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Palmitoylated TMX and Calnexin Target to the Mitochondria-Associated Membrane (MAM)

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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.)

1st Editorial Decision 19 April 2011

Thank you very much for submitting your paper on the characterization of a putative MAM-targeting motif for consideration to The EMBO Journal editorial office.
I do enclose the scientific assessment from three scientists for your information. As you will see, all rank the potential interest in your findings as relatively high. However, they also raise concerns on the conclusiveness of the data, at least at the current stage of analysis. Both, ref#1 and #2 raise concerns about the biochemical fractionations with ref#2 proposing to redo some of the work in more appropriate cell lines to improve the resolution on TMX1-3. This should also include better controls of the antibody. Ref#3 goes a step further in requesting the characterization of additional sequences/processes contributing to MAM-targeting. On balance, we still would like to offer you the chance to address these critical points as well as other issue broad up be the referees during a single round of major revisions.

Please do not hesitate to contact me with in case of further questions or indeed possible extension of the revision dedline (preferably via E-mail).

Finally, I do have to formerly remind you that it is EMBO_J policy to allow a one round of revision only and that the final decision on acceptance or rejection depends on the content and strength of the ultimate version of your study.

Yours truly,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1:

This is a very interesting paper on an emerging topic in cell biology, i.e. the elucidation of the specialized signaling domain at the interphase of mitochondria and ER. The biochemical isolation of a characterized membrane fraction (MAM) has opened the way to the direct identification of resident proteins, and now critical issues can be tackled (protein composition of MAMs, targeting signals, etc.). The current paper allows a significant step forward, by demonstrating that palmitoylation of critical residues is a signal for clustering ER proteins to the MAM. The experiments appear accurately performed and well presented, and the conclusions are supported by the presented data. I think the paper would be strengthened by addressing the following issues:

1. The subcellular fractionations of Fig. 1 E are a bit strange. ER proteins (ERO1α, CNX) are virtually absent in the microsomal fraction, then appear most abundant in the crude mitochondrial fractions, and decrease in both pure mitochondrial and MAM. This does not make sense, and one wonders both about the purity of the fractions and protein degradation. The issue must be solved, also using EM analyses.

2. The distribution of TMXs in the A375P and HeLa is not the same. TMX3, used in the rest of the paper as the prototype of non-MAM TMX, in fact appears strongly enriched in the MAM fraction in A375P (not in HeLa cells). Is there a tissue specific difference? And how do the authors explain it?

3. Of all MAM markers, CNX is the one I am least enthusiastic about, as many groups report it to be a ubiquitous chaperone, with no selective clustering in defined ER domains. Is the palmitoylation site found also in other MAM markers, and can the data be corroborated with another protein?

4. A number of signaling proteins are reported to be present in MAM (Bcl-2 family members, PML, p53), and the great interest in this cell domain depends also on the emerging idea that it controls signaling events controlling cell fate. Do these protein contain palmitoylated residues? And does inhibition of palmitoylation affect their subcellular distribution?

5. Finally, MAM's importance is related the crosstalk between the two organelles. How is the sorting of mitochondrial proteins affected by palmitoylation? Are also these proteins palmitoylated? Or does the clustering of ER proteins guide also the mitochondrial proteins? Monitoring VDAC isoforms, that have different distribution in mitochondrial, could be very beneficial for addressing this point.

Referee #2:

The MS of Lynes et al is part of a series of works by the Simmen's group, which focuses on targeting of proteins to the mitochondria associated ER membranes (MAM). This is an important subject considering the fundamental role of MAMs in cell signaling and metabolism and the lack of information on how proteins become localized at the MAM. This MS is concerned with the differential localization of transmembrane members of the protein disulfide isomerase (PDI) family of ER oxidoreductases (TMX1-4) in the ER membrane. Several lines of evidence are provided that both for TMX1 and calnexin, another ER protein that shows MAM localization, palmitoylation of specific cysteine residues is responsible for MAM targeting. Most of the studies seem to be carefully conducted but a number of points require clarification.

-Evaluation of endogenous TMX isoforms is totally dependent on antibodies that seem to provide weak signal. The validation of the antibodies is missing. It would be important to show the bands' molecular weight and a broad molecular weight range of the blots so the specificity of the antibodies can be assessed. Please consider that these antibodies are also used for immunofluorescence, where the different molecular weight proteins are not separated from each other. Surprisingly, the anti

TMX1 antibody does not seem to be listed in the methods.

