nature portfolio

Peer Review File

Bone controls browning of white adipose tissue and protects from diet-induced obesity through Schnurri-3-regulated SLIT2 secretion

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The manuscript makes several significant conclusions. One major finding, which is well supported by the evidence, is that the adaptor protein Schnurr 3, a regulator of bone development, is involved in producing bone derived endocrine signal which regulates adipose browning and metabolism. Two other conclusions are that Shn3 site of action is localized to bone tissue, and that the secreted protein Slit2 is a major bone-derived signal that regulates adipose browning and metabolism. These latter two conclusions would be highly significant for the field. However, the results presented raise concerns about key points, and will need additional experiments to support the conclusions of the paper.
1. Shn3 site of action: better characterization of bone-specific conditional knockout. The section on the tissue specific deletions of Shn3 needs clarification of key information to reach the conclusion that Shn3 is required in bone tissue to produce a systemic signal. This experiment uses a Shn3 floxed allele crossed with Osx-Cre. The crucial missing information is to characterize the effectiveness and selectivity of this conditional knockout allele.
a. Effectiveness of Shn2 knockout. The paper does not show that Shn3 is knocked out specifically in the osteoblast lineage, which would need to be verified on the level of preferably Shn3 protein (or mRNA). Cre lines vary in their effectiveness in deleting a floxed gene in all cells of a tissue type, so this needs to be characterized for this particular floxed gene.
b. Bone selective knockout of Shn2 osx conditional knockout. This would be strengthened if it was shown to knockout Shn3 expression specifically in bone, while leaving Shn3 expression intact in other relevant tissues. It is not stated whether Shn3 is expressed in adipose tissue, but this is also relevant either way. While the parallel Adipo-Cre experiment gave a negative result in terms of impact on metabolism and obesity, this conditional knockout was also not characterized in terms of Shn3 expression.
2. Slit2 as a bone-derived endocrine factor. The experiments on Slit2 lead to the conclusion that Slit2 is produced by bone, is increased by Shn3 deficiency, produces the majority of circulating Slit2 protein, and that this acts as a signal on adipose tissue. These are significant findings for the paper and for the field, and conflict in part with prior publications. There are several key points that will need additional information and likely key experiments to be added to support these conclusions.
A.. A general point is that the SLIT2 protein can act either as a full length intact protein, or as one or both of two major isoforms formed by proteolytic cleavage, the N and C fragments. A prior study found Slit2 as an adipose-derived autocrine signal that promotes beige adipocyte metabolism. This was specifically detected as adipose-secreted Slit2-C. It is further important to note that previous studies provide evidence that Slit2-C is more soluble and thus more likely to contribute to Slit2 levels in serum. The manuscript should clarify which forms of Slit2 were detected. Related to Slit2 protein detection, no data is presented on which fragment or full Slit2 is produced by osteoblasts, detected in serum, or biologically active on adipose tissue.
B. Detection and quantification of Slit2 protein levels in various tissues and mutants. Slit2 protein detection is problematic with many commercially available reagents, particularly antibody-based detection “specific” for Slit2. The reported Slit2 protein secretion from osteoblasts in Fig 5 depends on a ELISA kit from Novus. The manufacturer website information on this kit is minimal, and there are no prior publications cited for the efficacy or selectivity of this kit. Although this company produces 11 different antibodies against Slit2 protein, the antibody used for the ELISA kit does not seem to be described on the company website. The ELISA results in the current paper also present data on the quantification of

SLIT2 levels, but it is not mentioned the source or the fragment of SLIT2 protein used as a standard. Of the 11 NOVUS antibodies on the company website, the best characterized antibody, NBP2-20398, has several western blots, but with differing band patterns and sizes. None of the antibody assays have a proper negative control of knockout tissue, and neither does the ELISA kit documentation mention a validation with knockout tissue. While in the Novus western blots the top band is potentially full length Slit2 and detected in transfected cell lines and in mouse and rat brain tissue, the blots shown have smaller cross reacting bands which differ in sizes on different blots. These smaller bands do not match with either the expected N or C fragment sizes. The presence of cross-reacting bands likely represent other proteins which confound interpretations of an ELISA assay. An important point is that bone, serum, and adipose are distinct tissues with potentially different or different levels of cross-reacting epitopes.
C. Separating the expression and function of Slit2 in bone and adipose. Slit2 C is produced by adipose tissue (Svennson paper cited), so it is not at all clear how the Slit2 OSX conditional mutant would alter adipose levels of Slit2 C or adipose metabolism. There is little discussion to reconcile the current findings with the prior study. Perhaps serum Slit2 is the dominant form of Slit2. In that case, a key experiment is to show that adipose Slit2 protein is significantly derived from bone and/or serum, which would involve assaying Slit2 protein levels in adipose to compare levels between Slit2 OSX conditional mutants and Cre-only controls.

Reviewer #2 (Remarks to the Author):

Brief Summary: In this study, the authors utilize a Schinurri-3 deficient mouse model to attempt to understand the relationship between bone metabolism and energy homeostasis driven by adipose tissue, and the role they play in browning of white adipose tissue and diet-induced obesity.

Key results: The authors show that Shn3-/- mice have an overall higher bone mass phenotype and are resistant to diet-induced obesity, have reduced insulin resistance, and tolerate glucose better than control mice. Additionally, the authors discovered that browning of white adipose tissue was a driving factor of the resistance to diet-induced obesity, because when Shn3-/- mice were exposed to thermoneutral temperatures, the diet-induced obesity profile was comparable to control mice. Using an elegant mouse model to determine if the effects of Shn3 were attributable to either osteoblasts or adipocytes, they deleted Shn3 using a Cre-lox system in osteoblasts (Shn3Osx) or adipocytes (Shn3Adipoq) of mice and challenged them both with a high fat diet. They observed that Shn3Osx and not Shn3Adipoq mice mimicked the resistant diet-induced obesity phenotype, a truly significant finding. The researchers performed an adipose transplant experiment by grafting white adipose tissue from wild type mice into Shn3Osx mice, and results showed increased browning of the transplanted white adipose tissue, from which the authors concluded that osteokines are responsible for the browning of this white adipose tissue. Using RNA sequencing data from their previous study, the authors analyzed differentially expressed genes in Shn3-deficient osteoblasts and found that SLIT2 was significantly upregulated. With this information, they generated a mouse model, which harboured a deletion of Slit2 in osteoblasts (Slit2Osx) and observed that the white adipose tissue of Slit2Osx mice did not undergo browning and they were no longer resistant to diet-induced obesity. The authors emphasize SLIT2 as a novel osteokine produced by osteoblasts which induces white adipose tissue browning in a SHN3-dependent manner. Finally, researchers utilized gene therapy to silence Shn3 and

successfully delivered the microRNA plasmid to the skeleton of high fat diet-fed wild type mice. Once again, they observed that these mice recapitulated resistance to diet-induced obesity. This is a valuable study because the researchers convincingly identified a potential target for treating both osteoporotic and obesity-related disorders.

