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Acetylcholine-synthesizing macrophages in subcutaneous fat are regulated by β2-adrenergic signaling

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 13th Aug 2020

Thank you for submitting your manuscript entitled "Acetylcholine-synthesizing macrophages in subcutaneous fat are regulated by β2-adrenergic signaling" (EMBOJ-2020-106061) to The EMBO Journal. Please accept my apologies for the extended duration of the review process due to the summer holidays and the current COVID-19 pandemic. Your study has been sent to three referees for evaluation and we have now received reports from them, which are enclosed below for your information.

As you can see, while the referees find your work potentially interesting, they also raise major points that need to be addressed before they can support publication in The EMBO Journal. In particular, the reviewers stress the lack of physiological functional data and critical controls and also find that both the nature of the newly identified macrophage population and its relationship with previously described populations would need to be better investigated.

We agree with the referees that these are important points and addressing these and the other reviewers' requests will be essential to pursue publication of this study in The EMBO Journal. Strong support from the referees would also be needed for publication here. Given the overall interest of your study, I would like to invite you to submit a revised version of the manuscript according to the referees' requests. I should add that it is The EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

I realize that addressing all the referees' criticisms will require time and additional efforts that might also be technically challenging. I would therefore understand if you were to choose not to undergo an extensive revision here and rather pursue a submission elsewhere, in which case please inform us about your decision at your earliest convenience.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://emboj.embopress.org/about#Transparent_Process

We generally grant three months as standard revision time. As we are aware that many laboratories cannot function at full capacity owing to the COVID-19 pandemic, we may relax this deadline. Also, we have decided to apply our 'scooping protection policy' to the time span required for you to fully revise your manuscript and address the experimental issues highlighted herein. Nevertheless, please inform us as soon as a paper with related content published elsewhere.

Before submitting your revised manuscript, deposit any primary datasets (and computer code, where appropriate) produced in this study in an appropriate public database (see http://msb.embopress.org/authorguide#dataavailability). Please remember to provide a reviewer password, in case the datasets are not yet public. The accession numbers and database names should be listed in a formal "Data Availability" section (placed after Materials & Method). Provide a "Data availability" section even if there are no primary datasets produced in the study.

Feel free to contact me if you have any questions about the submission of the revised manuscript to The EMBO Journal. I thank you again for the opportunity to consider this work for publication and look forward to your revision.

Referee #1:

Acetylcholine has previously been shown to promote thermogenesis by the same group. The authors found that macrophages are the major population that responds to cold stress and expresses ChAT. Genetic deletion of ChAT selectively in macrophages completely abolished the acetylcholine level induced in SVF and resulted in defective thermogenic gene expression in iWAT. Finally, the authors identify that b2-adrenergic receptor signaling licenses the induction of ChAT in macrophages.

This paper is an interesting follow-up to the previous findings and characterizing a novel neurotransmitter-producing function of adipose tissue resident macrophages will be of interest to the field. Overall the data is strong and experiments are well controlled. One concern is the lack of functional physiological data in the manuscript. Since the authors have previously shown that nicotinic acetylcholine receptor signaling in iWAT affects whole body metabolism (obesity and energy expenditure) these parameters could be explored in the context of macrophage-specific ChAT knockout as well.

Major concerns:

1. The authors should provide functional tests regarding the thermogenic ability of LyzM-cre; ChAT fl/fl mice in CE, particularly the energy expenditure (VO2/VCO2), body temperature, and the authors should provide H&E staining of the iWAT tissue and examine their browning. Also, UCP1 protein levels by western blot in these conditions should be determined.

2. Similarly, it was not addressed in the manuscript whether b2-adrenergic signaling in macrophages controls functional thermogenesis via acetylcholine production. The authors should measure SVF acetylcholine levels in beta-less and b2 agonist conditions.

3. Figure 4M presents an interesting in vitro system to understand macrophage-adipose interaction via acetylcholine. The authors should further examine this direct effect by using different genotypes of SVF cells and fat explants, for example, using LyzM-cre; ChAT fl on the top SVF fraction, and bottom explants from Chrna2 KO mice.

4. From Figure 1G, it appears that only a very small proportion of macrophages are ChAT+ (about 2-4% of all adipose macs). This could be very interesting as it may suggest a functionally dedicated macrophage population for acetylcholine production. The authors should further characterize this specific population, for example, does ChAT+ macrophage highly express ADRB2 (and highly responsive to b2 agonist) as opposed to ChAT- macrophages?

Minor concerns:

1. From Figure 1B, it appears that frequency of ChAT+ macrophages is much lower than T/B cells, although only macrophages increase the proportion of ChAT+ fraction following CE. What proportion of total ChAT+ fraction pre- and post- CE is comprised of macrophages? What are the numbers of ChAT+ macrophage, B and T cells in SVF pre- and post-CE? Does the MFI of ChAT differ in macrophages pre- and post-CE? Examining these data from different perspectives will help understand whether b2-Adrenergic receptor signaling promotes ChAT+ macrophage expansion or

ChAT expression.

2. In Figure 2D, the authors should compare side by side thermogenic gene levels in RT and CE, to have a better idea of whether ChAT ablation in macrophages completely abolishes thermogenic gene induction in CE (or reducing their expression to the same level of RT).

3. In figure 3G,H and EV3, the authors should convert deltaCt value to normalized relative expression values.

4. In figure 3K and figure 4I, J, L, it is not clear how ChAT+ macrophage quantification by flow cytometry was performed. Are these plots obtained by running equal numbers of BMDMs and the histogram was plotted from gated ChAT-GFP+ population? If so, the gating strategy should be clarified in the figure legends. The authors should also quantify the % of ChAT-GFP+ macrophages in total BMDMs, in these conditions.

5. Several typos in figure EV5. C - should be b2 agonist, not antagonist; D - should be BMDM, not ChAT-GFP iWAT.

Referee #2:

The authors follow here on their previous work that has shown that hematopoietic cells residing within the stromal-vascular fraction of subcutaneous fat express choline acetyltransferase (ChAT) and thus serve as a local source of acetylcholine (Jun et al., 2018). Here, using ChATBAC-eGFP reporter model, they report that acetylcholine-synthesizing macrophages reside in subcutaneous fat and that using macrophage-specific (Chatfl/fl;LysM-Cre), T cell-specific (Chatfl/fl;Cd4-Cre) and B cell-specific (Chatfl/fl;Mb1-Cre) loss of ChAT in macrophages, but not in T cell or B cells, compromises the adaptive thermogenic capacity of subcutaneous fat. They finally show that ChAMs link adrenergic signaling to beige fat activation and ChAMs function selectively via activation of the β2-AR. Although interesting, several aspects of the data presented here limit the enthusiasm of the reviewer. See below my comments:

Multiple studies have reported the presence of several macrophage subsets in the adipose tissue with differences in phenotype, gene expression profile and functions. This study adds another one simply based on choline acetyltransferase expression without putting it in parallel with previous published work, which is not correct. The authors need to better understand the nature of such population (tissue-resident, monocyte?) as well as its relationship with previously described populations (See for example Chakarov et al, Science 2019).

On "An adaptive increase in ChAM abundance is evident following acute cold exposure", how is this happening? Local proliferation? Monocyte recruitment?

The claim that Chatfl/fl;LysM-Cre is macrophage-specific is an overstatement as Lyz M is expressed also in monocytes and neutrophils, the latter being ChAT-GFP in the figure EV1 (see the CD11b+CD64-F4/80- cells). The authors need to address this. They also need to show deletion specificity and efficacy controls.

The use of bone marrow-derived macrophages (BMDM) is a concern as the authors did not show any data supporting that these in vitro generated cells are related to their ChAMs beyond

expressing ChAT. How relevant they are to ChAMs?

On Figure EV2A, it is a major concern to see that the authors did not acquire the same amount of cells when comparing WT vs ChAT-eGFP.

Figure 1G flow cytometry data are barely convincing as well as in general flow cytometry plots showing ChAT-eGFP+ profile.
On the various flow cytometry gating strategies for ChAT-eGFP cells and immune cell populations, authors should show absolute numbers with statistical analsyis, not just a plot (ex Figure 3K or Figure 4, I, J and L).
Can the authors clarify that they carefully removed any lymphoid organ from IWAT, VWAT or BAT.

