

Drug-induced cis-regulatory elements in human hepatocytes affect molecular phenotypes associated with adverse reactions

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this manuscript, the authors identified new PXR-responsive enhancers for multiple genes, including UGT1A1, CYP24A1 and TSKU, by using the HepG2 cell line overexpressing PXR (referred to as ShP51 cells). They then confirmed the activity of these newly identified enhancers by either using luciferase reporter assays (transient transfection of the reporter constructs into the ShP51 cells), or by deleting the enhancers using CRISPR/Cas9 in the same ShP51 cells. Identification of new enhancers will improve our understanding of the complex transcriptional regulation by PXR.

In the CRISPR/Cas9-mediated deletion studies (e.g, Fig 2G), individual deletion of the known enhancer (e.g., PBREM for UGT1A1) and the newly identified enhancer (e.g., DPE15 or PBREM-like for UGT1A1) appeared to almost abolish the PXR induction of UGT1A1. Does this suggest that PBREM and PBREM-like enhancer functions redundantly? Have the authors tried to delete both the PBREM and PBREM-like enhancers and evaluate the functional outcome? Same questions go to the newly identified enhancers for CYP24A1 and TSKU.

All the experiments were performed in HepG2 cells overexpressing PXR (ShP51). Have the authors considered cell models with endogenous PXR, such as HepaRG cells? Utilization of such cell models will further confirm the physiological relevance of the new enhancers reported in this manuscript.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

The manuscript presents a study aiming to establish an association between drug-inducible cis-regulatory elements (CREs) in hepatocytes with pregnane X receptor (PXR)-mediated induction of drug metabolizing enzymes and their consequences on adverse drug reactions. The authors utilized a combination of transcriptional profiling via CAGE and GWAS data to identify several CREs around the UGT1A1, CYP24A1, and TSKU genes. The deletion of these CREs (CRISPR/Cas9 knockout) directly altered the regulation of their gene expression (mRNA levels) in an in vitro model of ShP51 cell line, which is derived from HepG2 cells with stable expression of human PXR. The experimental data are solid to support the findings on the roles of CREs in the transcriptional regulation of the targeted genes. However, the manuscript has several fundamental problems that need to be addressed.

1. The main aim of the study is to establish an association between drug-inducible CREs in human hepatocytes with adverse drug reactions (ADRs) as the title stated. The drug used in this study is rifampicin, an antimicrobial class of drugs to manage and treat diverse mycobacterial infections. The authors failed to provide information on what major ADRs can be caused by rifampicin in human patients and how they measured the ADRs in the in vitro system they used for the study which can mimic the ADRs in human population. Therefore, the findings in this manuscript didn't support their conclusion in the title.

2. The authors misused the term "drug response" in this manuscript. "Drug response" refers a person's unique response to a drug with results from the coordination of pharmacokinetic and pharmacodynamics processes for that drug on efficacy and

- toxicity. If rifampicin is the drug for the study, “drug response” should be focused on efficacy of rifampicin on antimicrobial infections or rifampicin-induced toxicity. In the current study, rifampicin was selected because it has capability to activate PXR and induce expression of drug-metabolizing enzymes, such as UGT1A1 and CYP3A4. “Drug induction” or “enzyme induction” should be an appropriate term to replace “drug response” in this study.
3. In line 84-85, the authors use vitamin D deficiency as a well-documented ADR of PXR activators but cite two references using phenytoin and phenobarbital. If rifampicin is the selected model drug in the study, the authors should provide supporting evidence on vitamin D deficiency as one of the major ADRs of rifampicin. Several studies provide such evidence.
 4. The selection of UGT1A1 for the study is poorly justified. If the purpose of the study is to establish an association between drug-inducible CREs with ADRs. Induction of UGT1A1 by rifampicin will help to reduce but not cause accumulation of bilirubin. Induction of UGT1A1 will also help to reduce irinotecan-induced neutropenia.
 5. Fig. 3 didn't show DPE128 can influence the induction of expression of CYP24A1 by rifampicin.
 6. The selection of TSKU for the study is poorly justified. TSKU is not a common abbreviation, and it is not defined in the first place. The functions of TSKU is not introduced in the metabolism of vitamin D. It is hard to explain why knockdown of TSKU can directly affect the expression of other CYP enzymes in the vitamin D metabolism. Is it a direct effect or just a consequence?
 7. All data on the induction of gene expression by rifampicin were only shown at mRNA levels by RT-PCR without support on protein levels.
 8. How were controls handled in the luciferase reporter assay? Were mutated or scrambled enhancer sequences used as negative controls?
 9. It is suggested to use a PXR knockdown or inhibitor to confirm whether the observed regulatory changes in gene expression are directly mediated by PXR activation.
 10. Identified CREs are suggested to test in other liver cell models or primary hepatocytes to confirm their broader relevance. Since HepG2-derived ShP51 cells were used, validating these results in primary human hepatocytes or other cell lines would help ensure the CREs are universally relevant to drug metabolism.
 11. The manuscript is not presented in a logical flow. The figures are not referred to in the text in the order they presented. It is not easy for readers to follow.
 12. The nomenclature for genes, mRNAs, and proteins are inconsistent with italic (genes) and non-italic (mRNAs and proteins).
 13. The reference style doesn't match the journal's style.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)

