

A noncanonical role of roX RNAs in autosomal epigenetic repression

Corresponding Author: Professor Qing Ma

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)

Ma and her team have provided key insight into how the roX non-coding RNAs function on autosomes. The combination of genomic approaches and validation has identified the first mechanism for the roX RNAs in repressing autosomal gene expression.

However, several new analyses will be key to complete the story:

1) The authors identified a specific class of GA-rich motifs on both the X and autosomes. Therefore, it is likely that there is a sequence specific mechanism of identification of these sites. These motifs are bound by the CLAMP protein which binds both the X and autosomes.

Is CLAMP bound at the autosomal roX sites in addition to the X-linked sites?

2) The autosomal derepression is very interesting. Are these genes equally distributed across chromosomes or located in heterochromatic regions? Meller and colleagues had previously shown that genes in heterochromatin or near it were derepressed by the loss of the roX RNAs throughout the genome.

3) Do genes on autosomes regulated by roX have sex-biased expression in wild type larvae? Why do these genes specifically need to be repressed by the roX? The authors can speculate about this.

Reviewer #2

(Remarks to the Author)

In this manuscript, the authors used a multi-omics approach to investigate the potentially non-canonical roles of the roX non-coding RNAs in Drosophila. The roX1/2 RNAs play an essential role in X chromosome dosage compensation in Drosophila. Here, the authors have shown that roX1/2 binds to autosomes and that this binding overlaps with H3K27me3 signals. The roX-bound autosomal genes showed a functional enrichment of developmentally expressed genes. Finally, the authors claimed that the roX non-coding RNA physically interacts with members of the Polycomb Repressive Complex (PRC) using ChIRP-MS (Chromatin Isolation by RNA Purification-Mass Spec).

The binding of roX1/2 to the autosomal genes has been previously reported by several studies, including the one using ChIRP-Seq (Valsecchi et al., NatComm 2018). However, if true, the potential interaction between the roX RNA and the PRC proteins should be of great interest. I have several major concerns with this finding.

Major comments.

1. It is not convincing whether the interaction between roX1/2 and PRC members is the biological truth or an artifact. In Figure 5B, the authors have shown that many proteins involved in the TCA cycle physically interact with the roX RNA. However, many of these genes are located in the mitochondria (e.g., NADH dehydrogenases and cytochrome genes),

whereas roX RNA presumably functions in the nucleus to regulate gene expression. Likewise, many of the translation-related proteins in the figure should be located in the cytosol, which makes me wonder how robust the result is.

It is also unclear whether the interaction between roX and NURF55 or E(bx) is direct or not. The authors did not use DNase or ethidium bromide to eliminate possible detection of proteins that are simply bridged by DNA (= indirect interaction.) The result in Figure 5D is intriguing, but it's less clear whether the interaction occurs in the nucleus or not at the scale given.

2. Without such evidence of direct interaction between roX RNA and PRC members, it is impossible to say whether roX binding and H3K27 trimethylation are occurring in the same cell or not. This is because most of the results are based on maceration of the whole larva, which has highly heterogeneous cell populations. In other words, we cannot distinguish if roX binding is happening in tissue A and H3K27me3 is happening in tissue B.

3. In the same line, if all roX binding sites are marked by H3K27me3 on autosomes, I would expect stronger repression of genes like *caudal* in Figure 3D in the control, and more dramatic de-repression of it upon knockout of roX RNA genes, which was not the case. In the same figure, it is not clear whether Figure 3D is from RNA-Seq or not. Please provide adjusted p-values for the differences.

4. Also in Figure 5, I am concerned that the upregulation of autosomal genes in the roX-KO is the overfitting artifact of the linear modeling of DESeq2. The authors can test this possibility by performing the same analysis after removing all X-linked genes at the count matrix level.

5. ChIRP-Seq of roX RNA, as well as its role in the regulation of developmental genes, has been well described by Asifa Akhtar's lab (Valsecchi et al., NatComm 2018). The authors even used their data. It is quite surprising that the authors never introduced the work or even discussed it. How does the authors' result differ from what Asifa Akhtar's group reported?

One thing I noticed was this; the authors stated, "Autosomal roX peaks exhibited no colocalization with MSL proteins or H4K16ac 133 modification."

This observation is inconsistent with the observation of Valsecchi et al. who reported overlapping peaks of MSL2 and roX2 near the TSS of autosomal genes. Such an inconsistency should be confirmed or argued and then discussed. Also, the authors' statement above is not based on any statistical test.

Minor comments.

1. Figure 5C apparently shows that the result is not normally distributed. In this case, using Student's t-test will inflate the p-value. The authors should use the Mann-Whitney U test, which is likely to make the result insignificant.

2. MOF is a member of the NSL complex in addition to its involvement in the MSL complex and activates not only X-linked genes but also autosomal genes. From this point of view, it is not clear why the authors didn't observe a downregulation of autosomal genes with MOF knockdown in Figure 3B. For the same reason, it is unexpected that MOF binding does not display peak shapes on autosomes in both WT and roX-KO.

3. Line 157, "In males, X -chromosomal roX peaks is known to exhibit pronounced H4K16ac signals (Fig. 1C)." Could you add a reference to this statement? It seems that the H4K16ac signal is simply lower at the TSS because of the low nucleosomal density at that position.

4. In Figure 3B and the corresponding text, it is less appropriate to compare the roX-KO result with the knockdown results of the MSL complex components because (A) the latter are knocked down with varying degrees of target removal and (B) the latter used a homogeneous population, unlike the former.

5. Please rearrange Figure 2. Now the order is A-D-B-C, which is very confusing.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

In a revised manuscript, the authors have addressed most of my concerns. The only weakness I still see is the mass spectrometry result, which captured non-nuclear but either cytoplasmic or mitochondrial proteins along with roX RNAs. The authors provided alternative evidence to support this conclusion, which was very helpful. However, since this observation is less expected by the general reader, a paragraph is needed in the Discussion section to address this. Below are my additional comments to improve the manuscript.

1. As mentioned above, the mass spectrometry result needs additional discussion.

2. The importance of this study does not lie in the binding of roX to autosomes, but in its interaction with PRC proteins, since the former has already been demonstrated by the Aktar group. The authors are advised to emphasize this importance throughout the manuscript. Another novelty is that roX colocalizes with CLAMP at the autosomes. This observation deserves more emphasis.

3. There are several supplementary figures that I found very useful and should be of interest to many readers. These include supplemental figure S2 (the CLAMP figure), S5, S8, and S9. I recommend that these figures be included in the main text.

Reviewer #3

(Remarks to the Author)

This very interesting study that documents a functional co-localization of roX RNA and PRC subunits at autosomal sites. The authors have done a solid job responding to reviewer comments, but some issues need to be clarified and a few gaps in discussing their findings remain.

Response to question 1. Analysis of CLAMP binding to autosomal sites reveals co-enrichment with roX1 and 2, but the authors fail to speculate on the significance of this finding. Enrichment of PRC subunits at autosomal roX sites is also presented. Is a role for CLAMP at PRC sites, or in deposition of repressive marks, implied?

Response to question 2. It is likely that many readers will have the concern brought up in Q2, namely, how does the regulation of autosomal genes by roX described in this study differ from what has previously been reported. Inclusion in the manuscript of a short, accurate paragraph contrasting current and previous studies is necessary to dispel confusion.

Response to question 3. The authors now include a very nice analysis revealing the female expression bias of autosomal genes that recruit roX. Would the authors please indicate how many genes contributed to this study and clarify what the colors in Sup. Fig. 5C represent. The + and – symbols are cryptic.

Additional comments related to manuscript.

Line 76. Relocation of the roX transcripts upon heat shock is noted, but I think the authors should mention that the MSL proteins also travel to autosomal sites.

Line 145. The phrase “. . roX occupancy on autosomes is not associated with dosage compensation.” should be changed to “. . roX occupancy on autosomes does not reflect recruitment of a functional MSL complex.”

Line 200. The statement that roX RNAs show sex-specific expression is not absolutely correct as roX1 is highly expressed in early embryos of both sexes. In light of the findings of this study it would be very interesting to (in the future) investigate a possible role for roX1 in embryonic PRC function.

The authors choose to manipulate roX in the male germ line in an unsuccessful effort to demonstrate a shift in H3K27me3 levels. The choice of this tissue is baffling as the roX RNAs are not expressed, or expressed at extraordinarily low levels, in the germ cells.

Response Fig. 13 needs a more detailed legend. It currently suggests that roX 2 colocalizes with MOF on autosomes, but I suspect that is not what the authors intended.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The authors have addressed all my concerns.

Reviewer #3

(Remarks to the Author)

I am satisfied with the current version of this manuscript.

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

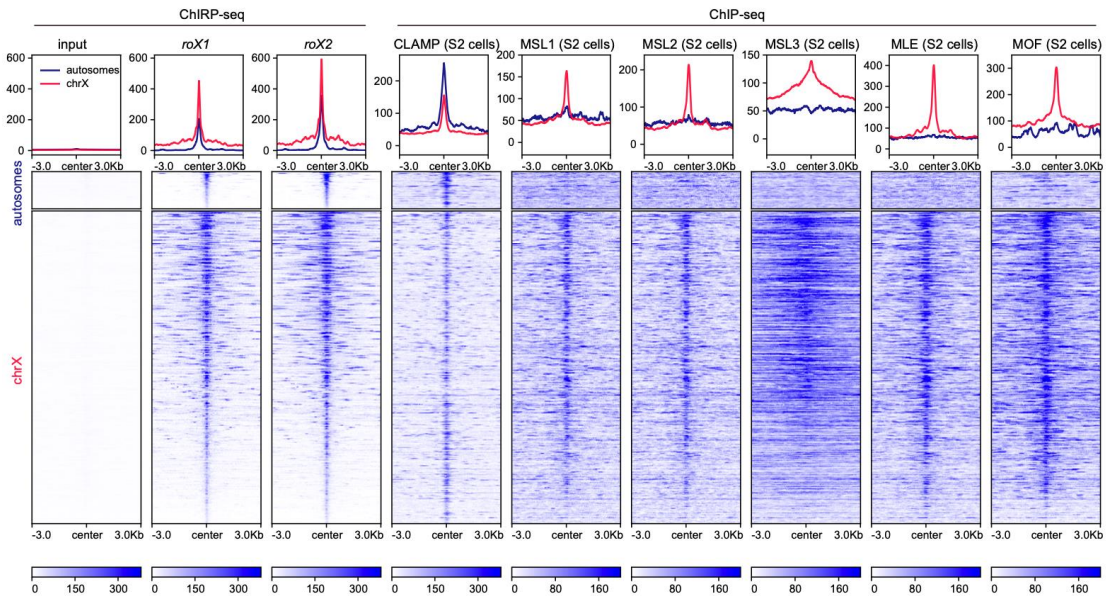
Ma and her team have provided key insight into how the roX non-coding RNAs function on autosomes. The combination of genomic approaches and validation has identified the first mechanism for the roX RNAs in repressing autosomal gene expression.

