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A splicing factor switch controls hematopoietic lineage specification of pluripotent stem cells

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 13th May 2020

Dear Dr. Shi,

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all referees think that the findings are of interest, but they also have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication in EMBO reports. As the reports are below, and I think all points need to be addressed, I will not detail them here.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and/or in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

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to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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See also our guide for figure preparation:
http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

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

4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

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5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq and array data) are deposited in an appropriate public database. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated and deposited').

See also: http://embor.embopress.org/authorguide#datadeposition

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

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

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

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

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

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

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

Referee #1:

This manuscript reports evidence for a SRSF2-NUMB-NOTCH regulatory axis that controls HEP specification, mediated by what the authors term a "splicing factor switch" that occurs during differentiation of HEP. The authors analyzed RNA-seq data derived from in vitro culture of a human ESC cell line to show that substantial changes in expression of spliceosomal factors as well as SR proteins occur at the to stage of HEP formation, and that changes in alternative splicing of key genes such as NUMB occur concomitantly. Interestingly, the splicing inhibitor pladienolide B (PLB) blocked HEP formation, strongly inhibited the splicing factor switch, and inhibited the switch in NUMB alternative splicing. Additional experiments showed that over-expression of SRSF2, which has predicted binding sites in the regulated NUMB exon, can prevent the skipping of exon 12 needed for formation of NUMB-S, at the same time substantially rescuing the differentiation of HEP cells. Impressively, over-expression of NUMB-S also rescues HEP differentiation.

The role of post-transcriptional RNA processing in early hematopoiesis is clearly very important but poorly understood. These studies offer the potential for novel insights into this area. The authors report an intriguing alternative splicing switch in the NUMB gene that appears critical for HEP formation. This splicing event has been reported in other contexts and previously shown to be important in Notch signaling, but to my knowledge the demonstration of its importance in early hematopoiesis is novel. This result seems important and novel. However, in this reviewer's opinion, some of the regulatory steps proposed in this pathway are interesting but they rest heavily on correlations and lack some of the specific experimental validations needed to validate the models. They also fail to discuss other studies of NUMB splicing that are relevant to this work. The work would benefit from collaboration with an expert in alternative splicing.

1. The conclusion that a single alternative splicing switch in the NUMB gene plays a prominent role in HEP formation is rather remarkable: over-expression of NUMB-S can substantially overcome the (partial) differentiation block caused by PLB treatment (Fig. 4F, 4G). This is a very nice result. The caveat is that we don't get what the level of NUMB-S protein over-expression is - is it much higher than the normal level would be?

2. The regulation of NUMB-S splicing by SRSF2 is not definitive. The evidence is mostly based on correlations between SRSF2 expression and the balance of alternative splicing isoforms NUMB-L vs NUMB-S. The authors say there are predicted SRSF2 binding sites in exon 12, consistent with SRSF2 acting as an enhancer protein to increase exon 12 splicing and expression of NUMB -L; decrease of SRSF2 expression would then be consistent with skipping of exon 12 and expression of NUMB-S. However, predictions of splicing factor binding are imperfect and no details about the sites are provided. Proof of direct regulation by SRSF2 would require data linking SRSF2 binding to regulation, perhaps in a minigene splicing reporter model, or by CLIP-seq experiments to measure actual binding in wild type vs binding site-mutated exon 12, with accompanying splice data. Otherwise, it could well be that SRSF2 influences NUMB splicing indirectly or only weakly at endogenous levels.

3. Related to point #2, a deficiency of the paper is the lack of discussion of previous studies of NUMB splicing regulation. Other papers on NUMB splicing have identified splicing factors including SRSF1 as regulators of what appears to be the same NUMB exon (Rajendran, et al., 2016). In fact, even the authors' data in Figure 1F seems to show that down-regulation of SRSF1 correlates better with the appearance of HEP cells than SRSF2. Please discuss whether SRSF1 might be the major regulator. Also, the data for stage-specific changes in expression of SRSF2 are not consistent in Figure 1F vs 1G. In Fig. 1F, it appears that the greatest down-regulation of SRSF2 occurs in the transition from CD31+/CD34+ to CD43+, but in Fig. 1G the significant change is indicated between APLNR and CD31+/CD34+.

4. Regarding the effects of PLB: the authors present the data as if PLB is inducing a reverse splicing switch opposite to the naturally occurring switch in HEP. On p.12 they say "after the inhibition of splicing, the upregulation of SRSF2 contributed to the HEP defects". In Fig. 3B, when comparing genes differentially expressed in DMSO- vs PLB-treated cells, the differences are labeled as "PLB-UP". There may be similar language in other places. Unless I misunderstand the experiment, a more accurate interpretation might be that PLB blocks the normal differentiation-associated splicing transitions from occurring. Do the authors really think that PLB reverts the phenotype in cells that have already switched, rather than acting as an inhibitor to prevent the switches?

5. In my opinion the analysis of potential downstream effects is very preliminary, and the cause and effect relationships not firmly established. The authors described a reverse correlation between

NUMB-S and the transcription factor HES1, and suggest that HES1 might act as a downstream target of NUMB-S (p. 14). They report that HES1 over-expression severely abolished HEP formation, analogous to the effects of PLB treatment, and they suggest that NUMB-S might be a repressor of HES1 expression. This would be interesting but it seems very speculative. In fact, they report that several key components of the NOTCH signaling pathway are up-regulated during HEP formation, and that NOTCH signaling was strongly associated with differentially expressed splicing events (Fig. 5A). Can the authors rule out an entirely different cause and effect relationship, whereby changes in expression or splicing of the transcription factors is the upstream event that alters expression of SRSF2 (or SRSF1?) that ultimately regulates NUMB splicing?

6. A lot of important methological details are missing. How did the authors identify changes in alternative splicing? Figure 3B: what cutoff values used for counting alternative splicing events? Fig. 3F: how many fold is SRSF2 over-expressed? We have no idea.

Minor issues:

1. Figure 1B is said to represent "all the expressed splicing factors". What is the list of genes included in this analysis? Is that what is shown in "Supplementary information supp 2"? This should be mentioned in the text.

2. On page 7, the authors describe a splicing factor switch in both major and minor spliceosomes. Given the fact that much of the machinery is shared between these two spliceosomes, I wonder whether it is the shared factors that switch?

3. p.8: what does it mean to say that the spliceosome is structure in an orderly manner during hematopoietic differentiation?

4. Figure 3D: the red/blue scale is confusing due to its similarity to the Z-score scales in other parts of the figures.

5. There are some grammatical and /or spelling errors that requiring careful editing. A few that I noticed are the following:
Throughput is spelled wrong in Figure 1A.
The title for Figure legend 3 needs editing.
"Neglectable" on p. 11 maybe should be "negligible"
What is "inferior" alternative splicing?

Referee #2:

The manuscript by Li et al reports on alternative splicing during human ESC hematopoietic differentiation. Alternative splicing (AS) was previously shown to affect the differentiation process of adult bone marrow hematopoietic lineages via different mechanisms, including AS of introns which controls protein translation as transcripts retaining introns do not leave the nucleus, and via generation of alternative transcription factor isoforms that drive alternative differentiation outcomes. In addition, mutations in proteins involved in the splicing process have been detected in leukemia and clonal hematopoiesis, suggesting a role in maintenance of normal hematopoiesis. A role for AS in hematopoietic development at the early embryonic stage was still largely unknown, with the exception of RUNX1 for which alternative isoforms have been linked to different

hematopoietic outcomes. Here the authors assess alternatively spliced mRNAs during human ESC differentiation into hematopoietic progenitors, analysing different stages along this pathway including APLNR+ mesoderm, CD31+CD34 hemogenic endothelial progenitor cells (HEPs) and CD43+ hematopoietic progenitor cells. They found that widespread AS occurred over the entire hematopoietic trajectory, with exon skipping most prevalent. Expression of splicing factors was reported to be reduced during differentiation of mesoderm in HEPs, with a switch in the expression of family members making up specific splicing complexes. Next they assessed the effects of inhibiting splicing during hematopoietic development using a chemical (PLB) that targets SF3B1 thus inhibiting the spliceosome. This inhibitor was found to affect the generation of HEPs in culture. This was reported to be associated with perturbation in AS and a block of the splicing factor switch. This was complemented with experiments in which splicing factors were overexpressed, which also led to a decrease in HEP generation. To assess the mechanism, they next identified alternatively spliced isoforms in the mesoderm to HEP transition that were affected by PLB treatment. One of the 26 identified was NUMB, known to play a role in cell fate decisions. NUMB switched from a long (in mesoderm) to a short isoform (in HEPs). Generation of NUMB-S was negatively affected when PLB was added to the cultures. Forced expression of NUMB-S in combination with PLB treatment rescued the HEP phenotype. As the slicing factor SRSF2 was downregulated upon differentiation from mesoderm to HEPs, it was tested whether its acts in the splicing of NUMB. Indeed it favoured generation of NUMB-L. As NUMB is a NOTCH antagonist the role of NOTCH signalling in HEP generation was assessed. The NOTCH inhibitor DAPT showed a dose-related effect on HEP generation suggesting that NOTCH levels need to be within a tightly controlled range (which is well known, e.g. work of the Bigas and Medvinsky labs). Assessing downstream NOTCH signalling genes, there appeared to be a negative correlation between NUMB-S and HES1 and when HES1 was overexpression, no HEPs were generated. The authors concluded that the NUMB-S isoform controls the strength of NOTCH signalling, possibly by repressing HES1. In summary, the manuscript starts with a general profiling of AS in human hematopoietic differentiation, shows there is a switch of splicing factors during this process and narrowed down on the role for one of the underlying alternatively spliced genes, NUMB a NOTCH antagonist to providing an additional example of an alternatively spliced gene affecting embryonic hematopoiesis, in addition to RUNX1.

General comments:
The experimental strategy and design is not consistently well-described and the statistical analysis is not always clearly explained: none of the graphs show the number of samples used in each comparison and this information is not always stated in the figure legend; the t-test might not always be the right choice; and the description of the analysis performed is not always sufficient (or present at all). This should be addressed.

Specific comments:
1. Fig 1A: CD31+CD34+ is not a phenotype specific for hemogenic endothelium in human in vitro differentiation. CD31+CD34+ cells include both vascular and hemogenic endothelium. CD73 could have been used to separate these populations (e.g. Ditadi et al., Nat Cell Biology 2015). This does not undermine the effect described in this population in the rest of the paper, but HEP is possibly not the right term to define these cells.

2. Fig 1C - RNA-seq heatmap of splicing factors: How were clusters determined? The two clusters do not match up with the displayed dendrogram. The dendrogram looks somewhat messed up, as the highest branch is overlapping with another branch near the top. One could argue there are 4 clusters (extra group at the top and bottom). The argument for 2 clusters should be clarified, e.g. could be backed up with an elbow plot or silhouette plot.

3. It is stated that there was a cytotoxic effect of PLB when used throughout the differentiation process (8 days, data not shown), but no effect when used only at 2-3 days intervals (Fig 2A). The authors should include the data on PLB cytotoxicity in the supplementary figure. It is also important to show viability staining on the experiments in Fig 2 to support that there are no problems with cytotoxicity with shorter PLB incubations. This would affect the conclusions of the manuscript.

4. Fig 2D-E relating to apoptosis and cell cycle analysis with PLB spliceosome inhibition, showing PLB specifically effects HEP production: Assays were performed at day 5, which is at the end of the PLB sensitive period of differentiation. Could this be too late, and sensitive cells at this point have died out? Earlier time points could be beneficial. This might also help to understand whether the consequence of PLB spliceosome inhibition is the activation of a specific cell-death program in HEP or the abrogation of mesoderm to HEP differentiation; Either way, is this affecting vascular and hemogenic endothelium in the same way (see minor points for comment on phenotypic identification of HEPs)? Is it specific for hemogenic endothelium? (in this regard, could the remaining CD31+CD34+ cells represent one of the two populations in fig. 2C?). Finally FACS plots should be included, to show gating strategy.

5. Fig S2D - a quantification of the LDL uptake experiment is needed to support the conclusion.

6. Fig 2F - the experimental strategy is not clearly explained. Is the APLNR to HEP differentiation performed while inhibiting the spliceosome with PLB (as suggested by the schematic) or there is an inhibition step first, followed by differentiation (as suggested in the main text)?

7. Fig 3A - experimental strategy not clearly explained: What are the cells sorted and collected? Are the cells FACS-sorted at day 5? If so, what is their phenotype? Are they HEP cells or are they stuck to APLNR+ stage?

8. Fig 3C - Heatmap of splicing factors showing HEP + PLB shows similar profile to APLNR+. It would be nice to see this as scatter plot and correlation of expression values. Does PLB treatment correlate with APLNR+ genome wide, or only at splicing factors?

9. Fig 3F - Inducible SRSF2 schematic does not show FLAG. Presumably it does, otherwise the western is referring to a different construct. Were stable transfected lines generated? This is not clearly explained neither in the main text nor in the methods. If not, how were the cells overexpressing SRSF2 selected? If Puromycin was used for selection, why do only a fraction of cells express GFP (in Fig.4F). If GFP was used to identify the overexpressing population, please provide the frequency of GFP+ cells analysed in supplementary figure 3 (and in all the overexpression experiments based on the same system).

10. Fig 3F and S3C-G - only one out of four factors (SRSF2) recapitulates the decreased HEP generation, which is in agreement with its significant and exclusive upregulation in response to PLB treatment. This seems a little light to conclude that "In summary, the disturbed splicing as a result of the impediment of the splicing factor switch leads to impaired HEP formation"?

11. Fig 4F - FACS gates showing SSC vs GFP, then CD34 CD31... Intended to show NUMB_S induction recovers phenotype caused by PLB. However, as the GFP intensity of the NUMB-S vector is weaker than the empty vector, the SSC vs GFP gate does not capture the same population, and is enriched for higher GFP levels. This could bias the result. On the basis of what were the gates set? FMOs should be shown in the supplementary. Axis of the dotplots should be better labelled with fluorescent intensities.

12. Fig 5C-D - This is already known and published (including the use of DAPT, Uenishi et al., Nat Communications 2018) and should be acknowledged in the main text.

13. The conclusion that "NUMB-S controls the precise strength of NOTCH signalling and the HEP differentiation process, potentially by repressing HES1 expression" needs the addition that the conclusion is based exclusively on the observation of the two factors independently. But there is no evidence supporting a direct or indirect effect of NUMB-S on HES1. The model in Fig 5J suggests otherwise and should be adapted.

Minor points:
Fig 1H: "No changed genes" is somewhat confusing; "Number of changed genes" would be clearer.

Fig 2A and 2C - units of measurement are missing on the quantification of flow cytometry experiments (frequencies of ...? Absolute number?)

Fig 3D - not clear what labels refer to what Venn circles

Fig 4C - Sashimi plot: Needs a y axis scale to allow comparison of relative transcript levels. Also needs exons labelled

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Referee #3:

In this manuscript, the authors investigate the role of post-transcriptional RNA processing in hematopoietic development using a hPSC-based model. The authors apply a combination of wet lab approaches and bioinformatics analysis to identify novel players linked to the differentiation of mesodermal cells to hemogenic endothelial progenitor cells. Their investigation led to the identification of a widespread mechanism whereby a dynamic alternative splicing reprograming occurs during hematopoietic development. They uncovered a splicing regulatory axis consisting of SRSF2-NUMB-NOTCH which seems to be an important contributor of hemogenic endothelial progenitor cells differentiation.

The manuscript is clear, concise, well written and figures are, in general, well-constructed. Furthermore, claims made by the authors are supported by the data generated from well-designed experiments. However, I feel that the manuscript could benefit from some minor clarifications/adjustments, as follows:
-The authors analysed the alternative splicing program of human hematopoietic development from ESCs and identified a splicing factor switch occurring during HEP generation (Figure 1). Have the author considered to examine the impact of alternative splicing events in splicing factors that could explain their switch in expression? In other words, is there an orchestrated AS program during differentiation that regulate splicing factors expression (eg. intron retention)?

- The authors use pladienolide B (PLB), a natural inhibitor of the spliceosome which targets SF3B1 but this compound is also cytotoxic and induced apoptosis, even at a very low dose during hematopoietic differentiation process (data not shown by the authors). Have the authors considered the use of siRNA/shRNA targeting specifically SF3B1 to corroborate the data obtained with PLB treatment?

