nature portfolio

Peer Review File

A Compendium of Chromatin Interaction Maps in the Giant Panda Genome

Corresponding Author: Professor Yan Li

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)
The manuscript entitled “A Compendium of Chromatin Interaction Maps in the Giant Panda Genome” by Pengliang Liu et al. presents a comparative analysis of bulk RNA-seq and Hi-C data obtained by the authors for nine giant panda tissues. This is a highly descriptive study, presenting an overview of differences between tissues at various levels: transcriptomic, promoter-enhancer interactions, TADs, compartments, etc. The multi-tissue transcriptomes for the giant panda were published before but the multi-tissue 3D genome is novel for the giant panda and might be of interest to the community and the wider field. The most valuable and interesting part, in my opinion, is the evolutionary analysis presented in two last sections of the Results: ‘3D chromatin maps enhance the biological interpretations of adaptive evolution’ and ‘Chromosome conformation reveals the potential function of accelerated regions in giant pandas’. While the manuscript is clearly written and the presented analysis is overall robust, I have several major concerns that need to be considered before this work can be published.

1) The greatest limitation of this study is the absence of biological replicates. The authors analyzed only one giant panda specimen (only one replicate per tissue). Therefore, the authors cannot perform (and do not perform) any traditional statistical analyses such as differential expression analysis (which is technically impossible when there are no replicates). They can only operate in terms like ‘more expressed’ or ‘less expressed’, and the whole manuscript is written in such a manner. Which is technically correct but it is sad that the majority of statements presented in the manuscript cannot be confirmed by proper statistics. Very few analyses in the manuscript are confirmed with p-values.
2) In Fig. 1a, the authors compare the extent of transcription across nine tissues. However, they do not perform downsampling to exactly the same sequencing coverage before this analysis. Therefore, higher extent of transcription could be potentially explained by higher sequencing coverage in a particular tissue.
3) A similar concern about the transcriptional complexity analysis (Fig. 1b): higher transcriptional complexity is usually explained by the better quality of the sequencing library. The authors need to demonstrate that the quality of all libraries is comparable to make sure that this is not a follow-up of a technical feature.
4) At line 187, the authors state that “The genes embedded in the stable B compartment were primarily involved in synapse functions”. Do they have an interpretation why it happens?
5) What background did they use for the GO enrichment analysis presented at line 262 (and other similar analyses, e.g., at line 268)? Genes within TADs are not random genes per se (as genes active in a particular tissue are concentrated at TAD boundaries), and if they aim to make a statement about particular functions of tissue-specific TADs, a proper background would be all genes within TADs in the particular tissue.
6) At line 299, the authors state that “Tissue-specific PEIs tend to have longer lengths (190 kb vs. 130 kb) …”. Maybe these ‘Tissue-specific PEIs’ are just weaker PEIs, therefore sometimes they are not detected (because PSYCHIC is not so sensitively detects weaker PEIs)? And that's why they appear as tissue-specific? It is difficult to prove that the contacts the authors call 'tissue-specific PEIs' are really absent in some tissues. Maybe they are present but just below the sensitivity threshold? And this point is in line with the observed longer length of 'tissue-specific PEIs' -- longer contacts are always weaker in Hi-C data.
7) At line 346, what test did the authors use to demonstrate ‘significantly enriched’ genomic regions? What was the p-value?

Suggestions:
1) It would be very interesting to cluster chromosomes in Fig. 2c. In humans, chromosomes that have Nucleolus organizer regions (NORs) are usually located in close proximity to each other, forming a pronounced separate cluster. Is it the same in

pandas? It would be also very interesting to compare how pronounced this cluster is in different tissues (i.e., compare distances between NORs chromosomes across tissues).

Minor issues:
1) Minor English editing is needed. I am not a native speaker, but it looks like something is wrong with phrases on lines 124, 184, 243, 353, 391, 409, 410, 413, 415, 418, 428, 464, 532, and probably others.
2) The last section of the Results ('Chromosome conformation reveals the potential function of accelerated regions in giant pandas') needs major English editing.
3) There is a missed section number on line 385.
4) In section ''Chromosome conformation reveals the potential function of accelerated regions in giant pandas', there are ARs and ncGARs. In the related Fig. 7, there are GARs and ncGARs. Either the figure or the text need to edited for consistency.

Reviewer #2

(Remarks to the Author)
In this work, Liu and collaborators present an integrated analysis of 3D genome architecture and gene expression across nine tissues (kidney, liver, lung, muscle, large and small intestine and subcutaneous and visceral adipose tissue) of an adult female giant panda.
This manuscript presents well-supported statements, underpinned by the generation of approximately 300 million valid chromatin contacts per tissue, alongside matched transcriptional profiles obtained via RNA-seq. These data were processed into high-resolution (10 kb) Hi-C interaction matrices, representing an impressive resource for a non-model mammalian species such as the giant panda. Importantly, all code and analytical pipelines used in the study are well documented in the Methods section and made accessible, ensuring transparency and reproducibility of the analyses.
Using these datasets, authors claim this is the first systematic investigation of tissue-specific alterations in 3D genome organization in this species, revealing changes at different structural levels: i) A/B compartmentalization; ii) topologically associating domains (TADs); and iii) promoter-enhancer interactions. While the core hypotheses and concepts explored in this work (being the tissue-specificity of chromatin architecture and its regulatory implication) have already been discussed in mammalian model organisms, this study still provides important validations. Its significance lies not in conceptual novelty but in the generation of a high-quality, multi-tissue dataset for a non-model species. This type of dataset is essential to expand our understanding of genome regulation within mammalian species and is a valuable resource for future comparative genomic projects, conservation efforts, and studies of panda-specific biology. Notably, this data could also serve as a platform for investigating age-related changes, disease mechanisms, or environmental adaptation in this endangered species and may help to understand others.
The major limitation of this study, as author stated, is that the sample size is relatively small (only one individual), however this is a limitation inherent to work in endangered or protected species. Noteworthy, the data quality compensates for this to a considerable extent and the inclusion of clear examples to illustrate the claims is appreciated and strengthens the manuscript.
Overall, this is a well-executed and valuable study that contributes important genomic resources for a non-model and conservation-relevant species. While the overarching concepts are not novel, the quality of the datasets, the clarity of the presentation, and the integrative analysis across tissues make this work a meaningful addition to the field. Provided the authors address the minor concerns I am detailing below, I support the publication of this article.

Specific points:

• In the introduction (line 49), the authors state that global reorganization of 3D genome architecture across tissues or cell types has been studied in "several animals," but they only cite mammalian species (humans, mice, and pigs). To strengthen this section and better contextualize their contribution, I recommend the authors include examples of 3D genome studies in non-mammalian species (e.g., Drosophila, zebrafish, birds), where significant chromatin reorganization has also been described. I provide some examples:
o Chicken: 10.1093/nar/gky1103
o Drosophila: 10.1016/j.cell.2017.03.024
o Zebrafish: 10.1101/gr.269860.120

• In line 67, the following sentence is written: "we analysed the RNA-seq libraries from nine tissues of an adult female giant panda, including heart, kidney, large intestine, liver, lung, muscle, small intestine, subcutaneous and visceral adipose tissues ". For the sake of clarity, I will suggest writing large and small intestine together, as the two adipose tissues are mentioned together.

• The authors report (line 142) that in each tissue, over 60% of chromatin contacts are intrachromosomal (cis contacts), noting this is consistent with findings in other mammals. However, this observation has already been clearly described in previous studies of mammalian non-model species that are not cited in the current manuscript, such as: 10.1016/j.celrep.2022.111839 and 10.1186/s12915-022-01301-7

• In the section discussing compartment switching regions (line 189), the authors refer to codes such as "1A8B" and "8A1B" to describe tissue-specific compartment changes. While these are defined in the figure caption, it would improve readability and clarity if a brief explanation were included directly in the main text. This would help readers better follow the argument without needing to refer back and forth to the figure, particularly since these codes are central to the interpretation of tissue specificity.

• The authors conclude that there is a "high degree of plasticity" in A/B compartments across giant panda tissues (line 222). However, based on the data presented, approximately 70% of compartments remain stable across tissues. This seems inconsistent with the characterization of overall high plasticity. A more precise and accurate interpretation would be to state that specific regions of the genome exhibit high plasticity in compartment status, which are likely to be functionally significant.

• A Pearson correlation of 0.70 between the number of promoter–enhancer interactions (PEIs) and gene expression levels is reported at line 289, which they interpret as consistent with an additive enhancer effect. While this is a reasonable starting point, I wonder if this analysis may overlook the influence of enhancer redundancy (a phenomenon in which multiple enhancers can compensate for one another without leading to proportional changes in gene expression). I am curious whether the presence of such redundant enhancers might partially explain why the correlation is moderate rather than higher (e.g., closer to 0.9). As an exploratory suggestion, it could be informative to assess whether the correlation increases when excluding genes whose PEI numbers change but whose expression remains stable across tissues. This might provide a clearer picture of enhancer contribution to gene regulation.

• The authors state that PEIs "usually work in a tissue-specific manner" based on the finding that approximately 37.38% of PEIs are unique to a single tissue (line 297). However, this implies that the majority (over 60%) are shared across multiple tissues. Therefore, it would be more accurate to say that a substantial subset of PEIs is tissue-specific, rather than suggesting this is the usual behavior.

• The authors report that, on average, each promoter is associated with nine putative enhancers based on their chromatin interactome maps (line 305). However, given that the detected enhancers have a mean size of 150 kb and the highest resolution of the Hi-C dataset is 10 kb, I am concerned about the potential for inflated PEI counts due to resolution limits and interaction noise.
I suggest the authors address the possibility of false positives in their PEI calls and clarify whether any filtering or confidence scoring was applied to distinguish high-confidence interactions.

• The authors report that tissue-specific enhancers exhibit significantly higher enrichment scores compared to stable enhancers (line 351). While statistical significance is noted, it would greatly improve interpretability if the authors also reported the effect size (for example, the fold change in enrichment scores between tissue-specific and stable enhancers).

• The authors note that the distances between ncGAR enhancers and their putative target genes are “slightly longer” than those of other PEIs (line 409). For clarity and interpretability, I recommend including the actual average or median distances for both ncGAR and non-ncGAR PEIs.

Reviewer #3

(Remarks to the Author)
Summary:
In this study, Liu et al. systematically profiled the transcriptome and 3D genome architecture of nine tissue types from a female giant panda. The authors described that the A/B compartment switching are correlated with the gene expression across the tissues. The authors further revealed that TAD boundaries and promoter-enhancer interactions (PEIs) are highly dynamic across tissues, with tissue-specific TADs and PEIs closely associated with differential gene expression and functional specialization across tissues. Lastly, the authors demonstrated that tissue-specific enhancers are significantly enriched for adaptive selection signatures in different populations of giant panda, suggesting that tissue-specific gene regulation has played a key role in the species’ adaptive evolution. While this work provides a prominent and valuable reference for giant panda evolutionary research by systematically characterizing chromosome architecture, substantive methodological limitations, unsupported claims, and interpretive overstatements compromise its key findings. Specific comments follow:

Major:

1.A major concern is that the study focuses on a single individual without biological replicates. This reduces confidence the authors' conclaims, as all the observed effects could represent individual-specific variation or technical artifacts rather than consistent biological patterns.

2.To determine the tissue-type specificity and diversity at A/B compartment distribution, the authors calculated and compared those A/B compartment switch across tissues at 20-kb resolution, which is quite different from to previous genome-wide compartment profiles at typical resolutions ranging from 40 kb to 500 kb in human cells. Higher resolution for analysis requires deeper sequencing depth (e.g., billion level unique mapped valid read pairs as the author mentioned (Rowley et al. Molecular Cell 2017)). One concern is that the authors need to provide more evidence to support the matched quality of Hi-C library and check the robustness of AB compartment identification at different resolutions.

3.The authors claimed that “~62.14% of TAD boundaries were changed in at least one tissue” appears inconsistent with established literature demonstrating relative TAD conservation across mammalian tissues. Without replicates, the high proportion of dynamic boundaries likely reflects technical noise rather than biological reality. I recommend removing the TADs analysis, as the data cannot robustly support the conclusion.

