nature portfolio

# Peer Review File

# Extensive enhancer crosstalk controls PPARG2 activation during adipogenesis

Corresponding Author: Professor Susanne Mandrup

**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)
This is a rigorous, well-written, and highly impactful manuscript that provides novel insights about the complex regulatory mechanisms of PPARG2 activation during adipogenesis. The authors identify an extensive, locus-wide "highly interconnected enhancer (HICE) community" that drives PPARG2 activation. This community is primed in undifferentiated cells and undergoes dynamic remodeling and increased connectivity during adipogenesis. Through carefully designed inducible CRISPR/Cas9 genome editing experiments, the authors show that a subset of enhancers are essential for efficient PPARG2 induction and adipocyte differentiation. Strikingly, the deletion of the super-enhancer E+102 almost completely prevented PPARG2 activation and was highly correlated with reduction in lipid accumulation, linking enhancer molecular biology to cellular physiology. Overall, this work represents a strong advance in understanding PPARG2 regulation and its relevance to metabolic health. I only have a few minor suggestions that can hopefully further strengthen the work.

1. Instead of “race” the authors should use “ancestry” as think that’s what they mean.

2. More information about the selected GWAS SNPs would be helpful to better understand the results. In supp tables 1 and 2, please include nominal p-values for the listed SNPs. The methods state that SNPs with $p<0.05$ were selected. This is quite permissive. If the SNP p-values do not surpass genome-wide significance, then they are unlikely to be the functional SNP in the GWAS signal.

3. The enhancer variant to target gene prediction approaches are good, but it would be more convincing if there’s also eQTL data to support the relationships. Is that the case? It’s not critical to show this and there may be missing eQTL data or low-powered studies unable to detect relevant eQTL, but some comment on this would be helpful.

4. More information should be provided about the SNPs that are the focal point of discussion in the enhancer communities. Based on the GWAS data, are any of the SNPs in the credible set? If so, what is the PIP? Such information would help to establish a stronger argument that the relevant SNPs are good candidates for causal SNPs in the signal.

Reviewer #2

(Remarks to the Author)
This manuscript presents a comprehensive and well-executed investigation into the enhancer regulation of the PPARG locus during adipogenesis. By integrating chromatin conformation analyses, CRISPR/Cas9-mediated enhancer deletions, and ChIP-seq experiments, the authors demonstrate that multiple enhancers act in a cooperative yet non-redundant manner to control PPARG2 expression. The study highlights the particular importance of enhancer E+102 in establishing enhancer connectivity and transcriptional output, while also implicating C/EBPβ and PPARγ as key regulators of enhancer activity. Furthermore, by linking these mechanistic insights to GWAS-identified variants associated with metabolic disease, the work provides an important translational perspective. Overall, the findings are novel, convincing, and of broad interest to the fields of adipocyte biology, enhancer function, and transcriptional regulation.

Minor Comments
1. Overall, the data convincingly support the idea that E+102 plays an important role in the enhancer network regulating PPARG2. However, as exemplified by the statement on page 10 (lines 244–247), “during adipogenesis, all super-enhancer

constituents, E+102 in particular, become more connected to the PPARG2 TSS (Fig. 2b)," the evidence presented in Figure 2b does not appear entirely clear-cut to justify such a strong emphasis. Nevertheless, given that E+102 emerges as a key enhancer from multiple lines of analysis, it would greatly strengthen the manuscript if the authors could discuss possible mechanistic explanations for why E+102, among all super-enhancer constituents, is activated earlier and more robustly. Such a discussion would help to reconcile the somewhat modest support from connectivity data with the overall conclusion of its essential role as a "seed enhancer."

2. In Figure 6b, the ChIP-seq data suggest that deletion of E+2 may also reduce C/EBPβ occupancy at several enhancers, even if the effect is less pronounced compared to E+102 deletion. This makes it somewhat unclear on what basis the authors describe certain changes as "significant." Since the term appears in several parts of the manuscript, it would be helpful if the authors could clarify how "significant" is defined in their analyses—for example, whether based on statistical testing, fold-change thresholds, or other criteria. A brief explanation would make the interpretation of the data more transparent and consistent.

3. The overlap between enhancers and GWAS-identified variants shown in Figure 7 is highly interesting and adds an important translational dimension to the study. However, it would strengthen the manuscript if the Discussion addressed more explicitly how these variants are thought to act. Specifically, do the authors consider that the variants alter enhancer function itself, for example by affecting chromatin accessibility or enhancer–promoter interactions? Or are they more likely to primarily influence transcription factor binding, such as that of PPARγ or C/EBPβ? Alternatively, is the precise mechanism still unclear? Even a brief speculative discussion of these possibilities would enhance the interpretation and clinical relevance of the findings.

