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Peer Review File

Microbiota-mediated induction of beige adipocytes in response to dietary cues

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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:

Referee #1

(Remarks to the Author)
Tanoue, Nagayama, Roochana, Zimmerman et al. have investigated the mechanisms by which a low-protein diet influences the degree of inguinal white adipose tissue "browning"/Ucp1 expression. They find that a diet low in essential amino acids induces Ucp1 expression in inguinal white adipose tissue through several pathways, including bile acid signaling through FXR, hepatic FGF21 production, and adrenergic signaling through b3-receptors. They also identify specific members of the microbiota that are at least in part necessary and sufficient for this phenomenon.

The study is extensive and comprehensive, and the manuscripts describes a whole series of interesting insights. Admittedly, several aspects of the study are somewhat expected based on the extensive literature on the relationships between Ucp1 expression with FXR signaling, FGF21 signaling, and adrenergic signaling. However, the authors also present several new observations, such as the role of bacterial nrfA upstream of FGF21-mediated Ucp1 induction. Nonetheless, various aspects of the study should be improved, including a few instances where the data shown in the manuscript is not completely in line with the authors' interpretations. Examples are described in detail below.

My first concern is that certain aspects of the mechanism summarized in Extended Data Fig. 18 are insufficiently supported by the data presented in the manuscript (see comments 1-4 below).

1) The claim that bile acids act on FXR expressed by ASPCs to trigger differentiation into beige adipocytes is insufficiently supported by the data shown in the manuscript:

a) Adipoq-Cre mice are used to demonstrate that adipocyte-intrinsic FXR signaling is required for the effect of low-protein diet on Ucp1 expression. However, ASPCs do not robustly express adiponectin. The authors could use their single-cell sequencing data to identify a population expressing both Adipoq and Nr1h4 which would the most likely candidate for mediating the reduction in Ucp1 expression that is shown in Fig. 3c.

b) Related to the point above, the authors should use a more suitable Cre line that specifically targets ASPCs in inguinal white adipose tissue, such as Prx1-Cre, to directly test whether FXR signaling in ASPCs contributes to browning of inguinal white adipose tissue.

c) The Pseudotime values in Extended Fig. 9c and 9d do not match, which makes it a bit difficult to follow the authors' argument of a differentiation trajectory towards beige adipocytes. To substantiate this claim, a fate mapping approach (again using a Cre line specific for ASPCs) would be more suitable to support the authors' conclusions.

2) The discussion section and the cartoon in Extended Data Fig. 18 suggest that the FXR-dependent and FGF21-dependent mechanisms are independent and "operate in parallel, each ostensibly contributing to the differentiation and accumulation of beige adipocytes" (lines 505 and 506). This conclusion is indeed in line with the observation that bile acid levels are unchanged in FGF21 KO mice, and that FXR deficiency does not influence FGF21 levels. However, there seems to be no induction of Ucp1 in the absence of FGF21 (Fig. 3f). If the pathways are indeed parallel, why would FXR signaling not be

active in the absence of FGF21? The same issue applies to experiments with the 20-mix community (Fig. 4j) and the experiments utilizing tungsten. In the case of the latter, why would bile acids not be able to induce Ucp1 when bacterial Fdh is inhibited by tungsten treatment?

3) A similar issue relates to the conclusion that b3-adrenergic signaling serves as a common downstream mechanism to both adipose tissue FXR signaling and hepatic FGF21 production. The authors do not show that these pathways in fact induce sympathetic signaling in inguinal white adipose tissue. The impact of FGF21 on sympathetic signaling has been explored in the literature, but it would be important to examine if and how hepatic FXR signaling leads to sympathetic input into iWAT, e.g. by measuring noradrenaline levels and innervation patters, the latter of which the authors have done for germ-free mice.

4) A minor question to the authors is about the rationale behind first selecting 20 members of the murine microbiome, followed by a more extensive investigation of human-derived species. Which members of the 20-mix consortium are required for Ucp1 induction, and do they share functional properties with the consortia isolated from humans?

My second concern relates to the technical validity and interpretation of some of the experiments shown in the manuscript (see comments 5-7 below).

5) Throughout the manuscript, several conclusions are based on p-values that seem to be affected by sample size. In some cases, the authors interpret a non-significant p-value to indicate the absence of a meaningful different; in other cases, the authors (reasonably) judge group to be different even with $p>0.05$. It would be preferable if the authors either increased the samples sizes or strictly followed the statistical interpretation. Examples include Fig. 2b and Extended Data Fig. 1b.

6) The point above point is not only important with respect to statistical rigor, but in some cases might actually affect some of the authors' conclusions. For instance, in Fig. 2b, an unbiased observer may conclude that LPD still induces Ucp1 in germ-free mice, but the absence of the microbiome generally reduces Ucp1 levels (regardless of the diet), which is well-described in the literature. Since this is such a central conclusion of the manuscript, it would be better if these results were statistically robust. This concern is emphasized by the results shown in Extended Data Fig. 6b as well as Fig. 2g, where it also seems that germ-free mice show a generally reduced expression of Ucp1 and associated genes, but a diet low in essential amino acids does lead to a substantial induction, which would argue that LPD and microbiota have additive effects on browning.

7) I perhaps missed it, but I was not able to find information about how the low-protein diet was given the germ-free mice? Has the diet been autoclaved? If so, can the authors rule out that changes in nutrient composition that have resulted from the sterilization procedure may have affected the results?

My final concern relates to the physiological effects of the phenomena described in the manuscript (see comments 8-10 below).

8) Several metabolic benefits of low-protein diet are presented in Fig. 1, but it is unclear if they are mediated by the pathways the authors investigate in the subsequent figures. It is even unclear if there are dependent on browning of inguinal white adipose tissue or the presence of the microbiota. This would be important to clarify, since it is entirely possible that the reported metabolic benefits are due to "off-target" effects of low-protein diet that are unrelated to what is studied in the rest of the manuscript.

9) Throughout the manuscript, Ucp1 is used as a marker for iWAT browning. In would be important, at least in some cases, to investigate the functional impact of Ucp1 induction in terms of oxygen consumption rate or energy expenditure as the authors have done in Fig. 1. Adding these readouts for all of the numerous interventions presented in the manuscript would be extremely time- and resource-demanding, but a few select examples would be needed to verify that iWAT Ucp1 induction is of sufficient magnitude to impact whole-body energy consumption at the different steps of the investigated mechanisms.

10) A question that this manuscript in my opinion would not need to answer comprehensively, but should at least address, is why the described microbiome changes happen in response to low-protein diet feeding. For instance, why would the absence of essential amino acids lead to enhanced activation of the dissimilatory nitrate reduction to ammonium pathway? And how does this in turn trigger hepatic FGF21 release? Again, clarifying all of these mechanistic gaps would probably require a separate study, but a putative teleological explanation could shed light on the logic behind this proposed pathway.

Referee #2

(Remarks to the Author)
In this study Tanoue et al demonstrate that low protein diet interacts with specific members of the gut microbiota to increase browning in subcutaneous adipose tissue, which contribute to metabolic improvements using human observational and mouse mechanistic studies. It is a true tour de force approach using a magnitude of mouse models and careful dissection of signaling mechanisms. The manuscript is clearly written and data are logically introduced and presented. There are a few minor suggestions that potentially can improve the manuscript further.

1. Does the low protein diet affect fecal energy content and energy extraction?
2. Can the responses to low protein diet in Figure 1i be statistically evaluated?
3. Related to Figure 3. Are insulin levels at fasting and during the GTT altered following low protein diet?
4. Do low protein diets affect Fgf21 expression in the adipose tissue?

5. Low protein diet reduces body weight in wild-type mice, but does it affect body weight in FGF21 and FXR deficient mice? In mice colonized with the different combinations of bacteria where browning was observed?
6. Does tungsten alter food and water intake?
7. The conclusion that the microbiota is responding to differences in nitrogen abundance upon feeding low protein diet is aligned with DOI: 10.1126/science.abh295 where the authors identified the microbiota to be important for nitrogen recycling. Perhaps worth to expand on the biological/evolutionary importance on how the microbiota senses nitrogen abundance in the gut and the resulting impact on the host.

Referee #3

(Remarks to the Author)
A. Summary of the key results:
Chronic cold exposure is the most potent method of thermogenic activation. The authors propose diet-based interventions as an alternative with fewer cardiovascular side effects. They cite studies linking diet and microbiota to browning but acknowledge gaps in understanding. Their findings convincingly show that LPD induces Ucp1 expression in adipocytes via circulating Fgf21 and Nr1h4.

Functional relevance remains unproven. Even a 100-1,000-fold increase in Ucp1 mRNA barely approaches UCP1 levels in classical BAT (PMID: 28109720), and the study provides no evidence linking thermogenic gene expression (Ucp1, Fgf21, Nr1h4) to energy expenditure in vivo. Measuring energy expenditure in genetic models, including Ucp1 KO mice, could clarify their functional role. However, a major weakness of the manuscript is the lack of metabolic data supporting relevance and the inappropriate methods used for analyzing metabolic data. My specific concerns are outlined below.

B. Originality and significance:
The authors demonstrate that LPD induces Ucp1 expression in subcutaneous white fat through bile acids, FXR, Fgf21, and ADRB3.

C. Data & methodology:
C1. Energy expenditure normalization is incorrect. The timing of measurements after the diet switch is unclear, and reporting only a 2-hour phase “during the day (12:00 pm–2:00 pm) and night (12:00 am–2:00 am)” is highly unorthodox. A sustained thermogenic effect should accompany significant weight loss; if not, alternative explanations such as impaired nutrient absorption or reduced food intake must be considered.
C2. Glucose dosing (1.2 g/kg) was based on body weight but should be normalized to lean mass or kept identical across groups. This is because a lighter mouse will receive less glucose and thus appear to have better glucose intolerance, but this is just because there was less glucose to clear. Typically differences in weight in obesity is due to fat mass,

D. Appropriate use of statistics and treatment of uncertainties:
D1. The VO2 is plotted repeatedly for only 5 mice. Therefore, repeated measures from the same mice artificially inflate sample size (~40×). Repeated measures from the same mouse are not independent observations; which violates the assumption of independence in statistical analysis.
D2. VO2 data is improperly normalized.

E. & F. Conclusions and suggested improvements:
1) Fig. 1E & Extended Data Fig. 3A – VO2 normalization by body weight introduces artifacts. VO2 or Energy expenditure (EE) should be plotted as ml/h or kcal/h per mouse, or analyzed using ANCOVA with lean/total body mass as a covariate. Excellent reviews on this topic have been written (PMID: 20103710; PMID: 22205519). A 2-hour VO2 phase is insufficient. Full data, including RER and movement, should be reported to assess robustness.

2) Extended Data Fig. 3a – The authors note in the fig legend that “Each data point represents an individual mouse at one time point (5 mice per group x 39 time points = 195 points per phase)”. Therefore, repeated measures from the same mice artificially inflate sample size (~40×). Repeated measures from the same mouse are not independent observations; which violates the assumption of independence in statistical analysis. This type of analysis exaggerates variability in the data, masking real effects or creating false positives. VO2 data should be plotted as mouse-wise averages (n = 5). This brings up another point, where an n = 5 is a very small sample size to convincingly show that the VO2 is reproducible with a relevant effect size.

3) The authors focus on Ucp1 expression but provide no functional evidence that its induction drives weight loss. The lack of proper VO2 normalization (see comment #1) further weakens this claim. If LPD-induced Ucp1 activation truly increases energy expenditure and drives weight loss, this effect should be evident before significant weight changes occur. However, Ucp1 mRNA was only shown following long-term LPD treatment. Additionally, genetic inactivation of Ucp1 or its regulators (e.g., Fgf21) should mitigate the effect. For instance, while Nr1h4fl/fl;Adipoq-Cre mice exhibit reduced Ucp1 expression on LPD (Fig. 3c), there is no functional evidence demonstrating the significance of this reduction.

4) Extended Data Fig. 3b – Glucose tolerance test (GTT) normalization by body weight is problematic. Since muscle takes up the lion’s share of glucose, glucose should be dosed by lean mass. Another approach is to just pick one dose of glucose (based on the control group) and just administer the same amount to all animals, as this is how this test is done in humans. Papers have been written about this (PMID: 29143855).

