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SIRT3-dependent deacetylation exacerbates acetaminophen hepatotoxicity

Zhongping Lu, Mohammed Bourdi, Jian H. Li, Angel M. Aponte, Yong Chen, David B. Lombard, Marjan Gucek, Lance R. Pohl and Michael N. Sack

Corresponding author: Michael N. Sack, National Institutes of Health

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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 18 February 2011

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, although all the referees find the topic of interest and in principle suitable for us, referees 2 and 3 consider the study preliminary for publication here at this stage. All of them request a number of technical improvements of the data, and the last two referees consider that further experiments are needed to provide convincing support for your model of the role of SIRT3-mediated deacetylation in acetaminophen toxicity.

Given that all referees provide constructive suggestions on how to make the work more conclusive, I would like to give you the opportunity to revise your manuscript. If the referee concerns can be adequately addressed, we would be happy to accept your manuscript for publication. However, please note that it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

Revised manuscripts must be submitted within three months of a request for revision unless previously discussed with the editor; they will otherwise be treated as new submissions. Revised manuscript length must be a maximum of 28,500 characters (including spaces). When submitting your revised manuscript, please also include editable TIFF or EPS-formatted figure files, a separate PDF file of any Supplementary information (in its final format) and a letter detailing your responses to the referees.

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. In the meantime, do not hesitate to get in touch with me if I can be of any assistance.

With best wishes,

Editor
EMBO Reports

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The authors investigated the role of the mitochondrial deacetylase SIRT3 in the pathogenesis of APAP-induced liver injury. The authors observed reduced liver injury in SIRT3-/- mice and identified a number of mitochondrial proteins that were acetylated in SIRT3-/- mice. One of the proteins, ALDH2, was further characterized as a SIRT3 substrate and as a target for NAPQI binding and inactivation. The authors concluded that protein deacetylation during APAP exposure may provide additional binding opportunities for NAPQI and thus enhances toxicity.

Overall, this is a well designed study providing interesting and highly novel findings. Most of the conclusions are justified by the data provided. A few issues need to be addressed:

1. The authors argue that SIRT3 activation opens more possibilities for NAPQI protein adduct formation. NAPQI protein binding is considered a very early event in the pathophysiology (30 min - 2 h with a dose of 300 mg/kg)). However, "beneficial" effects of SIRT3-deficiency are only observed between 8 and 24 h. How do the authors explain this delay in effect?

2. Supplemental Figure 3: the discussed data on mitochondrial respiration were not provided and could not be evaluated.

3. Supplemental Figure 5: The GSH and GSSG values are questionable. The values provided for fed mice (15 nmol GSH/mg liver protein) should be between 45 nmol/mg (8 µ mol/g liver) in the evening and 65 nmol/mg (12 µmol/ g liver) in the morning. For fasted mice these levels are around 25 nmol/mg protein (4-5 µmol/g liver). In addition, the GSSG values are way too high. In general, the hepatic GSSG content of a normal mouse liver is around 0.5% of GSH (GSH/GSSG ratio: 200). The authors need to re-evaluate their measurements. They underestimate the glutathione levels and have serious oxidation during work-up of the samples.

Referee #2 (Remarks to the Author):

In this manuscript, Zhongping et al. report that SIRT3, an NAD+ dependent class III HDAC, deacetylates mitochondrial aldehyde dehydrogenase 2 (ALDH2), and SIRT3 knockout mice are protected from acetaminophen (APAP) hepatotoxicity. The authors further show that deacetylation increases APAP toxic metabolite binding on ALDH2 and inhibits its enzymatic activity.

Their identification and characterization of a novel SIRT3 target, ALDH2, is of significant importance and reveals a cross-talk between mitochondrial posttranslational modifications in therapeutic drug administration. While the observations described are of interest, the results presented are preliminary and the following points need to be addressed before publication.

