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Tumor suppressor SIRT3 deacetylates and activates manganese superoxide dismutase (SOD2) to scavenge ROS

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Transaction Report:

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

1st Editorial Decision 19 October 2010

Thank you for the submission of your research manuscript to our editorial office. We have now received the evaluation of the expert reviewers and I am pasting them below.

I would prefer not to repeat the reports here in detail, but you will see that, while all referees agree on the potential interest of the findings, they also feel that in some instances additional work is needed.

Referee 1 states that stronger data should be provided to show that SIRT3 directly deacetylates SOD2 at K68 and that the evidence linking nutrient starvation and ROS to SIRT3 and SOD2 activity would need to be strengthened. This referee also suggests to measure mitochondrial superoxide levels rather than ROS. In addition, both referees 1 and 2 also point out some technical issues (including missing controls) that would need to be addressed before the manuscript can be published. Referee 2 remarks that the study relies heavily on the use of protein overexpression and that therefore additional evidence would be required to demonstrate that endogenous SOD2 is regulated by SIRT3 in the proposed way in vivo. This referee, together with referee 3, also feels that it should be clarified why a SOD2 mutant that cannot be acetylated does not display higher activity.

Overall, given these evaluations, the reviewers constructive comments and the potential interest of the study, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the referees (as outlined above and in their reports) must be addressed, especially with regard to the strengthening of the physiological significance of the findings and the

proof that SIRT3 directly deacetylates SOD2. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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We also welcome the submission of cover suggestions or motifs that might be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready.

Yours sincerely

Editor
EMBO reports

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Reviewers General Comments

This manuscript by Chen et al., 2010 "Tumor suppressor SIRT3 deacetylates and activates manganese superoxide dismutase (SOD2) to scavenge ROS" presents results demonstrating present work suggesting that MnSOD is acetylated at lysine 68 and that this specific lysine acetylation directs MnSOD2 activity. This statement is based on data showing that MnSOD lysine 68 is acetylated and can be hyperacetylated when treated with somewhat non-specific agents that pan inhibit most cellular lysine deacetylases. It is also shown that SIRT3 binds to and directs both the acetylation status and dismutase activity of MnSOD. Finally, data is presented that SIRT3 expression is governed by intracellular ROS levels that subsequently regulates MnSOD activity via a post translation modification involving acetylation.

It has previously been shown that SIRT3 directs intracellular ROS levels, immortalization of fibroblasts in vivo and mammary tumorigenesis in vivo, and MnSOD contains an acetyl lysine at position 68 in MnSOD has been previously identified to increase with CR (Schwer et al., Aging Cell 2009). However, the observation that MnSOD is regulated by a specific reversible acetyl lysine that is regulated by SIRT3 has not been previously described, and therefore this manuscript represents a novel finding into understanding the function of SIRT3 in mitochondrial biochemistry and biology. However, there are some issues with the data presented (see below) therefore in its current form the conclusions drawn by the authors are not supported by the experiments. As it stands, this manuscript requires improvement before it can justify the stated conclusions.

Some general comments in regards to this manuscript: some of the data is of sub-optimal quality that will need to be redone. In addition, some of this data presented has already been published and the authors might consider moving this data into the supplemental section and focus on the new and innovative data. In addition, the figure legends should provide a little more information about how the experiments were done to help the readers follow the results.

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Reviewers, Specific Comments:

1. The quality of some the westerns blots in the manuscript are sub-optimal and should be repeated. Specifically, Figure 1A, top panel where the blot is very hazy. Figure 1B, top panel the differing widths of the lanes prevents determine in changes in acetylation. Figure 2A where it is difficult to determine if there are any changes in immunoreactive protein levels. Figure 3A contains too much background that prevents clearly identifying protein levels. Figure 3D contains bands with significant variability in band width prevent clear identification of differences. Figure 4D, top panel needs to be improved.

2. There is no direct data in the manuscript that Sirt3 deacetylates MnSOD lysine 68 and this must be addressed if this manuscript is going to be reconsidered of publication. The only data addressing this issue is shown in the Supplemental Section, Fig. 1a showing mass spec data. However, this data only shows that MnSOD lysine 68 is acetylated and does not show that is changes.

3. The authors do show in figure 3A that siRNA knockdown of SIRT3 decrease MnSOD acetylation as well as decreases MnSOD activity. There are several significant issues with this experiment and panel that is critical to the conclusions made by the authors. First, the quality of the western is sub-optimal and this reviewer finds it difficult to determine if there is an increase in MnSOD acetylation in lanes 3 and 4. In addition, (and most importantly) this experiment only shows that MnSOD acetylation increases (lane 2 is the only lane where a difference is seen) and does not identify the specific lysine. These samples should be removed from the gel and mass spectrometry must be done to show the specific lysine that is hyperacetylated when SIRT3 is decreased by siRNA.

4. In the panels in figure 2 the investigators have failed to re-blot with MnSOD for loading control. Or for the IP western experiments the samples must be divided into equal fractions and determine equal amounts of IPed protein. In addition, for these experiments the authors must do western blotting to determine that there are equal amounts of total MnSOD and SIRT3 in the cell transfected with the different Sirtuin (T1-T7) cells and determine the difference between endogenous and exogenous MnSOD and SIRT3.

5. Figure 2B, bottom panel the y axis states "Relative SOD activity"? Is this correct? The figure legend states "K68 is the major site of SOD2 acetylation. Flag tagged wild-type and mutant SOD2 were expressed in HEK293T and affinity purified" suggest the y axis should be total MnSOD acetylation.

6. There are several issues that need to be addressed for the data panels presented in figure 4 since this is critical to the authors conclusions. First, figures 4A and 4B have been previously published in a similar or in some cases using much cleaner models that siRNA showing that SIRT3 determines and directs intracellular ROS levels.

7. In addition, figures 4A and 4B are measuring ROS levels and this is no specific enough for this work. The authors are suggesting changes in the regulation of MnSOD that converts superoxide to hydrogen peroxide. Thus, the correct experiments to do are the determination of mitochondrial superoxide levels.

8. The experiments in figures 4C-F are not specific and fail to directly connect SIRT3 and MnSOD activity. In addition, these experiments do not connect lysine 68 to the changes in MnSOD activity and this should be addressed. These experiments show that treatment with: (1) nutrient deprivation and DNMQ alter MnSOD acetylation; and (2) nutrient deprivation and DNMQ alter MnSOD alters MnSOD activity however, this does not a priori show that nutrient deprivation and DNMQ changes in MnSOD directly directs MnSOD activity since these treatments may be working via other potential pathways either in combination with SIRT3 and/or via another signaling factor. Control experiments must be added to make a more rigorous connection between these conditions, SIRT3, and MnSOD activity.

Closing Points:
In closing, the authors present a very novel finding that are very strongly supported by this reviewer and this work is potential extremely important to both the fields of sirtuins and MnSOD mitochondrial biology. However; the manuscript required the addition of significantly more control

data before it can justify the stated conclusions. In addition, the data identifying MnSOD lysine 68 in this process need additional data that will require either Mass Spectrometry data or a MnSOD lysine 68 specific antibody.

Referee #2 (Revision Comments):

To my opinion, the paper can be accepted only if all the major revisions will be done. The fact that the experiments were performed in vitro on overexpressed protein, significantly weaken the physiological significant of the results.

Referee #2 (Remarks to the Author):

Chen et al. follow the role of the mitochondrial deacetylase SIRT3 on SOD2 activity. The authors showed that when overexpressed in HEK293 cells, SOD2 is acetylated. SOD2 deacetylation by SIRT3 promotes its enzymatic activity which potentially results in a decrease in ROS levels. Therefore the findings might be relevant to the potential role of SIRT3 in longevity. This is an interesting manuscript that increases our knowledge on SIRT3. The following points should be addressed:

Major:

All of the experiment with SOD2 are done with tagged overexpressed protein. The authors must present an in vivo evidence that SOD2 is a substrate for SIRT3. My suggestion is to perform IP with Ab against Ace and western with Ab against SOD2 in SIRT3 KO vs. wildtype cells and in SIRT3 overexpression vs. wild type cells.

The role of deacetylation in inhibiting SOD2 is not clear. The K to R mutation that mimics deacetylated SOD2 should show in increase in SOD2 activity. Yet in figure 3c, the activity of K68R is not higher than wild type.