5) Fig. 1f – Weight loss upon LPD lacks food intake data, except for a brief 10-hour window (Extended Data Fig. 3b). Cumulative food intake is needed to assess energy balance. Since a major claim of the paper is that LPD induces thermogenesis through Ucp1 in beige fat to combat obesity, differences in food intake and nutrient absorption (with bomb calorimetry) must be ruled out.

6) Body Composition – Lean mass loss is unaccounted for. Since LPD reduces protein intake, its contribution to weight loss must be assessed.

7) Fig. 1f – HFD-to-LPD transition involves multiple dietary changes. A more controlled switch (e.g., HFD to HFD+LPD) would clarify LPD-specific effects, after all, they show that this combination still induced Ucp1 in Fig. 1a.

8) The authors should assess muscle mass loss on the LPD, as reduced muscle mass could contribute to increased Ucp1 expression through excess heat dissipation. Since experiments were conducted at room temperature, it is possible that LPD-induced muscle loss impairs shivering capacity, leading to heightened cold perception, increased sympathetic activation, and secondary Ucp1 upregulation. The authors should rule out the possibility that Ucp1 induction results secondarily from compromised muscle thermogenesis without contributing at all to the weight loss.

9) Percent changes (e.g., Fig. 1f, 2d, Extended Data Fig. 3b) should be supplemented with absolute body weight and body composition (fat vs. lean mass).

10) Rapid weight loss (Fig. 1f) suggests malabsorption. Fecal energy content (e.g., bomb calorimetry) should be measured.

11) The timing of O_2 measurement or GTT post-diet switch is also unclear.

12) Fig. 1A – Fold-change in Ucp1 induction is unclear. The control diet should be set to 1, with other conditions expressed relative to it.

13) Extended Data Fig. 18 – claims FXR promotes “beige fat biogenesis,” but de novo adipogenesis was not tested.

14) Prior work (PMID: 23404943) found no Ucp1 induction with LPD but is not discussed.

15) Extended Data Fig. 2d – Errors in terminology and interpretation:
a. “Glycolytic beige” should replace “glycogenic beige.”
b. Eno1, ldhb, and Pdha are general glycolysis markers, not specific to g-beige cells.
c. No evidence supports Phospho1’s role in the futile creatine cycle; prior work ruled it out as the phosphatase of the FCC.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
The authors have provided a thorough revision work which has addressed the large majority of my previous concerns. In those cases where the authors have not been able to provide compelling new mechanistic insights, they have appropriately toned down their conclusions.

I would only like to point out that my comments about the model (previous Ext. Data Fig. 18) were not meant to discourage the authors from including a cartoon summary. Rather, I referenced it to point out instances where the model was not fully supported by experimental data shown in the manuscript. Given the complexity of the pathways that the authors describe in this study, I would encourage them to re-include a cartoon summary, since it can be very helpful for the reader to obtain a graphical overview of the findings, but would suggest making sure the cartoon is perfectly in line with the data.

Referee #2

(Remarks to the Author)
The authors have satisfactorily addressed the concerns raised. The manuscript has improved considerably, and I have no further comments.

Referee #3

(Remarks to the Author)
Tanoue et al. have made notable improvements to their manuscript. However, several substantive issues remain that warrant further clarification or revision. While the authors now emphasize that their primary focus is on how a low-protein diet

(LPD) interacts with the gut microbiota to influence gene expression in adipose tissue, the structure and presentation of the manuscript continue to place substantial emphasis on Ucp1 expression and adipose browning. This is evident from the framing of the Introduction—which highlights the metabolic benefits of browning—as well as from the prominence of Ucp1 mRNA data throughout the first six pages and beyond. This creates a disconnect between the authors' stated focus in their rebuttal and the way the paper is currently written. Either the manuscript should be reframed to reflect this revised focus more accurately, or additional evidence should be presented to support the functional relevance of browning in the phenotype.

Remaining Concerns
1. The authors report increased Ucp1 mRNA levels with LPD, but this is presented per total RNA. Since LPD reduces total fat mass, it's likely that the total UCP1 content per animal is substantially lower than the figures suggest. UCP1 protein levels should be normalized per whole depot to account for tissue mass differences (see PMID: 35167098). Moreover, despite the emphasis on UCP1, the authors acknowledge in the discussion that there is no causal evidence linking browning to the phenotype. This warrants either a reframing of the manuscript to de-emphasize browning or additional mechanistic evidence supporting its relevance.

2. The authors report an approximate 1,000 kcal/kg increase in fecal energy content in the LPD group. However, no data are provided on total fecal mass. To accurately calculate total energy loss, caloric density (kcal/g) must be multiplied by total fecal output. Furthermore, this energy deficit must be contextualized over the duration of the experiment to determine whether it explains the observed body weight changes.

3. In Lines 162–164, the authors suggest that LPD induces functionally heterogeneous beige adipocyte subsets (lipolytic vs. glycolytic). This conclusion is not supported by the data. There is no single-cell transcriptomic or proteomic analysis to distinguish such subsets. Moreover, thermogenic adipocytes typically upregulate glycolysis while preserving lipid stores. Thus, the evidence for a distinct "glycolytic beige" population is speculative and not justified by the presented results.

4. The authors conclude that Adipocyte Progenitor Cells (APCs) are critical for Ucp1 induction based on Adipoq-driven FXR deletion impairing Ucp1 expression. However, Adipoq-Cre targets mature adipocytes, suggesting that LPD regulates Ucp1 expression in existing adipocytes, not through adipogenesis. Furthermore, the requirement for the β3-adrenergic receptor indicates that mature adipocytes are likely the direct target of upstream signals. Without lineage tracing, the claim that APCs are essential remains unsupported and possibly contradicted by the data.

5. In line 435, the manuscript references "creatine kinase levels," but this appears to be a measurement of plasma CK. This must be clearly stated in the text to avoid confusion with intracellular creatine kinase involved in thermogenesis.

Version 2:

Reviewer comments:

Referee #3

(Remarks to the Author)
Tanoue et al. have made notable improvements to their manuscript. Importantly, they have softened their tone and clarified that establishing a causal role for Ucp1 in their phenotype is not the primary focus. The new data showing that LPD requires Fgf21 (Fig. 3d) is a valuable addition.
While normalization per depot is not used by most, it is a valid design used by leading investigators in the UCP1 field (see PMID: 35167098, 28109720). For those familiar with these methodologies, this represents a thoughtful and rigorous addition, so I commend the authors for including multiple normalization approaches.

Remaining, minor concerns

1. The statement, "As canonical interscapular brown adipocytes..., beige adipocytes are thought to assume a dominant role in thermogenesis," overstates the evidence. Is there strong support that beige adipocytes play a "dominant" thermogenic role relative to classical BAT? The authors should clarify or provide supporting references, as the current phrasing implies that beige adipocytes are more thermogenically active than classical brown adipocytes in adult mice.
2. The statement, "Therefore, elucidating the mechanisms that drive beige adipocyte induction could shed light on why the beiging program exists in humans…", requires a supporting citation. The literature remains divided on whether human BAT corresponds to mouse beige or brown adipose tissue. The reality is probably more nuanced. Deeper neck regions in human BAT more closely resemble mouse BAT, whereas more superficial regions exhibit beige-like features. If the authors are referring to the latter, this should be clarified and referenced. Otherwise, the current text could be interpreted as implying that human white adipose tissue can readily undergo beiging, which remains uncertain. Happy to be proven wrong though, but if so, this needs citation support.
3. It appears that pathway enrichment analyses were conducted using all mouse genes as the background reference (Extended Data Fig. 1e, f). However, enrichment should ideally be calculated relative to genes expressed in BAT, as using the full genome can artificially inflate the significance of pathways enriched in highly expressed BAT genes. I recommend cross-referencing datasets such as TRAP-seq data from the Rosen group (Roh et al., 2017) for more accurate interpretation. I leave it to the authors' discretion.
4. I agree that collecting total fecal mass throughout the experiment is challenging. However, the available data could be

extrapolated to estimate cumulative values over longer durations. While approximate, such estimates would still be informative and worth considering. This is a minor suggestion and I leave it to the authors' discretion.

Referee #1 (Remarks to the Author):

Tanoue, Nagayama, Roochana, Zimmerman et al. have investigated the mechanisms by which a low-protein diet influences the degree of inguinal white adipose tissue "browning"/Ucp1 expression. They find that a diet low in essential amino acids induces Ucp1 expression in inguinal white adipose tissue through several pathways, including bile acid signaling through FXR, hepatic FGF21 production, and adrenergic signaling through b3-receptors. They also identify specific members of the microbiota that are at least in part necessary and sufficient for this phenomenon.
The study is extensive and comprehensive, and the manuscripts describes a whole series of interesting insights. Admittedly, several aspects of the study are somewhat expected based on the extensive literature on the relationships between Ucp1 expression with FXR signaling, FGF21 signaling, and adrenergic signaling. However, the authors also present several new observations, such as the role of bacterial nrfA upstream of FGF21-mediated Ucp1 induction. Nonetheless, various aspects of the study should be improved, including a few instances where the data shown in the manuscript is not completely in line with the authors' interpretations. Examples are described in detail below.

We greatly appreciate these thoughtful and constructive comments for improving our manuscript. We have carefully considered these concerns and made revisions accordingly. Revised contents in the text are highlighted in blue font, and we hope our responses have adequately addressed the reviewer's comments.

My first concern is that certain aspects of the mechanism summarized in Extended Data Fig. 18 are insufficiently supported by the data presented in the manuscript (see comments 1-4 below).

In response to the reviewer's concern, we have removed the summary figure (**previous Extended Data Fig. 18**) and revised the text to avoid overemphasizing our working model regarding the mechanisms by which FXR and FGF21 signalling contribute to beige fat development.

1) The claim that bile acids act on FXR expressed by ASPCs to trigger differentiation into beige adipocytes is insufficiently supported by the data shown in the manuscript:
a) Adipoq-Cre mice are used to demonstrate that adipocyte-intrinsic FXR signaling is required for the effect of low-protein diet on Ucp1 expression. However, ASPCs do not robustly express adiponectin. The authors could use their single-cell sequencing data to identify a population expressing both Adipoq and Nr1h4 which would the most likely candidate for mediating the reduction in Ucp1 expression that is shown in Fig. 3c.
b) Related to the point above, the authors should use a more suitable Cre line that specifically targets ASPCs in inguinal white adipose tissue, such as Prx1-Cre, to directly test whether FXR signaling in ASPCs contributes to browning of inguinal white adipose tissue.

We agree that the *Adipoq* gene is broadly expressed in iWAT cells, making Adipoq-Cre mice less than ideal for studying ASPC differentiation [please note that *Adipoq* expression was also detectable, albeit at low levels or in a limited number of cells, in ASPCs (see **Extended Data Fig. 9a**)].

To address this concern, we newly generated and analysed *Dpp4*-Cre × *Nr1h4*$^{flox/flox}$ mice, as *Dpp4* was expressed relatively specifically by ASPCs in iWAT (**Fig. 2g**), consistent with previous reports (such as Science 364, eaav2501, 2019). In *Dpp4*-Cre × *Nr1h4*$^{flox/flox}$ mice, beige adipocyte induction in response to a LPD was significantly impaired (**new Fig. 2e**), closely resembling the phenotype observed in *Nr1h4*$^{-/-}$ mice (**Fig. 2b**). While *Dpp4*-Cre drives *Nr1h4* deletion in *Dpp4*-expressing cells across multiple tissues and is therefore not exclusive to ASPCs, these findings strongly support the notion that FXR in Dpp4-expressing cell populations, including ASPCs in iWAT, is a critical mediator of the bile acid-driven browning response.

As the reviewer points out, more studies are required to fully prove our hypothesis of bile acid-mediated ASPC differentiation into beige cells, and we agree. For this reason, we have carefully revised the manuscript to avoid overstating any conclusions.

c) The Pseudotime values in Extended Fig. 9c and 9d do not match, which makes it a bit difficult to follow the authors' argument of a differentiation trajectory towards beige adipocytes. To substantiate this claim, a fate mapping approach (again using a Cre line specific for ASPCs) would be more suitable to support the authors' conclusions.

We thank the reviewer for pointing out the mismatch in pseudotime values. In the new **Extended Data Fig. 9b-d**, we have corrected the values and reconstructed the figure panels accordingly.

