

SIRT6 Overexpression Counteracts Chromatin Aging in the Male Murine Liver

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

A version of this paper was originally rejected for publication by Nature Communications, however that decision was reconsidered after appeal by the authors.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this manuscript, Nagar, Cohen and colleagues investigate the role of SIRT6, a NAD⁺-dependent deacetylase, in modulating age-related chromatin changes in the liver of mice. The authors employ a multi-omics approach, using ATAC-seq and RNA-seq to assess chromatin accessibility and transcriptional changes in young and old wild-type (WT) and SIRT6-overexpressing transgenic (TG) mice. They demonstrate that aging leads to increased global chromatin accessibility and altered histone modifications, particularly a reduction in H3K9me3 and an increase in H3K9ac, which correlate with inflammatory gene expression and metabolic dysfunction. SIRT6 overexpression in both young and old mice preserves chromatin structure in a youthful state, maintaining proper transcriptional programs and mitigating age-associated inflammation and metabolic decline. Importantly, the study reveals that SIRT6 can reverse age-related chromatin changes when induced later in life through AAV-mediated gene delivery, suggesting its potential as a therapeutic target for rejuvenation and the treatment of age-related diseases. The findings underscore the importance of SIRT6 in maintaining chromatin integrity and tissue identity during aging, providing insights into the mechanisms underlying the biology of aging and potential interventions.

Overall this is a very thorough, rigorously performed study, and the findings are dramatic, robust, and important. They should be of interest to the broad readership of Nature Communications.

There are a few relatively minor critiques.

1. In Figure 1b, the observations that chromatin changes in old WT compared to young WT do not occur in old TG vs young WT is compelling, suggesting old TG chromatin has “young” features. Although this comparison is key, it would be interesting to see the direct comparison of the Old vs Young TG samples; are there differences?

2. Related to this, in 1C, the authors conclude that the age-associated increases in accessibility compared to young WT mice does not occur in old TG mice. This effect is dramatic. Nonetheless, there does appear to be a difference between old vs young TG mice, with young TG LINE-1 showing decreased accessibility compared to WT young. The authors should discuss whether this is significant and what it may represent. Could there be deleterious changes of SIRT6 overexpression in young mice? This information could speak to whether therapeutic SIRT6 activation is better performed early or throughout life, or only later.

3. The ChIP-seq changes in H3K9 acetylation correlate strongly with the rejuvenating effects of SIRT6 on gene expression, and the authors conclude that their results show that SIRT6 exerts its protective effects primarily through H3K9ac deacetylation. While this is a fair conclusion, the data are still correlative. Since H3K9 acetylation is a very general marker of active gene expression, the changes observed could in theory be indirect, secondary to any mechanism that increases expression of the observed genes. To really implicate SIRT6-dependent deacetylation of H3K9ac in the mechanism, one would want to see SIRT6 localization to the sites of H3K9ac changes. In addition, it would be reassuring to see specificity for lack of changes of other acetylation sites. While the authors do show that H3K56ac patterns do not correlate well, H3K56ac is an unusual modification not as well linked to gene expression, and indeed for SIRT6 has been studied originally in the context of DNA damage chromatin changes. Comparison of other acetylation sites that are transcriptional activation markers and not SIRT6 substrates would be important to strengthen support for models involving direct SIRT6 H3K9 deacetylation.

4. Minor: Text cites Supplementary Fig. S8k for data showing H3K9ac changes at the ETS binding sites; this should be Supp Fig. S7k.

Reviewer #2

(Remarks to the Author)

In the present manuscript by Nagar et al., entitled "SIRT6 Overexpression counteracts chromatin aging", the authors used a multiomics approach to investigate the role of the histone deacetylase SIRT in maintaining and/or reverting age-related effects on liver chromatin. Previous research has implicated that during aging, declining SIRT6 expression and activity contribute to metabolic dysfunction in the liver, including increased susceptibility to hepatic steatosis, insulin resistance, and impaired stress responses. The role of SIRT6 with respect to chromatin architecture has not been investigated carefully in the aging liver and this is the topic of the manuscript. Overall, we are surprised to see that over-expression of a histone deacetylase has very mild effects in all of the datasets, which are often over-interpreted with respect to the role of SIRT6 in reverting age-related changes. There also seems to be an issue with data quality and quantification, which makes an interpretation very difficult (see below). In addition, the authors do not discuss - at least sufficiently - several recent papers (PMID: 26541291, PMID: 36082605, PMID: 25437555 and PMID: 37154113) that have used similar approaches (albeit not in a SIRT6 context) and have partly observed very different effects in the aging liver (see below).

Main points

- We find it surprising that the distribution of opening and closing peaks are almost identical in all genomic regions (Supplementary Fig. 1d). This is in stark contrast to a previously published dataset (PMID: 36082605), in which only promoters and 5'UTRs showed an increase in accessibility while the other genomic regions were characterised by closing peaks. How can this difference be explained?
- The analysis of histone modifications is not convincing. The western blot quantifications are not understandable (Supplementary Figure 8). How were they quantified (i.e. what is meant by H3Kx/H3, relative to Poinceau)? The H3K9ac for instance shows a very even signal as does the H3 - how can this result in a 2-fold enrichment in case of old? The differences in the ChIP are also minor. While the PCA shows some differences between the samples, only PC2 differentiates the sample by a small margin - however, this only explains a minor difference in the data. To be more convincing, this analysis requires differential peak analysis accompanied by screen shots for representative regions. The analysis of global changes (Fig. 4b) only show marginal differences and is also not ideal since ChIP was performed without an external spike-in to normalise, which is essential when comparing global changes in levels. The PCA plots also indicate a large variability between the samples and indicate the absence of any statistically significant changes. Like with the ATAC-seq, it is important to provide a table with all relevant sequencing metrics, such as number of reads, % alignment and number of called peaks.
- A similar concern about western blot quantifications regard Fig. 5c and SFig. 8c. If the quantification is correct, SIRT6 overexpression by AAV leads to a marked decrease in H3K27me3, a phenotype not observed in the trans gene. Can the authors explain this discrepancy? Finally, AAV delivery of SIRT6 reverts some of the chromatin alterations observed in the old mice and pathway level seem to correspond well. It would be good to see an overlap of genes rescued (e.g. as Venn) and some browser shots.
- For ATAC-seq data it is not sufficient to report average number of reads. Please provide a table specifying number of reads, % mapped reads, number of peaks called and FRiP score. In addition, it is essential to show how reproducible the datasets are, i.e. using PCA or correlation plots.

Minor points:

- Please mention the mouse strain directly in the manuscript (and not only refer to previous publications)
- Fig.1c/ There are very specific regions within the LINE-1 element on which SIRT6 has a clear effect - are these regulatory regions and/or have specific features? Would be nice to see a browser shot.
- For a more direct comparison of SIRT6 over-expression, it might be good to include the GO-terms of rescued (closed sites) in the main Figure (now: Supplementary Figure 2a)

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)

In this manuscript, Nagar et al. utilized a multi-omics approach (including ATAC-seq, methylome, and RNA-seq in the male mouse liver to look at the effect of aging on the chromatin. The authors focused on Sirt6, which is a histone deacetylase, because studies have shown that Sirt6 KO mice have an accelerated aging phenotype whereas Sirt6 overexpression in mice led to increases in both lifespan and healthspan. In order to study the effect of Sirt6 on age-associated chromatin changes, the authors generated a SIRT6 TG mouse line in which Sirt6 is overexpressed throughout the body. Using these mice (along with corresponding WT mice) ATAC-seq and RNA-seq was performed on livers from young and old SIRT6 TG and WT mice. The one of the main takeaways from the ATAC-seq experiments is that chromatin landscape of the old SIRT6 TG mice remained more young-like compared to their age matched WT littermates. Interestingly, they did not observe an increase in accessibility at LINE1 elements in the old SIRT6 TG mice like was present in the old WT mice. Overall, this suggests that SIRT6 preserves the integrity of global chromatin architecture, including LINE-1 regions, during aging. The authors then focused on DNA methylation of young and old SIRT6 TG and WT mice. Consistent with the ATAC-seq results old SIRT6 TG mice were able to ameliorate the significant decrease in DNA methylation that was observed in old WT mice. Through analysis using GREAT and GSEA (ATAC-seq and RNA-seq, respectively), the authors observed that aging led to an increase in inflammation pathways and a downregulation in metabolic pathways, both of which were rescued in the old SIRT6 TG mice. In order to look into the TFs that may be driving these changes with aging it was discovered that aging led to increase ETF binding with a corresponding decrease in LETF binding, which again was ameliorated by Sirt6 overexpression. This result suggests that SIRT6 may be involved in maintaining tissue identity which is key for tissue function and preventing cancer formation. The next section of the manuscript focused on H3K9ac and H3K56ac (both of which are targets for SIRT6) and their role in age-related chromatin changes. The authors discovered H3K9ac as a critical regulator of chromatin during aging, with SIRT6 conferring protection primarily through its deacetylation (with minimal effect of age on H3K56ac). The last set of experiments utilized a liver-specific Sirt6 overexpression to see if short-term overexpression (1 month) at old age is enough to rescue age-related chromatin changes. Interestingly, after one month of overexpression, SIRT6 was able to rejuvenate the chromatin landscape in the liver. The manuscript is closed by highlighting that SIRT6 may be a promising rejuvenation therapeutic which may have translation potential to combat aging and age-related diseases.

Overall, this paper details a comprehensive multi-omic study to understand the effect of Sirt6 on the chromatin landscape with aging. However, direct mechanistic evidence linking SIRT6 to the observed chromatin landscape rejuvenation in SIRT6 TG mice is missing, and both the main text and title should more explicitly acknowledge that only male animals were used in this study. Finally, there are a number of outstanding points that should be addressed to make sure the analysis is statistically supported and reproducible by other labs in the future.

Major comments

1. This study uses only male mice. While this is not inherently a concern (especially since only male mice have been reported to experience significant benefit from Sirt6 overexpression), it is important that both the title, abstract and main text (including the discussion) clearly state this fact (it is only stated once in the main text). Additionally, any conclusions drawn from the data should acknowledge that the findings are based solely on male animals and would likely not extend to females.
2. The main conclusion of this study is that SIRT6 can rejuvenate the chromatin landscape in old mice to a more youthful state. However, the authors do not directly investigate the mechanism by which SIRT6 drives this effect. Instead, they assume changes in H3K9ac, H3K18ac, and H3K56ac levels are the direct results of SIRT6 activity changes (although they are only indirect proxies for SIRT6 activity). Since other histone deacetylases can also act at these sites, these marks cannot be specifically attributed to SIRT6 function. Is there a reason the authors did not take a more direct approach—such as SIRT6 ChIP-seq—to assess SIRT6 binding and activity? Demonstrating a direct role for SIRT6 in shaping the chromatin landscape would significantly strengthen the study's mechanistic impact. If this is not feasible, we recommend toning down all claims of direct effects in the manuscript.
3. What ages were used for the H3K9ac and H3K56ac ChIP-seq experiments? It is not explicitly stated in the main text, methods, or figure legends. In addition, there is an inconsistency in the ages that used for omics profiling in the SIRT6 TG mice. For ATAC-seq, their liver tissue ages were young (5-7 months old) and old (18-21 months); whereas, for genome DNA methylation in liver tissue, the ages were young (3-4 months) and old (23-24 months). In figure 5 description, they specify the age as “24-month-old C57BL/6 male mice” and it is marked sacrificed at 25 months. This adds to the inconsistency of the age description of the mice. The differences must be explicitly explained and the age choices justified for robustness and interpretability.
4. Validation of SIRT6 overexpression in the liver of the SIRT6 TG mice should be included in this manuscript even if this mouse model has been validated and published previously (especially since overexpression transgenes tend to get silenced over generations). It would also be interesting to include the expression of known SIRT6 modulated genes (in the RNA-seq dataset) to ensure that the known activity of this protein is maintained in the SIRT6 TG mice.
5. To ensure reproducibility of the analyses (and accuracy of the reviewing process), there is a lot of information that should be included in the manuscript:
 - a. All analytical scripts/code should be deposited to a repository such as GitHub or made available as a supplemental file. Right now, the code is not available for reviewing.
 - b. For reproducibility purposes, version numbers for all software and R packages used (including cutadapt, R, ChIPseeker, EdgeR, csaw, HOMER, bagfootr, Metagene2, etc.), as well as date of access or versions for all databases (TRRUST, L1Base2, ...), should be explicitly included in the methods.
 - c. Based on the text, the authors used log fold change thresholding (see bottom of page 4 for instance) to determine their list of differentially regulated chromatin regions. Fold-change filtering, if not implemented into the statistical model used to identify differential genes/peaks, leads to poor FDR control and is not appropriate (see PMC2654802). Since fold change

filtering leads to statistical issues, all of its use must be removed and all analyses should be repeated without it (or alternate analytical tools that include logFC thresholding in the statistical testing must be used).

d. In the methods, the authors say they used both edgeR and DESeq2 for data processing. This is extremely puzzling, as these tools have fully redundant purposes -however, the specific way they were each used is not explained. Please elaborate on the use of both and why both were used in this case instead of a single tool.

e. In the statistics section, the authors say they used “paired or unpaired two-tailed Student’s t-test”. Was a normality test used to determine that the data fit the normality hypothesis? If yes, please mention why test was used to determine normality. If the data does not satisfy the normality assumption, please redo all tests using nonparametric equivalents.

f. “during their active hours (dark)” – this window is a 12 hour window, during which many changes (including in chromatin and gene regulation) can occur and have been documented. Please provide a shorter/more precise time window for long-term reproducibility purposes.

g. “integrity number (RIN) was assessed by TapeStation”. Was a threshold applied for downstream processing? If yes, this must be explicitly stated.

h. Western blot antibodies: Please also provide the dilution that was used for these experiments so that the methods can be reproducible.

Minor comments

1. In the opening sentences of the introduction, the authors mention both life expectancy (lifespan) and healthspan. For clarity, it would be helpful to briefly define these terms and distinguish between them, emphasizing that lifespan refers to the total length of life, while healthspan refers to the period of life spent in good health.

2. There is no clear introduction to the SIRT6 TG mouse model. For clarity, the authors should briefly describe the genetic construct and relevant features of the SIRT6 transgenic mice when the model is first introduced in the opening paragraph of the Results section. This would help readers unfamiliar with the model better understand the study’s context rather than rely on readers to go to the original paper describing the model.

3. It is unclear why the authors chose to focus specifically on the liver in this study. If there is a particular rationale—such as the liver’s metabolic relevance, known sensitivity to aging, or prior links to SIRT6 function—it would be helpful to briefly state this in the introduction to provide readers with a clearer foundation for the study’s focus.

4. The authors report that LINE-1 chromatin accessibility increases with age and is reduced by SIRT6 overexpression, based on ATAC-seq data. Did the authors also examine LINE-1 transcription levels in the RNA-seq datasets? If so, it would be informative to know whether SIRT6 overexpression similarly reduces LINE-1 transcript levels to those observed in young mice (and not just remodels the chromatin around it).

5. It would be interesting to check the expression of other enzymes responsible for H3K9, H3K18, and H3K56 acetylation (using either the RNA-seq dataset or western blot) to see how SIRT6 OE impacts their expression. Would also be interesting to look at their activity levels in the SIRT6 TG mice.

6. Supplementary Figure 9 is currently labeled as Supplementary Figure 8 in the supplementary file.

7. The authors label their transgenic mice as SIRT6 TG without clarifying that the TG means transgenic. This abbreviation needs to be explicitly defined at first use .

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this revised manuscript, Nagar, Cohen, and colleagues investigate the role of SIRT6, a NAD⁺-dependent deacetylase, in modulating age-related chromatin changes in the liver of male mice. The authors employ a multi-omics approach, using ATAC-seq, RNA-seq, and ChIP-seq to assess chromatin accessibility and transcriptional changes in young and old wild-type (WT) and SIRT6-overexpressing transgenic (TG) mice. They demonstrate that aging leads to increased global chromatin accessibility and altered histone modifications, particularly an increase in H3K9ac, which correlate with inflammatory gene expression and metabolic dysfunction. They propose that SIRT6 overexpression in both young and old mice preserves chromatin structure in a youthful state, maintaining proper transcriptional programs and mitigating age-associated inflammation and metabolic decline. Moreover, the study reveals that SIRT6 can reverse age-related chromatin changes when induced later in life through AAV-mediated gene delivery, suggesting its potential as a therapeutic target for rejuvenation and the treatment of age-related diseases. The findings underscore the importance of SIRT6 in maintaining chromatin integrity and tissue identity during aging, providing insights into the mechanisms underlying the biology of aging and potential interventions.

In this revision, the authors have attempted to address the concerns raised by reviewers by tempering the generality of their conclusions in several areas and providing new data in response to specific questions. However, some of the additions and clarifications have raised additional concerns.

Overall, this study has the potential to be a thorough and rigorous investigation, which is important and of interest to the broad readership of Nature Communications. Nevertheless, the remaining concerns should be addressed before publication.

