Riboflavin transporter SLC52A1, a target of p53, suppresses cellular senescence by activating mitochondrial complex II

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Editor-in-Chief: Matthew Welch

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.)
RE: Manuscript #E21-05-0262
TITLE: Riboflavin transporter SLC52A1, a target of p53, suppresses cellular senescence by activating mitochondrial complex II

Dear Prof. Kamada:

Your manuscript, entitled "Riboflavin transporter SLC52A1, a target of p53, suppresses cellular senescence by activating mitochondrial complex II" has been seen by two referees, whose verbatim comments are attached. Both referees felt that your findings, in principle, would be of interest to our MBoC readership. However, both also felt that additional experiments or edits were required to support your conclusion that SLC52A1 acts via p53 to suppress senescence. The main concern was to firm up data regarding the effects of SLC52A1 on p53 and AMPK by quantifying the results in Fig. 4 more convincingly. Additionally, they would like additional methods to ensure there are changes in senescence and how did this affect the U2OS line.

In sum, we would be happy to consider a revised manuscript that satisfies the joint concerns of the referees. Normally, if additional experiments are needed a paper is rejected. However, these experiments can be performed relatively easily and follow directly from your current results. Therefore, we look forward to receiving your revised manuscript, together with a letter indicating the changes you've made and your responses to the referees.

Sincerely,

Jody Rosenblatt
Monitoring Editor
Molecular Biology of the Cell

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Dear Prof. Kamada,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.
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Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

Mitochondrial dysfunction is a known factor associated with SnC. An interesting previous observation was the different mitochondrial response/activity during the time course of SnC establishment: transient increase during the early SnC stimulation but eventually silent in the end. Nagono et al showed in this manuscript that SLC52A1, a riboflavin transporter, acts upstream of the transient mitochondrial activation by increasing the supply of FAD to the OXPHOS complex II. The reviewer is impressed by the clear logic and beautiful dataset presented in this manuscript. Although this is an important paper to the field of SnC, the reviewer does have some technical concerns on some limitations of the current manuscript which probably will be strengthened by a few additional experiments.

1. The manuscript only use beta-gal and BrdU as the means to show changes of SnC. However, it is a common practice in the field to measure the status of p16/p21, SASP, or secreted oxidized HMGB1. Without these markers, it is possible that the relative slight increase of beta-gal (e.g., fig1B) is caused by other survival pathways not end up with SnC. Same concern for BrdU assay that the reduction of BrdU incorporation could be just cell cycle arrest, but not senescence.
2. The author showed in fig.3 about 2 folds increase of mitochondrial MMP after 5hr of Etoposide treatment, however, it is unclear to the reviewer whether the SnC phenotype showed in figure 1-2 was correlated with this time course or not. From the description in the method, the SnC assay was also done after 5 days of Etoposide treatment. This leads to the contradiction that both...
MMP/mitochondria activity increase and SnC establishment happen at the same time, while the authors tend to conclude that somehow MMP/mitochondrial activity is a feedback suppressor of SnC establishment. It will be important to examine both MMP and SnC establishment/status in the same cell to know if there is indeed reverse correlation between MMP and SnC status.

3. For fig.3K, the authors should explain why TTFA did not neutralize the effect of SLC52A2 OE? It seems block MMP did not block much of the SLC52A2 OE effect.

4. The quantification of AMPK and p52 in figure 4 is not convincing due to the lack of statistics. For example, the change of AMPK in fig.4A upon SLC52A1 OE is almost invisible and the lack of statistics further question whether there is any effect. Also, it was not described if such measurement was a result of normalization to the total AMPK. The authors probably want to also measure the ATP/AMP ratio as a separate way to confirm the potential AMPK activation. Similar to the AMPK activity, the statistics of p53 western blot was not described. The reviewer again cannot tell much difference from such blot showed in figure 4A.

5. The reviewer is not sure why the relative abundance of p-P53 is important. The p53 activity is correlated with the amount of p53 proteins that established by the S15, therefore more p-p53, higher activity of p53 in cell cycle arrest and establishment of SnC, therefore the logic of use the relative abundance of p-p53 compared to the total p53 as the indication of cell cycle arrest is not clear. Without such explanation, it is not clear why slightly lower p53 in figure 4A rescue the cells from SnC establishment.

