Heterogeneous Nuclear Ribonucleoprotein H1 Coordinates with Phytochrome and the U1 snRNP Complex to Regulate Alternative Splicing in *Physcomitrella patens*

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REPORT: (The report shows the major requests for revision and author responses. Minor comments for revision and miscellaneous correspondence are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

TPC2017-00719-BR 1st Editorial decision – *declined* Oct. 13, 2017

While all agree that light regulation of alternative splicing is an important and timely question, the reviewers argue that the claims are not supported by the current experiments. That said, we would be willing to re-consider a new manuscript that fully addresses the concerns raised during this review process.

---------- Reviewer comments:

[Reviewer comments shown below along with author responses]

Reviewer #1

Using the moss *P. patens*, the authors report that PHY4, an ortholog of Arabidopsis phyB red/far-red photoreceptor interacts with RNP-H1, a splicing factor in a red-light dependent manner. In addition, RNP-H1 interacts with the splicing-related factor PRP39, which in turn associates with U1 snRNP. Using RNA-seq analysis, they report that *prp39* mutants have alternative splicing defects when compared to the WT. However, this paper could have been interesting, but many conclusions are not supported and justified by the data. In addition, this manuscript is raising more questions than it is able to answer. Below are my detailed critiques:

Point 1. This manuscript has several grammatical and sentence structure errors. Written English needs to be improved significantly.

RESPONSE: We appreciate the comment. The English writing was thoughtfully checked in the latest manuscript.

Point 2. It is suggested that red-light activated PHY4 strongly interacts with RNP-H1 (Figure 1A) in a Y2H qualitative (visual) assay without any quantitation. However, it is difficult to draw this conclusion using a heterologous system, because in the dark and far-red there is still interaction between them.

RESPONSE: We appreciate the comment. A quantitative yeast two-hybrid (Y2H) assay was done in the latest manuscript (Figure 1B). We found that in yeast, red light (RL) indeed significantly promotes the interaction between PpPHY4 and PphnRNP-H1. Since in the Y2H assay, proteins of interest were located in the nucleus, we believe this

is the reason that we detected weak interactions in the dark and far red light conditions. Since PpPHY4 locates in the cytoplasm of the plant cell under darkness (Supplemental Figure 2), it interacts with nuclear PphnRNP-H1 after RL activation in planta, as we found (Figure 1C).

Point 3. Can PRP39 interact with PHY4, or is its interaction limited to RNP-H1?

RESPONSE: We tested the interaction between PpPHY4 and PpPRP39 by using both BiFC and Y2H and could not detect the interaction.

Point 4. Figure 1B: It is known that phy's translocate to the nucleus in red light. What is the localization of RNP-H1 so that the BiFC presented in this experiment could be evaluated accordingly. Some RNP's are known to shuttle between the nucleus and the cytoplasm.

RESPONSE: PphnRNP-H1 locates only in the nucleus, as shown in Supplemental Figure 2. However, since the phytochromes translocate into the nucleus after receiving light, the interaction between PpPHY4 and PphnRNP-H1 occurs in the nucleus, as we have observed.

Point 5. It is suggested that light regulates alternative splicing (AS), and one of the mechanism is the regulation of this process by PHY4 through its interaction with the RNP-H1 and PRP39 complex. The authors fail to isolate loss-of-function mutants for RNP-H1. In Figure 4, using RNA-seq, it is indicated that the *prp39* mutant has alternative splicing defects in the light when compared with the WT. However, the *phy4* mutant was not included in this experiment to show that PRP39 indeed functions downstream of PHY4 and they both have similar AS defects. This omission is scientifically not appropriate, and therefore the data presented here does not support the conclusion and the message that PHY4 controls AS via RNP-H1 and PRP39 in the light.

RESPONSE: In our latest version of manuscript, we reported the results from a *hnrnp-h1* mutant we recently obtained. We performed genome-wide analyses to compare the AS pattern in WT, *hnrnp-h1* and *phy4*. We also compared the AS profiles in the *prp39-1* mutant with WT, *hnrnp-h1* and *phy4*. In the AS analyses, we found that PpPRP39-1, PphnRNP-H1 and PpPHY4 co-regulated a significant number of IR events, and therefore we concluded that PpPHY4 controls AS via PphnRNP-H1 and PpPRP39-1 when plants receive light.

Point 6. What is the phenotype when RNP-H1 is overexpressed? Does it phenocopy PHY4 overexpression, as shown in Figure 5? This information will be important, as *rnp-h1* mutants are not available.

RESPONSE: We appreciate the comment. Since we have obtained the *hnrnp-h1* mutant, we tested the phenotype of the *hnrnp-h1* mutant and found that it is similar to that of the *phy4* mutant (Figure 2).

Point 7. Can it be tested that PHY4, RNP-H1, and PRP39 are in the same complex at the same time in the light?

RESPONSE: We thank the reviewer for the comment. Currently, we do not have a PRP39 overexpressing line or PRP39 antibody to test for complex formation between these proteins. We are working on this.

Reviewer #2:

The manuscript "Light activates signaling cascade of phytochrome, hnRNP and U1 snRNP to regulate pre-mRNA splicing in *Physcomitrella patens*" by Shih et al. describes the identification of splicing-related factors that can interact with phytochrome in Physcomitrella. Furthermore, the authors generate a mutant in one of these factors and compare the patterns of intron retention, a type of alternative splicing (AS), between wild type and mutant in darkness and upon red light exposure. This work aims at identifying the molecular basis of light-regulated AS, which was recently shown to be widespread in Physcomitrella and other plant species. I think that the topic of this work is of major interest, and another recent publication (Xin et al., PNAS, 2017) has identified a direct link between phytochrome signalling and a splicing regulator in Arabidopsis. However, the experimental evidence provided in the manuscript by Shih et al. is from my point of view not sufficient to support their conclusions, and many experiments would require further controls and the use of alternative experimental strategies. For more details, please see below. Moreover, a direct link between hnRNP1 and AS is lacking, and the seemingly overall moderate differences in light-dependent splicing patterns in comparison of wild type and the mutant in PPR39-1 do not support a major role of this factor in this process.

Point 1. Most of the authors' conclusions are built on protein interaction studies, using either yeast-two hybrid or BiFC assays. However, quantitative analyses are mostly absent or, when performed, not conclusive. From my point of view, none of the current data are sufficient to support the authors' conclusions, and extensive further experiments would be needed. In detail, the authors show single pictures of yeast colonies growing in the presence of PCB or PEB in darkness, red light, or far-red light (Fig. 1A). Yeast growth for PHY4/hnRNP-H1 can be seen under all conditions, while the positive control PHY4/PIF3 only gives growth in the presence of PCB and red light. A higher density of colonies is seen for PHY4/hnRNP1 in the PCB/RL condition; however, a quantitative assay would be needed to prove that there is a significant and reproducible difference in the interaction under different conditions.

RESPONSE: In this latest manuscript, we performed a Y2H liquid culture assay to quantify the interaction under different light condition. Under RL, the interaction between PpPHY4 and PpnRNP-H1 indeed was significantly enhanced.

