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The intracellular redox state is a core determinant of mitochondrial fusion.

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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 28 February 2012

Thank you for the submission of your research manuscript to our editorial office. We have now received the full sets of reviews on your study.

As the reports are pasted below I would prefer not to repeat them here in detail, but to only summarize the main points raised by the referees. You will see that all referees agree on the potential interest of the findings. However, they also agree that more work is needed in some places to provide stronger evidence for the claims made. All reviewers point out technical issues, including the need for additional statistical analysis and controls, that would need to be addressed. Both referees 2 and 3 state that more direct evidence should be provided that GSSG promotes mitochondrial fusion by oxidizing mitofusins and suggest generating cysteine mutants of mitofusins to test whether they still respond to GSSG. Reviewers 1 and 3 also feel that stronger proof for homomeric oligomer formation would be needed and that the data on the mobility shift of Mfn2 upon GSSG treatment would need to be provided.

Overall, given the reviewers constructive comments and the potential interest of the study, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the referees (as outlined above and in their reports) must be addressed. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that therefore, acceptance or rejection of the manuscript will depend on the completeness of your

responses included in the next, final version of the manuscript.

I look forward to seeing a revised form of your manuscript when it is ready.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

General Comments:

Previous work has demonstrated that ROS can provoke mitochondria to fuse. In this work, Shutt et al. investigate the molecular mechanism of this hyperfusion phenomenon. They argue that GSSG changes the oxidation state of mitofusins, which is correlated with greatly increased mitochondrial fusion in an in vitro reaction. When GSSG levels are high, the authors propose that mitofusins form oligomers via disulfide bonds that facilitates fusion. GTP hydrolysis seems important for generation of these oligomeric species, particularly those formed in cis.

That mitofusins can be oxidized and activated as a result of oxidative stress is interesting and of general interest to molecular biologists and those studying mitochondrial dynamics. Although the biochemical consequences of complex formation that lead to increased fusion remain unclear, I would recommend publication in EMBO Reports contingent upon the authors addressing specific concerns about the data quality and interpretations presented in this work. Two additional experiments are suggested that would strengthen the manuscript.

Specific Comments:

a) The 'control' and 'GSSG' samples in Figure 2C are presumable different glycerol gradients. Therefore, I am unconvinced that a 1-2 fraction shift between 'control' and 'GSSG' is real and not due to variation between glycerol gradients. In any case, quantification of bands and graphing intensity over fraction number is certainly called for here. Finally, the authors provide no interpretation of this shift of Mfn2 in GSSG - what could it mean?

b) The GTPgammaS glycerol gradient can be removed from Figure 2C - a simple SDS page gel of protein samples demonstrating the effect of GTPgammaS is preferred (as is done in Figure 3), since a gel with untreated samples loaded in a nearby lane would be internally controlled for possible poor transfer of large oligomers from the gel to the membrane.

c) Tiron has a catastrophic effect on mitochondrial fusion in Figure 1 - more severe than the defect caused by the ROS scavenger Tempol. Couldn't these data be interpreted as a need for iron(III) in the fusion reaction?

d) The authors show the whole blot for the Bax control to demonstrate a lack of Bax oligomerization in GSSG. The author's should also show the whole blot for Drp1, Slp-2, Hsp60, and VDAC, demonstrating a lack of oligomers, rather than cutting out specific portions of the gel. The blots for proteins not relevant for mitochondrial fusion and division could potentially be moved to the supplemental data.

e) The quality of the Mfn1 blot in Figure 2B must be improved if the goal is to show oligomerization of Mfn1 in GSSG when MFN2 is depleted. Perhaps soaking in BME would be helpful, as performed in Supp. Figure 1B, where the transition of Mfn1 to oligomeric form is more clear.

f) The description of some control samples is incomplete. As an example, what is the "Negative Control" used in Figure 1B? Does the term "Basal" in Figures 1B, 1C, and 1D refer to without cytosol? If so, it would be better to label those items as "minus cytosol."

g) What does the asterisk represent in Figure 2B? What are the horizontal lines on the side of this gel? These should be explained in a figure legend.

h) In Supp. Figure 1, I do not see any discernible difference between the mobility of AMS and GSSG-treated mitofusin and the mitofusin in the nearby lanes, particularly in comparison to the AMS-untreated mitochondria. To strengthen this point, the authors should re-run the gel using different conditions/gel concentration.

i) It is unclear whether the oligomers that form are homomeric or are heteromeric. Homomeric interaction can be examined by expressing epitope-tagged versions of mitofusins in cells, followed by precipitation and immunoblotting with relevant antibodies after a reducing SDS-PAGE gel. Such an experiment might indicate whether there might be a novel protein to which mitofusins are covalently linked during oxidative stress and increase the impact of the paper.

j) An additional experiment demonstrating that disulfide bond formation is important for fusion is ask whether pre-treatment of mitochondria with iodoacetate blocks fusion. Iodoacetate can react with cysteines to prevent them from forming disulfide bonds.

