Cell Genomics Transparent Peer Review Record

Stable and robust Xi and Y transcriptomes drive cell-type-specific autosomal and Xa responses *in vivo* and *in vitro* in four human cell types

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Summary

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Data freely available: YES

Code freely available: YES

Referees' reports, first round of review

Reviewer #1:

SUMMARY OF THE STUDY:
In this study from David Page's lab, the authors investigate how abnormal sex-chromosome complements affect autosomal and active X-chromosome expression. Male as well as female cells normally contain only a single active X (Xa), and its complement is either a gene-poor Y-chromosome (in males) or an inactive X (Xi, in females) - however notably, some genes escape X-inactivation and thus transcribe from Xi to variable degrees, and some parts of Y/X are shared (i.e. recombining pseudoautosomal regions). The authors utilized RNA-seq on human CD4-positive T-cells and monocytes collected and sorted from peripheral blood from individuals with naturally occurring sex-chromosome aneuploidy (1, 2, or 3 X-chromosomes or 0, 1, or 2 Y-chromosomes), and they investigated RNA-expression levels transcriptome-wide. By linear modeling they quantitatively analyzed the influence of the Xi and the Y chromosome copy numbers on gene expression in the two types of human immune cells in vivo. The results indicate that a stable group of genes on the inactive X (Xi) and Y chromosomes consistently influence autosomal and active X (Xa) gene responses in a manner that varies with cell type. The findings could be important for unraveling some of the molecular foundations of sex-related traits and for understanding phenotypes (or the lack thereof) in individual with sex-chromosome aneuploidies.

OVERALL ASSESSMENT:
The choice of methodology, cell system, and number of individuals investigated is sound, boosting N individuals T-cells = 76, N individuals for monocytes = 72. Additionally, the authors reused similar data from fibroblasts and lymphoblastoid cell lines previously published by the team. To me, the overall conclusions are unsurprising given the authors' previous in vitro work, but the present study is still very valuable, not least because of the use of in vivo samples, which strengthens the conclusions derived from in vitro studies. I am positive about this work, as it is well-executed and provides highly valuable in vivo data. Still, there is an element of salami slicing and the current study does not offer so much novelty over their previous two manuscripts exploring the same ideas (albeit in vitro). Where this manuscript could have had an extra edge is in relation to immune function, but these insights are brief and, if anything, show that the effect sizes in the previous studies are somewhat overestimated compared to in vivo settings. Immune reactivity is known to be affected by the sex-chromosome complement —possibly driven by genes identified in the present study, which provides an opportunity. However, such an in-depth study would likely require single-cell RNA-seq analyses and is probably out of reach with the current data set.

SPECIFIC POINTS:
1. Throughout many figure panels it is not possibly to distinguish the sex (male/female) neither sex-chromosome complement of samples plotted. For each panel this must be fixed by appropriate coloring and/or data-point shape. For example, Fig.1D plots XIST expression in cells of 1, 2, 3 X-chromosomes buy sex is not indicated.

2. X-chromosome inactivation (XCI) is assumed and modelled to be subjecting each additional X copy to silencing in the individuals, i.e. one single Xa + nXi. This assumption is according to previous studies that demonstrated that each additional X added tends to be inactivated in cells (mainly tested in mouse). However, there may be exceptions to this rule in vivo, as well as inter-individual and cell-type variation in degree of silencing (variable escape affecting 15-20% of genes) as well as complete loss of XCI in

fractions of cells. The authors should clarify how/if they addressed this issue, and in best case provide the average numbers of Xa and Xi chromosomes in the different samples. Potential Xa-number variation among samples could moreover open up for interesting follow-ups.

3. XCI bias: Is XCI random in the individuals with X-chromosome aneuploidy (as in normal female XX cells) or is skew towards a specific copy more common in these individuals? The current data sets provide a unique opportunity to address this question by allelic analysis of the RNA-seq data.

4. It would be extremely interesting to see how Xa genes that have escapee copies on Xi or conserved chrY homologs respond when additional Xi/Y copies are present and transcribing in the same cell. I.e., is the dose of the Xa allele affected by expression from additional alleles? I understand that allelic resolution is limited in human samples, but can the question be addressed at least in some individuals and genes?