Point 2. Most BiFC data (Fig. 1B, 2B, 3B) are shown as single pictures taken from different cells under different conditions. However, the variability of the results inherent to BiFC experiments cannot be assessed from these data and controls are lacking. As an alternative, the authors should show either time courses of the light response for individual cells or perform quantitative analyses of numerous cells. Immunoblots are lacking, but would be needed in order to exclude the possibility that light exposure affects the steady state levels of the interaction partners.

RESPONSE: We thank the reviewer for the comment. For the BiFC data in Fig.1, under dark conditions, there was no signal to detect, so what we observed under light conditions are not false positive signals. The reviewer suggested that we should do a time course experiment; however, time courses of the light response for individual cells is not possible in this experiment since under dark conditions, PpPHY4 and PphnRNP-H1 do not interact. Therefore, we cannot target an individual cell under the microscope and detect the fluorescence signal of the specific cell after light irradiation. As for the rBiFC experiments, it is a time course experiment for quantification. The number of cells observed for quantification was described in the figure legend. Immunoblots for checking the steady state level of the two proteins may not be practical, since we were using a transient expression system.

Point 3. The authors have included a quantitative analysis in Fig. 2D. However, the data brings up more questions. Based on the other data, there is no substantial interaction between hnRNP-H1 and PRP39 in darkness. So what is measured in the ratiometric assay in Fig. 2D under darkness should be background fluorescence in the YFP channel. The authors then observe a less than two-fold increase from background level after 4 h red light exposure in WT cells, while no light effect is seen in phy-deficient mutants. I think this weak increase would only make sense if there is already substantial interaction occurring in darkness, which would be in contrast to the results from Fig. 2B.

RESPONSE: We appreciate the comment. In the text, we did mention that PphnRNP-H1 interacts with PpPRP39-1 under both dark and red light conditions, and that is why we further used rBiFC to quantify the interaction. In the latest version of manuscript, we have revised the writing to make it clear. We also put BiFC photos in Supplemental Figure 9 and used only rBiFC data in Figure 4.

Point 4. Furthermore, are the BiFC ratios in darkness identical for the three genotypes? 4 h of darkness would be a better control than R0 (if this is what D refers to), as split YFP has a tendency to re-assemble. The interaction of hnRNP1-H1 and PRP39 in the yeast two-hybrid assay also suggests that this interaction can occur in a PHY-independent manner.

RESPONSE: We transiently expressed the proteins in cells, and therefore differences in BiFC ratios in darkness among the three genotypes are expected but should not be too high. Because the purpose of this experiment was to investigate whether RL promotes protein-protein interactions in different lines, whether the ratios in darkness are identical or not is not critical. We also detected YFP and RFP fluorescence in samples under darkness for 0, 1, and 4 hours. The fluorescence signals remained the same after longer incubation times in the dark.

Point 5. For Fig. 3, it remains unclear why from the candidates showing interaction in the yeast two hybrid, only U1C was tested via BiFC and whether this interaction can be regulated in a light-dependent manner.

RESPONSE: We choose U1C is because PpPRP39-1 and PpU1C showed the strongest interaction compared to other U1 components. Since our purpose was to define whether PpPRP39-1 is a U1 component or not, we believe the interaction tested between PpPRP39-1 and PpU1C is sufficient to make the conclusion.

Point 6. The key experiment following from the interaction between PHY4 and hnRNP-H1 would have been to analyse light-dependent AS patterns in a hnRNP-H1 mutant. However, the authors were not able to obtain a knockout in this gene and instead of trying to obtain a knockdown, they generated and analysed a mutant in PRP39-1. From the display of the data, I find it difficult to conclude how many of the light-regulated AS changes in the WT are altered in the mutant. Fig. 4 shows 6 clusters of events, based on the patterns of intron retention changes upon light exposure in the WT. The text says that "One batch of samples was then selected for analysis." (l. 260-261). Does this mean that only one replicate was used for the further analysis? This would certainly reduce the robustness of the data. Fig. 4B shows average IR levels for the 6 clusters; however, in order to be able to assess variability regarding the patterns in the WT and the mutant, it would be more informative to plot normalized levels for all events separately. The qPCR data show that all of the 4 tested AS events still change in response to light in the *prp39-1* mutant, albeit the response is weaker or the pattern is different. A statistical analysis would be needed in order to assess significant differences. Furthermore, what does the description of "pooled RNA...were analysed in triplicate for quantitative RT-PCR..." from the legend mean? At least three independent biological samples need to be analysed separately by quantitative RT-PCR and then the data combined. Furthermore, the *phy4* mutant should be included in the qPCR experiment. Based on the current data, I would not conclude that PRP39-1 plays an important role in light-regulated AS. Otherwise, there should be many AS events that show similar patterns in the WT and mutant in darkness, while light can trigger a shift in AS only in the WT. Based on the graphs in Fig. 4B, most events (or at least the averages) show the same or just a weaker response in the mutant compared to the WT (1, 2, 3, 6) or have already a very different rate of intron retention in darkness (4, 5).

RESPONSE: We appreciate this comment. In our latest manuscript, results from the *hnrnp-h1* mutant are included, and we performed RNA sequencing to check the global patterns of AS for comparison between WT and mutant lines. We collected three biological replicates to generate the RNA-seq data, and we also investigated AS in the *phy4* mutant.

Point 7. The mutants generated during the course of this work would require further characterisation. The conclusion that *prp39-1* is a knockout is based on the absence of a 300 bp RT-PCR product, which is visible as a very faint band besides several other bands in the WT (SFig. 4). Is the corresponding region within the deleted area or downstream of it? Information on the PHY4OE line is provided in SFig. 7 (which is not referenced in the text). The immunoblot with a tag-specific antibody reveals that the transgene is expressed. However, in order to be able to refer to this line as an overexpression mutant, a direct comparison of PHY4 levels between WT and the mutant would be needed.

RESPONSE: We revised SFig. 4 (Supplemental Figure 10 in the latest manuscript) and used primers to amplify full-length *PpPRP39*-1 in WT and the *prp39-1* mutant. The results showed that the transcription of *PpPRP39-1* is indeed disturbed. We thank the reviewer for pointing out that we did not refer to the PHY4OE line in text. The related part in the text was revised, and the construction and validation of the PHY4OE line are now shown in Supplemental Figure 4. Since we do not have a PpPHY4-specific antibody, checking the expression level of PpPHY4 in the PpPHY4 overexpression line and WT line is currently not possible.

Point 8. Figure 5 shows differences in the phototropism of WT, *phy4*, and *prp39-1*. Did the authors analyse whether general growth is unchanged, to exclude the possibility that this may have an effect on the phototropism?

RESPONSE: Although the mutants might have growth defects in the long-tern, in the phototropism experiment, we observed phototropism within a very short time (1 and 4 hours).

Point 9. The yeast two-hybrid screen for the identification of proteins interacting with hnRNP-H1 did not result in the identification of PHY4. Was PCB included? The authors should comment on this aspect.

RESPONSE: In the Y2H screening, we did not feed the yeast cells with PCB since our screening design was to identify the hnRNP-H1 interacting partner involved in pre-mRNA splicing. Another reason why it is not easy to identify phytochromes in Y2H screening is due to the size of phytochrome proteins, which makes it difficult to obtain full-length (or at least partial) cDNA in the library. This is also an important reason why we did pair-wise screening with full-length phytochromes and splicing regulators in the beginning of the study.

