The cellular function of ROP GTPase prenylation is important for multicellularity in the moss *Physcomitrium patens*

Liang Bao, Junling Ren, Mary Nguyen, Arkadiusz Slawomir Slusarczyk, Julie M. Thole, Susana Perez Martinez, Jinling Huang, Tomomichi Fujita and Mark P. Running
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Original submission

First decision letter

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MS TITLE: Cellular function of ROP GTPase prenylation is important for multicellularity in the moss Physcomitrium patens

AUTHORS: Liang Bao, Junling Ren, Mary Nguyen, Arkadiusz Slawomir Slusarczyk, Julie Thole, Susana Perez Martinez, Jinling Huang, Tomomichi Fujita, and Mark Running

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This manuscript investigated the cellular defects of mutants defective in a geranylgeranylation enzyme (ggb) as well as mutants of ROP GTPases, putative substrates of GGB in the moss physcomitrium patens. By over-expressing prenylatable (CVIM for farnesyltransferase and CVIL for geranylgeranylation) as well as nonprenylatable (SVIL) forms of ROP GTPases, authors showed that the prenylatable form could rescue some of the defects of ggb including tip growth, multicellularity, and formation of gametophore. The data support that defects of GGB (resulting in unicellular form) is likely due to reduced activity of ROP as a result of a lack of prenylation. This is an interesting study which provides novel insights into how cellular properties are connected to multicellularity as well as the key role of prenylation for ROP function in these cellular properties.

Comments for the author

Main comments
It will help the readers if authors provide a summary diagram at the end (in Discussion). In the diagram, the relationships between the two different enzymes (PFT and PGGT-I) and their target modification sequences as well as how they impact the corresponding YFP-ROP (CVIM, CVIL, SVIL) transgenes are clearly illustrated and summarized.
In the TEM images of Figure 1, it is hard for this reviewer see what the authors described in the manuscript text regarding cell surface disruption such as ‘cell walls attach to 50% area of the cell.’ In fact, it is hard to determine if Figure 1B shows two cells or a single cell. Authors should provide some aids such as arrows outlines, labels to help readers understand the data.
In manuscript text, authors indicated that cell plate formation in ggb and ROP-amiRNA was defective. But this is not shown in Figure 2C as author implied.

Minor comments
In Figure 3, labeling of each panel (especially the three different forms of ROP) is small and hard to see.
Why does over-expressing YFP-ROP(CVIL or CVIM) in wild type cause cell swelling? This appears similar to (instead of opposite of) the ggb loss-of-function mutant phenotype. Some discussion or interpretation will be helpful.
Why did the authors use an inducible (XVE) system to express ROP-amiRNA instead of ROP-null mutants previously reported? Some explanation will be helpful.
In Figure 3AB, the restoration of filament growth is not as clear as the manuscript text implies. Would the filament formation be more obvious if the moss is grown for a longer period of time?
In the summary (Line 29-30), authors stated “the developmental transition from one dimension to two dimensions, to three dimensions in P. patens.” What does it mean? Do you mean growth dimension? Are filaments one dimension? What is two and three dimension then?

Reviewer 2

Advance summary and potential significance to field

The article by Bao et al builds on a previous study from the same laboratory describing that prenylation is required for cell elongation, adhesion and differentiation in the moss Physcomitrium patens. Here, the authors present various experiments suggesting that prenylation of ROP proteins, and notably ROP4, is necessary for maintaining multicellularity and cell expansion in moss. The work is related to a very hot topic in biology (ie. the evolution of multicellularity), and is highly novel and interesting, but it suffers from a significant number of shortcomings that should be addressed.

Comments for the author

1. Figure S1 : Please provide genotyping (PCR) data to confirm presence or integration of the transgene in the moss genome
2. Line 132-133 : "Five ROP-amiRNA lines were obtained, each showing cell elongation defects upon induction by -estradiol". Data are not shown in the paper, please add data in SI.

3. Line 146-147 : The authors propose that “cytokinesis is not affected, although the orientation of cell plate formation was defective in ggb mutants and ROP-amiRNA plants". Data supporting this claim are not shown.
4. Line 157 : "We have generated three YFP-tagged PpROP4 variants: YFP-PpROP4CVIM, with a consensus farnesyltransferase target motif; YFP- PpROP4CVIL, with a geranylgeranylation motif; and nonprenylatable YFP-PpROP4SVIL". The strategy is not clear : where the motifs modifications of the original coding sequence or were they added after the coding sequence? The mode of action of these additional (?) motifs and expected phenotypes caused by ROP variant overexpression should be explicitly described.
5. Line 164 : "YFP-PpROP4SVIL overexpression does not affect cell morphology in WT and ggb mutants". Unless the SVIL variant is a line in which the prenylation motif is dead and this motif is necessary for ROP function, this result is surprising given the expected effect of ROP overexpression on cell morphology (see Ito et al. 2014 - http://dx.doi.org/10.1016/j.gene.2014.04.057). Could you explain the absence of phenotype in this line?
6. Line 178 : Data presented in Figure S3 are very hard to interpret as protoplasts are not individualized, therefore the conclusions of this experiment are not credible. See Figure 3 from Thole et al 2014 in comparison. This experiment should be repeated and tropic responses should be quantified.
7. Line 166 : Data presented in Figure 3 are very interesting but have not been quantified. The authors should show adequate measurements supporting the following claims : “YFP-PpROP4CVIM overexpression induces cell swelling in WT and triggers tip growth and cell adhesion to form filamentous cells in ggb mutants”.
8. Line 204 (Discussion) : The results presented in Figure 1 are not convincing and do not fully support the claim that breaking of cell walls in ggb mutants is caused by defects in cell expansion. The authors should think of better way to show cell expansion defects.
9. Line 208 : The authors do not explain whether and at which position ROP4 is prenylated in WT, which weakens their conclusions about the contribution of ROP prenylation to the “loss of multicellularity” phenotype of ggb mutants. They could use CRISPR-mediated gene editing to generate rop4 variants in which the prenylation sites have been removed or modified to be non-functional. Along the same line, it is not clear why the authors have not tried to complement their ROP-amiRNA line with WT ROP4, and prenylatable and non-prenylatable ROP4 variants. This would demonstrate the extent to which prenylation is necessary for ROP function.
10. Line 104 : "We could identify two distinct stages in ggb mutants, an early stage when the surface of cell wall attaches to more than 50% area of the cells, and a later stage when the surface of cell wall detaches from more than 50% of cells (Fig. 1A,B). This indicates that the detachment of cells could be due to unregulated cell expansion." The causal relationship between observed defects and cell expansion is not clear, observed defects could be caused by other problems, e.g. cell wall integrity. Could you explain your choice please?
11. Figure 3 : It is not clear at all that YFP protein fusions are over-expressed in the different transgenic lines, notably in the middle and right panels of Figure 3B. The authors should use western blot or quantify YFP fluorescence intensity to demonstrate that YFP fusions are overexpressed, or at least they should demonstrate by quantitative RT-PCR that the different transgenes are overexpressed in comparison with untransformed controls.
12. Experiment shown in Figure 1C is not clear. What is the rationale for this experiment? Please explain in the main text what you did and observed.
13. Fig S2 : Please correct typos in the legend.
14. Line 188 : Data shown in Figure 3C should be quantified and the ROP4SVIL OE line should be included in the experiment.
15. Line 192 : The aim and conclusions of the experiment are not very clear. What is the rationale of the experiment? What do you conclude from the results?
16. Line 227 : Are the authors talking about the molecular or biological function of ROPs here? The negative results with human HsRAC1 and HsKRAS4b should be discussed further. What are the limits of this experiment? The discussion about CaaL and CaaX motifs is interesting and probably justifies the choice of Arabidopsis and human proteins in the experiment shown in Figure 4, but this should be introduced earlier in the results section.

