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Developmental and Functional Heterogeneity of White Adipocytes within a Single Fat Depot

Kevin Y. Lee, Quyen Luong, Rita Sharma, Jonathan M. Dreyfuss, Siegfried Ussar, and C. Ronald Kahn

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Transaction Report:

(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. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 23rd Mar 2018

Thank you for submitting your manuscript for consideration by the EMBO Journal. Three referees have been assigned to your manuscript; we have received reports from all of them, which I copy below. In light of these comments, I am afraid we decided that we cannot offer publication in The EMBO Journal.

As you will see, the referees appreciate that the analysis extends previous work. However they also raise major concerns with the analysis that I am afraid preclude publication here. In more detail, the referees #1 and #2 state that in light of earlier work by your group and others, the conceptual advance provided is limited. Further, referee #2 is concerned as the in vivo physiological relevance of your findings is not sufficiently supported by the data in his/her view. Referee #3 states that the results are too preliminary and would need more differential functional validation.

Given these negative opinions from good experts on the field, and that we need strong support from the referees to move on, I am afraid we cannot offer to publish your study in The EMBO Journal.

REFEREE REPORTS:

Referee #1:

The paper by Lee et al., investigates the important topic of heterogeneity of white adipose tissue by

comparing gene expression profiles of 24 clonal cell lines made from subcutaneous and perigonadal adipose tissue of one ImmortomouseTM mouse. The authors performed PC analysis on the microarray data and grouped these cell lines into three types. They performed in vitro experiments to compare metabolism and responses to external stimuli such as TNFa, insulin, and growth hormone, between these three types of preadipocyte cell lines. They then chose three Cre lines to represent each of the three preadipocyte cell lines (based on the gene expression profiles), and performed lineage tracing studies. The authors provided evidence showing all three of the chosen Cre lines can give rise to preadipocytes and mature adipocyptes, and the percentages of contribution vary between the lines and depots. This is a descriptive and well-written manuscript, investigating the topic of preadipocyte heterogeneity in white adipose tissue. There are a few suggestions for clarifying experimental and conceptual issues.

Major points:
1. The authors grouped the cell lines into three types. Type one is solely perigonadal in origin while subcutaneous and perigonadal depots were found in both Type 2 and 3. Based on data shown in FigEV1C and D, Type 2 (or Cluster 2) is mainly subcutaneous in origin as only one clone (PGF3.2) in Type 2 is perigonadal in origin. Type 3 (Cluster 3) is consisted of 4 subcutaneous and 4 perigonadal origin preadipocyte cell lines (Fig EV1C). However, it is not clear whether multiple clonal lines and if both perigonadal or subcutaneous origin clones were used in the assays performed representing Type 3.
It has been shown previously that subcutaneous and visceral (including perigonadal) adipose tissue are different in metabolism and response to external stimuli etc. This therefore dampers the novelty of the current study as the result becomes comparisons between subcutaneous and visceral depots.

2. The authors chose Wt1-CreERT2 to represent Type 1, Mx1-Cre to represent Type 2, and Tagln-Cre to represent Type 3, based on gene expression profiling data between the three types of preadipocytes. It is not uncommon that the Cre line does not reflect the expression of the gene of interest. In this case, Wt1-CreERT2 is a knockin at the Wt1 locus and so is the Tagln-Cre. The Tagln-Cre (which is also known as SM22-Cre) used in the current study is a better choice compared to the one that is used previously (Berry et al 2006). However, the current Tagln-Cre has been reported not truthfully represent SM22 expression. For example, this line does not express Cre recombinase in embryonic SMC and cardiac myocytes (Zhang 2006), suggesting that this Cre line may not 100% reflect the expression of Tagln gene. The same question applies to the use of Mx1-Cre. This is important as a lot of the data shown in this manuscript were linking the types of the preadipocyte cell lines (grouped by gene expression profiles) and the three Cre lines used. The authors need to provide evidence that the Cre activity correlates with the expression of the genes (e.g. Mx1-Cre preadipocytes express Mx1 gene, and no Tagln expression and vice versa etc).
3. The authors interpret the cells highlighted using the different three Cre lines, give rise to preadipocytes, and mature adipocytes and therefore represent three distinct populations. Tagln-Cre (SM22-Cre) has been shown previously to highlight pericytes. Mx1-Cre has been shown to highlight MSCs. Wt1-CreERT2 has been used to highlight mesothelium. Pericytes, MSCs, and mesothelium have all been shown previously to be distinctive sources of adipocytes. Again, this reduces the novelty of the current study.
4. The authors performed three tests to compare the response of the three types of preadipocytes to external stimuli (Fig 4I). The difference shown in Fig 4I is small. Better quantification of the data will help clarify the doubts.
5. The links between Type and Cluster need to be clarified. For example, the manuscript describes the result as 'Type' while the Figs are using 'Clusters' (Fig Ev1C and D). There are 4 clusters in Fig1B, and only three are shown in Fig Ev1C and D (where is Cluster 4?). Comparing the clone name, Cluster 1 in Fig Ev1C is the same as Cluster 4 in Fig Ev1B. This part needs clarification.
6. The authors nicely show the cultured preadipocytes from ImmortomouseTM retain depot-specific gene expression. Do the clonal preadipocytes still retain the depot-specific gene expression?

