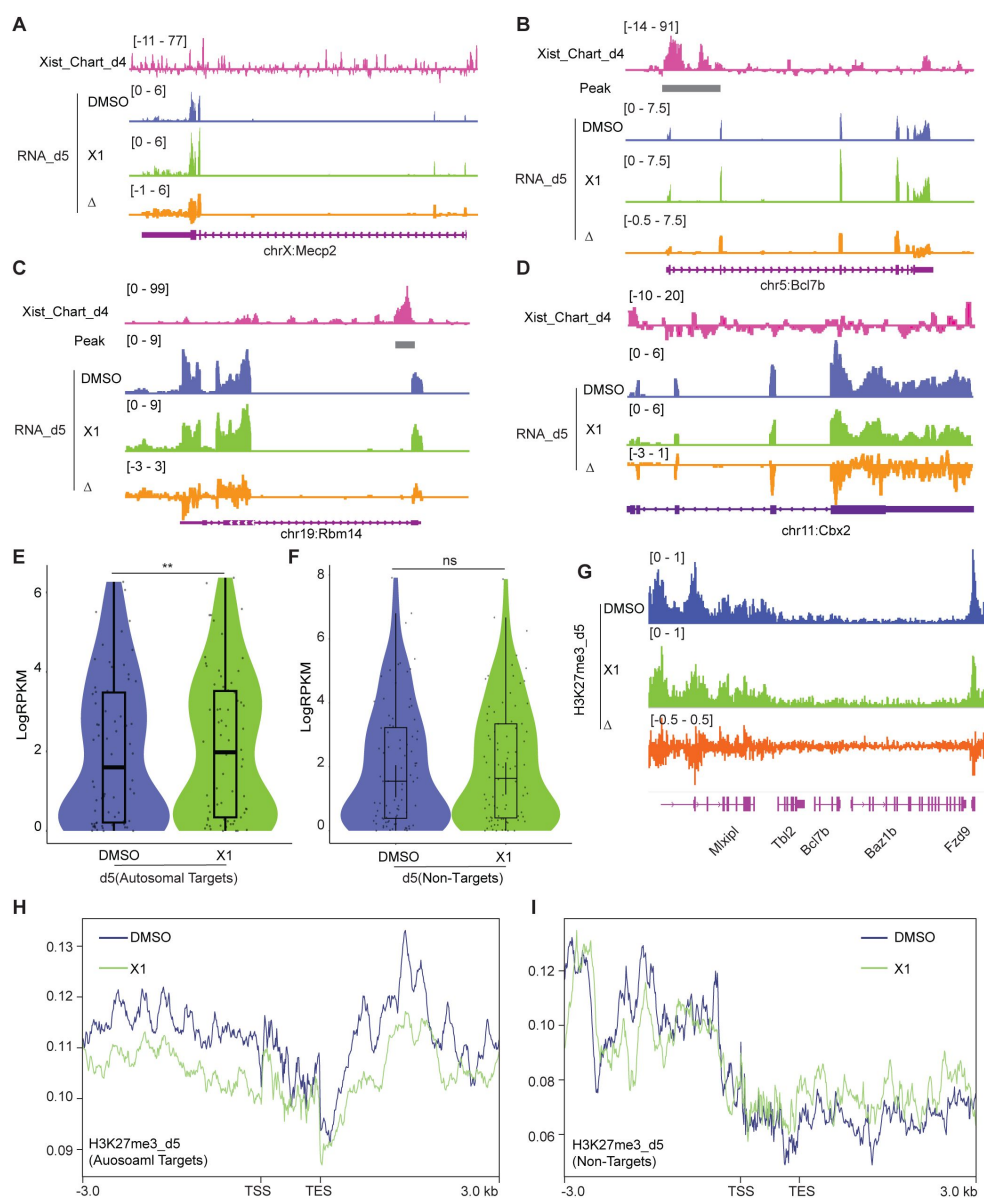


Figure 5.

Treating cells with the X1 inhibitor of Xist RNA perturbs autosomal target genes.

A-D. RNA-seq track depicting *Mecp2* (A) (an X-linked gene), *Bcl7b* (B) and *Rbm14* (C) (Xist-regulated autosome target), and *Cbx2* (D) (autosomal gene without Xist binding) expression in DMSO (control) and Xist inhibitor (X1) treated differentiated WT female ES cells at day 5 of differentiation. Change in coverage (Δ) is shown below (X1 - DMSO). The Xist peak on autosomes is from CHART at day 4.

E. Evaluation of gene expression levels for Xist autosomal targets in DMSO (control) and X1 treated WT female ES cells at day 5 of differentiation. The Xist peak on autosomes is from CHART at day 4. P-values are determined using the Wilcoxon rank sum test.

F. Evaluation of gene expression levels for Xist non-targets on autosomes in in DMSO (control) and X1 treated WT female ES cells at day 5 of differentiation. P-values are determined using the Wilcoxon rank sum test.

G. ChIP-seq track for H3K27me3 on *Bcl7b* (an Xist autosomal target, within a 160 kb window) in DMSO (control) and X1 treated WT female ES cells at day 5 of differentiation. Change in coverage (Δ) is shown below (X1 - DMSO).

H-I. Profile plot displaying H3K27me3 levels for Xist-autosomal targets flanking genes (H) (within a 100 kb window) or Xist non-targets (I) in DMSO (control) and X1 treated WT female ES cells at day 5 of differentiation. The Xist autosomal target is from CHART at day 4.

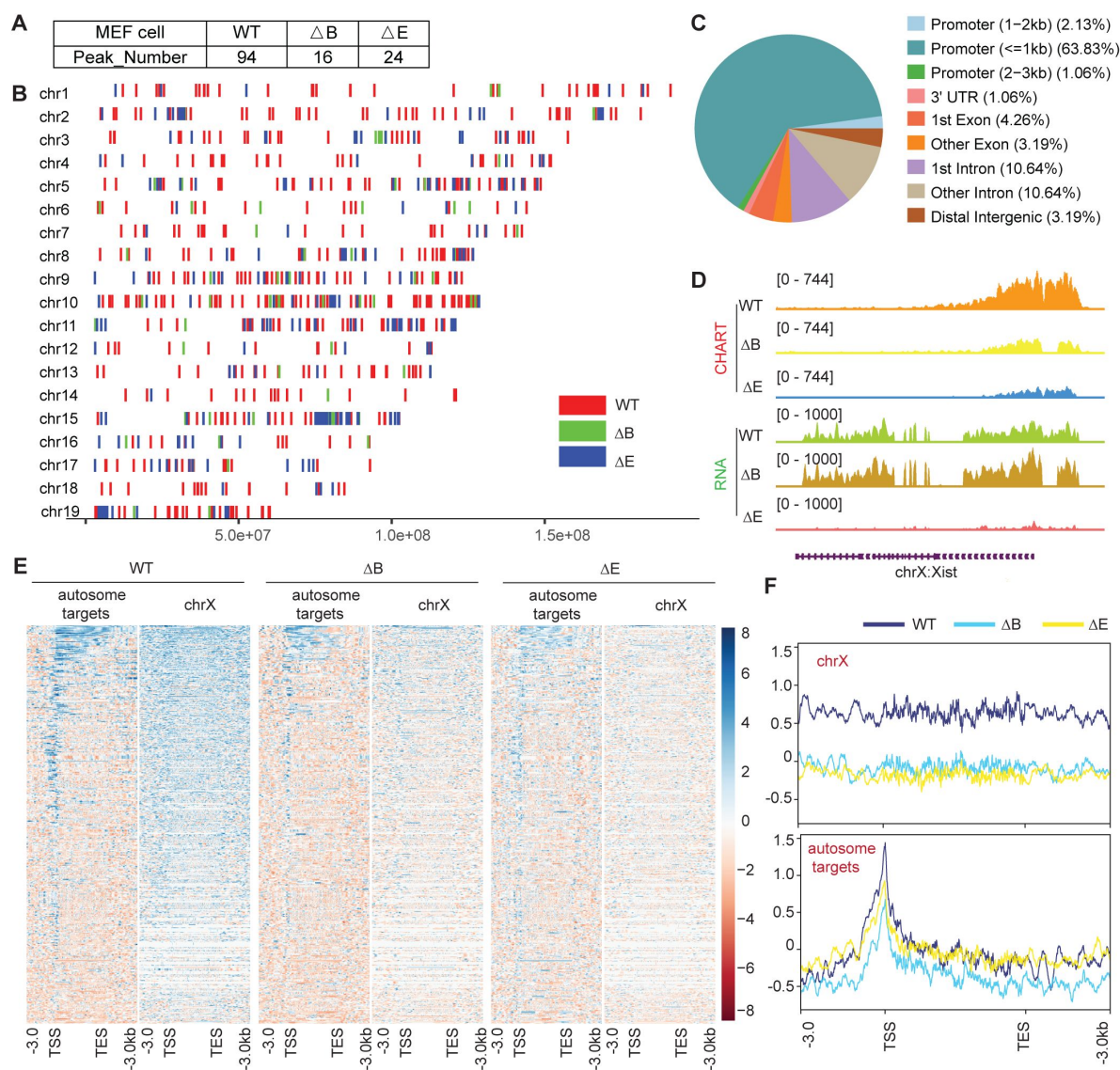


Figure 6.

Xist RNA also binds autosomal genes in post-XCI MEF cells.

- A.** Xist peak number (MACS2 peak calling) on autosomes in WT, Δ RepB, and Δ RepE female MEF cells.
- B.** Xist peak patterns (MACS2 peak calling) on autosomes in WT, Δ RepB, and Δ RepE female MEF cells.
- C.** Feature annotation of Xist binding loci on autosomes in WT female ES cells.
- D.** Representative CHART-seq and RNA-seq track patterns of Xist in WT, Δ RepB, and Δ RepE female MEF cells.
- E-F.** Heatmaps (E) and average profiles (F) depicting Xist coverage on autosome targets and chromosome X in WT, Δ RepB, and Δ RepE female MEF cells.

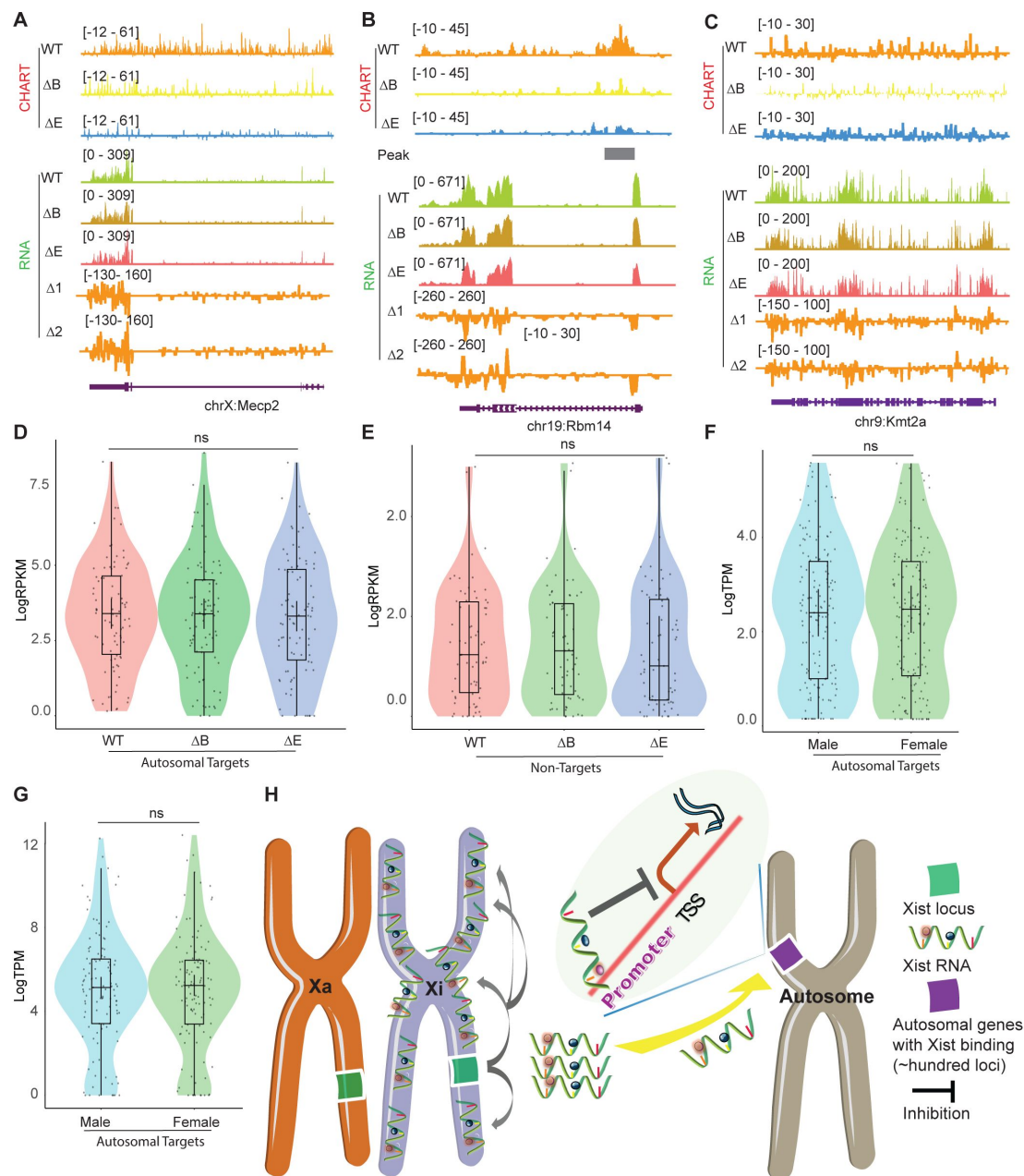


Figure 7.