However, several new analyses will be key to complete the story:

Response: Thank you for acknowledging the significance of our work. We have taken your feedback into careful consideration and have already integrated several new experiments and analyses to enrich the completeness of our study.

1) The authors identified a specific class of GA-rich motifs on both the X and autosomes. Therefore, it is likely that there is a sequence specific mechanism of identification of these sites. These motifs are bound by the CLAMP protein which binds both the X and autosomes. Is CLAMP bound at the autosomal roX sites in addition to the X-linked sites?

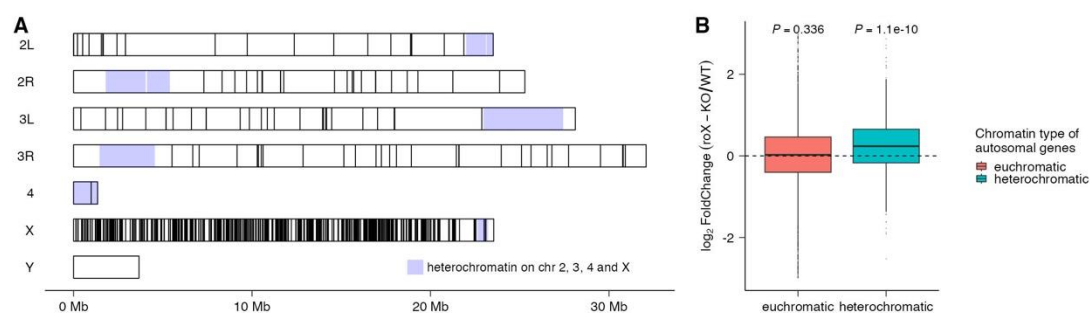
Response: Thank you for your helpful comment. Following your suggestion, we conducted additional analysis on the binding intensity of the CLAMP protein. Our findings indeed demonstrate robust CLAMP binding signals at both X-linked and autosomal roX binding sites, aligning with the presence of the enriched GA-rich DNA motif. This observation suggests that the mechanism of roX binding on autosomes may also involve the interaction with the CLAMP protein, akin to its binding on the X chromosome. Although it has been reported that CLAMP can directly interact with MSL2 and MLE *in vitro*¹⁻⁵ and recruit MSL proteins to the X chromosome⁶, we did not observe colocalization of MSL proteins on the autosomal roX-bound regions. We have included this new data analysis as Response Figure 1 and incorporated it into the revised manuscript as Supplementary Figure 2A (decribed on Page 6, Lines 229-236). Thank you for bringing attention to this aspect, which strengthens the comprehensiveness of our study.



Response Figure 1, added as Supplementary Figure 2A. CLAMP shows enrichment on *roX* binding sites. Enrichment signals of CLAMP and MSL proteins from S2 cells were plotted on *roX* peaks.

2) The autosomal derepression is very interesting. Are these genes equally distributed across chromosomes or located in heterochromatic regions? Meller and colleagues had previously shown that genes in heterochromatin or near it were derepressed by the loss of the *roX* RNAs throughout the genome.

Response: We appreciate your interest in our findings. To address the query regarding the distribution of *roX*-bound genes across the chromosomes and their association with heterochromatic regions, we compared the localization of *roX* peaks with previously defined heterochromatin regions in the paper you mentioned⁷. Our examination revealed that *roX* peaks are not equally distributed across the chromosomes and are predominantly localized outside of heterochromatin regions (Response Figure 2A). In our RNA-seq analysis, most genes located in heterochromatin regions show upregulated expression (Response Figure 2B). Given that *roX* RNAs do not directly bind to heterochromatin regions, it is plausible that the observed regulation of genes in these regions is a secondary regulation mediated by *roX* target genes. Thank you for highlighting this aspect for further consideration.

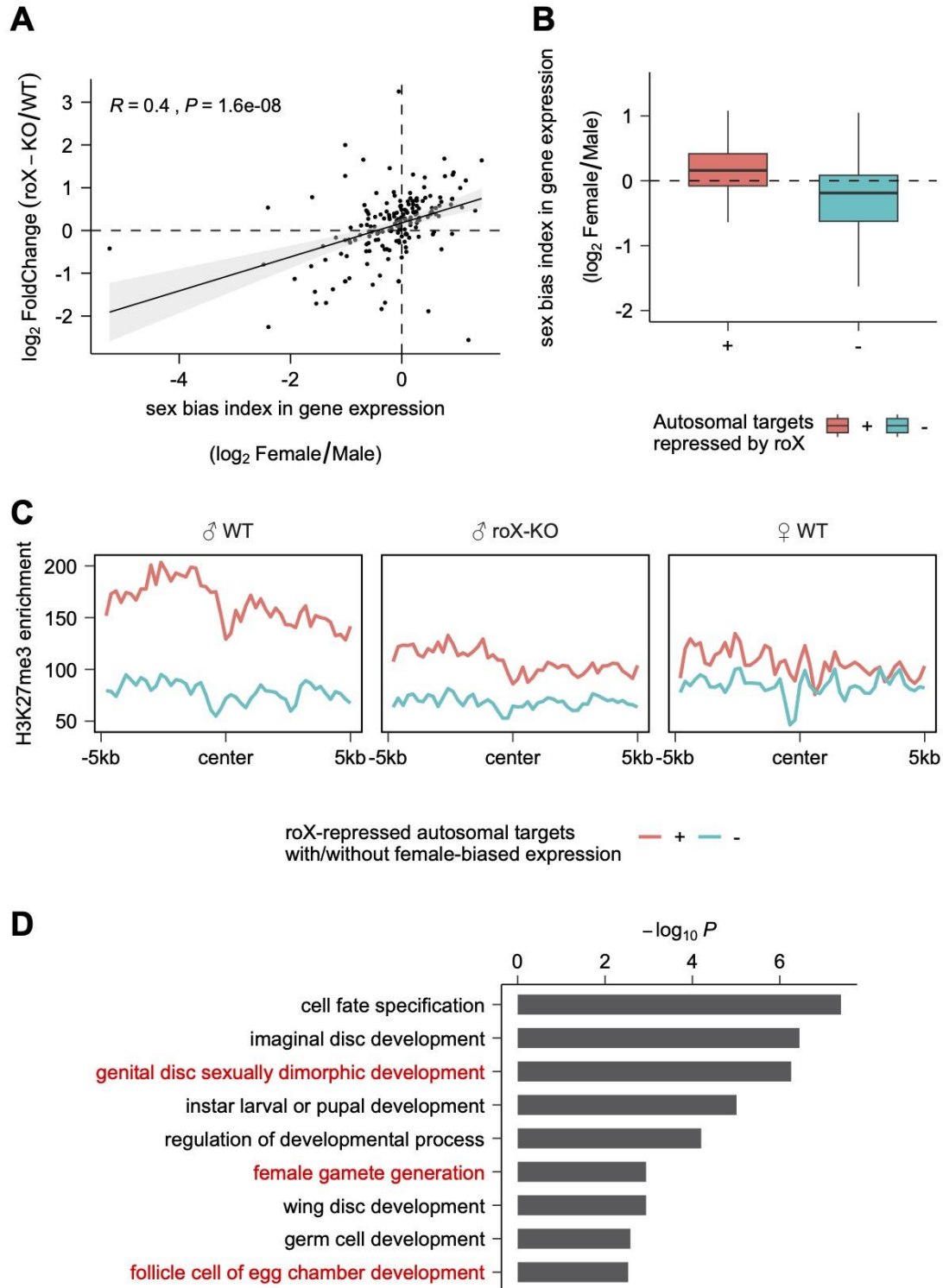


Response Figure 2. Autosomal heterochromatic genes are not directly regulated by *roX*. (A) Autosomal regions that are bound by *roX* RNAs are unevenly distributed across the chromosomes and are located outside of the heterochromatin regions. Genomic localizations of *roX* peaks and heterochromatin regions are shown in black and purple respectively. (B) Genes located in heterochromatin regions on autosomes show upregulated expression in *roX*-KO larvae, as revealed by RNA-seq. Normalization was performed for autosomal genes only.

3) Do genes on autosomes regulated by *roX* have sex-biased expression in wild type larvae? Why do these genes specifically need to be repressed by the *roX*? The authors can speculate about this.

Response: Thank you for your insightful suggestion. We appreciate the opportunity to address this important aspect of our study. In response to your query, we conducted an analysis of the sex bias index (female versus male in gene expression) for *roX*-bound autosomal genes. Our analysis revealed a correlation between their expression changes after *roX* loss and the sex bias index: the greater the female-biased index of the genes, the more they are upregulated in *roX*-KO (Pearson's correlation coefficient $R = 0.4$, $P = 1.6 \times 10^{-8}$, Response Figure 3A). Specifically, we found that *roX*-repressed genes predominantly exhibited female-biased expression in wild-type larvae (Response Figure 3B). Additionally, these *roX*-repressed, female-biased autosomal genes were associated with *roX*-mediated H3K27me3 deposition in males (Response Figure 3C) and were enriched in Gene Ontology terms related to female development (Response Figure 3D).

Based on these findings, we speculate that *roX* may repress autosomal gene expression to prevent inappropriate female differentiation and development in male organs. This suggests that *roX*-mediated repression of autosomal genes plays a critical role in ensuring proper sexual development in male *Drosophila*. In a broader context, this mechanism of sex-specific lncRNA regulation is not unique to *Drosophila*. For example, in mammals, the female-specific expression of *Xist* RNA has been implicated in female-biased autoimmune diseases, through the formation of *Xist* ribonucleoproteins⁸ or XCI escape⁹. Therefore, understanding the role of sex-specific lncRNAs like *roX* and *Xist* provides valuable insights into their crucial functions in sexual development and disease. We have added this new data to the revised manuscript in Supplementary Figure 5 and its description to Pages 5-6, Lines 199-211, and discussed it on Page 9, Lines 365-375.



Response Figure 3, added as Supplementary Figure 5. *roX* RNAs directly repress autosomal genes involved in female development. (A) Scatter plot showing expression changes of *roX*-bound autosomal genes after *roX* loss and sex ratio in their expression (female versus male). (B) A majority of autosomal genes directly repressed by *roX* show female-biased expression. (C) *roX* directly repressed autosomal female-biased genes are associated with higher H3K27me3 levels in males on *roX*-binding sites. These H2K27me3 levels declined after *roX* loss. (D) Enriched GO terms of *roX* directly repressed, female-biased autosomal genes as shown in (C). Terms related to female development are highlighted in red.

