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Peer Review File

Lineage tracing of nuclei in skeletal myofibers uncovers distinct transcripts and interplay between myonuclear populations

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Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The goal of this study was the identification of expression signature of newly fused nuclei in the myofiber. Newly fused nuclei are essential for the physiology of the muscle, but it is currently unclear what exact function they provide. The study successfully identifies a gene signature of newly fused nuclei. It also shows that newly fused nuclei in development and after muscle overload are distinct but also show a small overlap of genes that are preferentially expressed in 'new nuclei' in both conditions (H19 and Igf2). Furthermore, it provides evidence that newly fused nuclei differ in de novo generated fibers and pre-existing fibers. While I find the paper of interest and timely, I feel that the authors have made little or no effort to present their data in a reader-friendly and understandable manner. Extreme examples of this are Fig. 7 and the supplemental tables that are named in an abstruse manner (e.g. 454115_0_supp_849411_s0cjm0.xlsx). The take home messages of Figs. 7 do not seem to go beyond stating that differentially expressed genes exist under the various conditions; what these could mean for muscle biology is not sufficiently addressed.

A major technical concern is the use of particular Pax7rtTA and Pax7CreERT2 alleles for lineage tracing and conditional mutagenesis. In these alleles, Pax7 coding sequences are disrupted and they result in Pax7 heterozygosity, which affects muscle biology. Appropriate controls need to be used, but genotypes of controls are often not given or the ones used are inappropriate (wildtype).

Comments and concerns

1. In part, the study relies on the re-analysis of previously published (Petrany et al., DOI: 10.1038/s41467-020-20063-w) snRNAseq data sets. For instance, Fig. 3 in Petrany et al. and Fig. 1 in the new study uses the same datasets. In the previous study all nuclei were compared, whereas the new analysis includes only myonuclei (MuSC and myofiber nuclei), which is mentioned. The new analysis defines two new clusters called Dev1 and Dev.2, that are later shown to correspond to newly fused nuclei (Fig. 2). Two genes preferentially expressed in these clusters are H19 and Igf2 that are both transcribed from the H19/IGF2 imprinted gene cluster. H19 and Igf2 turn out as 'markers' for newly fused nuclei. The potential functional significance of these genes should be discussed in light of the previous literature on H19/Igf2 and their roles in the muscle. Are there any implications for our understanding of muscle functions?

2. New data are used starting with Fig. 2. Fig. 2 introduces a newly developed lineage tracing technology that relies on histone-GFP-labeling of progenitors and their descendent nuclei after fusion using a Pax7rtTA allele. Further, data are verified using a myomaker mutation in MuSC, which precludes fusion (MymkloxP/loxP; Pax7CreERT2 (MymkscKO)). These models are used to demonstrate that H19 and Igf2 are preferentially expressed in newly fused nuclei. The analysis shown in Fig. 2 was done at P28, and the DEV population of nuclei reflects thus accretion during development.
It is important to note that the Pax7 coding sequence is disrupted by rtTA and CreERT2 sequences in the Pax7rtTA and Pax7CreERT2 alleles, and animals that carry these alleles are therefore heterozygous for Pax7. Pax7 heterozygous mutations have effects on MuSC numbers, muscle growth and regeneration (https://doi.org/10.7554/eLife.33337; https://doi.org/10.1073/pnas.1307680110 and personal communication by other researchers that did not publish the problems, which arose with these alleles). The use of such alleles needs to be well controlled to exclude artefacts. The genotype of controls for Fig. 2g needs to be Mymk+/+; Pax7CreER, and the animals need to be treated with tamoxifen since tamoxifen also affects the skeletal muscle.

3. In Fig. 3 data, bulk RNA-seq of the GFP- positive and -negative nuclei from P28 muscles are shown. For the GFP-labeling/ lineage tracing, Pax7rtTA; TREH2B-GFP and doxycycline treatment were used. Histone-GFP-positive and -

negative nuclei of the same animals are compared. In addition, bulk RNA-seq of histone-GFP-positive and -negative nuclei from animals after muscle overload and Sham operated animals are shown. The genotype of Sham operated animals is not given. For this control, Pax7rtTA; TREH2B-GFP animals treated with doxycycline should be used to exclude artefacts caused by Pax7 heterozygosity and/or doxycycline. Considering the fact that the Millay lab has previously demonstrated the importance of newly fused nuclei in muscle overload, it is well possible that the number of MuSC and changes in their behavior (due to Pax7 heterozygosity) affects the muscle response to overload. Moreover, doxycycline is known to affect muscle function. The conclusion of the figure is that differentially regulated genes in newly fused nuclei in development and after muscle overload exist.
If it's difficult to compare differentially expressed genes in the figures due to a lack of gene names on the volcano plots. It seems reasonable to request that the authors provide examples of gene names on the plots to enhance the comprehensibility of the results. The authors should consider including gene ontology analysis of differentially expressed genes (GFP+ versus GFP-) not only for the developmental condition, but also for muscle overload in their paper.

4. Extended Data Fig. 2: A number of previous reports indicated that fusion to muscle fibers might occur preferentially at the ends. Pax7rtTA-dependent GFP labeling should allow to directly test this hypothesis. Are nuclei at the ends of the fibers preferentially labeled by GFP?

5. Fig. 4 further analyses the transcriptional response to muscle overload using snRNA-seq data (genotype/treatment of sham operated mice? See comment #3). Three distinct populations of myonuclei respond to overload, one that preexists in the muscle (Atf3) and two that correspond to newly fused nuclei (unclassified A and B). The Atf3 population seems to correspond to the Enah population previously described by the Millay lab, and to the nuclei expressing the repair signature characterized by others. A comparison of Atf3, Enah and repair signature populations should be provided and discussed. Unclassified A and B differ in the expression of Tnnt1/2 and they express Myh3 (better known as embryonic myosin that is expressed in newly generated fibers in disease/after injury). This should be mentioned when Myoh3 is mentioned the first time (page 10). What is known about the two Tnnt genes? Are they differential expressed in normal conditions? Do they have different functions? Are there any implications for our understanding of muscle biology?

6. Fig. 5 verifies Atf3, H19, Igf2 and Tnnt1/2 expression in muscle with and w/o overload. Genotypes of controls are not provided. Appropriate genotypes need to be used with the appropriate treatments to exclude artefacts (see comments 2 and 3).

