



Proper timing of a quiescence period in precursor prospermatogonia is required for stem cell pool establishment in the male germline

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MS TITLE: Proper timing of a quiescence period in precursor prospermatogonia is required for stem cell pool establishment in the male germline

AUTHORS: Jon Oatley, Guihua Du, Melissa Oatley, Nathan Law, Colton Robbins, and Xin Wu

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

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

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

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

Reviewer 1*Advance summary and potential significance to field*

In this study, Du et al. investigated the function of mitotic arrest in prospermatogonia during embryonic development, and its requirement for establishment of the postnatal spermatogonial stem cell (SSC) pool.

This study builds on previous work from the Koopman, Page and Oatley labs (Spiller et al., 2010; Hu et al., 2013; Yang et al., 2013). The authors utilised a cre-lox conditional knockout mouse model, where cell cycle regulator Rb1 was ablated in prospermatogonia using Blimp1-Cre in order to disrupt timing of their quiescence period during embryonic development and prior to establishment of the SSC pool. Using immunofluorescence, the authors characterised RB1/pRB1 activity at various stages of male germline development. They found that phosphorylated-RB1 (inactive form) immunostaining corresponded to periods of active proliferation of prospermatogonia. Next, using their conditional knockout model, the authors found that Blimp1-Cre-mediated Rb1 ablation resulted in aberrant proliferation of prospermatogonia at E14.5-E16.5 followed by significant loss of prospermatogonia at E18.5 and complete male germline ablation by P3.5-6.5.

The authors concluded that proper cell cycle regulation in prospermatogonia and correct timing of mitotic arrest in these cells is essential for establishment of the SSC pool. The authors then performed single cell RNA-sequencing to compare control versus Rb1 conditional knockout prospermatogonia at E14.5. The authors found that prospermatogonia normally arrest in G0/G1 phase, but upon loss of Rb1, these cells bypass the G1/S checkpoint and cannot enter mitotic arrest. Finally, the authors sought to investigate the functional relevance of the prospermatogonial quiescence period. Here, the authors analysed previously published scRNA-seq datasets and ultimately found dynamic regulation of genes involved in oxidative phosphorylation and meiosis during prospermatogonial development. The authors concluded that the period of quiescence in prospermatogonia facilitates a metabolic shift to oxidative phosphorylation and suppresses the initiation of meiosis.

To my knowledge, this is the first study to investigate the role of Rb1 ablation in prospermatogonia prior to their period of mitotic arrest. Previous studies by Hu et al. (2013) and Yang et al. (2013) used a Ddx4-Cre-mediated Rb1 knockout model, which results in Rb1 ablation after prospermatogonia have entered mitotic arrest. Therefore, this study provides important novel insight into the role of mitotic arrest timing in prospermatogonia and subsequent establishment of the SSC pool. In addition, the authors provide some insight into the regulatory mechanisms that occur during this quiescent phase. Understanding the mechanisms underlying establishment of the SSC pool is essential for the field of reproductive biology. In addition, understanding the role of quiescence periods in defining adult stem cell populations has important broader relevance to the fields of developmental and stem cell biology.

Comments for the author

The authors demonstrate an important role for RB1 and quiescence timing in prospermatogonia and subsequent establishment of the male germline. However, there are some additional data that are important to include before I would consider this study suitable for publication in Development. In addition, some data could be presented differently to improve clarity for the reader, and some areas require further clarification.

Major concerns:

1) Validation of the conditional knockout has not been provided. Although the Blimp1Cre;Rb1cKO mice show an obvious phenotype compared to controls, no data has been presented to confirm loss of RB1 protein in germ cells (and timing of this loss). Given that this model is used to ablate Rb1 at a time point prior to prospermatogonia entering mitotic arrest, it is essential to demonstrate that this has in fact occurred as expected, at what time point it has occurred, and with what level of efficiency. Ideally, this should be presented as a time point series of immunofluorescence analysis to verify that TRA98-positive germ cells are RB1-negative. These results could be supplemented by western blot analyses. Similarly, it should be demonstrated that isolated prospermatogonia from Blimp1Cre;Rb1cKO that are analysed by scRNA-seq are a pure (or at least mostly pure) population of Rb1 KO cells.

- 2) Details of statistical analyses are limited and the specific tests used for various quantifications have not been described in the figure legends. This is particularly important where there are low n-numbers (especially Figure S1A that states that n=2 for some conditions). What were the tests for/assumptions made about data normality?
- 3) On Page 5, paragraph 2, the authors state “both germ cell number and proliferation were found to be no different between control and Rb1-cKOBlimp1 mice at E13.5 (Fig. S2A and S2B)”;
- 4) Can the authors explain why Rb1 cKO cells have not clustered together in their scRNA-seq analyses despite the striking cell cycle/proliferative phenotype that has been presented in Figure 3?
- 5) The authors should provide additional validation of the scRNA-seq data presented in Figures 4 and 5. The cell cycle phenotype described in Figure 4 could be validated by flow cytometric analysis. Given that the authors isolate prospermatogonia by FACS based on tdTomato-expression, they should be able to adopt a similar strategy to perform cell cycle analyses on these cells. Furthermore, some validation of the metabolic changes described in Figure 5 should be performed, at least using immunofluorescence for markers based on scRNA-seq findings. The authors could consider showing these as a developmental time series, as well as control vs Rb1 cKO. Additional meiotic markers would also improve this figure.

Minor comments and questions:

- 1) Paragraph one of the Introduction requires citations.
- 2) Page 4, paragraph 1: which cell types show RB1/pRB1 positivity? Only germ cells?
- 3) Figure 1: This figure would benefit from the inclusion of split colour channel images, or at least zoom windows with some annotations. It is difficult to appreciate the data given the low magnifications and merged colours presented.
- 4) Given the expression/role of BLIMP1 in embryonic (Robertson et al., 2007 Development) and immune cell development (Mackay et al., 2016 Science; Turner et al., 1994 Cell), do Blimp1Cre;Rb1cKO have any other phenotype besides that of the germline?
- 5) Recently, BLIMP1 has been shown to have important roles within uterine tissues during pregnancy (Goolam et al., 2020 Nature Comms). The authors may wish to consider this study in light of their findings that show significantly reduced litter sizes in Blimp1Cre;Rb1cKO females.
- 6) The authors show that the percentage of proliferating prospermatogonia still drops to ~5% at E18.5 in Blimp1Cre;Rb1cKO mice, suggesting that another factor besides RB1 is regulating the cell cycle at this time point. Do the authors have any suggestions regarding the mechanism at this time point?
- 7) Regarding Figure 4A: can the authors present a UMAP/clustering analysis to demonstrate the differences between control and Blimp1Cre;Rb1cKO samples?
- 8) Regarding Figure 4E: The results text states “genes associated with promoting the G1/S transition were upregulated in Rb1-cKOBlimp1;tdTomato+ within each of the clusters (Fig. 4E). Figure 4E does not appear to show a comparison between control and cKO conditions.
- 9) Regarding Figure 5C: the authors nicely show dynamic regulation of OXPHOS genes during development. Do the authors also see an opposite trend for genes involved in glycolysis?
- 10) Regarding Figure 5F: do elevated levels of meiotic markers persist after E16.5 in Blimp1Cre;Rb1cKO mice? Could the authors stain at a slightly later time point, prior to complete loss of all germ cells? E.g. P1.5?

- 11) Can the authors speculate on why prospermatogonia lacking Rb1 undergo apoptosis, rather than continuing to proliferate or forming tumours?
- 12) Some details in the Material and Methods are not consistent with the data presented. Antibodies against FOXO1 and ZBTB16 are described but there are no stains for these markers presented. A section detailing flow cytometric analysis (including cell cycle analysis) is included, but this does not appear to correspond to any of the data presented. A TUNEL assay is described under “Cell viability analysis” but these data are not found in the manuscript.
- 13) Regarding Figure 6 legend: A brief description of the model presented would be beneficial for the reader.
- 14) Regarding Figure S3 legend: there is a mislabelling of “E)”, “F)” and “G)” in the text.

