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A CMC1-Knockout Reveals Translation-independent Control of Human Mitochondrial Complex IV Biogenesis

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Transaction Report:

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

1st Editorial Decision 23 August 2016

Thank you for the submission of your research manuscript to our journal. I am making a decision on behalf of my colleague Martina who is not in the office this week. We have now received the full set of referee reports on your manuscript that is pasted below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, they all also indicate that the data are not sufficiently convincing and point out inconsistencies that would need to be addressed and clarified. Given the (partial) overlap of the concerns and the overall number I think that all of them should be addressed.

We would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. Given the 7 main figures I suggest that you layout your manuscript as a full article for which there are no length limitations.

REFEREE REPORTS

Referee #1:

This manuscript reports the characterization of human CMC1 and its role in assembly of respiratory chain complex IV (CIV). CMC1 has been characterized to some degree in yeast, yet its function in CIV biogenesis is poorly understood. The authors use TALEN knockout cells and other biochemical approaches to suggest that CMC1 acts in the early steps of CIV assembly in a subcomplex that stabilizes mtDNA encoded COX1. The authors show that CMC1 forms a complex with other assembly factors (COA3 and COX14) along with COX1 and this precedes the addition of other CIV subunits and assembly factors. The authors conclude that this complex regulates the stability of COX1 rather than through translational regulation of COX1 as seen in yeast. This makes sense given the transcriptional regulation that is found in yeast while human transcripts lack UTRs for this role. There is a lot to take in with this manuscript and certainly complex IV assembly is complicated. This does detract somewhat from the main message in the manuscript. It is also still unclear as to the precise mechanistic function of CMC1 and the fact that complex IV can still assembly in its absence points to this protein playing a more peripheral function.

Other comments:

1.For Fig. 1, the levels of COX1 loss and also complex IV should be carefully quantified since there seems to be some variability between experiments. Figures 1 and 2 could also be merged and the authors could remove the work related to the generation of the cell lines.
2. It is surprising that loss of CMC1 seems to mostly affect the levels of Cox6b and Cox3 (Fig3B) - certainly more so than COX1. How do the authors explain this? Is Cox6b actually in the residual Complex IV in CMC knockout cells?
3.The pull down of newly translated COX1 is an important result to justify the main conclusions into the role of CMC1. However, the data shown in Fig 4B is quite weak with very little enrichment of COX1 over background. As it stands, this is not convincing.
4.In Fig. 4H, why is there not an equivalent enrichment of CMC1 associated with the complex IV subcomplex when COX1 accumulates?
5.Likewise, in fig 7C, why is the CMC1 in the subcomplex so much greater when Cox20 is knocked out while COX1 is not and the opposite is seen in Fig 7A?

Referee #2:

This thorough study documents for the first time that human CMC1 is a COX1-specific assembly factor. Studies by this group in yeast demonstrated that yeast Cmc1 is a COX-assembly factor, but the precise step in COX assembly was not defined. The present human cell study shows in a convincing manner that CMC1 binds newly synthesized COX1 along with two COX1 chaperones COA3 and COX14. The studies support a model in which CMC1 remains associated with newly synthesized COX1 until the peripheral subunits COX4 and COX5a bind. CMC1 is localized in the intermembrane space in association with the membrane, so its association with an early COX1 assembly intermediate is novel and significant. The study utilized HEK293T cells with the CMC1 locus disrupted by TALEN technology. Cells lacking CMC1 retain ~60% of intact cytochrome oxidase CIV, so CMC1 is not a critical assembly factor. The investigators nicely show that CIV in the KO cells can be restored with CMC1-FLAG expression. A key observation is the visualization of the CMC1/COX1 assembly intermediate (subCIV) in Fig. 4D. One complicating aspect of the data is that the subCIV band observed on BN-PAGE is a composite of different subcomplexes. The missing experiment in Fig. 4 is the COX1 BN immunoblot in CMC1 KO cells. That isn't shown until Fig 5E and that reveals that the subCIV component is equally abundant in CMC1 KO cells. This suggests that the CMC1 component of the "subCIV" band is minor. Whereas CMC1 is not a critical component in COX1 maturation, COX14 is shown to be, but not COA3. The lack of any effect of COA3 depletion is unexpected. It would be important to test CIV activity in the COA3 depleted cells to assess whether it has any effect. The COA3 siRNA is shown in Fig 5A to be very effective, so this is baffling.

It is very convincing that CMC1, COA3 and COX14 form an early COX1 assembly intermediate.

Using a COX1 cybrid cell line that is COX1 deficient, the CMC1 subCIV band is attenuated, suggesting that the CMC1 BN complex is Cox1-dependent. Steady-state levels of CMC1 should be shown in the cybrid line to assess whether steady-state abundance of CMC1 is COX1-dependent. Likewise, is CMC1 attenuated in doxycycline-treated cells.

