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Analysis of acetylation stoichiometry suggests that SIRT3 repairs nonenzymatic acetylation lesions

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Hartmut Vodermaier

1st Editorial Decision 18 March 2015

Thank you for submitting your manuscript on acetylation stoichiometry in liver tissue for consideration by The EMBO Journal. We have now received comments from four expert referees, copied below for your information. From these reports, I unfortunately have to conclude that we cannot publish the manuscript in its present form; however it is also apparent that the study has the potential to become a much more suitable candidate for an EMBO Journal article after a number of key issues raised by the referees may have been satisfactorily addressed.

One main concern is that for publication in a broad general journal like this one, the paper would require extensive re-writing to make it much less technical, e.g. by better segregating purely methodological descriptions from experimental results into respective manuscript 'Material & Methods' (currently very short!) and 'Results' sections. Moreover, the wider biological significance of the present work (which is clearly acknowledged by the expert referees) to a general readership would have to be more clearly laid out, especially in more explicit and accessible title and abstract. Furthermore, our 'article' format should provide an opportunity to re-balance main and expanded view figures (currently four vs. eleven!) to facilitate reading.

A related major problem is the insufficient description and explanation of experimental and mathematical concepts, as pointed out by referees 2 and 4. In particular the potential formula issue raised by referee 2 currently represents an overriding concern whose satisfactory clarification would be absolutely essential for further consideration.
Finally, referees 1 and 3 bring up several related queries concerning the biological results and insights from the present analyses. These may to a certain extent be addressed through in-depth discussion and/or improved presentation (e.g. for ref 1.2 or the related points ref 1.4/ref 3.1), but at least some of them would also appear to warrant data re-analysis and/or further experimentation, such as ref 1.3, ref 3.3, or the related points ref 1.1/ref 3.2.

Should you be able to satisfactorily address these main issues, taking into account also the various other more specific comments and suggestions from the referees, then we should be able to consider a revised manuscript further for publication. Please note however that it is our policy to allow only a single round of major revision, making it important to carefully respond to all points raised during this round; I would in this case be open to discussing an extension of our standard three-months revision time if needed. As always at The EMBO Journal, competing manuscripts published elsewhere during such an official revision period would have no negative impact on our final assessment of your revised study.

Thank you again for the opportunity to consider this work publication, and please do not hesitate to contact me should you have any questions regarding the referee reports or in case you would like to pre-discuss revision strategies/requirements. I look forward to hearing from you/receiving your revision.

REFEREE REPORTS:

Referee #1:

Summary

The manuscript by Weinert and colleagues builds upon their previous work quantifying acetyl-lysine stoichiometry in yeast by applying this method to the analysis of mammalian tissues and cell lines. The findings conclude that the majority of protein acetylation in tissues is of low stoichiometry and these estimates are even lower in rapidly dividing cultured cells. Their examination of acetylation in the fasted ATGL-KO tissues further validates and extends previous findings demonstrating that mitochondrial protein acetylation is heavily dependent on fat oxidation and that acetylation also affects fat oxidation proteins. These findings are interesting and represent an important advance in the field of acetylation and its regulation. However, the manuscript and its conclusions could be improved by addressing minor technical and editorial points.

Major comments

1. Regarding the stoichiometry at SIRT3-targeted sites. The study incorporates previously published analysis of relative acetyl-lysine changes in the absence of SIRT3 into their data analysis. While this is moderately useful in its current form, it would be interesting to use this data with the current stoichiometry data to estimate what the acetylation stoichiometry would be at SIRT3 regulated sites in the absence of SIRT3. For example, Hebert et al. reported that HMGCS2 K310 undergoes a 4-fold increase in acetylation in the absence of SIRT3. The current paper's data reports an acetyl-lysine stoichiometry for HMGCS2 K310 of 0.29-0.54%. Taking the Hebert, et al. data into account, this could mean that in the absence of SIRT3, the stoichiometry of K310 is between 1.16-2.16%. Thus, in the absence of SIRT3, the acetylation stoichiometry could now approaching functionally relevant levels. Especially if this is amplified across a pathway. This type of analysis of all putative SIRT3-regulated sites would be very interesting and would significantly enhance the primary data as well as the discussion/conclusion points implicating SIRT3 as a protein repair factor.

2. Regarding reactivity vs. stoichiometry. On page 10, the authors say that increased labeling of Sirt3-regulated sites suggests that they may be more reactive with AcP. However, they also assert that the increased labeling is because the Sirt3-regulated sites have lower stoichiometry? Which is it? This needs to be clarified, as the premise was the increased labeling indicated lower stoichiometry, independent of reactivity. Doesn't the notion that some classes of sites are more reactive call in to question the ability to relate this indirect assessment to stoichiometry? It seems impossible to know when a given site would be labeled due to a "reactivity effect" compared a "stoichiometry effect". This, at minimum, needs to be discussed.

3. Regarding the finding that "mitochondrial acetylation occurs at a higher median stoichiometry than cytoplasmic or nuclear acetylation". This point could be clarified considering histones and transcription factors probably have the highest acetylation stoichiometry in the cell. Indeed, previous work by this group found that histones and transcription factors have higher stoichiometry in yeast. Thus, it would be helpful to sub-divide cytoplasmic/nuclear proteins into functional categories and then discuss acetylation stoichiometry to address this confusion.

4. Regarding "insubstantial acetylation". The insubstantial stoichiometry at sites implicated in regulating protein function raises the possibility that acetylation does not occur at high enough levels to impact protein function through modification of individual sites, as described by the authors. However, no functional measurements were performed in this manuscript; thus, this language seems overly dismissive of several untested biological possibilities. For example, low acetylation does not mean that it cannot impact protein function, just because we cannot measure it. Or, it doesn't preclude the possibility that it could influence a pathway (as the authors briefly described). Additionally, low stoichiometry, even if non-enzymatic doesn't imply that this process was not selected for in evolution, and therefore provided some fitness advantage. The authors further describe the finding that "most acetylation occurs at an insubstantial stoichiometry, including SIRT3-regulated sites and other putative regulatory sites", but don't discuss the possibility that SIRT3 is required to maintain low stoichiometry at those site. Finally, most of the studies in this manuscript were performed in fed, "basal" conditions. Indeed, papers by the Alt and Auwerx groups showed early on that mice lacking SIRT3 don't have a phenotype under non-stressed conditions. Thus, future studies aimed at determining the stoichiometry under conditions (stressed, or otherwise) where SIRT3 is known to play a functional/physiological role, would be warranted. Together, the data presented as-is are an important advance for the field, but the interpretations of the data should be viewed in light of known vs. unknown biology.

Minor Comments

5. The authors use metabolic proteins and mitochondrial proteins interchangeably, which should be avoided. For example, the introduction incorrectly states that no acetyl-transferases are known to target metabolic proteins; while true for mitochondrial proteins, this is untrue for metabolic proteins in the cytoplasm.

6. The authors state that "A recent review suggested that nonenzymatic acetylation may represent a type of "carbon stress" which SIRT3 counteracts to ensure metabolic fidelity in mitochondria (Wagner & Hirschey, 2014). One way to test this idea is to determine the stoichiometry of acetylation on mitochondrial proteins and at SIRT3-regulated acetylation sites." However, this notion should be further explained. How exactly does determining the stoichiometry of acetylation test the carbon stress hypothesis?

7. An excessive amount of field-specific jargon is present, especially in the first half of the manuscript, which should be avoided to make the material more understandable to a broad readership

8. On page 9, the authors describe at length that unmodified peptides and acetyl peptides having no difference in ionization efficiency. [Indeed, acetylation neutralizes a lysine's charge, but causes a missed cleavage, making for another lysine at the c-term. Therefore, unmodified and acetyl peptides have a charge-carrying amine at the N and C term; the expectations would be for them to ionize similarly on average.] Why is this surprising?

Referee #2:

The authors employed quantitative MS and partial chemical acetylation strategy to estimate acetylation stoichiometry in mouse liver tissue. They claimed that mitochondrial proteins were acetylated at a very low stoichiometry and deduced that acetylation of mitochondrial proteins are mainly through spontaneous acetylating lysines by acetyl-CoA, which is of higher concentrations in mitochondria than in other compartments of the cells. They substantiated this conclusion by showing that fasting increased liver mitochondrial protein acetylation is dependent on fatty acids oxidation, which provide acetyl-CoA during fasting. The authors further proposed that SIRT3 functions as a protein repair factor instead of a regulator of metabolic enzymes. These findings and claims are seemingly novel but argue against most conclusions of many current reports. Looking into the detail of the study, this reviewer found that the mathematic model the author used to calculate the acetylation stoichiometry is mistakenly built and therefore, led to a wrong conclusion.

Major concerns:

1. In Figure 1A, it is hard to understand how chemical acetylation by AcP to sites with different initial acetylation stoichiometry resulted in the same 5% absolute increase in acetylation levels? The increase of chemical acetylation should be a function of amount of unacetylated lysine: C1=C*(1-I), in which C1 is the chemical acetylation stoichiometry, C* is AcP acetylation efficiency and I is the initial acetylation stoichiometry. The authors mistakenly set C1 as a fixed C that is independent of initial acetylation stoichiometry for all peptides.

After chemical acetylation, the total acetylation stoichiometry should be :
F1 = I+ C1 =I+ C* 1-I

The authors gave that F=I+C, which is correct in formula but with the C misinterpreted.

Now, the correct calculation for CP:

for peptides with only one lysine residue,

CP1= (1-F)/(1-I) ={1-[I+C*(1-I)]}/(1-I) = [1-I-C*(1-I)]/(1-I) = (1-I)*(1-C)/(1-I)=1-C

And for peptides with only two lysine residues,

CP2 = (1-F)/(1-I) = {1-[I+2*C*(1-I)-C* C*(1-I)]}/(1-I) = (1-C)2

Therefore, the CP value the authors used to estimate acetylation stoichiometry should be a fixed value, it is determined only by the efficiency of chemical acetylation and the number of number of lysines but have nothing to do with initial
acetylation stoichiometry I.

