

Transcriptional stochasticity reveals multiple mechanisms of long non-coding RNA regulation at the Xist – Tsix locus

Corresponding Author: Professor Gregor Neuert

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In their manuscript Kesler and Neuert have collected an impressive data set with careful absolute quantification of transcription at the Xist/Tsix antisense locus using smFISH. The study discusses a series of models that have been suggested over the years of how Tsix might regulate Xist. For each model they perform an analysis to test whether their data set would be consistent with the model or not. Although this approach is promising, it is a bit simplistic at times, since probably multiple mechanisms are at play and it would have been useful to disentangle their contributions more carefully. Moreover, the data set is not presented in sufficient detail to really access its quality. In general the reported findings are consistent with previous reports and add some interesting new insights, e.g. that transcriptional interference occurs at intermediate transcription rates. Although the manuscript does not present a big advance in understanding the Xist/Tsix locus, this quantitative data set will nevertheless be useful for the X inactivation community, if shared and documented properly. Moreover, the approach is promising also for analyzing other antisense loci.

Major points:

- Towards the end of the introduction the authors state "... the mechanisms of Tsix regulation of Xist remains unclear". I feel this is an overstatement, since a series of studies have shed light into the mechanisms. These studies are later introduced in the results section, but I feel they warrant being mentioned in the introduction to define the state of knowledge prior to the current study.
- The repeat A region has been suggested in the past to give rise to a separate transcript (PMID: 18974356). This should be mentioned in the introduction and also been taken into account when interpreting the results.
- On page 4 it is stated that "None of these previous studies demonstrated simultaneous expression and binding of Tsix and Xist within the same cells..." This is not correct. For example an in vivo time course study (PMID 30842444) reported that the majority of epiblast cells at E5.0 co-expressed Xist and Tsix from the same allele (monoallelic Xist and biallelic Tsix).
- On p. 4 it would be useful to give some more information on the culture conditions and differentiation protocol, since these have strong effects on the Xist patterns observed. Also the percentage of Xist positive cells should be mentioned in the text (at the moment it is only in a supplemental figure).
- On p. 5 it is stated that RepA and Xist spots are not expected to co-localize, if Xist binds to Tsix. However, RNA-FISH includes a denaturation step that might separate Xist and Tsix. I feel that prediction is a too strong statement, in particular since this argument is later used to state that Tsix does not bind to Xist. If the authors want to keep this argument in the manuscript, it requires a positive control experiment, maybe using an antisense pair known to interact, showing that probe hybridization is indeed prevented, if the antisense transcript is present.
- The authors have collected an amazing data set! It would be useful to see some more of it in the figures, such as the distribution of the number of Xist and Tsix transcripts for each time point.
- In the first section of the results, the fact that no co-localization of Xist and Tsix is observed is used as an argument against a direct interaction. It would be important to discuss in that section that rapid degradation through RNAi could preclude detection of co-localization.
- In Fig. S5 it would be useful to add information on how many transcription sites are plotted in each graph (n) to understand how robust the analysis is.
- To my knowledge Fig. 3A is the first time that single Tsix transcripts are observed away from the transcription site. To support this observation it would be helpful to report what percentage of transcripts / how many transcripts per cell are found outside of transcription sites.

- The data presentation in Fig. 3C+D, 4C+D, 5G+H is not easy to interpret. It would be useful to also show the individual distributions. Also a different representation of the 2-dimensional plot might help, either a scatter plot and/or in log scale. In addition, it would be good to report a contingency table for Xist/Tsix positive/negative in Fig. 4 + 5 and use a Fisher's exact test.
- For the data shown in Fig. 5G+H it would be useful to report how many intronic signals for Xist and Tsix colocalize.
- On p. 7/8 the authors investigate, whether Tsix might regulate Xist through transcriptional interference, and a negative correlation between Xist and Tsix signals at their transcription sites is taken as evidence for TI. However, such a negative correlation would also be expected, if Xist silences Tsix in the context of X inactivation. And could Xist RNA-mediated Tsix silencing be the reason, why few differences between Tsix 3' and 5' probes are observed?
- To distinguish between Xist-mediated silencing of Tsix and TI, it would be useful to compare Xist intronic and exonic probes. Specifically, one could analyze only alleles with Xist exonic signals (where Tsix silencing should occur) and then compare co-localization of intronic Xist and Tsix (3' and 5') on these alleles.
- To illustrate the accuracy of the spot detection / image segmentation, it would be helpful to show the segmentation for the example images in Fig. S3, S4, S8, S9, S10
- Fig. S11 shows that Tsix 5' signal is often observed without a Tsix 3' signal, which is taken as an indication for low processivity. Such a different could however also arrive from differential probe sensitivity. It would be helpful to perform this analysis separately in alleles with and without an Xist intronic signal. In those without the Xist intronic signal co-localization of Tsix 3' and 5' should be higher compared to those with an intronic signal where TI would occur.
- For most experiments in the manuscripts, it is not clear whether they were reproduced in independent biological replicates. For the main findings, it is important to show that they are reproducible.
- The data set generated in the study will be very useful for the community. It should therefore be provided in supplemental tables to allow reanalysis.
- Multiple analyses use somewhat arbitrary thresholds of 2 and 8 molecules. The analyses should be repeated with different threshold values to show that the results do not depend on the exact threshold used.
- The image analysis software should be made available as a Github repository
- The media formulation used to culture the ES cells is unusual, since it does not contain FBS, but is also not based on N2/B27 as other 2i media. It should be explained how this formulation was developed and potentially a reference should be added.

Reviewer #2

(Remarks to the Author)

Comment:

Xist and Tsix are two overlapping antisense-transcribed noncoding genes, with Tsix identified as an inhibitor of Xist during X chromosome inactivation (XCI). However, the specific mechanism remains unclear. This manuscript explores Tsix's regulatory mechanisms on Xist transcription at the single-cell level using smRNA-FISH. The authors extensively examined Tsix-mediated Xist transcriptional repression and concluded that it doesn't occur via direct antisense RNA-RNA interaction. Instead, they propose different regulatory mechanisms at the Xist/Tsix locus, contingent upon nascent transcription levels.

At moderate nascent transcription levels, co-transcription of antisense regions and Tsix-mediated H3K36me3 deposition at the Xist promoter repress Xist transcription. However, at high Xist transcription levels, evidence suggests transcriptional interference between opposite DNA strands. The study is intriguing, but several aspects need clarification.

1. The authors extensively examined Tsix and Xist expression with RNA-FISH, but probe specificity is unspecified. Including negative controls in cells lacking Tsix or Xist would be beneficial.
2. In Fig. 2D, while most dots showed strong colocalization between RepA and Xist, some lacked clear colocalization. An explanation for this would be helpful.
3. The study used ESC cells. Will these observations vary across cell types?
4. Quantification of negative correlation in Fig. 3 and Fig. 4 is puzzling. Figure 3's small spots of Tsix and Xist distinctly separate at the whole-cell level, indicating a negative correlation. However, the manuscript claims a lack of negative correlation. Conversely, in Fig. 4, where one of the two large spots (transcription sites) show Xist-Tsix colocalization, the authors conclude a negative correlation. The conclusions are unclear and inconsistent with the images.
5. The manuscript categorized sites based on nascent transcription levels (<2 transcripts as low, 2-8 as medium, and >8 as high). How were these cutoff values chosen?
6. Presenting separate channels in an image could better illustrate H3K36me3 levels.
7. The proposed model suggests Tsix-mediated H3K36me3 deposition at moderate transcription levels represses Xist transcription. It's unclear if Tsix transcripts are necessary for this repression or if active transcription alone suffices. Investigating Tsix transcript knockdown effects using methods like antisense oligonucleotides, where H3K36me3 remains unaffected, could clarify this.

8. "Previous studies utilized multiple mutagenesis strategies including deleting Tsix's promoter region, inserting a strong promoter to drive Tsix, or prematurely stopping Tsix transcription to demonstrate that Tsix regulates Xist in cis." Performing ChIP-seq of H3K36me3 on these mutants could confirm if Tsix transcription levels affect H3K36me3 modifications.

Reviewer #3

(Remarks to the Author)

Kesler and Neuert present an RNA FISH-based analysis of the Xist/Tsix locus in differentiating mouse embryonic stem cells. In the first portion of their study, the authors investigate a model from some of the earliest papers from that characterize Tsix, in which Tsix was proposed to function by base-pairing with Xist. While the field accepted this model as unlikely to be true quite some time ago, it was reassuring to see that the results presented in Figures 2 and 3 were consistent with prior data that demonstrated Tsix functions in cis and not in trans, independent of Dicer. In the second portion of the study, the authors investigate in more detail the mechanism by which Tsix might inhibit transcription of Xist. They observe through RNA FISH that higher levels of Tsix transcription correlate with lower levels of Xist production, consistent with expectations that Tsix functions via transcriptional interference. The authors also observe that their ability to detect H3K36me3 IF signal colocalizing with Xist RNA FISH signal varies with the overall levels of Xist and Tsix in the cell. From these data, the authors propose that two separate mechanisms govern repression of Xist by Tsix and vice versa – at high levels of Xist transcription, the authors propose a pure transcriptional interference mechanism, and at medium levels of Tsix transcription, the authors propose H3K36me3-mediated repression. What I appreciated most about this study was the detailed RNA FISH analyses of Xist and Tsix, which nicely supports transcriptional interference of Xist by Tsix. I was less convinced of the model that a separate K36-based mechanism governs repression specifically when Tsix or Xist are expressed at medium overall levels. Specific comments for the authors to consider are listed below:

1) In the introduction, the authors report percentages of the number of noncoding versus protein coding transcripts that in my opinion are inaccurate and/or overly speculative. The authors should consider examining their sources of information and adjust their statements accordingly.