Referee #3:

Summary.
Knights et. al. examine the non-neuronal cholinergic circuitry in adipose tissue and find that 'tissue-resident' macrophages are an essential source of acetylcholine responsible for regulating thermogenic activation in subcutaneous fat. Using a combination of genetic and pharmacological approaches the authors propose that b2-AR activation in a small subset of macrophages results in increased acetylcholine production which in turn controls adaptive thermogenesis in the iWAT. The authors do not however characterize the iWAT or the acetylcholine producing macrophages sufficiently. It is not obvious why the authors claim that ChAT GFP+ macrophages are resident, what these cells express, or where they are located in the adipose tissue. These are particularly important questions as they may explain how a relatively small number of cells may expand in response to b-AR to regulate thermogenic transcripts in the whole iWAT.

Major concerns.
Activation of b-ARs on macrophages increases the number of ChAT GFP+ cells. What contributes to this increase? Is it proliferation of GFP+ cells or expression of ChAT by previously GFP- cells?

Only a very small fraction of macrophages (~3%) express ChAT. How do the authors explain the increase in thermogenic transcripts in whole iWAT? Do the ChAT GFP+ macrophages have a special anatomical location in the iWAT?

Are there any morphological changes associated with increased number of ChAT GFP+ macrophages in the iWAT? Can the authors detect differences in body temperature as the result of blocking ChAT GFP+ macrophage expansion?

Are there ChAT GFP+ macrophages in the iBAT? How do b-AR agonists affect iBAT activity?

Could the authors demonstrate the expression pattern of LysMCre and VavCre in the iWAT? There are at least two VavCre lines, one targets all immune cells while the other targets only bone marrow-derived cells similar to Flt3Cre.

Oftentimes, the data is not represented in a clear manner. For example, in the text the authors claim that "thermogenic gene activation in iWAT was uncompromised during the response to acute cold exposure in T cell and B cell-specific knockouts". However, in Figures 2D,G, and J the qPCR values are not represented on the same graph (or normalized appropriately) as such it is impossible to discern if the expression of Ucp1, Dio2, and other genes are increased in response to cold exposure in the iWAT and iBAT of control mice, or how these changes are altered in LysMCre; ChATf/f mice. The authors current representation precludes the evaluation of critical controls for this experiment.
Additionally, throughout the manuscript the authors switch between representing their qPCR data as delta CT values or as relative mRNA levels. It would simplify the data presentation if the authors would represent all the qPCR results in the same manner as log expression: 2^(-deltaCT).

Minor concerns.
The introduction begins with claiming that adipose tissue has been "long held to be a static site of energy storage". This is somewhat misleading as original descriptions of this tissue have all focused on the dynamic nature of this organ.

Figure legends are not descriptive, e.g. in Figure EV1A it is not clear what the bar graph represents. Also, throughout the manuscript it is not clear how qPCR results were normalized.

In Figure EV2, the authors should provide a FMO for F4/80 and include exclusion markers for neutrophils and eosinophils.

The immunofluorescence images are of low quality. It is difficult to make any conclusions based on the presented images.

Response to the reviewers:

We are very grateful to the reviewers for their insightful comments and constructive suggestions on our manuscript. We have substantially expanded our investigation and experimentally addressed reviewers' concerns. A significant amount of new data was generated and included in the revised manuscript that now includes 4 figures, 5 expanded view figures, 3 expanded view tables and 1 expanded view movie.

Key points made to the revised manuscript are as follows:

1. Physiological significance of acetylcholine-producing macrophage-mediated thermogenesis.

(a) We examined thermogenic function of inguinal adipose tissues from cold-exposed control (*ChAT*$^{fl/fl}$) and macrophage-specific ChAT-deleted (*ChAT*$^{fl/fl}$;*LysM-Cre*) mice. Cold-induced activation of UCP1 protein and respiration was significantly lower in inguinal fat of *ChAT*$^{fl/fl}$;*LysM-Cre* mice than that of *ChAT*$^{fl/fl}$ controls.

(b) Systemic energy expenditure was evaluated in *ChAT*$^{fl/fl}$ and *ChAT*$^{fl/fl}$;*LysM-Cre* mice at room or cold temperature. While whole-body oxygen consumption rate was comparable between genotypes at room temperature, it was lower in *ChAT*$^{fl/fl}$;*LysM-Cre* mice than controls under acute cold exposure.

Therefore, inguinal thermogenic defects caused by blocking acetylcholine production in macrophages render compromised energy balance upon cold exposure.

2. Comprehensive characterization of acetylcholine-producing macrophages responsible for beige thermogenesis.

(a) Fate mapping approaches using a newly generated reporter mouse (ChAT-eGFP;*Cx3cr1*^{CreER}-RFP) revealed that the majority of acetylcholine-synthesizing macrophages in subcutaneous fat are tissue-resident, with some contribution from bone marrow-derived cells (infiltrating).

(b) We profiled the distribution of ChAT-expressing immune cells in inguinal adipose tissue with or without lymph node, and in lymph node alone. $ChAT^+$ immune cells were widely distributed throughout the whole inguinal adipose tissue. Acetylcholine-synthesizing macrophages were mainly present in the inguinal adipose tissue rather than the inguinal lymph node.

(c) We carried out RNA-seq using $ChAT^-$ and $ChAT^+$ macrophages from cold-exposed ChAT-eGFP mice. The transcriptome signature analysis indicated that activated $ChAT^+$ macrophages have enriched expression of genes encoding proteins regulating neurotransmitter signaling.

Therefore, our expanded study revealed a much more comprehensive characterization of this functionally distinct macrophage subpopulation that secretes acetylcholine in response to thermogenic cues in subcutaneous fat and contribute to energy dissipation.

Changes to the manuscript are highlighted in yellow. We have restructured some parts of the figures and some figure panels have now been moved in expanded view figures to make figures flow better. For clarity, we did not highlight individual reference number changes or figure panel changes due to restructuring of the text and the figures. As requested, we have converted deltaCt values to normalized relative expression throughout the manuscript. Additionally, histogram representations of BMDM flow cytometry have been removed throughout and these data are now presented with bar figures for clearer demonstration of quantification. When necessary, additional experiments were carried out to increase n number to enable appropriate statistical analysis. We also included the percentage of ChAT-$eGFP^+$ BMDMs in additional to total number as requested. Because flow cytometry experiments were performed on an equal number of events (cells), the number of ChAT-$eGFP^+$ cells will be directly proportional to the percentage of ChAT-$eGFP^+$ cells, similar appearance graphs were obtained in these experiments.

Our point-by-point responses to the reviewers' comments are below:

Referee #1:

Acetylcholine has previously been shown to promote thermogenesis by the same group. The authors found that macrophages are the major population that responds to cold stress and expresses ChAT. Genetic deletion of ChAT selectively in macrophages completely abolished the acetylcholine level induced in SVF and resulted in defective thermogenic gene expression in iWAT. Finally, the authors identify that b2-adrenergic receptor signaling licenses the induction of ChAT in macrophages.

This paper is an interesting follow-up to the previous findings and characterizing a novel neurotransmitter-producing function of adipose tissue resident macrophages will be of interest to the field. Overall the data is strong and experiments are well controlled. One concern is the lack of functional physiological data in the manuscript. Since the authors have previously shown that nicotinic acetylcholine receptor signaling in iWAT affects whole body metabolism (obesity and energy expenditure) these parameters could be explored in the context of macrophage-specific ChAT knockout as well.

We thank this reviewer for their nice comments about our manuscript and raising important points. We characterized the metabolic phenotypes of macrophage-specific ChAT deleted mice under cold at the tissue and whole-body levels.

Major concerns:

1. The authors should provide functional tests regarding the thermogenic ability of LyzM-cre; ChAT fl/fl mice in CE, particularly the energy expenditure (VO2/VCO2), body temperature, and the authors should provide H&E staining of the iWAT tissue and examine their browning. Also, UCP1 protein levels by western blot in these conditions should be determined.

We agree with the reviewer that functional assessment would strengthen our conclusions.

(a) We analyzed thermogenic function of inguinal adipose tissues (IWAT) from cold-exposed *ChAT*$^{fl/fl}$ control and *ChAT*$^{fl/fl}$;*LysM-Cre* mice. *ChAT*$^{fl/fl}$;*LysM-Cre* mice exhibited impaired

activation of UCP1 protein in IWAT after acute cold exposure (CE) at 4°C for 6 h. UCP1 protein expression was detectable in IWAT of *ChAT*$^{fl/fl}$ controls, whereas not in that of *ChAT*$^{fl/fl}$;*LysM-Cre* mice (Fig 2I). With this, thermogenic defects caused by macrophage-specific ChAT deletion were demonstrated at the mRNA and protein levels.