A second subCIV complex lacking CMC1 contains COX4 and COX5a, yet the abundance of this subcomplex is not so dependent on CMC1. An intriguing observation shown in Fig. 4H is that with attenuation of COX4-1 or COX5a, the abundance of the COX1 subCIV complex is markedly enhanced. The most likely subcomplex that accumulates would be predicted to be the CMC1-COX1 subcomplex, but the CMC1 abundance in the 120 kDa band doesn't increase so significantly. COA3 expands, so the component must be a COX1, COA3, COX14 subcomplex, but it isn't clear that CMC1 is present. The authors conclude on p. 10 that at least 2 COX1 subassembly complexes can be seen on BN gels. Perhaps there are more than 2!

The investigators attenuate COA3 and COX14 and observe the expected attenuation in COA3-COX1 subcomplexes. It is very unclear why the efficient siRNA attenuation in COA3 had no effect on CIV assembly (Fig. 5B). Again, CIV activity would be important to test to assess whether COA3 is important for human COX1 maturation. It is also surprising that COX11 or MITRAC7 siRNA did not lead to any attenuation in assembled CIV in Fig. 6C. Is this reproducible or substantiated in other studies?

In summary, this is a thorough study with high significance and novelty. This will be an important contribution to the field. The studies are well-executed, although a few studies are missing. The authors are encouraged to submit a revised manuscript with the suggested studies completed prior to a final decision.

Referee #3:

The manuscript "A CMC1-Knockout Reveals Translation-independent Control of Human Mitochondrial Complex IV Biogenesis" by Bournes et al. studies the role of the CMC1 in the context of cytochrome oxidase (COX) assembly. CMC1 is a twin-CX9X protein and therefore localizes to the mitochondrial intermembrane space where it stabilizes early assembly intermediates of COX. With a significant body of work the authors support their conclusions well, exploring an early step in COX assembly, which is differently regulated in yeast. The manuscript is very well presented as expected from this group. The points below should be addressed - I have only one real concern, i.e., the role of COX4 in the early assembly complex as outlined in point #4.

1) Table 1 was missing in the combined version that I reviewed.

2) In figure 2 and subsequent figures one KO cell line is used. Please mention in the text, based on quantitative Western analysis, how much reduction is seen in COX levels. This should also be discussed in the context of results shown in figure 2 A and B. It appears that COX is rate-limiting, a result that is important by itself and should be included/discussed.

3) Results section, when first talking about the 120 kDa complex: please mention molecular weights of all tested candidates. Please discuss how the molecular weights add up to the 120 kDa mass as you go along identifying the individual components.

4) Figure 5E and Figure 6F and Figure 7A: the COX4 panels do not make much sense. The authors previously concluded that COX4 is not part of the 120 kDa complex (e.g., 4C), but that clearly does not seem to be the case based on the multiple other experiments (the model in Fig. 7F should be changed, too). Is it possible that COX4 binds less tightly compared to the other components and is sometimes lost - perhaps conditions in Figs. 5E, 6F, and 7A were suitable to retain subunit IV in the complex, however, such a scenario might complicate the interpretation of the size of the 120 kDa complex; it should noted that although BN-PAGE is a mild method, Coomassie still acts as a (mild) detergent, and conditions in individual experiments might slightly differ, possibly explaining the different experimental results. In Figure 6F the authors show COX4 running at a lower weight, however, in contrast to subunits that are added later (e.g., COX2 in Fig 6E) it is detectable and gives a robust band, suggesting that it is part of the complex. These findings have to be critically

reevaluated and discussed. Regarding point #3 above it is also unlikely that the authors really have all components identified that constitute the 120 kDa complex. CMC1 (101AA), COX14 (57 AA), COX1 (513AA), and COA3 (57 AA) do not even come close to the reported molecular weight of 120 kDA. Adding COX4 and perhaps Va would bring this number closer (please discuss size predictions and their implications). In addition, it would be good to reprobe the blots shown Figs. 5E, 6F, and 7A with a COX5A antibody. For future work it might be a good idea to pull down the 120 kDa complex and to analyze it by mass spectrometry in order to identify additional components that may be part of the complex.

Minor:

-Page 4: COX4-1 is introduced here without further explanation; it might be useful to include a sentence on page 3 (where the COX subunits are listed) and to mention that several of the COX subunits are expressed as tissue specific isoforms, introducing the COX4-1/COX4-2 pair up front.

-Figure 2C: To this reviewer it seems that complex 3 levels are increased when CMC1-FLAG is expressed. Perhaps worth discussing in the context of supercomplex formation. For experiments as the one shown here it would be good to quantitate the Western blots and to report significant changes in the text of the manuscript.