First revision

Author response to reviewers' comments

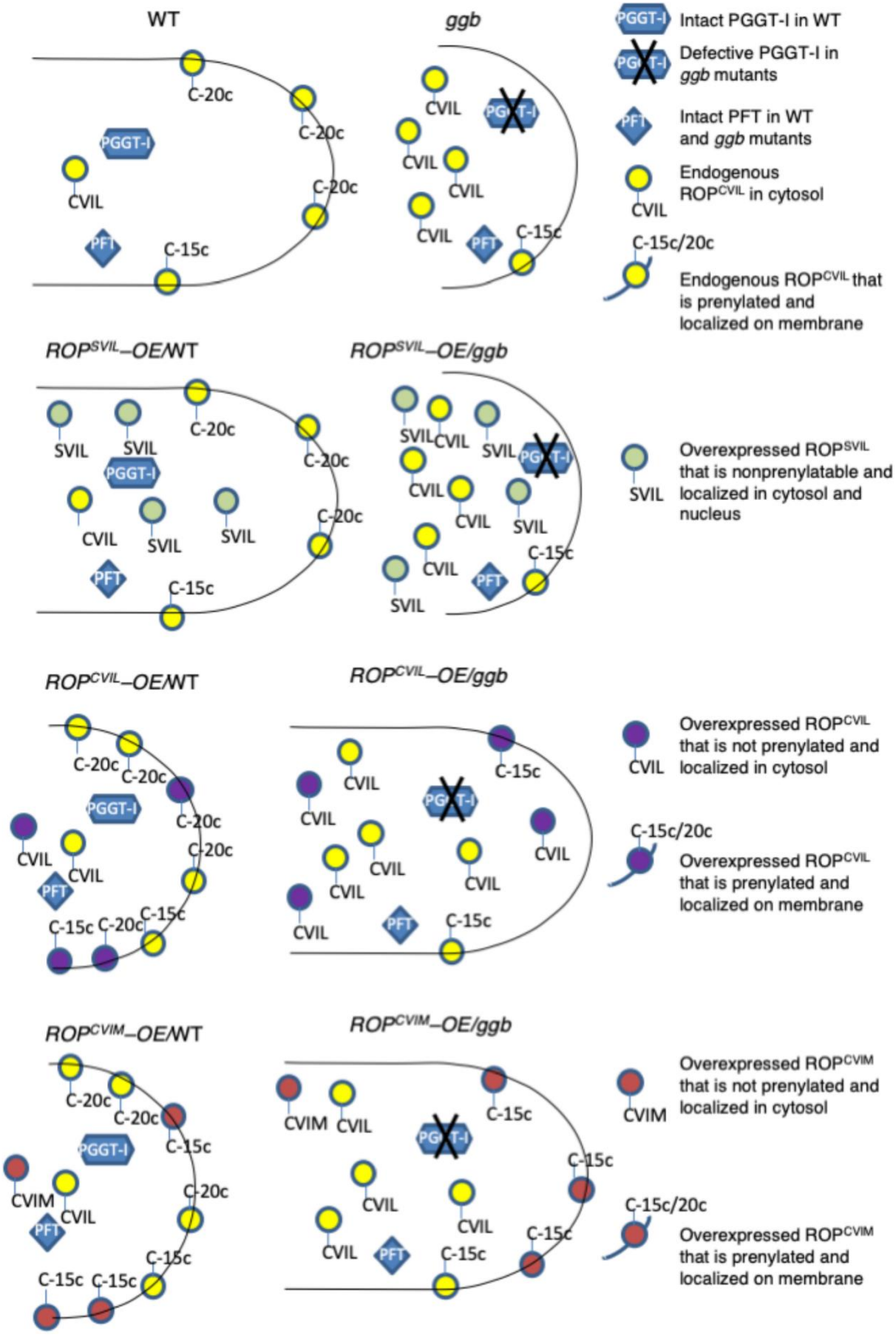
Reviewer 1 Advance Summary and Potential Significance to Field:
This manuscript investigated the cellular defects of mutants defective in a geranylgeranylation enzyme (ggb) as well as mutants of ROP GTPases, putative substrates of GGB in the moss physcomitrium patens. By over-expressing prenylatable (CVIM for farnesyltransferase and CVIL for geranylgeranylation) as well as nonprenylatable (SVIL) forms of ROP GTPases, authors showed that the prenylatable form could rescue some of the defects of ggb including tip growth, multicellularity, and formation of gametophore. The data support that defects of GGB (resulting in unicellular form) is likely due to reduced activity of ROP as a result of a lack of prenylation. This is an interesting study which provides novel insights into how cellular properties are connected to multicellularity as well as the key role of prenylation for ROP function in these cellular properties.

>>Thank you very much for your positive response and for the comments to our manuscript. Here are our responses to your comments and we hope this has answered your questions adequately.

Reviewer 1 Comments for the Author:
Main comments

#1, It will help the readers if authors provide a summary diagram at the end (in Discussion). In the diagram, the relationships between the two different enzymes (PFT and PGGT-I) and their target modification sequences, as well as how they impact the corresponding YFP-ROP (CVIM, CVIL, SVIL) transgenes are clearly illustrated and summarized.

>> We thank the reviewer's suggestion regarding the target modification sequences and impact on transgenes. We had a model figure in the Manuscript, the Supplemental Fig4. We have now revised the model with carbon modification (15c for 15 carbon farnesyl group and 20c for 20 carbon geranylgeranyl group) and impact on ROP localization (membrane vs cytosolic localization) and on development of transgenes. With the permission from the editor we can put it into the main text as Figure 5.



#2, In the TEM images of Figure 1, it is hard for this reviewer see what the authors described in the manuscript text regarding cell surface disruption such as ‘cell walls attach to 50% area of the cell.’ In fact, it is hard to determine if Figure 1B shows two cells or a single cell. Authors should provide some aids such as arrows, outlines, labels to help readers understand the data.

>>We thank the reviewer for this comment. We understand that the TEM does not show how much of the surface cell wall is detached. Therefore, we incorporated the calcofluor white images for live imaging to the new figure S1, showing that more than half of cell surface was detached (Dash lines below each image indicate the broken surface cell walls on the expanding cells, and orange lines indicate the cells beneath the cell wall surface in *ggb*). Because of this, we have also added the description of methods regarding the live imaging by the calcofluor white staining.