5. The authors use the wording "primary cells", which becomes a little confusing if not carefully reading the Methods section. For most, "primary cells" means cells collected in vivo and briefly cultured in vitro before analysis. If all cells used in the study are truly collected and analyzed directly from human blood without any round of ex-vivo culturing the authors should not term them "primary", but in vivo cells. The use of true in vivo cells without ex vivo culturing is a strength.

6. It would be interesting to see XACT expression in samples of various sex-chromosome aneuploidy. Is XACT expressed in any of the states? An XACT expression plot could be added to the supplementary. This is of interest for the X community to know, including if XACT is not expressed at all.

Reviewer #2:

In the manuscript titled " Stable and robust Xi and Y transcriptomes drive cell-type-specific autosomal and Xa responses in vivo and in vitro in four human cell types" the authors present new findings regarding gene expression regulation following changes in sex-chromosomal dosage. By studying naturally occurring sex chromosome constitutions, in two human immune cell types, the authors are following-up on their recently published work and addressing similar questions with an in-vivo outlook, adding physiological relevance to their well described discoveries.
Main Findings described in the text:
* Changes in sex-chromosome expressed genes as a response to sex chromosome dosage, are steady across all cell types tested, in-vitro and in-vivo.
* In contrast, autosomal genes respond to the same changes in a more cell-type specific manner.
* Autosomal response to sex chromosome dosage is purportedly uncoupled from age and hormonal profile.
* ZFX/Y factors might play a central role in the regulation of the autosomal gene response sex chromosome dosage.
This reviewer would like to point out the high level of readability of both the text and the figures and legends in the manuscript. Also, we should appreciate how the authors clearly address the limitations of their own studies alone the text.
The findings presented here, join an array of recently published works in underlying the importance of studying gene expression changes with allele-specific resolution. Even more so under the lens of immune system cell types, where sex-specific differences are eminent.

Nonetheless, there are several issues the authors should address before the MS can be considered for publication:

1. The authors chose to highlight TLR7 and CD40LG as examples of X-linked non-responsive genes. To this reviewer it was unclear if this result is also true in the non-immune cell types? Also, can the authors comment on the possible consequences of over-expression of these genes in the immune cell types? are genes falling in this category might be subjected to stricter regulation in cell-type specific manner? A more thorough discussion on the subject is needed.
2. On page 7 of the MS, the authors state: "These findings agree with prior expectations…". Where are these expectations coming from? the known expression patterns of these regions is not mentioned or referred to anywhere.
3. Can the authors clarify their conclusion that: "Changes in sex-chromosomal expression were remarkably constant in vivo and in vitro across all four cell types examined", when actually, the vast majority of X-linked genes are cell-type specific responders (Figure 2D).
4. In page 8, authors claim that their analysis revealed that the "identification of significantly responsive X-chromosomal genes was approaching saturation, with the majority of significantly responsive genes identified but additional genes likely to be identified with additional samples". Isn't reaching saturation indicate that no new genes will be identified with the addition of samples?
5. Figure 2E: this panel only shows correlation between the in-vivo cell types. where is the correlation between in-vivo Vs. in-vitro?
6. The authors address the issue of XCI escape in Figure 2F. But, given the nature of XCI escapees to very across different tissues, is inferring from the previous published cell types is the right way to study them in the current system?
7. Consistently along the MS, monocytes exhibit remarkably lower numbers of changing genes. Does it correlate to a difference in the total gene number expressed in both cell types? does this observation has any biological meaning that the authors can comment about?
8. The overall result paragraph titled: "Autosomal responses to sex chromosome dosage in vivo" is lacking any meaningful insights about the genes that are changing? any known sex-biased genes? biological relevant genes? what do we know other than number of genes changing? Also, this reviewer is missing a concluding sentence to this part.
9. This reviewer suggests the authors will be more careful when describing the ZFX/ZFY effects, given that the data is driven from a completely different cell types, without a thorough check of genome stability and ploidy described. (or should attempt getting this information in their own systems)
10. Regarding the effect of hormonal environment: did the authors checked the hormonal profile of their samples? if yes, where is the data? if not, it will be good if they can provide it.
11. Figure S10C, D: how come the total number of ZFX and/or ZFY knockdown genes is different in the two panels?
12. Figure 6: It's interesting that autosomal responses to Xi and Chr Y dosage exhibit greater cell-type specificity. Did the authors further investigate this phenomenon to explore potential mechanisms?
13. Figure 6: authors should make sure that numbers in the text match the numbers in the figure.