Referee #2:

1. Do the contents of this manuscript report a single key finding?

YES

2. Is the main message supported by compelling experimental evidence?

No

If NO, please indicate IN ORDER OF PRIORITY which additional experiments are ESSENTIAL (including controls and statistical analyses, and/or those experiments of low technical quality that must be repeated). Could they be conducted within three months?

The authors provide provocative data suggesting that GSSG promotes fusion by covalent modification of the mitofusins. As it stands, the in vitro fusion assay provides strong correlations, but direct evidence is lacking. For instance, GSSG could affect lipids or other proteins that act as accessory factors to the fusion reaction.

1) The authors need to identify the Mfn2 cysteines modified by GSSG and show that mutants do not respond. Modifications appear to map to the heptad repeats, which should narrow down possibilities.

2) The authors show that GSSG stimulates mitochondrial fusion in the absence of added cytosol (Fig. 1), but it is unclear whether this also holds for the Mfn2 band shift. Better evidence should be provided that GSSG crosslinks mitofusins through disulfide bridges.

3) As the authors discuss, several reports have demonstrated that mitochondrial fusion can occur following cell stress. They also show fused mitochondria after oxidant challenge. However, whether antioxidants prevent hyperfusion following milder forms of cell stress (e.g. serum starvation) needs to be examined.

4) Figure 4A+B - The changes in the images are subtle; zoomed images are needed to discern

hyperfusion. Are the graphs from one experiment or a compilation of several experiments? As there are no error bars, we have to assume this is from one and should be repeated to know the variation and if the changes are meaningful. It is also assumed the experimenter is blinded to the condition for the quantification but this needs be indicated.

3. Have similar findings been reported elsewhere (e.g. on a closely related protein; in another organism or context)?

No

GSSG induced Mfn2 oligomerization and mitochondrial fusion is a novel and exciting finding.

4. Is the main finding of general interest to molecular biologists?

Yes

The identification of how redox state may regulate the mitochondrial fusion machinery and the implications for cell survival should be of interest to most molecular biologists.

5. After appropriate revision, would a resubmitted manuscript be most suited for publication:

in EMBO reports

6. Please add any further comments you consider relevant:

Figure 2C - Legend contains "probed as indicated" but there are no labels.

The statistics and what the error bars indicate need to be explained in the paper.

Suppl. 1 - "*bME soak before transfer" should have the * removed or removed entirely since it makes it look like panel A lane with the 1 mM GSSG was soaked in bME.

Suppl. 2 - The exact experimental conditions are unclear. Are all the gels run under reducing conditions?

Referee #3:

Using their novel in vitro fusion assay, along with cell culture studies, Shutt et al. report an interesting finding that mitochondrial fusion is influenced by the levels of intracellular oxidised glutathione. They show that Mitofusins (and Opa1) are oxidised by increased GSSG and suggest that this induces the stress-activated hyperfusion state of mitochondria. Up to now, most studies into the regulation of mitochondrial dynamics have focussed on fission. The work is of physiological importance because it shows a new mode of regulation of the fusion machinery and provides new molecular information into the development of the hyperfused state. The work is of significant interest to a wide readership since it brings encompasses different research fields (oxidative stress and mitochondrial function) and also leaves questions open for future studies. In saying that, there are still some open ended questions and some clarifications required in the manuscript.
1. Figure 1A and 1B are confusing since the control and basal bars are not clear. In panel A, the control is 100% which, according to the legend should represent mitochondria containing added cytosol, although the y-axis indicates it is measured against the basal rate. However, panel B includes a basal bar (set at 100% - mitochondria alone?) and a cytosol bar (the latter being about 250%). It would be easiest to include a basal bar in Fig. 1A to maintain consistency with all other bar graphs in the figure. The concentration of GSSG used in Fig 1E should also be stated.