- Could the author explain (and clarify in the text) their choice of exon 9 of LAS1L, ATP5F1C and

HDAC7 (Figure 2 and S2) as markers of splicing defect after PLB treatment? Why not including intron retention as markers of splicing defect instead of exon inclusion? Furthermore, if I interpreted the data in the figures correctly, DMSO treatment seems to have more effect (for LAS1L and HDAC7) than with the two concentration of PLB tested. Any comment from the authors on that particular point? (maybe I did misunderstand something and if that's the case I do apologise for this comment).

-In Figure 4H, the authors detected (in silico) SRSF2 binding sites within NUMB-Exon12 and tested the effect of PLB treatment or/and SRSF2 overexpression on the expression of NUMB isoforms. The treatments led to the reduction of the level of the NUMB-S transcripts (Figure 4I) and a concomitant increase of the NUMB-L transcripts (Figure. 4J). They therefore concluded that SRSF2 functions in the regulation of NUMB alternative splicing. Have the author thought of inducing the knock down of SRSF2 to see if it induces the up-regulation of NUMB-Small isoform? Furthermore, it would be really essential to confirm that SRSF2 actually binds to exon 12 (and to which binding sites) before they could claim that SRFS2 is the direct player regulating NUMB-Exon12 splicing (using mini-gene reporter or Protein-RNA pull-down assay).

RESPONSE TO CRITIQUES

We thank the editors and reviewers for their instructive comments and recommendations. Below, we have articulated the point-by-point responses and revisions implemented, including new experimental data that addresses each of the reviewers' comments .

Referee #1:

This manuscript reports evidence for a SRSF2-NUMB-NOTCH regulatory axis that controls HEP specification, mediated by what the authors term a "splicing factor switch" that occurs during differentiation of HEP. The authors analyzed RNA-seq data derived from in vitro culture of a human ESC cell line to show that substantial changes in expression of spliceosomal factors as well as SR proteins occur at the stage of HEP formation, and that changes in alternative splicing of key genes such as NUMB occur concomitantly. Interestingly, the splicing inhibitor pladienolide B (PLB) blocked HEP formation, strongly inhibited the splicing factor switch, and inhibited the switch in NUMB alternative splicing. Additional experiments showed that over-expression of SRSF2, which has predicted binding sites in the regulated NUMB exon, can prevent the skipping of exon 12 needed for formation of NUMB-S, at the same time substantially rescuing the differentiation of HEP cells. Impressively, over-expression of NUMB-S also rescues HEP differentiation.

The role of post-transcriptional RNA processing in early hematopoiesis is clearly very important but poorly understood. These studies offer the potential for novel insights into this area. The authors report an intriguing alternative splicing switch in the NUMB gene that appears critical for HEP formation. This splicing event has been reported in other contexts and previously shown to be important in Notch signaling, but to my knowledge the demonstration of its importance in early hematopoiesis is novel. This result seems important and novel. However, in this reviewer's opinion, some of the regulatory steps proposed in this pathway are interesting but they rest

heavily on correlations and lack some of the specific experimental validations needed to validate the models. They also fail to discuss other studies of NUMB splicing that are relevant to this work. The work would benefit from collaboration with an expert in alternative splicing.

Response:

Regarding the SRSF2-NUMB-NOTCH axis, we included additional data to support our proposed model. Although these details are articulated in the point-by-point responses, here is a summary of the major experimental additions:

1) We used minigene reporter to confirm that SRSF2 could bind and promote the generation of *NUMB_L* isoform in response 2. This new data was incorporated as Fig. 5B-D and described on page 14, lines 343-354 (highlighted in yellow).

2) We overexpressed HES1 in 293T cells and found that no alteration of SRSF2 expression and no alterations of *NUMB* exon 12 splicing were detected, ruling out the possibility that HES1 regulates *NUMB* splicing indirectly via modulating SRSF2 expression in response 5. This new data was incorporated as Fig. EV5G and H and described on page 16, lines 400-402 (highlighted in yellow).

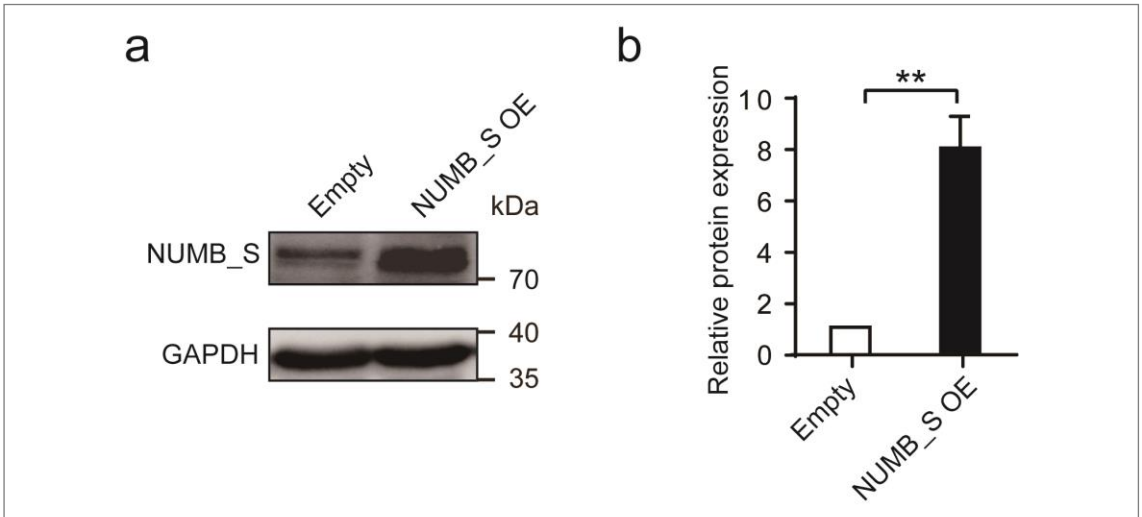
Regarding prior work on *NUMB* splicing, we have summarized the references relevant to *NUMB* splicing in the revised discussion section on page 19, lines 463-468 (highlighted in yellow).

Regarding the suggestion about including an expert in alternative splicing, the alternative splicing analysis in this study was supervised by Dr. Hong-Dong Li, a co-author and expert in alternative splicing.

Query 1: The conclusion that a single alternative splicing switch in the NUMB gene plays a prominent role in HEP formation is rather remarkable: over-expression of NUMB-S can substantially overcome the (partial) differentiation block caused by PLB treatment (Fig. 4F, 4G). This is a very nice result. The caveat is that we don't get what the level of NUMB-S protein over-expression is - is it much higher than the normal level would be?

Response 1:

To determine the expression level of NUMB_S, we conducted Western blotting from the NUMB_S stable overexpression hESC cell line (GFP^+), in which NUMB_S expression is regulated by a DOX-dependent system, as detailed in the manuscript. During day 2.5 to 5 of hematopoietic differentiation, 1 μg/ml DOX was added to induce NUMB_S overexpression. At day 5, we FACS-sorted GFP^+ cells and measured NUMB_S protein level with an anti-NUMB antibody by Western blotting (Fig. 4E). The NUMB_S protein was overexpressed approximately 8-fold relative to NUMB detected in the cells containing the empty vector. This new data was incorporated as Fig. 4E and described on page 13, lines 320-324. The figures are also shown below.

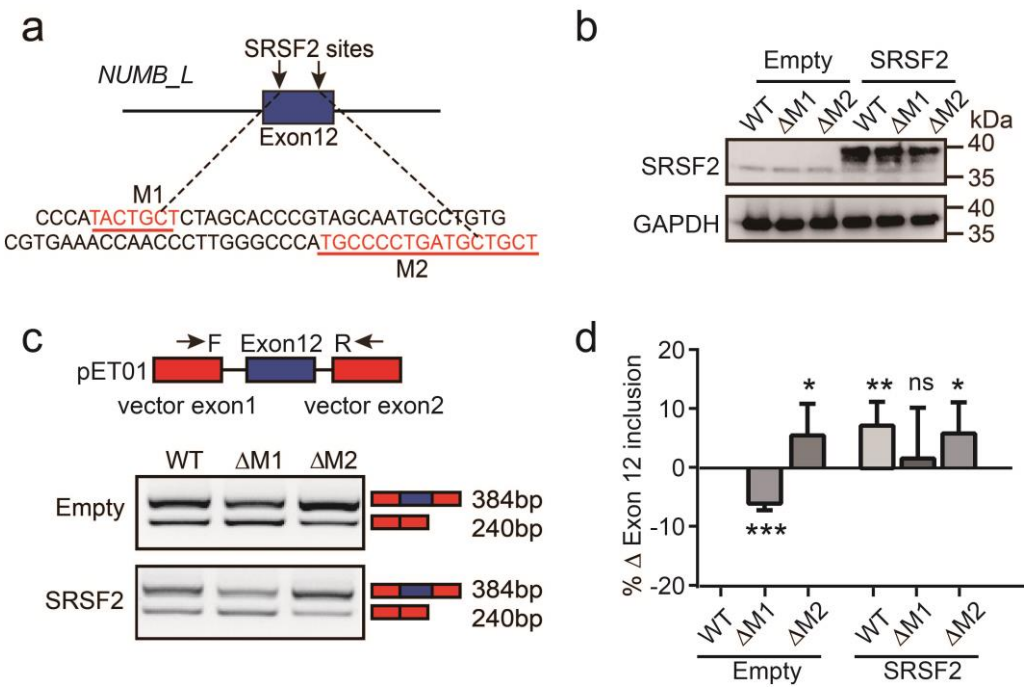


NUMB_S protein overexpression. (a) Western blotting measures the NUMB_S overexpression in GFP^+ cells at day 5 of hematopoietic differentiation. GAPDH serves as a loading control. (b) The quantification of the NUMB_S protein level.

Query 2: The regulation of NUMB_S splicing by SRSF2 is not definitive. The evidence is mostly based on correlations between SRSF2 expression and the balance of alternative splicing isoforms NUMB-L vs NUMB-S. The authors say there are predicted SRSF2 binding sites in exon 12, consistent with SRSF2 acting as an enhancer protein to increase exon 12 splicing and expression of NUMB-L; decrease of SRSF2 expression would then be consistent with skipping of exon 12 and expression of NUMB-S. However, predictions of splicing factor binding are imperfect and no details about the sites are provided. Proof of direct regulation by SRSF2 would require data linking SRSF2 binding to regulation, perhaps in a minigene splicing reporter model, or by CLIP-seq experiments to measure actual binding in wild type vs binding site-mutated exon 12, with accompanying splice data. Otherwise, it could well be that SRSF2 influences NUMB splicing indirectly or only weakly at endogenous levels.

Response 2:

As shown in the revised Fig. 5, there were two putative SRSF2 binding motifs on *NUMB* exon 12 (M1 and M2, labeled as red) by RBPmap (http://rbpmap.technion.ac.il/) (Fig. 5A). To further validate the potential of SRSF2 to regulate splicing of *NUMB* exon 12 via these motifs, we performed a minigene splicing reporter assay. We constructed a truncated NUMB reporter, encompassing a genomic fragment from 540 nucleotides upstream to 507 nucleotides downstream of *NUMB* exon 12. This reporter was cloned into an ExonTrap pET01 vector containing two constitutive exons, as reported previously (Rajendran *et al*, 2016). We also constructed two mutant NUMB reporters by deleting each motif (ΔM1 or ΔM2), respectively. After co-transfection of each NUMB reporter (WT, ΔM1 or ΔM2) with SRSF2 expression plasmids into 293T cells simultaneously, we found that SRSF2 promoted the generation of *NUMB_L* isoform via M1 but not M2 (Fig. 5B-D). This new data was incorporated as Fig. 5B-D and described on page 14, lines 343-354 (highlighted in yellow). The figures are also shown below.



SRSF2 represses the *NUMB_S* isoform generation. (a) The putative SRSF2 binding motifs on *NUMB* exon 12 (labeled as red), predicted by RBPmap software. M1 refers to motif 1 and M2 refers to motif 2. (b) Western blotting showing the overexpression of SRSF2 in 293T cells, which were co-transfected with NUMB reporter WT, ΔM1 or ΔM2. ΔM1 or ΔM2 represent plasmids deleting M1or M2 binding site. (c) A minigene splicing reporter assay showing SRSF2 promotes the generation of *NUMB_L* isoform via M1 but not M2 binding site. The upper panel indicating the pET01 vector information, which contains two constitutive exons. (d) The quantification of the exon 12 inclusion/ exclusion of (c).

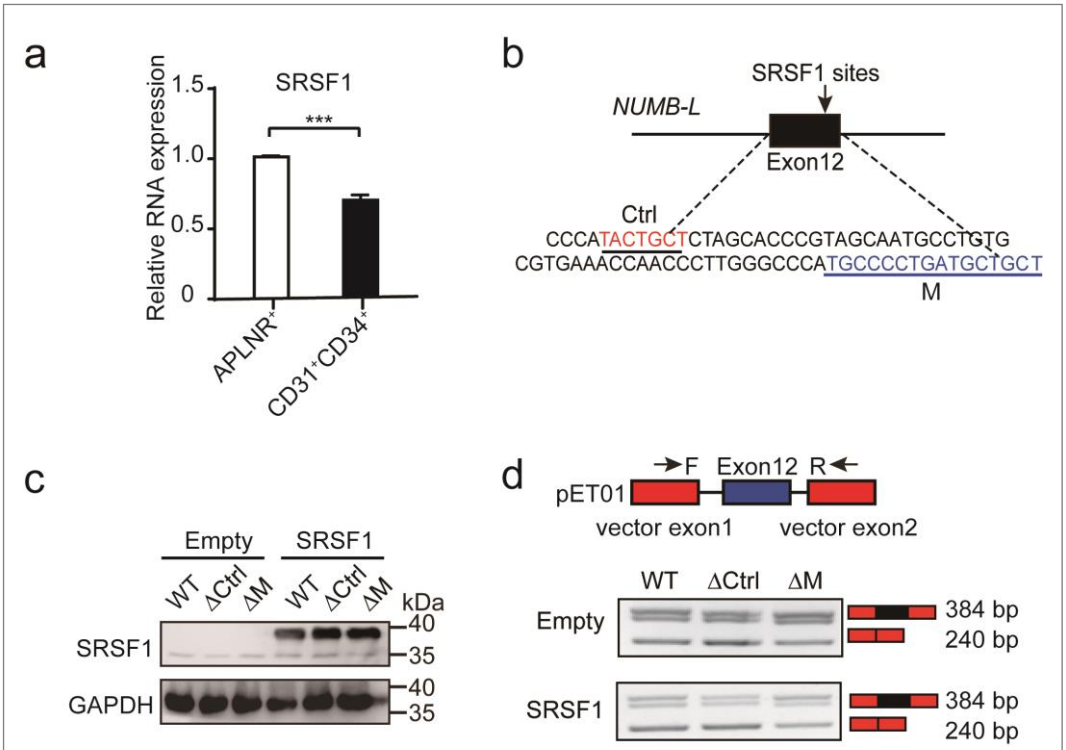
Query 3: Related to point #2, a deficiency of the paper is the lack of discussion of previous studies of NUMB splicing regulation. Other papers on NUMB splicing have identified splicing factors including SRSF1 as regulators of what appears to be the same NUMB exon (Rajendran, et al., 2016). In fact, even the authors' data in Figure

1F seems to show that down-regulation of SRSF1 correlates better with the appearance of HEP cells than SRSF2. Please discuss whether SRSF1 might be the major regulator. Also, the data for stage-specific changes in expression of SRSF2 are not consistent in Figure 1F vs 1G. In Fig. 1F, it appears that the greatest down-regulation of SRSF2 occurs in the transition from CD31+/CD34+ to CD43+, but in Fig. 1G the significant change is indicated between APLNR and CD31+/CD34+.

Response 3:

1) We have discussed the prior studies relevant to NUMB exon 12 splicing regulation, including SRSF1 and PTBP1(Rajendran *et al.*, 2016), SRSF3(Ke *et al*, 2018), RBM10(Bechara *et al*, 2013), RBM4(Tarn *et al*, 2016), RBFOX2 and SRPK2 (Lu *et al*, 2015), etc, in the Discussion on page 19, lines 463-468 (highlighted in yellow).