2) At its core, this study is examining a mechanism of cis-repression that occurs on a single allele, presumably after Xist has been upregulated on one X chromosome. I was looking for a bit more validation of the experimental system and the decision to study Xist/Tsix at D2 of differentiation. I would recommend that the authors place the data in Figure S2 into the main manuscript (Figure 1). It would also be useful to know what fraction alleles express Xist and Tsix at the timepoint selected, and the relationship between Tsix expression and Xist cloud formation. I am guessing that Tsix expression is essentially never observed on alleles that harbor Xist clouds, but it would be useful to know quantitatively whether or not that is the case.

3) I note that the manuscript did not include a seminal citation in this field, Gayen and Kalantry, PMID: 25981039, which disproved a large body of early work and demonstrated that Tsix functions to repress Xist on the active X after upregulation on the Xi, and does not play a role in choice of Xi. A reference to this paper should feature prominently in the manuscript and the authors should review its conclusions if they were not aware of the work.

4) The methods section was lacking many key details and forced the reader to look in previously published papers to understand how the experiments and analyses were carried out. The paper would benefit from a thorough description of all methods used, to the extent that a reader could reproduce the study, even for the methods that have already been published. While I am not certain whether Nature Comm. is one, many journals now require such descriptions. A full description of methods will improve rigor, reproducibility, and interpretability.

5) The image in Fig 3A and 4A is identical. This may have been intentional, but in case not, the authors should investigate.

6) The axes on the joint probability heatmaps took me some time to understand. A simple fix would be to label the units clearly – something like "# of RNA molecules detected by allele" would have helped. Also it took me some time to understand the authors meaning of [bin = #]. What I believe the authors intended was that this number specified the "bin size" or "step size" on the axes. Again a minor point, but adding a word or two more to describe the graph would have increased the clarity.

7) My lingering concern with the conclusions of the work are that the H3K36 IF data are correlative. The authors are heavily interpreting the inability to detect H3K36 at high levels of Xist to indicate causality, when in reality the absence of K36 at that condition could be due to other reasons. One possibility is that K36 at the 22kb Xist locus is below the limit of detection in their assay, and the reason that the authors can detect K36 at lower levels of Xist transcription is because under those conditions, the Xist gene body is clustering with other K36-rich loci to boost the signal above the limit of detection. High levels of Xist represses Tsix and most other genes on the X chromosome and would be expected to reduce the overall levels of regional K36 signal. Indeed, I struggle to rationalize how any highly transcribed gene, including Xist, would lack K36 over its entire gene body specifically under the condition in which it was highly transcribed. K36 is a modification that correlates positively with transcription and splicing, and at the highest levels of Xist transcription, both of those activities are abundant at the Xist locus. Thus I am concerned that the lack of K36 colocalization with Xist intronic RNA at high levels of Xist transcription is not related to lack of K36 at the gene body and is due to some other reason. One possibility (in addition to the 22kb signal being below the limit of detection) is that at high levels of Xist, its splicing occurs at a distance from its own locus. These are challenging assays to perform, but can the authors look for previously published data that would harbor allele-specific H3K36 ChIP-Seq profiles in cells that express Xist and harbor an inactive X chromosome? A prediction of the authors model would be that on the Xist-expressing allele in cells that have undergone XCI, H3K36 signal would be absent.

Conversely, the lack of K36 detection when Xist is lowly expressed seems most likely to be due to K36 IF signal over its own locus being below the limit of detection. And more than detecting K36 over the entire locus, what the authors are intending to do is correlate presence of the modification specifically over the Xist promoter, which Ohhata and colleagues demonstrated is the critical site for repression by Tsix (PMID: 26370508). But detecting K36me3 over a 1kb segment of DNA seems well beyond the limit of what is possible by the microscopy systems being employed in this work.

Thus, in the absence of an orthogonal assay, in a static system, in a low resolution IF assay that is unlinked to any DNA sequence, I do not believe that the authors are able to conclude that K36 represses Xist only when Xist is transcribed at medium levels.

When Xist is highly expressed, it would be safe to assume that Xist is repressing Tsix using SPEN and Polycomb, and the authors would need to disprove those models before concluding that transcriptional interference is the most relevant mechanism.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

It is much appreciated that the authors have provided additional details on their data set in the form of additional supplemental figures. Unfortunately, this reveals a series of problems in the data set and that the main conclusions are not robustly supported by the experimental data. Therefore I am not convinced that the manuscript with the current data sets is ready for publication.

Major comments

1. Efficiency of Xist upregulation appears highly variable between different experiments. The data set shown in Fig. 2a, where >50% cell express Xist at day 2 is apparently not representative. In Fig. 3c-d and Suppl. Fig. 15, 18, 39 cells make very little Xist, often <10%. This suggests that cells lost an X chromosome or the differentiation failed. These experiments should be repeated, since the data quality is poor.
2. Although most experiments were apparently performed in independent replicates, these were merged for most analyses. This is unacceptable, since it is crucial to show that the observations are reproducible. The analyses in Fig. 5 and Fig. 6 both show some unexpected associations (differences in TI between cells with different Xist levels; different association with H3K36me3 staining). To rule out that these are just random fluctuations, it is crucial to perform the analyses on independent biological replates and not merge all the cells for the analysis. Given the low quality of some replicates (very little Xist expression) this might be difficult. But in this case additional measurement must be made, since otherwise no robust conclusions are possible.
3. In the rebuttal letter the authors mention that the spot-detection software will only be published together with a future methods paper. Since the software is a crucial part of this manuscript, I believe that that software must be made available together with the manuscript and not in the future.

Minor comments

4. Section "Transcriptional Interference of Xist and Tsix at high nascent transcription": It should be mentioned that the negative correlation between Xist and Tsix might arise from Xist-mediated silencing of Tsix, since this observation is expected and not surprising (see comments from the first round of review).

5. The H3K36me3 data from Gjaltema et al used CUT&TAG not CUT&Run.

6. In Suppl. Fig. 41, data for an inactive X at day 0 of differentiation is shown. Usually, both X chromosomes are active in undifferentiated ESCs, how is this possible?

(Remarks on code availability)

I could not access the code through the link.

Also, in the rebuttal letter the authors mention that the spot-detection software will only be published together with a future methods paper. Since the software is a crucial part of this manuscript, I believe that that software must be made available together with the manuscript and not in the future.

Reviewer #2

(Remarks to the Author)

The revised manuscript shows significant improvement, with additional analyses and experiments addressing previous feedback. However, a few aspects still require clarification.

1. The authors have demonstrated the specificity of the Xist and Tsix probes by hybridizing them to human male Jurkat cells. However, it would be more appropriate to test these probes in mouse cells lacking Xist expression or with very low Xist expression, considering the substantial divergence between human and mouse genomes.

2. The authors included an analysis of H3K36me3 CUT&RUN in mouse mESCs during differentiation, revealing reduced H3K36me3 at Xist promoter regions on the Xist-expressing Xi. However, it remains unclear whether this reduction is specific to the Xist site or a general feature of this chromosome. A metaprofile showing H3K36me3 variations over genes on the Xi during differentiation should be provided. Additionally, it would be helpful to investigate whether other antisense gene pairs exhibit a similar H3K36me3 pattern during differentiation, either on the X chromosome or autosomes.

3. Genome browser views (e.g., IGV) should be included to visualize H3K36me3 signals over the listed example genes in the CUT&RUN results. Please indicate the specific genomic sites exhibiting the varied H3K36me3 signals. A brief description of the H3K36me3 CUT&RUN data analysis is needed. Additionally, it would be valuable to conduct statistical analyses to compare the changes and describe the fold change of H3K36me3.

4. The panels labeled "Pgk1" and "Rlim" in Supplementary Figure 41 lack information regarding the promoter or gene body. Please provide this information.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)

The authors have addressed my comments and I would support publication of this manuscript. The additional experiments support the conclusions that separate mechanisms dependent on lncRNA transcription levels can regulate antisense genes. As a note for the future, I found the inclusion of red, scored text in the revised manuscript to be a distraction that made it more difficult for me to read and assess the authors' changes.

(Remarks on code availability)

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I want to thank the authors for their explanations and clarifications. Some of these should also be included in the manuscript:

1. Bin size: It is not clear what the lowest bin in Fig. S15 represents (0-10 molecules?). I would consider to state this in the figure legend.
2. Fig. 5i-n and similar: The figure legend should contain a description of what the line and shaded area represent.
3. Regarding the analysis in Fig. 5i-n, the colocalization mentioned in the text, I would suggest to provide a measurement of uncertainty, e.g. std dev, also in the text.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

The manuscript has shown improvement. There are still some issues that need to be addressed.

1. The CUT&Tag data presented in Supplementary Figures 41-42 indicate a reduction in H3K36me3 levels at the Xist promoter region during differentiation in both XXΔXic (Xist is expressed) and XO (Xist is not expressed) mESCs. If H3K36me3 represses Xist, it is unclear why the decrease in H3K36me3 during differentiation in XO cells does not lead to an increase in Xist expression? This discrepancy requires clarification. The inclusion of XO in this context may add confusion.

Additionally, it would be beneficial to include RNA-seq tracks and expression levels of both Xist and Tsix in Supplementary Figures 41-42 to demonstrate whether Xist expression negatively correlates with H3K36me3 levels.

2. The statistical analyses depicted in Supplementary Figure 41 should be conducted to compare different stages of differentiation in order to assess whether the alterations in H3K36me3 levels are statistically significant during differentiation. Comparing across different genotypes (XO VS XXΔXic) are confusing and the XO data do not support the model that Xist is

repressed by H3K36me3.