Reviewer 2

Advance summary and potential significance to field

Oatley et al. characterized the development of prospermatogonia in the Rb1-cKO mouse, and addressed how ablation of cell cycle arrest led to stem cell pool establishment in the male germline. The authors showed that Rb1-cKO male failed to initiate mitotic arrest during fetal development led to prospermatogonial apoptosis and germline ablation. Using scRNA-sequencing analysis, they further demonstrated that a metabolic shift occurred, and inhibition of meiotic entry was impaired in Rb1-cKO prospermatogonia. From those data, the authors proposed that proper timing of a quiescence period in prospermatogonia is required for SSC pool establishment. Overall data are convincing and the manuscript addresses important questions. Presented data are sufficient to support conclusions of the authors, and further provide a significant insight into the relation between cell cycle quiescence and meiotic inhibition in prospermatogonia. Thus, the manuscript could be open for the fields and are supposed to deserve publication, if they could address following minor concerns.

Comments for the author

(1)Page 3, Line 26.

Spiller et al previously analyzed male germ cell proliferation in in Rb1-KO fetal testis, and proposed that Rb modulates male germ cell entry into G1/G0 arrest. The authors should more discuss the differences and similarities between observations in two studies.

(2)Page 4, Fig1

The authors showed that Phos-RB1 was undetectable when the germ cells entered quiescence. Immunostaining of RB1 should be shown to see whether RB1 was expressed but not phosphorylated. Also the authors should mention whether p107 and p130 were expressed in the Rb1-cKO prospermatogonia.

(3) Page 4, Line15-16 The authors stated that the active state of RB1 aligns with mitotic entry...(Page 4, Line15-16). This sentence is confusing, because RB1 acts as a repressor of E2F-mediated transcription and phosphorylation of RB1 leads to its dissociation from E2F. Thus, phosphorylated RB1 is in inactive state in terms of its function in E2F repression rather than in active state.

(5) Page 8, Fig5

The authors showed that STRA8 (Fig5E, F) was ectopically expressed at E15.5-16.5, and genes associated with meiosis were upregulated in Rb1-cKO prospermatogonia (Fig5D). This surprising finding led them to conclude that inhibition of meiotic initiation was compromised in Rb1-cKO prospermatogonia. However they did not confirm predictions from scRNA-seq datasets with functional data. To further validate this interesting observation and developmental trajectory predicted by scRNA-seq, it would strengthen their idea if they examine whether any sign of meiotic events, such as homolog synapsis, meiotic recombination or axis formation, indeed occur in E15.5-16.5 Rb1-cKO prospermatogonia by immunostaining of spread chromosome.

(6)FigS3 legend. The second (E) should be (F). (F) should be (G).

(7)Reference. Yang, QE et al is duplicated.

(8)Page4. In the title “Impacts of disrupted during.....” Disrupted what?

Reviewer 3

Advance summary and potential significance to field

This study provides interesting insight into the role of RB1 in the mitotic arrest that occurs during spermatogonial development. The authors performed conditional knock-out of RB1 in germ cells at the PGC stage and observed increased proliferation from E14.5 to E18.5 and increased apoptosis at E16.5 in the conditional knock-out. However, as these characteristics were also observed in the conditional knock-out of RB1 before (BOR 2010, Spiller et al), and after quiescence but at a later stage (PNAS, 2013, Hu et al.; BOR 2013, Yang et al). This might rather be a question of timing rather than a specific role in mitotic arrest. Whilst Figures 2 and 3 are not wholly novel, Figures 4 and 5 provide a new scRNA-sequencing experiment that was not done in previous studies. The trajectory analysis using the published data provides information about the changes in expression of cell cycle, oxidative phosphorylation, meiotic related genes amongst others. However comparison to the differential expression analysis in control and RB1 conditional knock-out at E14.5 is made difficult by the fact that they used pseudo-time in the trajectory analysis. Furthermore, the use of only one timepoint makes limits their analysis what is really happening in the RB1 conditional knock-out. Inclusion of control and knock-out scRNA-sequencing data at different timepoints would bypass the problem of comparing pseudo-time and actual time and enable accurate assessment of the changes in gene expression over time that occur during mitotic arrest and postnatal cell cycle re-entry in the knock-out.

In conclusion, although their signal-cell analysis approach is potentially interesting additional experiments are needed to become an accept in Development. The reviewers feel that this additional data will make this study substantially stronger and add to its novelty. The presence of many mistakes in this paper; including imprecise language missing data, incorrect figure references, figures without statistical tests and duplicated references, weaken and add doubt to the reliability of this study. Attention to detail will be required for subsequent revisions.

Comments for the author

Major comments

1. (Figure 1) Despite the authors plan to “explore the relationship of RB1 activity and proliferation of prospermatogonia”, they do not evaluate and discuss the relationship between pRB1 and Ki67 staining. It would also be good to state that pRB1 is the inactive form of the protein for those new to this field. Also, there is no indication of the number of mice that they analysed.
2. (Figure 2) “<69%” is confusing as 69 is a precise value. Either quote the exact value e.g. 69% or 69.2% for example or even include the error margin e.g. $69.2 \pm 0.5\%$. Same in Figure 3 (-86%).
3. (Figure 2, 3, S1) The number of individual mice used in the experiment is not adequately described, such as $n=3-24$ in Fig 2, $n=3-4$ in Fig.3 and $n=2-24$ in Fig.S1. Please clearly specify the number of mice used at each age point. In addition, an n of 2 is insufficient for published data. The authors should not directly compare two groups with such different n values. It is unclear whether two groups for particular condition have the same n value.
4. (Figure S1B) There were significantly smaller litter sizes from KO females according to Figure S1B. Therefore, the authors cannot conclude that no PGC cell death occurred.
5. (Figure 3) Although the authors observed CASPASE-3 in Figure 3, the authors described the method of TUNEL assay in materials and methods. Which one method was used?
6. (Figure 4) In regards to the quality control of the scRNA-seq data, please make it clearer what the “>300 genes detected” and “<5000 genes” are. Are the authors filtering out samples with more than 5000 genes? How does this relate to the 17,149 and 17,237 gene averages?
7. (Figure S3B) Please make it clearer what you did to go between the sequencing, filter and germ cell lists.

8. It would be useful/interesting if you cluster the cells according to their expression of the markers you use in Figure 4E and see if clusters 0-4 separate from each other.
9. Figure S3G and 4F are very similar. The authors should combine this into one figure and include error bars.
10. The authors state “As a result, a greater proportion of germ cells transitioned from G0/G1 to S phase with Rb1 inactivation across all clusters based on CellCycleScoring (Fig. 4F).” But cluster 1 has a very small difference. They should include a statistical test. In the legend the authors should state that this is based on CellCycleScoring.
11. For the statement “In particular, genes associated with promoting the G1/S transition were upregulated in Rb1-cKOBlimp1;tdTomato+ within each of the clusters (Fig. 4E)”, was this just Ccne1 and Rrm2 or did the authors identify more genes?
12. The authors should show the results of gene ontology analysis for each cluster in the main figure (in summarised form), not just in a supplementary table.
13. The authors nicely show the cut off in Figure S3C, what is the cut off in Figure S3D?
14. The time points of individual experiments are few and not well coordinated. For instance, their E14.5 single-cell analysis showed that cluster 0 and 2 dominantly expressed meiosis related genes, which is roughly half of the total cells. However, the authors performed Stra8 staining at E16.5, and found that only 15% of the E16.5 prospermatogonia is positive for Stra8 (Fig 5F). At the same time, the apoptotic cells at E16.5 are already 45% of total prospermatogonia. It is highly possible that the majority of the cells die before entering meiosis. Therefore, to determine the fate of cells that have lost Rb1, control and knock-out scRNA-sequencing data at different timepoints, including E16.5 and E18.5, would bypass the problem and provide more an accurate picture of cell fates.
15. (Figure 4, 5) In these scRNA-seq analyses, did the authors eliminate mitochondrial RNA reads?
16. (Figure 4, 5) The author concluded that “the period of mitotic arrest in prospermatogonia facilitates a metabolic shift to an OXPHOS stage and inhibition of meiotic initiation”. However, since nuclear- and mitochondrial-encoded OXPHOS transcript levels do not increase concordantly (Nature, 2016 533: 499), the increase of nuclear derived mRNA of the OXPHOS related genes does not mean upregulation of OXPHOS pathway. The cellular mitochondrial content changes due to apoptosis (Nat commun 2018, 9: 389). Thus, the authors instead need to check the metabolic shift of OXPHOS functionally at E14.5, E16.5 and E18.5.
17. For the section “To build a developmental trajectory of prospermatogonia, we first integrated publicly available scRNA-seq datasets from isolated prospermatogonia spanning from E12.5 to SSC establishment at P3.5 (GEO accession no. GSE119045 and GSE124904; (Law et al., 2019; Nguyen and Laird, 2019) (Fig. 5A)”. The authors should state what they show in Figure 5A. They state that germline development is asynchronous but Figure 5A shows that the developmental stages cluster well with each other and in Figure 5C the different timepoints broadly align along pseudotime. As they are comparing this data with their own E14.5 timepoint and to what is previously known about different developmental states, using pseudotime makes this more difficult so using actual time would make this easier.
18. (Figure 5) For the sentence “These findings suggest that inhibition of meiotic initiation and a shift in metabolic activity normally occurs in prospermatogonia during the quiescent period.”. Oxidative phosphorylation is just one type of metabolic activity in the cell and could simply be an indicator of the cell’s activity. Therefore, the finding that during quiescence oxidative phosphorylation is reduced is not especially novel. Also, did you identify any other metabolic related terms, e.g. carbohydrate, fatty acid, nucleotide metabolism to support this metabolic shift?
19. It would be more helpful to conclude what you actually found in the result. e.g.: (p5 Second paragraph) The statement “Collectively, these findings suggest that proper cell cycle regulation during the prospermatogonial stage of development to ensure correct timing of mitotic arrest is critical for establishment of the SSC pool and laying of the foundation for continuity of the spermatogenic lineage in postnatal life.” is very sweeping and is the sort of statement the authors would have at the end of the paper. They have not measured cell cycle regulation in this section, you only infer from RB1s role in the cell cycle. They also did not look at the timing of mitotic arrest. There are large chunks of the discussion that only mention about previous findings and sound rather intro-like. To make the discussion more concise, the focus should be on the contributions of this study to the wider field not on this and the previous RB1 knock-out studies. It would be useful to include some statements in the introduction to give the reader more context for the study.

