EMBO reports

FAM111A Induces Nuclear Dysfunction in Disease and Viral Restriction

Minghua Nie, Martina Oravcová, Yasaman Jami-Alahmadi, James a Wohlschlegel, Eros Lazzerini Denchi and Michael Boddy
DOI: 10.15252/embr.202050803

Corresponding author(s): Michael Boddy (nboddy@scripps.edu)

Review Timeline:

Submission Date:	4th May 20
Editorial Decision:	3rd Jun 20
Revision Received:	18th Sep 20
Editorial Decision:	28th Oct 20
Revision Received:	17th Nov 20
Accepted:	20th Nov 20

Editor: Martina Rembold

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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 3rd Jun 2020

Dear Dr. Boddy

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, the referees also note that the study lacks a clear focus and currently seems to represent a compilation of different findings related to FAM111A and I agree with this assessment. I would therefore suggest to focus your manuscript on the disease-related mutations and their effect on nuclear shape and apoptosis and the interaction with nuclear pore-associated proteins. You could either remove the data on RNF4, which I would suggest since it seems least connected to the rest of the study and the screen that identified this interaction will be published elsewhere, or you remove the part on viral infection.

If you decide to keep the data on RNF4, please make sure to include a minimal description of the screen conditions you used to identify FAM111A as interaction partner for RNF4 in the methods section.
If you keep the part on nuclear barrier and SV40 infection, it is not mandatory to test other viruses but the data should be discussed more in the context of what has been published.
The identification of direct FAM111A substrates would certainly strengthen the study, but it is not mandatory.
Furthermore, the data should be strengthened along the lines suggested by the referees (such as testing of the disease mutations in other cell lines and further apoptosis assays).

Taken together, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We invite you to submit your manuscript within three months of a request for revision. This would be September 3rd in your case. Yet, given the current COVID-19 related lockdowns of laboratories, we have extended the revision time for all research manuscripts under our scooping protection to allow for the extra time required to address essential experimental issues. Please contact me if you wish to discuss the time needed and the revisions further (martina.rembold@embo.org).

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section is missing.
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages
https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
()

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>).

Specifically, we would kindly ask you to provide public access to the the mass spec dataset.

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Method) that follows the model below (see also <

https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

9) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

10) Regarding data quantification:
- Please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (biological or technical replicate), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
IMPORTANT: Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates. If the data rely on a smaller number of replicates, scatter blots showing individual data points are recommended.
- Graphs must include a description of the bars and the error bars (s.d., s.e.m.).
- Please also include scale bars in all microscopy images.

11) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public

in this case."

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely

Martina Rembold, PhD
Editor
EMBO reports

****

Referee #1:

Nie et al. focus on one hit from the BioID experiment designed to identify interactors of RNF4, a Sumo targeted ubiquitin ligase, FAM111A. FAM111A is mutated in KCS2 and OCS ,severe developmental disorders and has been previously shown to restrict viral replication. Authors show that overexpression of disease-associated mutants results in cellular death which is largely independent of caspases but depends on FAM111A protease activity. The major phenotype that is associated with death are nuclear envelope abnormalities and the authors find that nucleoporins and GANP are FAM111A interactors, although they do not show that they are substrates of FAM111A.

This is largely descriptive study of FAM111A function. Without identification of the substrates, it will be difficult to gain insight into the mechanism of this important protease during normal celllular growth or during viral infection. The data are supportive of the conclusions put forth by the authors. Before publication, I would like to see some additional controls and improvement to the figures as delineated below:

1. The BioID experiments (Fig 1A, 4C) lack the non-biotin added experimental controls.
2. Suggested improvements to the figures:
Figure 1-please label that RNF4 has a myc tag in A and gfp in B. This is unclear and although present in the legend, would improve the figure.
Figure 3- graph in B needs axis description
Figure 4 A- needs more explanation about what is being reported. I assume this is peptide numbers but needs to be made clearer both in the table and in the legend.
4D- Western is suboptimal and should be repeated to clearly show GANP signal throughout a lane.