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

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have appropriately addressed the comments raised from last review, by providing new experimental data.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

The authors are very responsive to my comments. All comments are appropriately addressed. I don't have further concerns.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)

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

Researchers who co-review manuscripts.

(Remarks on code availability)

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We sincerely thank the reviewers for their thorough review of our manuscript and their insightful comments. We greatly appreciate the opportunity to improve the manuscript. Each comment has been carefully considered, and we have revised the manuscript to address all concerns raised.

In response to the reviewers' feedback, we conducted additional experiments to further strengthen our findings:

- Evaluated the protein-level impacts of individual enhancer deletions through LC-MS/MS
- Examined combinatorial effects of PBREM and PBREM-like with their double knockout
- Demonstrated the enhancer activities in HepaRG cells, which endogenously express PXR
- Validated the regulatory role of DPE128 on CYP24A1 in hepatocyte-derived cells
- Confirmed PXR involvement in the target gene activation via siRNA-mediated knockdown

We believe these additional experiments, conducted in response to the reviewers' comments, makes the manuscript more robust and coherent.

Please find our point-by-point responses below. For clarity, reviewer comments are indented in blue, and our response are indicated in black. Texts in the revised manuscript are in purple, with newly added sentences underlined and deleted text indicated using strikethrough.

Reviewer #1 (Remarks to the Author):

In this manuscript, the authors identified new PXR-responsive enhancers for multiple genes, including UGT1A1, CYP24A1 and TSKU, by using the HepG2 cell line overexpressing PXR (referred to as ShP51 cells). They then confirmed the activity of these newly identified enhancers by either using luciferase reporter assays (transient transfection of the reporter constructs into the ShP51 cells), or by deleting the enhancers using CRISPR/Cas9 in the same ShP51 cells. Identification of new enhancers will improve our understanding of the complex transcriptional regulation by PXR.

(Response)

We are pleased to find your recognition of the value of our study.

In the CRISPR/Cas9-mediated deletion studies (e.g, Fig 2G), individual deletion of the known enhancer (e.g., PBREM for UGT1A1) and the newly identified enhancer (e.g., DPE15 or PBREM-like for UGT1A1) appeared to almost abolish the PXR induction of UGT1A1. Does this suggest that PBREM and PBREM-like enhancer functions redundantly? Have the authors tried to delete both the PBREM and PBREM-like enhancers and evaluate the functional outcome? Same questions go to the newly identified enhancers for CYP24A1 and TSKU.

(Response)

We appreciate this stimulating question, and agree that double knockout of the enhancers will provide valuable insight to their combinatorial impact. As correctly noted, the deletion of either the PBREM or the PBREM-like enhancer almost abolish PXR-mediated UGT1A1 induction, indicating

that these enhancers cannot compensate for each other's absence and are therefore non-redundant. This experimental result can be explained in either of two possible regulatory relationships: mutual dependence, or independent activity. In the mutual dependence model, both enhancers must be active to drive target gene expression, meaning a double knockout would have no greater impact than a single knockout. Conversely, in the independent activity model, where each enhancer functions separately, a double knockout would further reduce UGT1A1 levels compared to a single knockout.

We generated double knock-out cells targeting both PBREM and PBREM-like enhancers to address this question. The results support the independent activity model, as we observed that the double knockout further reduced UGT1A1 induction compared to a single knock-out alone (Fig. S4B). We did not explore combinatorial enhancer relationships for CYP24A1 and TSKU, as the experiments in our study involves a single enhancer per gene.