Major Points

1. The authors claim that acetylation of ALDH2 competes with a modification by a toxic product of APAP, N-acetyl-p-benzoquinoneimine (NAPQI), at the same residue. However, they do not mention which lysines are modified by acetylation or NAPQI and do not substantiate the claim that the two modifications are indeed competing for the same residue. Identification of the sites of acetylation and modification should be conducted by mass spectrometry and mutation of the relevant site(s)

should be studied in detail, including enzymatic activity assays (Fig 3A), and NAPQI modification assay (Fig. 4A).

2. The data presented do not fully convince that SIRT3 is important in APAP toxicity. The authors should show (1) acetylation status of ALDH2 after 8 or 24 hour APAP treatment. (2) enzymatic activity of ALDH2 after 8 or 24 hour APAP treatment as well as short-term (1 hour) APAP treatment (Fig 3A).

3. The authors mentioned another target for SIRT3 which is responsible for acetaminophen induced liver injury in figure 4C, despite the fact that ALDH2 knockdown completely overcomes SIRT3 knock-out in acetaminophen induced liver injury (Fig 3E). Additional discussion is necessary if they claim SIRT3 has multiple targets.

Minor Points

1. Fig 1A: The authors show ALT level after overnight fasting. Please show that in fed condition as well. Also, please clarify time course of these drug treatments.
2. Fig 1B: Histology is not sufficient to quantify necrosis. Additional quantitative experiments will strengthen authors' conclusion.
3. Fig 2C: Please show Input SIRT3.
4. Fig 3A: Is fasting preceded by APAP treatment in this experiment? Please describe experimental conditions.
5. Fig 3A and 3B: Long-term (8 or 24 hour) APAP treatment is necessary.
6. Fig3F: Histology is not sufficient to quantify necrosis. Additional quantitative experiments are required.
7. Fig 4A and 4B: SIRT3 knockdown is necessary in these experiments.

Referee #3 (Remarks to the Author):

In this manuscript, Lu et al. set out to solve the paradox where fasting or CR exacerbates the toxicity of APAP. They identified ALDH2 as a novel SIRT3 substrate and proposed an interesting model in which acetylation and NAPQI antagonistically regulate ALDH2 activity. Fasting induces SIRT3 mediated deacetylation of ALDH2, leading to increased NAPQI binding. Binding of NAPQI in turn reduces ALDH2 activity, which contributes to APAP induced liver injury. The finding in this manuscript are novel, and the experiments clearly presented, including solid data from both in vitro and in vivo experiments that overall support the author's hypothesis. Nevertheless, there are few concerns that the authors need to address for the manuscript to warrant publication.

Major comments:

- Since a major mechanism proposed to exacerbate AILI during fasting is depletion of reduced gluthathione, it is surprising that the authors find no difference between WT and SIRT3 KO animals (mainly when their phenotype appears to depend on the presence of reduced levels of 4-HNE, indicating less oxidation). The authors should comment about these results in the Discussion, in the context of their "protective" phenotype. For instance, is there an alternative pathway that ALDH2 is regulating? Is the NAD/NADH ratio altered, which will indicate this pathway as the dominant one in their phenotype? It is clear from their ALDH2 activity assay that they do observe less capacity to reduce NAD in vitro in the SIRT3 deficient extracts, but they should measure NAD/NADH ratio in vivo, as they did for GSH/GSSG.

- A major oversight in the manuscript is that the entire model for fasting induced liver toxicity is based on the assumption that increased SIRT3 activity upon fasting causes decreased acetylation of ALDH2, which in turn will enhance NAPQI binding. The authors did not show that, indeed, fasting decreases ALDH2 acetylation in WT livers, a major point to support their hypothesis.

- Although the effect of ALDH2 knockdown on liver ALT production seems clear, this could be an indirect effect on liver toxicity, given the known protective effects of ALDH2 against oxidation. A more rigorous assay will be to test whether these SIRT3-/- ALDH2 knockdown cells exhibit more production of 4-HNE, similar to WT, an experiment that should be easy to perform.

- In Figure 4, the authors nicely show that APAP adducts bind to ALDH2, and treatment with nicotinamide, which increases ALDH2 acetylation, diminish binding. In support of their in vivo results, it will be reassuring to see that the SIRT3 deficient livers' samples (for which they already performed nice ALDH2 IPs, as shown in Fig.2) also exhibit diminished binding of APAP adducts.

