Ovarian carcinoma immunoreactive antigen-like protein 2 (OCIAD2) is a novel complex III specific assembly factor in mitochondria

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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.)

RE: Manuscript #E21-03-0143
TITLE: Ovarian carcinoma immunoreactive antigen-like protein 2 (OCIAD2) is a novel metazoan specific complex III assembly factor

Dear Agnieska,

Thanks a lot for submitting your work to MBoC. Your manuscript has now been evaluated by two experts; their reports are attached below. As you can see, both agree that your study is very interesting and an important contribution to the field. However, the two reviewers also suggest further experiments to strengthen your study. Specifically, further characterization of CIV assembly intermediates in absence of OCIAD2 and a few more control experiments are requested. These experiments can be performed relatively easily and follow directly from your current results.

We would be happy to consider a revised manuscript that satisfies the joint concerns of the referees. Please submit, together with your revised manuscript, a letter indicating the changes you've made and your responses to the referees.

Sincerely,
Martin

Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Chacinska,

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.

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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):

This manuscript by Chojnacka et al., convincingly demonstrates the role of OCIAD2 in promoting the correct assembly of complex III in human cells. This paper constitutes a significant contribution to the field of respiratory chain biogenesis as, compared to other complexes, not very many assembly factors for complex III are currently known. However, here are some suggestions to improve and solidify the data shown in the paper.

- Abstract: Related to the comment above, there are not very many factors known to be involved in complex III assembly. If you compare how many are known for complex IV, the number is much lower for complex III. Please remove "many factors" and substitute it with "several accessory proteins are involved as assembly factors" (or something similar).

- Introduction: Classically the narrative has been that mammalian CIII is composed of eleven subunits However these subunits are encoded by 10 different genes, as subunit 9 is just the cleaved N-terminal fragment of UQCRFS1 that remains retained in between UQCRC1 and UQCRC2. There are recent evidences pointing out to the fact that 'subunit 9' is not stoichiometric and that the function of TTC19 is to promote the removal this fragment to maintain CIII2 activity (Bottani et al., 2017 and Fernandez-Vizarra & Zeviani, 2018 (PMID: 29243944)). In line with this point, the function of the three UQCRFS1 assembly factors is very different. LYRM7, or Mzm1 in yeast, is the chaperone that keeps UQCRFS1 stable in the mitochondrial matrix but is not involved in the incorporation of the subunit (Cui et al., 2012 (PMID: 22927643); Sanchez et al., 2013). BCS1L translocates UQCRFS1 from the matrix to the inner membrane and there are more recent references proving this function that can be cited (Wagener et al., 2012 (PMID: 22575765); Tang et al., 2020 (PMID: 32042153)). The lack of TTC19 does not prevent the incorporation of UQCRFS1 and TTC19 binds CIII2 only when UQCRFS1 is present, therefore it is not involved in this process (Bottani et al., 2017). Regarding the human assembly model, the paper by Protasoni et al., 2020 shows some data pointing out to an interaction of CYC1, UQCR10 and UQCRH forming assembly intermediates that accumulate when they are not incorporated in the assembly pathway and this differs from the yeast assembly model.