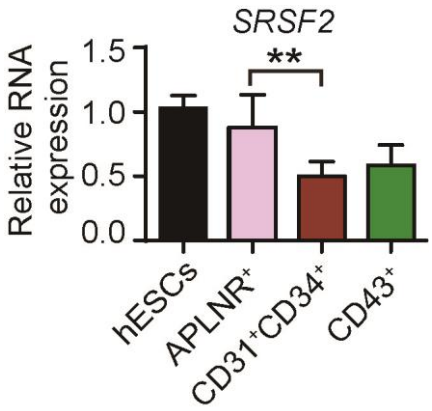
2) We agree with the reviewer that SRSF1 is also significantly downregulated during $APLNR^+$ to $CD31^+CD34^+$ differentiation, which was confirmed by RT-qPCR. To address the possible role of SRSF1 on *NUMB* exon 12 splicing, we also predicted SRSF1 putative binding motif(s) on exon 12 (labeled as red, M; which is also the M2 of SRSF2). By applying the same minigene splicing reporter assay as demonstrated for SRSF2 (Response 2), we found that SRSF1 promoted the *NUMB_S* isoform generation via this motif, consistent with the previous study (Rajendran et al., 2016). Thus, SRSF1 promotes *NUMB_S* generation via this motif (which we named as M2 in SRSF2 minigene assay), whereas SRSF2 suppresses *NUMB_S* via M1 motif. These results suggest that, besides SRSF2, SRSF1 is another important regulator during hematopoietic differentiation, and this merits further investigations. We incorporated the discussion on page 19, lines 468-474(highlighted in yellow). The figures are shown below.



SRSF1 promotes the *NUMB_S* isoform generation. (a) The mRNA expression of *SRSF1* measured by RT-qPCR in FASC-purified APLNR$^+$, CD31$^+$CD34$^+$ cells on day 2 and 5 differentiation, respectively. (b) The putative SRSF1 binding motifs on *NUMB* exon 12 (labeled as red), predicted by RBPmap. Ctrl serves as a negative control and M refers to SRSF1 binding motif. (c) Western blotting showing the over expression of SRSF1 in 293T cells, detected after co-transfection with each NUMB reporter (WT, ΔCtrl or ΔM). ΔCtrl or ΔM represent plasmids deleting specific putative binding site. (d) A minigene splicing reporter assay showing SRSF1 promoted the generation of *NUMB_S* isoform via M.

3) To illustrate the kinetics of SRSF2 during hematopoietic differentiation induced from hESCs, we harvested hESCs on day 0 as well as FACS-purified APLNR$^+$, CD31$^+$CD34$^+$, and CD43$^+$ cells on days 2, 5 and 8, respectively, and conducted RT-qPCR with at least 3 additional biological replicates. As shown in the updated Fig.

1G (n=6), we detected reduced APLNR$^+$ cell generation of CD31$^+$CD34$^+$ cells. The difference between the RNA-Seq and RT-qPCR might associate with distinct normalized strategy between them. The figure is also shown below.



The mRNA expression of splicing regulator *SRSF2* during hematopoietic differentiation was measured with RT-qPCR. The *ACTB* gene was used as a control.

Query 4: Regarding the effects of PLB: the authors present the data as if PLB is inducing a reverse splicing switch opposite to the naturally occurring switch in HEP. On p.12 they say "after the inhibition of splicing, the upregulation of SRSF2 contributed to the HEP defects". In Fig. 3B, when comparing genes differentially expressed in DMSO- vs PLB-treated cells, the differences are labeled as "PLB-UP". There may be similar language in other places. Unless I misunderstand the experiment, a more accurate interpretation might be that PLB blocks the normal differentiation-associated splicing transitions from occurring. Do the authors really think that PLB reverts the phenotype in cells that have already switched, rather than acting as an inhibitor to prevent the switches?

Response 4:

1) Thank you for pointing this out. We agree it is likely that PLB prevents the

splicing factor switch. We have revised all related descriptions throughout the entire manuscript. For example, “after the inhibition of splicing, the upregulation of SRSF2 contributed to the HEP defects” has been revised as “After inhibition of splicing with PLB, we observed the decreased generation of EPC and the sustained high-level of SRSF2” on page 13, lines 336-337 (highlighted in yellow).

2） We changed the label “PLB-UP” to “PLB-high” (Fig. 3D)

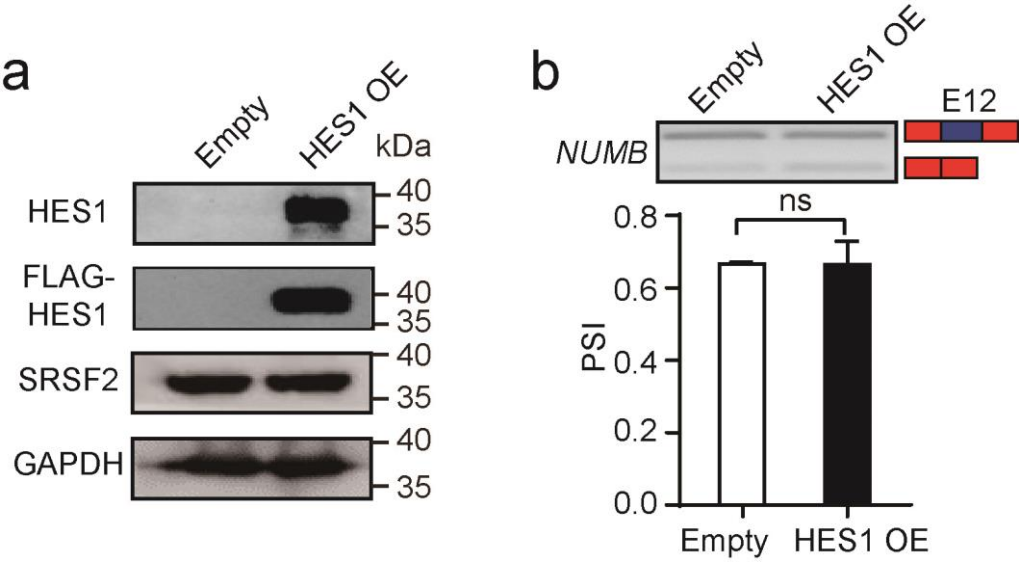
Query 5: In my opinion the analysis of potential downstream effects is very preliminary, and the cause and effect relationships not firmly established. The authors described a reverse correlation between NUMB-S and the transcription factor HES1, and suggest that HES1 might act as a downstream target of NUMB-S (p. 14). They report that HES1 over-expression severely abolished HEP formation, analogous to the effects of PLB treatment, and they suggest that NUMB-S might be a repressor of HES1 expression. This would be interesting but it seems very speculative. In fact, they report that several key components of the NOTCH signaling pathway are up-regulated during HEP formation, and that NOTCH signaling was strongly associated with differentially expressed splicing events (Fig. 5A). Can the authors rule out an entirely different cause and effect relationship, whereby changes in expression or splicing of the transcription factors is the upstream event that alters expression of SRSF2 (or SRSF1?) that ultimately regulates NUMB splicing?

Response 5:

1) To strengthen the cause-and-effect relationship between SRSF2, NUMB and HES1, we tested whether SRSF2 binds and regulates NUMB exon 12 splicing with minigene reporter system (Fig. 5A-D), as indicated in response 2.

2) Considering that HES1 only has one protein-coding isoform (NP_005515.1), we ruled out the possibility that changes in its splicing could be the upstream event to alter SRSF2 expression and indirectly to regulate NUMB splicing. To further

determine the mechanistic relationship between SRSF2 and HES1, we overexpressed HES1 in 293T cells, and assessed SRSF2 expression upon PLB treatment. No alteration of SRSF2 expression and no alterations of NUMB exon 12 splicing were detected (Fig. EV5G and H). This analysis provided evidence against the possibility that HES1 regulates NUMB splicing indirectly via modulating SRSF2 expression. This new data was incorporated as Fig. EV5G and H and described on page 16 (highlighted in yellow), lines 400-402. The figures are also shown below.



HES1 overexpression did not alter SRSF2 expression and *NUMB* exon 12 splicing. (a) Western blotting showing the expression of HES1 (detected by both endogenous HES1 antibody as well as anti-FLAG antibody) and SRSF2 in 293T cells. GADPH acts as a loading control. (b) The splicing of *NUMB* exon 12 after HES1 overexpression.

3) We did not assess the impact of other key NOTCH components on SRSF1 or SRSF2 expression and NUMB splicing, and this may merit further investigation.

Query 6: A lot of important methological details are missing. How did the authors identify changes in alternative splicing? Figure 3B: what cutoff values used for counting alternative splicing events? Fig. 3F: how many fold is SRSF2 over-expressed? We have no idea.

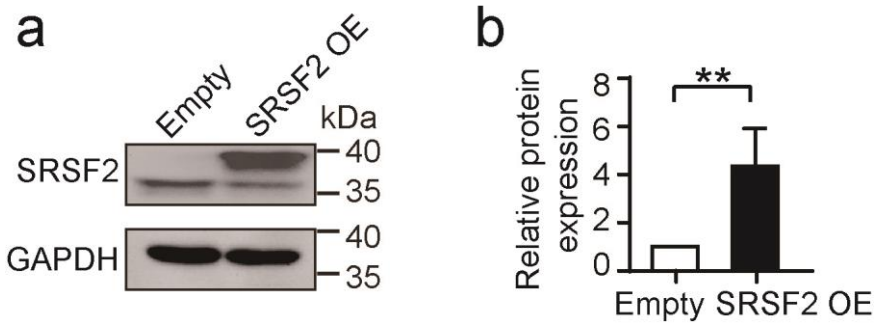
Response 6:

1) We expanded the description of important methodological details and data processing. This information was incorporated into Materials and Methods on pages 21-28, lines 530-704.

2) To identify changes in alternative splicing, we applied rMATS (version 4.0.2) with the default setting after pairwise comparison between two consecutive differentiation stages. This is described on page 26, lines 659-662.

3) For Fig 3B, to identify differential splicing events, we filtered the genes with FPKM >1 in at least one differentiation stage. Among them, the cutoffs of delta PSI > 0.2 & FDR < 0.05 were used to define differentially alternative splicing events. This is described on page 10, lines 250-252 and page 44, lines 1050-1052.

4) To determine the expression level of SRSF2, we conducted Western blotting from the SRSF2 stable overexpression hESC cell line (GFP^+), in which SRSF2 expression is regulated by a DOX-dependent system, as detailed in the manuscript. During day 2.5 to 5 of hematopoietic differentiation, 1 μg/ml DOX was added to induce SRSF2 overexpression. At day 5, we FACS-sorted GFP^+ cells and measured SRSF2 protein level with an anti-SRSF2 antibody by Western blotting (Fig. 4E). The SRSF2 protein was overexpressed approximately 4-fold relative to SRSF2 detected in the cells containing the empty vector. This new data was incorporated as Fig. 3G and described on page 11, lines 278-280. The figures are also shown below.



SRSF2 protein overexpression. (a) Western blotting measures the SRSF2 overexpression in GFP^+ cells at day 5 of hematopoietic differentiation. GAPDH serves as a loading control. (b) The quantification of the SRSF2 protein level.

213

214 Minor issues:

215 Query 1: Figure 1B is said to represent "all the expressed splicing factors". What is
216 the list of genes included in this analysis? Is that what is shown in "Supplementary
217 information supp 2"? This should be mentioned in the text.

218 Response 1:

219 We provided the list of expressed splicing factors in the revised Table EV1.

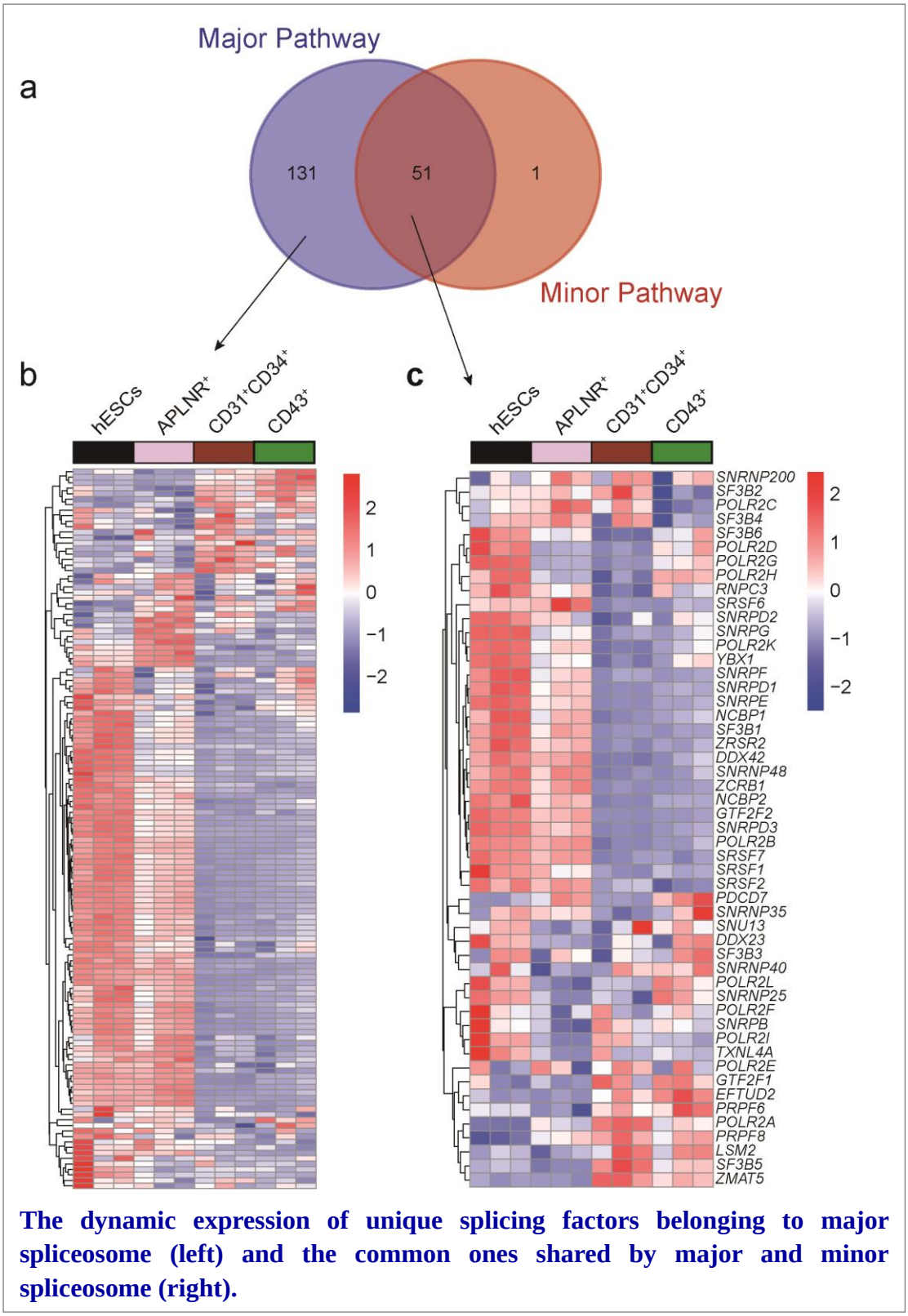
220 Query 2: On page 7, the authors describe a splicing factor switch in both major and
221 minor spliceosomes. Given the fact that much of the machinery is shared between
222 these two spliceosomes, I wonder whether it is the shared factors that switch?

223 Response 2:

224 According to the reviewer's suggestion, we analyzed the dynamics of unique splicing
225 factors of major spliceosome and the common ones shared by major and minor
226 spliceosome. The majority of the splicing factors, including both the shared and

227 unique ones, showed the switching pattern from APLNR$^+$ to CD31$^+$CD34$^+$ transition.

228 The figures are shown below.



The dynamic expression of unique splicing factors belonging to major spliceosome (left) and the common ones shared by major and minor spliceosome (right).

Query 3. p.8: what does it mean to say that the spliceosome is structure in an orderly manner during hematopoietic differentiation?

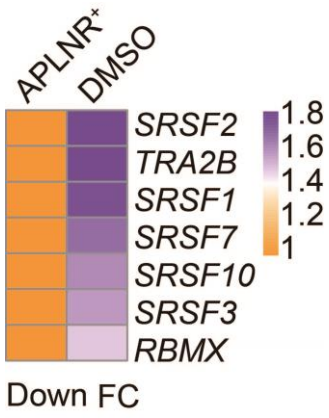
Response 3

We have deleted: “the spliceosome is structure in an orderly manner during hematopoietic differentiation” in the sentence and revised it as: “In summary, these results reveal that splicing factor expression is tightly controlled during hematopoietic differentiation”, and incorporated this statement on page 8, lines 195-196.

Query 4. Figure 3D: the red/blue scale is confusing due to its similarity to the Z-score scales in other parts of the figures.

Response 4:

To improve comprehension, we changed the scale to orange and purple in the updated Figure 3D. The figure is also shown below.



The heatmap illustrates the expression of seven splicing regulators. All gene expression was normalized to APLNR$^+$ cells. The purple color represents the reduced fold change.

