

Manuscript EMBOR-2013-38128

## FIP5 phosphorylation during mitosis regulates apical trafficking and lumenogenesis

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### Review timeline:

Submission date:	18 October 2013
Editorial Decision:	12 November 2013
Revision received:	16 December 2013
Editorial Decision:	09 January 2014
Revision received:	16 January 2014
Accepted:	17 January 2014

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### 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.)

Editor: Nonia Pariente

1st Editorial Decision

12 November 2013

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Thank you for your patience while your study has been under peer-review at EMBO reports. We have now received the three enclosed reports on it. As you will see, although all referees find the topic of the study interesting and the experiments well carried out in general, there are some outstanding issues that must be addressed before we can consider your study for publication.

As the reports are rather detailed, I will not belabor on them here. The most important things to address are strengthening the data regarding GSK-3 mediated FIP-5 phosphorylation in cells, and evidence of FIP-5 as a major GSK-3 target in lumen formation. In addition, there are various requests for further colocalization experiments and quantification of the data (which should be done on at least three independent experiments), and the cell-cycle dependent FIP-5 phosphorylation will need to be assessed in MDCK cells, all of which will help to strengthen the study.

If the referee concerns can be adequately addressed, we would be happy to accept your manuscript for publication. However, please note that it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

Revised manuscripts must be submitted within three months of a request for revision unless

previously discussed with the editor; they will otherwise be treated as new submissions. Revised manuscript length must be a maximum of 28,500 characters (including spaces). When submitting your revised manuscript, please also include editable TIFF or EPS-formatted figure files, a separate PDF file of any Supplementary information (in its final format) and a letter detailing your responses to the referees.

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

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, do not hesitate to get in touch with me if I can be of any assistance.

#### REFEREE REPORTS:

Referee #1:

Li et al. perform a rigorous examination of early steps in lumen formation using an MDCK model system. They focus on the earliest steps in the formation of the apical membrane initiation site (AMIS), which is intimately linked to the cell cycle. Their most intriguing finding is that the Rab11 effector FIP5 is reversibly phosphorylated during metaphase, possibly by GSK3, and that the dephosphorylation of FIP5 is apparently necessary to promote SNX18-dependent fission of exocytic vesicles. I have the following comments/suggestions:

Major

1. The model presented in Fig. 5B appears to indicate that the Rab11a/Fip5 endosomes are contributing to the membrane that is forming along the cleavage furrow - and will eventually form the AMIS. While Rab11a has previously been implicated in cytokinesis, the authors appear to argue on pages 5-6 that AMIS formation, as marked by ZO-1 and Cingulin, precedes the arrival of Rab11a/Fip5 vesicles, which subsequently accumulate at the AMIS. While I appreciate that cytokinesis and AMIS formation are likely to be intimately linked, and that the earliest events in AMIS formation probably occur simultaneous with cytokinesis, this is not clear from the data presented in the results. At present the data in Fig. 1 and Fig. S1 are not particularly revealing for the following reasons: (1) It is unclear in Fig. 1A why only one of the forming daughter cells has an accumulation of FIP5: (2) Because the cells are not obviously synchronized it is difficult to infer much from the results and timeline presented in Fig. 1D-G. (3) Fig. S1A - it appears that the cells have undergone division, but without AMIS formation, and in Fig. S1C it looks like FIP5/Crb3A are at the AMIS in one cell, but not the other. In sum, it would be helpful if the authors simultaneously imaged FIP5 and Cingulin, perhaps coupled with a non-specific membrane probe such as DiI or FM4-64. This should allow the reader to better assess when AMIS formation completes (relative to cell division) and when FIP5 is recruited to the AMIS.

2. My other major concern is the putative role for GSK-3 in phosphorylation of FIP5T276. As a general comment, *in vitro* kinase assays are not always conclusive when it comes to identifying the physiological kinase. Indeed, the experiments in Fig. S2A appear to indicate that the GSK-3 inhibitor only has a marginal effect on the extent of FIP5-GFP that is phosphorylated. Without quantitation and some measure of statistical significance it is difficult to assess whether there is an effect *in vivo*. In addition, the data in Fig. S5B-D indicate a potential role for GSK-3 in lumen formation, but there is no evidence presented that FIP-5 is the only or main target of GSK-3. This is important because GSK-3 has numerous targets in the cell.

