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Skeletal myosin light chain kinase regulates skeletal myogenesis by phosphorylation of MEF2C

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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. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 05 November 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. Please let me first apologise again for the delay in getting back to you with a decision - unfortunately one of the referees' reports was very late in coming in. However, we do now have the comments from all three referees, which are enclosed below. As you will see, all reviewers express significant interest in your work and are broadly in favour of publication. Referees 2 and 3, however, raise a critical issue that would need to be addressed first - namely that it would be essential to show that your major findings hold in a more physiologically relevant system. Referee 2 encourages the use of C2C12 cells, while referee 3 requests that some analysis in primary cells is included. I would strongly encourage you to repeat at least some of the critical experiments in primary cells, and to complement this with C2C12 cell culture work where appropriate (as suggested by referee 2). Referee 1 also raises a number of more technical concerns that are clearly spelled out and would need to be addressed.

In the light of the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as a standard revision time, and as a matter of policy, we do not

consider any competing manuscripts published during this period as negatively impacting on the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

In this paper, using biochemical approaches, Al Madhoun et al. have identified MLCK as a novel interactor of MEF2C protein during skeletal muscle cell differentiation. They show that MLCK is able to phosphorylate MEF2C in vitro and ex vivo and identified the phosphorylation site. Using mutants, they show that phosphorylation of MEF2C on this site is required for skeletal muscle but not cardiac muscle differentiation in P19 cells. Moreover, they show that the mutant protein is not able to induce histone aceylation and HAT recruitment on the skeletal myogenin promoter, although it has a wild type phenotype for the cardiac GATA4 promoter.
This is a solid paper, addressing an important issue (molecular basis of cell commitment), and the results are intriguing (in particular the difference between the GATA4 and myogenin promoters with regard to MEF2C phosphorylation). This reviewer, however, has some concerns that need to be addressed.

Major points:

In figure S1, a validation by Western blot of MLCK in the complex is required.
Inhibitors have various degrees of specificity. In addition to ML7, the authors should use an MLCK siRNA to further support their model.
In Figure 3D, at day 9 of differentiation, the level of cardiac α-actin should result from both cardiac and skeletal differentiation, and thus should not be as inhibited as the skeletal markers. The authors should comment on this point.
The experiment in Figure S3 is totally obscure. Is it that, contrary to what is stated in the legend, MHC was monitored at day 6 (panel A) and day 9 (panel B)? In that case, in Panel B, we should see the addition of cardiac and skeletal differentiation, and not only skeletal. The authors should comment on this point.
In figure 5A, ML7 does not impact on differentiation induced by MyoD alone. Does that imply that endogenous skMLCK is not involved under these conditions?
In figure 6B, the decrease of p300 binding to MEFT80A mutant as compared to the MEF2C is not obvious. It should be quantified.

Minor:

In Figure S1C, the label for time of differentiation is missing.
The sentence "Cardiac α-actin is expressed in both embryonic cardiac and skeletal muscle", p 10, should be rephrased: Cardiac α-actin is expressed in both cardiac and skeletal embryonic muscle.

The authors should define MHC+ve

Referee #2:

This manuscript reports on the identification of a novel MEF2C kinase - skMLCK. The authors convincingly demonstrate that skMLCK-directed phosphorylation of threonine 80 in MEF2C promotes interactions with p300 and PCAF acetyltransferases, and this is required for induction of muscle gene expression.

This work is novel, important and the experiments are well executed and described. As such, the manuscript is in principle suitable for publication on high impact journals, such as EMBO. It would be meaningless to ask for additional details or redundant evidence that might delay the publication of this interesting work. Therefore, I would just like to invite the authors to improve the quality of their discovery by performing two experiments. Note that even if not turning into positive data, at least knowing the outcome of these experiments would place the authors in the position of commenting and critically discuss the data.

1) The activation of endogenous skMLCK in a muscle cell line (e.g. C2C12) should be evaluated during proliferation and differentiation, to show a differentiation-induced activation of skMLCK.
2) The definitive demonstration of a crucial role of skMLCK in skeletal myogenesis could be provided by inhibiting the expression (by siRNA) and/or activity (by the specific inhibitor, ML-7) of skMLCK in C2C12 cells or MyoD-converted 10T1/2 fibroblasts and determine the impact on the differentiation ability of these cells, by scoring for the formation of MyHC-positive myotubes or the expression of endogenous myogenin. This should be compared with the p38 alpha and beta inhibitor SB, which blocks the myogenic program.

Minor points

1) Are the authors sure that the antibody used in ChIP (Fig. 6A) is against H3-K4 acetylated? In the same experiment they show that H3-K4 is methylated; therefore, it is unlikely that H3-K4 could be both methylated and acetylated.

