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APP substrate ectodomain defines amyloid-β peptide length by restraining γ-secretase processivity and facilitating product release

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1st Editorial Decision **1st Jun 2023**

Dear Lucia,

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.

As you can see from the comments, the referees appreciate the analysis and find it a good fit for The EMBO Journal. They raise a number of constructive comments that I would like to ask you to resolve in a revised version. Please also take into consideration referee #1's comments regarding readability. Let me know if we need to discuss anything further.

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: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

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

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
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IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
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We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (30th Aug 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

The paper shows that the strength of the interaction between the extracellular domain of C99 and gamma secretase determines its cleavage activity, which may trigger the toxic Aβ production that induces familial Alzheimer's disease, providing useful information for future drug discovery research. I think it's a well thought out paper. I enjoyed reading it. On the other hand, this reviewer was a bit involved in the field, and the authors tried to make it readable, so I was able to draw some analogies, but many readers may find it difficult to read. I would like to point out a few things to improve the readability. There may be some misunderstanding by this reviewer, but the data is quite complex, and they should be careful in their wording. There are some broad descriptions and inaccuracies scattered throughout the manuscript. In addition, there seems to be some subjective expressions and some discrepancies between claims and data, which I will point out below.
This may not be a problem for the authors, but the legends of the supplementary figures were not included in the merged file, and I had a trouble finding them, so I hope they will be included in the file together.

There is no statistical processing section in the method. Authors must specify what software was used and how the analysis was performed. This case is a rather potentially fatal problem with this paper.

Although the nature of the data makes this somewhat unavoidable, the graphs are made using various measurement standards, and it is difficult to grasp the consistency of the data. It is also confusing that the vertical axis setting of a similarly visible figure often changes. In particular, Figure 1D would be easier to relate to the next figure if it were described in terms of secreted Aβ measurements.

The statement in Figure S1B that the expression levels of the substrates are the same in wild-type and mutant is somewhat exaggerated. Their data seem to show some variation. If they just want to compare expression levels, they may need to find a way to do that. Their metabolism may change with the cleavage activity of GSECs, so why not use inhibitors, etc. for comparison? As a small detail, is it AICD at the bottom of the main band or is it some other metabolite? I think it is important to know if it is a metabolic pattern other than GSECs, since there is a band pattern that is not seen in the wild type.

I agree with what the author is trying to say in Figure 1D, and it seems difficult to set up a significant difference setting for this figure, but strictly speaking, it would be desirable to explain it in terms that show what has been statistically treated and what has not. Figure 1D is so detailed that it is difficult to know where to look, so the S26-A30, and A21-D23 that the author wants to focus on, could be surrounded by a dotted line, for example.

For Figures 1D and E, this reviewer recommends that the difference between the graphs be explained in the text, as there appears to be a discrepancy until it is understood that there is a difference between the percentage representation of the controls and the absolute comparison. Overall, the data in this paper are difficult to understand and should be explained in detail.

Figure S1D is not well explained by the legend, and I am not sure if what is shown in the upper and lower panels is the same and if there is a GSEC treatment. My understanding was that the amount of substrate was shown, but that is not very useful information since it can be adjusted during addition. if you measured AICD, I think you should present the AICD data.

The authors generalize in their description of Figure 2B that canonical Aβ is reduced, but I do not think this is the correct description, since some Aβ species are not reduced. It may be necessary to rethink a wording that allows the authors to distinguish whether they want to talk about each molecular species of canonical Aβ or the total amount of Aβ.

Shouldn't the principle of ELISA with 4G8 antibody used in Figure 2A be explained in the text? It may be difficult to read and understand this area without specialized knowledge.

While I almost agree with the authors' assertion in Figure 2F and elsewhere that multiple mutations synergistically increase short Aβ species, the reality appears to be a bit more complicated than described in the text, since Aβ20 and similar short Aβs that appear to be the end-product are not increased compared to D23V. A description of a more rational reason for focusing on the 20-29 species of Aβ might be helpful. Maybe it is also the limit of Aβ visible in 4G8 and shorter Aβ is not visible? Maybe that limitation should also be discussed in the text. For Figure 2F, if I apply the author's definition of short Aβ species, shouldn't they be connected by equals, rather than D23V+K28V It's a minor detail, but Figure 4B shows that the mutant is also affected by the elevated temperature and surfactant, so counteract may be an overstatement. If this data could be expressed in terms of differences in sensitivity of mutants to stress, etc., the author's argument would be easier to follow.

