

REST/NRSF preserves muscle stem cell identity by repressing alternate cell fate

Corresponding Author: Dr Vahab Soleimani

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Sahinyan et al. report that deletion of the Repressor Element 1-Silencing Transcription factor (REST) gene in muscle stem cells (MuSCs) results in derepression of non-muscle genes, reduced MuSC number in resting and regenerative conditions, and defective muscle regeneration consequent to MuSC apoptosis. Predicted REST binding sites are enriched at H3K4me3-marked promoter of non-expressed genes which are activated upon REST deletion.

1. REST Cut&Tag (or equivalent assay) would identify predicted REST binding sites which are indeed occupied by REST in MuSCs.
2. Ideally H3K27me3 maps should be generated to evaluate overlap between H3K4me3+/REST+ regions and bivalent (H3K4me3/H3K27me3+) promoters. H3K27me3 may be evaluated in REST-KO MuSCs. Alternatively, publicly available H3K27me3 MuSCs datasets may be employed for the analysis.
3. Presentation of GO terms (5-10 top, ranked according to p-value and gene list of the individual GO terms) of genes up- and down-regulated in REST-KO MuSCs would help in interpreting the biological significance of REST deletion.
4. Figure 4f. As acknowledged in the manuscript, being expressed in MuSCs, DCX is not a neurogenic-specific gene. Staining for a truly neurogenic-specific protein, not expressed in MuSCs, would address whether gene dysregulation in REST-KO results in productive protein synthesis of the corresponding non-muscle transcripts.
5. Figure 5b,c. Venn diagrams are difficult to read. A matrix might better illustrate expression of cell lineage-specific genes.
6. Lack of increased chromatin accessibility and transcriptional activation of muscle atrophy genes suggests that reduced muscle fiber size is likely due to ineffective MuSC contribution to muscle growth rather than protein catabolism. As such, the term muscle atrophy should be substituted with "reduced muscle growth" or analogous terms.
7. It is theoretically possible that apoptosis is induced by REST-dependent deregulation of anti-apoptotic genes. This can be determined by establishing whether anti-apoptotic genes are occupied by REST in MuSCs and whether their expression is up-regulated in REST-KO MuSCs.

Reviewer #2

(Remarks to the Author)

REST (also known as NRSF) and Myogenesis

REST (also known as NRSF) was discovered 30 years ago as a factor that represses neuronal genes. It is a well-characterized molecule that recruits coREST and, via coREST, histone deacetylases and other co-factors. Thus, REST functions in the epigenetic regulation and represses genes, typically neuronal genes. Around a dozen epigenetic factors

were already reported to be essential for the proper function of muscle stem cells and their derivatives, and REST can now be added to the ever-growing list.

While REST is most famous for repressing neuronal genes, it is well documented that it represses other genes, the exact ones depend on the context. REST is expressed broadly and maybe ubiquitously and is known to function in a multitude of cell types. In many cells types, genetic analyses have defined genes repressed by REST.

Sahinyan et al. report in their manuscript the function of REST in the myogenic lineage using genetic tools, i.e., Pax7cre, Pax7ERT2Cre, and REST^{fllox}. It is noteworthy that even the first analysis of a global knockout of REST done in 1998 (DOI: 10.1038/2431) demonstrated a disorganized myotome and a downregulation of myogenin.

The authors find deficits in myogenic cells (i.e., freshly isolated MuSC, myoblasts generated from MuSC expanded in vivo, and myofibers): myofibers are smaller in diameter, MuSC are reduced in numbers but appear to be otherwise phenotypically unaffected, but their derivatives (e.g., myoblasts) are prone to apoptosis. In three cell types, changes in gene expression are defined, and differentially expressed genes are detected. The authors provide a systematic analysis of this, but already a short glimpse at the table of de-repressed genes indicates that neuronal genes are most prominent (e.g., a large number at the top of the list that is sorted according to fold change gene expression). Moreover, there seems to be considerable overlap in the de-regulated genes in the three myogenic cell types. Finally, the authors find that the chromatin of upregulated genes gains in accessibility (ATAC-Seq) and loses repressive histone marks.

While the molecular analysis of the mutant phenotype relies on current state-of-the-art techniques, the histological, anatomical, and cell biological analysis is limited. The results presented were somewhat predictable: Neuronal genes and some genes of other lineages were upregulated in all three myogenic cell types with ablated REST. The authors claim that the changes observed are caused by deficits in MuSC, which is not supported by the data.

Specific Points:

1) The authors performed Cut&Tag of H3K4me3 and observed that 16% of the genes that are not expressed in MuSCs were annotated to H3K4me3 peaks. I assume from this that the genes expressed in MuSC are used for the entire comparison. Is it correct that the data shown in Fig 1i, j relied on a comparison of genes expressed in freshly isolated MuSC? How are the non-muscle genes defined? Muscle genes should be defined as the sum of genes expressed in deep quiescent MuSC, freshly isolated MuSC, myoblasts, and fibers. How many of the genes annotated as 'non-muscle' are expressed in these other types of myogenic cells?

2) In general, the anatomical, histological, and cell biological analysis of the REST mutants is incomplete, making the assessment of phenotypic changes impossible. For instance, after Pax7CreERT2 induced recombination of REST in juvenile mice, the animals underwent one or two rounds of injury, and subsequently reduced numbers of MuSC (around 50%) and small myofibers (not quantified) were observed. Deficits in differentiation might have caused the fiber deficits, or fiber growth might have been impaired. None of these potential mechanisms are analyzed.

3) A major point of the authors is the claim that the anatomical deficits observed in the muscle/myogenic lineage of REST mutants are due to a deficit in MuSC, but the presented data do not support this statement. The molecular data indicate that all myogenic stages (MuSC, myoblasts, and fibers) require REST, and the authors cannot even exclude that progenitors are also affected. Thus, the phenotypic changes might be caused by deficits in either progenitors, MuSC, myoblasts, or myofibers or a combination thereof (for the REST-cKO mutant), or in MuSC, myoblasts, or myofibers or a combination thereof (for the iCKO mutant). A better anatomical, histological, and cell biological analysis is needed to distinguish the different options.

4) The authors write: "smfRNA-Seq showed a modest change with 160 significantly differentially expressed genes.... Given the lower expression of REST in myofibers compared to MuSCs and primary myoblasts (Extended Data Fig. 3c), the modest alteration in gene expression of myofibers suggests that REST may not play a prominent role in the myofiber transcriptome." This argument is not valid. In MuSC, 226 differentially expressed genes were detected, and in fibers, 160 differentially expressed genes, nevertheless the authors dismiss the myofiber changes as irrelevant, and assign the deficits to MuSC. A brief look at the table of significantly changed genes shows that the fold changes in myofibers are very significant.

5) It is difficult to compare datasets obtained by bulk RNA-seq and smfRNA-Seq; please discuss whether smfRNA-Seq data have a similar depth as the bulk RNA-seq.

6) Similarly, the authors state that muscle fibers show a reduced diameter and argue that the absence of deregulated genes like *Murf* indicates that the change in diameter "is most likely due to the altered numbers and function of REST-cKO MuSCs, rather than changes in the myofiber itself." Fiber diameter is not only regulated by atrophy, and other mechanisms but inappropriate degradation of muscle proteins might cause a reduced diameter. The observed changes in gene expression in myofibers support the hypothesis that myofibers are directly affected.

7) Although MuSC are argued to be affected, apoptosis is analyzed exclusively in myoblasts (e.g., MyoD⁺ cells). Increased apoptosis is observed for activated MuSC on fibers (these cells co-express MyoD/Pax7 after 48 hrs culture, e.g., should be called myoblasts) or cultured myoblasts derived from primary cells. If the authors want to argue that MuSC undergo apoptosis, they should analyze MuSC.

8) There seems to be considerable overlap in the deregulated genes in the three myogenic cell types. Please also compare genes affected in myotubes to those deregulated in MuSC/myoblasts. How large is the overlap?

9) The authors describe ATAC-seq on single myofibers: "PCA of smfATAC-Seq shows that the chromatin state of the myofibers between the WT and REST-cKO do not show a distinct signature (Extended Data Fig. 5c)." To me, the PCA seems to indicate that the smfATAC-Seq data on WT fibers are not very reproducible; i.e., one wt dataset is very different from the REST-cKO, the other is not. The number of datasets should be extended. #

10) The examples tracks of smfATAC-Seq data are shown for genes that are not significantly changed in the fibers. Please provide examples of tracks for genes that are significantly changed. Also, it would have been interesting to know if the changes in the myofiber expression are associated with changes in histone modifications (i.e., recruitment of HDACs).

11) Also, please use the ATAC-Seq data obtained with MuSC to provide examples of ATAC-Seq tracks of genes whose expression is not changed in MuSC.

Reviewer #3

(Remarks to the Author)

In this manuscript, the authors showed that the master epigenetic regulator REST1 plays a crucial role in maintaining muscle stem cell (MuSC) identity and survival. They first observed that many non-muscle lineage genes in freshly sorted MuSCs retain the active chromatin mark H3K4me3, despite being transcriptionally repressed. Analysis of the DNA sequences under the H3K4me3 peaks of these genes revealed an enrichment for the REST binding motif. Then, using a genetic mouse model, they found that REST deletion leads to a depletion of the MuSC pool, defective muscle regeneration, and muscle atrophy. Further profiling studies indicate that REST deletion alters MuSC chromatin and transcription states, leading to a loss of myogenic identity. While this study solidly demonstrates REST1's role in safeguarding muscle stem cell identity with comprehensive phenotype and profiling data, the manuscript's quality could be further improved by enhancing the logical flow and delving deeper into the mechanistic studies.

Below are suggestions for the authors to consider in order to further improve their manuscript:

1. Profiling of histone marks in Figure 1: Given its foundational role in this study, the interesting target genes identified in the sequencing results should be validated using Cut&Tag-qPCR. This is crucial to address potential false positives inherent in high-throughput sequencing.
2. The transition to investigating REST is somewhat ambiguous, even though the authors mention finding enrichment for the REST binding motif. Motif analysis likely revealed enrichment for other transcription factors as well, which should be shown in the paper, at least in an extended data figure. A clearer rationale for focusing on REST over other transcription factors is needed.
3. The authors state, "We defined a list of 134 genes as being expressed or not expressed at a read per million (RPM)>5 or RPM<1 respectively." Clarification is needed on how genes with $1 < \text{RPM} < 5$ is categorized.
4. The authors provide extensive profiling data (smfRNA-seq, smfATAC-seq, SMART-seq, and ATAC-seq) on the chromatin and transcription state changes following REST depletion. However, these data are largely descriptive. The key question of how REST functions to regulate muscle stem cell identity is not deeply explored. Specially, how REST, together with other corepressors, represses the expression of non-muscle lineage genes with epigenetic active mark and the down-stream factor(s) of REST involved in this process are still not clear.
5. REST deletion in the genetic mouse model is only validated by qPCR (Extended Data Fig. 4b). Given the potential discrepancies between RNA and protein levels, it would be better to confirm REST protein levels via western blot or immunohistochemistry. The authors showed that REST deletion leads to apoptosis of muscle cells in Figure 6. Given that they have sequencing data for the samples as well, does the analysis of this sequencing data support the conclusion? Are any apoptosis-related biological processes enriched in REST-cKO cells?
6. Different numbers of replicates were used for various sequencing methods (e.g., ATAC-seq n=4, RNA-seq n=3, H3K4me3 n=2). The rationale behind these choices should be included in the Statistics section of the Methods part.
7. The results and discussion sections are too lengthy and difficult to follow. The results section should be concise, particularly the descriptive parts. The discussion often repeats results and should be condensed.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In Figure 1d,e,f, tracks for REST CUT&RUN should be presented along those of histone marks.

Reviewer #2

(Remarks to the Author)

Several concerns already raised in my original review have not been addressed.

The manuscript suffers from shortcomings in the context of existing data on Rest in myogenic cells. Moreover, the authors' primary conclusions, specifically regarding the functional consequences of Rest ablation in MuSCs, are inadequately supported by the presented data. These issues must be addressed before this work can be considered for publication.

1) Rest Ablation and Its Consequences:

The introduction fails to appropriately mention prior findings that Rest ablation results in the expression of neuronal genes in the myotome, a disorganized myotome, and the downregulation of myogenic differentiation markers (DOI: 10.1038/2431). These findings should be mentioned to establish the relevance of this study within the broader field.

2) Unsupported Claims on MuSC Functional Impairment and Apoptosis:

The authors claim throughout the manuscript (Abstract, Introduction, Results, and Discussion) that Rest mutant MuSCs are functionally impaired and undergo apoptosis. However, the data presented do not support this assertion. Instead, the findings indicate that apoptosis occurs in Rest mutant myoblasts—cells derived from MuSCs—in bulk cultures of primary myogenic cells, or when MuSC associated with myofibers are cultured on the fibers for 48 h. These cells, which express MyoD, are clearly myoblasts, not MuSCs.

Additionally, the authors incorrectly state that cells in fiber culture are located in their niche. Activated cells in 48-hour fiber culture are positioned outside the basal lamina (<https://doi.org/10.1002/stem.178>) and therefore cannot be considered as residing in their niche.

If the authors are unable to provide direct evidence that MuSCs undergo apoptosis or are otherwise functionally impaired, they must revise their claims accordingly. At best, they can state that changes in gene expression are observed in MuSCs, but there is no data to support functional impairment. Consequently, any claims regarding MuSC functional impairment should be removed from the manuscript.

