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Identification of TOR-responsive slow cycling neoblasts in planarians

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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 16th Apr 2020

Dear Dr. Pearson

Thank you for the submission of your research manuscript to our journal. I apologize for the delay in handling your manuscript. We have only recently received the full set of referee reports (copied below) and I have also discussed the reports further with the referees.

As you will see, the referees' opinion on your study are divided, although they actually raise very similar concerns. Neither referee 1 nor referee 3 are convinced that the identified cells are truly quiescent since these cells show sensitivity to radiation. The referees point out that these cells might rather represent a subpool of slowly cycling G1 cells. Referee 2 supports this view in his/her further feedback and referee 3 again emphasized that the conclusions on quiescence need to be toned down. Referee 1 further noted during the discussion that these smaller cells might have to grow in G1 before entering S phase, which would fit with the dependence on TOR signalling and suggested to critically revisit the data and consider alternative explanations. Regardless of whether these cells are truly quiescent or slowly cycling, both referee 2 and 3 consider the description of piwi+ cells capable of retaining BrdU after a long chase interesting and important for the field. On balance, I would therefore like to give you the chance to revise your study for EMBO reports. I am also happy to discuss the revision further.

Should you decide to embark on such a revision, please address all referee concerns in a complete point-by-point response. Acceptance of the 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 or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We invite you to submit your manuscript within three months of a request for revision. This would be July 16th in your case. Yet, given the current COVID-19 related lockdowns of laboratories, we have extended the revision time for all research manuscripts under our scooping protection to allow for the extra time required to address essential experimental issues. Please contact us to discuss the time needed and the revisions further.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section is missing.
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages
https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare

your figures.

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 (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
()

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Please add a formal "Data Availability" section (placed after Materials & Method) that follows the model below (see also <
https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>).
The Data availability section is used to provide access to primary datasets deposited in a public database. In case no such data have been generated, the Data Availability section is still required but it can state: "No primary datasets have been generated and deposited" (or similar).

Data availability

The datasets (and computer code) 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. ***

8) We would also encourage you to include the source data for figure panels that show essential

data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

9) 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 .

10) Regarding data quantification:
- Please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
IMPORTANT: Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates. If the data rely on a smaller number of replicates, scatter blots showing individual data points are recommended.
- Graphs must include a description of the bars and the error bars (s.d., s.e.m.).
- Please also include scale bars in all microscopy images.

11) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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

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

Martina Rembold, PhD
Editor
EMBO reports

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Referee #1:

This piece of work attempts to establish another new definition within the neoblast field, this time of G0 or quiescent neoblasts. Previous work from multiple sources argues strongly against quiescence, or even any population of stem cells that cycle slowly enough so as to potentially represent a distinct population in planarians. None of the data in this paper provides any evidence in support of a slow cycling population. Instead the data fits very well with TOR dependent control of G1 stem cell "growth", which is actually very interesting and the authors might want to pursue this.

1. While label retention in this case is evidence that a particular stem cell has undergone less division than most other cells, it is not clear evidence that it is quiescent, on the contrary without measuring and modelling the dilution of BrdU signal with cell divisions overtime it is not accurate to use phrases such as slow cycling in this context. Instead the authors first need to perform experiments that measure the relationship between label retention and the cell cycle. This could be done by pulsing and transplanting stem cells of a known number into irradiated hosts, and then measuring the expansion of the renewing population alongside label retention overtime. It could simply be, for example, that based on their detection assay and methods the authors are observing cells that underwent 5 cycles (BrdU+ve) or 6 or more cycles of cell division (BrdU-ve). This is not quiescence or evidence for a G0 population.
2. These experiments (suggested above) would indicate how many divisions of BrdU can still be detected in the average labelled neoblast and the distribution of divisions vs positive BrdU detection in this assay would be known. This would allow them see if two distributions of cell cycle behavior were required to explain the data, or rather one broader function described all the data.
3. The authors present double labeling of cells showing that cycling can occur to incorporate a second label, and therefore the BrdU label is in fact retained after at least one division. Rather than supporting their supposition this provides evidence against positive BrdU labelling being indicative of slow cycling as these cells have clearly divided at least once between analogue exposures. This data also demonstrates the stochastic nature of label retention that must be modelled. They do not attempt to model the nature of BrdU label loss/retention that would be required to provide more than anecdotal/potential evidence of truly slow cycling cells that could be a separate quiescent population.
4. There is no evidence double labelled cells are still neoblasts, in fact by the relative peripheral position they are likely progeny. The authors need to prove they are neoblasts not just say they are. This could be done by co-labelling with a stem cell marker, otherwise these double labelled cells should be referred to at the very least as "post-mitotic progeny cells or potentially still stem cells)
5. The data in Figure 1b is important. The authors should present all raw data points across the different animals indicating how many cells were counted in each animal. Additionally, the toxicity of BrdU needs to be assessed, so we need to know if these animals now have proportionally less piwi cells compared to untreated animals. It is also entirely possible that BrdU just slows cycling (not preventing it). The authors have the means to test this by measuring the difference between single and dual labelled animals.
6. A crucial point about these data is that at no stage is a relationship between the 2N RNA low cells and the BrdU label retaining cells established. As FACs and then BrdU detection of cells after pulse labeling is possible in planarians I can't understand why the authors didn't attempt this to establish their claims? T
7. Despite being allegedly slow cycling/quiescent these cells are in fact also radiosensitive like the fast cycling neoblast population, this does not fit with any understanding for how radiation works to target cycling cells, and in one of the main reasons why potentially slow cycling cancer cells are not

targeted by radiation.
8. The work on the smaller stem cells and the relationship with TOR is logical and interesting and the authors might wish to pursue this angle rather than try to introduce the concept of quiescent cells without proper data.
9. The work using the TSPAN+ antibody is unclear and picking just some of the number from this analysis doesn't help anyone to understand what is going. Clearly this reagent (not surprisingly) is not the goldilocks marker of pluripotent stem cells that it was originally presented as. The cell cycle kinetics of TSPAN protein detection are interesting, but to then make statements about pluripotency based of this makes no sense. The summary Figure 5 is wrong with respect to the author own data in the panels above.

Referee #2:

In this manuscript, Molinaro and colleagues present a novel analysis of planarian stem cells using BrdU/Edu and RNA-binding dyes to characterize quiescent progenitor subpopulations in planarians. They find evidence for label-retaining cells within the neoblast population and propose these slow-cycling cells are kept in reserve and activated for regeneration through Tor signaling activation. First, they track BrdU retention over time in a pulse chase experiment to find long-term label retaining piwi1+ cells that themselves are still capable of division as determined by double labeling with EdU. They reasoned that such relatively quiescent cells might have lower RNA content, and FACS purification of cells with this characteristic express factors related to quiescence. Further, they find that severe injuries like amputation cause a time-dependent increase in the size of the G0/RNA(low) cells as well as length and number of cell projections. Using nocodazole, they could chase G0 cells into G2/M arrested cells after amputation, arguing the injury-induced activation is functionally relevant for regenerative proliferation. They find that Tor inhibition prevented the cell size increase of G0/RNA(low) cells in regeneration and also the depletion of the G0/RNA(low) pool in the nocodazole chase assay. Finally, the TSPAN expressing cells G0 population after injury is enriched for TSPAN

Neoblasts are an intriguing cell type underlying the robust regeneration in planarians. Altogether, the experiments are of high quality and the message will be of considerable interest to regeneration researchers. I have only a few minor comments below for the authors to consider.