However, the authors gave a formula:

CP = (1-F)/(1-I) = (1-I-C) / (1-I)

and deduced :

I = (C+CP-1)/C

But, the experimental CP (i.e. CP1 as we gave above) should be 1-C, and if we replace CP in the authors' formula with CP1 (i.e., the experimental CP), it turned out that:

I = (C+CP-1)/C
=(C+CP1-1)/C
=(C+1-C-1)/C
=0

This well explained why the authors calculated that all the acetylation stoichiometry of known lysine sites in metabolic enzymes are all close to 0% (Figure 3B).

Moreover, if there are two lysine sites in the peptides, according to the author's equation:

I = (C+CP-1)/C
= (C+CP2-1)/C
=(C+(1-C)2-1)/C
=C-1

The calculated I should be negative value. However, the authors didn't gave such information in the manuscript. The reviewer suggest the authors check such information from the data and test whether this conclusion is correct.

In summary, this reviewer conclude that the authors used wrong assumption and reached wrong conclusion. The reviewer would also suggest the authors to re-examine the results of their published paper (B.T. Weinert et al., Molecular System Biology, Jan. 014) since similar strategy was used.

Minor points:

1. In this study and another study carried out by the same group of major authors, the in vitro acetylation assays were very poorly controlled. The trace amount of acetylation in proteins caused by commercial acetyl-CoA may simply due to there exists lysine modifying contaminants, such as acetyl-anhydride or acetyl-chloride in the commercial acetyl-CoA. Therefore, it is premature to conclude that acetyl-CoA spontaneously acetylates lysine side chain. More rigid control experiments are absolutely needed to exclude the contaminants effect before the authors can reach current conclusion.

2. It is incorrect to say that no mitochondria acetyltransferase was found in the discussion, in fact, Fan et al. had identified ACAT1 as a possible mitochondrial acetyltransferase (Mol. Cell. 2014 Feb 20;53(4):534-48)

3. If, like the authors claimed in this manuscript and in their previous papers that acetylation happens only at very low stoichiometry in metabolic enzymes, the influence of acetylation on metabolism should be trivial and dispensable. It is very hard to understand why cells express SIRT3, which is abundant in mitochondria, to deal with such trivial effects. The claim that SIRT3 functions as a repair factor of metabolism is, therefore, weakly funded.

Referee #3:

This manuscript by Weinert and colleagues tackles the difficult issue of measuring the absolute levels of acetylation on mitochondrial proteins by MS. Using several mutually complementary techniques, they find that acetylation levels at most sites are extremely low, <1%. This is an extremely surprising finding, given the dozens of papers already in the literature that purport to elucidate functional roles for acetylation on specific mitochondrial proteins.

This careful, thoughtful manuscript will be of general interest to the readership of EMBO, and will no doubt spark controversy and vigorous, healthy debate within the mitochondrial acetylation field. It has the potential to be a landmark paper and to significantly alter the way we think about mitochondrial acetylation. However, I do have some comments, mostly related to places in the manuscript that I believe could use additional experimentation, or at least discussion:

1. As the authors note, mice lacking the mitochondrial deacetylase SIRT3 show defects under stress conditions, including high fat diet (HFD; see Hirschey et al., Nature 2011). Can the authors assess absolute stoichiometry of acetylation under HFD conditions?

2. Similarly, it is a missed opportunity for the authors not to have directly assessed acetylation in SIRT3 KO animals. One could always argue that acetylation is low in wild-type animals because SIRT3 is a very efficient deacetylase. I am aware that many of the acetylation sites quantified in this study are not apparently SIRT3 target sites, as identified in a prior study (Hebert et al., Mol Cell 2013). However, in the absence of actually measuring acetylation in the SIRT3 KO in parallel with wild type, this remains a formal possibility.

3. It is very interesting that ATGL KO mice showed reduced mitochondrial acetylation. Ideally, the authors would cross these animals to the SIRT3 KO, and test rescue of the phenotypes observed in the latter mutant. This could be done by KD studies in cells instead. Acknowledging that these would represent long-term studies, probably outside the scope of the current work, can the authors at least measure activities or function of a couple of well-characterized SIRT3 targets in the ATGL mutant (complex I? SOD2?) to test whether they show increased activity, relative to wild-type control?

Minor points:

1. Some of the supplementary figures (e.g. E2) are reproduced very small, at the corner of the page.

2. For the statement "Calorie restriction causes a global increase in mitochondrial acetylation ...", the authors also need to cite Schwer et al., Aging Cell 2009.

Referee #4:

The manuscript from Weinert et al. entitled "Acetylation stoichiometry in liver tissue and at SIRT3-regulated sites" describes the use of several quantitative proteomics methods to study the acetylation stoichiometry. Although they measure in liver tissue in the context of SIRT3-regulation as it is clearly stated in the title, these qualifiers just make the title a bit awkward since it is quite likely that the findings in this interesting manuscript are farther reaching. As such, I recommend using a more generic title.

The authors carry out a very thorough analysis using SILAC- and TMT-based quantitative proteomics methods, and AQUA with targeted MS for the confirmation of some of the findings. This use of several quantitative proteomics methods, which do not really provide complementary information, but just corroborate the initial findings makes this manuscript rather technology heavy, which a) is not necessary and b) raises the question whether this manuscript in its current form is appropriate for EMBO Journal. This notion is further supported by a difficult to read first part of the result section, that i) is badly structured and ii) often lacks clarity. In this context, it is not helpful at all that e.g. the crucial definition of the stoichiometry, which should be "degree of chemical acetylation/(acetyl ratio - 1)", features incorrect bracketing. Instead the authors write twice (in figure 1 and on page 5) "(degree of chemical acetylation/acetyl ratio - 1)"; on page 10, they finally use the right formula). (Minor point: it would be helpful if the authors were to stick to one wording: either 'acetyl ratio' or 'ratio increased acetylation').

It is also not clear to this reviewer as to why the authors always provide various different "AcP-treated/control" ratios as y-axis (in figure 2 alone, there are 4 flavors thereof: i) log2 ratio; ii) median ratio 100mM; iii) median TMT ratio; and iv) median SILAC ratio). It might be helpful if always the acetylation stoichiometry ratios were given (and the appropriate subheader clarifies the context).

On page 12, the authors introduce in addition to the three quantitative LC/MS methods for quantifying the acetylation stoichiometry a fourth method, based on iBAQ, to confirm their results. Unfortunately, the authors don't really introduce and/or explain the 'abundance-corrected acetylated peptide intensity (ACI)'. At this moment, it is not even clear whether the authors merely want to say that the SILAC and TMT-based findings are consistent with their findings from a previous publication or whether they truly do some additional data analysis. For the former, this section is too long, for the latter, this section lacks clarity.

The authors seem to overdo it a bit when using four different proteomics methods for estimating the acetylation stoichiometry; unfortunately, this scientific rigor is missing when they compare the outcome of the (post-mitotic) tissue samples with actively dividing cells in culture. Since at least two major parameters are correlated/changed at the same time (dividing vs. post-mitotic cells; cell culture vs. tissue), the authors should be very careful drawing any conclusion as to whether changes are due to the cells being in culture or whether it is due to the fact that they actively dividing). Thus, it would be useful if the authors used a comparison with only one variable changed at a time.

While all the interpretations and conclusions drawn from the results make sense and are very interesting, the authors unfortunately stop short proposing (and possibly) carrying out experiments to test some of the resulting hypotheses. Instead, there is a (too) lengthy (though very thorough) discussion about the effect of calorie restriction on protein acetylation and currently available inconsistent findings, which might be due to the fact that acetylation site mutants can result in overestimating modification stoichiometries as the authors rightly point out towards the end of the manuscript.

An important aspect for the authors is the role of SIRT3; they talk a lot about SIRT3-regulated sites and use the notation (SIRT3 >2 etc.). Unfortunately, the authors completely miss to properly introduce in the main text a) the notation and b) where the 'SIRT3-regulated' sites are coming from. Instead, the readers have to rely on the legend of Figure 2 to finally be informed. Given the importance of these aspects, a proper introduction in the main text on page 6 is absolutely necessary.

In summary, the authors have beautiful and exciting data, which allows them to formulate very interesting hypotheses (which are unfortunately not tested). However, the manuscript is badly written and structured, with too much emphasis on the (excellent, but badly explained) analytical aspects, such that the main message gets lost since many readers will get stuck in the hard to understand first half of the result section. I strongly suggest that the authors:

i) significantly shorten and streamline the technical aspect of acetylation stoichiometry measurements by e.g. removing the TMT-part and the ACI part (especially since the ACI part is not properly described anyway),
ii) make sure all formulas are correct and try to clarify the explanations of their math and the ideas behind the math;
iii) more clearly structure the first half of the results section,
iv) tone down the conclusions drawn from the tissue vs. cell culture experiments,
v) and instead expand on a discussion about how to test their hypothesis and carry out some of these hypothesis tests.
vi)

Then, the manuscript should be considered for publication in EMBO Journal.

1st Revision - authors' response 16 June 2015

Response to reviewers' comments

We would like to thank the reviewers for critically evaluating our manuscript. Their comments helped us to improve technical aspects of this work, such as how we calculate stoichiometry, and helped us to focus the data analysis and discussion to provide a more coherent presentation of our data. Here we summarize some of the major points raised by the reviewers, and the most substantial changes made to the revised manuscript.

