Cardiomyocyte ploidy is dynamic during postnatal development and varies across genetic backgrounds

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MS TITLE: Cardiomyocyte ploidy is dynamic during postnatal development and varies across genetic backgrounds

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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. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

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

Swift et al compared cardiomyocyte numbers and ploidy classes in C57BL/6J and A/J strains and found that cardiomyocyte numbers plateau after the 1st postnatal week in B6 mice, but unexpectedly increase in A/J mice at 4-6 weeks. This unexpected rise in numbers was accompanied by an increase in the MNDC percentage between 3-4 wks. Corresponding to this time frame, the authors did not detect an increase in EdU incorporation in A/J mice following a single EdU injection, and suggest that there may even be a decrease in EdU incorporation compared to B6 mice when using a serial EdU injection strategy. These data, in conjunction, with a calculation that 8N cardiomyocytes decrease in numbers over this time frame are used to infer that "ploidy reversal" led to the increase in cardiomyocyte numbers.
Mechanistically, the authors show that Tnni3k deletion partially recapitulates ploidy dynamics of A/J mice. The authors also reanalyze their prior mapping data from the collaborative cross to identify Runx1 as a potential regulator of ploidy states, with supportive functional data in a Runx1 overexpression micee. Overall this paper is quite interesting and suggests new concepts about the timing and route for postnatal cardiomyocyte proliferation that could be quite important to regenerative biology. However, concepts like ploidy reversal are inferred rather than directly shown and are based on significant, but marginally so, results from experiments that are notorious for high levels of variability (dissociation and counting of cardiomyocyte numbers, ploidy determination based on DAPI intensity etc). Impact is limited because the phenomenon of ploidy modification is not related back to regenerative capacity, which was the premise for studying cardiomyocyte ploidy dynamics.

Comments for the author

Major comments

1. The major finding is a capacity for "polyploid" reversal in cardiomyocytes from A/J mice. These conclusions are indirect, based on a nominal difference in extrapolated changes of 8N cardiomyocytes (Fig 2G, H) and a relative absence of EdU labeling (Fig 1F, 2D). Without a more direct or orthogonal method to show ploidy reversal (see PMID 31866222), these conclusions are more suggestive. At a minimum, the authors should be more cautious in their interpretation of the data.

2. Have the authors really ruled out a round of proliferation between P21-P28 to account for changes in cardiomyocyte numbers? A daily EdU labeling strategy at this age range, followed by a ploidy analysis would be more convincing.

3. The significance of ploidy reversal is suggested to be related to regenerative capacity, but no data is shown regarding regenerative capacity or injury-induced cardiomyocyte proliferation in P21 A/J mice or with Runx1 overexpression. Note that an increase in injury induced cardiomyocyte cycling was shown for Tnni3k-/- mice in the Patterson et al, Nature Genetics manuscript.

4. I don't think the scRNA-seq data adds much to the manuscript. To this reviewer this data raises more questions than it answers. For example, how did the authors do doublet discrimination for scRNA-seq data on polyploid cells? Cluster 10 has endothelial markers, like Erg – how is this explained? The relationship of CM clusters identified here to CM4 does not seem robust. The major intent was to identify a signaling network that underlies ploidy dynamics. Thus, the scRNA-seq should, at a minimum, have histologic examples of Runx1, Tead, Erg, and Ki67 colocalization in cardiomyocytes.

Minor comments.
1. The number of time points used, differing labeling protocols, and strains make this a very confusing paper. Please make sure to label each panel with the time point and strain used.

2. Several typos regarding strain name, Edu instead of EdU throughout

3. Stacked bar charts are hard to interpret. A more effective method for data presentation should be considered.

4. Conclusions about Runx1 and Ki67 are difficult to understand with such few cells per field in fig 4D-J.

5. “Classic” staining of PCM1+ nuclei shown is not classic (or at least too low mag to assess) and data supporting ploidy bias with PCM1 labeling is not very strong. Unclear why the authors even mention this point, as their approach for sc-RNASeq is reasonable as is.