4.Loop calling methods are highly variable under different parameters or methods, it is quite necessary to ensure the precision of promoter-enhancer interactions either by experiments or analysis alone. The author neither provide the analysis robustness of PEI or loop identification with various typical approaches to ensure the false positive rate of PEI identification, nor the widely used APA plot analysis, which seems not rigorous. As shown in Fig. 5G-H, no visually discernible looping structures are apparent at claimed PEI loci in the Hi-C contact heatmaps.

5.In Fig. 6 C-D, the high variability in the contact matrices shows that the reported EP interactions may represent stochastic noise rather than biologically significant signals.

Minor:
1.The definitions of tissue-specific PCGs and tissue-enchanced PCGs are not clear and could confuse readers. The 'specific' and 'enhanced' genes were summaritzed in supplementary figure 7 and showed firaction difference wihin one specifc tissue types. However, the author have not clarify those difference(e.g. genomic profiles or functions) of gene set with different difinitions. Are these terms synonymous or distinct? Better definitions or discriptions of those should be provided with rationales.

2.No statistics of significance was made or shown for those genes in the tissue-specific A compartment in Supplementary Fig14 boxplot as similar as in Supplementary Fig21 boxplot, better to add since the author mentioned the tissue specific correlation.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Biology initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors answered all my comments in detail, and I have no further reservations regarding this manuscript. My main concern - the absence of biological replicates - remains unresolved, but the authors clearly explain why it is impossible to obtain more replicates. Therefore, I think it is up to the Editor to decide whether it is acceptable to publish a study without replicates in this case.

Minor note: I would suggest to include the downsampling analysis in the manuscript (not only in the rebuttal letter). It is a technical but important analysis.

Reviewer #2

(Remarks to the Author)
The revisions the authors made to their manuscript, "A Compendium of Chromatin Interaction Maps in the Giant Panda Genome" have broadly addressed my comments. I am largely convinced by the new data analysis and revised text, and I support publication.

As I stated in my previous review, regardless of the data limitation, I think this work is important because it generates a high-quality, multi-tissue dataset for a non-model species. This type of dataset is essential to expand our understanding of genome regulation within mammalian species and is a valuable resource for future comparative genomic projects, conservation efforts, and studies of panda-specific biology. Notably, this data could also serve as a platform for investigating age-related changes, disease mechanisms, or environmental adaptation in this endangered species and may help to understand others.

Reviewer #3

(Remarks to the Author)
All of my concerns have been addressed.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Biology initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who

co-review manuscripts.

Dear editor and reviewers,

Thank you for your consideration of our manuscript for publication in ***Communication Biology***.

We sincerely appreciate the thoughtful and constructive comments from the four anonymous reviewers, and your assistance in improving the manuscript. We have gone through all the reviewers' comments in detail and believe that we have addressed their questions and concerns. Below we provide a copy of the reviewers' comments with our point-to-point responses.

We look forward to hearing a positive response from you.

Best regards,

Yan Li

Detailed responses to Reviewers

Below, all critique and suggestions provided by reviewers are cited in gray italics, and our responses are in black. All revisions in the manuscript are marked in red.

Reviewer 1:

The manuscript entitled "A Compendium of Chromatin Interaction Maps in the Giant Panda Genome" by Pengliang Liu et al. presents a comparative analysis of bulk RNA-seq and Hi-C data obtained by the authors for nine giant panda tissues. This is a highly descriptive study, presenting an overview of differences between tissues at various levels: transcriptomic, promoter-enhancer interactions, TADs, compartments, etc. The multi-tissue transcriptomes for the giant panda were published before but the multi-tissue 3D genome is novel for the giant panda and might be of interest to the community and the wider field. The most valuable and interesting part, in my opinion, is the evolutionary analysis presented in two last sections of the Results: '3D chromatin maps enhance the biological interpretations of adaptive evolution' and 'Chromosome conformation reveals the potential function of accelerated regions in giant pandas'. While the manuscript is clearly written and the presented analysis is overall robust, I have several major concerns that need to be considered before this work can be published.

Response:

We appreciate the reviewer's comments and helpful suggestions for our manuscript. Below we provide a copy of the reviewers' comments with our point-to-point responses.

Comment 1-1:

The greatest limitation of this study is the absence of biological replicates. Thel authors analyzed only one giant panda specimen (only one replicate per tissue). Therefore, the authors cannot perform (and do not perform) any traditional statistical analyses such as differential expression analysis (which is technically impossible when there are no replicates). They can only operate in terms like 'more expressed' or 'less expressed', and

the whole manuscript is written in such a manner. Which is technically correct but it is sad that the majority of statements presented in the manuscript cannot be confirmed by proper statistics. Very few analyses in the manuscript are confirmed with p-values.

Response:

We acknowledge that the lack of biological replicates is an important limitation. This constraint stems from strict legal protections for giant pandas under Chinese law, which prohibits killing or collecting tissue from live specimens and mandates non-invasive research methods. For our study, the tissue samples were obtained from a giant panda that tragically died accidentally, which limited our ability to acquire multiple biological replicates.

However, we have conducted various analyses to validate the reliability of our data. For the transcriptome data, we merge our data with the previously published RNA-seq data of five giant panda tissues from five individuals (four females and one male) [1], including heart, liver, spleen, lung, and kidney. Clustering analysis indicated that the samples of the same tissues are clustered together (**Fig. R1**). For the same tissue, our data are highly correlated with previously published RNA-seq data. This finding give confidence in the reliability of our RNA-seq data. For gene expression analysis, we first normalized for sequencing depth across samples. We then identified tissue-specific genes and housekeeping genes by the tau score (τ) with a stringent cutoff ($\tau > 0.80$ for tissue-specific genes, $\tau < 0.20$ for housekeeping genes). To further validate the reliability of these genes, we mapped our identified tissue-specific and housekeeping genes to the above-mentioned public RNA-seq data [1]. We found that the identified tissue-specific and housekeeping genes in this study also exhibit the tissue-specific and housekeeping expression patterns in previously published RNA-seq data of giant pandas, respectively, which helped confirm the reliability and reproducibility of our results (**Supplementary Fig. 6**). In addition, functional enrichment results indicated that the tissue-specific genes are linked to functions that are highly specific to particular tissues, aligning with our existing understanding of the roles of tissue-specific genes. Indeed, in response to the concerns about individual

differences, we also leveraged public data to analyze potential transcriptional differences between male and female individuals. The analysis revealed that only few genes exhibited differential expression between male and female individuals (ranging from 2 to 10, FDR ≤ 0.01, $|\log_2 FC| \geq 2$) (**Fig. R2**). This suggests that, in the context of large-scale tissue-specific transcriptional differences, individual variation has a relatively minor impact. Together, these results confirmed the reliability of our RNA-seq.

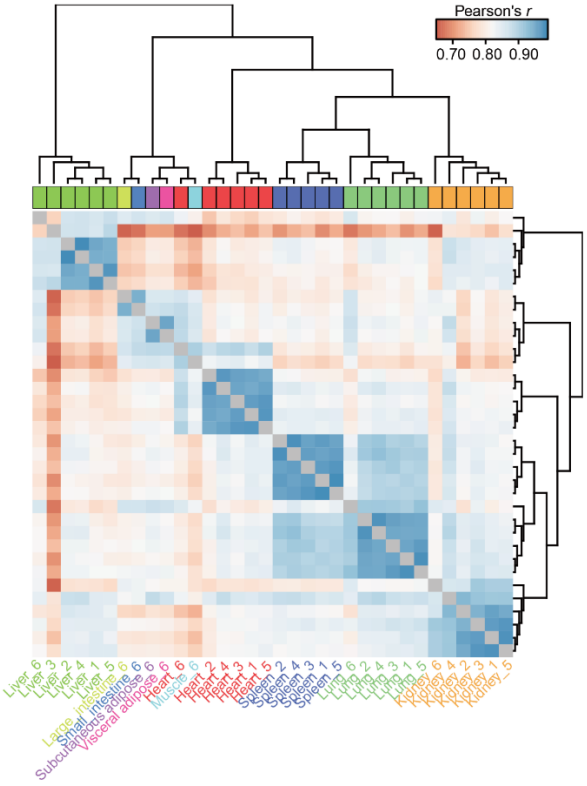


Fig. R1 Pearson's correlation coefficient heatmap of gene expression comparisons between pairwise samples. Sample suffix with '_6' represents the individual used in our study. The suffix with '_5' or '_6' represents the female sample and suffix with '_1' to '_4' represent the male sample.

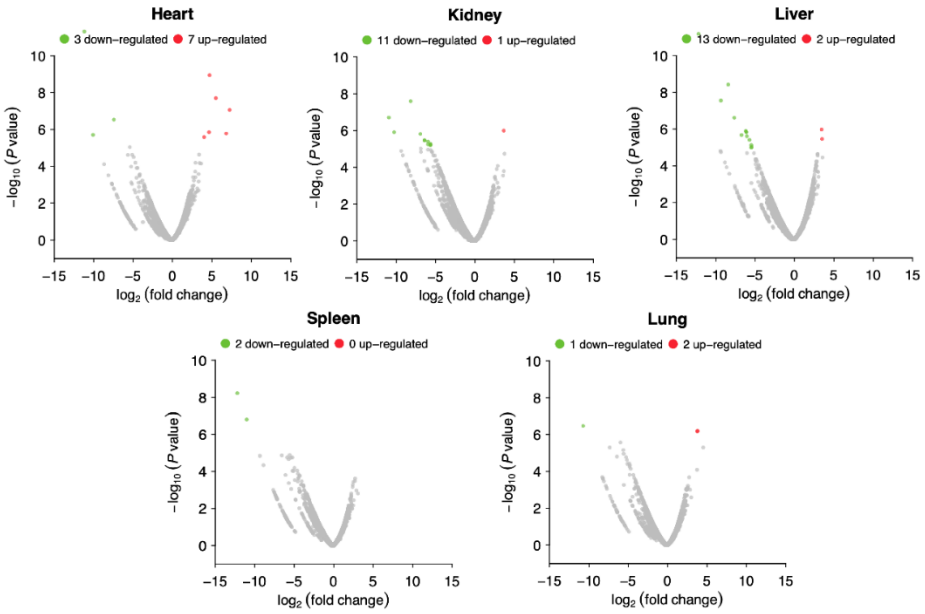
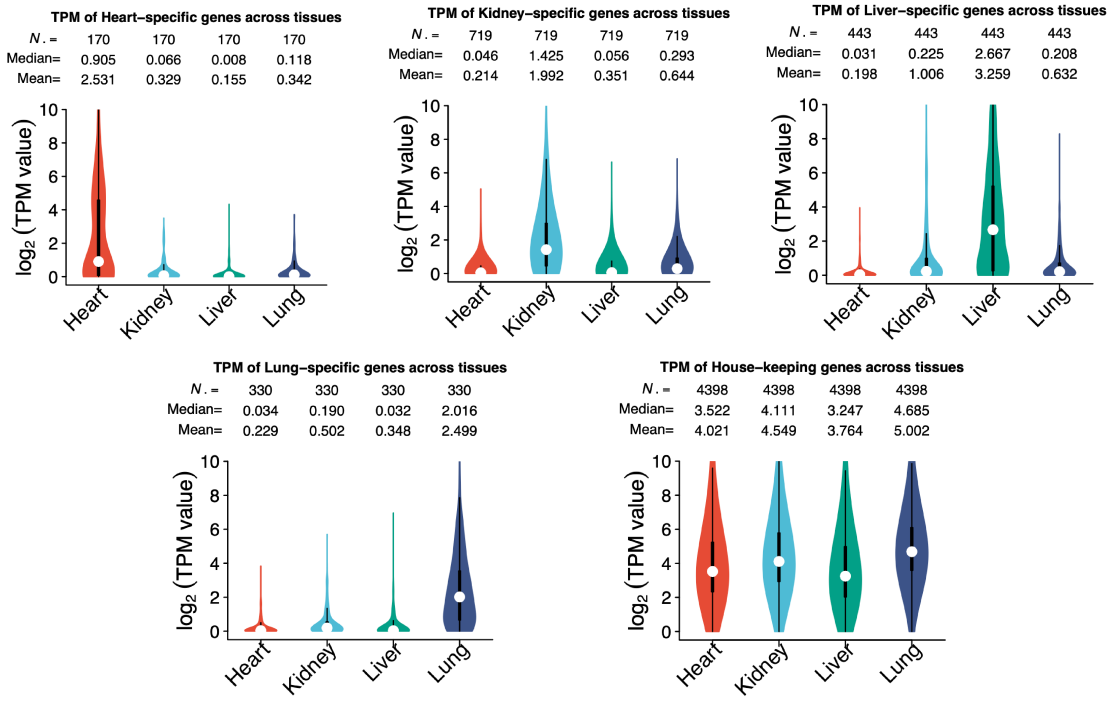


Fig. R2 Volcano plots showing the differentially expressed genes (cutoff: FDR ≤ 0.01, |log_2FC| ≥ 2) for the sex comparison of each tissue.