4. Several enhancer–enhancer interaction labels appear to be missing in Fig. 4a. Please revise to ensure all relevant interactions are clearly annotated.

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.

Reviewer #4

(Remarks to the Author)
Cetnarowska et al. investigate how PPARG2 is activated during adipocyte differentiation using human stromal progenitors (hMSC-TERT4) as model. Using Hi-C, Capture-C, ChIP-seq, and CRISPR/Cas9 enhancer deletions, the authors show that the PPARG locus contains a highly interconnected enhancer community. They demonstrate that multiple enhancers act in a non-redundant and cooperative manner, with promoter-proximal and super-enhancer constituents being essential for PPARG2 induction. Feedback regulation by PPARγ and C/EBPα further amplifies enhancer activity. Finally, they integrate GWAS data and deep learning predictions, showing that key enhancers overlap with non-coding variants linked to metabolic disease.
Overall, this is an excellent manuscript that provides important mechanistic insight into the regulation of PPARG2, a master regulator of adipogenesis. The study is highly relevant to both basic biology and metabolic disease. The manuscript has only a few limitations. E.g. it relies on immortalized hMSC-TERT4 cells, which may not fully capture in vivo adipose biology. Functional readouts are limited to mRNA expression and lipid accumulation; additional assays such as insulin sensitivity, adipokine secretion, or metabolic flux analyses could further support the conclusion that enhancer deletions affect adipocyte function. The integration of GWAS data was not further experimentally validated. Also, the work focuses on only the PPARG2 locus, thereby limiting its potential broader conclusion.

Despite these caveats, the manuscript is very strong, methodologically rigorous, and conceptually innovative. I believe it is already highly mature and suitable for publication after only minor revisions.

Some typos:
line 75: communitiesis -> communities
line 220: three independent clones not transfected > three independent clones transfected
line 574: studes -> studies
line 575: distance-depednent > distance-dependent
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Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed all comment. Congratulations on this important study!

Reviewer #2

(Remarks to the Author)
I consider that the authors have adequately addressed all points I previously raised. I have no further essential comments.

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.

Reviewer #4

(Remarks to the Author)
The authors made considerable efforts to revise the manuscript and have satisfyingly addressed the reviewers' comments. In my view, the quality of the manuscript has further improved and is of excellent quality, sufficient for publication.

**Reviewer #1 (Remarks to the Author):**

This is a rigorous, well-written, and highly impactful manuscript that provides novel insights about the complex regulatory mechanisms of PPARG2 activation during adipogenesis. The authors identify an extensive, locus-wide "highly interconnected enhancer (HICE) community" that drives PPARG2 activation. This community is primed in undifferentiated cells and undergoes dynamic remodeling and increased connectivity during adipogenesis. Through carefully designed inducible CRISPR/Cas9 genome editing experiments, the authors show that a subset of enhancers are essential for efficient PPARG2 induction and adipocyte differentiation. Strikingly, the deletion of the super-enhancer E+102 almost completely prevented PPARG2 activation and was highly correlated with reduction in lipid accumulation, linking enhancer molecular biology to cellular physiology. Overall, this work represents a strong advance in understanding PPARG2 regulation and its relevance to metabolic health. I only have a few minor suggestions that can hopefully further strengthen the work.

**Response:** We thank the reviewer for her/his positive feedback and for recognizing the importance of our studies.

1. Instead of “race” the authors should use “ancestry” as think that’s what they mean.

**Response:** We thank the reviewer for recognizing this mistake, which we have now corrected in the manuscript.

2. More information about the selected GWAS SNPs would be helpful to better understand the results. In supp tables 1 and 2, please include nominal p-values for the listed SNPs. The methods state that SNPs with p<0.05 were selected. This is quite permissive. If the SNP p-values do not surpass genome-wide significance, then they are unlikely to be the functional SNP in the GWAS signal.

