The TIM22 complex mediates the import of Sideroflexins and is required for efficient mitochondrial one-carbon metabolism.

Thomas Jackson, Daniella Hock, Kenji Fujihara, Catherine Palmer, Ann Frazier, Yau Low, Yilin Kang, Ching-Seng Ang, Nicholas Clemons, David Thorburn, David Stroud, and Diana Stojanovski

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

RE: Manuscript #E20-06-0390
TITLE: The TIM22 complex mediates the import of Sideroflexins and is required for efficient mitochondrial one-carbon metabolism.

Dear Dr. Stojanovski:

Your paper entitled "The TIM22 complex mediates the import of Sideroflexins and is required for efficient mitochondrial one-carbon metabolism" has been read by two expert referees. They both found the work interesting. However, they both suggested revisions, in some cases addressing the same points, involving additional data that may require re-review.

Among the points raised, I think it is particularly important to "compare the AGK-deficient cells to AGK-deficient cells re-expressing WT AGK and kinase-dead mutant of AGK" as pointed out by referee #1 (point 2), and elaborated upon by referee #2 (point 6).

Please examine the reviewer comments carefully. If you would like to submit a revised version of the paper, please include with your revised manuscript a point by point summary of your responses to each of the comments, and the revisions you make to the paper. I will reserve the possibility of having the revised manuscript re-reviewed, hopefully by the same two referees.

Thank you for submitting this interesting work to Molecular Biology of the Cell.

Sincerely,
Thomas D. Fox

Sincerely,

Thomas Fox
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Stojanovski,

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 haveopted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at mboc@ascb.org.

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
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Reviewer #1 (Remarks to the Author):

Acylglycerol kinase (AGK) is a mitochondrial lipid kinase but it was also shown to be a peripheral component of the mitochondrial inner membrane translocase complex, TIM22, which is required for the stability of TIM22. The lipid kinase activity of AGK appears to be not required for its TIM22 function. Mutations in AGK cause the Sengers syndrome mitochondrial disease. In this manuscript proteomic analyses was conducted in cells derived from Sengers syndrome patients and from cells in which AGK was deleted. Not surprisingly the protein abundance of the TIM22 substrates, the mitochondrial carriers SLC25A proteins were decreased. Unexpectedly, the abundance of the five trans membrane SFXN proteins was also decreased. The authors suggest that the SFXN proteins, which are serine carriers in the IMM, are also TIM22 substrates. Consistently, AGK deficient cells are more dependent on extracellular serine. The most important conclusion from this manuscript is that SFXN proteins are targets of TIM22. In addition, the authors observed reduction in enzymes involved in the mitochondrial one carbon metabolism and proteins components of complex I. However, some concerns should be addressed before publication.

Specific points

1. The authors should determine if patients' derived cells and AGK deficient cells are impaired in respiration.
2. It is important that the authors should compare the AGK-deficient cells to AGK-deficient cells re-expressing WT AGK and kinase-dead mutant of AGK.
3. The authors should corroborate the data in Figs. 1 D, 1E and 2D with immunoblotting.
4. Fig. 5A - it is not clear why Tim22 depletion affects only MTHFD2 protein level and not SHMT2 protein level.
5. The authors should consider the possibility that one carbon metabolism was impaired also because of the reduction in SLC25A38.
6. The authors should determine if the AGK depleted cells are dependent on extracellular glycine.
7. Although the authors deduced from their data that the one carbon metabolism in the mitochondria is impaired, the manuscript could be improved using isotopically labeled serine and measure labelled formate.

Reviewer #2 (Remarks to the Author):

The manuscript by Jackson et al. is a continuation of Stojanowski-group's interesting research on mitochondrial import machinery and its complex connections with metabolism. Here, they characterize mitochondrial proteomic consequences of Tim22 complex defects, and especially its subunit AGK, a lipid kinase, the functional defects of which they recently linked to explain Sengers syndrome.

Excitingly, in this manuscript, they link AGK to a recently described anabolic mitochondrial stress response, involving mitochondrial one-carbon metabolic enzymes. They find that AGK is required for import and/or assembly of serine transporters, SHMT2 and mitochondrial THF dehydrogenases. They decided to further characterize the recently discovered serine transporters, SFXN proteins, the functions of which in cell physiology are still rather unknown. Utilizing AGK-mutant patient cells and different KO cell models they find wide changes in mitochondrial proteomes, significantly overlapping in the different cell systems. The results especially highlight the 1C metabolic enzymes and complex I subunits as TIM22 substrates /dependent on AGK function. As the authors state, their results function as a resource of the wide effects of AGK lipid kinase in remodeling mitochondrial proteomes, with special emphasis on 1C metabolism and oxphos CI. The text is well written, and results seem solid. I have some specific comments.