Other comments:

1. What are the large protein species at the bottom of the western blots in S2B-C? If these are immunoglobulin chains then they should be marked as such.

2. page 5, second paragraph, second line, last word - substitute the word or for and.

3. Page 6, first paragraph. While likely to be true, no evidence is provided in Fig. S1A-D that FIP5 rides the central spindle microtubules (or any microtubules for that matter). Furthermore, this same paragraph states that GP135 accumulation at the apical pole of the daughter cells depends on the formation of the AMIS, "demonstrating that AMIS formation around the midbody during late telophase is required to ensure the fidelity of the central spindle-dependent targeting of FIP-5 endosomes." Again, this may very well be true, but no experiments address this issue directly.
4. Fig. 2E is confusing. Why does addition of GSK-3 to 6His-Phospho-T276 cause an increase in peptide binding to SNX-18? Does this indicate that GSK-3 phosphorylates other residues that may promote SNX18-peptide interaction?
5. Fig. S3C-D. I may be mistaken, but it seems that inhibiting GSK-3 would prevent phosphorylation and degradation of beta-catenin. If true, why does there seem to be less beta-catenin in D versus C?
6. The GP135 staining in Fig. 3D looks non-descript, almost cytoplasmic.
7. The text does not mention Fig. 3L when discussing the FIP5S188 mutant. Fig. 3E should be annotated so that it is clear that FIP5-SA refers to S188A.
8. It is unclear why the authors switch to polarized, filter-grown MDCK cells in Fig. 4A-B. Because of the cleavage plane of a multilayered epithelium preserves the apical domain, it is unclear that AMIS formation is occurring under these conditions.
8. Page 12, second paragraph, penultimate line - do the authors mean apical exocytic carrier?
9. Please define what MDCK-MIIR cells are.

Referee #2:

In this paper by Li et al. describe a novel mechanism for the regulation of Rab11/FIP5 endosomes targeting to the AMIS during cell cycle. For the first time, they show that GSK3 $\beta$  phosphorylates Rab11 effector FIP5 in a specific threonine residue T276, and this phosphorylation controls FIP5 binding to sorting nexin 18 (SNX18). FIP5 phosphorylation takes place specifically during metaphase, and the protein is then dephosphorylated during telophase, when the endosomes are targeted to the midbody during cell scission to initiate lumen formation. Functionally, phosphorylation of FIP5 switches the binding to SNX18 at the recycling endosome, which is required for its function during early lumen formation in MDCK cysts.

Altogether the proposed mechanistic pathways seem to be correct, the experimental design is accurate and clear, and the all the data satisfactorily validated. I believe that this paper represents a substantial advance in understanding the mechanisms for vesicle trafficking during the initial steps of epithelial lumen formation. The methods and results sections are very well detailed. The experiments are generally well performed, and clearly explained in the main text, figures and legends. There are some minor points that should be addressed before publication:

- 1-The authors propose that FIP5 mechanism depends on the interaction of this protein with SNX18. However, they fail to show any picture of their subcellular co-localization. The authors must show SNX18 co-localizations with cells expressing FIP5 wt and mutants. If their conclusions are correct, SNX18 staining in recycling endosomes should be reduced in FIP5 TD-expressing cells, while SNX18 staining in endosomes should be dispersed in cells expressing FIP5-TA.
- 2- Some of the data present in Fig. 2 need to be properly quantified. The authors need to quantify the interaction of FIP5 GFP, TD-GFP and TA-GFP with kif3A-T and SNX18 (Fig. 2C).
- 3-The quantifications of FIP5 phosphorylation during cell cycle by western-blot were performed in cultures of synchronized HeLa cells. The authors should also demonstrate this specific phosphorylation in MDCK cells in metaphase. MDCK cells from different cell-cycle phases could

be FACS-sorted by DNA content, and analyzed by western-blot to verify that G2/M cells contain more p-T276 FIP5 than G1/S cells.