-Make a mention in the figure legend what the difference is between panels 1 and 2 in figure 2E (overexposure of the latter, correct?). Third panel: why did the authors use two antibodies here? Using the complex I antibody only would be more informative.

-Page 8: "Here, consistently with the BN-PAGE data, the steady state levels of all CIV subunits, including COX1, decreased in the absence of CMC1 (Fig. 3B)." Does this statement hold up for subunit IV? See also point #4 above.

Page 9: Format Mick reference.

-Bar diagrams in Figures 4B and 7D: error bars are missing.

-Figure 5B legend: state how long knockdown was performed. In addition, explain the difference between the top and second panel in the legend (overexposure?).

1st Revision - authors' response 31 October 2016

Answer to Reviewers

Referee #1:

This manuscript reports the characterization of human CMC1 and its role in assembly of respiratory chain complex IV (CIV). CMC1 has been characterized to some degree in yeast, yet its function in CIV biogenesis is poorly understood. The authors use TALEN knockout cells and other biochemical approaches to suggest that CMC1 acts in the early steps of CIV assembly in a subcomplex that stabilizes mtDNA encoded COX1. The authors show that CMC1 forms a complex with other assembly factors (COA3 and COX14) along with COX1 and this precedes the addition of other CIV subunits and assembly factors. The authors conclude that this complex regulates the stability of COX1 rather than through translational regulation of COX1 as seen in yeast. This makes sense given the transcriptional regulation that is found in yeast while human transcripts lack UTRs for this role. There is a lot to take in with this manuscript and certainly complex IV assembly is complicated. This does detract somewhat from the main message in the manuscript.

The main points of this manuscript are now summarized in the second paragraph of the discussion.

It is also still unclear as to the precise mechanistic function of CMC1 and the fact that complex IV can still assembly in its absence points to this protein playing a more peripheral function.

We agree that the function of CMC1 on COX1 metabolism is not essential and therefore its role could be either redundant or involved in the support or catalysis of a COX1 maturation step. This has been commented the discussion.

Other comments:

1. For Fig. 1, the levels of COX1 loss and also complex IV should be carefully quantified since there seems to be some variability between experiments. Figures 1 and 2 could also be merged and the authors could remove the work related to the generation of the cell lines.

We hesitated to transfer the description of the KO-CMC1 cell lines generation to the supplementary data because it can be very useful to the general reader to have it in the main document. Since the editor suggested us to layout the manuscript as a full article for which there are no length limitations, we decided to maintain it in the main article. As the phenotype of KO-CMC1 is partial, we think it is interesting to show that several independent clones have decreased complex IV levels. We have added the quantification of CIV and CIV subunits (Fig 2C-D and 3C) as suggested by the reviewer.

2. It is surprising that loss of CMC1 seems to mostly affect the levels of Cox6b and Cox3 (Fig3B) - certainly more so than COX1. How do the authors explain this? Is Cox6b actually in the residual Complex IV in CMC knockout cells?

We added the quantification of CIV subunits in KO-CMC1 (Fig 3C). As stated by the reviewer, COX6b, COX2 and COX3 are indeed more decreased (20-30% residual) than COX1 or COX4 (70-50%). COX2, COX3 and COX6b are late assembly CIV subunits and are not stable when unassembled. Therefore, their steady-state levels are expected to be consistent with the CIV steady-state level (30%, Fig2C). On the contrary, COX1 and COX4 are early CIV assembly proteins and are more stable as part of early step CIV subassemblies. Thus, their steady-state levels will correspond to the addition of the proteins correctly assembled in CIV and in the different sub-CIV present in the cells (Fig 5E). In KO-CMC1 cells extracted with lauryl maltoside, we observed at least two sub-CIV containing COX1 but, for example, not COX3, which is even clearer when cells are extracted with a mild detergent like digitonin (new Fig 3B). Additionally, COX4 forms a dimer with COX5a before assembly, which is quite stable even when CIV is impaired in HEK293T cells. Similar steady-state protein level profile (COX2 and COX6b lower than COX1 and COX4) was observed upon depletion of COX10 and COX11 (Fig 6E) for the same reasons as for KO-CMC1. COX2 and COX6b are stable only when assembled in CIV whereas some COX1 and COX4 accumulate in early CIV assembly intermediates.

3. The pull down of newly translated COX1 is an important result to justify the main conclusions into the role of CMC1. However, the data shown in Fig 4B is quite weak with very little enrichment of COX1 over background. As it stands, this is not convincing.

