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SIRT3-dependent GOT2 acetylation status affects the malate-aspartate NADH shuttle activity and pancreatic tumor growth

Hui Yang, Lisha Zhou, Qian Shi, Yuzheng Zhao, Huaipeng Lin, Mengli Zhang, Shimin Zhao, Yi Yang, Zhi-Qiang Ling, Kun-Liang Guan, Yue Xiong and Dan Ye

Corresponding author: Dan Ye, Fudan University

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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.)

Editor: Andrea Leibfried

1st Editorial Decision 07 August 2014

Thank you for submitting your manuscript entitled 'GOT2 acetylation enhances its interaction with MDH2 and the malate-aspartate NADH shuttle activity'. I have now received the reports from all referees.

As you can see below, all referees find your manuscript potentially interesting. However, they raise various concerns, most importantly, all three referees question the physiological relevance of your findings which reflects the very preliminary state of the study. This makes it difficult to envision eventual publication of your manuscript in our journal.

However, conditioned on satisfactorily addressing the physiological concerns as well as the other relevant points, I would be happy to consider a revised version of the manuscript. Referees #2 and #3 provide constructive comments, and an analysis along the lines suggested by these referees would be very important for further consideration here.

Please contact me in case of further questions. I should also add that it is EMBO Journal policy to allow only a single round of revision and that it is therefore important to address all concerns raised at this stage.

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

REFEREE COMMENTS

Referee #1:

Yang and colleagues identify and analyze the acetylation-dependent interaction of mitochondrial of GOT2 with MDH2 to control cellular redox state and cancer growth both in vitro and in vivo.

Major comments:
Figs. 4 and 5: How does the GOT2-knockdown/shGOT2 *without* reconstitution affect the parameters analyzed?

Minor comments:
EGCG is not a *specific* GPDH inhibitor, but has been shown to exert many other functions. The text should be changed accordingly.

For reasons of visibility, the title of the ms. should possibly contain the terms "sirtuin/SirT3-dependent" and "cancer growth/proliferation".

Referee #2:

This manuscript by Yang and colleagues describes how acetylation of the mitochondrial GOT2 protein regulates NADH shuttling from cytosol to mitochondria. This acetylation event, occurring at three lysine residues, regulates the association between GOT2 and MDH2, and is controlled by SIRT3.

This is an interesting paper, and the experiments are well executed. Nevertheless, I have some conceptual questions related to the in vivo physiological role of GOT2 acetylation, and a few technical issues that need clarification.

Major concerns
(1) Considering the intimate link between cellular compartments when it comes to redox potential, how does constitutive (in)active GOT2 alter the cytosolic and nuclear redox state and sirtuin activity in these other compartments?
(2) The experiments in figure 4 suggest that acetylated GOT2 (3KQ-mutated; mimicking inactive SIRT3) reduces ROS levels. On the other hand, there is a wealth of publications in which active SIRT3 reduces ROS. How to reconcile these two points? What happens to SOD2 acetylation and activity when expressing GOT2-3KQ? Does knockdown of mitochondrial NADK2, the enzyme responsible for generating mitochondrial NADPH, block the antioxidant response?
(3) The in vivo physiological role of GOT2 acetylation is insufficiently clarified. It is much appreciated that the authors tested different concentrations of glucose and glutamine, but why do these two substrates behave the same even though glutamine does not need to glycolysis and transport of NADH? How does GOT2 acetylation change in tissues of fasted mice? During fasting SIRT3 is getting active, which would lead to deacetylation and inactivation of GOT2.
(4) Related to the previous point: in figure 4, the constitutively active 3KQ construct increased the mitochondrial NADH/NAD+ ratio, a state in which SIRT3 would get inactivated leading to self-amplification of the GOT2 activity. How would this regulation again be counterbalanced?
(5) Does pharmacological activation of SIRT3 using NMN or NR result in GOT2 deacetylation? Does this reduce the proliferative capacity of the pancreatic tumor cells?
(6) In all experiments where GOT2-MDH2 association is analyzed, the authors specifically link this to GOT2 acetylation, even though they acknowledge the fact that MDH2 is also acetylated at several lysine residues. It would be good to show specific MDH2 acetylation as well in these experiments.
(7) The constitutively (in)active mutants appear to be used very selectively. The deacetylated 3KR mutant is used only in the HEK cells, while to acetylated 3KQ construct is only used in the Panc-1 experiments. It would be helpful if both constructs were used at the same time to elegantly demonstrate that both have opposite roles in the regulation of GOT2 function and downstream physiology.

Minor concerns:
(8) What happens to cellular/mitochondrial respiration when 3KR and 3KQ are expressed in cells?
(9) There is a typo in the abstract: glutmate → glutamate

Referee #3:

A large number of metabolic enzymes have been identified by acetyl-proteomics studies, and this group has functionally characterized the role of acetylation of several of these. Yang et al now demonstrate that Got2 is acetylated at 3 sites, and that this enhances Got2 interaction with MDH2. Sirt3 is the deacetylase. Functionally, this promotes the malate-aspartate shuttle, promoting net transfer of NADH into mitochondria, ATP production, and NADPH production. 3K acetylation of Got2 promotes cell proliferation and promotes tumor growth. Got2 acetylation is increased in human pancreatic tumors, correlating with reduced Sirt3. Overall, the data supports the findings and the manuscript makes an important addition to our knowledge of how metabolism supports proliferation and the role of metabolic enzyme acetylation. My main concerns relate to establishing the physiological significance of this acetylation.

Major points:
1. A weakness of the paper is that it relies predominantly on overexpression studies, and interaction of the endogenous Got2 and MDH is not shown. If there are not suitable antibodies for IP of the endogenous proteins, perhaps an alternative technique such as proximity ligation assay could be attempted to demonstrate this.
2. Both Sirt3 and low glucose or glutamine are shown to counter the acetylation of Got2. To close the loop, can the glucose- and glutamine-dependent regulation of Got2 acetylation be ascribed to Sirt3? Could deacetylation in low glc/gln be blocked by NAM or Sirt3 knock down?
3. In Figure 4, Got2 3KQ promotes mitochondrial NADH levels, ATP production, increased stress resistance and proliferation, etc compared to wt. While these studies inherently rely on ectopic protein expression, to help establish the physiological significance of Got2 acetylation, a couple of experiments could be done. First, these measurements could be conducted with the 3KR mutant, which fails to interact with MDH. If acetylation of the wt Got2 is physiologically important, one would predict that the 3KR mutant would show higher cytosolic NADH, reduced ATP, reduced proliferation etc compared to wt. Second, these experiments could also potentially be done under high and low glucose, although glucose will regulate the whole metabolic program and manipulation of Got2 acetylation status in isolation may or may not be sufficient to mediate changes in these parameters in response to changes in glucose availability.

Minor points:
1. AOA is described as a specific malate-aspartate shuttle inhibitor and EGCG as a specific GPDH inhibitor. These inhibitors may be more generally inhibiting glutamine utilization due to their functions in inhibiting transaminases and GDH, respectively. Conclusions from this experiment should be drawn more cautiously.
2. Please check the validity of the statistics on Figures 5 G and H. It looks like the level of AcGot2 and Sirt3 are set to 1 for all normal samples, which artificially removes any variability between the normal samples, inflating the p value. Also G and H are not designated in the Figure legend.

