

Myf6/MRF4 is a Myogenic Niche Regulator Required for the Maintenance of the Muscle Stem Cell Pool

Felicia Lazure, Darren Blackburn, Aldo Corchado, Korin Sahinyan, Nabila Karam, Ahmad Sharanek, Duy Nguyen, Christoph Lepper, Hamed Najafabadi, Theodore Perkins, Arezu Jahani-Asl, and Vahab Soleimani

DOI: 10.15252/embr.202051585

Corresponding author(s): Vahab Soleimani (vahab.soleimani@mcgill.ca)

Review Timeline:

Submission Date:	19th Oct 19
Editorial Decision:	28th Nov 19
Revision Received:	4th Feb 20
Editorial Decision:	31st Mar 20
Revision Received:	24th Aug 20
Editorial Decision:	15th Sep 20
Revision Received:	16th Sep 20
Accepted:	18th Sep 20

Editor: Esther Schnapp

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Dr. Soleimani,

Thank you for your patience while your manuscript was peer-reviewed at EMBO reports. We have now received the full set of referee reports that is pasted below.

As you will see, all referees acknowledge that the findings are potentially interesting. However, referees 1 and 3 point out that the data as they stand are rather preliminary and that significant revisions will be required for consideration of the study for publication here. I think that especially all points raised by referee 1 are important and should be addressed. I.e., whether MRF4 specifically (as opposed to the other myogenic factors) has a role in adult muscle, and whether it is expressed and acts from within myofibers or satellite cells, or both. Referee 3 raises similar points. If you have any questions regarding the reports please let me know and we can discuss this further.

I would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of major revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further.

Regarding data quantification, please specify the number "n" for how many independent experiments (or specify the number of mice and of fibers) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

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

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- 2) Your manuscript contains statistics and error bars based on n=2 or on technical replicates. Please use scatter blots in these cases. No error bars can be calculated if n=2.

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

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See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are

collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2' etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

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

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

4) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

5) a complete author checklist, which you can download from our author guidelines <<https://www.embopress.org/page/journal/14693178/authorguide>>. Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<<https://orcid.org/>>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines <<https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>>

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* Note - All links should resolve to a page where the data can be accessed. *

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at <<https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>>.

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Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at <https://www.embopress.org/page/journal/14693178/authorguide#referencesformat>

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

In this manuscript the authors show that Myf6/MRF4 is required for the transcription from differentiated skeletal myofibers of cytokines that activate intracellular signaling cascades implicated in muscle stem cell activation (i.e. EGFR, LIF-STAT3 and VEGFR - otherwise defined as myokines in the text). They also show that Myf6/MRF4 Myf6 knockout (KO) mice, although born with a normal muscle stem cell compartment, undergo progressive reduction in their stem cell pool during postnatal life. Integrative analysis of ChIPseq and RNAseq datasets indicates that impaired EGFR and STAT3 signaling in MRF4-deficient muscles leads to upregulation of the p38 MAP kinase pathway in muscle stem cells, followed by spontaneous break from quiescence and eventually depletion of muscle stem cell pool. Finally, delivery of recombinant EGF and LIF rescues muscle stem cell pool defects in Myf6 knockout animals.

While the data reported in this manuscript are potentially of great interest and might reveal new insights into the regulation of myofiber-specific functions by individual myogenic bHLH factors, additional experiments are required to convincingly support the author's conclusions and clarify some fundamental issues.

At this stage this manuscript remains too speculative and suffers from important experimental flaws - see below.

Major Points

1) The data showing that MRF4 is the only myogenin bHLH expressed in differentiated myotubes is quite surprising and not convincing on many aspects. To the knowledge of this reviewer, it has not been shown previously that myofibers express only MRF4 among the myogenic bHLH factors. The RNA expression profile from whole mouse hindlimb skeletal muscles shown in Fig. 1a is not informative in this regard. To support the author conclusion, it is necessary that they show both RNA and protein expression profiles of the 4 bHLH myogenic factors in a time course from primary human and mouse adult myoblasts cultured in growth medium, and then along the whole process of incubation in differentiation medium. This experiment allows to better appreciate the dynamic expression patterns of these proteins by direct comparison with each other, and determine whether during myoblast differentiation into myotubes MyoD, Myf5 and Myogenin are all silenced, while MRF4 remains the only one expressed. To further validate this finding, the authors should perform nuclear staining of all 4 factors by immunofluorescence in muscle sections from human biopsies or mice.

2) One intrinsic limitation of the experimental setting this manuscript is that it does not determine the relative contribution to the proposed regulatory network from the MRF4 expressed in myofibers or in satellite cells. The authors infer most of their conclusions based on experiments performed in single fibers, with their associated satellite cells, from MRF4 KO mice. In the proposed model MRF4 deficiency in myofibers leads to impaired activation of satellite cells; however, it is unclear whether MRF4 deficiency in satellite cells also contributes to the altered response to cytokines and signaling activation. To address this issue, I highly recommend that the authors perform transwell experiments of co-culture between myotubes and satellite cells from wild type and MRF4 mice in all possible combinations. If the author's hypothesis is correct, then transwell co-culture with MRF4 null myotubes should be sufficient to impair wild type satellite cell activation. Obviously, the best way to address this issue would have been to generate conditional satellite cell- or myofiber-specific MRF4 knockout mice, which at this point I understand would require too much time.

An additional advantage of transwell co-culture between highly purified cell types (myotubes and satellite cells) is to eliminate the potential contribution of contaminating cell types (inflammatory cells, FAPS, etc etc) present in whole muscles as alternative source of MRF4-activated cytokines. This concern applies especially to the experiments in vivo of satellite cell (from luciferase mice) transplantation into MRF4 KO muscles.

3) ChIPseq data obtained by MRF4 overexpression raise concern, although moderate (given the support from data of KD and RNAseq), on the physiological meaning of the data. A comparative ChIPqPCR between MRF4, Myf5 and MyoD on specific sites of interest from the same cell line is absolutely required to address a key issue here - whether MRF4 has a unique ability to bind certain sites and activate target genes (i.e. myokines)

4) Although the authors claim that MyoD, Myf5 and myogenin are not expressed in differentiated muscles, their ChIPseq binding profile in myotubes has been published and is publicly available. The authors might want to perform a comparative analysis between these ChIPseq datasets and their MRF4 ChIPseq data, to determine whether MRF4 is the unique bHLH factors that binds the regulatory elements of satellite cell activating cytokines, and possibly identify the molecular determinants of this unique function.

5) As the spontaneous activation of p38 signalling in satellite cells appears to be essential for spontaneous exit from quiescence, experiments of functional rescue with the p38 inhibitor should be made

6) There is no mention or discussion of previous important papers reporting on other proposed functions of MRF4 that are at odd with the current manuscript - i.e. Moretti et al. 2016 Nat Comm

reported that MRF4 represses MEF2- mediated muscle gene expression and MRF4 knockdown in adult muscle induces hypertrophy; Suelves et al. 2004 EMBO reported that MRF4 represses muscle gene expression upon p38 phosphorylation. These apparently conflicting data should be at least discussed

7) Re-injury experiments should be performed to support evidence of functional depletion of satellite cells

8) It is unclear how the authors could succeed generating primary myoblast cell lines over-expressing MRF4-CTAP, given that satellite cells are notoriously prone to differentiate into myotubes (even when cultured in growth medium) and it is hard to propagate them over 3-4 passages. Further details in the protocol used should be provided.

9) In the text Fig. 1B erroneously indicates data reported in Fig. 1C, and Fig. 1C erroneously indicates data reported in Fig. 1D.

10) As none of the pathways perturbed in MRF4 null satellite cells seems to be implicated in maintenance of quiescence (they are rather involved in activation and differentiation) it is unclear why satellite cells from MRF4 null mice spontaneously break quiescence. The authors should at least provide an explanation.

Referee #2:

Overall this is a very nice work, testing an interesting problem, that adds to the field of muscle regeneration and stem cell niche.

The experiments are well-planned and executed, results convincing.

I have only suggestions to improve the quality of this work.

Summary:

The statement that "although Myf6 knockout mice are born with a normal muscle stem cell compartment, they undergo a progressive reduction in their stem cell pool during postnatal life because of reduced production of key myokines such as EGF, VEGFA and LIF" is the working hypothesis and should be presented as such, to be later demonstrated by the experiments reported.

Results:

1. Reduced VEGF expression should also affect endothelial cells. It takes a simple IF on growing, steady state and regenerating muscle to test whether a reduced microvasculature contributes to the observed phenotype.

2. Authors show that in Myf6 ko mice, MuSC break quiescence. It would be interesting to follow the fate of these inappropriately activating cells: do they proliferate? Do they fuse with existing healthy fibers (as it happened during post-natal growth? Authors exclude apoptosis or senescence. So,

what does it happen? In this respect, the images shown in figure 5 A-G are puzzling: the muscle fibers at 3 months seem clearly larger in the KO mouse, but at the other time points the difference is small if it exists at all. A morphometric analysis would complement these data. If activated MuSC differentiate one would expect larger fibres, otherwise the fate of these activated cells remains puzzling (if they do not die).

Discussion:

The discussion tends to repeat results in too much detail. It may be slightly shortened.

Minor point: at least in the PDF downloaded pages start from 48 rather than from 1.

Referee #3:

The study from Lazure and colleagues describe a large collection of observations that suggest a role of MRF4/Myf6 in the maintenance of the quiescent MuSC pool in adult muscle by regulating myokines such as EGF. The authors should comment on why they think Myf6 function is important though, since Myf6-null mice do not have a phenotype other than fewer MuSCs later in life. The data are interesting and, upon revision, certainly contribute to understanding the role of Myf6 in adult muscle.

In its present form though, the manuscript is quite long in general, with figure legends overly detailed. Several statements lack proper reference/s and authors miss many relevant and important citations. Quantification, numbers, and related statistics can be confusing and need amending.

General Comments

- The authors should better state why they think Myf6 function is important, since Myf6-null mice are viable, with normal-sized and functional muscles, can regenerate their muscle upon injury with WT-efficiency and produce MuSCs which proliferate and differentiate well. The Myf6 KO phenotype could be better explored (regeneration in later life, CSA and growth etc).
- Could use MRF4(Myf6) throughout to avoid confusion, although I understand that Myf6 is the mouse gene and MRF4 the human. Please check the Guidelines for Formatting Gene and Protein Names and correct where required to reduce confusion.
- As the authors state in their previous publication presenting the ChTAP-seq protocol, an appropriate functional validation assay should be done showing DNA binding of the tagged Myf6 with electrophoretic shift assay (EMSA) on some targets, although ChIP-Seq data is provided.
- The Myf6-null is constitutive, so MuSc will also lack Myf6 function. They explore their functions ex-vivo and find no differences, but should also examine self-renewal by giving the proportions of Pax7, MyoD or Pax7/MyoD expressing satellite cells (already presented in Fig 7h), rather than just the total cell number. Indeed, 'Pax7 high' are counted in Fig 7k, and with EGF, LIF, but how is 'Pax7 high' defined? Again counting Pax7, MyoD or Pax7/MyoD expressing satellite cells would be much more informative (e.g. DOI: 10.1083/jcb.200312007). Myf6 KO data on satellite cells should all be displayed together, as it appears in multiple figures. The authors should carefully detail Myf6 expression profile in MuSc and also refer to the literature, for example Machado and colleagues (doi.org/10.1016/j.celrep.2017.10.080) report Myf6 mRNA in satellite cells, also the Comai and recent Zammet reviews (doi.org/10.1016/j.semcd.2017.11.011.).
- Westerns to validate down-regulation of suggested Myf6 target genes in-vivo on muscle fibers is desirable to confirm observations. Same applies for up-regulation of p38 pathway.

- P38 is also a very early step in MuSC activation too, so should be discussed (e.g. doi.org/10.3389/fcell.2016.00091).
- The Myf6 KO data is convincing, but it would be useful to have a rescue experiment with ectopic myf6 expressed in myf6-null cells (myogenin too as a control) to see that VEGF, VEGFA, LIF are up-regulated.
- MuSC numbers in Myf6-KO drop in older mice (it would be useful to actually have the means, as hard to discern from the graphs in Fig 5). The authors should investigate regeneration when MuSC numbers are clearly depleted, rather than at 8 weeks as presented in Figure S3. Was there any differences in regeneration dynamics though, as only the 3 week point is illustrated.
- There appears to be different fiber CSAs visible in Figure 5 panel (5e) and (5g) and Fig 6? If this is representative. CSA measurements are required to resolve this objectively. A study on Mrf4 shRNA-driven phenotype from Moretti et al. (doi:10.1038/ncomms12397) reports effects on adult muscle growth. The authors should cite this paper and discuss their findings in the context of Moretti et al., and adult muscle growth.
- Are all MuSC depleted/affected equally? For example, using Myf5-YFP negative MuSC v Myf5-YFP expressing, as Figure 7n indicates that self-renewal occurs before activation. Does the RNA-Seq in Table S4 segregate these two populations in wt, are these changed in myf6 KO?
- The phenotype in MuSC in Myf6-null is reminiscent of Notch inactivation in satellite cells, which should be explored, or at least discussed (e.g. Stem Cells. 2012 Feb;30(2):232-42. doi: 10.1002/stem.773 and Stem Cells. 2012 Feb;30(2):243-52. doi: 10.1002/stem.775).
- In text "This observation that differentiation of muscle stem cells creates a physical niche whereby myokines (ligands) are produced in myofibers while their respective receptors are expressed in their associated MuSCs suggests the existence of a myokine-mediated communication network between myofiber and MuSCs." Figure 2 shows Myoblast and Myotubes, NOT MuSCs. To sustain such a statement authors need to show levels of myokines and receptor mRNA in quiescent MuSCs vs Fibers. Muscle Fiber isolation followed by MuSCs stripping could be useful. Is qsc gene expression detailed in Machado (doi.org/10.1016/j.celrep.2017.10.080) informative in this context? An interpretation of this data is creation of a niche by differentiating myotubes to direct MuSC self-renewal, this should be explored.
- After regeneration in transplanted mice, would the number of MuSC again be expected to drop through later life?
- What is being counted/measured in each panel should be made clearer in the figure legends, as should the N value, and what is actually being compared using statistical testing.