Minor points

1. The authors should state p values rather than generalising with **.

2. The tabs in Table S1 refer to incorrect or non-existent figures. These names must be updated.
 3. Page 7: Mki67 should be Ki67.
 4. The authors should include in materials and methods that the single cell trajectory analysis was performed on the GEO data and specify the source.
 5. References
 - Hu et al. is duplicated.
 - Nguyen et al. missing journal, issue and page numbers.
 - Nicholls et al. missing issue and page numbers.
 - Nikolic et al. missing issue and page numbers.
 - Varuzhanyan et al. missing page numbers.
 - Yang et al. is duplicated and page numbers are incorrect in the first instance.
-

First revision

Author response to reviewers' comments

We thank all the reviewers for their efforts in assessing our manuscript and the constructive comments that were made. We have attempted to address all concerns by revising the text, adding new data, or providing reasoning for why a change was not made. All revisions made to the manuscript text are highlighted.

Response to Reviewer #1

Comment 1: Validation of the conditional knockout has not been provided. Although the *Blimp1Cre*; *Rb1cKO* mice show an obvious phenotype compared to controls, no data has been presented to confirm loss of RB1 protein in germ cells (and timing of this loss). Given that this model is used to ablate *Rb1* at a time point prior to prospermatogonia entering mitotic arrest, it is essential to demonstrate that this has in fact occurred as expected, at what time point it has occurred, and with what level of efficiency. Ideally, this should be presented as a time point series of immunofluorescence analysis to verify that TRA98-positive germ cells are RB1-negative. These results could be supplemented by western blot analyses. Similarly, it should be demonstrated that isolated prospermatogonia from *Blimp1Cre*; *Rb1cKO* that are analysed by scRNA-seq are a pure (or at least mostly pure) population of *Rb1* KO cells.

Response: We agree with the reviewer that the time and extent of Cre recombination are important considerations. Given the evidence already present in the peer reviewed literature of *Blimp1-Cre* transgene activity in PGCs specifically starting at E6.25 and the efficiency of recombining other floxed genes between the developmental age points of E6.5 through 13.5 (timepoints prior to sex determination and mitotic arrest of prospermatogonia) having been measured at 75-100% (see examples Ohinata et al., 2005, Nature; Li et al., 2015, EMBO J), we do not believe that demonstrating this for *Rb1* conditional knockout in PGCs is necessary and have added a sentence to the results section to solidify this point (lines 108-110). We have no reason to suspect that the *Blimp1-Cre* did not effectively cause recombination of *Rb1*-floxed alleles and thereby inactivate *Rb1* in PGCs prior to the prospermatogonial period and initiation of mitotic arrest. Also, if the *Blimp1-Cre* activity on *Rb1*-floxed alleles occurred after mitotic arrest in prospermatogonia, a phenotype similar to using *Ddx4-Cre* would have been observed but this was not the case as a distinctly different spermatogenic disruption was discovered. Also, nearly 100% of seminiferous tubule cross-sections lacked spermatogenesis in the *Blimp1-Cre*; *Rb1*-floxed adult mice, thus demonstrating an extremely high, if not complete, penetrance of recombination in all PGCs. For these reasons, logic dictates that the inactivation of the *Rb1* gene occurred in PGCs prior to the prospermatogonial period and initiation of mitotic arrest. To solidify the point, we have added new data (Figure S3B) of *Rb1* gene expression (measured by scRNA-seq analysis) at E14.5 from control and *Blimp1Cre*; *Rb1* cKO testes that clearly shows undetectable *Rb1* transcripts in 100% of the germ cells at this age point which is prior to initiation of the quiescent period in prospermatogonia. These data have also been presented in the results section, lines 192-198.

Comment 2: Details of statistical analyses are limited and the specific tests used for various quantifications have not been described in the figure legends. This is particularly important where there are low n-numbers (especially Figure S1A that states that n=2 for some conditions). What were the tests for/assumptions made about data normality?

Response: A description of statistical analyses used in the study is presented in the materials and methods section and each test used for a dataset is now indicated in the figure legends. Please note that the only quantitative data we had presented with an n-value less than 3 was for body weights of Rb1-cKO mice (Figure S1A) and statistical comparisons with controls were not made. We have now incorporated new data from additional animals to the graph in order to increase the n-value and perform statistical analysis. Also, we have revised the methods section (lines 552-557) to indicate that all quantitative data are presented as mean \pm SEM and differences between experimental groups were determined statistically using the Student's *t*-test (independent-samples T test) or nonparametric test (Mann-Whitney U test) function of SPSS software when the data were of a normal distribution or non-normal distribution, respectively.

Comment 3: On Page 5, paragraph 2, the authors state “both germ cell number and proliferation were found to be no different between control and Rb1-cKO Blimp1 mice at E13.5 (Fig. S2A and S2B)”; however, there has been no quantification presented to support this claim. This could be performed using tdTomato⁺ mice that are used for scRNA-seq experiments in this study.

Response: As suggested, new data for quantification of germ cell proliferation at E13.5 has been added in Fig S2.

Comment 4: Can the authors explain why Rb1 cKO cells have not clustered together in their scRNA-seq analyses, despite the striking cell cycle/proliferative phenotype that has been presented in Figure 3?

Response: Previous studies have revealed major heterogeneity of transcriptome profiles for the germ cell population in wild-type mice at the embryonic/fetal age points of E12-18 (Nguyen et al., 2019, Curr Top Dev Biol; Law et al., 2019, Nature Comms). Thus, we are not surprised to have found heterogeneity still exists with the Rb1-cKO germ cell population at E14.5. A sentence has been added to the results section (lines 226-230) to clarify this point.

Comment 5: The authors should provide additional validation of the scRNA-seq data presented in Figures 4 and 5. The cell cycle phenotype described in Figure 4 could be validated by flow cytometric analysis. Given that the authors isolate prospermatogonia by FACS based on tdTomato-expression, they should be able to adopt a similar strategy to perform cell cycle analyses on these cells. Furthermore, some validation of the metabolic changes described in Figure 5 should be performed, at least using immunofluorescence for markers based on scRNA-seq findings. The authors could consider showing these as a developmental time series, as well as control vs Rb1 cKO. Additional meiotic markers would also improve this figure.