Reviewer #2 (Remarks to the Author):

In this manuscript, the authors used a multi-omics approach to investigate the potentially non-canonical roles of the roX non-coding RNAs in *Drosophila*. The roX1/2 RNAs play an essential role in X chromosome dosage compensation in *Drosophila*. Here, the authors have shown that roX1/2 binds to autosomes and that this binding overlaps with H3K27me3 signals. The roX-bound autosomal genes showed a functional enrichment of developmentally expressed genes. Finally, the authors claimed that the roX non-coding RNA physically interacts with members of the Polycomb Repressive Complex (PRC) using ChIRP-MS (Chromatin Isolation by RNA Purification-Mass Spec).

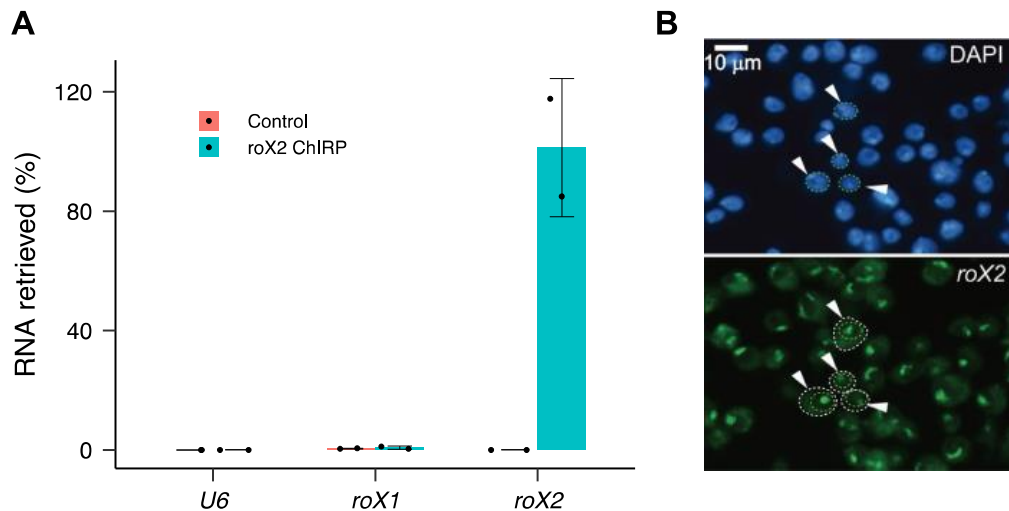
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Major comments.

1. It is not convincing whether the interaction between roX1/2 and PRC members is the biological truth or an artifact. In Figure 5B, the authors have shown that many proteins involved in the TCA cycle physically interact with the roX RNA. However, many of these genes are located in the mitochondria (e.g., NADH dehydrogenases and cytochrome genes), whereas roX RNA presumably functions in the nucleus to regulate gene expression. Likewise, many of the translation-related proteins in the Supplementary Figure should be located in the cytosol, which makes me wonder how robust the result is.

Response: We appreciate your insightful questions regarding the robustness of our ChIRP-MS data. We successfully enriched MSL dosage compensation components as positive controls, which is consistent with the expected function of dosage compensation for roX and MSL proteins. To ensure the reliability of our findings, we included strict negative controls with RNase A treatment to exclude non-specific protein retrieval. And RT-qPCR confirmed that in roX2 ChIRP-MS, almost all roX2 RNA, but not U6 or roX1 RNAs, can be specifically retrieved (Response Figure 4). These ensured the reliability of our findings.

Regarding the detection of cytoplasmic proteins in our ChIRP-MS data, it is not uncommon to enrich some cytoplasmic proteins in nucleus-localized lncRNA ChIRP studies, as demonstrated by *Xist/XIST* ChIRP-MS data enriching translation-related or mitochondria-localized proteins^{10,11}. Moreover, data from the Human Protein Atlas¹² indicate that many cytoplasm-localized proteins can also exhibit localization in other organelles, including the nucleus. We also re-evaluated the localization of roX RNAs using published roX2 FISH results in S2 cells¹³. The results revealed distinct nuclear speckles consistent with roX's predominant localization on the X chromosome. Intriguingly, some signals were observed outside the nucleus, suggesting potential cytoplasmic localization of roXs, which was also confirmed by a subsequent biochemical fractionation study¹⁴. While this raises interesting questions about the cytoplasmic functions of roX1/2, we acknowledge that our study does not delve into this aspect.



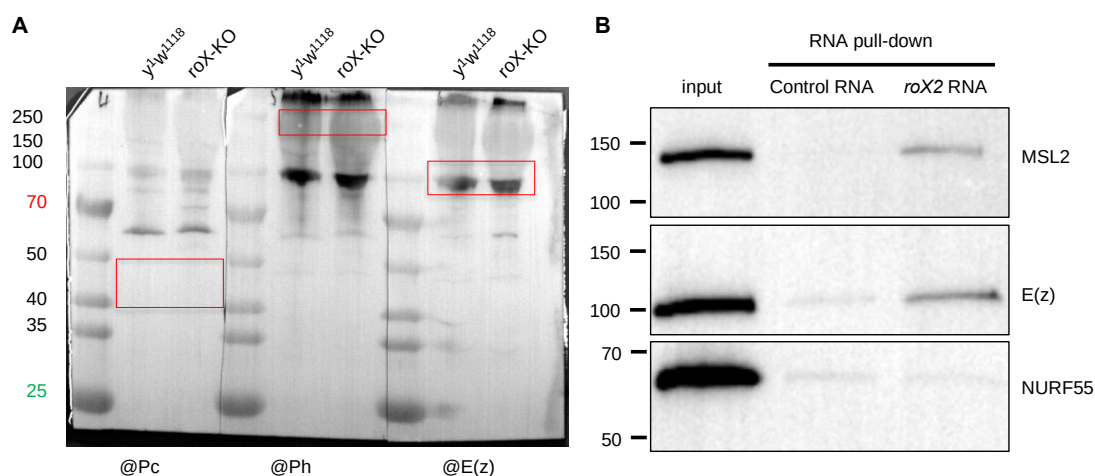
Response Figure 4. *roX2* ChIRP specifically enriched *roX2* transcript. **(A)** Relative RNA abundance of *U6*, *roX1* and *roX2* RNAs in *roX2* ChIRP products. Mean $\pm$ SD values are shown. **(B)** Localization of *roX2* RNAs outside the nucleus. This panel is adapted from Kim et al., PLoS Genet (2018) ¹³

It is also unclear whether the interaction between roX and NURF55 or E(bx) is direct or not. The authors did not use DNase or ethidium bromide to eliminate possible detection of proteins that are simply bridged by DNA (= indirect interaction.) The result in Figure 5D is intriguing, but it's less clear whether the interaction occurs in the nucleus or not at the scale given.

Response: Thank you for your insightful comments. Although we observe a prominent colocalization of *roX* autosomal peaks and PRC2 components (Figure 4C, D), and our ChIRP-MS and TriFC indicated interactions, we acknowledge the reviewer's concern that we could not determine whether the interaction between *roX* and PRC2 subunits is direct or mediated by other proteins and DNA. To address this, we intended to perform RNA immunoprecipitation, CLIP, or RNA antisense purification (RAP), with DNase treatment. Unfortunately, we could not obtain antibodies that specifically recognize PRC proteins in *Drosophila* (Response Figure 5A), making it challenging to perform RNA immunoprecipitation, CLIP or RAP for PRC proteins. As an alternative, we investigated the direct interaction between *roX* and PRC components *in vitro*. We transcribed *roX* RNA *in vitro* and expressed His-tagged PRC2 subunits in *E. coli*, followed by RNA biotinylation and pull-down assays. Consistent with previous findings, we observed a direct interaction between the *roX* and MSL2. For NURF55, we detected similar enrichment levels in both the Poly(A)₂₅ control RNA and *roX* pull-down, suggesting that while NURF55 can interact with *roX*, it also binds nonspecifically to other RNAs. For E(z), we detected greater enrichment in the *roX* pull-down experiment compared to the control RNA (Response Figure 5B), indicating that E(z) might interact directly with *roX* but also has nonspecific RNA-binding properties, similar to mammalian EZH2 and SUZ12 ¹⁵⁻¹⁸. Regarding Figure 5D, *roX1* RNA shows interaction with NURF55 mainly in para-nucleus regions as a condensed speckle, while ESC shows interaction both within and outside nucleus. These observations may reflect distinct regulatory mechanisms for future study. We have added a zoomed-in image in Figure 5D to better illustrate the localization of *roX*-PRC2 interactions.

In summary, we confirmed *roX*-PRC2 interaction by colocalization, ChIRP-MS, TriFC and *in vitro* RNA pull-down. We also demonstrated the *roX*-mediated regulation of H3K27me3 and gene repression.

Although there are still many unsolved questions and debates about PRC2 specificity and regulatory mechanisms in RNA-mediated gene expression, we are committed to further investigating these areas. We have incorporated this new data into our revised manuscript (Supplementary Figure 8) on Pages 7-8, Lines 286-293 and discussed these findings on Page 10, Lines 387-392.