The double-knockout experiment is included in the revised manuscript as below.

(page 11, line 231-236)

As deleting either of the enhancers almost abolishes the expression of UGT1A1, we next asked their combinatorial effect. We found that homozygous deletion of both PBREM and PBREM-like further reduced the expression (Fig. S4A, B). Given that our luciferase assays found comparable regulatory activities of PBREM-like with the minor alleles of PBREM (Fig. S4C), our experiments demonstrate that PBREM-like play a role of an independent enhancer of UGT1A1 as a functional copy of PBREM, beyond its sequence similarity to the well-known enhancer.

All the experiments were performed in HepG2 cells overexpressing PXR (ShP51). Have the authors considered cell models with endogenous PXR, such as HepaRG cells? Utilization of such cell models will further confirm the physiological relevance of the new enhancers reported in this manuscript.

(Response)

We appreciate this relevant comment and agree on the significance of utilizing cell models with endogenous PXR for assessing the physiological relevance of the enhancers. HepaRG cells are indeed a well-established model for this purpose, and we decided to conduct experiments using HepaRG cells.

We confirmed the intronic enhancer of UGT1A1 by using CRISPR interference in differentiated HepaRG cells. By delivering dCas9-KRAB and specific guide RNAs via lentiviral vectors, we demonstrated a significant reduction in rifampicin-induced UGT1A1 expression, confirming the functional role of this enhancer under physiological conditions (Fig. S4E). It was not feasible to examine PBREM-like with CRISPR interference due to its sequence similarity to PBREM, and with CRISPR/Cas9 knockout due to the limited proliferative capacity of differentiated HepaRG cells.

The validation experiments in HepaRG is included in the revised manuscript as below.

(page 11, line 244-246)

We further confirmed that its silencing by CRISPR interference (CRISPRi) similarly decreased the expression of UGT1A1 in both ShP51 cells and differentiated HepaRG cells (Fig. S4D, E).

Reviewer #4 (Remarks to the Author):

The manuscript presents a study aiming to establish an association between drug-inducible cis-regulatory elements (CREs) in hepatocytes with pregnane X receptor (PXR)-mediated induction of drug metabolizing enzymes and their consequences on adverse drug reactions. The authors utilized a combination of transcriptional profiling via CAGE and GWAS data to identify several CREs around the UGT1A1, CYP24A1, and TSKU genes. The deletion of these CREs (CRISPR/Cas9 knockout) directly altered the regulation of their gene expression (mRNA levels) in an in vitro model of ShP51 cell line, which is derived from HepG2 cells with stable expression of human PXR. The experimental data are solid to support the findings on the roles of CREs in the transcriptional regulation of the targeted genes. However, the manuscript has several fundamental problems that need to be addressed.

(Response)

We are pleased to find your recognition of the value of our study.

1. The main aim of the study is to establish an association between drug-inducible CREs in human hepatocytes with adverse drug reactions (ADRs) as the title stated. The drug used in this study is rifampicin, an antimicrobial class of drugs to manage and treat diverse mycobacterial infections. The authors failed to provide information on what major ADRs can be caused by rifampicin in human patients and how they measured the ADRs in the in vitro system they used for the study which can mimic the ADRs in human population. Therefore, the findings in this manuscript didn't support their conclusion in the title.

(Response)

We sincerely appreciate raising this important point. We have revised our manuscript to describe ADRs associated with rifampicin in human patients, and how the ADR associated molecular phenotypes are examined.

(page 4, line 86-90)

Vitamin D deficiency is a well-documented adverse effect of rifampicin and the other PXR activators described above¹⁴⁻¹⁹, such as phenytoin and phenobarbital while its molecular basis remains to be understood. Since PXR functions as a nuclear receptor regulating the expression of downstream genes, the shared adverse effect may result from unanticipated unexpected alterations in gene expression triggered by its activation.

(page 6, line 127-131)

...we narrowed down the candidate regions to the ones directly targeted by PXR and associated with ADR-linked phenotypes. We assessed their impact on molecular phenotypes associated with ADR through evaluating their regulatory activities of ADR associated genes by using CRISPR/Cas9 knockout, luciferase reporter assay, and siRNA knockdown experiments.