Regarding the suggestion to trace the ASPC fate following LPD feeding, we agree that such experiments would provide valuable insight. We view this as an important direction for future work. Generating the appropriate animal models and conducting lineage tracing studies would require substantial time and resources. We believe that such studies are beyond the scope of the current work and would require a dedicated manuscript to address comprehensively.

The primary objective of this study is to explore the potential for functional modulation of the gut microbiota through dietary intervention. We hope the reviewer will agree that our current data already provide valuable insight into the host response to bacterial and dietary cues.

In response to the reviewer's concerns, we have carefully revised the manuscript to clearly state the need for direct lineage tracing and to avoid overemphasizing our proposed model of ASPC-to-beige cell differentiation.

2) The discussion section and the cartoon in Extended Data Fig. 18 suggest that the FXR-dependent and FGF21-dependent mechanisms are independent and "operate in parallel, each ostensibly contributing to the differentiation and accumulation of beige adipocytes" (lines 505 and 506). This conclusion is indeed in line with the observation that bile acid levels are unchanged in FGF21 KO mice, and that FXR deficiency does not influence FGF21 levels. However, there seems to be no induction of Ucp1 in the absence of FGF21 (Fig. 3f). If the pathways are indeed parallel, why would FXR signaling not be active in the absence of FGF21? The same issue applies to experiments with the 20-mix community (Fig. 4j) and the experiments utilizing tungsten. In the case of the latter, why would bile acids not be able to induce Ucp1 when bacterial Fdh is inhibited by tungsten treatment?

We appreciate the reviewer's insightful comments and fully agree. Based on our observations that bile acid levels remained unchanged in *Fgf21*$^{-/-}$ mice and that *Fgf21* expression was unaffected by *Nr1h4* (FXR) deficiency (**Fig. 3d, e**), it is likely that the FXR and FGF21 pathways function in parallel. As deficiency of either *Fgf21* or *Nr1h4* impaired beige adipocyte induction (**Fig. 2b, Fig. 3c**), we proposed a model in which both pathways are required for the optimal browning response, whereas activation of either pathway alone is insufficient. Nevertheless, in response to the reviewer's suggestions and to further support this model, we performed four additional experiments:

1. Given that deletion of the ammonia-producing NrfA enzyme in *Bilophila* markedly impaired host *Fgf21* expression and browning response (**Fig. 7f, g**), we investigated the role of ammonia in beige cell induction. We found that ammonia concentration was increased in the portal (but not peripheral) circulation of SPF mice as well as gnotobiotic mice colonized with WT *Bilophila* and *R. timonensis* following LPD feeding (**new Fig. 7h**). In contrast, no such increase was observed in GF mice or in gnotobiotic mice colonized with *R. timonensis* and a Δ*nrfA Bilophila* mutant (**new Fig. 7h**). Supplementing the drinking water with ammonium chloride (NH_4Cl) restored hepatic *Fgf21* and

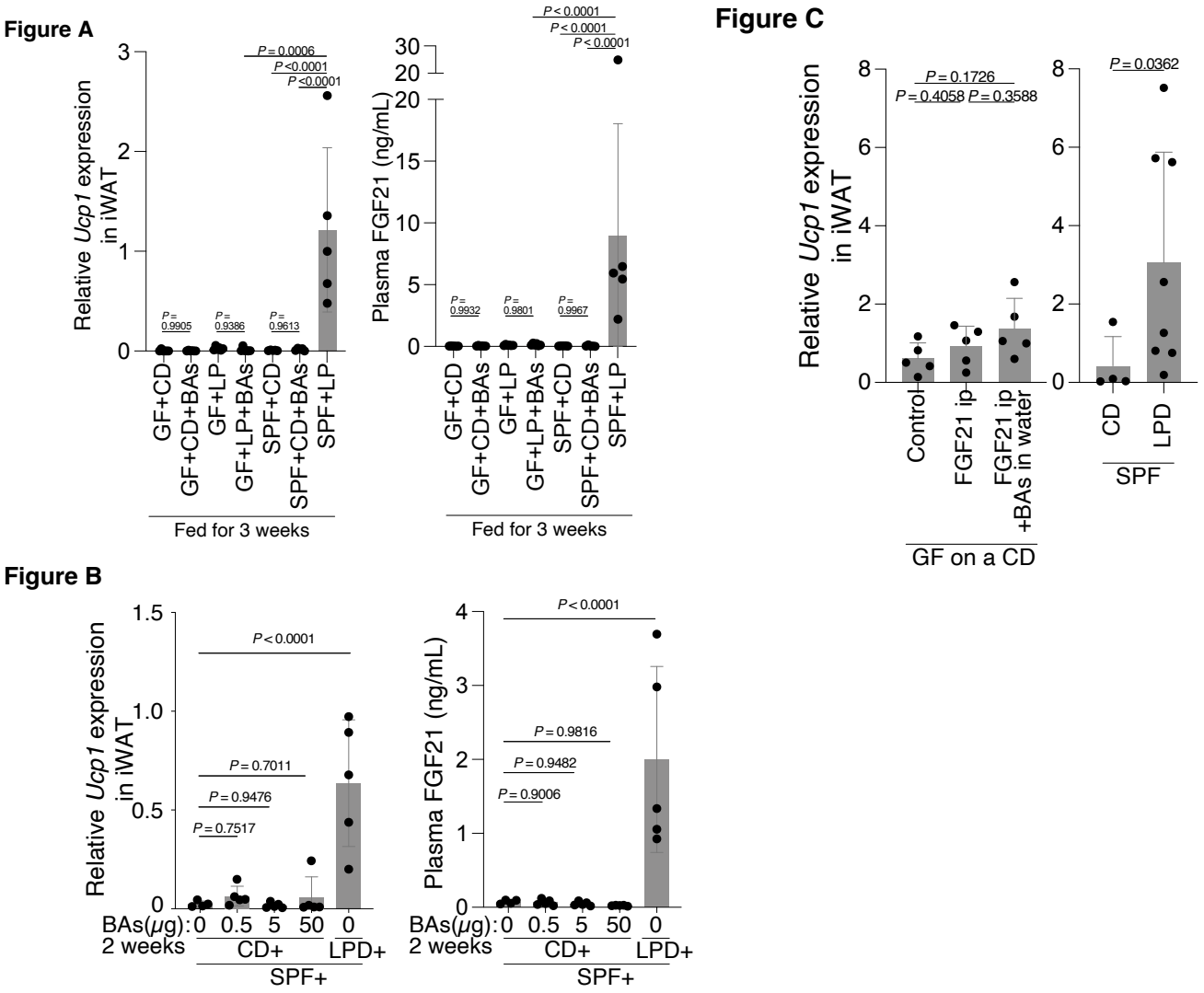
iWAT *Ucp1* expression in *R. timonensis* and Δ*nrfA Bilophila* co-colonized mice (**new Fig. 7i**). These new results suggest that microbiota-derived ammonia enters the portal circulation and promotes hepatic *Fgf21* expression, thereby contributing to iWAT browning.

2. To assess whether ammonia directly modulates hepatocyte function to induce *Fgf21*, we developed hepatocyte organoids derived from five human donors, as recently described (Nature, 2025 doi: 10.1038/s41586-025-08861-y). Consistently, ammonia stimulation led to a dose-dependent increase in expression of *FGF21* in all hepatocyte organoids, but not of genes involved in bile acid biosynthesis such as *CSAD* (**new Fig. 7j**). These findings suggest that microbiota-derived ammonia can directly induce hepatic expression of *FGF21* without notably altering the expression of genes in the bile acid synthesis pathway (supporting a model in which the ammonia-FGF21 and bile acid-FXR axes are independently regulated).

3. Leveraging our single-cell RNAseq dataset, we found that *β-Klotho* (a critical FGF21 receptor component) was expressed in a limited number of FXR-expressing ASPC cells (**Extended Data Fig. 9**). Additionally, we profiled iWAT gene expression in *Fgf21*$^{-/-}$, *Nr1h4*$^{-/-}$, and wild-type mice fed either a control or a low-protein diet by bulk RNA sequencing (**new Extended Data Fig. 11b**). In both *Fgf21*$^{-/-}$ and *Nr1h4*$^{-/-}$ mice, expression of *Ucp1*, *Elovl3*, and other beige adipocyte signature genes was markedly suppressed compared to wild-type controls. Additionally, we identified distinct sets of differentially expressed genes between genotypes. For example, *Acsl5* and *Pdk4* (both related to lipid metabolism) were preferentially downregulated in *Nr1h4*$^{-/-}$ mice, whereas *Arhgef37* and *Dusp4* (related to intracellular signalling) were preferentially downregulated in *Fgf21*$^{-/-}$ mice (**new Extended Data Fig. 11b**). These findings suggest that FGF21 and FXR signalling control different gene expression programs in iWAT, and that both pathways are likely required for maximal induction of the browning program.

4. We administered a mixture of bile acids (BAs: CA, CDCA, UCA, UDCA, and 7oxoDCA) via drinking water to GF and SPF mice for 3 weeks. This intervention failed to increase *Ucp1* expression in iWAT or plasma FGF21 levels (**Figure A, below**). Similar results were observed when the bile acid mixture was administered intraperitoneally every three days for two weeks in SPF mice on a control diet (**Figure B**). Likewise, repeated intraperitoneal injections of recombinant FGF21 alone did not significantly increase *Ucp1* expression (**Figure C**). These results suggest that activation of either the bile acid-FXR pathway or the FGF21 pathway alone is insufficient to drive beige adipocyte induction. Furthermore, co-administration of bile acids (orally) and recombinant FGF21 (ip) in GF mice resulted in only modest, non-significant *Ucp1* induction (**Figure C**), suggesting that, in addition to FXR and FGF21, other signalling pathways may contribute to LPD-induced WAT browning.

Collectively, these new results support our model that FXR and FGF21 act through parallel, non-redundant pathways that collectively contribute to beige adipocyte induction in iWAT. We have incorporated these new data into the revised manuscript. In addition, in response to the reviewer's concerns, we have removed the previous Extended Data Fig. 18 and revised the text to avoid overemphasizing our suggested model regarding the mechanisms of FXR and FGF21 in browning.



3) A similar issue relates to the conclusion that b3-adrenergic signaling serves as a common downstream mechanism to both adipose tissue FXR signaling and hepatic FGF21 production. The authors do not show that these pathways in fact induce sympathetic signaling in inguinal white adipose tissue. The impact of FGF21 on sympathetic signaling has been explored in the literature, but it would be important to examine if and how hepatic FXR signaling leads to sympathetic input into iWAT, e.g. by measuring noradrenaline levels and innervation patters, the latter of which the authors have done for germ-free mice.

We greatly appreciate the reviewer's insightful comments and fully agree with the suggestions. In response, we performed time-course whole-mount immunofluorescence staining of iWAT from *Fgf21*$^{-/-}$ and *Nr1h4*$^{-/-}$ SPF mice fed either a control or low-protein diet, detecting tyrosine hydroxylase (TH) and UCP1 (**new Extended Data Fig. 13**). In SPF wild-type or heterozygous mice, LPD feeding induced fine, dense, TH-positive sympathetic neuronal structures in regions enriched with UCP1-positive cells (near the inguinal lymph nodes). This innervation was detectable as early as one week after LPD initiation, preceding or coinciding with *Ucp1* upregulation (though precise temporal correlation of these events remains technically challenging). In contrast, both *Fgf21*$^{-/-}$ and *Nr1h4*$^{-/-}$ mice exhibited markedly reduced sympathetic innervation, resembling the pattern observed in wild-type GF mice or SPF mice fed a control diet (**new Fig. 3h**, **new Extended Data Fig. 13**). These

findings demonstrate that both FXR and FGF21 are essential for promoting sympathetic input into iWAT during LPD-induced browning.

In *Nr1h4*-deficient mice, *Ucp1* induction in response to a LPD was impaired but not completely abolished. Notably, however, the density of sympathetic neurons in iWAT was markedly reduced compared to wild-type control mice (**new Fig. 3h**, **new Extended Data Fig. 13**). This was an intriguing and unexpected finding. Since hepatic secretion of FGF21 is normal in FXR-deficient mice (**Fig. 3e**), we had anticipated that sympathetic innervation would be preserved. These results suggest that FXR and FGF21 signalling play nonredundant roles and converge on sympathetic innervation promoting beige adipocyte induction. We find these results are highly compelling and believe that they could open new avenues for future investigation.