The experiments were done in 293/T only. It is not clear how broad the phenomenon is. The authors should repeat some of the experiment in another cell line.

The title is confusing, the findings of this paper are about cancer. Therefore, please delete the words tumor suppressor.

In figure 3A, an additional siRNA sequence against SIRT3 and unspecific siRNA should be included.

Please show an endogenous IP between SIRT3 and SOD2

Minor:

Please show the levels of SOD2 acetylation in cells treated with TSA or NAM only.

Figure 1B
A reference to material methods on the manner of SOD activity measured is missing from the results section

Figure 2C
The indicated band of endogenous SIRT3 by IP may be slightly too weak

Figure 2D
please include a western analysis of the input.

Figure 3A
When treated by NAM only, the level of acetylated SOD2 is slightly stronger than in cells expressing siRNA for SIRT3 only. This may imply that other sirtuins (SIRT5?) also deacetylates

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SOD2 in the absence of SIRT3.

Figure 4C
After 4 h, the level of Ac-SOD2 decrease but its activity is the same as the 0 and 2h. Could it be that another unknown PTM regulates is regulates SOD2 activity after 8h?

Figure 2A legend; change SA to TSA.

Referee #3 (Remarks to the Author):

Dr. Chen and colleagues have performed a full factorial designed study to identify and characterize the role of SIRT3 in deaceylating and activating the mitochondrial superoxide dismutase SOD2 via direct deacetylation of lysine68.Using protein protein interaction studies, pharmacologic and siRNA inhibition of protein function and biochemical assays the authors show convincingly that SOD2 is a direct substrate of SIRT3 and this deacetylation activated SOD2 activity with a blunting of cellular ROS levels.

Comment:

1. Do the authors have a reason why the acetylation resistant arginine mutation does not show high intrinsic SOD activity?
2. Page 3, 3 lines from the bottom, should the final word on this line not be mitochondria instead of cytosol?
3. Page 5, 6 lines from the bottom - should it read treatment of cells with TSA and NAM instead of TSA and NAD?
3. Which antibody was used to show the change in acetylation of SOD2, or was this the aceylated lysine band the correlated in MW to where SOD2 should run?
4. The authors state that a role of SIRT3 in the oxidative stress response has not been directly shown, however, a paper has recently been published in Free Radical Biology and Medicine in this regard by Bao J et al. This should be stated.
5. Fig 2B. Does the IB-alpha Flag mean that the proteins were immunopreciptated with FLAG to show the protein protein interaction?

1st Revision - authors' response 08 February 2011

First of all, I would like to thank you for the efforts in handling and for offering us the opportunity to revise our manuscript "**Tumor suppressor SIRT3 deacetylates and activates manganese superoxide dismutase (SOD2) to reduce cellular ROS levels**". We have now completed all of the experiments to address reviewers' comments. Extensive experimentations have been carried out during the revision. Among them are four major additions.

First, we performed three experiments to show that SIRT3 directly deacetylates SOD2 at K68, an in vitro deacetylation assay by SIRT3 (new Fig. 2F), in vivo SIRT3 knocking down (new Fig 3E) and iTRAQ experiment (new Fig 3G). Together, these results provide strong support to the conclusion that SIRT3 deacetylates SOD2 at K68.

Second, we performed two experiments to strengthen the conclusion linking nutrient starvation and ROS to SIRT3 and SOD2 activity. We show that nutrient deprivation does not affect SOD2 acetylation level and activity when SIRT3 is knocked down (new Fig 4D). Next, we demonstrate that nutrient deprivation deacetylates and activates wild type SOD2, but had no effect on either acetylation levels or activities of K68Q and K68R mutants (new Fig 4E). These two lines of evidence linked nutrient deprivation effect on SOD2 activity to SIRT3 and lysine 68 of SOD2.

Third, we have measured mitochondria ROS levels in HEK293T cells and found that mitochondrial ROS was reduced by nearly 55% by the overexpression of SIRT3 and by 84% by the coexpression of both SIRT3 and SOD2, while overexpression of Sirt3 in SOD2 knock down cells caused no significant mitochondrial ROS reduction. This result supports that SIRT3 enhances the activity of SOD2 to control the mitochondrial ROS level (new Fig 4A).

Lastly, to provide additional evidence to demonstrate that endogenous SOD2 is regulated by SIRT3 in the proposed way in vivo, we demonstrate a significant increase of endogenous SOD2 after SIRT3 depletion (new Fig 3F). We also demonstrate a physical association of endogenous SOD2 and SIRT3 (new Fig. 2E).

The referees also asked additional, more specific questions that are answered below in the point-by-point response, including additional new experimental results. We

hope that these responses would satisfy the reviewers and that you will find the paper suitable for publication.

We look forward to hearing from you soon.

Point-by-point response

Referee #1

“This manuscript by Chen et al., 2010 "Tumor suppressor SIRT3 deacetylates and activates manganese superoxide dismutase (SOD2) to scavenge ROS" presents results demonstrating present work suggesting that MnSOD is acetylated at lysine 68 and that this specific lysine acetylation directs MnSOD2 activity. This statement is based on data showing that MnSOD lysine 68 is acetylated and can be hyperacetylated when treated with somewhat non-specific agents that pan inhibit most cellular lysine deacetylases. It is also shown that SIRT3 binds to and directs both the acetylation status and dismutase activity of MnSOD. Finally, data is presented that SIRT3 expression is governed by intracellular ROS levels that subsequently regulates MnSOD activity via a post translation modification involving acetylation.

It has previously been shown that SIRT3 directs intracellular ROS levels, immortalization of fibroblasts in vivo and mammary tumorigenesis in vivo, and MnSOD contains an acetyl lysine at position 68 in MnSOD has been previously identified to increase with CR (Schwer et al., Aging Cell 2009). However, the observation that MnSOD is regulated by a specific reversible acetyl lysine that is regulated by SIRT3 has not been previously described, and therefore this manuscript represents a novel finding into understanding the function of SIRT3 in mitochondrial biochemistry and biology.

We appreciate the reviewer’s recognition of the novelty of our study.

“However, there are some issues with the data presented (see below) therefore in its current form the conclusions drawn by the authors are not supported by the experiments. As it stands, this manuscript requires improvement before it can justify the stated conclusions.

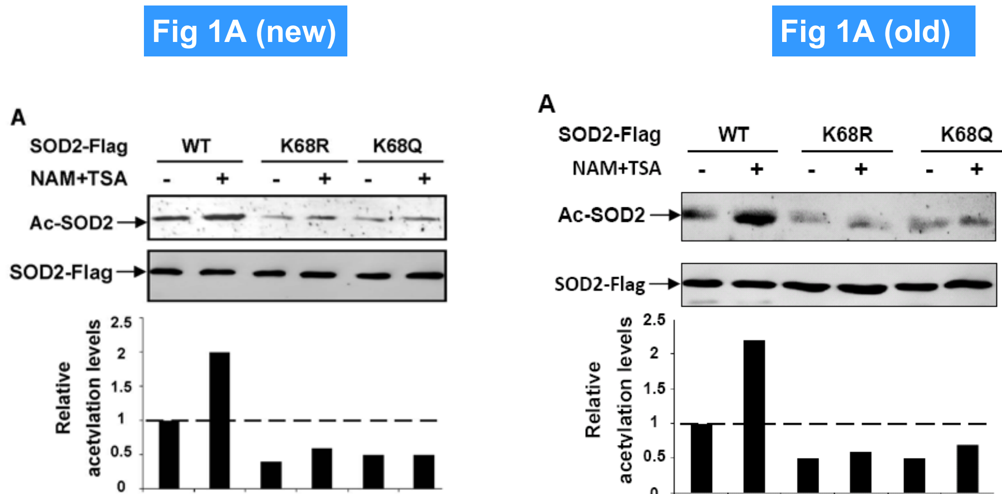
Some general comments in regards to this manuscript: some of the data is of sub-optimal quality that will need to be redone. In addition, some of this data presented has already been published and the authors might consider moving this data into the supplemental section and focus on the new and innovative data. In addition, the figure legends should provide a little more information about how the experiments were done to help the readers follow the results.”

Please see below point-by-point response.

