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A rare human centenarian variant of SIRT6 enhances genome stability and interaction with Lamin A

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **8th Feb 2022**

Dear Vera,

Thank you for submitting your manuscript for consideration by the EMBO Journal. Below is the official report with all four reviewer comments.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all four reviewers. And I look forward to speaking with you soon to discuss your plan.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

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

I have attached a guide for revisions for your convenience.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Kelly M Anderson, PhD
Editor
The EMBO Journal
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Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (9th May 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

Link Not Available

Referee #1:

In the current manuscript, the authors report the identification of a SIRT6 allelic variant (centSIRT6), harboring the amino acid substitutions N308K and A313S, in a subgroup of Ashkenazi Jewish centenarians that reduces genome instability. Given this link to centenarians and the clear role of SIRT6 as a key promoter of longevity in mice, the authors characterized the functional relevance of centSIRT6. Interestingly, centSIRT6 displays increased ADP-ribosylation but reduced deacylase activity and exert higher anti-aging functions as compared to wild-type SIRT6. The authors describe that centSIRT6 has improved capabilities towards LINE-L1 retrotransposon repression, DNA damage repair, and Lamin A regulation.
Given the importance of SIRT6 in longevity pathways, the identification and characterization of centSIRT6 is a remarkable finding and may have multiple implications in understanding the role of Sirtuins in human aging. However, the manuscript has several major scientific and technical issues that decrease considerably the relevance of this evidence. The main problem is that the story lacks depth and consistency. The authors perform many studies at different levels but do not really connect them in a coherent model. In the present form of the manuscript, the link between LINE-L1 silencing, Lamin A regulation, DNA repair and the imbalance between both SIRT6 enzymatic activities may perfectly be separated mechanisms. Moreover, all these interesting observations are underdeveloped. If the authors would have developed in detail any of these specific observations the work would have improved considerably.
The manuscript has also technical problems as several important experiments described in the manuscript lack proper controls and many experiments are poorly described both in the figure legends and in the methods section.

Here I detail these major issues:

1) I have some concerns regarding the targeted sequencing analysis in the centenarian populations:
a) Table 1: In the main text, it is stated association of rs350845 with living beyond 100 years and referenced to Table 1 with P = 0.049. However, the P-value in the table is 0.009. This needs further clarification.

b) In the second paragraph of the Results section states: "In addition, two rare missense variants in perfect linkage were observed, rs183444295 (A313S) and rs201141490 (N308K), aka centSIRT6, which had nearly double the allele frequency among centenarians (1.0%) compared to both study controls (0.55%) as well as a separate AJ cohort within GnomAD which did not contain centenarians (0.60%), although this difference was not statistically significant (P=0.7, P=0.5, respectively) due to lack of power. "
I have two issues here: First, lack of power cannot be assumed as causative of lack of statistical significance. Second, where are these P=0,7 and P=0,5 in Table 1? The authors should include more info in the table and/or in the legend.

c) Regarding Fig EV2F the authors state that "It shows a significant increase in lifetime" but no statistical test has been performed between samples, only the mean and SEM are shown.

2) I am quite confused regarding the SIRT6 deacetylation differences between WT and centSIRT6. The authors show that centSIRT6 has a weaker deacylase activity towards myristoylation and deacetylation. In the case of deacetylation, the authors show a clear decrease in the ability of centSIRT6 to deacetylate H3K9ac, H3K18ac, and H3K56ac but mass spectrometry analysis of the levels of these marks show no clear differences in cells expressing WT, centSIRT6, and the individual substitution. The authors conclude that the decrease in deacetylation activity is not enough to alter the global levels of the PTM. This raises several questions:
a) The authors seem to assume that only because global levels of the marks are not altered centSIRT6 does not have an altered function due to decreased deacetylation. However, these marks are regulated by many enzymes and the impact of SIRT6 is restricted to specific functions. The authors should test the effect of centSIRT6 in previously described SIRT6 functions where deacetylation of these marks is important. For instance, NF-KB response (H3K9ac), pericentric heterochromatin (H3K18ac) or DNA damage in S-phase (K56ac).
b) Figure 1E-F, 2D, EV3A lack two essential controls: i) histone H3. The quantification should be performed normalizing H3K9ac levels with total H3 levels; ii) the assay lacks a control without SIRT6. In both cases, a western blot of the levels of SIRT6 in these experiments should be included. In the case of Fig 2D, a positive control demonstrating Paraquat treatment really generates oxidative stress is required.
c) How do the authors reconcile the contradictory evidence between Fig1E-F and 2A-B? The authors monitored the deacetylation activity of SIRT6 protein variants at different time points including 60 minutes which is the time point also used in the experiment in Fig 1E-F. In sharp contrast to 1E-F, the authors did not observe differences between wild-type SIRT6 and N308K or A313S mutants, observed in Fig1E-F. Moreover, a control without SIRT6 should be also included.
d) Linked to point c, quantification of centSIRT6 H3K9Ac at 60 min does not represent the shown blot (clear band whereas quantification shows value near 0). It's also difficult to see asterisks because of color and they are on top of the line. Quantification of how many independent experiments is not stated in the figure legend. Same case for 2B.

3) Based on Fig.4A centSIRT6 and N308K are both more efficient in LINE-L1 repression. However, whether this function has an impact on retrotransposition activity and what is the molecular mechanism is totally unexplored by authors. Some specific issues:
a) The authors state that they looked at an evolutionarily active L1MdA1 LINE-1 family. This information should be in the main text and M&M in addition to the figure legend. Based on M&M information, authors say that they use primers targeting ORF1 and ORF2 sequences to differentiate LINE-1 subfamilies. Because ORF1 and ORF2 sequences are quite conserved, how do authors confirm the family specificity? In my opinion, qPCR results from Fig.4A indicate that centSIRT6 and N308K repress all LINE-L1 elements but it does not answer whether they do so in the youngest and putative dangerous one.
b) Does centSIRT6 activity also impact retrotransposition activity itself? Authors should perform classical retrotransposition assays with GFP reporters.
c) What is the molecular pathway involved in LINE-L1 silencing? The authors previously showed that SIRT6 KAP-1 ribosylation is important for heterochromatin formation at LINE-1 loci. Is s KAP1 ribosylation increased with centSIRT6?

4) Regarding the ADP-ribosylation experiments:
a) I am not sure I understand the mechanism claimed by the authors regarding Lamin ADP-ribosylation. The authors claim that the increased binding induces higher levels of ADPRibosylation in LaminA. However, the differences in ADPribosylated LMNA are mild (fig 6F). Can centSIRT6 ADPribosylate LMNA in vitro more than WT SIRT6? Are the differences observed in Lamina interactors in presence of centSIRT6 due to increased Lamin ADPRibosylation or due to increased binding between LaminA and centSIRT6? If the authors claim that ADP-ribosylation is involved this should be minimally demonstrated. For instance, what would be the effect of a centSIRT6 mutant deficient in ADP-ribosylation?
b) Linked to this, the authors state that many proteins identified in SIRT6 and LMNA IPS are ADP-ribosylated. If the authors claim that SIRT6 is directly involved in this ADPribosylation, they should show that centSIRT6 enhances ADPribosylation of one or several of these targets identified by MS by IP-WB.
c) As stated in the text, LMNA has been reported to be ADPribosylated on several residues. Can ADPribosylation be blocked by point mutations in these sites? If so, what happens if these mutants are overexpressed in centSIRT6 cells?

d) Figure 3C: total PARP1 blot is missing.

5) Regarding the DNA repair assays:
a) The authors show that NHEJ and HR are both increased in centSIRT6 in classical GFP reporter assays. However, their H2Ax dynamics show only a mild effect by this allelic variant. Authors should perform analysis with a marker of HR, such as rad51, and NHEJ, such as 53BP1 (to my surprise foci analysis is only shown in Figure EV5C).
b) In Figure EV4A, it is important to include percentages of GFP+ and RSD + cells in dot plots to give readers all information. Also, the figure legend should explain how analysis is performed. I assumed that black cells of dot blots are either negative or dead?
c) In Figure EV4D, dot plots must include percentages of populations to provide readers with all information.

6) It's difficult to interpret some data on telomerase-immortalized human Fibroblasts. The authors have used different concentrations of cumate to induce similar levels of SIRT6 expression in stable cell lines expressing SIRT6 variants (Fig. EV3B) and stated in the figure legend "These concentrations were used in experiments with these cells". The concentration of cumate used to induce the expression of SIRT6 WT is circa 8 times higher than the one used to induce CentSIRT6. This raises the concern that different concentrations of cumate may cause altered cellular responses independently of SIRT6. This should be addressed by the authors. Loading controls are missing also in this figure.

MINOR ISSUES:

1) The majority of figure legends lack information of the number of replicates of each experiment.
2) Page 5, line 11 Fig.1C should be instead Fig.1D
3) The authors often refer to allele when speaking about proteins which is not adequate. Protein variants or at least protein encoded by the centSIRT6 allele should be rather used.
4) Fig 2C. Review Y axis format, label on top of values. There seems to be an effect on K9Ac. Is the statistical analysis missing?
5) Fig.3A. A control showing the absence of specific bands in cells that do not express SIRT6 is needed. In this assay, it is not clear what the authors used to assess monoADPr. Since they assessed the incorporation of biotin-labelled NAD+ as stated in the figure legend, I assume that the authors used an anti-biotin Antibody (unfortunately no explanation is provided in the figure legend). On the other hand, in the material and methods section, the authors rather described radioactive NAD+. This should be clarified.
6) Fig3A-B. Detection of self-ribosylation of sirt6 using biotin-labeled NAD is not detailed in the methods. Regarding Fig3B: is this performed using biotin-NAD? SDS-PAGE of a dot blot?
7) In EV2C it is not clear what was exactly measured in this experiment. Additional explanations would be required either in the figure legend or Material & Methods.

Referee #2:

Simon et al present an examination of two rare missense mutations found in Sirt6 and demonstrate that these mutations confer potentially beneficial alterations to the function of Sirt6. They methodically assay the changes in function that the mutations confer either individually or in conjunction and show even though these mutations are in linkage the combination of the two effects is not always additive. The authors use several models (human cell lines, fibroblasts, and ESC-derived MSCs. Each analysis was a thoughtful experiment to address the hypothesis and overall, it is a well-designed and well written manuscript presenting an exciting novel haplotype overexpressed in an aged population that confers improved DNA damage response / genome maintenance.