3) Interpretation of PCA Results (Extended Data Fig. 5c):

The authors state: "PCA of smfATAC-Seq shows that the chromatin state of the myofibers between the WT and REST-cKO do not show a distinct signature." This statement is misleading. The PCA plot reveals that one WT dataset is notably distinct from REST-cKO, whereas the other is not. The authors should explicitly state that the PCA does not significantly separate REST-cKO and WT myofibers. Furthermore, they must discuss the possibility that this lack of separation is due to the heterogeneity of muscle fibers and acknowledge the need for a larger dataset to draw any definitive conclusions.

In summary, these deficiencies undermine the strength of the manuscript's conclusions and must be rectified to ensure accurate interpretation and presentation of the findings.

Reviewer #3

(Remarks to the Author)

In this revised manuscript, the authors have made substantial efforts to address the reviewers' comments and suggestions. The manuscript has been significantly improved through the inclusion of new data, particularly the CUT&Tag data for REST and H3K27me3 in freshly sorted muSCs, as well as additional analyses, clarifications, and explanations. The text has also been carefully edited to enhance readability and improve the overall flow. This reviewer has no further concerns.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The authors have failed to address my concern experimentally but continue to claim that MuSCs undergo apoptosis. Instead, they present data that suggest that myoblasts (or myogenic progenitors) undergo apoptosis and/or exhibit impaired differentiation in REST mutants. The text in the abstract, introduction, and throughout the manuscript must be revised to accurately reflect these findings.

Alternatively, the authors could address this point in the discussion (e.g. a paragraph on limitations of the study).

1) The authors repeatedly claim that MuSCs undergo apoptosis in REST mutants (e.g., Abstract: "Consequently, MuSCs enter cell death by apoptosis and the stem cell pool is depleted"), yet they never actually analyze apoptosis in MuSCs. Instead, their data assess apoptosis in myoblasts.

In my previous assessment, I already pointed out that the claims must either be revised or supported by experiments directly demonstrating that MuSCs undergo apoptosis. The authors argue that assessing apoptosis in MuSCs in vivo is difficult due to their low numbers.

2) The authors argue in their rebuttal that the MuSC pool is depleted upon REST deletion, and that they provided evidence from two different mutant models. While it is correct that the MuSC pool is reduced, the mechanism underlying this depletion remains unclear—specifically, whether MuSCs themselves undergo apoptosis or whether their progenitors do.

In the Pax7^{cre} mutants, REST deletion occurs around mid-gestation, yet the effects are only analyzed in adulthood. This makes it impossible to determine when the deficit arises. The significantly smaller fiber diameter suggests that fiber growth was impaired, likely due to a loss of myogenic progenitor cells (myoblasts) or a differentiation defect in progenitors that interfered with fiber development. A loss of myogenic progenitor cells could have also compromised the formation of the

postnatal MuSC pool. Thus, the analysis of Pax7cre mutants does not demonstrate that MuSCs lacking REST undergo apoptosis—rather, it shows that the myogenic lineage is disrupted and that apoptosis occurs in myogenic progenitor cells (myoblasts) when REST is absent.

Similarly, in the Tamoxifen-inducible Cre/REST mutants, MuSCs are only analyzed after injury. The authors did not conduct experiments tracking MuSC pool dynamics following REST deletion at different time points in an unchallenged state. Instead, they only examine the MuSC pool after 1–2 rounds of injury, where depletion is observed. Given the provided data, it is highly plausible—if not most likely—that transient amplifying MyoD+ MuSCs (i.e., myoblasts) were depleted, which in turn disrupted the replenishment of the quiescent MuSC pool.

Additionally, the authors show that REST-cKO cells undergo apoptosis in fiber culture assays, where MuSCs become activated, express MyoD, and thus transition into transient amplifying myoblasts. Therefore, the claim that MuSCs undergo apoptosis also remains unsubstantiated by this experiment. The authors must revise the text of their manuscript to accurately reflect their findings.

Additional points:

1) The Cre line used (Pax7cre, Jax, #010530) induces Rest deletion throughout the entire myogenic lineage, as it is expressed early in development in myogenic progenitors that eventually give rise to MuSCs. However, the authors misleadingly imply that the mutation is introduced only in MuSCs (e.g., page 11: “creating an exon 4 genetic deletion of REST in Pax7-expressing MuSCs”).

To avoid misinterpretation, the authors should explicitly state at the beginning of the paragraph that Rest mutation occurs in the entire myogenic lineage, not just in MuSCs. This distinction is crucial for accurately interpreting the results and understanding the potential effects of Rest loss at different stages of muscle development.

Version 3:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The authors write (line 286)

This data corroborates that the previously observed loss of the MuSC pool is not the result of a defect during embryogenesis, but rather an effect on the adult MuSCs.

In my opinion, these data indicate that apoptosis of myogenic progenitors (myoblasts) during development and activated MuSC (myoblasts) during regeneration is responsible for the loss of the MuSC pool. If the authors want to argue that developmental deficits are not present, they should provide data.

That the loss of MuSC pool is an effect on the adult MuSCs is also not substantiated by data, as I pointed out previously. Please provide such data, or abstain from arguing that MuSC are affected.

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We thank all reviewers for their insightful comments and critic of our manuscript. As indicated in point-by-point response to reviewers, we have performed new experiments, conducted additional analyses and extensively revised the manuscript.

Reviewer #1 (Remarks to the Author):

Sahinyan et al. report that deletion of the Repressor Element 1-Silencing Transcription factor (REST) gene in muscle stem cells (MuSCs) results in derepression of non-muscle genes, reduced MuSC number in resting and regenerative conditions, and defective muscle regeneration consequent to MuSC apoptosis. Predicted REST binding sites are enriched at H3K4me3-marked promoter of non-expressed genes which are activated upon REST deletion.

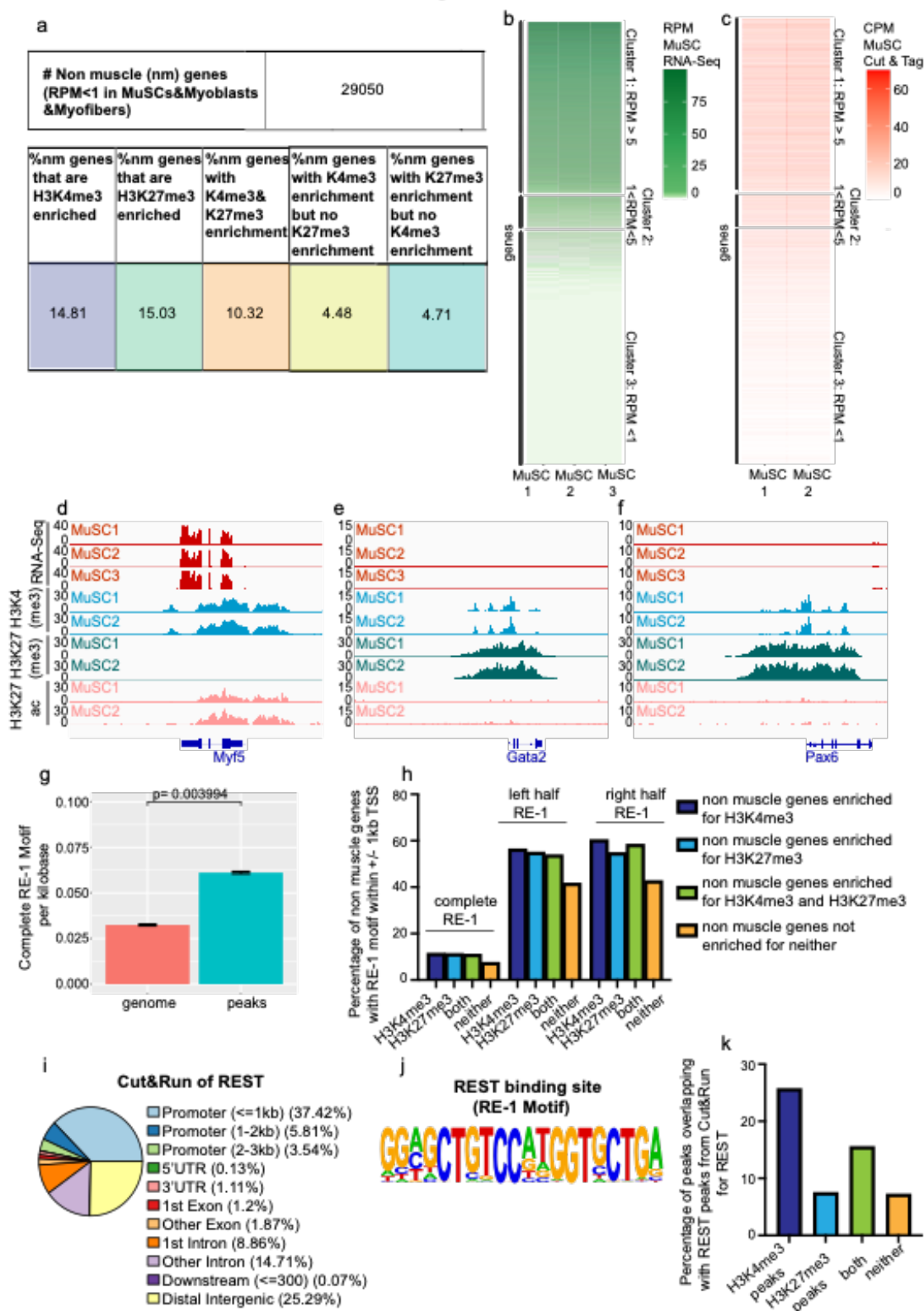
1. REST Cut&Tag (or equivalent assay) would identify predicted REST binding sites which are indeed occupied by REST in MuSCs.

We thank the reviewer for this valuable suggestion. To investigate the REST binding sites, we have now performed Cut&Run for REST in cultured myoblasts derived from *in vitro* expansion of muscle stem cells. We have opted to perform Cut&Run as opposed to Cut&Tag since Cut&Run is generally more recommended for investigation of transcription factors due to improved sensitivity and reduced possible background compared to Cut&Tag.

To identify REST binding sites, we have successfully performed Cut&Run for REST in low passage cultured myoblasts and validated the known REST binding sites. Computational analysis of REST peaks showed high enrichment of the RE-1 motif within the REST peaks as well as the REST binding mostly in the promoter and enhancer regions as shown by the peak annotation analysis (Fig. 1i,j). Furthermore, we intersected the Cut&Run for REST with histone mark data which revealed a substantial overlap between the number of REST peaks and H3K4me3 and H3K27me3 enriched peaks for non-muscle genes indicating an important role for REST regulation of non-expressed genes that retain an active or bivalent chromatin state in MuSCs (Fig. 1k). Please see below Fig. 1i-k and page 9 on the manuscript which now reads as:

Peak annotation analysis revealed that most of the REST peaks were in the promoter and enhancer regions as expected, given its known regulatory role in gene repression (Fig. 1i) and that REST binding sites contained the RE-1 motif (Fig. 1j). We then investigated the overlap of REST binding sites with the histone marks demarcating active or bivalent promoters on non-muscle genes. We found that approximately 25% of the REST peaks overlapped with the H3K4me3 peaks within the TSS of non-muscle genes and 16% of the REST peaks overlapped with the common peaks between H3K4me3 and H3K27me3 on the promoter of non-muscle genes (Fig. 1k). Overall, our new data corroborates our previous assertion that REST may be playing an important role in remodeling the chromatin state of the non-myogenic lineage genes.

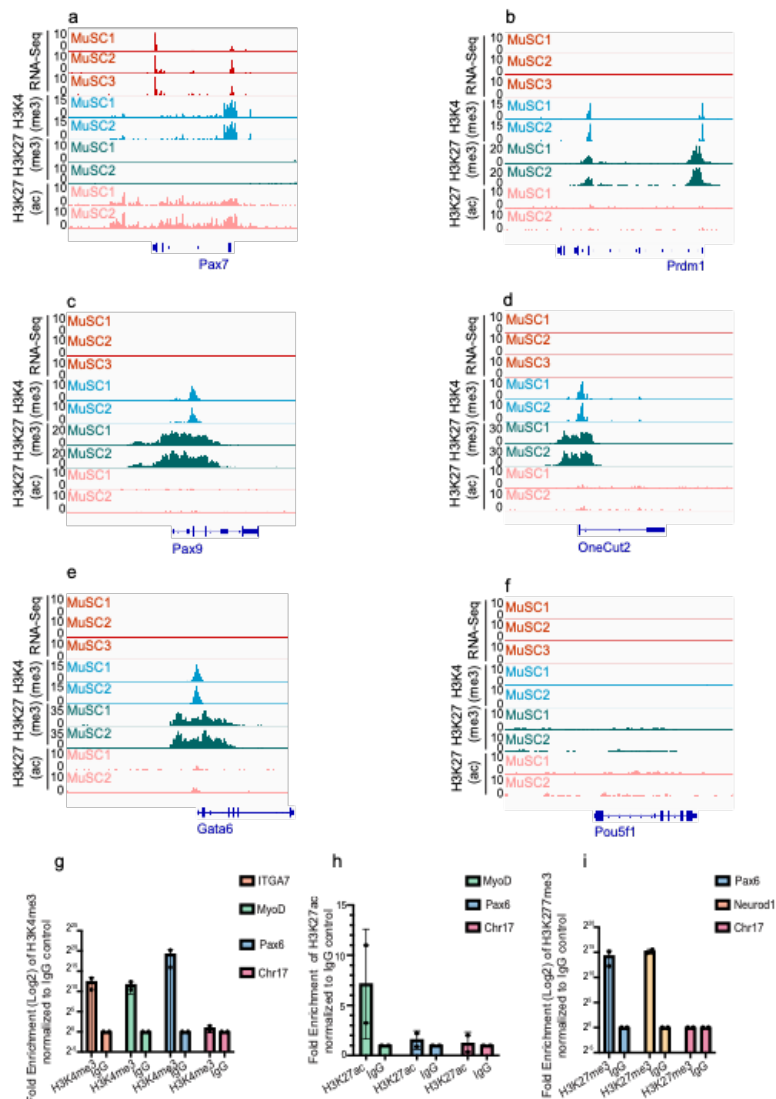
Figure 1



2. Ideally H3K27me3 maps should be generated to evaluate overlap between H3K4me3+/REST+ regions and bivalent (H3K4me3/H3K27me3+) promoters. H3K27me3 may be evaluated in REST-KO MuSCs. Alternatively, publicly available H3K27me3 MuSCs datasets may be employed for the analysis.