7. Fig. 6 defines newly fused myonuclei in de novo and pre-existing myofibers, which are distinguished due to their differences in fiber diameter. In Fig 4e unclassified A and B seem to express similar levels of Myh3, and Fig. 5a shows similar levels/distribution in central/peripheral nuclei. In Fig 5b, levels/distribution of the Myh3 transcripts are very distinct. Please comment.

8. In Fig. 7 another genotype, wildtype, is used to compare conditional myomaker mutant cells versus wildtype with and w/o overload. Wildtype is an inappropriate genotype for this analysis- the data should be repeated with tamoxifen treated Pax7rtTA;Mymk+/+ as controls to make sure that the effects observed are not due to Pax7 heterozygosity or tamoxifen. See also points 2 and 3 above. Moreover, the text describing Fig. 7 is abstruse and needs editing. What are the implications of the differentially expressed genes for our understanding of muscle biology?

Reviewer #2

(Remarks to the Author)
In their manuscript `Lineage tracing of newly accrued nuclei in skeletal myofibers uncovers distinct transcripts and interplay between nuclear populations`, Sun et al. use mouse genetics to study transcriptional diversity in pre-existing versus newly accrued myonuclei during postnatal development as well as overload. The manuscript is very interesting and has high novelty that could significantly move the field forward. I do feel though that the authors over-interprete their findings at few occasions and propose that they tune down their statements at some places. Additionally, few additional control experiments could further strengthen the data and conclusions.

MAIN COMMENTS

Line 136: The authors state that the H2B-GFP protein is long-lived. Can the authors provide data on the half-life of the H2B-GFP protein in the current model? How long after Dox does the GFP disappear? Is there a possibility to underestimate the number of fused nuclei in some of the experiments with a ‘longer’ timespan due to loss of GFP expression? This is relevant, cause otherwise it is very difficult to make a statement on pre-existing myonuclei versus newly recruited.

Line 140: 9% of GFP+ cells is not VCAM1+ Lin-. Can the authors perhaps speculate in the discussion section to which populations do those belong (and whether or not this could affect the interpretation of further experiments)?

Line 143: Why did the authors label for 12 days continuously? If the Pax7+ cells get labeled after 5days of Dox, there is no necessity to keep injecting Dox. First, this further reduces the ability to have mRNA trafficking and second, it is known that Dox induces a stress response (activating ATFs) and as such could affect gene expression. Can the authors explain WHY they chose this experimental set-up or show similar GFP+ myonuclei after shorter Dox treatments (for instance P12-17?)?

Line 145: There is no reason to assume that H2B-GFP moves through the syncytium when it already localized into the nucleus before fusing with the fiber unless there is still mRNA present (potentially due to recent Dox treatment?). It is therefore unclear why the authors make a claim based on the observation that some myonuclei remain unlabeled (which by no means is evidence of lack of movement). I suggest to remove this statement.

Line 160: Stating that H19 and IGF2 are enriched in recently fused myonuclei is an overstatement at that point in the manuscript. They are present in Dev1. Suggest to rephrase.

Line 165: It seems that the fibers that have newly fused myonuclei are in general positive for H19, so this might interfere with the quantification. Can the authors also quantify signal intensity in GFP+ versus GFP- myonuclei within the same H19+ fiber?

Line 185: did the authors identify some nuclear proteins within their dataset that could potentially be used to identify newly fused myonuclei?

Line 215: again, it is not clear why Dox treatment was maintained after overload. The authors should elaborate on this. Also, using the MOV model, it is not clear to me why EDL is analyzed (since one would expect very little contribution in EDL which is not overloaded…).

Line 220: I suggest to remove the statement `highlighting the reliability of the tracing approach`. One does not want to benchmark a model based on estimations. Suggest to rephrase to `numbers similar to previous estimations`.

Line 335: Besides the observation that the Tnnt2+ myofibers are smaller, there is in fact no evidence provided that those are truly de novo formed ones. One way to further support the hypothesis that Tnnt2 is expressed in de novo formed myofibers is to evaluate whether 100% of the myonlucei in those fibers are GFP+. As long as this evidence is not presented, I feel there is not sufficient evidence to prove the clain. I suggest to tune down (or present the evidence).

Line 465: the authors discuss the presence of a global sensing mechanism in fibers where pre-existing myonuclei respond to the accrual of new ones. Is it possible that external factors related to the Mymk phenotype (for instance continued mechanical overload) contribute to the altered response and not just the mere presence or not of the new myonuclei? The authors could add this to the discussion

Do the authors have any ideas/data on possible differences in myonuclei fusion with this model in slow vs fast fibers/muscles. The authors used various muscles with very pronounced fiber type compositions depending on the experiment. While no additional experiments are required, some hypotheses could be added to the discussion section. Along this line, TNNT isoforms are also fiber type specific. How can the results regarding Tnnt1/Tnnt2 as markers for newly fused nuclei be related to that? Could these markers be different depending on which muscle (or fiber type) you are studying?

Minor comments

The presented volcano plots are not that informative since no specific genes are annotated on the plots. Especially because multiple genes are discussed in detail in the main text. These genes should also be labelled in the respective volcano plots.

Figure 1:
- Line 100: Not all claims are completely clear based on the presented plots. Especially figure 1B is not convincing. For example, differences between the Dev1 and Dev2 clusters are not obvious. Also, the difference between both Dev clusters and the MuSC or IIx/IIb clusters is not as clear compared to the interpretation given in the text. I would suggest playing around with different colour schemes or plot types (e.g., feature plot) to make this plot better, or adapt interpretations accordingly.

- Differences between the Dev and other clusters are presented in figures 1D and 1F, but are not further discussed (possibly intentionally at this point in the manuscript). Could GSEA or over-representation analysis help to start dissecting gene signatures in the Dev clusters?

- Figure 1F: The description of the different coloured bars should be added to the figure caption. Distinct gene profiles of the Dev clusters are again very hard to see from the current plot. A different colour scheme, as compared to the current ‘all-purple’ scheme, could potentially help.

- Overall, while figure 1 is useful as a starting point in the manuscript, some claims are on the edge of being an overinterpretation based on the current plots.