1. The authors clarify that the Western blot data in Supplementary Figure 8, showing global H3K9ac and other histone marks, were normalized first to Ponceau staining and then to total H3. They argue that this method is more accurate than what is detected by eye, which shows minimal differences in H3K9ac, H3K56ac, and other marks with age or genotype. However, the rationale for introducing Ponceau normalization is unclear, as standard practice typically involves simply

normalizing to total H3. Generally, robust differences can be observed visually, even with unequal H3 levels, which can be estimated by eye. Additionally, it is concerning that the quantified H3K9ac levels do not appear to differ between old WT and old TG samples (Figure 8d), which seems contrary to the authors' major conclusions. It is plausible that global differences in these marks may not be detectable if aging-dependent changes are relatively localized. The authors should quantify the fraction of total H3K9ac genomic peaks altered by either aging or SIRT6 to determine if this aligns with the subtle, variable, or perhaps nonexistent differences in the global Western analysis.

2. In response to previous reviews, the authors have added new supporting data for their ChIP-seq analyses in Supplementary Figure 9, including differential peak analysis and three representative IGV snapshots. However, there is no description of what each example is intended to illustrate. I assume the first two examples show H3K9ac peaks that decrease in old WT and are reverted in old TG mice. However, there is no clear example of an increase in H3K9ac in old WT mice that is absent in TG mice, which is the main biological change the authors propose occurs in old mice and is rescued by SIRT6. The third example may intend to demonstrate this, but it is not clear, and quantification would be necessary to validate the claim. It is concerning that the authors did not provide at least one additional clear example.

3. The authors have now included PCA analyses for both ATAC-seq experiments (Figures 1c and 11e). It is troubling that PCA did not reveal differences for the AAV-SIRT6 overexpression experiments (Figure 11e). Furthermore, for the aging and SIRT6-TG experiment (Figure 1c), there is considerable variability across the different samples, particularly among the WT (old and young) mice.

Reviewer #2

(Remarks to the Author)

The revision of the manuscript addressed some of our concerns. In our opinion, the most critical aspects have not been answered:

- We thank the authors for clarifying how the western blot data was normalized. While we appreciate that ImageJ might pick up differences that are hidden from the human eye, a two-fold difference in intensity as reported for many of the modifications should be easily visible if blotted in the linear range of the antibody. This is particularly important as also the ChIP-seq data does not support a global change. Thus, these data is still not very convincing.

- The authors refer to the publication PMID: 38484732 that used a similar method to quantify global changes in ChIP-seq profiles and state that this was done without spike-in as well. This is true. Nevertheless, this is far from ideal and might misrepresent the data, given the normalisation procedure of ChIP-seq to overall read counts. In addition, the cited paper also reports a far more dramatic change in modification levels, in contrast to the minor change reported here ($<0.1 \log_2(\text{norm. counts} + 1)$)

- We were surprised to see that the differential peak calling for the ChIP-seq data revealed a large number of differential peaks, given that the PCA clearly shows intermixing of the individual ChIP-seq datasets. It is not understandable how the lack of variance between the samples can result in such large number of differential peaks. We tried to inspect the uploaded datasets, but were not able to look at them since they are currently still private.

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)

The authors mostly addressed my concerns in this revised version.

There is a remaining point that the authors refused to correct because they deem it "common practice" and thus refuse to hear the argument that it is statistically flawed and incorrect. Thus, my issue with $\log_2\text{FC}$ thresholding remains (as the authors refused to remove it). To note, just because other people do something wrong from a statistical standpoint, that is not sufficient reason to continue to do something wrong when alerted to the issues inherent with the practice. If the authors want increased stringency (which they argue), they can easily apply a more stringent FDR threshold (which would pose no problems in terms of statistics) to focus on the most robust results. As it stands, the practice of $\log\text{FC}$ thresholding is problematic (and the fact that it is widespread and not corrected after peer-review will continue to lead to these issues compounding). As an example for why "common practice" is not a good rationale - common practice until much too recently was to only use on sex of animals in biomedical research, because "female data is too noisy". This has been proven demonstrably false, yet remained common practice (like $\log\text{FC}$ thresholding).

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this revision, the authors have clarified a number of issues and updated data in response to my review as well as those of

the other reviewers. These changes have addressed my concerns. The study's findings are important and of interest to the broad readership of Nature Communications, and I support its publication

Reviewer #2

(Remarks to the Author)

In the second revision the authors have now included the suggestions of two reviewers and removed the WB normalization to Poinceau. They now write that there is no big differences upon aging in the marks analyzed. However, now H3K9ac shows an almost two-fold increase in levels (Supplementary Figure 8a and d). We agree with reviewer 1 that such an increase should be easily visible on a western blot, which it is not. Thus, despite the fact that the initial statement has been removed from the manuscript, the issue around WB quantifications remains unsolved.

Also, the question around ChIP-seq peaks and the PCA remains unsolved. The authors argue that there can be differential peaks present despite the fact that PCA shows intermixing. The authors argue that we might refer to the PCA for H3K56ac (Figure 4a,b). However, we are concerned about both ChIP-seq PCAs. Yes, there is a slight separation of data points in PC2 for H3K9ac (for half of the old WT samples) which in any case only explains less than 10% of the variance in the data. The authors need to at least show snapshots of the individual ChIP-seq tracks as part of the figure for selected genes as done for the ATAC-seq data in Figure 1d or Supplementary Figure 12c. In addition, the links provided by the authors for the private GEO data works now, but unfortunately only CSV files describing normalised peak files are available. This is not sufficient. The authors need to upload fastq files and ideally, bigwig files to allow an easy inspection of the data.

Finally, the manuscript moves from global to local changes as an explanation for the effect of SIRT6. This case would usually demand for a more direct assessment for the role of SIRT6 and a ChIP-seq dataset to overlay the effect on chromatin marks with SIRT6 occupancy. We acknowledge that this was asked before and deemed technically impossible by the authors. However, this needs to be explicitly mentioned as a limitation in the discussion.

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)

The authors have now addressed my comments satisfactorily.

Version 3:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

please see uploaded pdf

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 4:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors' changes in the text and figures appropriately address the remaining concerns.

Reviewer #5

(Remarks to the Author)

Below, I state my opinion on the points of contention between the reviewer and the authors.

“...the variability seen in the PCA plots is also visible in the genomic tracks. This poses a problem since the effects seen are very minor (see examples below). Even if the differences were statistically significant, it is very difficult to imagine that they have biological meaning...”

In my opinion, the variability between replicates is entirely reasonable for typical CHIP-seq experiments. It is more difficult to determine what constitutes a major/minor difference and whether this can have a biologically meaningful effect. I would highlight that the data in question is from a bulk dataset: a small change in CHIP-seq coverage could simply mean that a small number of cells is affected. But a minority of cells is very capable of having major biological ramifications. Thus, I would consider a result noteworthy as far as it is statistically significant.

“Some of the peaks presented in the browser shots 4c do not necessarily represent functional peaks. For instance, the one picked for ClpB is not showing the promoter peak (as would be functionally relevant for H3K9ac), but some minor peak downstream of the start site...”

This line of reasoning is based on the understanding of which regions are typically decorated by H3K9ac under healthy conditions. However, it is not a given that H3K9ac should only be present at promoters, especially in pathological conditions or in aging, and it also does not mean that K9ac cannot have an effect outside of promoters. There is vast research on the loss of fidelity in chromatin organization during aging. This includes increase in cryptic transcription. Ectopic acetylation marks may well play a role in this. Finally, the above statement assumes perfect annotation of gene models and regulatory elements. If a peak is consistent across replicates but does not coincide with a known promoter, it is worth considering there may be an unknown regulatory element.

“Therefore, especially for a representative figure, it is not necessary that the same individual mice be shown across all loci.”

I strongly disagree with this point. A figure is not representative of the underlying data if the points shown are cherry-picked. In conclusion, I believe that the CHIP-seq data analyzed in the manuscript is of good quality and allows drawing conclusions on the effect of aging and Sirt6 OE. However, comparisons should include more statistical tests, to avoid reliance on visualizations alone. Coverage plots (Such as Figure 4C) could be accompanied by boxplots and respective statistics. Because the genomic context of differential peaks was discussed, I also recommend plotting the genomic context (genic or intergenic, promoter, enhancer, exon, intron... for example, using ChipSeeker) of differential peaks and determining if there is enrichment for any particular region by comparing to all peaks as background. It would be interesting to know if differential peaks are enriched at gene bodies, for example.

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Point by point response to Reviewer comments

We thank the reviewers for their constructive and insightful comments, which have helped us to significantly improve the manuscript.

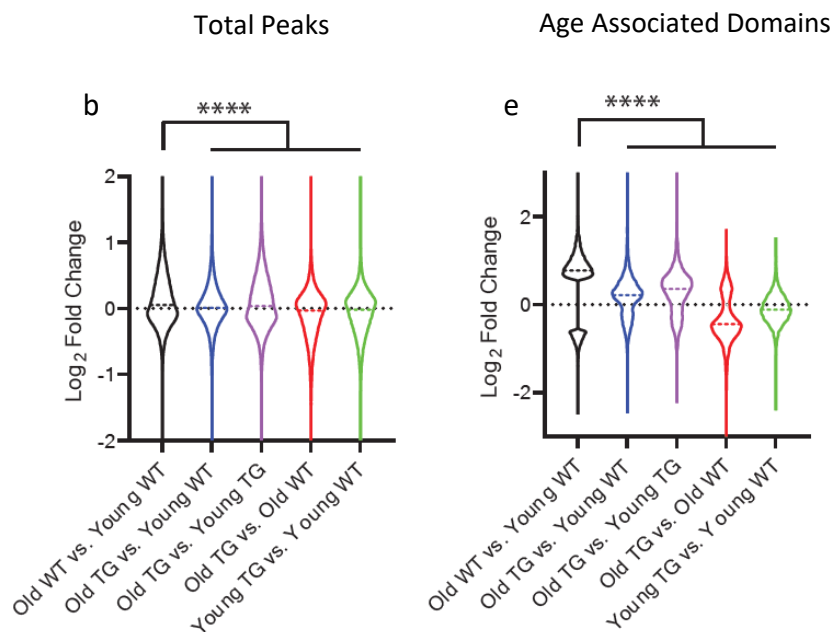
Below, please find a detailed, point-by-point response to each of the reviewers' comments.

Reviewer 1.

We thank this referee for the warm compliments and describing our research as "a very thorough, rigorously performed study", and claiming that "the findings are dramatic, robust, and important. They should be of interest to the broad readership of *Nature Communications*". Their constructive suggestions have enabled us to significantly strengthen the manuscript. A detailed point-by-point answer to their comments is provided below.

1. In Figure 1b, the observations that chromatin changes in old WT compared to young WT do not occur in old TG vs young WT is compelling, suggesting old TG chromatin has "young" features. Although this comparison is key, it would be interesting to see the direct comparison of the Old vs Young TG samples; are there differences?

We thank the reviewer for this interesting comment and have added the comparison in figures 1b,e (please find below). This reveals that there are indeed differences, however they are less pronounced than between young and old WT mice. This suggests an intriguing possibility that while SIRT6 TG mice do age, they do so at a slower rate than WT mice. The revised manuscript now addresses these findings: "Notably, AADs accessibility was slightly increased in old TG mice compared to young TG mice, suggesting that they do age but at a relatively slow rate".



2. Related to this, in 1C, the authors conclude that the age-associated increases in accessibility compared to young WT mice does not occur in old TG mice. This effect is dramatic. Nonetheless, there does appear to be a difference between old vs young TG mice, with young TG LINE-1 showing decreased accessibility compared to WT young. The authors should discuss whether this is significant and what it may represent. Could there be deleterious changes of SIRT6 overexpression in young mice? This information could speak to whether therapeutic SIRT6 activation is better performed early or throughout life, or only later.

We thank the reviewer for this insightful comment. We have emphasized the effect in young TG mice in the results of the revised manuscript: "Interestingly, LINE-1 regions were even less accessible in young SIRT6 TG than in young WT mice." This is in line with previous publications (cited in our revised manuscript) showing that SIRT6 represses LINE-1 (PMID: 30853213, PMID: 25247314).

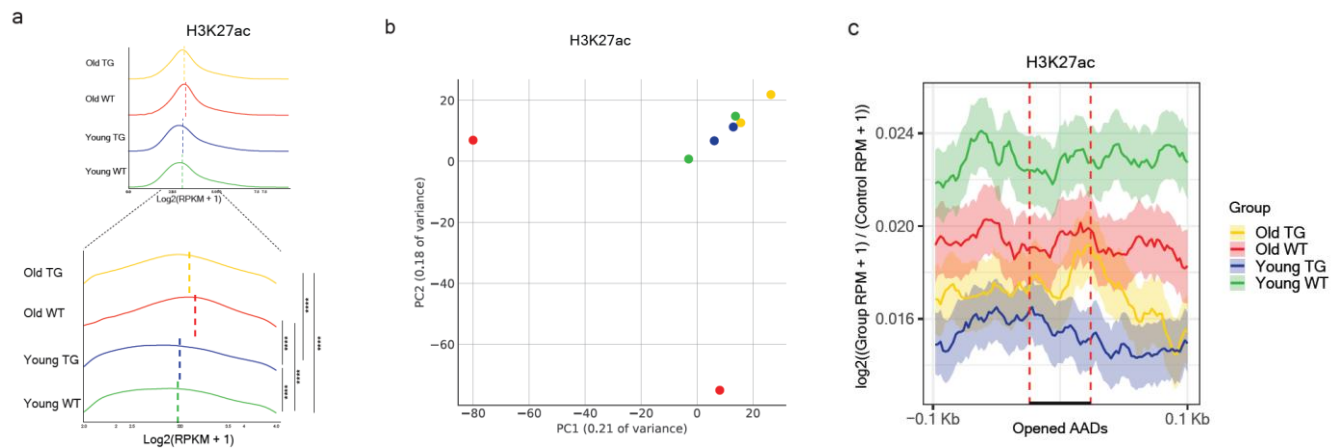
In addition, the following text has been added to the Discussion, following the description of the results obtained from the AAV-injected mice:

"This also raises the question of whether it is better to induce SIRT6 overexpression at an earlier or later stage of life. In most cases, such as LINE-1 and general chromatin accessibility, H3K9ac levels and inflammation, our results show that young SIRT6 TG mice seem to be even "younger" than young WT mice (Fig. 1,4, Supplementary Fig. 2c). It is difficult to identify clear detrimental effects of SIRT6 in young mice. Hence, it is worth exploring whether induced SIRT6 overexpression is even more beneficial if performed at a young age."

3. The ChIP-seq changes in H3K9 acetylation correlate strongly with the rejuvenating effects of SIRT6 on gene expression, and the authors conclude that their results show that SIRT6 exerts its protective effects primarily through H3K9ac deacetylation. While this is a fair conclusion, the data are still correlative. Since H3K9 acetylation is a very general marker of active gene expression, the changes observed could in theory be indirect, secondary to any mechanism that increases expression of the observed genes. To really implicate SIRT6-dependent deacetylation of H3K9ac in the mechanism, one would want to see SIRT6 localization to the sites of H3K9ac changes. In addition, it would be reassuring to see specificity for lack of changes of other acetylation sites. While the authors do show that H3K56ac patterns do not correlate well, H3K56ac is an unusual modification not as well linked to gene expression, and indeed for SIRT6 has been studied originally in the context of DNA damage chromatin changes. Comparison of other acetylation sites that are transcriptional activation markers and not SIRT6 substrates would be important to strengthen support for models involving direct SIRT6 H3K9 deacetylation.

We thank the reviewer for raising these important points. In regards to the lack of changes of other acetylation sites, we performed an H3K27ac ChIP-seq. H3K27ac is a well-established marker of transcriptional activation and is not a known SIRT6 substrate. Indeed, we observed age and SIRT6 dependent changes in H3K27ac signal (Revised supplementary Fig. 10 a-b, please find below). However, no correlation between H3K27ac and the changes in chromatin accessibility in opened AADs was observed (Revised supplementary Fig. 10 c, please find below), as H3K27ac levels decreased with age in these regions, emphasizing the specific role of

H3K9ac in maintaining the chromatin in a young-like state in old age. These results have been added to the revised manuscript, and the results section now states: “To test the specificity of the observed effects, we examined H3K27ac ChIP-seq profiles and found no correlation with chromatin accessibility changes upon aging or SIRT6 overexpression”.



Regarding SIRT6 direct deacetylation of H3K9ac, we agree that a SIRT6 ChIP-seq would strengthen this claim. Thus, we made several attempts to perform SIRT6 ChIP-seq on our liver samples, using different commercial antibodies, sonication conditions etc. However, as hinted at by reviewer #4 and the editor, SIRT6 ChIP-seq is known to be highly challenging and indeed the signal in these experiments was too low and comparable to the IgG control. We also attempted to overcome this challenge by using FLAG-SIRT6 ChIP-seq in the AML12 murine hepatocyte cell line with an anti-FLAG antibody to map SIRT6 binding sites, but unfortunately this experiment did not identify significant peaks either. Thus, we agree with the reviewer’s perspective and have accordingly toned down any statements implying a direct effect in the revised manuscript. For example, in the results: “These results highlight H3K9ac as a key regulator of chromatin during aging, with SIRT6 exerting its protective effects primarily through reducing H3K9ac levels” instead of “through H3K9ac deacetylation” in the old manuscript. Another example from the discussion: “Our ChIP-seq results highlight H3K9ac as a more dominant factor in both chromatin aging and the effects of SIRT6 in the murine liver, as evidenced by PCA analysis and the correlation between ChIP-seq signals and AADs, although it is possible the effect of SIRT6 on H3K9ac is indirect (Fig. 4c-d).”

4. Minor: Text cites Supplementary Fig. S8k for data showing H3K9ac changes at the ETS binding sites; this should be Supp Fig. S7k.