Reviewer #3:

The manuscript entitled "Light signaling cascade of phytochrome, hnRNP and U1 snRNP to regulate pre-mRNA splicing in *Physcomitrella patens*" from Shin and collaborators aims to unravel the underlying mechanisms acting in alternative splicing regulation by light. The authors claim that a phytochrome (PHY4) interacts with an hnRNP protein to modulate splicing. According to their interpretations, hnRNP-H1 binds PRP39 in a red light (and PHY4)-dependent manner and regulates the splicing of several genes in response to light. The manuscript is well written and the reasoning behind the experimental design is clear. I have, however, some concerns about the interpretations of the results and the experimental design I would like the authors to dissipate. If after these clarifications and controls the main message stands, I think the conclusions of the manuscript would be of relevance and interest for the splicing community in general.

Point 1. In general, I think the results and conclusions of the manuscript would be stronger if the authors add Co-IP data. In fact, they have constructs expressing the relevant proteins with HA and c-Myc tags, so they could easily do this.

RESPONSE: We thank the reviewer for the valuable suggestion. We understand that co-IP is a protein-protein interaction technique that can provide information on complex formation. We have been trying to optimize the conditions of co-IP for the Physcomitrella system, especially when working with photoreceptor phytochromes. Meanwhile, we believe that with at least 2 protein-protein interaction techniques tested in vitro and in vivo, pairwise interactions between these protein factors are clear enough for us to draw the current conclusion.

Point 2. I found it a bit weird that the authors showed hnRNP-H1 as an interactor of PpPHY4 and they didn't do more investigation of this factor. Even though the authors claim that knocking out this gene wasn't possible, they should try to knockdown this gene at least. Instead, they pursued "a downstream pathway" that is not, in my opinion, reflecting that much of a light-regulated response (see also Main Concern 5).

RESPONSE: We thank the reviewer for the suggestion. The *hnrnp-h1* mutant is now available since we performed CRISPR/Cas9 mutagenesis. Both physiology and genome-wide studies of PphnRNP-H1 were done and presented in the latest manuscript. For physiology studies, *hnrnp-h1* showed defects in RL-mediated phototropism and had a similar phototropism pattern to the *phy4* mutant (Figure 2). By using genome-wide analysis, we found that *hnrnp-h1* showed a significant AS defect compared to WT after the plants received light (Figure 3).

Point 3. In a similar direction, besides the hnRNP-H1 knockdown line(s), it would be great if the authors add double mutant lines like *prp39-phy4* and compare the affected AS events with those of the individual mutants (*prp39* and *phy4*). This could help to determine epistasis and cross-talk between different factors (see also Main Concern 5).

RESPONSE: We thank the reviewer for the suggestion. The *prp39-phy4* double mutant is not available at the current stage on our hand. Since the *hnrnp-h1* mutant is now available, we did experiments with *hnrnp-h1* to investigate its roles at both the physiological and molecular levels. In the latest manuscript, we concluded that PphnRNP-H1 is involved in RL-regulated AS and phototropism. We believe that clarifying the relationship between these components in light-mediated splicing regulation with genetic and molecular studies, such as co-IP and the generation of double mutants, can be integrated into another follow-up story, which does not affect the conclusion we made in the current manuscript.

Point 4. According to a recently published paper from the Matzke lab (DOI: 10.1534/genetics.117.300149), in *Arabidopsis thaliana*, the PRP39 protein works by promoting the recognition of GT-AG 5'ss-3'ss pairs, meaning that it would -most probably- increase intron splicing. This is partially consistent with the results of the present manuscript if we consider that PpPRP39's interaction with U1 components could be enhancing 5'ss recognition. On the other hand, hnRNPs in different systems are known to repress splicing instead of boosting its efficiency. Intriguingly, the interaction between PphnRNP-H1 and PpPRP39 proposed here, dependent on red light and PpPHY4, would (considering the model in fig. 6) change the activity of the spliceosome, rendering more intron retention. I think the authors are losing here a great opportunity to understand the mechanisms underlying these results. By analyzing interactors in pairs, they lost the potential effects of one interaction on the other interactions. I would rather guess that PpPRP39 interaction with the spliceosomal components is mutually exclusive with its interaction with PphnRNP-H1. I would suggest that the authors test the model in which PpPHY4 in red light moves to the nucleus and binds to PphnRNP-H1. This interaction somehow potentiates the binding of PphnRNP-H1 to PpPRP39. This latter interaction

is then repressing the binding of PpPRP39 to the spliceosomal components and diminishing the recognition of some 5'ss, ergo increasing intron retention. This model is consistent with the results in figure 5, since the PpPHY4OE is similar in phenotype to the *prp39-1* mutant. The model of the authors in figure 6 is, in fact, not considering the results of the *prp39* mutant. In this sense, I strongly encourage the authors to do Co-IPs of the different factors including, or not, PpPHY4 and using red light, far red and darkness. The authors must acknowledge that Phytochromes can be interconverted (Pr - Pfr) in vitro.

RESPONSE: We cannot agree more with the valuable comment the reviewer provided. We are putting efforts intensively into generating transgenic lines that would allow us to do co-IP and genetic experiments to investigate the relationship between these components and the function of protein interactions in light-mediated splicing regulation. As we answered for the last comment, these will be integrated into another follow-up story.

Point 5. With respect to the function of PpPRP39, I am not convinced that the provided evidence shows that this protein is particularly involved in a pathway of splicing regulation by light, as the authors claim. From the 3571 events reported to change in figure 4, only cluster 5 (305 events) seems to have an abolishment of the light responses. We could add that cluster 1 (762 events) has a response to 1 h of light that is smaller than that of WT moss, but still, the response is there, and cluster 4 (289 events), where the light responses seem to be completely different in the *prp39* mutant and the WT lines. However, the responses to light are still present. Hence, I don't see a strong reason to say that PRP39 is involved in the light regulation of splicing from this data. It is clear that PpPRP39 interacts with PphnRNP-H1 in a red light-dependent manner (Fig. 2); however, the analysis of the mutant shows that this factor (PRP39) has more general effects. To understand those effects related to light, the authors should use a knockdown (siRNA, miRNA) line for PphnRNP-H1 or directly use the *phy4* mutant line data for splicing and take those events affected in this mutant line and also affected in the *prp39* mutant. In this way, they could have a more accurate picture of what these factors are doing to modulate gene expression in response to light. Moreover, I would like to strongly encourage the authors to study other alternative splicing events than intron retention, since it does not seem that PRP39 is significantly relevant to this particular type of splicing event.

RESPONSE: We thank the reviewer for the suggestion. The *hnrnp-h1* mutant is available now. We have done genome-wide analyses using mRNA sequencing to detect the differential AS in WT and compared the AS patterns in *hnrnp-h1*, *prp39-1* and *phy4*. Indeed, the results show that a large proportion of RL-responsive IR events are mis-regulated in *phy4* and *hnrnp-h1* Figure 3) and well as *prp39-1* (Figure 5). In this manuscript, we analyzed exon skipping and alternative donor/acceptor site events. It is obvious that *hnrnp-h1* also regulates these events under light conditions (Supplemental Figure 7).