(Remarks on code availability)

Version 3:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I appreciate that the authors made an attempt to clarify their legends. Unfortunately, there is still important information missing. In Fig. S15 it is still not clear what the lowest bin presents. The legend now state that bin size is 20. Then the lowest bin should be 0-19? But this is not in agreement with the plots. That should be clarified. Similarly, in Fig. 5i-n the legend now states that mean and std dev are shown. But it not clear how these are calculated (individual cells, or independent biological replicates? And if so how many?).

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

The authors have addressed all of my comments. The quality of the figures has improved. I have no additional questions or suggestions.

(Remarks on code availability)

Version 4:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have adressed my concerns.

(Remarks on code availability)

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Response to Reviewers and specific changes in the manuscript:

Reviewer Comments are given in *Italic black text*. Authors' responses are provided in blue plain text.

We thank all the reviewers for their thorough review and insightful comments, which have significantly improved the manuscript. We have addressed all the points raised with new experiments and analyses.

Response to reviewer #1

1. *In their manuscript Kesler and Neuert have collected an impressive data set with careful absolute quantification of transcription at the Xist/Tsix antisense locus using smFISH.*

Response: We thank Reviewer #1 for devoting time to evaluate our article and recognizing the quality of the data.

2. *The study discusses a series of models that have been suggested over the years of how Tsix might regulate Xist. For each model they perform an analysis to test whether their data set would be consistent with the model or not. Although this approach is promising, it is a bit simplistic at times, since probably multiple mechanisms are at play and it would have been useful to disentangle their contributions more carefully.*

Response: We agree with the reviewer that potentially many more models are possible to understand the regulation of Xist and Tsix. We also don't claim that the models we studied are the only models. But to our knowledge nobody has tried to systematically test several models against each other for any sense -antisense pair or for Xist and Tsix. To get started somewhere we focused on models that have been described in several publications that either support or refute the model. Our thinking is that for a specific model certain basic conditions need to be fulfilled for the specific model to be valid. For example, for model 1 of binding of Xist to Tsix, this requires first that Xist and Tsix are expressed in the same cell. Second, that Xist and Tsix are physically near to bind to each other. There are most likely other conditions that need to be fulfilled to fully understand this model. However, we believe that if these two conditions are not met then we think it is very likely that Xist and Tsix do not bind to each other. The same rational was applied in the design for testing any of the other models.

We have updated the manuscript on page 3, line 12-13 which reads now:

"... for which we agnostically tested several of the proposed mechanisms of Xist and Tsix mutual regulation."

We agree with the reviewer that multiple mechanisms are likely at play, as shown in Figures 5 & 6. If different mechanisms play roles in different cells, our single-cell analysis indirectly indicates the prevalence of a specific mechanism by examining the fraction of cells within it. For instance, in Figure 5I-L, out of 669 Xist transcription sites, 334 do not show a difference in colocalization with Tsix 3' or Tsix 5'. This suggests that in 77% of cells, transcriptional interference is likely not a prevalent mechanism.

We emphasize that testing one model at a time does not reject other models; it simply evaluates the validity of the tested model. This manuscript aims to determine which proposed mechanism is most relevant. Future research might explore how prevalent a specific mechanism is, given the potential variability in gene expression and cell states. However, rigorously answering this question is beyond the scope of this current study and would require a separate manuscript.

3. *Moreover, the data set is not presented in sufficient detail to really access its quality.*

Response: We have now added Figures to the supplementary material with marginal, joint probability distributions, and scatter plots of RNA transcript counts for Xist and RepA and Xist and Tsix. For the colocalization analysis in Figure 2G-I, this is the most rigorous way to present the data. For Figures 3-5, we have now separated the joint probability distributions into marginal distribution for each replica experiments to

provide more details about the data. For Figure 6, we added marginal distribution to the supplementary materials to provide more insight. In summary, we have added the marginal distributions for each replica experiment in the form of supplementary Figures 8, 9, 10, 15, 18, 28, 37-40. In the original submission we included links to the full raw data, processed data, codes for analyzing the images, and codes for generating the figures. These links will be public after acceptance of the manuscript. We are also in the process to publish the RNA-FISH spot detection pipeline in a separate manuscript and we plan to publish a data descriptor of the data for easy reuse.

4. *In general the reported findings are consistent with previous reports and add some interesting new insights, e.g. that transcriptional interference occurs at intermediate transcription rates.*

Response: We observed transcriptional inference at high transcription rates and not at low or intermediate transcription rates (see Fig. 5, Supplemtray Fig. 28, 33).

5. *Although the manuscript does not present a big advance in understanding the Xist/Tsix locus, this quantitative data set will nevertheless be useful for the X inactivation community, if shared and documented properly. Moreover, the approach is promising also for analyzing other antisense loci.*

Response: We thank the reviewer for acknowledging the quality of the data and importance of the approach for other antisense loci. In the original submission we included links to the full raw data, processed data, codes for analyzing the images, and codes for generating the figures. These links will be public after appetence of the manuscript. We are also in the process to publish the RNA-FISH spot detection pipeline in a separate manuscript and we plan to publish a data descriptor of the data for easy reuse.

Major points:

6. *Towards the end of the introduction the authors state "... the mechanisms of Tsix regulation of Xist remains unclear". I feel this is an overstatement, since a series of studies have shed light into the mechanisms. These studies are later introduced in the results section, but I feel they warrant being mentioned in the introduction to define the state of knowledge prior to the current study.*

Response: Thank you for pointing this out. We have updated the mentioned section at end of the introduction of the manuscript and rewrote the section. We have deliberately described the individual mechanisms in the result section to directly compare the current knowledge to our experimental design and observed results.

On page 3, line 25-27, the updated text reads now:

"... there are many proposed mechanisms for Tsix regulation of Xist, some of which have conflicting evidence or even a complete lack of evidence³³."

In addition, we updated the introduction and shortly reviewed the models in the introduction.

On page 3, lines 29-34 and page 4, lines 1-4, we added the following text:

The mechanisms involved in this context are categorized into two types: those related to transcripts (Fig. 1a-c) and those concerning transcription (Fig. 1d-e). Transcript-based mechanisms encompass the RNA interference (RNAi) system, which can trigger the degradation of double-stranded RNA, a process that involves the binding of Xist and Tsix within the same cell (Fig. 1a)³⁴. Additionally, the binding of Tsix to Xist may block crucial proteins from associating with Xist, which is necessary for X-chromosome inactivation (XCI) (Fig. 1b)¹⁶. Tsix may also serve as a structural framework to attract proteins that suppress Xist's function (Fig. 1c)³⁵. On the other hand, transcription-based mechanisms include transcriptional interference (TI), where collisions between polymerases can impede the transcription process (Fig. 1d)³⁶. Furthermore, certain proteins are known to attach to polymerase tails, leading to alterations in chromatin during the transcriptional activity (Fig. 1e)^{14,37-40}.

7. *The repeat A region has been suggested in the past to give rise to a separate transcript (PMID: 18974356). This should be mentioned in the introduction and also been taken into account when interpreting the results.*

Response: Thank you for pointing this out and providing this important reference. We have now updated the introduction and discussion with this information.

On page 4, lines 4-5, we added the following text:

“In addition, Xist contains a repeat A region that is reported to give rise to a separate transcript²⁴. “

On page 11, line 20-22 we added the following text:

“Our analysis, which shows a high degree of colocalization, co-expression, and correlation between Xist and RepA, suggests that RepA is not an independent transcript but instead a segment of the Xist transcript (Fig. 2, Supplementary Fig. 8, 11, 13).”

8. *On page 4 it is stated that “None of these previous studies demonstrated simultaneous expression and binding of Tsix and Xist within the same cells...” This is not correct. For example an in vivo time course study (PMID 30842444) reported that the majority of epiblast cells at E5.0 co-expressed Xist and Tsix from the same allele (monoallelic Xist and biallelic Tsix).*

Response: Thank you for pointing out this study. We have now updated statement to include the information from the cited study.

On page 4, lines 25-27, the text now reads:

“Although earlier studies showed Xist and Tsix signals in the same cells^{22,45}, none of these previous studies demonstrated simultaneous colocalization of individual Tsix and Xist transcripts within the same cells and outside the transcription site, which would be necessary for either of these mechanisms.”

9. *On p. 4 it would be useful to give some more information on the culture conditions and differentiation protocol, since these have strong effects on the Xist patterns observed. Also the percentage of Xist positive cells should be mentioned in the text (at the moment it is only in a supplemental figure).*

Response: We have now added more information about the culture and differentiation conditions and the Xist positive cells in the result section.

On page 4, line 34 and page 5, line 1-2, we have now added:

“... are grown in 2i media to stay in the pluripotent state (0.5 % of Xist cloud positive cells) (Supplementary Fig. 3) and then non directionally differentiated for two days by removing 2i media (52 % of Xist cloud positive cells) (Fig. 2a), ...”

We also added an additional Supplementary Fig. 3 of cells with Xist and Tsix exonic probes at day 0 before differentiation showing no Xist clouds and all cells with Tsix expression.

10. *On p. 5 it is stated that RepA and Xist spots are not expected to co-localize, if Xist binds to Tsix. However, RNA-FISH includes a denaturation step that might separate Xist and Tsix. I feel that prediction is a too strong statement, in particular since this argument is later used to state that Tsix does not bind to Xist. If the authors want to keep this argument in the manuscript, it requires a positive control experiment, maybe using an antisense pair known to interact, showing that probe hybridization is indeed prevented, if the antisense transcript is present.*

Response: Thank you for pointing this out. In our RNA-FISH experiment, we don't use a heat-induced denaturation step, but we do use formamide to reduce secondary structures during the hybridization of probes to the RNA. We have rewritten the relevant section and updated Figure 2 to reflect these changes. However, our conclusion that Tsix does not bind to Xist remains strongly supported by the lack of Xist and Tsix

colocalization. Even if previously bound Xist and Tsix had separated, they should still be close to each other because the cells were initially cross-linked with formaldehyde.