Xist autosomal binding does not alter gene expression in MEF Cells.

A-C. Representative CHART-seq and RNA-seq track patterns of Xist autosomal binding genes such as *Mecp2* (A) and *Rbm14* (B), and autosome genes without Xist binding such as *kmt2a* (C) in WT, $\Delta RepB$, and $\Delta RepE$ female MEF cells. Change in coverage ($\Delta 1$ and $\Delta 2$) is shown below ($\Delta 1$ for $\Delta RepB$ -WT, and $\Delta 2$ for $\Delta RepE$ -WT ♀).

D-E. Gene expression levels for Xist targets (D) or non-targets (E) on autosomes in WT, $\Delta RepB$, and $\Delta RepE$ female MEF cells show no obvious changes. P-values are determined using the Wilcoxon rank sum test.

F-G. Gene expression levels for Xist targets on autosomes in male and female MEF cells show no obvious changes. P-values are determined using the Wilcoxon rank sum test.

H. Schematic of the Xist autosomal binding pattern influences the gene expression. During the differentiation process of female ES cells, the Xist is specifically expressed from the Xist loci of one X chromosome (Xi). It then binds to the Xist loci of the Xi *in cis*, spreading across this chromosome and effectively silencing the expression of most of its genes. Additionally, some Xist complex will also bind to hundreds of loci on autosomes, thereby inhibiting the expression levels of target genes located there.

Discussion

Together with a backdrop of studies on ability of Xist RNA to diffuse and bind chromatin in trans [15](#), [23](#), [25](#), the results of our current work challenges the conventional narrative that Xist operates exclusively *in cis* on the Xi. We find that autosomal Xist targets are not numerous, possibly limited to only ~100 in both pluripotent stem cells and in somatic cells. On autosomes, Xist does not spread and instead covers only a narrow region corresponding to single genes. The genes tend to be active genes, with Xist specifically targeting the promoters of those genes. Genetic analysis coupled to transcriptomic analysis showed that Xist down-regulates the target autosomal genes without silencing them. This effect leads to clear sex difference — where female cells express the ~100 or so autosomal genes at a lower level than male cells in the mouse ES cell differentiation (**Figure 7H**). Thus, our findings redefine the scope of Xist's functional repertoire and provide insights into the broader landscape of epigenetic regulation during cellular differentiation. Xist RNA therefore plays a more complex role than previously envisaged, with several implications and caveats.

First, the observed consistent binding of Xist to both the X chromosome and autosomes in female ES cells prompted further exploration of the intricate dynamics of Xist during cellular differentiation. Our categorization of genes based on their expression levels revealed a compelling correlation between Xist binding on autosomes and the expression levels of associated genes. Notably, Xist exhibited a preference for binding to active gene regions, as evidenced by the significantly higher expression of genes within the 10-kb range of Xist binding regions. The parallel upregulation of X-linked and autosomal genes in Δ RepB and male ES cells suggests a regulatory role of Xist in gene expression beyond its canonical function on the X chromosome. Therefore, the precise temporal modulation of Xist binding merits further investigation to elucidate its regulatory significance and during distinct stages of cell differentiation or development, particularly in understanding its autosomal targets which could have implications for gene regulation and cellular function.

Second, a recent study using an alternative Xist pulldown method, RAP-seq, also revealed the capability of Xist to bind to autosomes in naive human pluripotent stem cells (naive hPSCs) and mediate gene regulation [25](#). While we found that, on autosomes, Xist does not spread and instead covers only a narrow region corresponding to single genes, the previous study demonstrated broader regions of binding [25](#). Furthermore, our CHART experiments demonstrate Xist binding to autosomes in post-XCI cells (e.g., MEF), whereas the previous study found no significant binding beyond the stem cell stage [25](#). Our analysis also indicates that Xist's autosomal targets tend to be Polycomb-depleted relative to neighboring genes, whereas the prior human study observed a positive correlation between XIST RNA and PRC2 marks. These discrepancies may arise from differences in the Xist pulldown methods (CHART versus RAP) employed by the two studies or from inherent differences between mouse and human systems.

Third, we considered the possibility that the binding of Xist to autosomes could merely be a consequence of Xist diffusion following saturation of binding sites on the Xi, rather than any programmed event during development. We are inclined to reject this notion and propose that Xist binding to autosomes is specifically programmed. Upon deleting RepB, the binding of Xist to the X chromosome weakens, but concomitantly, its binding to autosomal targets also diminishes (**Figure 2C**). This suggests that Xist binding to autosomes is contingent upon Repeat B and is deliberate, rather than due to random Xist diffusion alone (in which case we would have expected increased autosomal binding). Additionally, following treatment with the Xist inhibitor X1, an increase in the expression of autosomal targets is observed (**Figure 5C**), implying that the regulation of autosomes by Xist is not merely an overflow effect of Xist saturating the X chromosome.

In summary, our study advances understanding of Xist-mediated epigenetic regulation by highlighting unexplored interactions with autosomes. The identified correlations between Xist binding and gene expression involvement pose intriguing questions regarding the regulatory mechanisms governing these processes. These insights contribute to the evolving paradigm of Xist biology, underscoring the need for continued exploration of the complexity of epigenetic control mechanisms. Future investigations will delve deeper into the functional consequences of Xist binding on autosomes and explore the potential downstream effects on cellular differentiation, development, and diseases associated with its dysregulation, such as cancer, immunity, and neuron development [25](#)[45](#)[49](#). A comprehensive understanding of Xist's influence beyond X chromosomes is crucial, particularly laying the groundwork for future exploration of Xist as a potential therapeutic target.

Methods

Cell lines

The wild-type and Xist's Repeat B or E deletion MEF cell lines (M. musculus/M. castaneus F1 hybrid) has been previously described and generated [32](#). Additionally, male Xist transgenic (Xist TG) MEF cells, previously denoted as “♂X+P” [23](#) were included in the study. MEFs were cultured in medium comprising DMEM, high glucose, GlutaMAX™ Supplement, pyruvate (Thermo Fisher Scientific), 10% FBS (Sigma), 25 mM HEPES pH 7.2-7.5 (Thermo Fisher Scientific), 1x MEM non-essential amino acids (Thermo Fisher Scientific), 1x Pen/Strep (Thermo Fisher Scientific), and 0.1 mM βME (Thermo Fisher Scientific), maintained at 37°C with 5% CO₂.

The wild-type and Xist's Repeat B deletion female ES cell line (M. musculus/M. castaneus F2 hybrid) harboring a mutated Tsix allele has been previously established [32](#)[50](#). The male ES cell line was previously referred to as J1⁵⁰. ES cells were grown on γ-irradiated mouse embryonic fibroblast (MEF) feeder cells. Culture conditions included DMEM, high glucose, GlutaMAX™ Supplement, pyruvate (Thermo Fisher Scientific), 15% Hyclone FBS (Sigma), 25 mM HEPES pH 7.2-7.5, 1x MEM non-essential amino acids, 1x Pen/Strep, 0.1 mM βME, and 500 U/mL ESGRO recombinant mouse Leukemia Inhibitory Factor (LIF) protein (Sigma) at 37°C with 5% CO₂.

ES cell differentiation

Undifferentiated ES cells were initially cultivated on γ-irradiated MEF feeders for 3 days (day 0). Subsequently, the ES cell colonies were trypsinized (Thermo Fisher Scientific), and the feeders were removed. ES cells were then transitioned to a medium devoid of LIF and cultured in suspension for 4 days to form embryoid bodies (EBs). On day 4, the EBs were carefully settled onto gelatin-coated plates and allowed to undergo further differentiation until they were harvested on day 14.

CHART-seq

Xist CHART-seq methodology was conducted in accordance with a previously described protocol [51](#). Briefly, the cells were collected and suspended in PBS at a concentration of 2.5 million cells/mL. Subsequently, cross-linking was performed using 1% formaldehyde at room temperature for 10 min, followed by quenching with 0.125 M glycine for an additional 10 min. After two washes with ice-cold PBS, cells were pelleted and snap-frozen in liquid nitrogen. For CHART, 25 million cells were thawed on ice and resuspended in 1 mL ice-cold sucrose buffer (10 mM HEPES pH 7.5, 0.3 M sucrose, 1% Triton X-100, 100 mM potassium acetate, 0.1 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 1 mM DTT, 1x protease inhibitor cocktail, 10 U/mL SUPERase•In™ RNase Inhibitor). The cell suspension was rotated at 4°C for 10 min, followed by dilution with 2 mL of cold sucrose buffer. Douncing was performed using an RNaseZAP treated 15-mL glass Wheaton Dounce tissue grinder 20 times with a tight pestle. The nuclear suspension was carefully layer on top of a cushion

of 7.5 ml glycerol buffer (10 mM HEPES pH 7.5, 25% glycerol, 1 mM EDTA, 0.1 mM EGTA, 100 mM potassium acetate, 0.5 mM spermidine, 0.15 mM spermine, 1 mM DTT, 1x cOmplete EDTA-free protease inhibitor cocktail, and 5 U/mL RNase inhibitor) in a new 15 ml tube, and centrifuged at 1,500 g for 10 min at 4°C. The pellet was resuspended in 3 mL of PBS and cross-linked with 3 ml 6% formaldehyde for 30 min at room temperature with rotation. Afterwards, nuclei were pelleted by centrifugation at 1,000 g for 5 min at 4°C and washed three times with ice-cold PBS, and resuspended in 1 mL ice-cold nuclear extraction buffer (50 mM HEPES, pH 7.5, 250 mM NaCl, 0.1 mM EGTA, 0.5% N-lauroylsarcosine, 0.1% sodium deoxycholate, 5 mM DTT, 10 U/mL RNase inhibitor), rotated for 10 min at 4°C, and then centrifuged at 400 g for 5 min at 4°C and resuspended in 230 µL cold sonication buffer (50 mM HEPES pH 7.5, 75 mM NaCl, 0.1 mM EGTA, 0.5% N-lauroylsarcosine, 0.1% sodium deoxycholate, 0.1% SDS, 5 mM DTT, 10 U/mL RNase inhibitor) to a final volume of ~270 µL. The nuclei were sonicated in a microtube using a Covaris E220 sonicator (140 W peak incident power, 10% duty factor, 200 cycles/burst, 4°C, 300 s). Sonicated chromatin was centrifuged at 16,000 g for 20 min at 4°C, and transfer the supernatant (~220 µL) to new tube and add 170 µL sonication buffer to a final volume of ~390 µL, which was pre-cleared by 60 µL MyOne Streptavidin C1 beads (Thermo Fisher Scientific) in 640 µL 2x hybridization buffer (50 mM Tris pH 7.0, 750 mM NaCl, 1% SDS, 1 mM EDTA, 15% formamide, 1 mM DTT, 1 mM PMSF, 1x cOmplete EDTA-free protease inhibitor cocktail, 100 U/mL RNase inhibitor) at room temperature for 1 h with rotation. Pre-cleared chromatin was divided into two CHART reactions for Xist and control capture, and 1% was saved as an input sample. For each CHART reaction, 36 pmol of antisense (Xist) or sense (control) biotinylated capture probes (pooled probe as previously described) were used²¹. Hybridization was performed at room temperature with rotation overnight, and 120 µL C1 beads were added to the samples and incubated at 37°C for 1 h with rotation. The beads were washed once with 1x hybridization buffer (33% sonication buffer, 67% 2x hybridization buffer) at 37°C for 10 min, five times with wash buffer (10 mM HEPES pH 7.5, 150 mM NaCl, 2% SDS, 2 mM EDTA, 2 mM EGTA, 1 mM DTT) at 37°C for 5 min, and twice with elution buffer (10 mM HEPES pH 7.5, 150 mM NaCl, 0.5% NP-40, 3 mM MgCl₂, 10 mM DTT) at 37°C for 5 min. 1% of the final wash was saved as an “RNA pulldown” sample. CHART-enriched DNA was eluted twice in 200 µL of elution buffer supplemented with 5 U/µL RNase H (New England BioLabs) at room temperature for 20 min. The “input” sample and CHART DNA were treated with 0.5 mg/mL RNase A (Thermo Fisher Scientific) at 37°C for 1 hr with rotation and then incubated with 1% SDS, 10 mM EDTA, and 0.5 mg/mL proteinase K (Sigma) at 55°C for 1 hr. Reverse crosslinking was performed using 150 mM NaCl (final concentration 300 mM) at 65°C overnight. DNA was purified using phenol-chloroform and further sheared to ~300bp fragments using Covaris E220e (140 W peak incident power, 10% duty factor, 200 cycles/burst, 120 s, 4°C). Sonicated DNA was purified using 1.8x Agencourt AMPure XP beads (Beckman Coulter). Input and CHART DNA libraries were prepared according to the protocol of the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina (NEB E7645S). Libraries were sequenced on Novaseq S4, generating approximately 30 million 150-nt paired-end reads per sample.

Data source and processing

Raw data for CHART-seq and bulk RNA-seq generated in this study have been deposited in the Gene Expression Omnibus (GEO) database with accession number GSE271096 and GSE271097, respectively. ChIP-seq data utilized in this study were sourced from a study conducted by David et al. in 2020³³ with GEO accession number: GSE135389. Additionally, RNA-seq and ChIP-seq data specifically pertaining to Xist inhibitor X1 were obtained from a dataset published in 2022³⁶ with GEO accession number: GSE141683. RNA-seq data for Xist TG in mES cells were acquired from another dataset published in 2017³⁵ with GEO accession number: GSE92894. RNA-seq data for male and female MEF cells were acquired from two published dataset with GEO accession number: GSE246699 and GSE118443. The data were processed and re-analyzed consistently in this study.