4- The authors should include molecular weight markers in their blots (Figure 2, 4, S2 and S3) for better understanding of the experiments.

5- There is no reference in the text of the recovery of the shFIP5 phenotype with FIP5SA (Fig. 3L, last bar in the graph). It should indicate "but FIP5SA did rescue the KD of endogenous FIP5 in cyst formation"

Referee #3:

This manuscript represents an important contribution to the expanding literature on mechanisms regulating polarity and their relationship to spindle dynamics during divisions. The findings here provide new information on the regulation of Rab11-FIP5 by GSK-3 and the impact of phosphorylation on interactions of FIP5 with SNX18. My comments are relatively minor on an otherwise excellent manuscript.

1) Some of the findings in Figure 1 confirm and extend those in an earlier paper on endosomes and MDCK cells (Hobby-Henderson, et al. Traffic, 2003) which should be acknowledged.

2) The findings of the assay of SNX association with FIP5 are the only panel without quantitation, Since this assay is key to the findings, the results Figure 2C should be quantitated.

3) On p9, the formation of multilumen cysts in the T276D cells is noted as a dominant negative phenotype for lumen formation. While it is certainly aberrant, it seems that a lack of lumen would be more consistent with dominant negative.

4) The discussion of Serine 188 phosphorylation in FIP5 in the results section seems out of place since no data are presented related to Serine 188 phosphorylation. This would be better placed in the discussion.

Minor:

1) p8 first para: "Resent" should be recent.

1st Revision - authors' response

16 December 2013

Reviewer #1

1. *"The model presented in Fig. 5B appears to indicate that the Rab11a/Fip5 endosomes are contributing to the membrane that is forming along the cleavage furrow - and will eventually form the AMIS. While Rab11a has previously been implicated in cytokinesis, the authors appear to argue on pages 5-6 that AMIS formation, as marked by ZO-1 and Cingulin, precedes the arrival of Rab11a/Fip5 vesicles, which subsequently accumulate at the AMIS. While I appreciate that cytokinesis and AMIS formation are likely to be intimately linked, and that the earliest events in AMIS formation probably occur simultaneous with cytokinesis, this is not clear from the data presented in the results.*

*(1) It is unclear in Fig. 1A why only one of the forming daughter cells has an accumulation of FIP5"*

Previous work from our and other laboratories has shown that in non-polarized cells, endosomes are delivered to the cleavage furrow in an asymmetric manner, first from one daughter cell and later from the other daughter cell. Our time-lapse images in Fig.1G clearly show that in MDCK cells, FIP5-endosomes are also transported asymmetrically. Thus, it is likely that the image in Fig. 1A showed the cell where FIP5-endosomes has been delivered to one side only. This apparent

asymmetry is one of the reasons that we did FIP5-GFP time-lapse imaging.

*“(2) Because the cells are not obviously synchronized it is difficult to infer much from the results and timeline presented in Fig. 1D-G.”*

One cannot synchronize MDCK cells embedded in Matrigel. Thus, all time-lapse images are done using non-synchronized cells. For this reason, we did not attempt to actually measure the time required for cells to undergo mitosis. We would also like to point out that Fig. 1D-G does show that FIP5-endosomes are first recruited to the centrosomes (at metaphase and anaphase) and later move to the midbody at telophase.

*“(3) Fig. S1A - it appears that the cells have undergone division, but without AMIS formation, and in Fig. S1C it looks like FIP5/Crb3A are at the AMIS in one cell, but not the other.”*

In Fig. S1A the cell is still at late telophase. The arrows point to the midbody. In Fig. S1C, both cells have FIP5/Crb3a endosomes accumulated around the centrosomes. We edited the Results section to make it more clear.