We fully agree that unravelling the molecular underpinnings of how FXR signalling in ASPCs leads to sympathetic remodelling in iWAT is an important topic. However, given the substantial scope of experiments already included in this study, and the complexity of the mechanism in question, which would require an additional in-depth investigation beyond the current framework, we would like to leave this issue for future studies.

4) A minor question to the authors is about the rationale behind first selecting 20 members of the murine microbiome, followed by a more extensive investigation of human-derived species. Which members of the 20-mix consortium are required for Ucp1 induction, and do they share functional properties with the consortia isolated from humans?

Our investigation of the human-derived strains suggested that activation of the dissimilatory nitrate reduction to ammonium (DNRA) pathway plays an important role in *Fgf21* induction. Based on this observation, we revisited our panel of 20 mouse-derived strains. Genome sequencing revealed that *Adlercreutzia* sp. (St.1H8) and *Parvibacter caecicola* (St.1B6) both encode lipoprotein signal peptide (LSP)-containing *nrfA* homologues (**new Extended Data Fig. 19a**). We cultured these strains under protein-restricted conditions (10% GAM) and found that these *nrfA*-carrying strains, especially *Adlercreutzia* sp. (St.1H8), were able to produce ammonia *in vitro* (**new Extended Data Fig. 19b**).

Additionally, we evaluated the bile acid conversion capacities of the 20 strains. We found that *Faecalibaculum rodentium* (St.1C4) was capable of converting tauro-CA to CA, 7oxoDCA and 3oxoCA, indicating carriage of BSH, 7αHSDH, and 3αHSDH enzymes by this strain (**new Extended Data Fig. 19c**).

Based on these new findings, we generated a mouse-derived five-strain consortium (mu5-mix) consisting of these three strains (*Adlercreutzia* sp. St.1H8, *P. caecicola* St.1B6 and *F. rodentium* St.1C4) plus two additional strains (T*uricibacter* sp. St.80E1 and *Blautia* sp. St.27G3) that were shown to be critical to the beige cell induction capacity of the parental 20-mix (**Fig. 4e, f**). Administering a LPD to mu5-mix-colonized mice resulted in robust iWAT *Ucp1* induction, comparable to that observed in mice colonized with the parental 20-mix (**new Fig. 4k**). Exclusion of the two *nrfA*-carrying strains from mu5-mix (yielding mu3-mix) abrogated this browning capacity (**new Fig. 4k**). These findings suggest that adipose tissue browning can be induced by diverse bacterial communities provided they possess both ammonia-producing and bile acid-metabolizing capabilities, functions that appear conserved across human- and mouse-derived strains.

My second concern relates to the technical validity and interpretation of some of the experiments shown in the manuscript (see comments 5-7 below).

5) Throughout the manuscript, several conclusions are based on p-values that seem to be affected by sample size. In some cases, the authors interpret a non-significant p-value to indicate the absence of a meaningful different; in other cases, the authors (reasonably) judge group to be different even with p>0.05. It would be preferable if the authors either increased the samples sizes or strictly followed the statistical interpretation. Examples include Fig. 2b and Extended Data Fig. 1b.

All statistical analyses have been carefully revised. Exact *p*-values are now reported.

6) The point above point is not only important with respect to statistical rigor, but in some cases might actually affect some of the authors' conclusions. For instance, in Fig. 2b, an unbiased observer may conclude that LPD still induces Ucp1 in germ-free mice, but the absence of the microbiome generally reduces Ucp1 levels (regardless of the diet), which is well-described in the literature. Since this is such a central conclusion of the manuscript, it would be better if these results were statistically robust. This concern is emphasized by the results shown in Extended Data Fig. 6b as well as Fig. 2g, where it also seems that germ-free mice show a generally reduced expression of Ucp1 and associated genes, but a diet low in essential amino acids does lead to a substantial induction, which would argue that LPD and microbiota have additive effects on browning.

As the reviewer insightfully points out, *Ucp1* induction was occasionally observed in GF mice following LPD feeding. However, the extent of this induction varied across experiments and was not robust. We examined mice of various genetic backgrounds and sources, as well as control and low-protein diets from different providers. However, the underlying cause of this variation remains unclear. In contrast, the response of GF mice to LPD feeding was consistently and significantly lower than SPF mice. In our previous submission, we did not elaborate on these observations and instead focused on the differences between SPF and GF mice due to space constraints. In the revised manuscript, we have carefully discussed the differences in response to the low-protein versus control diets in GF mice. Additionally, data from several experiments examining the response of GF mice to LPD feeding are now transparently presented in the newly added **Supplementary Information 1**.

7) I perhaps missed it, but I was not able to find information about how the low-protein diet was given the germ-free mice? Has the diet been autoclaved? If so, can the authors rule out that changes in nutrient composition that have resulted from the sterilization procedure may have affected the results?

We thank the reviewer for pointing out this inadvertent omission. This should have been properly stated in the Methods section. The sterilization was performed using radiation (50 Gy). Therefore, heat-induced changes in food components that may have been introduced by autoclave sterilization were avoided.

My final concern relates to the physiological effects of the phenomena described in the manuscript (see comments 8-10 below).

8) Several metabolic benefits of low-protein diet are presented in Fig. 1, but it is unclear if they are mediated by the pathways the authors investigate in the subsequent figures. It is even unclear if there are dependent on browning of inguinal white adipose tissue or the presence of the microbiota. This would be important to clarify, since it is entirely possible that the reported metabolic benefits are due to "off-target" effects of low-protein diet that are unrelated to what is studied in the rest of the manuscript.

We thank the reviewer for this crucial and valuable feedback. As this reviewer points out, the effects of a low-protein diet are broad and extend far beyond beige cell induction. Therefore, it is entirely possible that we are observing effects beyond beige cell induction, especially in previous Figure 1. While we had intended to carefully describe this caveat in the original manuscript, we now recognize that our original explanation was insufficient. In the revised version, we have carefully expanded our explanation to address these points more thoroughly.

Additionally, in accordance with the editor's suggestion, we have removed the experimental data on low-protein diets in patients with fatty liver disease. This decision was made due to the small sample size and the strong possibility that effects beyond beige cell induction were contributing to the observed phenotype. In the future, we plan to recruit more patients to present a more robust dataset.

9) Throughout the manuscript, Ucp1 is used as a marker for iWAT browning. In would be important, at least in some cases, to investigate the functional impact of Ucp1 induction in terms of oxygen consumption rate or energy expenditure as the authors have done in Fig. 1. Adding these readouts for all of the numerous interventions presented in the manuscript would be extremely time- and resource-demanding, but a few select examples would be needed to verify that iWAT Ucp1 induction is of sufficient magnitude to impact whole-body energy consumption at the different steps of the investigated mechanisms.

In several experiments, we included expression of not only *Ucp1* but also other genes representing mitochondrial function and lipid metabolism, such as *Elovl3* and *Cox7a1*, among others. Additionally, as a proxy for assessing the impact on whole-body energy expenditure, we monitored body weight changes. These data have now been incorporated into the revised manuscript as **new Extended Data Fig. 8b** (for *Nr1h4*$^{-/-}$ mice) and **new Extended Data Fig. 11a** (for *Fgf21*$^{-/-}$ mice).

10) A question that this manuscript in my opinion would not need to answer comprehensively, but should at least address, is why the described microbiome changes happen in response to low-protein diet feeding. For instance, why would the absence of essential amino acids lead to enhanced activation of the dissimilatory nitrate reduction to ammonium pathway? And how does this in turn trigger hepatic FGF21 release? Again, clarifying all of these mechanistic gaps would probably require a separate study, but a putative teleological explanation could shed light on the logic behind this proposed pathway.

We appreciate this reviewer's insightful comments and completely agree. As mentioned above, our new experimental results suggest that ammonia likely acts on liver cells to induce hepatic FGF21 production. However, we were unable to elucidate the precise mechanisms by which specific members of the gut microbiota sense environmental essential amino acid levels and compensate by upregulating nitrite reductase (*nrfA*). We agree with the reviewer that these are fascinating and important questions that warrant further investigation in future studies.

This work represents nearly eight years of effort, involving multiple researchers and extensive *in vivo* experimentation. As the field of diet-microbiota-multiorgan interactions rapidly expands, our findings naturally raise additional mechanistic questions. However, we believe it is not feasible to address all of them within a single study. We hope the mechanistic insights presented here are judged as both rigorous and sufficient within the scope of this manuscript.

Referee #2 (Remarks to the Author):

In this study Tanoue et al demonstrate that low protein diet interacts with specific members of the gut microbiota to increase browning in subcutaneous adipose tissue, which contribute to metabolic improvements using human observational and mouse mechanistic studies. It is a true tour de force approach using a magnitude of mouse models and careful dissection of signaling mechanisms. The manuscript is clearly written and data are logically introduced and presented. There are a few minor suggestions that potentially can improve the manuscript further.

We greatly appreciate the positive feedback and thoughtful suggestions for improving our manuscript. We have revised the manuscript accordingly. New content is highlighted in blue font in the revised manuscript, which we hope has adequately addressed this reviewer's comments.

1. Does the low protein diet affect fecal energy content and energy extraction?

To address this question, we measured faecal energy content by bomb calorimetry and found no significant difference between mice fed a low-protein versus a control diet (**new Extended Data Fig. 1l**).

To assess nutrient absorption, we orally administered a bolus of ^{13}C-labeled palmitic acid to mice and measured uptake into the plasma by LC-MS. We found no significant difference in plasma ^{13}C-palmitic acid concentrations between LPD- and control diet-fed mice (**new Extended Data Fig. 1m**).

Together, these findings suggest that LPD feeding does not significantly impair intestinal energy absorption.

2. Can the responses to low protein diet in Figure 1i be statistically evaluated?

We appreciate this reviewer's feedback and agree that statistical analysis is essential, even in this small clinical dataset. The reduction in body weight and BMI were found to be statistically significant.

However, due to the small sample size and the potential effects beyond beige cell induction, the editor suggested that it may be best to remove this clinical data from the manuscript. While we still believe that demonstrating the metabolic benefits of reduced protein intake in humans is valuable, we have decided to omit these data from the revised manuscript. Moving forward, we plan to recruit more patients to generate a more robust dataset in future studies.

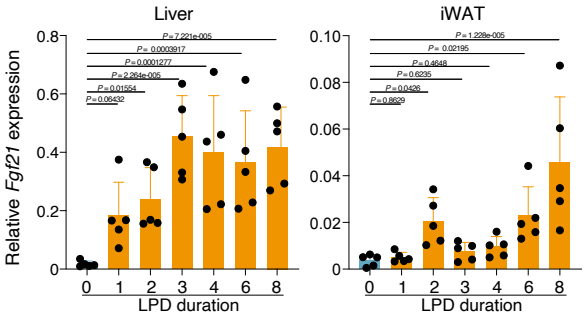
3. Related to Figure 3. Are insulin levels at fasting and during the GTT altered following low protein diet?

Thank you for your valuable feedback. In response, we measured blood insulin levels before and 30 minutes after glucose administration during an OGTT in LPD fed mice. Insulin levels were significantly reduced in the setting of LPD feeding, further supporting an improvement in insulin sensitivity. These data have been incorporated into the revised manuscript as **new Extended Data Fig. 1k**.

4. Do low protein diets affect Fgf21 expression in the adipose tissue?

This is an important point, especially in light of a recent study showing that adipocyte-specific FGF21 overexpression promotes metabolic health and extends longevity even under high-fat diet conditions (Cell Metab. 2025; S1550-4131(25)00267-0). In our study, *Fgf21* levels in adipose tissue were consistently elevated in response to LPD feeding (see **figure** below, for reviewer only). However, both the absolute levels (please see Y axes) and the magnitude of induction were much lower than those

observed in the liver. Therefore, in the context of a low-protein diet, the contribution of this low-level, adipose-derived FGF21 remains unclear, and we have chosen not to include these data in the main manuscript. (Should the reviewer or editor recommend including these data in the manuscript, we would be glad to do so.)



5. Low protein diet reduces body weight in wild-type mice, but does it affect body weight in FGF21 and FXR deficient mice? In mice colonized with the different combinations of bacteria where browning was observed?

We examined body weight in *Nr1h4* (FXR)-deficient and *Fgf21*-deficient mice. Upon LPD feeding, both *Nr1h4* and *Fgf21*$^{-/-}$ mice exhibited blunted upregulation of *Ucp1* and other beige marker genes in iWAT, and a concomitant increase in body weight compared to wild-type mice (**Extended Data Figs. 8b and 11a**).