Supplementary Fig .6. The expression pattern of our identified tissue-specific and housekeeping PCGs in the RNA-seq data of a previous article

For the Hi-C data, many results also proved the reliability of our dataset. (1) The characterizations of our identified 3D genome structures in line with the findings of previous studies in other mammalian species. For example, (i) the A compartments

were enriched for GC content and PCGs compared with the B compartments[2]; (ii) the TAD boundaries were enriched for the transcription start sites of PCGs[3,4]; (iii) the most PEIs occur within the same TAD[5]. (2) Consistent with previous results[2,4,6], the 3D genome structures also highly correlated with the transcriptional activity of PCGs. For instance, the PCGs with higher AB index or more PEIs tend to have higher expression levels[6].

Taken together with the other reviewers' comments (**Comment 2-8, 3-4**), we have also added the verification analysis of the reliability of our identified PEI structures. Briefly, we performed aggregating peak analysis for the identified PEIs in each tissue, which indicated the accuracy of PEI calling (APA score>2.05). Moreover, to confirm our identified PEIs have biological implications, we checking the published human H3K27ac peak data from the ENCODE database. We found that 48,742 our identified enhancer anchors (54.1%) in the giant panda overlapped with the identified human peak regions. Additionally, tissue-specific enhancers in the giant panda generally correspond with those in humans. Furthermore, we randomly selected one enhancer associated with the promoter of gene (*ARRDC3*), for validation in HEK-293T cells utilizing the Dual-Luciferase reporter assay. The results demonstrated a statistically significant increase in transcriptional activity for the tested enhancers compared to the controls. A more detailed response can also be found in the response to **Comment 2-8 and 3-5**.

Altogether, these results support the reliability of our RNA-seq and Hi-C data.

Comment 1-2:

In Fig. 1a, the authors compare the extent of transcription across nine tissues. However, they do not perform downsampling to exactly the same sequencing coverage before this analysis. Therefore, higher extent of transcription could be potentially explained by higher sequencing coverage in a particular tissue.

Response:

Indeed, the transcript quantification was performed by using kalisto software[7]. The

transcript abundance was estimated directly in TPM (Transcripts Per Kilobase Million). The TPM measurement includes normalization for transcript length and sequencing depth across samples. We have modified the relative description in the '**Methods**' section to make it easier for readers to follow (**Main text, Page 21, Lines 595-596**).

"The TPM measurement includes normalization for transcript length and sequencing depth across samples"

Moreover, we also analyzed the correlation between the sequencing depth and the number of detected genes in the tissues. Our findings revealed no clear correlation between them (r = 0.063) (**Fig. R3**), suggesting the efficacy and reliability of our approach in adjusting for sequencing depths.

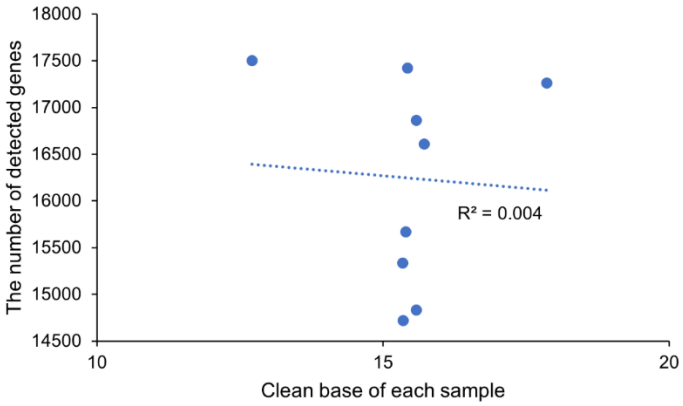


Fig. R3 The relationship between the sequencing depth and the number of detected genes in the giant panda tissues.

Indeed, due to the nature of our experimental design or available data, comparisons were performed without biological replicates. In this case, we used the edgeR package with a fixed biological coefficient of variation (BCV) to estimate dispersion, following the guidelines provided by the developers of edgeR [8].

Specifically, for each pairwise comparison, we performed normalization using the TMM method, and set the BCV to 0.1, which is a conservative estimate commonly used for moderately variable biological systems. This allowed us to perform exact tests despite the absence of replicates.

We acknowledge that DEG results without replicates must be interpreted with caution, and as such, our analysis focuses on exploratory identification of potentially interesting genes, which can serve as candidates for further validation. To increase robustness, we also applied stringent thresholds (FDR < 0.05 and |log2FC| > 1) to define differentially expressed genes

Comment 1-3:

A similar concern about the transcriptional complexity analysis (Fig. 1b): higher transcriptional complexity is usually explained by the better quality of the sequencing library. The authors need to demonstrate that the quality of all libraries is comparable to make sure that this is not a follow-up of a technical feature.

Response:

As we mentioned before, the sequencing depth were normalized in our study. To further verify the reliability, we also perform downsampling for our RNA-seq data and examined the transcriptional complexity across tissues. We found the results were consistent with our previous findings in the manuscript (**Fig. R4**), for example, the transcriptome complexity of large and small intestine was higher than that in other tissues, while the muscle had the lowest transcriptome complexity.

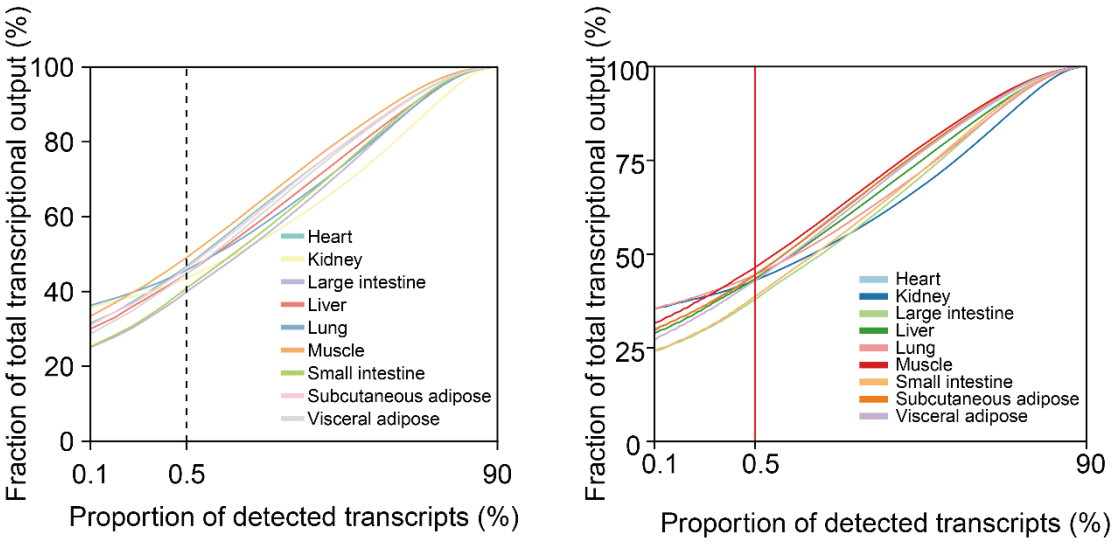


Fig. R4. Distribution of PCG abundance in each tissue by using our previous method (left panel) and the downsampling method (right panel). The x-axis represents the proportion of detected

PCGs, sorted from the most to the least abundance, with the vertical line (red) indicates the top 0.5% of highest abundance PCGs. The y-axis represents the accumulated fraction of expressed transcripts relative to the total number of transcripts in each tissue.

Comment 1-4:

At line 187, the authors state that "The genes embedded in the stable B compartment were primarily involved in synapse functions". Do they have an interpretation why it happens?

Response:

It is known that the synapses are information-processing units of the brain. A previous study has shown that the expression levels of synaptic genes are higher in the brain than in other tissues in humans[9]. Considering that the 9 tissues collected in our study did not include nervous-system tissues (e.g., brain). Therefore, we believe that the observed enrichment of synapse-related genes in stable B compartments possibly due to these genes is mainly functional in nervous system rather than in peripheral tissues.

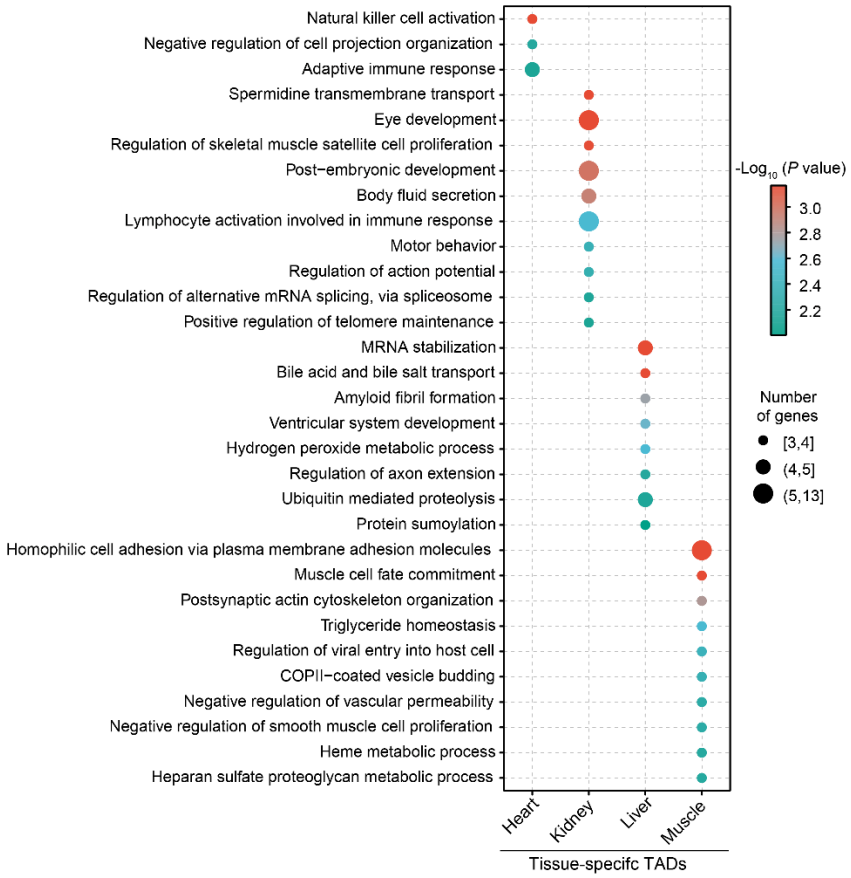
Comment 1-5:

What background did they use for the GO enrichment analysis presented at line 262 (and other similar analyses, e.g., at line 268)? Genes within TADs are not random genes per se (as genes active in a particular tissue are concentrated at TAD boundaries), and if they aim to make a statement about particular functions of tissue-specific TADs, a proper background would be all genes within TADs in the particular tissue.

Response:

Thanks for this valuable suggestion. In the TAD comparison, a subset of genes within TADs in the particular tissue was used as background in the functional enrichment analysis. We found that many previously identified enriched pathways remain present. Notably, "muscle cell fate commitment" was significantly enriched in muscle-specific TADs, "body fluid secretion" was significantly enriched in kidney-specific TADs, and "bile acid and bile salt transport" was significantly enriched in liver-specific TADs. We have modified the corresponding figure (**Supplementary figure 18c**).

c



Supplementary Fig. 18. c Functional enrichment for the PCGs located within the tissue-specific TADs. Dot color represents the $-\log_{10}$ (*P* value) calculated by one-sided accumulative hypergeometric test, and the size of the dot represents the number of PCGs enriched in the category.