We agree with the reviewer. We think the enrichment of newly synthesized COX1 in CMC1FLAG immunoprecipitation is indeed relatively weak for technical and biological reasons. The CMC1-COX1 complex is short-lived in wild-type cells, because CMC1 is released from the CMC1-COX1-COA3-COX14 complex prior to the incorporation of COX4. We did this experiment after a 30 min pulse, but do not actually know the turnover of the CMC1-COX1 complex. The experiment was repeated three times and the quantification showed statistically significant COX1 immunoprecipitation with CMC1FLAG. We have substituted the Fig 4B for another repetition of the experiment that is clearer. Also, we have added the missing error bars in the quantification bar graph. Now, we rationalized that the CMC1-COX1 co-immunoprecipitation should be enriched in wild-type cells treated with COX4 siRNA, since the assembly of COX1 is blocked and CMC1 complex is accumulated in these conditions. As expected, newly synthesized COX1 is well enriched in CMC1FLAG immunoprecipitates (new figure 4K) thus strongly supporting our conclusion that CMC1 interacts with newly-synthesized COX1.

4.In Fig. 4H, why is there not an equivalent enrichment of CMC1 associated with the complex IV subcomplex when COX1 accumulates?

In original Fig 4H (now Fig 4I), we present the accumulation of COX1-containing subcomplexes when expression of either COX4 or COX5 was silenced for 3 days. We have now added a new panel (Fig 4J) where the accumulation of the COX1, CMC1 and COA3 proteins is even clearer, following 8 days of knockdown. In these conditions, there is an accumulation of the CMC1-containing complex but it indeed seems greater for COX1-and COA3-containing complexes on Fig 4I. We interpret these data as resulting from the accumulation of several complexes of similar size, with slightly different composition. We know that the COX1/COX14/COA3 complex is stable in KO-CMC1 cells and it is possible that the amount of CMC1 is limiting for the formation of the COX1/COX14/COA3/CMC1 complex, which could explain the differences mentioned by the reviewer. In support of this possibility, on the BN-PAGE analyses of COX4 or COX5 knockdown cell extracts, the signal for CMC1 is always sharper than that of COX1 and COA3, which are always more diffused (Fig 4I and J), probably because the latter detects two subcomplexes, COX1/COX14/COA3 and COX1/COX14/COA3/CMC1 the first one being slightly smaller.

5.Likewise, in fig 7C, why is the CMC1 in the subcomplex so much greater when Cox20 is knocked out while COX1 is not and the opposite is seen in Fig 7A?

For clarification, we have included a more exposed panel for COX1 signal in Fig 7C, where we can observe that actually COX1 is also increased in sub-CIV in KO-COX20, as observed in Fig 7a for COX2 or COX3 cybrids.

Referee #2:

This thorough study documents for the first time that human CMC1 is a COX1-specific assembly factor. Studies by this group in yeast demonstrated that yeast Cmc1 is a COX-assembly factor, but the precise step in COX assembly was not defined. The present human cell study shows in a convincing manner that CMC1 binds newly synthesized COX1 along with two COX1 chaperones COA3 and COX14. The studies support a model in which CMC1 remains associated with newly synthesized COX1 until the peripheral subunits COX4 and COX5a bind. CMC1 is localized in the intermembrane space in association with the membrane, so its association with an early COX1 assembly intermediate is novel and significant. The study utilized HEK293T cells with the CMC1 locus disrupted by TALEN technology. Cells lacking CMC1 retain ~60% of intact cytochrome oxidase CIV, so CMC1 is not a critical assembly factor. The investigators nicely show that CIV in the KO cells can be restored with CMC1-FLAG expression. A key observation is the visualization of the CMC1/COX1 assembly intermediate (subCIV) in Fig. 4D. One complicating aspect of the data is that the subCIV band observed on BN-PAGE is a composite of different subcomplexes. The missing experiment in Fig. 4 is the COX1 BN immunoblot in CMC1 KO cells. That isn't shown until Fig 5E and that reveals that the subCIV component is equally abundant in CMC1 KO cells. This suggests that the CMC1 component of the "subCIV" band is minor. Whereas CMC1 is not a critical component in COX1 maturation, COX14 is shown to be, but not COA3. The lack of any effect of COA3 depletion is unexpected. It would be important to test CIV activity in the COA3 depleted cells to assess whether it has any effect. The COA3 siRNA is shown in Fig 5A to be very effective, so this is baffling.

We agree with the reviewer that COA3 knockdown is expected to induce a defect in CIV assembly. The discrepancy is explained because in the original Fig 5B, the silencing was performed for only 4 days, and in our experience with HEK293T cells, the turnover of most preexisting CIV requires approximately 4-7 days. Another limiting factor when working with siRNA is the residual amount of protein that is synthesized (even if small, as seen if Fig 5A for COA3, it can potentially accomplish some function). Independently, the main point of the original figure was to show that CIV subassemblies containing newly synthesized COX1 are highly decreased in COA3-silenced cells compared to the control. To clarify the confusion, we have modified the figure. Now, in Fig 5A, we have included the quantification of the steady-state levels of CIV subunits and assembly factors in COA3-or COX14-silenced cells. COX1 and COX2 are mildly decreased after 3 days of knockdown and strongly decreased following 7 days of knockdown. On the other hand, CMC1 levels are essentially unaffected after 3 days and decreased after 7 days, arguing for instability of these proteins if they cannot find their natural interacting partners. In Fig 5B, we now present the BN-PAGE profile at day 3 and day 7 of knockdown. In both cases, the CMC1/COA3/COX1 complex is strongly attenuated whereas the CIV defect is clear after 7 days but mild after 3 days.