Response: Regarding the cell cycle phenotype reflected by data presented in Figure 4, we agree that flow cytometric analysis of prospermatogonia in control and Rb1 cKO mice at E14.5 could be used to validate the scRNA-seq data. However, to achieve this the prospermatogonial population needs to be labeled and while we had devised an approach to accomplish this for scRNA-seq with tdTomato expression, generating a sufficient number of E14.5 Rb1 cKO and control fetuses to provide adequate numbers of cells for cell cycle assays is not feasible for us at present. Obtaining enough cells for scRNA-seq analysis was a heroic effort due to the well-known low fecundity of Blimp1-Cre mice. Due to restrictions brought about by the Covid19 pandemic, we were forced to scale back our mouse colony to maintenance levels and therefore don't have the resources available to initiate another major scale up needed for generating animal numbers that would be sufficient for flow cytometric based cell cycle analysis. In addition, because cells in the G0/G1 phase are negative for Ki67, we believe that the results presented in Figures 3A and 3B complement the cell cycle scoring analysis from scRNA-seq data and together these findings support a conclusion of altered cell cycle regulation in prospermatogonia due to lack of Rb1.

We also agree that adding other measures of OXPHOS activity in Rb1 cKO prospermatogonia would reinforce our conclusion about disruption in metabolic shifts. However, beyond use of transcriptome

profiling to make predictions of metabolic changes, measuring metabolic activity in prospermatogonia is extremely difficult and we are not aware of an assay that would be compatible due to limitations in the number of cells that are needed. We do appreciate the reviewer's suggestion of using immunofluorescence but do not agree that this approach would yield results that could be validly interpreted. The scRNA-seq data indicates that OXPHOS activity occurs in both control and Rb1 cKO prospermatogonia but is altered in the cKO cells. As the difference in activity is not binary, we do not believe that immunofluorescence is an adequate quantitative approach for measuring these types of changes in cellular processes. In an effort to address the reviewer's concern, we did attempt to perform some metabolic pathway biochemical assays, including measuring extracellular oxygen consumption and glycolysis. Unfortunately, we learned that these types of assays are not feasible, at least in our hands, with the limited number of cells that can be obtained during fetal development in mice. The approaches require at least 50,000 cells/sample; on average only ~2,000 prospermatogonia can be obtained per E14.5 day fetus via FACS, thus a minimum of 25 fetuses are needed to generate a single sample and with appropriate biological and technical replication ~200 fetuses would be needed for Rb1 cKO cells and ~200 fetuses for control cells. These numbers are simply beyond our resources to generate. Because of these limitations, we have not performed additional validation experiments for metabolic pathways; however, we have revised the text to temper the conclusions that have been drawn and point out that additional experimentation will be needed in future studies to verify that disruption of entry into mitotic arrest alters a metabolic shift during prospermatogonial development. The revisions are in lines 284-297 and 409-416 in the results and discussions section.

Comment 6: Paragraph one of the Introduction requires citations.

Response: Citations have been added as suggested.

Comment 7: Page 4, paragraph 1: which cell types show RB1/pRB1 positivity? Only germ cells?

Response: Based on immunofluorescent staining, both germ cells and a subset of somatic cells appear to express RB1/pRB1 which is consistent with findings of previous studies (Western et al., 2008, Stem cells; Spiller et al., 2010, Biol Reprod). A sentence has been added to the paragraph to clarify this point, lines 87-91.

Comment 8: Figure 1: This figure would benefit from the inclusion of split color channel images, or at least zoom windows with some annotations. It is difficult to appreciate the data given the low magnifications and merged colors presented.

Response: Figure 1 has been revised as suggested.

Comment 9: Given the expression/role of BLIMP1 in embryonic (Robertson et al., 2007 Development) and immune cell development (Mackay et al., 2016 Science; Turner et al., 1994 Cell), do Blimp1Cre;Rb1cKO have any other phenotype besides that of the germline?

Response: The only prominent phenotypic difference of the Blimp1-Cre;Rb1-floxed cKO mice that we observed was sterility in adulthood due to lack of germline. The cKO mice were otherwise healthy with a lifespan and mating behavior that was not different compared to controls. A sentence has been added to the results section that conveys these observations, lines 110-112.

Comment 10: Recently, BLIMP1 has been shown to have important roles within uterine tissues during pregnancy (Goolam et al., 2020 Nat Comms). The authors may wish to consider this study in light of their findings that show significantly reduced litter sizes in Blimp1Cre;Rb1cKO females.

Response: We appreciate the reviewer bringing this recent study to our attention. The reduction in litter size observed for Blimp1-Cre;Rb1-floxed cKO females is similar to what we reported previously for Ddx4-Cre;Rb1-floxed females (Yang et al., 2015, PLoS Genet). Thus, by deductive reasoning we infer that the fertility phenotype of Blimp1-Cre;Rb1-floxed cKO females was mainly caused by the dysregulation of follicle growth. However, we cannot rule out that Rb1 inactivation in Blimp1 expressing uterine cells contributed to the phenotype and have added a sentence in the results section to account for this, lines 125-128.

Comment 11: The authors show that the percentage of proliferating prospermatogonia still drops to ~5% at E18.5 in *Blimp1Cre;Rb1cKO* mice, suggesting that another factor besides RB1 is regulating the cell cycle at this time point. Do the authors have any suggestions regarding the mechanism at this time point?

Response: This is an interesting question which certainly warrants further exploration; however, we feel it is outside the scope of our current study. We used cKO of Rb1 in prospermatogonia to delay the timing of mitotic arrest, which was clearly accomplished, rather than fully ablate it. There are several potential mechanisms for why prospermatogonia still undergo mitotic arrest by E18.5 even in the absence of Rb1 activity including roles for other family members such as *p107* and *p130*, as well as other cell cycle regulators such as cyclin D-*Cdk4/6*, *Cdkn2a* and *Cdkn2b*. Although explaining how a mitotic arrest could still occur, even after a disruption of the normal timing is beyond the scope of our current study, we have added discussion of this observation to the revised manuscript, lines 338-345.

Comment 12: Regarding Figure 4A: can the authors present a UMAP/clustering analysis to demonstrate the differences between control and *Blimp1Cre;Rb1cKO* samples?

Response: Both UMAP representation of individual libraries and graph-based clustering for prospermatogonia isolated from control or Rb1 cKO mice have been added to Figure 4.

Comment 13: Regarding Figure 4E: The results text states “genes associated with promoting the G1/S transition were upregulated in *Rb1-cKO;Blimp1;tdTomato+* within each of the clusters (Fig. 4E). Figure 4E does not appear to show a comparison between control and cKO conditions.

Response: The reviewer is correct, and we had incorporated the wrong version of Figure 4E in the initial submission. The correct version of the Figure has been included in the revised manuscript.

Comment 14: Regarding Figure 5C: the authors nicely show dynamic regulation of OXPHOS genes during development. Do the authors also see an opposite trend for genes involved in glycolysis?

Response: Yes, the outcomes of scRNA-seq indicate increased glycolysis in the Rb1 cKO prospermatogonia; evidence by elevated expression of glycolytic enzyme genes such as *Eno1*. These data have been incorporated into the revised manuscript as a new supplemental Figure S6 and presented on lines 295-297.

Comment 15: Regarding Figure 5F: do elevated levels of meiotic markers persist after E16.5 in *Blimp1Cre;Rb1cKO* mice? Could the authors stain at a slightly later time point, prior to complete loss of all germ cells? E.g. P1.5?

Response: Based on the data presented in Figure 3D and E, greater than 85% of seminiferous tubules are devoid of germ cells at P1.5 in the *Blimp1-Cre;Rb1 cKO* mice and the remaining are undergoing apoptosis. Thus, to address the reviewer's question, we analyzed E18.5 testes and found that ~6% of prospermatogonia were STRA8+ which represents a drop by roughly half compared to E16.5. These data have been incorporated as a new supplemental Figure (Figure S7) and presented on lines 313-317.

Comment 16: Can the authors speculate on why prospermatogonia lacking Rb1 undergo apoptosis, rather than continuing to proliferate or forming tumors?