Reviewer 2

Advance summary and potential significance to field

It was previously shown that cardiomyocytes are heterogeneous in their ploidy and this affects their capacity to proliferate during heart regeneration or after myocardial infarction. The authors investigated how development of cardiomyocytes affects their ploidy in different genetic backgrounds: C57Bl/6J and A/J.
Interestingly, animals from these two strains are born and reach adulthood with similar numbers of cardiomyocytes but their ploidy and developmental progression differ significantly. The authors compared the cardiomyocytes in C57Bl/6J and A/J at different time points after birth and showed that cardiomyocytes in A/J display a dynamic pattern with an increase in the mononucleated diploid cardiomyocyte pool via ploidy reversal of polyploid cardiomyocytes finishing cytokinesis. In the second half of the manuscript, the authors returned to their previous GWAS studies using the hybrid mouse diversity panel and found a locus on chromosome 16 associated with the frequency of mononuclear cardiomyocytes polymorphisms between the C57Bl/6J and A/J strains. They further used a candidate approach and discovered that Runx1 overexpression is sufficient to induce cytokinesis and increase the expansion of mononucleated diploid cardiomyocytes. They also provided single nuclei RNAseq data to compare cardiomyocytes from C57Bl/6J and A/J mice.
This work is highly significant and their data are of high quality.

Comments for the author

This manuscript is nicely written and was a pleasure to read although more details in the description might help with interpretation of the data.

1. The authors stated that only litters with 3-8 pups were used. For Figure 1 and supplemental Table 1, are some data (N=6-8) using pups from the same/one litter? Is so, are these data representative? Furthermore, how does litter size affect the development of the pups and does this contribute to the potential difference? The authors do not have to repeat the experiments but should indicate the number of litters and litter sizes in addition to animal numbers.

2. For the heart weight/body weight ratio in supplemental Figure 1 the authors should consider adding body weight data in the chart. The heart weight of C57Bl/6J and A/J seems the same but C57Bl/6J has higher heart weight/body weight ratio at P7. Does this indicate C57Bl/6J pups are smaller? The authors indicated that N=3-17 animals. Are the animals from the same litters and how many litters are included?

3. It seems that some data points in Figure 1, 2,3, 4 and supplemental Figure 2 have large variation. The authors might consider scoring the data from males and females separately for P21, 4 and 6 weeks sample, especially since the sex should be easily distinguished at these stages.

4. In Figure 5D, the authors showed genes associated with different cardiomyocyte clusters. However, many are not cardiomyocyte genes. The authors also stated that cluster 9 has more genes associated with leukocyte lineages and may represent a cluster of doublets. This raises concerns whether the single nuclei RNAseq and data analyses were performed properly. Otherwise, these datasets can be very useful resources.

5. The authors should provide cluster markers for their datasets and differentially expressed genes as supplemental data in Excel files.
6. The authors should show the clusters where Runx1 and Tinn3k are expressed as Feature plots in the CM clusters unless they are not detected due to the sequencing depth. If so, the authors can also state it.

Minor comments.
1. The authors discussed the difference between their results vs the data from Naqvi et al (2014). They should note that Naqvi only used male mice in their studies.

2. cTnT staining is not visible in Fig. 1E.

Reviewer 3

Advance summary and potential significance to field

The

Comments for the author

This manuscript describes an interesting observation that certain genetic strains of mice apparently increase their fraction of mononuclear diploid cardiomyocytes after birth and up to weeks after DNA synthesis has been completed. Based on prior data from the senior author and a reanalysis of the mouse hybrid diversity panel they identify 2 loci that are likely relevant for determining the fraction of mononuclear diploid cardiomyocytes. Based on genetic data, they identified Tnni3k as the main contributor to this phenotype and show that when Tnni3k is deleted from C57Bl/6j mice this increases the fraction of mononuclear diploid cardiomyocytes. The second locus contains a gene, Runx1, that the authors imply to have an effect as well. They overexpress Runx1 in C57Bl/6j cardiomyocytes and show that this indeed enhances the fraction of mononuclear diploid cardiomyocytes suggesting this gene contained within the second locus might indeed be responsible. Finally, the authors perform single nuc RNA sequencing of C57Bl/6j and A/J mice to identify clusters of cardiomyocytes that might be responsible for the delayed cytokinesis. They identified 1 cluster that is derived from A/J mice that shows a similar pattern of enhanced gene expression as a prior publication.
When analyzing the upstream regulators likely activated in this cluster, they identify Tead1, which is a Yap target.

