EMBO reports

The regulation of glucose and lipid homeostasis via PLTP as a mediator of BAT-liver communication

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1st Editorial Decision 23rd Jan 2020

Dear Shingo,

Thank you for submitting your manuscript to our journal. We have now received three referee reports, which are copied below.

Please accept my apologies for this unusual delay in getting back to you. It took longer than usual to receive the full set of referee reports due to the recent holiday season.

As you can see, the referees express interest in the analysis. However, they also raise a number of concerns that need to be addressed to consider publication here. In particular, referees find that the causality of the findings and the BAT originality of PLTP require better support. I find the reports informed and constructive, and believe that addressing the concerns raised will significantly strengthen the manuscript.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1. A data availability section providing access to data deposited in public databases is missing (where applicable).
2. Your manuscript contains statistics and error bars based on n=2 or on technical replicates. Please use scatter plots in these cases.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page with page numbers, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper. For more details on our Transparent Editorial Process, please visit our website:
https://www.embopress.org/page/journal/14693178/authorguide#transparentprocess
You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

4) a complete author checklist, which you can download from our author guidelines (<http://embor.embopress.org/authorguide>). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (<http://embor.embopress.org/authorguide>).

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <http://embor.embopress.org/authorguide#expandedview>.

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) We would also encourage you to include the source data for figure panels that show essential data.

Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available <http://embor.embopress.org/authorguide#sourcedata>.

8) Regarding data quantification, please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates.
Please also include scale bars in all microscopy images.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Kind regards,

Deniz

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

This study identifies phospholipid transfer protein (PLTP) as a new batokine that has beneficial metabolic effects. Increased circulating levels of PLTP protected mice against high fat diet, enhanced glucose tolerance, and improves lipid homeostasis. These changes were accompanied by increased synthesis and excretion of bile acids in the liver, which in turn enhances glucose uptake and thermogenesis in BAT. The topic is indeed very interesting, results are novel, and the study is well performed. However, I have some considerations:
1. Overall, the results indicate that in mice, higher PLTP levels exert a beneficial action in terms of adiposity, and glucose/lipid homeostasis. However, as the authors indicate, circulating PLTP activity is increased in obese and insulin resistant subjects. Taking into account that they found PTLP as a candidate using thermogenic adipocytes from humans, how do the authors reconcile these results?

2. In line with the previous point, a recent study not cited in the manuscript indicates that PLTP deficiency attenuates high fat diet induced obesity and insulin resistance (PMID: 31220615). Again, these results are somehow difficult to reconcile with previous findings. There is some discussion about the role of PLTP coming from adipocytes and the fact that using AAVs to over-express PLTP may not necessarily reflect in vivo phenotypes of genetic null mice. Given this apparent controversy, I would highly recommend to perform a loss-of-function study silencing PLTP in the liver of mice to check whether they gain more fat and/or show alterations in lipid and glucose metabolism.

3. Another intriguing aspect is that the authors initially suggest PLTP as a batokine with potential implications in metabolism, but then AAVs are infused in the tail vein, therefore mainly over-expressing PLTP in the liver as it is acknowledged in the manuscript. This generated some doubts

about how the BAT-liver loop would work. One possibility to solve this question might be to inject the AAVs in the BAT of DIO mice to see if the over-expression of PLTP in BAT reproduces the main findings (body weight, GTT, ITT, lipid profile) of the current animal model. The expression of PLTP should be also measured (liver versus BAT) in animal models where the gene is manipulated.

Referee #2:

The manuscript by Sponton and collaborators reports several findings in relation to the biological actions of PLTP on energy metabolism, expression in BAT, effects on liver and indirect effects, via bile acids, on BAT.
The findings are considerably novel and the work is essentially well done. However, there are several relevant points with the manuscript:

a) the manuscript constains several over-statements establishing cause-and-effect relationships at several points of the manuscript where only associational observations are reported. This is specially remarkable, for example, for the title, which should be modified. What is stated in title is just a suggestion coming from the data, perhaps true or not in strict sense. The same happens with the last conclusive Figure, where at least question marks should be included. The authors show evidence that BAT releases PLTP and PLTP is a molecule exerting several effects, but it not shown anywhere in the experiments shown that it is just the PLTP originating in BAT what causes the multiple metabolic effects of PLTP. There are other examples throughout the manuscript. The manuscript should be revised thoroughly to state "suggestions" when only associational findings are reported.
b) Another weakness of the manuscript is that, for PLTP effects, it relies largely on pharmacological over-expression in mice using AAV-driven PLTP. Considering that this is a tool to increase PLTP levels based on enhanced artificial expression in liver, and that most of the target effects of PLTP are just found in liver, the possiblity of some artifacts due to forced expression of PLTP in liver itself cannot be ruled out. Complementary data would be needed to strengthen the biological role of PLTP on hepatic function. In this sense, the manuscript would be stongly improved if testing the effects of recombinant PLTP in hepatocytes in order to determine cell-autonomous effects (in the sense of what is done for brown adipocytes).