“1. The quality of some the westerns blots in the manuscript are sub-optimal and should be repeated. Specifically, Figure 1A, top panel where the blot is very hazy.”

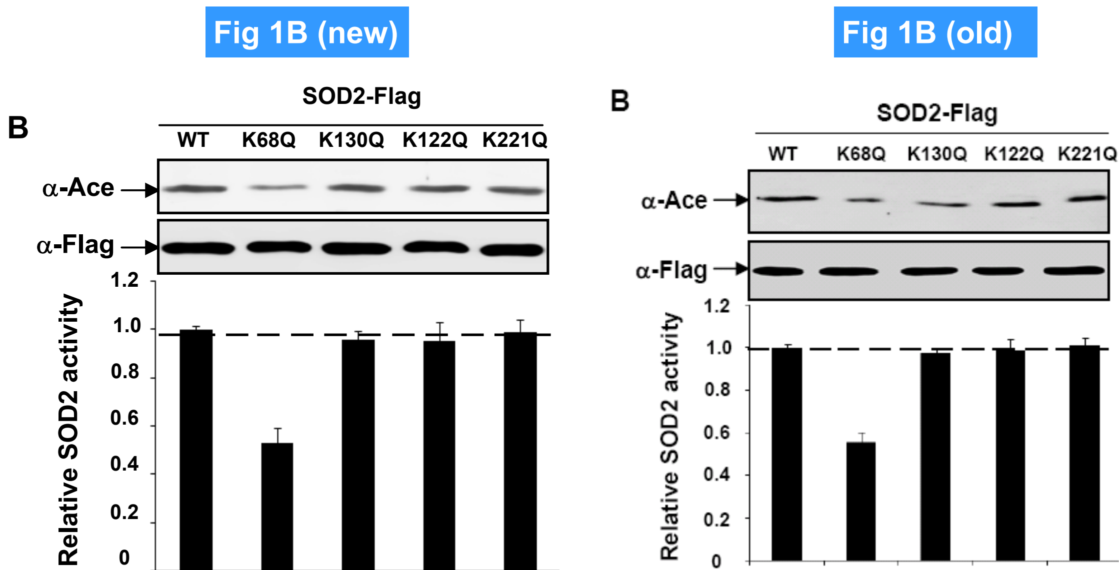
Response:
We have repeated this experiment and obtained a higher quality blot with consistent result that deacetylase inhibitors increase acetylation level of wild type SOD2 but not

K68 mutant SOD2. The new result is included in revised Fig. 1A and also copied below along with the old Fig. 1A for the convenience to review.



“Figure 1B, top panel the differing widths of the lanes prevents determine in changes in acetylation.”

Response:
We have re-run a SDS-PAGE of previously saved lysate and repeated the immunobloting experiment. The re-run produced better result that has replaced the old Fig. 1B and is copied below.



“Figure 2A where it is difficult to determine if there are any changes in immunoreactive protein levels.”

Response:
We have re-run the previously saved lysates of TSA treated cells on SDS-PAGE and performed immunoblotting experiment. The repeating experiment gave rise to a better illustration that there were little changes in the acetylation levels of SOD2 (left panel of new figure). We also quantified this result to draw a clear conclusion that NAM, not TSA, treatment increased SOD2 acetylation levels.

Fig 2A (new)

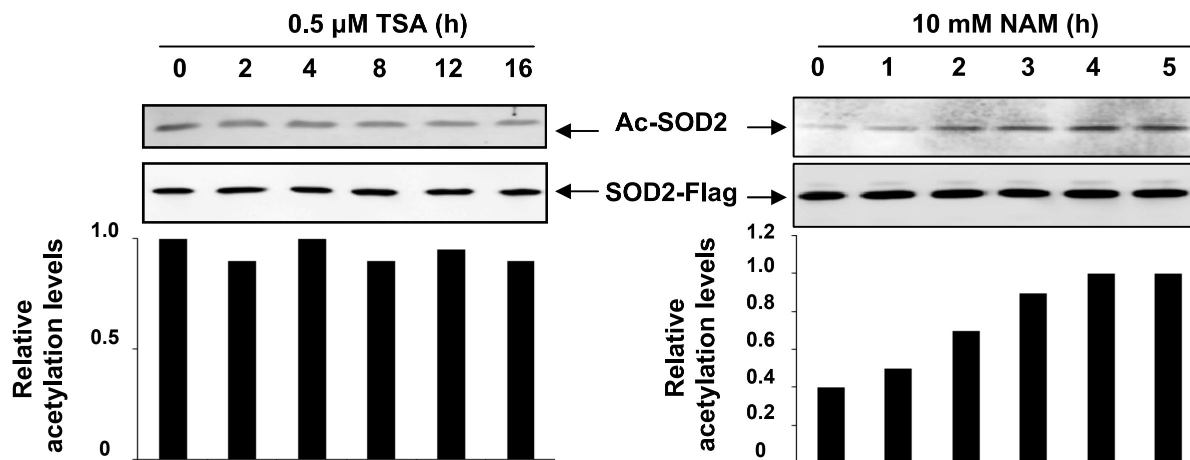
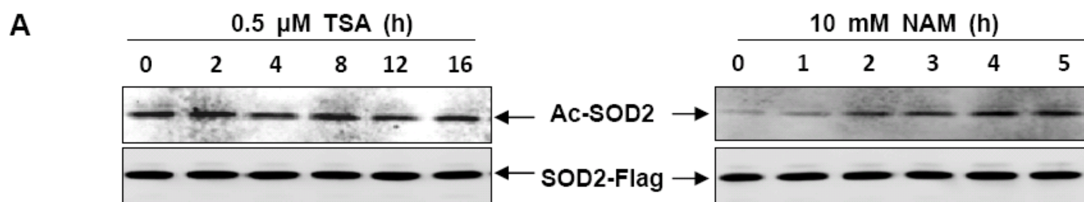
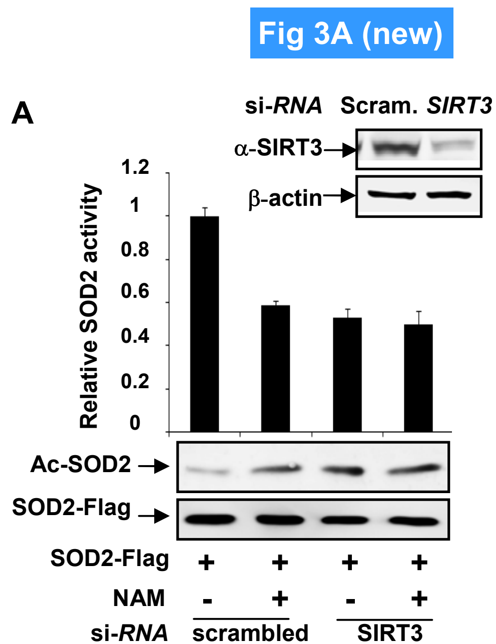


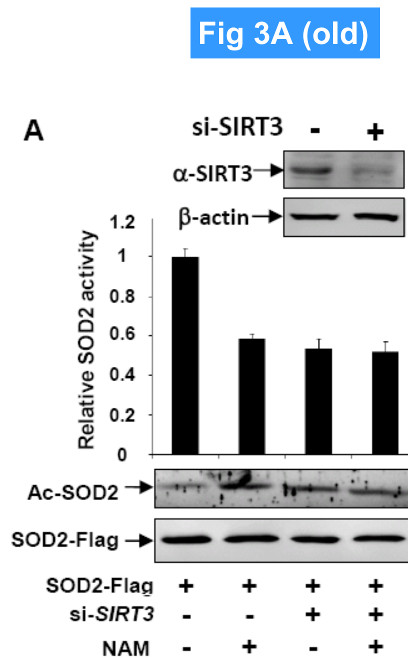
Fig 2A (old)



"Figure 3A contains too much background that prevents clearly identifying protein levels."