(b) We adopted acute cold exposure to activate immune ChAT signaling and beige thermogenesis, however it is not sufficient to induce IWAT remodeling detectable by H&E staining. Thus, we did not include H&E stained IWAT images of ChAT knockout mice exposed to cold. Alternatively, we analyzed IWAT oxygen consumption rate (OCR) to examine the consequence of ChAT deficiency at the functional level in IWAT. *ChAT*$^{fl/fl}$ and *ChAT*$^{fl/fl}$;*LysM-Cre* mice were kept at 4°C for 6 h and treated with 1 mg/kg formoterol for 30 min to amplify β_2 AR-dependent ChAT signaling under cold. Basal and oligomycin-insensitive OCR of IWAT was significantly lower in *ChAT*$^{fl/fl}$;*LysM-Cre* mice than *ChAT*$^{fl/fl}$ controls after cold challenge with β_2 AR agonist treatment (Fig 2J).

(c) We evaluated systemic energy metabolism in *ChAT*$^{fl/fl}$ and *ChAT*$^{fl/fl}$;*LysM-Cre* mice at room temperature (RT) and 4°C for 6 h using CLAMS. While whole-body OCR and energy expenditure were comparable between genotypes at RT, they were lower in *ChAT*$^{fl/fl}$;*LysM-Cre* mice than controls under cold exposure (Fig 2K and EV3D). However, no differences were detected in physical activity and gene expression of muscle shivering thermogenic markers between genotypes at both RT and cold (Fig EV3E and F). We also measured core body temperature of *ChAT*$^{fl/fl}$ and *ChAT*$^{fl/fl}$;*LysM-Cre* mice after 6 h CE using a rectal probe, however a snapshot of body temperature did not show differences between genotypes (Fig EV3C).

These further functional investigations greatly strengthened our hypothesis that the inguinal thermogenic defects caused by blocking acetylcholine production from macrophages renders compromised systemic energy balance upon cold.

2. Similarly, it was not addressed in the manuscript whether b2-adrenergic signaling in macrophages controls functional thermogenesis via acetylcholine production. The authors should measure SVF acetylcholine levels in beta-less and b2 agonist conditions.

To test β_2 adrenergic receptor (AR)-dependent acetylcholine secretion from inguinal stromal vascular fraction (SVF), we analyzed acetylcholine levels in PBS incubated with inguinal SVF

from β_2WT or β_2KO mice treated with 1 mg/kg β_2 AR agonist formoterol for 2 h. The elevation of acetylcholine secretion after formoterol treatment was only seen in inguinal SVF of β_2WT mice, whereas not in that of β_2KO mice. These new data were included in the revised manuscript (Fig 4F).

3. Figure 4M presents an interesting in vitro system to understand macrophage-adipose interaction via acetylcholine. The authors should further examine this direct effect by using different genotypes of SVF cells and fat explants, for example, using LyzM-cre; ChAT fl on the top SVF fraction, and bottom explants from Chrna2 KO mice.

We further experimentally expanded our investigation into the interaction between acetylcholine-producing macrophages and beige adipocytes. To confirm β_2 AR-dependent activated ChAT signaling in macrophages can activate the thermogenic response of neighboring beige adipocytes, we co-cultured inguinal SVF cells from β_2KO or *ChAT*$^{fl/fl}$;*LysM-Cre* mice with IWAT explants from β-less mice in the absence or presence of the β_2 AR agonist formoterol. Induction of *Ucp1* gene expression by formoterol was seen in co-culture with IWAT SVF cells from WT mice, whereas not when β_2 AR or ChAT was absent (Fig EV5M). Therefore, these data highlight that macrophages activated by β_2 AR signaling communicate with beige adipocytes via acetylcholine to mediate thermogenesis.

It has previously been reported that β_2 AR agonist may directly activate thermogenic fat via a PRDM16 dependent mechanism (Ohyama *et al*, 2016). To differentially demonstrate the cause for the activation of thermogenic genes in the IWAT explant in the lower chamber of our coculture system, we intentionally used IWAT from β-less mice, which will not respond to β_2 AR agonist but only acetylcholine from the upper chamber. Along this line, it would be difficult to interpret the results from experiments using explant IWAT from Chrna2 KO mice, since direct response to β_2 AR agonist through PRDM16 was still expected. Therefore it is not included in the present study.

4. From Figure 1G, it appears that only a very small proportion of macrophages are ChAT+ (about 2-4% of all adipose macs). This could be very interesting as it may suggest a functionally dedicated macrophage population for acetylcholine production. The authors should further characterize this specific population, for example, does ChAT+ macrophage highly express

ADRB2 (and highly responsive to b2 agonist) as opposed to ChAT- macrophages?

We are grateful to the reviewer for these comments. We have now extended our characterization of the ChAT-expressing macrophage population in subcutaneous fat. Cholinergic adipose macrophages are distributed throughout subcutaneous fat, but are largely absent from the inguinal lymphoid tissue (Fig 1C). Bulk RNA sequencing analysis of ChAT-eGFP^{+} macrophages revealed strong enrichment for pathways related to neurotransmitter transport/secretion and adrenergic signaling, compared to ChAT-eGFP^{-} macrophages. Genes necessary for acetylcholine signaling were more highly expressed in ChAT^{+} macrophages, providing evidence for a defined functional niche of ChAMs in subcutaneous adipose tissue (Fig 1K-M and EV2J).

Minor concerns:

1. From Figure 1B, it appears that frequency of ChAT+ macrophages is much lower than T/B cells, although only macrophages increase the proportion of ChAT+ fraction following CE. What proportion of total ChAT+ fraction pre- and post- CE is comprised of macrophages? What are the numbers of ChAT+ macrophage, B and T cells in SVF pre- and post-CE? Does the MFI of ChAT differ in macrophages pre- and post-CE? Examining these data from different perspectives will help understand whether b2-Adrenergic receptor signaling promotes ChAT+ macrophage expansion or ChAT expression.

These are important considerations, and we have now updated our figures to include these data. As shown now in Fig 1I, ChAT-eGFP^{+} macrophages are of a higher proportion of total ChAT-eGFP^{+} cells in IWAT during cold exposure, compared to room temperature. This is reflected in the total number of ChAT-eGFP^{+} macrophages too, with no discernable changes in the lymphocyte ChAT-eGFP^{+} fractions, or other CD45^{+} ChAT^{+} cells following cold (Fig EV2F-H). In addition, ChAT-eGFP MFI is higher in macrophages following cold exposure (Fig 1I).

2. In Figure 2D, the authors should compare side by side thermogenic gene levels in RT and CE, to have a better idea of whether ChAT ablation in macrophages completely abolishes thermogenic gene induction in CE (or reducing their expression to the same level of RT).

This is a very good suggestion. Accordingly, Figure 2E (originally Fig 2D) now includes a side-by-side comparison of the effects of macrophage-specific ChAT deficiency in IWAT at RT and cold. Cold-induced transcriptional activation of thermogenic genes was significantly lower in IWAT of *ChAT*$^{fl/fl}$;*LysM-Cre* mice compared to that of *ChAT*$^{fl/fl}$ controls. During revision, we thoroughly went through all original data files and noticed that Ppargc1a and Dio2 was switched in the original figure (Fig. 2D, CE). We have corrected it in the revised figure (now Fig 2E). We apologize for the mistake. Additionally, the figures that present gene expression analyses of IWAT of T cell or B cell ChAT knockout mice and BAT from these three models have been reformatted to have thermogenic gene expression levels side by side at RT and CE (Fig 2F, 2G, 2L, Fig EV3G, 3H).

3. In figure 3G,H and EV3, the authors should convert deltaCt value to normalized relative expression values.

As requested, we have converted deltaCt values to normalized relative expression throughout the manuscript. When necessary, additional experiments were carried out to increase n number to enable statistical analysis. Relevant figures in the revised manuscript are Fig 3J, Fig 3K, Fig EV2K, Fig EV2L and EV5L.