It is very convincing that CMC1, COA3 and COX14 form an early COX1 assembly intermediate. Using a COX1 cybrid cell line that is COX1 deficient, the CMC1 subCIV band is attenuated, suggesting that the CMC1 BN complex is Cox1-dependent. Steady-state levels of CMC1 should be shown in the cybrid line to assess whether steady-state abundance of CMC1 is COX1-dependent.

The steady-state levels of CMC1 and COA3 is shown in Fig 7B. We have now included the quantification of the bands in this figure. CMC1 and COA3 steady-state levels are decreased in COX1 cybrid cells but not in COX2 and COX3 cybrids. CMC1 and COA3 steady-state abundance is partially dependent on COX1 (50% decrease).

Likewise, is CMC1 attenuated in doxycycline-treated cells?

We added a panel F in Fig 4 with the steady-state levels of COX1, CMC1 and COA3 in cells treated with doxycycline for 24 hours as in Fig 4G. The quantification of several repetitions has been also included. CMC1 and COA3 steady-state levels remained unaffected after 24 hours doxycycline treatment. Furthermore, we also included the same experiment after 6 days of doxycycline treatment (Fig 7F) where CMC1 and COA3 steady-state levels are actually decreased. In conclusion, the permanent absence of COX1 adversely affects the stability of its interacting partners CMC1 and COA3, as observed in COX1 cybrid cells.

A second subCIV complex lacking CMC1 contains COX4 and COX5a, yet the abundance of this subcomplex is not so dependent on CMC1. An intriguing observation shown in Fig. 4H is that with attenuation of COX4-1 or COX5a, the abundance of the COX1 subCIV complex is markedly enhanced. The most likely subcomplex that accumulates would be predicted to be the CMC1-COX1 subcomplex, but the CMC1 abundance in the 120 kDa band doesn't increase so significantly. COA3 expands, so the component must be a COX1, COA3, COX14 subcomplex, but it isn't clear that CMC1 is present. The authors conclude on p. 10 that at least 2 COX1 subassembly complexes can be seen on BN gels. Perhaps there are more than 2!

See answer to reviewer 1, point 4.

The investigators attenuate COA3 and COX14 and observe the expected attenuation in COA3-COX1 subcomplexes. It is very unclear why the efficient siRNA attenuation in COA3 had no effect on CIV assembly (Fig. 5B). Again, CIV activity would be important to test to assess whether COA3 is important for human COX1 *maturation.*

Agree. See explanation on previous page in the response to your first point.

It is also surprising that COX11 or MITRAC7 siRNA did not lead to any attenuation in assembled CIV in Fig. 6C. Is this reproducible or substantiated in other studies?

Data for original Figs 6B, 6C and 6E were obtained in cells silenced for only 3 days, which can explain the absence of CIV attenuation, as explained above for COA3silenced cells. The goal of the experiment, however, was to explore the possibility that these proteins could be part of the CMC1 complex. Our data clearly showed that this is not the case (original Fig 6B, C and D). However, as the 3-day silencing experiment was clearly confusing for the reader, we have changed the figure and now present the same experiment at longer time of knockdown. For MITRAC7, the Rehling's group has shown a mild 50% decrease in CIV activity following knockdown. In our hands, although we obtained a very efficient knockdown of the protein (Fig 6B) we did not detect any obvious effect on CIV even after 7 days of silencing (Fig 6B and 6C). As CMC1, this protein is probably not essential for CIV assembly. However, the Rehling's group has convincingly shown that MITRAC7 interacts with COX1 in a complex with COX4. We have maintained this information in this new version of the article as it provides an additional evidence that CMC1 is released from the early COX1 intermediate prior to the incorporation of COX4. CMC1 does not co-immunoprecipitate with MITRAC7 (Fig 6D) and the CMC1 complex is unaffected in the absence of MITRAC7 (no shift of size or loss of stability). Although the function of human COX11 has not yet been established, it is expected that it will participate in copper deliver to COX1 as its bacterial and yeast counterparts, and therefore to be essential for COX1 maturation and CIV assembly. Here, we have substituted the original data of a short (3 days) COX11 knockdown for a longer one (7 days). New Fig 6E presents the steady-state levels of various CIV subunits following 7 days of silencing, as well as the