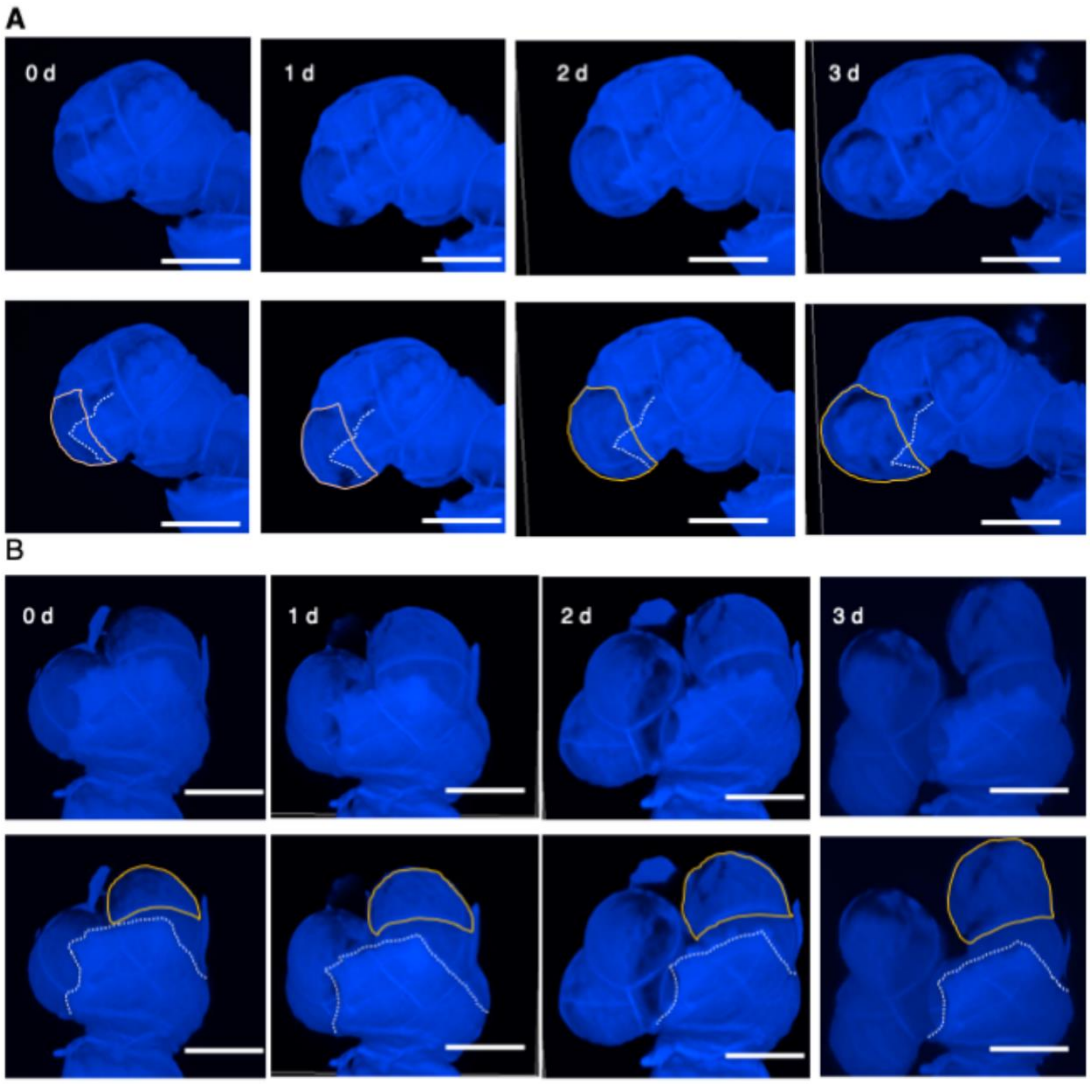


Fig. S1| 3-D time series (three days in total) showing the development of *ggb*. Two plants are shown for early **(A)** and later **(B)** development of cell separation. Note that, the area of cell wall surface covering the cell was gradually decreased from day 0 to day 3. White dash lines below each image indicate the broken surface cell walls on the expanding cells, and orange lines indicate the cells beneath the cell wall surface in *ggb*. Scale bar: 20 μm.

#3, In manuscript text, authors indicated that cell plate formation in ggb and ROP-amiRNA was defective. But this is not shown in Figure 2C as author implied.

>>In WT, all newly formed cell plates in protonemata connect two side walls. However, in *ggb* mutants and *ROP-amiRNA* plants, some newly formed cell plates connect the cross walls. To show this more clearly, we added two more images regarding cell plate formation defects (marked in the images) in *ggb* (A) and *ROP-amiRNA* (B) plants as the new figure S3.

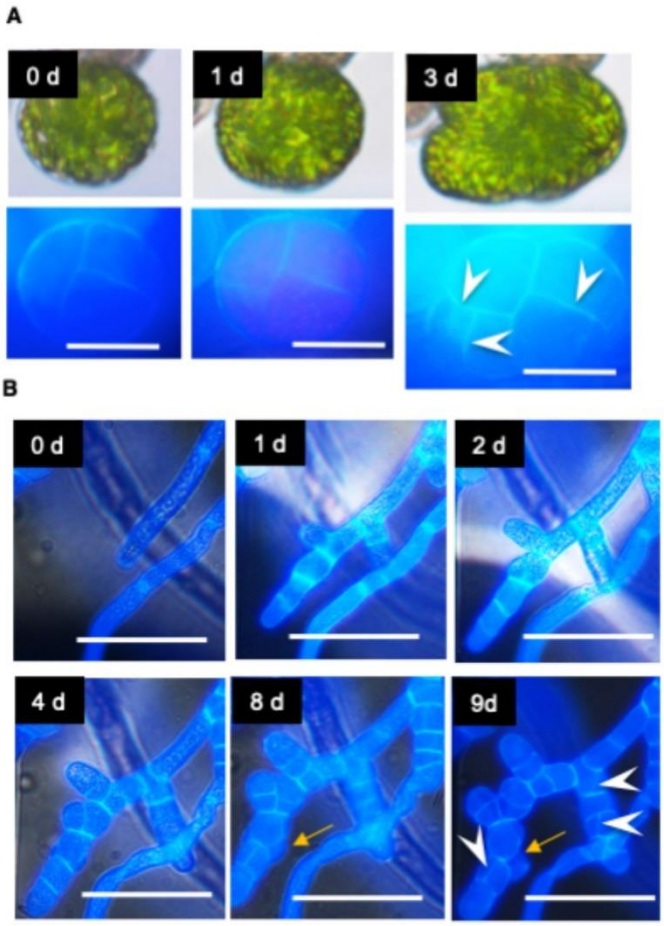


Fig. S3| Defect of cell plate orientation in *ggb* mutants and *ROP-amiRNA* plants. Live imaging of *ggb* mutants for 3 days **(A)** and ROP-*amiRNA#26* induced by 1 μM ß-estradiol for 9 days **(B)**. Note that cell separation occurred on 8 days after inducible knockdown (yellow arrows). Arrow heads show randomized cell division orientation that were shown in last day.
Scale bar:20 μm **(A)** and 100 μm **(B)**.

Minor comments

#4, In Figure 3, labeling of each panel (especially the three different forms of ROP) is small and hard to see.
>>We thank the reviewer for pointing this out. We have increased the font size from 9 to 10.5 in Figure 3. For the Figure 4, S5 and S6 that contain labels of the three forms of ROP as well, we also have increased the font size to be 10.5. We also put the labels outside of the images to increase the contrast of the labels.