Figure 2B: The text states 2 weeks of Dox administration, as compared to 16 days in the figure. I would suggest making this consistent. Would it also be helpful to highlight some GFP-positive and GFP-negative nuclei with arrows?

Line 115: At first appearance in the text, it would be good to further discuss H19 and Igf2 (type of RNA, annotated functions, …).

Line 231: ‘Many of these DEGs are shared, …’. A Euler plot or Venn diagram would help the reader to interpret these data.

Line 266: Write abbreviations (NMJ, MTJ) in full to make it easier for reader.

Line 280-281: ‘The Atf3 population was not enriched for H19, Igf2, Myh3, or Myh8 but instead was enriched for Atf3, Flnc, Enah, and Ankrd1’. Nrap and not Ankrd1 is highlighted in the dot plot (fig 4E).

Figure 6
- Line 340-343: Not that clear in figure 4E that unclassified A nuclei are similar to type IIA and ATF3+ nuclei, as stated in the text. Similar for statement that unclassified B are similar to type IIB. I would rephrase (tune down) these statements.

Line 724: Dot plot demonstrates upregulated (should be altered?) genes in Dev. Nuclei.

Line 1055: Further explanation of DESeq2 steps needed (statistical model, any surrogate variable modelling, which QC steps, …).

snRNAseq data processing:
- Supplementary plot with number of features, number of UMIs and % mitochondrial genes could be useful as QC metrics
- Clustering: describe how many PCs were used and why

Writing errors

Line 136: ‘a long-half life’ instead of ‘a long half-life’

Line 217: ‘dystropin’

Line 231: ‘DEGS’ instead of ‘DEGs’

Line 472: ‘the extent’

Line 911: ‘75% of the lateral …’

Line 991: ‘uuclei’

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Most of the points I raised in my initial review have been appropriately addressed. However, I have two remaining concerns:

1) Genotype of Controls: While the genotype is mentioned in the figure legend, it is not immediately noticeable. I suggest providing the genotype information directly in the figures themselves, in addition to the legend, to ensure clarity.
2) Pax7 Haploinsufficiency: Although there are no reported developmental or overload-related issues associated with Pax7 haploinsufficiency in the literature, this aspect may not have been thoroughly investigated. Furthermore, the significant deficits in regeneration reported by Relaix et al. (DOI: 10.1186/s13287-023-03506-1) and other studies suggest that Pax7 haploinsufficiency impacts myogenesis more than what could be described as modest. I recommend addressing this point in the Discussion section.

Reviewer #2

(Remarks to the Author)
The authors have addressed my comments -I have no further questions and congratulate them on their nice work.

We thank the reviewers for the thorough review of our manuscript and thoughtful suggestions. We carefully considered each comment to improve the manuscript either through addition of new data, alterations of text, or in some cases we provide answers specifically to reviewers but do not add them to the revised version of the manuscript. Below, we provide a point-by-point response to each comment.

Reviewer #1 (Remarks to the Author):

The goal of this study was the identification of expression signature of newly fused nuclei in the myofiber. Newly fused nuclei are essential for the physiology of the muscle, but it is currently unclear what exact function they provide. The study successfully identifies a gene signature of newly fused nuclei. It also shows that newly fused nuclei in development and after muscle overload are distinct but also show a small overlap of genes that are preferentially expressed in 'new nuclei' in both conditions (H19 and Igf2). Furthermore, it provides evidence that newly fused nuclei differ in de novo generated fibers and pre-existing fibers. While I find the paper of interest and timely, I feel that the authors have made little or no effort to present their data in a reader-friendly and understandable manner. Extreme examples of this are Fig. 7 and the supplemental tables that are named in an abstruse manner (e.g. 454115_0_supp_849411_s0cjm0.xlsx). The take home messages of Figs. 7 do not seem to go beyond stating that differentially expressed genes exist under the various conditions; what these could mean for muscle biology is not sufficiently addressed.

A major technical concern is the use of particular Pax7rtTA and Pax7CreERT2 alleles for lineage tracing and conditional mutagenesis. In these alleles, Pax7 coding sequences are disrupted and they result in Pax7 heterozygosity, which affects muscle biology. Appropriate controls need to be used, but genotypes of controls are often not given or the ones used are inappropriate (wildtype).

Comments and concerns

1. In part, the study relies on the re-analysis of previously published (Petrany et al., DOI: 10.1038/s41467-020-20063-w) snRNAseq data sets. For instance, Fig. 3 in Petrany et al. and Fig. 1 in the new study uses the same datasets. In the previous study all nuclei were compared, whereas the new analysis includes only myonuclei (MuSC and myofiber nuclei), which is mentioned. The new analysis defines two new clusters called Dev1 and Dev.2, that are later shown to correspond to newly fused nuclei (Fig. 2). Two genes preferentially expressed in these clusters are H19 and Igf2 that are both transcribed from the H19/IGF2 imprinted gene cluster. H19 and Igf2 turn out as 'markers' for newly fused nuclei. The potential functional significance of these genes should be discussed in light of the previous literature on H19/Igf2 and their roles in the muscle. Are there any implications for our understanding of muscle functions?

This is an excellent point by the reviewer as we indeed did not comment on specific genes in the original version of the manuscript. We think our work is not yet at the level to make definitive claims about the functions of genes contributed by newly fused nuclei, and it will take significant work to design systems to functionally understand these factors. For instance, many studies on H19/Igf2 in the literature focus on their role in myogenesis and it is difficult to uncouple those roles to a potential role in myofibers after contribution of newly fused nuclei. Nonetheless, we added a paragraph to the discussion to highlight the well-documented roles of H19 and Igf2, and also mentioned some molecular functions when they initially appear in the results section.

2. New data are used starting with Fig. 2. Fig. 2 introduces a newly developed lineage tracing technology that

relies on histone-GFP-labeling of progenitors and their descendent nuclei after fusion using a Pax7rtTA allele. Further, data are verified using a myomaker mutation in MuSC, which precludes fusion (MymkloxP/loxP; Pax7CreERT2 (MymkscKO)). These models are used to demonstrate that H19 and Igf2 are preferentially expressed in newly fused nuclei. The analysis shown in Fig. 2 was done at P28, and the DEV population of nuclei reflects thus accretion during development.
It is important to note that the Pax7 coding sequence is disrupted by rtTA and CreERT2 sequences in the Pax7rtTA and Pax7CreERT2 alleles, and animals that carry these alleles are therefore heterozygous for Pax7. Pax7 heterozygous mutations have effects on MuSC numbers, muscle growth and regeneration (https://doi.org/10.7554/eLife.33337; https://doi.org/10.1073/pnas.1307680110 and personal communication by other researchers that did not publish the problems, which arose with these alleles). The use of such alleles needs to be well controlled to exclude artefacts. The genotype of controls for Fig. 2g needs to be Mymk+/+; Pax7CreER, and the animals need to be treated with tamoxifen since tamoxifen also affects the skeletal muscle.

