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Upregulated function of mitochondria-associated ER membranes in Alzheimer disease

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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 03 September 2011

Thank you for submitting your manuscript entitled 'Presenilins are negative regulators of ER-mitochondrial communication' for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

Despite the rather significant potential interest of your findings, we are unfortunately not able to publish your manuscript at this time.
I apologize for the slow process in this case, due to delays during the summer travel period.

Based on the data presented in this manuscript, you propose that presenilins may have a negative role in MAMs and thus ER-mitochondrial exchange. Surprisingly, sporadic AD cases also affect MAMs, likely independently of presenilin.

The three referees all share our appreciation of the potential interest of these findings, but they also all raise significant issues that preclude publication.

While referee 1 is positively disposed 'so that others can be alerted and validate', the referee

concludes that the evidence remains circumstantial and that there is no proof that PS inhibits ER/mitochondrial crosstalk. Three major experiments are suggested: measure Calcium transfer; 3D imaging (electron tomography); obtain some mechanistic understanding of how PS affects ER/mitochondrial crosstalk, in particular in the context of Mfn2.

Referee 2 also highlights the unknown mechanism. Notably, the referee is concerned that if sporadic AD patients have the same defect, it is unlikely to be related to PS, concluding that this represents a 'major weakness of the study'. In our view this finding does not invalidate the proposed link of PS to MAM and MAM to AD, but it would strongly enhance the study if there was evidence how these effects arise in non-familial AD patients.

Referee 3 echoes the potential interest, but states that the 'main idea is not really tested', noting a lack of mechanism. The referee finds that the key experiment is the EM data, but that this is not definitive as it was only done in DKO rather than FAD cells. The referee lists a number of missing controls and suggests to check total mitochondria numbers. Like referee 1, Mfn2 is discussed: PS1/2 loss of function is known to decrease Mfn2 levels, which is at odds with conclusions drawn.

In summary, the current dataset has a number of deficiencies which detract from the conclusiveness of the data:

1) definitive evidence that PS affects crosstalk/contacts.
2) lack of any mechanism - given the potential link of Mfn2 to PS in MAMs.
3) how is MAM/crosstalk affected in sporadic AD?

Given these limitations, which would require extensive experimental revision to be addressed successfully, and The EMBO Journal's policy to only invite revisions on manuscripts that can be executed successfully within about three months, I am afraid that we cannot encourage a resubmission. We do acknowledge the great potential interest of these findings and hope that further data will lend definitive support to the conclusions drawn. We are certainly happy to discuss progress with this project in the future.

Thank you for the opportunity to consider this interesting manuscript. I am sorry that we cannot be more positive at this time, but we hope nevertheless that you will find our referees' comments helpful.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

In this paper, Area-Gomez and colleagues show that presenilin (PS) are enriched at MAMs that display the feature of a "lipid raft". Cells lacking (PS) display increased ER-mitochondrial transport of cholesterol, a feature that suggests that PS are negative regulators of ER-mitochondria communication.

Their conclusion is supported by evidence showing that

1) MAMs and lipid raft (LR) share common features, in terms of resistance to detergents (they are both resistant to TritonX), cholesterol content (they both have higher cholesterol content as compared to that found in the cytoplasm) and ACAT activity (they both display higher ACAT activity, which results in higher conversion of free cholesterol to cholesterol esters, CE). In this respect, they reckon, MAMs resemble LR biophysical characteristics and activity.

2) In MEFs lacking PS1, PS2 or both, the levels of both cholesterol and CE is greater as compared

to wt cells and the trafficking of phospholipids back and forward from the ER to the mitochondria is increased. This is also true in cells from AD patients (both familiar and sporadic).

3) In MEFs lacking PS1, PS2 or both, the length of contacts between ER and mitochondria is increased.

4) In MEFs lacking the structural MAM component MFN2, conversely the ER-mitochondrial phospholipids trafficking is decreased, like the production of the intracellular APP fragment AICD. Based on these observations, the authors conclude that PS are negative regulators of ER-mitochondrial communication, since in cells lacking Presinilins, the cross talk between ER and mitochondria is increased. In such way, Presinilins have opposite effects on MAM-related functions as compared to Mfn2, which was previously found to positively regulate MAM-mediated ER-mitochondria interactions.

The paper is well written and indeed very interesting in its contents and observations. The idea that in AD (and perhaps in other neurodegenerative diseases), increased cross talk between ER-mitochondria might be pathogenic is intriguing. This is a very important novel concept that must be brought to the attention of the broad readership of the EMBOJ so that it can be scrutinized by other laboratories and if proven correct, used as a basis for the development of rational therapies of AD. However, at this point the evidence presented here is still circumstantial and does not prove that PSare negative regulators of ER-Mitochondria cross talk. In more details, the following experiments are required:
1) The trafficking of Phospholipids between ER and mitochondria has been used to measure the degree of communication between both organelles in wt and Presinilins mutant cells. The authors should also measure ER-mitochondria calcium transfer in order to corroborate this data. Presinilins mutant cells might display a general higher metabolism, which might affect the synthesis of PtdSer and therefore its trafficking back and forward from the ER.
2) The authors measure the degree of interaction by quantifying ER-mitochondria contacts by EM. Conventional EM does not take into account of the ER-mitochondria conformation in 3D. In order to be quantitative, the length of mitochondrial-ER contacts should have been measured upon 3D reconstruction of EM acquisitions (electron tomography), or by live fluorescence imaging of the false colocalization between the two organelles described in Rizzuto et al Science 1998
3) The biggest unresolved question is the molecular mechanism by which PS negatively regulate ER-Mitochondria cross talk In other word, how can the absence of Presenilins physically increase the tether? Authors should explore if for example levels of MAM Mfn2 are reduced in PS deficient cells, and whether PS knockdown has any effect on ER-mitochondria transfer in Mfn2-/- cells.

Minor point: please revise typos (for example, "and?").

Referee #2

In this manuscript, the authors extend their previous study in which they demonstrated that presenilins (PS) and gamma-secretase activity reside in intracellular lipid rafts associated with mitochondrial-associated ER membranes (MAM), by demonstrating through biochemical and imaging approaches, functional consequences of deleting or mutating PS on MAM functions. They conclude that PS secretase function is regulated by their being present in MAM, and MAM functions are in turn regulated by the presence of secretase-competent PS there.

The studies seem carefully considered, and a strong case is made to support the conclusion that presenilins regulate MAM functions, perhaps by altering, through unknown mechanisms, the physical relationship between mitochondria and ER.

1. Surprisingly, to this reviewer, similar biochemical abnormalities as seen in PS deficient or mutant cells were observed in fibroblasts from sporadic Alzheimer's disease patients, who presumably have normal PS levels and function. Thus, the story here is confusing. The authors dismiss this in the Discussion as suggesting they have described a possibly overarching mechanism present in all forms of AD, but the focus of all the experiments and interpretations is on PS, so such a trivial discussion of this aspect is insufficient. The only way the story could be, as the title indicates, one about presenilins, is if the authors can link the observations from the SAD cells to PS, and this has not

been done, which represents the major weakness of the study.

2. In Figure 3D, what is the significance of each dot ?

3. In the tritiated serine incorporation experiments, how did serine get into the cells? Was there any difference in the uptake rates between the different cell lines, and how were these incorporated into the analyses?

4. Errors bars are lacking in Fig 5A; what was the concentration used in Fig 5B?

5. In the left gel in Fig 7C, it appears that either the gel has been digitally processed or that lanes from two different gels have been placed side by side to give the appearance they are parallel lanes of the same gel.