- Results: The biochemical characterization of the localization and interaction partners of OCIAD2 is beautifully done.
In Figure 2A, why are U2OS cells used for the localization of FLAG-OCIAD2 instead of the HEK293 cells used in the proteomics analyses?
Figure 3A: There is no specific information, but I assume the growth of the OCIAD2-KO cells was done in regular glucose medium. If the growth is not compared between glucose and 'respiratory' galactose medium, slower proliferation is not necessarily indicative of a mitochondrial defect. It could be due to off-target effects of the CRISPR/Cas9 gene editing and/or of the selection of clones that simply grow slower than the parental cells. To evaluate the relationship of growth defects and mitochondrial dysfunction, please perform the growth curves both in glucose and galactose and compare the differences in doubling times in the two media.
The activity measurements performed in the permeabilized cells (Figure 4C-E) are not correct in order to make the point that the authors want to make. The oxygen consumption measurements using pyruvate and malate still depend on the electron transfer through CIII, therefore even if there is not a CI deficiency per se, the respiration still appears reduced. The way to assess the electron transfer from reduced ubiquinol through CIII in an 'isolated' way, is to add rotenone to inhibit CI and then add the substrates to reduce the Q-pool and to make sure that this happens completely, succinate is added together with glycerol-3-phosphate. And then antimycin A is added to subtract the non-specific oxygen consumption. The oxygen consumption rates in the presence of glycerol-3-phosphate shown in Figure 4D are lower than those in the presence of malate+pyruvate (Figure 4C), and this is very odd. Please, re-evaluate the method used for the measurements. CIV-linked activity can also be evaluated by respirometry in the same permeabilized cells by adding TMPD and ascorbate after inhibition with antimycin A, and then inhibit with cyanide or azide. See for example the protocols in Hofhaus et al., 1996 (PMID: 8965720), this is an old protocol but it can be adapted to the use of high-resolution respirometers, and Doerrier et al. 2018 (PMID: 29850993). Then, in order to determine whether there is a real CI deficiency, or the reduced respiration is only the consequence of an isolated CIII deficiency (as suggested by the subsequent experiments), the enzymatic activity of CI (NADH:CoQ oxidoreductase) and CIII (Decylubiquinol:cytochrome c oxidoreductase) should be evaluated using a spectrophotometric kinetic assay, similarly to what was done for CIV activity.
Figure 5: The fact that the steady-state levels of the subunits are unchanged but the amounts of assembled CIII2 and III2+IV are reduced could be explained by the fact that there is an accumulation of subassembled species that are not detected by the antibodies in the 1D BN-PAGE and western blot analyses. This is a normal occurrence, and it would be good to run a 2D gels and western blot to detect the presence of sub-assemblies containing the different subunits, which would also give an idea what stage of the assembly process is blocked in the absence of OCIAD2. The BN-PAGE analyses suggest that there is no CI defect, so going back to the previous point, it would be interesting to confirm this by measuring the isolated enzymatic activity. The patterns shown here are in agreement with the idea that when the total amounts of CIII2 are low they preferentially associate within the supercomplexes as show in Lapuente-Brun et al., 2013 (PMID: 23812712) and Tropeano et al., 2020 (PMID: 31825807).
Being that a good antibody against OCIAD2 is available to the authors, and/or cell lines expressing FLAG-OCIAD2 have also been generated, it would be interesting to see what complex/complexes the protein forms by BN-PAGE and immunodetection. This would complement nicely the results shown in Figure 6A, which suggest that OCIAD2 binds to fully assembled CIII2

containing UQCRFS1, both in the 'free' form and in association in the supercomplexes.
- Discussion: The paragraph stating that there is a reduction in the activities of CI and CIII in the absence of OCIAD2 will need to be re-evaluated in view of the new measurements.

Reviewer #2 (Remarks to the Author):

In this work, Chojnacka et al. report the identification and functional characterization of the human OCIAD2 protein. The authors clearly demonstrate that OCIAD2 is located in the mitochondrial inner membrane, that it interacts with respiratory chain components and that its depletion in human cultured cells affects mitochondrial morphology, as well as respiratory chain function through the decreased activities of complexes I and III, and mitochondrial bioenergetics. According to the authors this effect mainly occurs at the level of respiratory chain complex III, since they observe a huge decrease of both CIII2 and supercomplex III2+IV with normal respirasome (or supercomplex I+III2+IV) levels. I mainly have some concerns related to data interpretation and presentation, and some technical aspects of this work must be improved in order to reach the right conclusions.

-From the data presented, I agree that the loss of OCIAD2 influences mitochondrial bioenergetics probably at the respiratory chain level. What is less clear to me is the unique functional effect of OCIAD2 on complex III (CIII) assembly. In this regard, it is necessary to show the effect of OCIAD2 loss on CIV assembly on the BN gels from figures 5A (digitonin) and 6A. In addition, please show the co-localization pattern of the OCIAD2 antibody on the same BN gels.

-Is the normal CIII assembly pattern and CIII activity rescued by over expression of OCIAD2FLAG in the KO cells? Again, with which structures does over expressed OCIAD2FLAG co-localize?

- According to the authors, CI assembly is normal in OCIAD2-KO cells. Why is there then a decrease in CI activity?

- It seems that, in OCIAD2-KO cells, CIII2 is preferentially stabilized in the respirasomes rather than in dimeric CIII or in SC III2+IV. Why? Can the authors speculate on a mechanism? Is there any other respiratory chain assembly factor behaving the same way? The authors compare the role of OCIAD2 with that of SCAFI, but the loss of the latter protein leads to the disappearance of SC III2+IV and to the parallel accumulation of CIII2, contrary to the observations in OCIAD-KO cells.