1. Reviewers #1 and #4, and the editor felt that the manuscript was overly technical and suggested restructuring it to make it understandable to a broad readership. We have extensively revised the introduction and discussion sections, and moved purely technical details (such as details of how we calculated median CP ratios and how we performed AQUA quantification) from the results to the materials and methods section. We further removed technical details and figures that were unnecessary or redundant, for example, the section on abundance-corrected intensity (ACI) was removed as we now include orthogonal validation based on 100% chemical acetylation.

2. Reviewer #2 pointed out an error in our formula for calculating acetylation site stoichiometry, and suggested to analyze the data using a corrected formula. Based on the reviewer's feedback we revised our equation, and further showed that we could calculate stoichiometry using two independently derived equations that provided identical results. We further revised Figure 1 to more clearly explain how we calculated stoichiometry estimates in this study.

3. In order to provide further support for the accuracy of our method we performed additional orthogonal validation using comprehensive (100%) chemical acetylation. We spiked-in known quantities of comprehensively acetylated proteins (akin to AQUA) in our samples to measure stoichiometry at more than 300 acetylation sites. These data provide further orthogonal validation for our stoichiometry estimates based on partial chemical acetylation.

4. Reviewers #1, #3, and #4 suggested that we investigate acetylation stoichiometry in SIRT3 KO animals and under conditions of dietary manipulations. In response to these suggestions we have predicted acetylation stoichiometry in SIRT3 KO animals, calorie-restricted SIRT3 KO animals, and calorie-restricted wild-type animals (Figure 3D). We further investigated how fasting affected acetylation levels at SIRT3-targeted sites in the liver, heart, brain, and brown adipose tissue of wild-type and ATGL-deficient mice (Figure 4). Analysis of these data, and previously published studies (Appendix Fig S5), enabled us to assess the impact of SIRT3 on its targets under steady state and dietary manipulations.

5. In response to reviewer #1, comment #3 we present a new analysis of high stoichiometry (>1%) acetylation in the results section (Figure 3B and 3C).

6. Based on all reviewer feedback the discussion section has been extensively edited to avoid reviewing much of the published literature and to instead focus on how our stoichiometry data help in better understanding the importance of SIRT3 in suppressing acetylation of mitochondrial proteins.

Below, we provide a detailed point-by-point response to the reviewers' comments.

Referee #1:

Summary
The manuscript by Weinert and colleagues builds upon their previous work quantifying acetyl-lysine stoichiometry in yeast by applying this method to the analysis of mammalian tissues and cell lines. The findings conclude that the majority of protein acetylation in tissues is of low stoichiometry and these estimates are even lower in rapidly dividing cultured cells. Their examination of acetylation in the fasted ATGL-KO tissues further validates and extends previous findings demonstrating that mitochondrial protein acetylation is heavily dependent on fat oxidation and that acetylation also affects fat oxidation proteins. These findings are interesting and represent an important advance in the field of acetylation and its regulation. However, the manuscript and its conclusions could be improved by addressing minor technical and editorial points.

We thank the reviewer for his/her assessment and insightful comments. Following the reviewers recommendation we have estimated stoichiometry of SIRT3 targets under different nutritional conditions, and this has greatly helped to assess the impact of SIRT3 under these conditions.

Major comments

1. Regarding the stoichiometry at SIRT3-targeted sites. The study incorporates previously published analysis of relative acetyl-lysine changes in the absence of SIRT3 into their data analysis. While this is moderately useful in its current form, it would be interesting to use this data with the current stoichiometry data to estimate what the acetylation stoichiometry would be at SIRT3 regulated sites in the absence of SIRT3. For example, Hebert et al. reported that HMGCS2 K310 undergoes a 4-fold increase in acetylation in the absence of SIRT3. The current paper's data reports an acetyl-lysine stoichiometry for HMGCS2 K310 of 0.29-0.54%. Taking the Hebert, et al. data into account, this could mean that in the absence of SIRT3, the stoichiometry of K310 is between 1.16-2.16%. Thus, in the absence of SIRT3, the acetylation stoichiometry could now approaching functionally relevant levels. Especially if this is amplified across a pathway. This type of analysis of all putative SIRT3-regulated sites would be very interesting and would significantly enhance the primary data as well as the discussion/conclusion points implicating SIRT3 as a protein repair factor.

We thank the reviewer for this suggestion, we have performed the suggested analysis and the results are presented in Figure 3D. These data support the notion that acetylation reaches an appreciable stoichiometry in SIRT3 KO animals that is likely sufficient to mediate the metabolic effects seen in those animals, particularly if multiple proteins and acetylation sites are affected in a common metabolic pathway. The relevant manuscript text is as follows;

"SIRT3-deficient animals have reduced metabolic function, presumably due to increased mitochondrial protein acetylation. In order to systematically investigate acetylation stoichiometry in SIRT3-deficient animals we used previously determined changes in liver protein acetylation in SIRT3 KO, calorie-restricted SIRT3 KO, and calorie-restricted wild-type mice (Hebert et al, 2013) to predict acetylation stoichiometry under these conditions (Fig 3D and Dataset EV1). SIRT3-targeted sites (S3>2) were predicted to have a median 4-fold increase in acetylation stoichiometry in SIRT3 KO animals, this increase was slightly greater (5-fold) in calorie-restricted SIRT3 KO animals. Sites that were most affected by loss of SIRT3 (S3>8) were predicted to have the greatest increase in acetylation stoichiometry, ~11-fold in SIRT3 KO animals and ~20-fold in calorie-restricted SIRT3 KO animals (Fig 3D). Glycine N-acyltransferase (GLYAT) K127 is ~57-fold elevated in both fed and calorie-restricted SIRT3 KO animals (Hebert et al, 2013). We estimated that this lysine is ~1.5% acetylated in wild-type animals, suggesting that acetylation stoichiometry could be as high as 87% in the absence of SIRT3. In addition, we predicted that hydroxymethylglutaryl-CoA lyase (HMGCL) K48 is ~16% acetylated and methylmalonyl-CoA epimerase (MCEE) K152 is ~9% acetylated in SIRT3 KO animals. In total, 48 SIRT3-targeted sites were predicted to have high (>1%) stoichiometry in SIRT3 KO animals and 57 SIRT3-targeted sites were predicted to have high stoichiometry in calorie-restricted SIRT3 KO animals. In contrast, only 13 SIRT3-targeted sites were predicted to have high stoichiometry in calorie-restricted wild-type animals. SIRT3-targeted sites were not substantially altered by calorie restriction in wild-type animals, therefore, the stoichiometry we predicted at these sites was similarly unaltered (Fig 3D, compare WT to WT calorie-restricted). Notably, sites not regulated by SIRT3 were slightly increased (median 2.3-fold) by calorie restriction in wild-type animals, while the median stoichiometry of SIRT3-targeted sites was unchanged. These analyses suggest that acetylation reaches at sufficient stoichiometry in SIRT KO animals to account for the metabolic defects seen in these animals, particularly if multiple proteins and acetylation sites are affected in a common metabolic pathway."

2. Regarding reactivity vs. stoichiometry. On page 10, the authors say that increased labeling of Sirt3-regulated sites suggests that they may be more reactive with AcP. However, they also assert that the increased labeling is because the Sirt3-regulated sites have lower stoichiometry? Which is it? This needs to be clarified, as the premise was the increased labeling indicated lower stoichiometry, independent of reactivity. Doesn't the notion that some classes of sites are more reactive call in to question the ability to relate this indirect assessment to stoichiometry? It seems impossible to know when a given site would be labeled due to a "reactivity effect" compared a "stoichiometry effect". This, at minimum, needs to be discussed.

The reviewer raises an important issue that affects the precision of our stoichiometry estimates. As explained below we now account for greater reactivity at SIRT3-targeted sites and we further indicate that reactivity differs for individual sites, which affects the accuracy of our stoichiometry calculations. The measured increase in acetylation at any given site is a function of both reactivity and initial stoichiometry, increased acetylation cannot be attributed to reactivity or stoichiometry alone. The reactivity at individual positions determines the degree (%) of partial chemical acetylation; we estimated the degree of acetylation by using the median CP ratio for different classes of sites. Since SIRT3-targeted sites showed greater reactivity (lower median CP ratios), we used the median CP ratios

for these sites in order to calculate their stoichiometry. Thus, we accounted for the difference in reactivity at these sites, as indicated in the revised manuscript.

*"In order to more accurately estimate stoichiometry at SIRT3-targeted sites we used the CP ratios shown in **Figure 1E** to calculate stoichiometry at these sites (0.94 for S3>2, 0.93 for S3>4, and 0.92 for S3>8)."*

However it is likely that individual positions differ in their reactivity and we cannot account for these differences. This possibility is indicated in the manuscript as follows.

"We consider these calculations to be estimates, since the CP ratios for individual sites were not determined and the degree of chemical acetylation at individual sites may be variable."

The inability to account for reactivity affects the precision of our measurements, however, our validation experiments using AQUA peptides and our new validation experiments using 100% acetylation (where lysine reactivity should not matter as all sites should be 100% acetylated) show that our estimates based on partial (5%) chemical acetylation are mostly accurate.

3. Regarding the finding that "mitochondrial acetylation occurs at a higher median stoichiometry than cytoplasmic or nuclear acetylation". This point could be clarified considering histones and transcription factors probably have the highest acetylation stoichiometry in the cell. Indeed, previous work by this group found that histones and transcription factors have higher stoichiometry in yeast. Thus, it would be helpful to sub-divide cytoplasmic/nuclear proteins into functional categories and then discuss acetylation stoichiometry to address this confusion.