In addition, to evaluate the impact of the hu4 strain consortium, GF mice were inoculated with faeces from participant T07, whose microbiota lacked browning capacity (see **Fig. 5c**) and then fed a HFD for 4 weeks. The mice were subsequently treated with a LPD alone or in combination with two oral doses of the hu4 strains (**new Fig. 6b**). While a LPD alone was somewhat efficacious, combination treatment with the hu4 strains led to significantly greater body weight loss, reduced white adipose tissue mass, improved glucose tolerance, increased *Ucp1* expression in both iWAT and BAT, and decreased plasma cholesterol, triglyceride, and ALT levels (**new Fig. 6b**). Notably, these effects occurred with minimal reduction in muscle mass (**new Extended Data Fig. 16b**).

6. Does tungsten alter food and water intake?

The tungsten supplementation experiments were conducted using GF isolators, which precluded the use of metabolic cages. Thus, since all studies were carried out in standard cages, we were unable to precisely quantify food and water intake. Nonetheless, based on careful daily observation, we determined that mice in the 0.1% tungsten treatment group did not exhibit any noticeable changes in food or water consumption. In contrast, mice in the 0.5% tungsten group exhibited mildly reduced appetite. These observations have now been included in the Methods section of the revised manuscript.

7. The conclusion that the microbiota is responding to differences in nitrogen abundance upon feeding low protein diet is aligned with DOI: 10.1126/science.abh2950 where the authors identified the microbiota to be important for nitrogen recycling. Perhaps worth to expand on the biological/evolutionary importance on how the microbiota senses nitrogen abundance in the gut and the resulting impact on the host.

We thank the reviewer very much for this insightful comment. We were aware of the Science paper on nitrogen recycling during hibernation in squirrels. As the reviewer points out, their findings are

indeed closely aligned with ours, demonstrating that hosts and microbiota cooperate to conserve nitrogen under conditions of nitrogen limitation.

In the case of squirrel hibernation, urease-expressing gut microbes play a critical role in converting urea into CO_2 and NH_3 in the intestine, with the resulting NH_3 subsequently transported to the liver and reincorporated into amino acids. Under low-protein diet conditions, we did not observe increased urease expression among gut bacteria. Instead, we found a pronounced upregulation of nitrite reductase. Nevertheless, both pathways result in NH_3 production, suggesting that microbial responses to nitrogen deficiency may be conserved across physiological contexts and species.

In accordance with the reviewer's suggestion, we have incorporated this discussion into the revised manuscript. We agree that much remains to be understood regarding how the gut microbiota senses nitrogen availability and how these microbial responses influence host physiology. We now highlight this as an important direction for future research in the manuscript.

Referee #3 (Remarks to the Author):

A. Summary of the key results:
Chronic cold exposure is the most potent method of thermogenic activation. The authors propose diet-based interventions as an alternative with fewer cardiovascular side effects. They cite studies linking diet and microbiota to browning but acknowledge gaps in understanding. Their findings convincingly show that LPD induces Ucp1 expression in adipocytes via circulating Fgf21 and Nr1h4.
Functional relevance remains unproven. Even a 100-1,000-fold increase in Ucp1 mRNA barely approaches UCP1 levels in classical BAT (PMID: 28109720), and the study provides no evidence linking thermogenic gene expression (Ucp1, Fgf21, Nr1h4) to energy expenditure in vivo. Measuring energy expenditure in genetic models, including Ucp1 KO mice, could clarify their functional role. However, a major weakness of the manuscript is the lack of metabolic data supporting relevance and the inappropriate methods used for analyzing metabolic data. My specific concerns are outlined below.
B. Originality and significance:
The authors demonstrate that LPD induces Ucp1 expression in subcutaneous white fat through bile acids, FXR, Fgf21, and ADRB3.
C. Data & methodology:
C1. Energy expenditure normalization is incorrect. The timing of measurements after the diet switch is unclear, and reporting only a 2-hour phase "during the day (12:00 pm–2:00 pm) and night (12:00 am–2:00 am)" is highly unorthodox. A sustained thermogenic effect should accompany significant weight loss; if not, alternative explanations such as impaired nutrient absorption or reduced food intake must be considered.
C2. Glucose dosing (1.2 g/kg) was based on body weight but should be normalized to lean mass or kept identical across groups. This is because a lighter mouse will receive less glucose and thus appear to have better glucose intolerance, but this is just because there was less glucose to clear. Typically differences in weight in obesity is due to fat mass,
D. Appropriate use of statistics and treatment of uncertainties:
D1. The VO2 is plotted repeatedly for only 5 mice. Therefore, repeated measures from the same mice artificially inflate sample size (~40×). Repeated measures from the same mouse are not independent observations; which violates the assumption of independence in statistical analysis.
D2. VO2 data is improperly normalized.

We thank the reviewer very much for these thoughtful and detailed comments. As the reviewer correctly notes, even though *Ucp1* mRNA expression was markedly increased in iWAT following LPD feeding, the levels did not approach those observed in classical brown adipose tissue. Also, we acknowledge that induction of *Ucp1* and other beige-associated genes alone does not constitute definitive evidence of meaningful thermogenesis and increased energy expenditure. A rigorous causal evaluation, such as using models deficient in beige adipogenesis or performing metabolic phenotyping, will be essential to clarify the role of Ucp1-expressing beige fat induced by LPD feeding. However, such experiments are beyond the scope of the current study, and these questions would be best addressed in future investigations dedicated specifically to adipose thermogenic biology.

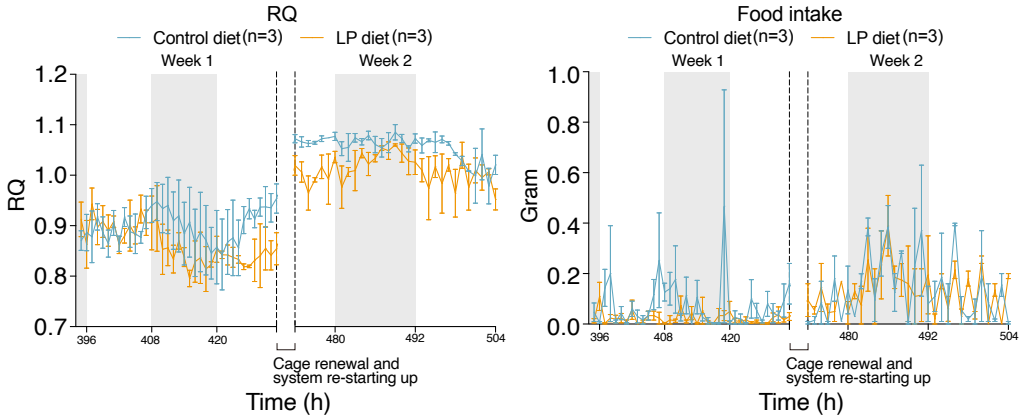
In response to the reviewer's concern, while we believe that the potential metabolic benefits of reduced protein intake in both mice and humans are of substantial interest, we have opted to exclude data from metabolic cage experiments (including VO_2 data), the MAFLD model, and the LPD human trial involving patients with fatty liver disease from the revised manuscript. We have, however, retained the data showing that dietary protein restriction and colonization with SPF microbiota or specific members of the microbiota led to significant reductions in body weight and improvements in glucose tolerance and fatty liver disease in mice (**Extended Data Fig. 2a-e, new Extended Data Fig. 2f-j** and **new Fig. 6b**). These datasets are critically important to illustrate the connection between dietary protein restriction, the gut microbiota, and weight loss.

While we appreciate the reviewer's comment, we respectfully note that the primary objective of this manuscript is not to define the thermogenic or metabolic capacity of beige adipocytes, but rather to elucidate how dietary protein restriction interacts with the gut microbiota to influence adipose tissue gene expression, structural remodelling, and putative function. We do not assert that the metabolic benefits of LPD feeding are solely attributable to WAT browning or *Ucp1* induction. Rather, we suggest that WAT browning may contribute in part to these effects but is unlikely to be the sole driver. We have revised the manuscript to more clearly define the scope and objectives of the study, to avoid overinterpreting *Ucp1* induction in iWAT as a definitive functional outcome, and to minimize the potential for misinterpretation regarding the primary focus of our work.

Suggested improvements:
1) Fig. 1E & Extended Data Fig. 3A – VO2 normalization by body weight introduces artifacts. VO2 or Energy expenditure (EE) should be plotted as ml/h or kcal/h per mouse, or analyzed using ANCOVA with lean/total body mass as a covariate. Excellent reviews on this topic have been written (PMID: 20103710; PMID: 22205519). A 2-hour VO2 phase is insufficient. Full data, including RER and movement, should be reported to assess robustness.
2) Extended Data Fig. 3a – The authors note in the fig legend that "Each data point represents an individual mouse at one time point (5 mice per group x 39 time points = 195 points per phase)". Therefore, repeated measures from the same mice artificially inflate sample size (~40×). Repeated measures from the same mouse are not independent observations; which violates the assumption of independence in statistical analysis. This type of analysis exaggerates variability in the data, masking real effects or creating false positives. VO2 data should be plotted as mouse-wise averages (n = 5). This brings up another point, where an n = 5 is a very small sample size to convincingly show that the VO2 is reproducible with a relevant effect size.

We completely agree that any claims regarding changes in energy expenditure or thermogenesis must be supported by appropriate normalization and careful interpretation of VO_2 data, and that overinterpretation of metabolic phenotypes can arise from flawed analysis of indirect calorimetry data. We acknowledge the reviewer's concern as part of a broader methodological challenge in the field.

As shown in the **figure** below (for reviewer only), in the first to second week following LPD initiation (for about 3 days), the respiratory quotient decreased despite no detectable change in food intake.



Nonetheless, in response to the reviewer's feedback, and to minimize the risk of misinterpretation, we have decided to omit the metabolic cage data including VO_2 data (previous Fig. 1e and Extended Data Fig. 3a), from the revised manuscript. This decision reflects our intention to avoid overinterpreting *Ucp1* induction in iWAT, but rather to focus on the primary objective of our study, to investigate which dietary compositions influence WAT browning and to elucidate the underlying mechanisms. Our

findings indicate that dietary protein restriction modulates microbiota function, which in turn activates FXR and FGF21 signalling pathways, enhances sympathetic innervation, and promotes beige cell induction.

3) The authors focus on Ucp1 expression but provide no functional evidence that its induction drives weight loss. The lack of proper VO2 normalization (see comment #1) further weakens this claim. If LPD-induced Ucp1 activation truly increases energy expenditure and drives weight loss, this effect should be evident before significant weight changes occur. However, Ucp1 mRNA was only shown following long-term LPD treatment. Additionally, genetic inactivation of Ucp1 or its regulators (e.g., Fgf21) should mitigate the effect. For instance, while Nr1h4fl/fl;Adipoq-Cre mice exhibit reduced Ucp1 expression on LPD (Fig. 3c), there is no functional evidence demonstrating the significance of this reduction.

We appreciate this reviewer's insightful comments and fully agree. In response, we examined the time course of Ucp1 induction and remodelling of tyrosine hydroxylase-positive sympathetic neurons by whole-mount immunostaining following LPD initiation. In SPF wild-type or heterozygous mice, LPD feeding induced fine, dense, TH-positive sympathetic neuronal structures in regions enriched with UCP1-positive cells (near the inguinal lymph nodes). This innervation was detectable as early as one week after LPD initiation, preceding or coinciding with *Ucp1* upregulation (though precise temporal correlation of these events remains technically challenging). In contrast, both *Fgf21*$^{-/-}$ and *Nr1h4*$^{-/-}$ mice exhibited markedly reduced sympathetic innervation, resembling the pattern observed in wild-type GF mice or SPF mice fed a control diet (**new Fig. 3h**, **new Extended Data Fig. 13**).

The body weight changes also followed similar kinetics with differences from the CD group becoming apparent within 1-2 weeks after LPD initiation (**Extended Data Fig. 1e** and **Fig. 1i**). While it remains challenging to determine the precise contribution of beige adipocytes to body weight loss, based solely on temporal kinetics, our findings are consistent with the notion that beige fat contributes, at least in part, to body weight regulation.