*“In sum, it would be helpful if the authors simultaneously imaged FIP5 and Cingulin. This should allow the reader to better assess when AMIS formation completes (relative to cell division) and when FIP5 is recruited to the AMIS.”*

As suggested, we have imaged FIP5-RFP and cingulin-GFP. The new data clearly show that the AMIS forms at telophase before FIP5-endosomes leave the centrosomes. As FIP5-endosomes are delivered to the AMIS, the new lumen forms and expands. The new data are shown in Supplemental figure 1G.

*2. “My other major concern is the putative role for GSK-3 in phosphorylation of FIP5T276. As a general comment, in vitro kinase assays are not always conclusive when it comes to identifying the physiological kinase. Indeed, the experiments in Fig. S2A appear to indicate that the GSK-3 inhibitor only has a marginal effect on the extent of FIP5-GFP that is phosphorylated. Without quantitation and some measure of statistical significance it is difficult to assess whether there is an effect in vivo. In addition, the data in Fig. S5B-D indicate a potential role for GSK-3 in lumen formation, but there is no evidence presented that FIP-5 is the only or main target of GSK-3. This is important because GSK-3 has numerous targets in the cell.”*

As suggested, we repeated the IP experiment two more times and quantified the pFIP5-T276 signal upon the use of GSK-3 inhibitor. As the data show, GSK-3 inhibitor reduced the signal by over 60%. Why is there not a complete absence of the signal? It is likely that the concentration of the inhibitor that we used in the experiments do not completely block GSK-3 activity. Generally, MDCK cells really did not “like” to be treated with the inhibitor, since it had a dramatic effect on the cells’ ability to form adherence junctions. For this reason, we opted only to treat cells for 1 hour at relatively low GSK-3 inhibitor concentrations. We felt that these data in combination with the IP experiments using FIP5-T276A mutant (no phosphorylation signal detected) and our in vitro phosphorylation assays do demonstrate that GSK-3 is very likely a kinase that phosphorylates FIP5 at T276.

We completely agree with the reviewer that FIP5 is not the only target of GSK-3. GSK-3 is known to phosphorylate many targets, including  $\beta$ -catenin. The same issue applies to almost any kinase, because most kinases function by phosphorylating multiple targets. Thus, we did not mean to state that during epithelial polarization and lumen formation, FIP5 is the only target of GSK-3. Our data do show that GSK-3 acts, at least in part, in phosphorylating FIP5 and inhibiting FIP5/SNX18 binding. We edited the manuscript to make this more clear.

Minor points:

*1. “What are the large protein species at the bottom of the western blots in S2B-C? If these are immunoglobulin chains then they should be marked as such.”*

Yes, those are IgG heavy chains. The blots in S2B-C were marked to make that clear.

2. “page 5, second paragraph, second line, last work - substitute the word or for and”

Corrected.

3. “Page 6, first paragraph. While likely to be true, no evidence is provided in Fig. S1A-D that FIP5 rides the central spindle microtubules (or any microtubules for that matter). Furthermore, this same paragraph states that GP135 accumulation at the apical pole of the daughter cells depends on the formation of the AMIS, “demonstrating that AMIS formation around the midbody during late telophase is required to ensure the fidelity of the central spindle-dependent targeting of FIP-5 endosomes.” Again, this may very well be true, but no experiments address this issue directly.”

We agree with the reviewer that while our data suggest that FIP5-endosomes are transported along central spindle microtubules to the forming AMIS, we do not show data directly testing that. As a result, we removed these statements from the manuscript. We do show (Fig. S1E-F) that the AMIS appears to form (as shown by CGN staining in Fig. S1F) before the delivery of gp135 to the midbody. However, since these data come from fixed cell imaging, we do not have time-lapse data to support it. As a result, we re-wrote the statement regarding the timing of AMIS formation and gp135 delivery to make it “weaker”.

4. “Fig. 2E is confusing. Why does addition of GSK-3 to 6His-Phospho-T276 cause an increase in peptide binding to SNX-18? Does this indicate that GSK-3 phosphorylates other residues that may promote SNX18-peptide interaction?”