Referee #2:

The FAM111A protein is a serine protease that has been implicated in viral restriction and two genetic disorders. The current study examines the pathological effects of the KCS/OCS mutants of FAM111A. They suggest that FAM111A protease activity is cytotoxic, and this is more dramatic for

the disease mutants. They suggest that this hyperactivity is associated with impacts on nuclear integrity and cell cycle progression and viability. There are a number of very intriguing observations about FAM111A interactions, impacts on cellular function, and the effects on viral restriction. The most interesting new information comes from Figure 5 which addresses the impact of FAM111A on nuclear barrier function during SV40 infection. The rest of the paper leads up to this but is less impactful. All figures are convincing in themselves but overall these observations are not all tightly linked. But if they could be pulled into a coherent model it would strengthen the study.

1) Figure 1 demonstrates that FAM111A binds the STUbl enzyme RNF4, consistent with other reports, and suggests that SUMOylated FAM111A is turned over by functional RNF4. The link to the rest of the study could be strengthened.

2) Figure 2 analyses the disease mutations. Over-expression of these mutants inhibited cell cycle progression, and this was lost for mutations of the protease site. It is not clear why these experiments were done in 293 cells? This cell line already expresses viral oncoproteins that alter cell cycle progression. The results would be more convincing if repeated in other cell lines. Finally, Figure 2C suggests cleavage of PARP, a marker of apoptosis.

3) Figure 3 looks at the effect of adding caspase inhibitor which blocked PARP cleavage but not cytotoxicity. FAM111A also changes nuclear morphology in a way that is dependent on protease activity but not caspases. The R569H mutant appears in a perinuclear structure that looks like an aggresome. Is this seen in other cell types?

4) Figure 4 uses proximity labeling to identify proteins bound by FAM111A using the protease-dead S541A mutant. This identified PCNA and other replication proteins, and a number of nuclear pore proteins. Is there any evidence that any of these nuclear pore components are cleaved in a FAM111A-dependnet manner?

5) Figure 5 tests the impact on SV40 infection by transfection of wild-type or host range mutants into U2OS cells. The LT antigen of the host range mutants begins to accumulate in the cytoplasm over time and this was lost for FAM111A depletion. Similar observations were made with 2XRFP-NLS reporter, suggesting a global nuclear barrier function is compromised. Has this been extended to other viruses impacted by FAM111A? How do these findings fir in with previous papers on viral restriction and host range?

Referee #3:

This is an interesting manuscript describing the characterisation of the FAM111A protease. Most of the data is of good quality and the conclusions drawn largely justified. My major criticism is a lack of focus in the manuscript-the larger initial portion deals with properties of wt and mutant FAM111A in human tumour cell lines but then in the final section it goes on to look at the relationship between the protease and SV40 T and host range mutants of SV40. My feeling is to lose the SV40 material (Figure 5) and concentrate on a shorter manuscript.
I have a number of criticisms which should be addressed:
1. An initial table summarising the properties of the FAM111A mutant proteins would be useful.
2. In figure 3C and lines 248-250 the authors say that the phenotype observed is dependent on R569H and S541A had no effect. Yet the IF images in 3C are quite different when R569H is expressed on its own or together with S541A-does this mean that S541A neutralises the effect of

R569H?
3. The authors use PARP cleavage as their only measure of apoptosis. As they suggest that the apoptotic induction is due to the profound cytotoxic effects of FAM111A and FAM111AR569H it would be most interesting to examine other markers of apoptosis such as annexin V staining to see if they also show apoptosis induction.
4. The western blot in Figure4D is of poor quality and needs to be repeated.

Response to Reviewer 1:

– We are truly grateful to both you and the Editor for the constructive suggestions that have helped us create a more focused and impactful article. We have rigorously addressed all Reviewers comments in our carefully revised manuscript, which now contains additional supporting data. Below is a detailed point-by-point response to your comments, but first I highlight some of the key changes:

1. *We removed the RNF4/SUMO data as suggested, allowing better focus on the related phenotypes caused by FAM111A patient mutants and polyomavirus replication.*
2. *We present new data on a likely target of FAM111A protease activity. We find that the central channel nucleoporin NUP62, which is essential for transport and gating of the nuclear pore, is depleted in the presence of hyperactive FAM111A, e.g. the KCS2 patient mutant FAM111A^{R569H}. This provides a plausible link between FAM111A hyperactivity, nuclear pore redistribution, and loss of nuclear barrier function.*
3. *We have added new data demonstrating the cytotoxicity of the FAM111A KCS2/OCS patient mutants in U2OS cancer cells and the "normal" hTERT1 immortalized RPE cell line (in addition to our existing analysis in HEK293 cells).*
4. *New FACS data are included on U2OS and RPE cell lines. They show that FAM111A KCS2/OCS patient mutants also disrupt cell cycle progression in these cell lines, with cells accumulating in S phase, as seen in HEK293 cells. Thus, FAM111A hyperactivity broadly disrupts S phase.*
5. *We added data showing that hyperactive FAM111A also induces caspase 3 cleavage (activation), in addition to our existing PARP cleavage data. This result further supports that hyperactive FAM111A can induce apoptosis.*