**Response:** We thank the reviewer for this helpful comment. We agree that the original wording conflated two different analytical steps and did not adequately separate (i) variant inclusion criteria from (ii) secondary trait annotation criteria in the CMD-Knowledge Portal. We have now modified the Methods (pages 33-35) and relevant Results sentences to clarify the following three points:
(1) Variant inclusion is based on genome-wide significant (GWS) GWAS signals for common variants and large WES/WGS consortia for rare variants. Common variants were obtained by identifying all genome-wide significant cardiometabolic GWAS index SNPs ($p < 5\times10^{-8}$) within the PPARG TAD across CMD-KP, DIAGRAM, CARDIoGRAMplusC4D, and the GWAS Catalog. These index variants were then expanded to high-LD proxies ($r^2 > 0.8$, CEU), as is standard at established loci.
Rare and low-frequency variants were obtained from CARDIoGRAMplusC4D WES/WGS and UK Biobank CardioMetabolic Consortium CHD analyses. As expected for variants with very low allele counts, such variants cannot achieve genome-wide significance due to limited power, but they constitute bona fide PPARG-locus associations from large sequencing resources. Rare variants were not LD-expanded.
(2) We agree with the reviewer that variants whose p-values are far weaker than their LD index SNP have a lower statistical prior for causality. We therefore now report all variant-specific p-values in Supplementary Tables 1 and 2.
(3) The previously mentioned “$p < 0.05$” applied only to PheWAS trait annotation, not variant selection. This threshold was used exclusively to identify secondary phenotypes nominally associated with variants already present in PPARG GWAS or sequencing-based loci. It did not influence which variants were included in the analysis. This is now clarified explicitly in the Methods.

As requested, Supplementary Tables 1 and 2 now include each variant's association p-value, enabling readers to evaluate the relative strength of evidence for each variant.
Finally, we emphasize that the goal of this section is not to declare locus-level associations, these are already well established for *PPARG*, but rather to integrate:
(i) Variants across the frequency distribution with prior support for cardiometabolic trait associations from large-scale variant association studies, (ii) Enformer-predicted regulatory effects, (iii) enhancer maps and ChIP-seq, (iv) and, thanks to this reviewer, now fine-mapping and QTL evidence to prioritize candidate functional variants within the *PPARG* regulatory landscape.

3. The enhancer variant to target gene prediction approaches are good, but it would be more convincing if there's also eQTL data to support the relationships. Is that the case? It's not critical to show this and there may be missing eQTL data or low-powered studies unable to detect relevant eQTL, but some comment on this would be helpful.

**Response:** We appreciate reviewer's feedback, and now we have evaluated QTLs for the top-scoring Enformer predicted regulatory variants (Supplementary Table 11). The data revealed that we indeed find colocalized eQTLs for eight common variants, including rs7647481 and rs7649970 located in enhancer E-1. We have addressed this important point in more detail in the results section (page 19). As expected, due to limited power we did not detect a colocalized eQTL for the rare variant rs181025382 (MAF=0.0001, CEU) in enhancer E-102.

4. More information should be provided about the SNPs that are the focal point of discussion in the enhancer communities. Based on the GWAS data, are any of the SNPs in the credible set? If so, what is the PIP? Such information would help to establish a stronger argument that the relevant SNPs are good candidates for causal SNPs in the signal.

**Response:** We appreciate this excellent point and have now queried the FinnGen data-server to retrieve available QTL ($p < 5e-8$) and fine-mapping information of the variants. Since the posterior inclusion probabilities (PIP) of variants depends on the number of variants in the corresponding LD blocks we used FinnGen's default threshold of 0.01 to identify fine-mapping associations. In a new Supplementary Table 10, we have included variants fine-mapped to phenotypes and their PIP from the top-scoring Enformer predicted regulatory variants. This information is now also included in the results section (page 18).

**Reviewer #2 (Remarks to the Author):**

This manuscript presents a comprehensive and well-executed investigation into the enhancer regulation of the PPARG locus during adipogenesis. By integrating chromatin conformation analyses, CRISPR/Cas9-mediated enhancer deletions, and ChIP-seq experiments, the authors demonstrate that multiple enhancers act in a cooperative yet non-redundant manner to control PPARG2 expression. The study highlights the particular importance of enhancer E+102 in establishing enhancer connectivity and transcriptional output, while also implicating C/EBPβ and PPARγ as key regulators of enhancer activity. Furthermore, by linking these mechanistic insights to GWAS-identified variants associated with metabolic disease, the work provides an important translational perspective. Overall, the findings are novel, convincing, and of broad interest to the fields of adipocyte biology, enhancer function, and transcriptional regulation.

**Response:** We thank the reviewer for her/his positive feedback and for recognizing the novelty and broad interest to the field.