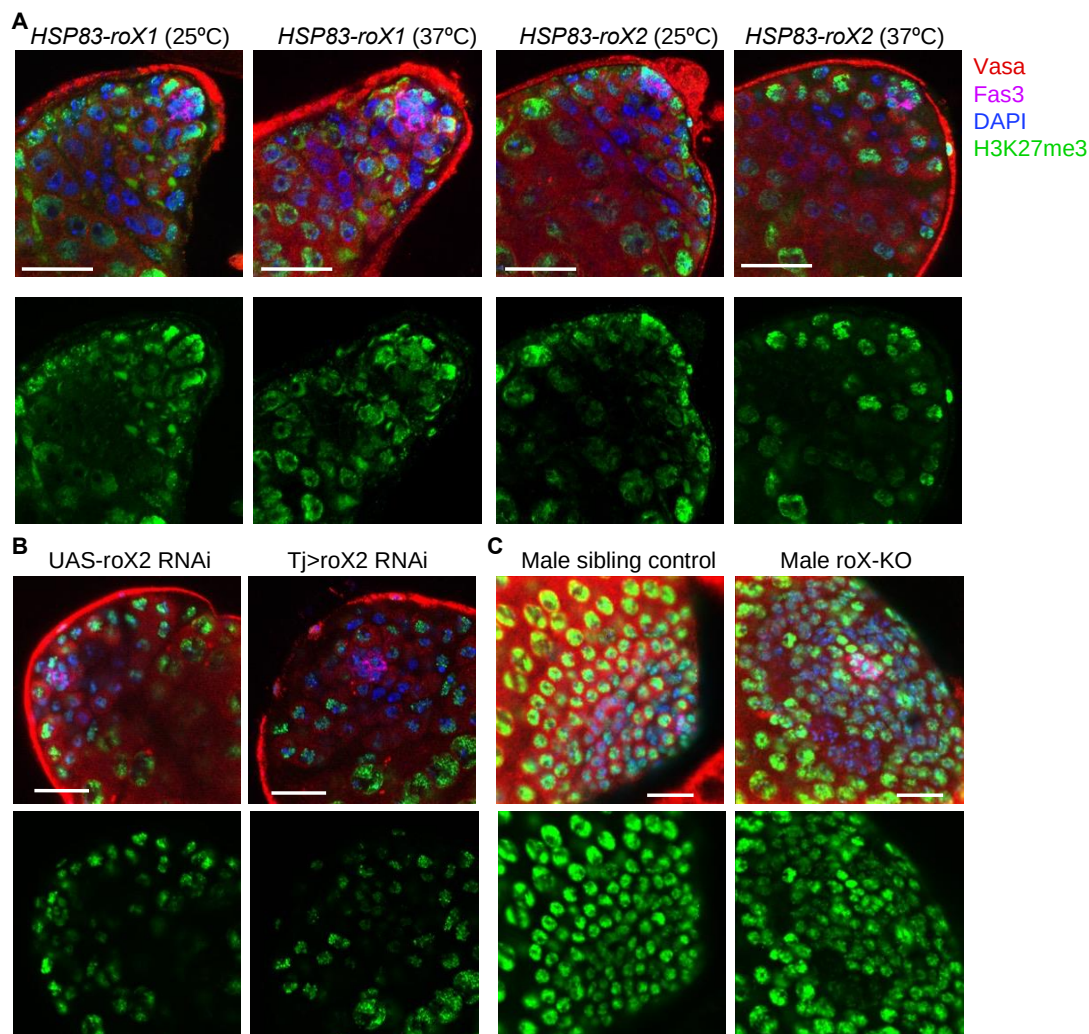


Response Figure 5. *roX2* interacts with some PRC2 proteins *in vitro*. (A) PRC antibodies failed to detect specific protein bands using protein extracts from *Drosophila* larvae. Larval protein extracts from *y*^{1w1118} and *roX*-KO populations were resolved by western blot using anti-Pc, anti-Ph and anti-E(z) sera respectively. (B) Detection of PRC2 proteins in *roX2* RNA pull-down products. *In vitro* transcribed and biotinylated *roX2* RNA was incubated with purified recombinant His-tagged protein from *E. coli*. After pull-down with streptavidin beads, the elutants were resolved by western blot using an anti-His antibody. MSL2, which can interact with *roX2*, serves as a positive control. Poly(A)₂₅ RNA is used as a control. This panel has been added as Supplementary Figure 8.

2. Without such evidence of direct interaction between *roX* RNA and PRC members, it is impossible to say whether *roX* binding and H3K27 trimethylation are occurring in the same cell or not. This is because most of the results are based on maceration of the whole larva, which has highly heterogeneous cell populations. In other words, we cannot distinguish if *roX* binding is happening in tissue A and H3K27me3 is happening in tissue B.

Response: Thank you for your insightful comment. The evidence from both the PIRCh-seq and RT&Tag assays, which demonstrated the enrichment of *roX* RNAs in H3K27me3 immunoprecipitants (Figure 2C, D), suggests that *roX* binding and H3K27me3 occur in the same cell. Additionally, our ChIP-seq data demonstrate that the intensity of H3K27me3 on autosomes correlates with proximity to *roX* binding sites, and the extent of H3K27me3 loss upon *roX* removal also correlates with proximity to *roX* binding sites (see Figure 2F, G), providing additional support for the involvement of *roX* RNAs in H3K27me3 regulation. In *roX*-KO larvae, where all cells lack *roX* genes, we observed a loss of H3K27me3 on autosomal *roX*-bound regions (Figure 2B), further indicating a causal role of *roX* RNAs in H3K27me3 regulation. While multimodal profiling of H3K27me3 and *roX* RNA occupancy by single-cell ChIP-seq of H3K27me3 and single-cell *roX* ChIRP-seq in the same single cells would be ideal, such methods are currently unfeasible. We also attempted immunostaining of H3K27me3 in *Drosophila* testes with cell type-specific *roX* manipulations. However, since *roX* RNAs regulate only a very small subset of H3K27me3 peaks,

discerning subtle H3K27me3 variations in individual cells from immunostaining is difficult, as immunostaining reflects global H3K27me3 levels in a single cell (Response Figure 6).



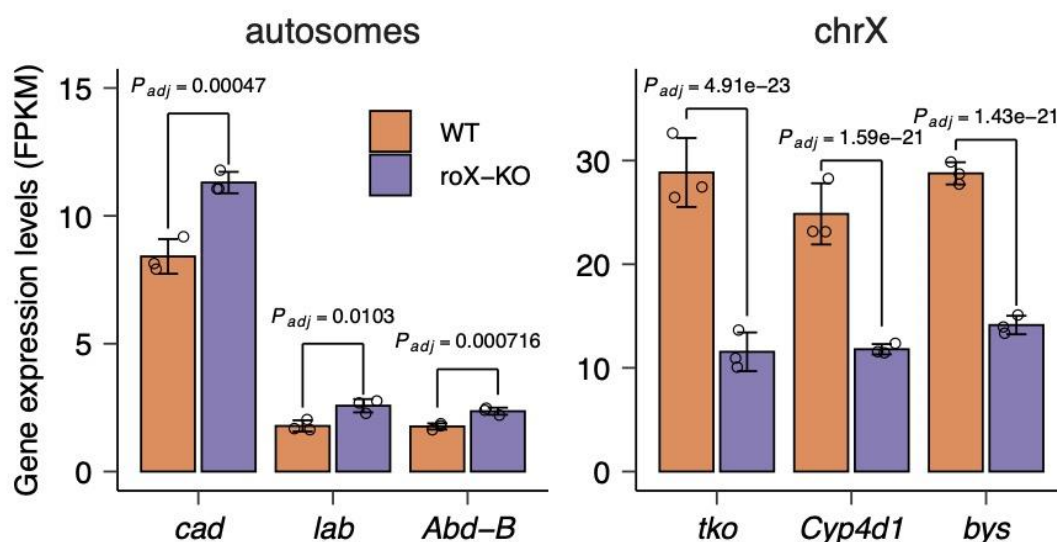
Response Figure 6. H3K27me3 immunostaining signals do not show obvious variations with changes in *roX* expression. Immunostaining of the apex of male testes from *roX* overexpression and knockout (or knockdown) lines was performed. Vasa (red), Fas3 (magenta), and DAPI (blue) channels indicate germline cells, hub cells and nuclei respectively. H3K27me3 signals are shown in green. (A) Immunostaining of adult testes from heat-induced *roX* overexpression lines (*HSP83-roX1* and *HSP83-roX2*). *Drosophila* flies were normally grown at 25°C, and heat shock was performed at 37°C to induce *roX* overexpression. (B) Immunostaining of adult testes from cyst stem cell (CySC)-specific knockdown of *roX2*. UAS-*roX2* RNAi is used as control. (C) Immunostaining of larval testes from *roX1 roX2* double knockout (*roX-KO*) and their male sibling control (FM7i-GFP/Y).

3. In the same line, if all *roX* binding sites are marked by H3K27me3 on autosomes, I would expect stronger repression of genes like *caudal* in Figure 3D in the control, and more dramatic de-repression of it upon knockout of *roX* RNA genes, which was not the case. In the same figure, it is not clear whether Figure 3D is from RNA-Seq or not. Please provide adjusted p-values for the differences.

Response: We appreciated for your insightful observation regarding the potential correlation between *roX*-binding, H3K27me3 and gene regulation. In *roX-KO* larvae, autosomal genes like *caudal* (*cad*) show

only slight but significantly upregulation with decreased H3K27me3, while some other genes do not exhibit significant activation (Response Figure 7 and Figure 3D). While there isn't a pronounced activation of all genes, it is common and reasonable, as many previous examples have shown that H3K27me3 variation does not immediately lead to expression changes¹⁹⁻²¹. For example, in mouse embryonic stem cells (ESCs), the loss of PRC2 and H3K27me3 did not lead to immediate gene upregulation in undifferentiated ESCs, as the differentiation signal and corresponding transcription factors are not yet available^{19,20}. Similarly, elevated H3K27me3 levels in ESCs with *Mcm2* mutation are also not coupled with gene repression²¹. Therefore, it's reasonable to infer that loss of H3K27me3 might cause no or subtle activation for certain genes.

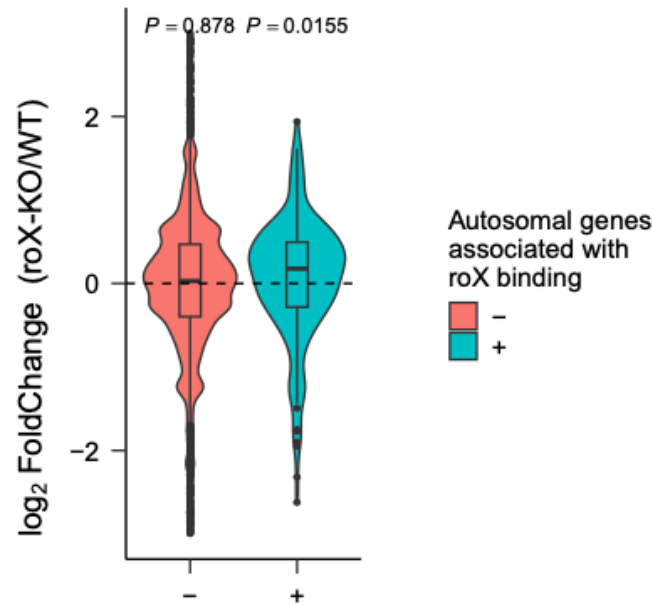
Additionally, we confirm that Figure 3D is based on RNA-seq data, and we have now provided the adjusted p-values for the differences in the revised manuscript.



Response Figure 7, revised in Figure 3D. Gene expression levels in roX-KO and wild type larvae revealed by RNA-seq. FPKM values from RNA-seq analyses for each gene are plotted ($n = 3$) and the adjusted P values using Benjamini and Hochberg method are indicated.

4. Also in Figure 5, I am concerned that the upregulation of autosomal genes in the roX-KO is the overfitting artifact of the linear modeling of DESeq2. The authors can test this possibility by performing the same analysis after removing all X-linked genes at the count matrix level.

Response: We appreciate the reviewer's attention to this matter. To address the concerns raised, we conducted additional analysis as suggested by removing all X-linked genes at the count matrix level and focusing solely on autosomal genes. The results revealed that only roX-bound genes exhibited a significant upregulation in the roX-KO condition (Response Figure 8), supporting that roX RNAs play a repressive role in autosomal gene expression. We have added this new analysis to the revised manuscript in Supplementary Figure 5F and its description to Page 6, Lines 216-221.