4. In figure 3K and figure 4I, J, L, it is not clear how ChAT+ macrophage quantification by flow cytometry was performed. Are these plots obtained by running equal numbers of BMDMs and the histogram was plotted from gated ChAT-GFP+ population? If so, the gating strategy should be clarified in the figure legends. The authors should also quantify the % of ChAT-GFP+ macrophages in total BMDMs, in these conditions.

For all BMDM flow cytometry data, an equal number (50,000) of BMDMs, as defined by the gating strategy featured in Fig EV4B, were analyzed to generate total abundances of ChAT-eGFP^{+} BMDMs. We have clarified this in the figure legend for each relevant figure panel (Fig 3, 4, EV4 and EV5). In addition to total numbers, we have now included frequency (%) of ChAT-eGFP^{+} BMDMs corresponding to each figure panel. To increase clarity, following the advice of the reviewers (#1 and #2), histogram representations of BMDM flow cytometry have been removed throughout and these data are now presented with bar graphs for clearer demonstration of quantification. When necessary, additional experiments were carried out to increase n number to enable statistical analysis.

5. Several typos in figure EV5. C - should be b2 agonist, not antagonist; D - should be BMDM, not ChAT-GFP iWAT.

Thank you for noticing these – we apologize for these mistakes and have corrected them and carefully proofread the revised manuscript.

Referee #2:

The authors follow here on their previous work that has shown that hematopoietic cells residing within the stromal-vascular fraction of subcutaneous fat express choline acetyltransferase (ChAT) and thus serve as a local source of acetylcholine (Jun et al., 2018). Here, using ChATBAC-eGFP reporter model, they report that acetylcholine-synthesizing macrophages reside in subcutaneous fat and that using macrophage-specific (Chatfl/fl;LysM-Cre), T cell-specific (Chatfl/fl;Cd4-Cre) and B cell-specific (Chatfl/fl;Mb1-Cre) loss of ChAT in macrophages, but not in T cell or B cells, compromises the adaptive thermogenic capacity of subcutaneous fat. They finally show that ChAMs link adrenergic signaling to beige fat activation and ChAMs function selectively via activation of the β2-AR. Although interesting, several aspects of the data presented here limit the enthusiasm of the reviewer. See below my comments:

Further studies have been carried out to experimentally address the concerns raised by this reviewer.

1. Multiple studies have reported the presence of several macrophage subsets in the adipose tissue with differences in phenotype, gene expression profile and functions. This study adds another one simply based on choline acetyltransferase expression without putting it in parallel with previous published work, which is not correct. The authors need to better understand the nature of such population (tissue-resident, monocyte?) as well as its relationship with previously described populations (See for example Chakarov et al, Science 2019).

We thank the reviewer for encouraging further characterization and contextualization of acetylcholine-synthesizing adipose macrophages. Our RNA-seq data revealed that ChAMs exhibit a distinct gene expression and pathway enrichment profile for neurotransmitter regulation, acetylcholine signaling and adrenergic receptor signaling (Fig 1K-M, EV2J). Comparative analysis with other described populations, including Lyve1$^{hi/lo}$/MHC$^{hi/lo}$/Cx3cr1$^{hi/lo}$ interstitial macrophages (Chakarov *et al*, 2019), Trem2^{+} lipid-associated macrophages (Jaitin *et al*, 2019), Ly6C and CD9 macrophages in obese adipose tissue (Hill *et al*, 2018) and sympathetic neuron-associated macrophages (Pirzgalska *et al*, 2017) yielded no definitive overlap between ChAMs and other published tissue macrophages subtypes. Future studies will further demonstrate how ChAMs may functionally interact with other cell types within the same tissue niche, including other functionally distinct macrophages. However, these may fall beyond

the scope of current study. We have incorporated these important points in our discussions. (page 13).

2. On "An adaptive increase in ChAM abundance is evident following acute cold exposure", how is this happening? Local proliferation? Monocyte recruitment?

Upon cold exposure, we saw increased proliferation (Ki67) and a corresponding increase in the total number of cholinergic macrophages, while ChAT expression (MFI) in cholinergic macrophages was also elevated in cold (Fig 1I-J). Using the inducible fate-mapping model ChAT-eGFP;*Cx3cr1*^{CreER}-RFP, we demonstrated that less than a quarter of ChAT-eGFP^{+} macrophages are labeled RFP^{+} (Fig 3G-I), providing evidence that these cells are predominantly, but not exclusively, tissue-resident. To reflect these conceptual findings, we have removed the terminology 'tissue-resident' from the abstract (strikethrough and yellow highlight).

3. The claim that Chatfl/fl;LysM-Cre is macrophage-specific is an overstatement as Lyz M is expressed also in monocytes and neutrophils, the latter being ChAT-GFP in the figure EV1 (see the CD11b+CD64-F4/80- cells). The authors need to address this. They also need to show deletion specificity and efficacy controls.

We appreciate this comment, and acknowledge the potential caveats of *LysM-Cre* for highly specific deletion in macrophages. In response to this comment, and that of reviewer #3 (comment #5), we performed detailed profiling of *LysM-Cre* specificity and efficacy in IWAT using ChAT-eGFP;*LysM-Cre*-RFP mice (Fig EV3A-B). We observed RFP^{+} labeling in several myeloid cell types, as expected from prior studies in other tissues (Abram *et al*, 2014; Shi *et al*, 2018). RFP^{+} labeling was most effective in IWAT macrophages (~95% RFP^{+}), with ~75% of neutrophils labeled. Importantly, we also used this mouse line to demonstrate that *LysM-Cre* is highly effective at deleting in ChAT-eGFP^{+} macrophages (Fig EV3B), and showed a complete absence of ChAT-expressing neutrophils and eosinophils (Fig EV1E). Thus, despite *LysM-Cre* exhibiting varying specificities towards non-macrophage myeloid cell types, the absence of ChAT in these cells (and highly effective labeling of ChAT+ macrophages) render this a useful and informative model for our study's purposes.

4. The use of bone marrow-derived macrophages (BMDM) is a concern as the authors did not

show any data supporting that these in vitro generated cells are related to their ChAMs beyond expressing ChAT. How relevant they are to ChAMs?

We concede the notion that BMDMs may not completely represent primary tissue macrophages and these cells can only be used as a proxy for undertaking mechanistic studies *in vitro* with potential caveats (Li *et al*, 2019). That being said, we did detect *Chat* transcript in BMDMs and identified a subpopulation of ChAT-eGFP^{+} BMDMs by flow cytometry (Fig EV4B). BMDMs, like primary IWAT macrophages, highly express the β_2-AR, with minimal relative expression of β_1-AR and β_3-AR (Fig 3J and K). We showed that ChAT is induced in BMDMs through activation of the β_2-AR, in the same manner as β_2 agonism increases ChAT expression in primary macrophages isolated from IWAT (Fig 4G-M). Together, these data do suggest the data from BMDMs provided some supportive evidence for the molecular insights into how ChAT is regulated in macrophages, as long as these observations were further validated by other systems.

5. On Figure EV2A, it is a major concern to see that the authors did not acquire the same amount of cells when comparing WT vs ChAT-eGFP.

We agree with this reviewer and have replaced this misleading figure with new data. This figure with an equal starting number of cells for the WT and ChAT-eGFP sample is now Fig EV1B.

6. Figure 1G flow cytometry data are barely convincing as well as in general flow cytometry plots showing ChAT-eGFP+ profile. On the various flow cytometry gating strategies for ChAT-eGFP cells and immune cell populations, authors should show absolute numbers with statistical analsyis, not just a plot (ex Figure 3K or Figure 4, I, J and L).

We have made several improvements to our flow cytometry data presentation throughout the manuscript in order to address these valid concerns. For flow cytometry experiments, graphs showing total cell numbers as well as graphs showing percentages have been included (with mean ± SEM and accompanying statistical analyses), and n number has been increased to permit appropriate statistical analyses when necessary. The applicable graphs can be found in Figures 1, 3, 4 and EV2-5. Further, we have included gating schematics with FMO controls in Figures EV1 and EV4 to provide more clarity on our gating strategies.