We thank the reviewer for noting this error and apologize for the mistake. It has been corrected, and in the revised manuscript it now appears as Supplementary Fig. 9d.

Reviewer #2.

We thank this reviewer for their interesting comments that enabled us to significantly strengthen the manuscript. A detailed point-by-point answer to these comments is provided below.

In the present manuscript by Nagar et al., entitled “SIRT6 Overexpression counteracts chromatin aging”, the authors used a multiomics approach to investigate the role of the histone deacetylase SIRT in maintaining and/or reverting age-related effects on liver chromatin. Previous research has implicated that during aging, declining SIRT6 expression and activity contribute to metabolic dysfunction in the liver, including increased susceptibility to hepatic steatosis, insulin resistance, and impaired stress responses. The role of SIRT6 with respect to chromatin architecture has not been investigated carefully in the aging liver and this is the topic of the manuscript. Overall, we are surprised to see that over-expression of a histone deacetylase has very mild effects in all of the datasets, which are often over-interpreted with respect to the role of SIRT6 in reverting age-related changes. There also seems to be an issue with data quality and quantification, which makes an interpretation very difficult (see below). In addition, the authors do not discuss - at least sufficiently - several recent papers (PMID: 26541291, PMID: 36082605, PMID: 25437555 and PMID: 37154113) that have used similar approaches (albeit not in a SIRT6 context) and have partly observed very different effects in the aging liver (see below).

Regarding the relatively mild effect of SIRT6, it seems that SIRT6 is not a promiscuous histone deacetylase and acts only on specific sites. Even so, we still observed a genome wide effect of SIRT6 on histone acetylation and chromatin structure.

We thank the reviewer for drawing our attention to the recent papers, all of which have been discussed in the revised manuscript.

Regarding PMID: 26541291 performed RNA-seq on young and old mouse livers. Their results were similar to those uncovered in our RNA-seq, and this has now been acknowledged in the results section:

"Gene Set Enrichment Analysis (GSEA) between old and young WT mice revealed an upregulation of the pro-inflammatory interferon-alpha response, and a downregulation of pathways related to fatty acid metabolism and oxidative phosphorylation (Fig. 2d,f and supplementary Table 8). This is consistent with the pathways identified based on the ATAC-seq data (Fig. 2a,b) and with previously published RNA-seq data from the aged murine liver (PMID: 26541291)."

Regarding PMID: 36082605 and PMID: 25437555, see first main point below. PMID: 25437555 performed MNase-seq of young and old murine livers. In the first main point below, we show how their results align with ours, and this is included in the revised manuscript.

Regarding PMID: 37154113, who performed ATAC-seq on young and old mouse livers, we have added the following to the discussion, comparing their results to ours:

"Interestingly, changes in ETS and LETF binding with age are consistent with the findings of Yu et al. However, while they observed a significant increase in AP-1 TFs binding with age, this was not found in our dataset (PMID: 37154113)."

Main points

1. We find it surprising that the distribution of opening and closing peaks are almost identical in all genomic regions (Supplementary Fig. 1d). This is in stark contrast to a previously published dataset (PMID: 36082605), in which only promoters and 5'UTRs showed an increase in accessibility while the other genomic regions were characterised by closing peaks. How can this difference be explained?

We thank the reviewer for referring us to Tessarz group's paper. Indeed, we showed that increased chromatin accessibility loci are not limited to promoters and 5' UTR regions, whereas their identified genomic regions were. This difference could be explained by the groups of mice studied. Bozukova et al. examined only mice that can be classified as "healthy agers," as they excluded animals showing any signs of inflammation, fibrosis, and/or pathological steatosis, common phenotypes of aging (PMID: 31427197). In contrast, our study did not exclude such animals and therefore represents the general aged population. In addition, consistent with our findings, another study by Aviv Regev's group (PMID: 25437555) also observed that only a small fraction of changes in nucleosome occupancy in the aging liver were proximal to the TSS. Finally, our study focused on a slightly older group of mice and used a larger sample size, which might also explain the differences between the studies. The revised discussion now includes the following paragraph addressing this point:

"Interestingly, our study revealed a similar pattern of changes in chromatin accessibility across all genomic features, with the highest percentage of AADs found in introns, followed by distal intergenic and promoter regions (Supplementary figure 1b-d). While this is in line with a previous publication showing that only a small subset of changes in nucleosome occupancy in the aging liver are proximal to the TSS (PMID:25437555), it is contrast to the findings of Bozukova et al., who observed increased chromatin accessibility with age only in the promoter and 5' UTR regions. A possible explanation for this discrepancy may be that Bozukova et al. studied only mice that can be classified as "healthy agers", as they excluded mice with any signs of inflammation, fibrosis and/or pathological steatosis, common phenotypes of aging (PMID: 31427197), while our study did not exclude such animals. Additionally, our study focused on slightly older groups of mice and used a larger sample size."

2. The analysis of histone modifications is not convincing. The western blot quantifications are not understandable (Supplementary Figure 8). How were they quantified (i.e. what is meant by H3K α /H3, relative to Poinceau)? The H3K9ac for instance shows a very even signal as does the H3 – how can this result in a 2-fold enrichment in case of old? The differences in the ChIP are also minor. While the PCA shows some differences between the samples, only PC2 differentiates the sample by a small margin – however, this only explains a minor difference in the data. To be more convincing, this analysis requires differential peak analysis accompanied by screen shots for representative regions. The analysis of global changes (Fig. 4b) only show marginal differences and is also not ideal since ChIP was performed without an external spike-in to normalize, which is essential when comparing global changes in levels. The PCA plots also indicates a large variability between the samples and indicate the absence of any statistically significant changes. Like with the ATAC-seq, it is important to provide a table with

all relevant sequencing metrics, such as number of reads, % alignment and number of called peaks.

We thank the reviewer for drawing our attention to this matter. Apparently, we failed to sufficiently clarify how these measurements were performed, which might have led to this comment. Regarding the WB quantification, each modified and non-modified histone ran on different gels in parallel, and each band was first normalized to the corresponding ponceau lane as a loading control. Afterwards, to compare the intensity of the specific modification in each sample, we divided the normalized-to-ponceau-intensity of each modified-histone band by the normalized-to-ponceau-intensity of the corresponding non-modified histone. For the convenience of the reader, Source data is provided including all ponceau images and Supplementary figure 8 includes a representative ponceau image as well.

Regarding the H3K9ac quantification, we agree that without compatible quantification it may be hard to see the abovementioned changes. The human eye may not identify changes that are found by the ImageJ software, especially as these need to be normalized to the ponceau and non-modified histone. All of our blots can be found in the Source data for the reviewers' impressions.

Regarding the ChIP-seq changes, as requested by the reviewer, we incorporated a differential peak analysis accompanied by representative screenshots for selected genomic regions (Supplementary Fig. 9a-c, found below). These analyses further demonstrate the age-associated increase in H3K9ac levels, which is reversed by SIRT6 overexpression in old mice.

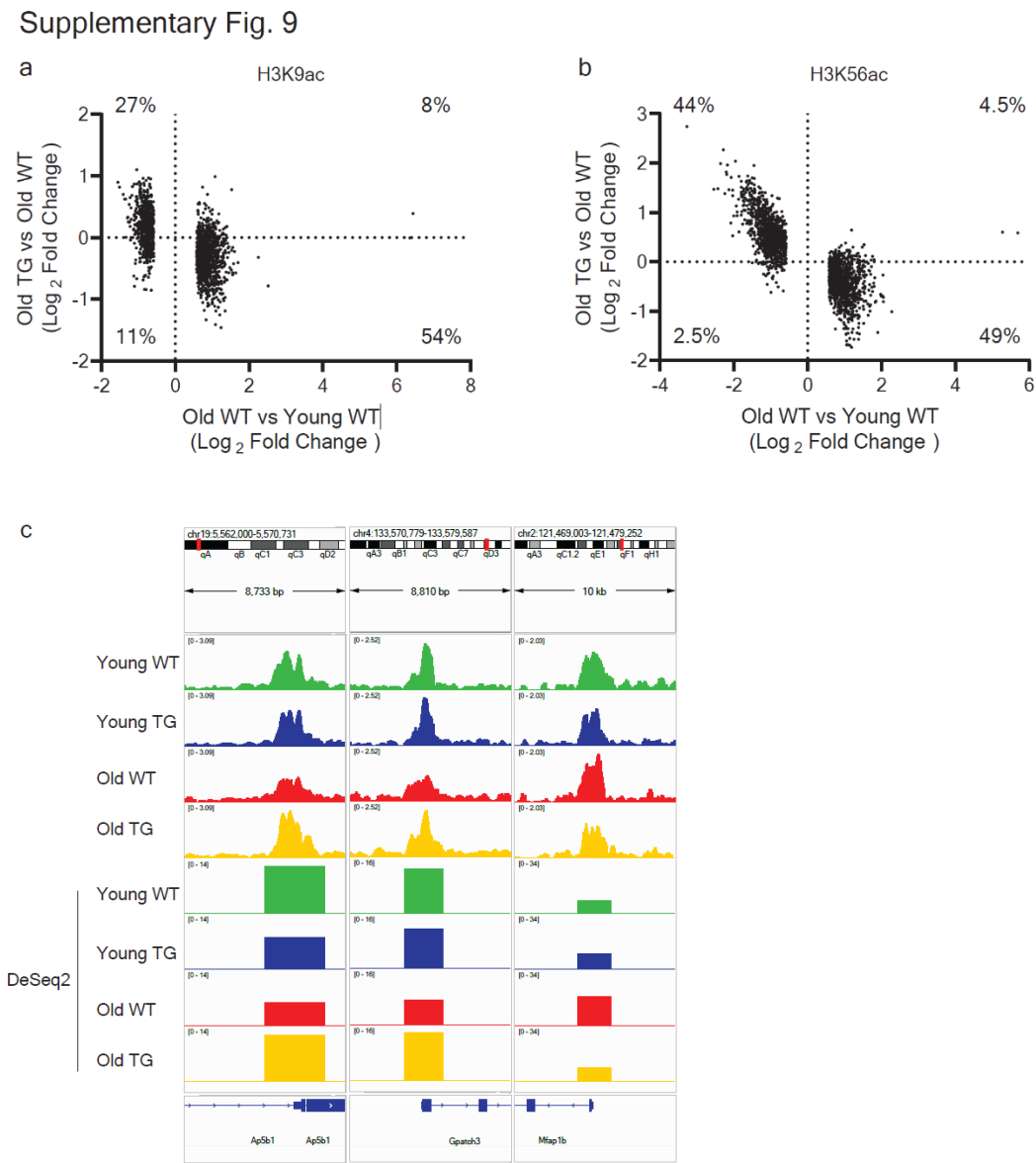
All ChIP-seq libraries in this study were prepared in parallel using identical antibody lots and chromatin inputs, with comparable sequencing depth and quality control. Genome-wide signal quantification was performed using DeSeq2 normalized counts, and full distributions of H3K9ac and H3K56ac were compared across conditions, revealing the same trend observed in the western blot analyses. Although we did not perform spike-in normalization, we do not believe this compromises the reliability of our results. Indeed, high-impact papers have performed this analysis despite the lack of spike-in normalization (e.g. PMID: 38484732).

Altogether, these statistically significant changes in H3K9ac are sufficient to distinguish between the different groups as reflected in the PCA. Thus, the differential analysis together with the PCA, WB, and global changes analysis support our conclusion that H3K9ac is upregulated with age, a phenotype reversed by SIRT6 overexpression.

In addition, we have added all sequence metrics for both ATAC-seq and ChIP-seq and they can be found in the supplementary table file (Supplementary tables 1, 20, 23 and 25).

The revised manuscript now includes this information:

“Given that H3K9ac and H3K56ac are direct substrates of SIRT6, we performed ChIP-seq of both marks on the same liver tissues used for both the ATAC-seq and the RNA-seq (Supplementary Table 19). Differential analysis of old WT vs. young WT found 1972 and 2341 differential peaks for H3K9ac and H3K56ac, respectively (p-value < 0.05, |fold change| > 1.5). The majority (62%) of differential H3K9ac peaks were upregulated with age, while differential H3K56ac peaks were more evenly divided between up- and downregulation (Supplementary Fig. 9a-b). For both marks, the comparison of old SIRT6 TG vs. old WT mice showed the reverse pattern for the same peaks. Representative IGV snapshots for H3K9ac are shown in Supplementary Fig. 9c.”



3. A similar concern about western blot quantifications regard Fig. 5c and SFig. 8c. If the quantification is correct, SIRT6 overexpression by AAV leads to a marked decrease in H3K27me3, a phenotype not observed in the trans gene. Can the authors explain this

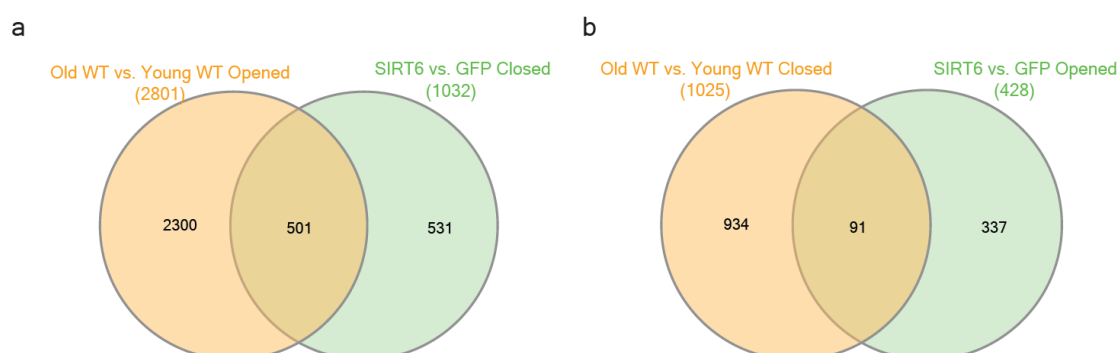
discrepancy? Finally, AAV delivery of SIRT6 reverts some of the chromatin alterations observed in the old mice and pathway level seem to correspond well. It would be good to see an overlap of genes rescued (e.g. as Venn) and some browser shots.

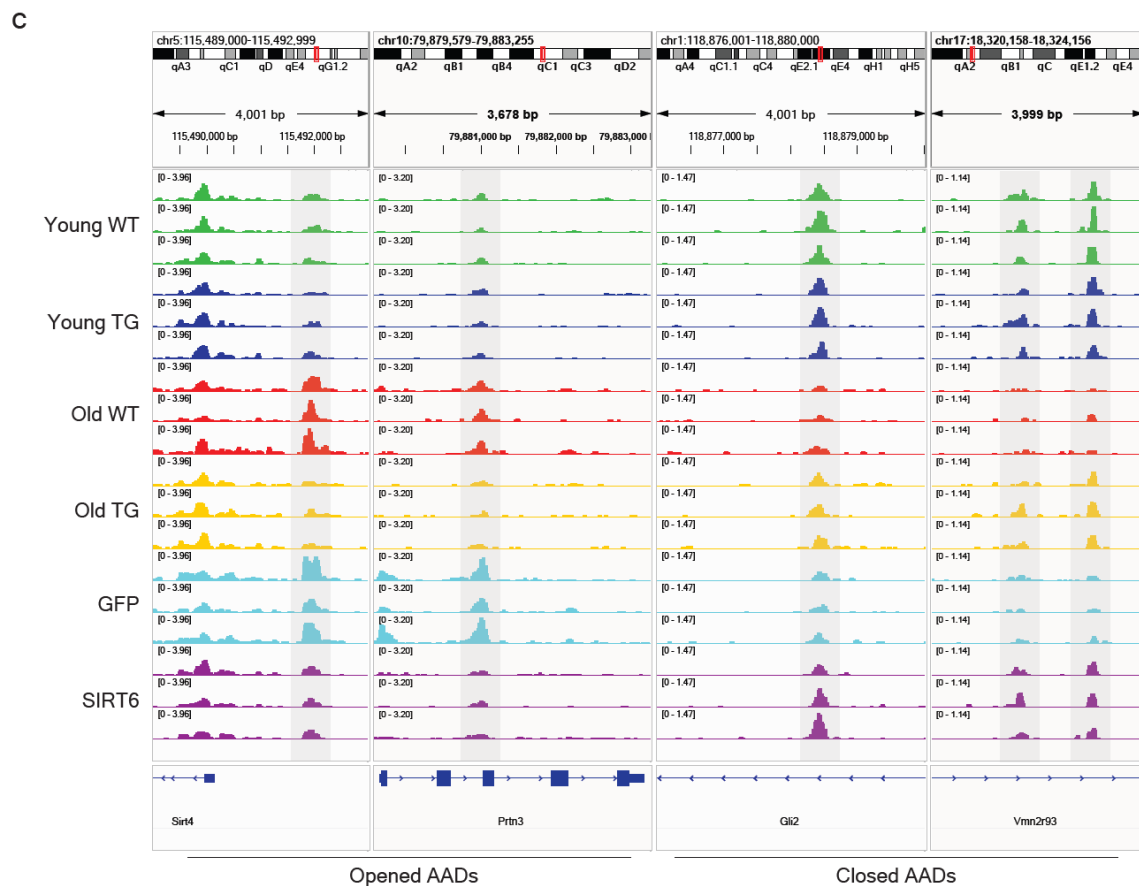
We thank the reviewer for this insightful comment about the difference in H3K27me3 levels between TG mice and AAV injected mice. Indeed, late-life induction of SIRT6 led to a significant decrease in H3K27me3 levels but lifelong SIRT6 expression did not. However, this apparent discrepancy can likely be explained by differences in the age of induction, and the duration and scope of expression. While the SIRT6 TG mice overexpress the gene systemically throughout life and were analyzed at 18–21 months of age, the AAV vector was injected at 24 months and restricted to hepatocytes for only one month. It is therefore reasonable to assume that both the shorter duration and the limited tissue specificity contributed to the different effect observed upon late-life induction. The revised manuscript now includes the following paragraph in the discussion emphasizing this topic:

“Interestingly, while no change was observed in the levels of H3K27me3 in old SIRT6 TG compared to old WT mice, induced overexpression of SIRT6 caused a significant reduction in H3K27me3 levels. Unlike lifelong SIRT6 expression studied at 18–21 months, late-life induction was performed at 24 months for just one month. Earlier induction and/or longer expression may achieve outcomes closer to those observed in SIRT6 TG mice. It also must be taken into account that hepatocyte specific overexpression of SIRT6, as in the late-life induction, does not completely mimic whole body SIRT6 overexpression, as in the lifelong overexpression, likely due to systemic effects.”