- In the in vitro deacetylation assay (Fig.2D), the authors might include SIRT3HY (already used in another experiment) and SIRT5, another mitochondrial Sirtuin with deacetylation activity, as negative controls, to confirm that the effect they see is specific for SIRT3 (of note, fasting is known to modulate other sirtuins as well, therefore such additions will be excellent controls for their model).

- Fig 1A shows that at 8 hrs of APAP, there is no difference in ALT between SIRT3 WT and KO liver samples. Yet, Fig 3E shows significant differences between the same samples (shScr). The authors should explain this discrepancy.

Minor comments

- There are multiple grammar mistakes throughout the manuscript. The authors should have their manuscript carefully edited before resubmission. Some examples: on page 3, the sentence "is, to our knowledge, has not been investigated", is grammatically incorrect. Same page "in the context of acetaminophen induced liver injury (AILI), may be is due in part, to protein lysine residue..." is also grammatically improper. On page 9, end of Discussion ""ALDH2 is as a functional substrate"

1st Revision - authors' response 10 May 2011

Reviewer 1, Comments:

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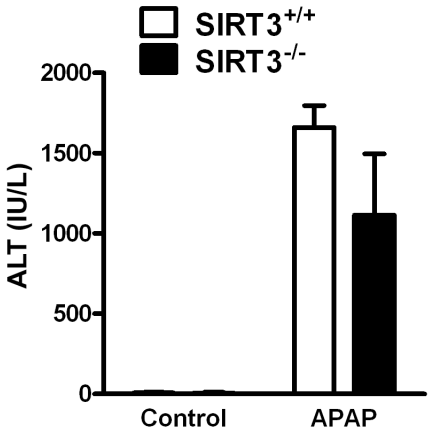
The systemic markers of the acute liver injury in response to an acute toxic dose of acetaminophen are usually measured by hepatocyte release of ALT into the circulation. The temporal release of ALT into the circulation relative to the binding of toxic adducts to hepatic proteins has previously been defined (Roberts et al, 1991), and shows that adduct binding peaks within the first four hours and that the hepatocytes exposed to these adducts then undergo cell death. The release of ALT into the circulation reflects this cell death and begins to rise at about 4 hours and peaks between 24 to 48 hours depending on the numbers of hepatocytes killed. This has now been more clearly delineated in the discussion in emboldened text – final paragraph on page 8 and the beginning of page 9.

The early beneficial effects noted in the SIRT$^{-/-}$ mice include the attenuation of 4-HNE adducts binding to mitochondrial proteins, already seen at 1 hour following APAP administration and the new data showing that the knockdown of ALDH2 in the SIRT3$^{-/-}$ mice reverse this ameliorative effect (New Figure 3F). In parallel, we also assayed ALDH2 activity at 4 hours and show similar results to what we found at 1 hour with higher enzyme activity in the SIRT3 null mice. This is included in the revised Figure 3A. Additional data to support the early benefit is the relatively better maintained mitochondrial respiration in the knockout mice. This is measured at 4 hours and is shown as Supplemental Figure 3. We also repeated the APAP toxicity study after an overnight fast to assay ALT levels 4 hours after the administration of APAP. This data is not included in the final report as it mirrors the 8 hour data with only a trend to lower ALT levels in the knockout mice. These data at 4 hours does, however, show the incremental rise in ALT levels, with levels between baseline and 8 hours post-injury, and are shown here for comparison (see next page). Hence, in line with the pathophysiology described above, we propose that these ameliorative early events are also reflected in the attenuation of the later biomarkers of acute acetaminophen induced hepatotoxicity.



Serum ALT levels in mice comparing APAP to vehicle control in fasted mice. The levels were drawn 4 hours after intraperitoneal APAP administration.

2. Supplemental Figure 3: the discussed data on mitochondrial respiration were not provided and could not be evaluated.

We apologize for this. The incorrect figure was inserted into the supplement and this has now been corrected in the revised Supplemental Figure 3.