Minor comments:

Does the retained BrdU label have an approximately typical nuclear size? It looks a bit smaller in some images but might just be the

How is the RNA(low) gate set? I might be missing it, but it's not clear to me from the methods and seems to be important for the interpretation of the experiments. In some cases this RNA(low) selected region appears as a complete "branch" shape (as in Fig EV1.E) but in others looks like the bottom half of a population (as in Fig 2A). Perhaps a relief plot or plot without the gate markings

would make this more clear, but in any case more description of the method would be ideal.

Line 247: Wenemoser and Reddien 2010 also implicated Tor in proliferative activation (see supplement of that paper).

Is the cell size measurement influenced by abundance of RNA as measured by the dye staining?

At authors discretion, I would suggest modifying the title of Figure 5 and perhaps the model as well. It is very interesting that TSPAN+ cells are among the G0 cells, raising the possibility that a source of self-renewing pluripotent TSPAN+ cells are these G0 cells described here. Many of the total TSPAN+ cells are in G1, however, which could be the progeny of slow-cycling G0 TSPAN+ cells. Alternatively, it seems equally possible that most neoblast populations have a G0 cohort. Also, it is not yet clear from the literature whether the only pluripotent neoblasts are TSPAN+.

Referee #3:

This manuscript from Molinaro et al sets out to characterize subpopulations of neoblasts in planarians. Planarians have a heterogeneous stem cell population that contains pluripotent cells and organ progenitor cells. In a long sought-after result, the authors demonstrate that some piwi-1+ stem cells are capable of retaining BrdU signal after a 5-week long chase. This result is great, and confirms that not all stem cells are cycling constantly, as was suggested in an early paper from Newmark and Sanchez Alvarado.

The authors also characterize the dynamics of these cells by implementing Pyronin Y staining, which labels double-stranded RNA and can distinguish G0 from G1 cells based on RNA content, finding that Pyronin Y defines a distinct cell population of 2N cells with low RNA content. Profiling of these cells with either bulk or single-cell RNA seq revealed that they have enriched levels of cyclin G2, and decreased levels of genes required for replication and cell cycle advancement. Based on these characteristics, the authors define these cells as G0, suggesting that they are quiescent.

Functional experiments with nocodazole show that the G0 cells, while small in number as compared to the rest of the population, shift into the S/G2/M population when cell cycle progression is inhibited. This progression appears to require the activity of the TORC1 complex, which is shown very clearly with the cell size measurements used in the manuscript. Additionally, the authors attempt to correlate injury-induced cell cycle progression by measuring levels of TSPAN-1 protein, a marker for pluripotent cells. To my knowledge, the behavior of these TSPAN-1-positive cells has not been previously examined during regeneration, and unfortunately the characterization here results in an unsatisfying conclusion to the manuscript.

Overall, this manuscript beautifully identifies slow-cycling stem cells in planarians, and applies new methods that improve and refine our resolution of the cell cycle profile of this complex stem cell population. Combining these experiments with RNA-seq data is potentially a very powerful approach. However, whether this newly identified G0 population is in fact quiescent remains poorly supported by the data in the paper, mostly because these cells are lost so rapidly after radiation.

Major comments:
1. Normally, non-cycling, quiescent cells (e.g. +4 cells in the intestine) possess some degree of radioresistance because they do not divide frequently. If this newly characterized G0 population

indeed represents a "quiescent" or slowly cycling population, then it should be at least somewhat resistant to radiation at early timepoints. Instead, Figure 2A shows a rapid decline in this population, suggesting that they are rapidly susceptible to radiation induced cell death. This calls into question whether this population is truly quiescent, and suggests that it may be a subset of G1 cells. One way of resolving this could be to remove the language of "quiescence" from the description of these cells. However, analysis of other characteristics of these cells would help too. For example, demonstrating that long-term labeling with BrdU localizes to this quiescent population. Or, further characterization of cyclin G2. This could be done either with gene expression in vivo, or in FACS, or with knockdown to demonstrate the function of these "G0" cells.

2. The molecular characterization in Figure EV4, where single cells express genes that normally define specific fates, is presented very briefly. To someone not entirely familiar with how representative this data might be, it seems like a very small number of cells is used to support this claim. This data is used to support the notion that these G0 cells have unspecified fates, but it may also be possible that this signal is noise from a few selected cells in a larger data set.

3. The attempt to correlate TSPAN-1 dynamics with the G0 population is unclear. Partly this is because the numbers don't correspond between the text and the figure. But also, in Figure 5B, there is an overall 5% increase in TSPAN-1+ cells after amputation that is not replicated in Figure 5C, making it difficult to interpret how representative this data might be. Also, the molecular characterization in Figure 5D and 5E is confusing and does not illuminate a clear trend in the data.

4. The slight but reproducible increase in G0 cell size after amputation, as monitored by plasma membrane staining with CellMask, signals a cell state transition that likely correlates with cell cycle progression. The authors find that amputation also induces an overall increase in process length. The significance of these morphological characteristics is unclear because their dynamics have not been previously described during regeneration (to my knowledge). This should be acknowledged in the text.

5. For sorting experiments, throughout the paper, only one experiment is shown and there is no indication of how many experiments have been done to determine the reproducibility of the findings. This information needs to be included.

We would like to begin by thanking the reviewers for their constructive comments. We have made substantial changes to the manuscript to address their concerns. A summary of the major changes is provided below, followed by point-by-point responses to the reviewers' comments.

Summary of major changes:

- We now refer to our cells of interest as "RNA^{low}" neoblasts rather than "G0" neoblasts and have toned down the language of quiescence throughout the manuscript.
- We have extended the EdU timecourse in Figure 2C to address concerns about the relatively slow cycling nature of RNA^{low} cells.
- We have re-organized the figures to increase the clarity of data presentation. This includes demoting ancillary data to the Appendix, as well as the following major changes:
    - Additional sublethal irradiation data is provided to show gate depletion (Figure EV2).
    - Simplification of and decreased emphasis on the data relating to TSPAN-1 and lineage potential. These data have been demoted to an Extended View figure (Figure EV3).
- We have added a new Results section, "*Lrig-1 is required to restrict RNA^{low} neoblast growth at homeostasis*", to present newly generated data characterizing the function of the tumour suppressor *Lrig-1,* which is enriched in RNA^{low} neoblasts. Here we demonstrate that *Lrig-1* is required to prevent RNA^{low} neoblast growth at homeostasis and for sustained regeneration (Figure 6). We have also added a new figure to the appendix providing negative data on *cyclin G2* (Appendix Figure S5), and a new Extended View figure showing that differentiation is not affected by *Lrig-1* or *cyclin G2* knockdown (Figure EV5).
- We have updated our model figure to reflect these changes.