The reviewer correctly points out that many acetylation sites on histones and transcriptional regulators have functional roles and likely occur at higher stoichiometry than sites modified non-specifically. Indeed, we previously found that in budding yeast high stoichiometry sites primarily occurred on such proteins, but the number of high stoichiometry sites was small and the majority of sites found on nuclear proteins were of low stoichiometry. Here we detected few high stoichiometry sites on nuclear proteins, likely due to the specialized composition of liver tissue, which is very rich in mitochondria. However, in this study our focus was on the stoichiometry of acetylation on mitochondrial and metabolic proteins, and at SIRT3-targeted sites in particular. In order to investigate the types of proteins with high (>1%) stoichiometry acetylation in our data we performed Gene Ontology enrichment analysis (Figure 3B), and we have added further text to discuss high stoichiometry acetylation sites as follows;

"In yeast, high stoichiometry acetylation sites were associated with nuclear proteins involved in chromatin organization and transcription (Weinert et al, 2014). In this study we estimated acetylation stoichiometry on a known regulatory site in the nucleus, structural maintenance of chromosomes protein 3 (SMC3) K106, which was 4% acetylated (Rolef Ben-Shahar et al, 2008; Zhang et al, 2008). However, most of the high stoichiometry sites we detected were on mitochondrial proteins, perhaps because the abundance of mitochondrial proteins in liver tissue limited the dynamic range of our analysis. Of the 4,322 sites with stoichiometry estimates, 344 occurred on proteins associated with the

UniProt keyword "nucleus" while 1,532 occurred on proteins associated with the keyword "mitochondrion" (Dataset EV1). Although we detected known regulatory acetylation sites on histone N-terminal domains, these sites occurred on multiply acetylated peptides for which we were unable to accurately estimate stoichiometry and were therefore excluded from our analysis.
High stoichiometry acetylation (>1% acetylated) was overrepresented on mitochondrial proteins, acyltransferases, lyases, butanoate metabolism, and valine, leucine and isoleucine degradation (Fig 3B). Overrepresentation of high stoichiometry acetylation sites on mitochondrial proteins is consistent with our observation of significantly higher median stoichiometry in this organelle (Fig 3A). We further observed that high stoichiometry sites were frequently found on enzymes that catalyze reactions involving acetyl-groups, such as AACS (K391, 13% acetylated), NAA30 (K235, 8% acetylated), NAA50 (K34, 5% acetylated), ACSM1 (K200, 5% acetylated), KEG1 (K128, 4% acetylated), and GLYAT (K127, 1.5% acetylated). These findings suggest that enzymes that transfer acetyl groups may accumulate higher levels of acetylation due to autocatalysis or higher local concentrations of acetyl-metabolites. A recent study similarly found that acetyl-CoA acetyltransferase 1 (ACAT1) catalyzed lysine acetylation of pyruvate dehydrogenase (PDHA1) K321 and pyruvate dehydrogenase phosphatase (PDP1) K202, mediating the Warburg effect in EGF-treated and immortalized cells (Fan et al, 2014). We determined acetylation stoichiometry at PDHA1 K321 by SILAC (0.84% acetylated) and TMT (0.39% acetylated). The low stoichiometry found at this position in liver tissue is consistent with the idea that PDHA1 K321 acetylation is only found after EGF stimulation or in immortalized cell lines (Fan et al, 2014)."

4. Regarding "insubstantial acetylation". The insubstantial stoichiometry at sites implicated in regulating protein function raises the possibility that acetylation does not occur at high enough levels to impact protein function through modification of individual sites, as described by the authors. However, no functional measurements were performed in this manuscript; thus, this language seems overly dismissive of several untested biological possibilities. For example, low acetylation does not mean that it cannot impact protein function, just because we cannot measure it. Or, it doesn't preclude the possibility that it could influence a pathway (as the authors briefly described). Additionally, low stoichiometry, even if non-enzymatic doesn't imply that this process was not selected for in evolution, and therefore provided some fitness advantage. The authors further describe the finding that "most acetylation occurs at an insubstantial stoichiometry, including SIRT3-regulated sites and other putative regulatory sites", but don't discuss the possibility that SIRT3 is required to maintain low stoichiometry at those site. Finally, most of the studies in this manuscript were performed in fed, "basal" conditions. Indeed, papers by the Alt and Auwerx groups showed early on that mice lacking SIRT3 don't have a phenotype under non-stressed conditions. Thus, future studies aimed at determining the stoichiometry under conditions (stressed, or otherwise) where SIRT3 is known to play a functional/physiological role, would be warranted. Together, the data presented as-is are an important advance for the field, but the interpretations of the data should be viewed in light of known vs. unknown biology.

We agree that the term "insubstantial" makes a qualitative judgment that the data does not support and we have revised this term to "very low".

Our conclusion that SIRT3 is required to maintain low stoichiometry acetylation is now clearly mentioned in the abstract and in the discussion.

The reviewers suggestion that we investigate acetylation stoichiometry under stressed conditions has been addressed by the new analyses where we have predicted acetylation stoichiometry in calorie-restricted animals (Figure 3D), we have investigated changes in acetylation at SIRT3-targeted sites in fasted animals (Figure 4), and we have analyzed previously published data describing acetylation changes in fasted and obese mice (Figure E7). These analyses support the notion that SIRT3 is likely important to suppress acetylation under these conditions.

Minor Comments

5. The authors use metabolic proteins and mitochondrial proteins interchangeably, which should be avoided. For example, the introduction incorrectly states that no acetyl-transferases are known to target metabolic proteins; while true for mitochondrial proteins, this is untrue for metabolic proteins in the cytoplasm.

We agree with the reviewer´s point, and edited the manuscript to more clearly refer to mitochondrial proteins.

6. The authors state that "A recent review suggested that nonenzymatic acetylation may represent a type of "carbon stress" which SIRT3 counteracts to ensure metabolic fidelity in mitochondria (Wagner & Hirschey, 2014). One way to test this idea is to determine the stoichiometry of acetylation on mitochondrial proteins and at SIRT3-regulated acetylation sites." However, this notion should be further explained. How exactly does determining the stoichiometry of acetylation test the carbon stress hypothesis?

The manuscript has been edited to further explain how measuring stoichiometry tests this hypothesis, both in the introduction and in the discussion section.

"A recent review suggested that nonenzymatic acetylation may represent a type of "carbon stress" which SIRT3 counteracts to ensure metabolic fidelity in mitochondria (Wagner & Hirschey, 2014). If this were the case we would expect that SIRT3 suppresses acetylation to very low stoichiometry under both basal conditions and the conditions that cause increased acetylation of mitochondrial proteins, such as calorie restriction and fasting."

"The results presented in this study provide support for the idea that SIRT3 repairs nonenzymatic acetylation lesions on mitochondrial proteins in order to preserve their normal function. Most importantly we found that SIRT3 suppressed acetylation to a very low stoichiometry at targeted sites. This suggests that SIRT3 is unlikely to regulate protein function by further deacetylating mitochondrial proteins, as the stoichiometry of acetylation is already very low, and acetylation at SIRT3-targeted sites typically inhibits enzymatic activity (Ghanta et al, 2013; Lin et al, 2012). We found that fasting did not result in reduced acetylation at SIRT3-targeted sites and these findings are consistent with previously published proteome-wide studies investigating mitochondrial acetylation in calorie-restricted, fasted, and obese mice (Hebert et al, 2013; Still et al, 2013). In light of our stoichiometry estimates, these results indicate that these dietary manipulations do not substantially alter acetylation levels at SIRT3-targeted sites generally. In contrast, loss of SIRT3 results in substantially increased acetylation at targeted sites that exceeds any changes observed at these same sites in fasted, calorie-

restricted, or obese wild-type animals, indicating that the role of SIRT3 in suppressing acetylation is greater than the effect of dietary manipulations on acetylation levels at these sites (for example, see Fig 3D). We found that SIRT3-targeted sites were similarly sensitive to fasting-induced acetylation in vivo and nonenzymatic acetylation in vitro, in a manner that was proportional to the degree of increased acetylation in SIRT3 KO animals. These data suggest that the increased acetylation at these sites in the absence of SIRT3 is a function of their inherent reactivity and the nonenzymatic origin of acetylation. This idea is further supported by our observation that fasting-induced acetylation at SIRT3-targeted sites was suppressed by loss of ATGL. Together, our data indicate that SIRT3 suppresses acetylation to a very low stoichiometry under all conditions analyzed, and suggest that a constitutive function of SIRT3 is to remove nonenzymatic acetylation lesions from mitochondrial proteins. The mitochondrial sirtuin deacylase SIRT5 suppresses both succinylation and glutarylation in mitochondria (Du et al, 2011; Park et al, 2013; Rardin et al, 2013a; Tan et al, 2014), and both of these modifications may occur nonenzymatically (Tan et al, 2014; Wagner & Payne, 2013; Weinert et al, 2013b), suggesting that sirtuin deacylases may have a general role in protecting mitochondrial proteins from reactive acyl-CoA metabolites."

7. An excessive amount of field-specific jargon is present, especially in the first half of the manuscript, which should be avoided to make the material more understandable to a broad readership

The first part of the manuscript has been extensively edited to make the manuscript better understandable to a broad readership, and most of the specific technical details have been moved to the materials and methods section.

8. On page 9, the authors describe at length that unmodified peptides and acetyl peptides having no difference in ionization efficiency. [Indeed, acetylation neutralizes a lysine's charge, but causes a missed cleavage, making for another lysine at the c-term. Therefore, unmodified and acetyl peptides have a charge-carrying amine at the N and C term; the expectations would be for them to ionize similarly on average.] Why is this surprising?

We agree with the reviewer that this observation is not necessarily surprising, however we felt that it may not be clear to researchers outside the field and it has not (to our knowledge) been demonstrated. The implication of this observation is that if acetylation were occurring at stoichiometric levels we should be able to detect it without affinity enrichment (as we show in Figure E2). Regardless, we removed this discussion from the revised manuscript to avoid overly technical points that distract readers from the core message, and our data shows that acetylation occurs at low stoichiometry without relying on this additional information.

