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Life span extension by targeting a link between metabolism and histone acetylation in Drosophila

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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.)

1st Editorial Decision 26 August 2015

Thank you for the submission of your research manuscript to our journal. We have now received the enclosed reports on it.

As you will see, the referees acknowledge that the findings are potentially interesting and novel. However, they also suggest several experiments to strengthen the study, which is especially important for the proposed core pathway. All referees also point out that the experiments and data need to be much more carefully described and presented. I think referee 3 raises the most crucial points, and all major points should be addressed. The optional suggestion would certainly also be a good addition, but is optional. The common concerns of referee 1 and 2 should also be addressed. While analyzing the transcriptional changes induced by histone acetylation, as suggested by referee 2, would be an interesting addition, referee 3 indicates in the cross-comments that this is not the highest priority. Please let me know if you would like to discuss the revisions further.

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

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the issue further. You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 35,000 characters (including spaces and references) and 5 main plus 5 expanded view figures. The results and discussion section must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. Commonly used materials and methods can further be moved to the supplementary information, however, please note that materials and methods essential for the understanding of the experiments described in the main text must remain in the main manuscript file. For a normal article there are no length limitations, it should have more than 5 main figures, the results and discussion section must be separate and the entire materials and methods included in the main manuscript file.

Regarding data quantification, can you please specify the number "n" for how many independent experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information is currently incomplete and must be provided in the figure legends.

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

REFEREE REPORTS

Referee #1:

Peleg and colleagues present an exiting and causal connection between the level of Acetyl-CoA (which integrates various nutrient pathways), histone acetylation and longevity. The paper should be published after revision.

Major:
-The manuscript suffers from rather sloppy writing (sometimes inscrutable phrasing, several errors in terms of punctuation, grammar, wrong references to figures, etc.)
- Supplemental Information is very poor
- legends to Supplemental Figures are missing
- Supplemental Methods, information about used strains are missing
- Methods need to be more elaborate about execution of fly experiments
- Methods suggest that fly lifespan determinations were at least partly (Figure 2C) performed with a mixed population of males and females, which is no common practice. Please repeat this experiment. Methods and/or figures and corresponding figure legends need to be more precice about that.
- Information on determination of fly physical activity are missing
- Choosing lower ambient temperatures as a model to study a specific connection of AcCoA metabolism, epigenetics and life span extension seems odd, as low temperatures probably affect a multitude of pathways and reduce overall metabolism. Other established life span extending regimens as caloric restriction would seem to be more fitting to study the effects of energy metabolism on epigenetics and aging and would be more relevant to humans.

Minor:

- Data sets in Figure 1C seem torn apart for no obvious reason, other than circumvent statistical tests with more strict post-hoc multiple comparison analysis.
- In Figure 3 the same data sets for midlife flies at 25{degree sign}C are used in both figures A and E, while it is not clear if they are derived from the same experiment.
- Given that Mariño et al (Mol Cell, 2014) recently published that interfering with AcCoA metabolism by targeting ACLY in mammalian cells affects protein acetylation and autophagy, which has repeatedly been shown to be highly relevant for aging, this paper seems worth citing.

Referee #2:

This is an interesting manuscript but some controls and a few simple experiments are needed to complete the manuscript. Without such data the manuscript appears incomplete and too preliminary for publication in EMBO Reports.
Specific comments:
* characterization of heterozygous mutants (atcpl and chm) should be done by qPCR for demonstrating whether indeed these mutants are haploinsufficient.
* Because the initial lifespan analyses were done with mixed populations of males and females, I would like to see lifespan for females of the atcpl and chm mutants (currently only lifespan for males has been done for atcpl and chm mutants)
*What are the transcriptional changes induced by histone acetylation? Are there gene classes involved in metabolism that are regulated?
*What is the physical activity measued in this study? This should be better specified. Why the physical activity of the control in fig.1 is different from the physical activity of the control in figure 4? Again, I would like to know exactly how this test is done and what is measured.
*What is the lifespan and ac-k levels resulting from over expression of chm and atcpl?
*Measurements are done in old age only in Fig1. To be complete and a useful comparison to the midlife timepoint, epigenetic changes should be assessed also in old age.