Figure 1

- Most data in Figure 1 could be supplementary.
- Panel (1c) clarification on the selection of MEF2A binding sites (among that of other muscle transcription factors) is required in text. Authors do not mention the study from Moretti et al. reporting the investigation of Myf6 in adult muscle. The report of Myf6/MEF2 duelling is worth mentioning here and in general in the manuscript.
- Panel (1e) No standard deviation? P=0?

Figure 2

- Panels (2d-2g) refer to siRNA on primary myotubes. This is NOT 'in-vivo', please edit Page 49, as this is misleading.
- Panel (2c) it is not mentioned in the text.

Figure 3

- Panel (3c) N=?

- Panel (3d) As per legend: "Depletion of Myf6 transcript by siRNAs in differentiated primary myotubes (5DM)"
- In figure 3 the authors show a decrease in VEGFA and EGF protein levels in the serum of KO animals compared to heterozygous Myf6+/- mice. This is the only time heterozygous Myf6+/- data is presented. It is important to determine whether there is any phenotype in heterozygous Myf6+/- mice.
- Please check as figure legend for panel (3e) is confusing.

Figure 4

- Panel (4f) is every dot presenting a fiber or a mouse? Please indicate N. If every dot is a fiber please show separated columns, one per animal, followed by an ANOVA analysis. This is common issue in figures.
- Panels (4g, h, i) same as per (4f)
- Panel (4g) how is "EGFR high" and "EGFR low" defined?

Figure 5

- Are CSAs different between nulls and wt?
- (5b, d, f, h) same as per 4f, the authors should indicate values per animal. If N=3 as written in legend, graphs (and analysis) should present 3 values for KO and 3 values for WT?
- (5j, k) same issue with data in the graph. The authors must indicate values per animal, not per fiber with all animals pooled together.

Figure 6

- Panel (6e) similar to fig.5e (same stage) fiber CSA appears larger in null than WT. Please measure.
- Panels (6b, d, f) data in the graph. The authors should indicate values per animal. I guess here every dot is a picture (?). If N=3 (or 5) as written in legend, graphs (and analysis) should present at least 3 values for KO and 3 values for WT.
- Same in (6l, o, j, v).
- Panel (6 l, j) Fusion index is normally calculated as the number of nuclei within MyHC-positive myotubes with {greater than or equal to} 2 nuclei as a percentage of the total nuclei present as reported in other studies (e.g. doi.org/10.1038/ncomms10871). Percentage of myonuclei in total number of nuclei per field of view" can be defined as a 'differentiation index'. Please amend. What is the N=?, please state and correct stats accordingly.
- What is the difference between (6l) and (6m) and (6o) and (6p), legend is unclear. (6l) and (6o) may use incorrect stats. Please correct according to biological replicates.
- Panel (6u). Legend is unclear. Have the same fibers been treated several times with EGF or different fibers have been suggested to different time points. What is the picture actually showing?
- Please correct (6v) to report biological replicates and stats accordingly.

Figure 7

- Panel (7l) should be joined to (6u) and possibly to (2l and 2m)
- Panel (7m) Stats needs revision as per previous comment.
- Panel (7k) Authors should define "PAX7 high"
- Panel (7n) authors should show whether Myf6 is expressed from myofiber, MuSCs or both

Referee #1:

In this manuscript the authors show that Myf6/MRF4 is required for the transcription from differentiated skeletal myofibers of cytokines that activate intracellular signaling cascades implicated in muscle stem cell activation (i.e. EGFR, LIF-STAT3 and VEGFR - otherwise defined as myokines in the text). They also show that Myf6/MRF4Myf6 knockout (KO) mice, although born with a normal muscle stem cell compartment, undergo progressive reduction in their stem cell pool during postnatal life. Integrative analysis of ChIPseq and RNAseq datasets indicates that impaired EGFR and STAT3 signaling in MRF4-deficient muscles leads to upregulation of the p38 MAP kinase pathway in muscle stem cells, followed by spontaneous break from quiescence and eventually depletion of muscle stem cell pool. Finally, delivery of recombinant EGF and LIF rescues muscle stem cell pool defects in Myf6 knockout animals.

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At this stage this manuscript remains too speculative and suffers from important experimental flaws - see below.

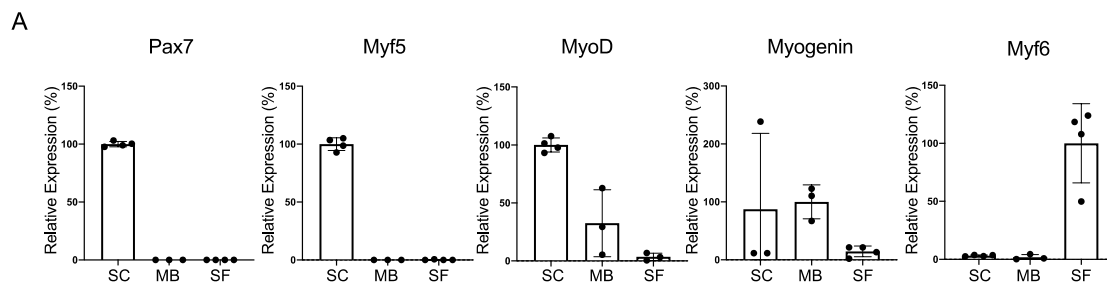
Major Points

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Response: We now provide additional expression data (EV1A) which shows the expression profile of the four myogenic factors in satellite cells, myoblasts and in single fibers stripped of their associated stem cells by trypsin. This new data clearly indicates that Myf6 is not expressed in

satellite cells or in proliferating myoblasts. Our data also shows that Myf6 is the only myogenic regulatory factor (MRF) that is expressed in non-injured muscle fiber. The temporal pattern of gene expression shown in New Figure EV1A is in agreement with numerous studies over the past three decades that have studied the temporal expression of MRFs during various stages of muscle differentiation (Smith et al. 1994 J. Cell Physiol.; Montarras et al. 1991 New Biol.; Hinterberger et al. 1991 Dev. Biol.; Bentzinger et al. 2012, Building muscle: molecular regulation of myogenesis. Cold Spring Harb. Perspect. Biol.), just to cite a few classic papers. Consistent with these studies our data clearly show that Myf6 is the predominant myogenic regulatory factor that is expressed in fully differentiated muscle fibers. Importantly, our new expression data from the muscle fiber in Figure EV1A is based on our new protocol which assays single muscle fibers after removal of mononuclear satellite cells (Blackburn et al. 2019. JBC).

Figure EV1



(New Figure EV1A)

2) One intrinsic limitation of the experimental setting this manuscript is that it does not determine the relative contribution to the proposed regulatory network from the MRF₄ expressed in myofibers or in satellite cells. The authors infer most of their conclusions based on experiments performed in single fibers, with their associated satellite cells, from MRF₄ KO mice. In the proposed model MRF₄ deficiency in myofibers leads to impaired activation of satellite cells; however, it is unclear whether MRF₄ deficiency in satellite cells also contributes to the altered response to cytokines and signaling activation. To address this issue, I highly recommend that the authors perform transwell experiments of co-culture between myotubes and satellite cells from wild type and MRF₄ mice in all possible combinations. If the author's hypothesis is correct, then transwell co-culture with MRF₄ null myotubes should be sufficient to impair wild type satellite cell activation. Obviously, the best way to address this issue would have been to generate conditional satellite cell- or myofiber-specific MRF₄ knockout mice, which at this point I understand would require too much time.

An additional advantage of transwell co-culture between highly purified cell types (myotubes and satellite cells) is to eliminate the potential contribution of contaminating cell types (inflammatory cells, FAPS, etc etc) present in whole muscles as alternative source of MRF₄-activated cytokines. This concern applies especially to the experiments in vivo of satellite cell (from luciferase mice) transplantation into MRF₄ KO muscles.

Response: As shown in Figure EV1A, clearly there is no expression of Myf6 transcript in the sorted satellite cells. Analysis of our RNA-Seq data from Myf6-KO and WT counterparts also shows that

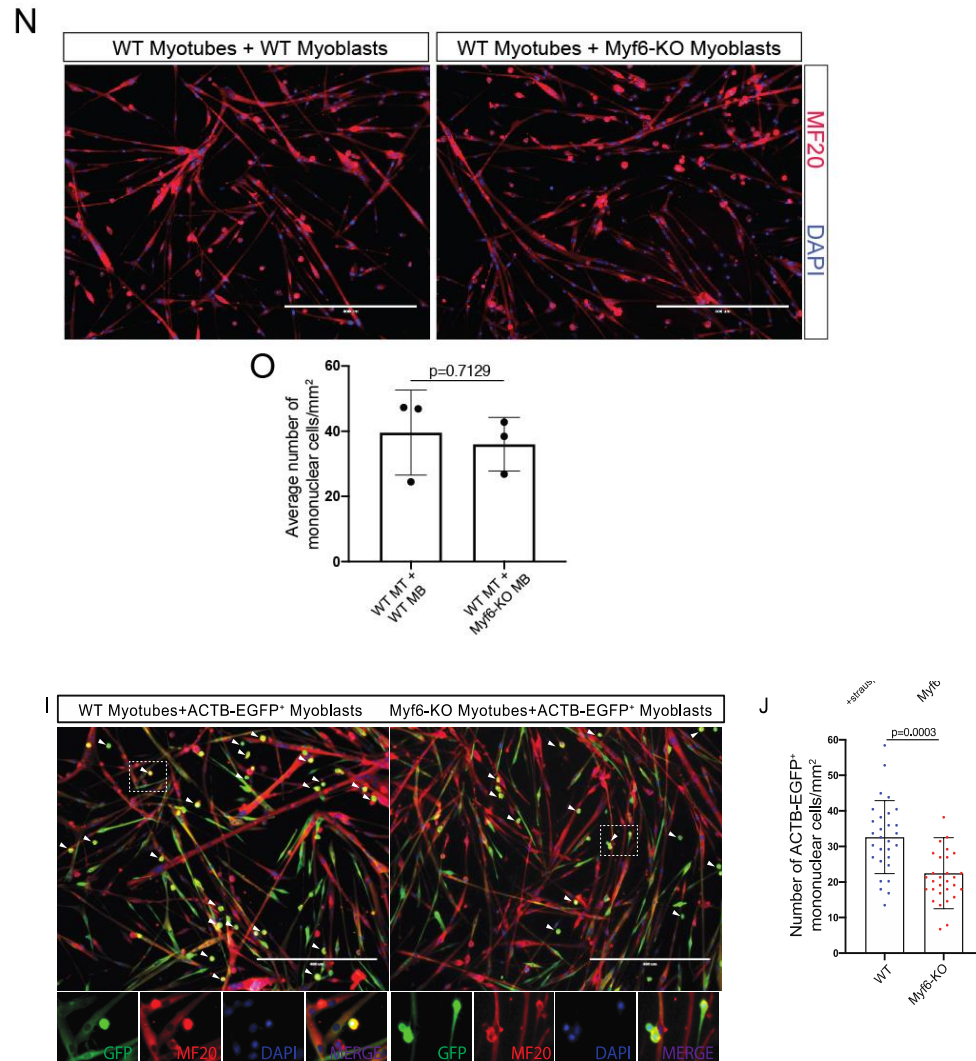
Myf6 is not expressed in satellite cells. Furthermore, our data in Figure 5 and Figure 6 clearly shows that Myf6 is not required for embryonic or neonatal muscle development. In Figure 5 we show that the satellite cell pool is similar between Myf6-KO and WT up to postnatal day 21 (P21). However, as mice aged the satellite cell pool gets diminished. On the other hand, we have provided experimental evidence in Figure 2 and Figure 3 that genetic deletion of Myf6 leads to the downregulation of key signaling molecules (myokines). In Figure 6 and Figure EV 4 we provide extensive experimental evidence that satellite cells from Myf6-KO mice have a defective quiescent state. We show that a significant fraction of these satellite cells activate p38 MAPK and MyoD and enter into myogenic differentiation.

In Figure 7L-O and Figure EV 4E-H we show that satellite cells from Myf6-KO have no defect in activation, proliferation or cell survival, strongly supporting our conclusion that genetic deletion of Myf6 has no satellite cell specific phenotype in adult muscle stem cells *ex vivo*. Additionally, Figure 7B-D unequivocally proves that Myf6-KO muscle is an unfavorable niche to donor transplanted satellite cells.