On page 5, line 14-16 reads now:

“As an example of a colocalized signal in RNA FISH is the hybridization of probes to Xist and RepA (Fig. 2b insert, right, Supplementary Fig. 11-14). Although we expected colocalization between Xist and RepA, previous studies showed that colocalization is not perfect⁴⁶.”

11. *The authors have collected an amazing data set! It would be useful to see some more of it in the figures, such as the distribution of the number of Xist and Tsix transcripts for each time point.*

Response: Thank you for these kind words. We have plotted the marginal distributions and generated new Supplementary Fig. 8-10, 15, 18, 39, 40 to show the data in more detail. Fig. 2 and 6 already have the number of cells and transcripts. We now updated captions for Fig. 3 with the number of cells and transcripts and for Fig. 4 & 5 we now added the number of cells, number of transcription sites, and number of transcripts.

12. *In the first section of the results, the fact that no co-localization of Xist and Tsix is observed is used as an argument against a direct interaction. It would important to discuss in that section that rapid degradation through RNAi could preclude detection of co-localization.*

Response: Thank you for pointing this out. We have now included statements addressing this point in the results section in addition to the discussion section.

On page 6, lines 10-13, we added:

“The hypothesis that RNAi-induced rapid RNA degradation is responsible for non-detection is not supported by our prior findings. We have established that RNA-FISH can effectively identify RNA molecules that have lifetimes of less than three minutes^{52,53}, suggesting that RNAi degradation speed should not hinder detection.”

On page 11, lines 20-24, we added:

“Our analysis, which shows a high degree of colocalization, co-expression, and correlation between Xist and RepA, suggests that RepA is not an independent transcript but instead a segment of the Xist transcript (Fig. 2, Supplementary Fig. 8, 11, 13). It is improbable that rapid RNA degradation via RNAi would prevent detection in our assays. Previous research has demonstrated that RNA-FISH is capable of detecting even short-lived RNA species with lifespans shorter than three minutes^{52,53}”

13. *In Fig. S5 it would be useful to add information on how many transcription sites are plotted in each graph (n) to understand how robust the analysis is.*

Response: We have now updated Supplementary Fig. 16 (previously S5) with the number of cells, transcription sites, and transcripts.

14. *To my knowledge Fig. 3A is the first time that single Tsix transcripts are observed away from the transcription site. To support this observation it would be helpful to report what percentage of transcripts / how many transcripts per cell are found outside of transcription sites.*

Response: Thank you for this suggestion, and we included supplementary Fig. S9 showing the percentage of cells with Xist and Tsix transcripts are outside of transcription sites.

15. *The data presentation in Fig. 3C+D, 4C+D, 5G-H is not easy to interpret. It would be useful to also show the individual distributions. Also a different representation of the 2-dimensional plot might help, either a scatter plot and/or in log scale. In addition, it would be good to report a contingency table for Xist/Tsix positive/negative in Fig. 4 + 5 and use a Fisher's exact test.*

Response: Thank you for this suggestion. We added marginal distributions and different ways of graphing the data in Supplementary Fig. 8-10, 15, 18, 39, 40. For example, in Supplementary Fig. 8, Replica 1, the Xist = 0, Tsix = 0 bin is yellow which corresponds to a probability of 0.65. In comparison, the scatter plot makes this information inaccessible because all the data point overlap without containing any information about the probability. We often see scatter plots for co-expression data in other publications. But this is a qualitative metric and difficult to interpret if there is a high density of data point for a specific bin size. We prefer the joint probability distributions because it has quantitative information on the co-expression of two transcript and their probability for each RNA expression level, information that is lost in the scatter plot. We updated the scaling on the color bar in Fig. 3c,d, 4c,d, 5g,h to make it more accessible.

The Fisher exact test would make sense if the data is presented in a binary format as suggested by the reviewer (Xist / Tsix positive or negative). Respectfully, the whole point of the manuscript is to utilize the full richness of the data in the form of an distribution. Also choosing a threshold to generate binary data for a contingency table will introduce another bias. Reducing these distributions to binary data would undermine the quality of the results.

16. *For the data shown in Fig. 5G+H it would be useful to report how many intronic signals for Xist and Tsix colocalize.*

Response: We added this information to the Figure 5 caption.

17. *On p. 7/8 the authors investigate, whether Tsix might regulate Xist through transcriptional interference, and a negative correlation between Xist and Tsix signals at their transcription sites is taken as evidence for TI. However, such a negative correlation would also be expected, if Xist silences Tsix in the context of X inactivation. And could Xist RNA-mediated Tsix silencing be the reason, why few differences between Tsix 3' and 5' probes are observed?*

Response: It appears that there is unfortunately some confusion. We did not intend to argue that negative correlation of Xist and Tsix intron signal by itself is evidence for transcriptional interference. Instead, we argue that a stronger negative correlation in the overlapping region of Xist and Tsix (where transcriptional interference can occur) compared to the non-overlapping region of Tsix (where transcriptional interference cannot occur) is evidence for transcriptional interference. The reviewer is correct that little difference between Tsix 5' and 3' probes indicate a lack of transcriptional interference and perhaps another mechanism such as Xist silencing of Tsix, which is why we argue that transcriptional interference only occurs at high transcription where there is a noticeable difference between the Tsix 5' and 3' probes. We added clarifying statements regarding this in the text to avoid confusion.

On page 8, lines 17-18, we added:

"To better understand the source of this negative correlation, we analyzed the significant variability in nascent transcription, which is a consequence of stochastic gene expression, a fundamental property of single cells (Supplementary Fig. 18)."

On page 8, lines 21-22, we added:

"...to gain nascent transcription dependent insights into transcriptional interference in addition to negative correlations."

18. *To distinguish between Xist-mediated silencing of Tsix and TI, it would be useful to compare Xist intronic and exonic probes. Specifically, one could analyze only alleles with Xist exonic signals (where Tsix silencing should occur) and then compare co-localization of intronic Xist and Tsix (3' and 5') on these alleles.*

Response: We believe our specific design of experiments with Xist intron and Tsix 3' intron or Tsix 5' intron probes address this question. Because only at high nascent Xist expression do we see a difference in colocalization between Tsix 5' and Tsix 3' with Xist.

In addition, we have generated new probes with for the Xist exon in the 488nm channel (FITC) with the goal to measure Xist intron (CY5) and Tsix 3' (TMR) or Tsix 5' (TMR) intron signal. Because of the high background in the 488nm channel and the resulting low signal to noise, we were not able to analyze the experiments. Furthermore, we tested Xist intron in the TF594 channel and Tsix intronic probes in the TMR channel, which would have allowed us to measure the Xist exon signal in the Cy5 channel. But because of the strong nascent transcription of Tsix, we did get cross talk into the Xist intro signal which cannot be corrected for.

19. *To illustrate the accuracy of the spot detection / image segmentation, it would be helpful to show the segmentation for the example images in Fig. S3, S4, S8, S9, S10*

Response: Thank you for this suggestion. We added segmentation examples for the images as Supplementary Fig. 5-7, 12-14, 21-23, 25-27, 30-32. We also previously published the cell and nuclear segmentation results in Kesler et al, Scientific Reports 2019. We also prepare another manuscript that benchmarks our spot detection algorithm, TrueSpot, against other commonly used spot detection software and we outperform them in most cases. The restricted link to the TrueSpot software can be found on Zenodo, at the link below. The software will be freely available upon the publication of the TrueSpot software.
<https://zenodo.org/records/13345321?preview=1&token=eyJhbGciOiJIUzUxMiIsImIhdCI6MTcyNDEwNDI5NSwiZXhwIjoxNzUzOTE5OTk5fQ.eyJpZCI6ImQwNzEzODExLW11YWMTNDhhYi05MDgyLTkzNDkxZTc5MGFjYiIsImRhdGEiOnt9LCJyYW5kb20iOiJIZGJlYmNIZThjOGVhYTAzYTc0OTcwZTVhOGUwZTE2NyJ9.YukEzgC1qz2PCPyA2RoiZrsSRKGGile0we9bmQhmWIqSZPI-DrHgWsqBZeplaJheQZAQ0HDepAOMT55eYxmPGA>

20. *Fig. S11 shows that Tsix 5' signal is often observed without a Tsix 3' signal, which is taken as an indication for low processivity. Such a different could however also arrive from differential probe sensitivity. It would be helpful to perform this analysis separately in alleles with and without an Xist intronic signal. In those without the Xist intronic signal co-localization of Tsix 3' and 5' should be higher compared to those with an intronic signal where TI would occur.*

Response: Thank you for this suggestion. Because we can detect an individual intronic transcript outside the transcription site (see Supplementary Fig. 23, 27, 32), we are certain that the lack of colocalization is not due to probe sensitivity. We also like to stress that nascent transcription as is shown in Figures 4 & 5, are quantified as the number of nascent transcripts and not as an intensity value. To convert the intensity of the transcription site into the number of nascent RNA molecules, we determined the mean intensity of an individual intronic signal outside the transcription site shown in Supplementary Fig. 16.

21. *For most experiments in the manuscripts, it is not clear whether they were reproduced in independent biological replicates. For the main findings, it is important to show that they are reproducible.*

Response: We updated the results section to indicate that our experiments were reproduced in independent biological replicates.