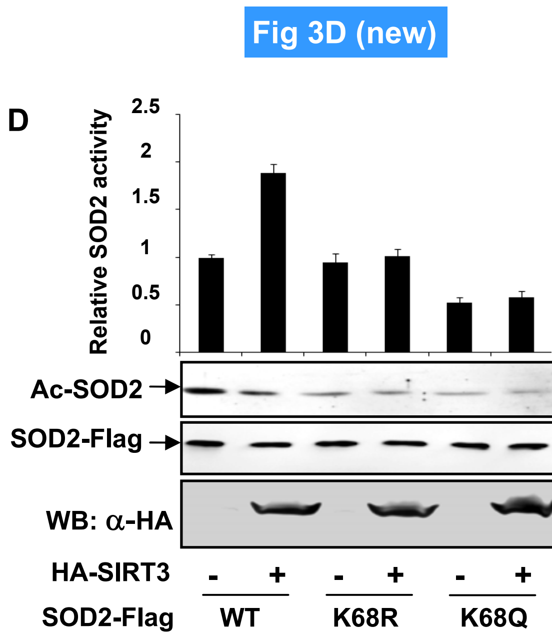
Response:
We have repeated this experiment using previously saved lysate and obtained a result with cleaner background. The old figure is replaced by new data.

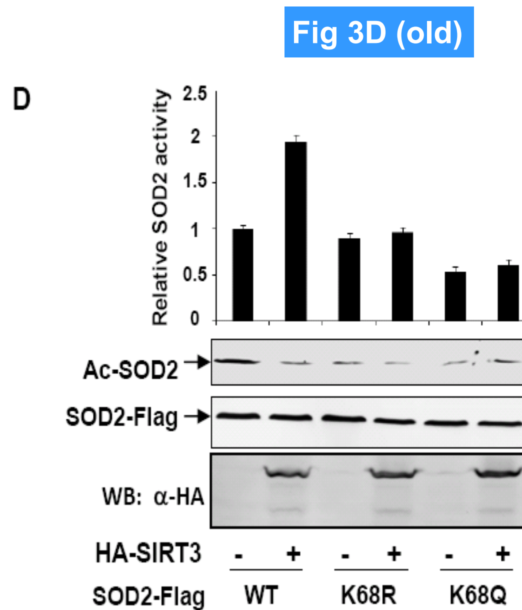




"Figure 3D contains bands with significant variability in band width prevent clear identification of differences."

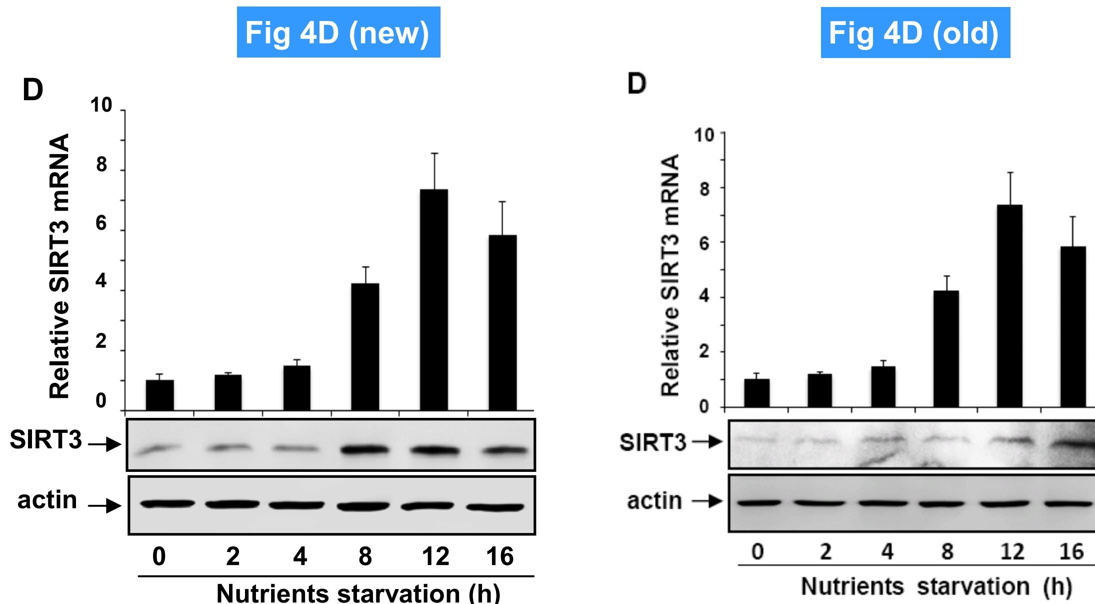
Response:
We have repeated this experiment and obtained the blot with uniform band width and consistent with our previous results.





"Figure 4D, top panel needs to be improved."

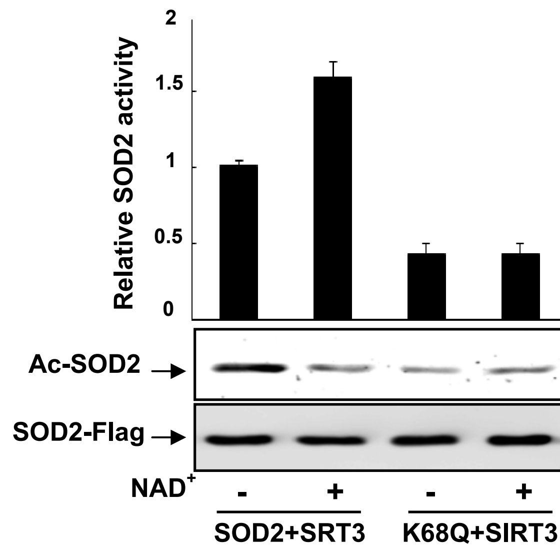
We have re-run the previously saved lysates on SDS-PAGE and performed immunoblotting experiment using anti-Sirt3 and anti-actin antibodies. The repeating experiment produced an image with better quality and the figure is included in revision as Fig. 4D.



" 2. There is no direct data in the manuscript that Sirt3 deacetylates MnSOD lysine 68 and this must be addressed if this manuscript is going to be reconsidered of publication. The only data addressing this issue is shown in the Supplemental Section, Fig. 1a showing mass spec data. However, this data only shows that MnSOD lysine 68 is acetylated and does not show that is changes."

Response:
We have performed three experiments, an in vitro deacetylation assay by SIRT3, in vivo SIRT3 knocking down and iTRAQ experiment to address this critical issue. In the in vitro deacetylation experiment, we ectopically expressed wild-type and K68Q mutant SOD2 in 293T cells, treated transfected cells with NAM (to increase SOD2 acetylation) and immunoprecipitated SOD2 and incubated the SOD2 precipitate with separately immunopurified SIRT3, followed by enzyme assay of SOD2 activity and immunoblotting to determine the SOD2 acetylation. While incubation with SIRT3 resulted in a clear decrease of acetylation and increase of enzyme activity of wild-type SOD2 in a NAD^{+}-dependent manner, SIRT3 exhibited little effect on both acetylation and enzyme activity of K68Q mutant SOD2. This result, which is now included as Fig. 2F in the revised paper, together with the result of SIRT3 knocking down and iTRAQ experiment described below, provide strong support to the conclusion that SIRT3 deacetylates SOD2 at K68.

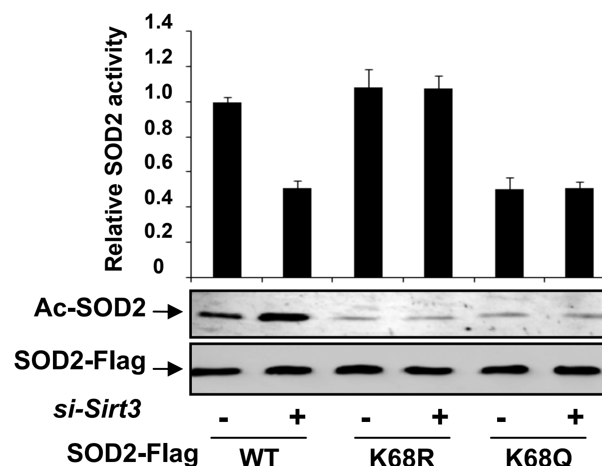
New Fig 2F



"3. The authors do show in figure 3A that siRNA knockdown of SIRT3 decrease MnSOD acetylation as well as decreases MnSOD activity. There are several significant issues with this experiment and panel that is critical to the conclusions made by the authors. First, the quality of the western is sub-optimal and this reviewer finds it difficult to determine if there is an increase in MnSOD acetylation in lanes 3 and 4. In addition, (and most importantly) this experiment only shows that MnSOD acetylation increases (lane 2 is the only lane where a difference is seen) and does not identify the specific lysine. These samples should be removed from the gel and mass spectrometry must be done to show the specific lysine that is hyperacetylated when SIRT3 is decreased by siRNA."