Reviewer #2 (Remarks to the Author):

Title: Riboflavin transporter SLC52A1, a target of p53, suppresses cellular senescence by activating mitochondrial complex II
Manuscript Number: E21-05-0262
Riboflavin is an essential vitamin for cellular growth and development in the form of FAD and FMN as cofactors of several flavozymes that play crucial role in the processes of bioenergetics, redox cycles, DNA repair mechanisms, protein folding, chromatin remodeling, apoptosis, etc. There are three different riboflavin transporters in the humans plays key role in maintaining body homeostatic levels of riboflavin. Though, much attention has been given already to elucidate the role of SLC52A2 and SLC52A3 genes in the normal physiology and their implications in the diseased conditions, because of their involvement in the intracellular transport of riboflavin. This study is majorly focused on addressing the involvement of SLC52A1 gene in the cellular senescence, which could provide new knowledge about the role of the SLC52A1 gene. In this context, the present investigation deserves some attention and the authors have very well addressed the proposed aims with sufficient data. The experiments are carefully performed, data are presented well and described clearly. However, few major concerns have been raised and can be addressed and modified by the authors accordingly.

Comments:

In the Figure 1E, Authors have shown that knockdown of SLC52A1 promoted the etoposide-induced activation of SA-β-gal and the loss of proliferative capability. Though SLC52A1 is silenced by siRNAs, there could be compensatory protective functions from the contributions of other riboflavin transporters such as SLC52A2 and SLC52A3. It would be better if it is clarified be performing experiments or citing relevant literatures.

Page no 7: Effect of riboflavin on replicative senescence was performed only with normal Hs68 cell
line but not in U2OS cell line. Yet the author concludes by saying that 'riboflavin uptake has an anti-
senescent effect at a relatively early stage in a broad variety of cell types'. What was the reason for
not performing this experiment in the U2OS cell line?

In Figure 3A, I could see there is no change in the levels of FAD with the U2OS cells even after
silencing SLC52A1 gene? What could be the reason for this, which can be discussed in the revised
manuscript.
Reviewer #1 (Remarks to the Author):

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1. The manuscript only use beta-gal and BrdU as the means to show changes of SnC. However, it is a common practice in the field to measure the status of p16/p21, SASP, or secreted oxidized HMGB1. Without these markers, it is possible that the relative slight increase of beta-gal (e.g., fig1B) is caused by other survival pathways not end up with SnC. Same concern for BrdU assay that the reduction of BrdU incorporation could be just cell cycle arrest, but not senescence.

We wish to thank the reviewer for the comment. We agree that it is important to add an additional marker for convincingly demonstrating the senescence status. Since we measured p21 status of the cells overexpressing SLC52A1 or treated with riboflavin in the former version of the manuscript (Figure 1D and 2B), we have extended these experiments to the cases of SLC52A1 knockdown and complex II inhibition, both are key experimental conditions exploring the SLC52A1 role in senescence. The results show that SLC52A1 knockdown or complex II inhibition enhanced the etoposide-induced upregulation of p21, that is, the p21 expression is correlated with the extent of senescence determined by SA-β-gal and BrdU proliferation assays. These results consolidate our findings that SLC52A1 impairs senescence by activating complex II. These new data have been added as Figures 1I and 3M, and the manuscript has been modified accordingly.
2. The author showed in fig.3 about 2 folds increase of mitochondrial MMP after 5hr of Etoposide treatment, however, it is unclear to the reviewer whether the SnC phenotype showed in figure 1-2 was correlated with this time course or not. From the description in the method, the SnC assay was also done after 5 days of Etoposide treatment. This leads to the contradiction that both MMP/mitochondria activity increase and SnC establishment happen at the same time, while the authors tend to conclude that somehow MMP/mitochondrial activity is a feedback suppressor of SnC establishment. It will be important to examine both MMP and SnC establishment/status in the same cell to know if there is indeed reverse correlation between MMP and SnC status.

We thank the reviewer for this constructive comment. Since MMP was increased with a peak at 5 days of etoposide treatment and decreased thereafter (at day 7) as pointed out by the reviewer, all of the senescence assays were done at 7 days of etoposide as described in Figure legends. However, we agree that it is important to examine both MMP and senescence status in the same cell. Therefore, we have performed a simultaneous analysis of SA-β-gal and MMP in the same cell. The results show that MMP was significantly higher in the SA-β-gal-negative cells than the SA-β-gal-positive cells (i.e. there is a reverse correlation between MMP and senescent status), supporting our conclusion that MMP/mitochondrial activity is a feedback suppressor of senescence establishment. These new data have been added as Figure 3N.

3. For fig.3K, the authors should explain why TTFA did not neutralize the effect of SLC52A2 OE? It seems block MMP did not block much of the SLC52A2 OE effect.