Is the decrease in total Aβ in the mutant in Figure 4D 'detrimental'? Also, the explanation in this figure is somewhat difficult to read, so why not be more specific instead of mutation effect, etc.?

I feel that it would be easier to compare Fig. 4F if you could describe the action of the C99 mutant with PSEN in the Naive state. It is painstaking to pick up the said data from Figure 1.

I did not understand the discussion of the interaction with PSEN1 loop 1 in the Figure 4F explanation. Isn't this somewhat over-speculation, and shouldn't we be looking at the entire molecular species, such as Aβ37, etc., rather than just talking about Aβ40?

I would like to ask a question because it is difficult for me to evaluate Figure S4D, but the differences seen in this data seem minor to the untrained eye, can you make sense of them? Maybe there is a citation or other reference that would be helpful. Is

the data such that statistical processing is not possible?
Figure 5 is great data, but can't you put the effect of Inhibitor X on the mutant as a control?
I am sorry to even mention this, but after reading this paper, it is difficult to understand what this figure is trying to show when looking at Figure 6C. I think the readability of this paper would be improved if you could create a graphical abstract where the conceptual part can be understood at a glance.

Minor point
Reading this paper, it seems to me that Notch and C99 are cleaved by a similar mechanism, I was wondering how GSM differentiates between the substrates?
82E1 is an antibody from IBL.
If the ELISA was purchased, can the authors fill in the code? Will the detection antibody be 6E10?
The gene transfer procedures and cell culture procedures should be described in detail.

Referee #2:

Koch and colleagues provide a major advance for our understanding of how the protease gamma-secretase cleaves its substrates. The study is novel and very timely as some pharma companies strive to bring a new generation of gamma-secretase modulators (GSMs) back into clinical trials for Alzheimer's disease. The study demonstrates that the hydrophobicity of the juxtamembrane domain (JMD) of a substrate determines how it is cleaved and processed by gamma-secretase. While previous studies already pointed to a role of the JMD, this current study is the first one to systematically address the role of the JMD. The study includes important control experiments (e.g. Notch in addition to APP) and applications (e.g. for understanding the mechanism of FAD mutations and the action of GSMs) and demonstrates how deleterious FAD mutations may be alleviated in a smart and targeted manner. The collection of numerous mutants and experiments finally leads to an attractive model explaining how gamma-secretase proceeds mechanistically in cleaving its substrates.

A few points need to be addressed for improving the manuscript.

Major points: none

Minor points:
1. Introduction and discussion are written for scientists with a very close scientific interest, but will be difficult to follow for scientists outside this field. Try to put both manuscript parts into the bigger context of intramembrane proteolysis and highlight better for non-specialists the major advance of the manuscript.
2. Clarify - or at least speculate - why some of the mutants increase total Abeta production. This finding is not explained by the model in figure 6, which focuses instead on the processivity.
3. Some previous studies which introduced mutations in the JMD of APP need to be cited and their work needs to be compared to the new findings in this manuscript (e.g. Pagnon de la Vega Sci Transl Med 2021; Tian Nature StructMolBiol 2010).

Referee #3:

In this paper, the authors focused on the extracellular region of APP as a factor affecting the cleavage mode of γ-secretase. They found that the hydrophobicity of the ECD sequence, including K28, regulates the binding of γ-secretase to its substrates, and that increasing hydrophobicity leads to the production of short Aβ. This study integrates structural biology as well as enzymology and molecular and cellular biology in its analysis. They revealed a new aspect of the enzymatic properties of γ-secretase.

The paper is worthy of acceptance if the following points are analyzed/modified.