-Please add also a CII antibody to all the BN gels to serve as loading control.

- The authors should also introduce other prevailing theories of respirasome functions, including the idea that respirasome formation may prevent aggregation of the inner mitochondrial membrane (https://doi.org/10.1016/j.cmet.2017.03.009) and facilitate completion of CI assembly (doi: 10.15252/embj.2019102817 and doi: 10.1016/j.cmet.2012.01.015).

Minor point:

- Please avoid repetitive results in the two columns of Figure 2C, integrate all data in just 1 column.

E21-03-0143

Chojnacka, Elancheliyan, et al.

Point-by-point response to the comments raised on the initial submission:

We thank the editor and the reviewers for their valuable comments and suggestions. We are happy to learn that our discoveries are found to be significant and potentially suitable for publication in Mboc journal. We think that constructive criticism and suggestions helped to improve our manuscript and allowed us to present a more insightful scientific data. We put a significant effort to address all the issues raised by the reviewers. We introduced new experimental data that are added to revised figures 3C, 4C-E, 5A, 5C, 6A and as new supplementary figures section: S3, S4, S5 in our revised manuscript. We rearranged the figure 2C and further text descriptions were introduced in the revised manuscript according to reviewers comments. In the below text, we addressed all the points raised by reviewer as a point by point response. We strongly believe that the revised manuscript provides enough support for our conclusions and meets journals high requirements for publication in MBoC.

****Reviewer's comments****

Reviewer#1(Remarks to the Author):

This manuscript by Chojnacka et al., convincingly demonstrates the role of OCIAD2 in promoting the correct assembly of complex III in human cells. This paper constitutes a significant contribution to the field of respiratory chain biogenesis as, compared to other complexes, not very many assembly factors for complex III are currently known. However, here are some suggestions to improve and solidify the data shown in the paper.

1) Abstract: Related to the comment above, there are not very many factors known to be involved in complex III assembly. If you compare how many are known for complex IV, the number is much lower for complex III. Please remove "many factors" and substitute it with "several accessory proteins are involved as assembly factors" (or something similar).

A: As suggested by reviewer, the following correction was included in the first phrase of the abstract.

"Assembly of the dimeric complex III ($CIII_2$) in the mitochondrial inner membrane is an intricate process in which several accessory proteins are involved as assembly factors." (line 38)

2) Introduction: Classically the narrative has been that mammalian CIII is composed of eleven subunits However these subunits are encoded by 10 different genes, as subunit 9 is just the cleaved N-terminal fragment of UQCRFS1 that remains retained in between UQCRC1 and UQCRC2. There are recent evidences pointing out to the fact that 'subunit 9' is not stoichiometric and that the function of TTC19 is to promote the removal this fragment to maintain CIII2 activity (Bottani et al., 2017 and Fernandez-Vizarra & Zeviani, 2018 (PMID: 29243944)). In line with this point, the function of the three UQCRFS1 assembly factors is very different. LYRM7, or Mzm1 in yeast, is the chaperone that keeps UQCRFS1 stable in the mitochondrial matrix but is not involved in the incorporation of the subunit (Cui et al., 2012 (PMID: 22927643); Sanchez et al., 2013). BCS1L translocates UQCRFS1 from the matrix to

the inner membrane and there are more recent references proving this function that can be cited (Wagener et al., 2012 (PMID: 22575765); Tang et al., 2020 (PMID: 32042153)). The lack of TTC19 does not prevent the incorporation of UQCRFS1 and TTC19 binds CIII2 only when UQCRFS1 is present, therefore it is not involved in this process (Bottani et al., 2017). Regarding the human assembly model, the paper by Protasoni et al., 2020 shows some data pointing out to an interaction of CYC1, UQCR10 and UQCRH forming assembly intermediates that accumulate when they are not incorporated in the assembly pathway and this differs from the yeast assembly model.