Regarding SIRT6 reverting some of the chromatin alterations observed in the old mice, per the reviewer’s important suggestion, we have added Venn diagrams of rescued genes as well as IGV snapshots (revised Supplementary fig. 11 a-c, below):

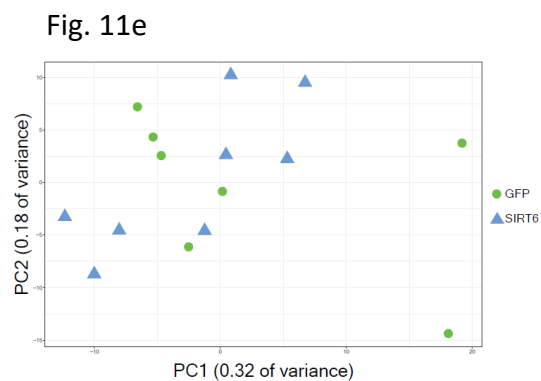
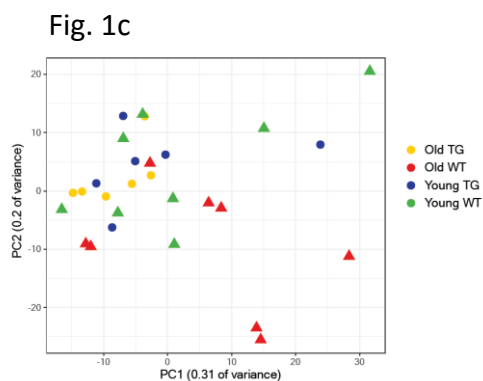
Supplementary Fig. 11





4. For ATAC-seq data it is not sufficient to report average number of reads. Please provide a table specifying number of reads, % mapped reads, number of peaks called and FRiP score. In addition, it is essential to show how reproducible the datasets are, i.e. using PCA or correlation plots.

We thank the reviewer for pointing out this important matter. All sequence metrics for both ATAC-seq and ChIP-seq can be found in the revised supplementary table file (Supplementary tables 1, 20, 23 and 25). We have also added PCA plots for both ATAC-seq analyses to the revised manuscript in supplementary figures 1c and 11e (found below, aging and SIRT6 TG mice on the left and SIRT6 AAV-injected mice on the right).



Minor points:

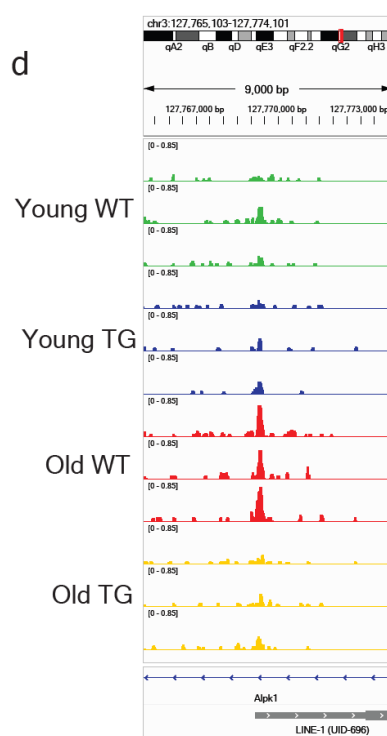
5. Please mention the mouse strain directly in the manuscript (and not only refer to previous publications)

We thank the reviewer for this constructive comment. We have added the specific mouse strain to the first paragraph of the results as follows:

“Researching the role of SIRT6 in this context was enabled through the use of our previously published SIRT6 transgenic (TG) mice. These C57Bl/6J^{0laHsd} mice exogenously overexpress SIRT6 controlled by a b actin promoter and a cytomegalovirus (CMV) enhancer, with a b globin 3' UTR (PMID: 20047575).”

6. Fig.1c/ There are very specific regions within the LINE-1 element on which SIRT6 has a clear effect - are these regulatory regions and/or have specific features? Would be nice to see a browser shot.

We thank the reviewer for this insightful comment. We couldn't find any specific feature in this region. However, we did notice that this region is just upstream to the 6 kb LINE-1 elements. We have added a representative IGV snapshot (Supplementary Fig. 1d).



7. For a more direct comparison of SIRT6 over-expression, it might be good to include the GO-terms of rescued (closed sites) in the main Figure (now: Supplementary Figure 2a)

We thank the reviewer for highlighting the importance of this panel and we have included it in the main figure (now Figure 2c).

Reviewer #4.

We thank this reviewer for finding our study “a comprehensive ... study” and for their important comments that enabled us to significantly improve the manuscript. A detailed point-by-point answer to these comments is provided below.

Major Comments

1. This study uses only male mice. While this is not inherently a concern (especially since only male mice have been reported to experience significant benefit from Sirt6 overexpression), it is important that both the title, abstract and main text (including the discussion) clearly state this fact (it is only stated once in the main text). Additionally, any conclusions drawn from the data should acknowledge that the findings are based solely on male animals and would likely not extend to females.

We thank the reviewer for drawing our attention to this important matter and agree that such clarification is crucial in general and specifically in aging research due to sex-dependent aging patterns. Therefore, we have updated the title of the manuscript to "SIRT6 Overexpression Counteracts Chromatin Aging in the Male Murine Liver". Additionally, we have stressed that the experiments have been performed only on male mice in all relevant places both in the introduction and in the results. Finally, given the difference between males and females in the effect of SIRT6 overexpression on lifespan, the revised manuscript now includes the following paragraph in the discussion:

"Notably, the positive effect of SIRT6 overexpression on lifespan is more pronounced in male than in female mice (PMID: 34050173). Thus, we decided to focus this study only on male mice to understand how SIRT6 overexpression leads to this dramatic effect. As a result, it remains unclear whether SIRT6's major role in regulation of global chromatin structure revealed in this study is sex dependent."

2. The main conclusion of this study is that SIRT6 can rejuvenate the chromatin landscape in old mice to a more youthful state. However, the authors do not directly investigate the mechanism by which SIRT6 drives this effect. Instead, they assume changes in H3K9ac, H3K18ac, and H3K56ac levels are the direct results of SIRT6 activity changes (although they are only indirect proxies for SIRT6 activity). Since other histone deacetylases can also act at these sites, these marks cannot be specifically attributed to SIRT6 function. Is there a reason the authors did not take a more direct approach—such as SIRT6 ChIP-seq—to assess SIRT6 binding and activity? Demonstrating a direct role for SIRT6 in shaping the chromatin landscape would significantly strengthen the study's mechanistic impact. If this is not feasible, we recommend toning down all claims of direct effects in the manuscript.

We thank the reviewer for raising this important point. We made several attempts to perform SIRT6 ChIP-seq on our liver samples, using different commercial antibodies, sonication conditions etc. However, as hinted at by the reviewer and the editor, SIRT6 ChIP-seq is known to be highly challenging and indeed the signal in these experiments was too low and comparable to the IgG control. We also attempted to overcome this challenge by using FLAG-SIRT6 ChIP-seq in the AML12 murine hepatocyte cell line with an anti-FLAG antibody to map

SIRT6 binding sites, but unfortunately this experiment did not identify significant peaks either. Thus, we agree with the reviewer's perspective and have accordingly toned down any statements implying a direct effect in the revised manuscript. For example, in the results: "These results highlight H3K9ac as a key regulator of chromatin during aging, with SIRT6 exerting its protective effects primarily through reducing H3K9ac levels" instead of "through H3K9ac deacetylation" in the old manuscript. Another example from the discussion: "Our ChIP-seq results highlight H3K9ac as a more dominant factor in both chromatin aging and the effects of SIRT6 in the murine liver, as evidenced by PCA analysis and the correlation between ChIP-seq signals and AADs, although it is possible the effect of SIRT6 on H3K9ac is indirect (Fig. 4c-d)."

3. What ages were used for the H3K9ac and H3K56ac ChIP-seq experiments? It is not explicitly stated in the main text, methods, or figure legends. In addition, there is an inconsistency in the ages that used for omics profiling in the SIRT6 TG mice. For ATAC-seq, their liver tissue ages were young (5-7 months old) and old (18-21 months); whereas, for genome DNA methylation in liver tissue, the ages were young (3-4 months) and old (23-24 months). In figure 5 description, they specify the age as "24-month-old C57BL/6 male mice" and it is marked sacrificed at 25 months. This adds to the inconsistency of the age description of the mice. The differences must be explicitly explained and the age choices justified for robustness and interpretability.

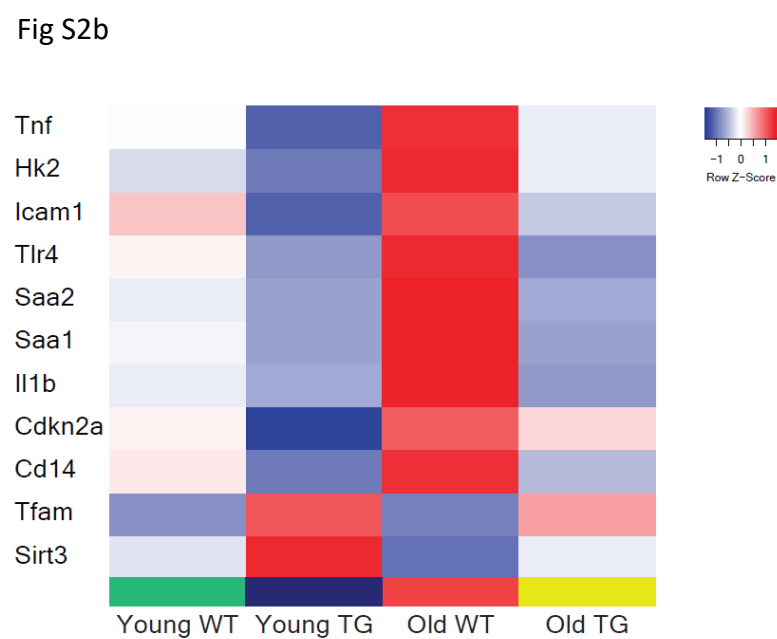
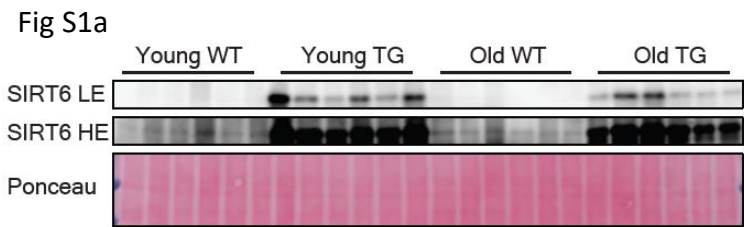
We thank the reviewer for this important comment. The H3K9ac and H3K56ac experiments were performed on livers from the same mice used for the ATAC-seq and RNA-seq. This omission has now been corrected and explicitly stated in the revised manuscript in the results section: "Given that H3K9ac and H3K56ac are direct substrates of SIRT6, we performed ChIP-seq of both marks on the same liver tissues used for both the ATAC-seq and the RNA-seq."

Regarding the small difference in the ages used for the DNA methylation and ATAC-seq experiments, this is the result of using a mice cohort from another experiment that grew under identical conditions. However, we do not believe that the minor age differences reduce the reliability of our results.

Concerning the AAV injected mice, we thank the reviewer for highlighting the lack of clarity in the text. The injection was performed at 24 months of age and the mice were sacrificed after 1 month at 25 months. Therefore, the revised manuscript now states in figure legend 5: "24-month-old C57BL/6 male mice were injected with a liver-specific AAV expressing GFP or SIRT6, and 1-month post-injection (at 25 months), the livers were collected and subjected to ATAC-seq".

4. Validation of SIRT6 overexpression in the liver of the SIRT6 TG mice should be included in this manuscript even if this mouse model has been validated and published previously (especially since overexpression transgenes tend to get silenced over generations). It would also be interesting to include the expression of known SIRT6 modulated genes (in the RNA-seq dataset) to ensure that the known activity of this protein is maintained in the SIRT6 TG mice.

We thank the reviewer for this important comment. The revised manuscript now includes validation of SIRT6 overexpression (Supplementary Fig. 1a, please see below. LE = low exposure, HE = high exposure) and the expression of known SIRT6 modulated genes from the RNA-seq (Supplementary Fig. 2b, please see below), demonstrating that SIRT6's overexpression and activity is preserved in our transgenic model.



5. To ensure reproducibility of the analyses (and accuracy of the reviewing process), there is a lot of information that should be included in the manuscript:

a. All analytical scripts/code should be deposited to a repository such as GitHub or made available as a supplemental file. Right now, the code is not available for reviewing.

We thank the reviewer for this comment. The computational analysis was conducted by The Ilana and Pascal Mantoux Institute for Bioinformatics at Weizmann Institute. Since this is an external commercial bioinformatics service provider, they usually do not provide their code. Yet, exceptionally, after discussion with them, detailed description of the analytical workflow, including commands, parameters, and software versions used, is provided, ensuring full transparency and reproducibility (please see attached file “Comprehensive summary of the analysis.pdf”). In addition, they kindly agreed that if further information is required it is possible to directly contact the bioinformatician who analyzed our data (eviatar.weizman@weizmann.ac.il).

b. For reproducibility purposes, version numbers for all software and R packages used (including cutadapt, R, ChIPseeker, EdgeR, csaw, HOMER, bagfootr, Metagene2, etc.), as well as date of access or versions for all databases (TRRUST, L1Base2, ...), should be explicitly included in the methods.

We thank the reviewer for this important comment. All version numbers have now been included in the revised methods section under bioinformatic analyses.

c. Based on the text, the authors used log fold change thresholding (see bottom of page 4 for instance) to determine their list of differentially regulated chromatin regions. Fold-change filtering, if not implemented into the statistical model used to identify differential genes/peaks, leads to poor FDR control and is not appropriate (see PMC2654802). Since fold change filtering leads to statistical issues, all of its use must be removed and all analyses should be repeated without it (or alternate analytical tools that include logFC thresholding in the statistical testing must be used).

We thank the reviewer for raising this important methodological comment and for referencing Li & Tibshirani (2008; PMCID: PMC2654802). We fully agree that post hoc fold-change thresholding, when applied without appropriate statistical correction, can in some cases compromise FDR control. However, our analysis follows widely accepted and recently published practices (Jin et al., 2025, *Nature Communications*, PMID: 40097444; Ruzicka et al., 2024, *Science*, PMID: 38781388; Shi et al., 2025, *Nature*, PMID: 40437094; Narang et al., 2024, *Nature Communications*, PMID: 39198446; Chang et al., 2023, *Cancer Cell*, PMID: 38039962; Hill et al., 2024, *Nature Communications*, PMID: 38228644), all of which adopted comparable analytical frameworks where log₂ fold-change thresholds are applied together with FDR cutoffs. These studies used similar pipelines and applied fold-change filtering after statistical testing, reflecting a community-wide standard. In addition, Li & Tibshirani themselves note that “If the FDR is controlled at a certain level using p-values, but a fold-change cutoff is applied as well, then the expected FDR must decrease, but by how much is unclear.” Thus, adding a fold-change cutoff is in fact more stringent than filtering by FDR alone. We would also like to emphasize that the main purpose of using a fold-change cutoff in our study was to focus on the most biologically relevant sites. Given the consistency of our approach with current literature and best practices in the field, we believe that our use of fold-change filtering is appropriate.

d. In the methods, the authors say they used both edgeR and DESeq2 for data processing. This is extremely puzzling, as these tools have fully redundant purposes -however, the specific way they were each used is not explained. Please elaborate on the use of both and why both were used in this case instead of a single tool.

We thank the reviewer for this comment. Indeed, this is a mistake – only DESeq2 was used for data processing. This has been corrected and the mention of edgeR has been omitted from the revised manuscript.

e. In the statistics section, the authors say they used “paired or unpaired two-tailed Student’s t-test”. Was a normality test used to determine that the data fit the normality hypothesis? If yes,

please mention why test was used to determine normality, If the data does not satisfy the normality assumption, please redo all tests using nonparametric equivalents.

We thank the reviewer for the important comment. We have performed a Shapiro-Wilk normality test to ensure that the data fit the normality hypothesis. When this was not the case, non-parametric equivalents were used. This has now been clarified in the methods section and in the revised figure legends.

f. “during their active hours (dark)” – this window is a 12 hour window, during which many changes (including in chromatin and gene regulation) can occur and have been documented. Please provide a shorter/more precise time window for long-term reproducibility purposes.