This is an innovative paper that identifies a potentially interesting and important phenomenon, namely that cardiomyocytes can undergo delayed cytokinesis and that this can give rise to an increase in cardiomyocytes. The main message of the manuscript is that cardiomyocytes can have separation in time from completion of DNA synthesis to mitosis to cytokinesis, and the authors identify 2 potentially important regulators of this process.

Although of interest, below are some points that could further strengthen the paper:
1. The main weakness of the paper is that there is no direct evidence of delayed cytokinesis, and that the quantifications are based on reasonable, but correlative data. Furthermore, there is no clear evidence or mechanism for how Tnni3k or Runx1 regulate cytokinesis. In vitro experiments that show direct regulation of cytokinesis of cardiomyocytes would strengthen the manuscript.
2. Runx1 is a marker of poor outcome after myocardial infarction and hypertrophy (PMID: 34190420, 29030345). Clearly, Runx1 expression is increased in cardiomyocytes after injury, but this is associated with poor outcome. There are a number of important questions to answer related to this: 1) Is Runx1 expression after MI sufficient to induce cytokinesis and result in an increase in cardiomyocyte numbers? 2) Is there a specific time-window within which Runx1 overexpression will result in delayed cytokinesis? 3) can you perform cell culture experiments to better characterize the role of Runx1 expression in the regulation of cytokinesis.
3. It appears that the experiments in Figure 4I-4K are underpowered (especially panel K). Can the authors add additional experiments to ensure that the results presented are reliable and correct?
4. To better connect the Tnni3k and Runx1 results to the single-nuc RNA sequencing results, can the authors test whether manipulation of Tnni3k/Runx1 leads to gene expression observed in cluster 10?
5. Do Tnni3k deletion and Runx1 overexpression have additive effects?

First revision

Author response to reviewers' comments

We thank all the reviewers for their helpful comments and timely reviews of our manuscript. We have now addressed all the concerns raised by reviewers to the best of our ability. Some of the major changes we made include:

- softening of language surround “ploidy reversal” and changing our terminology from “cytokinesis” to “completion of cell division”
- Removal of the single nucleus RNA-seq data
- Addition of a “regeneration” experiment for *Runx1* overexpression with function, scar, and cardiomyocyte proliferation all assessed.

Additional ways in which we have addressed the concerns are discussed below, in line with each comment. Finally, because the single nucleus RNA-seq data have been removed, some changes to authorship were made.

Reviewer 1 Advance Summary and Potential Significance to Field:

Swift et al compared cardiomyocyte numbers and ploidy classes in C57BL/6J and A/J strains and found that cardiomyocyte numbers plateau after the 1st postnatal week in B6 mice, but unexpectedly increase in A/J mice at 4-6 weeks. This unexpected rise in numbers was accompanied by an increase in the MNDC percentage between 3-4 wks. Corresponding to this time frame, the authors did not detect an increase in EdU incorporation in A/J mice following a single EdU injection, and suggest that there may even be a decrease in EdU incorporation compared to B6 mice when using a serial EdU injection strategy. These data, in conjunction, with a calculation that 8N cardiomyocytes decrease in numbers over this time frame are used to infer that “ploidy reversal” led to the increase in cardiomyocyte numbers. Mechanistically, the authors show that Tnni3k deletion partially recapitulates ploidy dynamics of A/J mice. The authors also reanalyze their prior mapping data from the collaborative cross to identify Runx1 as a potential regulator of ploidy states, with supportive functional data in a Runx1 overexpression micee. Overall, this paper is quite interesting and suggests new concepts about the timing and route for postnatal cardiomyocyte proliferation that could be quite important to regenerative biology. However, concepts like ploidy reversal are inferred rather than directly shown and are based on significant, but marginally so, results from experiments that are notorious for high levels of variability (dissociation and counting of cardiomyocyte numbers, ploidy determination based on DAPI intensity, etc). Impact is limited because the phenomenon of ploidy modification is not related back to regenerative capacity, which was the premise for studying cardiomyocyte ploidy dynamics.