Additionally, we assessed body weight changes in *Fgf21*$^{-/-}$ and *Nr1h4*$^{-/-}$ mice, both of which exhibited greater body weight gain than wild-type controls during LPD feeding (**new Extended Data Fig. 8b** and **Extended Data Fig. 11a**). We also evaluated body weight change in two groups of mice fed the same diet and colonized with identical microbiota, differing only in the presence or absence of the human-derived four-strain (hu4) consortium. Specifically, germ-free mice were first colonized with microbiota from participant T07, fed a high-fat diet for 4 weeks, and then treated with a LPD alone or in combination with two oral doses of the hu4 strains. Compared to LPD alone, combination treatment with the hu4 strains resulted in significantly greater body weight loss, reduced fat mass, and increased *Ucp1* expression in iWAT, without causing severe muscle mass loss (**new Fig. 6b** and **new Extended Data Fig. 16b**). These results support the notion that WAT browning, triggered by functional alterations in specific microbiota members and subsequent activation of FXR and FGF21 signalling, contributes, at least in part, to the metabolic benefits afforded by LPD feeding. However, we do not assert that WAT browning is the sole mediator of these effects. It remains challenging to establish a direct causal relationship between the induction of beige fat markers such as *Ucp1* in iWAT and the observed metabolic outcomes. We hope the reviewer will agree that fully resolving this question is outside the scope of the current study and would be more appropriately and comprehensively addressed in future investigations that are more focused on brown and beige adipose tissue biology. In the revised

manuscript, we have clarified these limitations and carefully revised the relevant sections to more accurately reflect the scope of our study and minimize potential misinterpretation.

4) Extended Data Fig. 3b – Glucose tolerance test (GTT) normalization by body weight is problematic. Since muscle takes up the lion's share of glucose, glucose should be dosed by lean mass. Another approach is to just pick one dose of glucose (based on the control group) and just administer the same amount to all animals, as this is how this test is done in humans. Papers have been written about this (PMID: 29143855).

We thank the reviewer for this important comment. In our study, we conducted the OGTT using a protocol that has been utilized in several previous publications, including STAR Protocols 3:101171 (2022), Methods Mol Biol. 1339:247-254 (2015), and Cell 172:731-743.e12 (2018). We agree that normalizing glucose dosing to lean mass may more accurately reflect glucose utilization, particularly by skeletal muscle, a major site of glucose uptake. However, accurately and consistently measuring lean mass presents technical challenges, especially when applied across large cohorts of gnotobiotic animals. Furthermore, given that brown and beige adipocytes also contribute to glucose consumption, we are not certain that lean mass-based adjustment fully captures the relevant physiology, nor that is represents the optimal method for comparing glucose utilization between groups of mice that differ in the number and activity of beige/brown adipocytes.

Regarding the alternative approach of administering a fixed glucose dose to all animals, we appreciate the reviewer's suggestion and recognize its conceptual merit. In human clinical settings, fixed glucose dosing is generally appropriate as it allows comparison to established reference values rather than direct comparison between patient groups. Moreover, individuals with diabetes or prediabetes are typically evaluated using additional markers such as HbA1c and other clinical parameters. In contrast, the optimal glucose dosing strategy for OGTT in rodent models remains a subject of ongoing debate. While various approaches exist, body weight-normalized dosing remains widely used in the field. We have added a note in the Methods section acknowledging this discussion and now cite suggested literature (Diabetologia 61:526-538, 2018) to provide context.

If the reviewer remains concerned and considers the OGTT data unacceptable, we are willing to omit these data from the revised manuscript.

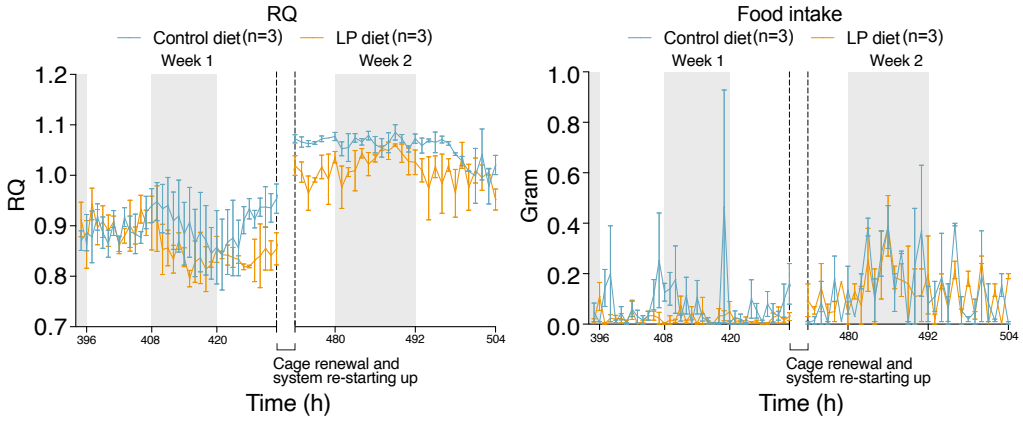
5) Fig. 1f – Weight loss upon LPD lacks food intake data, except for a brief 10-hour window (Extended Data Fig. 3b). Cumulative food intake is needed to assess energy balance. <u>Since a major claim of the paper is that LPD induces thermogenesis through Ucp1 in beige fat to combat obesity</u>, differences in food intake and nutrient absorption (with bomb calorimetry) must be ruled out.

We thank the reviewer for this important comment and apologize for the lack of clarity in how our major claim was presented. We would like to emphasize once again that the central focus of this study is <u>not</u> to show that a LPD combats obesity by inducing thermogenesis via *Ucp1* upregulation in beige fat, but rather that a LPD promotes *Ucp1* expression through functional modulation of the gut microbiota.

While a comprehensive assessment of energy balance, including detailed measurements of cumulative food intake, nutrient absorption, and energy expenditure and excretion, would indeed enhance interpretation of our metabolic data, we encountered several technical challenges in addressing this

point. We painstakingly repeated metabolic cage experiments; however, we found it extremely challenging to obtain accurate measurements of cumulative food intake throughout the experiments, as mice frequently scattered food from their containers. This issue was further compounded in gnotobiotic settings, which are central to our study, where precise food intake measurements within vinyl isolators are technically difficult. Therefore, as noted above, the VO_2 data in the previous Fig. 1f has been removed to avoid potential misinterpretation.

Nevertheless, based on careful daily observation, LPD-fed mice did not exhibit any noticeable changes in food or water consumption compared to control diet-fed mice. Importantly, after two weeks of LPD feeding, the respiratory quotient decreased while food intake remained largely unchanged (at least during periods when reliable measurements were obtained in metabolic cages) (**Figure for reviewer only**), suggesting that reduced food intake is unlikely to be a primary driver of the observed LPD-induced weight loss.



Furthermore, LPD-induced weight loss was evident even under high-fat dietary conditions (**new Extended Data Fig. 2f**).

Moreover, oral introduction of the browning-inducing hu4-mix to gnotobiotic mice, colonized with the browning-non-inducing T-07 microbiota, significantly augmented LPD-mediated weight loss compared to hu4-mix-untreated controls, despite identical dietary treatment (**new Fig. 6b**).

We also examined mice colonized with *R. timonensis* and the Δ*nrfA Bilophila* strain, and fed the same LPD with or without ammonium chloride (NH_4Cl) supplementation in drinking water. Deletion of *nrfA* in *Bilophila* significantly impaired hepatic *Fgf21* expression and iWAT browning response. Notably, supplementation with ammonia restored both hepatic *Fgf21* and iWAT *Ucp1* expression, despite identical dietary treatment (**new Fig. 7i**).

As suggested by the reviewer, we also examined faecal caloric content by bomb calorimetry. Faecal caloric content did not differ between SPF mice fed a control vs a low-protein diet (**new Extended Data Fig. 1l**). To further assess nutrient absorption, we orally administered a bolus of ^{13}C-labeled palmitic acid to LPD-fed mice and measured uptake into the plasma by LC-MS. We found no significant difference in plasma ^{13}C-palmitic acid concentrations between low-protein and control-diet fed mice (**new Extended Data Fig. 1m**).

Collectively, these findings indicate that neither diet aversion nor nutrient malabsorption are primary drivers of LPD-induced weight loss and WAT browning.

6) Body Composition – Lean mass loss is unaccounted for. Since LPD reduces protein intake, its contribution to weight loss must be assessed.

To address this concern, we assessed fat and lean volume using MRI and measured the weights of iWAT and gastrocnemius muscle in SPF mice fed either a low-protein or control diet. LPD feeding led to significant reductions in body weight, total fat mass, and iWAT mass (**new Extended Data Fig. 1e, g, h, j**). In contrast, gastrocnemius muscle mass and total lean mass were largely unaffected (**new Extended Data Fig. 1f, i, j,** and **Extended Data video 2**). These findings indicate that the observed *Ucp1* upregulation is unlikely to be due to pathological muscle wasting.

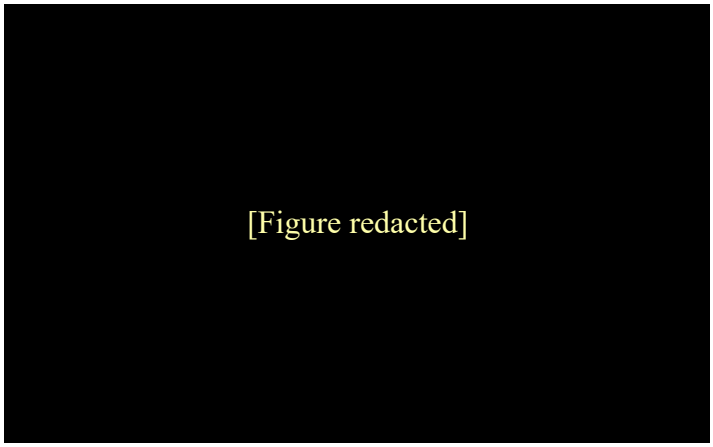
7) Fig. 1f – HFD-to-LPD transition involves multiple dietary changes. A more controlled switch (e.g., HFD to HFD+LPD) would clarify LPD-specific effects, after all, they show that this combination still induced Ucp1 in Fig. 1a.

We thank the reviewer for this insightful comment. In response, we conducted the suggested experiment using SPF mice, transitioning their diet from a high-fat diet (HFD) to a high-fat, low-protein diet (HF/LPD) containing 32% kcal fat. Consistent with our previous findings, this dietary shift resulted in a substantial induction of *Ucp1* expression and metabolic improvements, confirming that the low-protein component is a key driver of the observed effects, even in the context of a high-fat diet (HF/LPD, **new Extended Data Fig. 2f-j**).

8) The authors should assess muscle mass loss on the LPD, as reduced muscle mass could contribute to increased Ucp1 expression through excess heat dissipation. Since experiments were conducted at room temperature, it is possible that LPD-induced muscle loss impairs shivering capacity, leading to heightened cold perception, increased sympathetic activation, and secondary Ucp1 upregulation. The authors should rule out the possibility that Ucp1 induction results secondarily from compromised muscle thermogenesis without contributing at all to the weight loss.

We have included data on muscle mass (assessed by weighing the gastrocnemius muscle) and total lean mass (evaluated by MRI) in the revised manuscript. In some experiments, LPD feeding resulted in a modest reduction in muscle mass. However, overall lean mass was largely preserved (**new Extended Data Fig. 1f, i, j**), and the reduction in fat mass was consistently more pronounced (**Extended Data Fig. 1g, h, j,** and **Extended Data video 2**). As demonstrated in the **Extended Data video 1**, mice housed at room temperature exhibited normal behaviour with no signs of impaired mobility or shivering, suggesting that muscle loss was not severe enough to induce browning in iWAT.

[Text redacted]



To further address the reviewer's concern, we conducted additional experiments under thermoneutral conditions (30 °C). Even in these settings, robust *Ucp1* upregulation was observed in response to LPD feeding, to levels comparable to those observed in mice maintained at room temperature (**new Fig. 1d**). While we cannot entirely rule out contributions by the mechanisms proposed by the reviewer, our findings suggest that *Ucp1* induction is unlikely to be merely a byproduct of weight loss or compromised muscle-based thermogenesis.