We thank the reviewer for this important comment. The mice were sacrificed at ZT 16-18. This has now been stated explicitly in the methods section: “Mice were sacrificed by cervical dislocation after 4 hours of fasting during their active hours (dark) at ZT 16-18”.

g. “integrity number (RIN) was assessed by TapeStation”. Was a threshold applied for downstream processing? If yes, this must be explicitly stated.

We thank this reviewer for this constructive comment. Indeed, a threshold of $RIN \geq 7$ was applied. The revised methods section now states this information: “Only samples whose RIN was above 7 were taken for sequencing”.

h. Western blot antibodies: Please also provide the dilution that was used for these experiments so that the methods can be reproducible.

We thank the reviewer for this comment. All primary antibodies were diluted 1:1000, and secondary antibodies were diluted 1:15,000. The revised manuscript includes this information in the methods section: “All primary antibodies were diluted 1:1000 in 5% BSA(w/v). Secondary antibodies were diluted 1:15,000 in 5% skim milk (w/v)”.

Minor comments

1. In the opening sentences of the introduction, the authors mention both life expectancy (lifespan) and healthspan. For clarity, it would be helpful to briefly define these terms and distinguish between them, emphasizing that lifespan refers to the total length of life, while healthspan refers to the period of life spent in good health.

We thank the reviewer for this constructive comment. The revised introduction now clarifies how healthspan differs from lifespan: “Understanding the biology of aging may provide insights needed to prolong healthspan – the period of life spent in good health.”

2. There is no clear introduction to the SIRT6 TG mouse model. For clarity, the authors should briefly describe the genetic construct and relevant features of the SIRT6 transgenic mice when the model is first introduced in the opening paragraph of the Results section. This would help readers unfamiliar with the model better understand the study’s context rather than rely on readers to go to the original paper describing the model.

We thank the reviewer for this important comment. The opening paragraph of the results now includes the following explanation:

"Researching the role of SIRT6 in this context was enabled through the use of our previously published SIRT6 transgenic (TG) mice (Supplementary Fig. 1b). These C57Bl/6JOLA^{Hsd} mice exogenously overexpress SIRT6 controlled by a β actin promoter and a cytomegalovirus (CMV) enhancer, with a β globin 3' UTR."

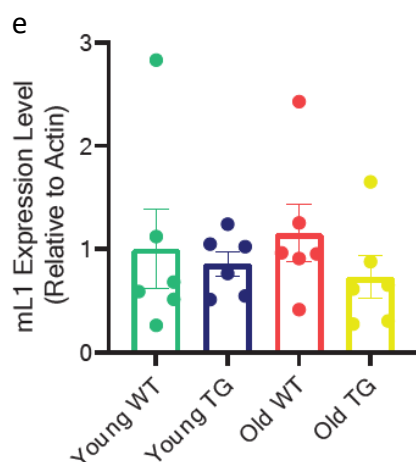
3. It is unclear why the authors chose to focus specifically on the liver in this study. If there is a particular rationale—such as the liver's metabolic relevance, known sensitivity to aging, or prior links to SIRT6 function—it would be helpful to briefly state this in the introduction to provide readers with a clearer foundation for the study's focus.

We thank the reviewer for bringing this omission to our attention. The final paragraph of the revised introduction now includes the following explanation of the rationale for performing our experiment specifically on the liver:

"In order to discover the role of SIRT6 in regulation of global chromatin with age, we decided to focus our study on the liver, as it is a major metabolic organ whose function is known to decline with age and be preserved in mice overexpressing SIRT6 (PMID: 34050173, PMID: 31851938, PMID: 29112863, PMID: 31427197)."

4. The authors report that LINE-1 chromatin accessibility increases with age and is reduced by SIRT6 overexpression, based on ATAC-seq data. Did the authors also examine LINE-1 transcription levels in the RNA-seq datasets? If so, it would be informative to know whether SIRT6 overexpression similarly reduces LINE-1 transcript levels to those observed in young mice (and not just remodels the chromatin around it).

We thank the reviewer for this interesting comment. We have performed RT-PCR of LINE-1 transcript levels in our livers and found that in comparison to young mice, there is a similar, albeit non-significant, trend of increased LINE-1 levels in old WT but not old SIRT6 TG mice. This is included in the revised manuscript as Supplementary Fig. 1e (please see below).

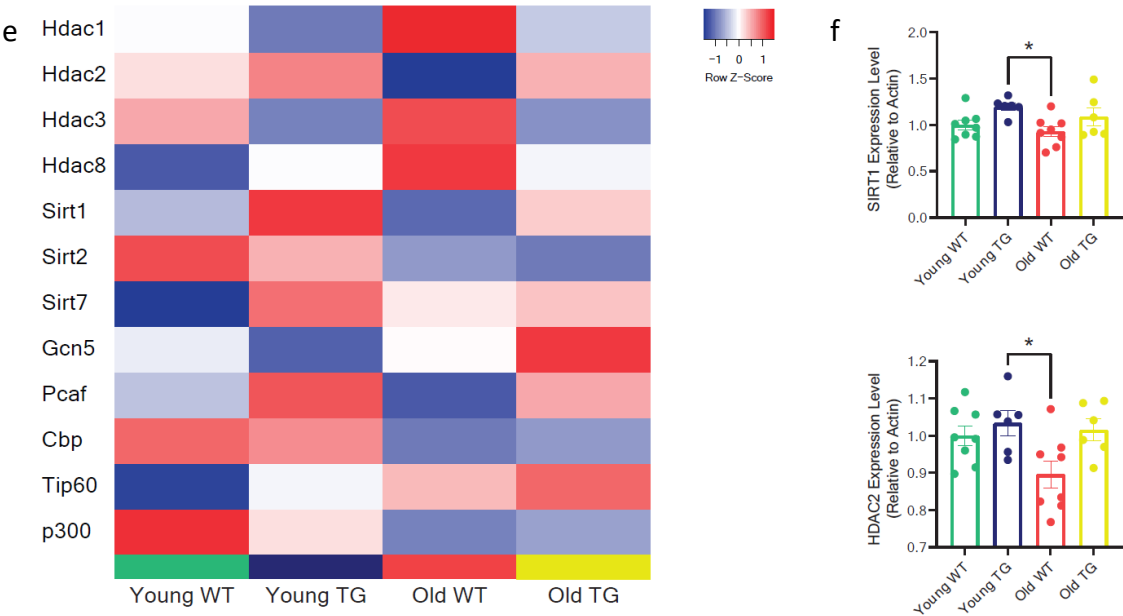


5. It would be interesting to check the expression of other enzymes responsible for H3K9, H3K18, and H3K56 acetylation (using either the RNA-seq dataset or western blot) to see how

SIRT6 OE impacts their expression. Would also be interesting to look at their activity levels in the SIRT6 TG mice.

We thank the reviewer for this insightful comment. We have checked the expression levels of the relevant enzymes in our RNA-seq data. Of these, only SIRT1 and HDAC2 exhibited trends that could explain the observed changes in histone modifications. However, RT-PCR found no significant change (when comparing young WT to old WT or old WT to old SIRT6 TG) in their expression levels. This data is now included in the revised manuscript as Supplementary Fig. 9e-f (see below).

Unfortunately, we do not see how it is possible to examine the activity levels of enzymes in a transgenic mouse model.



6. *Supplementary Figure 9 is currently labeled as Supplementary Figure 8 in the supplementary file.*

We thank the reviewer for noticing this, it has been corrected in the revised manuscript.

7. *The authors label their transgenic mice as SIRT6 TG without clarifying that the TG means transgenic. This abbreviation needs to be explicitly defined at first use.*

We thank the reviewer for this constructive comment. The abbreviation is now defined at first use in the results section of the revised manuscript: “Researching the role of SIRT6 in this context was enabled through the use of our previously published SIRT6 transgenic (TG) mice”.

We would like to thank the reviewers again for their important comments that enabled us to significantly improve our manuscript, and are looking forward to reading our revised manuscript in *Nature Comm.*

Point by point response to reviewers' comments

Reviewer 1

The reviewer raised several remaining concerns that should be addressed before publication:

1. *“The authors clarify that the Western blot data in Supplementary Figure 8, showing global H3K9ac and other histone marks, were normalized first to Ponceau staining and then to total H3. They argue that this method is more accurate than what is detected by eye, which shows minimal differences in H3K9ac, H3K56ac, and other marks with age or genotype. However, the rationale for introducing Ponceau normalization is unclear, as standard practice typically involves simply normalizing to total H3. Generally, robust differences can be observed visually, even with unequal H3 levels, which can be estimated by eye. Additionally, it is concerning that the quantified H3K9ac levels do not appear to differ between old WT and old TG samples (Figure 8d), which seems contrary to the authors' major conclusions. It is plausible that global differences in these marks may not be detectable if aging-dependent changes are relatively localized. The authors should quantify the fraction of total H3K9ac peaks altered by either aging or SIRT6 to determine if this aligns with the subtle, variable, or perhaps nonexistent differences in the global Western analysis.”*

We thank the reviewer for this important comment. The reviewer noted that the western blot for total H3K9ac does not reveal major global differences and emphasized that this has no impact on the main conclusion of the study. We agree that normalization solely to total H3, rather than to both H3 and Ponceau, reveals no significant changes in H3K9ac levels. However, as Reviewer 1 correctly points out, our conclusions do not rely on global acetylation changes, but on chromatin alterations at specific aging-associated loci.

The differential H3K9ac peaks identified by ChIP-seq between young and old WT liver account for only 5.8% of all peaks, a level well below the sensitivity of Western blot detection. Nonetheless, SIRT6 overexpression restores 87.5% of these age-associated H3K9ac changes to youthful levels. This strong, locus-specific effect directly supports our mechanistic model. In the revised manuscript we:

- normalize H3K9ac, and all other modifications, solely to total H3, (Fig 4a, Fig S8, Fig. 5c, Fig. S11c)
- clarify that the biology uncovered pertains to **localized** regulation of H3K9ac within AADs, and
- emphasize that this local action is precisely what SIRT6 is known to mediate and what our data robustly demonstrate. As demonstrated in this paragraph added to the discussion:

“While Western blot analysis indicated that global H3K9ac levels do not significantly differ between old WT and old SIRT6 TG mice (Supplementary Fig. 8d), our ChIP-seq data reveals a critical, locus-specific regulation that is obscured in bulk analyses. We observed that the majority (63%) of differential H3K9ac peaks are actually downregulated with age globally. However, a specific subset of genomic regions, the opened AADs, exhibits a distinct age-dependent increase in H3K9ac which drives chromatin opening and pro-inflammatory gene expression. Importantly, SIRT6 overexpression specifically suppresses this focal accumulation of H3K9ac at these deleterious loci, maintaining them in a repressed, youthful state. This demonstrates

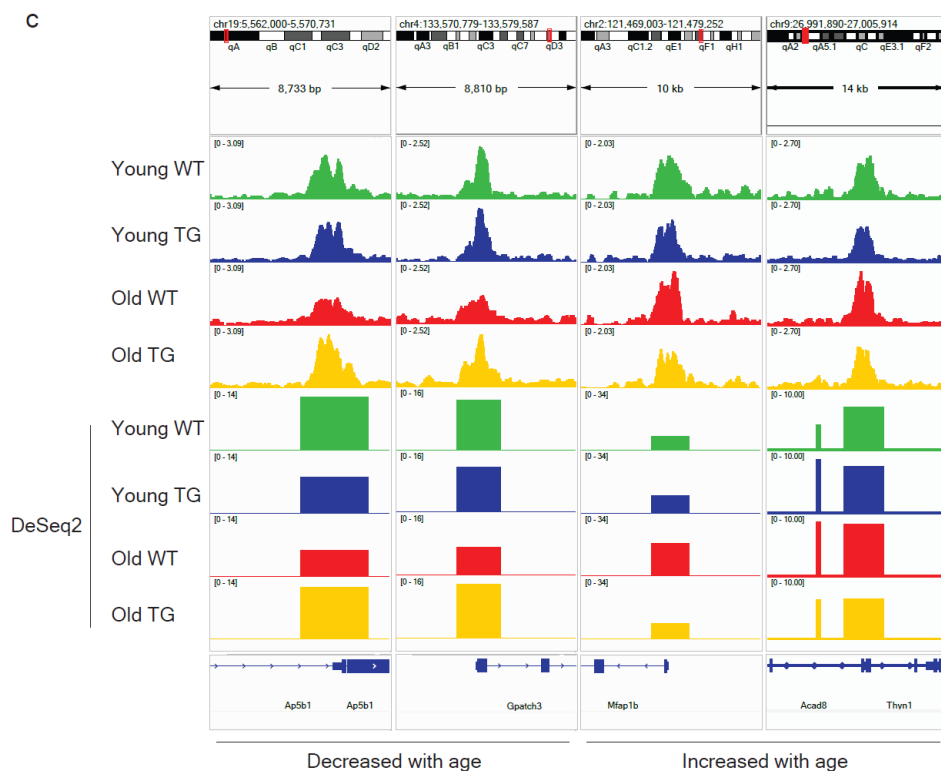
that SIRT6's protective mechanism is not a non-specific, global deacetylation, but rather a targeted preservation of chromatin structure at key regulatory sites. WB global readout averages H3K9ac over the entire genome and is therefore relatively insensitive to locus-specific changes. Consequently, this specific rescue effect is diluted by the broader, bidirectional histone landscape changes in whole-lysate quantification, highlighting the contribution of our high-resolution ChIP-seq in characterizing specific functional epigenetic alterations."

These changes will make the manuscript clearer without affecting any scientific conclusion.

2. "In response to previous reviews, the authors have added new supporting data for their ChIP-seq analyses in Supplementary Figure 9, including differential peak analysis and three representative IGV snapshots. However, there is no description of what each example is intended to illustrate. I assume the first two examples show H3K9ac peaks that decrease in old WT and are reverted in old TG mice. However, there is no clear example of an increase in H3K9ac in old WT mice that is absent in TG mice, which is the main biological change the authors propose occurs in old mice and is rescued by SIRT6. The third example may intend to demonstrate this, but it is not clear, and quantification would be necessary to validate the claim. It is concerning that the authors did not provide at least one additional clear example."

We appreciate the reviewer's comment on the clarity of Supplementary Fig. 9c. Although DeSeq2 quantifications were provided, the presentation lacked clear titles and visual emphasis. In the attached revised Supplementary Fig. 9c, we:

- added clear snapshot labels,
- included a more illustrative example showing increased H3K9ac in old WT mice that is attenuated in SIRT6-TG mice.



3. *“The authors have now included PCA analyses for both ATAC-seq experiments (Figures 1c and 11e). It is troubling that PCA did not reveal differences for the AAV-SIRT6 overexpression experiments (Figure 11e). Furthermore, for the aging and SIRT6-TG experiment (Figure 1c), there is considerable variability across the different samples, particularly among the WT (old and young) mice.”*

We thank the reviewer for this insightful comment. The reviewer noted variability among WT samples in the PCA. This heterogeneity is a well-established feature of aging biology. To account for this inherent variability, we intentionally used a relatively large cohort (n=6–8 animals), which provides more reliable estimates of aging-related changes than typical sample sizes in the field. In the AAV-SIRT6 experiment, the limited PCA separation is likely due to the advanced age of the mice and the short, one-month treatment window. Under these biologically conservative conditions, we did not expect full chromatin reprogramming, yet still observed significant and consistent changes at AADs. The revised results section now explicitly emphasize this point.

Regarding the transgenic mice ATAC-seq the revised manuscript now states: “Although principal component analysis (PCA) revealed only moderate separation between the old WT mice and the other groups (Supplementary Fig. 1c), analysis of the log₂ fold change distribution of all identified peaks indicated an overall increase in chromatin accessibility in old WT compared to young WT mice (Fig. 1b).”

And regarding the AAV8-injected mice ATAC-seq, the revised manuscript now states: “While PCA did not distinguish between groups and overall chromatin accessibility remained unchanged (Supplementary Fig. 11e-f), SIRT6-injected mice showed slightly decreased accessibility in AADs (Supplementary Fig. 11g).”

Reviewer 2

1. *“We thank the authors for clarifying how the western blot data was normalized. While we appreciate that ImageJ might pick up differences that are hidden from the eye, a two-fold difference in intensity as reported for many of the modifications should be easily visible if blotted in the linear range of the antibody. This is particularly important as also the ChIP-seq data does not support a global change. Thus, these data is still not very convincing.”*

We thank the reviewer for this important comment. The reviewer noted that the western blot for total H3K9ac does not reveal major global differences and emphasized that this has no impact on the main conclusion of the study. We agree that normalization solely to total H3, rather than to both H3 and Ponceau, reveals no significant changes in H3K9ac levels. However, as Reviewer 1 correctly points out, our conclusions do not rely on global acetylation changes, but on chromatin alterations at specific aging-associated loci.