We appreciate Reviewer 1’s intrigue for the striking developmental phenotype we uncovered. We also agree with the reviewer that there is/was an inference to myocardial regeneration and so have now added a major MI study to the manuscript. Please see new Figure 5.

Reviewer 1 Comments for the Author:

Major comments
The major finding is a capacity for “polyploid” reversal in cardiomyocytes from A/J mice. These conclusions are indirect, based on a nominal difference in extrapolated changes of 8N cardiomyocytes (Fig 2G, H) and a relative absence of EdU labeling (Fig 1F, 2D). Without a more direct or orthogonal method to show ploidy reversal (see PMID 31866222), these conclusions are more suggestive. At a minimum, the authors should be more cautious in their interpretation of the data.

We agree with the reviewer. While we believe the cell division experiment offered in Figures 2B- F are quite strong for demonstrating a delayed completion of cell division, the subsequent conclusion that these diploid CMs come from a polyploid source is less direct. We have removed any claims of “ploidy reversal” from the results section, softened our conclusions there, and raised this solely as a possible explanation within the discussion. The only mention of “ploidy reversal” can now be found in lines 321-326.

Have the authors really ruled out a round of proliferation between P21-P28 to account for changes in cardiomyocyte numbers? A daily EdU labeling strategy at this age range, followed by a ploidy analysis would be more convincing.

We understand the reviewer's concern. We have attempted two additional strategies to see if we were missing a regional population by our original 2-chamber analysis: 1) we did EdU injections and instead sectioned by 4-chamber view; and 2) we did as you suggest and looked for EdU-positive cardiomyocytes by single cell suspension. In both additional attempts we only find rare cardiomyocytes which mathematically could not possibly explain the increase in MNDCM population. We've now added language to the manuscript (see Lines 138-141).

One remaining possibility that would explain an increase in CM number in the absence of EdU incorporation is our EdU-administration protocol. We only performed EdU administrations during normal work hours. If a "burst" of proliferation occurs overnight, it is possible we would miss it. We have addressed this possibility in the discussion (see lines 336-338).

The significance of ploidy reversal is suggested to be related to regenerative capacity, but no data is shown regarding regenerative capacity or injury-induced cardiomyocyte proliferation in P21 A/J mice or with Runx1 overexpression. Note that an increase in injury induced cardiomyocyte cycling was shown for Tnni3k-/- mice in the Patterson et al, Nature Genetics manuscript.

We agree with the reviewer about the inference our data raise for myocardial regeneration. Both A/J and Tnni3k were tested in the Nature Genetics paper where both showed enhanced CM cell division after MI. To address this concern, we have added an MI study comparing Myh6- CreER+ controls to Myh6-CreER+;Runx1 overexpression mice. We demonstrate that following MI adult Myh6-CreER+;Runx1 overexpression mice display: 1) transient improvements in cardiac function; 2) transient reduction scar size; 3) increased CM cell cycle at both 14- and 28- days post MI; and 4) increased completion of CM cell division as quantified by our single cell methodology. We have added all these data to a new Figure 5. Also, see lines 280-306 and 389-400 addressing these results within the text of the manuscript.

I don't think the scRNA-seq data adds much to the manuscript. To this reviewer, this data raises more questions than it answers. For example, how did the authors do doublet discrimination for scRNA-seq data on polyploid cells? Cluster 10 has endothelial markers, like Erg – how is this explained? The relationship of CM clusters identified here to CM4 does not seem robust. The major intent was to identify a signaling network that underlies ploidy dynamics. Thus, the scRNA-seq should, at a minimum, have histologic examples of Runx1, Tead, Erg, and Ki67 colocalization in cardiomyocytes.

We appreciate the reviewer's feedback that this experiment did not "[add] much to the manuscript." As a result, we have now removed the single nucleus RNA-seq (sn-RNAseq) data from this manuscript.

Minor comments.

1. The number of time points used, differing labeling protocols, and strains make this a very confusing paper. Please make sure to label each panel with the time point and strain used.

Very sorry for the confusion. We have added labels to all panels and ensured our axis and figure legends are clear.

2. Several typos regarding strain name, Edu instead of EdU throughout

We have fixed these errors. Thank you.

