

## Extracellular interface between APP and Nicastrin regulates A $\beta$ length and response to $\gamma$ -secretase modulators

Dieter Petit, Manuel Hitzenberger, Sam Lismont, Katarzyna Marta Zoltowska, Natalie S. Ryan, Marc Mercken, François Bischoff, Martin Zacharias, Lucía Chávez-Gutiérrez

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### Review timeline:

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Revision received:	4th Mar 2019
Editorial Decision:	25th Mar 2019
Revision received:	2nd Apr 2019
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Editor: Karin Dumstrei

### Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

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1st Editorial Decision

7th Feb 2019

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

As you can see both referees find the analysis well done and interesting. Referee #1 mentions the limitation that you haven't looked at Abeta species longer than Abeta42. Having looked at the comments and the manuscript I do find the study important and would like to ask you address the raised concerns. If you have looked at Abeta species longer than Abeta42 then please include such analysis, if not then no need to carry out such studies. The concerns raised by referee #2 should be straight-forward to address.

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### REFeree REPORTS:

Referee #1:

Petit and colleagues show the role of Nicastrin in the regulation of gamma secretase (GSEC) activity by introducing a number of mutations. The authors find that the extracellular domain of Nicastrin affects the production of Abeta species with different lengths, i.e. with different degrees of pathogenicity, via changing stability of the GSEC/APP-Abeta complex.

The experiments are well designed, and the results are clear and convincing. The only weakness which we see in the manuscript is that the authors have not analyzed and quantified the Abeta species longer than Abeta42 because such experimental results would provide essential mechanistic information that is schematized in Figure 1A.

Without such data, it seems to us that the manuscript is more suitable for publication in EMBO Molecular Medicine rather than EMBO J.

Referee #2:

This paper takes a rigorous and detailed structure/function approach to addressing the mechanisms that control the stability of the complex formed between gamma secretase and its substrate. The overall conclusion is that a specific residue in nicastrin (I241/2) comes into close proximity to the substrate, and that this interaction controls both stability of the complex and thereby, processivity of the enzyme. The link between complex stability and processivity was the topic of an important earlier paper by the same group.

This is significant and interesting work about a topic of broad interest. The overall impact of the message is slightly diminished by the recent publication of a structure of gamma secretase with its Notch substrate, which also identified the proximity of that residue of nicastrin to the substrate. But the current paper not only confirms the structural conclusion - more importantly, it provides a rigorous and broadly convincing functional analysis. I therefore do not consider this paper to be badly 'damaged' by the earlier structural publication.

In general the data are convincing. Some of the effects are subtle, but they are mostly carefully controlled and statistically validated. Moreover, the key ideas are tested in several ways and this adds up to conclusions that I find compelling.

There are some issues that need to be addressed.

The whole paper is a bit wordy and repetitive. Some of the results and ideas are quite complex, so it is important that the story is told as clearly as possible. One problem is that the story does not always flow well. Results are anticipated in the text and there are several repetitive passages. Some basic editing and trimming would make a difference, including thinking hard about the narrative flow.

Figure 1A would be improved by indicating the topology of the substrate in the membrane with respect to the enzyme. For the reader to understand the paper at all, they must have a really clear mental picture of how the complex looks.

The description of the in vitro attempts to show that complex stability correlates with processivity (Fig 2B) is confusing and not really needed. The data were unclear and superseded by the next approach of using in vivo thermal stability, where the data are clear.

In Fig 3B, the effect of I241E is obvious, but I don't see that the data supports a statistically significant effect for N242E.

The inverted salt bridge experiment is elegant and the result convincing.

I found the whole section on GSMs to be confusing and unnecessarily long and rambling. The overall conclusions are a little clearer when repeated in the discussion, but the results section needs work. I'd suggest that shortening and tightening would make it easier to follow. In parallel, the GSM data in Fig 6 are hard to follow and can probably be presented more effectively.

The discussion is also unduly long and repetitive. I think there is way too much simple reiteration of the results. It is nearly half way through before there is any substantial 'discussion' of the data.

A general concern, which they do raise, is why there have been no human mutations identified in the relevant nicastrin residues. They point out that the majority of FAD cases do not have mutations in presenilin or APP, but has nicastrin been properly screened? If the answer is simply that there may be nicastrin mutants and they have not been properly looked for, then fine. But if we know that nicastrin is never mutated in FAD, isn't that a substantial problem for their hypothesis? This could do with slightly more discussion.