Minor Comments

1. Overall, the data convincingly support the idea that E+102 plays an important role in the enhancer network regulating PPARG2. However, as exemplified by the statement on page 10 (lines 244–247), "during adipogenesis, all super-enhancer constituents, E+102 in particular, become more connected to the PPARG2 TSS (Fig. 2b)," the evidence presented in Figure 2b does not appear entirely clear-cut to justify such a strong emphasis. Nevertheless, given that E+102 emerges as a key enhancer from multiple lines of analysis, it would greatly strengthen the manuscript if the authors could discuss possible mechanistic explanations for why E+102, among all super-enhancer constituents, is activated earlier and more robustly. Such a discussion would help to reconcile the somewhat modest support from connectivity data with the overall conclusion of its essential role as a "seed enhancer."

**Response:** We agree with the reviewer that the important role of E+102 is intriguing and cannot be explained by connectivity alone. In the Discussion in the original submission (page 25), we stated "The difference in importance of super-enhancer constituents cannot be explained solely by connectivity, as all constituents interact with the promoter-proximal enhancers and the *PPARG2* TSS. However, the importance of these enhancers appears to be closely linked to their effect on the activity of other enhancers in the locus." Later in the Discussion (page 24), we suggested that "the obligate role of E+102 may be due to its dual functions, acting as a seed enhancer in the super-enhancer and as a key target enhancer for feedback action by PPARγ (and possibly other adipogenic TFs)".
We have now further elaborated on this discussion to propose possible molecular mechanisms leading to E+102 being the first and most strongly activated enhancer constituent. First, the affinity of the E+102 sequence for early proadipogenic transcription factors is likely to be important. At day 1 of adipogenesis, where the super-enhancer constituents are not yet DNase accessible, the early proadipogenic transcription factor C/EBPβ appears to bind with similar strength to E+102, E+107 and E+111 (Figure 6a), indicating that early C/EBPβ occupancy cannot explain the difference on occupancy. The combination of other early proadipogenic transcription factors binding to E+102 compared with other super-enhancer constituents are likely to be important for the special role of E+102.

2. In Figure 6b, the ChIP-seq data suggest that deletion of E+2 may also reduce C/EBPβ occupancy at several enhancers, even if the effect is less pronounced compared to E+102 deletion. This makes it somewhat unclear on what basis the authors describe certain changes as "significant." Since the term appears in several parts of the manuscript, it would be helpful if the authors could clarify how "significant" is defined in their analyses—for example, whether based on statistical testing, fold-change thresholds, or other criteria. A brief explanation would make the interpretation of the data more transparent and consistent.

**Response:** We thank the reviewer for pointing this out. In all ChIP-seq analyses of binding to the 9 enhancers, "significant" refers to statistical significance (EdgeR differential binding analysis (DiffBind)) between binding in cells with deletion of enhancer E+2 or E+102 compared to control cells (p-value <0.05). We did not apply multiple testing corrections, given the small, hypothesis-driven set of tests. Cell lines with deletion of enhancer E+2 show fewer sites with a significant decrease of C/EBPβ occupancy at day 1 compared to cell lines with deletion of enhancer E+102. Similarly, we assessed MED1 differential binding at day 3 of adipogenesis in cell lines with deletion of enhancers: E+2, and E+102 compared to control cells (Fig. 5c). The statistical testing is indicated in the relevant figure legends as well as in the Methods section.

3. The overlap between enhancers and GWAS-identified variants shown in Figure 7 is highly interesting and adds an important translational dimension to the study. However, it would strengthen the manuscript if the Discussion addressed more explicitly how these variants are thought to act.

Specifically, do the authors consider that the variants alter enhancer function itself, for example by affecting chromatin accessibility or enhancer–promoter interactions? Or are they more likely to primarily influence transcription factor binding, such as that of PPARγ or C/EBPβ? Alternatively, is the precise mechanism still unclear? Even a brief speculative discussion of these possibilities would enhance the interpretation and clinical relevance of the findings.

**Response:** We thank the reviewer for raising this point. The listed variants are predicted to affect enhancer accessibility, based on the H3H27ac signal. We have now explained more explicitly (page 18) that we expect causal variants to affect the affinity of transcription factor binding to their target enhancers, thereby affecting enhancer activity as assessed by H3K27ac and affecting *PPARG2* promoter activity.

4. Several enhancer–enhancer interaction labels appear to be missing in Fig. 4a. Please revise to ensure all relevant interactions are clearly annotated.

**Response:** We thank the reviewer for alerting us to Figure 4a, which we agree was difficult to understand. We had only indicated the interactions that are significantly affected by one or more enhancer deletions on day 0 or day 10 of adipogenesis. We have now revised **Figure 4a** such that all interactions detected between the enhancers are indicated on the figure, the interactions which are significantly affected by enhancer deletions are indicated with bold font, whereas interactions that are unaffected by deletions are indicated in grey color. Moreover, we replaced crosses with 'NA', to indicate interactions that cannot be assessed as they involve the deleted enhancers.