Referee #3:

This manuscript describes a link between metabolism and histone acetylation that modifies aging in Drosophila. Overall, this manuscript is noteworthy in my view because of both conceptual and technical strengths: 1) central finding is interesting and novel, that metabolic alterations as flies age can provoke epigenetic alterations that modify lifespan and 2) mass spectrometry approaches are used to define the responsive histone lysines, providing more precision than conventional Western blots. With reasonable revisions, I expect that the manuscript should be suitable for publication in EMBO reports. In particular, a few more experiments are needed to really nail down the core pathway described.

Major points:
1. The relationship between acetyl-CoA, histone acetylation, and lifespan seems clear from the data, but I'm confused about how OCR fits in. One might anticipate that the increase in OCR is driving the acetyl-CoA and histone acetylation increase, and, indeed, Fig 3 seems to support this interpretation, but the butyrate data in Fig 2 seems to argue that the histone acetylation changes are instead driving the changes in OCR. This is confusing, since we have no idea as to how the histone acetylation changes might be doing this. While authors could delve into the underlying gene expression and epigenomic changes (or perhaps it is due to metabolic enzyme acetylation?), that seems well beyond the scope of the current manuscript. Instead, can authors do more experiments to clarify the pathway? Is the midlife change in oxygen consumption driving the acetyl-CoA and histone acetylation changes or vice versa? Could the atpcl and chm mutants help tease this out?

2. I'm also not clear on whether we are really looking at the same or a different mechanism for butyrate as with acetyl-CoA-dependent promotion of histone acetylation. Specifically, is H4K12ac increased by butyrate? This is not specifically presented in Fig S3.

3. Please provide more information about the atpcl mutant. Why is enzymatic activity still ~90% intact? With only a small reduction in activity, isn't it surprising that effects on histone acetylation and longevity are seen? Also, it's important to confirm that this small reduction in activity is sufficient to reduce acetyl-CoA levels. Finally does atpcl mutation suppress histone acetylation generally (in both young and midage flies) or specifically the induction seen with mid-age?

4. Can the atpcl and chm mutants be crossed to gain insight into whether they are in the same pathway or whether they more likely impact lifespan and H4K12ac through different mechanisms?

Optional:
1. The authors conduct an acetyl-proteomics analysis demonstrating increased acetylation on many proteins in midlife vs young flies. With the ATPCL mutant flies, there is an opportunity here to

determine which of these acetylation sites are responsive to acetyl-CoA availability. While a growing number of acetylated proteins have been shown to be responsive to conditions such as glucose availability or feeding/fasting, to my knowledge there has been no acetyl-proteomics study that has defined which proteins are acetylated in response to acetyl-CoA availability. If the authors could generate such a dataset, it would be broadly useful to the field and would add additional impact to the paper. That said, I wouldn't require it for the current manuscript.

Cross-comments from referee #1:

It looks to me that the reviewers have all made fair points. In my view, tightening up data on the core pathway should be higher priority than extending data to transcriptional profiles, etc. Though I agree that such info would be very interesting, it will be quite a bit of work to really nail down a satisfying mechanism for how histone aceytlation is mediating the described effects.

Cross-comments from referee #2:

I agree with reviewer #1 that new lifespan analyses should be done and better documented by reporting median and maximum lifespan, number of animals analyzed, number of batches analyzed, and statistical analyses by log-rank tests (these are things that are all typically reported in aging papers). Other methods (such as for measurement of physical activity) should also be better described. Overall, I echo reviewer#1 in the desire of seeing more consistency in the way experiments are done.

Concerning reviewer #3:
Point 1) I cannot think of any clean and conclusive experiment to test the causal interactions between OCR, acetyl-CoA, and histone acetylation apart delving into the underlying gene expression changes induced by histone acetylation to see whether any key set of metabolic genes is regulated and thus responsible for changes in OCR. Therefore I think they should do RNAseq analyses as I think these are important for the paper.

Point 2) valid suggestion

Point 3) I agree, it's hard to believe that the atpcl mutant is indeed such with just a small (10%) reduction in enzymatic activity (that's why I asked for qPCR validation)

Point 4) this is a valid experiment but the authors should be careful in controlling the genetic background of the double-mutant stock and make sure they have an isogenic control Optional exp) I would propose to do this but keep it as optional.