Other additional specific points are:

Results:

- The data on PLTP expression in BAT/brown/beige adipocytes as provided are somewhat poor and should be enhanced, essentially because the attribution of PLTP to be a batokine and the negative data on regulation by thermogenic stimuli deserve clarification. Does browning of scWAT (appearance of beige cells) increase PLTP at that depot (experiment at Suppl. Fig 1, shown only for BAT)?, do thermogenic agonists induce or not PLTP secretion from brown adipocytes?. Otherwise, ChIP-Seq are "binding data" not "functional data" and the final conclusive sentence at page 6, end of first paragraph, should be toned down.

- Clarify at sub-sections of Fig4 legend whether "PLTP-treated" means AAV-injected (if so, as expected from the long time PLTP treatment procedure, it should be made explicit).

- Discussion:

- A key point in the Discussion is the comparison of current data with data at the aP2-driven PLTP KO mouse model in the Jiang et al. paper. That model is expected to impair the PLTP expression and release in BAT. The way that this is discussed should be improved, the lack of coincidence between the loss-of-function approach and the currently reported approach may support a pharmacological role of PLTP but not necessarily supports conclusions on PLTP physiology on the basis of the current study.

- in addition to the paper mentioned reporting brown adipocyte secretomics data, at least three papers appeared recently with this same proteomics approach to brown adipocyte secretome (Ali Kahn et al. 2018; Villarroya et al., 2019, Deshmukh et al., 2019), one of them (Deshmukh et al, 2019) using human cells. It would be worth to confront, either if succintly, the current data with those existing data.

Referee #3:

In this new study from Sponton et al, the authors set out to identify novel "BatoKines," brown adipose tissue derived secreted factors. As the authors note, there is ample evidence to support the notion that BAT can regulate nutrient homeostasis beyond its effects on thermogenesis. Nevertheless, the mechanisms underlying these effects have been unclear. As such, this study is timely and addresses an important topic in adipose tissue biology and energy metabolism.
The authors combine proteomics with transcriptomics to identify candidate secreted molecules from human brown adipocytes. Of the list of candidates enriched in brown adipocytes (vs. human white adipocytes), they focus on phospholipid transfer protein (PLTP). AAV expression of PLTP impact body weight, energy expenditure, and cholesterol/lipid metabolism. They conclude the PLTP mediates a mechanism of inter-organ communication between the liver and BAT to control energy metabolism.

Overall, the paper is well-written and address an important area in the field. The effects are quite strong, providing sufficient evidence that PLTP impacts energy metabolism. Nevertheless, there are a number of issues that require clarification and further experiments to improve the study.

1) Regarding the initial screen: the author should comment on the extent of differentiation if BAT and WAT cell lines. The concern is whether some of the hits represent actual adipocyte-derived factors, or instead are emanating from undifferentiated precursors. Some of the hits are indeed stromal derived factors (e.g. PDGFRL, PDGFC, TIMP4). Some additional gene expression analysis of purified adipocytes vs. SVF may help.

2) Energy expenditure was analyzed before body weights diverged. This provides compelling evidence that PLTP directly impacts energy expenditure. However, many of the other analyses (Figure 3 and 4) seem to be performed at later time points where body weights have already diverged. The authors should examine some of these end-points at time point prior to body weights diverging. The question here is whether the effects on the liver/lipid metabolism are secondary to body weight changes/energy expenditure. Along these lines, have the authors given PLTP to UCP1 KO mice?

3) The in vivo experiments as designed do not necessarily implicated BAT-derived PLTP as a major

regulator of energy metabolism. Have the authors considered delivering an AAV directly to BAT? Perhaps one carrying an shRNA or gRNA? Another approach may be to measure circulating PLTP levels in BAT-less mice.

4) Minor: It is stated in the text that PLTP levels are around 5-10 ug/mL in plasma. These data should be shown.

Point-by-Point Response to the Reviewers:

We are profoundly grateful to all three reviewers for their comprehensive and thoughtful appraisal of our work, and for providing items of specific critique that we have been able to address, hopefully to their satisfaction. First, we would like to start by addressing the two major comments raised by the reviewers.

Major Point #1: The significance of BAT as a source of circulating PLTP levels:

We appreciate the comments from the reviewers regarding the importance of BAT, relative to other organs, as a source of circulating PLTP. To address this important question, we analyzed the circulating PLTP levels in a BAT-deficient mouse model, UCP1-Cre x PPARg$^{flox/flox}$ mice, which we have recently developed (Yoneshiro et al., 2019). As shown in Figure 1 (right), we found that BAT-ablation mice (*Ucp1*-Cre x PPAR$\gamma^{flox/flox}$) showed significantly lower levels of plasma PLTP activity than did control mice (PPAR$\gamma^{flox/flox}$ mice) by 58.1%. The data are consistent with the expression profile that BAT expresses PLTP at the highest level among other tissues examined in the study (Fig. 1F). These results indicate that BAT is indeed a major source of circulation PLTP, and reinforce our conclusion that PLTP is a batokine. The new result is now shown in **Fig. 1G**.

Major Point #2: The role of BAT-derived PLTP.