Point 6. The authors claim that photoreceptor effects are fast; however they observed that PphnRNP-H1 and PpPRP39 interact after 4 h of red light treatment. What is the point of view of the authors about this contradiction? Moreover, if the effective interaction they can detect and quantify occurs after 4 h of red light treatment, why do the authors give importance to what happens after 1 h in terms of alternative splicing regulation? Once more, I think a Co-IP for these 2 factors could give the authors more sensitivity in order to properly assess the timing of the interactions.

RESPONSE: As shown in rBiFC experiments (Figure 4D), a light-promoted interaction of PphnRNP-H1 and PpPRP39-1 can be observed after 1 hr of RL irradiation. Such enhancement requires the primary action of photo-activated phytochromes, which takes only minutes to translocate into the nucleus upon light irradiation. We therefore believe that photoreceptor-mediated splicing regulation can be fast and occurs in 1 hr.

Point 7. It would be nice to know the stability/levels of the different proteins in the dark and after 1 and 4 h of red light treatment. Even though it seems quite clear that these proteins do interact with each other I would encourage the authors to evaluate if the light-induced interactions (in particular the interaction coupling PHY4 with hnRNP-H1; and hnRNP-H1 with PRP39) are dependent on a change in the accumulated proteins or if they are related to the de novo synthesis of one of the interaction partners. In this sense, it would be mechanistically insightful, and complementary to the Co-IPs, to check if the effects on splicing/interactions require protein synthesis (i.e.: using cycloheximide to inhibit translation).

RESPONSE: We thank the reviewer for the comment. We believe the light-induce interaction between PHY4 and hnRNP-H1 is mainly caused by the light-triggered nuclear localization of phytochromes. However, for the interaction

of hnRNP-H1 and PRP39, the mechanism the reviewer proposed may play an important role in controlling the interaction. Investigating this hypothesis can be integrated into our ongoing study, which focuses on clarifying the relationship between these components in light-mediated splicing regulation with genetic and molecular studies, such as co-IP and the generation of double mutants.

Point 8. In fig. 2B it seems that NLS-mCherry (control) expression changes quite a lot between dark, 1 h light and 4 h. Did the authors analyzed this? Is protein stability and/or expression having a role in the reported effects?

RESPONSE: We used particle bombardment to transiently express NLS-mCherry protein (Supplemental Figure 9 in the revised manuscript). One shortcomings of using particle bombardment is that we cannot control the quantity and ratio of plasmids introduced into the cells; therefore the expression level may vary among cells. We therefore chose rBiFC to ensure that every transformant expresses same amount of nYFP, cYFP and RFP, and further used RFP as internal control.

Point 9. Do the authors treat all the introns together or do they divide those with GT-AG from those with AT-AC sites?

RESPONSE: We did not separate GT-AG and AT-AC sites for analysis.

TPC2018-00421-BR 1st Editorial decision – *declined* **Aug. 7, 2018**

We sincerely apologize for the length of time that it was in review; there was one reviewer that took far longer than we ask for and is typical for manuscripts at this journal, but we felt that their input was important. Your submission has been evaluated by members of the editorial board as well as expert reviewers in your field, and we regret to inform you that we are not able to recommend publication of this manuscript this stage. We have not made this decision lightly, as there are still serious concerns from reviewer 2 and 3 regarding the analysis and the interpretation of the results. There are still many experiments missing to support the claims of your paper, and these additional experiments will need more time than typically allowed for a second revision. However, the reviewers agree that if the results are well-supported, it would be a really interesting case of light-dependent alternative splicing. Both reviewers may be interested and willing to assess your manuscript again if you would choose *The Plant Cell* for a further revised version. Having said that, we would be willing to reconsider a new manuscript that fully addressed the concerns raised during this review process.

In addition, we find that the writing suffers from some problems with English grammar and awkward phrasing. We strongly encourage the use of a professional editing service to ensure that the work is presented in the best manner and reaches the broadest possible audience. There are many such services available; two that we can recommend are Plant Editors (planteditors.com) or Science Editors Network (scienceeditorsnetwork.com).

---------- Reviewer comments:

[Reviewer comments shown below along with author responses]

TPC2019-00314-RA Submission received **Apr. 27, 2019**

Reviewer comments on previously declined manuscript and **author responses:**

Reviewer #1:

In this manuscript by Shih et al., the authors show that in moss, *P. patens*, PHY4, an ortholog of Arabidopsis phyB red/far-red photoreceptor interacts with RNP-H1, a splicing factor in a red-light dependent manner in the nucleus. In addition, RNP-H1 interacts with the splicing-related factor PRP39, which in turn associates with U1 snRNP. Using transcriptome analysis, they report that *prp39* mutants have alternative splicing defects when compared to the WT.

I had reviewed the earlier version of this manuscript. This version of the manuscript has improved a lot, and the necessary experiments have been performed to justify the claims. I have one minor comment below, which I think the authors can easily address with the materials they have.

Point 1. Does the *ppr39* mutant have defects in phototropism similar to those of the *phy4* and *hnrnp-h1* mutants shown in Figure 2? The data from this experiment will further strengthen the claim that PPR39 functions in the same pathway as phy4 and RNP-H1.

RESPONSE: We thank the reviewer for the comment. Actually, the phototropism of *prp39-1* behaves opposite to the phototropism pattern of *phy4* and *hnrnp-h1*. In the latest manuscript, we provided new data showing that different from the red light-promoted interaction between PRP39 and hnRNP-H1, the interaction between PRP39 and U1C is inhibited by red light, suggesting that light differentially regulates PRP39-hnRNP-H1 and PRP39-U1C interactions. A study from the Matzke group (Kanno et al. Genetics. 2017. 207. 1347-1359) showed that PRP39 in plants promotes the recognition of 5'ss-3'ss pairs, and thus may enhance splicing. In our study, we show that hnRNP-H1 mainly functions in repressing splicing and causes intron retention. We therefore propose that hnRNP-H1 and PRP39 regulate alternative splicing of unknown gene(s) involved in moss phototropism. It is possible that under light conditions, AS isoforms of the unknown gene(s) are produced to positively regulate phototropism. hnRNP-H1 plays a positive role in producing the AS isoforms and phototropic response. PRP39, which enhances splicing, then functions as a negative regulator of isoform formation for the unknown gene(s) and phototropism. Because there is too much speculation in the proposed mechanism without clear evidence to support it, we decided not to address this question in the current manuscript.

Reviewer #2:

Shih et al. have submitted a revised version of their manuscript entitled "Light activates signaling cascade of phytochrome, hnRNP and U1 snRNP to regulate pre-mRNA splicing in *Physcomitrella patens*". Compared to the first submission, the latest manuscript version includes several new data sets and analyses. Major changes are the addition of quantitative yeast interaction data (Fig. 1B) and the analysis of an *hnRNP-h1* mutant. While some of the additions have strengthened the authors' conclusions, such as the light-dependent interaction of PHY4 and PphnRNP-H1 in yeast, other edits bring up new questions, and several of the original reviewer concerns, such as the absence of experiments investigating protein accumulation, remain unaddressed.