1st Revision - authors' response 11 November 2015

We would like to thank the referees for their encouraging and constructive criticism. We have now added a detailed method section to the manuscript, which we would like to submit as an article. Importantly, based on the referees' suggestions, we have performed RNA-seq experiments of young and midlife flies in both a wildtype and *chm* background. In addition, we have analyzed the metabolome of wildtype as well as *atpcl* mutant flies (Figures 4 and 5), which support our initial findings of a link between metabolism and gene expression during the early stages of aging. The revised data helped us to develop a model of how these two physiological processes are connected as flies age. A graphical summary of this model is now included as a new illustration (Figure 6).

Below we provide a point-by-point response of the referee's comments and suggestions:

Referee #1:

Peleg and colleagues present an exiting and causal connection between the level of Acetyl-CoA (which integrates various nutrient pathways), histone acetylation and longevity. The paper should be published after revision.

We would like to thank the reviewer for his/her appreciation of our work.

Major:

The manuscript suffers from rather sloppy writing (sometimes inscrutable phrasing, several errors in terms of punctuation, grammar, wrong references to figures, etc.). Supplemental Information is very poor, legends to Supplemental Figures are missing. Supplemental Methods, information about used strains are missing. Methods need to be more elaborate about execution of fly experiments.

We apologize if the original manuscript's writing was a bit preliminary. We have now extensively edited the manuscript to enhance its readability and to better describe our findings. We made sure to correct all grammatical and typographical errors and had the manuscript proofread by a native speaker. In addition, we made sure that all figures are referenced correctly and all necessary information about supplemental figures, used strains and methods is provided in the revised manuscript.

Methods suggest that fly lifespan determinations were at least partly (Figure 2C) performed with a mixed population of males and females, which is no common practice. Please repeat this experiment. Methods and/or figures and corresponding figure legends need to be more precice about that.

The reviewer raises an important point. Indeed, most of our lifespan determinations were done in mixed populations, in which males were further counted and analyzed biochemically. The rationale was to create a more physiological condition where males are not physically separated from females during their life. This is now explicitly stated for each figure describing life span determinations and also in the methods describing the procedure for life span determination. In addition to the analysis of males in mixed populations, we have also determined the lifespan in male-only populations for the wildtype, *+/atpcl* mutants and *+/chm* mutant lines, which are shown as Figure S4 in the revised manuscript. Using both experimental designs, the mutant males display significantly increased lifespan. These data support our hypothesis that the early stages of aging are induced by changes in the metabolome linked with the epigenetic landscape.

Information on determination of fly physical activity are missing.

This information is now included in the revised manuscript. (page 20)

Choosing lower ambient temperatures as a model to study a specific connection of AcCoA metabolism, epigenetics and life span extension seems odd, as low temperatures probably affect a multitude of pathways and reduce overall metabolism. Other established life span extending regimens as caloric restriction would seem to be more fitting to study the effects of energy metabolism on epigenetics and aging and would be more relevant to humans.

We agree with the reviewer that caloric restriction would be an alternative way to physiologically increase lifespan. However, the impact of caloric restriction in *Drosophila* males is rather low [1]**, making it difficult to get robust and reliable data. As lowering temperature resulted in the most robust lifespan extension, we chose this condition to investigate the molecular changes that occur during physiological lifespan extension. We have explained this in the revised manuscript:**

> ***In Drosophila several physiological conditions have been shown to affect life span ranging from caloric restriction*** [1]**, *to raising flies in complete darkness*** [2]**, *to raising flies at***

> ***reduced temperature*** [3]***. As the reduced temperature resulted in the most robust lifespan extension in D. melanogaster males (Figure 3B), we used this regimen to analyze the molecular changes during physiological lifespan extension. (page 10)***

Minor:
Datasets in Figure 1C seem torn apart for no obvious reason, other than circumvent statistical tests with more strict post-hoc multiple comparison analysis.

The experiments comparing the OCR between young and old and between young and midlife flies were done separately, hence we would prefer to compare the data individually. In the revised manuscript, we have split the two datasets (new Figure 1B and 1D) to be more coherent with the way we discuss the data in the main text.

In Figure 3 the same datasets for midlife flies at 25{degree sign}C are used in both figures A and E, while it is not clear if they are derived from the same experiment.