1. Interpretation of the effect of the D23 mutant
In Figure 1, the authors find D23F and K28F as important mutants in the mutant screen. These mutants are central "mutant" to this paper. Interestingly, D23F resulted more processive cleavage of Aβ than that observed in K28F mutant. In Figure 2, D23 is also more influential when comparing valine substitutions. Furthermore, the effect of the D23V+K23V mutant is similar to that of D23V; one could argue that this counteracts the effect of K23V. Can these results really be explained solely by the "hydrophobicity of the extracellular region"? The reviewer was not sure whether effects of these residues are similar. In particular, the LVFFAED, including D23, is a region where a major structural change occurs when Aβ becomes amyloid. The LVFFAED also contains several mutations associated with familial Alzheimer's disease. Considering that the Uppsala mutation in particular is a large deletion in this region, authors should discuss carefully about the pathological role of D23 and K23. From this point of view, this paper mainly focused on the analysis of K28. Thus, the term "substrate ECD" should be used more accurately throughout the paper, including the title. For example, juxtamembrane region in ECD would be more appropriate.

2. The results of the D23 mutant
In Figures 4F and G, the results of D23F with ins113T should also be shown and discussed.

3. Compatibility of D23 and K28 effects in Notch
The results of Notch cleavage in Figure 3 indicate the importance of K28; given the charges of H and K residues and the characteristics of D residue, H17D mutation should be analyzed in this experiment for the general argument of "hydrophobicity of the ECD".

4. The effect of GSM
The authors have been found that GSM is involved in the stability of γ-secretase-substrate binding. In this regard, it is important to know whether or not the effect on the substrate side can be generalized as a mechanism of increased processivity. A paper from the same group reported on the binding mode of GSM and showed that the V236W mutation has a similar effect to that of GSM (Petit et al., EMBO J 2022). It would be interesting to clarify whether this V236W effect is due to the increased substrate binding related to the ECD hydrophobicity.

VIB-KU LEUVEN CENTER FOR BRAIN & DISEASE RESEARCH

Date
September 1st, 2023

KU LEUVEN

Dear Dr. Karin Dumstrei,

We are pleased to submit the revised version of our original manuscript entitled "*APP substrate ectodomain defines Aβ length by restraining γ-secretase processivity and facilitating product release*" for consideration at the EMBO Journal.

We have made appropriate changes in the manuscript following the critic and suggestions of the reviewers, and the publisher (as suggested in the EMBO author guidelines). In addition, we created a synopsis and graphical abstract, both files are accompanying the revised manuscript.

Finally, we addressed point-by-point the comments of the reviewers in the accompanying 'Response to Reviewers' letter.

Thank you for your consideration.

Yours sincerely,

Lucía Chávez Gutiérrez on behalf of all other co-authors.

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We are thankful to the reviewers for their valuable feedback. Their questions and comments helped us to improve this report. Here, we address point-by-point their comments:

Referee#1:

The paper shows that the strength of the interaction between the extracellular domain of C99 and gamma secretase determines its cleavage activity, which may trigger the toxic Aβ production that induces familial Alzheimer's disease, providing useful information for future drug discovery research. I think it's a well thought out paper. I enjoyed reading it. On the other hand, this reviewer was a bit involved in the field, and the authors tried to make it readable, so I was able to draw some analogies, but many readers may find it difficult to read.

1. I would like to point out a few things to improve the readability. There may be some misunderstanding by this reviewer, but the data is quite complex, and they should be careful in their wording. There are some broad descriptions and inaccuracies scattered throughout the manuscript. In addition, there seems to be some subjective expressions and some discrepancies between claims and data, which I will point out below.

Thank you for your feedback, we have revised extensively our figures, figure legends and manuscript text to present and discuss the data as clear as possible. We also added data (see points below) to better support the key concepts of our study.

This may not be a problem for the authors, but the legends of the supplementary figures were not included in the merged file, and I had a trouble finding them, so I hope they will be included in the file together.

We apologise for the omission, the supplementary data and figures are included in the revised manuscript.

2. There is no statistical processing section in the method. Authors must specify what software was used and how the analysis was performed. This case is a rather potentially fatal problem with this paper.