2. Fig. 2: additional text is required to clarify whether cytosol has been added to the assay or not. In Fig 2C, the glycerol gradients do not show a strong Mfn2 oligomer in contrast to what is seen in Fig. 2A. A control "total" sample would be important to ensure that the oligomeric species has not simply aggregated or been degraded during the experiment (or was reduced in this experiment). Personally, I don't think that Fig. 2C is necessary and the important role of GTPgammaS treatment in inhibiting the oligomer is clearly presented in Fig. 3C. In addition, the supplemental Fig 2 does not show information that adds to the study as the shift in the movement of the oxidised species is minor and a control marker protein has not been employed. The authors also state reducing gels were used yet the figure legend states that non-denaturing gels were employed. Reducing this section would free up text for additional details.
3. In Fig. 3A, the effect of GSSG on fusion is not as significant as in Fig. 1E (450% vs 140%). As the error bars included are not broad, one would expect them to be similar. Clarification is needed here.
4. The fact that the oligomeric Mfn1 or Mfn2 species is not reduced in signal following knockdown (Fig. 2B) argues against the formation of hetero-oligomers of Mfns. However, the authors have not presented any evidence that indicates that the oligomers are in fact exclusively composed of Mitofusins. Can't the oligomers be also between Mfns and another regulatory molecule(s)? While the model presented in Fig. 4D could be correct, additional discussion is required.
5. The panels of mitochondrial morphology in Fig. 4A are not very clear. Additional panels showing close-ups of the fused mitochondrial forms would be useful. Also, Opa1 should be shown to determine whether the cellular oxidative stresses induce oxidation there as well. Should this not be the case, I would remove Opa1 data from the manuscript.
6. I am not convinced by the AMS treatment in Supp Fig. 1A as the mobility of Mfn2 following GSSG treatment does not appear to differ to that of control (+AMS). I suggest that this is repeated or removed.
7. The authors could more definitively show that oxidation of cysteine residues in Mfns are required for hyperfusion by expressing cysteine mutants in mitofusin double knockout cells. Mfn1 and Mfn2 have two conserved Cys residues in HR2 only so the mutations are not too difficult to make. While this experiment would require some effort, it would be highly instructive.
8. Other comments:
-It is not clear what the error bars represent on the bar graphs and how many experiments were conducted.
-In the various figures, the 2 in H2O2 should be subscript and uM and ug/ul must be changed to µM and µg/ µl.

1st Revision - authors' response 04 July 2012

Response to Reviewers

Reviewer 1:

We thank the reviewer for their support of our work, and we have addressed their specific concerns with both new experiments and adjustment of our conclusions where suggested.

R1: *a) The 'control' and 'GSSG' samples in Figure 2C are presumable different glycerol gradients. Therefore, I am unconvinced that a 1-2 fraction shift between 'control' and 'GSSG' is real and not due to variation between glycerol gradients. In any case, quantification of bands and graphing intensity over fraction number is certainly called for here. Finally, the authors provide no interpretation of this shift of Mfn2 in GSSG - what could it mean?*

HM: The reviewer is correct to be concerned with variation between gradients. Initially we had included gradients that were analyzed using both non-reducing (former Figure 2C) and reducing gels (former Supp Fig 2). For clarity, we now only show the results from the GSSG treated samples using a non-reducing gel where we compare the migration of the monomeric form of Mfn2 to the oligomeric form. In this case, we clearly see a ~2 fraction shift of the oligomeric forms of Mfn2 into the gradient relative to the monomer, which has been quantified as suggested by the reviewer.

Our only point from this data is that the oligomers do reflect a higher molecular weight complex of Mfn2, providing a second method to confirm this (in addition to the shift on a 2D non-reducing gel).

R1: *b) The GTPgammaS glycerol gradient can be removed from Figure 2C - a simple SDS page gel of protein samples demonstrating the effect of GTPgammaS is preferred (as is done in Figure 3), since a gel with untreated samples loaded in a nearby lane would be internally controlled for possible poor transfer of large oligomers from the gel to the membrane.*

HM: We have removed the GTPgS data from the glycerol gradients in Figure 2 as suggested as it is superfluous with Figure 3. Again, we had initially included this to further confirm that the higher molecular weight oligomers were not formed in the presence of GTPgS.

R1: *c) Tiron has a catastrophic effect on mitochondrial fusion in Figure 1 - more severe than the defect caused by the ROS scavenger Tempol. Couldn't these data be interpreted as a need for iron(III) in the fusion reaction?*

HM: It is interesting to consider a role for iron in the regulation of mitochondrial fusion. However, since there are many essential iron-sulfur complex-containing enzymes within the electron transport chain, we are afraid that the gross manipulation of iron may simply result in a loss of electrochemical potential, which is a known fusion blocker. Instead, we replaced the iron chelating scavenger Tiron (which acts both as an iron chelator and superoxide scavenging molecule) with a more specific antioxidant Trolox, a water soluble vitamin E analogue, which also strongly reduces fusion. We don't make any arguments on the difference between the relative inhibitions; we simply note that contrary to hydrogen peroxide treatment both antioxidants inhibit fusion, thus setting the stage to look at the effects of glutathione, the primary regulator of redox stress in the cell.

R1: *d) The authors show the whole blot for the Bax control to demonstrate a lack of Bax oligomerization in GSSG. The author's should also show the whole blot for Drp1, Slp-2, Hsp60, and VDAC, demonstrating a lack of oligomers, rather than cutting out specific portions of the gel. The blots for proteins not relevant for mitochondrial fusion and division could potentially be moved to the supplemental data.*

HM: We agree that the full blot is more informative and have included them in the supplemental data as suggested due to space constraints.

R1: *e) The quality of the Mfn1 blot in Figure 2B must be improved if the goal is to show oligomerization of Mfn1 in GSSG when MFN2 is depleted. Perhaps soaking in BME would be helpful, as performed in Supp. Figure 1B, where the transition of Mfn1 to oligomeric form is more clear.*

HM: We have repeated this experiment as suggested to show the Mfn1 oligomers more clearly.