Referee #2:

The authors employed quantitative MS and partial chemical acetylation strategy to estimate acetylation stoichiometry in mouse liver tissue. They claimed that mitochondrial proteins were acetylated at a very

low stoichiometry and deduced that acetylation of mitochondrial proteins are mainly through spontaneous acetylating lysines by acetyl-CoA, which is of higher concentrations in mitochondria than in other compartments of the cells. They substantiated this conclusion by showing that fasting increased liver mitochondrial protein acetylation is dependent on fatty acids oxidation, which provide acetyl-CoA during fasting. The authors further proposed that SIRT3 functions as a protein repair factor instead of a regulator of metabolic enzymes. These findings and claims are seemingly novel but argue against most conclusions of many current reports. Looking into the detail of the study, this reviewer found that the mathematic model the author used to calculate the acetylation stoichiometry is mistakenly built and therefore, led to a wrong conclusion.

We are grateful to the reviewer for carefully evaluating our work, and thank him/her for pointing out an error in our formula. We have corrected the error identified by the reviewer, and estimated stoichiometry using the revised formula. We also calculated stoichiometry using a previously published equation, and used an independent approach to estimate stoichiometry at >300 sites. Together, these results confirm our conclusions that most acetylation sites in our samples occurred at low levels.

Major concerns:

1. In Figure 1A, it is hard to understand how chemical acetylation by AcP to sites with different initial acetylation stoichiometry resulted in the same 5% absolute increase in acetylation levels? The increase of chemical acetylation should be a function of amount of unacetylated lysine: C1=C*(1-I), in which C1 is the chemical acetylation stoichiometry, C* is AcP acetylation efficiency and I is the initial acetylation stoichiometry. The authors mistakenly set C1 as a fixed C that is independent of initial acetylation stoichiometry for all peptides.

After chemical acetylation, the total acetylation stoichiometry should be:
F1 = I+ C1 =I+ C*（1-I）

The authors gave that F=I+C, which is correct in formula but with the C misinterpreted.

Now, the correct calculation for CP:

for peptides with only one lysine residue,

CP1= (1-F)/(1-I) ={1-[I+C*(1-I)]}/(1-I) = [1-I-C*(1-I)]/(1-I) = (1-I)*(1-C)/(1-I)=1-C

And for peptides with only two lysine residues,

CP2 = (1-F)/(1-I) = {1-[I+2*C*(1-I)-C* C*(1-I)]}/(1-I) = (1-C)2

Therefore, the CP value the authors used to estimate acetylation stoichiometry should be a fixed value, it is determined only by the efficiency of chemical acetylation and the number of number of lysines but have nothing to do with initial acetylation stoichiometry I.

However, the authors gave a formula:

CP = (1-F)/(1-I) = (1-I-C) / (1-I)

and deduced :

I = (C+CP-1)/C

But, the experimental CP (i.e. CP1 as we gave above) should be 1-C, and if we replace CP in the authors' formula with CP1 (i.e., the experimental CP), it turned out that:

I = (C+CP-1)/C
=(C+CP1-1)/C
=(C+1-C-1)/C
=0

This well explained why the authors calculated that all the acetylation stoichiometry of known lysine sites in metabolic enzymes are all close to 0% (Figure 3B).

Moreover, if there are two lysine sites in the peptides, according to the author's equation:

I = (C+CP-1)/C
= (C+CP2-1)/C
=(C+(1-C)2-1)/C
=C-1

The calculated I should be negative value. However, the authors didn't gave such information in the manuscript. The reviewer suggest the authors check such information from the data and test whether this conclusion is correct.

In summary, this reviewer conclude that the authors used wrong assumption and reached wrong conclusion. The reviewer would also suggest the authors to re-examine the results of their published paper (B.T. Weinert et al., Molecular System Biology, Jan. 014) since similar strategy was used.

We thank the reviewer for pointing out two important considerations: (1) that 5% acetylation would be relative to the non-acetylated portion of the protein; and (2) that doubly acetylated peptides will have an acetylation level that is much lower (0.25%). While the reviewer correctly pointed out these issues, the formula that he/she derives is different from that presented in our text. We indicated that we calculated stoichiometry using the following equation; stoichiometry = % chemical acetylation / (Ratio increased acetylation – 1). We did not use the equation (I = (C+CP-1)/C) as derived by the reviewer.

We believe that the model previously presented in Figure 1A led to this confusion and we have updated Figure 1 to more clearly present our method and the equation used to calculate stoichiometry.

In order to address the reviewer's concerns we derived the correct equation as follows:
I = initial stoichiometry, F = final stoichiometry, C = % chemical acetylation
F = I + C(1-I) *here we account for the point raised by reviewer #2 that "*The increase of chemical acetylation should be a function of amount of unacetylated lysine: C1=C*(1-I)*"
We measured the ratio of increased acetylation = (F/I), and the degree chemical acetylation = C
Using the above equation and solving for I in terms of (F/I) and C we derived the following equation;
$I = C / ((F/I) - (1-C))$
For 5% chemical acetylation this equals;
$I = 0.05 / ((F/I) – 0.95)$
For comparison, the equation that we used previously was;
$I = 0.05 / ((F/I) – 1)$

In light of the reviewer's comments we now correctly find that 5% chemical acetylation should result in a CP ratio of 0.95 for all sites. We realized that we could also calculate stoichiometry using the equation presented in Olsen et al (Science Signaling, 2010) and it is this equation that we now present in Figure 1C. Stoichiometry calculated using this equation gave an identical result to stoichiometry calculated using the equation we derived above. Thus, we verified our calculations by using two independently derived equations that gave identical results.

Equation derived here: $I = C / ((F/I) – (1-C))$
Where I = initial stoichiometry, F = final stoichiometry, C = % chemical acetylation

Equation from Olsen: $I = ((1-Cp) / ((F/I)-1)) / (1+ ((1-Cp) / ((F/I)-1)))$
Where CP ratio Cp = 1-C

If C = 0.05 and F/I = 10 both equations give the same result of 0.55%

In order to address the second point raised by the reviewer (that doubly acetylated peptides will have a much lower degree of chemical acetylation), we removed doubly acetylated peptides from our analysis (a small portion of the analyzed sites occurred on such peptides).

In order to provide additional support for our findings we further validated our results using 100% chemical acetylation to estimate stoichiometry (Figure 2D). These new results support the accuracy of our stoichiometry estimates based on partial (5%) chemical acetylation.

As suggested by the reviewer we have revisited our previous stoichiometry calculations (B.T. Weinert et al., Molecular System Biology, Jan. 2014) and found that using the correct equation had little impact on our stoichiometry estimates. Regardless, we are drafting a corrigendum to rectify the error in the formula and updating the relevant figures of this previous publication.

Minor points:

1. In this study and another study carried out by the same group of major authors, the in vitro acetylation assays were very poorly controlled. The trace amount of acetylation in proteins caused by commercial acetyl-CoA may simply due to there exists lysine modifying contaminants, such as acetyl-anhydride or acetyl-chloride in the commercial acetyl-CoA. Therefore, it is premature to conclude that acetyl-CoA spontaneously acetylates lysine side chain. More rigid control experiments are absolutely needed to exclude the contaminants effect before the authors can reach current conclusion.

We agree with the reviewer that commercial acetyl-CoA preparations may contain unknown acetylating agents and we wish to point out that we did not perform in-vitro acetylation with acetyl-CoA in this study. Furthermore, we think that the strongest evidence for nonenzymatic acetylation by acetyl-CoA is not based on in vitro reactions, rather it is based on the global and uniform fluctuations in low-level acetylation that depend on acetyl-CoA metabolism in distinct (mitochondrial versus non-mitochondrial) compartments as shown in our previous work in yeast (Weinert et al, Mol Sys Biol 2014) , the study by Pougovkina et al (Hum Mol Genet. 2014), and in this study using mouse tissues in an ATGL KO mouse model.

2. It is incorrect to say that no mitochondria acetyltransferase was found in the discussion, in fact, Fan et al. had identified ACAT1 as a possible mitochondrial acetyltransferase (Mol. Cell. 2014 Feb 20;53(4):534-48)

We have revised the manuscript and include discussion of this interesting study in our revised manuscript. The following was added to the introduction section of the manuscript.

"While thousands of acetylation sites have been identified on mitochondrial proteins, few acetyltransferases have been implicated in their regulation (Fan et al, 2014; Scott et al, 2012),"

The study by Fan et al (Mol. Cell. 2014 Feb 20;53(4):534-48) is further discussed in the results section and the discussion section.

"A recent study similarly found that acetyl-CoA acetyltransferase 1 (ACAT1) catalyzed lysine acetylation of pyruvate dehydrogenase (PDHA1) K321 and pyruvate dehydrogenase phosphatase (PDP1) K202, mediating the Warburg effect in EGF-treated and immortalized cells (Fan et al, 2014). We determined acetylation stoichiometry at PDHA1 K321 by SILAC (0.84% acetylated) and TMT (0.39% acetylated). The low stoichiometry found at this position in liver tissue is consistent with the idea that PDHA1 K321 acetylation is only found after EGF stimulation or in immortalized cell lines (Fan et al, 2014)."

"… with the exception of a single study (Fan et al, 2014), a regulatory axis between enzyme-catalyzed acetylation and SIRT3-mediated deacetylation has not been demonstrated."

3. If, like the authors claimed in this manuscript and in their previous papers that acetylation happens

only at very low stoichiometry in metabolic enzymes, the influence of acetylation on metabolism should be trivial and dispensable. It is very hard to understand why cells express SIRT3, which is abundant in mitochondria, to deal with such trivial effects. The claim that SIRT3 functions as a repair factor of metabolism is, therefore, weakly funded.