We compared histone H4 acetylation in young, midlife and cold flies in at least three biological replicates batches. Each of the batches compared young, midlife and cold flies. In the figures, we included the biological data from all the batches. For a better comparison, we indeed used the acetylation patterns of midlife flies in Figure 3A to compare it to young flies and in Figure 3E to compare it to midlife flies kept at reduced temperatures. We feel that this increases the comprehensibility of our data, but could of course change the arrangement. To prevent ambiguities, we have now explicitly stated this fact in the revised figure legend:

> ***Data for the midlife flies were normalized to young flies as shown in panel A (page 29).***

Given that Mariño et al (Mol Cell, 2014) recently published that interfering with AcCoA metabolism by targeting ACLY in mammalian cells affects protein acetylation and autophagy, which has repeatedly been shown to be highly relevant for aging, this paper seems worth citing.

We fully agree with the reviewer and apologize if this citation did not make it into the EMBO Reports submission of our manuscript. We have of course now included this citation in our revised manuscript.

We thank referee #1 for their useful criticism and hope to have provided adequate answers and new data that address their recommendation.

Referee #2:

This is an interesting manuscript but some controls and a few simple experiments are needed to complete the manuscript. Without such data the manuscript appears incomplete and too preliminary for publication in EMBO Reports.

We would like to thank the reviewer for his/her feedback and for his/her suggestion. We believe our additional experiments strengthen our revised manuscript.

Specific comments:

** characterization of heterozygous mutants (atcpl and chm) should be done by qPCR for demonstrating whether indeed these mutants are haploinsufficient.*

We thank the reviewer for this comment. Both *atpcl* and *chm* mutant are lethal as homozygotes and thus we use the heterozygotes. We have now performed RT-PCR for the heterozygote *chm* and observed a ~50% reduction of mRNA quantitation that spans the deleted exons (8-10), but very small reduction in exon 1 area (Figure S4). In addition, following poly-A selection and RNA-seq analysis (Figure 4), we observe a general 50% reduction across all the exons of chm in the mutant, suggesting that only about 50% of mRNA of the chm gene are processed in the mutant.

The *atpcl* allele (#11055 Bloomington) has a P-element insertion in the first intron, which results in a 20% reduction of *atpcl* mRNA as tested by RT-PCR (Figure S4). This could account for the approximately 20% effect on ATPCL activity that we observe. We had to use such a rather weak allele as ATPCL is an important enzymatic activity. We have now explained this in the revised manuscript:

> ***As expected, flies carrying one hypomorphic atpcl allele, which has reduced levels of atpcl RNA due to a P-element insertion in the first intron*** [42]***, display a ~20% reduction in ATPCL activity (Figure 4A). (page 11)***

Because the initial lifespan analyses were done with mixed populations of males and females, I would like to see lifespan for females of the atcpl and chm mutants (currently only lifespan for males has been done for atcpl and chm mutants)

Thank you for the suggestion. We now include the survival curve for the females. The *atpcl* female mutants indeed live longer compared to wildtype females. In case of the *chm* mutant females, we also observe a lifespan extension when *chm* females are kept together with wildtype males. Interestingly, this lifespan extension is not observed when *chm* mutant females are kept in the presence of *chm* mutant males (survival curves attached). The nature of this unexpected finding is unclear and may be due to the higher activity of midlife *chm* mutant males when compared to the wildtype ones (Figure 5F). Although we think this is an interesting finding, we feel that an in-depth analysis of this effect would be beyond the scope of this manuscript.

We mention our observations in the revised manuscript:

> ***It is worth mentioning that the chm mutant allele is homozygous lethal and the beneficial effect on lifespan is more pronounced in males than in females, suggesting that Chameau has additional functions that remain to be fully understood. (page 16).***

What are the transcriptional changes induced by histone acetylation? Are there gene classes involved in metabolism that are regulated?

Thank you for this suggestion. We have now incorporated these experiments into the manuscript to provide information on the mechanism by which *chm* influences aging. In agreement with previous, age-associated transcriptional changes, we show that around 20% of mRNA transcripts are significantly altered in midlife flies compared to young flies (Fig. 5). Interestingly, in *chm* midlife flies only 13% of the transcripts are significantly changed when compared to *chm* young flies. Further, of the 973 shared genes that change their expression in wildtype as well as in *chm* flies upon aging, the upregulated ones show a higher fold change in wildtype flies compared to *chm* flies. We show this dataset in the new Figure 5 and discuss it extensively on page 17 of the revised manuscript.