#5, Why does over-expressing YFP-ROP(CVIL or CVIM) in wild type cause cell swelling? This appears similar to (instead of opposite of) the ggb loss-of-function mutant phenotype. Some discussion or interpretation will be helpful.

**>>We believe this is explained by the possibility that balanced function of YFP-ROP(CVIL or CVIM) is required for cell morphology, and incorporated it into our model in figure 5.
A previous paper from the Fujita lab (Ito, 2014) showed that overexpressed wildtype ROP with intact CVIL also causes cell swelling; we have added this reference to the paper.**

Ito, K., Ren, J. and Fujita, T. (2014). Conserved function of Rho-related Rop/RAC GTPase signaling in regulation of cell polarity in *Physcomitrella patens*. *Gene* **544**, 241-247.

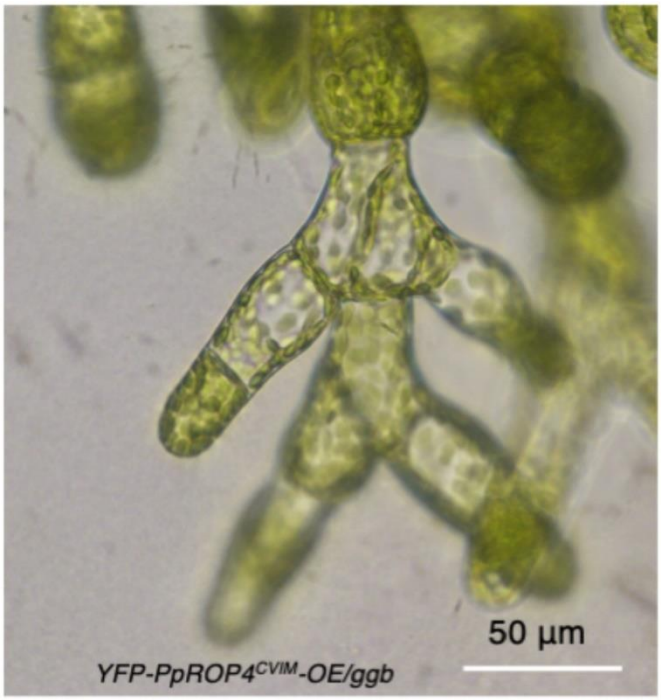
#5, Why did the authors use an inducible (XVE) system to express ROP-amiRNA instead of ROP-null mutants previously reported? Some explanation will be helpful.

>>A previous report from Gushima's lab said ROP-null mutants cannot survive in their current biology paper (Yi and Goshima, 2020). When commencing this study, we suspected that if all ROPs are mutated, the result would be lethality as well. In contrast, in the *ggb* mutant background, ROPs are still partially functional because of PFT. We therefore decided to use ROP-knockdown.
An inducible (XVE) system would allow us to get stable transgenic mutants of ROP- knockdown line when ROP-amiRNA is not induced in wild type background. Moreover, our data is consistent with the results of ROP-null reported in the Benzanilla lab (Cheng et al., 2020), and they showed that ROP-null can survive and showed a *ggb*-like phenotype.

Yi, P. and Goshima, G. (2020). Rho of Plants GTPases and cytoskeletal elements control nuclear positioning and asymmetric cell division during *Physcomitrella patens* branching. *Curr Biol* **30**, 2860-2868.e2863.
Cheng, X., Mwaura, B. W., Chang Stauffer, S. R. and Bezanilla, M. (2020). A fully functional ROP fluorescent fusion protein reveals roles for this GTPase in subcellular and tissue-level patterning. *Plant Cell* **32**, 3436–3451.

#6, In Figure 3AB, the restoration of filament growth is not as clear as the manuscript text implies. Would the filament formation be more obvious if the moss is grown for a longer period of time?

>>Yes. *YFP-PpROP4^{CVIM}-OE/ggb* can have slightly longer filaments if induction is for a longer period of time. But not as long as that of WT. The tip growth of *ggb* mutants is restored but not completely restored. For example, while the cell length of apical cell in WT can be up to 100 to 150 µm, the length of apical cells in *YFP-PpROP4^{CVIM}-OE/ggb* can be from 30 to 60 µm even after three weeks of induction.



#7, In the summary (Line 29-30), authors stated "the developmental transition from one dimension to two dimensions, to three dimensions in P. patens." What does it mean? Do you mean growth dimension? Are filaments one dimension? What is two and three dimension then?

>> We thank the reviewer for pointing this out. We added an explanation for 1D, 2D and 3D in the text. "The linear organization of protonemal cells without branches are one dimensional, the protonemal cells with branches are two dimensional and the gametophores are three dimensional (Moody et al., 2021)."

Moody, L. A., Kelly, S., Clayton, R., Weeks, Z., Emms, D. M. and Langdale, J. A. (2021). NO GAMETOPHORES 2 Is a Novel Regulator of the 2D to 3D Growth Transition in the Moss Physcomitrella patens. *Curr Biol* **31**, 555-563.e554.

Reviewer 2 Advance Summary and Potential Significance to Field:
The article by Bao et al builds on a previous study from the same laboratory describing that prenylation is required for cell elongation, adhesion and differentiation in the moss Physcomitrium patens. Here, the authors present various experiments suggesting that prenylation of ROP proteins, and notably ROP4, is necessary for maintaining multicellularity and cell expansion in moss. The work is related to a very hot topic in biology (ie. the evolution of multicellularity), and is highly novel and interesting, but it suffers from a significant number of shortcomings that should be addressed.

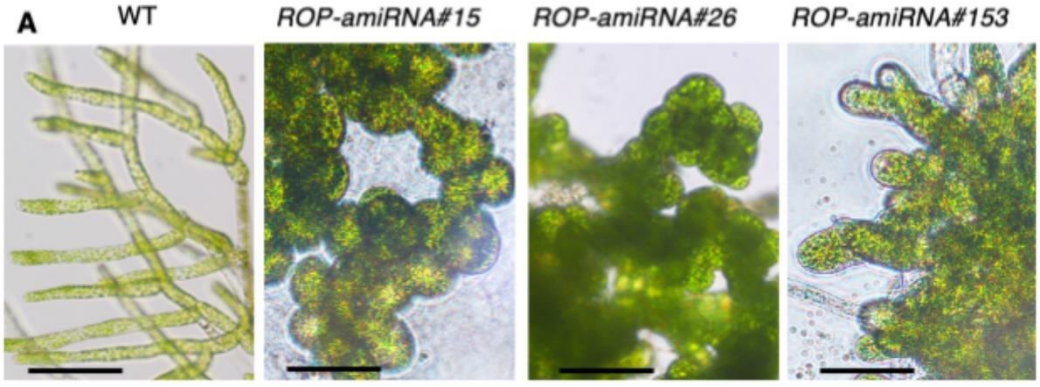
>> Thank you very much for your positive evaluation and the interest in the multicellularity. Hereafter, we reply all of your comments and we hope this has answered your questions adequately.

Reviewer 2 Comments for the Author:
1. Figure S1 : Please provide genotyping (PCR) data to confirm presence or integration of the transgene in the moss genome

>>We performed genotyping to confirm the integration of the transgene, but we do not have access to the original gel images. Because the phenotype was seen in multiple independent lines upon β-estradiol induction, is stable, and displays the expected phenotype based on published data, we are confident that the phenotype results from integration of the transgene.