**Referee #1**

*The experiments are well designed, and the results are clear and convincing. The only weakness which we see in the manuscript is that the authors have not analyzed and quantified the A $\beta$  species longer than A $\beta$ 42 because such experimental results would provide essential mechanistic information that is schematized in Figure 1A.*

We agree with the reviewer that the analysis of longer amyloid  $\beta$  (A $\beta$ ) species is of a significant importance, especially when analysing the effects of strongly destabilizing AD-linked mutations on GSEC function. Such analysis has not been included in the initial version of the manuscript since we have recently demonstrated that A $\beta$ 43 is produced at low levels, relative to A $\beta$ 38, A $\beta$ 40 and A $\beta$ 42 peptides, in wild-type or mild destabilizing FAD-linked conditions (Veugelen *et al*, 2016) that are similar to the degree of destabilization observed here for mutant NCTs. In these conditions, A $\beta$  (38+40)/42 ratio reliably reflects the efficiency of the 4<sup>th</sup> GSEC catalytic turnover and overall GSEC processivity of APP. Nevertheless, following the referee's suggestion, we have now quantified the A $\beta$ 43 peptide levels in conditioned medium collected from wild-type or respective mutant NCT GSECs expressed in mouse embryonic fibroblasts (cell-based thermoactivity assays at 42°C presented in Figure 3B). This analysis has been included in the revised version of the manuscript (Figure S2).

**Referee #2**

*This is significant and interesting work about a topic of broad interest. The overall impact of the message is slightly diminished by the recent publication of a structure of gamma secretase with its Notch substrate, which also identified the proximity of that residue of nicastrin to the substrate. But the current paper not only confirms the structural conclusion - more importantly, it provides a rigorous and broadly convincing functional analysis. I therefore do not consider this paper to be badly 'damaged' by the earlier structural publication.*

We are grateful to the reviewer for noting that our manuscript provides a rigorous and broadly convincing functional analysis of the mechanisms governing GSEC-mediated proteolysis. We, however, disagree that the overall impact of the message is diminished by the recent publication of the structure of GSEC with the Notch substrate. The recent GSEC-Substrate co-structures show the here tested NCT region in proximity to the substrates, but does not identify this interaction as functionally relevant, which is highlighted and mechanistically explained by our study. In our opinion the structural data strengthen our conclusions and the two (complementary) approaches provide novel, functional insights into the role of the extracellular NCT-APP interface in the regulation of GSEC proteolytic mechanisms.

*The whole paper is a bit wordy and repetitive. Some of the results and ideas are quite complex, so it is important that the story is told as clearly as possible. One problem is that the story does not always flow well. Results are anticipated in the text and there are several repetitive passages. Some basic editing and trimming would make a difference, including thinking hard about the narrative flow.*

We agree with the referee that the story is complex, and thus it is of a great importance to tell it as clearly as possible. Therefore, we have carefully revised the manuscript while taking into account all the suggestions and shortened the manuscript to improve the narrative flow.

*Figure 1A would be improved by indicating the topology of the substrate in the membrane with respect to the enzyme. For the reader to understand the paper at all, they must have a really clear mental picture of how the complex looks.*

Following the reviewer's suggestion, the Figure 1A has been modified and the orientation of the enzyme and the substrate relative to the membrane has been indicated. In addition, the N- and C-termini of the substrate have been marked.

*The description of the in vitro attempts to show that complex stability correlates with processivity (Fig 2B) is confusing and not really needed. The data were unclear and superseded by the next approach of using in vivo thermal stability, where the data are clear.*

We believe that the data obtained in the cell-free system provide insights into the influence of the detergent on the stability of the membrane-embedded E-S complexes. However, we understand the point of the reviewer, that cell-based data is clear and entirely support the conclusions. We also acknowledge that the results in detergent add an additional level of complexity to the manuscript, and thus following the reviewer's suggestion, have removed them from the revised version.

*In Fig 3B, the effect of I241E is obvious, but I don't see that the data supports a statistically significant effect for N242E.*

The reviewer is correct that there was no statistical analysis on the graph for the NCT- N242E GSEC mutant. The significance of the NCT- N242E substitution relates to the 'rescuing' effect that this mutant exerts on the thermal-induced decrement observed for the wild-type GSEC at 42°C, relative to 37°C (A $\beta$ (38+40)/A $\beta$ 42 ratios in Figure 3). In the revised manuscript, we have improved the presentation of the data by adding the statistical analysis requested by the reviewer:

The lack of the detrimental influence of the temperature on the stability of the NCT N242E-Substrate complex evidences the stabilizing effect of the N242E amino acid substitution. Accordingly, we measure a statistically significant difference between the A $\beta$ (38+40)/A $\beta$ 42 ratios generated by wild-type and mutant NCT-N242E GSECs at 42°C ( $p=0,0174$ , one-way ANOVA followed by Dunnett's multiple comparison test).