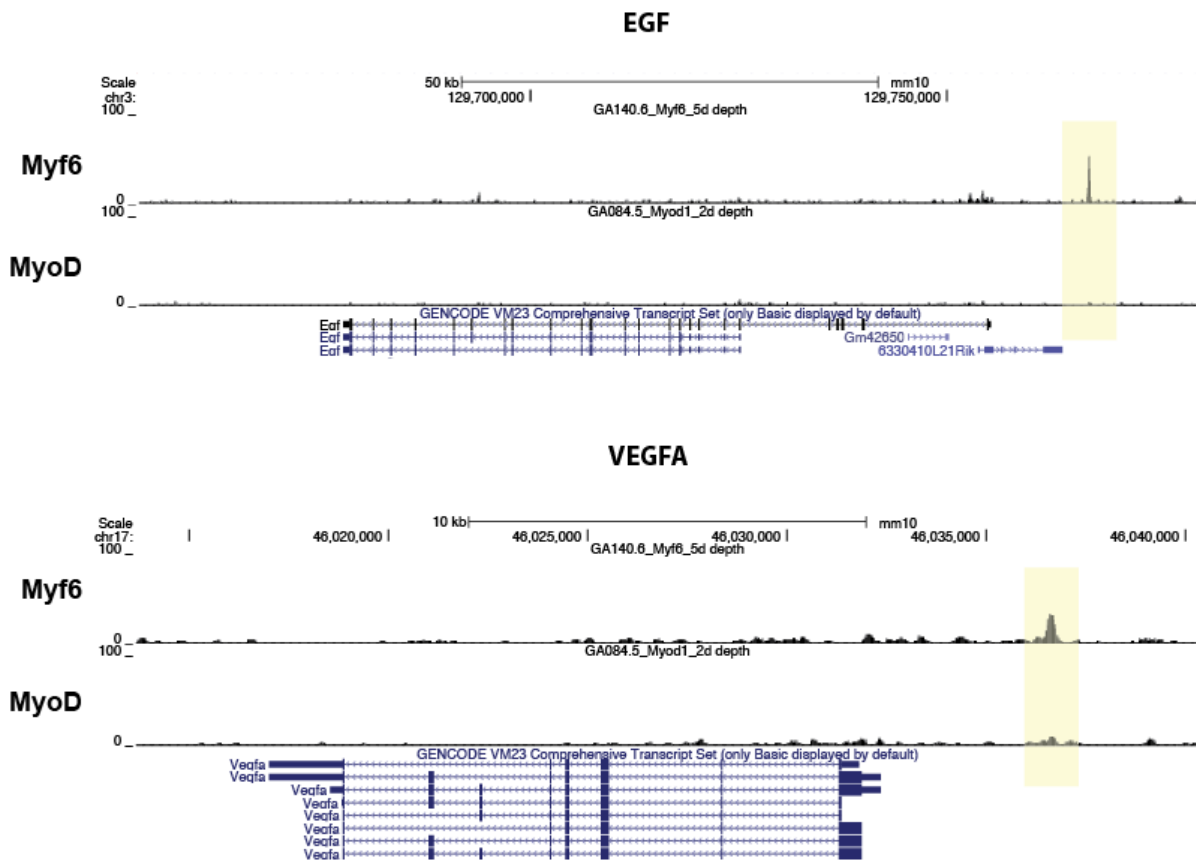
We performed new co-culture experiments as follows: we isolated satellite cells from WT mice by FACS using ITGA7⁺Lin⁻ approach as described in the manuscript. These cells were cultured and differentiated for 2 days. Afterwards, the myotubes were co-cultured with equal numbers of either WT or Myf6-KO myoblasts for an additional 2 days in differentiation media. We saw no significant difference in the number of unfused mononuclear cells between WT and Myf6-KO seeded myoblasts. This experiment shows that Myf6-KO myoblasts have equal differentiation capacity to those of their WT counterparts, in a WT environment (New Figure 7N-O).

Next, we performed new co-culture experiment where WT and Myf6-KO myotubes were seeded with equal numbers of myoblasts isolated from ACTB-EGFP⁺ mice. After two days in differentiation media, we observed that the number of mononuclear ACTB-EGFP⁺ cells remaining unfused were significantly lower in the presence of Myf6-KO myotubes compared to their WT counterparts (New Figure EV4I-J). These co-culture experiments together with donor *in vivo* transplantation experiments (Figure 7A-D) indicate that Myf6 in myofiber/myotubes regulates muscle stem cell quiescence.

Taken together, the new figure of gene expression profile of MRFs show that Myf6 is only expressed in differentiated muscle cells and not in satellite cells. Figure EV1A together with Figure EV4E-H show that Myf6-KO myoblasts have no defect in cell survival and Figure 6I which shows that Myf6-KO cultured satellite cells are fully differentiation competent. Together, these data clearly indicate that Myf6 exerts its function on satellite cells from the myofiber.



3) ChIP-seq data obtained by MRF₄ overexpression raise concern, although moderate (given the support from data of KD and RNA-seq), on the physiological meaning of the data. A comparative ChIP qPCR between MRF₄, Myf₅ and MyoD on specific sites of interest from the same cell line is absolutely required to address a key issue here - whether MRF₄ has a unique ability to bind certain sites and activate target genes (i.e. myokines)



Response: As shown above, Myf6 has the strongest ability to bind to the enhancer region of myokines like EGF and VEGFA, compared to MyoD in differentiating myotubes. Moreover, in mature muscle fibers, Myf6 but not Myf5, MyoD or Myogenin is expressed (New Figure EV1A). Therefore, transcriptional regulation of key myokines such as EGF and VEGFA is strictly under Myf6 control in muscle fibers (Figure above, Figure 2, Figure 3, Figure 4, Figure EV1A).

4) Although the authors claim that MyoD, Myf5 and myogenin are not expressed in differentiated muscles, their ChIPseq binding profile in myotubes has been published and is publicly available. The authors might want to perform a comparative analysis between these ChIPseq datasets and their MRF4 ChIPseq data, to determine whether MRF4 is the unique bHLH factors that binds the regulatory elements of satellite cell activating cytokines, and possibly identify the molecular determinants of this unique function.

Response: As mentioned before, we provide new expression data showing that in fully differentiated myofibers Myf6 is the predominant MRF that is expressed. The expression of the other three MRFs namely Myf5, MyoD and Myogenin is negligible in myofiber (Figure EV1A). As shown above, comparative analysis of MyoD and Myf6 shows that Myf6 but not MyoD binds to enhancer regions of EGF and VEGFA in multinucleated myotubes. In Figure 2 and Figure 3 we show

that Myf6 is indispensable for the expression of key myokines. These data indicate that Myf6 has a unique and novel function among MRFs to transcriptionally regulate myokines such as EGF within the myofibers.

5) As the spontaneous activation of p38 signalling in satellite cells appears to be essential for spontaneous exit from quiescence, experiments of functional rescue with the p38 inhibitor should be made

Response: An experimental blockade using p38 inhibitors would be informative to show if Myf6 function in maintaining quiescence is principally through inhibition of p38 signaling. However, that experiment, although important, would be beyond the scope of this manuscript. Our data in Figure 6Q-T shows that application of recombinant EGF to cultured myoblasts blocks p38 phosphorylation and blocks the expression of Myogenin, suggesting EGF is an inhibitor of p38 phosphorylation in muscle cells.

6) There is no mention or discussion of previous important papers reporting on other proposed functions of MRF₄ that are at odd with the current manuscript - i.e. Moretti et al. 2016 Nat Comm reported that MRF₄ represses MEF2- mediated muscle gene expression and MRF₄ knockdown in adult muscle induces hypertrophy; Suelves et al. 2004 EMBO reported that MRF₄ represses muscle gene expression upon p38 phosphorylation. These apparently conflicting data should be at least discussed.

Response: We have referenced and discussed those studies on Myf6. Notably, our results are in agreement with previously published work by Dr. Olson's group which shows that mice with a homozygous deletion of Myf6 are born normal.

With regards to the Moretti et al. 2016 paper where they show that *in vivo* administration of hairpin RNAi leads to increase in myofiber size, we observed slight increase in myofiber size in Myf6-KO mice but it does not reach statistical significance after careful measurement of muscle fibers up to 3 months of age. Clearly, Myf6-KO mice have no defect in the satellite cell compartment up to postnatal day 21 (P21). Thereafter, satellite cells break quiescence and enter into the myogenic differentiation program. The slight increase in fiber size is most likely due to spontaneous fusion of satellite cells. The lack of a significant difference in fiber size in our study may be due to the fact that Moretti et al. performed transient depletion of Myf6, whereas we have used a knockout mouse model.

With regards to Suelves et al. 2004, their work focuses on the effect of p38-induced phosphorylation on the function of Myf6 *in vitro*. As discussed by the authors, p38 activity is almost undetectable in uninjured adult skeletal muscle (Aronson et al. 1998). Therefore, p38 activity in differentiated myofibers would have a negligible effect on our *in vivo* studies using resting mice.

7) Re-injury experiments should be performed to support evidence of functional depletion of satellite cells

Response: We already performed an injury experiment and show that Myf6-KO satellite cells are fully capable of differentiating and forming new muscle fibers (Figure 7E). Importantly, the MuSC pool is completely replenished after injury (Figure E-G). Consistent with previous work, our data shows that Myf6 function during differentiation is redundant (Rawls et al. 1998. Development). Comparative analysis of Myf6 and MyoD ChIP-Seq shows that the core myogenic differentiation network is shared between Myf6 and MyoD (Figure EV5), consistent with previous study by Dr. Eric Olson's group (Rawls et al. 1998. Development). The core finding in our manuscript is that in adult resting muscle Myf6 regulates key myokines, such as EGF, in the muscle fiber which prevent satellite cells from premature differentiation (Figure 2, Figure 4, Figure 6).

8) It is unclear how the authors could succeed generating primary myoblast cell lines over-expressing MRF4-CTAP, given that satellite cells are notoriously prone to differentiate into myotubes (even when cultured in growth medium) and it is hard to propagate them over 3-4 passages. Further details in the protocol used should be provided.

Response: We have previously successfully generated numerous cTAP-tagged primary cell lines for MyoD, Myf5, Snai1, E47, Pax3, Pax7, HDAC1, HDAC2 and published the results in the following journals: [Soleimani et al. 2012, Developmental Cell](#); [Soleimani et al. 2012 Molecular Cells](#); [Soleimani et al. 2013. Nature Protocol](#); [Soleimani et al. 2018 Nucleic Acids Research](#). Moreover, we have established clear SOP to express the cTAP-tagged protein at near physiological levels (Soleimani et al. 2012 Mol. Cell; Soleimani et al. 2012 Dev. Cell). Therefore, we clearly have no problem in generating cTAP-tagged myogenic constructs and generating primary cell lines. We have performed similar quality control criteria to Myf6-cTAP primary cell lines used in this study.

9) In the text Fig. 1B erroneously indicates data reported in Fig. 1C, and Fig. 1C erroneously indicates data reported in Fig. 1D.

Fixed.

10) As none of the pathways perturbed in MRF4 null satellite cells seems to be implicated in maintenance of quiescence (they are rather involved in activation and differentiation) it is unclear why satellite cells from MRF4 null mice spontaneously break quiescence. The authors should at least provide an explanation.

Response: EGFR signaling has been implicated in asymmetric muscle stem cell division (Wang et al 2019. Cell Stem Cell). VEGFA has been implicated in maintaining muscle stem cell quiescence, (Verma et al. 2018. Cell Stem Cell). Stat3 signaling has been shown to be required for maintenance of muscle stem cell pool, (Zhu et al. 2016). Our data clearly shows that genetic deletion of Myf6 in myofibers leads to impairment in EGFR and STAT3 signaling in satellite cells. Importantly, application of recombinant EGF and LIF restores defects in EGFR and STAT3 signaling, respectively. Taken together, these data strongly support the hypothesis that transcriptional regulation of key myokines such as EGF by Myf6 from the myofiber plays a critical role in muscle stem cell quiescence and maintains the muscle stem cell pool.

Referee #2:

Overall this is a very nice work, testing an interesting problem, that adds to the field of muscle regeneration and stem cell niche.

The experiments are well-planned and executed, results convincing.

I have only suggestions to improve the quality of this work.

Thank you!

Summary:

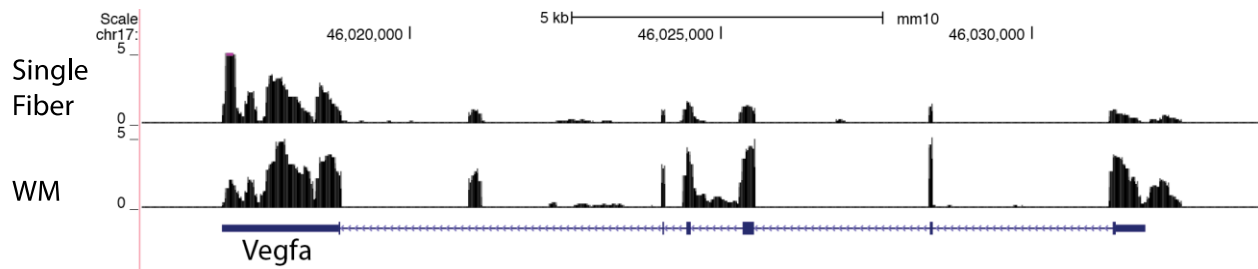
The statement that "although Myf6 knockout mice are born with a normal muscle stem cell compartment, they undergo a progressive reduction in their stem cell pool during postnatal life because of reduced production of key myokines such as EGF, VEGFA and LIF" is the working hypothesis and should be presented as such, to be later demonstrated by the experiments reported.

Response: Thank you very much for your appreciation of our manuscript. In the revised manuscript we have reworded the text to take your specific comment into consideration.

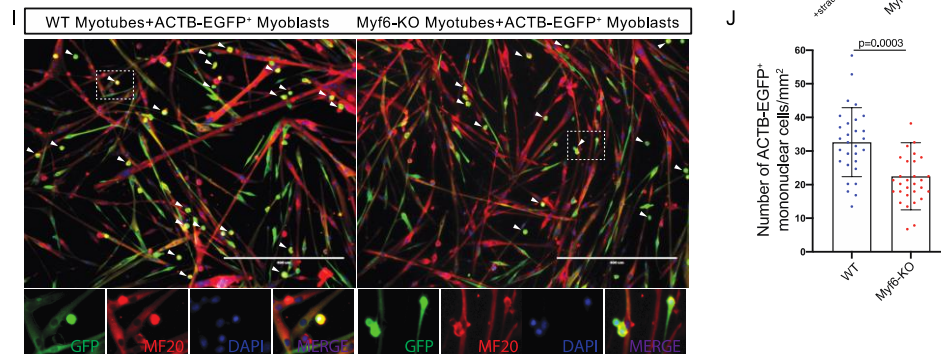
Results:

1. Reduced VEGF expression should also affect endothelial cells. It takes a simple IF on growing, steady state and regenerating muscle to test whether a reduced microvasculature contributes to the observed phenotype.

Response: We agree with the reviewer that reduced VEGFA from the myofiber might have effects beyond the muscle stem cell pool. Our comparative analysis of myofiber and whole muscle using SMART-Seq clearly shows that both myofiber and whole muscle express VEGFA (data from Blackburn..., Soleimani. 2019. JBC, figure below). Since there is higher VEGFA expression in the whole muscle compared to a single myofiber (normalized counts), this indicates that there are other cell types in skeletal muscle that also act as a source of VEGFA. For example, satellite cells are known to produce VEGFA (Verma et al. 2018. Cell Stem Cell). Likewise, our unpublished scRNA-Seq data shows that fibro/adipogenic progenitors (FAPs) also produce a significant amount of VEGFA. Accordingly, we performed FACS analysis of endothelial cells between Myf6-KO and WT counterparts using (CD11b⁻/CD45⁻/CD31⁺) gating strategy (Crouch and Doetsch, 2018. Nat. Protoc.) and found no significant difference in the number of endothelial cells between Myf6-KO and WT counterparts at 6 weeks of age. It is possible that reduced VEGFA can potentially affect endothelial cell numbers in older mice, but such experiments, although important, are outside of the scope of this manuscript.