Another important point raised by the reviewers is the functional contribution of BAT-derived PLTP. As suggested by the reviewers, we addressed this question by overexpressing PLTP in the BAT *via* directly injecting AV expressing PLTP into the BAT (Fig.2A, below). Subsequently, we analyzed the circulating PLTP activity, plasma bile acids, and adiposity. As shown below, we found that BAT-specific PLTP expression was sufficient to increase plasma PLTP activity (Fig.2B) and plasma bile acid levels (Fig.2C). Importantly, we found that BAT-specific PLTP expression significantly decreased white fat mass (Fig.2D). These suggest that BAT-derived PLTP sufficiently alters systemic bile acid balance and reduces adiposity. These new results are shown in **Fig. EV5**.

Referee #1:

This study identifies phospholipid transfer protein (PLTP) as a new batokine that has beneficial metabolic effects. Increased circulating levels of PLTP protected mice against high fat diet, enhanced glucose tolerance, and improves lipid homeostasis. These changes were accompanied by increased synthesis and excretion of bile acids in the liver, which in turn enhances glucose uptake and thermogenesis in BAT. The topic is indeed very interesting, results are novel, and the study is well performed. However, I have some considerations:

Comment-1: Overall, the results indicate that in mice, higher PLTP levels exert a beneficial action in terms of adiposity, and glucose/lipid homeostasis. However, as the authors indicate, circulating PLTP activity is increased in obese and insulin resistant subjects. Taking into account that they found PTLP as a candidate using thermogenic adipocytes from humans, how do the authors reconcile these results.

Response: First of all, we would like to thank the reviewer for his/her positive and thorough comments.

Consistent with the previous report in humans (Dullaart et al., 1994; Kaser et al., 2001; Murdoch et al., 2000), we found that plasma PLTP levels were significantly higher in obese mice relative to those in lean mice (**Fig. EV2B**).

It is conceivable that increased PLTP levels under obese conditions is an "adaptive" response to enhance the clearance of excess cholesterol and lipids. Many examples of such adaptive responses can be found in the regulation of metabolic hormones under obese conditions: to name a few, administration of GDF15 reduces body-weight gain and improves glucose homeostasis, whereas its circulating levels increase in obesity. Another example is that pharmacological activation of FGF21 leads to enhanced thermogenesis

and improved systemic glucose/lipid homeostasis, whereas higher circulating FGF21 levels are found in obesity. Thus, we consider that the result of higher PLTP levels in obesity support, rather than contradict, the beneficial effects of PLTP in systemic glucose and lipid homeostasis. We discussed this point in our revised manuscript (**Page 13**).

Comment-2: In line with the previous point, a recent study not cited in the manuscript indicates that PLTP deficiency attenuates high fat diet induced obesity and insulin resistance (PMID: 31220615). Again, these results are somehow difficult to reconcile with previous findings. There is some discussion about the role of PLTP coming from adipocytes and the fact that using AAVs to over-express PLTP may not necessarily reflect in vivo phenotypes of genetic null mice. Given this apparent controversy, I would highly recommend to perform a loss-of-function study silencing PLTP in the liver of mice to check whether they gain more fat and/or show alterations in lipid and glucose metabolism.

Response: This is a valid point. We discussed the following points that reconcile this apparent discrepancy between ours and the recent paper by Song et al. (Song et al., 2019) (also see **Page 13**).

1) The paper by Song et al. analyzed glucose tolerance and insulin sensitivity in mice after 12 weeks of high-fat and high-caloric diet feeding (60% kcal fat, 20% kcal % carbohydrate). The authors showed that whole-body PLTP KO mice gained significantly less body-weight than did controls at 6 weeks and thereafter. At 12 weeks of high-fat diet, the body-weight difference between KO vs. WT mice was over 10 g (KO mice were smaller than WT). Thus, the "improved" glucose tolerance and insulin sensitivity found in KO mice were most likely a metabolic consequence of lower body-weight, rather than the role of PLTP *per se*.

2) To address the extent to which PLTP alters glucose tolerance *independent* of its anti-obesity action, we performed new experiments that examined glucose tolerance in mice received PLTP vs. control at an earlier time point (6 weeks of HFD) before the body-weight difference diverged. We found that PLTP treatment significantly improved glucose tolerance even at the time when the difference between PLTP vs. control did not yet diverge (**New Fig. 3D**, **Fig. EV3A**). Furthermore, we showed that PLTP treatment increased bile acid levels in the plasma (**Fig. 4D**), the liver (**Fig. 4E**), and the faces (**Fig. 4F**) even when no difference was seen in the body-weight on a regular chow diet. Similarly, PLTP treatment reduced plasma cholesterol levels under the condition in which body-weight difference was not seen (**Fig. 4B**). Also, PLTP significantly increased whole-body energy expenditure prior to the divergence in body-weight (**Fig. 2E**). Collectively, these data suggest that the effect of PLTP on improving glucose tolerance and lipid homeostasis is not a metabolic consequence of the body-weight loss.

3) As we discussed in the manuscript, a previous study reported using aP2-Cre conditional PLTP knockout mice that *aP2*-Cre x *Pltp* KO mice exhibited a modest but significant reduction in plasma PLTP activity, high-density lipoprotein, phospholipids, and apolipoprotein A-I levels (Jiang et al., 2015). This is consistent with our gain-of-function studies *in vivo*.