Referee #3

The manuscript "Presenilins are negative regulators of ER-mitochondrial communication" is a follow-up on their previous work (Area-Gomez E, et al. 2009). The previous paper showed that presenilins are enriched in endoplasmic reticulum membranes associated with mitochondria (MAM). In this new study, they further showed that MAM displays the characteristics of intracellular lipid rafts, and that the loss of presenilin function results in increased ER-mitochondrial communication, as measured by cholesteryl ester and phospholipid synthesis, and correlates with a greater area of physical contact between ER and mitochondria. Based on these results the authors propose that presenilins are negative regulators of ER-mitochondrial communication. The proposal is potentially very interesting, and provides a novel opinion about the AD pathogenesis.
Unfortunately most data in the paper are not directly related to the proposal and the main idea is not really tested in the paper. Also, mechanism of presenilin actions in MAM is not explained. Most of the data are based on the study of cholesteryl ester and phospholipid synthesis, without related control experiments and these data do not support ER-mitochondrial communication. The key experiment is the EM study which show increased ER-mitochondrial contacts in DKO cells. But these results only obtained with DKO cells, not with FAD cells and the quality of data is questionable. So the conclusion about presenilins are negative regulators of ER-mitochondrial communication is not solid and much additonal experimenst will be needed to advance this claim.

Major comments:

1. Fig 2 showed increased cholesteryl ester synthesis in PS-mutant cells and AD patient fibroblasts, which maybe not due to increase ER-mitochondrial contacts, maybe just because increased ACAT expression in PS-mutant cells and AD patient fibroblasts, since there is report that ACAT-1 mRNA levels increased significantly in AD fibroblasts (Pani A, et al, 2009), the control experiment to compare the ACAT expression level is very important.

2. Similarly, fig4 and fig5 showed elevated phospholipid synthesis in PS-mutant cells and AD patient fibroblasts, which are also not supportive data for increasing ER-mitochondrial communication if without the control experiment to test the PtdSer synthase level.

3. Fig 6 EM data is a direct data to support the increased ER-mitochondrial contacts in presinilin-mutant MEF, but from fig6F, looks like the author observed more mitochondrial contacts in DKO cells than WT cells, is this due to there are more mitochondrial number in DKO cells? Since the mitochondrial fission/fusion also have effect on ER-mtiochondrial contact, the author should also analyze the total mitochondrial number in the EM images.
Also in the study (Zampese E et al, 2011), data were showed that overexpression of PS1-A246E did not significantly increase the area of ER-mitochondria colocalization, and down-regulation of endogenous PS1 by specific siRNA did not significantly modify ER-mitochondria tethering, which are opposite with this study.
It is also unclear if similar increase in mito-ER contacts is observed in PS1-FAD cells, for example in cells expressing PS1-A246E. If PS1 playing a "structural role" in supporting ER-mito junctions, it is not clear if this role is impaired by FAD point mutations, such as A246E. If it is not impaired then the proposed mechanism is only relevant for PS DKO cells, not for FAD cells.

4. in fig7, the author use MFN2 KO cell as a ER-mitochondrial contacts deficient cell, but in the study (Zampese E et al, 2011), data showed that knocking down PS1 or PS2 decrease MFN2 expression level, so please check the MFN2 expression level in PS KO cells and PS1KD cells, if MFN2 also decreased, how to explain the increase ER-mitochondrial contacts?

Also, MFN2 experiment on Fig 7 does not really test the main hypothesis of the paper. An appropriate experiment would be to knockdown MFN2 in PS DKO or PS1-FAD cells and observed reversal of phenotypes.

Minor comments:
Please check spelling and grammar: Page6 line10 "an and? involvement of presenilins in ER-mitochondrial communication."

Appeal 07 September 2011

I am writing in regard to the Journal's decision not to publish our manuscript. Naturally we are disappointed, but after reading the reviewers' comments, I would like to ask you to reconsider the "finality" of that decision. In particular, we feel not only that we can address most of the reviewers' concerns relatively quickly, but also note that each of the referees accepted the punch line of the paper, namely, that alterations in MAM function play a role in AD. On the other hand, we feel that we did not communicate clearly enough some aspects of the work, and this failure on our part may have influenced the reviewers' assessments.

As you summarized succinctly, all three reviewers were positive in their assessment of the quality of the work, and felt that it was of sufficient interest for publication, but that there were gaps that needed addressing. These fell into three broad categories:

(1) Lack of certain controls, such as ACAT1 and phosphtidylserine synthase levels, mitochondrial numbers, and examination of function in Mfn2-knockdown FAD cells. We agree with the reviewers that these are key controls, and they are relatively easy to provide.

(2) More EM and/or other evidence of increased ER-mitochondrial contact are required. We already have data that was not included in the manuscript showing such increased contact in FAD cells, using high-resolution quantitative immunocytochemistry, and would be happy to corroborate this by EM.

(3) Finally, lack of a mechanism to explain how mutations in presenilins affect ER-MAM crosstalk. We did not attempt try to explain the entire mechanism by which presenilins operate to separate ER from mitochondria, as we feel that this is beyond the scope of only one paper. However, by identifying a new concept connecting presenilin function with subcellular organelle communication and its relationship to neurodegeneration, we feel that we have inserted at least the first brick into this edifice.

Taken together, I would like to request that you reconsider the decision, and allow us to respond to the referees' concerns.

2nd Editorial Decision 16 September 2011

Thank you very much for discussing your plans for a revision of your recent manuscript entitled 'Presenilins are negative regulators of ER-mitochondrial communication' to The EMBO Journal.

I am sorry for the slow response, but I have just returned from a couple of back-to-back meetings with limited scope for e-mail communication.

We certainly tried to note in our decision letter that we share the referee's undoubted opinion that this dataset is potentially of significant interest.

The formal rejection was based on our policy to only explicitly commit to a revision, if it has a high chance of success after a nominal revision period of around three months. In this case, the revisions required would in all likelihood have exceeded this time period and we therefore decided to close the file.

However, it appears that you already have data to support the key concern that the microscopic evidence for the role of PS in MAM formation was not sufficiently strong. Furthermore, you appear open to address some of the other experimental issues, including assessment of the role of Mfn2.

It is not clear to us if you may be able to add data to show that the bridges are also affected in sporadic AD independently of PS1 or 2.

I agree that a detailed molecular mechanistic understanding is beyond the scope of the current manuscript.

In summary, we are certainly interested to reconsider a substantially revised manuscript that addresses the key points raised by the referees experimentally. However, we would have to evaluate a more detailed revision plan (or simply a revised version of the manuscript when it is available) before being able to commit to re-review. We would only undertake further peer review, if we felt the referee issues were addressed in some detail and by experimentation where necessary.

A resubmission would be formally treated as a new submission, and we would have to assess the level conceptual advance at the time of resubmission.

Yours sincerely,

Editor
The EMBO Journal

Additional correspondence (author) 16 September 2011

Thank you for agreeing to reconsider the possibility of re-submitting. We gladly accept your challenge to submit a manuscript that is deemed to be a significant contribution to the field and which is worthy of publication in EMBO J.

Resubmission 16 January 2012

We are most appreciative of your willingness to look at a second version of our manuscript on ER-mitochondrial communication in AD.

Even though the punch line is unchanged, we have revised the paper significantly, and have addressed essentially every issue raised by the reviewers (relevant changes in the manuscript are highlighted in yellow). In truth, many of the criticisms were both fair and helpful, and in fact, the new data generated to address these criticisms solidify our conclusions even more strongly than before.