Response Figure 8, added as Supplementary Figure 5F. Upregulation of roX-bound autosomal genes in roX-KO male larvae (normalization for autosomal genes only).

5. ChIRP-Seq of roX RNA, as well as its role in the regulation of developmental genes, has been well described by Asifa Akhtar's lab (Valsecchi et al., NatComm 2018). The authors even used their data. It is quite surprising that the authors never introduced the work or even discussed it. How does the authors' result differ from what Asifa Akhtar's group reported?

One thing I noticed was this; the authors stated, "Autosomal roX peaks exhibited no colocalization with MSL proteins or H4K16ac 133 modification."

This observation is inconsistent with the observation of Valsecchi et al. who reported overlapping peaks of MSL2 and roX2 near the TSS of autosomal genes. Such an inconsistency should be confirmed or argued and then discussed. Also, the authors' statement above is not based on any statistical test.

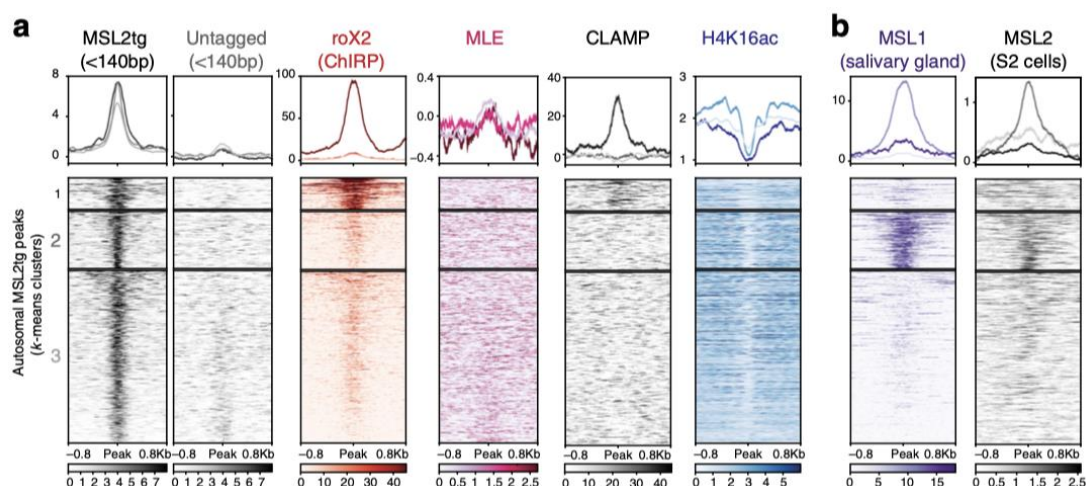
Response: Thank you for bringing up these important points. Regarding the research by Valsecchi et al. (2018)²², our results do not contradict their findings; in fact, our data are consistent. While they focused on autosomal MSL2 bindings, we concentrated on autosomal roX bindings. Unlike the situation on the X chromosome, where they exhibit high colocalization, autosomal MSL2 and roX bindings are almost mutually exclusive. In their study, Valsecchi et al. identified MSL2 targets using ChIP-seq with FLAG-tagged MSL2 in male larvae (Response Figure 9a below, which is original Figure 2a in Valsecchi et al., 2018). They observed autosomal binding of MSL2, with the majority of autosomal MSL2-FLAG binding sites (Cluster 2 and 3) showing no significant roX co-binding. Only a very small subset of sites (Cluster 1) exhibiting co-binding with roX (Response Figure 9a). However in the same paper, Valsecchi et al. demonstrated that the Cluster 1 peaks identified by MSL2-FLAG are not identified by ChIP-seq of endogenous MSL2 in S2 cells and they are not bound by MSL1 in salivary glands (Response Figure 9b,

which is original Figure 2b in Valsecchi et al., 2018). These suggest minimal, if any, colocalization of MSL2 and *roX* on autosomes, unlike the situation on the X chromosome.

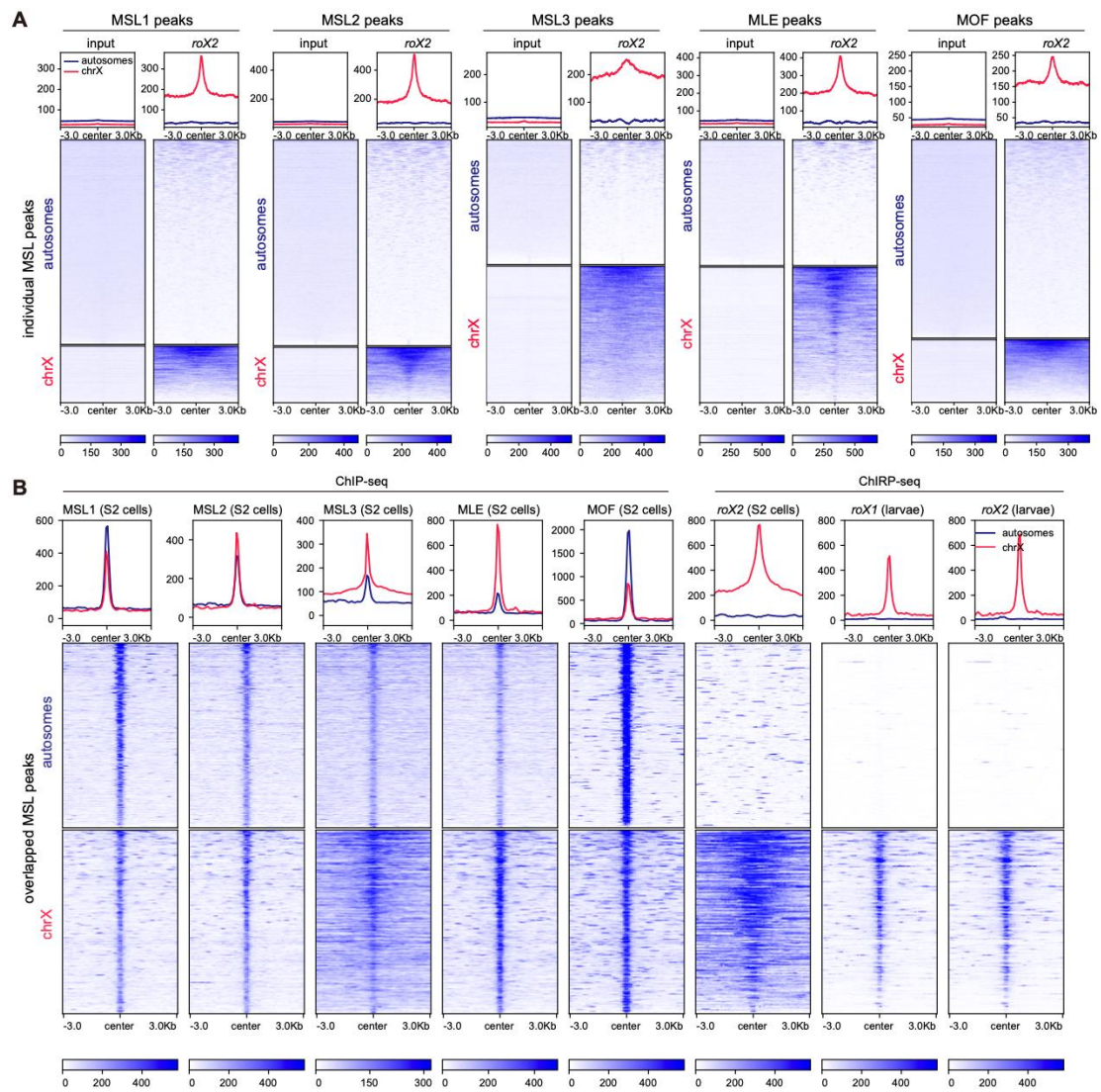
We utilized MSL2 ChIP-seq data from wild type and *roX*-KO male larvae²³, and from S2 cells²⁴, which showed minimal MSL2 binding on autosomal *roX* binding regions (Figure 1C in our manuscript, Response Figure 1 (added as Supplementary Figure 2A)). Specifically, less than 10% of autosomal *roX* peaks overlapped with MSL2 binding sites in S2 cells, and no overlap was observed in larvae MSL2 ChIP-seq (Response Figure 11, added as Supplementary Figure 3C, D). This is in stark contrast to the situation found on the X chromosome ($P = 2.39 \times 10^{-13}$ for S2 cells and $P = 1.86 \times 10^{-8}$ for larvae, Fisher's exact test, Response Figure 11, added as Supplementary Figure 3, C and D). Conversely, on autosomal MSL peaks, *roX* RNAs showed minimal enrichment (Response Figure 10, added as Supplementary Figure 2, B and C). Both Valsecchi et al.'s and our findings consistently suggest that the colocalization of *roX* and MSL2 on autosomes is limited, if it occurs at all.

Regarding H4K16ac modifications, Valsecchi et al. showed that autosomal MSL2 binding regions actually show very modest or no H4K16ac levels (Response Figure 9a and Figure 1e in Valsecchi et al., 2018). Similarly in our study, only 8% of the autosomal *roX* peaks overlapped with H4K16ac, in contrast to 89% on the X chromosome ($P = 3.24 \times 10^{-65}$, Fisher's exact test, Response Figure 11, added as Supplementary Figure 3D). Thus, there is no contradiction between our observations and those of Valsecchi et al.

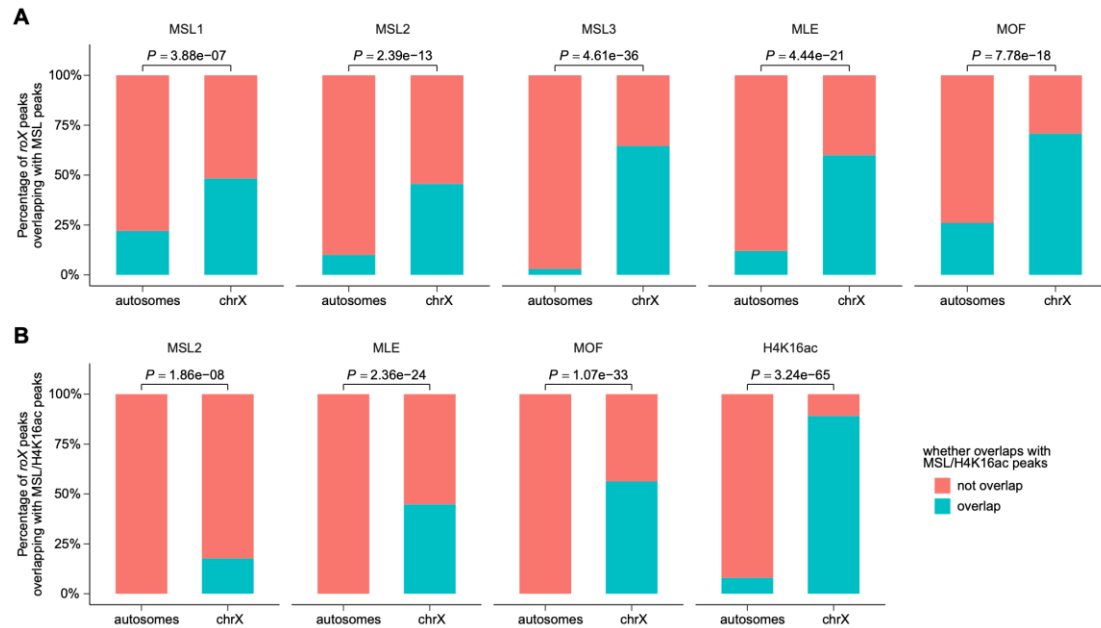
We appreciate the reviewer for highlighting the need for statistical tests. We have revised our manuscript and figures to include above quantification and statistical tests to support our observations (Response Figure 11, added as Supplementary Figure 3). Additionally, we have added a comparison of our data to Valsecchi et al., 2018, in the Discussion section (Page 9, Lines 347-357). We hope these explanations address your concerns and clarify any discrepancies.