Below, the reviewers' comments are shown in **blue bold font** followed by our responses in black.

Referee #1:

This piece of work attempts to establish another new definition within the neoblast field, this time of G0 or quiescent neoblasts. Previous work from multiple sources argues strongly against quiescence, or even any population of stem cells that cycle slowly enough so as to potentially represent a distinct population in planarians. None of the data in this paper provides any evidence in support of a slow cycling population. Instead the data fits very well with TOR dependent control of G1 stem cell "growth", which is actually very interesting and the authors might want to pursue this.

Before addressing the specific points below, we would like to begin by addressing the reviewer's general comments above.

First, it should be stated that heterogeneity in neoblast division rates has been previously observed in other planarian species (e.g. *Girardia tigrina* in work by Saló & Baguñà, 1984) and Platyhelminthes (e.g. *Macrostomum* in work by Nimeth *et al*, 2004).

Second, to our knowledge, the only study in the molecular era to directly test the hypothesis that some planarian neoblasts may cycle slower than others was that of Newmark and Sánchez Alvarado in 2000. This foundational work, which introduced the use of BrdU to planarians, was instrumental in propelling the study of neoblasts into the molecular era, providing the first method for tracing neoblast progeny

and interrogating neoblast cell cycle dynamics. In what was the first molecular experiment to test the idea of slow cycling neoblasts in planarians, continuous labeling of neoblasts with BrdU led to the conclusion that a slow cycling neoblast population was unlikely to be a prominent population in the flatworm, as 99% of neoblasts were $BrdU^+$ after 3 days of repetitive BrdU injections. In the 20 years since this seminal work, several groups have continued to build upon this foundation by carrying the study of neoblasts through rounds of technological advancements, leading to many key discoveries including the identification of molecular markers for neoblasts (*piwi-1*) (Reddien *et al*, 2005) and the detailed description of the robust proliferative response undergone by neoblasts in response to various injury contexts (Wenemoser & Reddien, 2010). In light of this growing body of knowledge, we recognize two important caveats with respect to the interpretation of the continuous BrdU labeling experiment by Newmark and Sánchez Alvarado. First, administration of BrdU was performed by injecting animals several times per day for 3 consecutive days. This repetitive injuring and persistent presence of BrdU, a cytotoxic compound, could conceivably result in some amount of neoblast apoptosis or otherwise negatively impact the health of the neoblast compartment, which may be sufficient to activate the slow cycling population. Indeed, we find that RNA^{low} neoblasts respond very rapidly to injury (Figure 3), especially when the neoblast compartment is specifically depleted (Figure 3D). The second caveat is that molecular markers for planarian neoblasts had not been described at the time of the original work, and so neoblast identity was deduced solely by morphology. From more recent work on neoblast heterogeneity (Wagner *et al*, 2011) we can appreciate that the neoblast population marked by expression of *piwi-1* is morphologically heterogeneous. Thus, it is possible that some neoblasts may have been excluded due to their variable morphology.

In revisiting the hypothesis that some neoblasts are slow cycling, we first looked to build on the work of Newmark and Sánchez Alvarado by using BrdU to assess neoblast division rates, but in a way that would also take into consideration work from the subsequent two decades. Thus, we administered BrdU by feeding to avoid any confounding effects of injury and assessed the kinetics of BrdU label dilution specifically within neoblasts by using *piwi-1* as a marker. The fact that we detected a small number of $BrdU^+$*piwi-1*$^+$ cells even 5 weeks after administration indicated that some neoblasts dilute the label, and thus cycle, more slowly than others.

From there, we took a systematic approach to confidently identify slow-cycling neoblasts by considering conserved features of quiescent stem cells in other systems. We began by using a common FACS strategy used in other systems for isolating cells in the G0 phase to try to further resolve putative G0 neoblasts, which we now refer to as RNA^{low} neoblasts in consideration of comments from all three reviewers. These RNA^{low} neoblasts fulfilled all morphological and functional criteria typical of quiescent stem cells that we assayed. These include:

1) low transcriptional activity (Darzynkiewicz *et al*, 1980; Eddaoudi *et al*, 2018; Kim & Sederstrom, 2015). Notably, backgating revealed that these RNA^{low} cells reside in the Hoechst side population (X2 gate). This draws interesting parallels to other systems, in which quiescent multipotent adult stem cells can also be isolated from the Hoechst side population (for example, quiescent HSCs) (Golebiewska *et al*, 2011; Weksberg *et al*, 2008). Please see Figures 2A and EV1A.

2) low proliferative activity in homeostasis (assayed by both EdU label retention and expression of cell cycle genes) (Conboy *et al*, 2007; Shinin *et al*, 2006; Buczacki *et al*, 2013; Cotsarelis *et al*, 1990). Please see Figures 2C and EV1B and C.

3) classic morphology of quiescent stem cells: small size and high nucleus:cytoplasm ratio (Rumman *et al*, 2015; Li *et al*, 2015). Notably, we have added additional data (Figure 6) demonstrating that this small size is maintained by the tumour suppressor *Lrig-1*, which has known roles in maintaining quiescence in other systems (Powell *et al*, 2012; Jensen & Watt, 2006; Jensen *et al*, 2009; Simion *et al*, 2014; Nam & Capecchi, 2020). Please see Figures 2B and 6C.

4) response following depletion of the rapidly cycling stem cell pool (Daynac *et al*, 2013; Yamamoto *et al*, 2013). Please see Figures 3D and EV2.

5) characteristic TOR-dependent growth phenotype in response to injury (Conlon & Raff, 1999; Gan & DePinho, 2009; Rodgers *et al*, 2014). Please see Figure 5C-F.

6) cell cycle entry during regeneration (Cheung & Rando, 2013; Cho *et al*, 2019; Rumman *et al*, 2015). Please see Figure 5B and F.

We believe these data strongly support the presence of a relatively slow-cycling neoblast population in planarians. However, we acknowledge that we cannot formally exclude the possibility that the RNA^{low} gate represents a population of small G1 neoblasts. Thus, we have replaced the term "G0 neoblast" with "RNA^{low} neoblast", substantially weakened our language with respect to quiescence in the text, and explicitly stated this caveat in the Discussion section (line 419).

1. While label retention in this case is evidence that a particular stem cell has undergone less division than most other cells, it is not clear evidence that it is quiescent, on the contrary without measuring and modelling the dilution of BrdU signal with cell divisions overtime it is not accurate to use phrases such as slow cycling in this context. Instead the authors first need to perform experiments that measure the relationship between label retention and the cell cycle. This could be done by pulsing and transplanting stem cells of a known number into irradiated hosts, and then measuring the expansion of the renewing population alongside label retention overtime. It could simply be, for example, that based on their detection assay and methods the authors are observing cells that underwent 5 cycles (BrdU+ve) or 6 or more cycles of cell division (BrdU-ve). This is not quiescence or evidence for a G0 population.