Response: Apoptosis of prospermatogonia is a well-known to occurs during perinatal development and has been speculated to be a means of eliminating those cells with compromised genetic integrity (Wang et al., 1998, Biol Reprod; Bejarano et al., 2018, Front Cell Dev Biol; Nguyen et al., 2019). Indeed, a recent study demonstrated that prospermatogonia with developmentally defective genetic and epigenetic integrity are eliminated by apoptosis in the mouse (Nguyen et al., 2020, Nat Cell Biol). Based on these previous findings, we speculate that an intrinsic biosensing mechanism initiates apoptosis in prospermatogonia that fail to enter mitotic arrest at the appropriate time during fetal development or have compromised genetic integrity due to Rb1 deficiency. These perspectives have been included in the discussion section of the revised manuscript, lines 346-355.

Comment 17: Some details in the Material and Methods are not consistent with the data presented. Antibodies against FOXO1 and ZBTB16 are described but there are no stains for these markers presented. A section detailing flow cytometric analysis (including cell cycle analysis) is included, but this does not appear to correspond to any of the data presented. A TUNEL assay is described under “Cell viability analysis” but these data are not found in the manuscript.

Response: These descriptions were carried over from a different version of the manuscript and have been removed in the revised version.

Comment 18: Regarding Figure 6 legend: A brief description of the model presented would be beneficial for the reader.

Response: We appreciate this comment and have added a brief description in Figure 6 legend as suggested.

Comment 19: Regarding Figure S3 legend: there is a mislabeling of “E)”, “F)” and “G)” in the text.

Response: The labeling has been corrected in the revised manuscript.

Response to Reviewer #2

Comment 1: Page 3, Line 26. Spiller et al previously analyzed male germ cell proliferation in in Rb1-KO fetal testis, and proposed that Rb modulates male germ cell entry into G1/G0 arrest. The authors should more discuss the differences and similarities between observations in two studies.

Response: The purpose of the current study was not to explore the role of Rb in regulating cell cycle progression of prospermatogonia, rather the focus was to determine what the ramifications are for not entering mitotic arrest at the appropriate time in development. We have revised the introduction to clarify the importance of the Spiller et al findings and the purpose of the current study, lines 61-70.

Comment 2: Page 4, Fig1 The authors showed that Phos-RB1 was undetectable when the germ cells entered quiescence. Immunostaining of RB1 should be shown to see whether RB1 was expressed but not phosphorylated. Also, the authors should mention whether p107 and p130 were expressed in the Rb1-cKO prospermatogonia.

Response: In Figure 1, immunostaining for RB1 and phospho-RB1 is presented in separate cross-sections of testicular tissue at E12.5-P6.5. The staining indicates that RB1 is present in prospermatogonia throughout development but phospho-RB1 is detectable during the period of proliferation from E12.5-13.5 and becomes low to undetectable at E14.5-P1.5 coincident with the period of mitotic arrest. Note that because both primary antibodies for RB1 and pRB1 were made in rabbits, we were unable to conduct co-immunofluorescent staining. We have revised the results section to clarify the findings, lines 87-97.

Regarding the expression of *p107 (Rbl1)* and *p130 (Rbl2)* in *Rb1-cKO* prospermatogonia, based on scRNA-seq analysis there was no difference compared to *Rb1* sufficient prospermatogonia. These findings are consistent with those of Spiller et al., 2010, Biol Reprod who reported no change in expression of either gene in the germ cells of Rb1 null mice at either at E14.5 or E16.5. We have reflected these findings in the revised manuscript, lines 196-198 and with new data in Figure S3B.

Comment 3: Page 4, Line 15-16 The authors stated that the active state of RB1 aligns with mitotic entry. This sentence is confusing, because RB1 acts as a repressor of E2F-mediated transcription and phosphorylation of RB1 leads to its dissociation from E2F. Thus, phosphorylated RB1 is in inactive state in terms of its function in E2F repression rather than in active state.

Response: We have revised the statement to indicate that the active vs. inactive state of RB1 aligns with cell cycle progression and arrest in prospermatogonia during late fetal and early neonatal development, lines 95-97.

Comment 4: Page 8, Fig5 The authors showed that STRA8 (Fig5E, F) was ectopically expressed at E15.5-16.5, and genes associated with meiosis were upregulated in Rb1-cKO prospermatogonia

(Fig5D). This surprising finding led them to conclude that inhibition of meiotic initiation was compromised in Rb1-cKO prospermatogonia. However, they did not confirm predictions from scRNA-seq datasets with functional data. To further validate this interesting observation and developmental trajectory predicted by scRNA-seq, it would strengthen their idea if they examine whether any sign of meiotic events, such as homolog synapsis, meiotic recombination or axis formation, indeed occur in E15.5-16.5 Rb1-cKO prospermatogonia by immunostaining of spread chromosome.

Response: We agree with the reviewer that examining chromosomes for meiotic events would complement the predictions made by STRA8 staining and scRNA-seq analyses and in doing so strengthen the conclusion of impaired meiotic inhibition in Rb1-cKO prospermatogonia. Unfortunately, we have been unable to generate a sufficient number of new cKO mice for this assessment due to COVID19 mandated reductions in animal numbers. Thus, we are unable to fully address the reviewer's comment with experimental evidence and instead have revised the text to soften our interpretation. We now state (lines 313-317) that the ectopic expression of STRA8 and significant increase in expression of genes related to meiosis indicate that Rb1-cKO prospermatogonia prematurely initiate a differentiation pathway.

Comment 5: FigS3 legend. The second (E) should be (F). (F) should be (G).

Response: We have revised the manuscript accordingly.

Comment 6: Reference. Yang, QE et al is duplicated.

Response: The duplicated reference has been deleted.

Comment 7: Page4. In the title "Impacts of disrupted during....." Disrupted what?

Response: The subtitle has been revised accordingly.

Response to Reviewer #3

Comment 1: (Figure 1) Despite the authors plan to "explore the relationship of RB1 activity and proliferation of prospermatogonia", they do not evaluate and discuss the relationship between pRB1 and Ki67 staining. It would also be good to state that pRB1 is the inactive form of the protein for those new to this field. Also, there is no indication of the number of mice that they analyzed.

Response: Sentences have been added to the first paragraph of the results section (lines 82-85) to clarify the active versus inactive states of RB1 and relationship of Ki67 expression with cell cycle status. The images of immunostaining in Figure 1 are representative of 3 different animals at each age point; this information is now included in the legend of Figure 1.

Comment 2: (Figure 2) "<69%" is confusing as 69 is a precise value. Either quote the exact value e.g. 69% or 69.2% for example or even include the error margin e.g. $69.2 \pm 0.5\%$. Same in Figure 3 (~86%).

Response: As suggested, the exact values have been included.

Comment 3: (Figure 2, 3, S1) The number of individual mice used in the experiment is not adequately described, such as n=3-24 in Fig 2, n=3-4 in Fig.3 and n=2-24 in Fig.S1. Please clearly specify the number of mice used at each age point. In addition, an n of 2 is insufficient for published data. The authors should not directly compare two groups with such different n values. It is unclear whether two groups for particular condition have the same n value.

Response: The number of mice used for each experiment has been clarified throughout the manuscript and additional replicates have been added to the datasets with n=2.

Comment 4: (Figure S1B) There were significantly smaller litter sizes from KO females according to Figure S1B. Therefore, the authors cannot conclude that no PGC cell death occurred.

Response: We agree and the conclusionary statement has been revised.

Comment 5: (Figure 3) Although the authors observed CASPASE-3 in Figure 3, the authors described the method of TUNEL assay in materials and methods. Which one method was used?

Response: We apologize for the error and have revised the Materials and Methods for correctness.

Comment 6: (Figure 4) In regards to the quality control of the scRNA-seq data, please make it clearer what the “>300 genes detected” and “<5000 genes” are. Are the authors filtering out samples with more than 5000 genes? How does this relate to the 17,149 and 17,237 gene averages?

Response: We appreciate the reviewer catching this as there were several typos. The description has been revised clarity.

Comment 7: (Figure S3B) Please make it clearer what you did to go between the sequencing, filter and germ cell lists.

Response: As suggest, the text has been revised for clarity; lines 199-213.

Comment 8: It would be useful/interesting if you cluster the cells according to their expression of the markers you use in Figure 4E and see if clusters 0-4 separate from each other.