Response Figure 9, original figures from Figure 2a and b in Valsecchi et al. (2018)²². Limited colocalization between *roX* and MSL on autosomes. (a) Binding profiles of *roX2* RNA, MSL proteins, CLAMP protein and H4K16ac near the identified autosomal MSL2tg binding sites. Most of the identified MSL-FLAG binding sites (Cluster 2 and 3) exhibit little if any enrichment of *roX2* signals, with only Cluster 1 showing strong *roX2* enrichment. (b) The Cluster 1 targets are not bound by MSL1 in salivary glands and MSL2 in S2 cells.



Response Figure 10, added as Supplementary Figure 2B. Colocalization of *roX* RNAs and MSL proteins on the X chromosome but not the autosomes in S2 cells. *roX2* RNAs show enrichment only on the X-chromosomal MSL peaks. *roX* ChIP-seq signals on individual (**A**) or overlapped (**B**) MSL peaks are shown.

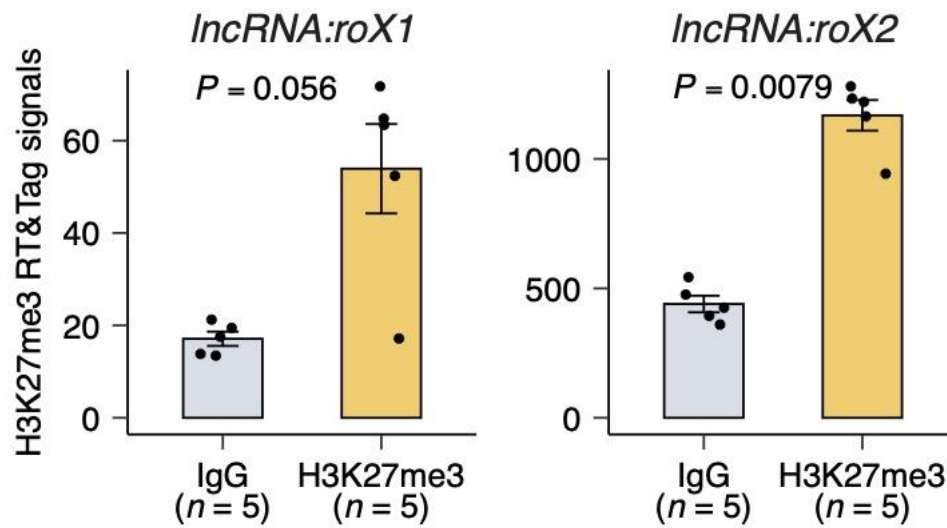


Response Figure 11, added as Supplementary Figure 3. MSL proteins and H4K16ac are not enriched on autosomal *roX* binding sites. Stackplots showing the percentage of autosomal *roX* peaks that overlap with MSL binding sites and in S2 cells (A) and male larvae (B). Significance levels are determined using the Fisher's exact test. NA: not applicable.

Minor comments.

1. Figure 5C apparently shows that the result is not normally distributed. In this case, using Student's t-test will inflate the p-value. The authors should use the Mann-Whitney U test, which is likely to make the result insignificant.

Response: We appreciate the reviewer's keen observation and helpful suggestions. It seems there may be a misunderstanding, as Figure 5C is a schematic representation, not reflecting a statistical distribution. We assume the reviewer is referring to Figure 2C instead. In Figure 2C, we initially employed Student's t-test to assess the enrichment of *roX* RNAs in H3K27me3 RT&Tag. Upon your recommendation, we have now utilized the Mann-Whitney U test (Wilcoxon rank sum test), which appropriately accounts for the non-normal distribution of the data. As a result, we found that one group (*roX1*) is indeed insignificant ($P = 0.056$), while *roX2* still exhibits significant enrichment in H3K27me3 RT&Tag data (Mean $\pm$ SD values are shown, $P = 0.0079$, Response Figure 12). This aligns with our conclusion derived from PIRCh-seq data, where *roX2* demonstrated a higher enrichment of H3K27me3 (Figure 2B, now rearranged as Figure 2C). We have updated Figure 2C (now rearranged as Figure 2D in revised manuscript) to reflect these changes accordingly.

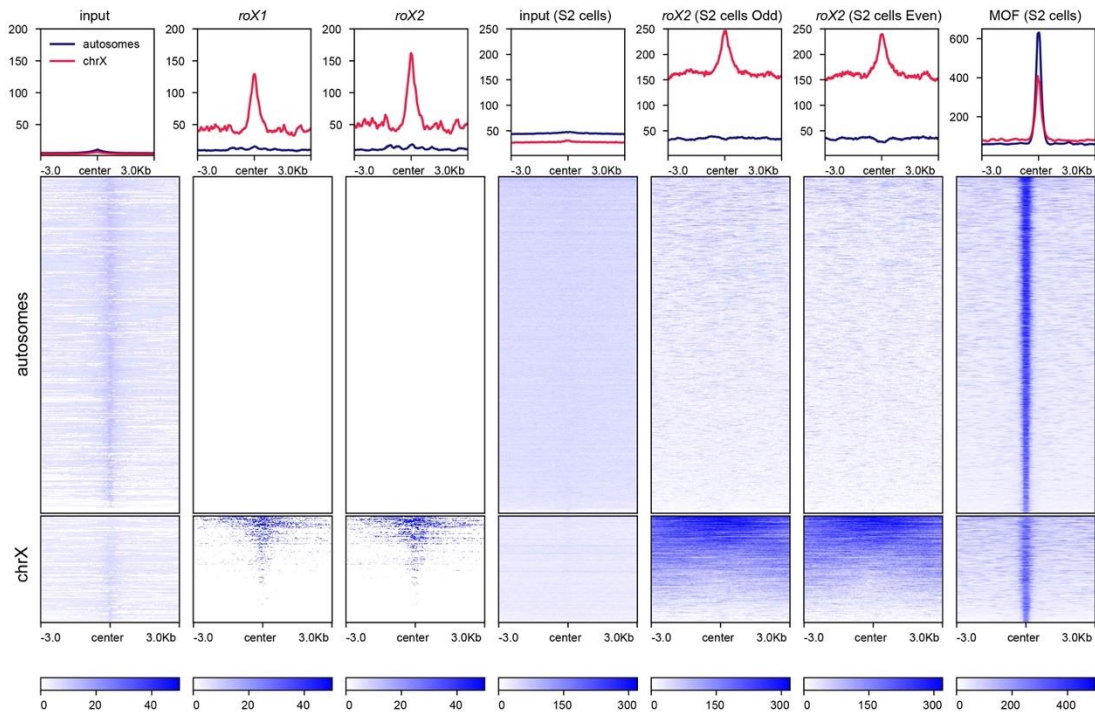


Response Figure 12, revised Figure 2D. Enrichment of *roX* RNAs in H3K27me3 RT&Tag. Significance levels are determined using the two-sided Wilcoxon test.

2. MOF is a member of the NSL complex in addition to its involvement in the MSL complex and activates not only X-linked genes but also autosomal genes. From this point of view, it is not clear why the authors didn't observe a downregulation of autosomal genes with MOF knockdown in Figure 3B. For the same reason, it is unexpected that MOF binding does not display peak shapes on autosomes in both WT and *roX*-KO.

Response: Thank you for your valuable feedback. In Figure 3B, our focus was specifically on the expression of *roX*-bound autosomal genes, which did not exhibit significant changes upon MOF knockdown. This observation aligns with the limited overlap between *roX* and MOF binding on autosomes, as illustrated in Figure 1C-D and supported by literature²². Since *roX* and MOF bind and regulate different genes on autosomes, it is reasonable that MOF binding does not display peak shapes on *roX*-associated autosomal peaks and *roX*-bound autosomal genes remain unchanged with MOF knockdown (Supplementary Figure 5E). These findings support our conclusion that *roX* binding and *roX* regulation appear to be independent of MOF and the MSL complex on autosomes. We have updated the labeling in Figure 3B to emphasize the expression changes of *roX*-bound genes. For better understanding, we have split the original Figure 3B into new Figure 3B showing only *roX*-KO RNA-seq data and Supplementary Figure 5E showing *msl* knockdown RNA-seq data.

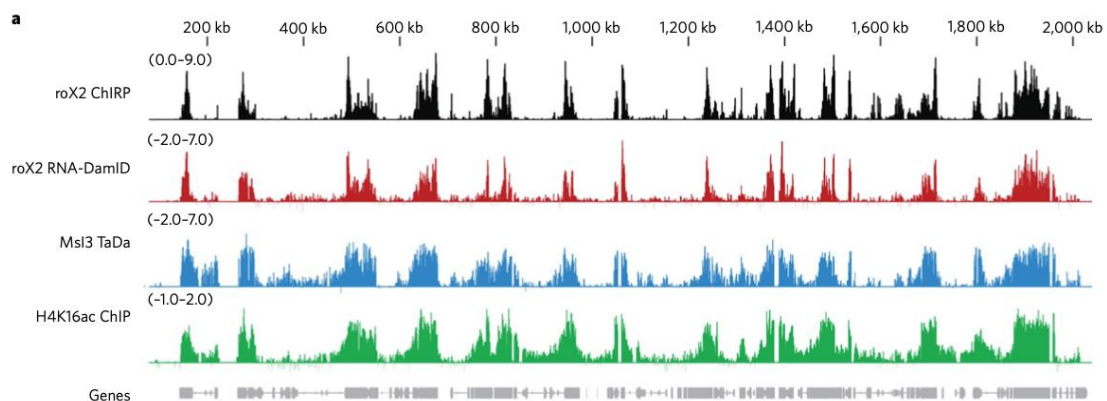
To further clarify the limited overlap between autosomal *roX* peaks and MSL complex, we plotted binding profiles of MSL complex components on *roX* binding sites and observed clear enrichment of all MSL proteins, including MOF, on *roX* binding sites on the X chromosome but not on autosomes (Response Figure 1). Additionally, plotting *roX* binding on MOF peaks revealed almost no binding signals of *roX* on autosomal MOF binding sites (Response Figure 13).