A: As suggested by reviewer, we included the following lines to the introduction section to elaborate our narrative:

"Following import into mitochondria, UQCRFS1 is bound and stabilized in the mitochondrial matrix by the LYRM7 chaperone (Mzm1 in yeast) (Cui *et al.*, 2012; Sanchez *et al.*, 2013). Bcs1/BCS1L translocates UQCRFS1 from the matrix to helps in the incorporation of UQCRFS1 into premature $CIII_2$ (Wagener and Neupert, 2012; Tang *et al.*, 2020)" (lines 122-126)

"Finally, TTC19 interacts with fully assembled $CIII_2$ to facilitate the removal of subunit 9 and the cleaved N-terminal fragments of UQCRFS1 that remain bound between UQCRC1 and UQCRC2 (Bottani *et al.*, 2017)." (lines 128-130)

"it has been shown in a recent protemic data of human cell line with $CIII_2$ assembly defect that CYC1, UQCR10 and UQCRH form assembly intermediates that accumulate during the failure of incorporation into pre-$CIII_2$, which is not described in yeast (Protasoni *et al.*, 2020)." (lines 133-136)

- Results: The biochemical characterization of the localization and interaction partners of OCIAD2 is beautifully done.

3) In Figure 2A, why are U2OS cells used for the localization of FLAG-OCIAD2 instead of the HEK293 cells used in the proteomics analyses?

A: We opted to use U2OS cells because of its microscopy friendly nature compared to HEK293 cells. We added the growing conditions and mentioned U2OS cells used only for microscopy in the Material and Method section.

4) Figure 3A: There is no specific information, but I assume the growth of the OCIAD2-KO cells was done in regular glucose medium. If the growth is not compared between glucose and 'respiratory' galactose medium, slower proliferation is not necessarily indicative of a mitochondrial defect. It could be due to off-target effects of the CRISPR/Cas9 gene editing and/or of the selection of clones that simply grow slower than the parental cells. To evaluate the relationship of growth defects and mitochondrial dysfunction, please perform the growth curves both in glucose and galactose and compare the differences in doubling times in the two media.

A: As suggested by reviewer, we performed the proliferation assay in galactose medium. The result confirms slower proliferation in both glucose and galactose medium in absence of OCIAD2 (revised figure 3C)

5) The activity measurements performed in the permeabilized cells (Figure 4C-E) are not correct in order to make the point that the authors want to make. The oxygen consumption measurements using pyruvate and malate still depend on the electron transfer through CIII, therefore even if there is not a CI deficiency per se, the respiration still appears reduced. The way to assess the electron transfer from reduced ubiquinol through CIII in an 'isolated' way, is to add rotenone to inhibit CI and then add the substrates to reduce the Q-pool and to make sure that this happens completely, succinate is added together with glycerol-3-phosphate. And then antimycin A is added to subtract the non-specific oxygen consumption. The oxygen consumption rates in the presence of glycerol-3-phosphate shown in Figure 4D are lower than those in the presence of malate+pyruvate (Figure 4C), and this is very odd. Please, re-evaluate the method used for the measurements. CIV-linked activity can also be evaluated by respirometry in the same permeabilized cells by adding TMPD and ascorbate after inhibition with antimycin A, and then inhibit with cyanide or azide. See for example the protocols in Hofhaus et al., 1996 (PMID: 8965720), this is an old protocol but it can be adapted to the use of high-resolution respirometers, and Doerrier et al. 2018 (PMID: 29850993). Then, in order to determine whether there is a real CI deficiency, or the reduced respiration is only the consequence of an isolated CIII deficiency (as suggested by the subsequent experiments), the enzymatic activity of CI (NADH:CoQ oxidoreductase) and CIII (Decylubiquinol:cytochrome c oxidoreductase) should be evaluated using a spectrophotometric kinetic assay, similarly to what was done for CIV activity.

A: In our previous protocol, complex III was stimulated with Glycerol-3-P without the presence of any other substrates. Indeed the oxygen consumption was really low. Therefore, we followed your suggestion and performed both the protocols described by Hofhaus *et al*., 1996 and Doerrier *et al*. 2018. OCIAD2-KO cells showed reduced O_2 consumption rate when complex I and III were stimulated and no oxygen consumption alteration when complex IV was stimulated compared to control using both protocols (revised figure 4C and 4D). Indeed, complex III alteration could suggest a misleading interpretation on a possible alteration in complex I activity related to oxygen consumption. Also, no other results from our research indicated a possible alteration in complex I. Therefore, we followed your suggestion and checked the enzymatic activity of CI and CIII. OCIAD2-KO presented reduced activity of complex III with no alteration of complex I (revised figure 4E). Therefore, we are grateful for the insight as it solved an issue that intrigued us.