Minor concerns:
The mutations A313S and N308K were described as being in perfect linkage with one another, but could the authors expand on the haplotype blocks that this haplotype belongs to- is it in linkage disequilibrium with the other eQTLs of Sirt6? A figure with the blocks defined across the Sirt6 gene would be helpful. Was this haplotype found as homozygous in the centenarians?

In regard to levels of expression of the mutant allele in conferring altered potential, it would be relevant to show the titration that was used to determine the cumate doses.

One of the exciting models used was the generation of hESCs with the centSIRT6 that were then differentiated into MSCs. (Please confirm in the text that these are mesenchymal stem cells). Given that different tissues may have unique response to SIRT6, it would be interesting to examine cell specific phenotypes of this centSIRT6 allele vs WT, as in the mMSCs it has been shown that SIRT6 plays a critical role in oxidative stress. The authors should examine if the centSIRT6 expression has any effect on the NRF2 pathway (qPCR of NRF2, HO-1, NQO1 etc) and or perform an oxidative stress challenge on these cells. While it is appreciated that these cells cannot be titrated to have equal levels of Sirt6 expression (like in the inducible system)

this model would be perhaps more reflective of the allele behavior in native cells.

Referee #3:

Simon et al.
"A rare human centenarian variant of SIRT6 enhances genome stability and interaction with Lamin A"

The study conducted by Simon et al. claims that a SITR6 allele containing two linked substitutions (N308K/A313S), which is enriched in Ashkenazi Jewish (AJ) centenarians, brings some beneficial phenotypes such as increased suppression of LINE1 retrotransposons, enhanced DNA double-strand break repair, more robust cancer cell killing, compared to the wild-type allele. These phenotypes are likely mediated by its enhanced mono-ADP ribosyltransferase (mADPr) activity and stronger interaction with Lamin A/C (LMNA). The data they presented in this manuscript are very interesting and stimulating. On the other hand, however, mechanistic evidence is relatively weak. Additionally, the data from fibroblasts or peripheral blood mononuclear cells (PBMCs) of AJ centenarians could further strengthen their claims, if these cells are available. Thus, the authors should address the following points to strengthen their conclusions.

Major points:

1) The mechanistic evidence supporting their notion that increased mADPr activity of centSIRT6 is responsible for beneficial cellular phenotypes should be provided. For example, is increased suppression of LINE1 retrotransposons dependent on KAP1? Is KAP1 more mono-ADP-ribosylated in cent SIRT6-expressing cells? It is also unclear how enhanced interaction between centSIRT6 and LMNA is connected to these beneficial cellular phenotypes. Currently, the results are just an association.

2) It is fair to speculate that these beneficial cellular phenotypes, such as suppression of LINE1 retrotransposons, DNA double strand break repair, capacity to kill cancer cells, could potentially have an impact to median lifespan or healthspan. However, at this moment, it is not clear whether any of these phenotypes is mechanistically connected to longevity in mammals. In this regard, the frequency of these linked substitutions is just 1.0% among AJ centenarians, meaning that 99% of AJ centenarians cannot be explained by these linked substitutions. Thus, what is the evidence or the logic to claim that these linked substitutions contribute to extreme longevity? Dr. Gorbunova previously used fruit flies to address this critical question in their Cell paper. Can they conduct this particular experiment for centSIRT6?

Minor points:

1) In Figure 1D, the authors claim that centSIRT6 confers a slight reduction in deacylase catalytic efficiency. Is this difference statistically significant between WT Vmax/Km and Cent Vmax/Km ?

2) In Figures 1E-F and 2A-B, different NAD+ concentrations were used (1mM vs. 5mM). Why did the authors have to use such high concentrations? And why were the concentrations different? If there is any particular reason for these conditions, the authors should explain it in the manuscript.

3) In Figure 2A, the graph of Cent at 15min does not look consistent with the WB result. Based on the WB result presented, the graph should show about a half of 0 timepoint. They should correct either one.

4) In Figure 2D, it seems strange that H3K9ac and H3K18ac in WT cells did not change at all before and after cumate induction. Does cumate-induced SIRT6 not have any effect on acetylation H3K9ac and H3K18ac? Additionally, the description regarding this experimental condition is not precise enough. The authors should provide a bit more details, such as incubation time after cumate addition and a time course of cumate and paraquat treatment.

5) In Figure EV2, even though centSIRT6 has a more open conformation compared to the wild type, it is unclear how this conformational change impacts to other features of centSIRT6. Do the authors attempt to claim that this structural change results in stronger interaction with LMNA?

Additional suggestions:

1) There are several simple typos. "Fig 1C" on page5, line 7 and line 11 is "Fig 1D ". "Fig 4F,G,Fig EV4D" on page 7, line 24 is "Fig4G, Fig EV4D".

2) In discussion, the authors wrote "Ashkenazi Jews who exhibited extreme phenotype (>100 years old)" on page 11, line 20. On the other hand, in Materials and Methods, they wrote that "a centenarian is defined as a healthy individual living independently at 95 years of age or older" on page 14, line 23. They should be consistent.

3) Figure 2C, vertical title "Relative Fraction" is overlapping with number.

4) In table 1, Alt and Ref alphabet in rs183444295 and rs201141490 seems to be wrong. As for rs183444295, "T" in Alt and "G" in Ref seems to be correct. As for rs201141490, "G" in Alt and "C" in Ref seems to be correct.

Referee #4:

A rare human centenarian variant of SIRT6 enhances genome stability and interaction with Lamin A

Single nucleotide polymorphisms (SNPs) in SIRT6 have been linked to human longevity, however these SNPs are typically in non-coding regions of the genome. In this study, the authors investigated potential SNPs associated with human longevity in a small Ashkenazi Jewish (AJ) population. This population, although small, is useful for genetic studies as it is more controlled than the broader datasets. They identified two SNPs in SIRT6 associated with human longevity (although not at a high significance due to the sample size restrictions) and found that a dual allele, dubbed the centenarian SIRT6 allele (centSIRT6), contained both SNPs. The authors then tested this centSIRT6 allele and found that it shifts the balance of deacetylation and mono-ADP-ribosylation activity of SIRT6 in vitro. This helped to validate the SNP findings from the AJ control and centenarian analysis. The variety of in vitro cell lines and methods used were well-designed and lend strength to their findings. They perform multiple methods of testing to support each of their conclusions about centSIRT6 activity and its interaction with LMNA. This work lays the foundation for future in vivo analysis of LMNA and SIRT6 manipulation as a means to prolong lifespan. There are some clarifications needed in the manuscript to support the author's conclusions.

Major Concerns:

1. The authors should list the criteria they used to determine a "healthy" individual. Were there any exclusion criteria (e.g. BMI) besides age? The age range and gender information for the control and centenarian groups should also be listed. If potential confounding variables (i.e. gender, BMI) for human longevity are unknown, the authors should mention this in their discussion section.

Minor Concerns:

1. There are quite a few areas with repeated sentences and high repetition that can be removed from the manuscript text.

2. The asterisks in figure 2 overlap with the figure itself and aren't visible.

3. There is no mention of the p-value, sample size, or the error bars in the Figure 2 legend.

4. Are there error bars for Figure 3B?

5. It may be a PDF issue, but Figure 4C is cut off and may need to have the size reduced.

6. There is no mention of the blots in Figure 4B and C in the legend.

7. There is no mention of the colored asterisks for Figure 4G in the legend.

8. It would be helpful to make the error bars in color for Figure 4F and G so that each group can be visualized.

9. The legend for Figure 4 states that there is another statistical method used but there is no mention of another method in the legend.

10. There is a mention of colored asterisks in the legend for Figure 5A, but there are none in the figure itself, only black asterisks.

11. Error bars are not mentioned in the legend for Figure EV2 C and G. The label E is also partially cut off by the figure itself.

12. There is no mention of error bars, the p-value or statistical test used in the legend for Figure EV3. Also the normalization is not mentioned for C in this figure.

13. The figure legend for Figure EV5 mentions NS but there is no NS in the figure itself. The legend does not state the sample size for the majority of the panels.

Referee #1

(Report for Author)
In the current manuscript, the authors report the identification of a SIRT6 allelic variant (centSIRT6), harboring the amino acid substitutions N308K and A313S, in a subgroup of Ashkenazi Jewish centenarians that reduces genome instability. Given this link to centenarians and the clear role of SIRT6 as a key promoter of longevity in mice, the authors characterized the functional relevance of centSIRT6. Interestingly, centSIRT6 displays increased ADP-ribosylation but reduced deacylase activity and exert higher anti-aging functions as compared to wild-type SIRT6. The authors describe that centSIRT6 has improved capabilities towards LINE-L1 retrotransposon repression, DNA damage repair, and Lamin A regulation.
Given the importance of SIRT6 in longevity pathways, the identification and characterization of centSIRT6 is a remarkable finding and may have multiple implications in understanding the role of Sirtuins in human aging. However, the manuscript has several major scientific and technical issues that decrease considerably the relevance of this evidence. The main problem is that the story lacks depth and consistency. The authors perform many studies at different levels but do not really connect them in a coherent model. In the present form of the manuscript, the link between LINE-L1 silencing, Lamin A regulation, DNA repair and the imbalance between both SIRT6 enzymatic activities may perfectly be separated mechanisms. Moreover, all these interesting observations are underdeveloped. If the authors would have developed in detail any of these specific observations the work would have improved considerably.
The manuscript has also technical problems as several important experiments described in the manuscript lack proper controls and many experiments are poorly described both in the figure legends and in the methods section.

Here I detail these major issues:

1) I have some concerns regarding the targeted sequencing analysis in the centenarian populations:
a) Table 1: In the main text, it is stated association of rs350845 with living beyond 100 years and referenced to Table 1 with P = 0.049. However, the P-value in the table is 0.009. This needs further clarification.
We thank the reviewer for pointing out this typo. The nominal P-value of rs350845 should be 0.009 as shown in the table. We have corrected the text to reflect this.

b) In the second paragraph of the Results section states: "In addition, two rare missense variants in perfect linkage were observed, rs183444295 (A313S) and rs201141490 (N308K), aka centSIRT6, which had nearly double the allele frequency among centenarians (1.0%) compared to both study controls (0.55%) as well as a separate AJ cohort within GnomAD which did not contain centenarians (0.60%), although this difference was not statistically significant (P=0.7, P=0.5, respectively) due to lack of power."
I have two issues here: First, lack of power cannot be assumed as causative of lack of statistical significance. Second, where are these P=0,7 and P=0,5 in Table 1? The authors should include more info in the table and/or in the legend.