Comment 1-6:

At line 299, the authors state that "Tissue-specific PEIs tend to have longer lengths (190 kb vs. 130 kb) …". Maybe these 'Tissue-specific PEIs' are just weaker PEIs, therefore sometimes they are not detected (because PSYCHIC is not so sensitively detects weaker PEIs)? And that's why they appear as tissue-specific? It is difficult to prove that the contacts the authors call 'tissue-specific PEIs' are really absent in some tissues. Maybe they are present but just below the sensitivity threshold? And this point is in line with the observed longer length of 'tissue-specific PEIs' -- longer contacts are always weaker in Hi-C data.

Response:

Yes, for the PEI that was not detected by PSYCHIC software, it cannot be simply assumed that it does not exist. Therefore, we have modified the description of ‘tissue-specific PEIs’ to ‘tissue-specific enhanced PEIs’ in revised manuscript (**Main text, Page 12, Lines 329-341**). Moreover, we have added aggregating peak analysis (APA) in this section, which demonstrated the accuracy of the identification of tissue-specific enhanced PEIs (**Supplementary Fig. 23**). Finally, we have removed the description about the length of 'tissue-specific enhanced PEIs' in the revised manuscript.

The changes made in the manuscript are as follow (**Page 12, Lines 327-333**):

“We next performed a comparative analysis to characterize the dynamics of PEIs among tissues. Approximately 37.38% of PEIs (71,077 of 190,126 PEIs) were identified in only one tissue and are thus classified as tissue-specific enhanced PEIs (**Fig. 5a**). This finding indicates that a substantial subset of PEIs works in a tissue-specific manner, consistent with observations documented in other mammalian studies[48,49]. APA validated the accuracy of the identification of tissue-specific enhanced PEIs (**Supplementary Fig. 23**).”

Supplementary Fig. 23. Normalized Hi-C signals around tissue-specific PEIs in each giant panda tissue at 10 kb resolution.

Comment 1-7:

At line 346, what test did the authors use to demonstrate 'significantly enriched' genomic regions? What was the p-value?

Response:

We calculated the ratio of the selection signatures located within enhancer regions and compared with the randomly selected regions. *P* value is calculated by chi-square test. We have added the corresponding description as follows (**Main text, Page 14, Lines 381-383**):

“We found that the genomic regions under selection were significantly enriched in enhancers (1184 of 3741, 31.65%, chi-square test, *P* = 0.019, compared to random regions).”

Comment 1-8:

It would be very interesting to cluster chromosomes in Fig. 2c. In humans, chromosomes that have Nucleolus organizer regions (NORs) are usually located in close proximity to each other, forming a pronounced separate cluster. Is it the same in pandas? It would be also very interesting to compare how pronounced this cluster is in different tissues (i.e., compare distances between NORs chromosomes across tissues).

Response:

We thank the reviewer for this excellent and insightful suggestion. Following the reviewer's advice, we performed a comprehensive bioinformatic analysis, and our results strongly confirm that a similar, pronounced clustering occurs in the giant panda across all tissues studied. Below, we detailed our analytical approach and key findings.

(1) Identification of NOR-bearing chromosomes in the giant panda

We annotated the giant panda reference genome using RepeatMasker (v4.2.0) to identify ribosomal DNA (rDNA) sequences. Regions with dense, contiguous annotations of 5.8S, 18S, and 28S rRNA genes were defined as Nucleolus Organizer Regions (NORs). This analysis predicted that giant panda chromosomes 4, 7, 11, 12, 13, and 16 contained NORs. This distribution differed from that in humans, where NORs were located on acrocentric chromosomes 13, 14, 15, 21, and 22, likely reflecting species-specific genomic architecture.

(2) Analysis of NOR-chromosome spatial clustering

To quantitatively assess the spatial proximity of these chromosomes, we extracted the normalized interaction contacts matrix with 1Mb resolution across nine tissues. For each tissue, we classified all inter-chromosomal contact pairs into three categories, which were NOR vs. NOR (Interactions between two NOR chromosomes), NOR vs.

Non-NOR (Interactions between a NOR chromosome and a non-NOR chromosome), and Non-NOR vs. Non-NOR (Interactions between two non-NOR chromosomes). The normalized interaction contacts for each pair were used as a proxy for spatial distance, with higher value indicating shorter distance.

(3) Tissue-specific comparison of clustering prominence

We compared the normalized interaction strengths among the three categories across all nine tissues using the Wilcoxon rank-sum test (a non-parametric test for two independent samples). The results were highly consistent and striking. The median interaction strength of the NOR vs. NOR group was extremely significantly higher ($P < 0.0001$) than that of both the NOR vs. Non-NOR group and the Non-NOR vs. Non-NOR group in the nine tissues analyzed (**Supplementary Fig. 11**).

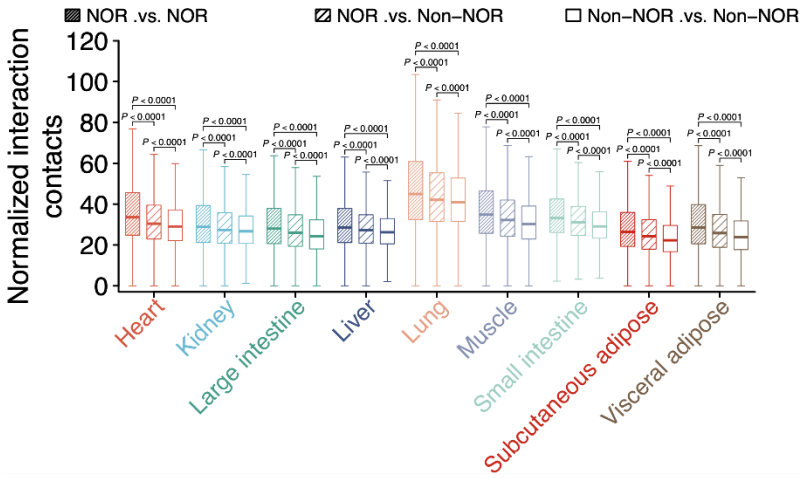
Furthermore, the interaction strength of the NOR vs. Non-NOR group was also extremely significantly higher ($P < 0.0001$) than that of the Non-NOR vs. Non-NOR group. This clear statistical hierarchy (NOR vs. NOR > NOR vs. Non-NOR > Non-NOR vs. Non-NOR) provided robust evidence that NOR-chromosomes formed a distinct, tight cluster in the 3D nuclear space.

Our analysis revealed that this pronounced clustering of NOR chromosomes was a consistent and ubiquitous feature across all nine tissues examined. The statistical significance was identical ($P < 0.0001$) in every case, indicating that the phenomenon was not tissue-specific but rather a fundamental aspect of giant panda nuclear organization.

In summary, we demonstrated that despite the difference in genomic location of NORs, the giant panda exhibited a strong tendency for NOR chromosomes to cluster together, mirroring the phenomenon observed in humans. This suggested a conserved mechanism of nucleolar-associated chromosome organization in mammals.

We have added these results to the manuscript as follows (**Main text, Page 6, Lines 153-159**).

"Notably, we found that chromosomes containing nucleolus organizer regions (NORs) tend to be located close to each other (as indicated by the contact frequency between the chromosomes), compared to the distances observed between chromosomes with NORs and those without, as well as between pairs of chromosomes lacking NORs (*P* < 0.0001, Wilcoxon rank-sum test) (**Supplementary Fig. 11**). The same trend was observed in all detected giant panda tissues (**Supplementary Fig. 11**)."



Supplementary Fig. 11. Normalized interaction strengths between two NOR chromosomes (NOR vs. NOR), NOR chromosome and non-NOR chromosome (NOR vs. Non-NOR), and two non-NOR chromosomes (Non-NOR vs. Non-NOR).

Comment 1-9:

Minor English editing is needed. I am not a native speaker, but it looks like something is wrong with phrases on lines 124, 184, 243, 353, 391, 409, 410, 413, 415, 418, 428, 464, 532, and probably others.

Response:

We have thoroughly checked and polished the English of our manuscript with the help of a native English speaker.

Comment 1-10:

The last section of the Results ('Chromosome conformation reveals the potential function of accelerated regions in giant pandas') needs major English editing.

Response:

We have carefully polished and improved the English writing.

Comment 1-11:

There is a missed section number on line 385.

Response:

We have corrected this issue.

Comment 1-12:

In section "Chromosome conformation reveals the potential function of accelerated regions in giant pandas', there are ARs and ncGARs. In the related Fig. 7, there are GARs and ncGARs. Either the figure or the text need to edited for consistency.

Response:

We are sorry for our oversight. We have checked and corrected all (**Main text, Pages 15-16, Lines 432-442**).

Reviewer 2:

In this work, Liu and collaborators present an integrated analysis of 3D genome architecture and gene expression across nine tissues (kidney, liver, lung, muscle, large and small intestine and subcutaneous and visceral adipose tissue) of an adult female giant panda.

This manuscript presents well-supported statements, underpinned by the generation of approximately 300 million valid chromatin contacts per tissue, alongside matched transcriptional profiles obtained via RNA-seq. These data were processed into high-resolution (10 kb) Hi-C interaction matrices, representing an impressive resource for a non-model mammalian species such as the giant panda. Importantly, all code and analytical pipelines used in the study are well documented in the Methods section and made accessible, ensuring transparency and reproducibility of the analyses.

Using these datasets, authors claim this is the first systematic investigation of tissue-specific alterations in 3D genome organization in this species, revealing changes at different structural levels: i) A/B compartmentalization; ii) topologically associating domains (TADs); and iii) promoter-enhancer interactions. While the core hypotheses and concepts explored in this work (being the tissue-specificity of chromatin architecture and its regulatory implication) have already been discussed in mammalian model organisms, this study still provides important validations. Its significance lies not in conceptual novelty but in the generation of a high-quality, multi-tissue dataset for a non-model species. This type of dataset is essential to expand our understanding of genome regulation within mammalian species and is a valuable resource for future comparative genomic projects, conservation efforts, and studies of panda-specific biology. Notably, this data could also serve as a platform for investigating age-related changes, disease mechanisms, or environmental adaptation in this endangered species and may help to understand others.

The major limitation of this study, as author stated, is that the sample size is relatively small (only one individual), however this is a limitation inherent to work in endangered or protected species. Noteworthy, the data quality compensates for this to a considerable

extent and the inclusion of clear examples to illustrate the claims is appreciated and strengthens the manuscript.

Overall, this is a well-executed and valuable study that contributes important genomic resources for a non-model and conservation-relevant species. While the overarching concepts are not novel, the quality of the datasets, the clarity of the presentation, and the integrative analysis across tissues make this work a meaningful addition to the field. Provided the authors address the minor concerns I am detailing below, I support the publication of this article.

Response:

We appreciate the reviewer's comments and helpful suggestions for our manuscript. Below we provide a copy of the reviewers' comments with our point-to-point responses.

Comment 2-1:

In the introduction (line 49), the authors state that global reorganization of 3D genome architecture across tissues or cell types has been studied in "several animals," but they only cite mammalian species (humans, mice, and pigs). To strengthen this section and better contextualize their contribution, I recommend the authors include examples of 3D genome studies in non-mammalian species (e.g., Drosophila, zebrafish, birds), where significant chromatin reorganization has also been described. I provide some examples:

o Chicken: 10.1093/nar/gky1103

o Drosophila: 10.1016/j.cell.2017.03.024

o Zebrafish: 10.1101/gr.269860.120

Response:

We appreciate the suggestion. We have now expanded the citations of relevant work in non-mammalian species, including chicken, goose, zebrafish and Drosophila (**Main text, Pages 2, Lines 49-54**).

"Global reorganization of the 3D genome organizations between tissues or cell types

has been studied in multiple animal models, encompassing both mammalian species (such as humans[19,20], mice[21,25,26], pigs[27]) and non-mammalian species (including chickens[24,28], pigeons[29], Drosophila[30,31] and zebrafish[32]. These studies have contributed to a more detailed and sophisticated mechanistic understanding of phenotypic differences."

Comment 2-2:

In line 67, the following sentence is written: "we analysed the RNA-seq libraries from nine tissues of an adult female giant panda, including heart, kidney, large intestine, liver, lung, muscle, small intestine, subcutaneous and visceral adipose tissues ". For the sake of clarity, I will suggest writing large and small intestine together, as the two adipose tissues are mentioned together.