Response:
To address these issues, we first repeated the immunoblotting experiment using previously saved lysate and obtained improved image (see new Fig. 3A shown above). Next, we performed a knocking down experiment to seek an additional support for SIRT3-mediated deacetylation at K68 of SOD2. We found that in cells depleted for SIRT3 by siRNA, the acetylation level of wild-type SOD2 was increased and the enzyme activity of SOD2 was decreased, but neither the acetylation nor the enzyme activity of both K68Q and K68R mutants SOD2 was affected (see newly added Fig. 3E).

New Fig 3E

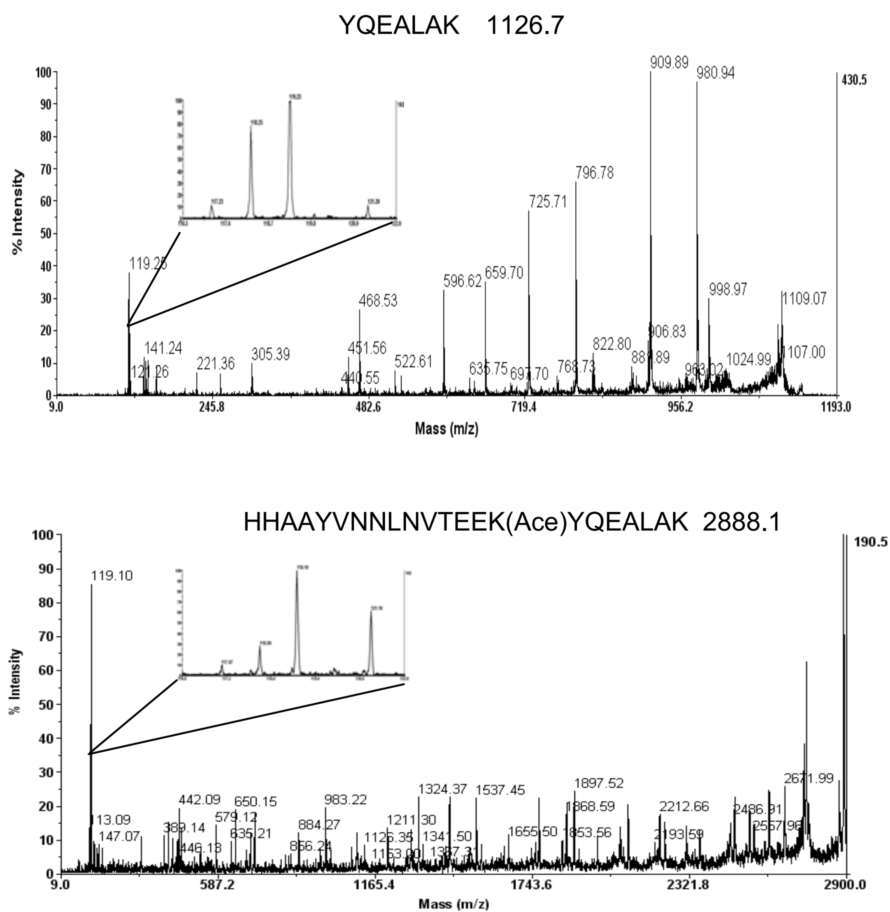


At last, we gel-purified SOD2 immunopurified from cells singly transfected, co-transfected with SIRT3 and with siRNA targeting SIRT3, and performed quantitative mass spectrometric analyses (i-TRAQ). This experiment indicates that the ratio of K68-unacetylated vs. K68-acetylated peptides was increased by the coexpression of SIRT3 and conversely decreased by the SIRT3 knocking down (see newly added Fig. 3G and Fig. S2). These new results demonstrate that SIRT3 directly deacetylates K68 of SOD2.

New Fig 3G

SOD2 peptide sequences	**Relative peptide abundance (%)**		
	SOD2	SOD2 + SIRT3	SOD2 + siSIRT3
YQEALAK	45.8 ± 1.5	64.3 ± 1.6	12.3 ± 1.1
HHAAYVNNLNVTEEK(Ac)YQEALAK	54.2 ± 1.5	35.7 ± 1.6	87.7 ± 1.1

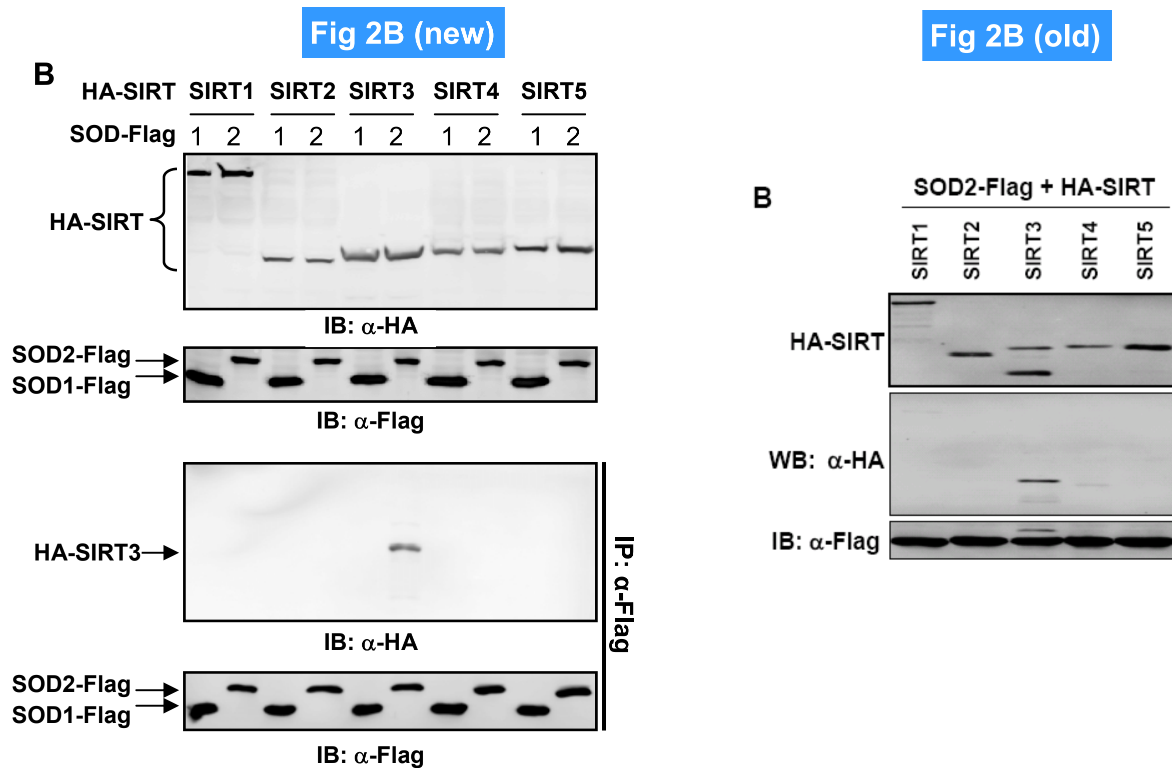
New Fig S2



"4. In the panels in figure 2 the investigators have failed to re-blot with MnSOD for loading control. Or for the IP western experiments the samples must be divided into

equal fractions and determine equal amounts of IPed protein. In addition, for these experiments the authors must do western blotting to determine that there are equal amounts of total MnSOD and SIRT3 in the cell transfected with the different Sirtuin (T1-T7) cells and determine the difference between endogenous and exogenous MnSOD and SIRT3."

Response:
We have repeated this experiment and verified that similar amounts of SIRTs and MnSOD were expressed in cells. A control panel is added to show that equal amount of immunoprecipitated MnSOD was used to detect its binding to SIRT. SOD1, which showed no binding to any of 5 SIRTs tested, was also included as an additional control. The new result supports our previous conclusion that MnSOD binds specifically to SIRT3 and is included in the new Fig. 2B.