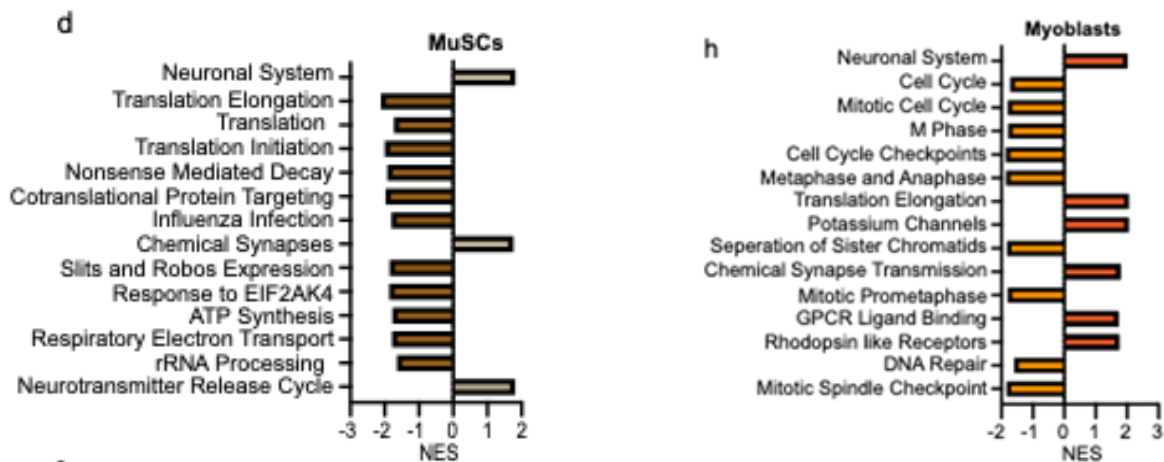
We thank the reviewer for their comment. We have now performed Cut&Run for H3K27me3 on freshly sorted MuSCs. The findings of this new data are shown in Fig. 1 and Extended Data Fig. 2 and are discussed on pages 5-9. Briefly, we have determined that approximately 10% of the non-muscle genes were enriched for both H3K4me3 and H3K27me3 marks, indicating a bivalent chromatin state. Additionally, we determined that the non-muscle genes that contained the active chromatin mark also contained the H3K27me3, indicating their bivalent state (Fig. 1d-f, Extended Data Fig. 2a-f). Furthermore, we have shown that approximately 10% of the non-muscle genes that have the bivalent chromatin state contain the RE-1 (REST binding motif), further validating the key role REST plays in regulating the expression of non-muscle lineage genes.

Extended Data Figure 2



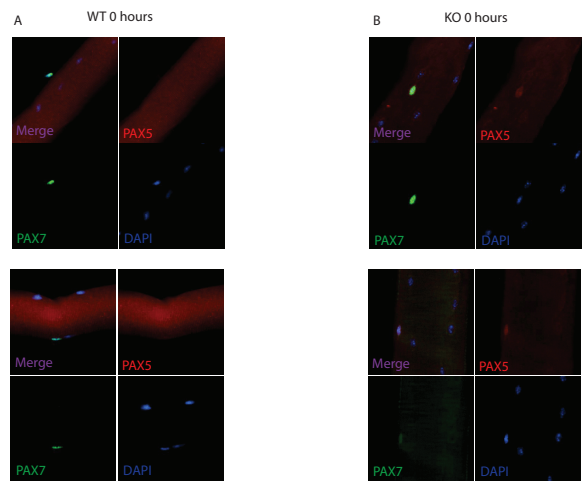
3. Presentation of GO terms (5-10 top, ranked according to p-value and gene list of the individual GO terms) of genes up- and down-regulated in REST-KO MuSCs would help in interpreting the biological significance of REST deletion.

We have performed Gene Set Enrichment Analysis on the differentially expressed genes between REST-cKO and WT MuSCs and myoblasts which are shown in Fig. 4d,h respectively. These represent the top significant Reactome Pathways (p-adj <0.1).



4. Figure 4f. As acknowledged in the manuscript, being expressed in MuSCs, DCX is not a neurogenic-specific gene. Staining for a truly neurogenic-specific protein, not expressed in MuSCs, would address whether gene dysregulation in REST-KO results in productive protein synthesis of the corresponding non-muscle transcripts.

We agree with the reviewer that in addition to neuronal cells, DCX is also expressed in non-neuronal cells. Indeed, recent research from our own lab shows that DCX is an early activation gene in muscle stem cells (Blackburn et al 2024 iScience). Therefore, expression of DCX in REST-KO muscle stem cells suggests defective quiescence state. To show a representative non-muscle lineage gene that is repressed by REST, we have performed IF staining of Pax5 on myofiber associated MuSCs at 0 hr which is shown in figure below. Pax5 is an important transcription factor for the lymphoid tissue development and is not expressed in WT MuSCs but gain expression upon deletion of REST as previously shown in Fig. 5e. IF staining (shown below) of MuSCs for Pax5 suggests that the changes in gene expression in REST-cKO are correlated with the protein changes where Pax5 gains expression in the REST-cKO MuSCs.



5. Figure 5b,c. Venn diagrams are difficult to read. A matrix might better illustrate expression of cell lineage-specific genes.

We thank the reviewer for their suggestion. Please see below the matrix representation of this figure where the number of differentially expressed genes that are expressed in each representative tissue but not in MuSCs are shown. While the matrix shows the number of differentially expressed genes that are common between the tissues, it does not demonstrate the number of differentially expressed genes uniquely in that tissue. For that reason, we think a Venn diagrams may better represent and encompass this information.

Column1	Brain	Heart	Kidney	Liver	Pancreas	Thymus	Digestive System
Brain	115	7	16	4	4	10	34
Heart	7	12	8	3	1	9	11
Kidney	16	8	27	9	2	8	21
Liver	4	3	9	13	2	3	10
Pancreas	4	1	2	2	5	0	5
Thymus	10	9	8	3	0	19	12
Digestive Sys	34	11	21	10	5	12	55

6. Lack of increased chromatin accessibility and transcriptional activation of muscle atrophy genes suggests that reduced muscle fiber size is likely due to ineffective MuSC contribution to muscle growth rather than protein catabolism. As such, the term muscle atrophy should be substituted with “reduced muscle growth” or analogous terms.

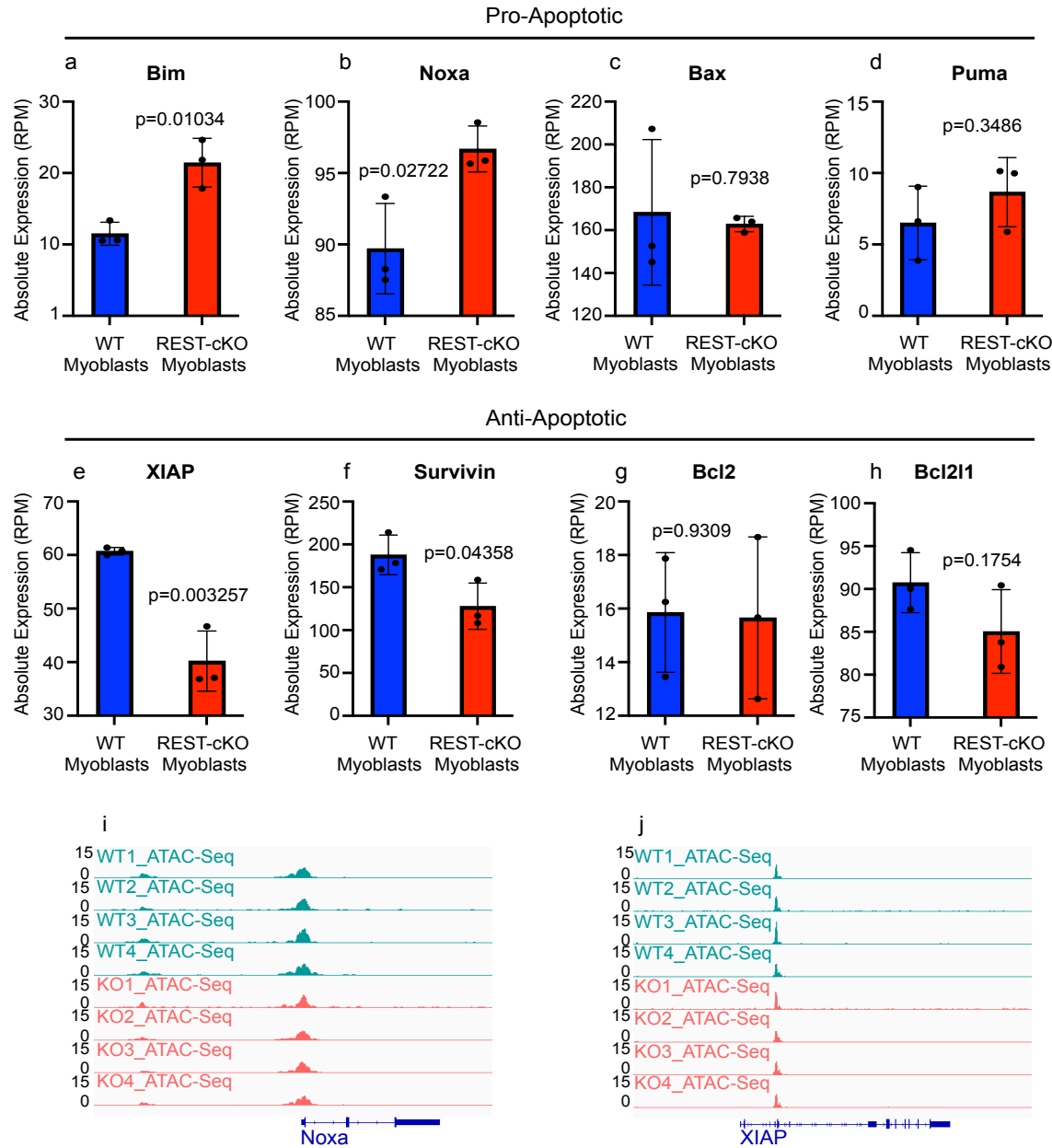
We have now edited the text to incorporate the suggested change whenever appropriate. Please see the edited section title on page 10 which now reads as “ Genetic deletion of REST leads to depletion of the MuSC pool and subsequent reduction in myofiber size in adult mice” as well as the edited text on page 12 : “ Overall, this data shows that the genetic deletion of REST leads to depletion of the MuSC pool, defective muscle regeneration and reduced myofiber sizes, indicating that REST is required for proper MuSC function and muscle health.” In addition please see the edited texts on page 12 “ Decrease in myofiber size is not associated with the myofiber specific transcriptomic and epigenetic changes in REST-cKO mice ” and on page 13 “ Given the significant reduction in the muscle size observed in REST-cKO mice, we investigated the transcriptomic and epigenetic alterations in the myofibers upon REST deletion to assess the contribution of the myofiber mediated changes to the reduced myofiber size that is observed.” Finally, please see the text on page 15 : “ Despite a significant reduction in the myofiber sizes suggesting muscle atrophy in the REST-cKO mice, neither the chromatin accessibility nor the gene expression of atrophy associated genes in the myofibers change. This data suggests that the observed changes in the muscle cross sectional areas are most likely the result of reduced muscle growth due to the altered numbers and function of REST-cKO MuSCs, rather than the changes in the myofibers themselves.”

7. It is theoretically possible that apoptosis is induced by REST-dependent deregulation of anti-apoptotic genes. This can be determined by establishing whether anti-apoptotic genes are occupied by REST in MuSCs and whether their expression is up-regulated in REST-KO MuSCs.

We have now investigated the gene expression and chromatin data in REST-cKO and WT myoblasts more closely in terms of the apoptotic and anti-apoptotic genes. While some of the pro-apoptotic genes had significantly increased expression upon REST deletion such as *Bim* and *Noxa* (Additional Figure a,b), other pro-apoptotic genes did not show a significant change in expression including *Bax* and *Bak* genes (Additional Figure c,d). Similarly, some of the anti-apoptotic genes showed downregulation in the REST-cKO such as *Survivin* and *XIAP* (Additional Figure e,f) while other anti-apoptotic genes such as *Bcl2* and *Bcl2l1* (Additional Figure g,h) did not show a significant change in their expression. Additionally, chromatin accessibility of the above mentioned genes do not show a change upon REST deletion as shown by the IGV tracks of ATAC-Seq on WT and REST-cKO MuSCs (Additional Figure i,j). This data suggest that induction of apoptosis in REST-KO cells is the result of muscle cell identity due to deregulated gene expression profile rather than direct inhibition of apoptotic pathway genes by REST.