Genetic targeting of TMX1 and 4 (siRNA?) and documentation of the changes in the respective bands/immunostaining would offer the best validation of the results described in Figs1-3 would be.

-Fig1
CaCo2 and M2 would be better than HeLa for the study of TMX1,2 and TMX3 respectively
Fig1E Why is not complex 2 is enriched in pure mito?

-Fig2 specificity of the immunofluorescence is unclear. Some antibodies also show nuclear signal.

-Fig5 Is the targeting of TMX2 to the MAM is also dependent on palmitoylation?

-Fig6A
IP3R3 do not seem to be MAM localized.
In the 2BP treated samples SERCA2B seems to show more MAM localization.

-Anti complex 2 listed twice in methods

-page 19 para 2: "32,700 rpm" please provide the force in g-s

Referee #3:

The paper by Lynes et al., EMBOJ-2011-77673, reports about palmitoylation as a new mechanism to accumulate preferentially two ER transmembrane proteins, the oxido-reductase TMX and the chaperone calnexin, in the MAM domain. The evidence, obtained mostly by gradient centrifugation and confocal/electron microscopy of HeLa cells, was strengthened by the use of mutated forms and chimerae of the proteins and by the use of an inhibitor of palmitoylation. As a whole the evidence reported in the paper demonstrates convincingly the involvement of palmitoylation in the MAM enrichment of the two proteins. However, as recognized in the paper, palmitoylation is a widespread process that induces multiple, diverse effects and involves a large number of proteins, some of which also resident in the ER. The specificity of the effect illustrated in the paper, which is not given a mechanistic explanation except for the possible involvement of multiple palmitoylating enzymes, the PATs, should be thoroughly re-considered to make the paper really convincing. Moreover the paper is redundant. It could be easily condensed of at least 20%.

Major points

1. as already mentioned, depending on the ER resident proteins palmitoylation does induce not only the enrichment in the MAM domain but also other effects Therefore, at variance with what repeatedly reported in the text (see for example page 14), palmitoylation cannot be fully responsible for the MAM enrichment, but requires the co-operation of other process(es), most likely not ltjoise inducing the simple ER retention. In the discussion the need of a mechanistic co-operation is acknowledged: "ER domain targeting requires a combination of multiple targeting sequences. In the case of calnexin for example, this includes an acidic cluster in the cytosolic domain that interacts with PACS-2 (Myhill et al, 2008); in the case of TMX, this includes a RQR motif reminiscent of the RQR ER retention motif of TMX4 (Roth et al, 2009)". This is a very important aspect of the work that should be further investigated. Is palmitoylation still effective when the other signals are shut down?

2. The enrichment of TMX and calnexin in MAMs is evident but moderate, no more than 50% maximun. Therefore this enrichment is not a real targeting. Most likely it is the result of a dynamic equilibrium among the various ER domains. This aspect should be discussed.

3. A problem already discussed in the paper is the relationship between MAMs and membrane rafts. In the case of calnexin the two domains appear to dissociate, therefore the concept that palmitoylation works because it drives the protein to rafts is unlikely. This conclusion is not surprising since 1. MAMs are large, and rafts small; 2.the ER contains little cholesterol: is it enough

to account for the detergent resistance of MAMs?; 3. resistance to detergents is a very crude criterion for raft identification. These aspects should be strengthened with new experiments evidence or greatly attenuated.

Minor Points

1. page 6, line 11: A375P cells express not only TMX4 but also the other TMXs, this should be specified.
2. Line 24: from Fig. 1C: also TMX3 appears enriched in the MAMs of A375P cells.
3. Page 7, line 16.: overlapping of TMX with mitotracker. Here the evidence is not very strong. To be convincing it should be emphasized that not all mitochondria re enwrapped by MAMs. In addition , panel C of Figure 2 is far from convincing: the overlapping of calnexin with mitotracker is certainly less extensive than that of TMX shown in panel A. Moreover, the phenotype of cells in panel C appears quite different from that of A and B. Panel C should be replaced. The same is needed in Fig. 5. About the Manders coefficient: how many images were analyzed?
4. Electron microscopy. The images are of poor quality and the significance of the results is unclear. How many images and how many particles were counted? In my opinion the impact of these images is quite limited. Quantitation emerges already from confocal microscopy. I would recommend removing the EM.
5. Page 9, line 2: "the majority of the anti-myc signal" definition is valid only for one construct, 141. For the other two, 411 and 441, the MAM signal is less and much less than 50%.
6. Line 5: why the negative data with TMX4 are not shown? This is an important result