R1: *f) The description of some control samples is incomplete. As an example, what is the "Negative Control" used in Figure 1B? Does the term "Basal" in Figures 1B, 1C, and 1D refer to without cytosol? If so, it would be better to label those items as "minus cytosol."*

HM: The figures and legends have been changed to better reflect the negative control conditions and eliminate the confusion about basal reaction (i.e. lacking cytosols)

R1: *g) What does the asterisk represent in Figure 2B? What are the horizontal lines on the side of this gel? These should be explained in a figure legend.*

HM: The asterisk was meant to indicate a slightly faster migration of the monomeric Mfn2 with GSSG treatment, presumably due to an intramolecular disulfide bond. The lines indicate the migration of the molecular weight marker and the relevant sizes are now indicated.

R1: *h) In Supp. Figure 1, I do not see any discernible difference between the mobility of AMS and GSSG-treated mitofusin and the mitofusin in the nearby lanes, particularly in comparison to the AMS-untreated mitochondria. To strengthen this point, the authors should re-run the gel using different conditions/gel concentration.*

HM: As other reviewers were also unconvinced, we have removed this data and topic of discussion from the paper.

R1: *i) It is unclear whether the oligomers that form are homomeric or are heteromeric. Homomeric interaction can be examined by expressing epitope-tagged versions of mitofusins in cells, followed by precipitation and immunoblotting with relevant antibodies after a reducing SDS-PAGE gel. Such an experiment might indicate whether there might be a novel protein to which mitofusins are covalently linked during oxidative stress and increase the impact of the paper.*

HM: We appreciate the reviewer's interest in the full characterization of the oligomeric complex observed in the presence of GSSG. The reviewer would like us to perform mass spec analysis on immunoprecipitated Mfns 1 and 2 under non-reducing (vs. reducing) conditions in the presence of GSSG, and functionally establish their roles in mitochondrial fusion. Indeed we have been working on this approach, but we feel that the identification and characterization of novel mitofusin interacting proteins is beyond the scope of this first manuscript. We believe that the demonstration that the oligomers require only one of the 2 Mfns is already rather unexpected, providing new insights into the types of complex reactions that will occur during mitochondrial fusion. Indeed, the interactions of Mfn2 with components at the ER will complicate our analysis since we will need to investigate the dual functions of Mfn2 partners in ER contacts and mitochondrial fusion, both of which may be modulated by disulfide transitions. We hope the reviewer will agree that these concepts will take a great deal of time and work to unravel.

R1: *j) An additional experiment demonstrating that disulfide bond formation is important for fusion is ask whether pre-treatment of mitochondria with iodoacetate blocks fusion. Iodoacetate can react with cysteines to prevent them from forming disulfide bonds.*

HM: This is an excellent suggestion by the reviewer. We have performed a dose response to iodoacetate on both control and GSSG conditions and see inhibition as expected, with the GSSG reaction being more sensitive to iodoacetate. This experiment demonstrates the importance of free cysteines both for fusion in general and for the stimulation by GSSG.

Reviewer 2

We thank the reviewer for agreeing that our work represents a novel and exciting finding that will be of interest to molecular biologists. We believe that the constructive comments and suggestions made by the reviewer have greatly improved our study, and hope that the reviewer agrees.

R2: *1) The authors need to identify the Mfn2 cysteines modified by GSSG and show that mutants do not respond. Modifications appear to map to the heptad repeats, which should narrow down possibilities.*

HM: We thank the reviewer for encouraging us to complete the scanning mutagenesis of conserved cysteine residues in Mfn2. We generated 8 different cysteine to alanine mutations of residues conserved in other Mfns. Only one, C684A, led to a loss of 3 (of 4) oligomeric species of Mfn2. This shows the importance of C684 in the generation of most forms of the oligomer. The remaining higher order species may require a combinatorial mutation of two cysteines, which we could not complete given the time constraints of the revision period. We did examine the most obvious combination, that of the two conserved residues flanking the C-terminal heptad repeat (C684 and C700), but did not see any additional loss of oligomers. In order to examine the functional requirement for C684, we compared the transfection of C684A relative to wild type Mfn2 into Mfn2-/- mouse embryonic fibroblasts. The mitochondrial morphology in these MEFs includes a mixture of fragmented and rod-like (intermediate) phenotypes, as described initially by the Chan group (JCB 2004). Although the wild type Mfn2 did lead to the appearance of cells with hyperfused mitochondria, the mutant C684 failed to rescue. This further indicates a critical requirement for the cysteines at the base of the heptad repeat in the process of mitochondrial fusion.

R2: *2) The authors show that GSSG stimulates mitochondrial fusion in the absence of added cytosol (Fig. 1), but it is unclear whether this also holds for the Mfn2 band shift. Better evidence should be provided that GSSG crosslinks mitofusins through disulfide bridges.*

HM: The gels are from reactions performed in the absence of cytosols, consistent with the oligomers consisting primarily of mitochondrial (and/or peripherally attached) proteins and not cytosolic proteins.
We have provided an additional figure (Supplemental Figure 1A) demonstrating that the shifted bands of Mfn2 and Opa1 are sensitive to reducing conditions. In addition we have identified C684 as being essential for the formation of a subset of the oligomers (see point 1 above).