We agree with the reviewer that the BrdU label retention data presented in Figure 1, in and of itself, is not clear evidence for a quiescent neoblast population. We would like to emphasize that the point of this experiment was to test the hypothesis that neoblast division rate is uniform. From the finding that some *piwi-1*$^{+}$ neoblasts retain the BrdU label even at 5 weeks post-pulse, we rejected this hypothesis and concluded that division rates within the neoblast population are actually variable (and much more so than previously thought). We apologize that this hypothesis was not clearly stated in our original manuscript and have added this clarification to the revised text.

To further illustrate this point, based on the example provided by the reviewer above, if all neoblasts cycled at the same rate the fraction of $BrdU^{+}$ cells would drop very quickly from its peak (after the 5^{th} division in this example) down to zero after the 6^{th} division. Instead, however, we see a very gradual loss of BrdU signal within the neoblast population, indicating that the division rate is variable. Within the same time period, some neoblasts have diluted the label beyond detection while others have not; that is to say the rate of division in some neoblasts is slower. To the reviewer's point, we are not, with these data, claiming that the neoblasts that remain $BrdU^{+}$ at 5 weeks post-pulse make up a discreet cell

population; rather, we are simply highlighting that there is a variable rate of division within the population.

2. These experiments (suggested above) would indicate how many divisions of BrdU can still be detected in the average labelled neoblast and the distribution of divisions vs positive BrdU detection in this assay would be known. This would allow them see if two distributions of cell cycle behavior were required to explain the data, or rather one broader function described all the data.

We thank the reviewer for this suggestion. For the purposes of our study, we were interested in the BrdU dilution kinetics at the population level, rather than at the level of individual neoblasts. We agree that the number of divisions required to dilute the BrdU label would be interesting to know; however, the suggested experiments are non-trivial and are outside the scope of this manuscript.

3. The authors present double labeling of cells showing that cycling can occur to incorporate a second label, and therefore the BrdU label is in fact retained after at least one division. Rather than supporting their supposition this provides evidence against positive BrdU labelling being indicative of slow cycling as these cells have clearly divided at least once between analogue exposures. This data also demonstrates the stochastic nature of label retention that must be modelled. They do not attempt to model the nature of BrdU label loss/retention that would be required to provide more than anecdotal/potential evidence of truly slow cycling cells that could be a separate quiescent population.

The purpose of our double-pulse experiment was not to show that the BrdU label is retained after at least one division, which is already known. The point, as stated in the text, was to demonstrate that the presence of BrdU does not *prevent* future division, i.e. the long-term retention of BrdU that we observe is likely not due to cell cycle arrest. From the work of Newmark & Sánchez Alvarado (2000), the current assumption in the planarian field is that 99% of neoblasts cycle every 3 days; in our experiment, we made a point to separate administration of BrdU and EdU by a much longer period (14 days) to specifically demonstrate that cells with relatively slower cell cycle rates are not arrested.

4. There is no evidence double labelled cells are still neoblasts, in fact by the relative peripheral position they are likely progeny. The authors need to prove they are neoblasts not just say they are. This could be done by co-labelling with a stem cell marker, otherwise these double labelled cells should be referred to at the very least as "post-mitotic progeny cells or potentially still stem cells)

The presence of two thymidine analogs within the same cell is definitive evidence in and of itself that the double labelled cells were, at the time of incorporation, still neoblasts. Whether these cells were still neoblasts at the time of fixation is irrelevant to our hypothesis and the interpretation thereof.

To explain further, neoblasts are known to be the only cycling cells in planarians; thus, in order for a cell to be positive for both BrdU and EdU, it *must* have been a neoblast at the time of BrdU administration and *must have remained* a neoblast at the time of EdU administration. Whether this double positive cell then goes on to differentiate is irrelevant to the point we are making from this experiment. Still, from numerous previous reports using thymidine analogs to label neoblasts in planarians (Newmark &

Sánchez Alvarado, 2000; Reddien *et al*, 2005; Eisenhoffer *et al*, 2008), it is known that the vast majority (>90%) of the analog signal is present within neoblasts at 1 day post-administration. The pattern of EdU signal in Figure 1C is consistent with detection within neoblasts. Thus, as we clearly state in the text, we concluded from this experiment that "BrdU$^+$ neoblasts are still able to proliferate at least 14 days from the initial labeling".

5. The data in Figure 1b is important. The authors should present all raw data points across the different animals indicating how many cells were counted in each animal. Additionally, the toxicity of BrdU needs to be assessed, so we need to know if these animals now have proportionally less piwi cells compared to untreated animals. It is also entirely possible that BrdU just slows cycling (not preventing it). The authors have the means to test this by measuring the difference between single and dual labelled animals.

At the reviewer's request, we have provided the source data for Figure 1B as a supplemental file (Source data – Figure 1B.xlsx). While it is possible that the incorporation of BrdU may have cytotoxic effects on neoblasts, BrdU has been used extensively in planarians and many previous reports (Eisenhoffer *et al*, 2008; Zhu & Pearson, 2018; Zhu *et al*, 2015; van Wolfswinkel *et al*, 2014; Currie *et al*, 2016; and many others, including this manuscript) have demonstrated that BrdU$^+$ neoblasts are capable of proliferating and differentiating. Nonetheless, if the BrdU was toxic, some BrdU$^+$ cells would die. Thus, if anything, the proportion of BrdU-retaining neoblasts may be underrepresented in our data.

With regards to the concern that BrdU slows cycling, our data in Figure 1B speak against this hypothesis, as we observed a sharp decline in the proportion *piwi-1*$^+$ cells that are BrdU$^+$ at early timepoints following administration, indicating that most BrdU$^+$ cells divide rapidly to dilute the label. Additionally, the label retention experiments in Figure 2C were performed using EdU instead of BrdU and we observed nearly identical dilution kinetics between the S/G2/M gate and the data in Figure 1B.

6. A crucial point about these data is that at no stage is a relationship between the 2N RNA low cells and the BrdU label retaining cells established. As FACs and then BrdU detection of cells after pulse labeling is possible in planarians I can't understand why the authors didn't attempt this to establish their claims? T

We agree with the reviewer that establishing a relationship between BrdU label retaining neoblasts and RNA^{low} neoblasts would increase the cohesiveness of the data in Figure 1 with the subsequent figures. Unfortunately, while BrdU immunostaining on dissociated cells is possible in planarians, coupling this with our FACS strategy is problematic, as the incorporation of BrdU results in quenching of the Hoechst fluorescence (Mozdziak *et al*, 2000). Thus, to address this concern we have doubled the length of the EdU label retention timecourse presented in Figure 2C, demonstrating that RNA^{low} neoblasts do not exhibit significant label dilution for at least 14 dpp.

7. Despite being allegedly slow cycling/quiescent these cells are in fact also radiosensitive like the fast cycling neoblast population, this does not fit with any understanding for how radiation works to target cycling cells, and in one of the main reasons why potentially slow cycling cancer cells are not targeted by radiation.