Authors' response to the first round of review

We thank the Reviewers for their thoughtful comments and helpful feedback on our manuscript. In response, we made numerous changes to the text, as described below in our point-by-point replies. Our revised manuscript also includes modifications to two main text figures (Figures 2 and 5) and two new supplemental figures (Figures S4 and S12).

Reviewer #1:

SUMMARY OF THE STUDY:

In this study from David Page's lab, the authors investigate how abnormal sex-chromosome complements affect autosomal and active X-chromosome expression. Male as well as female cells normally contain only a single active X (Xa), and its complement is either a gene-poor Ychromosome (in males) or an inactive X (Xi, in females) - however notably, some genes escape X-inactivation and thus transcribe from Xi to variable degrees, and some parts of Y/X are shared (i.e. recombining pseudoautosomal regions). The authors utilized RNA-seq on human CD4positive T-cells and monocytes collected and sorted from peripheral blood from individuals with naturally occurring sex-chromosome aneuploidy (1, 2, or 3 X-chromosomes or 0, 1, or 2 Ychromosomes), and they investigated RNA-expression levels transcriptome-wide. By linear modeling they quantitatively analyzed the influence of the Xi and the Y chromosome copy numbers on gene expression in the two types of human immune cells in vivo. The results indicate that a stable group of genes on the inactive X (Xi) and Y chromosomes consistently influence autosomal and active X (Xa) gene responses in a manner that varies with cell type. The findings could be important for unraveling some of the molecular foundations of sex-related traits and for understanding phenotypes (or the lack thereof) in individual with sex-chromosome aneuploidies.

OVERALL ASSESSMENT:

The choice of methodology, cell system, and number of individuals investigated is sound, boosting N individuals T-cells = 76, N individuals for monocytes = 72. Additionally, the authors reused similar data from fibroblasts and lymphoblastoid cell lines previously published by the team. To me, the overall conclusions are unsurprising given the authors' previous in vitro work, but the present study is still very valuable, not least because of the use of in vivo samples, which strengthens the conclusions derived from in vitro studies. I am positive about this work, as it is well-executed and provides highly valuable in vivo data. Still, there is an element of salami slicing and the current study does not offer so much novelty over their previous two manuscripts exploring the same ideas (albeit in vitro). Where this manuscript could have had an extra edge is in relation to immune function, but these insights are brief and, if anything, show that the effect sizes in the previous studies are somewhat overestimated compared to in vivo settings. Immune reactivity is known to be affected by the sex-chromosome

complement —possibly driven by genes identified in the present study, which provides an opportunity. However, such an in-depth study would likely require single-cell RNA-seq analyses and is probably out of reach with the current data set.

We appreciate the Reviewer's request that we explore more deeply the implications of our findings for immune function. In response to this request, we have added analyses, text, and display items, as follows: We now highlight an autosomal gene, *FCGR2B*, which we find to be positively Xi-responsive in monocytes and which has been shown previously to 1) exhibit female-biased expression and 2) be associated with systemic lupus erythematosus, a femalebiased autoimmune disease. We have added an accompanying panel for *FCGR2B* in Figure 5. In addition, we now delve deeper into the gene set enrichment analysis (associated with Figure 5) and present the "leading edge" genes that are responsible for a number of the significant enrichments in a new supplementary figure (Figure S12).

SPECIFIC POINTS:

1. Throughout many figure panels it is not possibly to distinguish the sex (male/female) neither sex-chromosome complement of samples plotted. For each panel this must be fixed by appropriate coloring and/or data-point shape. For example, Fig.1D plots XIST expression in cells of 1, 2, 3 X-chromosomes buy sex is not indicated.

We appreciate the comment and understand the interest in clarifying the sex of the individuals from whom cells were isolated. Our decision to graph the data without reference to donor sex was a deliberate one, motivated by the following biological and technical considerations:

On the biological side: gene expression responses to Chr X dosage were similar when linearly modeled using female samples only or male samples only. We initially had shown this 'female subset/male subset' analysis for autosomal gene responses, and we have now added subset analyses for NPX and PAR gene responses to Chr X dosage in CD4+ T cells and monocytes (see new supplementary Figure S4).

On the technical side: plotting all donors together reflects the design of our analytic (linear) model, where females and males of all six karyotypes were included in estimating the responses of individual genes to sex chromosome dosage.