1) The authors find complex I to be especially downregulated as a consequence of AGK deficiency but report no significant effects in the other oxphos enzymes. Complex I is the enzyme with the highest number of mtDNA encoded subunits, and depletion of serine amino acid as a consequence of AGK decrease would affect its synthesis most. What is the serine content of CI compared to other complexes? Could the downregulation be just a consequence of decreased serine availability? Could serine supplementation affect CI subunit amounts?

2) The authors study the localization of SFXN1 and 2 and confirm them to be inner membrane proteins. What about SFXN3? Why was this protein not studied for localization, while it was included in the following experiments?

3) The authors refer several times to recent studies reporting upregulation of mitochondrial 1C cycle as a response to mitochondrial DNA expression diseases. This novel stress response has been reported in different model systems, from mice to patients with deletions of mtDNA or defects of mt-translation. However, the authors find the response-related mt-1C-cycle enzymes to be decreased in AGK cells, i.e. the opposite to previous findings. They discuss that this might be a stress response by itself, which I don't find such an attractive possibility. I find a more likely explanation to be a mere transporter defect, or a function related to AGK lipid kinase. Actually, the authors have an exciting opportunity to test for the importance of the mtISR response for cells: can their AGK-KO cells upregulate mtISR? If they block mitochondrial translation by actinonin, is cell viability affected? This stress typically upregulates MTHFD2 highly, and if the enzyme cannot be transported to mitochondria in AGK-deficiency, how do the cells respond? Please test.
4) The authors state that GEO analysis indicated upregulation of catabolic processes (FAO, amino acid catabolism) and suggest that this is an attempt to compensate for oxphos deficiency (Complex I). However, typically oxphos deficiency causes further downregulation of respiratory chain and fatty acid oxidation, and increased reliance on glycolysis, and therefore induction of mitochondrial biogenesis is studied as a treatment strategy. How do the authors explain this? Do they find transcriptional upregulation of mitochondrial biogenesis inducers, such as PGC1alpha? Is mitochondrial mass increased?
6. What role do the authors suggest the AGK lipid kinase activity to play in regulating mitochondrial proteome? Previously, they published that AGK kinase activity was dispensable for its function as a Tim22 subcomplex. However, would the proteome of from cells with a kinase-dead enzyme be identical with that of WT? Are there subclasses of mitochondrial proteins that would require lipid phosphorylation to occur e.g for their assembly while being transported? This would be interesting especially concerning Complex I. As the authors have studied the kinase-dead enzyme before, they may have the cell line available, and the proteome could be studied. This experiment would be highly interesting and increase the information and novelty content of the paper further.

Minor comments:
Introduction: "Mitochondrial diseases are genetic disorders that arise due to defective ATP production within the mitochondrion". There is actually very little evidence that ATP production would be the causative factor in mitochondrial diseases. It is true that ATP synthesis is compromised, but the wide tissue specific symptoms indicate more complex disease mechanisms, which very likely involve the processes described in this manuscript - wide effects to tissue-specific metabolism in the mitochondria and the cytoplasm. Please modify the sentence.

Figure 6E, graphs in the middle and right. Does the "control" in these curves actually mean AGK-KO and SFNX1KO in full medium, and then these KO cells in -ser and -ser +formate? Please revise if so, because to use the word "control" first as a true WT cell line (left graph) and then for KO cells in normal medium is confusing. Please clarify.

THE UNIVERSITY OF MELBOURNE

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18 November, 2020

Dear Prof. Fox,

Thank you for your comments regarding our manuscript "The TIM22 complex mediates the import of Sideroflexins and is required for efficient mitochondrial one-carbon metabolism". We have addressed the majority of concerns raised by both reviewers, including your request to include additional data using the AGK kinase-dead mutant. Please find a point-by-point response below.

Reviewer 1:
[1] The authors should determine if patients' derived cells and AGK deficient cells are impaired in respiration.
We have performed several experiments to analyse respiration in both patient cells and AGK^{KO} HEK293 cells. Oxygen consumption rates using a Seahorse analyser (Supplementary Figure 1D) show that AGK^{KO} cells have statistically significant reductions in both basal and maximal oxygen consumption rates, confirming a functional defect in respiration. This data corroborates previously published findings, from our group (Kang et al., 2017) and others (Vukotic et al., 2017). Interestingly, the AKG kinase mutant (AGK^{G126E}) was only partially able to rescue the respiration defect. As an additional measure to assess respiration in AGK^{KO} HEK cells, we assessed ATP synthesis following supplementation with substrates for Complex I or Complex II (Supplementary Figure 1E). Consistent with the relatively mild OCR defects, we did not detect a significant decrease in rates of ATP synthesis under any conditions.