This appears to be a misunderstanding, perhaps resulting from our unclear presentation. We wish to clarify that we do not conclude that the role of SIRT3 as repair factor means that its functions are trivial. On the contrary, we claim that SIRT3 is required to suppress acetylation to low levels. In the absence of SIRT3 acetylation is substantially increased at hundreds, if not thousands of sites. Many previous studies have established that SIRT3-deficient animals have metabolic problems, most notably under conditions that increase mitochondrial acetylation. Therefore, SIRT3 function, as a repair factor, is most important under these conditions. These ideas are consistent with the new data analyses included in the revised manuscript.

Referee #3:

This manuscript by Weinert and colleagues tackles the difficult issue of measuring the absolute levels of acetylation on mitochondrial proteins by MS. Using several mutually complementary techniques, they find that acetylation levels at most sites are extremely low, <1%. This is an extremely surprising finding, given the dozens of papers already in the literature that purport to elucidate functional roles for acetylation on specific mitochondrial proteins.

This careful, thoughtful manuscript will be of general interest to the readership of EMBO, and will no doubt spark controversy and vigorous, healthy debate within the mitochondrial acetylation field. It has the potential to be a landmark paper and to significantly alter the way we think about mitochondrial acetylation. However, I do have some comments, mostly related to places in the manuscript that I believe could use additional experimentation, or at least discussion:

We thank the reviewer for his/her encouraging comments, and for very helpful suggestions. We agree with the reviewer that there is little knowledge about acetylation site stoichiometry in the literature, and our results will spark healthy debate about how acetylation and SIRT3 affect metabolism in mitochondria.

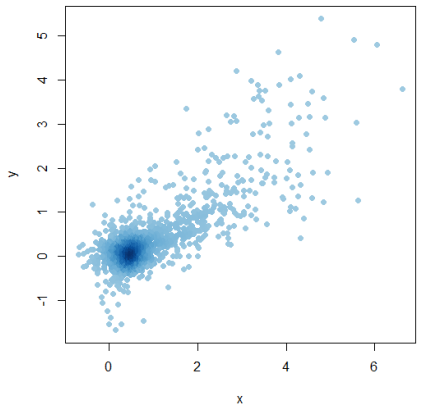
1. As the authors note, mice lacking the mitochondrial deacetylase SIRT3 show defects under stress conditions, including high fat diet (HFD; see Hirschey et al., Nature 2011). Can the authors assess absolute stoichiometry of acetylation under HFD conditions?

In order to address this, and other reviewer comments, we analyzed previously published changes in liver lysine acetylation in calorie-restricted, fasted, and obese (high fat diet) mice. We also analyzed acetylation levels at SIRT3-targeted sites in our own fasted mice, to better understand how dietary changes affect acetylation at SIRT3-targeted sites in wild-type (SIRT3 containing) animals. These new data are presented in Figures 3 and 4. We show that, in wild type animals, acetylation levels are only modestly affected by calorie restriction, fasting, and obesity, consistent with our hypothesis that SIRT3

suppresses acetylation under these conditions. As the reviewer notes, SIRT3 deficiency has a greater impact under HFD, we believe this is consistent with a major role of SIRT3 as a protein repair factor removing acetylation from mitochondrial proteins.

2. Similarly, it is a missed opportunity for the authors not to have directly assessed acetylation in SIRT3 KO animals. One could always argue that acetylation is low in wild-type animals because SIRT3 is a very efficient deacetylase. I am aware that many of the acetylation sites quantified in this study are not apparently SIRT3 target sites, as identified in a prior study (Hebert et al., Mol Cell 2013). However, in the absence of actually measuring acetylation in the SIRT3 KO in parallel with wild type, this remains a formal possibility.

Two previous studies have measured increased acetylation in the liver tissue of SIRT3 KO mice (Hebert et al, Mol Cell 2013 and Rardin et al PNAS 2013). Throughout the manuscript we used the sites defined by Hebert et al because their data set is more extensive. However, we found similar stoichiometry when using sites defined by Rardin et al (Figure 3B). Furthermore, the SIRT3-targeted sites defined in these two independent studies are highly significantly (P<2e-16) correlated (Pearsons correlation of 0.78, see plot below, x = Hebert et al, y = Rardin et al). Therefore, we consider it unnecessary to map SIRT3-targeted acetylation sites a third time. Based on the increased acetylation in SIRT3 KO animals we have predicted acetylation stoichiometry at these sites (Figure 3C and Table E1).



3. It is very interesting that ATGL KO mice showed reduced mitochondrial acetylation. Ideally, the authors would cross these animals to the SIRT3 KO, and test rescue of the phenotypes observed in the latter mutant. This could be done by KD studies in cells instead. Acknowledging that these would represent long-term studies, probably outside the scope of the current work, can the authors at least measure activities or function of a couple of well-characterized SIRT3 targets in the ATGL mutant (complex I? SOD2?) to test whether they show increased activity, relative to wild-type control?

The experiment suggested by the reviewer is reasonable and interesting; however we do not expect enzyme activity to be altered by reduced acetylation in ATGL KO animals in the presence of SIRT3. Furthermore, assaying enzymatic activity in ATGL KO mice would be compounded by the effects of

ATGL KO that are independent of acetylation levels, and any differences would be difficult to specifically attribute to reduced acetylation in these animals. Due to these complications in interpretation of results and time constraints of revision we feel that this interesting suggestion is better suited for future studies.

Minor points:

1. Some of the supplementary figures (e.g. E2) are reproduced very small, at the corner of the page.

The supplementary figures are now presented in the manuscript supplementary appendix at a sufficient size and with more optimal placement.

2. For the statement "Calorie restriction causes a global increase in mitochondrial acetylation ...", the authors also need to cite Schwer et al., Aging Cell 2009.

The referred to statement was removed from the revised manuscript, however, the reference has been added to a similar statement in the results section

"Interestingly, while calorie restriction and fasting cause a similar global increase in mitochondrial acetylation in liver tissue (Hebert et al, 2013; Pougovkina et al, 2014; Schwer et al, 2009), acetylation at SIRT3-targeted sites remains unchanged in calorie-restricted animals."

Referee #4:

The manuscript from Weinert et al. entitled "Acetylation stoichiometry in liver tissue and at SIRT3-regulated sites" describes the use of several quantitative proteomics methods to study the acetylation stoichiometry. Although they measure in liver tissue in the context of SIRT3-regulation as it is clearly stated in the title, these qualifiers just make the title a bit awkward since it is quite likely that the findings in this interesting manuscript are farther reaching. As such, I recommend using a more generic title.

The authors carry out a very thorough analysis using SILAC- and TMT-based quantitative proteomics methods, and AQUA with targeted MS for the confirmation of some of the findings. This use of several quantitative proteomics methods, which do not really provide complementary information, but just corroborate the initial findings makes this manuscript rather technology heavy, which a) is not necessary and b) raises the question whether this manuscript in its current form is appropriate for EMBO Journal. This notion is further supported by a difficult to read first part of the result section, that i) is badly structured and ii) often lacks clarity. In this context, it is not helpful at all that e.g. the crucial definition of the stoichiometry, which should be "degree of chemical acetylation/(acetyl ratio - 1)", features incorrect bracketing. Instead the authors write twice (in figure 1 and on page 5) "(degree of chemical acetylation/acetyl ratio - 1)"; on page 10, they finally use the
right formula). (Minor point: it would be helpful if the authors were to stick to one wording: either 'acetyl ratio' or 'ratio increased acetylation').

We thank the reviewer for his/her thoughtful comments. Following the reviewer´s (and editorial) suggestions we have extensively revised the manuscript to make it less technical and better understandable to broad readership. We apologize for the error in presenting the formula, in the revised manuscript the formula used to calculate stoichiometry is now more clearly defined in Figure 1C.

It is also not clear to this reviewer as to why the authors always provide various different "AcP-treated/control" ratios as y-axis (in figure 2 alone, there are 4 flavors thereof: i) log2 ratio; ii) median ratio 100mM; iii) median TMT ratio; and iv) median SILAC ratio). It might be helpful if always the acetylation stoichiometry ratios were given (and the appropriate subheader clarifies the context).

The figures have been edited to provide greater consistency

On page 12, the authors introduce in addition to the three quantitative LC/MS methods for quantifying the acetylation stoichiometry a fourth method, based on iBAQ, to confirm their results. Unfortunately, the authors don't really introduce and/or explain the 'abundance-corrected acetylated peptide intensity (ACI)'. At this moment, it is not even clear whether the authors merely want to say that the SILAC and TMT-based findings are consistent with their findings from a previous publication or whether they truly do some additional data analysis. For the former, this section is too long, for the latter, this section lacks clarity.

The section was based on new data and the ACI data were used to demonstrate that relative stoichiometry estimated using a completely different approach agreed with our estimates based on partial chemical acetylation (SILAC or TMT). However the section using ACI has been removed from the revised manuscript since we now provide additional orthogonal validation of absolute, not relative, stoichiometry using 100% chemical acetylation (Figure 2D).

The authors seem to overdo it a bit when using four different proteomics methods for estimating the acetylation stoichiometry; unfortunately, this scientific rigor is missing when they compare the outcome of the (post-mitotic) tissue samples with actively dividing cells in culture. Since at least two major parameters are correlated/changed at the same time (dividing vs. post-mitotic cells; cell culture vs. tissue), the authors should be very careful drawing any conclusion as to whether changes are due to the cells being in culture or whether it is due to the fact that they actively dividing). Thus, it would be useful if the authors used a comparison with only one variable changed at a time.