Response:

We have revised this sentence as suggested (**Main text, Page 3, Lines 69-72**):

"we analysed the RNA-seq libraries from nine tissues of an adult female giant panda, including heart, kidney, liver, lung, muscle, large and small intestine, subcutaneous and visceral adipose tissues."

Comment 2-3:

The authors report (line 142) that in each tissue, over 60% of chromatin contacts are intrachromosomal (cis contacts), noting this is consistent with findings in other mammals. However, this observation has already been clearly described in previous studies of mammalian non-model species that are not cited in the current manuscript, such as: 10.1016/j.celrep.2022.111839 and 10.1186/s12915-022-01301-7

Response:

Thanks for the reviewer's suggestion. We have supplemented the relevant references (**Main text, Page 6, Lines 147-150**):

"In each tissue, more than 60% chromatin contacts occurred within one chromosome

(i.e., intrachromosomal contacts, also known as *cis* contacts) (**Supplementary Fig. 10a**), in line with in line with previous findings for other mammalian and non-mammalian species[41,42].”

Comment 2-4:

In the section discussing compartment switching regions (line 189), the authors refer to codes such as “1A8B” and “8A1B” to describe tissue-specific compartment changes. While these are defined in the figure caption, it would improve readability and clarity if a brief explanation were included directly in the main text. This would help readers better follow the argument without needing to refer back and forth to the figure, particularly since these codes are central to the interpretation of tissue specificity.

Response:

Thank you very much for this helpful suggestion. We added following explication in the ‘Results’ (**Main text, Page 8, Lines 210-212**), hope this improvement will help readers understand our paper more easily:

“‘XA/YB’ represents the genomic region where X tissue displays A compartment status and the other Y tissues display B compartment status.”

Comment 2-5:

The authors conclude that there is a "high degree of plasticity" in A/B compartments across giant panda tissues (line 222). However, based on the data presented, approximately 70% of compartments remain stable across tissues. This seems inconsistent with the characterization of overall high plasticity. A more precise and accurate interpretation would be to state that specific regions of the genome exhibit high plasticity in compartment status, which are likely to be functionally significant.

Response:

Thanks very much for your constructive suggestion. We have revised our original description as follows (**Main text, Page 9, Lines 242-245**):

"Altogether, through cross-tissue comparisons, we were able to identify the specific regions of the genome in the giant panda that exhibit high plasticity in compartment status, which correlated with gene expression changes and closely related to tissue- or system-specific functions."

Comment 2-6:

A Pearson correlation of 0.70 between the number of promoter–enhancer interactions (PEIs) and gene expression levels is reported at line 289, which they interpret as consistent with an additive enhancer effect. While this is a reasonable starting point, I wonder if this analysis may overlook the influence of enhancer redundancy (a phenomenon in which multiple enhancers can compensate for one another without leading to proportional changes in gene expression). I am curious whether the presence of such redundant enhancers might partially explain why the correlation is moderate rather than higher (e.g., closer to 0.9). As an exploratory suggestion, it could be informative to assess whether the correlation increases when excluding genes whose PEI numbers change but whose expression remains stable across tissues. This might provide a clearer picture of enhancer contribution to gene regulation.

Response:

We appreciate the suggestion. We applied the 'tau' score to evaluate the fluctuation of gene expression among the tissues. As suggested, we excluded genes whose PEI numbers change but expression level remains stable across tissues (i.e., tau score<0.1) and recalculated the correlation coefficient. However, the correlation coefficient did not change significantly (Pearson's r = 0.71). Moreover, even the cutoff threshold becomes more stringent (tau score < 0.15), there were no notable changes in the correlation coefficients (Pearson's r = 0.71). Indeed, regulatory pathways of gene expression are very complex, with multiple factors in each regulatory step. Thus, we believe that the changes of PEIs are contributory but not fully explanatory in the expression patterns of genes among giant panda tissues.

We also added this corresponding description in the manuscript (**Main text, Pages 11-**

11, Lines 313-315):

"This finding suggests that while PEIs contribute to the observed patterns of gene expression in giant panda tissues, they do not entirely explain the variability in expression levels."

Comment 2-7:

The authors state that PEIs "usually work in a tissue-specific manner" based on the finding that approximately 37.38% of PEIs are unique to a single tissue (line 297). However, this implies that the majority (over 60%) are shared across multiple tissues. Therefore, it would be more accurate to say that a substantial subset of PEIs is tissue-specific, rather than suggesting this is the usual behavior.

Response:

Thanks for the reviewer's suggestion. We have revised our original description as follows (**Main text, Page 12, Lines 328-331**):

"Approximately 37.38% of PEIs (71,077 of 190,126 PEIs) were identified in only one tissue and are thus classified as tissue-specific enhanced PEIs (**Fig. 5a**). This finding indicates that a substantial subset of PEIs works in a tissue-specific manner, consistent with observations documented in other mammalian studies[56,57]."

Comment 2-8:

The authors report that, on average, each promoter is associated with nine putative enhancers based on their chromatin interactome maps (line 305). However, given that the detected enhancers have a mean size of 150 kb and the highest resolution of the Hi-C dataset is 10 kb, I am concerned about the potential for inflated PEI counts due to resolution limits and interaction noise.

I suggest the authors address the possibility of false positives in their PEI calls and clarify whether any filtering or confidence scoring was applied to distinguish high-confidence interactions.

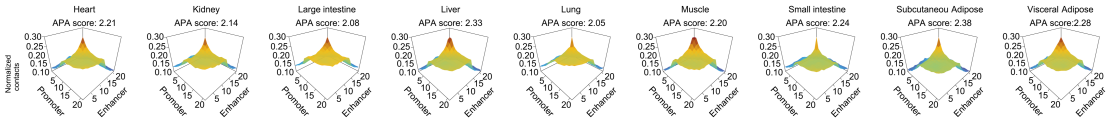
Response:

Thanks for the reviewer's suggestion. Indeed, we assign a statistical significance score (FDR) for each PEI and used stringent criteria to minimize false-positive identifications of PEIs in this study (FDR ≤ 0.001). Specifically, by subtracting the background model from the Hi-C data according to the PSYCHIC algorithm, we obtain the "residual" over-representation map. Statistical significance score (*P* values) is assigned using a log-Normal distribution fitted to the residuals in a 2Mb window surrounding each promoter, then corrected for multiple hypotheses (FDR). We have added the relevant description in the 'Result' section (**Main text, Page 11, Lines 300-302**).

"We employed PSYCHIC algorithm[52] to identify promoter-enhancer interactions (PEIs) and implemented rigorous filtering criteria to reduce false positives (FDR ≤ 0.001)."

Moreover, to assess the quality of PEI detection, we have also added aggregating peak analysis (APA) for the identified PEIs in each tissue, which demonstrated the accuracy of PEI calling (APA score>2.05) (**Supplementary Fig. 20**). The related description has been added to the revised Results (**Main text, Page 11, Lines 305-307**).

"Aggregating peak analysis (APA) indicated the accuracy and reliability of PEI calling (APA score>2.05) (**Supplementary Fig. 20**)"



Supplementary Fig. 20. Normalized Hi-C signals around the identified PEIs in each giant panda tissue at 10 kb resolution.

Additionally, to further assess the biological activity of our predicted enhancers, we conducted supplementary analyses. Initially, we downloaded comprehensive human H3K27ac peak data from the ENCODE database, encompassing 734 files and a total of 60,495,343 peaks derived from diverse human tissues and cell lines. Subsequently, we mapped 90,170 enhancers identified in giant pandas to the human Hg38 genome. Of these, 48,742 enhancers (54.1%) exhibited overlap with the human peak regions

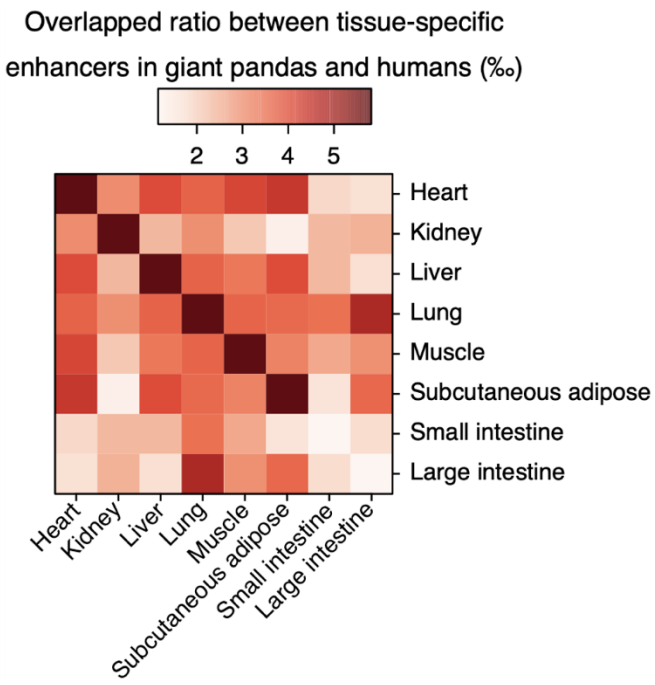
obtained, suggesting that our predicted enhancer regions demonstrate a considerable degree of reliability.

Furthermore, to investigate the conservation of tissue-specific enhancers across species, we organized the downloaded human peak data according to tissue type. Our analysis focused on eight tissues: heart, liver, kidney, lung, muscle, small intestine, large intestine, and subcutaneous adipose tissue. Utilizing bioinformatic techniques, we identified tissue-specific enhancers for these eight tissues in both giant pandas and humans. Subsequently, we mapped the tissue-specific enhancer regions from the giant panda genome to the human Hg38 genome and assessed their overlap ratios.

As illustrated in the figure below (**Supplementary Fig.30**), with the exception of the large and small intestines, giant panda tissue-specific enhancers were primarily situated within regions corresponding to human tissue-specific enhancers. This observation indicates a notable degree of conservation of tissue-specific enhancers between humans and giant pandas. The observed discrepancy in the large and small intestines can likely be attributed to the giant panda's specialized bamboo diet, which leads to significant differences in their digestive system relative to that of humans.

We have added the related contents in the '**Discussion**' section (**Main text, Page 19-20, Lines 551-559**):

"Moreover, at the PEI level, analysis of H3K27ac peak data from the ENCODE database revealed that 48,742 enhancer anchors (54.1%) overlapped with the identified human peak regions. Additionally, tissue-specific enhancers in the giant panda generally correspond with those in humans, with the notable exception of the large and small intestines (**Supplementary Fig.30**). This observation indicates a high degree of conservation of these enhancers between the species, with the differences in intestinal enhancers likely attributable to the panda's specialized bamboo diet and its impact on the digestive system. The validity of the putative enhancers interacting with promoters was confirmed through a Dual-Luciferase reporter assay."



Supplementary Fig.30. Overlapped ratio between tissue-specific enhancers in giant pandas and humans.

Comment 2-9:

The authors report that tissue-specific enhancers exhibit significantly higher enrichment scores compared to stable enhancers (line 351). While statistical significance is noted, it would greatly improve interpretability if the authors also reported the effect size (for example, the fold change in enrichment scores between tissue-specific and stable enhancers).

Response:

We appreciate the suggestion. We have added the following detailed information regarding the fold change in enrichment scores between tissue-specific and stable enhancers in the manuscript (**Main text, Page 14, Lines 385-388**).

"We found that the enrichment scores (selection signatures of Qinling population) for tissue-specific enhancers were significantly higher than those for stable enhancers (average fold change = 2.09, from 1.47 to 2.46) (**Fig. 6a**)"

Comment 2-10:

The authors note that the distances between ncGAR enhancers and their putative target genes are "slightly longer" than those of other PEIs (line 409). For clarity and interpretability, I recommend including the actual average or median distances for both ncGAR and non-ncGAR PEIs.

Response:

We apologize for not having provided more specific information. We have added the corresponding information in the manuscript (**Main text, Page 16, Lines 445-447**).

"The distance between ncGAR enhancers and target genes are slight longer than other PEIs (200 kb vs. 170 kb of non-ncGAR PEIs, $P = 1.11 \times 10^{-11}$, Wilcoxon rank-sum test)."