R2: *3) As the authors discuss, several reports have demonstrated that mitochondrial fusion can occur following cell stress. They also show fused mitochondria after oxidant challenge. However, whether antioxidants prevent hyperfusion following milder forms of cell stress (e.g. serum starvation) needs to be examined.*

HM: In our cell-free system, the addition of antioxidants or GSH completely abolishes basal and cytosol-stimulated fusion, as we show in Figure 1. Similarly we now demonstrate that the cysteine specific drug iodoacetate blocked both basal and GSSG-stimulated fusion in the absence of cytosol. Together these data indicate that free cysteines represent a core requirement for fusion, whether it is a basal reaction or when stimulated by either GSSG or cytosol. Given the large and historic literature that has demonstrated the role for GSH/GSSG as a core sensor for ROS and "stress" under a wide variety of conditions, we consider that our data provides compelling evidence for glutathione pathway as a mechanistic sensor driving stress-induced mitochondrial fusion.

However, the reviewer makes a specific point concerning cytosols from starved cells that should be addressed. We have previously shown that the addition of cytosols from forskolin treated cells stimulated fusion over control cytosols by ~2 fold (BMC Biol 2010). This was the first demonstration that mitochondria actively hyperfuse upon PKA stimulation (in addition to the inhibition of Drp1 and mitochondrial fission). In response to the current question, we attempted to generate enough cytosol from starved cells to expand our previous drug treatments and test whether the antioxidants also abolish starvation-induced fusion. Sadly however, we encountered technical difficulties in successfully starving the cells and obtaining the stimulatory cytosols to test these ideas. With more time, we will continue to pursue these questions in our future studies and apologize to the reviewer for not addressing this point within the current manuscript. However, we remain confident that our work will find resonance with many scientists interested in the mitochondrial response to stress.

R2: *4) Figure 4A+B - The changes in the images are subtle; zoomed images are needed to discern hyperfusion. Are the graphs from one experiment or a compilation of several experiments? As there are no error bars, we have to assume this is from one and should be repeated to know the variation and if the changes are meaningful. It is also assumed the experimenter is blinded to the condition for the quantification but this needs be indicated.*

HM: We apologize for the low resolution of the images and have added additional panels showing zoomed images of the morphology as requested. The morphology experiment has been repeated 3-4 times with the same trends observed. Blinded quantification and error bars were determined from coverslips, counting at least 30 cells for each treatment in triplicate. The text has been altered to indicate the methods used.

R2: *Figure 2C - Legend contains "probed as indicated" but there are no labels.*

HM: This figure has been moved to supplemental and the figure legend modified to indicate that the blot was probed for Mfn2.

R2: *The statistics and what the error bars indicate need to be explained in the paper.*

HM: We have added to the text that Student t-test was used to determine p-values and error bars represent standard deviation.

R2: *Suppl. 1 - "*bME soak before transfer" should have the * removed or removed entirely since it makes it look like panel A lane with the 1 mM GSSG was soaked in bME.*

HM: This figure has been removed entirely as the Mfn1 oligomers are now clearly visible in Figure 2B.

R2: *Suppl. 2 - The exact experimental conditions are unclear. Are all the gels run under reducing conditions?*

HM: Supplemental figure 2 has been removed from the paper as suggested by reviewers.

Reviewer 3

We thank the reviewer for their encouraging remarks that our work will be of significance interest to a wide readership. We hope we have clarified some of the open questions and concerns held by the reviewer, as outlined below:

R3: *1. Figure 1A and 1B are confusing since the control and basal bars are not clear. In panel A, the control is 100% which, according to the legend should represent mitochondria containing added cytosol, although the y-axis indicates it is measured against the basal rate. However, panel B includes a basal bar (set at 100% - mitochondria alone?) and a cytosol bar (the latter being about 250%). It would be easiest to include a basal bar in Fig. 1A to maintain consistency with all other bar graphs in the figure. The concentration of GSSG used in Fig 1E should also be stated.*

HM: The figures have been adjusted to avoid confusion between basal (mitochondria alone) and cytosol-containing reactions. The concentrations of GSSG used in figure 1E is now indicated. Note figure 1E has been replaced with a new figure showing fusion in the absence of cytosols, but also with the addition of iodoacetate.