What is the physical activity measured in this study? This should be better specified. Why the physical activity of the control in fig.1 is different from the physical activity of the control in figure 4? Again, I would like to know exactly how this test is done and what is measured.

We apologize to the reviewer for not explaining this method adequately in the initial manuscript. Briefly, we flipped young and midlife males into marked vials and measured the number of flies in each section after a fixed time. The method is now described on page 20. The reason for the differences between Figure 1 and 4 are that we wanted to get a better resolution in midlife experiments and therefore divided the vial in 5 (rather than 4) sections ('1' bottom, '5' top). In addition, to detect the difference between wildtype and *chm* flies at midlife, their location was measured after eight seconds instead of three seconds. If needed, we would be very happy to include the recorded videos for these experiments and add them as supplementary material.

What is the lifespan and ac-k levels resulting from over expression of chm and atcpl?

Thank you for this suggestion. We had intended to carry out such assays. However, we were unable to recover any viable flies or larvae upon crossing a UAS-Chameau containing fly strain with a Gal4-tub driver line, which suggests that a global overexpression of *chm* results in early lethality, making it impossible to investigate lifespan or ac-K levels in adult tissues. Further, since ATPCL is a key metabolic enzyme, which is highly regulated on multiple levels (something we show in our manuscript), a strong increase in enzymatic activity is difficult to achieve and to interpret. Although ATPCL overexpression could be an interesting experiment, we feel that such an experiment would require many additional analyses that go beyond the scope of the current analysis and manuscript.

**Measurements are done in old age only in Fig1. To be complete and a useful comparison to the midlife timepoint, epigenetic changes should be assessed also in old age.*

We agree with the reviewer that this would be an interesting investigation. However, the main purpose of the study was a detailed investigation of the epigenetic changes that occur at the early stages of aging, before many age-associated maladies set in. We feel that studies that investigate epigenetic changes in old flies (7-weeks) suffer from the fact that only 25% of all flies in a healthy population reach old age, which may be due to distinct gene expression and epigenetic states, that predisposes these flies to an extended lifespan. Though it would be very exiting to decipher the nature of such an epigenetic state, it was not the scope of the manuscript.

We thank referee #2 for their useful criticism and hope to have provided adequate answers and new data that address their recommendation.

Referee #3:
This manuscript describes a link between metabolism and histone acetylation that modifies aging in Drosophila. Overall, this manuscript is noteworthy in my view because of both conceptual and technical strengths: 1) central finding is interesting and novel, that metabolic alterations as flies age can provoke epigenetic alterations that modify lifespan and 2) mass spectrometry approaches are used to define the responsive histone lysines, providing more precision than conventional Western blots. With reasonable revisions, I expect that the manuscript should be suitable for publication in EMBO reports. In particular, a few more experiments are needed to really nail down the core pathway described.

We thank the reviewer for his/her encouraging feedback regarding the conceptual and technical aspects of our manuscript.

Major points: Point 1: The relationship between acetyl-CoA, histone acetylation, and lifespan seems clear from the data, but I'm confused about how OCR fits in. One might anticipate that the increase in OCR is driving the acetyl-CoA and histone acetylation increase, and, indeed, Fig 3 seems to support this interpretation, but the butyrate data in Fig 2 seems to argue that the histone acetylation changes are instead driving the changes in OCR. This is confusing, since we have no idea as to how the histone acetylation changes might be doing this. While authors could delve into the underlying gene expression and epigenomic changes (or perhaps it is due to metabolic enzyme acetylation?), that seems well beyond the scope of the current manuscript. Instead, can authors do more experiments to clarify the pathway? Is the midlife change in oxygen consumption driving the acetyl-CoA and histone acetylation changes or vice versa? Could the atpcl and chm mutants help tease this out?