Minor points

1. There is quite a bit emphasis on the presence of factors inhibiting adipogeneis based on the result that the clonal preadipocyte cells lines can differentiate, while the original visceral pool of cells does not differentiate. SVF culture is a very mixed population of cells. It is likely that the culturing condition (i.e. preadipocyte media) selects the clones with greater adipogenic potential. As a result, it might lead to the impression that clonal cells differentiate better due to the absence of inhibitors

that are present in the pool of cells. Could the authors provide evidence (as it is described in the result section, page7) by analyzing gene expression profiles in the clones that do not have adipogenic potential? (or simply down-tune the statement and leave it in discussion only)
2. Page 8, extreme variation in media acidification rates were observed between cell lines and it is mentioned that this is unrelated to differentiation capacity and the rate of proliferation. Please provide evidence of these statements.
3. How does Fig2C differ from Fig Ev1C? (duplication of information?)
4. For various assays/tests performed for comparison between the three different Types of preadipocytes, how many different clones from each Type were used? (i.e.is there clone to clone variation within the same Type?)
5. Church C et al (2015) showed negative result on the approach of generating of preadipocyte cell lines using ImmortomouseTM. Could the authors discuss the difference in methods between the current study and the previous study.
6. In Discussion, there are some confusions about the Prx1-Cre model (page 20) and the expression of Prx1 in Typ1-3 preadipocytes. The Prx1-Cre line is a transgene and does not reflect endogenous Prx1 expression.
7. There are some typos and some methods are missing (e.g. triglyceride measurement).

Referee #2:

Lee et al. present the results of an interesting and timely study on the heterogeneity of white adipocytes within a single fat depot. This group of investigators has pioneered this concept in recent years through a number of publications. In this particular study, the authors derive immortalized cell preadipocyte cell lines from white fat tissue obtained from the immortomouse model. The authors found that within a single visceral depot multiple preadipocyte cell lines can be derived, each giving rise to functionally distinct adipocytes. This concept has significant implications as it may suggest that the heterogeneity of white adipocytes, rather than the overall abundance, may influence energy metabolism in obesity.

Overall, the manuscript is well-written and the data is nicely presented. The major concerns lies in 1) the degree of conceptual advance: this idea of adipocyte heterogeneity within a single depot (subcutaneous WAT) was previously made by Lee et al. in their recent Diabetes paper. This new study extends this idea to visceral fat, however, this point (along with the WT1 Cre lineage tracing data) was made in the referenced Chau et al. Nature Cell Biology paper).

2) the strength of the in vivo data: the conclusions are largely based on the functional and molecular analysis of immortalized cell lines. The lineage tracing analyses suggest that precursor and adipocyte heterogeneity exists naturally in vivo but this should be further explored. In particular, it is not clear that the three different Cre drivers are truly marking unique populations that correlate to the cell populations found in immortalized SVF. Alternatively, the activity of the different Cre lines could simply be mosaic.

There are two suggested experiments that could significantly improve the study by strengthening the novelty and conclusions:

a. Examine whether the indicated functional preadipocyte/adipocyte heterogeneity exists within primary cells (not immortalized). The prediction would be that labeled cells isolated from the individual lineage tracing models would represent molecularly distinct preadipocytes who expression matches the correlating immortalized cell lines. Moreover, do these labeled precursors from the lineage tracing models give rise to functionally distinct adipocytes (again, correlating with the immortalized cell lines). This would significantly strengthen the conclusion that heterogeneity naturally exists.

b. A simple experiment that could raise the novelty and significance of the work would be to examine whether the proportions of cells (precursors and adipocytes) labeled by the individual Cre lines is altered by high fat diet feeding. This would lend credence to the idea that obesity is associated with changes in adipocyte heterogeneity.

Referee #3:

In this manuscript, the authors report highly interesting but strictly preliminary findings on the developmental and functional heterogeneity of white preadipocytes/adipocytes within fat depots. They analyzed gene expression profiles and adipogenesis potentials of clonal preadipocyte cell lines derived from white adipose tissues of transgenic mice expressing temperature-sensitive SV40T antigen (Immortomouse). The authors provide some evidence to suggest that there are at least three preadipocyte/adipocyte subpopulations in white adipose tissues. These three groups of white preadipocytes/adipocytes show distinct gene expression profiles and can be tracked by differential expression of three genes: Wilms' tumor 1 (Wt1), transgelin (Tagln), and myxovirus 1 (Mx1). Using lineage tracing analyses, the authors showed that these preadipocyte/adipocyte subpopulations appear to be present in vivo and differ in their abundance in different fat depots. However, detailed functional characterization of these distinct groups of white adipocytes in vivo is lacking in the current manuscript. Further definitive evidence needs to be provided to show that these groups of adipocytes isolated from mice by FACS are indeed functionally distinct at least in cell culture.

Major comments:
The authors clustered three different types of preadipocyte cell lines based on the gene expression profiles in microarray analyses in Figure 2. Type 1 preadipocytes have a distinct gene expression pattern from other types, but the difference between Type 2 and Type 3 is rather unclear. While Wt1 is highly enriched in Type 1 compared to Type 2 and 3, Tagln and Mx1 do not look specifically enriched in each type of preadipocytes. Functional studies of these three distinct groups of preadipocytes and adipocytes isolated by FACS from mouse fat depots are essential for validating the findings obtained from clonal cell lines. These analyses should at least include 1) gene expression profiles of preadipocytes and adipocytes, and 2) functional analyses of adipocytes as done in Figure 4.

Minor comment:
1. In Figure 2D, several genes besides Wt1 are clearly enriched in Type 1 preadipocytes. Please comment whether they are good markers for the Type 1 preadipocytes.
2. The legend for Figure 7 overlaps with 7B and should be rearranged.
3. Typos: there are multiple types especially in the Materials and Methods section.

Authors' correspondence 2nd April 2018

We are writing to ask you to reconsider your decision on our manuscript "Developmental and Functional Heterogeneity of White Adipocytes within a Single Fat Depot" (EMBOJ-2018-99291). While we appreciate the reviewers' comments and suggestions, and your offer to transfer the paper to Life Science Alliance, we believe the reviewers have underestimated the novelty of this paper and that a chance for revision should be warranted.