Respiration measurements in Sengers patient derived fibroblasts was performed using Seahorse in both patient fibroblasts and three control cell lines (Supplementary Figure 2E). A slight decrease in basal and maximal OCR was observed for P1, however the results lay mostly within the variation observed across the three controls analysed. The absence of a large respiration defect in the patient fibroblasts is consistent with the absence of a significant reduction in the steady state level of Complex I in these cells.

[2] It is important that the authors should compare the AGK-deficient cells to AGK-deficient cells re-expressing WT AGK and kinase-dead mutant of AGK.
Numerous experiments have now been included employing the AGK kinase-dead mutant. Label-free quantitative proteomics for mitochondria isolated from control, AGK^{KO,} AGK^{KO+WT} and AGK$^{KO+G126E}$ HEK293 cells has been performed. For most classes of protein downregulated in the AGK^{KO} cells, including SLC25 carrier proteins, sideroflexins, and Complex I subunits and assembly factors, re-expression of either WT or kinase-dead AGK was sufficient to complement protein levels (Figure 1E, Supplementary Figure 6B). This suggests that most changes observed in the AGK^{KO} cells result from impairment of TIM22 complex activity, rather than loss of the AGK lipid kinase activity. In addition to proteomics we have included the

kinase-dead mutant in additional biochemical analysis including: seahorse analysis (Supplementary Figure 1D); BN-PAGE analysis of respiratory chain complexes and assembly intermediates (Supplementary Figure 1C); and SDS-PAGE analysis of complex I subunits and assembly factors (Supplementary Figure 1A, 1B).

[3] The authors should corroborate the data in Figs. 1 D, 1E and 2D with immunoblotting.
Figure 1D (new 1C) demonstrates reduced levels of mitochondrial carrier proteins (SLC25) in the AGK^{KO} HEK293 cells. This is corroborating previously published data from our group (Kang et al., 2017) and others (Vukotic et al., 2017) which have been referenced in text. Kang et al., 2017 contains immunoblots showing reduced levels of ANT3 (SLC25A6) and GC1 (SLC25A22) via both SDS-PAGE (Figure 2A) and BN-PAGE (Figure 2E). Vukotic et al., 2017 contains an immunoblot showing reduced levels of ANT3 (SLC25A4) via SDS-PAGE (Figure 2A).

Figure 1E (new 1D) depicted the levels of proteins across OXPHOS complexes. We have included SDS-PAGE and corresponding quantifications of subunits and assembly factors from Complex I, as well as representative subunits from Complexes II, III and IV (Supplementary Figure 1A, 1B); and BN-PAGE analysis of respiratory chain complexes and assembly intermediates (Supplementary Figure 1C).

Figure 2D (new 2C) depicted levels of enzymes in 1C metabolism. We have performed SDS-PAGE and quantifications to corroborate the changes to the levels of MTHFD2, SHMT2, and SFXN1 in both patient fibroblasts (Figure 2E, 2F). In addition, we have performed qPCR on both patient fibroblasts (Supplementary Figure 3B) and show that the reduction in MTHFD2 and SHMT2 is due to transcriptional regulation, while the changes to SFXN1 are not transcriptionally regulated.

[4] Fig. 5A - it is not clear why Tim22 depletion affects only MTHFD2 protein level and not SHMT2 protein level.
Figure 5 focuses on the Sideroflexins as substrates of the TIM22 complex. To gauge some insight into this query we assessed if either MTHFD2 or SHMT2 require the TIM22 complex for import into mitochondria (Supplementary Figure 3A). Radiolabelled MTHFD2 and SHMT2 were imported into mitochondria isolated from WT or AGK^{KO} HEK cells and both proteins exhibited normal import kinetics into both cell lines suggesting they are not substrates of AGK or the TIM22 complex. Indeed, the analysis highlight proteolytic processing of both proteins suggesting they are TIM23 substrates.