Referee #1:

Nie et al. focus on one hit from the BioID experiment designed to identify interactors of RNF4, a Sumo targeted ubiquitin ligase, FAM111A. FAM111A is mutated in KCS2 and OCS ,severe developmental disorders and has been previously shown to restrict viral replication. Authors show that overexpression of disease-associated mutants results in cellular death which is largely independent of caspases but depends on FAM111A protease activity. The major phenotype that is associated with death are nuclear envelope abnormalities and the authors find that nucleoporins and GANP are FAM111A interactors, although they do not show that they are substrates of FAM111A.

This is largely descriptive study of FAM111A function. Without identification of the substrates, it will be difficult to gain insight into the mechanism of this important protease during normal cellular growth or during viral infection.

– Excitingly, during revision, we have identified the nucleoporin NUP62 as a likely substrate of FAM111A protease activity. Depletion of this gating/transport nucleoporin by FAM111A in a caspase-independent manner is striking. It could begin to explain the effects of FAM111A on nuclear barrier function, pore distribution, and DNA replication in disease and viral restriction.

The data are supportive of the conclusions put forth by the authors. Before publication, I would like to see some additional controls and improvement to the figures as delineated below:

1. The BioID experiments (Fig 1A, 4C) lack the non-biotin added experimental controls.

– In revising the manuscript, we have removed Fig. 1A and 1B. We have now added Fig. 4E, which includes a minus-biotin condition as requested. We typically do not use this as a control because endogenous biotin still allows for considerable labeling, as demonstrated by the residual pull down of myc-BirA-FAM111A in Fig. 4E. Nevertheless, without biotin added to the medium, insufficient GANP is labeled under these conditions to be detected by western blot.*

2. Suggested improvements to the figures:
Figure 1-please label that RNF4 has a myc tag in A and gfp in B. This is unclear and although present in the legend, would improve the figure.

–In revising the manuscript, we have removed RNF4 data from Figure 1 (as suggested by the Editor and a Reviewer).

Figure 3- graph in B needs axis description

– We have added the axis description to the graph, which is now Fig. 3C

Figure 4 A- needs more explanation about what is being reported. I assume this is peptide numbers but needs to be made clearer both in the table and in the legend.

– You are correct, these are peptide spectral counts. We have added a clearer description in both the table footer and legend to indicate this.

4D- Western is suboptimal and should be repeated to clearly show GANP signal throughout a lane.

– We have repeated the relevant components of the original Fig. 4D, which is now included as Fig. 4E along with the minus-biotin control.

Response to Reviewer 2:

– We are truly grateful to both you and the Editor for the constructive suggestions that have helped us create a more focused and impactful article. We have rigorously addressed all Reviewers comments in our carefully revised manuscript, which now contains additional supporting data. Below is a detailed point-by-point response to your comments, but first I highlight some of the key changes:

1. *We removed the RNF4/SUMO data as suggested, allowing better focus on the related phenotypes caused by FAM111A patient mutants and polyomavirus replication.*
2. *We present new data on a likely target of FAM111A protease activity. We find that the central channel nucleoporin NUP62, which is essential for transport and gating of the nuclear pore, is depleted in the presence of hyperactive FAM111A, e.g. the KCS2 patient mutant FAM111A^{R569H}. This provides a plausible link between FAM111A hyperactivity, nuclear pore redistribution, and loss of nuclear barrier function.*
3. *We have added new data demonstrating the cytotoxicity of the FAM111A KCS2/OCS patient mutants in U2OS cancer cells and the "normal" hTERT1 immortalized RPE cell line (in addition to our existing analysis in HEK293 cells).*
4. *New FACS data are included on U2OS and RPE cell lines. They show that FAM111A KCS2/OCS patient mutants also disrupt cell cycle progression in these cell lines, with cells accumulating in S phase, as seen in HEK293 cells. Thus, FAM111A hyperactivity broadly disrupts S phase.*
5. *We added data showing that hyperactive FAM111A also induces caspase 3 cleavage (activation), in addition to our existing PARP cleavage data. This result further supports that hyperactive FAM111A can induce apoptosis.*