We thank the reviewers for bringing up this important issue, which is not only relevant for our manuscript but also the field in general. The Pax7 alleles we used here (Pax7rtTA and Pax7CreER) do indeed disrupt the Pax7 coding sequence and result in heterozygous mice, which may cause issues in muscle in certain contexts. A recent paper (PMID 37833800) is the most appropriate to consider since it compares the various Pax7-CreER alleles. They showed that Pax7-CreER mice have normal MuSC numbers and respond to regeneration normally in the absence of tamoxifen. However, upon treatment with tamoxifen diet, Pax7-CreER mice exhibit fewer MuSCs and delayed regeneration. These data suggest there may be an interaction between the Pax7-CreER allele and tamoxifen diet during the response to cardiotoxin-induced muscle regeneration. Given these concerns, we did consider our system and the proper controls. We acknowledge the concern by the reviewer and would like to take the effort to explain why we think this issue is unlikely to impact our results, and also highlight the addition of control experiments in the revised manuscript.

a) Regarding Figure 2g mentioned by the reviewer, the control group is *Mymk*$^{loxP/loxP}$ (+tamoxifen) and this is now stated in the figure legend. We performed a control experiment comparing H19 levels in *Mymk*$^{loxP/+}$; *Pax7*^{CreER} or *Mymk*$^{loxP/+}$ mice during development (Extended Data Fig. 2). We used *Mymk*$^{loxP/+}$ mice here because there is no impact on fusion, and thus serves as an ideal control. We found no major difference in expression of H19 due to the presence of *Pax7*^{CreER} and thus conclude that gene expression in this system is not due to Pax7 disruption. It should also be noted that our goal here is to block fusion in *Mymk*$^{loxP/loxP}$; *Pax7*^{CreER} (+tamoxifen) mice and test if H19/Igf2 mRNA is altered. We understand the reviewers concern that differences in H19/Igf2 levels in myofibers could be due to a myogenesis defect caused by Pax7 heterozygosity, but we have shown this *Mymk*$^{loxP/loxP}$; *Pax7*^{CreER} efficiently blocks fusion (PMIDs: 25085416, 28186492, 31012848) and thus any modest effect of Pax7 heterozygosity is non-consequential.
b) It should also be noted that there is no evidence that there are issues with the Pax7 alleles during development or overload, which is the focus of our study and has a different reliance on MuSCs compared to toxin-induced regeneration. Indeed, mice with the Pax7-rtTA allele that we describe in this manuscript exhibit the level of myonuclear accretion (the ultimate endpoint of myogenesis) that is expected based on reports in WT animals (PMIDs: 33293533, 20175910). We observed 20% GFP+ myonuclei after labeling from postnatal (P) day 12 and analyzing at P28, which is what has been observed previously. A similar result was found during overload (both in forming new myofibers and fusing into existing myofibers) indicating that Pax7-rtTA does not significantly interfere with myogenesis in the systems we are studying.

c) Our gene expression analysis with the Pax7rtTA; TRE-GFP mouse model involved comparisons of GFP+ (newly labeled) and GFP- (pre-existing) myonuclei from the same mice, thereby ensuring any Pax7 disruption would uniformly affect both populations, minimizing its influence on the differential expression we observed.

3. In Fig. 3 data, bulk RNA-seq of the GFP- positive and -negative nuclei from P28 muscles are shown. For the GFP-labeling/ lineage tracing, Pax7rtTA; TREH2B-GFP and doxycycline treatment were used. Histone-GFP-positive and -negative nuclei of the same animals are compared. In addition, bulk RNA-seq of histone-GFP-positive and -negative nuclei from animals after muscle overload and Sham operated animals are shown. The genotype of Sham operated animals is not given. For this control, Pax7rtTA; TREH2B-GFP animals treated with doxycycline should be used to exclude artefacts caused by Pax7 heterozygosity and/or doxycycline. Considering the fact that the Millay lab has previously demonstrated the importance of newly fused nuclei in muscle overload, it is well possible that the number of MuSC and changes in their behavior (due to Pax7 heterozygosity) affects the muscle response to overload. Moreover, doxycycline is known to affect muscle function. The conclusion of the figure is that differentially regulated genes in newly fused nuclei in development and after muscle overload exist.

Thanks for bringing this to our attention. All our sham samples came from Pax7rtTA; TRE-H2B-GFP mice that received doxycycline treatment. PCM1+GFP- nuclei, representing pre-existing myonuclei, were used for analysis, while PCM1+GFP+ nuclei, which would signify newly fused myonuclei, were not detected as there was no active fusion at the sedentary stage in adult muscle within the 2-week timeframe of our detection window. We noted the specific genotype of the sham animals in the figure legends.

If it's difficult to compare differentially expressed genes in the figures due to a lack of gene names on the volcano plots. It seems reasonable to request that the authors provide examples of gene names on the plots to enhance the comprehensibility of the results. The authors should consider including gene ontology analysis of differentially expressed genes (GFP+ versus GFP-) not only for the developmental condition, but also for muscle overload in their paper.

Excellent point. Gene names have been added to volcano plots. Regarding gene ontology for GFP+ vs GFP- nuclei in the overload condition, it will not work well because there are too few genes that are differentially regulated between the populations to make gene ontology worthwhile.

4. Extended Data Fig. 2: A number of previous reports indicated that fusion to muscle fibers might occur preferentially at the ends. Pax7rtTA-dependent GFP labeling should allow to directly test this hypothesis. Are nuclei at the ends of the fibers preferentially labeled by GFP?