3. Supplemental Figure 5: The GSH and GSSG values are questionable. The values provided for fed mice (15 nmol GSH/mg liver protein) should be between 45 nmol/mg (8 µ mol/g liver) in the evening and 65 nmol/mg (12 µmol/ g liver) in the morning. For fasted mice these levels are around 25 nmol/mg protein (4-5 µmol/g liver). In addition, the GSSG values are way too high. In general, the hepatic GSSG content of a normal mouse liver is around 0.5% of GSH (GSH/GSSG ratio: 200). The authors need to re-evaluate their measurements. They underestimate the glutathione levels and have serious oxidation during work-up of the samples.

The reviewer is correct. We repeated the glutathione measurements on fresh samples using a different assay (Bioxytech GSH/GSSG-412 kit – Oxis Research). We show that the fasted levels of GSH after an overnight fast were 24.7 versus 23 nmol/mg protein in the wildtype and knockout mice respectively and these levels dropped to 3.5 and 3.7 nmol/mg protein respectively four hours after APAP administration. We also employed a second approach to measure glutathione levels by sending fresh snap frozen liver samples from fasted wildtype and knockout mice to a commercial metabolomics laboratory (Metabolon). They showed that the GSH and GSSG levels were the same in the liver in the wildtype and knockout mice. These data differ from that obtained in mice that underwent long term caloric restriction (Someya et al, 2010), and these differences will have to be

investigated. As these data add additional insight into the distinct SIRT3 mechanism identified in this study, we have added these data as a new Figure 4D, and in the results section at the end of the 1st results paragraph on page 4.

Additionally, we have included new text to point out the importance of the glutathione system in APAP injury. This concept has been added to the discussion in emboldened text on page 9, 2nd paragraph and states that - '**These data do not dismiss the role of glutathione depletion in APAP toxicity, as evidenced by greater injury in both genotypes when comparing fasted to fed mice. Rather, this study expands our understanding of the pathophysiology of APAP toxicity by uncovering a novel functional role for ALDH2 in attenuating acetaminophen hepatotoxicity and the role of acetylation in altering NAPQI binding to mitochondrial proteins.'**

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In this manuscript, Zhongping et al. report that SIRT3, an NAD+ dependent class III HDAC, deacetylates mitochondrial aldehyde dehydrogenase 2 (ALDH2), and SIRT3 knockout mice are protected from acetaminophen (APAP) hepatotoxicity. The authors further show that deacetylation increases APAP toxic metabolite binding on ALDH2 and inhibits its enzymatic activity.

Their identification and characterization of a novel SIRT3 target, ALDH2, is of significant importance and reveals a cross-talk between mitochondrial posttranslational modifications in therapeutic drug administration. While the observations described are of interest, the results presented are preliminary and the following points need to be addressed before publication.

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1. The authors claim that acetylation of ALDH2 competes with a modification by a toxic product of APAP, N-acetyl-p-benzoquinoneimine (NAPQI), at the same residue. However, they do not mention which lysines are modified by acetylation or NAPQI and do not substantiate the claim that the two modifications are indeed competing for the same residue. Identification of the sites of acetylation and modification should be conducted by mass spectrometry and mutation of the relevant site(s) should be studied in detail, including enzymatic activity assays (Fig 3A), and NAPQI modification assay (Fig. 4A).

Thank you, we believe that the studies performed to address this point have markedly strengthened the Report. We used an antibody directed at acetylated lysine residues to immunoprecipitate trypsin-digested peptides from liver mitochondria from the wildtype and knockout mice. The peptides were used for LCMSMS to identify peptide sequences and to find the 42 dalton shift associated with the conjugation of acetyl groups to lysine. The two reproducible alterations found in the knockout mice compared to the wildtype were acetylation of residues K370 and K377. The spectral shift identified at position K377 is shown as the new Figure 4E. Lysine residue mutagenesis/substitution studies were performed to mimic deacetylation (K to R) and acetylation (K to Q), and showed that the ALDH2 K377 is a principal lysine residue modifying NAPQI binding. This is shown in the new Figure 4F, where the K377Q mutation markedly inhibited NAPQI binding to ALDH2 and discussed in the last results section on page 8.