4) Studies by us and others found that adipose tissue (BAT) express PLTP at high levels relative to other organs (note: liver express much lower levels of PLTP than adipose tissues - see **Fig. 1F**). Thus, silencing PLTP in the liver would not cause a major effect on systemic PLTP levels. We will address the role of BAT-derived PLTP by genetically deleting PLTP by UCP1-Cre in the future.

Comment-3: Another intriguing aspect is that the authors initially suggest PLTP as a batokine with potential implications in metabolism, but then AAVs are infused in the tail vein, therefore mainly over-expressing PLTP in the liver as it is acknowledged in the manuscript. This generated some doubts about how the BAT-liver loop would work. One possibility to solve this question might be to inject the AAVs in the BAT of DIO mice to see if the over-expression of PLTP in BAT reproduces the main findings (body

weight, GTT, ITT, lipid profile) of the current animal model. The expression of PLTP should be also measured (liver versus BAT) in animal models where the gene is manipulated.

Response: This is an important point. As discussed in Major point #1 and #2, our new data show that **1)** BAT is a major source of circulating PLTP levels, and **2)** BAT-derived PLTP via overexpression of PLTP in the BAT sufficiently increases bile acids and decreases adiposity. These data reinforce the paper.

Referee #2:

The manuscript by Sponton and collaborators reports several findings in relation to the biological actions of PLTP on energy metabolism, expression in BAT, effects on liver and indirect effects, via bile acids, on BAT. The findings are considerably novel and the work is essentially well done. However, there are several relevant points with the manuscript:

Comment-1: The manuscript contains several over-statements establishing cause-and-effect relationships at several points of the manuscript where only associational observations are reported. This is especially remarkable, for example, for the title, which should be modified. What is stated in title is just a suggestion coming from the data, perhaps true or not in strict sense. The same happens with the last conclusive Figure, where at least question marks should be included. The authors show evidence that BAT releases PLTP and PLTP is a molecule exerting several effects, but it not shown anywhere in the experiments shown that it is just the PLTP originating in BAT what causes the multiple metabolic effects of PLTP. There are other examples throughout the manuscript. The manuscript should be revised thoroughly to state "suggestions" when only associational findings are reported.

Response: We thank the reviewer's comments and suggestions. As discussed in Major point #1 and #2, our new data show that 1) BAT is a major source of circulating PLTP levels, and 2) BAT-derived PLTP sufficiently increases bile acids and decreases adiposity. These data support the overall conclusions; meanwhile, we agree with the reviewer and should refrain overstatement of the results.

Thus, we changed the title to "The regulation of glucose and lipid homeostasis via PLTP as a mediator of BAT-liver communication". Also, we changed the summery figure, such that it reflects the results, rather than a proposed model (**New Fig. 6**).

Comment-2: Another weakness of the manuscript is that, for PLTP effects, it relies largely on pharmacological over-expression in mice using AAV-driven PLTP. Considering that this is a tool to increase PLTP levels based on enhanced artificial expression in liver, and that most of the target effects of PLTP are just found in liver, the possiblity of some artifacts due to forced expression of PLTP in liver itself cannot be ruled out. Complementary data would be needed to strengthen the biological role of PLTP on hepatic function. In this sense, the manuscript would be strongly improved if testing the effects of recombinant PLTP in hepatocytes in order to determine cell-autonomous effects (in the sense of what is done for brown adipocytes).

Response: Our data suggest that BAT-derived PTLP, induced by overexpression of PTLP directly in BAT, sufficiently increased plasma bile acid levels and reduces adiposity (see **Major point #2**). Moreover, overexpression of an enzymatic deficient PLTP ($PLTP^{M159E}$ mutant) failed to change body weight, systemic lipid and glucose homeostasis (see **Fig. 5**). Thus, the beneficial effect of PTLP is not an artifact of its overexpression in the liver.

It is important to note that PTLP itself does not act on hepatocytes. Our data suggest that the effect is mediated through regulating plasma cholesterol levels (via its enzymatic activity) and subsequent increases in bile acids to clear excess cholesterol. In fact, our new data show that recombinant PLTP (rPLTP) at

physiological concentrations, which we observed in mice, did not alter the expression of cholesterol/FA metabolism genes in cultured hepatocytes (**New Fig. EV3F**). Note that these genes are significantly reduced in mice treated with PLTP *in vivo* (**Fig. 4C**). We discussed these points in the revised manuscript (**Page 9-10**).

Comment-3 (Results): The data on PLTP expression in BAT/brown/beige adipocytes as provided are somewhat poor and should be enhanced, essentially because the attribution of PLTP to be a batokine and the negative data on regulation by thermogenic stimuli deserve clarification. Does browning of scWAT (appearance of beige cells) increase PLTP at that depot (experiment at Suppl. Fig 1, shown only for BAT)? do thermogenic agonists induce or not PLTP secretion from brown adipocytes? Otherwise, ChIP-Seq are "binding data" not "functional data" and the final conclusive sentence at page 6, end of first paragraph, should be toned down.

Response: As discussed in the Major point #1, our new data show that BAT is a major source of circulating PLTP levels. We also provided new data that beige adipocytes, isolated from mice expressing PRDM16 in adipocytes (as a genetic model to enhance beige fat biogenesis), releases high levels of PLTP (**New Fig 1H**).