We agree with the reviewer that we cannot rule out the possibility of a correlation between the allele and centenarian status due to unrelated mechanisms such as bottleneck or drift with the results of this test alone. To obtain additional evidence for the causative contribution of the centSIRT6 allele to the centenarian phenotype, we also evaluated whether there was independent evidence of

centSIRT6 allele enrichment within the larger GnomAD database itself and discovered centSIRT6 to be among the most enriched alleles among the 75+ cohort (<5th percentile). Given that *SIRT6* is associated with longevity in a variety of organisms, we were motivated to follow up with functional characterization with the current population level evidence.

For accuracy, we will change "due to lack of power" to "due to limited population size".

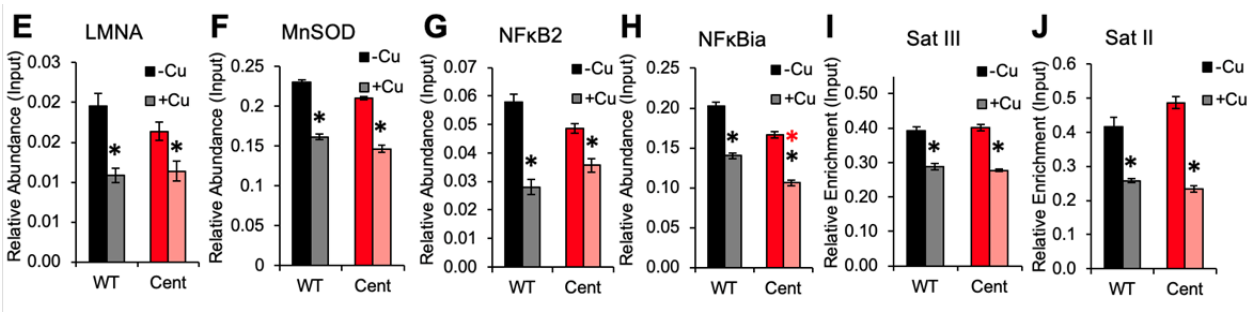
We apologize to the reviewers for the P-value typos. The values in Table 1 are correct (P=0.30 and 0.27), and the text in the Results has been updated.

c) Regarding Fig EV2F the authors state that "It shows a significant increase in lifetime" but no statistical test has been performed between samples, only the mean and SEM are shown.

Thank you for indicating this oversight. This information is now included in the manuscript in the figure legend for Fig EV2F:
"It shows a significant increase in lifetime τ (1.96 ± 0.02 ns), compared to the wild type SIRT6 (1.75 ± 0.01 ns). Error bars indicate SEM (n = 3-5). Asterisk indicates p<0.05, Student's t-test."

2) I am quite confused regarding the SIRT6 deacetylation differences between WT and centSIRT6. The authors show that centSIRT6 has a weaker deacylase activity towards myristoylation and deacetylation. In the case of deacetylation, the authors show a clear decrease in the ability of centSIRT6 to deacetylate H3K9ac, H3K18ac, and H3K56ac but mass spectrometry analysis of the levels of these marks show no clear differences in cells expressing WT, centSIRT6, and the individual substitution. The authors conclude that the decrease in deacetylation activity is not enough to alter the global levels of the PTM. This raises several questions:
a) The authors seem to assume that only because global levels of the marks are not altered centSIRT6 does not have an altered function due to decreased deacetylation. However, these marks are regulated by many enzymes and the impact of SIRT6 is restricted to specific functions. The authors should test the effect of centSIRT6 in previously described SIRT6 functions where deacetylation of these marks is important. For instance, NF-KB response (H3K9ac), pericentric heterochromatin (H3K18ac) or DNA damage in S-phase (K56ac).

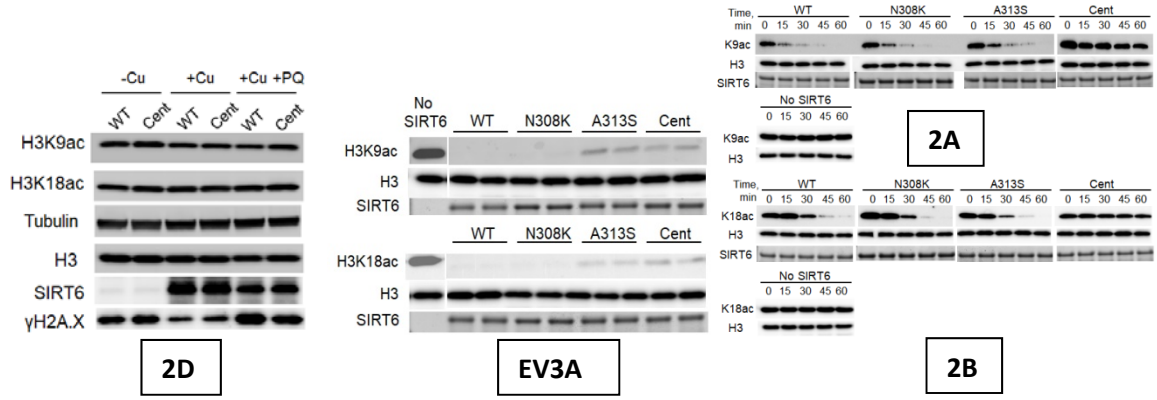
We thank the reviewer for this suggestion. We have included ChIP qPCR data from H3K9 targets (LMNA, MnSOD, NFκB2, NFκBia) and H3K18 targets (Sat II, Sat III) (PMID: 19135889, PMID: 21738489, PMID: 27043296) with and without SIRT6 induction (-/+ Cu) in the revised Figure 2E-J.. Looking at these specific, known targets of SIRT6 deacetylase activity, we do see that cumate (-) and the cumate (+) cells have differential histone acetylation status, with those with SIRT6 showing reduced acetylation (Fig2E-J). However, similar to what was observed at the global level, there is little to no difference between the two SIRT6 variants in live cells. We did not assay H3K56ac as reliable antibodies for this PTM are not available and we do not assess it in our other assays.



b) Figure 1E-F, 2D, EV3A lack two essential controls: i) histone H3. The quantification should be performed normalizing H3K9ac levels with total H3 levels; ii) the assay lacks a control without SIRT6. In both cases, a western blot of the levels of SIRT6 in these experiments should be included. In the case of Fig 2D, a positive control demonstrating Paraquat treatment really generates oxidative stress is required.

All except 2D are *in vitro* reactions that were prepared from a master mix with equal amounts of H3. We have provided the requested controls to Figure 2D and EV3A, including total H3 and the no SIRT6 for EV3A. We have also included the "no SIRT6" controls for Fig 2A, B.

We included a control to show that paraquat treatment generates oxidative stress by performing Western blot for γH2AX. We observed higher levels of gamma γH2AX upon paraquat treatment (γH2AX is also higher in cells that do not express SIRT6).



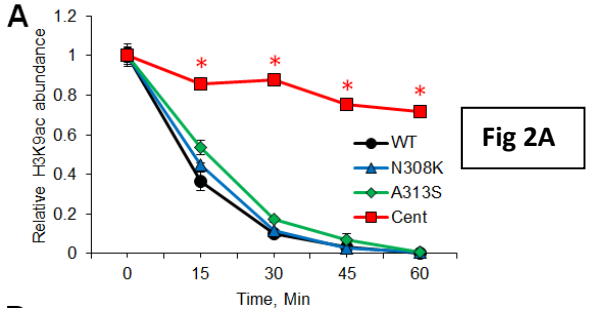
c) How do the authors reconcile the contradictory evidence between Fig1E-F and 2A-B? The authors monitored the deacetylation activity of SIRT6 protein variants at different time points including 60 minutes which is the time point also used in the experiment in Fig 1E-F. In sharp contrast to 1E-F, the authors did not observe differences between wild-type SIRT6 and N308K or A313S mutants, observed in Fig1E-F. Moreover, a control without SIRT6 should be also included.

Both figure sets show that centSIRT6 has reduced deacetylase activity. In Figure 1E-F we used a much lower concentration of NAD+ than that employed in Figure 2A, B, which could explain the differences. The higher concentration of NAD+ utilized in Figure 2A, B was employed to negate the potential of NAD+ being a limiting reagent. We have removed 1E-F as this data is redundant with 2A, B and not as informative.

We have provided "no SIRT6" controls for Fig 2A, B (see the Response above).

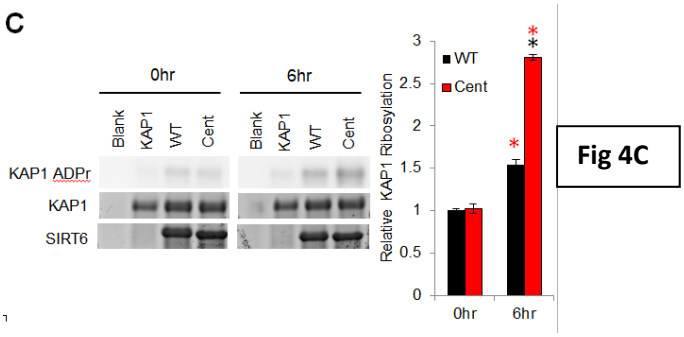
d) Linked to point c, quantification of centSIRT6 H3K9Ac at 60 min does not represent the shown blot (clear band whereas quantification shows value near 0). It's also difficult to see asterisks because of color and they are on top of the line. Quantification of how many independent experiments is not stated in the figure legend. Same case for 2B.

The reviewer is correct in the observation of the blot not matching the quantification. We found the error and fixed the quantification. For clarification of quantification, after normalization to H3 input, time 0 m values for each sample are used as the reference starting point of 100% or a relative value of "1".
We adjusted the positions of asterisks to make them more visible. Independent experiments were conducted in triplicate for these results. We have provided this information in the figure legends.