Data, methodology, and statistics: The data is sound, and the methods are well-described and justified. Generation of mouse models is backed up by referring the reader to previous publications and sample sizes for each experiment are acceptable. Appropriate statistical tests are used throughout the study.

Suggested Improvements:

Thank you for submitting this manuscript for review. After careful consideration, I would recommend that you address the following comments and make the following major and minor changes:

Major:

1. The aim of the study is clear, but the hypothesis is not, please state it so that it is obvious to the reader.
2. In extended data, the caption for Figure 2 mentions that g) shows H&E staining of pancreas islet, but this is not present in the figure. This must be fixed.
3. Mitochondrial bioenergetics are very important to study with regards to metabolism and browning; therefore, I believe that this study would benefit from mitochondrial respiration experiments of the adipose tissue.
4. In figure 4f, the authors only show mRNA expression of Ucp-1, when in all other figures, they provide expression of various thermogenic markers. Other markers should be included, or an explanation for why they were not included should at least be provided.
5. Were Slit2 levels measured in the serum of mice that were treated with rAAV9-amiR-Shn3 to further support the SHN3-SLIT2 axis? The authors should consider doing this.
6. In the discussion, it is mentioned that “we still did not eliminate the possibility that additional osteoblast secreted factors in addition to SLIT2 contribute to the energy metabolism
phenotype of Shn3-deficient mice”. What is your plan to address this? Please expand on this.

Minor:

1. Only male mice were used in this study, do the researchers have an explanation for this?
2. Although the authors refer the reader to a previous publication that showcases the RNA sequencing experiment, it would be beneficial to provide a short description in the methods section clarifying how genes encoding for secreted proteins were filtered for.
3. In the caption for Figure 3, please provide the number of mice used to examine thermogenic gene expression (Figure 3o) and lipolytic gene expression (Figure 3p).
4. In figure 4b, do the authors have an explanation for why other thermogenic markers such as Pgc1a and Cox8b did not increase in the in vitro study?
5. I suggest re-wording the figure title for Figure 6 to say that the SHN3 depletion protected mice from development of DIO.

Reviewer #3 (Remarks to the Author):

In this study, the authors demonstrated that osteoblast-specific Osx-Cr;Shnf/f as well as systemic Shn3-/- mice exhibited not only augmented osteoblast activity and bone formation but also resistance against HFD-induced obesity, and improved glucose homeostasis and insulin sensitivity associated with enhanced browning of WAT. Through RNA-seq transcriptional profiling, robust enhancement of Slit2 expression and SLIT2 protein excretion from cultured Shn3-deficient osteoblasts was noted. AAV-mediated Shn3 silencing also showed similar phenotypes.
Methodologies are largely sound and the results bear novel and interesting findings. There are some concerns that need to be addressed by the authors.

Major comments
1. Because of the intrinsic skeletal defects in Osx-Cre mice, the authors stated to use Osx-Cre mice as controls (lines 252-254). However, in some metabolic studies, they used Shn3f/f mice as controls (Figure 3c lower panel, 3e-3p, Figure 4b-4f). Because weight gain in Shnf/f mice was larger than that in Osx-Cre (Figure 3a), differences in those parameters between Osx-Cre and Shnf/f mice as controls may make a difference in the interpretation of the results. It is fair to use the same mice line as controls throughout the study. Otherwise, as shown in the upper panel of Figure 3c, the authors should show results using both Osx-Cre and Shnf/f as controls.
2. Slit2 was originally reported to be expressed in beige cells, and not a full-size 180 kDa SLIT2 but 50 kDa C-terminal fragment was shown to promote adipose thermogenesis (Ref. 22). What is the size of SLIT2 secreted from osteoblasts and present in the serum? In addition, in order to clarify the possibility that “osteoblasts are the major source of circulating SLIT2 and the key cell type determining overall serum SLIT2 levels” (lines 330-332), it is important to demonstrate serum SLIT2 levels in Apn-Cre;Slit2f/f in addition to Osx-Cre;Slit2f/f mice.
3. Serum SLIT2 levels in Figure 5d are only 1/10 of those in Figure 5c. These results again raise a concern that Osx-Cre mice are very different from WT, Shn3f/f or Slit2f/f mice, and cast doubt about the validity of using Osx-Cre mice as controls. The authors should demonstrate serum SLIT2 levels in Slit2f/f mice.
4. SHN3 is an intracellular adaptor protein that controls stabilization of beta-catenin downstream Wnt signaling. Therefore, gene deletion or AAV-mediated depletion of Shn3 in osteoblasts enhances Wnt signaling via stabilization of beta-catenin. Thus, the question arises whether enhanced SLIT2 production by SHN3 depletion in osteoblasts is mediated via enhanced Wnt signaling. If this is the case, maneuvers that enhance Wnt signaling can also enhance Slit2 expression in osteoblasts.
5. SLIT family proteins bind to Robo (Roundabout) receptors to exert their actions mainly in the developing nervous system (Ref.31). Therefore, if SLIT2 acts directly in adipocytes, it is important to demonstrate whether these cells express Robo receptors.

Minor comments
1. The unit of blood glucose in Figure 3e is mg/dL and that in extended Figure 3e and 3f is mmol/L. For better comparison, they should be standardized.
2. Significant differences between groups are shown between time points in Figures 1a, 3a, 3e, 3f, 5e, and 6e. However, such presentation is not easy to follow, and there appear to be difference in significance levels at different time points in some Figures. It is better to present significance level at each time point.

Point-by-point response:

Reviewer #1 (Remarks to the Author):

The manuscript makes several significant conclusions. One major finding, which is well supported by the evidence, is that the adaptor protein Schnurr 3, a regulator of bone development, is involved in producing bone derived endocrine signal which regulates adipose browning and metabolism. Two other conclusions are that Shn3 site of action is localized to bone tissue, and that the secreted protein Slit2 is a major bone-derived signal that regulates adipose browning and metabolism. These latter two conclusions would be highly significant for the field. However, the results presented raise concerns about key points, and will need additional experiments to support the conclusions of the paper.

Authors' response:
We appreciate the reviewer's judgement that the findings presented here are "highly significant for the field". The reviewer has raised several important points below that have been addressed with new data, overall significantly strengthening this manuscript. Please see our responses below.

1. Shn3 site of action: better characterization of bone-specific conditional knockout. The section on the tissue specific deletions of Shn3 needs clarification of key information to reach the conclusion that Shn3 is required in bone tissue to produce a systemic signal. This experiment uses a Shn3 floxed allele crossed with Osx-Cre. The crucial missing information is to characterize the effectiveness and selectivity of this conditional knockout allele.
a. Effectiveness of Shn3 knockout. The paper does not show that Shn3 is knocked out specifically in the osteoblast lineage, which would need to be verified on the level of preferably Shn3 protein (or mRNA). Cre lines vary in their effectiveness in deleting a floxed gene in all cells of a tissue type, so this needs to be characterized for this particular floxed gene.