1st Revision - authors' response 04 November 2014

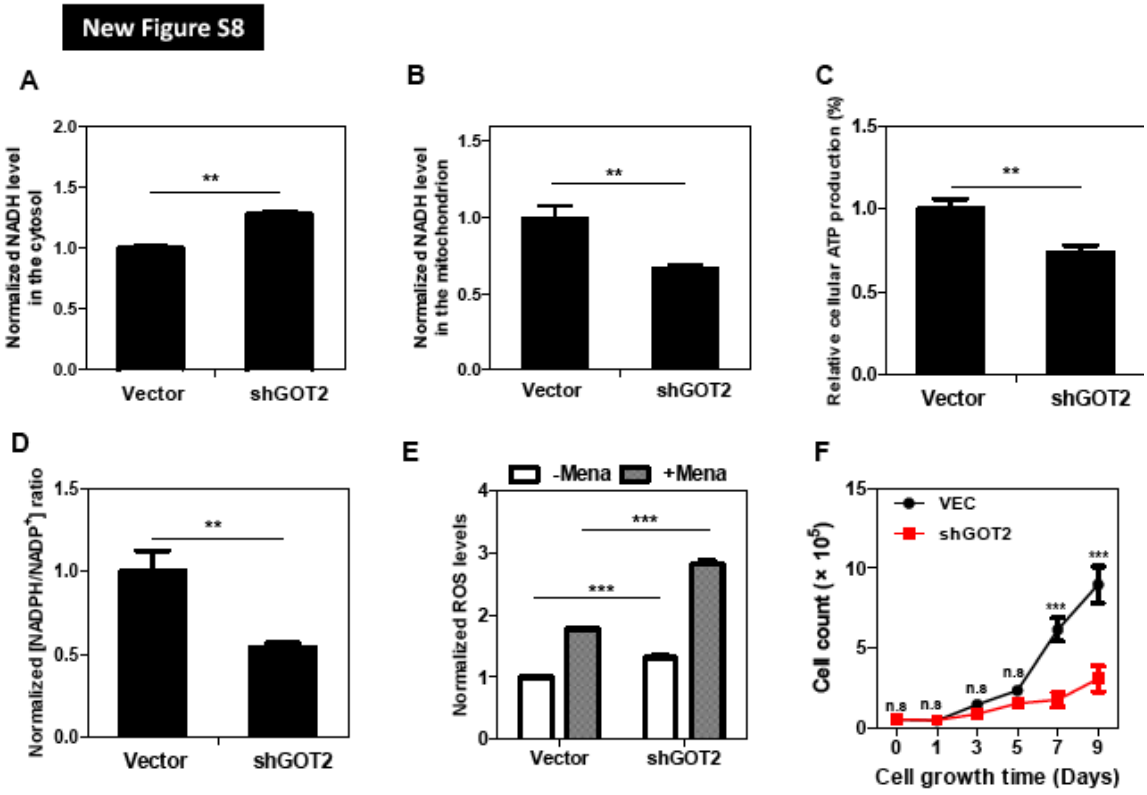
Referee #1:

We appreciate the reviewer's efforts of evaluating our work and constructive comments.

Major comments:
*Figs. 4 and 5: How does the GOT2-knockdown/shGOT2 *without* reconstitution affect the parameters analyzed?*

Response: The effect of *GOT2* knockdown/shGOT2 without putting-back on most of the

parameters analyzed in Figure 4 have been presented in original supplementary Fig. S6 (new supplementary Fig. S8A-E). In addition, we also found that *GOT2* knockdown significantly ($p<0.001$) decreased cell proliferation in Panc-1 cells (new supplementary Fig. S8F). These results support the conclusion that GOT2 increases pancreatic tumor cell proliferation by stimulating the cytosolic to mitochondral NADH transfer, ATP production, and NADPH production. **Figure S8**



Minor comments:
*EGCG is not a *specific* GPDH inhibitor, but has been shown to exert many other functions. The text should be changed accordingly.*

Response: According to the reviewer's suggestion, we have deleted "specific" and stated that EGCG can exert multiple functions, including inhibiting GPDH. Thanks for pointing this out.

For reasons of visibility, the title of the ms. should possibly contain the terms "sirtuin/SirT3-dependent" and "cancer growth/proliferation".

Response: We have revised the title of the manuscript to "SIRT3-dependent acetylation of GOT2 enhances the malate-aspartate NADH shuttle activity to promote pancreatic tumor growth".

Referee #2:

This manuscript by Yang and colleagues describes how acetylation of the mitochondrial GOT2 protein regulates NADH shuttling from cytosol to mitochondria. This acetylation event, occurring at three lysine residues, regulates the association between GOT2 and MDH2, and is controlled by SIRT3.

This is an interesting paper, and the experiments are well executed. Nevertheless, I have some conceptual questions related to the in vivo physiological role of GOT2 acetylation, and a few technical issues that need clarification.

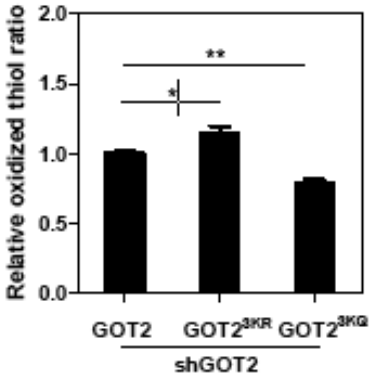
We are grateful to reviewer's appreciation on our study and constructive comments. Below, we address this reviewer's concerns point-by-point.

Major concerns:
(1) Considering the intimate link between cellular compartments when it comes to redox potential, how does constitutive (in)active GOT2 alter the cytosolic and nuclear redox state and sirtuin activity in these other compartments?

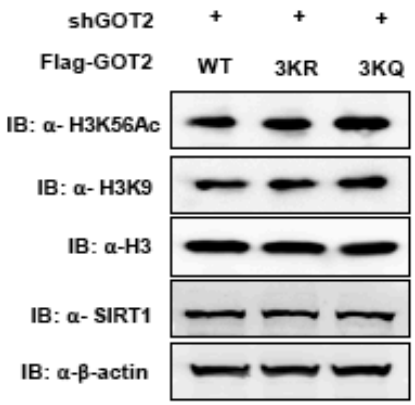
Response: It is technically challenging to measure the nuclear redox state in the cell. To measure the cytosolic redox status, we have established wild-type or 3K mutant rescued Panc-1 cells stably expressing cytosolic redox-sensitive green fluorescent protein 1 (roGFP1), which allows real-time visualization of thiol-disulfide metabolic state in the cytosol of living cells (Dooley et al., 2004. PMID: 14985369). We found that the 3KR rescued cells exhibited significantly ($p<0.05$) higher levels of disulfide (oxidized thiol), while the 3KQ rescued cells displayed significantly ($p<0.01$) lower levels of disulfide when compared to wild-type controls (new supplementary Figure S10). These data suggest that constitutive (in)active GOT2 alters the cytosolic redox status.

To determine the sirtuin activity in the nuclear and cytosolic compartments, we have examined the acetylation level of substrates of SIRT1 and SIRT2, which are present in the nucleus and cytosol, respectively. We found that the acetylation levels of H3K9 and H3K56, which are known substrates of SIRT1 (Nakahata et al., 2008. PMID: 18662547; Yuan et al., 2009. PMID: 19411844), did not differ between wild-type and 3KR/Q rescued cells (new supplementary Fig. S11A). Moreover, we found that the K100 acetylation level of PGM2, a direct substrate of SIRT2 (Xu et al., 2014. PMID: 24786789), did not differ between wild-type and 3K rescued cells (new supplementary Fig. S11B). Collectively, these results indicate that constitutive (in)active GOT2 may not globally affect the SIRT1 and SIRT2 activity in the nuclear and cytosolic compartments.

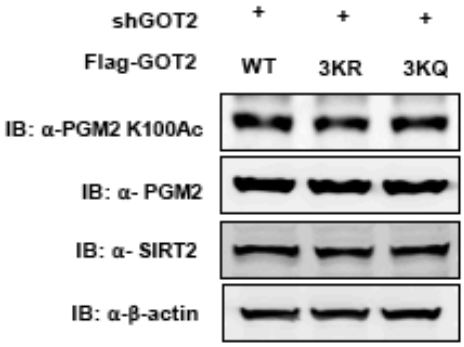
New Figure S10



New Figure S11A



New Figure S11B



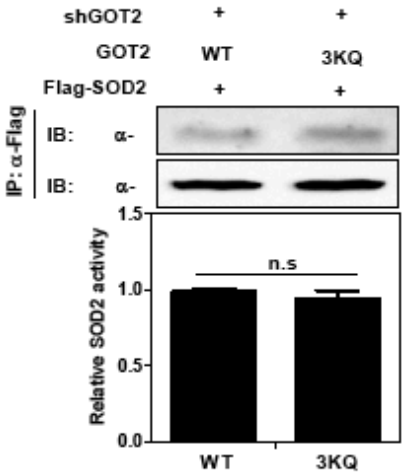
(2) The experiments in figure 4 suggest that acetylated GOT2 (3KQ-mutated; mimicking inactive SIRT3) reduces ROS levels. On the other hand, there is a wealth of publications in which active SIRT3 reduces ROS. How to reconcile these two points? What happens to SOD2 acetylation and activity when expressing GOT2-3KQ? Does knockdown of mitochondrial NADK2, the enzyme responsible for generating mitochondrial NADPH, block the antioxidant response?

Response: SIRT3 has multiple substrates, including SOD2 (Chen et al., 2011. PMID: 21566644), IDH2 (Yu et al., *J Biol Chem*. 2012. PMID: 22416140), and GOT2. Therefore, 3KQ mutant GOT2 cannot mimic inactive SIRT3. Supporting this notion, we have examined the acetylation level and enzyme activity of Flag-tagged SOD2 in wild-type and 3KQ GOT2 rescued cells, and found that constitutive active GOT2 (3KQ-mutated) did not affect SOD2 acetylation and activity (refer to 'Result to the reviewer' as shown below).