Consistent with the previous studies that PLTP is a target of PPARs (Tu and Albers, 1999, 2001), we show that both PRDM16 and PPARγ are co-recruited to the regulatory regions of the *Pltp* gene locus together with high H3K27ace and H3K4me marks (*i.e.,* active transcription marks), suggesting that this recruitment is not merely a "binding", but also associated with transcriptional activation. Furthermore, our transcriptome data (Ohno et al., 2012) found that a synthetic PPARg agonist, rosiglitazone, increased PLTP expression. We discussed these points in the revised manuscript (**Page 6**).

Comment-4 (Results): Clarify at sub-sections of Fig4 legend whether "PLTP-treated" means AAV-injected (if so, as expected from the long time PLTP treatment procedure, it should be made explicit).

Response: We apologize for the confusion. We revised the legend of Fig 4 and other figures, accordingly.

Comment-5 (Discussion): A key point in the Discussion is the comparison of current data with data at the aP2-driven PLTP KO mouse model in the Jiang et al. paper. That model is expected to impair the PLTP expression and release in BAT. The way that this is discussed should be improved, the lack of coincidence between the loss-of-function approach and the currently reported approach may support a pharmacological role of PLTP but not necessarily supports conclusions on PLTP physiology on the basis of the current study.

Response: We revised the discussion regarding discrepancies between the studies (**Page 12-13**).

Comment-6 (Discussion): In addition to the paper mentioned reporting brown adipocyte secretomics data, at least three papers appeared recently with this same proteomics approach to brown adipocyte secretome (Ali Kahn et al. 2018; Villarroya et al., 2019, Deshmukh et al., 2019), one of them (Deshmukh et al, 2019) using human cells. It would be worth to confront, either if succintly, the current data with those existing data.

Response: This is an important point. Two studies (Ali Khan et al., 2018; Deshmukh et al., 2019) indeed found PLTP in their secretome data, while the study by Villarroya et al. (Villarroya et al., 2019) did not find PLTP. This could be due to the differences in 1) mouse vs. human brown adipocytes, and 2) experimental conditions (thermogenic activation by cAMP vs. non-thermogenic activation). We included the results in the revised manuscript (**Page 5**).

Referee #3:

In this new study from Sponton et al, the authors set out to identify novel "BatoKines," brown adipose tissue derived secreted factors. As the authors note, there is ample evidence to support the notion that BAT can regulate nutrient homeostasis beyond its effects on thermogenesis. Nevertheless, the mechanisms underlying these effects have been unclear. As such, this study is timely and addresses an important topic in adipose tissue biology and energy metabolism.
The authors combine proteomics with transcriptomics to identify candidate secreted molecules from human brown adipocytes. Of the list of candidates enriched in brown adipocytes (vs. human white adipocytes), they focus on phospholipid transfer protein (PLTP). AAV expression of PLTP impact body weight, energy expenditure, and cholesterol/lipid metabolism. They conclude the PLTP mediates a mechanism of inter-organ communication between the liver and BAT to control energy metabolism.
Overall, the paper is well-written and address an important area in the field. The effects are quite strong, providing sufficient evidence that PLTP impacts energy metabolism. Nevertheless, there are a number of issues that require clarification and further experiments to improve the study.

Comment-1: Regarding the initial screen: the author should comment on the extent of differentiation if BAT and WAT cell lines. The concern is whether some of the hits represent actual adipocyte-derived factors, or instead are emanating from undifferentiated precursors. Some of the hits are indeed stromal derived factors (e.g. PDGFRL, PDGFC, TIMP4). Some additional gene expression analysis of purified adipocytes vs. SVF may help.

Response: We would like to thank the reviewer for his/her positive and thoughtful comments. We confirmed that the majority of the candidates (87.5%, 14/16) were expressed in differentiated brown adipocytes at higher levels than stromal cells. We included a table showing the expression levels of each candidate in purified adipocytes vs. SVF (**New Table EV 1**).

Comment-2: Energy expenditure was analyzed before body weights diverged. This provides compelling evidence that PLTP directly impacts energy expenditure. However, many of the other analyses (Figure 3 and 4) seem to be performed at later time points where body weights have already diverged. The authors should examine some of these end-points at time point prior to body weights diverging. The question here is whether the effects on the liver/lipid metabolism are secondary to body weight changes/energy expenditure. Along these lines, have the authors given PLTP to UCP1 KO mice?

Response: This is an important point. To address the question, we performed new experiments that examined glucose tolerance in mice received PLTP vs. control at an earlier time point before the body-weight difference diverged. We found that PLTP treatment significantly improved glucose tolerance even at the time (6 weeks of HFD) when the difference between PLTP vs. control did not yet diverge (**New Fig. 3D**, **Fig. EV3A**). Furthermore, we showed that PLTP treatment increased bile acid levels in the plasma (**Fig. 4D**), the liver (**Fig. 4E**), and the faces (**Fig. 4F**) even when no difference was seen in the body-weight on a regular chow diet. Similarly, PLTP treatment reduced plasma cholesterol levels under the condition in which body-weight difference was not seen (**Fig. 4B**). Also, PLTP significantly increased whole-body energy expenditure prior to the divergence in body-weight (**Fig. 2E**). Collectively, these data suggest that the effect of PLTP on improving glucose tolerance and lipid homeostasis is not a metabolic consequence of the body-weight loss.