Query 5: There are some grammatical and /or spelling errors that requiring careful editing. A few that I noticed are the following:

Throughput is spelled wrong in Figure 1A.

The title for Figure legend 3 needs editing.

"Neglectable" on p. 11 maybe should be "negligible"

What is "inferior" alternative splicing?

Response 5:

We appreciate the careful review. We have gone through the manuscript and corrected spelling and optimized grammar.

1) We corrected “Throughput” in the updated Figure 1A.

2) We revised the title of Figure legend 3 to “Disruption of the splicing factor switch impacts EPC specification” on page 38, lines 1041-1042.

3) "Neglectable" on p. 11 was changed to "little" (page 12, line 283).

4) We deleted “inferior” in the revised manuscript.

Referee #2:

The manuscript by Li et al reports on alternative splicing during human ESC hematopoietic differentiation. Alternative splicing (AS) was previously shown to affect the differentiation process of adult bone marrow hematopoietic lineages via different mechanisms, including AS of introns which controls protein translation as transcripts retaining introns do not leave the nucleus, and via generation of alternative transcription factor isoforms that drive alternative differentiation outcomes. In addition, mutations in proteins involved in the splicing process have been detected in leukemia and clonal hematopoiesis, suggesting a role in maintenance of normal hematopoiesis. A role for AS in hematopoietic development at the early embryonic stage was still largely unknown, with the exception of RUNX1 for which alternative isoforms have been linked to different hematopoietic outcomes. Here the authors assess alternatively spliced mRNAs during human ESC differentiation into hematopoietic progenitors, analyzing different stages along this pathway including APLNR+ mesoderm, CD31+CD34 hemogenic endothelial progenitor cells (HEPs) and CD43+ hematopoietic progenitor cells. They found that widespread AS occurred over the entire hematopoietic trajectory, with exon skipping most prevalent. Expression of splicing factors was reported to be reduced during differentiation of mesoderm in HEPs, with a switch in the expression of family members making up specific splicing complexes. Next they assessed the effects of inhibiting splicing during hematopoietic development using a chemical (PLB) that targets SF3B1 thus inhibiting the spliceosome. This inhibitor was found to affect the generation of HEPs in culture. This was reported to be associated with perturbation in AS and a block of the splicing factor switch. This was complemented with experiments in which splicing factors were overexpressed, which also led to a decrease in HEP generation. To assess the mechanism, they next identified alternatively spliced isoforms in the mesoderm to HEP transition that were affected by PLB treatment. One of the 26 identified was NUMB, known to play a role in cell fate decisions. NUMB switched from a long (in

mesoderm) to a short isoform (in HEPs). Generation of NUMB-S was negatively affected when PLB was added to the cultures. Forced expression of NUMB-S in combination with PLB treatment rescued the HEP phenotype. As the slicing factor SRSF2 was downregulated upon differentiation from mesoderm to HEPs, it was tested whether its acts in the splicing of NUMB. Indeed, it favored generation of NUMB-L. As NUMB is a NOTCH antagonist the role of NOTCH signaling in HEP generation was assessed. The NOTCH inhibitor DAPT showed a dose-related effect on HEP generation suggesting that NOTCH levels need to be within a tightly controlled range (which is well known, e.g. work of the Bigas and Medvinsky labs). Assessing downstream NOTCH signaling genes, there appeared to be a negative correlation between NUMB-S and HES1 and when HES1 was overexpression, no HEPs were generated. The authors concluded that the NUMB-S isoform controls the strength of NOTCH signaling, possibly by repressing HES1. In summary, the manuscript starts with a general profiling of AS in human hematopoietic differentiation, shows there is a switch of splicing factors during this process and narrowed down on the role for one of the underlying alternatively spliced genes, NUMB a NOTCH antagonist to providing an additional example of an alternatively spliced gene affecting embryonic hematopoiesis, in addition to RUNX1.

General comments:

The experimental strategy and design is not consistently well-described and the statistical analysis is not always clearly explained: none of the graphs show the number of samples used in each comparison and this information is not always stated in the figure legend; the t-test might not always be the right choice; and the description of the analysis performed is not always sufficient (or present at all). This should be addressed.

Response to general comments:

We thank the reviewer for pointing out these issues. In the revised manuscript, we

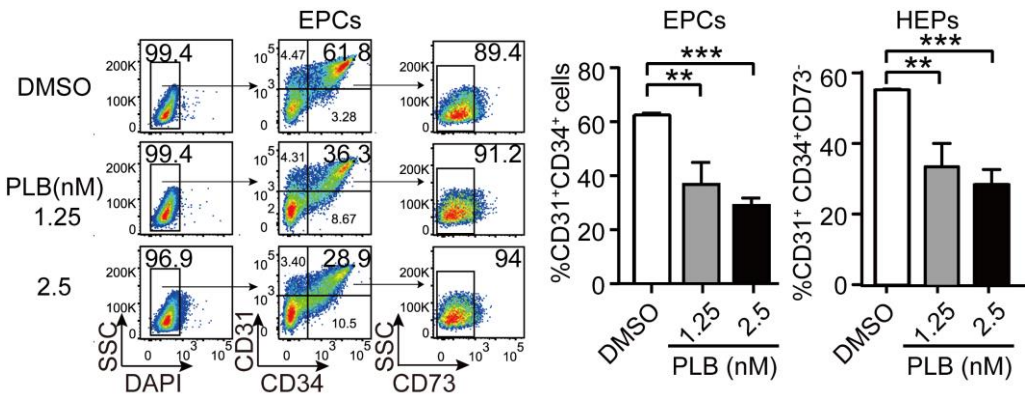
improved the description of experiments and analyses (Materials and Methods, pages 21-28, lines 530-704). Additionally, we more optimally conducted and presented the statistical analysis. P-values between two or three groups were determined by t-test, while multiple comparisons were calculated by ANOVA or the Wilcoxon signed-rank test (pages 27-28, lines 696-704). The sample numbers and statistical methods are labeled on each panel of the revised figure legends (highlighted with yellow).

Specific comments:

Query 1: Fig 1A: CD31+CD34+ is not a phenotype specific for hemogenic endothelium in human in vitro differentiation. CD31+CD34+ cells include both vascular and hemogenic endothelium. CD73 could have been used to separate these populations (e.g. Ditadi et al., Nat Cell Biology 2015). This does not undermine the effect described in this population in the rest of the paper, but HEP is possibly not the right term to define these cells.

Response 1:

We agree with the reviewer's comments. In the revised manuscript, we redefined $CD31^{+}CD34^{+}$ cells as endothelial progenitor cells (EPCs). We also defined HEPs as $CD31^{+}CD34^{+}CD73^{-}$ cells (incorporated into page 6, lines 122-125) and provided the experimental data regarding the influence of PLB on HEP ($CD31^{+}CD34^{+}CD73^{-}$) generation. This new data was incorporated into Figure 2G and discussed on page 10, lines 234-238. The figures are also shown below.

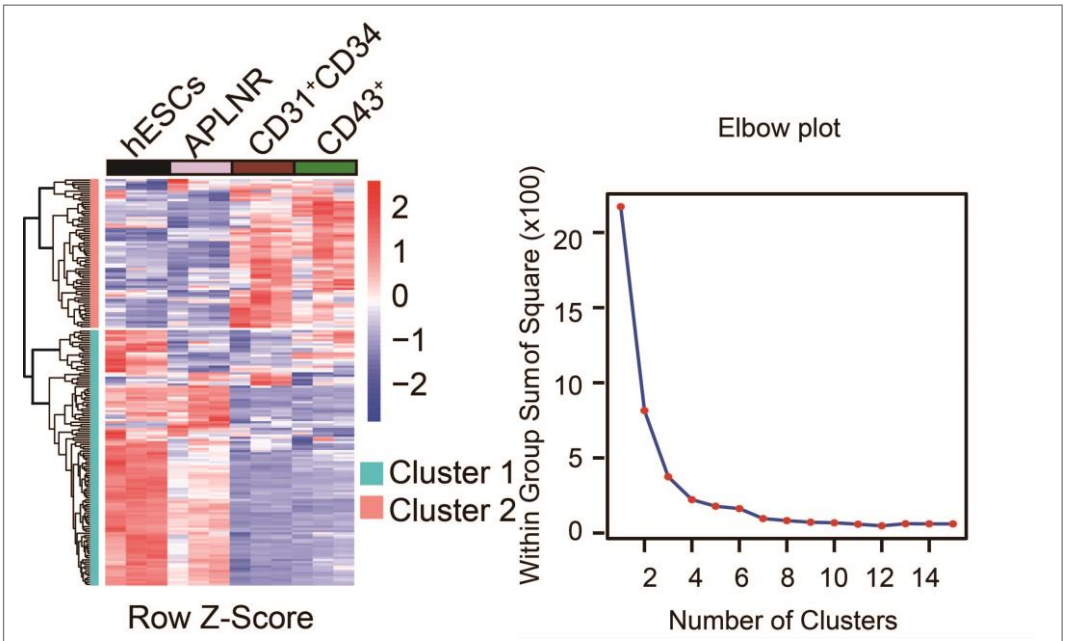


PLB treatment during day 2.5 to 5 reduced EPC and HEP generation on day 5 of hematopoietic differentiation.

Query 2. Fig 1C - RNA-seq heatmap of splicing factors: How were clusters determined? The two clusters do not match up with the displayed dendrogram. The dendrogram looks somewhat messed up, as the highest branch is overlapping with another branch near the top. One could argue there are 4 clusters (extra group at the top and bottom). The argument for 2 clusters should be clarified, e.g. could be backed up with an elbow plot or silhouette plot.

Response 2

The dendrogram was optimally presented in the updated Figure 1C. The clusters were defined based on unsupervised hierarchical clustering with Pheatmap package (default setting) in R, which segregated the splicing factors of the major spliceosome into two primary clusters (Fig. 1C), in accord with the elbow analysis (Fig. EV1H). The detailed methods are described on page 8, lines171-174 and in Material and Methods on page 27, lines 685-688. The figure is also shown below.



The left heatmap illustrates the row normalized expression of components within the major spliceosomal machinery using unsupervised hierarchical clustering. The right figure showing the number of clusters predicted by elbow method.

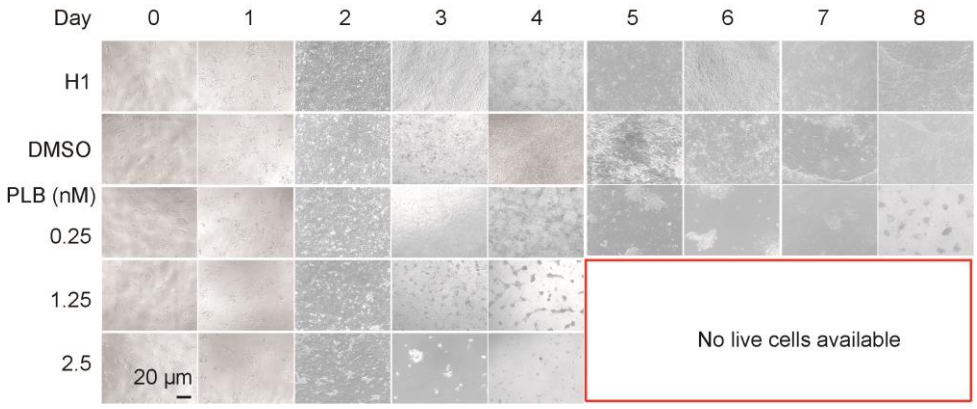
352

353 Query 3: It is stated that there was a cytotoxic effect of PLB when used
354 throughout the differentiation process (8 days, data not shown), but no effect when
355 used only at 2-3 days intervals (Fig 2A). The authors should include the data on
356 PLB cytotoxicity in the supplementary figure. It is also important to show
357 viability staining on the experiments in Fig 2 to support that there are no problems
358 with cytotoxicity with shorter PLB incubations. This would affect the conclusions
359 of the manuscript.

360 Response 3:

361 1) To investigate the cytotoxicity of PLB during hematopoietic differentiation, the
362 cells were treated with different concentrations of PLB (0.25, 1.25 and 2.5 nM)
363 throughout the differentiation process and cell morphology was analyzed from day

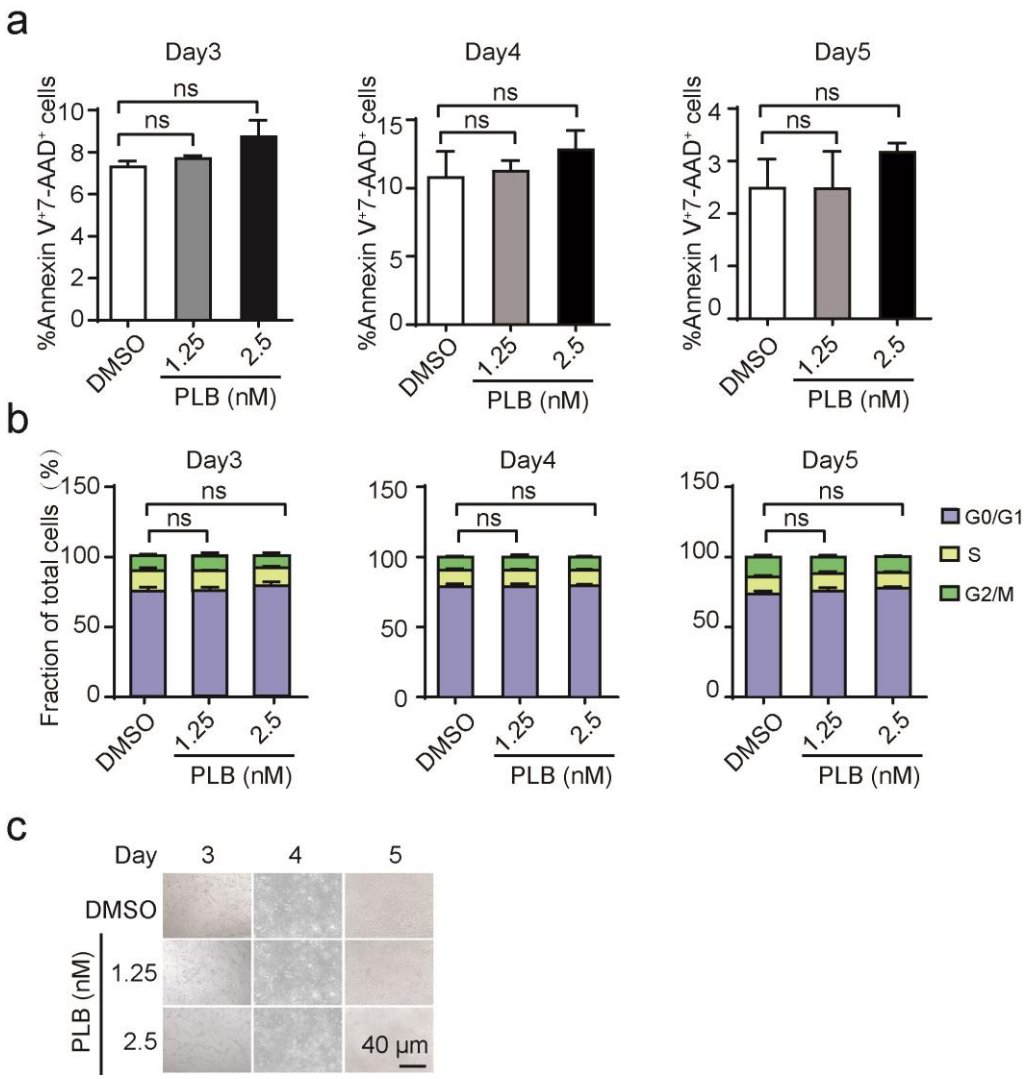
0 to 8 (Fig. EV2A). At day 5 of differentiation, we observed a large number of cell death at the higher doses of 1.25 and 2.5 nM. Even at the lower dose of 0.25 nM, starting from day 5 the cells could not differentiate with aberrant morphology. Again, this finding confirmed the cytotoxic effect of PLB throughout the differentiation process. This new data was incorporated as Fig. EV2A and discussed on page 9, lines 201-204. The figure is also shown below.



The morphology of cells treated with PLB throughout the hematopoietic differentiation.

2) To assess whether a shorter PLB treatment is cytotoxic (day 2.5-5 treatment), we measured the apoptosis, cell cycle and cell morphology on days 3, 4 and 5 of differentiation. There was no detectable cytotoxicity with the shorter PLB incubation. This new data was incorporated as Fig. 2E-F and Fig. EV3D-F and discussed on page 9, lines 217-219. The figures are also shown below.