Our data suggests that depletion of MTHFD2 following Tim22 KD is unlikely due impaired biogenesis. Koufaris and Nilsson, 2018, showed that MTHFD2 has a shorter protein half-life than other 1C metabolism enzymes, including SHMT2. Tim22 knock-down experiments were performed for 72 hours, and we suggest that the limited duration of the stress meant that the rapidly regulated MTHFD2 was depleted, while SHMT2, which has a longer half-life remained stable. This is supported by qPCR data (Supplementary Figure 7B) where the levels of MTHFD2 transcript is not significantly altered between control and AGK^{KO} cells, hinting towards a protein turnover event.

[5] The authors should consider the possibility that one carbon metabolism was impaired also because of the reduction in SLC25A38.
SLC25A38 is the mitochondrial glycine carrier. Glycine can be utilised as a source of 1C units through the glycine cleavage system, and it is possible that impaired glycine import could lead to an insufficiency of 1C units and subsequent impairment of 1C metabolism. If AGK^{KO} cells are utilising extracellular glycine as a source of 1C units, we would expect that growth in glycine free media would produce a proliferation defect that would be rescuable through addition of formate. Control and AGK^{KO} cells were grown in the absence of extracellular glycine, but we observed no growth defect for AGK^{KO} cells (Figure 6F), suggesting that glycine is not a major source of 1C units for these cells. Accordingly the reduction in SLC25A38 levels are unlikely to underpin the impairment of 1C metabolism observed in AGK^{KO} cells. Indeed, we detected no changes to the levels of SLC25A38 in any of our proteomic datasets.

[6] The authors should determine if the AGK depleted cells are dependent on extracellular glycine.

See Reviewer 1 comment [5].

[7] Although the authors deduced from their data that the one carbon metabolism in the mitochondria is impaired, the manuscript could be improved using isotopically labelled serine and measure labelled formate.
We agree that that serine labelling as a means to measure formate would be a wonderful addition to this manuscript, however this approach possesses numerous experimental challenges that make it difficult for those not experienced in the technique. Given this, we feel this request is beyond the scope of this current study, as it would require significant time and experimental work to establish the technique and/or establishment of new collaborations required.

Reviewer 2:
[8] The authors find complex I to be especially downregulated as a consequence of AGK deficiency but report no significant effects in the other oxphos enzymes. Complex I is the enzyme with the highest number of mtDNA encoded subunits, and depletion of serine amino acid as a consequence of AGK decrease would affect its synthesis most. What is the serine content of CI compared to other complexes? Could the downregulation be just a consequence of decreased serine availability? Could serine supplementation affect CI subunit amounts?
Mitochondrial translation of Complex I subunits was analysed in control and AGK^{KO} HEK293 cells using pulse-SILAC labelling to track the synthesis of mtDNA encoded proteins (Figure 6G). We see no defect in the rate of synthesis of ND1, ND2, ND4, or ND5, suggesting that the observed Complex I defect arises through a different mechanism, likely impaired biogenesis of Complex I subunits or assembly factors that utilise TIM22 complex for their import.

[9] The authors study the localization of SFXN1 and 2 and confirm them to be inner membrane proteins. What about SFXN3? Why was this protein not studied for localization, while it was included in the following experiments?
This has now been included (Figure 4D).

[10] The authors refer several times to recent studies reporting upregulation of mitochondrial 1C cycle as a response to mitochondrial DNA expression diseases. This novel stress response has been reported in different model systems, from mice to patients with deletions of mtDNA or defects of mt-translation. However, the authors find the response-related mt-1C-cycle enzymes to be decreased in AGK cells, i.e. the opposite to previous findings. They discuss that this might be a stress response by itself, which I don't find such an attractive possibility. I find a more likely explanation to be a mere transporter defect, or a function related to AGK lipid kinase. Actually, the authors have an exciting opportunity to test for the importance of the mtISR response for cells: can their AGK-KO cells upregulate mtISR? If they block mitochondrial translation by actinonin, is cell viability affected? This stress typically upregulates MTHFD2 highly and if the enzyme cannot be transported to mitochondria in AGK-deficiency, how do the cells respond?
See Reviewer 1 comment [4] which shows transport of MTHFD2 or SHMT2 does not rely on the TIM22 complex and therefore does not underpin the observed reduction in 1C enzymes.

As suggested, we performed a dose-response experiment that shows AGK^{KO} cells do not have any increased sensitivity to actinonin, suggesting that the 1C dysfunction observed does not compromise the ability of the cells to resist this particular mitochondrial stress (Supplementary Figure 7A). qPCR data demonstrates that the mtISR response, including transcriptional upregulation of MTHFD2 and SHMT2, is intact in both WT and AGK^{KO} HEK293 cells (Supplementary Figure 7B). Thus, the observed downregulation of MTHFD2 following stress in HEK cells involves another mechanism, such as increased intra-

mitochondrial turnover. As indicated above, Koufaris and Nilsson 2018 demonstrated that MTHFD2 is a rapidly regulated protein with a short half-life.