Additionally, we evaluated muscle mass changes in two groups of mice fed the same diet and colonized with identical microbiota, differing only in the presence or absence of the human-derived four-strain (hu4) consortium. Specifically, germ-free mice were first colonized with microbiota from participant T07, fed a high-fat diet for 4 weeks, and then treated with a LPD alone or in combination with two oral doses of the hu4 strains. Compared to LPD alone, combination therapy with the hu4 strains resulted in significantly greater body weight loss, reduced fat mass, and increased *Ucp1* expression in iWAT (**new Fig. 6b**). Notably, these effects occurred with minimal reduction in muscle mass and without a significant increase in creatine kinase levels (**new Extended Data Fig. 16b**). These findings suggest that a LPD exerts a more pronounced effect on reducing fat mass than on muscle mass, and that this effect is mediated, at least in part, by WAT browning induced by specific microbiota members.

9) Percent changes (e.g., Fig. 1f, 2d, Extended Data Fig. 3b) should be supplemented with absolute body weight and body composition (fat vs. lean mass).

We have included fat and lean masses in **Extended Data Fig. 1f, g**.

10) Rapid weight loss (Fig. 1f) suggests malabsorption. Fecal energy content (e.g., bomb calorimetry) should be measured.

We thank the reviewer for this important comment. As suggested, we measured faecal energy content by bomb calorimetry and found no significant difference between mice fed a low protein versus a control diet (**new Extended Data Fig. 1l**).

To further assess the possibility of nutrient malabsorption, we orally administered a bolus of ^{13}C-labeled palmitic acid to mice and measured uptake into the plasma by LC-MS. We found no significant difference in plasma ^{13}C-palmitic acid concentrations between low-protein and control diet-fed mice (**new Extended Data Fig. 1m**).

Together, these findings suggest that a LPD does not significantly alter intestinal energy absorption.

11) The timing of O_2 measurement or GTT post-diet switch is also unclear.

We have removed these VO_2 data from the manuscript. We now indicated the timing of GTT experiments.

12) Fig. 1A – Fold-change in Ucp1 induction is unclear. The control diet should be set to 1, with other conditions expressed relative to it.

We appreciate the reviewer's suggestion and have now included the fold-change data in **new Extended Data Fig. 1a**. However, additional normalization (such as expressing *Ucp1* levels as fold-change) can introduce unintended bias, particularly by masking biologically meaningful differences in basal expression levels. Therefore, throughout the manuscript, we have chosen to present gene expression data normalized to expression of a reference gene, which we believe better preserves the integrity of the data and permits fairer comparisons across conditions.

13) Extended Data Fig. 18 – claims FXR promotes "beige fat biogenesis," but de novo adipogenesis was not tested.

We have removed the previous Extended Data Fig. 18 from the revised manuscript.

14) Prior work (PMID: 23404943) found no Ucp1 induction with LPD but is not discussed.

We thank the reviewer for pointing this out. However, the experimental setup in that study differs substantially from ours and does not address the role of the microbiota, and as such we have selected to limit discussion of their findings in our current manuscript.

15) Extended Data Fig. 2d – Errors in terminology and interpretation:
a. "Glycolytic beige" should replace "glycogenic beige."

We thank the reviewer for pointing out this error. We have revised the manuscript accordingly.

b. Eno1, ldhb, and Pdha are general glycolysis markers, not specific to g-beige cells.
c. No evidence supports Phospho1's role in the futile creatine cycle; prior work ruled it out as the phosphatase of the FCC.

We have revised the manuscript accordingly.

Revisions to the text are highlighted in blue font and modified figures are indicated by blue rectangles. We hope that our responses have adequately addressed all reviewers' comments.

Referee #1

The authors have provided a thorough revision work which has addressed the large majority of my previous concerns. In those cases where the authors have not been able to provide compelling new mechanistic insights, they have appropriately toned down their conclusions.

I would only like to point out that my comments about the model (previous Ext. Data Fig. 18) were not meant to discourage the authors from including a cartoon summary. Rather, I referenced it to point out instances where the model was not fully supported by experimental data shown in the manuscript. Given the complexity of the pathways that the authors describe in this study, I would encourage them to re-include a cartoon summary, since it can be very helpful for the reader to obtain a graphical overview of the findings, but would suggest making sure the cartoon is perfectly in line with the data.

We greatly appreciate this suggestion. We agree that a well-designed summary figure could be very helpful for readers. However, given the complexity of the pathways described and the need to ensure full consistency with our current data, we opted not to include a summary schematic at this stage. We would like to avoid oversimplifying our findings and introducing any potential points of confusion.

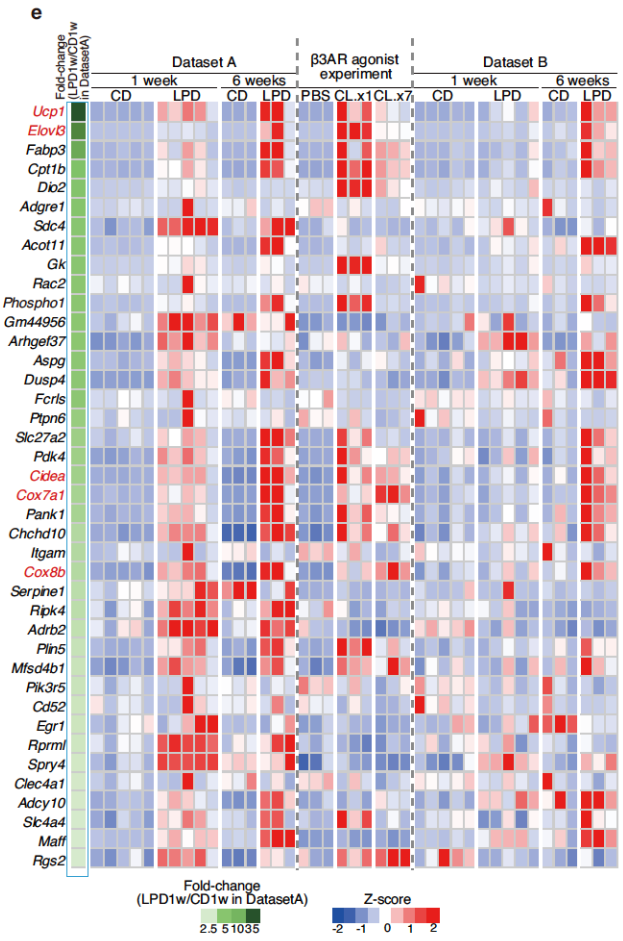
#3 (Remarks to the Author):

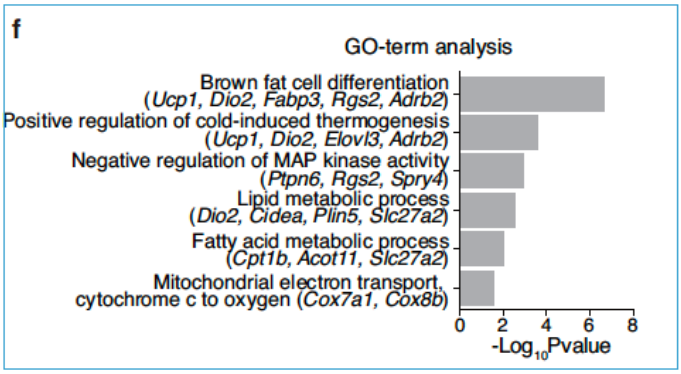
Tanoue et al. have made notable improvements to their manuscript. However, several substantive issues remain that warrant further clarification or revision. While the authors now emphasize that their primary focus is on how a low-protein diet (LPD) interacts with the gut microbiota to influence gene expression in adipose tissue, the structure and presentation of the manuscript continue to place substantial emphasis on Ucp1 expression and adipose browning. This is evident from the framing of the Introduction—which highlights the metabolic benefits of browning—as well as from the prominence of Ucp1 mRNA data throughout the first six pages and beyond. This creates a disconnect between the authors' stated focus in their rebuttal and the way the paper is currently written. Either the manuscript should be reframed to reflect this revised focus more accurately, or additional evidence should be presented to support the functional relevance of browning in the phenotype.

We agree that a causal role for UCP1-mediated browning in the weight loss phenotype cannot be established without *Ucp1* knockout models and comprehensive metabolic phenotyping. Accordingly, we have more carefully revised the text to avoid over-interpretation of the UCP1 data and to ensure that our conclusions remain firmly grounded in the data presented. Specifically, we have completely restructured the narrative of the **Abstract** and **Introduction** to clarify the conceptual foundation of the study, focusing on the diet- and gut microbiota-initiated processes rather than the effects downstream of adipose browning. In the **Results** and **Discussion** sections, we have reframed *Ucp1* expression as a molecular marker of adipose remodeling in response to a LPD and microbiota-induced functional alterations rather than a direct causal agent of thermogenesis.

In response to the comment regarding "the prominence of Ucp1 mRNA data throughout the first six pages," we have now added data demonstrating that a LPD induces upregulation of multiple mitochondrial and beige adipocyte markers in addition to *Ucp1* (as presented in new **Extended Data Fig. 1e, f**; see below). We have also added qPCR data for beige adipocyte signature genes including *Elovl3* and *Cox7a1* in multiple experiments, as space permits. We would be happy to include additional gene expression data and iWAT H&E staining if the Reviewer feels that these would strengthen the manuscript and if space permits.

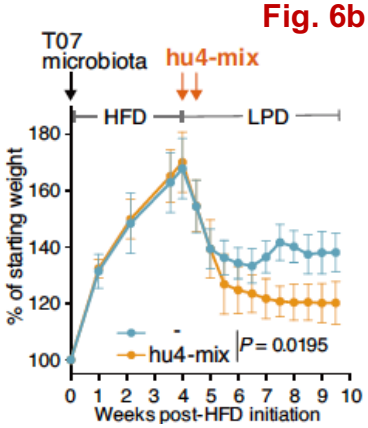
Extended Data Fig. 1e, f





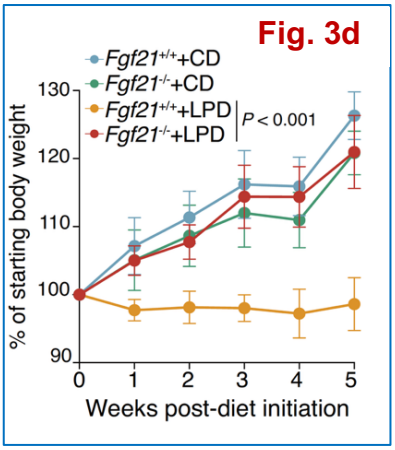
We also took advantage of this opportunity to revisit the original motivation of our study – namely, to uncover the mechanistic underpinnings by which microbial and dietary factors intersect to regulate host physiology. Guided by this motivation, we sought to determine whether, and by what mechanisms, environmental cues and microbiota-derived signals influence host metabolism. The downstream metabolic consequences are secondary to this conceptual framework and beyond the primary scope of our study. As such, our identification of both strain- and gene-level contributions of the gut microbiota to beige adipocyte induction remains highly relevant and meaningful, and further characterization of downstream phenotypes should be the basis of future work.

Fig. 6b



In particular, our data showing that oral administration of four human-derived bacterial strains significantly alters body weight in mice harboring a complex T07 donor-derived microbiota (**Fig. 6b**, included in the most recent revision) represents a novel and important finding – that specific microbiota members can influence body weight regulation, even though the precise contribution of beige adipocytes to systemic metabolic control is not definitively demonstrated. We have placed greater emphasis on these microbiota-related findings throughout the revised manuscript, and we hope that these revisions align with the Reviewer's perspective.

Additionally, we recognize that we should have more carefully considered the possibility that interactions between LPD feeding and the microbiota operate through both UCP1-dependent and UCP1-independent mechanisms. To address this, we have added new data showing that *Fgf21*-deficient mice are refractory to the weight-reducing effects of a LPD (new **Fig. 3d**), underscoring the key role of the FGF21 pathway in regulating body weight in our model. Notably, recent reports indicate that the anti-obesity effects of FGF21 are mediated, at least in part, via UCP1-independent pathways (*Cell Metab. 2015 21:731-8, Mol Metab. 2017, 6:1454-1467, Biochem Pharmacol. 2024, 221;16042*). Therefore, the combined effects of a LPD, FGF21 induction, and WAT browning likely lead to both UCP1-dependent and UCP1-independent metabolic outcomes. These new data and discussion points have been incorporated into the revised manuscript.