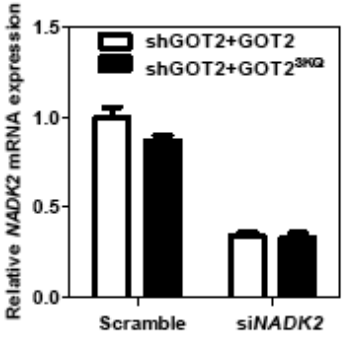
We have previously shown that *SIRT3* knockdown increases the acetylation level of SOD2 and inhibits the activity of SOD2 to scavenge ROS (Chen et al., 2011. PMID: 21566644). In this study, we show that *SIRT3* knockdown increases the acetylation level of GOT2, leading to enhanced protein interaction between GOT2 and MDH2 and stimulating the malate-aspartate NADH shuttle and thereby increasing NADPH and suppressing ROS. In SIRT3-inactive cells where the activity of anti-oxidative enzyme SOD2 is inhibited, whether or not GOT2 acetylation represents a compensatory mechanism to defend against harmful ROS still needs further investigation.

To address the reviewer's second question, we have utilized siRNA to deplete *NADK2* gene, and found that knocking-down *NADK2* could not block the anti-oxidant response in 3KQ mutant GOT2 rescued cells (new supplementary Figure S9), indicating that the anti-oxidant effect of GOT2 3K acetylation may be independent of *NADK2*.

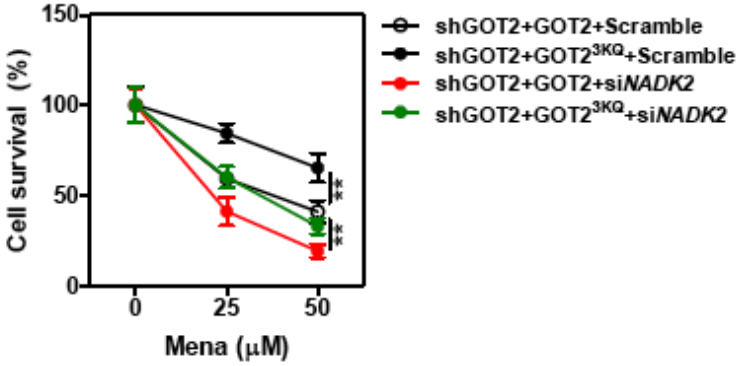
Result to the reviewer



New Figure S9A



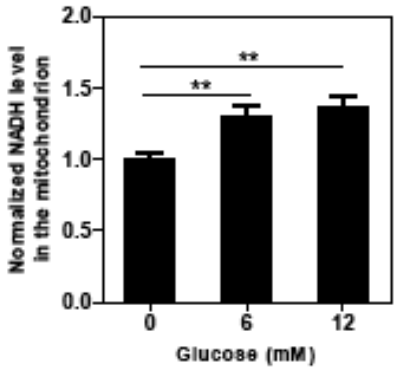
New Figure S9B



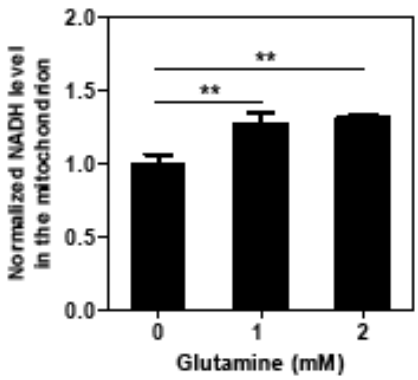
(3) The in vivo physiological role of GOT2 acetylation is insufficiently clarified. It is much appreciated that the authors tested different concentrations of glucose and glutamine, but why do these two substrates behave the same even though glutamine does not need to glycolysis and transport of NADH? How does GOT2 acetylation change in tissues of fasted mice? During fasting SIRT3 is getting active, which would lead to deacetylation and inactivation of GOT2.

Response: Both glucose and glutamine are the major carbon sources in cell culture. High concentration of glucose or glutamine indicates energy/nutrient sufficiency to the cell, stimulating overall energy metabolism, including both glycolysis (by glucose) and TCA (by glutamine). It is thus possible that some of the metabolic parameters, such as NADH, might behave similarly to both glucose and glutamine. Supporting this notion, we found that NADH level in the mitochondrion was indeed significantly increased by glucose or glutamine treatment in Panc-1 cells (new supplementary Figure S6A and S6B). Upon the reviewer's request, we have determined the Got2 K159 acetylation level in the tissues of overnight fasted mice, and found that Sirt3 protein expression was up-regulated in the liver and white adipose tissues during fasting, leading to reduced levels of Got2 K159 acetylation (new Figure 3H, 3I).

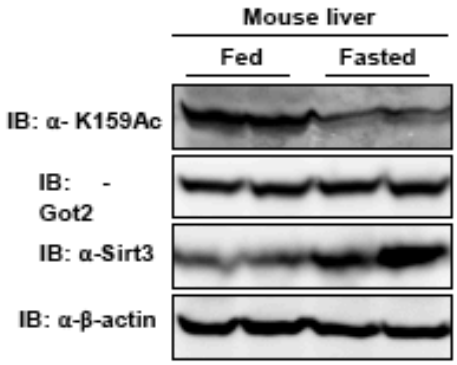
New Figure S6A



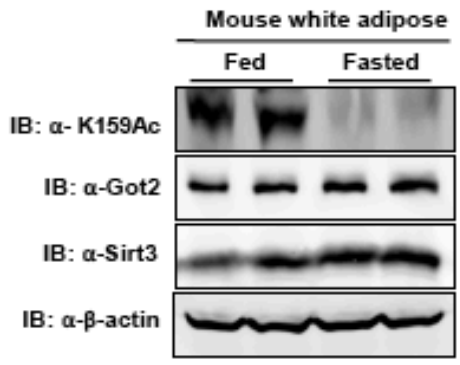
New Figure S6B



New Figure 3H



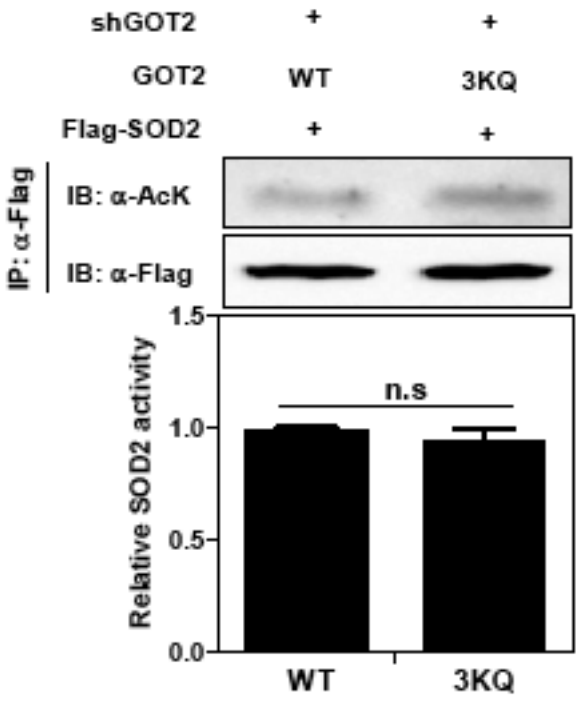
New Figure 3I



(4) Related to the previous point: in figure 4, the constitutively active 3KQ construct increased the mitochondrial NADH/NAD+ ratio, a state in which SIRT3 would get inactivated leading to self-amplification of the GOT2 activity. How would this regulation again be counterbalanced?

Response: In Figure 4, our original data demonstrated that the constitutively active 3KQ mutant GOT2 increased the mitochondrial NADH level, supporting the importance role of GOT2 in the net transfer of NADH from the cytosol to the mitochondrial compartment. We have never shown either NAD+ or the NADH/NAD+ ratio in the mitochondrion in the GOT2 3KQ rescued cells.
Because the mechanism of SIRT3 activity regulation is largely unknown, one cannot simply predict SIRT3 activity based on the NAD+ concentration even if it is changed. Moreover, our data demonstrated that constitutive active GOT2 (3KQ-mutated) did not affect SOD2 acetylation and activity, indicating that constitutive active GOT2 may not substantially affect SIRT3 activity (refer to the figure below). In fact, SIRT3 is known to affect multiple proteins in cellular regulation. The multi-step linear prediction may not be applicable in this case.

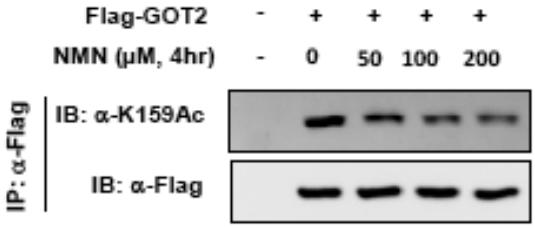
Result to the reviewer



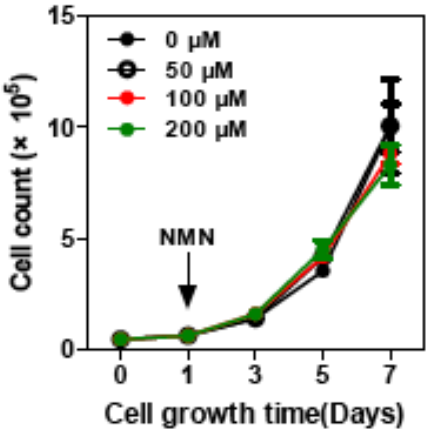
(5) Does pharmacological activation of SIRT3 using NMN or NR result in GOT2 deacetylation? Does this reduce the proliferative capacity of the pancreatic tumor cells?