[11] The authors state that GEO analysis indicated upregulation of catabolic processes (FAO, amino acid catabolism) and suggest that this is an attempt to compensate for oxphos deficiency (Complex I). However, typically oxphos deficiency causes further downregulation of respiratory chain and fatty acid oxidation, and increased reliance on glycolysis, and therefore induction of mitochondrial biogenesis is studied as a treatment strategy. How do the authors explain this? Do they find transcriptional upregulation of mitochondrial biogenesis inducers, such as PGC1alpha? Is mitochondrial mass increased?
We have included a qPCR experiment demonstrating that *PGC1a* is not transcriptionally upregulated in patient fibroblasts (Supplementary Figure 2C). We also analysed mitochondrial content in the context of whole cell lysates via western blot and determined that there was no increase in the proportion of mitochondrial proteins relative to non-mitochondrial proteins (Supplementary Figure 2B).

[12] What role do the authors suggest the AGK lipid kinase activity to play in regulating mitochondrial proteome? Previously, they published that AGK kinase activity was dispensable for its function as a Tim22 subcomplex. However, would the proteome of from cells with a kinase-dead enzyme be identical with that of WT? Are there subclasses of mitochondrial proteins that would require lipid phosphorylation to occur e.g for their assembly while being transported? This would be interesting especially concerning Complex I. As the authors have studied the kinase-dead enzyme before, they may have the cell line available, and the proteome could be studied. This experiment would be highly interesting and increase the information and novelty content of the paper further.
Related to reviewer 1 comment [2].

Label-free quantitative proteomics for mitochondria isolated from control, $AGK^{KO,}$ AGK^{KO+WT} and $AGK^{KO+G126E}$ HEK293 cells confirmed that known substates of TIM22, including SLC25 carriers, sideroflexins, and Complex I subunits/assembly factors, do not rely on AGK kinase-activity for their biogenesis (Figure 1E, Supplementary Figure 6B). Biochemical analysis on AGK^{KO} cells re-expressing WT or kinase-dead AGK confirms that the kinase-dead mutant is able to rescue the levels of Complex I subunits as assessed by SDS-PAGE (Supplementary Figure 1A, 1B). We have also included seahorse oxygen consumption rate analysis, which demonstrated that kinase-dead AGK was only able to partially rescue the OCR defect observed in the AGK^{KO} cells (Supplementary Figure 2D).

Minor comments:

[13] Introduction: "Mitochondrial diseases are genetic disorders that arise due to defective ATP production within the mitochondrion". There is actually very little evidence that ATP production would be the causative factor in mitochondrial diseases. It is true that ATP synthesis is compromised, but the wide tissue specific symptoms indicate more complex disease mechanisms, which very likely involve the processes described in this manuscript - wide effects to tissue-specific metabolism in the mitochondria and the cytoplasm. Please modify the sentence.
This sentence has been modified.

[14] Figure 6E, graphs in the middle and right. Does the "control" in these curves actually mean AGK-KO and SFNX1KO in full medium, and then these KO cells in -ser and -ser +formate? Please revise if so, because to use the word "control" first as a true WT cell line (left graph) and then for KO cells in normal medium is confusing. Please clarify.
We thank the reviewer for this observation. The 'control' line on each plot does indeed represent growth of the specified cell line in full media, and we have changed the labelling to reflect this.

RE: Manuscript #E20-06-0390R
TITLE: "The TIM22 complex mediates the import of Sideroflexins and is required for efficient mitochondrial one-carbon metabolism."

Dear Dr. Stojanovski:

Your revised manuscript entitled "The TIM22 complex mediates the import of Sideroflexins and is required for efficient mitochondrial one-carbon metabolism" has been read by the same two referees who saw the original submission.

As you can see, Referee #1 is satisfied that paper is ready to be accepted. Referee #2, however, has raised several questions regarding the revised paper, dealing with interpretive issues.

Please consider carefully the comments of #2 and address them in the Discussion. In your response, please include a point by point summary of any revisions you make to the paper.

Thank you again for submitting this interesting work to Molecular Biology of the Cell.

Sincerely,
Thomas D. Fox
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Stojanovski,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) 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.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

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 version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

Authors of Articles and Brief Communications whose manuscripts have returned for minor revision ("revise only") are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

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

Reviewer #1 (Remarks to the Author):

The revised manuscript addressed all my previous concerns.