Referee #2:

The FAM111A protein is a serine protease that has been implicated in viral restriction and two genetic disorders. The current study examines the pathological effects of the KCS/OCS mutants of FAM111A. They suggest that FAM111A protease activity is cytotoxic, and this is more dramatic for the disease mutants. They suggest that this hyperactivity is associated with impacts on nuclear integrity and cell cycle progression and viability. There are a number of very intriguing observations about FAM111A interactions, impacts on cellular function, and the effects on viral restriction. The most interesting new information comes from Figure 5 which addresses the impact of FAM111A on nuclear barrier function during SV40 infection. The rest of the paper leads up to this but is less impactful. All figures are convincing in themselves but overall these observations are not all tightly linked. But if they could be pulled into a coherent model it would strengthen the study.

– We are pleased that the Reviewer recognizes the intriguing results in our paper. We have refocused the paper on the overlapping phenotypes caused by FAM111A hyperactivity, due to KCS2/OCS patient mutations or viral challenge. The identification of nucleoporins e.g. NUP62, as likely FAM111A targets (see point 4 below) underpins a unifying model for how FAM111A hyperactivity could impact nuclear structure and function in disease and viral restriction.

1) Figure 1 demonstrates that FAM111A binds the STUbl enzyme RNF4, consistent with other reports, and suggests that SUMOylated FAM111A is turned over by functional RNF4. The link to the rest of the study could be strengthened.

– We have removed the original Fig. 1 RNF4-SUMO data, as both the Editor and a Reviewer deemed them least integrated with the rest of the study. We now focus on the related phenotypes caused by the FAM111A KCS2/OCS patient mutants and viral challenge.

2) Figure 2 analyses the disease mutations. Over-expression of these mutants inhibited cell cycle progression, and this was lost for mutations of the protease site. It is not clear why these experiments were done in 293 cells? This cell line already expresses viral oncoproteins that alter cell cycle progression. The results would be more convincing if repeated in other cell lines. Finally, Figure 2C suggests cleavage of PARP, a marker of apoptosis.

– We initially used HEK293 due to their very low levels of endogenous FAM111A (Fig. EV1). This made them a useful line for studying ectopic expression of FAM111A constructs, which are expressed at lower levels than endogenous FAM111A in many other lines e.g. Fig. 3A. As requested, we have generated new inducible cell lines for FAM111A expression and added the new data to Fig. 2 and Fig. EV2. We show FACS analyses of U2OS and hTERT1-RPE cells, both of which have similarly high levels of endogenous FAM111A (Fig. EV1). Importantly, as seen for HEK293 cells, we again see that FAM111A KCS2/OCS patient mutants cause RPE and U2OS cells to accumulate in S phase. This suggests a broad and largely genotype-independent disruption of replication by FAM111A mutants, as U2OS are cancer cells and RPE are a non-cancer-derived immortalized cell line.

3) Figure 3 looks at the effect of adding caspase inhibitor which blocked PARP cleavage but not cytotoxicity. FAM111A also changes nuclear morphology in a way that is dependent on protease activity but not caspases. The R569H mutant appears in a perinuclear structure that looks like an aggresome. Is this seen in other cell types?

– We believe the Reviewer is referring to the Mab414 signal in what is now Figs. 4C and EV4 (originally Fig. S2). This antibody detects a number of FG-repeat containing nucleoporins and is commonly used to detect nuclear pore distribution by IF. The perinuclear "aggregates" are actually a result of nucleoporin redistribution caused by FAM111A mutant expression. Notably, we have extended our analysis to U2OS and RPE cell lines, and again see a similar disruption of nucleoporin distribution by the FAM111A KCS2 patient mutant (Fig. EV4B).

4) Figure 4 uses proximity labeling to identify proteins bound by FAM111A using the protease-dead S541A mutant. This identified PCNA and other replication proteins, and a number of nuclear pore proteins. Is there any evidence that any of these nuclear pore components are cleaved in a FAM111A-dependnet manner?