22. *The data set generated in the study will be very useful for the community. It should therefore be provided in supplemental tables to allow reanalysis.*

Response: We have provided all the raw and processed data as an electronic supplement.

23. *Multiple analyses use somewhat arbitrary thresholds of 2 and 8 molecules. The analyses should be repeated with different threshold values to show that the results do not depend on the exact threshold used.*

Response: We added Supplementary Fig. 28 & 37 with more thresholds to show that the results do not depend on the exact threshold.

24. The image analysis software should be made available as a Github repository

Response: We have shared the preliminary dropbox links in the original submission in the Code and Data Sharing section. We also added github links to the codes and we plan to submit a separate manuscript on the image analysis software and another manuscript with the computational protocol. In addition, we prepared another manuscript that benchmarks our spot detection algorithm, TrueSpot, against other commonly used spot detection software and we outperform them in most cases. The restricted link to the TrueSpot software can be found on Zenodo, at the link below. The software will be freely available upon the publication of the TrueSpot software.

(<https://zenodo.org/records/13345321?preview=1&token=eyJhbGciOiJIUzUxMiIsImhhbmhhdC16MTcyNDEwNDI5NSwiZXhwIjoxNzUzOTE5OTk5fQ.eyJpZC16ImQwNzEzODEwLW11YW50MTNDhhYi05MDgyLTkzNDkxZTc5MGFjYiIsImRhdGEiOnt9LCJyYW5kb20iOiJlZGJlYmNlZThjOGVkyTAzYTc0OTcwZTVhOGUwZTE2NyJ9.YukEzgC1qz2PCPy2RoiZrsSRKGile0we9bmQhmWlqSZPI-DrHgWsqBZeplaJheQZAQ0HDepAOMT55eYxmPGA>)

25. The media formulation used to culture the ES cells is unusual, since it does not contain FBS, but is also not based on N2/B27 as other 2i media. It should be explain how this formulation was developed and potentially a reference should be added.

Response: We thank you for catching this, because the 2i media formulation was based on N2/B27, but these ingredients were missing in the methods section.

This has been updated on page 14, lines 21.

“...0.5x N2 Supplement (Gibco 17502-048), 0.5x B27 supplement (Gibco 17504044), ...”

Reviewer #2 (Remarks to the Author):

Comment:

Xist and Tsix are two overlapping antisense-transcribed noncoding genes, with Tsix identified as an inhibitor of Xist during X chromosome inactivation (XCI). However, the specific mechanism remains unclear. This manuscript explores Tsix's regulatory mechanisms on Xist transcription at the single-cell level using smRNA-FISH. The authors extensively examined Tsix-mediated Xist transcriptional repression and concluded that it doesn't occur via direct antisense RNA-RNA interaction. Instead, they propose different regulatory mechanisms at the Xist/Tsix locus, contingent upon nascent transcription levels.

At moderate nascent transcription levels, co-transcription of antisense regions and Tsix-mediated H3K36me3 deposition at the Xist promoter repress Xist transcription. However, at high Xist transcription levels, evidence suggests transcriptional interference between opposite DNA strands. The study is intriguing, but several aspects need clarification.

1. The authors extensively examined *Tsix* and *Xist* expression with RNA-FISH, but probe specificity is unspecified. Including negative controls in cells lacking *Tsix* or *Xist* would be beneficial.

Response: To address this concern, we used a two-pronged approach. First, we blasted all the probes against the human genome to test if the probes could cross-react. We found that this is not the case. Second, we tested all the Xist and Tsix probes used in the manuscript, by hybridizing them at the same concentrations to human male Jurkat cells. We find no enriched fluorescent signal for any of the RNA-FISH probe sets that would indicate off target probe binding. We compare these probe signals in human cells to mESC at day 2 of differentiation. The

intensities are plotted at the same scale. The experiment in Jurkat cells demonstrate that the probes are specific. We added Supplementary Fig. 2 showing the images of Jurkat cells in comparison to mESC with the probes used in this study. We choose this approach because we do not have a Xist or Tsix knockout cell line available in our lab.

2. In Fig. 2D, while most dots showed strong colocalization between RepA and Xist, some lacked clear colocalization. An explanation for this would be helpful.

Response: We thank the reviewer for asking for this clarification. We explain this lack of colocalization that some of the transcripts might be partially degraded that would then result in only the RepA probes binding. As pointed out in previous studies by Raj et al. Nature Methods 2008, colocalization is never 100% and our colocalization fraction is similar to previously published positive controls for colocalization.

On page 5, line 15-16, we added:

“Although we expected colocalization between Xist and RepA, previous studies showed that colocalization is not perfect⁴⁶.”

3. The study used ESC cells. Will these observations vary across cell types?

Response: The experimental design is not limited to mESC or to Xist and Tsix, and can be applied to other mRNA and long non-coding RNA and cell types for which RNA-FISH probes can be designed. However, demonstrating this versatility is outside the scope of the current study.

4. Quantification of negative correlation in Fig. 3 and Fig. 4 is puzzling. Figure 3's small spots of Tsix and Xist distinctly separate at the whole-cell level, indicating a negative correlation. However, the manuscript claims a lack of negative correlation. Conversely, in Fig. 4, where one of the two large spots (transcription sites) show Xist-Tsix colocalization, the authors conclude a negative correlation. The conclusions are unclear and inconsistent with the images.

Response: We agree with the reviewer that the images of Xist and Tsix mature transcripts, dispersed throughout the cell, give the incorrect impression of anti-correlation. We used this image intentionally to highlight the importance of analyzing nascent transcription. This image demonstrates allelic expression of Xist and Tsix, and sometimes, nascent co-expression. Due to space constraints, full-size images of these cells are provided in supplementary Fig. 4-7.

To rigorously test this impression, we analyzed the data and quantified the correlation in Fig. 3. We found that Xist and Tsix are not negatively correlated, and more importantly, their correlation is not different from random. This conclusion is based on high-quality images and a large number of single cells. One possible reason for the lack of correlation is that transcription occurred in the past, leading to a loss of correlation. To obtain a direct readout of transcription, we focused on the site of transcription in Fig. 4, as only cells with active transcription will show transcription sites. In this analysis, we found a clear negative correlation between nascent Xist and Tsix expression, distinct from random results.

On page 6, lines 23-24, we added:

“Therefore, we tested whether there was negative correlation between mature and dispersed Xist and Tsix transcripts.”

On page 7, lines 9-12, we added:

“Because transcription sites are identifiable as spots brighter than individual transcripts, we were able to quantify the presence of Xist and Tsix specifically at the transcription sites and their correlation using the data generated in the previous section (Figures 4A, Supplementary Fig. 4-7, 9, 10, 16, 18).”

5. *The manuscript categorized sites based on nascent transcription levels (<2 transcripts as low, 2-8 as medium, and >8 as high). How were these cutoff values chosen?*

Response: We choose these cutoffs to have a sufficient number of cells in each of the three expression ranges (low, medium, high). To demonstrate that the cutoffs do not change the interpretation of the results, we generated Supplementary Fig. 28 with different cutoffs that show that our results are not dependent on specific cutoff values.

6. *Presenting separate channels in an image could better illustrate H3K36me3 levels.*

Response: This is a helpful suggestion, but we could not fit it into the main figure. Instead, we have added Supplementary Fig. 36 with separate channels.

7. *The proposed model suggests Tsix-mediated H3K36me3 deposition at moderate transcription levels represses Xist transcription. It's unclear if Tsix transcripts are necessary for this repression or if active transcription alone suffices. Investigating Tsix transcript knockdown effects using methods like antisense oligonucleotides, where H3K36me3 remains unaffected, could clarify this.*

Response: Thank you for this interesting suggestion. We have thought about this kind of experiment in the past, but decided not to pursue it because a recent publication shows that antisense oligonucleotides ASO also interfere with the act of transcription (Lai et al., Molecular Cell, Volume 77, Issue 5, 5 March 2020, Pages 1032-1043.e4 ; J.-S. Lee & Mendell, Molecular Cell, Volume 77, Issue 5, 5 March 2020, Pages 1044-1054.e3).

8. *“Previous studies utilized multiple mutagenesis strategies including deleting Tsix's promoter region, inserting a strong promoter to drive Tsix, or prematurely stopping Tsix transcription to demonstrate that Tsix regulates Xist in cis.” Performing ChIP-seq of H3K36me3 on these mutants could confirm if Tsix transcription levels affect H3K36me3 modifications.*

Response: Thank you for your insightful suggestion. Although we do not have expertise in performing ChIP or ChIP-seq experiments, we sought to address your query by analyzing publicly available allele-specific CUT & RUN datasets for H3K36me3 in mouse embryonic stem cells (mESCs) during differentiation (0, 2, and 4 days; Gjaltema et al., Mol Cell 2022). Our analysis revealed a reduction in H3K36me3 at the Xist promoter during differentiation, particularly on the Xist-expressing inactive X chromosome. To explore whether the observed reduction in H3K36me3 might be due to the inactivation of X-linked genes, we examined the H3K36me3 levels on other X-linked genes like Pcg1 and Rlim, which are known to inactivate early. These genes would similarly exhibit reduced H3K36me3 levels if the alternative interpretation was valid. However, we did not observe any reduction of H3K36me3 on the Xist-expressing inactive X chromosome, suggesting that the reduction in H3K36me3 at the Xist promoter is not linked to Tsix transcription levels or the inactivation of these X-linked genes. Therefore, our findings do not support the hypothesis that Tsix transcription levels directly influence H3K36me3 modifications at the Xist promoter.