Response: As suggested, Figure 4E has been replaced with clustering based on expression of marker genes.

Comment 9: Figure S3G and 4F are very similar. The authors should combine this into one figure and include error bars.

Response: We appreciate the reviewers opinion on combining the data but have chosen to keep them separate because Figure S3G is intended to show the heterogeneity of germ cells at E14.5 due to differences in cell cycle progression, whereas Figure 4F is intended to show disruption of the G₁/S transition in germ cells lacking *Rb1*. The text has been revised to clarify this point, lines 226-230. In addition, error bars have been added to Figure 4F, as suggested.

Comment 10: The authors state “As a result, a greater proportion of germ cells transitioned from G₀/G₁ to S phase with *Rb1* inactivation across all clusters based on CellCycleScoring (Fig. 4F).” But cluster 1 has a very small difference. They should include a statistical test. In the legend the authors should state that this is based on CellCycleScoring.

Response: As suggested, statistical assessment has been conducted and the Figure legend has been revised.

Comment 11: For the statement “In particular, genes associated with promoting the G₁/S transition were upregulated in *Rb1*-cKOBlimp1;tdTomato+ within each of the clusters (Fig. 4E)”, was this just *Ccne1* and *Rrm2* or did the authors identify more genes?

Response: An incorrect version of Figure 4E was included in the original submission and we have replaced it with the correct version in the revised manuscript.

Comment 12: The authors should show the results of gene ontology analysis for each cluster in the main figure (in summarized form), not just in a supplementary table.

Response: As suggested, the data for gene ontology analysis has been included as a new Table 1.

Comment 13: The authors nicely show the cut off in Figure S3C, what is the cut off in Figure S3D?

Response: Description of the cut-offs for both Figures have been added to the text, lines 199-213, and therefore removed from the Figures themselves.

Comment 14: The time points of individual experiments are few and not well coordinated. For instance, their E14.5 single-cell analysis showed that cluster 0 and 2 dominantly expressed

meiosis related genes, which is roughly half of the total cells. However, the authors performed Stra8 staining at E16.5, and found that only 15% of the E16.5 prospermatogonia is positive for Stra8 (Fig 5F). At the same time, the apoptotic cells at E16.5 are already 45% of total prospermatogonia. It is highly possible that the majority of the cells die before entering meiosis. Therefore, to determine the fate of cells that have lost Rb1, control and knock-out scRNA-sequencing data at different timepoints, including E16.5 and E18.5, would bypass the problem and provide more an accurate picture of cell fates.

Response: We appreciate the reviewer's perspective but disagree with the suggested approach. The age point of E14.5 was chosen for conducting scRNA-seq because it is when interruption of normal entry to the quiescent period occurs, thus we were able to study genesis of the programming disruption. In contrast, while potentially informative, scRNA-seq analysis at E16.5 and 18.5 would explore the ramifications of disrupted entry into quiescence during development which would likely be secondary to the root cause that initiated at E14.5. This point has been clarified in the text, lines 181-186. In addition, we chose to examine STRA8 expression at the protein level in E16.5 prospermatogonia to assess the ramifications of disrupted entry into quiescence on a normal timeline at E14.5. This point has been clarified in the text, lines 305-308.

Comment 15: (Figure 4, 5) In these scRNA-seq analyses, did the authors eliminate mitochondrial RNA reads?

Response: We did not specifically remove mitochondrial RNA reads, rather quality control of the sequencing data removed cells with high mitochondrial reads that would suggest they were dead or dying. This description has been added to the results section, lines 206-208.

Comment 16: (Figure 4, 5) The author concluded that "the period of mitotic arrest in prospermatogonia facilitates a metabolic shift to an OXPHOS stage and inhibition of meiotic initiation". However, since nuclear- and mitochondrial-encoded OXPHOS transcript levels do not increase concordantly (Nature, 2016 533: 499), the increase of nuclear derived mRNA of the OXPHOS related genes does not mean upregulation of OXPHOS pathway. The cellular mitochondrial content changes due to apoptosis (Nat commun, 2018, 9: 389). Thus, the authors instead need to check the metabolic shift of OXPHOS functionally at E14.5, E16.5 and E18.5.

Response: We appreciate the reviewers concern with the OXPHOS assessment based solely on scRNA-seq analysis and agree that biochemical endpoints of analysis should be included in order to draw firmer conclusions. However, we point out that the downstream assessment of altered OXPHOS activity included filtering of cells based on the percentage of mitochondrial gene expression so that all libraries were similar (see new Table S3 and description on lines 284-297). In addition, according to the data presented in Figure 3G, there was no difference in the percentage of prospermatogonia with cleaved Caspase-3 between wild-type and Rb1 cKO fetuses at E14.5, thus we do not believe that changes in cellular mitochondrial content due to apoptosis was a confounding factor in our assessment of metabolic state from the scRNA-seq data. This point has been clarified in lines 181-186. Regarding the need for biochemical endpoint analysis of OXPHOS activity in prospermatogonia, we agree but, as addressed in the response to Reviewer 1 - Comment 5, beyond use of transcriptome profiling to make predictions of metabolic changes, measuring metabolic activity in prospermatogonia is extremely difficult and we are not aware of an assay that would be compatible due to limitations in the number of cells that are needed. In an effort to address both Reviewers' comments, we did attempt to perform some metabolic pathway biochemical assays, including measuring extracellular oxygen consumption and glycolysis. Unfortunately, we learned that these types of assays are not feasible, at least in our hands, with the limited number of cells that can be obtain during fetal development in mice. The approaches require at least 50,000 cells/sample; on average only ~2,000 prospermatogonia can be obtain per E14.5 day fetus via FACS, thus a minimum of 25 fetuses are needed to generate a single sample and with appropriate biological and technical replication ~200 fetuses would be needed for Rb1 cKO cells and ~200 fetuses for control cells. These numbers are simply beyond our resources to generate. Because of these limitations, we have not performed additional validation experiments for metabolic pathways; however, we have revised the text to temper the conclusions that have been drawn and point out that additional experimentation will be needed in future studies to verify that disruption of entry into mitotic arrest alters a metabolic shift during prospermatogonial development. The revisions are in lines 284-297 and 409-416 in the results and discussions section.

Comment 17: For the section “To build a developmental trajectory of prospermatogonia, we first integrated publicly available scRNA-seq datasets from isolated prospermatogonia spanning from E12.5 to SSC establishment at P3.5 (GEO accession no. GSE119045 and GSE124904; (Law et al., 2019; Nguyen and Laird, 2019) (Fig. 5A)”. The authors should state what they show in Figure 5A. They state that germline development is asynchronous but Figure 5A shows that the developmental stages cluster well with each other and in Figure 5C the different timepoints broadly align along pseudotime. As they are comparing this data with their own E14.5 timepoint and to what is previously known about different developmental states, using pseudotime makes this more difficult so using actual time would make this easier.

Response: We appreciate the reviewer’s perspective and have tried to revise the section accordingly. However, we disagree with the notion of using actual time rather than pseudotime for making the comparison of scRNA-seq datasets from E12.5 and P3.5 to the E14.5 dataset that was generated in the current study. The previous scRNA-seq studies (Law et al. 2019, Liao et al. 2019, Nguyen et al. 2020, Tan et al. 2020, and others) indicate that although developmental ages tend to cluster together after dimensional reductions such as a UMAP (as in Figure 5A of the current manuscript), there is heterogeneity among each developmental age with regard to developmental status. For example, not all germ cells enter mitotic arrest simultaneously; full mitotic arrest of the entire germ cell population spans ~3-4 developmental days in the mouse. As a result of this heterogeneity, there can be overlap between developmental ages that is resolved with pseudotime ordering and is otherwise masked by other standard analyses. Furthermore, pseudotime ordering better represents temporal shifts in gene expression while accounting for heterogeneity at individual ages. Figure 5C illustrates that developmental ages not only overlap with respect to developmental potential, but also indicates that “actual time” is generally recapitulated while accounting for heterogeneity among each developmental age. Finally, the separation of ages in a UMAP can also be attributed to technical differences in gene detection rates, sequencing depth, etc. Pseudotime ordering utilizes a modeling algorithm that better mitigates these technical factors while retaining heterogeneity.