1st Revision - authors' response 18 August 2011

(begins on next page)

Reviewer #1

This reviewer expressed his/her overall positive impression of the manuscript by stating that "the experiments appear accurately performed and well presented, and the conclusions are supported by the presented data." At the same time, she/he asked us to strengthen our manuscript with the following points (summarized before our reply and indicated in italics, references are given at the end of this letter):

1. *There was a concern about the purity and quality of our Percoll gradient fractions*: To address these points, we have expanded and repeated our Percoll gradient fractionation, now shown in Figure 1E and Supplemental Figure 4. We have optimized the separation between pure mitochondria and the MAM by a precise, reproducible selection of the fractions on the Percoll gradient. This is evidenced by **i.** the absence of the rough ER marker ribophorin-2 labeling in either fraction, **ii.** the majority of complex II labeling in the pure mitochondria fraction and **iii.** the improved isolation of MAM proteins, including TMX and calnexin, in the final MAM fraction. To investigate the purity of the presented fractions, we have performed electron microscopy on these fractions (Supplemental Figure 4). This showed that i. microsomes contain ribosomes (frequently present as clusters) and membraneous structures, ii. pure mitochondria contain mitochondria (e.g., upper left and lower right corner), and iii. the MAM fractions are largely devoid of intact mitochondria, but instead contain membraneous structures of different quality and aspect.

2. *TMX3 shows a distinct distribution between HeLa and A375P cells*: This observation by the reviewer is indeed correct. TMX3 appears to be more mobile within the ER than the other transmembrane oxidoreductases that we have examined. In Caco2 cells, its distribution covers all domains of the ER without any enrichment. To describe these findings better, we have added the following sentence: "In contrast, TMX4 was found in all ER fractions, whereas TMX3 showed a variable distribution, depending on the cell line." The explanation for these observations could lie in cell line specific TMX3 lipid or phosphorylation modifications or protein-protein interactions, but we have chosen not to investigate the reasons for this mobility any further in this manuscript, since they are likely not connected to TMX, TMX4 or calnexin.

3. *Calnexin is a ubiquitous chaperone with clustering in defined ER domains. Is the palmitoylation site also found in other MAM markers, and can the data be corroborated with another protein?* For the first point, we have two explanations. Firstly, besides our own published research papers (Bui et al, 2010; Gilady et al, 2010; Myhill et al, 2008), we are not the only laboratory that has observed the enrichment of calnexin on the MAM and a lesser amount of calnexin on other domains of the ER. i. Chevet et al. have published twice that calnexin is mostly on ER heavy membranes, and less so on microsomes (Delom et al, 2007a; Delom et al, 2007b), ii. Hayashi and Su showed in Figure 1G of their Cell paper from 2007 that calnexin, but not PDI or CYP450R is enriched on the MAM (Hayashi & Su, 2007), iii. Stone and Farese showed in Figure 4A of their JBC paper from 2009 that the microsome/MAM distribution of calnexin and DGAT2 is indistinguishable and that both are enriched on the MAM (Stone et al, 2009), iv. calnexin is more MAM-enriched than the IP3R, as reported by (Wieckowski et al, 2009) and v. whereas PDI is found in microsomes and does not show overlap with MAM proteins PSS1/2, calnexin shows extensive overlap (Stone & Vance, 2000). Together, this plethora of published information is highly consistent with our assessment that calnexin is indeed enriched on the MAM.