This is an interesting point raised by both reviewer 1 and reviewer 3. It is true that cycling cells exhibit higher sensitivity to irradiation than quiescent cells, and although all neoblasts are ablated following a lethal dose of irradiation (60 Gy), previous work has established that following a sublethal dose (12.5 Gy), some neoblasts survive and go on to repopulate the neoblast compartment (Lei *et al*, 2016). In our study, we found that RNA^{low} neoblasts exhibited a growth response following sublethal irradiation, similar to the response following amputation. In light of the reviewers' comments on this topic, we took a closer look at the kinetics of gate depletion following sublethal irradiation (data are provided in the new Figure EV2). Although the differences in the rate of depletion were not statistically significant between the RNA^{low} and other gates, there is a clear trend in the data which suggests that the RNA^{low} gate is depleted more slowly than the S/G2/M gate. The G1 gate is depleted the slowest, which is consistent with the presence of progeny cells and is in agreement with the data in Figure 2. As we now note in the Discussion, due to the rapid response by RNA^{low} neoblasts to sublethal irradiation (1 day), it is difficult to confidently assess the radio-sensitivity of this population, as both cell death and cell cycle entry are possible explanations for the observed gate depletion, and it will be interesting to pursue this topic further in future work. At present, these data are not sufficient to reject the hypothesis that RNA^{low} neoblasts are less sensitive to irradiation, and taken together with the other characteristics of RNA^{low} neoblasts that we have described in this study and summarized above (especially EdU label retention), we are confident in our description of these cells as relatively slow-cycling neoblasts.

8. The work on the smaller stem cells and the relationship with TOR is logical and interesting and the authors might wish to pursue this angle rather than try to introduce the concept of quiescent cells without proper data.

We thank the reviewer for their positive view of our data on TOR signaling as it related to RNA^{low} neoblasts.

9. The work using the TSPAN+ antibody is unclear and picking just some of the number from this analysis doesn't help anyone to understand what is going. Clearly this reagent (not surprisingly) is not the goldilocks marker of pluripotent stem cells that it was originally presented as. The cell cycle kinetics of TSPAN protein detection are interesting, but to then make statements about pluripotency

based of this makes no sense. The summary Figure 5 is wrong with respect to the author own data in the panels above.

We agree with the reviewer that the presentation of the TSPAN-1 data was confusing and apologize for the lack of clarity. Currently, very little is known about the expression profiles or markers of pluripotent neoblasts, and the recent paper by Zeng *et al* (2018) is currently the best available resource for interrogating this cell population. As such, we feel it is important to build on this report. However, due to its recent nature, there are still many open questions regarding the specificity of TSPAN-1 as a marker for pluripotency, as we have noted. Thus, we have greatly simplified our data and interpretations regarding TSPAN-1 expression. These data are now limited to providing a description of homeostatic TSPAN-1 expression within the FACS gates described in our study. These data have been moved to Figure EV3.

Referee #2:

In this manuscript, Molinaro and colleagues present a novel analysis of planarian stem cells using BrdU/Edu and RNA-binding dyes to characterize quiescent progenitor subpopulations in planarians. They find evidence for label-retaining cells within the neoblast population and propose these slow-cycling cells are kept in reserve and activated for regeneration through Tor signaling activation. First, they track BrdU retention over time in a pulse chase experiment to find long-term label retaining piwi1+ cells that themselves are still capable of division as determined by double labeling with EdU. They reasoned that such relatively quiescent cells might have lower RNA content, and FACS purification of cells with this characteristic express factors related to quiescence. Further, they find that severe injuries like amputation cause a time-dependent increase in the size of the G0/RNA(low) cells as well as length and number of cell projections. Using nocodazole, they could chase G0 cells into G2/M arrested cells after amputation, arguing the injury-induced activation is functionally relevant for regenerative proliferation. They find that Tor inhibition prevented the cell size increase of G0/RNA(low) cells in regeneration and also the depletion of the G0/RNA(low) pool in the nocodazole chase assay. Finally, the TSPAN expressing cells G0 population after injury is enriched for TSPAN

Neoblasts are an intriguing cell type underlying the robust regeneration in planarians. Altogether, the experiments are of high quality and the message will be of considerable interest to regeneration researchers. I have only a few minor comments below for the authors to consider.

We thank the reviewer for seeing the merit of our study and for the constructive comments below.

Minor comments:

Does the retained BrdU label have an approximately typical nuclear size? It looks a bit smaller in some images but might just be the

The size of the BrdU label ranges from filling the nucleus to occupying only a small nuclear area. In general, at early chase periods (e.g. 1 day), the vast majority of $BrdU^+$ cells have a BrdU signal that fills the nucleus. At later time points we observe a larger range of BrdU signal sizes, likely reflecting signal dilution due to cell division. This is the case for the EdU signal as well.

To illustrate this more clearly, we have now included example images in Figure 2D of EdU detection in dissociated cells at 1 and 14 dpp.

How is the RNA(low) gate set? I might be missing it, but it's not clear to me from the methods and seems to be important for the interpretation of the experiments. In some cases this RNA(low) selected region appears as a complete "branch" shape (as in Fig EV1.E) but in others looks like the bottom half of a population (as in Fig 2A). Perhaps a relief plot or plot without the gate markings would make this more clear, but in any case more description of the method would be ideal.

We thank the reviewer for bringing this to our attention. We have now added the following description for setting the FACS gates to the Methods section *FACS isolation of RNAlow neoblasts, TSPAN-1 staining and irradiation*:

"For all experiments, the RNA^{low} gate was set using samples from uninjured animals. Generally, the upper boundary of the RNA^{low} gate corresponded with the lower boundary of the S/G2/M gate. Additionally, lethally irradiated controls were used to confirm the boundary between the RNA^{low} gate (depleted by 1 dpi) and the G1 gate (largely still present at 1 dpi)."

Line 247: Wenemoser and Reddien 2010 also implicated Tor in proliferative activation (see supplement of that paper).

We thank the reviewer for catching this and have added the citation.

Is the cell size measurement influenced by abundance of RNA as measured by the dye staining?

No, our cell size measurements are not directly influenced by RNA abundance. For example, when comparing RNA^{low} cells from intact and regenerating animals, the same gated area (which is set based on the intact animals) is used for both samples, and yet the cell size measurements in the regenerating sample are larger.

At authors discretion, I would suggest modifying the title of Figure 5 and perhaps the model as well. It is very interesting that TSPAN+ cells are among the G0 cells, raising the possibility that a source of self-renewing pluripotent TSPAN+ cells are these G0 cells described here. Many of the total TSPAN+ cells are in G1, however, which could be the progeny of slow-cycling G0 TSPAN+ cells. Alternatively, it seems equally possible that most neoblast populations have a G0 cohort. Also, it is not yet clear from the literature whether the only pluripotent neoblasts are TSPAN+.