Although you intimated that this version might become a new submission rather than a revision, I have nevertheless added a "response to the reviewers" letter to the front of the pdf, as I think this might help you understand our thinking regarding the initial reviews, and how this in turn helped shape the newer version. As you may recall, the comments fell into 3 broad areas:

(1) Lack of some controls, especially the analysis of the expression of various genes associated with cholesterol and phospholipid metabolism. These have been analyzed, and we can now say that the increase in cholesterol ester and phospholipid synthesis is not due to any increase in the expression of these genes.

(2) More EM and ER-mitochondrial contact data. These have been provided, and support our initial contention regarding increased contacts in presenilin-mutant MEFS, and in FAD and SAD fibroblasts.

(3) Lack of a mechanism. As I noted in my previous letter to you, we feel that this is well beyond the scope of this manuscript. However, in responding to the suggestion from two referees to knock down mitofusin-2 in our presenilin-mutant cells, we discovered (to our surprise, actually) that the loss of Mfn2 could reverse the presenilin-induced MAM phenotype, implying that the two genes affect the same pathway regulating ER-mitochondrial connectivity, which indeed does speak to a potential mechanism.

You will also notice that we have changed the title from "Presenilins are negative regulators of ER-mitochondrial communication" to "Increased ER-mitochondrial communication in Alzheimer disease". We made this change to avoid confusion (as articulated best by Referee #2) regarding the specific role of presenilins in regulating ER-mito cross-talk as compared to the more general phenomenon uncovered by us indicating that upregulated cross-talk can cause AD, be it by mutated presenilins or by APP (in the case of FAD) or by currently unknown causes (in the case of SAD).

I appreciate your patience with us over the last few months.

Response to Reviewers

We thank the reviewers for their comments and suggestions, many of which were most helpful. We feel that the revised manuscript is now much improved.

Comments of Reviewer #1

The trafficking of Phospholipids between ER and mitochondria has been used to measure the degree of communication between both organelles in wt and Presinilins mutant cells. The authors should also measure ER mitochondria calcium transfer in order to corroborate this data.

While nobody has measured phospholipid or cholesterol ester metabolism in presenilin-mutant cells, there is a vast literature on changes in calcium homeostasis in both FAD and SAD, and the role of altered presenilin function in this process (see, for example, Small (2009) Neurochem. Res. 34:1824; Bezprozvanny and Mattson (2008) Trends Neurosci. 31:454), including altered calcium signalling in presenilin-mutant MEFs (e.g. Brunello et al (2009) J. Cell. Mol. Med. 13:3358; Tu et al. (2006) Cell 126:981), as well a growing literature indicating the role of MAM in regulating Ca2+ homeostasis (e.g. Csordas et al (2010) Mol. Cell 39:121, Giacomello et al. (2010) Mol. Cell 38:280). Moreover, many of the papers analyzed the very same cells and PS mutations that we studied here. Thus, we feel that it is unnecessary to re-document here the altered calcium homeostasis in presenilin-mutant or AD cells, as this is well established in the literature.

Conventional EM does not take into account of the ER-mitochondria conformation in 3D. In order to be quantitative, the length of mitochondrial-ER contacts should have been measured upon 3D reconstruction of EM acquisitions (electron tomography), or by live fluorescence imaging of the false colocalization between the two organelles described in Rizzuto et al Science 1998.

We have now performed a "co-localization" study looking for ER-mitochondrial apposition of fluorescent markers (now shown in Fig. 6), and it supports the EM data.

The biggest unresolved question is the molecular mechanism by which PS negatively regulate ERMitochondria cross talk In other word, how can the absence of Presenilins physically increase the tether? Authors should explore if for example levels of MAM Mfn2 are reduced in PS deficient cells, and whether PS knockdown has any effect on ER mitochondria transfer in Mfn2-/- cells.

We analyzed the expression of Mfn2 in PS-mutant cells, and they were unchanged (see Supplemental Fig. 3B). On the other hand, knockdown of PS in Mfn2-KO cells reversed aberrant MAM function, as did knockdown of Mfn2 in PS-KO cells (shown in Fig. 9). This surprising result (at least to us) implies that the two genes may affect the same regulatory process. We thank the reviewer for suggesting this experiment.

Comments of Reviewer #2

Surprisingly, to this reviewer, similar biochemical abnormalities as seen in PS deficient or mutant cells were observed in fibroblasts from sporadic Alzheimer's disease patients, who presumably have normal PS levels and function. Thus, the story here is confusing. The authors dismiss this in the Discussion as suggesting they have described a possibly overarching mechanism present in all forms of AD, but the focus of all the experiments and interpretations is on PS, so such a trivial discussion of this aspect is insufficient. The only way the story could be, as the title indicates, one about presenilins, is if the authors can link the observations from the SAD cells to PS, and this has not been done, which represents the major weakness of the study.

We agree with the reviewer that we sent a mixed message. Our view had always been that upregulated ER-mito cross-talk is a general feature of AD cells, and is not confined to presenilins alone. We have therefore recast the paper in a number of ways, starting with the title. We have also changed the tenor of the interpretation of the results, and elements of the discussion, to reflect this point of view. We believe that AD is an ER-mito communication problem, and that defects in presenilins are just one trigger: we have seen the same phenotype in APP-mutant cells, and in SAD cells where PS's and APP are normal, but in which γ- secretase, for unknown reasons, is not. As γ-secretase is the underlying pathogenetic theme in all forms of AD, perhaps it is the altered activity of this complex that is the key player in regulating MAM.

In the tritiated serine incorporation experiments, how did serine get into the cells? Was there any difference in the uptake rates between the different cell lines, and how were these incorporated into the analyses?

Serine enters cells via the ASCT1 neutral amino acid transporter (gene SLC1A4), which transports alanine, serine, cysteine, and threonine. With respect to Ser uptake, we acknowledge that different cell lines can have different rates of uptake. However, we performed 3H-Ser incorporation in vitro on isolated crude mitochondria (see Fig. 4C), thus obviating the transport issue, and the results were consistent with the in vivo data. Moreover, the pulse-chase data also circumvent the uptake issue, as we track the rate of intracellular conversion of 3H-Ser into 3H-PtdSer and 3H-PtdEtn (Fig. 4B) after the 3H-Ser has entered the cell.

Errors bars are lacking in Fig 5A; what was the concentration used in Fig 5B?

There are no error bars because the Cin-sensitivity curve shown in the figure was just an example, showing all four cell lines assayed side-by-side in a single experiment, merely to illustrate how cinnamycin affects cell viability and how it can be used to assay for MAM function as a complement to the 3H-PtdEtn data. We have replicated this result, and also have multiple data points (with error bars) comparing Cin-sensitivity between WT and DKO MEFs at single concentrations of Cin (typically 1 μM). We can provide these data, if desired.

In the left gel in Fig 7C, it appears that either the gel has been digitally processed or that lanes from two different gels have been placed side by side to give the appearance they are parallel lanes of the same gel.

Yes, the gel is processed, but not from two different experiments/blots. Rather, the WT and DKO lanes were separated by other [irrelevant] lanes, and we just eliminated those and juxtaposed the two lanes of interest. We can provide the original films, if desired.

Comments of Reviewer #3

Unfortunately most data in the paper are not directly related to the proposal and the main idea is not really tested in the paper. Also, mechanism of presenilin actions in MAM is not explained. Most of the data are based on the study of cholesteryl ester and phospholipid synthesis, without related control experiments and these data do not support ER-mitochondrial communication. The key experiment is the EM study which show increased ER-mitochondrial contacts in DKO cells. But these results only obtained with DKO cells, not with FAD cells and teh quality of data is questionable. So the conclusion about presenilins are negative regulators of ER-mitochondrial communication is not solid and much additonal experimenst will be needed to advance this claim.