– Excitingly, during revision we have indeed detected FAM111A-dependent depletion of a nucleoporin. Using the Mab414 antibody in western analysis we could readily detect NUP62, which is the strongest signal in whole cell lysates. Intriguingly, the NUP62 signal is strongly depleted by expression of the FAM111A KCS2 patient mutant, FAM111A^{R569H} (Fig. 4F). Moreover, this effect is independent of caspases, as Z-VAD-FMK has no impact on NUP62 depletion. It is notable that NUP62 is a central channel nucleoporin involved in gating and transport through the nuclear pore, whose depletion could contribute to the loss of nuclear barrier function we observe. Therefore, although more work is needed (such as in vitro assays, beyond the scope of the current study), these results point to processing of nucleoporins as a

function/effect of hyperactive FAM111A.

5) Figure 5 tests the impact on SV40 infection by transfection of wild-type or host range mutants into U2OS cells. The LT antigen of the host range mutants begins to accumulate in the cytoplasm over time and this was lost for FAM111A depletion. Similar observations were made with 2XRFP-NLS reporter, suggesting a global nuclear barrier function is compromised. Has this been extended to other viruses impacted by FAM111A? How do these findings fir in with previous papers on viral restriction and host range?

– We used the SV40 polyomavirus as a testbed for our analysis of FAM111A-mediated restriction, as it is the best characterized viral system and also has available tools/mutations to analyze e.g. replication dependency (as in Fig. 5). FAM111A was first identified as a restriction factor for SV40 host range mutants, and later shown to work with RFC1 and PCNA to restrict VACV replication (now fully discussed in the paper). These are the only viruses to date known to be impacted by FAM111A, hence we discuss our results in the context of SV40 and VACV restriction. We did not broadly discuss viral restriction as it has many layers unrelated to the role of FAM111A and nuclear barrier function. That is, the SV40 LT (C-terminus) and VACV SPI-1 have specific interactions with FAM111A, presumably to inactivate this particular restriction factor and allow replication. There are other regions of LT, and other factors like SPI-1, that target numerous distinct cellular responses to viral challenge, from entry into the cell to encapsidation. It will certainly be interesting for others studying VACV to apply our findings to their field, but due to the need to generate new restrictive cell lines and other tools for this analysis, we believe it is beyond the scope of our study.

Response to Reviewer 3:

– We are truly grateful to both you and the Editor for the constructive suggestions that have helped us create a more focused and impactful article. We have rigorously addressed all Reviewers comments in our carefully revised manuscript, which now contains additional supporting data. Below is a detailed point-by-point response to your comments, but first I highlight some of the key changes:

1. *We removed the RNF4/SUMO data as suggested, allowing better focus on the related phenotypes caused by FAM111A patient mutants and polyomavirus replication.*
2. *We present new data on a likely target of FAM111A protease activity. We find that the central channel nucleoporin NUP62, which is essential for transport and gating of the nuclear pore, is depleted in the presence of hyperactive FAM111A, e.g. the KCS2 patient mutant FAM111A^{R569H}. This provides a plausible link between FAM111A hyperactivity, nuclear pore redistribution, and loss of nuclear barrier function.*
3. *We have added new data demonstrating the cytotoxicity of the FAM111A KCS2/OCS patient mutants in U2OS cancer cells and the "normal" hTERT1 immortalized RPE cell line (in addition to our existing analysis in HEK293 cells).*
4. *New FACS data are included on U2OS and RPE cell lines. They show that FAM111A KCS2/OCS patient mutants also disrupt cell cycle progression in these cell lines, with cells accumulating in S phase, as seen in HEK293 cells. Thus, FAM111A hyperactivity broadly disrupts S phase.*
5. *We added data showing that hyperactive FAM111A also induces caspase 3 cleavage (activation), in addition to our existing PARP cleavage data. This result further supports that hyperactive FAM111A can induce apoptosis.*

Referee #3:

This is an interesting manuscript describing the characterisation of the FAM111A protease. Most of the data is of good quality and the conclusions drawn largely justified. My major criticism is a lack of focus in the manuscript-the larger initial portion deals with properties of wt and mutant FAM111A in human tumour cell lines but then in the final section it goes on to look at the relationship between the protease and SV40 T and host range mutants of SV40. My feeling is to lose the SV40 material (Figure 5) and concentrate on a shorter manuscript.

– We appreciate the Reviewers comments and have shortened/focused the manuscript. We have removed the section on RNF4/SUMO as on balance it was viewed to be the least integrated component of the manuscript (echoed by the Editor). Indeed, activation of FAM111A by the KCS2/OCS patient mutations and during viral restriction causes overlapping phenotypes e.g. nuclear pore redistribution, disruption of replication, and loss of nuclear structure/function.