3. Stacked bar charts are hard to interpret. A more effective method for data presentation should be considered.

While other methods for data presentation might be easier to interpret in some cases, we believe our use of stacked bar charts is necessary to comprehensively interpret shifts in CM

nucleation/ploidy classes. Where visualization of individual populations are both necessary and helpful, we do present those data with separate, additional panels. For example, all ploidy classes are presented in Figure 1C as a stacked bar chart, but just the MNDCM (1X2N) population is presented in the next panel, Figure 1D. Similarly, the somewhat confusing panel 2G is accompanied by 2H where we quantify the change in CM number from 3-4 weeks for our readers. We believe this is the most thorough and honest presentation of our data. We welcome recommendations from the reviewer, if they have specific alternative ideas for presenting these data.

4. Conclusions about Runx1 and Ki67 are difficult to understand with such few cells per field in fig 4D-J.

Admittedly, Runx1-positive cardiomyocytes are a rare cell. We have reduced the results to two simplified graphs (4E and 4F) and reciprocally simplified the conclusions we draw from it.

5. "Classic" staining of PCM1+ nuclei shown is not classic (or at least too low mag to assess) and data supporting ploidy bias with PCM1 labeling is not very strong. Unclear why the authors even mention this point, as their approach for sc-RNASeq is reasonable as is.

Because the sn-RNAseq data have been removed, we have also removed this detail.

Reviewer 2 Advance Summary and Potential Significance to Field:
It was previously shown that cardiomyocytes are heterogeneous in their ploidy and this affects their capacity to proliferate during heart regeneration or after myocardial infarction. The authors investigated how development of cardiomyocytes affects their ploidy in different genetic backgrounds: C57Bl/6J and A/J. Interestingly, animals from these two strains are born and reach adulthood with similar numbers of cardiomyocytes but their ploidy and developmental progression differ significantly. The authors compared the cardiomyocytes in C57Bl/6J and A/J at different time points after birth and showed that cardiomyocytes in A/J display a dynamic pattern with an increase in the mononucleated diploid cardiomyocyte pool via ploidy reversal of polyploid cardiomyocytes finishing cytokinesis. In the second half of the manuscript, the authors returned to their previous GWAS studies using the hybrid mouse diversity panel and found a locus on chromosome 16 associated with the frequency of mononuclear cardiomyocytes polymorphisms between the C57Bl/6J and A/J strains. They further used a candidate approach and discovered that Runx1 overexpression is sufficient to induce cytokinesis and increase the expansion of mononucleated diploid cardiomyocytes. They also provided single nuclei RNAseq data to compare cardiomyocytes from C57Bl/6J and A/J mice. This work is highly significant and their data are of high quality.

We appreciate the reviewer's overall enthusiasm for our study and recognition for the rigor of our approach. Thank you.

Reviewer 2 Comments for the Author:
This manuscript is nicely written and was a pleasure to read although more details in the description might help with interpretation of the data.

1. The authors stated that only litters with 3-8 pups were used. For Figure 1 and supplemental Table 1, are some data (N=6-8) using pups from the same/one litter? Is so, are these data representative? Furthermore, how does litter size affect the development of the pups and does this contribute to the potential difference? The authors do not have to repeat the experiments but should indicate the number of litters and litter sizes in addition to animal numbers.

In Figure 1, most experiments are comprised of multiple litters. Thus, conclusions have not been drawn from a single aberrant litter. Regarding litter size, we were concerned that litter size would impact postnatal growth parameters; thus, we attempted to control this as best possible, while still accommodating the fact that A/J mice are known to be poor breeders due to a hypoproduction of eggs by some females. In our re-evaluation of all experiments for the sake of answering this question, we did come across two occasions when a litter of 9 and 10 were used. In both cases, some animals were removed at very early time points (P1 and P5, respectively). Thus, animals from these litters that were used at later postnatal time points were raised within the

indicated parameters. Nevertheless, we have updated our methods section to reflect these new numbers (please see line 426). We have run some statistics looking at data that were derived from "small" litters (3-4 pups) versus "larger" litters (8-10 pups), and we did not detect any notable relationships between the size of litters and the parameters we measured.

To address this concern for the benefit of our readers, we have now added an additional Supplemental Table referenced in the methods section indicating the N, number of litters from which the data were derived, and number of pups in each litter. Please see lines 430-432 and Supplemental Table 3.