3) Based on Fig.4A centSIRT6 and N308K are both more efficient in LINE-L1 repression. However, whether this function has an impact on retrotransposition activity and what is the molecular mechanism is totally unexplored by authors. Some specific issues:

We previously published that SIRT6 mono-ADP ribosylates Kap1 and facilitates recruitment of Kap1 and HP1 to L1 5'UTRs to promote heterochromatin formation and silencing. See PMID: 25247314 and PMID: 30853213. We performed Kap1 riboslyation assay and show enhanced ribosylation by the Cent SIRT6, which is now provided in revised Fig 4C.



4) The authors state that they looked at an evolutionarily active L1MdA1 LINE-1 family. This information should be in the main text and M&M in addition to the figure legend. Based on M&M information, authors say that they use primers targeting ORF1 and ORF2 sequences to differentiate LINE-1 subfamilies. Because ORF1 and ORF2 sequences are quite conserved, how do authors confirm the family specificity? In my opinion, qPCR results from Fig.4A indicate that centSIRT6 and N308K repress all LINE-L1 elements but it does not answer whether they do so in the youngest and putative dangerous one.

We thank the reviewer for catching a mistake in this experiment's description. We clarify in the M&M as well in the text on Page 7 that we are using primers that target the evolutionary active L1HS LINE1 family, not the L1MdA1 family as was originally cited. This information is now in the text and the figure legend for this figure. This family is active in humans and known to be an endogenous mutagen (PMID: 27016617).

These primers are unique the L1HS family, and while some portions of the ORF1 and ORF2 reading frames of LINE1s are conserved, there is a great deal of sequence variability between different families.

b) Does centSIRT6 activity also impact retrotransposition activity itself? Authors should perform classical retrotransposition assays with GFP reporters.

We performed the retrotranspostion assay with the GFP reporter, as the reviewer requested. centSIRT6 allele inhibited retrotransposition significantly greater than the WT SIRT6 allele. The data is shown in in revised Fig 4B.

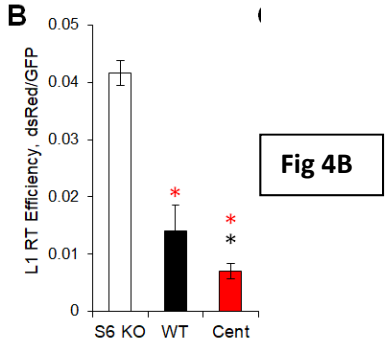


Fig 4B

c) What is the molecular pathway involved in LINE-L1 silencing? The authors previously showed that SIRT6 KAP-1 ribosylation is important for heterochromatin formation at LINE-1 loci. Is s KAP1 ribosylation increased with centSIRT6?

We performed Kap1 riboslyation assay and show enhanced ribosylation by the centSIRT6 in revised Fig 4C.

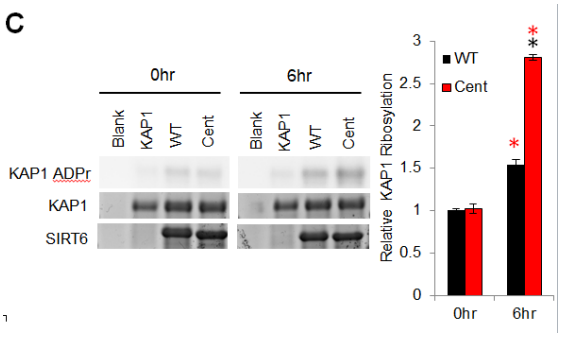


Fig 4C

4) Regarding the ADP-ribosylation experiments:
a) I am not sure I understand the mechanism claimed by the authors regarding Lamin ADP-ribosylation. The authors claim that the increased binding induces higher levels of ADPRibosylation in LaminA. However, the differences in ADPribosylated LMNA are mild (fig 6F). Can centSIRT6 ADPribosylate LMNA in vitro more than WT SIRT6? Are the differences observed in Lamina interactors in presence of

centSIRT6 due to increased Lamin ADPRibosylation or due to increased binding between LaminA and centSIRT6? If the authors claim that ADP-ribosylation is involved this should be minimally demonstrated. For instance, what would be the effect of a centSIRT6 mutant deficient in ADP-ribosylation?

The centenarian phenotype is ~20% increase in lifespan. As such, we expect only "mild" changes in the SIRT6 enzymatic activities observed in otherwise healthy individuals. Our previous attempts to establish *in vitro* Lamin A ribosylation conditions have proven difficult due to the complexity of *in vivo* chromatin compared to purified LMNA lacking PTMs and cofactors that may be necessary. We have included this limitation in the discussion (page 13 of the manuscript, and see below). We agree with the reviewer that we cannot distinguish whether Lamin A interactome changes due to increased centSIRT6 binding or increased SIRT6 ribosylation. We have emphasized this in the Discussion (see above). The experiment proposed by the reviewer would be difficult to quantify because SIRT6 enzymatic activity mutants have altered interactomes that would impact the interaction with Lamin A.

"Questions remain as to how centenarian SIRT6 allele together with LMNA enhance genome stability and contribute to longevity. As LMNA enhances SIRT6 enzymatic activities (PMID: 26549451), the stronger interaction may result in higher SIRT6 activity. Additionally, centSIRT6 may enhance LMNA function through stronger binding or as a result of a differential interactome of ribosylated LMNA. Previous attempts to induce ribosylation of purified LMNA by SIRT6 in vitro have been unsuccessful, possibly due to the lack of PTMs and the 3D nuclear organization that may be necessary. As ribosylations are notoriously difficult to detect by mass spectrometry, more work will need to be done to identify the specific residues on LMNA modified by SIRT6."

b) Linked to this, the authors state that many proteins identified in SIRT6 and LMNA IPS are ADP-ribosylated. If the authors claim that SIRT6 is directly involved in this ADPribosylation, they should show that centSIRT6 enhances ADPribosylation of one or several of these targets identified by MS by IP-WB.

We thank the reviewer for this suggestion. While we do provide evidence for enhanced ribosylation with previously identified targets (PARP1, SIRT6-self, and now KAP1), it is prudent to interrogate this on the other identified targets. We examined the ribosylation status of both vimentin and TAF15 (RBP56) via IP of said targets and probing with anti-ribosylation antibodies. We found both to be more ribosylated in IPs from centSIRT6 cells, shown in new Fig EV6A, B, C.

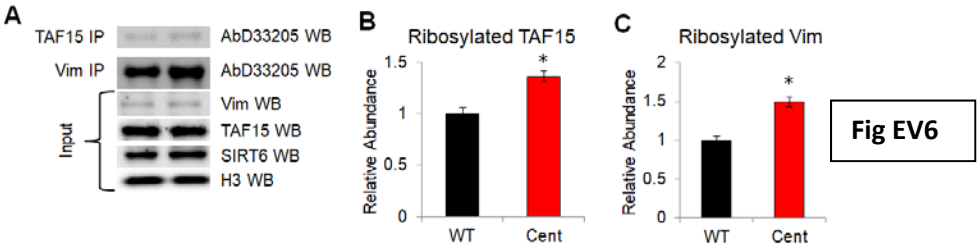


Fig EV6

c) As stated in the text, LMNA has been reported to be ADPribosylated on several residues. Can ADPribosylation be blocked by point mutations in these sites? If so, what happens if these mutants are overexpressed in centSIRT6 cells?

Detecting protein ribosylations by mass spectrometry is notoriously difficult due to the low stability of this modification. Many sites of LMNA ribosylation have been mapped by others; however, they are generally attributed to PARP1. The methods employed and published do not distinguish mono- versus poly-ADPr. Since we were unable to establish *in vitro* ribosylation reaction for Lamin A we were unable to map the residue mono-ADP ribosylated by SIRT6. As there are 14+ known ribosylation sites on LMNA, this task is a rather large undertaking. Additionally, LMNA overexpression is known to alter chromatin state, and OE of LMNA mutants would be non-physiological.

d) Figure 3C: total PARP1 blot is missing.

We have included PARP1 loading control (Fig 3C). These *in vitro* reactions were prepared from a master mix with equal amounts of PARP1.

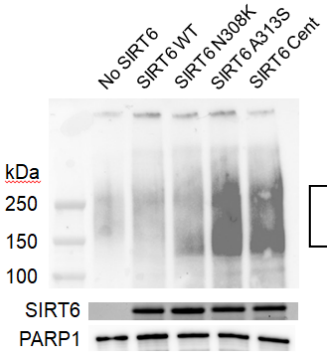


Fig 3C

5) Regarding the DNA repair assays:
a) The authors show that NHEJ and HR are both increased in centSIRT6 in classical GFP reporter assays. However, their γH2Ax dynamics show only a mild effect by this allelic variant. Authors should perform analysis with a marker of HR, such as rad51, and NHEJ, such as 53BP1 (to my surprise foci analysis is only shown in Figure EV5C).

We show improved DNA repair by two different approaches (γH2AX foci and GFP reporter assays), with GFP reporter assays being the most quantitative measure of a completed DNA repair reaction. Foci staining of the repair kinetics with additional markers are labor intensive, and would not reveal further insights.

b) In Figure EV4A, it is important to include percentages of GFP+ and RSD + cells in dot plots to give readers all information. Also, the figure legend should explain how analysis is performed. I assumed that black cells of dot blots are either negative or dead?

Analysis was described in the M&Ms. We have also provided percentages of populations in EV4A and EV4D. Black dots are live cells negative for GFP and DsRed.

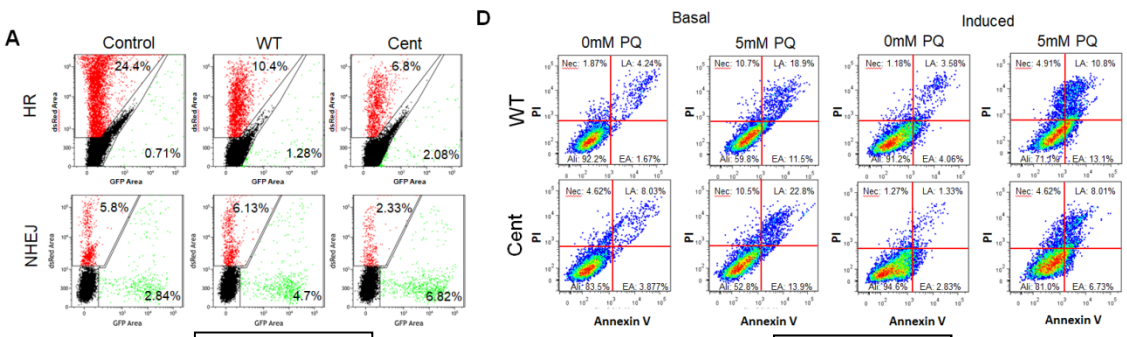


Fig EV4A

Fig EV4D

c) In Figure EV4D, dot plots must include percentages of populations to provide readers with all information.