Reviewer 3

In this study, Liu et al. systematically profiled the transcriptome and 3D genome architecture of nine tissue types from a female giant panda. The authors described that the A/B compartment switching are correlated with the gene expression across the tissues. The authors further revealed that TAD boundaries and promoter-enhancer interactions (PEIs) are highly dynamic across tissues, with tissue-specific TADs and PEIs closely associated with differential gene expression and functional specialization across tissues. Lastly, the authors demonstrated that tissue-specific enhancers are significantly enriched for adaptive selection signatures in different populations of giant panda, suggesting that tissue-specific gene regulation has played a key role in the species' adaptive evolution. While this work provides a prominent and valuable reference for giant panda evolutionary research by systematically characterizing chromosome architecture, substantive methodological limitations, unsupported claims, and interpretive overstatements compromise its key findings. Specific comments follow:

Response:

We appreciate the reviewer's comments and helpful suggestions for our manuscript. Below we provide a copy of the reviewers' comments with our point-to-point responses.

Comment 3-1:

A major concern is that the study focuses on a single individual without biological replicates. This reduces confidence the authors' conclaims, as all the observed effects could represent individual-specific variation or technical artifacts rather than consistent biological patterns.

Response:

We acknowledge that the lack of biological replicates is an important limitation. This constraint stems from strict legal protections for giant pandas under Chinese law, which prohibits killing or collecting tissue from live specimens and mandates non-invasive research methods. For our study, the tissue samples were obtained from a giant panda that tragically died accidentally, which limited our ability to acquire multiple

biological replicates.

However, we have conducted various analyses to validate the reliability of our data. For the transcriptome data, we merge our data with the previously published RNA-seq data of five giant panda tissues from five individuals (four females and one male) [1], including heart, liver, spleen, lung, and kidney. Clustering analysis indicated that the samples of the same tissues are clustered together (**Fig. R1**). For the same tissue, our data are highly correlated with previously published RNA-seq data. This finding give confidence in the reliability of our RNA-seq data. For gene expression analysis, we first normalized for sequencing depth across samples. We then identified tissue-specific genes and housekeeping genes by the tau score (τ) with a stringent cutoff ($\tau > 0.80$ for tissue-specific genes, $\tau < 0.20$ for housekeeping genes). To further validate the reliability of these genes, we mapped our identified tissue-specific and housekeeping genes to the above-mentioned public RNA-seq data [1]. We found that the identified tissue-specific and housekeeping genes in this study also exhibit the tissue-specific and housekeeping expression patterns in previously published RNA-seq data of giant pandas, respectively, which helped confirm the reliability and reproducibility of our results (**Supplementary Fig. 6**). In addition, functional enrichment results indicated that the tissue-specific genes are linked to functions that are highly specific to particular tissues, aligning with our existing understanding of the roles of tissue-specific genes. Indeed, in response to the concerns about individual differences, we also leveraged public data to analyze potential transcriptional differences between male and female individuals. The analysis revealed that only few genes exhibited differential expression between male and female individuals (ranging from 2 to 10, FDR ≤ 0.01, $|\log_2 FC| \geq 2$) (**Fig. R2**). This suggests that, in the context of large-scale tissue-specific transcriptional differences, individual variation has a relatively minor impact. Together, these results confirmed the reliability of our RNA-seq.

For the Hi-C data, many results also proved the reliability of our dataset. (1) The characterizations of our identified 3D genome structures in line with the findings of previous studies in other mammalian species. For example, (i) the A compartments

were enriched for GC content and PCGs compared with the B compartments[2]; (ii) the TAD boundaries were enriched for the transcription start sites of PCGs[3,4]; (iii) the most PEIs occur within the same TAD[5]. (2) Consistent with previous results[2,4,6], the 3D genome structures also highly correlated with the transcriptional activity of PCGs. For instance, the PCGs with higher AB index or more PEIs tend to have higher expression levels[6].

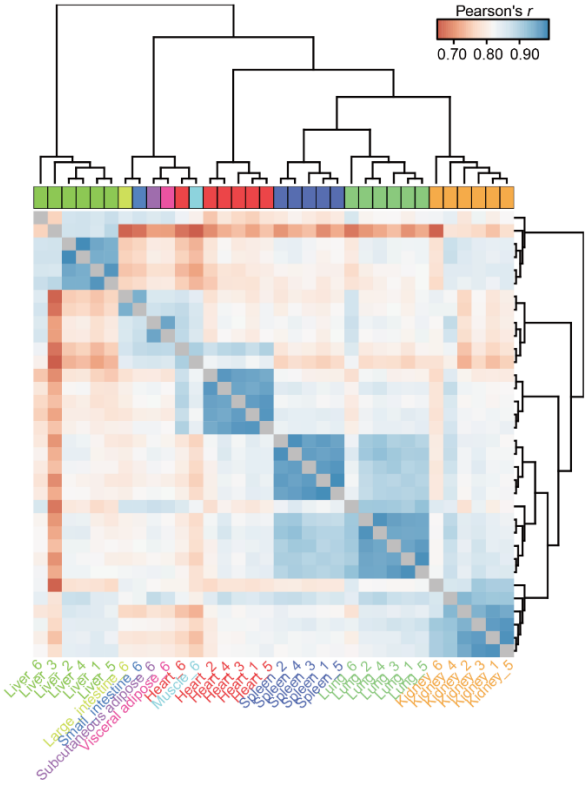


Fig. R1 Pearson's correlation coefficient heatmap of gene expression comparisons between pairwise samples. Sample suffix with '_6' represents the individual used in our study. The suffix with '_5' or '_6' represents the female sample and suffix with '_1' to '_4' represent the male sample.

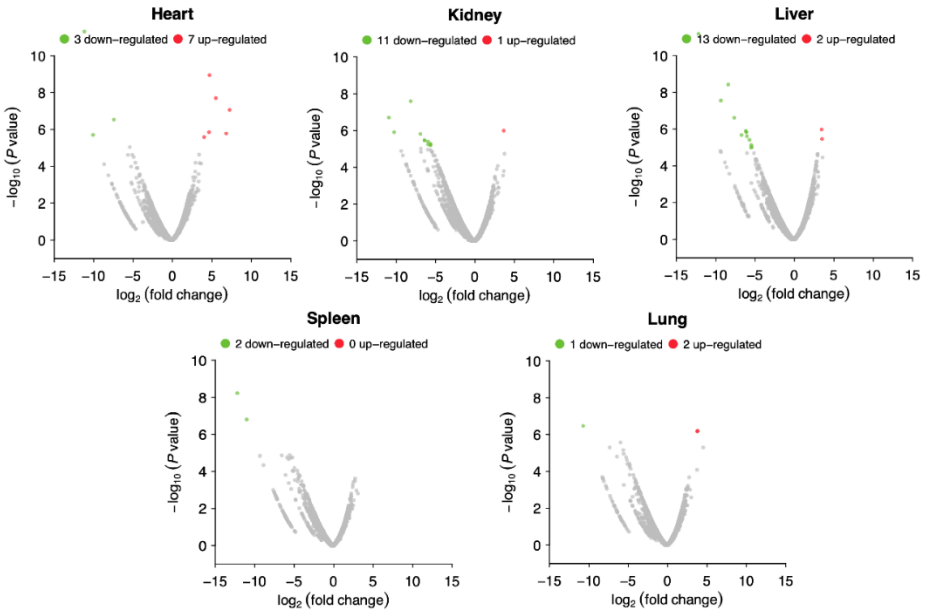
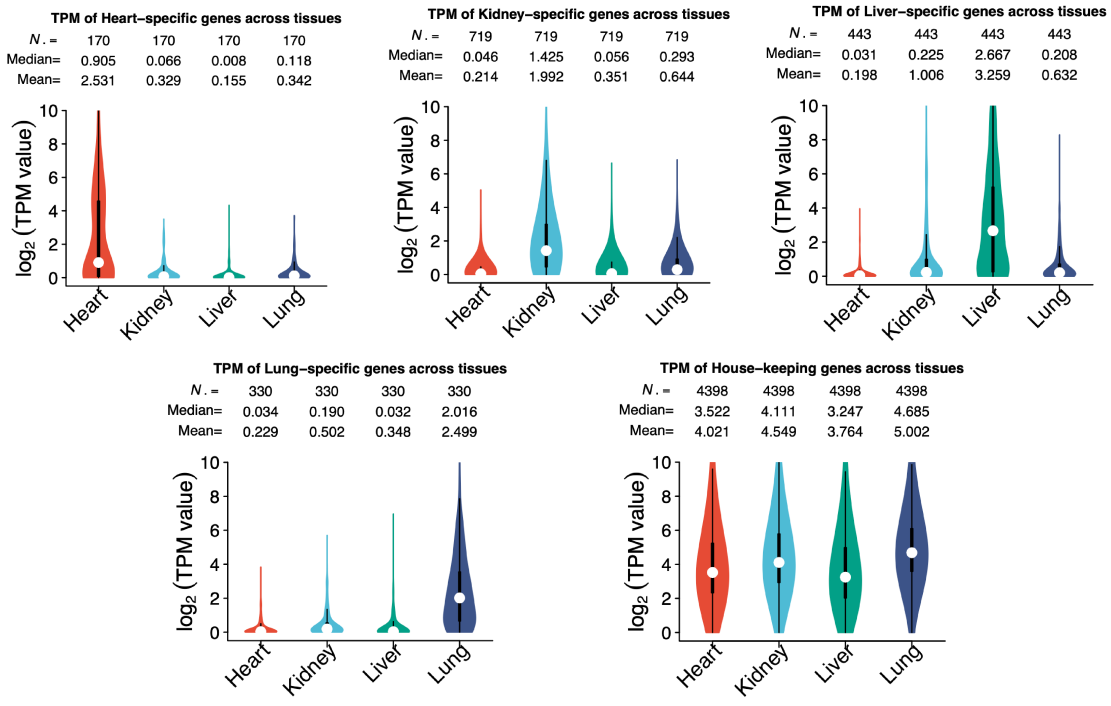


Fig. R2 Volcano plots showing the differentially expressed genes (cutoff: FDR ≤ 0.01, |log_2FC| ≥ 2) for the sex comparison of each tissue.



Supplementary Fig .6. The expression pattern of our identified tissue-specific and housekeeping PCGs in the RNA-seq data of a previous article

Taken together with the other reviewers' comments (**Comment 2-8, 3-4**), we have also added the verification analysis of the reliability of our identified PEI structures. Briefly, we performed aggregating peak analysis for the identified PEIs in each tissue,

which indicated the accuracy of PEI calling (APA score>2.05). Moreover, to confirm our identified PEIs have biological implications, we checking the published human H3K27ac peak data from the ENCODE database. We found that 48,742 our identified enhancer anchors (54.1%) in the giant panda overlapped with the identified human peak regions. Additionally, tissue-specific enhancers in the giant panda generally correspond with those in humans. Furthermore, we randomly selected one enhancer associated with the promoter of gene (*ARRDC3*), for validation in HEK-293T cells utilizing the Dual-Luciferase reporter assay. The results demonstrated a statistically significant increase in transcriptional activity for the tested enhancers compared to the controls. A more detailed response can also be found in the response to **Comment 2-8 and 3-5**.

Altogether, these results support the reliability of our RNA-seq and Hi-C data.

Comment 3-2:

To determine the tissue-type specificity and diversity at A/B compartment distribution, the authors calculated and compared those A/B compartment switch across tissues at 20-kb resolution, which is quite different from to previous genome-wide compartment profiles at typical resolutions ranging from 40 kb to 500 kb in human cells. Higher resolution for analysis requires deeper sequencing depth (e.g., billion level unique mapped valid read pairs as the author mentioned (Rowley et al. Molecular Cell 2017)). One concern is that the authors need to provide more evidence to support the matched quality of Hi-C library and check the robustness of AB compartment identification at different resolutions.