Reviewer #2 (Remarks to the Author):

I appreciate the new experimentation added, but wonder why conclusions of these experiments remain unclear or absent. Especially, I find the discussion of the stress responses still insufficient.

1. Authors: "We see no defect in the rate of synthesis of ND1, ND2, ND4, or ND5, suggesting that the observed Complex I defect arises through a different mechanism, likely impaired biogenesis of Complex I subunits or assembly factors that utilise TIM22 complex for their import."

Rev: Did you find any indications of impaired CI biogenesis or, in proteomics, deficient assembly factor candidates?

2. The authors have now included more data of SFXN3 to Figure 4C. While SFNX1 and 2 clearly are accessible to protein kinase upon disruption of the outer membrane, SFNX3 is cleaved but partially present. Furthermore, a smaller molecular weight Tim22 appears unlike SFNX1 and 2, where Tim22 is absent in the same conditions. These are not commented, but the authors only state: "For both SFXN1 and SFXN2, the C-terminal FLAG tag was only accessible to externally added proteinase K following hypoosmotic disruption of the outer membrane (Figure 4C, lane 4), confirming that SFXN1, SFXN2 and SFXN3 localise to the inner mitochondrial membrane with their C-termini facing the intermembrane space." Please clarify.

3. Authors: "AGKKO cells do not have any increased sensitivity to actinonin, suggesting that the 1C dysfunction observed does not compromise the ability of the cells to resist this particular mitochondrial stress (Supplementary Figure 7A)."

The new data of actinonin exposure indicate that cells with AGK deficiency can upregulate mitochondrial 1C cycle -related stress responses (MT-ISR) transcriptionally. However, the authors do not analyse protein levels, even if they make a general point that MTHFD2 is imported into mitochondria AGK-KO cells, but rapidly turned over - to explain the low abundance of the protein in their proteomics experiments. Is MTHFD2 protein turned over after actinonin response too, when its expression is especially increased? In previous publications of mt-stress, cited in the paper, MTHFD2 transcript levels faithfully correspond to protein levels. A conclusion of the current observations and previous articles suggests that presence of AGK in mt-stress could stabilize MTHFD2. Is AGK protective to MTHFD2 turnover? The point of Koufaris and Nilsson showing that MTHFD2 is a rapidly regulated protein with a short half-life, cited by the authors, does not appear to apply for mt-stress. These aspects should be properly discussed.

4. My previous point 11: The authors state that GEO analysis indicated upregulation of catabolic processes (FAO, amino acid catabolism) and suggest that this is an attempt to compensate for oxphos deficiency (Complex I). However, typically oxphos deficiency causes further downregulation of respiratory chain and fatty acid oxidation, and increased reliance on glycolysis, and therefore induction of mitochondrial biogenesis is studied as a treatment strategy. How do the authors explain this? Do they find transcriptional upregulation of mitochondrial biogenesis inducers, such as PGC1alpha? Is mitochondrial mass increased?
Au: We have included a qPCR experiment demonstrating that PGC1a is not transcriptionally upregulated in patient fibroblasts (Supplementary Figure 2C). We also analysed mitochondrial content in the context of whole cell lysates via western blot and determined that there was no increase in the proportion of mitochondrial proteins relative to non-mitochondrial proteins (Supplementary Figure 2B).
My current criticism: The authors' do not respond to my original question and conclude nothing of these new experiments. This is another example of an opposite response in AGK deficiency to what is typical for mitochondrial stress physiology. If oxphos is deficient, FAO and amino acid catabolism are downregulated, not upregulated, because FAO requires a functional respiratory chain. FAO increase and mitochondrial mass increase go hand in hand in physiology. Does AGK have a role in turning off FAO and mitobiogenesis upon oxphos dysfunction? Is peroxisomal FAO upregulated? I find that such unexpected findings of opposite metabolic behavior of the cell are interesting, if real, and should be somehow clarified and properly discussed.

THE UNIVERSITY OF MELBOURNE

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4 January, 2021

Dear Prof. Fox,

Thank you for your email of December 22, 2020. As indicated in this email we have now addressed the comments of Reviewer 2 in text and in a point by point response below.

We hope this satisfies any concerns you may have and those of Reviewer 2.