(page 19, line 418-426)

Our genome-wide quantitative assessment revealed a number of drug-inducible, PXR-mediated, and trait-associated enhancers in human hepatocytes. The results not only aligned closely with the anticipated outcomes to drug exposure but also provided insights into the complex regulatory mechanisms underlying drug responses. Our focused experiments further demonstrated that several of these enhancers affect molecular phenotypes associated with ADR, the expression of drug-induced genes associated with ADR, such as neutropenia and Vitamin D deficiency. The novel enhancers, regulatory alleles, and a molecular cascade leading to ADR contribute a precise understanding of the noncoding elements of the human genome underlying drug responses.

2. The authors misused the term “drug response” in this manuscript. “Drug response” refers a person’s unique response to a drug with results from the coordination of pharmacokinetic and pharmacodynamics processes for that drug on efficacy and toxicity. If rifampicin is the drug for the study, “drug response” should be focused on efficacy of rifampicin on antimicrobial infections or rifampicin-induced toxicity. In the current study, rifampicin was selected because it has capability to activate PXR and induce expression of drug-metabolizing enzymes, such as UGT1A1 and CYP3A4. “Drug induction” or “enzyme induction” should be an appropriate term to replace “drug response” in this study.

(Response)

We sincerely appreciate this sensible comment and agree that the term “drug induction” is more appropriate to describe the activation of gene expression following drug treatment in this context. We have revised the manuscript accordingly to ensure the use of precise terminology throughout.

(page 7, line 137-138)

The human hepatocellular carcinoma cell line HepG2 is a widely used model system, but it is not necessarily suitable for studying drug ~~responses~~ induction as it is

(page 16, line 349-350)

In this study, we systematically analyzed drug-inducible CREs to elucidate the genomic basis of both anticipated and unanticipated drug ~~responses~~ induction.

(page 18, line 403-405)

we also found that two SNPs in DPE128 genetically associated with vitamin D levels alter the regulatory activity (Fig. 3C). These results support the contribution of the super-enhancer to drug ~~responses~~ induction.

3. In line 84-85, the authors use vitamin D deficiency as a well-documented ADR of PXR activators but cite two references using phenytoin and phenobarbital. If rifampicin is the selected model drug in the study, the authors should provide supporting evidence on vitamin D deficiency as one of the major ADRs of rifampicin. Several studies provide such evidence.

(Response)

We appreciate pointing out this. We now included citations to the following studies, describing ADRs of rifampicin, as well as the ones of the other PXR activators, tamoxifen and dexamethasone.

14 Leung, C., Warner, J., Harris, M. & Nourse, C. Symptomatic Hypocalcemia Secondary to Rifampicin-induced Hypovitaminosis D. *Pediatr Infect Dis J* 35, 822-823 (2016).

15 Sheng, L. et al. Effects of repeated administration of rifampicin and isoniazid on vitamin D metabolism in mice. *Steroids* 104, 203-207 (2015).

18 Kim, H. J. et al. Changes in serum hydroxyvitamin D levels of breast cancer patients during tamoxifen treatment or chemotherapy in premenopausal breast cancer patients. *Eur J Cancer* 50, 1403-1411 (2014).

19 Skversky, A. L., Kumar, J., Abramowitz, M. K., Kaskel, F. J. & Melamed, M. L. Association of glucocorticoid use and low 25-hydroxyvitamin D levels: results from the National Health and Nutrition Examination Survey (NHANES): 2001-2006. *J Clin Endocrinol Metab* 96, 3838-3845 (2011).

(page 4, line 86-87)

Vitamin D deficiency is a well-documented adverse effect of rifampicin and the other PXR activators described above ¹⁴⁻¹⁹, such as phenytoin and phenobarbital

4. The selection of UGT1A1 for the study is poorly justified. If the purpose of the study is to establish an association between drug-inducible CREs with ADRs. Induction of UGT1A1 by rifampicin will help to reduce but not cause accumulation of bilirubin. Induction of UGT1A1 will also help to reduce irinotecan-induced neutropenia.

(Response)

We appreciate this constructive comment and agree that the selection of UGT1A1 needs be well justified. The motivation to study UGT1A1 is that the extent of UGT1A1 activation can influence the risk of ADRs. We have now explicitly clarified this point in the manuscript.