We acknowledge many of the specific points raised by the reviewers, most of which could be easily addressed in a revision. However, we disagree with reviewers' suggestion that this manuscript does represent a significant conceptual advance or that these adipocyte populations have already been described. Indeed, in this manuscript, we identify three adipocyte subpopulations (two of which are totally novel); determine their contributions in vivo to multiple fat depots (not just the two most commonly used depots); and define important functional differences between these subpopulations. This has not been done before and is novel in that it indicates that white adipose tissue is not just heterogeneous in terms of developmental lineage, but also that the adipocytes in a single fat depot are heterogenous in terms of function. This is a significant advance in the field and a very important advance conceptually.

We are willing to perform many of the additional molecular and functional experiments to further validate of these adipocyte subpopulations as requested by the reviewers, which we agree would strengthen the manuscript. These would be relatively straightforward to perform. In fact, we have attached a more detailed response to the reviewers, including the experimental plan to improve this

manuscript in a point-by-point manner. We believe that the manuscript will be strengthened by incorporating these changes, and that if revised, would make a paper we would all be proud to see published in The EMBO Journal.

Thank you very much for your reconsideration of your decision. We hope that you will agree to give us a chance for revision.

Editor's correspondence 7th May 2018

Thank you for contacting me regarding our decision and for your patience with my response, which got delayed due to internal discussions in the team, as well as getting back to the referees regarding your point-by-point response.

We realise that you would - judging from the information provided in the rebuttal letter - be potentially able to address the issues raised by the referees in a revised version of the manuscript. This view was shared by referees #2 and #3 who stated that your suggested revision experiments would significantly strengthen the study.

Overall, we would thus invite you to work towards a re-review. Accordingly, we would - given that you addressed all the experimental issues with compelling data - be prepared to ask the referees for further input.

Please note however, that in particular the functional proof of in vivo physiological relevance of your new stratification scheme and findings has been a major concern of all three referees. Thus, this will in our view be a core aspect to be considered.

Since a large number of key experiments have been suggested, and given the importance of the question addressed, it would be essential for you to provide a definitive and accurately described dataset in the revised version.

Please contact me if you have any questions, need further input on the referee comments or if you consider engaging in a compelling revision, in which case we would not close the file.

However, please note, that since the results of your experiments are entirely open at this stage, we cannot in any way predict the outcome of a re-submission, or make any promises towards publication.

1st Revision - authors' response 27th Jul 2018

We would like to thank the reviewers for their careful reading and useful suggestions. We have also clarified the text, reanalyzed the results, and displayed additional data as suggested by the reviewers. Of greatest importance, we have performed both gene expression analysis and metabolic studies on the primary preadipocytes isolated from the lineage tracing mouse models. These results are now added as two new multi-panel figures (Figure 5, EV5) that strongly support our original findings. The specific changes and additions are enumerated below.

Referee #1:

The paper by Lee et al., investigates the important topic of heterogeneity of white adipose tissue by comparing gene expression profiles of 24 clonal cell lines made from subcutaneous and perigonadal adipose tissue of one ImmortomouseTM mouse. The authors performed PC analysis on the microarray data and grouped these cell lines into three types. They performed in vitro experiments to compare metabolism and responses to external stimuli such as TNFa, insulin, and growth hormone, between these three types of preadipocyte cell lines. They then chose three Cre lines to represent each of the three preadipocyte cell lines (based on the gene expression profiles), and performed lineage tracing studies. The authors provided evidence showing all three of the chosen Cre lines can give rise to preadipocytes and mature adipocytes, and the percentages of contribution vary between the lines and depots. This is a descriptive and well-written manuscript, investigating the topic of preadipocyte heterogeneity in white adipose tissue. There are a few suggestions for clarifying experimental and conceptual issues.

Major points:

1. The authors grouped the cell lines into three types. Type one is solely perigonadal in origin while subcutaneous and perigonadal depots were found in both Type 2 and 3. Based on data shown in FigEV1C and D, Type 2 (or Cluster 2) is mainly subcutaneous in origin as only one clone (PGF3.2) in Type 2 is perigonadal in origin. Type 3 (Cluster 3) is consisted of 4 subcutaneous and 4 perigonadal origin preadipocyte cell lines (Fig EV1C). However, it is not clear whether multiple clonal lines and if both perigonadal or subcutaneous origin clones were used in the assays performed representing Type 3.

It has been shown previously that subcutaneous and visceral (including perigonadal) adipose tissue are different in metabolism and response to external stimuli etc. This therefore dampers the novelty of the current study as the result becomes comparisons between subcutaneous and visceral depots.

While a number of studies have shown that subcutaneous and visceral fat are different, this manuscript goes much further in that we now identify heterogeneity of adipocytes within a single depot. This comes not only from the single cell experiments mentioned above, but also from the *in vivo* lineage tracing experiments shown in Figures 7 and 8. This needs to be taken into account with both the qPCR experiments (Figure 3) and experiments determining the phenotypes of the adipocyte types shown in Figures 4A-I in which all of the Type 1, 2, and 3 cells were utilized, independent of whether they came from subcutaneous or perigonadal clones. Thus, this paper does more than just reiterating the differences between depots, it shows that many of the differences in gene expression and phenotype are dependent on adipocyte type and independent of depot of origin. These points have been clarified in the revised manuscript.