Furthermore, Gene Set Enrichment analysis of the RNA-Seq of REST-cKO and WT cells do not indicate apoptosis as a significantly enriched pathway in the DEG between the KO and the WT (Fig. 4d,h). However, GSE analysis does indicate significant changes in processes such as cell cycle, cell cycle checkpoints, ETC, translation and nonsense mediated decay (Fig. 4d,h) all of which can affect cell fate and ultimate induction of apoptosis. Therefore, the above data indicates that REST may not directly regulate the apoptotic pathway but may protect MuSCs against apoptosis by maintaining their myogenic lineage identity by repressing alternate pathways. When REST is genetically deleted in MuSCs, non-muscle lineage genes gain expression and cells go in a state of lineage confusion which triggers apoptosis mediated cell death. While REST does not directly regulate the transcription of apoptosis related genes, loss of REST in MuSCs consequently leads to cell death as we have clearly shown by *in vitro* and *in vivo* experiments.

Additional Figure



Reviewer #2 (Remarks to the Author):

REST (also known as NRSF) and Myogenesis

REST (also known as NRSF) was discovered 30 years ago as a factor that represses neuronal genes. It is a well-characterized molecule that recruits coREST and, via coREST, histone deacetylases and other co-factors. Thus, REST functions in the epigenetic regulation and represses genes, typically neuronal genes. Around a dozen epigenetic factors were already reported to be essential for the proper function of muscle stem cells and their derivatives, and REST can now be added to the ever-growing list.

While REST is most famous for repressing neuronal genes, it is well documented that it represses other genes, the exact ones depend on the context. REST is expressed broadly and maybe ubiquitously and is known to function in a multitude of cell types. In many cells types, genetic analyses have defined genes repressed by REST.

Sahinyan et al. report in their manuscript the function of REST in the myogenic lineage using genetic tools, i.e., Pax7cre, Pax7ERT2Cre, and RESTflox. It is noteworthy that even the first analysis of a global knockout of REST done in 1998 (DOI: 10.1038/2431) demonstrated a disorganized myotome and a downregulation of myogenin.

The authors find deficits in myogenic cells (i.e., freshly isolated MuSC, myoblasts generated from MuSC expanded in vivo, and myofibers): myofibers are smaller in diameter, MuSC are reduced in numbers but appear to be otherwise phenotypically unaffected, but their derivatives (e.g., myoblasts) are prone to apoptosis. In three cell types, changes in gene expression are defined, and differentially expressed genes are detected. The authors provide a systematic analysis of this, but already a short glimpse at the table of de-repressed genes indicates that neuronal genes are most prominent (e.g., a large number at the top of the list that is sorted according to fold change gene expression). Moreover, there seems to be considerable overlap in the de-regulated genes in the three myogenic cell types. Finally, the authors find that the chromatin of upregulated genes gains in accessibility (ATAC-Seq) and loses repressive histone marks.

While the molecular analysis of the mutant phenotype relies on current state-of-the-art techniques, the histological, anatomical, and cell biological analysis is limited. The results presented were somewhat predictable: Neuronal genes and some genes of other lineages were upregulated in all three myogenic cell types with ablated REST. The authors claim that the changes observed are caused by deficits in MuSC, which is not supported by the data.

Specific Points:

1) The authors performed Cut&Tag of H3K4me3 and observed that 16% of the genes that are not expressed in MuSCs were annotated to H3K4me3 peaks. I assume from this that the genes expressed in MuSC are used for the entire comparison. Is it correct that the data shown in Fig 1i, j relied on a comparison of genes expressed in freshly isolated MuSC? How are the non-muscle

genes defined? Muscle genes should be defined as the sum or genes expressed in deep quiescent MuSC, freshly isolated MuSC, myoblasts, and fibers. How many of the genes annotated as ‘non-muscle’ are expressed in these other types of myogenic cells?

In the initial analysis, the non-expressed genes were based on the RNA-Seq of freshly sorted MuSCs where non expressed genes included those that had RPM<1 in MuSCs. However, now we have redone all of the analysis with a novel list of non- muscle genes as suggested. This new non-muscle genes are based on our RNA-Seq of freshly isolated MuSCs, cultured myoblasts and myofibers. The non-muscle genes are defined as those that have RPM<1 in MuSCs, myoblasts and myofibers. Meaning these genes do not have any appreciable expression in muscle stem cells, the downstream progenitors or fully differentiated muscle fibers. The new analysis with the new list has yielded very similar results to our previous analysis where 14.81% of non-muscle genes were annotated to H3K4me3 (Fig. 1a). Additionally, we have now added Cut&Run of H3K27me3 and performed similar analysis (Fig. 1a). The new list of non-muscle genes were also used in the remainder of the analysis that are shown in Fig. 1a,g,h and Extended Data Fig. 1b,c. Please see edited Fig. 1 and Extended Data Fig. 1.

Extended Data Figure 1

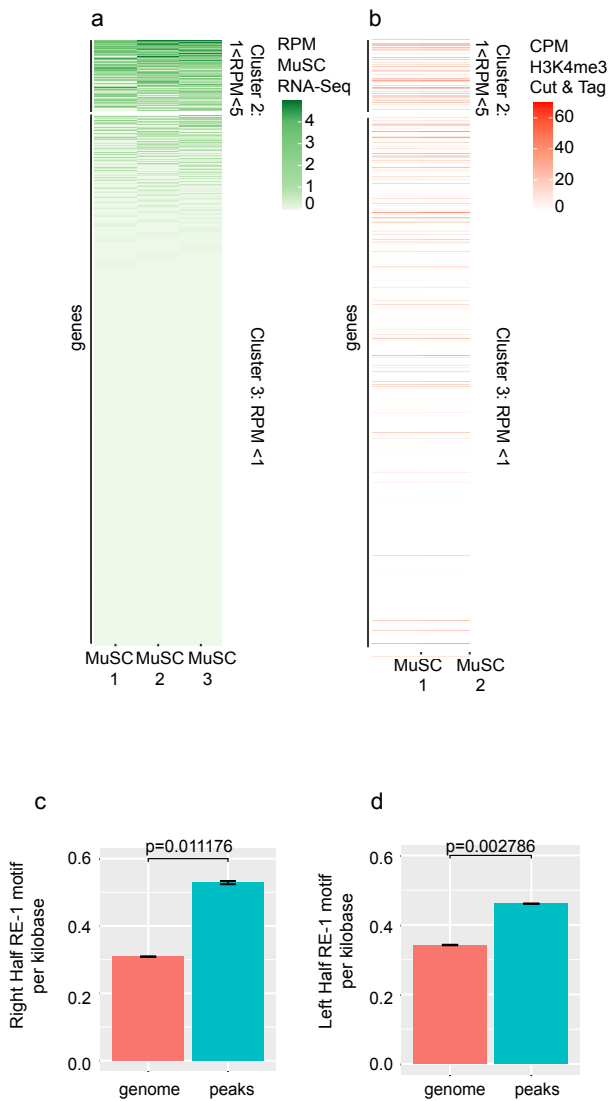
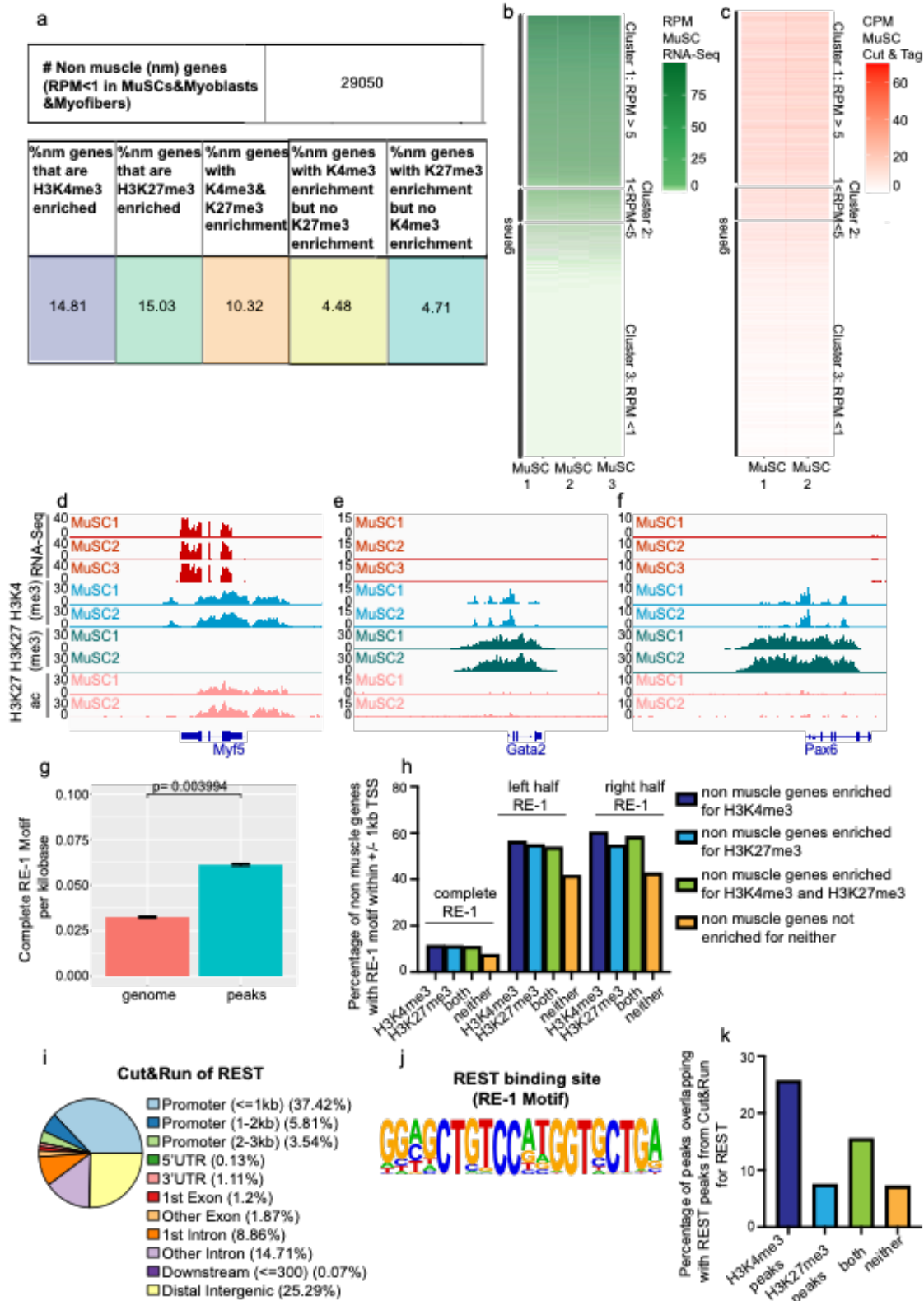


Figure 1



2) In general, the anatomical, histological, and cell biological analysis of the REST mutants is incomplete, making the assessment of phenotypic changes impossible. For instance, after Pax7CreERT2 induced recombination of REST in juvenile mice, the animals underwent one or two rounds of injury, and subsequently reduced numbers of MuSC (around 50%) and small myofibers (not quantified) were observed. Deficits in differentiation might have caused the fiber deficits, or fiber growth might have been impaired. None of these potential mechanisms are analyzed.

REST is expressed in all stages of muscle cells (i.e., stem cells, proliferating progenitors and differentiated muscle fibers) where it represses non-muscle lineage genes. However, differential analysis of gene expression between REST-KO and WT in all conditions shown that the highest level of significantly deregulated genes occurs between REST-KO myoblasts and their WT counterparts as indicated in new Extended Data Fig. 7d. Therefore, as we indicate throughout the manuscript loss of REST leads to deficit in muscle stem cell pool and impairs differentiation because REST-KO muscle stem cells and myoblasts undergo cell death as a result of simultaneous expression of numerous non-muscle lineage genes and loss of cell identity. We have clearly demonstrated this pattern by numerous *in vivo* and *in vitro* analysis.

3) A major point of the authors is the claim that the anatomical deficits observed in the muscle/myogenic lineage of REST mutants are due to a deficit in MuSC, but the presented data do not support this statement. The molecular data indicate that all myogenic stages (MuSC, myoblasts, and fibers) require REST, and the authors cannot even exclude that progenitors are also affected. Thus, the phenotypic changes might be caused by deficits in either progenitors, MuSC, myoblasts, or myofibers or a combination thereof (for the REST-cKO mutant), or in MuSC, myoblasts, or myofibers or a combination thereof (for the icKO mutant). A better anatomical, histological, and cell biological analysis is needed to distinguish the different options.

As we explained earlier, REST is required for the repression of non-muscle lineage genes in all myogenic stages including proliferating progenitors. As shown in new Extended Data Fig. 7d, the number of differentially expressed genes between REST-KO and WT is as follow: muscle stem cells, 224 genes, myoblasts 1510 genes and myofibers 165 genes. Therefore, although REST plays an important role in in all myogenic stages, it has the highest effect in progenitors based on the number of differentially expressed genes between WT and KO counterparts.

4) The authors write: "smfRNA-Seq showed a modest change with 160 significantly differentially expressed genes.... Given the lower expression of REST in myofibers compared to MuSCs and primary myoblasts (Extended Data Fig. 3c), the modest alteration in gene expression of myofibers suggests that REST may not play a prominent role in the myofiber transcriptome." This argument is not valid. In MuSC, 226 differentially expressed genes were detected, and in fibers, 160 differentially expressed genes, nevertheless the authors dismiss the myofiber changes as irrelevant, and assign the deficits to MUSC. A brief look at the table of significantly changed genes shows that the fold changes in myofibers are very significant.