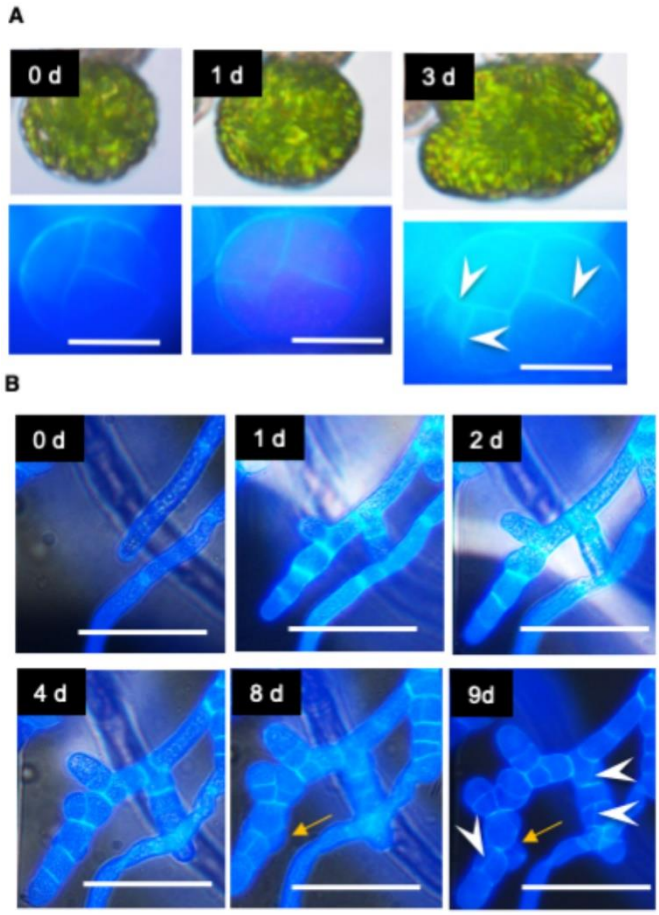
2. Line 132-133 : "Five ROP-amiRNA lines were obtained, each showing cell elongation defects upon induction by -estradiol". Data are not shown in the paper, please add data in SI.

>>We have two lines #15 and #26 and also other lines that showed cell elongation defects upon β-estradiol induction, but #15 and #26 showed more severe effect upon induction. So we added the pictures of #15 and #153 as in Fig S2.



3. Line 146-147 : The authors propose that “cytokinesis is not affected, although the orientation of cell plate formation was defective in ggb mutants and ROP-amiRNA plants". Data supporting this claim are not shown.

>>This was also pointed out by the first reviewer. We will copy the response from the above. In WT, all newly formed cell plates in protonemata connect two side walls. However, in *ggb* mutants and *ROP-amiRNA* plants, some newly formed cell plates connect the cross walls. To show this more clearly, we added two more images regarding cell plate formation defects (marked in the images) in *ggb* (A) and *ROP-amiRNA* (B) plants as the new figure S3.



4. Line 157 : "We have generated three YFP-tagged PpROP4 variants: YFP-PpROP4CVIM, with a consensus farnesyltransferase target motif; YFP- PpROP4CVIL, with a geranylgeranylation motif; and nonprenylatable YFP-PpROP4SVIL". The strategy is not clear : where the motifs modifications of the original coding sequence or were they added after the coding sequence? The mode of action of these additional (?) motifs and expected phenotypes caused by ROP variant overexpression should be explicitly described.

>>The wildtype form of ROPs in *P. patens* is terminated with CVIL at the C-terminal. We have changed one amino acid of the CVIL motif to produce ROP-CVIM or ROP-SVIL. To mutate the CVIL motif, we have added the corresponding sequences in the reverse primers and introduced the point mutation by PCR. We thank the reviewer's comment and realized we need to explicitly describe the strategy. We have revised this in the Method section.

"YFP-PpROP4^{CVIL}, YFP-ROP4^{CVIM} and YFP-PpROP4^{SVIL} were amplified by using pCAM- YFP-PpROP4 as a template and the CVIL, CVIM and SVIL point mutations at the c-terminal were introduced by using a corresponding reverse primer (**Table S1**)." **We also highlighted the primers that were used for introducing the point mutation in the reverse primers and marked the CVIL, CVIM and SVIL motif in red.**

CVIM would be farnesylated by PFT in *ggb* mutants, but both geranylgeranylated by PGGT-I and farnesylated by PFT in WT.
Similarly, CVIL would be both geranylgeranylated by PGGT-I and farnesylated by PFT in WT, but in the *ggb* mutants, CVIL can also be farnesylated.
However, SVIL can neither be geranylgeranylated by PGGT-I and farneylated by PFT because the Cysteine is changed to Serine. We have now explained this in the legend of Fig 5.

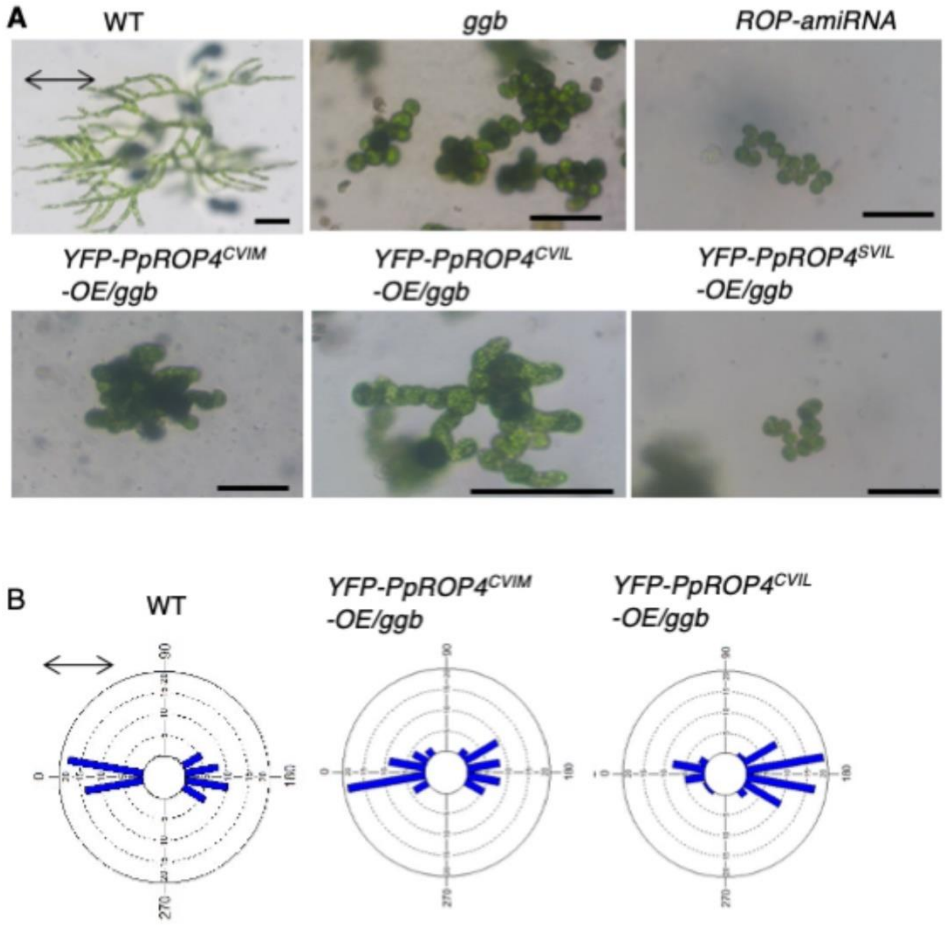
5. Line 164 : "YFP-PpROP4SVIL overexpression does not affect cell morphology in WT and ggb mutants". Unless the SVIL variant is a line in which the prenylation motif is dead and this motif is necessary for ROP function, this result is surprising given the expected effect of ROP

overexpression on cell morphology (see Ito et al. 2014 - http://dx.doi.org/10.1016/j.gene.2014.04.057). Could you explain the absence of phenotype in this line?