2)Page 9, line 8 - the authors state that HEK-293 do not contain exogenous MLCK activity. The should show it by measuring kinase activity of MLCK.

Referee #3:

The authors provide convincing evidence that skMLCK is involved in the conversion of P19 cells to skeletal myogenesis. A requirement for the phosphorylation of MEF2C on T80 is well supported. The lack of phosphorylation on MEF2C T80 appears to prevent recruitment of p300/CAF to acetylate histones.The authors adequately explain the possibilities of a lack of a phenotype in mice that are null for skMLCK. In general the data support the authors' conclusions. The manuscript is well written and critical controls are provided to support the primary observations. I have only one overall comment. All of the experiments are performed in highly artificial systems. The authors state in the first paragraph of the discussion that skMLCK may regulate the decision of progenitor cells to commit to a myoblast vs. a satellite cell fate. The authors need to perform at least one experiment on primary cells to demonstrate that the data are applicable to a system that is representative for muscle development or regeneration.

1st Revision - Authors' Response 06 March 2011

Referee #1:

We thank this reviewer for stating that this is a solid paper, addressing an important issue.

Major points:

Reviewer: In figure S1, a validation by Western blot of MLCK in the complex is required.

We have performed a western blot of MLCK in the complex for Fig. S1, confirming that skMLCK was co-purified with tagged MEF2C but not TAP-alone.

Reviewer: Inhibitors have various degrees of specificity. In addition to ML7, the authors should use an MLCK siRNA to further support their model.

We agree with the reviewer that under usual circumstances a siRNA approach would have been ideal. However, since there was no obvious muscle development phenotype in mice lacking skeletal MLCK, we predict that there would be compensation by the smooth or cardiac muscle MLCK isoforms. It is likely that ML-7 was able to show an effect in our experiments because it can inhibit all isoforms of MLCK. Furthermore, the concentrations used are far below the concentrations at which ML-7 inhibits other kinases. According to the manufacturer (http://www.emdchemicals.com/life-science-research/ml-7-hydrochloride/EMD_BIO-475880/p_uuid?ProductID=PwGb.s1OzZcAAAEiLTxCeVC_), the primary target for ML-7 is MLCK with Ki = 300nM, the secondary targets are PKA (Ki = 21uM) and PKC (Ki = 42 uM). We have included a discussion of this point on pages 12, 20, and 24.

Reviewer: In Figure 3D, at day 9 of differentiation, the level of cardiac a-actin should result from both cardiac and skeletal differentiation, and thus should not be as inhibited as the skeletal markers. The authors should comment on this point.

We used serum that supported skeletal muscle development better than cardiac muscle development. This can be seen by the modest 2-fold upregulation of cardiac a-actin on day 6, representing cardiomyogenesis, compared to about a 7-fold upregulation in the wild type cells on day 9, representing cardiac and skeletal myogenesis. The % loss of cardiac a-actin was about 70% in the mutant, while the loss of myogenin was about 87%. These numbers are fairly similar and indicate a low background of cardiomyogenesis.

However, to further support our data, we extended the qPCR analysis to study MHC3 and MHC6, which are more predominantly expressed in skeletal or cardiac muscle, respectively (Fig. 1) and have added these results to Fig. 3D. In P19[MEFT80A] cells, MHC3 was not upregulated when compared to the wild type or MEFT80D mutant cells. This lack of upregulation is similar to the lack of upregulation seen for cardiac a-actin, supporting the high level of skeletal myogenesis under these conditions compared to cardiac myogenesis. In contrast, an equivalent, but low level of upregulation was observed for MHC6 and cardiac a-actin when examined on day 6, indicating similar low levels of cardiomyogenesis for the three cell lines. This has been included in the results on page 10.

Reviewer: The experiment in Figure S3 is totally obscure. Is it that, contrary to what is stated in the legend, MHC was monitored at day 6 (panel A) and day 9 (panel B)? In that case, in Panel B, we should see the addition of cardiac and skeletal differentiation, and not only skeletal. The authors should comment on this point.

Figure S3 Panels A and B show cardiac and skeletal muscle obtained on day 9. A low overall percentage of cells differentiate into muscle (15% for cardiac muscle and 5% for skeletal muscle) and usually cardiac and skeletal muscle develop in different regions of the coverslip. Thus, the pictures shown are from the same experiment, just different regions of the same slide. The differences in morphology distinguish cardiac versus skeletal muscle and were used to count cardiac and skeletal myocytes. We have not been able to identify appropriate antibodies that can discern P19- or ES-derived cardiac from skeletal muscle. We have used and published this approach previously (Kennedy et al., BMC Biology, 2009, 7:67). In Panel B, GATA-4 is a cardiomyogenic

factor and its expression was not reduced by ML-7. Only the myogenic regulatory factors were significantly decreased, in agreement with the results from immunofluorescence. We have clarified this point in the figure legend.