Many thanks and I hope you have had a restful holiday period.
Diana

Reviewer #2:
1. Authors: "We see no defect in the rate of synthesis of ND1, ND2, ND4, or ND5, suggesting that the observed Complex I defect arises through a different mechanism, likely impaired biogenesis of Complex I subunits or assembly factors that utilise TIM22 complex for their import. "
Rev: Did you find any indications of impaired CI biogenesis or, in proteomics, deficient assembly factor candidates?
Identification of TMEM126A/B, TIMMDC1 and NDUFA11 (known Complex I assembly factors) as putative TIM22 complex substrates is one of the key conclusions of the paper. This is discussed extensively in the text. Following revision new experiments were added to explore the Complex I connection. This includes, Figure 1 (D and E) – we show reduced levels of complex I subunits in AGK-KO HEK cells that can be rescued with expression of WT or kinase dead. Supplementary Figures 1 – western blotting showing reduced levels of some CI subunits on SDS-PAGE, disrupted assembly factors on BN-PAGE. We also included new data that confirmed a respiration defect via reduced OCR in AGK knock-out cells.

2. The authors have now included more data of SFXN3 to Figure 4C. While SFNX1 and 2 clearly are accessible to protein kinase upon disruption of the outer membrane, SFNX3 is cleaved but partially present. Furthermore, a smaller molecular weight Tim22 appears unlike SFNX1 and 2, where Tim22 is absent in the same conditions. These are not commented, but the authors only state: "For both SFXN1 and SFXN2, the C-terminal FLAG

tag was only accessible to externally added proteinase K following hypoosmotic disruption of the outer membrane (Figure 4C, lane 4), confirming that SFXN1, SFXN2 and SFXN3 localise to the inner mitochondrial membrane with their C-termini facing the intermembrane space." Please clarify.
By protein kinase we assume the reviewer means protease and by "smaller molecular weight of Tim22 we assume they are referring to Tom22 and not Tim22".

The profile highlighted by the reviewer is due to lack of complete swelling of the mitochondrial outer membrane. To highlight this we have now indicated on page 12: "For SFXN1, SFXN2 and SFXN3 the C-terminal FLAG tag was only accessible to externally added proteinase K following hypoosmotic disruption of the outer membrane ***(Figure 4C, lane 4)*** ***(note inefficient rupturing of the outer membrane in SFXN3 panel as indicated by incomplete digestion of Tim29, hence incomplete removal of FLAG signal)****, confirming that SFXN1, SFXN2 and SFXN3 localise to the inner mitochondrial membrane with their C-termini facing the intermembrane space."*

The lower molecular weight species of Tom22 has now been addressed with the following statement in the Figure legend for Figure 4C indicating: " indicates a proteolytic fragment of Tom22 sometimes detected due to incomplete proteolytic processing."*

3. Authors: "AGKKO cells do not have any increased sensitivity to actinonin, suggesting that the 1C dysfunction observed does not compromise the ability of the cells to resist this particular mitochondrial stress (Supplementary Figure 7A). "

The new data of actinonin exposure indicate that cells with AGK deficiency can upregulate mitochondrial 1C cycle -related stress responses (MT-ISR) transcriptionally. However, the authors do not analyse protein levels, even if they make a general point that MTHFD2 is imported into mitochondria AGK-KO cells, but rapidly turned over - to explain the low abundance of the protein in their proteomics experiments. Is MTHFD2 protein turned over after actinonin response too, when its expression is especially increased? In previous publications of mt-stress, cited in the paper, MTHFD2 transcript levels faithfully correspond to protein levels. A conclusion of the current observations and previous articles suggests that presence of AGK in mt-stress could stabilize MTHFD2. Is AGK protective to MTHFD2 turnover? The point of Koufaris and Nilsson showing that MTHFD2 is a rapidly regulated protein with a short half-life, cited by the authors, does not appear to apply for mt-stress. These aspects should be properly discussed.
*To address this question we have included quantification of protein levels following actinonin treatment in Control and AGK^{KO} cells (****Supplementary Figure 7C****). The data shows (as indicated in our proteomics) that MTHFD2 is down in AGK^{KO} cells compared to control cells. Following actinonin treatment (although the transcript goes up in both Control and AGK^{KO} cells) the protein levels of MTHFD2 go down in both Control and AGK^{KO}, suggesting that AGK does not stabilise the protein in mt-stress as indicated by Reviewer 2. A detailed study of the mechanisms controlling MTHFD2 levels in different cellular contexts and during different stresses would require further biochemical characterisation and is beyond the scope of this work. To speculate extensively on specific mechanisms based on our current data would require over-interpretation.*