>>Yes. The YFP-PpROP4^{SVIL} terminated with SVIL is a mutation from the wildtype C of CVIL to S of SVIL. Because the C for cystine is the amino acid that is prenylated, mutation from Cystine to serine will result in a protein that cannot be prenylated.
In the paper (Ito et al. 2014 - http://dx.doi.org/10.1016/j.gene.2014.04.057), the authors used the overexpressed wildtype form of PpROP2, which is terminated with CVIL, and they found overexpression of wildtype form of PpROP2 caused cell swelling and depolarized growth of tip-growing apical cells; this result can only be obtained when ROP is prenylated. We note that two of the authors of the Ito paper (Junling Ren and Tomomichi Fujita) are also coauthors in this paper.

6. Line 178 : Data presented in Figure S3 are very hard to interpret as protoplasts are not individualized, therefore the conclusions of this experiment are not credible. See Figure 3 from Thole et al 2014 in comparison. This experiment should be repeated and tropic responses should be quantified.

>>We previously quantified the data but we did not include it in the paper. Since the reviewer asked, we have incorporated quantification data as part B of into what is now the Figure S5 (the previous figure S3)



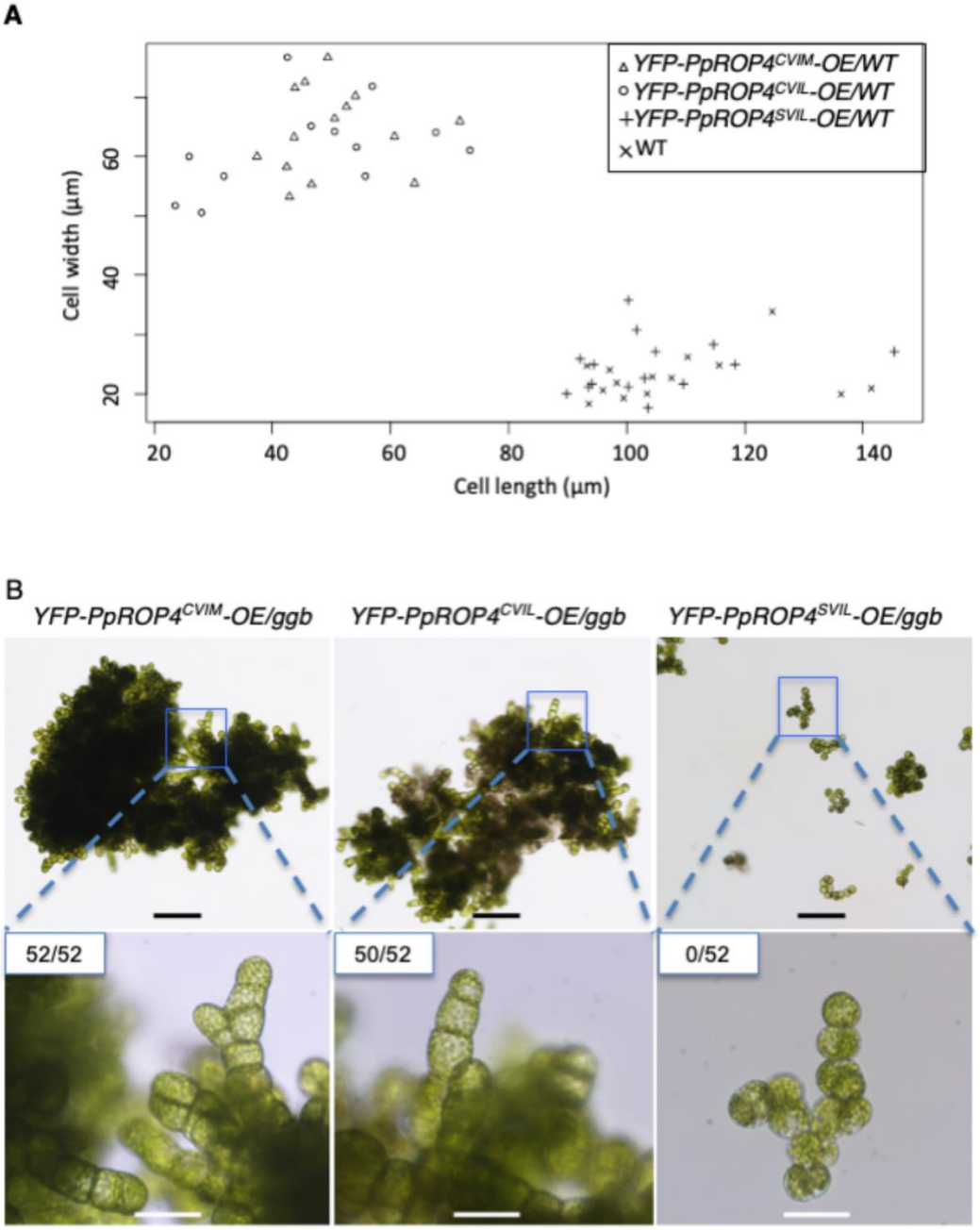
B, Protonemata distribution of WT (n=61) and PpROP4^{CVIM}/*ggb* (n=70) and PpROP4^{CVIL}/*ggb* (n=75) under polarized white light.

7. Line 166 : Data presented in Figure 3 are very interesting but have not been quantified. The authors should show adequate measurements supporting the following claims : “YFP-

PpROP4CVIM overexpression induces cell swelling in WT and triggers tip growth and cell adhesion to form filamentous cells in ggb mutants".

>>We thank the reviewer's comments and we added the quantitative data to figure 3 showing the cell length and the cell width of *YFP-PpROP4^{CVIM}-OE/WT* and *YFP-PpROP4^{CVIL}-OE/WT*, *YFP-PpROP4^{SVIL}-OE/WT* and WT. As we can see from the scatter plot below (A), while the cell width is greater in *YFP-PpROP4^{CVIM}-OE/WT* and *YFP-PpROP4^{CVIL}-OE/WT* compared to *YFP-PpROP4^{SVIL}-OE/WT* and WT, the cell length is smaller in *YFP-PpROP4^{CVIM}-OE/WT* and *YFP- PpROP4^{CVIL}-OE/WT* compared to *YFP-PpROP4^{SVIL}-OE/WT* and WT.