Furthermore, to establish a causal relationship, we collected microscopy data for Xist and H3K36me3 with and without Setd2 inhibition after 2 days of differentiation. The Setd2 inhibitor was used to reduce the levels of H3K36me3 in the cells. According to the model proposed in our paper, reducing H3K36me3 should lead to an increase in Xist transcription. Alternatively, if Xist expression itself causes a reduction in H3K36me3, then lowering H3K36me3 should have no effect on Xist levels. Our analysis showed that H3K36me3 was indeed reduced by the Setd2 inhibitor (Supplementary Fig. 43 a, b). Additionally, we observed that Tsix nascent transcription was reduced (Supplementary Fig. 43 c, d), while Xist nascent transcription remained unchanged (Supplementary Fig. 43 e, f). Notably, we found that the fraction of cells with nascent Xist expression increased upon drug treatment (Supplementary Fig. 43 g), whereas the fraction of cells with nascent Tsix expression did not

change (Supplementary Fig. 43 h). Based on these findings, we conclude that reducing H3K36me3 increases the fraction of cells expressing nascent Xist. We have included these results in Supplementary Fig. 43.

Reviewer #3 (Remarks to the Author):

Kesler and Neuert present an RNA FISH-based analysis of the Xist/Tsix locus in differentiating mouse embryonic stem cells. In the first portion of their study, the authors investigate a model from some of the earliest papers from that characterize Tsix, in which Tsix was proposed to function by base-pairing with Xist. While the field accepted this model as unlikely to be true quite some time ago, it was reassuring to see that the results presented in Figures 2 and 3 were consistent with prior data that demonstrated Tsix functions in cis and not in trans, independent of Dicer. In the second portion of the study, the authors investigate in more detail the mechanism by which Tsix might inhibit transcription of Xist. They observe through RNA FISH that higher levels of Tsix transcription correlate with lower levels of Xist production, consistent with expectations that Tsix functions via transcriptional interference. The authors also observe that their ability to detect H3K36me3 IF signal colocalizing with Xist RNA FISH signal varies with the overall levels of Xist and Tsix in the cell. From these data, the authors propose that two separate mechanisms govern repression of Xist by Tsix and vice versa – at high levels of Xist transcription, the authors propose a pure transcriptional interference mechanism, and at medium levels of Tsix transcription, the authors propose H3K36me3-mediated repression. What I appreciated most about this study was the detailed RNA FISH analyses of Xist and Tsix, which nicely supports transcriptional interference of Xist by Tsix. I was less convinced of the model that a separate K36-based mechanism governs repression specifically when Tsix or Xist are expressed at medium overall levels. Specific comments for the authors to consider are listed below:

1) In the introduction, the authors report percentages of the number of noncoding versus protein coding transcripts that in my opinion are inaccurate and/or overly speculative. The authors should consider examining their sources of information and adjust their statements accordingly.

Response: Thank you for this suggestion. We have examined our sources of information and removed speculative estimates.

On page 2, line 2-4, we changed the text to:

“Protein-coding genes make up only a small portion of the human genome while transcribed noncoding genes encompass a much larger fraction of the human genome ^{1,2}.”

2) At its core, this study is examining a mechanism of cis-repression that occurs on a single allele, presumably after Xist has been upregulated on one X chromosome. I was looking for a bit more validation of the experimental system and the decision to study Xist/Tsix at D2 of differentiation. I would recommend that the authors place the data in Figure S2 into the main manuscript (Figure 1).

Response: Thank you for this information. We have incorporated Figure S2 into the main manuscript as Fig. 2A and added Supplementary Fig. 10 showing the fraction of alleles expressing Xist and Tsix.

As a justification for the day 2 time point, we added on page 5, line 4-8, the following text:

“We chose the day 2 time point because both Xist and Tsix show variable expression, which is necessary to analyze their co-expression within the same cell and allele. Time points before day 2 show less Xist expression because Xist is not upregulated yet, making it significantly harder to analyze both Xist and Tsix co-expression. Likewise, time points after day 2 show less expression variation in Tsix because Tsix gets repressed.”

It would also be useful to know what fraction alleles express Xist and Tsix at the timepoint selected, and the relationship between Tsix expression and Xist cloud formation. I am guessing that Tsix expression is essentially never observed on alleles that harbor Xist clouds, but it would be useful to know quantitatively whether or not that is the case.

Response: In Fig. 4, we analyzed the number of transcripts in the Xist cloud and in the Tsix transcription site for each allele by selecting Xist and Tsix transcription sites that are less than 1µm apart (See supplementary Fig. 42). If one assumes that a Xist cloud has the intensity of at least 1 full length Xist RNA molecules, then one can use the joint probability distribution in Fig. 4c and compute the fraction of cell with and without an Xist cloud. For each of these fractions we can now determine the distribution of Tsix expression as shown in Supplementary Fig. 10.

3) *I note that the manuscript did not include a seminal citation in this field, Gayen and Kalantry, PMID: 25981039, which disproved a large body of early work and demonstrated that Tsix functions to repress Xist on the active X after upregulation on the Xi, and does not play a role in choice of Xi. A reference to this paper should feature prominently in the manuscript and the authors should review its conclusions if they were not aware of the work.*

Response: Thank you for this suggestion. We have included this seminal citation on page 4, line 21.

4) *The methods section was lacking many key details and forced the reader to look in previously published papers to understand how the experiments and analyses were carried out. The paper would benefit from a thorough description of all methods used, to the extent that a reader could reproduce the study, even for the methods that have already been published. While I am not certain whether Nature Comm. is one, many journals now require such descriptions. A full description of methods will improve rigor, reproducibility, and interpretability.*

Response: We have updated the method section to include more of the experimental and analytical details of the work so it can be more readily replicated.

On page 14, line 21 we added:

“...), 0.5x N2 Supplement (Gibco 17502-048), 0.5x B27 supplement (Gibco 17504044),...”

On page 15, lines 17-26, we added:

“After cell permeabilization, use BSA coated tips to transfer cells to a low bind tube. After centrifugation and EtOH aspiration, resuspend cells in 100µl wash buffer (PBS with 10% formamide), centrifuge at 300g, aspirate wash buffer completely, and then resuspend cells in 50 µl hybridization buffer (10% dextran (Sigma Aldrich, D8906), 10 mM vandy-ribonucleoside complex (NEB, S1402S), 0.02% RNase-free BSA (Ambion, AM2616), 1mg /ml E.coli tRNA (Sigma, R4251), 2x SSC (Ambion, AM9763), 10% formamid (Ambion, AM9342)) that contains RNA-FISH probes (1:1000 dilution) and antibody if necessary and incubate at 37C overnight. After hybridization resuspend cells in 1ml wash buffer, centrifuge at 300g, aspirate solution, resuspend cells in 1ml wash buffer with DAPI (Sigma, D9564, 150 ng/ml) and secondary antibody if required. Incubate for 30min at 37C. Repeat wash step one more time without DAPI and antibodies. ⁵²”

On page 15, lines 32-33, we added:

“...at 100x with a 500ms exposure time, at 300nm per z-step, from the top to the bottom of the cells ⁵⁰”

On page 16, lines 10-12, we added:

“The DAPI signal of the nucleus was automatically thresholded to maximize the number of non-overlapping signals presenting individual nuclei. The nuclei are used as seeds to segment the cell boundary in 2D automatically.”

5) *The image in Fig 3A and 4A is identical. This may have been intentional, but in case not, the authors should investigate.*

Response: Thank you for noticing this detail. This was intentional to show that we are investigating the same data, but different aspects of the data in these two figures, and we added a mention of this reasoning in the text on page 7, lines 9-12.

“Because transcription sites are identifiable as spots brighter than individual transcripts, we were able to quantify the presence of Xist and Tsix specifically at the transcription sites and their correlation using the data generated in the previous section (Fig. 4a, Supplementary Fig. 4-7, 9, 10, 16, 18).”

6) *The axes on the joint probability heatmaps took me some time to understand. A simple fix would be to label the units clearly – something like “# of RNA molecules detected by allele” would have helped. Also it took me some time to understand the authors meaning of [bin = #]. What I believe the authors intended was that this number specified the “bin size” or “step size” on the axes. Again a minor point, but adding a word or two more to describe the graph would have increased the clarity.*

Response: Thank you very much for this suggestion. We updated the figure caption to clarify that the quantification is allele specific. We also added updated Fig. 3 c,d and 4 c,d to increase clarity.

7) *My lingering concern with the conclusions of the work are that the H3K36 IF data are correlative. The authors are heavily interpreting the inability to detect H3K36 at high levels of Xist to indicate causality, when in reality the absence of K36 at that condition could be due to other reasons. One possibility is that K36 at the 22kb Xist locus is below the limit of detection in their assay, and the reason that the authors can detect K36 at lower levels of Xist transcription is because under those conditions, the Xist gene body is clustering with other K36-rich loci to boost the signal above the limit of detection. High levels of Xist represses Tsix and most other genes on the X chromosome and would be expected to reduce the overall levels of regional K36 signal. Indeed, I struggle to rationalize how any highly transcribed gene, including Xist, would lack K36 over its entire gene body specifically under the condition in which it was highly transcribed. K36 is a modification that correlates positively with transcription and splicing, and at the highest levels of Xist transcription, both of those activities are abundant at the Xist locus. Thus I am concerned that the lack of K36 colocalization with Xist intronic RNA at high levels of Xist transcription is not related to lack of K36 at the gene body and is due to some other reason. One possibility (in addition to the 22kb signal being below the limit of detection) is that at high levels of Xist, its splicing occurs at a distance from its own locus. These are challenging assays to perform, but can the authors look for previously published data that would harbor allele-specific H3K36 ChIP-Seq profiles in cells that express Xist and harbor an inactive X chromosome? A prediction of the authors model would be that on the Xist-expressing allele in cells that have undergone XCI, H3K36 signal would be absent. Conversely, the lack of K36 detection when Xist is lowly expressed seems most likely to be due to K36 IF signal over its own locus being below the limit of detection. And more than detecting K36 over the entire locus, what the authors are intending to do is correlate presence of the modification specifically over the Xist promoter, which Ohhata and colleagues demonstrated is the critical site for repression by Tsix (PMID: 26370508). But detecting K36me3 over a 1kb segment of DNA seems well beyond the limit of what is possible by the microscopy systems being employed in this work.*

Response: We thank the reviewer for the suggestion of looking at allele-specific profiles of H3K36me3 to distinguish between different interpretations of our data. We analyzed a CUT&RUN dataset of H3K36me3 that utilized mutants so one sample would be an Xist-expressing chromosome (inactive X) and the other would not express Xist (active X) (Gjaltema et al. Mo Cell 2022). This analysis supports the prediction that H3K36me3 is reduced at the Xist promoter during differentiation, especially for the Xist-expressing inactive X. To address the possibility that the reduced H3K36me3 localization with Xist was due to inactivation of X-linked genes, we also investigated Pcg1 and Rlim, which are genes that are expected to inactivate early and would have reduced H3K36me3 levels if the alternative interpretation was correct. We did not see any reduction of H3K36me3 on the Xist-expressing inactive X, which does not support this hypothesis.