quantification of the data. Depletion of COX11 induces a strong decrease in COX2 and COX6b levels (down to residual 2535%) and a milder attenuation of COX1 and COX4 (residual 70-50%). We show that COX11 is indeed necessary for CIV assembly in human cells. Additionally, in Fig 6H-I, the BN-PAGE analysis of 7-day COX11 knockdown cells shows a clear decrease in holo-CIV and an accumulation of the CMC1/COX1/COA3 complex, but also some COX4 sub-CIV complex at the same levels. In the absence of COX11, CIV assembly stalls at an early step that induces the accumulation of the CMC1/COX14/COX1/COA3 complex, a portion of which undergo release of CMC1 and incorporation of at least COX4 to yield a COX14/COX1/COA3/COX4 complex. This data suggest that copper insertion into COX1 is not an absolute requirement for COX4 interaction with COX1. Either copper insertion into CIV assembly line occurs after COX4 incorporation or COX4 undergo some aberrant protein-protein interaction that prevents productive holo-CIV assembly.

In summary, this is a thorough study with high significance and novelty. This will be an important contribution to the field. The studies are well-executed, although a few studies are missing. The authors are encouraged to submit a revised manuscript with the suggested studies completed prior to a final decision.

Referee #3:

The manuscript "A CMC1-Knockout Reveals Translation-independent Control of Human Mitochondrial Complex IV Biogenesis" by Bournes et al. studies the role of the CMC1 in the context of cytochrome oxidase (COX) assembly. CMC1 is a twin-CX9X protein and therefore localizes to the mitochondrial intermembrane space where it stabilizes early assembly intermediates of COX. With a significant body of work the authors support their conclusions well, exploring an early step in COX assembly, which is differently regulated in yeast. The manuscript is very well presented as expected from this group. The points below should be addressed -I have only one real concern, i.e., the role of COX4 in the early assembly complex as outlined in point #4.

1) Table 1 was missing in the combined version that I reviewed.

The missing table was included in this revised version.

2) In figure 2 and subsequent figures one KO cell line is used. Please mention in the text, based on quantitative Western analysis, how much reduction is seen in COX levels.

Quantification were added in Fig 2C-D and Fig 3B and commented in the text.

This should also be discussed in the context of results shown in figure 2 A and B. It appears that COX is rate-limiting, a result that is important by itself and should be included/discussed.

We agree with the reviewer that the fact that a ~45% decrease in CIV activity limits cell respiration to ~70% supports the notion that there is a low reserve cytochrome c oxidase capacity in cultured HEK293T cells, in agreement with previous cyanide titration experiments by the Attardi's group that had shown that in several cell lines, the CIV activity capacity is in low excess (16-40%) with respect to that required to support the endogenous respiration rate. This is now mentioned in page 7 of the revised version.

3) Results section, when first talking about the 120 kDa complex: please mention molecular weights of all tested candidates. Please discuss how the molecular weights add up to the 120 kDa mass as you go along identifying the individual components.

A paragraph was added in the discussion, stating the size of the various proteins and discussing this composition of the ~120kDa complex.

4) Figure 5E and Figure 6F and Figure 7A: the COX4 panels do not make much sense. The authors previously concluded that COX4 is not part of the 120 kDa complex (e.g., 4C), but that clearly does not seem to be the case based on the multiple other experiments (the model in Fig. 7F should be changed, too). Is it possible that COX4 binds less tightly compared to the other components and is sometimes lost -perhaps conditions in Figs. 5E, 6F, and 7A were suitable to retain subunit IV in the

complex, however, such a scenario might complicate the interpretation of the size of the 120 kDa complex; it should noted that although BN-PAGE is a mild method, Coomassie still acts as a (mild) detergent, and conditions in individual experiments might slightly differ, possibly explaining the different experimental results. In Figure 6F the authors show COX4 running at a lower weight, however, in contrast to subunits that are added later (e.g., COX2 in Fig 6E) it is detectable and gives a robust band, suggesting that it is part of the complex. These findings have to be critically reevaluated and discussed. Regarding point #3 above it is also unlikely that the authors really have all components identified that constitute the 120 kDa complex. CMC1 (101AA), COX14 (57 AA), COX1 (513AA), and COA3 (57 AA) do not even come close to the reported molecular weight of 120 kDA. Adding COX4 and perhaps Va would bring this number closer (please discuss size predictions and their implications). In addition, it would be good to reprobe the blots shown Figs. 5E, 6F, and 7A with a COX5A antibody. For future work it might be a good idea to pull down the 120 kDa complex and to analyze it by mass spectrometry in order to identify additional components that may be part of the complex.