We respectfully disagree with the assertion that the data in general and the cholesterol/phospholipid assays in particular, do not support the conclusion that upregulated MAM is a culprit in AD. Both biochemical assays, and especially the 3H-Ser incorporation assay, have been accepted as "gold standards" for MAM integrity and function by the people who initially discovered MAM and analyzed its function and properties in the '90s, prominent among them Jean Vance (University of Alberta, Edmonton), Dennis Voelker (National Jewish Health, Denver), and Gunther Daum (University of Graz, Austria). Coupled with our new data using the 3H-Ser incorporation assay to show the opposite effect when Mfn2, a known MAM protein that is a positive regulator of ER-mito connectivity, is ablated, as well as the reversal of phospholipid synthesis when Mfn2 is knocked down in presenilin-KO cells (and vice versa, when Ps1/2 is knocked down in Mfn2-KO cells), we feel that the data in support of a presenilin-MAM connection are actually quite solid.

With respect to the EM work, we note that ultrastructural analysis can only support, but not prove, the key findings of our paper. It is the biochemistry (see above) that lies at the heart of the evidence for ER-mito upregulation, irrespective of what the pictures look like. Nevertheless, we have now generated more EM's and have also performed fluorescent ER-mito co-localization experiments on FAD and SAD cells, and have obtained results similar to those obtained in the PS-KO MEFs, namely, that the number of contacts (Fig. 6) and the area of contact (Fig. 7 and Supplemental Fig. 8) between ER and mitochondria is significantly increased.

Fig 2 showed increased cholesteryl ester synthesis in PS-mutant cells and AD patient fibroblasts, which maybe not due to increase ER-mitochondrial contacts, maybe just because increased ACAT expression in PS-mutant cells and AD patient fibroblasts, since there is report that ACAT-1 mRNA levels increased significantly in AD fibroblasts (Pani A, et al, 2009), the control experiment to compare the ACAT expression level is very important.

In our hands, using qRT-PCR, ACAT1 is not increased in PS-mutant fibroblasts (comparing 2 controls with 6 FAD's). The difference with Pani's results may be technical, as Pani performed "standard" RT-PCR and measured the steady-state level of ACAT1 by Western blot, whereas we performed dynamic qRT-PCR using "real-time" amplification, which we believe reflects more accurately what is really going on.

Similarly, fig4 and fig5 showed elevated phospholipid synthesis in PS-mutant cells and AD patient fibroblasts, which are also not supportive data for increasing ER-mitochondrial communication if without the control experiment to test the PtdSer synthase level.

As with ACAT1, we also performed qRT-PCR on PTDSS1, PTDSS2, and PISD and found no increase.

Fig 6 EM data is a direct data to support the increased ER-mitochondrial contacts in presinilin-mutant MEF, but from fig6F, looks like the author observed more mitochondrial contacts in DKO cells than WT cells, is this due to there are more mitochondrial number in DKO cells? Since the mitochondrial fission/fusion also have effect on ER-mtiochondrial contact, the author should also analyze the total mitochondrial number in the EM images.

We are not claiming that the total number of mitos contacting ER are increased (in any event, EM is not the way to look for numbers of organelles), but rather that the area of contact is increased. With respect to mito fission/fusion, the mitochondria are indeed fragmented in PS-mutant cells (already reported in the literature - see, for example, Wang et al. (2009) J. Neurochem. 109:153), but we have not shown any pictures of mitos in this manuscript, as it is peripheral to the message we are trying to deliver here. We indeed think that the analysis of mitochondrial dynamics and function is crucial to our understanding of AD, and have a separate manuscript in the works focusing exclusively on this topic.

Also in the study (Zampese E et al, 2011), data were showed that overexpression of PS1-A246E did not significantly increase the area of ER-mitochondria colocalization, and down-regulation of endogenous PS1 by specific siRNA did not significantly modify ER-mitochondria tethering, which are opposite with this study.

We were aware of Zampese's results, and our goal was not to contradict this one paper. We note however, that our results are consistent with, and in agreement with, the findings from numerous other papers looking at, for example, calcium homeostasis in PS1-mutant cells (e.g. Brunello et al (2009) J. Cell. Mol. Med. 13:3358; Tu et al. (2006) Cell 126:981). We do not know why Zampese disagrees with the literature, but we note that from a conceptual point of view, it is hard to envision a model of AD pathogenesis that encompasses PS2 but not PS1, which after all, is the more "severe" mutation. We note, of course, that at least with respect to PS2, we reproduced Zampese's results quite closely, and we and they agree that ER-mito connectivity is important in AD.

It is also unclear if similar increase in mito-ER contacts is observed in PS1-FAD cells, for example in cells expressing PS1-A246E. If PS1 playing a "structural role" in supporting ER-mito junctions, it is not clear if this role is impaired by FAD point mutations, such as A246E. If it is not impaired then the proposed mechanism is only relevant for PS DKO cells, not for FAD cells.

Data showing increased ER-mito contacts by EM in FAD-PS1(A246E) (and in SAD) are shown in Fig. 7F and Supplemental Fig. 8. As noted above, we also show this by fluorescent ER-mito co-localization (Fig. 6).

In fig7, the authors use MFN2 KO cell as a ER-mitochondrial contacts deficient cell, but in the study (Zampese E et al, 2011), data showed that knocking down PS1 or PS2 decrease MFN2 expression level, so please check the MFN2 expression level in PS KO cells and PS1KD cells, if MFN2 also decreased, how to explain the increase ERmitochondrial contacts?

In our hands, Mfn2 levels in PS-KO cells are unchanged (see Supplemental Fig. 3B). We note that from a purely conceptual point of view, the ablation of Mfn2 decreases ER-mito contacts (as shown by de Brito et al, 2008); if so, the reduction in Mfn2 levels in the PS2-knockdown cells shown by Zampese should have blunted the increase in ER-mito contacts due to the reduction in PS2, but it did not, in contradiction with de Brito et al.

Also, MFN2 experiment on Fig 7 does not really test the main hypothesis of the paper. An appropriate experiment would be to knockdown MFN2 in PS DKO or PS1-FAD cells and observed reversal of phenotypes.

We thank the reviewer for this suggestion. See Fig. 9 and the comment to Reviewer #1 above.

3rd Editorial Decision 11 March 2012

Thank you for re-submitting your revised manuscript for consideration by the EMBO Journal. It has now been seen by the same three referees who reviewed your previous manuscript (although the reports are unfortunately renumbered, so that previous ref 1 is now ref 2, previous ref 2 is ref 3 and previous ref 3 is ref 1). The comments are shown below.

As you will see, all three referees continue to see potential interest in your dataset, but none of the referees is convinced that the current manuscript is close to publication at this point.

We acknowledge that referee 1 (previous ref 3) raises a number of new issues that were not raised in the previous round, including the need for a detailed mechanistic understanding of a role for presenilin (although you argue that the relevant target may actually be g-secretase, see ref 3) and replication of the work in neurons - certainly a legitimate point but not one that was raised in the last round.

Indeed, referee 3 (previously no. 2) also raises a number of specific issues for the first time that are relevant to experiments previously displayed. We certainly do not encourage that additional issues are raised on previous experiments, but feel that these points were raised in good faith and that they are important, since they are related to the strength of the data displayed, and we would therefore not wish to overrule these issues. Importantly, ref 3 does not accept your argument that both familial and sporadic AZ show the same effect, since it is not due to PS but potentially defective g-secretase. The referee is surprised by this important hypothesis and wants to see it tested experimentally (g-secretase inhibitors and rescue with secretase dead cells).