(page 10, line 208-211)

The activation level of UGT1A1 is known to influence various phenotypes, including the risk of neutropenia in irinotecan treatment and the metabolic clearance of the endogenous toxin bilirubin. We here focused on the investigation of unrevealed noncoding elements that could alter its expression.

5. Fig. 3 didn't show DPE128 can influence the induction of expression of CYP24A1 by rifampicin.

(Response)

We appreciate this critical comment. As correctly noted, there has been no demonstration that DPE128 regulate CYP24A1 expression in hepatocyte-derived cells, although this relationship was

previously shown in colorectal cancer derived cells by a previous study (J Biol Chem, 285:15599-15610, 2010).

In response to this comment, we conducted a CRISPR interference experiment in our model system, which result successfully demonstrated their regulatory relationships (Fig. 3B). This observation is included in the revised manuscript as below.

(page 13, line 295-299)

Regulatory activity of the enhancer cluster on CYP24A1 was only demonstrated in a cell line derived from colorectal adenocarcinoma⁵⁷, and we assessed the regulatory activity in ShP51 cells. We found that the CRISPRi-based repression of DPE128 significantly reduced the expression of CYP24A1 (Fig. 3B), suggesting its regulatory activity in a model system of hepatocyte.

6. The selection of TSKU for the study is poorly justified. TSKU is not a common abbreviation, and it is not defined in the first place. The functions of TSKU is not introduced in the metabolism of vitamin D.

(Response)

We appreciate this constructive comment and agree that the selection of TSKU needs be justified. Involvement of TSKU with vitamin D metabolism was implicated by a GWAS study, and we revised the manuscript to reflect this point, as well as to introduce the full name of TSKU and its known molecular function.

(page 9, line 197-198)

...and others located upstream of tsukushi, small leucine rich proteoglycan (TSKU) (DPE70–72, Fig. 4A).

(page 14, line 314-316)

Of the enhancer candidates overlapping SNPs associated with vitamin D levels, three (DPE70–72) were identified at 10.2 kb, 9.4 kb, and 0.5 kb upstream of TSKU which encodes a liver-enriched secreted protein that possibly regulates energy expenditure⁶².

It is hard to explain why knockdown of TSKU can directly affect the expression of other CYP enzymes in the vitamin D metabolism. Is it a direct effect or just a consequence?

(Response)

Thank you for raising this interesting point. TSKU is reported to function as a hepatokine, a signaling molecule secreted by the liver, rather than a transcription factor, and its role in influencing CYP enzymes is likely indirect. We have revised the manuscript to clarify this point further.

(page 15, line 342-346)

Given the three latter genes are involved in producing the active form of vitamin D, our results suggested that TSKU upregulates the levels of vitamin D by repression of negatively impacting genes (CYP24A1) and activation of positively impacting genes (CYP27A1 and CYP27B1). These

relationships are likely indirect, by considering TSKU have a role as a secreted protein⁶², not as a transcription factor.

7. All data on the induction of gene expression by rifampicin were only shown at mRNA levels by RT-PCR without support on protein levels.

(Response)

We appreciate this critical comment. We agree with the relevance of studying protein levels in the assessment of cellular and/or individual phenotypes. In this revision, we additionally evaluated the protein levels in the deletion clones of the novel enhancers using LC-MS/MS.

Our results revealed a significant reduction of UGT1A1 protein levels across all deletion clones targeting its enhancers, after the treatment of rifampicin (Fig. S3A). This is consistent with our analysis of mRNA expression levels. Conversely, the TSKU protein level showed a slight increase in the deletion clones of TSKU-up (Fig. S3B). This unexpected observation implies the presence of additional regulatory mechanisms, potentially involving negative feedback at translational or post-translational levels. The data is deposited in PRIDE under accession PXD060446 (token for reviewer: 7Mr5lm6kYFBG), and our observation is included in the revised manuscript as below.

(page 10, line 227-229)

Homozygous deletion of PBREM-like (Δ PBREM-like 1 and 2; Fig. 2D, F) showed a significant reduction of UGT1A1 expression as well (Fig. 2G). We also observed significant reduction of the UGT1A1 protein levels in these deletion clones (Fig S3A), consistent with the corresponding mRNA levels.

(page 11, line 241-244)

We found that homozygous deletion (Δ Intronic enh. 1–3; Fig. 2H, I) substantially decreased both basal and drug-induced expression of UGT1A1 (Fig. 2J). We also observed significant reduction of the UGT1A1 protein levels in the deletion clones (Fig S3A).