2. The authors chose Wt1-CreERT2 to represent Type 1, Mx1-Cre to represent Type 2, and Tagln-Cre to represent Type 3, based on gene expression profiling data between the three types of preadipocytes. It is not uncommon that the Cre line does not reflect the expression of the gene of interest. In this case, Wt1-CreERT2 is a knockin at the Wt1 locus and so is the Tagln-Cre. The Tagln-Cre (which is also known as SM22-Cre) used in the current study is a better choice compared to the one that is used previously (Berry et al 2006). However, the current Tagln-Cre has been reported not truthfully represent SM22 expression. For example, this line does not express Cre recombinase in embryonic SMC and cardiac myocytes (Zhang 2006), suggesting that this Cre line may not 100% reflect the expression of Tagln gene. The same question applies to the use of Mx1-Cre. This is important as a lot of the data shown in this manuscript were linking the types of the preadipocyte cell lines (grouped by gene expression profiles) and the three Cre lines used. The authors need to provide evidence that the Cre activity correlates with the expression of the genes (e.g. Mx1-Cre preadipocytes express Mx1 gene, and no Tagln expression and vice versa etc).

We would like to thank the reviewer for this suggestion and have now added these experiments, which we feel have greatly improved the manuscript. Specifically, we have now performed both gene expression analysis and metabolic studies on the primary preadipocytes isolated from Wt1-CreERT2, Tagln-Cre, and Mx1-Cre mice (Figures 5, EV5E-G). These data show that the Cre activity correlates with the expression of the genes, and that gene expression of most of the differentially expressed genes in the Immortomouse clones are reflected in this primary preadipocyte population. In addition, we also provide new functional analysis that the phenotypic differences between the preadipocyte/adipocyte subpopulations, including triglyceride accumulation and OXPHOS vs. glycolytic metabolism are retained in these isolated subpopulations. Taken together, these data strongly indicate that the three subpopulations we have identified in the clonal analysis are also present *in vivo*.

3. The authors interpret the cells highlighted using the different three Cre lines, give rise to preadipocytes, and mature adipocytes and therefore represent three distinct populations. Tagln-Cre (SM22-Cre) has been shown previously to highlight pericytes. Mx1-Cre has been shown to highlight MSCs. Wt1-CreERT2 has been used to highlight mesothelium. Pericytes, MSCs, and mesothelium have all been shown previously to be distinctive sources of adipocytes. Again, this reduces the novelty of the current study.

While the reviewer is correct in stating that mesothelium, pericytes, and mesenchymal stem cells have been shown to give rise to adipocytes, none of these have been studied together in a single report. Thus, from the literature, one is left with the impression that each of these represents the unique (and sometime only) source of preadipocytes. Our study defines how each of these potential precursor pools contributes differentially to both different adipose depots and different functional characteristics of mature adipocytes. We also identify populations missed in previous studies. For example, as the reviewer correctly noted, utilizing a different Tagln-Cre model, Berry et al concluded that Tagln precursors did not contribute to the white adipocyte lineage, however, this is clearly the not case when multiple adipose depots are studied in detail. Similar comments apply to

the Mx-1 derived adipocytes, which we observe are almost exclusively located in the subcutaneous adipose tissue, with the highest concentrations in the scapular white fat, a depot rarely studied. Our data also suggest that these cells are not from the hematopoietic lineage and represent a novel adipocyte subpopulation. Finally, we not only show that there are distinct origins of adipocytes, but also distinct function of adipocytes of different lineage origin, something not addressed in any of the previous studies. We think this speaks strongly to the novelty of our study. These point have been clarified in the current study.

4. The authors performed three tests to compare the response of the three types of preadipocytes to external stimuli (Fig 4I). The difference shown in Fig 4I is small. Better quantification of the data will help clarify the doubts.

The Western blots have been quantified as suggested (Figure EV3). The quantification show that significant differences in TNFα and GH, but not inulin-mediated mediated signaling between the adipocyte subpopulations.

5. The links between Type and Cluster need to be clarified. For example, the manuscript describes the result as 'Type' while the Figs are using 'Clusters' (Fig Ev1C and D). There are 4 clusters in Fig1B, and only three are shown in Fig Ev1C and D (where is Cluster 4?). Comparing the clone name, Cluster 1 in Fig Ev1C is the same as Cluster 4 in Fig Ev1B. This part needs clarification.

We apologize for the confusion and have clarified the text and figures as suggested. The adipocyte types were identified by clustering analysis of microarray data, and the terms “Type” and “Cluster” are used interchangeable in the manuscript and figures. This has been clarified in the revision. Experimentally, the adipocyte types in this manuscript were also defined by taking the *consensus* of the three independent clustering algorithms, depicted in Figures 2C, EV1C, and EV1D. The algorithm in EV1D placed a single clone, SCF3.9 into its own cluster. Only clonal cell lines that were similarly grouped together by all three clustering algorithms (depicted in the blue, red, and green shading) were are identified as an adipocyte type.

6. The authors nicely show the cultured preadipocytes from ImmortomouseTM retain depot-specific gene expression. Do the clonal preadipocytes still retain the depot-specific gene expression?

We thank the reviewer for the suggestion. We have analyzed the expression of Tbx15, Shox2, HoxA5, and HoxC8 in primary preadipocytes isolated from our Wt1-CreERT2, Tagln-Cre, and Mx1-Cre reporter mice (Figure EV5 A-D). Interestingly, we find that Tbx15 and HoxA5 are more highly expressed in Type 2 and Type 1 preadipocytes, respectively. On the other hand, the expression of Shox2 and HoxC8 and are highly expressed in subcutaneous or visceral preadipocytes, respectively, and are independent of the subpopulations we have identified. Taken together, these data suggest that some, but not all depot-specific differences in gene expression may reflect the differential cellular contribution of the subpopulations to adipose tissue depots.