2. Authors show that in Myf6 ko mice, MuSC break quiescence. It would be interesting to follow the fate of these inappropriately activating cells: do they proliferate? Do they fuse with existing healthy fibers (as it happened during post-natal growth? Authors exclude apoptosis or senescence. So, what does it happen? In this respect, the images shown in figure 5 A-G are puzzling: the muscle fibers at 3 months seem clearly larger in the KO mouse, but at the other time points the difference is small if it exists at all. A morphometric analysis would complement these data. If activated MuSC differentiate one would expect larger fibres, otherwise the fate of these activated cells remains puzzling (if they do not die).



Response: Satellite cells from Myf6-KO mice spontaneously exit quiescence (Figure 6) and undergo differentiation (Figure 6I). They have no defect in proliferation (Figure 7L), have no defect in differentiation (Figure 6I), they do not undergo apoptosis (EV4G), and do not undergo senescence (EV4E). The spontaneous exit of Myf6-KO satellite cells from quiescence and their entry into differentiation contribute to modest, albeit not statistically significant, increase in myofiber size (Figure 5, Figure 7E-F, Figure EV3). Our new co-culture experiment where we seeded tg-ACTB-EGFP⁺ myoblasts onto differentiated myotubes from Myf6-KO and WT shows that Myf6-KO myofibers induce the fusion of cultured tg-ACTB-EGFP⁺ myoblasts with pre-existing fibers more efficiently than their WT counterpart. Taken together, these experiments demonstrate that myotubes/myofibers from Myf6-KO trigger differentiation of associated muscle stem cells.

Discussion:

The discussion tends to repeat results in too much detail. It may be slightly shortened.
Done!

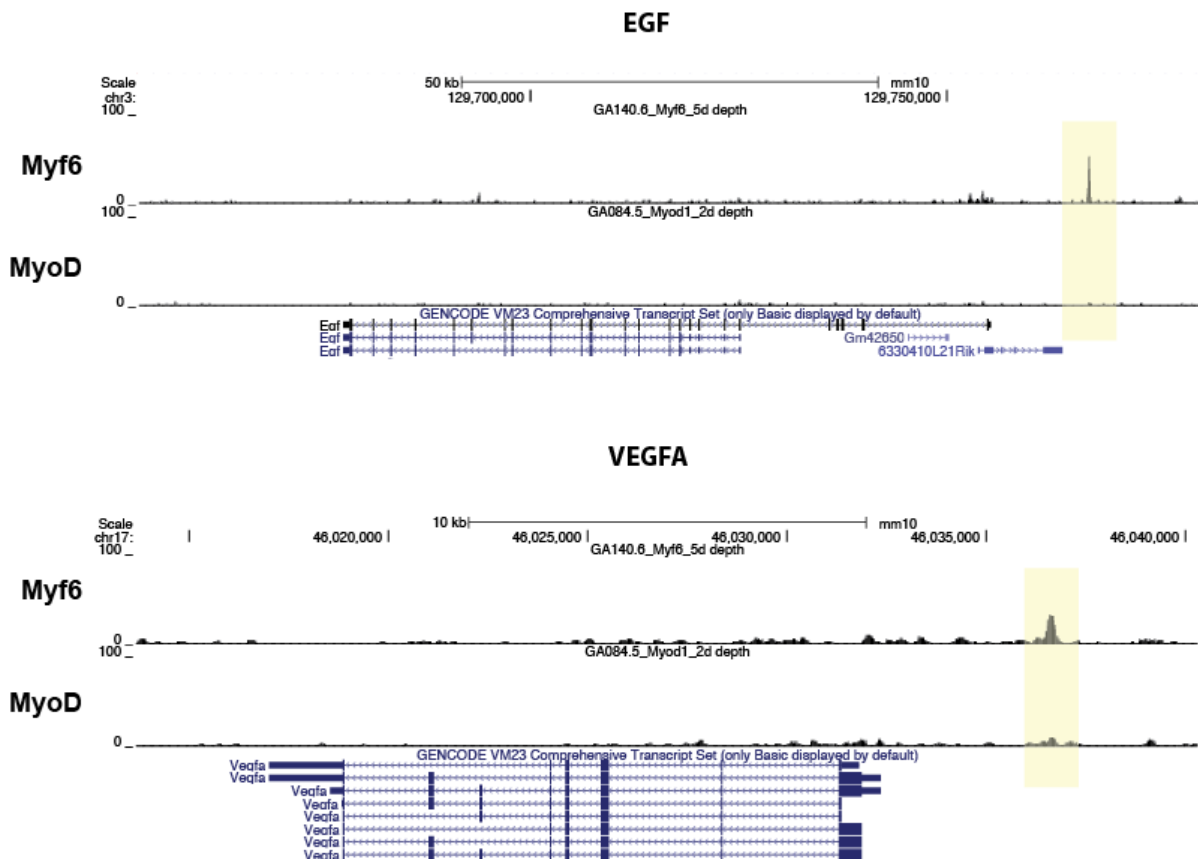
Minor point: at least in the PDF downloaded pages start from 48 rather than from 1.

Done!

Referee #3:

The study from Lazure and colleagues describe a large collection of observations that suggest a role of MRF₄/Myf6 in the maintenance of the quiescent MuSC pool in adult muscle by regulating myokines such as EGF. The authors should comment on why they think Myf6 function is important though, since Myf6-null mice do not have a phenotype other than fewer MuSCs later in life. The data are interesting and, upon revision, certainly contribute to understanding the role of Myf6 in adult muscle.

Response: We thank the reviewer for his/her appreciation of our manuscript. However, our careful analysis of the muscle stem cell compartment in adult mice over multiple developmental time points (P7, P21, 3 months, 10 months of age) revealed that Myf6-KO muscle stem cells break quiescence and enter into myogenic differentiation prematurely. Consequently, by three months of age, the stem cell pool in Myf6-KO shows a significant reduction in numbers. The importance of our manuscript is that we demonstrate a novel function for Myf6 in maintaining muscle stem cell quiescence by a myokine signaling pathway from the muscle fiber. This function is unique to Myf6 among all the MRFs. We provide the first direct evidence that a myogenic differentiation factor regulates stem cell quiescence by establishing communication between myofibers and their associated stem cells.



In its present form though, the manuscript is quite long in general, with figure legends overly detailed. Several statements lack proper reference/s and authors miss many relevant and important citations. Quantification, numbers, and related statistics can be confusing and need amending.

Response: Thank you for your comments. We have edited the revised manuscript to address these specific points.

General Comments

- The authors should better state why they think Myf6 function is important, since Myf6-null mice are viable, with normal-sized and functional muscles, can regenerate their muscle upon injury with WT-efficiency and produce MuSCs which proliferate and differentiate well. The Myf6 KO phenotype could be better explored (regeneration in later life, CSA and growth etc).

Response: Despite the lack of a developmental defect in Myf6-KO mice, Myf6 plays an important role in the maintenance of the muscle stem cell pool in adulthood. Therefore, the focal point of our manuscript is on the description of a novel function of Myf6 as a regulator of stem cell quiescence via myokine signaling from the muscle fiber. Our data regarding the viability and muscle fiber size

of Myf6-knockout mice is consistent with previous work by Dr. Eric Olson's group and also is in agreement with the work by Moretti et al. 2016 with regards to increase in fiber size.

Could use MRF₄(Myf6) throughout to avoid confusion, although I understand that Myf6 is the mouse gene and MRF₄ the human. Please check the Guidelines for Formatting Gene and Protein Names and correct where required to reduce confusion.

We use Myf6 as this is the nomenclature for the mouse gene. However, in the title we kept Myf6/MRF₄ because in human it is called MRF₄.

- As the authors state in their previous publication presenting the ChTAP-seq protocol, an appropriate functional validation assay should be done showing DNA binding of the tagged Myf6 with electrophoretic shift assay (EMSA) on some targets, although ChIP-Seq data is provided.

Response: We believe experiments shown in Figure EV1 completely address this point raised by the reviewer. We have not only functionally validated ChTAP-Seq for numerous previously published proteins (MyoD, Myf5, E47, HDAC1, HDAC2, Snai1, Pax3, Pax7), but also, as shown in Figure EV1, Myf6-cTAP is expressed, has clear nuclear localization and more importantly it has robust *in vivo* activity on its target sequence in Luciferase assays (Figure EV1E). Lastly, Myf6-cTAP clearly binds to the expected muscle specific genes, as shown by ChIP-Seq assay (Figure 1). The use of EMSA for *in vitro* binding is an additional assay. However, it involves use of radioactive material and we lack the license and equipment to do this particular experiment. We believe the experiments in Figure 1 and Figure EV1 are sufficient to demonstrate the functionality of Myf6-cTAP.

The Myf6-null is constitutive, so MuSc will also lack Myf6 function. They explore their functions *ex vivo* and find no differences, but should also examine self-renewal by giving the proportions of Pax7, MyoD or Pax7/MyoD expressing satellite cells (already presented in Fig 7h), rather than just the total cell number. Indeed, 'Pax7 high' are counted in Fig 7k, and with EGF, LIF, but how is 'Pax7 high' defined? Again counting Pax7, MyoD or Pax7/MyoD expressing satellite cells would be much more informative (e.g. DOI: 10.1083/jcb.200312007). Myf6 KO data on satellite cells should all be displayed together, as it appears in multiple figures. The authors should carefully detail Myf6 expression profile in MuSc and also refer to the literature, for example Machado and colleagues (doi.org/10.1016/j.celrep.2017.10.080) report Myf6 mRNA in satellite cells, also the Comai and recent Zammet reviews (doi.org/10.1016/j.semcd.2017.11.011.).

Using RNA-Seq and qPCR, we show that Myf6 expression is negligible in muscle stem cells and nonexistent in proliferating myoblasts (Figure EV1A). This inconsequential expression implies that there is no Myf6 protein produced in muscle stem cells. Furthermore, the lack of a phenotype with regards to apoptosis, senescence and proliferation in Myf6-knockout cultured myoblasts further supports the notion that Myf6 is not expressed at the protein level, and therefore has no function in muscle stem cells.

To determine the numbers of PAX7^{high} and PAX7^{low} cells when imaging EDL myofibers, images were captured at 60% signal intensity. Once all channels were overlaid, the GFP channel for PAX7 staining was set to 40% intensity. If no signal was visible at that threshold, cells were counted as

PAX7^{low}. If a signal was visible at that threshold, cells were counted at PAX7^{high}. The imaging threshold was determined by focusing on a field of view containing cells with varying signal intensities, and adjusting the settings such that the brightest cells are visible after image capture whereas the dimmer cells are not. A similar method was used to determine numbers of pEGFR^{high} and pSTAT3^{high} cells. Equal imaging parameters were used between experimental and control sample groups.

- Westerns to validate down-regulation of suggested Myf6 target genes in-vivo on muscle fibers is desirable to confirm observations. Same applies for up-regulation of p38 pathway. We have provided ELISA for key Myf6 target genes (Figure 3F-I) and performed immunostaining for phospho p38, which are both at protein level (Figure 6). Crucially our ELISA data shows that even in the serum of Myf6-KO mice, the levels of EGF and VEGFA are significantly lower than those of heterozygous mice. These experiments indicate that Myf6 is a master regulator of key myokines. We believe, for secreted factors, Western blots or immunofluorescent analyses are less efficient than ELISA. Therefore, we validated downregulation of key Myf6 targets by that technique.

- P38 is also a very early step in MuSC activation too, so should be discussed (e.g. doi.org/10.3389/fcell.2016.00091).

Response: We agree that p38 is involved in early activation and precedes activation of MyoD as shown previously. Similarly, our data shows that muscle stem cells from Myf6-KO activate p38 MAPK and enter into myogenic differentiation prematurely (Figure 6K-P).

- The Myf6 KO data is convincing, but it would be useful to have a rescue experiment with ectopic myf6 expressed in myf6-null cells (myogenin too as a control) to see that VEGF, VEGFA, LIF are up-regulated.

Response: Thank you for acknowledging the validity of our data. We have done rescue experiments of EGFR and STAT3 signaling by adding recombinant ligands on cultured myofiber-associated muscle stem cells (Figure 4D-I). Since Myf6 is not expressed in satellite cells or in proliferating myoblasts, generating a cell line over expressing Myf6 may not be a good way to draw any conclusions for the following reasons: 1) over expression will be out of context (i.e, forcing the expression of a transcription factor in a cell stage that normally does not express the factor), 2) the out of context expression of Myf6 may not be sufficient to override constraints in chromatin on genes such as EGF, VEGFA, etc. For example, even though MyoD is a master regulator of muscle cell differentiation, expression of MyoD either at physiological levels or by forced expression does not induce the expression of muscle differentiation genes in proliferating myoblasts (Soleimani et al. 2012. Molecular Cell).

- MuSC numbers in Myf6-KO drop in older mice (it would be useful to actually have the means, as hard to discern from the graphs in Fig 5). The authors should investigate regeneration when MuSC numbers are clearly depleted, rather than at 8 weeks as presented in Figure S3. Was there any differences in regeneration dynamics though, as only the 3 week point is illustrated.

Response: We have analyzed the muscle stem cell pool at multiple time points during development such as postnatal day 7 (P7), postnatal day 21 (P21), at three months of age and at 10 months of age (Figure 5). Our data clearly show that the muscle stem cell compartment is completely normal up to the postnatal day 21 (P21) (Figure 5). Thereafter, the stem cell pool undergoes reduction due to the impaired state of muscle stem cell quiescence and their precocious entry into myogenic differentiation in mice lacking Myf6 (Figure 6).

We have shown that the defect in the muscle stem cell pool of Myf6-knockout mice is only present in the resting, steady state. Muscle stem cells from Myf6-KO mice have no cell-intrinsic defects, and are completely capable of contributing to the regeneration of injured muscle and self-renewing (Figure 7E-G). Furthermore, myogenesis occurs similarly between Myf6-KO and WT animals in early post-natal development, as shown by cross-sections taken at post-natal days 7 and 21 (Figure 5). Taken together, this data suggests that the regeneration dynamics between WT and Myf6-KO mice would be similar at any given time point.