Referee 2 (previous 1) notes that the experiment underlying fig 9 needs to be repeated in an effort to make the data more significant. Note that we did not see the detailed description of any of the statistical information provided, as required by our guide to authors (see also ref 3).
The referee also wants to see it tested if PS1/2 rescues the lack of ER and mitochondria tethering in Mfn2-/- cells. I expect you may also wish to extend this to g-secretase, noting your response to ref 3.

As you know, it is EMBO Journal policy to allow only a single round of substantial experimental revision. Addressing the key referee criticisms would require a lot of additional experimentation. It is also likely beyond the scope of your present study, judging by the rebuttal you sent.

I cannot therefore encourage a further resubmission. However, if you do feel that you have addressed the essential experimental issues raised in the future and would like to discuss a further evaluation at this journal (with a subset of the same referees), I would be open to discuss this - of course I cannot commit to a further round of review at this time and we would understand if you preferred to submit elsewhere.

Thank you for the opportunity to re-consider your work for publication. I am sorry that we could not be more positive at this time.

Yours sincerely,

Editor
The EMBO Journal

Referee #1

The manuscript submitted by Area-Gomez, et al., entitled "Increased ER-mitochondrial communication in Alzheimer's disease" is a revised manuscript, incorporating the suggestions of the previous reviewers. The current report addresses many of the reviewers' concerns with additional experiments and a more focused case for the role of Alzheimer's disease related presenilin mutations in the augmentation of ER-mitochondrial co-operation or MAM function, specifically in the metabolism and transfer of phospholipids. The concept is novel, however the study is primarily descriptive with no real insight as to how wild type or mutant presenilin effects ER-mitochondrial function. For example, how do presenilin mutations facilitate PE/PS synthesis? Do presenilins catalyze a biosynthesis step? Which domain(s) of presenilin are important for this function?

Some issues:

1) The authors use the terms ER-mitochondrial "communication" and "connection". When they say communication, they mean function? When they say connection, do they mean contacts?
2) Figure 3C. What would expressing A246E in MEFs do to lipid production? Or expressing A246 in DKO MEFs? MEFs expressing A246E and WT PS are the missing controls for this experiment.
3) Figure 6. Can the authors provide representative images for all the genotypes in Fig B?
4) For bigger impact and relevance to the neurobiology of AD, parallel studies should have been performed in neurons. Do the authors have any data to suggest that this increase in MAM connectivity and function occurs in neurons?
5) Some images are still not of good quality, in particular some of the LipidTox staining (eg. Fig3C, D). Would the authors be able to provide clearer images?

Referee #2

I appreciated the effort put by the authors in addressing my main concerns. I am still a little bit confused by the experiments performed on the Mfn2 knockout cells and by the controversy with Zampese et al. Zampese shows that overexpression of PS1 increases, whilst its silencing decreases ER-mito contacts. The authors are acknowledging and discussing appropriately the discrepancies, and the fact that in their hands Mfn2 works to modulate tethering can offer some strength to their

conclusion. However, in the light of this published paper, some simple additional experiments are needed to clarify the issues and to convince the reader that the Area-Gomez paper is the one to believe.

First, in Fig 9 the error bars are gigantic. Please repeat the experiment to reach an acceptable n and SEM. Second, the missing experiment at this point is to verify if ablation of PS1/2 reverts the lack of tethering (measured by IF) between ER and mitochondria in Mfn2-/- cells. This would nicely corroborate the findings of lipid metabolism and tell us that the increase upon silencing of PS1/2 in Mfn2 cells shown here depends on the reversal of the distance between the organelles.

Referee #3

1. I remain perplexed by the seemingly similar results in SAD and FAD cells, with or without PS expression. The authors have suggested that whereas PS and APP may be normal in SAD, a common pathogenic feature of all AD is altered gamma secretase activity. Is this true? What is the evidence that gamma secretase activity is altered in the common forms of AD? because I am not aware of it.

If as the authors suggest, gamma secretase activity lies at the heart of all the observations, then it would be important to target the secretase activity directly. First, by determining whether non FAD, secretase-dead PS can rescue. Second, by using specific pharmacological inhibitors of secretase activity.

Secretase activity is generally believed to be absent until PS associates with other components of the complex. Association of the complex is believed to coincide with its trafficking out of the ER. The authors need to comment here. In addition, they should determine whether other components of the secretase complex are present in MAM, which is predicted by their model.

2. Regarding the authors' responses to my comments about doctoring gels. If lanes have been removed for presentation purposes, then it is NECESSARY to put a line between the lanes to show the reader that they were not originally adjacent lanes in the gel.

3. Regarding the responses to errors, yes, all experiments need to be analyzed and the statistics presented, even if a representative figure is shown.

4. The statement, bottom of page 5, that PS1 plays a more significant role in FAD is incorrect. There are more known mutations in PS1 that cause FAD, but the phenotypes of mutant PS2 and PS1 are the same.

5. Many figures are scatter-grams, but it is not clear what each point represents. Is each point a different patient cell line? If this is the case, then why are the number of patients cells presented different in different figures? Furthermore, does it imply that the particular experiment was run only once for each patient in each paradigm, i.e. there is no way to estimate the error associated with the measurement for each cell line?

6. There needs to be more information regarding analyses of the fluorescent images. It is not clear if fields were randomly selected, how many fields were chosen, and how many cells were counted.

7. The authors suggest on p6, regarding data in Fig3, that there are more lipid droplets in cells with mutant PS2 and APP....where are the data?

8. The authors conclude on p9 regarding Fig 8A that Mfn2 KO decrease phospholipid synthesis by 6-70%, whereas the data shown indicate that the inhibition is 20%.

9. The new title does not really reflect what has been studied. I think MAM is a focus and should be included, and ER-mito communication is bigger than what has been examined here, so is inappropriate.

Additional correspondence (author) 13 March 2012

Thank you for your letter of March 11. After reading your comments and those of the reviewers, we are still interested in pursuing submission to EMBO J, mainly because essentially all of the reviewers' comments can be addressed relatively easily. (We counted several specific items, but only 2 would require work beyond data already in hand or corrections that are easy to make, and those two sets of data could be produced by April 30.)

Quite frankly, I get the sense from your letter that you and the Journal are interested in our findings, but that the reviewers are expressing a degree of skepticism about our results/conclusions that mitigate against publication. I have already noted that their comments are easy to address, so perhaps their private comments to you indicate otherwise. In that case, there is not much we can do to change their minds, and we will submit elsewhere. However, if the door is still open, we would like to continue our committment to EMBO J.

I am attaching a short "bullet" list of the reviewer comments, with our one-line response to each. We feel that a few of them address real issues of substance, but that, at bottom, the majority actually reflect the reviewers' discomfort with this new way at looking at AD.

In any case, I really appreciate the supportive tone of your letter and look forward to hearing from you. If you wish, I would be happy to discuss these issues with you over the phone.