We agree with the reviewer that the Pax7rtTA model developed here can also give positional information as to where fusion occurs and how nuclei move within the syncytium. However, our main goal in this manuscript was to understand gene expression. While we have provided some evidence in our manuscript about the position of newly fused nuclei during development (Figure 2b) and muscle overload (Extended Data Figure 3b), these myofibers are imaged at a given time point and thus may not reveal where fusion occurs since nuclei could move within the syncytium after fusion. Given that caveat, our data shows that newly fused nuclei are evenly distributed across myofibers, from the central part to the ends, in response to overload stimulation.

This implies that the incorporation of new nuclei into the fiber does not follow a predetermined pattern aimed at the ends or center of the muscle fiber, but deeper analysis (intravital imaging) is needed to substantiate this claim and that will require significantly more work. We respectfully argue that the question of where fusion occurs is outside the primary scope of this manuscript.

5. Fig. 4 further analyses the transcriptional response to muscle overload using snRNA-seq data (genotype/treatment of sham operated mice? See comment #3). Three distinct populations of myonuclei respond to overload, one that preexists in the muscle (Atf3) and two that correspond to newly fused nuclei (unclassified A and B). The Atf3 population seems to correspond to the Enah population previously described by the Millay lab, and to the nuclei expressing the repair signature characterized by others. A comparison of Atf3, Enah and repair signature populations should be provided and discussed. Unclassified A and B differ in the expression of Tnnt1/2 and they express Myh3 (better known as embryonic myosin that is expressed in newly generated fibers in disease/after injury). This should be mentioned when Myoh3 is mentioned the first time (page 10). What is known about the two Tnnt genes? Are they differential expressed in normal conditions? Do they have different functions? Are there any implications for our understanding of muscle biology?

Thanks for the opportunity to clarify our experimental design, data, and interpretations related to Figure 4. Here we used HSA^{rtTA}; $TRE^{H2B\text{-}GFP}$ mice (no Pax7-targeted alleles) and the sham operated mice are also HSA^{rtTA}; $TRE^{H2B\text{-}GFP}$, which we have now noted in the results and figure legends.

We agree that the Atf3 population identified after overload could be similar to the Enah population we identified at postnatal (P) day 21 and the repair signature from dystrophic mice described by others. As requested, we compared the top 200 upregulated genes in the Atf3 population with the top 200 upregulated genes in the Enah population and found that 65 genes are shared. However, gene ontology for those 65 genes and gene ontology for the non-shared genes yield similar terms, so it is unclear to us how to use this information within our manuscript. We could simply state that we are able to verify enrichment of genes from the Atf3+ MOV population with the P21 Enah+ population, but we do not think this is a major point of the manuscript. We tried to analyze the repair signature from dystrophic muscle but found that only a small number of cells express Atf3 and that Flnc is not that specific to a certain population, likely because there is a lot of ongoing damage and repair in dystrophic muscle. It should also be noted that there are numerous analyses that can be performed with RNA-seq, which is why we have made the data accessible so users can do analyses of specific interest.

We acknowledge that we do not mention differences in unclassified A and B when Myh3 is first mentioned. The rationale for this is that when we initially discuss Myh3 we are describing the results from the snRNAseq after overload from a general perspective. Then we transition into determining which of the emerging populations in overload are derived from newly fused nuclei, then we describe the differences between unclassified A and B to support the claim that the syncytial environment can impact gene expression of newly fused nuclei. As we wrote the manuscript, we considered discussing differences in unclassified A and B first, but could not find an easy way to transition to the next steps and thus decided to mention differences between unclassified A and B later. We agree with the reviewer that there are multiple approaches about how and where to discuss differences in unclassified A and B, and these approaches are relatively stylistic and likely dependent on each reader, therefore we would like to keep the flow of the results as is.

Regarding the differential expression of Tnnt genes, we now give more background on the function of these genes and discuss implications for the muscle in the discussion. As mentioned above, we felt our data does not

yet lead to a definitive understanding for the function of particular genes contributed by newly fused nuclei, but we also understand that this reviewer would like us to explore possible implications of our findings, which we provide in the revised version of the manuscript (in the results section when describing Fig. 5b). Troponin T is a crucial component of the troponin complex necessary for the contraction of skeletal and cardiac muscle fibers. Tnnt1 and Tnnt2 genes encode for troponin T variants that are preferentially expressed in different muscle fiber types. Specifically, Tnnt1 is responsible for coding the slow-twitch skeletal muscle variant of troponin T. In contrast, Tnnt2 is associated with the cardiac muscle's troponin complex, where it connects to tropomyosin and anchors to thin filaments, a role it also plays in embryonic skeletal muscle cells but is not predominant in adult skeletal muscle fibers of either slow or fast twitch. An important consideration is the near absence of type I slow-twitch fibers in fully matured plantaris muscle, and it has been noted that stimulating muscle overload does not cause a switch in fiber type from fast to type I. Our results indicate an enrichment of Tnnt1 in newly fused myonuclei, which could reflect a temporary reorganization of the sarcomere complex to meet the short-term contraction needs during muscle overload adaptation.

6. Fig. 5 verifies Atf3, H19, Igf2 and Tnnt1/2 expression in muscle with and w/o overload. Genotypes of controls are not provided. Appropriate genotypes need to be used with the appropriate treatments to exclude artefacts (see comments 2 and 3).

All the control samples used in Fig 5b were from *Mymk*$^{loxP/loxP}$ littermates that received the same tamoxifen treatment, which is now stated in the figure legend. Similar to our response to comment #2, we think Pax7 heterozygosity is unlikely to cause these differences in gene expression in myonuclei because we are completely blocking fusion in Mymk^{scKO} mice. Moreover, in the revised version of the manuscript we found no major difference in expression of H19 due to the presence of *Pax7*^{CreER} during development and thus conclude that gene expression in this system is not due to Pax7 disruption. (Extended Data Fig. 2b).

7. Fig. 6 defines newly fused myonuclei in de novo and pre-existing myofibers, which are distinguished due to their differences in fiber diameter. In Fig 4e unclassified A and B seem to express similar levels of Myh3, and Fig. 6a shows similar levels/distribution in central/peripheral nuclei. In Fig 6b, levels/distribution of the Myh3 transcripts are very distinct. Please comment.