Authors' response:
This is an important point, and we fully agree that the efficiency of Cre-mediated tissue-specific deletion should be determined. In additional data generated to address this comment, we find that *Shn3* expression was efficiently ablated in skeletal tissue but not iWAT in $Shn3^{Osx}$ mice relative to $Shn3^{f/f}$ controls (Supplementary Fig. 3a-b). In addition, we note that Xu et al. Nat Med 2018 also provides validation of the skeletal phenotype of $Shn3^{Osx}$ mice and a direct comparison of the phenotypes obtained with other skeletal-targeting cre lines, showing that multiple skeletal targeting cre lines provide the same phenotype, thereby providing complementary phenotypic validation. Others have also examined the sites of Osx-cre activity using lineage reporters and found similar patterns of activity, including in studies comparing the targeting of skeletal tissue to peripheral adipose depots (Chen et al. PLOS One 2014).

b. Bone selective knockout of Shn3 osx conditional knockout. This would be strengthened if it was shown to knockout Shn3 expression specifically in bone, while leaving Shn3 expression intact in other relevant tissues. It is not stated whether Shn3 is expressed in adipose tissue, but this is also relevant either way. While the parallel Adipo-Cre experiment gave a negative result in terms of impact on metabolism and obesity, this conditional knockout was also not characterized in terms of Shn3 expression.

Authors' response:
We agree with the reviewer that determination of Cre targeting is important for this study. *Shn3* expression is widely present in multiple organ/tissues (GSE10246) including in adipose tissue[1]. As requested, we have analyzed *Shn3* expression in inguinal white adipose tissues (iWAT) of $Shn3^{Osx}$ mice and $Shn3^{f/f}$ mice. This confirmed that Osx-cre

only deleted *Shn3* in skeletal but not iWAT tissue (Supplementary Fig. 3b). The converse result was observed in *Shn3*^{Adq} mice and *Shn3*$^{f/f}$ mice, finding that Adipo-Cre only deleted *Shn3* in adipose tissue but not osteolineage skeletal cells (Supplementary Fig. 3c-d).

2. Slit2 as a bone-derived endocrine factor. The experiments on Slit2 lead to the conclusion that Slit2 is produced by bone, is increased by Shn3 deficiency, produces the majority of circulating Slit2 protein, and that this acts as a signal on adipose tissue. These are significant findings for the paper and for the field, and conflict in part with prior publications. There are several key points that will need additional information and likely key experiments to be added to support these conclusions.
A.. A general point is that the SLIT2 protein can act either as a full length intact protein, or as one or both of two major isoforms formed by proteolytic cleavage, the N and C fragments. A prior study found Slit2 as an adipose-derived autocrine signal that promotes beige adipocyte metabolism. This was specifically detected as adipose-secreted Slit2-C. It is further important to note that previous studies provide evidence that Slit2-C is more soluble and thus more likely to contribute to Slit2 levels in serum. The manuscript should clarify which forms of Slit2 were detected. Related to Slit2 protein detection, no data is presented on which fragment or full Slit2 is produced by osteoblasts, detected in serum, or biologically active on adipose tissue.

Authors' response:
We agree that these are important points, and addressing these questions has been a major focus of our efforts during revisions. To clarify which forms of SLIT2 were produced by osteoblasts, we performed immunoblotting using an anti-SLIT2 antibody validated in the prior study discussed by the reviewer here (Cat No. ab134166 from Abcam, as used in Svensson KJ, et al. Cell Metab. 2016) showing that osteoblasts can generate both full length SLIT2 and the same SLIT2 C-terminal fragment (SLIT2-C) reported in Svensson et al. (Fig. 5b). Further consistent with the findings in Svensson et al., only SLIT2-C is present in both serum and osteoblast supernatant, and this finding was further validated by mass spectrometry analysis (Fig. 5b-c and Supplementary Figure 4c). We have modeled our presentation of the mass spectrometry data on the presentation of similar data in Svensson et al. for clarity and ease of comparison. Deletion of *Slit2* in osteoblasts but not adipocytes *in vivo* led to a significant decrease in circulating SLIT2-C in serum samples (Fig. 5g-h and Supplementary Figure 4e-f), indicating that bone is a major source of the total circulating SLIT2-C. Thus, we believe that these results are overall in line with and supportive of the results of Svensson et al., albeit now providing new information that bone is a major source of total circulating SLIT2-C, in addition to the local production of SLIT2-C within adipose tissue reported in Svensson et al.

B. Detection and quantification of Slit2 protein levels in various tissues and mutants. Slit2 protein detection is problematic with many commercially available reagents, particularly antibody-based detection specific for Slit2. The reported Slit2 protein secretion from osteoblasts in Fig 5 depends on a ELISA kit from Novus. The manufacturer website information on this kit is minimal, and there are no prior publications cited for the efficacy or selectivity of this kit. Although this company produces 11 different antibodies against Slit2 protein, the antibody used for the ELISA kit does not seem to be described on the company website. The ELISA results in the current paper also present data on the quantification of SLIT2 levels, but it is not mentioned the source or the fragment of SLIT2 protein used as a standard. Of the 11 NOVUS antibodies on the company website, the best characterized antibody, NBP2-20398, has several western blots, but with differing band patterns and sizes. None of the antibody assays have a proper negative control of knockout tissue, and neither does the ELISA kit documentation mention a validation with knockout tissue. While in the Novus western blots the top band is potentially full length Slit2 and detected in transfected cell lines and in mouse and rat brain tissue, the blots shown have smaller cross reacting bands which differ in sizes on different blots. These smaller bands do not match with either the expected N or C fragment sizes. The presence of cross-reacting bands likely represent other proteins which confound interpretations of an ELISA assay. An important point is

that bone, serum, and adipose are distinct tissues with potentially different or different levels of cross-reacting epitopes.

Authors' response:

Thank you for raising this critical question, and we share the reviewer's interest in assessing the ability of those commercial antibodies, as well as ELISA kits, to detect the various SLIT2 cleavage fragments known to be present. However, we were unable to obtain further information from Novus Biological other than the public-facing information mentioned by the reviewer. Herein, we sought to evaluate the performance and assay characteristics of the commercial ELISA kit (Kit No. NBP2-82435, Novus Biologicals) used in this study. Importantly, this additional validation of the ELISA assay is complemented during revisions by additional SLIT2 immunoblotting and mass spectrometry data. We believe that the combination of all three methods, each providing consistent and complementary information, allows for a robust conclusion regarding SLIT2 secretion by bone cells.