**Reviewer #4 (Remarks to the Author):**

Cetnarowska et al. investigate how PPARG2 is activated during adipocyte differentiation using human stromal progenitors (hMSC-TERT4) as model. Using Hi-C, Capture-C, ChIP-seq, and CRISPR/Cas9 enhancer deletions, the authors show that the PPARG locus contains a highly interconnected enhancer community. They demonstrate that multiple enhancers act in a non-redundant and cooperative manner, with promoter-proximal and super-enhancer constituents being essential for PPARG2 induction. Feedback regulation by PPARγ and C/EBPα further amplifies enhancer activity. Finally, they integrate GWAS data and deep learning predictions, showing that key enhancers overlap with non-coding variants linked to metabolic disease.
Overall, this is an excellent manuscript that provides important mechanistic insight into the regulation of PPARG2, a master regulator of adipogenesis. The study is highly relevant to both basic biology and metabolic disease.

**Response:** We thank the reviewer for her/his positive feedback, and for noting its importance for both basic biology and metabolic disease.

The manuscript has only a few limitations. E.g. it relies on immortalized hMSC-TERT4 cells, which may not fully capture in vivo adipose biology. Functional readouts are limited to mRNA expression and lipid accumulation; additional assays such as insulin sensitivity, adipokine secretion, or metabolic flux analyses could further support the conclusion that enhancer deletions affect adipocyte function. The integration of GWAS data was not further experimentally validated. Also, the work focuses on only the PPARG2 locus, thereby limiting its potential broader conclusion.

**Response:** We appreciate the reviewer's thoughtful comments and agree that the study has a few limitations.
**Model system.** Our work relies on adipogenic differentiation of immortalized hMSC-TERT4 cells, which does not fully recapitulate *in vivo* adipose biology. We now state this explicitly in the Discussion.
**Functional readouts.** The aim of this study was to investigate transcriptional regulation at the *PPARG* locus, not the downstream effects of limiting PPARG expression. We have focused on mapping chromatin accessibility and interactions, transcription factor occupancy, *PPARG2* expression, and lipid accumulation. Assays such as insulin sensitivity, adipokine secretion, or metabolic flux could be valuable follow-ups in model systems (ideally *in vivo*) that reach a more mature adipocyte state than is the case for hMSC-TERT4. To assess the downstream effects of modulating *PPARG* expression, we have measured expression of a few adipogenic marker genes: *CEBPB* (which is upstream of PPARγ), *FABP4*, *CEBPA* (which are PPARγ target genes) at day 10 of differentiation in control and enhancer-deleted hMSC-TERT4-iCas9-4 lines. These data are now provided as a new Supplementary Fig. 5g.
**GWAS integration/allelic effects.** We acknowledge this limitation. However, an experimental validation of GWAS associations for variants located within studied enhancers and predicted to affect *PPARG2* expression is beyond the scope of this study. Our primary aim was to focus on the mechanistic workings of the enhancer community in the *PPARG* locus and to characterize the contribution of individual enhancers to *PPARG2* activation and adipogenesis.
**Locus focus.** In our hMSC-TERT4 model system, differentiation leads to induced expression of only the *PPARG2* isoform (Supplementary Figure 1b). We acknowledge that this limits generalization beyond *PPARG2*; however, as stated on page 4, it also provides a clean framework to investigate cooperativity within the enhancer network regulating the adipocyte-specific *PPARG2* promoter without interference from the *PPARG1* promoter.

Despite these caveats, the manuscript is very strong, methodologically rigorous, and conceptually innovative. I believe it is already highly mature and suitable for publication after only minor revisions.

**Response:** We thank the reviewer for her/his very positive feedback.

Some typos:

line 75: communitiesis -> communities
line 220: three independent clones not transfected > three independent clones transfected
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**Reviewer #1 (Remarks to the Author):**

The authors have addressed all comment. Congratulations on this important study!

**Response:** We thank the reviewer for her/his positive feedback and for recognizing the importance of our studies.

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I consider that the authors have adequately addressed all points I previously raised. I have no further essential comments.

**Response:** We thank the reviewer for her/his positive feedback.

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**Response:** We thank the reviewer for co-revieweing the manuscript, and the Nature Communications initiative that supports peer-review training and recognition for Early Career Researchers.

**Reviewer #4 (Remarks to the Author):**

The authors made considerable efforts to revise the manuscript and have satisfyingly addressed the reviewers' comments. In my view, the quality of the manuscript has further improved and is of excellent quality, sufficient for publication.

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