We agree with the reviewer regarding the UCP1-dependent vs. independent action because recent studies suggest that glucose uptake by BAT remains active in UCP1 KO mice (Ikeda et al., 2017; Olsen et al., 2017). We aim to address this question in the future.

Comment-3: The in vivo experiments as designed do not necessarily implicated BAT-derived PLTP as a major regulator of energy metabolism. Have the authors considered delivering an AAV directly to BAT?

Perhaps one carrying an shRNA or gRNA? Another approach may be to measure circulating PLTP levels in BAT-less mice.

Response: According to the suggestion, we performed new studies that determine the effect of BAT-derived PLTP on systemic PLTP and energy metabolisms. Please see **Major point #1** and **Major point #2**. Our new data demonstrate that BAT is a major source of PLTP in the circulation and controls systemic energy balance.

Comment-3 (Minor): It is stated in the text that PLTP levels are around 5-10 ug/mL in plasma. These data should be shown.

Response: Our revised manuscript includes the plasma PLTP concentration in lean and diet-induced obese mice (**New Fig. EV2B**).

References

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1st Revision - Editorial Decision 15th May 2020

Dear Shingo,

Thank you for submitting your revised manuscript. It has now been seen by all of the original referees.

As you can see, the referees find that the study is significantly improved during revision and recommend publication.

Referee #2 requests additional controls on the new mouse models used in the study. I have further discussed these comments with referee #1. If you have additional data at hand/add data to address these concerns, it would strengthen the manuscript. If not, please respond the comments of referee #2 textually and include the requested discussion points regarding the shortcomings of the mouse model, as also suggested by referee #1 in his/her additional comments.

Before I can accept the manuscript, I also need you to address some minor points below:

• Please separate the provided source data into one file per figure.
• We noted that Figure 2I is currently missing a scale bar.
• The EV Tables need to be removed from the Article file.
• The legends of Fig 6 and EV figures need to be removed from the figure files.
• Papers published in EMBO Reports include a 'Synopsis' to further enhance discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the key findings of the paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb.
• In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.
• Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your minor revision.

Kind regards,

Deniz

--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

The new experiments support the conclusions and I have no further comments.

Referee #2:

The revised manuscript by Sponton et al. incorporates additional experiments that address the most relevant points in relation to previous submission: investigating directly the actual possibility hat BAT is the main source of PLTP, and determining whether BAT-originating PLTP exerts the biological actions reported.
In this sense the manuscript is clearly improved.
The only general point is that, as the authors introduce two totally novel models in the context of their PLTP-related investigation, in both cases some additional, complementary, data are needed in order that the experimentation described was really sound in relation to the objectives and the conclusions obtained.
1. Experiments with the UCP1-CRE driven PPARg deletion, as model of BAT ablation. The authors need to expand the data in order to reinforce that the observations found for PLTP are attributable to BAT-deletion itself and not to side effects of BAT ablation. BAT ablation may cause side-effect outside BAT. In the reference Yonsehiro et al. indicated , the overall description of the overall phenotype of those mice is ver succinct.However, another report using this same model (Xiao et al., Arterioscler Thromb Vasc Biol. 2018;38:1738-1747.) report relevant indirect consequences at sites other than BAT, e.g. induction of "beiging" of WAT. Therefore, it is specifically needed to characterize whether there is any change in PLTP expression in other tissues, particularly WAT, as a consequence of BAT ablation. Additional information on the actual extent of BAT ablation achieved in the model (even in Supplementary) would help for consistency of the overall data.
2. Experiments with AAV-driven expression of PLTP in BAT. Some additional data are needed to interpret the results. The reduction of iWAT mass only one week post-injection is impressive. It would be needed to show whether this is just loss of fat (reduced adipocyte size) or any other changes in WAT plasticity what is elicited (simple microscopy pictures would reveal this) and account for the reduction. On the other hand, data on food intake and, in fact, energy balance, in this experiment should be provided.
These are few minor additions required but they are relevant to sustain the conclusions proposed by the authors from these two novel experiments and models, key for overall conclusion of the study.

Referee #3:

The authors addressed all of the concerns. Overall, the paper is well written and the study is novel and important for the field.

Point-by-Point Response to the Reviewer

Referee #2:

The revised manuscript by Sponton et al. incorporates additional experiments that address the most relevant points in relation to previous submission: investigating directly the actual possibility that BAT is the main source of PLTP, and determining whether BAT-originating PLTP exerts the biological actions reported. In this sense the manuscript is clearly improved. The only general point is that, as the authors introduce two totally novel models in the context of their PLTP-related investigation, in both cases some additional, complementary, data are needed in order that the experimentation described was really sound in relation to the objectives and the conclusions obtained.