*We have modified page 15 to include: "Surprisingly, the ISR appeared to be intact in the AGK^{KO} HEK cells, which were able to transcriptionally upregulate SHMT2, MTHFD2, SLC7A11 and DDIT3 to the same extent as control cells (**Supplementary Figure 7B**), although this did not correlate with an increase in the protein abundance of SHMT2 and MTHFD2 (**Supplementary Figure 7C**). In fact, for MTHFD2, protein levels in both control and AGK^{KO} HEK cells were reduced following treatment with actinonin (**Supplementary Figure 7C**). Although most studies of mitochondrial stress signalling show agreement between changes in transcript and protein abundance for MTHFD2, it appears that in some contexts this does not strictly apply. As we previously confirmed that MTHFD2 and SHMT2 do not require the TIM22 complex for their import **(Supplementary Figure 3A)**, their reduced abundance following actinonin treatment or in various systems of TIM22 complex dysfunction suggests an additional mechanism, such as turnover within mitochondria. In support of this, studies of MTHFD2 have confirmed that it has a particularly short half-life and is subject to rapid regulation (Koufaris and Nilsson, 2018)."*

4. My previous point 11: The authors state that GEO analysis indicated upregulation of catabolic processes (FAO, amino acid catabolism) and suggest that this is an attempt to compensate for oxphos deficiency (Complex I). However, typically oxphos deficiency causes further downregulation of respiratory chain and fatty acid oxidation, and increased reliance on glycolysis, and therefore induction of mitochondrial biogenesis is studied as a treatment strategy. How do the authors explain this? Do they find transcriptional upregulation of mitochondrial biogenesis inducers, such as PGC1alpha? Is mitochondrial mass increased?
Au: We have included a qPCR experiment demonstrating that PGC1a is not transcriptionally upregulated in patient fibroblasts (Supplementary Figure 2C). We also analysed mitochondrial content in the context of whole cell lysates via western blot and determined that there was no increase in the proportion of mitochondrial proteins relative to non-mitochondrial proteins (Supplementary Figure 2B).

My current criticism: The authors' do not respond to my original question and conclude nothing of these new experiments. This is another example of an opposite response in AGK deficiency to what is typical for mitochondrial stress physiology. If oxphos is deficient, FAO and amino acid catabolism are downregulated, not upregulated, because FAO requires a functional respiratory chain. FAO increase and mitochondrial mass increase go hand in hand in physiology. Does AGK have a role in turning off FAO and mitobiogenesis upon oxphos dysfunction? Is peroxisomal FAO upregulated? I find that such unexpected findings of opposite metabolic behavior of the cell are interesting, if real, and should be somehow clarified and properly discussed.
The gene ontology analysis presented in Figure 2 was undertaken to summarise a considerable amount of data in a transparent manner for readers. Questions pertaining to a role of AGK in turning off FAO or of peroxisomal FAO being upregulated cannot be addressed without further experimentation and are beyond the scope and message of this manuscript. Without further biochemical characterisation we are limited in how much we can speculate. We believe the discussion in this manuscript has discussed all aspects of key findings in a conservative manner, without over-interpretation of our data or other published data. We would expect this of a journal with the integrity and high standards of MBoC.

To clarify, we did not suggest that upregulation of metabolic processes in the patient fibroblasts is "an attempt to compensate for oxphos deficiency (complex I)" as suggested by reviewer 2 – rather, we suggested that it represents a response to "TIM22 complex dysfunction and consequent bioenergetic impairment." It is worth noting that Sengers syndrome is distinct from most mitochondrial diseases in that it is not directly caused by mutations in the OXPHOS machinery. The primary impact in Sengers syndrome appears to be a loss of metabolite carrier proteins in the inner membrane, and it is entirely possible that this leads to different stresses than a genetic lesion in the electron transport chain (the cause of classical mitochondrial disease). It seems plausible that the loss of various carrier proteins, including the ADP/ATP transporters, results in insufficient ATP synthesis and the activation of AMPK, a process which is known to induce a compensatory increase in mitochondrial biogenesis and energy generating mitochondrial metabolism via the phosphorylation of PGC1a. Additionally, depletion of sideroflexins and subsequent impairment of 1C metabolism (a process independent of OXPHOS deficiency) can also lead to AMPK activation.

RE: Manuscript #E20-06-0390RR
TITLE: "The TIM22 complex mediates the import of Sideroflexins and is required for efficient mitochondrial one-carbon metabolism."

Dear Dr. Stojanovski:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Sincerely,
Thomas Fox
Monitoring Editor
Molecular Biology of the Cell

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