Response: To address the reviewer's question, we have treated GOT2-overexpressing Panc-1 cells with increased concentrations of NMN, and found that pharmacological activation of SIRT3 by NMN could reduce the K159 acetylation level of GOT2 in a dose-dependent manner (new supplementary Figure S14A). However, NMN treatment did not significantly affect cell growth in Panc-1 cells (new supplementary Figure S14B).

New Figure S14A



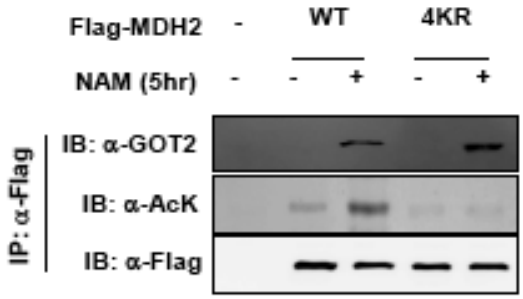
New Figure S14B



(6) In all experiments where GOT2-MDH2 association is analyzed, the authors specifically link this to GOT2 acetylation, even though they acknowledge the fact that MDH2 is also acetylated at several lysine residues. It would be good to show specific MDH2 acetylation as well in these experiments.

Response: We have previously identified four major regulatory acetylation sites in MDH2, including K185, K301, K307, and K314 (Zhao et al. 2010. PMID: 20167786). NAM increased the acetylation level of wild-type MDH2, but not the acetylation-deficient 4KR mutant MDH2. However, both the wild-type and the 4KR mutant MDH2 exhibited enhanced protein interaction with endogenous GOT2 upon NAM treatment (new supplementary Figure S1). These results suggest that acetylation of MDH2, unlike that of GOT2, does not affect the NAM-induced (presumably acetylation dependent) interaction between GOT2 and MDH2.

New Figure S1

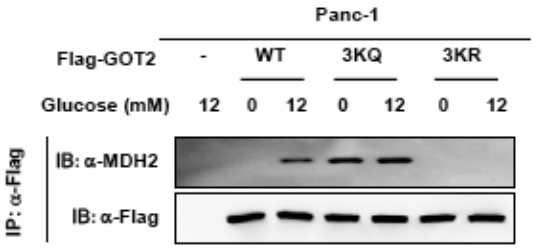


(7) The constitutively (in)active mutants appear to be used very selectively. The deacetylated 3KR mutant is used only in the HEK cells, while to acetylated 3KQ construct is only used in the Panc-1 experiments. It would be helpful if both constructs were used at the same time to elegantly demonstrate that both have opposite roles in the regulation of GOT2 function and downstream physiology.

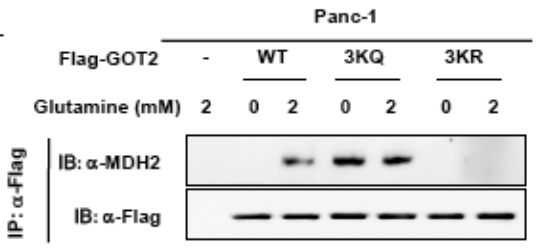
Response: Upon the reviewer's request, we have determined the effect of both 3KQ and 3KR mutations on regulating GOT2-MDH2 protein interaction in Panc-1 cells. Our data demonstrated that, as seen in HEK293T cells, the acetylation-mimetic 3KQ mutant GOT2 displayed stronger association with endogenous MDH2 as compared to wild-type GOT2, and this protein interaction was not affected by glucose or glutamine treatment

(new Figures 2G and 2H). In contrast, deacetylation-mimetic 3KR mutant GOT2 was incapable to bind with endogenous MDH2 in cells regardless of the glucose or glutamine status (new Figures 2G and 2H). These results indicate that acetylation regulation of MDH2-GOT2 binding is not specific to a particular cell type. They also suggest that acetylation-mimicking and disrupting mutants have opposite effects on the regulation of GOT2 as determined by its association with MHD2.

New Figures 2G



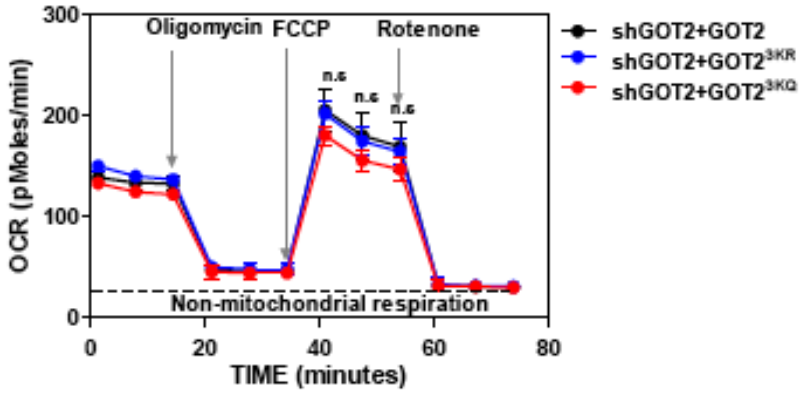
New Figures 2H



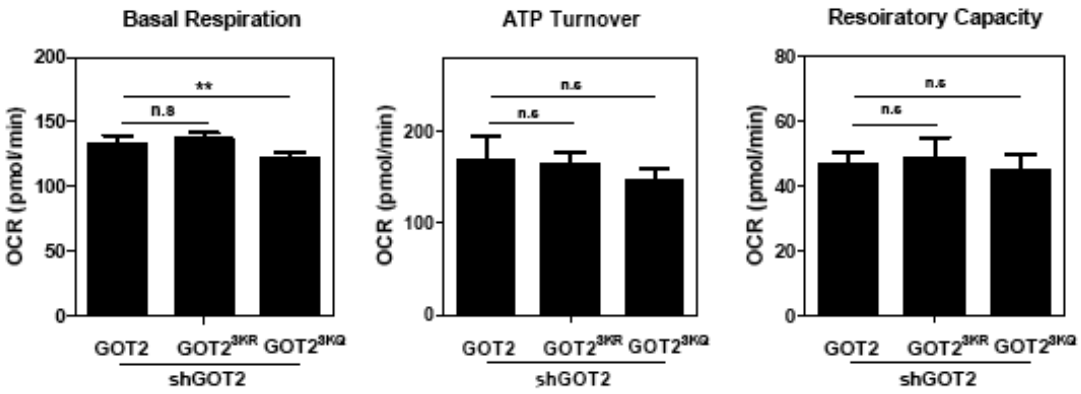
Minor concerns:
(8) What happens to cellular/mitochondrial respiration when 3KR and 3KQ are expressed in cells?

Response: To address the reviewer's question, we have determined the oxygen consumption rate (OCR) in wild-type and 3K mutant rescued cells, and found that the rate of basal respiration was significantly decreased in rescued cells expressing 3KQ mutant (new supplementary Figure S12).

New Figure S12B



New Figure S12C,D,E



(9) There is a typo in the abstract: glutmate → glutamate

Response: This has been corrected. We thank the reviewer for pointing this out.

Referee #3:

A large number of metabolic enzymes have been identified by acetyl-proteomics studies, and this group has functionally characterized the role of acetylation of several of these. Yang et al now demonstrate that Got2 is acetylated at 3 sites, and that this enhances Got2 interaction with MDH2. Sirt3 is the deacetylase. Functionally, this promotes the malate-aspartate shuttle, promoting net transfer of NADH into mitochondria, ATP production, and NADPH production. 3K acetylation of Got2 promotes cell proliferation and promotes tumor growth. Got2 acetylation is increased in human pancreatic tumors, correlating with reduced Sirt3. Overall, the data supports the findings and the manuscript makes an important addition to our knowledge of how metabolism supports proliferation and the role of metabolic enzyme acetylation. My main concerns relate to establishing the physiological significance of this acetylation.

We are grateful to reviewer's appreciation on our study and constructive comments. Below, we address this reviewer's concern point-by-point.