7. Can the authors clarify that they carefully removed any lymphoid organ from IWAT, VWAT or BAT.

Our standard protocol does not involve removal of the lymph node. To assess the contribution of lymphoid tissue to the ChAT-eGFP$^+$ profile, we microdissected out and digested the inguinal lymph node using an established protocol that the Maillard lab has extensive experience with (Chung *et al*, 2017). We compared resident ChAT-eGFP$^+$ cell types to the IWAT depot in the presence or absence of the lymph node. By and large, ChAT-eGFP$^+$ cells distribute throughout the depot with the exception that ChAT-eGFP$^+$ macrophages were more prominent in the fat rather than the lymph node (Fig 1C).

Given the very low number of ChAT$^+$ cells in VWAT and BAT, even with the lymph node in the fat tissue (original Fig 1C, new Fig 1D), we did not further characterize the distribution in these two depots in this regard.

Referee #3:

Summary.
Knights et. al. examine the non-neuronal cholinergic circuitry in adipose tissue and find that 'tissue-resident' macrophages are an essential source of acetylcholine responsible for regulating thermogenic activation in subcutaneous fat. Using a combination of genetic and pharmacological approaches the authors propose that b2-AR activation in a small subset of macrophages results in increased acetylcholine production which in turn controls adaptive thermogenesis in the iWAT. The authors do not however characterize the iWAT or the acetylcholine producing macrophages sufficiently. It is not obvious why the authors claim that ChAT GFP+ macrophages are resident, what these cells express, or where they are located in the adipose tissue. These are particularly important questions as they may explain how a relatively small number of cells may expand in response to b-AR to regulate thermogenic transcripts in the whole iWAT.

We thank this reviewer's constructive suggestions and have significantly expanded our investigation.

Major concerns.

1. Activation of b-ARs on macrophages increases the number of ChAT GFP+ cells. What contributes to this increase? Is it proliferation of GFP+ cells or expression of ChAT by previously GFP- cells?

This is an important consideration when studying diverse and dynamic immune cell types such as macrophages. Using flow cytometry, we found that cold temperature induces both an increase in expression (MFI) of ChAT in adipose macrophages and also an increase in number of ChAT-eGFP^{+} macrophages (Fig 1I), which was corroborated by our observation that Ki67 is higher in ChAT^{+} macrophages following cold (Fig 1J and EV2I). Together, these data provide evidence that ChAT is up-regulated in cholinergic macrophages, and that these cells undergo proliferation, in response to cold.

2. Only a very small fraction of macrophages (~3%) express ChAT. How do the authors explain the increase in thermogenic transcripts in whole iWAT? Do the ChAT GFP+ macrophages have a special anatomical location in the iWAT?

We found that ChAT-eGFP^{+} macrophages are dispersed throughout the subcutaneous adipose tissue (Fig 1A, 1C, Fig EV1A and Movie EV1), and less abundant in the lymph node (Fig 1C). The distribution within the fat depot and vicinity to the thermogenic fat cells enable acetylcholine secreted from these ChAMs to conveniently act on neighboring beige adipocytes, which express CHRNA2, the receptor to acetylcholine. Blocking acetylcholine synthesis in macrophages completely ablates any cold-induced increase in acetylcholine secretion in IWAT (original Fig 1H, new Fig 2H), which is important for thermogenic activation. Given this, we postulate that the absence of acetylcholine production by macrophages impairs the ability of adipocytes in the IWAT to activate their expression of important beige adipocyte genes like *Ucp1* and *Dio2* in response to environmental cues (Fig 2E) and oxygen consumption within IWAT and in the whole body (Fig 2J, 2K).

3. Are there any morphological changes associated with increased number of ChAT GFP+ macrophages in the iWAT? Can the authors detect differences in body temperature as the result of blocking ChAT GFP+ macrophage expansion?

Please see detailed discussion in response to question #1 from reviewer #1.

Because acute cold exposure is not sufficient to induce IWAT remodeling detectable by H&E staining, we alternatively analyzed the metabolic phenotypes of *ChAT*$^{fl/fl}$ and *ChAT*$^{fl/fl}$;*LysM-Cre* mice in IWAT by assessing UCP1 protein levels and tissue oxygen consumption rate (OCR). We also evaluated systemic energy metabolism of *ChAT*$^{fl/fl}$ and *ChAT*$^{fl/fl}$;*LysM-Cre* mice by measuring core body temperature and monitoring whole-body OCR using CLAMS. These data now included in Fig 2I-2K, Fig EV3C, 3D.

4. Are there ChAT GFP+ macrophages in the iBAT? How do b-AR agonists affect iBAT activity?

Very few ChAT-eGFP^{+} macrophages were observed in interscapular BAT (Fig 1D, Fig EV5B). To study the effect of β-AR agonism, we treated ChAT-eGFP mice with the β_2 AR agonist formoterol but found no induction of ChAT-eGFP^{+} cells (including macrophages) as we observed in IWAT (Fig EV5A-B). This is consistent with our previous report that CHRNA2 signaling mediates a beige fat selective response that is predominantly present in IWAT, but not in classical brown fat in the interscapular depot (Jun *et al*, 2018).

5. Could the authors demonstrate the expression pattern of LysMCre and VavCre in the iWAT? There are at least two VavCre lines, one targets all immune cells while the other targets only bone marrow-derived cells similar to Flt3Cre.

To better characterize the *LysM-Cre* and *Vav-iCre* drivers in IWAT, we generated ChAT-eGFP;*LysM-Cre*-RFP and ChAT-eGFP;*Vav-iCre*-RFP mice. This allowed us to assess specificity and efficiency of recombination (RFP^{+} labeling) in IWAT-resident cell types, and also to analyze labeling in ChAT-eGFP^{+} cells.

Vav-iCre was highly specific to CD45^{+} IWAT cells, including T cells, B cells and macrophages (Fig EV2A-B). It was also highly effective at labeling ChAT-eGFP^{+} cell types, which we have shown are >98% CD45^{+} (Fig EV2C-D). *LysM-Cre* was broadly specific across the myeloid cell types, with the highest proportion of labeling in IWAT macrophages, followed by neutrophils and a lesser proportion of dendritic cells and eosinophils (Fig EV3A). Importantly, *LysM*-Cre was highly effective at labeling ChAT-eGFP^{+} macrophages (Fig EV3B). It is worth noting that ChAT-

eGFP^{+} neutrophils and eosinophils were not detected in IWAT (Fig EV1E, Fig EV3B), consistent with our previous report (Jun *et al.*, 2018).

6. Oftentimes, the data is not represented in a clear manner. For example, in the text the authors claim that "thermogenic gene activation in iWAT was uncompromised during the response to acute cold exposure in T cell and B cell-specific knockouts". However, in Figures 2D,G, and J the qPCR values are not represented on the same graph (or normalized appropriately) as such it is impossible to discern if the expression of Ucp1, Dio2, and other genes are increased in response to cold exposure in the iWAT and iBAT of control mice, or how these changes are altered in LysMCre; ChATf/f mice. The authors current representation precludes the evaluation of critical controls for this experiment.
Additionally, throughout the manuscript the authors switch between representing their qPCR data as delta CT values or as relative mRNA levels. It would simplify the data presentation if the authors would represent all the qPCR results in the same manner as log expression: 2^(-deltaCT).

To better represent and improve interpretability of qPCR data assessing thermogenic activation, we have updated Figures 2E, 2F, 2G, 2L, EV2G and EV2H to include a side-by-side comparison of the effects of ChAT deficiency in IWAT or BAT at RT and CE. With respect to representation of qPCR data, we have converted deltaCt values to normalized relative expression (2^{-ddCt}) throughout the manuscript. Relevant figures in the revised manuscript are Fig 3J, Fig 3K, Fig EV2K, Fig EV2L and EV5L.

Minor concerns.
1. The introduction begins with claiming that adipose tissue has been "long held to be a static site of energy storage". This is somewhat misleading as original descriptions of this tissue have all focused on the dynamic nature of this organ.

We have now changed the wording and tone of this sentence to say “Adipose tissue is a dynamic endocrine organ known to actively function in response to environmental and endogenous cues to regulate systemic metabolism and energy expenditure”.

2. Figure legends are not descriptive, e.g. in Figure EV1A it is not clear what the bar graph represents. Also, throughout the manuscript it is not clear how qPCR results were normalized.

We have improved the clarity of descriptions provided in figure legends throughout the manuscript, in accordance with figure caption guidelines from EMBO Journal. A clause has been added to the legend for each relevant figure panel describing qPCR results, stating how expression was normalized and what method was utilized (2^{-ddCT}).