As the change in acetylation does not change ALDH2 activity itself, but rather functions as an allosteric modifier of NAPQI binding, we did not measure change in enzyme activities in response to the mutation of the identified lysine residues. In this respect we have deleted the text in the conclusion of the report suggesting that the modification of this residue directly alters enzyme activity.

2. The data presented do not fully convince that SIRT3 is important in APAP toxicity. The authors should show (1) acetylation status of ALDH2 after 8 or 24 hour APAP treatment. (2) enzymatic activity of ALDH2 after 8 or 24 hour APAP treatment as well as short-term (1 hour) APAP treatment (Fig 3A).

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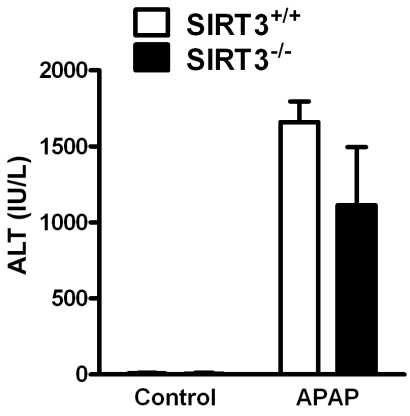


Serum ALT levels in mice comparing APAP to vehicle control in fasted mice. The levels were drawn 4 hours after intraperitoneal APAP or vehicle (control) administration.

As the temporal pathophysiology of an acute APAP overdose shows that the hepatocytes not irreversibly damaged by the insult begin to recover after the first few hours, we did not extend these assays further to for example 8 or 24 hours. Also, the initial study that demonstrated that ALDH2 activity was blunted in response to APAP administration, shows that activity is maximally decreased by 4 hours and rebounds by 8 hours (Landin et al, 1996).

3. The authors mentioned another target for SIRT3 which is responsible for acetaminophen induced liver injury in figure 4C, despite the fact that ALDH2 knockdown completely overcomes SIRT3 knock-out in acetaminophen induced liver injury (Fig 3E). Additional discussion is necessary if they claim SIRT3 has multiple targets.

This point is well taken. Text has been added to the discussion to address this, and to contextualize our findings with the prior evidence of the important role of depletion of reduced glutathione in this injury. This is discussed in emboldened text on page 9, 2nd paragraph and states that – '**These data do not dismiss the role of glutathione depletion in APAP toxicity, as evidenced by greater injury in both genotypes when comparing fasted to fed mice. Rather, this study expands our understanding of the pathophysiology of APAP toxicity by uncovering a novel functional role for ALDH2 in attenuating acetaminophen hepatotoxicity and the role of acetylation in altering NAPQI binding to mitochondrial proteins. Also, although the knockdown of ALDH2 nullifies the ameliorative effects of SIRT3 deficiency in this pathophysiology, our data cannot exclude the role of the other SIRT3 mitochondrial targets identified in this (supplemental Table 1) and other studies. Future characterization of these additional SIRT3 targets in this toxicity warrants investigation.'**

Moreover, as the potential for other targets is only theoretical at this time it was probably premature to have included a model schematic highlighting other targets. The model schematic (Prior Figure 3C) has now been removed.

Minor Points

1. Fig 1A: The authors show ALT level after overnight fasting. Please show that in fed condition as well. Also, please clarify time course of these drug treatments.

The inclusion of data in the fed state further highlighted the role of SIRT3 and this was very useful data to acquire – Thank you. This is now discussed in the first paragraph of the results section on page 4 and shown as the revised Figure 1A.

The APAP toxicity was administered as a single bolus dose, which reflects a large proportion of overdose effects in patients. This has been highlighted in the results section, in the methods and in the figure legends to clarify this point.

2. Fig 1B: Histology is not sufficient to quantify necrosis. Additional quantitative experiments will strengthen authors' conclusion.