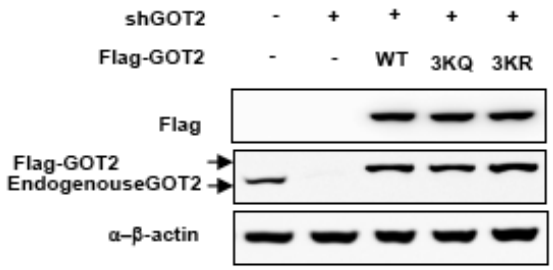
Major points:
1. A weakness of the paper is that it relies predominantly on overexpression studies, and interaction of the endogenous Got2 and MDH is not shown. If there are not suitable antibodies for IP of the endogenous proteins, perhaps an alternative technique such as proximity ligation assay could be attempted to demonstrate this.

Response: Testing the interaction between endogenous GOT2 and MDH2 is technically challenging due to the lack of antibodies suitable for IP of the endogenous protein. We have tried very hard with multiple antibodies, including GOT2 antibodies (Proteinintech, 14800-1-AP; Abcam, ab113693) and MDH2 antibodies (Cell Signaling Technology, 8610S; Abcam, ab110317), but failed to find suitable ones.
Alternatively, we have generated stable cells with endogenous GOT2 depletion and Flag-tagged wild-type or 3K mutant GOT2 rescue at a level similar to endogenous GOT2 protein (new supplementary Figure S7). In these stable cells, we determined the protein association between wild-type or 3K mutant GOT2 with endogenous MDH2 in cells treated with different concentrations of glucose or glutamine (new Figures 2G and 2H).

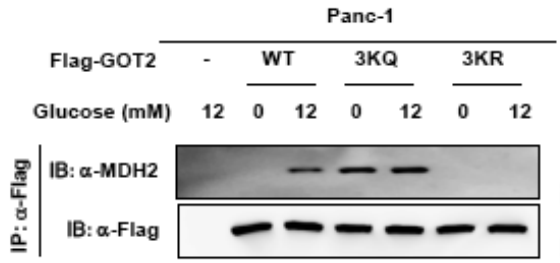
These results provide evidence for the regulation of GOT2-MDH2 interaction by acetylation under physiological conditions.

Moreover, we have determined the Got2 K159 acetylation level in the tissues of overnight fasted mice, and found that Sirt3 protein expression was up-regulated in the liver and white adipose tissues during fasting, leading to reduced levels of Got2 K159 acetylation (new Figure 3H, 3I). Both are in vivo results without involving any 'overexpression', strongly supporting the physiological relevance and significance of SIRT3-dependent regulation of GOT2 acetylation.

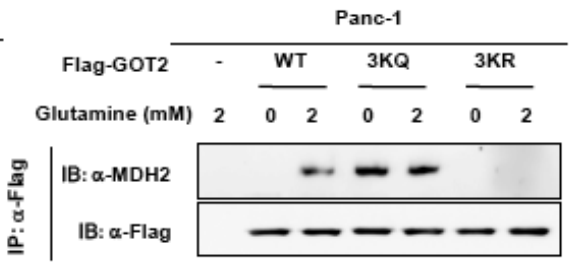
New Figure S7



New Figures 2G

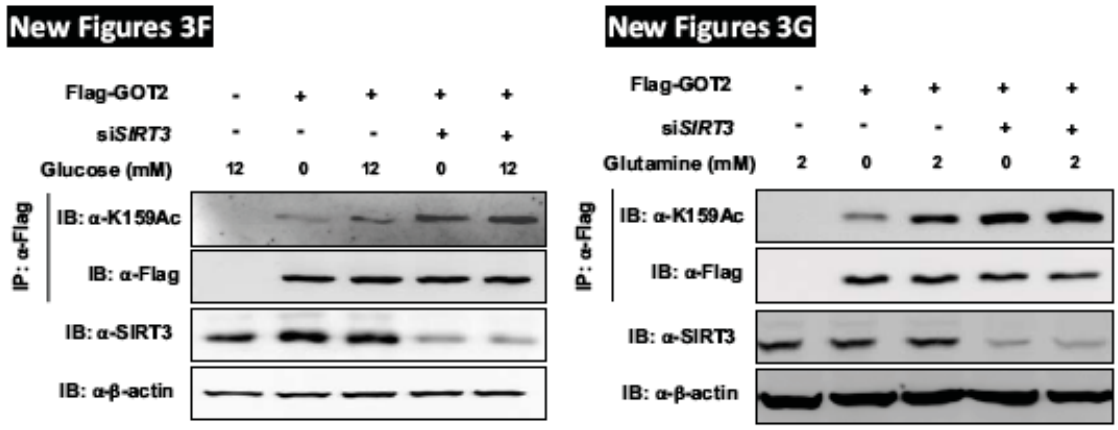


New Figures 2H



2. Both Sirt3 and low glucose or glutamine are shown to counter the acetylation of Got2. To close the loop, can the glucose- and glutamine-dependent regulation of Got2 acetylation be ascribed to Sirt3? Could deacetylation in low glc/gln be blocked by NAM or Sirt3 knock down?

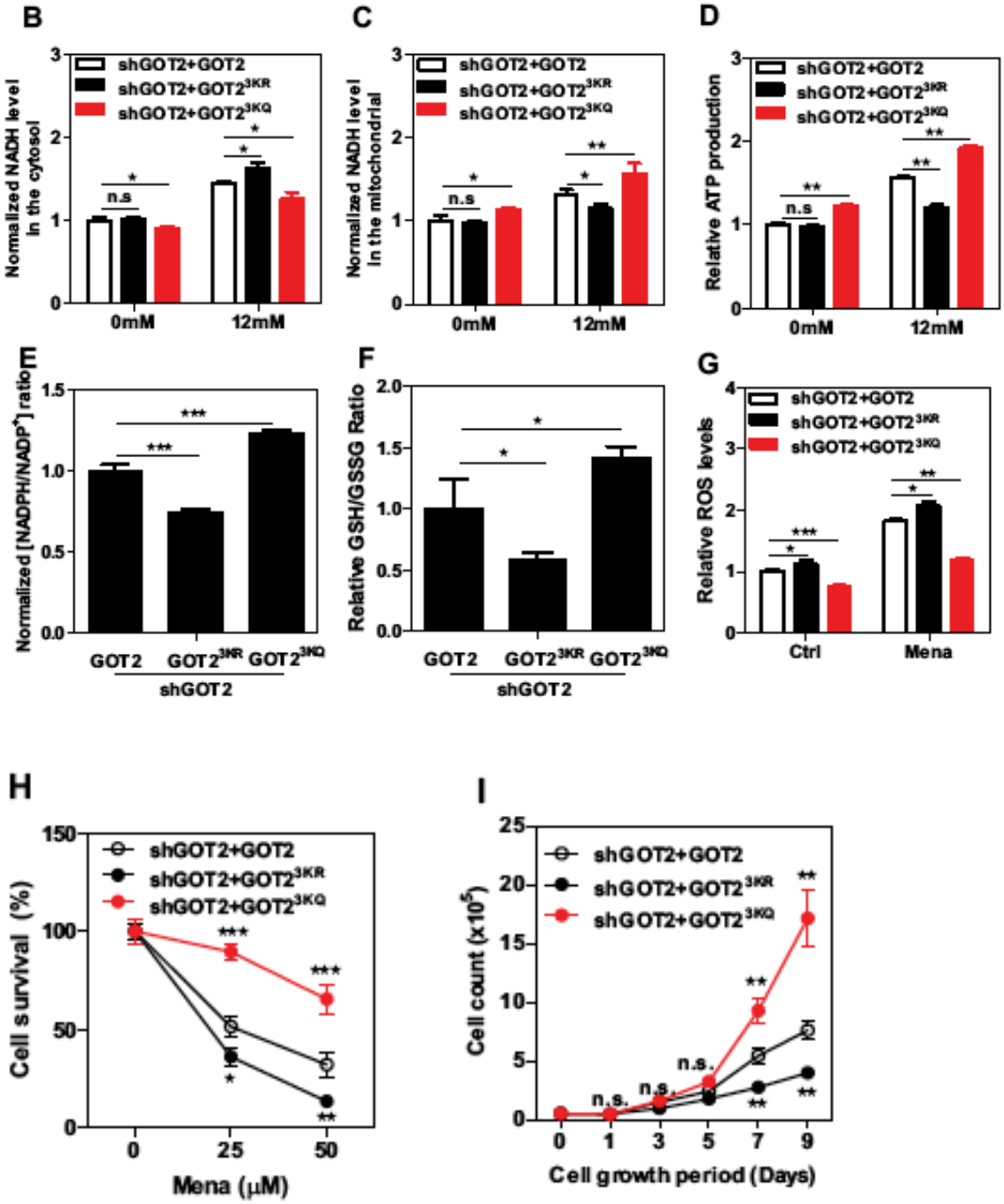
Response: To address this reviewer's comment, we have determined the effect of SIRT3 knock-down on affecting the glucose- and glutamine-dependent regulation of GOT2 acetylation in HEK293T cells. We found that the effect of high glucose and glutamine on changing the K159 acetylation level of GOT2 was diminished by the depletion of SIRT3 (new Figure 3F, 3G).