Several pieces of data demonstrate that COX4 or COX5a are not part of the CMC1 complex: 1-COX4 and COX5a do not co-immunoprecipitate with CMC1, even when using extraction conditions milder than for BN-PAGE analyses. 2-The stabilities of COX4 and COX5a are reciprocally interdependent. However, when COX4 or COX5a are silenced and then absent from the cell, the CMC1 complex is still accumulated (Fig 4I-J) and does not shift size on BN-PAGE. It should lose around 30 kDa if these proteins were part of the CMC1 complex. In new Fig 5E, the BN-PAGE analysis of CIV sub-complexes in KO-CMC1 shows the presence of at least three different complexes: a) CMC1/COA3/COX1/COX14 in the WT; b) COA3/COX1/COX14 in the KO, and c) COA3/COX1/COX14/COX4/COX5a in both cell lines. CMC1 is not essential for CIV assembly since in its absence some 30% normal CIV biogenesis occurs, which explains the detection of some COA3/COX1/COX14/COX4/COX5a complex in KO-CMC1 cells. In the figure, this complex seems indeed in the COX4 panel as abundant in the WT and in the KO line, although the latter was more loaded (see in the loading control) to facilitate clear detection of the COX1-and COA3-containing sub-complexes in both cell lines on the same gel. Abundance of COX4-containing complexes is actually lower in KO-CMC1 than in the WT. Unfortunately, our COX5a antibody is too weak for BN-PAGE analysis of sub-complexes, it would be indeed interesting. The 120 kDa CMC1 complex contains at least CMC1 (12 kDa), COA3 (12 kDa), COX1 (40 kDa) and COX14 (7 kDa) which do not add up to 120 kDa. Among several possibilities, the stoichiometry of these proteins could differ from 1:1:1:1, additional unidentified proteins could be present, or the migration on BN-PAGE of this particularly hydrophobic protein complex could differ from its real molecular weight. The clarification of this point warrants future work.

Minor:

-Page 4: COX4-1 is introduced here without further explanation; it might be useful to include a sentence on page 3 (where the COX subunits are listed) and to mention that several of the COX subunits are expressed as tissue specific isoforms, introducing the COX4-1/COX4-2 pair up front.

We have included a paragraph in page 10 introducing the two COX4 isoforms and the explanation of the data (Fig 4H) showing that, as expected, only the normoxic isofom COX4-1 is relevant for CIV assembly in HEK293T in our growth conditions.

-Figure 2C: To this reviewer it seems that complex 3 levels are increased when CMC1FLAG is expressed. Perhaps worth discussing in the context of supercomplex formation. For experiments as the one shown here it would be good to quantitate the Western blots and to report significant changes in the text of the manuscript.

Complex III is not reproducibly increased in KO-CMC1. Quantification of the data has been included in Fig 2.

-Make a mention in the figure legend what the difference is between panels 1 and 2 in figure 2E (overexposure of the latter, correct?). Third panel: why did the authors use two antibodies here? Using the complex I antibody only would be more informative.

In original Fig 2E, the NDUFA9 antibody that we used (and previously reused several times in the laboratory) was slightly contaminated by some COX1 antibody. We have now changed this figure by another repetition using a fresh NDUFA9 antibody. In this new figure, two different expositions are presented for COX1 and CORE2 and mentioned in the legend.

-Page 8: "Here, consistently with the BN-PAGE data, the steady state levels of all CIV subunits, including COX1, decreased in the absence of CMC1 (Fig. 3B)." Does this statement hold up for subunit IV? See also point #4 above.

Quantification of the data has been included in Fig 3B, which shows that the decrease in COX4 and COX1 steady-state levels are milder (70-50%) than for COX2, COX3 and COX6b (20 to 35%). Also see answer to reviewer 1, point 1.

Page 9: Format Mick reference.

Corrected.

-Bar diagrams in Figures 4B and 7D: error bars are missing.

Error bars were added.

-Figure 5B legend: state how long knockdown was performed. In addition, explain the difference between the top and second panel in the legend (overexposure?).

This figure was modified and the legend consequently adjusted. The requested information was included.

2nd Editorial Decision 23 November 2016

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see all three referees are very positive about the study and support its publication in EMBO reports. Referee 3 however is not yet fully convinced that COX4 is not part of the Cox1-Cmc1 sub-complex. I have discussed this matter further with referee 1 and 2 who consider the presented data sufficient to support your assertion that the Cmc1-containing Cox1 complex lacks Cox4 and Cox5. It is therefore not a prerequisite to perform the experiments suggested by referee 3 for acceptance but if you can reprobe the blots and supply the corresponding data in the revised manuscript, it would certainly further strengthen this point. Apart from this, there are a few things that we need from the editorial side before we can proceed with the official acceptance of your study.

- You have submitted information concerning the antibodies used as supplemental material. Please note that all information on materials and methods must be part of the main manuscript. Please incorporate this table into the Materials & Methods section and modify the corresponding reference to this information in the Author Checklist.