*I found the whole section on GSMs to be confusing and unnecessarily long and rambling. The overall conclusions are a little clearer when repeated in the discussion, but the results section needs work. I'd suggest that shortening and tightening would make it easier to follow. In parallel, the GSM data in Fig 6 are hard to follow and can probably be presented more effectively.*

The effect of the GSEC modulators (GSMs) on the generation of various A $\beta$  species by respective wild-type and NCT mutant GSECs is complex. We have now carefully revised the respective fragment of the manuscript and improved the data presentation in the Figure 6 and the complementary Figure S4.

*The discussion is also unduly long and repetitive. I think there is way too much simple reiteration of the results. It is nearly half way through before there is any substantial 'discussion' of the data.*

We acknowledge the referee's suggestions to edit the discussion by reducing the reiteration of the results and avoiding repetition. All these comments have been taken into account and we have modified the discussion accordingly.

*A general concern, which they do raise, is why there have been no human mutations identified in the relevant nicastrin residues. They point out that the majority of FAD cases do not have mutations in presenilin or APP, but has nicastrin been properly screened? If the answer is simply that there may be nicastrin mutants and they have not been properly looked for, then fine. But if we know that nicastrin is never mutated in FAD, isn't that a substantial problem for their hypothesis? This could do with slightly more discussion.*

To the best of our knowledge, no familial Alzheimer's disease (FAD)-causing mutations have been found in the *NCSTN* gene. However, this does not exclude the possibility of the existence of such genetic changes because FAD patients are not screened for mutations in this gene. We believe that pathogenic mutations in *NCSTN* could have been missed, especially in the light of the fact that their existence could be very rare. We discuss this point more amply in the revised version of the manuscript.

2nd Editorial Decision

25th Mar 19

Thanks for submitting your revised manuscript to The EMBO Journal. Your revision has now been re-reviewed by referee #2 and as you can see from the comments below, the referee appreciates the introduced changes.

There are just some final things to sort out:

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REFEREE REPORT:

Referee #2:

The authors have made a good effort to reply to the reviews.

2nd Revision - authors' response

2nd Apr 2019

*The authors performed all requested editorial changes.*

**YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓**

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding author name: Dr. Lucía Chávez-Gutiérrez

Journal Submitted to: The EMBO Journal

Manuscript Number: EMBOJ-2019-101494

### Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

#### A- Figures

##### 1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if  $n < 5$ , the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

##### 2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple  $\chi^2$  tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

#### B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	In all analyses the data of at least 3 independent experiments was used to perform statistics.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	In order to identify outliers, the Grubbs test (Graphpad Prism software) was used on each group of data. Using an $\alpha = 0.01$ . 1 outlier (data point 357,91% compared to WT) was identified and excluded for the I241E dataset (presented in figure 2B).
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No randomization procedure was used.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No blinding procedure was used.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	All presented analyses meet the assumptions of the applied statistical test. To test for normal distribution the D'Agostino-Pearson normality test was employed (Graphpad Prism software).
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	There are significant differences in SD between tested groups according to the one-way ANOVA and Bartlett's tests. However since the data follow normal distribution, the ANOVA test should compensate for the heterogeneity between the tested groups.

#### C- Reagents

#### USEFUL LINKS FOR COMPLETING THIS FORM

<http://www.antibodypedia.com>  
<http://1degreebio.org>  
<http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo>  
  
<http://grants.nih.gov/grants/olaw/olaw.htm>  
<http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm>  
<http://ClinicalTrials.gov>  
<http://www.consort-statement.org>  
<http://www.consort-statement.org/checklists/view/32-consort/66-title>  
  
<http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun>  
  
<http://datadryad.org>  
  
<http://figshare.com>  
  
<http://www.ncbi.nlm.nih.gov/gap>  
  
<http://www.ebi.ac.uk/ega>  
  
<http://biomodels.net/>  
  
<http://biomodels.net/miriam/>  
<http://jij.biochem.sun.ac.za>  
[http://oba.od.nih.gov/biosecurity/biosecurity\\_documents.html](http://oba.od.nih.gov/biosecurity/biosecurity_documents.html)  
<http://www.selectagents.gov/>

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	This information is provided in the materials and methods section
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All stable cell lines were derived from mEF KO cell lines, the original reports are cited in the materials and methods section. All cell lines were tested at least 3 times for mycoplasma contamination and all lines were negative.

\* for all hyperlinks, please see the table at the top right of the document

#### D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

#### E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

#### F- Data Accessibility

18. Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'.  Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

#### G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA
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