3. In Figure 4, Got2 3KQ promotes mitochondrial NADH levels, ATP production, increased stress resistance and proliferation, etc compared to wt. While these studies inherently rely on ectopic protein expression, to help establish the physiological significance of Got2 acetylation, a couple of experiments could be done. First, these measurements could be conducted with the 3KR mutant, which fails to interact with MDH. If acetylation of the wt Got2 is physiologically important, one would predict that the 3KR mutant would show higher cytosolic NADH, reduced ATP, reduced proliferation etc compared to wt. Second, these experiments could also potentially be done under high and low glucose, although glucose will regulate the whole metabolic program and manipulation of Got2 acetylation status in isolation may or may not be sufficient to mediate changes in these parameters in response to changes in glucose availability.

Response: Upon the reviewer's request, we have determined the effect of the 3KR mutant GOT2 on the parameters analyzed in Figure 4. We found that 3KR mutation, which disrupts GOT2-MDH2 binding, led to significantly increased NADH level in the cytosol (new Figure 4B), reduced NADH level in the mitochondrion (new Figure 4C), and reduced ATP production (new Figure 4D) in cells upon high glucose (12 mM) treatment. Moreover, 3KR rescued cells exhibited reduced NADPH/NADP ratio (new Figure 4E), reduced GSH/GSSG ratio (new Figure 4F), increased ROS level (new Figure 4G), reduced cell survival under oxidative stress (new Figure 4H), and reduced cell growth (new Figure 4I). In the same assay, 3KQ mutation, which mimics acetylation and increases GOT2-MDH2 binding, resulted in opposite effects.

New Figures 4B, C, D, E, F, G, H, I



Minor points:

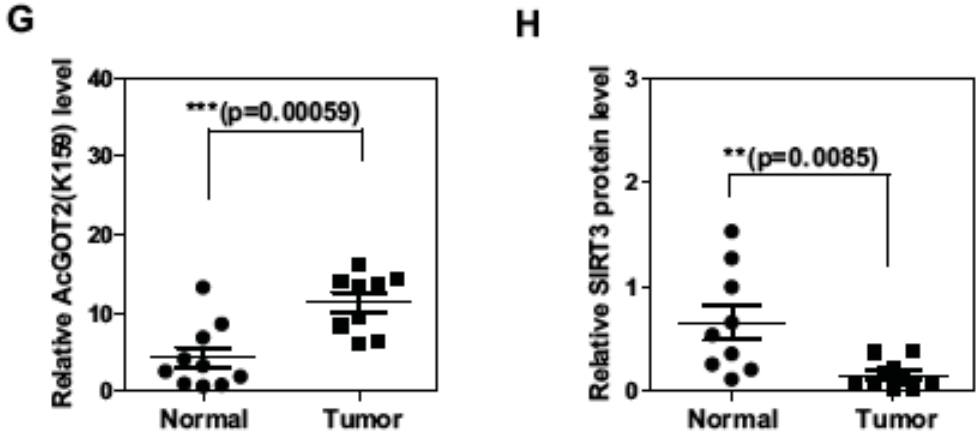
1. AOA is described as a specific malate-aspartate shuttle inhibitor and EGCG as a specific GPDH inhibitor. These inhibitors may be more generally inhibiting glutamine utilization due to their functions in inhibiting transaminases and GDH, respectively. Conclusions from this experiment should be drawn more cautiously.

Response: Following reviewer's suggestion, we have adjusted the statement about AOA and EGCG in the text of our revised manuscript. Thank you for pointing this out.

2. Please check the validity of the statistics on Figures 5 G and H. It looks like the level of AcGot2 and Sirt3 are set to 1 for all normal samples, which artificially removes any variability between the normal samples, inflating the p value. Also G and H are not designated in the Figure legend.

Response: As the reviewer suggested, we have re-quantified the levels of GOT2 K159 acetylation and SIRT3 protein in Figure 5G and H, setting one normal sample as '1' and showing the variability between the normal samples. Based on this, we have recalculated the p value (new Figures 5G and 5H). Moreover, the figure legend of Figure 5G and H has been modified.

New Figures 5G, 5H



Additional correspondence (editor) 21 November 2014

Thank you for submitting the revised version of your manuscript entitled 'GOT2 acetylation enhances its interaction with MDH2 and the malate-aspartate NADH shuttle activity' to us. I have now received the reports from two of the original referees.

As you can see below, while referee #3 supports now publication, referee #2 points out that the results are not holding up to your hypotheses. The physiological relevance of GOT2 acetylation/deacetylation for cell proliferation remains still unclear, and I therefore would at this point have no other choice but to reject your work.

However, we implemented a pre-decision consultation with our authors to give you the chance to position yourself in response to the referee's comments. I could therefore offer to look at a point-by-point response to the remaining concerns and to run it by the critical referee. If you see yourself not in a position to better illustrate the physiological significance of your findings, it would be however in your best interest to seek publication elsewhere at this stage. Please let me know how you would like to proceed. Thank you very much.

2nd Editorial Decision 04 December 2014

Thank you for submitting your manuscript for consideration by the EMBO Journal.
Not having heard from you after my pre-decision consultation, I am now taking a decision on your manuscript based on the referees' reports. As you can see below, referee #2 still thinks that the results are not holding up to your hypotheses. The physiological relevance of GOT2 acetylation/deacetylation for cell proliferation remains still unclear.

Given the necessity for further support for a physiological relevance for publication here, as outlined in my decision letter from 7th Aug 14, and the fact that the EMBO Journal can only afford to accept papers which receive enthusiastic support, I am afraid I have no other choice but to return your manuscript to you.
As outlined before, I would be prepared to re-assess an amended manuscript should you be able to address the remaining concerns.

Thank you in any case for the opportunity to consider this manuscript. I am sorry I cannot be more positive on this occasion, but I hope nevertheless that you will find our referees' comments helpful.

REFEREE COMMENTS

Referee #2:

The new set of experiments improved the manuscript at some points, for instance more convincingly demonstrating the mechanistic link between GOT2 and MDH2. The physiological evidence is still very limited, however, because no in vivo association could be shown. The physiological relevance of two SIRT3-dependent but apparently opposing systems remains unclear, with SOD2 scavenging ROS when SIRT3 is active and GOT2 inducing ROS defense when SIRT3 is inactive.

Furthermore I have three concerns based on the new experiments:
1) The respiration data (new Fig S12B) show lower respiration in 3KQ cells. Can the authors exclude that the increased mitochondrial NADH levels (new Fig 4C) are caused by reduced OXPHOS flux and therefore reduced NADH oxidation at the level of respiratory chain complex I?
2) The NMN supplementation studies seem to demonstrate GOT2 deacetylation (new Fig S14A), but proper controls are lacking to conclude that cell growth is not inhibited (new Fig S14B). For instance, the timing of both experiments is totally different: the former after 4h, the latter continuing for days. Are NAD+ and GOT2 deacetylation still up over this prolonged period? The conditions for the NMN experiments should also be described in the materials and methods.
3) Finally, the main conclusion of the manuscript hinges on the deacetylation of GOT2 and how acetylation state determines pancreatic tumor growth. Nevertheless, if one would accept the lack of proper controls highlighted in my previous point and follow the authors' logic, it is surprising that increasing GOT2 deacetylation with NMN does not reduce cell growth. This seems to be in full contradiction with their main point.

Referee #3:

The authors have addressed my concerns and I recommend publication.

Additional correspondence (author) 07 January 2015

First of all, we would like to thank you for the efforts of handling our manuscript (EMBOJ-2014-89557R) and giving us the opportunity to respond to the referee's comments. We are happy to see that referee #3 is satisfied with the revision. Thus, two of three referees are now positive. Referee #2 still has some concerns, including some misconception concerning the 'opposing' effect of SIRT3-mediated deacetylation on regulating GOT2 and SOD2, two enzymes known to be involved in cellular redox regulation, which we have addressed below in the attached point-by-point response,.

To address your comment and expand the physiological relevance of GOT2 (de)acetylation for cell proliferation, we have conducted additional experiments to address the effect of GOT2 3K acetylation on cell survival and cell growth during glucose depletion.

With these new experiments and clarification, I hope that you will find the paper is improved and offer us an opportunity to submit a revision.

We look forward to hearing from you soon.

Additional correspondence (editor) 09 January 2015

Thank you for your message and for providing a point-by-point response to the remaining concerns regarding your manuscript. I have now read your response and I appreciate that you added further data.
Based on the response provided, I can offer you to submit a revised version to us, which I will then send again to referee #2. You can send the manuscript by email to me or via our online submission system as a new submission.