We first validated the linearity of our ELISA assays through using recombinant Slit2 N-terminal (aa26-900, Cat.No 5444-SL, R&D systems) and Slit2 C-terminal protein fragments (aa1114-1521, 9430-SL-050, R&D systems) in a spike and recovery study. The results are shown as below (Figures 1 and 2 for reviewers. The X axis represents the concentration in ng/mL of each of the recombinant proteins. The Y axis represents assay signal, here expressed as the raw OD value.).

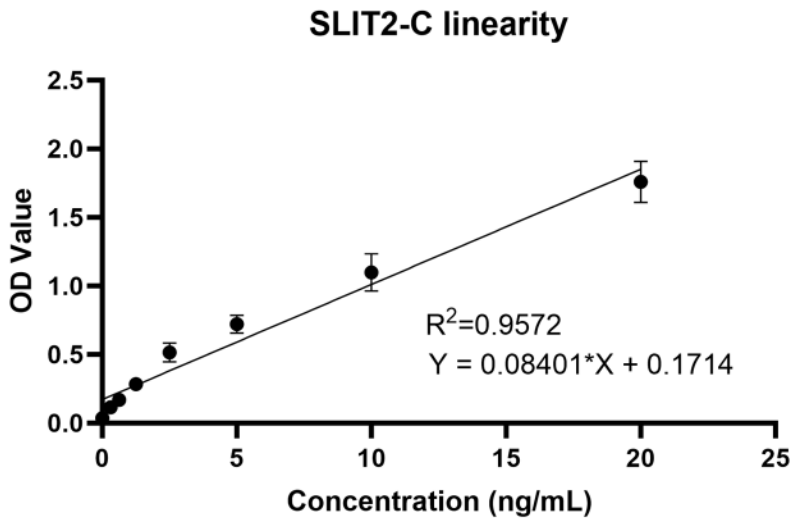


Figure 1 for reviewers.

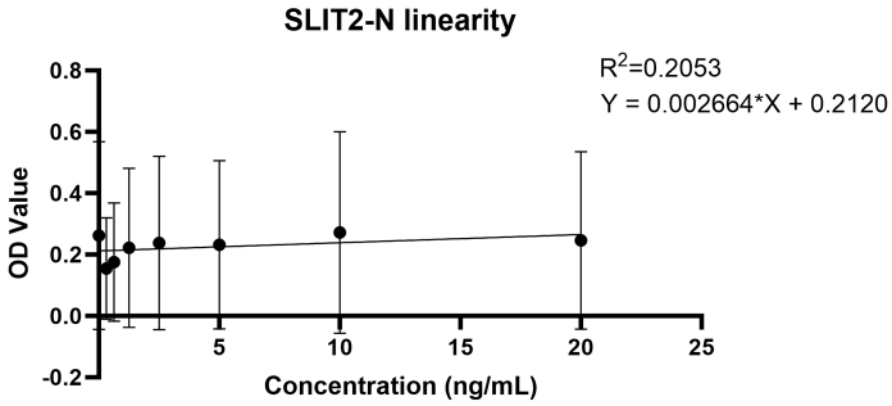


Figure 2 for reviewers.

These studies showed a linear and dose dependent signal in response to C-terminal but not N-terminal SLIT2. Next, we used a dilutional linearity study on murine serum (Figure 3 for reviewers), where demonstrating linearity of dilution is an important step in establishing that signal observed is due to the true presence of analyte. We note that both the spike and recovery and dilutional linearity assessment methods are core methods used for clinical immunoassay validation as standard approaches (e.g., as elaborated in detail in Andreasson et al. Frontiers Neurology 2015 and as more briefly summarized in https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-elisa/spike-recovery-linearity-assessment.html). Overall, these data provide evidence that the ELISA used here is likely to detect the SLIT2-C fragment and strengthens the overall confidence in the validity of the ELISA utilized here. In addition to the validation performed above, we also provided genetic evidence for the specificity of the ELISA used, noting that *Slit2*^{osx} mice display a marked reduction in ELISA signal. Lastly and most importantly, during revisions we have added additional immunoblotting and mass spectrometry-based methods as applied to both osteoblast conditioned medium and serum from the genetically altered mouse lines studied here as complementary approaches. As described in more detail below, these approaches confirm the results of the ELISA studies, overall offering multiple lines of evidence that osteoblasts are a major source of circulating serum SLIT2-C.

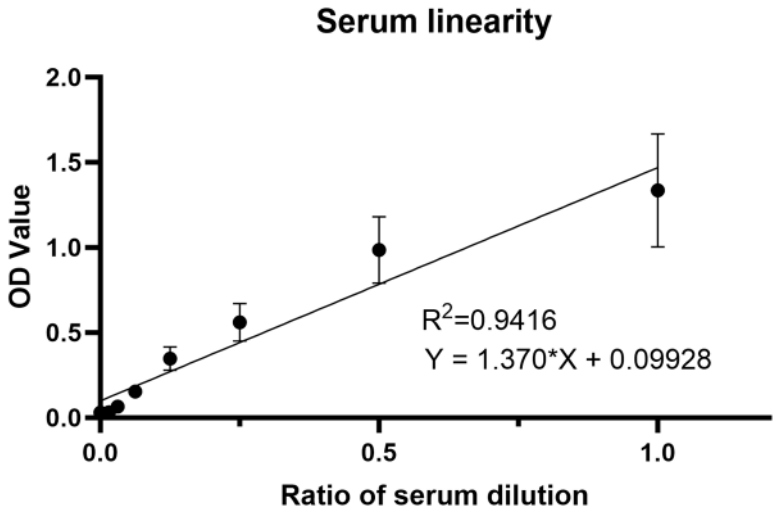


Figure for reviewers. 3

C. Separating the expression and function of Slit2 in bone and adipose. Slit2 C is produced by adipose tissue (Svennson paper cited), so it is not at all clear how the Slit2 OSX conditional mutant would alter adipose levels of Slit2 C or adipose metabolism. There is little discussion to reconcile the current findings with the prior study. Perhaps serum Slit2 is the dominant form of Slit2. In that case, a key experiment is to show that adipose Slit2 protein is significantly derived from bone and/or serum, which would involve assaying Slit2 protein levels in adipose to compare levels between Slit2 OSX conditional mutants and Cre-only controls.

Authors' response:

We thank you for raising these important issues. While the issue of how the function of bone-derived SLIT2 relates to SLIT2 produced locally in adipose tissue is complex and difficult to fully resolve within the scope of this study, we do feel it important to emphasize that our results here do not conflict with those in Svennson et al. and indeed overall raise the possibility that systemic bone-derived SLIT2 and local adipose-derived SLIT2 could play complementary roles in regulation of systemic energy metabolism. Additionally, many elements of the present report reinforce mechanistic elements of the Svennson report, such as the findings in that study on the role of a SLIT2 C-terminal fragment or the adipose browning effect and overall metabolic impact of SLIT2. This has been better communicated with an additional paragraph in the discussion section focused on the relationship of this study to the results in Svennson et al.