CHART-seq and ChIP-seq analysis

Initial data preprocessing involved removing adaptors using trim_galore/cutadapt (versions 0.4.3/1.7.1). Subsequently, trimmed reads were prepared for alignment. Alignment of the genomes of *Mus musculus* (mus) and *Mus castaneus* (cas) was performed using NovoAlign (version 4.03). Aligned reads were then mapped back to the reference mm10 genome using SNPs to generate a BAM file ²⁸[52](#). Samtools (version 1.11) was employed for random sampling to ensure uniform library sizes across the samples. Utilizing DeepTools (version 3.1.2), input-subtracted ChIP and CHART coverage profiles were created, resulting in bigwig. To discern the peaks of Xist CHART on the autosomes, chrX reads were initially excluded from the BAM file. MACS2 (version 2.1) was used for peak calling using default parameters. The resultant peak files from two replicates were merged using bedtools (version 2.30) intersect. Bedtools window (version 2.30) facilitated the creation of gene lists based on peak sizes, spanning 10, 20, 50, and 100 kilobases. DeepTools was used to conduct comparative analyses of coverage, replicate correlation analysis, represented through either profile or heatmap plots, for genomic regions associated with Xist, H3K27me3, or H2A119ub across distinct categories of gene lists. IDR (Irreproducible Discovery Rate, version 2.0.2) was used to check the reproducibility of peaks identified in replicates. Intervene (version 6.0.2) was used to generate the Venn diagram of the peak.

RNA-seq

Total RNA was extracted using TRIzol (Thermo Fisher Scientific), and rRNA was selectively removed using the NEBNext rRNA Depletion Kit (New England BioLabs), according to the manufacturer's instructions. RNA-seq libraries were prepared using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England BioLabs), which enabled the creation of strand-specific RNA-seq libraries. Libraries were sequenced on Novaseq S4, generating ~30 million 150-nt paired-end reads per sample.

RNA-seq analysis

Raw RNA-seq data underwent adaptor removal and trimming using trim_galore/cutadapt (versions 0.4.3/1.7.1). RNA-seq data were mapped to three different genomes: C57BL/6J (mm10), *M. musculus* (mus), and *M. castaneus* (cas). By employing Subread (version 2.0.2) featureCounts ⁵²[52](#), counts per gene were computed, while deepTools were utilized to generate bigwig files, providing a comprehensive overview of read coverage across the genomes. For expression quantification, both Reads Per Kilobase Million (RPKM) and log transformed RPKM values were calculated to establish a robust foundation for downstream analysis.

Quantification and Statistical Analysis

In the experimental design, two replicates were used for both the CHART and RNA-seq analyses. Statistical analyses were conducted, and the corresponding p-values are transparently reported in the figures and their respective legends. The significance levels are denoted by asterisks, where *, **, ***, and **** represent $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively.

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Statement of financial interest

JTL is an advisor to Skyhawk Therapeutics, a cofounder of Fulcrum Therapeutics, and a non-executive Director of the GSK.

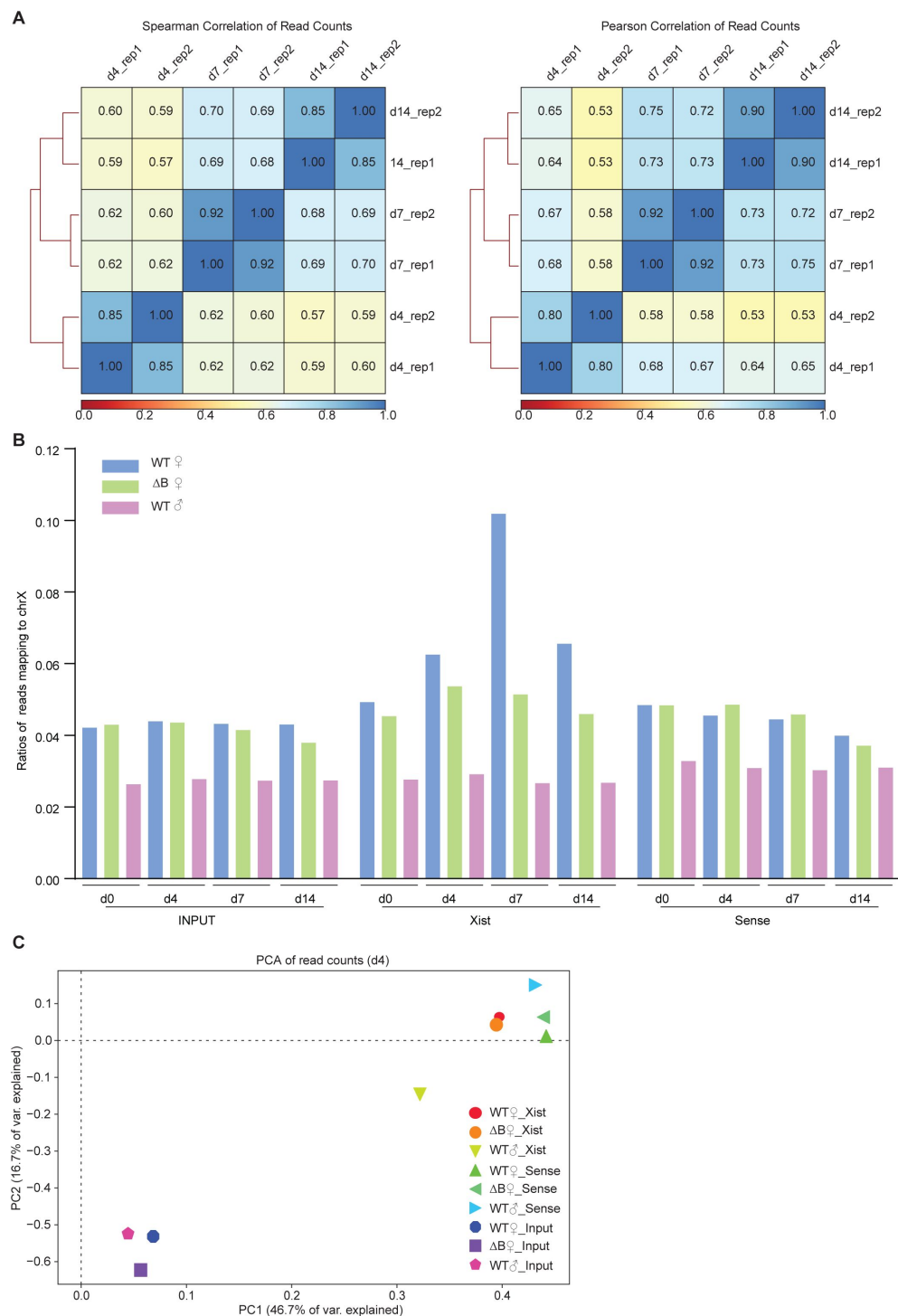


Figure S1.

Xist binds autosomal genes in trans.

- A.** Spearman (rank-based relationship) and Pearson (linear relationship) correlation analysis show that a strong positive correlation between the two biological CHART sequencing.
- B.** Xist CHART-seq reads percent of autosomes and Chromosome X in WT and Δ RepB female ES cells at day 0, day 4, day 7, and day 14. ES day 7 exhibits the highest Xist coverage. Sense probe and male ES cells are used as a control.
- C.** Principal Component Analysis (PCA) of CHART-seq reads include Xist, sense, and input in WT and Δ RepB female, and male ES cells at day 4. Samples clustering closer together share similar genome-wide coverage patterns.

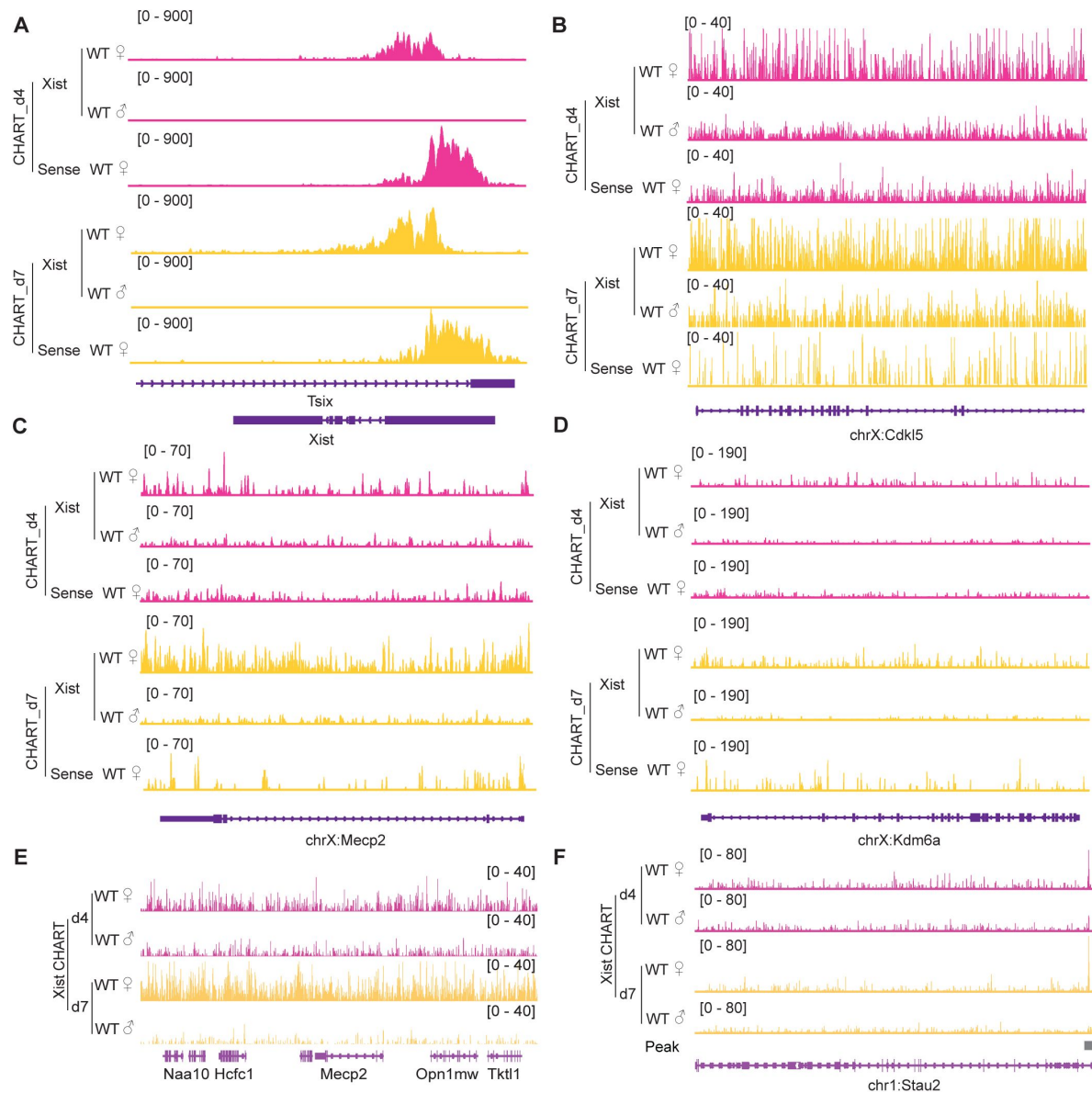


Figure S2.

Xist binds X chromosome during female ES cell differentiation.

A-D. Representative binding signals of Xist RNA on Xist locus (A), genes subjective to XCI such as *Cdkl5* (B), *Mecp2* (C), and escapee genes (*Kdm6a*) (D) in WT female ES cells at day 4 and day 7. WT male ES cells and sense probe are used as control.

E. Representative consecutive binding signals of Xist on chromosome X (~320 kb) in WT female ES cells at day 4 and day 7. WT male ES cells are used as control.

F. Representative site-specific binding of Xist on autosome locus (*Stau2*) in WT female ES cells at day 4 and day 7, WT male ES cells are used as control.

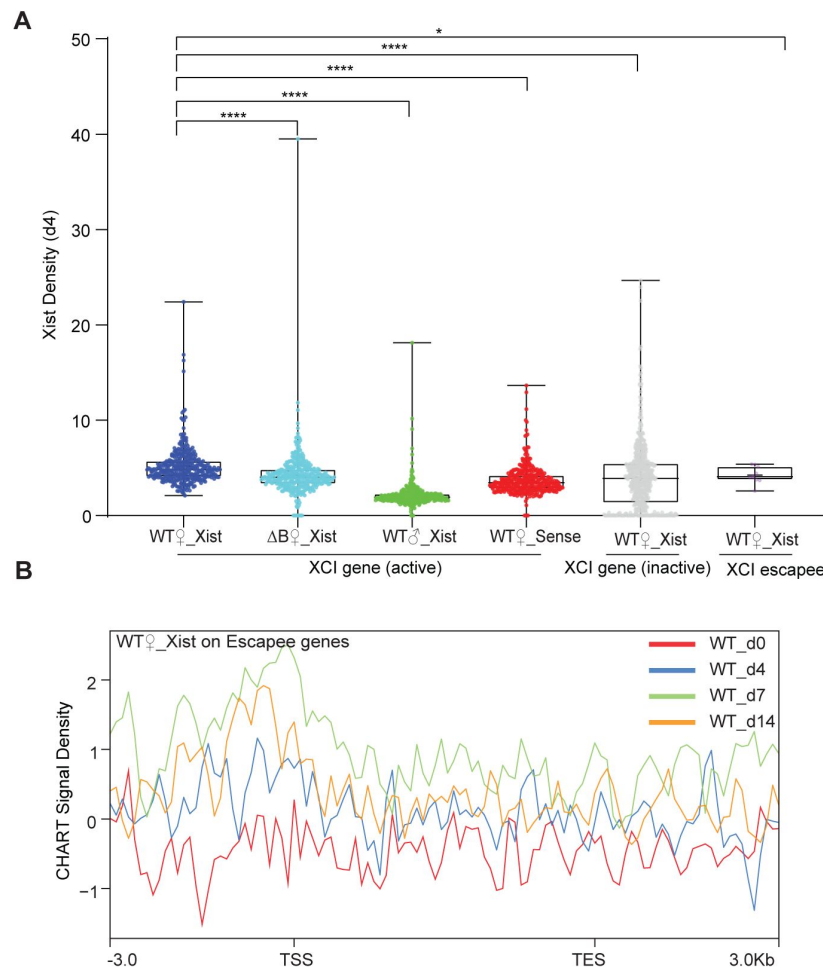


Figure S3.