Resubmission - authors' response 19 January 2015

Referee #2:

'The new set of experiments improved the manuscript at some points, for instance more convincingly demonstrating the mechanistic link between GOT2 and MDH2. The physiological evidence is still very limited, however, because no in vivo association could be shown.'

Response: As mentioned in our previous response letter, due to the lack of antibodies suitable for IP of the endogenous GOT2 and MDH2 proteins, it is technically challenging to test in vivo association between endogenous GOT2 and MDH2. To circumvent this problem, we have generated stable cells with depletion of endogenous GOT2 and re-expression of Flag-GOT2 *at a level similar to endogenous GOT2* (Figure S7). In these cells, the interaction between Flag-GOT2 and endogenous MDH2 is not detectable in cells starved for either glucose or glutamine, but is readily detectable in cells cultured in the presence of either glucose (12 mM) or glutamine (2 mM) (Figures 2G and 2H). Therefore, the glucose/glutamine-regulated GOT2-MDH2 association correlates closely with Flag-GOT2 acetylation (Figures 2A and 2B). We believe that these experiments strongly link the regulation of GOT2-MDH2 complex formation by acetylation with physiological stimuli.

'The physiological relevance of two SIRT3-dependent but apparently opposing systems remains unclear, with SOD2 scavenging ROS when SIRT3 is active and GOT2 inducing ROS defense when SIRT3 is inactive.'

Response: The fact that one SIRT enzyme (SIRT3) participates in the regulation of two proteins (GOT2 and SOD2) that have different or opposite effects (on ROS defense) is not unexpected. There are limited *SIRT* genes (total of seven including four, SIRT4, SIRT5, SIRT6, and SIRT7, that may not participate in lysine deacetylation), while as many as 4,500 proteins have been identified to be acetylated. Thus, each SIRT protein has to participate in the deacetylation of a large number of substrate proteins, including those that may possess seemingly different or even opposite cellular effects. This is no different from some other enzymes that control multiple substrates such as phosphptase PP2A. Of estimated 151 PP2A substrates are p53 and its negative regulator, MDM2 E3 ligase, both became functionally activated following PP2A-mediated dephosphorylation (e.g. *Mol Cell*. 2002. PMID: 11983168; *Oncogene*. 2004. PMID: 14712210; *EMBO J*. 2007. PMID: 17245430). Likewise, p53 activates the expression of many genes, some (e.g. p21) protecting cells by stopping their proliferation whereas others (e.g. Bax and PUMA) causing apoptosis.

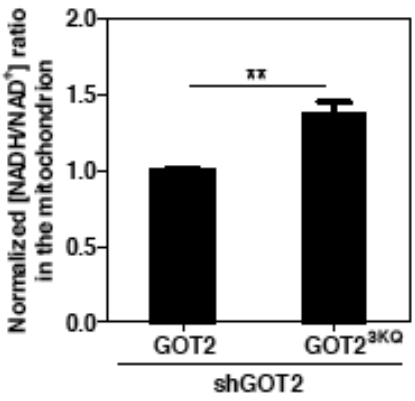
A case at point is that SIRT3 can deacetylate multiple substrates that may have different functions in ROS production and scavenge. Perhaps, the specific regulation is not at the level of SIRT3. Previous studies have identified several metabolic enzymes as direct substrates of SIRT3, including SOD2 (*EMBO Reports*. 2011. PMID: 21566644), IDH2 (*JBC*. 2012. PMID: 22416140), GOT2 (*Nature*. 2013. PMID: 23535601), and ALDH2 (*EMBO Reports*. 2011. PMID: 21720390). All fours enzymes can contribute, directly or indirectly, to ROS defense: SOD2 scavenges ROS through catalyzing the detoxification of superoxide into oxygen and hydrogen peroxide; IDH2 is a major source of NADPH that is the key metabolite for maintaining redox status; GOT2 converts Glu and OAA to α-KG and Asp to promote a serial of reactions, including malate decarboxylation and production of reductant NADPH; and ALDH2 possesses the antioxidant property by oxidizing and detoxify aldehydes. Notably, deacetylation by SIRT3 stimulates the activity of SOD2 and IDH2, but inhibits the activity of ALDH2. Moreover, as shown in this study, deacetylation by SIRT3 may not affect the enzyme activity of GOT2, but can impair GOT2-MDH2 association and therefore inhibit the malate-aspartate shuttle activity. These examples reveal the complexity of SIRT3 in regulating enzymes involved in cellular redox regulation. Considering the large number of acetylated proteins (>4,500) and small number of SIRT subfamily of deacetylases (<7), our findings and reports by others also suggest that the functional consequence of an individual SIRT in cell regulation is unlikely determined by one enzyme-one substrate-one phenotype mode. In the current study, we have demonstrated that the main function of SIRT3-mediated GOT2 deacetylation is to regulate its binding with MDH2 and thus the malate-aspartate NADH shuttle activity and ATP production. Reduced activity of GOT2 in ROS defense following SIRT3-deacetylation, we believe is offset by the reduced GOT2-MDH2 association, malate-aspartate shuttle and ATP production. These discussions have been added into the revised paper.

"*The physiological evidence is still very limited"* (referee #2)

Response: The major physiological role of GOT2 is to control the net transfer of cytosolic NADH through the semipermeable mitochondria inner membrane to maintain a high rate of glycolysis. In the previous version of our manuscript, we demonstrated that acetylation mimetic substitution at three lysine residues of GOT2 (referred to as GOT23KQ) enhanced GOT2-MDH2 binding, resulting in increased mitochondrial NADH and decreased cytoplasmic NADH (Figures 4B and 4C). In both assays, mitochondrial and cytosolic NADH was determined separately. To further strengthen this critical finding, we directly measured the ratio of NADH/NAD+ in the mitochondrion, which reflects the oxidation-reduction status of NADH at the level of respiratory chain complex I and is thus more physiological relevant. We found that the ratio of NADH/NAD+ was significantly increased (by 1.4-fold; $p < 0.01$) in the mitochondria of cells in which endogenous GOT2 was depleted and the 3KQ mutant was ectopically expressed (knock-down and put-back experiment) (new Figure 4D). This result provides further support to the physiological relevance of GOT2 acetylation for regulating mitochondrial NADH/NAD+ redox.

To the best of our knowledge, the current study represents the first report of acetylation of GOT2, the first report of regulation of the GOT2-MDH2 complex formation and thus the malate-aspartate shuttle activity/function by a posttranslational modification, and the first report of posttranslational regulation of the malate-aspartate shuttle in the cell in response to metabolic changes. These findings are supported by our in vivo results showing that GOT2 acetylation is changed in cultured cells exposed to high glucose/glutamine and in mouse tissues during fasting, and that GOT2 acetylation is broadly increased in human pancreatic cancer samples. As such, we would respectively argue that the physiological significance of the study is high and the evidences are strong.

New Figure 4D



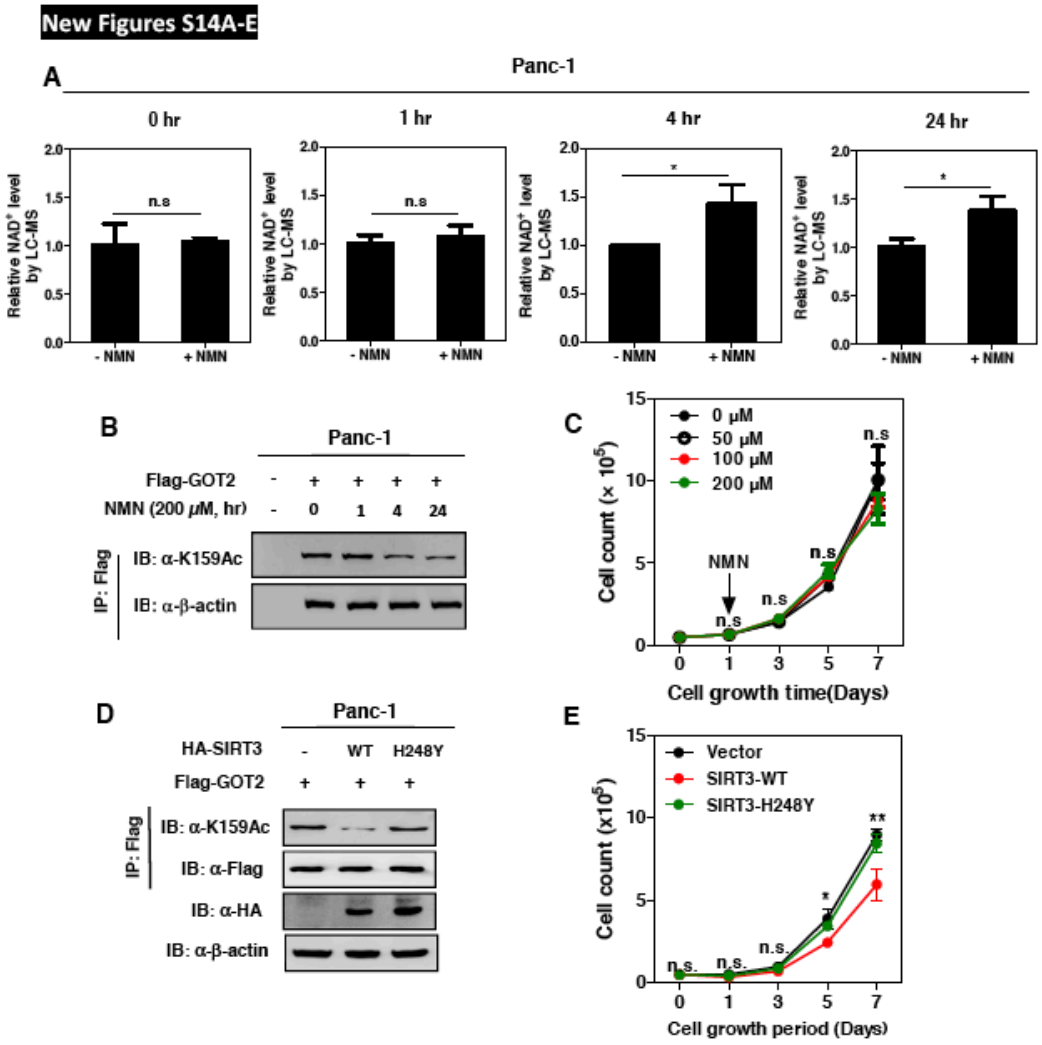
'Furthermore I have three concerns based on the new experiments:
1) The respiration data (new Fig S12B) show lower respiration in 3KQ cells. Can the authors exclude that the increased mitochondrial NADH levels (new Fig 4C) are caused by reduced OXPHOS flux and therefore reduced NADH oxidation at the level of respiratory chain complex I? '