We made the following changes in the lines below:

Abstract:
"Complete loss of OCIAD2 using gene editing in HEK293 cells resulted in abnormal mitochondrial morphology, a substantial decrease of both CIII2 and supercomplex III_2+IV, and reduction in CIII enzymatic activity." (lines 44-46)

Results:
"To confirm this reduction in maximal respiration of OCIAD2-KO cells, we assessed CI, CIII and IV activities coupled to oxygen consumption by two different protocols as described in Hofhaus *et al.,* 1996 and Doerrier *et al.,* 2018. Mitochondria depleted of OCIAD2 exhibited decreased oxygen consumption driven by pyruvate, malate and glutamate (CI substrate electron donors), and by succinate and glycerol-3-phosphate in the presence of rotenone (CIII substrate electron donor). No alteration was observed in CIV activity (Figure 4C). Using the second protocol, mitochondria depleted of OCIAD2 exhibited a similar decreased oxygen consumption related to CI and CIII activity, with no alteration in CIV activity (Figure 4D). As the oxygen consumption related to CI activity depends on the electron transfer through CIII,

we wondered whether CIII was the only ETC component altered in this situation. Therefore, the enzymatic activity of uncoupled CI, CIII and CIV were examined. Mitochondria lacking OCIAD2 exhibited decreased enzymatic activity for CIII, but no alterations in CI and CIV activity (Figure 4E and S4C)." (lines 241-253)

"Nevertheless, a reduction in maximal respiration and spare capacity, coupled to a reduction in CIII activity, indicates that OCIAD2 depleted cells have less capacity to cope with metabolic stress" (lines 256-258)

Discussion:

"We also observed a reduction in the oxygen consumption activities of CI and CIII, which is in line with the well-established correlation between severe CIII deficiency and CI impairment (Lamantea *et al.*, 2002; Acin-Perez *et al.*, 2004; Barel *et al.*, 2008; Tucker *et al.*, 2013; Wanschers *et al.*, 2014; Feichtinger *et al.*, 2017; Protasoni *et al.*, 2020). Nevertheless, the enzymatic activity of ETC complexes in OCIAD2-KO cells confirmed reduced activity only in CIII, indicating no compromise of CI." (lines 343-348)

6) Figure 5: The fact that the steady-state levels of the subunits are unchanged but the amounts of assembled CIII2 and III2+IV are reduced could be explained by the fact that there is an accumulation of subassembled species that are not detected by the antibodies in the 1D BN-PAGE and western blot analyses. This is a normal occurrence, and it would be good to run a 2D gels and western blot to detect the presence of sub-assemblies containing the different subunits, which would also give an idea what stage of the assembly process is blocked in the absence of OCIAD2. The BN-PAGE analyses suggest that there is no CI defect, so going back to the previous point, it would be interesting to confirm this by measuring the isolated enzymatic activity. The patterns shown here are in agreement with the idea that when the total amounts of CIII2 are low they preferentially associate within the supercomplexes as show in Lapuente-Brun et al., 2013 (PMID: 23812712) and Tropeano et al., 2020 (PMID: 31825807).

A: We performed 2D gels analysis for WT and OCIAD2-KO cells. The result shows assembly of complex III subunits such as UQCRC2 and RIESKIE into $CIII_2$ and $CIII_2$+IV is disrupted in OCIAD2-KO cells. However, we did not find any accumulation of CIII sub-assemblies blocked by absence of OCIAD2 (new supplementary figure S3B). We added the following lines in the manuscript.

"To further study the influence of OCIAD2 on CIII and to find the intermediate sub-assemblies blocked by deletion of OCIAD2, we performed 2D gel analysis. However, no accumulation of intermediates was found. Nevertheless, CIII subunits in $CIII_2$ and $CIII_2$+IV were significantly reduced (Figure S3B)." (lines 285-289)

The answer for enzymatic assay is addressed in the previous comment.

7) Being that a good antibody against OCIAD2 is available to the authors, and/or cell lines expressing FLAG-OCIAD2 have also been generated, it would be interesting to see what complex/complexes the protein forms by BN-PAGE and immunodetection. This would complement nicely the results shown in Figure 6A, which suggest that OCIAD2 binds to fully

assembled CIII2 containing UQCRFS1, both in the 'free' form and in association in the supercomplexes.