Our apologies for a confusing explanation of the experiment. Fig. 2E shows the binding of recombinant purified 6His-FIP5 to GST-SNX18 in the presence or absence of purified GSK-3. The top blot with anti-FIP5 antibody was to visualize the total 6His-FIP5 bound to GST-SNX18. As it is shown in the blot (compare lanes 5 and 6), the presence of GSK-3 decreased the amount of FIP5 bound to GST-SNX18. In the bottom panel, we probed the same membrane with an anti-pFIP5-T276 antibody that detects only FIP5 phosphorylated at T276. Thus, despite the fact that FIP5 is bound to SNX18 in lane 5, no pFIP5-T276 signal was detected since FIP5 is not phosphorylated. In lane 6, there is some signal of FIP5 phosphorylation which weakens but does not completely block the binding. In retrospect, the bottom blot is not very informative and just confuses the explanation. Thus, we removed it from the figure.

5. “Fig. S3C-D. I may be mistaken, but it seems that inhibiting GSK-3 would prevent phosphorylation and degradation of beta-catenin. If true, why does there seem to be less beta-catenin in D versus C?”

The reviewer is absolutely correct: GSK-3 inhibition prevents  $\beta$ -catenin degradation and results in its translocation to the nucleus. An increase in the total level of  $\beta$ -catenin can be seen in Fig. S3B. Fig. S3C-D are actually meant to demonstrate nuclear translocation (compare the levels of catenin in the nucleus between Fig. S3C and D). We edited the manuscript to make that more clear.

6. “The text does not mention Fig. 3L when discussing the FIP5S188 mutant. Fig. 3E should be annotated so that it is clear that FIP5-SA refers to S188A.”

We edited the manuscript to discuss Figure 3L. We also edited the legend for Fig. 3 to make it clear that FIP5-SA refers to FIP3-S188A mutant.

7. “It is unclear why the authors switch to polarized, filter-grown MDCK cells in Fig. 4A-B. Because of the cleavage plane of a multilayered epithelium preserves the apical domain, it is unclear that AMIS formation is occurring under these conditions.”

We decided to include filter-grown MDCK cells for a couple of reasons. First, we wanted to make sure that FIP5-T276 gets phosphorylated during cell division regardless of whether MDCK cells are grown in 2D or 3D cultures. Second, it is a bit easier to see the dramatic difference between dividing and interphase cells in filter-grown cultures. We do agree with the reviewer that it is extremely important to also show that FIP5-T276 phosphorylation is also increased in 3D cultures, which we show in Fig 4. D-F. We edited the manuscript to make it more clear.

8. “Page 12, second paragraph, penultimate line - do the authors mean apical exocytic carrier?”

Yes, we did mean apical exocytic carriers and have corrected it in the manuscript.

9. “Please define what MDCK-MIIR cells are”

Our apologies for the mistake. By MDCK-MIIR cells, we meant MDCK-II cells and have corrected it in the manuscript.

#### Reviewer #2

1. “The authors propose that FIP5 mechanism depends on the interaction of this protein with SNX18. However, they fail to show any picture of their subcellular co-localization.”

We do have an anti-SNX18 antibody that works well for IF and WB. However, despite our attempts, we were never able to visualize SNX18 associating with endosomes. To large extent, it is due to the fact that SNX18 is predominately cytosolic and only transiently associates with membranes during vesicle scission. It seems to be a common feature for many SNX proteins. Indeed, closely related SNX9 (mediates vesicles scission from the plasma membrane) works pretty much the same way and is only transiently recruited to the plasma membrane during vesicle scission. The association of SNX9 with the vesicles could only be shown while using TIRF microscopy to eliminate cytosolic SNX9 fluorescence. Unfortunately, this is not an option for us since TIRF microscopy cannot be used to visualize endocytic organelles, especially in cells embedded in 3D matrix.

2. “Some of the data present in Fig. 2 need to be properly quantified. The authors need to quantify the interaction of FIP5 GFP, TD-GFP and TA-GFP with kif3A-T and SNX18 (Fig. 2C).”

As suggested, quantification has been added to Figure 2C.