For the heart weight/body weight ratio in supplemental Figure 1 the authors should consider adding body weight data in the chart. The heart weight of C57Bl/6J and A/J seems the same but C57Bl/6J has higher heart weight/body weight ratio at P7. Does this indicate C57Bl/6J pups are smaller? The authors indicated that N=3-17 animals. Are the animals from the same litters and how many litters are included?

Thank you for the suggestion; we have now added a separate graph for each of these three phenotypes, which are now visible in Supplemental Figure 1. As reviewer 2 correctly predicted, C57BL/6J pups are nominally smaller at P7 and their hearts are weighing in as nominally larger. Thus, the HW/BW does appear different at this timepoint, but this difference is not maintained at later time points. As above, multiple litters contribute to the HW and BW measurements. The total N and number of litters for each genotype at each time point are now easily viewable in Supplemental Table 3.

2. It seems that some data points in Figure 1, 2,3, 4 and supplemental Figure 2 have large variation. The authors might consider scoring the data from males and females separately for P21, 4 and 6 weeks sample, especially since the sex should be easily distinguished at these stages.

We have evaluated each of these phenotypes taking sex into account. Aside from heart weight and body weight differences in young adults (≥6 weeks) we do not observe any overt differences of any phenotype across the sexes in these strains. Notably, even heart weight and body weight are not different at ≤4 weeks. We have now clarified this in the methods section – see lines 427-430.

3. In Figure 5D, the authors showed genes associated with different cardiomyocyte clusters. However, many are not cardiomyocyte genes. The authors also stated that cluster 9 has more genes associated with leukocyte lineages and may represent a cluster of doublets. This raises concerns whether the single nuclei RNAseq and data analyses were performed properly. Otherwise, these datasets can be very useful resources.

Per the suggestion of Reviewer 1, we have now removed the sn-RNAseq. We will be certain to consider this comment when we pursue publication of these data.

4. The authors should provide cluster markers for their datasets and differentially expressed genes as supplemental data in Excel files.

Same as above.

5. The authors should show the clusters where Runx1 and Tinn3k are expressed as Feature plots in the CM clusters unless they are not detected due to the sequencing depth. If so, the authors can also state it.

Same as above.

Minor comments.
1. The authors discussed the difference between their results vs the data from Naqvi et al (2014). They should note that Naqvi only used male mice in their studies.

We have now added this to the discussion (line 335).

2. cTnT staining is not visible in Fig. 1E.

We have made improvements to the picture to address this.

Reviewer 3 Advance Summary and Potential Significance to Field:
The

Reviewer 3 Comments for the Author:
This manuscript describes an interesting observation that certain genetic strains of mice apparently increase their fraction of mononuclear diploid cardiomyocytes after birth and up to weeks after DNA synthesis has been completed. Based on prior data from the senior author and a reanalysis of the mouse hybrid diversity panel, they identify 2 loci that are likely relevant for determining the fraction of mononuclear diploid cardiomyocytes. Based on genetic data, they identified Tnni3k as the main contributor to this phenotype and show that when Tnni3k is deleted from C57Bl/6j mice this increases the fraction of mononuclear diploid cardiomyocytes.
The second locus contains a gene, Runx1, that the authors imply to have an effect as well. They overexpress Runx1 in C57Bl/6j cardiomyocytes and show that this indeed enhances the fraction of mononuclear diploid cardiomyocytes, suggesting this gene contained within the second locus might indeed be responsible. Finally, the authors perform single nuc RNA sequencing of C57Bl/6j and A/J mice to identify clusters of cardiomyocytes that might be responsible for the delayed cytokinesis. They identified 1 cluster that is derived from A/J mice that shows a similar pattern of enhanced gene expression as a prior publication. When analyzing the upstream regulators likely activated in this cluster, they identify Tead1, which is a Yap target.

This is an innovative paper that identifies a potentially interesting and important phenomenon, namely that cardiomyocytes can undergo delayed cytokinesis, and that this can give rise to an increase in cardiomyocytes. The main message of the manuscript is that cardiomyocytes can have separation in time from completion of DNA synthesis to mitosis to cytokinesis, and the authors identify 2 potentially important regulators of this process. Although of interest, below are some points that could further strengthen the paper:

We thank the reviewer for their general support and appreciation of the innovation and striking phenomenon.