Blinded reading of the liver histology slides were performed in the previously published scale (Nakagawa et al, 2008). This data is now included as the new Figure 1C.

3. Fig 2C: Please show Input SIRT3.

Thank you this has now been included.

4. Fig 3A: Is fasting preceded by APAP treatment in this experiment? Please describe experimental conditions.

These mice were fasted overnight prior to APAP administration and this is now described in the results section in emboldened text on page 5 in the last paragraph, and in the methods section on page 10.

5. Fig 3A and 3B: Long-term (8 or 24 hour) APAP treatment is necessary.

Our study was designed to investigate the effect of mitochondrial protein acetylation on the acute binding of the APAP toxic metabolite NAPQI to mitochondrial proteins. The more chronic administration of APAP, while of important scientific interest, would introduce a multitude of new pathophysiologic variables that would need to be defined. We hope that the additional data added to this study is acceptable to the reviewer and would petition that this suggestion will be undertaken as a separate and future study.

6. Fig3F: Histology is not sufficient to quantify necrosis. Additional quantitative experiments are required.

We have, removed the representative images from the report and replaced those with the new data showing that the knockdown of ALDH2 in the SIRT3$^{-/-}$ mice resulted in increased 4-HNE adduct binding in response to APAP. These data are more direct evidence of the acute ameliorative effect of functioning ALDH2 in reducing acetaminophen induced liver injury. The histological data in response to ALDH2 knockdown has now been moved into the supplemental data (Figure S4B).

7. Fig 4A and 4B: SIRT3 knockdown is necessary in these experiments.

This study has now been performed and is discussed in the results section in emboldened text on page 7 at the end of the second paragraph and is shown as the new Figure 4C.

Reviewer 3, Comments:

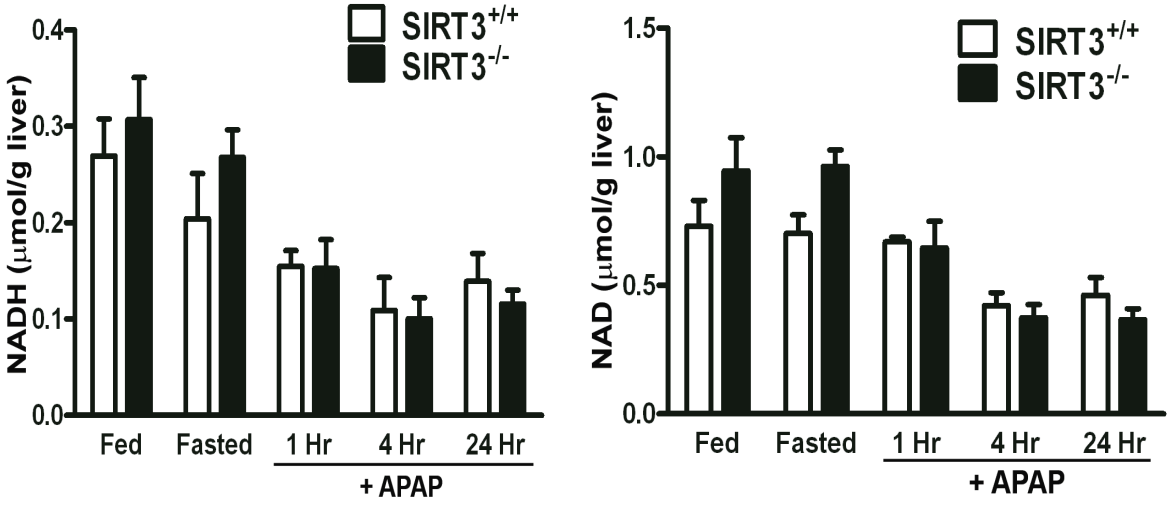
In this manuscript, Lu et al. set out to solve the paradox where fasting or CR exacerbates the toxicity of APAP. They identified ALDH2 as a novel SIRT3 substrate and proposed an interesting model in which acetylation and NAPQI antagonistically regulate ALDH2 activity. Fasting induces SIRT3 mediated deacetylation of ALDH2, leading to increased NAPQI binding. Binding of NAPQI in turn reduces ALDH2 activity, which contributes to APAP induced liver injury. The finding in this manuscript are novel, and the experiments clearly presented, including solid data from both in vitro and in vivo experiments that overall support the author's hypothesis. Nevertheless, there are few concerns that the authors need to address for the manuscript to warrant publication.