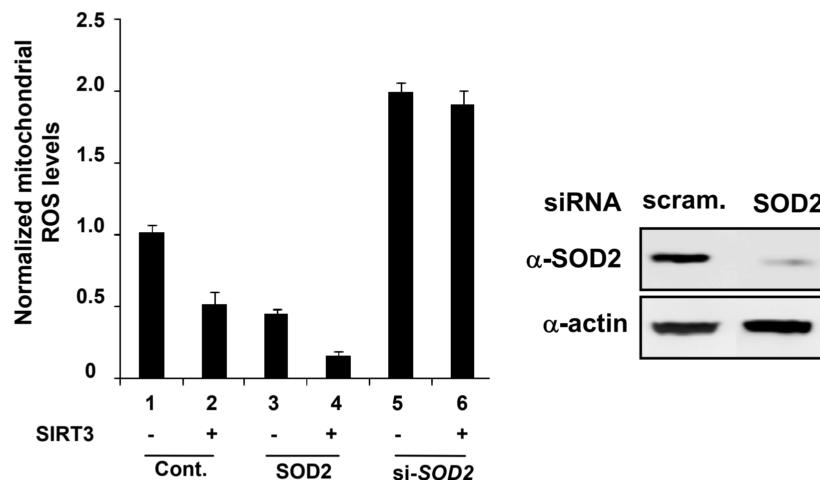
"5. Figure 2B, bottom panel the y axis states "Relative SOD activity"? Is this correct? The figure legend states "K68 is the major site of SOD2 acetylation. Flag tagged wild-type and mutant SOD2 were expressed in HEK293T and affinity purified" suggest the y axis should be total MnSOD acetylation."

Response:
The reviewer was likely referring to Figure 1B. Yes, the Y-axis was labeled correctly as "Relative SOD activity". We have modified the figure legend to avoid confusion.

“6. There are several issues that need to be addressed for the data panels presented in figure 4 since this is critical to the authors conclusions. First, figures 4A and 4B have been previously published in a similar or in some cases using much cleaner models that siRNA showing that SIRT3 determines and directs intracellular ROS levels. In addition, figures 4A and 4B are measuring ROS levels and this is no specific enough for this work. The authors are suggesting changes in the regulation of MnSOD that converts superoxide to hydrogen peroxide. Thus, the correct experiments to do are the determination of mitochondrial superoxide levels.”

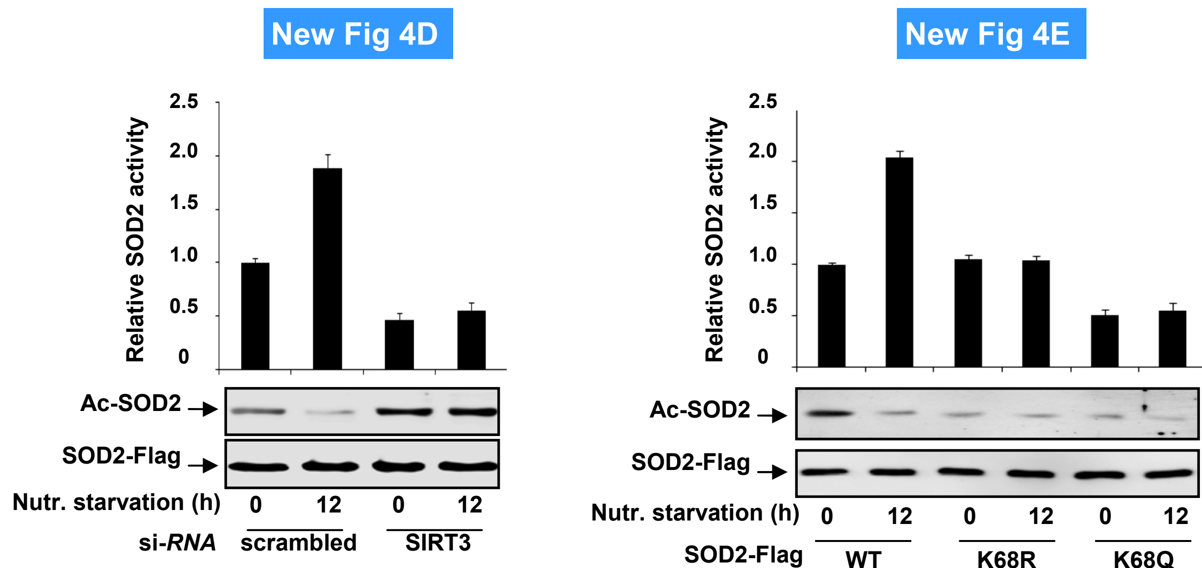
Response:
Following the reviewer’s suggestion, we moved old Fig. 4A and 4B into supplemental data. Furthermore, we have measured mitochondria ROS levels in HEK293T cells with either SOD2 ectopically expressed or knocked down and in combination with SIRT3 coexpression. We found that mitochondrial ROS was reduced by near 55% by the overexpression of SIRT3 and by 84% by the coexpression of both SIRT3 and SOD2, while overexpression of Sirt3 in SOD2 knock down cells cause no significant mitochondrial ROS reduction. This result supports that SIRT3 enhances the activity of SOD2 to control the mitochondrial ROS level. The new result is used as Fig. 4A in the revised manuscript.

New Fig 4A



“7. The experiments in figures 4C-F are not specific and fail to directly connect SIRT3 and MnSOD activity. In addition, these experiments do not connect lysine 68 to the changes in MnSOD activity and this should be addressed. These experiments show that treatment with: (1) nutrient deprivation and DNMQ alter MnSOD acetylation; and (2) nutrient deprivation and DNMQ alter MnSOD alters MnSOD activity however, this does not a priori show that nutrient deprivation and DNMQ changes in MnSOD directly directs MnSOD activity since these treatments may be working via other potential pathways either in combination with SIRT3 and/or via another signaling factor. Control experiments must be added to make a more rigorous connection between these conditions, SIRT3, and MnSOD activity.”

Response:
To address these issues, we first performed an experiment to show that nutrient deprivation does not affect SOD2 acetylation level and activity when SIRT3 is knocked down (new Fig 4D). Next, we compared the acetylation levels and relative enzyme activities of wild-type SOD, K68Q and K68R mutants under normal and nutrient deprivation conditions. We found that nutrient deprivation deacetylates and activates wild type SOD2, but had no effect on either acetylation levels or activities of K68Q and K68R mutants (new Fig 4E). These two lines of evidence linked nutrient deprivation effect on SOD2 activity to SIRT3 and lysine 68 of SOD2.



"In closing, the authors present a very novel finding that are very strongly supported by this reviewer and this work is potential extremely important to both the fields of sirtuins and MnSOD mitochondrial biology. However; the manuscript required the addition of significantly more control data before it can justify the stated conclusions. In addition, the data identifying MnSOD lysine 68 in this process need additional data that will require either Mass Spectrometry data or a MnSOD lysine 68 specific antibody."

Response:
We are grateful for the reviewer's appreciation and constructive suggestions and hope that the extensive new experiments have addressed the reviewer's concerns satisfactorily.

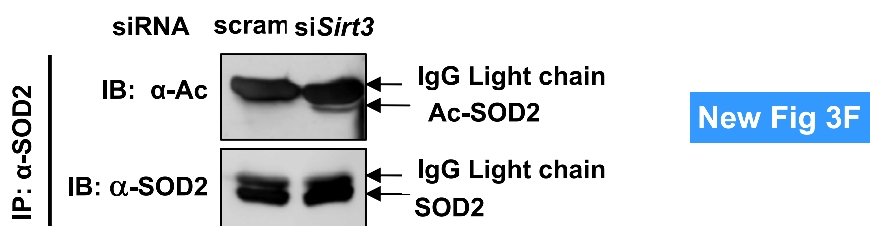
We have previously immunized two rabbits, but unfortunately both failed in producing anti-SOD2(K68)-acetylated antibody. Instead, we focused our efforts during the revision on using quantitative mass spectrometry technology (i-TRAQ) and provided more biochemical data to demonstrate that acetylation at K68 is critically important for the regulation of SOD2 activity by SIRT3 as described in detail above.