This is an astute point from the reviewer. We agree with the reviewer that Figure 4e shows similar expression of Myh3 in unclassified A and B myonuclei, which is also true for Fig. 6c. We think the reason Figure 6b exhibits differential staining of Myh3 is due to distribution of the mRNA, which is associated with the overall volume and cytoplasmic components in de novo and pre-existing myofibers. In cross-section, it is obvious that Myh3 expression is stronger in de novo myofibers, which we think is because the volume of the myofiber is less and there are fewer mature sarcomeres that could inhibit distribution. In contrast, in pre-existing myofibers have larger volumes and mature sarcomeres that could inhibit distribution. Finally, Myh3 mRNA is present in all nuclei that have formed a de novo myofiber, thereby leading to an increased concentration relative to a pre-existing myofiber that has Myh3-negative and Myh3-positive nuclei. Because this is a nuance of studying gene expression in syncytial cells with different volumes and nuclei, we would like to exclude this discussion from the manuscript, so as not to detract from the main message.

8. In Fig. 7 another genotype, wildtype, is used to compare conditional myomaker mutant cells versus wildtype with and w/o overload. Wildtype is an inappropriate genotype for this analysis- the data should be repeated with tamoxifen treated Pax7rtTA;Mymk+/+ as controls to make sure that the effects observed are not due to Pax7 heterozygosity or tamoxifen. See also points 2 and 3 above. Moreover, the text describing Fig. 7 is

abstruse and needs editing. What are the implications of the differentially expressed genes for our understanding of muscle biology?

We agree with the reviewer that the text describing Fig. 7 is difficult. We have made major revisions to Figure 7 and now show the analysis through a scatter plot, which should allow for improved reader comprehension and streamlined interpretations. We also took this opportunity to remove wildtype from our description because that is not correct. The mice analyzed were Pax7rtTA; TRE-GFP. Three of the sham controls in this analysis were Pax7rtTA;TRE-GFP mice that received dox but not overload, and are the same samples used in Figure 3. In addition, one sham was *Mymk*$^{loxP/loxp}$ + tamoxifen. This detail is now listed in the figure legends. The relative similarity between these sham samples is further suggestion that Pax7 heterozygosity and tamoxifen does not have a major impact on gene expression. It should also be noted that our result of dysregulated transcription in the absence of fusion is consistent with a previous report (PMID: 34109314), further indicating that our results are not due to improper controls.

Reviewer #2 (Remarks to the Author):

In their manuscript `Lineage tracing of newly accrued nuclei in skeletal myofibers uncovers distinct transcripts and interplay between nuclear populations`, Sun et al. use mouse genetics to study transcriptional diversity in pre-existing versus newly accrued myonuclei during postnatal development as well as overload. The manuscript is very interesting and has high novelty that could significantly move the field forward. I do feel though that the authors over-interprete their findings at few occasions and propose that they tune down their statements at some places. Additionally, few additional control experiments could further strengthen the data and conclusions.

MAIN COMMENTS

Line 136: The authors state that the H2B-GFP protein is long-lived. Can the authors provide data on the half-life of the H2B-GFP protein in the current model? How long after Dox does the GFP disappear? Is there a possibility to underestimate the number of fused nuclei in some of the experiments with a 'longer' timespan due to loss of GFP expression? This is relevant, cause otherwise it is very difficult to make a statement on pre-existing myonuclei versus newly recruited.

This is an excellent question from the reviewer and they are correct that we do not know the half-life of H2B-GFP in our model. In designing our experiments, we relied on comprehensive published studies that have monitored H2B-GFP expression in mice. One study (PMID 23023126) found that satellite cells retain H2B-GFP for an extended period. Specifically, when doxycycline was administered in the adult to induce H2B-GFP in satellite cells, the fluorescence remained observable in a subset of cells for at least 84 weeks. Additionally, another study we referenced in the original version of the manuscript (PMID 19060879) provides compelling evidence that slow-cycling stem cells—a state akin to that of post-mitotic myonuclei—can preserve the GFP label for up to a year. Since we monitored H2B-GFP myonuclei over a shorter timeframe (one to two weeks) compared to the more prolonged periods assessed in the two studies we mention above, we think it is unlikely that there is an underestimation of the number of newly fused nuclei. Additionally, the number of the GFP+ myonuclei at the time of collection in our study aligns with the data from previous research quantifying myonuclear accretion using alternate mouse models. Nonetheless, to help alleviate the reviewer's concern we assessed the number of GFP+ myonuclei at P42 after labeling from P12 to P28. Here we found a similar number of GFP+ myonuclei at P42 compared to P28 (the time point at which gene expression was analyzed)

indicating that the myonuclei do not lose GFP over this two-week chase. These data are shown in Extended Data Fig. 1d.

It is also possible that there are newly fused myonuclei that exhibit very low levels of H2B-GFP expression due to proliferation prior to fusion into the myofiber, and these would be incorrectly classified as pre-existing myonuclei. In this case, one would expect that these myonuclei would be EdU+. To directly test this, we treated Pax7rtTA;TRE-GFP mice with EdU during muscle overload and assessed how proliferation correlates to H2B-GFP expression in myonuclei. Here we found the vast majority of myonuclei were either EdU^+ GFP^+ or EdU^- GFP^+ with a small fraction of EdU^+ GFP^- (Extended Data Fig. 3e).

Overall, these new experiments further indicate that while no system can capture all events, we are not severely underestimating the number of fused nuclei due to loss of GFP expression from an early fusion event or due to loss of the GFP label from proliferation prior to fusion.

Line 140: 9% of GFP+ cells is not VCAM1+ Lin-. Can the authors perhaps speculate in the discussion section to which populations do those belong (and whether or not this could affect the interpretation of further experiments)?

Thank you for highlighting this aspect of our work. We are unable to speculate on the identity of those cells but do not think they impact interpretations. We suspect that it is due to potential heterogenous expression of VCAM1 in the MuSC population. It is possible that the GFP+ VCAM- cells are indeed MuSCs but have low level of VCAM1.

Line 143: Why did the authors label for 12 days continuously? If the Pax7+ cells get labeled after 5days of Dox, there is no necessity to keep injecting Dox. First, this further reduces the ability to have mRNA trafficking and second, it is known that Dox induces a stress response (activating ATFs) and as such could affect gene expression. Can the authors explain WHY they chose this experimental set-up or show similar GFP+ myonuclei after shorter Dox treatments (for instance P12-17?)?