- There appears to be different fiber CSAs visible in Figure 5 panel (5e) and (5g) and Fig 6? If this is representative. CSA measurements are required to resolve this objectively. A study on Mrf4 shRNA-driven phenotype from Moretti et al. (doi:10.1038/ncomms12397) reports effects on adult muscle growth. The authors should cite this paper and discuss their findings in the context of Moretti et al., and adult muscle growth.

Response: We have performed additional counts of fiber size (number of fibers per field of view). Our data is in complete agreement with the previous study from Dr. Eric Olson's group that the function of Myf6 is redundant in muscle differentiation. However, we do see a slight increase in fiber size in Myf6-KO mice, although the increase in fiber size does not reach statistical significance, this trend is in agreement with Moretti et al. 2016. We speculate that the slight increase in fiber size is because of premature activation and fusion of satellite cells from Myf6-KO mice.

- Are all MuSc depleted/affected equally? For example, using Myf5-YFP negative MuSC v Myf5-YFP expressing, as Figure 7n indicates that self-renewal occurs before activation. Does the RNA-Seq in Table S4 segregate these two populations in wt, are these changed in myf6 KO?

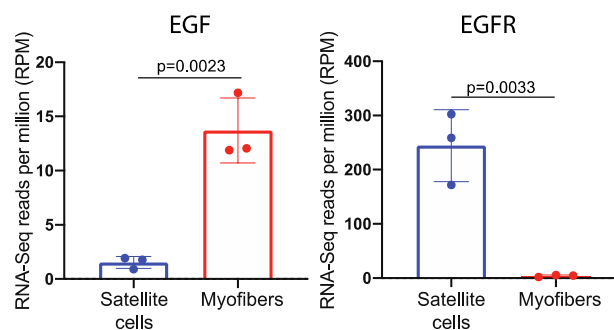
Response: This is a very good point but falls outside of the scope of this manuscript. We know that the satellite cell compartment is heterogeneous (Kuang et al. 2007. Cell). However, whether all satellite cells or a subset are more affected by the loss of Myf6 and perturbations in myokine signaling in adult mice requires more extensive analysis and also requires development and analysis of specific mouse models.

- The phenotype in MuSC in Myf6-null is reminiscent of Notch inactivation in satellite cells, which should be explored, or at least discussed (e.g. Stem Cells. 2012 Feb;30(2):232-42. doi: 10.1002/stem.773 and Stem Cells. 2012 Feb;30(2):243-52. doi: 10.1002/stem.775).

Response: This is a very good point! We have shown that VEGFA is under the direct control of Myf6 in muscle fibers and is reduced in the serum of Myf6-KO mice. Therefore, one would expect that reduced VEGFA may lead to activation of satellite cells due to impaired recruitment of endothelial cells which in turn provide Notch ligands for their quiescence, based on the study of Verma et. al. 2018. Cell Stem Cell. However, in addition to myofibers, FAPs and satellite cells also express VEGFA. Therefore, alternative sources of VEGFA exist in skeletal muscle. In the analysis of our RNA-Seq data from freshly sorted muscle stem cells of Myf6-KO and WT counterparts we see that upregulation of p38 MAPK pathway is the most significant and obvious upregulated pathway. On the other hand, similar analysis did not show convincing evidence for a significant change in Notch target genes.

- In text "This observation that differentiation of muscle stem cells creates a physical niche whereby myokines (ligands) are produced in myofibers while their respective receptors are expressed in their associated MuSCs suggests the existence of a myokine-mediated communication network between myofiber and MuSCs." Figure 2 shows Myoblast and Myotubes, NOT MuSCs. To sustain such a statement authors need to show levels of myokines and receptor mRNA in quiescent MuSCs vs Fibers. Muscle Fiber isolation followed by MuSCs stripping could be useful. Is qsc gene expression detailed in Machado (doi.org/10.1016/j.celrep.2017.10.080) informative in this context? An interpretation of this data is creation of a niche by differentiating myotubes to direct MuSc self-renewal, this should be explored.

Response: We now include comparison of gene expression profile between muscle stem cells and myofiber (Figure below). We also include whole transcriptome profile using our newly published method (single myofiber RNA-Seq, Blackburn et al. 2019. JBC) to show that myofibers, stripped of their associated satellite cells produce myokines. As can be seen in the following figure, the key myokine EGF and its receptor EGFR show a near perfect inverse expression pattern between satellite cells and muscle fibers.



- After regeneration in transplanted mice, would the number of MuSc again be expected to drop through later life?

Response: We clearly see a decrease in the muscle stem cell pool at three months, and a further decrease at 10 months. With everything being equal, transplantation of luciferase expressing donor muscle stem cells into the muscles of Myf6-KO mice (Figure 7) shows significant impairment in

retainment of engrafted donor stem cells. We would predict that the muscle stem cells would continue to decrease in the transplanted mice. This experiment alone provides strong evidence that the muscle niche in Myf6-KO mice is not capable of maintaining stem cell pool (Figure 7B-D).

- What is being counted/measured in each panel should be made clearer in the figure legends, as should the N value, and what is actually being compared using statistical testing.

Response: In the methods section and in figure legends we clearly say that we always use two tailed t-test. n values now always mention the number of mice and/or the number of cross-sections taken from each mouse, fields of view, or fibers.

Figure 1

- Most data in Figure 1 could be supplementary.

Response: Figure 1 shows a key summary of Myf6 binding data in fully differentiated myotubes. This experiment was done using a novel method that we developed for DNA binding proteins for which there are no ChIP-grade antibodies (Soleimani et al. 2013. Nature Protocols). The figure is a key summary of how many binding sites Myf6 has (Figure 1A), the most significant transcription factor binding motifs (Figure 1B), position of the best sites within sequence under all Myf6 peaks (Figure 1C), and GO terms analysis of genes associated with Myf6 peaks. We believe all the panels in Figure 1 are necessary to show the function of Myf6 in differentiating skeletal muscle cells.

- Panel (1c) clarification on the selection of MEF2A binding sites (among that of other muscle transcription factors) is required in text. Authors do not mention the study from Moretti et al. reporting the investigation of Myf6 in adult muscle. The report of Myf6/MEF2 duelling is worth mentioning here and in general in the manuscript.

Response: Mef2a binding site is the result of unbiased analysis of 12,885 sequences under Myf6 peaks by MEME (Bailey et al. 2009 Nucleic Acids Research). It's the second most highly enriched transcription factor binding motif under Myf6 peaks after E-box sequence.

- Panel (1e) No standard deviation? $P=0$?

Response: The standard deviation is so low it doesn't show up. P-value is almost zero such that it is rounded up to zero. However, we agree with the reviewer that p-value should not be rounded up to zero. Therefore, in the revised version we indicate p-value as $p < 2.2 \times 10^{-16}$, standard p-value in similar analysis.

Figure 2

- Panels (2d-2g) refer to siRNA on primary myotubes. This is NOT 'in-vivo', please edit Page 49, as this is misleading. [Done](#).
- Panel (2c) it is not mentioned in the text. [Done](#).

Figure 3

- Panel (3c) N=?

Response: N=3. Done.

- Panel (3d) As per legend: "Depletion of Myf6 transcript by siRNAs in differentiated primary myotubes (5DM)"
- In figure 3 the authors show a decrease in VEGFA and EGF protein levels in the serum of KO animals compared to heterozygous Myf6^{+/-} mice. This is the only time heterozygous Myf6^{+/-} data is presented. It is important to determine whether there is any phenotype in heterozygous Myf6^{+/-} mice.

Response: The use of Hets for our ELISA experiments is very conclusive in showing that knockout of Myf6 leads to a significant reduction in EGF and VEGFA protein in serum of Myf6-KO mice. The hets and WT mice have same phenotype.

- Please check as figure legend for panel (3e) is confusing.

Response: Figure 3E shows the Myf6 ChIP-Seq peak occupancy within +/-100kb of the transcription start sites (TSS) of genes coding for known myokines corresponding to panel 3D. Color scale ranges from black (zero peaks) to bright red (up to six peaks).

Figure 4

- Panel (4f) is every dot presenting a fiber or a mouse? Please indicate N. If every dot is a fiber please show separated columns, one per animal, followed by an ANOVA analysis. This is common issue in figures.

Response: Every dot represents a myofiber. We now indicate all requested number in the figure legends.

WT (n=24 fibers from n=3 mice) , Myf6-KO (n=22 fibers from n=3 mice) and Myf6-KO EDL

- Panels (4g, h, i) same as per (4f)

Fig 4g: WT (n=37 fibers from n=3 mice), Myf6-KO (n=34 fibers from n=3 mice) and Myf6-KO

Fig 4h: WT (n=18 fibers from n=3 mice), Myf6-KO (n=18 fibers from n=3 mice) and Myf6-KO EDL myofibers treated with LIF (n=11 fibers from n=3 mice).

Fig 4i: WT (n=29 fibers from n=3 mice), Myf6-KO (n=22 fibers from n=3 mice)

- Panel (4g) how is "EGFR high" and "EGFR low" defined?

We now provide a section in the revised manuscript on definition of EGFR- Pax7 high.

Figure 5

- Are CSAs different between nulls and wt?

We measured the number of fibers/mm² to look at fiber size in figure EV3, and do not see a significant difference between Myf6-KO and WT mice, despite a modest trend.

- (5b, d, f, h) same as per 4f, the authors should indicate values per animal. If N=3 as written in legend, graphs (and analysis) should present 3 values for KO and 3 values for WT?

These are now mentioned in the figure legend for Fig 5:

Figure 5b: (n=30 fields of view from n=3 animals)

Figure 5d: (n=27 fields of view from n=3 animals).

Figure 5f: (n=52 fields of view from n=5 animals).

Figure 5h: (n=60 fields of view from n=3 animals).

- (5j, k) same issue with data in the graph. The authors must indicate values per animal, not per fiber with all animals pooled together.

5J: Myf6-KO (n=41 fibers from n=3 mice) from Myf6-KO mice and (n=31 fibers from n=3 mice) from WT mice

5L (the quantification of data shown in 5K): (n=38 fibers from n=3 mice) from Myf6-KO mice (n=67 fibers from n=3 mice) from WT mice.

Figure 6

- Panel (6e) similar to fig.5e (same stage) fiber CSA appears larger in null than WT. Please measure.

We did measure, see figure EV3. There is no statistically significant difference between WT and Myf6-KO fiber size, despite a minor trend.

- Panels (6b, d, f) data in the graph. The authors should indicate values per animal. I guess here every dot is a picture (?). If N=3 (or 5) as written in legend, graphs (and analysis) should present at least 3 values for KO and 3 values for WT.

Figure 6b: (n=15 fields of view from n=3 animals).

Figure 6d: (n=28 fields of view from n=3 animals) for Myf6-KO mice and (n=31 fields of view from n=3 animals) for WT mice.

Figure 6f: (n=30 fields of view from n=5 mice).

- Same in (6l, o, j, v).

6l: (n= 88 myofibers) for Myf6-KO and (n= 86 myofibers) for WT mice.

6o: (n=56 myofibers) for Myf6-KO and (n=67 myofibers) for WT mice.

6j: (n=54 fields of view from n=3 mice) for Myf6-KO and (n=81 fields of view from n=4 mice) for WT mice.

6v: (n=19 fibers in EGF-treated and n=16 from untreated control myofibers).

- Panel (6 l, j) Fusion index is normally calculated as the number of nuclei within MyHC-positive myotubes with {greater than or equal to}2 nuclei as a percentage of the total nuclei present as reported in other studies (e.g. doi.org/10.1038/ncomms10871). Percentage of myonuclei in total number of nuclei per field of view" can be defined as a 'differentiation index'. Please amend. What is the N=?, please state and correct stats accordingly.

Going by this description, we did measure the fusion index, not differentiation index. Our figure is the ratio of nuclei in myotubes (two or more nuclei) versus total number of nuclei. In the Figure 6J

we have counted n=54 fields of view from Myf6-KO myotubes from three mice and n=81 fields of view from myotubes of four WT mice.

What is the difference between (6l) and (6m) and (6o) and (6p), legend is unclear. (6l) and (6o) may use incorrect stats. Please correct according to biological replicates.

In 6l-O, each point represents a fiber. In 6m-p, each point represents a mouse. In the revised version we have indicated the number of fibers, fields of view, number of biological replicates accordingly.

- Panel (6u). Legend is unclear. Have the same fibers been treated several times with EGF or different fibers have been suggested to different time points. What is the picture actually showing?

The same fibers were treated multiple times with EGF at different time points. The picture is showing the number of Pax7⁺ cells after the full 48 hours of sequential EGF treatments. We now mention that the fibers were treated sequentially.

- Please correct (6v) to report biological replicates and stats accordingly.
Figure 6v: n=16 fibers for WT and n=19 fibers for WT treated with EGF.

Figure 7

- Panel (7l) should be joined to (6u) and possibly to (2l and 2m)

Fig 7l is showing EdU incorporation, which fits with the point that Myf6-KO mice have a defective MuSC niche and not an intrinsic defect in the satellite cells themselves. We therefore believe that panel 7l should remain where it is.

- Panel (7m) Stats needs revision as per previous comment.

Two tailed t-test were performed between Myf6-KO and WT at each different time point: 6 hours (WT n=75 fields of view from n=3 mice, Myf6-KO n=72 fields of view from n=3 mice), 12 hours (WT n=82 fields of view from n=3 mice, Myf6-KO n=82 fields of view from n=3 mice), and 24 hours (WT n=70 fields of view from n=3 mice, Myf6-KO n=65 fields of view from n=3 mice) of EdU incorporation.

- Panel (7k) Authors should define "PAX7 high"

We now define Pax7high in the materials and methods under "Immunofluorescence Imaging".

- Panel (7n) authors should show whether Myf6 is expressed from myofiber, MuSCs or both
Panel 7n has been moved to the "synopsis" section. We now provide gene expression data of satellite cells, myoblasts and single muscle fiber and show that Myf6 is expressed in muscle fiber but not in adult satellite cells or myoblasts.