We agree with this assessment and in response to this suggestion, as well as comments from the other reviewers, we have decided to remove TSPAN-1 from our model and have greatly simplified the TSPAN-1 figure (now Figure EV5) to serve as a simple description of homeostatic TSPAN-1 expression in the

different FACS populations.

Referee #3:

This manuscript from Molinaro et al sets out to characterize subpopulations of neoblasts in planarians. Planarians have a heterogeneous stem cell population that contains pluripotent cells and organ progenitor cells. In a long sought-after result, the authors demonstrate that some piwi-1+ stem cells are capable of retaining BrdU signal after a 5-week long chase. This result is great, and confirms that not all stem cells are cycling constantly, as was suggested in an early paper from Newmark and Sanchez Alvarado.

The authors also characterize the dynamics of these cells by implementing Pyronin Y staining, which labels double-stranded RNA and can distinguish G0 from G1 cells based on RNA content, finding that Pyronin Y defines a distinct cell population of 2N cells with low RNA content. Profiling of these cells with either bulk or single-cell RNA seq revealed that they have enriched levels of cyclin G2, and decreased levels of genes required for replication and cell cycle advancement. Based on these characteristics, the authors define these cells as G0, suggesting that they are quiescent.

Functional experiments with nocodazole show that the G0 cells, while small in number as compared to the rest of the population, shift into the S/G2/M population when cell cycle progression is inhibited. This progression appears to require the activity of the TORC1 complex, which is shown very clearly with the cell size measurements used in the manuscript. Additionally, the authors attempt to correlate injury-induced cell cycle progression by measuring levels of TSPAN-1 protein, a marker for pluripotent cells. To my knowledge, the behavior of these TSPAN-1-positive cells has not been previously examined during regeneration, and unfortunately the characterization here results in an unsatisfying conclusion to the manuscript.

Overall, this manuscript beautifully identifies slow-cycling stem cells in planarians, and applies new methods that improve and refine our resolution of the cell cycle profile of this complex stem cell population. Combining these experiments with RNA-seq data is potentially a very powerful approach. However, whether this newly identified G0 population is in fact quiescent remains poorly supported by the data in the paper, mostly because these cells are lost so rapidly after radiation.

We thank the reviewer for seeing the merit of our study. The concerns raised by the reviewer are legitimate and highly constructive, and in addressing them we feel they have greatly strengthened our manuscript.

Major comments:

1. Normally, non-cycling, quiescent cells (e.g. +4 cells in the intestine) possess some degree of radioresistance because they do not divide frequently. If this newly characterized G0 population indeed represents a "quiescent" or slowly cycling population, then it should be at least somewhat

resistant to radiation at early timepoints. Instead, Figure 2A shows a rapid decline in this population, suggesting that they are rapidly susceptible to radiation induced cell death. This calls into question whether this population is truly quiescent, and suggests that it may be a subset of G1 cells. One way of resolving this could be to remove the language of "quiescence" from the description of these cells. However, analysis of other characteristics of these cells would help too. For example, demonstrating that long-term labeling with BrdU localizes to this quiescent population. Or, further characterization of cyclin G2. This could be done either with gene expression in vivo, or in FACS, or with knockdown to demonstrate the function of these "G0" cells.

The reviewer makes several interesting points in this comment and we thank them for their thoughtful suggestions. We have taken several steps to address these concerns, beginning with replacing the "G0 neoblast" name with "RNA^{low} neoblast" and weakening the language of quiescence throughout the manuscript.

With regards to the radioresistance concern, we direct the reviewer to our response to reviewer 1's comment #7. As we explain there, we see that RNA^{low} neoblasts undergo a growth response following sublethal irradiation, similar to the response following amputation. We have also added new data to Figure EV2, which suggest that the RNA^{low} gate is depleted more slowly than the S/G2/M gate following sublethal irradiation. Because it is difficult to say with certainty why the RNA^{low} gate becomes depleted following irradiation, we have also extended our EdU label retention timecourse in Figure 2C out to 14 days and have found that, while S/G2/M cells have significantly diluted the label during this time, RNA^{low} cells have not. Taken together, we are confident in our description of RNA^{low} cells as relatively slow-cycling neoblasts.

We also took the reviewer's suggestion to follow up on *cyclin G2* (*ccng2*) as a potential regulator of the RNA^{low} population. We also tested *Lrig-1* in these experiments, as *Lrig-1* is a known regulator of quiescent stem cells in other systems and was highly enriched in our bulk RNA-seq data from the RNA^{low} gate. While we did not observe a homeostatic phenotype following *ccng2* knockdown (Appendix Figure S5), we found that *Lrig-1* knockdown resulted in robust growth of RNA^{low} neoblasts at homeostasis (Figure 6). We also observed hyperproliferation in these animals. Interestingly, upon amputation, *Lrig-1* knockdown animals exhibited delayed regeneration. We speculate in the Discussion that these phenotypes are consistent with a failure to reserve RNA^{low} neoblasts for regeneration, and it will be interesting to test this hypothesis in future work.

2. The molecular characterization in Figure EV4, where single cells express genes that normally define specific fates, is presented very briefly. To someone not entirely familiar with how representative this data might be, it seems like a very small number of cells is used to support this claim. This data is used to support the notion that these G0 cells have unspecified fates, but it may also be possible that this signal is noise from a few selected cells in a larger data set.

We apologize for the brevity and in light of the reviewer's concerns (with this and comment #3 below) we have lessened our emphasis on the potency of RNA^{low} neoblasts. For this analysis, we used a published dataset comprised of 96 single X1 cells (i.e. cells with >2N DNA content). We chose this dataset because it was generated using the same protocol as our scRNAseq data of RNA^{low} cells. Although this dataset is quite small, we found that known markers of different lineages were largely mutually exclusive in their expression – if an X1 cell expressed markers of the neural lineage, it likely did

not also express markers of the epithelial lineage. This marker specificity has also been observed in high-throughput sequencing of neoblasts (Fincher *et al*, 2018; Zeng *et al*, 2018). However, this does not seem to be the case in RNA^{low} neoblasts, as we often observe co-expression of markers of different lineage within the same cell. Of course, due to our rather crude FACS strategy, we suspect that there is heterogeneity within the RNA^{low} gate that we have yet to appreciate, and high-throughput scRNAseq of RNA^{low} neoblasts would make for an interesting future direction.

3. The attempt to correlate TSPAN-1 dynamics with the G0 population is unclear. Partly this is because the numbers don't correspond between the text and the figure. But also, in Figure 5B, there is an overall 5% increase in TSPAN-1+ cells after amputation that is not replicated in Figure 5C, making it difficult to interpret how representative this data might be. Also, the molecular characterization in Figure 5D and 5E is confusing and does not illuminate a clear trend in the data.

We apologize for the lack of clarity and the confusion with regards to data presentation. This confusion was caused by our decision to exclude data on TSPAN-1 expression in the PM gate from the main figure and place it in the supporting EV figure. We have now ensured that all data for each experiment are presented together in the same figure to avoid similar confusion in our revised manuscript.