377

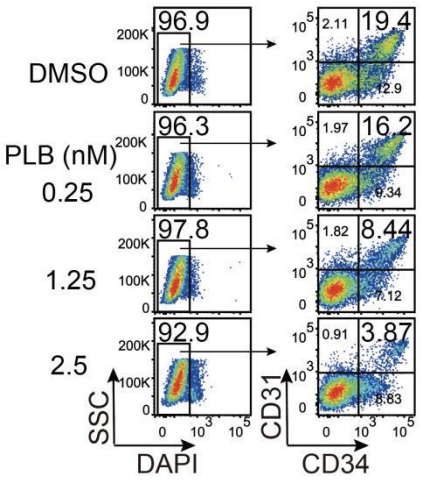


Evaluation of the effects of shorter PLB treatment (from day 2.5 to 5) during hematopoietic differentiation. The apoptotic level measured by annexin $V^+7\text{-}AAD^+$ by flow cytometry (a), the cell cycle measured with PI staining by flow cytometry (b) as well as cell morphology (c) of DMSO and PLB treated cells on day 3, 4 and 5 of differentiation.

378

379 3) DAPI staining was included to monitor cell viability for all FACS analyses in this
380 study. We have incorporated the DAPI staining into Fig. 2C and 2G, Fig. EV2B
381 and 2C. In general, the cell viability > 90% in all of the FACS analyses. The

representative figures are also shown below.



Representative FACS plots with DAPI staining included.

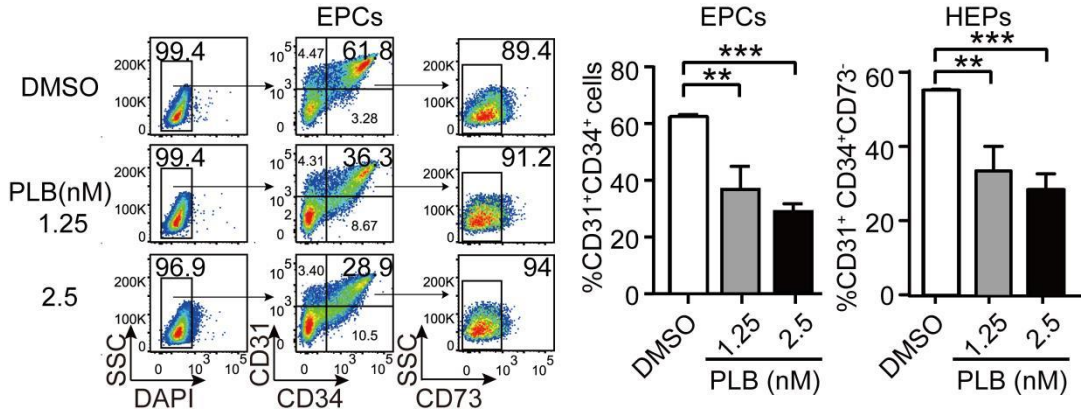
Query 4: Fig 2D-E relating to apoptosis and cell cycle analysis with PLB spliceosome inhibition, showing PLB specifically effects HEP production: Assays were performed at day 5, which is at the end of the PLB sensitive period of differentiation. Could this be too late, and sensitive cells at this point have died out? Earlier time points could be beneficial. This might also help to understand whether the consequence of PLB spliceosome inhibition is the activation of a specific cell-death program in HEP or the abrogation of mesoderm to HEP differentiation; Either way, is this affecting vascular and hemogenic endothelium in the same way (see minor points for comment on phenotypic identification of HEPs)? Is it specific for hemogenic endothelium? (in this regard, could the remaining CD31+CD34+ cells represent one of the two populations in fig. 2C?). Finally, FACS plots should be included, to show gating strategy.

Response 4:

1) Please refer to response 3. To rule out the possibility that it is too late to test cell

cycle and apoptosis, we measured the apoptosis, cell cycle and cell morphology on days 3, 4 and 5 of differentiation after PLB treatment from day 2.5 to 5 (Fig. 2E, F and Fig. EV3D, E and F). We did not detect increased apoptotic level at the early times, and therefore PLB impairs mesoderm to HEP differentiation. This new data was incorporated into Fig. 2E, F and Fig. EV3D, E and F and discussed on page 9, lines 217-219.

2) Please refer to response 1. We tested the impact of PLB on $CD31^{+}CD34^{+}$ $CD73^{-}$ HEPs (Fig. 2G). Similar to the $CD31^{+}CD34^{+}$ EPCs, the generation of HEPs decreased significantly. This new data was incorporated into Figure 2G and discussed on page 10, lines 234-238. The figures are also shown below.



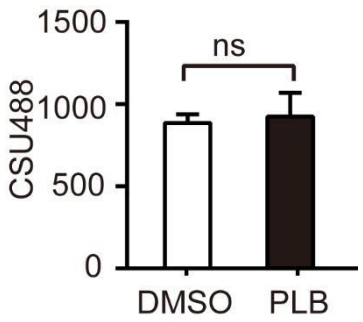
PLB treatment during day 2.5 to 5 reduced EPC and HEP generation on day 5 of hematopoietic differentiation.

3) FACS plots with the gating strategies were incorporated into the Fig. 2C, 2G, 3H, 3K, 4F and 6H, as well as Fig. EV2B, 2C, 3J, 4I and 5F.

Query 5: Fig S2D - a quantification of the LDL uptake experiment is needed to support the conclusion.

Response 5:

We quantified the LDL fluorescence intensity with Volocity 3D image analysis software, and there was no impact of PLB on the intensity. This new data was incorporated as Fig. EV3H and discussed on page 9, lines 219-221.

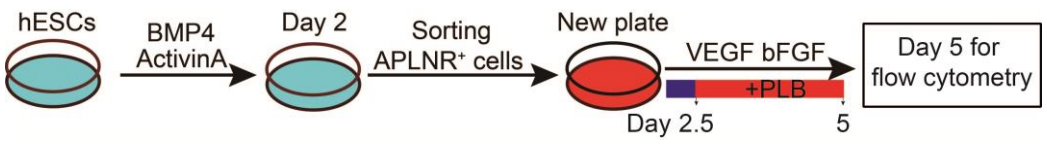


Quantification of LDL uptake based on the LDL fluorescence intensity by Volocity 3D image analysis software.

Query 6: Fig 2F - the experimental strategy is not clearly explained. Is the APLNR to HEP differentiation performed while inhibiting the spliceosome with PLB (as suggested by the schematic) or there is an inhibition step first, followed by differentiation (as suggested in the main text)?

Response 6

Cells were cultured with PLB throughout the APLNR to EPC differentiation (day 2.5-5). To increase comprehension, we revised the schematic (Fig. 2G) and incorporated the relevant text on page 10, lines 231-233. The figure is also shown below.

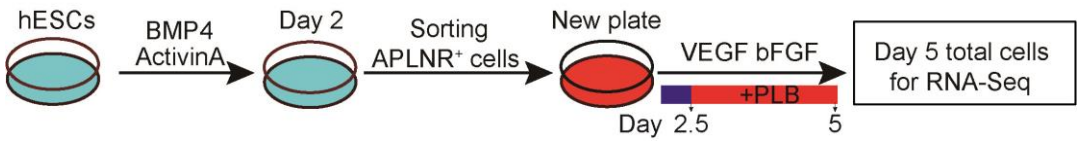


Schematic diagram of the experiment.

Query 7: Fig 3A - experimental strategy is not clearly explained: What are the cells sorted and collected? Are the cells FACS-sorted at day 5? If so, what is their phenotype? Are they HEP cells or are they stuck to APLNR+ stage?

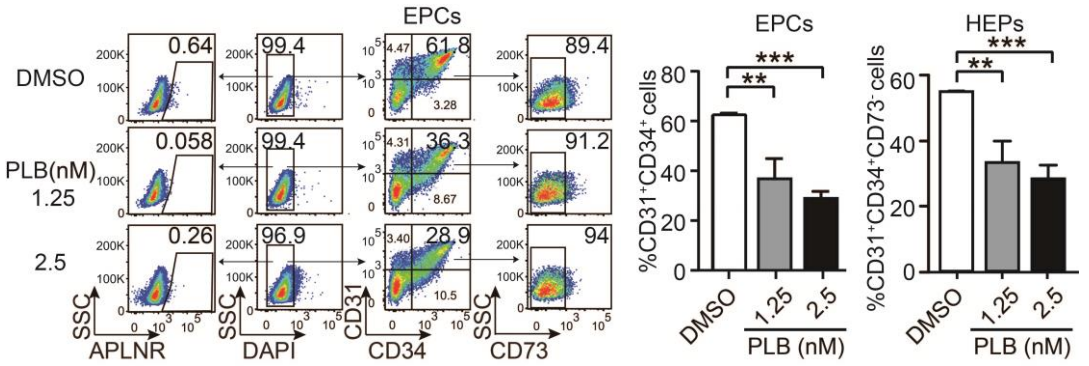
Response 7:

1) On day 2, we purified APLNR$^+$ cells by flow cytometry and induced them to undergo hematopoietic differentiation. After 12 hours of induction (at day 2.5), PLB was added until day 5. All cells on day 5 were collected for RNA-Seq analysis (Fig. 3A). We revised the schematic (Fig. 3A) and the relevant text on page 10, lines 245-248. The figure is also shown below.



Schematic diagram of the experiment.

2) To determine the differentiation stage of the cells at day 5, we tested the presence of APLNR$^+$ mesoderm cells, CD31$^+$CD34$^+$ EPC and CD31$^+$CD34$^+$CD73$^-$ HEPs. Little to no APLNR$^+$ cells could be detected on day 5 of differentiation. The percentage of CD31$^+$CD34$^+$ EPCs and CD31$^+$CD34$^+$CD73$^-$ HEPs were reduced significantly upon PLB treatment, demonstrating that PLB impaired APLNR$^+$ generation of EPC and HEP cells. This new data was incorporated into revised Fig. 2G and discussed on page 10, lines 234-238. The figures are also shown below.

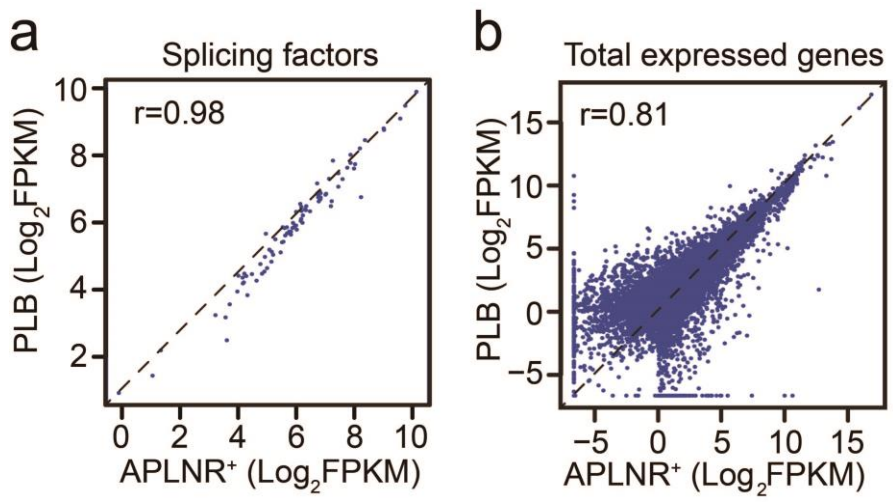


PLB treatment during day 2.5 to 5 reduced EPC and HEP generation on day 5 of hematopoietic differentiation.

Query 8: Fig 3C - Heatmap of splicing factors showing HEP$^+$ PLB shows similar profile to APLNR+. It would be nice to see this as scatter plot and correlation of expression values. Does PLB treatment correlate with APLNR+ genome wide, or only at splicing factors?

Response 8:

We generated the scatter plot with splicing factors in the heatmap and all expressed genes between APLNR$^+$ cells and PLB-treated cells at day 5 of differentiation. The expression correlation coefficient (r) of the splicing factors and all expressed genes was 0.98 and 0.81, respectively. So, the transcriptome of PLB treated-cells on day 5 was analogical to that of APLNR$^+$ cells, especially the expression of the splicing factors. This new data was incorporated as Fig. EV4B and C and discussed on page 11, lines 258-260. The figures are also shown below.



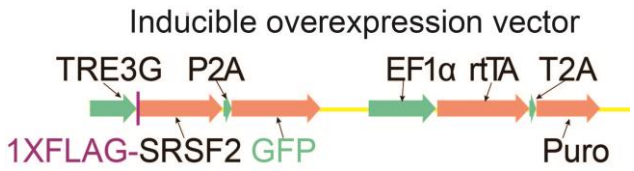
The scatter plot showing the correlation of splicing factors (a) and all expressed gene (b) between APLNR$^+$ cells and PLB-treated cells at day 5 of differentiation. r refers to the correlation coefficient.

458

459 Query 9: Fig 3F - Inducible SRSF2 schematic does not show FLAG. Presumably it
460 does, otherwise the western is referring to a different construct. Were stable
461 transfected lines generated? This is not clearly explained neither in the main text nor
462 in the methods. If not, how were the cells overexpressing SRSF2 selected? If
463 Puromycin was used for selection, why do only a fraction of cells express GFP (in
464 Fig.4F). If GFP was used to identify the overexpressing population, please provide the
465 frequency of GFP$^+$ cells analysed in supplementary figure 3 (and in all the
466 overexpression experiments based on the same system).

467 Response 9:

468 1) Thank you for pointing this out. The FLAG was missing in the prior version of
469 the schematic, and we corrected this (Figure EV4F). The figure is also shown
470 below.



The DOX-inducible overexpression vector structure of SRSF2.

2) We generated a stable transfected hESC cell line. The transfected ESCs were selected with 1μg/ml puromycin for at least 10 consecutive days under self-renewal conditions (Fig. EV4F). The procedure to generate the stable overexpression cell line is described in Materials and Methods on page 23, lines 574-585.

3) Although GFP^+ cells account for 70-90% at the onset of differentiation in each stable overexpression cell line, we often detect a trend of decreased percentage of GFP^+ cells during hematopoietic differentiation, consistent with previous studies (Wang *et al*, 2018a; Wang *et al*, 2018b). This might result from dramatic chromatin remodeling and consequent gene silence during hESCs differentiation.

Query 10: Fig 3F and S3C-G - only one out of four factors (SRSF2) recapitulates the decreased HEP generation, which is in agreement with its significant and exclusive upregulation in response to PLB treatment. This seems a little light to conclude that "In summary, the disturbed splicing as a result of the impediment of the splicing factor switch leads to impaired HEP formation"?

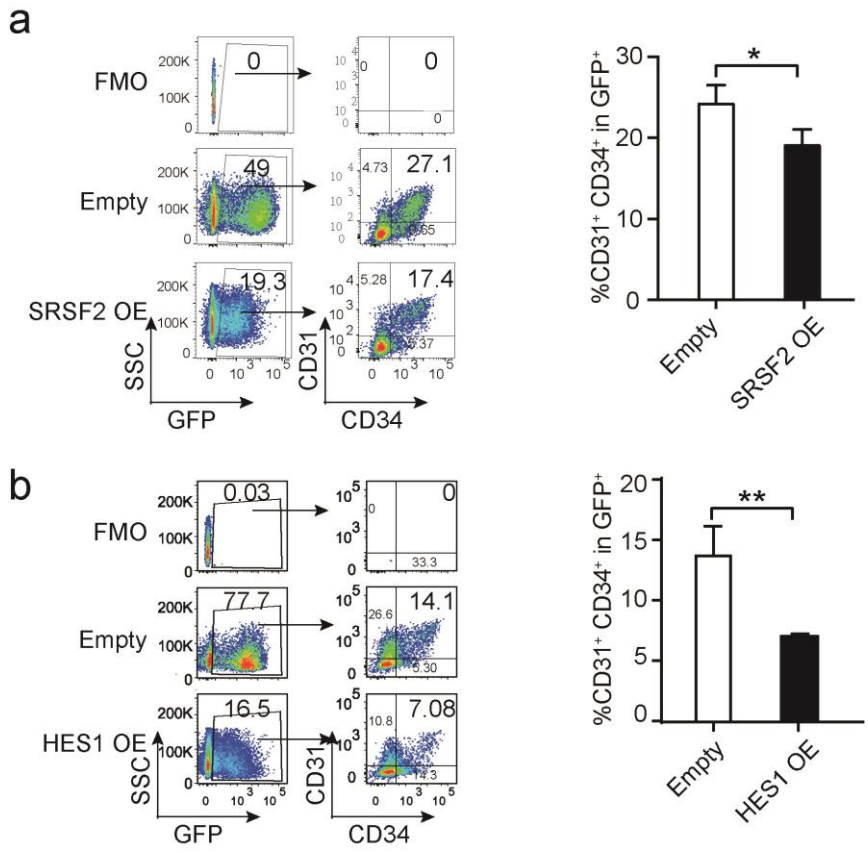
Response 10:

Based on the recommendation, we revised the conclusion to: “In summary, inhibition of the splicing factor switch contributes to impaired EPC formation”. This new description was incorporated on page 12, lines 293-294.