Reviewer: In figure 5A, ML7 does not impact on differentiation induced by MyoD alone. Does that imply that endogenous skMLCK is not involved under these conditions?

The reviewer raises a very interesting point that we had not originally addressed. We performed qPCR analysis for skMLCK expression and found that skMLCK is expressed at very low levels in 10T1/2 fibroblasts and is upregulated >4-fold by MyoD and MEF2C expression, compared to MyoD alone. Accordingly, ML-7 did not inhibit the MyoD-induced differentiation because the skMLCK levels were low. We have addressed this on page 15. Please note that the original figure 5 has been renamed Figure 6 in the new version of the manuscript.

Reviewer: In figure 6B, the decrease of p300 binding to MEFT80A mutant as compared to the MEF2C is not obvious. It should be quantified.

Western blots were quantified using the Image J program. The p300 CoIP band intensities were normalized to the intensity of their corresponding control ß-actin bands and then to total p300 for each sample. The relative intensities are indicated directly on the figure. Please note that Fig. 6B is now Fig. 7B.

Minor:

Reviewer: In Figure S1C, the label for time of differentiation is missing.

The label has been added.

Reviewer: The sentence "Cardiac a-actin is expressed in both embryonic cardiac and skeletal muscle", p 10, should be rephrased: Cardiac a -actin is expressed in both cardiac and skeletal embryonic muscle.

The sentence has been removed and we have emphasized that MHC3 appears to be predominantly skeletal muscle-specific.

Reviewer: The authors should define MHC+ve

MHC+ve has been defined on pg. 10.

Referee #2:

We thank the reviewer for pointing out that our work is novel, important, and that the experiments are well executed.

Reviewer: 1) The activation of endogenous skMLCK in a muscle cell line (e.g. C2C12) should be evaluated during proliferation and differentiation, to show a differentiation-induced activation of skMLCK.

As requested, we have identified a differentiation-induced activation of skMLCK in C2C12 myoblasts by western blot analysis. We have included this data in Fig. S1F and in the results on pg 6.

Reviewer: 2) The definitive demonstration of a crucial role of skMLCK in skeletal myogenesis could be provided by inhibiting the expression (by siRNA) and/or activity (by the specific inhibitor, ML-7) of skMLCK in C2C12 cells or MyoD-converted 10T1/2 fibroblasts and determine the impact on the differentiation ability of these cells, by scoring for the formation of MyHC-positive myotubes or the expression of endogenous myogenin. This should be compared with the p38 alpha and beta inhibitor SB, which blocks the myogenic program.

To address the broader relevance of a role for skMLCK, we first examined if ML-7 could inhibit C2C12 myoblast differentiation. We found that it did not inhibit myogenin upregulation or MHC expression. However, our results in P19 and ES cells indicated a role for skMLCK in regulating commitment to the muscle lineage. Thus, we hypothesized that C2C12 cells may not be affected because they are already committed myoblasts, expressing MyoD. Next, we analyzed the activation of quiescent satellite cells during growth in tissue culture. In agreement with our findings in ES and P19 cells, ML-7 inhibited MyoD and Myf-5 upregulation during the activation of muscle satellite cells. Furthermore, we observed a very high level of MEF2C in the quiescent satellite cell, which is consistent with it playing a role in this process. Finally, we compared the inhibition by ML-7 with the inhibition by SB. We were able to reproduce the recent findings of Palacios et al. (Palacios et al., Cell Stem Cell, 2010, **7**(4)**:** 455-469) showing that SB inhibited the downregulation of Pax7, resulting in enhanced proliferation of the activated satellite cell and leading to higher Myf-5 levels. Thus, the comparison of the two inhibitors was very interesting and completely in line with their expected biochemical function. This data has been added in a new Figure 5, in the results on pages 13-14, in the discussion on page 17, 19-21, and we have expanded our model (Fig. 8) to include a comparison of myogenesis in ES and satellite cells, showing the similarities of the two models.

Minor points

Reviewer: 1) Are the authors sure that the antibody used in ChIP (Fig. 6A) is against H3-K4 acetylated? In the same experiment they show that H3-K4 is methylated; therefore, it is unlikely that H3-K4 could be both methylated and acetylated.

We thank the reviewer for pointing this out as we had made a mistake. The antibody used was directed against acetylated H3K14. We have corrected this throughout the manuscript.

Reviewer: 2) Page 9, line 8 - the authors state that HEK-293 do not contain exogenous MLCK activity. They should show it by measuring kinase activity of MLCK.