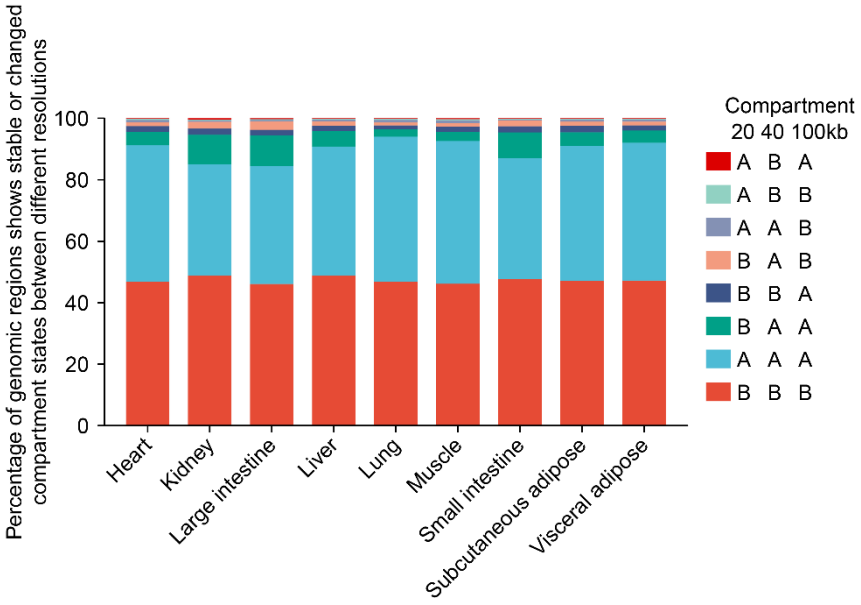
Response:

A previous study showed that precise A/B compartments (also termed as compartmental domains) can be observed at high resolution (e.g., 5-kb [11]). To make a more precise annotation of A/B compartments in giant panda tissues, we identified small A/B compartments at 20-kb resolution. Overall, a good correspondence was observed when we performed comparisons of compartment calls made at 20-kb, 40-kb and 100-kb resolution (~92.22% and ~91.60% of A/B compartments at 20-kb were assigned the same compartment type at 40-kb and 100-kb in each sample,

respectively) (**Supplementary Fig. 12d**).

We have added the corresponding description in the '**Results**' section as follows (**Main text, Page 7 Lines 177-182**):

"To further ensure reliability, we identified compartments at 40 and 100 kb resolutions, which are standard for compartment calling. Comparisons of compartment calls at 20, 40, and 100 kb resolutions showed strong consistency, with approximately 92.22% and 91.60% of A/B compartments at 20 kb matching the same type at 40 kb and 100 kb, respectively (**Supplementary Fig. 12d**)."



Supplementary Fig. 12d The percentage of genomic regions shows stable or changed compartment states between different resolutions in each sample.

We have also added the statistical information of the maximum resolution for each Hi-C library. The analysis of matrix resolution was conducted as previously described[12], where matrix resolution is defined as the smallest locus size at which 80% of loci exhibit at least 1000 intra-chromosomal contacts. The average maximum Hi-C resolution across the nine tissues is 5.26 kb, with a range from 4.52 to 6.02 kb. This information has been included in **Supplementary Table 3.** In the studies referenced earlier, Rowley et al. utilized billions of uniquely mapped valid read pairs and conducted analyses at a very high resolution, such as 5 kb[11]. Although our

dataset is comparatively smaller, it is adequate for identifying A/B compartments at a 20 kb resolution.

Comment 3-3:

The authors claimed that "~62.14% of TAD boundaries were changed in at least one tissue" appears inconsistent with established literature demonstrating relative TAD conservation across mammalian tissues. Without replicates, the high proportion of dynamic boundaries likely reflects technical noise rather than biological reality. I recommend removing the TADs analysis, as the data cannot robustly support the conclusion.

Response:

Actually, previous studies have demonstrated notable differences suggesting tissue and cell-type specificity concerning topologically associating domain (TAD) boundaries. Dixon et al. (2012) were among the first to report on TAD structures, revealing that approximately 51% of TAD boundaries differed between mouse embryonic stem (ES) cells and cortex, and around 47% differed between human ES cells and IMR90 cells[3]. Similarly, Schmitt et al. identified 3,010 distinct TAD boundaries, representing approximately 60% of all TAD boundaries, across seven different cell lines and 14 human tissues[13]. These findings are consistent with our study, which identified 2,971 altered TAD boundaries (~62.14% of all TAD boundaries) across giant panda tissues. In contrast, our earlier research showed that TAD boundaries are largely stable across pig populations (domestic vs. wild boars, >95% stability)[4] and at varying nutritional levels (~80% stability during weight changes)[14].

To further confirm, we have also conducted a differential analysis of TAD boundaries using an alternative established method, namely the directionality index algorithm[3]. Similarly to our previous analysis, we observed that approximately 58.28% of TAD boundaries exhibited variation among giant panda tissues. A detailed examination of a wide array of genomic loci revealed that TAD boundaries are indeed modified across different giant panda tissues (**Fig. R5**). These observations, coupled with the significant differences in gene expression across tissues within tissue-specific TADs

compared to those within stable TADs, support the reliability of the identified alterations in TAD boundaries across tissues.

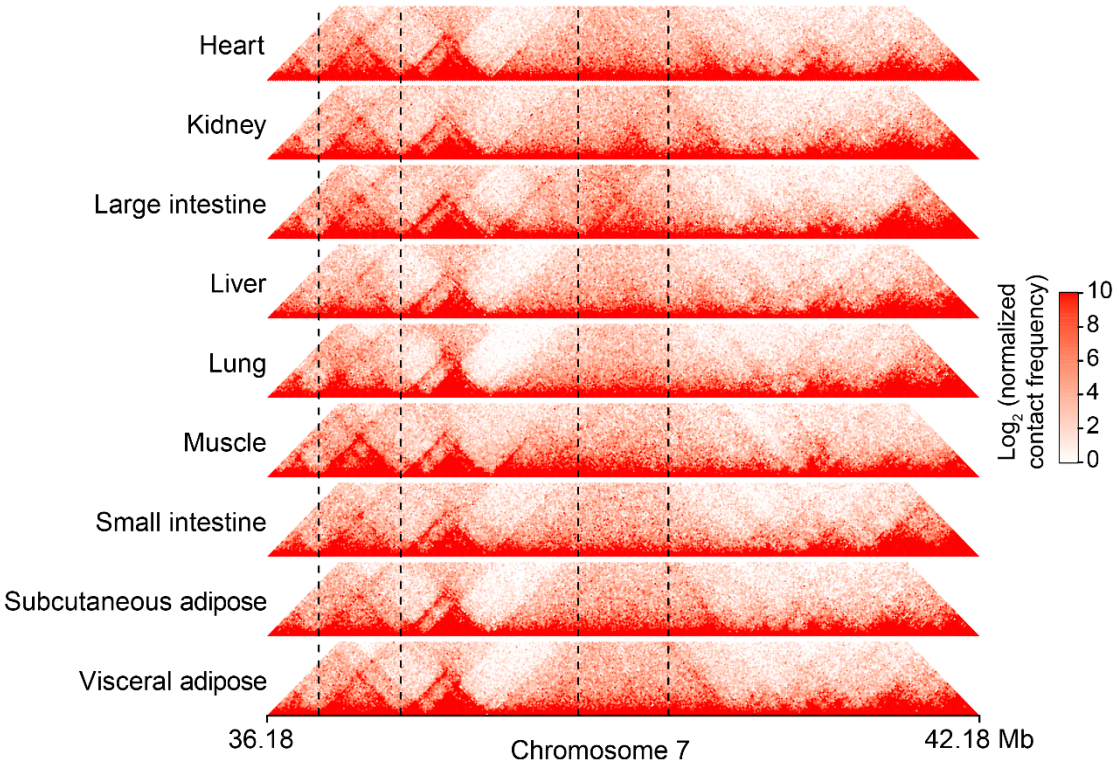


Fig. R5. Representative TAD boundaries change among giant panda tissues.

Comment 3-4:

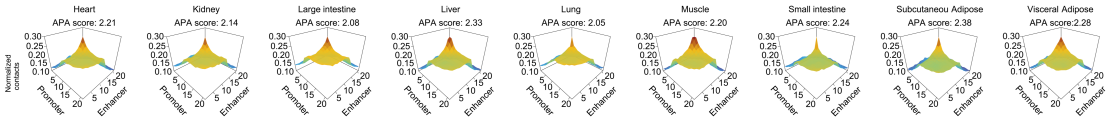
Loop calling methods are highly variable under different parameters or methods, it is quite necessary to ensure the precision of promoter-enhancer interactions either by experiments or analysis alone. The author neither provide the analysis robustness of PEI or loop identification with various typical approaches to ensure the false positive rate of PEI identification, nor the widely used APA plot analysis, which seems not rigorous. As shown in Fig. 5G-H, no visually discernible looping structures are apparent at claimed PEI loci in the Hi-C contact heatmaps.

Response:

We are thankful for this comment. Indeed, in the previous version of the manuscript, we did not identify the loops, but rather PEIs. The putative PEI structure

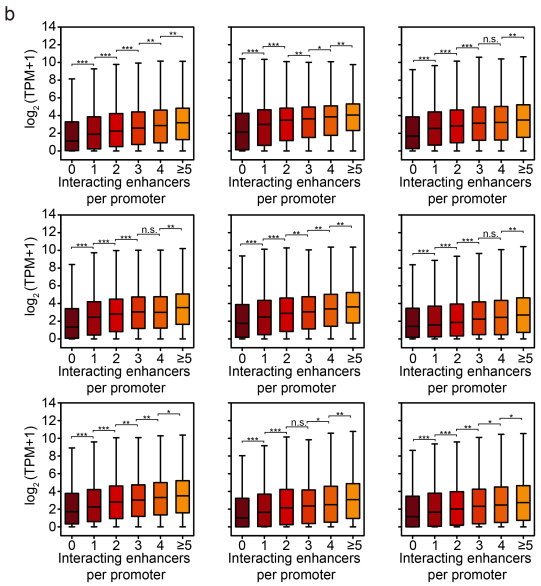
was identified using PSYCHIC, which has been successfully applied to identify PEI structures in multiple species, such as pig[14], chicken[15] and Drosophila[16]. Moreover, we used a relatively stringent criteria to filter for highly reliable PEIs (FDR ≤ 0.001). To further assess the quality of PEI detection, we have also added aggregating peak analysis (APA) for the identified PEIs in each tissue, which demonstrated the accuracy of PEI calling (APA score>2.05) (**Supplementary Fig. 20**). The related description has been added to the revised '**Results**' section as follows (**Main text, Page 11, Lines 305-307**).

"Aggregating peak analysis (APA) indicated the accuracy and reliability of PEI calling (APA score>2.05) (**Supplementary Fig. 20**)"



Supplementary Fig. 20. Normalized Hi-C signals around the identified PEIs in each giant panda tissue at 10 kb resolution.

Furthermore, the positive correlation between the gene expression and the number of PEIs again confirms the reliability of our identification of PEIs (**Supplementary Fig. 21b**).



Supplementary Fig. 21. b Gene expression level with different numbers of interacting enhancers. *P* values were calculated using the Wilcoxon rank-sum test.

Previous studies have shown that the promoter-enhancer interactions not only occur between loop anchors, many PEIs are occurred within or spanning the loop domains. We have added the identification of loops in each tissue of giant panda. We found about 29.62% PEIs were located within or spanning loop domains, while only ~6.82% were located in loop anchors. Therefore, PEI is usually manifest in chromatin interaction map as focal interaction point, and may not have a typical loop structure in the chromatin interaction map.

Comment 3-5:

In Fig. 6 C-D, the high variability in the contact matrices shows that the reported EP interactions may represent stochastic noise rather than biologically significant signals.