Thank you for noticing this, we have added a section on statistical analysis to the revised manuscript.

3. Although the nature of the data makes this somewhat unavoidable, the graphs are made using various measurement standards, and it is difficult to grasp the consistency of the data. It is also confusing that the vertical axis setting of a similarly visible figure often changes. In particular, Figure 1D would be easier to relate to the next figure if it were described in terms of secreted Aβ measurements.

We revised our graphs for consistency and clarity. Regarding the analysis of Aβ products, we used Aβ profiles and/or ratios (e.g. Figures 1D, 1E) to visualize and quantify changes in GSEC processivity because they are independent of substrate concentration and overall endopeptidase activity. Profiles/ratio values thus inform about enzyme processivity and can be interpreted independent of total secreted Aβ. Nevertheless, the measured total secreted Aβ levels are presented as supplementary data.

4. The statement in Figure S1B that the expression levels of the substrates are the same in wild-type and mutant is somewhat exaggerated. Their data seem to show some variation. If they just want to compare expression levels, they may need to find a way to do that. Their metabolism may change with the cleavage activity of GSECs, so why not use inhibitors, etc. for comparison? As a small detail, is it AICD at the bottom of the main band or is it some other metabolite? I think it is important to know if it is a metabolic pattern other than GSECs, since there is a band pattern that is not seen in the wild type.

Thank you for this comment, it enables to clarify an important point. We acknowledge that substrate expression levels show some fluctuations and therefore we now refer to them as 'robust'. The important point here is that substrate expression levels are not a limiting factor for Aβ production. The fact that WT/mutant substrate expression levels are robust, though not exaltedly the same, make this point clear. For instance, Figures 1 and EV1 show that drastic decreases in Aβ levels caused by some mutants, such as D23F or K28F, are not due to low substrate levels.

Related to the bands detected by western blotting with the anti-Flag ab: we expressed the direct substrate of GSEC (APP_{C99}-3XFLAG) in our cell assays; however APP_{C99} may be processed by the alpha-secretase to generate lower

molecular weight substrates (e.g. APP_{C83}). These shorter APP CTF fragments are detected as lower bands. AICD migrates at an even lower molecular weight (see EV1D). Although these APP substrates are likely processed by GSEC, by measuring only Aβ peptides (not p3 peptides) we follow exclusively the processing of the overexpressed WT/mutant APP_{C99} substrates. For clarity, we added molecular weight markers to the figures EV1B and EV1D, these figures show the different molecular weights between APP substrates and products (AICD).

5. I agree with what the author is trying to say in Figure 1D, and it seems difficult to set up a significant difference setting for this figure, but strictly speaking, it would be desirable to explain it in terms that show what has been statistically treated and what has not. Figure 1D is so detailed that it is difficult to know where to look, so the S26-A30, and A21-D23 that the author wants to focus on, could be surrounded by a dotted line, for example.

Thank you for this comment. We have enlarged Figure 1D and underlined the key regions in the substrate to improve data presentation and clarity. In addition, statistical analysis of WT/mutant peptides is presented in Figure EV1C.

6. For Figures 1D and E, this reviewer recommends that the difference between the graphs be explained in the text, as there appears to be a discrepancy until it is understood that there is a difference between the percentage representation of the controls and the absolute comparison. Overall, the data in this paper are difficult to understand and should be explained in detail.

We revised the manuscript for clarity and hope that these aspects are now clear.

7. Figure S1D is not well explained by the legend, and I am not sure if what is shown in the upper and lower panels is the same and if there is a GSEC treatment. My understanding was that the amount of substrate was shown, but that is not very useful information since it can be adjusted during addition. if you measured AICD, I think you should present the AICD data.

We apologise for the confusion, we have revised the figure and corresponding legend to improve clarity.

8. The authors generalize in their description of Figure 2B that canonical Aβ is reduced, but I do not think this is the correct description, since some Aβ species are not reduced. It may be necessary to rethink a wording that allows the authors to distinguish whether they want to talk about each molecular species of canonical Aβ or the total amount of Aβ.