Xist binds XCI genes during differentiation.

A. Xist CHART signal coverage on X chromosome genes (XCI-active/inactive, and escapee) in WT female ES cells at day 4. Sense and male ES cells are used as control. P-values are determined using the Wilcoxon rank sum test.

B. Average profile of Xist CHART signal on XCI escapee genes (day 0, 4, 7, and 14) shows that Xist accumulated in the upstream of the promoter but depleted in the escapee gene body.

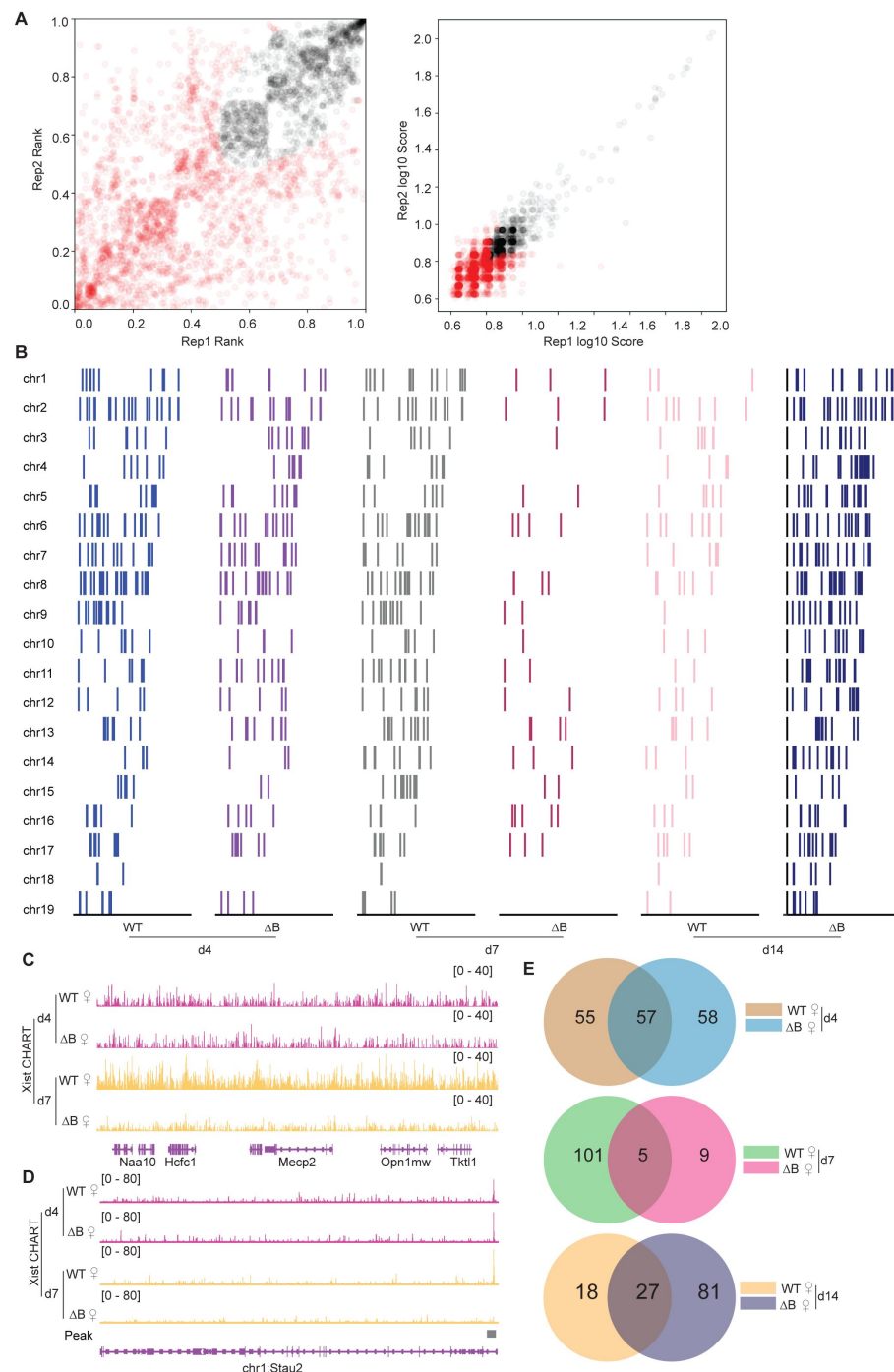


Figure S4.

Xist binds select autosomal genes in trans.

A. IDR (Irreproducible Discovery Rate) analysis to assess the reproducibility of the peaks detected across biological replicates (day 4). The results showed a strong correlation between the replicates, with an IDR threshold of 0.05 (red point > 0.05).

B. Xist peak pattern (MACS2 peak calling) on autosomes in WT and Δ RepB female ES cells at day 4, day 7, and day 14.

C. Representative consecutive binding signals of Xist on chromosome X (~320 kb) in WT and Δ RepB female ES cells at day 4 and day 7.

D. Representative site-specific binding of Xist on autosome locus (*Stau2*) in WT and Δ RepB female ES cells at day 4 and day 7.

E. The Venn diagram illustrates the overlap of peak sites identified in WT and Δ RepB female ES cells at day 4, day 7, and day 14.

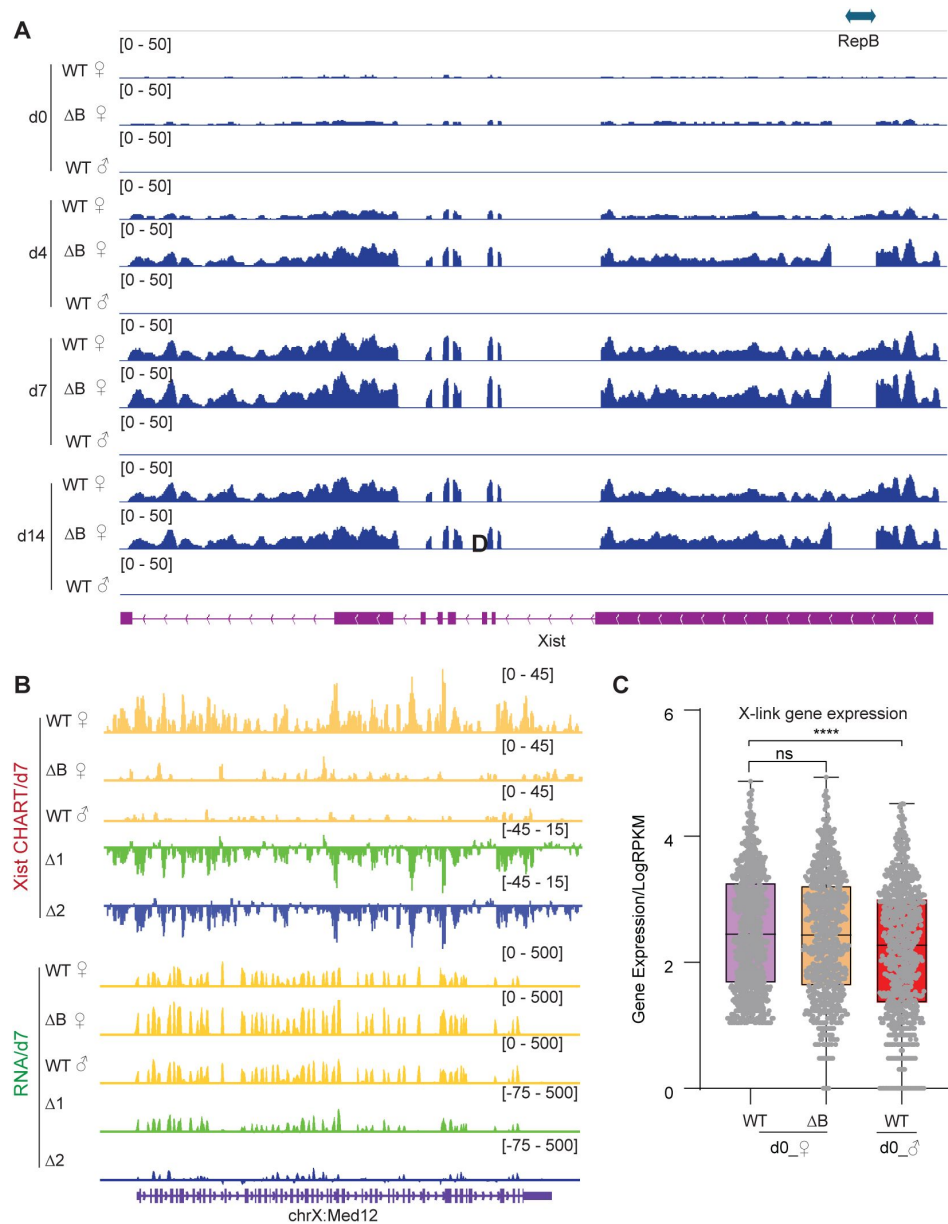


Figure S5.

Xist binds X-linked genes in a RepB-dependent manner.

A. RNA-seq track patterns of Xist in WT, ΔRepB female, and male ES cells at day 0, day 4, day 7, and day 14.

B. Exemplifying CHART-seq and RNA-Seq patterns of an X-linked gene (*Med12*) at day 7. Change in coverage (Δ1 and Δ2) is shown below (Δ1 for ΔRepB ♀ - WT ♀, and Δ2 for WT ♂ - WT ♀).

C. Evaluation of gene expression levels for X-linked genes in WT and ΔRepB female, and male ES cells at day 0. P-values are determined using the Wilcoxon rank sum test.

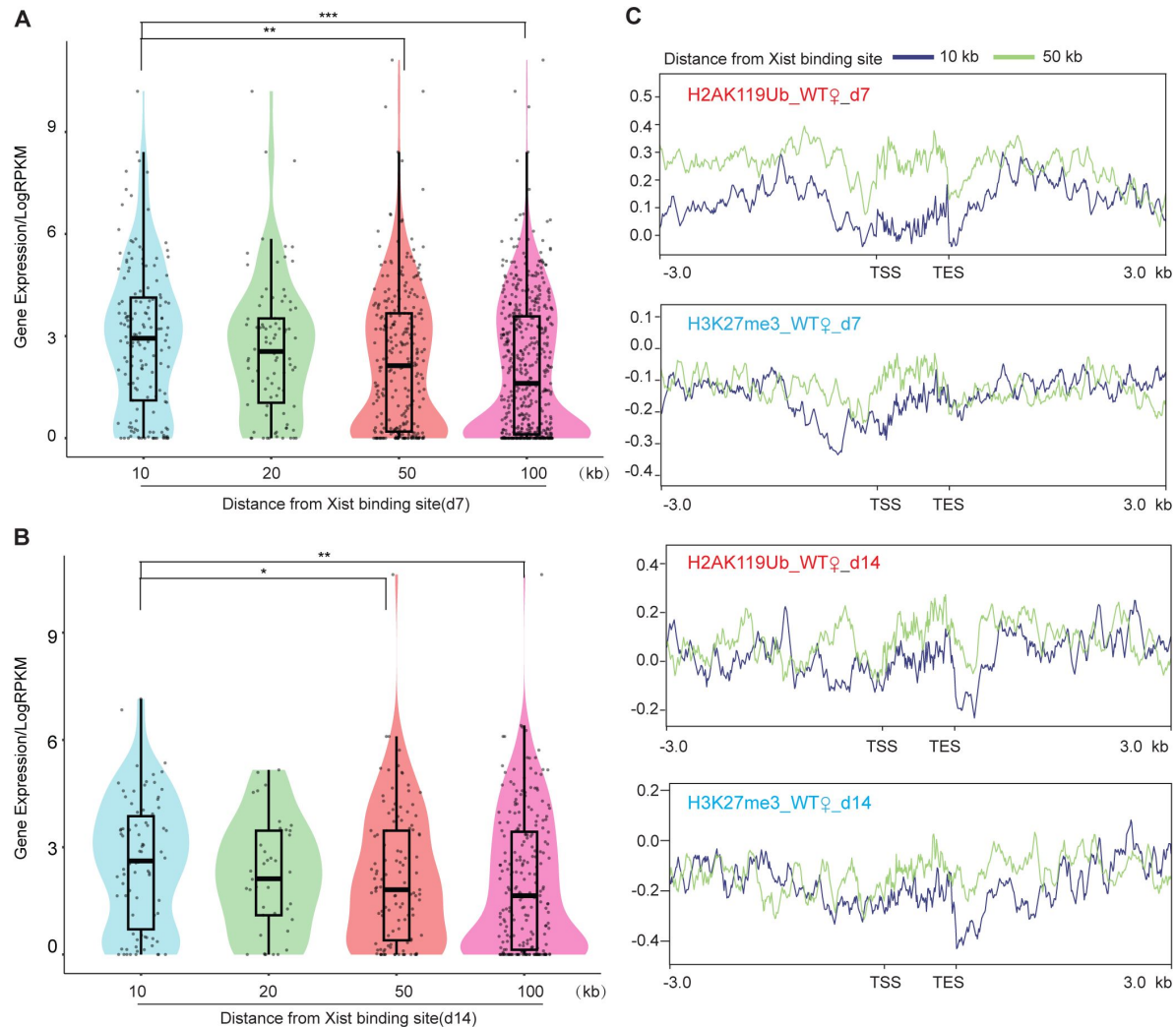


Figure S6.