We have included these percentages in the revised figures, as well as population labels for the Annexin V stains in EV4D (See above Response to Point 5b).

6) It's difficult to interpret some data on telomerase-immortalized human Fibroblasts. The authors have used different concentrations of cumate to induce similar levels of SIRT6 expression in stable cell lines expressing SIRT6 variants (Fig. EV3B) and stated in the figure legend "These concentrations were used in experiments with these cells". The concentration of cumate used to induce the expression of SIRT6 WT is circa 8 times higher than the one used to induce CentSIRT6. This raises the concern that different concentrations of cumate may cause altered cellular responses independently of SIRT6. This should be addressed by the authors. Loading controls are missing also in this figure.

We have now included the H3 loading controls for EV3B. We assessed cumate's impact on cell viability and growth and have included this data in figure EV3D, E. Cumate had no effect on cell viability or proliferation rate.

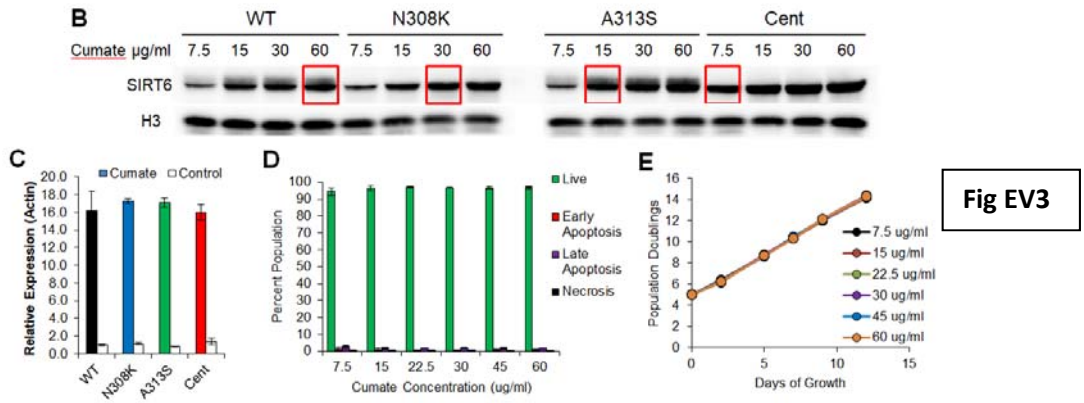


Fig EV3

MINOR ISSUES:

1) The majority of figure legends lack information of the number of replicates of each experiment.
We have now provided this information in all the figure legends.

2) Page 5, line 11 Fig.1C should be instead Fig.1D

Thank you for noting this typo. It has been corrected.

3) The authors often refer to allele when speaking about proteins which is not adequate. Protein variants or at least protein encoded by the centSIRT6 allele should be rather used.
Corrected as suggested.

4) Fig 2C. Review Y axis format, label on top of values. There seems to be an effect on K9Ac. Is the statistical analysis missing?

We have corrected the axis alignment issue. As indicated in the text, the difference in K9Ac is not statistically significant.

5) Fig.3A. A control showing the absence of specific bands in cells that do not express SIRT6 is needed. In this assay, it is not clear what the authors used to assess monoADPr. Since they assessed the incorporation of biotin-labelled NAD+ as stated in the figure legend, I assume that the authors used an anti-biotin Antibody (unfortunately no explanation is provided in the figure legend). On the other hand, in the material and methods section, the authors rather described radioactive NAD+. This should be clarified.

Fig 3A is an *in vitro* experiment, no cells. We have included a control without NAD.

We used HRP-conjugated Streptavidin (Sigma) for detection of biotin-NAD+. This information is now in the M&M. Experiment was also run using radio-labeled NAD+ (giving the same results). However, radio-labeled NAD+ has become commercially unavailable, and we removed this method from the manuscript.

6) Fig3A-B. Detection of self-ribosylation of sirt6 using biotin-labeled NAD is not detailed in the methods. Regarding Fig3B: is this performed using biotin-NAD? SDS-PAGE of a dot blot?

We have provided a more detailed description of the biotin-labeled NAD+ assay on page 23. We used SDS-PAGE gels:

SIRT6 Ribosylation Assay

Three µg of SIRT6 was combined with 50 mM Tris-HCl pH=7.5, 1 mM DTT, 10 µM ZnCl2, 150 mM NaCl, 25uM Biotin-NAD+ (Tocris 6573), and ddH2O up to 20 µl. All reagents were combined on ice in a master mix except for SIRT6. Master mix was aliquoted into reaction tubes and SIRT6 was added and incubated at 37°C for 3h. Reaction were quenched with 1 volume of 2x Laemmli buffer with BME. Samples were boiled and then run on SDS-PAGE gel and then transferred to PDPF membrane. Membranes were probed using HRP-streptavidin (Sigma RABHRP3).

7) In EV2C it is not clear what was exactly measured in this experiment. Additional explanations would be required either in the figure legend or Material & Methods.

Figure EV2C shows thermostability of the purified proteins. The Method is described on Page 23:

Thermostability

Four µM SIRT6 protein was combined with 1x SYPRO Orange dye in storage buffer (50 mM Tris-HCl pH=7.5, 150 mM NaCl, 1mM DTT, 5% glycerol) to 50 µl. Samples were placed in qRT-PCR plate and run on a BioRad CFX Connect Real Time machine using a Melt Curve protocol (30°C-75°C, 0.5°C steps in 10 s intervals).

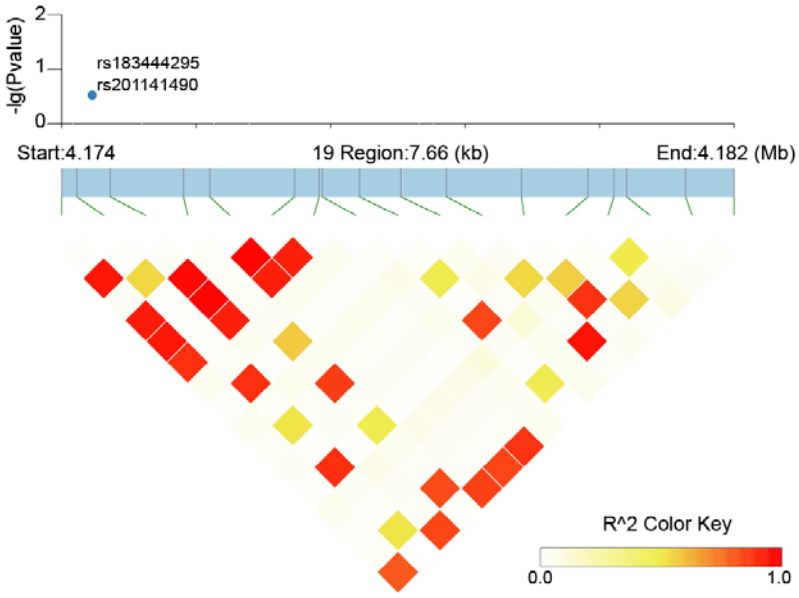
Referee #2

(Report for Author)
Simon et al present an examination of two rare missense mutations found in Sirt6 and demonstrate that these mutations confer potentially beneficial alterations to the function of Sirt6. They methodically assay the changes in function that the mutations confer either individually or in conjunction and show even though these mutations are in linkage the combination of the two effects is not always additive. The authors use several models (human cell lines, fibroblasts, and ESC-derived MSCs. Each analysis was a thoughtful experiment to address the hypothesis and overall, it is a well-designed and well written manuscript presenting an exciting novel haplotype overexpressed in an aged population that confers improved DNA damage response / genome maintenance.

Minor concerns:
The mutations A313S and N308K were described as being in perfect linkage with one another, but could the authors expand on the haplotype blocks that this haplotype belongs to- is it in linkage disequilibrium with the other eQTLs of Sirt6? A figure with the blocks defined across the Sirt6 gene would be helpful. Was this haplotype found as homozygous in the centenarians?

We thank the reviewer for the suggestion. This was a pooled-seq experiment, and while we could observe the co-occurrence of A313S and N308K in the same amplicon, we could not do the same with rs350845. However, we checked if there were any LD blocks within *SIRT6* gene (hg19, chr19:4,174,106-4,182,596) using data from large database 1000 Genomes (shown below, blue dot indicates the position of SIRT6 variants), but there was no obvious haplotype block in *SIRT6* gene.

Centenarians carrying the SIRT6 variants are all heterozygous carriers.



In regard to levels of expression of the mutant allele in conferring altered potential, it would be relevant to show the titration that was used to determine the cumate doses.

The titration is provided in Figure EV3B, C.

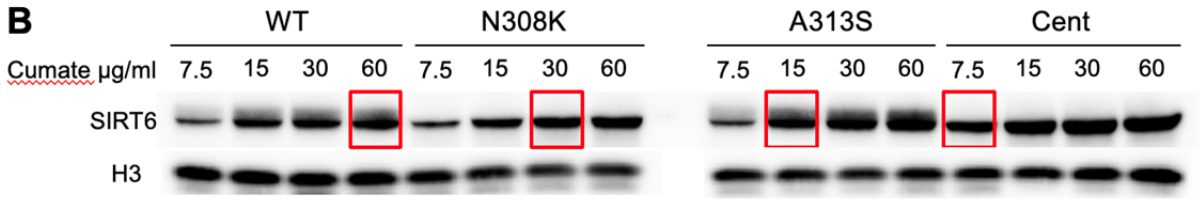


Fig EV3B

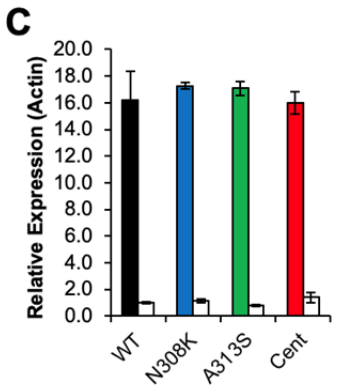


Fig EV3C

One of the exciting models used was the generation of hESCs with the centSIRT6 that were then differentiated into MSCs. (Please confirm in the text that these are mesenchymal stem cells). Given that different tissues may have unique response to SIRT6, it would be interesting to examine cell specific phenotypes of this centSIRT6 allele vs WT, as in the mMSCs it has been shown that SIRT6 plays a critical role in oxidative stress. The authors should examine if the centSIRT6 expression has any effect on the NRF2 pathway (qPCR of NRF2, HO-1, NQO1 etc.) and or perform an oxidative stress challenge on these cells. While it is appreciated that these cells cannot be titrated to have equal levels of Sirt6 expression (like in the inducible system) this model would be perhaps more reflective of the allele behavior in native cells.