Referee #2:

Chen et al. follow the role of the mitochondrial deacetylase SIRT3 on SOD2 activity. The authors showed that when overexpressed in HEK293 cells, SOD2 is acetylated. SOD2 deacetylation by SIRT3 promotes its enzymatic activity which potentially results in a decrease in ROS levels. Therefore the findings might be relevant to the potential role of SIRT3 in longevity. This is an interesting manuscript that increases our knowledge on SIRT3. The following points should be addressed:"

"All of the experiment with SOD2 are done with tagged overexpressed protein. The authors must present an in vivo evidence that SOD2 is a substrate for SIRT3. My suggestion is to perform IP with Ab against Ace and western with Ab against SOD2 in SIRT3 KO vs. wildtype cells and in SIRT3 overexpression vs. wild type cells."

Response:
To determine the effect of SIRT3 on the acetylation level of SOD2 in vivo, we immunoprecipitated endogenous SOD2 from 293T cells transfected with either scrambled siRNA or siRNA targeting SIRT3, followed by immunoblotting with anti-acetyllysine antibody. This experiment demonstrates a significant increase of endogenous acetylation of SOD2 after SIRT3 depletion and the data is presented in new Fig 3F (and included below).



New Fig 3F

"The role of deacetylation in inhibiting SOD2 is not clear. The K to R mutation that mimics deacetylated SOD2 should show in increase in SOD2 activity. Yet in figure 3c, the activity of K68R is not higher than wild type."

Response:

The result that enzyme activity of K68R mutant is not higher than the wild-type SOD2 likely reflects that the stereo structure of K68, in addition to serving a negative regulatory function as the site of acetylation, may also play an important role for SOD2 catalytic activity. In other words, the structural alteration caused by the K68R mutation reduces the catalytic activity loss of SOD2 that offsets the gained catalytic activity caused by disruption of acetylation at this position.

"The experiments were done in 293/T only. It is not clear how broad the phenomenon is. The authors should repeat some of the experiment in another cell line."

Response:
We have previously done similar experiments in HeLa (Figures 4C, 4D and 4E, and also Supplement Figure 3) and got results.

"The title is confusing, the findings of this paper are about cancer. Therefore, please delete the words tumor suppressor."

Response:
Genetic analysis of *Sirt3* mutant mice has recently demonstrated that it functions as a tumor suppressor (Kim et al. 2010 *Cancer Cell* 17:41-52). Our study demonstrates that SIRT3 is a major SIRT that deacetylates and activates SOD2 in the removal of ROS. Since increased ROS is closely linked to tumorigenesis, we think that including the term 'Tumor suppressor' is appropriate.

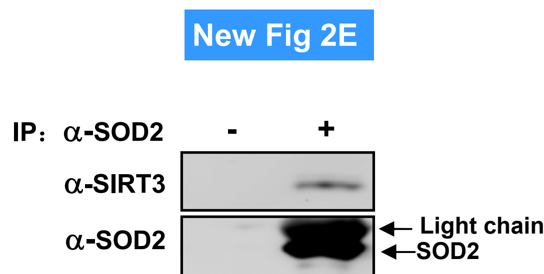
"In figure 3A, an additional siRNA sequence against SIRT3 and unspecific siRNA should be included."

Response: Yes we did use a scrambled siRNA as control, and we have changed the label of newly made Fig 3A.

"Please show an endogenous IP between SIRT3 and SOD2"

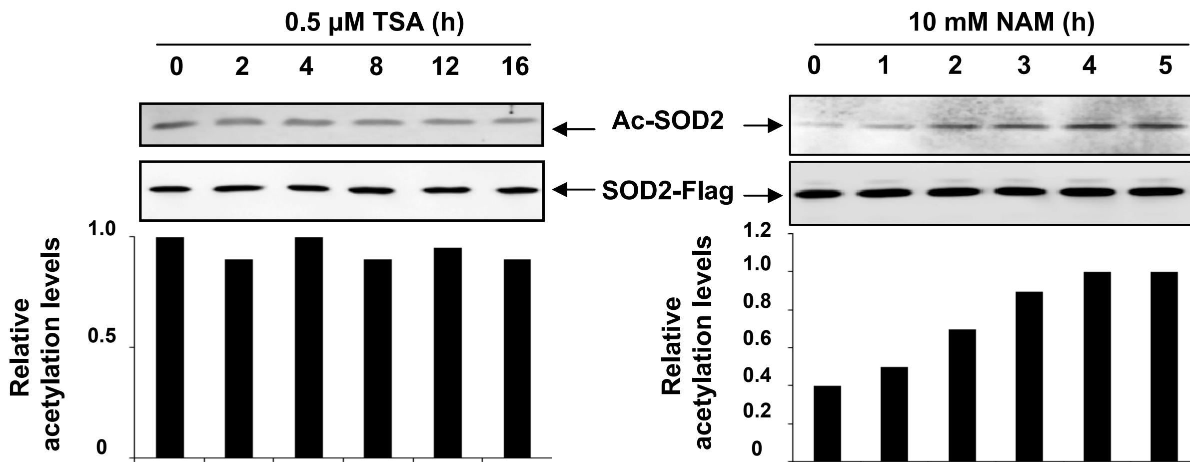
Response:
We used anti-SOD2 antibody to immunoprecipitate endogenous SOD2 and detected that association of SIRT3 in SOD2 immunoprecipitate. The new data is included as Fig. 2E in revised manuscript (copied below).

New Fig 2E



"Minor:
Please show the levels of SOD2 acetylation in cells treated with TSA or NAM only."

Response:
This data is presented in Fig. 2A of revised figures.

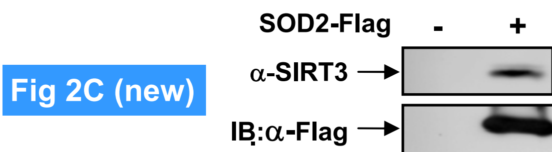


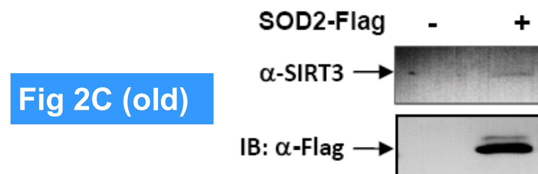
"Figure 1B. A reference to material methods on the manner of SOD activity measured is missing from the results section"

Response:
We have added it.

"Figure 2C. The indicated band of endogenous SIRT3 by IP may be slightly too weak"

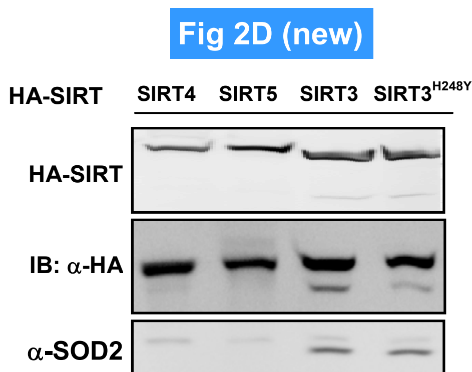
Response:
We have repeated this experiment and obtained a better result.

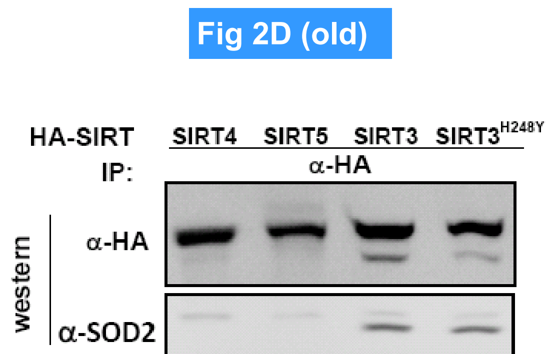




"Figure 2D, please include a western analysis of the input."

Response: The input western blotting is added





"Figure 3A. When treated by NAM only, the level of acetylated SOD2 is slightly stronger than in cells expressing siRNA for SIRT3 only. This may imply that other sirtuins (SIRT5?) also deacetylates SOD2 in the absence of SIRT3."

Response:
We agree with the referee, that other mitochondrial Sirtuin such as SIRT5 could also deacetylate SOD2, but want to focus on SIRT3 in this paper.

"Figure 4C. After 4 h, the level of Ac-SOD2 decrease but its activity is the same as the 0 and 2h. Could it be that another unknown PTM regulates is regulates SOD2 activity after 8h?"

Response: We have re-run the SDS-PAGE of previously saved lysate and performed immunobloting experiment using anti-acetyllysine twice. The new result demonstrates a similar kinetics between deacetylation and increase of enzyme activity and is included as Fig. 4C in the revision.