At the same time, we agree with this reviewer that calnexin is ubiquitous within the ER. In fact, we detect signals for calnexin in all ER fractions, albeit of significantly lower level outside of the MAM. It is possible that specific stress situations lead to a loss of calnexin MAM enrichment, thus explaining increased levels of calnexin on domains not identical with the MAM. For instance, the block of palmitoylation shows such a reduction clearly in Figure 6.
For the second point, we have extensively scanned all currently available proteomic studies that list the palmitoylated proteome (Dowal et al, 2011; Forrester et al, 2011; Kang et al, 2008; Yount et al, 2010). These scans have led to the identification of mouse heme oxygenase-1 and human VDAC1/2 as well as human GRP75. As answer to this reviewer's question, heme oxygenase-1 is indeed dependent on palmitoylation for enrichment to the MAM, whereas the mitochondrial VDACs and GRP75 are not (Figure 8). We have thus successfully corroborated palmitoylation as being important for MAM targeting of ER membrane proteins.

4. *Do Bcl-2 family members, PML or p53 contain palmitoylated residues? Does inhibition of palmitoylation alter their intracellular distribution?* We have scanned the same set of databases and our own screen (Fahlman & Berthiaume, unpublished observations), but the only candidate protein within this group that we could identify is the Bcl2 family member Bcl2L12. However, there is no evidence that Bcl2L12 localizes to the MAM. Instead, we have analyzed whether a set of Bcl2 family proteins localize to the MAM and other regions of the ER. This group comprises Bim and Puma, but we were unable to detect palmitoylation of either. Instead, we indeed identified GRP75 as a palmitoylated protein. Contrary to heme oxygenase-1 however, this novel MAM palmitoylated protein did not shift to other domains of the ER. Due to these experiments, our results therefore suggest that palmitoylation is mostly affecting the MAM localization of membrane ER proteins, but not of peripherally associated ER proteins.

5. *Are mitochondrial proteins, in particular proteins of the VDAC family palmitoylated?* This is a very useful suggestion. We have examined the distribution of VDAC1 and 2 to domains of the ER. Consistent with some published papers, we have detected only small amounts of these on the ER. However, the inhibition of palmitoylation led to a small shift of VDAC1 and VDAC2 into domains of the rough ER, suggesting that the equilibrium of VDAC between the ER and mitochondria could be affected by palmitoylation.

Reviewer #2

This reviewer stated that "most of the studies seem to be carefully conducted, but a number of points require clarification." Our reply is given for each paraphrased point indicated in italics, references are given at the end of this letter:

1. *The validation of the antibodies is missing. It would be important to show a broad molecular weight spectrum of the blots. Surprisingly, the anti-TMX antibody is not listed in the methods. Genetic targeting and documentation of the changes in the respective bands/immunostaining would offer the best validation:* We provide the name of the supplier (Sigma) in the revised manuscript. We now also provide a quality control of the antibodies as they have been used in the manuscript, rabbit anti-TMX (IF and Western) and mouse anti-TMX4 (IF), using siRNA and shRNA. The rabbit anti-TMX4 antibody used for Western blots has been validated in Supplemental Figure 1 of (Roth et al, 2009). We now also show a large section of a gel for the anti-TMX antibody, demonstrating it does not cross-react with many bands. The rabbit anti-TMX4 antibody used for Western blots has been described in (Roth et al, 2009).

2. *CaCo2 would be better than HeLa for the study of TMX1, 2 and 3, respectively*: We now show the Optiprep gradient results for CaCo2 in Supplemental Figure 3.

3. *Why is complex II not enriched in pure mitochondria (Figure 1E)*: As an extension to our response to reviewer #1 point 1, we now have improved separation for mitochondrial and MAM proteins. Mitochondrial proteins are now enriched in pure mitochondrial fractions, with only residual amounts in MAM fractions. Similar distribution has previously been observed by Scorrano and de Brito for Tomm20 (Figure 1e (de Brito & Scorrano, 2008).

4. *Figure 2: Why do some antibodies also show nuclear background signal?* On some preparations (e.g., 2C, but not 2A, D), anti-TMX does show nuclear background. We currently do not know the reason for this, but by providing Supplemental Figure 1, we are confident to have provided sufficient evidence for antibody specificity.

5. *Is targeting of TMX2 also dependent on palmitoylation?* TMX2 is not known to be palmitoylated, so it likely uses a different mechanism.

6. *IP3R3 do not seem to be MAM-localized*: Indeed, this appears to be a surprising finding, but it has been published previously by multiple laboratories (Giorgi et al, 2010; Szabadkai et al, 2006; Wieckowski et al, 2009) that IP3Rs are MAM-localized, but not MAM-enriched.