Thanks for giving us the opportunity to explain our rationale for labeling. We carefully analyzed newly fused myonuclei during two distinct phases of muscle growth, both involving proliferation, differentiation, and fusion of myogenic progenitors. It is possible that labeling for 5 days would have been sufficient, but we wanted to ensure that the GFP signal remained as strong as possible to capture as many newly fused nuclei as possible. One concern that we wanted to mitigate is the timing between Dox treatment and fusion. For instance, if we only treated with Dox for 5 days from P12 to P17, if the cells did not activate and fuse to myofibers, they would be undergoing a loss of expression of GFP mRNA because Dox was removed. More specifically, a satellite cell that is labeled during the initial 5 days may sit there for another 10 days prior to fusion and during this 10 days GFP would become diminished. Thus, we continued the doxycycline treatment to reinforce GFP expression until the Pax7 expression was fully discontinued. We attempted to better explain the rationale in the text.

While Dox can impact gene expression, we used Pax7rtTA;TRE-GFP mice that received Dox as a control, and we compared GFP+ and GFP- myonuclei within the same animal so both sets of myonuclei were exposed to Dox, and these controls are now clearly stated in the revised version of the manuscript.

Line 145: There is no reason to assume that H2B-GFP moves through the syncytium when it already localized into the nucleus before fusing with the fiber unless there is still mRNA present (potentially due to recent Dox treatment?). It is therefore unclear why the authors make a claim based on the observation that some

myonuclei remain unlabeled (which by no means is evidence of lack of movement). I suggest to remove this statement.

We removed this statement. Our goal here was to highlight the contrast in this system to traditional Cre-based lineage tracing approaches, but we agree the reviewer that the statement is out of context as previously constructed.

Line 160: Stating that H19 and IGF2 are enriched in recently fused myonuclei is an overstatement at that point in the manuscript. They are present in Dev1. Suggest to rephrase.

Agreed. We rephrased this statement.

Line 165: It seems that the fibers that have newly fused myonuclei are in general positive for H19, so this might interfere with the quantification. Can the authors also quantify signal intensity in GFP+ versus GFP- myonuclei within the same H19+ fiber?

We appreciate the alternative quantification method as a valid option, and we used this approach but decided to not include it in the manuscript because we felt analysis on sections was more reliable. An issue with isolated myofibers is that we were not able to exclude the possibility that a small number of GFP+ nuclei were not inside myofibers and instead in the satellite cell compartment. We tried combining RNAscope with dystrophin or Pax7 immunostaining but this did not work well. This is also the reason that our other quantification of the percentage of GFP+ nuclei was not done on isolated myofibers.

Line 185: did the authors identify some nuclear proteins within their dataset that could potentially be used to identify newly fused myonuclei?

This is an interesting thought. We have not deeply investigated this possibility and agree that if there was an enrichment of nuclear proteins, those could be used to identify newly fused nuclei without using the complex genetics that we describe in this manuscript. We looked through our sequencing results and did not find nuclear proteins that were obviously enriched. However, our sequencing data is publicly available for the field to pursue this question.

Line 215: again, it is not clear why Dox treatment was maintained after overload. The authors should elaborate on this. Also, using the MOV model, it is not clear to me why EDL is analyzed (since one would expect very little contribution in EDL which is not overloaded…).

The single fiber analyses for the MOV study detailed in this manuscript were conducted on fibers taken from the plantaris muscle. We corrected this mistake in the results and methods sections, and we thank the reviewer for catching this mistake. We also added a few sentences to explain our rationale for maintaining Dox treatment after overload, and the rationale here is similar to our description during development (discussed in response to reviewer's concern related to Line 143).

Line 220: I suggest to remove the statement `highlighting the reliability of the tracing approach`. One does not want to benchmark a model based on estimations. Suggest to rephrase to `numbers similar to previous estimations`.

Agreed, this statement has been removed.

Line 335: Besides the observation that the Tnnt2+ myofibers are smaller, there is in fact no evidence provided that those are truly de novo formed ones. One way to further support the hypothesis that Tnnt2 is expressed in de novo formed myofibers is to evaluate whether 100% of the myonlucei in those fibers are GFP+. As long as this evidence is not presented, I feel there is not sufficient evidence to prove the claim. I suggest to tune down (or present the evidence).

Thank you for this comment and we also contemplated how to better substantiate this concept in the original version of the manuscript. We attempted an additional RNAscope analysis on longitudinal sections, but it was too difficult to follow individual myofibers throughout the section. Since we are not able to provide new data, we tuned down this claim as suggested by the reviewer. Instead of 'likely' de novo myofibers, we say 'potentially' de novo. We also added this sentence to the results section – 'However, our characterization of *de novo* and pre-existing myofibers are only based on size, thus we are unable to definitively conclude that *Tnnt2*$^{+}$ myofibers are *de novo*.'

Line 465: the authors discuss the presence of a global sensing mechanism in fibers where pre-existing myonuclei respond to the accrual of new ones. Is it possible that external factors related to the Mymk phenotype (for instance continued mechanical overload) contribute to the altered response and not just the mere presence or not of the new myonuclei? The authors could add this to the discussion.

This is an excellent point by the reviewer and we have added this possibility to the discussion.

Do the authors have any ideas/data on possible differences in myonuclei fusion with this model in slow vs fast fibers/muscles. The authors used various muscles with very pronounced fiber type compositions depending on the experiment. While no additional experiments are required, some hypotheses could be added to the discussion section. Along this line, TNNT isoforms are also fiber type specific. How can the results regarding Tnnt1/Tnnt2 as markers for newly fused nuclei be related to that? Could these markers be different depending on which muscle (or fiber type) you are studying?

We added comments in the discussion related to the above points.

Minor comments

The presented volcano plots are not that informative since no specific genes are annotated on the plots. Especially because multiple genes are discussed in detail in the main text. These genes should also be labelled in the respective volcano plots.

Genes are now added to the volcano plots.

Figure 1:
- Line 100: Not all claims are completely clear based on the presented plots. Especially figure 1B is not convincing. For example, differences between the Dev1 and Dev2 clusters are not obvious. Also, the difference between both Dev clusters and the MuSC or IIx/IIb clusters is not as clear compared to the interpretation given

in the text. I would suggest playing around with different colour schemes or plot types (e.g., feature plot) to make this plot better, or adapt interpretations accordingly.