Point 1. My major concern with the present study is the outcome of the alternative splicing studies in comparison of wild type and *phy4* as well as the other mutants. Assuming that photoreceptor-mediated alternative splicing plays an important role in red-light responsive splicing changes in Physcomitrella, I would expect a clear reduction in the number of splicing changes in the *phy4* mutant as well as the *hnrnp-h1* mutant compared to the wild type. However, as shown in Fig. 3A, an even higher number of red light-dependent splicing changes are seen in *phy4* (1764) than in the wild type (1578). The number of changes in *hnrnp-h1* shows an only slight reduction compared to the wild type. While redundant signalling pathways may explain why many splicing changes from the wild type are still visible in *phy4*, the large number of events that change in response to red light specifically in *phy 4* but not in the wild type (1560 events) is completely unexpected and may indicate a limited robustness of the analysis pipeline.

RESPONSE: We thank the reviewer for the comment. The larger number of light-responsive IR events in *phy4* was also high to us. We re-analyzed the RNA-seq data using a much more stringent filter; however, the *phy4* mutant still had a higher number of red light-responsive IR events. What we speculated is exactly like the reviewer suggested: there are other parallel pathways to regulate red light-responsive alternative splicing, possibly through other phytochromes in Physcomitrella. It could be that other phytochromes compensate for or even raise the photoactivities in the *phy4* mutant. In the newly revised version, we modified the way of presenting data by simply comparing red light responsiveness in the mutants for those differential IR events originally identified from the wild type. With this, it is clear that a major portion of red light-responsive IR events are misregulated in the *phy4* and *hnrnp-h1* mutants and that those co-regulated by both PHY4 and hnRNP-H1 can be defined. How other moss phytochromes control alternative splicing will be further studied.

Point 2. As described before, I'm concerned about the robustness of the analysis for detecting light-dependent splicing changes. Along this line, I'm surprised that the R1 and R4 time points look so different (e.g. Fig. 3B) ,and I'm wondering what the biological meaning of this short-lived changes may be.

RESPONSE: We thank the reviewer for the comment. We believe that after receiving light for 4 hours, secondary regulation such as differential expression of splicing factor genes already simultaneously occurs together with the primary regulation induced by phytochromes. AS patterns are complicated because of multiple feedback regulation at 4 hours or longer of light exposure. This is the reason that IR events showing dramatic responses at R1 become

less prominent at R4. In the revised manuscript, we decided to focus on the immediate change at R1 to make the point clear.

The dramatic IR response after 1 hour of red light irradiation from the dark suggest that plants require immediate, robust changes in the transcriptome to respond to changing light conditions. Regulation at the pre-mRNA splicing step provides the most efficient way to alter transcript or protein abundance. In our case, rapid induction of IR by light for a subset of ribosomal protein gene transcripts may transiently shoot down the activity of some ribosome species for translation control.

Point 3. A red light-mediated interaction between PHY4 and hnRNP-H1 in plant cells is proposed based on showing one picture each of BiFC signals for a transformed cell from a dark sample or RL sample (Fig. 1C). Showing a time course of the red light response for the same cell would be more informative. Other than stated in the authors' response to this comment from my previous review, I think transformed cells can be identified in dark samples using the cotransformed mCherry. If this does not work, the authors could switch to stably transformed lines.

RESPONSE: This is a good suggestion but has some technical difficulty for moss cells; however, we still managed to do this with the previously established orchid petal system (Lee et al. The Plant Cell. 2012). As shown in supplemental figure S3, we can observe the red light-induced interaction between PHY4 and hnRNP-H1 in the same cell.

Point 4. The authors show in Fig. 2D clearly reduced bending angles at R1 for *phy4* and *hnrnp-h1* compared to wild type. At R4, *phy4* still has a much lower bending angle than the wild type, while *hnrnp-h1* shows no difference to the wild type. The striking difference between R1 and R4 for the two mutants is at least unexpected and would need to be tested by analysing additional time points between 1 and 4 h.

RESPONSE: We thank the reviewer for pointing out this issue. We went back to re-analyze the bending phenotype and realized that we did not count the angles of negatively grown protonema, which is also a phenotype we should consider. In the newest version of manuscript, we updated Figure 2D by counting the bending angles for all protonema as described previously (Mittmann, et al. PNAS. 2004) . Once taking negative phototropism into account, the bending angles of *phy4* and *hnrnp-h1* are much lower than WT in both R1 and R4. This result suggests the *hnrnp-h1* mutant also has defects in phototropic responses at R4, with abnormal negative phototropism under lateral red light.

Point 5. The absence of immunoblot analyses leaves it open if the increase in BiFC signal upon light exposure may result from increased protein accumulation, e.g. due to changes in protein turnover.

RESPONSE: We thank the reviewer for the comment. We checked the protein abundance of *hnRNP-H1* in the dark and under red light. As shown in Figure 1, the protein abundance of *hnRNP-H1* was not affected by red light compared to the dark.

Point 6. The manuscript would still require language editing.

RESPONSE: After we revised the manuscript, we sent it to the English editor recommended by the journal. We believe that the writing has been improved.

Point 7. The authors refer to an Act5P:cMyc-PHY4 line as an PHY4 overexpressor. However, in the absence of data comparing the levels of endogenous PHY4 and ectopic cMyc-PHY4, e.g. on the transcript level, overexpression is not proven. The immunoblot analysis with an anti-cMyc antibody is limited to detection of the ectopic protein.

RESPONSE: In Supplemental Figure S5C of the revised manuscript, we included the result to show the transcript level of *PHY4* in wild type, *phy4* and PHY4 overexpression lines. The results clearly indicate that PHY4 is overexpressed.

Point 8. Line 154 - 157: "The phytochrome-interacting pattern of PphnRNP-H1 is similar to that of Arabidopsis Phytochrome Interacting Factor 3 (AtPIF3), a transcription factor (TF) known to interact with phytochromes in a RL-dependent manner (Ni et al., 1998)." This is an overstatement, at least on the quantitative level, given fold-changes of approximately 1.5 and 10 for hnRNP-H1 and PIF3, respectively.

RESPONSE: We thank the reviewer for the comment. Indeed, this part is over-stated. We already revised this paragraph in the latest manuscript.

Reviewer #3:

As I said in the previous version, the manuscript entitled "Light activates signaling cascade of phytochrome, hnRNP and U1 snRNP to regulate pre-mRNA splicing in *Physcomitrella patens*" from Shih (sorry for the typo in my previous review) and collaborators aims to unravel the underlying mechanisms acting in alternative splicing regulation by light. The authors claim that a phytochrome (PHY4) interacts with an hnRNP protein to modulate alternative splicing, mainly intron retention. According to their interpretations, hnRNP-H1 binds the pre-mRNA-processing factor 39-1 (PRP39) in a red light (and PHY4)-dependent manner and regulates the splicing of several genes in response to light. In turn, PpPRP39-1 associates with the core component of U1 small nuclear RNP to regulate splicing. Even though the authors made clear improvements to the manuscript, I still have some concerns about the interpretations of the results and the experimental design.

Point 1. Figure 1 shows that PHY4 interacts with hnRNP-H1 independently of the chromophore (phycocyanobilin) and the light condition. However, when PHY4 is in the Pfr form (after absorbing RL), the interaction seems stronger. In any case, the interaction between these 2 proteins is completely different from that of PHY4 and PIF3, which clearly depends on light conditions.