- normalize H3K9ac, and all other modifications, solely to total H3, (Fig 4a, Fig S8, Fig. 5c, Fig. S11c)
- clarify that the biology uncovered pertains to **localized** regulation of H3K9ac within AADs, and
- emphasize that this local action is precisely what SIRT6 is known to mediate and what our data robustly demonstrate. As demonstrated in this paragraph added to the discussion:

“While Western blot analysis indicated that global H3K9ac levels do not significantly differ between old WT and old SIRT6 TG mice (Supplementary Fig. 8d), our ChIP-seq data reveals a critical, locus-specific regulation that is obscured in bulk analyses. We observed that the majority (63%) of differential H3K9ac peaks are actually downregulated with age globally. However, a specific subset of genomic regions, the opened AADs, exhibits a distinct age-dependent increase in H3K9ac which drives chromatin opening and pro-inflammatory gene expression. Importantly, SIRT6 overexpression specifically suppresses this focal accumulation of H3K9ac at these deleterious loci, maintaining them in a repressed, youthful state. This demonstrates that SIRT6’s protective mechanism is not a non-specific, global deacetylation, but rather a targeted preservation of chromatin structure at key regulatory sites. WB global readout averages H3K9ac over the entire genome and is therefore relatively insensitive to locus-specific changes. Consequently, this specific rescue effect is diluted by the broader, bidirectional histone landscape changes in whole-lysate quantification, highlighting the contribution of our high-resolution ChIP-seq in characterizing specific functional epigenetic alterations.”

These changes will make the manuscript clearer without affecting any scientific conclusion.

2. *“The authors refer to the publication PMID: 38484732 that used a similar method to quantify global changes in ChIP-seq profiles and state that this was done without spike-in as well. This is true. Nevertheless, this is far from ideal and might misrepresent the data, given the normalisation procedure of ChIP-seq to overall read counts. In addition, the cited paper also reports a far more dramatic change in modification levels, in contrast to the minor change reported here ($<0.1 \log_2(\text{norm. counts} + 1)$)”*

We thank the reviewer for this important comment. The reviewer noted that global quantification from ChIP-seq is imperfect without spike-ins. Since these data do not contribute to our main conclusions, we removed those panels entirely.

3. *“We were surprised to see that the differential peak calling for the ChIP-seq data revealed a large number of differential peaks, given that the PCA clearly shows intermixing of the individual ChIP-seq datasets. It is not understandable how the lack of variance between the samples can result in such large number of differential peaks. We tried to inspect the uploaded datasets, but were not able to look at them since they are currently still private.”*

We thank the reviewer for this interesting comment. The reviewer raised a conceptual point about the coexistence of limited PCA separation and numerous differential peaks. We appreciate the opportunity to clarify this. Although the separation between groups in H3K9ac PCA was moderate, there is still evidence of separation between the old WT mice and the other groups (Fig 4b, left panel). However, no separation was observed in H3K56ac PCA (Fig 4b, right panel) which lead to the reviewer concern. As documented in previous studies (e.g., PMID: 40087566, 39471208, 30348083), PCA highlights the dominant sources of variation across all peaks, whereas differential peak calling identifies statistically significant, coordinated changes within specific subsets of peaks. Thus, small but highly consistent group-specific changes, such as those we report, may result in many significant differential peaks even when PCA separation is subtle. In our H3K56ac dataset, only ~3500 of ~79,000 peaks were differentially expressed, consistent with an aging phenotype that affects specific chromatin regions rather than the global epigenomic landscape. The revised results now explicitly address it:

“Despite the lack of separation found in the H3K56ac PCA, differential analysis of old WT vs. young WT found 4627 and 3554 differential peaks for H3K9ac and H3K56ac, respectively (p-value < 0.05; Supplementary Table 22).”

We regret that the reviewer was unable to inspect our dataset., we are providing the datasets’ tokens again here for the reviewer’s convenience:

Experiment	#	Link	Token
SIRT6 TG ATAC-seq	GSE294103	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE294103	itcfkoyejtaljyz
SIRT6 TG RNA-seq	GSE294067	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE294067	adyxiuigtiprwh
SIRT6 TG ChIP-seq	GSE294085	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE294085	evqtosquzmtmfgb
SIRT6 AAV8 ATAC-seq	GSE294084	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE294084	otmjciqmxfgbzqn
SIRT6 TG Methylome	GSE296168	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE296168	cxanwcqidrwbvwx

Reviewer 4

1. “There is a remaining point that the authors refused to correct because they deem it “common practice” and thus refuse to hear the argument that it is statistically flawed and incorrect. Thus, my issue with log2FC thresholding remains (as the authors refused to remove it). To note, just because other people do something wrong from a statistical standpoint, that is not sufficient reason to continue to do something wrong when alerted to the issues inherent with the practice. If the authors want increased stringency (which they argue), they can easily apply a more stringent FDR threshold (which would pose no problems in terms of statistics) to focus on the most robust results. As it stands, the practice of logFC thresholding is problematic (and the fact that it is widespread and not corrected after peer-review will continue to lead to these issues compounding). As an example for why “common practice” is not a good rationale - common practice until much too recently was to only use on sex of animals in biomedical research, because “female data is too noisy”. This has been proven demonstrably false, yet remained common practice (like logFC thresholding).”

We thank the reviewer for this constructive comment. The reviewer expressed concern about our use of both an FDR cutoff and a log2 fold-change threshold. In response, we reanalyzed the data using a more stringent FDR cutoff (FDR < 0.05) and removed the fold-change filter, as recommended. We reviewed the updated results and found that all major conclusions remain unchanged. The full updated analysis is now integrated into the revised manuscript (Fig. 1e-h, Fig. 2a-b, Fig. 3, Fig. 5 d-h and all corresponding supplementary figures). We have included revised key figures as appendix to this letter.

We would like to thank the reviewers again for their important comments that enabled us to significantly improve our manuscript, and are looking forward to reading our revised manuscript in *Nature Comm.*

Revised Key Figures

Fig. 1f

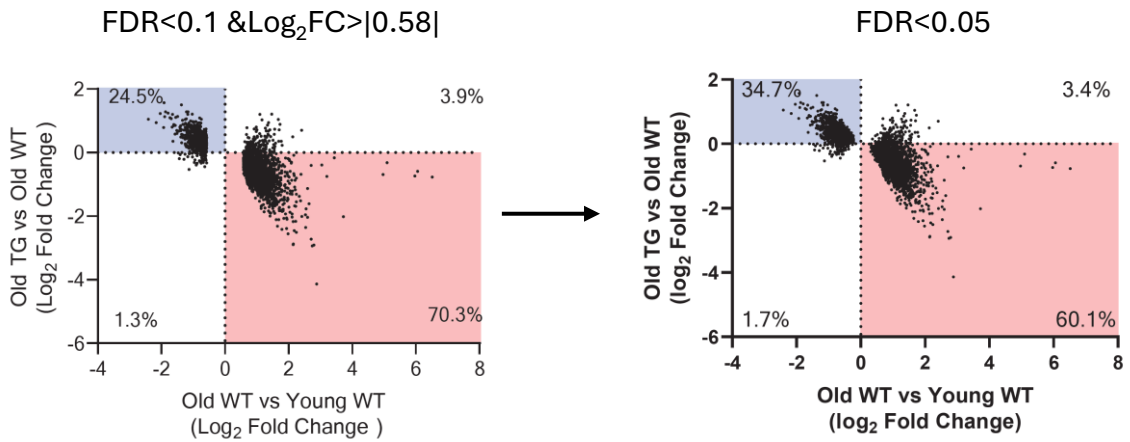


Fig. 2a

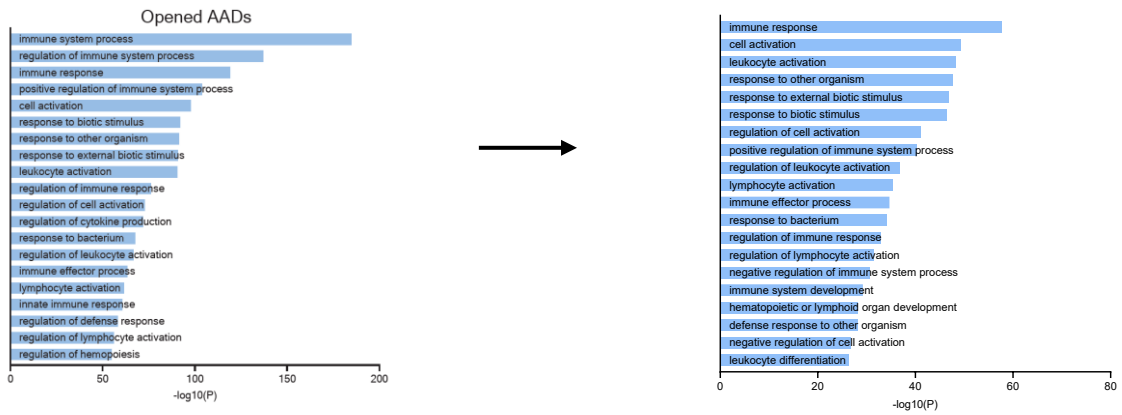


Fig. 2b

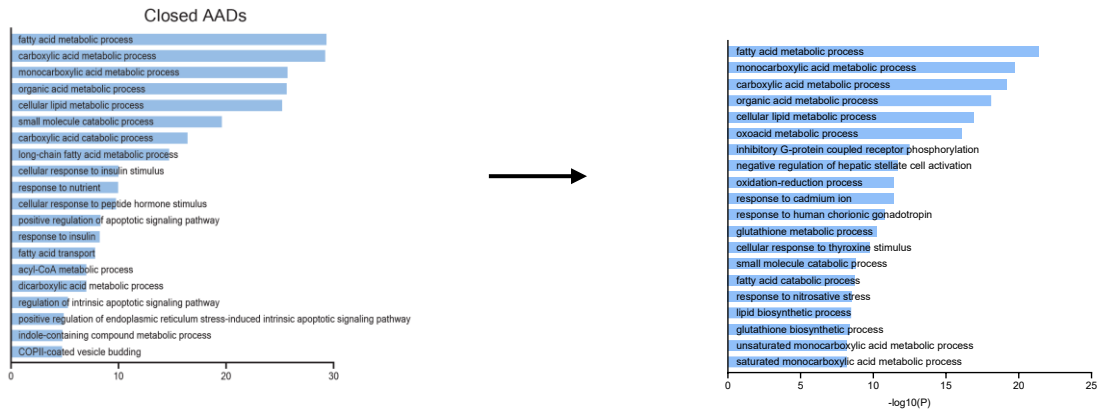


Fig. 3a

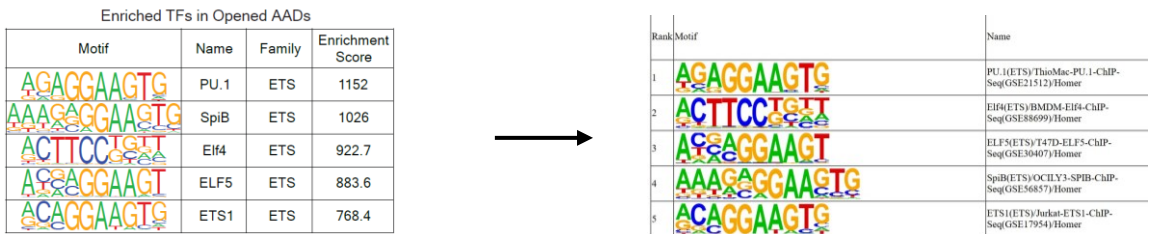
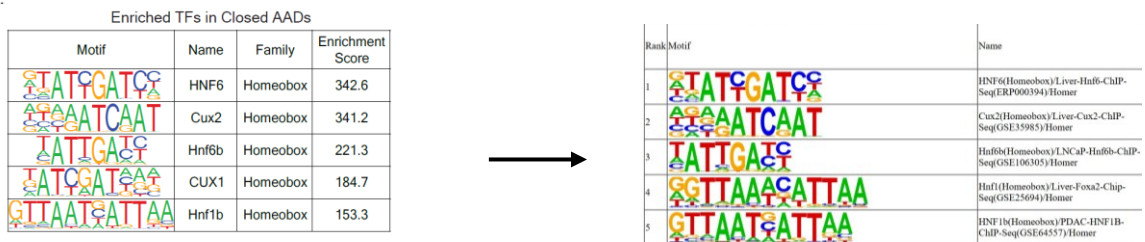


Fig. 3b



Point by Point Response

Reviewer #1 (Remarks to the Author):

In this revision, the authors have clarified a number of issues and updated data in response to my review as well as those of the other reviewers. These changes have addressed my concerns. The study's findings are important and of interest to the broad readership of Nature Communications, and I support its publication.

We thank the reviewer for their previous insightful comments that enabled us to significantly improve our manuscript and for supporting the publication of our paper whose findings they described as "important and of interest to the broad readership of Nature Communications".

Reviewer #2+3 (Remarks to the Author):

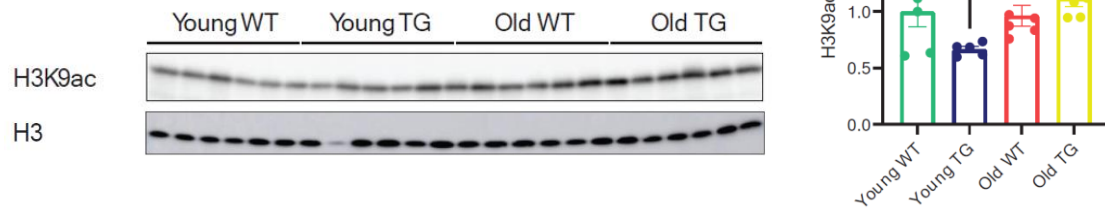
In the second revision the authors have now included the suggestions of two reviewers and removed the WB normalization to Poinceau. They now write that there is no big differences upon aging in the marks analyzed. However, now H3K9ac shows an almost two-fold increase in levels (Supplementary Figure 8a and d). We agree with reviewer 1 that such an increase should be easily visible on a western blot, which it is not. Thus, despite the fact that the initial statement has been removed from the manuscript, the issue around WB quantifications remains unsolved.

We thank the reviewers for this important comment. Due to the confusion around the quantification of the H3K9ac western blot, which, as noted by the editor, may derive from the lower resolution of the blot, we decided to rerun it. As can be seen in the revised figure below, the new blot provided a higher resolution image whose quantification revealed no significant differences in global H3K9ac levels with age or upon SIRT6 overexpression in old mice. The new blot and quantification are now incorporated in the revised manuscript, and the revised text now acknowledges these results.

On page 9, in the results section: "Among these, H3K56ac showed a trend toward an increase in old WT compared to young WT mice, while age had no effect on H3K9ac in WT mice."

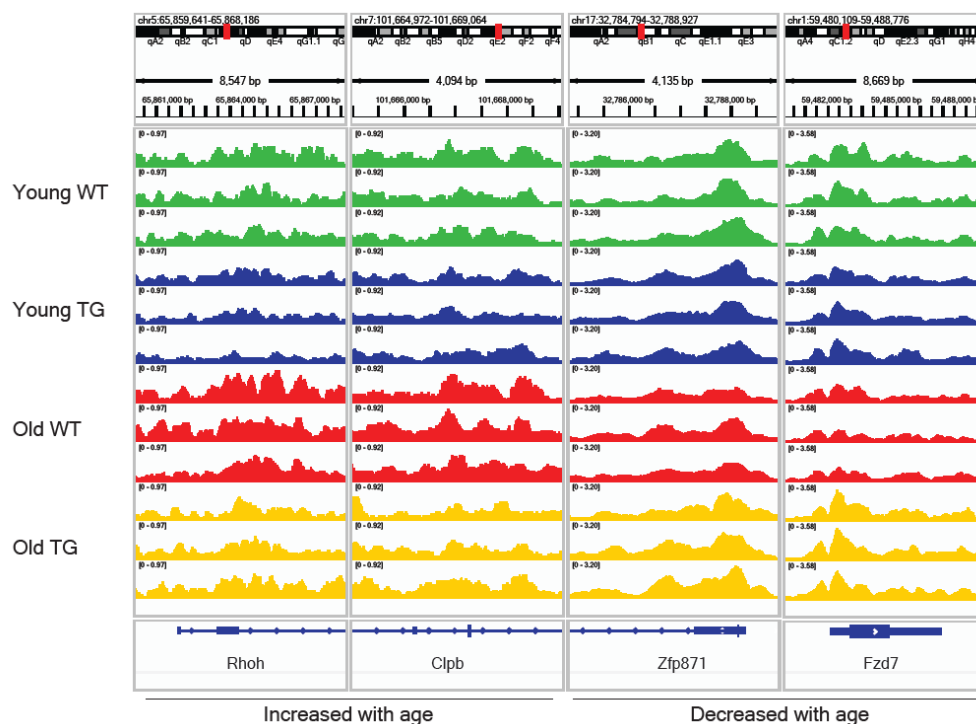
And on page 13, in the discussion: "While Western blot analysis indicated that global H3K9ac levels do not change significantly with age or upon SIRT6 overexpression in old mice...".

From Revised Supplementary Fig. 8a,d



Also, the question around ChIP-seq peaks and the PCA remains unsolved. The authors argue that there can be differential peaks present despite the fact that PCA shows intermixing. The authors argue that we might refer to the PCA for H3K56ac (Figure 4a,b). However, we are concerned about both ChIP-seq PCAs. Yes, there is a slight separation of data points in PC2 for H3K9ac (for half of the old WT samples) which in any case only explains less than 10% of the variance in the data. The authors need to at least show snapshots of the individual ChIP-seq tracks as part of the figure for selected genes as done for the ATAC-seq data in Figure 1d or Supplementary Figure 12c. In addition, the links provided by the authors for the private GEO data works now, but unfortunately only CSV files describing normalised peak files are available. This is not sufficient. The authors need to upload fastq files and ideally, bigwig files to allow an easy expection of the data.