I have a number of criticisms which should be addressed:
1. An initial table summarising the properties of the FAM111A mutant proteins would be useful.

– We have added a diagram of the FAM111A protein and indicated the positions of mutations used in the study, including those found in KCS2 or OCS patients (Fig. 1A). This helps put the mutations and effects in clearer context, thank you.

2. In figure 3C and lines 248-250 the authors say that the phenotype observed is dependent on R569H and S541A had no effect. Yet the IF images in 3C are quite different when R569H is expressed on its own or together with S541A- does this mean that S541A neutralises the effect of R569H?

– We have attempted to clarify our results and meaning in the text and new Fig. 4C and Fig. EV4 (Fig. 3C and S2 in initial submission). What we show is that the protease dead mutant S541A does not cause the phenotypes seen with hyperactive R569H FAM111A. Moreover, by combining the R569H and S541A mutations in the same FAM111A construct we neutralize the effects of R569H. That is, it is the protease activity of FAM111A R569H that is toxic and causes the nuclear pore redistribution. Now that we have identified NUP62 as a likely substrate at the nuclear pore, this makes good sense with the phenotypes caused by FAM111A hyperactivity.

3. The authors use PARP cleavage as their only measure of apoptosis. As they suggest that the apoptotic induction is due to the profound cytotoxic effects of FAM111A and FAM111AR569H it would be most interesting to examine other markers of apoptosis such as annexin V staining to see if they also show apoptosis induction.

– We have added caspase 3 cleavage as an additional measure of apoptosis induction in our new Fig. 4F. This data mirrors that of PARP cleavage, showing relatively weak induction of apoptosis, as compared to the high cytotoxicity of hyperactive FAM111A. We also do not detect a significant sub-G1 population in our FACS analyses (e.g. Fig 2B), indicating only weak induction of apoptosis by FAM111A. Importantly, as discussed in the paper, we do not believe apoptosis is the primary cause of FAM111A-induced phenotypes, rather it is a later (or stochastic) consequence of FAM111A hyperactivity in some cells. Indeed, we identify NUP62 processing as a FAM111A-dependent but caspase-independent event, which could start to explain the impact of FAM111A on nuclear pore distribution and nuclear barrier function (Fig. 4C & F, Fig. 5).

4. The western blot in Figure4D is of poor quality and needs to be repeated.

– We have repeated the relevant components of the original Fig. 4D, which is now included as Fig. 4E, along with a minus-biotin control (per Reviewer 1).

1st Revision - Editorial Decision 28th Oct 2020

Dear Dr. Boddy

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, all referees are very positive about the study and request only minor changes to clarify text and some of the findings. If patient cells are available, the cleavage of Nup62 should be analyzed, otherwise it should be critically discussed.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

- Your article will be published in our Reports section. To match the short format of our Scientific reports I kindly ask you to combine the Results and Discussion section and to keep our character limit of 25,000 plus/minus 2,000 in mind (excludes references and materials and methods).

- Please reformat the reference list to match journal style. The year should be in brackets and et al should be used if there are more than 10 authors (i.e., list the first 10 authors followed by et al).

- You might have seen the related manuscript from the lab of Niels Mailand that was published recently in EMBO reports, which I suggest to cite (Hoffmann et al, EMBO Rep (2020): 21:10

- EV figure legends: please remove the word 'supplementary' from the figure title.

- Please rename the table files to 'Table EV#' and please also correct the callout to these tables accordingly.

- You uploaded an .xls file called 'biorxiv077594-file001.xls' as dataset. I assume this lists the interacting proteins identified in your BioID-based proteomics assay. If so, please call it "Dataset EV1" and please provide a legend in the first tab of the .xls file. Please also add a callout to the Dataset somewhere in the manuscript text.

- Please note that the Author Checklist will be published with the Review Process File. Therefore, please remove the reviewer password from field F-18.

- Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-600 pixels large (width x height) in .png format. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

We look forward to seeing a final version of your manuscript as soon as possible.

Kind regards,

Martina Rembold

Martina Rembold, PhD

Senior Editor
EMBO reports

****

Referee #1:

I do not have further comments. I was overall positive the first time around. The authors addressed my questions and also added data on NUP62 as a likely substrate of FAM111A protease This study complements the recently published manuscript from the Mailand group.