1. The main weakness of the paper is that there is no direct evidence of delayed cytokinesis, and that the quantifications are based on reasonable, but correlative data. Furthermore, there is no clear evidence or mechanism for how Tnni3k or Runx1 regulate cytokinesis. In vitro experiments that show direct regulation of cytokinesis of cardiomyocytes would strengthen the manuscript.

We recognize that our single cell suspension methodology for assessing cell division is retrospective in nature, but it is definitive – the only way a EdU+ cell can be both mononuclear and diploid is if complete cell division has taken place. Catching the actual transient event of cytokinesis or abscission is very difficult, even with some of the *in vitro* methodologies offered by other groups. We did try some *in vitro* experiments with isolated P1 CMs from wildtype and RunxOE mice, both with and without Nrg1 stimulation. Our output was looking for Aurora Kinase B stain on fixed cells, and we had trouble identifying sufficient Aurora Kinase B localized to the cleavage furrow in any condition to comfortably draw a conclusion. There are many reasons such experiments may not have "worked" – they are technically challenging, mouse CMs are more difficult to culture than rat, inappropriate time point, *in vitro* may not recapitulate what we see *in vivo*, etc. Unfortunately, we are not equipped to perform video microscopy on live cultures in this lab, and we are concerned that these challenging experiments are not the best way to approach the question anyway.

With this said, this concern by the reviewer brings to our attention an error on our side. We do not think, nor do we test, if these molecules (Tnni3k nor Runx1) play a direct role on the process of "cytokinesis" per say. However, our choice of language in the previous version insinuated exactly this. With the substantial data we have provided, all we can claim is that cell division has taken

place. To address this concern, we have changed our language throughout the manuscript. We now say “completion of cell division” rather than “cytokinesis”. We have highlighted every instance in the manuscript where our language has been adjusted, which we hope the reviewer agrees more appropriately fits the conclusions we can carefully draw from the experiments we performed without overstating our findings or inferring cytokinesis where it has not been directly tested.

2. Runx1 is a marker of poor outcome after myocardial infarction and hypertrophy (PMID: 34190420, 29030345). Clearly, Runx1 expression is increased in cardiomyocytes after injury, but this is associated with poor outcome. There are a number of important questions to answer related to this: 1) Is Runx1 expression after MI sufficient to induce cytokinesis and result in an increase in cardiomyocyte numbers? 2) Is there a specific time-window within which Runx1 overexpression will result in delayed cytokinesis? 3) can you perform cell culture experiments to better characterize the role of Runx1 expression in the regulation of cytokinesis.

Thank you for these questions and suggestions regarding Runx1. We will address each individually:
1) Yes, Runx1 induction after MI is sufficient to increase completion of cell division in CMs. We now provide a full MI study in response to both this question and concerns raised by Reviewer 1. Please see Figure 5 for all MI results, and panel 5J for the assessment of cell division after MI by our single cell suspension method. Assessing CM number in a fibrotic scenario presents many additional challenges and concerns, therefore we have not attempted to do this, nor do we see data such as these being regularly offered by the field (in post-MI studies, specifically).
2) We believe the added MI study performed on adult mice from 8-10 weeks of age also addresses your question about a time window. Yes, Runx1 is sufficient in other time windows. Of course, this does not exhaust the possibility that Runx1 might be insufficient on its own in an untested scenario.
3) Finally, regarding *in vitro* experiments, this is exactly the experiment we attempted above in response to your first question.

McCarroll et al. (PMID 2903345) showed deletion of *Runx1* improved function post-MI; that is not to say that overexpression must have the opposite result. Considering Runx1 is a transcriptional regulator, perhaps CM overexpression or depletion exerts phenotypic effects via different mechanisms, both resulting in beneficial phenotypes. We try to address this responsibly in our discussion (See lines 389-400).

3. It appears that the experiments in Figure 4I-4K are underpowered (especially panel K). Can the authors add additional experiments to ensure that the results presented are reliable and correct?