This is an important point that we have clarified in the revised manuscript.
Briefly, we show that all tested mutations, excepting the K28R, significantly lower the generation of canonical peptides, as determined by the summation of these peptides (Fig. EV2B). However, none of these mutations decrease total secreted Aβ levels (Figure 2A), pointing to a shift in profiles. Interestingly, the Aβ total / Aβ (37+38+40+42) ratio showed a linear correlation with the hydrophobicity of the aa substitution (Figure 2C), indicating that this is a relevant feature for processivity. In the revised version we discuss the overall shift in profiles as the key observation, and tried not to go in detailed discussion of specific canonical peptides as this makes the interpretation (unnecessarily) complex.

9. Shouldn't the principle of ELISA with 4G8 antibody used in Figure 2A be explained in the text? It may be difficult to read and understand this area without specialized knowledge.

We have added a brief explanation in the text and in figure legends., thank you for the suggestion.

10. While I almost agree with the authors' assertion in Figure 2F and elsewhere that multiple mutations synergistically increase short Aβ species, the reality appears to be a bit more complicated than described in the text, since Aβ20 and similar short Aβs that appear to be the end-product are not increased compared to D23V. A description of a more rational reason for focusing on the 20-29 species of Aβ might be helpful. Maybe it is also the limit of Aβ visible in 4G8 and shorter Aβ is not visible? Maybe that limitation should also be discussed in the text. For Figure 2F, if I apply the author's definition of short Aβ species, shouldn't they be connected by equals, rather than D23V+K28V <G25V-K28V (4xV)? The data tends to be interpreted with subjective and changing definitions based somewhat on the author's assertions, and an objective description seems necessary.

Thank you for raising this point. We used the 4G8 or 6E10 antibodies in the IPs of Aβ peptides from CM, prior MS-based detection. Indeed, a limitation in these analyses is that the antibodies may not be able to efficiency recognize Aβ species shorter than Aβ20 since the integrity of their epitopes could be compromised by the processing. We discuss this limitation in the revised manuscript and also added that the MS-based profiles represent estimates of relative proportions.
In the evaluation of mutation-driven changes in Aβ production emphasis should be put not only on the shorter peptides, but also on the overall peptide profile. The D23V mutation generates very short N-terminal fragments as well as ~10% of Aβ38 and Aβ40 peptides. These relatively 'longer' peptides are not generated from the double D23V-K28V mutant substrate, implying a more efficient processing of the double relative to the single mutant substrate.
Please see point 1 in the comments raised by reviewer 3.

11. It's a minor detail, but Figure 4B shows that the mutant is also affected by the elevated temperature and surfactant, so counteract may be an overstatement. If this data could be expressed in terms of differences in sensitivity of mutants to stress, etc., the author's argument would be easier to follow.

We adapted the wording and clarified in the text that the mutations enhance the generation of shorter Aβ species in comparison to the WT.

12. Is the decrease in total Aβ in the mutant in Figure 4D 'detrimental'? Also, the explanation in this figure is somewhat difficult to read, so why not be more specific instead of mutation effect, etc.?

An increasing body of evidence suggest that elevated APPCTF levels contribute to neurodegeneration in Alzheimer's disease (Lauritzen *et al*, 2021). Pathogenic mutations in APP or PSEN1 may impair the global processing of APP, lead to increased APPCTF levels and potentially contribute to endosomal dysfunction, as shown in (Kwart *et al*, 2019). Impairments in the global GSEC processing of APP can thus be considered as detrimental.
Our studies are focussed on GSEC processivity and we mostly discuss the implications of the generated data for the sequential mechanism. However, we have added in the discussion a short paragraph on this specific point.

13. I feel that it would be easier to compare Fig. 4F if you could describe the action of the C99 mutant with PSEN in the Naive state. It is painstaking to pick up the said data from Figure 1.

Figure 4F presents the effects of WT or mutant APP substrates on Aβ processing by different FAD-linked PSEN1/GSEC complexes. For clarity reasons, we did not add all the conditions in WT cells, which are described in Figure 1D. We believe that the addition of these data to the figure makes it very complex.