We thank the reviewer for their comment. In the transcriptome of single myofibers, we do not observe a significant change in the expression of muscle specific genes between the REST-cKO

and WT. We have shown that the differences in the transcriptome in the REST-cKO myofibers are driven by the changes in the neurogenic program given the known role of REST. Given the very little change in the muscle specific transcriptome along with the relatively lower expression of REST in the myofibers (Extended Data Fig. 4c), we predict that the role of REST in myofibers is primarily the repression of non-myogenic genes.

We have now edited the text to further clarify this point on page 13 which now reads as: “Differential gene expression analysis between the myofibers of WT and REST-cKO mice revealed 165 significant differentially expressed genes (absolute $\log_2FC > 1$, $p\text{-adj} < 0.05$). However, quantification of the expression of muscle specific genes such as muscle creatine kinase (*Ckm*, (Fig. 3d, Extended Data Figure. 6a), actin alpha 1 (*Acta 1*) and myosin heavy chain 4 (*Myh4*) (Extended Data Figure. 6a) in myofibers revealed no significant difference in the REST-cKO and WT myofibers. This suggests that REST may not be playing a prominent role in myofiber transcriptome possibly due to its lower expression in myofibers compared to MuSCs and primary myoblasts (Extended Data Fig. 4c).”

5) It is difficult to compare datasets obtained by bulk RNA-seq and smfRNA-Seq; please discuss whether smfRNA-Seq data have a similar depth as the bulk RNA-seq.

smfRNA-seq and bulk RNA-seq have comparable depth and cover full gene transcript. We have made extensive comparative analysis of these technologies in our previous publication (Lazure et al 2020 EMBO Reports; Blackburn et al 2019 JBC; Lazure et al 2023 Nature Communications).

6) Similarly, the authors state that muscle fibers show a reduced diameter and argue that the absence of deregulated genes like *Murf* indicates that the change in diameter "is most likely due to the altered numbers and function of REST-cKO MuSCs, rather than changes in the myofiber itself." Fiber diameter is not only regulated by atrophy, and other mechanisms but inappropriate degradation of muscle proteins might cause a reduced diameter. The observed changes in gene expression in myofibers support the hypothesis that myofibers are directly affected.

Our single myofiber RNA-Seq from WT and REST-cKO mice show that the main differences in the myofibers are driven from the neurogenic gene programs being upregulated in the KO. The expression of muscle specific genes or muscle atrophy genes do not show a difference upon REST deletion. Therefore, we use the term “fiber growth” instead of atrophy for clarity. Please see the edited texts on pages 12,13 and 15.

7) Although MuSC are argued to be affected, apoptosis is analyzed exclusively in myoblasts (e.g., MyoD⁺ cells). Increased apoptosis is observed for activated MuSC on fibers (these cells co-express MyoD/Pax7 after 48 hrs culture, e.g., should be called myoblasts) or cultured myoblasts derived from primary cells. If the authors want to argue that MuSC undergo apoptosis, they should analyze MuSC.

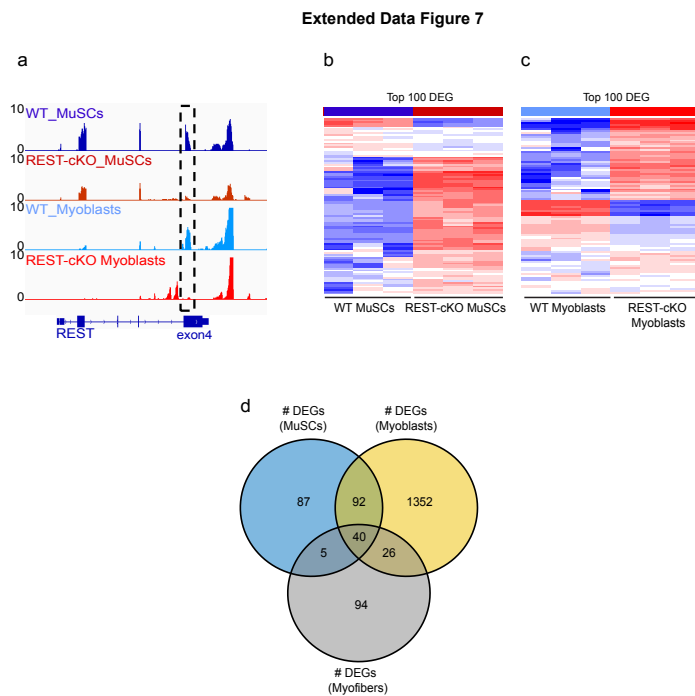
We understand reviewer’s concerns and for that reason we have added IF staining of myofiber associated MuSCs for the apoptosis marker in addition to IF staining on cultured myoblasts and Annexin V staining of myoblasts. Therefore, we have assayed for apoptosis in both muscle stem cells and cultured myoblasts. While another experiment to conclusively show the apoptosis in

MuSCs would be to perform annexin V assay along with the FACS isolation, this approach is technically challenging because one cannot obtain sufficient freshly sorted muscle stem cells from REST-KO mice for a conclusive analysis. That is why we decided to look at myofiber associated MuSCs which still represents the cells within their niche.

8) There seems to be considerable overlap in the deregulated genes in the three myogenic cell types. Please also compare genes affected in myotubes to those deregulated in MuSC/myoblasts. How large is the overlap?

We have now compared the overlapping differentially expressed genes between MuSCs, myoblasts and myofibers. Please see Extended Data Fig. 7d below and the manuscript page 16 where it now reads as follows:

“Further analysis of the RNA-Seq data revealed large transcriptomic changes in both quiescent MuSCs and cycling primary myoblasts, although more dramatically in myoblasts. For instance, there are 224 significant differentially expressed genes (absolute $\log_2FC > 1$, $p\text{-adj} < 0.05$) between WT and REST-cKO MuSCs while there are 1510 significant differentially expressed genes between WT and REST-cKO myoblasts (Extended Data Fig. 7b,c,d). It is worth mentioning that out of the 224 DEG in MuSCs, 132 of them are shared with the DEG in myoblasts of which 40 of them are shared with the DEG in myofibers (Extended Data Fig. 7d). In contrast, from the 1510 DEG in myoblasts, 158 are shared with DEG in MuSCs and myofibers and the majority of the DEG (1352) are unique to myoblasts (Extended Data Fig. 7d). This data indicates that the genetic deletion of REST results in transcriptomic changes in quiescent MuSCs, cycling primary myoblasts and in the myofibers, although the most prominent transcriptomic changes are observed in the context of myoblasts, which may be due to the higher expression of REST in myoblasts compared to MuSCs and myofibers (Extended Data Fig. 4c).”

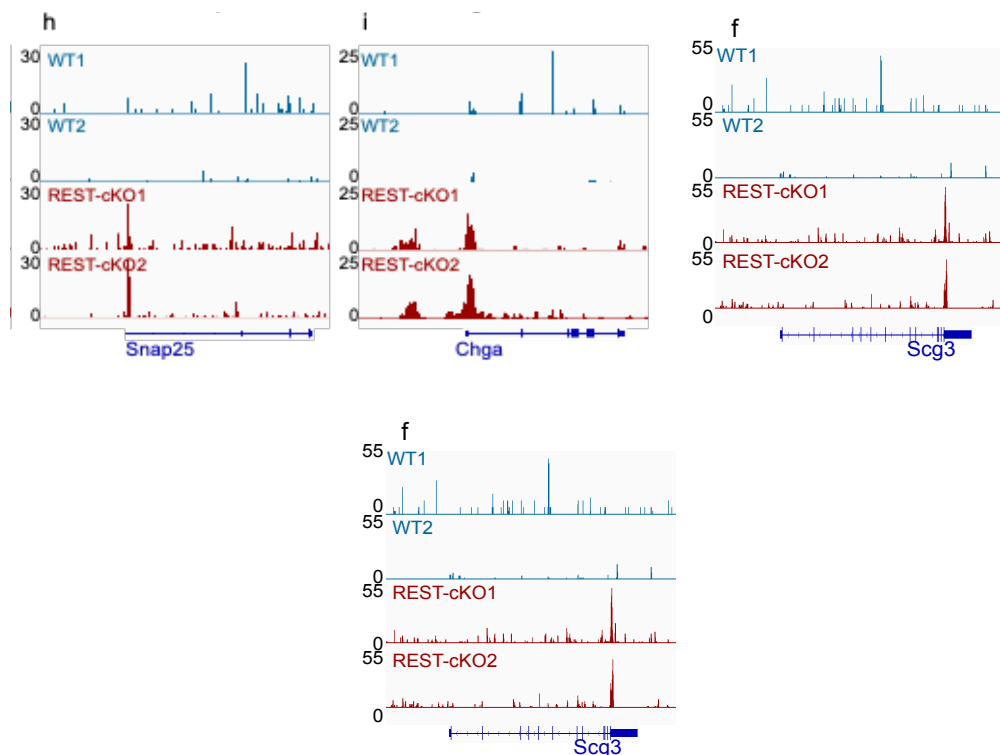


9) The authors describe ATAC-seq on single myofibers: "PCA of smfATAC-Seq shows that the chromatin state of the myofibers between the WT and REST-cKO do not show a distinct signature (Extended Data Fig. 5c)." To me, the PCA seems to indicate that the smfATAC-Seq data on WT fibers are not very reproducible; i.e., one wt dataset is very different from the REST-cKO, the other is not. The number of datasets should be extended. #

We performed ATAC-seq on single myofibers to see the extent of chromatin alteration in REST-KO myofibers and to make a comparison between chromatin state and gene expression output. We agree with the reviewer that the PCA plot does not significantly separate the REST-KO and WT myofibers. This can be explained by the fact that there is significant heterogeneity in muscle fibers. On the other hand, the extent of chromatin alteration in REST-KO myofibers is not grossly different than WT.

10) The examples tracks of smfATAC-Seq data are shown for genes that are not significantly changed in the fibers. Please provide examples of tracks for genes that are significantly changed. Also, it would have been interesting to know if the changes in the myofiber expression are associated with changes in histone modifications (i.e., recruitment of HDACs).

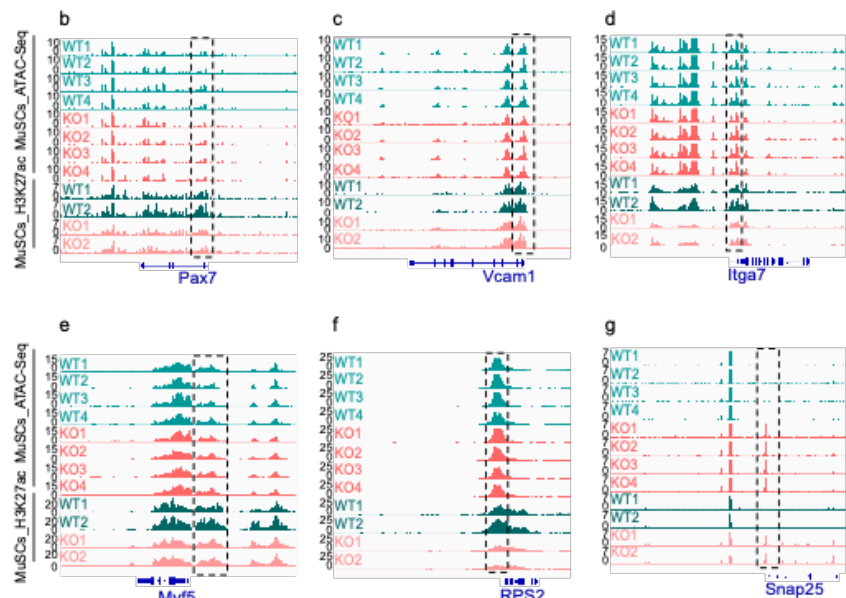
We have shown example tracks of genes that change their accessibility in the REST-cKO myofibers in Figures 3h and 3i but we have now added more tracks in the Extended Data Figure 6f and edited the result on the manuscript page 14 : “ However, similar to the gene expression data, representative neuronal genes such as *Snap25*, *Chga* and *Scg3* showed an increase in accessibility in the REST-cKO condition (Fig. 3h,i, Extended Data Fig. 6f).” Please see Fig. 3 and Extended Data Fig. 6 below.



11) Also, please use the ATAC-Seq data obtained with MuSC to provide examples of ATAC-Seq tracks of genes whose expression is not changed in MuSC.

We have now added ATAC-Seq tracks of genes expressed in MuSCs that don't change their expression and accessibility upon REST deletion such as *Pax7*, *Vcam1*, *Itga7*, *Myf5* and the housekeeping *RPS2*. Please see Extended Data Figure 12b-f and edited manuscript on page 22 that is as follows:

“After determining the overall chromatin change in the REST-cKO, we then focused on locus specific alterations in the chromatin. We first validated the accessibility of specific genes that do not change in their expression upon REST deletion, such as MuSC specification factor *Pax7* and other MuSC expressed genes such as *Vcam1*, *Itga7* and *Myf5* as well as the housekeeping gene *RPS2* (Extended Data Fig. 12b-f).”