We added this analysis as Supplementary Fig. S41.

We also thank the reviewer for noting the correlative nature of our analysis. To establish causality, we also collected Xist and H3K36me3 microscopy data with and without a Setd2 inhibitor after 2 days of differentiation and Setd2 inhibition, which would lower the amounts of H3K36me3 in the cell. If our model in the paper is correct, then decreasing H3K36me3 should increase the transcription of Xist. If an alternative model is true, where Xist expression causes reduction of H3K36me3, then Xist levels should be unaffected. Our analysis shows that H3K36me3 was reduced by the Setd2 inhibitor (Supplementary Fig. 43 A,B), that Tsix nascent transcription was

reduced (Supplementary Fig. 43 C,D), whereas Xist nascent transcription did not change (Supplementary Fig. 43 E,F). Instead that the fraction of cell with nascent Xist increases upon drug treatment (Supplementary Fig. 43 G), whereas the fraction of cell with nascent Tsix did not change (Supplementary Fig. 43 H). From these experiments we conclude that lowering H3K36me3 increases the fraction of cells with Xist nascent expression.

We have added Supplementary Fig. 43 with these results.

In the main manuscript we added the following text on page 10, lines 19-29:

“..., a pattern that was previously observed in allele-specific population experiments (Supplementary Fig. 41)⁶⁵. We then compared these results to experiments where Setd2 was inhibited during two days of differentiation. We quantified the number of H3K36me3 spots in the cells, the intensity of Tsix and Xist intronic signals, and the fraction of cells with nascent Xist and Tsix expression (Supplementary Fig. 43). Our findings showed that Setd2 inhibition reduces the number of H3K36me3 spots in cells (Supplementary Fig. 43 a, b), which in turn decreases the intensity of nascent Tsix expression (Supplementary Fig. 43 c, d). However, there was no reduction in nascent Xist expression (Supplementary Fig. 43 e, f). Interestingly, we observed an increase in the fraction of cells with nascent Xist expression upon Setd2 inhibition (Supplementary Fig. 43 g), while no change was detected in the fraction of cells with nascent Tsix expression under the same conditions (Supplementary Fig. 43 h).”

And on page 13, lines 7-12 we added:

“Through Setd2 inhibitor studies (Supplementary Fig. 43), we observed that the intensity of Tsix transcription sites decreased, suggesting that the number of polymerases transcribing Tsix is reduced. However, the number of cells initiating Tsix transcription did not change. In contrast, while the intensity of Xist transcription sites remained unchanged during Setd2 inhibition, the fraction of cells exhibiting nascent Xist expression increased. This indicates that Setd2 regulates the frequency of polymerase initiation responsible for transcribing Xist.”

And on page 15, lines 5-7, we added:

“Setd2 inhibitor studies were conducted using 500 nM of SETD2i (inhibitor EPZ-719, MedChemExpress) in 2i media during two days of differentiation, as previously described. RNA-FISH probes and H3K36me3 antibodies were used as outlined above.”

8. Thus, in the absence of an orthogonal assay, in a static system, in a low resolution IF assay that is unlinked to any DNA sequence, I do not believe that the authors are able to conclude that K36 represses Xist only when Xist is transcribed at medium levels.

Response: We do not argue that K36 represses Xist only when Xist is transcribed at medium levels, but that we see the greatest enrichment of K36me3 at medium levels. This is an important distinction, and we think this is an interesting observation that warrants future investigation in future studies.

9. When Xist its highly expressed, it would be safe to assume that Xist is repressing Tsix using SPEN and Polycomb, and the authors would need to disprove those models before concluding that transcriptional interference is the most relevant mechanism.

Response: We thank the reviewer for this suggestion. TI is only one mechanism and we do not argue that this is the main mechanism. SPEN and Polycomb silencing are not excluded. Understanding how SPEN and Polycomb are recruited by Xist to silence Tsix is beyond the scope of the current project and requires carefully executed research in the future.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

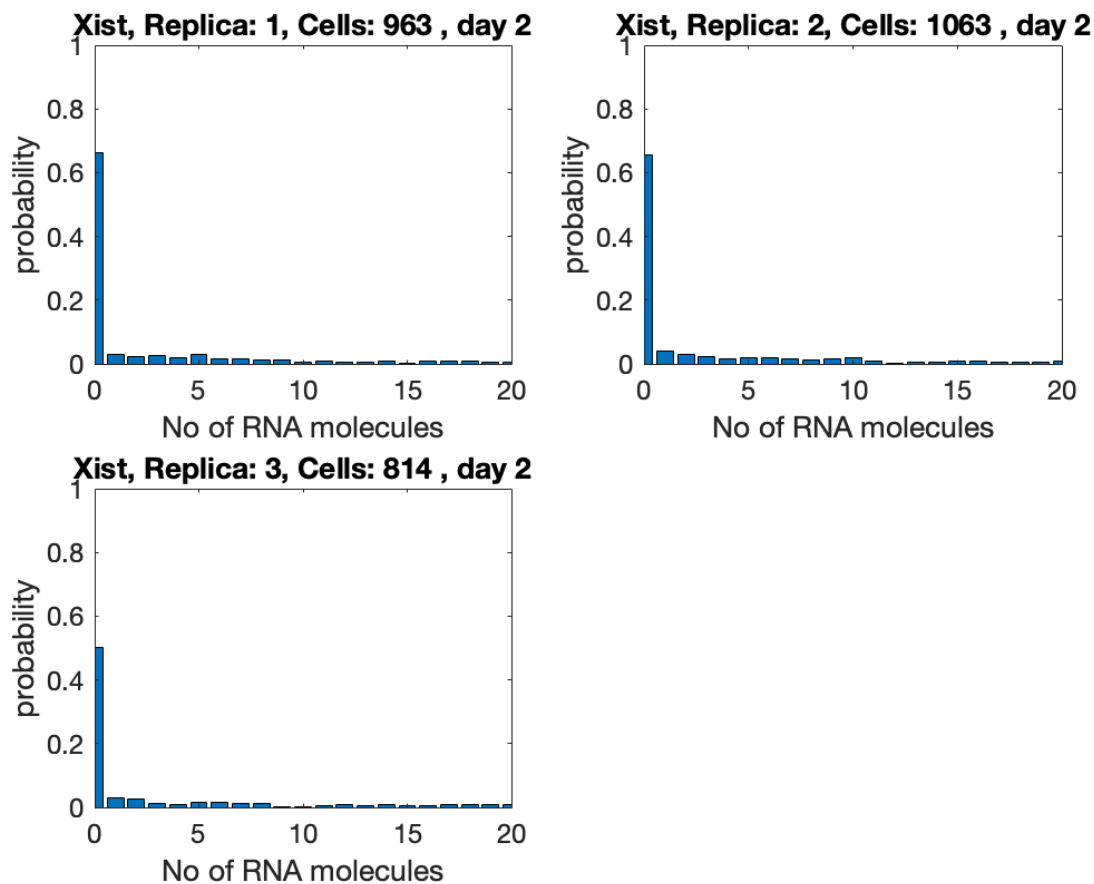
It is much appreciated that the authors have provided additional details on their data set in the form of additional supplemental figures. Unfortunately, this reveals a series of problems in the data set and that the main conclusions are not robustly supported by the experimental data. Therefore I am not convinced that the manuscript with the current data sets is ready for publication.

Response: Thank you for acknowledging the improvements made to the manuscript.

Major comments

1. Efficiency of Xist upregulation appears highly variable between different experiments. The data set shown in Fig. 2a, where >50% cell express Xist at day 2 is apparently not representative. In Fig. 3c+d and Suppl. Fig. 15, 18, 39 cells make very little Xist, often <10%. This suggests that cells lost an X chromosome or the differentiation failed. These experiments should be repeated, since the data quality is poor.

Response: Thank you for bringing this to our attention. We realize there may have been some confusion. To clarify, the data presented in Figures 2a, 3c, 3d, and S15 correspond to exonic Xist and Tsix transcripts, while the data in Supplementary Figures 18 and 39 represent intronic transcripts. For the exonic Xist data, a bin size of 20 Xist exon-labeled transcripts was used to compare the joint probability distributions in Figure 3 with the marginal distributions shown in Supplementary Figure S15. We have also replotted the Xist data from Supplementary Figure S15, using a bin size of 1 Xist transcript, which shows a similar fraction of cells expressing Xist as in Figure 2a.