Genes bound by Xist exhibit higher expression levels and lower H3K27me3 and H2AK119ub binding levels.

A-B. The analysis of gene expression within the 10, 20, 50, and 100-kilobase binding regions of Xist is performed in WT female ES cells at day 7 (B), and day 14 (C), respectively. P-values are determined using the Wilcoxon rank sum test.

C. Average profile plots showing H3K27me3 and H2AK119ub coverage over genes within the 10 and 50-kilobase binding regions of Xist in WT female ES cells.

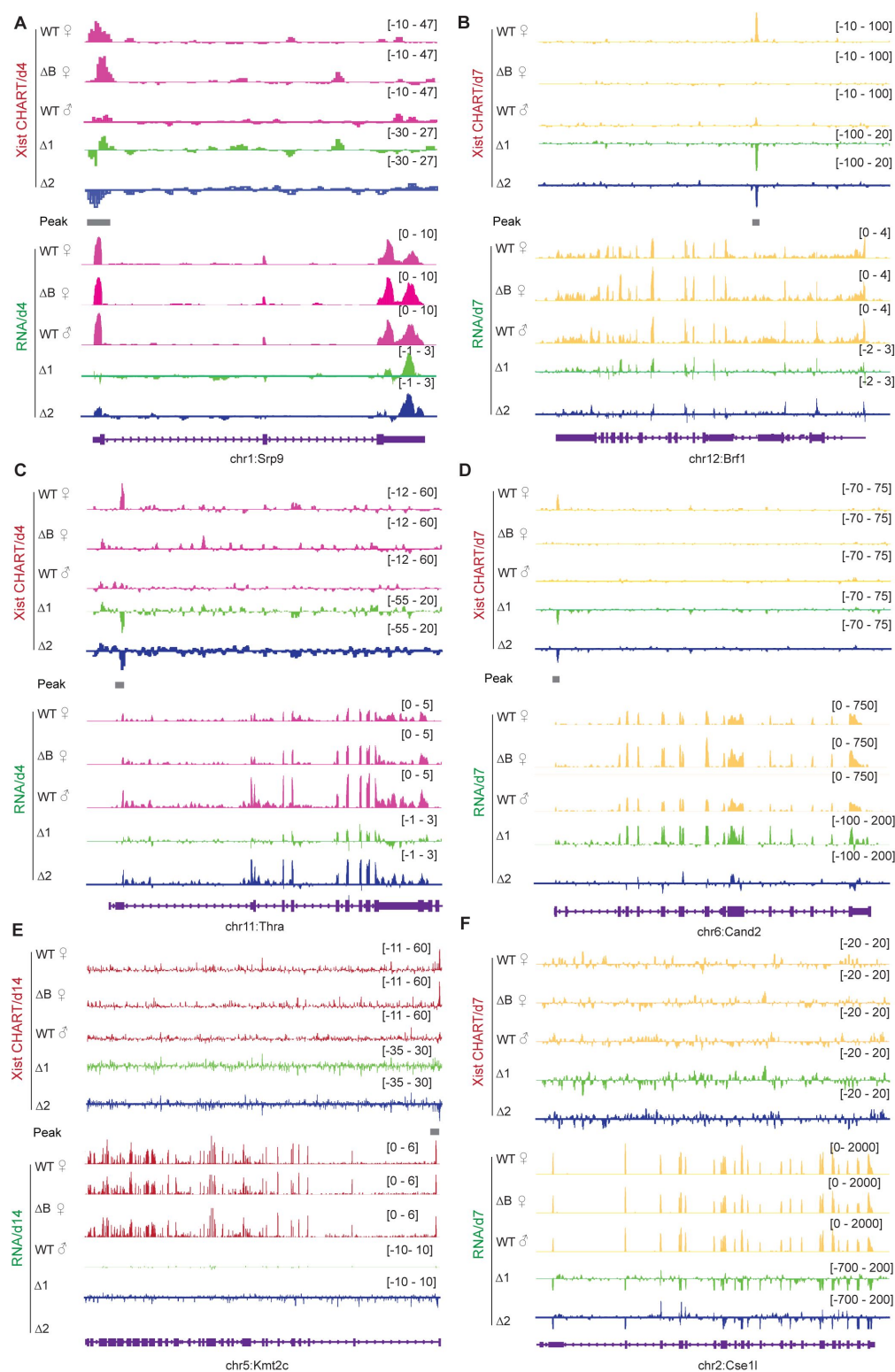


Figure S7.

Example of Xist binding on autosomal genes and influence on gene expression.

This figure illustrates CHART-seq and RNA-seq patterns of autosomal genes, including *Srp9*, *Brf1*, *Thra*, *Cand2*, and *Kmt2c*, which exhibit Xist binding on different days. *Ces1l*, which lacks Xist binding, is used as a control. Change in coverage ($\Delta 1$ and $\Delta 2$) is shown below ($\Delta 1$ for $\Delta \text{RepB} \text{♀} - \text{WT} \text{♀}$, and $\Delta 2$ for $\text{WT} \text{♂} - \text{WT} \text{♀}$).

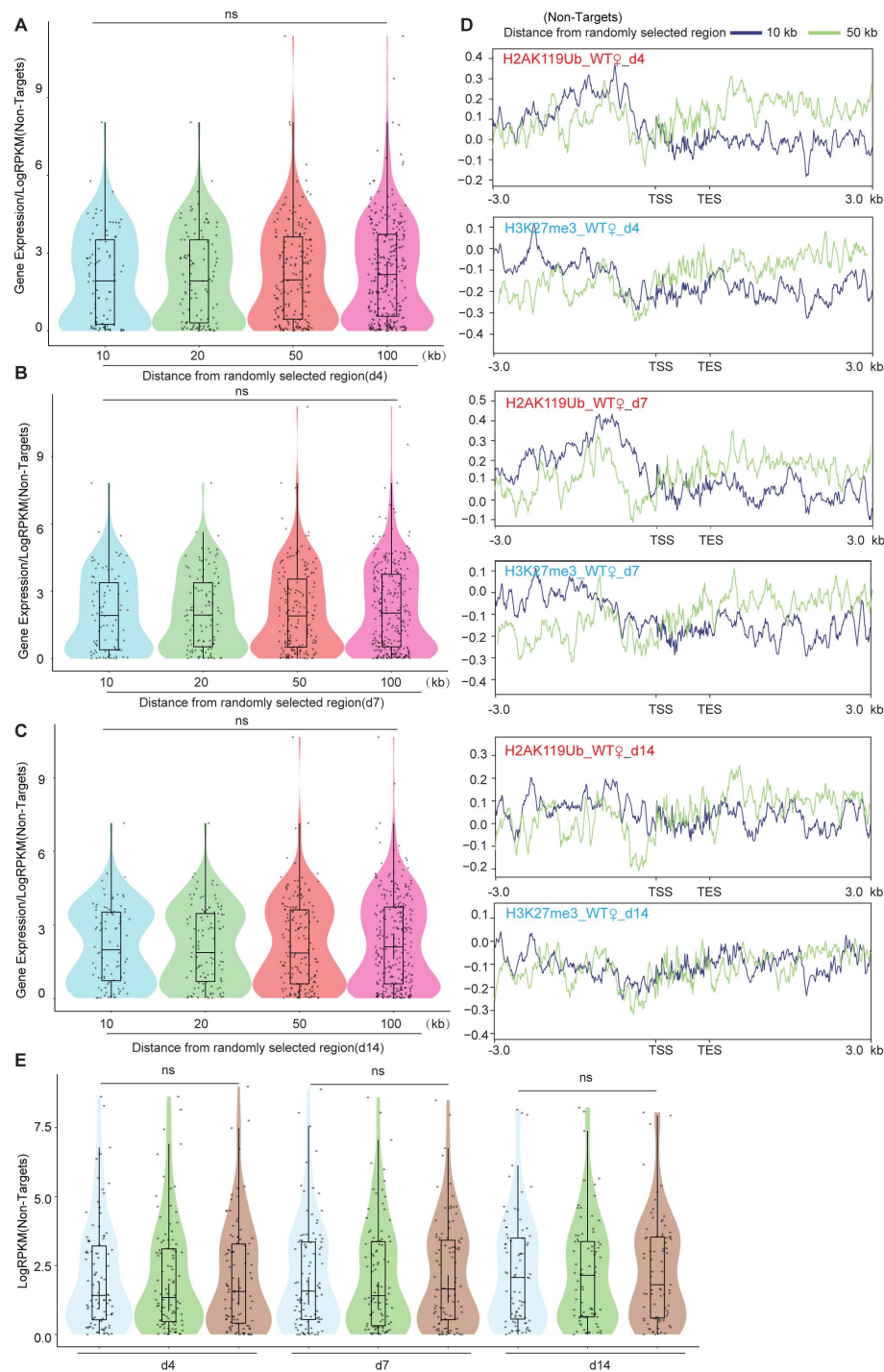


Figure S8.

Genes not bound by Xist exhibit no changes in gene expression or differences in H3K27me3 and H2AK119ub signals.

A-C. The analysis of gene expression within the 10, 20, 50, and 100-kilobase randomly selected regions is performed in WT female ES cells at day 4 (A), day 7 (B), and day 14 (C), respectively. P-values are determined using the Wilcoxon rank sum test.

D. Average profile plots showing H3K27me3 and H2AK119ub coverage over genes within the 10 and 50-kilobase randomly selected regions in WT female ES cells at different time points.

E. Assessing gene expression levels of Xist non-targets on autosomes in WT, Δ RepB female ES cells, and male ES cells at different time points. P-values are determined using the Wilcoxon rank sum test.

Figure S9.

Xist overexpression inhibits select autosomal genes and X-linked genes.

RNA-seq track shows the *Med14* (X-linked gene) (A) and *Bcl7b* (an Xist autosomal target gene) (B) expression levels in differentiated ectopic Xist overexpressed ES cell lines (Tg), the control (Ctrl) was doxycycline-treated wildtype cell. Change in coverage (Δ) is shown below (Tg - Ctrl).

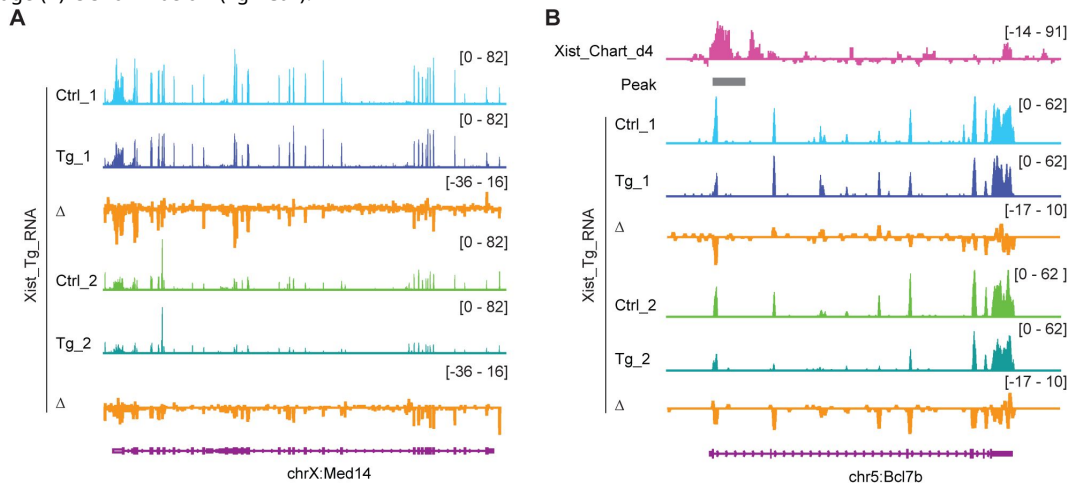
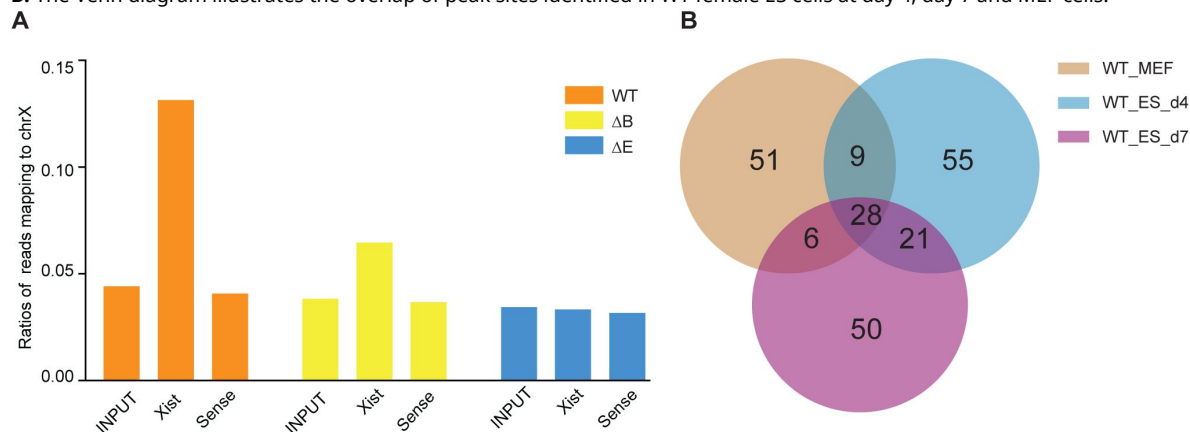


Figure S10.

Xist has overlapped autosomal binding peaks in differentiated ES and MEF cells.

A. Coverage of CHART-Seq reads (input, Xist, and sense control) on Chromosome X in WT, Δ RepB, and Δ RepE female MEF cells.