Reviewer #3 (Remarks to the Author):

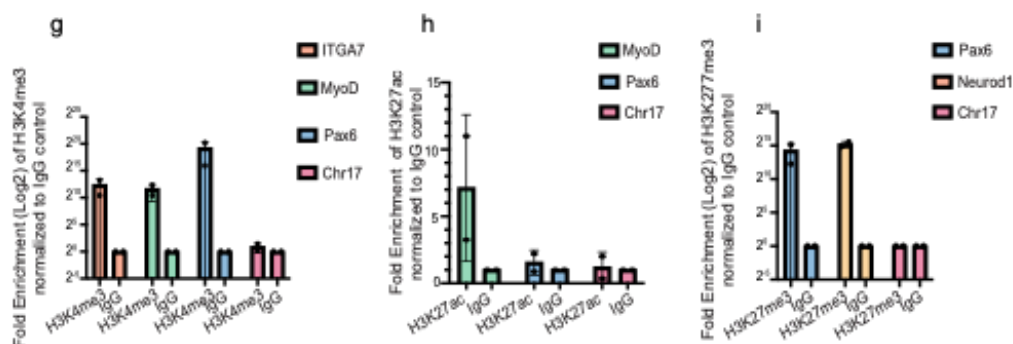
In this manuscript, the authors showed that the master epigenetic regulator REST1 plays a crucial role in maintaining muscle stem cell (MuSC) identity and survival. They first observed that many non-muscle lineage genes in freshly sorted MuSCs retain the active chromatin mark H3K4me3, despite being transcriptionally repressed. Analysis of the DNA sequences under the H3K4me3 peaks of these genes revealed an enrichment for the REST binding motif. Then, using a genetic mouse model, they found that REST deletion leads to a depletion of the MuSC pool, defective muscle regeneration, and muscle atrophy. Further profiling studies indicate that REST deletion alters MuSC chromatin and transcription states, leading to a loss of myogenic identity. While this study solidly demonstrates REST1's role in safeguarding muscle stem cell identity with comprehensive phenotype and profiling data, the manuscript's quality could be further improved by enhancing the logical flow and delving deeper into the mechanistic studies.

Below are suggestions for the authors to consider in order to further improve their manuscript:

1. Profiling of histone marks in Figure 1: Given its foundational role in this study, the interesting target genes identified in the sequencing results should be validated using Cut&Tag-qPCR. This is crucial to address potential false positives inherent in high-throughput sequencing.

We thank the reviewer for their suggestion. We have now validated the Cut&Tag of H3K4me3 by qPCR analysis of select targets (Extended Data Fig. 2g). We validated the enrichment for the TSS of *MyoD* and *ITGA7* (marker genes expressed in MuSCs) compared to IgG control. We also validated the enrichment of H3K4me3 on the promoter of a non-muscle gene, *Pax6*. As a negative control, we used a negative control region with no genomic features on Chr17 qE5 as internal control. (Extended Data Fig. 2g). We have also performed qPCR on our Cut&Tag libraries of H3K27ac (Extended Data Fig. 2h) which showed enrichment for *MyoD* compared to IgG control but lacked enrichment for the non-muscle gene *Pax6* which is consistent with our data that non expressed genes in MuSCs retain the active/bivalent H3K4me3 mark but lack the H3K27ac which is associated with active gene expression. In addition, we have also validated our novel Cut&Run libraries of H3K27me3 with qPCR which showed enrichment for the non-muscle genes *Pax6* and *Neurod1* compared to the IgG controls.

Please see Extended Data Fig. 2g-i below for further details as well as the edited text on the manuscript page 7 which now includes the following text: “We also further validated some targets of Cut&Tag assay with quantitative PCR (qPCR) which showed enrichment of H3K4me3 for MuSCs genes such as *MyoD* and *ITGA7* as well as the non-muscle gene, *Pax6* (Extended Data Fig. 2g). Similarly, we validated the Cut&Tag of H3K27ac which showed enrichment for *MyoD* but lacked enrichment for non-muscle expressed genes such as *Pax6*, as expected (Extended Data Fig. 2h). Finally, qPCR validation of the H3K27me3 libraries also showed enrichment for the non-muscle genes such as *Pax6* and *Neurod1* (Extended Data Fig. 2i). Overall, these data demonstrate that many non-muscle genes retain the bivalent or the active H3K4me3 mark despite their transcriptionally repressed state in MuSCs, indicating the presence of another repressive mechanism for their regulation in MuSCs.”



2. The transition to investigating REST is somewhat ambiguous, even though the authors mention finding enrichment for the REST binding motif. Motif analysis likely revealed enrichment for other transcription factors as well, which should be shown in the paper, at least in an extended data figure. A clearer rationale for focusing on REST over other transcription factors is needed.

Our initial focus on REST was guided by bioinformatic analysis of gene promoters enriched for H3K4me3 that remained repressed in muscle cells. Analysis of the sequences under H3K4me3 peaks in this set of genes identified 97 putative TF binding sites with $p < 0.01$. We then took a candidate approach, narrowing our focus to repressive transcription factors expressed in muscle cells. This rationale has been included in the revised version of the manuscript.

3. The authors state, "We defined a list of 134 genes as being expressed or not expressed at a read per million (RPM) > 5 or < 1 respectively." Clarification is needed on how genes with $1 < \text{RPM} < 5$ is categorized.

We thank the reviewer for their comment. We have now edited the text on page 6 to clarify which now reads as: "Focusing on the presence of H3K4me3 specifically on genes not expressed in MuSCs, we classified gene expression levels using MuSC RNA-Seq data into three groups: expressed (RPM > 5), not expressed (RPM < 1), and ambiguous expression ($1 < \text{RPM} < 5$)."

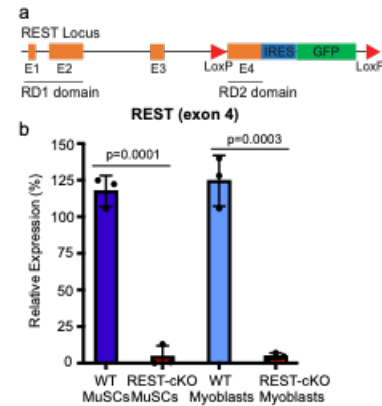
4. The authors provide extensive profiling data (smfRNA-seq, smfATAC-seq, SMART-seq, and ATAC-seq) on the chromatin and transcription state changes following REST depletion. However, these data are largely descriptive. The key question of how REST functions to regulate muscle stem cell identity is not deeply explored. Specially, how REST, together with other corepressors, represses the expression of non-muscle lineage genes with epigenetic active mark and the down-stream factor(s) of REST involved in this process are still not clear.

We now provide additional data on *in vivo* binding of REST in muscle cells coupled with Cut&Run data for H3K27me3. Integration of this data with ATAC-seq, extensive gene expression analyses and multiple *in vivo* and *in vitro* experiments establishes a novel role for REST in regulating muscle cell identity by repressing genes expression of alternate cell fates. While we recognize that REST recruits various chromatin modifiers (such as RCOR1/2, Sin3a/b, HDACs, LSD1, and G9a) to regulate chromatin structure and gene expression, a comprehensive analysis of all these factors in muscle cells is beyond the current manuscript's scope.

5. REST deletion in the genetic mouse model is only validated by qPCR (Extended Data Fig. 4b). Given the potential discrepancies between RNA and protein levels, it would be better to confirm REST protein levels via western blot or immunohistochemistry.

We thank the reviewer for their comment. We have also shown REST exon 4 deletion (which codes for repressive domain) from the RNA-Seq on REST-cKO MuSCs and myoblasts in Extended Data Fig. 5a below which clearly demonstrates the exon 4 deletion. REST possesses 2

repressive domains, RD1 and RD2 which elicit the repression. Exon 4 deletion results in the ablation of the RD2 and renders REST non-functional for gene repression as previously shown. REST can still be transcribed and may be detected at the protein level but exon 4 ablated REST is non-functional. This can be seen from our RNA-Seq data where known targets of REST gain significant expression in the REST-cKO (Fig. 4e,i). Additionally, ATAC-Seq on REST-cKO cells also showed gain of accessibility on the promoters of known REST target genes (Fig. 5i-k). This demonstrates that our mouse model of exon 4 ablation successfully elicits REST deletion in Pax7 expressing MuSCs.

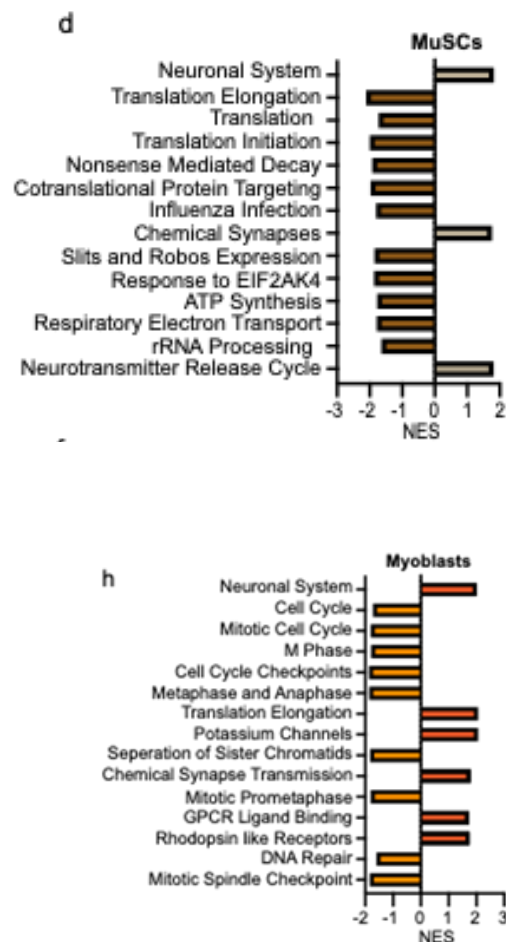
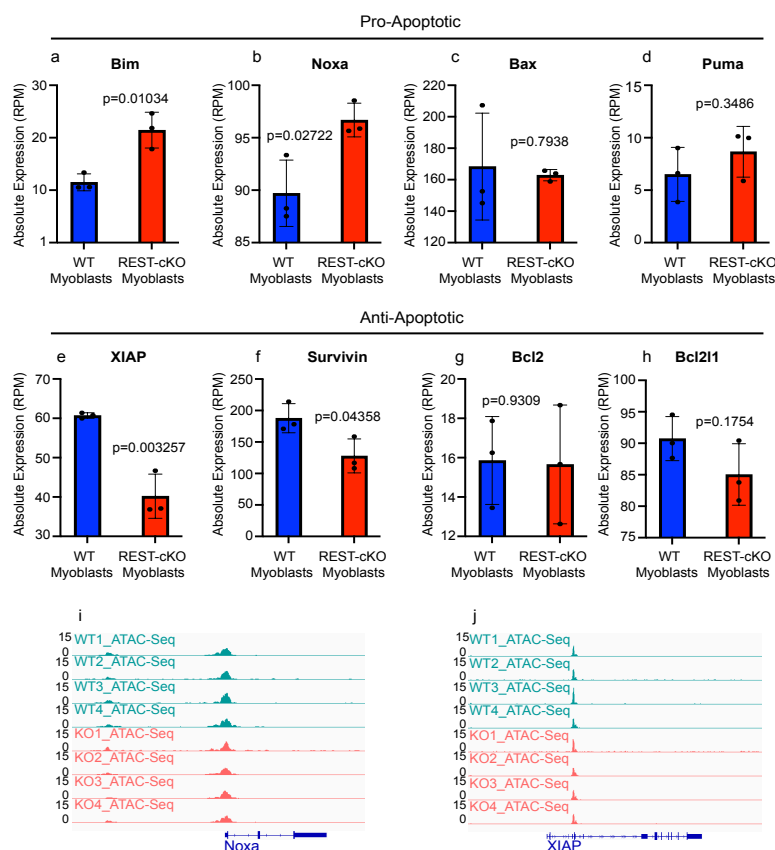


The authors showed that REST deletion leads to apoptosis of muscle cells in Figure 6. Given that they have sequencing data for the samples as well, does the analysis of this sequencing data support the conclusion? Are any apoptosis-related biological processes enriched in REST-cKO cells?

We thank the reviewer for their comment. We have now investigated the gene expression and chromatin data in REST-cKO and WT myoblasts more closely in terms of the apoptotic and anti-apoptotic genes. While some of the pro-apoptotic genes had significantly increased expression upon REST deletion such as *Bim* and *Noxa* (Additional Figure a,b), other pro-apoptotic genes did not show a significant change in expression including *Bax* and *Bak* genes (Additional Figure c,d). Similarly, some of the anti-apoptotic genes showed downregulation in the REST-cKO such as *Survivin* and *XIAP* (Additional Figure e,f) while other anti-apoptotic genes such as *Bcl2* and *Bcl2l1* (Additional Figure g,h) did not show a significant change in their expression. Additionally, chromatin accessibility of the above mentioned genes do not show a change upon REST deletion as shown by the IGV tracks of ATAC-Seq on WT and REST-cKO MuSCs (Additional Figure i,j). This data suggest that induction of apoptosis in REST-KO cells is the result of muscle cell identity due to deregulated gene expression profile rather than direct inhibition of apoptotic pathway genes by REST.

Furthermore, Gene Set Enrichment analysis of the RNA-Seq of REST-cKO and WT cells do not indicate apoptosis as a significantly enriched pathway in the DEG between the KO and the WT (Fig. 4d,h). However, GSE analysis does indicate significant changes in processes such as cell cycle, cell cycle checkpoints, ETC, translation and nonsense mediated decay (Fig. 4d,h) all of which can affect cell fate and ultimate induction of apoptosis. Therefore, the above data indicates that REST may not directly regulate the apoptotic pathway but may protect MuSCs against apoptosis by maintaining their myogenic lineage identity by repressing alternate pathways. When REST is genetically deleted in MuSCs, non-muscle lineage genes gain expression and cells go in a state of lineage confusion which triggers apoptosis mediated cell death. While REST does not directly regulate the transcription of apoptosis related genes, loss of REST in MuSCs consequently leads to cell death as we have clearly shown by *in vitro* and *in vivo* experiments.