We thank the reviewers for this constructive comment and agree that additional snapshots would be beneficial. Thus, to support the claim of age and SIRT6 dependent changes in H3K9ac, we have added IGV snapshots of individual tracks from the H3K9ac ChIP-seq to the revised manuscript, and they now appear as Fig. 4c (please see below).



Regarding the GEO, all ChIP-seq bigwig files have been uploaded and are now available for review. This, as stated by the reviewers, will allow an easy assessment of the data. Fastq files were uploaded upon first submission of our manuscript. Unfortunately, according to GEO policy, fastq files cannot be downloaded even with a reviewer's token. At this stage, we prefer the dataset not to be fully public prior to an editorial decision. They will be available for downloading as soon as the data become public. Per our request, the GEO provided us with an index list describing the SRA metadata for all our uploaded fastq files for the reviewer's convenience (see table below):

Experiment	#	Link	Token	SRA Metadata
SIRT6 TG ATAC-seq	GSE294103	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE294103	itcfkoyejtaljyz	https://ftp-trace.ncbi.nlm.nih.gov/sra/review/SRP577387_20260127_130320_1c545807b6673a8a3d6067c3fcb0d82a
SIRT6 TG RNA-seq	GSE294067	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE294067	adyxiuigptiprwh	https://ftp-trace.ncbi.nlm.nih.gov/sra/review/SRP577249_20260127_125252_89ecb4c4c1e7ec4bf70059e8aa3fa8c8
SIRT6 TG ChIP-seq	GSE294085	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE294085	evqtosquzmtf gb	https://ftp-trace.ncbi.nlm.nih.gov/sra/review/SRP577283_20260127_130151_c4afd240a0f04d81902c01067aa98956
SIRT6 AAV8 ATAC-seq	GSE294084	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE294084	otmjciqmxfgbzqn	https://ftp-trace.ncbi.nlm.nih.gov/sra/review/SRP577284_20260127_125922_05abb210c6c31429af84e383e4762732

SIRT6 TG Methylome	GSE296168	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE296168	cxanwcqidrbvwx	https://ftp-trace.ncbi.nlm.nih.gov/sra/review/SRP582805_20260127_130415_0bd1c46678d1bf0a3945bfcf3369ad13
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Finally, the manuscript moves from global to local changes as an explanation for the effect of SIRT6. This case would usually demand for a more direct assessment for the role of SIRT6 and a ChIP-seq dataset to overlay the effect on chromatin marks with SIRT6 occupancy. We acknowledge that this was asked before and deemed technically impossible by the authors. However, this needs to be explicitly mentioned as a limitation in the discussion.

We thank the reviewers for this significant comment. We have now acknowledged this limitation in the discussion, on page 13:

"Although we mapped local H3K9ac changes at specific loci in response to SIRT6 overexpression, we did not generate SIRT6 occupancy data to directly overlay binding with these chromatin changes, which we acknowledge as a limitation."

Reviewer #4 (Remarks to the Author):

The authors have now addressed my comments satisfactorily.

We thank the reviewer for their previous important comments which helped us significantly improve the quality of our manuscript, and for finding the revised manuscript satisfactory.

Point by Point Response to Reviewers

Comment #1

"We agree that it was a good idea to rerun some of the western blots. However, given the results we still feel that the text misrepresents the data. Quote (page 9, 2nd paragraph): "Among these, H3K56ac showed a trend toward an increase in old WT compared to young WT mice, while age had no effect on H3K9ac in WT mice. Interestingly, this increase in H3K56ac was mitigated in old SIRT6 TG mice. As these changes were not statistically significant, this global analysis failed to provide a robust explanation for the observed changes in AADs." There are two issues with this statement. The authors push too hard as there is not a trend in the data for H3K56ac to be slightly upregulated, which makes the second part even more misleading as SIRT6 cannot mitigate an effect that was not present in the first place. The toned-down follow up sentence does not compensate here."

We thank the reviewers for accepting our rerun gel. Yet, the reviewers' comment about H3K56ac is not entirely clear. As described in the text, we highlighted that the slight upregulation in H3K56ac in age was not statistically significant and did not draw any conclusions from this result. This was, in our opinion, simply the description of the results, and we definitely did not "push" any conclusion. Given that H3K56ac is a known SIRT6 substrate, we considered it essential to describe these results due to their importance for the aging field. However, per the reviewers' comment, we removed all claims of a trend in H3K56ac from the manuscript and include only the following line: "This global analysis failed to provide a robust explanation for the observed changes in AADs".

Comment #2

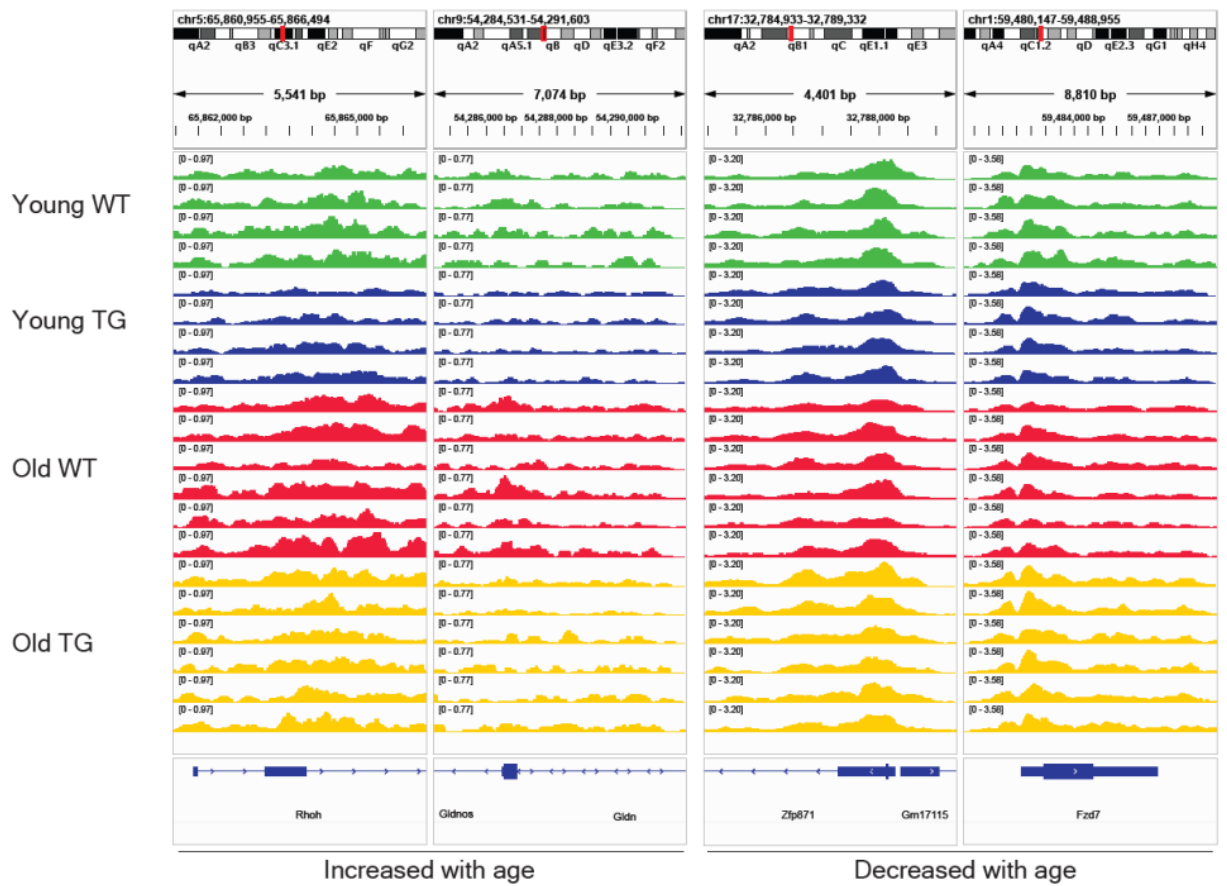
"More worrying is the H3K9ac genomic data. We thank the authors for making it available now. We inspected the tracks on the IGV browser and there are a number of concerns. Firstly, the variability seen in the PCA plots is also visible in the genomic tracks. This poses a problem since the effects seen are very minor (see examples below). Even if the differences were statistically significant, it is very difficult to imagine that they have biological meaning, certainly not explaining the effects seen on chromatin architecture by ATAC-seq, which puts into question the model of SIRT6 function the authors would like to push. Some of the peaks presented in the browser shots 4c do not necessarily represent functional peaks. For instance, the one picked for ClpB is not showing the promoter peak (as would be functionally relevant for H3K9ac), but some minor peak downstream of the start site (see Review-Figure 1). Even more worrying is the apparent use of different tracks to highlight apparent increases/decreases in different genomic loci. The browser shot always depicts three tracks, while uploaded are up to six repeats that were also used for differential peak calling. Review-Figure 2 strongly suggests that at least for the two examples used, different tracks were used at the different loci. This is unacceptable, particularly given the variability between experiments."

We thank the reviewers for these comments. In short, the reviewers raised two claims. 1- The ChIP-seq data is highly variable and therefore it is impossible to draw biological meaning out of it, specifically as one out of the 4 shown loci was within the gene body and not in a promoter region. 2- We chose to show three out of the four to six mice in each group, and different mice were used for each peak instead of showing the same mice for all. Before diving into the differential ChIP-seq analyses, we would like to emphasize that our manuscript's main claim is not based on the differential analysis (in fact it was only added upon the reviewers' request). We claim that the age-dependent change in chromatin, specifically of opened AADs in old mice, is accompanied by an elevation in the global signal of H3K9ac that is mitigated by SIRT6

overexpression. Although those changes are minor at each specific AAD site, the global effect across all opened AADs is significant and robust, as was demonstrated by the metagene plot (Fig.4 d). Therefore, focusing on specific peaks as suggested by the reviewer, does not capture the global trend across opened AADs that forms the basis of our conclusion.

Regarding the comment about variability, high variability is a well-documented phenomenon of aging (Meyer et al., *Nature Aging*, 2025, Martinez-Jimenez et al. *Science* 2017, and Bahar et al. *Nature*, 2006). Therefore, we purposefully used a large sample size (n=6 in old mice) to account for the variability in the population. Indeed, despite the expected variability, we uncovered global significant changes in the H3K9ac signal in AADs, revealing a common general effect of aging and SIRT6. As to the biological significance of the results, we do not see why these changes in H3K9ac levels cannot have caused changes in chromatin architecture and accessibility as observed by ATAC-seq. Indeed, the ATAC-seq fold changes were in the same magnitude as those of the ChIP-seq. As with the ChIP-seq, the power of the ATAC-seq is derived from the genome-wide scale of the effect. Furthermore, in regard to the functionality of the peaks, we never claimed that all changes were found at promoters. Thus, we chose to show a peak that is located in the gene body as an example of a non-promoter peak. Importantly, although the main effect of H3K9ac on gene expression may be via its levels in the promoter, H3K9ac in the gene body is known to increase transcription as well (Etchegaray et al. *Molecular Cell*, 2019). It is also worth emphasizing that since the peak is situated within the gene body, which is occupied by H3K9ac, the “background” represents this real biological H3K9ac signal, and not just technical noise. Indeed, this “background” is much lower in intragenic regions. Finally, out of the four peaks represented in Fig. 4c, three are localized to the promoter. However, per the reviewers' request to show promoter localized peaks, we have replaced this peak with a promoter localized peak in the revised manuscript (Fig. 4c; please see below).

Regarding the second comment on showing different tracks for different loci. While we appreciate the reviewers' concern, we believe the comment places disproportionate emphasis on an aspect that does not constitute a genuine limitation of the work. In short, we showed four sites on four different chromosomes. As is inherent to the nature of omics analyses, not all genes/proteins/metabolites etc. behave the same in each mouse. Therefore, especially for a representative figure, it is not necessary that the same individual mice be shown across all loci. As our experiment included many samples, the statistically significant differential signal arises from all samples as a group, as can be inspected in the data we have uploaded (Supplementary Table S21). Representative tracks that correspond to the statistically significant change were chosen for each locus. The fact that those tracks are representative was mentioned several times in the manuscript. For that reason, we did not deem it necessary to name each track specifically. We apologize if the reviewers find this misleading. In the revised manuscript the figure now displays tracks from all samples, in consistent order across loci (Fig. 4c; please see below).



Point by Point Response to Reviewers

Reviewer #1 (Remarks to the Author):

“The authors' changes in the text and figures appropriately address the remaining concerns.”

We would like to thank the reviewer for their insightful comments throughout the review process which helped us significantly improve our manuscript.

Reviewer #5 (Remarks to the Author):

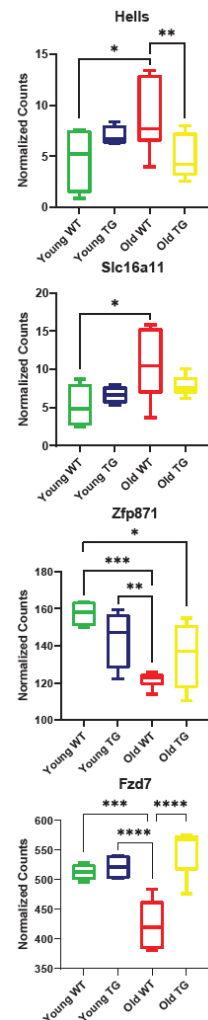
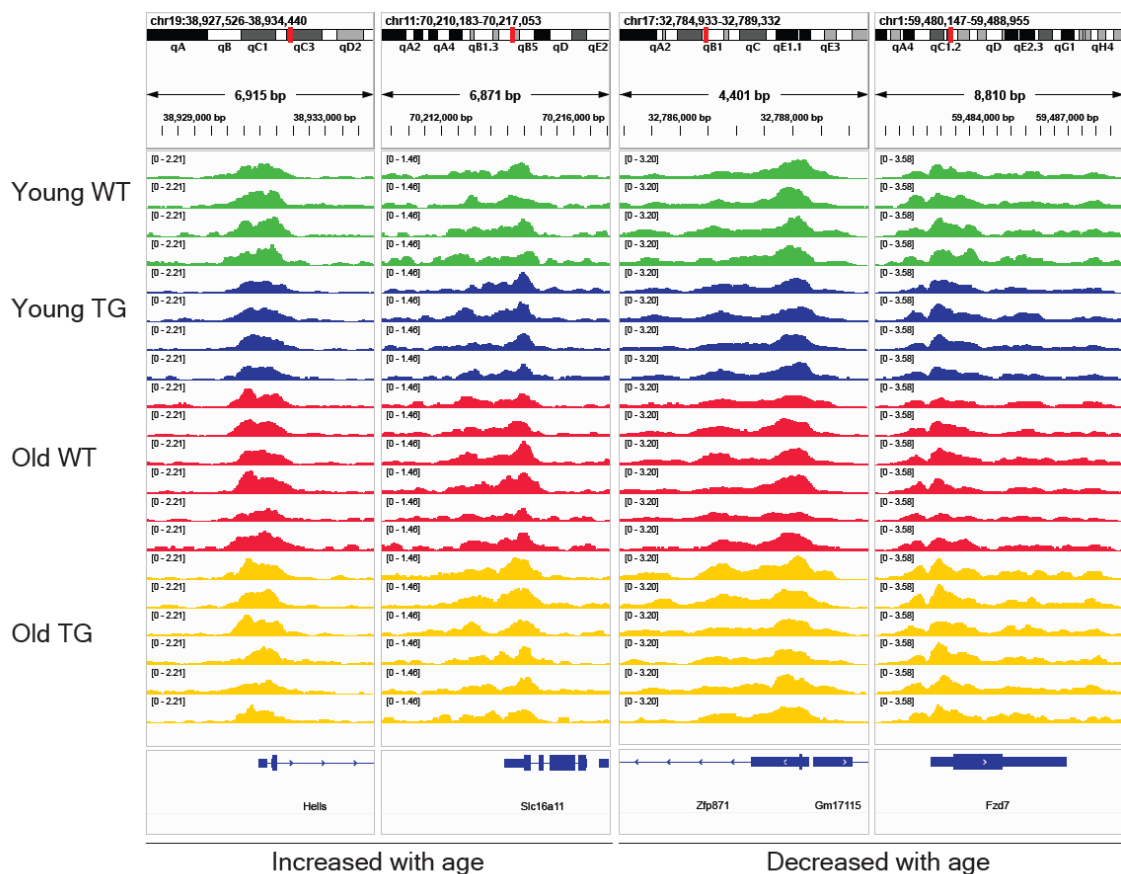
“Below, I state my opinion on the points of contention between the reviewer and the authors. ... In my opinion, the variability between replicates is entirely reasonable for typical CHIP-seq experiments. It is more difficult to determine what constitutes a major/minor difference and whether this can have a biologically meaningful effect. I would highlight that the data in question is from a bulk dataset: a small change in CHIP-seq coverage could simply mean that a small number of cells is affected. But a minority of cells is very capable of having major biological ramifications. Thus, I would consider a result noteworthy as far as it is statistically significant.... If a peak is consistent across replicates but does not coincide with a known promoter, it is worth considering there may be an unknown regulatory element... A figure is not representative of the underlying data if the points shown are cherry-picked.