Major comments:
- Since a major mechanism proposed to exacerbate AILI during fasting is depletion of reduced gluthathione, it is surprising that the authors find no difference between WT and SIRT3 KO animals (mainly when their phenotype appears to depend on the presence of reduced levels of 4-HNE, indicating less oxidation). The authors should comment about these results in the Discussion, in the context of their "protective" phenotype. For instance, is there an alternative pathway that ALDH2 is regulating? Is the NAD/NADH ratio altered, which will indicate this pathway as the dominant one in their phenotype? It is clear from their ALDH2 activity assay that they do observe less capacity to reduce NAD in vitro in the SIRT3 deficient extracts, but they should measure NAD/NADH ratio in vivo, as they did for GSH/GSSG.

We agree that we should not have diminished the role of reduced glutathione in this pathophysiology, but should have communicated that a deacetylation mechanism is additionally operational in this toxicity. The discussion text has been changed to address this as described in the 2nd paragraph on page 9. The discussion is as follows: '**These data do not dismiss the role of glutathione depletion in APAP toxicity, as evidenced by greater injury in both genotypes when comparing fasted and fed mice. Rather, this study expands our understanding of the**

pathophysiology of APAP toxicity by uncovering a novel functional role for ALDH2 in attenuating acetaminophen hepatotoxicity and the role of acetylation in altering NAPQI binding to mitochondrial proteins.'

As suggested we measured NAD and NADH levels in our laboratory in response to feeding, fasting and to APAP administration in fasted mice. These metabolites again did not show any significant differences (see below). Whether these data reflect that NAD and NADH levels do not play a dominant role in this SIRT3 mediated effect on this pathophysiology, or that the sensitivity of the assay used (NAD/NADH Assay Kit - abcam) to measure these metabolites is not sufficiently sensitive to elucidate differences, or that we measured whole cell levels and the changes are exclusive to the mitochondria have not been determined to date. Nevertheless, as the NAD/NADH data does not appear to shed additional insight into the mechanism underscoring the SIRT3 effect described in this study, we have elected to not include these data in the report.



- A major oversight in the manuscript is that the entire model for fasting induced liver toxicity is based on the assumption that increased SIRT3 activity upon fasting causes decreased acetylation of ALDH2, which in turn will enhance NAPQI binding. The authors did not show that, indeed, fasting decreases ALDH2 acetylation in WT livers, a major point to support their hypothesis.

In our original report, we did not make clear that the initial screening 2D-gel electrophoresis study (Figure 2A) was performed after an overnight fast. This has now been described in the results section on page 4 (2nd paragraph, 3rd line). These data have also been confirmed in a new study shown as the new Figure 2E and is discussed in the results section on page 5 (end of 1st paragraph).

- Although the effect of ALDH2 knockdown on liver ALT production seems clear, this could be an indirect effect on liver toxicity, given the known protective effects of ALDH2 against oxidation. A more rigorous assay will be to test whether these SIRT3-/- ALDH2 knockdown cells exhibit more production of 4-HNE, similar to WT, an experiment that should be easy to perform.

Thank you, this was an excellent suggestion and this study has now been performed. As you suspected we do see that the knockdown of ALDH2 in the SIRT3$^{-/-}$ mice results in increased 4-HNE adduct levels in response to APAP. These data are now shown in the new figure 3F and discussed in the results section on the top of page 7.

- In Figure 4, the authors nicely show that APAP adducts bind to ALDH2, and treatment with nicotinamide, which increases ALDH2 acetylation, diminish binding. In support of their in vivo results, it will be reassuring to see that the SIRT3 deficient livers' samples (for which they already performed nice ALDH2 IPs, as shown in Fig.2) also exhibit diminished binding of APAP adducts.