One possibility raised by our findings is that bone-derived and local adipo-lineage produced SLIT2 could be subject to differential transcriptional regulation, allowing these two distinct sources of SLIT2 to each exert dominant effects under distinct conditions. In line with this, we primarily study the role of bone-derived SLIT2 after high fat diet exposure, whereas in the Svennson et al. report, high fat diet is noted to suppress SLIT2 expression. During revisions, we have strengthened our data on this point, adding immunoblotting data showing osteoblasts tested ex vivo from high-fat-diet (HFD) exposed mice are still able to secrete SLIT2-C protein, whereas a much lower level of SLIT2-C signal was detected ex vivo in primary adipocytes from HFD-fed mice (Supplementary Fig. 4b, left). Similarly, we have generated complementary in vivo data through both immunoblotting and qPCR, that bone tissue retained robust SLIT2 expression in the setting of HFD exposure whereas adipose tissue exhibited an overall lower level of local SLIT2 expression (Supplementary Fig. 4b, right). We believe that these data are broadly consistent with both the major findings of our present report, that bone derived SLIT2 regulates energy metabolism after HFD exposure, and the Svennson report, that HFD suppresses adipolineage SLIT2 expression. These also offer potential biologic rationale for the dual expression of metabolically active SLIT2 in both bone and adipose tissue, as SLIT2 expression at these two sites is differentially regulated and may therefore be responsive to different physiologic and pathologic contexts.

Additionally, during revisions we have analyzed *Slit2* levels in public tissue expression data (GSE10246, Lattin JE, Schroder K, Su AI, Walker JR et al. Expression analysis of G Protein-Coupled Receptors in mouse macrophages. *Immunome Res.* 2008 Apr 29;4:5.), with external transcriptional data supporting our observations that bone produces markedly high amounts of SLIT2 relative to other tissues (Supplementary Figure 4a).

The experiment suggested here to measure levels of serum-derived SLIT2 protein in adipose tissue is would be an excellent approach to advance towards the goal of understanding how locally produced and systemic (bone produced) SLIT2-C relate and each differentially impact local SLIT2. However, in pilot studies modeled on approaches in the recent Mittenbühler et al, Cell Metab 2023, we found that the obtainable ECF volumes were very scant and posed a considerable challenge for sensitive measurement of SLIT2 levels.

To summarize, we here present new data that bone and adipose expression of SLIT2 are independent and likely to be differentially regulated, suggesting that these two sources of SLIT2 are likely to play distinct biologic roles.

Reviewer #2 (Remarks to the Author):

Brief Summary: In this study, the authors utilize a Schinurri-3 deficient mouse model to attempt to understand the relationship between bone metabolism and energy homeostasis driven by adipose tissue, and the role they play in browning of white adipose tissue and diet-induced obesity.

Key results: The authors show that Shn3-/- mice have an overall higher bone mass phenotype and are resistant to diet-induced obesity, have reduced insulin resistance, and tolerate glucose better than control mice. Additionally, the authors discovered that browning of white adipose tissue was a driving factor of the resistance to diet-induced obesity, because when Shn3-/- mice were exposed to thermoneutral temperatures, the diet-induced obesity profile was comparable to control mice. Using an elegant mouse model to determine if the effects of Shn3 were attributable to either osteoblasts or adipocytes, they deleted Shn3 using a Cre-lox system in osteoblasts (Shn3Osx) or adipocytes (Shn3Adipoq) of mice and challenged them both with a high fat diet. They observed that Shn3Osx and not Shn3Adipoq mice mimicked the resistant diet-induced obesity phenotype, a truly significant finding. The researchers performed an adipose transplant experiment by grafting white adipose tissue from wild type mice into Shn3Osx mice, and results showed increased browning of the transplanted white adipose tissue, from which the authors concluded that osteokines are responsible for the browning of this white adipose tissue. Using RNA sequencing data from their previous study, the authors analyzed differentially expressed genes in Shn3-deficient osteoblasts and found that SLIT2 was significantly upregulated. With this information, they generated a mouse model, which harboured a deletion of Slit2 in osteoblasts (Slit2Osx) and observed that the white adipose tissue of Slit2Osx mice did not undergo browning and they were no longer resistant to diet-induced obesity. The authors emphasize SLIT2 as a novel osteokine produced by osteoblasts which induces white adipose tissue browning in a SHN3-dependent manner. Finally, researchers utilized gene therapy to silence Shn3 and successfully delivered the microRNA plasmid to the skeleton of high fat diet-fed wild type mice. Once again, they observed that these mice recapitulated resistance to diet-induced obesity. This is a valuable study because the researchers convincingly identified a potential target for treating both osteoporotic and obesity-related disorders.

Data, methodology, and statistics: The data is sound, and the methods are well-described and justified. Generation of mouse models is backed up by referring the reader to previous publications and sample sizes for each experiment are acceptable. Appropriate statistical tests are used throughout the study.

Authors' response:
We thank the reviewer for judging that this work represents "a truly significant finding" and also for the constructive suggestions that follow. We believe that addressing these suggestions over the course of revisions has greatly improved the manuscript. Please see our responses below.

Suggested Improvements:

Thank you for submitting this manuscript for review. After careful consideration, I would recommend that you address the following comments and make the following major and minor changes:

Major:

1. The aim of the study is clear, but the hypothesis is not, please state it so that it is obvious to the reader.

Authors' response:

We have edited the manuscript to now include clear hypothesis statements in both the introduction (lines 166-169) and results (lines 305-307).

2. In extended data, the caption for Figure 2 mentions that g) shows H&E staining of pancreas islet, but this is not present in the figure. This must be fixed.

Authors' response:

We thank the reviewer for pointing out this omission. This result has been added to the revised manuscript in Supplementary Fig 2f.

3. Mitochondrial bioenergetics are very important to study with regards to metabolism and browning; therefore, I believe that this study would benefit from mitochondrial respiration experiments of the adipose tissue.

Authors' response:

We fully agree that the mitochondrial bioenergetics are critical to our study and we share the reviewer's interest in evaluating how skeletal SHN3-deficiency impacts mitochondrial respiration of primary iWAT cells. **This result has been added during revisions as Figure 2h.** Primary iWAT cells from Shn3-KO mice displayed increased oxygen consumption, specifically elevations in spare respiratory capacity, maximal respiration and ATP production. We believe that this data markedly improves the manuscript by now showing direct functional evidence for the adipose browning effect mediated by the skeletal SHN3/SLIT2 axis identified here.

4. In figure 4f, the authors only show mRNA expression of Ucp-1, when in all other figures, they provide expression of various thermogenic markers. Other markers should be included, or an explanation for why they were not included should at least be provided.

Authors' response:

We thank the reviewer for this suggestion. We repeated the study in **revised Fig 4f** and updated all panels to include a broader set of thermogenic genes that parallel the thermogenic markers utilized in other figures. All of the thermogenic marker genes were increased in the SHN3-conditional knockout group.