Brief response to reviewers' comments

Reviewer #1 (previous reviewer #3)
Q: How do presenilin mutations facilitate phospholipid synthesis?
A: A great question, but not in the scope of the paper.
Q: Do presenilins catalyze a phospholipid biosynthesis step?
A: No. Presenilins are aspartyl proteases.
Q: Which domains of presenilins are important for phospholipid metabolism.
A: An excellent question, but outside the scope of the paper.
Q: When the authors say connection, do they mean contacts?
A: Yes.
Q: What would expressing A246E in MEFs do to lipid production?
A: The data are already presented in Fig. 3.
Q: Can the authors provide representative images for all the genotypes in Fig 6B?
A: Yes.
Q: Does increased MAM connectivity and function occur in neurons?
A: Another great question, but again, outside the scope of the paper.
Q: Would the authors be able to provide clearer images of LipidTox staining?
A: Yes

Reviewer #2 (previous reviewer #1)
Q: Why should I believe you rather than Zampese et al?
A: We do not pretend to rebut Zampese, but can easily comment of the issues.
Q: Repeat experiments in Fig. 9 to improve statistical significance.
A: Not a problem.

Reviewer #3 (previous reviewer #2)
Q: What is the evidence that g-secretase activity is altered in SAD?
A: All cases of AD show increased Ab_{42}:Ab_{40} ratios, indicative of altered g-secretase activity.
Q: Test the hypothesis with (a) g-secretase inhibitors and (b) rescue with secretase-dead cells.
A: An excellent suggestion - we already have data using (a) that support our hypothesis.
Q: Are the other components of the g-secretase complex present in MAM?
A: Yes. Already published in Area-Gomez et al., 2009.
Q: Please show that gels were not "doctored" using lines between the lanes.
A: Will do.
Q: Analyze all relevant experiments with appropriate statistics.
A: Will do.
Q: The FAD phenotypes of PS1 vs PS2 are the same.

A: Incorrect. Age of onset and clinical course are less pronounced in FAD-PS2.

Q: Are the data points in the scattergrams from single patients?

A: We will clarify this point (we do have mutiple data points from individual patients).

Q: Can you provide more technical information about the fluorescent images?

A: Yes.

Q: Where are the data on the lipid droplets in APP- and PS2-mutant cells?

A: FAD-PS2 is in Supplemental Fig. S6. We will add FAD-APP as well, and more information if needed.

Q: Discrepancy between text and Fig. 8 regarding % reduction in phospholipid synthesis.

A: Yes, our error. Will fix.

Q: The title "...ER-mitochondrial communication"...) is inappropriate.

A: Debatable, but we are open to suggestions.

Additional correspondence (editor) 20 March 2012

Thank you for your note and the useful 'response-in-brief' to the referee comments, which I have now been able to review.

As we noted in our decision letter, we also regard the further reaching questions asked by one of the referees as very interesting, but since they were not initially raised, we will not hold you to them. You appear to be in a position in principle to address many of the issues. I highlight in particular the important question regarding sporadic vs. familiar AD, for which you appear to have data already. From the brevity of your response, I could not follow in how far you may be able to address the following issue I raised in the decision letter: 'The referee also wants to see it tested if PS1/2 rescues the lack of ER and mitochondria tethering in Mfn2-/- cells. I expect you may also wish to extend this to g-secretase, noting your response to ref 3. '

I should clarify that we have abolished confidential referee comments at the EMBO Journal some time ago (see attached article), so our response is based on what you saw, with one exception - the referee fill out a table summarizing interest, novelty and technical quality. I append the information below FYI, so that you have complete transparency.

Technical Quality Adequate High Adequate
Novelty High High High
General Interest Low High High

If you can substantially address all the key issues outlined in our correspondence, we would indeed aim to return a revised manuscript to the referees. Of course, we can only publish this study with adequate referee endorsement.

1st Revision - authors' response 09 May 2012

Attached please find our re-submission.

In brief, we have responded to everything that has been requested of us. With respect to the major critique regarding the role of gamma-secretase activity itself as the regulator of MAM behavior, we have including new data showing that DAPT, a highly specific inhibitor of gamma-secretase activity, affects MAM function, but surprisingly, not ER-mitochondrial connectivity (which is why I asked you for a postponement of our resubmission, so as to double-check this result) (see Fig. 10). We also, of course, used a genetic approach to address this issue, by asking if "catalytically dead" D385A PS1 constructs could rescue MAM function and connectivity in presenilin-mutant cells. We obtained data supporting a role for gamma-secretase activity in MAM function, but upon reflection realized that this was a multivariable experiment which cannot provide a definitive answer to the question (mainly because D385A mimics a PS-knockdown phenotype). This is all noted in the results.

I want to thank you for the opportunity to re-submit, and believe that the manuscript now conforms to EMBO J's exacting standards. You have been most patient with us, and I am most appreciative of that.

Response to reviewers' comments

We thank the reviewers for their comments. As before, we think that the comments were helpful and that they have made the manuscript even stronger.

Comments of Referee #1:

The manuscript submitted by Area-Gomez, et al., entitled "Increased ER-mitochondrial communication in Alzheimer's disease" is a revised manuscript, incorporating the suggestions of the previous reviewers. The current report addresses many of the reviewers' concerns with additional experiments and a more focused case for the role of Alzheimer's disease related presenilin mutations in the augmentation of ER-mitochondrial cooperation or MAM function, specifically in the metabolism and transfer of phospholipids. The concept is novel, however the study is primarily descriptive with no real insight as to how wild type or mutant presenilin effects ER-mitochondrial function.

We feel that beyond describing a new phenomenon, we actually have obtained some insight into an unexpected biological role of presenilins that also provides a new way of understanding the pathogenesis of AD. It is true that we do not know how presenilin mutations facilitate upregulated MAM function (as exemplified by increased phospholipid synthesis), but our data, and especially the experiments showing that Mfn2 can restore MAM function in Ps-mutant cells, and our new data in this revision that γ-secretase catalytic activity plays a role in MAM function but not in ER-mitochondrial communication, are actually a start at helping us understand mechanism, as they are pointing us towards thinking about a "machine" that regulates ER-mitochondrial connections/distance. We feel that answering detailed questions about mechanism are well beyond the scope of this manuscript, but ones which will certainly be addressing in the future.

For example, how do presenilin mutations facilitate PE/PS synthesis?

We do not know, and feel that this question is outside the scope of the paper. However, we have provided new data suggesting that the presenilin protein itself mat be regulating the distance between ER and mitochondria and hence phospholipid transport between the two organelles (see Fig. 10).

Do presenilins catalyze a biosynthesis step?

While we do not know the answer to this unequivocally, almost certainly the answer is No, as presenilins are (1) aspartyl proteases and (2) intramembrane rhomboid-like proteases.

Which domain(s) of presenilin are important for this function?

If the reviewer is asking about the domains responsible for maintaining the proper ER-mitochondrial distance, we do not know. However, we note that among the dozen or so FAD cells that we checked, the pathogenic mutations were spread along the entire length of the protein, implying that there is no specific domain responsible. Of course this is an extremely interesting question, but again, is outside the scope of the paper.

The authors use the terms ER-mitochondrial "communication" and "connection". When they say communication, they mean function? When they say connection, do they mean contacts?

Yes, we used words interchangeably, mainly for stylistic purposes. However, we do differentiate between MAM functionality within the ER, and ER-mitochondrial communication. We do not mean to imply that different physical distances between mitochondria and ER may affect different MAM functions, although that may indeed be the case (see Csordas et al., 2010).

Figure 3C. What would expressing A246E in MEFs do to lipid production? Or expressing A246 in

DKO MEFs? MEFs expressing A246E and WT PS are the missing controls for this experiment.

Lipid production in FAD-PS1(A246E) fibroblasts (AG06840 and AG06848) are shown in Fig. 3. Lipid production in PS1-KD MEFs, as measured by lipid droplet formation, was rescued by overexpression of WTPS1 but not by PS1-A246E. The relevant control (rescue with WT-PS1) is also shown in the figure. As for expressing A246E in the DKO, we did not see the point of doing this experiment, because we would still have the effect of missing Ps2 (i.e. if it rescues, all well and good, but if it doesn't it could be because Ps2 is missing). We think that A246E expression in PS1-KD cells is the appropriate scenario to analyze the effect of mutations in Ps1 on lipid production.