3. “The quantifications of FIP5 phosphorylation during cell cycle by western-blot were performed in cultures of synchronized HeLa cells. The authors should also demonstrate this specific phosphorylation in MDCK cells in metaphase. MDCK cells from different cell-cycle phases could be FACS-sorted by DNA content, and analyzed by western-blot to verify that G2/M cells contain more p-T276 FIP5 than G1/S cells.”

As suggested, we FACS-sorted MDCK cells at G1/G0 and G2/M phases and tested the levels of FIP5 and pFIP5-T276 by western blotting. In agreement with our previous conclusions, we have detected a higher level of pFIP5-T276 in G2/M cells. The new data is now in Figure 4G.

4. “The authors should include molecular weight markers in their blots (Figure 2, 4, S2 and S3) for better understanding of the experiments.”

As suggested, molecular weight markers have been added.

5. “There is no reference in the text of the recovery of the shFIP5 phenotype with FIP5SA (Fig. 3L, last bar in the graph). It should indicate “but FIP5SA did rescue the KD of endogenous FIP5 in cyst formation”

The manuscript has been edited to include the proposed statement.

#### Reviewer #3

1. “Some of the findings in Figure 1 confirm and extend those in an earlier paper on endosomes and MDCK cells (Hobdy-Henderson, et al. Traffic, 2003) which should be acknowledged.”

As suggested, the citation has been added to the manuscript.

2. *“The findings of the assay of SNX association with FIP5 are the only panel without quantitation, Since this assay is key to the findings, the results Figure 2C should be quantitated.”*

As suggested, quantification has been added to Fig. 2C.

3. *“On p9, the formation of multilumen cysts in the T276D cells is noted as a dominant negative phenotype for lumen formation. While it is certainly aberrant, it seems that a lack of lumen would be more consistent with dominant negative.”*

By “dominant negative” here, we meant T276D disrupts *single lumen* formation despite the existence of endogenous FIP5, while T276A does not have such effects. We did not mean to state that the phenotype of multi-lumen formation is a dominant negative effect compared to single lumen formation. We edited the manuscript to make it more clear.

4. *“The discussion of Serine 188 phosphorylation in FIP5 in the results section seems out of place since no data are presented related to Serine 188 phosphorylation. This would be better placed in the discussion.”*

The revised manuscript now contains data regarding FIP5-S188 phosphorylation and apical lumen formation (Figure 3E and L; Supplemental Figure 3A). As a result, we have left the discussion regarding FIP5-S188 phosphorylation in the Results section of the manuscript.

5. *“p8 first para: “Resent” should be recent.”*

“Resent” has been replaced with “Recent”.

2nd Editorial Decision

09 January 2014

I have now received feedback on your revised study from the three referees. As you will see, all referees are now positive about the study, although referee 1 asks that you revise one point of the text. I am thus happy to write with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once the remaining concern of referee 1 and a few other issues have been addressed, as follows.

- There are a few missing details in the figure legends regarding the analyses performed. In figures 2C, D, E and 3L, please indicate how many independent experiments were analyzed, what value is being represented (mean, median) and what type of errors (SD, SE...), as well as the statistical test applied and P values where necessary. The supplementary figure legends also lack these details; please go through these in detail and provide all necessary information; they should be as comprehensive as the legends to the main figures.

- Please provide figure 5 in a smaller format and with a consistent label size throughout both panels, in preparation for publication.

- Please indicate in the Material and Methods section of the main text that there are supplementary methods available online.

Once all remaining corrections have been attended to, you will receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Many thanks for your contribution to EMBO reports.

REFeree REPORTS:

Referee #1:

The authors have addressed the majority of my concerns. However, please note the following:

1. Page 8, lines 6-8 - the authors report that FIP5S188A is apically enriched, "suggesting that FIP5-S188 phosphorylation is required for FIP5 targeting during lumen formation." I don't think this conclusion is supported by the data.

Referee #2:

The authors have successfully answered all my points and the article is now ready for publication in EMBO Reports

Referee #3:

I believe that the authors have adequately responded to the criticisms leveled by the reviewers.

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2nd Revision - authors' response

16 January 2014

Final revision received.

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3rd Editorial Decision

17 January 2014

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.

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.

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