Comment 18: (Figure 5) For the sentence “These findings suggest that inhibition of meiotic initiation and a shift in metabolic activity normally occurs in prospermatogonia during the quiescent period.”. Oxidative phosphorylation is just one type of metabolic activity in the cell and could simply be an indicator of the cell’s activity. Therefore, the finding that during quiescence oxidative phosphorylation is reduced is not especially novel. Also, did you identify any other metabolic related terms, e.g. carbohydrate, fatty acid, nucleotide metabolism to support this metabolic shift?

Response: We appreciate the reviewer’s perspective on whether observing an alteration in OXPHOS activity is not especially novel but disagree with the point. Several studies have shown that OXPHOS activity is linked with both spermatogonial and PGC differentiation even when the cells are in a different state of proliferation (for examples, Lord T and Nixon B, 2020, Dev Cell; Hayashi et al., 2017, PNAS), and induction of OXPHOS activity triggers loss of embryonic stem cell pluripotency (Vega-Naredo et al., 2014, Cell Death Diff). We have clarified this point in the Discussion section, lines 430-437.

Comment 19: It would be more helpful to conclude what you actually found in the result. e.g.: (p5, Second paragraph) The statement “Collectively, these findings suggest that proper cell cycle regulation during the prospermatogonial stage of development to ensure correct timing of mitotic arrest is critical for establishment of the SSC pool and laying of the foundation for continuity of the spermatogenic lineage in postnatal life.” is very sweeping and is the sort of statement the authors would have at the end of the paper. They have not measured cell cycle regulation in this section, you only infer from RB1s role in the cell cycle. They also did not look at the timing of mitotic arrest. There are large chunks of the discussion that only mention about previous findings and sound rather intro-like. To make the discussion more concise, the focus should be on the contributions of this study to the wider field not on this and the previous RB1 knock-out studies. It would be useful to include some statements in the introduction to give the reader more context for the study.

Response: We appreciate the reviewer’s opinion about how our manuscript is structured and have tried to revise accordingly.

Comment 20: The authors should state p values rather than generalising with **.

Response: A standard practice in biological statistics is to use * for designation of significant differences at $P \leq 0.05$ and ** at $P \leq 0.01$. This is our preferred approach to presentation of statistical assessment for quantitative datasets.

Comment 21: The tabs in Table S1 refer to incorrect or non-existent figures. These names must be updated.

Response: The table has been updated.

Comment 22: Page 7: Mki67 should be Ki67.

Response: Revision has been made.

Comment 23: The authors should include in materials and methods that the single cell trajectory analysis was performed on the GEO data and specify the source.

Response: As suggested, these pieces of information have been added.

Comment 24: References

- Hu et al. is duplicated.
- Nguyen et al. missing journal, issue and page numbers.
- Nicholls et al. missing issue and page numbers.
- Nikolic et al. missing issue and page numbers.
- Varuzhanyan et al. missing page numbers.
- Yang et al. is duplicated and page numbers are incorrect in the first instance.

Response: Revisions have been made.

Second decision letter

MS ID#: DEVELOP/2020/194571

MS TITLE: Proper timing of a quiescence period in precursor prospermatogonia is required for stem cell pool establishment in the male germline

AUTHORS: Jon M Oatley, Guihua Du, Melissa Oatley, Nathan Law, Colton Robbins, and Xin Wu

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

As you will see, the referees appreciate to your efforts to revise the manuscript along with their comments. Nonetheless they still retain a few substantial concerns in results as represented by comments by Reviewer 1 and 3. They recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and

where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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

Reviewer 1

Advance summary and potential significance to field

The authors have made substantial improvements to their manuscript, including the inclusion of new data quantification of previously presented data and changes to the manuscript text. The authors have made an effort to address all of my previous comments, despite the challenges presented by the COVID-19 pandemic.

Overall, the authors have provided novel insight into the mechanisms underlying establishment of the SSC pool. I would agree that this manuscript is suitable for publication in Development if the minor comments below are addressed appropriately.

Comments for the author

- 1) In Supplementary Figure S7, the immunofluorescence images presented in panel A appear to show an equivalent, if not higher, number of STRA8+ cells in the control condition. This is not representative of the quantification presented in panel B, where the control values are all zero. There are clear TRA98+/STRA8+ double-positive cells in each condition. Maybe there has been an error in the preparation of this figure?
- 2) Regarding Figure S3B, the dot plot only depicts data for Rb1. Data for p107 and p130 are not shown, as described in the figure legend and in the results text (lines 198-201).
- 3) In Figure 4 and Figure S1, the figure legend describes the use of asterisks to denote statistical significance; however, some of the panels display the exact p-values.

Reviewer 2

Advance summary and potential significance to field

The authors showed revised data and appropriate corrections in the manuscript, which overall sufficed my previous questions and concerns.

Comments for the author

Regarding my comment 4 on ectopic expression of STRA8 and meiotic genes in E16.5 Rb1-cKO prospermatogonia, although the authors rephrased their statement (lines 313-317), that sentence was rather unclear to imply what may have happened. Rather, it would be better to state "inhibition of meiotic entry could be compromised in Rb1-cKO prospermatogonia."

Reviewer 3

Advance summary and potential significance to field

The authors responded to many of my concerns adequately. However, I still have the following concerns.

Comments for the author

(1) Comments 16

The reviewer thinks that validation of OXPHOS related events is necessary for this study. The reasons are, firstly, there is generally a time difference between changes in protein and gene expression levels. Secondly, more specific to Rb study, it has already known that the mitochondrial pathway is controlled by post-transcriptional regulation and the effects of Rb loss on the proteome are significantly different from the transcriptional changes. Proliferative cells typically show biased toward aerobic glycolysis even in the presence of oxygen because of their high energy demand for rapid growth. In this condition, the effects of Rb loss upon OXPHOS are known to be cell type/status specific, the OXPHOS is upregulated in some cell types but downregulated in the others. Thus, validation is necessary. The limitation of the number of cells obtained from in vivo is not a problem. The cytometric assessments of mitochondria can be performed using fluorescent probes, and immune-histochemical analyses of the OXPHOS molecules they have identified as being upregulated is reasonably possible. The measurement of ATP levels is also useful to prove the metabolic changes they claim.

(2) Comments 18

There is a huge leap of logic in this newly added argument (Line 430-437). What they are observing in this paper is the response of OXPHOS in an artificial environment when the Rb gene is deleted. It is not hard to imagine that the studies in the cited references share only the term “OXPHOS”, but differ in the state of the cell cycle some referring to PGCs during mitotic cell division stage, others to metabolic studies of meiosis in spermatogenic cells, and probably differ in the expression levels of many metabolic, cell cycle genes and Rb-related molecules between them. Therefore, it is clear that they are not able to be compared. Also, since this study only observed the OXPHOS-related gene expression without metabolomic analyses, the argument that “a metabolic shift occurred” is an overestimation. Also, Vega-Naredo paper did not use ES cells. They analyzed OXPHOS using P19 embryonal carcinoma cell line. Besides, a part of the information of the newly cited paper (Varuzhanyan et al.) is missing.

(3) Fig. S6: The authors state in the text that Eno1 expression was downregulated in Rb1 cKO (Line 295-297), but Fig. S6 appears to show its upregulation. What is a functional relevance of the Eno1 behaviour in prospermatogonia lacking Rb?

In addition to the above comments, I have some additional suggestions regarding their comments on this revision.

(4) To strengthen their study to investigate the ramifications of disrupted entry into quiescence, it might be useful to provide additional examples of meiotic genes using their Rb1-cKOblimp1. Assessing whether multiple meiotic markers, rather than one are affected would lead to a more accurate picture of the consequences. Can they present UMAP expression plot for some representative genes, including Stra8, to show the difference between control and cKO at E14.5?

(5) Again, as for the OXPHOS genes, can the authors present more information such as expression of some representative genes on UMAP feature plot, comparing control and cKO, because this is one of the major conclusions of this work?

(6) Fig. 4D: Please indicate in the legend how many genes are shown in the heatmap.

(7) Fig. 5C: Please indicate in the legend how many genes are contained in each group.

(8) I would suggest reporting exact p-values in addition to ** as this is more informative and is becoming a more common practice in biology.