Figure 6. Can the authors provide representative images for all the genotypes in Fig B?

Yes. Please see revised Fig. 6A.

For bigger impact and relevance to the neurobiology of AD, parallel studies should have been performed in neurons. Do the authors have any data to suggest that this increase in MAM connectivity and function occurs in neurons?

As the reviewer can appreciate, this important issue is logistically difficult to address, at least in humans. We are working on addressing this issue in mice, and hope to include data on this question in a future manuscript.

Some images are still not of good quality, in particular some of the LipidTox staining (eg. Fig3C, D). Would the authors be able to provide clearer images?

We have revised the photos in Fig. 3C to show the LipidTox pattern in WT, DKO, DKO+WT-Ps1, and DKO+A246E-Ps1. As for Fig. 3D, perhaps the file that the reviewer received was of lower quality, but the quality of the photos in our hands looks quite good (and is certainly good in the original tiff file), and we would like to leave it as is.

Comments of Referee #2:

I appreciated the effort put by the authors in addressing my main concerns. I am still a little bit confused by the experiments performed on the Mfn2 knockout cells and by the controversy with Zampese et al. Zampese shows that overexpression of PS1 increases, whilst its silencing decreases ER-mito contacts. The authors are acknowledging and discussing appropriately the discrepancies, and the fact that in their hands Mfn2 works to modulate tethering can offer some strength to their conclusion. However, in the light of this published paper, some simple additional experiments are needed to clarify the issues and to convince the reader that the Area-Gomez paper is the one to believe.

We do not view the issue as Area-Gomez vs Zampese, as we do not see it as our task to refute someone else's work, but to present our view of the issue, and let the reader make his or her own judgments. Moreover, with respect to the "big picture," it is a little bit of "apples vs oranges." Zampese maintains that downregulation of PS1 does not affect ER-mito connections as measured by calcium transport. As noted in our previous response to the reviewers, this observation goes against the literature, which shows quite clearly that PS1-mutant cells (KO, KD) have increased cytosolic and mitochondrial calcium peaks (see, for example, Tu et al., 2006), and that Ca2+ is increased in PS1-FAD cells (many refs). Moreover, if upregulated MAM and its associated increase in calcium is an underlying cause of AD, it would be difficult to understand why, as opposed to PS2 mutations, PS1 mutations do not affect calcium derived from ER-mito connections and yet still cause disease. In addition, it would also be difficult to reconcile these data with the higher number of PS1 mutations (and patients) over PS2 mutants, and the lethality of Ps1-KO but not Ps2-KO mice. With respect to the details of Zampese that differ from our work, we reiterate that it is more appropriate for the reader, not us, to decide as to the relative merits of each paper. However, if the reviewer is really interested in knowing how we view the differences between the two papers, we are willing to provide chapter and verse.

First, in Fig 9 the error bars are gigantic. Please repeat the experiment to reach an acceptable n and SEM.

We agree with the reviewer on this point. We have repeated the experiments and the data now are indeed "tighter." They continue to support the idea that Mfn2 and presenilins act in "opposite" ways with respect to ER-mitochondrial communication, which we feel is a truly interesting mechanistic insight.

Second, the missing experiment at this point is to verify if ablation of PS1/2 reverts the lack of tethering (measured by IF) between ER and mitochondria in Mfn2-/- cells. This would nicely corroborate the findings of lipid metabolism and tell us that the increase upon silencing of PS1/2 in Mfn2 cells shown here depends on the reversal of the distance between the organelles.

We have repeated this experiment, adding a control to check for tethering between ER and mitochondria. See Fig. 9.

Comments of Referee #3:

I remain perplexed by the seemingly similar results in SAD and FAD cells, with or without PS expression. The authors have suggested that whereas PS and APP may be normal in SAD, a common pathogenic feature of all AD is altered gamma secretase activity. Is this true? What is the evidence that gamma secretase activity is altered in the common forms of AD? because I am not aware of it.

The similarity in results between SAD and FAD merely suggests a common underlying mechanism for both, which we would suggest is related to increased MAM function and ER-mitochondrial communication. As described in the manuscript, that increase can be caused by many factors, three of which are mutations in PS1, PS2, and APP - causing FAD - but there may be dozens of other factors in SAD patients causing the same increase, via mechanisms currently unknown: ApoE4 may be one, SORL1 another, clusterin a third, and so on.

The reviewer is correct that the literature implicates mainly the familial forms of FAD in showing altered γ-secretase activity; the documented decrease in Aβ42:40 ratio in CSF in sporadic cases has been ascribed to kinetic effects (e.g. deposition vs clearance) rather than to any intrinsic change in the site of APP processing (although there are papers implying altered activity in SAD [e.g. Kakuda et al., 2012; Placanica et al., 2009]).

(We note, however, that over the years altered γ-secretase activity, as measured by either total Aβ or by the Aβ42:40 ratio, has become one of the definitions of the disease, and is presumably the cause of amyloid plaque accumulation in both patients and animal models.) Our focus is on upregulated ER-mito communication and MAM function as the common denominator of both SAD and FAD, not on what triggers these effects. Accordingly, we have modified the text to clarify this point of view, and to eliminate any confusion regarding γ-secretase activity as being such a common denominator.

Lastly, in order to emphasize our view that our findings apply not only to FAD but also to SAD, we have included one more figure in the paper (Fig. 11): a scheme - admittedly highly simplistic - of AD pathogenesis.

We hope that this will clarify what we have in mind.

If as the authors suggest, gamma secretase activity lies at the heart of all the observations, then it would be important to target the secretase activity directly. First, by determining whether non FAD, secretase-dead PS can rescue. Second, by using specific pharmacological inhibitors of secretase activity.

We have now performed these experiments using the known γ-secretase inhibitor DAPT (see Fig. 10).

Secretase activity is generally believed to be absent until PS associates with other components of the complex. Association of the complex is believed to coincide with its trafficking out of the ER. The authors need to comment here. In addition, they should determine whether other components of the

secretase complex are present in MAM, which is predicted by their model.

We are not claiming that γ-secretase in MAM is operating in the absence of the other components. In fact, we have found that, along with the presenilins, nicastrin, APH-1, and PEN-2 are present in the MAM (Area-Gomez et al., 2009). Moreover, we note that Vetrivel et al., 2005 (and others) have shown that, along with the presenilins, nicastrin, APH-1, and PEN-2 are present in detergent-resistant membranes, which is consistent with our results here, and have also noted the technical problems associated with analyzing MAM. As suggested by the reviewer, we have now commented on this point in the Discussion.

Regarding the authors' responses to my comments about doctoring gels. If lanes have been removed for presentation purposes, then it is NECESSARY to put a line between the lanes to show the reader that they were not originally adjacent lanes in the gel.

We regret the oversight. This has now been fixed.

3. Regarding the responses to errors, yes, all experiments need to be analyzed and the statistics presented, even if a representative figure is shown.

This comment was mainly in regard to the curves shown in Fig. 5A. We have now added an extra panel to this figure giving the overall data set (with error bars and statistics); it indicates that PS1 and DKO cells are indeed significantly more sensitive to cinnamycin than are WT MEFs. In addition, statistical methods are noted in all the figure legends, where appropriate.

4. The statement, bottom of page 5, that PS1 plays a more significant role in FAD is incorrect. There are more known mutations in PS1 that cause FAD, but the phenotypes of mutant PS2 and PS1 are the same.