Should the reader be interested in parsing the responses of individual genes in females or males, donor metadata and normalized read counts are provided in the supplementary data and accompanying github page.

2. X-chromosome inactivation (XCI) is assumed and modelled to be subjecting each additional X copy to silencing in the individuals, i.e. one single Xa + nXi. This assumption is according to previous studies that demonstrated that each additional X added tends to be inactivated in cells (mainly tested in mouse). However, there may be exceptions to this rule in vivo, as well as interindividual and cell-type variation in degree of silencing (variable escape affecting 15-20% of genes) as well as complete loss of XCI in

fractions of cells. The authors should clarify how/if they addressed this issue, and in best case provide the average numbers of Xa and Xi chromosomes in the different samples. Potential Xa-number variation among samples could moreover open up for interesting follow-ups.

The Reviewer raises the possibility that the human immune cells in our study might not follow the n-1 rule. We are entirely confident that they do follow the n-1 rule on both historical and empirical grounds.

First, on historical grounds, the generality of the n-1 rule is well established across eutherian mammals, including humans; indeed, the still-current understanding of the n-1 rule first emerged from human studies, in 1961 (Harnden, The Lancet), and has been confirmed by decades of analysis by many investigators (see, for example, the identification of the *XIST* gene by Brown and colleagues, Nature 1991).

Second, on empirical grounds, our current dataset and linear modeling unequivocally affirm the n-1 rule. If cells with multiple X chromosomes possessed two (or more) Xa chromosomes, then expression of most or all expressed X-chromosomal genes should increase with the number of X chromosomes. In reality, we observe that expression of most expressed X-chromosomal genes is unchanged as the number of X chromosomes increases. This argues strongly against the presence of two (or more) Xa chromosomes in the immune cells in our study.

3. XCI bias: Is XCI random in the individuals with X-chromosome aneuploidy (as in normal female XX cells) or is skew towards a specific copy more common in these individuals? The current data sets provide a unique opportunity to address this question by allelic analysis of the RNA-seq data.

With the bulk RNA-Seq data that we have available, estimating allelic ratios of Xa versus Xi expression would be possible only if multiple individuals displayed significantly skewed XCI, such that either the maternal or paternal Chr X is Xa in most cells. While such significant skewing is not uncommon in cultured cell lines, it is rarely observed in cells taken directly from the blood. Thus, our dataset is not well suited to the allelic analysis envisioned by the Reviewer. While otherwise of great interest, the question of skewing is tangential to our present study, as our conclusions do not rely upon XCI being random or skewed.

4. It would be extremely interesting to see how Xa genes that have escapee copies on Xi or conserved chrY homologs respond when additional Xi/Y copies are present and transcribing in the same cell. I.e., is the dose of the Xa allele affected by expression from additional alleles? I understand that allelic resolution is limited in human samples, but can the question be addressed at least in some individuals and genes?

This is another interesting biological question, but again our human dataset is not well suited to the allelic analyses envisioned by the Reviewer.

5. The authors use the wording "primary cells", which becomes a little confusing if not carefully reading the Methods section. For most, "primary cells" means cells collected in vivo and briefly cultured in vitro before analysis. If all cells used in the study are truly collected and analyzed directly from human blood without any round of ex-vivo culturing the authors should not term them "primary", but in vivo cells. The use of true in vivo cells without ex vivo culturing is a strength.

We appreciate the Reviewer's highlighting this strength of the study, i.e., that we studied cells collected directly from human blood with no culturing outside the body.

We will look to the editor's advice on the semantic question of how one should refer to cells collected directly from the body. In the meantime, we have reviewed the literature regarding the terms "primary cells" (as distinct from "primary cell cultures") and "cells *in vivo*". Based on our reading of the literature, and pending editorial input, we have retained the term "primary cells", which is understood (by our reading of the literature) to refer to cells isolated directly from the body (but no longer in the body, where one would instead refer to "cells *in vivo*"). Again, we will defer to the editor on this semantic question.

6. It would be interesting to see XACT expression in samples of various sex-chromosome aneuploidy. Is XACT expressed in any of the states? An XACT expression plot could be added to the supplementary. This is of interest for the X community to know, including if XACT is not expressed at all.

XACT is indeed an interesting gene. Unfortunately, like a minority of X-linked protein-coding genes, *XACT* is not expressed in any of the four cell types studied, so we have no new light to shed on its biology.