R3: *2. Fig. 2: additional text is required to clarify whether cytosol has been added to the assay or not. In Fig 2C, the glycerol gradients do not show a strong Mfn2 oligomer in contrast to what is seen in Fig. 2A. A control "total" sample would be important to ensure that the oligomeric species has not simply aggregated or been degraded during the experiment (or was reduced in this experiment). Personally, I don't think that Fig. 2C is necessary and the important role of GTPgammaS treatment in inhibiting the oligomer is clearly presented in Fig. 3C. In addition, the supplemental Fig 2 does not show information that adds to the study as the shift in the movement of the oxidised species is minor and a control marker protein has not been employed. The authors also state reducing gels were used yet the figure legend states that non-denaturing gels were employed. Reducing this section would free up text for additional details.*

HM: We apologize for the confusing labels we had used within the figures. In all cases we have clarified whether cytosol was present or absent, which is indicated in the new figure legends. In terms of the levels of oligomers observed on the glycerol gradients, the reviewer is correct to consider that some may be lost in the sample preparation compared to what is observed in a simple 2D non-reducing gel. As pointed out by reviewer 1, we have simplified our presentation of the glycerol gradients to make a single point – that the oligomeric forms of Mfn2 with the GSSG-treated samples are shifted by two fractions relative to the monomer (observed on the same gel). As requested by the reviewer, we have removed the gradient fractionation of the control and GTPgS treated samples, and the fractionation resolved on reducing gels in Supplemental 2.

R3: *3. In Fig. 3A, the effect of GSSG on fusion is not as significant as in Fig. 1E (450% vs 140%). As the error bars included are not broad, one would expect them to be similar. Clarification is needed here.*

HM: The experiment in Figure 3A did not include an initial tethering step where mitochondria are pelleted. For most experiments we pellet the mitochondria on ice to promote tethering (and ultimately fusion) by favoring the formation of trans interactions. However, in Figure 3 we were testing the ability of the Mfn2 oligomers to form in the absence of trans interactions, so this step was

omitted lowering the overall fusion signal. The text has been altered to better reflect this discrepancy for the readers. Additionally, these experiments were performed on different days with different preparations of mitochondria, which can also lead to some variability in the absolute levels of stimulation. However, the trends are consistent with every batch of mitochondria we have prepared.

R3: *4. The fact that the oligomeric Mfn1 or Mfn2 species is not reduced in signal following knockdown (Fig. 2B) argues against the formation of hetero-oligomers of Mfns. However, the authors have not presented any evidence that indicates that the oligomers are in fact exclusively composed of Mitofusins. Can't the oligomers be also between Mfns and another regulatory molecule(s)? While the model presented in Fig. 4D could be correct, additional discussion is required.*

HM: We agree it is not possible from our data to conclude that heteroligomers between Mfn1 or Mfn2 and another unidentified protein do not occur. Although we did not state specifically that the oligomers are homotypic, our model depicts this. We have expanded the discussion and removed the model in order to clearly outline the possibility of unknown protein(s) being involved. We did include a simpler model of Mfn2 oligomers forming in either cis or trans to introduce the dilution experiments in Figure 3, which does not explicitly describe potential interactors, but we again considered this in the legend.

R3: 5. The panels of mitochondrial morphology in Fig. 4A are not very clear. Additional panels showing close-ups of the fused mitochondrial forms would be useful. Also, Opa1 should be shown to determine whether the cellular oxidative stresses induce oxidation there as well. Should this not be the case, I would remove Opa1 data from the manuscript.

HM: We have added additional panels with insets showing zoomed images of the morphology as requested. Initially we included Opa1 to make another point about the specificity of the GTPgS treatment on the dissolution of the Mfn2 oligomers, while GTPgS stabilized the Opa1 oligomers. It was meant as an internal control for both the gradients and the effect of GTPgS on the oligomers. However, since we have removed the other glycerol gradients from the study (see above and other reviewers), we have removed it as suggested.

R3: *6. I am not convinced by the AMS treatment in Supp Fig. 1A as the mobility of Mfn2 following GSSG treatment does not appear to differ to that of control (+AMS). I suggest that this is repeated or removed.*

HM: Although we are convinced that there is a small downward shift in mobility of Mfn2 upon AMS treatment, we appreciate that this is difficult to quantify and we have removed it from the paper.

R3: *7. The authors could more definitively show that oxidation of cysteine residues in Mfns are required for hyperfusion by expressing cysteine mutants in mitofusin double knockout cells. Mfn1 and Mfn2 have two conserved Cys residues in HR2 only so the mutations are not too difficult to make. While this experiment would require some effort, it would be highly instructive.*

HM: As discussed earlier in response to reviewer 2 we have generated 8 different cysteine mutants, identifying C684 at the junction of the second heptad repeat as a critical residue required for the generation of 3 out of 4 of the GSSG-induced Mfn2 oligomers. Transfection of this construct into Mfn2-/- MEF cells did not lead to the generation of hyperfused mitochondria, where the wild type Mfn2 did result in significant rescue.

R3: 8. Other comments:
-It is not clear what the error bars represent on the bar graphs and how many experiments were conducted.

HM: Figure legends now indicate that graphs are representative of multiple experiments and error bars represent standard deviation from replicate samples in a given experiment.

-In the various figures, the 2 in H2O2 should be subscript and uM and ug/ul must be changed to µM and µg/ µl.

HM: We have changed to text in the figures to include the proper symbols.