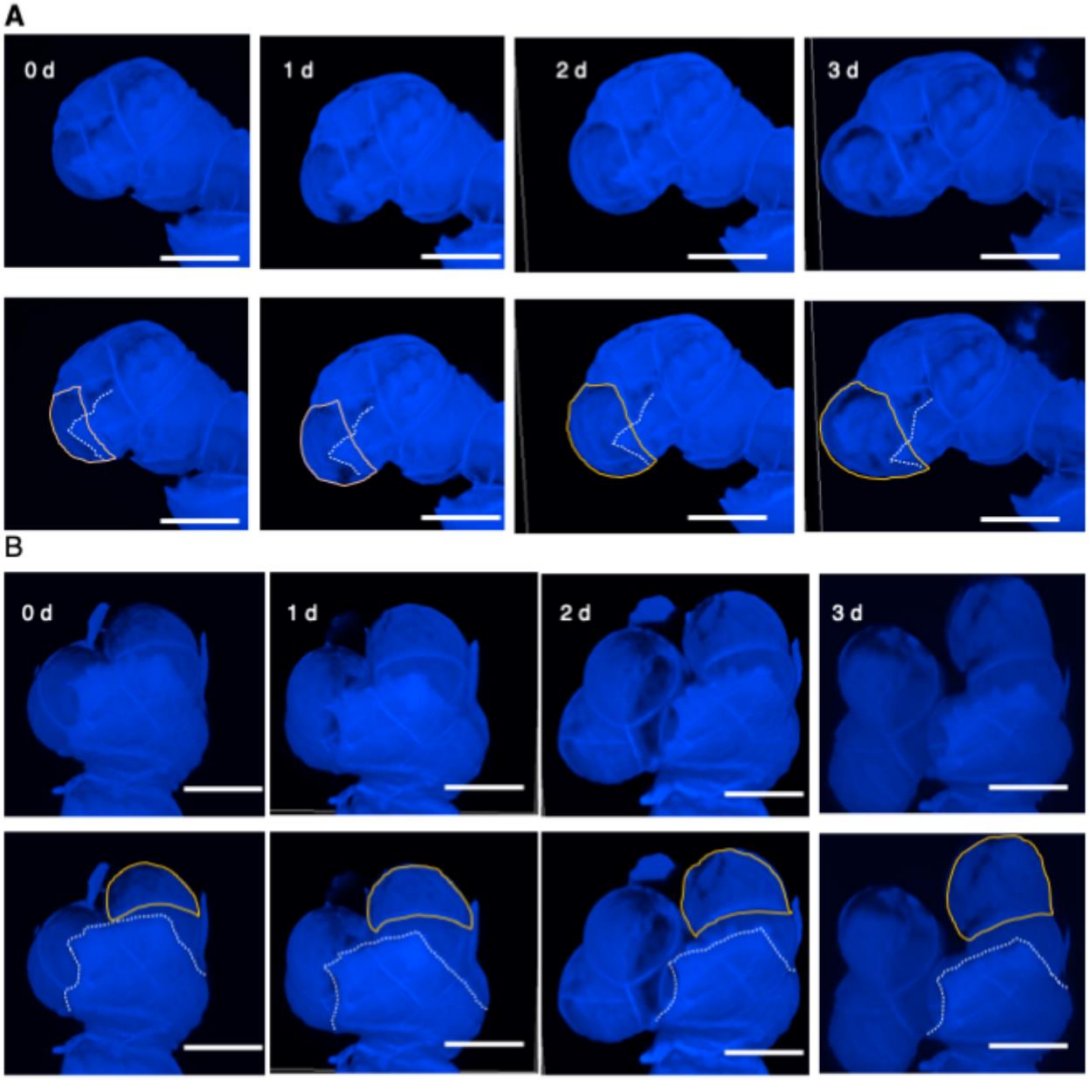
To show the tip growth of ggb mutants are rescued (forming filamentous cells) by expressing YFP-PpROP4^{CVIM} and YFP-PpROP4^{CVIL}, we have counted 52 plants each of *YFP-PpROP4^{CVIM}- OE/ggb* and *YFP-PpROP4^{CVIL}-OE/ggb* and *YFP-PpROP4^{SVIL}-OE/ggb* that contain at least three linear cells connected. Most plants in *YFP-PpROP4^{CVIM}-OE/ggb* and *YFP-PpROP4^{CVIL}-OE/ggb* formed filamentous cells while *YFP-PpROP4^{SVIL}-OE/ggb* did not. This figure was listed as Fig S5.



8. Line 204 (Discussion) : The results presented in Figure 1 are not convincing and do not fully support the claim that breaking of cell walls in ggb mutants is caused by defects in cell expansion. The authors should think of better way to show cell expansion defects.

>> **We understand the reviewer's concern, in that Figure 1 showed breaking of cell walls using SEM and TEM, which may be subject to artifacts. We added a live imaging figure (the new Figure S1) to additionally support our conclusion that tearing occurs due to cell expansion.**

In the live imaging staining the cell wall by calcofluor white, we could see the broken cell wall surface was torn.



9. Line 208 : The authors do not explain whether and at which position ROP4 is prenylated in WT, which weakens their conclusions about the contribution of ROP prenylation to the "loss of multicellularity" phenotype of *ggb* mutants. They could use CRISPR-mediated gene editing to generate rop4 variants in which the prenylation sites have been removed or modified to be non-functional. Along the same line, it is not clear why the authors have not tried to complement their *ROP-amiRNA* line with WT ROP4, and prenylatable and non-prenylatable ROP4 variants. This would demonstrate the extent to which prenylation is necessary for ROP function.

>> **Thank you for your comments. For readers unfamiliar with the prenylation processes, we added clarification in the text to reflect previous findings that prenylation only occurs on cysteines within five (and nearly always four) amino acids of the C terminus:** "There are two prenylation enzymes, farnesyltransferase (PFT) and geranylgeranyltransferase I (PGGT-I), that can recognize the C-terminal CaaX/CaaL four amino acid sequence (CaaX-box, where C is the prenylated cysteine, a is usually an aliphatic amino acid, and X is usually alanine, cysteine, glutamine, methionine, or serine for PFT and almost always leucine, namely CaaL box, for PGGT-I)" **Also, based on previous studies of ROP and other GTPases, ROP can only be prenylated on the C of the CVIL at the C terminus. This is consistent with the similarity in phenotype between the *ggb* mutant and loss of function Rop mutants (Cheng et al., 2020), since prenylation is required for the function of ROPs. We did not use a CRISPR-mediated system, but we used point-mutation in the CaaL motif, and overexpression of ROP with the CVIL or CVIM is active, but not with SVIL. We have thus demonstrated that loss of prenylation of ROP is the reason for the *ggb* phenotype. Further complementation experiments, such as with a miRNA-resistant ROP4, are not feasible in the time frame provided for revision, but would also likely not alter the conclusion that prenylation of the CVIL cysteine is necessary for ROP**

function given the evidence we present.

Cheng, X., Mwaura, B. W., Chang Stauffer, S. R. and Bezanilla, M. (2020). A fully functional ROP fluorescent fusion protein reveals roles for this GTPase in subcellular and tissue-level patterning. *Plant Cell* **32**, 3436–3451.