The identification of post-translational modifications on proteins extracted from whole tissue is sometimes limited by the percent occupancy of these modifications in the whole organ sample. When we immunoprecipitated with ALDH2, we did not find significant adduct binding by immunoblot analysis. The inverse, experiment, which has proven successful, when the immunoprecipitation of the target protein has not, as for other post-translational modifications such as acetylation (Ahn et al, 2008; Hirschey et al, 2010), was unfortunately not successful here. It appears that we were unable to precipitate out hepatic proteins using the antibody directed at NAPQI adducts.

- In the in vitro deacetylation assay (Fig.2D), the authors might include SIRT3HY (already used in another experiment) and SIRT5, another mitochondrial Sirtuin with deacetylation activity, as negative controls, to confirm that the effect they see is specific for SIRT3 (of note, fasting is known to modulate other sirtuins as well, therefore such additions will be excellent controls for their model).

We did overexpress the murine full length SIRT5 to assess the specificity of SIRT3 in deacetylating ALDH2. Two sets of experiments were performed. We show in the new Figure 2F that SIRT5 does not deacetylate ALDH2 and in the new Figure 4D that the overexpression of SIRT5 does not modulate NAPQI binding to ALDH2.

- Fig 1A shows that at 8 hrs of APAP, there is no difference in ALT between SIRT3 WT and KO liver samples. Yet, Fig 3E shows significant differences between the same samples (shScr). The authors should explain this discrepancy.

We cannot be absolutely sure why this is the case, but do point out that the susceptibility to APAP is greater in both genotypes mice following the lentiviral infections (Page 6, 2 lines from the bottom). Whether the greater susceptibility to APAP hepatotoxicity unmasks greater protection by protein acetylation may be a putative mechanism underlying the demonstration of significance at 8 and 24 hours in the lentiviral infection ALDH2 knockdown study, is possible?

Minor comments

- There are multiple grammar mistakes throughout the manuscript. The authors should have their manuscript carefully edited before resubmission. Some examples: on page 3, the sentence "is, to our knowledge, has not been investigated", is grammatically incorrect. Same page "in the context of acetaminophen induced liver injury (AILI), may be is due in part, to protein lysine residue..." is also grammatically improper. On page 9, end of Discussion ""ALDH2 is as a functional substrate"

We apologize for this and believe that we have corrected all of the grammatical errors.

Reference List

Ahn BH, Kim HS, Song S, Lee IH, Liu J, Vassilopoulos A, Deng CX, Finkel T (2008) A role for the mitochondrial deacetylase Sirt3 in regulating energy homeostasis. *ProcNatlAcadSciUSA* **105:** 14447-14452

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2nd Editorial Decision 23 May 2011

Thank you for the submission of your revised manuscript. I have now heard back from referees 1 and 3, to whom I sent the revised version of your manuscript. Referee 2 was unfortunately not available, but referee 3 assessed also your responses to referee 2. I am happy to say that, as you will see below, they are both fully supportive of the study. Thus, we will accept it for publication, once a few formal issues below have been addressed.

I am happy to be the bearer of good news and look forward to receiving your final manuscript file and figures.

With best wishes,

Editor
EMBO reports

Referee reports

Referee #1:

The authors have satisfactorily addressed the reviewers' comments.

Referee #3:

In this revised version of their manuscript, Lu and colleagues did an outstanding job in addressing satisfactorily all the reviewers concerns. I believe the manuscript present now a convincing argument providing novel insights into the mechanisms underlying fasting-enhanced acetaminophen-induced liver failure. This manuscript will attract interest from a broad audience, and as such it is now suitable for publication in Embo Reports.
Minor comment:

- On the beginning of page3 of the rebuttal letter, the authors mentioned that they have now included the data on GSH/GSSH levels in new Figure 4D, however this figure shows the non-effect of SIRT5 over-expression on NAPQI binding. I believe the authors meant Fig.1D. This should be corrected as well on page 4 of the manuscript.