RESPONSE: We thank the reviewer for the comment. Indeed, background interaction was observed when PHY4 and hnRNP-H1 were expressed in yeast cells under dark conditions. We tried different methods to reduce the background interaction in yeast but failed. We believe that it is due to the nature of the artificial Y2H system, which forces two target proteins to localize in the nucleus for interaction. In the case of PHY4 and hnRNP-H1, some yeast proteins may associate with both to induce the expression of reporter genes. However, with functional chromophore (PCB), red light indeed promotes the interaction. With the chromophore analog (PEB) that locks phytochromes in the Pr form, the red light-induced interaction was also blocked. This evidence supports the notion that the interaction between PHY4 and hnRNP-H1 depends on the functional chromophore and red light. From in-planta experiments, it is clear that their interaction only occurs in the nucleus after 1 hour of red light irradiation when PHY4 is photoactivated and targeted to the nucleus.

In the revised manuscript, we updated Figure 1 by adding quantitative data for BiFC. We also managed to observe the interaction in the same cell with the previously established orchid petal system (Lee et al. The Plant Cell. 2012). As shown in Supplemental Figure S3, we can observe the red light-induced interaction between PHY4 and hnRNP-H1 in the same cell. With more than two protein-protein interaction techniques and quantification for the interaction, we believe the red light-dependent interaction between PHY4 and hnRNP-H1 occurs.

Point 2. Figure 2 is showing that the *phy4* and *hnrnp-h1* mutants have different responses to light. In 2C and 2D, we can see that, even though there might be a deficiency at 1 h in RL, at the 4 h time point, the *hnrnp-h1* mutant behaves like the WT plants (and clearly different from the *phy4* mutant).

RESPONSE: We thank the reviewer for pointing out this issue. Similar to what we responded to Reviewer #2, we went back to re-analyze the bending phenotype and realized that we did not count the angles of negatively grown protonema, which is also a phenotype we should consider. In the newest version of manuscript, we updated Figure 2D by counting the bending angles for all protonema as described previously (Mittmann, et al. PNAS. 2004) . Once taking negative phototropism into account, the bending angles of *phy4* and *hnrnp-h1* were lower than WT in both R1 and R4. This result suggests that the *hnrnp-h1* mutant also has defects in phototropic responses at R4, with abnormal negative phototropism under lateral red light.

Point 3. The lack of correlation between the responses of the *phy4* and *hnrnp-h1* mutants is again evident in figure 3. The behavior of the analyzed IR events is practically the same at 4 h in RL in the *hnrnp-h1* mutant and in the WT plant. However, the *phy4* mutant is showing different responses. Similar conclusions can be drawn from Suppl. Fig. 7.

RESPONSE: We thank the reviewer for the comment. We believe that after receiving light for 4 hours, secondary regulation such as differential expression of splicing factor genes already simultaneously occurs together with the primary regulation induced by phytochromes. AS patterns are complicated because of multiple feedback regulation after 4 hours. This is the reason that IR events show dramatic responses at R1 but less prominent responses at R4. Although IR patterns in WT and the *hnrnp-h1* mutant at R4 look more similar compared to that of *phy4*, there are still differences. This also indicates that PHY4, the primary factor of the light-sensing process, play a major role in splicing regulation. The dramatic IR response after 1 hour of red light irradiation from the dark suggests that plants

require immediate, robust changes in the transcriptome to respond to the changing light conditions. In the revised manuscript, we decided to focus on the immediate change at R1 to make this point clear.

Point 4. Furthermore, the authors are missing the opportunity to analyze their results in a better way. What is relevant for their question is whether those events that are changing in WT plants are still changing (or not) in the mutants. The Venn diagrams in 3A is not directly answering this question. The authors should check the particular responses in the mutants of all the affected events in WT plants.

RESPONSE: We thank the reviewer for the good suggestion. We re-analyzed the data as the reviewer suggested. The new version of RNA-seq analysis results is shown as Figure 3 in the latest version of manuscript. We modified the way of presenting data by simply comparing red light responsiveness in the mutants for those differential IR events originally identified from the wild type. With this, it is clear that a major portion of red light-responsive IR events are misregulated in *phy4* and *hnrnp-h1*, and those co-regulated by both PHY4 and hnRNP-H1 can be defined.

Point 5. Then the authors move forward and find that PRP39-1 interacts with hnRNP-H1 (Fig. 4A). Even though it is clear the interaction exists, differences between RL and Dark are not clear at all. YFP signal from the rBiFC constructs (4B) is present in every tested condition (4C and 4D) and from a biological point of view, it is not clear how a not significant 1.3 fold-change (statistics are missing but the errors are almost overlapped between D and R1 in the WT) would make a difference. So, taken together, these results are not supporting the claims nor the model in figure 7 nor the Abstract's claim saying that "PphnRNP-H1 binds with higher affinity to a splicing factor, pre-mRNA-processing factor 39-1 (PpPRP39-1), in the presence of red light-activated phytochromes." Analyses of combined interactions are missing, and results showing the different studied components acting in the same pathway are missing too. In conclusion, the evidence presented is not supporting the conclusions, and the authors would need to provide further data to confirm their claims.

RESPONSE: We thank the reviewer for the comment. The interaction between hnRNP-H1 and PRP39-1 occurs in all conditions, even in the dark, but is rapidly enhanced by red light. Although red light-enhanced interaction at R1 is around 1.3 fold, it does not necessarily mean the impact of the interaction is also weak. Especially when we compare the rBiFC results from WT and *phy4*, it is obvious PHY4 is required for promoting the interaction between hnRNP-H1 and PRP39-1. What we propose is the interaction between hnRNP-H1 and PRP39-1 could be more transient than others. In the new submission, we provided new evidence to show that the interaction between PRP39-1 and U1C can be suppressed by red light and the interaction at a certain degree requires PHY4.

Point 6. The results presented in Suppl. Fig 1 are, in my opinion, not completely straightforward. While it is clear that PHY4 interacts with At-PIF3 (control), the interactions with hnRNPs are all weaker than this control. In particular, the one the authors selected (Pp3c7_8760, PphnRNP-H1) is not that different from others like Pp3c12_3660, so it is not clear why the authors go after this particular candidate. I was previously asking for validation of these interactions by other means (i.e.: co-IP), but there is still a need of further evidence in this direction. Fig. 1 has similar issues. The authors claim co-IP has some issues but should not be different from co-IP in any other system. However, even though my interpretations are different from those of the authors', I also think the interactions are conclusive. I am not convinced on how light regulates these interactions. Furthermore, a technique like co-IP would be useful to know how the different interaction pairs affect the other interactions. This is a key question that remains unexplored in the manuscript.

RESPONSE: We thank the reviewer for the comment. PIF3 is a well-characterized TF that strongly binds to phytochromes; therefore, it is used as a positive control. Compared to PIF3, the interaction between PHY4 and hnRNP-H1 is relatively weaker and possibly more transient. It does not necessarily mean the impact of the interaction is also weak.

We updated Figure 1 by adding more quantitative data for BiFC. We also managed to observe the interaction in the same cell with the previously established orchid petal system (Lee et al. The Plant Cell. 2012). As shown in Supplemental Figure S3, we can observe the red light-induced interaction between PHY4 and hnRNP-H1 in the same cell. With more than two protein-protein interaction techniques tested in vitro and in vivo and quantification for the interaction, we believe that the red light-dependent interaction between PHY4 and hnRNP-H1 occurs.