In this manuscript, Lazure et al. use a combination of approaches and datasets to provide evidence that MRF4 is the only myogenic basic helix-loop-helix (bHLH) transcriptional activator expressed in mature, differentiated skeletal myofibers and is specifically required for the activation of several myokines implicated in the paracrine control of muscle stem cell activation.

While this reviewer appreciates that MRF4-regulated expression and release of myokines could provide a novel layer of regulation of muscle stem cells, by cross talk with myofibers, this study contains multiple flaws and inconsistencies that preclude the evaluation of most of the results presented in this manuscript.

I note that this is the Reviewer 1 of the initial submission of this manuscript, and this assessment takes into account the author's point-to-point reply to the 3 referees. In this regard, the authors have made an effort to reply to the multiple concerns raised by this reviewer and reviewers 2 and 3; however, not all the referee's points have been satisfactorily addressed and there are still important issues that remain unsolved.

Below are the major concerns that remain unsolved in the revised manuscript and some suggestions on how to address them. I also encourage the authors to modify the manuscript by trimming the existing data and selecting the most accurate evidence that could support the main author's conclusion - that is, MRF4 regulates myofiber's output of regulatory factors for muscle stem cells.

1) MRF4 vs other bHLH myogenic factors and control of gene expression in myofibers: while this reviewer appreciates from public datasets that MRF4 is the most expressed myogenic bHLH factor in differentiated myofibers, as the authors claim, this is not sufficient to support the conclusion that MRF4 accounts for the entire control of gene expression in adult myofibers, given that MRF4 knock out mice are viable and do not show any obvious phenotype. I believe this apparent discrepancy could be explained by the functional compensation and redundancy between the myogenic bHLH factors that has not been experimentally addressed by the authors. The experimental setting used by the authors does not adequately address this issue (see specific points below).

Response: We agree that there is functional compensation by other MRFs during muscle cell differentiation. This is also consistent with our comparative ChIP-Seq data of MyoD and Myf6 (Fig. EV5). However, in the resting myofibers Myf6 is the predominant MRF that is expressed (Fig. EV1). We included RNA-Seq data of single myofibers and qPCR analysis to support this conclusion in the revised manuscript. Second, during muscle injury myogenic differentiation is activated and obviously there is compensation from other myogenic factors such as MyoD that account for lack of differentiation phenotype in Myf6-KO muscle as shown in Fig. 7E,F. Thirdly, and consistent with reviewer's comment we show that the core myogenic differentiation network is common between Myf6 and MyoD (Fig. EV5), suggesting that during differentiation there is compensation from other MRFs. In this manuscript we show that the myofiber acts as ligand and muscle stem cells as a receptor. The novel role of Myf6 in myofibers is to regulate the

expression of ligands. Since under steady state Myf6 is the predominant MRF expressed in the myofiber (Fig. EV1), genetic deletion of Myf6 abrogates ligand/receptor communication, hence leading to depletion of muscle stem cell pool in adult muscle under steady state. We have focused on two key signaling pathways, namely EGFR and STAT3 to show that loss of Myf6 leads to impaired activation of both pathways. We show that loss of Myf6 in muscle fiber leads to impairment in activation of both of these signaling pathways which play critical roles in the maintenance of the muscle stem cell pool and regulation of asymmetric division as shown by recent literature (Wang et al. 2019; Zhu et al. 2016). In the context of ligand/receptor communication between myofiber and associated muscle stem cells, transcriptional regulation of key ligands required for muscle stem cell function is controlled by Myf6 within the muscle fiber. Importantly, this function of Myf6 is not related to any potential role for Myf6 in adult muscle stem cells as our data clearly shows that muscle stem cells and myoblasts do not express Myf6. Therefore, Myf6 establishes a ligand/receptor interaction between myofibers and their associated stem cells via myokine signaling.

2) Cell culture and MRF4 over-expression: the authors rely on data generated from MRF4-tagged over-expression in cultures of myoblasts generated from muscle stem cells isolated from wild type mice, propagated in culture, transduced with retroviral delivery of tagged-MRF4 and then cultured again under antibiotic selection. This protocol raises multiple concerns. First, although the authors claim that they have already published the generation of myoblasts from primary muscle stem cells isolated by FACS from adult muscles, the biological rationale is still missing, as muscle stem cells tend to spontaneously differentiate when cultured, even in the presence of high serum concentration. It is hard to believe that after multiple rounds of propagation, retroviral infection and puromycin selection, the authors could effectively generate differentiation-competent myoblasts. As a matter of fact, there is no evidence in the manuscript that these cells exist. The authors should show clear evidence of homogeneous cultures of myoblasts and their differentiated progenies (multinucleated myotubes), by providing phase contrast pictures of wide fields representative of these cultures, and use immunostaining for mutually exclusive identity markers (Pax7, MyoD, myogenin, MyHC). The data presented in Fig. EV1 show just some random nuclear staining without any phenotypic or molecular indications that these nuclei belong to myoblasts or myotubes or other cell types, and this is largely insufficient. Second, MRF4 overexpression is not the proper way to determine the physiological role of MRF4 in the control of gene expression. As MRF4 target genes are bound by endogenous MRF4, the over-expressed MRF4 will clearly bind off-target genes. ChIP-qPCR validation is mandatory in this case, but has not been performed by the authors. As the authors have available MRF4 null mice, this reviewer is wondering why they did not use myoblasts from these mice, instead of wild type cells, to replace MRF4 with the exogenous gene. Third, the relative role of MRF4 versus endogenous bHLH is also not properly addressed, given that the authors did not perform a direct comparison of chromatin binding of exogenous tagged-MRF4 and other bHLH factors (i.e. MyoD) in the same cell types - see specific point below. Again, comparative ChIPqPCR validation should have been performed on target genes with antibodies against taggedMRF4, MyoD, Myf5, and myogenin.

Response: First, the ChTAP-Seq approach (Soleimani et al. 2013, Nat. Protocols) we have taken to map genome-wide expression of Myf6 is the same approach that we have taken to generate data for numerous other myogenic factors such as Pax3, Pax7 (Soleimani et al. 2012. Dev. Cell), MyoD, Myf5, Snai1, HDAC1, HDAC2 (Soleimani et al. 2012. Mol. Cell). The validity of this method has already been experimentally demonstrated and peer reviewed in the above publications from our lab. We have no problem with generating cultured myoblasts over expressing myogenic factors as demonstrated by our published work just cited. Second, due to the lack of a ChIP-grade antibody against Myf6, ChTAP-Seq is a robust and viable alternative which we have successfully used to map Myf6 binding sites. Third, Myf6 ChIP is only a screen and the overwhelming *in vivo* and *in vitro* data presented in the manuscript to support our conclusion stand on its own even in the absence of ChIP-Seq data. Lastly, we have validated key Myf6 myokine targets by loss of function analysis such as qPCR (Fig. 2D-G), myotubes secretome (Fig. 3F,G) and blood serum (Fig. 3H,I). I would also like to point out that there is no ChIP grade antibody against Myf6 (MRF4). Therefore, performing an endogenous ChIP qPCR using Myf6 antibody is impossible.

3) Explanation of MRF4 null phenotype: the lack of an overt phenotype is likely due to the compensatory effect by other bHLH factors, which means that MRF4 represses them and that upon MRF4 deficiency de-repression of these factor drives the compensation. Interestingly, this compensation does not seem to occur with myokines. Thus, it seems to me that the authors should focus on the understanding of this selective phenotype shown by MRF4 null muscles - e.g. loss of myofiber-muscle stem cell control, presumably by failure of MRF4 to transcriptionally activate the expression of myokines from myofibers. This is the major novelty of this work and it raises the interesting issue of why in myofibers the transcription of myokines is specifically and non-redundantly regulated by MRF4, but not other bHLH factors. Of course, a comparative analysis with MyoD- or Myf5-null muscles would also be necessary to support this contention.

Response: Our RNA-Seq of Myf6-KO and WT and ELISA assay (Fig. 3) clearly shows what the reviewer is pointing out. This conclusion is also clearly outlined in the synopsis figure. As to why in resting myofiber the transcription of myokines is specifically regulated by Myf6 is because in resting myofiber Myf6, but no other MRFs is expressed (Fig. EV1). What makes Myf6 unique in regulating myokines in muscle fiber is the unique temporal expression of Myf6 which is restricted to the mature myofiber (Fig. EV1). In other words, in resting myofibers, Myf6 is the predominant myogenic factor that is expressed! The expression of other remaining myogenic factor (Myf5, MyoD and Myogenin) is minimal – at the background level. In our datasets available through this GitHub link (https://github.com/ahcorcha/Lazure_et_al_2020/tree/master/data/bigwigs) one can clearly see that only single myofibers and whole muscle but not muscle stem cells express Myf6. With regards to the suggestion that a comparative analysis with MyoD and Myf6-null mice is required to support our conclusion, we respectfully disagree. In the Myf6-KO mice myokine expression is significantly down regulated (Fig. 3), clearly indicating that none of the other MRFs can compensate for Myf6 function. This is because myofibers only expresses Myf6. I would also like to draw the attention

of the reviewer to Fig. 7 where we show that everything else being equal, Myf6-KO muscle is incapable of maintaining transplanted muscle stem cells. This data clearly indicates that Myf6-KO muscle is an unfavorable niche for stem cells.

4) Genome wide data and analysis. 1) ChIPseq: Although multiple ChIPseq datasets are presented, this reviewer found that the GEO link provided shows that the only ChIPseq data generated by the authors is actually just one experimental replicate with anti-Tag antibodies against tagged-MRF4 (in this regard, the authors should rectify the information in the public repository about the antibodies used, as it is stated anti-Myf6-MRF4, while it should instead be specified that the ChIPseq has been done with an antibody against an over-expressed tagged-Myf6-MRF4). One replicate with over-expressed tagged protein and without validation with ChIPqPCR analysis on gene of specific interest is not acceptable to support the multiple claims made by authors in this manuscript. Moreover, only Myf6-MRF4 peak calls and two differential expression analyses results were found in the GEO link, with no ChIP-seq or RNA-seq tracks in bigWig format (bigWig files of RNA-seq data are provided as a separate GitHub link). Raw data are not accessible. It is therefore impossible to assess the data quality. Finally, the comparisons of ChIPseq profiles shown in this manuscript are between the over-expressed tagged-MRF4 (in the myoblast cell line generated by the authors) and publicly available datasets, which have been generated from other cell types. This is not an accurate comparison, especially when made among myogenic bHLH factors that share the same DNA binding motif. Likewise, RNA-seq tracks are compared with gene expression microarrays generated from different cell lines (Fig. 2 and 3, and Fig. EV2) and this comparison is definitely inaccurate.

Response: As mentioned earlier we performed Myf6 ChIP-Seq following the same protocol as we have done with multiple other myogenic factors (Soleimani et al. 2013, Nat. Protoc, Soleimani et al. 2012, Dev. Cell; Soleimani et al. 2012, Mol. Cell). All of the ChIP-Seq datasets that the reviewer is referring to were generated by VDS (The corresponding author on this manuscript). I am sorry the reviewer has problems with login to GEO. We have verified with NCBI and logged in to access data from multiple computers and we do not see any problem with accessing data. In the GitHub page we provided RNA-Seq data for WT satellite cells, Myf6-KO satellite cells, single myofibers, whole muscle of Myf6-KO and WT mice under resting and injury condition. We have made this data publicly available so that the reviewers can access it freely without needing password. We provided BigWig files in response to specific request from the editorial team. All of the raw data is also deposited in GEO. Regarding the last point raised in Figure EV2, we are not comparing RNA-Seq to microarray, we just show them side by side. I don't see any problem with showing different datasets side by side.

5) MRF4-target myokines and their effect on muscle stem cells: this analysis is very diffuse, with an initial identification, from ChIP-seq and RNA-seq data, of certain potential MRF4-target myokines in myofibers (EGF, VEGF, LIF), which is followed by analysis of their downstream signaling pathways activated in muscle stem cells that range from STAT3 and EGFR to p38 signalling in WT or MRF4 KO cells. Overall, the analysis turns to be diffuse and not conclusive. A coherent and complete investigation of just one axis between MRF4-activated myokines in

myofibers and the engagement of the specific downstream signal transduction pathway that regulates muscle stem cell activity is highly recommended. In this regard, I also recommend that the authors refer to just one mechanism of control of muscle stem cells by MRF4-activated myokines - either maintenance of quiescence or activation. It is still unclear the interpretation of the biological outcome of MRF4 deficiency. For instance, the authors show that MRF4 null myofibers are defective in EGF, VEGF and LIF expression in unperturbed conditions, and this results in deficient activation of STAT3 and EGFR signaling in muscle stem cells. These signaling have been implicated in the activation, division polarity and differentiation of satellite cells, but not maintenance of quiescence; however, the MRF4 deficient muscle stem cells show spontaneous loss of quiescence and activation, leading to an overall loss of number. It is also unclear why is the p38 activated in MRF4 null muscle stem cells - what is the MRF4-regulated myokine in myofibers that negatively represses p38 signaling in muscle stem cells and how?

Response: We respectfully disagree with the reviewer on this point. Going from the binding of Myf6, to the ligands required for activation of those signaling pathways, followed by validation and gain/loss of function and its effect on myofiber associated stem cells is a very focused and logical progression. The key signaling pathways we refer to in the manuscript are known to play essential roles in regulating muscle stem cell function. For example, VEGFA signaling has recently been implicated in maintaining muscle stem cell quiescence (Verma et al. 2018). EGFR has been shown to regulate muscle stem cells asymmetric cell division (Wang et al. 2019) and STAT3 has been shown to regulate the muscle stem cell pool (Zhu et al. 2016). With regards to why in Myf6-KO muscle p38MAPK is activated and what might be responsible to block p38, we have already shown in the revised manuscript that EGF alone can block activation of p38 (Fig 6). Therefore, loss of Myf6 in muscle leads to abrogation of ligand/receptor communication. This communication is required to maintain muscle stem cell quiescence.