Second revision

Author response to reviewers' comments

Response to Reviewer 1 Comments

Comment: In Supplementary Figure S7, the immunofluorescence images presented in panel A appear to show an equivalent, if not higher, number of STRA8+ cells in the control condition. This is not representative of the quantification presented in panel B, where the control values are all zero. There are clear TRA98+/STRA8+ double-positive cells in each condition. Maybe there has been an error in the preparation of this figure?

Response: We thank the reviewer for recognizing this error in figure preparation and have corrected it in the revised manuscript.

Comment: Regarding Figure S3B, the dot plot only depicts data for Rb1. Data for p107 and p130 are not shown, as described in the figure legend and in the results text (lines 198-201).

Response: We apologize for this error and have now included the data for Rb1, p107, and p130.

Comment: In Figure 4 and Figure S1, the figure legend describes the use of asterisks to denote statistical significance; however, some of the panels display the exact p- values.

Response: The figures have been revised for consistency.

Response to Reviewer 2 Comments

Comment: Regarding my comment 4 on ectopic expression of STRA8 and meiotic genes in E16.5 Rb1-cKO prospermatogonia, although the authors rephrased their statement (lines 313-317), that sentence was rather unclear to imply what may have happened. Rather, it would be better to state “inhibition of meiotic entry could be compromised in Rb1-cKO prospermatogonia.”

Response: We agree with this comment and have revised the text accordingly.

Response to Reviewer 3 Comments

Comment: The reviewer thinks that validation of OXPHOS related events is necessary for this study. The reasons are, firstly, there is generally a time difference between changes in protein and gene expression levels. Secondly, more specific to Rb study, it has already known that the mitochondrial pathway is controlled by post- transcriptional regulation and the effects of Rb loss on the proteome are significantly different from the transcriptional changes. Proliferative cells typically show biased toward aerobic glycolysis even in the presence of oxygen because of their high energy demand for rapid growth. In this condition, the effects of Rb loss upon OXPHOS are known to be cell type/status specific, the OXPHOS is upregulated in some cell types but downregulated in the others. Thus, validation is necessary. The limitation of the number of cells obtained from in vivo is not a problem. The cytometric assessments of mitochondria can be performed using fluorescent probes, and immune-histochemical analyses of the OXPHOS molecules they have identified as being upregulated is reasonably possible. The measurement of ATP levels is also useful to prove the metabolic changes they claim.

Response: We appreciate the reviewer's opinion that validation of altered OXPHOS activity in Rb1 deficient germ cells is needed. However, as we attempted to rationalize in the first revision of the manuscript, conducting this at the protein or biochemical level for germ cells in E14.5 mouse testes in a rigorous quantitative manner is extremely difficult. Respectfully, we disagree with the reviewer that measurement of mitochondria via cytometric analysis would yield interpretable data that validates or refutes the conclusion of altered OXPHOS activity made by gene expression analysis. Prospermatogonia at E14.5 in both the control and Rb1 cKO mice contain mitochondria, thus it is not a binary assessment, rather the difference indicated by gene expression analysis is OXPHOS activity. We feel that to validate the indications from gene expression, a rigorous quantitative biochemical analysis is needed which cannot be substituted for with fluorescent

probes or immunohistochemical staining which lack elements needed for true quantitation. To generate biochemical data for making measures such as ATP levels requires a number of cells that is not reasonable for the context of our experimentation. We cannot simply examine whole E14.5 testes because the measurements would include somatic and germ cells. Thus, the germ cell population needs to be separated from the somatic cell population and to provide sufficient numbers of cells for biochemical assays would require >500 E14.5 fetuses for just a single sample. We are unable to conduct this type of experimentation and unfortunately cannot satisfy the reviewer's request. Rather, we have pointed out the limitations of our analysis for altered OXPHOS activity being based on gene expression only and indicated that validation at the biochemical level is needed for concrete conclusions to be made (lines 293-295, 411-416, and abstract lines 9-12).

Comment: There is a huge leap of logic in this newly added argument (Line 430-437). What they are observing in this paper is the response of OXPHOS in an artificial environment when the Rb gene is deleted. It is not hard to imagine that the studies in the cited references share only the term "OXPHOS", but differ in the state of the cell cycle, some referring to PGCs during mitotic cell division stage, others to metabolic studies of meiosis in spermatogenic cells, and probably differ in the expression levels of many metabolic, cell cycle genes and Rb-related molecules between them. Therefore, it is clear that they are not able to be compared. Also, since this study only observed the OXPHOS-related gene expression without metabolomic analyses, the argument that "a metabolic shift occurred" is an overestimation. Also, Vega-Naredo paper did not use ES cells. They analyzed OXPHOS using P19 embryonal carcinoma cell line. Besides, a part of the information of the newly cited paper (Varuzhanyan et al.) is missing.

Response: We agree that a strong conclusion about a metabolic shift occurring during prospermatogonial development is not fully supported by gene expression analysis alone and have softened this stance in the discussion section (lines 435- 439). We now imply that inactivation of Rb1, which disrupted the normal quiescence period timeframe, led to altered expression of genes associated with OXPHOS activity and these data suggest that a shift in metabolic activity occurs during prospermatogonial development, similar to changes that occur with differentiation of other types of stem cells. Also, we have revised for accuracy how the Vega-Naredo paper is discussed (lines 430-432). The abstract has also been revised (lines 9-12) to soften the conclusion and we no longer refer to a metabolic shift occurring during prospermatogonial development, rather, we indicate that the outcome of gene expression profiling indicate that OXPHOS activity was disrupted in prospermatogonia that did not initiate quiescence on a normal timeline in development. Taken together, we feel that these revisions appropriately reflect the limitations of our study in not being able to validate alteration of metabolic processes with biochemical assays.

Comment: Fig. S6: The authors state in the text that Eno1 expression was downregulated in Rb1 cKO (Line 295-297), but Fig. S6 appears to show its upregulation. What is a functional relevance of the Eno1 behavior in prospermatogonia lacking Rb?

Response: We appreciate the reviewer for pointing out the mistake and have revised the text to reflect that Eno1 expression was upregulated in Rb1 cKO cells. The enzyme ENO1 plays a key role in glycolysis, thus the upregulation of expression for the encoding Eno1 gene supports our conclusion of altered OXPHOS activity in prospermatogonia lacking Rb1. We have revised the text (lines 296-298) to reiterate this notion.

Comment: To strengthen their study to investigate the ramifications of disrupted entry into quiescence, it might be useful to provide additional examples of meiotic genes using their Rb1-cKOBlimp1. Assessing whether multiple meiotic markers, rather than one, are affected would lead to a more accurate picture of the consequences. Can they present UMAP expression plot for some representative genes, including Stra8, to show the difference between control and cKO at E14.5?

Response: We have added a new dotplot to Figure S7 of expression for several genes associated with meiosis and referenced to it in the results section (line 305).

Comment: Again, as for the OXPHOS genes, can the authors present more information such as expression of some representative genes on UMAP feature plot, comparing control and cKO, because this is one of the major conclusions of this work?

Response: We have added a new dotplot to Figure S6 of expression for several genes associated with OXPHOS and referenced to it in the results section (line 292).

Comment: Fig. 4D: Please indicate in the legend how many genes are shown in the heatmap.

Response: We have revised the figure legend as suggested.

Comment: Fig. 5C: Please indicate in the legend how many genes are contained in each group.

Response: We have revised the figure legend as suggested.

Comment: I would suggest reporting exact p-values in addition to ** as this is more informative and is becoming a more common practice in biology.

Response: We have made this revision as suggested.

Third decision letter

MS ID#: DEVELOP/2020/194571

MS TITLE: Proper timing of a quiescence period in precursor prospermatogonia is required for stem cell pool establishment in the male germline

AUTHORS: Jon M Oatley, Guihua Du, Melissa Oatley, Nathan Law, Colton Robbins, and Xin Wu

ARTICLE TYPE: Research Article

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

Reviewer 3

Advance summary and potential significance to field

This study is informative about the role of Rb1 in the transition from the fetal to the neonatal periods.

Comments for the author

Although the authors did not validate the reduction of the OXPHOS pathways, they appropriately addressed most of my concerns. I believe the manuscript is publishable with the revised manuscript.