Comment: Experiments with the UCP1-CRE driven PPARg deletion, as model of BAT ablation. The authors need to expand the data in order to reinforce that the observations found for PLTP are attributable to BAT-deletion itself and not to side effects of BAT ablation. BAT ablation may cause side-effect outside BAT. In the reference Yonsehiro et al. indicated the overall description of the overall phenotype of those mice is ver succinct. However, another report using this same model (Xiao et al., Arterioscler Thromb Vasc Biol. 2018;38:1738-1747.) report relevant indirect consequences at sites other than BAT, e.g. induction of "beiging" of WAT. Therefore, it is specifically needed to characterize whether there is any change in PLTP expression in other tissues, particularly WAT, as a consequence of BAT ablation. Additional information on the actual extent of BAT ablation achieved in the model (even in Supplementary) would help for consistency of the overall data.

Response: We are grateful to reviewer #2 for the thoughtful comments on our revised manuscript. Regarding the possible contribution of beige adipocytes in BAT-less mice (UCP1-Cre x *Pparg* KO mice), we consider the following:

1. As the reviewer pointed out, the paper by Xiao et al. showed that the mRNA expression of *Ucp1*, *Cidea*, *Cox8*, and *Elovl3*, in the inguinal WAT of KO mice were higher by ~3 fold relative to that in WT control mice. On the other hand, many other beige-selective markers, including *Prdm16, Pgc1a, Dio2, Ebf2*, and *Cited*, were not different between KO and control mice. Thus, the paper does not determine if the UCP1-specific ablation of PPARg stimulates beige fat biogenesis (*i.e.,* the number of beige adipocytes) or simply stimulates the expression of *Ucp1* and several other genes in existing adipocytes. The histological data (shown in Fig.4B) of the paper do not detect any visible sign of increased multi-locular beige adipocytes in the inguinal WAT of KO mice, implying the possibility that UCP1-specific ablation of PPARg induces a compensatory activation of *Ucp1* mRNA expression in exiting white adipocytes, rather than increasing the number of beige adipocytes *per se*.
2. In our study, we found that the iBAT mass was significantly reduced in BAT-less mice (UCP1-Cre x PPARg$^{flox/flox}$) relative to the littermate control mice (PPARg$^{flox/flox}$). However, no difference was seen in the tissue mass of WAT, liver, and muscle between the genotypes (Fig.

1 below). Thus, the reduction of circulating PLTP levels, as observed in the BAT-less mice, is due to reduced BAT mass.

3. Even in the scenario in which compensatory increased beige fat biogenesis occurs in BAT-less mice, the contribution of increased beige adipocytes to circulating PLTP level would be negligible because our data show that circulating PLTP levels in BAT-less mice was significantly lower than those in control mice by 58.1% (shown in **Figure 1G**).

4. Due to COVID19, we could not examine the expression of PLTP levels in the inguinal WAT of BAT-less mice; however, the conclusion of the study, *i.e.,* BAT is a major source of circulating PLTP, remains well supported regardless of the outcomes.

These points were discussed in the main text (Page 5-6) and also cited the paper by Xiao et al. The data in Fig.1 (below) are now shown in **Fig. EV1B**.

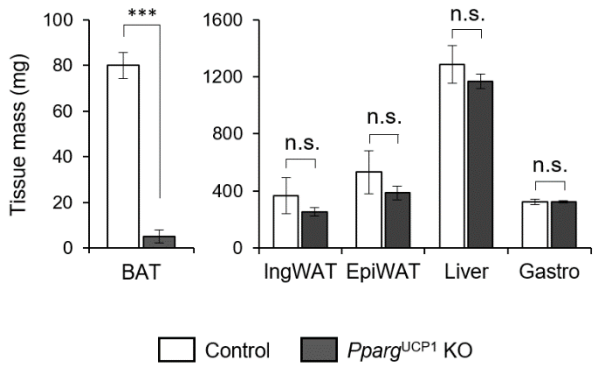

Figure 1. Body and tissue weights of *Pparg*^{UCP1} KO mice (n=4) and control mice (n=3). *** *P*<0.001 by unpaired t-test. n.s., not significant.

Comment: Experiments with AAV-driven expression of PLTP in BAT. Some additional data are needed to interpret the results. The reduction of iWAT mass only one-week post-injection is impressive. It would be needed to show whether this is just loss of fat (reduced adipocyte size) or any other changes in WAT plasticity what is elicited (simple microscopy pictures would reveal this) and account for the reduction. On the other hand, data on food intake and, in fact, energy balance, in this experiment should be provided. These are few minor additions required but they are relevant to sustain the conclusions proposed by the authors from these two novel experiments and models, key for overall conclusion of the study.

Response: We agree with the reviewer that the food intake and histological analysis of WAT are relevant, while the reduction of fat mass is most likely due to increased energy expenditure, rather than reduced food intake given the following points:

1. BAT-selective PLTP overexpression lead to an increase in circulating PLTP activity by ~2 fold (see **Fig. EV5C**). This increase was equivalent to or even less than the increase in circulating PLTP levels by tail-vein injection of AAV-PLTP (see **Figure 5D**).

2. We found that a 2-fold increase in circulating PLTP activity did not alter food intake and physical activity of mice, whereas it significantly increased whole-body energy expenditure and reduced fat mass (see **Figure 2D and 2E**).
3. As shown in Fig. 2 (below), we found that WAT from mice with ~2-fold increase in circulating PLTP activity reduced adipocyte size in the inguinal WAT, while no feature of lipodystrophy or adipose tissue inflammation was found in the WAT.