Reviewer #2:

In the manuscript titled " Stable and robust Xi and Y transcriptomes drive celltype-specific autosomal and Xa responses in vivo and in vitro in four human cell types" the authors present new findings regarding gene expression regulation following changes in sexchromosomal dosage. By studying naturally occurring sex chromosome constitutions, in two human immune cell types, the authors are following-up on their recently published work and addressing similar questions with an in-vivo outlook, adding physiological relevance to their well described discoveries.

Main Findings described in the text:

* Changes in sex-chromosome expressed genes as a response to sex chromosome dosage, are steady across all cell types tested, in-vitro and in-vivo.
* In contrast, autosomal genes respond to the same changes in a more cell-type specific manner. * Autosomal response to sex chromosome dosage is purportedly uncoupled from age and hormonal profile.
* ZFX/Y factors might play a central role in the regulation of the autosomal gene response sex chromosome dosage.

This reviewer would like to point out the high level of readability of both the text and the figures and legends in the manuscript. Also, we should appreciate how the authors clearly address the limitations of their own studies alone the text.

The findings presented here, join an array of recently published works in underlying the importance of studying gene expression changes with allele-specific resolution. Even more so under the lens of immune system cell types, where sex-specific differences are eminent.

Nonetheless, there are several issues the authors should address before the MS can be considered for publication:

1. The authors chose to highlight TLR7 and CD40LG as examples of X-linked non-responsive genes. To this reviewer it was unclear if this result is also true in the non-immune cell types?
Also, can the authors comment on the possible consequences of over-expression of these genes in the immune cell types? are genes falling in this category might be subjected to stricter regulation in cell-type specific manner? A more thorough discussion on the subject is needed.

CD40LG is not expressed in fibroblasts or LCLs; *TLR7* is expressed in LCLs, where its ΔE_X value is 0.07. X-chromosomal genes with immune-related functions, such as *TLR7* and *CD40LG*, have been previously hypothesized to contribute to female-biased autoimmunity and other sex differences in the immune system. These hypotheses are grounded in the belief that these Xchromosomal immune-related genes are more highly expressed in females than in males. We chose to highlight *TLR7* and *CD40LG* as non-responsive genes to demonstrate that, at a cellpopulation resolution, these genes do not show higher expression with more X chromosomes. Thus, our findings suggest that other factors likely contribute to female-biased autoimmunity and to other sex differences in the immune system.

2. On page 7 of the MS, the authors state: "These findings agree with prior expectations…". Where are these expectations coming from? the known expression patterns of these regions is not mentioned or referred to anywhere.

We have added appropriate references to expression patterns for PAR genes and NPX genes with NPY homologs in the text.

3. Can the authors clarify their conclusion that: "Changes in sex-chromosomal expression were remarkably constant in vivo and in vitro across all four cell types examined", when actually, the vast majority of X-linked genes are cell-type specific responders (Figure 2D).

We thank the Reviewer for this insightful comment, and agree that the original Figure 2D was misleading. To address this concern, we have replaced the potentially confusing UpSet plot in Figure 2 with a proportional bar plot that better conveys the intended message: expressed PAR genes and expressed NPX genes with NPY homologs consistently showed significant responses to Xi dosage across all four cell types.

4. In page 8, authors claim that their analysis revealed that the "identification of significantly responsive X-chromosomal genes was approaching saturation, with the majority of significantly responsive genes identified but additional genes likely to be identified with additional samples". Isn't reaching saturation indicate that no new genes will be identified with the addition of samples?

We have clarified this in the text. We did not intend to suggest that all X-chromosomal genes have been identified, but that the addition of further samples to the dataset will reveal fewer and fewer newly statistically significant genes (that is, the curve is "approaching saturation", but not yet fully saturated).

5. Figure 2E: this panel only shows correlation between the in-vivo cell types. where is the correlation between in-vivo Vs. in-vitro?

The correlation between in-vivo and in-vitro is shown in Figure S5.

6. The authors address the issue of XCI escape in Figure 2F. But, given the nature of XCI escapees to very across different tissues, is inferring from the previous published cell types is the right way to study them in the current system?