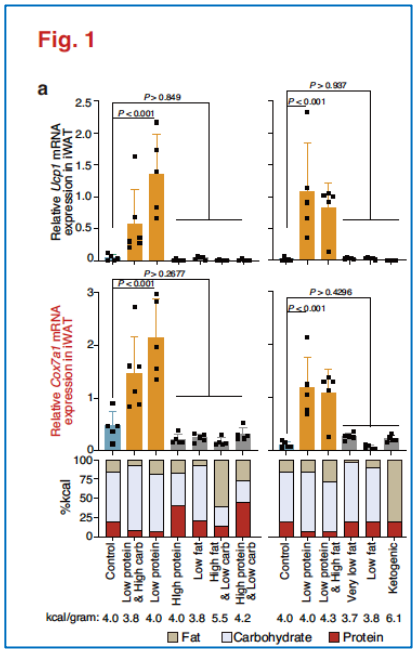
1. The authors report increased Ucp1 mRNA levels with LPD, but this is presented per total RNA. Since LPD reduces total fat mass, it's likely that the total UCP1 content per animal is substantially lower than the figures suggest. UCP1 protein levels should be normalized per whole depot to account for tissue mass differences (see PMID: 35167098). Moreover, despite the emphasis on UCP1, the authors acknowledge in the discussion that there is no causal evidence linking browning to the phenotype. This warrants either a reframing of the manuscript to de-emphasize browning or additional mechanistic evidence supporting its relevance.

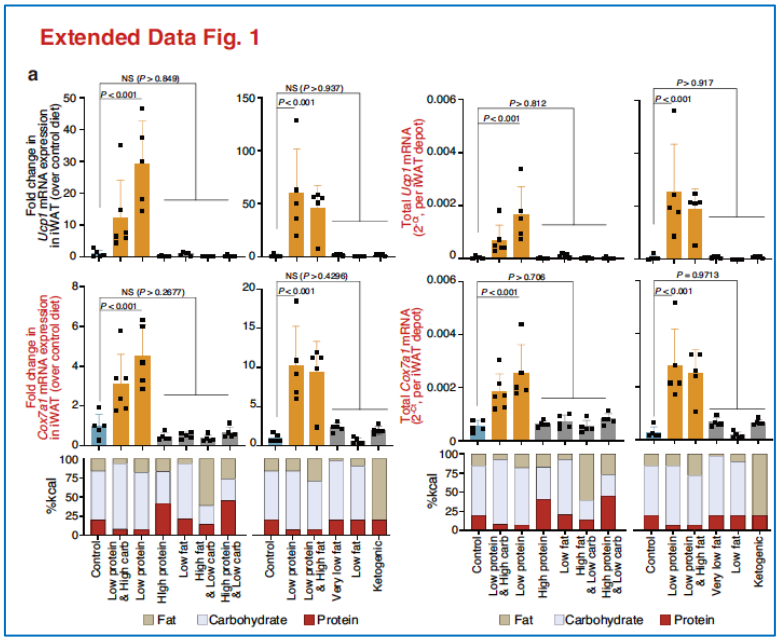
We appreciate the Reviewer's thoughtful comments, which reflect long-standing and important considerations in the field. We agree that Ucp1 mRNA is highly dynamic and may primarily reflect sympathetic input rather than thermogenic capacity. However, we would like to highlight that in addition to Ucp1 induction at the mRNA level, we also show induction at the protein level by both Western blotting (**Extended Data Fig. 1c**) and immunostaining, including whole-mount analyses that clearly demonstrate the presence of beige adipocytes (**Fig. 3i, Extended Data Fig. 13**). The Reviewer may have inadvertently overlooked these data

Additionally, we were surprised by this Reviewer's suggestion to normalize expression data on a per-depot basis, especially given that a different normalization strategy (fold-change over control) was emphasized in the previous round of review. Per-depot normalization does not seem to be the standard in the field, so at first we were unsure whether it would be the most appropriate method. Nevertheless, we fully acknowledge the concerns and methodological caveats brought up by the Reviewer. Luckily, we had extracted RNA from the entire iWAT depot (rather than a fragment) in most experiments and thus were able to analyze total per-depot *Ucp1* mRNA expression levels. Accordingly, as suggested, we have now included data showing total per-depot *Ucp1* and well as *Elovl3* and *Cox7a1* mRNA expression, quantified as $2^{-[Ct]}$ in multiple figure panels including new **Extended Data Fig. 1a**.

In the spirit of full transparency, we have included multiple metrics of mRNA expression in **new Fig.1a** and **Extended Data Fig. 1**: 1) relative abundance (the most commonly-used and, in our view, the most accurate), 2) fold-change, and 3) per-depot (as suggested by the Reviewer). Importantly, our conclusions remain consistent across all methods. Throughout the revised manuscript, we have shown mRNA expression data in two formats (relative abundance and total per-depot expression) where possible. While we recognize that presenting the data in multiple formats could add complexity for readers, we have done so as requested to ensure transparency and completeness.

While we agree that evaluation of whole-animal UCP1 protein content could be informative, this is not feasible within the scope of our current study. We have revised the text to more clearly acknowledge the crux of the issue brought up by this Reviewer, namely that *Ucp1* induction could either be a driver or a consequence of weight loss. Importantly though, either scenario would be consistent with our central finding that a LPD reshapes the microbiota to influence adipose biology.





2. The authors report an approximate 1,000 kcal/kg increase in fecal energy content in the LPD group. However, no data are provided on total fecal mass. To accurately calculate total energy loss, caloric density (kcal/g) must be multiplied by total fecal output. Furthermore, this energy deficit must be contextualized over the duration of the experiment to determine whether it explains the observed body weight changes.

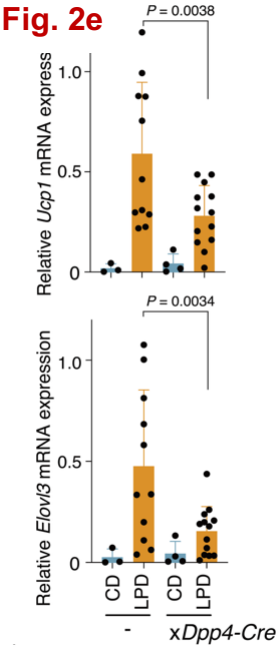
We agree with the Reviewer that a complete energy balance study would ideally include both faecal caloric density and total faecal output. However, collecting total faecal mass over the course of experiments that range in length from weeks to months is logistically challenging and, in our view, would contribute little insight beyond what is already provided by caloric density measurements. We have therefore revised the text to present faecal caloric density as a complementary observation only, without suggesting a quantitative contribution to change in body weight.

3. In Lines 162–164, the authors suggest that LPD induces functionally heterogeneous beige adipocyte subsets (lipolytic vs. glycolytic). This conclusion is not supported by the data. There is no single-cell transcriptomic or proteomic analysis to distinguish such subsets. Moreover, thermogenic adipocytes typically upregulate glycolysis while preserving lipid stores. Thus, the evidence for a distinct "glycolytic beige" population is speculative and not justified by the presented results.

We agree that our previous wording was speculative and as such we have removed the term "glycolytic beige cells" from the revised manuscript, instead simply reporting changes in gene expression without inferring distinct cell subsets.

4. The authors conclude that Adipocyte Progenitor Cells (APCs) are critical for Ucp1 induction based on Adipoq-driven FXR deletion impairing Ucp1 expression. However, Adipoq-Cre targets mature adipocytes, suggesting that LPD regulates Ucp1 expression in existing adipocytes, not through adipogenesis. Furthermore, the requirement for the β3-adrenergic receptor indicates that mature adipocytes are likely the direct target of upstream signals. Without lineage tracing, the claim that APCs are essential remains unsupported and possibly contradicted by the data.

Fig. 2e



We respectfully disagree with the Reviewer's concern regarding the role of APCs. The FXR-expressing cells described in our study co-express canonical adipose progenitor cell markers including *Dpp4*, *Col1a1*, *Pgdfra*, *Wnt2*, *Cd34*, and *Dcn* **(Fig. 2g, Extended Data Fig. 9a)**, corroborating their identity as APCs. Moreover, we had included new data in the previous round of revisions directly demonstrating the specific contribution of APC-expressed FXR by generating and analyzing a new mouse model in which FXR is conditionally knocked out in *Dpp4*-expressing cells (which include APCs but exclude other adipocyte-lineage cells) (**Fig. 2e**), although this experiment may not have been fully appreciated by the Reviewer. Consistent with our previous data, these mice exhibit significantly attenuated *Ucp1* and *Elovl3* induction compared to FXR-sufficient controls upon LPD feeding. Together, these data support a mechanistic role for APCs. While lineage tracing may also be a valuable tool, given the specificity and consistency of the data already presented, we feel that it would not substantially alter the interpretation of our findings.

5. In line 435, the manuscript references "creatine kinase levels," but this appears to be a measurement of plasma CK. This must be clearly stated in the text to avoid confusion with intracellular creatine kinase involved in thermogenesis.

We thank the Reviewer for this comment. We have amended this sentence accordingly.

Response to Referee #3:

Tanoue et al. have made notable improvements to their manuscript. Importantly, they have softened their tone and clarified that establishing a causal role for Ucp1 in their phenotype is not the primary focus. The new data showing that LPD requires Fgf21 (Fig. 3d) is a valuable addition. While normalization per depot is not used by most, it is a valid design used by leading investigators in the UCP1 field (see PMID: 35167098, 28109720). For those familiar with these methodologies, this represents a thoughtful and rigorous addition, so I commend the authors for including multiple normalization approaches.

Remaining, minor concerns

1. The statement, "As canonical interscapular brown adipocytes..., beige adipocytes are thought to assume a dominant role in thermogenesis," overstates the evidence. Is there strong support that beige adipocytes play a "dominant" thermogenic role relative to classical BAT? The authors should clarify or provide supporting references, as the current phrasing implies that beige adipocytes are more thermogenically active than classical brown adipocytes in adult mice.

We thank the reviewer for raising this important point. We agree that the original wording could be interpreted as overstating the relative thermogenic contribution of beige adipocytes compared with classical brown adipose tissue. To avoid any potential overinterpretation, we have removed this description from the revised manuscript.

2. The statement, "Therefore, elucidating the mechanisms that drive beige adipocyte induction could shed light on why the beiging program exists in humans…", requires a supporting citation. The literature remains divided on whether human BAT corresponds to mouse beige or brown adipose tissue. The reality is probably more nuanced. Deeper neck regions in human BAT more closely resemble mouse BAT, whereas more superficial regions exhibit beige-like features. If the authors are referring to the latter, this should be clarified and referenced. Otherwise, the current text could be interpreted as implying that human white adipose tissue can readily undergo beiging, which remains uncertain. Happy to be proven wrong though, but if so, this needs citation support.

We appreciate the reviewer's careful and nuanced comment regarding the current understanding of human adipose tissue heterogeneity. We agree that the relationship between human BAT and mouse beige or brown adipocytes is complex and remains an active area of investigation. To avoid implying conclusions that extend beyond the available evidence, we have removed this statement from the manuscript.

3. It appears that pathway enrichment analyses were conducted using all mouse genes as the background reference (Extended Data Fig. 1e, f). However, enrichment should ideally be calculated relative to genes expressed in BAT, as using the full genome can artificially inflate the significance of pathways enriched in highly expressed BAT genes. I recommend cross-referencing datasets such as TRAP-seq data from the Rosen group (Roh et al., 2017) for more accurate interpretation. I leave it to the authors' discretion.

We appreciate the reviewer's insightful suggestion. Our goal was to perform pathway enrichment analysis using a background that would minimize any a priori assumptions, and we therefore used all mouse genes as the reference set. In our view, restricting the background to BAT-expressed genes potentially introduces additional bias rather than reduce it.

While we agree that TRAP-seq datasets are valuable resources, we were cautious about relying on a single published dataset from an earlier study for background definition. For these reasons, we have retained our current approach. Nevertheless, we acknowledge the reviewer's point and appreciate this perspective.

4. I agree that collecting total fecal mass throughout the experiment is challenging. However, the available data could be extrapolated to estimate cumulative values over longer durations. While approximate, such estimates would still be informative and worth considering. This is a minor suggestion and I leave it to the authors' discretion.

We thank the reviewer for this constructive suggestion. To address this point, we have added two additional time points (days 32 and 37) to the revised manuscript, allowing for a more consistent assessment. These data are now presented in the new Extended Data Fig. 2f.