5. Were Slit2 levels measured in the serum of mice that were treated with rAAV9-amiR-Shn3 to further support the SHN3-SLIT2 axis? The authors should consider doing this.

Authors' response:

We agree that the reviewer's suggestion would strengthen the study. During revisions, we have measured the levels of SLIT2-C in the circulation of the AAV-treated murine models. According to the recommendations of reviewer #1, we utilized immunoblots to evaluate the SLIT2 level in sera instead of an ELISA-based approach, in part as this allows discrimination of full-length versus active fragments of SLIT2. As shown in Supplementary Figure 5b-c, circulating SLIT2-C levels were increased in AAV9-amiR-Shn3 treated mice relative to mice treated with a AAV9-amiR-Ctrl control. Thus, postnatal depletion of SHN3 in bone can not only elevate bone mass but also increase SLIT2 production, thereby resisting the development of diet-induced-obesity.

6. In the discussion, it is mentioned that we still did not eliminate the possibility that additional osteoblast secreted factors in addition to SLIT2 contribute to the energy metabolism phenotype of Shn3-deficient mice§. What is your plan to address this? Please expand on this.

Authors' response:
We thank the reviewer for this suggestion, and in the revised manuscript, we have added additional discussion on this point. In brief, we point out how identification of SLIT2 can facilitate further identification of additional osteokines by examining SHN3, SLIT2-compound deficient osteoblasts for additional effects on energy metabolism, such as additional WAT browning effects as a starting point to identify additional osteokines. Likewise, another complementary approach could include examining mice that are genetically not capable of WAT browning for additional effector mechanisms of bone regulation of energy metabolism and then linking those effector mechanisms to their bone derived mediators. A discussion of these points has been added to lines 484-498 in our revised manuscript.

Minor:

1. Only male mice were used in this study, do the researchers have an explanation for this?

Authors' response:
We agree with the reviewer that consideration of whether the skeletal SHN3/SLIT2 axis has sex dependent effects is important. Our initial focus on male mice was driven by a desire to maximize the ability to compare between experiments in this report. In revised Supplementary fig 1c-d, we have now added new data showing that female HFD exposed *Shn3*$^{-/-}$ versus WT control mice show a similar phenotype as their male counterparts.

2. Although the authors refer the reader to a previous publication that showcases the RNA sequencing experiment, it would be beneficial to provide a short description in the methods section clarifying how genes encoding for secreted proteins were filtered for.

Authors' response:
Genes that were differentially expressed were rank ordered based of the degree of differential expression and then manually screened for secreted factors. The top 7 differentially expressed genes encoding for secreted factors were then visualized by a heatmap and then further scrutinized for additional study based 1) deprioritizing matrix proteins that are unlikely to reach systemic circulation other than short propeptides/telopeptide fragments, 2) prioritizing genes with a robust absolute degree of expression in osteoblasts, and 3) prioritizing factors known to have a metabolic regulatory activity in other contexts. This is now better described in the revised methods section.

3. In the caption for Figure 3, please provide the number of mice used to examine thermogenic gene expression (Figure 3o) and lipolytic gene expression (Figure 3p).

Authors' response:
Thank you for bringing up this issue. We have added this information to our revised manuscript figure legends.

4. In figure 4b, do the authors have an explanation for why other thermogenic markers such as Pgc1a and Cox8b did not increase in the in vitro study?

Authors' response:
We thank the reviewer for pointing this out. Upon re-reviewing the data with a focus on this point, we thought it possible that only a moderate degree of beige differentiation was seen in the original experiment and that this may have masked observing the expected increase in the full range of regulated thermogenic markers. To address this, we have fully repeated this study in the revised Fig 4b, achieving a more robust overall degree of thermogenic

differentiation in both groups and thereby finding that all of the thermogenic marker genes are upregulated by exposure to osteokines from SHN3-deficient osteoblasts.

5. I suggest re-wording the figure title for Figure 6 to say that the SHN3 depletion protected mice from development of DIO.

Authors' response:

We appreciate this suggestion and have accordingly re-worded the title of Figure 6 to "AAV-9 mediated SHN3 depletion protected mice from development of DIO".

Reviewer #3 (Remarks to the Author):

In this study, the authors demonstrated that osteoblast-specific Osx-Cr;Shnf/f as well as systemic Shn3-/- mice exhibited not only augmented osteoblast activity and bone formation but also resistance against HFD-induced obesity, and improved glucose homeostasis and insulin sensitivity associated with enhanced browning of WAT. Through RNA-seq transcriptional profiling, robust enhancement of Slit2 expression and SLIT2 protein excretion from cultured Shn3-deficient osteoblasts was noted. AAV-mediated Shn3 silencing also showed similar phenotypes. Methodologies are largely sound and the results bear novel and interesting findings. There are some concerns that need to be addressed by the authors.

Authors' response:

We are grateful for the reviewer's judgement that the work is novel and interesting and for the constructive suggestions that follow. These have been addressed over the course of manuscript revisions as detailed below, which we believe has substantially improved the overall quality of the manuscript.

Major comments

1. Because of the intrinsic skeletal defects in Osx-Cre mice, the authors stated to use Osx-Cre mice as controls (lines 252-254). However, in some metabolic studies, they used Shn3f/f mice as controls (Figure 3c lower panel, 3e-3p, Figure 4b-4f). Because weight gain in Shnf/f mice was larger than that in Osx-Cre (Figure 3a), differences in those parameters between Osx-Cre and Shnf/f mice as controls may make a difference in the interpretation of the results. It is fair to use the same mice line as controls throughout the study. Otherwise, as shown in the upper panel of Figure 3c, the authors should show results using both Osx-Cre and Shnf/f as controls.

Authors' response:

We thank you for raising this critical issue. We agree that having identical control groups throughout the study would facilitate comparison between experiments. During revisions we have added additional control groups to address this issue, we have added a new cohort of mice using *Shn3* floxed allele controls (revised Fig. 3a, 3c-d) and have placed the prior data using Osx-cre only controls for the same study into supplementary Fig 3i-l. Both cohorts share the same phenotype and support the same conclusions. Regardless of whether an osx-cre only control or a Shn3 floxed allele control without cre is used, SHN3-ablation in bone is able to drive adipose tissue browning and reduced body weight gain in the DIO animal model.

Similarly, we also added *Slit2* floxed controls for studies in the *Slit2*^{osx} mice. As seen in the revised Fig 5i, these repeated studies confirm the original results with the *Slit2*^{Osx} mice gaining increased weight in response to HFD relative to Slit2$^{f/f}$ controls. Similarly, secondary endpoints such as increases in total adiposity and decreases in the expression of thermogenic marker genes in *Slit2*^{Osx} mice were also repeated with these new controls. To avoid confusion and potential issues associated with merging these new cohorts with the previous data, we have placed

our previous data for this study into revised Supplementary Figure 4n-q, so that both data utilizing osx-cre controls and *Slit2* floxed allele controls is available. Similar to the above, both the use of osx-cre only controls or Slit2 floxed allele without cre controls provide the same conclusion, that conditional knockout of SLIT2 in bone is able to drive increased body weight gain and decreased thermogenesis in HFD-exposed mice.