Query 11: Fig 4F - FACS gates showing SSC vs GFP, then CD34 CD31... Intended to show NUMB_S induction recovers phenotype caused by PLB. However, as the GFP intensity of the NUMB-S vector is weaker than the empty vector, the SSC vs GFP gate does not capture the same population, and is enriched for higher GFP levels. This could bias the result. On the basis of what were the gates set? FMOs should be shown in the supplementary. Axis of the dotplots should be better labelled with fluorescent intensities.

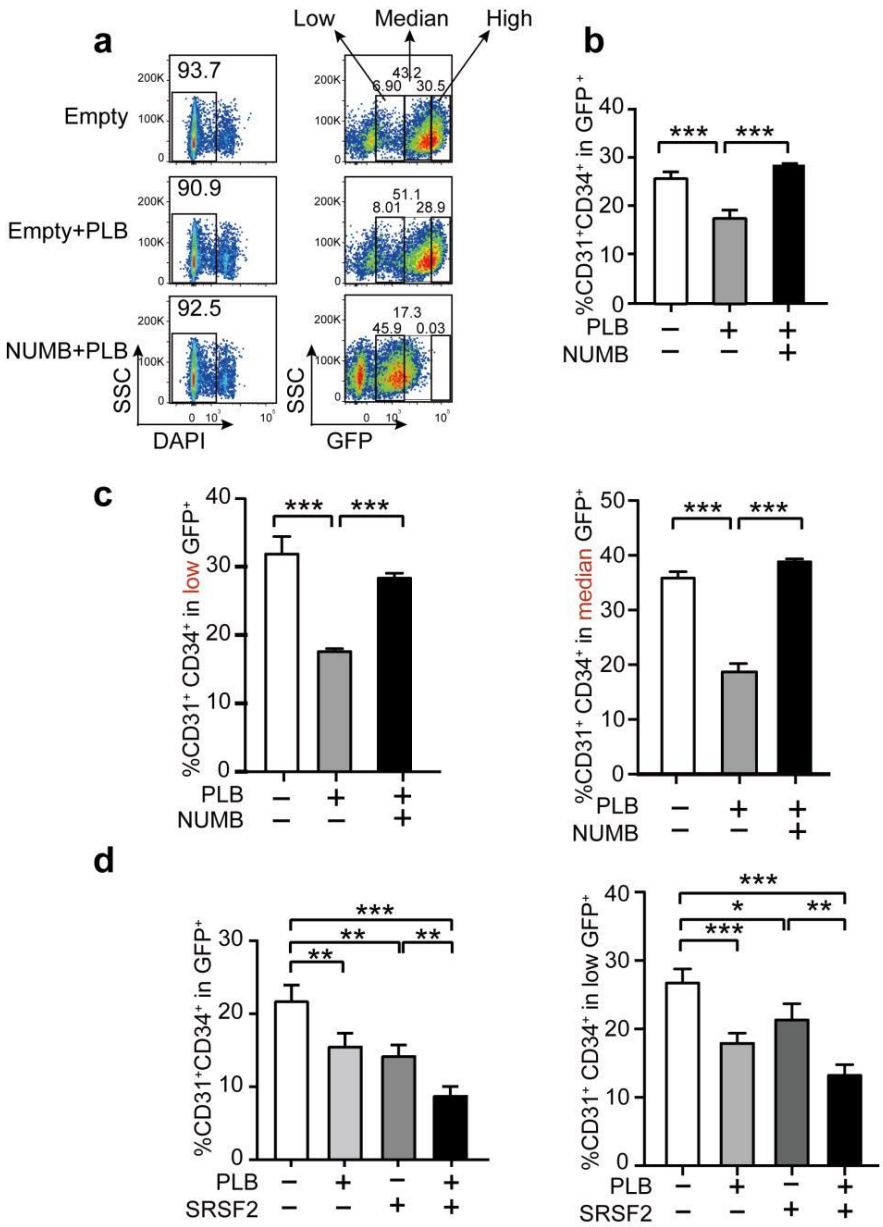
Response 11:

1) The gating strategy in the prior version was based on isotype IgG controls. We agree with the reviewer that the FMO controls are better negative controls than IgG. We repeated the SRSF2 and HES1 overexpression experiments and this yielded the same results as with the IgG control. The figures are shown below.



The frequency of CD31$^+$CD34$^+$ cells with ectopic expression of SRSF2 (a) and HES1 (b). FMO controls were included.

2) We parsed the GFP$^+$ population based on its fluorescent intensity. The similar trend was detected when comparing the GFP population with coordinate fluorescent intensity, which helps to exclude the possible biased data interpretation. The data is shown below.



The frequency of CD31$^+$CD34$^+$ cells with varied GFP fluorescent intensity. (a) The GFP$^+$ cells were divided into the low, median and high subpopulation based on the GFP fluorescent intensity. (b)The percentage of CD31$^+$CD34$^+$ cells in GFP$^+$ population on day 5 of differentiation treated with DMSO or PLB alone or in combination of PLB and DOX (to induce NUMB overexpression) from day 2.5 to 5. (c) The percentage of CD31$^+$CD34$^+$ cells in low (left) and median (right) GFP$^+$ population in (a). (d) The percentage of CD31$^+$CD34$^+$ cells in total (left) and low (right) GFP$^+$ population upon PLB treatment with or without SRSF2 overexpression.

508

3) We updated all FACS plots in the figures with labeled axes. This new data was incorporated into Fig. 2C and G, Fig. 3H and K, Fig. 4F, Fig. 6H, Fig. EV2B and C, Fig. EV3J, Fig. EV4I, and Fig. EV5F.

Query 12: Fig 5C-D - This is already known and published (including the use of DAPT, Uenishi et al., Nat Communications 2018) and should be acknowledged in the main text.

Response 12:

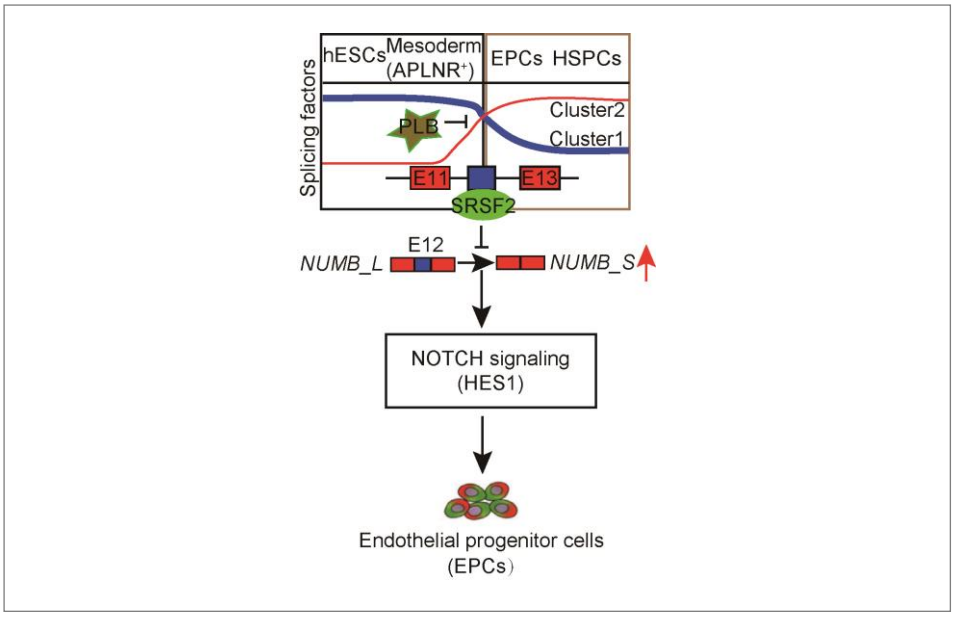
We incorporated the reference and discussion in the main text on page 19, lines 480-481.

Query 13： The conclusion that "NUMB-S controls the precise strength of NOTCH signaling and the HEP differentiation process, potentially by repressing HES1 expression" needs the addition that the conclusion is based exclusively on the observation of the two factors independently. But there is no evidence supporting a direct or indirect effect of NUMB-S on HES1. The model in Fig 5J suggests otherwise and should be adapted.

Response 13:

1) We edited the conclusion as follows “These results suggest that HES1 might function downstream of NUMB_S, directly or indirectly”. We incorporated the description in the main text on pages 15-16, lines 394 -395.

2) The model of Fig. 6J was revised as recommended. The figure is also shown below.



530

531 Minor points:

532 Fig 1H: "No changed genes" is somewhat confusing; "Number of changed genes"
533 would be clearer.

534 Response 1:

535 We revised it as “genes without DE” instead. Please find it in the updated Fig. 1H.

536 Fig 2A and 2C - units of measurement are missing on the quantification of flow
537 cytometry experiments (frequencies of ...? Absolute number?)

538 Response 2:

539 We relabeled the graphs as normalized frequency of $CD43^+$ or $CD31^+CD34^+$ in the
540 revised Fig. 2A, 2D and 6C.

541 Fig 3D - not clear what labels refer to what Venn circles

542 Response 3:

543 We matched the color of legends on the circles and Venn circles (revised Fig. 3D).

544 Fig 4C - Sashimi plot: Needs a y axis scale to allow comparison of relative transcript
545 levels. Also needs exons labelled

546 Response 4:

547 Y axis scale of Sashimi plot and *NUMB* exons were supplied in the Fig. 4C.

548

549 Referee #3:

550 In this manuscript, the authors investigate the role of post-transcriptional RNA
551 processing in hematopoietic development using a hPSC-based model. The authors
552 apply a combination of wet lab approaches and bioinformatics analysis to identify
553 novel players linked to the differentiation of mesodermal cells to hemogenic
554 endothelial progenitor cells. Their investigation led to the identification of a
555 widespread mechanism whereby a dynamic alternative splicing reprograming occurs
556 during hematopoietic development. They uncovered a splicing regulatory axis
557 consisting of SRSF2-NUMB-NOTCH which seems to be an important contributor of
558 hemogenic endothelial progenitor cells differentiation.
559
560 The manuscript is clear, concise, well written and figures are, in general,
561 well-constructed. Furthermore, claims made by the authors are supported by the data
562 generated from well-designed experiments.

563 We appreciate the reviewer's positive comments.

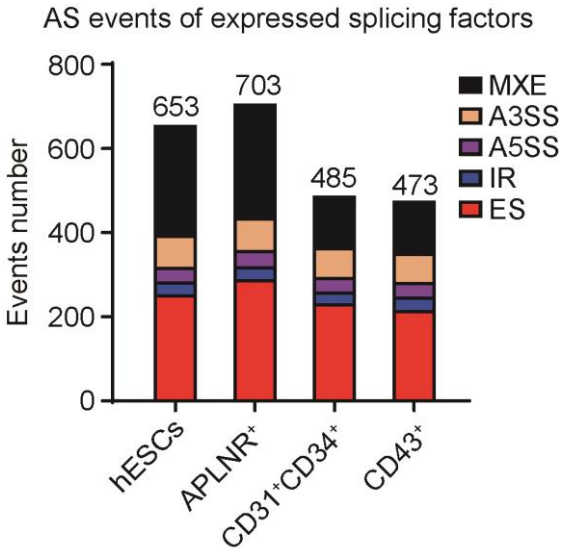
564 However, I feel that the manuscript could benefit from some minor
565 clarifications/adjustments, as follows:
566

567 Query 1: The authors analysed the alternative splicing program of human
568 hematopoietic development from ESCs and identified a splicing factor switch
569 occurring during HEP generation (Figure 1). Have the author considered to examine
570 the impact of alternative splicing events in splicing factors that could explain their
571 switch in expression? In other words, is there an orchestrated AS program during
572 differentiation that regulate splicing factors expression (eg. intron retention)?"

573 Response 1:

574 Thanks for the constructive suggestion. We have analyzed the splicing events of

expressed splicing factors of the 4 stages of human hematopoietic development from ESCs. The data shown that MXE decreased from APLNR$^+$ to CD31$^+$CD34$^+$ cells. So, the splicing factor switch might associate with the reduced MXE splicing events from APLNR$^+$ to CD31$^+$CD34$^+$ cells. The figure was attached below.



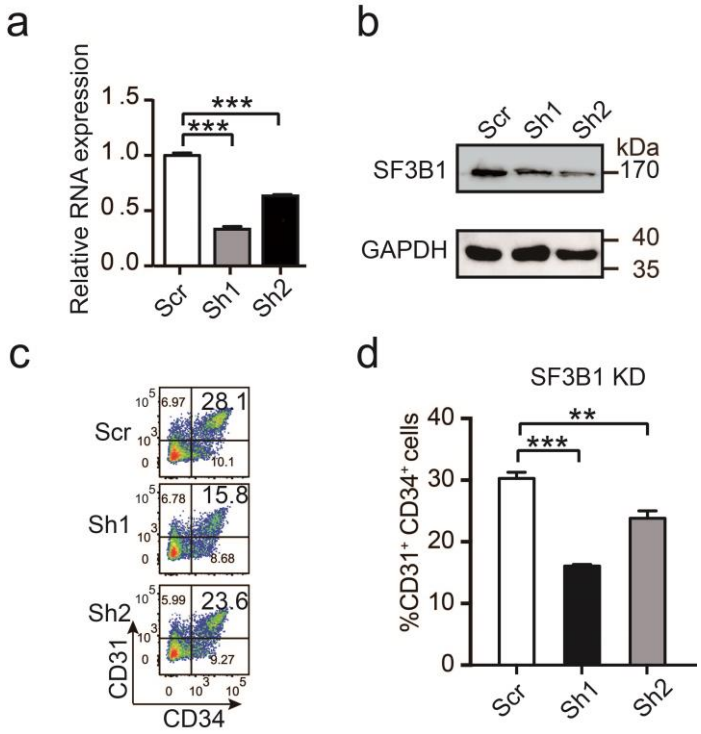
The alternative splicing events of expressed splicing factors during hematopoietic differentiation.

Query 2: The authors use pladienolide B (PLB), a natural inhibitor of the spliceosome which targets SF3B1 but this compound is also cytotoxic and induced apoptosis, even at a very low dose during hematopoietic differentiation process (data not shown by the authors). Have the authors considered the use of siRNA/shRNA targeting specifically SF3B1 to corroborate the data obtained with PLB treatment?

Response 2:

We generated stable cell lines with DOX-inducible expression of SF3B1 shRNAs and SF3B1 downregulation was confirmed by RT-qPCR (reduced to 33-63%, $P<0.001$) and Western blotting with 1 μg/ml DOX induction. This new data was incorporated as

589 Fig. EV3I. Consistent with the inhibitory effect of PLB on $CD31^+CD34^+$ cells,
590 Downregulation of SF3B1 impaired $CD31^+CD34^+$ generation. This new data was
591 incorporated as Fig. EV3J and K and discussed on page 9, lines 224-229. The figures
592 are also shown below.



Knockdown of SF3B1 impairs the production of $CD31^+CD34^+$ EPCs. (a) The mRNA level of *SF3B1* measured by RT-qPCR in empty control and shRNAs-stable cells. (b) The western blotting showing the expression of SF3B1. (c) Reprehensive FACS plots showing the generation of $CD31^+CD34^+$ EPCs after SF3B1 depletion. (d) The bar graph demonstrating the quantification of $CD31^+CD34^+$ EPCs after SF3B1 depletion.

593

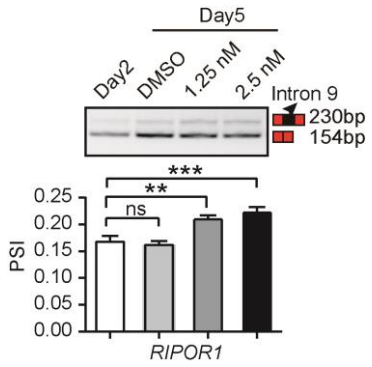
594 Query 3: Could the author explain (and clarify in the text) their choice of exon 9 of
595 LAS1L, ATP5F1C and HDAC7 (Figure 2 and S2) as markers of splicing defect after
596 PLB treatment? Why not including intron retention as markers of splicing defect
597 instead of exon inclusion? Furthermore, if I interpreted the data in the figures

598 correctly, DMSO treatment seems to have more effect (for LAS1L and HDAC7) than
599 with the two concentration of PLB tested. Any comment from the authors on that
600 particular point? (maybe I did misunderstand something and if that's the case I do
601 apologize for this comment).