B. The Venn diagram illustrates the overlap of peak sites identified in WT female ES cells at day 4, day 7 and MEF cells.



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Reviewer #1 (Public review):

Summary:

The manuscript by Yao S. and colleagues aims to monitor the potential autosomal regulatory role of the master regulator of X chromosome inactivation, the Xist long non-coding RNA. It has recently become apparent that in the human system, Xist RNA can not only spread in cis on the future inactive X chromosome but also reach some autosomal regions where it recruits transcriptional repression and Polycomb marking. Previous work has also reported that Xist RNA can show a diffused signal in some biological contexts in FISH experiments.

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Based on this experimental design, the authors make several bold claims:

- (1) Xist binds to about a hundred specific autosomal regions.
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- (6) Xist binding to autosomal regions also occurs in somatic cells but does not lead to gene repression.

Together, these claims suggest that Xist might play a role in modulating the expression of autosomal genes in specific developmental and cellular contexts in mice.

Strengths:

This paper deals with an interesting hypothesis that Xist ncRNA can also function at autosomal loci.

Weaknesses:

The revised manuscript now includes many additional bioinformatic analyses to support the premise that Xist RNA targets a specific set of about 100 promoters and attenuates their expression in the early stages of differentiation. I have previously raised significant concerns about the bioinformatic analyses and the robustness of the data, especially those linked to CHART-seq datasets. Despite some improvements, fundamental problems with the analysis remain, precluding a conclusion on whether Xist RNA binds specific autosomal promoters. The main concerns include:

(1) The authors nicely explain the use of biological replicates; however, they still fail to provide the sufficient analysis I requested on d0 and sense probes. While some quantification is presented in Figures 1E and 1F, the peak calling I asked for has still not been performed. In the response document, the authors report that about 600 peaks were identified in d0 female ESCs compared to about 100 in differentiated conditions. They explain this by the well-known phenomenon of having a background of differentiated cells in d0. In my opinion, this reasoning is flawed. With 98% of cells not inducing Xist in the culture, it is unimaginable why 600 peaks would be detected in the peak calling analysis. Rather, this demonstrates a high background in the CHART peak calling. To assess this further, I have reanalyzed d7 CHART datasets and found robust enrichment of the sense probe on promoters of genes, even stronger than the antisense probe. MACS peak calling also identifies a robust number of peaks on the sense probe. Indeed, even though Figure 1F shows low sense probe enrichment, this is because it focuses on the anti-sense peaks only. An opposite effect is observed when focusing on all genes or on sense-peaks. Therefore it is tough to decide which of the signal is truly due to Xist binding and what is an inherent problem with the CHART signal. These results cast serious doubts on the biological conclusions of this work and point to a very high background level of promoter signal in both sense and antisense samples.

(2) The authors do not address the conundrum of their results: how is it possible to have a genome-wide autosomal accumulation of Xist signal at promoters (see Figures 1A and 1B), while simultaneously specifically affecting only 100 promoters in the genome? The signal is either general (as Figures 1A and 1B suggest) or specific (as implied by the peak calling), but it cannot be both. Current data points to the fact that CHART has a bias for the most open parts of the chromatin.

(3) The text is still very confusing when it comes to Polycomb. Some experiments point to the fact that there are few PRC1/2 marks at putative Xist autosomal binding sites (Figure 3C), while the use of X1 induces the loss of PRC2 marks. I still find this internally contradictory. The authors sadly do not address my concerns with additional analysis. Their current data indicate that upon Xist upregulation, Xist-RNA binds to autosomal regions that are highly expressed and devoid of Polycomb. These loci then become transcriptionally attenuated and gain some (but low) level of PRC2 in a Xist-dependent fashion. If this model is true, then all these regions should not have Xist in d0 of differentiation and should also have slightly lower levels of PRC2. The argument that there is a low level of Xist in 2-5% of cells should not be a problem because most of the signal will come from the 98% of cells not expressing Xist (as seen in Figure 1A). Without timepoint 0, the whole premise of the paper is difficult to interpret. Either the d0 samples are good enough, or the system is so leaky that it is nearly impossible to identify Xist-specific effects. Males are a useful control but are obviously a genetically very different line with distinct epigenetic and signaling statuses. It is crucial to

compare the timing of repression/PRC accumulation to conclude if and how Xist is functional on these loci.

(4) The authors did not address my concerns about the transcriptional analysis. I believe that the control genes are not selected properly. This analysis should not have been performed on just 100 randomly selected regions/genes. Instead, bootstrapping of 100 randomly selected regions/genes should be done, e.g., 1000 times. Additionally, one should only sample from expressed genes to have a comparable control gene set. For example, in Figures 4D and 4E, the distribution of control regions is entirely different. To stress again, relying on a set of 100 randomly selected genes/regions is not statistically robust; controls have to be matched, and bootstrapping has to be performed. Finally, each timepoint uses a different set of autosomal targets. There is a need to visualize the same set of genes across all timepoints (including d0). For example, are genes bound by Xist at d7 highly expressed at d0 and then attenuated only at d7? What happens to them at d14 (see points from 3)? The arguments about d0 heterogeneity are again not convincing (nor is Figure 3H, which shows a different set of genes).

(5) Transcriptional analysis is often shown only as tracks however the reads for key example genes have to be quantified properly and not just visualized or amalgamated in a violin plot.

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Reviewer #2 (Public review):

Summary:

To follow-up on recent reports of Xist-autosome interaction the authors examine female (and male transgenic) mESCs and MEFs by CHARTseq. Upon finding that only 10% of reads map to X, they sought to identify reproducible alternative sites of Xist-binding, and identify ~100 autosomal Xist-binding sites in active chromatin regions. They demonstrate a transient down-regulation of autosomal expression. They utilize published male transgenic inducible Xist mESC data to support their findings. In their system, inhibition of Xist reduces autosomal impact.

Strengths:

The authors address a topical and interesting question with a series of models including developmental timepoints and utilize unbiased approaches (CHARTseq, RNAseq). For the CHARTseq they have controls of both sense probes and male cells; and indeed do detect considerable background with their controls. The use of 'metagene' plots provides a visual summation of genic impact. They compare with published data.

Weaknesses:

The revised text and rebuttal clarified my confusion of the 'follow-up' analyses (Figure 4) compared to published datasets. Further, the figure legends have been improved.

While the controls were a strength, it appears that when focussed on bound regions, the background (from sense probes) is now also substantially higher than global background (compare 1E to 1A/B). Thus, why do these autosomal targets enrich for the sense probes, and how to distinguish from such background for the ΔB experiments? If male and sense are both controls, then why is sense lower for males than females, doesn't this suggest Xist impact? While authors note d0 might detect Tsix, the signal is only slightly reduced by d14 and never equivalent. Indeed, the new PCA (S1C) does show as noted that female Xist interactions are distinct from sense and male, but the male signal is even more distinct from sense probes.

It would have been preferable to see the dispersion of the Xist RNA cloud in these ΔB cells, rather than a reference.

Only 2 replicates were used, but there were multiple time-points: D0, D4, d7, d14; further, the correlation analysis showed good reproducibility, and in response to reviews they note that 2 replicates are standard of practice.

The conclusion that RepB is "required for localization to the ~100 genes" is based on density (panel 2E); however, these autosomal targets retain enrichment at TSSs (panel 2A) and indeed the text suggests they are the same sites, suggesting that in fact the choice of autosomal region binding is not RepB dependent. Thus, this remains unresolved for me.

The introduction is clear, and the senior author is a leader in the field; however, by this reviewer's count 19 of the 52 references include the senior author.

Better descriptors for the supplemental Excel files would be helpful.

Aim achievement: The authors do identify autosomal sites with enrichment of chromatin marks and evidence of silencing. Their revised text clarifies many issues, although this reviewer still remains unconvinced that the autosomal targeting is repB-dependent.

The impact of Xist on autosomes is important for consideration of impact of changes in Xist expression with disease (notably cancers). Knowing the targets (if consistent) would enable assessment of such impact.

<https://doi.org/10.7554/eLife.101197.2.sa2>

Reviewer #3 (Public review):

Summary:

Yao et al use CHART to identify chromatin associated with Xist in female mouse ESCs, and, as control, male ESCs at various timepoints of differentiation. Besides binding of Xist to X chromosome regions they found significant binding to autosomes, concentrating mostly on promoter regions of around 100 autosomal genes, as elucidated by MACS. The authors went on to show that the RepB repeat is mostly responsible for these autosomal interactions using a female ESC line in which RepB is deleted. Evidence is provided that Xist interacts with active autosomal genes containing lower coverage of repressive marks H3K27me3 and H2AK119ub and that RepB dependent Xist binding leads to dampening of expression, but not silencing of autosomal genes. These results were confirmed by overexpression studies using transgenic ESCs with doxycycline-inducible Xist as well as via a small molecule inhibitor of Xist (X1), inducing/inhibiting the dampening of autosomal genes, respectively. Finally, using MEFs and Xist mutants RepB or RepE the authors provide evidence that Xist is bound to autosomal genes in cells after the XCI process but appears not to affect gene expression. The data presented appear generally clear and consistent and indicate some differences between human and mouse autosomal regulation by Xist. Thus, these results are timely and should be published.

Strengths:

Regulation of autosomal gene expression by Xist is a "big deal" as misregulation of this lncRNA causes developmental defects and human disease. Moreover, this finding may explain sex-specific developmental differences between the sexes. The results in this manuscript identify specific mouse autosomal genes bound by Xist and decipher critical Xist regions that mediate this binding and gene dampening. The methods used in this study are

appropriate, and the overall data presented appear convincing and are consistent, indicating some differences between human and mouse autosomal regulation by Xist.

Comments on revisions:

In the revised manuscript, the authors have addressed my previous criticisms satisfactorily. Moreover, the manuscript has been much improved with new confirmatory results and additional control experiments. This, combined with more detailed descriptions/explanations facilitates data interpretation, making the paper more transparent and easier to read.

<https://doi.org/10.7554/eLife.101197.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Reviewer #1 (Public review):

Summary:

The manuscript by Yao S. and colleagues aims to monitor the potential autosomal regulatory role of the master regulator of X chromosome inactivation, the Xist long non-coding RNA. It has recently become apparent that in the human system, Xist RNA can not only spread in cis on the future inactive X chromosome but also reach some autosomal regions where it recruits transcriptional repression and Polycomb marking. Previous work has also reported that Xist RNA can show a diffused signal in some biological contexts in FISH experiments.

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Based on this experimental design, the authors make several bold claims:

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- (6) Xist binding to autosomal regions also occurs in somatic cells but does not lead to gene repression.*

Together, these claims suggest that Xist might play a role in modulating the expression of autosomal genes in specific developmental and cellular contexts in mice.

Strengths:

This paper deals with an interesting hypothesis that Xist ncRNA can also function at autosomal loci.

Weaknesses: The claims reported in this paper are largely unsubstantiated by the data, with multiple misinterpretations, lacking controls, and inadequate statistics. Fundamental flaws in the experimental design/analysis preclude the validity of the findings. Major concerns are listed below: (1) The entire paper is based on the CHART observation that Xist is specifically targeted to autosomal promoters. Overall, the data analysis is flawed and does not support such conclusions. Importantly the sense WT and the 0h controls are not used, nor are the biological replicates.

We respectfully disagree with Rev1 but nevertheless thank the reviewer for making some suggestions that helped to strengthen our manuscript. We have provided new experiments and analyses in the revised manuscript. Please see responses below.

Rev1 seems to have missed or misunderstood some key experiments. In fact, the sense WT and 0h controls were shown. Furthermore, we included at least two biological replicates for each experiment.

We used both male ES cells (which do not express Xist) and sense probes as key negative controls, as outlined in Figure S1. Crucially, we only analyzed peaks that were reproducible between biological replicates. The Xist CHART peaks in differentiating female ES cells were significantly enriched above the “background” defined by the sense probe and male controls. Specifically, in comparison to undifferentiated female ES cells (day 0) where both X chromosomes are active and Xist is not induced, Xist CHART robustly pulled down the X chromosome during cell differentiation (day 4, day 7, and day 14). In contrast, male ES cells showed no significant pull-down of the X chromosome, and the sense group also exhibited markedly reduced binding (new Figure S1B). Furthermore, Principal Component Analysis (PCA) of CHART-seq reads (day 4 as an example) include Xist, sense, and input in WT and Δ RepB female, further confirmed that the sense probe CHART was clearly distinguishable from Xist CHART signals. Please see revised Figure S1C. Together, these findings underscore the specificity and robustness of our CHART results.

Data is typically visualized without quantification, and when quantified, control loci/gene sets are erroneously selected. Firstly, CHART validation on the X in FigS1 is misleading and not based on any quantifications (e.g., see the scale on Kdm6a (0-190) compared to Cdkl5 (0-40)). If scaled appropriately, there is Xist signal on the escapee.

Rev1 may have misread the presented data. In the example raised by Rev1, Fig. S1 is inherently quantitative: e.g., a ratio is a number in Fig. S1A (now Fig. S1B) and all gene tracks in Fig. 1B-E are shown with scales. We showed X-linked genes in Fig. S1 (now Fig. S2) as a control to demonstrate that the CHART worked and that Xist accumulated over time from day 0 to day 14. Our new Figure 1B demonstrates the Xist accumulation in graph format.

Our paper focuses on Xist autosomal binding sites. Thus, the X-linked examples were placed in the supplement. Escapee genes do in fact accumulate Xist at their promoter regions and this finding is consistent with data published by Simon et al. (2013, Nature). It was therefore not desirable in this paper to reanalyze X-linked genes, including escapees. Nevertheless, to address the reviewer’s concerns, we present new data in new Figure S3A. Here we analyzed the density of Xist binding across X-linked genes, including both active and inactive genes, as well as escapee genes. From this quantitative analysis, it should be clear that escapees do bind Xist. However, from the metagene plots in Figure S3B, we confirm the previous conclusion that escapees bind Xist at high levels just upstream of the promoter and that there is a depletion of Xist in the escapee gene body, consistent with a barrier preventing Xist from moving into the active gene.