We respectfully disagree. The age of onset and the progression are more severe in FAD-PS1 than in FADPS2 (see, for example, Jayadev et al., 2010, which is referenced in the paper**)**.

5. Many figures are scatter-grams, but it is not clear what each point represents. Is each point a different patient cell line? If this is the case, then why is the number of patients cells presented different in different figures? Furthermore, does it imply that the particular experiment was run only once for each patient in each paradigm, i.e. there is no way to estimate the error associated with the measurement for each cell line?

We have revised the relevant panels in each of the main text figures to now indicate which data points are from FAD patients with mutations in PS1 (circles), PS2 (triangles) and APP (squares). As for multiiple experiments per cell line, we have revised Table S1 to show how many times each experiment was performed.

Where multiple experiments (e.g. 3H-Ser incorporation assays) were performed on the same cell line, we have included the average result for that cell in the main text figure, rather than "subdividing" the data points for that cell line, as by doing so the figure would rapidly become cluttered, rendering it difficult to understand. Obviously, when analyzing a cell line more than once, we obtain a range of values, but suffice it to say that in our hands the single average data points were on the whole quite reflective of the entire data set.

6. There needs to be more information regarding analyses of the fluorescent images. It is not clear if fields were randomly selected, how many fields were chosen, and how many cells were counted.

We now note this in the Methods.

7. The authors suggest on p6, regarding data in Fig3, that there are more lipid droplets in cells with mutant PS2 and APP....where are the data?

These are now indicated in Fig. 3D (triangle [PS2] and square [APP]).

8. The authors conclude on p9 regarding Fig 8A that Mfn2 KO decrease phospholipid synthesis by

6-70%, whereas the data shown indicate that the inhibition is 20%.

Our error; we thank the reviewer for catching it.

9. The new title does not really reflect what has been studied. I think MAM is a focus and should be included, and ER-mito communication is bigger than what has been examined here, so is inappropriate.

We agree with the reviewer's overall philosophical point. We have modified the title accordingly.

4th Editorial Decision 22 June 2012

Thank you for submitting your interesting revised manuscript a few weeks ago. It has now been seen by referees 2 and 3 and their comments are enclosed below. As you will see, both referees recommend publication enthusiastically, pending minor textural revision. In particular, we agree with referee 2 (as discussed previously) that it will be very useful to include a fair discussion of discrepancies with the 2011 Zampese paper - I would suggest that this can be based on a shortened version of your letter, which I found clear, informative, balanced and constructive.

Given the referees' positive recommendations, I would like to invite you to submit a final manuscript for publication. We will proceed with formal acceptance and publication as soon as we have the final revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your fascinating dataset for publication. I look forward to your revision.

I appreciate that the process in this case was slower than you should have expected from this journal. However, I do hope that the review process improved the overall dataset significantly and this will raise the impact of the work significantly. We certainly appreciated your informative and constructive rebuttals and revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #2

Authors did a great job in addressing my previous concerns. I am convinced by their experiments and I think that this is a very important piece of work that must be brought to the attention of the readership of The EMBOJ.
I also appreciated the extensive discussion of the discrepancies with the results of Zampese et al; I think that at least some discussion of the issues of overexpression and of seemingly internally contradictory results by that group shall be included in the discussion, not to twist the arm of the reader, but to present the full picture.
I suggest that the Authors rewrite the abstract to shorten it and make it more appealing to a broader readership.

Finally, I think that the authors satisfactorily answered to the comments by reviewer 1.

a. They clearly show a role for PS in the modulation of the distance between ER and mitochondria, a key aspect (as testified by the experiment with Mfn2-/- cells) in determining efficiency of PS/PE biosynthesis; I think that it is difficult that PS1/2 are catalyzing a biosynthetic step, rather they are facilitating the apposition of the membranes that is required for the efficient synthesis.
b. Clearly, a detailed definition of the domains of PS involved is out of the scope of this data heavy manuscript. In conjunction with the newly added data, this is a focused story that shows how mutated (or absent) PS causes decreased distance between mitochondria and ER. This theme will be crucial in the years to come as a pathogenetic determinant of AD.

Referee #3

I am satisfied with the authors' responses and revisions and new data. I suggest two things that should be noted in a final revised manuscript.

First, there is evidence of FAD PS gain of function regarding calcium signaling from the ER through InsP3 receptors (Cheung, K.-H., L. Mei, D.-O. D. Mak, I. Hayashi, T. Iwatsubo and J. K. Foskett. 2010. Gain-of-function enhancement of IP3 receptor modal gating by familial Alzheimer's disease-linked presenilin mutants in human cells and mouse neurons. Science Signaling 3:ra22)

Second, the last line of the abstract should insert the word "may" to be a little more careful regarding the conclusions.

2nd Revision – authors' response 27 June 2012

Comments of Referee #2:

I also appreciated the extensive discussion of the discrepancies with the results of Zampese et al; I think that at least some discussion of the issues of overexpression and of seemingly internally contradictory results by that group shall be included in the discussion, not to twist the arm of the reader, but to present the full picture.
We have added a section in the Discussion briefly outlining the discrepancies between our work and that of Zampese et al. We did not go into great detail, other than to mention some conceptual inconsistencies, while focusing mainly on the pitfalls of overexpression experiments and adding a passing comment on their *Mfn2* results. We hope that we have gotten the point across in a fair, concise, firm, but polite way.

I suggest that the Authors rewrite the abstract to shorten it and make it more appealing to a broader readership.
As requested, we have shortened the Abstract, by about 20%, but think that it still encapsulates the main punch lines of the paper for *EMBO J*'s broad audience.

Comments of Referee #3:
First, there is evidence of FAD PS gain of function regarding calcium signaling from the ER through InsP3 receptors (Cheung, K.-H., L. Mei, D.-O. D. Mak, I. Hayashi, T. Iwatsubo and J. K. Foskett. 2010. Gain-offunction enhancement of IP3 receptor modal gating by familial Alzheimer's disease-linked presenilin mutants in human cells and mouse neurons. Science Signaling 3:ra22)
We appreciate the referee's point, and are absolutely open to the possibility that some dominant PS mutations may cause true gain of function. However, in our view Cheung et al (2010) is not the best example of this. While the paper indeed claims gain-of-function, our reading of the paper tells a slightly different story, especially in light of the results in our manuscript. We think that the increases in PS-mediated calcium trafficking found by Cheung et al (2010) are more easily explained by haploinsufficiency (called by some a "gain of negative function") than by gain-of-function. Cheung et al. concluded that the changes were a gain of function because the increased trafficking was independent of γ-secretase activity. However, they came to this conclusion based on the measurement of Ca2+ levels in cells expressing "catalytically-dead" PS1 constructs, which, as we noted in our previous response to the reviewers (and which we discuss in our manuscript), gives a misleading result, as these constructs mimic haploinsufficient PS1. We have nevertheless decided to cite Cheung et al (2010) anyway, because they actually use the phrase "gain-of-function" in their title, but we cite them in a context that is more nuanced. We now also cite a

paper by De Strooper et al. (1998) which we think supports the gain-of-function view more compellingly.
Maybe the best way to view this is to note that the issue is a matter of some debate (see, for example, Wolfe (2007) EMBO Rep. 8:136-140) and that sometimes it is devilishly difficult to determine how a dominant mutation acts (look at the trouble the ALS people are having). This seems to be especially true in the case of AD.

Second, the last line of the abstract should insert the word "may" to be a little more careful regarding the conclusions.
Done.