We thank the reviewer for these thoughtful comments and apologize for the confusion in interpreting our data. We have now tried to clarify these points in the revised manuscript. The observed OCR increase upon SB/TSA treatment shown in Figure 2 is indeed rapid and transient, and unlikely due to changes in gene expression induced by histone acetylation. We rather hypothesize that the increase of OCR is due to an increase in protein acetylation and that these acetylation changes alter the enzymatic activity of the acetylated proteins. The large increase in young flies compared to midlife flies is likely due to a higher ground state of acetylation and activity of these enzymes. We hope this clarifies our model.
We have now explicitly stated this in the *Results* section:

> ***… adding these KDAC inhibitors to isolated heads resulted in a rapid increase of OCR (Figure 2C and S2D), which subsided after multiple OCR measurements. Heads prepared from midlife flies show a less pronounced increase of OCR (Figure 2C). This may be due to the higher ground state protein acetylation of these enzymes in midlife, due to elevated acetyl-CoA levels (Figure 2A and B). (page 8)***

and in our *Discussion*:

> ***Our observation that a treatment of isolated fly heads with KDAC inhibitors increases OCR within minutes suggests that such a direct feed forward mechanism might indeed exist. The finding that midlife flies have a higher ground state of acetylation and are less susceptible to a stimulation by KDAC inhibitors argues for similar acetylation events triggered by KDAC inhibitor treatment and aging. (page 14)***

To better understand the molecular processes, we did indeed delve into the analysis of the gene expression changes between young and midlife flies in wildtype and *chm* flies. Similar to what other researchers have seen in diverse model systems, we observe age-related changes in gene expression. As we do not see an enrichment of a particular GO term, we interpret at least part of these as increased transcriptional noise. This increase is substantially attenuated in *chm* flies, which led us to develop a model that is now shown as a new Figure 6 in the revised manuscript.

I'm also not clear on whether we are really looking at the same or a different mechanism for butyrate as with acetyl-CoA-dependent promotion of histone acetylation. Specifically, is H4K12ac increased by butyrate? This is not specifically presented in Fig S3.

As we discussed above in our response to point 1 of the reviewer, we realized that we did not explain the experiments properly, which resulted in difficulties to understand the interpretation we provide for our results. Indeed, in contrast to the experiments shown in Figure 2, where we measured the effect of SB on the OCR, the experiment described in Figure S3 shows the outcome of a daily SB feeding for a week (start with SB food at day 4, and tested in day 11). In general, histone acetylation of mono-, di-, tri- and tetra-acetylation are increased by extensive SB consumption, suggesting that the histone acetylation is 'pushed' towards poly-acetylated states, including H4K12ac. This is in agreement with the fact that SB is a broad inhibitor of KDACs, and that it is not specific for any particular acetylation site on histones.

Point 3: Please provide more information about the atpcl mutant. Why is enzymatic activity still ~90% intact? With only a small reduction in activity, isn't it surprising that effects on histone acetylation and longevity are seen? Also, it's important to confirm that this small reduction in activity is sufficient to reduce acetyl-CoA levels. Finally does atpcl mutation suppress histone acetylation generally (in both young and midage flies) or specifically the induction seen with mid-age?

The *atpcl* mutant (#11055 Bloomington) carries a hypomorph allele, which results in a moderate decrease of approximately 20% in atpcl mRNA. Nevertheless, it is homozygote lethal, which demonstrates the importance of this enzyme, which is why we tested the heterozygous flies. By using a targeted analysis, we confirm that midlife *atpcl* mutants have indeed a 60% reduced level of acetyl-CoA as shown in figure 4C. This is more pronounced in midlife flies than in young flies, which is also supported by the fact that an *atpcl* mutation has a much stronger effect on the general metabolome in midlife flies than in young flies (new figure 4B), which is why we analysed the histone modifications in midlife rather than young flies (new figure 4D).

Point 4: Can the atpcl and chm mutants be crossed to gain insight into whether they are in the same pathway or whether they more likely impact lifespan and H4K12ac through different mechanisms?

We would like to thank the reviewer for this suggestion. We have performed the suggested experiment. As reviewer 2 suggested in point 4 of his cross comments,"the authors should be careful in controlling the genetic background of the double-mutant stock", we have crossed the flies as follows:

1) cyo/chm (strain 1) x cyo/atpcl (strain 2)

vs.

2) cyo/+ (strain 1) x cyo/atpcl (strain 2)

Thus, we created similar genetic background hybrids. Interestingly, we observe similar life span of the double mutant flies compared to the *atpcl* mutant (new Figure 5G). Based on these data, we speculate that the two enzymes likely operate in the same pathway (new Figure 6). Alternatively, it is possible there are other yet unknown factors that limit a further extension of lifespan.