2. Slit2 was originally reported to be expressed in beige cells, and not a full-size 180 kDa SLIT2 but 50 kDa C-terminal fragment was shown to promote adipose thermogenesis (Ref. 22). What is the size of SLIT2 secreted from osteoblasts and present in the serum? In addition, in order to clarify the possibility that osteoblasts are the major source of circulating SLIT2 and the key cell type determining overall serum SLIT2 levels (lines 330-332), it is important to demonstrate serum SLIT2 levels in Apn-Cre;Slit2f/f in addition to Osx-Cre;Slit2f/f mice.

Authors' response:

We agree that the reviewer here raises a critical point that has been a major focus of efforts during revisions. During revisions, we performed both immunoblotting and mass spectrometry in order to identify the forms of Slit2 secreted from osteoblasts that are present in serum. Indeed, as shown in **revised Figure 5b-c and revised Supplementary Figure 4a-c**, a C-terminal SLIT2 fragment was produced by osteoblasts and is the major form of SLIT2 detected in serum (SLIT2-C, matching the C-terminal fragment in the reference discussed by the reviewer here.)

Per the reviewer's suggestion, we also generated Adipoq-Cre Slit2$^{f/f}$ mice (*Slit2*^{Adipoq} mice) and compared their circulating Slit2-C levels to those of littermate controls as presented in the **revised Supplementary Figure 4e**. We found that depletion of *Slit2* in adipolineage cells did not impact effect global circulating levels of Slit2-C level. This data is also consistent with our finding that circulating SLIT2 levels are markedly reduced in *Slit2*^{osx} mice and that bone is therefore the major source of circulating SLIT2.

3. Serum SLIT2 levels in Figure 5d are only 1/10 of those in Figure 5c. These results again raise a concern that Osx-Cre mice are very different from WT, Shn3f/f or Slit2f/f mice, and cast doubt about the validity of using Osx-Cre mice as controls. The authors should demonstrate serum SLIT2 levels in Slit2f/f mice.

Authors' response:

Thank you for raising this important question. During revisions, we utilized both immunoblotting and ELISA assays to confirm serum SLIT2 levels in *Slit2*$^{f/f}$ mice to provide an additional control group to compliment the osx-cre control and address this concern. Firstly, we demonstrated that, in a continuation of the response immediately above, SLIT2-C was the major serum form of SLIT2 present in Slit2$^{f/f}$ mice using immunoblotting (Fig 5g and Supplementary Fig 4e). Furthermore, as shown in Figure 5h, we found that circulating average Slit2 level in HFD-fed *Slit2*$^{f/f}$ mice shows a mean level of 8.231ng/mL, which is close to our previous results (10.63ng/mL in HFD exposed WT mice). Thus, comparison to either Osx-cre or *Slit2*$^{f/f}$ controls provide the same conclusion, that *Slit2*^{osx} mice display marked reductions in circulating SLIT2 levels, establishing bone as the major source of circulating SLIT2. Regarding Osx-cre, it is appreciated in the field that Osx-cre mice display a mild delay in bone formation (e.g., Huang and Olsen, Transgenic Res 2015), however Osx-cre remains likely the most widely utilized bone targeting cre due to its high efficiency and action early in the osteoblast lineage. Therefore, it is anticipated that Osx-cre mice can display mild phenotypes and it is therefore generally highly encouraged to utilize Osx-cre mice as the control of choice for studies utilizing this line. However, for this reason, we do believe that it is important to retain the Osx-cre controls as these allow for exclusion that any phenotypes observed could be due to confounding effects of the cre allele. Currently, being able to provide both the *Slit2*$^{f/f}$ and osx-cre controls is ideal as this will provide both points of comparison.

4. SHN3 is an intracellular adaptor protein that controls stabilization of beta-catenin downstream Wnt signaling. Therefore, gene deletion or AAV-mediated depletion of Shn3 in osteoblasts enhances Wnt signaling via stabilization of beta-catenin. Thus, the question arises whether enhanced SLIT2 production by SHN3 depletion in osteoblasts is mediated via enhanced Wnt signaling. If this is the case, maneuvers that enhance Wnt signaling can also enhance Slit2 expression in osteoblasts.

Authors' response:

We share the reviewer's interest in understanding how SHN3 regulates SLIT2 production. Per the reviewer's suggestion, we evaluated the role of Wnt signaling in regulating SLIT2 synthesis, with new data added to **revised Supplementary Figure 4d**. We utilized WNT3a as a direct WNT agonist. In line with the reviewer's suggestion, we observed a striking elevation in SLIT2-C production in differentiated osteoblasts, in line with the increase in SLIT2 production in SHN3-deficient osteoblasts. Thus, WNT signaling does indeed regulate osteoblast SLIT2 production as the reviewer proposed.

5. SLIT family proteins bind to Robo (Roundabout) receptors to exert their actions mainly in the developing nervous system (Ref.31). Therefore, if SLIT2 acts directly in adipocytes, it is important to demonstrate whether these cells express Robo receptors.

Authors' response:

We share the reviewer's interest in identifying how SLITs act in adipolineage cells. Since classical Slit-Robo signaling primarily acts through ROBO1 and ROBO2, we focused on assessing Robo1/2 expression in adipose tissue to address this point. As shown in **revised Supplementary Figure 4i**, we found that iWAT expresses both Robo1 and Robo2. These expression levels were not changed in SHN3-deficient mice.

Minor comments

1. The unit of blood glucose in Figure 3e is mg/dL and that in extended Figure 3e and 3f is mmol/L. For better comparison, they should be standardized.

Authors' response:

We thank the reviewer for this helpful suggestion. We have changed this in the revised manuscript in Supplementary Figure 3k and 3l.

2. Significant differences between groups are shown between time points in Figures 1a, 3a, 3e, 3f, 5e, and 6e. However, such presentation is not easy to follow, and there appear to be difference in significance levels at different time points in some Figures. It is better to present significance level at each time point.

Authors' response:

We thank you for this recommendation to improve manuscript clarity. We had changed the presentation of statistical significance levels at these different time points and also similarly modified our data presentation in other figures to improve consistency and clarity. Now significance levels are indicated above each time point. Please see our revised Fig 1a, 2h, 3a, 3b, 3e, 3f, 5i, 6e and Supplementary Fig 4m.