Minor points

1. There is quite a bit emphasis on the presence of factors inhibiting adipogeneis based on the result that the clonal preadipocyte cells lines can differentiate, while the original visceral pool of cells does not differentiate. SVF culture is a very mixed population of cells. It is likely that the culturing condition (i.e. preadipocyte media) selects the clones with greater adipogenic potential. As a result, it might lead to the impression that clonal cells differentiate better due to the absence of inhibitors that are present in the pool of cells. Could the authors provide evidence (as it is described in the result section, page7) by analyzing gene expression profiles in the clones that do not have adipogenic potential? (or simply down-tune the statement and leave it in discussion only) 2. Page 8, extreme variation in media acidification rates were observed between cell lines and it is mentioned that this is unrelated to differentiation capacity and the rate of proliferation. Please provide evidence of these statements.

This is an important point, which has been clarified in the revised discussion. We have now also included a citation to a study published in Nature while this paper was in revision that focused only on this feature of visceral preadipocytes not to differentiate until separated into individual clones. In this study, the authors identified a subpopulation of CD142+ cells do actually repress adipogenic capacity both in vitro and in vivo (Schwalie PC, et al. Nature 2018).

With regard to the acidification point, the picture depicting the varied media acidification of the clonal cell lines was performed when the cells were 100% confluent and not proliferating. The figure legend has been changed to clarify this point. A graph depicting that the media acidification rates of the clones was unrelated to differentiation capacity has been added as suggested (Figure EV1A).

2. How does Fig2C differ from Fig Ev1C? (duplication of information?) 4. For various assays/tests performed for comparison between the three different Types of preadipocytes, how many different

clones from each Type were used? (i.e.is there clone to clone variation within the same Type?) 5. Church C et al (2015) showed negative result on the approach of generating of preadipocyte cell lines using ImmortomouseTM. Could the authors discuss the difference in methods between the current study and the previous study.
Figures 2C, 1EVB, and 1EVC depict the three independent clustering algorithms we utilized and are not a duplication of information. As we stated earlier (See response to major point 1) all of the clones were used were used for the phenotypic analysis. We agree with the previous studies that Immortomouse preadipocytes have can have limited adipogenic capacity (Church C, et al. 2015), especially when studied as pooled cells isolated from the visceral fat depots (Figure 1). However, in contrast to the previous study, we flow sorted single preadipocytes and generated clonal cell lines. These cell lines were highly and reproducibly adipogenic, suggesting the presence of anti-adipogenic cells within the pooled depot-specific cell lines, as described in point 1 above. This is another novel aspect of this study.
6. In Discussion, there are some confusions about the Prx1-Cre model (page 20) and the expression of Prx1 in Typ1-3 preadipocytes. The Prx1-Cre line is a transgene and does not reflect endogenous Prx1 expression.
We agree with the reviewer's point that the Prx1 transgene may not reflect endogenous Prx1 expression and will change the discussion accordingly.
7. There are some typos and some methods are missing (e.g. triglyceride measurement).
We apologize for any typos and omissions, and will correct these errors.

Referee #2:

Lee et al. present the results of an interesting and timely study on the heterogeneity of white adipocytes within a single fat depot. This group of investigators has pioneered this concept in recent years through a number of publications. In this particular study, the authors derive immortalized cell preadipocyte cell lines from white fat tissue obtained from the immortomouse model. The authors found that within a single visceral depot multiple preadipocyte cell lines can be derived, each giving rise to functionally distinct adipocytes. This concept has significant implications as it may suggest that the heterogeneity of white adipocytes, rather than the overall abundance, may influence energy metabolism in obesity.
Overall, the manuscript is well-written and the data is nicely presented.
We thank the reviewer for these generally positive comments.
The major concerns lies in
1) the degree of conceptual advance: this idea of adipocyte heterogeneity within a single depot (subcutaneous WAT) was previously made by Lee et al. in their recent Diabetes paper. This new study extends this idea to visceral fat, however, this point (along with the WT1 Cre lineage tracing data) was made in the referenced Chau et al. Nature Cell Biology paper).
While this paper certainly builds on previous work in the field by us and others (as do all papers), we disagree with the reviewer's statement that this manuscript does not represent a significant degree of conceptual advance. Indeed, in our previous paper, we demonstrated how the expression of T-box15 regulates metabolism in a subset of adipocytes within the subcutaneous adipose tissue, and the report from Chau et al. demonstrated that the mesothelium was shown to give rise to a subpopulation of visceral adipocytes. However, this manuscript represents a notable advance, as we identified two new subpopulations of adipocytes, determined the *in vivo* contribution of all three types of preadipocytes to multiple adipose depots, and demonstrated that these adipocyte subpopulations have differences in metabolism and differential responses to exogenous stimuli, such as insulin, growth hormone and inflammatory cytokines. None of these points had been shown in any previous study by us or others.
2) the strength of the in vivo data: the conclusions are largely based on the functional and molecular analysis of immortalized cell lines. The lineage tracing analyses suggest that precursor and adipocyte heterogeneity exists naturally in vivo but this should be further explored. In particular, it is not clear that the three different Cre drivers are truly marking unique populations that correlate to the cell populations found in immortalized SVF. Alternatively, the activity of the different Cre lines could simply be mosaic.
As suggested by the reviewer, we have now performed both gene expression analysis a on the primary preadipocytes isolated from Wt1-CreERT2, Tagln-Cre, and Mx1-Cre mice (Figures 5A-I). These data show that the Cre activity correlates with the expression of the genes, and that gene expression of most of the differentially expressed genes in the clonal cell lines are reflected in this primary preadipocyte population. In addition, we have also provide functional analysis that the

phenotypic differences between the preadipocyte/adipocyte subpopulations, including triglyceride accumulation and OXPHOS vs. glycolytic metabolism are retained in these isolated subpopulations (Figure 5J-N; EV5E-G). Taken together, these data strongly indicate that the adipocytes labelled by the Cre lines are not due to mosaic activity, but are in fact, the three subpopulations we have identified in the clonal analysis.