Optional:
Point 5: The authors conduct an acetyl-proteomics analysis demonstrating increased acetylation on many proteins in midlife vs young flies. With the ATPCL mutant flies, there is an opprtunity here to determine which of these acetylation sites are responsive to acetyl-CoA availability. While a growing number of acetylated proteins have been shown to be responsive to conditions such as glucose availability or feeding/fasting, to my knowledge there has been no acetyl-proteomics study that has defined which proteins are acetylated in response to acetyl-CoA availability. If the authors could generate such a dataset, it would be broadly useful to the field and would add additional impact to the paper. That said, I wouldn't require it for the current manuscript.

We think this is an exciting suggestion, which we would like to follow up experimentally in the future.

We thank referee #3 for their useful criticism and hope to have provided adequate answers and new data that address their recommendation.

Cross comments

Cross-comments from referee #1:
It looks to me that the reviewers have all made fair points. In my view, tightening up data on the core pathway should be higher priority than extending data to transcriptional profiles, etc. Though I agree that such info would be very interesting, it will be quite a bit of work to really nail down a satisfying mechanism for how histone aceytlation is mediating the described effects.

We thank the reviewer for his/her cross comment. We decided to incorporate gene expression profiles of the *chm* flies, as we believe it adds another interesting dimension to our working model and supports our conclusions.

Cross-comments from referee #2:
I agree with reviewer #1 that new lifespan analyses should be done and better documented by reporting median and maximum lifespan, number of animals analyzed, number of batches analyzed, and statistical analyses by log-rank tests (these are things that are all typically reported in aging papers). Other methods (such as for measurement of physical activity) should also be better described. Overall, I echo reviewer#1 in the desire of seeing more consistency in the way experiments are done.

Thank you. We have now added documentation and statistical analysis of the life span experiments, including log-rank and the time points for 10% death of the pre-mortality plateau phase. The rationales behind the experiments, including the physical activity assays, are now described in the method section.

Concerning reviewer #3:
Point 1) I cannot think of any clean and conclusive experiment to test the causal interactions between OCR, acetyl-CoA, and histone acetylation apart delving into the underlying gene expression changes induced by histone acetylation to see whether any key set of metabolic genes is

regulated and thus responsible for changes in OCR. Therefore I think they should do RNAseq analyses as I think these are important for the paper.

Thank you. We completely agree with this comment, which is why we have included the RNA-seq experiments in the revised manuscript. Based on our working model, we emphasize that the age-associated changes in the transcriptome is as a result of increased H4K12ac levels, which in turn are induced by increased acetyl-CoA levels, which is the by-product of an increased metabolic activity. We do not observe a particular set of metabolic genes being upregulated, which is why we believe that the increase in OCR is not necessarily caused by changes in gene expression but rather by higher protein acetylation. This is further supported by the kinetics of the effect.

With regards to the effect of SB on OCR, we notice a rapid increase of OCR upon SB treatment. The increase occurs between 10 to 60 minutes, peaking at 30 minutes. As such, we believe that our experiments in Figure 2 suggest that SB and TSA might mediate the increase of OCR by protein acetylation. This notion is supported by the lower increased of OCR in midlife flies, which have already higher basal protein acetylation levels. Nonetheless, it has been shown that SB and TSA also impact histone acetylation levels that might provide feedback to the mitochondria to increase its activity via other pathways.

It is evident from the survival curve that flies treated with SB (which have increased OCR and histone acetylation) have significantly shorter lifespan. These data prompted us to test whether lowering acetyl-CoA production via ATPCL mutation, or whether lowering H4K12ac via *chm* mutation, would lead to increased lifespan. In support of our previous data, both *atpcl* and *chm* mutants indeed display lower H4K12ac and increase life span.

Point 2) valid suggestion.

We have addressed this point as outlined in our response to reviewer 3.

Point 3) I agree, it's hard to believe that the atpcl mutant is indeed such with just a small (10%) reduction in enzymatic activity (that's why I asked for qPCR validation).

We have performed a RT PCR experiment, which shows a moderate reduction of 20% in atpcl mRNA and included the result as a new supplemental Figure S4a.

Point 4) this is a valid experiment but the authors should be careful in controlling the genetic background of the double-mutant stock and make sure they have an isogenic control Optional exp) I would propose to do this but keep it as optional.