10. Line 104 : "We could identify two distinct stages in ggb mutants, an early stage when the surface of cell wall attaches to more than 50% area of the cells, and a later stage when the surface of cell wall detaches from more than 50% of cells (Fig. 1A,B). This indicates that the detachment of cells could be due to unregulated cell expansion." The causal relationship between observed defects and cell expansion is not clear, observed defects could be caused by other problems, e.g. cell wall integrity. Could you explain your choice please?

>> We cannot completely rule out other reasons, but uncontrolled cell expansion is the most likely explanation, because we see the surface of cell wall is torn apart in regions of cell expansion and not in other regions (again visible in live images that were added as the new Figure S1).

11. Figure 3: It is not clear at all that YFP protein fusions are over-expressed in the different transgenic lines, notably in the middle and right panels of Figure 3B. The authors should use western blot or quantify YFP fluorescence intensity to demonstrate that YFP fusions are overexpressed, or at least they should demonstrate by quantitative RT-PCR that the different transgenes are overexpressed in comparison with untransformed controls.

>>**We thank the reviewer's comments on the expression level. We are satisfied by the YFP levels in micrographs and the rescue of the tip growth phenotype of *ggb* mutants. As we can see from Figure 3B, the YFP signal is located to the plasma membrane in *YFP-PpROP4*^{CVIL}*/ggb* and *YFP-PpROP4*^{CVIM}*/ggb* and to cytosol and nucleus in *YFP-PpROP4*^{SVIL}*/ggb*. Only YFP-PpROP4^{CVIL} and YFP-PpROP4^{CVIM} rescued the tip growth defects of the *ggb* mutants. Therefore, the overexpression of ROPs are sufficient and we are confident the expression level is enough because we can see enough signals in the fluorescence microscope and phenotypic restoration.**

12. Experiment shown in Figure 1C is not clear. What is the rationale for this experiment? Please explain in the main text what you did and observed.
>> For Figure 1C, we wanted to investigate if the cause of cell separation is due to uncontrolled cell expansion.
We thank the reviewer's suggestions, and we have added to the description of how to interpret the result in the text.
"Comparison of microsphere staining patterns between different time points allows us to detect the area of expansion on cell surface over time periods. No change of staining patterns on an area indicates no cell expansion on that area of cell surface, while a change of staining patterns indicates cell expansion."

To show this clearly, we added live imaging data (new Figure S1). Also because of this, we have added methods for the live imaging experiment in the Method section.

13. Fig S2 : Please correct typos in the legend.
>>Yes, we have corrected the typos. Thank you.

14. Line 188 : Data shown in Figure 3C should be quantified and the ROP4SVIL OE line should be included in the experiment.

>>For Fig 3C, we have grown 4 independent lines of *ROP4*^{CVIL}*-OE/ggb*, *ROP4*^{CVIM}*-OE/ggb* and *ROP4*^{SVIL}*-OE/ggb* on ß-estradiol (inducing) plates. Every transgenic line showed a consistent phenotype in terms of gametophore development:
***ROP4*^{CVIL}*-OE/ggb*: did not rescue the gametophore development**

***ROP4^{CVIM}-OE/ggb*: rescued the gametophore development**
and *ROP4^{SVIL}-OE/ggb*: did not rescue the gametophore development. We added a picture of *ROP4^{SVIL}-OE/ggb* in Fig 3C.
15. Line 192 : The aim and conclusions of the experiment are not very clear. What is the rationale of the experiment? What do you conclude from the results?

>> **We wanted to test if ROPs from vascular plants have a conserved function with PpROP4. We also wanted to test if other RHO family G proteins, for example from human, could have a similar effect. We thank the reviewer for pointing out that the rational for this experiment was not clear, and we have modified the heading, in order to describe that prenylation of AtROP1 rescues *ggb* (in which most PpROP function is lost) but other RHO family G proteins cannot, showing conservation of ROP function among distant plants but that conservation of RHO-family small GTPase function is not observed among eukaryotes in general.**

"Expressing prenylatable *Arabidopsis* AtROP1 rescues multicellular defects of *ggb* mutants while Human HsRAC1 and HsKRAS4b do not"

16. Line 227 : Are the authors talking about the molecular or biological function of ROPs here? The negative results with human HsRAC1 and HsKRAS4b should be discussed further. What are the limits of this experiment? The discussion about CaaL and CaaX motifs is interesting and probably justifies the choice of Arabidopsis and human proteins in the experiment shown in Figure 4, but this should be introduced earlier in the results section.
>>We thank the reviewer for pointing this out. We have added a sentence describing this before we show the results.

"ROPs belongs to the Ras superfamily of small GTPases that contains five major families: Ras, Rho, Arf/Sar, Ran, and Rab, and plant ROPs are most closely homologous to RACs of the Rho family (Rojas et al., 2012), but ROPs form a single, distinct clade (Eklund et al., 2010). Thus we wanted to test if the prenylation/ROP module function for multicellularity is conserved by expressing *Arabidopsis* AtROP1 and Human HsRAC1 and HsKRAS4b in *ggb* mutants. AtROP1 and HsRAC1 belong to the Rho family and HsKRAS4b belongs to the RAS family (Rojas et al., 2012)."

We also added a line in the Discussion mentioning the significance: "suggesting the conserved function of ROPs between *Arabidopsis* and *P. patens* but not a general conservation among Rho GTPases or other GTPases of the RAS superfamily in eukaryotes."

Second decision letter

MS ID#: DEVELOP/2021/200279

MS TITLE: Cellular function of ROP GTPase prenylation is important for multicellularity in the moss Physcomitrium patens

AUTHORS: Liang Bao, Junling Ren, Mary Nguyen, Arkadiusz Slawomir Slusarczyk, Julie Thole, Susana Perez Martinez, Jinling Huang, Tomomichi Fujita, and Mark Running
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The authors have satisfactorily addressed all of my comments. The work uncovers the role of prenylation of ROP GTPase on multicellularity, which is novel and significant.

Comments for the author

The only minor comment for this revised manuscript is Figure S2BC legend; it does not mention which ROP-amiRNA line was analyzed with the qRT-PCR. It maybe helpful to give the gene IDs of ROP1, 2, 3, and 4 in the legend as well.
The inclusion of the summary model (Figure 5) is very helpful to the readers.

Reviewer 2

Advance summary and potential significance to field

The article by Bao et al builds on a previous study from the same laboratory that described that prenylation is required for cell elongation, adhesion and differentiation in the moss Physcomitrium patens. Here, the authors present various experiments suggesting that the prenylation of ROP proteins, and notably ROP4, is necessary for maintaining multicellularity and cell expansion in moss. The work is related to a very hot topic in biology (ie. the evolution of multicellularity), and is highly novel and interesting.

Comments for the author

The authors have done a sincere effort to address all the comments raised by reviewer 1 and myself, and the revised version of the manuscript reads very well.
I have only a few minor remarks :
1. It would be nice to explain in the legend of Figure S6B what the new data show (eg. angle of filament growth with respect to unidirectional light?) and indicate the unit on the plots.
2. Page 9 : “the rescued gametophore is substantially smaller…” should be changed to “rescued gametophores are substantially smaller…”
3. Page 9 : “ROPs belongs to…” should be changed to “ROPs belong to…”
4. Page 11 : “suggesting the conserved function of ROPs between Arabidopsis and P. patens”. I guess the authors are talking about the “molecular function” of ROPs, this should be clarified.