2nd Editorial Decision 23 July 2012

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, the referees are now all positive about its publication in EMBO reports. They have a few suggestions for additional changes, but these mostly appear to be minor changes to the text/discussion. Under point 3, referee 1 suggests to include additional data and I would encourage you to do so if you already have these data at hand. If not, we would not make this a pre-requisite for acceptance and you could also more carefully word the respective text. In addition, please note the comment on the statistics and clarify this point.

I am writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication the few minor issues/corrections as outlined above have been addressed.

If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you again for your contribution to EMBO reports.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

General Comments:

Here, Schutt et al. have submitted a manuscript of improved quality and have satisfied most of my concerns about the original manuscript. The addition of experiments designed to find the relevant cysteines affected by GSSG is a nice addition to the paper. There are a few concerns that I list below, but otherwise the manuscript is interesting and warrants publication in EMBO Reports.

Specific Comments:

1) The authors state: "Although Mfn heterodimers are not a requisite, the appearance of multiple oligomeric species could represent multimers of Mfns with altered mobility due to distinct disulfide species, or could reflect the presence of additional, unknown partners within these complexes."

I fail to see how the glycerol gradient in Figures 2C and 2D distinguish between these two possibilities. Changed sedimentation in a glycerol gradient could certainly result from new members joining a complex, but it could also be caused by a complex with no additional subunits changing conformation due to new disulfide bonds.

2) In the figure legends of Figure 1 and Figure 3, the authors state that reactions or data are 'representative' of at least three independent experiments. This language must be clarified. Either one representative experiment is shown (in which case error bars wouldn't be present), or the data are averaged. Providing the precise number of replicates is important here.

3) The authors state in their rebuttal that, "The reviewer would like us to perform mass spec

analysis..." This is certainly not the case. Without performing mass spec, one can at least determine if Mfn2 or Mfn1 is forming disulfide bridges with itself (which is an important question and would be a nice addition to the paper). Co-express Mfn2-HA and Mfn2-myc in the same cell, treat +/- GSSG, boil in SDS +/- DTT, dilute, and pull down with anti-myc beads. See if the HA-tagged version precipitates in the condition of +GSSG -DTT.

4) In Figure 1E, the authors should simply place the concentrations of iodoacetate below the bars on the graph. The triangle doesn't properly represent the increases in iodoacetate concentration, which are logarithmic.

5) In Figure 2C, the fact that Mfn2 protein is being assayed should be put right on the figure.

6) The authors write: "However at higher concentrations of mitochondria (10mg/ml), trans oligomers may occur in the presence of GSSG even without GTP hydrolysis." However, one alternative possibility for the inability of GTPgammaS to block oligomer formation at 10mg/ml mitochondria could be the generation of GTP during the fusion reaction that would compete with GTPgammaS.

ATP is added directly to the reaction according to Schauss et al. Also, succinate is added to the reaction, energizing the mitochondria and providing even more ATP. Mitochondria contain nucleoside-diphosphate kinase, which can convert ATP to GTP (normally this works in the opposite direction). Therefore more mitochondria may lead to enough GTP produced during the reaction so that oligomer formation can occur.

Referee #2:

The authors addressed my concerns satisfactorily. Particularly the identification of one of the oxydized cysteines is a significant addition to the manuscript.

Referee #3:

The authors have adequately addressed the concerns raised (except they still have to change the ug/ul in Fig. 3C and D to µg/µl). The manuscript reads well and the data is quite clear. As it stands, this manuscript is a very important advance into our understanding of how mitochondrial fusion is regulated and I congratulate the authors for uncovering this interesting finding.

2nd Revision - authors' response 25 July 2012

Response to Reviewers.

We thank the reviewers for their support, and for their help in improving our study. We respond to each final comment below.

Referee #1:

1) The authors state: "Although Mfn heterodimers are not a requisite, the appearance of multiple oligomeric species could represent multimers of Mfns with altered mobility due to distinct disulfide species, or could reflect the presence of additional, unknown partners within these complexes." I fail to see how the glycerol gradient in Figures 2C and 2D distinguish between these two possibilities. Changed sedimentation in a glycerol gradient could certainly result from new members joining a complex, but it could also be caused by a complex with no additional subunits changing conformation due to new disulfide bonds.

HM: We understand the reviewers' frustration with the fact that we have not precisely defined the total composition of proteins within the higher molecular weight oligomers. As we stated, they

could be comprised either of homomers or multimers with additional, unknown proteins. We agree that the sedimentation shift in the glycerol gradient cannot distinguish between these possibilities, which was why we did not attempt to conclude either from the experiment. The glycerol gradient simply validates the fact that the oligomeric form of Mfn2 is in a unique, higher molecular weight complex from the monomeric form of Mfn2. The reviewer argues that this complex may not reflect increased molecular weight, and only the formation of new disulfides, reflecting a conformational change. We humbly suggest that when new disulfides are made, this constitutes a new complex that goes beyond a conformational change. If disulfides are generated within the same molecule of Mfn2, we would observe a slight downward shift in molecular weight on the non-reducing gel, which could be considered a conformational change. We do observe this, however the appearance of the larger high molecular weight forms clearly indicates a new, higher molecular weight complex stable to detergent that was not present in the control reaction.