We thank the reviewer for this comment. We have confirmed that MSCs are mesenchymal stem cells in the revised manuscript. It was reported in previous studies that NRF2-mediated antioxidant response pathway is repressed in SIRT6-deficient human MSCs (PMID: 26768768) and mouse embryonic fibroblasts (PMID: 31216030). However, based on qRT-PCR (new Fig EV5E) and RNAseq (new Fig EV5F) on human MSCs expressing WT SIRT6 or centSIRT6, we found no significant changes in the transcriptional levels of NRF2 and its downstream targets (HO1, NQO1, GCLC, GCLM and TXN).

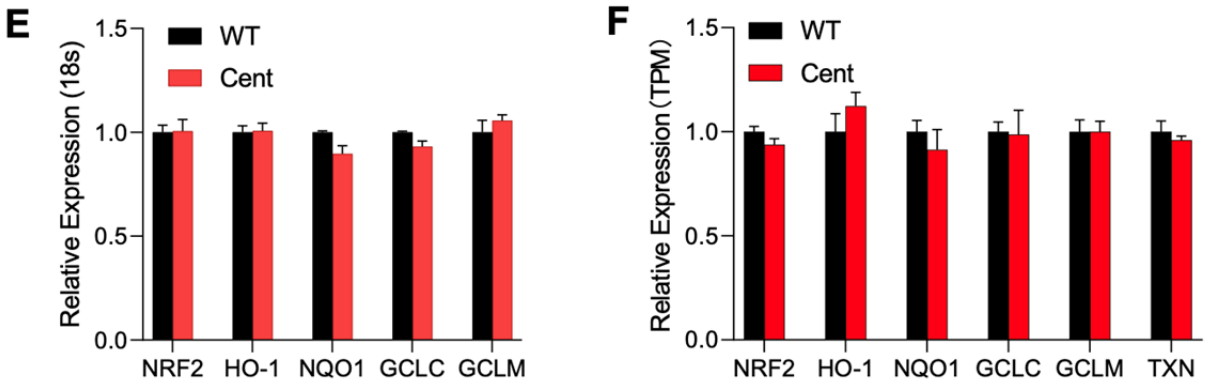


Fig EV5

Referee #3

(Report for Author)
Simon et al.
"A rare human centenarian variant of SIRT6 enhances genome stability and interaction with Lamin A"

The study conducted by Simon et al. claims that a SITR6 allele containing two linked substitutions (N308K/A313S), which is enriched in Ashkenazi Jewish (AJ) centenarians, brings some beneficial phenotypes such as increased suppression of LINE1 retrotransposons, enhanced DNA double-strand break repair, more robust cancer cell killing, compared to the wild-type allele. These phenotypes are likely mediated by its enhanced mono-ADP ribosyltransferase (mADPr) activity and stronger interaction with Lamin A/C (LMNA). The data they presented in this manuscript are very interesting and stimulating. On the other hand, however, mechanistic evidence is relatively weak. Additionally, the data from fibroblasts or peripheral blood mononuclear cells (PBMCs) of AJ centenarians could further strengthen their claims, if these cells are available. Thus, the authors should address the following points to strengthen their conclusions.

Fibroblasts and peripheral blood mononuclear cells (PBMCs) from AJ centenarians are not available. Immortalized blood cells were the only cell type preserved from this cohort; these cells were collected over a long period of time and show significant batch effects depending on the time of isolation and immortalization. Although the functional analysis using centenarian-derived cells could be supportive, since longevity is a complex trait, the phenotypes observed in centenarian-derived cells from genetically diverse individuals could not be ascribed to the SIRT6 variants alone. Therefore, we prefer to use isogenic cell models.

Major points:

1) The mechanistic evidence supporting their notion that increased mADPr activity of centSIRT6 is responsible for beneficial cellular phenotypes should be provided. For example, is increased suppression of LINE1 retrotransposons dependent on KAP1? Is KAP1 more mono-ADP-ribosylated in cent SIRT6-expressing cells? It is also unclear how enhanced interaction between centSIRT6 and LMNA is connected to these beneficial cellular phenotypes. Currently, the results are just an association.

To address this question, we have performed KAP1 ribosylation assays with WT and centSIRT6 (new Fig 4C). We found centSIRT6 to demonstrate enhanced KAP1 riboslyation compared to WT SIRT6, which may explain the enhanced suppression of LINE1 elements.

We do not claim that LINE1 suppression is connected to enhanced LMNA binding, though this is an attractive hypothesis. LMNA has been reported to enhance SIRT6 enzymatic activity (PMID: 26549451) which may directly benefit LINE1 suppression and DNA repair.

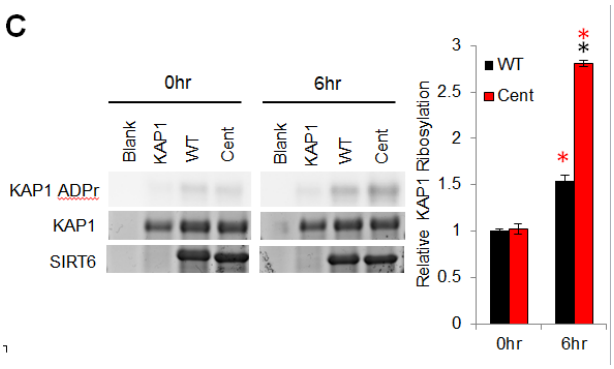


Fig 4C

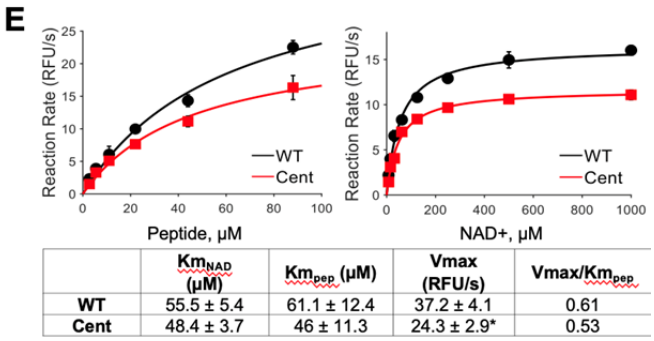
2) It is fair to speculate that these beneficial cellular phenotypes, such as suppression of LINE1 retrotransposons, DNA double strand break repair, capacity to kill cancer cells, could potentially have an impact to median lifespan or healthspan. However, at this moment, it is not clear whether any of these phenotypes is mechanistically connected to longevity in mammals. In this regard, the frequency of these linked substitutions is just 1.0% among AJ centenarians, meaning that 99% of AJ centenarians cannot be explained by these linked substitutions. Thus, what is the evidence or the logic to claim that these linked substitutions contribute to extreme longevity? Dr. Gorbunova previously used fruit flies to address this critical question in their Cell paper. Can they conduct this particular experiment for centSIRT6?

We thank the reviewer for the thoughtful comments. Unfortunately, unlike the differences in the SIRT6 core domain that we evaluated in fruit flies in the *Cell* paper, the centenarian mutations are located in the variable C-terminus of SIRT6 that is not conserved even in the mouse. Hence, we cannot assay its effect on lifespan in a model system.

Minor points:

1) In Figure 1D, the authors claim that centSIRT6 confers a slight reduction in deacylase catalytic efficiency. Is this difference statistically significant between WT Vmax/Km and Cent Vmax/Km ?

We have now provided this statistic (new Fig 1E). The difference in the Vmax/Km ratio between the WT and centSIRT6 is not statistically significant, which does not affect our argument that the Centenarian variant confers its advantages exclusively through ribosylation.



	Km_{NAD} (μM)	Km_{pep} (μM)	Vmax (RFU/s)	$Vmax/Km_{pep}$
WT	55.5 ± 5.4	61.1 ± 12.4	37.2 ± 4.1	0.61
Cent	48.4 ± 3.7	46 ± 11.3	24.3 ± 2.9*	0.53

Fig 1E

2) In Figures 1E-F and 2A-B, different NAD+ concentrations were used (1mM vs. 5mM). Why did the authors have to use such high concentrations? And why were the concentrations different? If there is any particular reason for these conditions, the authors should explain it in the manuscript.

Higher NAD+ concentration was used in 2A-B to ensure that NAD+ is not a limiting reagent. We have decided to remove Fig 1E-F, as they are redundant with 2A-B and only provide a single time point.

3) In Figure 2A, the graph of Cent at 15min does not look consistent with the WB result. Based on the WB result presented, the graph should show about a half of 0 timepoint. They should correct either one. **The reviewer is correct in the observation of the blot not matching the quantification. We found the error and fixed the quantification.**

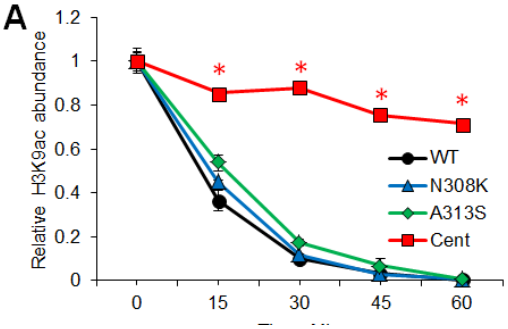


Fig 2A

4) In Figure 2D, it seems strange that H3K9ac and H3K18ac in WT cells did not change at all before and after cumate induction. Does cumate-induced SIRT6 not have any effect on acetylation H3K9ac and H3K18ac? Additionally, the description regarding this experimental condition is not precise enough. The authors should provide a bit more details, such as incubation time after cumate addition and a time course of cumate and paraquat treatment.