Overall, we agree with the reviewer that the TSPAN-1 data were confusing and difficult to interpret. As such, we have greatly simplified these data, now including only a description of homeostatic TSPAN-1 expression in Figure EV3.

4. The slight but reproducible increase in G0 cell size after amputation, as monitored by plasma membrane staining with CellMask, signals a cell state transition that likely correlates with cell cycle progression. The authors find that amputation also induces an overall increase in process length. The significance of these morphological characteristics is unclear because their dynamics have not been previously described during regeneration (to my knowledge). This should be acknowledged in the text.

To our knowledge, the reviewer is correct in saying that the extension of projections is not a common morphological response of slow cycling stem cells to injury. We have taken the reviewer's suggestion and have added a statement in the Discussion to acknowledge this point, and speculate on a potential role for these projections in cell migration (see paragraph 3 of the Discussion).

5. For sorting experiments, throughout the paper, only one experiment is shown and there is no indication of how many experiments have been done to determine the reproducibility of the findings. This information needs to be included.

We thank the reviewer for bringing this to our attention and have added statements of n values to each figure legend.

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1st Revision - Editorial Decision 26th Oct 2020

Dear Dr. Pearson

Thank you for the submission of your revised manuscript to EMBO reports. It was sent back to referee #1 and #3 and we have now received their reports (copied below).

As you can see, the referees find that the study has been significantly improved during revision and support publication after some remaining issues have been addressed. It might be advisable to focus and rewrite the manuscript to provide a clearer message and maybe a focus on TOR and to discuss the Lrig1 RNAi data in a more extensive manner. All statements on quiescence should be avoided. Please also provide point-by-point response to the remaining concerns.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

- Please add callouts to Figure 6D and to the panels of Figs EV2,4+5 panels in the text.

- Figure 4A: you have now stated that the images from the intact animals have been duplicated from Figure 2B. Please also add a statement whether the intact and amputated animals/images are from the same experiment.

- Figure S4A: please define the size of the scale bar in the legend
- Figure S4B: please define the number of animals or cells (n)
- Figure S5B, C: Please define the nature of the error bars and the statistical test used

- Table 1: Please note that we can only typeset black and white tables. Please replace the red color in Table 1 for another highlight.

- Data references: You cite Molinaro & Pearson 2016 and Fincher et al 2018 as "normal" and as "Data reference". This is in principle fine but please note that you need two different reference types in your reference list: the "Data reference" needs to refer directly to the Dataset and the standard reference refers the reader to the published manuscript.

Here is the example we listed on our Guide to Authors (see also https://www.embopress.org/page/journal/14693178/authorguide#referencesformat)

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- Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results. Please send us a

draft of this text along with the revised manuscript.

We look forward to seeing a final version of your manuscript as soon as possible.

With kind regards,

Martina Rembold

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

By focusing on interpreting the interesting experiments they have performed and by bringing them together more coherently the paper is much improved by the relatively simple adjustments made. The addition of new data certainly adds to the scope and broader interest. I appreciate the authors explaining the double labelling experiments more clearly in there response, which I think, along with the radiation data, demonstrates that the smaller slower cycling cells nonetheless behave as part of the whole population of stem cells. They do not have distinct slower cycling properties, rather they need to grow a little before they join the larger G1 population and, crossing the experimenter defined FACS gate, and dthen divide again as appropriate. The fact they respond to the extra demands of regeneration is good to know, but not surprising. I am glad that there is no need to evoke the G0 quiescence concept.

A few minor comments the authors could address in a final version.

1) Should they consider the logical argument that all stem cells require TOR for division not just the smaller ones? I am unclear why TOR signaling wouldn't drive division of the whole population?
2) Do small cells require TOR signalling to allow them to grow in G1? Could this explain the phenotypes presented more clearly?
3) They mention a link between projections, TSPAN-1 and stem cell migration. What is known about planarian stem cell migration in planarians? Do migrating cells have similar projections and are these cells small?

Referee #3:

Characterization of the activity, molecular composition, and biology of neoblasts will help to illuminate the nature of this heterogeneous cell type and hopefully attribute specific functions to subsets of these stem cells. This paper adopts a new approach to describe and characterize a newly identified subset of neoblasts. The changes made in this revised version (simplifying TSPAN, EdU time course) are helpful. However, the biological significance and possible function of this subset of neoblasts still remains somewhat unresolved. Even though the authors have removed statements claiming that RNAlow cells are quiescent, this theme is still present in the writing, and is

distracting, considering that the data supporting these cell cycle transitions are not fully supported and carried through all of the figures. Together, the message of this manuscript still comes across as fragmented, especially with the addition of the Lrig1 RNAi data, which is somewhat unsatisfying. One option would be to eliminate this data to make the emphasis on TOR more clear, and limit the scope and assumptions made in the paper (see comments below).

Fate of RNAlow cells.
The assumption the authors make is that RNAlow cells, 48 hours after injury, progress into the cell cycle. However, as the authors state, they may also be dying. If this is the case, it undermines the assumption about the cell size changes and what they mean. The authors should resolve this (either by lineage tracing) or labeling for apoptosis within the RNAlow gate.

Cell area changes.
This morphological characteristic is noted 48 hours after amputation, 1 day after sublethal radiation, and in Lrig1(RNAi) knockdowns. Although it's clear that the functional significance of this cell growth remains unknown, the expansion seen in Lrig1(RNAi) animals is an opportunity to connect it with some downstream biology. Including cell area quantification 48hrs after amputation would help to clarify and simplify the interpretation of the regeneration results.

Below, the reviewers' comments are shown in **blue bold font** followed by our responses in black.

Referee #1:

By focusing on interpreting the interesting experiments they have performed and by bringing them together more coherently the paper is much improved by the relatively simple adjustments made. The addition of new data certainly adds to the scope and broader interest. I appreciate the authors explaining the double labelling experiments more clearly in there response, which I think, along with the radiation data, demonstrates that the smaller slower cycling cells nonetheless behave as part of the whole population of stem cells. They do not have distinct slower cycling properties, rather they need to grow a little before they join the larger G1 population and, crossing the experimenter defined FACS gate, and dthen divide again as appropriate. The fact they respond to the extra demands of regeneration is good to know, but not surprising. I am glad that there is no need to evoke the G0 quiescence concept.

We would like to thank the reviewer for their helpful feedback throughout the revision process and for their positive comments toward our new data.

A few minor comments the authors could address in a final version.

1) Should they consider the logical argument that all stem cells require TOR for division not just the smaller ones? I am unclear why TOR signaling wouldn't drive division of the whole population?