602 Response 3

603 1) The reason we selected genes of *LAS1L*, *ATP5F1C* and *HDAC7* is they displayed
604 marked alternative isoform usage during APLNR$^+$ to CD31$^+$CD34$^+$ transition (Fig.
605 EV3A-C). In addition, given that exon skipping is the most prevalent splicing
606 event during hematopoietic development from ESCs, we tested exon skipping (e.g.
607 exon 9) after PLB treatment. This new data was incorporated as Fig. EV3A-C and
608 discussed on page 9, lines 211-214.

609 2) We also verified the altered intron retention for gene *RIPOR1*. The figure is shown
610 below.



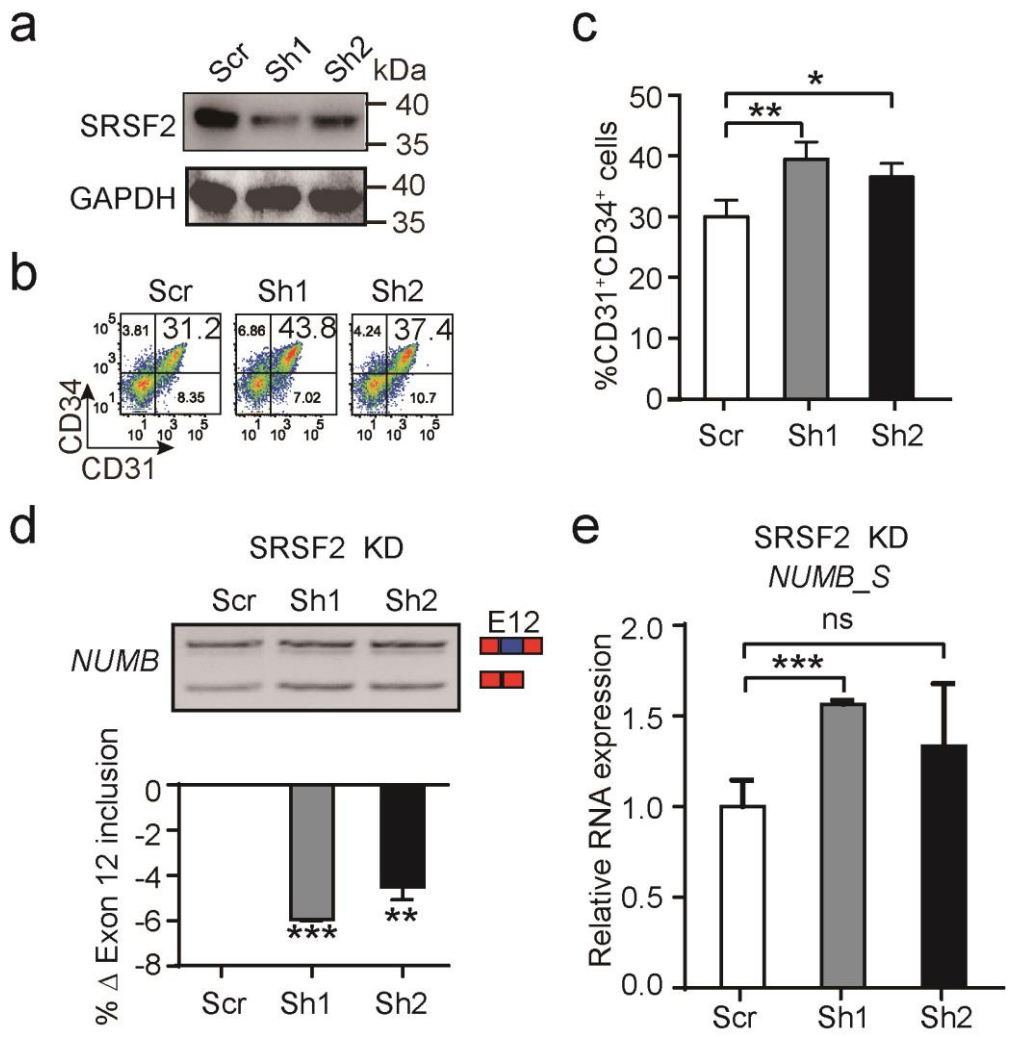
The intron retention of *RIPOR1*. The top panel is a representative RT-PCR electropherogram depicting the inclusion or exclusion *RIPOR1* exon 9 in cells at day 2 as well as cells at day 5 without or with PLB treatment with indicated concentrations, respectively. The quantification of PSI was presented in the bottom bar graph. P-values were calculated by one-way ANOVA followed by Dunnett's test.

3) The first lane indicates isoform expression on day 2 of differentiation, while the second lane (DMSO lane) shows isoform expression on day 5 of differentiation. This result demonstrates that with the hematopoietic differentiation from day 2 to 5, the expression of long isoforms of *LAS1L* and *HDAC7* were increased while the expression of short isoforms decreased. However, PLB blocked the isoform switch (lane 3 and 4).

Query 4: In Figure 4H, the authors detected (in silico) SRSF2 binding sites within NUMB-Exon12 and tested the effect of PLB treatment or/and SRSF2 overexpression on the expression of NUMB isoforms. The treatments led to the reduction of the level of the NUMB-S transcripts (Figure 4I) and a concomitant increase of the NUMB-L transcripts (Figure. 4J). They therefore concluded that SRSF2 functions in the regulation of NUMB alternative splicing. Have the author thought of inducing the knock down of SRSF2 to see if it induces the up-regulation of NUMB-Small isoform? Furthermore, it would be really essential to confirm that SRSF2 actually binds to exon 12 (and to which binding sites) before they could claim that SRFS2 is the direct player regulating NUMB-Exon12 splicing (using mini-gene reporter or Protein-RNA pull-down assay).

Response 4:

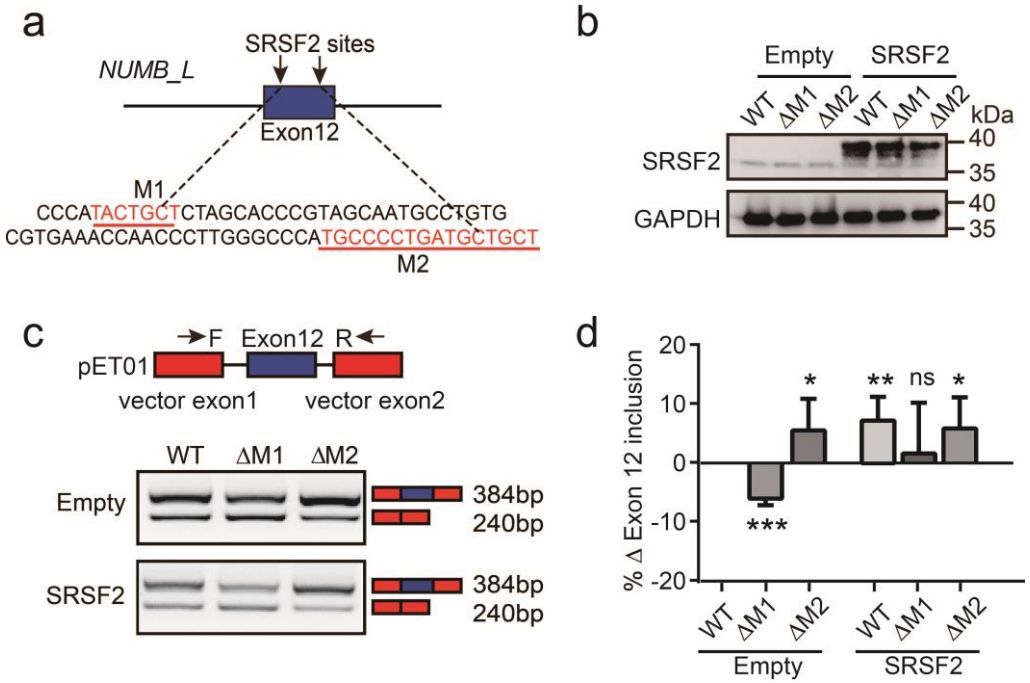
1) We generated stable cell lines with DOX-inducible expression of SRSF2 shRNAs and SRSF2 downregulation was confirmed by Western blotting with 1 μg/ml DOX induction. This new data was incorporated as Fig. 3J. Consistent with the inhibitory effect of PLB on $CD31^+CD34^+$ cells, downregulation of SRSF2 enhanced $CD31^+CD34^+$ generation (Fig. 3K and L) and upregulated *NUMB_S* expression (Fig. EV5D and E). This new data was incorporated as Fig. 3J-L discussed on pages 11-12, lines 285-294 and Fig. EV5D and E discussed on page 14, lines 361-364. The figures are shown below.



Knockdown of SRSF2 promotes the production of CD31$^+$CD34$^+$ EPCs. (a) The western blotting showing the expression of SRSF2 in empty vector control and shRNAs-infected cells with 1 μg/ml DOX. (b) Reprehensive FACS plots showing the generation of CD31$^+$CD34$^+$ EPCs after SRSF2 depletion in day 5-differentiated cells. (c) The bar graph demonstrating the statistical quantification of CD31$^+$CD34$^+$ EPCs after SRSF2 depletion. (d) The splicing of *NUMB_S* exon 12 upon SRSF2 depletion on day 5 of hematopoietic differentiation. (e) The expression of *NUMB_S* upon SRSF2 depletion on day 5 of hematopoietic differentiation.

637

2) As shown in the revised Fig. 5, there were two putative SRSF2 binding motifs on *NUMB* exon 12 (M1 and M2, labeled as red) by RBPmap (http://rbpmap.technion.ac.il/) (Fig. 5A). To further validate the potential of SRSF2 to regulate splicing of *NUMB* exon 12 via these motifs, we performed a minigene splicing reporter assay. We constructed a truncated NUMB reporter encompassing a genomic fragment from 540 nucleotides upstream to 507 nucleotides downstream of *NUMB* exon12. This reporter was cloned into an ExonTrap pET01 vector containing two constitutive exons, as reported previously (Rajendran *et al.*, 2016). We also constructed two mutant NUMB reporters by deleting each motif (ΔM1 or ΔM2), respectively. After co-transfection of each NUMB reporter (WT, ΔM1 or ΔM2) with SRSF2 expression plasmids into 293T cells simultaneously, we found that SRSF2 promoted the generation of *NUMB_L* isoform via M1 but not M2 (Fig. 5B-D). This new data was incorporated as Fig. 5A-D and described on page 14, lines 343-354 (highlighted in yellow). The figures are also shown below.



SRSF2 represses the *NUMB_S* isoform generation. (a) The putative SRSF2 binding motifs on *NUMB* exon 12 (labeled as red), predicted by RBPmap software. M1 refers to motif 1 and M2 refers to motif 2. (b) Western blotting showing the overexpression of SRSF2 in 293T cells, which were co-transfected with NUMB reporter WT, ΔM1 or ΔM2. ΔM1 or ΔM2 represent plasmids deleting M1or M2 binding site. (c) A minigene splicing reporter assay showing SRSF2 promotes the generation of *NUMB_L* isoform via M1 but not M2 binding site. The upper panel indicating the pET01 vector information, which contains two constitutive exons. (d) The quantification of the exon 12 inclusion/ exclusion of (c).

References:

Bechara EG, Sebestyén E, Bernardis I, Eyras E, Valcárcel J (2013) RBM5, 6, and 10 differentially regulate NUMB alternative splicing to control cancer cell proliferation. *Molecular cell* 52: 720-733

Ke H, Zhao L, Zhang H, Feng X, Xu H, Hao J, Wang S, Yang Q, Zou L, Su X *et al* (2018) Loss of TDP43 inhibits progression of triple-negative breast cancer in coordination with SRSF3. *Proceedings of the National Academy of Sciences of the United States of America* 115: E3426-e3435

Lu Y, Xu W, Ji J, Feng D, Sourbier C, Yang Y, Qu J, Zeng Z, Wang C, Chang X *et al* (2015) Alternative splicing of the cell fate determinant Numb in hepatocellular carcinoma. *Hepatology (Baltimore, Md)* 62: 1122-1131

Rajendran D, Zhang Y, Berry DM, McGlade CJ (2016) Regulation of Numb isoform expression by activated ERK signaling. *Oncogene* 35: 5202-5213

Tarn WY, Kuo HC, Yu HI, Liu SW, Tseng CT, Dhananjaya D, Hung KY, Tu CC, Chang SH, Huang GJ *et al* (2016) RBM4 promotes neuronal differentiation and neurite outgrowth by modulating Numb isoform expression. *Mol Biol Cell* 27: 1676-1683

Wang H, Liu C, Liu X, Wang M, Wu D, Gao J, Su P, Nakahata T, Zhou W, Xu Y *et al* (2018a) MEIS1 Regulates Hemogenic Endothelial Generation, Megakaryopoiesis, and Thrombopoiesis in Human Pluripotent Stem Cells by Targeting TAL1 and FLI1. *Stem cell reports* 10: 447-460

Wang M, Wang H, Wen Y, Chen X, Liu X, Gao J, Su P, Xu Y, Zhou W, Shi L *et al* (2018b) MEIS2 regulates endothelial to hematopoietic transition of human embryonic stem cells by targeting TAL1. *Stem cell research & therapy* 9: 340-340

1st Revision - Editorial Decision 16th Oct 2020

Dear Dr. Shi,

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now support the publication of your study in EMBO reports. However, referee #1 has some remaining points and some suggestions to improve the study, we ask you to address in a final revised manuscript.

Moreover, I have these editorial requests:

- I would suggest this slightly modified title:
A splicing factor switch controls hematopoietic lineage specification of pluripotent stem cells

- Please provide the abstract written in present tense.

- Regarding data quantification and statistics, please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (also of the EV figures). Please provide statistical testing where applicable.

- In panel D of Fig. EV3 the first two images of the middle column are identical (4 - DMSO and 1.25 PLB). Please check.

- As the Western blots shown are significantly cropped, could you provide the source data for all the blots (main and EV figures). The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. Please submit the source data (scans of entire blots) together with the revised manuscript. Please include size markers for scans of entire blots, label the scans with figure and panel number and send one PDF file per figure.

- It seems not all authors listed on the title page are mentioned in the author contributions section. Please check.

- Table EV1 needs to be a dataset. Please call this Dataset EV1 and update the call-outs. Please remove the legend of this dataset file from the manuscript text and add it as the first TAB to the excel sheet.

- Table EV2 needs then to be re-named as Table EV1. Please also update the call-outs. Please put the legend as general title to the table file (Sequences of all primers used), and remove that from the manuscript main text.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:

- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript. Please do that for corresponding author Zhou. Please find instructions on how to link the ORCID ID to the account in our manuscript tracking system in our Author guidelines:
http://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

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

Yours sincerely

Achim Breiling
Editor
EMBO Reports

Referee #1:

In this revised manuscript, the authors worked hard to address the critiques by addition of several new experiments. They also improved the discussion of their work in relation to previous studies by citation of additional literature. However, in my opinion, there are still some questions that need to be addressed before the novel model of a SRSF2-NUMB-NOTCH regulatory axis can be supported as a pathway to control HEP specification. Admittedly, validating all the major steps in a single paper is quite an arduous undertaking.

1. SRSF2. Regarding the critiques about SRSF2 as a regulator of NUMB-S splicing: the authors have partially strengthened this part of the model by adding new data. Using a minigene splicing reporter to study regulation of NUMB exon 12, they showed (Fig. 5D/E) that over-expression of SRSF2 promotes an increase in exon 12 inclusion in 293T cells, and that this effect requires the predicted SRSF2 binding site designated as M1. This is consistent with the model that reduced expression of SRSF2 during differentiation will lead to reduced exon 12 splicing, i.e., increased NUMB-S.

So, the new data better support the hypothesis that SRSF2 can regulate NUMB splicing via a binding site in exon 12. But I'm not completely convinced that the aggregate can yet support assigning SRSF2 a prominent role as a key component of a SRSF2-NUMB-NOTCH axis. There are a few issues.