Response Figure 13. Colocalization of *roX* and MOF occurs on the X chromosome, but not on autosomes. *roX* ChIRP-seq signals from larvae and S2 cells are plotted on MOF peaks.

3. Line 157, "In males, X -chromosomal *roX* peaks is known to exhibit pronounced H4K16ac signals (Fig. 1C)." Could you add a reference to this statement? It seems that the H4K16ac signal is simply lower at the TSS because of the low nucleosomal density at that position.

Response: Thank you for the valuable feedback. Previous studies have shown that *roX*-bound X-chromosomal regions show highly enriched H4K16ac signals^{22,25} (Response Figure 14). In addition, our analysis, as depicted in Figure 1C, indeed demonstrates that in males, X-chromosomal *roX* peaks demonstrate co-binding of MOF, MSL2, and MLE, where H4K16ac signals are highly enriched (Figure 1C). We have already added references to this statement in revised manuscript on Page 5, Line 173.



Response Figure 14, original figure from Figure 2a in Cheetham and Brand, *Nat Struct Mol Biol* (2018)²⁵. IGV tracks showing *roX* peaks on the X-chromosomes with pronounced H4K16ac signals.

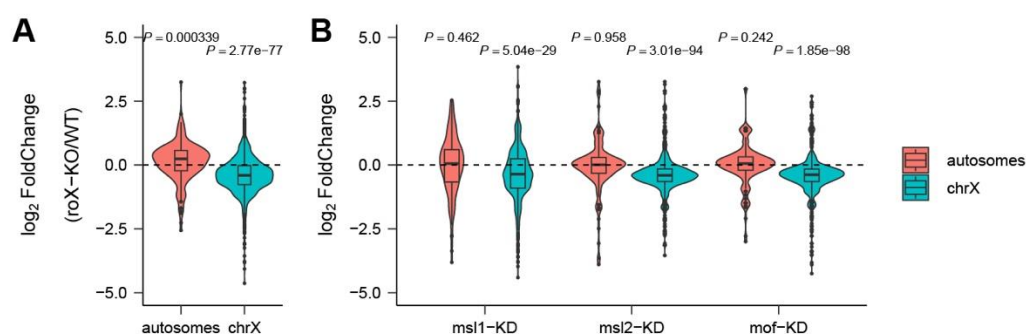
4. In Figure 3B and the corresponding text, it is less appropriate to compare the *roX*-KO result with the

knockdown results of the MSL complex components because (A) the latter are knocked down with varying degrees of target removal and (B) the latter used a homogeneous population, unlike the former.

Response: We appreciate the reviewer's insightful comment. Our aim in Figure 3B was to illustrate that autosomal genes associated with *roX* binding are not regulated by MSL proteins, in contrast to the situation on the X chromosome. While we acknowledge the differences between knockdown and knockout strategies in terms of target removal and population homogeneity, both approaches are utilized to elucidate the transcriptomic effects of specific regulators.

Indeed, we observed a significant downregulation of genes on *roX*-bound X chromosome genes with both *roX* knockout and MSL complex knockdown, confirming the effectiveness of these methods (Response Figure 15). However, autosomal genes associated with *roX* showed significant upregulation upon *roX* loss, while they remained unchanged with MSL complex knockdown (Response Figure 15). This suggests that autosomal genes associated with *roX* are not regulated by MSL proteins, contrasting the situation on the X chromosome.

To address these concerns, we have separated the knockout of *roX* and knockdown of MSL complex components into two distinct figure panels (Figure 3B and Supplementary Figure 5E). Additionally, we have modified the corresponding text to clarify these differences and ensure clarity for the readers in the revised manuscript on Page 5, Lines 193-195, 197-199, and Page 6, Lines 212-216.



Response Figure 15, revised as Figure 3B and Supplementary Figure 5E. Expression changes of *roX*-bound genes upon loss of *roX* RNAs (A) and *msl* knockdown (B).

5. Please rearrange Figure 2. Now the order is A-D-B-C, which is very confusing.

Response: Thank you for your feedback. We have rearranged Figure 2 to follow a more logical sequence, now presented as A-B-C-D. We believe this adjustment will enhance the overall readability and facilitate a better understanding of the presented data.

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<https://doi.org/10.1038/s41594-017-0006-4>

REVIEWER COMMENTS

Reviewer #2 (Remarks to the Author):

In a revised manuscript, the authors have addressed most of my concerns. The only weakness I still see is the mass spectrometry result, which captured non-nuclear but either cytoplasmic or mitochondrial proteins along with roX RNAs. The authors provided alternative evidence to support this conclusion, which was very helpful. However, since this observation is less expected by the general reader, a paragraph is needed in the Discussion section to address this. Below are my additional comments to improve the manuscript.

Response: Thank you very much for your thoughtful and constructive feedback, which has significantly helped improve our manuscript. We are pleased that you find we have addressed most of your concerns. We also agree that further discussion of the non-nuclear proteins captured in our roX mass spectrometry data will help clarify this aspect for readers. Below, we provide point-by-point responses to your comments and outline the revisions made accordingly.

1. As mentioned above, the mass spectrometry result needs additional discussion.

Response: Thank you for your helpful comments. In our previous response, we provided alternative evidence explaining the presence of some non-nuclear proteins in the roX ChIRP-MS data, and we are glad to hear that you found it helpful. In response to your suggestion, we have added a dedicated paragraph to the revised manuscript (Lines 395-407, Page 10) that elaborates on this observation. The new paragraph in the Discussion section reads:

"We observed the identification of several proteins predicted to localize outside the nucleus in our roX ChIRP-MS results. It is not uncommon to enrich some cytoplasmic proteins in nucleus-localized lncRNA ChIRP studies, as demonstrated by Xist/XIST ChIRP-MS data enriching translation-related or mitochondria-localized proteins^{1, 2}. Moreover, data from the Human Protein Atlas indicate that many cytoplasm-localized proteins can also exhibit localization in other organelles, including the nucleus³. We also re-evaluated the localization of roX RNAs using published roX2 FISH results in S2 cells⁴. The results revealed distinct nuclear speckles consistent with roX's predominant localization on the X chromosome. Intriguingly, some signals were observed outside the nucleus, suggesting potential cytoplasmic localization of roXs. A similar cytoplasmic localization of roX RNA is also observed in testes⁵. This raises interesting questions about the cytoplasmic functions of roX1/2, which warrant additional investigation."

2. The importance of this study does not lie in the binding of roX to autosomes, but in its interaction with PRC proteins, since the former has already been demonstrated by the Aktar group. The authors are advised to emphasize this importance throughout the manuscript. Another novelty is that roX colocalizes with CLAMP at the autosomes. This observation deserves more emphasis.

Response: Thank you for your valuable feedback and for highlighting the key aspects of our study. We fully agree that the main significance lies in the **autosomal repression** mediated by the interaction between roX and **PRC proteins**. To ensure this point is more clearly emphasized throughout the manuscript, we have made specific revisions.

In particular, we made the following updates and clarifications:

- In the **Abstract**, we modified the sentence to: *“This study uncovers the unconventional epigenetic repressive function of roX RNAs **with PRC interaction**.”* (Line 36, Page 1)
- In the **Introduction**, we added the sentence: *“These findings reveal the unconventional epigenetic repressive function of roX RNAs via PRC, and...”* in the summary section (Lines 105-106, Page 3).

Additionally, we would like to highlight that the following points were already included in the original manuscript, which emphasized the PRC-mediated repression:

- In the **Results** section under the subtitle “roX RNAs Interact with PRCs to Modulate H3K27me3 Deposition on Autosomes”, we stated: *“Whereas on autosomes, roX RNAs act in concert with PRCs to modulate repressive H3K27me3 deposition, thereby repressing gene transcription.”* (Lines 309-311, Page 8).
- In the **Discussion**, we previously noted: *“This study unveils an unexpected facet of roX function: its role in gene repression through interaction with PRC2, particularly on autosomes.”* (Lines 338-339, Page 9)

Regarding the novel observation of roX colocalization with CLAMP, we have also added the following paragraph to the **Discussion** section (Lines 408-413, Page 10):

“It is also noteworthy that CLAMP protein exhibits strong binding signals at roX binding sites, particularly in autosomal regions where MSL proteins are absent, while PRC proteins are enriched. In addition, CLAMP has been shown to co-immunoprecipitate with the PRC recruiter Psq^{6, 7}. This suggests that CLAMP may play a role in facilitating the genomic occupancy or the repressive function of the roX–PRC complex in autosomal regions.”

3. There are several supplementary figures that I found very useful and should be of interest to many readers. These include supplemental figure S2 (the CLAMP figure), S5, S8, and S9. I recommend that these figures be included in the main text.

Response: Thank you for your valuable suggestion, and we appreciate that you find these figures very useful and of interest to many readers. As recommended, we have moved supplemental figures S2 (the CLAMP figure), S5, and S8 into main Figures 1D, 4, and 6F respectively. Regarding Supplementary Figure S9, it presents our re-analysis of previously reported heat shock-induced roX relocalization. Given the space constraints and the fact that we did not extensively investigate this particular aspect of roX function, we have opted to retain it as a supplementary figure.

Reviewer #3 (Remarks to the Author):

This very interesting study that documents a functional co-localization of roX RNA and PRC subunits at autosomal sites. The authors have done a solid job responding to reviewer comments, but some issues need to be clarified and a few gaps in discussing their findings remain.

Response: Thank you for your positive comments on our study. We appreciate your thoughtful feedback and have carefully updated our manuscript to address the points raised and enhance the clarity and completeness of our findings.

Response to question 1. Analysis of CLAMP binding to autosomal sites reveals co-enrichment with roX1 and 2, but the authors fail to speculate on the significance of this finding. Enrichment of PRC subunits at autosomal roX sites is also presented. Is a role for CLAMP at PRC sites, or in deposition of repressive marks, implied?

Response: Thank you for your insightful comment. Our analysis revealed CLAMP binding on autosomal roX binding sites, where MSL proteins are absent but PRC proteins are enriched. Since CLAMP does not recruit MSL proteins to autosomes (Figure 1D) and it has been shown to co-immunoprecipitate with the PRC recruiter Psq^{6, 7}, it is possible that CLAMP facilitates the genomic occupancy or the repressive role of the roX–PRC complex at these autosomal sites. This interaction might involve novel complexes, regulatory mechanisms and functions that warrant further investigation. Exploring how roX and CLAMP recruit different complexes in autosomal versus X chromosomal sites will be an important next step in our research. We have added a paragraph to Discussion section of the revised manuscript (Lines 408-413, Page 10), as referenced in our response to above Reviewer #2, Question 2.