We clarified these points in the manuscript (Page 12).

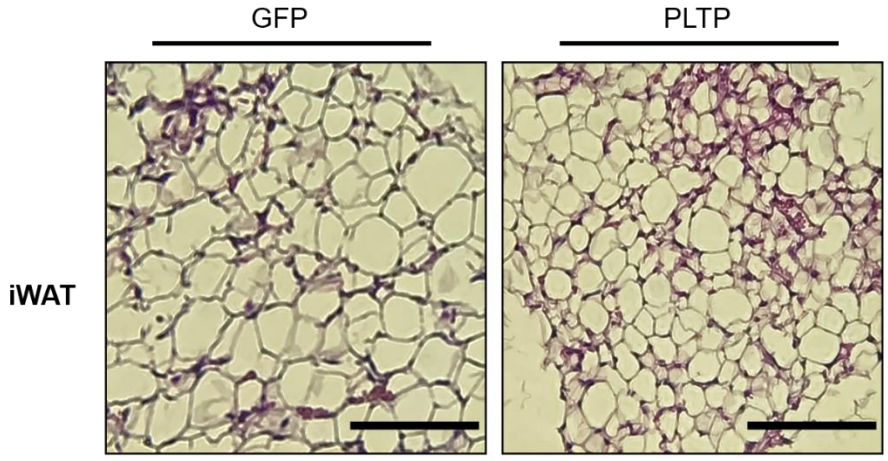

Figure 2. H&E staining of iWAT of mice administered with adenovirus containing either GFP or PLTP via tail-vein. Scale bar 150µm.

2nd Revision - Editorial Decision 12th Jun 2020

Dear Shingo,

Thank you for submitting your revised manuscript. I have now looked at everything and all looks fine. Therefore I am very pleased to accept your manuscript for publication in EMBO Reports.

Congratulations on a nice work!

Before we can transfer your manuscript to our production team, we need your approval on the following. We would like to change the statement under 'Data Availability Section' to the following: 'This study includes no data deposited in external repositories. All data is presented in the Source Data files'.

Kind regards,

Deniz
--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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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 iustified
- ➔ 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?	The sample size was determined by the power analysis with α = 0.05 and power of 0.8, developed by Cohen (1988), and based on our experience with experimental models, anticipated biological variables, and previous literatures. Sample numbers were described in the Figure legends.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For animal studies, the sample size was defined as described above.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Samples or animals were not excluded from the analysis. In some case (detailed in figure legends) statistical analysis were performed in n<5 due to technical issues during the experimental analysis. However, those facts do not compromise overall interpretation of the results
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.	mice were randomly assigned at the time of purchase or weaning to minimize any potential bias
For animal studies, include a statement about randomization even if no randomization was used.	as described above
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.	The lipidomic analysis and tissue or blood bile acids quantification were performed by authors that were not directly involved in sample acquisition. However, the analysis were not blinded. The metabolic studies in vivo were also not blinded. Significant changes in body weight between groups (particularly in high fat diet experiments) make blinded studies unfeasible.
4.b. For animal studies, include a statement about blinding even if no blinding was done	as described above
5. For every figure, are statistical tests justified as appropriate?	detailed description of sample size and statistical analysis performed are described in details in methods section and figure legends
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	the data in this study meet the statistical assumptions of the tests (described in details in methods section, e.g Shapiro-Wilk or Kolmogorov-Smirnov test for normality and Levene's test for homogeneity)

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

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Is there an estimate of variation within each group of data?	The standard error mean (S.E.M) was used in this study as representative of variance.
Is the variance similar between the groups that are being statistically compared?	Yes, the standard error mean is similar between groups in this study

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).	NA
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	the cell lines used in this study was reported by our group in previous study (Shinoda et al, 2015). The cell line was tested for mycoplasma contamination.

* 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.	C57Bl/6 mice (Stock No.000664) obtained from the Jackson laboratory. BAT-less mice (Ucp1-Cre x Ppargflox/flox) in C57BL/6 background was generated as previously described by Yoneshiro et al., 2019 (doi: 10.1038/s41586-019-1503-x). Mice were at 6-8 weeks old, unless otherwise specified. The mice had free access to food and water, 12 h light cycles, and were caged at 23 ˚C. Detailed information about the experimental mice in this study is described in methods section
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experimental procedures were performed in compliance with all relevant ethical regulations applied to the use of small rodents. The animal protocol was approved by the UCSF Institutional Animal Care and Use Committee.
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.	The study was performed following the guidelines established by the UCSF Institutional Animal Care and Use Committee. This minimizes animal distress, is effective and efficient; it is consistent with the recommendations of the American Veterinary Medical Association (AVMA) Guidelines on Euthanasia.

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	RNA-Seq data was previously described by our group (Shinoda et al, 2015). CTR and Ap2-PRDM16 secretome data are public available (Svensson et al, 2016). PRDM16 ChiP-Seq data (Harms et al, 2015), PPARg ChiP-Seq data (Siersbaek et al, 2012) and ENCODE H3K27ac and H3K4me3 (Yue et al, 2014) are also public available.
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).	NA
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.	No