The Reviewer is correct that the meta-analysis from which we classified genes "Xi-expressed" or "Xa-only expressed" utilized LCLs and fibroblasts and did not include allele-specific data from CD4+ T cells or monocytes. While we agree that allele-specific data from CD4+ T cells and monocytes would be ideal, the available data (our current data) are not amenable to allelespecific analysis. We thus utilized the previously available robust meta-analysis. We would argue that, although the original data was derived from LCLs and fibroblasts, it is clear that the same set of genes that are expressed from Xi in LCLs and fibroblasts show consistently stable
ΔE_X values in CD4+ T cells and monocytes (see Figure 2F, Figure S5, and Figure 6E-G). Ultimately, this underscores the major finding of our study regarding the stability of Xiexpressed genes across cell types.

7. Consistently along the MS, monocytes exhibit remarkably lower numbers of changing genes. Does it correlate to a difference in the total gene number expressed in both cell types? does this observation has any biological meaning that the authors can comment about?

The Reviewer correctly notes that monocytes have fewer genes with statistically significant responses to Xi or Chr Y dosage. While we have no definitive explanation, the monocyte dataset does have slightly fewer samples than the CD4+ T cell dataset (72 vs. 76 samples), and the monocytes express slightly fewer autosomal genes than CD4+ T cells (12,140 vs. 12,756 genes). Beyond these factors, it is possible that the autosomal transcriptome of monocytes is truly less responsive to Xi and Chr Y dosage than that of CD4+ T cells. This would be consistent with our power analysis of autosomal responses shown in Figure S7, and in any case it adds to the evidence that autosomal responses to Xi and Y dosage are cell-type-specific.

8. The overall result paragraph titled: "Autosomal responses to sex chromosome dosage in vivo" is lacking any meaningful insights about the genes that are changing? any known sex-biased genes? biological relevant genes? what do we know other than number of genes changing? Also, this reviewer is missing a concluding sentence to this part.

As described above in response to Reviewer 1, we now highlight a number of functionally relevant genes that are significantly impacted by Chr X and/or Chr Y dosage. The first example is *FCGR2B*, which is implicated in systemic lupus erythematosus, has higher expression in females, and significantly increases expression with Chr X (but not Chr Y) dosage. The second set of functionally relevant genes are derived from our gene set enrichment analysis. We have added further details about the "leading edge" genes that drive the significant enrichments in key immune pathways, including interferon alpha and interferon gamma response pathways.

9. This reviewer suggests the authors will be more careful when describing the ZFX/ZFY effects, given that the data is driven from a completely different cell types, without a thorough check of genome stability and ploidy described. (or should attempt getting this information in their own systems)

Yes, we acknowledge the limitations of our current analysis of ZFX and ZFY effects – namely, that our analysis compares ZFX targets in different cell types while the ideal experiment would compare the effects of *ZFX* and/or *ZFY* in CD4+ T cells and monocytes. We have revised the text to better reflect these limitations.

10. Regarding the effect of hormonal environment: did the authors checked the hormonal profile of their samples? if yes, where is the data? if not, it will be good if they can provide it.

Unfortunately, we do not have access to hormonal profiles for our donors.

11. Figure S10C, D: how come the total number of ZFX and/or ZFY knockdown genes is different in the two panels?

The analyses presented in Figures S10C and S10D are restricted to genes expressed in both fibroblasts and the given cell type (CD4+ T cells in S10C, monocytes in S10D). As different sets of genes intersect between CD4+ T cells and fibroblasts versus monocytes and fibroblasts, there are different numbers of genes plotted in each figure panel. We have clarified this in the accompanying figure legend.

12. Figure 6: It's interesting that autosomal responses to Xi and Chr Y dosage exhibit greater cell-type specificity. Did the authors further investigate this phenomenon to explore potential mechanisms?

We agree that mechanisms driving the cell-type-specific autosomal responses are of great interest, yet definitive mechanistic conclusions are beyond the scope of the present study. In the Discussion, we briefly hypothesize that cell-type-specific chromatin accessibility and expression of autosomal cofactors contribute to the cell-type-specific autosomal responses. We hope to pursue this experimentally in the future.

13. Figure 6: authors should make sure that numbers in the text match the numbers in the figure.

We have clarified how the numbers cited in the text correspond to Figure 6.

Referees' report, second round of review

Reviewer #1:

The authors have addressed each of my points to sufficient satisfaction and I have no additional substantial questions.

Authors' response to the second round of review

NA