There are two suggested experiments that could significantly improve the study by strengthening the novelty and conclusions:

a. Examine whether the indicated functional preadipocyte/adipocyte heterogeneity exists within primary cells (not immortalized). The prediction would be that labeled cells isolated from the individual lineage tracing models would represent molecularly distinct preadipocytes who expression matches the correlating immortalized cell lines. Moreover, do these labeled precursors from the lineage tracing models give rise to functionally distinct adipocytes (again, correlating with the immortalized cell lines). This would significantly strengthen the conclusion that heterogeneity naturally exists.

We would like to thank the reviewer for the suggested experiments and have performed these experiments as suggested. As mentioned in response to concern #2, we have performed both gene expression analysis and functional analysis on the preadipocytes isolated from Wt1-CreERT2, Tagln-Cre, and Mx1-Cre lineage tracing mice. These data show that the differentially expressed genes in the clonal cell lines are reflected in this primary preadipocyte population. Furthermore, these isolated preadipocytes/adipocytes recapitulate the phenotypic differences observed in the cell lines, including triglyceride accumulation and OXPHOS vs. glycolytic metabolism observed in the clonal cell analysis (Figure 5J-N; EV5E-G). Together, these data strongly indicate that the adipocytes labelled by the Cre lines represent the three subpopulations we have identified in the clonal analysis.

b. A simple experiment that could raise the novelty and significance of the work would be to examine whether the proportions of cells (precursors and adipocytes) labeled by the individual Cre lines is altered by high fat diet feeding. This would lend credence to the idea that obesity is associated with changes in adipocyte heterogeneity.

Again, we agree with the reviewer on this suggestion and believe that elucidating the response of adipocyte subpopulations in obesity is an important experiment. In fact, this work is currently underway. However, we feel that full characterization of these adipocyte subtypes and their response to obesity in terms of both physiology and cell biology will require a full study and not just a few panels in a figure in the current manuscript. Thus, considering all of the other new data in this paper, we feel that this is outside the scope of what should be presented here, which already includes 8 figures with 58 data panels, as well as 26 panels of supplemental data). For the information of the reviewer, we will comment that our preliminary evidence so far indicates that in HFD-induced obesity reduces the number of Type 1 and 2 adipocytes. This appears to be due to the fact that these adipocyte subpopulations are uniquely sensitive to the inflammatory response and thus are surrounded by macrophages and crown-like structures. As should be apparent, understanding the mechanisms underlying this finding will require many further experiments and thus is beyond the scope of this study.

Referee #3:

In this manuscript, the authors report highly interesting but strictly preliminary findings on the developmental and functional heterogeneity of white preadipocytes/adipocytes within fat depots. They analyzed gene expression profiles and adipogenesis potentials of clonal preadipocyte cell lines derived from white adipose tissues of transgenic mice expressing temperature-sensitive SV40T antigen (Immortomouse). The authors provide some evidence to suggest that there are at least three preadipocyte/adipocyte subpopulations in white adipose tissues. These three groups of white preadipocytes/adipocytes show distinct gene expression profiles and can be tracked by differential expression of three genes: Wilms' tumor 1 (Wt1), transgelin (Tagln), and myxovirus 1 (Mx1). Using lineage tracing analyses, the authors showed that these preadipocyte/adipocyte subpopulations appear to be present in vivo and differ in their abundance in different fat depots. However, detailed functional characterization of these distinct groups of white adipocytes in vivo is lacking in the current manuscript. Further definitive evidence needs to be provided to show that these groups of adipocytes isolated from mice by FACS are indeed functionally distinct at least in cell culture.

Major comments:

The authors clustered three different types of preadipocyte cell lines based on the gene expression profiles in microarray analyses in Figure 2. Type 1 preadipocytes have a distinct gene expression pattern from other types, but the difference between Type 2 and Type 3 is rather unclear. While Wt1

is highly enriched in Type 1 compared to Type 2 and 3, Tagln and Mx1 do not look specifically enriched in each type of preadipocytes. Functional studies of these three distinct groups of preadipocytes and adipocytes isolated by FACS from mouse fat depots are essential for validating the findings obtained from clonal cell lines. These analyses should at least include 1) gene expression profiles of preadipocytes and adipocytes, and 2) functional analyses of adipocytes as done in Figure 4.
We would like to thank the reviewer for this suggestion. We have now performed both gene expression analysis and metabolic studies on the primary preadipocytes isolated from Wt1-CreERT2, Tagln-Cre, and Mx1-Cre mice (Figures 5, EV5E-G) and think that these additional data have greatly strengthened the manuscript. These data show that the Cre activity correlates with the expression of the genes, and that gene expression of most of the differentially expressed genes in the Immortomouse clones are reflected in this primary preadipocyte population. In addition, we also provide functional analysis that the phenotypic differences between the preadipocyte/adipocyte subpopulations, including triglyceride accumulation and OXPHOS vs. glycolytic metabolism, are retained in these isolated subpopulations. Taken together, these data strongly indicate that the three subpopulations we have identified in the clonal analysis are also present *in vivo*.
Minor comment:
1. In Figure 2D, several genes besides Wt1 are clearly enriched in Type 1 preadipocytes. Please comment whether they are good markers for the Type 1 preadipocytes.
We agree with the reviewer's assessment that there are additional markers of Type 1 preadipocytes. For example, we show in Figure 3A-C and Figure 5A-C that Lrrn4 and Upk3b are also good markers for Type 1 preadipocytes.
2. The legend for Figure 7 overlaps with 7B and should be rearranged.
The figure has been rearranged as suggested.
3. Typos: there are multiple types especially in the Materials and Methods section.
We apologize for any errors and have now corrected the typos as suggested.