In conclusion, I believe that the CHIP-seq data analyzed in the manuscript is of good quality and allows drawing conclusions on the effect of aging and Sirt6 OE. However, comparisons should include more statistical tests, to avoid reliance on visualizations alone. Coverage plots (Such as Figure 4C) could be accompanied by boxplots and respective statistics. Because the genomic context of differential peaks was discussed, I also recommend plotting the genomic context (genic or intergenic, promoter, enhancer, exon, intron... for example, using ChipSeeker) of differential peaks and determining if there is enrichment for any particular region by comparing to all peaks as background. It would be interesting to know if differential peaks are enriched at gene bodies, for example.”

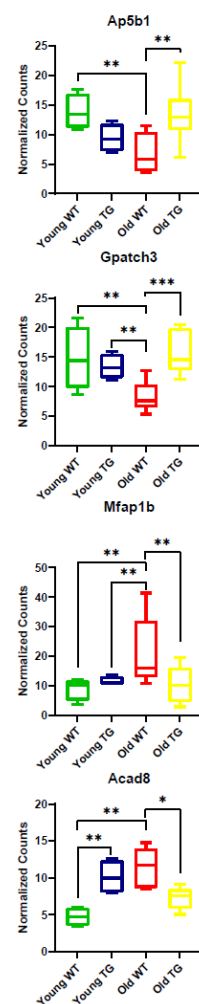
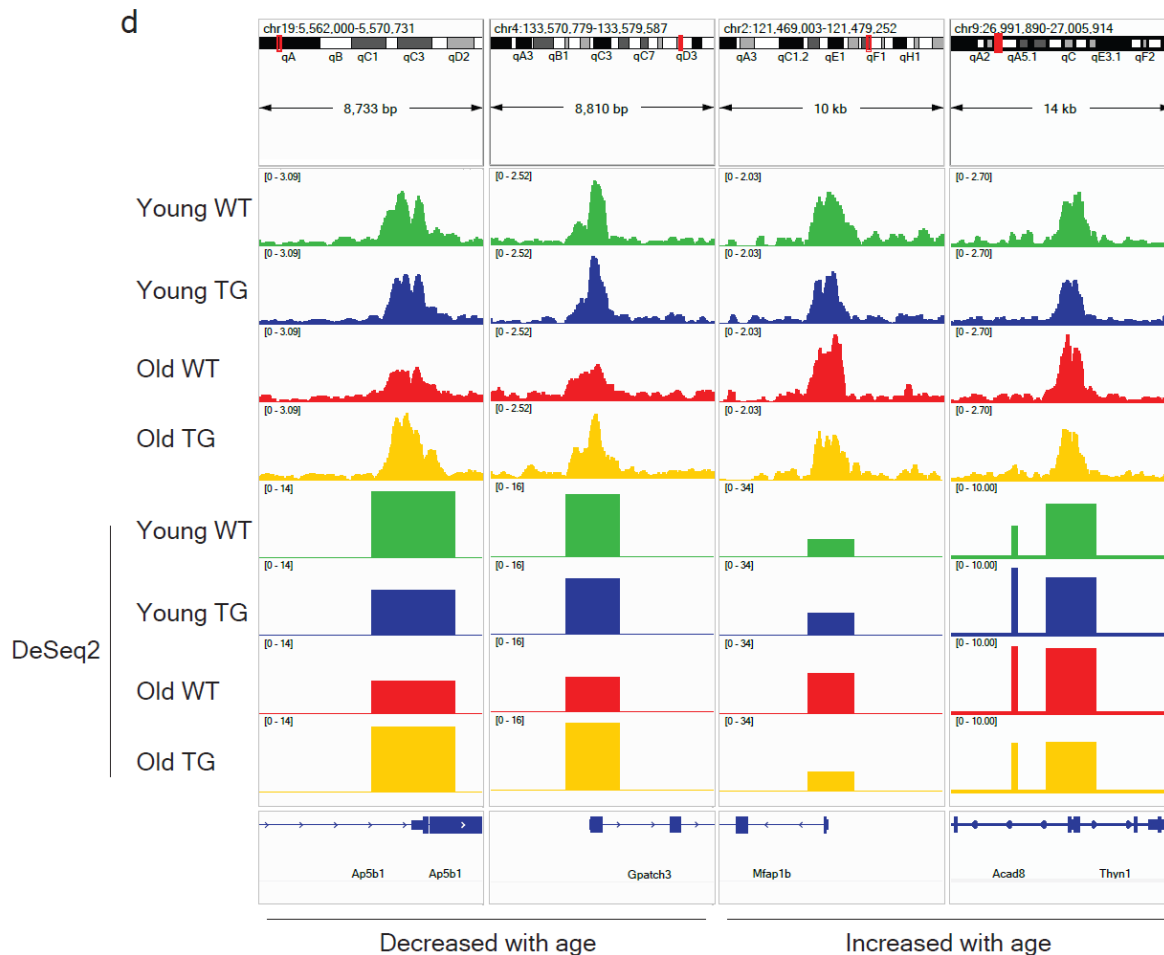
We thank the reviewer for these important and insightful comments, and for finding that our ChIP-seq data are of good quality and support our conclusions regarding the effects of aging and Sirt6 OE.

In response to the reviewer's requests, the revised manuscript now includes quantitative boxplots alongside the coverage plots (Fig. 4c, Supplementary Fig. 9d; please see below). In addition, in light of the reviewer's note that these data are derived from a bulk dataset, the upregulated peaks now include Hells and Slc16a11, two hepatocyte-expressed genes (PMID: 41689757 and PMID: 28666119, respectively), instead of the non-hepatocyte-expressed genes Rhoh and Gldn, to better account for bulk tissue heterogeneity.

Fig. 4c

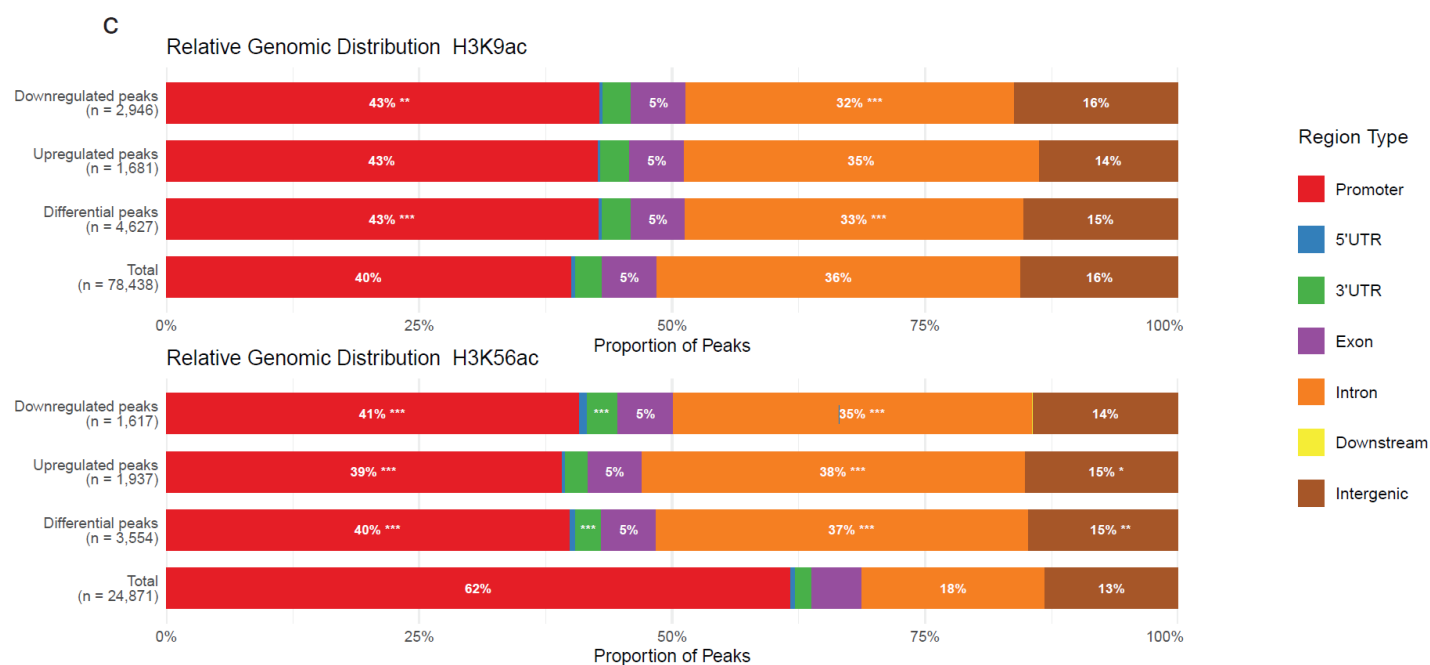


Supplementary Fig. 9d



Regarding the genomic context of the differential ChIP-seq peaks, the revised Supplementary Fig. 9c now includes an analysis of the genomic context of the differential peaks for both H3K9ac and H3K56ac (please see below). This analysis revealed that most peaks are located in promoters and introns. Interestingly, whereas H3K9ac showed only slight promoter enrichment among differential peaks relative to total peaks, H3K56ac differential peaks were significantly depleted at promoters and, conversely, enriched in intronic and intergenic regions. On pages 9-10 of the revised manuscript, we now describe these new findings: "Analysis of the genomic distribution of these marks revealed that H3K9ac differential peaks were slightly enriched at promoters (43%) compared to total H3K9ac peaks (40%) (Supplementary Fig. 9c). In contrast, while the vast majority of total H3K56ac peaks were located at promoters (62%), differential H3K56ac peaks were significantly depleted at promoters (~40%) and instead enriched in intronic and intergenic regions (Supplementary Fig. 9c)."

Supplementary Fig. 9c

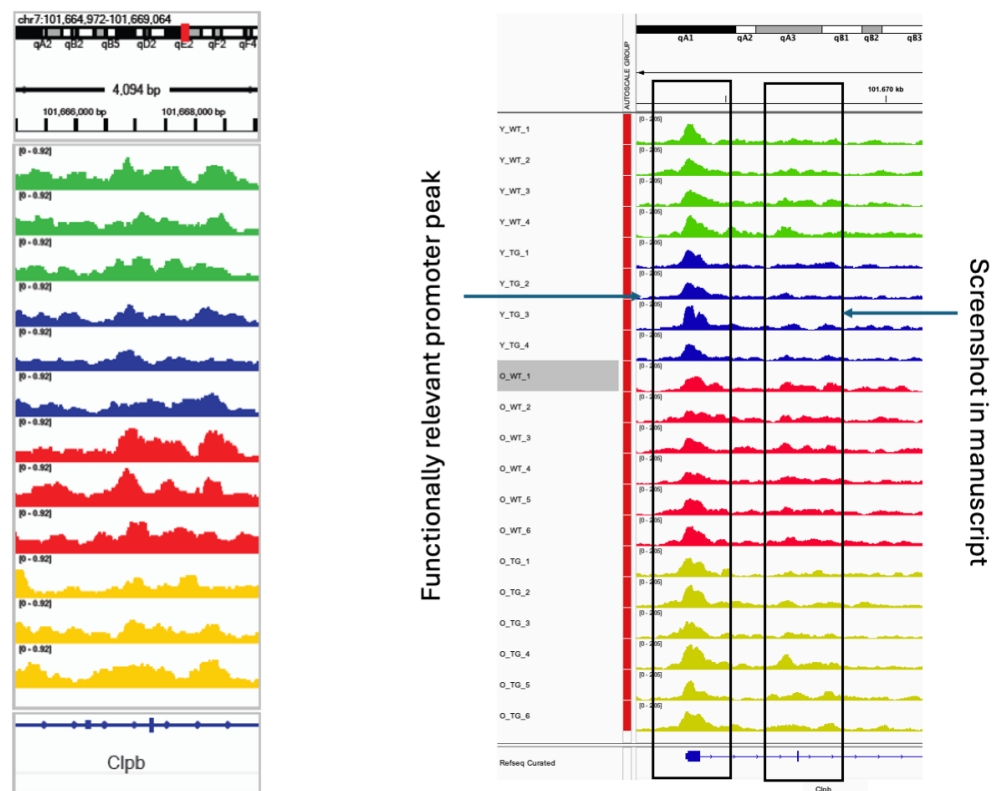


We would like to thank the reviewers and the editor again for their positive feedback and insightful comments, which helped us significantly improve our manuscript. We look forward to seeing our manuscript published in *Nature Communications*.

This revision of the manuscript by Nagar et al on the role of SIRT6 in liver aging has attempted to address some of our remaining concerns.

We agree that it was a good idea to rerun some of the western blots. However, given the results we still feel that the text misrepresents the data. Quote (page 9, 2nd paragraph): “Among these, H3K56ac showed a trend toward an increase in old WT compared to young WT mice, while age had no effect on H3K9ac in WT mice. Interestingly, this increase in H3K56ac was mitigated in old SIRT6 TG mice. As these changes were not statistically significant, this global analysis failed to provide a robust explanation for the observed changes in AADs.” There are two issues with this statement. The authors push too hard as there is not a trend in the data for H3K56ac to be slightly upregulated, which makes the second part even more misleading as SIRT6 cannot mitigate an effect that was not present in the first place. The toned-down follow up sentence does not compensate here.

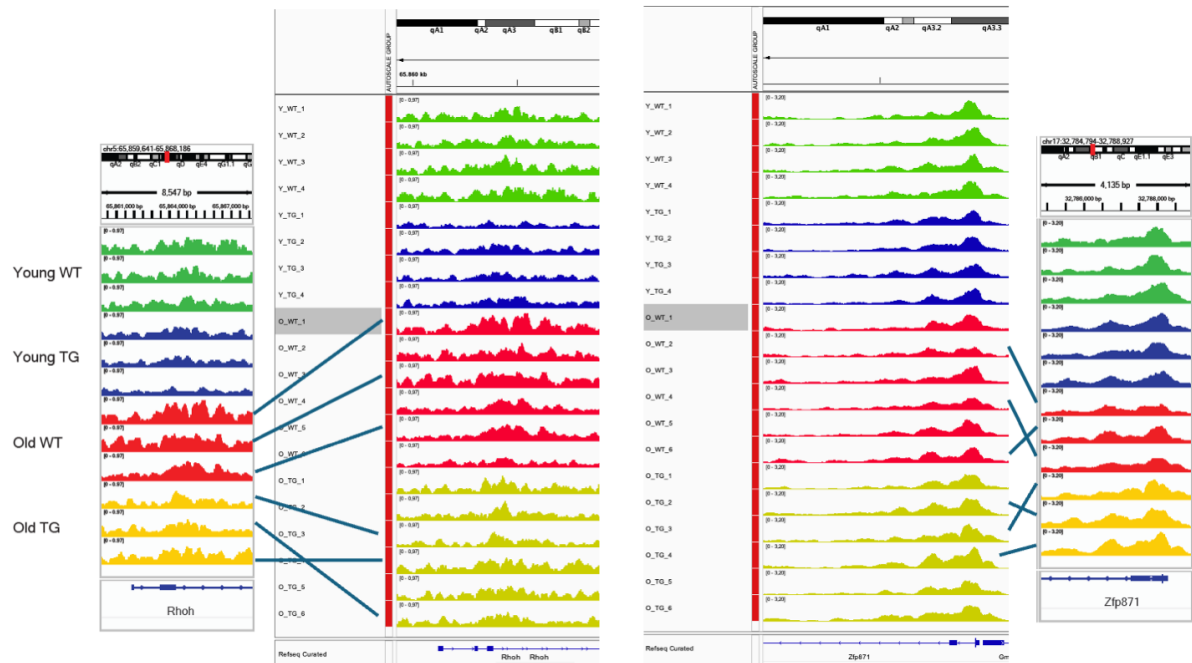
More worrying is the H3K9ac genomic data. We thank the authors for making it available now. We inspected the tracks on the IGV browser and there are a number of concerns. Firstly, the variability seen in the PCA plots is also visible in the genomic tracks. This poses a problem since the effects seen are very minor (see examples below). Even if the differences were statistically significant, it is very difficult to imagine that they have biological meaning, certainly not explaining the effects seen on chromatin architecture by ATAC-seq, which puts into question the model of SIRT6 function the authors would like to push. Some of the peaks presented in the browser shots 4c do not necessarily represent functional peaks. For instance, the one picked for ClpB is not showing the promoter peak (as would be functionally relevant for H3K9ac), but some minor peak downstream of the start site (see Review-Figure 1).



Review-Figure 1: Left is screenshot taken from Figure 4c, right is an IGV browser shot of all bw files provided by the authors. Important note: The numbering used by us does not necessarily have to correspond to the one used by the authors. Note that in some experiments the area used by the authors just barely enriches over background.

Even more worrying is the apparent use of different tracks to highlight apparent increases/decreases in different genomic loci. The browser shot always depicts three tracks, while uploaded are up to six repeats that were also used for differential peak calling. Review-Figure 2 strongly suggests that at

least for the two examples used, different tracks were used at the different loci. This is unacceptable, particularly given the variability between experiments.



Review-Figure 2: Matching Figure 4c screenshots with provided bw files. Matching was performed based on the peak shapes and strongly suggest that different tracks were used (cherry-picked) at different genomic locations.

Taken together, the authors try and convey a model throughout the text that is not supported by the data. At times, data is over-interpreted to corroborate a conclusion much more robust than the data can corroborate; this applies at least to the western blots and the CHIPseq data. We regret to conclude that the authors have not been able to address our concerns over the last three rounds of revisions.