Referee #2:

This revised version of the manuscript has been significantly improved by removing data which allows a more focused story and by addition of new data that identify a substrate for FAM111A protease activity. The paper reads very nicely. It does a much better job of bringing together disparate functions of FAM111A into a common theme. The observations are now compelling and the manuscript fits well with the EMBO Reports format.

Minor points:
1) Are the lower levels of hyperactive mutants in the blot of Figure 3A possibly due to auto-cleavage activity? This might be consistent with the recent Kojima paper. Is this also seen for the S541A mutants in Figure 2C? Why are these differences less noticeable in Figure 3B?
2) The Discussion is a little long and could be shortened to focus on the interpretations and implications rather more than restating findings.

Referee #3:

In this modified version of the manuscript the authors have addressed most, although not all, of the original criticisms. They have also added new data in an attempt to explain their original observations and make the paper less descriptive.

Unfortunately, the authors have not really answered one of my original criticisms and the new data is not totally convincing. I suggested that a second method should be used to look for apoptosis, in addition to PARP cleavage. The authors have shown caspase 3 activation as this alternative-this is not really valid as activated caspase 3 is probably responsible for PARP cleavage and so they are in the same pathway. Again, I would suggest using annexin V staining.

The new data showing degradation of Nup62 is interesting, but this raises problems. If Nup62 is a primary target for FAM111A why is it not picked up in BioID screen (Figure 4A)? The authors should also show what happens to at least one of the other identified Nups (Figure 4A)-is it also degraded? If KCS or OCS patient cells are available surely it is important, and relatively simple, to examine the level of Nup62 expression in them. This would establish whether Nup62 cleavage is, at least partially, responsible for the observed phenotype.

– Reviewers, thank you again for your time, suggestions, and positivity that have helped us produce a more impactful manuscript. We address your additional suggestions and questions below.

Referee #1:
I do not have further comments. I was overall positive the first time around. The authors addressed my questions and also added data on NUP62 as a likely substrate of FAM111A protease This study complements the recently published manuscript from the Mailand group.

– *Thank you! We are excited to have this more polished story published.*

Referee #2:
This revised version of the manuscript has been significantly improved by removing data which allows a more focused story and by addition of new data that identify a substrate for FAM111A protease activity. The paper reads very nicely. It does a much better job of bringing together disparate functions of FAM111A into a common theme. The observations are now compelling and the manuscript fits well with the EMBO Reports format.

– *Thank you for the constructive criticism that has helped us produce a much more focused and impactful report.*

Minor points:
1) Are the lower levels of hyperactive mutants in the blot of Figure 3A possibly due to auto-cleavage activity? This might be consistent with the recent Kojima paper. Is this also seen for the S541A mutants in Figure 2C? Why are these differences less noticeable in Figure 3B?

– *Indeed, we believe that the hyperactive mutants undergo auto-cleavage, as shown in both of the recent Kojima and Hoffmann (EMBO Rep 2020) papers. The difference between Fig 3A and 3B may reflect subtle differences in the times at which cells were harvested, or strength of induction for FAM111A expression. These could both impact the point at which auto-cleavage is strongly triggered. However, across multiple experiments, we generally see lower levels for the hyperactive FAM111A mutants, as seen by Kojima and Hoffmann.*

2) The Discussion is a little long and could be shortened to focus on the interpretations and implications rather more than restating findings.

– *During revision we have combined the Results and Discussion sections, and have trimmed the Discussion as suggested.*

Referee #3:
In this modified version of the manuscript the authors have addressed most, although not all, of the original criticisms. They have also added new data in an attempt to explain their original observations and make the paper less descriptive.

– *Thank you for recognizing the improvements to our manuscript, and for your time in making useful suggestions to get us to this point.*

Unfortunately, the authors have not really answered one of my original criticisms and the new data is not totally convincing. I suggested that a second method should be used to look for apoptosis, in addition to PARP cleavage. The authors have shown caspase 3 activation as this alternative-this is not really valid as activated caspase 3 is probably responsible for PARP cleavage and so they are in the same pathway. Again, I would suggest using annexin V staining.