Response to question 2. It is likely that many readers will have the concern brought up in Q2, namely, how does the regulation of autosomal genes by roX described in this study differ from what has previously been reported. Inclusion in the manuscript of a short, accurate paragraph contrasting current and previous studies is necessary to dispel confusion.

Response: Thank you for your insightful comment. We have carefully compared our study with previous work to highlight the distinctions and our findings.

First, earlier studies reported the localization of MSL proteins and roX RNAs on autosomes, as well as the relocalization of roX to autosomal regions following heat shock⁸⁻¹², which is consistent with our observations. However, these earlier studies did not focus on systematically analyzing the autosomal co-localization of roX with MSL, PRC proteins, or histone modifications, nor did they examine the functional consequences on gene expression at these sites. In contrast, our analysis provides a more comprehensive view, showing that while roX localizes to autosomal regions, its binding sites do not overlap with MSL proteins on autosomes (Figure 1). Furthermore, we discovered that roX colocalizes with PRC and primarily regulates gene repression on autosomes, a role that is distinct from its well-established function in gene activation on the X chromosome. This is thoroughly analyzed and explained in our previous response (Round one revision response to Q1, Response Figure 1), and supported by current Figures 1, 3 and 5, and Supplementary Figure 8.

Second, while Deng et al.¹³ observed changes in heterochromatin gene expression upon loss of roX, our analysis extended these findings by examining the chromosomal distribution of roX binding sites. Specifically, we investigated whether roX is enriched at heterochromatin regions as detailed in our previous round one revision response to Reviewer 1, Q2. Our findings demonstrated that roX binding peaks are not evenly distributed across chromosomes and are predominantly located outside heterochromatic regions (Round one revision Response Figure 2A). We have added as Supplementary

Figure 1B in the current manuscript. Since *roX* RNAs do not directly bind to heterochromatin, we hypothesize that the regulation of gene expression in these regions is likely an indirect effect, potentially mediated by *roX* target genes or MSL relocalization.

We have included additional paragraphs in the Discussion section to further clarify and compare our findings with previous studies (Lines 414-433, Page 11).

Response to question 3. The authors now include a very nice analysis revealing the female expression bias of autosomal genes that recruit *roX*. Would the authors please indicate how many genes contributed to this study and clarify what the colors in Sup. Fig. 5C represent. The + and – symbols are cryptic.

Response: Thank you for your valuable observation. We have now included the number of genes contributing to this analysis in the figure legend and clarified the color coding in Supplementary Figure 5C (current Figure 4C). Additionally, we have replaced the cryptic "+" and "-" symbols with more descriptive labels for clarity.

Additional comments related to manuscript.

Line 76. Relocation of the *roX* transcripts upon heat shock is noted, but I think the authors should mention that the MSL proteins also travel to autosomal sites.

Response: Thank you for your insightful feedback. You are correct that *roX* transcripts relocalize to autosomes following heat shock, as reported in previous studies¹¹. However, it is important to note that the autosomal sites to which *roX* re-localizes under heat shock conditions do not overlap with MSL proteins binding sites (Supplementary Figure 8).

While MSL proteins are indeed known to relocalize to autosomal regions^{8,12}, this occurs specifically in *roX* knockout larvae, not under heat shock conditions. In *roX* mutants, the MSL complex becomes less abundant on the male X chromosome and instead accumulates at pericentromeric heterochromatin regions on autosomes¹². We have added this information, along with the relevant references, in the Discussion section (Lines 424-433, Page 11) as you suggested.

Line 145. The phrase “. . . *roX* occupancy on autosomes is not associated with dosage compensation.” should be changed to “. . . *roX* occupancy on autosomes does not reflect recruitment of a functional MSL complex.”

Response: Thank you for the suggestion. We have revised the sentence accordingly.

Line 200. The statement that *roX* RNAs show sex-specific expression is not absolutely correct as *roX1* is highly expressed in early embryos of both sexes. In light of the findings of this study it would be very interesting to (in the future) investigate a possible role for *roX1* in embryonic PRC function.

Response: Thank you for your insightful comments. We agree that *roX1* shows relatively high expression in early embryos of both sexes¹⁴, while sex-specific *roX* expression emerges from late embryonic stages¹⁵. We also acknowledge the potential for future studies to explore the role of *roX1* in

embryonic PRC function. In light of your feedback, we have revised the statement to clarify that “*roX* RNAs exhibit a sex-specific expression pattern and function in males **from late embryonic stages onward** (Lines 202-203, Page 5).”

The authors choose to manipulate *roX* in the male germ line in an unsuccessful effort to demonstrate a shift in H3K27me3 levels. The choice of this tissue is baffling as the *roX* RNAs are not expressed, or expressed at extraordinarily low levels, in the germ cells.

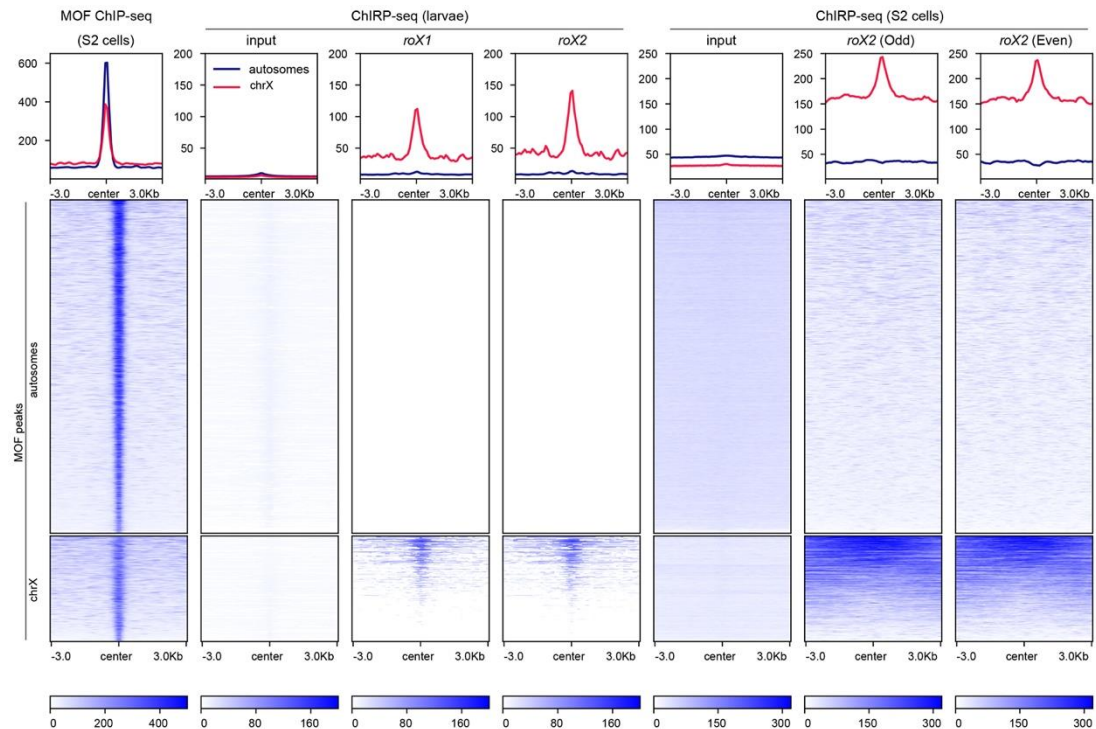
Response: Thank you for your thoughtful comment. We agree that *roX* is indeed expressed at low levels in germ cells^{5, 16}, making it an unsuitable system for knockdown experiments to detect H3K27me3 changes in germ cells. In our previous revision, we therefore focused on somatic cells in the testes, where *roX* expression is significantly higher^{5, 16}. Additionally, we attempted *roX* overexpression via heat shock in all cells to investigate potential changes in H3K27me3 levels. However, we recognize that *roX* RNAs regulate only a small subset of H3K27me3 peaks, which makes detecting subtle changes via immunostaining challenging, as this method reflects global H3K27me3 levels across individual cells. Therefore, while we attempted this approach to explore potential insights, we acknowledge its limitations in discerning fine-scale alterations. Based on these limitations and your insightful comments, we have chosen not to include the immunostaining data in the manuscript. We believe that the strong evidence from our PIRCh-seq, ChIP-seq, and RT&Tag data—demonstrating *roX* autosomal binding and its role in H3K27me3 regulation—provides a more robust foundation for our conclusions.

That being said, we recognize that *roX* RNAs regulate only a small subset of H3K27me3 peaks, making it challenging to detect subtle changes in H3K27me3 by immunostaining, which reflects global H3K27me3 levels in individual cells. Therefore, while we attempted this approach to explore potential insights, we acknowledge its limitations in discerning fine-scale alterations. In light of your comments and our own findings, we have opted not to include the immunostaining data in the manuscript. We believe that the strong evidence from our PIRCh-seq, ChIP-seq, and RT&Tag data—demonstrating *roX* autosomal binding and its role in H3K27me3 regulation—provides a more robust foundation for our conclusions.

Response Fig. 13 needs a more detailed legend. It currently suggests that *roX* 2 colocalizes with MOF on autosomes, but I suspect that is not what the authors intended.

Response: Thank you for your careful observation, and we apologize for the confusion. To clarify, previous Response Figure 13 displays the binding signals of *roX* at MOF-binding sites. The figure is intended to show the clear enrichment of *roX* RNAs at MOF-binding sites on the X chromosome, but not on autosomal sites. We believe the confusion may have arisen from the "input" panel, which shows weak signals on both the X chromosome and autosomes. However, it's important to note that the input signal is very low, and we adjusted the color scale to accurately reflect the signal strength. To further minimize any potential confusion, we have also rearranged the figure to place MOF ChIP-seq lane in front of *roX* ChIRP-seq lanes (current Response Figure 1).

Additionally, we have revised the figure legend to include more detailed explanations, which we have provided below. We hope these clarifications resolve any misunderstandings and address your concern.



Response Figure 1. Colocalization of *roX* and MOF occurs on the X chromosome, but not on autosomes. *roX* ChIRP-seq signals from larvae and S2 cells are plotted at MOF binding sites. MOF binding sites on the X chromosome show a high enrichment of *roX* RNAs in both larvae and S2 cells. In contrast, the autosomal MOF binding sites do not display any significant enrichment of *roX* signals.

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REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

The authors have addressed all my concerns.

Response: Thank you for your positive feedback on our revised manuscript.

Reviewer #3 (Remarks to the Author):

I am satisfied with the current version of this manuscript.

Response: Thank you for your recognition on our revised manuscript.