A: We performed BN-PAGE and probed the two different OCIAD2 antibody to see the complexes formation. However, the band signal is smeary from top to bottom of the blot. Therefore, it becomes inconclusive to predict its association with any complex (new supplementary figure S3A&C and revised figure 6A)

8) Discussion: The paragraph stating that there is a reduction in the activities of CI and CIII in the absence of OCIAD2 will need to be re-evaluated in view of the new measurements.

A: We re-evaluated our discussion section. We no longer claim CI enzymatic activity is reduced.

Reviewer #2 (Remarks to the Author):

In this work, Chojnacka et al. report the identification and functional characterization of the human OCIAD2 protein. The authors clearly demonstrate that OCIAD2 is located in the mitochondrial inner membrane, that it interacts with respiratory chain components and that its depletion in human cultured cells affects mitochondrial morphology, as well as respiratory chain function through the decreased activities of complexes I and III, and mitochondrial bioenergetics. According to the authors this effect mainly occurs at the level of respiratory chain complex III, since they observe a huge decrease of both CIII2 and supercomplex III2+IV with normal respirasome (or supercomplex I+III2+IV) levels. I mainly have some concerns related to data interpretation and presentation, and some technical aspects of this work must be improved in order to reach the right conclusions.

1) From the data presented, I agree that the loss of OCIAD2 influences mitochondrial bioenergetics probably at the respiratory chain level. What is less clear to me is the unique functional effect of OCIAD2 on complex III (CIII) assembly. In this regard, it is necessary to show the effect of OCIAD2 loss on CIV assembly on the BN gels from figures 5A (digitonin) and 6A. In addition, please show the co-localization pattern of the OCIAD2 antibody on the same BN gels.

A: As suggested by reviewer, we performed the BN-PAGE for CIV assembly using the buffer containing digitonin for isolated mitochondria from WT and OCIAD2-KO cells. However, we were not able to find the condition to detect changes in $CIII_2$+IV as shown with DDM solubilization (new figure S3A and revised figure 5A). And we also did not observe interaction of ^{FLAG}OCIAD2 with any CIV assembly (new supplementary figure S3C).

In addition, we mapped the BN-PAGE co-localization pattern of OCIAD2 with two different antibodies (new supplementary figure S3A and revised figure 5A).

2) Is the normal CIII assembly pattern and CIII activity rescued by over expression of OCIAD2FLAG in the KO cells? Again, with which structures does over expressed OCIAD2FLAG co-localize?

A: We followed the suggestion of reviewer and performed the rescue by transient overexpression of ^{FLAG}OCIAD2 for 5 days in OCIAD2-KO cells. However, we did not

observe the rescue of $CIII_2$ assembly in OCIAD2-KO cells (new supplementary figure S4A and S4B). We also tried to detect any possible rescue of complex III activity by high-resolution respirometry and enzymatic assay. No rescue was detected in both cases (new supplementary figure S4C and S4D). This could be likely due to inappropriate amount and/or insufficient time of expression and/or incomplete biogenesis of OCIAD2.

Importantly, we confirmed the phenotype of OCIAD2-KO cells, $CIII_2$ assembly defect using OCIAD2 RNAi (revised figure 5C). In addition, to strengthen our argument about OCIAD2 being responsible for growth and mitochondria morphological defects. We performed the proliferation assay and microscopy for cells treated with OCIAD2-RNAi. The proliferation of OCIAD2 RNAi treated cells significantly reduced after 48hrs and morphology is also severely affected (new supplementary figure S5A&B). Thus, the phenotype of KO and silencing of OCIAD2 are in agreement.

^{FLAG}OCIAD2 forms a smeary band signal from top to bottom of BN-PAGE. So, it is unpredictable to detect its co-localization with any particular complex (new supplementary figure S4B).

3) According to the authors, CI assembly is normal in OCIAD2-KO cells. Why is there then a decrease in CI activity?

A: It was an intriguing point of the initial manuscript. However, in light of the new high-resolution respirometry and enzymatic assay results, we no longer claim the reduction in complex I activity (revised figure 4C, 4D and 4E). As complex III is indeed affected, less electrons from complex I will reach complex IV generating a bias in the interpretation of the results. No alteration on complex I activity is detected by enzymatic assay confirming alteration only in complex III activity.