3. In Figure EV2, the authors should provide a FMO for F4/80 and include exclusion markers for neutrophils and eosinophils.

A revised comprehensive gating strategy figure has now been included with FMOs (now Fig EV1). We have also incorporated markers for neutrophils and eosinophils, with analysis demonstrating the absence of ChAT-eGFP^{+} neutrophils and eosinophils in IWAT (Fig EV1E), consistent with our previous report (Jun *et al.*, 2018).

4 .The immunofluorescence images are of low quality. It is difficult to make any conclusions based on the presented images.

We have now included higher resolution Adipo-clear immunofluorescence images (Fig EV1A).

Response letter reference list

Abram CL, Roberge GL, Hu Y, Lowell CA (2014) Comparative analysis of the efficiency and specificity of myeloid-Cre deleting strains using ROSA-EYFP reporter mice. *J Immunol Methods* 408: 89-100

Chakarov S, Lim HY, Tan L, Lim SY, See P, Lum J, Zhang XM, Foo S, Nakamizo S, Duan K *et al* (2019) Two distinct interstitial macrophage populations coexist across tissues in specific subtissular niches. *Science* 363: eaau0964

Chung J, Ebens CL, Perkey E, Radojcic V, Koch U, Scarpellino L, Tong A, Allen F, Wood S, Feng J *et al* (2017) Fibroblastic niches prime T cell alloimmunity through Delta-like Notch ligands. *J. Clin. Investig* 127: 1574-1588

Hill DA, Lim HW, Kim YH, Ho WY, Foong YH, Nelson VL, Nguyen HCB, Chegireddy K, Kim J, Habertheuer A *et al* (2018) Distinct macrophage populations direct inflammatory versus physiological changes in adipose tissue. *Proc Natl Acad Sci U S A* 115: E5096-E5105

Jaitin DA, Adlung L, Thaiss CA, Weiner A, Li B, Descamps H, Lundgren P, Bleriot C, Liu Z, Deczkowska A *et al* (2019) Lipid-Associated Macrophages Control Metabolic Homeostasis in a Trem2-Dependent Manner. *Cell* 178: 686-698 e614

Jun H, Yu H, Gong J, Jiang J, Qiao X, Perkey E, Kim DI, Emont MP, Zestos AG, Cho JS *et al* (2018) An immune-beige adipocyte communication via nicotinic acetylcholine receptor signaling. *Nat Med* 24: 814-822

Li C, Menoret A, Farragher C, Ouyang Z, Bonin C, Holvoet P, Vella AT, Zhou B (2019) Single cell transcriptomics based-MacSpectrum reveals novel macrophage activation signatures in diseases. *JCI Insight* 4: e126453

Ohyama K, Nogusa Y, Shinoda K, Suzuki K, Bannai M, Kajimura S (2016) A Synergistic Antiobesity Effect by a Combination of Capsinoids and Cold Temperature Through Promoting Beige Adipocyte Biogenesis. *Diabetes* 65: 1410-1423

Pirzgalska RM, Seixas E, Seidman JS, Link VM, Sanchez NM, Mahu I, Mendes R, Gres V, Kubasova N, Morris I *et al* (2017) Sympathetic neuron-associated macrophages contribute to obesity by importing and metabolizing norepinephrine. *Nat Med* 23: 1309-1318

Shi J, Hua L, Harmer D, Li P, Ren G (2018) Cre Driver Mice Targeting Macrophages. *Methods Mol Biol* 1784: 263-275

1st Revision - Editorial Decision 5th Jul 2021

Thank you for submitting your revised study. Please accept my apologies for the delay in getting back to you with our decision. The manuscript has been sent back to the original referees, but referee #3 did not accept to re-review it. We thus have obtained only two reports, which are appended below for your information.

As you can see, while referee #1 finds that his/her criticisms have been adequately addressed and recommends the study for publication, referee #2 still has major issues about the study. In particular, this reviewer stressed that the nature of ChAM population remains unclear and that you did not perform the requested side-by-side bioinformatic comparison. Also, s/he states that how acute cold exposure increases ChAM abundance would need to be further characterized.

Given these divergent opinions, we contacted and independent expert in the field, who finds that further characterisation of the nature of the ChAM population, as well as how cold exposure increases ChAM abundance would be out of the scope of this study. Nevertheless, the advisor recommends that you perform a side-by-side bioinformatic comparison using published datasets as requested by referee #2.

In addition, there are few editorial issues concerning the text and the figures that I need you to address before we can officially accept your manuscript.

Referee #1:

The authors have addressed my concerns. I recommend acceptance.

Referee #2:

The authors did not address the concerns that were raised. See comments below:

1. Multiple studies have reported the presence of several macrophage subsets in the adipose tissue with differences in phenotype, gene expression profile and functions. This study adds another one simply based on choline acetyltransferase expression without putting it in parallel with previous published work, which is not correct. The authors need to better understand the nature of such population (tissue-resident, monocyte?) as well as its relationship with previously described populations (See for example Chakarov et al, Science 2019).

"We thank the reviewer for encouraging further characterization and contextualization of acetylcholine-synthesizing adipose macrophages. Our RNA-seq data revealed that ChAMs exhibit a distinct gene expression and pathway enrichment profile for neurotransmitter regulation, acetylcholine signaling and adrenergic receptor signaling (Fig 1K-M, EV2J). Comparative analysis with other described populations, including Lyve1hi/lo/MHChi/lo/Cx3cr1hi/lo interstitial macrophages (Chakarov et al, 2019), Trem2+ lipid-associated macrophages (Jaitin et al, 2019), Ly6C and CD9 macrophages in obese adipose tissue (Hill et al, 2018) and sympathetic neuron-associated macrophages (Pirzgalska et al, 2017) yielded no definitive overlap between ChAMs and other published tissue macrophages subtypes. Future studies will further demonstrate how ChAMs may functionally interact with other cell types within the same tissue niche, including other functionally distinct macrophages. However, these may fall beyond the scope of current study. We have incorporated these important points in our discussions. (page 13)."

The authors did not performed a side by side bioinformatic comparison as requested. They could have used published datasets and compared them to their data.

2. On "An adaptive increase in ChAM abundance is evident following acute cold exposure", how is this happening? Local proliferation? Monocyte recruitment?

"Upon cold exposure, we saw increased proliferation (Ki67) and a corresponding increase in the total number of cholinergic macrophages, while ChAT expression (MFI) in cholinergic macrophages was also elevated in cold (Fig 1I-J). Using the inducible fate-mapping model ChAT-eGFP;Cx3cr1CreER-RFP, we demonstrated that less than a quarter of ChAT-eGFP+ macrophages are labeled RFP+ (Fig 3G-I), providing evidence that these cells are predominantly, but not exclusively, tissue-resident. To reflect these conceptual findings, we have removed the terminology 'tissue-resident' from the abstract (strikethrough and yellow highlight)."

Demonstrating that ChAT-eGFP+ are labeled using the inducible fate-mapping model ChAT-eGFP;Cx3cr1CreER-RFP after 7 days tamoxifen cannot allow to answer on tissue residence nor origins. The labelling could be a mix of monocyte contribution (labelled by tamoxifen) and recombination due to intrinsic CX3CR1 expression. The time frame of this experiment does not allow to answer such question. The authors should label for 5-6 days and then investigate the recombination months after labeling (and not 7 days) and observe for decay or not. Ultimately, they should label during embryonic development to assess embryonic origin and not monocyte origin but this is beyond the scope of the study.

7. Can the authors clarify that they carefully removed any lymphoid organ from IWAT, VWAT or BAT.

"Our standard protocol does not involve removal of the lymph node. To assess the contribution of lymphoid tissue to the ChAT-eGFP+ profile, we microdissected out and digested the inguinal lymph node using an established protocol that the Maillard lab has extensive experience with (Chung et al, 2017). We compared resident ChAT-eGFP+ cell types to the IWAT depot in the presence or absence of the lymph node. By and large, ChAT-eGFP+ cells distribute throughout the depot with the exception that ChAT-eGFP+ macrophages were more prominent in the fat rather than the lymph node (Fig 1C). Given the very low number of ChAT+ cells in VWAT and BAT, even with the lymph node in the fat tissue (original Fig 1C, new Fig 1D), we did not further characterize the distribution in these two depots in this regard."

I do not understand why the lymph nodes are not removed when authors are studying fat macrophages.... This is very puzzling and concerning.