2) In the figure legends of Figure 1 and Figure 3, the authors state that reactions or data are 'representative' of at least three independent experiments. This language must be clarified. Either one representative experiment is shown (in which case error bars wouldn't be present), or the data are averaged. Providing the precise number of replicates is important here.
HM: We apologize for the confusion. We now include clarification of this both in the methods and the figure legends. To be clear, the error bars in each experiment are calculated from either duplicates or triplicates within an experiment on the same day. The data obtained from these experiments was representative of at least 3 independent experiments.

3) The authors state in their rebuttal that, "The reviewer would like us to perform mass spec analysis..." This is certainly not the case. Without performing mass spec, one can at least determine if Mfn2 or Mfn1 is forming disulfide bridges with itself (which is an important question and would be a nice addition to the paper). Co-express Mfn2-HA and Mfn2-myc in the same cell, treat +/- GSSG, boil in SDS +/- DTT, dilute, and pull down with anti-myc beads. See if the HA-tagged version precipitates in the condition of +GSSG -DTT.

HM: We agree that this experiment is possible, and versions of this have been done previously by the Mihara group in 2004 (JCS), and the Nunnari group in 2011 (Mol Cell) who showed dimerized interactions in trans. However during the revision period, we unfortunately were not able to obtain the two differently tagged constructs to generate stable lines within our luciferase reporter sHeLa cells in order to mix the mitochondria as requested. We considered that a mass spec analysis would actually be a better strategy since there would be no need to limit ourselves to Mfn interactions and could identify unexpected new partners. We are working hard towards this end and again must apologize for not completing the experiment as requested.

4) In Figure 1E, the authors should simply place the concentrations of iodoacetate below the bars on the graph. The triangle doesn't properly represent the increases in iodoacetate concentration, which are logarithmic.

HM: We have changed the figure as requested.

5) In Figure 2C, the fact that Mfn2 protein is being assayed should be put right on the figure.

HM: We have changed the figure as requested.

6) The authors write: "However at higher concentrations of mitochondria (10mg/ml), trans oligomers may occur in the presence of GSSG even without GTP hydrolysis." However, one alternative possibility for the inability of GTPgammaS to block oligomer formation at 10mg/ml mitochondria could be the generation of GTP during the fusion reaction that would compete with GTPgammaS.
ATP is added directly to the reaction according to Schauss et al. Also, succinate is added to the reaction, energizing the mitochondria and providing even more ATP. Mitochondria contain nucleoside-diphosphate kinase, which can convert ATP to GTP (normally this works in the opposite direction). Therefore more mitochondria may lead to enough GTP produced during the reaction so that oligomer formation can occur.

HM: The reviewer is correct that at very high concentrations of mitochondria, many metabolic

changes can occur in the reaction. We do add succinate to fuel the mitochondria and the explanation proposed is certainly valid. We didn't include additional speculative explanations for the residual appearance of oligomers at 10mg/ml mitochondria and we would need to measure the levels of GTP in the reaction to confirm this. However, it still could be due to a mass action effect of so many mitochondria, that even elevated levels of GTP would not definitively explain the result. We thank the reviewer for their suggestion.

Referee #2:

The authors addressed my concerns satisfactorily. Particularly the identification of one of the oxydized cysteines is a significant addition to the manuscript.

HM: We thank the reviewer very much for their consideration and support of our manuscript.

Referee #3:

The authors have adequately addressed the concerns raised (except they still have to change the ug/ul in Fig. 3C and D to µg/µl). The manuscript reads well and the data is quite clear. As it stands, this manuscript is a very important advance into our understanding of how mitochondrial fusion is regulated and I congratulate the authors for uncovering this interesting finding.

HM: We have changed the font for the "micro" in Figure 3 as suggested, and apologize for that oversight. We thank the reviewer for their kind words of congratulations!

Correspondence - Editor 27 July 2012

Many thanks for submitting the revised version of the manuscript. I appreciate your clarification that the error bars have been calculated on duplicates or triplicates of one representative experiment.

I do, however, feel that it would be more appropriate to calculate the error bars based on all three independent experiments together, rather than on one representative experiment only, as replicates of one experiment are not truly independent. Also, error bars should not be calculated from 2 experiments only (regardless of whether the experiments are truly independent or technical replicates).

I would therefore kindly ask you to re-do the calculation of the error bars in figures 1 and 3 and modify the text, figure legends and figures accordingly.

It will be easiest if you simply send us the new files by email and we will then upload them to the rest of your manuscript.

Thank you very much in advance and I apologize for this inconvenience.

Yours sincerely,

Editor
EMBO reports

3rd Editorial Decision 08 August 2012

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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Yours sincerely,

Editorial Assistant
EMBO Reports