We completely agree with this point and have thus created the *chm/atpcl* double mutant in a similar hybrid background to the +/atpcl line. This is shown as a new Figure 5G.

References

1. Pletcher SD, Macdonald SJ, Marguerie R, Certa U, Stearns SC, Goldstein DB, Partridge L (2002) Genome-wide transcript profiles in aging and calorically restricted Drosophila melanogaster. *Curr Biol* **12**: 712–723.
2. Allemand R, Cohet Y, David J (1973) Increase in the longevity of adult Drosophila melanogaster kept in permanent darkness. *Exp Gerontol* **8**: 279–283.
3. Miquel J, Lundgren PR, Bensch KG, Atlan H (1976) Effects of temperature on the life span, vitality and fine structure of Drosophila melanogaster. *Mechanisms of Ageing and Development* **5**: 347–370.

2nd Editorial Decision 02 December 2015

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed reports from the referees. Referee 2 still has a few suggestions for how the study could be further improved, that I would like you to address and incorporate before we can proceed with the

official acceptance of your manuscript.

I noticed that the tests used to calculate p-values are not always specified in the respective figure legends, please add these.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

I look forward to seeing a final, revised version of your manuscript as soon as possible. Please let me know if you have any questions.

REFEREE REPORTS

Referee #1:

Good revision

Referee #2:

The authors have addressed most of my concerns but there are some issues that I would like to see addressed before publication:

* as originally requested by referee#1, the authors should display OCR data for young, midlife, and old flies (currently shown in fig. 1B and 1D) all in the same graph. Although I understand that these experiments were done separately, it should be easy to repeat this so that data for these time points can be generated at the same time and thus cross-compared and displayed on the same graph.

* Because fig.1 displays data also for old flies, it would be good that all panels in fig.1 display such data, i.e. that metabolite levels and enzymatic activities in fig. 1E-F are also shown for old flies in the same graphs.

* Fig2C: why the Y-axis starts from -5? The figure legend mentions a "dashed line" not shown here. Another (better) way to represent this data is to show also the controls (the sample with no SB addition, i.e. the vehicle controls).

* the experiment in fig. 5G is interesting but questionable because in the methods the authors say that the atpcl and chm mutants have been back-crossed each to "its own respective control" (which control is used for each of these mutants should be specified in the methods). Thefore it appears that the chm/atpcl flies have a different genetic background than the +/atcpl flies, making a comparison not possible in lifespan experiments. For such comparison, the 2 mutants should have been back-crossed against the same genetic background control. Therefore data in Fig. 5G should be removed from the manuscript.

* The authors should specify in all figure legends whether the data refers to males or female flies.

Referee #3:

This is a very nice paper that significantly advances our understanding of metabolic influence over aging. I now recommend publication of the revised manuscript.

2nd Revision - authors' response 04 December 2015

I have attached a revised manuscript where the tests used to calculate p-values are specified in all figure legends. We have also specified in all figure legends whether the data refer to males from mixed or homogenous populations. We agree with the reviewer that Fig2C profits from showing the vehicle controls and have changed the figure and the figure legend accordingly.

We think the aging experiments using the double mutant, which was suggested by two reviewers is actually an important experiment and shows that the two mutants affect the same molecular pathway. We would therefore rather like to keep it in the manuscript. In contrast to reviewer 2s impression, the two atpcl/chm and the +/atpcl flies share the same genetic background. We realized that we needed to do a better job to describe the strains used and now explicitly outline the strain backgrounds in the single and double mutants in the revised manuscript.

With regards to the first two points raised by reviewer 2, we agree that it would be very interesting to have more data on the old flies. However, this is not the prime topic of this manuscript. Although we agree with the reviewer that the oxygen consumption measurements are easy to repeat (in fact we have repeated them 35 times for the young flies and 24 or 12 times for the midlife and old flies), to perform the experiments in parallel would require at least two additional months to age flies in a controlled manner before such measurements could be done. As we had already outlined in the cover letter of the revision, the study of metabolic changes in old flies is not within the scope of the manuscript as we explicitly aimed to investigate the early stages of ageing rather than possible adaptive effects in old flies. Therefore we feel that performing these additional experiments as well as the determination of metabolite levels and enzyme activities in old flies does not address the questions we wanted to answer in this work.

3rd Editorial Decision 09 December 2015

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