Previous reports from our group and others have shown that SIRT6 overexpression from a strong CMV promoter reduces global H3K9ac levels. In this experiment, we used cumate induction system, which we calibrated to express SIRT6 at much lower, close to endogenous, levels (compare SIRT6 expression in Image A below from PMID: **21680843 to Fig EV3B SIRT6). This is likely the reason we do not see a strong change in H3 acetylation status upon cumate-induced SIRT6 expression.**

Given the Km of SIRT6, it has been argued that SIRT6 is a weak deacetylase and its activity is likely relegated to select targets. In order to explore this possibility further, we performed ChIP qPCR of known targets of SIRT6 H3 deacetylation. We have included ChIP qPCR data from H3K9 targets (LMNA, MnSOD, NFκB2, NFκBia) and H3K18 targets (Sat II, Sat III) (PMID: 19135889, PMID: 21738489, PMID: 27043296). Looking at these specific targets of SIRT6 deacetylase activity, we do see that cumate (-) and the cumate (+) cells have differential histone acetylation status, with those with SIRT6 showing lower acetylation (Fig2E-J). However, similar to what was observed at the global level, there is little to no difference between the two SIRT6 variants in live cells.

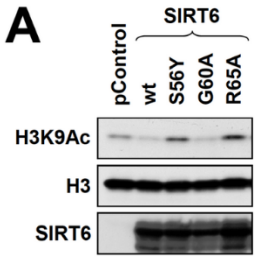
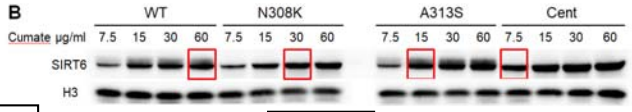


Image A
(From Mao et al, Science, 2011)



EV3B

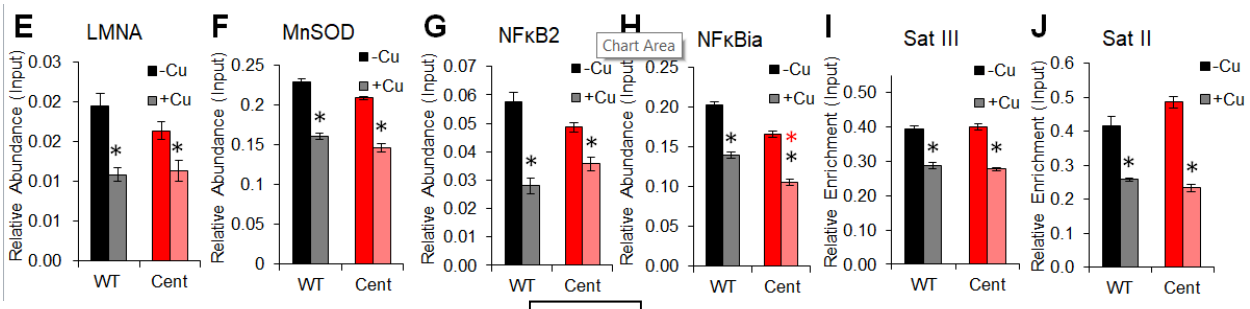


Fig 2

5) In Figure EV2, even though centSIRT6 has a more open conformation compared to the wild type, it is unclear how this conformational change impacts to other features of centSIRT6. Do the authors attempt to claim that this structural change results in stronger interaction with LMNA?

We do not claim that more open conformation of SIRT6 leads to stronger interaction with LMNA. However, it is tempting to speculate that it does.

Additional suggestions:

1) There are several simple typos. "Fig 1C" on page5, line 7 and line 11 is "Fig 1D ". "Fig 4F,G,Fig EV4D" on page 7, line 24 is "Fig4G, Fig EV4D".
Thank you! Corrected.

2) In discussion, the authors wrote "Ashkenazi Jews who exhibited extreme phenotype (>100 years old)" on page 11, line 20. On the other hand, in Materials and Methods, they wrote that "a centenarian is defined as a healthy individual living independently at 95 years of age or older" on page 14, line 23. They should be consistent.
We appreciate the reviewer for pointing it out. We altered the text to reflect the age of centenarians to >95.

3) Figure 2C, vertical title "Relative Fraction" is overlapping with number.
Corrected.

4) In table 1, Alt and Ref alphabet in rs183444295 and rs201141490 seems to be wrong. As for rs183444295, "T" in Alt and "G" in Ref seems to be correct. As for rs201141490, "G" in Alt and "C" in Ref seems to be correct.

We thank the reviewer for reviewing our manuscript very carefully. The alphabet suggested by reviewer was the changed nucleotides in codon, and the Alt/Ref alphabet we used in the table was consistent with dbSNP and was the nucleotide on the sense strand of reference genome (the coding sequence of SIRT6 was the antisense strand of the reference genome). For rs183444295 (aa. A313S, nt. Gcc/Tcc), The Ref allele from dbSNP is C (complementary base pairing of G) and Alt is A (complementary base pairing of T). We think it should be Ok to show Ref/ Alt alphabet that is consistent with dbSNP.

Referee #4

Report for Author:
A rare human centenarian variant of SIRT6 enhances genome stability and interaction with Lamin A

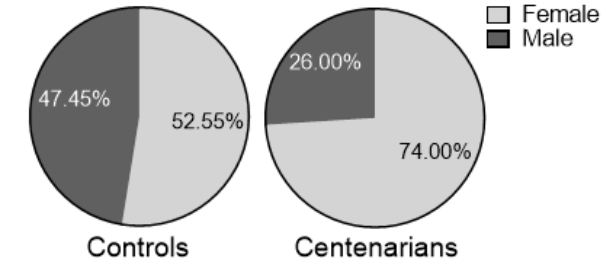
Single nucleotide polymorphisms (SNPs) in SIRT6 have been linked to human longevity, however these SNPs are typically in non-coding regions of the genome. In this study, the authors investigated potential SNPs associated with human longevity in a small Ashkenazi Jewish (AJ) population. This population, although small, is useful for genetic studies as it is more controlled than the broader datasets. They identified two SNPs in SIRT6 associated with human longevity (although not at a high significance due to the sample size restrictions) and found that a dual allele, dubbed the centenarian SIRT6 allele (centSIRT6), contained both SNPs. The authors then tested this centSIRT6 allele and found that it shifts the balance of deacetylation and mono-ADP-ribosylation activity of SIRT6 in vitro. This helped to validate the SNP findings from the AJ control and centenarian analysis. The variety of in vitro cell lines and methods used were well-designed and lend strength to their findings. They perform multiple methods of testing to support each of their conclusions about centSIRT6 activity and its interaction with LMNA. This work lays the foundation for future in vivo analysis of LMNA and SIRT6 manipulation as a means to prolong lifespan. There are some clarifications needed in the manuscript to support the author's conclusions.

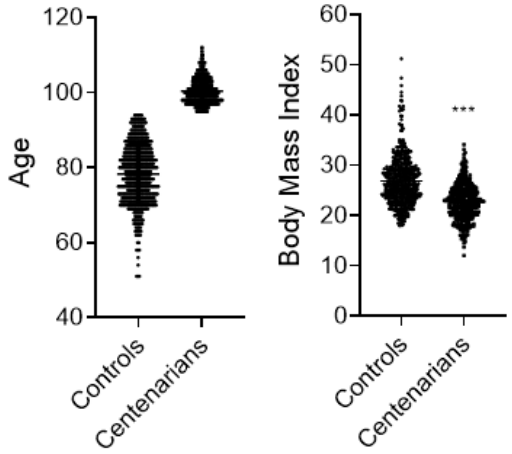
Major Concerns:

1. The authors should list the criteria they used to determine a "healthy" individual. Were there any exclusion criteria (e.g. BMI) besides age? The age range and gender information for the control and centenarian groups should also be listed. If potential confounding variables (i.e. gender, BMI) for human longevity are unknown, the authors should mention this in their discussion section.

We thank the reviewer for the thoughtful comments. The participants' ages were defined by birth certificates or dates of birth as stated on passports. Recruited centenarians were required to be living independently at 95 years of age as a reflection of good health. Health histories were obtained by a standardized questionnaire including height and weight. The ages of centenarians were 100.4±3.3 years (mean±SD) and the ages of controls were 78.3±8.1 years (mean±SD) at the time of sequencing. 72% of centenarians and 52.55% of controls were female. The BMIs of centenarians were 26.9±4.8 (mean±SD) and the BMIs of controls were 22.8±3.6 (mean±SD). Significantly lower BMIs in centenarians were observed ($p < 0.0001$), which is consistent with previous studies by the Barzilai group using the same cohort (smaller sample size than ours because more individuals were since recruited) (PMID: 14728640, PMID: 12403798). The plot of age, gender and BMI in this cohort are shown below. We have provided this information in the revised manuscript (Fig 1A).

Group	Female	Male	Age (mean±SD)	BMI (mean ± SD)
Control	289	261	78.3 ± 8.1	26.9 ± 4.8
Cent	333	117	100.4 ± 3.3	22.8 ± 3.6





Minor Concerns:

1. There are quite a few areas with repeated sentences and high repetition that can be removed from the manuscript text.

We thank the reviewer for indicating redundancies in the text. We have reviewed the manuscript for these repetitions and have consolidated the text where appropriate and in accordance with the requests of the other reviewers.

2. The asterisks in figure 2 overlap with the figure itself and aren't visible.
Thank you for pointing out these issues with the images. They have been corrected for visual clarity.

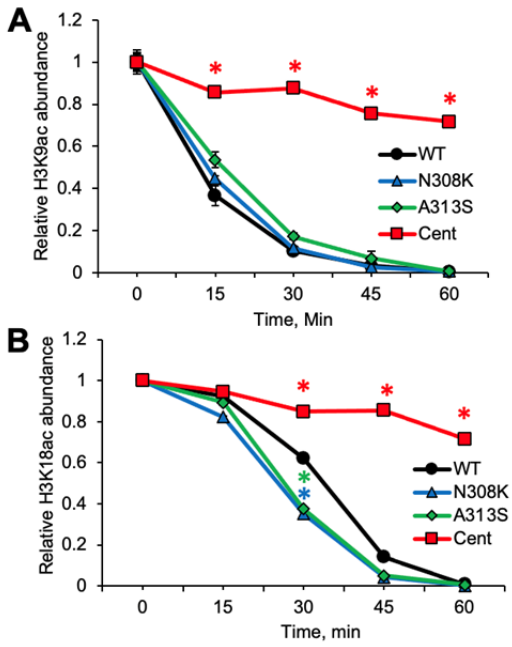


Fig 2AB

3. There is no mention of the p-value, sample size, or the error bars in the Figure 2 legend.
We appreciate the reviewer for indicating this oversight. We have provided this information in the figure legends.