14. I did not understand the discussion of the interaction with PSEN1 loop 1 in the Figure 4F explanation. Isn't this somewhat over-speculation, and shouldn't we be looking at the entire molecular species, such as Aβ37, etc., rather than just talking about Aβ40?

We performed further analyses on the intron 4 variant (data presented in Figure 4F-G and EV4E-F) and adapted this part of the results accordingly. We understand the reviewer's comment and removed the speculation on the effects of the PSEN loop 1 mutation in the revised manuscript.

15. I would like to ask a question because it is difficult for me to evaluate Figure S4D, but the differences seen in this data seem minor to the untrained eye, can you make sense of them? Maybe there is a citation or other reference that would be helpful. Is the data such that statistical processing is not possible?

The changes observed for the WT condition vs K28F (≥ 10 kcal/mol) would be considered a clear effect in these type of simulations. However, we understand that it is difficult to understand the weight of the changes and added p-values obtained by ANOVA.

16. Figure 5 is great data, but can't you put the effect of Inhibitor X on the mutant as a control?

Thank you for the suggestion. We assessed the effect of InhX treatment on the processing of WT and mutant substrates, the results are presented in Figure EV5D. In contrast to DAPT or semagacestat, the substitutions in APP ECD did not shift the inhibitory profiles of InhX.

17. I am sorry to even mention this, but after reading this paper, it is difficult to understand what this figure is trying to show when looking at Figure 6C. I think the readability of this paper would be improved if you could create a graphical abstract where the conceptual part can be understood at a glance.

We have created a graphical abstract and modified Figure 6C and corresponding legend. We hope that the implemented changes help the reader to understand the key concepts of this study.

Minor points:

- Reading this paper, it seems to me that Notch and C99 are cleaved by a similar mechanism, I was wondering how GSM differentiates between the substrates?

Available data support the notion that second generation GSMs modulate the processing of different substrates (Wanngren *et al*, 2012).However, it remains unclear if (and how) different GSEC substrates are modulated by these drugs (reviewed in (Luo & Li, 2022)). We find this topic very relevant and fascinating; however, we do not have data to address it. We also believe that it goes beyond the aims of this study.

- 82E1 is an antibody from IBL.

We purchased this antibody from Tecan, which acquired IBL. Reference numbers and information on reagents are listed in Materials and Methods.

- If the ELISA was purchased, can the authors fill in the code? Will the detection antibody be 6E10?

In the method section we specify the detection antibody as either JRF/AbN/25 or 6E10. The JRF/AbN/25 antibody was obtained through a collaboration with Janssen Pharmaceutica NV (Beerse, Belgium). The 4G8 ELISA is a custom MSD coated plate, the reference number is provided in the methods section of the revised manuscript.

- The gene transfer procedures and cell culture procedures should be described in detail.

We revised methods and added detailed information about these protocols.

Referee #2:

Koch and colleagues provide a major advance for our understanding of how the protease gamma-secretase cleaves its substrates. The study is novel and very timely as some pharma companies strive to bring a new generation of gamma-secretase modulators (GSMs) back into clinical trials for Alzheimer's disease. The study demonstrates that the hydrophobicity of the juxtamembrane domain (JMD) of a substrate determines how it is cleaved and processed by gamma-secretase. While previous studies already pointed to a role of the JMD, this current study is the first one to systematically address the role of the JMD. The study includes important control experiments (e.g. Notch in addition to APP) and applications (e.g. for understanding the mechanism of FAD mutations and the action of GSMs) and demonstrates how deleterious FAD mutations may be alleviated in a smart and targeted manner. The collection of numerous mutants and experiments finally leads to an attractive model explaining how gamma-secretase proceeds mechanistically in cleaving its substrates.

A few points need to be addressed for improving the manuscript.

Major points: none

Minor points:

- Introduction and discussion are written for scientists with a very close scientific interest, but will be difficult to follow for scientists outside this field. Try to put both manuscript parts into the bigger context of intramembrane proteolysis and highlight better for non-specialists the major advance of the manuscript.