As the reviewer pointed out, TTFA showed a slight, significant but not complete inhibitory effect on SLC52A1 overexpression (compare bars 7 with 8 in Figure 3K). Based on these results, we concluded that complex II is partially but not solely responsible for the SLC52A1-mediated senescence suppression as described the results section (P. 9, lines 12-14) and, at the same time, discussed the possibility that another FAD-dependent enzyme may also play a role in this mechanism (P. 11, lines 20, 21). To make this point clearer, we have revised the text as below (P. 9, lines 7-9):

“suppression of these senescence markers by SLC52A1 overexpression was significantly impaired by TTFA, although the effect on SA-β-gal was only modest (Figure 3, K and L)”
4. The quantification of AMPK and p52 in figure 4 is not convincing due to the lack of statistics. For example, the change of AMPK in fig.4A upon SLC52A1 OE is almost invisible and the lack of statistics further question whether there is any effect. Also, it was not described if such measurement was a result of normalization to the total AMPK. The authors probably want to also measure the ATP/AMP ratio as a separate way to confirm the potential AMPK activation. Similar to the AMPK activity, the statistics of p53 western blot was not described. The reviewer again cannot tell much difference from such blot showed in figure 4A.

We appreciate the reviewer’s comment on this point. We agree that it is important to show statistical significance in phosphorylation status of AMPK and p53. Therefore, we have repeated these experiments three times and demonstrated that both the phosphorylation of AMPK and p53 were significantly impaired by SLC52A1 overexpression and conversely enhanced by complex II inhibition (Figure 1 in this rebuttal letter). The phosphorylation levels of AMPK and p53 were normalized to their total protein levels as described in the legend of Figure 4. These results support our conclusion that SLC52A1-complex II impairs the AMPK-p53 activity in response to senescence-inducing stimuli. The results of statistical analysis have been added to Figure 4, A-C.
Figure 1. Complex II-mediated mitochondrial activation attenuates the AMPK-p53 pathway. (A) U2OS cells transfected with pcDNA3-HA-SLC52A1 and treated with 2 µM etoposide for 5 days (three batches, Batches 1-3) were subjected to immunoblot analysis. The phosphorylation levels of AMPK and p53 relative to their total protein
levels were quantified using NIH ImageJ software. Immunoblot images (left) and the results of their statistical analysis (right) are shown. (B, C) U2OS (B) and Hs68 (C) cells treated with 400 µM TTFA and etoposide at 2 and 0.5 µM, respectively, for 5 days (three batches, Batches 1-3) were subjected to immunoblot analysis. Immunoblot images (left) and the results of their statistical analysis (right) are shown. Data are mean ± s.d. (n = 3 independent cultures). Statistical significance is shown using the Student’s t-test analysis; *P < 0.05; **P < 0.01.

5. The reviewer is not sure why the relative abundance of p-P53 is important. The p53 activity is correlated with the amount of p53 proteins that established by the S15, therefore more p-p53, higher activity of p53 in cell cycle arrest and establishment of SnC, therefore the logic of use the relative abundance of p-p53 compared to the total p53 as the indication of cell cycle arrest is not clear. Without such explanation, it is not clear why slightly lower p53 in figure 4A rescue the cells from SnC establishment. Although p53 phosphorylation at S15 plays a key role in the stabilization and activation of p53, the p53 stability itself is also regulated by other phosphorylation events (e.g. T18, S20, and so on), acetylation, and glucosylation (e.g. reviewed by Gu B and Zhu WG. Int J Biol Sci. 8: 672-84, 2012). Because the main aim of these experiments was to determine whether AMPK activation leads to the p53 phosphorylation at S15 (the phosphorylation target of AMPK), normalization of phosphorylated p53 levels to their total protein was performed to exclude the p53-stabilizing effect of those post-translational modifications, which is considered to be the appropriate way to measure specific phosphorylation changes in this situation. To clarify this point, we have revised the text as below:

“For example, AMPK phosphorylates p53 at Ser15, leading to p53 stabilization and activation” (P. 10, lines 1, 2)

“suggesting that phosphorylation of AMPK and p53 was inhibited by SLC52A1 overexpression” (P. 10, lines 5, 6)

“These results suggest that phosphorylation of AMPK and p53 is impaired by complex II-dependent mitochondrial activation” (P. 10, line 12, 13)

Regarding the relationship between the p53 levels and cell cycle arrest, there is no discrepancy between the p53 phosphorylation level (regardless of whether relative or absolute abundances) and senescence status, given that p53 phosphorylation was
decreased by SLC52A1 overexpression and conversely increased by complex II inhibition (Figure 4, A-C).