4. Are there error bars for Figure 3B?
We have now provided error bars.

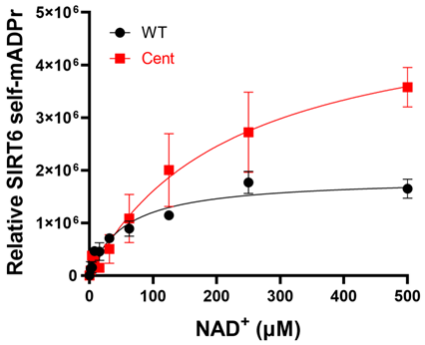


Fig 3B

5. It may be a PDF issue, but Figure 4C is cut off and may need to have the size reduced.
There was, indeed, an error in the PDF formatting. It has been corrected.

6. There is no mention of the blots in Figure 4B and C in the legend.
The blots are from transfected cell lines to demonstrate that SIRT6 levels were equivalent in the transiently transfected cells. Explanation of the blots are now provided (Fig 4D, E).

7. There is no mention of the colored asterisks for Figure 4G in the legend.
The colors correspond to the alleles in the legend, and indicate significant difference ($p<0.05$) from the WT control. This explanation is now provided in figure legends.

8. It would be helpful to make the error bars in color for Figure 4F and G so that each group can be visualized.
Thank you for the suggestion. Data is now presented with color-coordinated error bars to improve clarity.

9. The legend for Figure 4 states that there is another statistical method used but there is no mention of another method in the legend.
We apologize for the ambiguity and have clarified in the figure legends the tests used.

10. There is a mention of colored asterisks in the legend for Figure 5A, but there are none in the figure itself, only black asterisks.
We thank the reviewer for catching this. We have removed the mention of colored asterisks.

11. Error bars are not mentioned in the legend for Figure EV2 C and G. The label E is also partially cut off by the figure itself.
We have included the error bars explanation in the figure legend. Error bars represent standard deviation in Figure EV2C, G.

12. There is no mention of error bars, the p-value or statistical test used in the legend for Figure EV3. Also the normalization is not mentioned for C in this figure.

We have extensively clarified the error bars, stats, and p-value criteria for all figures in their appropriate legends. Figure EV3C was normalized to Actin expression. This information is included now in both the Y-axis label and in the figure legend.

13. The figure legend for Figure EV5 mentions NS but there is no NS in the figure itself. The legend does not state the sample size for the majority of the panels.

Thank you for pointing this out. It was an oversight that has been corrected. All experiments in Figure EV5A, B, E, F were performed in triplicate. Figure EV5C, was done with n=6. Fig EV5D represents 600+ scored nuclei from 10 fields per cohort.

Dear Vera,

Congratulations on a great revision! Overall the referees and I feel you have satisfied the majority of concerns and I thank you for your comprehensive response to the referee comments. That said, we ask that you please make the two changes recommended by Referee 3 and 4 in an updated manuscript. In addition, please address the following editorial changes:

1. Please update the "Data Availability" section with bullet points as described in the online EMBO author guide

2. Also please provide the accession IDs for PRIDE/ProteomeXchange

3. Please review our new policy on conflict of interest on the EMBO author guide website and update the title of this section to: Disclosure and competing interests statement

4. For references, only the first 10 author names should be displayed. In cases where there are more than 10 authors, add et al. after the 10th author name.

5. Please ask Dr. Suh to provide an ORCID ID in their EMBO profile

6. Please provide Grant numbers in the Funding information section

7. Please upload the main figures as individual, high-resolution figure files. Up to five EV figures (from the supplemental file) can also be uploaded as individual figure files and their legends should then be added to the manuscript, after the main figure legends.

8. Figure 4I is called out before Figure 4H in the manuscript text, please update

9. For Figure EV1, the callout should include EV1A, EV1B, and EV1C

10. For the 3 dataset EV legends please add the names and legends and remove colors.

11. We encourage the publication of source data, particularly for electrophoretic gels and blots and graphs, with the aim of making primary data more accessible and transparent to the reader. It would be great if you could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure or for graphs, an Excel spreadsheet with the original data used to generate the graphs. The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight marker; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files.

12. Attached is a word document from our publisher with their notes from their pre-publication check on your manuscript. Please take a look at the file and the comments regarding figure legends and respond to the issues.

13. Please change the title of the abstract to "Abstract" rather than "Summary"

Thank you again for the opportunity to consider your work for publication. I look forward to your revision.

Kind regards,

Kelly

Kelly M Anderson, PhD
Editor
The EMBO Journal
k.anderson@embojournal.org

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

Use the link below to submit your revision:

Link Not Available

Referee #1:

The new version of the manuscript has been improved with a considerable amount of new evidence. All this new data supports the authors' claims and have strengthened the work. The authors have addressed satisfactorily all my concerns and clarified/corrected all the doubts/mistakes I pointed out. An important issue to remark is that, in contrast to the original version, this revised version provides a more cohesive story where the different parts have been more convincingly linked. This said, there are some still some evidences that could benefit from further development, such as Lamin ADPribosylation. However, considering all the included evidence, this would not change the main conclusions of the Ms and it could be expanded in a another manuscript.

Referee #2:

The authors have adequately addressed the concerns raised.

Referee #3:

EMBOJ-2021-110393R
Simon et al.
"A rare human centenarian variant SIRT6 enhances genome stability and interaction with Lamin A"

In this revised manuscript, the authors adequately addressed all questions from this reviewer, except for the major comment #2. This reviewer does understand the limitation of the experimental system. Nonetheless, unfortunately, the effect of centSIRT6 on lifespan cannot be tested in a model system. Thus, in a strict sense, it remains unclear whether the enzymatic activity of centSIRT6 really contributes to the longevity of AJ centenarians.

In this regard, it is strongly suggested to tone down the last sentence in the first paragraph of the Discussion section: "Our findings suggest that increased SIRT6 activity also benefits human longevity."

With this minor textual change, this manuscript will be ready to be accepted for EMBO Journal.

Referee #4:

A rare human centenarian variant of SIRT6 enhances genome stability and interaction with Lamin A

Single nucleotide polymorphisms (SNPs) in SIRT6 have been linked to human longevity, however these SNPs are typically in non-coding regions of the genome. In this study, the authors investigated potential SNPs associated with human longevity in a small Ashkenazi Jewish (AJ) population. This population, although small, is useful for genetic studies as it is more controlled than the broader datasets. They identified two SNPs in SIRT6 associated with human longevity (although not at a high significance due to the sample size restrictions) and found that a dual allele, dubbed the centenarian SIRT6 allele (centSIRT6), contained both SNPs. The authors then tested this centSIRT6 allele and found that it shifts the balance of deacetylation and mono-ADP-ribosylation activity of SIRT6 in vitro. This helped to validate the SNP findings from the AJ control and centenarian analysis. The variety of in vitro cell lines and methods used were well-designed and lend strength to their findings. They perform multiple methods of testing to support each of their conclusions about centSIRT6 activity and its interaction with LMNA. This work lays the foundation for future in vivo analysis of LMNA and SIRT6 manipulation as a means to prolong lifespan. The authors have addressed prior reviewer concerns with updated experiments and text in the manuscript, and no major areas of concern remain.

However, one issue that should be addressed is the following:

1. The authors have included the statistical tests used for their data analysis. There are instances of using the student's t-test when comparing multiple groups, as well as two-way ANOVA. No mention occurs of any post-hoc analysis or correction for multiple comparisons. Can the authors confirm whether they used any correction or post-hoc analysis?

REV 3 Request: In this regard, it is strongly suggested to tone down the last sentence in the first paragraph of the Discussion section: "Our findings suggest that increased SIRT6 activity also benefits human longevity."

We agree with the reviewer's point regarding this sentence. We have now changed it to read: "*Our findings indicate that increased SIRT6 activity has a beneficial impact on cellular functions linked to longevity and may contribute to increased lifespan in centenarians*."

We believe this to be a more conservative assertion, while still emphasizing the novelty and potential implications of our findings.

REV 4 Request: The authors have included the statistical tests used for their data analysis. There are instances of using the student's t-test when comparing multiple groups, as well as two-way ANOVA. No mention occurs of any post-hoc analysis or correction for multiple comparisons. Can the authors confirm whether they used any correction or post-hoc analysis?

We now clarify in the Statistics section that all two-way ANOVAs were subsequently analyzed using a Bonferroni correction.

2nd Revision - Editorial Decision **8th Aug 2022**

Dear Vera,

Congratulations on an excellent manuscript, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal. Thank you for attending to the final editorial concerns in the latest version. Please note that I've made a few minor editorial changes, which you can check over at the proof stage.

I will now begin the final checks before submitting to the publisher next week. Once at the publisher, it will take about 3 weeks for your manuscript to be published online. As a reminder, the entire peer review process including referee concerns and your point-by-point response will be available to readers.

It has been a pleasure to work with you to get to the acceptance stage. I will be in touch throughout the final editorial process until publication. In the meantime, I hope you find time to celebrate!

Kind regards,

Kelly

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here:
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If you have any questions, please do not hesitate to call or email the Editorial Office. Thank you for your contribution to The EMBO Journal.

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Reporting Checklist for Life Science Articles (updated January 2022)

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	N/A

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods, Figures

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	N/A
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	N/A

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Materials and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	N/A
Please detail **housing and husbandry conditions**.	Yes	Materials and Methods

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	N/A
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	N/A

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Yes	Materials and Methods

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Materials and Methods

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	N/A
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	N/A

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Yes	Materials and Methods

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Materials and Methods
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	N/A
Include a statement about **blinding** even if no blinding was done.	Yes	Materials and Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	N/A
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figure Legends, Materials and Methods

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure Legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure Legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	N/A
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	N/A
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	N/A
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	N/A
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Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	N/A
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	N/A
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	N/A

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

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State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	N/A
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	N/A
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	N/A

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Materials and Methods
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	N/A
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	N/A
If publicly available data were reused, provide the respective **data citations in the reference list**.	Yes	Materials and Methods