As mentioned above, we have revised the manuscript for clarity. In addition, we highlighted in the discussion the novel concepts and model for GSEC processivity that our study presents.

⎫ Clarify - or at least speculate - why some of the mutants increase total Abeta production. This finding is not explained by the model in figure 6, which focuses instead on the processivity.

Our observations point to a link between the ectodomain of the substrate and the global endopeptidase activity. These findings are intriguing but preliminary; therefore, we prefer to be cautions with the interpretation. We note that total secreted Aβ levels, assessed here, are a proxy of the global activity levels, and that the analysis of the AICD levels (direct endopeptidase product) would be required to draw conclusions. In the revised manuscript, we do mention the intriguing modulation of global activity levels by the APP substrate ECD, but indicate that further research is needed to understand it.

⎫ Some previous studies which introduced mutations in the JMD of APP need to be cited and their work needs to be compared to the new findings in this manuscript (e.g. Pagnon de la Vega Sci Transl Med 2021; Tian Nature StructMolBiol 2010).

Thank you for the suggestion. We refer to and discuss these studies in the revised manuscript.

Referee #3:

In this paper, the authors focused on the extracellular region of APP as a factor affecting the cleavage mode of γ-secretase. They found that the hydrophobicity of the ECD sequence, including K28, regulates the binding of γ-secretase to its substrates, and that increasing hydrophobicity leads to the production of short Aβ. This study integrates structural biology as well as enzymology and molecular and cellular biology in its analysis. They revealed a new aspect of the enzymatic properties of γ-secretase.
The paper is worthy of acceptance if the following points are analyzed/modified.

1. Interpretation of the effect of the D23 mutant.
In Figure 1, the authors find D23F and K28F as important mutants in the mutant screen. These mutants are central "mutant" to this paper. Interestingly, D23F resulted more processive cleavage of Aβ than that observed in K28F mutant. In Figure 2, D23 is also more influential when comparing valine substitutions. Furthermore, the effect of the D23V+K23V mutant is similar to that of D23V; one could argue that this counteracts the effect of K23V. Can these results really be explained solely by the "hydrophobicity of the extracellular region"?

Thank you for your critic. We propose that Lys28 - APP_{C99} creates an energy barrier that disfavours further processing of the Aβ40 (~12 aa downstream) and Aβ38/42 peptides (10aa/14 aa). In addition to Lys28, other polar/charged extracellular residues contribute to this barrier and thereby collectively restrict processivity.
When the first barrier (Lys28) is overcome –likely a stochastic event – substrate threading continues and fuels further proteolysis, until the next energy barrier is reached (Glu22-Asp23).
The generation of relatively longer peptides (Aβ40/38) from the D23V substrate is likely due to the presence of Lys28 (first barrier). However, the D23V mutation not only lowers the initial barrier (thus facilitates further substrate threading), but also directly affects the 2^{nd} barrier (contributed by Glu22-Asp23 and potentially other polar/charged residues). The latter promotes even further threading, relative to the WT or K28V.
The K28V mutation only lowers the first barrier. The second barrier, still present, restricts further substrate threading and proteolysis, relative to the K28V+D23V or the single D23V. Therefore, single hydrophobic substitutions at position 28 do not promote generation of very short peptides (<Aβ28).
When K16 (likely the third barrier) is mutated together with K28 and D23 (K16V+D23V+K28V, newly added data to Figure 2F) even shorter peptides are generated, relative to the D23V+K28V.
These data support that extracellular residues contribute to the modulation of the sequential mechanism.
Please also see point 10 in the comments by reviewer 1.

The reviewer was not sure whether effects of these residues are similar. In particular, the LVFFAED, including D23, is a region where a major structural change occurs when Aβ becomes amyloid. The LVFFAED also contains several mutations associated with familial Alzheimer's disease. Considering that the Uppsala mutation in particular is a large deletion in this region, authors should discuss carefully about the pathological role of D23 and K23. From this point of view, this paper mainly focused on the analysis of K28. Thus, the term "substrate ECD" should be used more accurately throughout the paper, including the title. For example, juxtamembrane region in ECD would be more appropriate.