Reviewer #2 (Remarks to the Author):

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Manuscript Number: E21-05-0262
Riboflavin is an essential vitamin for cellular growth and development in the form of FAD and FMN as cofactors of several flavozymes that play crucial role in the processes of bioenergetics, redox cycles, DNA repair mechanisms, protein folding, chromatin remodeling, apoptosis, etc. There are three different riboflavin transporters in the humans plays key role in maintaining body homeostatic levels of riboflavin. Though, much attention has been given already to elucidate the role of SLC52A2 and SLC52A3 genes in the normal physiology and their implications in the diseased conditions, because of their involvement in the intracellular transport of riboflavin. This study is majorly focused on addressing the involvement of SLC52A1 gene in the cellular senescence, which could provide new knowledge about the role of the SLC52A1 gene. In this context, the present investigation deserves some attention and the authors have very well addressed the proposed aims with sufficient data. The experiments are carefully performed, data are presented well and described clearly. However, few major concerns have been raised and can be addressed and modified by the authors accordingly.
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In the Figure 1E, Authors have shown that knockdown of SLC52A1 promoted the etoposide-induced activation of SA-β-gal and the loss of proliferative capability. Though SLC52A1 is silenced by siRNAs, there could be compensatory protective functions from the contributions of other riboflavin transporters such as SLC52A2 and SLC52A3. It would be better if it is clarified by performing experiments or citing relevant literatures.
We thank the reviewer for this constructive comment. We agree that it would be important to investigate whether there is compensatory expression of other riboflavin transporters (SLC52A2 and SLC52A3). Therefore, we have examined the expression of SLC52A2 and SLC52A3 in the SLC52A1-depleted cells treated with etoposide. The results show that SLC52A1 knockdown did not affect the expression of SLC52A2 and SLC52A3, suggesting that SLC52A2 and SLC52A3 have no compensatory mechanism for the downregulation of SLC52A1 at least under our experimental conditions. These new data have been added as Figure 1F, and the manuscript has been modified accordingly.

**Page no 7:** Effect of riboflavin on replicative senescence was performed only with normal Hs68 cell line but not in U2OS cell line. Yet the author concludes by saying that 'riboflavin uptake has an anti-senescent effect at a relatively early stage in a broad variety of cell types'. What was the reason for not performing this experiment in the U2OS cell line?

The reviewer’s comment is correct. Because U2OS cells are a transformed cell line derived from a human osteosarcoma, the experiments suggested by the reviewer seem not to be appropriate in the sense that they are resistant to replicative senescence. Therefore, we have concluded that the statement “riboflavin uptake has an anti-senescent effect at a relatively early stage in a broad variety of cell types and stimuli” (P. 7, lines 8-10) is an overstatement and removed the words “in a broad variety of cell types and stimuli” from the original text to make this point clearer.

*In Figure 3A, I could see there is no change in the levels of FAD with the U2OS cells even after silencing SLC52A1 gene? What could be the reason for this, which can be discussed in the revised manuscript.*

We wish to thank the reviewer for the comment. Whereas the change in the FAD levels was effectively inhibited by knockdown of SLC52A1 in the presence of etoposide (compare bars 3 and 4 of Figure 3A), SLC52A1 knockdown alone did not affect the FAD levels in the static state (in the absence of etoposide) (compare bars 1 with 2). We observed that SLC52A1 knockdown alone had no significant effect on the SLC52A1 expression (Figure 1E) and senescence markers (SA-β-gal, BrdU proliferation assay, and the expression of p53 and p21; Figure 1, G-I), collectively suggesting that in the
static state, SLC52A1 does not take part in the riboflavin uptake and consequently cellular metabolism. This view is also consistent with our recent study demonstrating that senescence-inducing stimuli is essential for the induction of SLC52A1 expression (Nagano T et al., Sci Rep. 6: 31758, 2016). To clarify the effect of SLC52A1 knockdown on cellular functions in the static state, we have revised the text as below (P. 6, lines 3-5):

“knockdown of SLC52A1 promoted the etoposide-induced activation of SA-β-gal and the loss of proliferative capability, while SLC52A1 knockdown alone had no significant effect (Figure 1, G and H)”

All changes made:
Figures:
The results of statistical analysis for immunoblot data have been added to Figure 4, A-C.
The immunoblot images in Figure 4, B and C have been replaced by the images that more closely match the statistical results.

Several new panels were added:
The new Figure 1F shows that knockdown of SLC52A1 did not affect the expression of other SLC52A family (SLC52A2 and SLC52A3) in the presence of etoposide.
The new Figure 1I shows that etoposide-induced increase in p53 and p21 was enhanced by knockdown of SLC52A1.
The new Figure 3M shows that etoposide-induced p21 increase was enhanced by complex II inhibition.
The new Figure 3N shows that there is a reverse correlation between mitochondrial membrane potential and senescent status.

The text and figure legends were altered accordingly.
RE: Manuscript #E21-05-0262R

TITLE: "Riboflavin transporter SLC52A1, a target of p53, suppresses cellular senescence by activating mitochondrial complex II"

Dear Prof. Kamada:

We have received your revised submission and are satisfied with your responses to the reviewers and the revisions your team has made. Therefore, we would be happy to accept your manuscript to the Molecular Biology of the Cell.

Sincerely,
Jody Rosenblatt
Monitoring Editor
Molecular Biology of the Cell

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Dear Prof. Kamada:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

Within approximately four weeks you will receive a PDF page proof of your article.

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