- Every EMBO reports paper includes a 'Synopsis' to further enhance its discoverability. Synopses are displayed on the html version and they are freely accessible to all readers. The synopsis includes a short summary text of about two sentences as well as 2-4 one sentence bullet points that summarize the key findings of the paper. These should be complementary to the abstract, i.e., not repeat the same text. This is a good place to be more informative and include, as appropriate, key acronyms and quantitative or organism information. Could you please provide the text as well as a synopsis image (100-400 x 550 pixels) that summarizes the paper or shows a model?

We look forward to seeing a final version of your manuscript as soon as possible. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The authors have adequately addressed my concerns and I believe the manuscript is now suitable for publication.

Referee #2:

The revision is well-crafted. The additional studies e.g. prolonged shutdown of Cox11 reveals important new data. This is a solid study with ample novelty to justify publication. The study is highly significant.

Referee #3:

In the revised version of the manuscript the authors have addressed all of my concerns except for one point that was only partially addressed and relates to the role of COX4 in the 120 kDa complex. I would feel more comfortable if this issue had been discussed as part of the manuscript: Figures 5E, 6G, and 7A show that COX4 is present in the 120 kDa complex since the authors show a band with the COX4 antibody at 120 kDa. Assuming the antibody is specific (as judged from source files of Fig. 3C or Fig. 4H) how can the signals in Figs. 5E, 6G, and 7A be explained?
The authors argue that several lines of evidence suggest that COX4 (and COX5) is not part of the complex, but I am not convinced that this holds up:

The authors argue: "COX4 and COX5a do not co-immunoprecipitate with CMC1, even when using extraction conditions milder than for BN-PAGE analyses."

The IP experiment does not prove that COX4 and COX5 are not part of the complex. For example, antibody binding could interfere with binding to COX4 and COX5.

The authors argue: "However, when COX4 or COX5a are silenced and then absent from the cell, the CMC1 complex is still accumulated (Fig 4I-J) and does not shift size on BN-PAGE. It should lose around 30 kDa if these proteins were part of the CMC1 complex."

This is indeed a good point. However, over the course of the knockdown perhaps enough of COX4 and COX5 might have accumulated, enough to give a signal in the Western blot, since this is not a knockout experiment.

It would therefore be good to re-probe the blots shown in Fig. 4I/J with a COX4 (and COX5) antibody to answer this question definitely. At this point it is difficult to say which experiments are more meaningful, Figs. 5E, 6G, and 7A versus Fig 4I-J, but without the above control this reviewer still believes that COX4 (and perhaps COX5a) are part of the 120 kDa complex.

A minor point that I forgot to mention in my previous review is that COX5a is not a transmembrane protein (model in Fig. 7G) but instead exclusively localized to the matrix side on COX. Depending on the outcome of the control experiment above the model should be modified accordingly as well as the text of the manuscript.

2nd Revision - authors' response 25 November 2016

Answers to reviewers:

Regarding the remaining concern of reviewer 3, we have added the requested blot probed with anti-COX4 antibody in figure 4J. The results show that COX4 is not accumulated within the CMC1 complex, further strengthening our conclusions. On a second respect, we appreciate the observation regarding the matrix localization of human COX5, which now has been corrected in the model

presented in Fig 7.

3rd Editorial Decision 02 December 2016

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Antoni Barrientos
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2016-43103V1

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- If n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Data Analysis for in vitro and cell culture studies: the majority of experiments were done at least in triplicate. One was done in duplicate : mitochondrial proteins synthesis in cybrids mutants (Fig 7E), however the same aspect was tested in triplicate in another cell line (Fig 7D) and as been already published in again another. Quantitative data were presented as means ± STDEV and expressed in
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Does not apply
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Does not apply
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Does not apply
For animal studies, include a statement about randomization even if no randomization was used.	Does not apply
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Does not apply
4.b. For animal studies, include a statement about blinding even if no blinding was done	Does not apply
5. For every figure, are statistical tests justified as appropriate?	Yes, mentioned in figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normality of data distribution, adequacy of sample size and equality of variance in standard deviation were assumed.
Is there an estimate of variation within each group of data?	Does not apply
Is the variance similar between the groups that are being statistically compared?	Does not apply

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We provide a list of the antibody used in this study in table 2. We actually confirm the correctness of all the antibodies in this study. We show that the western signal dispear either the corresponding knockdown or knockout or we observe the expected size in both denaturated or native condition.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We profided the sources of all the cell lines tested in the material and methods tested, and used antibodies to confirm theirs authenticities. They were not tested for mycoplasma contamination.

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Does not apply
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Does not apply
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Does not apply

E- Human Subjects

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13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Does not apply

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F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Does not apply
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G- Dual use research of concern

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