Thank you for your comment. In the revised manuscript we elaborated more on the roles and potential interaction of D23 and K28, as well as discuss FAD-linked APP data relevant to this region.

2. The results of the D23 mutant.
In Figures 4F and G, the results of D23F with ins113T should also be shown and discussed.

The requested experiments were performed and are shown in Figure 4F-G. The results are in line with those generated from the analysis of other FAD-linked variants.

3. Compatibility of D23 and K28 effects in Notch.
The results of Notch cleavage in Figure 3 indicate the importance of K28; given the charges of H and K residues and the characteristics of D residue, H17D mutation should be analyzed in this experiment for the general argument of "hydrophobicity of the ECD".

As suggested by the reviewer, we analysed the H17D mutation and also included the H17Q. The results are presented in Figure 3 (see panel D and E) and discussed in the revised manuscript. They support the model presented by our study.

4. The effect of GSM.
The authors have been found that GSM is involved in the stability of γ-secretase-substrate binding. In this regard, it is important to know whether or not the effect on the substrate side can be generalized as a mechanism of increased processivity. A paper from the same group reported on the binding mode of GSM and showed that the V236W mutation has a similar effect to that of GSM (Petit et al., EMBO J 2022). It would be interesting to clarify whether this V236W effect is due to the increased substrate binding related to the ECD hydrophobicity.

In (Petit *et al*, 2022) we show that the V236W mutation increases GSEC-substrate complex stability by filling a (sub)pocket in PSEN1. The filling of this sub-pocket (by mutation or GSM) could displace water molecules from the critical, juxtamembrane E-S region; thus, increasing hydrophobicity and lowering the solvation penalty for burying polar residues in the substrate's ECD in the membrane. We speculate on this specific point in the revised discussion.

Dear Lucia,

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by referees #1 and 3. As you can see below, they are both very supportive of the revised version. Given this I am very pleased to let you know that we can accept the manuscript for publication here. Before sending you, the formal accept letter there are just a few editorial points that need to be resolved:

- The Funding info FWO - 1S23819N is missing in the MS file.

- Please remove the Authors Contributions from the manuscript. The 'Author Contributions' section is replaced by the CRediT contributor roles taxonomy to specify the contributions of each author in the journal submission system. Please use the free text box in the 'author information' section of the manuscript submisssion system to provide more detailed descriptions (e.g., 'X provided intracellular Ca++ measurements in fig Y')

- The call out is missing for Appendix Table S6

- the synopsis image is too large it should be 550 wide by [200-400]

- Figure legends should be moved after References

I have also run the revision by our publisher and they have the following comments:

- The figure legend style does not comply with the journal guidelines; the legends are provided in a 'Run-on' style.

- A separate 'Data Information' section is required for figure 2.

- Please note that in figures 2d; EV2c comparison for "*/**/***" has not been represented in the figure panel.

- Please define the annotated p values **** in the legend of figure 1e.

- Please indicate the statistical test used for data analysis in the legend of figure 1e.

- The error bars are not defined in the legends of figures 1e; 2e; 4d, e, g; 5c; EV2e.

- The information related to n is missing in the legend of figures 1e; 4d, e, g; 5c; EV2e.

- Although 'n' is provided, please describe the nature of entity for 'n' in the legend of figure 6b.

That should be all. Congratulations on a nice study!

Let me know if you have any further questions.

Best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

Use the link below to submit your revision:

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Referee #1:

I have reviewed the resubmitted version.
I think the authors did a good job of addressing the reviewers' questions in the first edition. I felt that the discussion points were well organized. I think it is a very excellent result and paper.

I do not have any suggestions for additional revisions on my part.

Referee #3:

The authors fulfilled our requests.

All editorial and formatting issues were resolved by the authors.

2nd Revision - Editorial Decision **25th Sep 2023**

Dear Lucia,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now looked at everything and all looks good!

I am therefore very pleased to accept the manuscript for publication here

Best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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The data shown in figures should satisfy the following conditions:
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- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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Each figure caption should contain the following information, for each panel where they are relevant:
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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Mutagenic primers listed in Appendix Table S6

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