7. *SERCA2b shows more MAM-enrichment upon 2-bromopalmitate treatment*: This is a correct observation by this reviewer. While we cannot explain this movement of SERCA2b (unique to this protein), we refer to this observation as follows: "The MAM amounts of these marker proteins were not reduced by the treatment of HeLa cells with 2-bromopalmitate, but only led to an increased MAM enrichment of SERCA2b (Figure 6A)."

8. *Anti-complex II is listed twice in Materials & Methods*: This has now been corrected.

9. *32700 rpm: Please provide g-force*: We now add that this corresponds to 100,000g.

Reviewer #3

In the eyes of this reviewer, "the evidence reported in the paper demonstrates convincingly the involvement of palmitoylation in the MAM enrichment of the two proteins." However, this reviewer asked us to thoroughly reconsider the specificity of the effect illustrated in the paper. We agree that our manuscript in its previous version did not address this issue. With our new Figure 8, we are however confident to provide such insight, since this figure demonstrates that the effect appears to be specific to ER membrane proteins, but is not or only partially reproduced for mitochondrial VDAC proteins or the cytosolic, peripheral MAM protein GRP75. Our reply is given for each paraphrased point indicated in italics, references are given at the end of this letter:

Major points

1. *Palmitoylation cannot be fully responsible for MAM enrichment, but requires the cooperation of other processes and sorting signals. This is a very important aspect of the work that should be further investigated. Is palmitoylation still effective when the other signals are shut down?* We agree with this reviewer that this is an important question. We have therefore undertaken the requested studies, which

are presented in Supplemental Figure 7. Since the CXXC mutant did not show altered distribution, we focused on the mutants of the PACS-2 interaction motif and the RQR ER retention motif of TMX. We now show that palmitoylation is not dependent on the presence of these motifs, suggesting, as we had suspected, that these mechanisms are complementary.

2. *The enrichment of calnexin and TMX is moderate, no more than 50% maximum, it is likely the result of a dynamic equilibrium among the various ER domains:* We refer to the described MAM localization mechanisms as targeting, but when speaking about a steady state scenario mention it as enrichment. Nevertheless, given the expected small relative size of the MAM versus the remainder of the ER, even a 50% amount on this domain is a very significant enrichment, leading to drastically lower concentrations of TMX and calnexin on other domains of the ER. We have added the following sentence in the discussion: "MAM-associated calnexin amounts to around 50% of its total, comparable to TMX; however, given the small relative size of the MAM, when compared to the remainder of the ER, this is a very significant enrichment." We agree with this reviewer (as also stated in the response to reviewer #1, point 3) that these chaperones and oxidoreductases are mobile within the ER. Furthermore, we are aware that chemical or homeostatic conditions could lead to changes of this equilibrium Such statements have also been tentatively published in papers of the Chevet laboratory dealing with calnexin, albeit without providing any mechanism (Delom et al, 2007a; Delom et al, 2007b). Moreover, palmitoylation is a reversible modification that offers itself for such a mechanism; consistent with this possibility, our results suggest that palmitoylated (although we cannot assume wild type proteins to be fully palmitoylated) and non-palmitoylated TMX and calnexin localize to different domains of the ER.

3. *A problem already discussed is the relationship between MAMs and membrane rafts. The concept that palmitoylation works, because it drives calnexin and TMX into rafts is unlikely. Is ER-associated cholesterol enough to account for the mechanism? These aspects should be strengthened with new experiments*. We agree with this reviewer that ER cholesterol is not very relevant for MAM enrichment, nor for the MAM structure, at least from the point of view of the proteins examined in this paper. We have added Supplemental Figure 8 that shows only a marginal effect for calnexin and TMX MAM enrichment upon cholesterol depletion. Therefore, DRMs containing TMX are likely not cholesterol-based. Nevertheless, given the demonstrated role of cholesterol for the sigma-1 receptor (Hayashi & Fujimoto, 2010), it is still possible that cholesterol regulates the formation of other MAM-associated DRMs.

Minor points

1. *A375P cells express not only TMX, but also the other TMXs; this should be specified*. We have now rephrased as follows: "This showed that in this selection, A375P was the only cell line that also expressed TMX4 in high amounts, as previously reported (Roth et al, 2009)."