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The revised manuscript is improved with significant new results that address most of the reviewer concerns, particularly the concerns about the Slit2 part of the study. The overall strengths of the study include thorough and crucial genetic experiments that demonstrate several significant advances for the field. These include a key function for Schnurr3 in regulation of bone-derived endocrine signals that regulate adipose metabolism, that Shn3 function is localized to bone, and evidence that at least one key bone-derived signal is the Slit2-C protein fragment found circulating in the blood. The Slit2-C detected in blood is largely sourced from bone tissue, based on key evidence using a bone specific knockout of Slit2. Building on these main conclusions, another interesting finding is that a transient interference of Shn3 function using a viral interference vector can increase Slit2-C levels in circulation, and that this causes obesity resistance to high fat diet. This work also adds to and complements a prior paper on Slit2-C produced in adipose tissue, with discussion added to propose how the two tissue sources of this signal may function.
A few minor points to add to the revised manuscript:
Add validation of ELISA assay for Slit2-C serum concentrations to Supplementary Figure. A significant improvement in the manuscript is the thorough characterization of the Slit2 ELISA assay. Unfortunately, the commercial supplier has failed to provide information on the specificity of the key antibody reagent. However, the authors have provided validation results using spiked samples to establish that the Slit2 ELISA is specific for the Slit2 C fragment (and not the N fragment), and that standard curves can be produced that allow the quantification of Slit2-C fragment concentrations in serum. This is an important new tool for researchers of Slit2, and the graphs and experiment description should be added to the appropriate supplementary figure. (In addition, the authors are requested to submit these results to the company so that other researchers would have access to this.) As an additional minor point, the methods fail to explain how serum is collected, processed, or the volumes used for either ELISAs or western blots: a description should be added to the methods section.
Improved characterization of Slit2-C protein levels by Western blotting. A key point that remains unknown with these Slit2-C antibody assays is to verify that the detected band(s) are lost in Slit2 knockout mutant tissue and/or serum. This would be the gold standard, particularly for an antibody which has significant cross reactivity with non-specific proteins in tissue lysates (although serum detection is more clear, as shown in the results). It is acknowledged that in this system, it is not possible to obtain knockout tissue or serum because Slit2 mutants are perinatal lethal, making obtaining adults impossible. This caveat for the Slit2 detection method should be acknowledged, at least in the methods or supplementary figures.

Reviewer #2 (Remarks to the Author):

Thank you for addressing comments and suggestions. A small note, it was mentioned in the rebuttal letter that the new mitochondrial respiration data were added in as Figure 2h, but they were placed in Supplementary Figures as Figure 2D. Was this intentional or an error?

Reviewer #3 (Remarks to the Author):

The authors have addressed all my comments satisfactorily, and the manuscript is much improved by the revisions and newly provided data. Importantly, the results now support the authors' conclusions and the proposed model very well. I now recommend the manuscript for publication.

Point-by-point response:

Reviewer #1 (Remarks to the Author):

The revised manuscript is improved with significant new results that address most of the reviewer concerns, particularly the concerns about the Slit2 part of the study. The overall strengths of the study include thorough and crucial genetic experiments that demonstrate several significant advances for the field. These include a key function for Schnurr3 in regulation of bone-derived endocrine signals that regulate adipose metabolism, that Shn3 function is localized to bone, and evidence that at least one key bone-derived signal is the Slit2-C protein fragment found circulating in the blood. The Slit2-C detected in blood is largely sourced from bone tissue, based on key evidence using a bone specific knockout of Slit2. Building on these main conclusions, another interesting finding is that a transient interference of Shn3 function using a viral interference vector can increase Slit2-C levels in circulation, and that this causes obesity resistance to high fat diet. This work also adds to and complements a prior paper on Slit2-C produced in adipose tissue, with discussion added to propose how the two tissue sources of this signal may function.

Authors' response:

We are grateful for the reviewer's judgement that our work had been largely improved after 1st round of revision. And we also appreciate the reviewer's guidance during the revision process. Please see our response below.

A few minor points to add to the revised manuscript:

Add validation of ELISA assay for Slit2-C serum concentrations to Supplementary Figure. A significant improvement in the manuscript is the thorough characterization of the Slit2 ELISA assay. Unfortunately, the commercial supplier has failed to provide information on the specificity of the key antibody reagent. However, the authors have provided validation results using spiked samples to establish that the Slit2 ELISA is specific for the Slit2 C fragment (and not the N fragment), and that standard curves can be produced that allow the quantification of Slit2-C fragment concentrations in serum. This is an important new tool for researchers of Slit2, and the graphs and experiment description should be added to the appropriate supplementary figure. (In addition, the authors are requested to submit these results to the company so that other researchers would have access to this.) As an additional minor point, the methods fail to explain how serum is collected, processed, or the volumes used for either ELISAs or western blots: a description should be added to the methods section.

Authors' response:

We thank the reviewer's recommendation to improve the present study and also future SLIT2 research. According to the reviewer's recommendation, we have added our Slit2 ELISA validation results to Supplementary Figure. 4d-f. We have also added to the methods section text explaining how we collected and processed serum samples for ELISAs and WB including the relevant serum volumes.

Improved characterization of Slit2-C protein levels by Western blotting. A key point that remains unknown with these Slit2-C antibody assays is to verify that the detected band(s) are lost in Slit2 knockout mutant tissue and/or serum. This would be the gold standard, particularly for an antibody which has significant cross reactivity with non-specific proteins in tissue lysates (although serum detection is more clear, as shown in the results). It is acknowledged that in this system, it is not possible to obtain knockout tissue or serum because Slit2 mutants are perinatal lethal, making obtaining adults impossible. This caveat for the Slit2 detection method should be acknowledged, at least in the methods or supplementary figures.

Authors' response:

We appreciate the reviewer providing guidance in improving our manuscript. As per the reviewer's recommendation, we have added the caveats regarding SLIT2 detection by WB in the methods section. We hope our findings will support the future studies of Slit2.

Reviewer #2 (Remarks to the Author):

Thank you for addressing comments and suggestions. A small note, it was mentioned in the rebuttal letter that the new mitochondrial respiration data were added in as Figure 2h, but they were placed in Supplementary Figures as Figure 2D. Was this intentional or an error?

Authors' response:

We apologize for this error and thank the reviewer for pointing this out. We have corrected this error and further checked all other figures and figure legends as well.

Reviewer #3 (Remarks to the Author):

The authors have addressed all my comments satisfactorily, and the manuscript is much improved by the revisions and newly provided data. Importantly, the results now support the authors' conclusions and the proposed model very well. I now recommend the manuscript for publication.

Authors' response:

We thank the reviewer for this judgement and for the guidance in improving the manuscript.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The revised manuscript has addressed the issues raised by the previous reviewers. As suggested in the previous review, the authors have added their results validating the ELISA assay for Slit2, and have also added text explaining potential limitations of the Slit2 antibody for western blot detection of Slit2 levels (specifically, the lack of testing Slit2 knockout samples).