Response: We cannot exclude this possibility, and future investigation is needed to determine how cells expressing 3KQ mutant GOT2 have reduced basal respiration. Nevertheless, we found a significant increase of NADH/NAD+ ratio in the mitochondrion of the 3KQ rescued cells (new Figure4D), indicating that the increased mitochondrial NADH level is mostly likely caused by the enhanced NADH transfer from cytosol to mitochondria, rather than reduced NADH oxidation at the level of respiratory chain complex I.

'2) The NMN supplementation studies seem to demonstrate GOT2 deacetylation (new Fig S14A), but proper controls are lacking to conclude that cell growth is not inhibited (new Fig S14B). For instance, the timing of both experiments is totally different: the former after 4h, the latter continuing for days. Are NAD+ and GOT2 deacetylation still up over this prolonged period? The conditions for the NMN experiments should also be described in the materials and methods.'

Response: In our original experiments, NMN has been replenished to the cells every day for a period of 6 days. This condition for the NMN supplementation experiments has been described in more detail in the revised paper.

To address reviewer's question, we performed LC-MS analysis to measure intracellular levels of NAD+ after different periods of NMN treatment. Our data demonstrated that NAD+ was significantly increased (by 1.4-fold; $p<0.05$) in Panc-1 cells after NMN treatment for 4 hrs, resulting in decreased GOT2 K159 acetylation (new Figures S14A and S14B). Moreover, intracellular NAD+ levels remained high in Panc-1 cells after NMN treatment for 24 hrs, leading to decreased GOT2 K159 acetylation (new Figures S14A and S14B). These results indicate that the effect of NMN on increasing NAD+ can be kept for a prolonged period, such as 24 hours.



In contrast to changes in cellular NAD+ and GOT2 K159 acetylation, cell growth was, however, not affected by NMN treatment (old Figure S14B → new Figure S14C). It has to be noted that NMN broadly activates NAD+-dependent enzymes, including SIRT3 and the other SIRTs. To more specifically test the effect of SIRT3 on cell proliferation, we generated stable Panc-1 cells co-overexpressing Flag-tagged GOT2 and HA-tagged wild-type or catalytic inactive mutant SIRT3, and growth of these stable cells were carefully monitored over a period of 6 days. As expected, stable overexpression of SIRT3, but not SIRT3H248Y mutant, decreased the K159 acetylation level of Flag-GOT2 (new Figure S14D). Moreover, stable overexpression of SIRT3, but not SIRT3H248Y mutant, significantly inhibited cell proliferation (new Figure S14E). These results suggest that SIRT3 suppresses pancreatic cancer cell proliferation, which is at least in part associated with SIRT3-mediated GOT2 deacetylation.

'3) Finally, the main conclusion of the manuscript hinges on the deacetylation of GOT2 and how acetylation state determines pancreatic tumor growth. Nevertheless, if one would accept the lack of proper controls highlighted in my previous point and follow the authors' logic, it is surprising that increasing GOT2 deacetylation with NMN does not reduce cell growth. This seems to be in full contradiction with their main point.'

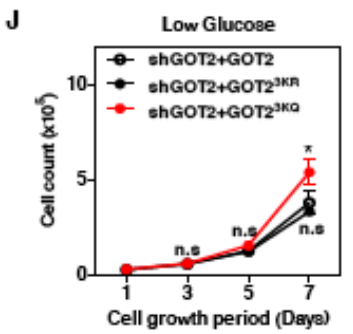
Response: In the previous version of our manuscript, we have shown that expression of deacetylation-mimetic 3KR mutant GOT2 suppresses the proliferation of pancreatic cells, while acetylation-mimetic 3KQ mutant GOT2 promotes cell proliferation (old Figure 4I). These results suggest that GOT2 3K acetylation promotes the net transfer of cytosolic NADH into mitochondria to stimulate ATP production

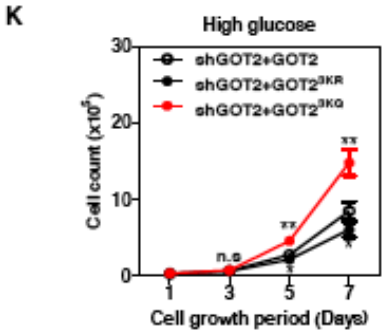
and increases NADPH production to suppress ROS, thereby increasing pancreatic cancer cell proliferation.

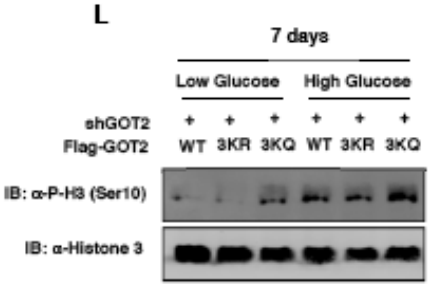
As mentioned above, the failure of NMN to influence cell growth is likely due to the fact that NMN broadly activates NAD+-dependent enzymes, including SIRT3 and the other SIRTs.

Earlier studies have reported that SIRT3 knockout increases the growth of MEF cells cultured in glucose, while SIRT3 overexpression represses the proliferation of human breast cancer CAMA1 cells (Finley et al. *Cancer Cell*. 2011. PMID: 21397863), indicating a tumor suppressor role of SIRT3. Supporting this notion, we found that SIRT3 overexpression led to decreased GOT2 acetylation and significantly inhibited cell proliferation in Panc-1 cells (new Figures S14D and S14E). Moreover, we found that SIRT3 expression was broadly reduced in pancreatic tumor samples (Figure 5H). Our current findings suggest a potential role of SIRT3 in tumor suppression in pancreatic cancer. These results are also consistent with our main conclusion that GOT2 acetylation at 3K promotes pancreatic cancer cell proliferation.
To provide further support to this conclusion and expand the physiological relevance of GOT2 acetylation, we performed additional experiments to examine the effect of GOT2 3K acetylation on cell proliferation and cell survival during glucose depletion. We found that acetylation-mimetic 3KQ mutant GOT2 promoted cell proliferation under the condition of low glucose (0.5 mM glucose) or high glucose (12 mM) (new Figures 4J and 4K). The observed growth advantage was in line with higher levels of histone H3 phosphorylation at Ser10 and reduced apoptosis in the 3KQ rescued cells under low glucose condition (new Figures 4L and 4M). These data are consistent with a cell growth promoting role of GOT2 acetylation even under stress conditions such as glucose depletion.

New Figures 4J-M







M

Low glucose (7 days)	Living cell (PI and Annexin V negative,%)	Apoptotic cell (PI and/or Annexin V positive,%)
shGOT2+GOT2	53.8	46.1
shGOT2+GOT2^{3KR}	52.2	47.7
shGOT2+GOT2^{3KQ}	71.5	28.5

3rd Editorial Decision 26 January 2015

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by one of the original referees (see comments below). I am happy to inform you that the referee is satisfied with the revisions and therefore has no further objections towards publication in The EMBO Journal.

REFEREE COMMENTS

Referee #2:

I have no further comments on the manuscript.