Overall, the manuscript does not seem suitable for publication in the present form, as it lacks scientific rigor, rationale and data supporting author's conclusions. Still, this reviewer notices that there are some convincing datasets that could stand alone to support some novel and interesting conclusion, when separated from the other inaccurate datasets. Most of the data ex vivo and in vivo generated with MRF4 KO mice, single fibers and satellite cells are convincingly supporting the author's hypothesis that in myofibers MRF4 is required to activate expression of myokines that regulate muscle stem cell activation. I suggest that the authors re-haul the whole manuscript by focusing on these data and adding essential experiments. For instance, starting with RNA-seq analysis in MRF4 KO fiber can identify MRF4-specific myokines, and this can be followed by biochemical validation (qPCR and ChIPqPCR) upon restoration of MRF4 (by delivery of tagged-MRF4) as compared with tagged-MyoD, as control. Then, the authors can focus on activation of one specific signaling triggered by one MRF4-regulated myokine in satellite cells in one specific condition (either quiescence or injury-mediated activation of muscle stem cells) and then show related experiments of rescue ex vivo and the in vivo data, which appear accurate and reproducible. This reviewer highly recommend that the authors limit the complexity of their experimental system (and claims to support) by narrowing down the study

on just one event regulated by MRF4 - either myofiber-released myokines that control muscle stem cell quiescence in resting conditions or satellite cell activation upon injury.

Response: We disagree with selectively narrowing down the effect of Myf6 to one molecule and one pathway and ignoring everything else. We think the manuscript represent an unbiased analysis of Myf6 function in muscle and should include key components regulated by Myf6. We have extensively validated the role Myf6-mediated regulation of myokines to EGFR and STAT3 pathways and provide compelling evidence that Myf6 functions as a major regulator of muscle stem cell quiescence by establishing a ligand/receptor interaction between myofiber and the associated muscle stem cells. The reviewer mentions “accurate” and “inaccurate” datasets but doesn’t say which one is which and on what basis certain datasets are inaccurate or accurate!

Dear Vahab,

Thank you for the submission of your revised manuscript. We have now received the comments from referee 1 whom I asked to assess your reply to all concerns, as the other 2 referees were unfortunately not available.

As you will see, the referee is not convinced that the data have been sufficiently and satisfactorily strengthened, and we can therefore not offer to publish your manuscript. I also attach the referee response to your reply to the comments below and hope that it makes our case clear. While we are interested in the novel findings reported in your study, these would have to be strengthened and the manuscript would have to be refocused on the most novel and interesting aspects. In the absence of such revisions, we have decided not to proceed with the handling of your manuscript at EMBO reports.

I am sorry to disappoint you on this occasion, and hope that the referee comments will be helpful in your continued work in this area.

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

In this manuscript, Lazure et al. use a combination of approaches and datasets to provide evidence that MRF4 is the only myogenic basic helix-loop-helix (bHLH) transcriptional activator expressed in mature, differentiated skeletal myofibers and is specifically required for the activation of several myokines implicated in the paracrine control of muscle stem cell activation.

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Point-by-point response to reviewer:

We thank the reviewer for in depth examination of our manuscript and for many of her/his important suggestions to improve the quality and readability of the manuscript. To address the reviewer's and editorial concerns in the revised version of this manuscript, we have incorporated the following new figures and panels: new Fig1A; new Fig2F; new Fig3J,K,L,M; new FigEV1C,D; new Fig EV2 E,F,G and new FigEV4A. These new experiments further corroborate our original conclusions that Myf6 has a critical and unique role in regulating myokines in the myofiber to prevent premature differentiation of their associated satellite cells. The manuscript conclusively shows that 1) Myf6 is required to establish a receptor/ligand relationship between myofibers and their associated satellite cells; 2) Myf6 is the principal MRF that is exclusively expressed in myofibers but not in adult satellite cells; 3) myofibers are the major sources of myokines such as EGF and VEGFA whose transcription is dependent on Myf6 in the fiber and 4) in the absence of Myf6 in the myofiber their associated satellite cells prematurely activate and differentiate by upregulating p38 MAPK and MyoD.

More specifically, and as requested by the reviewer and the editor, we have performed a rescue experiment, validated by both RT-qPCR and ELISA, showing that re-introduction of exogenous Myf6 through retroviral vector into the Myf6-null primary myoblasts isolated from the Myf6-KO mice by FACS can restore the expression of myokines. We have now confirmed the reintroduction of Myf6 into these cells and their ability to differentiate using qPCR and immunofluorescence. We have also performed ChIP-qPCR for H3K27Ac in myotubes, a regulatory histone mark of active chromatin and reanalyzed previous RNA-Seq data from whole muscle, single myofibers and satellite cells to further prove the contribution of the muscle fiber to myokine production. Finally, we have refocused the manuscript to center our findings on the EGF/EGFR/P38MAPK axis. We strongly believe that the extensive amount of experimental data presented in this revised manuscript provides unequivocal proof of our conclusion. Lastly, we have extensively edited the manuscript and refocused specifically on EGFR signaling pathway to demonstrate the importance of Myf6-regulated ligands within myofiber in establishing a receptor/ligand relationship between satellite cells and myofibers. We believe, this novel discovery will further enhance our knowledge on how skeletal muscle stem cells communicate with their niche environment.

1) MRF4 vs other bHLH myogenic factors and control of gene expression in myofibers: while this reviewer appreciates from public datasets that MRF4 is the most expressed myogenic bHLH factor in differentiated myofibers, as the authors claim, this is not sufficient to support the conclusion that MRF4 accounts for the entire control of gene expression in adult myofibers, given that MRF4 knock out mice are viable and do not show any obvious phenotype. I believe this apparent discrepancy could be explained by the functional compensation and redundancy between the myogenic bHLH factors that has not been experimentally addressed by the authors. The experimental setting used by the authors does not adequately address this issue (see specific points below).

Response:

We appreciate the reviewer's comments regarding relationship between Myf6 and other MRFs. In this refocused version of the revised manuscript we show that Myf6 transcriptionally regulates EGF which in turn prevents premature differentiation of satellite cells under normal physiological conditions by blocking p38 MAPK activation. This signaling pathway between myofiber and satellite cells is entirely regulated by Myf6. The proof of this novel regulatory pathway is further corroborated by the new rescue experiment in which exogenous Myf6 reintroduced into the Myf6-KO restores the expression of EGF.

2) Cell culture and MRF₄ over-expression: the authors rely on data generated from MRF₄-tagged over-expression in cultures of myoblasts generated from muscle stem cells isolated from wild type mice, propagated in culture, transduced with retriviral delivery of tagged-MRF₄ and then cultured again under antibiotic selection. This protocol raises multiple concerns. First, although the authors claim that they have already published the generation of myoblasts from primary muscle stem cells isolated by FACS from adult muscles, the biological rationale is still missing, as muscle stem cells tend to spontaneously differentiate when cultured, even in the presence of high serum concentration. It is hard to believe that after multiple rounds of propagation, retroviral infection and puromycin selection, the authors could effectively generate differentiation-competent myoblasts. As a matter of fact, there is no evidence in the manuscript that these cells exist.

We have now added additional primary cells lines over expressing Myf6 and we show that these cells are perfectly capable of myogenic differentiation as shown by immunofluorescence analysis with marker of terminal differentiation, MF20. Specifically, the following new experiment directly address the reviewer's concern:

new Fig EV1C: IF of Myf6-HF myotubes (staining for Flag, DAPI, transmitted)

new Fig EV1D: IF of Myf6-HF myotubes (staining for MF20, DAPI, transmitted)

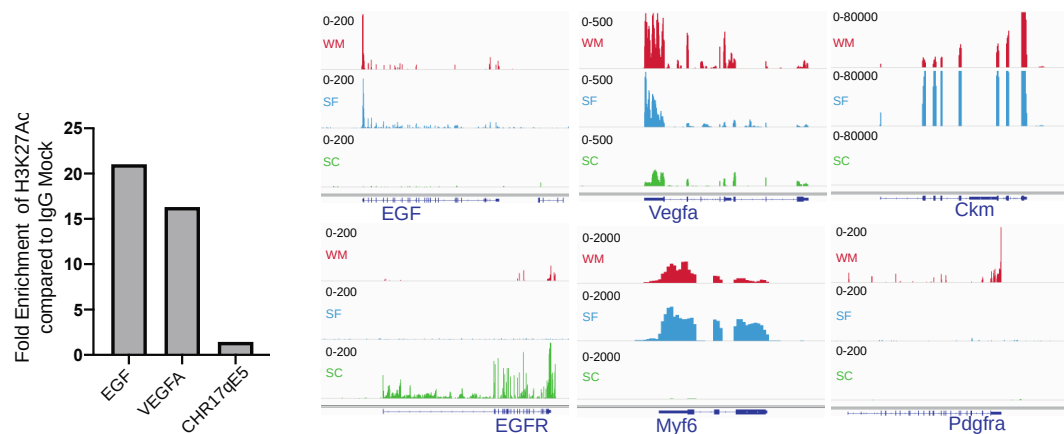
new Fig 3K: qPCR for Myf6 in Myf6-HF, WT, and Myf6-KO myotube cDNA.

These new experiments and new figures are proof that stable primary cell lines are capable of myogenic differentiation as we have shown in this manuscript.

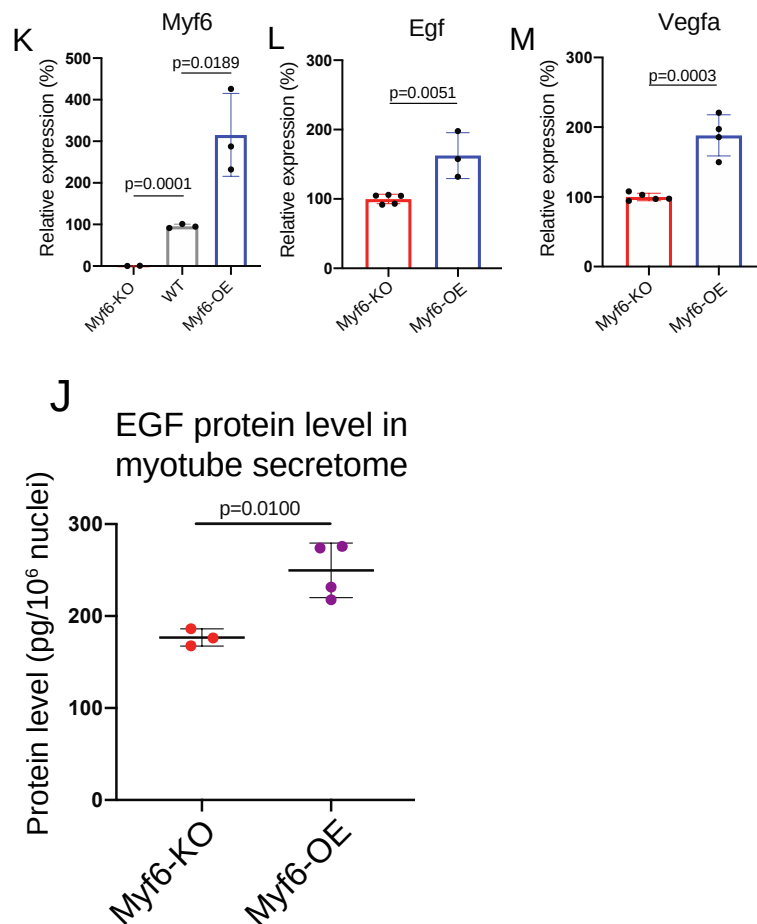
The authors should show clear evidence of homogeneous cultures of myoblasts and their differentiated progenies (multinucleated myotubes), by providing phase contrast pictures of wide fields representative of these cultures, and use immunostaining for mutually exclusive identity markers (Pax7, MyoD, myogenin, MyHC). The data presented in Fig. EV1 show just some random nuclear staining without any phenotypic or molecular indications that these nuclei belong to myoblasts or myotubes or other cell types, and this is largely insufficient. Second, MRF₄ overexpression is not the proper way to determine the physiological role of MRF₄ in the control of gene expression. As MRF₄ target genes are bound by endogenous MRF₄, the over-expressed MRF₄ will clearly bind off-target genes. ChIP-qPCR validation is mandatory in this case, but has not been performed by the authors. As the authors have available MRF₄ null mice, this reviewer is wondering why they did not use myoblasts from these mice, instead of wild type cells, to replace MRF₄ with the exogenous gene. Third, the relative role of MRF₄ versus endogenous bHLH is also not properly addressed, given that the authors did not perform a direct comparison of chromatin binding of exogenous tagged-MRF₄ and other bHLH factors (i.e. MyoD) in the same cell types - see specific point below. Again, comparative ChIPqPCR validation should have been performed on target genes with antibodies against taggedMRF₄, MyoD, Myf5, and myogenin.

Response: We have now provided immunofluorescent images of myotubes (5DM) over-expressing Myf6-HF (Figure EV1C-D), including staining for both FLAG and MF20. We also provide homogenous cultures of those stable primary cell lines stained PAX7 prior to their differentiation. These new experiments show that our Myf6-HF primary myoblasts are differentiation-competent. Additionally, we show by RT-qPCR (new Fig. 3K) of Myf6 expression in Myf6-KO, WT and Myf6-OE myotubes generated from Myf6-KO myoblasts, that Myf6-O/E myotubes are exogenously expressing Myf6-HF.

Regarding comparative analysis of ChIP for myogenic factors such as MyoD, Myf5 and Myog, we agree with the reviewer that such analysis is important but it is beyond the scope of this manuscript. However, as an additional confirmation that myofibers are an important source of myokines, we have performed ChIP-qPCR for H3K27Ac in myotubes, and show that the Transcription Start Sites (TSS) of EGF and VEGFA are active compared to a closed, negative control region of the genome (Chr17qE5). Additionally, we have now generated Integrative Genomics Viewer (IGV) tracks comparing EGF, EGFR and VEGFA expression from RNA-Seq of whole muscle, single fibers, and satellite cells, including positive controls (Myf6, CKM) and negative control (Pdgfra) for single fiber purity. These additional panels complement our UCSC ChIP-Seq tracks (figure 2C,D,E) for H3K4me3 and H3K4me1 in the regulatory regions of EGF, VEGFA, OSM and LIF, and further confirm one of our main findings that muscle fibers are a principal source of myokines.