(page 14-15, line 332-326)

We found a significant decrease of TSKU expression in the deletion mutants (Fig. 4D), which indicated that a portion of the large region play a role to increases TSKU expression. We observed slight increase of the TSKU protein levels in the deletion clones (Fig S3B), which suggested a presence of negative feedback at translational or post-translational levels.

8. How were controls handled in the luciferase reporter assay? Were mutated or scrambled enhancer sequences used as negative controls?

(Response)

We appreciate raising this important point and acknowledge the need for clarity. We have revised the manuscript as follows to ensure the experimental setup is explicitly described.

(page 22, line 484-486)

Data were normalized against the Renilla reniformis luciferase activity, which served as an internal control. An empty pGL4.26 vector was used as a negative control, with Firefly/Renilla ratio set to 1.

9. It is suggested to use a PXR knockdown or inhibitor to confirm whether the observed regulatory changes in gene expression are directly mediated by PXR activation.

(Response)

We appreciate this relevant suggestion. In response, we conducted siRNA-based knock-down experiments of PXR, and found the expression levels of UGT1A1, CYP24A1, and TSKU are significantly reduced (Fig. S2). This observation is included in the revised manuscript as below.

(page 9, line 200-205)

We performed siRNA knockdown experiments targeting PXR, and found a significant reduction in the expression levels of UGT1A1, CYP24A1, and TSKU by depletion of PXR, under the condition of rifampicin treatment (Fig. S2). Combined with the observed upregulation of these genes in response to rifampicin (Fig. 1B), the result implied that the induced CREs may influence their expressions through PXR.

10. Identified CREs are suggested to test in other liver cell models or primary hepatocytes to confirm their broader relevance. Since HepG2-derived ShP51 cells were used, validating these results in primary human hepatocytes or other cell lines would help ensure the CREs are universally relevant to drug metabolism.

(Response)

We appreciate this important comment. The same point was also raised by the referee #1, and we agree with the importance of using other cell models. HepaRG is a well-established model for liver cells, in particular the ones having endogenous PXR expressed, and we decided to conduct experiments using HepaRG cells.

We confirmed the intronic enhancer of UGT1A1 by using CRISPR interference in differentiated HepaRG cells. By delivering dCas9-KRAB and specific guide RNAs via lentiviral vectors, we demonstrated a significant reduction in rifampicin-induced UGT1A1 expression, confirming the functional role of this enhancer under physiological conditions (Fig. S4E). It was not feasible to examine PBREM-like with CRISPR interference due to its sequence similarity to PBREM, and with CRISPR/Cas9 knockout due to the limited proliferative capacity of differentiated HepaRG cells.

The validation experiments in HepaRG is included in the revised manuscript as below.

(page 11, line 244-246)

We further confirmed that its silencing by CRISPR interference (CRISPRi) similarly decreased the expression of UGT1A1 in both ShP51 cells and differentiated HepaRG cells (Fig. S4D, E).

11. The manuscript is not presented in a logical flow. The figures are not referred to in the text in the order they presented. It is not easy for readers to follow.

(Response)

Thank you for raising this point. We have revised the manuscript to ensure that the figures are referenced in the order in which they appear in the text.

12. The nomenclature for genes, mRNAs, and proteins are inconsistent with italic (genes) and non-italic (mRNAs and proteins).

(Response)

Thank you for pointing this out. We revised the manuscript to use italic font only for genes.

13. The reference style doesn't match the journal's style.

(Response)

Thank you for pointing this out. We have updated the references to ensure they fully comply with the journal's specified style.

Reviewer #5 (Remarks to the Author):

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

(Response)

We sincerely appreciate the time and effort of co-reviewing this manuscript

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have appropriately addressed the comments raised from last review, by providing new experimental data.

(Response)

We are grateful for your recognition of our revised manuscript with the new experimental data. We sincerely appreciate your constructive suggestions, which contributed to enhancing the quality of our work.

Reviewer #2 (Remarks to the Author):

The authors are very responsive to my comments. All comments are appropriately addressed. I don't have further concerns.

(Response)

We are pleased to find your assessment of our responses. We sincerely appreciate your positive and critical comments, which were highly valuable in improving our manuscript.