We thank the editor and the reviewers for their constructive comments and suggestions. We included additional bioinformatics analysis and addressed all concerns raised individually. We feel the manuscript is further improved and would be of great interest to the readership of EMBO Journal. All updates to the manuscript text have been highlighted in yellow.

Our point-by-point response to the comments are as follows:

Editorial:

-> Indicate the number of replicates used for calculating statistics.

We have now provided Source data in addition to stating the number of replicates used to calculate statistics in each corresponding Figure legend. To clarify this, in the Statistical Analysis subsection of the Methods, we have now included the statement:

"The number of replicates used for calculating statistics can be found in the corresponding legend of each Figure, in addition to Source Data."

-> Please upload Table EV1 - EV3 as separate individual Docx or xlsx files.

We have now uploaded Tables EV1, EV2 and EV3 as individual docx files.

-> Please zip the MovieEV1 file with its legend in docx or txt format.

We have now uploaded the MovieEV1 file (mp4) and corresponding legend (docx) together in a zipped folder.

-> We generally encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

Source data have now been uploaded as individual files for each Figure

-> Mass spectrometry primary datasets produced in this study need to be deposited in an appropriate public database (see http://msb.embopress.org/authorguide#dataavailability). The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Method).

We carefully reviewed the author guidelines and available public databases regarding the deposition of mass spectrometry primary datasets.

While public data repositories are available to deposit large-scale datasets from non-targeted mass spectrometry analysis such as proteomics, we applied *targeted mass spectrometry* analysis to measure secreted acetylcholine levels from inguinal stromal vascular fraction. Therefore, our mass spectrometry data do not reach the standard acceptable for available public databases. But we provided all the mass spectrometry primary data included in the manuscript in our source data file.

Referee #1:

The authors have addressed my concerns. I recommend acceptance.

We thank the reviewer for the nice comments.

Referee #2:

The authors did not address the concerns that were raised. See comments below:

1. Multiple studies have reported the presence of several macrophage subsets in the adipose tissue with differences in phenotype, gene expression profile and functions. This study adds another one simply based on choline acetyltransferase expression without putting it in parallel with previous published work, which is not correct. The authors need to better understand the nature of such population (tissue-resident, monocyte?) as well as its relationship with previously described populations (See for example Chakarov et al, Science 2019).

"We thank the reviewer for encouraging further characterization and contextualization of acetylcholine-synthesizing adipose macrophages. Our RNA-seq data revealed that ChAMs exhibit a distinct gene expression and pathway enrichment profile for neurotransmitter regulation, acetylcholine signaling and adrenergic receptor signaling (Fig 1K-M, EV2J). Comparative analysis with other described populations, including Lyve1hi/lo/MHChi/lo/Cx3cr1hi/lo interstitial macrophages (Chakarov et al, 2019), Trem2+ lipid-associated macrophages (Jaitin et al, 2019), Ly6C and CD9 macrophages in obese adipose tissue (Hill et al, 2018) and sympathetic neuron-associated macrophages (Pirzgalska et al, 2017) yielded no definitive overlap between ChAMs and other published tissue macrophages subtypes. Future studies will further demonstrate how ChAMs may functionally interact with other cell types within the same tissue niche, including other functionally distinct macrophages. However, these may fall beyond the scope of current study. We have incorporated these important points in our discussions. (page 13)."

The authors did not performed a side by side bioinformatic comparison as requested. They could have used published datasets and compared them to their data.

We have carried out a side by side bioinformatics comparison (Response Figure 1). In addition to the Science paper by Chakarov et al. that was specifically suggested by the reviewer, we also included a study by Pirzgalska et al (Nature Medicine, 2017). Chakarov et al. did have RNAseq analysis targeting fat-derived Lyve1^{hi} and Lyve1^{lo} macrophages, but the main focus of this study was to compare these two populations of macrophages across various tissues. Pirzgalska et al. identified a subpopulation of macrophages that mediate clearance of norepinephrine and are functionally involved in mediating thermogenic fat activation and energy homeostasis. Our side by side comparison using the datasets published from these two studies help to provide additional understanding of the commonalities and, diversity of macrophage subpopulations, and the unique features of ChAMs. We have now incorporated these analyses as part of Figure EV2 (with an updated Figure EV2 legend), and also included methods for these analyses in the “Bulk RNA sequencing” Materials and Methods subheading.

2. On "An adaptive increase in ChAM abundance is evident following acute cold exposure", how is this happening? Local proliferation? Monocyte recruitment?

"Upon cold exposure, we saw increased proliferation (Ki67) and a corresponding increase in the total number of cholinergic macrophages, while ChAT expression (MFI) in cholinergic macrophages was also elevated in cold (Fig 1I-J). Using the inducible fate-mapping model ChAT-eGFP;Cx3cr1CreER-RFP, we demonstrated that less than a quarter of ChAT-eGFP+ macrophages are labeled RFP+ (Fig 3G-I), providing evidence that these cells are predominantly, but not exclusively, tissue-resident. To reflect these conceptual findings, we have removed the terminology 'tissue-resident' from the abstract (strikethrough and yellow highlight)."

Demonstrating that ChAT-eGFP+ are labeled using the inducible fate-mapping model ChAT-eGFP;Cx3cr1CreER-RFP after 7 days tamoxifen cannot allow to answer on tissue residence nor origins. The labelling could be a mix of monocyte contribution (labelled by tamoxifen) and recombination due to intrinsic CX3CR1 expression. The time frame of this experiment does not allow to answer such question. The authors should label for 5-6 days and then investigate the recombination months after labeling (and not 7 days) and

observe for decay or not. Ultimately, they should label during embryonic development to assess embryonic origin and not monocyte origin but this is beyond the scope of the study.

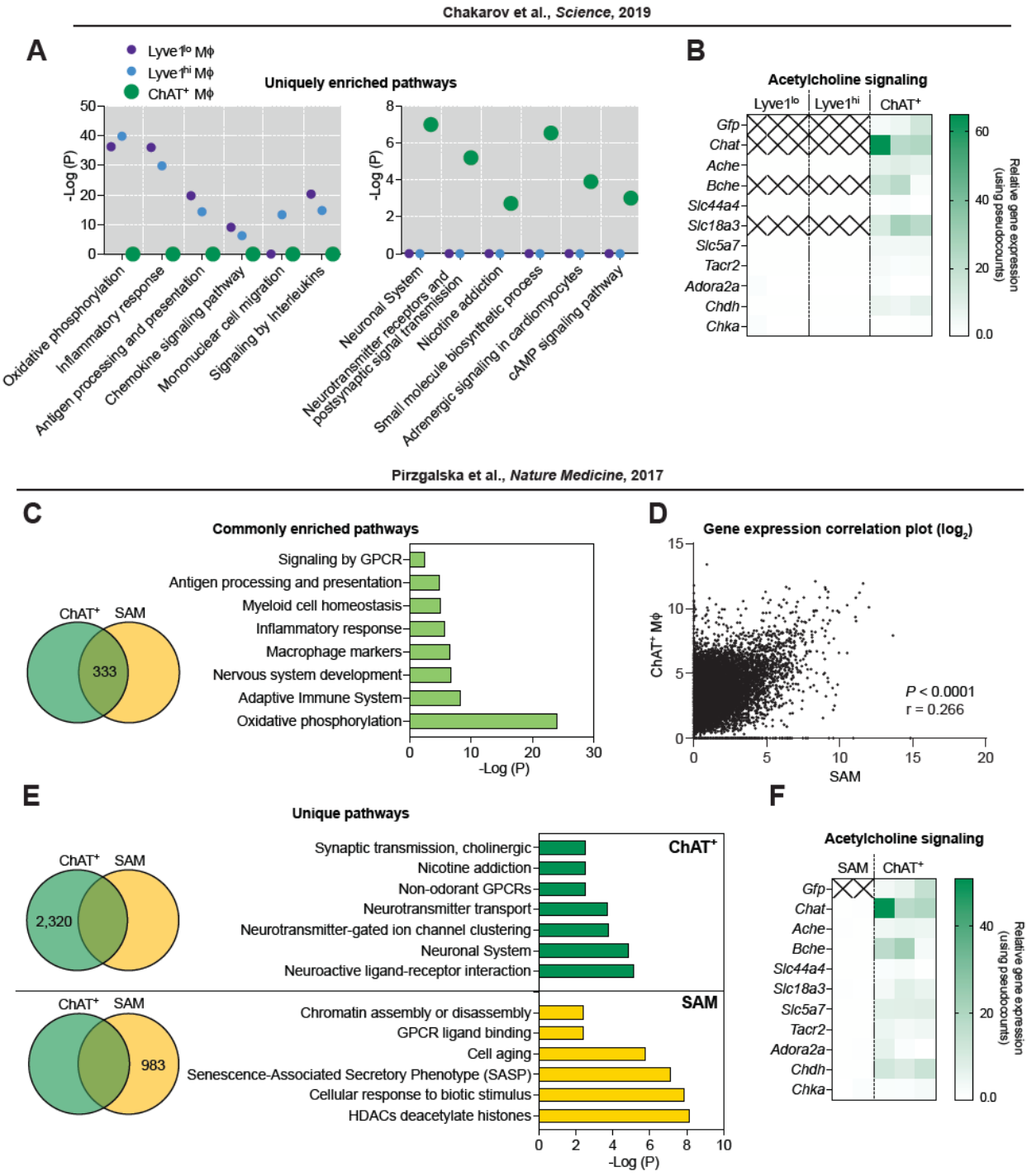
Future work will shed more light on how ChAMs are regulated under various conditions, such as how this population may contribute to long term subcutaneous adipose tissue remodeling during environmental changes or how ChAMs are involved in embryonic development. However, we feel that these experiments fall outside of the scope of the current study.