The reviewer is correct to point out that we could not conclude with certainty whether increased acetylation occurred due to growth arrest, or due to changes in metabolism, or both. In the revised manuscript we provide evidence that growth-arresting HeLa cells causes a similar increase in mitochondrial acetylation (Figure 5A) and we further acknowledge that the observed higher acetylation in tissues may not be due to the same mechanism by which growth-arrest induces higher acetylation, as follows:

"We suspect that growth-arrest causes increased acetylation by prolonging the exposure of proteins to acetyl-CoA, however, the precise mechanism remains unclear and it is possible that other physiological differences between cultured cells and tissues results in higher levels of mitochondrial acetylation in tissues."

While all the interpretations and conclusions drawn from the results make sense and are very interesting, the authors unfortunately stop short proposing (and possibly) carrying out experiments to test some of the resulting hypotheses. Instead, there is a (too) lengthy (though very thorough) discussion about the effect of calorie restriction on protein acetylation and currently available inconsistent findings, which might be due to the fact that acetylation site mutants can result in overestimating modification stoichiometries as the authors rightly point out towards the end of the manuscript.

An important aspect for the authors is the role of SIRT3; they talk a lot about SIRT3-regulated sites and use the notation (SIRT3 >2 etc.). Unfortunately, the authors completely miss to properly introduce in the main text a) the notation and b) where the 'SIRT3-regulated' sites are coming from. Instead, the readers have to rely on the legend of Figure 2 to finally be informed. Given the importance of these aspects, a proper introduction in the main text on page 6 is absolutely necessary.

The definition of SIRT3-targeted (SIRT3-regulated) sites is now included in the main manuscript text, as well as the appropriate figure legends.

In summary, the authors have beautiful and exciting data, which allows them to formulate very interesting hypotheses (which are unfortunately not tested). However, the manuscript is badly written and structured, with too much emphasis on the (excellent, but badly explained) analytical aspects, such that the main message gets lost since many readers will get stuck in the hard to understand first half of the result section. I strongly suggest that the authors:

i) significantly shorten and streamline the technical aspect of acetylation stoichiometry measurements by e.g. removing the TMT-part and the ACI part (especially since the ACI part is not properly described anyway),

We decided to keep the TMT data to show that our results were not substantially biased by our quantitative method, and because the estimates based on TMT quantification can add further confidence to estimates based on SILAC quantification (several examples are cited in the revised manuscript). However the data based on ACI has become redundant with orthogonal validation based on 100% acetylation (Figure 2D) and has been removed from the manuscript.

ii) make sure all formulas are correct and try to clarify the explanations of their math and the ideas behind the math;

The formulas have been correctly explained and displayed in the revised manuscript (please refer to our response to Reviewer #2, major comment #1), further details are also included in the revised methods section.

iii) more clearly structure the first half of the results section,

The first half of the results section has been restructured to remove excessive technical and methodological details

iv) tone down the conclusions drawn from the tissue vs. cell culture experiments,

Please refer to the detailed response to the concern above.

v) and instead expand on a discussion about how to test their hypothesis and carry out some of these hypothesis tests.

We have expanded the discussion and analysis specifically to focus on the important question of how dietary manipulations affect acetylation at SIRT3-targeted sites in wild-type (SIRT3 proficient) animals. These new analyses are presented in Figures 3 and 4.

vi) Then, the manuscript should be considered for publication in EMBO Journal.

2nd Editorial Decision 07 August 2015

Thank you for submitting your revised manuscript for our consideration. Please excuse the delay in getting back to you with a decision, due to limited availability of our referees at this time of the years. All four original referees have now assessed the study once more, as well as your response letter to the initial comments, and in light of their feedback we have decided to proceed forward towards publication of this work in The EMBO Journal. As you will see, only referee 2 retains substantial reservations from the technical point of view, but having further discussed these experimental issues (and your response to referee 2's original comments) in detail with referee 4 (who themselves did not provide a formal re-review report at this time), we concluded that the remaining concerns of referee 2 would not warrant further delays in the publishing process of this work. Before formal acceptance of the paper, I would however like to ask to still consider the remaining minor issues brought up by referees 1 and 3 and to incorporate appropriate changes (except for ref 1 point 2) into a final version of the manuscript.

When resubmitting a re-revised manuscript, please also provide us (in your resubmission cover letter) with 2-5 one-sentence 'bullet points', containing brief factual statements that summarize key aspects of the paper - they will accompany the online version of the article as part of a 'synopsis'. Please see the latest research articles on our website (emboj.embopress.org) for examples - I am happy to offer further guidance on this if necessary. In addition, I would encourage you to also 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 is fixed to 550 pixels in width and 150-400 pixels in height.

I look forward to receiving your final version, after which we should be able to swiftly proceed with formal acceptance and publication of the study. Should you have any further questions in this regard, please do not hesitate to get back to me.

REFEREE REPORTS:

Referee #1:

Reviewer 1 Weinert, et al. manuscript revision comments

The authors have adequately addressed the majority of this reviewer's comments including the use of previously published data to estimate acetylation stoichiometry in SIRT3 KO tissues, discussion of reactivity vs. stoichiometry, clarifying the stoichiometry results from cellular organelles in the context of previously published work, data interpretation, and word choice. Furthermore, the authors avoided excessive technical details in the text which clarifies the main message. Together, the revision by Weinert and colleagues has been substantially improved since the original submission and represents an important advance in this field. Included below are only additional minor comments on the revision, which could be considered to further improve the manuscript.

Major comments:

None

Minor comments:

1. The author's statement that "Another study examined liver acetylation in fasted and obese mice (Still et al, 2013), and these data similarly indicated that acetylation, and SIRT3-targeted sites, were not substantially altered by fasting or obesity in wild-type animals (Appendix FigS5B) - Page 14". Looking at Figure 4B-C in the Still et al paper, the majority of Sirt3 target sites trend towards (and a subset with statistically significance) a decrease in acetylation in both refeeding (or an increase with fasting) and obese mice. Perhaps the authors of the current manuscript should restate this sentence to indicate that the mean change across all Sirt3 target acetylation changes was not particularly striking, compared to a manipulation such as knocking out Sirt3 (Hebert et. al. and Rardin et. al.). However, the fact that these sites do increase in fasting is consistent with the conclusions of the current manuscript that Sirt3 target sites are particularly reactive with acetyl CoA. Also, the authors of the current study incorrectly refer to the Still et. al. data as high fat (Referee #3's point #1) diet-driven obesity in wild-type (Appendix Figure S6) mice. The Still et al work did not assess obesity in "wild type" mice, as these were leptin deficient Ob/Ob animals. Further, the obesity was not the result of high fat diet, as the Ob/Ob animals became obese due to overeating (hyperphagia) the same diet as the lean (wild type) animals.

2. Re: Response to Referee #3's Point #2. The inclusion of estimating the acetylation stoichiometry in Sirt3 KO mice is a nice addition to the paper and adds needed insight into what the current data may mean for understanding the physiology of Sirt3. While mitochondrial acetylation stoichiometry in general is quite low, Sirt3 is clearly critical for making this so. However, what still missing is directly measuring the acetylation stoichiometries in the Sirt3 KO animals. The methods are clearly quite accurate (as several approaches have been used and other reviewers have helped improve the calculations) and the manuscript is a significant contribution at present. However, this final piece of information is missing. If authors could obtain Sirt3 KO tissues and apply (at least one of) their methods to add information here for at least some sites (such as those suggested to go up to high stoichiometry in Sirt3 KO), the manuscript would be significantly stronger. However, the amount of additional work required is significant, and therefore could be considered for future studies.

3. Re: Stoichiometry calculations. It appears that the acetylation stoichiometry calculations (both by incomplete and complete in vitro acetyl labeling) rely on the assumption that a given lysine only exists as acetylated or unmodified. However, this is not necessarily true: lysines are subject to a wide number of PTMs, so if this assumption is indeed used, it should at least be stated that any lysines that are subject to additional PTMs (presumably most of them) would tend to have higher acetylation stoichiometry estimates [acetylK/(acetylK+unmodK)] than are true in reality [acetylK/(acetylK+unmodK + otherPTMs)]. For the AQUA approach, was total protein abundance assessed using peptides that mapped to other regions of the protein (not the same lysine in its unmodified form)? If so, this this gets around the worry about other PTMs on the modified K in question. The AQUA should definitely be done using at least two unmodified peptides from other regions of the protein, otherwise it can't be considered an intendent approach as it incorporates the same assumptions. This point was unclear and should, at minimum, be clarified.

Referee #2:

After revision, the reviewer is still puzzled by the explaination from the authors.
1. The formula is the basis for them to get their conclusion and they have used it in at least two

occasions. Moreover, they realized that the question was wrong. It is not convingcing that the authors only explained this problem as trivial as a typo. As a matter of fact, the rationale the authors used to derive the new formula is totally different from what they used to derive the original one. In the original formula, the authors claimed that it was hard for them to detect acetylated pepetide in the sample and thus they calculated based on levels of unacetylated peptides. In the new formula, they used the acetylated peptide levels to do the calculation. Can or can't the authors detect acetylated peptide from the original sample? This is serious enough to threat the relaibility of this study.

2. SIRT3 (like any other catalist or enzymes) only change the speed of deacetylating reaction but does not change the equilibrium of the reaction,which is determined by free energy change of the reaction. The free energy change need to be estimated for the deacetylating reaction to show that the acetylation level can reach such low level.

Overall, this reviewer is still doubtful to this work.

Referee #3:

The changes made to the manuscript by Weinert and colleagues have substantially strengthened it, and I support its publication in EMBO. I have a few very minor suggestions for the final version:

1. The word "indicates" in the title is too strong, in my opinion. The data presented in this manuscript notwithstanding, it is very difficult to conclusively rule out the existence of a mitochondrial acetyltransferase. I recommend changing this word to "suggests".