To address the reviewers' concerns on the effect of endogenous versus exogenous Myf6, we have now generated stable cell lines derived from Myf6-null myoblasts, where Myf6-HF has been reintroduced by retroviral infection. RT-qPCR analysis shows that the addition of Myf6 into Myf6-null cells rescues the expression of myokines EGF and VEGFA. Furthermore, ELISA using the secretome from these Myf6-OE myotubes shows a similar increase of EGF at the protein level. This new data further corroborates our original conclusions and shows that Myf6 is necessary and sufficient for transcriptional activation of myofiber-derived myokines.



3) Explanation of MRF₄ null phenotype: the lack of an overt phenotype is likely due to the compensatory effect by other bHLH factors, which means that MRF₄ represses them and that upon MRF₄ deficiency de-repression of these factor drives the compensation. Interestingly, this compensation does not seem to occur with myokines. Thus, it seems to me that the authors should focus on the understanding of this selective phenotype shown by MRF₄ null muscles - e.g. loss of myofiber-muscle stem cell control, presumably by failure of MRF₄ to transcriptionally activate the expression of myokines from myofibers. This is the major novelty of this work and it raises the interesting issue of why in myofibers the transcription of myokines is specifically and non-redundantly regulated by MRF₄, but not other bHLH factors. Of course, a comparative analysis with MyoD- or Myf5-null muscles would also be necessary to support this contention.

We agree with the reviewer that lack of differentiation phenotype in Myf6 null mice is likely due to compensation from other MRFs. However, as the reviewer points out regulation of Myokines within myofiber is uniquely controlled by Myf6 and hence in the absence of Myf6 myokine-mediated communication between myofiber and associated satellite cells breaks down. We have extensively edited the manuscript and refocused it to clearly demonstrate this unique function of Myf6.

4) Genome wide data and analysis. 1) ChIPseq: Although multiple ChIPseq datasets are presented, this reviewer found that the GEO link provided shows that the only ChIPseq data generated by the authors is actually just one experimental replicate with anti-Tag antibodies against tagged-MRF₄ (in this regard, the authors should rectify the information in the public repository about the antibodies used, as it is stated anti-Myf6-MRF₄, while it should instead be specified that the ChIPseq has been done with an antibody against an over-expressed tagged-Myf6-MRF₄). One replicate with over-expressed tagged protein and without validation with ChIPqPCR analysis on gene of specific interest is not acceptable to support the multiple claims made by authors in this manuscript. Moreover, only Myf6-MRF₄ peak calls and two differential expression analyses results were found in the GEO link, with no ChIP-seq or RNA-seq tracks in bigWig format (bigWig files of RNA-seq data are provided as a separate GitHub link). Raw data are not accessible. It is therefore impossible to assess the data quality. Finally, the comparisons of ChIPseq profiles shown in this manuscript are between the over-expressed tagged-MRF₄ (in the myoblast cell line generated by the authors) and publicly available datasets, which have been generated from other cell types. This is not an accurate comparison, especially when made among myogenic bHLH factors that share the same DNA binding motif. Likewise, RNA-seq tracks are compared with gene expression microarrays generated from different cell lines (Fig. 2 and 3, and Fig. EV2) and this comparison is definitely inaccurate.

1. **The Myf6 ChIP-Seq was performed as an initial screen. The requirement of Myf6 for adequate production of myokines has been validated through our extensive in vivo and in vitro experiments including whole muscle RNA-Seq, ELISA, immunofluorescence and qPCR, knockout/rescue experiments.**
2. **All ChIP-Seqs included in this manuscript (Myf6, MyoD, H3K4me3, H3K4me1, H3) have been performed by the corresponding author, and we therefore believe that is appropriate to display different tracks alongside each other.**
3. **We have edited all figures containing both microarray and RNA-Seq data. We now display the whole muscle RNA-Seq figure and the microarray panels in separate figures (Fig 2A, Fig 3D), or in separate panels (Fig EV3 B,D).**

5) MRF₄-target myokines and their effect on muscle stem cells: this analysis is very diffuse, with an initial identification, from ChIP-seq and RNA-seq data, of certain potential MRF₄-target myokines in myofibers (EGF, VEGF, LIF), which is followed by analysis of their downstream signaling pathways activated in muscle stem cells that range from STAT₃ and EGFR to p38 signalling in WT or MRF₄ KO cells. Overall, the analysis turns to be diffuse and not conclusive. A coherent and complete investigation of just one axis between MRF₄-activated myokines in myofibers and the engagement of the specific downstream signal transduction pathway that regulates muscle stem cell activity is highly recommended. In this regard, I also recommend that the authors refer to just one mechanism of control of muscle stem cells by MRF₄-activated myokines - either maintenance of quiescence or activation. It is still unclear the interpretation of the biological outcome of MRF₄ deficiency. For instance, the authors show that MRF₄ null myofibers are defective in EGF, VEGF and LIF expression in unperturbed conditions, and this results in deficient activation of STAT₃ and EGFR signaling in muscle stem cells. These signaling have been implicated in the activation, division polarity and differentiation of satellite cells, but not maintenance of quiescence; however, the MRF₄ deficient muscle stem cells show spontaneous loss of quiescence and activation, leading

to an overall loss of number. It is also unclear why is the p38 activated in MRF4 null muscle stem cells - what is the MRF4-regulated myokine in myofibers that negatively represses p38 signaling in muscle stem cells and how?

Response: we have now refocused our manuscript to highlight the involvement of Myf6 in the EGF/EGFR signaling pathway. We show that Myf6 is required for the production of EGF in the myofiber. We have moved data involving LIF/STAT3 signaling to the expanded view section (figure EV3). EGF signaling is directly related to p38MAPK activation, as shown by the block of differentiation upon EGF treatment (Fig 5Q-T). Overall, our manuscript now clearly focuses on how Myf6 regulates EGF, which blocks P38MAPK signaling in order to prevent premature differentiation and consequent exhaustion of the MuSC pool.

Once again we thank the reviewer for critical evaluation of our manuscript. We believe, the revised and refocused version of our manuscript which contain additional new experiments provides compelling evidence that Myf6 establishes a receptor/ligand relationship between myofiber and their associated satellite cells.

Dear Vahab,

Thank you for the submission of your revised manuscript. We have now received the enclosed comments from referee 1 who now supports the publication of your manuscript and we can therefore in principle accept it.

Only a few formal changes are still required:

- Please delete one keyword as only 5 keywords can be included.
- Arezu Jahani-Asl is missing from the Author Contributions, please add.
- Our Reference format changed to Harvard style, please correct. The EMBO reports style is also in EndNote.
- Please add the grant numbers to our online manuscript handling system when you upload the final manuscript version.
- A Table EV1 callout is missing, please add.
- Please add the EV Figure legends to the main Article file after the main Figure legends.
- The EV Tables with their legends should be uploaded as individual word or excel files.
- The source data needs to be uploaded as one file per figure.
- Tables EV1-3 should be regular EV tables and uploaded as individual files.
- Tables EV4 and EV5 should be Datasets (Dataset EV1 and EV2) and uploaded as individual excel files. All EV Tables and Datasets need a legend within their files.
- I attach to this email a related ms file with comments by our data editors. Please address all comments in the final manuscript.

I slightly modified the short summary and first bullet point. Please let me know whether you agree with the following:

Skeletal muscle fibers are a key signaling component of the muscle stem cell niche. Myf6-induced expression of myokines in skeletal muscle maintains myofiber-associated muscle stem cell pools by blocking their premature differentiation in adult mice.

- Myf6 transcriptionally promotes the expression of a vast array of myokines such as Epidermal Growth Factor EGF and VEGFA in the myofibers of adult mice.
- Genetic deletion of Myf6 impairs the activation of the EGFR signaling pathway in satellite cells.
- Mice with a homozygous deletion of Myf6 are born normal. However, Muscle stem cells from Myf6-knockout mice spontaneously break from quiescence, activate p38 MAPK and enter into myogenic differentiation in resting adult mouse muscle.

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

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

The authors have addressed most of the reviewer concerns and extensively rehailed the manuscript, which is now more focused on the most interesting discovery here - that is, the specific and non-redundant (among the myogenic bHLH factors) ability of Myf6//MRF4 to activate the expression of myofiber-derived signals that negatively regulate satellite cell activation. There are still few experimental details that do not completely convince this reviewer; however, I believe that at this stage it is fair to give to the authors the opportunity to publish their study and in particular their findings on a this novel myofiber/satellite cells cross-communication through the Myf6/MRF4-EGF-p38 signalling, as it will be of great interest to the scientific community in the field of skeletal myogenesis.

Response to Reviewer:

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[Response: Thank you!](#)

Response to editor:

Only a few formal changes are still required:

- Please delete one keyword as only 5 keywords can be included.

[Response: Done.](#)

- Arezu Jahani-Asl is missing from the Author Contributions, please add.

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[Response: Done.](#)

- Please add the grant numbers to our online manuscript handling system when you upload the final manuscript version.

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- A Table EV1 callout is missing, please add.

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- The EV Tables with their legends should be uploaded as individual word or excel files.

Response: Done.

- The source data needs to be uploaded as one file per figure.

Response: Done.

- Tables EV1-3 should be regular EV tables and uploaded as individual files.

Response: Done.

- Tables EV4 and EV5 should be Datasets (Dataset EV1 and EV2) and uploaded as individual excel files. All EV Tables and Datasets need a legend within their files.

Response: Done.

- I attach to this email a related ms file with comments by our data editors. Please address all comments in the final manuscript.

Response: We have now addresses all comments in the final version of the manuscript.

I slightly modified the short summary and first bullet point. Please let me know whether you agree with the following:

Response: Thank you! I agree with the changes you made.

Skeletal muscle fibers are a key signaling component of the muscle stem cell niche. Myf6-induced expression of myokines in skeletal muscle maintains myofiber-associated muscle stem cell pools by blocking their premature differentiation in adult mice.

- Myf6 transcriptionally promotes the expression of a vast array of myokines such as Epidermal Growth Factor EGF and VEGFA in the myofibers of adult mice.
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Dr. Vahab Soleimani
McGill University
Human Genetics
3640 rue University
Montreal, QC H3A 0C7
Canada

Dear Vahab,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
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Corresponding Author Name: Vahab D Soleimani
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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if $n \leq 5$, the individual data points from each experiment should be plotted and any statistical test employed should be justified.
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
 - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
 - are tests one-sided or two-sided?
 - are there adjustments for multiple comparisons?
 - exact statistical test results, e.g., P values = x but not P values < x;
 - definition of 'center values' as median or average;
 - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size were chosen based on numbers on the number of biological replicates published in similar studies.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	At least three biological biological replicates (three mice per group) were used in all relevant experiments
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Samples were analyzed blindly and where counting was done the counts were corroborated independently by two people
For animal studies, include a statement about randomization even if no randomization was used.	No randomization was used
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Yes. Samples were analyzed blindly.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The blinding was done at the stage of counting and analysis of results.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. Statistical significance was assessed by two tailed t-test unless specified otherwise
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibodies with Company and Catalog number Pax7:DSHB AB_528428 MyoD (G-1):Santa Cruz Sc-377460 Laminin: Sigma-Aldrich, L9393 MF20:DSHB, AB_2147781 EGFR:Cell Signaling42675 Phospho-EGFR (Y1068): Cell Signaling 22345 STAT3:Cell Signaling91395 STAT3:Cell Signaling49045 Phospho-STAT3 (Y705): Cell signaling, 91315 Phospho-STAT3 (Y705): Cell Signaling, 91455 Phospho-STAT3 (S727): Cell Signaling, 91365 GAPDH: Abcam ab9484 Tubulin: Sigma P8203 Ki67: Abcam AB15580 Anti-mouse ITGA7-Alexa647:R&D systemsFAB3518R Anti-mouse PE-CD45:Invitrogen 12-0451-82 Anti-mouse PE-CD11b: Invitrogen ebioscience 12-0112-82
	Anti-mouse PE-CD31:BD Pharmigen553373 Anti-mouse PE-Sca1/Ly6A-E:BD Pharmigen553108 Alexa Fluor 568 goat anti-mouse IgG1:Invitrogen A21124 Alexa Fluor 488 goat anti-rabbit IgG:Invitrogen A11008 Alexa Fluor 594 goat anti-mouse IgG2b: InvitrogenA21145 Alexa Fluor 594 goat anti-rabbit IgG:Invitrogen A11012 Alexa Fluor 568 donkey anti rabbit IgG: Invitrogen A10042 Alexa Fluor 488 goat anti mouse IgG1: Invitrogen A21121 p38 MAPK: Cell Signaling 92125 P-p38 MAPK (T180/Y182): Cell Signaling 92165 P-p38 MAPK (T180/Y182): Cell Signaling 45115 EGFR: BethylIHC-00005 P-EGFR (Y1068): Cell Signaling 37775 Anti-GFP Rabbit IgG:Invitrogen A11122 Anti-mouse Cleaved Caspase 3: Cell Signaling 96645
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines were tested for mycoplasma contamination HEK-293T: ATCC, CRL-1573 COS-7:ATCC, CRL-1651 Phoenix-ECO:ATCC, CRL-3214 HeLa:ATCC, CCL-2 C2C12:ATCC, CRL-1772

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	C57BL/6J: Jackson Laboratories000664 C57BL/6: Charles RiverC57BL/6NCrl Myf6tm1.1(cre/ERT2)Lepr/J: (Christoph Lepper laboratory)025669 (C57BL/6-Tg(CAG-EGFP)1Osb/J):Jackson Laboratories003291 Animals were housed at no more than 5 mice/cage with constant supply of food and water. We have primarily used mice of the ages: 1 week, 3 weeks, 3 months, 10 months of age, as indicated in the manuscript. The Myf6tm1.1(cre/ERT2)Lepr/J colony was maintained using heterozygous littermates. Both male and female mice were used in this study. Control and experimental conditions were sex-matched.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal procedures were approved by the McGill University Animal Care Committee (UAAC).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLOS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We have complied with these guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'.	A Data Availability section is provided. The primary ChIP-Seq and RNA-Seq data reported in this paper is deposited in NCBI and available through Geo Accession numbers GSE123836 and GSE133505.
Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Data has been deposited.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biocompare (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No
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