All X-linked loci should have been quantified and classified based on escape status; sense control should also be quantified, and biological replicates should be shown separately.

Please see above response.

Additionally, in the revised manuscript, we have examined the Irreproducible Discovery Rate (IDR) to validate the reproducibility of peaks between the two replicates in the revised version, and we included a representative example from female WT ES cells at day 4 (revised Figure S4A). The results showed a strong correlation between the replicates, with an IDR threshold of 0.05 (red point > 0.05). As described in the Methods section, to ensure reliable and robust peak identification, we performed peak calling (MACS2) separately on each replicate, and then used *bedtools intersect* to identify peaks that overlapped between the two replicates. This stringent process, including strict q-value settings in MACS2, ensures the reliability and reproducibility of the peaks presented in this study.

Secondly, and most importantly, Figure 1 does not convincingly show specific Xist autosomal binding. Panel A quantification is on extremely variable y-scales and actually shows that Xist is recruited globally to nearly all autosomal genes, likely indicating an unspecific signal. Again, the sense and 0h controls should have been quantified along with biological replicates.

Figure 1 shows heatmaps and corresponding metagenes for d0, d4, d7, and d14 female ES cells. Two biological replicates are analyzed. In our revised manuscript, we have used Pearson and Spearman correlation coefficients to measure the strength and direction of a relationship between two biological replicates and shown that the two replicates have high reproducibility (new Figure S1A). On d0, the Xist coverage on autosomes and X chromosome is low, but there is a clear increase on d4, d7, and d14, particularly at the TSS of autosomal genes, as shown by the metagene plots on in Figure 1A-B and the CHART density maps in new Figure 1E-F. We also show relative depletion of Xist signals in the male and sense negative controls.

Upon inspecting genome browser tracks of all regions reported in the manuscript (Rbm14, Srp9, Brf1, Cand2, Thra, Kmt2c, Kmt2e, Stau2, and Bcl7b), the signal is unspecific on all sites with the possible exception of Kmt2e. On all other loci, there is either a strong signal in the 0h ESC controls or more signal in some of the sense controls. This implies that peak calling is picking up false positive regions. How many peaks would have been picked up if the sense or the 0h controls were used for peak calling? It is likely that there would be a lot since there are also possible "peaks" (e.g., Fzd9) in control tracks.

The analysis cannot be performed by visual inspection. A statistical analysis must be performed to call signal above noise. This is why we performed peak-calling on two biological replicates and identified overlapping peaks using *bedtools intersect* to improve reliability. Significant peaks are noted as black bars under each track. As mentioned above, for our analysis, we focused on the top 100 peaks based on peak scores to ensure robustness. Xist has significantly higher signal compared to the sense probe in the Xist-autosomal peak regions (revised Figure 1E-F). Additionally, we conducted peak calling on undifferentiated ES cells (d0) and detected a significantly higher number of peaks (~600) compared to the differentiated states (d4 or d7) (~100).

Single-cell sequencing studies have shown that about 2% of undifferentiated mESCs express detectable Xist (Pacini et al., *Nat Commun*, 2021). The Xist peaks in "day 0" cells may be due to the differentiating population.

Further inspection of the data was not possible as the authors did not provide access to the raw fastq files. When inspecting results from past published experiments {Engreitz, 2013 #1839} reported regions were not bound by Xist.

On the contrary, we deposited the raw data files to GEO prior to the submission of the paper and included the reviewer link to access them. As of August 24, 2024, GEO publicly released these files, allowing for full inspection of the data.

Regarding the Engreitz publication, it is not recommended to compare our current study to their analysis for the crucial reason that the Engreitz study was not conducted under physiological conditions. The authors overexpressed the *Xist* gene in male ES cells. Because *Xist* RNA can silence genes in male cells as well, this ectopic overexpression normally leads to cell death — thus forcing examination of effects in a narrow time window before *Xist* can fully spread and act across the genome. Comparing our experiments (endogenous *Xist* expression in female ES cells) to the ectopic overexpression in male ES cells of Engreitz et al. should therefore not be undertaken.

Thirdly, contrary to the authors' claim, deleting the B repeat does not lead to a loss of autosomal signal. Indeed, comparing Fig1A and Fig2B side by side clearly shows no difference in the autosomal signal, likely because the autosomal signal is CHART background. Properly quantifying the signal with separate replicates as well as the sense and 0h controls is vital. Overall current data together with published results indicate that CHART peak calling on autosomes is due to technical noise or artefacts.

In our revised manuscript, we have included the quantitative results as mentioned above in the main and supplementary figure (new Figure 1E-F, Figure 2E-F, and S3A). The data clearly show an enrichment in the *Xist* CHART samples in differentiating female ES cells.

We believe the reviewer may be comparing the original Figure 1A and Figure 2A (not Figure 2B). As mentioned above, the analysis cannot be performed by visual inspection. Please see new Figure 2E and 2F. From these data, it should be clear that deleting RepB causes a decrease in *Xist* targeting to autosomal loci.

*(2) The RNA-seq analysis is also flawed and precludes strong statements. Firstly, the analysis frequently lacks statistical analysis (Fig3B, FigS2B-C) and is often based on visualizations (Fig 3D-G) without quantifications. Day 4 B-repeat deletion does not lead to a significant change in the expression of genes close to *Xist* signal (Fig3H, d14 does not fully show).*

Please see new revised Figure 3B and Figures S2B-C (now revised as Figures S6A and S6B).

Secondly, for all transcriptional analysis, it is important to show autosomal non-target genes, which is not always done.

In the revised manuscript, we included non-target genes for each analysis (new Figure 4E-F, 5D and 5F, 7C and 7E, S7F, S8).

*Indeed, both males and B repeat deletion will lead to transcriptional changes on autosomes as a secondary effect from different X inactivation status. The control set, if used, is inappropriate as it compares one randomly selected set of ~100 genes. This introduces sampling error and compares different classes of genes. Since *Xist* signal targets more active genes, it is important to always compare autosomal target genes to all other autosomal genes with similar basal expression patterns.*

Please see new Figure S8. We included 100 randomly selected non-target sites on autosomes for this comparative analysis. For consistency, we applied the same flanking regions (10 kb) in the analysis of both target and non-target genes. We believe that this selection method for nontargets is appropriate for two reasons: first, it allows us to control for Xist binding and non-binding; second, it ensures a similar number of genes in both groups, providing a robust foundation for statistical analysis.

(3) The ChIP-seq analysis also has some problems. The authors claim that there is no positive correlation between genes close to Xist autosomal binding (10kb) compared to those 50kb away (Fig 3C, S2D); however, this analysis is based entirely on metagene visualization. Signal within the Xist binding sites should be quantified (not genes close by) and compared to other types of genomic loci and promoters. Focusing on the 50kb group only as controls is misleading.

We believe the reviewer may have misunderstood our conclusions. As stated in the paper, we observed lower coverage of the histone marks H3K27me3 and H2AK119ub, associated with PRC2 and PRC1, respectively. Our conclusions regarding PRC1/2 support the RNA-seq results, indicating that Xist tends to bind to actively expressed genes. In other words, these genes exhibit lower levels of PRC-mediated silencing signals. This observation underscores the relationship between Xist binding and gene activity, highlighting that Xist preferentially associates with regions that are less subject to silencing by polycomb repressive complexes.

Secondly, the authors only look at PRC mark signal upon differentiation; what about the 0h timepoint, i.e., is there pre-marking?

Day 0 is not an appropriate timepoint for this analysis because Xist is not yet induced. There is also a small fraction of cells (<5%) that spontaneously differentiate and start to undergo XCI. Because of these reasons, the day 0 timepoint is considered somewhat heterogeneous and it would be difficult to make conclusions regarding Xist peaks in these samples.

Most worryingly, the data analysis is not consistent between figures (see Fig3C vs 5H-I). In Fig5, the group of Xist targets was chosen as those within 100kb of Xist binding, which would encompass all the control regions from Fig3C. In this analysis, the authors report that there is Xist-dependent H3K27me3 deposition, and in fact, here the Xist autosomal targets have more of it than the controls. Overall, all of this analysis is misleading, and clear conclusions cannot be made.

We believe that the reviewer may have also misunderstood the analysis in Figure 5. Figure 5 shows the effect of the Xist inhibitor, X1, on H3K27me3 and gene expression. X1 blocks reduces PRC2 targeting and gene silencing — consistent with X1's effect on RepA as published in Aguilar et al. 2022.

All in all, because the fundamental observation is not robust (see point 1), all subsequent analyses are also affected. There are also multiple other inconsistencies within the analysis; however, they have not been included here for brevity.

We again respectfully disagree with Rev1 but thank the reviewer for making suggestions that helped to strengthen our manuscript. We believe that the revised manuscript with new analyses is improved in part because of the reviewer's critical comments.

Reviewer #2 (Public review):

Summary:

To follow-up on recent reports of Xist-autosome interaction the authors examine female (and male transgenic) mESCs and MEFs by CHARTseq. Upon finding that only 10% of reads map to X, they sought to identify reproducible alternative sites of Xist-binding, and identify ~100 autosomal Xistbinding sites and show a transient impact on expression.

Strengths:

The authors address a topical and interesting question with a series of models including developmental timepoints and utilize unbiased approaches (CHARTseq, RNAseq). For the CHARTseq they have controls of both sense probes and male cells; and indeed do detect considerable background with their controls. The use of deletions emphasizes that intact functional Xist is involved. The use of 'metagene' plots provides a visual summation of genic impact.

Reviewer 2 has made some excellent suggestions. We have revised the manuscript accordingly and are grateful to the reviewer for the recommendations.

Weaknesses:

Overall, the result presentation has many 'sample' gene presentations (in contrast to the stronger 'metagene' summation of all genes). The manuscript often relies on discussion of prior X chromosomal studies, while the data generated would allow assessment of the X within this study to confirm concordance with prior results using the current methodology/cell lines.

Many of the 'follow-up' analyses are in fact reprocessing and comparison of published datasets. The figure legends are limited, and sample size and/or source of control is not always clear. While similar numbers of autosomal Xist-binding sites were often observed, the presented data did not clarify how many were consistent across time-points/cell types. While there were multiple time points/lines assessed, only 2 replicates were generally done.

We apologize for the deficiencies in the legend. The revised manuscript has corrected them.

We generated many new datasets with deep sequencing, with at least two biological replicates for each. Such experiments are extremely expensive by nature. Thus, two biological replicates are typically considered acceptable.

Additionally, we performed reanalysis of published datasets to test whether — *in the hands of other investigators* — cell lines expressing Xist also supported autosomal targeting. Figure 4 is a case in point. Here we examined Tg1 and Tg2, which respond to doxycycline to overexpress Xist from an ectopic site. Transcriptomic analysis showed significant downregulation of autosomal Xist targets, as exemplified by *Rbm14* and *Bcl7b* (new Figure 4C, S9B). In contrast, non-targets of Xist such as *Stau1* did not demonstrate significant changes in gene expression (new Figure 4E and 4G). Looking across all autosomal target genes, we observed a significant decrease in mean expression in the Xist overexpressing cell lines (new Figure 4D). The fact that the autosomal changes were also observed in datasets generated by other investigators greatly strengthen our conclusions.

Aim achievement:

The authors do identify autosomal sites with enrichment of chromatin marks and evidence of silencing. More details regarding sample size and controls (both treatment, and most importantly choice of 'non-targets' - discussed in comments to authors) are required to determine if the results support the conclusions.

Specific scenarios for which I am concerned about the strength of evidence underlying the conclusion:

I found the conclusion "Thus, RepB is required not only for Xist to localize to the X-chromosome but also for its localization to the ~100 autosomal genes" (p5) in contrast to the statement 2 lines prior: "A similar number of Xist peaks across autosomes in Δ RepB cells was observed and the autosomal targets remained similar". Some quantitative statistics would assist in determining impact, both on autosomes and also X; perhaps similar to the quintile analysis done for expression.

We have added the Xist coverage panel for day 4 and 7 in the identified Xist-autosomal peak regions (new Figure 1E-F, Figure 2E-F), as mentioned above. The results clearly demonstrate that the deletion of RepB decreases Xist binding to autosomes. Also, we showed that Δ RepB increased X-linked genes expression in our revised Figure 3D.

It is stated that there is a significant suppression of X-linked genes with the autosomal transgenes; however, only an example is shown in Figure 4B. To support this statement, a full X chromosomal geneset should be shown in panels F and G, which should also list the number of replicates.

Please see new Figure 4B.

As these are hybrid cells, perhaps allelic suppression could be monitored? Is Med14 usually subject to X inactivation in the Ctrl cells, and is the expression reduced from both X chromosomes or preferentially the active (or inactive) X chromosome?

If Rev2 is referring to Figure 4, the dataset used in Figure 4 comes from another research group and was previously published (Loda, A. et al. Nat Commun, 2017).

If Rev2 is referring to our ES cells, they are N2 cell lines. The X chromosomes are fully hybridized (Cas/Mus), but the autosomes are not fully hybridized (Ogawa et al., Science, 2008). *Med14* is subject to XCI and is expressed from the Xa, silenced on the Xi.

The expression change for autosomes after transgene induction is barely significant; and it was not clear what was used as the Ctrl? This is a critical comparator as doxycycline alone can change expression patterns.

We agree that there was a modest change in expression after transgene induction, but it is a significant change. Again, the dataset is from a published study where the authors generated doxycycline-responsive Xist transgenes (see above). The control in this case is Dox-treated wildtype cells. We now clarify these points.