The plots have been modified to larger dot sizes and a more distinct color scheme. We also changed the claim related to Fig. 1b is that Dev 1 and Dev 2 populations exhibit an enrichment for genes associated with a myonuclear transcriptional identity, and there are no direct comparisons of Dev1 and Dev2 with any other population. We used PHATE trajectory analysis to assess their state along the myogenic lineage, which is shown in Fig. 1c.

- Differences between the Dev and other clusters are presented in figures 1D and 1F, but are not further discussed (possibly intentionally at this point in the manuscript). Could GSEA or over-representation analysis help to start dissecting gene signatures in the Dev clusters?

The reviewer is correct that the lack of discussion is intentional as the major point of this figure is that a nuclear population is associated with development, which could indicate they are newly fused. An inherent challenge for bioinformatics is cluster identification and when we integrated we found that the Dev 1 cluster did not make it into a myogenic population and clustered with FAPs likely due to Ebf1 gene signature. This is why we focused on Dev 2 and chose not to compare Dev. 1 and Dev 2. We do not think further dissection between Dev 1 and Dev 2 is helpful for the main point of our manuscript.

- Figure 1F: The description of the different coloured bars should be added to the figure caption. Distinct gene profiles of the Dev clusters are again very hard to see from the current plot. A different colour scheme, as compared to the current 'all-purple' scheme, could potentially help.

The description of colored bars have been added to figure legend. We have adjusted the color scheme and size of the dots as suggested.

- Overall, while figure 1 is useful as a starting point in the manuscript, some claims are on the edge of being an overinterpretation based on the current plots.

We removed overinterpretations associated with this figure but kept the main point that we observe a unique cluster of nuclei mainly during development.

Figure 2B: The text states 2 weeks of Dox administration, as compared to 16 days in the figure. I would suggest making this consistent. Would it also be helpful to highlight some GFP-positive and GFP-negative nuclei with arrows?

We are now consistent with the timing of Dox treatment (16 days) between text and figure. We added arrows and arrowheads to highlight nuclei.

Line 115: At first appearance in the text, it would be good to further discuss H19 and Igf2 (type of RNA, annotated functions, …).

We have added further discussion to the text as requested. However, we do this at the stage when H19 and Igf2 are correlated with GFP+ nuclei and not at their first appearance (Fig. 1 when describing Dev population).

We did not feel it was appropriate to discuss within Fig. 1 because we are listing many genes and only focus on H19 and Igf2 later.

Line 231: 'Many of these DEGs are shared, …'. A Euler plot or Venn diagram would help the reader to interpret these data.

We have added a Venn diagram.

Line 266: Write abbreviations (NMJ, MTJ) in full to make it easier for reader.

This has been changed as requested.

Line 280-281: 'The Atf3 population was not enriched for H19, Igf2, Myh3, or Myh8 but instead was enriched for Atf3, Flnc, Enah, and Ankrd1'. Nrap and not Ankrd1 is highlighted in the dot plot (fig 4E).

We adjusted the text so it is consistent with the dot plot.

Line 340-343: Not that clear in figure 4E that unclassified A nuclei are similar to type IIA and ATF3+ nuclei, as stated in the text. Similar for statement that unclassified B are similar to type IIB. I would rephrase (tune down) these statements.

We agree and have tuned down our interpretations through adjustment/removal of text.

Line 724: Dot plot demonstrates upregulated (should be altered?) genes in Dev. Nuclei.

We changed to 'altered'.

Line 1055: Further explanation of DESeq2 steps needed (statistical model, any surrogate variable modelling, which QC steps, …).

These explanations have been added to the methods. QC was not performed within DESeq2 since QC was performed during processing, which is noted in the methods. Specific use of DESeq2 depended on the analysis and is available in the provided GitHub link.

snRNAseq data processing:
- Supplementary plot with number of features, number of UMIs and % mitochondrial genes could be useful as QC metrics
- Clustering: describe how many PCs were used and why

In the original version of the manuscript there was a supplementary table with features, UMIs and other QC metrics (Supplementary Table S7), which is the Cell Ranger output from data processing. In the revised version, we provide metrics on features, UMIs, and % mitochondrial genes in Extended Data Figure 5. The PC dimensions used to replicate the figures are available in the code available on GitHub. It should be noted that there is no standard determining the correct number of PCs through the SC transformed workflow in Seurat

and it's typically understood that higher number of PCs represent more subtle biological variation rather than technical artifacts as seen in the log normalized RNA-based processing.

Writing errors

Line 136: 'a long-half life' instead of 'a long half-life'

Line 217: 'dystropin'

Line 231: 'DEGS' instead of 'DEGs'

Line 472: 'the extent'

Line 911: '75% of the lateral …'

Line 991: 'uuclei'

Each writing error has been corrected and we thank the reviewers for catching these mistakes.

We thank the reviewers for careful review of our manuscript. We respond to each of their comments below in green font.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

Most of the points I raised in my initial review have been appropriately addressed. However, I have two remaining concerns:

We appreciate the reviewer indicated most of their concerns have been addressed.

1) Genotype of Controls: While the genotype is mentioned in the figure legend, it is not immediately noticeable. I suggest providing the genotype information directly in the figures themselves, in addition to the legend, to ensure clarity.

We have noted this suggestion.

2) Pax7 Haploinsufficiency: Although there are no reported developmental or overload-related issues associated with Pax7 haploinsufficiency in the literature, this aspect may not have been thoroughly investigated. Furthermore, the significant deficits in regeneration reported by Relaix et al. (DOI: 10.1186/s13287-023-03506-1) and other studies suggest that Pax7 haploinsufficiency impacts myogenesis more than what could be described as modest. I recommend addressing this point in the Discussion section.

It was difficult to address this concern about Pax7 haploinsufficiency in the discussion section due to flow. We agree it is possible that there could be impacts on myogenesis that have not been adequately investigated, and mentioned this caveat in the results sections.

Reviewer #1 (Remarks on code availability):

I am sorry but I am unable to review the code. A computational person should do this.

Reviewer #2 (Remarks to the Author):

The authors have addressed my comments -I have no further questions and congratulate them on their nice work.

We appreciate the positive comments from the reviewer.

Reviewer #2 (Remarks on code availability):

We have looked at the code in the initial revision and managed to download and use it.