2. Although most experiments were apparently performed in independent replicates, these were merged for most analyses. This is unacceptable, since it is crucial to show that the observations are reproducible. The analyses in Fig. 5 and Fig. 6 both show some unexpected associations (differences in TI between cells with different Xist levels; different association with H3K36me3 staining). To rule out that these are just random fluctuations, it is crucial to perform the analyses on independent biological replates and not merge all the cells for the analysis. Given the low quality of some replicates (very little Xist expression) this might be difficult. But in this case additional measurement must be made, since otherwise no robust conclusions are possible.

Response: All data presented in the manuscript were analyzed as independent experiments but plotted as means and standard deviations. Independent experiments are shown in Figures 2I & J, 3c & d, 4c & d, and 5g & h. Figures 5i-n present independent experiments as mean values with standard deviations to highlight the variation between replicates. The statistical tests used to compare the Tsix 5' and 3' data incorporate the independent replicate experiments to account for variation between experiments. In Figure 6, the solid line represents the mean, and the shaded area indicates the standard deviation from the biological replicate experiments. The CMH test uses the individual replicate experiments.

3. In the rebuttal letter the authors mention that the spot-detection software will only be published together with a future methods paper. Since the software is a crucial part of this manuscript, I believe that that software must be made available together with the manuscript and not in the future.

Response. We have made the code for determining the spot available on Zenodo and included the link in the manuscript.

Minor comments

4. Section "Transcriptional Interference of Xist and Tsix at high nascent transcription": It should be mentioned that the negative correlation between Xist and Tsix might arise from Xist-mediated silencing of Tsix, since this observation is expected and not surprising (see comments from the first round of review).

Response: Thank you for bringing this to our attention. We believe the negative correlation observed in the joint probability distribution (Figure 5g & h) arises from analyzing the entire dataset without differentiating based on expression levels, which is why we did the analysis based on different expression levels as detailed in Figure 5i–n.

In Figure 5i–n, we compare the joint expression of Xist and Tsix 5', as well as Xist and Tsix 3'. For each subgroup of cells, we observe roughly the same number of cells and transcription sites per cell. Transcription sites are only visible if polymerases have initiated transcription and the transcripts are detected after the first two exons of Tsix (see the schematic diagram in Figure 2b and Figure 5a & d). If Xist were silencing Tsix, we would expect a significant reduction in the fraction of Tsix transcription sites per cell compared to cells where silencing does not occur. However, none of our data supports this expectation. Therefore we conclude that Xist does not silence Tsix.

5. The H3K36me3 data from Gjaltema et al used CUT&TAG not CUT&Run.

Response: Thank you for pointing this out. We have now corrected the oversight.

6. In Suppl. Fig. 41, data for an inactive X at day 0 of differentiation is shown. Usually, both X chromosomes are active in undifferentiated ESCs, how is this possible?

Response: Thank you for pointing this out. We have now updated Supplementary Figure 41 with the genotype information.

Reviewer #1 (Remarks on code availability):

I could not access the code through the link.

Response: We have now made the codes public, and they should be accessible.

Also, in the rebuttal letter the authors mention that the spot-detection software will only be published together with a future methods paper. Since the software is a crucial part of this manuscript, I believe that that software must be made available together with the manuscript and not in the future.

Response: We have now made the codes public, and they should be accessible.

Reviewer #2 (Remarks to the Author):

The revised manuscript shows significant improvement, with additional analyses and experiments addressing previous feedback. However, a few aspects still require clarification.

Response: Thank you for acknowledging the improvements in the manuscript.

1. The authors have demonstrated the specificity of the Xist and Tsix probes by hybridizing them to human male Jurkat cells. However, it would be more appropriate to test these probes in mouse cells lacking Xist expression or with very low Xist expression, considering the substantial divergence between human and mouse genomes.

Response: In Supplementary Figure 3, at day 0, we observe very low Xist expression, as expected. In contrast, in Supplementary Figure 4, at day 2 of differentiation, Xist expression is significantly higher. This data clearly demonstrates the specificity of the probes.

2. The authors included an analysis of H3K36me3 CUT&RUN in mouse mESCs during differentiation, revealing reduced H3K36me3 at Xist promoter regions on the Xist-expressing Xi. However, it remains unclear whether this reduction is specific to the Xist site or a general feature of this chromosome. A metaprofile showing H3K36me3 variations over genes on the Xi during differentiation should be provided. Additionally, it would be helpful to investigate whether other antisense gene pairs exhibit a similar H3K36me3 pattern during differentiation, either on the X chromosome or autosomes.

Response: Thank you for pointing this out. We have now included more genes in the analysis in Supplementary Figure 41 and we have included screenshot from the UCSC Genome Browser comparing CUT&TAG data for H3K36me3, using data from Gjaltema et al., in Supplementary Figure 42-48.

3. Genome browser views (e.g., IGV) should be included to visualize H3K36me3 signals over the listed example genes in the CUT&RUN results. Please indicate the specific genomic sites exhibiting the varied H3K36me3 signals. A brief description of the H3K36me3 CUT&RUN data analysis is needed. Additionally, it would be valuable to conduct statistical analyses to compare the changes and describe the fold change of H3K36me3.

Response: We have now generated browser views for the listed genes showing H3K36me3 data and added them as Supplementary Figure 41-48. Additionally, we included a description of the CUT&TAG analysis in the supplementary methods section. We also performed statistical analysis on the fold changes shown in Supplementary Figure 41 and have added these values to the figure and its caption.

4. The panels labeled "Pgk1" and "Rlim" in Supplementary Figure 41 lack information regarding the promoter or gene body. Please provide this information.

Response: We have now added this information to Supplementary Figure 41.

Reviewer #3 (Remarks to the Author):

The authors have addressed my comments and I would support publication of this manuscript. The additional experiments support the conclusions that separate mechanisms dependent on lncRNA transcription levels can regulate antisense genes. As a note for the future, I found the inclusion of red, scored text in the revised manuscript to be a distraction that made it more difficult for me to read and assess the authors' changes.

Response: We thank the reviewer for supporting our manuscript for publication. We have now adjusted the track changes to make them less distracting.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

I want to thank the authors for their explanations and clarifications. Some of these should also be included in the manuscript:

1. Bin size: It is not clear what the lowest bin in Fig. S15 represents (0-10 molecules?). I would consider to state this in the figure legend.

Response: We have now defined the bin size in the figure legend.

2. Fig. 5i-n and similar: The figure legend should contain a description of what the line and shaded area represent.

Response: We have now defined the lines and shaded areas in the figure legend.

3. Regarding the analysis in Fig. 5i-n, the colocalization mentioned in the text, I would suggest to provide a measurement of uncertainty, e.g. std dev, also in the text.

Response: We have provided this information already in a form of a p-value using the Cochran-Mantel-Haenszel test. Please see yellow highlighted text on page 8, lines 29, 34 and page 9, line 4.

Reviewer #2 (Remarks to the Author):

The manuscript has shown improvement. There are still some issues that need to be addressed.

1. The CUT&Tag data presented in Supplementary Figures 41-42 indicate a reduction in H3K36me3 levels at the Xist promoter region during differentiation in both XXΔXic (Xist is expressed) and XO (Xist is not expressed) mESCs. If H3K36me3 represses Xist, it is unclear why the decrease in H3K36me3 during differentiation in XO cells does not lead to an increase in Xist expression? This discrepancy requires clarification. The inclusion of XO in this context may add confusion.

Response: We have generated a new Supplementary Figures 41 focusing on the XXΔXic genotype, excluding XO data for clarity. For each genomic region, we compare H3K36me3 CUT&TAG RPKM reads with strand-specific RNA RPKM reads to illustrate the correlation between H3K36me3 signals and RNA expression. In the H3K36me3 signal (Supplementary Figures 41a&b), we observe a decrease during differentiation (41a), an increase in Xist RNA expression (41b), and a decrease in Tsix expression (41c&b).

Additionally, it would be beneficial to include RNA-seq tracks and expression levels of both Xist and Tsix in Supplementary Figures 41-42 to demonstrate whether Xist expression negatively correlates with H3K36me3 levels.

Response: We updated the genome tracks in Supplementary Figures 42-50, focusing on the XXdXic genotype and added the RNA-seq data.

2. The statistical analyses depicted in Supplementary Figure 41 should be conducted to compare different stages of differentiation in order to assess whether the alterations in H3K36me3 levels are statistically significant during differentiation. Comparing across different genotypes (XO VS XXΔXic) are confusing and the XO data do not support the model that Xist is repressed by H3K36me3.

Response: We have performed statistical analysis (t-test) on the two replicate experiments of the CUT&TAG and RNA-seq datasets, and the results are presented in Supplementary Table 2.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

I appreciate that the authors made an attempt to clarify their legends. Unfortunately, there is still important information missing. In Fig. S15 it is still not clear what the lowest bin presents. The legend now state that bin size is 20. Then the lowest bin should be 0-19? But this is not in agreement with the plots. That should be clarified. Similarly, in Fig. 5i-n the legend now states that mean and std dev are shown. But it not clear how these are calculated (individual cells, or independent biological replicates? And if so how many?).

Response: We have updated the figure legends in Supplementary Figures 8, 10, 15, 18, 38, and 39 to clarify the definition of the bin sizes. Additionally, we have included the number of replicate experiments and the number of cells in each replicate experiment in Figure 5i–n. All these changes are highlighted in yellow.

Reviewer #2 (Remarks to the Author):

The authors have addressed all of my comments. The quality of the figures has improved. I have no additional questions or suggestions.

Response: We thank the reviewer for their valuable comments, which have helped to improve the manuscript.