2. Statistical analysis need to be provided in Fig. 4C.

3. More detail should be provided about the cell culture methods - e.g. how was the serum starvation performed in Fig. 5A?

2nd Revision - authors' response 14 August 2015

Response to reviewers´ comments

We would like to thank the reviewers for their feedback. Their comments and suggestions have undoubtedly improved the manuscript.

Referee #1:

Reviewer 1 Weinert, et al. manuscript revision comments

The authors have adequately addressed the majority of this reviewer's comments including the use of previously published data to estimate acetylation stoichiometry in SIRT3 KO tissues, discussion of reactivity vs. stoichiometry, clarifying the stoichiometry results from cellular organelles in the context of previously published work, data interpretation, and word choice. Furthermore, the authors avoided excessive technical details in the text which clarifies the main message. Together, the revision by Weinert and colleagues has been substantially improved since the original submission and represents an important advance in this field. Included below are only additional minor comments on the revision, which could be considered to further improve the manuscript.

Major comments:
None

Minor comments:
1. The author's statement that "Another study examined liver acetylation in fasted and obese mice (Still et al, 2013), and these data similarly indicated that acetylation, and SIRT3-targeted sites, were not substantially altered by fasting or obesity in wild-type animals (Appendix FigS5B) - Page 14". Looking at Figure 4B-C in the Still et al paper, the majority of Sirt3 target sites trend towards (and a subset with statistically significance) a decrease in acetylation in both refeeding (or an increase with fasting) and obese mice. Perhaps the authors of the current manuscript should restate this sentence to indicate that the mean change across all Sirt3 target acetylation changes was not particularly striking, compared to a manipulation such as knocking out Sirt3 (Hebert et. al. and Rardin et. al.). However, the fact that these sites do increase in fasting is consistent with the conclusions of the current manuscript that Sirt3 target sites are particularly reactive with acetyl CoA. Also, the authors of the current study incorrectly refer to the Still et. al. data as high fat (Referee #3's point #1) diet-driven obesity in wild-type (Appendix Figure S6) mice. The Still et al work did not assess obesity in "wild type" mice, as these were leptin deficient Ob/Ob animals. Further, the obesity was not the result of high fat diet, as the Ob/Ob animals became obese due to overeating (hyperphagia) the same diet as the lean (wild type) animals.

We thank the reviewer for his/her comments. We have updated the text to more accurately describe the acetylation changes in the paper by Still et al and to include the observation that SIRT3-targeted sites were also more affected by fasting in this paper, similar to our own results. The new text is included below.

*"Another study examining liver acetylation in fasted and obese mice (Still et al, 2013), showed that median acetylation levels were not substantially altered by fasting or obesity in wild-type animals (**Appendix Fig S5B**). A small number of acetylation sites were significantly affected by fasting and obesity (Still et al, 2013), and our analysis of their data showed that SIRT3-targeted sites were the most affected by fasting (**Appendix Fig S5B**), consistent with our own results (**Fig 4A** and **4B**). Together these data suggest that SIRT3-targeted sites are only modestly affected by fasting or calorie restriction, in contrast to the substantially increased acetylation seen in SIRT3 KO animals"*

We further thank the reviewer for pointing out the origin of the obesity in these animals which we incorrectly described as being due to high fat diet in our comments to reviewer #3. However, obesity was not attributed to high fat diet in the manuscript text and therefore no further changes were made to the manuscript.

2. Re: Response to Referee #3's Point #2. The inclusion of estimating the acetylation stoichiometry in Sirt3 KO mice is a nice addition to the paper and adds needed insight into what the current data may mean for understanding the physiology of Sirt3. While mitochondrial acetylation stoichiometry in general is quite low, Sirt3 is clearly critical for making this so. However, what still missing is directly measuring the acetylation stoichiometries in the Sirt3 KO animals. The methods are clearly quite accurate (as several approaches have been used and other reviewers have helped improve the calculations) and the manuscript is a significant contribution at present. However, this final piece of information is missing. If authors could obtain Sirt3 KO tissues and apply (at least one of) their methods to add information here for at least some sites (such as those suggested to go up to high stoichiometry in Sirt3 KO), the manuscript would be significantly stronger. However, the amount of additional work required is significant, and therefore could be considered for future studies.

We do not have access to SIRT3 KO tissues and therefore were not able to include these experiments in this revised manuscript. We agree that directly measuring acetylation stoichiometry in SIRT3 KO mouse liver would be excellent experiment for future studies.

3. Re: Stoichiometry calculations. It appears that the acetylation stoichiometry calculations (both by incomplete and complete in vitro acetyl labeling) rely on the assumption that a given lysine only exists as acetylated or unmodified. However, this is not necessarily true: lysines are subject to a wide number of PTMs, so if this assumption is indeed used, it should at least be stated that any lysines that are subject to additional PTMs (presumably most of them) would tend to have higher acetylation stoichiometry estimates [acetylK/(acetylK+unmodK)] than are true in reality [acetylK/(acetylK+unmodK + otherPTMs)]. For the AQUA approach, was total protein abundance assessed using peptides that mapped to other regions of the protein (not the same lysine in its unmodified form)? If so, this this gets around the worry about other PTMs on the modified K in question. The AQUA should definitely be done using at least two unmodified peptides from other regions of the protein, otherwise it can't be considered an intendent approach as it incorporates the same assumptions. This point was unclear and should, at minimum, be clarified.

The reviewer is correct to point of that the presence of additional modifications at the same position would cause us to overestimate acetylation stoichiometry at that position. However, we do not have information about stoichiometry of other PTMs on acetylated lysines and therefore could not account for this. We recognize this generic limitation and it is now acknowledged the results section.

"In addition, we cannot account for additional modifications at these positions, the presence of which would cause us to overestimate acetylation stoichiometry."

AQUA quantification was performed using a single unmodified peptide to determine protein abundance. For 4 of the 5 proteins we used an unmodified peptide containing one of the acetylation sites so that we could account for both the unmodified and acetylated forms when calculating protein abundance. The reviewer is correct to point out that we cannot account for other modifications at this position, which would result in underestimation of protein abundance. However, the same is true for any other unmodified peptide we might use to estimate protein abundance. In retrospect it would have been best to use several unmodified peptides to determine protein abundance, preferably using peptides generated by cleavage at two arginine residues (which are not as frequently modified as lysine residues are). These details are now provided in the methods section and the sequences of the AQUA peptides used are provided in Dataset EV3.

*"First we determined protein abundances for our proteins of interest using AQUA quantification of unmodified peptides (one unmodified peptide for each protein, see **Dataset EV3** for AQUA peptide sequences)."*

Referee #2:

After revision, the reviewer is still puzzled by the explaination from the authors.
1. The formula is the basis for them to get their conclusion and they have used it in at least two occasions. Moreover, they realized that the question was wrong. It is not convingcing that the authors only explained this problem as trivial as a typo. As a matter of fact, the rationale the authors used to derive the new formula is totally different from what they used to derive the original one. In the original formula, the authors claimed that it was hard for them to detect acetylated pepetide in the sample and thus they calculated based on levels of unacetylated peptides. In the new formula, they used the acetylated peptide levels to do the calculation. Can or can't the authors detect acetylated peptide from the original sample? This is serious enough to threat the relaibility of this study.

The reviewer raises several points that are important to address. In the reviewers initial comments he/she pointed out a problem in the equation we used to calculate stoichiometry. We acknowledged that it was an error (not a typo) and fixed the equation. Based on the reviewer's comments we also realized that we could use the equation described by Olsen et al to calculate stoichiometry. This equation gave identical results to our own equation, and we decided to present this equation in the revised manuscript because it was previously described and published. Both equations used the same data to calculate stoichiometry, the ratio of increased acetylation after partial chemical acetylation, and the global decrease in corresponding peptide abundance

that was used to infer the degree of partial chemical acetylation. This is thoroughly described in our previous response to reviewer #2.

Yes, we can detect endogenously acetylated peptides without partial chemical acetylation; acetylation at these sites was quantified by two methods, SILAC and TMT, and further detected through the use of AQUA peptides. Note that antibody enrichment of acetylated peptides was required to detect most of these sites, underlining their relative low abundance compared to unmodified peptides.

Finally, we validated our method using AQUA peptides and by using 100% chemical acetylation.

2. SIRT3 (like any other catalist or enzymes) only change the speed of deacetylating reaction but does not change the equilibrium of the reaction,which is determined by free energy change of the reaction. The free energy change need to be estimated for the deacetylating reaction to show that the acetylation level can reach such low level.

This is an interesting concept that can be addressed in a future study.

Overall, this reviewer is still doubtful to this work.

We thank the reviewer for his/her critical analysis and comments which have helped to improve the equation used to calculate stoichiometry and improved the accuracy of our results.

Referee #3:

The changes made to the manuscript by Weinert and colleagues have substantially strengthened it, and I support its publication in EMBO. I have a few very minor suggestions for the final version:

1. The word "indicates" in the title is too strong, in my opinion. The data presented in this manuscript notwithstanding, it is very difficult to conclusively rule out the existence of a mitochondrial acetyltransferase. I recommend changing this word to "suggests".

We agree with the reviewer and the title has been modified as suggested.

2. Statistical analysis need to be provided in Fig. 4C.

The distributions of acetylation site ratios were compared using Wilcoxen test and showed that SIRT3-targeted sites were significantly more acetylated after fasting compared with mitochondrial sites in both brain and brown adipose tissues. The Manuscript, figure legend, and figure have been updated with the new analysis.

3. More detail should be provided about the cell culture methods - e.g. how was the serum starvation performed in Fig. 5A?

This has been further clarified by adding:

"HeLa cells were serum starved for 48 hours by washing the cells twice with PBS before transferring into the above described DMEM median lacking dialyzed serum."