We understand that co-IP is a protein-protein interaction technique that can provide information on complex formation. It is, however, limited to protein interactions that are relatively static, and with noise from non-specific

and indirect binding. We have been trying to optimize the conditions of co-IP for the Physcomitrella system, especially when we are dealing with the photoreceptor phytochromes; however, it is still not so successful at this moment. This indicates that the interaction between PHY4 and hnRNP-H1 is transient, similar to kinase-substrate interactions. We think in the revised manuscript, we have provided more information other than what co-IP can produce.

Point 7. Before I was asking for a knock-down line or a mutant for hnRNP-H1. The authors are now introducing a CRISPR-CAS mutant for hnRNP-H1; however, they did not make the crosses in order to know if hnRNP-H1 is acting downstream of PHY4 or not. This is another key experiment missing.

RESPONSE: We thank the reviewer for the comment. To fulfill what the reviewer suggested, we have been trying to generate double mutants since the last submission. Genetic crossing is not practical in the Physcomitrella system so we tried to generate a PHY4 mutation in the *hnrnp-h1* mutant background or a hnRNP-H1 mutation in the *phy4* mutant background using traditional gene targeting and CRISPR/Cas9 systems. Unfortunately, after more than 20 rounds of transformation and selection experiments, we still could not obtain surviving transformants. Based on our long experience in generating moss mutants, we think double mutations are lethal to the plant. This also indicates that both genes together are required for the survival of mosses. We are still seeking other strategies to do this.

On the other hand, in this study, we did not intend to clarify whether these factors act at the upstream and downstream position of the same pathway. As a red light photoreceptor, it seems to be reasonable that phytochrome is the primary component for light sensing and delivers the signal into the nucleus for protein interactions and splicing regulation. Based on our new experimental results, we think protein-protein interactions between splicing factors initiated by phytochromes in the nucleus are transient (more like the kinase-substrate interaction) but play a critical role in modulating pre-mRNA splicing. As observed from the RNA-seq data, the loss of any of these factors including PHY4, hnRNP-H1 and PRP39-1 results in the mis-regulation of a big (but the same) group of light-responsive AS events. These results clearly indicate that hnRNP-H1 and PRP39-1 are regulated by light to modulate splicing, and PHY4 is the trigger.

We also believe that other signaling pathways, such as plastid signaling and energy status, are involved in the process of splicing regulation in parallel with phytochrome-mediated activities. The hnRNP-H1 as well as PRP39-1 and U1C identified in our study may play roles in these pathways. However, from physiological and RNA-seq analyses, we have confidence that these factors function (at least mainly) in the phytochrome-mediated pathway. Other phytochromes and unknown factors may also participate in the regulatory process. Work is needed to support these hypotheses.

Point 8. As above, I still have the question whether PRP39-1 acts downstream of PHY4. Generating the double mutant *prp39-phy4* could enable the authors to determine epistasis and cross-talk between different factors. I do not agree this could be part of a follow up story since with the evidence presented, we only have some degree of certainty that all these factors (in pairs) can interact ,and this is not enough to build a story.

RESPONSE: As we have responded for Comment 2, in this study, we did not try to clarify whether these factors act in the upstream and downstream position of the same pathway. We actually modified the title to better fit with the data we reported this time. Meanwhile, because we did not detect a protein-protein interaction between PHY4 and PRP39-1, determining epistasis and cross-talk of PHY4 and PRP39-1 through generating the double mutant may not be informative. We believe that with the protein-protein interaction, physiological and RNA-seq analyses here, the functional roles of the new factors we identified in phytochrome-mediated splicing regulation can be illustrated as a complete story.

Point 9. Based on a manuscript from the Matzke lab (DOI: 10.1534/genetics.117.300149), I was guessing that PpPRP39 interaction with the spliceosomal components is mutually exclusive with its interaction with PphnRNP-H1. The authors said they agree with this idea, but they do not provide any evidence in this, or in other direction. Hence, this is still an issue I think the authors should dissipate for the publication of this research. Something they could have done already is to check if the interaction between PRP39 and U1-C can be modulated by light, as they have all the materials for that.

RESPONSE: We truly thank the reviewer for the good suggestion. In the latest manuscript, we quantified the interaction between PRP39-1 and U1C by rBiFC and indeed their interaction, as the reviewer guessed, was inhibited

by red light and photo-activated PHY4 (see the updated Figure 6 in the manuscript). After red light treatment, PpPRP39 interacted less strongly with the spliceosomal component U1C, supporting the reviewer's prediction that light differentially regulates PRP39-hnRNP-H1 and PRP39-U1C interactions. We therefore propose the hypothesis that after photoactivated phytochromes interact with hnRNP-H1, affinity between hnRNP-H1 and PRP39 is increased. This will dissociate PRP39 from the U1 snRNP core component U1C and causes the decrease of U1 activity. As a result, light-responsive intron retention occurs.

Point 10. I am still not convinced from the evidence provided that PRP39 is involved in a pathway of splicing regulation by light, as the authors claim. Even though the authors added new data to clarify this, I would suggest that they analyze if those events affected in the WT are not affected in the different mutants. I would expect that some red light-responsive events remain unchanged in the *phy4* mutant. From those, I would also expect that many remain unchanged in the mutants of the downstream acting factors (hnRNP-H1, PRP39-1, etc.). This is an analysis that is missing in the manuscript.

RESPONSE: Again, we thank the reviewer for the suggestion. In the new version of manuscript, we re-analyzed our RNA-seq data as the reviewer suggested and found that a big proportion of IR events significantly changed in WT showed less responsiveness to red light in *phy4*, *hnrnp-h1* and *prp39-1*. These events may be co-regulated by the photoreceptor and splicing factors.

Point 11. In the previous version of this manuscript, I was commenting that PphnRNP-H1 and PpPRP39 interact after 4 h of red light treatment, and asking if the effective interaction the authors can detect and quantify is after 4 h of red light treatment, why do the authors give importance to what happens after 1 h in terms of alternative splicing regulation? The authors' answer is that the interaction can be observed at 1 h of RL. If this is correct, then we can consider the interaction is there also in the dark. As I said before, Figure 4 is not conclusive at all.

RESPONSE: The interaction between PphnRNP-H1 and PpPRP39 happens at all time points we tested (dark, R1 and R4), but red light promotes the interaction between the two proteins. We might not have made this point clear in the old version of the manuscript. Related paragraphs in the Results section have been revised.

Point 12. I still would like to know the stability/levels of the different proteins in the dark and after 1 and 4 h of red light. As I said before, it would be mechanistically insightful to check if the effects on splicing/interactions require protein synthesis (i.e., using cycloheximide to inhibit translation).

RESPONSE: We thank the reviewer for the comment. We have checked the protein abundance of hnRNP-H1 in the dark and under red light. As shown in Figure 1, protein abundance of hnRNP-H1 is not affected by red light compared to the dark.

Point 13. The authors replied they took all the introns together; I encourage them to analyze them (GT-AG and AT-AC) separately.

RESPONSE: We performed further analysis to determine splice site usage for all introns and light-responsive AS events in Physcomitrella. As shown in Figure 3, the percentage of AT..AC introns in Physcomitrella is very low, although it is slightly increased in light-regulated AS events. We therefore believe that the majority of light-regulated AS events still occur in canonical U1/U2 introns.