We have now added N to these experiments, and former panel 4K (now 4J) has now reached statistical significance. With Runx1 overexpression we see more MNDCMs (Fig 4H), we see more CMs that entered the cell cycle (i.e. are EdU+) completing cell division (Fig 4I), and we see an increase in total CMs (Fig 4J).

4. To better connect the Tnni3k and Runx1 results to the single-nuc RNA sequencing results, can the authors test whether manipulation of Tnni3k/Runx1 leads to gene expression observed in cluster 10?

Per the suggestion of Reviewer 1, we have now removed the sn-RNAseq. We will be certain to consider this comment when we pursue publication of these data.

5. Do Tnni3k deletion and Runx1 overexpression have additive effects?

This is an interesting question that we cannot fully answer at this time. Prior to the pandemic we did have this cross breeding and we got 2 litters from it collected at P21. From these two litters, two mice were both Tnni3k KO and Runx1 overexpression, and they had comparable frequency of MNDCMs as Runx1 overexpression on its own (~6% MNDCM). This preliminary experiment is underpowered, but might suggest that no, in the context of C57BL/6J mice there are not additive effects of the two alleles. Notably, this experiment was performed before we knew of the

expansion of MNDCMs that occurs in both A/J and Tnni3k KOs after P21. Thus, had we performed this experiment more recently, we would have collected at different time points. Unfortunately, we do not have these animals breeding at this time. We have now added a discussion point to the manuscript to address this interesting idea, please see lines 383-388.

Second decision letter

MS ID#: DEVELOP/2022/201318

MS TITLE: Cardiomyocyte ploidy is dynamic during postnatal development and varies across genetic backgrounds

AUTHORS: Samantha K Swift, Alexandra L Purdy, Mary E Kolell, Kaitlyn G Andresen, Caitlin Lahue, Tyler Buddell, Kaelin A Akins, Christoph D Rau, Caitlin O'Meara, and Michaela Patterson
ARTICLE TYPE: Research Article

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

Reviewer 1

Advance summary and potential significance to field

In their revision, Swift et al have substantially enhanced their manuscript and addresses my concerns. The revised manuscript adds adult MI experiments (and removed sn-RNA-seq experiments), showing a transient recovery of cardiac function with Runx1 overexpression. More importantly, they report an increase in cardiomyocyte cell cycle activation and completion with Runx1 overexpression establishing a connection to their neonatal experiments. The result is a very nice manuscript that suggests the concept that polyploid cardiomyocytes can have delayed cell cycle completion, with mechanistic insights on potential mediators of this phenomenon. The authors are to be congratulated on this very interesting work.

Comments for the author

To the author's question about alternative strategies for presenting the stacked bar-chart data, I see their point about the challenges in presenting data with such high dimensionality. One could consider breaking these complex charts into multiple plots to reduce the dimensionality.
For example, for Figure 1C, one could show two different plots (one for each strain). Each plot would have 4 lines (one for each ploidy class). This might make the temporal trends in ploidy class a little more apparent. Honestly, just a suggestion!

Reviewer 2

Advance summary and potential significance to field

The authors investigated how development of cardiomyocytes affects their ploidy in different genetic backgrounds: C57Bl/6J and A/J. Interestingly, animals from these two strains are born and reach adulthood with similar numbers of cardiomyocytes but their ploidy and developmental progression differ significantly. The authors compared the cardiomyocytes in C57Bl/6J and A/J at different time points after birth and showed that cardiomyocytes in A/J display a dynamic pattern with an increase in the mononucleated diploid cardiomyocyte pool via finishing cell division of polyploid cardiomyocytes. They discovered that Runx1 overexpression is sufficient to induce cytokinesis and increase the expansion of mononucleated diploid cardiomyocytes. Now they further linked this intriguing finding to adult mouse heart regeneration after MI by overexpressing Runx1.

Comments for the author

The authors have addressed my comments especially now scRNAseq data are removed.

Reviewer 3

Advance summary and potential significance to field

The addition of the MI experiment is somewhat helpful, although not extremely so.
The bimodal response, suggesting initial improvement in function, followed by a decline in function suggests a temporary induction of Runx1 (as is naturally occurring, but maybe at insufficient levels) might be preferable.
The mechanistic insights provided by the manuscript are minimal beyond the notion that cell cycle completion can occur well after cell cycle initiation.

Comments for the author

No further comments