We have included HEK293 cells in our western blot analysis of C2C12 myoblasts and myotubes and demonstrated that there isn't detectable skMLCK in HEK-293 cells. This is in agreement with the manufacturer's information (http://www.abnova.com/products/products_detail.asp?Catalog_id=H00085366-M01). We have included this finding in Fig. S1F and on page 9.

Referee #3:

We thank the reviewer for stating that we have provided convincing evidence that skMLCK is involved in P19 cell skeletal myogenesis.

Reviewer: The authors state in the first paragraph of the discussion that skMLCK may regulate the decision of progenitor cells to commit to a myoblast vs. a satellite cell fate. The authors need to perform at least one experiment on primary cells to demonstrate that the data are applicable to a system that is representative for muscle development or regeneration.

To address the broader relevance of a role for skMLCK, we first examined if ML-7 could inhibit C2C12 myoblast differentiation. We found that it did not inhibit myogenin upregulation or MHC expression. However, our results in P19 and ES cells indicated a role for skMLCK in regulating commitment to the muscle lineage. Thus, we hypothesized that C2C12 cells may not be affected because they are already committed myoblasts, expressing MyoD. Next, we analyzed the activation of quiescent satellite cells during growth in tissue culture. In agreement with our findings in ES and P19 cells, ML-7 inhibited MyoD and Myf-5 upregulation during the activation of muscle satellite cells. Furthermore, we observed a very high level of MEF2C in the quiescent satellite cell, which is consistent with it playing a role in this process. Finally, we compared the inhibition by ML-7 with the inhibition by SB. We were able to reproduce the recent findings of Palacios et al. (Palacios et al., Cell Stem Cell, 2010, **7**(4)**:** 455-469) showing that SB inhibited the downregulation of Pax7, resulting in enhanced proliferation of the activated satellite cell and leading to higher Myf-5 levels. Thus, the comparison of the two inhibitors was very interesting and completely in line with their expected biochemical function. This data has been added in a new Figure 5, in the results on pages 13-14, in the discussion on page 17, 19-21, and we have expanded our model (Fig. 8) to include a comparison of myogenesis in ES and satellite cells, showing the similarities of the two models.

2nd Editorial Decision 30 March 2011

Many thanks for submitting the revised version of your manuscript EMBOJ-2010-76061R. It has now been seen again by referees 1 and 3, both of whom are satisfied with your responses to their previous concerns, and are now fully supportive of publication (referee 3's brief comments on the revision are pasted below; referee 1 had no specific comments). I am therefore pleased to be able to tell you that we will be able to publish your manuscript in the EMBO Journal.

However, there are just a few issues from the editorial side first:

- In Figure 2A, it appears that the IgG control and skMLCK lanes have been spliced together. This is acceptable practise, provided that the lanes did originally come from the same blot, and it's just that intervening lanes have been removed for clarity. However, this does need to be clearly marked with a black line, and stated in the figure legend. Also, please can I ask you to send the original scan of this blot? This is something we routinely ask for in cases where we request such changes to figures and require for our records.
- In Figure 2B, the blots appear to be rather highly contrasted, and some of the bands look rather odd, with very defined borders (particularly in the Flag blot). This could be due to the contrast adjustments, or to file compression artefacts, but in any case, please can you replace these panels with less contrasted versions, and also to send me the original scans of the blots?
- In the legend to Figure 7B, you don't state the number of replicates from which you calculate the "% total p300".
- We now require "Author Contributions" and "Conflict of Interest" statements for all published manuscripts. Please can you add these below the "Acknowledgments" section in the manuscript text?

If you could make these changes and submit a revised version of the manuscript, and send me the original scans by email, I hope we should then be able to accept your study for publication in

EMBOJ.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #3:

The authors have addressed all of the concerns of the reviewers and the manuscript should be published.

2nd Revision - Authors' Response 13 April 2011

Thank you for all of your help with this manuscript. I have appended:

1) A pdf file "Fig. 2A" that contains the original scan of the blot. Further I have added a black line to mark the spliced region of the blot in the figure and stated clearly that the lanes came from the same blot, with intervening lanes removed for clarity.

2) A pdf file "Fig. 2B" that contains original scans from this figure, which also had had intervening lanes removed for clarity. Some of the bands were overexposed and the film itself is quite yellow, due to problems with the developing reagents at that point in time. I'm showing both light and dark exposures, with boxes around the scans that were used in the figure itself. We have chosen lower exposures of the Flag and His blots, which provide bands that are more realistic. I have added a black line to the original figure, showing the splice site and stated that intervening lanes were removed for clarity in the figure legend.

3) The number of replicates has been added to the figure legend for Fig. 7B

4) Author contributions and conflict of interest statements have been included with the manuscript.

Please let me know if you require anything further or if there is anything that isn't clear. Thanks again for your help.