First, it's not clear from the splicing reporter study in 293T cells that the magnitude of the SRSF2 effect is sufficient to explain NUMB-S splicing regulation during differentiation. Differentiation of APNLR+ (exon 12 PSI=74) was accompanied by a large decrease in SRSF2 expression in parallel with a large decrease in splicing: PSI=17 at day 5 of differentiation (PSI=17). This data is in Fig. 4C. If SRSF2 is the major regulator of that splicing switch, wouldn't we expect a strong effect also in 293T cells? But over-expression of SRSF2 in 293 cells only showed a modest increase in exon 12 splicing, with a change in PSI<10 (Fig. 5C/D).

Second, while the the effects of SRSF2 knockdown on NUMB splicing was not tested in the 293T cells, this activity was examined in differentiating hemogenic progenitor cells (Figure EV5). There appeared to be only a modest effect on NUMB splicing, e.g., parts D and E shows <10% change in exon 12 splicing. In that experiment the NUMB-S PCR band still appeared to be less abundant than the NUMB-L product (whereas NUMB-S should be more abundant than NUMB-L at day 5, see Fig. 4B).

On the other hand, the new data in Figure 3G and 3H seem to show that SRSF2 over-expression greatly depresses EPC production. This would support the authors' model, but I am a little worried about the huge difference in percentage of GFP+ cells in the empty vector vs SRSF2-over-expressing cells, and the position of the GFP+ gate in relation to the overall shape of the cell profile. Is it possible that the difference in profiles might complicate the interpretation of effects on EPC production? Is it possible that over-expression is so harmful to these cells that the GFP+ population is low due to killing of the cells?

2. HES1. Regarding the query about the mechanism of action of HES1: The authors responded to this query by adding data to rule out the possibility that HES1 acts upstream to regulate SRSF2 expression. That new data convincingly rules out that alternative model. However, I still worry whether the data quantitatively make sense to support NUMB-S regulation of HES1, and HES1 as the major regulator of NOTCH. For example, in Figure 6E, the treatment of cells with PLB only increases HES1 expression by maybe 25%, and forced expression of NUMB-S in PLB-inhibited cells mostly reduces that back to the baseline observed in cells differentiated in the absence of PLB. That doesn't seem like a strong regulator to me. However, I am not an expert in transcription, and could be convinced to accept the argument if there is literature showing that this order of magnitude change in HES1 is sufficient to modulate NOTCH.

3. Other literature. The paper is much improved with respect to discussing other literature about regulation of NUMB splicing. I would recommend one additional change, that is, to explain that the exon they refer to as exon 12 is in fact the same exon designated as exon 9 in some other studies, e.g., the paper by Rajendran (unless I am mistaken).

Minor issues:
1. Line 299-300: "Additionally, genes exhibiting isoform proportion changes (delta iso > 0.15; blue circle) or genes with differentially expressed isoforms" . The question is, what is the difference between isoform proportion changes and differentially expressed isoforms?

2. Line 338 "We ectopic expression of SRSF2 during EPCs generation". Something is missing in that sentence.

3. Line 393-4 "Moreover, the combined induction of NUMB_S and the inhibition of splicing with PLB restored HES1 expression (Fig. 6E)" [actually, restored lower expression]

Referee #2:

The authors have adequately addressed my concerns and the changes have improved the manuscript.

Referee #3:

The authors have successfully addressed all my comments/suggestions but also the ones from the two other referees; therefore, I recommend their manuscript for publication in EMBO reports.

Response for Reviewer

Referee #1:

In this revised manuscript, the authors worked hard to address the critiques by addition of several new experiments. They also improved the discussion of their work in relation to previous studies by citation of additional literature. However, in my opinion, there are still some questions that need to be addressed before the novel model of a SRSF2-NUMB-NOTCH regulatory axis can be supported as a pathway to control HEP specification. Admittedly, validating all the major steps in a single paper is quite an arduous undertaking.

1. SRSF2. Regarding the critiques about SRSF2 as a regulator of NUMB-S splicing: the authors have partially strengthened this part of the model by adding new data. Using a minigene splicing reporter to study regulation of NUMB exon 12, they showed (Fig. 5D/E) that over-expression of SRSF2 promotes an increase in exon 12 inclusion in 293T cells, and that this effect requires the predicted SRSF2 binding site designated as M1. This is consistent with the model that reduced expression of SRSF2 during differentiation will lead to reduced exon 12 splicing, i.e., increased NUMB-S.

So, the new data better support the hypothesis that SRSF2 can regulate NUMB splicing via a binding site in exon 12. But I'm not completely convinced that the aggregate can yet support assigning SRSF2 a prominent role as a key component of a SRSF2-NUMB-NOTCH axis. There are a few issues.

First, it's not clear from the splicing reporter study in 293T cells that the magnitude of the SRSF2 effect is sufficient to explain NUMB-S splicing regulation during differentiation. Differentiation of APNLR+ (exon 12 PSI=74) was accompanied by a large decrease in SRSF2 expression in parallel with a large decrease in splicing: PSI=17 at day 5 of differentiation (PSI=17). This data is in Fig. 4C. If SRSF2 is the major regulator of that splicing switch, wouldn't we expect a strong effect also in 293T cells? But over-expression of SRSF2 in 293 cells only showed a modest increase in exon 12 splicing, with a change in PSI<10 (Fig. 5C/D).

Second, while the effects of SRSF2 knockdown on NUMB splicing was not tested in the 293T cells, this activity was examined in differentiating hemogenic progenitor cells (Figure EV5). There appeared to be only a modest effect on NUMB splicing, e.g., parts D and E shows <10% change in exon 12 splicing. In that experiment the NUMB-S PCR band still appeared to be less abundant than the NUMB-L product (whereas NUMB-S should be more abundant than NUMB-L at day 5, see Fig. 4B).

Response:

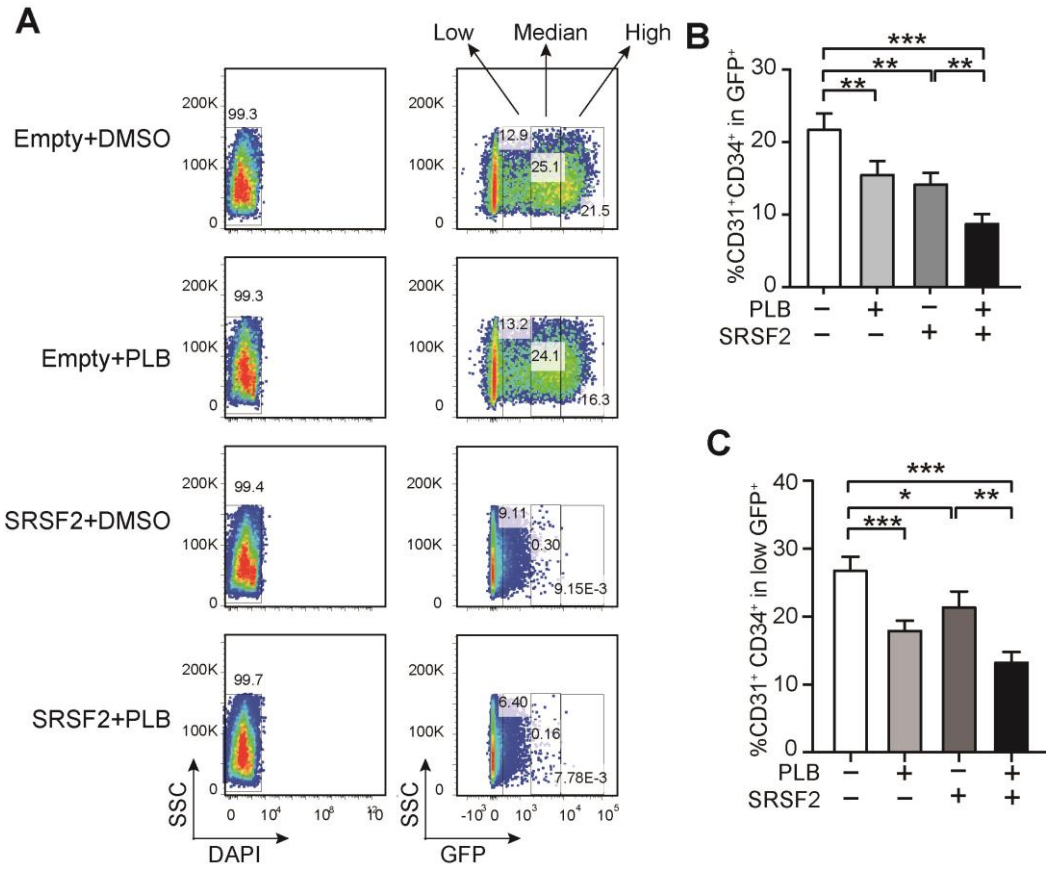
1) We really appreciate the careful review and the insightful comments.
2) Based on the minigene reporter assay, the overexpression of SRSF2 in 293T cells did not show the similar effect on NUMB splicing regulation as in EPCs. Also the knockdown of SRSF2 in EPCs displayed a modest effect on NUMB splicing. This could be partially explained by the possible cellular specificity of SRSF2 on NUMB splicing, e.g. 293T cells versus EPCs. Or it is likely that the level of overexpression or knockdown of SRSF2 could influence the effect on NUMB splicing.
3) Of course, we cannot rule out the possibility that other genes, such as SRSF1, SRSF3 or other yet unknown splicing factors, could be involved in the regulation of

NUMB splicing in EPCs as well. We have incorporated this possibility in the discussion section on page 19, lines 464-470 and toned down the role of SRSF2 on NUMB splicing in the entire manuscript.

On the other hand, the new data in Figure 3G and 3H seem to show that SRSF2 over-expression greatly depresses EPC production. This would support the authors' model, but I am a little worried about the huge difference in percentage of GFP+ cells in the empty vector vs SRSF2-over-expressing cells, and the position of the GFP+ gate in relation to the overall shape of the cell profile. Is it possible that the difference in profiles might complicate the interpretation of effects on EPC production? Is it possible that over-expression is so harmful to these cells that the GFP+ population is low due to killing of the cells?

Response:

1) To rule out the possibility that the difference in GFP^+ profiles might complicate the interpretation of effects on EPC production. We parsed the GFP^+ population based on its fluorescent intensity. The similar trend was detected when comparing the GFP population with coordinate fluorescent intensity, which helps to exclude the possible biased data interpretation. The data is shown below.



The frequency of CD31$^+$CD34$^+$ cells with varied GFP fluorescent intensity. (A) The GFP$^+$ cells were divided into the low, median and high subpopulation based on the GFP fluorescent intensity. (b)The percentage of CD31$^+$CD34$^+$ cells in total GFP$^+$ population on day 5 of differentiation treated with DMSO or PLB alone or in combination of PLB and DOX (to induce SRSF2 overexpression) from day 2.5 to 5. (C) The percentage of CD31$^+$CD34$^+$ cells in low GFP$^+$ population in (A).

2) We did not observe the increased cell death in the SRSF2 overexpressed cells during differentiation. Although GFP$^+$ cells account for 70-90% at the onset of differentiation in each stable overexpression cell line, we often detect a trend of decreased percentage of GFP$^+$ cells during hematopoietic differentiation, consistent with previous studies (Wang *et al*, 2018a; Wang *et al*, 2018b). We believe such decline could result from dramatic chromatin remodeling and consequent gene silence during hESCs differentiation.

2. HES1. Regarding the query about the mechanism of action of HES1: The authors responded to this query by adding data to rule out the possibility that HES1 acts upstream to regulate SRSF2 expression. That new data convincingly rules out that alternative model. However, I still worry whether the data quantitatively make sense to support NUMB-S regulation of HES1, and HES1 as the major regulator of NOTCH. For example, in Figure 6E, the treatment of cells with PLB only increases HES1 expression by maybe 25%, and forced expression of NUMB-S in PLB-inhibited cells mostly reduces that back to the baseline observed in cells differentiated in the absence of PLB. That doesn't seem like a strong regulator to me. However, I am not an expert in transcription, and could be convinced to accept the argument if there is literature showing that this order of magnitude change in HES1 is sufficient to modulate NOTCH.

Response:

Prior studies have shown that HES1 expression is exquisitely controlled. HES1 binds to its own promoter to repress its own expression in neural stem cells ((Hirata *et al*, 2002). Additionally, Notch ligand Dll1 activates Notch signaling and concomitantly induces HES1 expression ((Jarriault *et al*, 1995); (Ohtsuka *et al*, 1999)) and the activation of HES1, in turn, represses Dll1 expression in neural cells(Kageyama *et al*, 2008). Due to HES1 expression is precisely regulated by multiple negative feedback loops, we assume that the variation of its expression should often be modest. Thus, it is possible that 25% of HES1 induction could exert biological functions.

3. Other literature. The paper is much improved with respect to discussing other literature about regulation of NUMB splicing. I would recommend one additional change, that is, to explain that the exon they refer to as exon 12 is in fact the same exon designated as exon 9 in some other studies, e.g., the paper by Rajendran (unless I am mistaken).

Response:

The reviewer is right. The exon we referred to as exon 12 is the same exon designated as exon 9 in some other studies e.g., the paper by Rajendran. As suggested, we changed the exon 12 to 9 in the revised manuscript.

Minor issues:

1. Line 299-300: "Additionally, genes exhibiting isoform proportion changes (delta iso > 0.15; blue circle) or genes with differentially expressed isoforms". The question is, what is the difference between isoform proportion changes and differentially expressed isoforms?

Response 1:

Isoform proportion, which is calculated as the expression of an individual isoform to the sum of all isoforms belonging to the same gene, is a metric to quantify the activity of splicing at the isoform level (Monlong et al, 2014). Differentially expressed isoforms were identified by using DESeq2 (version 1.20.0) (Love et al, 2014) with default settings between two consecutive differentiation stages. Accordingly, differentially expressed isoforms may not exhibit isoform proportion changes. Their definition and description could be found in method section on page 26-27, lines 646-651 and lines 660-666.

2. Line 338 "We ectopic expression of SRSF2 during EPCs generation". Something is missing in that sentence.

Response 2:

Thanks for pointing it out. We changed the sentence to “Ectopic expression of SRSF2 during EPCs generation recapitulated the reduced generation of EPCs obtained with PLB treatment.”

3. Line 393-4 "Moreover, the combined induction of NUMB_S and the inhibition of splicing with PLB restored HES1 expression (Fig. 6E)" [actually, restored lower expression]

Response 3:

We changed the sentence to "Moreover, the combined induction of NUMB_S and the inhibition of splicing with PLB restored the HES1 expression to its normal level (Fig. 6E)" as suggested by the reviewer on page 15, line 391.

Referee #2:

The authors have adequately addressed my concerns and the changes have improved the manuscript.

Response： We thank for the reviewer for supporting our work.

Referee #3:

The authors have successfully addressed all my comments/suggestions but also the ones from the two other referees; therefore, I recommend their manuscript for publication in EMBO reports.

Response： We thank for the reviewer for supporting our work.

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Wang M, Wang H, Wen Y, Chen X, Liu X, Gao J, Su P, Xu Y, Zhou W, Shi L *et al* (2018b) MEIS2 regulates endothelial to hematopoietic transition of human embryonic stem cells by targeting TAL1. *Stem cell research & therapy* 9: 340-340

2nd Revision - Editorial Decision 12th Nov 2020

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China

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

Yours sincerely,

Achim Breiling
Editor
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Referee #1:

I thank the authors for their response to the critique. They present an attractive model that makes very important and novel points about the contribution of alternative splicing to differentiation of HEP. It is highly impressive that a single alternative splicing switch in the NUMB gene can substantially impact HEP formation. It is also clear that SRSF2 is a splicing factor that can modulate

NUMB splicing, and changes expression during differentiation in a manner consistent with a physiological role in regulating NUMB. My main concern is whether the SRSF2 is really the major regulator of NUMB splicing, to justify the "SRSF2-NUMB-NOTCH regulatory axis" terminology. The effects of SRSF2 expression on NUMB splicing in over-expression and knockdown assays were modest, regulating NUMB splicing much less than what occurs during HEP differentiation. My opinion is that the SRSF2-NUMB-NOTCH regulatory axis, while not entirely wrong, is probably an over-simplification. On that point, the discussion is improved by recognizing that other splicing factors may also play a role in the NUMB switch.

Minor point: there is a grammatical error in the abstract: "We identify a splicing factor switch links to the" (should be "linked").

****

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The data shown in figures should satisfy the following conditions:

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- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
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- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
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2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
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- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
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  - definition of 'center values' as median or average;
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

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

B- Statistics and general methods

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

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Is the variance similar between the groups that are being statistically compared?	YES

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7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The cell line information was provided on page 21 ,line 502 and tested for mycoplasma contamination.

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18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	YES,We " Data Availability " section on page 29, lines 701-703.
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