Based on previous work on TOR signaling in planarians as well as the long list of roles for TOR signaling in other systems (Meng *et al*, 2018; Hine 2018; Iglesias *et al,* 2019; Iglesias *et al*, 2019; González-Estévez *et al*, 2012; Peiris *et al*, 2012; Wenemoser & Reddien, 2010; Tu *et al*, 2012), it is certainly likely that TOR signaling is involved in many aspects of planarian stem cell regulation. For the purpose of our study we chose to focus on the RNA^{low} population specifically, but we do not at any point speak against a role for TOR signaling outside of this neoblast subset; indeed, the whole worm proliferation observed following TOR knockdown suggests a broad effect across all stem cells in planarians. To clarify this point in the manuscript, we have added the following to the Discussion:

"Thus, *in addition to its known regulatory functions on the neoblast compartment as a whole*, we propose that TORC1 plays a similar role in planarians and promotes regeneration *in part* by facilitating the "activation" of RNA^{low} neoblasts."

2) Do small cells require TOR signalling to allow them to grow in G1? Could this explain the phenotypes presented more clearly?

This is indeed what we have described in our study – the small, RNA^{low} cells require TOR signaling in order to grow, and subsequently enter the cell cycle. This idea is consistent with the morphological changes associated with the previously described "G_{alert}" transition phase undergone by quiescent stem cells in various mouse tissues in response to injury (Rodgers *et al,* 2014). Additionally, we do not believe

that RNA^{low} cells are simply G1 cells that happen to be small and require TOR to get up to size, because their small size appears to be actively maintained by Lrig-1 at homeostasis.

3) They mention a link between projections, TSPAN-1 and stem cell migration. What is known about planarian stem cell migration in planarians? Do migrating cells have similar projections and are these cells small?

We thank the reviewer for this interesting question and have adjusted the text to add more about this interesting topic. From recent work using a partial irradiation approach, planarian neoblasts from shielded regions of the animal were found to extend projections and migrate into adjacent lethally irradiated regions to repopulate the stem cell compartment. This migration was associated with the extension of projections in a seminal paper by Abnave *et al,* 2017. We have added more about this work to our Discussion paragraph on migration.

Referee #3:

Characterization of the activity, molecular composition, and biology of neoblasts will help to illuminate the nature of this heterogeneous cell type and hopefully attribute specific functions to subsets of these stem cells. This paper adopts a new approach to describe and characterize a newly identified subset of neoblasts. The changes made in this revised version (simplifying TSPAN, EdU time course) are helpful. However, the biological significance and possible function of this subset of neoblasts still remains somewhat unresolved. Even though the authors have removed statements claiming that RNAlow cells are quiescent, this theme is still present in the writing, and is distracting, considering that the data supporting these cell cycle transitions are not fully supported and carried through all of the figures. Together, the message of this manuscript still comes across as fragmented, especially with the addition of the Lrig1 RNAi data, which is somewhat unsatisfying. One option would be to eliminate this data to make the emphasis on TOR more clear, and limit the scope and assumptions made in the paper (see comments below).

We would like to thank the reviewer for their constructive feedback throughout the revision process. In response to the first round of reviewer comments, we greatly weakened the language of quiescence throughout the manuscript. It is important to note, however, that the rationale behind many of the experiments we performed was based heavily on testing the hypothesis that the RNA^{low} neoblasts may be a relatively quiescent population in planarians that utilize the conserved quiescence regulators TOR and Lrig-1. As such, it is not possible to outright remove all mentions of quiescence in the manuscript, though we have made every effort to present the data with unbiased language and clearly state that, while these cells have many conserved characteristics of quiescent stem cells, much work will be required to test this hypothesis in detail and uncover the mechanisms of their regulation. As this is a first report on this population of RNA^{low} neoblasts, we agree with the reviewer that many interesting questions regarding the specific functions of these cells in planarian regeneration remain to be answered, and we look forward to doing so in the future.

Fate of RNAlow cells.

The assumption the authors make is that RNAlow cells, 48 hours after injury, progress into the cell cycle. However, as the authors state, they may also be dying. If this is the case, it undermines the assumption about the cell size changes and what they mean. The authors should resolve this (either by lineage tracing) or labeling for apoptosis within the RNAlow gate.

To clarify, our comment that these cells may be dying was made specifically in reference to experiments involving irradiation. As we present in Figure 2A, the RNA^{low} gate is sensitive to a lethal dose of irradiation (60 Gy), as are all neoblasts. Following a sub-lethal dose of irradiation (12.5 Gy), we observed a slower depletion of the RNA^{low} gate (Figure EV2), and we also found that RNA^{low} neoblasts underwent a cell growth response in this context (Figure 3D). This growth response is analogous to that observed following amputation. Using NDZ cell cycle arrest experiments, we showed that the RNA^{low} cell growth response observed during regeneration is correlated with cell cycle entry (Figure 5B). Thus, one explanation for the depletion of the RNA^{low} gate following sub-lethal irradiation is that these cells have entered the cell cycle (thus moving into the G1 or S/G2/M gates). However, given the very rapid depletion of the RNA^{low} gate following lethal irradiation, presumably by cell death, it remains equally possible that RNA^{low} neoblasts are radio-sensitive at sub-lethal doses as well.

Outside of these irradiation experiments, we have not observed any evidence that RNA^{low} cells (or any large subset of neoblasts) die during homeostasis or regeneration. Indeed, the response of the neoblast population to injury has been extensively studied, and mass apoptosis specifically within this population has not been reported. Our NDZ experiment in *TOR(RNAi)* animals also supports that the response by RNA^{low} neoblasts at 48 hpa is not a result of cell death, as the RNA^{low} gate does not become depleted in this condition (Figure 5F). We have added a sentence to the Results section *RNA^{low} neoblasts enter mitosis in a TOR-dependent manner* to highlight this point.

Cell area changes.
This morphological characteristic is noted 48 hours after amputation, 1 day after sublethal radiation, and in Lrig1(RNAi) knockdowns. Although it's clear that the functional significance of this cell growth remains unknown, the expansion seen in Lrig1(RNAi) animals is an opportunity to connect it with some downstream biology. Including cell area quantification 48hrs after amputation would help to clarify and simplify the interpretation of the regeneration results.

We agree that following up on the *Lrig-1(RNAi)* phenotype is an interesting area of study and we are excited to do so in future work. Thus far, the data we provide implicates Lrig-1 in actively maintaining the small size of RNA^{low} neoblasts at homeostasis and demonstrates a role for Lrig-1 in regeneration; however, numerous questions remain. For example, what is the mechanism by which Lrig-1 restricts RNA^{low} neoblast size? Does Lrig-1 act exclusively on RNA^{low} neoblasts or does it function more broadly? Is the *Lrig-1(RNAi)* regeneration phenotype a direct consequence of Lrig-1's function within RNA^{low} neoblasts? Does the regeneration phenotype continue to worsen with additional rounds of regeneration? Could this be a result of stem cell aging in *Lrig-1(RNAi)* planarians? For the current study, we included the Lrig-1 data to demonstrate the utility of our FACS approach and RNAseq datasets for discovering interesting candidates involved in neoblast regulation; however, a more detailed study of the mechanisms of Lrig-1's role in RNA^{low} neoblast regulation and regeneration is outside the scope of this manuscript.

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2nd Revision - Editorial Decision 14th Dec 2020

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

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