2nd Editorial Decision 1st Sep 2018

Thank you for sending us the revised version of your manuscript EMBOJ-2018-99291R, and my apologies for the unusual delay in processing this manuscript, due to detailed discussions within the team in order to ensure a fair and balanced decision. Your manuscript has now been re-evaluated by two of the original referees and we have received reports from both of them, which I enclose below. In light of these comments, I am afraid we decided that we cannot proceed with publication in The EMBO Journal.

As you can see, the referees acknowledge that the study has improved. However they also state persistent issues with core aspects of the analysis that I am afraid preclude publication here. Both referees maintain that major concerns raised in the first round of review have not been conclusively addressed. In particular, the differentiation potential of the primary cell population remains unresolved. Also, proof for functional heterogeneity keeps being unclear. Therefore the referees are not supportive of publication.

As indicated earlier, we do agree with the referees that additional conclusive insights into the physiological in vivo relevance of the three proposed cell populations would be required to warrant publication in The EMBO Journal. I am sorry to see, that you have failed to convince the referees. Given these negative opinions from good experts in the field together with our further assessment, I regret to say, that we decided not to go further with this manuscript.

REFEREE REPORTS:

Referee #2:

The revised paper from Lee et al. is certainly improved and a number of critiques have been adequately addressed. However, there is an important issue that remains unresolved. A major conclusion is that distinct preadipocyte populations exist in the visceral fat tissue. Initially, this was suggested based largely on immortalized cell cultures. Multiple reviewers indicated the need to explore this with primary cells. Indeed, the authors isolated distinct primary cell populations using their genetic models and gene expression differences were nicely presented (and consistent with

previous in vitro results). The problem is that they then proceed to immortalize them again before studying their differentiation capacity. The question remains: do the 3 different labeled populations of primary (not immortalized) cells 1) undergo differentiation and 2) give rise to 3 unique fat cell types.

Referee #3:

In their revised manuscript, Lee et al. have addressed some of reviewers' concerns. The authors describe their newly performed experiments to try to address reviewers' comments and present the new figure 5 in the revised manuscript. Specifically, they have added both gene expression analysis and metabolic studies on the primary preadipocytes isolated from Wt1-CreERT2, Tagln-Cre, and Mx1-Cre mice crossed with reporter mice.
However, the major concerns remain. Authors claim that the differentially expressed genes and the phenotypic differences in the clonal cell lines are reflected in these primary preadipocyte populations. Although the data suggest that the three subpopulations in the clonal analysis are present in vivo, some results are inconsistent between the clonal cell lines and primary preadipocytes. For instance, while type 3 preadipocytes (marked by the expression of Mx1) show high levels of Cxcl12 in the clonal cell lines, Cxcl12 expression is not enriched in Mx1-positive primary preadipocytes and is even lower than that of Wt1-positive preadipocytes. More importantly, the functional analyses in the preadipocyte/adipocyte subpopulations have failed to reveal significant differences between Tagln-positive and Mx1-positive adipocytes. Overall, the study reports strictly preliminary findings in the developmental and functional heterogeneity of white preadipocytes/adipocytes within fat depots. Without functional studies of these groups of preadipocytes and adipocytes in mice, the manuscript is only describing the heterogeneity of white adipocytes within fat depots.

Minor comment:
There are multiple typos in Figures (e.g. "we;;" in Figure 4C, "Isoproteranol" in Figure 4E, and "26B4" in Figure 5C).

2nd Revision - authors' response 13th Sep 2018

We were obviously disappointed by your email concerning our manuscript "Developmental and Functional Heterogeneity of White Adipocytes within a Single Fat Depot" and are writing to ask you to reconsider your decision.

There are two major reasons we feel that the previous decision is unfair and inappropriate.

1. In our revision (which required many months of additional experimentation), we addressed all of the reviewers' initial comments, in many cases with considerable amounts of new data.

1. We believe that what is being asked by the reviewers has never been part of any previous publication in the field. Thus, while we appreciate the thoughts of the reviewers, we feel that their expectations for this manuscript are far beyond the scope of what has been done in any comparable studies.
As a reminder, in this study, we not only have identified 2 or 3 new adipocyte subpopulations, but we also demonstrate that these subpopulations differentially contribute to both the preadipocytes and mature adipocytes in different adipose depots. Furthermore, we have developed two separate cellular models to interrogate the phenotype of these cells. These phenotypic data distinguish this study from other recent papers on adipocyte heterogeneity, all of which were published in high impact journals [1-5], and none of which even begin to interrogate the functional and phenotypic differences between adipocyte subpopulations identified.

In addition, we do not believe that the concerns of referees regarding the immortalization of the primary GFP+ preadipocytes are warranted. The FACS protocol used to isolate preadipocytes (CD45-, CD31-, Ter119-, CD29+, CD34+, Sca1+ cells) [6], which the reviewer has questioned, has been used by many others and by us in numerous publications and is well documented to yield adipocyte precusors [4, 7, 8]. Furthermore, the data in the manuscript clearly demonstrate that the

primary GFP+ preadipocytes isolated via this procedure have differential gene expression (Figures 5A-I), and, most importantly, give rise to GFP+ adipocytes in vivo (Figure 7A). We do have data indicating that primary GFP+ preadipocytes from the Wt1- and Tagln- reporter mice (representing Type 1 and 2 preadipocytes) are adipogenic in vitro that could be added to the manuscript if you desire (Figure A attached below). However, we did not originally include it, since space is limited, and the adipogenic potential of FACS-sorted preadipocytes has been published many times. Thus, we thought it was more important to demonstrate that the preadipocytes retain adipogenic potential after immortalization.