7. Can the authors clarify that they carefully removed any lymphoid organ from IWAT, VWAT or BAT.

"Our standard protocol does not involve removal of the lymph node. To assess the contribution of lymphoid tissue to the ChAT-eGFP+ profile, we microdissected out and digested the inguinal lymph node using an established protocol that the Maillard lab has extensive experience with (Chung et al, 2017). We compared resident ChAT-eGFP+ cell types to the IWAT depot in the presence or absence of the lymph node. By and large, ChAT-eGFP+ cells distribute throughout the depot with the exception that ChAT-eGFP+ macrophages were more prominent in the fat rather than the lymph node (Fig 1C). Given the very low number of ChAT+ cells in VWAT and BAT, even with the lymph node in the fat tissue (original Fig 1C, new Fig 1D), we did not further characterize the distribution in these two depots in this regard."

I do not understand why the lymph nodes are not removed when authors are studying fat macrophages.... This is very puzzling and concerning.

We understand it is routine practice for experts studying immunology to remove lymph nodes from their resident tissue. The reason we do not remove fat lymph nodes when studying interaction between immune cells and beige adipocytes is largely due to the regional variations within the subcutaneous fat. This is recognized and documented phenomena in the field of thermogenic beige fat (Cell Metab. 2018, 27(1):226-236.e3, Nature, 2019, 565 (7738):180-185.). In particular, in the 2019 Nature paper (565 (7738):180-185), it was clearly shown that newly identified subtype glycolytic-beige fat specifically “localized near the lymph node” (Extended Data Figure 2). We carried out most of our experiments without removing lymph nodes, to minimize any unnecessary variation between experiments. We did appreciate the reviewer’s comments in this regard and feel analysis on tissue without lymph nodes and lymph node alone provided further insights to this study.



Response Figure 1. ChAMs are a fat-derived distinct macrophage population.
A-B – RNA-seq data for fat-derived Lyve1^{hi} and Lyve1^{lo} MΦ (Chakarov *et al*, 2019) were procured from the Gene Expression Omnibus (GEO) Series Accession GSE125667. (A) Biological pathway analysis of enriched genes in Lyve1^{lo}, Lyve1^{hi} (left) or ChAT-eGFP^{+} MΦ (right) was performed. Common biological pathways were not detected across the subpopulations. Lyve1^{lo} and Lyve1^{hi} MΦ highly expressed genes for immune regulation and/or inflammation, whereas ChAT-eGFP^{+} MΦ revealed enriched expression of genes in neuronal and adrenergic signaling. (B) Relative expression (pseudocounts) of genes relevant to acetylcholine signaling in Lyve1^{hi}, Lyve1^{lo} and ChAT-eGFP^{+} MΦ (n = 3). Counts for *Gfp*, *Chat*, *Bche* and *Slc18a3* were not available in the GSE125667 dataset.
C-F – RNA-seq data for subcutaneous fat-derived sympathetic neuron-associated MΦ (SAM) (Pirzgalska *et al*, 2017) were procured from the GEO Series Accession GSE103847. (C) Biological pathway analysis of commonly enriched genes in SAM and ChAT-eGFP^{+} MΦ. (D) Gene expression correlation plot between ChAT-eGFP^{+} MΦ and SAM. Spearman correlation coefficient (r) test did not indicate strong association in global gene expression between ChAT-eGFP^{+} MΦ and SAM. (E) Biological pathway analysis of uniquely expressed genes in ChAT-eGFP^{+} MΦ versus SAM. Transcriptomic comparison of ChAT-eGFP^{+} MΦ and SAM suggested overlapping molecular features in intrinsic macrophage marker

profiles or properties. However, ChAT-eGFP$^+$ MΦ was identified as a distinct population from SAM with an enrichment of neuronal signaling. (F) Relative expression (pseudocounts) of genes relevant to acetylcholine signaling in ChAT-eGFP$^+$ MΦ and SAM (n = 3 for ChAT-eGFP$^+$ MΦ, 2 for SAM). Counts for *Gfp* were not available in the GSE103847 dataset.

2nd Revision - Editorial Decision 30th Jul 2021

I am pleased to inform you that your manuscript has been accepted for publication in The EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Jun Wu
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2020-106061R1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample sizes are biological replicates and were chosen based on preliminary data or our previously published reports. Shapiro-Wilk testing was used to determine whether data were distributed normally then parametric two-tailed Student's t-tests used to assess statistical significance for two-group comparisons or a one-way analysis of variance (ANOVA) with Tukey's post-hoc testing for multiple comparisons involving one independent variable. P values are indicated as *P<0.05, **P<0.01 and ***P<0.001 unless otherwise specified.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We used the sample sizes based upon previously reported data, preliminary and pilot data
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data were excluded from the analyses
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Yes. Mice were matched for age and randomly assigned for the treatments.
For animal studies, include a statement about randomization even if no randomization was used.	For all animal experiments, the animals were randomly assigned at the time of purchase or weaning to minimize any possible bias. Age-matched mice were used for in vivo studies.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	CLAMS were performed by a staff scientist at the University of Michigan Animal Phenotyping Core without in-depth knowledge about the scope of the current work. RNA sequencing was performed by a staff scientist at the the Advanced Genomics Core of University of Michigan without in-depth knowledge about the scope of the current work.
4.b. For animal studies, include a statement about blinding even if no blinding was done	CLAMS were performed by a staff scientist at the University of Michigan Animal Phenotyping Core without in-depth knowledge about the scope of the current work. Three dimensional adipose imaging of adipose tissue was performed blinded.
5. For every figure, are statistical tests justified as appropriate?	We performed stasistical analysis as indicated in the respective figure legends and methods section of the manuscript.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The statisical analysis performed was also mentioned.
Is there an estimate of variation within each group of data?	No

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Company and catalog numbers (and clone numbers, when applicable) for all antibodies included in the study are provided in the manuscript expanded view table 3
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	ChAT-eGFP, Chat-Cre, Ai14, Chatfl/fl, Vav-iCre, LysM-Cre, Cd4-Cre, and Mb1-Cre and Cx3cr1-CreER mice were all obtained from Jackson Laboratories (stock nos. 007902, 031661, 007914, 016920, 008610, 004781, 022071and, 020505 and 020940 respectively). β-less mice were provided by Brad Lowell (Beth Israel Deaconess Medical Center, Boston). All mice were housed under 12-h light/12-h dark cycle (6 a.m.–6 p.m.) with a standard rodent chow diet unless otherwise specified.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal-related procedures were approved by and conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) at the University of Michigan.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We have followed the ARRIVE guidelines through the course of these studies and during reporting.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	A data availability section is included in the Methods which states that RNA sequencing data have been uploaded to NCBI Gene Expression Omnibus database at Accession No. GSE174345
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	RNA sequencing data has been deposited to the public database NCBI Gene Expression Omnibus at Accession No. GSE174345
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA