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γ-Secretase dysfunction at the core of Familial 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 20 January 2012

Thank you for submitting your manuscript to the EMBO journal. Your study has now been seen by three referees and their comments are provided below.

While referee #3 is not persuaded that we gain enough novel mechanistic insight into gamma-secretase mediated cleavage, referee #1 and 2 find that the present analysis is an important step forward and support publication here. Given the comments provided by referee #1 and 2, I will go with their overall recommendation and ask you to submit a suitably revised manuscript for our consideration. They raise a number of specific concerns that shouldn't involve too much additional work to resolve.

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 work for publication. I look forward to your revision.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

The manuscript by Chávez-Gutiérrez represents an important and very detailed piece of work regarding the mechanisms of AD causing mutations. These data revisit a debate regarding loss of versus gain of function of AD causing mutations in presenilin and APP using both in vitro assays and cell culture studies. The data set itself is comprehensive and of extremely high quality the authors should be commended for generating such a data set. For the most part the conclusions drawn are supported by the data and the manuscript is very well-written and well referenced. There are some concerns that need to be addressed. These are elaborated on below. Of great importance are the statistical issues as they potentially undermine an otherwise really elegant study .

1. The paper is largely written as if the Ihara model of gamma-secretase sequential cleavage is in fact not a model but a proven fact. Though I believe that the model provides an excellent framework to explain gamma-cleavage, it is a model and may not be entirely correct. First regards the issue of production lines. I think the data suggests that the initial epsilon cleavage can influence subsequent cleavages, but it's not clear that there is a one to one correlation. In other words there is some imprecision along the way. Also though a stepwise cleavage is suggested, does it always happen that way or can the secretase skip a step? An example of how this is reflected in the writing is that the term 4th cleavage is used, but really this is the final cleavage before substrate is released form γ-secretase, but it might be the third cleavage or second in some circumstances. So I would just ask the authors to consider how they are potentially using precise terms for a model of cleavage that may not be so precise. Of course the authors should still feel free to discuss ways in which their data support the model but that should be in the discussions not "pre-supposed" in the results....

2. I have often thought the LOF vs GOF argument has been overplayed as the human data does not support LOF. Some comment on the fact that human data does not support LOF would be appropriate to include. There is no Notch phenotype, there is no evidence for APP CTF accumulation etc.....

3. Detergent effects. Many point mutations alter the stability of a protein. So when PS/γ-secretase is solubilized by CHAPSO or other detergents it might artificially result in some LOF. Can the authors perform some experiments using non-solubilized members to exclude that the results are not being influenced by detergents. If not this caveat should be included in the discussion.

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5. Statistics. The description of the statistics is uneven and its not clear that appropriate tests are being used. For multiple comparisons between multiple mutants and controls ANOVAS not two-tailed t-tests are needed. In some cases statistics are lacking. Table 1 which is perhaps the meet of the manuscript has no statistical evaluation. So how much of a difference is needed to reach significance? Figure 3 is also problematic as the data do not reach statistical significance but are likely to be biologically meaningful if truly the rates are > 50% different. I think this is just variance and low n numbers but it really undermines the entire data package. Going back to Table 1 few of the differences are more then 2-fold....

6. I think the data on Abeta38 being potentially pathogenic is a little oversold. I think the data raise an intriguing point but there is no human data that I am aware of that shows 38 to be an important pathogenic species. I think it is worth raising the issue, but balancing this with some caveats....

Referee #2

This is an excellent study aimed at dissecting the biochemical mechanisms by which PSEN and APP mutations cause dominant, early-onset Alzheimer's disease. The results show quite convincingly that the general effect of these mutations is to alter gamma-secretase processing of APP substrate. The experiments performed are technically very challenging and time-consuming. Despite these challenges, no "short-cuts" were taken, and the experiments were apparently carried out rigorously.

Altogether, the results from this study provide a coherent picture of how various disease-causing mutations change Abeta production. Among the notable observations:

* PSEN1 and PSEN2 mutations do not consistently change the 1st proteolytic event that produces AICD but does consistently change the 4th proteolytic event that converts Abeta42 to Abeta38 and Abeta43 to Abeta40. This effectively refutes the notion that simple reduction of overall proteolytic activity (i.e., "loss of function") is a common feature of AD-causing presenilin mutations.

* APP mutations skew the product line from Abeta49 to Abeta48, increasing the proportion of Abeta42 and Abeta38.

* Surprisingly, Abeta38 supports Abeta42 aggregation more than does Abeta40. This has important implications for the development of gamma-secretase modulators that increase Abeta38 while decreasing Abeta42.

* Putative "Notch-sparing" gamma-secretase inhibitors show little or no APP/Notch selectivity in comparable in vitro assay systems. This has important implications for drug discovery efforts targeting gamma-secretase.

* Gamma-secretase modulators increase the 4th proteolytic event in the Abeta48 product line (i.e., conversion of Abeta42 to Abeta38).

All that being said, several suggestions are recommended below that would be critical for putting this study into proper context and perspective and clarifying some of the analyses and conclusions.

(1) The idea that this study is the first to describe qualitative (versus quantitative) changes in Abeta production is not accurate. The authors reference a now 10-year-old review article by Hardy and Selkoe to suggest that the amyloid hypothesis stresses quantitative changes in total Abeta or Abeta42. But the field has held for a number of years that the ratio of Abeta42/40 is the critical factor (e.g., Tanzi and Bertram, Cell, 2005). Proper references should be cited.

(2) Likewise, in the discussion section, proper citations should be included in the list of 3 levels by which PSEN1&2 mutations affect gamma-secretase. The variable effects on initial endoproteolysis as well as the consistent formation of longer Abeta peptides has recently been reported (Quintero-Monzon O et al., Biochemistry, 2011) and reports from laboratory of Yasuo Ihara have previously shown that PS1 mutations shift the first proteolytic event from AICD50 to AICD51 (i.e., from the Abeta49 product line to the Abeta48 product line) (Sato T, et al., JBC, 2003).

(3) In describing the results in Fig. 1 and Table 1, it is said that "gamma-secretase activities are normalized to enzyme levels". Exactly how this normalization was carried out should be described (e.g., based on levels of PSEN NTF?).

(4) How Abeta42 and Abeta43 were quantified for Fig. 2 is not clear, as the urea-based gel in panel C does not show resolution of these two peptides. Perhaps this panel can be expanded so that the readers can tell the degree of resolution.

(5) It is stated that the PS2 N141 mutation does not significantly affect endopeptidase activity (the first cut of APP), but Fig. 3 suggests otherwise, as does the quantification: the mutant is said in the text to be 46.6% as efficient as the wild type PSEN2. Or is the implication that the errors make the results not statistically significant?

Referee #3

This study deals with a central question in Alzheimer's disease (AD) research. Mutations in presenilin, the catalytic component of the protease gamma-secretase, cause familial forms of AD. However, their mechanism of action - although intensively investigated for many years - is still unsatisfactorily understood. The authors provide a comparative analysis of different presenilin mutations and carefully evaluate their effect on total gamma-secretase activity and on the individual cleavages of this multiply cleaving enzyme. The study is complemented by a similar analysis of

APP mutations and of gamma-secretase modulators, which are a major drug class for therapeutic tests against AD. From the analyses using in vitro, in vivo and biophysical experiments the authors conclude that presenilin (PS) mutations do not simply mediate a loss-of-signaling of different substrates and thereby cause neurodegeneration - which had been one previous hypothesis. Instead, the authors demonstrate that mutations in PS or APP affect gamma-cleavage in different ways, by reducing the final gamma-secretase cut or the "product line" used.

This is an interesting study of high technical quality, providing a comprehensive comparison of different mutations. It gives many beautiful insights, for example into the catalytic constants of gamma-secretase, the biophysical properties of Abeta38 and into the action of the gamma-secretase activators.

My major criticism, however, is that this study does not provide fundamentally new insights into the mechanism, by which the mutations or compounds affect the gamma-secretase cleavage. I am well aware that the final answer can most likely only be obtained by solving the structure of gamma-secretase, which still is a major task for the future. However, the principles emphasized here - reduction of 3rd/4th cleavage and a shift of product line - follow up on several previous studies in the recent past from different labs working on gamma-secretase or the related signal peptide peptidase family. Thus, I feel that the study would be better suited for a more specialized journal.

Minor comment:
The Km and vmax values should be carefully checked. For example, for the deltaE9 mutation the values for Notch are surprising: at 2.25 microM the velocity has nearly reached the plateau at around 30. It is hard to imagine that vmax can still reach 54 as indicated in the table. If it still is, then Km should be larger. At the half-maximal velocity (27) the substrate concentration of this green line would be around 1.5 and not 0.65. This, however, would strongly alter the efficiency ratio in figure 1E.

1st Revision - authors' response 13 February 2012

Referee #1

The manuscript by Chávez-Gutiérrez represents an important and very detailed piece of work regarding the mechanisms of AD causing mutations. These data revisit a debate regarding loss of versus gain of function of AD causing mutations in presenilin and APP using both in vitro assays and cell culture studies. The data set itself is comprehensive and of extremely high quality the authors should be commended for generating such a data set. For the most part the conclusions drawn are supported by the data and the manuscript is very well-written and well referenced. There are some concerns that need to be addressed. These are elaborated on below. Of great importance are the statistical issues as they potentially undermine an otherwise really elegant study

We thank the referee for his/her very kind words. Indeed, there is enormous work behind the data presented in this manuscript. The additional constructive criticisms have helped us to improve our study.

1. The paper is largely written as if the Ihara model of gamma-secretase sequential cleavage is in fact not a model but a proven fact. Though I believe that the model provides an excellent framework to explain gamma-cleavage, it is a model and may not be entirely correct. First regards the issue of production lines. I think the data suggests that the initial epsilon cleavage can influence subsequent cleavages, but it's not clear that there is a one to one correlation. In other words there is some imprecision along the way. Also though a stepwise cleavage is suggested, does it always happen that way or can the secretase skip a step? An example of how this is reflected in the writing is that the term 4th cleavage is used, but really this is the final cleavage before substrate is released form γ-secretase, but it might be the third cleavage or second in some circumstances. So I would just ask the authors to consider how they are potentially using precise terms for a model Of cleavage that may not be so precise. Of course the authors should still feel free to discuss ways in which their data support the model but that should be in the discussions not "pre-supposed" in the results....

The referee is right in saying that the Ihara model is not a proven fact. In accordance, we have

changed the introduction to make that clearer and indicated in the results section that our interpretation is based on a model that is not yet fully proven. On the other hand, our data fit remarkably well with this model and it is very difficult to describe our observations in complete neutral terms. We decided therefore for reasons of clarity to use terminology that refers to this model. In this regard, we would like to stress that only very few data (Czirr et al, 2008; Page et al, 2008) are present in the literature that are not fully explained by the model. Actually, both papers show dissociation between the effects of compounds on Abeta42 versus Abeta38, and we discuss in the manuscript that those data are not necessarily in contradiction with the Ihara model. Moreover and importantly, the evidence in support for the Ihara model is really impressive (Kakuda et al, 2006; Qi-Takahara et al, 2005; Sato et al, 2005; Takami et al, 2009; Yagishita et al, 2008; Yagishita et al, 2006), see also (Fukumori et al, 2010) and for a discussion see pp242-243 in (De Strooper & Annaert, 2010). We added these references to the paper.

We hope that the referee accepts that we continue presenting the data as we did before, but with clear statements and qualifiers indicating that the description of the data is based on the assumptions made by the Ihara model. In addition, we would like to point out that we are currently not aware of other models that provide insights into the catalytic mechanism of the complex.

2. I have often thought the LOF vs GOF argument has been overplayed as the human data does not support LOF. Some comment on the fact that human data does not support LOF would be appropriate to include. There is no Notch phenotype, there is no evidence for APP CTF accumulation etc.....

We agree with the referee, but the problem is that no reports are available that explicitly investigated the absence of APP-CTF accumulation or the complete absence of Notch phenotypes in FAD patients. Thus, it is difficult to use these negative arguments as valid reasons in the discussion. Thus, we rather focused our discussion on the recently published reports showing „real" LOF of γ-secretase activity (Li et al; Liu et al; Pink et al; Wang et al), as judged by the skin problems related to Notch signaling. As these patients do apparently not develop AD, this is a strong argument against the LOF hypothesis as proposed by Dr. Shen and others

3. Detergent effects. Many point mutations alter the stability of a protein. So when PS/γ-secretase is solubilized by CHAPSO or other detergents it might artificially result in some LOF. Can the authors perform some experiments using non-solubilized members to exclude that the results are not being influenced by detergents. IF not this caveat should be included in the discussion.

The referee is correct in pointing out this potential problem. We have partially addressed this issue by confirming the data (when possible) in intact cells (see figure 2E, 4G-H and suppl.4) and in fact, the predictions we made for both PSEN and APP hold true, indicating that detergent solubilization does not affect necessarily intrinsic properties of the enzyme. However, we are aware that the catalytic efficiencies determined in this condition might be

underestimated if point mutations in PSENs impact indeed protein stability. Nevertheless, if this applies to some FAD mutations, the additional LOF, induced by detergent would not change our main conclusions. On the contrary, this would favor even more a scenario where LOF is not necessary for the FAD pathogenic mechanisms.

Apart from the *in vivo* experiments that were already included in the previous version of the manuscript, we now include a couple of sentences in the discussion section about this specific point, as we cannot exclude the effect of detergent on the overall outcome of the results.

4. I am a little uncomfortable with the term carboxypeptidase activity used in some instances. I'd accept the initial term carboxypeptidase like activity be sued throughout.

We agree and have changed this in the text accordingly

5. Statistics. The description of the statistics is uneven and its not clear that appropriate tests are being used. For multiple comparisons between multiple mutants and controls ANOVAS not two-tailed t-tests are needed. In some cases statistics are lacking. Table 1 which is perhaps the meet of the manuscript has no statistical evaluation. So how much of a difference is needed to reach

significance? Figure 3 is also problematic as the data do not reach statistical significance but are likely to be biologically meaningful if truly the rates are > 50% different. I think this is just variance and low n numbers but it really undermines the entire data package. Going back to Table 1 few of the differences are more then 2-fold....

We thank the referee to his/her comment. We have redone all statistics in the manuscript and we feel that the manuscript has indeed improved. We have used ANOVA and Dunnett's post test analysis for multiple comparisons. As you can see in the corrected figures, the new analysis changed the level of significance in some cases, but it did not affect any of our conclusions. With regard to Fig 3, it is true that the data do not reach statistical significance although the trends suggest that the rate is different. Thus, we now included in the text the statistical significance given by a 2-tailed t-test (P value) which reaches 0.3 and a caution note. Further work to dissect this particular aspect of our data will not affect any of the conclusions of the current manuscript. We want to give emphasis to the fact that the N141I *PSEN2* modifies the γ-secretase activity as the FAD *PSEN1* mutations do. Our results on the carboxypeptidase like activity clearly show that FAD *PSEN*s consistently impair the 4th turnover of the complex and all tests are fully significant. We have also done statistical analysis for Table1, which is explained in the corresponding legend.

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The referee is right in the sense that we have for the time being no proof that these in vitro observations on the behavior of Abeta38 are relevant for the in vivo situation. In fact, this point made us to include these results in the supplementary section. In the manuscript, we only intended to make clear that the “innocence” of abeta38 is not proven, and that some caution and further work is needed. We have now included the caveats and qualifiers requested by the referee in the discussion.

Referee #2

This is an excellent study aimed at dissecting the biochemical mechanisms by which PSEN and APP mutations cause dominant, early-onset Alzheimer's disease. The results show quite convincingly that the general effect of these mutations is to alter gamma-secretase processing of APP substrate. The experiments performed are technically very challenging and time-consuming. Despite these challenges, no "short-cuts" were taken, and the experiments were apparently carried out rigorously. Altogether, the results from this study provide a coherent picture of how various disease-causing mutations change Abeta production. Among the notable observations:

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All that being said, several suggestions are recommended below that would be critical for putting this study into proper context and perspective and clarifying some of the analyses and conclusions.

We thank the referee for his/her appreciation of our work and are happy to take his/her suggestions into consideration in the new version of this manuscript.

(1) The idea that this study is the first to describe qualitative (versus quantitative) changes in Abeta production is not accurate. The authors reference a now 10-year-old review article by Hardy and Selkoe to suggest that the amyloid hypothesis stresses quantitative changes in total Abeta or Abeta42. But the field has held for a number of years that the ratio of Abeta42/40 is the critical factor (e.g., Tanzi and Bertram, Cell, 2005). Proper references should be cited.

We have adapted the manuscript to include these and other references.

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We have adapted the manuscript to include these and additional references

(3) In describing the results in Fig. 1 and Table 1, it is said that "gamma-secretase activities are normalized to enzyme levels". Exactly how this normalization was carried out should be described (e.g., based on levels of PSEN NTF?).

As stated in the Figure 1 legend: "γ-Secretase activities were normalized to presenilin CTF fragment levels or full length PS1 levels for the DE9 mutant". We have now made that point clearer by including it in the methodology section.

(*4) How Abeta42 and Abeta43 were quantified for Fig. 2 is not clear, as the urea-based gel in panel C does not show resolution of these two peptides. Perhaps this panel can be expanded so that the readers can tell the degree of resolution.*

Abeta38, Abeta40 Abeta42 and Abeta43 were quantified by ELISAs (please see methodology). In figures 2B, 3B and 4D, "soluble" refers to the sum of these four Abeta species, whereas sAbeta refers to AbetaX quantified from the extracellular media of cells overexpressing APP substrate (Fig. 2E, 4G and 4H).

Urea-based gels were only used to have a qualitative overview of all Abeta products in the reactions, but not for quantitative purposes. Panel 2C has been expanded in the new version.

(5) It is stated that the PS2 N141 mutation does not significantly affect endopeptidase activity (the first cut of APP), but Fig. 3 suggests otherwise, as does the quantification: the mutant is said in the text to be 46.6% as efficient as the wild type PSEN2. Or is the implication that the errors make the results not statistically significant?

Please, see point 5 of referee 1.

Referee #3

This study deals with a central question in Alzheimer's disease (AD) research. Mutations in presenilin, the catalytic component of the protease gamma-secretase, cause familial forms of AD. However, their mechanism of action - although intensively investigated for many years - is still unsatisfactorily understood. The authors provide a comparative analysis of different presenilin mutations and carefully evaluate their effect on total gamma-secretase activity and on the individual cleavages of this multiply cleaving enzyme. The study is complemented by a similar analysis of APP mutations and of gamma-secretase modulators, which are a major drug class for therapeutic tests against AD. From the analyses using in vitro, in vivo and biophysical experiments the authors conclude that presenilin (PS) mutations do not simply mediate a loss-of-signaling of different substrates and thereby cause neurodegeneration - which had been one previous hypothesis. Instead,

the authors demonstrate that mutations in PS or APP affect gamma-cleavage in different ways, by reducing the final gamma-secretase cut or the "product line" used.

This is an interesting study of high technical quality, providing a comprehensive comparison of different mutations. It gives many beautiful insights, for example into the catalytic constants of gamma-secretase, the biophysical properties of Abeta38 and into the action of the gamma-secretase activators.

My major criticism, however, is that this study does not provide fundamentally new insights into the mechanism, by which the mutations or compounds affect the gamma-secretase cleavage. I am well aware that the final answer can most likely only be obtained by solving the structure of gamma-secretase, which still is a major task for the future. However, the principles emphasized here - reduction of 3rd/4th cleavage and a shift of product line - follow up on several previous studies in the recent past from different labs working on gamma-secretase or the related signal peptide peptidase family. Thus, I feel that the study would be better suited for a more specialized journal.

A crucial point in the evaluation of the relevance of this study is to differentiate what has been suggested from what has been demonstrated in the literature. Whereas several studies have indeed analyzed FAD mutations in many different ways and conditions, none of them have reported data that proves what consistent effects FAD mutations cause. Here, we started by translating commonly used terms like LOF/GOF into “biochemical terms” to analyze quantitatively the effects of these mutations on γ-secretase function and/or APP processing. Our results not only put definitive numbers but also connect our observations to catalytic mechanisms. Thus, we feel that even when using technologies and models introduced by Ihara and colleges, we have moved the analysis of γ-secretase function to a higher level by asking different questions and addressing them with scientific rigor. For instance, we are convinced that the identification of the “regulatory points” where FAD mutations act will help on the development of novel strategies for the modulation of Abeta profiles. We were very happy with the summary made by referee 2, highlighting the points of major interest of our study, and we believe that many colleagues will share the interest on our findings, as we could see while we were generating the data.

Our final aim is to complement the current study with crystals of the enzyme as this will finally allow to resolve how exactly the mutations affect structure and function, but even then, an accurate interpretation of crystal data will most likely need the incorporation of functional analysis (we provided here) and modeling.

Minor comment:

The Km and vmax values should be carefully checked. For example, for the deltaE9 mutation the values for Notch are surprising: at 2.25 microM the velocity has nearly reached the plateau at around 30. It is hard to imagine that vmax can still reach 54 as indicated in the table. If it still is, then Km should be larger. At the half-maximal velocity (27) the substrate concentration of this green line would be around 1.5 and not 0.65. This, however, would strongly alter the efficiency ratio in figure 1E.

We thank referee 3 for his/her accurate comment. In fact, the values displayed for DE9 in table1 were not correct. We have carefully checked Table1 in the new version of the manuscript.

Czirr E, Cottrell BA, Leuchtenberger S, Kukar T, Ladd TB, Esselmann H, Paul S, Schubenel R, Torpey JW, Pietrzik CU, Golde TE, Wiltfang J, Baumann K, Koo EH, Weggen S (2008) Independent generation of Abeta42 and Abeta38 peptide species by gamma-secretase. *J Biol Chem* **283**(25)**:** 17049-17054

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Qi-Takahara Y, Morishima-Kawashima M, Tanimura Y, Dolios G, Hirotani N, Horikoshi Y, Kametani F, Maeda M, Saido TC, Wang R, Ihara Y (2005) Longer forms of amyloid beta protein: implications for the mechanism of intramembrane cleavage by gamma-secretase. *J Neurosci* **25**(2)**:** 436-445.

Sato T, Tanimura Y, Hirotani N, Saido TC, Morishima-Kawashima M, Ihara Y (2005) Blocking the cleavage at midportion between gamma- and epsilon-sites remarkably suppresses the generation of amyloid beta-protein. *FEBS Lett* **579**(13)**:** 2907-2912.

Takami M, Nagashima Y, Sano Y, Ishihara S, Morishima-Kawashima M, Funamoto S, Ihara Y (2009) gamma-Secretase: successive tripeptide and tetrapeptide release from the transmembrane domain of beta-carboxyl terminal fragment. *J Neurosci* **29**(41)**:** 13042-13052

Wang B, Yang W, Wen W, Sun J, Su B, Liu B, Ma D, Lv D, Wen Y, Qu T, Chen M, Sun M, Shen Y, Zhang X Gamma-secretase gene mutations in familial acne inversa. *Science* **330**(6007)**:** 1065 Yagishita S, Morishima-Kawashima M, Ishiura S, Ihara Y (2008) Abeta46 is processed to Abeta40 and Abeta43, but not to Abeta42, in the low density membrane domains. *J Biol Chem* **283**(2)**:** 733-738

Yagishita S, Morishima-Kawashima M, Tanimura Y, Ishiura S, Ihara Y (2006) DAPT-induced intracellular accumulations of longer amyloid beta-proteins: further implications for the mechanism of intramembrane cleavage by gamma-secretase. *Biochemistry* **45**(12)**:** 3952-3960.

2nd Editorial Decision 28 February 2012

Thanks for submitting your revised manuscript to the EMBO Journal. Your revision has now been seen by referee #1. As you can see below, referee #1 appreciates the introduced changes. I am therefore very pleased to proceed with the acceptance of the paper for publication here. You will receive the formal acceptance letter shortly.

Thank you for submitting your interesting manuscript to the EMBO Journal!

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORT

Referee #1:

The authors have done an excellent job revising the manuscript in response to the reviewer's concerns.