2. *Also TMX3 appears to be enriched in MAMs of A375P cells*. We have used TMX and TMX4 as a tool to discover the TMX MAM enrichment mechanism. While it is true that TMX3 shows MAM enrichment in some cell lines, it is not found in all cell lines. We have rephrased as follows: "In contrast, TMX4 was found in all ER fractions, whereas TMX3 showed a variable distribution, depending on the cell line."

3. *Panel C of figure 2 is far from convincing: the overlap of calnexin with mitochondria is certainly less extensive than that of TMX shown in panel A. The phenotype of cells in panel C appears quite different from panels A and B*. We are not quite certain what was meant with this comment, since

Figure 2C does not show overlap of calnexin with mitochondria, but rather its overlap with TMX. This panel, together with Figure 6 simply shows that both TMX and calnexin show overlap with mitochondria, but of a different kind; they also both only overlap partially with each other. Other than that, we agree that the cells in panel C look slightly different, but are within the bandwidth for HeLa cells in our hands. We also agree that overall, the overlap of calnexin with mitochondria is slightly less than for TMX, this set of data is shown in Figure 6.

4. *In my opinion, the impact of electron microscopy is quite limited*. Electron microscopy was used to cross-check our findings with another technology. We agree that this is not the strongest piece of evidence, which is why we have placed it as Figure 3 rather than into Figure 1.

5. *"As shown in Figure 4C, we detected the majority of the anti-myc signal in fraction 6 containing the MAM, whenever the cytosolic portion of TMX was present (constructs 141, 411, and 441)." This is valid only for one construct, 141*. We agree mostly with this assessment and have changed the sentence as follows: "As shown in Figure 4C, we detected a gradient of the anti-myc signal towards fraction 6 containing the MAM, whenever the cytosolic portion of TMX was present (constructs 141, 411, and 441)."

6. *Why was the negative data with TMX4 are not shown? This is an important result*. Unfortunately, it is not clear to us, which negative data is meant, since Figure 4 lists all constructs with results on all chimera involving TMX and TMX4. We are happy to provide this if this reviewer specifies what aspect is meant and on which page.

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2nd Editorial Decision 21 September 2011

Once more apologies for this delay in getting back with a final assessment caused by temporary unavailability of the last referee. With the text amendments requested by ref#3 already provided, I kindly ask you to consider the minor points raised by ref#2 and provide us with an ultimate version to your earliest convenience.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #2:

The MS was carefully revised and addresses my concerns. Some of the individual experiments are not overwhelming on their own, but as a whole, the results effectively support an interesting and important novel aspect of protein targeting to the ER-mitochondrial interface.

I think the paper would benefit from simplifying some of the primary figures and moving figure components to the supplemental material. For example several images shown in Fig2 and 3 can be moved to the supplemental material and the remaining panels can be unified to a single figure.

Minor points:
-Fig3 table; Please state how many particles were counted.
-Mitochondrial complex 2 is abbreviated as "Mito C2" in Fig 6 vs "C2" in Suppl Fig2.
-It would be practical to show molecular weight markers for the immunoblots.

Referee #3:

The revised version now includes several suggestions of reviewers and is therefore improved. Other suggestions, however, have not been given much attention. In addition, the paper is long and repetitive. A thorough reconsideration of the text appears therefore appropriate.

2nd Revision - authors' response 23 September 2011

I am pleased to resubmit the manuscript ID# **EMBOJ-2011-77673R2** entitled **"Palmitoylated TMX and Calnexin Target to the Mitochondria-Associated Membrane (MAM)"** by Lynes, Bui, Yap, Benson, Schneider, Ellgaard, Berthiaume and Simmen. Our paper has been revised and we have addressed the issues raised by the reviewers.

In brief, the most important changes and improvements to the manuscript are the following:

1. As requested by reviewer #2, we have fused Figures 2 and 3 and have moved less important data into the supplemental figures.

2. We now provide the number of particles that were used for the immuno EM quantification in the figure legend.

3. We have labeled Complex 2 uniformly as “C2”.

4. We have added molecular weight markers in Figure 1B, also using bigger gel excerpts.

5. We have cut the text by approximately 12.5% to address the concerns of reviewer #3.

Overall, we are confident to have addressed all points raised by the reviewers and look forward to your response.