– We apologize that we did not fully understand your suggestion. Our intention was simply to show that caspase-dependent apoptosis is weakly induced by FAM111A hyperactivity. The recent Hoffmann et al paper (EMBO Reports 2020) shows the same weak induction of caspase-dependent apoptosis by FAM111A patient mutants, and additionally shows that a small sub-G1 cell population is detected following extended expression of FAM111A. We do not see the sub-G1 population, but our ectopic FAM111A expression level is lower than theirs (nearer endogenous levels), so may not drive such extensive cell death. As now discussed in the revised manuscript, we do not believe that induction of apoptosis is a primary effect of FAM111A hyperactivity, and it does not account for FAM111A-induced nuclear barrier dysfunction or its disruption of replication (including viral replication).

The new data showing degradation of Nup62 is interesting, but this raises problems. If Nup62 is a primary target for FAM111A why is it not picked up in BioID screen (Figure 4A)? The authors should also show what happens to at least one of the other identified Nups (Figure 4A)-is it also degraded?

– *We have added the following to the discussion about NUP62: Such "missed" identifications occur with proximity labeling methods that require spatially available biotinylation sites, and also with mass spectrometry-based methods that more readily detect large and abundant proteins. Importantly, NUP62 is clearly depleted in a FAM111A-dependent but caspase-independent manner, making it the best candidate target of FAM111A protease activity described to date.*
– *Our data on GANP, whilst not directly demonstrating FAM111A-dependent depletion, recapitulate the BioID screen data, and indicate that like NUP62 it is a strong candidate FAM111A target. GANP, like the other very high-molecular weight NPC-associated factors, are difficult to detect in western analyses of total protein extracts, which precludes a similar approach to that used for NUP62.*

If KCS or OCS patient cells are available surely it is important, and relatively simple, to examine the level of Nup62 expression in them. This would establish whether Nup62 cleavage is, at least partially, responsible for the observed phenotype.

– *Unfortunately, to our knowledge KCS2/OCS patient cells are not available. Such an experiment would also be difficult to interpret due to the lack of an appropriate control, which is why others and we generated model KCS2/OCS cell lines. That is, an isogenic control from the same patient, expressing only wild-type FAM111A, is not possible. Therefore, the levels of the essential protein NUP62 in an isolated cell population would not be particularly informative.*

2nd Revision - Editorial Decision 20th Nov 2020

Dr. Michael Boddy
The Scripps Research Institute
Molecular Medicine
10550 N. Torrey Pines Road
La Jolla, CA 92037
United States

Dear Nick,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Kind regards,
Martina

Martina Rembold, PhD
Senior Editor
EMBO reports

****

THINGS TO DO NOW:

You will receive proofs by e-mail approximately 2-3 weeks after all relevant files have been sent to

our Production Office; you should return your corrections within 2 days of receiving the proofs.

Please inform us if there is likely to be any difficulty in reaching you at the above address at that time. Failure to meet our deadlines may result in a delay of publication, or publication without your corrections.

All further communications concerning your paper should quote reference number EMBOR-2020-50803V3 and be addressed to emboreports@wiley.com.

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Michael N. Boddy
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2020-50803V1

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 χ2 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?	The sample size was chosen according to standard practice in the field, based on whether it is sufficiently large to lead to statistically significant difference among groups/conditions.
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?	NA
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.	Yes. Multiple biological replicates were always performed with treatment administration process randomized. In some experiments with chemical treatments, chemicals obtained from different manufacturer were tested and compared to be sure there is no secondary effect due to quality differences.
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.	Yes. For example, in the experiments in which we counted the number of cells with certain subcellular localization features, random fields of cells were imaged. Cell counting was conducted after the imaging and image file names have been scrambled by another researcher to minimize subjective bias.
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?	For each figure that involves graph summarizing experimental observations, sample size, number of repeats, and statistical method were always described in figure legend.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Since the sample comparison used in our study all involves pair-wise comparison of two independent samples with no preassumption on which sample is greater, two-tailed, unpaired t-test was used in all our statistical analysis.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-report

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-tumo

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://www.selectagents.gov/

Is there an estimate of variation within each group of data?	Yes. Standard deviation or standard error of the mean was calculated for each group to estimate variation within each group.
Is the variance similar between the groups that are being statistically compared?	Generally yes, and the variances were taken into consideration in our statistical analysis.

C- Reagents

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).	Detailed information on antibodies (along with their supplier, catalog number, and dilution) used in this study was 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.	The cell lines used in this study are detailed in the Materials and Methods section. The cells are routinely (monthly or at a higher frequency) tested for mycoplasma contamination using a PCR-and/or an enzymatic-based method.

* 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	The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD020327.
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