Point 14. Suppl. Fig. S6 is showing that the mutants are not doing much about the splicing regulation. Most of the validated candidate splicing events behave in the mutants, mostly, slightly different from those in WT plants, whereas just some are showing defects in the response to 1 or 4 h of red light in particular mutants. These results are not consistent with the hypothesis of the authors that all these factors (mutated in the different lines) are involved in the same signaling pathway.

RESPONSE: We thank the reviewer for this comment. In the newest version manuscript, we re-analyzed the RNA-seq data. We found that although the majority of IR events showed similar patterns in WT and the mutants, their IR levels were very different. In *phy4* and *hnrnp-h1*, IR levels were not changed as dramatically as in the WT in response to red light. We picked up some candidate events from the new data for validation. Results from qRT-PCR are consistent with those of RNA-seq data.

Point 15. While interesting, the analysis in Suppl. Figure 7 should focus in those events that are affected in the WT plants but do not change in the mutants; those that are equally unaffected in both mutants are the relevant ones.

RESPONSE: We thank the reviewer for this comment. We changed the way to analyze RNA-seq data in the new version of manuscript. In Supplementary Figure 7, we checked the events that were affected in the WT but do not change in the mutants.

Point 16. The text can be further improved. I am not going into it in a point-by-point manner since I think the authors need to change several sections by providing more data, but I would recommend that the authors re-check the writing.

RESPONSE: After we revised the manuscript, we also sent it to the English editor recommended by the journal. We believe that the writing has been improved.

TPC2019-00314-RA 1st Editorial decision – *accept with minor revision* June 23, 2019

On the basis of the advice received, the board of reviewing editors would like to accept your manuscript for publication in The Plant Cell. This acceptance is contingent on revision based on the comments of our reviewers.

---------- Reviewer comments:

[Provided below along with author responses]

TPC2019-00314-RAR1 1st Revision received July 15, 2019

Reviewer comments on previous submission and **author responses**:

Reviewer #3

First of all, I would like to congratulate the authors for all their efforts, their hard work and resolution. I was checking the previous revisions and it is clear that the manuscript improved a lot and they addressed, or at least tried to address in many different ways, all our concerns. The new data and results, like those showing interactions using orchid petal cells, give the paper an extra by providing further and independent evidence that supports the manuscript's main conclusions. Moreover, the manuscript is now easier to follow and understand since the writing has been improved. After all these revisions, I am glad to say the authors have successfully addressed all my concerns. To the authors: Thanks for considering my opinion and thoughts about your work.

RESPONSE: We sincerely thank the reviewer for giving us all valuable suggestions throughout the manuscript from the very beginning of our submissions. Your comments greatly improved our manuscript. The abstract has been updated as suggested.

Reviewer #5:

In this manuscript, the authors show that *P. patens* phytochrome PHY4, the ortholog of Arabidopsis phytochrome B, interacts with the splicing regulator PphnRNP-H1 both in yeast and in moss cells, using different methodological approaches in vitro and in vivo. This manuscript continues the story from the work published by Wu et al. in 2014, where they show that phytochromes regulate alternative splicing in *P. patens* in response to red light. The interaction between PHY4 and PrphnRNP-H1 depends on red light. PphnRNP-H1 is important for phototropic responses in protonemata, and this provides an interesting physiological response as an outcome of the described interactions. Using mutants, the authors study the roles of PHY4 and PphnRNP-H1 in alternative splicing using RNA-seq approaches. Then, they investigate the interaction between PphnRNP-H1 and PpPRP39 and the interaction between PpPRP39-1 and spliceosomal components.

Point 1. To analyze alternative splicing changes, the authors used the following criteria: "RL-regulated IR events were considered to be RL-responsive when an event had a P value < 0.005." I think it would be more appropriate to use not only a statistical criteria, but also to consider an event to be RL-responsive if there is a fold-change above a certain % (for example, above 5%). This would help to look at changes that are biologically relevant, and not only statistically significant.

RESPONSE: We thank the reviewer for the suggestion. We did consider both statistics test and fold change when

we started to work on genome-wide analysis for alternative splicing (AS) several years ago. After a large-scale validation for AS events by qRT-PCR, we found that the statistics test already provides reasonable confidence in identification of light-responsive AS events. As shown in the Methods section, the statistics (Student t-test) consider the difference of IR levels (derived from three biological replicates) between D and R1 samples. Because a stringent P value was used, we think our criteria already take biological relevance of the changes into account.

As suggested, we also tried to adopt fold change above 5% as the second criterion. Only three events were deleted from the 1577 RL-responsive events in WT. We further analyzed the involvement of PHY4 and hnRNP-H1 in regulating RL-responsive events and found that the percentage of events showing PHY4 and hnRNP-H1 dependence remained the same (Figure R1). This conclusion still holds true when we tested various levels of fold changes as the cutoff (Figure R1). We therefore decided to keep our original criteria for defining RL-responsive events.

Point 2. The results in figure 4 are not as striking as the ones provided in figures 1-3. The interaction between PRP39 and H1 is not really clear from the images provided, and there is also an interaction between the two factors in dark conditions, before light irradiation. This makes it difficult to analyze the conclusions supported by the authors. I suggest that the statements concluded from these experiments should be toned down (and the abstract, revised) unless further experimental support is provided.

RESPONSE: We thank the reviewer for this comment. We did find that PpPRP39 and PphnRNP-H1 interact even in the dark. However, as we have pointed out in the Results and Discussion sections based on the quantitative data, the function of red light is to promote the interaction between these two proteins, and also to dissociate PRP39 from U1 snRNP. We agree with the reviewer that the term "RL-induced interaction" we used to conclude Figure 4 should be modified into "RL-promoted interaction". To further clarify this point, we also revised our model (Figure 7). We will then keep our statements in the Discussion and Abstract.

Point 3. In the Discussion, the authors analyze changes in alternative splicing regulation that can be dependent or independent of phytochromes. They suggest that phytochrome-dependent regulation of alternative splicing could be "more rapid and sensitive, (and) could be the primary event that occurs immediately after light exposure. Plastid signaling might be induced after longer light exposure". However, the work by Petrillo et al. shows changes in alternative splicing after 2, 4 or 6 hrs. of light irradiation. This sentence should be accordingly revised.

RESPONSE: We thank the reviewer for the comment. This part has been toned down slightly as suggested. However, we would like to point out some experimental differences between our study and the work by Petrillo et al. In their light conditions, 100 mmolm^{-2}s^{-1} was used in the case of red or blue light. Compared to our condition (5 mmolm^{-2}s^{-1} in our study), their light intensity was much higher and probably triggers many light responses in plant cells.

TPC2019-00314-RAR1 2nd Editorial decision – *acceptance pending* **July 20, 2019**

We are pleased to inform you that your paper entitled "hnRNP coordinates with phytochrome and U1 snRNP to regulate alternative splicing in *Physcomitrella patens*" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff. At this stage, your manuscript will be evaluated by a Science Editor with respect to scientific content presentation, compliance with journal policies, and presentation for a broad readership.

Final acceptance from Science Editor **August 6, 2019**