Additional Figure



6. Different numbers of replicates were used for various sequencing methods (e.g., ATAC-seq n=4, RNA-seq n=3, H3K4me3 n=2). The rationale behind these choices should be included in the Statistics section of the Methods part.

Thank you. We have now included the rationale in the manuscript methods section for each corresponding sub section.

7. The results and discussion sections are too lengthy and difficult to follow. The results section should be concise, particularly the descriptive parts. The discussion often repeats results and should be condensed.

We have now edited the results and discussion section extensively in the revised manuscript for clarity.

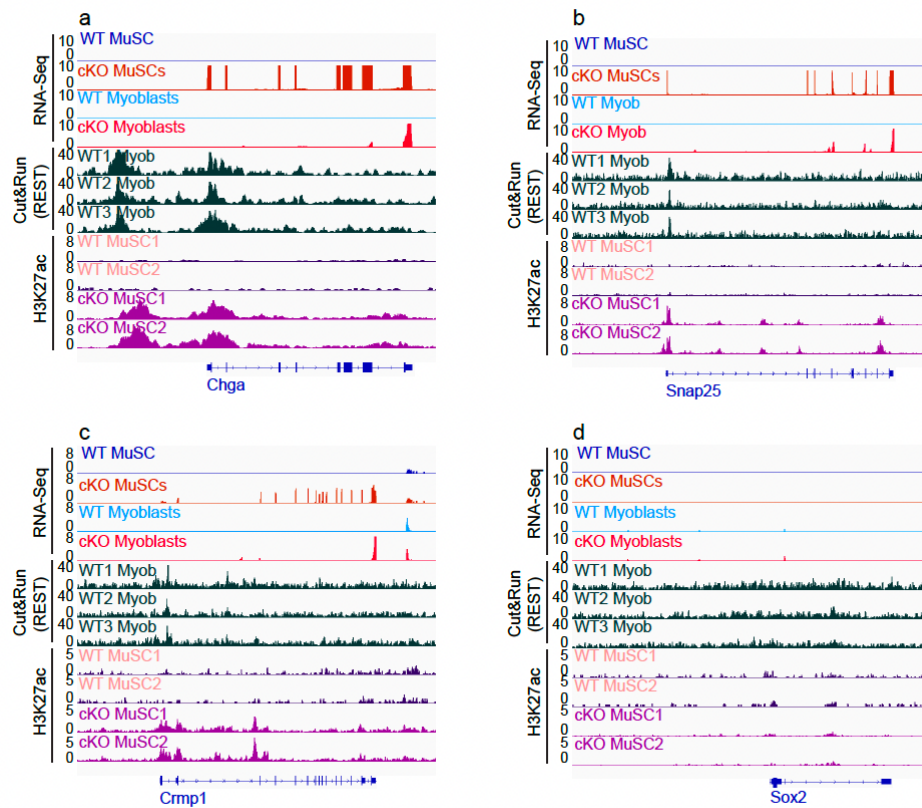
Point by point response to reviewers.

Reviewer #1 (Remarks to the Author):

In Figure 1d,e,f, tracks for REST CUT&RUN should be presented along those of histone marks.

We thank the reviewer for their constructive comments. Figure 1d-f is a representative figure showing distinct epigenetic state between genes that are expressed in muscle (i.e., *Myf5*) and genes that are not expressed in muscle (i.e., *Gata2* and *Pax6*) in which the latter genes maintain a bivalent chromatin state and remain repressed. Fig. 1d-f does not yet introduce the Cut&Run data for REST. To maintain coherence, we did not include REST tracks in Figure 1. However, we have now included a new figure (Extended Data Fig. 9) for the REST Cut&Run tracks for the top differentially expressed genes between WT and REST-cKO cells. This figure also includes RNA-Seq tracks for MuSCs and myoblasts, showing representative non-expressed genes in muscle cells gaining expression upon REST deletion concomitant with gain in acetylation of histone H3 Lysine 27 (H3K27ac). The data presented in this new representative figure shows that REST is directly involved in remodeling of heterochromatin on these genes and genetic deletion of REST leads to acquisition of acetylation mark, H3K27ac.

Extended Data Figure 9



Reviewer #2 (Remarks to the Author):

Several concerns already raised in my original review have not been addressed.

Point by point response to reviewers.

The manuscript suffers from shortcomings in the context of existing data on Rest in myogenic cells. Moreover, the authors' primary conclusions, specifically regarding the functional consequences of Rest ablation in MuSCs, are inadequately supported by the presented data. These issues must be addressed before this work can be considered for publication.

1) Rest Ablation and Its Consequences:

The introduction fails to appropriately mention prior findings that Rest ablation results in the expression of neuronal genes in the myotome, a disorganized myotome, and the downregulation of myogenic differentiation markers (DOI: 10.1038/2431). These findings should be mentioned to establish the relevance of this study within the broader field.

We thank the reviewer for their comment. We have now edited the text on manuscript to incorporate the information and the reference. Please see the edited text on page 5 which now reads as “REST is essential for embryonic development, as mice lacking REST exhibit embryonic lethality at embryonic day 11.5. This lethality is attributed at least in part to the ectopic activation of neurogenic gene programs in non-neuronal tissues, including the myotome. The misexpression of these neurogenic genes disrupts myotome organization and results in a reduction in the expression of key myogenic differentiation genes.”

2) Unsupported Claims on MuSC Functional Impairment and Apoptosis:

The authors claim throughout the manuscript (Abstract, Introduction, Results, and Discussion) that Rest mutant MuSCs are functionally impaired and undergo apoptosis. However, the data presented do not support this assertion. Instead, the findings indicate that apoptosis occurs in Rest mutant myoblasts—cells derived from MuSCs—in bulk cultures of primary myogenic cells, or when MuSC associated with myofibers are cultured on the fibers for 48. These cells, which express MyoD, are clearly myoblasts, not MuSCs.

Additionally, the authors incorrectly state that cells in fiber culture are located in their niche. Activated cells in 48-hour fiber culture are positioned outside the basal lamina (<https://doi.org/10.1002/stem.178>) and therefore cannot be considered as residing in their niche.

If the authors are unable to provide direct evidence that MuSCs undergo apoptosis or are otherwise functionally impaired, they must revise their claims accordingly. At best, they can state that changes in gene expression are observed in MuSCs, but there is no data to support functional impairment. Consequently, any claims regarding MuSC functional impairment should be removed from the manuscript.

We appreciate the reviewer's concern. However, our data demonstrate that the MuSC pool is depleted upon REST deletion in two independent models namely constitutive and inducible conditional mouse models, as shown through FACS isolation of MuSCs, TA cross-sectional staining of Pax7+ cells, and myofiber staining for Pax7+ cells at 0 hours (Fig. 2, Extended data Figure 5). Therefore, genetic deletion of REST leads to depletion of muscle stem cell pool. Due to the rarity of MuSCs and the cell death phenotype observed, it remains technically challenging to investigate the mechanism of cell death in MuSCs *in vivo*. To this end, we have performed an Annexin V assay on cultured primary myoblasts obtained from REST-cKO and WT and also stained myofiber-associated cells for Cleaved-Caspase 3 (marker of apoptosis). These

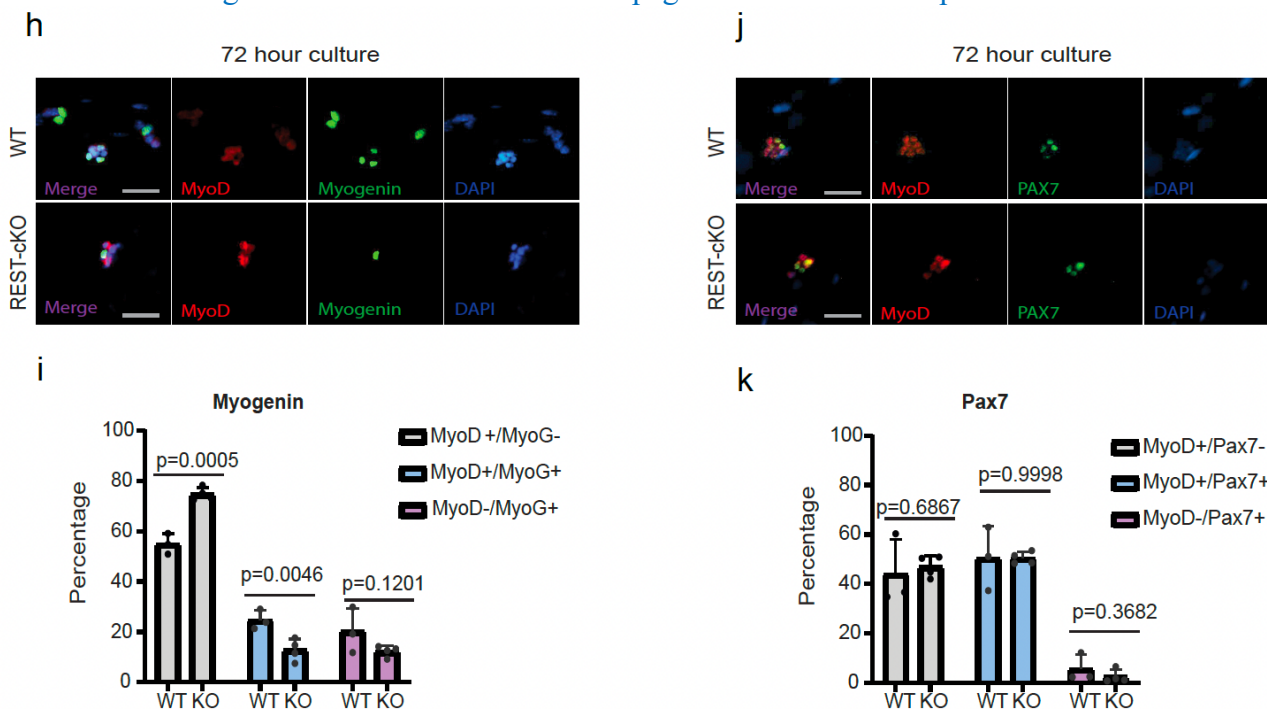
Point by point response to reviewers.

experiments revealed that REST-cKO cells undergo apoptosis-mediated cell death, providing an explanation for the depletion of MuSCs (Fig. 6).

We acknowledge that MuSCs cultured on myofibers do not fully recapitulate the characteristics of stem cells within their native niche. To address this, we specifically refer to them as “myofiber-associated MuSCs.” While it remains challenging to recreate the true *in vivo* niche *in vitro*, myofiber cultures offer the closest approximation that is technically possible. However, to ensure clarity, we have consistently referred to these cells as “myofiber-associated muscle stem cells” throughout the manuscript, as noted on pages 18 and 24.

In addition to undergoing apoptosis-mediated cell death, RNA-Seq analysis of REST-cKO MuSCs and myoblasts reveals functional defects in myogenic cells upon REST deletion. GSEA of differentially expressed genes highlights disruptions in multiple pathways critical for MuSC activation and differentiation (Fig. 4d, h). Furthermore, RNA-Seq data indicate a significant downregulation of key differentiation genes, suggesting impaired differentiation potential in REST-cKO MuSCs.

To further support these findings, we have added new figure on myofiber staining to directly assess differentiation defects following REST deletion. Myofiber-associated MuSCs were stained at 72 hours for MyoD and Myogenin, and REST-cKO cells exhibited significantly fewer MyoD⁺/Myog⁺ cells and significantly more MyoD⁺/Myog⁻ cells, indicating defective differentiation. Additionally, staining for Pax7 and MyoD revealed no significant differences in the proportions of Pax7⁺/MyoD⁺, MyoD⁺/Pax7⁻, or MyoD⁻/Pax7⁺ cells. These data are presented in Extended Data Fig. 8h-k below and discussed on page 17 of the manuscript.



3) Interpretation of PCA Results (Extended Data Fig. 5c):

The authors state: "PCA of smfATAC-Seq shows that the chromatin state of the myofibers between the WT and REST-cKO do not show a distinct signature." This statement is misleading.

Point by point response to reviewers.

The PCA plot reveals that one WT dataset is notably distinct from REST-cKO, whereas the other is not. The authors should explicitly state that the PCA does not significantly separate REST-cKO and WT myofibers. Furthermore, they must discuss the possibility that this lack of separation is due to the heterogeneity of muscle fibers and acknowledge the need for a larger dataset to draw any definitive conclusions.

We have now edited the text to address the reviewer's concern. The edited manuscript on page 15 now reads as "PCA of smfATAC-Seq does not significantly separate out the WT and REST-cKO single myofiber chromatin state possibly due to the intrinsic heterogeneity of myofibers (Extended Data Fig. 6c)."

In summary, these deficiencies undermine the strength of the manuscript's conclusions and must be rectified to ensure accurate interpretation and presentation of the findings.

We have now carefully addressed the concerns raised by the reviewer and included an additional new figure and edited the text in the revised manuscript to fully address their concerns.

Reviewer #3 (Remarks to the Author):

In this revised manuscript, the authors have made substantial efforts to address the reviewers' comments and suggestions. The manuscript has been significantly improved through the inclusion of new data, particularly the CUT&Tag data for REST and H3K27me3 in freshly sorted muSCs, as well as additional analyses, clarifications, and explanations. The text has also been carefully edited to enhance readability and improve the overall flow. This reviewer has no further concerns.