Response:

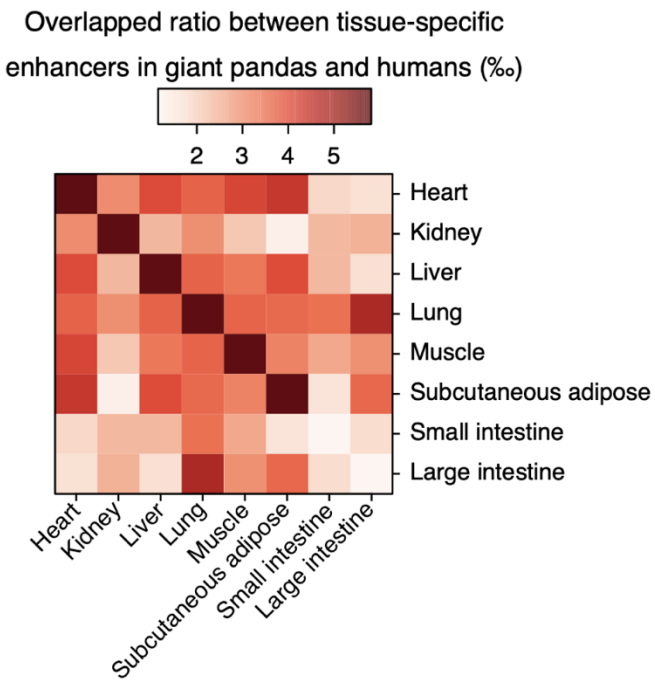
Together with the **comments 2-8 of Reviewer 2 and comments 3-4 of Reviewer 3**, the reviewers conveyed their concern about the reliability of our identified PEIs. To validate the biological significance of the predicted PEIs, we evaluated the biological activity of the predicted enhancer regions associated with promoters, referred to as enhancer anchors of PEIs. Initially, we procured an extensive dataset of human H3K27ac peaks from the ENCODE database, which included 734 files and encompassed a total of 60,495,343 peaks from various human tissues and cell lines. We then aligned 90,170 enhancers identified in giant pandas to the human Hg38 genome. Of these, 48,742 enhancers (54.1%) overlapped with the human peak regions, indicating that our predicted enhancer regions exhibit a substantial degree of reliability.

To further investigate the conservation of tissue-specific enhancers across species, we systematically organized the downloaded human peak data by tissue type. Our analysis concentrated on eight specific tissues: heart, liver, kidney, lung, muscle, small intestine, large intestine, and subcutaneous adipose tissue. Employing bioinformatic

techniques, we identified tissue-specific enhancers for these eight tissues in both giant pandas and humans. Subsequently, we mapped the tissue-specific enhancer regions from the giant panda genome to the human Hg38 genome and evaluated their overlap ratios. As depicted in the figure below (**Supplementary Fig.30**), with the exception of the large and small intestines, the tissue-specific enhancers in giant pandas predominantly correspond to regions associated with human tissue-specific enhancers. This finding suggests a significant degree of conservation of tissue-specific enhancers between humans and giant pandas. The observed differences in the large and small intestines are likely attributable to the giant panda's specialized bamboo diet, which results in substantial variations in their digestive system compared to that of humans.

We have added the related contents in the '**Discussion**' section (**Main text, Page 19-20, Lines 551-559**):

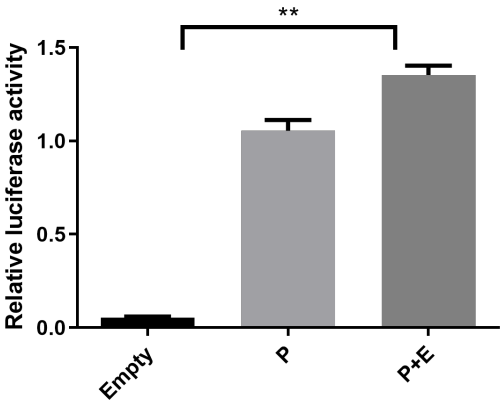
"Moreover, at the PEI level, analysis of H3K27ac peak data from the ENCODE database revealed that 48,742 enhancer anchors (54.1%) overlapped with the identified human peak regions. Additionally, tissue-specific enhancers in the giant panda generally correspond with those in humans, with the notable exception of the large and small intestines (**Supplementary Fig.30**). This observation indicates a high degree of conservation of these enhancers between the species, with the differences in intestinal enhancers likely attributable to the panda's specialized bamboo diet and its impact on the digestive system. The validity of the putative enhancers interacting with promoters was confirmed through a Dual-Luciferase reporter assay."



Supplementary Fig.30. Overlapped ratio between tissue-specific enhancers in giant pandas and humans.

Finally, to assess the biological significance of enhancer anchors of promoters, we randomly selected enhancer contacted with the promoter of gene (*ARRDC3*), for validation in HEK-293T cells utilizing the Dual-Luciferase reporter assay. The results demonstrated a statistically significant increase in transcriptional activity for the tested enhancers compared to the controls ($P < 0.05$, two-sided Student's t-test) (**Supplementary Fig.22**). The corresponding description is added to the manuscript as below (**Main text, Page 11-12, Lines 317-322**):

"To confirm the reliability of enhancers identified as interacting with promoters, we randomly selected enhancer associated with the promoter of gene (*ARRDC3*), for validation in HEK-293T cells utilizing the Dual-Luciferase reporter assay. The results demonstrated a statistically significant increase in transcriptional activity for the tested enhancers compared to the controls ($P < 0.05$, two-sided Student's t-test) (**Supplementary Fig. 22**)."



Supplementary Fig. 22. Validation of the enhancers identified as interacting with promoters utilizing a Dual-Luciferase Reporter Assay System in HEK-293T cells. Data are presented as mean ± SD (n = 3). *P*-values were determined using a two-sided Student's t-test.

Comment 3-6:

The definitions of tissue-specific PCGs and tissue-enchanced PCGs are not clear and could confuse readers. The 'specific' and 'enhanced' genes were summaritzed in supplementary figure 7 and showed firaction difference wihin one specifc tissue types. However, the author have not clarify those difference(e.g. genomic profiles or functions) of gene set with different difinitions. Are these terms synonymous or distinct? Better definitions or discriptions of those should be provided with rationales.

Response:

Actually, the definition of "tissue-specific" depends on arbitrary cut-off levels and many molecules previously identified as "tissue-specific" have been demonstrated to be expressed in several tissues[17]. Consequently, the tissue-specific PCGs were further categorized into two groups, as referenced in a prior study: (i) tissue-enriched PCGs and (ii) tissue-enhanced PCGs[17]. Tissue-enriched PCGs are characterized by expression levels in a particular tissue that are at least fourfold higher than those in all other analyzed tissues. In contrast, tissue-enhanced PCGs exhibit gene expression levels that are at least fourfold higher than the average expression levels across all other tissues.

We apologize for not making it clearer. We have revised our original description of this analysis, hope this improvement will help readers understand our paper more easily (**Main text, Page 4, Lines 108-115**).

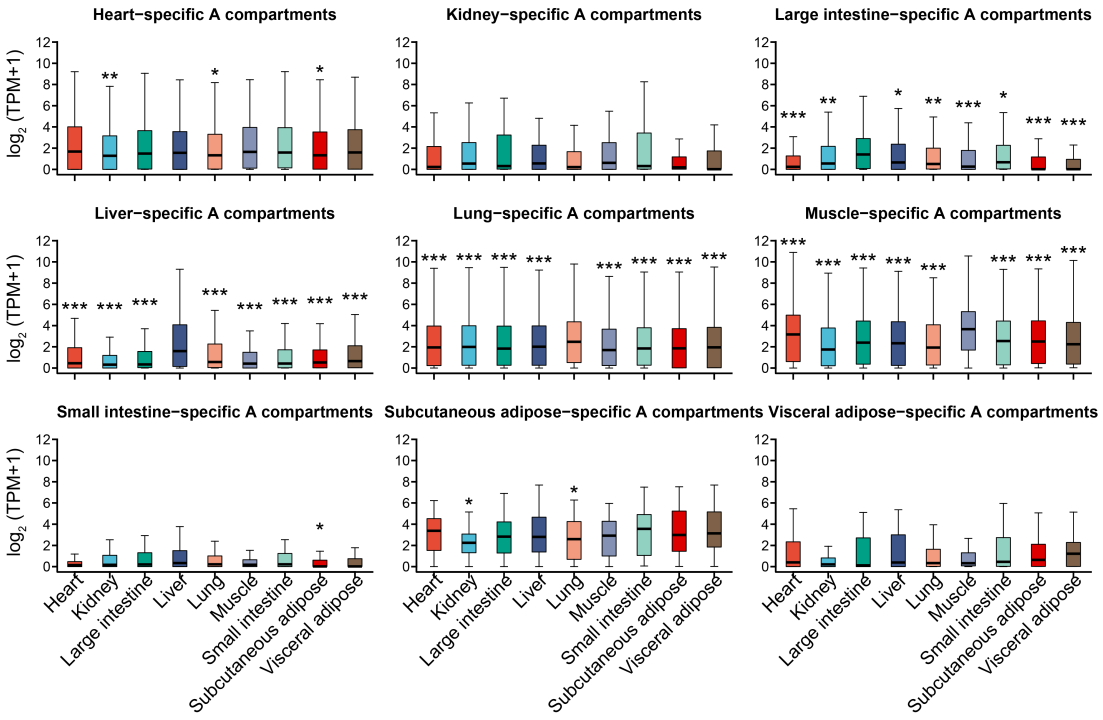
"Given that the definition of "tissue-specific" depends on arbitrary cut-off levels and many molecules previously identified as "tissue-specific" have been demonstrated to be expressed in several tissues. We further categorized the tissue-specific PCGs into two groups, as referenced in a prior study[34]: (i) tissue-enriched PCGs and (ii) tissue-enhanced PCGs. Tissue-enriched PCGs are characterized by expression levels in a particular tissue that are at least fourfold higher than those in all other analysed tissues. In contrast, tissue-enhanced PCGs exhibit gene expression levels that are at least fourfold higher than the average expression levels across all other tissues."

Comment 3-7:

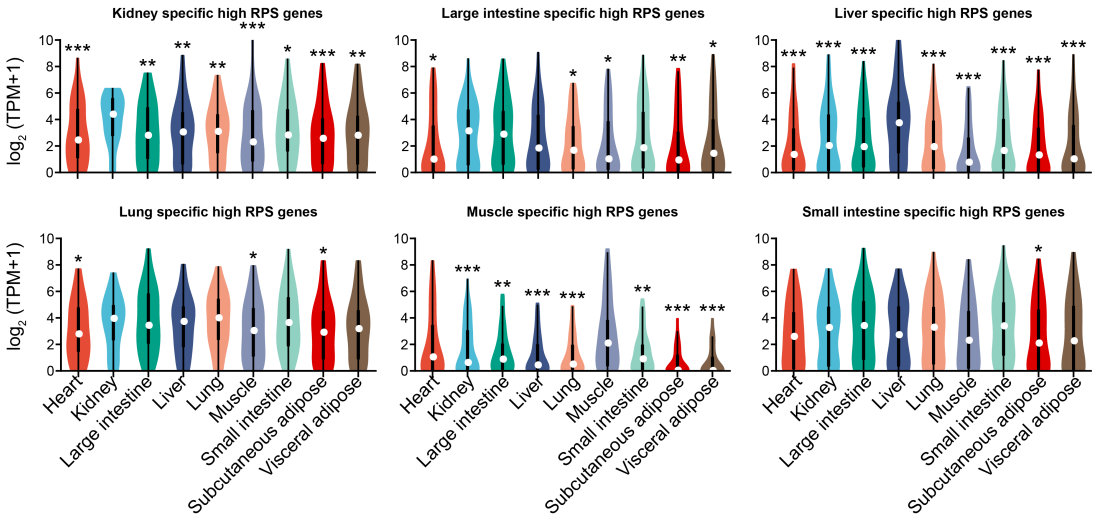
No statistics of significance was made or shown for those genes in the tissue-specific A compartment in Supplementary Fig14 boxplot as similar as in Supplementary Fig21 boxplot, better to add since the author mentioned the tissue specific correlation.

Response:

We apologize for not including the results of statistical tests in the initial version of the manuscript. Significance has been added based on indicated statistical test (see **Supplementary Fig. 15 and 25**).



Supplementary Fig. 15. Expression levels of PCGs embedded in tissue-specific A compartment regions across tissues. For a given tissue, the statistical significance of differences in gene expression of this tissue and other tissues were assessed by Wilcoxon rank-sum test. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.



Supplementary Fig. 25. Expression level for the tissue-specific high RPS PCGs across the nine giant panda tissues. Tissues (including heart, subcutaneous adipose and visceral adipose) with fewer than 35 tissue-specific high RPS PCGs were excluded from the functional

enrichment analysis. For the given tissue-specific high RPS genes, the statistical significance of differences in gene expression of this tissue and other tissues were assessed by Wilcoxon rank-sum test. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

Reviewer 4

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Biology initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response:

We appreciate the reviewer's comments and helpful suggestions for our manuscript.

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