4) It seems that, in OCIAD2-KO cells, CIII2 is preferentially stabilized in the respirasomes rather than in dimeric CIII or in SC III2+IV. Why? Can the authors speculate on a mechanism? Is there any other respiratory chain assembly factor behaving the same way? The authors compare the role of OCIAD2 with that of SCAFI, but the loss of the latter protein leads to the disappearance of SC III2+IV and to the parallel accumulation of CIII2, contrary to the observations in OCIAD-KO cells

A: In the previous version of the manuscript, we explored the possibility of OCIAD2 acting as a stabilizer of CIII as OCIAD2 absence does not affect steady-state levels of protein components of CIII; but rather the amount of fully assembled $CIII_2$. Also, OCIAD2 deletion does not affect steady-state levels of all detected OXPHOS proteins. So, we can further speculate in two possible mechanisms. Firstly, absence of OCIAD2 could lead $CIII_2$ and $CIII_2$+CIV destabilization and prone to degradation. Secondly, OCIAD2 could be involved in the balance between $CIII_2$, $CIII_2$+CIV, and respiratory supercomplex assembly. Because, in absence of OCIAD2, $CIII_2$ and $CIII_2$+CIV could be exclusively recruited to respiratory supercomplex.

One assembly factor which behaves similarly to this hypothesis is human Hypoxia Inducible Gene Domain family proteins HIGD1A, which regulates CIII and CIII-containing supercomplex biogenesis (Gómez *et al.*, 2020 - PMID: 32375044; Vercellino and Sazanov, 2021 PMID: 34621061). Finally, we apologize for any misunderstanding for a possible direct

link between OCIAD2 and SCAF1 function. We stated that "we can similarly propose that OCIAD2 could interact with $CIII_2$ and $CIII_2$+IV just as SCAF1 does". We never suggested that absence of OCIAD2 and SCAF1 display a similar phenotype.

We added a following lines to manuscript to address the above comment:

"The respirasomes are important mitochondrial architecture serving various purposes such as cell survival and averting aggregation (Milenkovic *et al.,* 2017). In OCIAD2-KO cells, the total amount of $CIII_2$ is low and seems to preferentially associate to the respirasomes (Lapuente-Brun *et al.,* 2013; Tropeano *et al.,* 2020)." (Lines 348-352)

5) Please add also a CII antibody to all the BN gels to serve as loading control.

A: We added CII blots to all BN-PAGE revised figures (5A, 5C and 6A).

6) The authors should also introduce other prevailing theories of respirasome functions, including the idea that respirasome formation may prevent aggregation of the inner mitochondrial membrane (https://doi.org/10.1016/j.cmet.2017.03.009) and facilitate completion of CI assembly (doi: 10.15252/embj.2019102817 and doi: 10.1016/j.cmet.2012.01.015).

A: We agree with reviewer for the above suggestion. So we included, the following lines in the discussion section.

"The biogenesis of the respirasome involves a CI assembly intermediate, which acts as a scaffold for the incorporation of CIII and CIV subunits (Moreno-Lastres *et al.,* 2012). However, CIII plays a central role in the formation of supercomplexes and misassembly of the CIII subunits affects biogenesis of CIV (Protasoni *et al.,* 2020). It is also known that the mitochondrial inner membrane is especially protein rich leading to tight protein packaging. Accordingly, proteins contact each other by weak interactions to avoid any deleterious effect of a strong interaction such as aggregation or protein nucleation (Milenkovic *et al.,* 2017). These weak interactions are highly coordinated by various assembly factors and we hypothesize that OCIAD2 could act as one of them, potentially exclusively for CIII." (Lines 375-383)

7) Please avoid repetitive results in the two columns of Figure 2C, integrate all data in just one column.

A: We excluded the repetitive information from revised figure 2C.

RE: Manuscript #E21-03-0143R
TITLE: "Ovarian carcinoma immunoreactive antigen-like protein 2 (OCIAD2) is a novel complex III specific assembly factor in mitochondria"

Dear Dr. Chacinska,

thanks a lot for sending the revised manuscript. You have addressed all the points raised by the two experts satisfactory and we are pleased to accept your manuscript for publication. Thanks a lot for submitting your interesting work to MBoC.

Sincerely,
Martin Ott
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Chacinska:

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.

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