We thank the reviewer for their time to review our manuscript and their thoughtful comments throughout the review process.

Point by point response to reviewers

Reviewer #2 (Remarks to the Author):

The authors have failed to address my concern experimentally but continue to claim that MuSCs undergo apoptosis. Instead, they present data that suggest that myoblasts (or myogenic progenitors) undergo apoptosis and/or exhibit impaired differentiation in REST mutants. The text in the abstract, introduction, and throughout the manuscript must be revised to accurately reflect these findings.

Alternatively, the authors could address this point in the discussion (e.g. a paragraph on limitations of the study).

We thank the reviewer for their insightful comments, particularly regarding the potential role of apoptosis in REST-deficient muscle stem cells (MuSCs) *in vivo*. We agree with the reviewer that our direct experimental evidence for the induction of apoptosis in REST-KO mice is on the proliferating primary myoblasts and the myofiber associated progenitors from the EDL myofibers (Figure 6). Therefore, we have edited the text throughout the revised manuscript to highlight this point. It is important to note, however, that the direct detection of apoptotic cells *in vivo* remains technically challenging due to the rare nature of muscle stem cells under homeostasis and the rapid and efficient clearance of apoptotic cells by the immune system. Even in tissues undergoing high levels of apoptosis, detecting apoptotic cells remain a challenge in the presence of a functional immune system *in vivo*. This phenomenon has been well-documented in previous studies (e.g., "Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance," *Nature*, 2009; "Phagocytosis of apoptotic cells in homeostasis," *Nature Immunology*, 2015; "Apoptotic cell clearance: basic biology and therapeutic potential," *Nature Reviews Immunology*, 2014).

Nevertheless, we performed immunofluorescent staining for Cleaved Caspase-3 on freshly isolated MuSCs from both constitutive (REST^{f/f}; Pax7^{Cre}) and inducible (REST^{f/f}; Pax7^{CreER}) conditional knockout mouse models under homeostatic (non-injured) conditions (Supplementary Fig. 15). Consistent with previous findings, we did not observe Cleaved Caspase-3 positivity in quiescent MuSCs in either model. However, robust staining was observed in the myogenic progenitor (myoblast) population and in myofiber-associated myoblasts in culture (Figure 6).

However, we cannot entirely exclude the possibility of apoptosis occurring in MuSCs *in vivo* because of their clearance by immune system and their low numbers. Nonetheless, we have revised the manuscript, from the title through the discussion, to clearly indicate that our experimental evidence points to the induction of apoptosis in progenitors and primary myoblasts in REST-KO mice. Furthermore, we have added a new paragraph discussing this issue on the limitations of the study.

1) The authors repeatedly claim that MuSCs undergo apoptosis in REST mutants (e.g., Abstract: "Consequently, MuSCs enter cell death by apoptosis and the stem cell pool is depleted"), yet they never actually analyze apoptosis in MuSCs. Instead, their data assess apoptosis in myoblasts. In my previous assessment, I already pointed out that the claims must either be revised or

Point by point response to reviewers

supported by experiments directly demonstrating that MuSCs undergo apoptosis. The authors argue that assessing apoptosis in MuSCs *in vivo* is difficult due to their low numbers.

As mentioned above, we have now performed Cleaved Caspase-3 staining on freshly isolated MuSCs from both the non-inducible and inducible REST-cKO mouse models (Supplementary Fig. 15). As expected, we did not observe apoptosis in the freshly sorted MuSCs, likely due to the rapid clearance of apoptotic cells by the immune system *in vivo*. However, since our experimental evidence demonstrates apoptosis occurring in the myogenic progenitor population, we have revised the manuscript throughout to reflect this updated interpretation. The specific changes made are outlined below:

-The Title: Now reads “REST/NRSF Preserves Muscle Stem Cell Identity by Repressing Alternate Cell Fate”

-The Abstract (Page 2): Edited to include: “Consequently, muscle progenitor cells go through apoptosis and muscle stem cell pool is depleted.”

-Introduction (Page 5): Now states: “We report that functional deletion of REST in myogenic lineage leads to altered epigenome in MuSCs, loss of MuSC identity, resulting in apoptosis-mediated loss of the muscle progenitors and eventual myofiber atrophy.”

-Results (Page 24): A new section entitled “Deregulation of gene expression patterns upon REST deletion leads to apoptosis of myogenic progenitor cells in adult mice” discusses the updates.

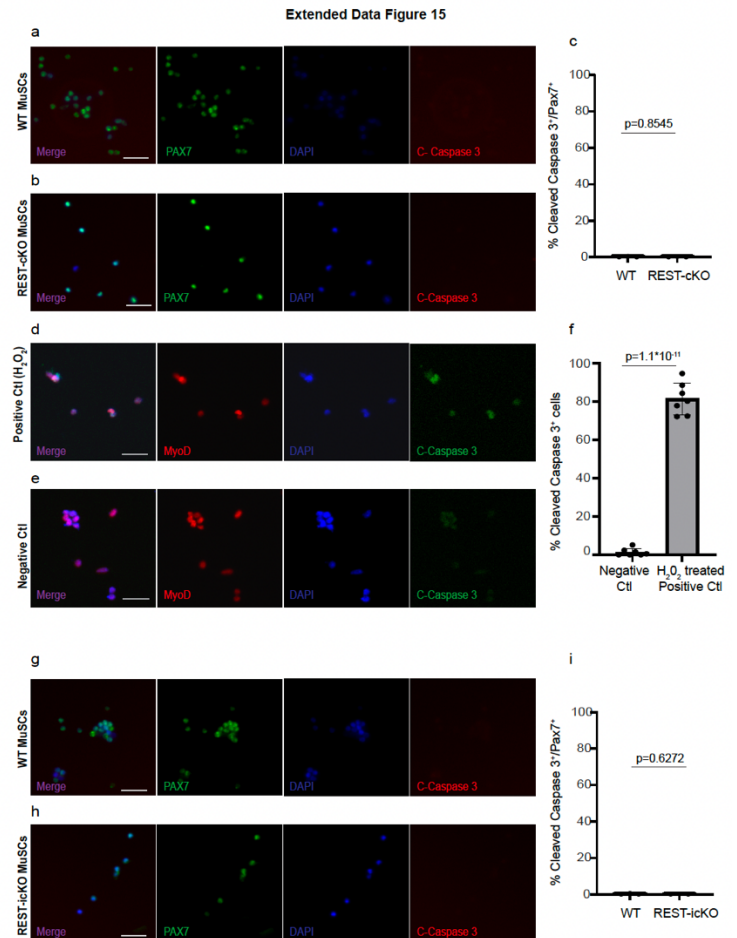
-Results (Page 24): Revised to read: “To further assess apoptosis of REST-cKO cells, we stained myofiber associated myoblasts at 48 hours for the presence of Cleaved-Caspase 3, another marker of apoptosis (Fig. 6d). We found a significant increase in the number of Cleaved-Caspase3⁺ myoblasts in the REST-cKO (Fig. 6d,e). Similar results were obtained when we performed immunostaining of WT and REST-cKO cultured primary myoblasts with the Cleaved-Caspase3 antibody, which again showed significantly more apoptotic cells in the KO condition (Fig. 6f,g). In contrast, Cleaved-Caspase3 staining of freshly sorted quiescent MuSCs showed no detectable apoptosis in either WT or KO mice, likely due to *in vivo* clearance of apoptotic cells by the immune system [83-85] (Supplementary Fig. 15).

-Results (Page 25): Now includes: “This data shows that the deregulated gene expression patterns in MuSCs and myoblasts due to loss of REST leads to apoptosis and the subsequent reduction of the muscle progenitors and reduction in the MuSC pool.”

-Discussion (Pages 27 and 30): Now states: “Genetic deletion of REST in myogenic cells leads to their death by apoptosis and subsequent reduction of their pool and myofiber growth.” and “The simultaneous expression of genes specific to a multitude of lineages leads to loss of cell identity in the MuSCs and their progenitors, compromising their function and resulting in the loss of the MuSC pool and reduction in the muscle fiber sizes.”

2) The authors argue in their rebuttal that the MuSC pool is depleted upon REST deletion, and that they provided evidence from two different mutant models. While it is correct that the MuSC pool is reduced, the mechanism underlying this depletion remains unclear—specifically, whether MuSCs themselves undergo apoptosis or whether their progenitors do.

In the Pax7cre mutants, REST deletion occurs around mid-gestation, yet the effects are only analyzed in adulthood. This makes it impossible to determine when the deficit arises. The significantly smaller fiber diameter suggests that fiber growth was impaired, likely due to a loss of myogenic progenitor cells (myoblasts) or a differentiation defect in progenitors that interfered with fiber development. A loss of myogenic progenitor cells could have also compromised the formation of the postnatal MuSC pool. Thus, the analysis of Pax7cre mutants does not demonstrate that MuSCs lacking REST undergo apoptosis—rather, it shows that the myogenic



Point by point response to reviewers

lineage is disrupted, and that apoptosis occurs in myogenic progenitor cells (myoblasts) when REST is absent.

Similarly, in the Tamoxifen-inducible Cre/REST mutants, MuSCs are only analyzed after injury. The authors did not conduct experiments tracking MuSC pool dynamics following REST deletion at different time points in an unchallenged state. Instead, they only examine the MuSC pool after 1–2 rounds of injury, where depletion is observed. Given the provided data, it is highly plausible—if not most likely—that transient amplifying MyoD⁺ MuSCs (i.e., myoblasts) were depleted, which in turn disrupted the replenishment of the quiescent MuSC pool.

Additionally, the authors show that REST-cKO cells undergo apoptosis in fiber culture assays, where MuSCs become activated, express MyoD, and thus transition into transient amplifying myoblasts. Therefore, the claim that MuSCs undergo apoptosis also remains unsubstantiated by this experiment. The authors must revise the text of their manuscript to accurately reflect their findings.

We agree that certain limitations of our study do exist, and we have clearly outlined them in the revised manuscript under the “limitations” on page 31. As pointed out by the reviewer, in the conditional non-inducible REST-cKO (REST^{fl/fl}; Pax7^{Cre}) model, REST is deleted during mid-gestation, while phenotypic outcomes are assessed in the adulthood. This temporal disconnect limits our ability to determine the precise developmental stage at which defects arise. The reduced myofiber sizes observed may reflect early impairments in myogenic progenitors’ proliferation or differentiation, rather than a direct consequence of REST deletion in adult MuSCs. Although we also utilized a tamoxifen-inducible REST knockout model to delete REST specifically in adult MuSCs, analysis was performed post-injury when MuSCs are already activated. Both of these two mouse models demonstrate that REST-cKO myoblasts and muscle progenitors undergo apoptosis, which may contribute to the regeneration defects observed. Although quiescent MuSCs did not show positive staining for apoptotic markers, we cannot fully rule out the occurrence of apoptosis in these cells. In the *in vivo* context, apoptotic MuSCs may have been rapidly cleared by the immune system, making their detection challenging. Nevertheless, our compelling *in vivo* and *in vitro* data strongly suggest that the REST complex regulates heterochromatin to repress alternative cell fates in myogenic cells, providing a key mechanism by which loss of REST leads to muscle stem cell depletion and impaired differentiation.

Additional points:

1) The Cre line used (Pax7cre, Jax, #010530) induces Rest deletion throughout the entire myogenic lineage, as it is expressed early in development in myogenic progenitors that eventually give rise to MuSCs. However, the authors misleadingly imply that the mutation is introduced only in MuSCs (e.g., page 11: “creating an exon 4 genetic deletion of REST in Pax7-expressing MuSCs”).

To avoid misinterpretation, the authors should explicitly state at the beginning of the paragraph that Rest mutation occurs in the entire myogenic lineage, not just in MuSCs. This distinction is

Point by point response to reviewers

crucial for accurately interpreting the results and understanding the potential effects of Rest loss at different stages of muscle development.

We thank the reviewer for highlighting this clarification. We have revised the manuscript accordingly to improve clarity. Specifically, on page 11, the text now reads:

“We used a genetic mouse model where exon 4 of REST is conditionally deleted in the myogenic lineage using the Cre-loxP system. REST^{f/f} mice were crossed with Pax7^{Cre} mice (Jax, #010530) creating an exon 4 genetic deletion of REST in Pax7 expressing myogenic cells (hereafter named REST-cKO) (Supplemental Fig. 5a).” Additionally, we have updated the text on page 27 to: “Genetic deletion of REST in myogenic cells leads to their death by apoptosis and subsequent reduction of their pool and impaired myofiber growth.”

Point by point response to reviewers

Reviewer #2 (Remarks to the Author):

The authors write (line 286)

This data corroborates that the previously observed loss of the MuSC pool is not the result of a defect during embryogenesis, but rather an effect on the adult MuSCs.

In my opinion, these data indicate that apoptosis of myogenic progenitors (myoblasts) during development and activated MuSC (myoblasts) during regeneration is responsible for the loss of the MuSC pool. If the authors want to argue that developmental deficits are not present, they should provide data.

That the loss of MuSC pool is an effect on the adult MuSCs is also not substantiated by data, as I pointed out previously. Please provide such data, or abstain from arguing that MuSC are affected.

We understand the reviewer's concern and have revised the manuscript to acknowledge the possibility of an embryonic defect. Please see the edited text on page 12 which now reads as "These data suggest that MuSC pool defect emerges postnatally. However, a caveat remains that since the reduced MuSC pool in the inducible model is observed following injury, we cannot fully rule out the potential embryogenic defects."