Finally, it is worth noting that we needed to immortalize the GFP+ preadipocytes to study these populations, since in some depots they are relatively rare. For example, Type 3 preadipocytes are only found in appreciable numbers in one type of fat pad (the scapular white fat pad) and, even in this depot, they comprise only about 20% of preadipocytes. Thus, digestion of scapular white fat yields only ~5000 Type 3 preadipocytes. So, to fulfill the reviewer's request, each single point would require the pooling of fat from about 10 mice, and a single experiment would require over 50 age- and sex-matched mice. This is totally unreasonable from a cost point of view and also would raise serious questions from our institutional animal research (IACUC) committee as to the ethics of sacrificing so many mice to make such a minor point, especially one that is already established in other data.

While I almost never argue with an Editorial decision, in this case we hope that you will reconsider the decision regarding our manuscript, we which feel is unfair and that the request for more data is out of proportion to anything being published in top journals elsewhere.

Thank you for all of your help with this paper and thank you very much for considering our request.

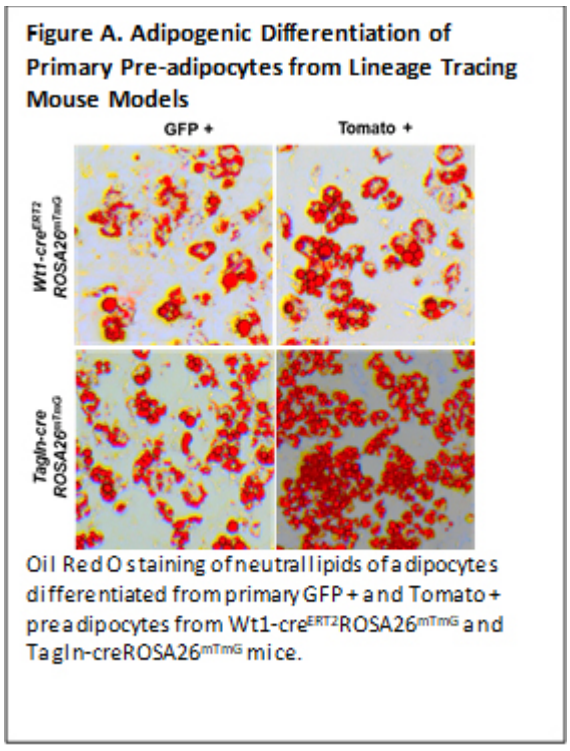

Figure A. Adipogenic Differentiation of Primary Pre-adipocytes from Lineage Tracing Mouse Models Oil Red O staining of neutral lipids of adipocytes differentiated from primary GFP + and Tomato + preadipocytes from Wt1-creERT2ROSA26mTmG and Tagln-creROSA26mTmG mice.

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2. Gavin, K.M., et al., De novo generation of adipocytes from circulating progenitor cells in mouse and human adipose tissue. FASEB J, 2016. 30(3): p. 1096-108.

3. Jiang, Y., et al., Independent stem cell lineages regulate adipose organogenesis and adipose homeostasis. Cell Rep, 2014. 9(3): p. 1007-22.

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8. Macotela, Y., et al., Intrinsic differences in adipocyte precursor cells from different white fat depots. Diabetes, 2012. 61(7): p. 1691-9.

3rd Editorial Decision 26th Oct 2018

Thank you for contacting me regarding our decision and for your patience with my response. My sincere apologies for the protraction due to detailed discussions in the team, as well as asking additional input from an arbitrating advisor, who got delayed, but whose comments I enclose below.

As you will see the arbitrator gave us overall positive feedback, in particular valuing the complementary data presented demonstrating adipogenic potential of primary pre-adipocytes purified from Wt1- and Tagln- GFP animals.
Based on this input together with our additional considerations we have now decided that the study is appropriate for publication in The EMBO Journal, pending amendment of the manuscript by the additional experiments illustrated.

Thus, we ask you to complement your manuscript with above analysis in a final minor revision and submit this version using the link provided below.

ARBITRATING ADVISOR'S COMMENTS:

I can understand the concerns of the reviewers. However the technical limitations raised by the authors in response to the requests of the reviewers are in my opinion legitimate. To answer properly the reviewers requests for some of the adipocytes populations identified it will be necessary an unreasonable number of mice. Also the cost required will be excessive for the improvement. So I think the authors could include in the manuscript the Figure A with data indicating that primary GFP+ pre-adipocytes from the Wt1- and Tagln- reporter mice (representing Type 1 and 2 pre-adipocytes) are adipogenic. This could make the paper acceptable. I think the paper is relevant and the huge amount of work included advances the field. Also the paper globally considered is elegant in its conception and well written.

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: C Ronald Kahn
Journal Submitted to: The EMBO Journal
Manuscript Number:

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 χ2 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.

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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?	page 30
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	page 30
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	page 30
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.	page 30
For animal studies, include a statement about randomization even if no randomization was used.	page 30
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.	page 30
4.b. For animal studies, include a statement about blinding even if no blinding was done	page 30
5. For every figure, are statistical tests justified as appropriate?	Yes. All data are shown as mean ± SEM in Fig 1-8 and EV1-EV7.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. Normal distribution was assessed for all data (page 30).
Is there an estimate of variation within each group of data?	Yes. All data are shown as mean ± SEM in Fig 1-8 and EV1-EV7.
Is the variance similar between the groups that are being statistically compared?	Yes

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).	Table EV2.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	pages 26, 30.

* 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.	Species, strain, gender, age, and modification reported in Figure Legends pages 38-46. Housing, husbandry conditions, and animal sources on page 26.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Page 26.
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.	Confirmed.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable.
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.	Not applicable.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not applicable.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable.
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.	Not applicable.
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.	Not applicable.

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	Page 30.
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).	All data shown.
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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.	Not applicable.

G- Dual use research of concern

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