Fig 4C (new)

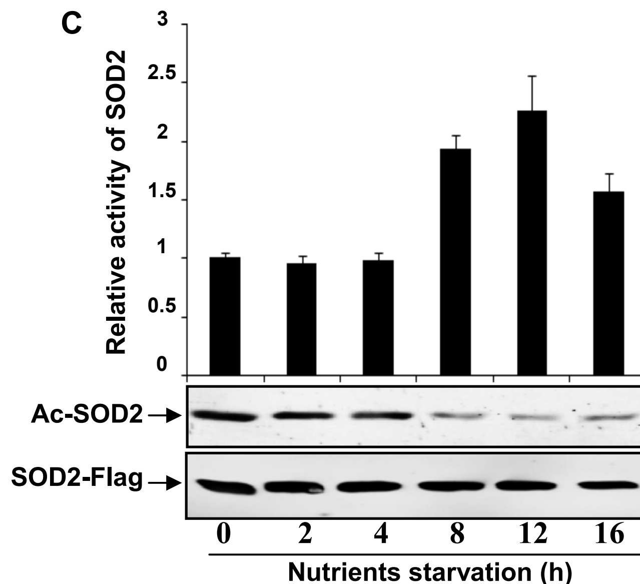
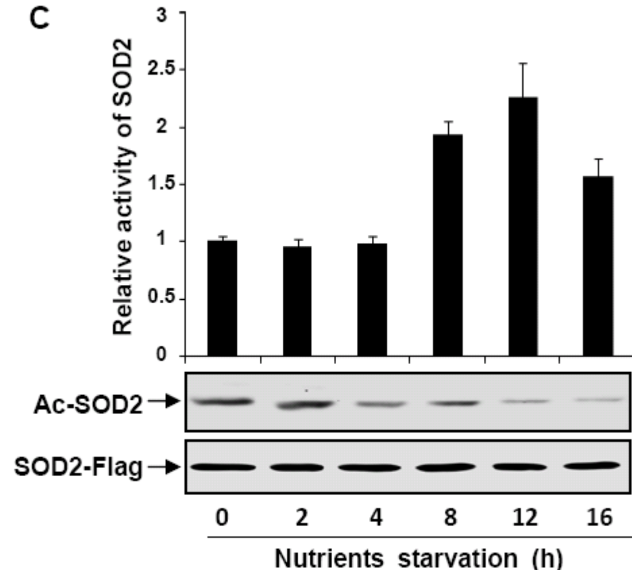


Fig 4C (old)



"Figure 2A legend; change SA to TSA."

Response:
We have corrected this mistake.

Referee #3:

Dr. Chen and colleagues have performed a full factorial designed study to identify and characterize the role of SIRT3 in deaceylating and activating the mitochondrial superoxide dismutase SOD2 via direct deacetylation of lysine68.Using protein protein interaction studies, pharmacologic and siRNA inhibition of protein function and biochemical assays the authors show convincingly that SOD2 is a direct substrate of SIRT3 and this deacetylation activated SOD2 activity with a blunting of cellular ROS levels.

"Do the authors have a reason why the acetylation resistant arginine mutation does not show high intrinsic SOD activity?"

Response:
The result that enzyme activity of K68R mutant is not higher than the wild-type SOD2 likely reflects that the stereo structure of K68, in addition to serving a negative regulatory function as the site of acetylation, may also play an important role for SOD2 catalytic activity. In other words, the structural alteration caused by K68R mutation reduces the catalytic activity loss of SOD2 that offsets the gained catalytic activity caused by disruption of acetylation at this position.

"Page 3, 3 lines from the bottom, should the final word on this line not be mitochondria instead of cytosol?"

Response:
It should be "in the cytotosol and mitochondria", and we have made changes in the revised text.

"Page 5, 6 lines from the bottom - should it read treatment of cells with TSA and NAM instead of TSA and NAD?"

Response:
It should be "TSA and NAM". We have corrected it.

"Which antibody was used to show the change in acetylation of SOD2, or was this the aceylated lysine band the correlated in MW to where SOD2 should run?"

Response:
It is our home made anti-acetyllysine Ab which was used in our previously published work (Zhao et al., *Science* 327, 1001-1004).

" The authors state that a role of SIRT3 in the oxidative stress response has not been directly shown, however, a paper has recently been published in Free Radical Biology and Medicine in this regard by Bao J et al. This should be stated."

Response:

We have cited this paper.

"Fig 2B. Does the IB-alpha Flag mean that the proteins were immunopreciptated with FLAG to show the protein protein interaction?"

Response: We missed one label "IP: α-Flag" to the left of the figure and have added to the revised Figure to avoid confusion.

2nd Editorial Decision 24 February 2011

Thank you for the submission of your revised manuscript to our offices. We have now received the enclosed reports from the referees that were asked to assess it. I am happy to tell you that both referees now support publication of your study in our journal.

Referee 1 only feels that the two recent publications on SIRT3 and MnSOD (Qiu et al, 2010; Tao et al., 2010) should be cited and, more importantly, that the differences between your results and the two other papers should be discussed (identification of different modified Lys residues and different consequences on MnSOD activity). I agree with this referee that such a discussion is very relevant and would ask you to include it in the final version.

On a more formal note, and as I have also indicated previously, with its 35,300 characters, the manuscript currently exceeds our length restrictions and I would kindly ask you to go through the text one more time to identify passages that might be shortened so that in the end, the manuscript does not contain more than 28,000 characters (including spaces and references). You would also need to make some space for the additional discussion as requested by referee 1. While basic methods and materials may be displayed in the supplementary section to free up space, methods and materials required for the repetition of the key experiments may not be displayed in the supplementary section only.

As I also indicated in one of my last emails, please provide information on how many times each experiment has been repeated. To ensure statistical significance, each experiment should ideally be repeated at least three independent times. If an experiment has only been done twice, it also does not make much sense to add error bars to the graphs.

I look forward to seeing a new revised version of your manuscript as soon as possible.

Please do not hesitate to contact me if you need any further information or help in preparing your final version.

I would like to thank you again for your cooperation during the review process and congratulate you on your study, which is very much improved.

Yours sincerely

Editor
EMBO reports

REFEREE REPORTS

Referee #1 (Remarks to the Author):

This manuscript by Chen et al., 2010 "Tumor suppressor SIRT3 deacetylates and activates manganese superoxide dismutase (SOD2) to scavenge ROS" presents results demonstrating MnSOD is deacetylated at lysine 68 by SIRT3 and this acetylation directs MnSOD2 activity. The authors have done an excellent job of address the concerns of the reviewers in this revision that were done by added multiple new experiments as well as repeating many experiments as well. Overall this manuscript is much improved.

However, some small additions to the text would improve this manuscript. In this regard, the authors really should address the two recent articles that were recently published in Cell Metabolism (Qiu et al., 2010) and Molecular Cell (Tao et al., 2010) that also show that Sirt3 deacetylates MnSOD. Specifically, I think the reviewers should address the differences in the specific lysines identified in each manuscript that for the Qui manuscript was lysines 53 and 89 and for the Tao manuscript lysine 122. In addition, this manuscripts suggest that these lysine activate enzymatic activity when mutated

to an arginine while these Chen et al., suggests that lysine 68 has a negative effect on enzymatic activity. I think such a discussion would be very well received by the field and would most certainly add to this manuscript. In addition, the PIs might discuss the idea that specific lysines may positively and negatively direct activity and these results match those seen for kinase / phosphorylation.

Referee #2 (Remarks to the Author):

The authors adressed most of my comments and I am quite satisfied with the revised manuscript.

2nd Revision - authors' response 15 March 2011

Attached is the final version that has included the important section of Methods as well as modified discussion regarding the other two papers on SIRT3-SOD2.

The total characters are under 30,000 and I am very grateful for the 2,000 characters space you offered-they were very much needed.

Thanks again for the efforts in editing our paper.

3rd Editorial Decision 15 March 2011

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

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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Finally, we provide a short summary of published papers on our website to emphasize the major findings in the paper and their implications/applications for the non-specialist reader. To help us prepare this short, non-specialist text, we would be grateful if you could provide a simple 1-2 sentence summary of your article in reply to this email.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Editor
EMBO reports