In the discussion there is the statement. "Genetic analysis coupled to transcriptomic analysis showed that Xist down-regulates the target autosomal genes without silencing them. This effect leads to clear sex difference - where female cells express the ~100 or so autosomal genes at a lower level than male cells (Figure 7H)." This sweeping statement fails to include that in MEFs there is no significant expression difference, in transgenics only borderline significance, and at d14 no significant expression difference. The down-regulation overall seems to be transient during development while targeting is ongoing?

Indeed, the Xist effects on autosomes seem to occur during cell differentiation in ES cells. While there is no apparent effect in MEFs, we cannot exclude effects on other somatic cells. Regardless of whether the effects are in early development or throughout life, the sex

differences may have life-long effects in mammals. The study conducted in human cells by the Plath lab also concluded that the differences primarily affect stem cells.

Finally, I would have liked to see discussion of the consistency of the identified genes to support the conclusion that the autosomal sites are not merely the results of Xist diffusion.

We address this in the third paragraph of the Discussion. Our main argument is that if autosomal binding were caused by diffusion, then RepB deletion or X1 treatment would have led to increased binding at autosomal sites, as Xist would bind less to the X chromosome. However, as demonstrated in our study, both treatments resulted in reduced Xist binding on both the X chromosome and autosomes. This finding suggests that the binding is specific and reliant on Xist's RepA and RepB domains, rather than being a passive diffusion process.

To examine overlap between the conditions (days of differentiation and WT/RepB cells), we generated Venn Diagrams as now shown in Figure S4E.

The impact of Xist on autosomes is important for consideration of impact of changes in Xist expression with disease (notably cancers). Knowing the targets (if consistent) would enable assessment of such impact.

We thank Rev2 for the very helpful review and for the forward-looking experiments. Indeed, the physiological changes brought on by autosomal targeting will be of future interest.

Reviewer #3 (Public review):

Summary:

Yao et al use CHART to identify chromatin associated with Xist in female mouse ESCs, and, as control, male ESCs at various timepoints of differentiation. Besides binding of Xist to X chromosome regions they found significant binding to autosomes, concentrating mostly on promoter regions of around 100 autosomal genes, as elucidated by MACS. The authors went on to show that the RepB repeat is mostly responsible for these autosomal interactions using a female ESC line in which RepB is deleted. Evidence is provided that Xist interacts with active autosomal genes containing lower coverage of repressive marks H3K27me3 and H2AK119ub and that RepB dependent Xist binding leads to dampening of expression, but not silencing of autosomal genes. These results were confirmed by overexpression studies using transgenic ESCs with doxycycline-inducible Xist as well as via a small molecule inhibitor of Xist (X1), inducing/inhibiting the dampening of autosomal genes, respectively. Finally, using MEFs and Xist mutants RepB or RepE the authors provide evidence that Xist is bound to autosomal genes in cells after the XCI process but appears not to affect gene expression. The data presented appear generally clear and consistent and indicate some differences between human and mouse autosomal regulation by Xist. Thus, these results are timely and should be published.

We thank Rev3 for the positive remarks and great suggestions. We have amended the manuscript per below.

Strengths:

Regulation of autosomal gene expression by Xist is a "big deal" as misregulation of this lncRNA causes developmental defects and human disease. Moreover, this finding may explain sexspecific developmental differences between the sexes. The results in this manuscript identify specific mouse autosomal genes bound by Xist and decipher critical Xist regions that mediate this binding and gene dampening. The methods used in this

study are appropriate, and the overall data presented appear convincing and are consistent, indicating some differences between human and mouse autosomal regulation by Xist.

Weaknesses:

(1) The figure legends and/or descriptions of data are often very short lacking detail, and this unnecessarily impedes the reading of the manuscript, in particular the figures would benefit not only from more detailed descriptions/explanations of what has been done but also what is shown.

We have included more detailed descriptions in the figure legends and throughout the manuscript.

This will facilitate the reading and overall comprehension by the reader. One out of many examples: In Fig S1B in the CHART data at d4 and d7 there is not only signal in female WT Xist antisense but also in female sense control. For a reader that is not an expert in XCI it would be helpful to point out in the legend that this signal corresponds to the lncRNA Tsix (I suppose), that is transcribed on the other strand.

We thank the reviewer for this excellent point. We have amended the Results section accordingly.

(2) Different scales are used in the lower panels of Figures 1A and 2A, which makes it difficult to directly compare signals between the different differentiation stages.

We have included a figure combining all timepoints — d0, d4, d7, and d14 WT female Xist CHART signals — on the X chromosome and autosomes to support our thesis. Please see new Figure 1B.

(3) In this study some of the findings on mouse cells contrast previously published results in human ESCs: 1) Xist binding occurs preferentially to promoters in mice, not in human. 2) Binding of Xist is mostly detected in polycomb-depleted regions in mice but there is a positive correlation between Xist RNA and PRC2 marks in human ESCs. These differences are surprising but may be very interesting and relevant. While I am aware that this might be a difficult task, it would be helpful to experimentally address this issue in order to distinguish whether species specific and/or methodological differences between the studies are responsible for these differences.

Indeed, our findings in mouse cells contrast with those observed in humans. As discussed in the manuscript, this discrepancy may be attributed to factors such as cell type, differentiation methods, and the Xist pull-down technique employed (our CHART method utilizes a 20 nt oligo library, whereas RAP uses long oligos). We agree that future work should investigate the underlying causes of these differences between mouse and human systems.

Recommendations for the authors:

Reviewer #2 (Recommendations for the authors):

For Figure 2: labelling ΔB on the panel A timeline (e.g. d0- ΔB) would make the results clearer for the audience. Panel B makes most sense beside panel E of Figure 1, so combine here and skip in Figure 1?

We have modified Figure 2A and thank Rev2 for this suggestion. As for the embedded tables: since we performed peak calling for WT and ΔB separately, we believe that showing both the

peak numbers and their corresponding peak patterns provides a clearer representation of the data.

I agree that at day 7 there appears to be a difference in X; but by day 14 this looks much more minimal - is it just time-shifted rather than altered? Perhaps this could be discussed. Autosomal binding sites show no change in number.

Day 7 exhibits the strongest Xist binding on the X chromosome, consistent with the de novo establishment phase of XCI when Xist is expressed at the highest levels (300 copies/cell during de novo XCI versus ~100 copies/cell during maintenance [Sunwoo et al., 2015 as cited]. Per our RNA-seq analysis here, we also observed highest Xist expression on day 7 and reduced levels on day 14 (Fig. S5A). This expression difference explains the reduced Xist CHART levels on day 14 compared to day 7.

While the X has previously been examined, it would seem beneficial to conduct the same expression analyses (Figure 3) for the X (perhaps supplemental), as the authors have the data 'in hand'. I feel comparison to X in the main figure for panels A and B would fit, while a similar analysis for the X for panel C could be supplemental, presumably supporting the published data to which this data is currently compared.

This is a good suggestion. Please find the new data in Figures 2E-F and 3D, which demonstrate that the RepB deletion inhibits Xist binding on the X chromosome, resulting in increased X-linked gene expression, as previously mentioned. Since Xist binds across the X chromosome, we did not perform peak calling as we did for the autosomes. Therefore, applying a similar analysis as in Figures 3A-B may not be appropriate in this case.

Such a direct comparison to X-data from the same study would be important. For panel H: How many replicates (2)? This should be in the legend. What is the change in median expression? Again, a supplemental figure showing impact on X-linked targets would be useful. Do male and female ESCs show an expression difference prior to differentiation (ie d0)? The data underlying this Figure should be in one of the supplementary tables, showing the full statistical tests and average change. The supplementary tables 8-12 list the WT target genes, not expression differences with the deletion. Again, given that the difference appears transient, might the ΔB cells be altered in rate of differentiation?

Panel H (revised Figure 3G) includes two replicates, and this has been added to the legends. We have provided a supplementary figure demonstrating that RepB increases the expression levels of X-linked genes on days 4, 7, and 14 (revised Figure 3D). Male and female ESCs show differences in the expression of X-linked genes, as both X chromosomes are active in females at this stage prior to differentiation (revised Figure S5C).

A supplementary table with statistical tests and average change information has been included in our revised version (Table S11).

On the other hand, these Xist-autosomal target genes displayed no significant differences between WT male, female, or ΔB female cells on day 0 — prior to onset of XCI and Xist expression. Please see new Figure 3H.

As for whether ΔB cells are altered in their rate of differentiation, the analysis by Colognori et al. 2019 indicates that ΔB cells differentiate similarly to WT cells. (In Figure 6 of Colognori et al. 2019, autosomal genes expressed similarly in WT and ΔB cells, whereas XCI is affected only in ΔB cells)

We have also modified the legends for our supplementary tables.

Why were the transgene lines examined upon neuronal differentiation rather than the same approach as in Figures 1-3? I would have thought neuronal differentiation might be more similar to d14, where limited changes remain? Could the authors clarify and discuss?

We apologize for the confusion. The Tg lines in Figure 4 came from a previously published study. We performed reanalysis of published datasets because we wanted to test whether — in the hands of other investigators — cell lines expressing Xist also supported autosomal targeting. Here we examined Tg1 and Tg2, which respond to doxycycline to overexpress Xist from an ectopic site. Transcriptomic analysis showed significant downregulation of autosomal Xist targets, as exemplified by *Bcl7b* and *Rbm14* (Figure 4C and S9B). In contrast, non-targets of Xist such as *Stau1* did not demonstrate significant changes in gene expression (Figure 4E and 4F). Looking across all autosomal target genes, we observed a significant decrease in mean expression in the Xist overexpressing cell lines (Figure 4D). The fact that the autosomal changes were also observed in datasets generated by other investigators greatly strengthen our conclusions. We have clarified this in the Results section.

Figure 5 - the legend should specify the number of replicates and clarify the blue/green (intuitive, but not specified). Are the 'target' / 'non-target' genes from d4 Chart (but the RNA from d5)? How are 'non-targets' defined - do they match the 'targets' in certain criteria (expression level, chromatin features, GC content)? Do they change per differentiation protocol?

We have modified the legends to clarify that the 'target' and 'non-target' genes are derived from the day 4 CHART-seq data, while the RNA data is from day 5, as that study sequenced day 5 and not day 4. Non-targets were randomly chosen based on (i) the absence of Xist binding and (ii) similar expression levels. Please see revised Figure S8.

It would be helpful to compare Xist expression levels across the various models, and the MEF model could be better described - are they polyploid as often happens?

We have included the Xist expression levels of ES cells and MEF cells in the revised version (revised Figure S5A, 6D). The transformed MEFs are indeed tetraploid, as is typical.

For 6A to be informative, one needs to know % mapping to X in ES timeline, which is in supplemental, so perhaps 6A should also be supplemental?

We have moved 6A to the supplemental figure.

It is odd that ΔB seems to have had more impact in MEFs, and I would like more discussion - but I also think I am missing something: "We observed that Xist signals were more substantially reduced on both the Xi and autosomal regions in ΔRepE MEFs compared to ΔRepB cells", yet in lower panel 6 G it looks like ΔB is LOWER than ΔE? Am I misinterpreting?

We apologize for the confusing writing. The revised text now reads: "To investigate, we utilized a deletion of Xist's Repeat E (ΔRepE), which was previously demonstrated to severely abrogate localization of Xist to the Xi 41,42. We reasoned that the severe loss of Xist binding might unmask a transcriptomic difference. As expected, we observed that Xist signals were somewhat more reduced on the Xi in ΔRepE MEFs compared to ΔRepB cells (Figure 6E-6F). Despite this reduction, peak coverages in autosomal target genes did not increase in ΔRepE MEFs (Figure 6E-6F). However, there was an overall decrease in the number of significant autosomal peaks in ΔRepE MEFs relative to WT cells (Figure 6A). Regardless, we observed no

significant transcriptomic differences in Δ RepE MEFs relative to WT MEFs (Figure 7A-7E). Additionally, further examination of RNA sequencing data from male and female MEF cells in two published studies 43,44 corroborated that the expression levels of these autosomal Xist targets did not exhibit significant changes (Figure 7F and 7G). Altogether, the analysis in MEFs demonstrates that Xist continues to bind autosomal genes in post-XCI somatic cells. However, autosomal binding of Xist in post-XCI cells does not overtly impact expression of the associated autosomal genes. Nonetheless, we cannot exclude more subtle changes that do not meet the significance cut-off.”

Overall, I would like to see how consistent these autosomal peaks are - I shudder to suggest Venn diagrams, but something to show whether there are day/lineage specific peaks and/or Δ repeat B/E resistant peaks.

We now present Venn diagrams comparing MEF, ES_d4, and ES_d7, showing approximately 50% overlap between MEF and ES cells (revised Figure S10B). This may be expected, as each timepoint is a different developmental stage of XCI, with expected gene expression differences.

Very minor comments:

It would be easier if the supplemental tables were tabs in 1 file!

We will defer to the editor on how best to format the supplemental tables.

Similar to the text, could gene names be included in the supplemental?

We have provided gene names in the supplemental files.

Figure 3 legend: should 'representing' be representative?

We have modified it.

"Xist patterns identified in human cells" p 5; it is challenging to follow human versus mouse, so specify or ensure correct use of XIST/Xist Indeed, we edited the manuscript accordingly.

Gene names should be italicized.

We have italicized gene names in our manuscript.

Ref. 38 lacks details (...).

We have updated the reference.

Peak-like characters - perhaps characteristics? P8

We have modified this.

Reviewer #3 (Recommendations for the authors):

On page 6, the 6th sentence in the first paragraph needs correction. "Consistent with Xist's behavior on the X chromosome."

We have modified the sentence. Thank you.

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